IDENTIFICATION AND CHARACTERIZATION OF NOVEL PLAYERS REGULATING PROTEIN QUALITY CONTROL AND DEGRADATION IN THE MAMMALIAN ENDOPLASMIC RETICULUM

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RICCARDO BERNASCONI

Dipl. Natw. ETH Zurich
born March 25th, 1983
citizen of Castel San Pietro (TI), Switzerland

accepted on the recommendation of

Prof. Dr. Markus Aebi, examiner
Prof. Dr. Yves Barral, co-examiner
Prof. Dr. Maurizio Molinari, co-examiner

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Abstract

The endoplasmic reticulum (ER) is the entry site of newly synthesized proteins in the secretory pathway. Nascent polypeptide chains are co-translationally N-glycosylated at Asn-X-Ser/Thr sequons and acquire the native conformation under the assistance of a multitude of ER-resident molecular chaperones and folding enzymes. A quality-control system surveys the lumen of the ER, insuring that only correctly folded and assembled proteins are transported through the secretory pathway at their final destination. Non-native proteins are actively retained in the folding compartment, while terminally misfolded polypeptides are dislocated into the cytosol where they are degraded by 26S proteasomes.

Processing of N-glycans, initially composed of 2 N-acetylglucosamine, 9 mannose and 3 glucose residues, plays an essential regulatory role both for folding and for ER-associated degradation (ERAD) of proteins synthesized in the ER lumen. Glycopolypeptides that have not acquired their native structure in due time, become accessible to ER-resident α1,2-mannosidases, which by de-mannosylating the peptide-bound N-glycan(s) activate the disposal pathway. In fact, mannose removal is indication of long retention in the ER lumen and represents a strong signal that labels folding-defective glycopolypeptides for degradation. To ensure removal of folding-defective proteins from the ER lumen, misfolded proteins must be extracted from the ER folding machinery, exported across the ER membrane under the assistance of multimeric protein complexes built around membrane-embedded E3 ubiquitin ligases, poly-ubiquitinylated and degraded by cytosolic 26S proteasomes. Failure of protein quality control and ERAD may lead to severe human diseases such as cystic fibrosis, hereditary lung emphysema, liver failure and neurodegenerative syndromes. Several key components and mechanisms regulating protein folding, quality control and degradation have been identified, but understanding of these events at the molecular level remains incomplete.

The aim of my project was the identification and characterization of novel factors/pathways regulating protein quality control and disposal in the mammalian ER.
Initially, we focused our study on the characterization of OS-9, a putative mammalian ortholog of the \textit{S. cerevisiae} protein Yos9p. We found that in mammalian cells, OS-9 is present in two isoforms, OS-9.1 and OS-9.2, generated by alternative splicing of the OS-9 gene. We reported that both OS-9 variants are transcriptionally induced upon activation of the Ire1/Xbp1 ER-stress pathway. Our data showed that OS-9.1 and OS-9.2 fail to associate with folding-competent proteins. Rather, they selectively bind folding-defective polypeptides thereby inhibiting transport of non-native conformers through the secretory pathway. Additionally, OS-9 substrate-binding and activity do not require an intact mannose 6-P homology domain (MRH). The intralumenal level of OS-9.1 and OS-9.2 inversely correlates with the fraction of a folding-defective glycopolypeptide, the Null\textsubscript{hong kong} (NHK) variant of \alpha 1-antitrypsin that escapes retention-based ER quality control. Thus, OS-9.1 and OS-9.2 play a dual role in mammalian ER quality control: firstly as crucial retention factors for misfolded conformers, and secondly as promoters of protein disposal from the ER lumen. It remains a matter of study if they only regulate disposal of glycopolypeptides, or whether they are involved in clearance from the ER lumen of both glycosylated and non-glycosylated polypeptides (Bernasconi, R., Pertel, T., Luban, J. & Molinari, M. (2008) A Dual Task for the Xbp1-responsive OS-9 Variants in the Mammalian Endoplasmic Reticulum: Inhibiting Secretion of Misfolded Protein Conformers and Enhancing their Disposal. \textit{J Biol Chem} 283, 16446-16454).

In a second study, we investigated the mechanisms regulating recognition and dislocation across the ER membrane of misfolded glycopolypeptides. In \textit{S. cerevisiae}, the presence of structural lesions in the luminal, transmembrane, or cytosolic domains determines the classification of misfolded polypeptides as ERAD-L, ERAD-M or ERAD-C substrates and results in selection of distinct degradation pathways. ERAD-L and ERAD-M substrates are cleared from the ER lumen by the HRD1 pathway, while ERAD-C substrates use the DOA10 pathway. These pathways comprise several luminal, transmembrane and cytosolic factors associated with the ER membrane-embedded E3 ligases Hrd1p and Doa10p, respectively. The mechanisms regulating recognition and dislocation of ERAD substrates across the mammalian ER membrane are much more complex (e.g. there are at least 7 ER-embedded E3 ligases) and poorly characterized. We found that disposal of soluble (non-transmembrane) misfolded proteins (that we define as ERAD-L\textsubscript{S} substrates)
and of membrane-tethered misfolded proteins (that we define as ERAD-L\textsubscript{M} substrates) with the same luminal lesion possess different molecular requirements. Clearance of ERAD-L\textsubscript{S} substrates is strictly dependent on the E3 ubiquitin ligase HRD1, the associated cargo receptor SEL1L and two interchangeable ERAD shuttles, OS-9 and XTP3-B. These ERAD factors become dispensable for degradation of the same polypeptides when membrane-tethered (ERAD-L\textsubscript{M} substrates). These findings revealed that, in contrast to budding yeast, tethering of mammalian ERAD-L substrates to the membrane changes selection of the degradation pathway (Bernasconi, R., Galli, C., Calanca, V., Nakajima, T. & Molinari, M. (2010) Stringent requirement for HRD1, SEL1L, and OS-9/XTP3-B for disposal of ERAD-LS substrates. J Cell Biol 188, 223-235).

In a third study, we characterized the involvement of an ER member of the peptidyl prolyl \textit{cis/trans} isomerases (PPIs) family, cyclophilin B (CyPB), in clearance of misfolded polypeptides from the mammalian ER lumen. \textit{Cis/trans} isomerization of peptide bonds preceding proline residues is a rate-limiting step for the attainment of the native and functional 3D structure. \textit{In vitro}, it has been widely shown that this reaction is catalyzed by PPIs. Nevertheless, the importance of PPIs during protein quality control in the ER of living cells has not been demonstrated yet. We found that CyPB, the most abundant archetypical ER-resident PPI, plays a crucial role in disposal from the ER of soluble (non-membrane) folding-defective polypeptides (ERAD-L\textsubscript{S} substrate) containing \textit{cis}-prolines. On the other hand, CyPB is dispensable for disposal of the same polypeptides when tethered at the ER membrane (ERAD-L\textsubscript{M} substrates) and for disposal of polypeptides lacking \textit{cis} proline residues. Thus, \textit{cis-to-trans} isomerization of peptidyl-prolyl bonds of misfolded polypeptides seems to contribute to efficient protein disposal from the mammalian ER (Bernasconi, R., Soldà, T., Galli, C., Pertel, T., Luban, J. and Molinari, M. (2010) Cyclophilin B promotes degradation of misfolded polypeptides from the mammalian endoplasmic reticulum SUBMITTED).

We have previously reported (Cali et al. 2008) that EDEM1, a short-living regulator of ERAD, is segregated from ER-resident long-living chaperones such as BiP and Calnexin, and is released from the ER in LC3-I-coated vesicles.
In a fourth study, we have showed that mouse hepatitis virus (MHV), a coronavirus (CoV), exploits the host cell machinery for the COPII-independent vesicular export from the ER of EDEM1 to co-opt cellular membranes for replication. In this study we have found that cell infection with MHV substantially delays EDEM1 turnover, causing its accumulation and of at least another short-living ER chaperone, OS-9, in the virus-induced double membrane vesicles (DMVs) containing the viral replication and transcription complexes (RTCs). DMVs are coated with non-lipidated LC3/Atg8 and LC3/Atg8 down-regulation, but not inactivation of the host cell autophagy, protects mammalian cells from CoV infection.

Il reticolo endoplasmatico (ER) è il sito dove le proteine appena sintetizzate entrano nella via secretoria. Catene polipeptidiche nascenti sono glicosilate co-traduzionalmente sulle catene laterali delle asparagine (Asn) in sequenze Asn-X-Ser/Thr e acquisiscono la conformazione nativa grazie all’assistenza di una moltitudine di chaperoni molecolari ed enzimi residenti nell’ER. Un controllo di qualità sorveglia le proteine prodotte nel lume dell’ER garantendo che solo proteine correttamente ripiegate e assemblate siano trasportate attraverso la via secretoria fino a raggiungere la destinazione finale. Proteine non native sono trattenute attivamente, mentre polipeptidi mal piegati vengono rapidamente rimossi dal lume dell’ER.

I glicani legati a proteine di nuova sintesi sono inizialmente composti da 2 N-acetilglucosamine, 9 mannosi e 3 glucosi. I saccaridi terminali sono progressivamente eliminati da glucosidasi e mannosidasi attive nel lume dell’ER. Questi processi giocano un ruolo essenziale nella biogenesi proteica reclutando lectine e ossidoriduttasi che facilitano l’acquisizione della struttura nativa, o reclutando fattori che deviano le proteine difettose verso il citosol per permetterne la degradazione (Degradazione associata all’ER (ERAD)). Glicopolipeptidi che non riescono ad acquisire per tempo la struttura nativa diventano accessibili alle α1,2-mannosidasi residenti nell’ER che rimuovono uno dopo l’altro 4-5 mannosi generando un segnale degradativo che determina estrazione dalla macchina di piegamento, dislocazione nel citosol, poli-ubiquitinazione e degradazione operata dal 26S proteasoma. Difetti nel ripiegamento, nel controllo di qualità o nella degradazione di proteine di nuova sintesi possono condurre a gravi malattie umane come la fibrosi cistica, l’enfisema polmonare ereditario, la cirrosi epatica e a svariate sindromi neurodegenerative. Molte componenti chiave e meccanismi che regolano il piegamento, il controllo di qualità e la degradazione delle proteine prodotte nell’ER sono state identificate. Ciononostante, la comprensione di questi eventi a livello molecolare rimane incompleta.
L’obiettivo del mio lavoro è stato quello di identificare e caratterizzare nuovi fattori/vie che regolano il controllo di qualità e la rimozione di proteine mal piegate dal lume dell’ER di mammiferi.

Inizialmente abbiamo caratterizzato OS-9, un ortologo nel mammifero della proteina di lievito S. cerevisiae Yos9p. Abbiamo dimostrato che in cellule di mammifero OS-9 è presente in due isoforme (OS-9.1 e OS-9.2), derivate dallo splicing alternativo del gene OS-9. Abbiamo riportato che entrambe le varianti di OS-9 sono indotte trascrizionalmente mediante l’attivazione della via Ire1/Xbp1 in risposta a stress dell’ER. I nostri dati mostrano che OS-9.1 e OS-9.2 non si associano a proteine native ma legano selettivamente polipeptidi con ripiegamento difettoso, inibendo il trasporto di conformazioni non native attraverso la via secretoria. Il legame con il substrato e l’attività di OS-9 non necessitano di un dominio lectinico omologo al mannosio 6-P funzionale. Abbiamo inoltre osservato che il livello intraluminale di OS-9.1 e OS-9.2 correla inversamente con la frazione di un glicopolipeptide parzialmente incapace di acquisire una corretta struttura tridimensionale, la variante Null\textsubscript{Hong Kong} (NHK) di α1-antitrypsin, che riesce ad evitare la ritenzione mediata dal controllo di qualità dell’ER.


In un secondo studio, abbiamo caratterizzato i meccanismi che regolano il riconoscimento e la dislocazione attraverso la membrana dell’ER di glicopolipeptidi piegati non nativi. In S. cerevisiae la presenza di lesioni strutturali luminali, transmembranali o citosoliche determina la classificazione di proteine non native in substrati ERAD-L, ERAD-M o ERAD-C e risultano nella selezione di vie degradative distinte. Substrati ERAD-L ed ERAD-M sono rimossi attraverso la via HRD1, mentre
substrati ERAD-C usano la via DOA10. Queste vie comprendono molte componenti luminali, transmembranali e citosoliche associate rispettivamente con le E3 ligasi Hrd1p o Doa10p.

Nelle cellule di mammifero, i meccanismi che regolano il riconoscimento e la dislocazione di substrati ERAD attraverso la membrana dell’ER sono molto più complessi (per esempio ci sono almeno 7 E3 ligasi nella membrana dell’ER) e sono scarsamente caratterizzati. Abbiamo dimostrato che la degradazione di proteine non ancorate alla membrana (da noi definite substrati ERAD-L_S) richiede l’intervento di fattori specifici che non sono richiesti per la degradazione delle stesse proteine quando sono ancorate alla membrana dell’ER (da noi definite substrati ERAD-L_M). La rimozione di substrati ERAD-L_S è strettamente dipendente dall’E3 ligase HRD1, dal cargo recettore associato SEL1L e da due ERAD shuttles intercambiabili, OS-9 e XTP3-B. L’intervento di questi fattori non è invece obbligatorio per i substrati ERAD-L_M. Questi dati rivelano che, rispetto a S. cerevisiae, la selezione della via di degradazione dipende dal legame o meno del substrato ERAD alla membrana (Bernasconi, R., Galli, C., Calanca, V., Nakajima, T. & Molinari, M. (2010) Stringent requirement for HRD1, SEL1L, and OS-9/XTP3-B for disposal of ERAD-LS substrates. J Cell Biol 188, 223-235).

In un terzo studio, abbiamo caratterizzato il coinvolgimento di un membro della famiglia delle peptidil-prolil cis/trans isomerasi (PPIs) residente nell’ER, la ciclofilina B (CyPB), in ERAD nelle cellule di mammifero.

L’isomerizzazione trans/cis di un legame peptidico che precede una prolina è una delle reazioni chimiche che determina la velocità dell’acquisizione della struttura tridimensionale nativa e funzionale. In vitro, numerosi dati dimostrano che tale reazione è catalizzata da PPIs. In vivo, l’intervento delle PPIs durante la biogenesi (ripiegamento, controllo della qualità o degradazione) di proteine espresse nell’ER non è stato ancora dimostrato.

I nostri risultati mostrano che CyPB, la più abbondante PPI che risiede nell’ER, gioca un ruolo cruciale nella degradazione di un polipeptide solubile (substrato ERAD-L_S) contenente proline in cis, mentre non interviene nella degradazione di proteine che non contengono legami polypeptidici in cis o nella degradazione di proteine ancorate alla membrana. Perciò, l’isomerizzazione da cis a trans di legami peptidil-prolil in polipeptidi non nativi sembra facilitare la loro rimozione dal lume dell’ER (Bernasconi,

In uno studio precedente (Calì et al. 2008), abbiamo riportato che EDEM1, un regolatore di ERAD a corta emivita, è selettivamente segregato da chaperoni dell’ER di lunga emivita come BiP e Calnexina, e lascia l’ER tramite vescicole ricoperte da LC3-I.

In un quarto studio, abbiamo mostrato come il virus dell’epatite murina (MHV), un coronavirus (CoV), sfrutta una via cellulare già esistente nella cellula infetta al fine di replicare. Tale via permette alla cellula di ridurre il contenuto luminale di chaperoni che regolano ERAD, per esempio EDEM1, OS-9 e SEL1L. Queste proteine vengono selettivamente rilasciate dall’ER in vescicole non rivestite da COPII come capita per le vescicole secretorie, ma da LC3/Atg8 non lipidato. Il contenuto di tali vescicole (da noi definite EDEMosomi) viene degradato da enzimi endo/lisosomiali. I nostri dati mostrano che l’infezione con MHV causa un accumulo di EDEM1, OS-9 e altri chaperoni coinvolti in ERAD a corta emivita in vescicole che il virus induce per incorporare complessi di trascrizione che sono necessari per la replicazione virale. Queste DMV (per Double Membrane Vesicles) derivanti dalle membrane dell’ER sono rivestite da LC3 non lipidato (proprio come gli EDEMosomi). La riduzione del livello intracellulare di LC3, ma non l’inattivazione dell’autofagia, protegge le cellule di mammifero da infezione di CoV. Il nostro studio ha identificato la via cellulare dirottata da CoV, una scoperta che potrebbe permettere lo sviluppo di nuove terapie volte a impedire la replicazione virale e descrive un nuovo ruolo della forma non lipidata di LC3 in un processo non correlato all’autofagia (Reggiori, F., Monastyrska, I., Verheije, M.H., Calì, T., Ulasli, M., Bianchi, S., Bernasconi, R., deHaan, C.H.M. and Molinari, M. (2010) Coronaviruses Hijack LC3-I-Positive EDEMosome Membranes for Replication. Cell Host & Microbe).
Table of Contents

1. INTRODUCTION 1

1.1 BIOSYNTHESIS OF CORE OLIGOSACCHARIDES AND THEIR TRANSFER FROM A LIPID DONOR ONTO NASCENT CHAINS 2
  1.1.1 The N-X-S/T sequon is a short and clearly defined acceptor sequence 2
  1.1.2 A large, hydrophilic and branched oligosaccharide structure is transferred to proteins 3

1.2 SEQUENTIAL PROCESSING OF THE OLIGOSACCHARIDE YIELDS SPECIFIC N-GLYCAN STRUCTURES THAT DIRECT PROTEIN FOLDING, EXPORT OR DEGRADATION 4
  1.2.1 Processing of Glc3Man3GlcNAc2 by glucosidase I 4
  1.2.2 Processing of Glc3Man3GlcNAc2 and of Glc3Man6GlcNAc2 by glucosidase II: Association with and release from calnexin and calreticulin 5
  1.2.3 Processing of Man6GlcNAc2 by UGT1: The calnexin/calreticulin cycle 7

1.3 GLYCAN-DEPENDENT EXPORT OF NATIVE PROTEINS FROM THE ER: THE CARGO RECEPTORS VIPL, ERGIC-53 AND VIP36 8

1.4 GLYCAN-DEPENDENT QUALITY CONTROL AND ERAD 9
  1.4.1 Generating the degradation signal: Processing of Man6GlcNAc2 by members of the GH family 47 10
  1.4.2 Interpreting the degradation signal: ER lectins Yos9p, OS-9 and XTP3-B 11

1.5 BIBLIOGRAPHY 12

2. A DUAL TASK FOR THE XBP1-RESPONSIVE OS-9 VARIANTS IN THE MAMMALIAN ER: INHIBITING SECRETION OF MISFOLDED PROTEIN CONFORMERS AND ENHANCING THEIR DISPOSAL 17

2.1 INTRODUCTION 19

2.2 RESULTS 20
  2.2.1 Two splice variants of OS-9 are expressed in mammalian cells 20
  2.2.2 OS-9 is a N-glycosylated protein of the ER lumen 21
  2.2.3 OS-9 is an Ire1/Xbp1-inducible ER-stress regulated gene 23
  2.2.4 OS-9 variants associate with NHK and inhibit secretion of extensively misfolded conformers from the ER lumen 24
  2.2.5 OS-9 variants do not associate with A1-antitrypsin and do not affect secretion of endogenous and ectopic native proteins 26
  2.2.6 Reduction of the intralumenal level of OS-9 reduces tightness of retention-based ER quality control 26
  2.2.7 OS-9 variants unproductively associate with non-glycosylated ERAD substrates 28
  2.2.8 A functional MRH domain is dispensable for substrate-association and for activity of OS-9 29

2.3 DISCUSSION 31

2.4 MATERIALS AND METHODS 33

2.5 BIBLIOGRAPHY 36

2.6 SUPPLEMENTARY FIGURES 38

3. STRINGENT REQUIREMENT FOR HRD1, SEL1L AND OS-9/XTP3-B FOR DISPOSAL OF ERAD-L5 SUBSTRATES 41

3.1 INTRODUCTION 43

3.2 RESULTS 46
  3.2.1 Consequences of HRD1 deletion on disposal of membrane-anchored and soluble BACE476 46
  3.2.2 HRD1 activity is required for efficient disposal of BACE476Δ 48
  3.2.3 Consequences of HRD1 deletion on disposal of membrane-anchored and soluble CD3-δ 49
  3.2.4 HRD1 and GP78 requirements for disposal of membrane-tethered and soluble BACE476 50
  3.2.5 HRD1 and GP78 requirements for efficient disposal of membrane-tethered and soluble CD3-δ 52
  3.2.6 SEL1L is required for efficient disposal of ERAD-L5 substrates 53
  3.2.7 OS-9 and XTP3-B are interchangeable ERAD shuttles required for efficient disposal of ERAD-L5 but not of ERAD-L6 substrates 55
  3.2.8 Down-regulation of SEL1L and HRD1 allows identification of OS-9.1 as delivery factor for BACE476Δ 56
3.2.9 Disposal of the classical ERAD-L5 substrate NHK and of its two ERAD-L5 variants NHKrace and NHKCD3 57
3.3 Discussion 60
3.4 Materials and Methods 64
3.5 Bibliography 67
3.6 Supplementary Figures 70

4. Cyclophilin B promotes degradation of misfolded polypeptides from the mammalian endoplasmic reticulum 75
4.1 Introduction 77
4.2 Results 78
4.2.1 Assessing the involvement of PPIs in ERAD 78
4.2.2 Involvement of the luminal immunophilin Cyclophilin B in the ERAD-L5 pathway 79
4.2.3 The enzymatic activity is required for Cyclophilin B-assisted acceleration of ERAD-L5 81
4.2.4 Cyclophilin B is only required for disposal of non-membrane tethered BACE457Δ containing cis peptidyl-prolyl bonds 82
4.3 Materials and Methods 85
4.4 Bibliography 87

5. Coronavirus hijack LC3-I-positive edemosome membranes for replication 89
5.1 Introduction 91
5.2 Results 93
5.2.1 MHV infection does not require an intact autophagy machinery 93
5.2.2 Non-lipidated LC3/ATG8 associates with CoV-induced DMVs 94
5.2.3 Analogies between MHV-induced DMVs and EDEMosomes 96
5.2.4 MHV infection interferes with ERAD tuning and results in accumulation of ERAD tuning substrates in the virus-induced DMVs 99
5.2.5 LC3-I is required for CoV replication 100
5.3 Discussion 103
5.4 Materials and Methods 105
5.5 Bibliography 108
5.6 Supplementary Figures 111

6. Concluding remarks and perspectives 117

Appendix 125
Curriculum Vitae 125
List of Publications 126
Acknowledgements 127
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BACE</td>
<td>Beta-site APP Cleaving Enzyme 1</td>
</tr>
<tr>
<td>CNX</td>
<td>Calnexin</td>
</tr>
<tr>
<td>CoV</td>
<td>Coronavirus</td>
</tr>
<tr>
<td>CRT</td>
<td>Calreticulin</td>
</tr>
<tr>
<td>DMVs</td>
<td>Double Membrane Vesicles</td>
</tr>
<tr>
<td>EDEM</td>
<td>ER Degradation Enhancing α-Mannosidase I-like protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-Associated Degradation</td>
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<tr>
<td>ERManI</td>
<td>ER α1,2-Mannosidase I</td>
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<tr>
<td>GI</td>
<td>α-Glucosidase I</td>
</tr>
<tr>
<td>GII</td>
<td>α-Glucosidase II</td>
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<tr>
<td>GH</td>
<td>Glycosyl Hydrolase</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetylglucosamine</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney cells</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>MHV</td>
<td>Mouse Hepatitis Virus</td>
</tr>
<tr>
<td>MRH</td>
<td>Mannose 6-phosphate Receptor Homology</td>
</tr>
<tr>
<td>OST</td>
<td>Oligosaccharyl Transferase</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein Disulfide Isomerase</td>
</tr>
<tr>
<td>PPI</td>
<td>Peptidyl-Prolyl cis/trans Isomerase</td>
</tr>
<tr>
<td>RTCs</td>
<td>Replication and Transcription Complexes</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucose:Glycoprotein Glucosyltransferase</td>
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The processing of N-linked glycans determines secretory protein homeostasis in the eukaryotic cell. Folding and degradation of glycoproteins in the endoplasmic reticulum (ER) are regulated by molecular chaperones and enzymes recruited by specific oligosaccharide structures. Recent findings have identified several components of this protein quality control system that specifically modify N-linked glycans thereby generating oligosaccharide structures recognized by carbohydrate-binding proteins, lectins. These latter direct newly synthesized polypeptides to the folding, secretion or degradation pathways. The “glyco-code of the ER” displays the folding status of a multitude of cargo proteins. Deciphering this code will be instrumental to understand regulation of protein homeostasis in eukaryotic cells and to intervene in such processes that have crucial importance for clinical and industrial applications.

This is part of a multi-author review published in Trends in Biological Sciences. My contribution was the construction of the figures.
1.1 Biosynthesis of core oligosaccharides and their transfer from a lipid donor onto nascent chains

Asparagine (N)-linked protein glycosylation is a covalent protein modification that occurs across the three domains of life: bacteria, archaea and eukaryotes. In eukaryotic cells, N-linked glycosylation constitutes the most prominent modification of secretory proteins. This complex biosynthetic pathway has best been studied in the fungus *Saccharomyces cerevisiae*, but it seems to be highly conserved in other fungal, plant and animal species (1). The pathway initiates at the ER membrane where a lipid carrier, dolichylpyrophosphate, serves as a membrane anchor for the assembly of an oligosaccharide. The bipartite biosynthesis of the oligosaccharide initiates at the cytoplasmic side of the ER membrane. Nucleotide-activated sugar donors serve as substrates for a series of different glycosyl transferases that lead to a mannose$_5$-N-acetylglucosamine$_2$ oligosaccharide. This lipid-bound oligosaccharide is translocated across the membrane (2) where an additional four mannose and three glucose residues are added. In the ER lumen, dolichylphosphomannose and dolichylphosphoglucose act as substrates for the individual glycosyl transferases. The transfer of the N-glycan precursor onto nascent polypeptide chains is catalyzed by the oligosaccharyltransferase (OST), a protein complex consisting of eight different subunits in yeast. OST utilizes the activated glucose$_3$-mannose$_9$-N-acetylglucosamine$_2$ (Glc$_3$Man$_9$GlcNAc$_2$) oligosaccharide donor as a substrate to covalently modify defined asparagine side chains within the acceptor sequence N-X-S/T (X cannot be a proline) (1). Most of the eukaryotic species studied transfer this specific oligosaccharide structure to proteins (Fig. 1).

N-glycosylation is in many aspects a unique covalent protein modification, but in the context of this review, the spatio-temporal aspect of the transfer reaction and the physico-chemical properties of the oligosaccharide are of central importance.

1.1.1 The N-X-S/T sequon is a short and clearly defined acceptor sequence

The N-X-S/T sequon can be found frequently in proteins. In contrast to other covalent post-translational modifications such as O-glycosylation or phosphorylation, no additional, large protein domain is required to define an N-linked glycosylation site. However, several studies suggest that the N-X-S/T sequence can only be
modified if it lies within a flexible domain of the polypeptide (3), suggesting that the peptide acceptor takes on a defined structure in the course of the modification reaction. Accordingly, the overall number of proteins and sites that are glycosylated can be increased by executing N-glycosylation before the folding process, during or immediately after the translocation of the nascent chain into the ER lumen (4). Indeed, in many eukaryotic systems, the OST is associated with the translocon and with the ribosome (5). It has also been proposed that defined subunits of the OST might act as chaperones or enzymes to modulate, or even prevent, the folding of the target proteins in order to facilitate N-glycosylation (6,7).

1.1.2 A large, hydrophilic and branched oligosaccharide structure is transferred to proteins
This hydrophilic structure itself (Fig. 1) affects the solubility and folding of proteins (1). More importantly, it can be modified by several ER-localized glycosyl hydrolases and one glucosyl transferase in a cascade of reactions that, due to the branched structure of the oligosaccharide, can result in a variety of structures that serve as ligands for carbohydrate binding proteins, lectins (Fig. 2).
These characteristics of N-glycans, the possibility to transfer them to different sites of proteins without the need of an extended primary protein acceptor sequence, the sequential processing of the oligosaccharide and the binding of specific lectins are a pre-requisite for the function of N-glycan structures as clearly defined signal molecules in the process of protein folding and quality control. This signal, either alone or in combination with specific properties of the covalently attached polypeptide, determines the fate of a glycoprotein in the ER. Accordingly, not all N-linked glycans of a given protein are used as such signals (8,9).

1.2 Sequential processing of the oligosaccharide yields specific N-glycan structures that direct protein folding, export or degradation

1.2.1 Processing of Glc$_3$Man$_9$GlcNAc$_2$ by glucosidase I
The tri-glucosylated form of the protein-bound oligosaccharide has a half-life of few seconds. The outermost glucose residue (glucose n, Fig. 1) might in fact be removed immediately after addition of the oligosaccharide onto the polypeptide nascent chain by glucosidase I. This α1,2 exo-glucosidase is a type II membrane protein member of the glycosyl hydrolase (GH) family 63 (10) that is associated with the translocon complex in close proximity to the OST (11). This observation supports the hypothesis that glucosidase I processing contributes to the efficiency of glycosylation by shifting the direction of the equilibrium of the OST reaction towards the product. So far, the Glc$_2$Man$_9$GlcNAc$_2$ structure generated by the glucosidase I has attracted little interest; indeed it was considered to be a transient trimming intermediate rapidly processed by glucosidase II to the more long-lived mono-glucosylated form of the protein-bound oligosaccharide. However, the recent characterization of malectin, a well-conserved ER-resident type I membrane protein as a putative Glc$_2$-high mannose-binding lectin (12), hints at a possible function of this processing intermediate of N-linked glycans in protein biogenesis and quality control (Fig. 2).
Figure. 2. Structures, generation and functions of polypeptide-bound N-glycans. Several different oligosaccharide structures are displayed on immature cargo polypeptides located in the ER lumen. Most of the oligosaccharide structures, as well as the glycosyl hydrolases involved in their generation are conserved in mammals and in S. cerevisiae. Mammalian cells are characterized by the presence of a re-glucosylating activity (UGT1) and a more extensive de-mannosylation of non-native polypeptides can occur, resulting in the removal of up to 4 α1,2-bonded mannose residues by one or more members of the GH family 47. The right-hand column illustrates the signal encoded by the combination of a given N-glycan structure and the associated polypeptide. Mammalian-specific features are highlighted with a gray background.

1.2.2 Processing of Glc$_2$Man$_9$GlcNAc$_2$ and of Glc$_1$Man$_9$GlcNAc$_2$ by glucosidase II: Association with and release from calnexin and calreticulin

The glucose residues m and l (Fig. 1) are removed by glucosidase II. This α1,3 exo-glucosidase is a luminal member of the GH family 31 and contains a catalytic α-subunit and a regulatory β-subunit (10). The β-subunit contains a mannose 6-phosphate receptor homology domain (MRH) (13) and comprises the conserved C-
terminal -XDEL sequence that retains the holoenzyme in the ER lumen. The \( \alpha \)-subunit alone possesses hydrolytic activity toward \( p \)-nitrophenyl \( \alpha \)-D-glucopyranoside (14,15), but the \( \beta \)-subunit is required for processing of the core oligosaccharide (14).

The regulation of glucosidase II function is not fully understood. In a fast and possibly concerted action with glucosidase I, glucosidase II first removes glucose m (Fig. 1) to generate Glc\(_1\)Man\(_9\)GlcNAC\(_2\), the ligand of the ER-resident lectin chaperones calnexin and calreticulin (Fig. 2). Calnexin is a type I transmembrane protein and calreticulin is its soluble homolog. They are composed of a globular carbohydrate-binding domain, which folds into a leguminous (L)-type lectin-like \( \beta \)-sandwich structure, and a proline-rich P-domain, which recruits the lectin-associated oxidoreductase ERp57 (16,17). ERp57 catalyzes the formation of native disulfide bonds in folding polypeptides. The different membrane topology of the two ER lectins determines their substrate specificity. The membrane-anchored calnexin preferentially interacts with polypeptide-bound mono-glucosylated glycans close to the membrane, whereas calreticulin prefers association with more peripheral glycans (18-20). Reduction of the physical constraints upon release from calnexin/calreticulin/ERp57 results in a structural collapse that often leads to attainment of the native polypeptide structure (21).

Upon release of the folding polypeptide from calnexin and calreticulin, glucosidase II removes the innermost glucose l (Fig. 1), thereby generating the Man\(_9\)GlcNAC\(_2\) structure. Although both reactions catalyzed by glucosidase II remove an \( \alpha_1,3 \)-linked glucose, the second cleavage event requires the transient separation and repositioning of the glucosidase II active site (22). This time window is possibly exploited by calnexin and calreticulin to associate with the folding polypeptide (11). When tested in isolated microsomes with chains arrested in the translocon, glucosidase II activity progresses with high efficiency only when a second N-linked glycan is available on the same polypeptide chain. It has been suggested that association of the \( \beta \)-subunit with the 6'-tetramannosyl branch of an oligosaccharide allows proper positioning of the \( \alpha \)-subunit to cleave glucose l from a distinct oligosaccharide in spatial proximity (11). However, both \textit{in vitro} (14,23,24) and \textit{in vivo} (25), the presence of a single N-linked glycan is sufficient for glucosidase II activity and for substrate association with calnexin and calreticulin. The rapidity of
the concerted action of glucosidase I and glucosidase II in living cells allows immediate association of polypeptide chains emerging in the ER lumen with calnexin, calreticulin and ERp57, thereby resulting in co-translational formation of native disulfide bonds (26,27).

1.2.3 Processing of Man$_9$GlcNAc$_2$ by UGT1: The calnexin/calreticulin cycle

Glycoproteins can fold properly after one single association with a lectin chaperone, as observed in mammalian cells (28) and in Schizosaccharomyces pombe (29). However, proper maturation of the glycoprotein might require more than a single association (30,31). The ER of multicellular eukaryotes, of certain fungi (e.g. S. pombe and Mucor rouxii) and of unicellular eukaryotes such as Trypanosoma cruzi (32) contains a folding sensor, the UDP-glucose:glycoprotein glucosyltransferase (UGT1 or UGGT), a member of the glycosyl transferase family 24 (10). UGT1 consists of a large N-terminal domain that binds non-native protein structures and a C-terminal carbohydrate transferase domain (33). This bi-functionality allows UGT1 to "inspect" polypeptides which display Man$_9$GlcNAc$_2$ oligosaccharides and to exclusively re-glucosylate the terminal mannose g (Fig. 1) in polypeptides which have a pseudo-native protein structure (34,35). It remains controversial whether UGT1 activity requires the proximity of the oligosaccharide to be re-glucosylated with a structural defect in the immature polypeptide chain (36,37). Regeneration of the Glc$_1$Man$_9$GlcNAc$_2$ oligosaccharide results in substrate re-association with calnexin/calreticulin/ERp57 for another round of folding-attempts (Fig. 2). The repeated removal and re-addition of glucose I by the counteracting actions of glucosidase II and UGT1 drives cycles of substrate release and re-association with the lectin chaperones, the so called calnexin/calreticulin cycle (38). During the off-phase, N-glycans are exposed to ER-resident α1,2-mannosidases belonging to the GH family 47 (39) that can remove, one-by-one, the terminal α1,2-bonded mannose residues. Progressive N-glycan de-mannosylation renders the associated polypeptide a weaker ligand for calnexin/calreticulin (40), a better substrate for the glucosidase II (23) and a suboptimal substrate for the UGT1 (41) (Fig. 3). Altogether, it facilitates the interruption of folding attempts and directs folding-defective polypeptides into the ER-associated degradation (ERAD) pathway (42).
Fig. 3. Oligosaccharide preferences of ER-resident glycosyl transferase, glycosyl hydrolases, glucosyl transferase and lectins. The figure summarizes the transfer of the core oligosaccharide on newly synthesized cargo polypeptides (OST), the progressive modification of the N-linked glycan by glycosyl hydrolases (glucosidases and mannosidases) and a glucosyl transferase (UGT1). Different oligosaccharides act as ligands for a series of ER-localized lectins (malectin, CNX, CRT, cargo receptors, OS-9 and XTP3-B splice variants). A color code shows arbitrary strength of affinities/activities of the given ER resident sugar binding/modifying protein for a given oligosaccharide structure (from white (no affinity/activity) to red (higher affinity/activity)). For cargo receptors and ERAD lectins also refer to (52). References are in the text.

### 1.3 Glycan-dependent export of native proteins from the ER: The cargo receptors VIPL, ERGIC-53 and VIP36

Glycopolypeptides are offered a time window to complete their folding program in the calnexin chaperone system (42). Upon attainment of the native structure, the majority of cargo proteins are exported from the ER in vesicles coated with cytosolic COatamer Protein II (COPII) that bud at ER exit sites (43). In mammalian cells, these transport vesicles undergo homotypic fusion to generate a stationary ER-Golgi intermediate compartment (ERGIC) from which cargo proteins reach the cis-Golgi in COPI-coated vesicles (44). By contrast, in yeast, COPII-coated cargo vesicles are directly delivered to the Golgi compartment (45). Transmembrane proteins can directly interact with the cytosolic COPII coat, whereas soluble cargo proteins can require specific receptors for recruitment into COPII-coated vesicles (46). ER export
of certain glycosylated proteins is facilitated by several leguminous L-type lectins located in the ER (VIPL), cycling between ER and ERGIC (ERGIC-53) or between ERGIC and cis-Golgi (VIP36) (47-49). Emp47p and Emp46p, yeast orthologs of ERGIC-53, have been proposed to act as cargo receptors between the ER and the Golgi in S. cerevisiae (50,51).

It is significant that VIP36 and VIPL preferentially associate with native proteins displaying high mannose glycans with three mannose residues, but not glucose, on the oligosaccharide branch A (i.e. Man$_9$ oligosaccharides, Man$_8$ structures lacking the terminal mannose residue on branch B or the one on branch C and Man$_7$ structures lacking both terminal mannoses on branches B and C) (49) (Fig. 3). These cargo export lectins have much lower affinity for extensively de-mannosylated glycans that, consistently, serve to tag the associated polypeptide for disposal rather than for export (52). A third cargo receptor, ERGIC-53, binds with lower affinity to a broader range of oligosaccharides, even if they are capped with a terminal glucose residue (49) (Fig. 3).

### 1.4 Glycan-dependent quality control and ERAD

Folding-defective polypeptides or components that do not incorporate into protein complexes must eventually be cleared from the ER folding environment. In both S. cerevisiae and higher eukaryotes, the importance of mannose trimming for ERAD of misfolded glycoproteins has been reported (9,53,54), supporting the suggestion of a mannose timer mechanism (55). The slowly acting ER $\alpha$1,2-mannosidase I (ERManI in mammals; Mns1p in yeast) was proposed to operate as such a molecular timer, with the Man$_8$GlcNAc$_2$ isomer B generated upon removal of the $\alpha$1,2-bonded mannose of the central oligosaccharide branch acting as the N-glycan degradation signal if displayed on terminally misfolded polypeptides (Fig. 2). More recent results suggest that more extensive de-mannosylation of misfolded polypeptides occurs (52) and that, at least in yeast, different members of the GH family 47 of $\alpha$1,2-mannosidases intervene sequentially to generate the ERAD signal (56). In mammalian cells, the GH family 47 of $\alpha$1,2-mannosidases comprises the ERManI and the ER degradation enhancer, mannosidase alpha-like proteins EDEM1,
EDEM2, EDEM3 (Htm1p in yeast) as well as the three Golgi-resident MAN1A, MAN1B and MAN1C (10).

1.4.1 Generating the degradation signal: Processing of Man$_9$GlcNAc$_2$ by members of the GH family

Recent analysis in *S. cerevisiae* revealed that removal of at least two $\alpha$1,2-linked mannose residues from the protein-bound oligosaccharide is required for degradation of glycoproteins. The N-glycan degradation signal is a terminal $\alpha$1,6-linked mannose, generated by processing of the C branch (removal of mannose k, Fig. 1) of a Man$_9$GlcNAc$_2$ glycan (56,57). This reaction is catalyzed by Htm1p (EDEM), which functionally depends on prior processing of the N-glycan by glucosidase I and II as well as Mns1p (ERManI) (56). Htm1p was found in a complex with the oxidoreductase Pdi1p. Similarly to the function proposed for the EDEM1-associated mammalian reductase ERdj5 (58), Pdi1p could be recruited to identify non-properly folded proteins as substrates for Htm1p or to promote unfolding of ERAD substrates to facilitate their dislocation across the ER membrane.

In higher eukaryotes further processing of Man$_9$GlcNAc$_2$ was observed as well, and generation of Man$_7$GlcNAc$_2$, Man$_6$GlcNAc$_2$ and Man$_5$GlcNAc$_2$ glycan structures was reported to precede or elicit degradation (59-64) (Fig. 2). Interestingly, in cell lines characterized by N-glycosylation with aberrant oligosaccharides lacking the cleavable mannose residues on branches B and C (e.g., the Chinese hamster ovary mutant lines B3F7 and MadIA214), processing of $\alpha$1,2-linked mannose residues is still required for glycoprotein disposal (64,65). In these cells the only cleavable mannose residues are those on the branch A that when modified with a terminal $\alpha$1,3-bonded glucose determine polypeptide retention by the calnexin chaperone system. Extraction from calnexin/calreticulin is only possible by endo-mannosidase cleavage of the A-branch or by preventing entry into the cycle due to exo-mannosidase removal of the re-glucosylatable mannose g (Fig. 1). These data highlight the importance of extraction of folding-defective glycopolypeptides from the calnexin/calreticulin chaperone system, which becomes irreversible, only upon branch A de-mannosylation (66). Based on the temporal order of N-glycan processing that is determined by the substrate specificity of the different glycosyl hydrolases (Figs. 2-3), we propose that removal of the terminal $\alpha$1,2-linked mannose
of the A branch ensures the exit from the calnexin/calreticulin cycle and is necessary, but not sufficient, for glycoprotein degradation. Additional trimming of the C branch generates the terminal misfolding signal, the α1,6-linked mannose, recognized by the human ER-degradation lectins OS-9 and XTP3-B (Yos9p) (67-69), which targets the N-glycoprotein for ERAD (Fig. 2).

1.4.2 Interpreting the degradation signal: ER lectins Yos9p, OS-9 and XTP3-B

The finding that the chemical structure of the N-glycan is important in the degradation of misfolded glycoproteins led to the proposal that a lectin receptor is involved in the recognition of ERAD substrates (54,70,71). Yos9p and mammalian OS-9.1/OS-9.2 and XTP3-B1/XTP3-B2 constitute a group of proteins that have lectin-like domains with homology to the mannose-6-phosphate receptor family and are implicated in the recognition of misfolded glycoproteins for degradation (68,69). *S. cerevisiae* Yos9p is part of the Hrd1p complex (named for HMG-CoA Reductase Degradation) to which it is bound via Hrd3p, a transmembrane protein with a large luminal domain, where it performs a proof-reading or gating function (72,73). Hrd3p recruits the misfolded proteins while Yos9p scans the substrate for the correct N-glycan structure, signaling terminal misfolding. Determination of the N-glycan substrate specificity of Yos9p revealed that it binds N-glycans that have a terminal α1,6-linked mannose (57). This corroborates the working model that this N-glycan structure, generated by Htm1p (EDEM) (56), is the N-glycan signal for glycoprotein degradation in yeast. Mammalian OS-9 and XTP3-B splice variants were also found in large complexes containing the HRD1 E3 ubiquitin ligase and SEL1L (orthologs of Hrd1p and Hrd3p, respectively) (67-69,74,75). The components of this mammalian complex are similar to those in *S. cerevisiae*, suggesting evolutionary conservation. The lectin activity of OS-9 is required for enhancement of glycoprotein ERAD, and like Yos9p, OS-9 can specifically bind N-glycans lacking the terminal α1,2-linked mannose from the C branch (67) (Fig. 3). However, a study using lectin mutants suggested that the MRH domains of OS-9 and XTP3-B might not be required for binding to ERAD substrates, but rather for interaction with SEL1L (69), which carries several N-glycans (76,77).
1.5. Bibliography

A dual task for the Xbp1-responsive OS-9 variants in the mammalian ER: Inhibiting secretion of misfolded protein conformers and enhancing their disposal

Riccardo Bernasconi, Thomas Pertel, Jeremy Luban and Maurizio Molinari

Institute for Research in Biomedicine, CH-6500 Bellinzona, Switzerland

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Normally, non-native polypeptides are not transported through the secretory pathway. Rather, they are translocated from the endoplasmic reticulum (ER) lumen into the cytosol where they are degraded by proteasomes. Here we characterize the function in ER quality control of two proteins derived from alternative splicing of the OS-9 gene. OS-9.1 and OS-9.2 are ubiquitously expressed in human tissues and are amplified in tumors. They are transcriptionally induced upon activation of the Ire1/Xbp1 ER-stress pathway. OS-9 variants do not associate with folding-competent proteins. Rather, they selectively bind folding-defective ones thereby inhibiting transport of non-native conformers through the secretory pathway. The intraluminal level of OS-9.1 and OS-9.2 inversely correlates with the fraction of a folding-defective glycoprotein, the Null_hong kong (NHK) variant of α1-antitrypsin that escapes retention-based ER quality control. OS-9 up-regulation does not affect NHK disposal, but reduction of the intraluminal level of OS-9.1 and OS-9.2 substantially delays disposal of this model substrate. OS-9.1 and OS-9.2 also associate transiently with non-glycosylated folding-defective proteins, but association is unproductive. Finally, OS-9 activity does not require an intact mannose 6-P homology domain (MRH).

Thus, OS-9.1 and OS-9.2 play a dual role in mammalian ER quality control: firstly as crucial retention factors for misfolded conformers, and secondly as promoters of protein disposal from the ER lumen.
2.1 Introduction

About 30% of the eukaryotic gene products are synthesized by ribosomes attached at the cytosolic face of the ER (1). The nascent polypeptide chains are translocated into the ER lumen where molecular chaperones and folding enzymes assist their maturation. Native proteins are transported at their intra- or extracellular destination through the secretory pathway. The ER lumen also contains chaperones and enzymes that retain and appropriately tag terminally misfolded proteins for destruction. Due to the facility of manipulating the yeast genome, many aspects and components of ERAD have been discovered in *S. cerevisiae* (2-4). Yos9p is no exception. It was initially reported that deletion of Yos9p from the yeast ER selectively inhibits degradation of glycosylated ERAD substrates (5). Subsequent work revealed that Yos9p is required for disposal of substrates with luminal folding-defects, while it is dispensable for disposal of proteins with defects in the transmembrane and cytosolic domains (6-10). Studies on the involvement of the mammalian ortholog OS-9 in ERAD have been hampered by data showing that OS-9 is a cytosolic protein (11). This study was followed by a series of publications in which experimental design and interpretation of the data were based on the assumption that OS-9 is a cytosolic protein (12-14).

Our analysis shows that OS-9 is a N-glycosylated protein expressed in two splice variants in the ER lumen. Transcription of both OS-9 variants is enhanced upon activation of the Ire1/Xbp1 pathway in cells exposed to acute ER stress. OS-9 variants do not associate with folding-competent proteins, but form non-covalent complexes with misfolded ones. OS-9 association prevents secretion from the ER of misfolded NHK conformers and facilitates NHK disposal. OS-9 variants play a crucial role in maintaining the tightness of retention-based ER quality control and in promoting disposal of misfolded proteins from the mammalian ER.
2.2 Results

2.2.1 Two splice variants of OS-9 are expressed in mammalian cells

The OS-9 gene is composed of 15 exons (17). Alternative splicing events that conform to the GT-AG rule could generate 4 variants of the protein. The full-length version OS-9.1, which comprises 667 amino acids in humans; OS-9.2, which lacks the entire exon 13 and is 55 residues shorter with a glutamate to glycine conversion in the splice region; OS-9.3, which lacks exon 13 and the final part of exon 11 and is 70 residues shorter; OS-9.4, which lacks the final part of exon 11 resulting in a 15-residues deletion (schematics in Fig. 1A and (18)). The expression of OS-9.1 and OS-9.2, two splice variants derived from a single transcript, has been shown in osteosarcoma cell lines (18).

To determine which splice variants are expressed in HEK293 cells, we amplified the total cellular cDNA pool with specific primers. Our analysis revealed expression of transcripts for OS-9.1 (Fig. 1B, amplification products of 461 in the left panel and 333 bp in the right panel) and OS-9.2 (Fig. 1B, amplification products of 296 bp in the left panel). Transcripts for variants 3 and 4 were not expressed (Fig. 1B, lack of amplification products of 416 and 251 bp in the left and of 288 bp in the right panel, respectively). To confirm expression of OS-9.1 and OS-9.2 at the protein level, HEK293 cells were detergent-solubilized and the proteins contained in the post nuclear supernatant were separated by SDS-PAGE, transferred onto PVDF membrane, and probed with polyclonal antibodies raised against OS-9. The antibodies recognized two polypeptide bands (Fig. 1C, lane 1). The upper band co-migrated with ectopically expressed OS-9.1 (Fig. 1C, lane 2) and the lower with OS-9.2 (Fig. 1C, lane 3). Similar results were obtained with mouse embryonic fibroblasts (MEF), thus showing that mammalian cultured cells contain two splice variants of OS-9: OS-9.1 and OS-9.2.
Figure 1. OS-9 is a glycosylated, stress inducible ER resident protein. A Schematic view of OS-9, numbering is for the human protein. The signal peptide (SP), the mannose 6-phosphate receptor homology domain (MRH) and the putative splice regions (grey part of exon 11 and the entire exon 13) are shown. B Whole cDNA pool from HEK293 cells was amplified with specific primers (depicted in panel A) to determine the expression of OS-9 splice variants. Theoretical lengths are shown on the right, the position of 100, 200, 300, 400 and 500 bp markers is shown on the left. C Expression of endogenous (lane 1) and of ectopic OS-9.1 (lane 2) and OS-9.2 (lane 3) is revealed by immunoblot (IB). D EndoH-treatment to confirm modification with N-linked oligosaccharides of the endogenous OS-9 variants revealed by IB and of ectopically expressed, labeled OS-9 variants immunoisolated (IP) from cell-lysates. E The presence of intramolecular disulfide bond was confirmed by changes in OS-9 mobility in non-reducing (NR) vs. reducing (R) gels. F Immunofluorescence showing co-localization between PDI (left panel) and ectopically expressed OS-9 (right panel). G Endomembranes were purified from metabolically labeled HEK293 cells. The select proteins (calreticulin (crt), calnexin (cnx), OS-9 and EDEM1) were immunoisolated from luminal (lanes 1-4) or membrane fractions (lane 5-8). The OS9 and EDEM1 mobility is shown in lanes 9 and 10, respectively. H HEK293, wt and Xbp1-/- MEF were exposed (+) or not (-) to 2.5 µg/ml Tun for 12 h to induce ER stress. The levels of OS-9, EDEM1, Synoviolin (Xbp1-dependent), Bip and Sel1L (ATF6-dependent) and Actin (loading control) have been assessed by semi-quantitative RT-PCR. Depletion of Xbp1 prevents stress induction of both OS-9 transcripts. I Quantitative RT-PCR analysis of induction of Bip, EDEM1, OS-9, Sel1L and Synoviolin upon ER stress in wt and Xbp1-/- MEF. Actin was used as endogenous control (n=2).

2.2.2 OS-9 is a N-glycosylated protein of the ER lumen

Yos9p, the yeast ortholog of mammalian OS-9 has an amino terminal signal sequence for protein targeting into the ER lumen and a C-terminal HDEL signal for ER-retrieval. Consistent with intralumenal localization, Yos9p displays 4 N-linked glycans, a hallmark of proteins synthesized in the ER. The human ortholog OS-9 has
been reported to be associated with the cytosolic face of the ER membrane (11). Instead, our results clearly showed that OS-9 variants are soluble proteins in the ER. First, as for the yeast protein, the hidden Markov model used by SignalP (19) shows that both the human and the mouse OS-9 have a cleavable N-terminal signal sequence, a characteristic of proteins translocated into the ER (20).

Second, endogenous and ectopically expressed OS-9.1 and OS-9.2 are N-glycosylated, a covalent modification of proteins translocated into the ER (21) that would not occur if OS-9 would face the cytosol as claimed in (11). For the endogenous proteins, this was established by subjecting detergent-lysates of HEK293 cells to Mock- or to EndoH-treatment. EndoH specifically removes N-linked oligosaccharides from ER proteins (22). Cellular proteins from Mock- and EndoH-treated detergent-lysates were separated in SDS-PAGE, transferred onto PVDF membranes and probed with antibodies raised against endogenous OS-9 (eOS9) (Fig. 1D, lane 1 (Mock) and 2 (EndoH)). EndoH-treatment reduced the apparent MW of both OS-9 variants of about 4 kDa compared to untreated samples. The faster electrophoretic mobility is consistent with removal of 1 N-linked glycan from asparagine 177, which is the only acceptor site for oligosaccharides in the OS-9.1 and OS-9.2 sequences. N-glycosylation was also confirmed for ectopically expressed OS-9 (rOS9). In this case, HEK293 cells were transfected with plasmids for expression of OS-9.1 (Fig. 1D, lanes 3-4) or OS-9.2 (lanes 5-6). Seventeen hr post-transfection, cells were metabolically labeled with $^{35}$S-methionine and -cysteine. Labeled OS-9.1 and OS-9.2 were immunoisolated with specific antibodies from detergent lysates and the immuno-complexes were mock-treated (lanes 3 and 5) or were incubated for 1 hr with EndoH (lanes 4 and 6). EndoH-treatment enhanced the electrophoretic mobility of the ectopically expressed proteins, thus confirming their glycosylated status (compare lane 3 with 4 in Fig. 1D for OS-9.1 and lane 5 with 6 for OS-9.2).

Third, the electrophoretic mobility of both OS-9 variants is slower when the proteins are separated under reducing (R, Fig. 1E) rather than under non-reducing (NR) conditions. This shows that OS-9 cysteines are engaged in intramolecular disulfide bonds (23), another hallmark of proteins translocated in the ER lumen. Fourth, immunofluorescence analysis in HEK293 cells revealed the co-localization of ectopically expressed OS-9 with protein disulfide isomerase (PDI), a canonical ER marker (Fig. 1F).
To determine whether OS-9 variants are membrane-bound or luminal proteins, HEK293 cells were metabolically labeled and extensively washed. Cells were broken with 10 passages through a 25G1 needle. Post nuclear supernatants were subjected to ultracentrifugation to separate endo-membranes from the cytosol. The endo-membrane pellet was washed twice to remove cytosolic contaminants and was subsequently extracted with carbonate to separate the luminal content (Fig. 1G, lanes 1-4) from the membrane-bound proteins (lanes 5-8). Calreticulin and EDEM1 served as luminal markers (24,25). Consistently, they mostly partitioned in the luminal fraction (Fig. 1G, lanes 1 and 4, respectively) and were only weakly detected in the membrane fraction (lanes 5 and 8). On the other hand, the ER membrane marker calnexin partitioned in the membrane fraction (lane 6) and was not found in the luminal fraction (lane 2). The distribution of OS-9 variants reflected the distribution of the luminal markers calreticulin and EDEM1 as shown by their enrichment in the luminal fraction (Fig. 1G, lane 3).

2.2.3 OS-9 is an Ire1/Xbp1-inducible ER-stress regulated gene

We next determined whether OS-9 transcription is induced in cells experiencing acute ER-stress. RT-PCR analysis in HEK293 and in MEF cells revealed that the basal level of OS-9.1 transcripts is substantially lower than that of OS-9.2 transcripts (Fig. 1H, lane 1 for HEK and lane 3 for MEF). This confirms data showing that transcripts for isoform 2 are the most abundantly expressed in human sarcomas (18). Cell exposure to tunicamycin, a potent ER-stress inducer, enhanced the expression of OS-9 transcripts without affecting the splicing reaction generating OS-9.2. OS-9.2 remained the most abundant splice variant amplified in both HEK293 (Fig. 1H, lane 2) and in MEF (Fig. 1H, lane 4) subjected to acute ER stress. As positive controls, tunicamycin also enhanced expression of EDEM1, BiP, Synoviolin and Sel1L transcripts (Fig. 1H). Deletion of the Ire1-activated transcription factor Xbp1 did not prevent induction of BiP and of Sel1L, two genes regulated by the ATF6 ER-stress pathway (26), but substantially inhibited induction of EDEM1, OS-9 and Synoviolin transcription (Fig. 1H, lanes 5-6 and also refer to (27) for Xbp1-regulation of Synoviolin). These data were confirmed by quantitative real-time RT-PCR analysis (Fig. 1I). These results are intriguing because in S. cerevisiae Yos9p forms a functional complex with Hrd1p and Hrd3p, the orthologs of Synoviolin and Sel1L, respectively. It seems therefore that components of the same functional complex are
regulated by distinct stress-induced pathways. Altogether, Fig. 1 shows that OS-9 variants are soluble, highly oxidized, N-glycosylated proteins of the ER lumen, which are transcriptionally induced upon activation of the Ire1/Xbp1 ER-stress pathway.

2.2.4 OS-9 variants associate with NHK and inhibit secretion of extensively misfolded conformers from the ER lumen

Human α1-antitrypsin is a serine protease inhibitor secreted from hepatocytes (28). The NHK variant (29) is a truncated, folding-defective form of α1-antitrypsin (30). We report that in transiently transfected mammalian cultured cells, only about 75% of ectopically expressed NHK becomes an ERAD substrate. About 25% of the newly synthesized NHK escapes ER retention and is secreted from cells as aberrant disulfide-bonded dimers (see below and Fig. S1).

To determine the function of OS-9 variants in the ER lumen, HEK293 cells expressing NHK were mock transfected (Fig. 2, lanes 1-3), transfected with OS-9.1 (lanes 4-6) or with OS-9.2 (lanes 7-9). The proteins were metabolically labeled for 10 min with $^{35}$S-methionine and cysteine. After various chase times with unlabeled amino acids, the labeled intracellular NHK (Figs. 2A-B) and the labeled secreted NHK escaping retention-based ER quality control (Figs. 2C-D) were immunoisolated with specific antibodies from cell-lysates and from the culture media, respectively. Quantifications were performed by band densitometry in reducing SDS-PAGE. These experiments revealed first, that OS-9.1 and OS-9.2 associate with NHK. In fact, they did co-precipitate with the ERAD substrate (arrows in Fig. 2A, lanes 4-9). Second, that co-expression of both OS-9 variants similarly delayed disappearance of labeled NHK from the ER lumen (Figs. 2A-B). In fact, after a 120 min of chase, 30% of the labeled NHK was still retained in cells with physiologic OS-9 content (Fig. 2A, lane 3 and Fig. 2B) vs. 55% in cells expressing high levels of OS-9.1 (lane 6) or of OS-9.2 (lane 9).

The intracellular persistence of labeled NHK did not result from substantial inhibition of NHK disposal in response to ectopic OS-9 expression. Rather, it resulted from the efficient inhibition of secretion of non-native NHK. In fact, while roughly 25% of the labeled NHK normally escapes retention-based ER quality control and is secreted in the extracellular media (reducing gel shown in Fig. 2C, lanes 1-3 and Fig. 2D, Mock), this percentage dropped to less than 5% when OS-9.1 (lanes 4-6) or OS-9.2 (lanes 7-9) were individually up-regulated (Fig. 2D). Note that non-native NHK is secreted
as monomers or non-covalent oligomers \( (\text{NHK}_{\text{MONOMER}}) \) and as disulfide-bonded dimers or oligomers \( (\text{NHK}_{\text{DIMER}}) \) as shown when the proteins are separated in non-reducing gels (Fig. S1).

**Figure 2.** Consequences of OS-9 up-regulation on secretion and degradation of NHK and on secretion of \( \alpha_1 \)-antitrypsin. 

**A** Radiolabeled NHK has been immunisolated after the indicated chase times from detergent-extract of cells with normal (lanes 1-3) or elevated levels of OS-9.1 (lanes 4-6) or OS-9.2 (lanes 7-9). Note the co-precipitation of ectopic OS-9.1 and of ectopic OS-9.2. 

**B** Quantification of intracellular NHK \((n=4)\). 

**C** Secretion of labeled NHK from cells with normal (lanes 1-3) or elevated content of OS-9.1 (lanes 4-6) or OS-9.2 (lanes 7-9). 

**D** Quantification of \( \alpha_1 \)-antitrypsin \((n=3)\). 

**E** Same as **A** for \( \alpha_1 \)-antitrypsin. 

**F** Quantification of **E**. 

**G** Same as **C** for \( \alpha_1 \)-antitrypsin. 

**H** Quantification of **G**. 

**I** NHK co-precipitates with OS-9.1 (lanes 4-6) and with OS-9.2 (lanes 7-9). 

**L** \( \alpha_1 \)-antitrypsin does not co-precipitate with OS-9 variants.
2.2.5 OS-9 variants do not associate with α1-antitrypsin and do not affect secretion of endogenous and ectopic native proteins

OS-9-mediated ER-retention was specific for misfolded conformers because OS-9 over-expression did not interfere with secretion of the amyloid precursor proteins (APP), an endogenous secretory protein of HEK293 cells (arrowhead in Fig. 2C) nor with secretion of ectopically expressed α1-antitrypsin (Figs. 2E-H). Consistent with a selective association of OS-9 variants with misfolded proteins (Fig. 2A), the immuno-isolation of α1-antitrypsin did not result in the co-precipitation of ectopic OS-9.1 and OS-9.2 (Fig. 2E). Moreover, immuno-isolation of OS-9 variants resulted in the co-precipitation of NHK (Fig. 2I, lanes 4-9), but not of α1-antitrypsin (Fig. 2L, lanes 4-9), thus confirming the specificity of the associations. Note that the fraction of labeled NHK co-precipitating with OS-9.1 (Fig. 2I, lanes 4-6) and with OS-9.2 (lanes 7-9) increased with progression of chase as if association would start only after a lag phase during which NHK is subjected to unproductive folding-attempts.

2.2.6 Reduction of the intraluminal level of OS-9 reduces tightness of retention-based ER quality control

Consistent with a role of OS-9 in ER retention of non-native polypeptides, OS-9 down-regulation upon specific RNA interference (Fig. 3A and Methods section) increased, by more than 50%, the secretion of misfolded NHK (from 25% of the labeled NHK in control cells (Fig. 2C, lanes 1-3 and Fig. 2D) and in cells expressing an inactive RNA duplex (Fig. 3B, lanes 1-4 and Fig. 3C, shluc), to almost 40% in cells expressing an OS-9-targeted interfering RNA (Fig. 3B, lanes 5-8 and Fig. 3C, shOS9). Interestingly, the reduction of the intraluminal OS-9 content also delayed disappearance of intracellular NHK thus hinting at a role of endogenous OS-9 in facilitating NHK disposal (Figs. 3D-E, shOS9 and comments to Fig. 4). OS-9 down-regulation did not change the intracellular fate of the folding-competent α1-antitrypsin (Figs. 3F-I) confirming the selectivity of OS-9 for aberrant polypeptides. The specificity of the results was confirmed by back-transfection of OS-9.1 and OS-9.2 in the cells subjected to RNA interference (Fig. S2).
The pleiotropic functions of OS-9 in the mammalian ER lumen are better appreciated upon analysis of the data shown in Figs. 2-3 as summarized in Fig. 4. In cells with physiologic OS-9 content, about 30% of the labeled NHK was still retained in the ER lumen 120 min after synthesis, 22% had been secreted from cells and 48% had been degraded (Fig. 4, Mock, average of 3-5 independent experiments). Prolongation of the chase (e.g. 240 min in Figs. 2B, D) did not increase much the amount of aberrant NHK escaping ER quality control (25%, Fig. 2D), but allowed slow disposal of the NHK fraction retained in the ER (Fig. 2B). Elevation of the intralumenal OS-9 level (Fig. 4, OS-9) significantly increased the fraction of protein retained in the ER (55%), mostly at the expense of the fraction escaping ER quality control (reduced from 22-25 to 4%).
The reduction of the intralumenal level of OS-9 (Fig. 4, shOS9) also increased the amount of NHK retained in the ER lumen (from 30 to 50%). In this case, however, at the expense of the part that should have been degraded, which was reduced from 48 to roughly 20%.

Thus, OS-9 fulfills dual function in the ER as ERAD regulator and as crucial operator of retention-based ER quality control. Ectopic expression of OS-9 in cells subjected to RNA interference re-established the phenotype of cells overexpressing OS-9 because the ectopic gene lacks the 3’ UTR targeted by the siRNA (Fig. 4, shOS9+OS9 and Fig. S2).

**Figure 4.** Intracellular (Retention), secreted and degraded NHK after a 120 min chase has been determined in cells with normal (Mock, shluc), high (OS9, shOS9+OS9) and reduced (shOS9) OS-9 content. This panel summarizes the data shown in Figs. 2-3.

**2.2.7 OS-9 variants unproductively associate with non-glycosylated ERAD substrates**

In *S. cerevisiae*, Yos9p associates with non-glycosylated polypeptides, but it does not affect their fate (5,8). To assess whether OS-9 associates and regulates disposal of non-glycosylated polypeptides from the mammalian ER, we performed the same experiments shown above for the tri-glycosylated protein NHK (Fig. 2) but we used the non-glycosylated NHK_{QQQ} as a model ERAD substrate. NHK_{QQQ} is a variant of NHK in which the asparagine residues of the three consensus sequences for N-glycosylation have been replaced by glutamine residues. Fig. 5 shows that both OS-9.1 and OS-9.2 did co-precipitate with NHK_{QQQ} (panel A). OS-9 association with this non-glycosylated ERAD substrate was unproductive because it did not affect kinetics of disappearance of the labeled protein from cells (Fig. 5B, note that NHK_{QQQ} is not secreted from cells). Down-regulation of OS-9 variants only weakly delayed ERAD of NHK_{QQQ} (Figs. 5C-D). The co-precipitation of labeled NHK_{QQQ} with OS-9 variants confirmed the specificity of the interactions (Fig. 5E). It seems relevant that kinetics
of this unproductive association between OS-9 variants and NHK_{QQQ} differ from the kinetics of the productive association between OS-9 variants and NHK. In fact, only for NHK, which is glycosylated and escapes to some extent retention-based ER quality control (Figs. 2C-D), the fraction of labeled protein associated with OS-9 increases during the chase (Fig. 2l).

**Figure 5.** Consequences of OS-9 up-regulation and down-regulation on degradation of NHK_{QQQ}. A Radiolabeled NHK_{QQQ} has been immunoisolated after the indicated chase times from detergent-extract of cells with normal (lanes 1-3) or elevated levels of OS-9.1 (lanes 4-6) or OS-9.2 (lanes 7-9). Note the co-precipitation of ectopic OS-9.1 and of ectopic OS-9.2. B Quantification of intracellular NHK_{QQQ}. C Intracellular content of labeled NHK_{QQQ} for cells with normal (lanes 1-3) or reduced (lanes 4-6) OS-9 levels. D Quantification of C. E NHK_{QQQ} co-precipitates with OS-9.1 (lanes 4-6) and with OS-9.2 (lanes 7-9).

### 2.2.8 A functional MRH domain is dispensable for substrate-association and for activity of OS-9

Next, we determined whether inactivation of the MRH domain upon mutation of the conserved arginine188 residue into an alanine (8,10) affected OS-9 association with substrates and OS-9 activity. To this end, cell lines with low intracellular level of OS-9...
(Fig. 3) were back-transfected with OS-9.2\textsubscript{R188A}. As shown above for the wild type protein, the mutated protein as well did co-precipitate with NHK (Fig. 6A, lanes 3-6). This confirmed data published for the yeast ortholog Yos9p (8), and showed that substrate association does not require an intact MRH domain. In mammalian cells, however, the mutation did also not affect the function of the protein as shown by the virtually complete inhibition of secretion of the folding-defective NHK in the extracellular media (Figs. 6C-D). The co-precipitation of labeled NHK with OS-9.2\textsubscript{R188A} confirmed the specificity of the interactions and the kinetics previously observed for productive association characterized by an increased complex formation at later chase times (Fig. 6E).

**Figure 6. Consequences of overexpression of OS-9 with mutated MRH domain.** A Consequences on the fate of NHK in OS-9.2\textsubscript{R188A} back-transfected shOS9 cells. B Quantification of A. C Secretion of labeled NHK. D Quantification of C. E NHK co-precipitates with OS-9.2\textsubscript{R188A} (lanes 4-6).
2.3 Discussion

Here we show that OS-9.1 and OS-9.2 are ER-resident glycoproteins. Their intraluminal level, which is increased upon activation of the Ire1/Xbp1 ER stress-pathway, inversely correlates with tightness of retention-based ER quality control. This was established by using NHK, a folding-defective, truncated form of the secretory protein α1-antitrypsin as model substrate. In transiently transfected HEK293 cells (Figs. 2, 3, 6 and Fig. S1) and in MEF (unpublished), only about 75% of ectopically expressed NHK becomes an ERAD substrate. About 25% of the newly synthesized, misfolded NHK escapes ER retention and is secreted from transiently transfected cells. Elevation of the intralumenal OS-9 content abolishes secretion of misfolded NHK, while reduction of the intralumenal OS-9 content results in enhanced secretion of non-native NHK. On the other hand, variations in the intralumenal OS-9 content do not affect secretion of folding-competent proteins.

The yeast ortholog Yos9p has been shown to regulate ERAD (6-10,31,32), and while this manuscript was in preparation, a role for OS-9 in mammalian ERAD has been shown (33). We report that the physiologic OS-9 content insures maximal ERAD efficiency because OS-9 induction does not further enhance ERAD capacity. Enhancement of ERAD capacity would probably require co-ordinate over-expression of several components of the human synoviolin complex (see below). OS-9 down-regulation moderately inhibits disposal of folding-defective polypeptides.

Christianson et al. recently reported that OS-9.1 does not bind transthyretin variants, which are non-glycosylated destabilized proteins. They concluded that OS-9 is not able to bind folding-defective luminal proteins lacking N-glycans (33). This must be substrate-specific because our data showed that OS-9.1 and OS-9.2 transiently bind the non-glycosylated ERAD substrate NHKQQQ. As reported for the yeast ortholog Yos9p (5,8), for OS-9 variants as well the association with non-glycosylated folding-defective proteins was non-productive. NHKQQQ is tightly retained in the ER lumen. It was therefore not possible to determine with this substrate whether OS-9 plays a role in preventing secretion of extensively misfolded non-glycosylated polypeptides, as it does with N-glycosylated ones.

The role of the OS-9’s MRH domain also remains unclear. Christianson et al. suggested an interesting model in which this domain is involved directly or indirectly
in a functional association with the transmembrane protein Sel1L in the synoviolin complex that regulates disposal from the ER lumen of polypeptides with luminal defects (33,34). A direct involvement of the MRH domain of OS-9 in a complex with Sel1L would be a clear difference with the yeast system in which formation of the complex does not require an intact MRH domain and is independent on Sel1L glycosylation (7,32). Our data showed that substrate binding and activity of OS-9 do not require a functional MRH domain. Hence, a direct role of the MRH domain in stabilizing a multi-protein complex regulating ERAD seems unlikely.

Yos9p and OS-9 belong to a luminal surveillance complex comprising Kar2p /BiP, Hrd3p/Sel1L and Hrd1p/Synoviolin (6-10,31,33). Analysis of transcriptional regulation of the mammalian orthologs of the yeast Hrd1p complex reveals that all components are inducible upon ER-stress, but only OS-9 variants and synoviolin require activation of the Ire1/Xbp1 pathway (Figs. 1H-I and (27)). We postulate that the “retention of misfolded” versus “facilitation of disposal” functions of mammalian OS-9 proteins might depend on formation of distinct multiprotein complexes and that activation of individual stress-response pathways (Ire1-regulated vs. ATF6-regulated) in specific tissues or under specific developmental or stress conditions could enhance one or the other function of OS-9.

Altogether, our data show that OS-9.1 and OS-9.2 play a dual role in the mammalian ER as ERAD promoters and as crucial operators of the retention-based ER quality control machinery that inhibits release of non-native conformers from the ER lumen into the secretory pathway.
2.4 Materials and Methods

**Antibodies, expression plasmids and OS-9 mutagenesis**-The pcDNA3 plasmids encoding mouse OS-9.1 and OS-9.2 were a kind gift of L. Litovchick. The nucleotide sequences of all plasmids used in this study were verified on both strands. Mutagenesis of the OS-9 MRH domain was carried out using a PCR-based site-directed mutagenesis Quick Gene® kit (Stratagene). The following primers were used: 5'-CTCAACGGGAAGC CCGCAGAAGCTGAAGTTCG-3' (sense) and 5'-CGAACTTCAGCTTCTGCGGGCTT CCCGTTGAG-3' (antisense).

The anti OS-9 used for immuno-precipitation, immuno-blots and immuno-fluorescence was from Novus Biologicals (BC100-519). The anti PDI for immuno-fluorescence was a commercial monoclonal antibody from ABR (MA3-018). The anti EDEM1 for immunoprecipitation was purchased from Santa Cruz Biotech Inc (C-19, sc-27391). The monoclonal anti APP (15) was a kind gift of P. Paganetti. For immunoblots, all primary antibodies were used at 1:1000-1:2000 dilutions. Secondary antibodies for immunoblots were HRP-conjugated anti-rabbit antibodies (1:5000). The ECL-Plus detection system was from Amersham. DNA preparations were obtained using commercially available purification kits (Sigma). Secondary antibodies for immunofluorescence (Alexa 488-labeled goat anti-rabbit, Alexa 594-labeled goat anti-mouse) were used at 1:100 dilutions.

**Cell Lines, transient transfections, metabolic labelling, analysis of data**-All cell lines used in the study were grown in DMEM supplemented with 10% FBS. Xbp1−/− cells were a kind gift of L. Glimcher. Cells at 80-90% confluence in a 6 cm tissue culture plate were transfected with the expression plasmid of interest (4 µg for single transfections, 6 µg total DNA for transfections with two plasmids) using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Experiments were performed 17 hr after transfection. Metabolic labelling is described in (16). Gels were exposed to BioMax (Kodak) films and scanned with an Agfa scanner. Relevant bands were quantified by ImageQuant software (Molecular Dynamics).

**Subcellular fractionation and separation of membrane vs. luminal content**-35S-labelled cells were extensively washed in a 10 cm dish with isotonic buffer. They were detached with a rubber policeman and resuspended in 800 µl of
homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 20 mM NEM, 1 mM EDTA and a cocktail of protease inhibitors, pH 7.4). Cells were broken with 10 passages through a 25G1 needle. Post nuclear supernatants were subjected to ultracentrifugation (45 min, 200’000 g in TLA 120.2) to separate endomembranes from the cytosol. The endomembrane-containing pellet was extensively washed, resuspended in 500 µl 100 mM Na$_2$CO$_3$ and incubated for 25 min on ice for carbonate extraction. After an additional ultra-centrifugation step (45 min, 200’000 g), the supernatant was harvested (ER lumen in Fig. 1G). The endomembrane fraction (ER memb in Fig. 1G) was washed once with 100 mM Na$_2$CO$_3$ (35 min, 200’000 g) and then resuspended in 800 µl of lysis buffer. Insoluble material was removed after 25 min on ice by 10 min centrifugation at 200’000 g. The ER luminal content and the endomembrane fraction were subjected to immunoprecipitation against calreticulin, calnexin, OS-9 and EDEM1.

**Immunofluorescence microscopy**-2x10$^5$ HEK293 cells were seeded in a 6 cm Petri dishes containing 1% alcian blue treated glass cover slips. Cover slips were rinsed with PBS, and cells were fixed in 3,7% formaldehyde. After 2 short washings with 10 mM Hepes serum-free medium, and two additional washing with PBS, the antigen accessibility was improved by a 20-min incubation with 0,05% saponin, 10% Goat serum, 10 mM Hepes and 15 mM glycine. Images were viewed on a Nikon eclipse E-800 fluorescent microscope, captured by a Hamamatsu EM-CCD Digital camera C9100 and analysed with the Open lab 3 software (Improvision, Inc., Lexington, MA).

**Semi quantitative and quantitative reverse transcriptase-polymerase chain reactions (RT-PCR)**-Primers used for identification of OS-9 splice variants expressed in HEK293 cells were as follows: primers amplifying all 4 putative splice variants (Fig. 1B left panel): CTTCCGTACAGACGGGACC (for), GGGCGGACAATTTT GATCT (rev); primers amplifying only OS-9.1 and OS-9.4 (Fig. 1B, right): CTTCCGTACAGACGGGACC (for), CTGATTAGGCGCTCCGAGA (rev). For analysis of variations in gene transcription upon ER stress, HEK293, wt and Xbp1$^{-/-}$ MEF were plated in 6-cm dishes without (mock) or with tunicamycin (Tun, 2.5 µg/ml). After 12 h cells were lyzed in TRlzol reagent (Invitrogen) and RNA was isolated according to the instructions of the manufacturer. Two µg of RNA were used for cDNA synthesis using SuperScriptII reverse transcriptase (Invitrogen) and oligo(dT) (Invitrogen). Semi
quantitative PCR was performed using TaqDNA polymerase (Invitrogen) with transcript-specific primers using three different cycle numbers for each gene, all within the linear phase of template amplification. Quantitative RT-PCR was performed using 7900HT Fast Real-Time PCR System. The PCR reactions were performed in a 10 µl Power SYBR Green PCR master mix (Applied Biosystem), 5 µl cDNA, 4 µl ddH₂O and 1 µl primer mix (0.5 µM final). The housekeeping gene b-actin was used as reference. Data were analyzed using the SDS 2.2.2 software. The primers were as follows: m-b-Actin, CTTTCTGGGTATGGAATCCT (for), GGAGCAATGATCTTGATCTT (rev); h-b-Actin, CTTCTGGGATGGTGACCTC (for), GGAGCAATGATCTTGATCTT (rev); m/h-Bip, GAGTCTTTCAATGGAAGA (for), CCAGTCAGATCAATGTACC (rev); m/EDEM1, TGGAGTTTGGATTCTGAG (for), CTGCAGTCCAGGGAAGAA (rev); h/EDEM1, AAGATTCCACCGTGACCA (for), GTATCATTGCTCCGGAAGGT (rev); m/h-OS-9, GGAGGAGCTGAGTGATGC (for), GTCTGGCTGTTGAGCTGTT (rev); m/Sel1L, CCTCGAGCAAGAGCAC (for), GCGGTCTCAATCCACATCA (rev); m-Synoviolin, CGTGTGGACTTTATGGAAACGC (for), CGGGTCGAGTGTGGATGAA (rev).

**Lentiviral transduction and stable cell line establishment**-VSVg pseudotyped lentiviral vectors were produced by transient transfection of 293FT cells (Invitrogen) with an HIV-1-based packaging vector, VSVg envelope vector, and transfer vector containing either the short hairpin for human OS-9 or Luciferase. Forty-eight hours post-transfection, viral supernatants were filtered through a 0.45 µm filter, and added to HEK293 cells. Transduced cells were selected with puromycin gradually, from 1-30 µg/ml over two weeks. OS-9 shRNA targeting sequence, targeting the 3’ UTR, is as follows: GCTGCCTACCTGGAGATTC.
2.5 Bibliography

2.6 Supplementary Figures

**Figure S1.** Radiolabeled secreted α1AT (left panel) and NHK (right panel) have been immunoisolated after the indicated chase times from detergent-extract of cells and analyzed on a non-reducing SDS-PAGE.

**Figure S2.** Consequences of OS-9 down-regulation on secretion and degradation of NHK and on secretion of α1-antitrypsin. A Intracellular content of labeled NHK from cells with reduced OS-9 level (shOS-9, lanes 1-3), back-transfected with OS-9.1 (shOS9+OS-9.1, lanes 4-6) or with OS-9.2 (shOS9+OS-9.2, lanes 7-9). B Quantification of A. C Same as A for secreted NHK. D Quantification of C. E NHK co-precipitates with OS-9.1 (lanes 4-6) and with OS-9.2 (lanes 7-9).
Stringent requirement for HRD1, SEL1L and OS-9/XTP3-B for disposal of ERAD-LS substrates

Riccardo Bernasconi¹, Carmela Galli¹, Verena Calanca¹, Toshihiro Nakajima³ & Maurizio Molinari¹,²

¹Institute for Research in Biomedicine, Via V. Vela 6, 6500 Bellinzona, Switzerland
²Ecole Polytechnique Fédérale de Lausanne, School of Life Sciences, 1015 Lausanne, Switzerland
³St. Marianna University School of Medicine, Kanagawa, 216-8512 Japan

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I performed all the experiments presented in this study.
Sophisticated quality control mechanisms prolong retention of protein folding intermediates in the endoplasmic reticulum (ER) until maturation, while sorting out terminally misfolded polypeptides for ER-associated degradation (ERAD). The presence of structural lesions in the luminal, transmembrane, or cytosolic domains determines the classification of misfolded polypeptides as ERAD-L, ERAD-M or ERAD-C substrates and results in selection of distinct degradation pathways. Here we show that disposal of Soluble (non-transmembrane) polypeptides with luminal lesions (ERAD-Ls substrates) is strictly dependent on the E3 ubiquitin ligase HRD1, the associated cargo receptor SEL1L and two interchangeable ERAD lectins, OS-9 and XTP3-B. These ERAD factors become dispensable for degradation of the same polypeptides when Membrane-tethered (ERAD-LM substrates). Our data reveal that, in contrast to budding yeast, tethering of mammalian ERAD-L substrates to the membrane changes selection of the degradation pathway.
3.1 Introduction

Accumulation of misfolded proteins hampers the function of the ER and elicits a variety of stress responses that might eventually result in cell death. The capacity to rapidly remove folding-defective polypeptides from the ER lumen is therefore crucial to maintain cell homeostasis (1-3). Polymerogenic, misfolded proteins such as ATZ, a folding-defective, aggregation-prone mutant of the secretory protein α1-antitrypsin, and serpins are removed from the ER upon activation of multiple proteasome-dependent and proteasome-independent disposal pathways including autophagy (4-8). Normally however, misfolded proteins produced in the ER are extracted from folding machineries and are dislocated across the ER membrane to be degraded by cytosolic 26S proteasomes in a series of tightly regulated events collectively defined as ER-associated protein degradation (ERAD) (9). Most of the polypeptides entering the ER lumen are modified with pre-assembled glucose$_3$-mannose$_9$-N-acetylglucosamine$_2$-oligosaccharides, which are covalently attached to asparagine side chains in Asn-Xxx-Ser/Thr sequons emerging in the ER lumen. Stepwise processing of protein-bound oligosaccharides eventually determines the fate of the covalently linked polypeptide chains (10). For instance, degradation of terminally misfolded glycoproteins from the ER requires extensive demannosylation of the protein-bound oligosaccharides, which precedes polypeptide dislocation across the ER membrane and degradation (11-13). Current models claim that dislocation across the ER membrane follows specific pathways regulated by luminal, transmembrane, and cytosolic complexes built around membrane-embedded E3 ubiquitin ligases (13-16). In S. cerevisiae, disposal of transmembrane proteins with cytosolic defects (ERAD-C substrates) requires intervention of the DOA10 pathway. Membrane anchored polypeptides with transmembrane lesions (ERAD-M substrates) as well as membrane-tethered and soluble polypeptides with lesions in the ER lumen (ERAD-L substrates) are cleared from the ER through the HRD1 pathway (17-23).

The mammalian system is more complex. The mammalian ER membrane contains several E3 ubiquitin ligases. Few of them (e.g. RNF5/RMA1, TEB4, TRC8, RFP2) are still poorly characterized and/or have a restricted number of substrates ((24-28)
and reviewed in (14)). Others such as Synoviolin/HRD1 and GP78 are better characterized and regulate, in a concerted action with a number of interacting partners, disposal of numerous folding-defective polypeptides used as model ERAD substrates (13-16).

Individual substrates follow preferential routes for dislocation across the mammalian ER membrane. Consistently, inactivation of the GP78 pathway is sufficient to substantially delay disposal of conventional ERAD substrates such as TCRα, CD3-δ and CFTRΔF508 (25,29-34), but it does not affect disposal of other classical ERAD substrates such as NHK or Ri332. On the other hand, inactivation of HRD1 or of components of the multimeric complex built around this E3 ligase has no consequence on disposal of GP78 clients (for example (25,35-39)), but specifically interferes with disposal of folding-defective polypeptides such as NHK, Ri332, Igμs, IgκLC, IgγHC, IgλLC ((35,40-48) and references therein). Since the reasons behind this substrate specificity are unknown, it is impossible to predict which pathway will be used by a given folding-defective polypeptide. ERAD substrates recruitment to HRD1 occurs either directly, or indirectly through transient associations of the HRD1 co-factor SEL1L with luminal acceptors such as OS-9, XTP3-B, EDEM1, BiP or GRP94 (41,45,47,49,50). Few of these components do not participate in the GP78 or in other dislocation complexes (25,45,48). Certainly therefore, the different composition of the complexes built around membrane-embedded E3 ubiquitin ligases determines substrate selection, but which substrate feature is relevant to determine whether a misfolded polypeptide will preferentially use one or the other ERAD pathway is unknown.

To better understand this, we compared the requirements for efficient disposal of two canonical, N-glycosylated, membrane anchored ERAD substrates (BACE476 (51) and CD3-δ (52)) with the requirements for efficient disposal of their variants lacking the transmembrane anchor (BACE476Δ and CD3-δΔ, Fig. 1). Our data reveal that only degradation of the soluble (non-transmembrane) variants of BACE476 and CD3-δ strictly depend on several participants of the HRD1 pathway regulating ERAD, namely the E3 ubiquitin ligase HRD1, the HRD1-associated cargo receptor SEL1L and the ERAD lectins OS-9 and XTP3-B. Disposal of the membrane-tethered variants of the same folding-defective polypeptides remained in fact unperturbed upon inactivation of HRD1, SEL1L and, significantly, OS-9 and
XTP3-B. Thus, in contrast to yeast (53,54), substrate de-mannosylation in the mammalian ER is not (only) required to generate a signal for disposal decoded by the ERAD lectins of the OS-9 family. In fact, at least when the folding-defective glycopolypeptide is tethered at the ER membrane, intervention of OS-9 and XTP3-B becomes dispensable for efficient disposal. Moreover, and again in contrast to yeast (22,23), the presence or the absence of a membrane anchor alters selection of the disposal pathway used by ERAD-L substrates in mammalian cells. This was confirmed by the finding that the crucial dependency on components of the HRD1 pathway for degradation of NHK, another classic ERAD-L substrate (55), was substantially relieved when the protein was anchored at the ER membrane. Our data lead us to group polypeptides with structural lesions in the ER lumen in two sub-classes, namely ERAD-L\textsubscript{S} substrates (for Soluble ERAD-L substrates whose disposal is strictly dependent on the HRD1 pathway) and ERAD-L\textsubscript{M} substrates (for Membrane-tethered ERAD-L substrates for which alternative ERAD pathways can be activated to insure efficient disposal). Our data further highlight the more significant complexity and the somewhat different mechanisms regulating protein quality control in the mammalian versus the budding yeast ER.

![Figure 1. Schematic representation of the 7 canonical ERAD substrates used in this study. BACE476, CD3-δ, NHK\textsubscript{BACE} and NHK\textsubscript{CD3} are type I membrane proteins (ERAD-L\textsubscript{M} substrates); BACE476\textsubscript{Δ}, CD3-δ\textsubscript{Δ} and NHK are the corresponding, soluble ERAD-L\textsubscript{S} substrates.](image-url)
3.2 Results

3.2.1 Consequences of HRD1 deletion on disposal of membrane-anchored and soluble BACE476

Availability of mouse embryonic fibroblasts (MEF) lacking HRD1 (56) prompted us to assess the involvement of this membrane-embedded E3 ubiquitin ligase in disposal of folding-defective polypeptides from the ER lumen. *Hrd1*−/− cells are more sensitive to ER stress-induced apoptosis, but they are not under ER stress ((56) and Fig S2C). Tissue-specific versions of the human beta-secretase (BACE501 (57)) are amongst the best-characterized ERAD substrates. For example, the type I membrane glycoprotein BACE476 and its soluble variant BACE476Δ originate from alternative splicing of the BACE transcripts resulting in a 25-residues deletion in the protein’s luminal ectodomain. This deletion prevents attainment of the native structure when the polypeptide is ectopically expressed in cultured mammalian cells (51). BACE variants are defined as canonical ERAD substrates because their degradation requires extensive de-mannosylation of the protein-bound oligosaccharides ((51) and Fig. S1), is accelerated in cells expressing high levels of EDEM1 and EDEM2 (58-60), is delayed in cells with low content of EDEM proteins (58) and is performed in the cytosol, upon P97-facilitated extraction, by 26S proteasomes ((51,61) and Fig. S1).

BACE476 (Fig. 2A) and BACE476Δ (Fig. 2B) were individually expressed in wild type MEF (wt, lanes 1-3) and in MEF lacking HRD1 (*Hrd1*−/−, lanes 4-6). Seventeen hours after transfection with appropriate expression plasmids, cells were pulsed for 10 min with 35S-methionine and cysteine and were subsequently chased in normal cell culture media for the times shown in Figs. 2A-B. At the end of each chase time, cells were detergent-solubilized and the residual amount of radiolabeled ERAD substrate was immunoisolated from post nuclear supernatants (PNS) with specific antibodies (Fig. S2A), separated on SDS-polyacrylamide gels and quantified. The kinetics of BACE476 disposal was essentially the same in cells with (Fig. 2A, lanes 1-3) and without HRD1 (Fig. 2A, lanes 4-6). In both cell lines, only about 30% of the initial amount of radiolabeled polypeptide was immunoisolated from PNS after a 6 hr chase (Fig. 2A, quantification). On the other hand, HRD1 deletion substantially delayed disposal of the same protein lacking a transmembrane
anchor. In fact, only about 20% of the initial amount of BACE476Δ was immunoisolated from lysates of wt cells after a 4 hr chase (Fig. 2B, lane 3 and quantification), while cells lacking HRD1 still contained about 80% of the initial amount of labeled protein (lane 6 and quantification). Therefore, HRD1 deletion selectively impaired disposal of the soluble variant of BACE476.

The labeled ERAD candidates had faster mobility at the end (Figs. 2A-B, lanes 3 and 6) than at the beginning of the chase (Figs. 2A-B, lanes 1 and 4). This is caused by the progressive de-mannosylation of ER retained misfolded polypeptides that reduces their apparent molecular weight. Extensive de-mannosylation interrupts futile folding-attempts in the calnexin chaperone system and promotes substrate deviation in the ERAD pathway (reviewed in (10)). Consistently, the progressive increase in BACE electrophoretic mobility and BACE disposal were inhibited by kifunensine, a specific inhibitor of α1,2-mannosidases (Figs. S1A-B, lanes 3). Deletion of HRD1 substantially delayed BACE476Δ disposal, without
affecting the enhancement of BACE mobility, hence the extensive de-
mannosylation that precedes BACE ERAD. This shows that, in the series of luminal
events leading to BACE destruction (i.e., 1) folding attempts phase in the calnexin
cycle (51), 2) extraction from the folding machinery facilitated by substrate de-
mannosylation (51,58), 3) delivery at the ER membrane, 4) dislocation across the
ER membrane), the deletion of HRD1 only interferes with late events, as expected
for a component of the dislocation machinery.

3.2.2 HRD1 activity is required for efficient disposal of BACE476Δ
To avoid spurious phenotypes possibly caused by uncharacterized differences
between wild type and knockout cell lines, we confirmed that HRD1 is required for
efficient disposal of BACE476Δ by reproducing the experiments shown in Figs. 2A-
B in a single cell line. To this end, we compared degradation of BACE476Δ in Hrd1−−/−
knockout cells mock-transfected (Mock, Fig. 2C, lanes 1-3) and in the same cell
line expressing an active (HRD1, Fig. 2C, lanes 4-6 and Fig. S2B) or an inactive
form of HRD1 (the C307S mutant HRD1* (62), Fig. 2C, lanes 7-9 and Fig. S2B). In
all experiments, BACE476Δ was efficiently retained in the ER and degraded as
confirmed by lack of BACE476Δ secretion (Fig. S2D). BACE476Δ had a half-life (t1/2)
longer than 240 min in cells lacking HRD1 (Fig. 2B, lanes 4-6 and Fig. 2C,
lanes 1-3, for determination of protein half-lives please refer to Methods). Back-
transfection of the active form of HRD1 accelerated disposal of BACE476Δ to a rate
(t1/2=150 min, Fig. 2C, lanes 4-6) approaching the disposal-rate measured in wild
type cells (Fig. 2B, lanes 1-3 and quantifications). In cells expressing the inactive
form of HRD1, disposal of BACE476Δ (t1/2>240 min, Fig. 2C, lanes 7-9) was as
slow as in cells lacking HRD1 (Fig. 2B, lanes 4-6 and Fig. 2C, lanes 1-3). We
concluded that an active HRD1 pathway is required for efficient degradation of
BACE476Δ from the mammalian ER. Experiments performed to assess
consequences of ectopic expression of active or inactive HRD1 on disposal of
BACE476 in cells lacking this E3 ubiquitin ligase confirmed dispensability of the
HRD1 activity when the same folding-defective ERAD substrate was tethered at the
ER membrane (Fig. S3A).
3.2.3 Consequences of HRD1 deletion on disposal of membrane-anchored and soluble CD3-δ

CD3-δ is a type I membrane glycoprotein, which is typically part of the T-cell receptor/CD3 complex involved in T-cell development and signal transduction. When expressed individually, the CD3-δ orphan subunit cannot attain its native structure and is degraded from the ER with mechanisms that have been thoroughly characterized. In particular, CD3-δ degradation requires de-mannosylation and is operated by cytosolic proteasomes (52). CD3-δ is a client of the E3 ubiquitin ligase GP78 (29,31-34) and it has been reported that RFP2, another E3 ligase contributes to its disposal (27). Down-regulation of HRD1 does not affect CD3-δ degradation (35), even though, it has recently been reported that down-regulation of HRD1 might result in increased intracellular levels of GP78 (63,64). It was therefore not unexpected that CD3-δ clearance from the ER lumen occurred with similar kinetics in wild type cells (Fig. 3A, lanes 1-3) and in cells lacking HRD1 (lanes 4-6).

The anchor-less variant of CD3-δ as well was efficiently retained in the ER as confirmed by lack of secretion in the extracellular media (Fig. S2E). As for the membrane-tethered version of the protein, CD3-δΔ degradation required de-mannosylation and was operated by cytosolic proteasomes. Significantly however, deletion of the CD3-δ membrane-anchor converted this GP78/RFP2 client protein to an HRD1 client. In fact, degradation of the anchor-less CD3-δΔ was defective in cells lacking HRD1 (Fig. 3B, lanes 4-6 and quantifications). HRD1-deletion inhibited CD3-δΔ disposal without affecting the extensive substrate de-mannosylation that signals prolonged ER retention of this folding-defective polypeptide.

As in the case of BACE476Δ, to avoid spurious phenotypes possibly caused by uncharacterized differences between wild type and knockout cell lines, we confirmed involvement of HRD1 by showing that CD3-δΔ disposal was defective in cells lacking HRD1 or expressing the inactive form of HRD1 (t_{1/2}>120 min, Fig. 3C, lanes 1-3 and 7-9, respectively), but progressed with wild type kinetics (t_{1/2}<60 min, lanes 4-6) upon back-transfection of active HRD1 in the knockout cells. The finding that an active HRD1 pathway is required for efficient degradation of CD3-δΔ but not for disposal of the same protein when associated with the ER membrane was confirmed by assessing the dispensability of HRD1 for CD3-δ disposal (Fig. S3B).
3.2.4 HRD1 and GP78 requirements for disposal of membrane-tethered and soluble BACE476

So far, we showed that deletion of HRD1 significantly interferes with disposal of the anchor-less variants of BACE476 and of CD3-δ (ERAD-L<sub>S</sub> substrates), leaving unaffected disposal of polypeptides with the same luminal domain, but tethered at the ER membrane (ERAD-L<sub>M</sub> substrates). This may indicate that HRD1 is not involved in disposal of membrane anchored ERAD-L substrates, or that upon HRD1 deletion surrogate pathways regulated by other membrane-embedded E3 ligases are engaged to insure disposal.

To verify this, we next assessed the consequences of the individual and combined down regulation of HRD1 and GP78 in clearance of membrane-tethered and soluble BACE476 from the ER. Down-regulation of HRD1 obtained by specific RNA interference (RNAi, Fig. 4A) slightly delayed disposal of BACE476 (Fig. 4B, lanes 4-6 and quantification siHRD1). Down-regulation of GP78 (Fig. 4A) had no effect (Fig. 4B, lanes 7-9 and quantification siGP78). However, the combined inactivation of both HRD1 and GP78 (Fig. 4A) substantially delayed BACE476 disposal (Fig.
The delay of BACE476 disposal obtained upon combined inactivation of the HRD1 and of the GP78 pathways was comparable to the delay obtained upon cell exposure to classical ERAD inhibitors such as kifunensine or PS341 (Fig. S1A).

Figure 4. Consequences of HRD1 and GP78 down-regulation on degradation of membrane-tethered and soluble variants of BACE476 and CD3-δ. A The efficiency of siRNA-based HRD1 and GP78 down-regulations were checked by immunoblot. Tubulin is a loading control. B Radiolabeled BACE476 was immunoisolated after the indicated chase times from cells expressing a scrambled siRNA (siSCR, lanes 1-3), a siRNA targeting HRD1 (siHRD1, lanes 4-6), GP78 (siGP78, lanes 7-9) or both HRD1 and GP78 (siHRD1/siGP78, lanes 10-12). Relevant bands were quantified and plotted. C Same as B for BACE476Δ. D Same as B for CD3-δ. E Same as B for CD3-δΔ.

Taken together, these data show that GP78 can replace the inactive HRD1 and that, vice versa, HRD1 can replace inactive GP78 to insure efficient disposal of BACE476. On the contrary, HRD1 down-regulation was sufficient to delay BACE476Δ disposal to a similar extent than α1,2-mannosidases or proteasome...
inactivation (Fig. 4C, lanes 4-6, quantifications and Fig. S1B). Down-regulation of GP78 had no effect on BACE476Δ degradation (lanes 7-9) and combined inactivation of the two E3 ubiquitin ligases (lanes 10-12) had the same inhibitory effect as the individual down-regulation of HRD1 (quantification). This is in agreement with a model claiming that the presence or the absence of a membrane anchor determines requirements for disposal of ERAD-L substrates. More precisely, deletion of the membrane anchor converted BACE476 from a HRD1/GP78 client into an obligate client of the HRD1 pathway.

3.2.5 HRD1 and GP78 requirements for efficient disposal of membrane-tethered and soluble CD3-δ

Next, we assessed the contribution of HRD1 and GP78 in clearance from the ER of CD3-δ and of CD3-δΔ. Down-regulation of HRD1 did not affect degradation of CD3-δ (Fig. 4D, lanes 4-6 and quantifications). Down-regulation of GP78 did delay CD3-δ disposal (Fig. 4D, lanes 7-9) and the additional inactivation of HRD1 did not further protect this folding-defective polypeptide from ERAD (lanes 10-12 and quantifications). These data are consistent with findings reporting that CD3-δ is a client of the GP78 machinery (29,31-34) and with data showing that CD3-δ disposal remains unaffected upon inactivation of the HRD1 pathway (Fig. 3A and (35)). The incomplete inhibition of CD3-δ disposal upon inactivation of GP78 (Fig. 4D) is consistent with data showing that at least another E3 ligase, RFP2 (27), contributes to CD3-δ clearance from the ER lumen.

In agreement with our model predicting that changes in substrate tethering to the membrane determine selection of the ERAD pathway regulating clearance of ERAD-L substrates from the ER, HRD1 down-regulation was sufficient to substantially delay CD3-δΔ disposal (Fig. 4E, lanes 4-6 and quantifications), while down-regulation of GP78 had no effect (lanes 7-9). Combined inactivation of the two ligases (lanes 10-12) had the same inhibitory effect on CD3-δΔ disposal as the individual down-regulation of HRD1. Thus, deletion of the membrane anchor converted CD3-δ from a GP78/RFP2 client into an obligate client of the HRD1 pathway.
3.2.6 SEL1L is required for efficient disposal of ERAD-L_S substrates

Our data show a stringent requirement of HRD1 for degradation of the ERAD-L_S substrates BACE476Δ and CD3-δΔ. The same polypeptides might exploit the HRD1 and/or the GP78 and/or other ERAD complexes for efficient disposal when anchored at the ER membrane. The complex built around HRD1 contains several proteins that are excluded from the GP78 complex. Amongst them, SEL1L plays a crucial role in protein disposal by recruiting ERAD substrates either directly, or indirectly through association with the ERAD lectins OS-9 and XTP3-B (41,45,47,49), through association with the member of the glycosyl hydrolase 47 family EDEM1 (50), or through association with luminal chaperones such as BiP or GRP94.

Our finding that only ERAD-L_S substrates were strictly dependent on active HRD1, prompted us to verify the involvement of SEL1L in clearance of ERAD-L_S versus ERAD-L_M substrates. To this end, kinetics of protein disposal was compared in mock-treated cells (siSCR in Fig. 5) and in cells with reduced expression of SEL1L (siSEL1L in Fig. 5). Down-regulation of SEL1L (Fig. 5A) only marginally slowed disposal of the ERAD-L_M substrate BACE476 (Fig. 5B, lanes 4-6 and quantifications) but substantially delayed degradation of the ERAD-L_S substrate BACE476Δ (Fig. 5C, lanes 4-6 and quantifications). Similarly, down-regulation of SEL1L had no effect on degradation of the ERAD-L_M substrate CD3-δ (Fig. 5D, lanes 4-6 and quantifications), but fully protected the anchor-less variant of the same folding-defective polypeptide from degradation (Fig. 5E, lanes 4-6 and quantifications). Taken together, these findings showed that down-regulation of SEL1L recapitulates the phenotype obtained upon deletion (Figs. 2-3) or down-regulation of HRD1 (Fig. 4). Inactivation of HRD1 or of SEL1L had little or no effect on disposal of ERAD-L_M substrates, but dramatically interfered with clearance from the ER lumen of the same polypeptides when detached from the ER membrane (ERAD-L_S substrates).
Figure 5. Disposal of soluble misfolded polypeptides relies on SEL1L and both OS-9+XTP3-B.
A The efficiency of siRNA-based SEL1L, OS-9 and XTP3-B down-regulations were checked by immunoblot. Tubulin is a loading control. B Radiolabeled BACE476 was immunoisolated after the indicated chase times from cells expressing a scrambled siRNA (siSCR, lanes 1-3), a siRNA targeting SEL1L (siSEL1L, lanes 4-6), OS-9 (siOS-9, lanes 7-9), XTP3-B (siXTP3-B, lanes 10-12) or both XTP3-B and OS-9 (siXTP3-B/siOS-9, lanes 13-15). Relevant bands were quantified and plotted. C Same as B for BACE476Δ. D Same as B for CD3-δ. E Same as B for CD3-δΔ.
3.2.7 OS-9 and XTP3-B are interchangeable ERAD shuttles required for efficient disposal of ERAD-L$_S$ but not of ERAD-L$_M$ substrates

The mammalian splice variants of OS-9 and XTP3-B constitute a group of proteins that have lectin-like domains with homology to the mannose-6-phosphate receptor family (65). So far, OS-9 or XTP3-B intervention in ERAD has been only shown for the model ERAD substrates NHK or Ri$_{332}$ (45-49,66). The individual down-regulation of these ERAD lectins has generally no or mild phenotypes and attempts to establish the involvement of OS-9 or XTP3-B variants in disposal of other glycosylated folding-defective polypeptides have been unsuccessful, thus questioning their relevance as general regulators of protein disposal from the mammalian ER. Our data confirm that individual down-regulation of OS-9 (Figs. 5A, B, E, lanes 7-9 and quantifications), or of XTP3-B (Figs. 5A, B, E, lanes 10-12 and quantifications), did not significantly delay disposal of BACE476, BACE476Δ, CD3-δ and CD3-δΔ. However, combined down regulation of OS-9 and XTP3-B specifically retarded disposal of the HRD1 client BACE476Δ (Fig. 5C, lanes 13-15 and quantifications) to a similar extent than down-regulation of SEL1L (lanes 4-6) and of HRD1 (Fig. 4C, lanes 4-6). Combined down regulation of OS-9 and XTP3-B also partially protected CD3-δΔ from disposal (Fig. 5E, lanes 13-15). Thus, OS-9 and XTP3-B have redundant activities in the ER lumen so that their combined inactivation might be required to substantially delay disposal. NHK is an exception to this rule because individual down-regulation of OS-9 is sufficient to significantly delay ERAD (46,47). Moreover, our data show that OS-9 and XTP3-B intervention is only required for disposal of ERAD-L$_S$ substrates that must be shuttled from the ER lumen to the ER membrane-embedded HRD1 complex for efficient clearance from the ER. For this reason we define OS-9 and XTP3-B as ERAD shuttles. Disposal of the same glycopolypeptides tethered to the ER membrane (ERAD-L$_M$ substrates) progressed unperturbed even upon substantial reduction of the cellular content of OS-9 and XTP3-B. This shows that the two ERAD shuttles do not intervene in disposal of membrane-tethered N-glycosylated polypeptides with luminal defects, or that they can efficiently be replaced by a surrogate factor upon their combined inactivation (see Discussion).
3.2.8 Down-regulation of SEL1L and HRD1 allows identification of OS-9.1 as delivery factor for BACE476Δ

In an attempt to identify ERAD factors interacting with ERAD-L<sub>S</sub> versus ERAD-L<sub>M</sub> substrates, BACE476Δ was tagged with a EFRH tetrapeptide (which is recognized by β1, a monoclonal antibody described in (67)) while BACE476 was HA-tagged. The two proteins were ectopically co-expressed in mock-treated cells (siSCR, Figs. 6A-B, lane 1) or in cells with reduced levels of HRD1 (lane 2), GP78 (lane 3), HRD1 and GP78 (lane 4), SEL1L (lane 6), OS-9 (lane 7), XTP3-B (lane 8), OS-9 and XTP3-B (lane 9). Cells were lysed under conditions that preserve many substrate:chaperone interactions (51). BACE476Δ-β1 (Fig. 6A) and BACE476-HA (Fig. 6B) were individually immunoisolated with the appropriate anti-tag antibody, together with their interacting partners. The amount of proteins separated in SDS-PAGE and blotted on a PVDF membrane was normalized to insure equal loading of BACE in each lane (lower panels in Figs. 6A-B). The PVDF membrane was probed with antibodies to GRP94, BiP and OS-9 to determine whether these ER chaperones claimed to be involved in ERAD were found in complexes sufficiently stable to survive the cell lysis and immunoprecipitation protocols. The case for GRP94 is unclear as variations in the amount of this chaperone co-precipitated with BACE476Δ-β1 (Fig. 6A) or BACE476-HA (Fig. 6B) were too small to be considered significant. For BiP as well variations were small. However, a stabilization of BACE476Δ:BiP complexes was observed upon inactivation of HRD1 (Fig. 6A, lane 2), of HRD1+GP78 (lane 4), of SEL1L (lane 6), and upon combined inactivation of OS-9+XTP3-B (lane 9). These variations were reproducible even when BACE476Δ-β1 was individually expressed in cells subjected to transient interferences (unpublished data). Stabilization of BACE476Δ:BiP complexes upon disassembly of the HRD1 dislocon shows that BiP contributes to delivery of terminally misfolded polypeptides to this machinery.

Our analysis also revealed a significant stabilization of BACE476Δ:OS-9.1 complexes in cells subjected to HRD1 (Fig. 6A, lane 2), HRD1+GP78 (lane 4, less evident), or SEL1L down-regulations (lane 6). Under the same conditions, and actually in the same cells since BACE476Δ-β1 and BACE476-HA were co-expressed, we were unable to detect stabilization of complexes between BACE476 and the ER chaperones tested (Fig. 6B).
The stabilization of BACE476Δ:OS-9.1 complexes (and possibly of BACE476Δ:XTP3-B complexes (an antibody to detect endogenous XTP3-B is unfortunately not available)) when SEL1L or HRD1 are not available for substrate delivery, is consistent with a sequential involvement of these components during channeling of BACE476Δ into the cytosol for disposal.

Figure 6. Trapping of BACE476Δ by OS-9.1 upon inactivation of the HRD1 pathway. A BACE476Δ was immunoisolated from detergent-extracts of cells incubated with a scrambled siRNA, and siRNA targeting HRD1, GP78, GP78 and HRD1 (lanes 1-4), SEL1L, OS-9, XTP3-B and XTP3-B+OS-9 (lanes 6-9, respectively). TCE is Total Cell Extract (lane 5). Proteins were separated in SDS-polyacrylamide gels and transferred on PVDF. The membranes were blotted with antibodies recognizing endogenous GRP94 (upper panel), BIP (second panel from the top), OS-9.1 and OS-9.2 (third panel from the top) and BACE476Δ as a loading control (lower panel). B Same as A for BACE476.

3.2.9 Disposal of the classical ERAD-L,S substrate NHK and of its two ERAD-L,M variants NHKBACE and NHKCD35

So far, we showed that deletion of the membrane anchor of ERAD-L substrates confers a strong dependency on the HRD1 machinery for degradation, independent on the mechanisms regulating disposal of the membrane-tethered version of the proteins under investigation. To challenge our model we decided to determine requirements for disposal of a soluble ERAD substrate and to verify how requirements would change upon addition of a transmembrane anchor. To this end, we selected a classic ERAD-L substrate, the NHK variant of the secretory protein α1-antitrypsin(55). As previously shown in other cell lines (46), we confirm that a
fraction of NHK escapes ER retention and is secreted extracellularly (about 15% of the labeled protein in HeLa cells (Fig. S2F)). The expectations were that disposal of this ERAD-L_S protein should substantially be delayed upon inactivation of the E3 ligase HRD1 and that the strict dependency on HRD1 should be relieved when the folding-defective polypeptide is converted in a membrane-tethered ERAD-L_M protein. Reduction of the intraluminal level of HRD1 (Fig. 7A, lanes 4-6) substantially inhibited NHK degradation, without affecting secretion of the protein (Fig. S2F). The combined inactivation of the GP78 pathway did not further protect the protein from disposal (Fig. 7A, lanes 10-12). The intervention of HRD1 was not surprising since both SEL1L (47) and our unpublished experiments) and OS-9 (46,47) have been shown to participate in NHK disposal. Thus, NHK behaves as a bona fide ERAD-L_S protein as defined above, which shows strict dependency on the HRD1 pathway for disposal.

Tethering of NHK at the ER membrane was obtained by appending the membrane anchor of BACE501 (NHK_BACE, Fig. 1) or the membrane anchor of CD3-δ (NHK_CD3δ, Fig. 1) at the C-terminus of NHK (Methods). The membrane-anchor of BACE501 does not contain misfolded or retention signals. Consistently, BACE501 is efficiently transported at the cell surface when the protein is ectopically expressed in cultured cells (57). On the other hand, it is not entirely clear whether the transmembrane domain of CD3-δ is free of ‘misfolding signal’. Anchoring of NHK_BACE and of NHK_CD3δ at the ER membrane was confirmed by subcellular fractionation as described in (59) (Figs. S5A-B, respectively). Both proteins were degraded from cells with slightly faster kinetics compared to their soluble counterpart (compare lanes 1-3 in Figs. 7A (NHK), 7B (NHK_BACE) and 7C (NHK_CD3δ)). Degradation was efficiently inhibited upon inactivation of ER mannosidases and cytosolic proteasomes (unpublished data). In agreement with our model, conversion of NHK in an ERAD-L_M substrate substantially relieved HRD1 dependency. In fact, disposal of both NHK_BACE (Fig. 7B) and NHK_CD3δ (Fig. 7C) was unaffected upon reduction of the intraluminal level of HRD1. Disposal of the membrane-tethered versions of NHK was somewhat delayed only upon inactivation of both the HRD1 and the GP78 pathways (lanes 10-12 in Figs. 7B-C) hinting at the possible intervention of alternative ERAD pathways in disposal of NHK when anchored at the ER membrane.
Figure 7. Involvement of HRD1 and GP78 in disposal of soluble and membrane-tethered NHK variants. A Radiolabeled NHK was immunoisolated after the indicated chase times from cells expressing a scrambled siRNA (siSCR, lanes 1-3), a siRNA targeting HRD1 (siHRD1, lanes 4-6), GP78 (siGP78, lanes 7-9) or both HRD1 and GP78 (siHRD1/siGP78, lanes 10-12). Relevant bands were quantified and plotted. B Same as A for NHK_BACE. C Same as A for NHK_CD36.
3.3 Discussion

The protein folding, quality control and disposal machineries operating in the mammalian ER lumen determine the fate of thousands of gene products each one characterized by unique structural, biophysical and biochemical properties. The covalent addition of pre-assembled glucose$_3$-mannose$_2$-N-acetylglucosamine$_2$-oligosaccharides on nascent polypeptide chains (Fig. 8A) and their processing generate unique signals decoded by ER resident lectins, glucosyl transferases and hydrolases that determine prolongation or interruption of folding and onset of disposal (10,68). Current models claim that a series of α1,2-mannosidases (MnsIp and Htmlp in *S. cerevisiae* (53,54), ERManI, EDEM proteins and/or Golgi mannosidases in mammalian cells (55,60,69,70) and reviewed in (68)), slowly remove terminal α1,2-bonded mannose residues (dark green in Fig. 8A) from oligosaccharides displayed on terminally misfolded polypeptides. De-mannosylated oligosaccharides recruit ERAD lectins such as Yos9p in *S. cerevisiae* and OS-9 and XTP3-B splice variants in mammals (45-49,53,54,66,71,72). ERAD lectins and luminal chaperones such as BiP, GRP94 and PDI deliver terminally misfolded polypeptides to “dislocons” at the ER membrane. Dislocons contain a membrane-embedded E3 ubiquitin ligase and several adaptor molecules with substrate-recognition/modifying domains facing the luminal and/or the cytosolic side of the ER membrane. Altogether, these multimeric protein complexes regulate the export of terminally misfolded polypeptides across the ER membrane and their polyubiquitylation that facilitates proteasomal intervention (reviewed in (13-16)).

In *S. cerevisiae*, the primary role of de-mannosylation of folding-defective polypeptides is the exposure of the α1,6-bonded mannose residue $j$ (Fig. 8A) that recruits the ERAD lectin Yos9p (53,54). Our data show that in mammalian cells, even though de-mannosylation is required for disposal of N-glycosylated ERAD-L$_S$ (for Soluble, detached from the ER membrane) and ERAD-L$_M$ (for Membrane-tethered) substrates, OS-9 and XTP3-B are only required for disposal of ERAD-L$_S$ substrates. We have defined OS-9 and XTP3-B as *ERAD shuttles* because they associate with soluble, misfolded polypeptides in the ER lumen and deliver them at dislocons embedded in the ER membrane. Our data show that OS-9 and XTP3-B
are interchangeable in this function, thus explains the mild, if any phenotype caused by their individual inactivation reported so far in the literature.

Figure 8. Structure and composition of N-linked glycans. **A** The asparagine (Asn)-linked core oligosaccharide is composed of two N-acetylglucosamines (squares), nine mannoses (circles, the cleavable α1,2-bonded mannoses are in dark green) and three glucoses (triangles). The linkages are indicated and letters a-n are assigned. A, B and C define the three oligosaccharide branches. **B** Aberrant oligosaccharide transferred to nascent polypeptide chains in B3F7 cells (76).

At least for ERAD-L<sub>M</sub> substrates, the primary role of de-mannosylation might not be the recruitment of OS-9 and XTP3-B because the two ERAD shuttles are dispensable for disposal of N-glycosylated polypeptides tethered at the ER membrane. One could envision that another lectin decodes the ERAD signal generated upon de-mannosylation of ERAD-L<sub>M</sub> substrates when OS-9 and XTP3-B are not available. If this would hold true, this yet-to-be-characterized factor cannot act as surrogate ERAD lectin for soluble, misfolded glycopolypeptides.

Alternatively, the primary role of de-mannosylation of ERAD-L<sub>M</sub> substrates could be to elicit extraction of folding-defective polypeptides from the calnexin chaperone system, which is a rate-limiting step in disposal of N-glycosylated proteins (10). The mammalian ER contains the quality control enzyme UDP-glucose:glycoprotein glucosyl transferase (UGT1) (73). The activity of UGT1 preserves the glucose residue I (**Fig. 8A**), which is required for the association of non-native polypeptides.
with the lectin chaperones calnexin and calreticulin (74). Retention of non-native polypeptides in the calnexin chaperone system protects them from degradation (68,75). Therefore, removal of mannose \( g \), which is the only hexose that can be re-glucosylated by the UGT1, causes the irreversible extraction of terminally misfolded polypeptides from the folding machinery and is crucial to initiate a series of events eventually leading to polypeptide disposal. Consistently, pharmacologic inactivation of \( \alpha 1,2 \)-mannosidases (51) or reduction of their intraluminal level (58) substantially delay release of terminally misfolded polypeptides from calnexin. As a further indication that removal of mannose \( g \) is relevant for mammalian ERAD, recent data have shown that substrate de-mannosylation is required in mutant cell lines characterized by addition on nascent chains of aberrant oligosaccharides in which mannose \( g \) is the only removable \( \alpha 1,2 \)-bonded mannose (Fig. 8B) (60,76,77). In S. cerevisiae, a functional ortholog for UGT1 and the possibility for non-native polypeptides to be retained in the calnexin cycle are missing. Removal of mannose \( g \) as a signal required for polypeptide disposal would therefore make little sense, while removal of mannose residues \( i \) and \( k \) is crucial because it allows an active segregation of misfolded polypeptides upon Yos9p association (68).

The mammalian ER membrane hosts several E3 ubiquitin ligases that might potentially contribute to dislocation/disposal of misfolded proteins from the ER (reviewed in (13-16)). Individual or combined inactivation of two of them, namely HRD1 and GP78, was sufficient to significantly delay disposal of classical ERAD-L substrates such as BACE, CD3-\( \delta \) and NHK variants. For folding-defective polypeptides detached from the ER membrane (ERAD-L\(_ S \) substrates), efficient disposal required HRD1, SEL1L and OS-9 or XTP3-B. The stringent requirement for components of the HRD1 pathway was relieved when the very same luminal domains were tethered to the ER membrane. Thus, ERAD-L\(_ M \) substrates do use, or can eventually engage, alternative ERAD pathways when the HRD1 pathway has been shut down. Such complexity is absent or has not been reported so far in the case of S. cerevisiae in which soluble and membrane-tethered ERAD-L substrates are degraded by a unique pathway built around the E3 ubiquitin ligase Hrd1p (17,19,22,23).

It can be envisioned that access to membrane-embedded dislocons might be facilitated by lateral diffusion in the lipid bilayer when folding-defective polypeptides
are tethered to the ER membrane. On the other hand, misfolded proteins floating in
the ER lumen might be more dependent on luminal ERAD shuttles (e.g., OS-9 and
XTP3-B) that direct them from the ER lumen to dislocons embedded in the ER
membrane. HRD1 accessory factors such as OS-9, XTP3-B and their membrane-
receptor SEL1L are excluded from complexes containing GP78 or other E3
ubiquitin ligases (25,45,48). Thus, the HRD1 complex seems better equipped to
recruit soluble folding-defective polypeptides at the ER membrane.
Notably, misfolded proteins lacking a transmembrane anchor might fall outside the
ERAD-Ls group and might show little or no dependency on conventional ERAD
shuttles that would channel them to the HRD1 complex. For example ATZ and
serpins do not belong to the ERAD-Ls group because they form polymeric deposits
that cells remove from the ER by activating multiple proteasome-dependent and
proteasome-independent disposal pathways (see Introduction and (4-8)). Another
example is ApoB, a protein that lacks a transmembrane anchor but it actually is
membrane-associated in the lipid-ligand deficient state that triggers its disposal (44)
and as such may engage alternative, ERAD shuttles-independent disposal
pathways.

It remains to be established what determines the ERAD pathway selection for
ERAD-Lm substrates. For example, why membrane anchored canonical ERAD
substrates such as CD3-δ (Figs. 3-5) require GP78 and/or RFP2 for efficient
disposal (27,29,31-34), while others such as BACE476 (Figs. 2, 4, 5), NHKbace and
NHKCD3δ (Fig. 7) can use both the HRD1 and the GP78 pathways. Certainly, our
observations are consistent with the emerging picture that protein quality control in
the mammalian ER conserves regulatory factors and basic mechanisms operating
in the ER of the budding yeast but is characterized by additional levels of
complexity. This is probably required to insure efficiency of protein biogenesis and
maintenance of homeostasis in the context of multicellular systems.
3.4 Materials and Methods

Expression plasmids, antibodies and inhibitors-The pClneo plasmids encoding for CD3-Δ and XTP3-B were a kind gift of S. Fang and C. Niehrs, respectively. Plasmids for expression of BACE, NHK and HRD1 variants are described in (46,56,58). CD3-Δ was prepared by inserting a STOP codon (primer sequence: GACTCGGGCACCATGGAGTTCCGACACTGAGCTGGTGTCATCTTC) before the membrane anchor of CD3-Δ using the site-directed mutagenesis kit (Stratagene). The membrane-tethered variants of NHK (NHK_{BACE} and NHK_{CD3δ}) were generated by appending at the protein C-terminus the amino acids PQTDESTLMIAYVMAMAICALFMLPLCLMVCMQWRCLRCLQRQHDDFADDISLLK (which correspond to residues 448 to 501 of BACE501) or VELDSGTMAVIFIDLALLALGVYCAGHETGRPSGAEVQALLKNEQNYQLRDREDTQYSRLGNWPRNKS (which correspond to residues 96 to 173 of CD3-δ). Transmembrane segments are in italic. DNA preparations were obtained using commercially available purification kits (Sigma). The nucleotide sequences of all plasmids were verified on both strands. Antibodies to BACE, NHK and OS-9 are described in (46). Antibody to Tubulin is from ABM. Antibodies to GP78 and SEL1L were a kind gift of Y. Ye and H. Ploegh. Antibodies to BiP, EDEM1 and GRP94 were from Santa Cruz, Sigma and ABR, respectively. The proteasome inhibitor PS341 was a king gift of Millenium Pharmaceuticals Inc and was used at a concentration of 9 μM. Kifunensine, was from TRC Inc and was used at a concentration of 100 μM.

Cell Lines, transient transfections, RNA interferences, metabolic labelling, immunoprecipitations immunoblots and analysis of data-Hrd1⁻/⁻ and wt MEF cells were grown in DMEM supplemented with 10% FBS. Cells at 80-90% confluence in a 6 cm tissue culture plate were transfected with the expression plasmid of interest (4 μg for single transfections, 6 μg total DNA for double transfections) using Lipofectamine 2000 (Invitrogen) according to the manufacturer instructions. Experiments were normally performed 17 hours after transfection. Expression of all proteins has been confirmed by immunoprecipitation (e.g. Fig. S2). For siRNA-based interference, HeLa cells were grown in MEM Alpha supplemented with 10% FBS. Cells at 50% confluence in a 3.5 tissue culture plate were transfected with siRNA duplex (Ambion Inc, 50 pmol/dish) using Lipofectamine
2000 according to the manufacturer instructions. Four hours after transfection, the medium was replaced with MEM Alpha supplemented with 1% of non-essential amino acids (GIBCO). Thirty hours after siRNA transfection, cells were transfected with the expression plasmids of interest. Experiments were performed 48 hours post-siRNA transfection. siRNA targeting sequences: HRD1: GGUGAUGGGCAA GGUGUUC; GP78: GACGGAUUCAAGUACCUUU; SEL1L: GGCUAUACUGUGGC UAGAA; OS-9.1 and OS-9.2: CAUCAUCCAGGAGACAGAG; XTP3-B1 and XTP3-B2: AGCAGUUGUUCUACAGAA. When detection of endogenous target proteins was impossible, the efficiency of siRNA was confirmed by the significant reduction in expression of ectopically expressed targets (i.e. HRD1, GP78 and XTP3-B).

Eighteen hours after transfection, cells were starved for 20 min in Met/Cys free medium, pulsed for 10 min with 50 µCi [S\textsuperscript{35}]Met/Cys and chased for the indicated times with MEM Alpha supplemented with 5mM cold Met/Cys. Postnuclear supernatant (PNS) was prepared by solubilization of cells in 400 µl/3.5 cm dish (or 800 µl/6 cm dish) ice-cold 2% CHAPS (Anatrace) in HEPES-buffered saline (HBS), pH 6.8, containing 20 mM N-ethylmaleimide and protease inhibitors. CHAPS-insoluble material was separated by centrifugation at 10’000 g for 10 min. Immunoprecipitations were performed by adding protein A beads (Sigma; 1:10, w/v swollen in HBS) with the selected antibody and incubated for 2h at 4°C. Immunoprecipitates were extensively washed (3x10 min) with 0.5% CHAPS in HBS, resuspended in sample buffer, boiled for 5 min and finally separated in SDS-PAGE. Gels were exposed to BioMax (Kodak) films and scanned with an Agfa scanner. Relevant bands were quantified by ImageQuant software (Molecular Dynamics). Due to the number of Petri dishes that can be handled in parallel, to analyze all conditions in a single transfection round, we had to perform three-points kinetics. Proteins half-lives given in the text were confirmed by separate, independent experiments using additional chase times. Immunoblots were performed using the SNAP i.d. protein detection system (Millipore). All primary antibodies were used at 1:200-1:333 dilutions. Secondary antibodies were HRP-conjugated and used at 1:10’000 dilutions. The ECL-Plus detection system was from Amersham.

Subcellular fractionation and separation of membrane vs. luminal content-
Metabolically labeled cells expressing NHK\textsubscript{BACE} and NHK\textsubscript{CD35} were extensively washed with isotonic buffer and resuspended in 800 µl of homogenization buffer (10
mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 20 mM NEM, 1 mM EDTA and a cocktail of proteases inhibitors, pH 7.4) (59). Cells were broken with 10 passages through a 25G1 needle. Post nuclear supernatants were subjected to ultracentrifugation (45 min, 200’000 g in TLA 120.2) and the endomembrane-containing pellet was extensively washed, resuspended in 500 µl 100 mM Na₂CO₃, incubated for 25 min on ice for carbonate extraction. After an additional ultracentrifugation step (45 min, 200’000 g), the supernatant was harvested. The endomembrane fraction was washed once with 100 mM Na₂CO₃ (35 min, 200’000 g) and then resuspended in 800 µl of lysis buffer. Insoluble material was removed after 25 min on ice by 10 min centrifugation at 200’000 g. The ER luminal content and the endomembrane fraction were subjected to immunoprecipitation against calnexin (CNX), calreticulin (CRT) and NHK_BACE or NHK_CD35.

**Semi quantitative reverse transcriptase-polymerase chain reactions (RT-PCR)**- MEF cells were mock-treated or incubated for 12 h with 2.5 µg/ml tunicamycin. Cells were lysed in TRIzol reagent (Invitrogen) and RNA was isolated according to the instructions of the manufacturer. Two µg of RNA were used for cDNA synthesis using SuperScriptII reverse transcriptase (Invitrogen) and oligo(dT) (Invitrogen). RT-PCR was performed using TaqDNA polymerase (Invitrogen) with transcript-specific primers: unspliced+spliced *Xbp1*, AAACAGAGTAGCAGCTCAGACTGC (for), TGGCTGGATGAAAGCAGGTT (rev); *BiP*, GAGTTCTTCAATGGCAAGGA (for), CCAGTCAGATCAAATGTACCC (rev); *b-Actin*, CTTTCTGGGTATGGAATCTT (for), GGAGCAATGATCTTGATCTT (rev).
3.5 Bibliography

3.6 Supplementary Figures

Figure S1. Consequences of inhibition of α1,2 mannosidases and proteasomes on disposal of ERAD-LM and ERAD-LS substrates. A Radiolabeled BACE476 was immunoisolated after 10 or 360 min of chase from lysates of cells mock-treated (lanes 1-2) or incubated with 100 µM of the mannosidase inhibitor kifunensine (KIF, lane 3) or with 9 µM of the proteasome inhibitor PS341 (lane 4). UT is untranslocated, non-glycosylated BACE476. Being in the cytosol, degradation of UT is inhibited upon inactivation of cytosolic proteasomes with PS341, but remains unaffected upon inactivation of luminal α1,2-mannosidases. Relevant bands were quantified and plotted. B Same as A for BACE476Δ. Error bars represent standard deviation (n=2).

Figure S2. Control of model ERAD substrates expression, UPR activity in Hrd1−/− cells and analysis of secretion of soluble ERAD substrates. A Immunoprecipitation of model ERAD proteins from radiolabeled cell lysates transfected with an empty plasmid (lane 1) or with plasmids for expression of BACE476 (lane 2), BACE476Δ (lane 3), NHKCD3δ (lane 4), NHKBACE (lane 5), NHK (lane 6), CD3-δ (lane 7) or CD3-δΔ (lane 8). Proteins were separated in a 15% SDS-polyacrylamide gel. B Western blots for mock and Hrd1−/− cells. C Immunoblot analysis of XBP1, BIP and Actin in wild-type (wt) and Hrd1−/− cells treated with Taxol and Tunicamycin. D Western blot analysis of intracellular (lanes 1-4) and extracellular (lanes 5-8) 476Δ proteins at 0', 90', 180', and 270' time points. E Western blot analysis of intracellular (lanes 1-4) and extracellular (lanes 5-8) CD3-δΔ proteins at 0', 25', 50', 75', and 100' time points. F Western blot analysis of intracellular (lanes 1-4) and extracellular (lanes 5-8) NHK proteins at 0', 60', 120', and 180' time points.
Please note that the low content in cysteine and methionine results in a weaker signal for CD3-δ and CD3-δΔ.

B HRD1 was immunoprecipitated from Hrd1−/− cells transfected with an empty plasmid (Mock, lane 1), or with plasmids for expression of HRD1 (HRD1, lane 2) or inactive HRD1 (HRD1*, lane 3). C wt and Hrd1−/− MEF cells were exposed or not exposed to 2.5 µg/ml tunicamycin (Tun) for 12 h to induce mild ER stress. The levels of unspliced and spliced Xbp1, BiP and Actin (loading control) have been assessed by semi-quantitative RT-PCR. D Secreted radiolabeled BACE476Δ was immunosolated after the indicated chase times from cells expressing a scrambled siRNA (siSCR, lanes 1-3), a siRNA targeting HRD1 (siHRD1, lanes 4-6), GP78 (siGP78, lanes 7-9) or both HRD1 and GP78 (siHRD1/siGP78, lanes 10-12). BACE476Δ is not secreted under these experimental conditions.

E same as D for CD3-δΔ. CD3-δΔ is not secreted under these experimental conditions.

F same as D for NHK. About 15% of labeled NHK is secreted from cells.

Figure S3. Involvement of HRD1 in disposal of membrane-anchored BACE476 and CD3-δ.

A Radiolabeled BACE476 was immunosolated after the indicated chase times from cells lacking Hrd1 and transfected with an empty plasmid (Mock, lanes 1-3), or with a plasmid for expression of wild-type HRD1 (HRD1, lanes 4-6) or inactive HRD1 (HRD1*, lanes 7-9). Relevant bands were quantified and plotted. B same as A for CD3-δ. Error bars represent standard deviation (n=2).

Figure S4. Loading control and BiP levels. Total cell extract of cells incubated with a scrambled siRNA (lane 1), and siRNA targeting HRD1 (lane 2), GP78 (lane 3), GP78 and HRD1 (lane 4), SEL1L (lane 5), OS-9 (lane 6), XTP3-B and (lane 7) and XTP3-B+OS-9 (lane 8) were separated in SDS-polyacrylamide gels and transferred on PVDF. The membrane was blotted with antibodies recognizing endogenous BiP (upper panel). Tubulin is shown as a loading control (lower panel). A protein cross-reacting with the anti-BiP antibody is shown with asterisk.
Figure S5. NHK\textsubscript{BACE} and NHK\textsubscript{CD3\delta} are membrane-tethered versions of NHK. A Endomembranes were purified from metabolically labeled cells. The select proteins (calnexin (CNX), calreticulin (CRT) and NHK\textsubscript{BACE}) were immunoisolated from membrane (Membr, lanes 1–3) or luminal fractions (Lumen, lanes 4–6). In lane 1 NHK\textsubscript{BACE} co-precipitates with calnexin. B same as A for NHK\textsubscript{CD3\delta}. In lane 1 NHK\textsubscript{CD3\delta} co-precipitates with calnexin.
Chapter 4

Cyclophilin B promotes degradation of misfolded polypeptides from the mammalian endoplasmic reticulum

Riccardo Bernasconi¹, Tatiana Soldà¹, Carmela Galli¹, Thomas Pertel¹,², Jeremy Luban¹,² & Maurizio Molinari¹,³

¹Institute for Research in Biomedicine, Via V. Vela 6, 6500 Bellinzona, Switzerland
²Department of Microbiology and Molecular Medicine, University of Geneva
   CH-1211 Geneva, Switzerland
³Ecole Polytechnique Fédérale de Lausanne, School of Life Sciences, CH-1015 Lausanne, Switzerland

Submitted work

Contribution:
I performed the majority of the experiments presented in this study.
Formation and reduction of covalent bonds between cysteine side chains and \textit{cis/trans} isomerization of peptide bonds preceding proline residues are rate-limiting steps for the attainment of the native and functional 3D structure of polypeptides synthesized in the endoplasmic reticulum (ER). These reactions are also rate-limiting for the unfolding of aberrant polypeptides that require retro-translocation (dislocation) across the ER membrane for proteasomal degradation (1). \textit{In vitro}, these reactions are catalyzed by protein disulfide isomerases (PDIs (2)) and peptidyl prolyl \textit{cis/trans} isomerases (PPIs (3,4)), respectively. Extensive experimental evidence has shown the importance of PDIs-assisted polypeptide folding and unfolding in living cells (5,6). Despite 20 years of PPI catalysis experiments \textit{in vitro}, the importance of PPIs for protein quality control in the ER of living cells has not been demonstrated. Here we present the first evidence of the involvement of a PPI in protein quality control by showing that cyclophilin B (CyPB), the most abundant archetypical ER-resident PPI, plays a crucial role in disposal from the mammalian ER of a non-membrane anchored folding-defective polypeptide (ERAD-L\text{S} substrate (7)) containing cis-prolines. These findings have importance for understanding protein quality control in the ER and have implications for treatment of protein-folding diseases.
4.1 Introduction

Most polypeptides entering the ER lumen are covalently modified at asparagine side chains with glucose₃-mannose₉-N-acetylglucosamine₂- oligosaccharides. Their maturation is assisted by a dedicated folding machinery comprising the oligosaccharide-binding chaperones calnexin and calreticulin and the oxidoreductase ERp57 (8). Processing of oligosaccharides displayed on misfolded conformers by ER-resident α1,2-mannosidases, with removal of up to 4 terminal mannose residues, irreversibly extracts folding-defective polypeptides from the lectin-operated folding machinery (9). Two ERAD shuttles, OS-9 and XTP3-B, transport soluble, extensively de-mannosylated terminally misfolded glycopolypeptides (ERAD-LS substrates) from the ER lumen to the site of dislocation across the ER membrane. They deliver ERAD-LS substrates to a multi-protein complex comprising the membrane receptor SEL1L, the associated E3 ubiquitin ligase HRD1 and an elusive dislocation (retro-translocation) channel (7,10). Extensively de-mannosylated membrane-tethered polypeptides with the same luminal defects (ERAD-LM substrates) are cleared from the ER lumen even upon inactivation of the ERAD-LS pathway (7).

Although the process of dislocation across the ER membrane is poorly defined, unfolding of aberrant polypeptide chains (11) and disassembly of disulfide-bonded protein aggregates (12) have been shown to facilitate it. A role in ERAD has been demonstrated for several members of the PDI superfamily (e.g. PDI, ERp57, ERp72, ERp29, ERdj5) thus implying that reduction of inter- and intra-molecular disulfide bonds plays a crucial role in ERAD by eliminating tertiary and quaternary structures that could impair transport across a putative proteinaceous membrane dislocan (reviewed in (5)). We hypothesized that the PPIs-catalyzed interconversion of cis into trans peptidyl-prolyl bonds could also facilitate dislocation across the ER membrane of ERAD substrates by eliminating turns in the polypeptide secondary structure.
4.2 Results

4.2.1 Assessing the involvement of PPIs in ERAD

To assess the involvement of PPIs in ERAD, we monitored consequences of cell exposure to cyclosporine (CsA), an inhibitor of immunophilin members of the PPIs family (13) on degradation of one ERAD-L_M (BACE457) and of one ERAD-L_S substrate (BACE457Δ). CsA-treatment was compared with cell exposure to a series of well-characterized ERAD inhibitors (thapsigargin (Tg, which inhibits the SERCA pump thus depleting luminal calcium (14)); kifunensine (Kif, an inhibitor of α1,2-mannosidases (15)); PS341 (a proteasome inhibitor (16))). BACE457 and BACE457Δ are splice variants of the human beta-site amyloid precursor protein-cleaving enzyme BACE501 (17), an aspartic protease involved in generation of the Ab peptide that forms plaques in the brain of Alzheimer disease patients. A 44-residue deletion in the ectodomain prevents attainment of the native structure and results in degradation from the ER lumen when the proteins are ectopically expressed in cultured cells (12,18). BACE457 and BACE457Δ contain 26 and 25 proline residues, respectively. In both proteins, the peptidyl-prolyl bonds preceding the proline residues at positions 84, 146 and 390 are in the cis configuration (http://swift.cmbi.ru.nl/servers/html/index.html).

Seventeen hours after cell transfection with appropriate expression plasmids, the ectopically expressed ERAD substrates were metabolically labeled for 10 min by incubating cells in a media containing 35S-methionine and -cysteine. The initial amount of labeled BACE457 (Figs. 1A-B, lane 1) or BACE457Δ (Figs. 1C-D, lane 1) was immunoisolated from cell lysates prepared after a 10 min of chase in the absence of radioactivity. To monitor ERAD, the residual amount of labeled BACE457 and BACE457Δ was immunoisolated after a 120 (Figs. 1A-B) or a 75 min of chase (Figs. 1C-D) from mock-treated cells (lane 2) or from cells exposed to CsA (lane 3), Tg (lane 4), Kif (lane 5) or PS341 (lane 6). Tg, KIF and PS341 substantially delayed disposal of both folding-defective glycoproteins (Figs. 1A-D). This was not surprising because calcium depletion induced by Tg dramatically affects the ER lumen environment, de-mannosylation, which is inhibited by Kif, is an absolute requirement for disposal of terminally misfolded polypeptides displaying N-linked oligosaccharides and the 26S proteasome, which is blocked by PS341, degrades the misfolded
glycopolypeptides dislocated from the ER lumen (1,10). CsA was as efficient as the conventional ERAD inhibitors Tg, Kif and PS341 in inhibiting disposal of BACE457Δ (Figs. 1C-D, lane 3 vs lane 2). However, CsA was ineffective at inhibiting degradation of BACE457, thus of the same misfolded polypeptide tethered to the ER membrane (Figs. 1A-B, lane 3 vs lane 2). We therefore identify CsA as the first selective inhibitor of the ERAD-Lₘ pathway.

4.2.2 involvement of the luminal immunophilin Cyclophilin B in the ERAD-Lₘ pathway

CsA is a cyclic undecapeptide produced by the fungus Tolypocladium inflatum gams. It is used in the clinic as an immunosuppressant to reduce the risk of graft rejection upon allogenic transplant and to improve short-term allograft survival (19). The PPI family member cyclophilin B (CyPB) is the ER-resident target of CsA (20). A role for CyPB (or of any other PPI family member) in catalysis of peptidyl prolyl cis/trans isomerization in protein biogenesis and/or quality control in the ER of living cells is not supported by experimental data, yet. To determine whether CyPB selectively
intervenes in disposal of ERAD-L<sub>S</sub> substrates, we compared degradation of the ERAD-L<sub>M</sub> substrate BACE457 (Figs. 2B-C) and of the ERAD-L<sub>S</sub> substrate BACE457Δ (Figs. 2D-E) in cells with normal level of CyPB (lanes 1-3), with reduced level of CyPB (lanes 4-6) or with reduced level of CyPA, a cytosolic target of CsA (lanes 7-9). Down-regulation of the target proteins upon specific RNA interference is shown in Fig. 2A.

![Image](image_url)

**Figure. 2.** Consequences of CyPB or CyPA down-regulation on BACE457 and BACE457Δ disposal from the ER. A Down-regulation of CyPB and of CyPA were assessed by immunoblot of total cell lysates. Tubulin is a loading control. B Radiolabelled BACE457 was immunoisolated at the end of the chase times from detergent-extracts of cells expressing a scambled siRNA (siSCR, lanes 1-3), a siRNA targeting CyPB (siCyPB, lanes 4-6) or CyPA (siCyPA, lanes 7-9) and exposed to CsA (lanes 3, 6 and 9). C Quantification of the labelled polypeptide bands. D same as B for BACE457Δ. E same as C for BACE457Δ. Error bars represent SD from the mean of at least two independent experiments.
The data shown in Figs. 2B-C confirmed that cell exposure to CsA does not significantly delay disposal of the membrane-tethered BACE457 from the ER lumen (compare lane 2 with 3). Down-regulation of CyPB (Figs. 2B-C, lanes 4-6) or of CyPA (lanes 7-9) had no significant consequences on BACE457 disposal. Thus, CyPB is dispensable for disposal of this ERAD-L_M substrate.

As shown in Figs. 1C-D, CsA substantially inhibited disposal of BACE457Δ (Figs. 2D-E, lane 3 versus lane 2). Consistent with the identification of CyPB as the intracellular target of CsA modulating ERAD-L_S, the down-regulation of CyPB substantially delayed BACE457Δ disposal (Figs. 2D-E, lane 5 versus lane 2). Exposure of cells with low intralumenal content of CyPB to CsA had a minor, additional inhibitory effect on BACE457Δ disposal (lane 6 versus lane 5). In contrast, the down-regulation of CyPA did not delay BACE457Δ disposal (lane 8 versus lane 2). CsA fully maintained the inhibitory effect on BACE457Δ disposal in cells with low levels of CyPA (lane 9). These data are consistent with a selective involvement of the luminal immunophilin CyPB in the ERAD-L_S pathway.

4.2.3 The enzymatic activity is required for Cyclophilin B-assisted acceleration of ERAD-L_S

We hypothesized that CyPB might facilitate BACE457Δ disposal by assisting the enzymatic conversion of peptidyl-prolyl bonds of BACE457Δ from the cis into the trans configuration. This would eliminate turns in the polypeptide chain thus facilitating protein dislocation across the ER membrane, which is required for ERAD and occurs through an elusive proteinaceous channel (10). To address this, we assessed whether the catalytic activity of CyPB is required for BACE457Δ disposal. To this end, an active and a catalytically inactive CyPB carrying a R62A mutation that substantially reduces the prolyl isomerization activity (21) were back-transfected in cells with a reduced content of the endogenous enzyme (Fig. 3A). Both CyPB and CYPBR62A carried three silent mutations in their coding sequence to render their transcripts resistant to the small interfering RNA used to down-regulate endogenous CyPB. Ectopic expression of CyPB in cells subjected to specific RNAi to reduce the intralumenal level of the endogenous protein re-established efficient disposal of BACE457Δ (Figs. 3B-C, lane 8 versus lane 5). In these cells, like in cells with normal content of endogenous CyPB (lanes 1-3), exposure to CsA substantially delayed BACE457Δ disposal (lane 9 versus lane 8). In contrast, ectopic expression of the
enzymatically inactive CyPB_{R62A} was not sufficient to recover BACE457Δ disposal in cells depleted of the endogenous enzyme (lane 11 versus lane 5). This indicates that the enzymatic activity is required for CyPB-assisted acceleration of ERAD-Lₜ and implies that enzymatic conversion of one or more of the cis peptidyl-prolyl bonds of BACE457Δ facilitates disposal of the terminally misfolded polypeptide.

**Figure 3.** Reversibility of the ERAD defect requires back-transfection of enzymatically active CyPB. A Down-regulation of CyPB and back-transfections of active or catalytically inactive (R62A) CyPB were assessed by immunoblot of total cell lysates. Tubulin is a loading control. B Radiolabelled BACE457Δ was immunoisolated at the end of the chase times from detergent-extracts of cells expressing normal levels of CyPB (siSCR, lanes 1-3), in cells with reduced level of CyPB (siCyPB, lanes 4-6), in cells with reduced level of CyPB back-transfected with active (siCyPB+CyPB, lanes 7-9) or inactive CyPB (siCyPB+CyPB_{R62A}, lanes 10-12). C Quantification of the labelled polypeptide bands. Error bars represent SD from at least two independent experiments.

4.2.4 Cyclophilin B is only required for disposal of non-membrane tethered BACE457Δ containing cis peptidyl-prolyl bonds

To test our model, we next replaced the three cis proline residues of BACE457Δ with alanine residues. While depletion of endogenous CyPB substantially delayed disposal of the wt BACE457Δ (**Figs. 3D-E**, lane 5 versus lane 2 and **Fig. 4A-B**, lanes
4-6 versus 1-3), degradation of BACE457ΔP84,146,390A remained unperturbed upon reduction of the intraluminal content of the immunophilin (Fig. 4C-D, lanes 4-6 versus 1-3). Thus, the enzymatic activity of CyPB is only required for disposal of non-membrane tethered BACE457Δ containing cis peptidyl-prolyl bonds.

All in all, our study identified CsA as the first selective inhibitor of the ERAD-L₅S pathway. Furthermore, we showed that CyPB, the ER-resident target of CsA, facilitates disposal from the mammalian ER of BACE457Δ, an ERAD-L₅S substrate (Fig. 5). CyPB is dispensable for disposal from the ER of the same misfolded polypeptide when this substrate is tethered to the ER membrane, thus confirming that tethering at the ER membrane changes the requirement for efficient polypeptide clearance from the mammalian ER lumen (7). Finally, we provide evidence that the
effect of CyPB depends on its catalytic activity and on the presence of cis proline residues in the ERAD substrate. These data are the first demonstration that cis to trans isomerization of peptidyl-prolyl bonds facilitates polypeptide disposal and the first demonstration of intervention of a member of the PPI superfamily in protein quality control in the ER.

Figure. 5. Involvement of CyPB in disposal of an ERAD-L5 substrate with cis proline residues. The ERAD shuttles OS-9 and XTP3-B might act as Man₅,₇-binding lectins to transport misfolded glycopolypeptides from the ER lumen to the membrane-anchored adaptor protein SEL1L, which is associated with the E3 ubiquitin ligase HRD1. The terminally misfolded glycopolypeptide has to be unfolded (e.g., reduction of aberrant disulfide bonds (by PDIs) and cis to trans isomerization of peptidyl-prolyl bonds (by PPIs, namely CyPB) in order to allow dislocation across the ER membrane through an elusive channel. Poly-ubiquitylation and degradation by the 26S-proteasome occur at the cytosolic side of the ER membrane.
4.3 Materials and Methods

Expression plasmids, antibodies and inhibitors-Plasmids and antibodies for BACE variants are described in (7,18). Plasmid for CyPB expression is described in (22). Primers for silent mutations that protect ectopic CyPB from RNAi (CyPB, 5'-AAAGACTGTCCA AAAACCGTAGACAATTTTGTGGCCTTAGCT-3'). Primers for generation of inactive CyPB_{R62A} (5'-GGCTACAAAAACAGCAAATTCCATGCTGTAAT CAAGGACTTCATG-3'). Primers for generation of BACE457_{ΔP84,146,390A}, which lacks cis prolines (5'-CCGTGGCAGCGCCCAGACG-3', 5'-GGCACCCGACCTGCC TGACGACTCCC-3', 5'-CAGCGGTGGAAGGCCTTTGTCACCTTGA-3'). Mutants were generated using the site-directed mutagenesis kit (Stratagene). DNA preparations were obtained using commercially available purification kits (Sigma). The nucleotide sequences of all plasmids were verified on both strands. Antibodies against CyPB, CyPA and Tubulin were from ABR, Biomol and ABM. The proteasome inhibitor PS341 was a kind gift of Millenium Pharmaceuticals Inc and was used at a concentration of 9 µM. Kifunensine (Toronto Research Chemicals Inc), thapsigargin (Sigma) and cyclosporine A (Bedford Labs) were used at a concentration of 100 µM, 300 nM and 20 µM, respectively.

Cell Lines, transient transfections, RNA interferences, metabolic labelling, immunoprecipitations, immunoblots and analysis of data-HeLa cells were grown in MEM Alpha supplemented with 10% FBS. Cells at 80-90% confluence in a 6 cm tissue culture plate were transfected with the expression plasmid of interest (4 µg for single transfections, 6 µg total DNA for double transfections) using Lipofectamine 2000 (Invitrogen) according to the manufacturer instructions. Experiments were normally performed 17 hours after transfection. For siRNA-based interference, HeLa cells at 50% confluence in a 3.5 tissue culture plate were transfected with siRNA duplex (Ambion Inc, 50 pmol/dish) using Lipofectamine 2000 according to the manufacturer instructions. Four hours after transfection, the medium was replaced with MEM Alpha supplemented with 1% of non-essential amino acids (GIBCO). Thirty hours after siRNA transfection, cells were transfected with the expression plasmids of interest. Experiments were performed 48 hours post-siRNA transfection. siRNA targeting sequences: CyPB: CAAAAACAGUGGAUAAUUU; CyPA:
CUGGAUUGCAGAGUUAAGU. Eighteen hours after transfection, cells were starved for 20 min in Met/Cys free medium, pulsed for 10 min with 50 μCi [35S]Met/Cys and chased for the indicated times with MEM Alpha supplemented with 5 mM cold Met/Cys. Postnuclear supernatant (PNS) was prepared by solubilization of cells in 400 μl/3.5 cm dish (or 800 μl/6 cm dish) ice-cold 2% CHAPS (Anatrace) in HEPES-buffered saline (HBS), pH 6.8, containing 20 mM N-ethylmaleimide and protease inhibitors. CHAPS-insoluble material was separated by centrifugation at 10'000 g for 10 min. Immunoprecipitations were performed by adding protein A beads (Sigma; 1:10, w/v swollen in HBS) with the selected antibody and incubated for 2h at 4°C. Immunoprecipitates were extensively washed (3x10 min) with 0.5% CHAPS in HBS, resuspended in sample buffer, boiled for 5 min and finally separated in SDS-PAGE. Gels were exposed to BioMax (Kodak) films and scanned with an Agfa scanner. Relevant bands were quantified by ImageQuant software (Molecular Dynamics). Immunoblots were performed by using the SNAP i.d. protein detection system (Millipore). All primary antibodies were used at 1:200-1:333 dilutions. Secondary antibodies were HRP-conjugated and used at 1:10'000 dilutions. The ECL-Plus detection system was from Amersham.
4.4 Bibliography

Coronavirus hijack LC3-I-positive EDEMosome membranes for replication

Fulvio Reggiori¹#, Iryna Monastyrska¹*, Monique H. Verheije²*, Tito Calì³,⁴, Mustafa Ulasli¹, Siro Bianchi³, Riccardo Bernasconi³, Cornelis A.M. de Haan²# & Maurizio Molinari³,⁵#

¹Department of Cell Biology and Institute of Biomembranes, University Medical Centre Utrecht, Utrecht, The Netherlands
²Virology Division, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands
³Institute for Research in Biomedicine, Bellinzona, Switzerland
⁴Department of Biochemistry, University of Padova, Padova, Italy
⁵Ecole Polytechnique Fédérale de Lausanne, School of Life Sciences, Lausanne, Switzerland

* Equal contribution

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Contribution:
For this work I have established that OS-9 is a short-living ER chaperone and that is segregated in so-called EDEMosomes.
Coronaviruses (CoV) are enveloped positive-stranded RNA viruses that infect the mammalian respiratory and gastrointestinal tracts with mechanisms that are poorly characterized. Here we show that mouse hepatitis virus (MHV) exploits the host cell machinery for the COPII-independent vesicular export from the endoplasmic reticulum (ER) of EDEM1, a short-living regulator of ER-associated degradation (ERAD), to co-opt cellular membranes for replication. Cell infection with MHV causes accumulation of EDEM1 and of at least another short-living ER chaperone, OS-9, in the virus-induced double membrane vesicles (DMVs) containing the viral replication and transcription complexes (RTCs). DMVs are coated with non-lipidated LC3/Atg8 and the down-regulation of this protein, but not inactivation of host cell autophagy, protects mammalian cells from CoV infection. Our study identifies the host cellular pathway hijacked by CoV paving the way for the development of new therapies to combat this family of viruses and describes a new autophagy-unrelated role for non-lipidated LC3-I.
5.1 Introduction

As an early and crucial event upon infection, CoV such as the severe acute respiratory syndrome (SARS) virus and MHV induce the formation of DMVs in host cells and target their RTCs on the limiting membranes of these structures (1-4). A recent analysis of SARS- and MHV-infected cells by electron tomography has revealed that these DMVs are part of a reticular network of modified ER membranes and contain double-stranded RNA (dsRNA) in their interior [(5) and references therein]. The ER origin of the DMVs is supported by the finding that 2 nonstructural proteins (nsp3 and nsp4) with transmembrane segments that are part of the RTCs become N-glycosylated (6-8). Moreover, nsp4 localizes to the ER when separately expressed and moves to the DMVs upon viral infection (9). While previous work has shown that the early secretory pathway and CoV replication are closely connected (9-11), the lack of ER, ER-Golgi intermediate compartment (ERGIC) or Golgi protein markers in CoV-induced DMVs, suggests that their biogenesis does not depend on the conventional routing of proteins through this transport pathway (9,10,12). Thus, even though CoV must hijack ER-derived host cell membranes for replication, the precise origin of the lipid bilayers of the DMVs, the host protein content and the identity of the cellular factors essential for DMV formation, remain mysterious (5).

The possible involvement of the autophagy machinery in the conversion of host membranes into DMVs has been reported. However, while Atg5, an essential component of the autophagy machinery (13,14), has been shown to be dispensable for MHV replication (15), contradictory immuno-fluorescence (IF) data report the presence (15,16) or the absence (2,12) of the autophagosome protein marker LC3/Atg8 on DMVs (2). In this study we have used MHV, the virus prototype for the investigation of the CoV biology, to unveil the origin of the virus-induced DMVs and to identify host cell factors essential for CoV replication.

In the ER, newly synthesized, unstructured polypeptides can attract the folding, as well as the degradation machineries that are operating in the lumen of this organelle (17,18). Under normal growth conditions therefore, the activity of the ERAD machinery must be maintained low to avoid premature interruption of folding programs and favour attainment of the native structure over degradation of immature
polypeptides (19). Consistently, it has been reported that EDEM1, a crucial regulator of ERAD (20,21), is selectively cleared from the ER to tune-down the ERAD activity (19). This post-translational regulation mechanism has been named ERAD tuning. It relies on the selective sorting of EDEM1 and probably other short-living ER chaperones in 200-800 nm vesicles called EDEMosomes. These vesicles, coated with non-lipidated LC3/Atg8 (LC3-I) (19), emerge from the ER through a COPII coat-independent mechanism (22) and deliver their content to the endosomal and/or lysosomal compartments for disposal in a series of poorly characterized events (17,19,22,23).

We have discovered that MHV exploits the pathway of EDEMosomes formation to generate the DMVs required for viral replication. In doing so, MHV interferes with the degradation of EDEM1 and OS-9, another short-living chaperone that we identify here as a second EDEMosome cargo, by trapping them into the DMVs. Our data are consistent with the model in which DMVs are generated from the host ER (5) and explain the absence of conventional ER-resident chaperones in their interior. In addition, we show that while the autophagy pathway is not essential for MHV infection, non-lipidated LC3-I coats CoV-induced DMVs and is essential for MHV replication.
5.2 Results

5.2.1 MHV infection does not require an intact autophagy machinery

To conclusively establish whether autophagy is required for MHV infection, we assessed the consequences of deleting ATG7, a gene essential for autophagy (24), on DMV biogenesis. IF analysis of two RTC components, nsp2 and nsp3, in MHV-infected wild type (ATG7+/+) and ATG7 knockout mouse embryonic fibroblasts (ATG7−/− MEF) revealed that these two proteins were similarly distributed to numerous punctuate structures in both cell lines (Fig. 1A). These puncta represent the virus-induced DMVs that contain viral dsRNA [co-localization in Fig. 1A and (5,8)]. The presence in both ATG7+/+ and ATG7−/− MEF of these double-membrane structures with a diameter of approximately 200-350 nm (5,25) was confirmed by conventional electron microscopy (Fig. 1B, arrowheads). Moreover, cells with and without ATG7 were equally susceptible to the MHV infection. This was established by assessing the virus replication using a recombinant luciferase-expressing MHV [Fig. 1C; (26)] and by determining the titer of a virus stock on these cells by using the mean Tissue Culture Infection Dose (TCID50) test (Fig. 1D). MHV replication in ATG7+/+ and ATG7−/− MEF during the course of an infection was also very similar (Fig. 1E). Thus, the conventional host cell autophagy is not required for formation of MHV-induced DMVs, nor for viral replication and production of viral progeny.
MHV replication does not change during the course of an infection. The experiment described in panel C was repeated in a time-course manner and luciferase expression was determined at 5h, 7h and 10h p.i.

5.2.2 Non-lipidated LC3/ATG8 associates with CoV-induced DMVs

Since contrasting data have been published on the presence (15,16) or the absence (2,12) of LC3/ATG8 on CoV-induced DMVs, we examined this issue. Our analysis by IF showed that endogenous LC3 extensively co-localized with the DMV protein markers nsp2 and nsp3 (Fig. 2A and quantification in Fig. 2C). This co-localization
was observed during the entire course of the MHV infection (Fig. S1). In contrast, ectopically expressed GFP-LC3, a conventional protein marker for autophagosome membranes (27,28), did not co-localize with nsp2 and nsp3 (Figs. 2B-C). These apparently conflicting data were confirmed in other cell lines (e.g. in HeLa cells, Fig. S2) and explain the contradictory data in the literature.

Figure 2. Autophagy-independent recruitment of LC3-I onto MHV-induced DMVs. A,B. Endogenous LC3 partially co-localizes with MHV-induced DMVs. HEK293 cells stably transfected (B) or not (A) with a plasmid expressing GFP-LC3 were infected with MHV-Srec and processed for IF at 7h p.i. C. Summary statistics of the samples shown in panels A and B expressed as the percentage of LC3 or GFP-LC3 puncta co-localizing with the nsp2/nsp3 signals. Error bars represent the standard error of the mean percentage from counting 40 cells in three independent experiments. The asterisk indicates that the two samples are significantly different (t_{df=78}=10.4, p<0.0001). D. LC3-I is recruited to DMVs independently from the autophagy machinery. Atg7^{+/+} and Atg7^{-/-} MEF were infected with MHV before being processed for IF at 7h p.i. E. Statistical analysis of the samples shown in (D) performed as described in (C). F. EDEM1 is present in nsp2- and LC3-I-positive membranes. HeLa-CEACAM1a cells were infected with MHV-nsp2GFP for 7h before fractionating a cell extract on a continuous Optiprep gradient. Ten fractions were collected from the top to the bottom of the gradient and probed with antibodies against EDEM1, GFP and LC3. The fractionation profile was confirmed by performing this experiment three times. G. Non-lipidated LC3-I associates with DMVs. HeLa-CEACAM1a cells were transiently transfected with a plasmid expressing C-terminally HA-tagged, non-lipidable LC3 before being infected with MHV. Cells were fixed at 7h p.i. and processed for IF. DMVs and LC3-HA were detected with antibodies against nsp2/nsp3 and HA, respectively.
LC3 is present in the cell predominantly in a cytoplasmic form (LC3-I) that upon autophagy induction is converted into an active lipitated form (LC3-II) by specific covalent linkage to the phosphatidylethanolamine present on autophagosomal membranes (27,28). The lipidation of LC3-I and the formation of LC3-II-coated autophagosomes require several proteins, including Atg7 (24). However, Atg7 was not necessary for the association of endogenous LC3 to DMVs (Fig. 2D and quantification in Fig. 2E). Consequently, Atg7 and LC3 lipidation are not essential for the formation of LC3-positive DMVs. Analysis of the protein content in DMVs induced upon MHV-nsp2GFP infection of HeLa cells and separated on continuous density gradients as described (19) showed the presence of LC3-I in the denser fractions containing the DMV protein marker nsp2-GFP (Fig. 2F, fractions 9 and 10). These fractions were clearly separated from the lighter autophagosomes containing LC3-II that floated at the top of the gradient. Moreover, IF analyses revealed that ectopically expressed C-terminally HA-tagged, non-lipidable LC3 localizes on DMVs (Fig. 2G). Taken all together, these data show that an intact host autophagy machinery is dispensable for the virus life cycle and lipidation is not required for LC3 association with the DMV membranes.

5.2.3 Analogies between MHV-induced DMVs and EDEMosomes
The ER origin of the MHV-induced DMVs (5,9), the absence of conventional ER markers in their membranes and lumen (9,10,12), their association with LC3-I and the fact that DMVs are stained with antibodies against endogenous LC3 but not with ectopically expressed GFP-LC3 (Figs. 2, S2) are features reminiscent of those describing the EDEMosomes (19). Significantly, the LC3-I coat distinguishes DMVs (this study) and EDEMosomes [(19) and Fig. S6] from autophagosomes, which are associated with LC3-II and can be decorated with GFP-LC3 (27,28).
As in the case of formation of the ER-derived, CoV-induced DMVs, it is unclear whether an active autophagy machinery is required for formation of ER-derived EDEMosomes and/or for disposal of EDEM1 [(19) vs. (23)]. To better understand this, we compared variations in the intracellular levels of EDEM1 and of p62, a canonical substrate of autophagy (29), under conditions that either inactivate [e.g. ATG7-deletion (Figs. 3A-B) or cell incubation with chloroquine (CQ, Figs. 3C-D) (24,27)] or activate autophagy [(e.g. rapamycin (rap, Figs. 3C-D) treatment (27)]. Deletion of ATG7 inhibits the turnover of p62 (30) thus substantially increasing the
intracellular level of this autophagy substrate (Fig. 3A, lane 2 vs lane 1). On the contrary, deletion of ATG7 did not result in substantial variations of the level of EDEM1 (Fig. 3A, lane 2 vs lane 1), thus showing that Atg7 and conventional autophagy are dispensable for EDEM1 turnover. Consistently, when wild type and ATG7−/− MEF were metabolically radiolabeled and chased for 10-90 min, the amount of residual endogenous EDEM1 decreased with similar kinetics in the two cell lines confirming similar rate of disposal in the presence or absence of Atg7 (Fig. 3B and quantifications). Cell exposure to CQ inhibited the p62 degradation resulting in higher levels of this protein, as expected for a substrate of autophagy [Fig. 3C, lane 2; (31)]. CQ delayed EDEM1 turnover and resulted in intracellular accumulation of EDEM1 [Fig. 3C, lane 2 vs. lane 1, and 3D, panel on the left; (19)]. Finally, induction of autophagy with rapamycin reduced the intracellular levels of p62 as expected for an autophagy substrate [Fig. 3C, lane 3; (31)], but increased those of EDEM1 (Fig. 3C, lane 3 vs. lane 1) by delaying its turnover (Fig. 3D, panel on the right). These results confirmed that, as reported above for CoV replication, the pathway regulating EDEM1 turnover is clearly distinct from autophagy. They also highlight another analogy between the ERAD tuning pathway and the CoV infection. Cell exposure to the autophagy-inducer rapamycin negatively affected both EDEM1 turnover (Fig. 3C-D) and MHV replication as shown by measuring the levels of both the N nucleocapsid levels and luciferase in cells infected with the recombinant luciferase-expressing MHV (Fig. S3). All together, these observations led us to hypothesize that MHV hijacks the ERAD tuning machinery to co-opt cellular membranes for DMVs generation.
Figure 3. Components of the ERAD tuning pathway are associated with DMVs. A,B. EDEM1 turnover does not depend on an intact autophagy machinery. (A) Cell extracts from Atg7+/+ (lanes 1) and Atg7−/− (lane 2) were separated by SDS-PAGE and Western blot membranes probed with antibodies against EDEM1, p62, LC3 and tubulin. Repetition of the analysis showed no significant differences in the level of EDEM1 in Atg7+/+ vs. Atg7−/− MEF. The percentages (left panel) indicate the relative EDEM1 levels in the knockout cells compared to wild type cells, and represent the average of two experiments. (B) Atg7+/+ and Atg7−/− MEF were metabolically labeled and chased for the times
indicated before lysis and immuno-isolation of EDEM1. The residual radiolabeled EDEM1 present in each lane was quantified and indicated below each band. Repetition of the analysis showed no significant differences in the turnover of EDEM1 in Atg7<sup>+/+</sup> vs. Atg7<sup>−/−</sup> MEF. C.D. CQ and rapamycin effects on EDEM1 turnover. (C) Atg7<sup>+/+</sup> MEF were untreated or treated with 100 mM CQ or 1 mM rapamycin (rap) for 4h before preparation of cell extracts and analysis as in panel A. The percentages (left panel) indicate the relative EDEM1 levels in drug-treated cells compared to mock-treated cells, and represent the average of two experiments. (D) Same as (B) to confirm in a pulse-chase radiolabeling experiment that CQ and rap delay EDEM1 turnover. EDEM1 and OS-9 co-localise with DMVs. HeLa cells were infected with MHV-Srec and processed for IF at 7h p.i. using antibodies against dsRNA and (E) EDEM1 or (F) OS-9. G. OS-9 co-localises with dsRNA in autophagy-deficient cells. Atg7<sup>+/+</sup> and Atg7<sup>−/−</sup> MEF infected with MHV-Srec were fixed at 7h p.i. and processed for IF using antibodies against OS-9 and dsRNA. H. Variations of EDEM1 and OS-9 cellular levels upon MHV infection. Cell extracts were analyzed by Western blot using antibodies against EDEM1 or OS-9. The percentages (left panel) indicate the relative EDEM1 and OS-9 levels in infected cells compared to control cells, and represent the average of two experiments.

5.2.4 MHV infection interferes with ERAD tuning and results in accumulation of ERAD tuning substrates in the virus-induced DMVs

As mentioned above, despite their ER origin (5), CoV-induced DMVs and EDEMosomes do not contain conventional ER chaperones or protein markers of the early compartments of the secretory pathway (9,12,19,25,32). By IF analysis of MHV-infected wild type and Atg7<sup>−/−</sup> knockout cells, we systematically assessed the presence in DMVs of several conventional protein markers of the early compartment of the secretory pathway essentially confirming that none of them was contained in the virus-induced DMVs (Fig. S4 and not shown). Only two antibodies stained the virus-induced dsRNA- or nsp2/nsp3-positive compartments, namely those recognizing EDEM1 and OS-9 (Fig. 3E-F). The presence of EDEM1 during the entire course of the infection (Fig. S5) confirmed our working model postulating that CoV hijack the ERAD tuning pathway to promote formation of their DMVs. Our model was further corroborated by the observation that cell infection with MHV substantially interfered with ERAD tuning, thus causing intracellular accumulation of EDEM1 and OS-9 (Fig. 3H, lane 2 vs lane 1 and quantifications), due to the re-localization and confinement of these chaperones into the lasting MHV-induced DMVs [refer to the EDEM1 fractionation profile of Fig. 2F and the IF data in Fig. 3E-F, S5)]. In non-infected cells, EDEM1 has an half-life of about 60 min (Fig. 3B,D and (19)). Since MHV infection induces a host translational shutoff (33), the persistence of EDEM1 (and OS-9) for several hours confirms the defective clearance of this protein in infected cells (Fig. 3H, S5). Entrapping of a fraction of cellular OS-9 in the DMVs was unexpected but significant. Like EDEM1, OS-9 is a regulator of protein disposal from the ER and it is expressed in two splice variants, OS-9.1 and OS-9.2 (34,35).
Immuno-detection of OS-9 in the MHV-induced DMVs (Fig. 3F for HeLa cells and Fig. 3G for ATG7+/+ and ATG7-/- MEF) led us to verify by subcellular fractionation whether OS-9 localizes to the same LC3-I-positive vesicular structures containing EDEM1 in non-infected cells (19). Our analysis showed that at steady state about 20% of OS-9.1 and a smaller amount of OS-9.2 were indeed found in LC3-I-positive EDEMosomes, which sedimented in fractions 7-9 of a continuous Optiprep gradient, while other conventional ER chaperones were fully excluded from these fractions [Fig. S6; (19)]. Despite their presence in DMVs, EDEM1 and OS-9 are not required for MHV replication. In fact, MHV infectivity was not affected by their knockdown as measured by examining the synthesis of the viral structural N protein, the DMV formation by IF and by assessing virus replication by either determining the TCID_{50} value of a virus stock on these cells or assaying the luciferase expression levels at different p.i. time points (Fig. S7). Taken together, these results support the hypothesis that CoV hijack the ERAD tuning machinery to generate the replicative DMVs.

5.2.5 LC3-I is required for CoV replication

The association of LC3-I with MHV-induced DMVs is supported by data showing that these vesicles are decorated with anti-LC3 antibodies in cells lacking Atg7 and LC3-II (Fig. 2D), they co-sediment with LC3-I but not LC3-II in density gradients (Fig. 2F) and they co-localize with LC3-HA (Fig. 2G). We therefore verified whether LC3 is required for viral replication, even though the autophagy protein Atg7, which is required for its covalent membrane association, is dispensable (Fig. 1). Efficient depletion of LC3A and LC3B was obtained by specific RNA interference and was confirmed with isoform-specific antibodies (Fig. 4A, lane 2). LC3 down-regulation protected cells from MHV infection as assessed by inhibition of the synthesis of the structural protein N (Fig. 4A) and by a substantial decrease in luciferase levels measured in both single time point and time-course experiments (Fig. 4B-C, respectively). The inhibition of MHV replication observed in LC3 knockdown cells was caused by a defect in DMVs biogenesis as no nsp2 and nsp3 signal was detected by IF (Fig. 4D). The initial DMVs and RTCs generated upon MHV infection are necessary for the massive synthesis of extra nsp proteins, which in turn leads to the formation of a multitude of additional DMVs and RTCs. Consequently, a defect in DMV biogenesis results in a severe block of nsp production. Crucially, back-
transfection of LC3 knockdown cells with the plasmid expressing C-terminally HA-tagged, non-lipidable LC3 restored MHV replication measured by monitoring N protein synthesis (Fig. 4E).

Figure 4. LC3-I is required for the formation of DMVs. A, B. LC3 is essential for CoV infection. HeLa-CEACAM1a cells were transfected with either siRNA directed against LC3A and LC3B (siLC3) or non-targeting (siSCR) siRNA. After 48h, cells were infected with MHV-2aFLS and analyzed at 7h p.i. This analysis has been repeated five times. (A) Cell lysates were analyzed by Western blot using antibodies against the viral N protein, LC3 and actin. The percentage indicates the relative N protein levels in LC3-depleted cells compared to control cells and represent the average of three experiments. (B) Luciferase activity was measured as described in Experimental Procedures and error bars indicate the standard error deviations for an experiment made in triplicate. C. The experiment described in panel B was repeated in a time-course manner and luciferase expression was determined at 5h, 7h and 10h p.i. This experiment has been repeated three times. D. DMV biogenesis is strongly reduced in absence of LC3. Localization of nsp2/nsp3 and LC3 was examined by IF. E. C-terminally HA-tagged, non-lipidable LC3 rescues the MHV replication defect in cells subjected to specific LC3 down-regulation. HeLa-CEACAM1a cells were transfected with either siRNA directed against LC3A and
LC3B (siLC3) or non-targeting (siSCR) siRNA. After 24h, one of the samples was transfected with a plasmid expressing C-terminally HA-tagged, non-lipidable LC3 while MHV was inoculated at 48h. Cell lysates were prepared at 7h p.i. and analyzed by Western blot using antibodies against the viral N protein, LC3, HA and actin. Note that the higher levels of LC3-I in the siLC3+LC3-HA lane are due to LC3-HA, which run as LC3-I in the used SDS-PAGE gel.
5.3 Discussion

During evolution, pathogens have developed molecular devices to exploit conserved cellular pathways to optimally infect, replicate and/or leave host cells. Until now, no host protein has been directly implicated in CoV replication. The ER origin, the unconventional association to LC3-I, the lack of a COPII coat that normally characterizes vesicles released from the ER, the absence of conventional ER protein markers, and the presence of EDEM1 and OS-9, are all characteristics that CoV-induced DMVs share with EDEMsomes. The fact that both CoV replication and EDEM1/OS-9 turnover rely on mechanisms that share several analogies leads us to postulate that CoV hijack the ERAD tuning machinery for the generation of DMVs, which provide the membranous support for viral RTCs.

Based on our data, our current working model is that one or more CoV nsp’s do associate with a still elusive EDEMsome cargo receptor that normally mediates segregation and vesicular export from the ER of EDEM1 and OS-9. Accordingly to what is known about other vesicular transport pathways (36), we postulate that the EDEMsome cargo receptor interacts with subunits of a cytosolic vesicle protein coat (non-lipidated LC3-I seems a good candidate for participating in this function). It is unlikely that the viral nsp’s recruit LC3-I at the cytosolic surface of DMVs because LC3-I is associated to EDEMsomes in uninfected cells (19) and because we have not found direct interaction between nsp’s and LC3-I (our unpublished data). Astonishingly, while EDEMsomes are transient structures that end their journey in late endosomes and/or lysosomes, MHV-induced DMVs are persistent cytoplasmic organelles (32). Therefore, the presence of either nsp’s or of other viral products inhibits the fusion of EDEMsomes/DMVs with the degradative compartments of the endosomal system. A main goal of future research is the identification of the elusive EDEMsome cargo receptor and the characterization of its involvement in CoV infection. It also remains to be established whether the ERAD tuning and CoV infection share mechanistic analogies with the recently characterized Atg5/Atg7-independent type of autophagy (37). Importantly, in our study we also show that blocking DMV formation by depleting LC3 severely impairs MHV infection. Consequently, future investigations on the involvement of viral proteins and ERAD tuning components as well as the unconventional intervention of certain factors of the
host cell autophagy machinery in the formation of CoV-induced DMVs, might provide valuable therapeutic targets.
5.4 Materials and Methods

**Cell lines and viruses**-HEK293, HeLa, HeLa-CEACAM1a [HeLa cells stably expressing the MHV receptor mCEACAM1a, (10)], murine LR7, shLUC HEK293, shEDEM1/shOS-9 HEK293 cells and, Atg7+/+ and Atg7−/− MEF [a kind gift of Masaaki Komatsu, The Tokyo Metropolitan Institute Medical Science, Tokyo, Japan, (24)] were grown in Dulbecco's modified Eagle's medium (Invitrogen, Breda, Netherlands) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (all from Invitrogen). The shLUC and shEDEM1/shOS-9 HEK293 cell lines stably silenced for luciferase and both splice variants of OS-9 plus EDEM1, respectively, were generated as described (34). Targeting sequences were GCTGCCTACCTG GAGATTCAG for OS-9 and ACAGATAGTCTCTTAGTTAT for EDEM1. The HEK293 cells stably transfected with a plasmid expressing GFP-LC3 (38), kindly provided by Sharon Tooze (Cancer Research UK, London, United Kingdom), were grown in the same medium supplemented with 0.5 mg/ml of G418 (Invitrogen). HeLa cells were transiently transfected with the plasmid expressing GFP-LC3 (a kind gift of Karla Kirkegaard) using Lipofectamine 2000 (Invitrogen) according to the manufacture’s protocol. LR7 mouse cells were used to propagate the wild type and recombinant MHVs (based on the A59 strain). The recombinant viruses expressing the firefly luciferase gene (MHV-2aFLS), nsp2-GFP (MHV-nsp2GFP) or the spike protein that gives the virus an extended host range (MHV-Srec) have been described elsewhere (10,26,39).

**Gene silencing with siRNA**-Sets of 3 different siRNA duplexes targeting different sites within the coding sequences of LC3A and LC3B were designed by and obtained from Applied Biosystems/Ambion (Nieuwerkerk a/d IJssel, The Netherlands). One day after seeding, the HeLa-CEACAM1a cells were transfected with 40 nM of siRNA using Lipofectamine 2000. Experiments were conducted at 48h post-transfection. Antibodies against LC3 (NanoTools, Teningen, Germany) were used to assess by Western blot the depletion of the targeted gene.

**Plasmid construction**-LC3B was amplified by PCR from cDNA (rzpd, Berlin, Germany) using appropriate primers and cloned into both the pDest-520 vectors
generating the pLC3B-HA plasmids. The 3’ primer used to create the C-terminally tagged version of LC3B was designed such that it mutates the penultimate glycine of LC3B into an alanine so that the resulting protein cannot be converted into LC3-II by proteolytic cleavage and subsequent lipidation. In addition, silent mutations were introduced into the LC3B nucleotide sequence in order to have the LC3B-HA protein fusion not knockdown by the employed siRNA duplexes.

**Immuno-fluorescence microscopy**-Cells were grown and processed for IF on coverslides as described (10). Fluorescence signals were visualized with a DeltaVision RT fluorescence microscope equipped with a CoolSnap camera (Applied Precision, Issaquah, WA). Images were generated by collecting a stack of 20 pictures with focal planes 0.20 µm apart in order to cover the entire volume of the cell and by subsequently deconvolving them using the SoftWoRx software (Applied Precision). A single focal plane is shown at each time. Primary immunological reactions were carried out with polyclonal anti-EDEM1 (Sigma, Zwijndrecht, Netherlands), anti-OS-9 (Novus Biologicals, Littleton, CO), anti-nsp2 and nsp3 (40) and anti-Sec23 (Affinity Bioreagents, Etten-Leur, Netherlands) antisera and monoclonal anti-LC3 (NanoTools), anti-HA (a kind gift of G. Bu, Washington University), anti-dsRNA K1 (English and Scientific Consulting Bt., Szirák, Hungary), anti-KDEL (Calbiochem, Nottingham, UK) and anti-ERGIC53 (Alexis Biochemicals, Zandhoven, Belgium) antibodies. All experiments were repeated two or three times, and the presented images show a representative fluorescence profile.

**Western blot analyses**-The cell extracts were prepared with lysis buffer (200 mM NaCl, 50 mM HEPES pH 6.8, 2% CHAPS and protease inhibitors) and boiling for 5 min in sample buffer. Proteins were then separated on a SDS-PAGE gel and transferred onto a PVDF membrane before being analyzed with specific antibodies against EDEM1, OS-9, N protein (a kind gift from Stuart Siddell, University of Bristol, United Kingdom), Erp57 (41), calnexin (a kind gift of A. Helenius, ETH Zurich, Switzerland), p62 (Progen Biotechnik, Heidelberg, Germany), mouse BiP (Stressgen, Ann Arbor, MI), tubulin (Applied Biological Materials, Richmond Canada) and actin (MP Biomedicals, Illkirch, France). Alexa Fluor680-conjugated goat anti-rabbit or rabbit anti-mouse secondary antibodies (Molecular Probes, Breda, Netherlands)
were used for the visualization of the immuno-blots using an Odyssey system (Li-Cor Biosciences, Lincoln, NB). In all Western blot analyses, tubulin and actin represent the loading controls.

**Miscellaneous procedures**- Electron microscopy, immuno-electron microscopy, the quantification of the virus replication using firefly luciferase-carrying virions MHV-2aFLS, the measurement of the TCID\textsubscript{50}, the subcellular fractionation on Optiprep gradients, pulse-chase radiolabeling experiments followed by immuno-precipitations and electron microscopy were conducted as previously described (10,19,42).

**Statistical analysis**- The number of punctate LC3 or GFP-LC3 fluorescent dots was counted in the central section of each cell. Proportions of LC3 or GFP-LC3 puncta co-localizing with nsp2/nsp3 were fitted with a generalized linear model in R version 2.8.1 (R Development Core Team 2009), using the glm() procedure and the family=quasibinomial option to correct for overdispersion. Differences between LC3 and GFP-LC3 were assessed with the t-test.
5.5 Bibliography


5.6 Supplementary Figures

Figure S1. Endogenous LC3 co-localization with MHV-induced DMVs during the course of an infection. A. HeLa-CEACAM1a cells were infected with MHV and processed for IF at 5h, 7h and 10h p.i. B. Summary statistics of the samples shown in panels A as the percentage of DMVs co-localizing with LC3. Error bars represent the standard error of the mean percentage from counting of 40 cells.

Figure S2. N-terminally GFP-tagged LC3 does not co-localise with DMVs in HeLa cells. HeLa cells were transfected with either a plasmid expressing GFP-LC3 (B) or mock-treated (A) and after 24h, infected with MHV-Srec. Cells were then fixed at 7h p.i and analysed by IF as in Figures 2A and 2B. This analysis has been performed twice.
Figure S3. Rapamycin treatment affects MHV replication in an autophagy-independent way. A,B. HeLa-CEACAM1a cells grown in presence of protease inhibitors (10 mg/ml E64d and 28 mg/ml pepstatin A) for 1h were inoculated with MHV-2aFLS and 10 µM rapamycin was added 1h p.i. Subsequently, cell extracts were prepared at 7h p.i. and used to analyze (A) the N protein synthesis by Western blot and (B) the virus replication by measuring luciferase expression. This analysis was repeated twice. C. The experiment described in panel B was repeated in a time-course manner and luciferase expression determined at 5h, 7h and 10h p.i. D. The experiment described in panel B was also performed in Atg7+/+ and Atg7-/- MEF, and repeated 3 times. It shows that rapamycin inhibits MHV replication independently of the presence of an intact autophagy machinery.

Figure S4. Protein markers for the early compartments of the secretory pathway do not co-localize with the MHV-induced DMVs. HeLa cells were infected with MHV-Srec and fixed at 7h p.i. before being processed for IF with antibodies against the dsRNA or nsp2/nsp3 and the KDEL tetrapeptide (ER), Sec23 (ER exit sites and COPII vesicles) or ERGIC-53 (ERGIC and cis-Golgi). These analyses have been carried out twice.
Figure S5. EDEM1 co-localization with MHV-induced DMVs during the course of an infection. A. HeLa-CEACAM1a cells were infected with MHV and processed for IF at 5h, 7h and 10h p.i. B. Summary statistics of the samples shown in panel A as the percentage of EDEM1 co-localizing with MHV-induced DMVs. Error bars represent the standard error of the mean percentage from counting 40 cells.

Figure S6. OS-9 is a new cargo protein of EDEMosomes. A cell extract (tot) obtained from MEF was fractionated on a Optiprep gradient and fractions were analyzed by Western blot with antibodies against EDEM1, OS-9, LC3, calnexin and ERp57. This subcellular fractionation experiment has been repeated 5 times with identical results.
Figure S7. EDEM1 and OS-9 are not required for MHV infectivity. A. Expression levels of EDEM1 and OS-9 in shLUC and shEDEM1/shOS-9 HEK293 cells assessed by Western blot. Calnexin was used as the loading control. B-C. The shLUC and shEDEM1/shOS-9 HEK293 cell lines were infected with MHV-Srec for 7h. Subsequently, cell extracts were prepared to assess the synthesis of the N structural protein (B), IF was performed to monitor DMV biogenesis (C) and the titer of a virus stock (D) was determined as described in Figure 1D. This experiment was performed twice. E. The shLUC and shEDEM1/shOS-9 HEK293 cell lines were also infected with MHV-2aFLSrec and luciferase expression was measured at 5h, 7h and 10h.
Chapter 6

Concluding Remarks and Perspectives

Nearly 30% of the proteins synthesized in the mammalian cells are co-translationally translocated into the ER. About 90% of them contain one or more Asn-Xxx-Ser/Thr sequon, which is an acceptor site for pre-assembled glucose$_3$-mannose$_2$-N-acetylglucosamine$_2$- oligosaccharides. Thus, the processes of folding and disposal in the ER have to deal with a very large number of N-glycosylated polypeptides. N-linked oligosaccharides and their processing play an important role in the regulation of protein biogenesis, quality control and degradation in the ER. The unique properties of the N-linked glycan are essential pre-requisites for the accomplishment of these essential tasks. In the course of eukaryotic evolution, a fine-tuned universal system, based on differential processing and recognition of N-linked glycans has evolved that, in combination with the folding factors in the ER, ensures proper folding of a multitude of different proteins.

The majority of the findings, proposed mechanisms and pathways available in the literature to describe protein biogenesis and quality control, derive from studies performed in the budding yeast or in mammalian cultured cells with a very limited number of so-called model proteins (i.e. ERAD substrates). This possibly results in an over-simplification of the events that happens inside the ER so that, potentially, several crucial factors/processes still remain to be fully understood or even discovered. We are convinced that the use of a more broad spectrum of model substrates, with different physical-chemical properties (e.g. different glycosylation status, different aggregation propensities, different secretion efficiencies,...) will lead to a more comprehensive understanding of the processes that regulate protein biogenesis in Eukarya. This will be instrumental to understand the regulation of protein homeostasis in eukaryotic cells and to intervene in such processes that have crucial importance for clinical and industrial applications.
With this in mind, we have generated a palette of folding-defective polypeptides, more precisely BACE476, BACE476Δ, BACE457, BACE457QQ, BACE457Δ, BACE457ΔQQ, NHK_{BACE}, NHK_{BACE-QQQ}, NHK_{CD3δ}, NHK_{CD3-QQQ}, NHK, NHK_{QQQ}, CD3-δ, CD3-δQQQ, CD3-δΔ, CD3-δΔQQQ.

We expect that the detailed characterization of the factors regulating quality control of the model substrates generated in our lab will lead to the better characterization of the mechanisms operating in the mammalian ER.

Although very much is known about the processes that assist folding of newly synthesized glycopolypeptides in the ER lumen, there are still a number of unanswered questions that need to be addressed in order to fully understand the complexity of the folding and the disposal processes. Few relevant questions are concisely discussed below.

**The quality control**

**The important role of the folding sensor UGT1**

Once released from CNX/CRT, proteins that attain their native structure are de-glucosylated by glucosidase II and are then transported to their final destination through the secretory pathway. Non-native or pseudo-native polypeptides are not released from the ER because the folding sensor UGT1 specifically re-glucosylates the terminal α1,2-bonded mannose residue on the oligosaccharide branch A thus causing re-association with CNX/CRT for another round of folding attempts.

So far, it is not fully understood how UGT1 can discriminate between native, pseudo-native and misfolded structures. Additionally, it is unknown if UGT1 re-glucosylates the N-glycan(s) only if this lies in close proximity to unfolded regions.

**ERAD: the important role of oligosaccharide de-mannosylation**

**Mannose trimming as a signal for destruction**

In *S. cerevisiae*, substrate de-mannosylation operated by Mns1p (an ERManI ortholog) and Htm1p (an EDEM ortholog) generates a degradation signal that consists of an exposed α1,6-bonded mannose residue on the C-branch of oligosaccharides displayed on non-native polypeptides. This signal is decoded by the ERAD lectin Yos9p. Thus, in the budding yeast, N-glycan de-mannosylation acts as a
signal for disposal, by recruiting an ERAD lectin that delivers terminally misfolded proteins to the dislocation complex at the ER membrane.

In mammalian cells, substrate de-mannosylation also occurs, even though the identification of the factor(s) involved in mannose removal is still ongoing (see also next section). Since the budding yeast has no UGT1, the function of de-mannosylation in mammalian cells could have another purpose.

In mammalian cells, the reglucosylation capacity of UGT1, (the capacity to retain misfolded polypeptides in association with CNX/CRT), is still significant for partially de-mannosylated oligosaccharides. Only removal of the terminal mannose residue on branch A results in the irreversible extraction of terminally misfolded glycopolypeptides from the folding environment. Thus, trimming of the A-branch appears to be crucial for protein disposal from the mammalian ER.

In fact, pharmacologic inhibition α1,2-mannosidases not only delays glycoprotein degradation in wild type cells, but also in cell lines that transfer truncated oligosaccharides (e.g. Glc₃Man₅GlcNAc₂ in B3F7 cells or Man₅GlcNAc₂ in MadIA214 cells, Fig. 1). In these cell lines, the only cleavable α1,2-bonded mannose residues are those on the A-branch and their removal is required to elicit disposal. Therefore, it is conceivable that the primary purpose of de-mannosylation in mammalian cells is to allow irreversible extraction of terminally misfolded polypeptides from the CNX/CRT chaperone system rather than (or in addition to) the generation of a signal for disposal that must be decoded by ERAD lectins. In agreement with this is our finding that the mammalian ERAD lectins are only required for efficient disposal of ERAD-L₅ glycoproteins, while they are dispensable for disposal of ERAD-L₆ glycoproteins.

Figure 1. High-mannose precursor structures from (A) normal and (B) Dol-P-Man-deficient (B3F7) cell lines.
The glycosyl hydrolase 47 family
The GH47 family comprises three subfamilies of proteins, including ERManI, three EDEM proteins (EDEM1-3) and three Golgi α1,2-mannosidases (Golgi MAN1A-C). The presence of 7 different α1,2-mannosidases in the early secretory pathway of mammalian cells makes it difficult to identify the one(s) actually intervening in the extensive substrate de-mannosylation that elicit clearance of misfolded polypeptides from the ER lumen. It has been generally accepted that ERManI cleaves the terminal mannose on branch B. However, there is still an ongoing debate on which factors(s) catalyzes the further oligosaccharide processing to the Man$_{5-6}$ structure. It is highly plausible that the ER-resident EDEMs proteins intervene in these trimming events. Consistently, it has been reported that EDEM1 and EDEM3 are active α1,2-mannosidases. Golgi α1,2-mannosidases could also be involved in extensive substrate de-mannosylation. In this case (i) ERAD substrates could cycle between the ER and early Golgi or (ii) ER-retained Golgi mannosidases could act upon ER-retained glycopolypeptides.

ERAD lectins vs ERAD shuttles
As already mentioned above, in S. cerevisiae substrate de-mannosylation generates a degradation signal that recruits the ERAD lectin Yos9p. In mammalian cells the tagging of misfolded polypeptides for disposal is more complex and many more homolog proteins and/or splice variants of given gene products play crucial roles. First of all, there are at least two Yosp9 orthologs: OS-9 and XTP3-B. Additionally, each of them is present in at least 2 splice variants. Both proteins possess lectin-like domains (1 in OS-9 and 2 in XTP3-B) but their involvement in ERAD as lectins remains controversial. It is possible that OS-9 and XTP3-B act as pure lectins that recognize extensively de-mannosylated oligosaccharides, it is possible that a dual mode of binding also requires recognition of misfolded protein-determinants, or that OS-9 and XTP3-B regulate disposal of misfolded polypeptides independent on the presence of oligosaccharides. Certainly, at least for some ERAD substrates, they seem to be functionally interchangeable.
**Non-glycosylated ERAD substrates**

**Disposal pathways for non-glycosylated ERAD substrates**

Very little is known about cellular factors and mechanisms that are involved in folding and particularly in disposal of non-glycoproteins. It will be interesting to determine how non-glycosylated polypeptides are extracted from the folding environment and if the disposal pathways that we characterized are conserved. Furthermore, factors considered pure lectins, like CNX/CRT and OS-9/XTP3-B, could still be involved to some extent for maturation and disposal of non-glycoproteins.

**Dislocation**

**Identification of the dislocation channel**

The dispute about the identity of the mammalian dislocation channel is far from the end. The identification of the dislocation channel(s) remains controversial and a long list of suspects is available in the literature. These include Sec61, Derlin proteins and E3 ubiquitin ligase family members.

**ER-resident factors**

**Glycosylation of ER-resident chaperones**

Several ER-resident factors involved in folding and disposal are glycosylated. Few of them that are involved in ERAD are even highly glycosylated (e.g. SEL1L has 5 N-glycans, EDEM1 5 N-glycans, EDEM2 4 N-glycans, EDEM3 7 N-glycans). The reason for that is still unknown. One can hypothesize that the glycosylation is important during the folding of these chaperones, for their stability or even for their interaction with components of multimeric functional complexes such as those composing the dislocation machineries built around membrane-embedded E3 ubiquitin ligases. Furthermore, since N-glycosylation is an energy-consuming process, one would suggest that these highly glycosylated proteins have a long half-life inside the ER lumen. In contrast, it seems that many of the glycosylated ER chaperones are characterized by an unconventional short half-life ($t_{1/2}$ of about 1-2 hr) (see next section). Intriguingly, orthologs of proteins that act as lectins in the budding yeast such as the mammalian OS-9 and XTP3-B, contain a consensus sequence for N-glycosylation in their sugar-binding pocket, which is in most cases occupied by an oligosaccharide. This could interfere with the lectin function of these proteins and
could indicate alternative mechanisms of action for these ERAD regulators in multicellular organisms.

Rapid turnover of a subset of ER-resident factors
Within the ER lumen there is a competition between the folding and the degradation machineries for incoming cargo. It has been established that many factors involved in ERAD (e.g. ERMan1, EDEM1, OS-9 and SEL1L) have a short half-life (between 1 and 2 hr), whereas conventional ER folding chaperones are long-living proteins (with half-lives of about 24 hr). In order to improve folding efficiency, it is conceivable that at steady state protein folding must have an advantage over protein disposal. For this to occur, ERAD factors must be maintained at low concentrations in the ER. One way to achieve this is to rapidly remove them from the folding environment. This could be obtained by (i) segregation of ERAD regulators from the long-living folding chaperones (for example by sub-compartmentalization) and/or by (ii) post-translational clearance of ERAD regulators from the ER lumen. Evidence suggests that the latter can be true, however the molecular mechanism(s) that regulates this rapid turnover of a particular subset of ERAD factors is still elusive. Interestingly, however, a class of pathogen that might hijack this pathway for production of progeny, the coronaviruses, has been identified.
Appendix

Curriculum Vitae

Personal Information

Name: BERNASCONI
First name: Riccardo
Date of birth: 25 March 1983
Nationality: Swiss
Civil status: single
Private address: Via Fontana – 6874 Castel San Pietro – Switzerland
E-mail: riccardo.bernasconi@irb.unisi.ch

Education

2007 – 2010 Ph.D. in Biology, ETH, Zurich (CH)
Supervisor: Prof. Dr. Markus Aebi
Work performed at the Institute for Research in Biomedicine, Bellinzona (CH)
Supervisor Dr. Maurizio Molinari
Academic title: Doctor of Sciences

2002 – 2006 M.Sc. in Biology, ETH, Zurich (CH)
Academic title: Dipl. Natw. ETH

1998 – 2002 High School, Liceo Cantonale di Mendrisio (CH)
Degree: Federal Matura Type C
List of Publications


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