Enhanced proteasome mediated protein degradation in calreticulin deficient cells: A prosurvival mechanism

by

Anton V. Uvarov

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In Partial Fulfillment of the Requirements
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DOCTOR OF PHILOSOPHY

Department of Biochemistry and Medical Genetics
Faculty of Medicine
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THE UNIVERSITY OF MANITOBA

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Enhanced Proteasome Mediated Protein Degradation in Calreticulin Deficient Cells: A Prosurvivial Mechanism

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Anton V. Uvarov

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

Doctor of Philosophy

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ABSTRACT

Calreticulin is a lectin type chaperone essential for intracellular calcium homeostasis. Deletion of the calreticulin gene compromises the overall quality control within the endoplasmic reticulum (ER), leading to activation of the unfolded protein response. However, the structure of the ER in calreticulin deficient cells (*crt-/-*) is not altered due to accumulation of misfolded proteins and general ER stress. Previous studies demonstrated that ER stress induces cytoplasmic localization of p53 and prevents p53-dependent apoptosis by a pathway involving PERK, GSK-3β and MDM2. Interestingly, calreticulin deficient cells also have diminished cell death rates in response to DNA damage induced by UV, staurosporine and etoposide. This could be attributed to reduced p53 protein levels.

The aim of this study was to determine whether the ubiquitin-proteasome pathway is activated in *crt-/-* cells as a possible compensatory mechanism for cell survival. Here we showed a significant increase in the expression of genes involved in ER associated degradation (ERAD) and activation of the ERAD process in general (ER export, ubiquitination and degradation) upon loss of calreticulin function. We demonstrated that the ubiquitination of two proteins processed in the ER, connexin 43 and A1AT NHK (α1-antitrypsin mutant) are increased in *crt-/-* cells. Furthermore, our results showed that the increased proteasome activity in the *crt-/-* cells could be reversed upon re-introduction of calreticulin or calsequestrin (a muscle calcium binding protein). We also illustrated that increased cytosolic Ca^{2+} enhances the proteasome activity. Our data showed that *crt-/-* cells are also more resistant to cell death induced by ER stress drugs such as tunicamycin

and thapsigargin. This effect is due to decreased p53 stability, its higher degradation rates via ubiquitin-proteasome pathway and its inactivation.

Our previous and present work indicates that calreticulin deletion severely affects intracellular protein folding, triggering the unfolded protein response (UPR) and increasing the degradation capacity of the cells. Activation of the ERAD pathway and cellular proteolytic machinery affects the turnover of ER proteins as well as some cytosolic factors (e.g. p53).

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LIST OF ABBREVIATIONS

aa amino acid

A1AT NHK α1-antitrypsin genetic variant-null Hong Kong

AIP1 Ask1 interacting protein 1

ASK1 apoptosis signal-regulating kinase 1

ATF6 activating transcription factor 6

BF brefeldin

BiP Grp78, ER chaperone

bZIP basic leucine zipper

CFTR cystic fibrosis transmembrane conductance regulator

CHOP C/EBP homologous protein

CNX calnexin

COPII coat protein complex II

CREB cAMP response element binding protein

CRE cyclic AMP response element

CRT calreticulin

Crt-/- calreticulin knockout

Cnx-/- calnexin knockout

CSQ calsequestrin

Cul cullin family proteins

Cx43 connexin43

DHFR-HC major histocompatibility complex class I heavy chain fused to dihydrofolate reductase

DMEM Dulbecco's Modified Eagle's Medium

DUB deubiquitinating enzyme

EDEM ER degradation enhancing α1,2 – mannosidase like protein

eIF2 eukaryotic translation initiation factor 2

ER endoplasmic reticulum

ERAD endoplasmic reticulum associated degradation

ER ManI ER α1,2 – mannosidase I

ERO1 ER oxidoreductin 1

ERSE ER stress response element

FBS fetal bovine serum

GADD34 growth arrest and DNA damage inducible protein 34

GFP-HC major histocompatibility complex class I heavy chain fused to green fluorescent protein

GI glucosidase I

GII glucosidase II

GLS Golgi localization sequence

GPI glycophosphatidylinositol anchor

HAUSP herpes virus-associated ubiquitin-specific protease

IC ionomycin

IP3R inositol tri-phosphate receptor

IRE1 inositol-requiring protein 1

JIK c-Jun N-terminal inhibitory kinase

JNK c-Jun N-terminal kinase

Kif kifunensine

MEF mouse embryonic fibroblasts

NQO1 NAD(P)H: quinine oxidoreductase 1

OGT O-GlcNAc transferase

OST oligosaccharyl transferase

PAAF1 proteasomal ATPase-Associated Factor 1

PCR polymerase chain reaction

PDI protein disulfide isomerase

PERK protein kinase RNA-like ER kinase

PKA cAMP-dependent protein kinase

PP1 protein phosphatase 1

PTP permeability transition pore

QC quality control

S1P site 1 protease

S2P site 2 protease

SAGA complex containing three classes of proteins – SPTs, ADAs, GCN5 and histone acetyl transferase

SCF-FBS Skp1-Cullin1-F-box protein-Roc1, F-box protein for sugar recognition

SR sarcoplasmic reticulum

TG thapsigargin

TN tunicamycin

TRAF2 tumor necrosis factor receptor-associated factor 2

TRB3 tribbles-related protein 3

UCH ubiquitin C-terminal hydroxylases

Ufd1-Npl4 ubiquitin fusion degradation 1 nuclear protein localization 4

Ufd2 ubiquitin fusion degradation 2

UGGT UDP-glucose: glycoprotein glucosyltransferase

ULP ubiquitin like protein specific protease

uORF upstream open reading frame

UPR unfolded protein response

Wt wild type

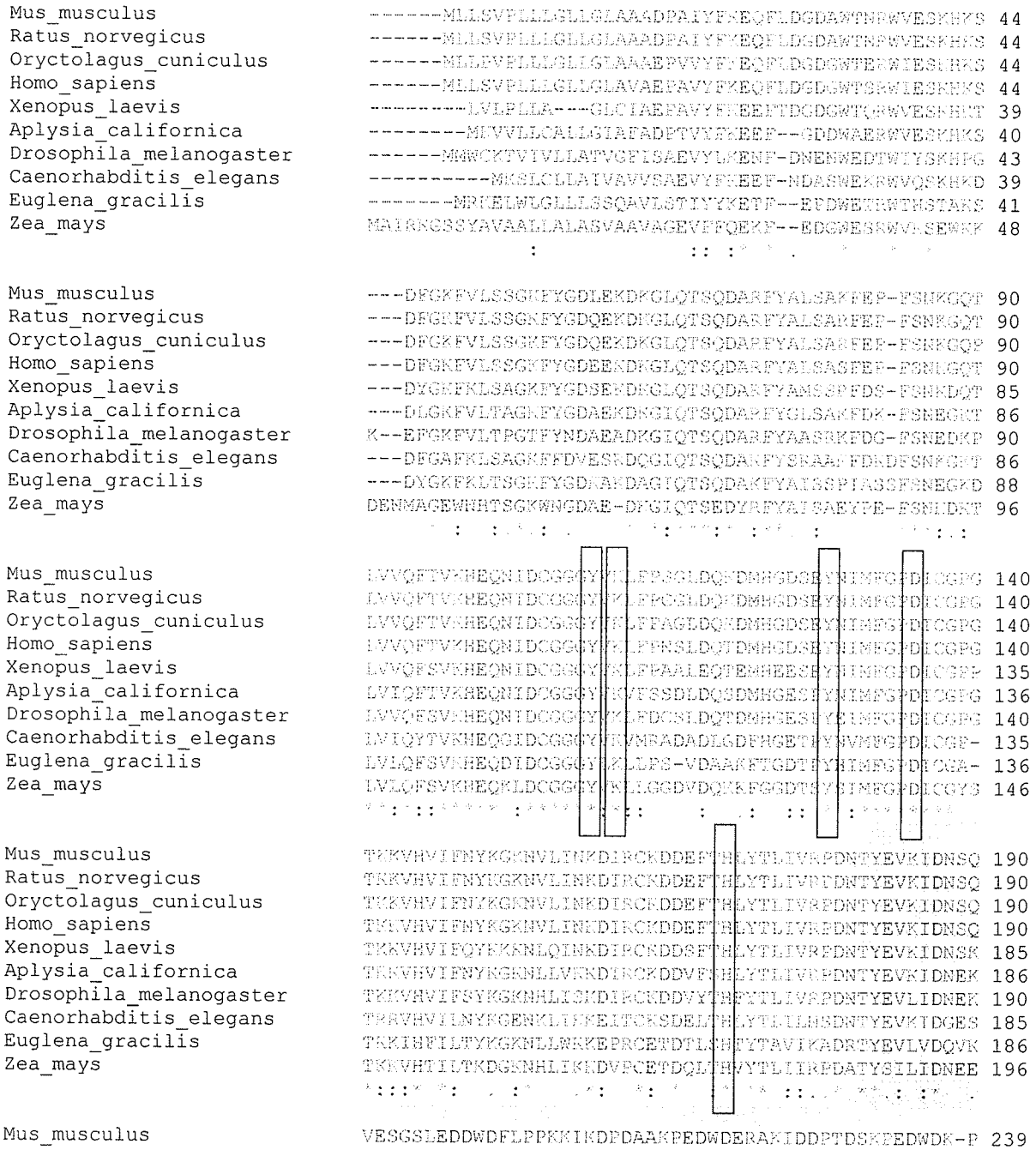
XBP1 X-box binding protein-1

A. LITERATURE REVIEW

1. CALRETICULIN – ER RESIDENT LECTIN TYPE CHAPERONE

1.1. Sequence and cellular localization

Calreticulin was originally identified as a Ca^{2+} binding protein of the muscle sarcoplasmic reticulum in 1974 (Ostwald and MacLennan, 1974). Later calreticulin was demonstrated to be the major calcium binding protein of the endoplasmic reticulum (ER) with widespread tissue distribution. It has a molecular mass of 46 kDa and is expressed in all eukaryotes except yeast and is absent in prokaryotes (Michalak et al., 1999). The amino acid sequence of calreticulin is highly homologous among different species (Figure 1). Calreticulin has been divided into three putative regions, the N-, P- and C-domains, with somewhat arbitrarily defined boundaries (Michalak et al., 1999). The N-terminal of calreticulin contains the signal sequence that is processed cotranslationally. The N-domain (*aa* 1-180) is the most conserved region of the protein among different species (Figure 1). It contains two cysteine residues (C88 and C120) that are required for disulfide bridge formation. The N-domain also contains highly conserved histidine residues which are important for Zn^{2+} binding. The region spanning *aa* 181-290 is known as P-domain. It is homologous to corresponding regions in other Ca^{2+}-binding chaperones such as calnexin (Bergeron et al., 1994) and calmegin (Watanabe et al., 1994). The P-domain of calreticulin represents a proline rich sequence with three repeats of the PXXIXDPDAXKPEDWDE sequence, followed by three repeats of the


Mus_musculus
Ratus_norvegicus
Oryctolagus_cuniculus
Homo_sapiens
Xenopus_laevis
Aplysia_californica
Drosophila_melanogaster
Caenorhabditis_elegans
Euglena_gracilis
Zea_mays

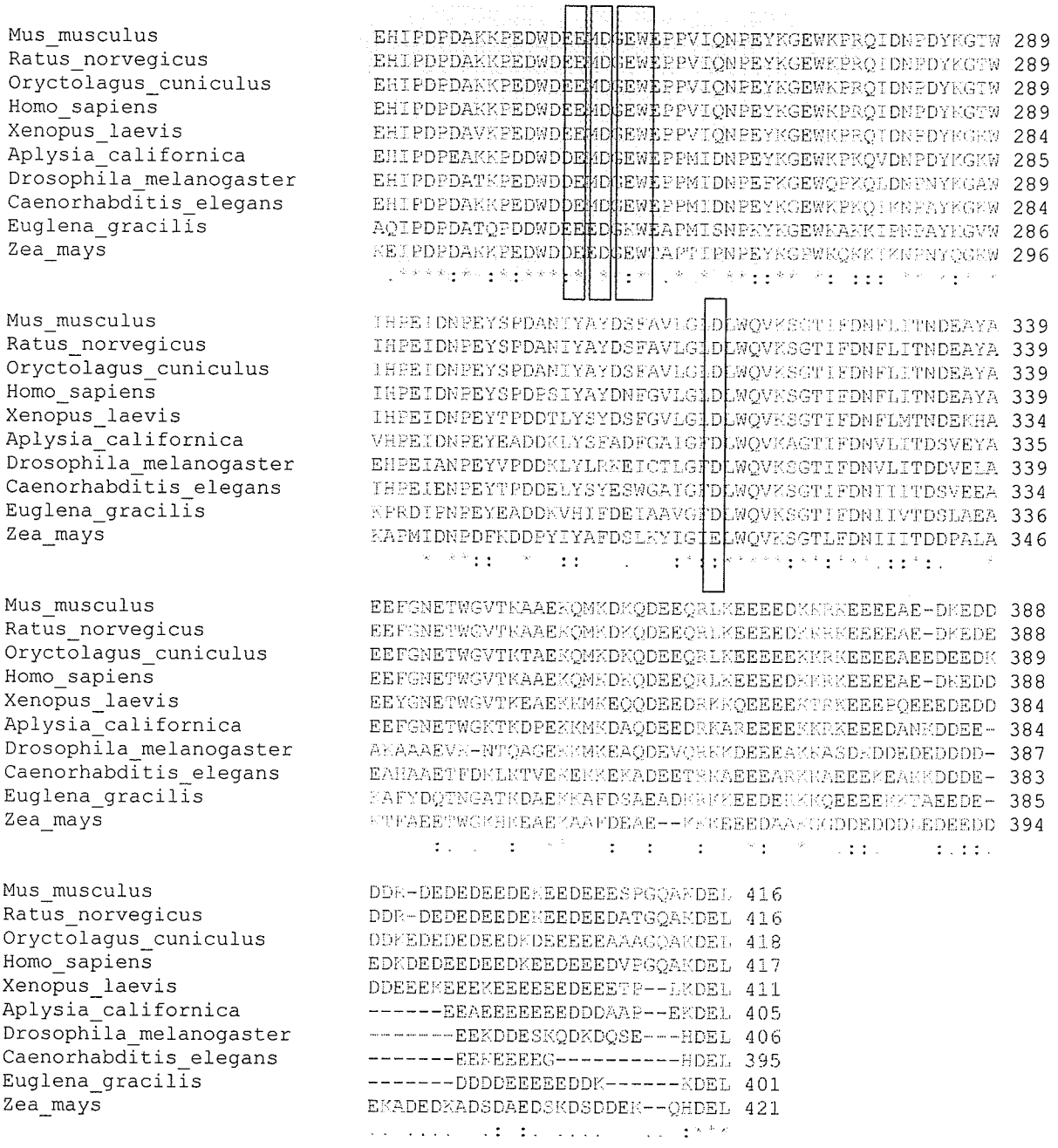


Figure 1. Multiple alignment of calreticulin aminoacid sequences from different species. Calreticulin domains are highlighted by colors: N domain – pink, P domain – green, C domain – yellow. Hydrophobic residues are highlighted in red, hydrophilic in green, negatively charged in blue, positively charged in pink. Amino acid residues described in Chapter 1.3. are highlighted in red boxes. H170, E256, D258, E260 and W261 highlighted in red boxes correspond to H153, E239, D241, E243 and W244 described in a literature review, due to the presence of N-terminal signal peptide (refer to original publication) . Alignment was created using ClustalW v.2.

GXWXPPXIXNPXYX sequence (Figure 1). It is also rich in negatively charged amino acids (*aa*) (E/D) that are essential for high affinity low capacity Ca^{2+} binding (K_d~1μM, B_{max}= 1 mol of Ca^{2+}/ mol of protein) (Baksh and Michalak, 1991). The C-terminal part of the protein (*aa* 290-416) is highly acidic and terminates with the ER retention sequence KDEL/H. The C-domain of calreticulin is homologous to the C-terminal region of calsequestrin, a Ca^{2+} -storage protein of muscle sarcoplasmic reticulum (SR) (Treves et al., 1990). Overall, the C-domain of calreticulin is designated as a high capacity, low affinity calcium binding region (K_d ~ 2mM, B_{max} > 25 mol of Ca^{2+}/mol of protein) (Baksh and Michalak, 1991).

Calreticulin is localized to the lumen of the ER and the nuclear envelope. However, recent reports suggested that calreticulin can be found at the cell surface of MEF cells (Gardai et al., 2005) or in the cytoplasm of CHO cells (Afshar et al., 2005). Despite multiple reports on cytoplasmic or cell surface function of calreticulin, the current dogma states that calreticulin acts only from within the ER. The controversy regarding calreticulin localization arose from observations showing direct interaction of purified calreticulin with α-integrin (Coppolino et al., 1995) and several steroid receptors including glucocorticoid receptor (Burns et al., 1994), androgen receptor (Dedhar et al., 1994) and vitamin D3 (Wheeler et al., 1995). Furthermore, overexpression of calreticulin was shown to suppress the activity of nuclear receptors (Burns et al., 1994). However, a subsequent study showed that overexpression of the cytoplasmic form of calreticulin could not inhibit the glucocorticoid receptor function (Michalak et al., 1996), suggesting an indirect effect of calreticulin residing in the lumen of the ER. The indirect effect could be mediated by the ability of calreticulin to regulate intracellular Ca^{2+} homeostasis and its

chaperone function. Alternatively, calreticulin could activate signalling molecules residing in the ER membrane such as IRE1 or PERK that could facilitate ER to nucleus signalling. More recently, calreticulin was shown to bind to thyroid transcription factor-1 (Perrone et al., 1999), glucose transporter-1 (Totary-Jain et al., 2005) and viral RNAs (Iakova et al., 2004) and therefore was able to modulate gene transcription or protein translation. These observations contribute to the controversy of calreticulin localization. A possible mechanism for the multiple localization of calreticulin was recently proposed by Afshar et.al (Afshar et al., 2005). This group showed that calreticulin can escape from the KDEL based ER retention system and retrotranslocate to the cytoplasm (Afshar et al., 2005). They also demonstrated that the retrotranslocation pathway was independent of endoplasmic reticulum associated degradation (ERAD) and is mediated by the function/structural feature of calreticulin C-domain (Afshar et al., 2005).

1.2. Three dimensional structure and domain organization

As discussed above, the calreticulin *aa* sequence is divided in to three putative domains. *In silico* prediction using homology modeling (ESyPred3D Web Server 1.0), suggests that the the N-domain of calreticulin is a highly folded globular structure encompassing eight anti-parallel β-strands connected by loops (Figure 2). Based on this prediction, the globular structure also includes the beginning of the C-domain (*aa* 290-341) Therefore, the original domain structure suggested by Opas et al. (Opas et al., 1991), does not exactly complement the predicted three dimensional structure.

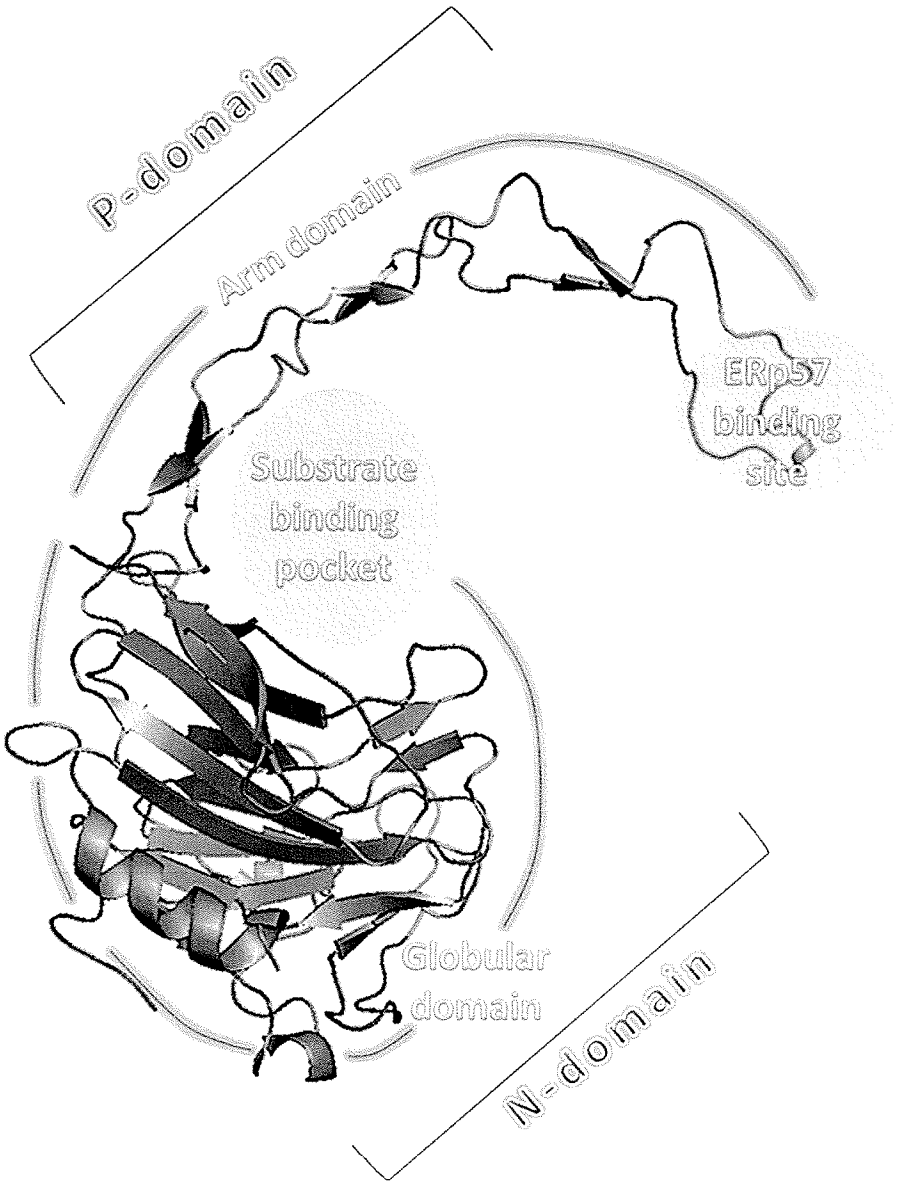


Figure 2. Calreticulin structure. Three dimensional model of the N- (globular) and P- (arm) domain of calreticulin was sketched using PyMOL v1.1 (DeLano Scientific) based on the NMR studies of the P-domain of calreticulin (Ellgaard et al., 2001) and XRD data of calnexin (Schrag et al., 2001) using homology modeling prediction (ESyPred3D Web Server 1.0).

To date, only the NMR structure of the calreticulin P-domain (*aa* 189-288) has been determined (Ellgaard et al., 2001a; Ellgaard et al., 2001b). The entire polypeptide chain of the P-domain represents an extended hairpin fold with two ends located in close proximity to each other (Figure 2). The structure is stabilized by three anti-parallel β-sheets that are formed by *aa* 189-192 and 276-279, 206-209 and 262-265, 223-226 and 248-251. Thus, consecutive β-strands are separated by 17 residues in the N-terminal part of the hairpin and by 14 residues in the C-terminal part. Several proline residues are clustered near the β-strands, preventing the formation of longer β-sheets. The hairpin loop (*aa* 227-247) and the two connecting regions between the β-sheets contain a hydrophilic cluster formed by conserved tryptophan residues (Ellgard et al., 2001a). Overall, the P-domain structure is conformationally flexible. This feature of P-domain appears to be crucial for the chaperone function of calreticulin.

The C-terminal part of calreticulin represents extended clusters of acidic residues that are flanked at regular intervals by one or more basic residues. In total, there are 37 acidic residues within the last 56 terminal *aa* of the protein (Figure 1). This region adopts an extended and flexible conformation due to strong electrostatic repulsion between negatively charged residues (Tan et al., 2006). Overall, calreticulin adopts a highly elongated structure with a globular N-domain and minimal interdomain interactions within the P-domain, allowing it to undergo local conformational changes. Structural plasticity is a key determinant of its biological role as a molecular chaperone, allowing it to transiently interact with high affinity with a large number of structurally different glycoproteins within the ER (Saito et al., 1999).

1.3. Biological functions of calreticulin

A large number of biological functions have been attributed to calreticulin including: chaperoning of the newly synthesized proteins in the ER (Trombetta and Helenius, 1998); anti-thrombotic effects at the cell surface (Kuwabara et al., 1995); regulation of intracellular Ca^{2+} homeostasis (Mery et al., 1996); modulation of gene expression (Burns et al., 1994); and regulation of cellular adhesion Coppolino et al., 1997).

As an ER chaperone calreticulin is involved in folding of glycoproteins and plays an important role in the ER quality control process (Hebert et al., 1995b; Trombetta and Helenius, 1998) establishing a calreticulin/calnexin cycle (described in details in section A.2.1.). Both of these chaperones are highly versatile and involved in folding and maturation of a variety of molecules, including ion channels, surface receptors, integrins, cell cycle proteins and transporters (Zhang et al., 1997). Calreticulin and calnexin share many substrates; however, there are some molecules that require presence of only one chaperone(Williams et al., 2006). These exceptions include influenza virus hemagglutinin (HA) that demonstrates significant folding defects when expressed in cells lacking calnexin (Molinari et al., 2004) and MHC class I molecules that are loaded with suboptimal peptides and show premature release at the surface of calreticulin deficient cells (Gao et al., 2002). It was demonstrated, that gene targeted deletion of calreticulin induces ER stress and activates UPR as evident by a significant increase in the level of BiP, calnexin, IRE1α, and PERK (Knee et al., 2003). On the other hand, Molinari etal., reported no significant change in the level of calnexin expression and only a modest decrease in the folding efficiency in *crt-/-* cells (Molinari et al., 2004). However, tryptic

analysis of microsomal protein fractions in calreticulin deficient cells further supports the accumulation of unfolded proteins in the lumen of the ER, suggesting the activation of UPR in these cells (Knee et al., 2003). Interestingly, there is no significant protein aggregation in the ER or changes in the ER structure (electron microscopic analysis) due to the presence of misfolded proteins (Uvarov and Mesaeli, 2008), indicative of efficient removal of improperly folded glycoproteins. Furthermore, it was demonstrated that in *crt-/-* cells many proteins are either malformed, e.g. bradykinin receptor (Nakamura et al., 2001) and MHC class I protein (Gao et al., 2002), or their level is significantly decreased, e.g. p53 (Mesaeli and Phillipson, 2004) and inositol tri-phosphate (IP_3) receptor (Nakamura et al., 2001).

It was also shown that as a lectin-like chaperone, calreticulin can interact with glycosylated C-terminal tail of SERCA2b and intraluminal loop of IP_3 receptor thus modulating Ca^{2+} transport and Ca^{2+} levels in the ER (Michalak et al., 1999). The luminal part of the ER/SR is known to be a major Ca^{2+} storage site with calreticulin and calsequestrin as the dominant Ca^{2+} binding proteins. Overexpression of calreticulin results in an increased amount of intracellular stored Ca^{2+} mainly due to high capacity binding in the C-terminal part of calreticulin (Mery et al., 1996). However, increased expression of calreticulin in the ER does not affect the cytoplasmic Ca^{2+} concentration (Mery et al., 1996). Interestingly, sustained overexpression of calreticulin in HEK 293 cells resulted in decreased activity of store operated channels, which correlated with an increase in total stored Ca^{2+} (Arnaudeau et al., 2002). In contrast, calreticulin deficient cells have a reduced capacity for Ca^{2+} storage in the ER, while the cytoplasmic Ca^{2+} concentration is significantly higher (Nakamura et al., 2001). Furthermore, previous

study demonstrated that bradykinin-induced calcium release from the ER is impaired in *crt-/-* cells (Mesaeli et al., 1999) which could be a result of improper Ca^{2+} handling within the ER and misfolding of bradykinin receptor in these cells (Nakamura et al., 2001).

Intracellular free Ca^{2+} regulates many signaling pathways, including those involved in cell survival, apoptosis, proliferation and differentiation (Berridge et al., 2003). For example, cardiac muscle differentiation is coordinated by GATA family of transcription factors that are activated in a calcineurin/ NF-AT dependent manner (Molkentin et al., 1998). Calcineurin is a Ca^{2+}/calmodulin dependent serine/threonine phosphatase that consists of catalytic subunit and regulatory subunit (Hemenway and Heitman, 1999). The activity of calcineurin is regulated by calmodulin binding to catalytic subunit and Ca^{2+} binding to regulatory subunit(Hemenway and Heitman, 1999). Increased cytosolic Ca^{2+} leads to calcineurin activation (Crabtree, 1999) which dephosphorylates NF-AT transcription factor, inducing its nuclear import (Crabtree, 1999). Nuclear NF-AT interacts with other transcription factors, such as GATA-4 leading to transcriptional activation of genes essential for cardiac development (Molkentin et al., 1998). Interestingly, nuclear translocation of NF-AT is compromised in calreticulin deficient cells (Mesaeli et al., 1999). Calreticulin gene knockout in mice has been shown to be embryonic lethal as a result of impaired cardiac development (Mesaeli et al., 1999); however, the phenotype could be rescued by overexpression of constitutively active calcineurin in the heart (Guo et al., 2002).

Calreticulin is also known to modulate some cellular functions outside of the ER, such as regulation of steroid sensitive gene expression (Burns et al., 1994) and cell adhesion

(Coppolino et al., 1997). Thus, calreticulin was shown to interact with the C-terminal cytoplasmic tail of α-integrin and modulate its function (Coppolino et al., 1997). Opas et al., have reported higher expression of N-cadherin and vinculin upon overexpression of calreticulin (Opas et al., 1996). In addition over-expression of calreticulin in epithelial kidney cells significantly increased cell migration (Hayashida et al., 2006). Overall, the effect of calreticulin overexpression on cell adhesion processes could be due to calreticulin effects on gene expression and/or cell signaling pathways, such as Wnt/β-catenin pathway (Michalak et al., 1999).

1.4. Essential amino acid residues

Leach et.al. (Leach et al., 2002) provided a general overview of calreticulin regions that are required for its major functions (oligosaccharide binding, native polypeptide binding and interaction with other ER chaperones). Six calreticulin deletion mutants (M1-M6) were assessed for binding to various substrates in *in vitro* experiments. The results of this study are summarized in Figure 3. This group demonstrated that the primary lectin binding site resides within the globular domain (*aa* 1-189/290-341) because deletion mutant M6 (which lacks *aa* 191-282) retained 75% of the oligosaccharide binding obtained for full length calreticulin, while the ability of M1 mutant (lacks C-domain negative tail) to bind oligosacharides was indistinguishable from the complete protein. There is a second lectin site located within the P-domain of calreticulin which has lower affinity and lacks specificity for monoglycosylated oligosaccharides (mutant M5, *aa* 191-282). Consistent with these data, a single mutation of calreticulin at *aa* Y109, K111, Y128, D135 or D317 completely abolished oligosaccharide binding, while mutation of *aa*

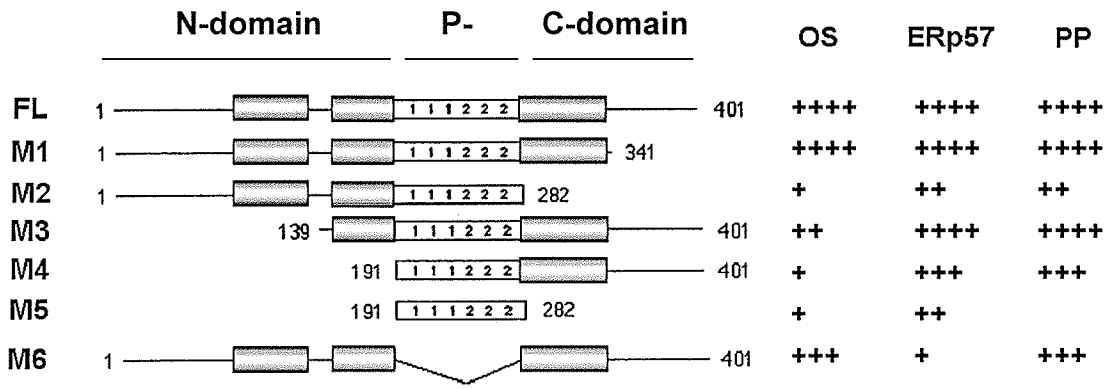


Figure 3. Determining calreticulin functional residues. Mapping of lectin, ERp57 binding and polypeptide binding sites of calreticulin using deletion mutants (adapted from Leach et al., 2002). FL – full length protein; M1 mutant – a mutant with the negatively charged tail of C–domain deleted; M2 mutant – the complete C–domain is deleted; M3 and M4 mutants – lack different portions of N-domain of calreticulin; M5 mutant – contains only P-domain; M6 mutant – P-domain is deleted. OS – relative oligosaccharide binding; ERp57 - relative ERp57 binding; PP – polypeptide binding (residual aggregation).

M131 and W319 showed only weak affinity for oligosaccharides (Kapoor et al., 2004; Thomson and Williams, 2005). Moreover, W319 (W302 in Martin et al., 2006) was also shown to be crucial for the structural organization of the carbohydrate binding pocket of calreticulin (Martin et al., 2006). In contrast, mutant M5 retained ~50% of binding to Erp57, suggesting that the calreticulin's site of interaction with ERp57 is centered within the P-domain (Figure 3). In accordance with these results, site specific mutagenesis demonstrated that residues E239, D241, E243, and W244 are essential for the formation of the ERp57 – calreticulin complex (Martin et al., 2006). It was also observed that in the presence of 100 μM Zn^{2+}, ERp57 can interact with the N-terminal portion of the calreticulin globular domain (Leach et al., 2002). Finally, the polypeptide binding site of calreticulin was localized within the globular domain of the protein (based on the differences between mutants M1/M2 and mutants M3/M4) (Figure 3).

Recently, Zn^{2+} was determined to be an important functional part of calreticulin (Guo et al., 2003). It was shown that Zn^{2+} - dependent conformational changes in calreticulin affects its ability to bind to unfolded proteins/glycoprotein substrates *in vitro*. Four histidine residues (H25, H82, H128, H153) within the N-domain are involved in the coordination of a Zn^{2+} ion (Guo et al., 2003). H153 was identified as a key determinant of substrate specificity and is found within the flexible loop that separates the globular region from the arm region of calreticulin. It is believed that Zn^{2+} binding at this site can affect the degree of flexibility of the loop structure and the shape of the substrate binding pocket (Guo et al., 2003).

Ca^{2+} and ATP are among other important modulators of calreticulin function and structure. Ca^{2+} binding to calreticulin is localized within the stretch of negatively charged

aa in the P-domain and within the negative tail of the C-domain. However, the particular residues required for Ca^{2+} are still undetermined. Interestingly, it was shown that calreticulin adopts more rigid structure upon Ca^{2+} binding whereas it is more loosely packed upon binding to Zn^{2+} (Tan et al., 2006). Moreover, removal of bound Ca^{2+} as well as reduction of the disulfide bond (disulfide bridge is formed between C88 and C120) of calreticulin significantly reduces lectin activity of this chaperone (Vassilakos et al., 1998).

2. GLYCOPROTEIN FOLDING, QUALITY CONTROL AND DEGRADATION

2.1. N-glycan processing and quality control within calreticulin/calnexin cycle

The ER is an organelle defined microscopically as an extended network of tubular membranes which is in continuity with the nuclear envelope. There are two types of ER: the rough ER to which ribosomes are attached and the smooth ER that represents a network of ribosome free tubules and is the exit site of secretory cargo vesicles. Additional functions of the ER include lipid metabolism, carbohydrate metabolism, drug detoxification and the initial steps of the protein secretory pathway (Baumann and Walz, 2001). Prior to secretion of proteins out of the ER, they are subjected to a quality control process. Protein folding is assessed by ER resident chaperones and if conformation is correct, the newly synthesized proteins exit the ER via COPII (coat protein complex II) transport vesicles (Vashist et al., 2001). Proteins that are not folded correctly are either retained in the ER for additional cycles of the quality control process or subjected to

proteolysis through the ERAD pathway (Meusser et al., 2005). Alternatively the unfolded proteins form aggregates inside the lumen of ER (Trombetta and Parodi, 2003).

The ER lumen is topologically equivalent to the extracellular space and therefore its environment is different from the cytosol (Sitia and Braakman, 2003). It has higher Ca^{2+} concentrations, higher protein concentrations (>100 mg/ml), a more oxidizing milieu and a specialized set of molecular chaperones and enzymes (Hwang et al., 1992). Moreover, there are protein modifications that take place only inside this compartment (signal peptide cleavage, disulfide bond formation, N-linked glycosylation and addition of glycophosphatidylinositol (GPI) anchors) (Schroder and Kaufman, 2005). This special environment and specific modifications are required for correct protein folding and quality control. The significance of the quality control (QC) system can be outlined by the following reasons: a) by preventing the premature exit of improperly folded proteins from the ER, it extends the exposure of those molecules to chaperone machinery and promotes them to adopt their correct conformation; b) the QC system ensures that misfolded proteins are not delivered to their terminal compartments (eg. plasma membrane) where they could alter cell function; c) correctly folded proteins exit QC and are transported outside of the ER lumen. The key structural features that are recognized by molecular chaperones are exposed hydrophobic regions, unpaired cysteine residues, specific carbohydrates and aggregates (Ellgaard and Helenius, 2003). Importantly, substrate recognition within the QC system is heavily dependent on structural fluctuations and the magnitude of the free energy barrier between native and non-native protein conformations. The lower the overall stability of a protein, the more frequently it exposes chaperone-binding sites (Kowalski et al., 1998a; Kowalski et al., 1998b).

The calnexin/calreticulin cycle is the major quality control system responsible for correcting the folding of almost all of the glycoproteins that are synthesized in the ER (Ellgaard et al., 1999; Molinari et al., 2004). As soon as the nascent protein enters the ER, specific asparagine residues within N-X-S(T) sequences are recognized by oligosaccharyl transferase (OST) and glycosylated with the pre-assembled $Glc_3Man_9GlcNAc_2$ moiety (Figure 4) (Wieland et al., 1987). The next step includes sequential cleavage of the outer α1,2-linked glucose residue by glucosidase I (GI) (Hettkamp et al., 1984) and of the first α1,3-linked glucose by glucosidase II (GII) (Burns and Touster, 1982). These modifications are accomplished co-translationally in a matter of minutes (Helenius and Aebi, 2004). Both calnexin and calreticulin are lectins that interact with the resulting monoglucosylated $Glc_1Man_9GlcNAc_2$ oligosaccharide of newly synthesized glycoproteins (Ou et al., 1995; Spiro et al., 1996). Calreticulin and calnexin are also known to interact with the thiol-disulfide oxidoreductase ERp57 via their P-domain (Figure 4). ERp57 was shown to form transient interchain disulphide bonds with calnexin- and calreticulin-bound glycoproteins (Oliver et al., 1997). Sequestration of glycoproteins within the calnexin/calreticulin cycle promotes correct folding (Hebert et al., 1996). It is believed that a folding glycoprotein is locked between the arm domain and the globular domain of calreticulin, thus reducing its tendency to aggregate (Williams, 2006). This interaction also brings glycoproteins in proximity to ERp57, which is an important cofactor that assists in correct disulfide bond formation during the ongoing protein folding process (Kang and Cresswell, 2002).

Other ER chaperones and folding enzymes, such as BiP and PDI, are also known to participate in the calreticulin/calnexin cycle and to assist in the folding of glycoproteins

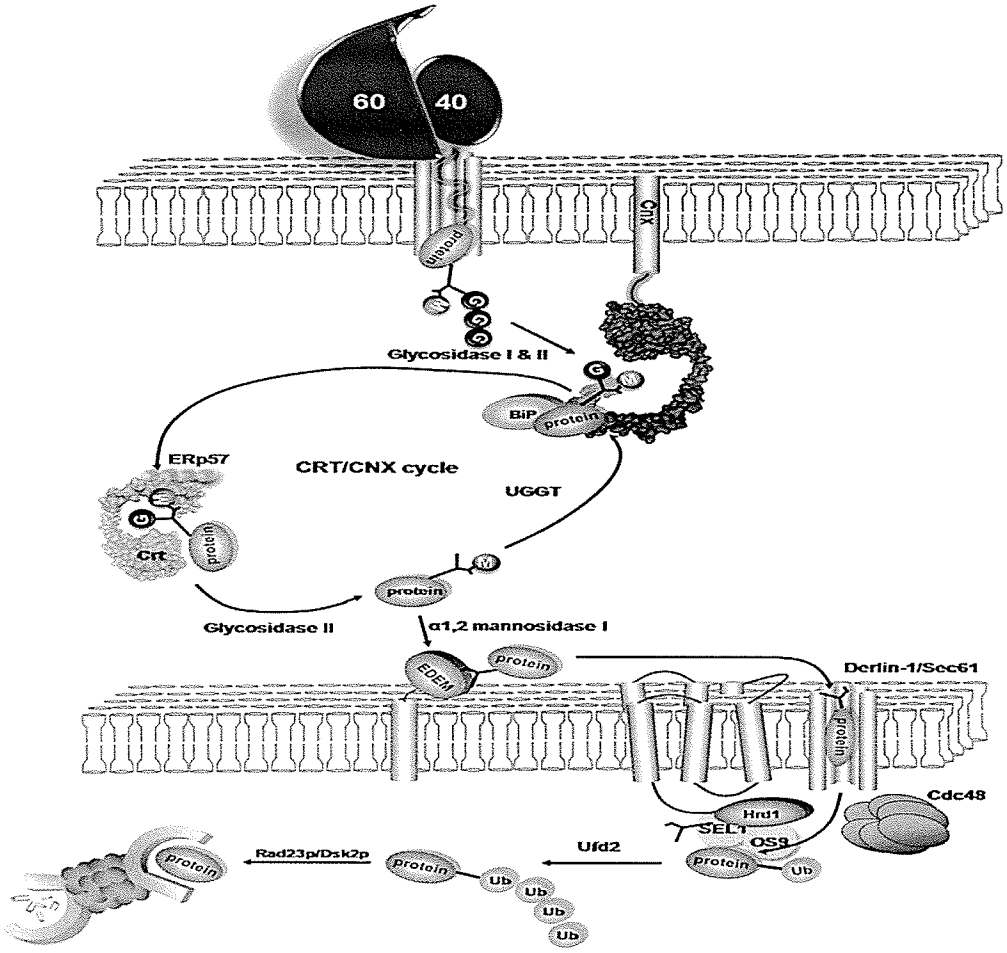


Figure 4. Glycoprotein quality control cycle coupled with endoplasmic reticulum associated degradation. A schematic diagram showing essential steps of protein co-translational synthesis, glycosylation, folding, ER export, ubiquitination and proteasomal degradation. Prior to entry of the newly synthesized glycoprotein into the quality control pathway, two terminal glucose residues are removed by glucosidases I and II, generating a glycoprotein that can enter the calreticulin/calnexin cycle. Cleavage of the remaining glucose of the maturing glycoprotein by glucosidase II allows the protein to exit the chaperone cycle. Incompletely folded proteins become substrates for UDP-glucose: glycoprotein glucosyltransferase (UGGT), while perminantly misfolded proteins are demannosilated by α1,2 – mannosidase I and recognized by EDEM protein. EDEM targets glycoproteins for ER associated degradation. ER to cytosol translocation is completed through the Sec61 channel with the help of the Cdc48 protein. After translocation to the cytosol, misfolded proteins are ubiquitinated by ER associated ubiquitin ligases, such as Hrd1 and Ufd2 and conveyed to the proteasome by accessory factors (e.g. Rad23 and Dsk2).

(Zhang et al., 1997) (Figure 4). Interestingly, glycosylation itself has a direct effect on folding. It is known that glycan addition rigidifies the protein structure by limiting the conformational space that is accessible to the polypeptide chain (Imperiali and O'Connor, 1999). Other effects of glycosylation include: formation of additional β-turns through the interaction between the N-acetyl group of the first GlcNAc residue and the polypeptide chain itself, as well as an increase in the solubility of folding intermediates (O'Connor and Imperiali, 1996). To exit the chaperone cycle, the glycoprotein is deglucosylated by glucosidase II (Figure 4). At this step, correctly folded glycoproteins can exit the ER (Hebert et al., 1995a). In contrast, incompletely folded proteins become substrates for UDP-glucose: glycoprotein glucosyltransferase (UGGT) which places a glucose moiety back onto the non-native glycoprotein and therefore promotes a new cycle of association with calnexin and calreticulin (Figure 4). These cycles of glucosylation/deglucosylation are repeated until the molecule is correctly folded or until it is targeted for degradation (Wu et al., 2003). However, deletion of UGGT does not lead to diminished quality control, indicating that this enzyme is not the primary factor that retains non-native proteins within the ER (Solda et al., 2007). UGGT functions as a molecular mechanism that delays the re-direction of misfolded polypeptides from the calnexin/calreticulin cycle to the ERAD pathway (Molinari et al., 2005). The selectivity of UGGT towards incompletely folded proteins must be based on general features common for misfolded proteins, such as exposed hydrophobic peptides or patches, excessive surface dynamics and other small local folding defects. *In vitro* studies have shown that partially ordered, molten globule-like structures appeared to be optimal for efficient recognition by UGGT (Caramelo et al., 2003). At the same time, the enzyme ignores glycoproteins in native and

random-coil conformations, as well as short glycopeptides and isolated glycans (Trombetta and Helenius, 2000).

2.2. Endoplasmic reticulum associated degradation

2.2.1. Substrate recognition

Proteins that are permanently misfolded are targeted for degradation by ERAD. The important switch/mechanism that shifts improperly folded substrates from the calreticulin/calnexin cycle to translocation and subsequent degradation is the process of mannose trimming (Wu et al., 2003). The terminal mannose residues on misfolded glycoproteins are trimmed by ER α1,2 – mannosidase I, giving rise to a specific $Man_8GlcNAc_2$ isomer (Herscovics, 2001) (Figure 4). Overexpression of ER ManI also stimulates trimming of additional mannose residues producing $Man_7GlcNAc_2$, $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$ (Hosokawa et al., 2003). A previous report showed that reglucosylation of a substrate by UGGT is less efficient with demannosylated proteins, thus preventing them from entry into additional folding cycles (Sousa et al., 1992). De-mannosylated glycoproteins are recognised by the ER degradation enhancing α1,2 – mannosidase like protein (EDEM) (Hosokawa et al., 2001) (Figure 4). Interestingly, overexpression of EDEM enhances ERAD, whereas inhibition of α1,2 – mannosidase I by kifunensine significantly downregulates ERAD (Hosokawa et al., 2003). It is still not completely known whether EDEM and its homologues (EDEM2, EDEM3) are just intermediate acceptors of misfolded glycoproteins prior to their translocation from the ER

or whether they are active α1,2 – mannosidases or molecular chaperones. Two recent studies demonstrated that intralumenal upregulation of EDEM and EDEM3 leads to increased mannose removal from folding-defective polypeptides (Hirao et al., 2006; Olivari et al., 2006). Furthermore, EDEM prevents disulfide bond mediated dimer formation (Hosokawa et al., 2006) and inhibits covalent aggregation of misfolded polypeptides (Olivari et al., 2006), thus functioning as a molecular chaperone. Overall, EDEM accelerates the degradation of misfolded substrates that are diverted from the QC process.

In contrast to glycoproteins that are processed by the calreticulin/calnexin cycle, misfolded, non-glycosylated ER proteins are recognised by BiP (luminal homolog of Hsp70) and subsequently by PDI (protein disulfide isomerase) or Eps1p, a membrane-anchored member of the PDI family which is expressed only in yeast (Wang and Chang, 2003). Interaction with PDI is a prerequisite for ER export of all substrates (even without disulfide bridges) (Gillece et al., 1999). The general function of BiP is to bind to the exposed hydrophobic regions of the folding intermediates and misfolded proteins, preventing their aggregation (Kabani et al., 2003). Moreover, the rate of proteolysis of ERAD substrates is correlated with the rate of their dissociation from BiP (Chillaron and Haas, 2000).

2.2.2. Translocation from the ER to the cytosol

Two main prerequisites for successful export of improperly folded proteins out of the ER are protein solubility and pre-/co-translocational unfolding. Solubility of

glycoproteins is partially achieved by glycosylation (Marquardt and Helenius, 1992). BiP activity, as well as J-domain containing proteins Jem1p and Scj1p (in yeast only) are also necessary to keep ERAD substrates in a soluble form (Kabani et al., 2003; Nishikawa et al., 2001). Furthermore, in order to be translocated across the ER membranes to the cytosol, proteins need to be dissociated from luminal chaperones and any other oligomeric complexes (Nishikawa et al., 2001), and the disulfide bonds need to be reduced (Fagioli et al., 2001). It is still not known whether the protein should be unfolded prior to translocation across the ER membrane. For example, cholera toxin was shown to be unfolded by PDI prior to translocation out of the ER (Tsai et al., 2001). In contrast, dislocation of bulky chimeric proteins, such as GFP-HC/DHFR-HC (major histocompatibility complex class I heavy chain fused to green fluorescent protein or dihydrofolate reductase) was shown to take place without unfolding, since both GFP and DHFR retained their native functions and properties (Fiebiger et al., 2002; Tirosh et al., 2003). These results suggest that the diameter of the translocon should be in the range of 40-60 Å in order to allow the passage of such large molecules, which is in accordance with the previously estimated size of the ER translocon (Hamman et al., 1997).

The ER export channel is still not fully characterised. Sec61, a protein-conducting channel that normally mediates protein import into the ER was suggested to function in reverse mode once it is associated with a different set of cofactors and substrate acceptors (Pilon et al., 1997) (Figure 4). Interestingly, it was demonstrated that the function of Sec61 as an import channel is reduced if there is a high demand for ER protein export following overexpression of a misfolded protein (Ng et al., 2000). It is also speculated that the protein translocation channel has two modes of action: it has to open laterally in

order to accommodate transmembrane proteins, and transversely to facilitate passage of soluble proteins. Another protein suggested to be a part of a translocation pore is the mammalian protein Derlin1 (Lilley and Ploegh, 2004) (Figure 4). A complex involving Derlin1 and Cdc48 was shown to be involved in the retrotranslocation and ERAD of the misfolded cystic fibrosis transmembrane conductance regulator (CFTR) (Sun et al., 2006). Other proteins that could possibly form an export channel include Hrd1 and TEB4 (Kreft et al., 2006; Plemper et al., 1999). Both of these proteins contain several transmembrane regions that could form a putative pore.

There are two protein factors that could serve as a driving force for ER protein translocation and may determine the direction of the process. These are Cdc48/p97 protein (Ye et al., 2001) and the 19S regulatory particle of the proteasome (Mayer et al., 1998). Cdc48 is a member of the AAA-ATPase family of chaperones and is involved in a number of diverse cellular pathways through interactions with different adaptor proteins (Figure 4). Two well characterized adaptors for Cdc48 are p47 and the Ufd1-Npl4 (ubiquitin fusion degradation 1 nuclear protein localization 4) complex (Meyer et al., 2002). Binding of these cofactors to the N-terminal domain of Cdc48p was shown to be mutually exclusive (Meyer et al., 2000). Interaction with p47 shifts Cdc48 function towards regulation of the post-mitotic fusion of Golgi membranes (Uchiyama et al., 2002). In contrast, the Ufd1-Npl4-Cdc48 complex binds ubiquitinated proteins and is important for the ERAD process (Meyer et al., 2002). The p97-Ufd1-Npl4 complex has been suggested to have a dual function. This complex can act as a dislocase that extracts proteins from the ER translocation channel in an ATP dependent manner (Ye et al., 2003). Indeed, the Ufd1-Npl4-Cdc48 complex was recently found in association with ER

membranes through interactions with Derlin1, ubiquitin ligase gp78 and the recently discovered VIMP protein (Ye et al., 2004). The Ufd1-Npl4-Cdc48 complex can also function as a segregase, acting downstream of the dislocation process (Braun et al., 2002). In this mode, Ufd1-Npl4-Cdc48 recognizes and binds to ubiquitinated proteins that have already crossed the ER membrane, thus separating them from associated non-ubiquitinated proteins and making them available for proteasomal degradation (Romisch, 2005).

Another important function of Cdc48 is to recruit the proteasome to the vicinity of the translocation channel and thus couple the processes of extraction from the ER and degradation (Braun et al., 2002). It was also demonstrated that the 19S regulatory particle of the proteasomal complex can function as an exclusive cytosolic factor required for the ER export process (Lee et al., 2004). Kalies et al. were able to co-precipitate subunits of the Sec61 complex with ER associated proteasome 19S particles (Kalies et al., 2005). Moreover, they demonstrated that ribosomes and proteasomes compete for binding to the ER membrane and have similar affinities for their receptor.

2.2.3. Ubiquitination and targeting to proteasomes

Ubiquitination of ERAD substrates is an important factor for their ER export. However, there are some differences in the sequence of dislocation/ubiquitination events for luminal and transmembrane proteins (the detailed mechanism of ubiquitination and proteasomal degradation will be described in section 2.3). Soluble ER luminal substrates are first transported in an ubiquitin-independent manner until their lysine residues are

exposed on the cytoplasmic side of the ER membrane and recognised by ubiquitin ligases (Romisch, 2005). Next, these proteins become ubiquitinated, allowing their association with cytoplasmic ubiquitin-binding factors that will escort them towards proteasomal complexes (Figure 4). In contrast, transmembrane ERAD substrates with cytoplasmic lysine residues are first ubiquitinated on their cytoplasmic tails, stimulating their interaction with ubiquitin-binding factors and thus promoting their dislocation from the ER membrane and translocation out of the ER lumen (Romisch, 2005).

Mammalian cells utilize several ubiquitin ligases (E3) in the process of ERAD including the ER resident ligases RMA1, gp78 (Morito et al., 2008), Hrd1 (Bays et al., 2001), TEB4 (Ravid et al., 2006), Trc8 (Kostova et al., 2007) and the non-ER resident ligases such as CHIP (Imai et al., 2005) and Parkin (Takahashi et al., 2003). The majority of ER bound ubiquitin ligases are known to associate with several cofactors. For example, SEL1 and OS9 interact with Hrd1 (Neuber et al., 2005) (Christianson et al., 2008) and ERASIN interacts with gp78 (Liang et al., 2006). These complexes, in association with the ubiquitin conjugating enzymes (E2) such as Ubc7p and Ubc6p /Cue1p (both yeast), carry out the ubiquitination of ERAD substrates (Biederer et al., 1997). Each E3 enzyme is specific for a particular set of substrates: luminal or transmembrane type, glycosylated or non-glycosylated. The SCF-FBS1 and SCF-FBS2 (SCF, Skp1-Cullin1-F-box protein-Roc1 and FBS, F-box protein for sugar recognition) represent two recently discovered ubiquitin ligase complexes that bind specifically to N-glycosylated proteins and contribute to their elimination via ERAD in the cytosol (Yoshida et al., 2002). The FBS1 and FBS2 components of these complexes recognize

mannose-terminated N-glycans and distinguish native from unfolded glycoproteins by sensing the exposed chitobiose structure (Yoshida et al., 2003).

E3 ubiquitin ligases mediate the initial oligoubiquitination of ERAD substrates while polyubiquitination, a process that is fundamental for proteasomal degradation, is catalyzed by E4 enzymes (Kostova et al., 2007). A recent study demonstrated that Cdc48 binds to both oligoubiquitinated substrates and Ufd2 (ubiquitin fusion degradation 2, Figure 4) (Richly et al., 2005). This interaction triggers the E4 activity of Ufd2 and subsequently 3-6 ubiquitin moieties are added to the substrate. The ubiquitin chain is then recognized by the Rad23 receptor protein (Figure 4), accompanied by binding to the Ufd2 enzyme (Richly et al., 2005). Rad23 has also been shown to interact with Png1, a cytoplasmic enzyme that is required for removing the N-linked glycans from the polyubiquitinated substrates (Kim et al., 2006). Deglycosylation is required prior to substrate-Rad23 interaction (Suzuki et al., 2002). Oligosaccharide chains are known to stabilize protein structure and therefore unfolding of specific domains by the 19S proteasome subunit may be hindered without initial deglycosylation (Suzuki et al., 2002). Finally, the Rad23-Png1-substrate complex dissociates from the Ufd2 enzyme and shuttles the polyubiquitinated proteins towards the 26S proteasome. This process is mediated through the interaction between Rad23 and the Rpn1 subunit of the 19S particle (Suzuki et al., 2001). The ubiquitin chains are recognised by Rpn10 and positioned for de-ubiquitination and subsequent entry into the proteolytic channel by the Rpt5 subunit (Verma et al., 2004).

2.3. Ubiquitin – proteasome system

The ubiquitin proteasome pathway (Figure 5) is required for the targeted degradation of a variety of proteins to facilitate their renewal as well as proteins that are unable to fold properly in the ER. This pathway is conserved from yeast to mammals (Groll and Clausen, 2003). The first part of the pathway is represented by the multi-step process of ubiquitination (ubiquitin is a highly conserved 76 amino acid protein) (reviewed in (Kerscher et al., 2006)). Ubiquitination is an ATP-dependent process carried out by three classes of enzymes in three sequential steps. At first, the ubiquitin activating enzyme (E1) forms a thio-ester bond with ubiquitin. Activated ubiquitin is then transferred to the ubiquitin conjugating enzyme (E2). At the last step, the E3 ubiquitin ligase covalently binds ubiquitin to a lysine residue of the target protein. There is a great diversity of E3 enzymes. They can be an individual protein or a multi-subunit complex. Cullin family proteins (Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5) have been recently shown to comprise the core module of such multi-subunit ensembles (Chew et al., 2007). Each E3 enzyme possesses specificity towards a particular target protein or a subset of proteins (Kerscher et al., 2006). The specificity of E3 ligases is based on conformational recognition of the substrate, post-translational modification of the substrate protein, or through the binding of specific cofactors to the substrate. In addition, it was demonstrated that polyubiquitination (addition of more than one ubiquitin moiety) could require the additional activity of certain ubiquitin-chain elongation factors also known as E4 enzymes (Koegl et al., 1999). These factors bind to oligoubiquitinated (containing a single ubiquitin residue) substrates and catalyse polyubiquitin-chain assembly in

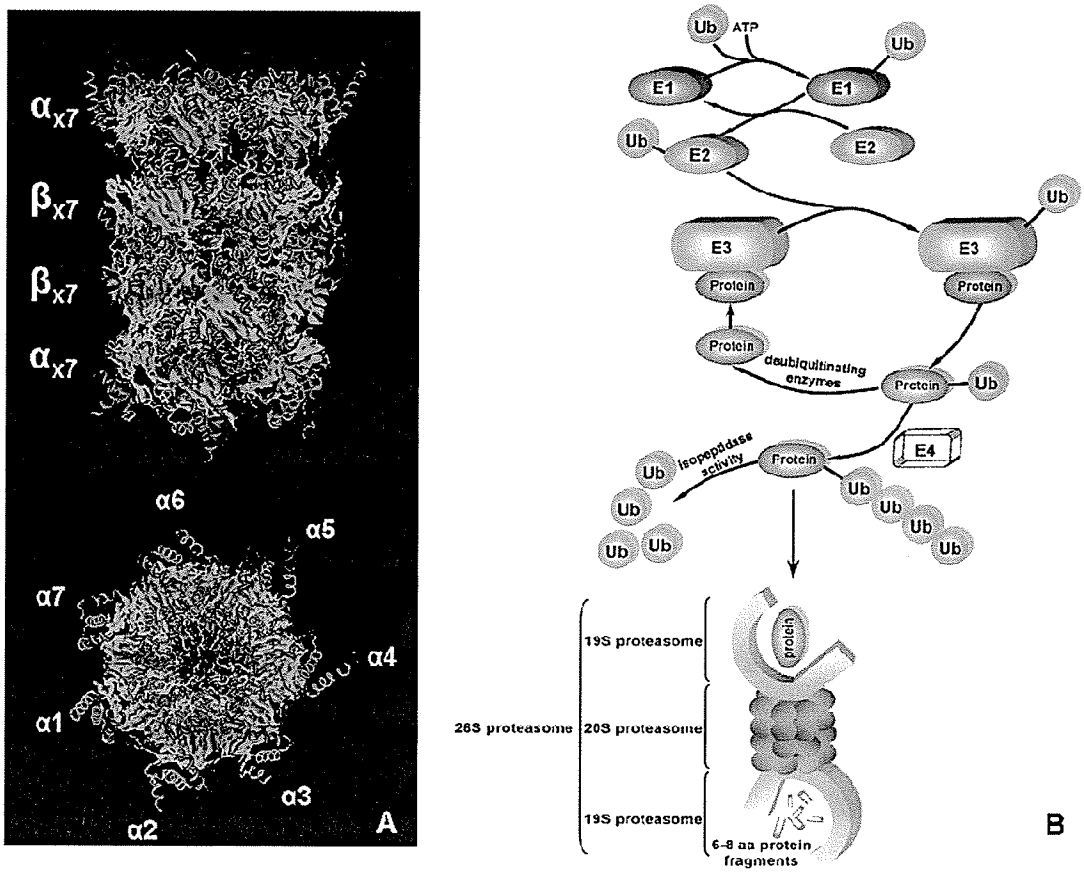


Figure 5. Ubiquitin – proteasome pathway. (A) Three dimensional structure of the 20S proteasome with secondary structure color alignment (α helices are in pink, β strands are in green). **(B)** A schematic illustration of the ubiquitin– proteasome system, showing the cascade of ubiquitin ligases (E1, E2, E3 and E4) and 26S proteasome. Protein degradation within this pathway begins with the ATP-dependent activation of ubiquitin by E1, followed by transfer of the ubiquitin to an E2, and finally attachment of the ubiquitin to a lysine residue in the target protein with the help of an E3 ligase. Once the ubiquitin-protein conjugate is formed, additional ubiquitin molecules are added to this potein, creating a polyubiquitin chain. Polyubiquitinated proteins are either recognized by the 26S proteasome and degraded in an ATP-dependent process, or they are disassembled by deubiquitinating enzymes to regenerate both the target protein and ubiquitin.

collaboration with E1, E2 and E3 enzymes. Ufd2 was recently discovered as an E4 enzyme participating in the process of ERAD (Tu et al., 2007). Polyubiquitinated proteins are recognized by the 19S subunits and unfolded into the 20S proteasome core particle for degradation. The recognition of polyubiquitinated substrates can be facilitated by polyubiquitin chain receptor proteins, such as Rad23 (Saeki et al., 2002). Alternatively, ubiquitin groups can be removed from the protein substrate by deubiquitinating enzymes (DUBs) or ubiquitin like protein -specific proteases (ULPs), thus stabilizing the target protein (Kerscher et al., 2006). Li et.al. identified herpesvirus-associated ubiquitin-specific protease (HAUSP) as a novel p53-interacting protein (Li et al., 2002). The same group demonstrated that HAUPS binds p53 and stabilizes it by direct de-ubiquitination, implying that HAUSP can function as a tumor suppressor (Li et al., 2002).

The proteasome (usually refered to as the 26S proteasome) is a 2.5 MDa complex consisting of approximately 32 different subunits and is found in both the nucleus and cytoplasm (Voges et al., 1999). As mentioned above, it is subdivided in two subcomplexes – the 670 kDa proteolytic core particle (20S) and the 900 kDa regulatory particle (19S). The 19S subunit is responsible for recognition, unfolding and translocation of ubiquitinated targets into the inner part of the complex for degradation. Topologically, the 20S core is arranged as four axially stacked heptameric rings (α1-7, β1-7, β1-7, α1-7), with multiple catalytic centers located within the hollow cavity of the cylinder (Figure 5) (Groll and Clausen, 2003). Substrates access the catalytic chamber through axial ports in the end rings of α subunits, although in the absence of activators, these channels are closed and the proteasome activity is repressed (Groll and Clausen, 2003). The

proteolytic activity of the complex is mainly localized to the inner β-rings (Heinemeyer et al., 2004). The eukaryotic proteasome posseses three distinct catalytic activities: a chymotrypsin-like activity with preference for tyrosine or phenylalanine, a trypsin-like activity with preference for arginine or lysine and a post-glutamyl hydrolyzing activity with preference for glutamate or aspartate (Heinemeyer et al., 2004). Peptide bond hydrolysis employs a core threonine residue of β-subunit as the catalytic nucleophile. Passage of the unfolded substrate through the proteolytic tunnel leads to the generation of short protein fragments (6-8 amino acid residues). The 20S core exists in at least two distinct forms (constitutive and immuno-) that differ in the composition of their catalytic subunits located on the β rings (Voges et al., 1999). Generally, the 26S proteasome is activated through the binding of the 19S (PA700) subcomplex to the end rings of the α subunits. Each 19S complex contains a number of ATPases and utilizes a large amount of ATP for its function (Rubin et al., 1998). PA28 and PA200 are other well described proteasome activators that are required for the hydrolysis of short peptides (Schmidt et al., 2005). Unlike PA700, PA28 and PA200 neither need ATP nor bind polyubiquitin chains, but primarily assist the proteasome in ubiquitin-independent proteolysis (Schmidt et al., 2005). PA28 and P200 bind to the outer rings of the 20S complex and enhance proteasome activity by relieving the occlusions at the proteasome pores (Glickman and Raveh, 2005). Endogenous proteasome inhibitors are less characterized and may require the presence of activators in order to affect proteasome activity. PAAF1 (Proteasomal ATPase-Associated Factor 1) is a proteasome inhibitor, functioning at the level of assembly/disassembly of the 26S complex (Park et al., 2005). Two proline rich proteins, PI31 and Pr39 were also demonstrated to repress the 20S function through blocking the

chamber cavity and preventing the binding of activators to the complex (Gaczynska et al., 2003).

The diversity of proteasomal complexes is further increased due to the presence of "hybrid proteasome complexes" (Dahlmann, 2005). Two different regulators can bind to opposite outer α rings of the 20S core, producing a structure with a different catalytic property (19S regulator-20S proteasome-PA28). Hybrid proteasomes may have a special function during the immune response. This process is likely to be highly regulated, although the mechanistic basis of it is unknown.

Overall, the protein degradation capacity inside the cell can be regulated by the number of proteasome complexes, the activity of the proteasome regulatory proteins, and the ubiquitin conjugating enzymes (Bajorek and Glickman, 2004; Schmidt et al., 2005). Recent reports suggested that ER stress (Hong et al., 2004a), or changes in intracellular concentrations of divalent cations (Menconi et al., 2004) can activate the ubiquitin-proteasome pathway. Thus, *in vitro* studies with purified 20S proteasomes demonstrated that addition of Ca^{2+} to the proteasome fractions increases the chymotrypsin-like activity of the 20S complexes (Reshetnyak et al., 2004). Ca^{2+} binding to the proteasome induces conformational changes in a cluster of highly conserved proteasome residues within the α subunits (α6 Y6, α7 D8, α6 P15, α7 Y25). These structural alterations promote a more open conformation and facilitate substrate access into the proteolytic channel, thus enhancing proteolysis. In addition to its effect on the catalytic function of proteasome, Ca^{2+} was shown to modulate the activity of a proteasome activator REG (Realini and Rechsteiner, 1996). The lysine/glutamic acid rich region of the 29-kDa subunit of human REG proteasomal activator binds Ca^{2+}. Ca^{2+}, in turn, was shown to reversibly inhibit the

enhanced peptidase activity of the complex between the proteasome and recombinant REG.

Proteasome activity can also be regulated by metabolic factors. Zhang et al. have shown that the 26S proteasome can be inhibited by the O-GlcNAc transferase (OGT), coupling proteasome function to glucose metabolism (Zhang et al., 2003). The Rpt2 ATPase of the mammalian 19S complex is modified with O-GlcNAc both *in vitro* and *in vivo*. This reversible modification was shown to inhibit the proteolysis of the transcription factor Sp1 and synthetic peptide substrates (Zhang et al., 2003). This study suggested that the O-GlcNAc modification of the proteasome allows the organism to adjust its metabolic needs by controlling the availability of amino acids and regulatory proteins. Recently, Zhang et.al. described another mechanism of metabolic control of proteasome activity, mediated by cAMP-dependent protein kinase (PKA) (Zhang et al., 2007). This study demonstrated that PKA can directly stimulate the chymotrypsin-like and trypsin-like activities of the 26S proteasome through phosphorylation of the Rpt6 ATPase subunit of the 19S complex. An increased level of cAMP, as seen during starvation, could increase proteasome activity, thus enhancing protein degradation to provide the glycogenic amino acids that could be used for gluconeogenesis. Altered proteasomal activity has also been shown to play a role in different pathophysiological conditions such as cancer (Bazzaro et al., 2006), AIDS (Crinelli et al., 1999; Crinelli et al., 1997), sepsis (Hobler et al., 1999), and skeletal muscle atrophy - cachexia (Mitch and Goldberg, 1996).

The proteasome also possesses some non-proteolytic functions during the processes of DNA transcription (Collins and Tansey, 2006), nucleotide excision DNA repair (Reed

and Gillette, 2007) and chromatin remodeling (Collins and Tansey, 2006). It was recently demonstrated that some 19S (PA700) subcomplexes are localized to gene promoters and serve as coactivators/ATPases for the SAGA (named for three classes of proteins it contains – SPTs, ADAs, GCN5 and histone acetyltransferase) transcription adaptor complex (Lee et al., 2005c). There is also genetic and biochemical evidence suggesting a role for the proteasome in the transcriptional elongation process, although the exact mechanism is still unknown (Ferdous et al., 2001). In addition, the 19S subunit was shown to be involved in epigenetic gene regulation in *Saccharomyces cerevisiae*. Methylation of histone H3 at active genes is an epigenetic mark that distinguishes active from silent chromatin (Bryk et al., 2002). Interestingly, methylation of H3 at lysine residues K4 and K79 depends on ubiquitination of histone H2B (Sun and Allis, 2002). Ezhkova et al. demonstrated that the proteasomal ATPase subunits Rpt4 and Rpt6 function to connect these two histone modifications. They reported that recruitment of Rpt4 and Rpt6 to chromatin is signaled by ubiquitination of histone H2B and also demonstrated that mutations in these ATPases specifically block the downstream methylation of histone H3 (Ezhkova and Tansey, 2004).

Additional functions have also been attributed to the ubiquitin protein. In order to efficiently target a protein for degradation, multiple ubiquitins should be linked together through K48 into a specific polyubiquitin chain (Pickart, 1997). In contrast, monoubiquitination is often utilized by the protein trafficking machinery (Gregory et al., 2003). Ubiquitination can also have an effect on transcriptional activity. Indeed, yeast transcription factor Met4 is ubiquitinated by SCF (Skip1/Cullin/F-box) ligase, leading to its inactivation (Kuras et al., 2002). Moreover, ubiquitination has been recently shown to

have an inhibitory effect on histone function. In yeast, Rad6 was shown to mediate ubiquitination of histone H2B, which is necessary for histone H3 methylation and transcriptional silencing (Robzyk et al., 2000). An additional level of complexity is added through the presence of alternative ubiquitin-like modifiers, such as SUMO, Nedd8, Atg8, etc. (Kerscher et al., 2006). For example, conjugation of the small ubiquitin-like protein SUMO-1 to p53 does not target p53 for degradation, but instead increases p53 transactivation ability (Gostissa et al., 1999). In contrast, Mdm2-dependent Nedd8 modification of p53 was shown to inhibit its transcriptional activity (Xirodimas et al., 2004). These findings outline the diverse mechanisms by which ubiquitin-like proteins and E3 ligases can differentially control the function and stability of their substrates.

3. UNFOLDED PROTEIN RESPONSE

3.1. Definition of the ER stress response

ERAD is one of several mechanisms that are activated in response to perturbations in luminal protein folding and homeostasis in the ER. The whole program of integrated cellular signalling in response to ER stress is well defined and is known as the unfolded protein response (UPR) (Schroder and Kaufman, 2005; Shen et al., 2004). The main purpose of this system is to transmit information about the protein folding imbalance in the ER to the cytoplasm and nucleus, eventually inducing the transcription of UPR genes. The responses elicited by induction of the UPR include: a) reduction in the protein load that enters the lumen of the ER through translational attenuation and decrease in

translocation rates; b) increased ER folding capacity achieved by transcriptional upregulation of the UPR target genes encoding ER luminal chaperones; c) removal of misfolded proteins and damaged ER via the mechanisms of ERAD and autophagy, respectively. These processes represent adaptive responses necessary for cell survival. If ER homeostasis cannot be re-established and the protein folding defect is not corrected, affected cells are eliminated from the organism through the induction of the apoptotic program (Szegezdi et al., 2006). The major UPR transducers that sense the changes inside the ER and initiate the required response are: inositol-requiring protein 1 (IRE1) (Cox et al., 1993); activating transcription factor 6 (ATF6) (Haze et al., 1999); and protein kinase RNA-like ER kinase (PERK) (Harding et al., 1999). These proteins represent ER transmembrane molecules that contain luminal and cytoplasmic domains. The luminal portion serves as a sensor for the protein folding environment, while the cytoplasmic domain functions as an effector that forwards the signal to the nucleus where it initiates transcriptional changes.

3.2. Signal transduction by IRE1

IRE1 is an ER transmembrane glycoprotein that contains both protein kinase and RNase activities in its cytoplasmic domain (Cox et al., 1993). To date, two mammalian homologs, IRE1α (Tirasophon et al., 1998) and IRE1β (Wang et al., 1998) have been identified. In response to perturbations in protein folding within the ER, IRE1 oligomerizes leading to trans-autophosphorylation of juxtaposed kinase domains (Shamu and Walter, 1996). Oligomerization is usually initiated by binding of unfolded proteins to

the IRE1 luminal sensor domain, or alternatively, can be triggered by the dissociation of an oligomerization-repressing chaperone (BiP and others) (Bertolotti et al., 2000). Autophosphorylation subsequently activates the IRE1 RNase activity (Tirasophon et al., 2000). The only known substrate of IRE1 in mammals is XBP1 (X-box binding protein-1) (Calfon et al., 2002). IRE1 cleaves the XBP1 mRNA twice, excising an intron fragment, which generates a frame shift. The 3' and 5' fragments are then ligated, presumably by tRNA ligase (Trl1 in yeast), generating a spliced mRNA (Sidrauski et al., 1996). XBP1 encodes a basic leucine zipper containing transcription factor. Interestingly, both the precursor mRNA and spliced mRNA of XBP1 are translated, generating two different proteins with different functions (Calfon et al., 2002). The XBP1 protein that is translated from spliced mRNA (XBP1s) encodes a potent activator of UPR target genes (Yoshida et al., 2001a), whereas the protein encoded by the precursor RNA (XBP1u) acts as a repressor of UPR target genes. XBP1 mRNA expression is elevated upon induction of UPR (Yoshida et al., 2001a), which in combination with IRE1 activation subsequently raises the levels of XBP1s. As ER stress declines, IRE1 is inactivated, leading to production of XBP1u (Yoshida et al., 2006). XBP1u represents an inhibitor of XBP1s signaling and also negatively regulates XBP1 transcription (feedback mechanism). Mammalian IRE1 was also demonstrated to be a crucial factor in the activation of the cell death machinery (Wang et al., 1998). IRE1, in complex with tumor necrosis factor receptor-associated factor 2 (TRAF2), is known to modulate c-Jun N-terminal kinase (JNK) signalling and promote the clustering of procaspase 12, as well as its activation by cleavage in response to ER stress (Urano et al., 2000). These events were shown to mediate the ER stress induced apoptotic signalling pathway. In addition, proapoptotic

factors Bax and Bak form a protein complex with the cytosolic domain of IRE1 and activate IRE1 signalling, providing a physical link between members of the core apoptotic pathway and the UPR (Hetz et al., 2006).

3.3. Signal transduction by PERK

PERK is another ER transmembrane protein that regulates translational attenuation in response to ER stress (Harding et al., 1999). Similar to IRE1, PERK contains a luminal sensor domain and a cytoplasmic kinase domain. Autophosphorylation of PERK is triggered by oligomerization of its luminal domains in response to the accumulation of unfolded proteins (Harding et al., 1999). PERK phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) at Ser51, which leads to inhibition of eIF2β and thus prevents eIF2 recycling to its active GTP-bound form (Harding et al., 1999). Lower levels of active eIF2 result in reduced formation of translation initiation complexes, leading to reduced recognition of AUG initiation codons and therefore general translational inhibition. This mechanism reduces the load of newly synthesized proteins and thus prevents the clogging of the already stressed ER lumen with misfolded products (Harding et al., 1999). Furthermore, PERK mediated eIF2 inhibition also triggers transcriptional activation of the UPR genes through translational upregulation of the ATF4 transcription factor (Vattem and Wek, 2004). The 5' untranslated region of the mouse ATF4 mRNA contains two upstream open reading frames (uORFs). The 5' proximal uORF1 is a positive-acting element that facilitates ribosome scanning and re-initiation at downstream coding regions within the ATF4 mRNA. The basis for this re-

initiation capacity is not yet defined. When eIF2-GTP is present at normal levels (non-stress conditions), ribosomes scanning downstream of uORF1 re-initiate at the next coding region, uORF2, an inhibitory element that blocks ATF4 expression. During stress conditions, reduction in the levels of eIF2-GTP increases the time required for the scanning ribosomes to become competent to reinitiate translation. This delayed re-initiation allows ribosomes to skip the inhibitory uORF2 and instead reinitiate at the ATF4-coding region. Increased levels of ATF4 lead to transcriptional activation of downstream targets (Lu et al., 2004). One of the activated genes is *GADD34* (growth arrest and DNA damage inducible protein 34). This gene encodes the substrate targeting subunit of protein phosphatase 1 (PP1) that dephosphorylates eIF2α, providing a negative feedback mechanism (Novoa et al., 2001).

3.4. Signal transduction by ATF6

ATF6 (p90ATF6) represents a novel class of metazoan-specific ER stress transducers. These are transmembrane proteins that contain a luminal stress-sensing portion and a basic leucine zipper (bZIP) transcription factor in the cytosolic domain (Haze et al., 1999). Two homologous transcription factors are known to exist in mammalian cells: ATF6α and ATF6β (also known as cAMP response element binding protein (CREB) related protein). ATF6 retention in the ER is mediated by binding to the BiP and calreticulin chaperones (Shen et al., 2002; Hong et al., 2004b). ATF6 possesses two independent and redundant Golgi localization sequences within its luminal region (GLS1 and GLS2) (Shen et al., 2002). BiP protein was shown to bind GLS1 of ATF6 and

therefore control its localization within the ER (Shen et al., 2002). Under conditions of ER stress, BiP is sequestered by unfolded proteins and is dissociated from ATF6, allowing it to translocate to the Golgi complex (Chen et al., 2002). ATF6 is also retained in the ER through interaction of its N-linked glycans with calreticulin (Hong et al., 2004b). During ER stress, newly synthesized ATF6 is underglycosylated and therefore is not recruited by calreticulin. However, it could still be retained within the ER lumen via heterodimerization with fully glycosylated ATF6 that is bound to calreticulin (Hong et al., 2004b). Interestingly, it was reported that ER-stress mediated by thapsigargin triggers an acute proteasomal degradation of the pre-existing pool of glycosylated ATF6, providing an escape mechanism for newly synthesized ATF6 (Hong et al., 2004a).

ATF6 activation is mediated by translocation to the Golgi complex, where it is cleaved by the Golgi resident site 1 protease (S1P) and site 2 protease (S2P) to generate a 50 kDa nuclear form of ATF6 (ATF6f) that acts as a bZIP transcriptional activator for ER-stress inducible target genes (Ye et al., 2000). ATF6f binds to the cyclic AMP response element (CRE) (Wang et al., 2000) and to the ER stress response elements (ERSE-I and ERSE-II) (Kokame et al., 2001). It was demonstrated that ER stress induces formation of the transcription factor complex ERSF that binds to the ERSE-I site (CCAAT-N_9-CCACG) and is required for mammalian UPR induction. This complex consists of the constitutive component NF-Y (CBF) bound to CCAAT and an inducible component (ATF6α/β) bound to CCACG (Yoshida et al., 2001b). ATF6 activates a subset of UPR target genes encoding molecular chaperones and protein disulfide isomerases as well as XBP1 (Yoshida et al., 2001a). Interestingly, calreticulin promoter harbors a *cis*-element containing ERSE motif required for ATF6 binding and

transcriptional induction of calreticulin gene (Yoshida et al., 1998). Many other ER-anchored transcription factors, such as bZIP transcription factors OASIS (Kondo et al., 2005), CREB3/Luman (Raggo et al., 2002), CREB4 (Stirling and O'Hare, 2006), CREB-H (Omori et al., 2001) and BBF2H7 (Kondo et al., 2007) are activated by ER stress and mediate the induction of the UPR pathway.

3.5. ER stress and cell death

The mechanism of cell death triggered by ER stress is not completely understood. There are two types of ER stress induced apoptosis: intrinsic (receptor-independent) and extrinsic (receptor-dependent). As a part of the intrinsic pathway, the multidomain proapoptotic Bax and Bak proteins were shown to localize to the ER, in addition to their mitochondrial localization (Zong et al., 2003). Moreover, ER stress was shown to induce conformational changes and oligomerization of Bax and Bak on the ER membrane, which leads to increased Ca^{2+} efflux from the ER and subsequent elevation of cytosolic Ca^{2+} concentrations (Scorrano et al., 2003). Leak of ER luminal Ca^{2+} is a critical step in the ER-induced apoptotic pathway that leads to calpain activation and procaspase-12 cleavage (Nakagawa and Yuan, 2000). Procaspase-12 expression is induced by the ATF6 transcription factor (Larner et al., 2006) and is cleaved by calpain (Nakagawa and Yuan, 2000) or caspase-7 (Rao et al., 2001). Activated caspase-12 cleaves Bap31 (Rao et al., 2004), resulting in mitochondrial permeabilization, cytochrome *c* release, and Apaf-1 mediated procaspase-9 processing (Shiraishi et al., 2006). Caspase-9 activates the executioner caspase, procaspase-3. Alternatively, caspase-12 mediated processing of

procaspase-9 can be induced through Apaf-1 and cytochrome c independent pathways (Rao et al., 2002). Ca^{2+} released from the ER is taken up by mitochondria (Crompton, 1999). Sustained Ca^{2+} accumulation in the mitochondrial matrix opens the permeability transition pore (PTP) which leads to dissipation of the electrochemical potential, matrix swelling, relocalization of Bax to mitochondria, the release of cytochrome *c* and apoptosome formation (Crompton, 1999). The physical link between the components of the intrinsic apoptotic pathway and the UPR is not fully established; however, it is suggested that Bax and Bak can form a protein complex with the cytosolic domain of IRE1α (Hetz et al., 2006).

Topologically, the ER lumen is equivalent to the extracellular space (Sitia and Braakman, 2003) and therefore the extrinsic apoptotic pathway in response to ER stress must be initiated at the ER membrane. As an ER membrane protein, IRE1 forms a trimeric complex with TRAF2 and apoptosis signal-regulating kinase 1 (ASK1) in response to ER stress (Nishitoh et al., 2002), thus playing a role in ER stress induced apoptosis. Formation of this complex is mediated by Ask interacting protein 1 (AIP1) (Luo et al., 2008). Under normal conditions, TRAF2 mediates procaspase-12 clustering; however, upon ER stress, TRAF2 forms a complex with IRE1 and is therefore released from procaspase-12 binding which is required for caspase-12 activation (Yoneda et al., 2001). TRAF2 and IRE1 interaction is also promoted by c-Jun N-terminal inhibitory kinase (JIK) (Yoneda et al., 2001). Formation of this heteromeric complex leads to activation of JNK, which turns on the apoptotic program. The existence of both intrinsic and extrinsic apoptotic pathways as a reaction to prolonged ER stress emphasizes the

intracellular and extracellular properties of the ER organelle and different outputs associated with its malfunction.

The other two UPR transducers PERK and ATF6 can also trigger proapoptotic signalling during prolonged ER stress. Signalling from ATF6 and PERK has a convergence point. C/EBP homologous protein, CHOP (also known as growth arrest and DNA damage inducible gene 153, GADD153) is known to be a downstream target of ATF6f (ATF6 signalling) and ATF4 (PERK signalling) transcription factors (Ma et al., 2002; Yoshida et al., 2000). CHOP expression is induced in response to DNA damage and ER stress conditions (Zinszner et al., 1998). CHOP-induced apoptosis is mediated through transcriptional upregulation of several genes including GADD34 (Marciniak et al., 2004), ER oxidoreductin 1 (ERO1α) and Tribbles-related protein 3 (TRB3) (Ohoka et al., 2005) as well as trancriptional repression of the Bcl2 prosurvival protein (McCullough et al., 2001). CHOP directly activates GADD34 and ERO1, thus promoting protein biosynthesis and oxidation within the stressed ER, leading to lethal consequences. TRB3 is a negative feedback regulator of ATF4-dependent transcription and negatively affects the stress response (Jousse et al., 2007). Overexpression of CHOP was shown to induce apoptosis in M1 myeloblastic leukemia cells (Matsumoto et al., 1996), while CHOP-/- mouse embryonic fibroblasts were demonstrated to be resistant to ER stress induced apoptosis (Zinszner et al., 1998) due to sustained repression of translation. Overall, it is suggested that ER stress mediated cell death mechanisms evolved in complex metazoans in order to improve the fitness of the whole organism during such stress. Damaged cells are removed by apoptosis and then replaced through regenerative programs.

4. ADDITIONAL MECHANISMS OF CELL REPROGRAMMING DURING ER STRESS

4.1. Control of p53 function during ER stress

The p53 tumor suppressor is one of the central players in the response of cells to various forms of stress including DNA damage (Lee et al., 1995), telomere attrition (Leri et al., 2003), oncogene activation (Wu et al., 1993), hypoxia (Liu et al., 2005) and loss of normal growth and survival signals (Perry and Levine, 1993). The main form of stress that activates p53 is genotoxic stress in the form of DNA double-strand breaks or stalled DNA replication forks (Enoch and Norbury, 1995). Lack of p53 can lead to the loss of genomic integrity and cancer development (Donehower et al., 1992). Activation of p53 can induce several effects, such as cell differentiation, senescence, DNA repair, cell cycle arrest and apoptotic cell death (Riley et al., 2008).

There are several mechanisms to control p53 activity, including regulation of its subcellular localization, regulation of protein stability, and post-translational modifications (Brooks and Gu, 2003). One of the primary mechanisms for p53 destabilization is mediated through it ubiquitination and degradation through the ubiquitin-proteasome system (Yang et al., 2004). To date, a number of E3 ubiquitin ligases involved in p53 ubiquitination have been identified, such as Mdm2, MdmX, Yin Yang 1, MTBP, Pirh2, and COP1 (Badciong and Haas, 2002; Brady et al., 2005; Sui et al., 2004). These enzymes are considered as negative regulators of the p53 protein. Phosphoinositide 3-kinase (PI3K)–Akt signaling is considered the major route for p53

downregulation. Akt promotes phosphorylation and translocation of the Mdm2 oncoprotein into the nucleus, where it ubiquitinates the p53 protein. In contrast, the PTEN tumor suppressor protein inhibits activation of Akt, thus restricting cytoplasmic Mdm2 localization and promoting p53 function (Mayo and Donner, 2002). It was also reported that p300 possesses ubiquitin ligase activity for the p53 protein and promotes it polyubiquitination, suggesting that the transcriptional regulator p300 could function as an E4 type ligase for p53 (Grossman et al., 2003). p53 stabilization is mostly achieved through p53 acetylation (Brooks and Gu, 2003; Ito et al., 2002). Acetylation of p53 occurs primarily at the same lysine residues that are involved in ubiquitination process, thus ensuring competition between ubiquitin ligases and acetyl transferases for the same residues (Brooks and Gu, 2003).

Proteasomal degradation of p53 may also be mediated independently of both MDM2 and ubiquitin (Asher et al., 2005). The ubiquitin-independent pathway is regulated by NAD(P)H: quinine oxidoreductase 1 (NQO1). Recently, it was shown that dicoumarol and several other coumarin and flavone inhibitors of NQO1 activity, which compete with NAD(P)H for binding to NQO1, induce ubiquitin-independent p53 degradation and provide an antiapoptotic effect (Tsvetkov et al., 2005). However, the biological significance of this system is not well defined.

As mentioned above, p53 is activated mainly in response to genotoxic stress. Therefore, there should be specific mechanisms in order to restrict p53 activation and its effects to conditions that threaten genomic integrity. ER stress typically activates cytoprotective pathways that help cells to adapt to these conditions and induces apoptosis when adaptation is not possible (Ma and Hendershot, 2004). One of the adaptive

mechanisms in response to ER stress includes p53 downregulation and diminished apoptosis. Baltzis et. al. demonstrated that the ER resident eIF2α kinase PERK mediates p53 proteasomal degradation (Baltzis et al., 2007). Phosphorylated eIF2α blocks protein biosynthesis while another PERK substrate GSK3β is translocated to the nucleus upon activation. Nuclear localized GSK3β phosphorylates p53 at S315 and S376 (Qu et al., 2004). These phosphorylation events induce p53 ubiquitination by Mdm2, followed by p53 cytoplasmic translocation and proteasomal degradation (Pluquet et al., 2005). Thus, ER stress triggers p53 downregulation independently of translational control (eIF2α phosphorylation). Interestingly, translation of p53 transcripts was unaffected under conditions of translational initiation blockage (Baltzis et al., 2007). It is possible that p53 transcripts can bypass the translational inhibitory effect of eIF2α phosphorylation in order to avoid total p53 loss. In accordance with this, human p53 mRNA was shown to contain an internal ribosome entrance site (Ray et al., 2006).

Previous studies from our laboratory demonstrated that p53 cytoplasmic relocation and subsequent decrease in p53 protein levels could be triggered by alterations in the level of the ER resident chaperone calreticulin (Mesaeli and Phillipson, 2004). Loss of calreticulin function has been shown to activate ER stress, resulting in increased expression of other ER chaperones and enhanced activity of IRE1α and PERK (Knee et al., 2003). However, recent data from our laboratory demonstrated that phosphorylation of GSK3β was significantly increased in calreticulin deficient cells (Jalali and Mesaeli, unpublished data), suggesting that GSK3β is inactivated and is therefore not involved in p53 downregulation.

4.2. ER stress induced apoptosis mediated by PUMA and NOXA

Prolonged exposure of cells to ER stress was recently shown to activate gene transcription of the BH3-only proteins PUMA and NOXA through p53 function (Li et al., 2006). Furthermore, cells deficient in NOXA or PUMA are more resistant to ER stress induced apoptosis (Li et al., 2006). In contrast, Baltzis et. al. demonstrated that prolonged ER stress in several tumor cell lines triggers p53 degradation and does not induce NOXA or PUMA transcription (Baltzis et al., 2007). While the controversy still exists, it is suggested that different cell lines can have different modes of GSK3β regulation and therefore may not have the same capacity to induce the GSK3β dependent p53 degradation pathway. Thus, different cell lines may utilize different mechanisms for the regulation of ER stress induced apoptosis.

B. RATIONALE

Calreticulin is a lectin chaperone that is essential for intracellular calcium homeostasis as well as for glycoprotein folding and maturation. Deletion of the calreticulin gene compromises the ER protein quality control pathway, leading to the activation of the unfolded protein response (Knee et al., 2003). Accumulation of aberrant proteins within the ER of calreticulin deficient (*crt-/-*) cells stimulates IRE1α and PERK kinases (Knee et al., 2003) leading to the activation of stress signalling cascades as well as transcriptional upregulation of luminal chaperones. Thus, expression of the calnexin chaperone is increased in *crt-/-* cells. Interestingly, calnexin – substrate association is severely reduced and protein folding is accelerated (Ellgaard et al., 1999; Molinari et al., 2004). Moreover, the structure of the ER in *crt-/-* cells is not altered due to accumulation of misfolded proteins, supporting the existence of a compensatory mechanism. ERAD is a multi-step process important for the efficient removal of unfolded and misfolded proteins from the lumen of the ER. To date, little is known about the involvement of ER resident lectin like chaperones, such as calreticulin or calnexin, in the process of ERAD. To establish the role of calreticulin in this pathway, we assessed the functionality of different ERAD steps in *crt-/-* cells.

ER stress induced apoptosis is one of the response mechanisms that are activated during prolonged exposure to a stress environment (Szegezdi et al., 2006). Removal of damaged cells ultimately improves the condition of the whole organism. Interestingly, calreticulin deficient cells demonstrate a compromised apoptotic response to DNA damage induced by UV (Mesaeli and Phillipson, 2004) or pharmacological agents such

as staurosporine and etoposide (Nakamura et al., 2000). This could be due to decreased p53 protein levels in *crt-/-* cells (Mesaeli and Phillipson, 2004). However, the mechanism of p53 downregulation in these cells is not established. A previous report from our laboratory demonstrated a significant increase in p53 promoter activation in *crt -/-* cells, illustrating that the observed decrease in p53 protein levels are not due to transcriptional downregulation of the p53 gene (Mesaeli and Phillipson, 2004). Qu et.al. have demonstrated that ER stress induces p53 cytoplasmic localization and degradation, preventing p53-dependent apoptosis (Qu et al., 2004). The same study reported that the observed decrease in p53 protein is due to ER stress mediated GSK-3β activation. Interestingly, GSK-3β is inactivated in *crt-/-* cells (Jalali and Mesaeli, unpublished data).

Regulation of intracellular Ca^{2+} homeostasis represents another important function of calreticulin. In *crt-/-* MEF cells, the resting free cytosolic Ca^{2+} concentration is significantly higher as compared to *wt* cells (Nakamura et al., 2001). These cells also have a lower ER capacity for Ca^{2+} storage, although the free ER Ca^{2+} concentration is not changed (Nakamura et al., 2001). Given that Ca^{2+} is a major second messenger within the cell, we were interested in whether the function of calreticulin as a Ca^{2+} regulator has a significant impact on the process of ERAD and other stress responsive pathways. Indeed, calcium is known to be an important regulator of protein-protein interactions within the ER. Moreover, it was demonstrated that the levels of intracellular Ca^{2+} can affect the rates of protein degradation within the cell (Menconi et al., 2004). Ca^{2+} has also been shown to activate the 20S proteasome *in vitro* (Reshetnyak et al., 2004).

Overall, the aim of our study was to investigate the response mechanism(s) that counterbalances the diminished protein folding capacity of the *crt-/-* cells.

C. HYPOTHESIS

"Enhanced activity of the ubiquitin-proteasome system and increased rates of p53 degradation contribute to the survival of *crt-/-* cells under conditions of ER stress".

To test our hypothesis we addressed the following experimental objectives:

1. To determine changes in the activity of the ubiquitin – proteasome system in *crt-/-* cells by examining:

1.1. total cellular ubiquitination in *wt* and *crt-/-* cells;

1.2. proteasome activity in *wt* and *crt-/-* cells;

1.3. the expression levels of proteasomal genes and/or proteasomal subunits;

1.4. intracellular localization of proteasomal complexes.

2. To investigate changes in the ERAD process upon loss of calreticulin or calnexin function.

3. To examine the mechanism of p53 downregulation in calreticulin deficient cells and to determine the role of ER stress in this process. This objective was achieved by examining:

3.1. p53 stability in *crt-/-* cells and the role of the proteasome in this process;

3.2. the levels of p53 ubiquitination between *wt* and *crt-/-* cells;

3.3. the effect of ER stress on p53 downregulation in *wt* and *crt-/-* cells.

4. To determine the role of the functional domain(s) of calreticulin in the results we obtained in objectives 1-3.

D. MATERIALS AND METHODS

1. CELL CULTURE AND TREATMENTS

Mouse embryonic fibroblasts (MEF) were isolated from 14 day old wild type (*wt*) and calreticulin knockout (*crt-/-*) mouse embryos as described previously (Mesaeli and Phillipson, 2004). Wild type (*wt*) and calnexin deficient (*cnx-/-*) cells were a generous gift of Dr. Michalak (University of Alberta, Edmonton, Canada). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal bovine serum (FBS, Invitrogen). For cell treatments, different inhibitors were dissolved in DMSO or water. The final concentration of DMSO in the media did not exceed 0.1% at any time. In control cells, 0.1% DMSO was added to the media. The inhibitors used were: ALLN (calpain inhibitor); wortmanin (PI3K inhibitor); AKT kinase inhibitor II; MDM2 inhibitor; MG132 (proteasome inhibitor); lactacystin (proteasome inhibitor); kifunensine (mannosidase inhibitor); thapsigargin (inhibitor of ER Ca^{2+}-ATPase); brefeldin A (disrupts Golgi) (all obtained from Calbiochem); tunicamycin (inhibitor of N-linked glycosylation); cycloheximide (inhibitor of translational elongation) and ionomycin (Ca^{2+} ionophore) (Sigma).

2. PLASMIDS

The pcDNA3.1 vector was purchased from Invitrogen. pcDNA3.1-CRT-HA, pcDNA3.1-CRT^{NP}, pcDNA3.1-CRT^{PC}, pSVL-Calsequestrin expression vectors were a

generous gift from Dr. Michalak (University of Alberta). pcDNA-human α1-antitrypsin genetic variant-null Hong Kong (hATNHK) plasmid was a generous gift from Dr. Sifers (Baylor College of Medicine, Houston). The pCMV-5A vector was purchased from Stratagene. To generate pCMV5A-BiP and pCMV5A-calnexin expression constructs, mouse cDNA encoding BiP and calnexin were amplified with Taq polymerase (Invitrogen) using specific primers (Table 1) and subcloned into BamHI/HindIII(or EcoRI) restriction sites of the pCMV5A expression vector.

3. SITE-DIRECTED PCR MUTAGENESIS

Site-specific mutagenesis of the Trp244 (to Ala) residue of calreticulin and calreticulin deletion mutants were performed using an overlap extension polymerase chain reaction technique (Ho et al., 1989). The PCR reaction was carried out using *Pfx* DNA polymerase (Invitrogen) and calreticulin primers listed in Table 1. pcDNA3.1-CRT-HA, pcDNA3.1-CRT^{NP}, pcDNA3.1-CRT^{PC} were used as templates during the amplification reaction. The first PCR reaction was carried out using primers for the 5' end of the calreticulin cDNA encoding the signaling peptide and the 3' region encoding Trp244 and surrounding residues (the primer containing T244A mutation). The second PCR reaction included the primers for the 3' end of calreticulin encoding the KDEL retention sequence and a midregion of calreticulin containing the mutation site. The 5'forward primer and 3' reverse primer were common for all three templates. The products of two PCR reactions were combined and used as templates for PCR reaction using primers for the 5' and 3' ends of calreticulin.

Table 1. List of oligonucleotide primers used through out this study.

Gene	Primer sequence
mBiPFBamH I	5' CGCGGATCCATGATGAAGTTCACTGTGG 3'
mBiPRHind III	5' CCCAAGCTTCAACTCATCTTTTTCTGAT 3'
mCANXFBamH I	5' CGCGGATCCATGGAAGGGAAGTGGTTAC 3'
mCANXREcoR I	5' CCCGAATTCCTCTCTTCGTGGCTTTCTG 3'
CRTstartF	5' ACTTAAGCTTGGTACCGAGCTC 3'
CRTW244AR	5' CGGCTCCGCCTCTCCGTCCATTTC 3'
CRTW244AF	5' GGAGAGGCGGAGCCGCCGGTGATT 3'
CRTendR	5' GGCCCTCTAGACTACAGCTCG 3'
Calnexin-F	5' CGCGGATCCATGGAAGGGAAGTGGTTAC 3'
Calnexin-R	5' GAGATGGAGGAGTGCTGGC 3'
EDEM-F	5' ATAGCGGCCGCATGCAATGGCGAGCGCTTG 3'
EDEM-R	5' GAAGACCAACCAGAGCACCG 3'
EDEM2-F	5' CTACGATGAGCTGAGACCTC 3'
EDEM2-R	5' CCTGAAGAACCTCCACTACT 3'
EDEM3-F	5' CAGCTGATGAGCTCATGCCT 3'
EDEM3-R	5' CAAGGCGTCATCCACATCG 3'
Hrd1-F	5' ATGTTCCGCACCGCAGTG 3'
Hrd1-R	5' CATAGTAGGCATGAGCCACC 3'
PSMA1/α6/C2-F	5' CTGTTTGGAGCCCTCAGG 3'
PSMA1/α6/C2-R	5' TGTGGTCAGGTCCTGCTC 3'
PSMA5/α5/zeta-F	5' TCGGTCCGAGTACGACAG 3'
PSMA5/α5/zeta-R	5' GGTCCCAGATGGGTCCAT 3'
PSMB3/β3/C10-F	5' CGTCATGGCCATGAAGGGA 3'
PSMB3/β3/C10-R	5' CGTTCAGCATGGCCTGAG 3'
PSMC3/Rpt5/S6a-F	5' ACGCCCGAGAGTCCAGTGA 3'
PSMC3/Rpt5/S6a-R	5' GGGCTGGATACCCAAGTT 3'
PSMC4/Rpt3/S6b-F	5' GACCTGGAGGACCTGTAC 3'
PSMC4/Rpt3/S6b-R	5' GGGAGGATCGATGCCAAT 3'
PSMD3/Rpn3/S3-F	5' ATGAAGCAGGAGGGCTCG 3'
PSMD3/Rpn3/S3-R	5' CGAGGACGAAACTGCAGGT 3'
PSMD4/Rpn10/S5a-F	5' CTGCAGGCCCAGCAGGAT 3'
PSMD4/Rpn10/S5a-R	5' GGCACCAAGACCCAGCAT 3'
P53-F	5' ATGACTGCCATGGAGGAG 3'
P53-R	5' TCAGTCTGAGTCAGGCCC 3'
GAPDH-F	5' GCCAAGGTCATCCATGACAAC 3'
GAPDH-R	5' GTCCACCACCCTGTTGCTGTA 3'

4. TRANSIENT TRANSFECTIONS

wt and *crt-/-* MEF cells were transiently transfected overnight with 15 μg of plasmid DNA (pCMV5A-BiP, pCMV5A-calnexin, pcDNA-hATNHK, pcDNA-CRT-HA, pcDNA-CRT^{NP}, pcDNA-CRT^{PC}, pcDNA-CRT^{W244A}, pcDNA-CRT^{NPW244A}, pcDNA-CRT^{PCW244A}, pSVL-Calsequestrin or their respective empty vectors) in 30 μl of Lipofectamine 2000 (Invitrogen) diluted in 1.5 ml of Opti-MEM (Invitrogen). The next day, the media was replaced with DMEM containing 10% FBS. 48 hours after transfection the cells were used for western blot or proteasome assay.

5. GENERATION OF STABLY TRANSFECTED HEK293 CELLS

HEK293 cells were seeded at 80–90% confluence in 10-cm Petri dishes. 24 hours later each plate was transfected with 15 μg of expression plasmid as described above. pcDNA-hATNHK expression plasmid contains a selection gene for Zeocin resistance. Selection media (DMEM containing 10% FBS and 400 μg/ml zeocin) was added to cells 48 h after transfection. Cells were grown in the selection media until separate colonies became visible (9 days). Individual Zeocin-resistant clones were then isolated and expanded to generate stable cell lines. Expression of A1AT NHK in the individual clones was assessed by immunoblot with rabbit polyclonal anti-α1-antitrypsin antibody (Dako).

6. siRNA TRANSFECTIONS

For siRNA transfections, 2.5 x 10^5 cells were plated in 6 well plates in DMEM media containing 10% FBS. The next day, the media was replaced with Opti-MEM media (Invitrogen) and cells were transfected with 80 pmols of calnexin siRNA or a control siRNA (siRNA-A, Santa Cruz Biotechnology) using Lipofectamine 2000. Six hours after transfection, the media was changed to DMEM supplemented with 2% FBS and cells were incubated for 24 hours. Cells were then used for proteasome activity and western blot analysis.

7. WESTERN BLOT

Cell lysates containing 30 μg of protein (or ubiquitin enriched proteins) were separated on a 10% SDS-polyacrylamide gel and transfered to a nitrocellulose membrane (Trans-Blot Transfer Medium, BIO-RAD) using wet transfer system (BIO-RAD). After blocking with 5% milk powder in PBS, the primary antibody was added to the nitrocellulose membrane and incubated for 3 hours. HRP-conjugated secondary antibodies (Jackson Laboratories) were used, followed by enhanced chemiluminescence solutions (Santa Cruz Biotechnology). Fluor-S Max (BioRad) was used to obtain the western blot images. The same membrane was stripped and reprobed with an antibody to cytoskeletal actin (Sigma) to control for equal loading. A rabbit polyclonal anti-ubiquitin antibody was purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibodies to the 20S proteasome α/β subunits and 19S regulatory Rpt3 subunit were obtained from

BIOMOL Int. The p44S10 subunit was detected using a goat polyclonal antibody (Santa Cruz Biotechnology). Calreticulin and calsequestrin expression was detected using polyclonal goat anti-calreticulin and polyclonal rabbit anti-calsequestrin antibodies, respectively (a generous gift from Dr. Michalak, University of Alberta). For analysis of expression of myc-tagged exogenous proteins (BiP, Calnexin), western blotting was carried out using a rabbit polyclonal antibody to c-myc (Santa Cruz Biotechnology). Anti-p53 mouse monoclonal antibody was from Oncogene Science (USA). Rabbit polyclonal anti-p53 antibody was from Santa Cruz (CA, USA).

8. UBIQUITIN PULL DOWN ASSAY

Total cellular ubiquitinated proteins were enriched using an ubiquitin enrichment kit (Calbiochem) following the manufacturer's recommendation. Briefly, cells were lysed using a buffer consisting of: 50 mM Hepes (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail, and 10 mM N-ethylmaleimide (NEM). Lysates were incubated with the affinity beads (GST-fusion protein containing the ubiquitin-associated domain of Rad23 conjugated to glutathione agarose) at 4°C for 4 hours with constant mixing. Beads were washed 3 times with ice-cold wash buffer (lysis buffer without NEM and protease inhibitors), re-suspended in loading buffer containing 250 mM Tris HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and bromophenol blue. Proteins were resolved on a 10% SDS-PAGE. The level of ubiquitinated proteins was then assayed by western blot with a rabbit polyclonal anti-ubiquitin antibody (Sigma) or rabbit polyclonal anti-p53 antibodies.

9. ANALYSIS OF ER PROTEIN UBIQUITINATION

To determine changes in the rate of ubiquitination of ER associated proteins in *wt* and *crt-/-* cells we used both an endogenous protein connexin 43 (Cx43) and an ectopically expressed protein - A1AT NHK (a well established substrate for ERAD). A plasmid containing the A1AT NHK was transiently transfected into both cell types as described above. 48 hours after transfection, cells were treated with 50 μM MG132 for 4 hours followed by ubiquitin pull down assay as described above. Cell lysates were then resolved on a 10% SDS acrylamide gel and western blot was carried out using rabbit polyclonal anti-α1-antitrypsin antibody (Dako).

To examine Cx43 ubiquitination, cells were scraped in ice-cold PBS followed by centrifugation for 30 seconds at 7500g, 4°C. Pellets were resuspended in hypotonic buffer containing 4 mM Hepes, pH7.9, 4 mM NaF, 0.2 mM $Na_4P_2O_7$, 0.2 mM Na_3VO_4, 0.05 mM Na_2MoO_4, 8 mM β-glycerophosphate, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 0.05% NP-40. Cell debris were removed by centrifugation for 30 sec at 13,000 rpm and the supernatants were collected for ubiquitin pull down assay as described above. To remove the ubiquitin moiety from the enriched ubiquitinated proteins, the samples were treated with 0.5 μg Isopeptidase T (ubiquitin C-terminal hydroxylase, Calbiochem) in a buffer containing 25 mM Hepes, pH 7.5 and 10 mM DTT at 25°C for 30 minutes. Isopeptidase T is a member of the high molecular weight group of ubiquitin C-terminal hydroxylases (UCHs) involved in the hydrolysis of isopeptide linkages of polyubiquitin chains and is often used to release proteins from ubiquitin chains. Ubiquitin moieties can

hinder antibody/antigen complex formation and ubiquitin removal was necessary to unmask the Cx43 antigen. Immunoblot analysis was carried out using a rabbit polyclonal anti-Cx43 antibody.

10. IMMUNOCYTOCHEMISTRY

To examine the localization of the 20S and 19S proteasome complexes, cells were plated on coverslips. The next day, cells were washed with PBS and fixed using 4% paraformaldehyde in PBS for 10 minutes. Cells were then washed with PBS to remove the formaldehyde, permeabilized with PBS containing 0.1% saponin, and blocked with blocking solution (PBS containing 0.1% saponin and 2% milk powder) for 30 minutes. Cover slips were then incubated with rabbit polyclonal antibodies to the 20S proteasome α/β subunits or the 19S regulatory Rpt3 subunit for 1 hour at room temperature. After a series of washes with PBS, FITC-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) was added to the coverslips and incubated for 1 hour at room temperature. The coverslips were then mounted on glass slides using Vectashield mounting media containing DAPI (nuclear stain, Vector Laboratories Inc., Burlingame, CA). Fluorescent images were obtained using a Zeiss epi-fluorescent microscope (Zeiss, ON).

11. QUANTITATIVE ANALYSIS OF PROTEASOME ACTIVITY

Cells were lysed using a buffer containing (final concentration): 25 mM Hepes (pH

7.5), 2.5 mM EDTA, 75 mM NaCl and 0.5% Triton X-100 (modified from the manufactures protocol). The proteasome activity in the cell lysate was assayed using a 20S proteasome assay kit (Calbiochem). Briefly, 100-200 μg of protein was incubated with the 10 μM fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC, (proteasome substrate III) in a 96-well microtiter plate at 37°C. Proteasome activity was measured by monitoring the release of free AMC (7-amido-4-methylcoumarin) using a Spectra Max Gemini fluorimeter and Soft Max Pro 4.0 software (Molecular Device). The kinetics of AMC release was measured by monitoring the increase in fluorescence in 2-minute intervals for 1 hour (excitation max. 380 nm and emission max. 460 nm). In order to test for substrate specificity, a group of cells were first treated with 50 μM MG132 (proteasome inhibitor) for 4 hours and then the proteasome activity was assayed.

12. SUBSTRATE OVERLAY ASSAY

To examine changes in the 26S proteasome, a substrate overlay assay was carried out as described by Hoffman and Rechsteiner (Hoffman and Rechsteiner, 1994). Briefly, cells were lysed using a buffer containing (final concentration): 25 mM Hepes (pH 7.5), 2.5 mM EDTA, 75 mM NaCl and 0.5% Triton X-100. Lysates (100 μg of protein) were resolved on non-denaturing gels (4.5% acrylamide gel, 2.3% sucrose, 90mM Tris, 80mM borate, 0.08 mM EDTA) in TBE buffer (90mM Tris, 80mM borate, 0.08 mM EDTA) at 50V for 16-20 hours. The separated proteins were analyzed for *in vitro* proteasome activity by incubating the gels with 200 μM Suc-LLVY-AMC, 2mM ATP for 30 minutes at 37 °C. The fluorescent images from each gel were acquired with a Fluor-S Max

(BioRad). 1.5 μg of commercially purified 20S proteasome (Calbiochem) was used as a reference.

13. RNA ISOLATION WITH TRIzol

wt and *crt-/-* cells were seeded at 80–90% confluence in 10-cm Petri dishes. Cells were lysed in a culture dish by adding 1 ml of TRIzol and passing the cell lysate several times through a pipette. Lysates were transferred to 1.5 ml microcentrifuge tubes and incubated at room temperature for 5 minutes. 0.2 ml of chloroform was then added to the lysates. The tubes were mixed for 15 seconds and then incubated at room temperature for 5 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4°C and the upper aqueous phase was transferred to a new tube. RNA was precipitated by adding 0.5 ml of isopropyl alcohol followed by 10-minute incubation at room temperature. Samples were then centrifuged at 12,000g for 10 minutes at 4°C, the supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol. Samples were centrifuged at 7,500g for 5 minutes at 4°C. The RNA pellet was air-dried for 15-20 minutes and then dissolved in 20μl of RNAse-free water. RNA samples were stored at -80°C.

14. RNA ISOLATION USING RNAEASY MINI KIT

To isolate RNA devoid of any trace of phenol we used the RNAeasy mini kit (Qiagen) following the manufacturer's protocol. Briefly, *wt* and *crt-/-* cells were seeded in 10-cm Petri dishes. Once 80-90% confluence was reached, cells were lysed by adding 0.6 ml of

RLT buffer (kit component) which contains guanidine thiocyanate. Cell lysates were then collected from each plate and transferred to a microcentrifuge tube. Next, 70% ethanol was added to the homogenized cell lysates and mixed by pipetting. The mixture was then applied to an RNAeasy mini column and centrifuged at 9,000g for 15 seconds. The RNAeasy column was then washed with RW1 buffer (kit component) followed by washing with RPE (kit component) buffer containing ethanol and centrifugation. To dry the column and to ensure that no ethanol is carried over during RNA elution, the column was additionally centrifuged at 9,000g for 2 minutes. RNA was eluted with 30µl of RNase free water and subsequent centrifugation at 9,000g for 1 minute. The concentration of the isolated RNAs was determined using a UV spectrophotometer (GeneQuant *pro* RNA/DNA calculator, Fisher).

15. REVERSE TRANSCRIPTASE PCR ASSAY

For semi-quantitative RT-PCR assay, total RNA was isolated using Trizol reagent as described above. Specific cDNA were amplified using Superscript II Reverse transcriptase and Taq polymerase (Invitrogen, Canada). Briefly, 1.5 µg total RNA isolated from *wt* and *crt-/-* cells was mixed with 500µg/ml Oligo(dT)15, 0.5 mM dNTP and sterile distilled water in a total volume of 12 µl. The mixture was heated to 65°C for 5 minutes and then quickly cooled on ice for 2 minutes. The samples were then mixed with 5 X First-Strand Buffer, 0.01M DTT and 1µl of SuperScript® II (RT) and incubated at 42°C for 50 minutes. The enzyme was then heat inactivated at 70°C for 15 minutes. For the PCR reaction, 1 µg of the synthesized cDNA was added to a mixture containing 0.2

mM dNTP, 0.4 μM of primer mix, 1.5 mM $MgCl_2$, 10 X PCR Buffer and 0.5 μl of *Taq* DNA polymerase. PCR for GAPDH (housekeeping gene) was carried out using the same cDNA and primers specific to mouse GAPDH as a control. The primers (Table 1) were designed based on published cDNA sequences. Amplified products were run on 1% agarose gels, stained with ethidium bromide and analysed by gel densitometry (Gel Doc, BioRad).

16. REAL TIME qPCR

For quantitative real time PCR experiments, total RNA was extracted using the Qiagen RNAeasy Kit (as described above). cDNA synthesis and PCR amplification were carried out using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad) and a Bio-Rad iCycler Thermal Cycler. Briefly, 200 ng of total RNA was added to a tube containing 200 nM of each forward and reverse primer, 0.25 μl iScript Reverse Transcriptase and 12.5 μl of 2 X SYBR Green RT-PCR reaction mixes. The final reaction volume was adjusted to 25 μl. Table I shows the list of primers (Sigma Genosys) used for quantitative real time PCR. Data were analyzed with iCycler 3.11 software. mRNA expression of each specific gene was normalized to expression of the GAPDH housekeeping gene. The C_t (threshold cycle or crossing point) is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. Expression of each gene relative to GADPH was calculated as the difference between the threshold values of the two genes (ΔC_t). The $\Delta\Delta C_t$ value describes the difference between the

average ΔC_t value of *crt-/-* cells and the average ΔC_t value of *wt* cells. The values are mean ± SE of three separate experiments.

17. ATP DETERMINATION

Total cellular ATP was extracted as described by Yang *et al.* (Yang et al., 2002). Cells were washed twice with PBS and then re-suspended in de-ionized water (200 µl) and boiled for 10 minutes. Total cellular ATP was measured using an ATP determination kit (Molecular Probes) following the manufacturer's protocol. Briefly, 10 µl of cell lysate was added to 100 µl of luciferin–luciferase reaction buffer (0.5 mM D-luciferin, 1.25 µg/ml firefly luciferase, 25 mM Tricine buffer, pH 7.8, 5 mM $MgSO_4$, 100 µM EDTA and 1 mM DTT) and the light emission was determined using a Lumat LB 9507 luminometer. The amount of ATP in the each sample was calculated from the standard curve and normalized to protein content. ATP standard solutions were made by diluting 5 mM ATP in ddH_2O.

18. CYTOTOXICITY ASSAY

To determine the effect of proteasome inhibitors on cell viability, equal numbers of *wt* and *crt-/-* cells were plated in 10 cm^2 plates and allowed to adhere overnight. The next day, cells were incubated with media containing 10 µM lactacystin for 16 hours at 37°C or 50 µM MG132 for 4 to 8 hours at 37°C. To examine the effect of ER stress on cell viability, *wt* and *crt-/-* cells were incubated with media containing 0.1 µM thapsigargin

(TG) or 1 μg/ml tunicamycin (TN) for 3 hours at 37°C. At the end of each treatment, cells were washed with PBS and the percentages of live/dead cells were calculated using a Live/Dead assay kit (Molecular Probes) following the manufacturer's protocols. Briefly, treated cells were collected by centrifugation and then mixed with an equal volume of Live/Dead working solution containing calcein AM (4 μM) and ethidium homodimer-1 (Etd-1, 8 μM) for 30 min at room temperature. Calcein AM is a cell permeable dye which is retained in the live cells, producing a green fluorescence. Etd-1 is only taken up by the damaged cells and its binding to DNA emits a red fluorescent signal. The number of live cells was calculated from the fluorescence readings at 538 nm for each sample using a Spectra Max Gemini plate reader and plotted as a percentage relative to control samples (no treatment). To examine the amount of dead cells by fluorescent microscopy, cells were cultured on sterile glass coverslips until a 90% cell density was obtained and then incubated with regular media or media containing 10 μM lactacystin for 12 hours. As a positive control for cell death, cells were treated with 70% EtOH for 30 minutes (killing all the cells). Coverslips were then washed with PBS and incubated with 4 μM Etd-1 for 45 minutes at room temperature. Coverslips were mounted onto microscope slides and images were obtained using a Ziess Axioscope equipped with a Texas Red filter.

19. METABOLIC RADIOLABELLING AND IMMUNOPRECIPITATION OF A1AT NHK

The role of calreticulin and calnexin in the processing and folding of A1AT NHK was determined using pulse chase experiments. *wt*, *crt-/-* and *cnx-/-* mouse embryonic fibroblasts were transiently transfected with 15 μg of pcDNA-hATNHK plasmid DNA.

The next day, cells were subcultured and plated equally on 10 cm^2 Petri dishes (in order to achieve equal levels of hATNHK expression) and incubated for 24 hours at 37°C. To investigate the effect of calreticulin overexpression on A1AT NHK maturation, HEK 293 cells stably expressing pcDNA-hATNHK were transiently transfected with pcDNA-CRT-HA plasmid.

For metabolic radiolabelling experiments, cells were incubated in Methionine-free DMEM (Gibco) for 30 minutes at 37°C followed by a 15-minutes pulse with L-[^{35}S]Met/Cys *in vitro* cell labelling mix (GE Healthcare). Cells were then washed twice with 1x PBS (Gibco) to remove unincorporated radiolabel and then chased for different time periods (0, 1, 2 hours) at 37°C with normal DMEM supplemented with 5mM L-methionine and 5mM L-cysteine. Cells were then lysed and collected by scraping in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50mM Tris-HCl, pH 8.0) at 4°C. For immunoprecipitation, all samples were cleared by centrifugation at 7,500 *g* for 5 minutes at 4°C prior to receiving an excess (50x) of rabbit anti human A1AT antibody (Dako). Lysates were then incubated overnight with constant rotation at 4°C. Immunocomplexes were captured by addition of 40 µl of Protein A agarose beads. Immunocomplexes were then washed three times with NP-40 lysis buffer. The samples were then resolved using 10% SDS PAGE. Gels were vacuum dried and exposed to X-OMAT scientific imaging film (Kodak). Autoradiographs were scanned (GS-800, Bio-Rad) and the bands corresponding to A1AT NHK were quantified by densitometry (Quantity One 4.5.0., Bio-Rad).

20. QUANTIFICATION OF DATA AND STATISTICAL ANALYSIS

Data quantification for the ubiquitin pull down experiments was obtained by densitometric quantification of the bands using Quantity One 4.5.0 analytical software (BIO-RAD). Statistical analysis was performed using Sigma Stat 3.11 and Sigma Plot 9.0. Statistical differences were assessed using Student's t-test (independent).

E. RESULTS

1. ENHANCED ACTIVITY OF THE UBIQUITIN-PROTEASOME SYSTEM IN CALRETICULIN DEFICIENT CELLS

1.1. Increased proteasome activity in calreticulin deficient cells

To determine the effect of the absence of calreticulin on the ubiquitin-proteasome pathway, we first examined changes in total cellular ubiquitination. Total cellular ubiquitinated proteins were enriched using ubiquitin affinity beads followed by western blot with anti-ubiquitin antibodies. As shown in Figure 6, there was no significant difference between the ubiquitination profiles of non-treated *crt-/-* and *wt* cells. Differential proteasome activities can exhibit a dramatic effect on the levels of total ubiquitinated proteins. To establish whether there were any changes in the rate of proteasome dependent degradation due to loss of calreticulin function, cells were treated with the proteasome inhibitor (MG132) for 4 hours. This treatment resulted in a significant increase in the level of ubiquitinated proteins in *wt* and *crt-/-* cells (Figure 6). Moreover, the percentage increase of ubiquitinated proteins in MG132 treated *crt-/-* cells was significantly higher than in MG132 treated *wt* cells (Figure 6, bar graph). This observation suggests that there is a higher rate of both ubiquitination and degradation of ubiquitinated proteins in *crt-/-* as compared to *wt* cells. Consequently, the increased proteasome activity in *crt-/-* cells results in faster degradation of ubiquitinated proteins, as seen by the equivalent levels of ubiquitinated proteins in untreated *wt* and *crt-/-* cells

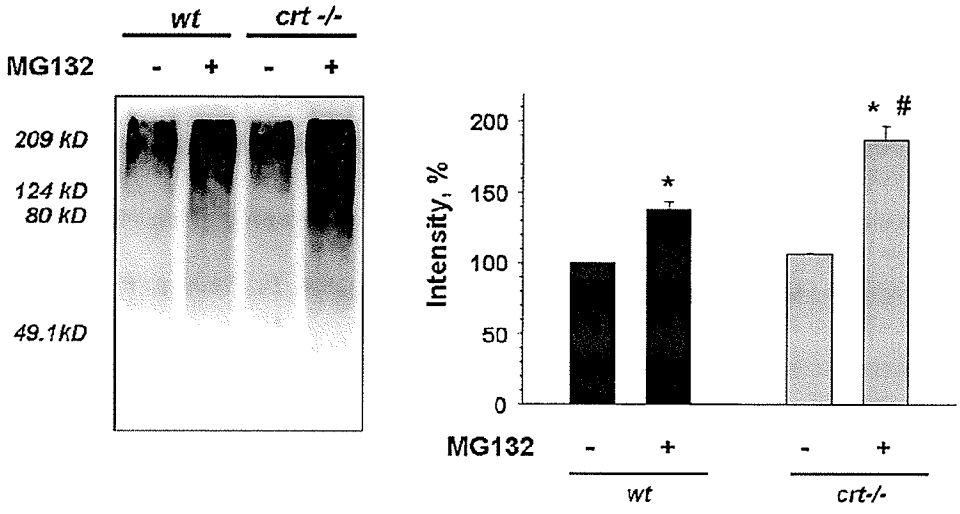


Figure 6. Increased level of ubiquitinated proteins in *crt-/-* cells following proteasome inhibition. Ubiquitinated proteins were enriched using ubiquitin affinity beads. Proteins were then resolved on 10% SDS page and followed by western blotting with an anti-ubiquitin antibody. Bar graph is quantification of total ubiquitinated proteins in the *wt* and *crt-/-* cells. The values are mean ± SE of three separate experiments.
* $p<0.05$ significantly different as compared to untreated cells, # $p < 0.05$ as compared to *wt* cells treated with 50 μM MG132.

(Figure 6, bar graph).

To study the changes in ubiquitination of ER associated proteins in the absence of calreticulin, we compared the rates of ubiquitination of Connexin43 (Cx43, endogenous protein) and A1AT NHK (α1-antitrypsin mutant) an exogenously expressed protein. Cx43 folding and initial oligomerization has been shown to be carried out in the ER (Laird et al., 1995), whereas A1AT NHK is a well known glycoprotein-ERAD substrate (Hosokawa et al., 2003). Both Cx43 and A1AT NHK are degraded via the ubiquitin proteasome pathway (Laing and Beyer, 1995; Shen et al., 2006). To assess the degree of A1AT NHK ubiquitination, *wt* and *crt-/-* cells were transiently transfected with A1AT NHK. Western blot analysis using an anti-A1AT antibody shows that both cell lines express similar levels of NHK protein (Figure 7A, lower panel). Next, transfected cells were treated with the MG132 proteasome inhibitor and total ubiquitinated proteins were enriched using ubiquitin affinity beads. Western blotting with an anti-A1AT antibody demonstrated that the levels of poly-ubiquitinated and mono-ubiquitinated NHK are increased in *crt-/-* cells as compared to *wt* cells (Figure 7A). To detect the levels of Cx43 ubiquitination, cell lysates were incubated with ubiquitin affinity beads followed by treatment with isopeptidase T in order to remove ubiquitin molecules from the proteins. As shown in Figure 7B the levels of ubiquitinated Cx43 were elevated in the absence of calreticulin. These data illustrate that the loss of calreticulin function increases ubiquitination and degradation of ER substrates.

To determine whether calreticulin deletion triggers any changes in the proteasome function, we measured proteasome activity in cell lysates prepared from *wt* and *crt-/-* cells in the presence or absence of MG132. Figure 8 shows a significant increase (~35%)

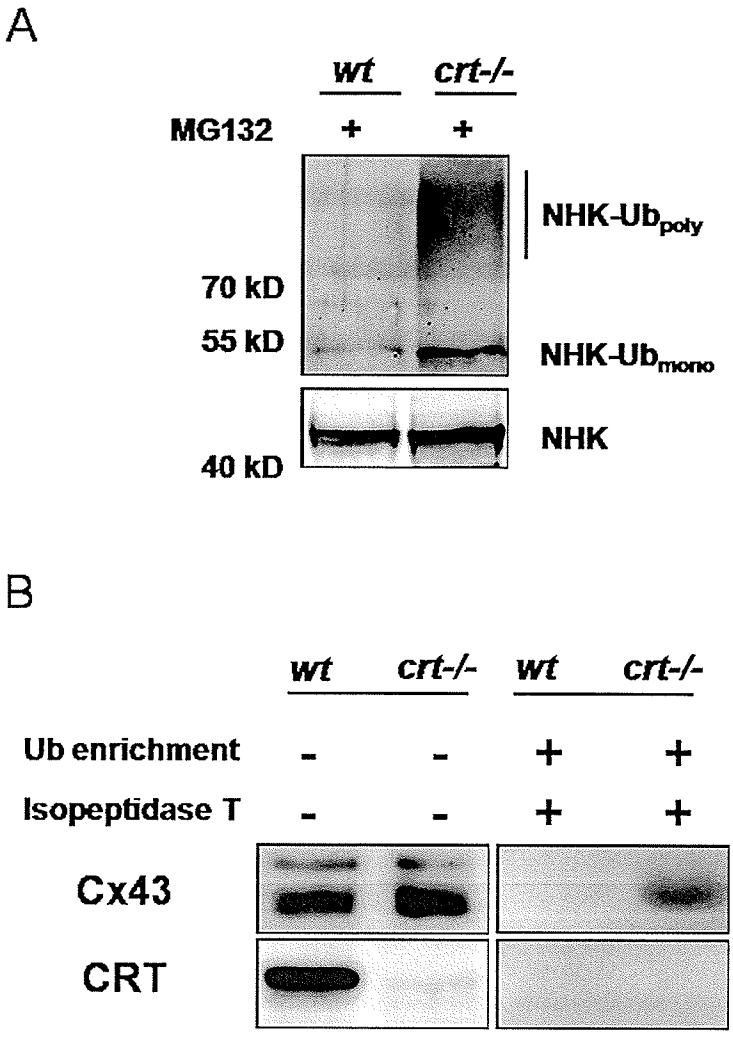


Figure 7. Ubiquitination of exogenously expressed mutant α1-antitrypsin (NHK) and endogenously expressed Connexin 43 (Cx43) in *crt-/-* and *wt* cells. Ubiquitinated proteins were enriched using ubiquitin affinity beads as described in *Materials and Methods*. **(A)** Upper panel shows western blot with anti-NHK antibody of ubiquitinated NHK (upper blot) and total non-ubiquitinated NHK (lower blot) in *wt* and *crt-/-* cells. **(B)** Western blot with rabbit anti-Cx43 antibody showing the level of total Cx43 (left panel) and ubiquitinated Cx43 in *wt* and *crt-/-* cells. The level of ubiquitinated Cx43 was examined after ubiquitin pull down and subsequent treatment with isopeptidase. Isopeptidase treatment is required in order to expose the antigen site that is blocked by ubiquitin chains.

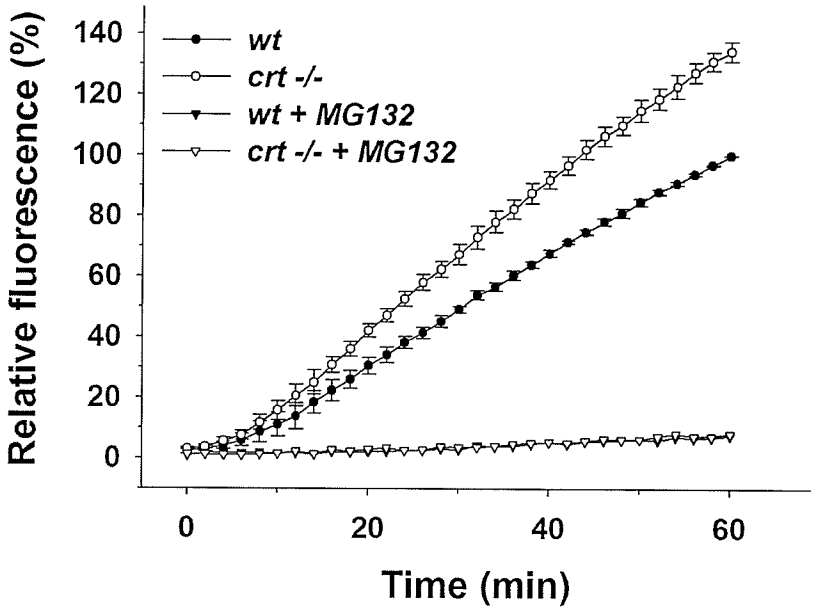


Figure 8. Changes in proteasome activity in *crt-/-* cells as compared to *wt* cells. Cells were treated with media containing 50μM MG132 or DMSO (solvent) for 4 hours followed by lysis as described in *Materials and Methods*. Chymotrypsin like activity of the proteasome was measured by monitoring the release of free AMC (amidomethylcoumarin) from the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC (Ex.: 380 nm; Em.: 460 nm). The values are mean ± SE of three separate experiments carried out in duplicates. The fluorescence values of *wt* + MG132 and *crt-/-* + MG132 are overlapping.

in proteasome activity of *crt-/-* cells as compared to *wt* cells in the absence of an inhibitor. Furthermore, pre-treatment of the cells with MG132 for 4 hours before the preparation of cell lysates, resulted in the complete inhibition of proteasome activity in both cell types (Figure 8). To distinguish between the activity of the 26S proteasome and the 20S proteasome, we carried out a substrate overlay assay that differentiates between the two complexes based on their molecular weight. Figure 9A shows the location of the 20S proteasome (purified protein, Calbiochem) and the 26S proteasome (from 100μg *wt* cell lysates) on a non-denaturing acrylamide gel after the substrate overlay assay. Figure 9A also shows that the 26S proteasome comprises the majority of the proteasome activity in the cell lysates, since the 20S proteasome activity was barely detectable (Figure 9A, arrowhead). As shown in Figure 9B, the enzymatic activity of the 26S proteasome is higher in *crt-/-* cell lysates as compared to the *wt* cells. Interestingly, no proteasome activity corresponding to the 20S complex was detected in the cell lysate from *wt* or *crt-/-* cells (Figure 9B, arrowhead).

1.2. Cells lacking calreticulin are more sensitive to proteasome inhibition and maintain high ATP levels

Previous reports have shown that *crt-/-* cells are more resistant to both UV and drug induced apoptosis (Mesaeli and Phillipson, 2004; Nakamura et al., 2000). Inhibition of proteasome activity has been shown to induce cell death in many cell types, including tumor cells (Bazzaro et al., 2006; Fribley et al., 2004). Therefore, we examined whether elevated proteasome activity is a compensatory mechanism essential for *crt-/-* cell

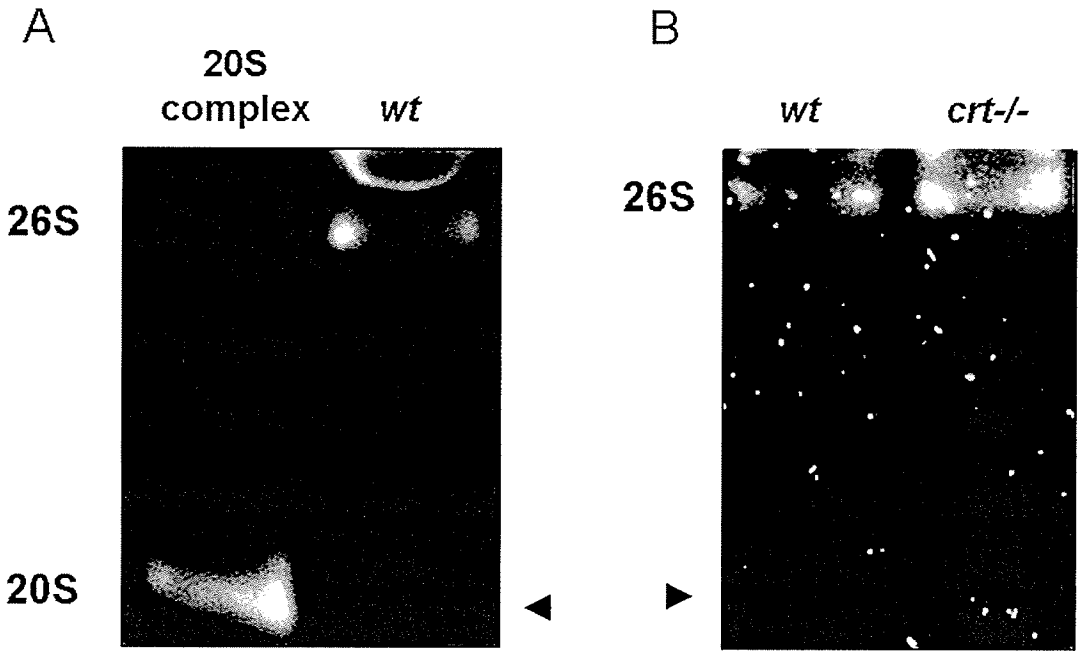


Figure 9. Substrate overlay assay to differentiate between the activity of 20S and 26S proteasome. **(A)** Commercially available purified 20S proteasome complex (lane 1) and cell lysates from *wt* cells (lane 2) were resolved on a non-denaturing gel. The activities of 20S and 26S proteasome were identified by a substrate overlay assay, based on different electophoretic mobilities. **(B)** Changes in proteasome activity in *crt-/-* cells as compared to *wt* cells. 100 µg of protein cell lysate from *wt* and *crt-/-* cells were prepared as above. The Suc-LLVY-AMC-hydrolysing activity of the samples was detected by the overlay technique. In A and B the sample loading wells are cut off in these images. The curved bands on top of the gel, is an artifact at the junction between stacking and separating gels. Gel image is a representative image of three separate experiments.

survival. *wt* and *crt-/-* cells were treated with 10 μM lactacystin for 16 hours and cell viability was assessed using a Live/Dead assay (see *Materials and Methods*). Figure 10A shows that cell viability was decreased following treatment with lactacystin in both cell types. However, *crt-/-* cells were more sensitive to this treatment. As an alternative approach, we examined the rates of cell death following proteasome inhibition using immunofluorescent staining. *wt* and *crt-/-* cells were grown on glass coverslips and treated with 10 μM lactacystin for 12 hours. Coverslips were washed with PBS and then stained with EthD-1 dye. As it is shown in Figure10B, the number of dead cells following proteasome inhibition was higher in the absence of calreticulin. Similar results were obtained after treatment with the MG132 proteasome inhibitor (Figure 11). Cell death was measured in *wt* and *crt-/-* cells after exposure to MG132 (for 4 or 8 hours). As shown in Figure 11, prolonged exposure to MG132 induced higher rates of cell death in both cell types, but the effect was significantly higher in *crt-/-* cells. In general, the cytotoxicity of MG132 was greater than that observed for lactacystin which could be due to side effects of MG132, such as changes in mitochondrial membrane permeability (Lee et al., 2005a; Lee et al., 2005b).

The proteasome is one of the major ATP consumers inside the cell and its activity is modulated by six conserved ATPase subunits of the 19S complex (Rpt1-6) (Glickman et al., 1998). ATPases are essential for the 26S complex assembly, gating of the translocation channel, substrate unfolding, and translocation of unfolded substrates into the central chamber (Rubin et al., 1998). Thus, we measured the intracellular ATP content in *crt-/-* cells to determine whether increased proteasome activity in these cells could be maintained by the cellular ATP content. As shown in Figure 12, the total ATP

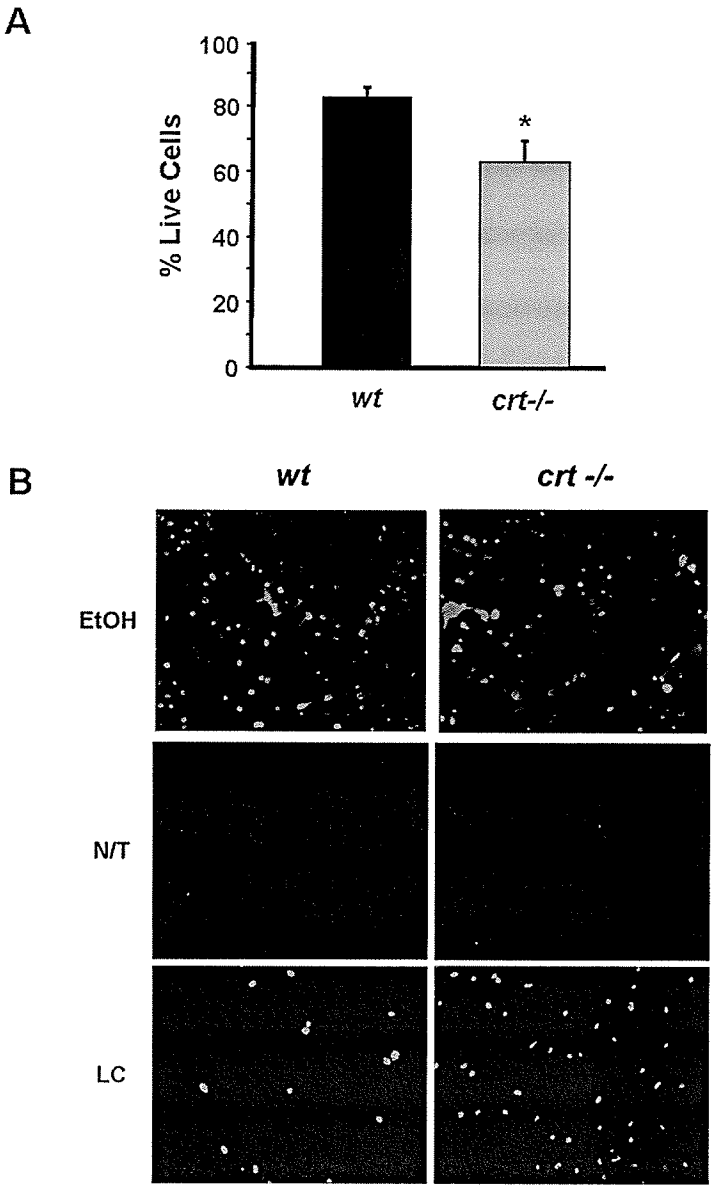


Figure 10. Increased susceptibility of *crt-/-* cells to cell death following treatment with proteasome inhibitor lactacystin. (A) Effect of lactacystin on cell viability. Cells were incubated in the presence or absence of 10 μM lactacystin (LC) for 16 hours followed by analysis with a Live/Dead assay kit. The values are mean ± SE of three separate experiments. * $p<0.05$ significantly different as compared to *wt* cells. **(B)** A representative fluorescent images showing the effect of lactacystin on cell death. *wt* and *crt-/-* cells were treated with 10 μM lactacystin for 12 hours or 70% EtOH (positive control) and the number of dead cells was assessed by labeling the cells with 4 μM ethidium homodimer-1 (Etd-1). The Etd-1 positive dead cells were visualized using a fluorescence microscope (Zeiss AxioScop 2). N/T represent non-treated cells.

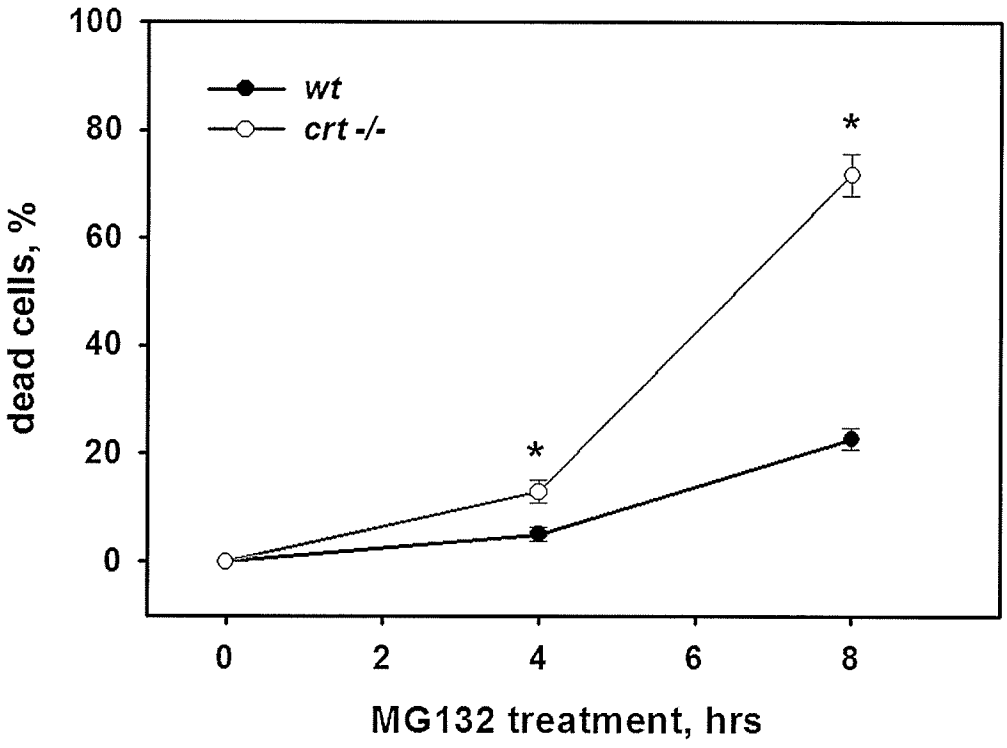


Figure 11. MG132 induced cell death. *crt-/-* cells are more sensitive to a proteasome inhibitor MG132 (50 μM) as measured by Etd-1 incorporation using fluorescence microplate reader. Etd-1 enters cells with damaged membranes and undergoes 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a fluorescence signal (ex/em ~ 495nm/ ~635nm). Etd-1 is excluded by the intact plasma membrane of live cells. Cell death was calculated as a percent of dead cells in MG132 treated *wt* or *crt-/-* cells relative to untreated cells, as described in *Materials and Methods*. The values are mean ± SE of three separate experiments. * $p < 0.05$ as compared to *wt* cells.

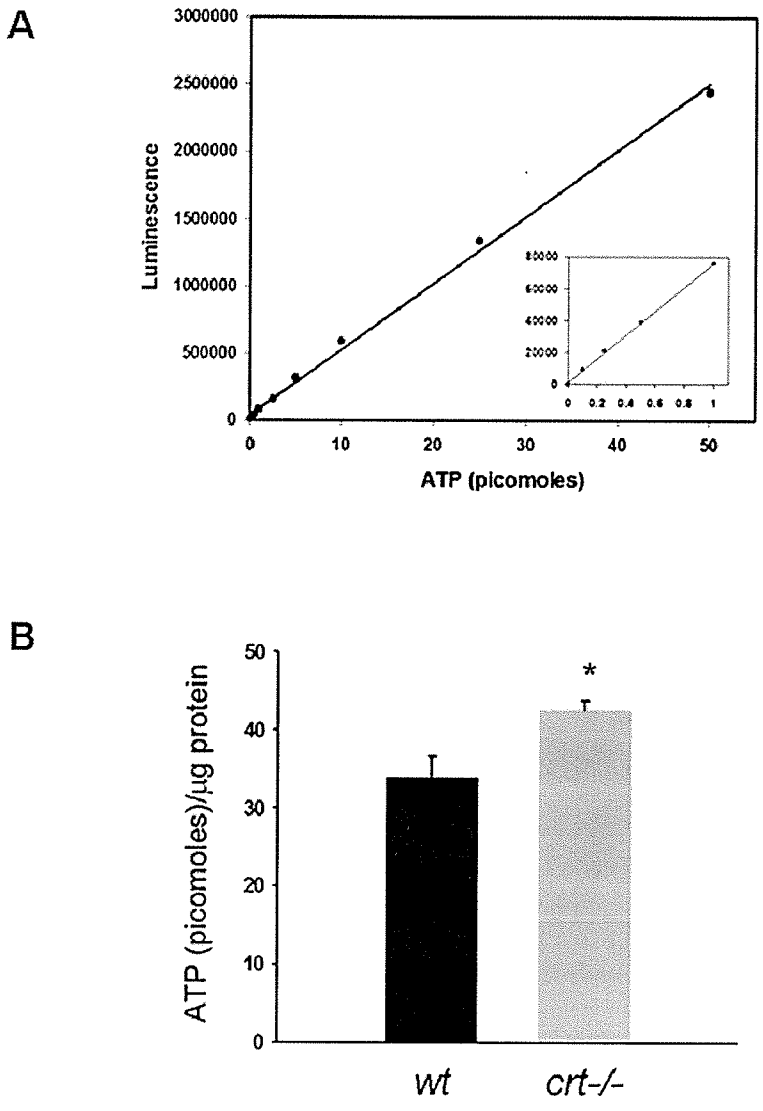


Figure 12. Determination of ATP content in *wt* and *crt-/-* cells. The amount of ATP in the experimental samples was calculated from a standard curve **(A).** Each reaction contained 1.25 µg/ml of firefly luciferase, 50µM D-luciferin, and 1 mM DTT. After a 10 minute incubation, luminescence was measured (arbitrary units). **(B)** ATP levels in *wt* and *crt-/-* cells as normalized to protein content in each sample. The values are mean ± SE of three separate experiments. * $p < 0.05$ as compared to *wt* cells.

levels were significantly higher in *crt-/-* cells as compared to *wt* cells.

1.3. mRNA levels of proteasome subunits in *crt-/-* cells

The increased proteasome activity in *crt-/-* cells could be mediated either by increased expression of the proteasome subunits or increased enzymatic activity of the proteasome complexes. To examine the changes in proteasome subunit expression, we carried out quantitative real-time PCR analysis of seven key proteasome subunits [α5, α6, β3 (component of the 20S complex), Rpt3, Rpt5 (ATPases of the 19S base complex), Rpn3 and Rpn10 (19S lid and base components required for ubiquitin recognition)]. Table 2 summarizes the data from three separate experiments for each subunit. Interestingly, the mRNA levels of α5, α6, β3, Rpt3, and Rpn3 subunits were reduced in *crt-/-* cells relative to *wt* cells (Table 2). Furthermore, there were no significant changes in the Rpt5 and Rpn10 gene expression (Table 2). Similar results were obtained using the semi-quantitative reverse transcriptase assay, where α5, α6, Rpt3, Rpn3 and Rpn10 appeared to be downregulated (Table 2). These results indicate that the increased proteasome activity in *crt-/-* cells is not due to the up-regulation of the mRNA level of different proteasome subunits and a subsequent increase in the number of 26S proteasome complexes. Indeed, a recent report on an ovarian cancer cell line showed that increased activity of the ubiquitin-proteasome pathway, and not increased expression of its components, augments the sensitivity of these cells to undergo apoptosis upon treatment with proteasome inhibitors (Bazzaro et al., 2006). To determine whether decreased mRNA corresponded with decreased protein, the protein levels of several proteasomal subunits (where

Table 2. Real time qPCR and reverse transcriptase PCR expression analysis of proteasomal subunits.

Gene/subunit	Relative mRNA expression levels	
	Real Time qPCR	Reverse Transcription PCR
	Ratio *crt-/-* / *wt*	Ratio *crt-/-* / *wt*
PSMA1/α6/C2	0.77 ± 0.10	0.65 ± 0.05
PSMA5/α5/zeta	0.73 ± 0.06	0.67 ± 0.10
PSMB3/β3/C10	0.52 ± 0.12	0.82 ± 0.19
PSMC3/Rpt5/S6a	0.92 ± 0.15	0.96 ± 0.00
PSMC4/Rpt3/S6b	0.73 ± 0.06	0.84 ± 0.09
PSMD3/Rpn3/S3	0.55 ± 0.09	0.69 ± 0.14
PSMD4/Rpn10/S5a	0.92 ± 0.16	0.83 ± 0.05

The values are given as normalized target gene expression of *crt-/-* versus *wt* cells ($2^{-\Delta\Delta ct}$, see *Materials and Methods*). The values are mean ± SE of three separate experiments.

antibodies for the mouse protein were commercially available) were determined in *wt* and *crt-/-* cells by Western blot analysis (Figure 13). As shown in Figure 13, there was no significant difference in the amount of Rpt3 (a subunit of 19S complex) or α/β subunits (20S complex). However, the level of the p44S10 protein (another subunit of the 19S complex) was slightly lower in *crt-/-* as compared to *wt* cells. These data suggest that the elevated proteasome activity observed in the *crt-/-* cells is not due to increased levels of the mRNAs encoding the proteasomal subunits or an increased levels of proteasomal proteins.

1.4. Mechanism of regulation of proteasome activity in *wt* and *crt-/-* cells

The increased proteasome activity in calreticulin deficient cells could be due to induction of ER stress or up-regulation of other ER chaperones (e.g. BiP or Calnexin) which have been reported to occur in *crt-/-* cells (Knee et al., 2003). First, we examined the effect of ER stress on proteasome activity. As shown in Figure 14, treatment with thapsigargin, that selectively inhibits the ER Ca^{2+}-ATPase and lowers calcium levels inside the ER (Rogers et al., 1995), had no effect on proteasome activity in *wt* or *crt-/-* cells. Furthermore, treatment of *wt* cells with tunicamycin, an inhibitor of N-linked glycosylation (Kuo and Lampen, 1974), caused only a slight increase (less than 10%) in the total proteasome activity (Figure 14). However, proteasome activity was significantly increased in *crt-/-* cells following tunicamycin treatment (Figure 14). Similar changes in proteasomal activity were observed when these cells were treated with brefeldin A, an agent that disrupts the Golgi structure and interrupts protein trafficking (Fujiwara et al.,

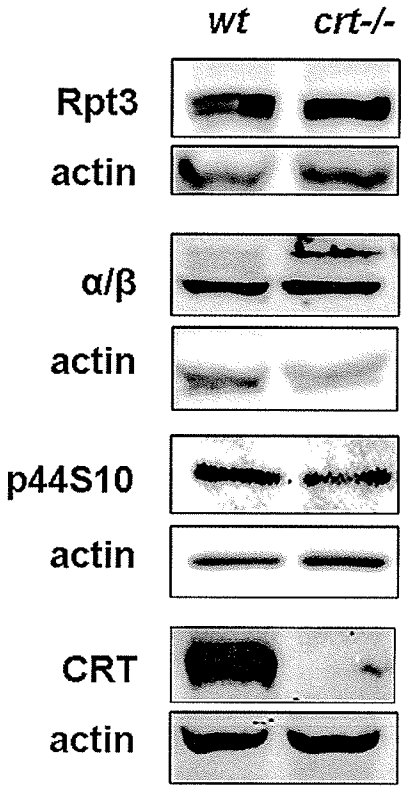


Figure 13. Analysis of expression of proteasomal subunits in *wt* and *crt-/-* cells. Western blots were carried out using cell lysates from *wt* and *crt-/-* cells and antibodies specific to Rpt3, α/β subunits, p44S10 and CRT. The same blots were stripped and probed with antibody to actin as a loading control. α/β subunits represent the 20S complex (core of the proteasome), while Rpt3 and p44S10 are subunits of 19S component (the lid part of the 26S proteasome). Images showing a representative western blot for each antibody from two separate experiments.

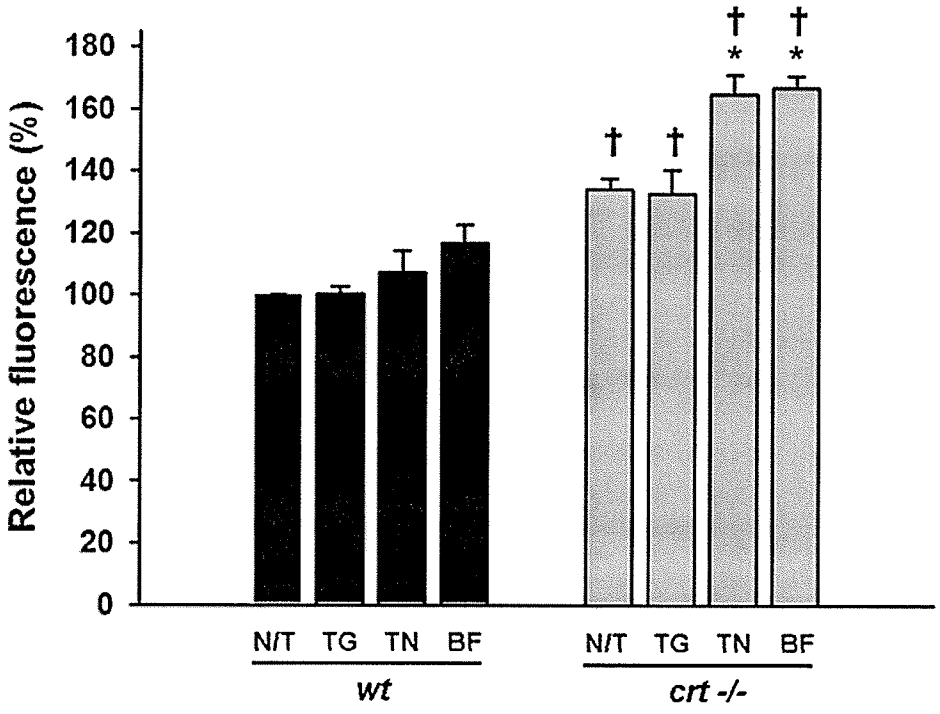


Figure 14. Changes in the proteasome activity following induction of ER stress. Chymotrypsin like activity of the proteasome was measured by first treating the cells with 0.1 μM thapsigargin (TG) 1 hour, 1μg/ml tunicamycin (TN) 3 hours, or 7 μM brefeldin A (BF) 3 hours, followed by preparation of cell lysate and proteasome assay (as described in *Materials and Methods*). The values are mean ± SE of three separate experiments. * $p<0.05$ significantly different as compared to untreated cells (N/T). † $p<0.05$ significantly different as compared to corresponding *wt* cells.

1988) (Figure 14). Next, we examined the effect of BiP and calnexin overexpression on proteasome function. *Wt* cells were transiently transfected with plasmids containing *myc* tagged BiP or calnexin. As shown in Figure 15, overexpression of either BiP or calnexin induced a slight increase (less than 10%) in proteasome activity in *wt* cells.

To further establish the mechanism of regulation of proteasome activity in *crt-/-* cells, we tested the two primary functions of calreticulin (a regulator of Ca^{2+} homeostasis and an ER chaperone (Michalak et al., 1999)). Recently, Ca^{2+} has been shown to activate the 20S proteasome *in vitro* (Reshetnyak et al., 2004). Moreover, intrinsic proteasome activity was shown to be significantly elevated upon treatment of L6 muscle cells with the Ca^{2+} ionophore A23187 (Menconi et al., 2004). Therefore, we tested whether the Ca^{2+} regulatory function of calreticulin can modulate proteasome activity. A previous report by Nakamura et.al. showed that the resting free cytosolic Ca^{2+} concentration in *crt-/-* cells is significantly higher than in *wt* cells (Nakamura et al., 2001). These cells also have a lower capacity for Ca^{2+} storage in the ER, although the free Ca^{2+} concentration in the ER is not changed (Nakamura et al., 2001). Therefore, we examined the effect of increasing cytosolic Ca^{2+} on the modulation of proteasome activity. Figure 16A shows that treating *wt* and *crt-/-* cells with the Ca^{2+} ionophore ionomycin induced a significant increase in the proteasome activity of *wt* cells but did not change the proteasome activity of *crt-/-* cells. This differential effect of ionomycin could be due to the higher basal level of cytosolic Ca^{2+} in *crt-/-* cells (~50% higher) which contributes to the elevated basal proteasomal activity and thus an ionophore could not induce any further increase. To determine whether Ca^{2+} induced changes in proteasome activity was due to the alterations in the function of the 26S proteasome complexes, we treated *wt* cells with the Ca^{2+}

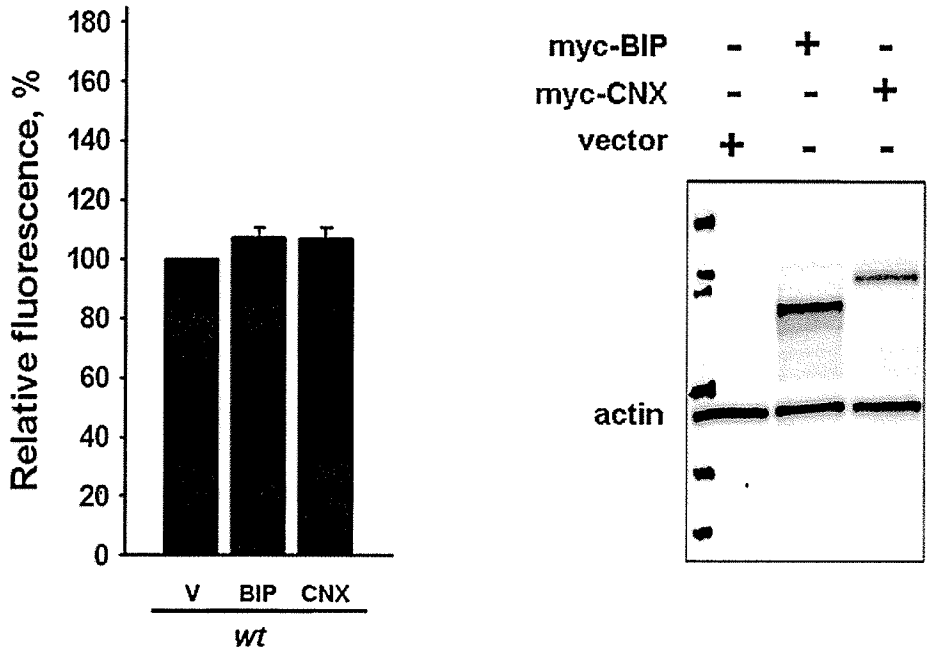


Figure 15. Overexpression of the ER chaperones, BiP or calnexin (CNX), in *wt* cells does not affect proteasome activity. *wt* cells were transiently transfected with *myc*-BiP or *myc*-CNX. After 48 hours cells were lysed and the proteasome activity was assayed. A representative western blot with anti-*myc* and anti-actin antibodies shows the level of expression of *myc*-BiP, *myc*-CNX and actin in the same cell lysate used to measure proteasome activity. Bar graphs are mean ± SE of three separate experiments.

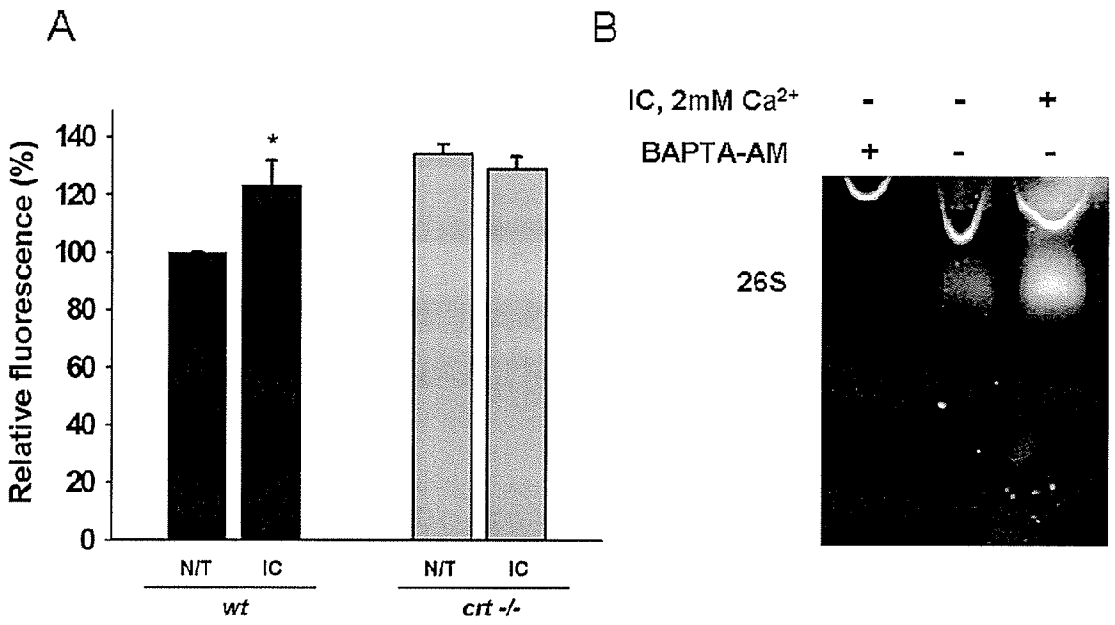


Figure 16. Effect of changes in intracellular Ca^{2+} on proteasome activity. (A) Chymotrypsin like activity of the proteasome was measured upon treatment of cells with 6 μM ionomycin (IC) or buffer control (N/T) in the presence of 2mM Ca^{2+} for 2 hours. Values are expressed as percentage of the proteasome activity in the *wt* cells. The values are mean ± SE of three separate experiments. * $p<0.05$ significantly different as compared to non-treated cells. **(B)** Effect of intracellular Ca^{2+} on the activity of 26S proteasome in *wt* cells was examined using substrate overlay assay. To chelate intracellular Ca^{2+}, cells were incubated with 50 μM BAPTA-AM in the presence of 0.02% pluoronic acid and 250μM sulfinpyrazone for 30 min. To increase cytosolic Ca^{2+}, cells were treated with 2 μM ionomycin in the presence of 2mM Ca^{2+} for 3 hours. Cell lysates isolated after each treatment were resolved on a nondenaturing gel followed by overlay assay (using Suc-LLVY-AMC as a substrate). The loading wells are cut off. The curved bands on top of the gel is a result of autofluorescence of a white precipitate formed on the gel. Gel image is a representative of three separate experiments.

chelator BAPTA-AM or the Ca^{2+} ionophore ionomycin, followed by a substrate overlay assay (Figure 16B). Our results demonstrated that chelation of intracellular Ca^{2+} by BAPTA-AM abolishes 26S proteasome activity (Figure 16B first lane), while elevation of intracellular Ca^{2+} mediated by ionomycin significantly increases the activity (Figure 16B last lane). Next, to determine whether changes in intracellular Ca^{2+} affect the localization of the proteasomes, thus changing their activity, we carried out immunocytochemical staining. Anti- α/β (20S) and anti-Rpt3 (19S) antibodies were used to locate proteasomal complexes in *wt*, *crt-/-* and ionomycin treated *wt* cells. Our results illustrate that the overall increase in proteasome activity in *crt-/-* cells, as well as the effect of increased cytosolic Ca^{2+} on proteasome activation in *wt* cells, is not due to changes in the localization of the 20S and 26S proteasome complexes (Figure 17).

Expression of exogenous calreticulin in *crt-/-* cells has been shown to decrease the basal cytosolic Ca^{2+} concentration close to that of *wt* cells (Nakamura et al., 2001). Thus we examined whether reintroduction of calreticulin in *crt-/-* cells has any effect on proteasome activity. As shown in Figure 18, calreticulin transfected *crt-/-* cells (CRT+*crt-/-*) showed significantly lower proteasome activity, as compared to the empty vector transfected *crt-/-* cells (110±2% for CRT+*crt-/-* vs. 134±3 for vector+*crt-/-*). Proteasome activity in CRT+*crt-/-* cells (110%) was still slightly higher than that of *wt* cells (100%, not shown), likely due to the fact that the expression level of calreticulin in CRT+*crt-/-* cells was less than that of *wt* cells (Fig. 19A). In addition, to ascertain the Ca^{2+} buffering role of the re-introduced calreticulin on proteasome function, we transfected *crt-/-* cells with calsequestrin (Figure 18, lower panel). Calsequestrin is a Ca^{2+} binding protein localized to the muscle sarcoplasmic reticulum. It is similar to calreticulin

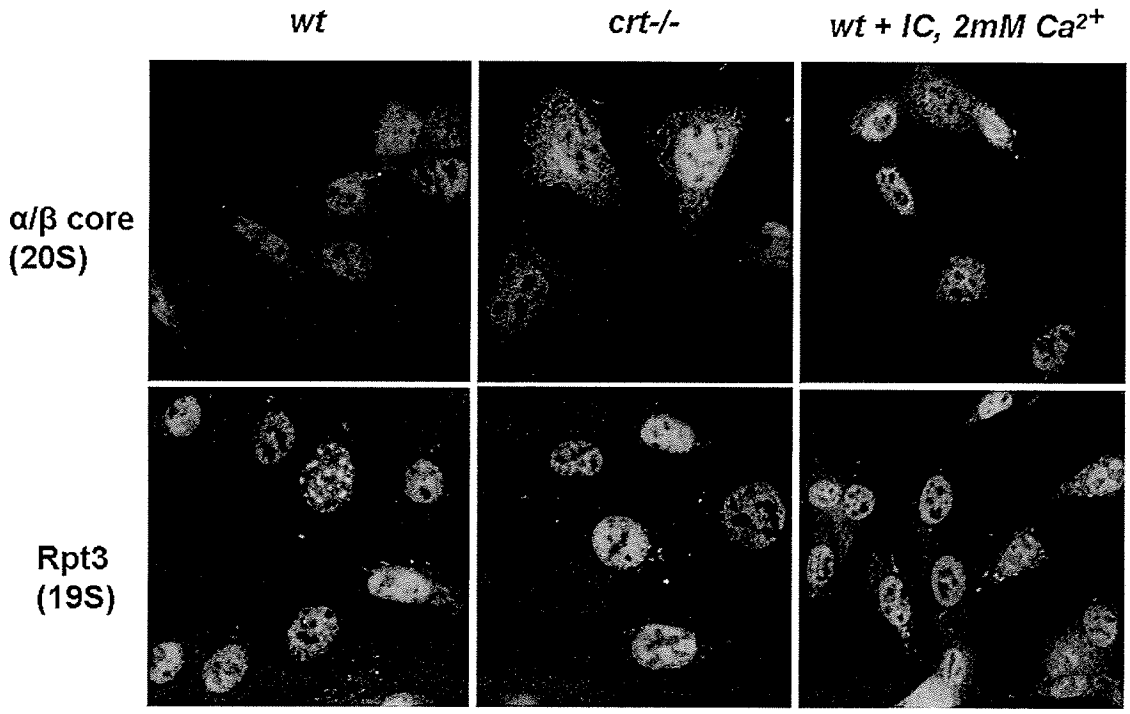


Figure 17. Localization of 20S proteasomal complexes and 19S regulatory subunits in *wt*, *crt-/-* and *wt* cells treated with calcium ionophore (IC). Immunofluorescent staining was carried out as described in *Materials and Methods* using anti-α/β antibodies for 20S and anti-Rpt3 for 26S proteasomes. The effect of increased intracellular Ca^{2+} on proteasome localization was examined by treating *wt* cells with 2 μM ionomycin in the presence of 2mM Ca^{2+} for 3 hours followed by immunocytochemical staining.

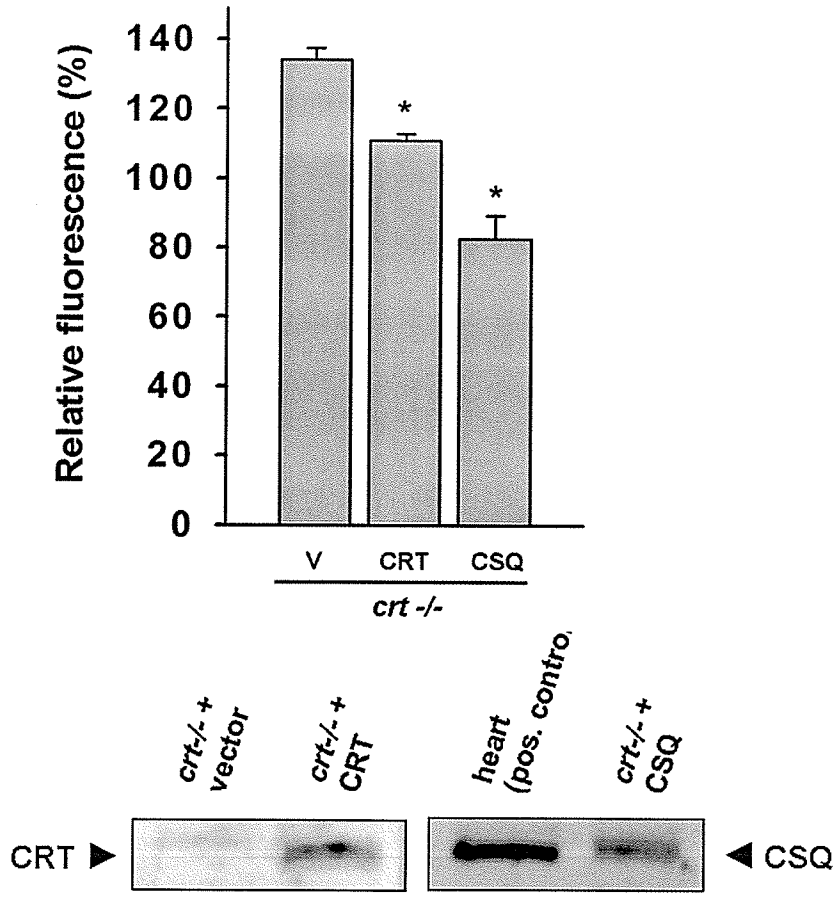


Figure 18. Effect of ectopically expressed calreticulin or calsequstrin on the proteasome activity in *crt-/-* cells. Chymotrypsin like activity of the proteasome was measured in *crt-/-* cells transiently transfected with a plasmid containing HA-calreticulin (CRT), calsequestrin (CSQ) or the empty vector (V). The lower panel is western blots with antibodies to calreticulin and calsequestrin showing the expression of the exogenous protein in the *crt-/-* cells. Mouse heart sample was used as a positive control for calsequestrin expression. The bar graph values are mean ± SE of four separate experiments expressed as percentage of *wt* cells. * $p<0.05$ significantly different as compared to vector transfected cells.

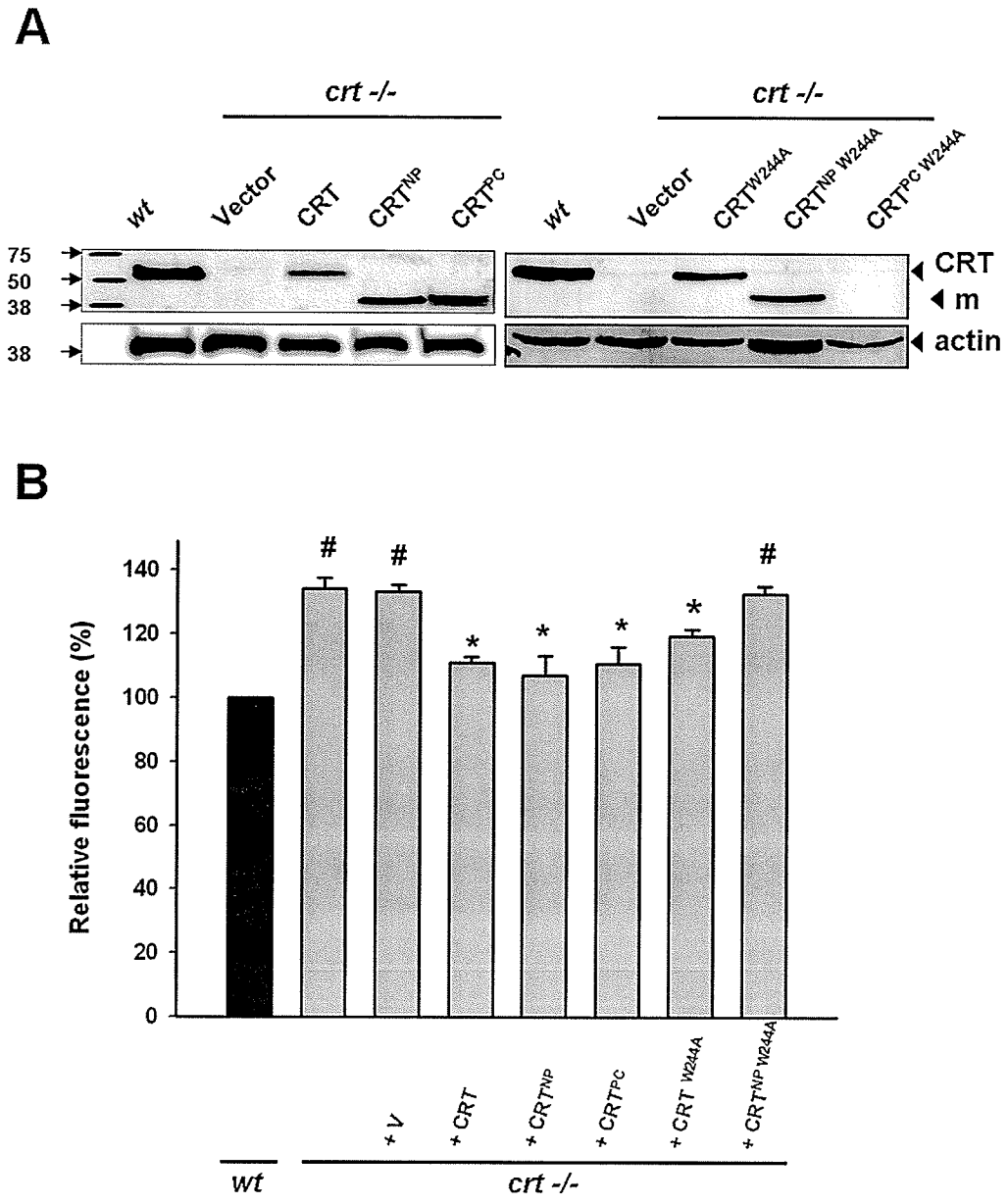


Figure 19. Changes in the proteasome activity upon reintroduction of calreticulin or deletion mutants of calreticulin in *crt-/-* cells. (A) Western blot analysis with anti-calreticulin antibody of the *wt*, *crt-/-* cells and *crt-/-* cells transiently transfected with expression vectors encoding calreticulin (CRT-*crt-/-*), calreticulin deletion mutants or point mutants of calreticulin (CRT^{NP}, CRT^{PC}, CRT^{W244A}, $CRT^{NP\text{-}W244A}$ and $CRT^{PC\text{-}W244A}$). 'm' represents the location of the calreticulin deletion mutants. Panel **(B)** shows proteasome activity in the *wt*, *crt-/-* or *crt-/-* transfected with (V), CRT, CRT^{NP}, CRT^{PC}, CRT^{W244A} or $CRT^{NP\text{-}W244A}$ cell lysates. The levels of activity were compared relative to non-transfected *crt-/-* cells. Bar graphs are the mean ± SE of three separate experiments. * $p<0.05$ significantly different as compared to the vector-transfected *crt-/-* cells. # $p<0.05$ significantly different as compared to the *wt* cells

in Ca^{2+} regulation, but unlike calreticulin it does not have a chaperone function (Beard et al., 2004). Calsequestrin is not endogenously expressed in MEF cells. Interestingly, ectopically expressed calsequestrin was able to significantly decrease the proteasome activity (82±6% for CSQ+*crt-/-*) in *crt-/-* cells (Figure 18), suggesting the importance of Ca^{2+} in the regulation of proteasome activity.

To differentiate between the Ca^{2+} buffering capacity (located in the C-domain of calreticulin) and the lectin-like chaperone (P-domain of calreticulin) function of the reintroduced calreticulin on proteasome function, we explored the effect of different calreticulin domains and their ability to modulate the activity of the ubiquitin-proteasome pathway. We used calreticulin deletion mutants that lack either the C-domain (CRT^{NP}) or the N-domain (CRT^{PC}) (Nakamura et al., 2001). Calreticulin mutants (CRT^{NP} and CRT^{PC}) or full length calreticulin were introduced into *crt-/-* cells and the resulting proteasome activity was measured (Figure 19). As shown in Figure 19B, both deletion mutants of calreticulin, CRT^{NP} (C-domain deletion) and CRT^{PC} (N-domain deletion) were able to reduce proteasome activity to that observed in CRT+*crt-/-* cells. The vector alone had no effect on proteasome activity (Figure 19B). All of the three expressed proteins (calreticulin, CRT^{NP} and CRT^{PC}) contain the P-domain of calreticulin, which suggest a role for this domain in the observed reduction of proteasome activity in the transfected *crt-/-* cells. The P-domain contains both the high affinity Ca^{2+} binding site and the lectin-like chaperone site of calreticulin (Michalak et al., 1999). Therefore, we examined the role of this domain in modulating proteasome activity by site directed mutation of Trp244 (W244A). This mutation has been shown to disrupt the chaperone function of calreticulin and its ability to bind ERp57 (Martin et al., 2006). Figure 19A

(top panel) shows that transfection of *crt-/-* with CRT^{W244A} or $CRT^{NP\text{-}W244A}$ resulted in the expression of proteins of the expected size. However, several attempts to express $CRT^{PC\text{-}W244A}$ in *crt-/-* cells failed to produce protein at the expected size, which could be attributed to decreased stability of this mutant protein. As shown in Figure 19B, expression of $CRT^{NP\text{-}W244A}$ (the function of P-domain is disturbed and C-domain is deleted) in *crt-/-* did not alter proteasome activity as compared to empty vector transfected *crt-/-* cells. On the other hand, introduction of CRT^{W244A} (the function of P-domain is disturbed while C-domain is present) resulted in decreased proteasome activity when compared to the empty vector transfected *crt-/-* cells. However, proteasome activity of *crt-/-* cells transfected with CRT^{W244A} was higher than in cells transfected with the native calreticulin. Overall these data suggest that both the P and C-domains of calreticulin are important for calreticulin's role as a modulator of proteasome activity.

1.5. Effect of calnexin depletion on proteasome activity

Calreticulin and calnexin are homologous ER chaperones which play an important role in the quality control of N-glycosylated protein folding (Helenius and Aebi, 2004). In *crt-/-* cells, the calnexin gene expression is increased (2.14 ± 0.41, Table 3) leading to increased calnexin protein levels (Figure 20, western blot and Knee et al., 2003). Increased calnexin expression in these cells could be a mechanism to further activate the UPR pathway in order to compensate for the absence of calreticulin. Therefore, we examined the effect of down regulation of calnexin on proteasome activity in these cells. Transfection of *wt* and *crt-/-* cells with an siRNA specific for calnexin significantly

decreased the calnexin protein level (by 60%) in both cell types as compared to cells transfected with non-targeting control siRNA-A (Figure 20, western blot). siRNA mediated partial downregulation of the calnexin protein in *wt* cells did not affect proteasome activity (Figure 20, bar graph). In contrast, downregulation of the calnexin protein with siRNA in *crt-/-* cells induced a further increase in proteasome activity (Figure 20, bar graph), suggesting that the loss of both lectin-like chaperones has a cumulative effect on the activation of proteasome activity. Our data in Figure 19 show that cells with partial expression of calreticulin (*crt-/-* + CRT) do not demonstrate any significant difference in proteasome activity as compared to *wt* cells. Similarly, it could be that the siRNA mediated decrease in calnexin levels in *wt* cells was not sufficient to trigger any significant changes in proteasome function. Therefore, we examined differences in proteasome activity upon complete loss of calnexin function using MEF cells isolated from homozygous calnexin knockout embryos (*cnx-/-*). As shown in Figure 21, total absence of calnexin protein expression triggers proteasome activity, mediating an even higher increase in its proteolytic function compared to calreticulin deficient MEF cells. The observed difference in proteasome activity between calnexin knock-down (siRNA) and *cnx-/-* cells could be due to different levels of the calnexin protein in these cells. Indeed, reintroduction of calnexin into *cnx-/-* cells by transient transfection partially restored its expression levels (Figure 21, western blot) and decreased proteasome activity close to that of *wt* cells (Figure 21).

Next, we examined the level of total ubiquitinated proteins in calnexin knockout cells. *wt* and *cnx-/-* cells were pre-treated with the MG132 proteasome inhibitor, followed by a ubiquitin pulldown assay. As shown in Figure 22, the levels of total ubiquitinated

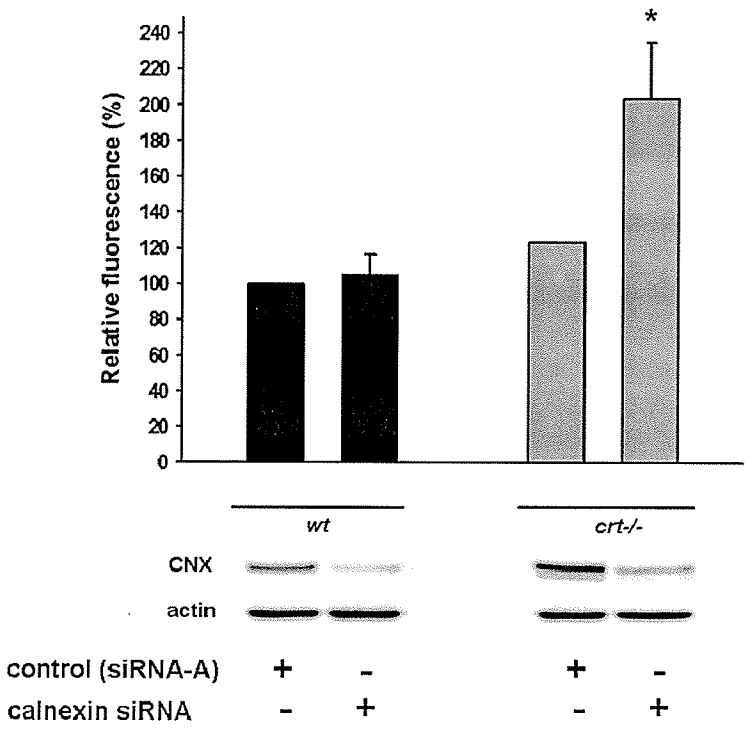


Figure 20. Effect of calnexin (CNX) depletion on proteasome activity in *wt* and *crt-/-* cells. Cells were transfected with an siRNA to mouse calnexin or a control non-targeting siRNA (siRNA-A). 24 hours after transfection, cells were lysed and the proteasome activity was measured (bargraph). The lower panel is a western blot showing the level of cellular calnexin after transfection with calnexin-siRNA or control siRNA. * $p<0.05$ significantly different as compared to siRNA-A transfected *crt-/-* cells.

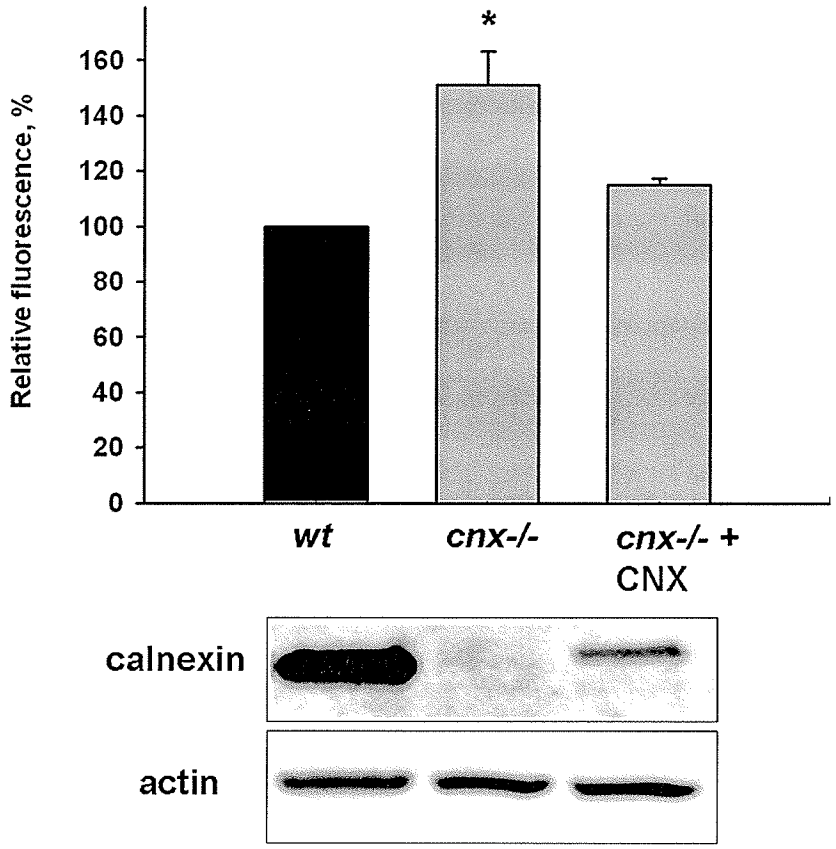


Figure 21. Changes in proteasome activity in *cnx-/-* cells as compared to *wt* cells. Chymotrypsin like activity was measured by monitoring the release of free AMC (amidomethylcoumarin) from the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC (Ex.: 380 nm; Em.: 460 nm). The values are mean ± SE of three separate experiments carried out in duplicates. * $p<0.05$ significantly different as compared to *wt* cells. Lower panel shows the expression levels of calnexin in these cells.

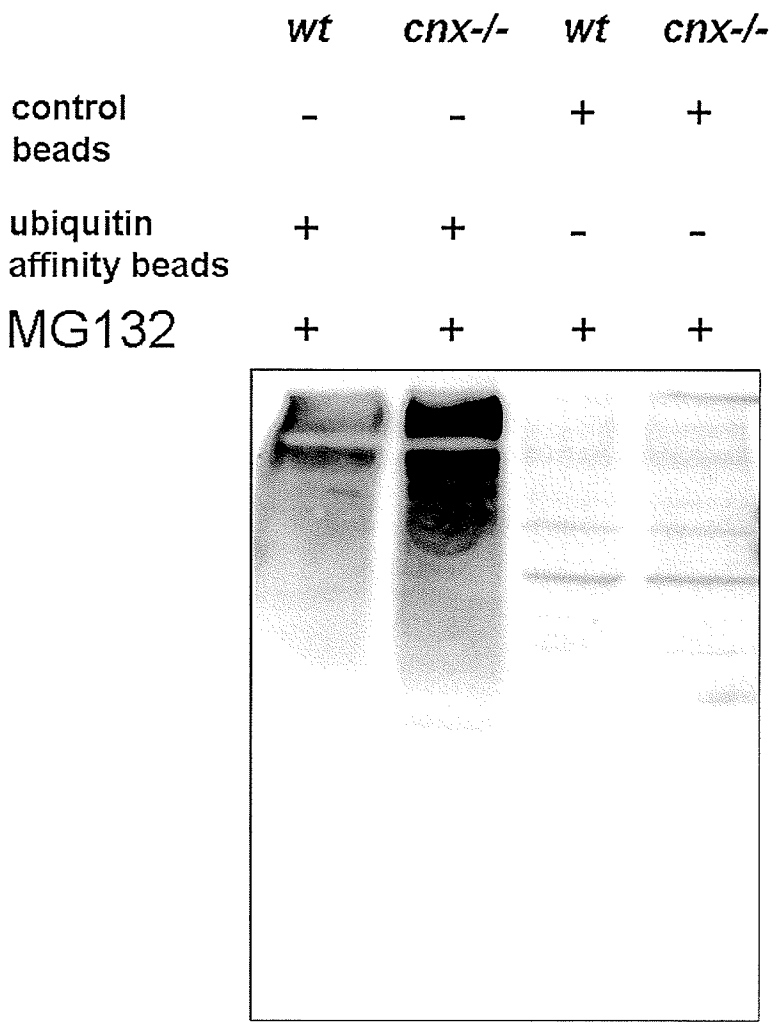


Figure 22. Increased level of ubiquitinated proteins in *cnx-/-* cells following proteasome inhibition. *wt* and *cnx-/-* cells were treated with 50 μM MG132 for 4 hours, followed by lysis as described in Materials and Methods. Ubiquitinated proteins were enriched using ubiquitin affinity beads. Proteins were then resolved on 10% SDS page and followed by western blot with anti-ubiquitin antibody. Western blot image is a representative of three separate experiments.

proteins in *cnx-/-* cells treated with MG132 were significantly higher than in *wt* cells, similar to calreticulin deficient cells (Figure 6). These data illustrate that the absence of either chaperone leads to increased activity of ubiquitin-proteasome pathway. Furthermore, depletion of luminal calreticulin and/or calnexin activates the ubiquitin-proteasome pathway.

2. LOSS OF CALRETICULIN INCREASES THE RATE OF ERAD

2.1. Inhibition of α1,2-mannose trimming in the ER lumen significantly lowers proteasome function

Translocation of misfolded proteins across the ER membrane regulates the availability of substrates for ERAD and therefore could be a limiting factor in this process. To enter ERAD the misfolded glycoproteins must dissociate from calnexin, a process which requires mannose trimming by α1,2-mannosidase I (Hosokawa et al., 2001). To examine the role of this process in proteasome regulation in *crt-/-* cells we used kifunensine, a specific inhibitor of α1,2-mannose residue trimming from N-glycans that was previously shown to suppress ER associated protein degradation (Tokunaga et al., 2000). Figure 23 shows that kifunensine treatment significantly reduced proteasome activity in both *wt* and *crt-/-* cells. Furthermore, kifunensine treated *crt-/-* cells have similar levels of proteasome activity as that of the non-treated *wt* cells (Figure 23). These data are supportive of a direct functional link between two separate stages of the ERAD pathway, translocation and degradation.

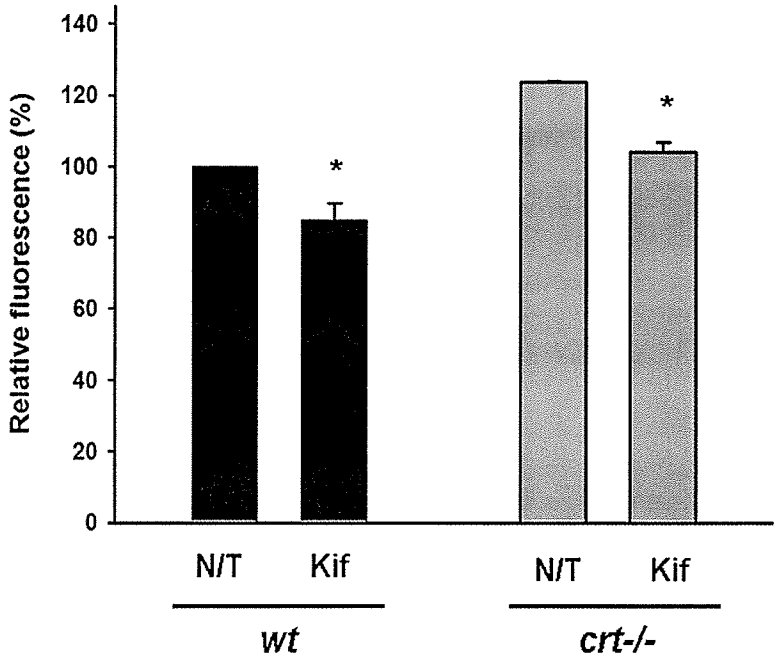


Figure 23. Effect of kifunensine on proteasome activity in *wt* and *crt-/-* cells. Kifunensine (Kif) was added to the cell cultures at a final concentration of 5 μg/ml. 4 hours later cells were lysed and proteasome activity was measured. The values are mean ± SE of three separate experiments. * $p<0.05$ significantly different as compared to non-treated cells (N/T).

2.2. Increase in transcription levels of major lumen/cytoplasm dislocation components in *crt-/-* cells

Efficient removal of misfolded proteins from the lumen of the ER of *crt-/-* cells requires enhanced recognition of misfolded proteins as well as their more efficient translocation from the ER to the cytoplasm, which could be achieved via increased expression of the proteins involved in these process, namely calnexin, EDEM and Hrd1. Calnexin and EDEM are lectins required for the recognition and dislocation of terminally misfolded glycoproteins, while Hrd1 is a multi-spanning RING-finger quality control ubiquitin ligase, assisting in the retrograde translocation of misfolded proteins. To date, three isoforms of EDEM (EDEM1, EDEM2 and EDEM3) have been isolated (Hirao et al., 2006; Oda et al., 2003; Olivari et al., 2005). Real-time PCR using mRNA isolated from *wt* and *crt-/-* cells revealed a significant increase in the mRNA levels of calnexin, EDEM1 and Hrd1 in *crt-/-* cells (Table 3). However, no changes were observed in the levels of EDEM2 and EDEM3. Interestingly, the loss of calnexin function in *cnx-/-* cells did not result in significant changes in the mRNA levels of Hrd1, EDEM1, EDEM2 and EDEM3 (Table 3). Furthermore, Molinari et.al. showed that there is no change in calreticulin expression in *cnx-/-* cells as compared to *wt* cells (Molinari et al., 2004).

Table 3. Real time RT-PCR analysis of proteins involved in ERAD proof-reading and protein translocation.

Gene	Relative mRNA expression	
	ratio *crt-/-* / *wt*	ratio *cnx-/-* / *wt*
Calnexin	2.14 ± 0.41 *	N/A
Hrd1	1.35 ± 0.1 *	0.77 ± 0.23
EDEM1	1.64 ± 0.16 *	1.25 ± 0.15
EDEM2	1.05 ± 0.13	1.22 ± 0.21
EDEM3	0.80 ± 0.11	1.14 ± 0.27

The values are given as normalized target gene expression of *crt-/-* (or *cnx-/-*) versus *wt* cells ($2^{-\Delta\Delta ct}$, see *Materials and Methods*). The values are mean ± SE of three separate experiments. * $p < 0.05$ significantly different as compared to *wt* cells.

2.3. Enhanced degradation of misfolded hA1AT NHK in *crt-/-* and *cnx-/-* cells

To examine whether calreticulin/calnexin depletion triggers any changes in the process of ERAD of misfolded glycoproteins, *crt-/-* and *cnx-/-* cells were transfected with the human α1-antitrypsin mutant (A1AT NHK) cDNA. A1AT is a serum glycoprotein belonging to the serine protease inhibitor superfamily and is synthesized primarily by hepatocytes. Mutations in A1AT lead to the development of chronic obstructive pulmonary disease, emphysema, liver cirrhosis and other severe pathologies (Carrell and Lomas, 2002). A1AT NHK is terminally misfolded and is therefore a secretion incompetent variant of A1AT (Sifers et al., 1989). A1AT NHK is not able to pass the quality control checkpoint and is thus subjected to proteasomal degradation (Hosokawa et al., 2003). Overexpression of human ER mannosidase I or EDEM was shown to accelerate the degradation of A1AT NHK, while kifunensine (ER mannosidase I inhibitor) completely inhibited its degradation (Hosokawa et al., 2003; Hosokawa et al., 2001). These results indicate that the A1AT NHK mutant is a valuable tool to assess changes in the ERAD process and its major components. Using this mutant, we determined whether cells deficient in calreticulin or calnexin display any changes in the rate of ERAD. *crt-/-* and *cnx-/-* cells were transiently transfected with A1AT NHK, pulse labelled with [^{35}S]-Met, Cys and then chased with unlabeled Met, Cys mixture for up to 2 hours. A1AT NHK was immunoprecipitated from the cell lysates and its levels were examined by autoradiography. As shown in Figures 24 and 25, in *wt* cells A1AT NHK protein was stable for 1 hour of chase, after which the level of radiolabelled protein was reduced by 10-20%. In contrast, loss of either chaperone, calreticulin (Figure 24) or

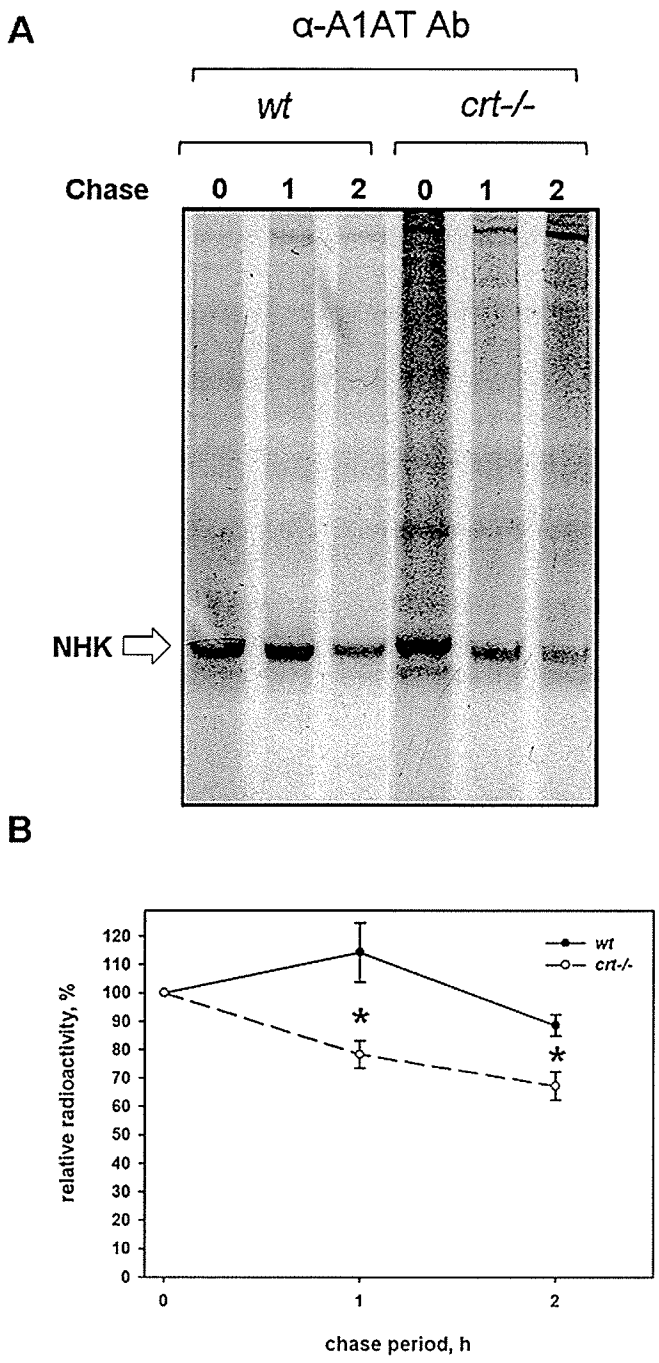


Figure 24. Degradation of A1AT NHK in *wt* and *crt-/-* cells. Panel **(A)** shows a representative autoradiogram of the immunoprecipitates using an antibody against the A1AT protein. **(B)** The intensity of bands corresponding to A1AT NHK from the autoradiogram was quantified and plotted according to chase time. The radioactivity of NHK at 0 hours chase was set arbitrarily to 100% The values are mean ± SE of three separate experiments. * $p < 0.05$ as compared to *wt* cells.

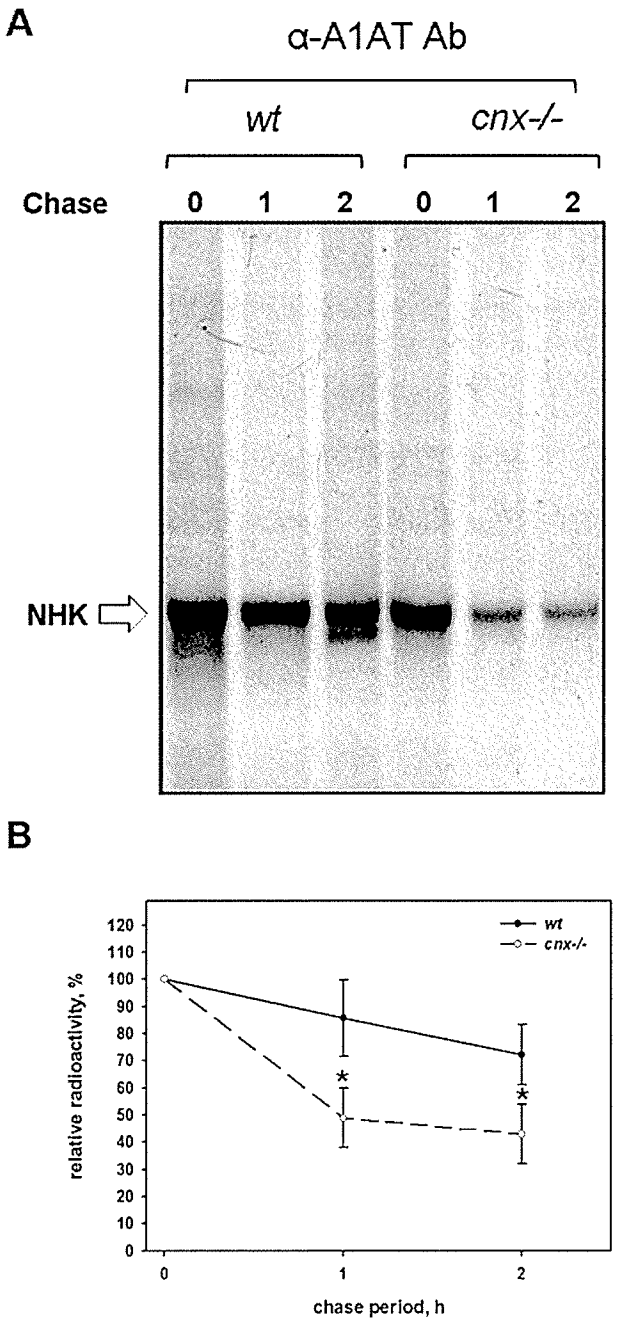


Figure 25. Degradation of A1AT NHK in *wt* and *cnx-/-* cells. Panel **(A)** shows a representative autoradiogram of the immunoprecipitates using an antibody against the A1AT protein. **(B)** The intensity of bands corresponding to A1AT NHK from the autoradiogram was quantified and plotted according to chase time. The radioactivity of NHK at 0 hours chase was set arbitrarily to 100% The values are mean ± SE of three separate experiments. * $p < 0.05$ as compared to *wt* cells.

calnexin (Figure 25) resulted in a rapid (1 hour) decrease in the A1AT NHK protein. Moreover, the rate of decrease of the A1AT NHK protein was faster in the absence of calnexin, with more than 50% of A1AT NHK been degraded after 1 hour of chase (Figure 25), as compared to a 20% decrease in the absence of calreticulin (Figure 24). Overall, our data demonstrate that there is a higher rate of degradation of terminally misfolded A1AT in cells with compromised folding machinery (*crt-/-* and *cnx-/-* cells), although it seems that the loss of calnexin has a more profound effect on the rates of NHK degradation. This could be due to the fact that calnexin functions not only as a chaperone for newly synthesized glycoproteins, but is also a part of the multisubunit gp78-p97/VCP-Derlin1-calnexin complex that is required for ERAD regulation (Ballar et al., 2007). Furthermore, loss of calreticulin function leads to the upregulation of calnexin expression (Knee et al., 2003), which may provide some compensation, while calnexin deletion does not affect calreticulin expression (Coe et al., 2008).

Next, we examined the effect of calreticulin overexpression on NHK stability. HEK 293 cells were stably transfected with A1AT NHK and then transiently transfected with the calreticulin-HA expression vector. Figure 26 shows that the rate of NHK degradation is diminished in cells overexpressing calreticulin as compared to HEK 293 cells transfected with the empty vector. Our data suggest that the level of ER luminal calreticulin/calnexin can affect the rate of degradation of misfolded glycoproteins (ex. NHK) going through the quality control pathway.

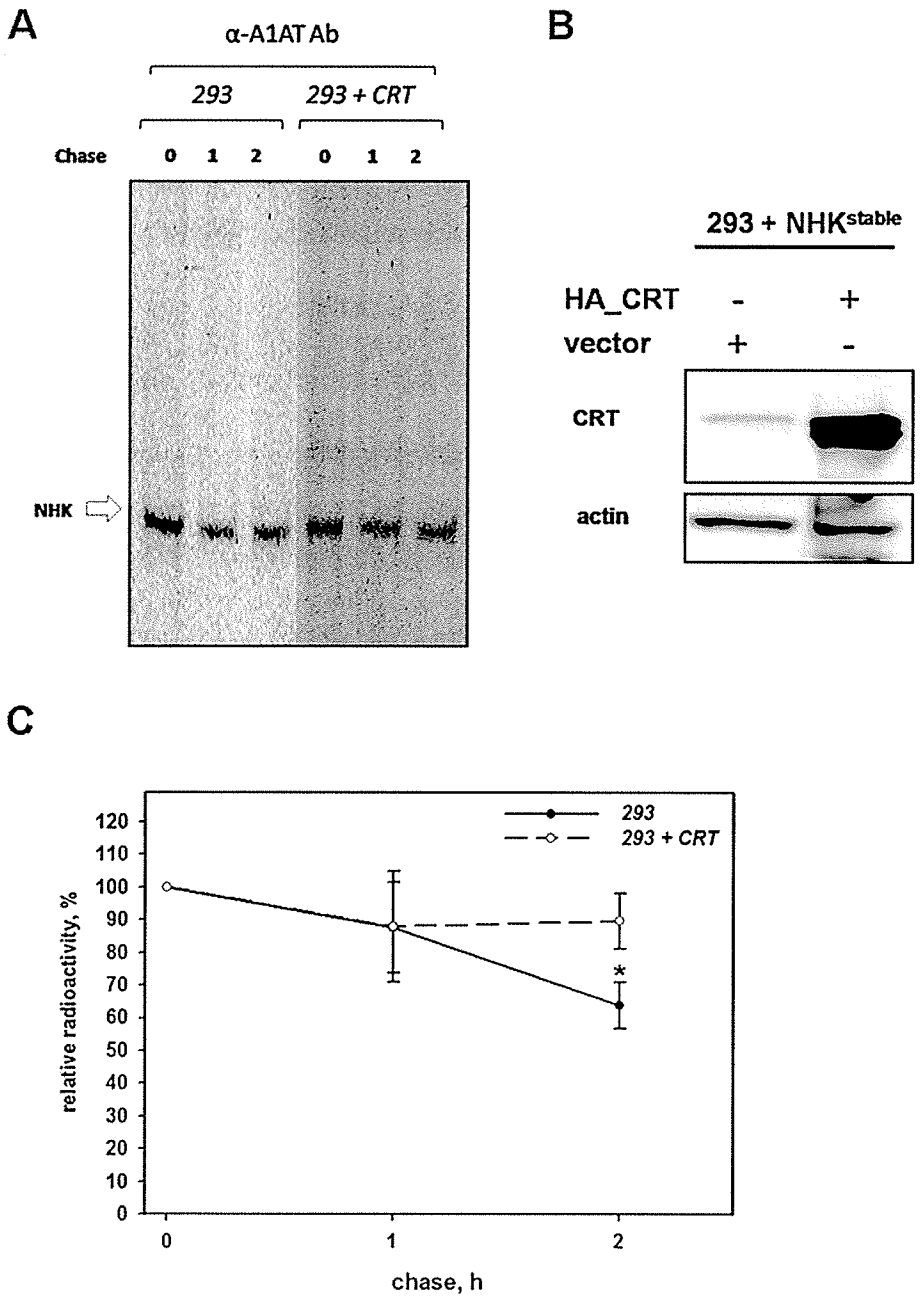


Figure 26. Degradation of A1AT NHK in HEK 293 and HEK 293+CRT cells. Panel **(A)** shows a representative autoradiogram of the immunoprecipitates using an antibody against the A1AT protein. **(B)** Western blot analysis showing calreticulin expression levels in HEK 293 cells stably transfected with CRT. **(C)** The intensity of bands corresponding to A1AT NHK from the autoradiogram was quantified and plotted according to chase time. The radioactivity of NHK at 0 hours chase was set arbitrarily to 100% The values are mean ± SE of three separate experiments. * $p < 0.05$ as compared to *293* cells.

3. p53 DEREGULATION IN CALRETICULIN DEFICIENT CELLS

3.1. p53 protein downregulation in *crt-/-* cells

Figure 27, as well as a previous report from our laboratory (Mesaeli and Phillipson, 2004), demonstrated that expression of the p53 protein is significantly reduced in *crt-/-* cells as compared to *wt* cells. This effect was not due to transcriptional regulation of the p53 gene, as the loss of calreticulin function resulted in a significant increase in p53 promoter activation (Mesaeli and Phillipson, 2004). Moreover, the level of p53 transcripts were not significantly different in both cell types, as shown by semi-quantitative RT-PCR (Figure 28). Next, we examined the effect of calnexin on cellular levels of the p53 protein. As shown in Figure 29A, knockdown of calnexin using siRNA did not alter p53 protein expression. Furthermore, loss of calnexin function in *cnx-/-* cells did not affect p53 protein levels (Figure 29B). These data suggest that decreased p53 protein in MEF cells is specific to the loss of calreticulin function.

3.2. The downregulation of p53 in *crt-/-* cells is Akt/MDM2 independent

One of the major pathways involved in the downregulation of the p53 protein, and therefore mediating resistance to apoptosis, is the phosphoinositide 3-kinase (PI3K) – Akt – MDM2 signalling pathway (Zhou et al., 2001). Recent data from our laboratory demonstrated that the activity of PI3K and Akt is significantly elevated in the absence of calreticulin (Jalali and Mesaeli, unpublished data). To test whether increased activity of

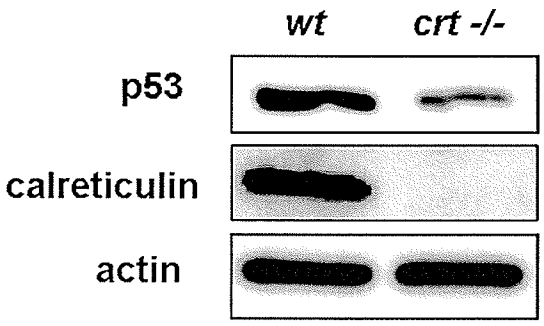


Figure 27. Downregulation of p53 protein levels in *crt-/-* cells. Western blot analysis showing the levels of p53 in *wt* and *crt-/-* cells. The same membrane was stripped and reprobed with antibodies to calreticulin and cytoskeletal actin. A representative western blot image of three separate experiments is shown.

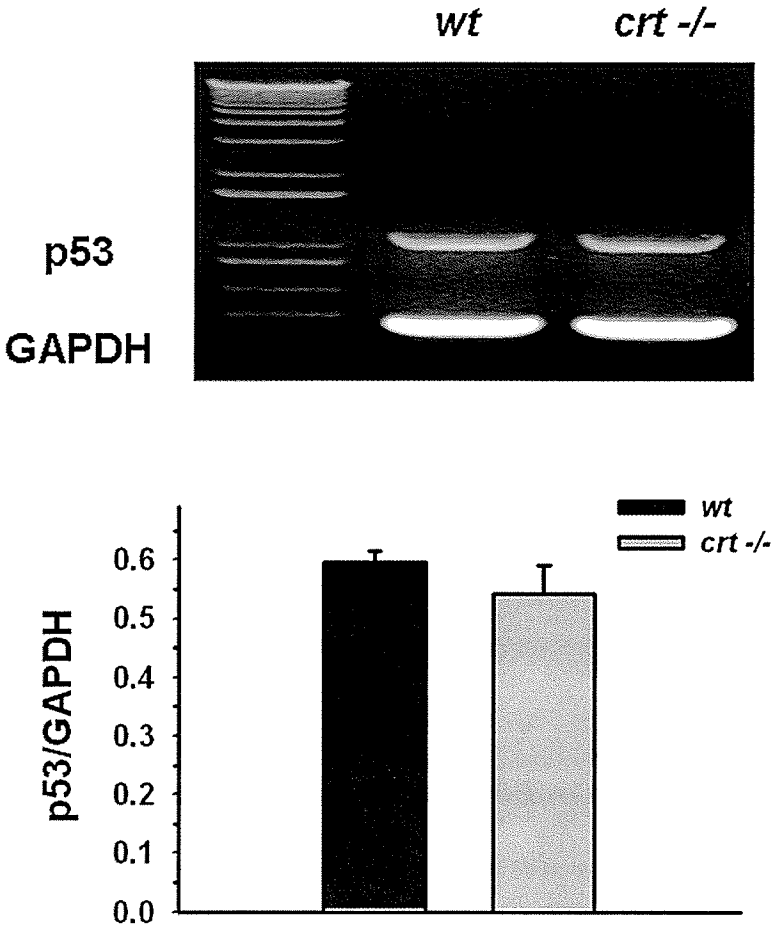


Figure 28. RT-PCR analysis of p53 mRNA levels in *wt* and *crt-/-* cells. Top panel – a representative image of an agarose gel showing the RT-PCR product. Lower panel shows quantification of the bands corresponding to p53 in *wt* and *crt-/-* cells. The data were obtained using Quantity One software, as described in *Materials and Methods*. The data are presented as the ratio of p53 signal normalized to GAPDH signal. The values are mean ± SE of three separate RT-PCR reactions from three different total RNA isolations.

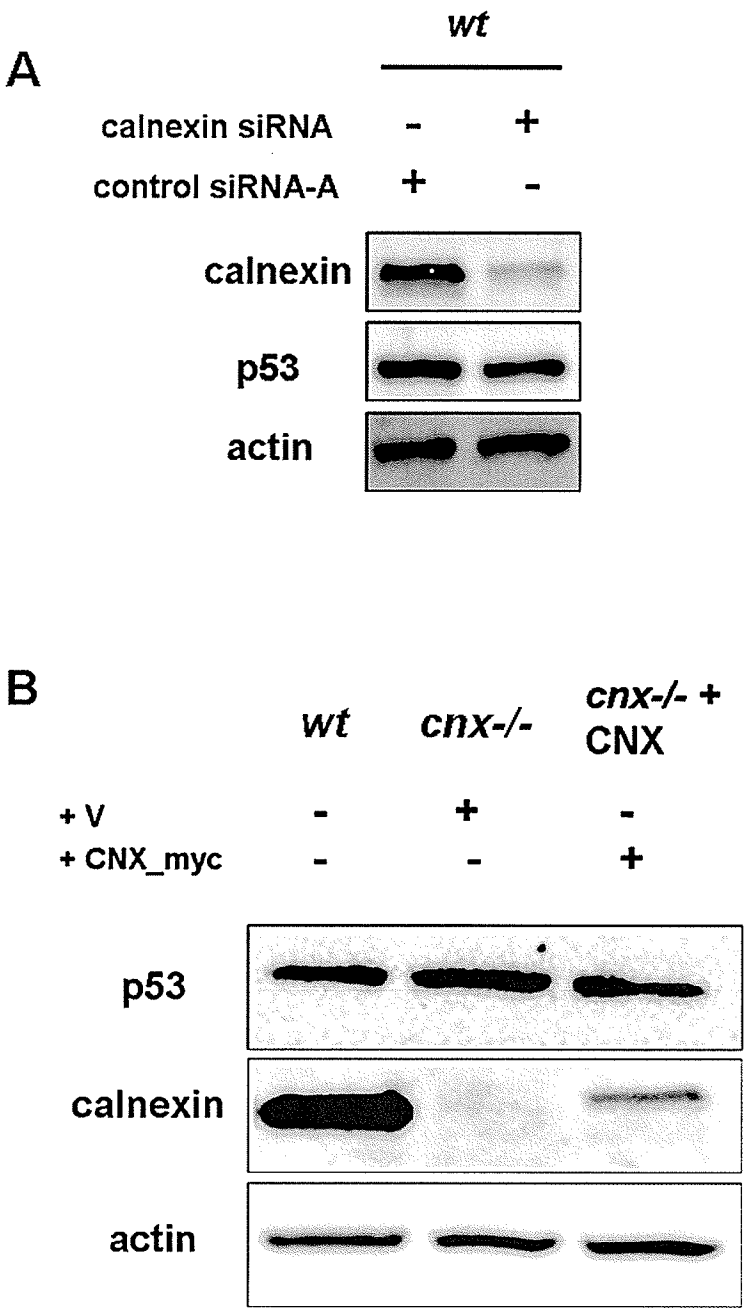


Figure 29. p53 protein expression levels in cells transfected with calnexin siRNA or *cnx-/-* cells. **(A)** A representative western blot showing the expression levels of p53, calnexin and actin in *wt* cells transfected with control siRNA-A or calnexin siRNA. **(B)** A representative western blot showing the expression levels of p53, calnexin and actin in *wt* *cnx-/-* cells and *cnx-/-* cells transiently transfected with exogenous calnexin. V – is an empty vector; CNX_myc is a calnexin expression plasmid.

these kinases in *crt-/-* cells triggers p53 downregulation, we used pharmacological inhibitors specific for the different components of the Akt pathway. As shown in Figure 30A, treating cells with wortmanin (PI3K inhibitor) or Akt inhibitor II (Akt inhibitor), both of which should prevent MDM2 phosphorylation, did not affect the levels of p53 protein in *wt* and *crt-/-* cells. Furthermore, inhibition of MDM2 function (treatment with MDM2 inhibitor) decreased p53 protein levels in *wt* and *crt-/-* cells to a similar extent (Figure 30A). Moreover, the ratio of p53 protein in *wt* cells relative to *crt-/-* cells was not significantly altered following these treatments (Figure 30B). Interestingly, inhibition of the proteasome using MG132 resulted in a significant increase in the level of p53 protein in *crt-/-* cells, with no significant change in the p53 protein level in *wt* cells (Figure 30A). Previous studies illustrated that p53 can also be a substrate for cleavage by the calcium-activated neutral protease, calpain and that treatment of cells expressing wild-type p53 with an inhibitor of calpain resulted in the stabilization of the p53 protein (Kubbutat and Vousden, 1997). However, no changes in p53 protein levels were detected upon treatment of *wt* or *crt-/-* MEF cells with ALLN (a calpain inhibitor) (Figure 30).

To determine the rate of p53 protein degradation in *wt* and *crt-/-* cells, cells were treated with 50μg/ml cycloheximide for different time intervals (to inhibit protein synthesis). Figure 31A shows that the rate of p53 degradation after continuous inhibition of protein synthesis was higher in *crt-/-* cells as compared to *wt* cells, illustrating a decrease in the half-life of p53 in the absence of calreticulin. Next, we examined the levels of ubiquitinated p53 in *wt* and *crt-/-* cells to investigate the role of the ubiquitin-proteasome pathway in p53 deregulation. *wt* and *crt-/-* cells were first treated with proteasome inhibitor. Total ubiquitinated proteins were enriched using ubiquitin affinity

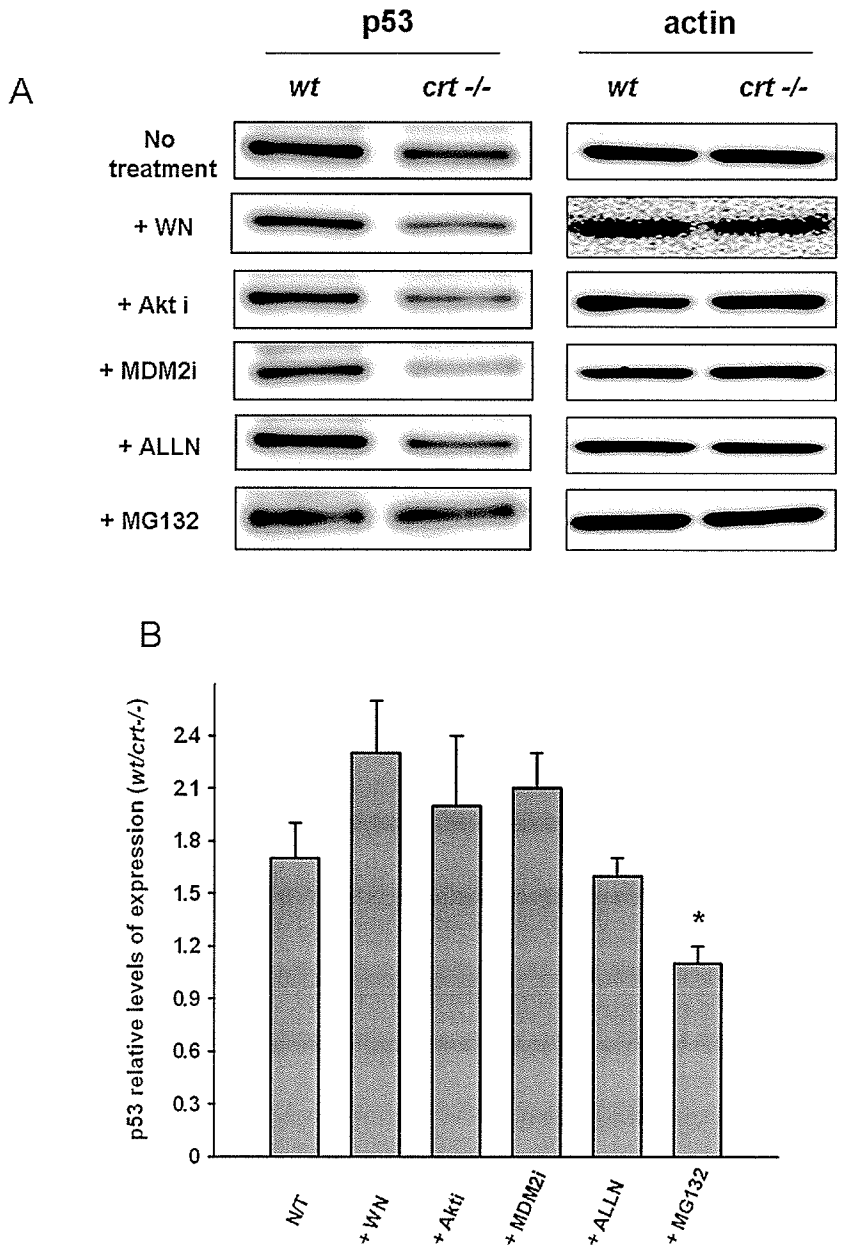


Figure 30. Decrease in p53 protein level in *crt-/-* cells is proteasome dependent and not due to alteration in PI3K – Akt signalling pathway. (A) p53 expression in *wt* and *crt-/-* cells untreated or treated with either 0.2μM wortmanin (WN, 1 hour), 10μM Akt inhibitor II (Akti, 2 hours), 40μM MDM2 inhibitor (MDM2i, 2 hours), 20μM calpain inhibitor (ALLN, 2 hours) or 50μM proteasome inhibitor (MG132, 4 hours) was analyzed by immunobloting. Band density was quantified and normalized to the actin signal from the same blot. **(B)** The ratio of p53 levels in *wt* to *crt-/-* cells after each treatment were calculated and presented as a bar graph. The values are mean ± SE of three separate experiments. * $p<0.05$ significantly different as compared to non-treated cells.

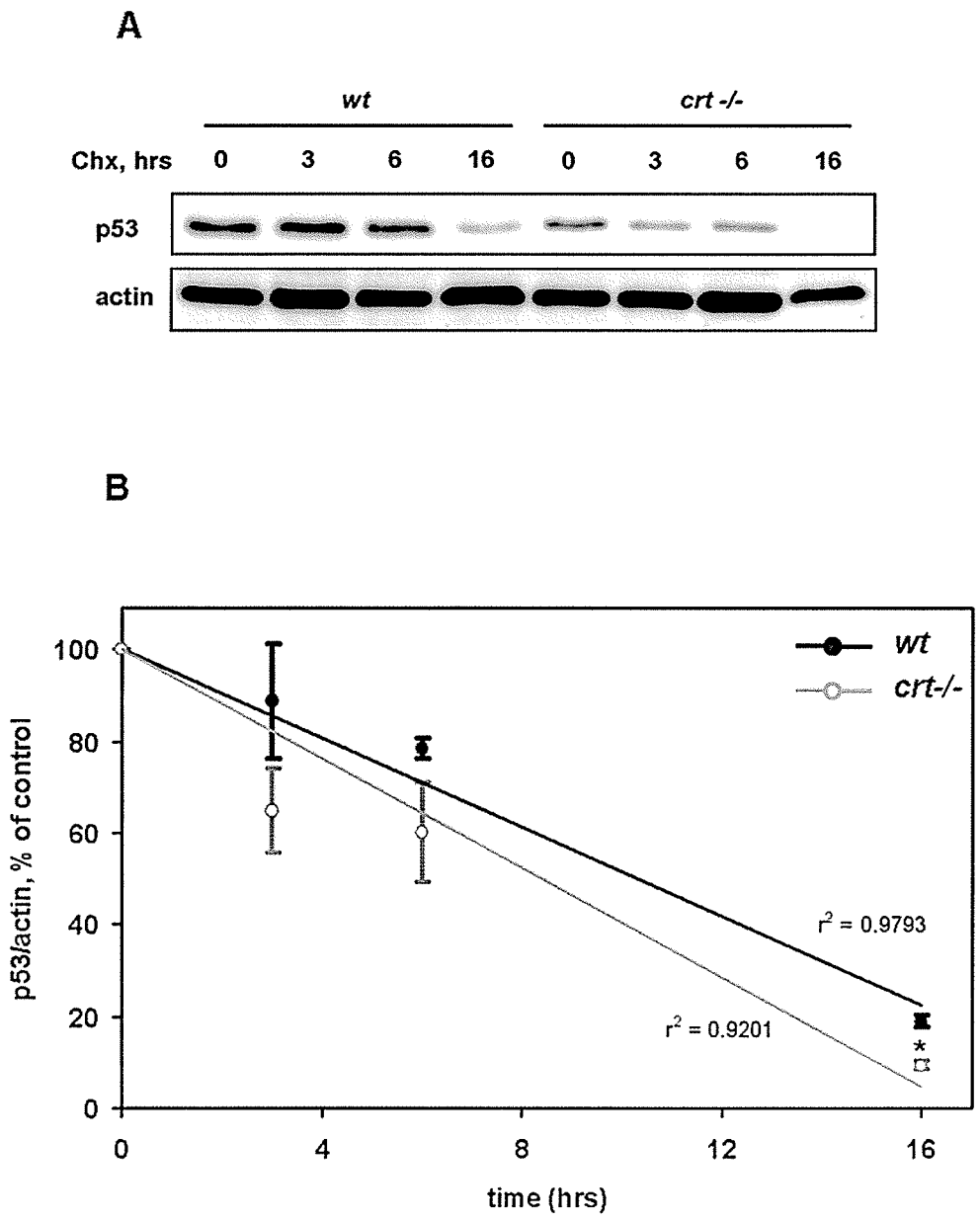


Figure 31. Decreased p53 protein stability in *crt-/-* cells. (A) *wt* and *crt-/-* cells were treated with cycloheximide (Chx, 50μg/ml) and then harvested at the indicated time points. A representative western blot with monoclonal anti-p53 and anti-actin antibodies is shown to demonstrate the protein expression levels. **(B)** The p53 band density at each time point was normalized to actin and plotted with respect to time. Data were fitted using linear regression (SigmaPlot 9.0). The values are mean ± SE of three separate experiments. The regression value is indicated in the graph. * $p < 0.05$ as compared to *wt* cells.

beads (see *Materials and Methods*) followed by westen blotting with a polyclonal anti-p53 antibody (Santa Cruz, CA). As shown in Figure 32, p53 ubiquitination is elevated in the absence of calreticulin. Our data suggest that the decrease in p53 protein in *crt-/-* cells is due to increased p53 degradation through the ubiquitin-proteasome pathway.

3.3. Endoplasmic reticulum stress induces p53 downregulation in *wt* and *crt-/-* cells

Knee et.al. reported that protein folding is accelerated and quality control is compromised in calreticulin-deficient cells, triggering ER stress and the unfolded protein response (Knee et al., 2003). ER stress has been shown to induce the cytoplasmic localization of p53 and to prevent p53-dependent apoptosis (Pluquet et al., 2005). Therefore, we examined the effect of ER stress on p53 expression levels in *wt* and *crt-/-* cells. Treatment of cells with tunicamycin (an inhibitor of N-linked protein glycosylation), thapsigargin (a SERCA pump inhibitor, calcium agonist), or brefeldin A (disrupts the Golgi structure and protein trafficking) led to a substantial decrease in p53 protein levels in both *wt* and *crt-/-* cells, as compared to untreated cells (Figure 33A,B). Interestingly, the level of p53 protein was significantly lower in *crt-/-* cells treated with different ER stress inducing agents (Figure 33B). Furthermore, disruption of the Golgi network and protein trafficking in *crt-/-* cells had the most severe outcome, indicating that the effects of stress conditions, such as compromised folding and processing, have an additive effect on p53 protein levels. Our data illustrate that p53 protein degradation in *crt-/-* cells is triggered by ER stress, with subsequent activation of the ubiquitin-

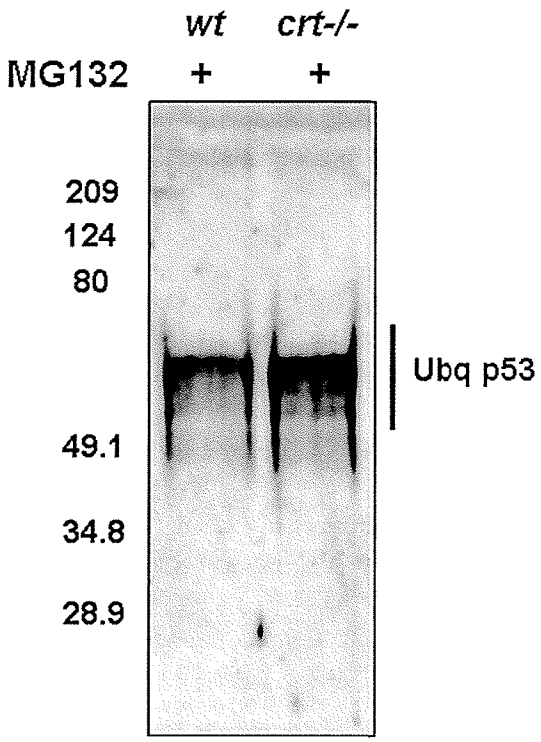


Figure 32. Western blot showing the levels of ubiquitinated p53 in *wt* and *crt -/-* cells. Cells were treated with 50μM MG132 for 4 hours. Ubiquitinated proteins were enriched using ubiquitin affinity beads. The affinity matrix after ubiquitin enrichment was incubated with SDS containing loading buffer and samples were run on SDS-PAGE. Western blot analysis was performed with polyclonal anti-p53 antibodies which recognize ubiquitinated p53.

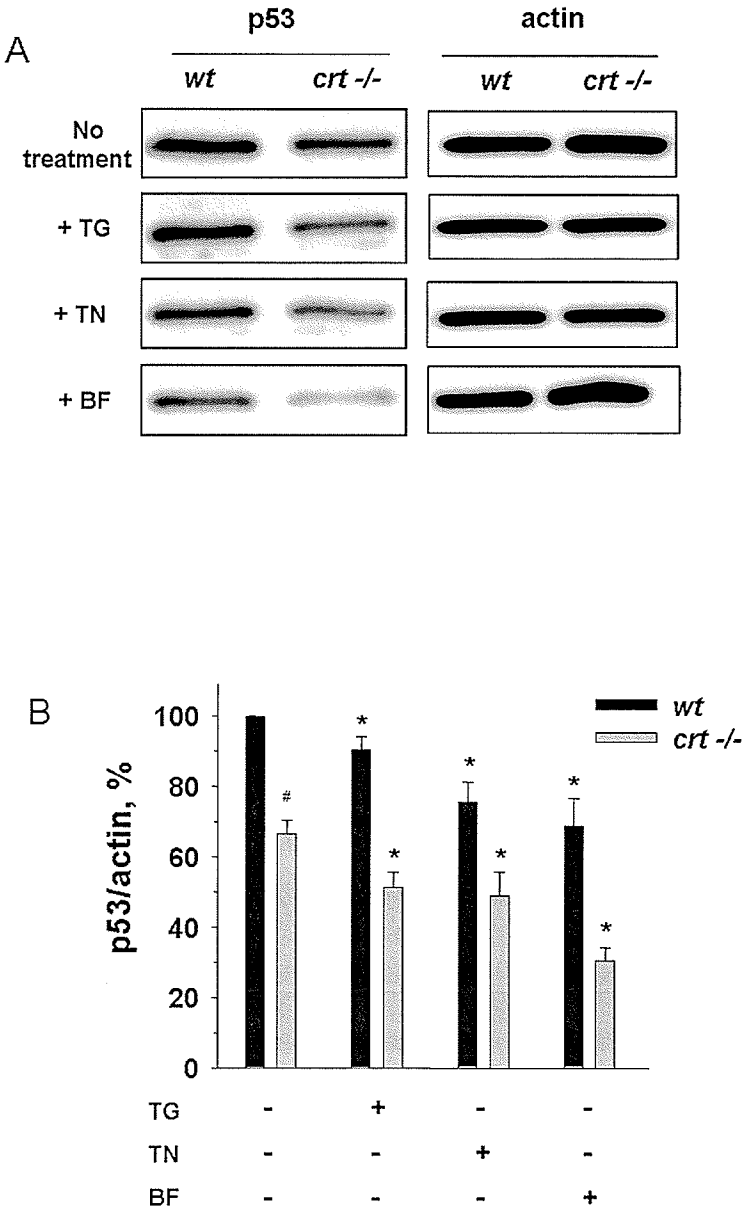


Figure 33. Pharmacologically induced ER stress downregulates p53 in both *wt* and *crt-/-* cells (A) Representative western blot showing changes in p53 protein after each treatment. Cells were untreated or treated with 0.1 μM thapsigargin (TG) for 1 hour, 1μg/ml tunicamycin (TN) for 3 hours, or 7 μM brefeldin A (BF) for 3 hours and western blot analysis was performed with a monoclonal anti-p53 antibody. p53 band density was quantified, normalized to actin band and plotted as bar graphs relative to untreated *wt* cells (panel **B**). The values are mean ± SE of three separate experiments. * $p < 0.05$ as compared to untreated cells. # $p < 0.05$ as compared to untreated *wt* cells (100%).

proteasome pathway. Futher ER stress via pharmacological agents results in further reduction of p53 protein levels in these cells.

3.4. *Crt-/-* cells are more resistant to cell death induced by ER stress

To test the role of the ER luminal environment on p53 function, we tested the sensitivity of *wt* and *crt-/-* cells to drug-induced cell death. Previous studies illustrated that *crt-/-* cells are more resistant to etoposide, staurosporine and UV induced apoptosis (Mesaeli and Phillipson, 2004; Nakamura et al., 2000). Figure 34 demonstrates that both tunicamycin and thapsigargin decrease the cell viability of *wt* and *crt-/-* cells. However, *wt* cells are less resistant to ER stress induced cell death when compared to *crt-/-* cells (Figure 34). The increased viability of *crt-/-* cells after continuous treatment with thapsigargin or tunicamycin could be attributed to a more pronounced decrease in p53 protein levels in these cells as compared to *wt* cells (Figure 33B).

3.5. Effect of changes in intracellular Ca^{2+} on p53 expression levels

Calreticulin functions as a regulator of Ca^{2+} homeostasis and an ER chaperone (for review see Michalak et al., 1999). In order to investigate the mechanism of regulation of p53 stability by calreticulin, we examined its two functions. In *crt-/-* MEF cells, the resting free cytosolic Ca^{2+} concentration is significantly higher as compared to *wt* cells (Nakamura et al., 2001). These cells also show a lower ER capacity for Ca^{2+} storage, although the free ER Ca^{2+} concentration is unchanged (Nakamura et al., 2001).

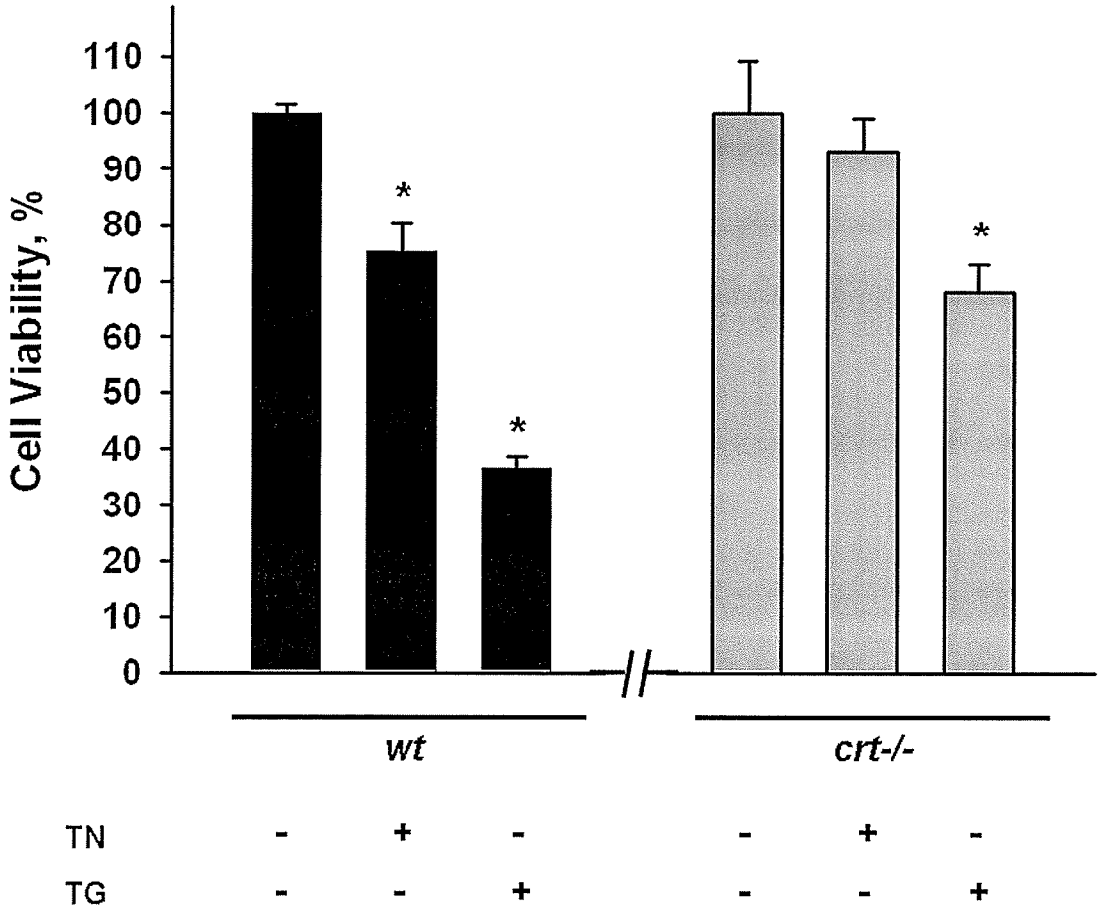


Figure 34. Effect of ER stress on cell death in *wt* and *crt-/-* cells. *wt* and *crt-/-* cells were treated with 0.1 μM thapsigargin (TG) for 3 hours or 1μg/ml tunicamycin (TN) for 3 hours. The percentage of live cells was calculated using a cytotoxicity assay, as described in *Materials and Methods*. * $p < 0.05$ as compared to untreated cells.

Reintroduction of calreticulin in *crt-/-* cells has been shown to decrease the basal cytosolic Ca^{2+} concentration close to that of *wt* cells (Nakamura et al., 2001). Therefore, we determined whether the reintroduction of calreticulin in *crt-/-* cells could rescue p53 levels in those cells. Figure 35 shows that calreticulin transfected *crt-/-* cells had significantly higher p53 protein levels as compared to empty vector transfected *crt-/-* cells. To further support the Ca^{2+} buffering role of the reintroduced calreticulin in the regulation of p53 stability, we transfected *crt-/-* cells with calsequestrin (Figure 35). Similar to calreticulin, calsequestrin was able to rescue p53 protein levels in *crt-/-* cells (Figure 35). Next, we examined the effect of increased cytoplasmic Ca^{2+} on p53 protein levels in *wt* cells, to mimic the conditions in *crt-/-* cells. Figure 36 shows that continuous treatment of *wt* cells with a Ca^{2+} ionophore, ionomycin, had no effect on p53 protein levels or its stability. However, the effect of the ionophore on p53 expression levels could be masked by the triggering of ionomycin-induced apoptosis and accompanied changes in p53 transcription levels (Ojcius et al., 1991; Takei and Endo, 1994).

3.6. Changes in p53 expression upon reintroduction of calreticulin or its deletion mutants in *crt-/-* cells

To differentiate between the Ca^{2+} buffering capacity and chaperone function of the exogenously expressed calreticulin on p53 stability in *crt-/-* cells, we explored the effect of overexpression of different calreticulin domains on p53 protein levels. Calreticulin deletion mutants (CRT^{NP} and CRT^{PC}) were introduced into *crt-/-* cells and p53 expression levels were measured by western blot (Figure 37). As shown in Figure 37, the empty

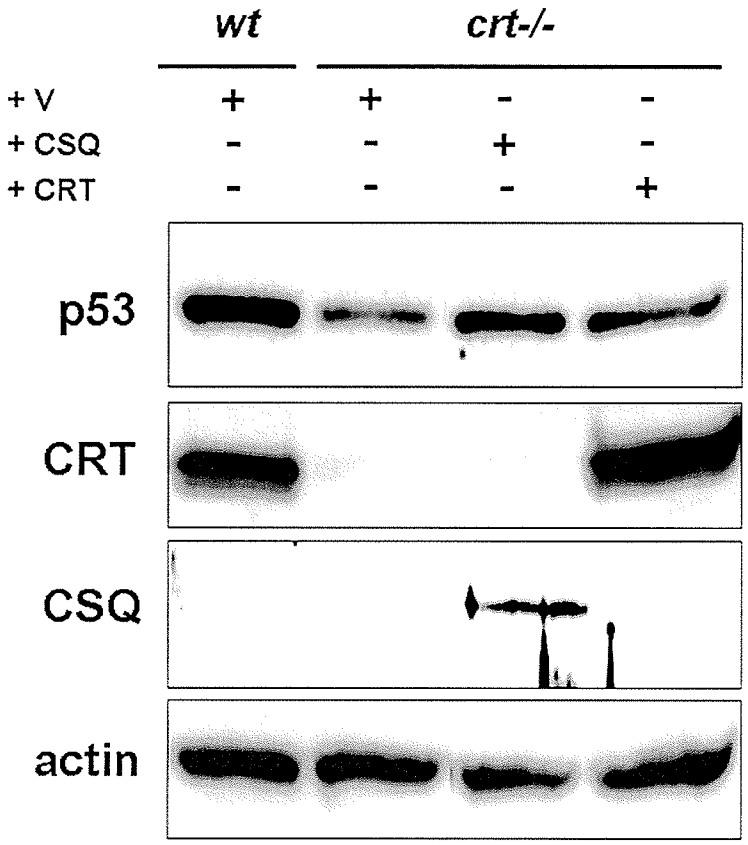


Figure 35. Effect of ectopic expression of calreticulin or calsequestrin on p53 protein levels in *crt-/-* cells. Western blot analysis showing the expression levels of p53, calreticulin, calsequestrin and actin in *wt* cells and *crt-/-* cells transiently transfected with a plasmid containing HA-calreticulin (CRT), calsequestrin (CSQ) or the empty vector.

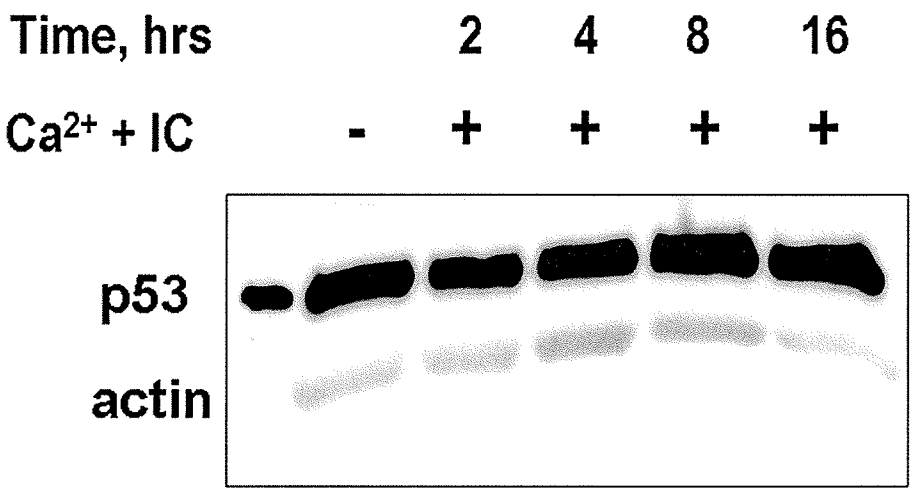


Figure 36. Effect of increased intracellular Ca^{2+} on p53 protein levels. Representative western blot showing the effect of increased intracellular Ca^{2+} on p53 protein levels in *wt* cells. *wt* cells were treated with 2 μM ionomycin (IC) in the presence of 2mM extracellular Ca^{2+} for 2 – 16 hours. Immunoblot with anti-p53 antibody was used to examine the levels of p53 protein at different time points.

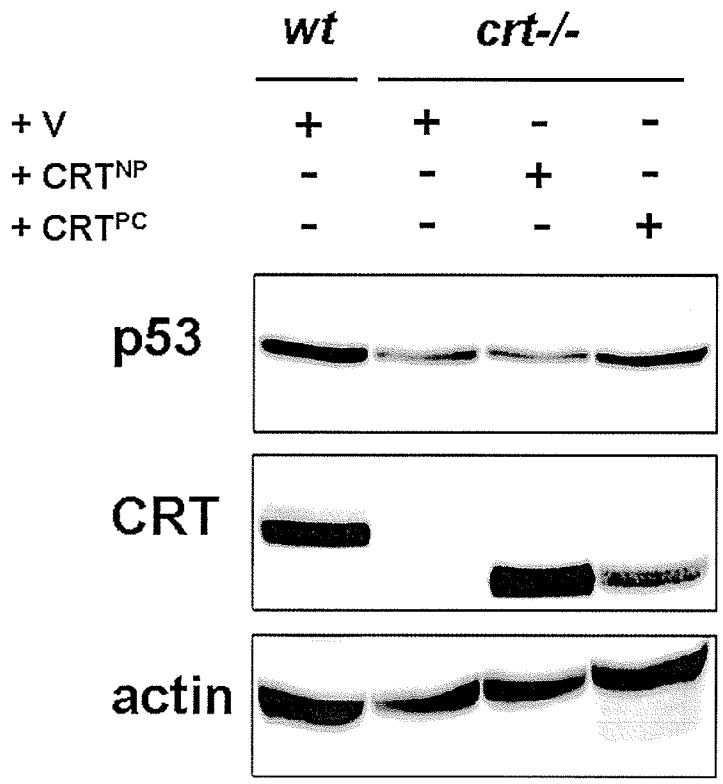


Figure 37. Changes in p53 expression upon reintroduction of calreticulin or its deletion mutants in *crt-/-* cells. Western blot analysis showing the expression levels of p53, calreticulin (CRT) and actin in *wt* and *crt-/-* cells transiently transfected with expression vectors encoding empty vector (V) or calreticulin deletion mutants (CRT^{NP}, CRT^{PC}). Western blot image is a representative of three separate experiments.

vector alone, as well as the CRT^{NP} (C-domain deletion) mutant, did not affect p53 protein levels. In contrast, CRT^{PC} (N-domain deletion mutant of calreticulin) was able to restore the expression levels of p53 in *crt-/-* cells, close to that observed in *wt* cells. These data suggest that the C-terminal calcium binding domain and not the N-terminal chaperone domain of calreticulin is important for modulation of p53 stability. To examine the role of the lectin like P-domain of calreticulin in the process of p53 degradation, we created an expression construct of calreticulin that is mutated at W244. Mutation of W244 to A in the tip of the P-domain of calreticulin has been shown to disrupt its chaperone function and its ability to bind ERp57 (Martin et al., 2006). As shown in Figure 38, expression of CRT^{W244A} in *crt-/-* cells resulted in a complete rescue of p53 expression levels, thus confirming our previous observations that the loss of the Ca^{2+} binding function of calreticulin, and not its chaperone function, affects p53 stability.

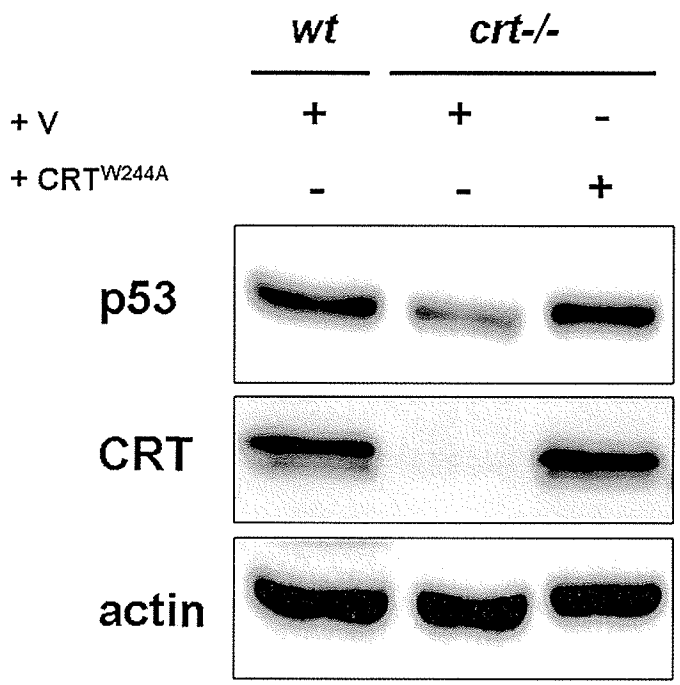


Figure 38. Effect of disruption of the calreticulin P domain structure on p53 protein levels. Representative western blots showing the expression levels of p53, calreticulin (CRT) and actin in *wt* and *crt-/-* cells transiently transfected with an expression vector encoding calreticulin with a point mutation in the P-domain (CRT^{W244A}) or with the empty vector (V).

F. DISCUSSION

1. ENHANCED FUNCTION OF THE UBIQUITIN-PROTEASOME PROTEOLYTIC PATHWAY AND INCREASED ERAD ACTIVITY IN *CRT-/-* CELLS

Calreticulin, in association with calnexin, plays an important role in the proper folding of nascent glycoproteins. Therefore, elimination of calreticulin could lead to the accumulation of misfolded glycoproteins in the ER, thereby clogging the ER protein folding machinery. However, in *crt-/-* cells the UPR is induced, resulting in a significant increase in the expression of other ER resident chaperones (Knee et al., 2003). Interestingly, *crt-/-* cells do not show any difference in the ultra-structure of the ER or any changes in the total levels of ER resident glycoproteins. There is another report, suggesting only modest changes in the levels of expression of other ER chaperones and accelerated protein folding in *crt-/-* cells (Molinari et al., 2004). This report is contradictory to another recently published work (Coe et al., 2008) and our observations. Our studies illustrate that *crt-/-* cells are able to survive ER stress via the activation of the ubiquitin-proteasome pathway. Indeed, proteasome inhibition in *crt-/-* cells results in a significantly higher accumulation of ubiquitinated proteins (Figure 6) and increased cell death (Figures 10 and 11) as compared to the control. Furthermore, we showed that ubiquitination of two ER substrates, Cx43 and A1AT NHK, is significantly increased in *crt-/-* cells (Figure 7). This increase in protein ubiquitination and degradation in *crt-/-* cells could be due to activation of the ERAD pathway as a compensatory mechanism in

order to retain the ER protein folding capacity and to eliminate the unfolded proteins accumulated in the ER.

To determine whether there are any changes in the rates of ERAD in *crt-/-* cells, we used the misfolded genetic variant null Hong Kong of α1-antitrypsin (A1AT NHK) which is a well established ERAD substrate (Termine et al., 2005). A1AT NHK association with calnexin was demonstrated by co-immunoprecipitation (Le et al., 1994). A previous report demonstrated that overexpression of human ER ManI in HEK 293 cells enhanced A1AT NHK degradation due to accelerated trimming of its N-glycans (Hosokawa et al., 2003). Furthermore, addition of kifunensine or lactacystin to this system prevented A1AT NHK degradation, indicating that A1AT NHK is processed through the ERAD pathway. Our studies revealed that A1AT NHK is polyubiquitinated in *crt-/-* cells (Figure 7A) and its stability is significantly reduced (Figure 24), suggesting that the process of ERAD is elevated in these cells. Similar data were obtained for *cnx-/-* cells (Figure 25), illustrating that the function of both calreticulin and calnexin is important in ER protein quality control. In contrast, overexpression of calreticulin in HEK 293 cells slightly decreased the degradation of A1AT NHK (Figure 26). It was previously demonstrated that EDEM interacts with and accelerates the degradation of misfolded A1AT NHK, acting as a lectin type chaperone (Hosokawa et al., 2001). The effect of calreticulin and calnexin on the process of ERAD is therefore opposite to that of EDEM. Calreticulin and calnexin function upstream of ER ManI, while EDEM acts as a downstream acceptor of mannosidase substrates (described above). Elevated levels of calreticulin and calnexin would cause misfolded substrates within the quality control cycle to be retained longer prior to mannose trimming. In contrast, higher levels of

EDEM would facilitate translocation across the ER membrane and subsequent ubiquitination of the misfolded glycoproteins.

ERAD is a multi-step process, important for the efficient removal of unfolded and misfolded proteins from the lumen of the ER. The ubiquitin-proteasome system represents a major component of this process (Werner et al., 1996). ER stress has been shown to trigger upregulation of several proteins known to be a functional part of ERAD, including calnexin, calreticulin (Knee et al., 2003), EDEM (Hosokawa et al., 2001; Molinari et al., 2003) and Hrd1 (Kaneko et al., 2002). Interestingly, in *wt* cells where the function of all ERAD components is conserved, induction of ER stress by treatment with thapsigargin, tunicamycin, or brefledin (Figure 14) induces only slight changes in proteasome activity. Similarly, Menendez-Benito et.al. recently reported normal proteasome levels and proteasome activity in human melanoma cells (MelJuSo) treated with tunicamycin or thapsigargin (Menendez-Benito et al., 2005). However, in calreticulin deficient cells the proteasome activity is significantly increased (Figures 8 and 9). Moreover, induction of additional ER stress in these cells using pharmacological agents such as tunicamycin and brefeldin resulted in a further increase in proteasome activity (Figure 14). This differential effect of ER stress inducing agents in *wt* and *crt-/-* cells could be due to the fact that the loss of calreticulin function disturbs the ER lumen homeostasis. Indeed, in *crt-/-* cells, the expression of several ER chaperones (e.g. calnexin, BiP, Grp94) is altered and the chaperone function of calnexin is compromised (Knee et al., 2003). Therefore, introduction of additional stress on the ER could elevate the compensatory response and trigger further activation of the proteasome.

The combined induction of ER chaperones (Knee et al., 2003), elevated expression of

proteins involved in ERAD and activation of the ubiquitin-proteasome system in *crt-/-* cells suggest the presence of a complex network designed to overcome the stress conditions induced upon the loss of ER chaperone function. This network is essential for cell survival. Overexpression of calnexin or BiP in *wt* cells had no significant effect on proteasome function (Figure 15), illustrating that the increased expression of ER chaperones in *crt-/-* cells is not the trigger of elevated proteasome activity. However, siRNA mediated knockdown of calnexin in *crt-/-* cells resulted in further proteasomal activation (Figure 20). The main function of calreticulin and calnexin within the ER is to retain the newly synthesized glycoproteins within the folding cycle and to assure their interaction with other chaperones such as PDI and ERp57 (Oliver et al., 1999). Interestingly, it was reported that in *crt-/-* cells, protein folding is accelerated due to diminished association between the newly synthesized protein substrate and calnexin (Knee et al., 2003; Molinari et al., 2005). It is therefore possible that the further elevation of proteasome activity in *crt-/-* cells with reduced calnexin expression could be due to accelerated and less efficient protein folding, as well as a significant decrease in protein ER retention time, resulting in overloading of the ERAD dislocation chain. As a result, the ubiquitin-proteasome system is triggered to meet the demands of increased protein disposal.

The rate of degradation of glycoproteins by ERAD is linked to trimming of terminal mannose residues from the B branch of the oligosaccharide (Fagioli and Sitia, 2001). Under experimental conditions, when the ER ManI is either inhibited by kifunensin or mutated, glycoprotein degradation is significantly slower (Liu et al., 1999). The same study demonstrated that intracellular protein turnover was arrested with kifunensine,

implicating the participation of ER ManI in the disposal process (Liu et al., 1999). Interestingly, inhibition of α1,2-mannosidase in *crt-/-* cells, and therefore indirect alteration of the retrograde transport of ER substrates, has a negative effect on proteasome activity (Figure 34). This suggests that proteasome function can be adjusted based on the amount of misfolded proteins in the ER export route. Collectively, our data indicate that the observed increase in calnexin expression (Knee et al., 2003), and other proteins involved in ERAD, compensates for the absence of calreticulin and facilitates the ER export and degradation of misfolded ER proteins.

2. MECHANISM OF REGULATION OF PROTEASOME ACTIVITY IN *CRT-/-* CELLS

Proteasomes are one of the major ATP consumers inside the cell (Kenniston et al., 2003). Thus, ATP availability could be a limiting factor affecting the rate of protein degradation (Glickman et al., 1998). Our data showed that *crt-/-* cells contain significantly higher levels of ATP as compared to *wt* cells (Figure 12). The increased ATP production in the absence of calreticulin could be supported by upregulation of insulin mediated glucose uptake and therefore increased energy metabolism (Jalali and Mesaeli unpublished data). Furthermore, a previous report showed that in *crt-/-* cells the resting free cytosolic Ca^{2+} concentration $[Ca^{2+}]_c$ was significantly increased (Nakamura et al., 2001) as compared to *wt* cells (148±10 nm vs. 100±12 nm). The levels of $[Ca^{2+}]_c$ usually correlate with the levels of mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) (Rutter et al., 1993). Increased $[Ca^{2+}]_m$ was shown to activate the F_1F_0 ATP synthase as well as electron

transport chain (Figure 39) (Territo et al., 2001). Moreover, elevated $[Ca^{2+}]_c$ has been suggested to activate the adenine nucleotide translocase (ATN) (Figure 39) (Gunter et al., 2000). Therefore, the elevated ATP levels in *crt-/-* cells may be induced by higher $[Ca^{2+}]_c$ in these cells. Increased ATP levels can in turn support the enhanced proteasome activity.

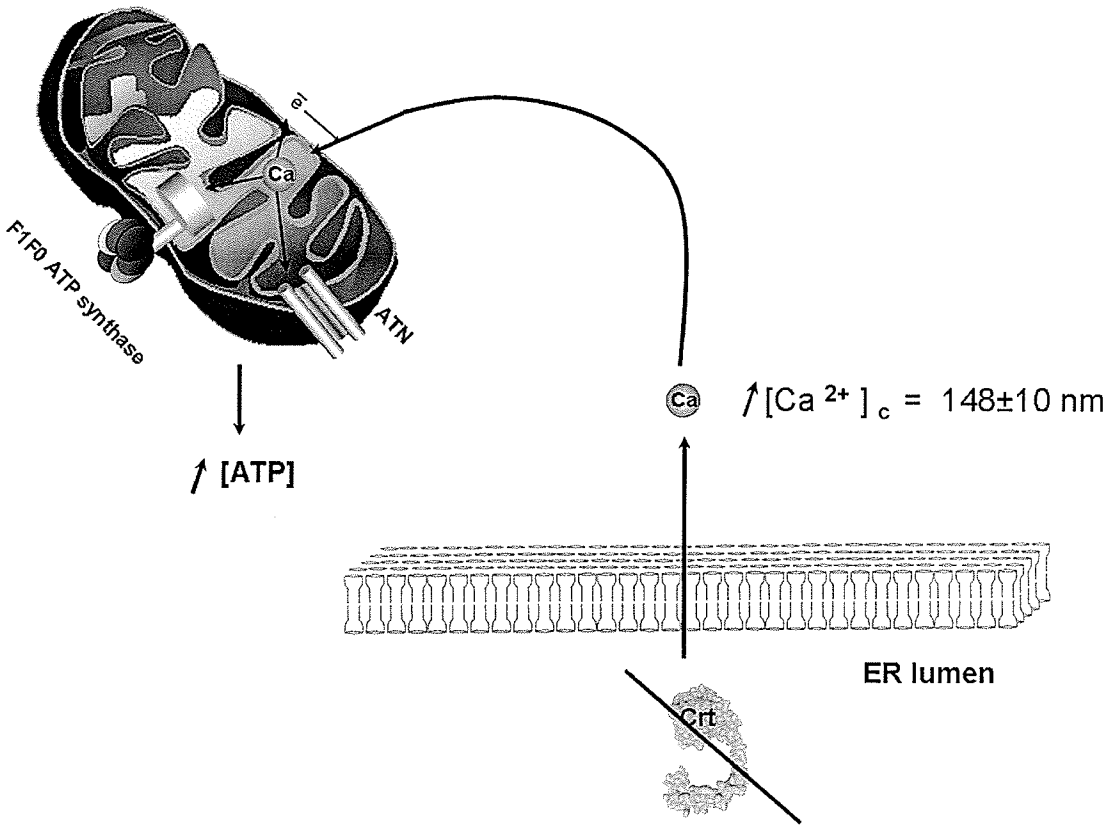


Figure 39. Effect of intracellular Ca^{2+} on ATP production in *crt-/-* MEFs. In *crt-/-* cells the resting free $[Ca^{2+}]_c$ was significantly increased, which is usually in parallel with the levels of mitochondrial Ca^{2+} ($[Ca^{2+}]_m$). Increased $[Ca^{2+}]_m$ was shown to activate F1F0 ATP synthase as well as electron transport chain. Furthermore, elevated $[Ca^{2+}]_c$ has been suggested to activate the adenine nucleotide translocase (ATN).

The activity of an enzyme can be regulated by changes in its protein expression or by changes in the activity of its catalytic unit. In our study, we observed no significant differences in the expression of several proteasome subunits (Figure 13). Moreover, we showed that the mRNA levels of several proteasome subunits were downregulated in *crt-/-* cells as compared to *wt* cells (Table 2), suggesting a possible feedback mechanism between the activity of the proteasomes and the regulation of their gene expression (Figure 40). Recent work by Meiners (Meiners et al., 2003) revealed the existence of an autoregulatory feedback mechanism in vascular smooth muscle cells that allows compensation for reduced proteasome activity by coordinated transcriptional activation of the proteasome genes and not by post-transcriptional events. A similar regulatory mechanism is known to exist in *Saccharomyces cerevisiae*, where the short-lived Rpn4 transcription factor, regulating the expression of genes encoding proteasomal subunits, is also known to be a substrate for the 26S proteasome (Xie and Varshavsky, 2001). Our data therefore suggest that increased proteasome activity in *crt-/-* cells could lead to proteolytic downregulation of a hypothetical transcriptional regulator with a subsequent decrease in the rates of proteasome gene transcription, thus explaining our results in Table 2.

To examine the mechanism of regulation of proteasome activity by calreticulin, we utilized truncated versions of calreticulin with either the N-domain deleted (PC-mutant) or the C-domain deleted (NP-mutant). Figure 19 showed that both NP- and PC- mutants of calreticulin were able to reduce the elevated proteasome activity in *crt-/-* cells, indicating that the function of the P-domain of the calreticulin is one of the extrinsic

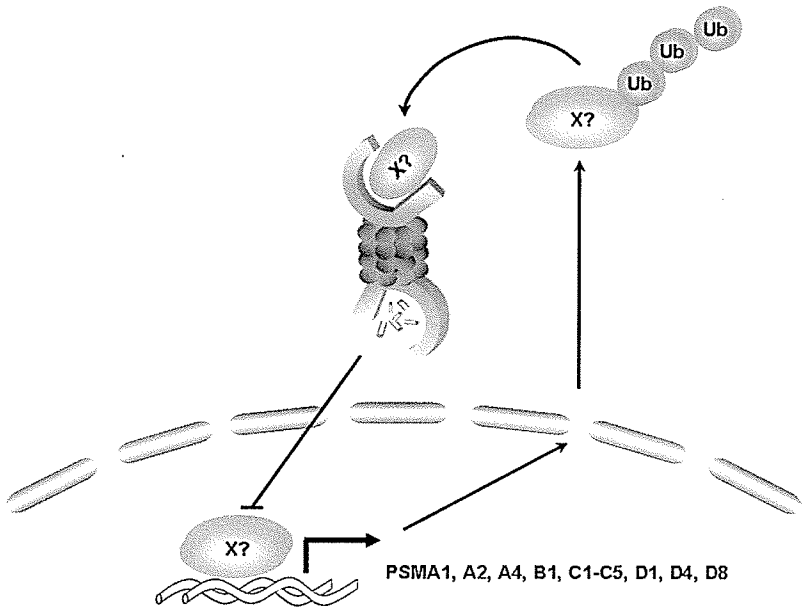


Figure 40. Possible mechanism for transcriptional downregulation of several proteasomal genes in *crt-/-* cells. Increased proteasome activity could lead to downregulation of a transcription factor (or transcription co-activator) involved in proteasome gene regulation creating a negative feedback loop.

modulators of proteasome activity. The P-domain of calreticulin contains both the lectin binding site and the protein-protein interaction site (Leach et al., 2002). The later function is important for recruitment of other ER chaperones required for the processing of newly synthesized proteins (Michalak et al., 1999). Mutation analysis of the calreticulin protein demonstrated that W244, located in the hairpin tip of the calreticulin P-domain, is important for binding of calreticulin to other ER resident chaperones (e.g. ERp57) (Martin et al., 2006). This mutation was shown to affect the structure of the P-domain of calreticulin, by possibly affecting the stability of this region and impairing carbohydrate binding to calreticulin (Martin et al., 2006) (Figure 41). Loss of ERp57 binding in the CRT^{W244A} and $CRT^{NP\text{-}W244A}$ mutants could therefore be crucial for luminal organization of the chaperone and folding network inside the cell, which could elucidate the failure of these mutants to reverse the increase in proteasome activity when introduced into *crt-/-* cells (Figure 19). Overall, these data illustrate that the organization and composition of the ER folding network could affect the ERAD process and its individual steps.

Protein-protein interactions between various components of the ER chaperone network are dependent on ER luminal Ca^{2+} levels and could be affected by the buffering capacity of the ER. Our studies also suggested that the loss of calreticulin's chaperone function in *crt-/-* cells may not be the only mechanism for proteasomal activation (Figure 16, 18). In addition to its chaperone function, calreticulin also plays an important role in the regulation of intracellular Ca^{2+} homeostasis (Michalak et al., 1999). Two recent reports have demonstrated that the free Ca^{2+} concentration inside the cell could affect proteasome activity both *in vitro* and *in vivo* (Menconi et al., 2004; Reshetnyak et al., 2004). The resting level of cytosolic Ca^{2+} in *crt-/-* cells is significantly higher compared

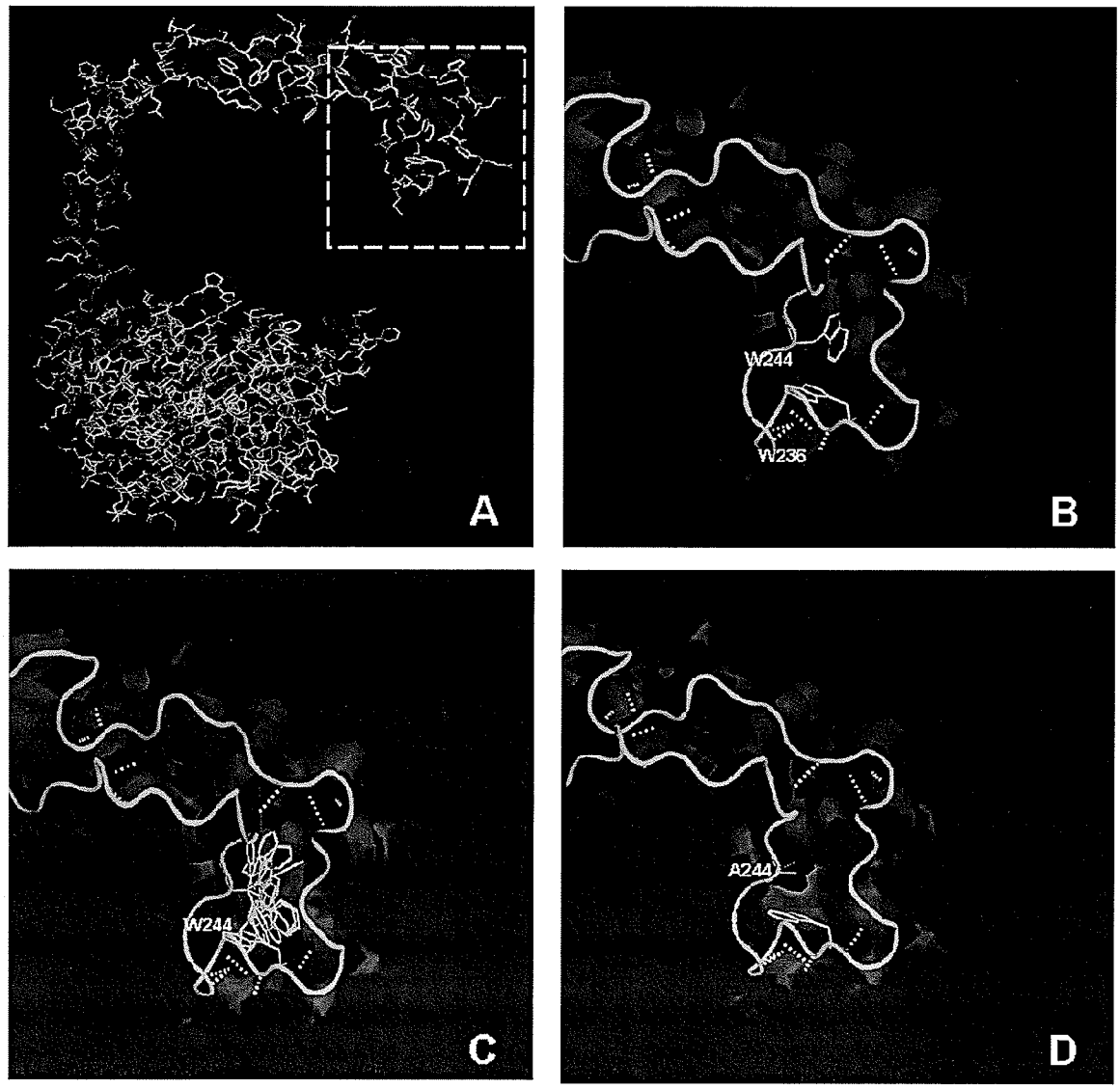


Figure 41. W244A mutation in calreticulin P domain. (A) Three dimensional structure of calreticulin with the tip of the P-domain highlighted. **(B)** W244 and W236 at the hairpin tip of the P-domain are critical for the formation of the protein complexes between calreticulin and other ER resident chaperones. **(C)** The indole ring of the W244 provide essential stability for the P-domain. **(D)** W244A mutant has a disturbed structure in this region, creating an unstable cavity.

to *wt* cells (Nakamura et al., 2001). Therefore, the increased level of cytosolic Ca^{2+} could facilitate enzymatic activation of the proteasomes in *crt-/-* cells. To examine this postulate we transiently transfected *crt-/-* cells with expression vectors encoding full-length calreticulin, the deletion mutant CRT^{PC} or calsequestrin. Previous studies showed that reintroduction of exogenous calreticulin can restore its endogenous function in *crt-/-* cells (Mesaeli and Phillipson, 2004), while the CRT^{PC} mutant restores the Ca^{2+} binding and lectin-like function (Nakamura et al., 2001). Calsequestrin, on the other hand, is a Ca^{2+} buffering protein associated with the sarcoplasmic reticulum of skeletal and cardiac muscle cells (Beard et al., 2004). Calsequestrin has a moderate affinity (Kd ~ 4-40 μM) and high capacity (40–50 mol Ca^{2+} mol^{-1} calsequestrin) for Ca^{2+}, but is not involved in protein chaperoning and maturation (Beard et al., 2004). Expression of these three constructs in *crt-/-* cells resulted in a significant decrease in proteasome activity (Figure 17 and Figure 19). Furthermore, experiments using the substrate overlay assay confirmed that quenching intracellular Ca^{2+} by BAPTA-AM abolishes 26S proteasome activity, while increasing intracellular Ca^{2+} by ionomycin increases this activity in *wt* cells (Figure 16). Overall, these data support the role of intracellular Ca^{2+} in the regulation of proteasome activity.

The general pattern of proteasome distribution within the cell is usually represented by uniform labelling of the cytoplasm, with negative shadows corresponding to vacuolar structures, and more intense labelling of the nucleus, with negative areas corresponding to the nucleoli (Reits et al., 1997). Overall, 1% - 14% of cytoplasmic proteasomes have been shown to be associated with the ER (Beyette and Mykles, 1992; Rivett et al., 1992). Furthermore, subcellular fractionation experiments demonstrated that within the ER

subpopulation, proteasomes are mostly localized to the smooth ER and the *cis*-Golgi (Palmer et al., 1996). It is possible that ERAD substrates are degraded by this group of membrane-bound proteasomes. Previous data indicate that proteasomes could be more active at some cellular locations than at others (Wojcik et al., 1996). Such regions, with increased proteasome-dependent proteolysis, are often called "proteolysis centers". Ultrastructurally, these centers appear as electron dense granules accumulated around the centrosomes in the vicinity of Golgi stacks (Wojcik, 1997). It is possible that the observed change in proteasome activity in *crt-/-* cells is triggered by changes in the localization of proteasomal complexes. However, our data demonstrated no significant difference in proteasome localization in *crt-/-* cells as compared to *wt* cells or *wt* cells treated with calcium ionophore (Figure 18). These data indicate that the observed changes in proteasome activity cannot be attributed to altered proteasome localization.

3. CHANGES IN THE ER ENVIRONMENT INDUCE ALTERATION IN PROTEASOME ACTIVITY

Proteasomes have traditionally been considered as passive machines with constant levels of component subunits, acting uniformly once the substrate proteins are identified. The regulation of proteolysis was thought to be based mostly on substrate recognition through polyubiquitination. In addition, proteasome regulation was considered to occur only at the level of gene expression and not at the level of its activity. However, it was recently demonstrated that there are more sophisticated ways to alter proteasome function and to match its activity to a particular cellular requirement (Hanna et al., 2007). For

example, a recent study showed that proteasome levels were not affected in response to stress triggered by ubiquitin depletion or cessation of ubiquitin recycling (Hanna et al., 2007). Instead, the tight regulation was achieved through a signal-dependent alteration in proteasome subunit composition (Hanna et al., 2007). The current hypothesis on the regulation of proteasome activity supports the existence of cellular components capable of sensing the changes in the intracellular environment and adjusting the proteasome function according to the physiological demands. Tight regulation between intracellular conditions and proteasome function was recently described (Yerlikaya et al., 2008). This group demonstrated that inhibition of protein degradation by the 26S proteasome leads to inhibition of protein synthesis, mediated through increased phosphorylation of eIF2alpha by the heme-regulated inhibitor kinase (HRI) (Yerlikaya et al., 2008). HRI therefore represents an example of a cellular sensor that can adjust protein degradation.

Our data summarized in Table 4 demonstrate the correlation between the conditions inside the ER and the activity of the proteasomes. Lack of calreticulin, calnexin or both chaperones inside the ER stimulates the proteolytic machinery. Calreticulin and calnexin act as folding machinery for the newly synthesized glycoproteins, retaining them within the ER and promoting their interaction with other chaperones (Ruddock and Molinari, 2006). Thus, upon the loss of these chaperones, retention of glycoproteins within the ER will be generally reduced. Moreover, the substrate – chaperone association with other components of the ER quality control cycle will be compromised, as described by other groups (Ellgaard et al., 1999; Molinari et al., 2004). As a result, more misfolded proteins will be produced and more substrates for the ERAD process will be available, triggering an increased demand for proteasome function (Table 4).

Table 4. Relative correlation between the conditions inside the endoplasmic reticulum (folding status, glycosylation status, concentration/availability of chaperones, activity of luminal enzymes, etc.) and the activity of the proteasomes.

ER lumen environment	Proteasome activity, ΔRF (% change) relative to control (untreated *wt*)
crt-/-	34.27 ± 3.23
cnx-/-	50.99 ± 12.99
crt-/- + CNX siRNA	103.92 ± 31.2
wt + Tunicamycin	7.44 ± 7.04
wt + Kifunensine	- 14.95 ± 4.67
wt + Thapsigargin	0.35 ± 2.32
crt-/- + Tunicamycin	64.81 ± 6.39
crt-/- + Kifunensine	3.96 ± 2.95
crt-/-+ Thapsigargin	32.84 ± 7.45

CRT stands for calreticulin, CNX stands for calnexin, ΔRF is a difference in relative fluorescence.

As a nascent protein enters the ER lumen, it is modified covalently by the addition of a pre-assembled glycan composed of two *N*-acetyl-glucosamine, nine mannose and three glucose residues. N-glycans are important for protein recognition by the ER chaperone system (Hammond et al., 1994). Tunicamycin inhibits the formation of *N*-acetyl-glucosamine containing lipid intermediates and therefore is widely used as an inhibitor of N-linked glycosylation. Our data revealed that tunicamycin treatment stimulates proteasome function in *crt-/-* cells (Table 4). The observed effect could be due to diminished interaction between newly synthesized, translocated proteins and lectin-type chaperones, leading to production of aberrant proteins and increasing the demand for proteasome function. Indeed, treatment of HepG2 cells (a human hepatoma cell line) with tunicamycin was shown to decrease the protein levels of apolipoprotein B-100 by enhancing its degradation (Liao and Chan, 2001). This degradation could be prevented by lactacystin, a specific irreversible proteasome inhibitor. Moreover, tunicamycin also increases apoB ubiquitination, suggesting that tunicamycin induces the ERAD of apolipoprotein B.

Kifunensine inhibits ER ManI and can therefore decrease the flow of substrates from the quality control system to the translocation sites (Fagioli and Sitia, 2001). The decrease in substrates available for translocation and ubiquitination could lead to decreased demand for proteasome function. Indeed, treatment of *wt* and *crt-/-* cells with kifunensine significantly reduced the proteasome activity (Table 4).

Luminal calcium is required for the ER chaperone network organization. However, depletion of ER calcium levels by thapsigargin treatment does not reveal any significant effect on proteasome activity in *wt* or *crt-/-* cells (Table 4). This observation suggests that

within the ER, defects in the processing of newly synthesized proteins due to inhibition of glycosylation and deglycosylation had a more detrimental effect on the overall folding machinery than an imbalance in ER Ca^{2+} handling. Thus, changes in ER glycosylation and deglycosylation could lead to significant alterations in proteasome function in order to support the demand for increased protein turnover.

4. A COMPENSATORY MECHANISM AS A RESPONSE TO DIMINISHED FOLDING POTENTIAL AND ER STRESS IN *CRT-/-* CELLS

Our data collectively elucidate that the function of calreticulin in the regulation of intracellular Ca^{2+} homeostasis, as well as its chaperone function contained within arm domain (P-domain) are important regulators of proteasome activity. Proteasome transformation is likely achieved through adaptation of its intrinsic activity, changes in subunit composition and/or changes in the regulation of the proteasome by PA28, PA700 and other factors. Similar proteasomal transformations were observed in aged muscle (Ferrington et al., 2005) and during retinal response to local inflammation and oxidative stress during age-related macular degeneration (Ethen et al., 2007). In our current studies, we described a cytoprotective mechanism in calreticulin deficient cells as a counterbalance for the diminished folding potential that facilitates the removal of misfolded proteins from the ER.

Loss of calreticulin function induces ER stress due to compromised quality control of glycoprotein folding. Accumulation of aberrant proteins induces Ire1α and PERK kinases in *crt-/-* cells (Knee et al., 2003), activating signalling cascades and transcriptional

upregulation of many luminal chaperones, including calnexin. As mentioned above, calnexin – substrate association is severely reduced and protein folding is accelerated in *crt-/-* cells (Knee et al., 2003; Molinari et al., 2004). The increased rate of protein folding and reduced time of association with chaperones could contribute to the increased incidence of protein misfolding. Elevated levels of calnexin, EDEM and Hrd1 in *crt-/-* cells (Table 3) could facilitate efficient dislocation of unfolded proteins to the cytosol and their subsequent ubiquitination. Our data also suggest that this stress response mechanism is transduced from the ER lumen to the cytoplasmic environment, particularly to the proteasomal complexes. Signalling may be mediated through changes in the ER luminal environment, such as folding status, glycosylation status, concentration and availability of chaperones, activity of luminal enzymes and/or changes in the concentrations of cytosolic calcium. The increase in proteasome activity is not a result of transcriptional upregulation (Table 2) but is a consequence of posttranslational events, such as changes in proteasome composition or changes in the intrinsic activity of the catalytic subunits. As a result, in *crt-/-* cells aberrant proteins are disposed more efficiently, the ER structure is maintained and the whole system reaches an adaptive equilibrium. Additional outcomes may include a shortened half-life or altered expression of several proteasome regulated proteins, as well as changes in the function and stability of extracellular proteins that are folded and processed within the ER.

5. *CRT-/-* CELLS ARE RESISTANT TO ER STRESS DUE TO DECREASE IN P53 PROTEIN STABILITY

ER stress induced apoptosis is a response mechanism devoted to eliminating cells that are not adapted to the new environment. It was recently demonstrated that calreticulin deficient cells have a compromised apoptotic response to DNA damage induced by UV (Mesaeli and Phillipson, 2004) or pharmacological agents such as staurosporine and etoposide (Nakamura et al., 2000). We also showed that *crt-/-* cells are more resistant to cell death triggered by exposure to tunicamycin and thapsigargin (Figure 34). p53 is a transcription factor important for the initiation of apoptosis in different cell types (Yonish-Rouach et al., 1991). Interestingly, regulation of p53 transcription was unaffected in *crt-/-* cells (Figure 28). Previous work from our laboratory has demonstrated that p53 protein levels are significantly downregulated in *crt-/-* cells. Our current results indicate that this is due to elevated proteasome activity (Figures 8 and 9), increased p53 ubiquitination (Figure 32) and therefore upregulation of p53 proteolytic degradation (Figure 31). These changes lead to p53 functional inactivation, partially explaining the diminished apoptotic response of *crt-/-* cells to UV. A previous report by Pluquet et.al. illustrated that ER stress induces p53 degradation by a pathway involving PERK and GSK3β (Pluquet et al., 2005). However, data from our laboratory showed that the loss of calreticulin function leads to GSK3β phosphorylation and inactivation (Jalali and Mesaeli unpublished data). Therefore, *crt-/-* cells utilize a different mechanism for p53 downregulation. We also demonstrated that the C-terminal Ca^{2+} binding domain of

calreticulin represents an important modulator of p53 stability (Figure 32), while the N- and P- domains are dispensable for this process (Figure 32, 33).

Ca^{2+} signalling has diverse cellular functions ranging from signal transduction to gene regulation. It was recently shown that S100B, an EF hand calcium binding protein, interacts with the C-terminal part of p53 (Rustandi et al., 2000), preventing it from PKC dependent phosphorylation and subsequent acetylation. Phosphorylation and acetylation of p53 promote different interactions with other proteins and with target gene regulatory elements (Brooks and Gu, 2003). It also disrupts interaction between p53 and its negative regulator, MDM2 (Colman et al., 2000). Thus, binding of S100B to p53 negatively affects p53 stability, leading to its functional inhibition. These results provide one of the possible links between Ca^{2+} signalling and oncogenic processes that are regulated by the p53 protein.

Calreticulin is not an exclusive chaperone known to affect p53 function. Previous studies showed that the Hsp70 family of chaperones directly interact with p53 and suppress its activity (Ehrhart et al., 1988; Nihei et al., 1993). Moreover, depletion of these chaperones (Hsp70, Hsp72, Hsp27, mot-2) results in p53 activation, triggering cellular senescence (Daugaard et al., 2005; Rohde et al., 2005). It is suggested that these chaperones serve as endogenous suppressors of a latent senescence program, keeping p53 under control and allowing cancer transformation to occur (Sherman et al., 2007). Interestingly, depletion of individual chaperones, like Hsp72 or Hsp27, did not compromise the ubiquitin proteosome system and chaperone machinery, as there was no accumulation of oxidized, ubiquitinated or unfolded protein species in these cells (O'Callaghan-Sunol et al., 2007; Sherman et al., 2007). These results indicate that the

effect of the deletion of Hsp70 related chaperones on p53 function is mediated through a specific mechanism and is not triggered by cellular proteotoxic stress. In contrast, calreticulin deletion severely compromises intracellular protein quality control, activating the UPR and increasing the degradation capacity of the cells. Activation of the ERAD pathway and cellular proteolytic machinery affects the turnover of ER proteins as well as the half-life of some cytosolic factors (e.g. p53). As a result, p53 stability is decreased, promoting cells to avoid cell death and to adapt to current stress conditions. Thus, our data demonstrated that as a chaperone regulating Ca^{2+} homeostatsis, calreticulin could function as an important modulator of p53 stability, function and the p53 dependent cellular response to genotoxic and proteotoxic stress.

G. CONCLUSIONS

Our results, in conjunction with previous data (Knee et al., 2003), have demonstrated that in *crt-/-* cells there are several responsive mechanisms that facilitate cell survival and provide long term adaptation during conditions of continuous ER stress. Loss of calreticulin resulted in accelerated glycoprotein folding and compromised quality control within the ER (Knee et al., 2003; Molinari et al., 2004). This triggers the activation of two major UPR transducers – PERK and Ire1 kinases (Knee et al., 2003). As a result, transcription of several ER resident chaperones and proteins involved in ERAD is increased, thus enhancing the ER export of misfolded proteins and their degradation. The activity of the ubiquitin-proteasome system is increased, enhancing the utilization of damaged proteins. We also demonstrated that *crt-/-* MEF cells have diminished rates of ER stress induced apoptosis compared to *wt* MEF cells or other cell types described in the literature (Li et al., 2006). This effect was due to increased cytoplasmic localisation of p53, as well as its increased ubiquitination and degradation. Interestingly, the mechanism of p53 downregulation in *crt-/-* cells is different from the recently described pathway of p53 degradation triggered by tunicamycin or thapsigargin (Baltzis et al., 2007). We demonstrated that the P-domain and Ca^{2+} binding C-domain of calreticulin modulate proteasome activity while the function of the C-domian is critical for the control of p53 stability in *crt-/-* cells.

H. FUTURE DIRECTIONS

As previously described, misfolded proteins are removed from the ER by retrotranslocation to the cytosol and degradation by the ubiquitin-proteasome system, a process known as ERAD. However, a small number of misfolded secretory proteins are known to be degraded by proteasome-independent ERAD mechanisms (Schmitz and Herzog, 2004). It was reported that a mutant form of yeast carboxypeptidase Y, when expressed in mammalian cells (Mancini et al., 2003), and the Z variant of α1-antitrypsin in liver cells (Cabral et al., 2000) are degraded by a proteasome-independent pathway. Interestingly, the trimming of the N-glycans appears to determine whether these proteins are degraded by proteasomes or by another protease. The majority of proteases that are responsible for proteasome-independent ERAD remain unidentified. However, a cysteine protease, ER-60, has been reported to degrade misfolded proteins within the ER (Otsu et al., 1995). It would be interesting to determine whether such alternative mechanisms are also activated in *crt-/-* cells.

Changes in the proteasome function can be attributed to changes in the proteasome subunit composition (Husom et al., 2004). Differences in proteasome assembly can be studied by proteomic approaches (Drews et al., 2007). The new top-down proteomic method allows analysis of intact proteasome complexes and subunits. In this technique, protein subunits are analyzed without protease treatment prior to mass spectrometry (MS). Recently, the top-down proteomics approach, based on electrospray ionization quadrupole-TOF MS and MS/MS experiments, has been used to study the structural organization of the 19S lid of the *Saccharomyces cerevisiae* proteasome (Sharon et al.,

2006). First, the accurate mass of the intact complex (noncovalent bonds preserved) was detected. Then, noncovalent bonds were disrupted by collision activated dissociation and the subunit organization of the 19S complex was determined. Using this modern approach we can study whether ER stress or the loss of calreticulin function can trigger significant alterations in the composition of proteasome complexes.

It would also be interesting to determine whether calreticulin knockdown in *cnx-/-* cells triggers further activation of the proteasome, similar to calnexin knockdown in *crt-/-* cells. Another important aspect of future studies is to find out the ubiquitin ligases that determine increased p53 ubiquitination in *crt-/-* cells. It is possible that ER associated ligases such as Hrd1 are able to use p53 as their substrate.

Our future work can thus provide new insights into the organization of the ERAD pathway as well as dynamic regulation of the proteasome composition. Alterations in proteolytic pathways are associated with numerous diseases and our data can give new information required for understanding the mechanisms of initiation and progression of these pathologies.

I. REFERENCES

Afshar, N., Black, B. E., and Paschal, B. M. (2005). Retrotranslocation of the chaperone calreticulin from the endoplasmic reticulum lumen to the cytosol. Mol Cell Biol *25*, 8844-8853.

Arnaudeau, S., Frieden, M., Nakamura, K., Castelbou, C., Michalak, M., and Demaurex, N. (2002). Calreticulin differentially modulates calcium uptake and release in the endoplasmic reticulum and mitochondria. J Biol Chem *277*, 46696-46705.

Asher, G., Tsvetkov, P., Kahana, C., and Shaul, Y. (2005). A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. Genes Dev *19*, 316-321.

Badciong, J. C., and Haas, A. L. (2002). MdmX is a RING finger ubiquitin ligase capable of synergistically enhancing Mdm2 ubiquitination. J Biol Chem *277*, 49668-49675.

Bajorek, M., and Glickman, M. H. (2004). Keepers at the final gates: regulatory complexes and gating of the proteasome channel. Cell Mol Life Sci *61*, 1579-1588.

Baksh, S., and Michalak, M. (1991). Expression of calreticulin in Escherichia coli and identification of its Ca2+ binding domains. J Biol Chem *266*, 21458-21465.

Ballar, P., Zhong, Y., Nagahama, M., Tagaya, M., Shen, Y., and Fang, S. (2007). Identification of SVIP as an endogenous inhibitor of endoplasmic reticulum-associated degradation. J Biol Chem *282*, 33908-33914.

Baltzis, D., Pluquet, O., Papadakis, A. I., Kazemi, S., Qu, L. K., and Koromilas, A. E. (2007). The eIF2alpha kinases PERK and PKR activate glycogen synthase kinase 3 to promote the proteasomal degradation of p53. J Biol Chem *282*, 31675-31687.

Baumann, O., and Walz, B. (2001). Endoplasmic reticulum of animal cells and its organization into structural and functional domains. Int Rev Cytol *205*, 149-214.

Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A., and Hampton, R. Y. (2001). Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nat Cell Biol *3*, 24-29.

Bazzaro, M., Lee, M. K., Zoso, A., Stirling, W. L., Santillan, A., Shih Ie, M., and Roden, R. B. (2006). Ubiquitin-proteasome system stress sensitizes ovarian cancer to proteasome inhibitor-induced apoptosis. Cancer Res *66*, 3754-3763.

Beard, N. A., Laver, D. R., and Dulhunty, A. F. (2004). Calsequestrin and the calcium release channel of skeletal and cardiac muscle. Prog Biophys Mol Biol *85*, 33-69.

Bergeron, J. J., Brenner, M. B., Thomas, D. Y., and Williams, D. B. (1994). Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. Trends Biochem Sci *19*, 124-128.

Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol *4*, 517-529.

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., and Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol *2*, 326-332.

Beyette, J. R., and Mykles, D. L. (1992). Immunocytochemical localization of the multicatalytic proteinase (proteasome) in crustacean striated muscles. Muscle Nerve *15*, 1023-1035.

Biederer, T., Volkwein, C., and Sommer, T. (1997). Role of Cue1p in ubiquitination and degradation at the ER surface. Science *278*, 1806-1809.

Brady, M., Vlatkovic, N., and Boyd, M. T. (2005). Regulation of p53 and MDM2 Activity by MTBP. Mol Cell Biol *25*, 545-553.

Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002). Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. Embo J *21*, 615-621.

Brooks, C. L., and Gu, W. (2003). Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol *15*, 164-171.

Bryk, M., Briggs, S. D., Strahl, B. D., Curcio, M. J., Allis, C. D., and Winston, F. (2002). Evidence that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in S. cerevisiae by a Sir2-independent mechanism. Curr Biol *12*, 165-170.

Burns, D. M., and Touster, O. (1982). Purification and characterization of glucosidase II, an endoplasmic reticulum hydrolase involved in glycoprotein biosynthesis. J Biol Chem *257*, 9990-10000.

Burns, K., Duggan, B., Atkinson, E. A., Famulski, K. S., Nemer, M., Bleackley, R. C., and Michalak, M. (1994). Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. Nature *367*, 476-480.

Cabral, C. M., Choudhury, P., Liu, Y., and Sifers, R. N. (2000). Processing by endoplasmic reticulum mannosidases partitions a secretion-impaired glycoprotein into distinct disposal pathways. J Biol Chem *275*, 25015-25022.

Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., and Ron, D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature *415*, 92-96.

Caramelo, J. J., Castro, O. A., Alonso, L. G., De Prat-Gay, G., and Parodi, A. J. (2003). UDP-Glc:glycoprotein glucosyltransferase recognizes structured and solvent accessible hydrophobic patches in molten globule-like folding intermediates. Proc Natl Acad Sci U S A *100*, 86-91.

Carrell, R. W., and Lomas, D. A. (2002). Alpha1-antitrypsin deficiency--a model for conformational diseases. N Engl J Med *346*, 45-53.

Chen, X., Shen, J., and Prywes, R. (2002). The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. J Biol Chem *277*, 13045-13052.

Chew, E. H., Poobalasingam, T., Hawkey, C. J., and Hagen, T. (2007). Characterization of cullin-based E3 ubiquitin ligases in intact mammalian cells--evidence for cullin dimerization. Cell Signal *19*, 1071-1080.

Chillaron, J., and Haas, I. G. (2000). Dissociation from BiP and retrotranslocation of unassembled immunoglobulin light chains are tightly coupled to proteasome activity. Mol Biol Cell *11*, 217-226.

Christianson, J. C., Shaler, T. A., Tyler, R. E., and Kopito, R. R. (2008). OS-9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD. Nat Cell Biol.

Collins, G. A., and Tansey, W. P. (2006). The proteasome: a utility tool for transcription? Curr Opin Genet Dev *16*, 197-202.

Colman, M. S., Afshari, C. A., and Barrett, J. C. (2000). Regulation of p53 stability and activity in response to genotoxic stress. Mutat Res *462*, 179-188.

Coppolino, M., Leung-Hagesteijn, C., Dedhar, S., and Wilkins, J. (1995). Inducible interaction of integrin alpha 2 beta 1 with calreticulin. Dependence on the activation state of the integrin. J Biol Chem *270*, 23132-23138.

Coppolino, M. G., Woodside, M. J., Demaurex, N., Grinstein, S., St-Arnaud, R., and Dedhar, S. (1997). Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. Nature *386*, 843-847.

Cox, J. S., Shamu, C. E., and Walter, P. (1993). Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell *73*, 1197-1206.

Crabtree, G. R. (1999). Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT. Cell *96*, 611-614.

Crinelli, R., Bianchi, M., Gentilini, L., Magnani, M., and Hiscott, J. (1999). Activation of the ubiquitin proteolytic system in murine acquired immunodeficiency syndrome affects IkappaBalpha turnover. Eur J Biochem *263*, 202-211.

Crinelli, R., Fraternale, A., Casabianca, A., and Magnani, M. (1997). Up-regulation of the ubiquitin-conjugating and proteolytic systems in murine acquired immunodeficiency syndrome. Eur J Biochem *247*, 91-97.

Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. Biochem J *341 (Pt 2)*, 233-249.

Dahlmann, B. (2005). Proteasomes. Essays Biochem *41*, 31-48.

Daugaard, M., Jaattela, M., and Rohde, M. (2005). Hsp70-2 is required for tumor cell growth and survival. Cell Cycle *4*, 877-880.

Dedhar, S., Rennie, P. S., Shago, M., Hagesteijn, C. Y., Yang, H., Filmus, J., Hawley, R. G., Bruchovsky, N., Cheng, H., Matusik, R. J., and et al. (1994). Inhibition of nuclear hormone receptor activity by calreticulin. Nature *367*, 480-483.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

Drews, O., Zong, C., and Ping, P. (2007). Exploring proteasome complexes by proteomic approaches. Proteomics *7*, 1047-1058.

Ehrhart, J. C., Duthu, A., Ullrich, S., Appella, E., and May, P. (1988). Specific interaction between a subset of the p53 protein family and heat shock proteins hsp72/hsc73 in a human osteosarcoma cell line. Oncogene *3*, 595-603.

Ellgaard, L., and Helenius, A. (2003). Quality control in the endoplasmic reticulum. Nat Rev Mol Cell Biol *4*, 181-191.

Ellgaard, L., Molinari, M., and Helenius, A. (1999). Setting the standards: quality control in the secretory pathway. Science *286*, 1882-1888.

Ellgaard, L., Riek, R., Braun, D., Herrmann, T., Helenius, A., and Wuthrich, K. (2001a). Three-dimensional structure topology of the calreticulin P-domain based on NMR assignment. FEBS Lett *488*, 69-73.

Ellgaard, L., Riek, R., Herrmann, T., Guntert, P., Braun, D., Helenius, A., and Wuthrich, K. (2001b). NMR structure of the calreticulin P-domain. Proc Natl Acad Sci U S A *98*, 3133-3138.

Enoch, T., and Norbury, C. (1995). Cellular responses to DNA damage: cell-cycle checkpoints, apoptosis and the roles of p53 and ATM. Trends Biochem Sci *20*, 426-430.

Ethen, C. M., Hussong, S. A., Reilly, C., Feng, X., Olsen, T. W., and Ferrington, D. A. (2007). Transformation of the proteasome with age-related macular degeneration. FEBS Lett *581*, 885-890.

Ezhkova, E., and Tansey, W. P. (2004). Proteasomal ATPases link ubiquitylation of histone H2B to methylation of histone H3. Mol Cell *13*, 435-442.

Fagioli, C., Mezghrani, A., and Sitia, R. (2001). Reduction of interchain disulfide bonds precedes the dislocation of Ig-mu chains from the endoplasmic reticulum to the cytosol for proteasomal degradation. J Biol Chem *276*, 40962-40967.

Fagioli, C., and Sitia, R. (2001). Glycoprotein quality control in the endoplasmic reticulum. Mannose trimming by endoplasmic reticulum mannosidase I times the proteasomal degradation of unassembled immunoglobulin subunits. J Biol Chem *276*, 12885-12892.

Ferdous, A., Gonzalez, F., Sun, L., Kodadek, T., and Johnston, S. A. (2001). The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. Mol Cell *7*, 981-991.

Ferrington, D. A., Husom, A. D., and Thompson, L. V. (2005). Altered proteasome structure, function, and oxidation in aged muscle. Faseb J *19*, 644-646.

Fiebiger, E., Story, C., Ploegh, H. L., and Tortorella, D. (2002). Visualization of the ER-to-cytosol dislocation reaction of a type I membrane protein. Embo J *21*, 1041-1053.

Fribley, A., Zeng, Q., and Wang, C. Y. (2004). Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. Mol Cell Biol *24*, 9695-9704.

Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. (1988). Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. J Biol Chem *263*, 18545-18552.

Gaczynska, M., Osmulski, P. A., Gao, Y., Post, M. J., and Simons, M. (2003). Proline- and arginine-rich peptides constitute a novel class of allosteric inhibitors of proteasome activity. Biochemistry *42*, 8663-8670.

Gao, B., Adhikari, R., Howarth, M., Nakamura, K., Gold, M. C., Hill, A. B., Knee, R., Michalak, M., and Elliott, T. (2002). Assembly and antigen-presenting function of MHC class I molecules in cells lacking the ER chaperone calreticulin. Immunity *16*, 99-109.

Gardai, S. J., McPhillips, K. A., Frasch, S. C., Janssen, W. J., Starefeldt, A., Murphy-Ullrich, J. E., Bratton, D. L., Oldenborg, P. A., Michalak, M., and Henson, P. M. (2005). Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell *123*, 321-334.

Gillece, P., Luz, J. M., Lennarz, W. J., de La Cruz, F. J., and Romisch, K. (1999). Export of a cysteine-free misfolded secretory protein from the endoplasmic reticulum for degradation requires interaction with protein disulfide isomerase. J Cell Biol *147*, 1443-1456.

Glickman, M. H., and Raveh, D. (2005). Proteasome plasticity. FEBS Lett *579*, 3214-3223.

Glickman, M. H., Rubin, D. M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V. A., and Finley, D. (1998). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell *94*, 615-623.

Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G. (1999). Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. Embo J *18*, 6462-6471.

Gregory, R. C., Taniguchi, T., and D'Andrea, A. D. (2003). Regulation of the Fanconi anemia pathway by monoubiquitination. Semin Cancer Biol *13*, 77-82.

Groll, M., and Clausen, T. (2003). Molecular shredders: how proteasomes fulfill their role. Curr Opin Struct Biol *13*, 665-673.

Grossman, S. R., Deato, M. E., Brignone, C., Chan, H. M., Kung, A. L., Tagami, H., Nakatani, Y., and Livingston, D. M. (2003). Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science *300*, 342-344.

Gunter, T. E., Buntinas, L., Sparagna, G., Eliseev, R., and Gunter, K. (2000). Mitochondrial calcium transport: mechanisms and functions. Cell Calcium *28*, 285-296.

Guo, L., Groenendyk, J., Papp, S., Dabrowska, M., Knoblach, B., Kay, C., Parker, J. M., Opas, M., and Michalak, M. (2003). Identification of an N-domain histidine essential for chaperone function in calreticulin. J Biol Chem *278*, 50645-50653.

Guo, L., Nakamura, K., Lynch, J., Opas, M., Olson, E. N., Agellon, L. B., and Michalak, M. (2002). Cardiac-specific expression of calcineurin reverses embryonic lethality in calreticulin-deficient mouse. J Biol Chem *277*, 50776-50779.

Hamman, B. D., Chen, J. C., Johnson, E. E., and Johnson, A. E. (1997). The aqueous pore through the translocon has a diameter of 40-60 A during cotranslational protein translocation at the ER membrane. Cell *89*, 535-544.

Hanna, J., Meides, A., Zhang, D. P., and Finley, D. (2007). A ubiquitin stress response induces altered proteasome composition. Cell *129*, 747-759.

Harding, H. P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature *397*, 271-274.

Hayashida, Y., Urata, Y., Muroi, E., Kono, T., Miyata, Y., Nomata, K., Kanetake, H., Kondo, T., and Ihara, Y. (2006). Calreticulin represses E-cadherin gene expression in Madin-Darby canine kidney cells via Slug. J Biol Chem *281*, 32469-32484.

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell *10*, 3787-3799.

Hebert, D. N., Foellmer, B., and Helenius, A. (1995a). Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. Cell *81*, 425-433.

Hebert, D. N., Foellmer, B., and Helenius, A. (1996). Calnexin and calreticulin promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes. Embo J *15*, 2961-2968.

Hebert, D. N., Simons, J. F., Peterson, J. R., and Helenius, A. (1995b). Calnexin, calreticulin, and Bip/Kar2p in protein folding. Cold Spring Harb Symp Quant Biol *60*, 405-415.

Heinemeyer, W., Ramos, P. C., and Dohmen, R. J. (2004). The ultimate nanoscale mincer: assembly, structure and active sites of the 20S proteasome core. Cell Mol Life Sci *61*, 1562-1578.

Helenius, A., and Aebi, M. (2004). Roles of N-linked glycans in the endoplasmic reticulum. Annu Rev Biochem *73*, 1019-1049.

Hemenway, C. S., and Heitman, J. (1999). Calcineurin. Structure, function, and inhibition. Cell Biochem Biophys *30*, 115-151.

Herscovics, A. (2001). Structure and function of Class I alpha 1,2-mannosidases involved in glycoprotein synthesis and endoplasmic reticulum quality control. Biochimie *83*, 757-762.

Hettkamp, H., Legler, G., and Bause, E. (1984). Purification by affinity chromatography of glucosidase I, an endoplasmic reticulum hydrolase involved in the processing of asparagine-linked oligosaccharides. Eur J Biochem *142*, 85-90.

Hetz, C., Bernasconi, P., Fisher, J., Lee, A. H., Bassik, M. C., Antonsson, B., Brandt, G. S., Iwakoshi, N. N., Schinzel, A., Glimcher, L. H., and Korsmeyer, S. J. (2006). Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. Science *312*, 572-576.

Hirao, K., Natsuka, Y., Tamura, T., Wada, I., Morito, D., Natsuka, S., Romero, P., Sleno, B., Tremblay, L. O., Herscovics, A., *et al.* (2006). EDEM3, a soluble EDEM homolog, enhances glycoprotein endoplasmic reticulum-associated degradation and mannose trimming. J Biol Chem *281*, 9650-9658.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene *77*, 51-59.

Hobler, S. C., Williams, A., Fischer, D., Wang, J. J., Sun, X., Fischer, J. E., Monaco, J. J., and Hasselgren, P. O. (1999). Activity and expression of the 20S proteasome are increased in skeletal muscle during sepsis. Am J Physiol *277*, R434-440.

Hoffman, L., and Rechsteiner, M. (1994). Activation of the multicatalytic protease. The 11 S regulator and 20 S ATPase complexes contain distinct 30-kilodalton subunits. J Biol Chem *269*, 16890-16895.

Hong, M., Li, M., Mao, C., and Lee, A. S. (2004a). Endoplasmic reticulum stress triggers an acute proteasome-dependent degradation of ATF6. J Cell Biochem *92*, 723-732.

Hong, M., Luo, S., Baumeister, P., Huang, J. M., Gogia, R. K., Li, M., and Lee, A. S. (2004b). Underglycosylation of ATF6 as a novel sensing mechanism for activation of the unfolded protein response. J Biol Chem *279*, 11354-11363.

Hosokawa, N., Tremblay, L. O., You, Z., Herscovics, A., Wada, I., and Nagata, K. (2003). Enhancement of endoplasmic reticulum (ER) degradation of misfolded Null Hong Kong alpha1-antitrypsin by human ER mannosidase I. J Biol Chem *278*, 26287-26294.

Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L. O., Herscovics, A., and Nagata, K. (2001). A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. EMBO Rep *2*, 415-422.

Hosokawa, N., Wada, I., Natsuka, Y., and Nagata, K. (2006). EDEM accelerates ERAD by preventing aberrant dimer formation of misfolded alpha1-antitrypsin. Genes Cells *11*, 465-476.

Husom, A. D., Peters, E. A., Kolling, E. A., Fugere, N. A., Thompson, L. V., and Ferrington, D. A. (2004). Altered proteasome function and subunit composition in aged muscle. Arch Biochem Biophys *421*, 67-76.

Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. Science *257*, 1496-1502.

Iakova, P., Wang, G. L., Timchenko, L., Michalak, M., Pereira-Smith, O. M., Smith, J. R., and Timchenko, N. A. (2004). Competition of CUGBP1 and calreticulin for the regulation of p21 translation determines cell fate. Embo J *23*, 406-417.

Imai, J., Hasegawa, H., Maruya, M., Koyasu, S., and Yahara, I. (2005). Exogenous antigens are processed through the endoplasmic reticulum-associated degradation (ERAD) in cross-presentation by dendritic cells. Int Immunol *17*, 45-53.

Imperiali, B., and O'Connor, S. E. (1999). Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. Curr Opin Chem Biol *3*, 643-649.

Ito, A., Kawaguchi, Y., Lai, C. H., Kovacs, J. J., Higashimoto, Y., Appella, E., and Yao, T. P. (2002). MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. Embo J *21*, 6236-6245.

Jousse, C., Deval, C., Maurin, A. C., Parry, L., Cherasse, Y., Chaveroux, C., Lefloch, R., Lenormand, P., Bruhat, A., and Fafournoux, P. (2007). TRB3 inhibits the transcriptional activation of stress-regulated genes by a negative feedback on the ATF4 pathway. J Biol Chem *282*, 15851-15861.

Kabani, M., Kelley, S. S., Morrow, M. W., Montgomery, D. L., Sivendran, R., Rose, M. D., Gierasch, L. M., and Brodsky, J. L. (2003). Dependence of endoplasmic reticulum-associated degradation on the peptide binding domain and concentration of BiP. Mol Biol Cell *14*, 3437-3448.

Kalies, K. U., Allan, S., Sergeyenko, T., Kroger, H., and Romisch, K. (2005). The protein translocation channel binds proteasomes to the endoplasmic reticulum membrane. Embo J *24*, 2284-2293.

Kaneko, M., Ishiguro, M., Niinuma, Y., Uesugi, M., and Nomura, Y. (2002). Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. FEBS Lett *532*, 147-152.

Kang, S. J., and Cresswell, P. (2002). Calnexin, calreticulin, and ERp57 cooperate in disulfide bond formation in human CD1d heavy chain. J Biol Chem *277*, 44838-44844.

Kapoor, M., Ellgaard, L., Gopalakrishnapai, J., Schirra, C., Gemma, E., Oscarson, S., Helenius, A., and Surolia, A. (2004). Mutational analysis provides molecular insight into the carbohydrate-binding region of calreticulin: pivotal roles of tyrosine-109 and aspartate-135 in carbohydrate recognition. Biochemistry *43*, 97-106.

Kenniston, J. A., Baker, T. A., Fernandez, J. M., and Sauer, R. T. (2003). Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA+ degradation machine. Cell *114*, 511-520.

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu Rev Cell Dev Biol *22*, 159-180.

Kim, I., Ahn, J., Liu, C., Tanabe, K., Apodaca, J., Suzuki, T., and Rao, H. (2006). The Png1-Rad23 complex regulates glycoprotein turnover. J Cell Biol *172*, 211-219.

Knee, R., Ahsan, I., Mesaeli, N., Kaufman, R. J., and Michalak, M. (2003). Compromised calnexin function in calreticulin-deficient cells. Biochem Biophys Res Commun *304*, 661-666.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635-644.

Kokame, K., Kato, H., and Miyata, T. (2001). Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. J Biol Chem *276*, 9199-9205.

Kondo, S., Murakami, T., Tatsumi, K., Ogata, M., Kanemoto, S., Otori, K., Iseki, K., Wanaka, A., and Imaizumi, K. (2005). OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes. Nat Cell Biol *7*, 186-194.

Kondo, S., Saito, A., Hino, S., Murakami, T., Ogata, M., Kanemoto, S., Nara, S., Yamashita, A., Yoshinaga, K., Hara, H., and Imaizumi, K. (2007). BBF2H7, a novel transmembrane bZIP transcription factor, is a new type of endoplasmic reticulum stress transducer. Mol Cell Biol *27*, 1716-1729.

Kostova, Z., Tsai, Y. C., and Weissman, A. M. (2007). Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. Semin Cell Dev Biol *18*, 770-779.

Kowalski, J. M., Parekh, R. N., Mao, J., and Wittrup, K. D. (1998a). Protein folding stability can determine the efficiency of escape from endoplasmic reticulum quality control. J Biol Chem *273*, 19453-19458.

Kowalski, J. M., Parekh, R. N., and Wittrup, K. D. (1998b). Secretion efficiency in Saccharomyces cerevisiae of bovine pancreatic trypsin inhibitor mutants lacking disulfide bonds is correlated with thermodynamic stability. Biochemistry *37*, 1264-1273.

Kreft, S. G., Wang, L., and Hochstrasser, M. (2006). Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). J Biol Chem *281*, 4646-4653.

Kubbutat, M. H., and Vousden, K. H. (1997). Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. Mol Cell Biol *17*, 460-468.

Kuo, S. C., and Lampen, J. O. (1974). Tunicamycin--an inhibitor of yeast glycoprotein synthesis. Biochem Biophys Res Commun *58*, 287-295.

Kuras, L., Rouillon, A., Lee, T., Barbey, R., Tyers, M., and Thomas, D. (2002). Dual regulation of the met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment. Mol Cell *10*, 69-80.

Kuwabara, K., Pinsky, D. J., Schmidt, A. M., Benedict, C., Brett, J., Ogawa, S., Broekman, M. J., Marcus, A. J., Sciacca, R. R., Michalak, M., and et al. (1995). Calreticulin, an antithrombotic agent which binds to vitamin K-dependent coagulation factors, stimulates endothelial nitric oxide production, and limits thrombosis in canine coronary arteries. J Biol Chem *270*, 8179-8187.

Laing, J. G., and Beyer, E. C. (1995). The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. J Biol Chem *270*, 26399-26403.

Laird, D. W., Castillo, M., and Kasprzak, L. (1995). Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. J Cell Biol *131*, 1193-1203.

Larner, S. F., Hayes, R. L., and Wang, K. K. (2006). Unfolded protein response after neurotrauma. J Neurotrauma *23*, 807-829.

Le, A., Steiner, J. L., Ferrell, G. A., Shaker, J. C., and Sifers, R. N. (1994). Association between calnexin and a secretion-incompetent variant of human alpha 1-antitrypsin. J Biol Chem *269*, 7514-7519.

Leach, M. R., Cohen-Doyle, M. F., Thomas, D. Y., and Williams, D. B. (2002). Localization of the lectin, ERp57 binding, and polypeptide binding sites of calnexin and calreticulin. J Biol Chem *277*, 29686-29697.

Lee, C. S., Han, E. S., Han, Y. S., and Bang, H. (2005a). Differential effect of calmodulin antagonists on MG132-induced mitochondrial dysfunction and cell death in PC12 cells. Brain Res Bull *67*, 225-234.

Lee, C. S., Han, E. S., Park, E. S., and Bang, H. (2005b). Inhibition of MG132-induced mitochondrial dysfunction and cell death in PC12 cells by 3-morpholinosydnonimine. Brain Res *1036*, 18-26.

Lee, D., Ezhkova, E., Li, B., Pattenden, S. G., Tansey, W. P., and Workman, J. L. (2005c). The proteasome regulatory particle alters the SAGA coactivator to enhance its interactions with transcriptional activators. Cell *123*, 423-436.

Lee, R. J., Liu, C. W., Harty, C., McCracken, A. A., Latterich, M., Romisch, K., DeMartino, G. N., Thomas, P. J., and Brodsky, J. L. (2004). Uncoupling retro-

translocation and degradation in the ER-associated degradation of a soluble protein. Embo J *23*, 2206-2215.

Lee, S., Elenbaas, B., Levine, A., and Griffith, J. (1995). p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. Cell *81*, 1013-1020.

Leri, A., Franco, S., Zacheo, A., Barlucchi, L., Chimenti, S., Limana, F., Nadal-Ginard, B., Kajstura, J., Anversa, P., and Blasco, M. A. (2003). Ablation of telomerase and telomere loss leads to cardiac dilatation and heart failure associated with p53 upregulation. Embo J *22*, 131-139.

Li, J., Lee, B., and Lee, A. S. (2006). Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. J Biol Chem *281*, 7260-7270.

Li, M., Chen, D., Shiloh, A., Luo, J., Nikolaev, A. Y., Qin, J., and Gu, W. (2002). Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature *416*, 648-653.

Liang, J., Yin, C., Doong, H., Fang, S., Peterhoff, C., Nixon, R. A., and Monteiro, M. J. (2006). Characterization of erasin (UBXD2): a new ER protein that promotes ER-associated protein degradation. J Cell Sci *119*, 4011-4024.

Liao, W., and Chan, L. (2001). Tunicamycin induces ubiquitination and degradation of apolipoprotein B in HepG2 cells. Biochem J *353*, 493-501.

Lilley, B. N., and Ploegh, H. L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. Nature *429*, 834-840.

Liu, X. H., Kirschenbaum, A., Yu, K., Yao, S., and Levine, A. C. (2005). Cyclooxygenase-2 suppresses hypoxia-induced apoptosis via a combination of direct and indirect inhibition of p53 activity in a human prostate cancer cell line. J Biol Chem *280*, 3817-3823.

Liu, Y., Choudhury, P., Cabral, C. M., and Sifers, R. N. (1999). Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. J Biol Chem *274*, 5861-5867.

Lu, P. D., Harding, H. P., and Ron, D. (2004). Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J Cell Biol *167*, 27-33.

Luo, D., He, Y., Zhang, H., Yu, L., Chen, H., Xu, Z., Tang, S., Urano, F., and Min, W. (2008). AIP1 is critical in transducing IRE1-mediated endoplasmic reticulum stress response. J Biol Chem.

Ma, Y., Brewer, J. W., Diehl, J. A., and Hendershot, L. M. (2002). Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. J Mol Biol *318*, 1351-1365.

Ma, Y., and Hendershot, L. M. (2004). The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer *4*, 966-977.

Mancini, R., Aebi, M., and Helenius, A. (2003). Multiple endoplasmic reticulum-associated pathways degrade mutant yeast carboxypeptidase Y in mammalian cells. J Biol Chem *278*, 46895-46905.

Marciniak, S. J., Yun, C. Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H. P., and Ron, D. (2004). CHOP induces death by promoting

protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev *18*, 3066-3077.

Marquardt, T., and Helenius, A. (1992). Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. J Cell Biol *117*, 505-513.

Martin, V., Groenendyk, J., Steiner, S. S., Guo, L., Dabrowska, M., Parker, J. M., Muller-Esterl, W., Opas, M., and Michalak, M. (2006). Identification by mutational analysis of amino acid residues essential in the chaperone function of calreticulin. J Biol Chem *281*, 2338-2346.

Matsumoto, M., Minami, M., Takeda, K., Sakao, Y., and Akira, S. (1996). Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. FEBS Lett *395*, 143-147.

Mayer, T. U., Braun, T., and Jentsch, S. (1998). Role of the proteasome in membrane extraction of a short-lived ER-transmembrane protein. Embo J *17*, 3251-3257.

Mayo, L. D., and Donner, D. B. (2002). The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. Trends Biochem Sci *27*, 462-467.

McCullough, K. D., Martindale, J. L., Klotz, L. O., Aw, T. Y., and Holbrook, N. J. (2001). Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol *21*, 1249-1259.

Meiners, S., Heyken, D., Weller, A., Ludwig, A., Stangl, K., Kloetzel, P. M., and Kruger, E. (2003). Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of Mammalian proteasomes. J Biol Chem *278*, 21517-21525.

Menconi, M. J., Wei, W., Yang, H., Wray, C. J., and Hasselgren, P. O. (2004). Treatment of cultured myotubes with the calcium ionophore A23187 increases proteasome activity via a CaMK II-caspase-calpain-dependent mechanism. Surgery *136*, 135-142.

Menendez-Benito, V., Verhoef, L. G., Masucci, M. G., and Dantuma, N. P. (2005). Endoplasmic reticulum stress compromises the ubiquitin-proteasome system. Hum Mol Genet *14*, 2787-2799.

Mery, L., Mesaeli, N., Michalak, M., Opas, M., Lew, D. P., and Krause, K. H. (1996). Overexpression of calreticulin increases intracellular Ca2+ storage and decreases store-operated Ca2+ influx. J Biol Chem *271*, 9332-9339.

Mesaeli, N., Nakamura, K., Zvaritch, E., Dickie, P., Dziak, E., Krause, K. H., Opas, M., MacLennan, D. H., and Michalak, M. (1999). Calreticulin is essential for cardiac development. J Cell Biol *144*, 857-868.

Mesaeli, N., and Phillipson, C. (2004). Impaired p53 expression, function, and nuclear localization in calreticulin-deficient cells. Mol Biol Cell *15*, 1862-1870.

Meusser, B., Hirsch, C., Jarosch, E., and Sommer, T. (2005). ERAD: the long road to destruction. Nat Cell Biol *7*, 766-772.

Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D., and Warren, G. (2000). A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. Embo J *19*, 2181-2192.

Meyer, H. H., Wang, Y., and Warren, G. (2002). Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. Embo J *21*, 5645-5652.

Michalak, M., Burns, K., Andrin, C., Mesaeli, N., Jass, G. H., Busaan, J. L., and Opas, M. (1996). Endoplasmic reticulum form of calreticulin modulates glucocorticoid-sensitive gene expression. J Biol Chem *271*, 29436-29445.

Michalak, M., Corbett, E. F., Mesaeli, N., Nakamura, K., and Opas, M. (1999). Calreticulin: one protein, one gene, many functions. Biochem J *344 Pt 2*, 281-292.

Mitch, W. E., and Goldberg, A. L. (1996). Mechanisms of muscle wasting. The role of the ubiquitin-proteasome pathway. N Engl J Med *335*, 1897-1905.

Molinari, M., Calanca, V., Galli, C., Lucca, P., and Paganetti, P. (2003). Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. Science *299*, 1397-1400.

Molinari, M., Eriksson, K. K., Calanca, V., Galli, C., Cresswell, P., Michalak, M., and Helenius, A. (2004). Contrasting functions of calreticulin and calnexin in glycoprotein folding and ER quality control. Mol Cell *13*, 125-135.

Molinari, M., Galli, C., Vanoni, O., Arnold, S. M., and Kaufman, R. J. (2005). Persistent glycoprotein misfolding activates the glucosidase II/UGT1-driven calnexin cycle to delay aggregation and loss of folding competence. Mol Cell *20*, 503-512.

Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell *93*, 215-228.

Morito, D., Hirao, K., Oda, Y., Hosokawa, N., Tokunaga, F., Cyr, D. M., Tanaka, K., Iwai, K., and Nagata, K. (2008). Gp78 Cooperates with RMA1 in ER-associated Degradation of CFTR{Delta}F508. Mol Biol Cell.

Nakagawa, T., and Yuan, J. (2000). Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol *150*, 887-894.

Nakamura, K., Bossy-Wetzel, E., Burns, K., Fadel, M. P., Lozyk, M., Goping, I. S., Opas, M., Bleackley, R. C., Green, D. R., and Michalak, M. (2000). Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. J Cell Biol *150*, 731-740.

Nakamura, K., Zuppini, A., Arnaudeau, S., Lynch, J., Ahsan, I., Krause, R., Papp, S., De Smedt, H., Parys, J. B., Muller-Esterl, W., *et al.* (2001). Functional specialization of calreticulin domains. J Cell Biol *154*, 961-972.

Neuber, O., Jarosch, E., Volkwein, C., Walter, J., and Sommer, T. (2005). Ubx2 links the Cdc48 complex to ER-associated protein degradation. Nat Cell Biol *7*, 993-998.

Ng, D. T., Spear, E. D., and Walter, P. (2000). The unfolded protein response regulates multiple aspects of secretory and membrane protein biogenesis and endoplasmic reticulum quality control. J Cell Biol *150*, 77-88.

Nihei, T., Sato, N., Takahashi, S., Ishikawa, M., Sagae, S., Kudo, R., Kikuchi, K., and Inoue, A. (1993). Demonstration of selective protein complexes of p53 with 73 kDa heat shock cognate protein, but not with 72 kDa heat shock protein in human tumor cells. Cancer Lett *73*, 181-189.

Nishikawa, S. I., Fewell, S. W., Kato, Y., Brodsky, J. L., and Endo, T. (2001). Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. J Cell Biol *153*, 1061-1070.

Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., and Ichijo, H. (2002). ASK1 is essential for endoplasmic

reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev *16*, 1345-1355.

Novoa, I., Zeng, H., Harding, H. P., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. J Cell Biol *153*, 1011-1022.

O'Callaghan-Sunol, C., Gabai, V. L., and Sherman, M. Y. (2007). Hsp27 modulates p53 signaling and suppresses cellular senescence. Cancer Res *67*, 11779-11788.

O'Connor, S. E., and Imperiali, B. (1996). Modulation of protein structure and function by asparagine-linked glycosylation. Chem Biol *3*, 803-812.

Oda, Y., Hosokawa, N., Wada, I., and Nagata, K. (2003). EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. Science *299*, 1394-1397.

Ohoka, N., Yoshii, S., Hattori, T., Onozaki, K., and Hayashi, H. (2005). TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. Embo J *24*, 1243-1255.

Ojcius, D. M., Zychlinsky, A., Zheng, L. M., and Young, J. D. (1991). Ionophore-induced apoptosis: role of DNA fragmentation and calcium fluxes. Exp Cell Res *197*, 43-49.

Olivari, S., Cali, T., Salo, K. E., Paganetti, P., Ruddock, L. W., and Molinari, M. (2006). EDEM1 regulates ER-associated degradation by accelerating de-mannosylation of folding-defective polypeptides and by inhibiting their covalent aggregation. Biochem Biophys Res Commun *349*, 1278-1284.

Olivari, S., Galli, C., Alanen, H., Ruddock, L., and Molinari, M. (2005). A novel stress-induced EDEM variant regulating endoplasmic reticulum-associated glycoprotein degradation. J Biol Chem *280*, 2424-2428.

Oliver, J. D., Roderick, H. L., Llewellyn, D. H., and High, S. (1999). ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin. Mol Biol Cell *10*, 2573-2582.

Oliver, J. D., van der Wal, F. J., Bulleid, N. J., and High, S. (1997). Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. Science *275*, 86-88.

Omori, Y., Imai, J., Watanabe, M., Komatsu, T., Suzuki, Y., Kataoka, K., Watanabe, S., Tanigami, A., and Sugano, S. (2001). CREB-H: a novel mammalian transcription factor belonging to the CREB/ATF family and functioning via the box-B element with a liver-specific expression. Nucleic Acids Res *29*, 2154-2162.

Opas, M., Szewczenko-Pawlikowski, M., Jass, G. K., Mesaeli, N., and Michalak, M. (1996). Calreticulin modulates cell adhesiveness via regulation of vinculin expression. J Cell Biol *135*, 1913-1923.

Ostwald, T. J., and MacLennan, D. H. (1974). Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. J Biol Chem *249*, 974-979.

Otsu, M., Urade, R., Kito, M., Omura, F., and Kikuchi, M. (1995). A possible role of ER-60 protease in the degradation of misfolded proteins in the endoplasmic reticulum. J Biol Chem *270*, 14958-14961.

Ou, W. J., Bergeron, J. J., Li, Y., Kang, C. Y., and Thomas, D. Y. (1995). Conformational changes induced in the endoplasmic reticulum luminal domain of calnexin by Mg-ATP and Ca2+. J Biol Chem *270*, 18051-18059.

Palmer, A., Rivett, A. J., Thomson, S., Hendil, K. B., Butcher, G. W., Fuertes, G., and Knecht, E. (1996). Subpopulations of proteasomes in rat liver nuclei, microsomes and cytosol. Biochem J *316 (Pt 2)*, 401-407.

Park, Y., Hwang, Y. P., Lee, J. S., Seo, S. H., Yoon, S. K., and Yoon, J. B. (2005). Proteasomal ATPase-associated factor 1 negatively regulates proteasome activity by interacting with proteasomal ATPases. Mol Cell Biol *25*, 3842-3853.

Perrone, L., Tell, G., and Di Lauro, R. (1999). Calreticulin enhances the transcriptional activity of thyroid transcription factor-1 by binding to its homeodomain. J Biol Chem *274*, 4640-4645.

Perry, M. E., and Levine, A. J. (1993). Tumor-suppressor p53 and the cell cycle. Curr Opin Genet Dev *3*, 50-54.

Pickart, C. M. (1997). Targeting of substrates to the 26S proteasome. Faseb J *11*, 1055-1066.

Pilon, M., Schekman, R., and Romisch, K. (1997). Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. Embo J *16*, 4540-4548.

Plemper, R. K., Bordallo, J., Deak, P. M., Taxis, C., Hitt, R., and Wolf, D. H. (1999). Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. J Cell Sci *112 (Pt 22)*, 4123-4134.

Pluquet, O., Qu, L. K., Baltzis, D., and Koromilas, A. E. (2005). Endoplasmic reticulum stress accelerates p53 degradation by the cooperative actions of Hdm2 and glycogen synthase kinase 3beta. Mol Cell Biol *25*, 9392-9405.

Qu, L., Huang, S., Baltzis, D., Rivas-Estilla, A. M., Pluquet, O., Hatzoglou, M., Koumenis, C., Taya, Y., Yoshimura, A., and Koromilas, A. E. (2004). Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3beta. Genes Dev *18*, 261-277.

Raggo, C., Rapin, N., Stirling, J., Gobeil, P., Smith-Windsor, E., O'Hare, P., and Misra, V. (2002). Luman, the cellular counterpart of herpes simplex virus VP16, is processed by regulated intramembrane proteolysis. Mol Cell Biol *22*, 5639-5649.

Rao, R. V., Castro-Obregon, S., Frankowski, H., Schuler, M., Stoka, V., del Rio, G., Bredesen, D. E., and Ellerby, H. M. (2002). Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. J Biol Chem *277*, 21836-21842.

Rao, R. V., Hermel, E., Castro-Obregon, S., del Rio, G., Ellerby, L. M., Ellerby, H. M., and Bredesen, D. E. (2001). Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. J Biol Chem *276*, 33869-33874.

Rao, R. V., Poksay, K. S., Castro-Obregon, S., Schilling, B., Row, R. H., del Rio, G., Gibson, B. W., Ellerby, H. M., and Bredesen, D. E. (2004). Molecular components of a cell death pathway activated by endoplasmic reticulum stress. J Biol Chem *279*, 177-187.

Ravid, T., Kreft, S. G., and Hochstrasser, M. (2006). Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. Embo J *25*, 533-543.

Ray, P. S., Grover, R., and Das, S. (2006). Two internal ribosome entry sites mediate the translation of p53 isoforms. EMBO Rep *7*, 404-410.

Realini, C. A., and Rechsteiner, M. C. (1996). Proposed role of a gamma-interferon inducible proteasome-regulator in antigen presentation. Adv Exp Med Biol *389*, 51-61.

Reed, S. H., and Gillette, T. G. (2007). Nucleotide excision repair and the ubiquitin proteasome pathway--do all roads lead to Rome? DNA Repair (Amst) *6*, 149-156.

Reits, E. A., Benham, A. M., Plougastel, B., Neefjes, J., and Trowsdale, J. (1997). Dynamics of proteasome distribution in living cells. Embo J *16*, 6087-6094.

Reshetnyak, Y. K., Kitson, R. P., Lu, M., and Goldfarb, R. H. (2004). Conformational and enzymatic changes of 20S proteasome of rat natural killer cells induced by mono- and divalent cations. J Struct Biol *145*, 263-271.

Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C., and Jentsch, S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell *120*, 73-84.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol *9*, 402-412.

Rivett, A. J., Palmer, A., and Knecht, E. (1992). Electron microscopic localization of the multicatalytic proteinase complex in rat liver and in cultured cells. J Histochem Cytochem *40*, 1165-1172.

Robzyk, K., Recht, J., and Osley, M. A. (2000). Rad6-dependent ubiquitination of histone H2B in yeast. Science *287*, 501-504.

Rogers, T. B., Inesi, G., Wade, R., and Lederer, W. J. (1995). Use of thapsigargin to study Ca2+ homeostasis in cardiac cells. Biosci Rep *15*, 341-349.

Rohde, M., Daugaard, M., Jensen, M. H., Helin, K., Nylandsted, J., and Jaattela, M. (2005). Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms. Genes Dev *19*, 570-582.

Romisch, K. (2005). Endoplasmic reticulum-associated degradation. Annu Rev Cell Dev Biol *21*, 435-456.

Rubin, D. M., Glickman, M. H., Larsen, C. N., Dhruvakumar, S., and Finley, D. (1998). Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome. Embo J *17*, 4909-4919.

Ruddock, L. W., and Molinari, M. (2006). N-glycan processing in ER quality control. J Cell Sci *119*, 4373-4380.

Rustandi, R. R., Baldisseri, D. M., and Weber, D. J. (2000). Structure of the negative regulatory domain of p53 bound to S100B(betabeta). Nat Struct Biol *7*, 570-574.

Rutter, G. A., Theler, J. M., Murgia, M., Wollheim, C. B., Pozzan, T., and Rizzuto, R. (1993). Stimulated Ca2+ influx raises mitochondrial free Ca2+ to supramicromolar levels in a pancreatic beta-cell line. Possible role in glucose and agonist-induced insulin secretion. J Biol Chem *268*, 22385-22390.

Saeki, Y., Saitoh, A., Toh-e, A., and Yokosawa, H. (2002). Ubiquitin-like proteins and Rpn10 play cooperative roles in ubiquitin-dependent proteolysis. Biochem Biophys Res Commun *293*, 986-992.

Saito, Y., Ihara, Y., Leach, M. R., Cohen-Doyle, M. F., and Williams, D. B. (1999). Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins. Embo J *18*, 6718-6729.

Schmidt, M., Hanna, J., Elsasser, S., and Finley, D. (2005). Proteasome-associated proteins: regulation of a proteolytic machine. Biol Chem *386*, 725-737.

Schmitz, A., and Herzog, V. (2004). Endoplasmic reticulum-associated degradation: exceptions to the rule. Eur J Cell Biol *83*, 501-509.

Schroder, M., and Kaufman, R. J. (2005). The mammalian unfolded protein response. Annu Rev Biochem *74*, 739-789.

Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003). BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. Science *300*, 135-139.

Shamu, C. E., and Walter, P. (1996). Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. Embo J *15*, 3028-3039.

Sharon, M., Taverner, T., Ambroggio, X. I., Deshaies, R. J., and Robinson, C. V. (2006). Structural organization of the 19S proteasome lid: insights from MS of intact complexes. PLoS Biol *4*, e267.

Shen, J., Chen, X., Hendershot, L., and Prywes, R. (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. Dev Cell *3*, 99-111.

Shen, X., Zhang, K., and Kaufman, R. J. (2004). The unfolded protein response--a stress signaling pathway of the endoplasmic reticulum. J Chem Neuroanat *28*, 79-92.

Shen, Y., Ballar, P., and Fang, S. (2006). Ubiquitin ligase gp78 increases solubility and facilitates degradation of the Z variant of alpha-1-antitrypsin. Biochem Biophys Res Commun *349*, 1285-1293.

Sherman, M. Y., Gabai, V., O'Callaghan, C., and Yaglom, J. (2007). Molecular chaperones regulate p53 and suppress senescence programs. FEBS Lett *581*, 3711-3715.

Shiraishi, H., Okamoto, H., Yoshimura, A., and Yoshida, H. (2006). ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1. J Cell Sci *119*, 3958-3966.

Sidrauski, C., Cox, J. S., and Walter, P. (1996). tRNA ligase is required for regulated mRNA splicing in the unfolded protein response. Cell *87*, 405-413.

Sifers, R. N., Shen, R. F., and Woo, S. L. (1989). Genetic control of human alpha-1-antitrypsin. Mol Biol Med *6*, 127-135.

Sitia, R., and Braakman, I. (2003). Quality control in the endoplasmic reticulum protein factory. Nature *426*, 891-894.

Solda, T., Galli, C., Kaufman, R. J., and Molinari, M. (2007). Substrate-specific requirements for UGT1-dependent release from calnexin. Mol Cell *27*, 238-249.

Sousa, M. C., Ferrero-Garcia, M. A., and Parodi, A. J. (1992). Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. Biochemistry *31*, 97-105.

Spiro, R. G., Zhu, Q., Bhoyroo, V., and Soling, H. D. (1996). Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver Golgi. J Biol Chem *271*, 11588-11594.

Stirling, J., and O'Hare, P. (2006). CREB4, a transmembrane bZip transcription factor and potential new substrate for regulation and cleavage by S1P. Mol Biol Cell *17*, 413-426.

Sui, G., Affar el, B., Shi, Y., Brignone, C., Wall, N. R., Yin, P., Donohoe, M., Luke, M. P., Calvo, D., and Grossman, S. R. (2004). Yin Yang 1 is a negative regulator of p53. Cell *117*, 859-872.

Sun, F., Zhang, R., Gong, X., Geng, X., Drain, P. F., and Frizzell, R. A. (2006). Derlin-1 promotes the efficient degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) and CFTR folding mutants. J Biol Chem *281*, 36856-36863.

Sun, Z. W., and Allis, C. D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature *418*, 104-108.

Suzuki, T., Park, H., Kwofie, M. A., and Lennarz, W. J. (2001). Rad23 provides a link between the Png1 deglycosylating enzyme and the 26 S proteasome in yeast. J Biol Chem *276*, 21601-21607.

Suzuki, T., Park, H., and Lennarz, W. J. (2002). Cytoplasmic peptide:N-glycanase (PNGase) in eukaryotic cells: occurrence, primary structure, and potential functions. Faseb J *16*, 635-641.

Szegezdi, E., Logue, S. E., Gorman, A. M., and Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep *7*, 880-885.

Takahashi, R., Imai, Y., Hattori, N., and Mizuno, Y. (2003). Parkin and endoplasmic reticulum stress. Ann N Y Acad Sci *991*, 101-106.

Takei, N., and Endo, Y. (1994). Ca2+ ionophore-induced apoptosis on cultured embryonic rat cortical neurons. Brain Res *652*, 65-70.

Tan, Y., Chen, M., Li, Z., Mabuchi, K., and Bouvier, M. (2006). The calcium- and zinc-responsive regions of calreticulin reside strictly in the N-/C-domain. Biochim Biophys Acta *1760*, 745-753.

Termine, D., Wu, Y., Liu, Y., and Sifers, R. N. (2005). Alpha1-antitrypsin as model to assess glycan function in endoplasmic reticulum. Methods *35*, 348-353.

Territo, P. R., French, S. A., Dunleavy, M. C., Evans, F. J., and Balaban, R. S. (2001). Calcium activation of heart mitochondrial oxidative phosphorylation: rapid kinetics of mVO2, NADH, AND light scattering. J Biol Chem *276*, 2586-2599.

Thomson, S. P., and Williams, D. B. (2005). Delineation of the lectin site of the molecular chaperone calreticulin. Cell Stress Chaperones *10*, 242-251.

Tirasophon, W., Lee, K., Callaghan, B., Welihinda, A., and Kaufman, R. J. (2000). The endoribonuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response. Genes Dev *14*, 2725-2736.

Tirasophon, W., Welihinda, A. A., and Kaufman, R. J. (1998). A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. Genes Dev *12*, 1812-1824.

Tirosh, B., Furman, M. H., Tortorella, D., and Ploegh, H. L. (2003). Protein unfolding is not a prerequisite for endoplasmic reticulum-to-cytosol dislocation. J Biol Chem *278*, 6664-6672.

Tokunaga, F., Brostrom, C., Koide, T., and Arvan, P. (2000). Endoplasmic reticulum (ER)-associated degradation of misfolded N-linked glycoproteins is suppressed upon inhibition of ER mannosidase I. J Biol Chem *275*, 40757-40764.

Totary-Jain, H., Naveh-Many, T., Riahi, Y., Kaiser, N., Eckel, J., and Sasson, S. (2005). Calreticulin destabilizes glucose transporter-1 mRNA in vascular endothelial and smooth muscle cells under high-glucose conditions. Circ Res *97*, 1001-1008.

Treves, S., De Mattei, M., Landfredi, M., Villa, A., Green, N. M., MacLennan, D. H., Meldolesi, J., and Pozzan, T. (1990). Calreticulin is a candidate for a calsequestrin-like function in Ca2(+)-storage compartments (calciosomes) of liver and brain. Biochem J *271*, 473-480.

Trombetta, E. S., and Helenius, A. (1998). Lectins as chaperones in glycoprotein folding. Curr Opin Struct Biol *8*, 587-592.

Trombetta, E. S., and Helenius, A. (2000). Conformational requirements for glycoprotein reglucosylation in the endoplasmic reticulum. J Cell Biol *148*, 1123-1129.

Trombetta, E. S., and Parodi, A. J. (2003). Quality control and protein folding in the secretory pathway. Annu Rev Cell Dev Biol *19*, 649-676.

Tsai, B., Rodighiero, C., Lencer, W. I., and Rapoport, T. A. (2001). Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. Cell *104*, 937-948.

Tsvetkov, P., Asher, G., Reiss, V., Shaul, Y., Sachs, L., and Lotem, J. (2005). Inhibition of NAD(P)H:quinone oxidoreductase 1 activity and induction of p53 degradation by the natural phenolic compound curcumin. Proc Natl Acad Sci U S A *102*, 5535-5540.

Tu, D., Li, W., Ye, Y., and Brunger, A. T. (2007). Inaugural Article: Structure and function of the yeast U-box-containing ubiquitin ligase Ufd2p. Proc Natl Acad Sci U S A *104*, 15599-15606.

Uchiyama, K., Jokitalo, E., Kano, F., Murata, M., Zhang, X., Canas, B., Newman, R., Rabouille, C., Pappin, D., Freemont, P., and Kondo, H. (2002). VCIP135, a novel essential factor for p97/p47-mediated membrane fusion, is required for Golgi and ER assembly in vivo. J Cell Biol *159*, 855-866.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and Ron, D. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science *287*, 664-666.

Uvarov, A. V., and Mesaeli, N. (2008). Enhanced ubiquitin-proteasome activity in calreticulin deficient cells: a compensatory mechanism for cell survival. Biochim Biophys Acta *1783*, 1237-1247.

Vashist, S., Kim, W., Belden, W. J., Spear, E. D., Barlowe, C., and Ng, D. T. (2001). Distinct retrieval and retention mechanisms are required for the quality control of endoplasmic reticulum protein folding. J Cell Biol *155*, 355-368.

Vassilakos, A., Michalak, M., Lehrman, M. A., and Williams, D. B. (1998). Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin. Biochemistry *37*, 3480-3490.

Vattem, K. M., and Wek, R. C. (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc Natl Acad Sci U S A *101*, 11269-11274.

Verma, R., Oania, R., Graumann, J., and Deshaies, R. J. (2004). Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. Cell *118*, 99-110.

Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu Rev Biochem *68*, 1015-1068.

Wang, Q., and Chang, A. (2003). Substrate recognition in ER-associated degradation mediated by Eps1, a member of the protein disulfide isomerase family. Embo J *22*, 3792-3802.

Wang, X. Z., Harding, H. P., Zhang, Y., Jolicoeur, E. M., Kuroda, M., and Ron, D. (1998). Cloning of mammalian Ire1 reveals diversity in the ER stress responses. Embo J *17*, 5708-5717.

Wang, Y., Shen, J., Arenzana, N., Tirasophon, W., Kaufman, R. J., and Prywes, R. (2000). Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. J Biol Chem *275*, 27013-27020.

Watanabe, D., Yamada, K., Nishina, Y., Tajima, Y., Koshimizu, U., Nagata, A., and Nishimune, Y. (1994). Molecular cloning of a novel Ca(2+)-binding protein (calmegin) specifically expressed during male meiotic germ cell development. J Biol Chem *269*, 7744-7749.

Werner, E. D., Brodsky, J. L., and McCracken, A. A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. Proc Natl Acad Sci U S A *93*, 13797-13801.

Wheeler, D. G., Horsford, J., Michalak, M., White, J. H., and Hendy, G. N. (1995). Calreticulin inhibits vitamin D3 signal transduction. Nucleic Acids Res *23*, 3268-3274.

Wieland, F. T., Gleason, M. L., Serafini, T. A., and Rothman, J. E. (1987). The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell *50*, 289-300.

Williams, D. B. (2006). Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum. J Cell Sci *119*, 615-623.

Wojcik, C. (1997). On the spatial organization of ubiquitin-dependent proteolysis in HeLa cells. Folia Histochem Cytobiol *35*, 117-118.

Wojcik, C., Schroeter, D., Wilk, S., Lamprecht, J., and Paweletz, N. (1996). Ubiquitin-mediated proteolysis centers in HeLa cells: indication from studies of an inhibitor of the chymotrypsin-like activity of the proteasome. Eur J Cell Biol *71*, 311-318.

Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. (1993). The p53-mdm-2 autoregulatory feedback loop. Genes Dev *7*, 1126-1132.

Wu, Y., Swulius, M. T., Moremen, K. W., and Sifers, R. N. (2003). Elucidation of the molecular logic by which misfolded alpha 1-antitrypsin is preferentially selected for degradation. Proc Natl Acad Sci U S A *100*, 8229-8234.

Xie, Y., and Varshavsky, A. (2001). RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc Natl Acad Sci U S A *98*, 3056-3061.

Xirodimas, D. P., Saville, M. K., Bourdon, J. C., Hay, R. T., and Lane, D. P. (2004). Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell *118*, 83-97.

Yang, N. C., Ho, W. M., Chen, Y. H., and Hu, M. L. (2002). A convenient one-step extraction of cellular ATP using boiling water for the luciferin-luciferase assay of ATP. Anal Biochem *306*, 323-327.

Yang, Y., Li, C. C., and Weissman, A. M. (2004). Regulating the p53 system through ubiquitination. Oncogene *23*, 2096-2106.

Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., Prywes, R., Brown, M. S., and Goldstein, J. L. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell *6*, 1355-1364.

Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature *414*, 652-656.

Ye, Y., Meyer, H. H., and Rapoport, T. A. (2003). Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. J Cell Biol *162*, 71-84.

Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T. A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature *429*, 841-847.

Yerlikaya, A., Kimball, S. R., and Stanley, B. A. (2008). Phosphorylation of eIF2alpha in Response to 26S Proteasome Inhibition is Mediated by the Heme-regulated Inhibitor (HRI) Kinase. Biochem J.

Yoneda, T., Imaizumi, K., Oono, K., Yui, D., Gomi, F., Katayama, T., and Tohyama, M. (2001). Activation of caspase-12, an endoplastic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. J Biol Chem *276*, 13935-13940.

Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature *352*, 345-347.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001a). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell *107*, 881-891.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., and Mori, K. (2000). ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. Mol Cell Biol *20*, 6755-6767.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., and Mori, K. (2001b). Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and 6beta that activates the mammalian unfolded protein response. Mol Cell Biol *21*, 1239-1248.

Yoshida, H., Oku, M., Suzuki, M., and Mori, K. (2006). pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response. J Cell Biol *172*, 565-575.

Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K., and Tai, T. (2002). E3 ubiquitin ligase that recognizes sugar chains. Nature *418*, 438-442.

Yoshida, Y., Tokunaga, F., Chiba, T., Iwai, K., Tanaka, K., and Tai, T. (2003). Fbs2 is a new member of the E3 ubiquitin ligase family that recognizes sugar chains. J Biol Chem *278*, 43877-43884.

Zhang, F., Hu, Y., Huang, P., Toleman, C. A., Paterson, A. J., and Kudlow, J. E. (2007). Proteasome function is regulated by cyclic AMP-dependent protein kinase through phosphorylation of Rpt6. J Biol Chem *282*, 22460-22471.

Zhang, F., Su, K., Yang, X., Bowe, D. B., Paterson, A. J., and Kudlow, J. E. (2003). O-GlcNAc modification is an endogenous inhibitor of the proteasome. Cell *115*, 715-725.

Zhang, J. X., Braakman, I., Matlack, K. E., and Helenius, A. (1997). Quality control in the secretory pathway: the role of calreticulin, calnexin and BiP in the retention of glycoproteins with C-terminal truncations. Mol Biol Cell *8*, 1943-1954.

Zhou, B. P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M. C. (2001). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol *3*, 973-982.

Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. (1998). CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev *12*, 982-995.

Zong, W. X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q. C., Yuan, J., and Thompson, C. B. (2003). Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol *162*, 59-69.