A confocal microscopy investigation of the release of chaperones from the endoplasmic reticulum during proteostasis stress

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A confocal microscopy investigation of the release of chaperones from the endoplasmic reticulum during proteostasis stress

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Master of Research

Of the

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DECLARATION

This thesis is submitted in accordance with the regulations of the University of Wollongong and in partial fulfilment of the requirements for the degree of Master of Research. It does not include any material published by another person except where due reference is made in the text. The experimental work described in this thesis is original work and has not been submitted for a degree or diploma at any other university.

Usha Purja

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ABSTRACT

ER chaperones are a critical component of cellular proteostasis that assist proteins to gain and maintain their proper functional folding state, and direct irreversibly misfolded proteins to degradation. Recent studies have demonstrated endoplasmic reticulum (ER) stress-induced release of many ER-resident chaperones from the ER into the cytosol, including binding immunoglobulin protein (BiP), calreticulin (CRT), protein disulfide isomerase (PDI), and the normally secreted chaperone clusterin (CLU). Although the physical pathway used by these chaperones to reach the cytosol from the ER is yet unknown, recently N-terminal arginylation of ER chaperone has been put forward as a mechanism of their release. Evidence is accumulating to suggest that these chaperones play cytoprotective roles in the cytosol and direct misfolded proteins for degradation. This study assessed whether it was possible to use confocal microscopy to image the ER stress-induced release of chaperones from the ER/Golgi to the cytosol. Following optimisation of cultured cell preparation, and image acquisition parameters, this was successfully achieved in both HEK293 and HeLa cells. In HeLa cells, chaperone release to the cytosol was demonstrated in response to three different inducers of ER stress - DTT, MG132, and brefeldin A. The results suggest that release of chaperones from the ER to the cytosol is a general cell response to ER stress. CNX was not released from the ER under these conditions, establishing that the results cannot be explained by a simple loss of ER membrane integrity. The results presented in this thesis are not in themselves conclusive, but when combined with future work using other techniques to interrogate the same phenomenon, should help establish the processes underpinning this potentially very important new proteostasis mechanism.
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<thead>
<tr>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>AFU</td>
<td>Arbitrary fluorescence unit</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>Atgs</td>
<td>Autophagy related genes</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>CLU</td>
<td>Clusterin</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone-mediated autophagy</td>
</tr>
<tr>
<td>CNX</td>
<td>Calnexin</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbondioxide</td>
</tr>
<tr>
<td>CP</td>
<td>Core particle</td>
</tr>
<tr>
<td>CPT</td>
<td>Camptothecin</td>
</tr>
<tr>
<td>CPY</td>
<td>Carboxypeptidase ysc Y</td>
</tr>
<tr>
<td>CRT</td>
<td>Calreticulin</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cells</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitinating enzymes</td>
</tr>
<tr>
<td>Der1</td>
<td>Derlin 1</td>
</tr>
</tbody>
</table>
DMEM: Dulbecco's Modified Eagle's Medium
DNA: Deoxyribonucleic acid
DTT: Dithiothreitol
E1: Ubiquitin activating enzyme
E2: Ubiquitin transferring enzymes
E3: Ubiquitin ligase
EDEM: ERAD-enhancing mannosidase-like proteins
EDTA: Ethylenediaminetetraacetic acid
EGFP: Enhanced green fluorescent protein
ER: Endoplasmic reticulum
eIF2α: Eukaryotic initiation factor 2 α
Gly: Glycine
GFP: Green fluorescent protein
GRP 94: Glucose regulated protein 94
hCLU: Human clusterin
HCMV: Human cytomegalovirus
HEK293 cells: Human Embryonic Kidney cells
HSPA8: Heat shock protein family A
IRE1: Inositol requiring enzyme 1
Lys: Lysine
LAMP2: Lysosome-associated membrane protein 2
LC3: Microtubule associated protein 1 light chain 3
LIF: Leica image file
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCS</td>
<td>Membrane contact sites</td>
</tr>
<tr>
<td>mCherry</td>
<td>Monomeric cherry</td>
</tr>
<tr>
<td>mEm</td>
<td>Monomeric emerald</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>MSDD</td>
<td>Multi-system degenerative disorder</td>
</tr>
<tr>
<td>NBR1</td>
<td>Neighbour of BRCA1 gene 1</td>
</tr>
<tr>
<td>OPTN</td>
<td>Optineurin</td>
</tr>
<tr>
<td>p62</td>
<td>polyubiquitin binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulphide isomerase</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein kinase RNA (PKR)-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGC1α</td>
<td>peroxisome proliferator-activated receptor α–coactivator 1</td>
</tr>
<tr>
<td>RAD23</td>
<td>Radiation sensitivity abnormal 23</td>
</tr>
<tr>
<td>R-BiP</td>
<td>Arginylated BiP</td>
</tr>
<tr>
<td>R-CRT</td>
<td>Arginylated CRT</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>Regulatory particle</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TIFF</td>
<td>Tagged image file format</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>UBA</td>
<td>Ubiquitin-associated</td>
</tr>
<tr>
<td>UBL</td>
<td>Ubiquitin-associated (UBA)-Ub-like modifier</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
</tr>
<tr>
<td>VCP</td>
<td>Valosin-containing protein</td>
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CHAPTER 1: INTRODUCTION

1.1 General Introduction

Proteins are complex biological macromolecules that take part in all cellular functions. They are synthesised on ribosomes as a linear chain of amino acids (aa). In order to be biologically active, each protein must fold into a specific three-dimensional structure called its native state (or conformation) within a biologically relevant time frame. The native state is the most thermodynamically stable, lowest free energy state (Dobson, 2003). The information required for any protein to acquire its native state is contained in its amino acid sequence. Although smaller proteins (<50 aa in length) can spontaneously fold into their native conformation, larger proteins (>100 aa in length) cannot efficiently fold unassisted. Many factors can influence protein folding such as the size of the protein, macromolecular crowding inside the cell, and the level of intrinsic disorder (Minton, 2005, Gregory et al., 2017, Dunker et al., 2008). The total number and size of proteins increases from bacteria to eukaryote. Mammalian cells synthesise more than 10,000 different proteins at any one time, with an average size greater than 100 aa in length (Klaips et al., 2018, Hartl et al., 2011). The complexity of protein structure increases in parallel with their size. Furthermore, larger proteins spend more time in partially folded intermediate state (Hartl et al., 2011) which makes them much more vulnerable to misfolding especially in the crowded intracellular environment (~ 300 g of protein per litre) (Minton, 2005). Intrinsically disordered proteins exist as dynamic ensembles that undergo rapid conformational change, some of which acquire a stable conformation only after binding to their specific ligands or other macromolecules while others never attain a stable conformation such proteins are vulnerable to misfolding and aggregation (Fonin et al., 2018, Dunker et al., 2008).

Cells have a complex, evolutionarily conserved network to combat all the above challenges and maintain proteome homeostasis (proteostasis) (Hartl et al., 2011, Sontag et al., 2017, Klaips et al., 2018). Proteostasis encompasses all those processes that ensure the correct synthesis, folding, localisation, and turnover of the proteome to maintain cell function and viability. Key elements include molecular chaperones, stress-response pathways such as the unfolded protein response (UPR), and degradative systems such as the ubiquitin proteasome system (UPS) and the autophagosome-lysosome system (autophagy). Molecular chaperones and their regulators are key components of proteostasis that help folding of de novo synthesised polypeptide chains and refolding of partially folded proteins to acquire their
Chapter 1  

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native state. During stress conditions (e.g. in the presence of foreign DNA or an excess of misfolded proteins), chaperones may be translocated to different locations in the cell. Although the roles of such stress-induced translocations are yet to be established, recent evidence suggests their involvement in directing irreversibly misfolded proteins to degradative pathways.

ER is an important organelle where folding of newly synthesised proteins destined to secretion, or for transport to the plasma membrane or organelles occurs (Stevens and Argon, 1999). It contains many ER-resident chaperones including members of the heat shock family (i.e., immunoglobulin binding protein/BiP, glucose regulated protein 94/GRP94, and co-chaperones), lectins (i.e., calnexin and calreticulin), and the protein disulphide isomerase (PDI) family (Ma and Hendershot, 2004). Although it is not clear why there are so many chaperones, each chaperone appears to have distinct roles that collectively assist unfolded proteins attain their native conformations and maintain proteostasis. BiP regulates ER stress signalling pathways in addition to binding to newly synthesised proteins. PDI helps unfolded proteins in forming disulphide bond and CNX and CRT bind/hold the partially folded proteins (Halperin et al., 2014). Under normal physiological conditions, these chaperones generally reside within the ER lumen. However, during cellular stress many ER chaperones including BiP, PDI, and CRT are found in non-ER compartments (Afshar et al., 2005, Wiersma et al., 2015, Cha-Molstad et al., 2015, Li et al., 2013, Zhang et al., 2010, Zhang et al., 2014).

Clusterin (CLU) is an ATP independent chaperone secreted from the cell under normal physiological conditions. It is also implicated in modulating transcriptional networks, pro-survival signalling, and inhibition of cell death pathways. Under certain stress conditions, it has been reported as present in the cytosol and mitochondria (Nizard et al., 2007, Li et al., 2013, Zhang et al., 2014). It is highly upregulated in cancer cells and Alzheimer's disease and inhibits cell death by inducing autophagosome biosynthesis and supressing pro-apoptotic signalling (Zhang et al., 2014, Li et al., 2013). CLU silenced human prostate cancer cells and mice showed attenuation of stress induced autophagy while cells over-expressing CLU showed enhanced autophagy (Zhang et al., 2014) indicating a cytoprotective role for CLU. Owing to its possible cytoprotective function, it is considered as a potential therapeutic target and clinical trials are currently underway (Wilson and Zoubeidi, 2017).
Although multiple reports describe the stress-induced release of ER-resident chaperones and CLU from the ER to the cytosol and suggest that this process is cytoprotective (Zhang et al., 2010, Gregory et al., 2017), the mechanism(s) by which these chaperones are released and the detail of their actions in the cytosol remain largely unknown.

1.2 Protein synthesis, folding, and misfolding

The amino acid sequence for proteins is encoded in the DNA (Pain, 1996), and this sequence provides the information needed for correct protein folding as it is being synthesised on the ribosome. For small proteins, the rate of synthesis may be slower than the rate of protein folding, allowing co-translational folding before completion of synthesis. Small proteins ~ 50 aa in length can fold before exiting the ribosome (Holtkamp et al., 2015), however, larger proteins (>100 aa), cannot complete folding during synthesis and exit the ribosome as a partially folded nascent polypeptide chain that undergoes post-translational folding in the cytosol or in specific cell compartments such as the ER, Golgi, or mitochondria (Dinner et al., 2000). Larger proteins often need the assistance of multiple chaperones to fold correctly and pass through thermodynamically different active intermediate states before reaching their native structure. The folding process is challenging and rate limiting: proteins that fail to fold within biologically relevant time frame are degraded (Wickner et al., 1999).

Each individual protein molecule has the potential to adopt a large number of conformations. The conformation adopted depends on many weak and non-covalent interactions that are selected stochastically based on the stability of interactions (Dobson et al., 1998). Usually native interactions are more stable than non-native interactions - this helps polypeptide chains find their lowest energy structure to result in the native conformation (Dobson, 2003). This implies that the larger a polypeptide chain, the more likely it is to spend more time in intermediately folded states before finally arriving at its native state. The intermediate state is thermodynamically unstable (contains high free energy) and kinetically active, searching for native interactions (Markossian and Kurganov, 2004). While residing in a partially folded state, proteins have solvent-exposed hydrophobicity and unstructured polypeptide backbones that make them vulnerable to non-native interactions that can lead to their misfolding and aggregation (Hartl et al., 2011, Sontag et al., 2017). This vulnerability to non-native folding is exaggerated in the case of metastable proteins that contain substantial unstructured regions or those proteins that need structural flexibility to function (Dunker et al., 2008). Another challenge to protein folding is the crowded cellular environment that increases the frequency
of macromolecular interactions, potentially favouring the aggregation of partially folded protein intermediates and misfolded proteins (Minton, 2005, Ellis and Minton., 2006). Therefore, proteins frequently require molecular chaperones to fold into their native state. Molecular chaperones constantly patrol for exposed hydrophobic residues on partially folded polypeptides or misfolded proteins and bind to them to prevent non-native binding events (Kim et al., 2013, Hartl et al., 2011).

Different non-physiological conditions such as elevated temperature, extreme pH, Ca\(^{2+}\) imbalance, glucose deprivation, and hypoxia can cause cellular stress. Under these conditions misfolded proteins can accumulate inside the cell (Fulda et al., 2010). Furthermore, transcriptional and translational errors resulting from DNA mutation or dysfunctional ribosomes can also lead to the accumulation of misfolded or dysfunctional proteins (Markossian and Kurganov, 2004).

1.3 Protein degradation

Protein misfolding is inevitable and nearly 20% of proteins fail to fold into native states (Wickner et al., 1999). Degradation of misfolded proteins is necessary to avoid damaging effects arising from them and to maintain protein homeostasis (Klaips et al., 2018). Two major degradative pathways namely the UPS and autophagy are tightly regulated to degrade such proteins. The relative contributions of each system can vary between organ and cell types. However, under normal physiological conditions, UPS is the major housekeeping degradative pathway that is responsible for ~ 80-90% of cellular proteolysis whereas autophagy is responsible for ~ 10-20% of cellular proteolysis (Cohen-Kaplan et al., 2016b). In contrast, autophagy is dominant over UPS in aged and stressed cells (Gamerdinger et al., 2011). Recent reports have shown an active role for co-chaperones in deciding the fates of misfolded proteins, specifically whether they should be delivered to the proteasome or autophagy (Cohen-Kaplan et al., 2016b, Gamerdinger et al., 2011).

1.3.1 The ubiquitin proteasome system

The UPS is a selective degradative system that comprises conserved sequential events; substrate recognition and ubiquitination (conjugation of Ub to substrate), shuttling of ubiquitinilated cargo to the proteasome, and its proteasomal degradation (Cohen-Kaplan et al., 2016b, Cohen-Kaplan et al., 2016c). Ubiquitin (Ub) is a 76 amino acid protein which when conjugated to a protein provides a degradation signal (degron). Three enzymes, namely E1
(the ubiquitin-activating enzyme), E2 (the ubiquitin-transferring enzyme), and E3 (the ubiquitin ligase) mediate substrate recognition and ubiquitination. Conjugation of Ub initiates with ATP dependent activation of Ub by E1. The activated Ub-E1 complex then transfers Ub to E2 to form an Ub-E2 complex. The last step of ubiquitination is binding of Ub-E2 complex with specific E3 ligase and transfer of Ub from E2 to lysine (Lys) residues of substrates. This forms an isopeptide bond between C-terminal glycine (Gly76) of Ub and a Lys residue of the substrate. Only one Ub (mono-ubiquitination) or a chain of Ub (polyubiquitination) may be attached to substrate. Polyubiquitination is formed by continuous attachment of additional Ub to either the N-terminal methionine residues of the previously attached Ub moiety or a Lys residue of the substrate. In general, when Ub is attached via its Lys44 or Lys11 residues to the substrate, this produces proteasomal degrons that are recognised and bound by a Ub-associated (UAB)-Ub-like modifier (UBL), such as radiation sensitivity abnormal 23 (RAD23). UBL binds to the ubiquitinated substrate through its C-terminal UBA domain and delivers it to proteasome (Cohen-Kaplan et al., 2016b, Kwon and Ciechanover, 2017).

The 26S proteasome consists of a 20S core particle (CP) and one or two 19S regulatory particle (RP) that cap the CP. UBL presents the ubiquitinated substrate to the 19S regulatory particle (RP) through its N-terminal UBA domain. This is followed by deubiquitination performed by deubiquitinating enzymes (DUBs), unfolding, and transfer to the proteolytic compartment of the 20S CP for degradation (Cohen-Kaplan et al., 2016b, Cohen-Kaplan et al., 2016a).

1.3.2 Autophagy

Autophagy (Greek word meaning: self-eating) is a process in which cytoplasmic materials are degraded within lysosomes. On the basis of mechanism(s) of cargo delivery to the lysosome, autophagy can be broadly divided into three types: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (autophagy) (Cohen-Kaplan et al., 2016b). Microautophagy is a non-selective lysosomal degradative pathway in which cytoplasmic contents including organelles are directly degraded within lysosomes. CMA, on the other hand, is highly selective and specifically degrades cytosolic misfolded proteins that contain an accessible KFERQ-like motif (Cuervo and Wong, 2014). These motifs are recognised and trafficked by HSPA8 (heat shock protein family A (Hsp70) member 8) to the lysosome. Lysosome-associated membrane protein 2 (LAMP2) at the lysosomal surface recognises and binds to the HSPA8-substrate complex. This binding induces conformational changes in
monomeric LAMP2 that then oligomerises to form a translocon channel through which the substrates are transported into the lysosomal lumen (Purnell et al., 2018, Cuervo and Wong, 2014).

Macroautophagy is the most predominant lysosomal degradative pathway in which the cytoplasmic cargo (autophagic cargo) is sequestered inside a double membrane vesicle, the autophagosome. The process consists of three conserved stages, nucleation, elongation and substrate sequestration, and finally fusion with the lysosome. Many proteins including Beclin-1, autophagy related genes (Atgs), Atg5 and Atg12, and microtubule associated protein 1 light chain 3 (LC3) are essential for the autophagosome formation (Cohen-Kaplan et al., 2016b, Dikic and Elazar, 2018). Autophagy begins with the formation of a small curved membrane sac, the phagophore, that extends in length to surround the autophagic cargo before fusing at its ends to completely enclose the cargo within a double membrane vesicle, the autophagosome. The autophagosome then moves along microtubules to a lysosome where the outer membrane of the autophagosome fuses with the lysosome to form an autolysosome. The autophagic cargo is released into the lysosomal lumen for degradation, and the degraded by products are later released into the cytosol for reuse by the cell (Purnell et al., 2018). Macroautophagy can be either selective or non-selective. Non-selective autophagy is constitutively active at a low basal level and sequesters bulk cytoplasmic contents whereas selective macroautophagy sequesters specific cargo such as intracellular bacteria or Ub-tagged proteins resistant to proteasomal degradation (Zhang et al., 2007, Kwon and Ciechanover, 2017, Cha-Molstad et al., 2015). In the case of specific macroautophagy, ubiquitinated substrate is recognised by an autophagic adapter such as the polyubiquitin binding protein (p62), neighbour of BRCA1 gene 1 (NBR1), or optineurin (OPTN), and presented to the autophagosome (Purnell et al., 2018). In general autophagic adaptor proteins contain two domains, a UBD (e.g. ZZ domain) that recognises and binds to ubiquitinated substrate and an LC3-binding domain (e.g. LIR) that binds to LC3 present on the autophagosome membrane (Cha-Molstad et al., 2015, Cohen-Kaplan et al., 2016c, Kwon and Ciechanover, 2017).

In addition to degrading ubiquitinated substrates, both pathways degrade non-ubiquitinated substrates (Ji and Kwon, 2017, Cohen-Kaplan et al., 2016b). The mechanism(s) underlying these non-canonical degradative pathways are still elusive. However, it is now recognised that both systems cross talk and act simultaneously in accordance with the need to maintain proteostasis (Ji and Kwon, 2017, Kwon and Ciechanover, 2017, Cohen-Kaplan et al., 2016a,
Cohen-Kaplan et al., 2016b). For example, valosin-containing protein (VCP)/p97 is a conserved hexameric ATPase that is known to mediate proteasomal degradation but it appears to play a role in autophagic degradation as well. In multi-system degenerative disorder (MSDD), loss of function of p97 due to mutation showed inhibition in autophagosome maturation and caused accumulation of p62 and lipidated LC3 (LC3 lipidation is essential for phagophore maturation and autolysosome formation) (Ju and Weihl, 2010, Tresse et al., 2010). The role of p62 in the degradative system is only partly understood. It is an autophagic/proteasomal substrate and an adaptor as well (Cohen-Kaplan et al., 2016a). It preferentially binds to the Lys63 linked Ub chains and presents the conjugated cargo for autophagy. However, its ubiquitination at Lys13 by Parkin (E3 ligase) makes it a substrate for proteasomal degradation (Song et al., 2016). Further details regarding substrate selectivity and adaptor preferences for degradation systems are yet to be uncovered.

1.4 Molecular chaperones and proteostasis

While it is unknown why there are so many different types of molecular chaperones (~180 in mammalian cells), each of them functions in a slightly different way (Hartl et al., 2011). They can be broadly divided into holdases and foldases on the basis of their ATP requirement. Holdases are ATP independent chaperones and hold proteins in stable soluble complexes, whereas foldases fold proteins in a process requiring ATP. Chaperones act to ensure native protein conformation and prevent aggregation. They do so by recognising and binding to hydrophobic regions (usually buried in the native conformation) exposed on unfolded or partially folded proteins (Hartl et al., 2011).

CLU is a holdase chaperone predominantly found in humans in plasma and cerebrospinal fluid (CSF). Its expression is upregulated during different conditions of stress including oxidative stress, heat, ionising radiation, pro-apoptotic insults, and proteotoxicity (Nizard et al., 2007). It is linked to numerous protein misfolding disorders including Alzheimer’s disease, familial amyloidotic polyneuropathy, and amyloidotic cardiomyopathy (Gregory et al., 2017). Under certain stress conditions, CLU escapes the secretory pathway and enters the cytosol (Nizard et al., 2007) and mitochondria (Zhang et al., 2014, Li et al., 2013). Although its role is yet to be established, recent evidence suggests that, like secreted CLU, intracellular CLU has a cytoprotective role (Zhang et al., 2014, Gregory et al., 2017).

BiP is a multifunctional ER resident chaperone that regulates $Ca^{2+}$ homeostasis and initiates the UPR, binds to misfolded or partially folded proteins, and facilitates both import and
export of polypeptide through the ER membrane translocon (Alder et al., 2005, Gething, 1999). Under conditions of ER stress, BiP is also found in the nucleus, mitochondria, cytosol and at the cell surface (Zhang et al., 2010, Tsai et al., 2015, Li et al., 2013, Zhang et al., 2014, Cha-Molstad et al., 2015). It appears to assist the release of CLU to the cytosol and also has cytoprotective function (Li et al., 2013, Zhang et al., 2014).

Many ER chaperones and CLU have been found physically associated with aggregated masses of disease-related proteins (Tanaka et al., 2004), consistent with the idea that these chaperones may act to inhibit diseased-associated protein aggregation. They are suggested to direct irreversibly misfolded or aggregated proteins to sites of degradation or spatial sequestration (Klaips et al., 2018, Sontag et al., 2017, Li et al., 2013, Zhang et al., 2014, Cha-Molstad et al., 2015). Recent reports suggest a close relationship between CLU and BiP. In MG132 treated LNCaP (human prostate cancer) cells, BiP over-expression was shown to promote the release of CLU from the ER to the cytosol (Li et al., 2013). Another study demonstrated that CLU knockdown in MCF-7 (human breast cancer) cells and cancer stem cells significantly decreased camptothecin-induced release of BiP into the cytosol (Arumugam et al., 2017) indicating an inter-dependent relationship between the release of the two chaperones.

### 1.5 Dysfunction of proteostasis in disease and aging

Many serious diseases such as dementia, cancer, Parkinson, Huntington and cardiovascular diseases, and cystic fibrosis have been linked to excessive protein misfolding and aggregation resulting from proteostasis failure (Klaips et al., 2018). Although the cause is unknown, proteostasis capacity declines with age and correlates with a corresponding increase in many protein aggregation related neurodegenerative diseases/disorders (Klaips et al., 2018, Martinez et al., 2017, Hartl et al., 2011) (Fig. 1.1).
Chapter 1

1.6 ER homeostasis

The ER is a critical organelle where transmembrane and secretory proteins (nearly 1/3rd of total proteins) enter to fold into their native state. It is also an important organelle for lipid and carbohydrate metabolism and has the highest resting concentration of Ca$^{2+}$ within the cell. Its unique oxidising environment is suitable for the formation of disulphide bonds important in the folding of some proteins. It also contains the Ca$^{2+}$ dependent chaperones BiP, CRT, and CNX which collectively act to promote protein folding. ER homeostasis involves many processes, including the regulated entry of newly synthesised polypeptides and generation of correctly folded proteins, refolding of partially unfolded or misfolded proteins, and the retrotranslocation of irreversibly misfolded proteins for degradation in the cytoplasm (ER associated protein degradation, ERAD). The ER is a dynamic organelle and it undergoes a constant structural and spatial re-organisation in response to different cellular conditions. Cellular insults can result in an excess accumulation of unfolded or misfolded proteins in the ER lumen perturbing ER homeostasis, a condition called ER stress. ER stress
can be caused by altered Ca\textsuperscript{2+} concentrations, modified protein glycosylation, foreign DNA, mutations, and aging. ER stress activates a signalling pathway called the UPR (Senft and Ronai, 2015, Kim et al., 2013, Walter and Ron, 2011).

1.6.1 Unfolded protein response (UPR)

The UPR can be induced by three stress sensor proteins spanning from the EE membrane; inositol requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA (PKR)-like endoplasmic reticulum kinase (PERK). Under normal physiological conditions, inside the ER lumen these sensors are bound to the ER chaperone BiP. When misfolded proteins accumulate in the ER lumen, BiP dissociates from these sensors and binds to misfolded proteins. Following dissociation from BiP, these sensors are activated and work together to selectively upregulate genes involved in increasing proteostasis capacity and transiently attenuating global protein translation to relieve protein load inside the ER (Walter and Ron, 2011).

**ATF6**: During ER stress, once dissociated from BiP, ATF6 is packed into transport vesicles and delivered to the Golgi where it is cleaved to remove its luminal and transmembrane domains by site 1 and 2 protease (S1P and S2P respectively). The resulting N-terminal cytosolic ATF6 then enters the nucleus to activate UPR target genes including those encoding BiP, PDI, and glucose regulated proteins 94 (GRP94) (Walter and Ron, 2011). ATF6 also stimulates ERAD and mitochondrial biogenesis via its effect on peroxisome proliferator-activated receptor α coactivator 1 (PGC1α) and associated co-activators (Senft and Ronai, 2015).

**PERK**: In response to ER stress, once dissociated from BiP, PERK is activated by a process involving dimerization and auto-phosphorylation. The phosphorylated PERK kinase selectively binds to the α-subunit of eukaryotic initiation factor 2 (eIF2α), resulting in the transient attenuation of protein synthesis. Phosphorylated eIF2α also selectively activates translation of specific stress-induced proteins, predominantly ATF4. ATF4 in turn induces the expression of other stress-induced genes including the C/EBP homologous protein (CHOP), growth arrest and DNA damage-inducible 34 (GADD34), and ERAD components (Ji and Kwon, 2017, Kim et al., 2013, Walter and Ron, 2011).

**IRE1**: Once dissociated from BiP, IRE1 is activated by oligomerization. The IRE1 RNAse domain splices and activates XBP-1 which is a transcription factor that enhances the
expression of chaperones, ERAD components and lipogenesis enzymes (Senft and Ronai, 2015).

Although transient UPR signaling may be beneficial in resolving ER stress and preserving cellular homeostasis, prolonged activation of the UPR will lead to the cell becoming committed to apoptosis as a mechanism to safeguard the whole organism from the defective cell(s) that cannot re-establish proteostasis (Walter and Ron, 2011, Rainbolt et al., 2014).

1.6.2 ER associated degradation (ERAD)

Protein folding is an inherently erroneous process. Misfolded proteins in the ER lumen are retrotranslocated to the cytosol via ERAD and degraded by the UPS or autophagy. There is no proteolytic system resident within the ER lumen. Therefore, misfolded proteins must be moved from the ER into the cytosol (Meusser et al., 2005, Oda et al., 2003). ERAD was initially studied in mutant yeast ER luminal protein carboxypeptidase ysc Y (CPY*) - proteasomal degradation of this mutant was found to be promoted by ubiquitin conjugating enzyme Ubc7p. It was speculated that CPY* entered the ER, where it was glycosylated, and then transported back from the ER lumen to the cytosol where it was conjugated to Ub and then degraded (Plemper et al., 1998).

Details of how the ER quality control systems discriminate between misfolded or partially folded intermediates and irreversibly misfolded ERAD substrates are yet to be established. One of the suggested mechanisms is simultaneous binding of EDEM (ERAD-enhancing a-mannosidase-like proteins) and CNX to the N-linked glycans (Man8GlcNAc2-oligosaccharides) that provides a degradation signal and subsequent retrotranslocation to the cytosol (Meusser et al., 2005). Excess hydrophobicity exposed on the surface of misfolded proteins is another suggested mechanism of recognition of ERAD substrates (Vembar and Brodsky, 2008).

1.6.2.1 ERAD channels

Translocons are ER transmembrane proteins that form a narrow ring like pore through which nascent polypeptides emerging from ribosomes enter the ER lumen (Johnson and van Waes, 1999). ERAD substrates are suggested to use the same translocon to reach the cytosol for degradation (Plemper et al., 1998). The Sec61 channel is a well-known component of the translocon which is believed to operate bidirectionally. It has been demonstrated that a mutated Sec61 gene in yeast caused defective ERAD and that misfolded luminal protein CPY* was associated with the luminal domain of the Sec61 channel (Pilon et al., 1998).
Human cytomegalovirus (HCMV) exploits the ERAD system to downregulate elements of the host’s immune defence. US2 is a membrane-anchored protein encoded by HCMV that binds to class I MHC molecules in the ER and induces its translocation to the cytosol and subsequent destruction by proteasome. In cells that express US2, the Sec61 channel was found to be essential for the dislocation of the MHC class I heavy chain into the cytosol (Wiertz et al., 1996). This study was further supported by the association of other subunits of the Sec61 translocon with ERAD substrate ApoB100 (Meusser et al., 2005). Sec61 complex is suggested to form a signal gated translocon channel either by self-oligomerisation or by cooperating with other additional factors. In other systems such as in peroxisome (PEX system), signal gated channels are formed transiently and can shuttle fully folded and oligomeric proteins across membranes. Sec61 may be able to form differently sized transient translocon channels to allow the movement of ERAD substrates from the ER into the cytosol (Johnson et al., 2001, Romisch, 2017). The Sec61 translocon comprises a Sec61 channel that is flanked by Sec62 and Sec63 subunits (Alder et al., 2005). BiP is known to assist the translocation of misfolded proteins from the ER lumen to the cytosol through Sec61 translocon channel (Alder et al., 2005). In BiP assisted misfolded protein translocation, interaction of BiP with Sec63 is essential for the retrotranslocation to occur. This interaction increases the affinity of binding of BiP to misfolded proteins in the ER lumen and causes an allosteric conformational change in the Sec61 channel (to open the channel) that allows passage of misfolded protein (Alder et al., 2005). The precise contribution of the Sec62 subunit in retrotranslocation is unknown, however, its knockdown produced decreased insertion of newly synthesised proteins into the ER indicating its role in co-translational translocation of de novo polypeptides (Lyman and Schekman, 1995). The possibility that CLU is also released to the cytosol via this translocon is supported by the observation that CLU release is assisted by BiP (Li et al., 2013) and knockdown of BiP decreases CLU in the cytosol but not the level of CLU mRNA (Li et al., 2013, Zhang et al., 2014, Arumugam et al., 2017). The possible role of Sec61 in the release of CLU to the cytosol is shown Fig. 1.2.
Derlin-1 (Der1) is another suggested translocon involved in transport of misfolded proteins from inside the ER to the cytosol (Knop et al., 1996). It is a multi-spanning ER membrane protein that is essential for degrading certain ERAD substrates. In yeast, deletion of the Der1 gene completely blocked release of two soluble ERAD substrates (CPY* and PrA*) (Knupp et al., 2018). However, later evidence suggested that Der1 associates with another channel Hrd1 to assist in the release process (Mehnert et al., 2014, Romisch, 2017).

Baldridge and Rapoport (2016) have demonstrated that transmembrane ubiquitin ligase Hrd1 is sufficient to retrotranslocate lumenal misfolded proteins and its autoubiquitination is the main signal for initiating translocation. In this study proteoliposomes containing a membrane anchored version of CPY* (CPY-TM) and Hrd1 were generated and it was shown that CPY-TM can cross the ER membrane via autoubiquitination of Hrd1 followed by ubiquitination of the substrate. Recently, the possibility that Sec61, Der1 and Hrd1 co-operate in translocating
different ERAD substrates has been proposed. In this proposal, ERAD substrates could exit the ER using one, two or all channels depending on the complexity and type of ERAD substrate (Romisch, 2017).

1.7 Inter-organellar dynamics during ER stress

The traditional view of intracellular organelles such as the ER, Golgi, and mitochondria is of discrete organelles with a relatively static structure. More recent observations indicate that these organelles are interconnected by membrane contacts sites (MCS) and undergo constant spatial and structural reorganization depending on cellular conditions (Kornmann, 2013, Rainbolt et al., 2014, Senft and Ronai, 2015). The ER and mitochondria are physically connected via an ER-mitochondria tethering complex that serves as a platform for inter-organellar communication that facilitates the transfer of metabolites such as Ca\(^{2+}\) and lipids (Kornmann, 2013). Because of their physical and functional relationship with the ER, mitochondria are highly sensitive to ER stress. ER stress can alter the concentrations of metabolites in mitochondria or induce stress signalling pathways that ultimately affect mitochondrial functions directing them towards either pro-survival or pro-apoptotic activities (Rainbolt et al., 2014).

In addition to its effects in ER homeostasis, activated PERK also induces the expression of various mitochondrial quality control factors such as LON (mitochondrial protease), HSPA9 (refolding of misfolded proteins), and PARKIN (removes damaged mitochondria via mitophagy). This signalling is accomplished via ATF4 and degradation of Tim 17A (a core subunit of the inner mitochondrial membrane translocase 23; TIM23) by phosphorylated eIF2\(\alpha\) that shuts down global translation and reduce mitochondrial protein import (Rainbolt et al., 2014). Recent reports suggest that in addition to its role in maintaining ER proteostasis, PERK is also involved in regulating mitochondrial proteostasis (Lebeau et al., 2018). A role for mitochondria in providing adaptive responses to ER stress has been reported by Knupp et al. (2018). One of the peculiar features of ER stress-initiated cell death is mitochondrial accumulation of cellular ROS due to insufficient mitochondrial electron transport. A recent report suggests a critical role for IRE1 and calcineurin in protecting the cell from ER stress by enhancing mitochondrial respiration (and decreasing ROS accumulation). Calcineurin is a Ca\(^{2+}\) dependent phosphatase which enhances the activity of PERK (Knupp et al., 2018). Under normal physiological conditions, the entry of calcium from the ER lumen into mitochondria stimulates ATP production by increased respiratory electron transport chain...
activity and decreased mitochondrial membrane potential (MMP). During ER stress, Ca\(^{2+}\) flux from the ER into mitochondria increases causing hyperpolarization of the MMP and increased ROS production. Knockdown of either IRE1 or calcineurin causes Ca\(^{2+}\) dysregulation, increased ROS accumulation and cell death (Knupp et al., 2018). ER stress and mitochondrial dysfunctions are intrinsically linked to many metabolic diseases such as cardiovascular disorders, type 2 diabetes (T2D), non-alcoholic fatty liver disease, and neurodegenerative diseases including Alzheimer’s disease, amyotrophic lateral sclerosis (ALS), and Parkinson’s diseases (Rainbolt et al., 2014, Lebeau et al., 2018).

1.8 Stress-induced release of ER chaperones and CLU into the cytosol

The ER chaperones BiP, PDI, CRT, and the normally secreted chaperone CLU, are stress inducible. It is now established that many ER chaperones and CLU can be released from the ER into the cytosol during ER stress (Alder et al., 2005, Afshar et al., 2005, Cha-Molstad et al., 2015, Nizard et al., 2007). ER stress can be induced in vitro using different agents such as dithiothreitol (DTT), MG132, and brefeldin A (BFA) (Nizard et al., 2007, Cha-Molstad et al., 2015, Gregory et al., 2017). DTT is a strong reducing agent that prevents disulphide bond formation (essential for proteins native conformation) and cause ER stress. MG132 is a proteasome inhibitor and causes accumulation of misfolded proteins in the cytosol. BFA inhibits transport of proteins from the ER to the Golgi and causes an accumulation of proteins inside the ER.

CRT was the first ER chaperone reported to occur outside the ER. It was discovered bound to a KXGFFKR sequence in the cytoplasmic tail of α−integrins (Afshar et al., 2005). Later reports suggested its existence at the tumour cell surface and extracellular space under conditions of stress (Wiersma et al., 2015, Afshar et al., 2005). Similarly, stress-induced localisation of BiP to different sub-cellular compartments including the cytosol, mitochondria, tumour cell surface, and nucleus has been reported and it is linked to a cell mechanism to relieve stress and maintain homeostasis (Ni et al., 2011, Li et al., 2013, Cha-Molstad et al., 2015). Stress-induced N-terminal arginylation of many ER resident chaperones and their subsequent release from the ER to the cytosol under conditions of stress has been extensively demonstrated by Cha-Molstad et al. (2015); BIP, CRT, PDI were demonstrated to translocate to the cytosol in response to ER stress induced by proteasome inhibition. In the same report, arginylated BiP in the cytosol was suggested to bind to and
direct cytosolic misfolded proteins to autophagy via interaction with the p62 autophagic adaptor.

The first detailed experimental demonstration of stress-induced release of CLU to the cytosol during conditions of ER stress in U-251 cell (human glioblastoma cell line) was shown using both biochemical and immunofluorescence assays (Nizard et al., 2007). In digitonin permeabilised cells, CLU specific antibody detected CLU in the cytosol using flow cytometry and confocal microscopy. It was observed that ER stress induced increased CLU levels in the ER and the cytosol. The release of CLU to the cytosol was further demonstrated using an enzymatic assay based on the action of an exclusively cytosolic enzyme, deubiquitinase (DUB). A chimeric protein (CLU-ubiquitin-GFP) was expressed and it was shown that this fusion protein underwent deubiquitination during ER stress confirming translocation of CLU into the cytosol (Nizard et al., 2007).

1.9 Mechanism of release of ER chaperones and CLU

Little is known about the mechanism(s) of release of ER chaperones and CLU into the cytosol. Recent reports strongly suggest the involvement of the “N-end rule pathway” in the release (Cha-Molstad et al., 2015). The “N-end rule pathway” is a regulator of in vivo protein half-life in which misfolded proteins containing an N-terminal degradation signal (N-degron) are recognised and bound by ubiquitin ligase through its recognition component (N-recogins). This is followed by polyubiquitination and proteolysis via the UPS or autophagy (Tasaki et al., 2012). It has been demonstrated that many ER chaperones including BiP, PDI, and calreticulin undergo arginine (Arg)-transfer RNA transferase (R-transferase) mediated N-terminal arginylation, resulting in their cytosolic transfer and turnover via autophagy. It has been shown that arginylated BiP (R-BiP) release is induced by proteasome inhibition (MG132) and thapsigargin. Once in the cytosol R-BiP gets ubiquitinated and interacts with the p62 ZZ domain via its N-terminal arginine. This interaction causes self-oligomerisation and aggregation of p62, followed by its increased association with LC3, leading to the subsequent selective lysosomal degradation of both ER chaperones and their associated cargo (Cha-Molstad et al., 2015).

Since CLU contains an N-terminal negatively charged aspartate residue (a potential N-degron), it may use the same “N-end rule pathway” to exit its secretory pathway and reach the cytosol. This is further supported by knockdown of ATE1 (the gene encoding R-transferase) that completely inhibits release of CLU to the cytosol under conditions of ER
stress (Wilson unpublished). A potential pathway used by CLU to reach the cytosol is depicted in Fig. 1.3.

![Figure 1.3 A potential pathway of CLU release from the ER lumen to the cytosol and subsequent degradation in stressed cells.](image)

Stress induced by different factors (e.g. cytosolic misfolded proteins) may induce post-translational modifications of ER chaperones and CLU which in turn lead to them being released to the cytosol. In the cytosol, ATE1-encoded arginine-transfer RNA transferase (transfers L-arginine from Arg-tRNAArg to protein N-terminus) arginylates these chaperones. The arginylated chaperones alone or in combination, or loaded with cytosolic cargo, are recognised by a specific adaptor that mediates their transfer to a degradative system (e.g. autophagy). Direct engulfment of cytosolic cargo-loaded chaperones by lysosomes may be another pathway (not shown).

1.10 Potential pathways of chaperones release from the ER

Although the pathways of chaperones release have yet to be elucidated, two major potential routes are: channel mediated and non-channel mediated pathways. The channel mediated pathway for the release of chaperones from the ER to the cytosol may be similar to the ERAD pathway described above (1.6.2.1).

1.10.1 Non-channel mediated pathway (s)

ER resident chaperones possess a C-terminal Lys-Asp-Glu-Leu (KDEL) sequence that differentiates them from the secretory cargoes in transport vesicles (Afshar et al., 2005, Wiersma et al., 2015). When the secretory cargoes are transferred from the ER to the Golgi,
many ER resident chaperones leave the ER along with the associated cargoes. These chaperones are recognised by KDEL receptors (docking stations) present at different stages on the secretory pathway and are returned to the ER lumen. One of the suggested pathways involved in ER chaperone release involves masking of the KDEL retrieval sequence by glycosylation so that it is no longer recognised by KDEL receptors (Ni et al., 2011). KDEL receptor saturation is another possible pathway. For instance, stressed HeLa cells do not increase the expression of KDEL receptors but do significantly increase BiP expression, raising the possibility that an excess of ER chaperones in response to ER stress may exceed the KDEL receptor capacity (Wiersma et al., 2015). Furthermore, in murine fibroblast cells (3T6), the binding of KDEL by KDEL receptors was found to be pH dependent - decreasing or increasing intracellular pH with Na⁺/H⁺ transport inhibitor and chloroquine respectively resulted in expression of HSP47 (an ER chaperones) at the cell surface (Sauk et al., 1998). It is important to note, however, that these studies do not provide a complete mechanism for how the chaperones are released from membrane compartments to reach the cytosol. The Golgi apparatus has a protein quality control system (Golgi protein quality control, GPQC) that routes defective lumenal or membrane proteins that escape the ER retention mechanism to the lysosome (Arvan et al., 2002). Non-ubiquitinated CRT was detected in the cytosol of MG132-treated Cos-7 cells, indicating that its release to the cytosol occurred via a ubiquitination-independent pathway (Afshar et al., 2005). Similarly, Golgi dependent and independent transport of misfolded proteins and ER chaperones from the ER to the lytic vacuole via multivesicular bodies have been reported in plants (Pimpl et al., 2006) and yeast (Armstrong, 2010, Kim and Klionsky, 2000, Teter et al., 2001, Teter and Klionsky, 2000, Thumm, 2000). The identity of non-channel mediated pathways for the release of ER chaperones to the cytosol has yet to be discovered.

### 1.11 Significance of release of ER chaperones and CLU into the cytosol

Although it appears that CLU and many ER chaperones are relocated into the cytosol in response to ER stress, the physiological significance of their translocation is yet to be established. Based on the emerging evidence, the release of chaperones into the cytosol appears to be part of a larger adaptive strategy to protect cells from acute stresses that lead to an excess accumulation of misfolded proteins in the cytosol (Gregory et al., 2017, Zhang et al., 2010, Li et al., 2013). The close association of ER chaperones with misfolded proteins and degradative systems detected in biochemical and immunofluorescence analyses suggest
they play a role in escorting misfolded proteins from the ER lumen and/or the cytosol for proteolysis. This protects the cell from proteotoxicity, which is implicated in many neurodegenerative disorders where protein misfolding and aggregation causes neuronal cell death (Gregory et al., 2017, Cox and Ecroyd, 2017). Multiple independent studies have shown that stress-induced release of ER chaperones into the cytosol increases cell survival. Recently a cytoprotective role for CLU in clearing intracellular misfolded TDP-43 (an ALS-associated aggregation-prone protein) and preserving neuronal cell viability was reported in a study using cultured neuronal cells and transgenic *Drosophila* (Gregory et al., 2017). In ER stressed cells, CLU showed extensive colocalisation with TDP-43 and reduced the number of cytoplasmic inclusions. A cytoprotective role for CLU was further demonstrated in transgenic *Drosophila* motor neurons, in which CLU dramatically cleared TDP-43 from axons, partially extended locomotor activity, and significantly extended fly lifespan. It was also shown that in *Drosophila* photoreceptor cells, co-expression of CLU with cytosolic aggregation-prone and disease-relevant proteins, (mutant R406W) human tau and Huntingtin-Q128, protected the cells from proteotoxicity, but only during ER stress (Gregory et al., 2017).

In cancer cells ER chaperones and CLU are also found on the cell surface, in other organelles such as mitochondria and the nucleus (Wiersma et al., 2015, Nizard et al., 2007, Ni et al., 2011, Li et al., 2013, Zhang et al., 2010, Zhang et al., 2014). Since chaperones are stress-inducible, they are highly upregulated during cancer therapy. The upregulated chaperones affect treatment by either inhibiting apoptosis or enhancing survival pathways (Zhang et al., 2014, Li et al., 2013). Recent reports describe a significant increase in the efficacy of an anti-cancer drug, camptothecin (CPT), in CLU knockdown cancer stem cells (CSC) and MCF-7 (human breast cancer cell). When treated with CPT these CLU knockdown cells showed significant necrosis (including increased expression of apoptotic signalling molecules such as Bak, Bax, and PARP, increased cytosolic Ca$^{2+}$, and reduction of cytosolic ATP) (Arumugam et al., 2017). In the same study, it was demonstrated that CLU knockdown significantly decreased CPT-induced release of BiP into the cytosol. Therefore, by inhibiting the release of ER chaperones and CLU to the cytosol, along with anti-cancer treatment, it may be possible to amplify proteotoxic stress and thereby enhance the efficacy of anti-cancer treatment to slow down cancer progression (Zhang et al., 2010, Arumugam et al., 2017).

ER chaperones and CLU play critical roles in proteostasis (Hartl et al., 2011, Klaips et al., 2018). Their release from the ER into the cytosol during ER stress may act to rescue cells from proteotoxicity associated with ER stress. Once in the cytosol, they either bind and
present misfolded proteins to degradative systems or induce autophagosome synthesis (Cha-Molstad et al., 2015, Gregory et al., 2017, Zhang et al., 2014). Although the physical pathway(s) by which these chaperones are released from the ER to the cytosol is unknown, many ER chaperones have been demonstrated to use the “N-end rule pathway” as part of this mechanism (Cha-Molstad et al., 2015). Very few studies have previously used western blot analysis or fluorescence imaging to measure ER stress-induced release of chaperones from the ER to the cytosol. This project developed confocal microscopy approaches to detect ER stress-induced release of ER chaperones to the cytosol.

1.12 Aims
The main aims of this study were:

1. To establish whether confocal microscopy can be used to detect the ER stress-induced release of ER chaperones from the ER/Golgi to the cytosol.
2. To optimise the many parameter settings used in confocal microscopy to provide the best possible images of ER chaperones release from the ER/Golgi to the cytosol.
3. To test whether the ER stress-induced release of ER chaperones from the ER/Golgi into the cytosol is a general cellular response to different inducers of ER stress.
CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Except for those products listed in table 2.1, all other items were purchased from Sigma-Aldrich (Sydney, Australia). All cells were sourced from the American Type Culture Collection (ATCC) (Virginia, U.S.A), and DH5α bacterial cells were sourced from Thermo Scientific, Sydney, Australia.

Table 2.1. Lists of products and their suppliers used in this project

<table>
<thead>
<tr>
<th>Products</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAfilter plasmid Midi Kits</td>
<td>QIAGEN (Hilden Germany)</td>
</tr>
<tr>
<td>Agarose</td>
<td>Amresco (Ohio, U.S.A.)</td>
</tr>
<tr>
<td>HyperLadder molecular weight markers and 5 x DNA Loading Buffer</td>
<td>Bioline (London, U.K.)</td>
</tr>
<tr>
<td>restriction enzyme HindIII and digestion buffer R</td>
<td>Fermentas (Maryland, U.S.A)</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), 0.05% trypsin-EDTA, and the transfection reagent Lipofectamine 2000</td>
<td>Invitrogen (Sydney, Australia)</td>
</tr>
<tr>
<td>Foetal calf serum (FCS)</td>
<td>Bovogen Biologicals (Victoria, Australia)</td>
</tr>
<tr>
<td>ER-ID® Red assay kit (GFP-CERTIFIED®)</td>
<td>Enzo Life Sciences, (Farmingdale, USA)</td>
</tr>
<tr>
<td>Mito Tracker Deep Red FM</td>
<td>Thermo Fisher Scientific (Massachusetts, U.S.A.)</td>
</tr>
<tr>
<td>MG132 and Brefeldin A (BFA)</td>
<td>Abcam (Cambridge, U.K.)</td>
</tr>
<tr>
<td>µ-slide 8 well</td>
<td>ibidi GmbH (Planegg/Martinsried, Germany)</td>
</tr>
</tbody>
</table>
2.2 Plasmid preparation

All plasmid constructs, unless specified, were obtained from Genscript (Piscataway, USA). A map of the pRc/CMV mammalian expression plasmid containing an insert encoding human clusterin (hCLU) is shown in Figure 2.1 A. This same plasmid vector was modified to house an expression insert encoding hCLU-GFP; this was constructed by adding a sequence encoding a short amino acid linker followed by enhanced green fluorescent protein (GGGGSGG-EGFP) at the 3’ end of the hCLU coding sequence. Another similar plasmid was constructed in the Wilson lab to encode for expression of hCLU fused at its C-terminus with the fluorescent protein mCherry. Wild type hCLU was cloned into a pCMV-mCherry backbone (Clontech) using XhoI and EcoRI restriction sites (pCMV-hCLU-mCherry; Figure 2.1B). Additional plasmids used in this study are listed in table 2.2. For transformation, ~ 100 ng of plasmid was added to 100 µL of chemically competent DH5α bacterial cells, incubated on ice for 30 min, heat shocked at 42°C for 30 sec, and then immediately returned to ice for a further 45 min. The cells, after adding 700 µl of lysogeny broth (LB), were incubated at 37°C for 1 h. On an LB agar plate containing 0.1 mg/ml ampicillin, 100 µL of transformation mixture was uniformly spread and then incubated at 37°C overnight. A single colony from the overnight culture was selected to inoculate 250 mL of LB media containing 0.1 mg/ml of ampicillin, which was then incubated at 37°C overnight on a shaker. Purification of plasmids from the overnight culture was done using Isolate II Plasmid Mini Kit (Bioline, Australia) as per the manufacturer’s instructions. Plasmid DNA concentrations were estimated by absorbance at 260 nm using the SPECTROstar® Nano spectrometer (BMG labtech, Ortenberg, Germany).
Figure 2.1 Maps of (A) pRC/CMV-HT7 (encoding hCLU) and (B) pRC/CMV-hCLU-mCherry mammalian expression plasmids. The blue arrow in (A) indicates an insert encoding hCLU. The red arrow in (B) indicates an insert encoding hCLU-mCherry. Source (A) Genscript and (B) Wilson lab.
Table 2.2 Lists of plasmids and their source details

<table>
<thead>
<tr>
<th>Plasmids encoding</th>
<th>Source details</th>
</tr>
</thead>
<tbody>
<tr>
<td>BiP-mCherry</td>
<td>Erik Snapp (Addgene plasmid # 62233; <a href="http://n2t.net/addgene:62233">http://n2t.net/addgene:62233</a>; RRID:Addgene_62233)</td>
</tr>
<tr>
<td>BiP-mGFP</td>
<td>Erik Snapp (Addgene plasmid # 62231; <a href="http://n2t.net/addgene:62231">http://n2t.net/addgene:62231</a>; RRID:Addgene_62231)</td>
</tr>
<tr>
<td>mEm-CRT-N-16</td>
<td>Michael Davidson (Addgene plasmid # 54023; <a href="http://n2t.net/addgene:54023">http://n2t.net/addgene:54023</a>; RRID:Addgene_54023)</td>
</tr>
<tr>
<td>mEm-CNX-N-14</td>
<td>Michael Davidson (Addgene plasmid # 54021; <a href="http://n2t.net/addgene:54021">http://n2t.net/addgene:54021</a>; RRID:Addgene_54021)</td>
</tr>
<tr>
<td>mEm-Golgi-7</td>
<td>Michael Davidson (Addgene plasmid # 54108; <a href="http://n2t.net/addgene:54108">http://n2t.net/addgene:54108</a>; RRID:Addgene_54108)</td>
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2.3 Cell culture and transfection

Both HEK293 cells and HeLa cells were maintained in T-25 flasks containing DMEM/F12 supplemented with 5 % (v/v) FCS (complete medium, hereafter). Cells were passaged when they were nearly 90% confluent (every 3-4 days), and were lifted using 0.05% Trypsin-EDTA (Invitrogen, Sydney, Australia). Throughout the study period, all cells were maintained at 37°C and 5 % (v/v) CO₂ in a Heracell 150i CO₂ incubator (Thermo Fisher Scientific, Massachusetts, USA).

For transient transfection, cells were seeded into a µ-slide 8-well Ibidi plate (ibidi GmbH, Germany) and incubated overnight (until they reached ~70 % confluency). The cells were then transfected with the desired plasmid encoding fluorescently tagged ER chaperones or hCLU using Lipofectamine™ 2000 following the manufacturer’s instructions. For co-transfection, both plasmids were mixed in the ratio of 1:1 to make the final recommended DNA concentration. The medium containing transfection mixture was replaced 4 h after its addition to cells with complete medium (this was the only change to the manufacturer’s protocol). All subsequent treatments were performed 24 - 48 h post-transfection.

2.4 Cell treatment, staining and fixing

Following transfection, cells were exposed to different treatments which induce ER stress, detailed in Table 2.3. For shorter treatments such as by DTT and BFA, cells were initially counterstained with ER-ID and Hoechst 33342 before the start of treatment. However, for
longer treatments such as MG132, cells were initially treated with MG132 and then counterstained with the ER and nuclear stains. For negative controls, cells were not treated with any ER stress inducers. After treatment and staining, cells were fixed with 1% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS) (i.e. 1% PFA/PBS), sealed with a transparent tape, and were finally imaged by confocal microscopy.

Table 2.3 Details of cell treatments to induce ER stress

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time h (post-transfection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM DTT</td>
<td>42-48 h</td>
</tr>
<tr>
<td>5 µg/ml BFA</td>
<td>42-48 h</td>
</tr>
<tr>
<td>10 µM MG132</td>
<td>32-48 h</td>
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2.5 Confocal microscopy and image processing

Fixed cells were imaged using a Leica SP8 confocal microscope system (Leica Microsystems, Germany). The excitation and emission settings used in this study are outlined in Table 2.3. For individual specimens, up to three colours of fluorescence were acquired using sequential scanning. For optimisation purpose, initial images were acquired at different pixel resolutions (i.e. 512x512, 1024x1024, 2048x2048, 4096x4096, and 8192x8192), different frame averaging (i.e. 2, 6, 8, 12, 20, and 30), and different numbers of z-steps. All fluorescence images were deconvoluted using Huygens essential wizard. Images were quantified by using pixel intensity quantification software provided with the confocal microscope system (LAS X 3.5.0.18371). Then, images in LIF (Leica image file) format were exported as TIFF (Tagged Image File Format) and image brightness was increased using Adobe Photoshop CC 2018. Finally, images were assembled using Adobe Illustrator CC 2018 for display in the Results section.
Table 2.3 Summary of excitation and emission wavelengths used for confocal microscopy

<table>
<thead>
<tr>
<th>Fluorescent label</th>
<th>Excitation (nm)</th>
<th>Emission wavelengths collected (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP/mEmerald</td>
<td>488</td>
<td>498-550</td>
</tr>
<tr>
<td>mCherry</td>
<td>552</td>
<td>600-725</td>
</tr>
<tr>
<td>ER-ID</td>
<td>552</td>
<td>625-725</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>405</td>
<td>415-550</td>
</tr>
<tr>
<td>MitoTracker Deep red FM</td>
<td>552</td>
<td>625-775</td>
</tr>
</tbody>
</table>
CHAPTER 3: RESULTS

Multiple studies have demonstrated ER stress-induced release of ER-resident chaperones and the normally secreted chaperone CLU into the cytosol in response to different chemical inducers of ER stress including DTT, MG132, and brefeldin A (BFA). A central aim of this project was to establish whether it was feasible to image ER stress-induced release of ER chaperones from the ER/Golgi into the cytosol. HEK293 cells that are commonly used for recombinant protein expression due to their ease of transfection were initially used. The cells were transiently transfected to express different ER-resident chaperones or CLU fused to fluorescent proteins (e.g. mGFP and mCherry), treated or not with different inducers of ER stress, and imaged by confocal microscopy. Initially, an attempt was made to image live cells in order to be able to follow real time events occurring inside cells during the induction of ER stress. However, stress caused by repeated laser scanning of cells, and physical movement of cells from their initial positions during treatment made this difficult. Therefore, imaging of cells after fixation with PFA/PBS was performed. To avoid undesired stress to cells in adjacent wells resulting from diffusion of formaldehyde fumes (Fig. 3.1), individual wells supplemented with PFA/PBS were sealed with transparent tape. Preliminary time course experiments showed that visible effects on ER and cell morphology were detected after 5 h of 1 mM DTT treatment. Therefore, cells were fixed after 2.5 and 5 h of DTT treatment for subsequent examination by confocal microscopy. Significant time was also invested in optimising acquisition settings for confocal microscopy to obtain the highest quality images possible. In HEK293 cells, the ER was found distributed throughout much of the volume of the cytoplasm making it difficult to locate “ER-free” cytosol in which ER chaperones released to the cytosol might be clearly imaged. Therefore, in later experiments, HeLa cells were used because these are larger than HEK293 cells, and in these cells the ER does not occupy as much of the cytoplasm as in HEK293 cells. Three different chemical agents, DTT, MG132, and BFA were used to induce ER stress to test whether the release of ER chaperones from the ER/Golgi into the cytosol is a general cellular response to ER stress. Subcellular structures of unstressed and stressed cells were compared and analysed. Finally, quantitative analysis using pixel intensity line graphs of images of cells expressing ER chaperone fusion proteins (e.g. BiP-mCherry, hCLU-mCherry) was performed to test whether the membranes surrounding the cell surface blebs contained either ER-ID or mEm-Golgi-7. This study was able to establish that confocal microscopy can be used to detect the ER stress-induced release
of ER chaperones from the ER/Golgi to the cytosol. This release may be a general cellular defence mechanism against acute ER stress.

3.1 Development of technique to chemically fix cells in individual wells during time course experiments

Conventionally, different chemicals such as aldehyde derivatives and alcohols have been used to fix cells. The intent behind using fixatives is to preserve the morphology of cellular structures without introducing any artefacts (Zeng et al., 2013). Higher concentrations of fixatives can introduce artefacts such as seen in the fixation-associated shrinkage of chondrocytes due to the high osmolarity of 4% (w/v) PFA/PBS (Loqman et al., 2010). Therefore, it is necessary to find the lowest concentration of fixative that can fix cells without causing any major changes in cell structures (Zeng et al., 2013). Previous experiments have shown that 1% PFA/PBS is suitable to fix cells without detectable effects on cell morphology (M. Wilson, pers commun). However, for time-course experiments in which cells in adjacent wells on a multi-well plate/slide have to be fixed at different points in time, it was important to establish if the presence of fixative in individual wells had any effects on live cells in adjacent wells. To examine this, individual wells containing cells on an 8-well Ibidi plate had the medium removed and replaced with 1% PFA/PBS at intervals of 30-60 min. Approximately 1 h after adding PFA/PBS to wells, some live cells in adjacent wells appeared rounded and some had detached from the base of the well. Over longer periods of time, the proportion of live cells in adjacent wells showing signs of stress progressively increased (Figure 3.1). Presumably this resulted from formaldehyde fumes diffusing from wells containing fixative to adjacent wells. Therefore, to avoid this problem, in future experiments transparent tape was used to seal wells to which fixative had been added.
Figure 3.1: Transmitted light images obtained by confocal microscopy of HEK293 cells. Control cells were fixed with 1% PFA/PBS immediately after removal from an incubator. The plate containing live cells in adjacent wells was then returned to the incubator for periods of 1-5 h (indicated above panels). Images were taken using a 63X oil immersion objective lens; scale bar indicates 20 µm except image at the bottom left (30 µm).

3.2 Effect of DTT on HEK293 cells

DTT is a potent reducing agent that chemically reduces disulfide bonds formed between cysteine residues to generate free sulfhydryl groups, an action that can interfere with the folding of proteins inside the endoplasmic reticulum (ER). It is widely used to induce ER stress (Li *et al.*, 2010, Oslowski and Urano, 2011, & Higa *et al.*, 2014). To identify a time point at which fixing DTT-stressed HEK293 cells would capture visible ER-stress induced changes, a preliminary time course experiment was performed. HEK293 cells seeded into an 8 well Ibidi plate were treated or not with 1 mM DTT for 2.5 h and 5 h, stained with ER-ID (stains the ER, red) and Hoechst 33342 (nuclear stain, blue), and fixed with 1% PFA/PBS. ER-ID is a small cell permeable organic probe that specifically stains the ER (Kadonosono *et al.*, 2017, Derry *et al.*, 2014, Raina *et al.*, 2014), however details regarding the specific component(s) of the ER it stains are not disclosed by the manufacturer. The wells containing
fixative were then sealed with transparent tape (to avoid formaldehyde fumes diffusing to other wells) and imaged by confocal microscopy. Unstressed HEK293 cells showed ER-ID fluorescence as a network of interconnected tubules distributed throughout the cytosol, excluding the nucleus (Fig. 3.2A). After 2.5 h of DTT stress, ER-ID fluorescence was found in two distinct locations; higher ER-ID fluorescence was detected as highly branched network of tubules, more concentrated towards the central region of the cell, adjacent to the nucleus, and lower intensity ER-ID fluorescence was found in cell surface blebs at the periphery of the cell (Fig. 3.2B, white star). Following 5 h of DTT treatment, these cell surface blebs were greater in number (Fig. 3.2C, white stars). Some of cell surface blebs did not show detectable ER-ID fluorescence. In addition, membrane surrounding cell surface blebs did not have detectable ER-ID fluorescence indicating that ER was inside these blebs which is a significant feature for this project. The areas in the cytosol that do not have ER-ID fluorescence (i.e. “ER-free” cytosol) are critical for this project to be able to image release of ER chaperones from the ER to the cytosol.
Figure 3.2: Single z-plane fluorescence and transmitted light images obtained by confocal microscopy of unstressed and DTT-treated HEK293 cells. Unstressed (A) and cells treated for 2.5 h (B) and 5 h (C) with 1 mM DTT were fixed and imaged. Two colours of fluorescence were acquired: ER-ID (ER stain, red) and Hoechst 33342 (nuclear stain, blue). All images were acquired at a pixel resolution of 1024x1024, frame averaging of 6, 100X oil immersion objective, and zoom factors of 1.34 (1), 1.46 (2), and 1.31 (3), 4.27 (4), 3.5 (5), and 4.5 (6). Transmitted light images shown at the far right, and overlays of 2 fluorescence images are shown in the other panels. Scale bars indicate 30 µm (1st lane), 10 µm (2nd and 3rd lanes). White stars indicate cell surface blebs.

3.3 Optimisation of confocal microscope parameters for image acquisition

Conventional biochemical methods (e.g. Western blotting of subcellular fractions) can be used to measure release of chaperones from the ER into the cytosol. These methods, however, are labour-intensive and cannot detect detailed structural changes in, for example,
the ER and Golgi during different treatments. Confocal microscopy has this ability. It can produce sharp single plane images devoid of out of focal plane light (a common problem in conventional widefield microscopy) or 3-dimensional (maximum projection) images obtained by “stacking” optical sections collected at different vertical (z) positions through the cell. Confocal microscopy can be used to image specific fluorescently labelled organelles or proteins of interest. The quality of images acquired, however, is greatly influenced by many different factors including the viability of the cells being imaged and multiple image acquisition parameter settings. All the parameters need to be optimised to obtain the most accurate and reliable image, otherwise, results can be misleading or of poor quality. In this study, significant time was initially invested to optimise cell viability prior to fixation (3.1) and image acquisition parameters. To begin, images were collected at a pixel resolution of 1024x1024, frame averaging of 8, and a 15-step z-series spanning a vertical distance of 11-19 µm, (depending on the size of the cell imaged). Maximum projection images were deconvoluted using Huygens essential wizard to give a sharp image with reduced background (Fig. 3.3B).
Figure 3.3: Maximum projection fluorescence images obtained by confocal microscopy of DTT treated HEK293 cells. Cells treated for 2.5 h with 1 mM DTT were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: ER-ID (ER stain, red), BiP-mGFP (BiP, green), and Hoechst 33342 (nuclear stain, blue). Scale bar indicates 10 µm. All images were acquired at a pixel resolution of 1024x1024, frame averaging of 6, 100X oil immersion objective, zoom factor of 5.38, and maximum projection of 15 z-series images collected across a vertical distance of 11.08 µm. All images in the lower row (B) were obtained after deconvoluting the corresponding images in the upper row (A) using Huygens essential wizard. All the fluorescence labels are indicated above the panels; overlays of all 3 fluorescence images are shown on the far right. White stars indicate cell surface blebs containing BiP-mGFP fluorescence. The regions of yellow fluorescence indicate colocalisation of ER-ID and BiP-mGFP fluorescence.

With the aim of trying to image the release of ER chaperones from the ER to the cytosol, it was noted that maximum projection images, which effectively display fluorescence collected from multiple z-planes spanning a distance of microns within cells, were sometimes less than ideal (Fig. 3.4A). Single z-plane fluorescence images were found to give better discrimination between the ER and the surrounding cytoplasm than maximum projection images (Fig. 3.4B). Therefore, most of the subsequent images collected in this study were single plane images.

Photobleaching is one of the major problems associated with fluorescence microscopy. Laser intensity set at 3% in preliminary tests (Appendix I) was found to be sufficient to detect ER-ID fluorescence and this fluorescence did not decrease even after exposure of the same cell to 3% laser for more than 40 times over a 6.5 h period. However, this laser power setting was
not sufficient in some cases, such as when collecting high resolution images of chaperone-fluorescent protein fusion proteins that have lower levels of fluorescence. In these cases, it was necessary to increase laser power to 25% (to obtain good signal:noise ratios). This increased level of laser intensity can cause photobleaching and toxicity to cells, an effect that is more significant when longer wavelength excitation light is used such as is needed to excite ER-ID (Fig. 3.4B far left). ER-ID fluorescence (excitation: 552, emission: 625-725) (Fig. 3.4B) significantly decreased after 9 exposures to 6% laser power, whereas the fluorescence of calreticulin (CRT, normally found in the ER lumen) fused to monomeric emerald (mEm-CRT-N-16) did not significantly decrease under the same conditions (i.e. single plane fluorescence image in Fig. 3.4B was taken from the 10th step in a 15-step z-series).

![Figure 3.4: Maximum projection and single z-plane fluorescence images, and transmitted light images obtained by confocal microscopy of DTT-treated HEK293 cells.](image)

Initially an image pixel resolution of 1024x1024, and frame averaging of 8 was used, however, in later experiments, pixel resolution of 4096x4096 and frame averaging of 12 was used to give even higher quality images (Fig. 3.5).
Figure 3.5: Single z-plane fluorescence images obtained by confocal microscopy of untreated and DTT-treated HEK293 cells. Unstressed cells (A, B, and C) and cells treated for 5 h with 1 mM DTT (D) were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: ER-ID (ER stain, red), mEmerald-calnexin-N-14 (calnexin/CNX, green), and Hoechst 33342 (nuclear stain, blue). Scale bars indicate 10 µm. All images were acquired at a pixel resolution of 4096x4096, and frame averaging of 5 (A), 10 (B), 12 (C), and 20 (D), a 100X oil immersion objective and zoom factors of 4.01(A), 4.56(B), 5(C), and 4.2(D). All images were deconvoluted using Huygens essential wizard. E. Enlarged views of insets from A (i), B (ii), C (iii), and D (iv). The regions of yellow fluorescence indicate colocalisation of ER-ID and mEm-CNX-N-14 fluorescence.

3.4 Confocal imaging of DTT-treated HEK293 cells

The primary goal of this study was to establish whether it was feasible to image the release of ER chaperones from the ER/Golgi into the cytosol in cells in response to ER stress. Initially, live cell-imaging was attempted, but due to problems with phototoxicity arising from repeated laser scanning of cells, and the cells moving during imaging (Appendix I), the decision was taken to image fixed cells in subsequent work. Cells were transiently transfected to express different ER chaperones, including BiP, CRT, and CNX, fused to fluorescent proteins (e.g. mGFP and mCherry). The transfected cells were treated or not for 5 h with 1 mM DTT to induce ER stress and counterstained with ER-ID (red) and Hoechst 33342 (blue) to stain the ER and nucleus respectively, and imaged after fixing with 1% PFA.

BiP has a high affinity for binding to misfolded proteins and it plays two major roles in the secretory and degradative pathways: (1) it binds to and traffics secretory proteins from the ER lumen to the Golgi for further post-translational modification, and (2) it binds to severely misfolded proteins in the ER and directs them to the cytosol for degradation. Although, the
exact pathway used by the normally ER-resident BiP to reach the cytosol is unknown, it has been reported to use the “N-end rule pathway” as a mechanism to exit from the ER lumen and reach the cytosol (Cha-Molstad et al., 2015). The first ER-resident chaperone studied in this project was BiP-mGFP. ER-ID fluorescence in untreated (unstressed) HEK293 cells was found as highly branched tubules widely distributed throughout the cells excluding the nucleus, whereas BiP-mGFP fluorescence was detected as interconnected structures mainly concentrated adjacent to the nucleus (Fig. 3.6A). Overlaid images of ER-ID and BiP-mGFP fluorescence showed extensive co-localisation (yellow fluorescence) (Fig. 3.6A, 3rd lane). After 5 h of DTT stress, however, the fluorescence of ER-ID and BiP-mGFP had discrete patterns of distribution. ER-ID fluorescence was found in two distinct locations; higher ER-ID fluorescence was more concentrated towards the central region of the cell, adjacent to the nucleus, and lower intensity ER-ID fluorescence was found at the cell periphery in cell surface blebs (Fig. 3.6B). In contrast, BiP-mGFP fluorescence was found mostly peripherally located in cell surface blebs of varying size and number (Fig. 3.6B, white star). The cell surface blebs in DTT stressed HEK293 cells were further analysed in single z-plane images (Fig. 3.6 C-D). As expected, in unstressed cells BiP-mGFP fluorescence extensively co-localised with ER-ID adjacent to the nucleus (Fig. 3.6C, white star). This suggests that in unstressed cells, BiP resides particularly inside those regions of the ER adjacent to the nucleus. In DTT-stressed cells, however, BiP-mGFP fluorescence was found both in the central regions of cell adjacent to the nucleus and at the periphery of the cells in cell surface blebs. Overlayed fluorescence images of ER-ID and BiP-mGFP fluorescence showed colocalisation of ER-ID and BiP-mGFP fluorescence (i.e. regions of yellow fluorescence) mostly in the central regions of cell proximity to the nucleus. However, in most of the membrane surrounding cell surface blebs containing diffuse BiP-mGFP fluorescence, there was no ER-ID fluorescence (Fig. 3.6D, white star) suggesting release of BiP-mGFP from the ER to the cytosol.
Figure 3.6: Single plane fluorescence images obtained by confocal microscopy of untreated and DTT-treated HEK293 cells. Unstressed cells and cells treated for 5 h with 1 mM DTT were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: ER-ID (ER stain, red), BiP-mGFP (BiP, green), and Hoechst 33342 (nuclear stain, blue). Scale bars indicate 50 µm (A-B), and 10 µm (C-D). All images were acquired at a pixel resolution of 1024x1024, frame averaging of 8, 100X oil immersion objective, zoom factors of 1 (A), 0.75 (B), 3.07 (C), and 5.59 (D), and deconvoluted using Huygens essential wizard. Fluorescence labels are indicated above the panels C-D. The regions of yellow fluorescence indicate colocalisation of ER-ID and BiP-mGFP. White stars indicate cell surface blebs containing diffuse BiP-mGFP fluorescence. The cells shown here are representative of > 100 HEK293 cells in three independent experiments.

Similar to BiP, CRT has also been reported to be released from the ER to the cytosol during ER stress (Afshar et al., 2005, Cha-Molstad et al., 2015). Therefore, HEK293 cells were transiently transfected to express CRT fused to monomeric emerald (mEm-CRT-N-16), treated with 1 mM DTT or not and imaged (Fig. 3.7). In unstressed HEK293 cells, mEm-CRT-N-16 fluorescence was detected as finely branched green fluorescence, mostly localised at the central regions of the cell, around the nucleus (Fig. 3.7B, 2nd lane from the left), and
ER-ID fluorescence was detected as interconnected tubules widely distributed throughout the cytoplasm, but absent from the nucleus (Fig. 3.7 C, 1st lane from the left). Overlayed fluorescence images of mEm-CRT-N-16 and ER-ID showed co-localisation of mEm-CRT-N-16 and the ER. In contrast, in DTT treated HEK293 cells mEm-CRT-N-16 fluorescence was detected both in tubular interconnected structures in the central region near to the nucleus and in cell surface blebs, similar to those seen for BiP-mGFP in DTT treated HEK293 cells (Fig. 3.6). Consistent with previous reports (Cha-Molstad et al., 2015, Afshar et al., 2005), these cell surface blebs containing diffuse mEm-CRT fluorescence suggest that CRT was released into the cytosol during ER stress.
Figure 3.7: Single z-plane fluorescence images obtained by confocal microscopy of untreated and DTT-treated HEK293 cells. Unstressed cells and cells treated for 5 h with 1 mM DTT were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: ER-ID (ER stain, red), mEm-CRT-N-16 (CRT, green), and Hoechst 33342 (nuclear stain, blue). Scale bars indicate 50 µm (A) and 10 µm for rest. All images were acquired at a pixel resolution of 1024x1024, frame averaging of 8, 100X oil immersion objective except A (40X oil immersion), and zoom factors of 0.75 (A), 6.06 (1), 5.51 (2), 4.1 (3), 3.43 (4), 4.59 (B), and 4.43 (C). All images were deconvoluted using Huygens essential wizard. Fluorescence labels are indicated above the panels; panels second from the right represent overlays of all 3 fluorescence images, transmitted light images are shown on the far right. White stars indicate cell surface blebs containing diffuse mEm-CRT-N-16 fluorescence. The regions of yellow fluorescence indicate colocalisation of ER-ID and mEm-CRT-N-16 fluorescence. The cells shown here are representative of > 100 HEK293 cells in three independent experiments.

Previously Cha-Molstad et al. (2015) reported that unlike BiP and CRT, CNX is not released from the ER into the cytosol during ER stress. Therefore, to test if CNX is released into the cytosol in response to DTT-induced ER stress, HEK293 cells were transiently transfected to
express CNX fused to monomeric emerald (mEm-CNX-N-14) (Fig. 3.8). In unstressed cells, mEm-CNX-N-14 and ER-ID fluorescence extensively co-localised (Fig. 3.8C). In DTT treated HEK293 cells, mEm-CNX-N-14 fluorescence was detected in two distinct locations; more intense mEm-CNX-N-14 fluorescence was detected in ring-like structures mostly at the cell periphery (Fig. 3.8, white star), and lower intensity mEm-CNX-N-14 fluorescence was detected proximal to the nucleus. ER-ID fluorescence, on the other hand, was detected both centrally and at peripheral regions, broadly corresponding to the distribution of mEm-CNX-N-14 fluorescence. Overlaid fluorescence images of mEm-CNX-N-14 and ER-ID showed apparent overlap at the peripheral ring-like structures, however, the intensity of mEm-CNX-N-14 fluorescence was dominant over that of ER-ID fluorescence. The increased level of CNX during ER stress can be expected because, like other ER chaperones, CNX is also stress inducible. The results suggest that during ER stress, CNX is not released from the ER into the cytosol in DTT-treated HEK293 cells, in agreement with Cha-Molstad et al. (2015). This is also supported by the results of western blot assays that do not detect the release of CNX to the cytosol in ER-stressed HEK293 cells (S. Satapathy, unpublished).
CLU is a secreted glycoprotein with chaperone activity. Recently, translocation of CLU from the ER to the cytosol during ER stress has been reported (Nizard et al., 2007, Zhang et al.,...
Therefore, imaging hCLU-mGFP fluorescence in HEK293 cells was attempted. However, due to the low intensity of hCLU-mGFP fluorescence compared to that of ER-ID, imaging hCLU-mGFP was challenging. A plasmid was constructed in the Wilson lab that encodes for expression of hCLU fused at its C-terminus with the fluorescent protein mCherry (pCMV-hCLU-mCherry; S. Satapathy, unpublished). HEK293 cells transiently transfected to express hCLU-mCherry and Golgi-7 fused at its C-terminus with the fluorescent protein monomeric emerald (mEm-Golgi-7) appeared to have comparable fluorescence intensity to one another. Therefore, cells transfected to express hCLU-mCherry were used in further experiments to study the intracellular distribution of CLU. In unstressed cells, mEm-Golgi-7 fluorescence was detected in interconnected membrane stacks restricted to a specific region not far from the nucleus whereas hCLU-mCherry fluorescence was detected in a finely branched tubular network widely distributed throughout the cytoplasm (Fig. 3.9B). This suggests that in unstressed cells, CLU is found in both the ER and the Golgi, consistent with its known passage through these compartments prior to its secretion (Nizard et al., 2007, Zhang et al., 2014). In DTT stressed cells, hCLU-mCherry fluorescence was detected in cell surface blebs (Fig. 3.9A and C, white star), similar to those seen for ER chaperones in Fig. 3.6-3.8. Interestingly, under the same conditions of ER stress, mEm-Golgi-7 fluorescence appeared in ring-like structures that co-localised or not hCLU-mCherry fluorescence (Fig. 3.9A and C, white star). There is a constant flow of vesicles from the ER to the Golgi and vice-versa. Therefore, it is expected that ER stress will induce physical changes in the Golgi (Soo et al., 2015). In DTT-treated HEK293 cells, the cell surface blebs containing diffuse hCLU-mCherry fluorescence (and lacking detectable mEm-Golgi-7 fluorescence) suggests release of CLU from the ER/Golgi during ER stress. Results presented in Nizard et al. (2007) suggest that an unknown fraction of the CLU released into the cytosol during ER stress originates from the Golgi.
Figure 3.9: Single z-plane fluorescence images obtained by confocal microscopy of untreated and DTT-treated HEK293 cells. Unstressed cells or cells treated for 5 h with 1 mM DTT were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: hCLU-mCherry (hCLU, red), mEm-Golgi-7 (Golgi-7, green), and Hoechst 33342 (nuclear stain, blue). Scale bars indicate 10 µm. All images were acquired at a pixel resolution of 4096x4096, frame averaging of 12, 100X oil immersion objective, and zoom factor of 0.75 (A), 3.48 (1), 3.45 (2), 6.82 (3), 3.25 (4), 3.77 (B) and 2.28 (C). All images were deconvoluted using Huygens essential wizard. Fluorescence labels are indicated above the panels; panels second from the right represent overlays of all 3 fluorescence images, transmitted light images are shown on the far right. Image brightness was increased using Photoshop to visualise the relatively low fluorescence of hCLU-mCherry. White stars indicate hCLU-mCherry fluorescence inside cell surface blebs. The regions of yellow fluorescence indicate colocalisation of mEm-Golgi-7 and hCLU-mCherry fluorescence. The cells shown here are representative of >100 HEK293 cells in three independent experiments.
3.5 Confocal imaging of DTT-treated HeLa cells

A primary aim of this study was to image stress induced release of chaperones from the ER/Golgi into the cell cytosol. Imaging of the ER stress-induced release of ER chaperones from the ER to the cytosol was difficult because, particularly in HEK293 cells, the ER network occupied much of the cytoplasm in healthy cells and dilated during ER stress making it even more difficult to visually resolve contents of the ER from the cytosol under these conditions. Therefore, a decision was made to examine HeLa (human cervical cancer) cells as they are larger in size than HEK293 cells and their ER does not extend throughout the cytoplasm to the same extent as in HEK293 cells (Fig. 3.10). To gain further insight into the changes that take place in the ER and Golgi compartments during ER stress, HeLa cells were transiently transfected to express mEm-Golgi-7, treated with 1 mM DTT for 5 h or not, and counterstained with ER-ID. ER-ID fluorescence in unstressed HeLa cells was detected in a branched tubular network widely distributed throughout much of the cell excluding the nucleus, whereas mEm-Golgi-7 fluorescence was localised in a stack of membrane sacs adjacent to the nucleus (Fig. 3.10A). In contrast, in DTT-treated HeLa cells, ER-ID fluorescence extensively co-localised with mEm-Golgi-7 fluorescence (Fig. 3.10B-C). This suggests that ER stress causes extensive re-organisation of the Golgi, and that the ER and Golgi membranes become “mixed” in stressed HeLa cells. The effect of ER stress on the Golgi was expected (explained in 3.4). In addition, cell surface blebs observed in transmitted light images (Fig. 3.9B-C; yellow arrow head, panels at the far right) were not associated with either ER-ID or mEm-Golgi-7 fluorescence, suggesting that the membrane surrounding these blebs was not derived from the either of these membrane compartments.
Figure 3.10: Single plane fluorescence images obtained by confocal microscopy of unstressed and DTT treated HeLa cells. Cells treated for 5 h with 1 mM DTT or not were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: ER-ID (red), mEm-Golgi-7 (green), and Hoechst 33342 (blue). All images were acquired at a pixel resolution of 4096x4096, frame averaging of 12, 100X oil immersion objective, and zoom factors of 1.98 (A), 1.92 (B), and 2.31 (C). All images were deconvoluted using Huygens essential wizard. Fluorescence labels are indicated above panels; panels second from the right represent overlays of all 3 fluorescence images, transmitted light images are shown on the far right. Scale bars indicate 10 µm. Yellow arrowheads indicate cytosol free of the ER. The cells shown here are representative of >20 HeLa cells examined in three independent experiments.

HeLa cells were treated with DTT to see whether results obtained earlier using HEK293 cells could be recapitulated in a different cell type. Hela cells were transfected to express BiP-mCherry or hCLU-mCherry, and mEm-Golgi-7. In unstressed HeLa cells mEm-Golgi-7 fluorescence was detected as membranous interconnected sac-like structures restricted near to the nucleus, while BiP-mCherry fluorescence was found widely distributed throughout the cytoplasm (Fig. 3.11A). Following DTT-induced stress, mEm-Golgi-7 fluorescence was detected as in two distinct locations. Higher intensity of mEm-Golgi-7 fluorescence was detected in interconnected membranous sacs localised near to the nucleus whereas lower intensity of mEm-Golgi-7 fluorescence was detected in finely branched structures distributed throughout the cytosol excluding the nucleus and BiP-mCherry fluorescence was found both
associated with presumptive ER throughout much of the cytoplasm, and inside cell surface blebs. BiP-mCherry fluorescence was detected in cell surface blebs that were surrounded by membrane that lacked any visible mEm-Golgi-7 fluorescence, suggesting that the membrane surrounding cell surface blebs containing hCLU-mCherry did not originate from the Golgi (Fig. 3.11B, white star). A similar pattern of changes in fluorescence were obtained when imaging DTT stressed HeLa cells transfected to express hCLU-mCherry and mEm-Golgi-7 (Fig. 3.11D). In unstressed HeLa cells hCLU-mCherry was detected both in the central regions of cell proximal to the nucleus and in cell surface blebs. In congruent with previous results in HEK293 cells (Fig. 3.9B), membrane surrounding cell surface blebs (containing hCLU-mCherry fluorescence) showed no mEm-Golgi-7 fluorescence suggesting that this membrane did not derive from the Golgi (Fig. 3.11D).
Figure 3.11: Single plane fluorescence images obtained by confocal microscopy of untreated and DTT-treated HeLa cells. Unstressed cells and cells treated for 5 h with 1 mM DTT were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: BiP-mCherry (BiP, red) or hCLU-mCherry (red), mEm-Golgi-7 (green), and Hoechst 33342 (nuclear stain, blue). Scale bars indicate 10 µm. All images were acquired at a pixel resolution of 4096x4096, frame averaging of 12, 100X oil immersion objective, and zoom factors of 3.46 (A), 3.07 (B), 2.76 (C), and 3.05 (D). All images were deconvoluted using Huygens essential wizard. Fluorescence labels are indicated above the panels; panels second from the right represent overlays of all 3 fluorescence images, transmitted light images are shown on the far right. Image brightness was increased using Photoshop to visualise the relatively low fluorescence of BiP-mCherry and hCLU-mCherry. White stars indicate BiP-mCherry (B) and hCLU-mCherry (C) fluorescence at cell surface blebs. The regions of yellow fluorescence indicate colocalisation of mEm-Golgi-7 and BiP-mCherry or hCLU-mCherry fluorescence. The cells shown here are representative of > 100 HeLa cells in three independent experiments.
3.6 Confocal imaging of MG132-treated HeLa cells

Recent reports suggest ER stress-induced release of ER chaperones is a defence mechanism to protect cells from acute challenges to proteostasis (Cha-Molstad et al., 2015, Gregory et al., 2017). Therefore, in order to show that the release of ER chaperones to the cytosol is a general cellular response to ER stress and is not specific to DTT treatment (section 3.5), HeLa cells were treated with other inducers of ER stress (MG132 and BFA). MG132 is a known proteasome inhibitor and it is widely used to induce ER stress (Nizard et al., 2007, Cha-Molstad et al., 2015, Afshar et al., 2005). Many studies have demonstrated MG132-induced release of ER chaperones (and CLU) from the ER to the cytosol. Therefore, further experiment was done to determine whether imaging MG132-treated HeLa cells can show release of BiP-mCherry and hCLU-mCherry to the cytosol. In transfected MG132-treated HeLa cells, as expected, BiP-mCherry fluorescence was visible in cell surface blebs and mEm-Golgi-7 fluorescence was detected in branched membranous structures mostly restricted to regions proximal to the nucleus (Fig. 3.12). The membrane of the cell surface blebs containing diffuse BiP-mCherry fluorescence did not appear to contain mEm-Golgi-7 fluorescence (Fig. 3.12A and C, white star) indicating that the blebs had not been directly derived from Golgi membrane and the chaperone was no longer contained inside a Golgi-membrane bound compartment.
**Figure 3.12:** Single z-plane fluorescence images obtained by confocal microscopy of MG132-treated HeLa cells. Cells treated for 16 h with 10 µM MG132 were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: BiP-mCherry (BiP, red), mEm-Golgi-7 (green), and Hoechst 33342 (nuclear stain, blue). Scale bars indicate 10 µm. Images were acquired at a pixel resolution of 4096x4096, frame averaging of 12, 40X (A) and 100X (rest) oil immersion objectives, and zoom factors of 0.75 (A), 2.81 (1), 2.43 (2), 3.48 (3), 3.35 (4), 2.14 (B), and 2.51 (C). All images were deconvoluted using Huygens essential wizard. Panels A and B show overlay images, and panel C (from left to right) shows individual fluorescence images for BiP-mCherry and mEm-Golgi-7, fluorescence overlay and transmitted light images. Fluorescence labels are indicated on top of panels B & C. The regions of yellow fluorescence indicate Image brightness was increased using Photoshop to enhance the relatively low fluorescence of BiP-mCherry. White stars indicate cell surface blebs containing diffuse BiP-mCherry fluorescence. The regions of yellow fluorescence indicate colocalisation of mEm-Golgi-7 and BiP-mCherry fluorescence. The cells shown here are representative of > 100 HeLa cells in three independent experiments.
A similar pattern of changes in fluorescence were obtained when imaging MG132-treated HeLa cells transfected to express hCLU-mCherry and mEm-Golgi-7 (Fig. 3.13). MG132 treated HeLa cells showed mEm-Golgi-7 fluorescence limited to regions near the nucleus and diffuse hCLU-mCherry fluorescence inside cell surface blebs. In the membrane surrounding these cell surface (Fig. 3.13A and C, white star), mEm-Golgi-7 fluorescence was not visible suggesting that hCLU-mCherry had been released into the cytosol (that protruded into the blebs). The pattern of changes in hCLU-mCherry fluorescence was similar to those observed for BiP-mCherry fluorescence during ER stress (Fig. 3.12) suggesting that both chaperones had been released into the cytosol during MG132 treatment.
Figure 3.13: Single z-plane fluorescence images obtained by confocal microscopy of MG132-treated HeLa cells. Cells treated for 16 h with 10 μM MG132 were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: hCLU-mCherry (red), mEm-Golgi-7 (green), and Hoechst 33342 (blue). Scale bars indicate 10 μm. Images were acquired at a pixel resolution of 4096x4096, frame averaging of 12, 100X oil immersion objectives except A (40X oil immersion), and zoom factors of 0.75 (A), 3.44 (1), 3.07 (2), 1.77 (3), 3.62 (4), 3.24 (B), and 3.62 (C). All images were deconvoluted using Huygens essential wizard. Panels A and B are overlay fluorescence images. C. Fluorescence labels are indicated above panel; panels second from the right represent overlays of all 3 fluorescence images, transmitted light images are shown on the far right. Image brightness was increased using Photoshop to enhance the relatively low fluorescence of hCLU-mCherry. White stars indicate cell surface blebs containing diffuse hCLU-mCherry fluorescence. The regions of yellow fluorescence indicate colocalisation of mEm-Golgi-7 and hCLU-mCherry fluorescence. The cells shown here are representative of > 100 HeLa cells in three independent experiments.
3.7 Confocal imaging of BFA-treated HeLa cells

BFA inhibits the trafficking of vesicles from the ER to the Golgi and thereby causes ER stress. Transfected HeLa cells were incubated with BFA (5 µg/ml in DMEM/F12 supplemented with 5% FCS) for 5 h, fixed as before, and then examined by confocal microscopy. Unstressed HeLa cells showed hCLU-mCherry fluorescence associated with a finely branched tubular network widely distributed throughout the cytoplasm and mEm-Golgi-7 fluorescence localised in a membrane stack adjacent to the nucleus (Fig. 3.14C). Following the 5 h BFA treatment, hCLU-mCherry fluorescence was detected in cell surface blebs that lacked visible mEm-Golgi-7 fluorescence (Fig. 3.14 A and C), suggesting that hCLU-mCherry had been released into the cytosol in response to ER stress induced by BFA treatment. In some cells, hCLU-mCherry fluorescence appeared in ring like structures that lacked visible mEm-Golgi-7 fluorescence (Fig. 3.14A and C). In BFA-treated HeLa cells, the presence of BiP-mCherry and hCLU-mCherry fluorescence in cell surface blebs lacking mEm-Golgi-7 fluorescence gives further support to the proposal that release of these chaperones to the cytosol is part of general cellular response to ER stress.
Figure 3.14: Single z-plane fluorescence images obtained by confocal microscopy of BFA-treated HeLa cells. Cells treated for 5 h with 5 μg/ml BFA were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: hCLU-mCherry (red), mEm-Golgi-7 (green), and Hoechst 33342 (blue). Scale bars indicate 10 μm. Images were acquired at a pixel resolution of 4096x4096, frame averaging of 12, 40X (A) and 100X (B-C) oil immersion objectives, and zoom factors of 0.75 (A), 3.39 (1), 3.77 (2), 3.87 (3), 2.28 (4), 3.43 (B), and 2.88 (C). All images were deconvoluted using Huygens essential wizard. Panels A and B are overlay fluorescence images. Fluorescence labels are indicated above panels; panels second from the right represent overlays of all 3 fluorescence images, transmitted light images are shown on the far right. Image brightness was increased using Photoshop to enhance the relatively low fluorescence of hCLU-mCherry. White stars indicate cell surface blebs containing diffuse hCLU-mCherry fluorescence. The regions of yellow fluorescence indicate colocalisation of mEm-Golgi-7 and hCLU-mCherry fluorescence. The cells shown here are representative of > 100 HeLa cells in three independent experiments.
3.8 Quantitative analysis of fluorescence in cell surface blebs of ER-stressed cells

The major aim of this study was to assess whether it was possible to image the ER stress-induced release of ER chaperones from the ER/Golgi to the cytosol. Owing to time limitations, experiments to simultaneously image ER, Golgi and chaperone fusion proteins were not performed. However, to further analyse whether BiP and CLU were released from the Golgi/ER during ER stress, cell surface blebs in deconvoluted, single z-plane fluorescence overlay images obtained by confocal microscopy were analysed using LASX software. The pixel intensity linegraph tool was chosen in which pixels along a drawn line are plotted on the X-axis, and the Y-axis plots fluorescence intensity. Using this software, a line (region of interest: ROI) is drawn over an overlay image, to generate the corresponding plot of fluorescence intensity versus position along the line. As a negative control, a random ROI was drawn outside of the cell (that had no visible fluorescence) and for a positive control a ROI passing through an area of the cell with visibly high fluorescence intensity was drawn. In DTT treated HEK293 cells transfected to express BiP-mGFP and ER-ID 5 different ROIs were drawn; a negative control (ROI03), a positive control (ROI05), and three tests ROIs (i.e. ROI01, ROI02, and ROI04), and analysed if release of BiP-mGFP from the ER can be shown (Appendix II). However, it was difficult to show release of BiP-mGFP from the ER into the cytosol because of difficulty in finding “ER free” cytosol in HEK cells as explained earlier (3.4).

In MG132-treated HeLa cells, five different ROIs were drawn; a negative control (ROI01), a positive control (ROI02), and test ROIs as ROI03, ROI04 and ROI05 (Fig. 3.15B). ROI01 was drawn outside of the cell where there was no visible fluorescence, whereas ROI02 was drawn within the cell where BiP-mCherry and mEm-Golgi-7 fluorescence was high (~28,000 and ~45,000 arbitrary fluorescence units, AFU, respectively). In all three test ROIs, mEm-Golgi-7 fluorescence was not detected (~ 0 AFU) whereas BiP-mCherry fluorescence was detected ~ 8,500 AFU (Fig. 3.15C). As expected, the intensity of BiP-mCherry fluorescence in the test ROIs was ~ 3X lower than in the positive control. This is consistent with the release of BiP-mCherry into the cytosol. Inside the ER, BiP-mCherry is localised within a small volume. Therefore the BiP-mCherry fluorescence appears (relatively) “bright”. However, if it is released into the cytosol, it is then diluted into a much larger volume and appears (per pixel) less bright. So the weak diffuse fluorescence observed within the cell surface blebs was expected. This interpretation is further supported by pixel intensity line
graphs. In all tested ROIs there was no “spike” in mEm-Golgi-7 fluorescence at the positions of the surrounding membrane (Fig. 3.15C, yellow arrow heads) (Fig. 3.15B), suggesting that the membrane surrounding these blebs was not derived from the Golgi.
Figure 3.15: Single z-plane transmitted light and fluorescence images obtained by confocal microscopy of MG132-treated HeLa cells and pixel intensity line graph. Cells treated for 16 h with 10 μM MG132 were fixed and imaged at 48 h post-transfection. A. Transmitted light image. Scale bar indicates 10 μm. B. Overlay image of three colours of fluorescence: BiP-mCherry (red), mEm-Golgi-7 (green), and Hoechst 33342 (blue). The image was acquired at a pixel resolution of 4096x4096, frame averaging of 12, 100X oil immersion objective, zoom factor of 3.07, and deconvoluted using Huygens essential wizard. C. Pixel intensity line graphs of the deconvoluted overlay image in B. Image brightness was increased using Photoshop to enhance the relatively low fluorescence of hCLU-mCherry. Yellow arrow heads indicate the positions corresponding to the membrane surrounding the bleb. The cell shown here is a representative of > 100 HeLa cells in three independent experiments.
MG132-treated HeLa cells transfected to express hCLU-mCherry were also analysed similarly; five different ROIs were drawn; a negative control (ROI01), a positive control (ROI02), and test ROIs as ROI3, ROI04 and ROI05 (Fig. 3.16B). ROI01, like before, was drawn outside of the cell where there was no visible fluorescence, whereas ROI02 was drawn within the cell containing high levels of fluorescence associated with mEm-Golgi-7 (~25,000 AFU). In three test ROIs (i.e. ROI03, ROI04, and ROI05), mEm-Golgi-7 fluorescence was not detected (~ 0 AFU) at the positions corresponding to the surrounding membrane whereas hCLU-mCherry fluorescence was detected inside the bleb (Fig. 3.16C), suggesting that hCLU-mCherry was released into the cytosol, part of which is contained within a cell surface bleb bounded by a membrane that is not derived from the Golgi.
Figure 3.16: Single z-plane transmitted light and fluorescence images obtained by confocal microscopy of MG132-treated HeLa cell and pixel intensity line graph. Cells treated for 16 h with 10 µM MG132 were fixed and imaged at 48 h post-transfection. A. Transmitted light image. Scale bar indicates 10 µm. B. Overlay image of three colours of fluorescence: hCLU-mCherry (red), mEm-Golgi-7 (green), and Hoechst 33342 (blue). The image was acquired at a pixel resolution of 4096x4096, frame averaging of 12, 100X oil immersion objective, zoom factor of 4.81, and deconvoluted using Huygens essential wizard. C. Pixel fluorescence intensity line graph of the image shown in B. Image brightness was increased using Photoshop to enhance the relatively low fluorescence of hCLU-mCherry. Yellow arrow heads indicate the positions of the membrane surrounding the bleb. The cell shown here is a representative of > 100 HeLa cells in three independent experiments.
BFA-treated HeLa cells were also analysed similarly; four different ROIs were drawn, a negative control (ROI01), a positive control (ROI02), and test ROIs (ROI03, ROI04 and ROI05) (Fig. 3.17B). ROI01 was drawn outside of the cell where there was no visible fluorescence and ROI02 was drawn passing through a region within the cell containing high levels of fluorescence associated with hCLU-mCherry (~ 22,000 AFU) and mEm-Golgi-7 (~ 40,000 AFU). In the two test ROIs, mEm-Golgi-7 fluorescence was not detected (~ 0 AFU) whereas hCLU-mCherry fluorescence was detected ~ 12,000 AFU (maximum) for ROI03 and 14,000 AFU (maximum) for ROI04 and ROI05 (Fig. 3.17C). As expected, the intensity of the diffuse hCLU-mCherry fluorescence inside the bleb was almost half than in the positive control. Inside the ER, the hCLU-mCherry fluorescence appears (relatively) “bright” as it is localised within a small volume of ER tubules. However, when it is released into the cytosol, it is diluted into a bigger volume and appears (per pixel) less bright. This interpretation is further supported by pixel intensity line graph analysis. In both test ROIs there was no “spike” in mEm-Golgi-7 fluorescence (Fig. 3.17C, yellow arrow heads) at the positions corresponding to the membrane surrounding the bleb (Fig. 3.17B), suggesting that the membrane surrounding the bleb is not derived from the Golgi.
Figure 3.17: Single z-plane transmitted light and fluorescence images obtained by confocal microscopy of BFA-treated HeLa cells and pixel intensity line graphs. Cells treated for 5 h with 5 µg/ml of BFA were fixed and imaged at 48 h post-transfection. A. Transmitted light image. Scale bar indicates 10 µm. B. Overlay image of three colours of fluorescence: hCLU-mCherry (red), mEm-Golgi-7 (green), and Hoechst 33342 (blue). The image was acquired at a pixel resolution of 4096x4096, frame averaging of 12, 100X oil immersion objective, zoom factor of 4.12, and deconvoluted using Huygens essential wizard. C. Fluorescence intensity line graphs of the deconvoluted overlay image shown in (B). Image brightness was increased using Photoshop to enhance the relatively low fluorescence of hCLU-mCherry. Yellow arrow heads indicate the positions corresponding to the membrane surrounding the bleb; the cell shown here is a representative of > 100 HeLa cells examined in three independent experiments.
DISCUSSION

4.1 Introduction

ER chaperones are a major component of the cellular proteostasis network that stabilise and/or fold de novo polypeptides or partially folded polypeptides, and direct irreversibly misfolded proteins to degradative pathways (Hartl et al., 2011). As their name suggest, they reside within the ER lumen under normal physiological conditions. However, during ER stress, some of these chaperones are found in the cytosol. Like ER-resident chaperones, the normally secreted chaperone CLU is also released into the cytosol during ER stress (Nizard et al., 2007, Li et al., 2013, Zhang et al., 2010, Zhang et al., 2014). Recent evidence suggests that this release is part of a cellular defence mechanism to combat ER stress related proteotoxicity. It has been proposed that, once in the cytosol, the released chaperones bind to misfolded proteins present there and direct them for degradation, stimulating autophagy (Cha-Molstad et al., 2015, Gregory et al., 2017, Zhang et al., 2014). Recently the “N-end rule pathway” has been implicated in the release of many ER chaperones from the ER to the cytosol during ER stress (Cha-Molstad et al., 2015), however the physical route by which the chaperones are released is unknown. One potential pathway is via the ER membrane translocon, however there is no direct evidence for its involvement. Therefore, alternative pathways are possible. ER stress is induced during many disorders including neurodegenerative diseases and cancer (Hartl et al., 2011, Klaips et al., 2018). Identifying the pathway(s) by which ER-resident chaperones and CLU are released from the ER to the cytosol in response to ER stress may help identify therapeutic targets to treat ER stress related diseases.

Very few studies have measured ER stress-induced release of these chaperones from the ER to the cytosol using western blot analysis or fluorescence imaging (Afshar et al., 2005, Nizard et al., 2007, Cha-Molstad et al., 2015, Decca et al., 2007). The current study developed confocal microscopy approaches to detect release of these chaperones to the cytosol in response to ER stress.

The project was commenced using HEK293 cells because they are easily transfected. To observe real-time changes occurring inside the cells during induction of ER stress, live cell-imaging was initially attempted. However, physical movement of cells during imaging made this very difficult (Appendix I). Therefore, the decision was taken to image cells after fixation with PFA/PBS. Individual well where PFA/PBS had been added was sealed with transparent
tape to avoid undesired stress to cells in adjacent wells resulting from diffusion of formaldehyde fumes (section 3.1). Preliminary time course experiments showed noticeable effects on the morphology of cells and the after treatment for 5 h with 1 mM DTT (section 3.2). Therefore, in subsequent work, cells were fixed after periods of 2.5 h and 5 h of DTT treatment. Optimisation of confocal microscopy acquisition parameters was also performed to obtain the highest quality images possible (section 3.3). Following 5 h of DTT treatment, HEK293 cells showed cell surface blebs containing diffuse ER chaperone associated fluorescence (Fig. 3.6-3.8). However, in these cells, ER was found distributed throughout much of the cytosol leaving very little “ER free” cytosol. Therefore, in later experiments, HeLa cells, which are physically larger than HEK293 cells and in which the ER occupies less of the cytoplasm, were used (Fig. 3.9).

The current study aimed to show that the release of ER chaperones to the cytosol is not specific to any one inducer of ER stress, but is a general cell response to ER stress. Three different agents were used to induce ER stress in HeLa cells, DTT, MG132, and BFA, and all three induced the release of ER chaperones and CLU from the ER/Golgi to the cytosol, consistent with this release being a general cellular defence response to acute ER stress. Pixel intensity line graph analysis of images of cells expressing ER chaperone fusion proteins was performed to show that the membrane surrounding cell surface blebs did not contain ER-ID or mEm-Golgi-7, confirming the interpretation that the chaperones had been released from the ER/Golgi to the cytosol. Therefore, this project successfully established that confocal microscopy can be used to detect the ER stress-induced release of ER chaperones from the ER/Golgi to the cytosol.

4.2 Confocal imaging of ER chaperone release to the cytosol in DTT-treated HEK293

To begin imaging stress-induced release of chaperones from the ER into the cytosol, HEK293 cells transiently transfected to express BiP-mGFP were treated (or not) with 1 mM DTT, and stained with ER-ID, before imaging BiP-mGFP and ER-ID fluorescence. In unstressed cells, BiP-mGFP fluorescence was found in a branched membrane compartment concentrated in the region surrounding the nucleus, and ER-ID fluorescence was detected in a branched membrane compartment distributed throughout much of the cytoplasm. Overlayed fluorescence images of BiP-mGFP and ER-ID showed extensive co-localisation in the perinuclear region (Fig. 3.6C, white star). This distribution is consistent with the expected localisation of BiP-mGFP within specific regions of the ER. BiP is a major ER chaperone
that plays a critical role in protein folding and regulating the UPR, directing misfolded proteins for degradation (Alder et al., 2005, Ni et al., 2011, Cha-Molstad et al., 2015). After DTT treatment, both BiP-mGFP and ER-ID fluorescence showed different patterns of distribution. BiP-mGFP fluorescence was found both in the perinuclear region and in cell surface blebs (Fig. 3.6D). ER-ID fluorescence, on the other hand, was found in two distinct locations. More intense ER-ID fluorescence was found in branched tubules concentrated towards the central region of the cell (adjacent to the nucleus) and lower intensity ER-ID fluorescence was found towards the periphery of the cells, and inside cell surface blebs. This change in ER structure revealed by ER-ID fluorescence in response to ER stress is in agreement with previous studies that have shown extensive changes and dilations of the ER during stress (Vembar and Brodsky, 2008, Xu et al., 2005). There was no apparent ER-ID fluorescence associated with the membrane surrounding most of the cell surface blebs (Fig. 3.6D, white star), consistent with the release of BiP-mGFP from the ER into the cytosol. This was expected because previously Cha-Molstad et al. (2015) have used cell fractionation and immunostaining to show release of BiP from the ER to the cytosol in HeLa cells during ER stress-induced.

Another ER chaperone CRT has also been reported to be released from the ER to the cytosol during ER stress (Cha-Molstad et al., 2015, Afshar et al., 2005). Therefore, to examine whether this release could be imaged by confocal microscopy, HEK293 cells were transiently transfected to express CRT fused to monomeric emerald (mEm-CRT-N-16) (Fig. 3.7). In unstressed cells, as expected, mEm-CRT-N-16 fluorescence was detected in branched structures localised in the perinuclear region and ER-ID fluorescence was detected in a highly branched tubular network widely distributed through the cytoplasm (Fig. 3.7B). In DTT treated HEK293 cells, more intense ER-ID fluorescence was detected in the perinuclear region of the cell and lower intensity ER-ID fluorescence was observed at cell periphery in cell surface blebs. There was no detectable ER-ID fluorescence in the membrane surrounding the cell surface blebs containing diffuse mEm-CRT-N-16, suggesting that CRT had been released into the cytosol in response to ER stress-induced by DTT.

Unlike BiP and CRT, CNX appears to remain inside the ER in HeLa cells during ER stress (Cha-Molstad et al., 2015). In order to confirm this observation using confocal microscopy, HEK293 cells were transiently transfected to express CNX fused to monomeric emerald (mEm-CNX-N-14), treated or not with DTT, and imaged (Fig. 3.8). In unstressed HEK293 cells, overlayed images of mEm-CRT-N-16 and ER-ID fluorescence showed CRT inside the
ER (Fig. 3.8B). In DTT treated HEK293 cells, mEm-CNIX-N-14 fluorescence was found in two distinct locations; more intense mEm-CNIX-N-14 fluorescence was detected in ring-like structures located mostly at the cell periphery (Fig. 3.8, white star), and less intense mEm-CNIX-N-14 fluorescence was detected in a branched membrane network concentrated in the perinuclear region. Overlayed fluorescence images of mEm-CNIX-N-14 and ER-ID showed extensive overlap at both these locations, however, the intensity of mEm-CNIX-N-14 fluorescence dominant that of ER-ID fluorescence. CNX is a stress-inducible chaperone. Therefore, the increased level of CNX during ER stress can be anticipated. This is consistent with mEm-CNIX-N-14 being retained inside the ER and not being released into the cytosol in DTT treated HEK293 cells, and agrees with Cha-Molstad et al. (2015) who also report that CNX is not released from the ER during stress. Western blot analyses of subcellular fractions prepared from DTT-treated HEK293 cells also confirm that, in contrast to BiP and CRT, CNX is not released from the ER in response to ER stress (S. Satapathy, unpublished). This is an important finding because it confirms that the release of the ER-resident chaperones BiP and CRT from the ER to the cytosol is not due to a stress-induced loss of integrity of the ER membrane but is a specific response of certain ER chaperones to ER stress.

In addition to ER chaperones, multiple studies have shown that ER stress induces the release of the normally secreted chaperone CLU from the ER into the cytosol (Li et al., 2013, Zhang et al., 2014, Nizard et al., 2007) and that BiP promotes CLU release under these conditions (Li et al., 2013). To image the DTT-induced release of CLU from the ER/Golgi into the cytosol, HEK293 cells transiently transfected to express hCLU-mCherry and mEm-Golgi-7 were imaged (the reasons for using hCLU-mCherry but not hCLU-mGFP is discussed in section 3.4). In unstressed HEK293 cells, mEm-Golgi-7 fluorescence was found localised in a single cluster of membrane sacs positioned a short distance from the nucleus whereas hCLU-mCherry fluorescence was found in a membrane network widely distributed throughout much of the cytoplasm (excluding the nucleus) (Fig. 3.8A). Overlayed fluorescence images of hCLU-mCherry and mEm-Golgi-7 showed extensive overlap between them (Fig. 3.9A). However, the intensity of hCLU-mCherry fluorescence was lower than that of mEm-Golgi-7. This pattern of fluorescence is consistent with CLU being inside the ER and the Golgi under normal physiological conditions. This distribution of CLU was expected because CLU is post-translationally modified in the ER and the Golgi before it is secreted (Nizard et al., 2007). In DTT-treated HEK293 cells, hCLU-mCherry fluorescence was found in two different locations; in interconnected tubular structures in the perinuclear regions and
inside cell surface blebs. In the overwhelming majority of cases, the membrane surrounding the cell surface blebs lacked detectable mEm-Golgi-7 fluorescence, consistent with the CLU having been released from the ER/Golgi into the cytosol. The re-organisation of the ER and the Golgi during ER stress was expected as recent studies suggest that the continual trafficking of vesicles from the ER to the Golgi and backwards from the Golgi to the ER is disrupted during ER stress (English and Voeltz, 2013, Lippincott-Schwartz et al., 2000).

As discussed earlier, nascent CLU polypeptide enters the ER where a series of post-translational modifications including disulfide bond formation and glycosylation occurs; it is further processed in the Golgi, including proteolytic cleavage into the alpha- and beta-chains. The ER is an oxidising environment, so when a strong reducing agent (e.g. DTT) is added to the cells, it inhibits the formation of disulfide bonds and results in the accumulation of unfolded and misfolded proteins inside the ER. Nizard et al. (2007) have demonstrated using selective permeabilisation and western blot analysis that CLU released into the cytosol in response to ER stress is comprised of both cleaved (mature) and uncleaved forms, suggesting that CLU may be released from both the ER and the Golgi. A limitation of using confocal imaging to investigate the release of CLU from the ER/Golgi is that it cannot distinguish between the two forms of CLU, however, it can detect the release to the cytosol.

In HEK293 cells, the ER was found to be distributed throughout most of the volume of the cytoplasm, extending outwards from the nucleus to just inside the cell membrane. Thus, in these cells there was very little “ER-free” cytoplasm to image, making it more difficult to resolve the fluorescence associated with chaperone fusion proteins released into the cytosol away from that of the ER (Fig. 3.6-3.8, and Appendix II). Therefore, in subsequent experiments, physically larger HeLa cells were used. In HeLa cells, the ER did not occupy as much of volume of the cytoplasm as in the smaller HEK293 cells.

4.3 Confocal imaging of DTT-treated HeLa cells

HeLa cells transiently transfected to express BiP-mCherry and mEm-Golgi-7 were treated (or not) with DTT and imaged. In unstressed HeLa cells, mEm-Golgi-7 fluorescence was detected as an elaborate tubular network localised centrally adjacent to the nucleus (Fig 3.10A, 2nd row from the left) whereas BiP-mCherry fluorescence was detected in a branched network distributed throughout much of the cytoplasm. This was expected because under normal physiological conditions BiP remains primarily in the ER lumen, but it is also present in the Golgi as it carries secretory cargo from the ER to the Golgi in COPII vesicles.
Following DTT treatment, BiP-mCherry fluorescence was concentrated in two distinct locations. Higher intensity BiP-mCherry fluorescence was detected associated with a branched perinuclear tubular network adjacent to the nucleus whereas less intense BiP-mCherry fluorescence was detected inside cell surface blebs (Fig. 3.11B, white star). However, mEm-Golgi-7 was detected in a localised membrane stack adjacent to the nucleus. Overlayed fluorescence images of BiP-mCherry and mEm-Golgi-7 showed extensive overlap between BiP-mCherry and mEm-Golgi-7 fluorescence in the regions near to the nucleus (Fig. 3.11B, 2nd lane from the right). However, mEm-Golgi-7 fluorescence was not detected in the membrane surrounding the cell surface blebs containing diffuse BiP-mCherry fluorescence (Fig. 3.11B). The intensity of BiP-mCherry fluorescence in the cell surface blebs was lower than that found in the presumptive ER close to the nucleus. This was expected because if BiP is released from the ER/Golgi into the cytosol, the resulting dilution will decrease the level of its fluorescence measured for each pixel (section 3.8).

To determine whether the results from DTT-treated HEK293 cells could be recapitulated in HeLa cells, DTT-stressed (or not) HeLa cells expressing hCLU-mCherry and mEm-Golgi-7 were imaged (Fig. 3.11C-D). In untreated HeLa cells, hCLU-mCherry was detected in a finely branched network distributed throughout much of the cytoplasm whereas mEm-Golgi-7 fluorescence was confined to a membrane stack positioned close to the nucleus (Fig. 3.11C). In DTT treated HeLa cells, hCLU-mCherry fluorescence was found associated with presumptive ER throughout much of the cytoplasm, and inside cell surface blebs. Similar to previous results (Fig. 3.9 C), hCLU-mCherry fluorescence was detected inside large cell surface blebs that lacked any detectable mEm-Golgi-7 fluorescence, suggesting that hCLU-mCherry had been released from the ER/Golgi to the cytosol (Fig. 3.11D).

4.4 Release of ER chaperones into the cytosol could be a general cellular response to stress

In order to show that the release of ER-resident chaperones and CLU during ER stress is not specific to DTT stress but is a general stress response used by cells, MG132 and BFA were used to induce ER stress in HeLa cells transiently transfected to express mEm-Golgi-7 and either BiP-mCherry or hCLU-mCherry. Owing to time constraints, simultaneous imaging of ER, Golgi, and ER chaperone fusion proteins was not performed. MG132 is a peptide aldehyde that inhibits the proteolytic activity of the 26S proteasome leading to a gradual accumulation of misfolded proteins in the cytosol and the induction of ER stress. BFA, on the
other hand, is a fungal metabolite that inhibits vesicular transport of proteins from the ER to the Golgi - this results in the accumulation of proteins in the ER lumen and induces ER stress (Nizard et al., 2007). In both MG132 and BFA treated HeLa cells, as expected, BiP-mCherry and hCLU-mCherry fluorescence was detected inside cell surface blebs that lacked detectable mEm-Golgi-7 fluorescence in the surrounding membranes, recapitulating the results obtained earlier in DTT-treated HeLa cells (Fig. 3.11-3.13). There were some significant differences in the cellular responses to these two inducers of ER stress. Stress-induced release of BiP-mCherry and hCLU-mCherry was more easily detected following MG132 treatment than DTT treatment. MG132 inhibits degradation of all proteins in the cytosol (including chaperones), therefore, this may be a possible reason why it was easier to image MG132-induced releases of ER chaperones than DTT-induced. The structural changes observed in mEm-Golgi-7 fluorescence in response to MG132 treatment are consistent with previous studies that showed dramatic changes in the structure of Golgi in response to proteasomal inhibition (Peanne et al., 2010).

Any two given fluorescently labelled species are referred to as colocalized when they are found within the same region(s) (Costes et al., 2004). However, visually, the perception of colocalisation (e.g. of red and green fluorescence) depends on the relative fluorescence intensities of the two labels. If the intensities of both red and green fluorescence are similar, as seen in Fig. 3.14C, regions of colocalization are easily seen in the merged image as appearing yellow. However, if the intensity of fluorescence of one colour is much greater than the other, regions of colocalization are far less obvious and the colour of the more intense fluorescence visually dominates (e.g. Fig. 3.14 1/2/3/4). To avoid such biases (and errors of interpretation), the fluorescence of cell surface blebs in ER-stressed cells were subjected to quantitative analyses.

4.5 Quantitative analysis of fluorescence in cell surface blebs of ER-stressed cells

To confirm the release of ER-resident chaperones and CLU from the ER/Golgi into the cytosol in response to ER stress, quantitative analysis of the fluorescence in cell surface blebs of ER-stressed cells was performed (Fig. 3.14-3.16, Appendix II)). For this, single z-plane fluorescence overlay images obtained by confocal microscopy were analysed using pixel-intensity line graphs in which pixels along a drawn line (called a region of interest, ROI) are plotted on the X-axis, and the fluorescence intensity plotted on the Y-axis. Accordingly, three different types of ROIs were drawn; a negative control ROI (a line drawn outside of the cell
where there is no detectable fluorescence), a positive control ROI (a line passing through a region of cell containing intense fluorescence), and two or three test ROIs (a line that passes through membrane surrounding cell surface blebs). However, in stressed HeLa cells, mEm-Golgi-7 fluorescence was not detected in the membrane surrounding the cell surface blebs containing either BiP-mCherry or hCLU-mCherry fluorescence. The intensity of BiP-mCherry and hCLU-mCherry fluorescence was greater in the ER/Golgi (positive control) than in the cell surface blebs, consistent with their release into the greater volume of the cytosol. This confirmed that the membrane surrounding the cell surface blebs did not originate from the Golgi membrane and the chaperones were no longer contained inside a Golgi-membrane bound compartment.

Images obtained after fixing cells cannot provide details of events occurring inside the cell during stress. One of the major problems encountered in this study was physical movement of HEK293 cells during live cell-imaging. In future, this can be optimised by testing different cell lines that are larger and more adherent than HEK293 cells, and also by using cell immobilisation techniques such as by coating the culture plate with fibronectin or poly-L-lysine. This technique, however, should be used with precaution so that cellular behaviour detected is not contributed by the immobilisation technique. Time did not allow for further study, however, it is recommended to in future simultaneously image the ER, Golgi and chaperones in cells undergoing ER stress. For example, ER chaperones tagged with mGFP (e.g. BiP-mGFP), CellLight-Golgi-red fluorescent protein (Golgi-RFP), and ER-Tracker Blue-White DPX (ThermoFisher). This would help clarify the roles of the ER and the Golgi in the stress-induced release of chaperones to the cytosol. The imaging of diffuse ER chaperones outside of the ER/Golgi and inside cell surface blebs during ER stress is a novel finding. The composition of these blebs, and their longer terms fate following acute cell stress is unknown and will require further work. Previous studies show a close association between ER stress and autophagy. Fluorescent-based biosensors indicating levels of LC3 or p62 can be used to identify autophagy (Cha-Molstad *et al.*, 2015). Whether the cell surface blebs observed in these experiments are associated with apoptosis could be assessed by staining cells with Annexin V-FITC (which binds to phosphatidyl serine exposed on the surface of apoptotic cells) or using Cell Event Caspase-3/7 Green Detection Reagent (which detects activated caspase 3/7).

**4.6 Mechanism of release**
Several studies had predicted a role for arginylation of the N-terminus of ER-resident chaperones in their release from the ER to the cytosol. For example, Reddy et al. (2003) demonstrated the N-terminal region of BiP was exposed to the cytosol using two different approaches: biochemical (e.g. limited trypsin digestion and sodium carbonate extraction) and western blot analysis. They performed limited trypsin digestion of microsomes (vesicles formed from the ER when cells are disrupted) isolated from CHO cells western blotting with anti-BiP antibody to detect three bands of ~ 78 kDa (mature BiP), 50 kDa and 35 kDa (cleaved forms). They further isolated the luminal and ER membrane fractions using sodium carbonate extraction method and detected BiP in both luminal and ER membrane. In the same experiment, they did not detect any CRT in the ER membrane fraction but CNX was detected only in this fraction. Based on these results, they suggested that a subfraction of BiP exists spanning the ER membrane with its N-terminus exposed to the cytosol.

Decca et al. (2007) identified arginylated CRT (R-CRT) in the cytosol using mass spectrometry. They reported N-terminal arginylation of CRT by ATE1-encoded arginine-transfer RNA transferase (R-transferase) at N-terminal glutamate (Glu) in response to thapsigargin. They reported that cytosolic R-CRT was induced with increased calcium chelator (EGTA) in the medium and decreased with the addition of Ca^{2+}. They suggested that release of CRT from the ER to the cytosol may have a role in the formation of stress granules in the cytosol. More recently Cha-Molstad et al. (2015) demonstrated that the ER chaperones BiP, CRT, and PDI follow the same “N-end rule pathway” to reach the cytosol from the ER. These chaperones were shown to be arginylated in response to cytosolic dsDNA or proteasome inhibition. They reported that BiP and PDI were arginylated at N-terminal Glu and Asp residues, respectively. Using an RNA interference assay, Cha-Molstad et al. (2015) showed that N-terminal arginylation of BiP by ATE1 was essential for BiP to reach the cytosol. They also reported that arginylation of BiP did not occur when the N-terminal Glu was mutated to valine and knock down of ATE1 in HeLa cells abolished arginylation of BiP. In fact, they performed subcellular fractionation in which R-BiP was retrieved from the cytosolic fraction. However, they did not demonstrate whether arginylation occurs at the cytosolic face of the ER membrane when the N-terminus of the ER chaperone is exposed to the cytosol or after complete translocation of the chaperones from the ER to the cytosol. CNX, on the other hand, does not appear to fit the criteria for the “N-end rule pathway” as it contains a positively charged N-terminal residue (a histidine). The apparent retention of CNX within the ER during ER stress in the current study is further supported by western blot
analysis of subcellular fractions of DTT-treated HEK293 cells that did not show CNX in the cytosol fraction (S. Satapathy, unpublished). This finding also suggests that CNX can be used as an ER marker during ER stress. CLU contains a negatively charged aspartate (Asp) at its N-terminus and ATE1 knockout in HEK293 cells completely inhibits CLU translocation from the ER to the cytosol during ER stress (S. Satapathy unpublished).

4.7 Possible pathway(s) of release

ER-resident chaperones and CLU may reach the cytosol via the ER membrane translocon, although there is no direct evidence that this is the case. The major translocon channels suggested to be involved in translocating misfolded proteins and ER chaperones from the ER to the cytosol are the Sec61 translocon, Der1, and Hrd1 (described in section 1.6.2.1). The translocon is suggested to open when required, for example, to allow nascent polypeptides to enter the ER. It can expand from an inner diameter of 9–15 Å in the ribosome-free state to a diameter of 40–60 Å in the active (ribosome-bound) state (Johnson and van Waes, 1999). The integrity of the ER membrane is maintained during the translocon inactive state by sealing of the lumenal side opening of the translocon by ADP bound BiP (Alder et al., 2005). An outstanding question here is whether the size of translocon is sufficient to allow passage of ER chaperones and CLU from the ER to the cytosol. At the time of entry, nascent polypeptides are in an extended linear chain form and can pass through the translocon pore. In case of the ER-resident chaperones BiP, CRT, and PDI, and CLU, it is unlikely that this channel can accommodate the fully folded conformation of these proteins. These chaperones are found in the cytosol in their fully mature states (Nizard et al., 2007, Afshar et al., 2005, Decca et al., 2007, Cha-Molstad et al., 2015).

Other possible pathways for the release of ER chaperones to the cytosol during ER stress remain to be defined but could involve lipid droplets forming in the ER membrane (Ploegh, 2007) or vesicular transport (Cha-Molstad et al., 2015). Currently, however, there is no comprehensive explanation for how these chaperones are released from the ER to the cytosol.
CONCLUSION

The release of ER-resident chaperones and CLU to the cytosol during ER stress may exert protective effects in disease states (Tsai et al., 2015, Gregory et al., 2017). Translocation of these chaperones to the cytosol is linked to ER stress caused by misfolded proteins in the cytosol (Ni et al., 2011, Cha-Molstad et al., 2015, Zhang et al., 2014). In cancer treatment, overexpression of these chaperones in response to drugs cause treatment resistance and enhances cancer progression (Li et al., 2013, Zhang et al., 2014). In fact, knockdown of CLU in human prostate cancer cell has been found effective in reducing cancer progress (Zhang et al., 2014) and in cancer stem cells facilitates programmed necrosis (Arumugam et al., 2017).

In transgenic Drosophila neurons expressing TDP-43 (an amyotrophic lateral sclerosis-associated and aggregation-prone protein), co-expression of CLU significantly reduced the levels of TDP-43 in motor neuron axons, partially extended locomotor activity, and significantly prolonged lifespan (Gregory et al., 2017). In this model, the neurons expressing TDP-43 were undergoing ER stress and the CLU was released to the cytosol. Therefore, understanding the mechanism and pathways used by ER chaperones to reach the cytosol may help us understand and treat cancers and neurodegenerative diseases such as amyotrophic lateral sclerosis, and Alzheimer’s and Parkinson’s diseases.

This study used confocal microscopy to detect ER stress-induced release of ER-resident chaperones and CLU from the ER/Golgi to the cytosol. Imaging of this release was more difficult in HEK293 cells, owing to their small size and widely distributed ER. Therefore, later work was performed using the larger HeLa cells, in which chaperone release to the cytosol was demonstrated in response to three different inducers of ER stress (DTT, MG132, and BFA). The results suggest that ER stress-induced release of ER-resident chaperones and CLU is a general cell response to ER stress. An important result to note is that CNX was not released from the ER under these conditions, establishing that the results cannot be explained by a simple loss of ER membrane integrity. The results presented in this thesis are not in themselves conclusive, but when combined with future work using other techniques to interrogate the same phenomenon, should help establish the processes underpinning this potentially very important new proteostasis mechanism.
REFERENCES


References


References


Appendix I. Live cell-imaging

HEK293 cells were stained with ER-ID (ER stain, red) and imaged by confocal microscopy. Constant temperature (37°C) and CO₂ (5%) were established before placing an Ibidi plate containing the sample into the sample chamber. Image acquisition parameters were set such that photobleaching from repeated laser exposure would be minimal (i.e. laser set at 3%, pixel resolution of 512*512, frame average of 2, zoom factor of 2.3), and images were collected at 10 min intervals for 6.5 h. Cells disappeared after every 2 h and position had to be re-set. Although, given parameters appeared to be non-toxic to the cell (as indicated by constant level of fluorescence throughout the whole treatment period), the physical movement of cells did not allow further live cell-imaging.

Appendix Fig. I: Single z-plane fluorescence and transmitted light images obtained by confocal microscopy of live HEK293 cells. Cells stained with ER-ID (ER stain, red) were imaged at every 10 min for 6.5 h. Scale bar indicates 25 µm. Images were acquired at a pixel resolution of 512x512, frame averaging of 2, oil immersion objectives of 63X, and zoom factor of 2.31. Image position was re-set after 2.5 h, 5 h, and 6.5 h.
Appendix II. Cell surface blebs in DTT-treated HEK293 cell

HEK293 cells were transiently transfected to express BiP-mGFP, treated with 1 mM DTT for 5 h, counterstained with ER-ID and Hoechst 33342, and imaged by confocal microscopy. At 5 h of DTT treatment, BiP-mGFP fluorescence was found mostly peripherally located in cell surface blebs (white stars) where there was no significant ER-ID fluorescence.

Appendix Fig. II: Single plane fluorescence images obtained by confocal microscopy of DTT-treated HEK293 cells. Cells treated for 5 h with 1 mM DTT were fixed and imaged at 48 h post-transfection. Three colours of fluorescence were acquired: ER-ID (ER stain, red), BiP-mGFP (BiP, green), and Hoechst 33342 (nuclear stain, blue). Scale bars indicate 10 µm. All images were acquired at a pixel resolution of 1024x1024, frame averaging of 8, 100X oil immersion objective, zoom factors of 2.76(1), 5.88(2), 5.5(3), and 6.72(4), and deconvoluted using Huygens essential wizard. The regions of yellow fluorescence indicate colocalisation of ER-ID and BiP-mGFP. White stars indicate cell surface blebs containing diffuse BiP-mGFP fluorescence. The cells shown here are representative of > 100 HEK293 cells in three independent experiments.
Appendix III. Analysis of ER chaperones in unstressed HEK293 cell

In DTT-treated HEK293 cells transfected to express BiP-mGFP, cell surface blebs containing diffuse BiP-mGFP were analysed for ER-ID fluorescence using pixel fluorescence intensity line graphs. Five regions of interest (ROI) were drawn. ROI01 was drawn outside the cell where there was no visible fluorescence (negative control), while other ROIs were drawn inside the cell; ROI drawn along the major portion of the cell (positive control), ROI03, ROI04, and ROI05 are test ROIs. As seen below, detecting ER stress-induced release of BiP-mGFP from the ER into the cytosol was difficult due to distribution of the ER throughout the cytoplasm, making it harder to find “ER-free” cytosol. Nevertheless, the images obtained suggest that the cell surface blebs containing BiP are not surrounded by membrane derived from the ER, consistent with BiP being released into the cytosol.
Appendix Fig. III: Single z-plane transmitted light and fluorescence images obtained by confocal microscopy of unstressed HEK293 cells and pixel intensity line graphs. Unstressed cells were fixed and imaged at 48 h post-transfection. A. Transmitted light and overlay non-convoluted image. Scale bar indicates 10 µm. B. Overlay image of three colours of fluorescence: ER-ID (red), BiP-mGFP (green), and Hoechst 33342 (blue). The image was acquired at a pixel resolution of 1024x1024, frame averaging of 8, 100X oil immersion objective, zoom factor of 2.63, and deconvoluted (B) using Huygens essential wizard. C. Pixel intensity line graph of the image shown in B. Image brightness was increased using Photoshop to enhance the relatively low fluorescence of BiP-mGFP. Yellow arrow heads indicate the positions corresponding to the membrane. The cells shown here are representative of > 100 HeLa cells examined in three independent experiments.
Appendix IV. Analysis of cell surface blebs in DTT-treated HEK293 cell

In DTT-treated HEK293 cells transfected to express BiP-mGFP, cell surface blebs containing diffuse BiP-mGFP were analysed for ER-ID fluorescence using pixel fluorescence intensity line graphs. Five regions of interest (ROI) were drawn. ROI01 was drawn outside the cell where there was no visible fluorescence (negative control), while other ROIs were drawn inside the cell; ROI02 drawn along the major portion of the cell (positive control), ROI03, ROI04, and ROI05 are test ROIs. As seen below, detecting ER stress-induced release of BiP-mGFP from the ER into the cytosol was difficult due to distribution of the ER throughout the cytoplasm, making it harder to find “ER-free” cytosol. Nevertheless, the images obtained suggest that the cell surface blebs containing BiP are not surrounded by membrane derived from the ER, consistent with BiP being released into the cytosol.
Appendix Fig. IV: Single z-plane transmitted light and fluorescence images obtained by confocal microscopy of DTT-treated HEK293 cells and pixel intensity line graphs. Cells treated for 5 h with 1 mM DTT were fixed and imaged at 48 h post-transfection. A. Transmitted light image. Scale bar indicates 10 μm. B. Overlay image of three colours of fluorescence: ER-ID (red), BiP-mGFP (green), and Hoechst 33342 (blue). The image was acquired at a pixel resolution of 1024x1024, frame averaging of 8, 100X oil immersion objective, zoom factor of 6.52, and deconvoluted using Huygens essential wizard. C. Pixel intensity line graph of the image shown in B. Image brightness was increased using Photoshop to enhance the relatively low fluorescence of BiP-mGFP. Yellow arrow heads indicate the positions corresponding to the membrane surrounding the bleb. The cells shown here are representative of > 100 HeLa cells examined in three independent experiments.
Appendix V. Analysis of cell surface blebs in DTT-treated HEK293 cell

In DTT-treated HEK293 cells transfected to express mEm-CNX-N-14, cell surface blebs containing diffuse mEm-CNX-N-14 were analysed for ER-ID fluorescence using pixel fluorescence intensity line graphs. Five regions of interest (ROI) were drawn. ROI01 was drawn outside the cell where there was no visible fluorescence (negative control), while other ROIs were drawn inside the cell; ROI02 drawn along the major portion of the cell (positive control), ROI03, ROI04, and ROI05 are test ROIs. As seen below, although ER stress caused increased mEm-Golgi-7 fluorescence and caused its re-distribution into peculiar patterns it did not induce release of mEm-Golgi-7 from the ER into the cytosol.
Appendix Fig. V: Single z-plane transmitted light and fluorescence images obtained by confocal microscopy of DTT-treated HEK293 cells and pixel intensity line graphs. Cells treated for 5 h with 1 mM DTT were fixed and imaged at 48 h post-transfection. A. Transmitted light image. Scale bar indicates 10 µm. B. Overlay image of three colours of fluorescence: ER-ID (red), mEm-CNX-14 (green), and Hoechst 33342 (blue). The image was acquired at a pixel resolution of 1024x1024, frame averaging of 8, 100X oil immersion objective, zoom factor of 3.54, and deconvoluted using Huygens essential wizard. C. Pixel intensity line graph of the image shown in B. Image brightness was increased using Photoshop to enhance the relatively low fluorescence. Yellow arrow heads indicate the positions corresponding to the membrane. Red arrows indicate fluorescence intensity peak of mEm-CNX-14 fluorescence as compared to ER-ID fluorescence. The cells shown here are representative of > 100 HeLa cells examined in three independent experiments.