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# Delineating the Mechanisms of Misfolded Endoplasmic Reticulum (ER) Luminal Protein Retrotranslocation for ER-Associated Degradation

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## Abstract

Secreted, plasma membrane, and resident proteins of the secretory pathway are synthesized in the endoplasmic reticulum (ER) where they undergo post-translational modifications, oxidative folding, and subunit assembly in tightly monitored processes. An ER quality control (ERQC) system oversees protein maturation and ensures that only those reaching their native state will continue trafficking into the secretory pathway to reach their final destinations. Proteins that fail quality control must be recognized and eliminated to maintain ER proteostasis. The ER-associated degradation (ERAD) was discovered nearly 30 years ago and entails the identification of improperly matured secretory pathway proteins and their retrotranslocation to the cytosol for degradation by the ubiquitin-proteasome system. While multiple ER-Associated Degradation (ERAD) components have been identified and their roles elucidated, it remains less clear how folded domains on ERAD clients complicate their extraction from the ER and degradation. To address this, we used several luminal ERAD substrates with well-defined structural properties. Deglycosylation and digitonin permeabilization assays were used to monitor client extraction from the ER. Our fully unfolded clients were released into the cytosol when the proteasome was inhibited. Conversely, ERAD clients possessing a single folded domain were completely retrotranslocated but were not released from the ER membrane without proteasome function. These clients were fully reduced, but still retained structure in their folded domain. Overall, our data argue that ubiquitinated clients with well-folded domains can be dislocated from the ER in a p97-dependent manner, but proteasome activity is required to fully release them from the cytosolic side of the ER membrane and the ERAD extraction machinery.To decipher key steps in the extraction of non-glycosylated ER luminal proteins, and to determine how Hrd1, p97, and the proteasome contribute to the retrotranslocation of clients, we developed a novel in-cell biotin-based reporter system. The bacterial BirA biotin ligase was tethered to FAM8A1, a component of the retrotranslocon that interacts with Hrd1, thus positioning the biotin ligase near the cytosolic exit site. The non-secreted immunoglobulin (Ig) κ light chain (NS1 κ LC), which is composed of a poorly folded N-terminal domain and a well-folded C-terminal domain was used in the biotinylation assays. NS1 was modified at either the N- (BAP-NS1) or C- (NS1-BAP) terminus with a biotin acceptor peptide (BAP) allowing us to detect cytosolic exposure of both ends of this ERAD client. We established that BAP-tagged NS1 constructs were still ERAD substrates and that BirA-tagged FAM8A1 still assembled with the retrotranslocon. We found that both termini of NS1 were readily biotinylated when the proteasome was inhibited by MG132 treatment, or when dominant negative constructs of Hrd1 and p97 were expressed. To differentiate between full extraction of NS1 and partial exposure of the termini to the cytosolic side of the ER, we permeabilize the plasma membrane with digitonin. Cytosolic proteins were released, but both biotinylated and non-biotinylated NS1 remained cell-associated. This argues that the biotinylated light chain was not fully extracted from the ER when NS1 degradation was inhibited with either MG132 or with the Hrd1 and p97 mutants. The digitonin permeabilized cells expressing BAP-NS1 were treated with proteinase K to assess how much of this client was exposed to the cytosolic side of the ER. We found that the NS1 κ light chain was only partially extracted when cytosolic ERAD components (proteasome and p97) were impaired. These data indicate that the termini of this ERAD client can be inserted into the retrotranslocon and “sample” the cytosolic side but require the action of an E3 ligase, the p97 complex and the proteasome to be fully extracted.



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# Delineating the Mechanisms of Misfolded Endoplasmic Reticulum (ER) Luminal Protein Retrotranslocation for ER-Associated Degradation

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## DEDICATION

Dedicated to my father.
It is because of you that I am here today
and why I can always move forward.

## ACKNOWLEDGEMENTS

My passion for science and my curiosity for how cells -the smallest units of life- work, led me to continue my education and delve into the highest university degree. For this, I arrived in Memphis six years ago. Right from my lab rotation I felt that the Hendershot Lab would become my new home. Dr. Linda M. Hendershot welcomed me, became my Ph.D. advisor, and has stayed by my side to this day. I am truly grateful to her for mentoring me all these years, and for teaching me the true values and ideals of a scientist, while always being a supportive and caring advisor. I am thankful for everything that I have learned from Dr. Hendershot, from our discussions about the technical aspects of my work, to all the scientific brainstorming that we did to "see" how those misfolded proteins eventually get out the ER! It is because of all that, why I had the chance to grow and mature both scientifically and personally. I will always cherish my time here.

Along with Dr. Hendershot, I am very thankful to Melissa Mann. Melissa has always been there on a professional and personal level. She has been very helpful from day one, and she is the one who taught me many of our lab's techniques, as well as the accuracy and dexterity that scientific experimentation should be performed. I will also never forget all the chats we had about culture, politics, food, music and so much more! It all made the long hours of work more fun. I am also thankful to all my colleagues within the Hendershot lab, both past and present, for making work place vibrant and for always being supportive, encouraging, and helpful at any given opportunity. I thank Dr. Julia Behnke and Dr. Matthias Feige, for training me when I first started, for their support, and their advice. Dr. Joel Otero was the one who passed the torch, or rather his project, to me. I am very grateful to Joel for his help, his assistance, and his pipet. I truly thank Dr. Viraj Ichhaporia for his support, his feedback and scientific input, but more importantly for his friendship. I thank Rachael Wood, my friend and fellow graduate student for her help, support, and the much-needed coffee breaks. Dr. Greg Poet, Dr. Kristine Pobre, Mary Carson Irvine, and Chris Melendez-Suchi, I really appreciate the support, feedback, and technical help that I have received from you. I must express a big thanks to Candace Hayes, the Rhodes student that I supervised the past year, who stayed with me throughout the long hours and complicated experiments, and assisted in producing great data and finalizing my project.

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Finally, I want to thank Sabato Leo, who supported me from the beginning, gave me strength and endured grad student life with me. Lastly, I am grateful beyond measure to my parents and brother, without whom I would have never been able to experience this life-changing journey.

## ABSTRACT

Secreted, plasma membrane, and resident proteins of the secretory pathway are synthesized in the endoplasmic reticulum (ER) where they undergo post-translational modifications, oxidative folding, and subunit assembly in tightly monitored processes. An ER quality control (ERQC) system oversees protein maturation and ensures that only those reaching their native state will continue trafficking into the secretory pathway to reach their final destinations. Proteins that fail quality control must be recognized and eliminated to maintain ER proteostasis. The ER-associated degradation (ERAD) was discovered nearly 30 years ago and entails the identification of improperly matured secretory pathway proteins and their retrotranslocation to the cytosol for degradation by the ubiquitin-proteasome system.

While multiple ER-Associated Degradation (ERAD) components have been identified and their roles elucidated, it remains less clear how folded domains on ERAD clients complicate their extraction from the ER and degradation. To address this, we used several luminal ERAD substrates with well-defined structural properties. Deglycosylation and digitonin permeabilization assays were used to monitor client extraction from the ER. Our fully unfolded clients were released into the cytosol when the proteasome was inhibited. Conversely, ERAD clients possessing a single folded domain were completely retrotranslocated but were not released from the ER membrane without proteasome function. These clients were fully reduced, but still retained structure in their folded domain. Overall, our data argue that ubiquitinated clients with well-folded domains can be dislocated from the ER in a p97-dependent manner, but proteasome activity is required to fully release them from the cytosolic side of the ER membrane and the ERAD extraction machinery.

To decipher key steps in the extraction of non-glycosylated ER luminal proteins, and to determine how Hrd1, p97, and the proteasome contribute to the retrotranslocation of clients, we developed a novel in-cell biotin-based reporter system. The bacterial BirA biotin ligase was tethered to FAM8A1, a component of the retrotranslocon that interacts with Hrd1, thus positioning the biotin ligase near the cytosolic exit site. The non-secreted immunoglobulin (Ig) κ light chain (NS1 κ LC), which is composed of a poorly folded N-terminal domain and a well-folded C-terminal domain was used in the biotinylation assays. NS1 was modified at either the N- (BAP-NS1) or C- (NS1-BAP) terminus with a biotin acceptor peptide (BAP) allowing us to detect cytosolic exposure of both ends of this ERAD client. We established that BAP-tagged NS1 constructs were still ERAD substrates and that BirA-tagged FAM8A1 still assembled with the retrotranslocon. We found that both termini of NS1 were readily biotinylated when the proteasome was inhibited by MG132 treatment, or when dominant negative constructs of Hrd1 and p97 were expressed. To differentiate between full extraction of NS1 and partial exposure of the termini to the cytosolic side of the ER, we permeabilize the plasma membrane with digitonin. Cytosolic proteins were released, but both biotinylated and non-biotinylated NS1 remained cell-associated. This argues that the biotinylated light chain was not fully extracted from the ER when NS1 degradation was inhibited with either MG132 or with

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| | |
|---|---|
| ADP | Adenosine Diphosphate |
| ATF | Activating Transcription Factor |
| ATG | Autophagy-related |
| ATP | Adenosine Triphosphate |
| BAP | Biotin Acceptor Peptide |
| BiP | Immunoglobulin binding protein |
| CCPG1 | Cell Cycle Progression 1 |
| CD4 | Cluster of differentiation 4 |
| CHX | Cycloheximide |
| CNX | Calnexin |
| ConA | Concanavalin A |
| COPII | Coatamer protein II |
| CP | Core Particle of the proteasome |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| CRT | Calreticulin |
| DG | De-glycosylation |
| DHFR | Dihydrofolate Reductase |
| EDEM | ER Degradation Enhancing α-Mannosidase-Like Protein |
| EGFP | Enhanced Green Fluorescent Protein |
| EM | Electron Microscopy |
| ER | Endoplasmic Reticulum |
| ER Man I | Endoplasmic Reticulum Mannosidase I |

| | |
|---|---|
| ERAD | Endoplasmic-Reticulum-Associated Degradation |
| ERAD-C | Endoplasmic-Reticulum-Associated Degradation for cytosolic proteins |
| ERAD-L | Endoplasmic-Reticulum-Associated Degradation for luminal proteins |
| ERAD-M | Endoplasmic-Reticulum-Associated Degradation for membrane proteins |
| ERdj | ER-Resident DnaJ-Like Proteins |
| ERQC | Endoplasmic Reticulum Protein Quality Control |
| GFP | Green Fluorescence Protein |
| Glc | Glucose |
| Grp | Glucose Regulated Protein |
| GTP | Guanosine Triphosphate |
| HC | Immunoglobulin Heavy Chain |
| HCMV | Human Cytomegalovirus |
| HIV | Human Immunodeficiency Virus |
| HMGR | Human 3-hydroxy-3-methylglutaryl-CoA Reductase |
| Hsc | Heat Shock Cognate Protein |
| Hsp | Heat Shock Protein |
| HXK | Hexokinase |
| Ig | Immunoglobulin |
| IP | Immunoprecipitation |
| IRE | Inositol-Requiring Enzyme |
| LC | Immunoglobulin Light Chain |

| | |
|---|---|
| LC3 | Microtubule-Associated Protein 1 Light Chain |
| LIR | LC3-interacting Region |
| LPL | Lipoprotein Lipase |
| Man | Mannose |
| mHC or miniHC | mini Heavy Chain, a truncated form of the Ig γ heavy chain |
| MHC-I | Major Histocompatibility Complex class I |
| MHC-II | Major Histocompatibility Complex class II |
| MRH | Mannose-6-phosphate receptor homology |
| MS | Mass Spectrometry |
| NBD | Nucleotide-Binding Domain |
| NEF | Nucleotide Exchange Factor |
| NGLY1 | N-glycanase 1 |
| NHK | Null Hong Kong variant of α1-antitrypsin |
| Npl4 | Nuclear protein localization 4 |
| NS1 | Non-secreted Ig κ Light Chain |
| OST | Oligosaccharyltransferase |
| OTU1 | Ubiquitin Thioesterase |
| PDI | Protein Disulfide Isomerase |
| PERK | Protein Kinase RNA-like Endoplasmic Reticulum Kinase |
| PPI | Peptidyl-Prolyl-Isomerase |
| RING | Really Interesting New Gene |
| RP | Regulatory Particle of the proteasome lid |
| SRP | Signal Recognition Particle |

| | |
|---|---|
| StrAv | Streptavidin |
| TCRα | T-Cell Receptor α chain |
| Ub | Ubiquitin |
| UBXD8 | Ubiquitin-like (UBX)-domain-containing protein |
| UDP | Uridine Phosphorylase |
| Ufd1 | Ubiquitin Recognition Factor in ER Associated Degradation 1 |
| UGGT | UDP-Glucose Glycoprotein Glucosyltransferase |
| UPR | Unfolded Protein Response |
| UPS | Ubiquitin Proteasomal System |
| VCP | Valosin-containing Protein |
| VIMP | Valosin-containing Protein-Interacting Membrane Protein |
| VLDL | Very Low-Density Lipoprotein |

# CHAPTER 1. INTRODUCTION[1]

## Endoplasmic Reticulum

The endoplasmic reticulum (ER) is the largest single membrane-delineated organelle in eukaryotic cells. The ER is the major site for protein synthesis and trafficking, lipid biogenesis, calcium storage and regulation, and it is also involved in stress signaling responses [1, 2]. The functional complexity of this organelle is also matched by an equally complex and dynamic architecture [1]. The ER is composed of a continuous membrane network that incorporates the nuclear envelope. The peripheral ER is organized in flattened sheets and branched tubules. Specific functions of the ER have been associated with the different structures. For instance, ribosomes bound to the cytosolic side of the ER, which appears rough in electron micrographs leading to it designation as rough ER (rER), are translating proteins, thus marking areas where protein synthesis occurs. On the other hand, the smooth (lacking ribosomes) ER defines the areas where lipid synthesis takes place, as well as the exit sites for proteins that will traffic to the Golgi and beyond.

## The ER Is a Hub for Protein Synthesis

Approximately one-third of the human proteome is synthesized in the ER. This accounts for proteins destined for secretion, cell surface expression, or residence in the secretory pathway itself. Most newly synthesized secretory and membrane proteins enter the ER co-translationally **(Figure 1-1)**. These proteins contain an N-terminal sequence rich in hydrophobic amino acids (ER signal sequence) that identifies them for targeting to the ER. This short sequence is recognized and bound by the signal recognition particle (SRP) as soon as it emerges from the ribosome. SRP binding to the signal sequence causes a temporary stall in translation, an event known as elongation arrest, and directs the ribosome to the ER membrane by docking to the SRP receptor [3, 4]. The interaction of the SRP receptor with the SRP positions the translating polypeptide at the Sec61 translocon channel [5]. Translation then resumes after disengagement of the SRP and polypeptides enter the ER lumen through the Sec61 channel or are inserted into the ER membrane [6]. In the ER lumen, which offers a unique oxidizing environment, newly synthesized polypeptides encounter a host of molecular chaperones and folding enzymes that both aid and monitor their progress in achieving their functional tertiary or quaternary state. Maturation of nascent ER proteins often involves the addition and modification of N-linked glycans, the formation of disulfide bonds, isomerization of peptidyl-prolyl bonds, and assembly into multimeric complexes [7]. Proteins that adopt their native conformation will be transported from the ER via COPII vesicles to their

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[1] Modified from final submission with permission. Oikonomou, C. and L.M. Hendershot, Disposing of misfolded ER proteins: A troubled substrate's way out of the ER. Mol Cell Endocrinol, 2019: p. 110630 https://doi.org/10.1016/j.mce.2019.110630 [34].

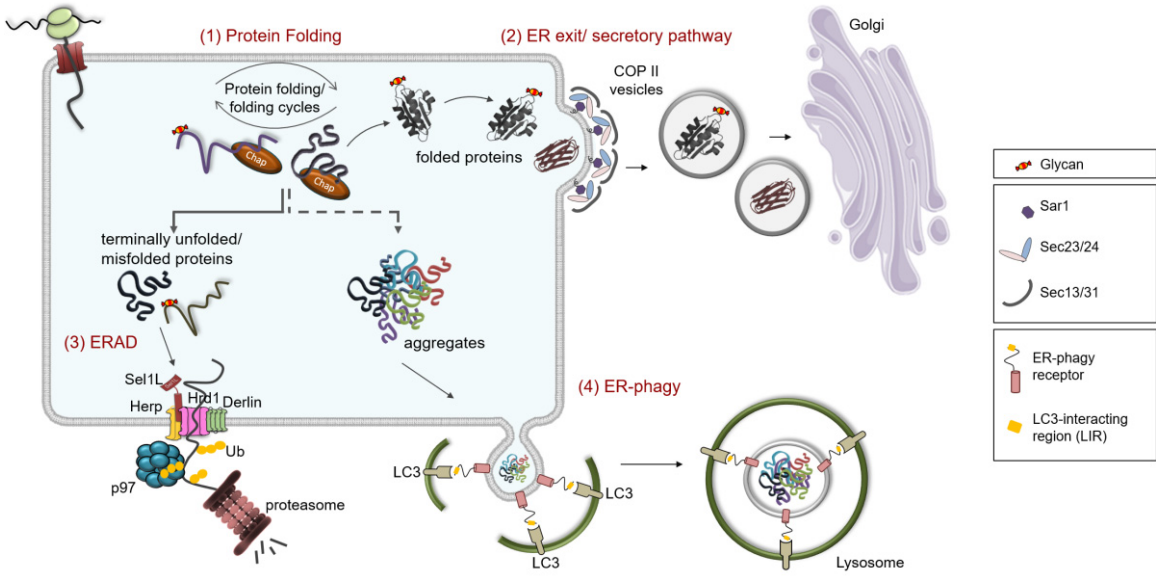



**Figure 1-1. ER-quality control (ERQC): from folding, to secretion or disposal of ER synthesized proteins**

(1) Newly synthesized proteins enter the ER lumen via the Sec61 translocon channel and begin to fold once in the lumen where their progress is both aided and monitored by molecular chaperones (chap). (2) Correctly folded and assembled proteins that pass ERQC are incorporated into COPII vesicles and are transported further along the secretory pathway to reach their functional destination, either in the secretory pathway itself, the cell surface, or the extracellular space. To maintain ER homeostasis, proteins that fail to obtain their proper native structure must be eliminated from the ER by one of two mechanisms: ER-associated degradation (ERAD) or ER-phagy. (3) Soluble ERAD clients are targeted for retrotranslocation to the cytosol via a channel (retrotranslocon) consisting of several multi-pass membrane proteins and auxiliary factors. As these clients emerge in the cytosol, they become poly-ubiquitinated allowing them to be recognized by the p97 AAA-ATPase that provides the energy for extracting the protein from the ER for delivery to 26S proteasome for degradation. (4) Large protein aggregates that are not easily handled by ERAD, are degraded by ER-phagy via the lysosomal pathway. This process is also dependent on receptors, which possess LC3-interacting regions (LIRs) that allow interactions with LC3-decorated autophagosome membranes and engulfment of the protein aggregates and delivery to lysosomes for degradation via standard autophagic pathways.

functional destinations, which can be other organelles of the secretory pathway, the cell surface, or the extracellular space.

## Molecular chaperones and folding enzymes in the ER

Molecular chaperones are proteins which interact with nascent proteins, in order to stabilize them in an unfolded state and prevent their aggregation, without having a direct folding capacity themselves. Their continued association with proteins provides an indication that the protein has not yet reached its native conformation. Two chaperone families reside in the ER lumen: the Heat Shock Protein (Hsp70/BiP) chaperones and the lectin chaperones. Both the lectins [8] and BiP [9] are part of larger complexes containing protein disulfide isomerases (PDIs) and peptidyl-prolyl isomerases (PPIs) that catalyze the folding reaction. These folding enzymes catalyze rate-limiting reactions during protein folding and favor certain folding pathways over others [10] [11].

### Heat shock protein chaperones

The ER-resident Hsp chaperone superfamily includes BiP/Grp78 (Hsp70), Grp170 (Hsp110) and the ERdj (Hsp40) co-chaperone family. A central component and one of the first factors that a nascent polypeptide chain will encounter in the ER is BiP (Binding Immunoglobulin Protein). BiP is an Hsp70 chaperone and the most abundant protein in the ER. It was initially identified due to its interaction with unassembled immunoglobulin heavy chains (Ig HCs). BiP binds directly to unfolded regions on non-glycosylated nascent chains or glycoproteins that do not possess a glycan near the region that is slow to fold [12, 13].

The ability of BiP to bind and release its substrates depends on its nucleotide-bound state and is regulated through the actions of its Hsp40/DnaJ co-chaperones, seven of which have been identified in mammals and three in yeast [14, 15]. Four of the mammalian ERdjs (ERdj3-6) bind directly to unfolded proteins and recruit BiP via their highly conserved J domains. BiP binds substrates in its ATP form, which triggers ATP hydrolysis to convert BiP to a form with high affinity for its substrate. Next, ADP release from BiP and ATP rebinding trigger discharge of substrates. For this latter step, two nucleotide exchange factors (NEFs) exist in the ER: Sil1 and the glucose-regulated protein of 170 kDa (Grp170) which belongs to the large Hsp70 family. Grp170 is also a chaperone itself and is able to bind unfolded proteins as well [16].

### Lectin chaperones and protein glycosylation

Many of the proteins expressed in the ER are glycosylated. For this modification, preassembled glycans (3x Glucoses, 9x Mannoses, and 2x N-acetylglucosamines – $Glc_3Man_9GlcNAc_2$) are covalently transferred *en bloc* to proteins by the oligosaccharyl transferase complex (OST) on asparagines in the N-X-S/T (where X can be any amino acid except proline) consensus motif [17]. Glycosylation restricts folding pathways resulting in a net positive effect on protein folding, enhances thermodynamic stability,

decreases a protein's aggregation propensity, and also operates as a quality control check point in glycoprotein maturation in the ER [18].

The lectin chaperones calnexin (CNX) and calreticulin (CRT) are mainly responsible for the fate of glycosylated proteins, monitoring and binding to mono-glucosylated N-linked glycans on nascent polypeptides [19]. These lectin chaperones are exclusively expressed in the ER, unlike the Hsp chaperones, which also have cytosolic orthologs. Calnexin, a type I ER membrane protein, and calreticulin, a luminal soluble paralog of calnexin, recognize and bind mono-glucosylated glycans [20-22]. Their binding cycles are controlled by the glucosidases and transferases that dictate the carbohydrate composition on maturing proteins in the ER [10]. Glucosidases I and II sequentially remove the first two glucoses thus generating mono-glucosylated glycans, which are in turn recognized by calnexin/calreticulin [10, 11]. Trimming of the single glucose by glucosidase II disrupts lectin binding. For proteins that have not folded, UDP-glucose-glycoprotein glucosyltransferase (UGGT) will recognize the exposed hydrophobic region near the N-linked glycan and add a single glucose moiety, allowing the unfolded glycoprotein to reenter the lectins cycle [23]. This allows them more chances to fold while avoiding aggregation. Once a glycoprotein has folded and buried hydrophobic regions it will exit the lectin cycle upon removal of the single glucose, allowing it to exit the ER and proceed in the secretory pathway. On the other hand, if folding does not occur after a certain amount of time, the glycan is further trimmed by mannosidases, which prevent it from being reglucosylated and signals for their disposal.

**Oxidoreductases**

Most proteins synthesized in the ER contain inter- and/or intra- chain disulfide bonds, which stabilize the structure, and are formed through the action of protein disulfide isomerases (PDIs), of which more than 20 exist in the mammalian ER. Disulfide bonds (S-S) can serve to stabilize a folded protein or promote the assembly of oligomeric protein complexes [7, 10]. While incorrect disulfides can be deleterious to a protein causing it to be unstable or aggregate and consequently be eliminated from the cell, other proteins require the formation of non-native disulfide bonds in order to ultimately achieve their native state. Protein disulfide isomerases can catalyze the oxidation of sulfhydryl groups of cysteines by acting as electron acceptors to create disulfide bonds in a polypeptide, but also the reduction of disulfide bonds by acting as electron donors [24]. This enzyme class can also catalyze the isomerization of disulfide bonds, which entails reduction of existing bonds and formation of new bonds in order to rearrange non-native or off-pathway disulfides. The redox potential of some resident ER PDI family members is such that they can only serve to reduce disulfide bonds, whereas others are highly oxidizing. In proteins with multiple domains, disulfide bonds can form independently on one domain while another domain can remain unfolded or in the process of folding or assembly with another protein domain [7]. Overall, through these functions, disulfide bonds assist the protein folding reaction and increase its fidelity.

### Peptidyl-prolyl cis-trans isomerases (PPI)

Native proteins usually contain peptide bonds in trans conformation with the exception of Xaa-Proline bonds that can be both trans and cis. Isomerization of the cis-trans bonds is a rate-limiting step of the polypeptide folding process and is catalyzed by peptidyl-prolyl cis-trans isomerases (PPI), which can either belong to the cyclophilin family or the FKBP family. A member of both families is present in the ER [10]. In fact, the rate-limiting step in Ig domain folding was found to be peptidyl-prolyl isomerization [25]. Furthermore, some proteins require additional modifications such as acylation, myristoylation, palmitoylation, and proline/lysine hydroxylation, which are performed by their corresponding enzymes. Modifications on proteins add to the stability, solubility and ultimately the overall functionality of the protein [26].

### Multiple domain proteins and assembly into multimeric complexes

Many ER synthesized proteins consist of multiple domains. Each separate domain is able to fold independently and be modified (e.g. glycosylated). Furthermore, various proteins expressed in the ER do not function alone but rather require assembly with partner proteins in multimers. Multimeric or oligomeric assembly usually occurs after some domains have been shaped, and in many cases, it completes the folding processes. Indeed, there is growing appreciation that often assembly does not take place between folded proteins but instead it drives the folding of the individual monomers [27, 28]. This template-assisted folding also serves to allow quality control of multimeric proteins. Well-studied examples of such proteins are the immunoglobulins, which require the synthesis of multiple protein chains and only once all chains are formed and assembled can the molecules remain stably folded and be expressed.

Overall, protein synthesis in the ER is a very carefully orchestrated process. Proteins are retained in the ER associated with the BiP or the calnexin/calreticulin complexes until they are folded. They will navigate through different intermediates with different free energy content with the assistance of various factors in the ER, until they reach the right fold thus burying the hydrophobic sequences that are recognized by BiP [29] and UGGT [30, 31], and until the correct local / distal interactions and modifications have been accomplished. Ultimately, properly folded and assembled proteins no longer interact with these chaperones and will exit the ER lumen via COP-II vesicles, proceed to the Golgi and continue into the secretory pathway to reach their final site of function [32].

## ER-Quality Control

The ER possesses complex and sophisticated systems to supervise the progress and success of protein biosynthesis and to distinguish between folding intermediates on the way to acquire their final fold, from terminally aberrantly folded proteins. These systems comprise the ER protein quality control (ERQC), which by monitoring protein

folding fidelity, ensures that only properly structured proteins will be expressed **(Figure 1-1)**. The two main chaperone families, the Hsp and the lectin chaperones are in the epicenter of ERQC. These chaperone families associate with and triage nascent polypeptides and folding intermediates and either promote polypeptide folding and continuation of folding cycles until they adopt their final conformation and proceed into the secretory pathway or identify folding-incompetent polypeptides and target them for elimination from the ER.

**Failing to fold**

Despite all concentrated efforts by the ER to effectively complete protein folding, in some cases, newly-synthesized polypeptides can still fail to obtain their correct native structure. The ER houses a vast number of factors that participate in secretory pathway protein synthesis and processing in addition to the synthesizing and newly-synthesized polypeptides which are being handled. Therefore, the ER proteome can reach an estimated concentration of 100mg/ml [33], and protein folding in this crowded milieu can be a challenging task. Furthermore, protein synthesis can be error prone due to mutations in the genetic information, mistakes in translation, failure to engage the right folding factors, failure to assemble with partner protein chains, or due to changes in ER environment (including changes in the concentration of ATP or in the redox flux of the ER); and that can give rise to an increased misfolded-protein load. Accumulating misfolded or terminally unfolded protein species is problematic not only for depriving cells from expressing functional proteins, but also because increased load of such proteins can lead to ER stress and because such species may form aggregates, which can be toxic for the cell overall. In fact, a number of chronic diseases including cystic fibrosis and neurodegenerative diseases have production and accumulation of aberrantly folded proteins as the basis of their pathogenesis. It is therefore crucial that misfolded and terminally unfolded protein species are eliminated in order to avoid toxicity and potentially futile folding attempts.

Secretory pathway proteins that are not ultimately able to fold must be identified, separated from proteins *en route* to folding, and degraded. Such proteins range from entirely unfolded and misfolded polypeptides, to multi-domain proteins with at least one domain un- /mis- folded, and to unassembled polypeptide chains that remain unstructured. It is not entirely clear how folding attempts versus decisions to degrade are controlled for various unfolded clients, although some aspects of this process are understood [34] **(Figure 1-2)**.

The biggest progress in this field has been the result of studies on glycoproteins, where in the ER lumen the structure of N-linked glycans dictates continued chances to fold, recognition for transport to the Golgi, or alternatively targeting for degradation **(Figure 1-2)** [35, 36]. Glycosylated ER proteins contain $Glc_3Man_9GlcNAc_2$ glycans. Progressive trimming and reforming of the glycans, dictates whether a protein will continue its folding cycles or if it will be recognized for degradation. Folded proteins possess $Man_9GlcNAc_2$ glycans, whereas the removal of mannose residues from

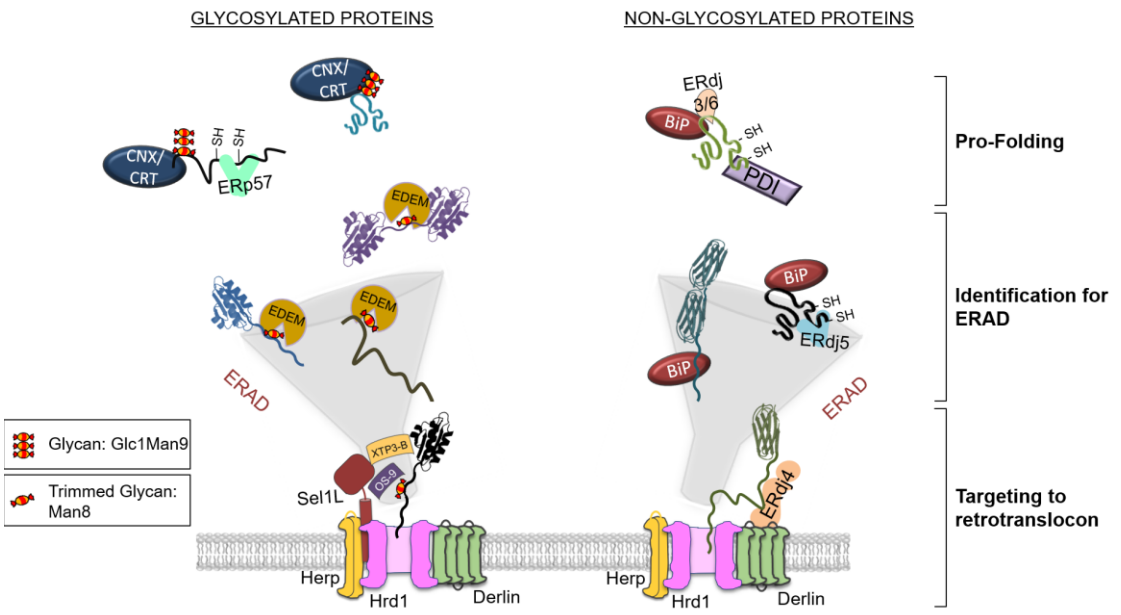



**Figure 1-2. ER-quality control: triaging proteins between folding and ERAD**

The N-linked glycans on nascent glycoproteins provide a recognition signal for the lectin chaperones calnexin (CNX) and calreticulin (CRT) that are associated with co-factors like the PDI family member ERp57, allowing them to undergo continued attempts to fold. Processing of the N-linked glycan by resident ER mannosidases removes the terminally unfolded glycoprotein from the folding cycle and allows it to be recognized by the EDEM proteins. The glycosylated ERAD client is transferred to two other luminal lectins, XTP3-B and OS-9 which pass the client to Sel1L, an integral membrane protein, which is associated with the retrotranslocation complex. In the case of non-glycosylated proteins, they bind to the Hsp70 chaperone BiP as they enter the ER, which interacts with pro-folding ER-localized DnaJ co-factors like ERdj3/ERdj6. By less well understood mechanisms, the critical decision of ERQC between folding and identification for degradation involves transfer to the pro-degradation co-chaperones ERdj4 and ERdj5. ERdj5 is a reductase that mediates further unfolding of clients for ERAD, while ERdj4 is associated with Derlin, a component of the retrotranslocon.

the N-linked glycan by the slow acting [37] α1,2-ER-mannosidase I (ER ManI) causes the client to exit the calnexin/calreticulin cycle. A protein whose N-glycan is trimmed from 9 to 8 mannoses is a substrate for both Golgi transport and ERAD revealing a possible competition between these outcomes, which is central to ERQC. Studies have shown that if the protein is folded it will be incorporated into COPII vesicles through its association with cargo receptors. However, if it is not folded, its hydrophobic regions in combination with the mannose-trimmed glycan will be recognized by ER degradation-enhancing α-mannosidase I-like proteins (Htm1p/Mnl1p in yeast and EDEM1-3 in mammals). Indeed, recently, *in vitro* experiments showed that EDEM1-2 had increased activity on denatured glycoproteins, but low mannosidase activity on free oligosaccharides and on glycoproteins. Additionally, a detected association of the mannosidases with different oxidoreductases that act on the glycoprotein substrates was proposed to serve for promoting unfolding of at least the region containing the glycan, which would in turn facilitate the enzymatic activity of the mannosidases [38]. Together these findings imply a model where the mannosidases function according to the "glycan code" that they read, but their actions are enhanced by the degree of unfolding of the polypeptide substrate (presumably by being able to directly bind such exposed regions on proteins) [38, 39]. Glycans that have been de-glucosylated and de-mannosylated signal for a terminally misfolded protein. The EDEM triad, targets terminally misfolded proteins for degradation by promoting binding to lectins OS-9 and XTP3-B that directs the glycoproteins to ubiquitin-proteasome system [40-43]. OS-9 (Yos9p in yeast) and XTP3-B are ER soluble mannose-specific lectins that recognize proteins with trimmed (by ERManI/ EDEM1-3) glycans via their mannose-6-phosphate receptor homology (MRH) domains [44], which preferentially bind glycans whose mannoses have been trimmed to the extent that the α1,6-mannose in arm C is exposed [41, 44, 45]. Moreover, it appears that compartmentalization of glycosylated ERAD clients in regions of the ER distinct from ER exit sites plays a role in the fidelity of ERQC [46].

In the case of non-glycosylated BiP clients the pivotal point in ERQC is less well understood, but a number of recent studies suggest that it likely involves the transfer of clients from pro-folding to pro-degradation ERdj co-chaperones **(Figure 1-2)** [14, 47], which all recognize exposed (unfolded) hydrophobic regions on proteins [48]. For instance, ERdj3 is associated with the translocon [49, 50], which suggests that it can engage nascent chain as they enter the ER lumen, binds stably to long-lived Ig heavy chains [9], has multiple interaction sites throughout two BiP clients [48]. Furthermore, depletion of ERdj3 accelerates the turnover of the PiZ mutant of α1-anti-trypsin [51]. All of these observations are consistent with a pro-folding function for ERdj3. However, in another study ERdj3 depletion resulted in the stabilization of a glucocerebrosidase mutant [52]. Closer examination revealed this mutant associated with calnexin instead, which does not demonstrate that ERdj3 was performing a pro-degradation function for this protein; only that calnexin provided a longer period for folding than the BiP/ERdj3 cycle. ERdj6 selectively binds unfolded vesicular stomatitis virus G protein and is released as the protein folds [53]. Neither ERdj3 nor ERdj6 are significantly up-regulated by ER stress and in fact nascent ERdj6 synthesized during stress is inefficiently targeted to the ER [54]. This in combination with the other studies described is compatible with a pro-folding role for these ERdj proteins instead of a pro-degradation one. Other studies

provide evidence that ERdj4 and ERdj5 assist BiP in targeting proteins for degradation. ERdj4 is associated with retrotranslocon components [55], and reduced expression of ERdj4 prolongs the half-life of disease-associated surfactant protein C mutants [56], pro-insulin [57], and epithelial sodium channels [58]. ERdj5 possess six thioredoxin-like domains in addition to a J domain, thus it is a member of both the PDI and ERdj super-families [59]. It functions primarily as a reductase in the ER where it serves to reduce folded domains or oligomeric structures so they can more readily be degraded [60]. Depletion of ERdj5 stabilizes the levels of surfactant protein C mutants [56], while its over-expression accelerates turnover of both the null Hong Kong (NHK) variant of α1-anti-trypsin and its non-glycosylated NHK-QQQ mutant [60, 61], it functions to reduce toxin subunits allowing them to enter the cytosol [62, 63], and it binds luminal degradation components. Both ERdj4 and ERdj5 bind rarer sequences on classic BiP substrates (the non-secreted κ light chain (NS1) and a truncated γ heavy chain (γ $V_H$-$C_H1$ or else miniHC)) that are predicted to be aggregation prone [48], necessitating the rapid degradation of these clients if the sites are not buried by folding. Further work will reveal more details on the mechanisms which regulate the recognition of terminally misfolded non-glycosylated proteins from folding intermediates and their separation for degradation.

**Responding to the problem**

In response to accumulation of incorrectly folded proteins in the ER, the cell will attempt to repair or eliminate such proteins in three ways: by inducing the unfolded protein response (UPR) as well as by employing the ER-associated degradation (ERAD) and autophagy. If unfolded proteins accumulate in the ER to a point that exceeds the available levels of molecular chaperones, in particular BiP, that prevent them from aggregating, a signal transduction program is activated termed UPR. This response aims to restore ER homeostasis [64, 65] and is increasingly the target of small molecule activators and suppressors [66]. In fungi, Ire1 is the single transducer of the UPR, but as organisms grew in complexity so did the response [67]. For instance, plants have two transducers Ire1 and ATF6 and metazoan have three Ire1, PERK and ATF6. The mammalian UPR is characterized by an up-regulation in ER chaperones, a combined decrease in translation and increase in degradation to limit the load of unfolded proteins dependent on chaperones, expansion of the ER volume to reduce their potential for aggregation. It also induces cell cycle exit to prevent the perpetuation of cells experiencing ER stress, and activation of apoptotic pathways if the stress is not resolved, although the tipping point between cell survival and death varies dramatically by tissue type. The basic outline and components of the UPR in several organisms are well-defined, and a major focus of research is shifted towards its roles in development [68] and disease [69]. The demonstration that unfolded proteins are the signal that activates the ER stress response was reported several years ago [70], as was the demonstration that BiP over-expression but not that of other ER chaperones could inhibit its activation [71]. However, the precise mechanism of UPR activation continues to be debated with two theories predominating and most recent studies focusing on Ire1 [72, 73]. Some contend that release of BiP from the transducers by competition with unfolded proteins provides

the critical activation signal [74-77] and others argue that unfolded proteins bind directly to the transducers causing them to cluster and activate in trans [68, 78]. Recent quantitative studies have convincingly shown that levels of an unassembled IgM heavy chain temporarily surpass levels of BiP leading to an acute activation of the UPR [79, 80].

### Removing aberrantly folded proteins

Two mechanisms exist to identify and remove unfolded/misfolded proteins or orphan subunits of multimeric complexes from the ER: ERAD and ER-phagy. Many components of these two pathways are conserved in organisms ranging from yeast to mammals. These pathways feed into distinct cellular hubs **(Figure 1-1)** widely employed for the degradation of proteins from all organelles, the proteasome and the lysosome (vacuole in yeast). The delivery of ER clients to the proteasome was identified ~30 years ago and is currently better understood, although cutting-edge research continues on ERAD providing a more detailed and mechanistic understanding of this pathway. ER-phagy on the other hand has been more recently discovered and as such our understanding of this pathway is currently more limited. In the next sections, I will first discuss ER-phagy and then ERAD, since it is the main focus of this thesis.

## ER-Phagy

Cells employ two main pathways to degrade proteins, one is by the ubiquitin-proteasome system (UPS) and the other is via autophagy and the lysosomal machinery. Both these ways are also employed to dispose of aberrantly folded ER proteins [81]. The autophagic pathway (ER-phagy) can remove large protein aggregates [82, 83] that are not easily handled by ERAD and transport them to the lysosome for degradation [84-86]. These processes can also extract damaged ER membranes, help shrink the ER after resolution of an ER stress response (RecovER-phagy), and maintain homeostasis of different types of ER membranes. While this area of research is fairly new, a number of the components and their functions are emerging. ER-phagy relies on many downstream components of traditional autophagic pathways including Atg/LC3 and a membrane source, which may or may not be ER membrane, for delivery of cargo to the lysosomes **(Figure 1-1)**. It is also dependent on receptors, proteins capable of bending or distorting the ER membrane, and proteins capable of membrane scission. In the case of mammals, the most upstream components vary by the type of ER-phagy.

The first component to be identified was FAM134B/RETREG1, which localizes to the ends of ER sheets and is important for the turnover of this type of ER membrane [87]. FAM132B inserts into the ER membrane post-translationally through a Reticulon Homology Domain (RHD), which does not fully enter the ER lumen and serves to bend the membrane. Some evidence suggests that atlastin 2, a member of the dynamin GTPase super-family, may provide the scission step in fragmentation of sheet ER [88]. FAM134B possesses an LC3-interacting region (LIR) at its C-terminus that allows it to

interact with LC3 decorated autophagosome membranes. It can play a role in the degradation of some luminal protein aggregates including the PiZ mutant of $\alpha_1$-anti-trypsin [89] and procollagen [82]. FAM134B does not enter the ER and thus must have interactors that traverse the ER membrane to recognize luminal clients. Reticulon 3 (RTN3) has numerous splice variants and the longest of these, RTN3L, serves to turnover or remodel ER tubules [84]. It also inserts into the ER membrane, but doesn't cross it, and provides the membrane bending function.

UPR activation leads to up-regulation of CCPG1, an integral membrane, non-canonical autophagy cargo receptor [90]. It possesses a single LIR in the cytosolic portion of the protein and two FIP200 interacting regions that together allow it to cluster and interact with the autophagic machinery. Its luminal region is poised to interact with misfolded or aggregated proteins, and studies in acinar pancreatic cells reveal a role for CCPG1 in proteostasis. As discussed above, one aspect of the UPR is to enlarge the ER to accommodate unfolded proteins. Once the stress is resolved, in mammalian cells a process referred to as RecovER-phagy is enlisted to re-establish ER homeostasis. Data argue that mammalian Sec62, a transmembrane protein that is a component of the Sec61 translocon [91], is repurposed to degrade excess ER membranes during recovery [92]. Data argue that Sec62 dissociates from the translocon and recruits the autophagic apparatus through the single LIR near its C-terminus. It is noteworthy that ER chaperones and folding enzymes that were up-regulated by the UPR are also taken up in the RecovER-phagy process, but ERAD components are not affected [92], allowing these two mechanisms of ER degradation to act simultaneously.

## ER-Associated Degradation

The identification of resident ER molecular chaperones in the mid-1980s and improved techniques to study the biosynthesis of secretory pathway cargo led a number of labs to discover that failure rates for achieving native protein structures could be quite high, particularly in the case of disease-associated mutant proteins [93, 94] as well as of incomplete assembly of multimeric proteins [95-97]. These unsuccessfully folded proteins or unassembled, orphan subunits were unable to be released form the ER and instead turned over rapidly [95, 98]. However, unexpectedly lysosomal inhibitors and agents that disrupted transport to the Golgi did not stabilize them, arguing they were not trafficking to Golgi and were neither degraded in the lysosome. A subsequent effort by multiple groups revealed that these proteins were extracted from the ER and degraded by the ubiquitin-proteasome system via a process that was termed ER-associated degradation (ERAD) [99, 100]; a pathway that was conserved from yeast to mammals[101]. In the ensuing years, the number of proteins classified as ERAD substrates has grown and many ERAD components have been identified through a combination of genetic and biochemical approaches [102-104]. Thus, we now have achieved a good understanding of the general ERAD process, even though certain specific events in the pathway remain obscure.

Three different branches of ERAD (ERAD-L, -M and -C) have been described for disposing of aberrantly folded proteins, according to the topology of the misfolded or unfolded segment in the protein. Luminal ER proteins with misfolded parts follow ERAD-L. On the other hand, integral membrane ER proteins can possess misfolded regions in their luminal, membrane-spanning, or cytosolic segments and will follow the ERAD-L, ERAD-M or ERAD-C correspondingly. These three ERAD branches use different components for identifying, targeting and dislocating their substrates.

**ERAD in homeostasis and disease**

Apart from the clearance of folding-defective proteins in the ER, ERAD plays an essential role in managing the levels of various proteins in a process that is highly regulated and occurs as a response to specific signals. One of the more fully characterized examples involves the ERAD-regulated levels of 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMGR), an ER-localized, multi-pass membrane protein that catalyzes the rate-limiting step of sterol biosynthesis [105, 106]. In a very simplified overview, sterol accumulation in the ER membrane alters transmembrane regions of HMGR and enhances its binding to membrane-embedded Insig-1/2 proteins, which in turn associate with ER membrane ubiquitin ligases (gp78 and Trc8 in humans and HRD1 in yeast), thereby promoting ubiquitination of HMGR and proteasomal degradation [107]. Conversely, lower membrane levels of sterol prevent these associations and targeting to the ubiquitin proteasome system, resulting in the stabilization of HMGR. Another enzyme in the pathway of sterol synthesis, squalene monooxygenese, is subject to ubiquitination (by Teb4 in mammals and Doa10a in yeast) in response to increased levels of cholesterol [108]. In another example of feedback regulation, levels of apolipoprotein B, which plays a critical role in forming and trafficking VLDL, is regulated by ERAD in response to reduced lipid availability or synthesis [109]. Defects in the degradation pathways of these proteins lead to a variety of diseases linked to lipid homeostasis and atherosclerosis. Similarly, ER stress and protein misfolding has been linked to a number of disorders of the liver and pancreas, and muscles [110], as well as systemic metabolism [111-113].

Several studies have focused on understanding the effect of various ERAD components in health and disease. Global deficiencies in ERAD proteins are embryonic lethal, and thus cell type-specific deficiencies in ERAD components have been used to provide insights into physiological ERAD functions and endogenous ERAD substrates [114]. Adipocyte-specific ablation of Sel1L, a critical ERAD component for glycoproteins, resulted in mice on a Western-type diet with postprandial hypertriglyceridemia. This was found to be due to a resulting ER accumulation and aggregation of lipoprotein lipase [115]. Other studies reported that Sel1L knock-out mice developed pancreatic insufficiency [116], whereas epithelial Sel1L was shown to be required for intestinal homeostasis [117]. Maybe less immediately obvious, loss of ERAD components can also affect proteins residing outside the ER. For instance, during ER stress associated with cirrhosis, Hrd1 targets NRF2, a transcription factor that protects against oxidative stress. Hepatocyte-specific deletion of Hrd1, led to a dramatic increase

in NRF2 levels and its targets in an experimental model for cirrhosis [118]. This finding highlights the pathological importance of cross-talk between ER stress and ERAD pathways.

## Exploiting ERAD

Interestingly, it has been demonstrated by various studies that the ERAD machinery is exploited by certain viruses and toxins to escape immunosurveilance and infect the host cells [119, 120]. In particular, the human cytomegalovirus (HCMV) induces the expression of the viral US2 and US11 in the ER, which select newly synthesized MHC-I and MHC-II for degradation. This way, proteins that would activate the host cell's immune response are eliminated in favor of the viral infection. Similarly, cells infected with HIV or herpes virus produce the proteins Vpu [121] and mK3 [122] respectively, which cause the degradation of CD4 and MHC-I in each case to promote infection. Pathogenic bacteria and their toxins also use the ERAD pathway for their own benefit. Cholera toxin from *Vibrio cholera* and Shiga toxin from *Shingella dysenteriae* behave in the same manner. These toxins are internalized by the cell and follow the secretory pathway in a retrograde manner. Once in the ER these toxins exploit the ERAD machinery in order to exit the ER to the cytosol where they exert their function [123-125].

It is therefore of no surprise that ERAD, as a fundamental component of ER homeostasis, is vital for cell and human health in general. In keeping with this, increasing evidence links this pathway to many diseases. So, a detailed knowledge of this pathway (the protein components and the steps in this process), its substrates and its functions, is necessary to completely understand how ER proteostasis is achieved, as well as to explain the etiology of ERAD-associated diseases.

## ERAD substrate exit from the ER: The retrotranslocon and substrate retrotranslocation

Once folding and/or assembly of nascent ER proteins has failed and clients for ERAD have been identified, they must be transported to the cytosol where the UPS resides. This requires a protein conducting channel, a source of energy for retrotranslocation and ubiquitin conjugation for proteasomal degradation. The retrotranslocon channel that accommodates the extraction of ERAD clients to the cytosol has been the focus of intensive genetic and biochemical research and has resulted in the identification of numerous retrotranslocon components [102, 126-128]. However, the channel's exact composition remains controversial. In an attempt to identify mammalian channel components, a transmembrane ERAD client was affinity purified and subjected to MS/MS analyses of co-purified proteins identified several proteins known to be involved in ERAD, as well as the multi-pass membrane proteins Derlins 1-3 [129, 130]. The Derlins are mammalian homologues of the yeast Der1p, which is essential for the degradation of some yeast ERAD clients [131]. Subsequently, photocrosslinking

experiments conducted in yeast with an ER luminal ERAD client identified interactions with Hrd1p (Hrd1 in mammals) at early stages of retrotranslocation [132] and major interactions with Hrd3p (a homologue of the mammalian SEL1 [133]) throughout the length of the polypeptide chain [134]. Hrd1p is a multi-pass membrane protein that oligomerizes and that possesses E3 ligase activity, both of these properties are required for its ERAD function **(Figure 1-3)** [132] , and over-expression of Hrd1p complemented the loss of other essential ERAD components including Hrd3p [132]. Reconstitution of proteoliposomes with Hrd1p was sufficient to retrotranslocate a membrane-anchored ERAD client when p97 was added [135]. Hrd1p was found to undergo auto-ubiquitination, resulting in a conformational change in the protein that allowed initiation of retrotranslocation of the substrate. A cryo-electron microscopy structure of Hrd1p bound to Hrd3p has been solved revealing multiple interactions between the luminal domains of these proteins, and the assembly of the transmembrane domains of Hrd1p into a funnel-like structure [136] that is reminiscent of the nascent polypeptide-conducting channel of Sec61 and prokaryotic SecYp [137], which serves to translocate nascent chains into the ER lumen. Conformational changes (presumably by its auto-ubiquitination) of Hrd1 can lead to a "gate open" state and to its transition to a protein-conducting mode. In combination these data are consistent with Hrd1 being the central part of the retrotranslocon.

CRISPR-mediated Hrd1 knockout in mammalian cells dramatically diminished the degradation of both luminal and membrane clients [138], although direct evidence for it being the major channel component have not yet been obtained. A large scale interaction map of the mammalian ERAD network was obtained by isolating 15 individual proteins that had been implicated in ERAD and performing MS/MS analysis on interacting proteins for each and then employed a multilayer approach that integrated proteomics, functional genomics, and gene expression data to delineate organization of the ERAD pathway in mammals [139]. This tour-de-force approach, together with various other biochemical studies, have established Hrd1 as a central hub, have linked luminal components of the pathway to cytosolic, downstream effectors of ERAD, and have identifed new components. More specifically, Hrd1 itself has been shown to nucleate many adaptor proteins that connect retrotranslocon components together but can also link substrate recognition to retrotranslocation **(Figure 1-4)**. Sel1L (Hrd3 in yeast) binds to the EDEM proteins, to OS-9 and XTP3-B, but also directly to Hrd1; thusly, it can act as a transporter of terminally misfolded proteins to the retrotranslocon [41, 45, 140-143]. Additionally, other integral membrane ERAD factors including Derlin, Herp, VIMP, and UBXD8 are organized around the Hrd1/Sel1L complex and facilitate substrate processing and delivery for retrotranslocation and recruitment of cytosolic ERAD components thus linking ER-membrane and cytosolic events [104]. It is noteworthy, that many other E3s have been identified to participate in ERAD. In mammals gp78, MARCH6, RNF145, RNF139 (also named TRC8) are involved in ERAD, but only a limited number of substrates have been associated with them [103]. Therefore, it seems plausible that the core of the retrotranslocon can be an ERAD-specific E3 ligase that will orchestrate different co-factors to regulate substrate extraction, or two ligases could function in parallel or in sequential steps to complete retrotranslocation; future experiments will shed further light on these processes.

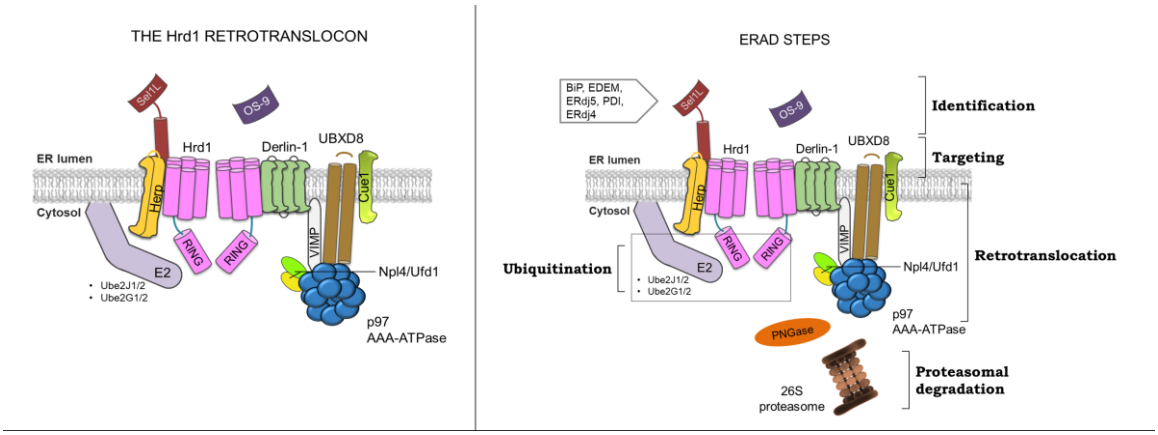
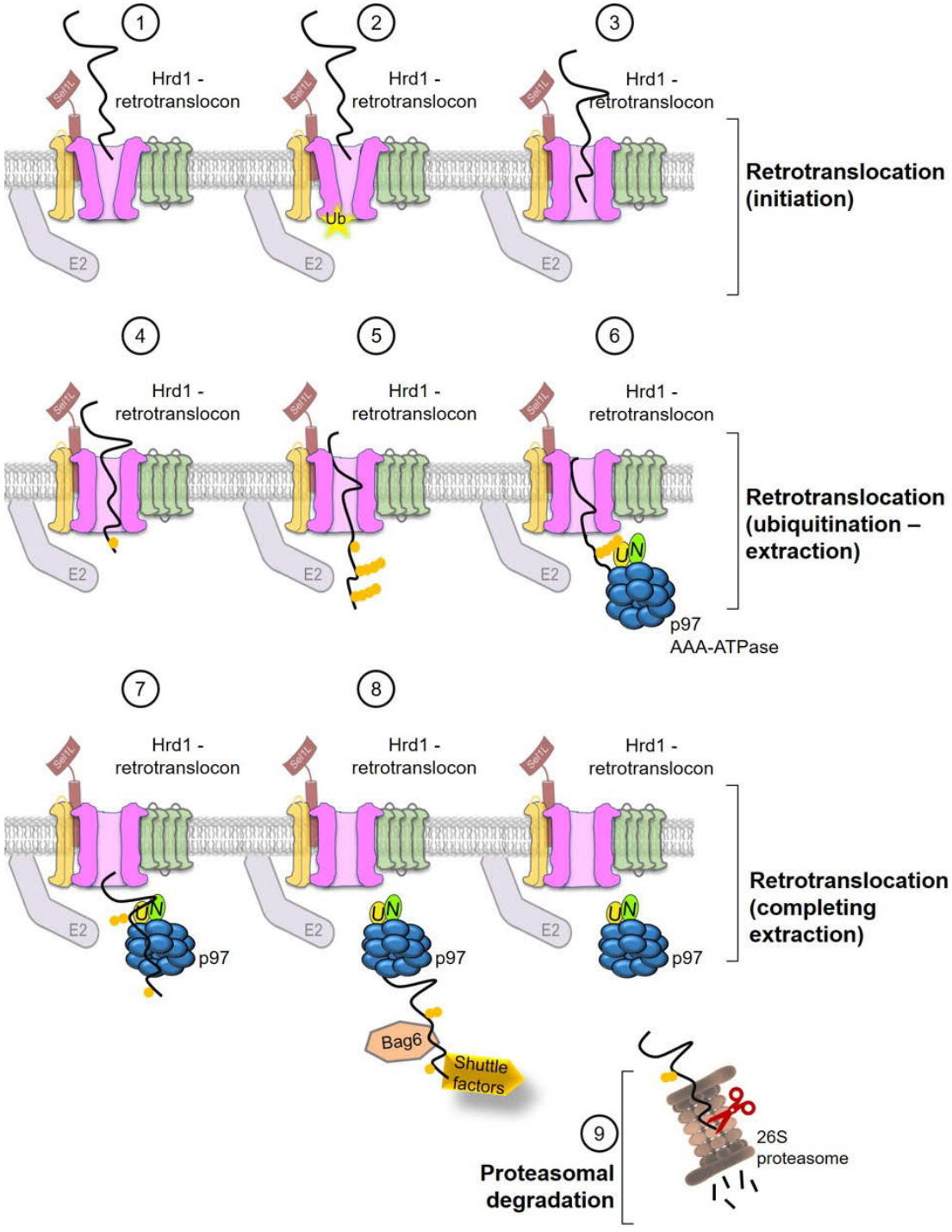



**Figure 1-3. The Hrd1 retrotranslocon channel and the general ERAD steps followed for protein degradation**

Left: A schematic representation of a Hrd1 retrotranslocon channel. Herp, Sel1L, and Derlin are organized around the Hrd1 dimer. The corresponding E2 ubiquitin conjugating enzyme is also near the cytosolically oriented RING Ub-ligase domain of Hrd1. The p97/Npl4/Ufd1 complex is recruited to the ERAD machinery at the cytosolic phase of the ER membrane via interactions with VIMP and UBXD8. Right: A general overview of the steps composing the ERAD pathway. ER luminal factors such as BiP, EDEMs, ERdj4/5 and PDIs identify ERAD clients. Clients are then delivered to Sel1L, OS-9, Derlin and/or Herp and are thus targeted for degradation. Hrd1 and its adaptor proteins also mediate the retrotranslocation of ERAD clients from the ER to the cytosol. In the cytosolic phase substrates become ubiquitinated and are recognized by the p97/Npl4/Ufd1 complex which via ATP hydrolysis completes ERAD client extraction from the ER. PNGase will the act on glycoproteins and will remove their glycans. Finally, clients will reach the proteasome where they will be degraded.

**Figure 1-4. General model for retrotranslocation of ERAD clients through a Hrd1 retrotranslocon channel**

(1) An ERAD client is targeted to a closed retrotranslocon via its association with Sel1L/Hrd3p, which interacts directly with the multi-pass E3 ligase Hrd1. (2) Auto-ubiquitination of Hrd1 results in conformational changes in it leading to an "opening" of the channel. (3) The polypeptide will be inserted into the Hrd1 channel and begin crossing the ER membrane via hydrophobic interactions with the TM regions of Hrd1 and sequential rounds of binding and release. (4) Once the substrate begins to emerge into the cytosol, it will be ubiquitinated by Hrd1 and associated E2 ubiquitin-conjugating proteins. (5) As the client continues to move through the channel, additional Ub chains are added and extended resulting in client poly-ubiquitination. (6) The AAA-ATPase, p97, is recruited to the ER membrane via its association with VIMP. Poly-ubiquitin chains on the ERAD client are recognized by the Ufd1/Npl4 (U/N) co-factors, which are located at the N-terminus of the p97. (7) Through conformational changes due to cycles of ATP binding and hydrolysis, p97 provides the necessary energy to extract ERAD clients from the ER membrane. (8) In many cases the ERAD client is then recognized by cytosolic chaperones and shuttling factors that deliver it to the proteasome. (9) Finally, the 26S proteasome receives ERAD substrates and again via cycles of ATP-hydrolysis translocates the polypeptide into its proteolytic core for degradation


1
2
3
Hrd1 -
retrotranslocon
Sel1L
Ub
E2
Retrotranslocation
(initiation)
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5
6
Retrotranslocation
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extraction)
UN
p97
AAA-ATPase
7
8
Retrotranslocation
(completing
extraction)
Bag6
Shuttle
factors
9
Proteasomal
degradation
26S
proteasome

## ERAD substrate ubiquitination occurs on multiple types of amino acids

As the ERAD substrate emerges into the cytosol it is ubiquitinated by ER-localized E3 ubiquitin ligases **(Figure 1-4)** of which nearly 40 have been identified in mammals thus far [144] and three in yeast [145], although the vast number of mammalian ERAD clients queried rely on the Hrd1 E3. In addition to canonical modification of lysines and the N-terminus of proteins, a variety of linkages used in constructing poly-Ub chains have been identified and mutational analyses have argued that Ub can be added to several other amino acids for both cytosolic and ERAD clients [146, 147]. For instance, mK3, a mouse γ-herpesvirus E3 modifies the cytosolic tail of the major histocompatibility chain to escape immune detection even when all lysines on the cytosolic tail are mutated [148]. The attachment of Ub chains occurred as long as serines, threonines, or cysteines were present, and susceptibility of these chains to pH or reducing agents was compatible with their attachment to these residues via hydroxyester or thioester bonds accordingly. Similar data were obtained for Hrd1-dependent ubiquitination of the non-secreted immunoglobulin κ light chain (NS1) [149] and the T cell receptor α chain [150] in mammalian cells, and on Doa10-dependent modification of a lysine-less version of the inner nuclear membrane protein Asi2 in yeast [151]. The E2s responsible for serine/threonine modification in mammals are Ube2J2 [152] and Ube2J1 [153], whereas Ubc6 and Ubc7 are necessary for non-lysine modification in yeast [151]. *In vitro* studies demonstrated that Ubc6 attached Ub to hydroxylated amino acids, whereas Ubc7 was responsible for Ub chain elongation [154]. This is consistent with K48 linkages on poly-Ub chains attached to serine or threonine in mammals [149]. The diversity in amino acids that can be modified by ERAD-specific E2/E3 pairs likely provides the flexibility to tag clients soon after they emerge into the cytosol and to ubiquitinate any of the ~6500 proteins that enter the ER but fail to fold.

## Providing the energy for retrotranslocation

In addition to playing a role in recognition by the proteasome, ubiquitination allows the ERAD client to be engaged by the p97/VCP (valosin-containing protein) AAA-ATPase complex (Cdc48 in yeast). p97 is an essential, highly conserved, homo-hexameric AAA+ ATPase with a vast array of cellular functions and is the only known energy source for the complete dislocation of ERAD clients [155-158]. p97 consists of an N-terminal domain (N domain) and two ATPase domains (D1 and D2 domains). Six monomers form a double-ring structure with the D1 ring at the top and the D2 ring at the bottom of the structure, thus creating also a central pore with 12 ATP binding sites in total **(Figure 1-5)**. The N domains provide binding sites for multiple distinct co-factors that regulate the complex's diverse functions, including Ufd1 and Npl4, which bind to the N domain of the D1 ring and recognize ubiquitinated substrates **(Figure 1-4)** [159, 160]. The p97/Ufd1/Npl4 complex (p97/UN) was reported to be able to bind both ubiquitinated and non-ubiquitinated ERAD substrates but had considerably higher affinity for ubiquitinated clients [161]. The N domain is the one to also associate with ERAD-associated ER membrane proteins VIMP and UBXD8 [129, 162], orienting p97 to the ER membrane so it can capture ubiquitinated-clients as they emerge from the ER [163].

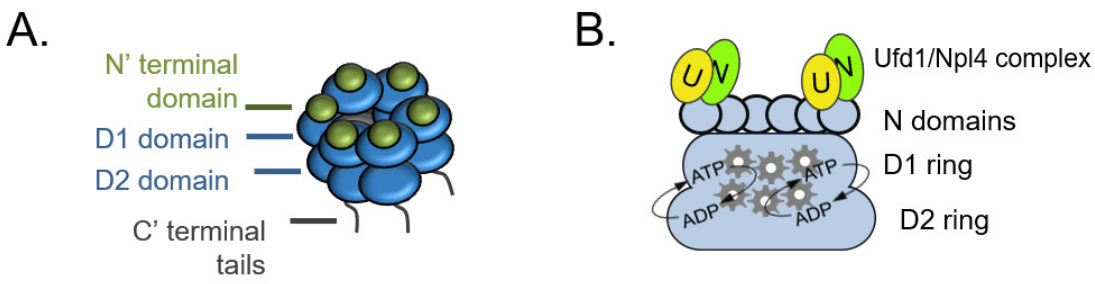



**Figure 1-5. Schematic representation of the p97 AAA-ATPase**

Basic schematic representation of p97. (A) Domains and organization of p97. (B) Cartoon of p97 with the Ufd1/Npl4 (U/N) co-factors bound and the ATPase centers indicated.

A recent structure of the N domain of D1 together with a minimal essential fragment of VIMP revealed that their interaction, and thus recruitment of p97 to the ER membrane, is modulated through nucleotide-dependent conformational changes in p97, which can serve to enhance or diminish the levels of p97 associated with the ER during cellular stresses [158]. This structure also explains why certain p97 mutations are pathogenic [164]. P97 is the only energy providing module known to date to accommodate the complete dislocation of ERAD clients. In total, the actions of p97 direct substrate remodeling, unfolding and extraction from membranes and macromolecular complexes, by ATP hydrolysis [156-158].

A number of studies have focused on how clients interact with p97 during the extraction process. Using normal mode analyses it was shown that the largest movements in the p97 structure occur between the D1:D2 rings supporting a model in which ERAD clients can be threaded between the two D rings, pass through the central cavity of the D2 ring, and exit from the distal side of D2 [165]. In this case, ATP-hydrolysis-mediated conformational changes of p97 result in an up-and-down movement of the N domains, which causes the substrate to be dislocated -pulled- from its source. A cryo-EM structure of full-length human p97 revealed multiple conformational states in the complex, some of which indicated the central cavity of D1 could be large enough to accommodate an unfolded polypeptide chain [159]. Recently, a photo-crosslinking study demonstrated interactions between an ERAD client and several points in the central cavities of the D1 and D2 rings, while they showed that the client exited from the D2 ring, arguing it had passed through both rings [166, 167]. In support of this model of clients passing through p97, a structure of the D1:D2 core of VAT, an archaeon p97 homologue, was obtained in which an unfolded VAT subunit was present throughout the central cavity formed by both NBD domains [168]. Lastly, a recent structure of yeast p97 complexed with a client captured images of a single Ub of the chain bound to Npl4 in an unfolded state and inserted through the D1 ring to the periphery of the D2 ring [169]. Substrate release from p97 has been shown to be dependent on ATP-hydrolysis by the D1 ring and did not occur spontaneously but rather needed the actions of OTU1, a de-ubiquitinating enzyme (DUB) [167, 170].

P97 also possesses unfoldase activity, which is critical to its function in ERAD as well, by processing and preparing the substrates for the proteasome. A recent example came from *in vitro* experiments with the photo-cleavable mEOS3.2 whose proteasome-mediated degradation was facilitated by p97 unfolding (measured by loss of fluorescence) [171].

**Delivery of ERAD substrates to the proteasome, deglycosylation and degradation**

The next step is the delivery of the ERAD substrates to the 26S proteasome for degradation **(Figure 1-4)**. One study using a p97 mutant to isolate stalled retrotranslocating chains reported immunoprecipitation of a proteasome subunit resulted in co-immunoprecipitation of Hrd1p, Hrd3p, luminal ERAD components, and p97 only when yeast with mutant p97 were used [172]. Although, in that case it was not defined if

this complex was maintained by direct interactions or via auxiliary factors. Proteins like hHR2 (Rad23 in yeast) which have both ubiquitin- and proteasome- binding domains might serve to transfer the substrate from p97 to the proteasome, thus acting as shuttling factors [103]. Cytosolic chaperones such as Hsc70 (Matsumura, David, & Skach, 2011) and Bag6 [173] can interact with ERAD clients in the cytosol after the p97 step by binding to hydrophobic regions on retrotranslocated substrates. Such interactions possibly serve to maintain substrate solubility and prevent aggregation in the aqueous cytosolic environment, but also to assist in substrate channeling to the degradation machinery. Further studies will determine the factors and mechanisms used to deliver ERAD clients to the proteasome after retrotranslocation.

Before their degradation, glycosylated ERAD proteins have their glycans removed in the cytosol after retrotranslocation. This is an enzymatic reaction performed by PNGase (peptide N-glycanase; NGLY1 or Ngly1 in human and mice) [174]. PNGase is membrane-associated on the cytosolic face of the ER, recruited and kept there by various protein-protein interactions. NGLY1 binds to the C-terminus of p97/VCP via its PUB domain (PNGase- and ubiquitin-related domain) [174] and can access polypeptides as they exit from the p97 complex. HR23B is another factor that can bind both PNGase and the proteasome thereby acting as a substrate "escort" [175, 176]. In this way, the events of retrotranslocation – deglycosylation – degradation are interconnected.

The proteasome, the principal proteolytic machine in eukaryotic cells, is the final destination of UPS. The proteasome is a large 2.5MDa protease located in the cytosol and nucleus of cells, which modulates the degradation of ubiquitinated proteins (either misfolded polypeptides or regulatory proteins). It is comprised of multiple subunits: the 20S core particle (CP), which is a barrel-like structure, and a 19S regulatory particle (RP) that caps both sides of the 20S form the 26S proteasome [177]. The 20S CP is formed by four stacks of hetero-heptameric rings that compose a channel structure. Two α and two β rings, according to their composition either of seven α (α1-7) or seven β (β1-7) subunits, form the CP. The 19S RP is organized in two sub-complexes, the lid and the base. The lid mainly has a scaffolding role, but also essential proteasomal DUB activity that is crucial for substrate processing and degradation; while the base is formed by six AAA-ATPases organized in a hetero-hexameric ring that makes a molecular motor, and four non-ATPases that act as scaffold proteins and ubiquitin receptors. The proteasome has three catalytic activities, the trypsin-like, the chymotrypsin-like and the peptidyl-glutamyl-peptide bond hydrolyzing activities, with the catalytic sites positioned in the β rings within the chamber of the CP. Substrates access the proteolytic core via the RP and proceeds to the CP in an axial way and an ATP-dependent manner.

Given the vast range of substrates that must be efficiently degraded by the proteasome, it must be highly promiscuous. Research that spans decades mirrors an effort to understand the structure and function of the 26S proteasome in detail [178-185]. *In vitro* EM and more recently cryo-EM studies have shed some light into structural characteristics and arrangements of the proteasome in good resolution. Such insights have helped to explain the mode of function of this degradation machine. Ubiquitinated substrates are recognized and recruited by receptors of the RP. The base of the 19S

particle is also where ubiquitin shuttle factors (such as Rad23 and Dsk2) are going to arrive. Unlike p97/VCP, the proteasome requires a flexible region on the substrate to bind [171]. Substrate binding to the base of the 19S caused rearrangement of the ATPases at the base of the 19S RP. These changes together with ATP-hydrolysis allow for removal of ubiquitin from the inserted polypeptide and induce substrate unfolding which in turn facilitates substrate movement towards the CP. In turn, this triggers structural changes on the CP which acquires a "gate open" conformation and thus moving the substrate into the proteolytic chamber where it is degraded into short peptides (usually of 7-9 amino acids). Repeated cycles of ATP binding and hydrolysis produce the energy required to "pull" substrates unfold them and translocate them along the axial channel into the proteolytic core of the 20S particle.

## Aim

While multiple components of the ERAD machinery and their roles have been identified and well-studied, how ERAD substrates move across the ER membrane to reach the cytosol for degradation remains obscure. Only a limited number of studies have focused on whether structural properties of ERAD clients themselves also affect their retrotranslocation. Our aim was to characterize the specific role of the major cytosolic ERAD protein components in substrate retrotranslocation from the ER lumen and release from the ER membrane for degradation. Additionally, we wanted to determine if and how structural properties of the ERAD clients, in the form of well-folded domains, add to the complexity of all such processes.

# CHAPTER 2. WELL FOLDED DOMAINS IN LUMINAL ERAD CLIENTS DICTATE ADDITIONAL REQUIREMENTS FOR THE FINAL STEPS OF THEIR DEGRADATION

## Introduction

The endoplasmic reticulum (ER) is the source of most proteins that will populate single membrane-bound organelles of the cell, reside at the cell surface, or be secreted into the extracellular milieu. It has been estimated that approximately one third of the human genome encodes such secretory pathway proteins, which play critical roles in every aspect of multi-cellular life. The ER provides a supportive environment for the folding of these proteins into functional tertiary or quaternary structures through the actions of a large number of resident molecular chaperones and their co-factors, as well as folding enzymes like protein disulfide isomerases and peptidyl-prolyl isomerases [7, 186, 187]. Nascent secretory proteins are usually retained in the ER until their maturation is complete to ensure that only functional proteins populate their required destination. Equally important to the proper maturation of nascent proteins in the ER is the ability to recognize those that fail, which is executed by the ER quality control (ERQC) system [188, 189], and target them for degradation in the cytosol in a process termed ER associated degradation (ERAD) [128, 190].

Understanding the cellular components of essential processes in ERAD has been the focus of a large number of studies and reviews [128, 166, 190-192]. While a number of steps in the pathway remain incompletely understood, a general overview of ERAD has been obtained. Prolonged exposure of hydrophobic patches on proteins, which should be buried within the folded protein, provides an element of recognition of ERAD clients. However, many of the same chaperones that assist in protein folding in the ER also play a role in recognizing those that do not mature properly [47], likely due to structural similarities between nascent proteins that enter the ER in an unfolded state and terminally unfolded proteins. The distinction between a nascent protein with the potential to fold and a protein that should be targeted for degradation is best understood for glycoproteins in which the N-linked glycan is the focus of monitoring both folding and an inability to fold [18]. As long as the glycan on the nascent protein is in the monoglucosylated state, the glycoprotein remains a client of the lectin chaperones calnexin/calreticulin where it retains the possibility to fold [193, 194]. Continued reglucosylation of the glycan occurs through the activity of UDP-glycosyl transferase, which recognizes hydrophobic patches on the unfolded client [23, 195]. However, once mannose residues on the glycan are trimmed by EDEM 1-3 [43, 196], the unfolded client is no longer modified by UDP-glycosyl transferase and instead becomes an ERAD substrate. More recently, an integral membrane complex composed of Slp1 and EMp65 was shown to serve as a “guardian” to protect nascent glycoproteins from ERAD [197]. The pivotal recognition process is less well understood for non-glycosylated BiP clients, but a more limited group of studies suggest that differential binding the DnaJ-like BiP co-factors may play a role [14].

Once the decision has been made to degrade an unfolded/misfolded client, it must be targeted and inserted into a protein channel, referred to as the retrotranslocon or dislocon, for extraction to the cytosol where it will be degraded by the proteasome. A number of components of the retrotranslocon have been identified, although it appears there is some heterogeneity in the composition of individual retrotranslocons [104, 129, 190, 198]. One of these components, Hrd1, is a multi-pass integral membrane protein that forms part of the channel itself [132, 199]. Hrd1 possesses E3 ubiquitin ligase activity with the RING domain oriented to the cytosol [200, 201]. Briefly, upon emerging into the cytosol, the ERAD client becomes poly-ubiquitinated, which can occur on a number of amino acids, including serine, threonine, and cysteine, in addition to the prototypical lysine residues [148-150, 202]. In addition to Hrd1, a limited number of other ER-associated E3 ligases have been identified [203]. The attached ubiquitin chain provides a recognition motif for the p97/VCP complex [204] that is associated with the ER membrane [162, 205]. The AAA-ATPase, p97, provides the energy to extract ERAD clients from the ER membranes for degradation by the proteasome. With the possible exception of the cholera toxin A1 subunit [206, 207], all integral membrane, as well as soluble, luminal ERAD clients examined thus far require the activity of p97 for their disposal.

While ERAD clients are often referred to as unfolded or misfolded proteins, it is important to note that most mammalian proteins are composed of multiple domains, which in many cases are able to fold independently and often assemble into multimeric complexes. Integral transmembrane proteins can possess regions that fail to fold in their luminal portion (ERAD-L), the membrane-spanning region (ERAD-M), or the cytosolic domain (ERAD-C), and studies have shown that the components necessary for the recognition and disposal of these different types of clients varies [208-210]. Similarly, soluble, luminal proteins can have a single domain that misfolds, which is sufficient to make them an ERAD client [211, 212]. Only limited studies have been conducted to assess how much the folded domains in these proteins contribute to the complexity of extracting them for degradation. To address this deficit, we employed a number of luminal ERAD clients for which biophysical and cell biological studies have determined the structural state of their various domains. These include glycosylated and non-glycosylated proteins that possess a single well-folded domain, as well as clients that do not possess any folded domains. We found that the activities of Hrd1 and p97 were required for retrotranslocation of all of these clients, and completely unfolded proteins were released into the cytosol without a requirement for proteasomal activity. Conversely, ERAD clients with a well-folded domain were fully retrotranslocated when proteasomal proteolytic activity was inhibited, but they retained structure in the cytosol and were not disengaged from the ER membranes, demonstrating a role for the proteasome in their complete extraction from the ERAD machinery.

## Materials and Methods

### Constructs and generation of mutants

The following well-characterized proteins were used as our main ERAD substrates: the non-secreted murine NS1 κ LC [211] in pSVL (and in pcDNA3.1 where indicated), the ubiquitination-deficient NS1-$V_L$STK$^-$ [149] in pSVL, the truncated murine γ1 Heavy Chain [28] tagged with 2xHA (mHC$^{HA}$) in pSVL, the human A6 TCR alpha chain (A6-TCRα) [27] in pcDNA3.1, the human hemagglutinin (HA)-specific TCR alpha chain (HA-TCRα) [27] in pcDNA3.1 and the human NHK mutant of α1-antitrypsin [213] in pcDNA3.1. Single N-linked glycosylation consensus sites (N-X-S/T) were engineered throughout our non-glycosylated clients using the Q5 mutagenesis kit (E0554S, NEB, Ipswich, MA) to monitor deglycosylation, which occurs after substrate presentation in the cytosol. When glycosylation sites were added to folded domains, the mutations were introduced on turns or loops (which were mapped from the available crystal structure for the $C_L$ domain of NS1 (UniProtKB - P01837) or from the predicted structure for the $V_H$ domain of mHC$^{HA}$) in order to minimize adverse effects on the natural folding of such domains.
To engineer NS1 constructs with a single N-linked glycan consensus sequence, the following changes were made:
NS1-N28 had a V30T substitution (For: GCCAGTGAGAATGTG**acc**ACTTATGTTTCCTGG, Rev: CCAGGAAACATAAGT**ggt**CACATTCTCACTGGC); NS1-N53 had a Y55T substitution (For: GCATCCAACCGG**acc**ACTGGGGTCCCC, Rev: GGGGACCCCAGT**ggt**CCGGTTGGATGC ); NS1-N100 had a G100N substitution (For: CACGTTCGGA**aac**GGGACCAAGC, Rev: TACGGATAGCTGTAACCC); NS1-N129 had a G129N substitution (For: AACATCTGGA**aat**GCCTCAGTCGTGTG, Rev: AACTGCTCACTGGATGGT); NS1-N157 had a V159T substitution (For: ACAAAATGGC**acc**CTGAACAGTTG, Rev: CGTTCACTGCCATCAATC); and NS1-N170 had a D170N substitution (For: GGACAGCAAA**aat**AGCACCTACA, Rev: TGATCAGTCCAACTGTTCAG);
To make NS1-$V_L$STK$^-$ constructs with a single glycan:
NS1-$V_L$STK$^-$ N28 had a V30T substitution (For: TGAGAATGTG**acc**GCTTATGTTGCCTGGTATCAACAGAGACCAGAG, Rev: GCGGCCCTGCAGGCCAAG); and NS1-$V_L$STK$^-$ N100 had G100N & A102T substitutions to allow for the N-glycan acceptor motif (For: **gacc**AGGCTGGAAATAAGACGGG, Rev: **ccatt**TCCGAACGCGTACGGATA);
In the case of introducing a glycan into mHC$^{HA}$:
mHC$^{HA}$-N55 had a R57S substitution (For: TAGCAACGGT**agc**ACTAATTACAATG , Rev: GGATTAATCTCTCCAATCC); and mHC$^{HA}$-N118 had a A118N substitution (For: CGTCTCCTCA**aac**TCCACCAAGG, Rev: GTGACCAGAGTCCCTTGG);
For A6-TCRα, a construct with a single glycan remaining on the Vα domain (A6-TCRα-N45 only) was engineered by eliminating the four glycosylation sites present in the Cα domain. These include: N167A (For: CAGCCAGACC**gcc**GTGTCCCAGA, Rev: TCGAAGTCGGTGAACAGG), N201A and N212A (For:

**tgcgccaacgccttcgcc**AACAGCATTATCCCAGAGGACACATTCTTCCC, Rev: **ggcgaagtcgctcttggc**GGACCAGGCCACGGCGCT), and N248A (For: GAACTTCCAG**gc**CCTGAGCGTGATC, Rev: AGGTTGGTGTCTGTCTCG).
A cytosolically expressed NS1 (ΔssNS1) was engineered by removing the ER targeting signal sequence (For: ACCGGATCGATCCCTCGACCTGCAGATGGGGAACATTGTAATGACCCAATCT CCCA, Rev: TGGGAGATTGGGTCATTACAATGTTCCCCATCTGCAGGTCGAGGGATCGATC CGGT),
and additionally, substituting alanines for Met 4, 11, 13 to eliminate alternative translation initiation products by Q5 mutagenesis: For: **aaatccgcttccgct**TCAGTAGGAGAGAGGGTC, Rev: **gggagattgggtagc**TACAATGTTCCCCATCTG.
Finally, wild type p97 and the ATP hydrolysis-defective mutant p97QQ, each in the pcDNA3 vector, were kind gifts from Dr. Yihong Ye (NIDDK, USA). The Hrd1 mutant deficient in ubiquitin ligase activity (Hrd1 C291S in pcDNA3) was generously supplied by Dr. Yuval Reiss (Proteologics, Israel).

**Cell culture and transfections**

293T human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM; 15-013-CV, Corning - cellgro, Manassas, VA) supplemented with 10% (v/v) fetal bovine serum (FBS; S11150, Atlanta biologicals, Flowery Branch, GA), 2mM L-glutamine (25-005-CI, Corning), and a 1% (v/v) antibiotic-antimycotic solution (25μg/ml amphotenicin B, 10,000μg/ml streptomycin, and 10,000 units of penicillin; Cellgro/Mediatech, Manassas, VA) (complete DMEM) at 37°C and 5% $CO_2$. 293T cells were plated 24hrs prior to transfection, which was performed using GeneCellin (GC5000, BioCellChallenge, Toulon, France) according to the manufacturer's protocol. For all analysis, 1μg of each indicated ERAD substrate was used per p60 dish. When p97WT, p97QQ or Hrd1C291S was co-expressed, 1.5μg of each plasmid was used and equal amounts of empty pcDNA3.1 vector were used in the control samples.
The P3U.1 murine plasmacytoma cells, which naturally synthesize the NS-1 κ LC, were grown in complete DMEM supplemented with 55μM 2-mercaptoethanol (21985023, Gibco, Grand Island, NY) at 37°C and 5% $CO_2$. The non-LC synthesizing variant, the Ag8.653 cell line was grown in the same conditions and was used as control cell line for the κ LC expressing P3U.1 cells.

**Lysis**

60 mm plates of transfected 293T cells were lysed in Nonidet P-40 lysis buffer (NP-40: 50mM Tris/HCl pH 7.5, 150mM NaCl, 0.5% Nonidet P40, 0.5% sodium deoxycholate, 0.1mM PMSF, 0.5X complete protease inhibitor tablets w/o EDTA). Lysates were clarified by centrifugation at 12,000g. For the experiments with the P3U.1 and Ag8.653 cells, $2x10^6$ cells were used, lysed in NP-40 lysis buffer and lysates were

processed as described above. For experiments where the oxidation status of clients was studied, the cells were lysed in complete NP-40 buffer additionally supplemented with 20mM NEthylmaleimide (NEM; E3876-5G, Sigma-Aldrich, St. Louis, MO).

**Digitonin permeabilization experiments**

293T cells transfected with the constructs of interest were grown in p60 dishes. After 24hrs the cells were treated with 10μM MG132 (CAS 133407-82-6 | Calbiochem | 1MG, Millipore, Burlington, MA) or DMSO (276855, Sigma-Aldrich) control for 3.5hrs and were then collected and washed once with PBS and once with KHM buffer (110mM KOAc, 20mM Hepes pH 7.2, 2mM MgOAc). The cells were then gently resuspended in 1ml KHM (intact cells) or KHM containing 40μg/ml final concentration of digitonin (D5628-1G, Sigma-Aldrich) (digitonin-permeabilized cells) and were incubated on ice for 5min. Intact and permeabilized cells were pelleted by centrifugation at 1000g for 5min at 4ºC. The resulting cell pellets were lysed in 0.5ml NP40 buffer (cells fraction). The supernatant (sup fraction) was collected for analysis after a second centrifugation step at 500g for 5min at 4ºC. Equivalent fractions of the cell lysate and supernatant were used for Western blotting. $2x10^6$ P3U.1 cells were plated and maintained in p60 dishes in their normal culture media containing 10μM MG132 or DMSO control for 3.5hrs. After this time, the P3U.1 cells were collected and permeabilized in the same way as described above.

**Deglycosylation experiments**

To assess ERAD substrate exposure to the cytosol we used deglycosylation as a measurement. 293T cells were plated on p60s, after 24hrs transfected with each of the indicated ERAD constructs and the next day were treated with 10μM MG132 proteasomal inhibitor for 3.5hrs (or DMSO control) or cells were co-transfected with each of the substrates together with the dominant negative form of p97 (p97QQ) or of Hrd1 (Hrd1C291S) or empty pcDNA3.1 (ctrl). For the cycloheximide (CHX) and/or MG132 chase, cells were treated with 10μM MG132 or 100μg/ml CHX for the indicated time points. The cells were finally lysed in 0.5ml of NP40 lysis buffer and 1.5% of lysate was analyzed by Western blotting. To examine the complete deglycosylation of our glycosylated proteins, we treated samples with Endo H (P0702L, NEB) or PNGase F (P0704S, NEB) according to the manufacturer's protocol. At the end, samples were mixed with 2x Laemmli sample buffer and analyzed.

**Assessment of disulfide bond content**

To monitor the oxidation status of the different ERAD clients, the respective DNA constructs were transfected in 293T cells. The next day, cells were treated with 10μM MG132 proteasomal inhibitor (or DMSO control) for 3.5hrs, washed once with PBS containing 20mM NEM and lysed in 0.5ml NP-40 supplemented with

0.1mM PMSF, 0.5× complete protease inhibitor tablets w/o EDTA and 20mM NEM. Input samples were kept, mixed with 2x non-reducing (without β-mercaptoethanol) Laemmli buffer and analyzed by Western blotting.

**Concanavalin A lectin experiments**

We used agarose bound Concanavalin A beads (AL-1003, VECTOR LABORATORIES, Burlingame, CA) to separate glycosylated and non-glycosylated species. 293T cells were grown in p60 dishes and transfected with the mono-glycosylated NS1 client (NS1-N100). After 24hrs the cells were treated with 10μM MG132 or DMSO control for 3.5hrs and were then collected and lysed in 0.5ml of NP-40 lysis buffer supplemented with 1mM $CaCl_2$, 1mM $MgCl_2$, 1mM $MnCl_2$, 0.1mM PMSF and 20mM NEM. Lysates were incubated o/n at 4ºC with 2:1 v/v ConA slurry. Samples were then spun down (500g for 5min) and the supernatant (unbound) was collected and mixed with 2x non-reducing Laemmli buffer for analysis of the non-glycosylated proteins, while the glycosylated proteins (bound) were eluted from the beads with 2x non-reducing Laemmli buffer. The resultant samples were analyzed by Western blotting.

**Partial proteolysis**

Stability against proteolytic digestion was assessed by partial proteolysis experiments. Cells expressing the indicated constructs were lysed in 0.5ml NP-40 devoid of protease inhibitors and PMSF. Proteinase K (V302B, PROMEGA, Madison, WI) was added to a final concentration of 20μg/ml. Digestion was performed on ice for 25min and was followed by 5min incubation with 5mM (final concentration) of PMSF (Sigma) to inactivate the Proteinase K. Next, 2x Laemmli sample buffer was added, and samples were boiled and immediately loaded onto SDS-PAGE gels for Western blotting. To test if the Proteinase K resistant fragments were glycosylated or not, after digestion with the Proteinase and quenching with PMSF, the samples were treated with Endo H. At the end of this reaction 2x Laemmli buffer was added and samples were immediately analyzed by Western blotting.

**Co-immunoprecipitation experiments**

In these experiments, $2x10^6$ κ LC expressing P3U.1 or control Ag8.653 cells were used. After the indicated treatments cells were lysed in 1ml NP-40 and the LCs were immunoprecipitated overnight with anti-κλ-conjugated agarose beads (SouthernBiotech, Birmingham, AL). LCs were then eluted from the beads with 2x sample buffer and their interacting proteins were analyzed by Western blotting. To assess association of LCs with Hsc70, cells were processed and analyzed in the same way as before except that in this case the NP-40 lysis buffer was additionally supplemented with 10mM $MgCl_2$, 6mg/ml glucose and 50U/ml hexokinase (HXK) to allow ATP depletion.

293T cells were transfected with either NS1 (pcDNA3.1) or NHK and after 24hrs were treated with 10μM MG132 or DMSO control for 3.5hrs. Cells were then lysed in 1ml NP-40 and NS1 and NHK were immunoprecipitated overnight, respectively with anti-κ or anti-α1-Antitrypsin antibody, and agarose beads (CA-PRI-0100, Repligen, Waltham, MA) for the final hour. Proteins were again eluted with 2x sample buffer and analyzed by Western blotting.

**Western blot analysis, imaging and quantification**

Samples were mixed with reducing or non-reducing sample buffer as indicated, separated on SDS-polyacrylamide gels (13% gels for all analysis, except for the studies of the oxidation status of our substrates where 15% gels used, and for the Proteinase K experiments where 12% gels were used), and then transferred to PVDF membranes (IPFL00010, Millipore). Membranes were fixed with methanol, blocked with gelatin wash buffer, and incubated overnight with the indicated primary immune reagents in blocking buffer followed by species-specific secondary reagents, also in blocking buffer. Western blots were developed using the Pierce™ ECL (32106, Thermo Fisher Scientific, Waltham, MA), and for quantitative analysis they were scanned with the LI-COR Fc Odyssey scanner (LI-COR, Lincoln, NE). Analysis and quantification were performed with the Image Studio Lite software.

**Antibodies**

The following antibodies were used for blotting: polyclonal goat anti-mouse κ LC (1050–01, SouthernBiotech); polyclonal rabbit anti-α1-Antitrypsin (for NHK, A0409-1VL, Sigma); monoclonal mouse anti-TCRα (TCRA 1145 ThermoFisher Scientific); mouse anti-GAPDH (MAB374, Millipore); goat anti- calreticulin (N-19) (sc-6468, Santa Cruz Biotechnology, Dallas, TX); rabbit anti-calnexin (SPC-127A, StressMarq, Victoria, Canada), mouse anti-Hsc70 (B-6) (sc-7298, Santa Cruz Biotechnology), mouse anti-VCP/p97 (ab11433, abcam, Cambridge, MA), and mouse anti-20S proteasome subunit alpha (C8) (PW8110, Biomol, Hamburg, Germany). HRP-conjugated goat anti-rabbit (sc-2054), HRP-conjugated donkey anti-goat (sc-2020), and HRP-conjugated goat anti-mouse (sc-2031) were purchased by Santa Cruz Biotechnology. The mouse monoclonal anti-HA epitope antibody was a generous gift from A. Reynolds, Vanderbilt University.

## Results

To begin our studies, we chose five luminal ERAD clients for which the folding status of their various domains had been determined by cell-based, and where possible, biophysical studies **(Figure 2-1)**. These included three proteins with one well-folded domain. The non-secreted NS1 immunoglobulin (Ig) $\kappa$ light chain possesses a variable domain ($V_L$) that is not able to fold properly and a well-folded constant domain ($C_L$) [211]. A second luminal client in this class was the A6 T-cell receptor α chain

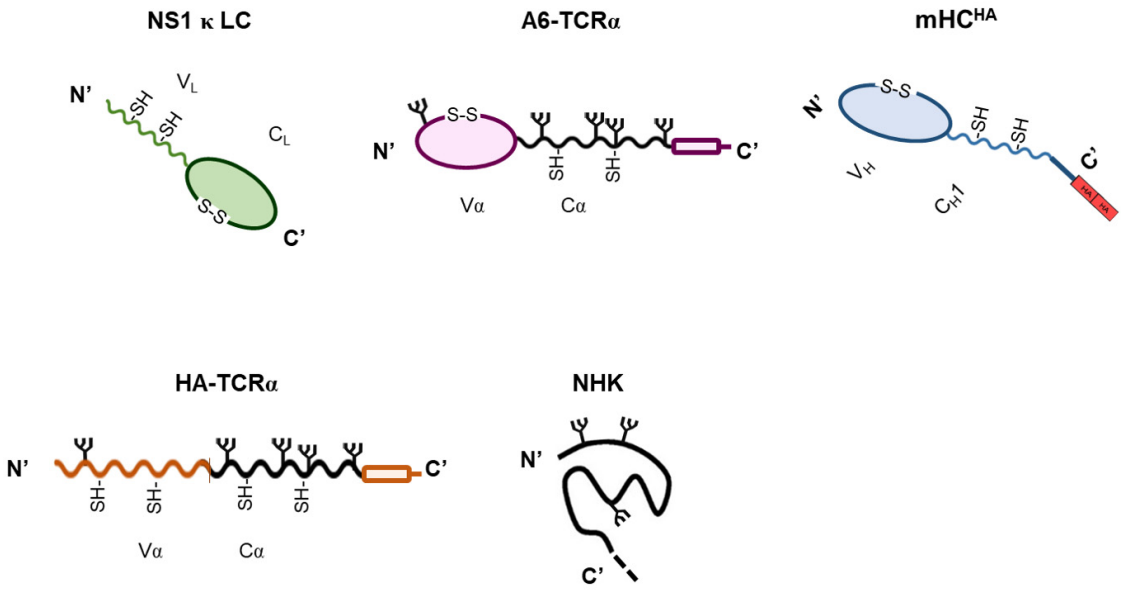



**Figure 2-1. Schematic representation of our model ERAD substrates**

The well-folded domains are represented as filled ovals, while unfolded segments are shown as wavy lines. Disulfide bonds (S-S), free cysteines (-SH), glycans (▉), and the HA epitope tag (red box) are indicated. Shown here from left to right are: the non-glycosylated NS1 κ light chain with the well-folded domain at the C' terminus, the A6-TCRα with 5 glycans and the folded domain at the N' terminus, the non-glycosylated 2x HA-tagged $mHC^{HA}$ with the well-folded domain at the N' terminus, the unfolded HA-TCRα with 5 glycans, and the unstructured NHK with 3 glycans (the dashed line represents the portion deleted from this protein).

(A6-TCRα) [214]. This protein possesses a variable region (Vα) that folds and forms its intradomain disulfide bond and a constant region (Cα), which is unoxidized and unstructured, as demonstrated by cell-based and biophysical assays [27]. The third client was a truncated γ1 heavy chain (mini-HC HA-tagged or $mHC^{HA}$) composed of a folded, oxidized variable domain ($V_H$) and a reduced, unstructured $C_H1$ constant domain, as observed in cell-based and biophysical assays [28, 215]. We also used two ERAD clients without a well-folded domain that have been the focus of previous studies. The HA-TCRα chain shares the unoxidized, unstructured Cα domain with A6-TCRα, but in this case it has a variable region that is also unable to undergo oxidative folding and is unstructured, as determined by NMR [27]. The second completely unfolded client was the null Hong Kong (NHK) variant of α1-antitrypsin, which is caused by a dinucleotide deletion resulting in a frame shift that deletes 61 amino acids from the C-terminus of this protein [213]. Although α1-antitrypsin has not been examined by the types of biophysical studies conducted on the immune proteins, this protein is not comprised of multiple domains that fold independently, and a deletion of this size is likely to have profound effects on the remaining portion of the protein. All five proteins have been the basis of many ERAD studies. These proteins include glycosylated and non-glycosylated clients, as well as two proteins with a folded domain at the N-terminus versus one with the folded domain at the C-terminus **(Figure 2-1)**. As classic ERAD substrates, the requirements for Hrd1, p97, and the proteasome in the turnover of our protein substrates have been well-established. Nonetheless, all clients were examined in pulse-chase experiments conducted in the either the presence or absence of proteasomal inhibitors to ensure that they behaved as ERAD clients in our hands (data not shown).

**Possession of well-folded domains impairs full release of luminal ERAD substrates into the cytosol, after proteasome inhibition**

To assess the localization of ERAD clients with well-folded domains when proteasomal degradation was inhibited, we transiently expressed these proteins in 293T cells and subjected them to digitonin extraction assays. Due to the relatively high cholesterol content of the plasma membrane compared to that of the ER, low concentrations of the detergent digitonin can disrupt the plasma membrane, while leaving the ER membrane intact [216]. Centrifugation of digitonin-treated cells allows cytosolic proteins to escape, while ER proteins remain cell associated. A readily detectable portion of our two completely unfolded glycoproteins, NHK and HA-TCRα, became deglycosylated (DG) in cells that had been treated with the proteasome inhibitor MG132 through the action of N-glycanase, which associates with p97 at the cytosolic side of the retrotranslocon and deglycosylates ERAD clients prior to their degradation [174, 217]. A significant portion of this pool was released by digitonin **(Figure 2-2A)**, arguing that these two clients were accumulating in the cytosol when the proteasome was inactive, which is in agreement with previous studies [218, 219]. The cytosolic protein GAPDH was completely released by digitonin treatment, whereas ER resident proteins calnexin/calreticulin and Grp170 remained cell-associated, demonstrating the integrity of the ER was intact. When the three ERAD clients with a folded domain were similarly

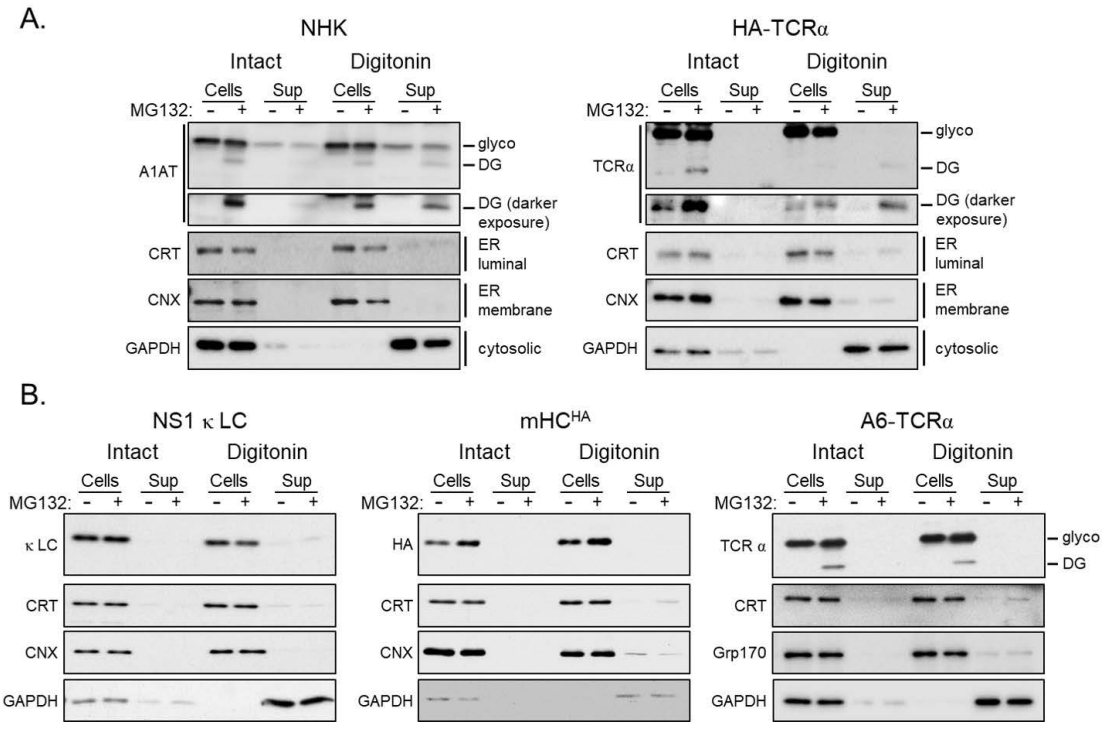



**Figure 2-2. Possession of well-folded domains impairs full release of ERAD substrates into the cytosol**

(A) 293T cells were transfected with vectors encoding either NHK or HA-TCRα, two ERAD clients without well-folded domains. Following treatment with 10μM MG132 (+) or DMSO (-) control for 3.5hrs, the cells were kept intact or were treated with digitonin for 5mins. In each case, after centrifugation, the cell pellet and the supernatant (cytosol in digitonin treated cells) were collected for direct analysis by western blotting with the indicated immune reagents. GAPDH was used to monitor release of cytosolic proteins, Calreticulin (CRT) and Grp170 to detect fractionation of luminal ER proteins and Calnexin (CNX) to mark ER membrane proteins. B) 293T cells expressing either NS1, $mHC^{HA}$ or the A6-TCRα were treated as in (A) and processed for western blotting. For all glycoproteins, the glycosylated (glyco) and deglycosylated (DG) forms are indicated. In panel (A), a darker exposure of the portion of the gel containing the deglycosylated form is also shown.

analyzed, they all remained cell associated, even though a portion of the A6-TCRα chain became deglycosylated (DG) in presence of MG132 **(Figure 2-2B)**.
The TCRα chain glycans are predominantly present on the unfolded Cα domain **(Figure 2-1)**, making it unclear how far this protein might extend into the cytosol when the proteasome was inhibited and raising the possibility that the proteasome might play a role in pulling ERAD clients with folded domain completely through the retrotranslocon.

**Substrates with well-folded domains are completely retrotranslocated across the ER membrane after proteasome inhibition as revealed by deglycosylation**

To determine how far the ERAD clients with a folded domain extended into the cytosol, we turned to the naturally unglycosylated NS1 protein and first engineered a single glycan acceptor sequence at three distinct sites within the unfolded $V_L$ domain **(Figure 2-3)**. This domain becomes reduced and ubiquitinated when the proteasome is inhibited [220], making it likely that this is the domain entering the retrotranslocon first. Each of the N-linked glycan consensus sites engineered in the $V_L$ domain was readily glycosylated, as shown by comparing the migration of each mutant with the parental NS1 and by Endo H treatment, which cleaved the glycans and restored mobility to that of the non-glycosylated NS1. MG132 treatment resulted in a pool that was deglycosylated, even in the case of the NS1-N100 construct, which had the glycan positioned at the $V_L$:$C_L$ boundary **(Figure 2-4A)**, revealing that the entire $V_L$ domain must reach the cytosol. We next engineered sites in the $C_L$ domain and were careful to choose positions within loops of the well-characterized Ig fold, as glycans at these positions should be less likely to interfere with domain folding **(Figure 2-3)**. All sites were glycosylated and treatment with MG132 also resulted in deglycosylation of a portion of each of these modified NS1 constructs **(Figure 2-4B)**. This indicated that even the well-folded $C_L$ domain was extracted from the ER and entered the cytosol. In all cases, co-expression of the p97QQ mutant, which was deficient in ATPase activity, or the ubiquitin-ligase deficient Hrd1C291S mutant prevented deglycosylation. We next tested if this was also true of our other ERAD clients with a well-folded domain. In the case of the A6-TCRα, we genetically removed the four N-linked sites on the Cα domain **(Figure 2-3)**, so it would be possible to readily determine if the remaining glycan on the well-folded Vα domain was deglycosylated upon proteasomal inhibition. Again, we found that mono-glycosylated A6-TCRα was deglycosylated **(Figure 2-4C)**, arguing that the folded domain was passing through the retrotranslocon. Similarly, engineering glycan recognition sequences on a predicted loop within the $V_H$ domain ($mHC^{HA}$-N55) or in the unfolded $C_H1$ domain ($mHC^{HA}$-N118) of the truncated γ1 HC produced clients that were readily glycosylated, and a portion of each was deglycosylated upon MG132 treatment **(Figure 2-4C)**. Together these data suggested that although the ERAD clients with folded domains were not released from cells with digitonin, they were able to fully pass through the retrotranslocon and reach the cytosolic side.

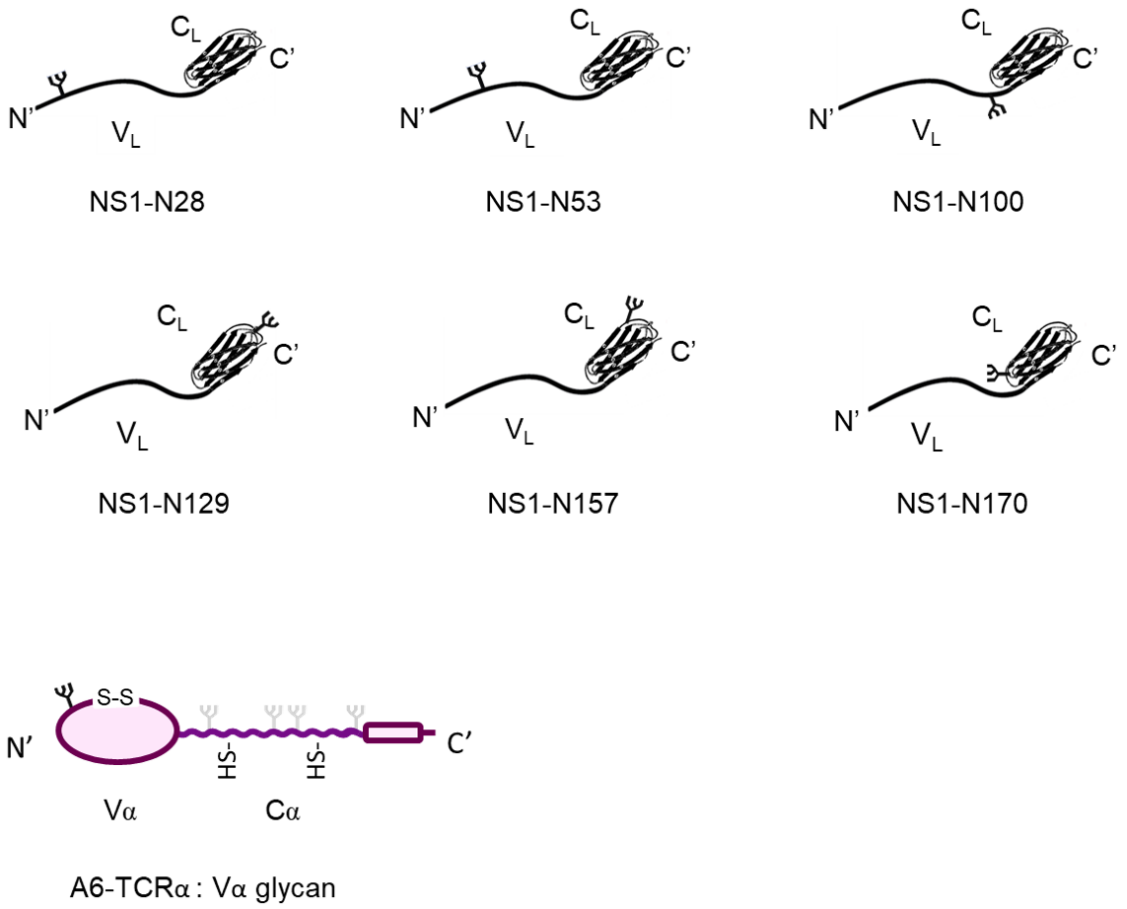



**Figure 2-3. Schematic representation of location of engineered and naturally occurring N-glycans**

Single N-linked glycosylation motifs (S/T-X-N) were engineered throughout the NS1 sequence at the indicated sites and named for the location of the modified asparagine. The $C_L$ domain is presented as a ribbon structure based on its crystal structure, and glycans were placed on the indicated loops or turns between β sheets of this domain. The 4 glycans on the unfolded Cα domain of the A6-TCRα were removed (greyed glycans) to allow only the single glycan on the folded Vα domain to remain on this protein. A single glycan was inserted into the $V_H$ and $C_H1$ domains of the truncated γ1 heavy chain ($mHC^{HA}$).

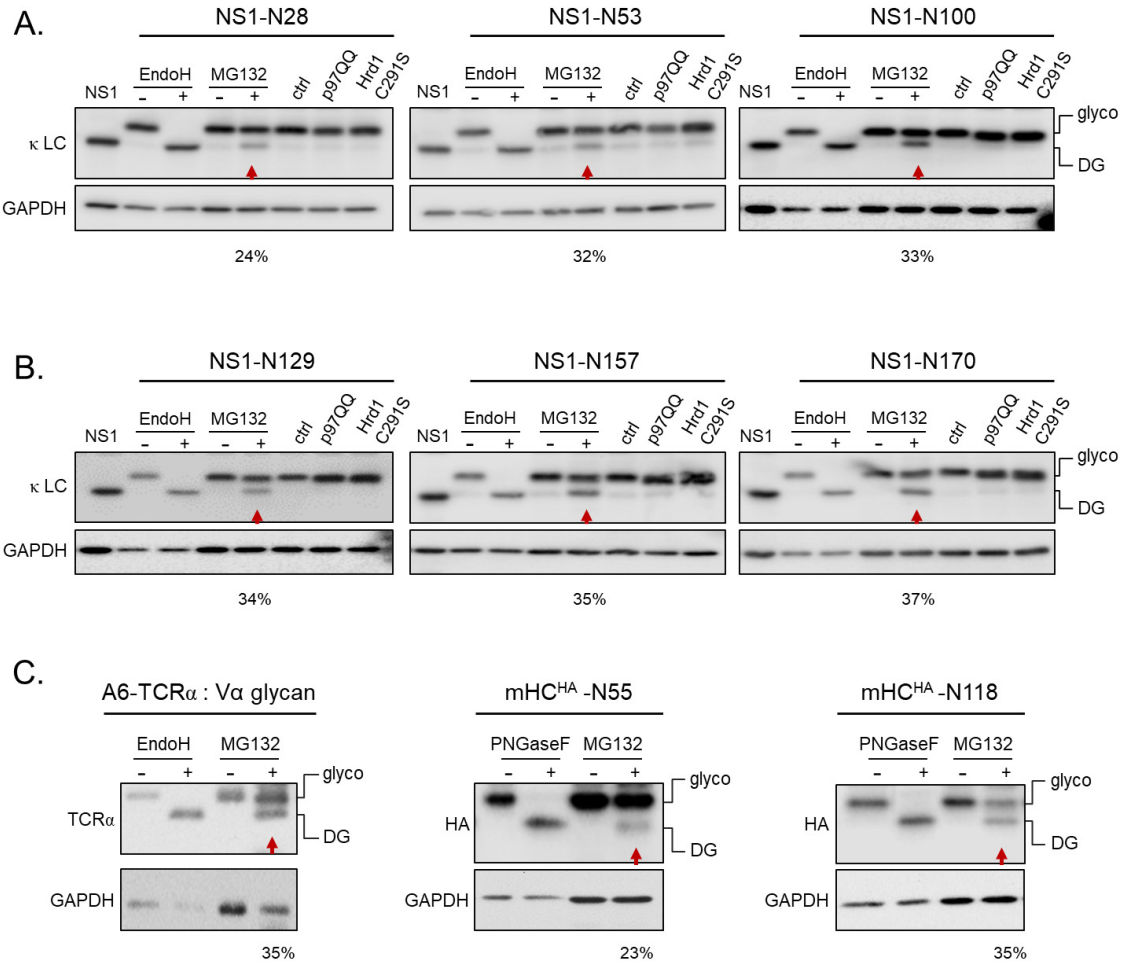



**Figure 2-4. Substrates with well-folded domains are completely retrotranslocated across the ER membrane after proteasome inhibition, as revealed by deglycosylation**

(A) Cells were transfected with the indicated NS1 constructs bearing a single N-linked glycan on the $V_L$ domain. Cell lysates were prepared 24hrs later and treated with (+) or without (-) Endo H to identify mobility of the glycosylated and non-glycosylated forms of these constructs. The parental, non-glycosylated NS1 was used as a control in each case. Cells were treated with 10μM MG132 (+) or DMSO control (-) for 3.5hrs, or cells were co-transfected with the indicated NS1 mutants and either empty pcDNA3.1 (ctrl), the p97QQ mutant, or the Hrd1C291S mutant. Lysates were directly analyzed by western blotting with anti-κ antibody. Red arrows point at DG species and the percent deglycosylated is shown below each panel. (B) Cells were transfected with NS1 constructs bearing a single glycan on the $C_L$ domain and processed as in (A). (C) Cells expressing, a mutant A6-TCRα construct in which the single glycan present on the Vα domain remained, or mutant $mHC^{HA}$ constructs possessing a single glycan on either its folded (N55) or unfolded (N118) domain were prepared as in (A) and an analyzed by western blotting with anti-TCRα or anti-HA respectively (DG: red arrow). In all cases, GAPDH was used as a loading control.

## Deglycosylation is dependent on ERAD substrate ubiquitination and retrotranslocation

In an effort to better understand this last finding and the systems being used, we performed several additional tests and control experiments. First, we explored the possibility that our "deglycosylated pool" of these partially folded ERAD clients was due to MG132 stabilizing a normally rapidly degraded population that never entered the ER. Cycloheximide chase experiments were conducted on cells expressing the NS1-N129 construct either in the presence or absence of MG132 **(Figure 2-5A)**. We found that over time the glycosylated form began to disappear and the deglycosylated form to appear when cells were treated with both cycloheximide and MG132, demonstrating a precursor-product relationship. We also queried whether proteasomal inhibition could affect the integrity of ER membranes in a way that allowed N-glycanase to interact with luminally-confined proteins. To examine this possibility, we expressed an NS1 variant with an engineered glycan in the $C_L$ domain (NS1-N157) and combined co-expression of wild-type or mutant p97 with MG132 treatment. We found that deglycosylation in the presence of proteasomal inhibition was completely blocked when the dominant negative p97 mutant was present **(Figure 2-5B)**, confirming that MG132 treatment did not allow N-glycanase to gain access to the ER lumen. Together these two approaches demonstrated that the deglycosylated species, which occurred after MG132 treatment, was not a result of stabilizing proteins that didn't enter the ER or of non-retrotranslocation-specific removal of glycans.

The lack of client deglycosylation observed with co-expression of the dominant negative Hrd1C291S mutant strongly suggested that ubiquitination was required for retrotranslocation. However, a recent study found that auto-ubiquitination of Hrd1 was critical to its activity in client dislocation [135]. Hence, we examined a variant of the NS1 LC that cannot be ubiquitinated due to mutation of all lysines, serines, and threonines in the $V_L$ domain (NS1-$V_L$-STK$^-$); the well-folded $C_L$ domain is left intact and is not ubiquitinated [149]. Using the digitonin release assay on cells with normal Hrd1 function, NS1-$V_L$-STK$^-$ was found to remain cell-associated **(Figure 2-5C)**. To determine if it was able to be dislocated from the ER upon proteasome inhibition, a single glycosylation site was engineered near the N-terminus of the unfolded $V_L$ domain (N28), and a separate one was introduced near the C-terminus of this domain (N100). When these constructs were co-expressed with the p97 and Hrd1 mutants, similar to studies with the NS1 constructs described in **Figure 2-4**, neither client was deglycosylated **(Figure 2-5D)**. However, inhibition of the proteasome did not lead to the appearance of a deglycosylated form of either NS1-$V_L$-STK$^-$ construct **(Figure 2-5D)**. Thus, client ubiquitination was indeed required for it to be recognized by cytosolic factors and pulled far enough into the cytosol for the glycan at N28 to become accessible to N-glycanase.

## Retrotranslocated domains are reduced

We took a closer look at the structural properties of the retrotranslocated pool of the clients with a well-folded domain to determine why they were not released by

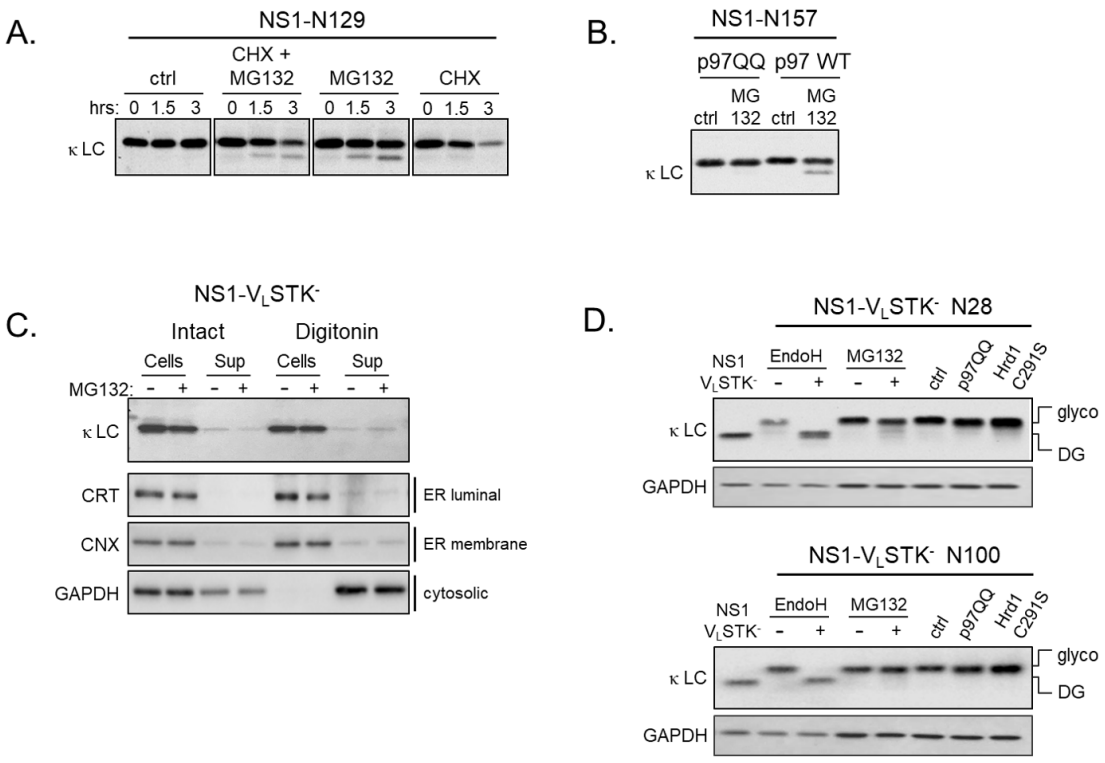



**Figure 2-5. Deglycosylation is dependent on ERAD substrate ubiquitination and retrotranslocation**

(A) Cells expressing the NS1-N129 mutant were subjected to cycloheximide (CHX) chase experiments coupled with or without MG132 treatment and lysed at the indicated time points and analyzed as described previously. Deglycosylated (DG) and glycosylated (glyco) species are indicated. (B) Cells were co-transfected with NS1-N157 and either p97 WT or ATPase inactive p97QQ mutant. The next day, they were treated with 10μM MG132 or DMSO (ctrl) for 3.5hrs. Lysates were collected and blotted with anti-κ antibody. (C) Cells expressing the non-glycosylated NS1-$V_L$STK$^-$ were treated with or without MG132 for 3.5hrs and were then either kept intact or permeabilized with digitonin as described previously. Lysates were analyzed by western blotting and blotted with anti-κ antibody. CRT, CNX and GAPDH were used as controls. (D) Cells transfected with the indicated NS1-$V_L$STK$^-$ constructs with a single N-linked glycan on the $V_L$ domain (at positions 28 or 100) were treated and analyzed as in **Figure 2-4** (A & B).

digitonin beginning with the oxidation status of NS1. Ig domains are a common module found in eukaryotic proteins that are synthesized in the ER and traffic through the secretory pathway. This module is particularly stable in the extracellular environment, due to the presence of an intradomain disulfide bond that secures the folded domain, and is the second most common structural motif found in metazoan proteins [221].The presence of the intramolecular disulfide bond can be detected by increased mobility on non-reducing SDS polyacrylamide gels [222, 223]. Under steady state conditions, the NS1 protein exists in a form in which both domains are oxidized (ox2), and a partially oxidized (ox1) form, in which only the $C_L$ domain possesses a disulfide bond [211]. We engineered a NS1 κ LC that did not have an ER targeting signal sequence (ΔssNS1) and was thus synthesized in the cytosol to use as a control. We found that the ΔssNS1 construct migrated the same under both reducing and non-reducing electrophoresis conditions, and its migration was not affected by MG132 treatment **(Figure 2-6A)**. This revealed that no disulfide bonds were formed in this protein (ox0) when synthesized in the reducing environment of the cytosol. When both the ΔssNS1 and the parental NS1 were run on the same non-reducing SDS polyacrylamide gel, we found that in the absence of MG132 NS1 populated the expected two redox forms (ox1 and ox2), which both migrated faster than the fully reduced ΔssNS1, and is consistent with previously reported data [211]. However, when the proteasome was inhibited, we observed an additional slower migrating band that co-migrated with the ox0 form observed with the cytosolically expressed ΔssNS1 construct **(Figure 2-6A)**, revealing that a pool of the $C_L$ domain was reduced under these conditions. Similarly, $mHC^{HA}$, which has a folded $V_H$ domain and an unstructured $C_H1$ domain [25] and thus exists only as an ox1 redox form [28], had a pool that became fully reduced with MG132 treatment **(Figure 2-6B)**.

As the fraction of each of these constructs that was deglycosylated upon proteasomal degradation was similar to the amount that became fully reduced, it was likely that they represented the same pool. To test this directly, we chose the NS1-N100 construct with the single engineered N-glycan in the $V_L$ domain. Proteasome inhibition results in the appearance of the deglycosylated form when the protein was analyzed under reducing conditions, and the control sample separates into the ox1 and ox2 forms on non-reducing gels. Endo H treatment increased the mobility of both species, demonstrating that both were glycosylated. When NS1-N100 isolated from control and MG132 treated cells was separated under non-reducing conditions, we observed a third species that migrated between the ox1 and ox2 isoforms from the proteasome inhibited cells, but slightly slower than the deglycosylated ox1 form generated by Endo H treatment **(Figure 2-6C)**.

To determine if this new species represented reduced and deglycosylated NS1-N100 protein, the glycosylated pool was separated from the deglycosylated one by incubation with Concanavalin A (ConA)-conjugated beads. The lectin binds to the N-linked glycan, allowing the glycosylated form of the protein to be readily isolated by centrifugation, leaving the deglycosylated form in the supernatant. All samples were electrophoresed under non-reducing conditions and the parental non-glycosylated NS1 was used as a control. The new species observed only upon MG132 treatment of cells expressing NS1-N100 co-migrated with the ox0 form of non-glycosylated NS1 and bind

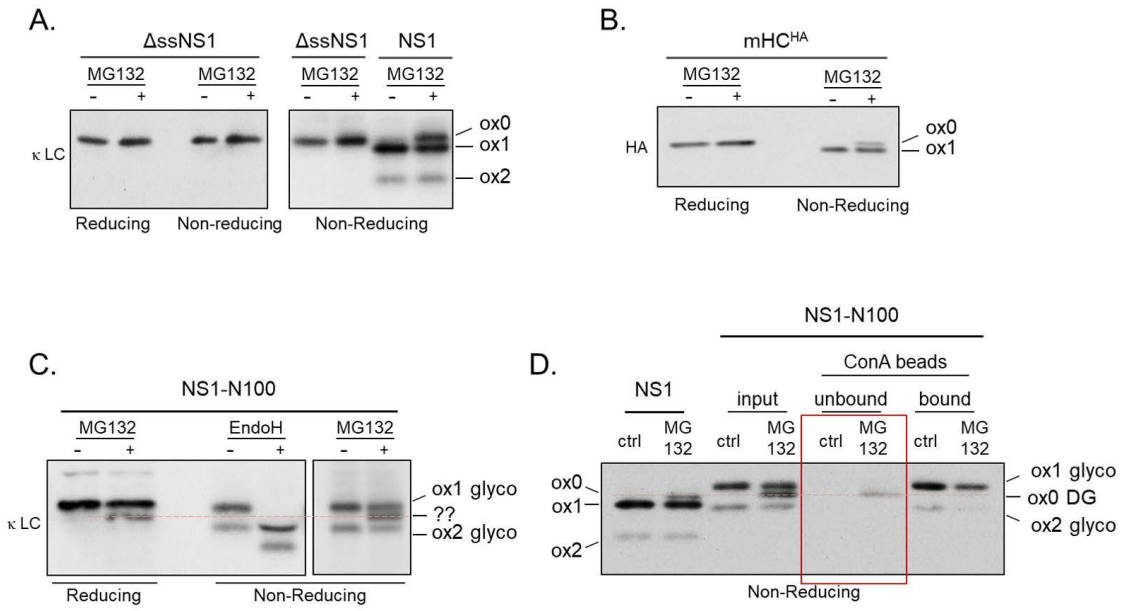



**Figure 2-6. All retrotranslocated domains are reduced**

(A) Cells were transfected with either the cytosolically expressed ΔssNS1 or with NS1. After 24hrs, cells were treated with or without MG132, and cell lysates were prepared in NP-40 buffer containing NEM. Samples were mixed with 2x Laemmli sample buffer containing β-mercaptoethanol (reducing) or without (non-reducing), electrophoresed, and analyzed by western blotting with anti-κ antibody. The different oxidation species (ox0, ox1, ox2) are indicated. (B) Cells transfected with $mHC^{HA}$ were treated and analyzed as in (A) and blotted with anti-HA antibody. (C) Cells expressing NS1-N100 were treated with (+) or without (-) MG132 as previously described. Lysates were analyzed under reducing and non-reducing conditions. A portion of the lysate from cells not treated with MG132 was also digested with Endo H to determine the migration of the deglycosylated ox1 and ox2 species. (D) 293T cells expressing NS1-N100 were treated with or without MG132 as previously described. Samples from the lysates (NP-40 with NEM) were kept as inputs, while the remainder of each test condition was incubated with ConA-conjugated beads. Equivalent samples from the portion that did not bind the beads (ConA-unbound) and from the eluate (ConA-bound) were collected. All samples were analyzed by western blotting under non-reducing conditions with anti-κ antibody. Migration of the NS1-N100s bands was compared with those of NS1 (in the first two lanes). Bands corresponding to the various redox states are indicated.

to ConA **(Figure 2-6D)**, demonstrating that the deglycosylated species appearing upon MG132 treatment was fully reduced. When our other NS1 constructs with a single glycan were similarly examined after MG132 treatment, we detected a third band migrating between the ox1 and ox2 forms in all of them (data not shown).

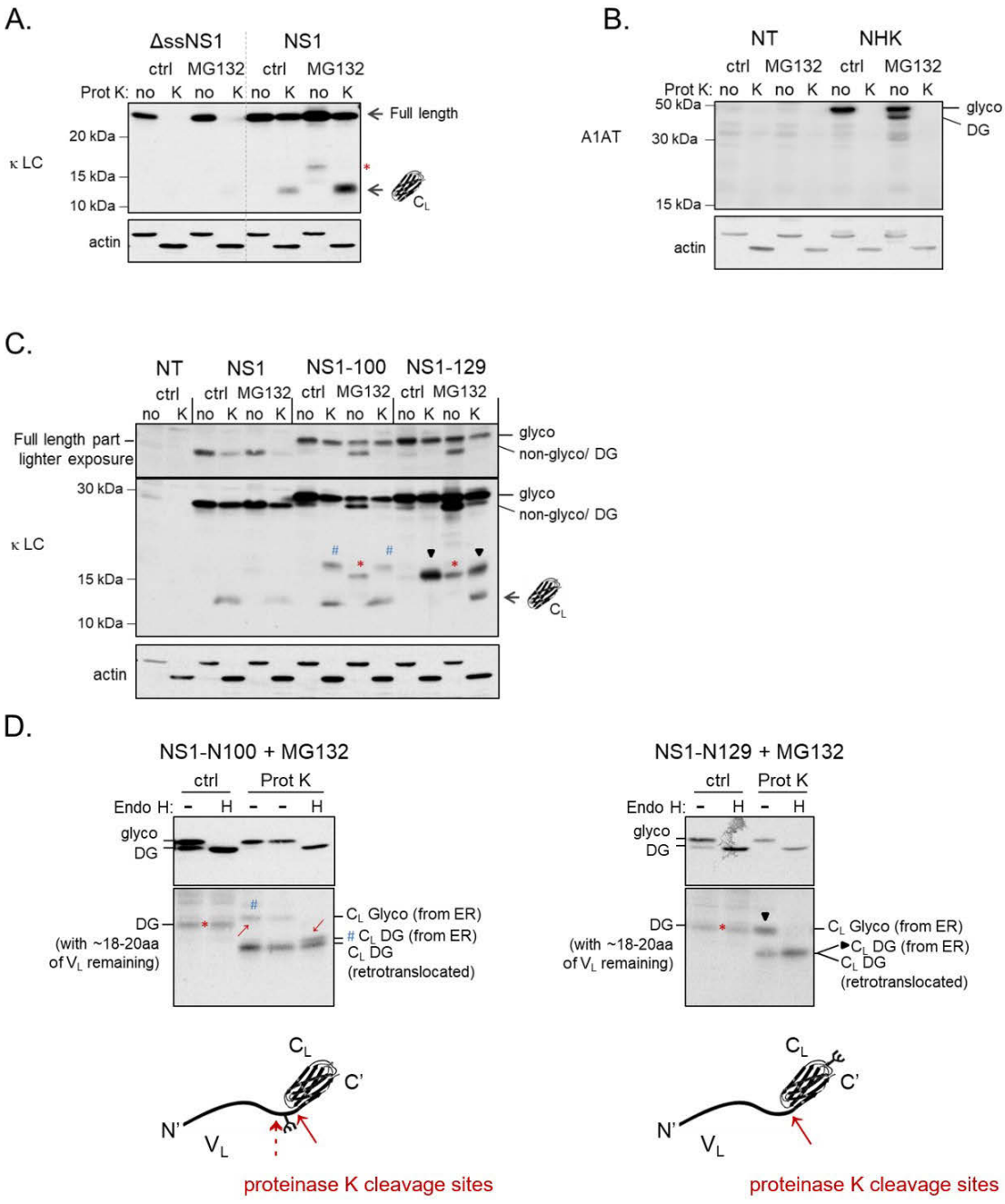
**The retrotranslocated and deglycosylated NS1 $C_L$ domain still retains structure as indicated by resistance to proteinase K digestion**

We were puzzled by our finding that the completely reduced forms of NS1 and $mHC^{HA}$ were retained in digitonin-permeabilized cells, whereas the fully reduced HA-TCRα and NHK were not. To better understand this difference, we asked if the proteins with well-folded domains might still retain structure in the absence of their disulfide bond after retrotranslocation, since a previous *in vitro* study found that reduction of a recombinant $C_L$ domain did not significantly alter its structure [224]. To test this, we relied on the NS1 constructs, because the immune serum is specific for the folded domain, and we could utilize the ΔssNS1 to control for possible spontaneous $C_L$ domain folding. Lysates were treated with limiting concentrations of Proteinase K to distinguish between unstructured and structured domains, and samples were electrophoresed on higher percent acrylamide gels to detect and resolve small fragments that might otherwise run with the dye front. The ΔssNS1 construct was entirely digested with Proteinase K, including its $C_L$ domain, arguing that this domain did not fold well in the reducing environment of the cytosol and in the absence of ER chaperones **(Figure 2-7A)**. Conversely, the parental NS1 construct had a readily detectable band after Proteinase K digestion migrating with an apparent molecular weight of ~12 kDa, consistent with it representing a complete $C_L$ domain. Similar examination of NHK revealed that it was fully susceptible to digestion by the same concentration of Proteinase K, in keeping with a lack of structure for this mutant **(Figure 2-7B)**. For all samples tested, this concentration of Proteinase K partially cleaved actin, our loading control, into a smaller, faster migrating fragment.

From this experiment conducted on whole cell lysates, it was not possible to establish if the protected $C_L$ band arose only from the ox1 form, which would have been in the ER lumen, or if it also included the retrotranslocated and reduced ox0 isoform. To distinguish between these possibilities, we similarly tested two of the NS1 constructs possessing a single N-linked glycan; the NS1-N100 with an engineered glycan at the boundary between the $V_L$ and $C_L$ domains and the NS1-N129 with the glycan in the $C_L$ domain. For the NS1-N100 construct, treatment of the control sample with Proteinase K resulted in two anti-κ-reactive fragments being protected; one of which co-migrated with the protected fragment in the parental non-glycosylated NS1 protein and thus represented the unglycosylated $C_L$ domain, and a slightly larger fragment (#), both of which likely originated from the ER-localized ox1 species **(Figure 2-7C)**. The same species were present after Proteinase K digestion of MG132-treated cells **(Figure 2-7A, C)**. Incubation of the sample from MG132-treated/Proteinase K digested lysates with Endo H resulted in increased migration of only the slower migrating species, which still migrated slightly slower than the non-glycosylated $C_L$ domain **(Figure 2-7D**, # and red arrows**)**.

**Figure 2-7. The retrotranslocated and deglycosylated $C_L$ domain maintains structure as indicated by resistance to Proteinase K digestion**

(A) Cells expressing ΔssNS1 or NS1 were treated with or without MG132 as before. Lysates (without protease inhibitors) were collected, incubated for 20mins on ice with (K) or without (no) Proteinase K, and then for 5mins with 5mM PMSF to inactivate the Proteinase K. Samples were mixed with 2x reducing sample buffer and directly analyzed by western blotting with anti-κ antibody. Actin was used as control for the Proteinase reaction. The migration of full-length NS1 and the $C_L$ domain are indicated, as is a novel species (*) observed with MG132 treatment but no Proteinase K. (B) Cells expressing the NHK ERAD client or control, non-transfected (NT) cells were analyzed as in (A). (C) Cells were transfected with NS1, NS1-N100 or NS1-N129 and treated with or without MG132 followed by incubation with or without Proteinase K as described before. Lysates were analyzed by western blotting. Non-transfected (NT) control cells were also included. Migration of (full-length glycosylated (glyco), full-length non-glycosylated (non-glyco), and deglycosylated (DG) forms are indicated, as is the band corresponding to the $C_L$ domain. Novel bands arising in the Proteinase K-treated lysates from cells expressing the glycosylated NS1 variants (# and ▼) and from samples obtained from cells only treated with MG132 (*) are also indicated. A lighter exposure of the top part of the gel is included to more readily distinguish the various full-length bands. (D) Samples from the MG132-treated lysates used in panel C (both without (ctrl) and with Proteinase K) were treated additionally with Endo H to test the glycosylation status of the Proteinase resulting fragments. Schematic representations of NS1-N100 and NS1-N129 are shown below each panel, and the site of Proteinase K cleavage for NS1-N129 is indicated as a solid red arrow and that for NS1-100 with a dotted red line. Fragment identities are indicated. The non-EndoH-treated, Proteinase K-digested sample for NS1-N100 was duplicated by mistake.


A.
ΔssNS1
NS1
ctrl MG132 ctrl MG132
Prot K: no K no K no K no K
Full length
20 kDa
κ LC
15 kDa
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$C_L$
actin
B.
NT
NHK
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Prot K: no K no K no K no K
50 kDa
glyco
A1AT
30 kDa
DG
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NS1
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no K no K no K no K no K no K no K
Full length part
lighter exposure
glyco
non-glyco/ DG
30 kDa
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κ LC
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NS1-N100 + MG132
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(with ~18-20aa
of $V_L$ remaining)
$C_L$ Glyco (from ER)
# $C_L$ DG (from ER)
$C_L$ DG
(retrotranslocated)
$C_L$
C'
N'
$V_L$
proteinase K cleavage sites
NS1-N129 + MG132
ctrl Prot K
Endo H: - H - H
glyco
DG
DG
(with ~18-20aa
of $V_L$ remaining)
$C_L$ Glyco (from ER)
$C_L$ DG (from ER)
$C_L$ DG
(retrotranslocated)
$C_L$
C'
N'
$V_L$
proteinase K cleavage sites

Furthermore, this data indicated that the glycan present at N100 partially interfered with Proteinase K cleavage at the $V_L$:$C_L$ junction giving rise to both a slightly larger deglycosylated species, as well as a non-glycosylated species cleaved at this junction. The NS1-N129 construct was more readily interpreted. This protein expressed in non-MG132 treated cells was partially protected from Proteinase K, resulting in a fragment slightly smaller (▼) than the glycosylated fragment generated for the NS1-N100 protein **(Figure 2-7C)**. This was compatible with it originating from the ER-localized ox1 isoform, in which the entire $V_L$ domain is digested and the protected $C_L$ domain is glycosylated. Proteinase K digestion of the MG132-treated samples produced two fragments, including one that co-migrated with the unglycosylated $C_L$ domain of NS1 and NS1-N100. Endo H digestion of this sample confirmed that this band was indeed already deglycosylated, since its mobility did not change, and therefore represented the retrotranslocated species still retaining structure. The slower migrating band (▼) was glycosylated and thus arose from the ER-localized ox1 isoform **(Figure 2-7D)**. Surprisingly, we observed an identical ~15kDa, anti-κ-reactive species, in MG132-treated samples from all three groups of κ LC expressing cells (NS1, NS1-N100 & NS1-N129) that had not been subjected to Proteinase K digestion **Figure 2-7A, C, D**, red asterisks). This band migrated slower than the deglycosylated $C_L$ domain and similar to the glycosylated $C_L$ domain, but its presence in the non-glycosylated NS1 protein argued that this represented an unglycosylated fragment that is larger than the $C_L$ domain alone. Indeed, digestion of this sample with Endo H revealed that it was not glycosylated **(Figure 2-7D**, red asterisks**)**. Based on its deduced molecular weight it must include ~30-35 amino acids of $V_L$ domain in addition to the 105 amino acid $C_L$ domain and represents an intermediate in proteasomal degradation. Together the data presented in this **Figure** strongly indicated that the deglycosylated ox0 isoform generated after MG132 treatment still possessed significant structure, even though the disulfide bond had been reduced.

**Retrotranslocated proteins with folded domains remained associated with p97 when the proteasome was inhibited**

Our digitonin experiments revealed that the presence of a well-folded domain in clients obstructed their full release from the ER membrane in absence of the degradative capacity of the proteasome, possibly due to the fact that they still retained structure. To determine what these retrotranslocated clients might be interacting with that could prevent their release from digitonin-solubilized cells, we shifted our studies to the P3U.1 mouse plasmacytoma cell line, which produces only a non-secreted κ LC and is the source of the NS1 construct used in our studies thus far [225]. This line produces very large amounts of the LC and was previously used effectively by us to identify associated proteins in co-immunoprecipitation experiments [220]. We first tested if MG132 treatment resulted in a fully reduced form of the LC in this line and that it remained cell-associated when digitonin was used to disrupt the plasma membrane **(Figure 2-8A)**. Having confirmed this was the case, we immunoprecipitated κ LCs from the P3U.1 cell line and used a LC loss variant line, the Ag8.653 cells [226], as a control. Samples were processed for western blotting to identify co-precipitating proteins by a candidate approach. We found that κ LCs associated with p97 and the 20S subunit of the

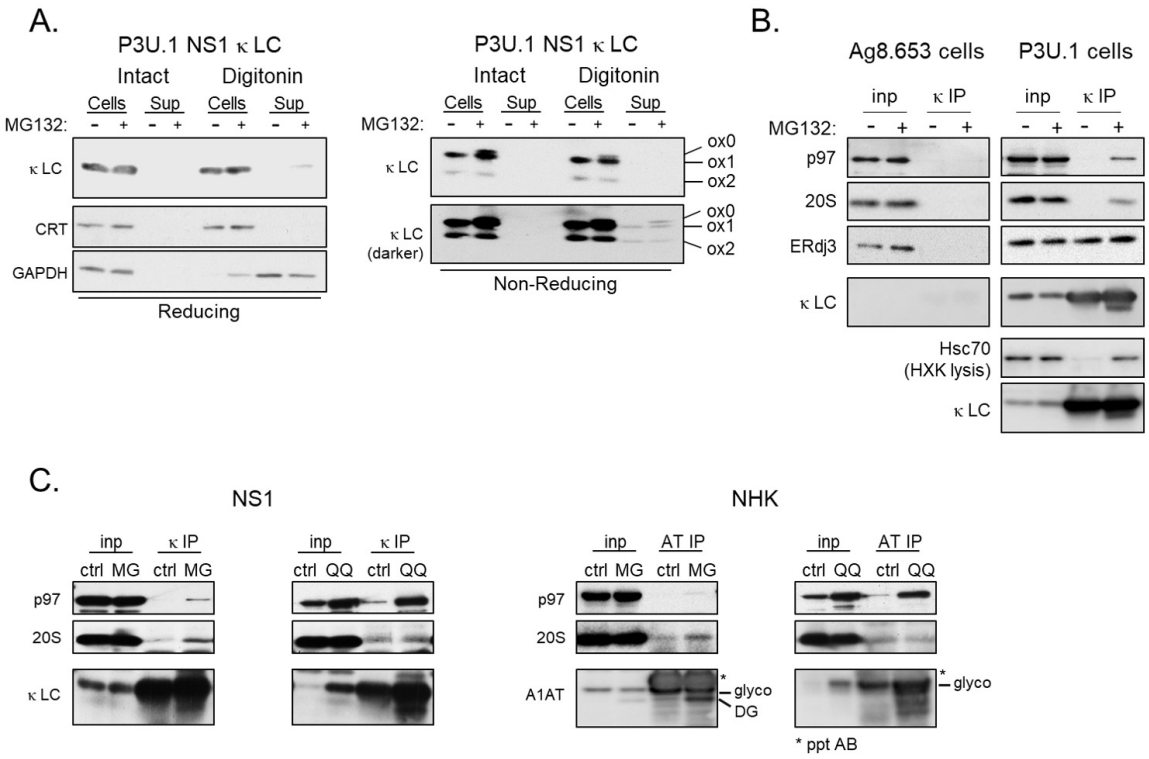


**Figure 2-8. Retrotranslocated proteins with folded domains remained associated with p97 when the proteasome was inhibited**

P3U.1 plasmocytoma cells, which constitutively express κ light chains, were treated with and without MG132 for 3.5hrs and either kept intact or permeabilized with digitonin as described previously **(Figure 1)**. Samples from all treatments and fractions were analyzed under reducing and non-reducing conditions. The ER luminal chaperone CRT and the cytosolic GAPDH were used as controls. Redox species are indicated. (B) κ light chain expressing P3U.1 and control non-light chain expressing Ag8.653 cells were treated with or without MG132 for 3.5hrs and lysed in NP-40 buffer. Light chains were immunoprecipitated with anti-LC-conjugated agarose beads. Immunoprecipitated material (IP) and total lysate samples (inputs – inp) were separated on reducing SDS-gels and analyzed by western blotting with an anti-p97, anti-20S proteasome subunit, anti-ERdj3, and anti-κ LC. A separate set of samples treated in the same way, were lysed in NP-40 lysis buffer supplemented with hexokinase (HXK) and were similarly immunoprecipitated and analyzed by western blotting with anti-Hsc70 and anti-κ LC antisera. (C) 293T cells expressing NS1 or NHK treated with or with MG132 as described before (left panels) or 293Ts co-expressing NS1 with p97QQ or NHK with p97QQ (right panels) were lysed in NP-40 lysis buffer. Lysates were immunoprecipitated with anti-κ or anti-α1-Antitrypsin antibodies and prepared for western blotting with the indicated antisera. The asterisk in the NHK panels indicates the immunoprecipitating antiserum, which migrates just above the glycosylated NHK protein.

proteasome when MG132 was used, but not in its absence, nor were they detected in anti-κ isolated material from the control Ag8.653 cells **(Figure 2-8B)**. Under both conditions, ERdj3 co-precipitated with the κ LC, in keeping with a pool of the LC remaining in the ER. When hexokinase was added to the lysing buffer to deplete ATP, we found the retrotranslocated κ LC bound to Hsc70 in MG132-treated cells **(Figure 2-8B** bottom panel**)**.

To assess if association with the membrane-tethered p97 protein was specific for ERAD clients with well-folded domains, we returned to the 293T cells and examined both the NS1 and the NHK produced from a high-expression vector (pcDNA3.1) **(Figure 2-8C)**. Immunoprecipitation of NS1 LC revealed readily detectable p97 and the 20S subunit of the proteasome in MG132-treated cells, whereas when NHK was isolated from cells treated with MG132, the 20S subunit co-precipitated but p97 was not clearly detected. When the p97QQ mutant was co-expressed with these clients **(Figure 2-8C** right panels**)**, we readily observed a dramatic increase in p97 associated with both of them, indicating that our inability to detect p97 binding to NHK was not due to a technical limitation of the assay. In both cases, enhanced binding to the mutant p97 reduced the client's interaction with the 20S subunit to near basal levels. In combination, our data argue that unfolded ERAD clients can be released from cytosolic ERAD factors (such as p97) after retrotranslocation without the requirement for proteasome function, whereas those with a well-folded domain are dependent on the proteasome to be fully released from the ER membrane.

## Discussion

While many of the cellular components that participate in ERAD have been identified and we have a good understanding of their functions in this process, there is scant information available as to how the presence of well-folded domains in soluble ERAD clients adds to the complexity of their extraction from the ER. A group of papers from the Sitia lab have examined the requirements for degradation of Ig μ heavy chain multimers [227] and μ heavy chain-TCRα chimeras [228], both of which possess multiple folded domains and are covalently assembled with subunits making them very complicated ERAD clients. In both cases, interchain disulfides were shown to be reduced prior to retrotranslocation. It is noteworthy that proteasome inhibition resulted in reduction of the inter- and intra-molecular disulfide bonds of the J chain that is covalently bound to the μ chain multimers and deglycosylation of this subunit, indicating that it was dislocated to the cytosol. However, the freed μ heavy chain multimers showed no evidence of deglycosylation, arguing that this dimeric ERAD client with oxidized domains at both its N- and C-termini was not retrotranslocated in the absence of proteasomal function [227]. Conversely, studies on NHK retrotranslocation, using either a split-GFP construct that only fluoresced once the two pieces combined in the cytosol [219] or proximity biotinylation methodology [229], revealed that this completely unfolded client was fully extracted to the cytosol when proteasomes were inhibited. Similar results were obtained with the unfolded HA-TCRα [218].

Here, we used relatively simple proteins with well-characterized structural elements to study the effects of a single well-folded domain on retrotranslocation and sought to establish the requirements for full extraction of these clients from the ER. We found that all three of our model proteins with a well-folded domain fully entered the cytosol (retrotranslocated) when the proteasome was inhibited, as indicated by complete deglycosylation of naturally occurring or engineered N-linked glycans dispersed throughout the protein. Nonetheless, these ERAD clients could not be extracted with digitonin arguing that they remained associated with the ER membranes. This was not true of our fully unfolded proteins, NHK and the HA-TCRα. Those proteins were released from cells with digitonin after proteasome inhibition revealing that the activity of p97 was sufficient to fully extract them from the ER after ubiquitination. In our study, retrotranslocation of the clients with a folded domain required both that they were ubiquitinated and the activity of p97. This finding is counter to a study using proximity biotinylation, which found that NS1 was modified by cytosolically expressed BirA even in the presence of the p97QQ mutant, as was the NS1 $V_L$-STK$^-$ mutant that cannot be ubiquitinated [229]. Conversely, they found that co-expression of a BiP trap mutant, which is not released from the client, inhibited biotinylation. Based on our data using these same mutants and constructs, it is possible that the proximity labeling experiments might be revealing continuous sampling of the cytosol by the termini of ERAD clients, which would require that BiP be released. In support of this possibility, an earlier study revealed that BiP release coincided with client retrotranslocation [230].

In our study, we found that the retrotranslocation of all three clients with a folded domain was accompanied by their full reduction. In the case of NS1, this is counter to our previous report [220] and that of another group [230], which were unable to detect a fully reduced species. In the present study, we were able to detect ox0 by increasing the concentration of polyacrylamide in our SDS gels to 15% and electrophoresing until the 20kDa marker reached the bottom of the gel. Although the size of the two Ig domains is very similar, reduction of the $C_L$ domain had a very small effect on migration compared to that of the $V_L$ domain. We further demonstrated that retrotranslocated NS1 retained structure in the reduced $C_L$ domain, as indicated by protease resistance. This is further supported by our previous finding that ubiquitination was confined to the $V_L$ domain with no modification detected on the $C_L$ domain [149]. Intriguingly, our studies detected a very sharp, anti-κ reactive intermediate migrating at ~15-16 kDa when MG132 was used without the addition of protease. Based on the molecular weight of this species and using the actual sequence of the NS1 protein, we calculated that this fragment was comprised of the ~12 kDa $C_L$ domain together with an additional ~30-35 amino acids of the $V_L$ domain. It has been estimated that the distance from the outside of the 20S proteasome to the proteolytic active site is ~70Å [231], which is equivalent to ~20 amino acids of an extended polypeptide chain. This is consistent with the $C_L$ domain stalling ~10-15 amino acids from the 20S proteasome particle. This argues that the $C_L$ domain represented an impediment to entry into the proteasome core, consistent with this domain retaining structure until likely unfolded by the AAA-ATPases found in the proteasome lid [179]. Unfortunately, we could not examine protease resistance or intermediates in degradation of the mHC$^{HA}$ or the A6-TCRα, because the antisera specific for these proteins can only recognize the unfolded domains.

Since the $C_L$ domain retains structure in the cytosol, it raises the question as to whether this domain, and presumably that of our other clients with a well-folded domain, was unfolded in order to pass through the retrotranslocon and then refolded in the cytosol or if it passed through this channel in a folded state. We found that when NS1 was translated in the cytosol (ΔssNS1) it was unable obtain enough structure to become resistant to Proteinase K. Thus, if well-folded domains need to be unfolded to pass through the retrotranslocon, they must retain some structure as a catalyst for refolding once they reach the cytosol. Although the dimensions of actively translocating retrotranslocons in cells have not been determined, there have been several studies that provide insights into this point. The addition of EGFP to an ERAD client revealed that fluorescence was retained throughout retrotranslocation, arguing that either it was not unfolded during extraction or that it refolded very rapidly [232]. In another study, DHFR was tethered to the N-terminus of the Class I MHC protein, which is an ERAD client in the absence of assembly with $\beta_2$ microglobulin. They found that addition of methotrexate to stabilize the DHFR moiety in a fully folded state did not impede retrotranslocation [233]. The narrowest cross-section of DHFR is 40 Å, whereas the dimensions of an Ig domain are 40Åx25Åx25Å, suggesting it might also be able to pass through the channel intact. In spite of a concerted effort, we were unable to determine if the Ig domains of our clients were reduced prior to inserting into the retrotranslocon. NHK dimers are reduced to monomers in the ER by the PDI family member ERdj5 prior to retrotranslocation [60]. However, neither reducing ERdj5 expression with shRNA nor exogenous over-expression had any effect on our clients (data not shown). Ig domains are comprised of 7-9 antiparallel β strands, in which strands 1-4 form one face of the structure and are disulfide bonded to the second part of the structure comprised of strands 5-7 with a Greek key topology [234]. It is conceivable that reduction of the bond between strands 2 and 6 inside the ER could allow sufficient unfolding to separate the two portions of the domain, allowing it to pass through a narrower channel while retaining enough structure to refold in the cytosol. We previously reported that reduction of the $V_L$ domain represents a rate-limiting step in the degradation of NS1 [149], but we have found mutation of cysteines forming the intra-domain disulfide bond in the $C_L$ domain does not accelerate the turnover of NS1 (unpublished data from our lab). This result could be compatible with the $C_L$ passing through the retrotranslocon intact or could suggest that unfolding of the $C_L$ domain is more rate-limiting than its reduction. A better understanding of retrotranslocon dimensions during the extraction process is needed to determine the limitations on client structure during the process.

Previous studies have revealed that two ERAD clients without well-folded domains can be entirely extracted from the ER membranes through the action of p97 [219, 229], whereas ERAD clients with multiple folded domains are not even retrotranslocated without additional requirements for proteasomal activity [227]. In our study, we discovered that a single folded domain did not block retrotranslocation but did prevent full release from ER membranes, raising the question of where these clients are held up. We found that a portion of NS1, but not NHK, remained associated with p97 when proteasomal degradation was inhibited. This suggested that while p97 is capable of pulling the folded domain through the retrotranslocon it is not able to release the substrate

independent of a fully functional proteasome. The mechanism by which ERAD clients are handled by p97 has been the subject of multiple studies and reviews [156, 157, 166], which have generated several models. It has been proposed that clients enter through the D1 ring, are threaded through the central cavity, and released from the D2 ring for further processing by the proteasome [161]. However, several structural and mutational studies have led to a second model in which D1 and D2 move apart from each other upon ATP binding resulting in the formation of a channel between them through which clients can access the D2 pore and pass through it [165]. Irrespective of the model, it is possible that a well-folded domain imposes an obstacle in this process and requires additional energy to allow the client to be released from p97. This energy could be provided by the AAA-ATPases residing in the base of the 19S proteasome lid. When the proteolytic activity of the proteasome is inhibited, these AAA-ATPase are likely to remain engaged with clients previously fed into the proteasome and thus become unavailable to further assist p97. On the other hand, proteins without well-folded domains were readily processed by p97 and could be released without additional aid from the proteasome.

In summary, our studies reveal that proteins with even a single well-folded domain pose additional constraints on the ERAD process compared to more unstructured proteins. Clients with a well-folded domain queried here were fully reduced, retrotranslocated, and reached far enough into the cytosol to become deglycosylated in the absence of proteasomal degradation, similar to fully unfolded proteins. However, these clients retained significant structure after retrotranslocation, likely causing them to remain associated with p97 at the cytosolic face of the ER membrane. Our findings with these three Ig domain-containing clients are likely relevant to many other proteins, as the Ig module represents the second most common structural motif in metazoan proteins and is particularly abundant in cell surface receptors and secreted proteins [221].

# CHAPTER 3. ER LUMINAL ERAD SUBSTRATES FIRST SAMPLE THE CYTOSOL BEFORE THEIR RETROTRANSLCOATION

## Introduction

Proteins that populate the secretory pathway, membrane proteins, and proteins to be secreted by the cell account for approximately one third of the human genome and are synthesized and fold in the endoplasmic reticulum (ER). A multitude of proteins flux through the ER, and thusly the ER must ensure fidelity of folding. Once nascent polypeptides enter in the ER lumen, they begin folding in a unique more complex environment with the assistance of several ER resident chaperones and their co-factors [14], as well as folding factors such as protein disulfide isomerases and peptidyl-prolyl isomerases [7, 186]. Proteins that have acquired their native conformation and have assembled into multimeric complexes if necessary are able to traffic further into the secretory pathway to reach their final destinations. On the other hand, certain physiological and pathological conditions can result in an imbalance between the protein-folding demand and the folding capacity. When acquisition of the correct fold fails, the arising misfolded, unfolded, or partially folded proteins must be recognized and eliminated from the ER via a process termed ER-associated degradation (ERAD).

ERAD is a multistep process by which incorrectly folded proteins are being recognized, extracted to the cytosol and degraded by the ubiquitin-proteasome system (UPS). ER luminal chaperons and lectins, such as BiP, calnexin/calreticulin, the EDEM lectins, OS-9 and XTP3-B, recognize unfolded proteins or regions in proteins and trimmed glycans, and target these species to the ER membrane for extraction to the cytosol and elimination by the ubiquitin proteasome system (UPS). A protein conducting channel is in place to accommodate ERAD client exit from the ER, a process termed retrotranslocation or dislocation. Research that spans decades has resulted in the identification of many proteins that participate in ERAD substrate retrotranslocation. To add complexity to this process, it has been shown that a variety of factors organize to serve as the channel for different substrates, thusly providing heterogeneity in the composition of individual retrotranslocons [104, 129, 190, 198]. The multi-pass integral ER membrane protein Hrd1 is one of the retrotranslocon components identified in most cases examined, and it has been argued that it forms part of the channel itself [132, 199]. Hrd1 is also an E3 ubiquitin ligase with its RING domain in the cytosolic phase of the ER [200, 201]. Once the ERAD clients are delivered to the retrotranslocon, they are inserted into the channel and shortly after they reach the cytosol, they become ubiquitinated. The AAA-ATPase, p97 together with its co-factors, which is recruited to the ER membrane via protein-protein interactions, recognizes and binds ubiquitinated proteins as they emerge out of the ER. ATP hydrolysis by p97 provides the energy required to pull and extract the ERAD clients from the ER. Finally, ERAD substrates are delivered to the 26S proteasome, the principal proteolytic machine in eukaryotic cells, for degradation.

It is easily understandable that retrotranslocation is a critical step in the ERAD pathway, since it is the step linking ER luminal and cytosolic events of this process.

However, the exact mechanism by which ERAD clients are retrotranslocated remains incompletely understood. Additionally, the available techniques used to study protein dislocation from the ER, traditionally, rely either on subcellular fractionation and analysis of the proteins in the different fractions, or in measuring ERAD substrate deglycosylation (for glycosylated proteins) or ubiquitination, events that occur only when the clients have reached well into the cytosol. Therefore, it is currently difficult to study intermediate steps in the process, test how each individual ERAD factor contributes in protein retrotranslocation, or to make the distinction between complete and partial substrate retrotranslocation. For this purpose, in-cell reporter-based assays are more suitable. Currently, two main systems exist: the site-specific biotinylation reporter assay [235, 236] and the split-GFP method [138, 219, 237]. Site-specific biotinylation in cells involves the attachment of biotin to retrotranslocating ERAD clients that have been tagged with the biotin acceptor sequence, by the BirA biotin ligase, which must be expressed in the cytosol. In this way, client biotinylation can be tracked and studied, and be compared with conditions that inhibit it. On the other hand, the split-GFP system is a fluorescence-based assay, in which retrotranslocation results in the reconstitution of GFP fluorescence from its fragments. The degree of biotinylation or of fluorescence serves as reporter for the localization and the quantity of dislocated substrates in living cells.

Both biotinylation and the split-GFP system have one of their main components in the cytosol (BirA or the incomplete GFP), and as a result may be missing some ERAD clients that have been at least partially presented in the cytosol but have not reached far enough to become modified. Here we focused on optimizing the biotinylation assay to make it more sensitive and detect clients immediately as they emerge out of the retrotranslocon. As a result, we chose to tether the BirA biotin ligase to the cytosolic phase of the retrotranslocon by fusing it to the N' terminus of FAM8A1, a novel component of the channel and a strong Hrd1 interactor [139]. By using our new biotinylation technique, we aimed to characterize the role of individual cytosolic ERAD protein components on substrate retrotranslocation and to determine the localization of misfolded, stabilized ERAD substrates when these components are non-functional. We demonstrate the establishment of a highly sensitive system that manages to modify and track ERAD clients as they exit from the retrotranslocon. The contribution of ubiquitination, p97, and the proteasome to substrate movement into the cytosol was examined, by separately inhibiting each of these functions. Our results support a model in which ERAD clients can partially dislocate and either sample the cytosol or remain in the partially dislocated state when all luminal components are functional even in absence of ubiquitination and all downstream events.

## Materials and Methods

### Constructs and generation of mutants

Cytosolic BirA (cyt BirA) and ER-localized BirA (ER BirA) in pcDNA3 were kind gifts from Dr. Oscar R. Burrone (ICGEB, Italy) and have been previously described

[235]. The N' terminally S-tagged FAM8A1 construct in pcDNA3.1+ was a generous gift from Dr. John Christianson (Nuffield Department of Medicine, UK). The well-characterized non-secreted murine NS1 κ LC [211] in pSVL was used as our main ERAD client.

The BirA DNA sequence was amplified by the cyt BirA construct and was ligated N' terminally of the S-FAM8A1 in pcDNA3.1+ at the HindIII site. The two BAP-tagged NS1 constructs were also provided by Dr. Oscar R. Burrone (ICGEB, Italy) in pcDNA3 vector additionally tagged with the V5 tag. With In-Fusion cloning we added the BAP tag to an NS1 construct at the lower expression pSVL vector, additionally omitting the 12 amino acid V5 tag in order to not have long unstructured segments that could potentially affect the substrates properties. To make N' terminally tagged NS1 (BAP-NS1) in pSVL, the vector was linearized by PCR after the ER targeting sequence and before the NS1 coding sequence (For: AACATTGTAATGACCCAATCTCCCAAATCC, Rev: CCCATCAGCTCCATATAACCAGAGC) and the BAP tag was amplified with primers with sequences overlapping in part with the destination vector (For: GGAGCTGATGGGGGCCTGAACGATATTTTC, Rev: GGGTCATTACAATGTTTTCGTGCCATTCGATTTTC). The two fragments were ligated according to the manufacturer's protocol. To make C' terminally tagged NS1 (NS1-BAP) in pSVL, the vector was linearized by PCR after the NS1 coding sequence and before the stop codon (For: TAGGACGTCAATAATCACTAGTGCGGCCG, Rev: ACACTCATTCCTGTTGAAGCTCTTGACAATGGG) and the BAP tag was amplified with primers with sequences overlapping in part with the destination vector (For: CAACAGGAATGAGTGTGGCCTGAACGATATTTTC, Rev: GATTATTGACGTCCTATTCGTGCCATTCGAT). The two fragments were again ligated according to the manufacturer's protocol.

ATP hydrolysis-defective p97QQ mutant in pcDNA3 vector, was a kind gift from Dr. Yihong Ye (NIDDK, USA). The Hrd1 mutant deficient in ubiquitin ligase activity (Hrd1 C291S in pcDNA3) was generously supplied by Dr. Yuval Reiss (Proteologics, Israel).

**Cell culture and transfections**

293T human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM; 15-013-CV, Corning - cellgro, Manassas, VA) supplemented with 10% (v/v) fetal bovine serum (FBS; S11150, Atlanta biologicals, Flowery Branch, GA), 2mM L-glutamine (25-005-CI, Corning), and a 1% (v/v) antibiotic-antimycotic solution (25μg/ml amphotenicin B, 10,000μg/ml streptomycin, and 10,000 units of penicillin; Cellgro/Mediatech, Manassas, VA) (complete DMEM) at 37°C and 5% $CO_2$. 293T cells were plated 24hrs prior to transfection, which was performed using GeneCellin (GC5000, BioCellChallenge, Toulon, France) according to the manufacturer's protocol. For all analysis, 1μg of each indicated ERAD substrate was used per p60 dish. 0.2ug of ER BirA and 0.2ug of cyt BirA were used unless otherwise indicated. 1ug FAM8A1 and BirA-FAM8A1 were transfected in cells, unless otherwise described. When p97QQ, Hrd1

C291S or Hrd1 wild-type were co-expressed, 1.5µg of each plasmid was used and equal amounts of empty pcDNA3.1 vector were used in the control samples.

**Pulse chase and cycloheximide chase experiments**

To analyze the turnover rate of our BAP-tagged NS1 constructs when the proteasome was inhibited, twenty-four hrs post-transfection, cells were treated with 10µM MG132 or DMSO (control) for 2hrs. Then cells were washed and pre-incubated in complete DMEM labeling media (Cellgro) supplemented with 10% dialyzed FBS for 30min, and pulse-labeled with 100 µCi/p60 of EasyTagTM EXPRESS35S Protein Labeling Mix for 30min. MG132 or DMSO were present throughout this hour. The cells were then washed and chased in complete media supplemented with 2 mM unlabeled Cys and Met for 1 h with DMSO or MG132. At the beginning of the chase period (0hrs), after 1.5hrs and after 3hrs of chase, cells were lysed in 1 ml of Nonidet P-40 lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1 mM PMSF, 1× Roche complete protease inhibitor tablets w/o EDTA). After clearing the lysate at 20,000 × g for 15 min at 4 °C, the supernatant was immunoprecipitated with the anti-κ light chain antibody overnight. Immune complexes were isolated with CaptivATM PriMAB Protein A agarose slurry, washed with Nonidet P-40 washing buffer (50 mM Tris/HCl, pH 7.5, 400 mM NaCl, 0.5% Nonidet P40 substitute, 0.5% sodium deoxycholate), eluted with 2x reducing Laemmli buffer and analyzed by SDS-PAGE. The resulting gels were incubated in Amplify (GE Healthcare, Pittsburgh, PA) supplemented with 3% glycerol for 30 min at room temperature before they were dried. Data were captured with the Typhoon FLA 9500 Scanner (GE Healthcare) and analyzed with the ImageQuantLT software.

For the cycloheximide (CHX) chase, cells co-transfected with the indicated constructs and treated without (control) or with 100µg/ml CHX for the time points specified. The cells were finally lysed in 0.5ml of NP40 lysis buffer and 1.5% of cleared lysates was mixed with 2x Laemmli sample buffer and analyzed by Western blotting.

**Biotinylation, cell lysis, and cell extract preparation**

Transfected 293T cells with the constructs of interest were incubated maintained in serum-free media on the experiment day. One hour (or otherwise as indicated) before lysis, 2.5mM of biotin were added to the cells in culture to allow biotinylation of our proteins of interest. Then, cells were washed with PBS supplemented with 20mM NEthylmaleimide (NEM; E3876-5G, Sigma-Aldrich, St. Louis, MO) in, pH 6.8, to remove free biotin and block BirA activity and then immediately lysed with 300µl/dish Nonidet P-40 lysis buffer (NP-40: 50mM Tris/HCl pH 7.5, 150mM NaCl, 0.5% Nonidet P40, 0.5% sodium deoxycholate, 0.1mM PMSF, 0.5X complete protease inhibitor tablets w/o EDTA) and 20mM NEM. Lysates were clarified by centrifugation at 12,000g and were mixed with 2x Laemmli sample buffer and boiled. Afterwards, samples were divided in two tubes, and one was incubated without while the other with 1µg

streptavidin (StrAv) (Sigma) for 20' at room temperature (RT) before electrophoresis. This incubation with StrAv is necessary for the separation of biotinylated from non-biotinylated proteins.

**Digitonin permeabilization experiments**

293T cells transfected with the constructs of interest were grown in p60 dishes. After 24hrs the culture media was replaced with serum-free media. Then the cells were treated for 3hrs with 10μM MG132 (CAS 133407-82-6 | Calbiochem | 1MG, Millipore, Burlington, MA) or DMSO (276855, Sigma-Aldrich) control where applicable. One hour before digitonin permeabilization, 2.5mM of biotin were added to each dish. Next, cells were collected and washed once with PBS and once with KHM buffer (110mM KOAc, 20mM Hepes pH 7.2, 2mM MgOAc). The cells were then gently resuspended in 1ml KHM (intact cells) or KHM containing 40μg/ml final concentration of digitonin (D5628-1G, Sigma-Aldrich) (digitonin-permeabilized cells) and were incubated on ice for 5min. Intact and permeabilized cells were pelleted by centrifugation at 1000g for 5min at 4ºC. The resulting cell pellets were lysed in 0.5ml NP40 buffer (cells fraction). The supernatant (sup fraction) was collected for analysis after a second centrifugation step at 500g for 5min at 4ºC. Equivalent fractions of the cell lysate and supernatant were mixed with 2x sample buffer, were boiled for 10min and were briefly let to cool. Samples were again incubated for 20min at RT with or without 1ug of StrAv before electrophoresis and analysis with western blotting.

**Partial proteolysis**

Stability against proteolytic digestion was assessed by partial proteolysis experiments on digitonin permeabilized cells. For this, cells expressing the indicated constructs and having been treated as indicated, were first treated with 2.5mM biotin for one hour before permeabilization with digitonin as described before. The permeabilized cells fraction was collected in KHM buffer and was split in two samples, one was incubated without and the other with 100ug/ml of proteinase K (V302B, PROMEGA, Madison, WI) for 30min on ice. After this incubation period, 5mM (final concentration) of PMSF (Sigma) was added for 5min in all samples to inactivate the Proteinase K. Cells were then lysed in 0.5ml NP-40. Lysates were cleared by centrifugation, mixed with 2x Laemmli sample buffer and boiled, before being divided in two and incubated with or without 1ug StrAv as described before. Samples were analyzed by western blotting.

**Western blot analysis, imaging and quantification**

Samples were separated on 12% SDS-polyacrylamide gels, and then transferred to PVDF membranes (IPFL00010, Millipore). Membranes were fixed with methanol, blocked with gelatin wash buffer, and incubated overnight with the indicated primary immune reagents in blocking buffer followed by species-specific secondary reagents, also

in blocking buffer. Western blots were developed using the Pierce™ ECL (32106, Thermo Fisher Scientific, Waltham, MA), and for quantitative analysis they were scanned with the LI-COR Fc Odyssey scanner (LI-COR, Lincoln, NE). Analysis and quantification were performed with the Image Studio Lite software.

### Antibodies

The following antibodies were used for blotting: polyclonal goat anti-mouse κ LC (1050–01, SouthernBiotech); mouse anti-GAPDH (MAB374, Millipore); goat anti-S tag (ab19321, abcam); rabbit anti-Hrd1 (AP2184A, ABGENT); chicken anti-BirA (GW20013F, SIGMA); mouse anti-20S proteasome subunit alpha (C8) (PW8110, Biomol, Hamburg, Germany); rabbit anti-Grp170; goat anti-calreticulin (N-19) (sc-6468, Santa Cruz Biotechnology, Dallas, TX); rabbit anti-calnexin (SPC-127A, StressMarq, Victoria, Canada); and rabbit anti-ERdj3. HRP-conjugated goat anti-rabbit (sc-2054), HRP-conjugated donkey anti-goat (sc-2020), HRP-conjugated goat anti-chicken (sc-2428), and HRP-conjugated goat anti-mouse (sc-2031) were purchased by Santa Cruz Biotechnology.

## Results

Protein biotinylation is an enzymatic reaction catalyzed by a biotin ligase. In cells, site-specific biotin-labeling of proteins can be achieved by co-expression and co-localization of the *E. coli*-derived biotin ligase BirA, and the protein of interest tagged with the appropriate biotin acceptor sequence (BAP). The BAP tag is a 15 amino acid long sequence (GLNDIFEAQKIEWHE) containing a single lysine that is the biotin acceptor. The BAP peptide is the minimal sequence required for efficient biotinylation by BirA after incubation with biotin **(Figure 3-1)**, when BirA and substrate are in proximity [238]. Fusion of BAP to target proteins and co-expression of BirA allow the biotinylation and study of the proteins of interest.

### BAP-tagged NS1 κ light chains are ERAD substrates and can be readily biotinylated by ER localized BirA

The non-secreted NS1 immunoglobulin (Ig) *κ* light chain (LC) is synthesized in the ER, and it is a known luminal ERAD substrate, for which the requirements for Hrd1, p97, and the proteasome in its turnover have been well-established [149, 220, 230]. NS1 possesses a variable domain ($V_L$) that is not able to fold properly and a well-folded constant domain ($C_L$) [211], with the characteristic Ig fold and that is further stabilized by a disulfide bond. Due to its misfolded $V_L$ domain, NS1 becomes a candidate for ERAD. We, therefore, chose this well-studied protein to add the BAP sequence at either the N' or C' terminus to convert it into a substrate for BirA. Tagging the N' or the C' terminus of NS1 with BAP **(**schematics on **Figure 3-2A** and **B)** allowed us to track the

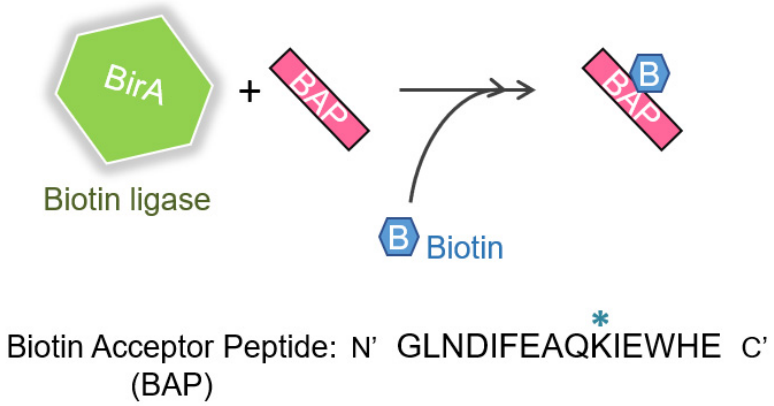


**Figure 3-1. Principle of the proximity biotinylation assay**

Biotinylation of the single lysine on the BAP sequence is achieved when the biotin ligase BirA is in close proximity to the BAP peptide and after addition of biotin. BirA is shown here as a green hexagon, the BAP tag is represented as a pink box and biotin is shown in blue.

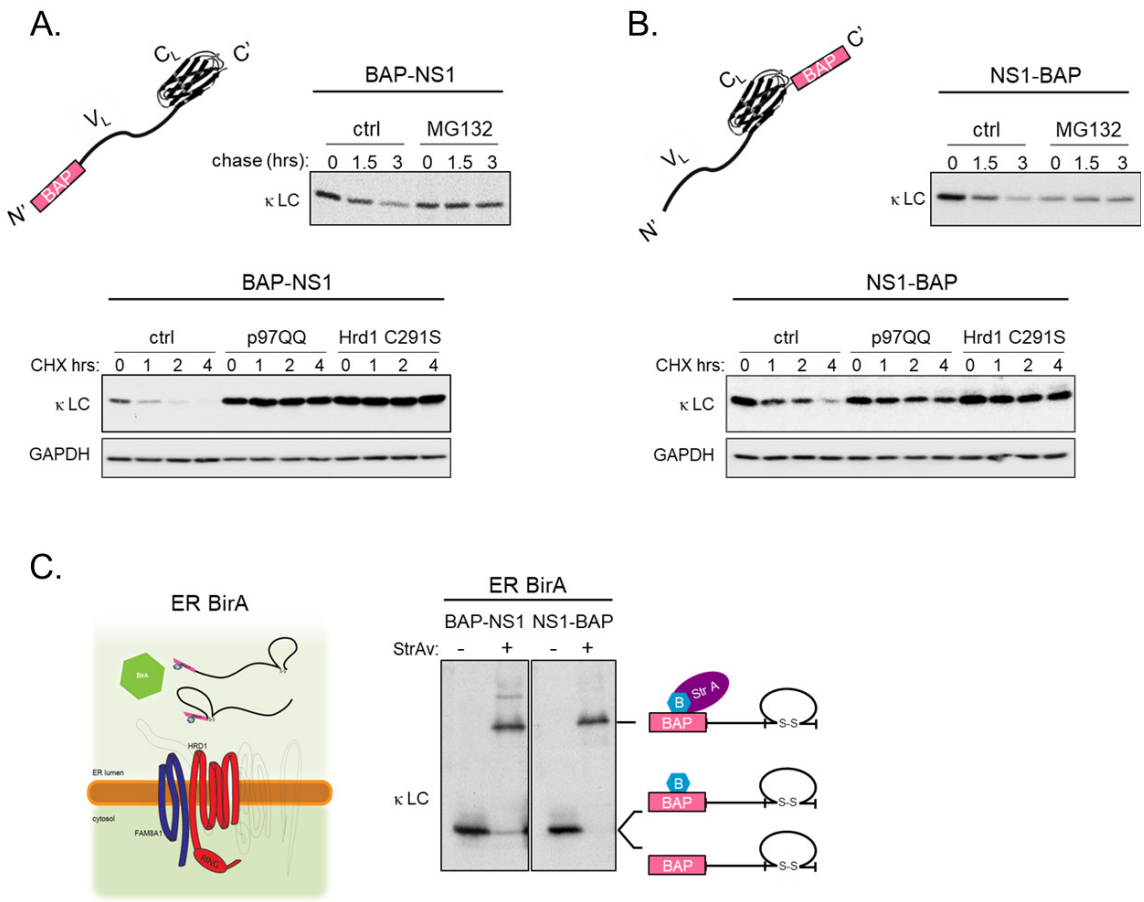


**Figure 3-2. BAP-tagged NS1 constructs remain ERAD substrates and are readily biotinylated by ER localized BirA**

(A) NS1 was N' terminally tagged with the BAP sequence to engineer the BAP-NS1 construct (top left schematic). 293T cells expressing BAP-NS1 were either pulse-labeled and chased in the absence (ctrl) or presence of 10μM MG132 for the indicated time points (top right panel) or were treated with 100μg/ml CHX and chased for the indicated times with or without co-expression of p97QQ or of Hrd1 C291S (bottom panel). (B) same as in A but for the C' terminally tagged NS1, the NS1-BAP. (C) Left: ER-localized BirA (ER BirA) allows the labeling of BAP-tagged proteins in the ER lumen. Right: 293Ts expressing ER BirA and either BAP-NS1 or NS1-BAP were labeled for 1hr with 0.25mM biotin, incubated with/without streptavidin (StrAv), and then analyzed in a western blot retardation assay to separate biotinylated (StrAv-bound) from non-biotinylated (non-StrAv-bound) proteins. BAP-tagged NS1 was detected with anti-κ LC antibody. As a visual example of how the different protein species migrate in the gel, schematics are shown on the right side of the western blot representing the BAP-NS1 (BAP-unfolded domain-folded domain as a circle). At the bottom BAP-NS1 and biotinylated BAP-NS1 are migrating the same without addition of StrAv. StrAv (shown as a purple oval) adds weight to the biotinylated protein causing a significant delay in migration – top band.

retrotranslocating client, and possibly to study directionality in the retrotranslocation process (whether the protein dislocates from its N' or C' terminus).

After engineering the two BAP-tagged NS1s, we tested that the new constructs remained ERAD clients and required both Hrd1 and p97 for their degradation, which was true of the untagged NS1 protein **(Figure 3-2A** and **B)**. For this, pulse-chase experiments were conducted on cells expressing either BAP-NS1 or NS1-BAP in the presence or absence of MG132. Both proteins expressed well and turned over with kinetics similar to untagged $\kappa$ light chain (half-life of ~1.5hrs) [220, 230]. For both tagged proteins, proteasome inhibition caused a reduction in protein turnover, which resulted in increased protein levels, indicating that both constructs are degraded by the 26S proteasome, much like NS1. Cycloheximide (CHX) chase experiments were performed in cells transfected with either BAP-tagged NS1 construct together with the ATPase deficient p97QQ mutant or the ubiquitination deficient Hrd1C291S mutant. Again, turnover of both BAP-tagged NS1 proteins was inhibited and a dramatic accumulation of both proteins occurred in the absence of the p97 and Hrd1 functions. Together these data indicate that BAP-NS1 and NS1-BAP can be effectively expressed in cells, with turnover rates similar to NS1, and they remain ERAD clients with degradation dependent on Hrd1, p97, and the proteasome, like NS1 [149].

We next performed an initial biotinylation test to examine the ability of our constructs to become biotinylated. To do so, we expressed BAP-NS1 or NS1-BAP in cells together with ER localized BirA **(Figure 3-2C)**. An ER targeting signal sequence was engineered at the N-terminus of BirA to direct expression of the ligase to the ER lumen. In this case, BirA has access to all proteins expressed in the ER but can only modify the target proteins that contain the BAP tag, after incubation with biotin.

To limit biotinylation to the experimental time course, cells were first pre-incubated in serum-free media for 2hrs to avoid biotin from other sources. A biotin pulse of 1hr resulted in almost complete biotinylation of our tagged clients. Biotin is rapidly and covalently attached to the BAP tag and biotinylation can be monitored by a western blot retardation assay. For this, lysates are incubated in the presence or absence of Streptavidin (StrAv). Streptavidin binds biotin with high affinity in a strong non-covalent bond, and the complex of StrAv-biotinylated protein is resistant to the SDS-PAGE denaturing conditions. Therefore, samples can be analyzed by SDS gel electrophoresis, where biotinylated proteins will separate from their non-modified counterparts due to a delay (retardation) in their migration caused by the increased molecular weight that StrAv adds to the protein [236, 239]. Biotinylation of the clients tagged at either end was readily detected and was specific to the presence of StrAv. Our results showed that co-localization of substrate and BirA allowed substrate biotinylation, as detected by retardation of the LC migration after incubation with StrAv and blotting with κ LC-specific antisera.

**Establishing a retrotranslocon-targeted in-cell proximity biotinylation system by fusing BirA to FAM8A1**

In order to specifically monitor clients as they emerged into the cytosol, as opposed to the cytosolically expressed BirA that was being used in other studies [229, 236], we sought retrotranslocon proteins that could accommodate BirA at their cytosolic domains without altering their functions. FAM8A1 emerged as the strongest candidate to fuse BirA to. FAM8A1 is a component of the ERAD machinery identified by a systems-level strategy that integrated proteomics and genomics approaches, which found that it interacted with Hrd1 [139]. It was also reported that FAM8A1 has a 1:2 stoichiometric relationship with Hrd1 and is critical for oligomerization of the Hrd1 complex [240, 241]. Further characterization of this novel Hrd1 interactor revealed that FAM8A1 is an integral membrane protein with three membrane spanning domains **(Figure 3-3A)**. Importantly for our studies, its long N' terminal region is cytosolically oriented and lacks structure. Consequently, we fused BirA at the N' terminus of FAM8A1 **(Figure 3-3B)**.

For our experiments we used a FAM8A1 construct that had been previously tagged with an N' terminal S-tag, so that the protein could be detected by anti-S tag antibody. BirA was added right before the S-tag, thusly making the construct: N' BirA-S-FAM8A1 C'. For simplicity reasons, however, herein after we omit mentioning the S-tag. To ensure that BirA-FAM8A1 still maintained its original localization and association with Hrd1 and that it was not an unstable protein, we performed some additional tests. Firstly, we transiently expressed the original FAM8A1 protein or BirA-FAM8A1 in cells together with Hrd1 and assayed the expression of our constructs as well as their ability to associate **(Figure 3-3C)**. The two FAM8A1 constructs were immunoprecipitated with anti-S antisera and analyzed by western blotting. Both FAM8A1 and BirA-FAM8A1 were expressed at similar levels, and in both cases Hrd1 co-immunoprecipitated, demonstrating that tagging the protein with BirA didn't affect its localization nor its association with Hrd1. Cycloheximide chase experiments revealed that fusing BirA to FAM8A1 did not destabilize the fusion protein, and the new construct was turning over with similar kinetics as FAM8A1 alone. Together these data indicate that BirA-FAM8A1 can be transiently expressed in cells and localize at the retrotranslocon, maintaining its association with Hrd1.

**Retrotranslocon targeted BirA is fully functional and more efficient in biotinylating cytosolically exposed ERAD substrates**

After successfully localizing BirA to the retrotranslocon, we next wanted to compare the new biotinylation system with the more commonly used cytosolic BirA. However, the first critical step before proceeding was to optimize the incubation time with biotin, and the concentration of the corresponding BirA constructs that would be sufficient to reliably modify retrotranslocating and fully retrotranslocated ERAD clients.

To test the time required for biotin labeling we used the BAP-NS1 ERAD client and examined the efficiency of biotinylating retrotranslocated clients after different times

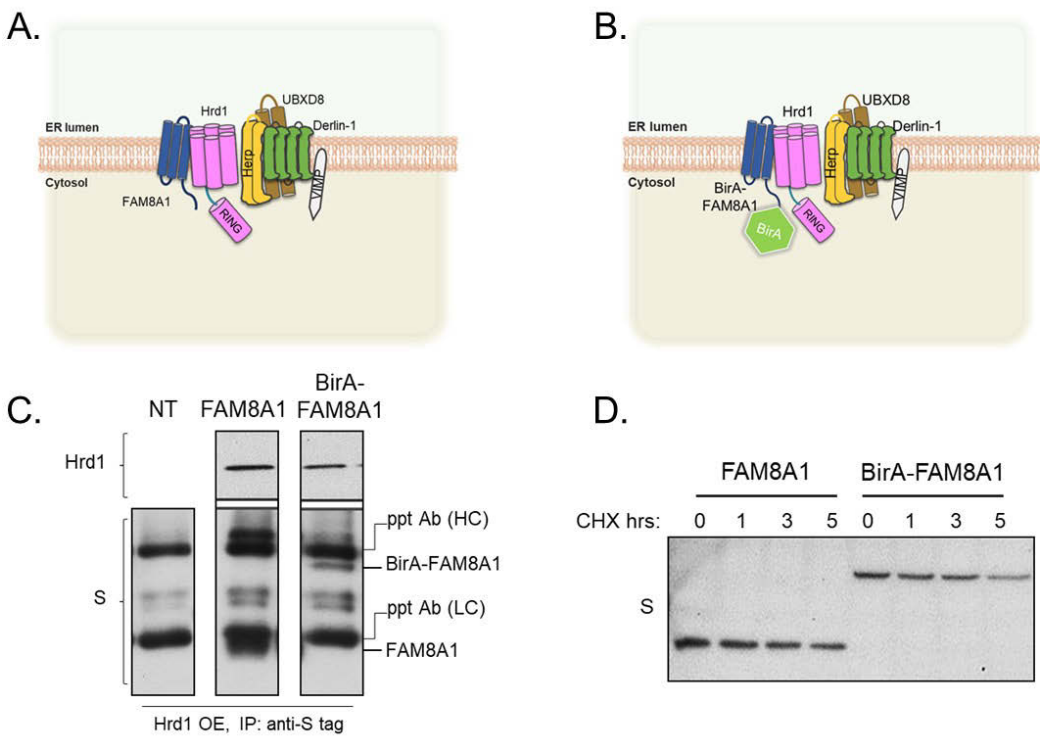


**Figure 3-3. Tagging BirA to FAM8A1 did not alter its expression, turnover rate, or its association with Hrd1**

(A) Simplified schematic of the Hrd1 retrotranslocon with its interactor proteins FAM8A1, Herp, Derlin-1, UBXD8, and VIMP. (B) Schematic showing the retrotranslocon-targeted BirA by fusing it to the cytosolic tail of FAM8A1. (C) The FAM8A1 construct had an N' terminal S-tag, which was maintained even after fusion of BirA to that terminus. Cells were transiently transfected with FAM8A1 or BirA-FAM8A1 together with Hrd1. Lysates were immunoprecipitated (IP) with anti-S antibody. Samples were subjected to SDS-PAGE and western blotting for Hrd1 and FAM8A1. The bands corresponding to Hrd1, FAM8A1, BirA-FAM8A1, and the precipitating antibody light chains (LC) and heavy chains (HC) are indicated. Non transfected (NT) cells were used as control. (D) Cells expressing FAM8A1 or BirA-FAM8A1 were treated with 100µg/ml CHX and were chased for the indicated time points. Lysates were analyzed by western blotting with anti-S antisera.

of incubation with biotin. Hence, BAP-NS1 was expressed in cells together with the cytosolically expressed BirA (cyt BirA) as described before [229]. In brief, ERAD substrates are retrotranslocated to the cytosol in order to access the ubiquitin-proteasome system for their degradation. When BirA is cytosolically expressed, it will be able to biotinylate ERAD clients that have been retrotranslocated. MG132 was additionally used to inhibit protein degradation and allow accumulation of the retrotranslocated and biotinylated proteins. Proteins that have not retrotranslocated during the time the proteasome is inhibited will not be biotinylated and that would indicate accumulation of clients in the ER lumen. Therefore, cells transiently transfected with both BAP-NS1 and cyt BirA, were treated with or without MG132 either for 3hrs or overnight **(Figure 3-4A)**. Biotin was added to the media either for the total amount of the MG132 treatment (overnight or for 3hrs), or only for 1hr at the end of the 3hr incubation with MG132. Next, samples were treated with or without StrAv, analyzed with a western blot retardation assay, and detected with anti-κ LC antibody. Results demonstrated detectable biotinylation in all cases after incubation with StrAv. This indicates that when the degradative capacity of the proteasome is inhibited, a portion of BAP-NS1 retrotranslocated. Overnight treatment with MG132 and biotin led to a large increase in the levels of both biotinylated and non-biotinylated BAP-NS1. Interestingly, incubation with biotin overnight also resulted in readily detectable biotinylation of κ light chains from control cells that had not been treated with proteasome inhibitor. Of note, overnight treatment of the cells with proteasome inhibitors led to cellular stress and unhealthy-looking cell culture; so, we refrained from performing such treatments. A three-hour proteasome inhibition also resulted in significant biotinylation without any modification occurring in non-MG132 treated samples. The amount of biotinylation was comparable whether biotin was present for the entire 3hr treatment or for one hour at the end of the treatment. Because the BAP-tagged NS1 had a half-life of ~1.5 hrs, we reasoned that a 3hr incubation with MG132 should provide a reasonable sized pool of retrotranslocated protein, and we chose to incubate the cells with biotin for one hour (1hr biotin pulse) at the end of any treatment before lysis.

To ensure that biotinylation did not affect the turnover of our clients, we compared the degradation rates of biotinylated and non-biotinylated BAP-NS1 in a CHX chase assay **(Figure 3-4B)**. Cells expressing BAP-NS1 and cyt BirA were treated with MG132 for 3hrs and with biotin for 1hr to allow biotinylated proteins to accumulate. After this time, the media was removed, cells were washed, and new media containing CHX was added. Lysates were collected at 0hrs, 2hrs and 4hrs after CHX addition, incubated with StrAv, and analyzed by western blot retardation assay. Data demonstrated that that biotinylation did not impede degradation, since both biotinylated and non-biotinylated NS1 turned-over. During the chase of biotinylated BAP-NS1, we observed a slight increase of the biotinylated species after 2hrs. This is an important finding, which reveals how non-biotinylated BAP-NS1 becomes biotinylated prior to degradation.

After having established the biotinylation assay, we wanted to compare our newly optimized retrotranslocon-oriented biotinylation system with the traditional cytosolic approach **(Figure 3-4C)**. In the same experiment, we verified the optimal BirA-FAM8A1 expression level needed to efficiently biotinylate retrotranslocated, BAP-tagged proteins.

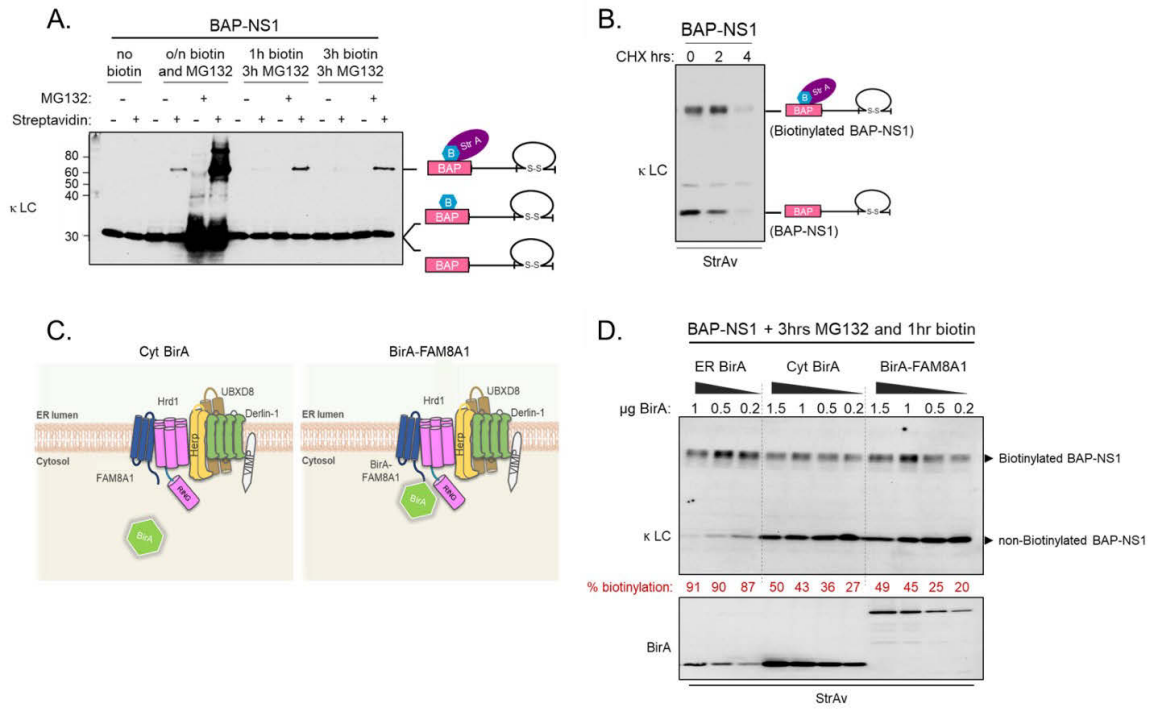


**Figure 3-4. Retrotranslocon-targeted BirA is fully functional and more efficient in biotinylating cytosolically exposed ERAD substrates**

(A) Cells were transfected with BAP-NS1 and cyt BirA and were treated with (+) or without (-) 10μM MG132 for the indicated times. Biotin was added to the culture media at a concentration of 0.25mM either for the total duration of MG132 treatment, or for only one hour at the end of the 3hr treatment. Lysates were collected and samples were briefly incubated in the presence (+) or absence (-) of StrAv. Samples were analyzed in a western blot retardation assay with anti-κ LC. The schematics show biotinylated and non-biotinylated BAP-NS1 bound or not to StrAv. (B) 293T cells expressing BAP-NS1 and cytosolic BirA were treated with MG132 for 3hrs and labeled for 1hr with biotin to allow for protein biotinylation. After this time, cells were washed and treated only with 100μg/ml CHX for the indicated times. Lysates were collected, incubated with StrAv and analyzed with a western blot retardation assay. (C) Schematics showing the two biotinylation systems for studying retrotranslocation. On the left panel the cytosolic BirA is shown, and on the right panel the retrotranslocon-tethered biotinylation system are presented. (D) Western blot retardation assay for a titration experiment to determine the concentration of BirA that leads to efficient biotinylation. Cells were transfected with BAP-NS1 and increasing amounts of DNA encoding the indicated BirA constructs. Samples were collected and analyzed after a 3hr MG132 (10μM) treatment and 1hr incubation with 0.25mM biotin. The amount of biotinylated BAP-NS1 is expressed as a percent of the total amount of LC detected (modified + unmodified). Samples were also blotted with anti-BirA antibody to test the protein levels of each BirA construct.

Cells were co-transfected with BAP-NS1 and either ER BirA, cyt BirA or BirA-FAM8A1 at various concentrations ranging from 1.5μg to 0.2μg of DNA. Cells were then subjected to proteasome inhibition with MG132 for 3hrs and were labeled with biotin for 1hr to allow protein biotinylation, which was calculated as % of modified protein compared with the total amount of LC. ER-localized BirA readily biotinylated the BAP-NS1 construct to ~90% at all concentrations. BirA-FAM8A1 was found to be more efficient in biotinylating BAP-NS1, even though it was expressed at lower levels compared to cytosolic BirA. Specifically, although only 0.2μg of cyt BirA were enough to reach protein levels comparable to 1.5μg of BirA-FAM8A1, it only managed to modify 27% of the total BAP-NS1. On the contrary, that amount of BirA-FAM8A1 resulted in biotinylation of almost 50% of the total LCs. This demonstrates that our retrotranslocon-targeted BirA system was more sensitive and achieved greater levels of biotinylation for an ERAD client, with lower protein levels. Finally, since transfecting 1.5μg of BirA-FAM8A1 DNA only marginally increased the amount of biotinylation observed compared to 1μg of DNA, we chose to proceed with this lower amount of DNA able to successfully modify the ERAD client.

**Substrates stabilized with proteasome inhibitors or by p97 and Hrd1 mutants are readily biotinylated at both termini, but are not fully extracted from the ER**

Having an optimized retrotranslocon-tethered biotinylation system in hand, we proceeded to examine how inhibition of p97 or Hrd1 would influence retrotranslocation of our client by measuring the biotinylation of both BAP-NS1 and NS1-BAP. We have shown that both BAP-tagged NS1 proteins were stabilized when the proteasome, p97 or Hrd1 were inhibited **(Figure 3-2A** and **B)**. We saw that NS1 was biotinylated on its N' terminus when the proteasome was inactive. Next, we wanted to understand if proteasomal inhibition permits C' terminal modification, possibly allowing complete retrotranslocation of this client from the ER without the requirement for an active proteasome. Furthermore, we investigated whether a client can move across the ER membrane in the absence of ubiquitination or p97.

Western blot retardation assays were performed with samples from cells co-transfected with BirA-FAM8A1 and either BAP-NS1 or NS1-BAP and either treated with MG132 for 3hrs or left untreated. Both constructs were also co-transfected with mutant p97 (p97QQ) or a Hrd1 construct deficient in Ub ligase activity (Hrd1 C291S) to assess the effect of impaired p97 and ubiquitination on ERAD client movement across the ER membrane during retrotranslocation **(Figure 3-5A** and **C)**. Surprisingly, a significant population of both termini was biotinylated under control conditions, indicating that a great number of LCs had reached the cytosol and was on the path to degradation. This also implies that there is a delay between biotinylation and degradation. Proteasome inhibition for 3hrs only modestly increased the biotinylation observed in control cells (up to 40% for BAP-NS1 and ~50% for NS1-BAP). Unexpectedly, both BAP-tagged NS1 constructs were biotinylated even when p97QQ or Hrd1 C291S were co-expressed. In fact, biotinylation in the presence of p97QQ or Hrd1 C291S was slightly greater than modification after MG132 treatment **(**graphs on **Figure 3-5A** and **C)**.

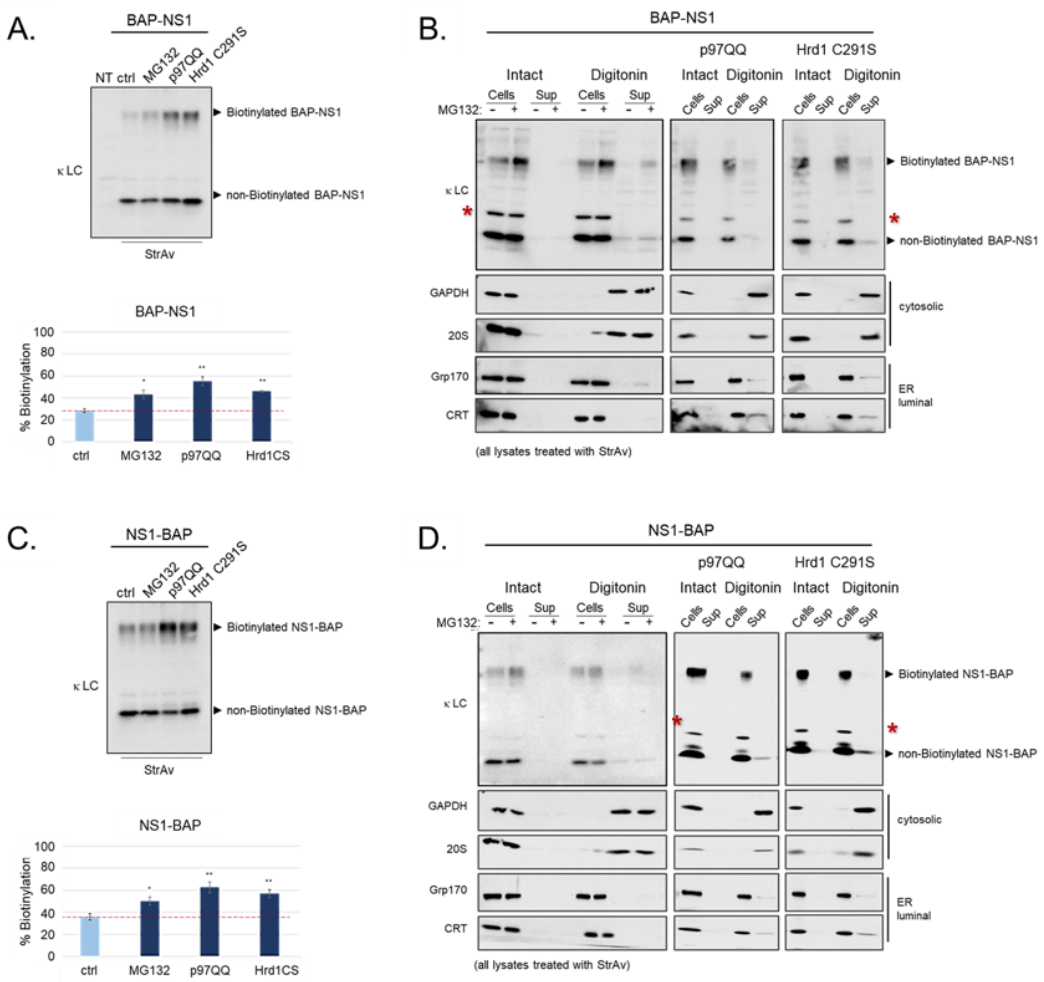


**Figure 3-5. Substrates stabilized with proteasome inhibitors or by p97 and Hrd1 mutants are readily biotinylated at both termini when cytosolic ERAD components are impaired, but are not fully extracted from the ER**

(A-C) Cells expressing BAP-NS1 (A) or NS1-BAP (C) were treated without (ctrl) or with 10μM MG132 for 3hrs or were co-transfected with the indicated mutants (p97QQ or Hrd1 C291S). Biotin was added in the cell culture for 1hr before lysis. Cell lysates were supplemented with StrA to allow visualization of biotinylation after western blotting (NT=non-transfected cell lysates). The graphs show the % of biotinylation in each case, calculated as before **(Figure 3-4D)**. Data represent the mean ± S.E.M derived from 4 separate experiments. (B-D) 293T cells were transfected with vectors encoding either BAP-NS1 (B) or NS1-BAP (D) and BirA-FAM8A1 and were either treated with 10μM MG132 (+) or DMSO (-) control for 3hrs, or co-transfected with p97QQ or Hrd1 C291S as indicated. Biotin was added for 1hr before lysis and the cells were kept intact or were treated with digitonin for 5mins. In each case, after centrifugation, the cell pellet (Cells) and the supernatant (Sup: cytosol in digitonin treated cells) were collected for analysis. After adding StrAv, lysates and supernatants were subjected to western blot retardation assay with anti-κ LC antibody. GAPDH and the 20S proteasome subunit were used to monitor release of cytosolic proteins, and Grp170 and Calreticulin (CRT) to detect fractionation of luminal ER proteins. *The red asterisks indicate a non-specific background band.

This was an intriguing result since retrotranslocation and the subsequent biotinylation of proteins in the absence of p97 function or ubiquitination was not anticipated. Biotinylation of both N' and C' terminal BAP can occur from either having the entire protein retrotranslocated in the cytosol, or from cells attempting to initiate retrotranslocation from both termini. NS1 can fully retrotranslocate when the proteasome is inhibited and the actions of all upstream proteasomal components remain functional. This agrees with the data generated using NHK (another ERAD substrate) using the split-GFP system [219]. Such movement is difficult to envision when the functions of p97 and Hrd1 are impaired. With the possible exception of the cholera toxin A1 subunit [206, 207], all proteins require the activity of p97 for their extraction from the ER. Hrd1 was found to be essential for substrate dislocation outside of the ER [237] and reconstituted proteoliposomes [199].

To better understand our unexpected biotinylation results, we aimed to determine where stabilized, biotinylated substrates (BAP-NS1 and NS1-BAP) accumulate. Consequently, we performed semi-permeabilization experiments on cells to separate cytosolic proteins from ER proteins. We transiently expressed BAP-NS1 or NS1-BAP in 293T cells along with BirA-FAM8A1, treated the cells with MG132 or co-expressed with either p97 or Hrd1 mutants, and subjected them to digitonin extraction assays after 1hr incubation with biotin **(Figure 3-5B and D)**. Low amounts of digitonin disrupt the plasma membrane making it semi-permeable, while leaving the ER membrane intact [216]. Centrifugation of digitonin-treated cells allows cytosolic proteins to escape, including fully retrotranslocated ERAD clients, while ER proteins remain cell associated. Samples from cell pellets and supernatants were collected and mixed with StrAv before western blotting. Cytosolic proteins GAPDH and the 20S proteasome subunit were completely released by digitonin treatment, whereas ER resident proteins Grp170 and calreticulin remained cell-associated, demonstrating the integrity of the ER remained intact.

In all cases, MG132, p97QQ, and Hrd1C291S resulted in a significant amount of biotinylation. Strikingly, both biotinylated and non-biotinylated NS1 species remained cell associated. Non-biotinylated NS1 was expected to be in the ER lumen and inaccessible to BirA-FAM8A1. However, biotinylated NS1, which would account for retrotranslocated proteins, was also found to be cell associated and didn't follow the pattern of cytosolic proteins. These data raise two possibilities: 1) NS1 is able to fully exit from the ER and become biotinylated (on both termini) independent of ubiquitination, p97 or the proteasome, but without being released into the cytosol or 2) NS1 can sample the cytosol and either remain in a partially retrotranslocated state or slide back into the ER lumen since downstream ERAD components responsible for completion of client extraction are inactive.

**Limited digestion of cytosolically exposed BAP-NS1 by proteinase K**

To follow up on our unforeseen results, we wished to determine whether NS1 is cytosolically exposed in its entirety, or if it is in fact localized in the ER lumen but can

partially slip through to the cytosol and return to the ER. In order to assess how much NS1 protein was exposed to the cytosol when the activities of the proteasome or p97 were compromised, we performed protease sensitivity assays **(Figure 3-6)**. Cells expressing BAP-NS1 and BirA-FAM8A1 were either treated with MG132 to inhibit the proteasome function or were additionally co-transfected with p97QQ. A 1hr biotin pulse preceded digitonin incubation to permeabilize the plasma membrane. Intact cells and non-transfected (NT) cells were included as controls. Next, 100ug/ml of proteinase K was added to the cells to allow digestion of cytosolically exposed proteins, and PMSF was added to stop the protease reaction Samples were lysed, denatured, mixed with StrAv, and analyzed by western blot retardation. To ensure our results were not masked by post-lysis protease digestion, we evaluated the states of two ER luminal proteins (Grp170 and ERdj3) which should not be accessed by proteinase K due to their location, and an ER membrane protein (calnexin, CNX) whose C' terminal tail is oriented cytosolically and thus is sensitive to protease digestion. Proteinase K cleaved the cytosolic domain of CNX causing a slight shift in the migration pattern due to the now smaller size of the protein. When cells were directly solubilized **(Figure 3-6** first panel**)**, all controls were completely digested by proteinase K. In lysates where NS1 is fully exposed to the proteinase there is an almost complete digestion of protein with only the $C_L$ domain being resistant. In all cases, no NS1 was digested by proteinase K when cells were kept intact, and the protein was inaccessible by proteinase K. However, in digitonin treated cells a 20% reduction in biotinylation was observed, reflecting some loss of mainly biotinylated BAP-NS1. Interestingly, inhibition of either p97 or the proteasome had the same effect on the loss of biotinylation. In both of our conditions of ERAD inhibition we observed that a significant number of biotinylated BAP-NS1 was protected from digestion by the protease, suggesting that either BAP-NS1 is localized in the ER lumen, or it is found in larger protein complexes in the cytosol, or both. Interestingly enough, when biotinylation was lost, it did not produce a smaller fragment corresponding to a CL domain alone, which indicates that the proteinase K has access only to a limited region of BAP-NS1, possibly at the N' terminus where the BAP tag is located.

## Discussion

Endoplasmic reticulum (ER)–associated degradation (ERAD) is the pathway responsible for disposal of misfolded and unfolded proteins from the ER by the ubiquitin-proteasome system. Retrotranslocation of ERAD substrates to the cytosol is a critical step in the pathway and it features the collective actions of several ERAD components. Despite having identified many ERAD components and having established a good understanding of their functions in this process, the mechanistic details of client movement across the ER membrane for retrotranslocation is lacking. Currently two reporter-based assays are available to study retrotranslocation and the intermediate steps in its process: the site-specific biotinylation reporter assay and the split-GFP system. Here we established an optimized retrotranslocon-tethered in-cell biotinylation system to study how ubiquitination, p97, and the proteasome contribute to ERAD client retrotranslocation and to determine where clients localize when these cytosolic ERAD components are compromised.

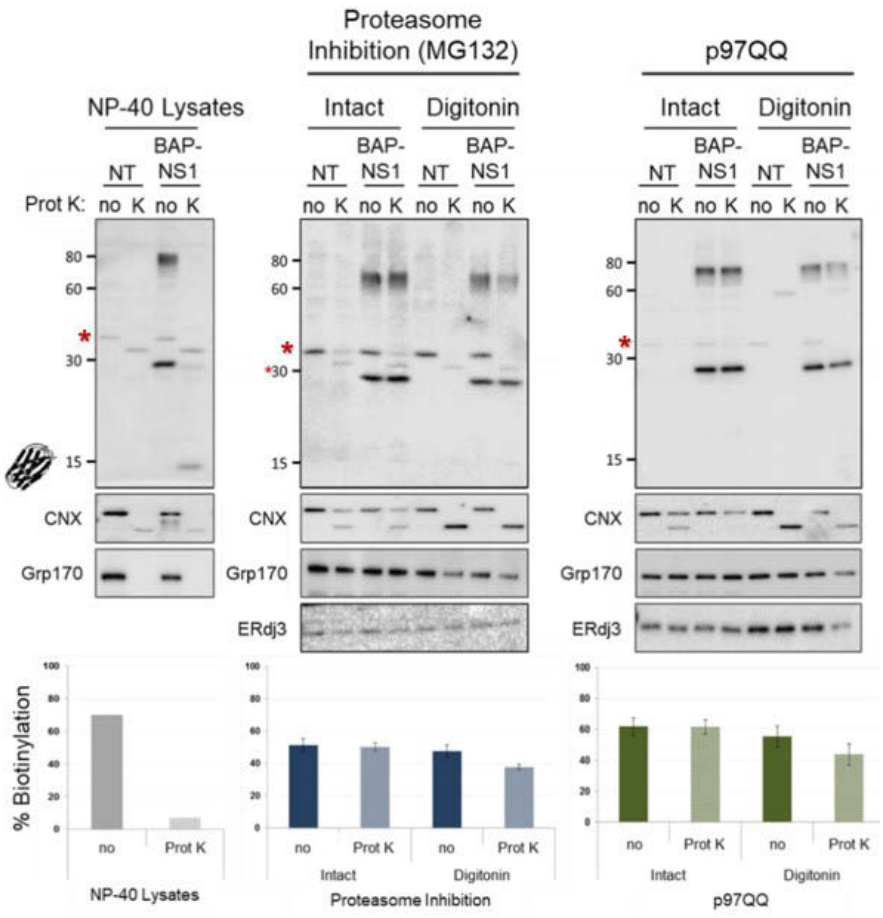


**Figure 3-6. Differential digestion of cytosolically exposed BAP-NS1 by proteinase K**

Biotinylation was performed as described before in BAP-NS1 transfected cells under all our previous conditions of ERAD inhibition (MG132 proteasomal inhibition or co-expression with p97 dominant negative mutant) and three types of samples were collected: lysates, intact cells, and digitonin treated semi-permeable cells. These samples were incubated with 100µg/ml proteinase K (K) or without (no) as control. Following proteinase incubation, PMSF (5mM) was added for 5mins to quench the reaction and cells were lysed with NP-40. Samples were mixed with 2x sample buffer and StrAv was added to all samples before western-blotting, allowing visualization of the biotinylated BAP-NS1 species. The transmembrane protein calnexin (CNX) and two ER luminal chaperones Grp170 and ERdj3 were used as controls to evaluate the efficiency of the proteinase K digestion. In all cases, corresponding non-transfected (NT) controls were included. The graphs represent the amount of biotinylated BAP-NS1 in each case (expressed as a percent of the total amount of LC detected (modified + unmodified)). Data represent the mean ± StDev derived from 2 different experiments. *Red asterisks indicate non-specific background bands.

We tethered the biotin ligase BirA to the cytosolic tail of FAM8A1, a novel Hrd1 interactor and a component of the retrotranslocon channel, without compromising its localization and association with Hrd1. This way, BAP-tagged ERAD clients become biotinylated as soon as they emerge from the retrotranslocon without the need for them to have traveled further into the cytosol. To start using this system and collect initial data, we tested one ERAD substrate which was BAP-tagged at either the N' or C' terminus. The 15 amino acid BAP tag is a small sequence [238]. We showed that it did not alter the tagged protein's properties or dependency on the same ERAD components for degradation. Our retrotranslocon-targeted BirA was more sensitive in biotinylating an ERAD client compared to the traditional system with "free" cytosolic BirA, as shown by the smaller amounts of BirA-FAM8A1 needed to achieve comparable biotinylation with cyt BirA. Even though we lack a negative control in our assay, we produced valuable data. Due to our unexpected biotinylation results we were initially puzzled and decided to put a halt to this system. In light of our further studies with NS1 and other proteins with folded domains and with unfolded proteins **(Chapter 2)** we are now able to better interpret the biotinylation data and draw compelling conclusions.

Our first unexpected result was biotinylation observed under control conditions with both Bap-NS1 and NS1-BAP. When ERAD is not inhibited in any way (control cells), biotinyated NS1 degrades normally. Therefore, the percent of biotinylation measured in control cells represents NS1 molecules that moved to the cytosol and were not degraded rapidly in one continuous action. This assumed lag between NS1 biotinylation and its degradation presents a curious finding as it counters our de-glycosylated data with the NS1 protein, in which we find in control, untreated samples, no significant de-glycosylation **(Chapter 2, Figure 2-4A** and **2B)**. Proteasome inhibition lead to biotinylation of both BAP-tagged NS1 proteins. This result is in agreement with previous data on NS1-BAP with cyt BirA [229]. Biotinylation of both N' and C' terminal BAP tags is possible if the protein has fully retrotranslocated to the cytosol and thus both ends can be modified, or if each terminus sampled the cytosol on the way to crossing the ER membrane even if extraction was not complete. In Chapter 2 we described how a folded domain does not impair retrotranslcoation and that NS1 can be fully extracted to the cytosol when the proteasome is inhibited. In this way, biotinylation of both BAP-tagged NS1 constructs can be understood.

Expression of p97QQ and Hrd1C291S impairs p97-mediated protein extraction and ubiquitination, which is essential for downstream interactions (eg: binding to p97). Therefore, we were surprised to find increased biotinylation of both BAP-NS1 and NS1-BAP when the p97 and Hrd1 mutants were co-expressed. We showed here that our two substrates, biotinylated and not, remained cell-associated in digitonin permeabilization experiments, and we saw previously that under both of these conditions there was no de-glycosylation. De-glycosylation is an enzymatic reaction that occurs in the cytosol by PNGase [174] after the protein has been significantly extrcated by p97. Since, with impaired Hrd1 or p97 there was no de-glycosylation observed when glycosylated NS1 was used **(Chapter 2, Figure 2-4)** but there was significant biotinylation, this indicates that biotinylation happens first. This is a fascinating result, which allows the interpretation of the observed increased biotinyation for both BAP NS1 constructs. The

NS1 κ light chain possesses an unfolded $V_L$ domain and a well-folded $C_L$ domain. It was previously shown that all ubiquitination occurs on the unfolded domain [149]. The fact that the N' terminal $V_L$ domain of NS1 is both unfolded and the recipient of ubiquitination, allows us to hypothesize that this domain may be inserted first in the retrotranslocon channel and reaches the cytosolic side first, independent of cytosolic ERAD functions. Therefore, biotinylation of BAP-NS1 can be explained in all cases **(Figure 3-7A, B** and **C)**. When the p97QQ is co-expressed, the protein can still become at least partially retrotranslocated, ubiquitinated and engage p97, but in absence of ATP-hydrolysis by p97, clients cannot complete extraction or be released in the cytosol. Even when the mutant Hrd1 is co-expressed, the proteins can reach the cytosolic side in attempt to engage the ubiquitin-proteasome system, ergo they can become biotinylated, but lack of ubiquitination prevents movement past that point. These findings agree with our de-glycosylation data, where glycosylated NS1 was not able to become de-glycosylated when p97QQ or Hrd1C291S were co-expressed **(Chapter 2, Figure 2-4A and 2B)**. Biotinylation of NS1-BAP was altogether unexpected, especially when the p97 or Hrd1 mutants were used. Considering that the protein would retrotranslocate from its N' terminus, that would require the entire protein to have reached the cytosol. Although that is the case when the proteasome is inhibited, it is difficult to favor that possibility in the absence of either ubiquitination or of p97's actions, especially also when taking into consideration the fact that we did not observe de-glycosylation of glycosylated NS1 with these conditions. However, a compelling alternative is that the short, unfolded BAP tag at the C' terminus of NS1 now provides an alternative way for dislocating this protein, which is additionally facilitated by the single lysine on the BAP tag that may be recognized by the cell as a potential ubiquitination site. In this case, the cell attempts to retrotranslocate NS1 from the C' terminal BAP tag but due to its small size that is unattainable and consequently this fragment only samples the cytosol **(Figure 3-7D)**, but ubiquitination and/or engagement of cytosolic ERAD factors is not feasible due to the length of this tag, so the protein slips into the ER lumen to attempt retrotranslocation from its N' terminus. This switch possibly requires time during which more molecules will try retrotranslocation and re-enter the ER lumen and that could also justify the increased biotinylation observed in all cases with NS1-BAP compared with BAP-NS1. These data are very intriguing since not only do they suggest sampling, but also, they have implications on the directionality for retrotranslocation, for which a long, unfolded region of a protein (no matter which termini it is located) is necessary to be able to be inserted in the channel, become ubiquitinated and engage p97 to complete extraction.

Finally, our results with proteinase K digestion provided us with evidence that support our models of sampling and retrotranslocation from the unfolded domain. We performed proteinase K sensitivity assays on BAP-NS1 in digitonin treated cells, and we showed that part of the $V_L$ domain had reached the cytosolic side of the ER in all cases tested of impaired ERAD, since the biotinylated species was susceptible to proteinase digestion. These data suggest that even in absence of proteasome or p97 function, an ERAD client can still be exposed in the cytosol (and so it is accessible by the proteinase), further supporting the concept of clients first sampling the cytosolic side when luminal ERAD components are functional. Interestingly, the loss of biotinylation matched the

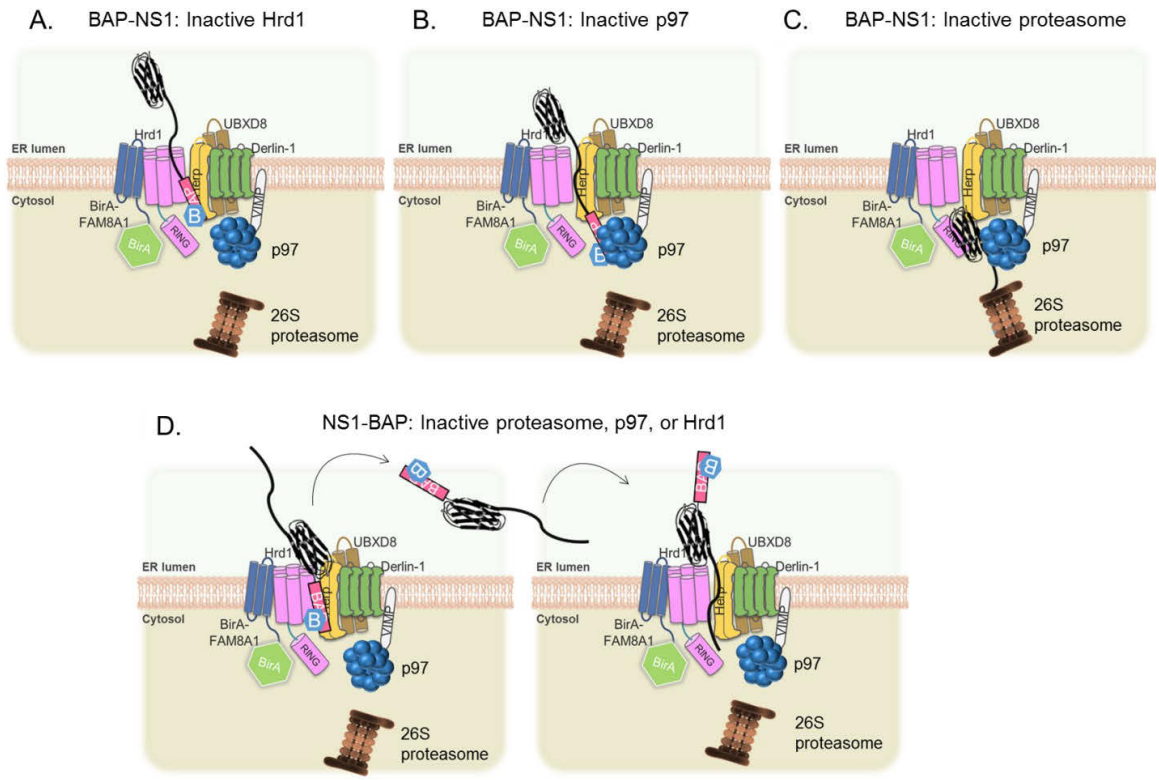


**Figure 3-7. Retrotranslocation models**

Schematics showing possible modes of retrotranslocation of BAP-NS1 (A, B and C) and NS1-BAP (D) as identified by our experiments with ubiquitination, p97 or proteasomal inhibition.

amount of de-glycosylation observed when the glycan was placed near the N' terminus of NS1, which in both cases was approximately 20%.Considering this, the region of the protein which samples the cytosol must not be significantly bigger than the 15-20 amino acids because proteinase digestion did not result in a lower band corresponding to a $C_L$ domain alone or several smaller fragments. Yet, some of the biotinylated protein was protected from proteinase digestion like the non-biotinylated ER localized species was. This hints that part of the biotinylated population may be accumulating in the ER when it is unable to retrotranslocate but after having reached the cytosol first (as the case with p97QQ would be). At the same time, partially retrotranslocated and fully retrotranslocated proteins can also be found in complexes and thus they will be protected from proteinase digestion. ERAD substrates are thought to form dynamic protein complexes at the cytosolic side of the ER membrane that can become stack when blocked [139, 162, 242], and so it is highly possible that BAP-NS1 in our case was also in complex with cytosolic ERAD factors including p97 and its co-factors, with or without the proteasome. As such, inhibition of the proteasome or p97 may have kept the client protected from proteinase K by remaining associated with it **(Figure 3-7B and C)**. In our study, examination of NS1-BAP with the proteinase K experiments is missing. That would add valuable information as to whether this protein when biotinylated would be sensitive at all to the proteinase. Having such data, we would be able to conclude more definitely about client sampling and accumulation in the ER when cytosolic ERAD components are inhibited.

In conclusion, our biotinylation data in combination with our previous knowledge on NS1 retrotranslocation **(Chapter 2)** allow us to make some very compelling conclusions on protein retrotranslocation and identify some intermediate steps in the process. Firstly, proteins attempt to cross the ER membrane when an unstructured region is available, and they will sample the cytosol. If the region is long enough and can accommodate ubiquitination, the protein will exit when cytosolic ERAD components are functional. When unfolded regions are not long enough, then according to our data the protein will re-enter the ER lumen and make a new attempt to be extracted in other ways. Finally, when cytosolic ERAD functions are impaired, clients either remain associated with cytosolic components or may slip back in the ER lumen, depending on which cytosolic interactions did or did not happen.

# CHAPTER 4. DISCUSSION

## Significance of ERAD and Importance of Understanding Retrotranslocation

The ER is the biosynthetic hub for secreted proteins, membrane proteins, and secretory pathway proteins. It offers a unique environment and houses chaperones and folding enzymes that promote folding and maturation of newly synthesized polypeptides. In many cases proteins are also assembled into multimeric complexes, which often completes folding of the individual polypeptide chains. ER-quality control systems (ERQC) monitor protein folding fidelity and only allow properly folded proteins to be expressed. Despite all of these resources devoted to protein folding, newly synthesized polypeptides can fail to acquire their native conformation. The extent of misfolding can range from only a sub-domain of a protein failing to fold to completely unfolded proteins. Terminally misfolded and unfolded proteins are retained in the ER and eventually disposed. The ER-associated degradation (ERAD) is a conserved pathway from yeast to mammals, dedicated to eliminating damaged and unassembled proteins from the ER [243]. ERAD also has an essential role in regulating levels of various ER proteins in response to specific signals. In this way, ERAD is critical for lipid homeostasis and metabolism [114]. Furthermore, it has been well-established that many viruses and toxins exploit the ERAD pathway to move through the secretory pathway in a retrograde manner and infect the host cell [119, 244]. The importance of ERAD is highlighted by the fact that genetic ablation of ERAD factors leads to embryonic lethality. Defects in this pathway can result in a toxic buildup of misfolded proteins and is associated with many human conditions such as aging, metabolic diseases, neurodegenerative diseases, and diabetes [245, 246]. Understanding the mechanistic aspects of ERAD is essential to explain the molecular signature of ERAD-associated diseases and to carve the path for novel therapeutic approaches.

Substrates for ERAD are proteins that are at least partially aberrantly folded. Three different ERAD branches exist which accommodate the topology of the misfolded region of their substrate proteins. Luminal ER proteins with misfolded parts follow ERAD-L, while integral membrane ER proteins with luminal, membrane-spanning, or cytosolic misfolded segments and will follow the ERAD-L, ERAD-M or ERAD-C correspondingly. Once recognized for degradation, ERAD clients must be transported to the cytosol in a process termed retrotranslocation or dislocation, where they encounter the ubiquitin-proteasome (UPS) system. Retrotranslocating an ERAD client requires a protein conducting channel, energy, and ubiquitination. All ERAD pathways converge in the cytosolic side, requiring ubiquitination (by various ERAD-associated ubiquitin ligases), mechanical force to perform client extraction from the ER membrane provided by the p97 AAA-ATPase, and active proteasome to effectively degrade ERAD clients. Research that spans decades, has led to identification and characterization of many ERAD components. However, critical aspects of ERAD remain obscure. Amongst the most important unanswered questions is how the ER accommodates movement of a misfolded client across the ER membrane. This question is of particular relevance for ERAD-L substrates, which have to entirely move to a different cell compartment. ER

luminal ERAD substrates must be directed to the ER membrane, inserted into the retrotranslocon channel, moved across the membrane, and be extracted to the cytosolic side. To complicate things further, many ERAD substrates are structurally diverse and contain one or more folded domains in addition to the misfolded region(s). In this project, we studied how folded domains on ERAD clients are processed for retrotranslocation and how the main cytosolic ERAD functions (ubiquitination, p97-mediated client extraction from the ER membrane, and proteasomal degradation) synchronize to eject ERAD clients to the cytosol for degradation, and we produced key mechanistic insights on the retrotranslocation of such domains.

## The Enigmatic Retrotranslocon

ERQC systems are in place to recognize the progression of folding for both glycosylated and non-glycosylated proteins. As such, ERQC identifies proteins unable to acquire their native conformation and targets them for degradation. At the epicenter of ERQC lie the lectin and the heat shock chaperones, which monitor glycosylated and non-glycosylated proteins correspondingly. These systems deliver ERAD clients to the luminal side of the ER membrane and prepare them for retrotranslocation. On the ER membrane, a putative channel is in place to accommodate dislocation of proteins to the cytosol. No clear consensus exists on the identity of such a channel or even on whether one channel can serve all ERAD clients. Several multi-spanning membrane proteins with verified contributions to the ERAD of different clients have been proposed as candidates for a retrotranslocon channel. These include the Sec61 component of the translocon through which newly synthesizing polypeptides enter the ER [99, 247], the Derlin proteins [130, 205], and ER-ubiquitin ligases themselves [132, 135]. Recently data from in vitro reconstituted proteoliposomes with purified components from yeast made a strong case that the Hrd1 E3 ubiquitin ligase is instrumental for client extraction in yeast, arguing it could form the channel or that it is an essential component of the retrotranslocon channel [135, 199]. These data were supported by a Cryo-EM structure of Hrd1, which revealed that this protein dimerizes and when in association with its partner Sel1L (another integral membrane protein), it adopts a funnel-like shape on the ER membrane [136]. This Hrd1 funnel "opens" to facilitate retrotranslocation following auto-ubiquitination of Hrd1 [135].

## How Is Retrotranslocation Initiated?

In this study, we used a variety of structurally different ERAD clients whose folded state has been described by biophysical experiments and with well-characterized requirements for Hrd1 and p97 for their degradation. The NS1 κ light chain is one of them. NS1 possesses an N' terminal unfolded variable ($V_L$) domain, which is responsible for targeting this protein for ERAD, and a well-folded C' terminal ($C_L$) domain. We tagged this protein with a 15 amino acid biotin acceptor peptide (BAP) either at the N' or the C' terminus and by using a retrotranslocon-tethered BirA system placed on the cytosolic side of the ER membrane. We monitored biotinylation of our client, as an

indication of cytosolic exposure when Hrd1, p97 or the proteasome were inhibited **(Chapter 3)**. We found that under all conditions both BAP-NS1 and NS1-BAP were readily biotinylated due to the BAP-tag coming in proximity with BirA on the cytosolic side, allowing its biotinylation. This finding was compatible with our digitonin permeabilization, proteinase K sensitivity, and de-glycosylation data for this client **(Chapters 2 and 3)**, supporting a model where NS1 can fully retrotranslocate from the ER membrane in absence of proteasomal function **(Chapter 3)**. In further support of our data, photo-crosslinking experiments with another well-characterized ERAD-L substrate, the unfolded sCPY* fused on its C' terminus with the well-folded DHFR, showed that Hrd1 interacted with multiple residues on the unfolded domain of this client up to the DHFR domain [132]. This suggests that the unfolded part of the fusion protein had been able to cross the membrane sampling the cytosol. The fact that a client can sample the cytosol, even in absence of ubiquitination (when the Hrd1 dominant negative mutant was co-expressed), the first event to occur on the cytosolic side, provides us with some interesting insights on retrotranslocation. Firstly, during retrotranslocation an unfolded region is necessary for the insertion of a protein into the retrotranslocon, and secondly, energy is required to begin the forward movement towards the cytosolic side.

A small unfolded segment of even a few amino acids (~15aa) may be enough for the cell to attempt retrotranslocation. That explains the observed biotinylation of BAP-tagged NS1 and especially of NS1-BAP **(Chapter 3)**. At this stage, after insertion in the channel, a client may be able to slide back and forth in the channel with energy input from ER luminal ERAD factors, probably until cytosolic factors also act to promote substrate movement to the cytosol. These data argue that insertion of an ERAD client into the retrotranslocon and initial movement across the ER membrane depends on an unfolded region and energy from the ER luminal factors. Who may be providing this energy to initiate retrotranslocation? One source of force for moving a client across the ER membrane could be the structural changes that occur on Hrd1 itself (and possibly its co-factors too that are organized around Hrd1 and altogether may form a channel – **Figure 1-3**). A second, and more direct, source of energy possibly comes from ER luminal chaperones. The Hsp families of chaperones (Hsp70s, Hsp40s, Hsp90s) with BiP as a main player, are bound to unfolded regions on proteins. Since unfolded parts on proteins usually keep hydrophobic regions exposed, chaperone binding prevents the aggregation and maintains solubility of such polypeptides. BiP possibly remains bound to unfolded regions until they are delivered to the retrotranslocon. At this point, a nucleotide exchange factor (either Grp170 or Sil1) will drive BiP back to its ATP-bound state, thus causing the release of the misfolded substrate into the retrotranslocon. Consecutive rounds of substrate binding and release by BiP in this region of the ER may be responsible for the first steps of retrotranslocation. In support of this argument, in the study of Sasset et al., ATPase deficient BiP mutants stabilized NS1 in the ER lumen without any biotinylation from cytosolic BirA [229]. Additionally, biotinylation was reported with the ubiquitination deficient BAP-tagged NS1-$V_L$STK$^{-}$BAP [229], which even though it cannot engage cytosolic factors since it cannot be ubiquitinated, it was able to be "pushed" into the channel by BiP that was attempting to extract this client. Overall, BiP's actions seem to help guarantee that misfolded proteins will be inserted to

the retrotranslocon once the client has committed to degradation and allow it to reach the ubiquitin-proteasome system and be eliminated from the ER.

## Is Substrate Unfolding Necessary for Retrotranslocation?

An unfolded segment on an ERAD substrate is essential to insert the polypeptide into the retrotranslocon and initiate movement across the ER membrane. However, ERAD has to be able to mediate the processing of whole proteins that in addition to the misfolded portion may also possess folded domains, which usually contain disulfide bonds, have assembled with other polypeptides, or are even intact molecules, as is the case with viral particles. This raises many intriguing questions such as whether there is a need for unfolding and reduction of ERAD substrates to facilitate their crossing the ER membrane for degradation, how do proteins with multiple domains pass through the retrotranslocon, and how structurally complex clients can be accommodated?

A few studies have addressed this topic. Efforts from the Sitia lab concentrated on understanding the requirements for degradation of Ig μ heavy chain multimers [227] and μ heavy chain-TCRα chimeras [228]. Both these proteins possess multiple folded domains and are covalently assembled with subunits making them structurally complex ERAD clients. In both cases, interchain disulfides were shown to be reduced prior to retrotranslocation. Proteasome inhibition also resulted in reduction of the inter- and intra-molecular disulfide bonds of the J chain glycoprotein that is covalently bound to the μ chain multimers, and in deglycosylation of this subunit, indicating that it was dislocated to the cytosol. However, the freed μ heavy chain multimers showed no evidence of deglycosylation, arguing that this dimeric ERAD client with oxidized domains at both its N- and C-termini was not retrotranslocated in the absence of proteasomal function (Mancini et al., 2000). In another study, the membrane MHC-I was fused with EGFP on its luminal side, and after proteasome inhibition this protein was found to be dislocated to the cytosol after proteasomal inhibition and accumulated as a de-glycosylated intermediate without any fluorescence loss. This suggested that either the EGFP protein crossed the ER membrane intact or that it was able to refold rapidly once in the cytosol [232]. Another fusion protein, the DHFR-MHC-I was also found to be free in the cytosol, in a de-glycosylated form, when proteasomal degradation was blocked; even when the DHFR domain was bound to the structure-stabilizing molecule methotrexate. This observation indicated that a very stably folded domain can possibly be accommodated by the retrotranslocon [233]. However, one should take into consideration that MHC-I heavy chains are transmembrane proteins that degraded in response to viral signals. Viruses express the proteins US2 and US11, which target MHC-I heavy chains for degradation in order to prevent an immune response from the host cells and promote infection. US2/US11 are localized in the ER membrane at the retrotranslocon and stimulate ubiquitination of MHC heavy chains, which leads to their degradation. Therefore, extraction of MHC-I may be mechanistically very different than retrotranslocation of ERAD-L substrates in that (a) it relies not only on the traditional retrotranslocon components but also on viral proteins and perhaps on Sec61, and (b) since it is an integral membrane protein, there is a possibility that it is easier to extract.

In the previous studies, the proteins that were retrotranslocated and found in the cytosol were either simple one domain or unfolded proteins, or heavy chains of the MHC-I complex that were degrading via the actions of viral proteins, probably in mechanistically different ways. In our study, we used proteins consisting of Ig domains (NS1, mHC and A6-TCRα), that possessed one unfolded and one folded (Ig fold) domain, and we demonstrated that all our clients were able to retrotranslocate with their folded domain maintaining structure **(Chapter 2)** but reduced. So, is protein unfolding necessary for retrotranslocation? Our data together with the previously published data, argue for a retrotranslocon channel that is able to accommodate certain folded domains, especially when they have unfolded regions that can be engaged by the ERAD factors. The narrowest cross-section of DHFR is 40Å [233], while an Ig domain measures 40Åx25Åx25Å, so at least domains of that size can be accommodated through the channel. Another alternative may be that the channel can adapt its size to the different substrates. For instance, one could envision a channel composed of a Hrd1-dimer/oligomer to be able to dilate according to the protein that has to be processed. Further research will shed more light to whether unfolding is really necessary for retrotranslocation and whether the channel has fixed dimensions or if it adaptable to its clients. Another interesting topic is how are more complex proteins with multiple folded domains being passed through the channel for extraction to the cytosol. An example of that is the Ig γ heavy chain, which possesses four domains, three of which are able to fold but one remains unfolded until the heavy chain can assemble with the light chain. In absence of light chains, γ heavy chain becomes an ERAD substrate. The unfolded domain in this case resides in the middle of the protein, and that possibly leads to the need of additional requirements for effective retrotranslocation of this client. Does this protein need to unfold more domains in order to have an unfolded terminal region to insert into the channel? Can retrotranslocation begin with an unfolded segment in the middle of a protein being inserted as a loop into the channel and then with the action of cytosolic factors the client can then be "pulled" out of the ER? Interestingly, the half-life of this ERAD client is ~12hrs (compared to NS1 for instance that has a half-life of 1.5hrs), which possibly reflects that there are indeed additional requirements for ERAD of more complex clients.

Despite the notion that the retrotranslocon could potentially accommodate large and/or folded domains, all evidence so far reports the need to reduce disulfide bonds on ERAD substrates. Retrotranslocated substrates are found to be devoid of inter- and intra-chain disulfides [228]. Removal of disulfide bonds also serves to separate oligomers in the ER and prepare the monomers for degradation to prevent aggregation. Such is the case for NHK, which is an unstructured protein that forms dimers that are broken by ERdj5-mediated reduction of inter-chain disulfides [60, 248]. Unlike the oxidizing environment in the ER lumen, the cytosol is reducing. Therefore, one possibility could be that once ERAD clients (as single polypeptide chains) become exposed to the cytosol, they become reduced. However, since the need to reduce inter-chain disulfides in the ER lumen has been well reported, and a variety of reductases reside in the ER lumen, another possibility is that reduction of ERAD substrates occurs in the ER itself. In this scenario, reduction would also serve to assist in fitting a client in the retrotranslocon for extraction to the cytosol. In our study we demonstrated clearly that after proteasome inhibition,

retrotranslocated proteins were reduced (that was the case for both NS1 and mHC). Interestingly, when we examined the oxidation status of our glycosylated (ER localized) and de-glycosylated (retrotranslocated) client we were not able to detect any glycosylated and reduced species. This indicates that if reduction happens in the ER lumen, it must occur right before or even during retrotranslocation. In **Chapter 2** we discussed how ERdj5, a J domain-containing protein, which interacts with BiP and also has reductase activity that is relevant for other ERAD substrates, did not seem to have any effect on our NS1 protein. However, over 20 PDI family members have been reported to participate in mammalian ERAD. We performed proteomics and searched for κ light chain interacting proteins with mass-spectrometry (MS). For this, we used our P3U.1 plasmocytoma cell line, and kept untreated and MG132-treated cells. From those samples we isolated the κ light chains and searched for their integrators in both cases **(Appendix)**. Interestingly, one of the proteins identified by MS was PDIA6 (or ERp5), a PDI family member. PDIA6/ERp5, which is not a very well-studied PDI, has been reported to be in complex with BiP, and so it would have access to BiP substrates [249, 250]. Indeed, NS1 is a well-known BiP substrate. This PDI family member was also found to interact with one other ERAD client RI332 [242]. Consequently, it seems that it may be a good candidate for reducing NS1 in the ER prior to retrotranslocation.

## The Implications of Ubiquitination During ERAD

As ERAD substrates emerge out of the retrotranslocon to the cytosolic side of the ER membrane, they become poly-ubiquitinated by ER-localized ubiquitin ligases. Indeed, at the center of the retrotranslocon is Hrd1, an E3 ubiquitin ligase with the ligase domain positioned outside of the ER membrane at the exit of the channel, and in the vicinity E2 ubiquitin conjugating enzymes reside. Several ERAD- associated E2 and E3s have been identified. The residues on a protein that can be modified for ERAD are also diverse, including the traditional lysines, but also serines, threonines, and cysteines **(Chapter 1)**. It seems that the different E2/E3 pairs that are formed are critical for the versatility of ERAD client ubiquitination. ERAD client ubiquitination in the cytosol during retrotranslocation serves three main purposes: (a) flags the protein for proteasomal degradation, (b) serves as leverage for binding to the p97 ATPase, and (c) keeps the substrate from moving backwards to the ER lumen. p97 binding to clients promotes the forward movement of the retrotranslocating protein and completes extraction of the clients from the ER membrane.

## Completing Retrotranslocation

The p97 AAA-ATPase is recruited to the cytosolic phase of the retrotranslocon on the ER membrane via interactions with protein components of the channel (namely VIMP and UBXD8) and recognizes ubiquitinated ERAD substrates as they emerge out of the ER [251]. P97 is the only energy providing module known to date to accommodate the complete dislocation of ERAD clients out of the ER. This protein consists of an N-terminal domain (N domain), and two ATPase domains (D1 and D2 domains), and it

forms a homohexameric double-ring structure **(Figure 1-5)**. The multiple co-factors that have been identified to bind to the N domain of p97, have been shown to also direct its functions [155]. For ERAD, the co-factors Ufd1 and Npl4 bind ubiquitinated substrates and bridge the interaction of substrates with p97. By ATP hydrolysis at the D1 and D2 domains, p97 undergoes conformational changes that result in client extraction from the ER membrane to complete their retrotranslocation. The force generated by ATP hydrolysis by p97 may be part of the answer to how domains that still maintain structure can pass across the ER membrane. Once clients have been extracted to the cytosolic side of the ER, rounds of de-ubiquitination and re-ubiquitination serve to process the substrate (maybe completely unfold it?) for its delivery to the proteasome. For this, du-ubiquitinating enzymes (DUBs) have been reported to be associated with p97. The ATPase activity of p97 also provides segregase, disaggregase, and unfoldase capacity to the complex. The final outcomes of p97's coordinated functions with ubiquitin are substrate remodeling, unfolding and extraction from membranes and macromolecular complexes.

After p97-mediated protein extraction, the next step for ERAD is proteasomal degradation. Two ways are being proposed for substrate delivery to the proteasome: (a) by a direct "handoff" mechanism, and (b) by shuttling factors. Even though in archaea a direct interaction of Cdc48/p97 with the proteasome was identified, suggesting that in that case there was a continuous passage for the substrate to be degraded, only limited data exist for eukaryotic cells. One study in yeast used MG132 or a p97 mutant to pause retrotranslocation and isolate stalled retrotranslocating chains and reported co-immunoprecipitation of a proteasome subunit with Hrd1p, Hrd3p, luminal ERAD components, and p97 [172]. One caveat of the study was that it was not defined if this complex was maintained by direct interactions or via auxiliary factors. A model where shuttling factors transfer the substrate from p97 to the proteasome has been more popular. Proteins like hHR2 (Rad23 in yeast) and Dsk2, which have both ubiquitin and proteasome binding domains might serve to transfer the substrate from p97 to the proteasome, thus acting as shuttling factors [103]. Cytosolic chaperones such as Hsc70 [252] and Bag6 [173] can interact with ERAD clients in the cytosol after the p97 step by binding to hydrophobic regions on retrotranslocated substrates. Such interactions possibly serve to maintain substrate solubility and prevent aggregation in the aqueous cytosolic environment, but also to assist in substrate channeling to the degradation machinery. Further studies will determine the factors and mechanisms used to deliver ERAD clients to the proteasome after retrotranslocation.

## Proteasomal Degradation

The final destination of ERAD clients is the proteasome, which is responsible for their degradation. The proteasome harbors ubiquitin binding particles on its 19S lid that recruit ubiquitinated substrates and accommodate them in such a way that unfolded termini of the substrate will be fed into the proteolytic chamber to initiate degradation. The lid of the proteasome, which is where the substrates are recruited to, also includes DUBs and ATPases, all of which mediate rounds of substrate unfolding and translocation

into the proteolytic core until the whole substrate protein has been eliminated. Efficient proteasomal degradation, in addition to polyubiquitin chains, requires a loosely folded or unfolded region on the substrate protein to engage first with the proteasomal unfolding and translocation machinery. Such a region also appears to serve as the degradation initiation site, and if it is located at a protein's termini, then the degradation efficiency improves [253-255]. Proteins with folded parts and without a loose domain fail to bind tightly to the proteasome, pose a kinetic barrier, and are degraded less efficiently [256, 257].

From the way the proteasome couples multiple functions (de-ubiquitination, ATP-hydrolysis, and conformational changes) with substrate unfolding and translocation to the proteolytic core, it seems that efficient substrate processing is highly dependent on maintaining these sequential actions intact. Indeed, unfolding of substrates, which was reported to be the rate-limiting step in the process [256], increases as the substrate translocates into the 26S proteasome and that in turn decreases substrate unfolding as the polypeptides move into the protease. This has possible implications on how the ATPase function can be intertwined with the degradative function of the proteasome, and if one function is interrupted then other functions will be inhibited as well. In accordance to this, recently it was shown that non-ideal substrates were effectively rejected by the proteasome, which depends on substrate-processing kinetic proofreading, to guarantee degradation fidelity.

## A Folded Domain on an ERAD Substrate Does Add Complexity to ERAD, and Poses Additional Demands for Processing in the Cytosol

### ERAD clients with a folded domain are trapped in the cytosol bound to the cytosolic ERAD machinery

In this study, we demonstrated that ERAD clients possessing a well-folded domain can cross the ER membrane and retrotranslocate with the actions of the p97 complex and in the presence of ubiquitination, but they maintain significant structure on their well-folded domain, even though it was reduced. Clients that remained partially folded in the cytosol, were not able to be released by the cytosolic ERAD machinery and as a result they were not detected in the cytosol, like our unfolded clients did, when the proteasome was inhibited. This finding sent us on a path to explore and understand why this distinction occurred, and where the folded domains were held up. Previous unpublished data from our lab had showed that in cells with ERdj3 knockdown γ heavy chain turnover was accelerated and association to p97 was dramatic. This led us to follow a targeted biochemical approach, isolate κ LCs from cells treated with or without MG132 to inhibit the proteasome, and test for known cytosolic ERAD components that may be bound to the LCs in each case **(Chapter 2)**. We found a dramatic increase in the binding of p97, the proteasome, and Hsc70. This finding reveals how LCs are being indeed held up at the last steps of the process. Our detection of Hsc70 binding to NS1 in co-IP experiments reveals that cytosolic chaperones bind retrotranslocated LCs to possibly

maintain them in a soluble state in the aqueous cytosolic environment. In addition to that, we also performed Mass-Spectrometry (MS) analysis to seek for other, maybe novel, cytosolic ERAD factors that may be bound to our LCs and cause them to become trapped when not unfolded. Again, we used samples from control and MG132-treated κ LC expressing cells. Our MS experiment, although at a pilot stage, revealed some very interesting data. When the proteasome was inhibited, κ LCs interacted with various components of the ubiquitin-proteasome system and with cytosolic chaperones. Ubiquitin was the top hit, and it was followed by the chaperones Hsc70 and Bag6. Subunits of the proteasome were also detected. These data agreed with our biochemical evidence for Hsc70 and proteasome binding. Curiously, we were not able to identify p97, or any of its co-factors on our MS. We also saw that in addition to Hsc70, Bag6 was also enriched in the samples from MG132 treated cells. Bag6 is a ubiquitin-like cytosolic chaperone that has been reported to bind dislocated clients to maintain their solubility, assist in the dislocation process and promote client delivery to the proteasome [173]. It usually functions downstream of p97 [258, 259].

Our data so far, point to the fact that retrotranslocated ERAD clients that possess structure in the cytosol (in our case in the form of a folded domain) have difficulty in being released from p97 or/and being transferred to the proteasome for degradation when the proteasomes are inactive. Two questions stem from our findings: (a) why the mighty ATPase p97 cannot release clients with a folded domain in absence of proteasomal function, while it has no difficulty doing so with unfolded clients, and (b) why proteasomal functions are important for the disengagement of folded domains from p97?

**P97 remains bound to clients with folded domains in absence of proteasomal functions**

During ERAD, the p97 ATPase binds poly-ubiquitinated clients and completes their extraction from ER. Simple, unfolded ERAD clients are being extracted from p97 and released into the cytosol when proteasomes are inactive. This argues that p97 has the ability to disengage from some of its substrates. But it seems that when a substrate is structurally more complex, p97 remains associated with it. In order to understand this inability of p97 to release complex clients, we need to examine how p97 binds its substrates and what is needed to release them.

Understanding the ways that p97 functions has been the focus of multiple labs. P97 has the capacity to extract proteins from membranes and large complexes, unfold proteins, but also act as a chaperone itself. A few models have been proposed as to how p97 binds and processes its clients (described in **Chapter 1**). In brief, clients have been proposed to either enter from the D1, travel through the D1 and D2 pores, and exit from the "trans" side of the D2 ring; or enter from the D1:D2 interface, enter the D2 pore, and exit again from the "trans" side of D2; or enter and exit from the D2 ring without ever interacting with the D2 ring (this last model is the least favorable for ERAD for topological reasons, since p97 is recruited to the ER membrane by protein-protein interactions and it is oriented with the D1 ring near the membrane). Very recently the

crystal structures of p97 in the act of unfolding a substrate and while unfolding ubiquitin were solved [260, 261]. In both papers, clients were shown to interact and "enter" in p97 from the D1 pore. Cooney et al. demonstrated how p97 uses ATP to undergo such structural changes to employ a hand-over-hand mechanism to translocate its substrates through its pore [261]. Twomey et al. demonstrated how the Ufd1/Npl4 co-factors by recognizing polyubiquitin chains on clients recruit clients to p97 [260]. Ubiquitin then becomes unfolded by p97 and starts translocating into p97's pore. In this way, p97 moves ubiquitin and then starts acting on unfolding and translocating the substrate that Ub is on. For their experiments, Twomey et al. used a simple fluorescent substrate and implied substrate translocating through p97 by loss of fluorescence. This extraordinary work by these two groups, has uncovered crucial details of p97's functions. However, further research is necessary to unravel how more complicated substrates are being processed, do they all follow this threading model or in other cases substrates are managed differently (maybe following the D1:D2 side access model), and how multi-domain clients would be handled (i.e. the γ heavy chain that possesses 3 folded domains). We cannot conclude from our studies how our substrates with folded domains were processed by p97, but we did show (with proteinase K experiments) how they were not completely unfolded. Lack of unfolding may be one reason why our clients remained associated with p97. Given that our clients remained partially folded in the cytosol, that could support a hypothesis where additional energy is required to pull them from p97 itself. This extra force may be provided by the proteasome itself. The proteasome does contain ATPases at the base of the 19S lid [177]. As such the proteasome may be able to directly grasp clients from p97 and immediately start processing them. When we inhibited the degradative function of the proteasome, we possibly caused a complete stall in all the proteasome's functions. That can explain why our LCs were bound to p97 but also to chaperones such as Bag6 that functions in steps between p97 and the proteasome. A second possibility could be the lack of appropriate DUBs (de-ubiquitinating enzymes). DUBs are critical to remove ubiquitin and ubiquitin chains from proteins. Short ubiquitin chains are required from the proteasome to engage substrates, and as one can imagine, a proteasomal substrate with long ubiquitin chains may not be favorable for proteasome engagement. Few DUBs are known to participate in ERAD [192, 262]. To complicate things further, the κ LCs that we have used in our study, but also the mHC, contain serine and threonine ubiquitination, as opposed to the traditional lysine ubiquitination. There is a possibility that this modification requires special DUBs to prepare these proteins for the proteasome. There is a possibility that either such DUBs cannot be recruited, or the proteasome itself can perform this action and release clients from p97, and so when the proteasome is inhibited de-ubiquitination and substrate release cannot happen.

**Are retrotranslocated ERAD clients with a folded domain found in a complex with both p97 and the proteasome?**

Our studies to identify cytosolic ERAD factors that interact with LCs when the proteasomes are inhibited revealed increased association of our LCs with p97 and the proteasome. In a separate experiment we saw that when the proteasomes were inhibited with MG132, we readily detected a degradation intermediate that corresponded to the $C_L$

domain with some extra amino acids of the unfolded $V_L$ domain **(Chapter 2)**. That was a sharp band on SDS-PAGE that most probably represented a population of LCs that bound to the proteasome and entered the proteolytic chamber for degradation but in the absence of the degradation capacity it remained trapped there. That is a very fascinating finding and it raises the question of whether our retrotranslocated clients remain bound to both p97 and the proteasome in a complex, or whether different pools of LCs are associated with p97 and others with the proteasome. If LCs are in the same complex with both p97 and the proteasome, that argues for a continuous and coupled process of retrotranslocation and degradation, which is stalled when we treat cells with MG132 and possibly causes LCs to pile up in all steps before p97. Neither in our biotinylation nor in our deglycosylation experiments did we observe complete modification of our clients (100% biotinylation or 100% deglycosylation) after MG132 treatment. That really suggests that only a few proteins became retrotranslocated and then the process is paused preserving retrotranslocated LCs in the cytosol and all the rest in the ER lumen. It is of no surprise then that long treatments with MG132 and prolonged inhibition of ERAD leads to UPR, ER and cell stress and even cell death. The other scenario is that some proteins remain bound to p97 and others are trapped in inactive proteasomes. This would support a model where the p97 and degradation steps are independent and shuttling factors (maybe Bag6) are required to link these two events. In this case, inhibiting the proteasome traps some clients there and some others with p97 since they cannot be delivered to the next step which now is a plugged proteasome. In both cases, eventually the cytosolic ERAD machinery will become saturated will clients that cannot be processed and that could result in plugging the exit sites and accumulation of ERAD clients in the ER.

## Future Perspectives

Our study revealed some very compelling insights on ERAD client retrotranslocation. Future research will shed light and advance our understanding of how this important pathway operates. Additional analysis of the retrotranslocon will explain whether one or many retrotranslocon channels exist to cater to all ERAD clients. What is the/their composition and how do they mechanistically function to pass proteins across the ER membrane. Achieving the crystal structure resolution of many ERAD components will be critical to understand the way they function. Understanding how ERAD clients are recognized and delivered to the retrotranslocon, and exactly how they are processed in the cytosol until they can be degraded will be of great value. That would be of particular importance when it comes to complex proteins with multiple domains and subunits. Would there be a difference with such proteins? Do ERAD factors have to invest more time and energy to process complicated clients and is that a reason why different proteins have different half-lives? The exciting field of ERAD has gone a long way so far, but there are still so many aspects of it that are awaiting to be uncovered.

## Conclusions

In total, we have shown here that retrotranslocation of ERAD substrates can be initiated by an unfolded region on the clients. The client will then sample the cytosol and after becoming ubiquitinated, it will engage the p97 complex, which will complete substrate extraction from the ER membrane and into the cytosol. Unfolded clients are then easily processed with less energy requirements. In absence of proteasomal degradation, such proteins can be released from the cytosolic ERAD machinery and accumulate in the cytosol. Conversely, clients that possessed one folded domain had different requirements for ERAD. A folded domain did not pose a barrier for retrotranslocation, but rather on substrate processing and release from p97. Our ERAD clients with folded domains were efficiently extracted from the ER by p97. On the cytosolic side of the ER membrane, those proteins were found reduced but with significant structure remaining on the folded domain. That resulted in the clients remaining associated with p97 in absence of the proteasome's functions. This implies additional energy requirements for these clients that are possibly provided by a fully active proteasome, which may be coupling its degradative function with its ATPase and unfoldase actions.

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## APPENDIX. MASS-SPECTROMETRY DATA ON K LIGHT CHAIN INTERACTING PROTEINS UNDER CONTROL CONDITIONS AND AFTER MG132 PROTEASOME INHIBITION

P3U.1 mouse plasmocytoma cell line

A.

| Reference | Name | SC -MG123 | SC +MG123 | SC NegCntrl |
|---|---|---|---|---|
| cu\|NS1_Cus | NS1 | 51 | **41** | 0 |

B.

| Reference | Name | SC -MG123 | SC +MG123 | SC NegCntrl |
|---|---|---|---|---|
| tr\|J3QK04\|J3QK04_MOUSE | Ub related | 0 | **53** | 0 |
| tr\|D3YYZ2\|D3YYZ2_MOUSE | Ub related | 0 | **51** | 0 |
| sp\|P63017\|HSP7C_MOUSE | Hsc71 | 26 | **44** | 3 |
| sp\|Q9Z1R2\|BAG6_MOUSE | Bag6 | 0 | **21** | 0 |
| sp\|P17156\|HSP72_MOUSE | Hsp70 | 15 | **17** | 0 |
| sp\|Q9QUM9\|PSA6_MOUSE | proteasome subunit | 0 | **2** | 0 |
| sp\|Q9Z2U0\|PSA7_MOUSE | proteasome subunit | 0 | **2** | 0 |
| sp\|P53657\|KPYR_MOUSE | Pyruvate Kinase PKLR | 0 | **2** | 0 |
| sp\|P46471\|PRS7_MOUSE | proteasome subunit | 0 | **2** | 0 |
| sp\|P62192\|PRS4_MOUSE | proteasome subunit | 0 | **2** | 0 |
| sp\|Q80TT8\|CUL9_MOUSE | Cullin-9 | 0 | **2** | 0 |

C.

| Reference | Name | SC -MG123 | SC +MG123 | SC NegCntrl |
|---|---|---|---|---|
| sp\|P20029\|GRP78_MOUSE | BiP | 151 | **109** | 2 |
| sp\|P17182\|ENOA_MOUSE | a-Enolase | 13 | **7** | 0 |
| sp\|P52480\|KPYM_MOUSE | Pyruvate Kinase PKM | 12 | **8** | 0 |
| sp\|Q8K2B3\|SDHA_MOUSE | Succinate dehydrogenase flavoprotein subunit | 12 | **3** | 4 |
| sp\|Q61696\|HS71A_MOUSE | Hsp70-1A | 11 | **8** | 0 |
| sp\|P16627\|HS71L_MOUSE | Hsp70-1L | 10 | **8** | 0 |
| sp\|P17751\|TPIS_MOUSE | Triosephosphate isomerase | 9 | **0** | 0 |
| sp\|P06151\|LDHA_MOUSE | LDHA | 7 | **3** | 0 |
| sp\|P11499\|HS90B_MOUSE | Hsp90-B | 6 | **4** | 0 |
| sp\|P08113\|ENPL_MOUSE | Endoplasmin | 5 | **2** | 0 |
| sp\|P35564\|CALX_MOUSE | Calnexin | 5 | **0** | 0 |
| sp\|Q922R8\|PDIA6_MOUSE | PDIA6/ERp5 | 2 | **0** | 0 |
| sp\|Q91X78\|ERLN1_MOUSE | Erlin-1 | 2 | **0** | 0 |

κ light chains (LC) were immunoprecipitated from P3U.1 plasmocytoma cells that had been treated without (-MG132) and with (+MG132) 10uM of the MG132 proteasomal inhibitor for 3hrs. Sample were then analyzed by mass-spectrometry and κ LC interacting proteins were identified in each case. Data show identified proteins for which the spectral counts (SC) deferred by 2 or more between our two conditions. The Ag8.8 cell line that does not express LCs was used as negative control. Panel (A) presents the SCs for the κ LC itself, (B) presents proteins that were identified to co-IP with κ LC after MG132 treatment, and (C) presents proteins that were identified to co-IP with κ LC in untreated cells.

# VITA

Christina Oikonomou, born in 1985, obtained her Bachelor of Science (Hons.) degree from Harokopio University of Athens, Greece, majoring in Nutritional and Clinical Dietetics. She then enrolled at Wageningen University in Wageningen, the Netherlands, and completed her Master of Science degree with a major in Molecular Nutrition (Metabolism and Nutritional Genomics) in 2011. After briefly working as a researcher at the "Alexander Fleming" Biomedical Sciences Research Center in Vari, Greece, she pursued doctorate studies at the University of Tennessee Health Science Center where she enrolled in August of 2013 as a Ph.D. student. She has been conducting her doctoral research on characterizing the molecular mechanisms of retrotranslocation for ERAD. She is expected to obtain her Ph.D. degree in December 2019.