PROTEIN FOLDING IN THE ER:
THE FATE OF β-SECRETASE N-GLYCOSYLATION MUTANTS

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Abstract

The endoplasmic reticulum (ER) houses a quality control machinery for newly synthesized proteins destined to the cellular plasma membrane, to endo- and exocytic compartments and to the extracellular space. About one third of the proteins synthesized in mammalian cells travel through the ER, where molecular chaperones, foldases and sugar-modifying enzymes assist them for the attainment of the native structure. The quality control insures, with rare exceptions, that only native proteins are released from the ER. Folding intermediates, orphan subunits of oligomeric complexes and misfolded polypeptides are retained in the compartment. Cell and organism homeostasis depends on the regulated balance between transport of native proteins to the site of destination and disposal of terminally misfolded structures.

Aim of my work was to better characterize the molecular basis of the events that regulate protein maturation and quality control in the mammalian ER. To this end, we generated soluble forms of Beta-site APP cleaving enzyme 1 (BACEs) with 4, 3, 2, 1 and 0 N-glycosylation sites and we expressed them in mammalian cells. Analysis of the fate of the five BACEs variants revealed a direct correlation between the number of N-glycans displayed on the nascent polypeptide chains and folding efficiency, folding rate and secretion. The presence of a single N-glycan was sufficient to recruit calnexin. Addition of 1 to 4 N-glycans progressively enhanced the dissociation rate from BiP and reduced the propensity of newly synthesized BACEs to enter aberrant soluble and insoluble aggregates. Finally, inhibition of the proteasome increased the yield of active BACEs secreted from cells. This suggests that a fraction of BACEs undergoes premature degradation that decreases the amount of the enzyme that can attain the native structure.

In a second study, we analyzed chaperone-regulated retention of non-native glycopolypeptides in the mammalian ER. Cycles of de-/re-glucosylation regulated by the counteracting activities of glucosidase II and UDP-glucose:glycoprotein glucosyltransferase (UGT1) are protracted as long as glycoproteins are un/misfolded.
Substrate cycling in the calnexin chaperone system was thought to be required for efficient ER retention of non-native polypeptides. Here we compared the fate of a glycoprotein, tsO45 G, expressed in mammalian cells with and without the re-glucosylation enzyme UGT1 (ugt1<sup>−/−</sup> cells). Folding of tsO45 G is characterized by a reversible, temperature-dependent defect. UGT1 is a central player of glycoprotein quality control in the ER. It re-glucosylates non-native glycopolypeptides thus inhibiting their release from the calnexin cycle. We show that the persistently misfolded tsO45 G protein expressed in wt and in ugt1<sup>−/−</sup> cells was eventually released from calnexin and entered disulfide-bonded aggregates associated with BiP/GRP78. Deletion of UGT1 did not cause release of non-native G protein from the ER but resulted in faster loss of folding competence upon formation of BiP-associated disulfide-bonded aggregates. Even in cells lacking UGT1, release of misfolded conformers from calnexin occurred after an unexpected long lag period. Thus, the first release from calnexin that initiates the cycling of misfolded glycopolypeptides in the calnexin chaperone system requires persistent glycoprotein misfolding. Our data also showed that misfolded conformers eventually released from calnexin in cells with and without UGT1 were not exported through the secretory pathway. Rather, they were trapped by the BiP chaperone system. We propose that retention-based ER quality control consists in two phases involving two distinct chaperone complexes, the calnexin and the BiP system.
Il reticolo endoplasmico (ER) è il luogo del controllo di qualità per le proteine sintetizzate dai ribosomi e destinate a raggiungere la membrana plasmatica, compartimenti endo- e esocitici e lo spazio extracellulare. Circa un terzo delle proteine sintetizzate nelle cellule di mammifero transita dall’ER, dove chaperones molecolari, foldasi e enzimi che modificano gli zuccheri sono dedicati al folding delle proteine e le assistono nell’ottenimento della forma nativa. Il controllo di qualità assicura con rare eccezioni che solo proteine native vengano rilasciate dall’ER. Strutture intermedie, subunità singole di complessi oligomerici e polipeptidi mal ripiegati sono ritenuti nel compartimento. L’omeostasi di cellula e organismo dipende dall’equilibrio regolato tra trasporto di proteine native al loro sito di destinazione e lo smaltimento di strutture considerate irreversibilmente mal ripiegate.

L’obiettivo del mio lavoro è stato quello di caratterizzare maggiormente le basi molecolari degli eventi che regolano maturazione e controllo di qualità delle proteine nell’ER di cellule di mammifero.
Per questo scopo abbiamo generato forme solubili di BACE (Beta-site APP cleaving enzyme 1) con 4, 3, 2, 1 e 0 siti di N-glicosilazione. Analisi riguardanti il destino delle cinque forme solubili di BACE (BACEs) espresse in cellule di mammifero hanno rivelato una correlazione diretta tra il numero di glicani presenti sulle catene polipeptidiche nascenti e l’efficienza di folding, la velocità di folding e la secrezione. La presenza di un singolo glicano è sufficiente per il reclutamento di calnexina. L’aggiunta di 1-4 glicani aumenta progressivamente la dissociazione dal chaperone molecolare BiP e diminuisce la tendenza di BACEs a formare aggregati aberranti. Inoltre l’inibizione del proteasoma promuove la secrezione di BACEs attiva. Questo suggerisce che una frazione di BACEs è sottoposta a una degradazione prematura che diminuisce la quantità di enzima che raggiunge la struttura nativa.

In un secondo studio abbiamo analizzato la retenzione regolata da chaperones di glicopolipeptidi non nativi nell’ER di mammifero. Cicli di de-/ri-glicosilazione regolati dalle attività opposte di glucosidasi II e UGT1 (UDP-glucose:glycoprotein
glucosyltransferase) vengono protratti finché i substrati mantengono uno stato di mal ripiegamento. Si pensava che i cicli nel sistema di calnexina fossero richiesti per una retenzione efficiente di polipeptidi non nativi nell'ER.

Qui abbiamo confrontato il comportamento di una glicoproteina, tsO45 G, espressa in cellule con e senza l'enzima di ri-glucosilazione UGT1 (cellule ugt1−/−). Il folding di tsO45 G presenta un difetto reversibile dipendente dalla temperatura. UGT1 è un importante membro del controllo di qualità delle glicoproteine nell'ER. Il suo compito è la ri-glucosilazione di polipeptidi non nativi che inibisce il rilascio dal ciclo di calnexina. I dati mostrano che la proteina tsO45 G persistentemente mal ripiegata nelle cellule wt e in quelle ugt1−/− viene infine rilasciata da calnexina e forma aggregati legati da ponti disolfuro e associati a BiP/GRP78. La delezione di UGT1 non causa il rilascio di proteina G non nativa dall'ER, ma risulta in un’accelerata perdita della capacità di ripiegamento correlata alla formazione degli aggregati associati a BiP. Anche in cellule senza UGT1, il rilascio da calnexina di strutture mal ripiegate avviene dopo un inaspettato lungo periodo di tempo. Quindi il primo rilascio da parte della lectina, quello che inizia i cicli di glicoproteine mal ripiegate nel sistema, richiede uno stato di mal ripiegamento persistente. I nostri dati mostrano anche che polipeptidi mal ripiegati rilasciati da calnexina in cellule con e senza UGT1 non vengono esportati attraverso la via secretoria. Al contrario vengono trattenuti dal sistema di chaperone di BiP. Proponiamo quindi che il controllo di qualità basato sulla retenzione consiste in due fasi che coinvolgono due complessi distinti di chaperones, il sistema di calnexina e quello di BiP.
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<th>Description</th>
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<tbody>
<tr>
<td>BACE</td>
<td>Beta-site APP Cleaving Enzyme 1, β-secretase 1, Memapsin-2, Aspartyl protease 2</td>
</tr>
<tr>
<td>BACEs</td>
<td>Soluble form of BACE</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane conductance Regulator</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary cells</td>
</tr>
<tr>
<td>CNX</td>
<td>Calnexin</td>
</tr>
<tr>
<td>COP I</td>
<td>Cytosolic Coat Protein I</td>
</tr>
<tr>
<td>COP II</td>
<td>Cytosolic Coat Protein II</td>
</tr>
<tr>
<td>CRT</td>
<td>Calreticulin</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>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-Golgi Intermediate Compartment</td>
</tr>
<tr>
<td>ERMαnI</td>
<td>ER α1,2-mannosidase I</td>
</tr>
<tr>
<td>GI</td>
<td>α-Glucosidase I</td>
</tr>
<tr>
<td>GII</td>
<td>α-Glucosidase II</td>
</tr>
<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>MHC</td>
<td>Major Histocompatibility Complex</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>TAP</td>
<td>Transporter Associated with antigen Processing</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucose:glycoprotein Glucosyltransferase</td>
</tr>
<tr>
<td>VIP36</td>
<td>Vesicular Integral Protein of 36 kDa</td>
</tr>
<tr>
<td>VIPL</td>
<td>VIP36-Like protein</td>
</tr>
</tbody>
</table>
About one third of the proteins synthesized in mammalian cells are co-translationally translocated in the endoplasmic reticulum (ER). Newly synthesized polypeptides emerging in the ER lumen are exposed to machineries that assist conformational maturation and to machineries designed to interrupt futile folding attempts and to destroy terminally misfolded proteins. To introduce the results sections, I summarize here recent progresses made in the characterization of protein folding, quality control and degradation in the mammalian ER.
1.1 Protein translocation and maturation in the mammalian ER

1.1.1 Arriving at the ER membrane

The ER lumen is site of maturation for all secretory proteins, for proteins destined for the plasma membrane and for the endocytic and exocytic compartments. Most of the proteins that start their journey throughout the cell in the ER are characterized by the presence of a short N-terminal hydrophobic address tag, the signal sequence (Blobel and Dobberstein, 1975). When this short sequence of about 20 residues emerges from the ribosome, it associates with an RNA/protein complex, the signal recognition particle (Walter and Blobel, 1982). Polypeptide elongation is substantially slowed until the ribosome engages a proteinaceous channel in the ER membrane, the Sec61 complex (Deshaies and Schekman, 1987). Only then synthesis is resumed and nascent chains are vectorially discharged across the ER membrane (Fig. 1.1) (Redman and Sabatini, 1966; Redman et al., 1966; Sabatini and Blobel, 1970). The highly hydrophobic signal sequence is integrated in the membrane and is normally removed from the nascent chains by a pentameric complex in the ER membrane, the signal peptidase (Hegde and Bernstein, 2006). Signal peptide cleavage often occurs co-translationally (Daniels et al., 2003). However, examples are known of proteins whose folding is facilitated by persistent anchoring of the N-terminus to the membrane resulting from a delayed cleavage of the signal peptide (e.g. the HIV glycoprotein gp160 (Li et al., 2000)).

1.1.2 Polypeptide translocation into the ER

The translocation channel consists of a heterotrimeric complex (Sec61αβγ) with the α-subunit forming the actual channel (Van den Berg et al., 2004). Several accessory proteins are also associated with the translocon, i.e. the translocating chain-associating membrane protein (TRAM), the translocon-associated complex (TRAP), the signal peptidase complex, the oligosaccharyl transferase and several ER-resident molecular chaperones such as BiP, GRP94, calnexin and calreticulin that associate with incoming nascent chains at the luminal side of the translocation pore (Fig. 1.1). The driving force for co-translational protein translocation is given by chain elongation and the passage through the Sec61 channel does not require nucleotide hydrolysis (Connolly and Gilmore, 1986). Polypeptide translocation may also occur
post-translationally. In this case, the translocation channel also comprises a tetrameric Sec62/Sec63 complex (Meyer et al., 2000; Tyedmers et al., 2000). Post-translational translocation requires ATP hydrolysis because it occurs by a ratcheting mechanism in which BiP/GRP78, an ATPase of the Hsp70 family inside the ER lumen, inhibits backward movement of the translocating chain (Matlack et al., 1999). The size of the aqueous channel serving for protein translocation is matter of some debate, with a proposed diameter ranging from 8 to 60Å (Rapoport, 2007). The ribosomal tunnel and the translocation pore can certainly accommodate α-helical structures (Mingarro et al., 2000; Whitley et al., 1996) (Fig. 1.1), but it remains unclear whether folding of larger structures is possible. An elegant series of experiments recently showed that folding of a fragment of the Semliki forest virus capsid protein is only possible when the polypeptide chain has moved beyond the translocon pore (Kowarik et al., 2002). These results are revealing because molecular modeling shows that a cylindrical pore with a diameter of 40-60Å should easily accommodate capsid folding. Thus, a diameter of 40-60Å for the active channel seems too large. On the other hand, it has been shown that several membrane segments do assemble within the translocation pore before integration into the ER membrane (Borel and Simon, 1996; Kida et al., 2007; McCormick et al., 2003; Meacock et al., 2002; Skach, 2007; Skach and Lingappa, 1993). These data highlight the remarkable flexibility of the translocation site in the ER membrane possibly resulting from the capacity of Sec61 complexes to co-ordinate their lateral openings in a large channel.

1.1.3 Emerging in the ER lumen
After a 100Å long ribosomal tunnel (Morgan et al., 2000), a short gap of about 20Å between the ribosome and the translocon and the 45Å long translocation pore, the nascent polypeptide chain emerges in the ER lumen (Fig. 1.1). Unstructured nascent chains that emerge from the translocation pore expose hydrophobic patches, unpaired cysteines and other aggregation-prone determinants that during the folding process will be buried inside the native conformers. The intervention of ER-resident molecular chaperones such as BiP/GRP78 (with several co-factors) (Blond-Elguindi et al., 1993; Haas and Wabl, 1983; Hendershot, 2004) or GRP94 (Argon and Simen, 1999; Melnick et al., 1994; Nieland et al., 1996) (Table 1.1) is crucial to maintain nascent chains in a folding-competent state. Similarly, the engagement of exposed
unpaired cysteines in mixed disulfides with members of the protein disulfide isomerase (PDI) superfamily such as PDI, ERp72 and ERp57 (Molinari and Helenius, 1999; Reddy and Corley, 1998; Sitia et al., 1990) (Table 1.2) hampers formation of non-native inter-molecular disulfides and facilitates the oxidative phases of polypeptide maturation.

Altogether, chaperone association inhibits aggregation of unfolded polypeptides and facilitates the activity of folding enzymes that catalyze rate-limiting reactions of the polypeptide folding (e.g. formation of inter- and intra-molecular disulfide bonds (Ellgaard and Ruddock, 2005) and cis/trans isomerization of peptidyl-prolyl bonds (Kiefhaber et al., 1990)). Chaperone association also retains non-native polypeptides in the ER lumen because chaperones display ER-retention or ER-retrieval sequences such as C-terminal KDEL-like signals for luminal chaperones and cytosolic KXXX signals for ER membrane proteins of type I (Ellgaard et al., 1999). ER-resident chaperones are often organized in multiprotein complexes (Meunier et al., 2002; Zhang and Herscovitz, 2003) that possibly form cages or local environments in which newly synthesized polypeptides explore conformations that eventually lead to the native state, which is the one with the lowest Gibbs free energy (Anfinsen, 1973).

<table>
<thead>
<tr>
<th>Family</th>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp70</td>
<td>GRP78/BiP</td>
<td>Conventional chaperone</td>
</tr>
<tr>
<td></td>
<td>ERdj1/Mtj1</td>
<td>Co-factors for BiP</td>
</tr>
<tr>
<td></td>
<td>ERdj2/hSec63</td>
<td>Substrate delivery</td>
</tr>
<tr>
<td>Hsp40</td>
<td>ERdj3/HEDJ/ERj3/ABBP-2</td>
<td>Enhancement of ATP hydrolysis</td>
</tr>
<tr>
<td></td>
<td>ERdj4/Mdj1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERdj5/JPDI</td>
<td></td>
</tr>
<tr>
<td>GrpE-like</td>
<td>BAP/Sil1</td>
<td>Co-factors for BiP</td>
</tr>
<tr>
<td></td>
<td>GRP170</td>
<td>Nucleotide exchange factors</td>
</tr>
<tr>
<td>Hsp90</td>
<td>GRP94/endoplasmin/ERp99</td>
<td>Conventional chaperone</td>
</tr>
</tbody>
</table>

Formation of inter- and intra-molecular disulfide bonds that covalently link cysteines is a common modification that starts immediately when nascent polypeptide chains enters into the ER lumen (Chen et al., 1995; Molinari and Helenius, 1999). Several members of the PDI superfamily, most of which play unknown roles in protein biogenesis and quality control, catalyze protein oxidation and reshuffling of non-
native disulfides. Family members contain Cys-X-X-Cys (CXXC) active site motifs in thioredoxin domains (Table 1.2). For example, PDI has two catalytic domains (a and a’) divided by two inactive thioredoxin-like, substrate-binding domains (b and b’) (Ellgaard and Ruddock, 2005). In family members acting as oxidases, thus promoting disulfides formation, the cysteines in the CXXC motif are disulfide-bonded. They act as an electron acceptor and leave the substrate-oxidation-reaction in a reduced state. Reductases disassemble disulfide bonds, for example, when protein unfolding is required for translocation across the ER membrane into the cytosol of toxin subunits or ERAD substrates (Bernardi et al., 2008; Molinari et al., 2002; Tsai et al., 2002; Wahlman et al., 2007). In this case, the CXXC motif is initially reduced and leaves the reaction in an oxidized state. Finally, isomerases, which play a crucial role in adjusting the unique set of native intramolecular disulfides, enter and leave the reaction with a reduced active site.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Active site motif</th>
<th>Postulated activity</th>
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<tbody>
<tr>
<td>ERdj5</td>
<td>CXHC, CXPC, CXPC, CXPC</td>
<td>reductase</td>
</tr>
<tr>
<td>PDIR</td>
<td>CXHC, CXHC, CXXC</td>
<td>inefficient catalyst, lacks essential Glu</td>
</tr>
<tr>
<td>ERp72</td>
<td>CXHC, CXHC, CXHC</td>
<td>oxidase/isomerase</td>
</tr>
<tr>
<td>ERp46</td>
<td>CXHC, CXHC, CXHC</td>
<td>oxidase</td>
</tr>
<tr>
<td>PDI</td>
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<td>oxidase/isomerase</td>
</tr>
<tr>
<td>PDIp</td>
<td>CXHC, CXHC</td>
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<td>PDILT</td>
<td>SXXS, SXXC</td>
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</tr>
<tr>
<td>ERp57</td>
<td>CXHC, CXHC</td>
<td>reductase/isomerase</td>
</tr>
<tr>
<td>P5</td>
<td>CXHC, CXHC</td>
<td>oxidase</td>
</tr>
<tr>
<td>ERp44</td>
<td>CXXS</td>
<td>Retention of Ero1</td>
</tr>
<tr>
<td>ERp18</td>
<td>CXHC</td>
<td>inefficient catalyst, lacks essential Glu</td>
</tr>
<tr>
<td>TMX</td>
<td>CXXC</td>
<td>?</td>
</tr>
<tr>
<td>TMX2</td>
<td>SXXC</td>
<td>?</td>
</tr>
<tr>
<td>TMX3</td>
<td>CXHC</td>
<td>oxidase</td>
</tr>
<tr>
<td>TMX4</td>
<td>CXXC</td>
<td>inefficient catalyst, lacks essential Glu</td>
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Figure 1.1 - Co-translational protein translocation into the ER lumen
Nascent chains are co-translationally injected into the ER lumen through the Sec61 translocon. The large and small ribosomal subunits at the cytosolic face of the ER membrane are labelled with 60S and 40S, respectively. Asparagine residues in appropriate sequons are covalently modified with the addition of pre-assembled oligosaccharides. The two N-acetylglucosamine residues are in yellow, the four $\alpha(1-2)$-bonded mannose residues in grey, and the three terminal glucose residues in red. The shape and colour code of the saccharide units in the protein-bound glycan are the same in Figures 1.1-1.5. Nascent chains associate with a variety of ER-resident molecular chaperones and folding enzymes. OST is oligosaccharyl transferase; GI and GII are glucosidase I and II, respectively; CNX is calnexin, PDI is protein disulfide isomerase.

Much less is known about one other rate-limiting reaction occurring during protein folding in the ER lumen, i.e. the isomerization of prolyl-peptidyl bonds. In particular, despite several ER-resident members of the peptidyl-prolyl cis/trans isomerases (PPI) superfamily have been described (Table 1.3), their involvement in polypeptide maturation in vivo is poorly understood.
### Table 1.3 - Members of the human PPI family

<table>
<thead>
<tr>
<th>Protein</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyclophilinB / CypB</td>
<td>Part of large multi-chaperone complexes in the ER</td>
</tr>
<tr>
<td>FKBP2 / FKBP13</td>
<td>ER-stress induced</td>
</tr>
<tr>
<td>FKBP7 / FKBP23</td>
<td>Modulation of BiPs ATPase</td>
</tr>
<tr>
<td>FKBP10 / FKBP65</td>
<td>Association with BiP-bound substrates</td>
</tr>
</tbody>
</table>

#### 1.1.4 Addition of pre-assembled oligosaccharides onto nascent chains

Most of the polypeptides emerging in the ER lumen receive N-glycans (Helenius and Aebi, 2004), but important exceptions do exist. For example, albumin, the most abundant secretory protein produced in the liver, is not glycosylated.

The oligosaccharyl transferase (OST), a dimer of 9 different subunits (Chavan et al., 2006) (Table 1.4) is strategically positioned at the exit of the translocation pore (Fig. 1.1). The OST scans the sequence of nascent polypeptide chains emerging from the translocation pore in search for asparagine-any amino acid but proline-serine/threonine (Asn-Xxx-Ser/Thr) consensus sequences (Li et al., 2008). The hydroxyl group of the serine or threonine residue in the consensus sequence is brought in close proximity to the amide group of the asparagine, which is activated (Imperiali and Hendrickson, 1995) to accept the covalent addition of a pre-assembled oligosaccharide whose composition (3 glucose, 9 mannose and 2 N-acetylglucosamine residues, Glc$_3$Man$_9$GlcNAc$_2$, Fig. 1.2) is conserved in plants, fungi and mammals (Parodi and Leloir, 1979). The OST transfers this pre-assembled oligosaccharide from a lipid donor in the ER membrane (dolichol-pyrophosphate) onto the nascent chain (Parodi et al., 1972) as soon as the acceptor asparagine has emerged for 40-45Å, corresponding to about 12-14 residues, from the translocation pore (Nilsson and von Heijne, 1993) (Fig. 1.1). The addition of oligosaccharides, bulky hydrophilic appendices that extend for about 30Å from the polypeptide backbone, dramatically changes the biophysical properties of unstructured nascent chains substantially increasing their solubility. As thoroughly discussed in the next sections, N-glycosylation will also determine the fate of the associated polypeptide chain in many different ways. For example, if a glycosylation site is located in the first 50 residues or so, then addition of the oligosaccharide sterically hinders binding of chaperones such as BiP to the polypeptide backbone. Instead, lectin-like chaperones will associate and assist glycoprotein maturation (Molinari and Helenius, 2000). If the
first N-glycan is more downstream in the sequence, then the polypeptide will associate with BiP or other peptide backbone-binding chaperones. In this case, the folding polypeptides may only subsequently be handed off to the lectin chaperone system (Hammond and Helenius, 1994; Molinari and Helenius, 2000; Pipe et al., 1998; Tomita et al., 1999; Wang et al., 2005). Finally, if the polypeptide is folding-defective, the slow removal of individual mannose residues from the N-glycan will tag it for extraction from the ER folding environment and degradation (Helenius, 1994) (Section 1.2.4).

**Figure 1.2 - Structure of core oligosaccharides**
The panel on the left shows the three-antennary oligosaccharide covalently attached to the side chain of an asparagine in the N-X-S/T consensus sequence for N-linked glycosylation. Branches A, B and C are those that display the terminal mannose residues A, B and C, respectively. The panel on the right shows the aberrant oligosaccharide used in cell lines with defective synthesis of mannosyl-phosphoryldolichol (e.g. B3F7).

### 1.1.5 Recruiting calnexin and calreticulin
Calnexin is a type I protein and calreticulin is its luminal paralog (Table 1.4). Both proteins are lectin chaperones, but calreticulin is also involved in calcium storage in the ER lumen because it contains a highly acidic C-terminal domain that binds 18 calcium ions with low affinity (Kd of about 2 mM (Baksh and Michalak, 1991)). Calnexin and calreticulin are retained in the ER lumen by a cytosolic KKXX and a luminal KDEL motif, respectively. They both contain a single carbohydrate-binding domain adopting a leguminous lectin-like β-sandwich fold (Schrag et al., 2001) and a long hairpin of 140Å for calnexin and of approximately 110Å for calreticulin, the proline-rich P-domain. The tips of the calnexin and calreticulin P-domains interact
with the b’ domain of the oxidoreductase ERp57 (Frickel et al., 2002; Leach et al., 2002; Pollock et al., 2004; Russell et al., 2004). ERp57 is therefore in the best position to catalyze maturation of newly synthesized glycoproteins for which, disulfide bond formation is a rate-limiting reaction (Molinari and Helenius, 1999; Oliver et al., 1997; Zapun et al., 1998).

Table 1.4 - Glycosylating, sugar processing and sugar binding proteins in the ER

<table>
<thead>
<tr>
<th>Protein</th>
<th>Family</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligosaccharyl transferase</td>
<td>Addtion of Glc$_3$Man$_9$GlcNAc$_2$-</td>
<td></td>
</tr>
<tr>
<td>Glucosidase I</td>
<td>GH Family 63</td>
<td>Removal of glucose-1</td>
</tr>
<tr>
<td>Glucosidase II</td>
<td>GH Family 31</td>
<td>Removal of glucose-2 and -3</td>
</tr>
<tr>
<td>Calnexin</td>
<td>lectin</td>
<td>Binding to Glc$_1$Man$_9$GlcNAc$_2$</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>lectin</td>
<td>Binding to Glc$_1$Man$_9$GlcNAc$_2$</td>
</tr>
<tr>
<td>UGT1</td>
<td>GT Family 24</td>
<td>Re-addition of glucose-3 on Mannose A</td>
</tr>
<tr>
<td>ERGIC-53, VIPL, VIP36</td>
<td>L-type lectin</td>
<td>High-mannose lectins</td>
</tr>
<tr>
<td>ER α1,2-mannosidase I</td>
<td>GH Family 47</td>
<td>Preferential removal of Mannose B</td>
</tr>
<tr>
<td>EDEM1</td>
<td>GH Family 47</td>
<td>Binding and processing of Man$_x$GlcNAc$_2$</td>
</tr>
<tr>
<td>EDEM2</td>
<td>GH Family 47</td>
<td>Binding and processing of Man$_x$GlcNAc$_2$</td>
</tr>
<tr>
<td>EDEM3</td>
<td>GH Family 47</td>
<td>Binding and processing of Man$_x$GlcNAc$_2$</td>
</tr>
<tr>
<td>OS-9.1, OS-9.2, erlectin</td>
<td>lectins?</td>
<td>ER-retention/ERAD</td>
</tr>
</tbody>
</table>

Few seconds after addition onto nascent chains, the N-linked oligosaccharide becomes accessible to the glucosidase I, a type II membrane glycoprotein of the glycosyl hydrolase (GH) family 63 (Table 1.4). The glucosidase I removes the outermost glucose-1 when the N-glycan is located 72 residues from the ribosome P-site, which is also the distance required for addition of the N-glycan (Fig. 1.1). This reaction is therefore immediate and shows that the OST and the glucosidase I active sites are in close proximity to each other (Deprez et al., 2005). The second glucose is also rapidly removed by another α-glucosidase, the glucosidase II (Fig. 1.1). This is a soluble heterodimeric glycanase of the GH family 31 (Table 1.4) composed of a catalytic α-subunit and a regulatory β-subunit (Trombetta et al., 1996). The glucosidase II also removes glucose-3. However, being glucose-3 differently oriented in space than glucose-2 (Petrescu et al., 1997), this last cleavage needs transient separation and repositioning of the glucosidase II active site. This time window is
possibly exploited by calnexin and calreticulin to associate with the mono-
glucosylated trimming intermediate of the N-linked glycan processing (Deprez et al.,
2005).
The globular sugar-binding domain of calnexin and calreticulin accommodates the
entire mono-glucosylated branch A of a protein-bound oligosaccharide (Fig. 1.2)
within a concave β-sheet in which the glucose ring sits on the side chain of
methionine-189 and is hydrogen bonded to tyrosine-165, lysine-167, tyrosine-186,
and glutamic acid-217 in calnexin (Schrag et al., 2001).
Calnexin and calreticulin association slows protein folding and renders it more
efficient (Hebert et al., 1996), for example by inhibiting formation of non-native
disulfide bonds. In some cases in fact, N-glycans are appropriately positioned so that
calnexin/calreticulin association will protect cysteines from premature oxidation. The
influenza virus hemagglutinin (HA) with its peculiar head-to-tail structure represents a
paradigmatic example of such a situation. The N-terminal cysteine-14 of the
hemagglutinin must covalently pair with the C-terminal cysteine-466 (Wilson et al.,
1981). With an average synthesis rate of 4 residues/sec, oxidation of cysteine-14 has
therefore to be delayed by about 100 sec for efficient maturation of the newly
synthesized protein. N-glycans at position 8, 22 and 38 insure co-translational
calnexin binding (Chen et al., 1995; Molinari and Helenius, 2000; Pieren et al., 2005)
that prevents immediate oxidation of cysteine-14 with one of the 8 cysteines
preceding the appropriate partner in the primary sequence.

1.1.6 Cycling in the calnexin chaperone system
Removal of the two outermost glucose residues occurs co-translationally and
generates the mono-glucosylated intermediate of the N-glycan trimming that
associates with calnexin/calreticulin and ERp57 (Fig. 1.3, step 1) (Degen et al., 1992;
Degen and Williams, 1991; Galvin et al., 1992; Hammond et al., 1994; Molinari and
Helenius, 1999; Oliver et al., 1997; Ou et al., 1993; Wada et al., 1991; Zapun et al.,
1998). Association can persist for several minutes and certainly continues after chain
termination. The finding that the innermost glucose-3 of a misfolded glycoprotein
retained in the ER lumen was removed and re-added (Suh et al., 1989) and the
identification of a large luminal protein member of the glycosyl transferase family 24,
the UDP-glucose:glycoprotein glucosyltransferase (UGT1, Table 1.4) that re-
glucosylates non-native glycoproteins (Parodi et al., 1984) led Ari Helenius and
coworkers to propose an elegant cycle of de-/re-glucosylation determining substrate dissociation/re-association with calnexin and calreticulin: the calnexin cycle (Hammond et al., 1994).

In this model, which has been better defined in the last 15 years but is still valid today, proteins eventually released from calnexin/calreticulin are de-glucosylated by glucosidase II. This prevents their immediate re-association with the lectin-like chaperones (Fig. 1.3, step 2 or step 3a). If the polypeptide has attained the native structure, it is released from the ER and is transported at the final intra- or extracellular destination through the secretory pathway (step 2). If the polypeptide is terminally misfolded and extensively de-mannosylated (Section 1.2.4), it is handed off to BiP/PDI to be prepared for translocation into the cytosol and degradation (steps 5-7) (Cabral et al., 2002; Molinari et al., 2002). However, if the polypeptide released from calnexin has pseudo-native conformation, UGT1 will selectively re-glucosylate the terminal mannose on the oligosaccharide branch A to promote re-association with calnexin and/or calreticulin and prolong retention in the folding environment (Fig. 1.3, step 4) (Caramelo et al., 2004).

Several mechanistic features of glycoprotein folding are conserved between mammals and yeast. The core oligosaccharide added onto nascent chains has the same composition and in both organisms is added onto Asn-Xxx-Ser/Thr glycosylation sequons. Several proteins involved in the mammalian calnexin/calreticulin chaperone system have functional orthologs in S. cerevisiae. GlsI, GlsII and Cne1p are the glucosidase I, glucosidase II and calnexin orthologs, respectively. Moreover, the yeast oxidoreductase Mpd1p interacts with Cne1p to form a functional complex that enhances the catalytic activity of the oxidoreductase (Kimura et al., 2005), similarly to the functional calnexin:ERp57 complex (Molinari and Helenius, 1999; Oliver et al., 1997; Zapun et al., 1998). However, other components are missing in S. cerevisiae, for example calreticulin and, most importantly, UGT1. Thus, polypeptide cycling on/off calnexin does only occur in the mammals and in more complex yeast strains such as S. pombe that possesses a UGT ortholog (Fanchiotti et al., 1998).
Figure 1.3 - The fate of folding-competent and folding-defective glycoproteins

The model shows the fate of newly synthesized, folding competent glycopolypeptides that are eventually transported at their site of activity through the secretory pathway (green arrows). Folding-defective polypeptides are trapped in a first phase of retention-based ER quality control (the calnexin cycle) and, eventually in a second phase of retention-based ER quality control (the BiP/PDI system). Terminally misfolded polypeptides are subjected to extensive de-mannosylation in the mammalian ER. ERManI is ER α1,2-mannosidase I; EDEMs stays for EDEM1, EDEM2 and EDEM3.
1.1.7 Leaving the ER
Properly folded and completely assembled proteins are eventually released from the ER in transport vesicles coated with Cytosolic Coat Protein II (COPII) that bud at ER exit sites (Gurkan et al., 2006). In yeast, COPII-coated cargo vesicles are directly delivered to the Golgi compartment (Bonifacino and Glick, 2004). In mammalian cells, it has been proposed that they undergo homotypic fusion to generate a stationary ER-Golgi intermediate compartment (ERGIC) from which cargo proteins reach the cis-Golgi in COPI-coated vesicles (Appenzeller-Herzog and Hauri, 2006; Aridor et al., 1995; Pepperkok et al., 1993). Transmembrane proteins can directly interact with the cytosolic COPII coat, while soluble cargo proteins may require specific receptors for recruitment in COPII-coated vesicles (Barlowe, 2003). It has recently been shown that ER export of some glycosylated proteins is facilitated by several leguminous-type lectins located in the ER (VIPL), cycling between ER and ERGIC (ERGIC-53) or between ERGIC and cis-Golgi (VIP36) (Kamiya et al., 2008) (Table 1.4). ERGIC-53 certainly is the best-characterized one. It is involved in export of several soluble cargo proteins such as pro-cathepsin Z and pro-cathepsin C (Appenzeller et al., 1999; Vollenweider et al., 1998), coagulation factors V and VIII (Zhang et al., 2003) and α1-antitrypsin (Nyfeler et al., 2008).

1.1.8 Deletion of individual members of the calnexin chaperone system
Several general (BiP, PDI, GRP94, CypB,…), substrate-specific (Hsp47 for collagen, tapasin for MHC class I loading complex, egasyn for β-glucuronidase, RAP for LDL receptors, …) and tissue-specific (PDILT, PDIp, calmegin, …) chaperones, enzymes and escorting factors contribute to polypeptide maturation in the mammalian ER (Hebert and Molinari, 2007). However, the mechanisms regulating maturation and quality control of N-glycosylated polypeptides in the calnexin chaperone system are the best studied and understood. For example, individual knockouts for each member of the glycoprotein-dedicated folding machinery have been generated. Cell lines lacking glucosidase I (Ray et al., 1991), glucosidase II (Reitman et al., 1982), calnexin (Denzel et al., 2002), calreticulin (Mesaeli et al., 1999), ERp57 (Garbi et al., 2006) and UGT1 (Molinari et al., 2005) are available. Most of them have been derived from mouse embryos and their characterization led to better understand the role of each factor in the process of glycoprotein maturation.
Glucosidases-deficiency

Glucosidase I and glucosidase II sequentially remove the outermost and the middle glucose immediately after transfer of the pre-assembled oligosaccharides from the dolichol lipid donor in the ER membrane onto nascent polypeptide chains. Their intervention is required to generate the mono-glucosylated trimming intermediate that binds to calnexin and calreticulin.

Lec23 is a ricin-resistant CHO cell line (Ray et al., 1991) expressing inactive glucosidase I characterized by a serine to phenylalanine amino acid substitution in the active site (Hong et al., 2004). PhaR2.7 is a mouse lymphoma cell line selected for resistance to the cytotoxic effects of Phaseolus vulgaris leukoagglutinating lectin (Reitman et al., 1982) characterized by the absence of glucosidase II activity. In these two cell lines, generation of the mono-glucosylated intermediate of N-glycan trimming does not occur and newly synthesized glycoproteins remain tri- (in Lec23) or di-glucosylated (in PhaR2.7). A similar phenotype is obtained upon cell exposure to castanospermine or deoxynojirimycin derivatives, which are specific inhibitors of the \( \alpha \)-glucosidases (Elbein, 1987).

Analysis of protein association with calnexin or calreticulin revealed that it was abolished or substantially perturbed in these cells and that glycoprotein folding and secretion was generally accelerated and less efficient (Branza-Nichita et al., 1999; Hebert et al., 1996; Kearse et al., 1994; Machold and Ploegh, 1996; Ora and Helenius, 1995; Zhang et al., 1997). Importantly, the viability of these cell lines revealed the dispensability of the calnexin chaperone system in cultured cells. However, the lethal outcome for a neonate 2-months after birth of an inherited glucosidase I deficiency named congenital disorder of glycosylation type IIb (De Praeter et al., 2000) and the embryonic lethality of the knockouts of other members of the calnexin cycle reveal a critical role for this chaperone system possibly restricted to specific organs or developmental phases.

Calreticulin- and calnexin-deficiency

Calreticulin and calnexin associate with most, if not all glycoproteins that are synthesized in the mammalian ER. The calreticulin knockout is embryonic lethal at E14.5. Lethality results from impaired cardiac development and defective execution of calcium-dependent signaling events and can fully be ascribed to the loss of the calcium-binding activity of calreticulin (Guo et al., 2002; Mesaeli et al., 1999).
Instead, calnexin knockout mice are carried to full term. About 50% of the newborns die within 48 hr and the survivors show growth defects and motor disorders associated with a dramatic loss of large myelinated nerve fibers (Denzel et al., 2002). Similarly to inactivation of α-glucosidases, deletions of calreticulin, calnexin (and calmegin, the testis-specific homologue of calnexin (Ikawa et al., 2001; Ikawa et al., 1997)) are well tolerated in cultured cells. Despite binding to a large number of nascent glycoproteins, their deletion only prevents maturation of few of them, thus proving the existence of surrogate folding machineries that can intervene when the chaperones of choice are busy or inactive.

Analysis of knockout cells revealed the crucial role of calreticulin in assembly and loading of class I MHC complexes with appropriate immunogenic peptides to be presented at the cell surface (Gao et al., 2002) (Fig. 1.4). It also revealed that maturation of influenza virus HA obligatorily requires calnexin assistance (Molinari et al., 2004; Pieren et al., 2005).

Calnexin and calreticulin associate with a distinct set of substrates (Danilczyk et al., 2000; Halaban et al., 1997; Keller et al., 1998; Otteken and Moss, 1996; Peterson et al., 1995; Pieren et al., 2005; Pipe et al., 1998; Van Leeuwen and Kearse, 1996) even though they share the same specificity for mono-glucosylated protein-bound oligosaccharides. A possible reason of substrate-selectivity is their different topology (Danilczyk et al., 2000). Surprisingly, maturation of calnexin substrates was not characterized by enhanced association with calreticulin upon calnexin-deletion (Pieren et al., 2005). Relevant exceptions were viral calnexin substrates expressed in infected cells. For example, the G protein of the vesicular stomatitis virus (VSV) did transiently associate with calreticulin upon calnexin-deletion in VSV-infected cells, but not when ectopically expressed in transfected cells. These data led to suggest that viral infections may subvert the normal glycoprotein recognition by the ER lectins (Pieren et al., 2005) and may explain why inactivation of the calnexin/calreticulin cycle affects viral replication and infectivity but not cell viability (Fischer et al., 1996; Gruters et al., 1987; Mehta et al., 1997; Mehta et al., 1998; Ouzounov et al., 2002; Wu et al., 2002).
**ERp57-deficiency**

ERp57 is a member of the PDI superfamily of ER-resident oxidoreductases (Table 1.2). It is associated with calnexin and calreticulin and facilitates oxidative maturation of newly synthesized N-glycosylated polypeptides (Ellgaard and Ruddock, 2005). ERp57-deletion is embryonic lethal but it does not impair cell viability and it does not elicit an acute unfolded protein response (Garbi et al., 2006; Soldà et al., 2006). This confirms that, even though most glycopolypeptides make use of the calnexin chaperone system, their maturation can proceed under the assistance of surrogate folding machineries.

ERp57 is also part of the MHC class I loading complex (Garbi et al., 2007; Hughes and Cresswell, 1998) where it is engaged in a stable disulfide bond with tapasin. Tapasin is an ER-resident protein that recruits MHC class I molecules to be loaded with antigenic peptides to the TAP peptide transporter located in the ER membrane (Fig. 1.4) (Peaper et al., 2005). It has been reported that the peptide-loading complex contains between 20% (steady state) and 85% (upon interferon-γ treatment) of the cellular ERp57 (Garbi et al., 2006; Peaper et al., 2005).

Analysis of primary B-cells and fibroblasts lacking ERp57 showed a strong reduction of recruitment of MHC class I in the peptide loading complex (Garbi et al., 2007). This was surprising because it had been assumed that tapasin was required and sufficient for this function. Comparison of wild type vs. knockout cells also revealed that the covalent association of ERp57 induces a conformational change in tapasin that enhances affinity for MHC class I molecules, prolongs their retention in the loading complex and possibly allows a better selection of the antigenic peptide (Garbi et al., 2006). A role of ERp57 in facilitating the conformational breathing required for high-affinity loading of peptides in the MHC class I antigen-binding cleft has also been proposed (Kienast et al., 2007; Wearsch and Cresswell, 2007).
Figure 1.4 - The antigen-loading complex
The model shows biogenesis and loading with immunogenic peptides of the MHC class I complex. Antigenic peptides are generated by cytosolic proteasomes and are imported in the ER lumen through the TAP complex. Note that in the loading complex ERp57 forms a stable, covalent complex with tapasin.

Back-transfection of mouse fibroblasts lacking ERp57 with substrate-trapping ERp57 mutants in which one or both CXXC active sites were mutated to CXXA, led to the identification of several cellular ERp57 substrates (Jessop et al., 2007). The two mutated catalytic sites were shown to be equally efficient in trapping substrate proteins, with the exception of tapasin and few others that only formed mixed disulfides with the active site of subdomain a (Jessop et al., 2007).

Analysis of protein maturation in cells lacking ERp57 revealed that ERp72 (Table 1.2) can act as a surrogate oxidoreductase in assisting maturation of glycoproteins (Soldà et al., 2006). ERp72 is the only member of the PDI superfamily that shares the residues that in ERp57 are involved in association with the calnexin and
calreticulin P-domains (Ellgaard and Ruddock, 2005). In contrast to ERp57, however, substrate association with calnexin and calreticulin was dispensable for ERp72 intervention (Soldà et al., 2006). On the mechanistic site, analysis of an obligate calnexin/ERp57 substrate, the influenza virus HA, revealed that deletion of ERp57 exclusively hampered the post-translational phase of HA maturation consisting in reshuffling of intra-molecular disulfide bonds to the native set. The co-translational phase consisting in the oxidation of cysteines emerging in the ER lumen progressed very efficiently even without ERp57 (Soldà et al., 2006). These findings and data showing that ERp57 is in the reduced state in the ER lumen at steady state (Antoniou and Powis, 2003; Jessop and Bulleid, 2004; Mezghrani et al., 2001) suggest that ERp57 acts as an isomerase/reductase, rather than as an oxidase.

**UGT1-deficiency**

UGT1 prolongs substrate retention in the calnexin chaperone system by re-glucosylating oligosaccharides on non-native polypeptides prematurely released from calnexin (Hammond et al., 1994). UGT1 deletion is embryonic lethal for most homozygous mice (E13), but few of them survive until birth (Molinari et al., 2005). Viability of cultured cells lacking UGT1 is apparently normal. Unbiased comparison of protein biogenesis in cells with and without UGT1 revealed the existence of cellular and viral glycoproteins that attain the native structure in a single round of association with calnexin. Kinetics of release from calnexin and folding efficiency of these proteins were the same in the two cell lines. It also showed that other proteins are normally subjected to multiple binding events to complete maturation. In fact, in cells lacking UGT1 they were prematurely released from calnexin and their folding efficiency dropped. The finding that UGT1-deletion substantially delayed release from calnexin of few cellular proteins and of the influenza virus HA was unpredicted. These data showed that UGT1 and/or a UGT1-associated folding enzyme (e.g. the seleno-cysteine-containing oxidoreductase Sep15 that forms a 1:1 complex with UGT1 (Labunskyy et al., 2007)) regulate the conformational maturation that might be required for release from calnexin of select substrates (Soldà et al., 2007). The data also implied that calnexin can act as a *bona fide* molecular chaperone that retains non-native glycoprotein conformers (even in cells lacking UGT1), a function that can be inferred from *in vitro* experiments (reviewed in (Williams, 2006)).
The consequences of UGT1-deletion on ER retention of misfolded conformers have been studied in cells expressing the tsO45 G protein (Gallione and Rose, 1985). At the permissive temperature (32°), the G protein rapidly attain the native structure in the calnexin chaperone system (t_{1/2} is about 15 min) (Hammond and Helenius, 1994). At the non-permissive temperature (39°), it remains associated with calnexin for at least 60 min (Hammond et al., 1994). This was ascribed to a rapid turnover (t_{1/2} of 5-10 min (Wada et al., 1997)) of the terminal glucose displayed by non-native G protein, which is repeatedly removed by glucosidase II to be then rapidly re-added by UGT1 (Hammond et al., 1994; Suh et al., 1989).

Surprisingly, deletion of UGT1 did not accelerate release of the misfolded tsO45 G protein from calnexin for at least 60 min, showing that the misfolded protein was not cycling on/off calnexin. Beyond the 60 min, a second phase of retention-based ER quality control was characterized by a slow release of the misfolded G protein that entered in BiP-associated disulfide-bonded aggregates. Deletion of UGT1 substantially accelerated entry of terminally misfolded polypeptides in this second phase (Molinari et al., 2005, Chapter 4). Evidently, cycling of misfolded glycopolypeptides in the calnexin chaperone system is activated very late as if repeated releases from dynamic constrictions caused by calnexin binding would only be exploited as a very last attempt to eventually fold defective polypeptides. Our data revealed that cycling in the calnexin chaperone system represents the first phase of retention-based ER quality control (Fig. 1.3). In cells with and without UGT1, the terminally misfolded tsO45 G (Molinari et al., 2005) or folding-defective ERAD candidates (Molinari et al., 2002) are eventually released from the calnexin cycle to be trapped in the second phase of retention-based quality control relying on BiP association. This phase precedes substrate dislocation into the cytosol for disposal (Fig. 1.3).

1.1.9 Deletion of other chaperones
The individual knockouts of calreticulin, UGT1, ERp57 are embryonic lethal at E13-14 while mice lacking calnexin die early after birth. The reason for lethality has been established only for calreticulin and is the loss of the calcium-binding function of this protein, rather than the loss of its chaperone activity (Guo et al., 2002; Mesaeli et al., 1999). In all cases, however, cultured cells show surprisingly mild phenotypes and do not show symptoms of acute unfolded protein response. This shows that for most
(but not all) cargo proteins translocated in the ER alternative folding machineries can be activated that efficiently support glycoprotein maturation.

Deletions of private chaperones allowed assessment of the function of the chaperone-of-interest in very specific processes. For example deletion of tapasin, a component of the antigen-loading complex, resulted in obvious phenotypes related to impaired antigen presentation (Garbi et al., 2000). Deletion of Hsp47, a collagen-specific chaperone, resulted in a clear defect in collagen biosynthesis (Nagai et al., 2000).

Deletion of tissue-specific chaperones has also been reported. An interesting case is deletion of calmegin, the testis-specific isoform of calnexin. Calmegin binds transiently to a large number of mono-glucosylated proteins synthesized in sperm cells, but its deletion only affects maturation of very few of them, namely fertilins (Ikawa et al., 2001; Ikawa et al., 1997). Consistently with lack of consequences on other glycoproteins maturation, sperm number, viability or motility are unaffected in calmegin-deficient mice, while the fertilins regulated sperm’s capacity to bind to the egg’s zona pellucida is lost. This causes male sterility (Ikawa et al., 1997; Nakanishi et al., 2004).

Deletion of conventional chaperones such as GRP94 (Wanderling et al., 2007) and BiP (Luo et al., 2006) has also been reported. Both deletions are embryonic lethal (E7.5 for GRP94 and E3.5 for BiP). Few or no data are available on phenotypes linked to the loss of their chaperone function. The studies are hampered, at least in the case of BiP, by the pleiotropic roles of this abundant ER protein that seals the translocon, acts as a molecular ratchet to facilitate post-translational protein import in the ER, contributes to calcium homeostasis and serves as a chaperone (Hendershot, 2004). The essential role of BiP is underscored by the very early lethality of the homozygous embryos and by the identification of BiP as the substrate of SubAB, one of the most potent bacterial toxins responsible for haemolytic uremic syndrome outbreaks (Montecucco and Molinari, 2006).

1.1.10 The fate of newly synthesized, folding-competent polypeptides: a summary

To summarize, the ER is the first station of the secretory pathway. Ribosomes attached at the cytosolic face of the ER membrane co-translationally insert two classes of proteins in the ER lumen: firstly, the ER-resident proteins that operate in
the ER lumen where they are retained by KDEL-like or KXXX-like sequences or through association with retained proteins; secondly, the transiting-cargo that will normally leave the ER lumen only upon acquisition of the correct tertiary and quaternary architecture.

Polypeptide maturation in the ER lumen is assisted by canonical chaperones that utilize ATP such as GRP94 and BiP with its several cofactors ERdj1-5, BAP/Sil1 and GRP170 (Table 1.1), by lectin chaperones (calnexin and calreticulin, Table 1.4) and by a variety of enzymes that facilitate protein folding. Some of them (one oligosaccharyl transferase, two α-glucosidases, one glucosyltransferase, and several α1,2-mannosidases, Table 1.4) add onto nascent chains and process oligosaccharides. Others, catalyze rate-limiting reactions such as covalently cross-linking cysteines (about 15 members of the PDI superfamily, Table 1.2) and cis/trans isomerization of peptidyl-prolyl bonds (at least 4 different members of the PPI superfamily, Table 1.3). The ER lumen also contains a series of substrate- and tissue-specific chaperones that take care of individual, or of a restricted population of clients (Hebert and Molinari, 2007). Also, specialized chaperones, lectins and enzymes localized in the ER lumen or in the ER-Golgi intermediate compartment such as ERGIC-53, ERp44, Ero1 may intervene to facilitate polymerization of oligomeric proteins (reviewed in (Anelli and Sitia, 2008)).

Exposure of hydrophobic patches, unpaired reactive cysteines or other less-well defined non-native structures may elicit association of a series of ER-resident proteins, whose binding inhibits exit of non-native cargo from the folding environment. Tightness of retention-based ER quality control is not absolute and secretion of unstable protein conformers that pass ER quality control but are subsequently misfolding outside the cell may occur (e.g. transthyretin mutants (Johnson et al., 2005; Sekijima et al., 2005)).

Upon successful completion of the folding program, the vast majority of native proteins are incorporated into COPII-coated vesicles to be released from the folding compartment (Gurkan et al., 2006). Concentration of certain glycoproteins in transport vesicles requires intervention of specialized lectins (Table 1.4). VIP36 and VIPL preferentially associate with native glycoproteins displaying three mannose residues, but no glucose on the oligosaccharide branch A; ERGIC-53 seems to have low affinity but broader specificity as it also binds mono-glucosylated N-glycans (Kamiya et al., 2008).
1.2 Substrate recognition and translocation into the cytosol for ERAD

1.2.1 ERAD substrates: not only terminally misfolded polypeptides

Genome replication is an accurate process with error-rates of less than 1 every $10^{10}$ nucleotides warranted by DNA editing and repair mechanisms (Branzei and Foiani, 2008; Jiricny, 2006). Transcription and translation, the latter with 1 error every 1000-10000 bases, are less efficient processes (Hebert and Molinari, 2007). It may therefore happen that, despite genetic integrity, individual nascent chains emerging in the ER lumen carry mutations, deletions or truncations that may prevent polypeptide folding. It may also happen that polypeptides with correct primary structure enter off-pathways eventually leading to irreversible misfolding. Moreover, certain polypeptides have an intrinsic low capacity to attain a transport-competent structure, the most evoked example being the cystic fibrosis transmembrane conductance regulator (CFTR) with a folding efficiency below 30% (Kopito, 1999). It is unclear how many of the newly synthesized polypeptide chains will not acquire a native structure. Values ranging from an amazing 30% (Schubert et al., 2000) to much less (Vabulas and Hartl, 2005) have been reported. In any case, efficient removal from the folding environment of these physiologic by-products of protein biogenesis is required to maintain ER homeostasis (Molinari and Sitia, 2005).

Under pathologic conditions such as ER stress or viral infection, the fraction of newly synthesized polypeptides successfully completing maturation may substantially drop. For example, acute ER stress may enhance the activity of the ER degradation machinery to such an extent that polypeptide disposal may start in advance of termination of polypeptide folding programs causing co- or immediate post-translational degradation (reviewed in (Molinari, 2007; Moremen and Molinari, 2006)). As a second example, viral gene products may associate and selectively target cellular proteins for destruction (e.g. the MHC class I heavy chain and the viral receptor CD4 are rapidly degraded in cells infected with the cytomegalovirus (HCMV) (Wiertz et al., 1997) and the human immunodeficiency virus (Willey et al., 1992), respectively). As a third example, negative feedback mechanisms do exist to adapt the intracellular level and activity of rate-limiting enzymes of specific metabolic pathways to the cellular demand for the pathway’s final product (e.g. the turnover of
the rate-limiting enzyme for cholesterol biosynthesis, the 3-hydroxy-3-methylglutaryl coenzyme A reductase (Gil et al., 1985), is selectively enhanced under conditions of high sterol levels (Chin et al., 1985; Hampton, 2002).

1.2.2 Understanding ERAD
Native proteins are rapidly released from the ER into the secretory pathway. Therefore, the vast majority of the cargo present in the ER lumen is either unfolded or misfolded. Unfolded chains are intermediates of a productive folding program that will eventually attain the native structure if retained long enough in the folding environment. Terminally misfolded chains, on the other hand, have irreparably failed their folding attempts and must rapidly be removed from the ER lumen and degraded, otherwise they will accumulate and will inhibit the compartmental capacity to deal with nascent chains incessantly emerging in the ER lumen. The efficient execution of these complex tasks is crucial for cell and organism survival and their manipulation may offer therapeutic approaches to cure or alleviate the symptoms of conformational diseases caused by defective protein folding (Aridor, 2007; Conn et al., 2007; Molinari, 2007; Yoshida, 2007).

The mechanisms regulating protein disposal from the mammalian ER have been established in some detail by careful analysis of the fate of several model substrates expressed in cultured cells. Amongst them, proteins that do not attain the native structure because they carry mutations (e.g. α1-antitrypsin Z (Wu et al., 1994)), deletions (e.g. the CFTR ΔF508 (Ward and Kopito, 1994) or β-secretase splice variants (Molinari et al., 2002)), or truncations (e.g. theNullhong kong variant of α1-antitrypsin (Liu et al., 1997)). Other examples are offered by orphan subunits of oligomeric complexes ectopically expressed in cultured cells (e.g. the asialoglycoprotein receptor subunit H2a (Wikstrom and Lodish, 1991), the T cell receptor α-subunit (Lippincott-Schwartz et al., 1988) or orphan immunoglobulin chains (Knittler et al., 1995; Okuda-Shimizu and Hendershot, 2007)) or cellular targets of viral gene products (e.g. the MHC class I molecules in cells expressing the HCMV immunoevasins US2 and US11 (Wiertz et al., 1997)). Moreover, yeast genetics in S. cerevisiae (Wolf and Schafer, 2005) paved the way for the identification of several ER-resident, transmembrane and cytosolic proteins that
regulate protein quality control. The mammalian system is much more complex, but several folding, quality control and ERAD regulators operating in mammalian cells have functional orthologs in yeast. In section 1.2.4 few differences between the yeast and the mammalian systems are highlighted.

Altogether, it is clear that the existing models on function of folding, quality control and degradation machineries have been generated upon analysis of a limited set of model proteins synthesized in select model systems. It still remains unclear how far we can go with generalizations of data collected from these studies.

1.2.3 A lag phase before destruction
Initially, there is no difference between a folding-competent and a folding-defective polypeptide. They are both inserted co-translationally within the ER lumen in an unfolded state and are retained for some time in the folding machinery. Folding-competent polypeptides eventually attain the native structure and escape chaperone-mediated retention in the ER lumen. Folding-defective ones are initially subjected to folding attempts, and are therefore normally not degraded immediately after synthesis. The phase of futile folding attempts can be visualized as a lag phase that precedes degradation onset (Amara et al., 1989; de Virgilio et al., 1999; Fagioli and Sitia, 2001; Le et al., 1990; Lippincott-Schwartz et al., 1988; Mancini et al., 2003; Molinari et al., 2002). Folding-defective glycoproteins spend most of this lag phase in the calnexin chaperone system. As long as folding-competent and folding-defective polypeptides are in the calnexin chaperone system, they are protected from premature degradation (Molinari et al., 2002).

How exactly folding intermediates necessitating longer retention in the folding machinery are distinguished from terminally misfolded conformers that must be extracted from the ER and degraded, is still a matter of intensive study. For glycoproteins, N-glycan processing plays a crucial role in these decisions (Molinari, 2007). Nascent chains are decorated with a pre-assembled 14-saccharides glycan (Glc$_3$Man$_9$GlcNAc$_2$-). **Fig. 1.3, NASCENT**. The initial removal of the two terminal glucose residues is an irreversible process that generates the mono-glucosylated trimming intermediate that recruits calnexin and calreticulin (**Fig. 1.3**, step 1). The removal of glucose-3 that follows substrate release from calnexin/calreticulin (**Fig. 1.3**, step 2 or step 3a) is on the other hand a reversible reaction. In fact, if the fully
de-glucosylated oligosaccharide is displayed on a polypeptide chain that elicits
UGT1-recognition, glucose-3 can be re-added to prolong retention of the folding
polypeptide in the calnexin chaperone system (and delay deviation of non-native
polypeptides into the ERAD pathway) (Fig. 1.3, step 4). During the off-phase, N-
glycans may eventually become accessible to ER mannosidases of the glycosyl
hydrolase 47 (GH47) family (these are $\alpha1,2$-mannosidases (Moremen and Molinari,
2006), Table 1.4). $\alpha1,2$-mannosidases can potentially remove 4 mannoses from
protein-bound oligosaccharide (Fig. 1.3, step 5). N-glycans with reduced number of
mannoses displayed on a polypeptide exposing non-native determinants that elicit
persistent association with ER-resident factors, for example BiP (Fig. 1.3, step 6)
represents a potent signal for polypeptide disposal (Fig. 1.3, step 7).

### 1.2.4 Extensive de-mannosylation to deviate misfolded proteins for ERAD

Native proteins are packaged into COPII-coated transport vesicles and are delivered
through the secretory pathway at their final intra- or extracellular destination.
Immature polypeptides must be retained in the ER lumen until achievement of their
native structure. Terminally misfolded polypeptides must be translocated into the
cytosol to be degraded by 26S proteasomes. Efficient execution of these three tasks,
i.e. secretion of native structures, retention of folding intermediates and disposal of
terminally misfolded polypeptides maintains ER homeostasis.

To channel terminally misfolded glycoproteins for degradation, the *futile*
cycles of release and re-association with calnexin/calreticulin regulated by the counteracting
activities of the glucosidase II and UGT1 must eventually be interrupted. Current
models claim that while temporary detached from calnexin, non-native
glycopolypeptides may become accessible to one (Cabral et al., 2001; Lederkremer
and Glickman, 2005) or more (Olivari and Molinari, 2007) ER-resident $\alpha1,2$-
mannosidases. These will remove one (mannose B (Cabral et al., 2001)) or,
sequentially, up to 4 $\alpha$-(1-2)-bonded mannose residues (Lederkremer and Glickman,
2005; Olivari and Molinari, 2007) (Fig. 1.3, step 3b). Substrate de-mannosylation
makes the associated polypeptide a weaker ligand for calnexin and calreticulin (Spiro
et al., 1996), a better substrate for glucosidase II (Totani et al., 2006) (conflicting
results have been published on this, though (Grinna and Robbins, 1980)) and a worst
substrate for UGT1 (Sousa et al., 1992). When a polypeptide with low mannose content is eventually released from calnexin and calreticulin the re-glucosylation necessary to re-associate becomes less and less efficient. Consistently, selective inhibition of ER α1,2-mannosidases with the alkaloid kifunensine retards release of folding-defective polypeptides from the calnexin cycle (Molinari et al., 2002) and delays ERAD (Liu et al., 1999; Su et al., 1993; Tokunaga et al., 2000).

*S. cerevisiae* lacks a UGT1 ortholog that delay onset of degradation by prolonging retention of non-native polypeptides in the calnexin chaperone system (Fernandez et al., 1994). Removal of a single mannose residue from the N-glycan branch B (Fig. 1.2) by the MnsI is apparently sufficient to tag terminally misfolded polypeptides for disposal (Jakob et al., 1998). Cumulating evidences indicate that requirements for mammalian ERAD may be different. Firstly, already in the early 90’s, it has been shown that extensive de-mannosylation precedes protein disposal from the mammalian ER (Su et al., 1993; Wikstrom and Lodish, 1991). This was recently confirmed by several studies showing that N-glycans on folding-defective polypeptides are extensively processed to Man$_{5-6}$ in the mammalian ER (Foulquier et al., 2004; Foulquier et al., 2002; Frenkel et al., 2003; Hosokawa et al., 2003; Kitzmuller et al., 2003; Lederkremer and Glickman, 2005; Su et al., 1993; Wikstrom and Lodish, 1991). Secondly, removal of a single mannose residue also characterizes the fate of native proteins that are released from the ER into the secretory pathway (Helenius and Aebi, 2001). It is therefore unlikely that this single mannose-processing event represents a strong signal for disposal from the mammalian ER. Furthermore, several studies have shown that mannose removal is still required for glycoprotein degradation in mannosyl-phosphoryl-dolichol-deficient cell lines (Cacan et al., 1992). These cells are characterized by addition onto nascent chains of incomplete oligosaccharides that only display removable α(1-2)-bonded terminal mannoses on branch A (Ermonval et al., 2001; Olivari et al., 2006) (Fig. 1.2, B3F7 mutant CHO line). Certainly, in the mammalian system, removal of mannose A (Fig. 1.2) has irreversible consequences because this saccharide is the only residue that can be re-glucosylated by UGT1 and its cleavage irreversibly extracts folding-defective polypeptides from the calnexin chaperone system (Fig. 1.3, steps 5-6).
Who is operating the extensive de-mannosylation of folding-defective polypeptides occurring in the mammalian ER? The mammalian ortholog of MnsI is the ER $\alpha 1,2$-mannosidase I (ERManI), a member of the GH47 family of $\alpha 1,2$-mannosidases. Similarly to the yeast protein, ERManI specifically removes mannose B from protein-bound oligosaccharides, unless its activity is tested in vitro at unphysiologically high concentrations. In this case, recombinant ERManI removes mannose B as well as other $\alpha(1\text{-}2)$-bonded mannoses (Herscovics et al., 2002). It is possible that this enzyme is enriched in specialized sub-regions of the mammalian ER and extensively de-mannosylates terminally misfolded polypeptides (Avezov et al., 2008; Lederkremer and Glickman, 2005). However, the specificity of $\alpha 1,2$-mannosidases is conferred by the dimension of the carbohydrate binding site where an arginine residue at the bottom of the catalytic site (Arg273 in the yeast MnsIp, Arg461 in human ERManI) plays a critical role in reducing the degree of freedom of the oligosaccharide entering the active site (Romero et al., 2000). $\alpha 1,2$-mannosidases with broader specificity (e.g. Golgi-resident $\alpha 1,2$-mannosidases or the $\alpha 1,2$-mannosidase of *Penicillium citrinum*) that can trim Man$_6$GlcNAc$_2$- to Man$_5$GlcNAc$_2$- are characterized by the presence of a smaller, uncharged residue at this position (Lobsanov et al., 2002). The conversion of MnsIp and ERManI in mannosidases that efficiently generate the Man$_5$GlcNAc$_2$- final product requires the replacement of Arg273 (or 461) with a leucine or a glycine (Romero et al., 2000).

An additional possibility is that other $\alpha 1,2$-mannosidases contribute to the generation of the ERAD signal in the mammalian ER. Likely candidates are three recently characterized ER-resident members of the GH47 family, namely EDEM1, EDEM2 and EDEM3 (Olivari and Molinari, 2007). Notably, the three EDEM proteins are characterized by the presence of a glycine residue at the bottom of their putative active sites. This would confer to them the capacity to generate Man$_5$GlcNAc$_2$- oligosaccharides. There is an ongoing debate whether the EDEM proteins are active mannosidases. Certainly, they conserve the structure of the catalytic site as well as all the catalytic residues present in the other mannosidases of the GH47 family (Karaveg et al., 2005; Moremen and Molinari, 2006). Moreover, it has been shown that up-regulation of EDEM1 and EDEM3 enhances de-mannosylation of ERAD substrates (Hirao et al., 2006; Olivari et al., 2006) and that this does not occur if the
ectopically expressed EDEM1 or EDEM3 carry a mutation of a single residue in their putative catalytic sites (Hirao et al., 2006; Olivari et al., 2006). It must also be mentioned that recombinant T. cruzi EDEM is an active α1,2-mannosidase that extensively processes Man$_9$-GlcNAc$_2$- to Man$_5$-GlcNAc$_2$- (Banerjee et al., 2007).

1.2.5 Disposal of non-glycosylated proteins
The fate of non-glycosylated proteins that enter the ER is much less clear compared to the fate of N-glycosylated ones. Certainly, folding-defective glycosylated and non-glycosylated polypeptides are both translocated into the cytosol to eventually be degraded by 26S proteasome. It can be postulated that folding-defective glycoproteins are subjected to two phases of retention-based ER quality control, the first in the calnexin chaperone system and the second in the BiP/PDI chaperone system (Fig. 1.3), while non-glycosylated polypeptides must by-pass the first phase and can only rely on the second (Molinari et al., 2005). Analysis of few folding defective polypeptides and of bacterial toxins that exploit the same machineries to invade the host cell cytosol revealed that recruitment of BiP and/or PDI immediately precedes translocation of terminally misfolded proteins into the cytosol (Gillece et al., 1999; Kabani et al., 2003; Molinari et al., 2002; Nishikawa et al., 2001; Tsai et al., 2001). Recent work showed that dislocation into the cytosol of non-glycosylated κ light chain requires derlin-1 and HERP (Okuda-Shimizu and Hendershot, 2007), while the glycosylated ERAD substrate Null$_{hong\, kong}$ requires derlin-2 and -3 (both forming a complex with EDEM1), but neither derlin-1 (Oda et al., 2006) nor HERP (Okuda-Shimizu and Hendershot, 2007). Since HERP is located in the ER membrane with most of its volume exposed to the cytosol (Kokame et al., 2000), its interaction with non-glycosylated ERAD substrates was proposed to be indirect or to require a partial dislocation of the misfolded protein into the cytosol (Okuda-Shimizu and Hendershot, 2007). If HERP plays the same role as its yeast ortholog Usa1p, then an implication of these data would be that in mammals, the soluble, misfolded NHK is dislocated into the cytosol and degraded without intervention of the Hrd1p/Synoviolin complex (see next section) because formation and stability of the complex should rely on the presence of HERP.
1.2.6 Directing ERAD substrates to the retro-translocation site

Both in yeast and in mammalian cells, the processes that regulate recognition and translocation of terminally misfolded polypeptides into the cytosol are ill defined. Substrate ubiquitylation facilitates proteasomal degradation and requires an activating, a conjugating and a ligating enzyme (E1, E2 and E3, respectively). Recently, many aspects and several factors involved in these processes have been characterized in *S. cerevisiae* where distinct machineries are involved in recognition, retro-translocation and polyubiquitylation of polypeptides displaying folding defects in their luminal, transmembrane or cytosolic domains (Vashist and Ng, 2004). These complex machineries are organized around two RING finger E3 ubiquitin ligases, Doa10p (TEB4/MARCH VI in mammals) and Hrd1p/Der3p (Synoviolin and gp78 in mammals) (Ismail and Ng, 2006; Kostova et al., 2007).

Membrane polypeptides with cytosolic lesions are targeted to the ERAD-C machinery (Doa10p complex). The Doa10p complex comprises the E2 (ubiquitin-conjugating) enzymes Ubc6p (Ube2j1 and Ube2j2 in mammals) and Ubc7p (Ube2g1 and Ube2g2) with their membrane-connector Cue1p as well as a substrate extractor complex containing the AAA-ATPase Cdc48p (p97 in mammals) with the Ufd1 and Npl4 co-factors and the connector Ubx2p (Carvalho et al., 2006; Li et al., 2007; Nakatsukasa et al., 2008; Vashist and Ng, 2004).

Transmembrane and soluble proteins with luminal defects are targeted to the ERAD-M and ERAD-L pathways (Hrd1p/Der3p complex, Fig. 1.5 shows one possible arrangement of the corresponding mammalian Synoviolin complex). The Hrd1p/Der3p (Synoviolin) complex contains the cytosolic factors also found in the Doa10p complex but several additional transmembrane (Der1p (derlin1-3), Usa1p (HERP), Hrd3p (Sel1L)) and luminal proteins (e.g. Yos9p (OS-9.1, OS-9.2, erlectin in mammals) and Kar2p (BiP) (Carvalho et al., 2006; Denic et al., 2006; Gauss et al., 2006)).
The mammalian system is much more complex and has a greater number of E3 enzymes involved in ERAD. Some of them are spanning the ER membrane, for example Synoviolin (Yagishita et al., 2008), gp78 (Fang et al., 2001) and TEB4 (Hassink et al., 2005). Synoviolin and gp78 are involved in disposal of several substrates from the ER lumen (Kostova et al., 2007; Morito et al., 2008) while for TEB4 no cellular substrate has been described, yet. Other mammalian E3 ubiquitin ligases are cytosolic proteins (e.g. parkin, CHIP and the SCF-Fbs1/Fbs2 complex involved in degradation of Pael-R (Imai et al., 2001), CFTR ΔF508 (Younger et al., 2006) and select glycoproteins (Yoshida et al., 2002), respectively).
Despite several highly cited publications seemed to have solved the issue, it remains unclear how misfolded proteins cross the ER membrane during their translocation into the cytosol. Several candidates such as Sec61 and derlin proteins as well as protein-independent mechanisms such as lipid droplets formation (Ploegh, 2007) could be involved in the process. For example, dislocating MHC class I heavy chains in cells expressing immunoevasins were cross-linked to Sec61 (Wiertz et al., 1996); yeast Sec61 mutants exhibit severe defects in degradation of soluble, but not of membrane-bound folding-defective polypeptides (Huyer et al., 2004; Pilon et al., 1997; Plemper et al., 1997; Walter et al., 2001); Sec61 is used for retro-translocation of toxin subunits that subvert the ERAD pathway to invade the host cell cytosol (Simpson et al., 1999), but derlin-1 also offers an essential contribution (Bernardi et al., 2008); Sec61 binds the 19S proteasomal subunit (Kalies et al., 2005); Sec61 is not part of the Hrd1p and Doa10p ubiquitin ligase complexes regulating cytosolic translocation of ERAD candidates (Ismail and Ng, 2006); antibodies to Derlin-1, but not against Sec61, inhibit disposal of select substrates (Wahlman et al., 2007). Der1p, which is part of the yeast Hrd1p complex, and the mammalian orthologs derlin-1, -2 and -3 have been implicated in retro-translocation (Lilley and Ploegh, 2004; Lilley and Ploegh, 2005; Oda et al., 2006; Wahlman et al., 2007; Ye et al., 2005; Ye et al., 2004).

1.2.7 The mammalian orthologs of Yos9p

In *S. cerevisiae*, Yos9p binds and targets terminally misfolded polypeptides to the Hrd1p complex (Bhamidipati et al., 2005; Buschhorn et al., 2004; Carvalho et al., 2006; Denic et al., 2006; Kim et al., 2005; Szathmary et al., 2005). Intriguingly, although several examples show that most of the components of yeast machineries are conserved in the mammalian system, for long time, it was believed that mammalian OS-9 was a cytosolic protein, associated with the cytosolic face of the ER membrane (Litovchick et al., 2002). This study was followed by several other publications, in which experimental design and interpretation of the results were based on the assumption that OS-9 was a cytosolic protein (Baek et al., 2005; Su et al., 1993; Wang et al., 2007). The different topology hampered the identification of OS-9 as the functional ortholog of Yos9p. Recent studies in the lab of Ron Kopito (Christianson et al., 2008) and in our lab (Bernasconi et al., 2008) demonstrated that mammalian OS-9 is a glycosylated ER-resident protein expressed in two splice
variants, OS-9.1 and OS-9.2. OS-9 variants associate with folding-defective glycoproteins, but not with native ones and have a dual function within the ER: they maintain the tightness of retention-based ER quality control by preventing forward transport of non-native conformers (this has been shown for the folding-defective Null<sub>hong kong</sub> variant of α1-antitrypsin (Bernasconi et al., 2008)) and they participate in disposal of misfolded proteins from the mammalian ER ((Christianson et al., 2008) and (Bernasconi et al., 2008)).

Our studies show that transcription of both OS-9 variants is enhanced upon activation of the Ire1/Xbp1 pathway in cells exposed to acute ER stress. Analysis of transcriptional regulation of the mammalian orthologs of the components 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, whereas Sel1L is regulated by ATF6 (Kaneko et al., 2007) and (Bernasconi et al., 2008). Our working model claims that retention of misfolded versus facilitation of disposal functions of mammalian OS-9 variants might depend on formation of multiprotein complexes, and that activation of individual stress-response pathways (Ire1-regulated versus ATF6-regulated) in specific tissues or under specific stress conditions could enhance one or the other function of OS-9.

Both OS-9 variants contain a mannose 6-phosphate receptor homology (MRH) domain whose function remains unclear. Christianson et al. suggested that this domain is involved directly or indirectly in a functional association with Sel1L (as shown in one possible model in Fig. 1.5). However, the presence of a N-linked glycan in the middle of the MRH domain (this glycan is absent in the yeast ortholog), clashes somewhat with a putative role of this domain in the assembly of the complex with Sel1L. Moreover the results of our group showing that OS-9 binds to non-glycosylated substrates and that substrate association with OS-9 and degradation are not affected by mutation of the MRH domain (Bernasconi et al., 2008), imply that OS-9 does not require a functional MRH domain. This would be consistent with the glycan code theory (Helenius and Aebl, 2004) that postulates a role of N-glycans in ERAD as a signal for disposal and not as a requirement for assembly of degradation complexes.
1.2.8 Macroautophagy

Macroautophagy is a unique intracellular process in which membrane-bound compartments engulf organelles and macromolecules and deliver them to lysosomes for destruction. Mammalian cells display a low level of constitutive autophagy (baseline autophagy) that regulates normal turnover of cytosolic components. Autophagic activity can strongly be induced upon nutrient deprivation to play pleiotropic roles in a variety of cytoprotective functions (Cuervo, 2004; Mizushima, 2005; Shintani and Klionsky, 2004; Yoshimori, 2007). Autophagy can also be induced upon ER stress to insure cell survival and to counterbalance ER proliferation (Bernales et al., 2006; Ogata et al., 2006; Yorimitsu et al., 2006). Macroautophagy is abolished upon deletion of the atg5 gene, which is essential for autophagosome formation (Hara et al., 2006; Komatsu et al., 2006; Mizushima et al., 2001). In cells subjected to several hours of nutrient deprivation (Kamimoto et al., 2006), or in which acute accumulation of insoluble protein aggregates triggers ER stress (Fujita et al., 2007; Kouroku et al., 2007), deletion of ATG5 results in accumulation of aberrant polypeptides in the ER lumen (reviewed in (Yorimitsu and Klionsky, 2007)). Thus, autophagy certainly contributes with proteasomal degradation to the clearance of otherwise indigestible protein aggregates accumulating in the cytosol or in the ER. Autophagy may also be activated under acute stress conditions (Klionsky, 2007). However, an involvement of autophagy in degradation of misfolded proteins from the ER at steady state must be an exception, rather than a rule. Several reports show in fact that lysosome and autophagy inhibitors do not normally affect ERAD (Klausner and Sitia, 1990; Meusser et al., 2005; Romisch, 2005; Ruddock and Molinari, 2006), which is, on the other hand, profoundly affected by interference with the activity of the ubiquitylating or proteasomal machineries.

1.2.9 Do autophagy-like processes regulate ERAD activity? The concept of ERAD tuning

ER-resident molecular chaperones and folding enzymes are long-living proteins. Exceptions, however, do exist. For example, it has been reported that the ER α1,2-mannosidase I, a crucial regulator of ERAD, is characterized by rapid turnover (Wu et al., 2007) and a recent published study of our group reveals that EDEM1 has unusual short half-life for an ER-resident protein (Cali et al., 2008).
An interesting electron microscopy and confocal immunofluorescence analysis recently revealed the presence of EDEM1 in small ER-derived vesicles that lack conventional ER markers and a recognizable cytosolic coat such as COPII. The destination or function of these vesicles has not been established, but the occasional presence of ERAD substrates led to propose a possible role of this vesicular transport out of the ER in removal of misfolded proteins from the ER lumen (Zuber et al., 2007). Results of our studies led to challenge this hypothesis and to postulate that a vesicular transport out of the ER regulates the rapid turnover of EDEM1, and possibly of other short-living ERAD regulators. The selective segregation of factors regulating protein disposal from the mammalian ER would reduce the competition between folding and degradation machineries operating in the ER lumen at the advantage of the protein folding process (Cali et al., 2008). The data show that in cells with defective EDEM1 turnover (e.g. cells lacking basal autophagy), the intraluminal level of this mannosidase is aberrantly elevated. This enhances disposal from the ER lumen of misfolded proteins and, most significantly, substantially shortens the time allocated to nascent polypeptides to attain the native structure, thus causing premature interruption of ongoing folding programs and unphysiologic disposal of folding-competent polypeptides (Cali et al., 2008). We postulate that at steady state, in unstressed cells, the ER protein folding machinery must be offered a kinetic advantage over the protein degradation machinery operating in the same compartment to deal with unstructured nascent chains. We propose that mechanisms that we define as ERAD tuning contribute to maintenance of ER homeostasis, which is required for optimal function of the ER protein factory. These post-translational mechanisms operate in mammalian cells to selectively remove from the ER folding compartment ERAD operators that, if present in excessive concentration, could prematurely interrupt productive polypeptide maturation.

### 1.2.10 The fate of folding-defective polypeptides: a summary

In the ER lumen, there is an intrinsic difficulty to distinguish terminally misfolded polypeptides to be selected for degradation from unstructured intermediates of the folding programs that will eventually complete their maturation. N-glycans appended to polypeptide chains may facilitate this task. Polypeptides entering a folding program expose oligosaccharides with nine mannose residues. This oligosaccharide structure is best suited for retention and/or cycling in the calnexin chaperone system that offers
appropriate folding conditions and prevents unwanted, premature degradation. Instead, folding-defective polypeptides that have spent already some time in unproductive folding attempts are eventually exposed to ER-resident α1,2-mannosidases that can sequentially remove up to 4 mannose residues. Extensive de-mannosylation inhibits and, upon removal of the mannose residue A, eventually fully prevents substrate retention in the calnexin chaperone system. It certainly therefore represents a strong signal for disposal from the mammalian ER.

Cumulating evidences show that the percentage of newly synthesized protein that is folded as opposed to degraded is strongly affected by the kinetic competition between conformational maturation and recognition for disposal in the ER lumen. As such, any change in protein folding versus degradation rate will determine the percentage of the newly synthesized proteins that will eventually acquire native structure (reviewed in (Brodsky, 2007; Hebert and Molinari, 2007; Molinari, 2007; Wiseman et al., 2007)). Several disease-linked polypeptide mutations do not compromise the function, but delay polypeptide maturation resulting in polypeptide disposal in advance of maturation. In all these cases, pharmacologic intervention can be hypothesized to accelerate maturation of the mutated polypeptide or to delay the onset of its disposal to such an extent that the folding program can successfully be completed ((Aridor, 2007; Conn et al., 2007; Molinari, 2007) and references therein).
Chapter 2

Focus and Aims of the Thesis

In the introductive chapter I presented part of the known mechanisms regulating protein folding in the mammalian ER. In the next two sections I show the investigations carried out during my doctoral studies. They focus on the role of N-glycans in protein folding and maturation in mammalian cells and on the retention-based quality control involving calnexin and BiP. These findings can be helpful in understanding and in possibly find therapeutic approaches to the highly debilitating conformational diseases that are caused by defects in the folding process of specific proteins.

In the first project we performed a systematic analysis aiming at understanding these mechanisms in cultured human cells. As model proteins we generated soluble forms of the human BACE (BACEs) with 4, 3, 2, 1 or 0 N-linked glycans and we expressed them in human cells. The experiments focused on understanding the relationship existing between glycosylation extent and secretion efficiency and rate, chaperone utilization, formation of by-products of the folding program and enzymatic activity. Moreover we determined whether inhibition of the polypeptides disposal enhances the fraction of newly synthesized chains that attain a native, transport-competent structure.

The second project is a study of the calnexin-based retention of non-native polypeptides in the ER. We infected cells with the Vesicular Stomatitis Virus that codifies for a glycoprotein with a reversible, temperature-dependent folding defect (tsO45 G protein). Experiments were performed in cells with and without the UDP-glucose:glycoprotein glucosyltransferase (UGT1), an enzyme that re-glucosylates non-native glycopolypeptides, preventing their release from the calnexin cycle. The study focused on the mechanisms of the retention-based ER quality control of terminally misfolded proteins and on the molecular chaperones involved.
Consequences of Individual N-glycan Deletions and of Proteasomal Inhibition on Secretion of Active BACE

BACE is an aspartic protease involved in the production of a toxic peptide accumulating in the brain of Alzheimer’s disease patients. After attainment of the native structure in the endoplasmic reticulum (ER), BACE is released into the secretory pathway. In order to better understand the mechanisms regulating protein biogenesis in the mammalian ER, we determined the fate of five variants of soluble BACE with 4, 3, 2, 1 or 0 N-linked glycans. The number of N-glycans displayed on BACEs correlated directly with folding and secretion rates and with the yield of active BACEs harvested from the cell-culture media. Addition of a single N-glycan was sufficient to recruit the calnexin chaperone system. Addition of 1 to 4 N-glycans progressively enhanced the dissociation rate from BiP and reduced the propensity of newly synthesized BACEs to enter aberrant soluble and insoluble aggregates. Finally, inhibition of the proteasome increased the yield of active BACEs secreted from human cultured cells.
3.1 Introduction

Systematic studies aiming at understanding mechanisms regulating protein biogenesis in living cells are rare and rely on a very short list of select model substrates (Pearse et al., 2008 and references therein). Characterization of the mechanisms that regulate protein folding, quality control and disposal from the ER lumen is crucial because defects in polypeptide maturation are often linked to highly debilitating conformational diseases (Aridor, 2007). The capacity to intervene in protein biogenesis will lead to development of therapeutic approaches aiming at delaying the progressive worsening of disease conditions, or even at reverting disease phenotypes by using chemical and pharmacological chaperones or compounds that target drugable regulators of the cell folding and disposal machineries. It will also facilitate more efficient and rentable production of recombinant proteins to be used in the clinics or in the industry (Baldi et al., 2007).

Here we performed a thorough analysis of the fate of a disease-regulating glycoprotein, the soluble form of human BACE (BACEs). BACEs was selected as model substrate because it can be produced in E. coli and refolded in active form from inclusion bodies (Hong et al., 2000; Sardana et al., 2004; Shimizu et al., 2008). In principle therefore, this protein can attain an active form independent of N-glycosylation.

The human BACE (EC 3.4.23.46, UniProtKB/Swiss-Prot entry P56817) is also named β-secretase, Memapsin-2 or Asp 2. It is a tetra-glycosylated type I transmembrane protein expressed in the human brain. BACE is an aspartic protease that sheds the amyloid precursor protein (APP) ectodomain (Hussain et al., 1999; Lin et al., 2000; Sinha and Lieberburg, 1999; Vassar et al., 1999; Yan et al., 1999). Subsequent cleavage of the APP membrane stub by the γ-secretase leads to the formation of the Alzheimer’s disease associated Aβ peptide (Aguzzi and Haass, 2003; Haass, 2004). The enzyme contains three disulfide bonds and two active aspartyl residues (Fig. 3.1A). Downstream of the signal peptide required for entry in the ER, a pro-peptide of 24 amino acids (Fig. 3.1A, residues 22-45) is present. Removal of this pro-segment in the Golgi compartment is carried out by furin or other
pro-protein convertase and is required to form the mature BACE (Benjannet et al., 2001; Bennett et al., 2000; Creemers et al., 2001; Shi et al., 2001).

**Figure 3.1 - BACEs features and structure**

A. Schematic representation of BACEs variants. Numbers represent amino acids residues of the polypeptide sequence. Relevant features are shown: signal peptide (SP), pro-peptide (Pro), the two catalytic aspartyl residues (D), the three native disulfide bonds and N-glycosylation sites (triangles).

B. Three-dimensional structure of the protease domain of BACE in the inhibitor-unbound (coral) and -bound state (green). Inhibitor OM00-3 (Hong and Tang, 2004) is shown by a blue stick model. Active-site flap (thick-thread line) movement is a well known conformational change for aspartic proteases (adapted from Hong et al., 2000).

The soluble forms of BACE (BACEs) used in this project (Fig. 3.1A) were generated by deletion of the transmembrane and the cytosolic domain. A tag to facilitate immunoisolation was added at the C-terminus (EFRH-tag). Removal of the different N-glycosylation sites was performed by site directed mutagenesis by substitution of the codon AAC (asparagine coding) with the codon CAA (glutamine coding). A crystallization-derived structure of free and of inhibitor-bound protease domain of BACE is shown in Fig. 3.1B (Hong et al., 2000).

Five variants of BACEs with 4, 3, 2, 1 or 0 N-linked glycans were ectopically expressed in human cells to study the dependency on the glycosylation state for parameters such as secretion rate and efficiency, chaperone use, formation of by-products of the folding program and enhancement of production yield upon manipulation of the ER folding and degradation capacity.
3.2 Results

3.2.1 A direct correlation between extent of BACEs glycosylation and secretion

To determine whether the extent of protein glycosylation affects protein folding and secretion efficiency, we analyzed the fate of BACEs displaying 0 (BACEs0) to 4 N-glycans (BACEs4). The polypeptides were ectopically expressed, individually, in HEK293 cells. Eighteen hours after transfection, cells were metabolically labeled for 10 min with $[^{35}S]$methionine and $[^{35}S]$cysteine. Incubations were prolonged after radioactivity washout for 5 to 240 min, when the secretion plateau was reached for all glycosylated mutants (Fig. 3.2, the chase was prolonged up to 480 min only for BACEs0, panel A). At the end of each chase time, the cell culture media were harvested and the secreted, labeled BACEs variants were immunoisolated with a specific antibody (Fig. 3.2, secreted). Cells were solubilized with 2% CHAPS, a zwitterionic detergent, and the lysates were centrifuged to separate detergent-soluble material (the post nuclear supernatants (PNS)) from detergent-insoluble material. The labeled BACEs variants present intracellularly at the end of each chase time were immunoisolated from the detergent-soluble fraction (Fig. 3.2, intracellular) and were separated in 10% polyacrylamide gels under reducing conditions. Labeled bands were quantified by densitometric analysis.

The amount of labeled BACEs immunoisolated from cell-lysates decreased (Figs. 3.2A-3.2E, intracellular) as a function of protein secretion with progression of the chase (Figs. 3.2A-3.2E, secreted). This showed that for all glycosylation mutants, a variable fraction of the newly synthesized, labeled protein was secreted in the extracellular media.

The amount of detergent-soluble labeled BACEs0 retained intracellularly decreased to about 25% of the initial amount after 240 min and to about 10% after 480 min of chase (Fig. 3.2A, intracellular). The amount of labeled BACEs0 immunoisolated from the cell culture media did not exceed the 5% of the initial synthesized pool of protein (Fig. 3.2A, secreted). Thus, secretion of non-glycosylated BACEs0 was very inefficient; the vast majority of newly synthesized BACEs0 underwent degradation or entered in detergent-insoluble aggregates (see below).
Figure 3.2 - Secretion rate and efficiency correlate with the extent of BACEs glycosylation

A Radioactively labeled BACEs0 has been immunoisolated from cell lysates (intracellular, lanes 1-5) or from the cell culture media (secreted, lanes 6-10) after the indicated chase times. Proteins have been separated in reducing SDS-PAGE and labeled BACEs0 has been quantified. BACEs0 immunoisolated after a 5 min chase is considered the initial amount (100%). The residual intracellular BACEs0 (25%) and the fraction of labeled BACEs0 harvested from the culture media after 240 min of chase (5%) are shown in lanes 3 and 8, respectively. Only for this mutant, chase was prolonged up to 480 min. B Same as A for BACEs1. C Same as A for BACEs2. D Same as A for BACEs3. E Same as A for BACEs4. F Quantifications of secretion of the five glycosylation mutants as shown in panel A-E (secreted). G Secretion efficiency of the five glycosylation mutants after 240 minutes for all mutants, average of at least three independent experiments.
The mono- and di-glycosylated BACEs variants disappeared with faster kinetics from the intracellular fractions (Figs. 3.2B-3.2C, intracellular). Secretion of BACEs1 (38%, Fig. 3.2B, secreted) and of BACEs2 (44%, Fig. 3.2C, secreted) was much more efficient compared to secretion of the non-glycosylated version of the model protein (5%, Fig. 3.2A, secreted).

The more extensively glycosylated forms of BACEs (BACEs3 (Fig. 3.2D) and BACEs4 (Fig. 3.2E)) disappeared even faster from the detergent-lysates and were secreted with the highest efficiency (about 60%, Figs. 3.2D and 3.2E).

These data demonstrated the existence of a direct correlation between the extent of BACEs glycosylation and the efficiency of secretion.

3.2.2 A direct correlation between extent of BACEs glycosylation and secretion kinetics

The data for the secretion rates and secretion efficiencies are summarized for the five glycosylation mutants in Figs. 3.2F and 3.2G, respectively. These data confirm the direct correlation existing between the extent of BACEs glycosylation and the secretion efficiency (Fig. 3.2G), both also correlating with the secretion rate (Fig. 3.2F). Secretion of the non-glycosylated mutant (BACEs0) was very slow and inefficient. BACEs1 and BACEs2 had similar secretion kinetics, with a $t_{50}$ (time of half-maximal secretion) of about 90 minutes. The more extensively glycosylated BACEs3 and BACEs4 showed a $t_{50}$ of about 60 minutes.

Thus, the five generated BACEs variants show significant differences in secretion efficiency and kinetics (Fig. 3.2).

As a control, to confirm that these differences depend on the extent of BACEs N-glycosylation and are not caused by the asparagine to glutamine substitutions, we expressed BACEs4 in presence of tunicamycin during starvation and pulse. This nucleoside antibiotic inhibits the first step in the synthesis of the dolichol-linked oligosaccharide, thus inhibiting protein N-glycosylation. The level of BACEs4 secretion under tunicamycin treatment was similar to BACEs0 secretion under normal conditions (data not shown). We can conclude that secretion efficiency of the generated BACEs variants depends on presence and number of N-glycans and not on the amino acid residue present at the glycosylation sites (asparagine vs. glutamine).
3.2.3 A direct correlation between extent of BACEs glycosylation and rate of acquisition of a DTT-resistant conformation

As a rule, newly synthesized polypeptides are released from the ER only upon attainment of the native structure (Ellgaard et al., 1999). Unfortunately, conformational antibodies discriminating native from non-native BACEs are not available. Thus, to confirm that kinetics and extent of secretion were dependent on the acquisition of the native structure, we monitored the rate of generation of DTT-resistant disulfides in the five glycosylation mutants. In most folded proteins, intramolecular disulfide bonds are solvent inaccessible and are not reduced by moderate DTT concentrations (Thornton, 1981).

At the end of each chase time shown in Fig. 3.3 and before lysis, cells were incubated for 5 min on ice in a medium containing 5 mM DTT. Cell incubation with this concentration of DTT readily reduces non-native, solvent exposed, intramolecular disulfides without affecting native disulfides (Tatu et al., 1993). The labeled BACEs mutants were immunoisolated from PNS and the proteins were separated under non-reducing conditions. Disulfide bonds in non-native conformers were fully reduced by the DTT-treatment on ice. Consequently, non-native conformers showed slower electrophoretic mobility (arrows sens in Fig. 3.3) when compared to native ones. In the latter, preservation of covalent bonds linking cysteines resulted in faster electrophoretic mobility (arrows res in Fig. 3.3). The DTT-sensitive and the DTT-resistant labeled polypeptide bands were quantified and the ratio DTT-resistant:DTT-sensitive was plotted (Fig. 3.3).
Figure 3.3 - Efficiency of attainment of DTT-resistant structure correlates with the extent of BACEs glycosylation

A At the end of the chase times indicated, cells were incubated for 5 min on ice with 5 mM DTT in PBS to reduce non-native disulfide bonds. After cell lysis, intracellular BACEs0 was immunoisolated and separated in non-reducing SDS-PAGE. The ratio DTT-sensitive BACEs0 (arrow sens) : DTT-resistant BACEs0 (res) is plotted. B Same as A for BACEs1. C Same as A for BACEs2. D Same as A for BACEs3. E Same as A for BACEs4.

For intracellular BACEs0, the conversion from the DTT-sensitive into the more compact DTT-resistant conformation was very slow and inefficient (Fig. 3.3A). This was consistent with the very slow and inefficient secretion shown for this protein in Figs. 3.2A and 3.2F. Even after a 90 min chase, about 80% of the intracellular BACEs0 was fully reduced upon exposure of cells to DTT. Addition of 1, 2, 3, and 4 N-glycans progressively accelerated the formation of DTT-resistant conformers that reached the 37, 62, 86 and 98% after the 80 min of chase shown in Figs. 3.3B-3.3E, respectively. As discussed above, the fraction of labeled, DTT-resistant form of the proteins eventually decreased during the chase because the native (and DTT-resistant) conformers are rapidly secreted in the extracellular media (Fig. 3.2).
3.2.4 An inverse correlation between number of glycans and persistence of BiP-binding

Biogenesis of BACE is characterized by sequential association with BiP first and with calnexin subsequently (Molinari et al., 2002; Molinari and Helenius, 2000; Pieren et al., 2005). To compare kinetics of BiP-association, lysis of cells expressing ectopic BACEs0, BACEs1, BACEs2 and BACEs4 was performed at the end of the chase times shown in Figs. 3.4A-3.4D in the presence of apyrase. Apyrase rapidly depletes cellular ATP and allows preservation of the non-covalent complexes engaging BiP and cellular substrates (Munro and Pelham, 1986).

Immunoisolation of BiP from cell lysates and protein separation in reducing gels revealed that association of BACEs0 with BiP was long lasting. For at least 30 min post-synthesis there was no release of BACEs0 from BiP (compare lane 1 and lane 2 in Fig. 3.4A). BACEs0 release from BiP progressed slowly during the chase and even 240 min of chase roughly 25% of the initial amount was still associated with the chaperone. In contrast, BACEs1 (54% of labeled protein released from BiP in 30 min, compare lanes 1 and 2 in Fig. 3.4B), BACEs2 (75% release in 30 min, Fig. 3.4C) and BACEs4 (85% release in 30 min, Fig. 3.4D) were rapidly and efficiently released from this luminal chaperone. Since BiP associates with hydrophobic regions in the polypeptide backbone (Blond-Elguindi et al., 1993; Hendershot, 2004), it is conceivable that the presence of bulky hydrophilic N-glycans reduces the room available for BiP to bind and concomitantly facilitates the recruitment of lectin chaperones (Molinari and Helenius, 2000). Also, since the presence of a single N-glycan was sufficient to substantially accelerate substrate release from BiP, it is possible that the presence of an oligosaccharide per se and/or the intervention of sugar processing enzymes (α-glucosidases) and lectins (e.g. calnexin) may sterically interfere with BiP binding and/or may contribute to BiP displacement from newly synthesized polypeptides.
Cells were solubilized in the presence of apyrase to rapidly consume cellular ATP thus preventing substrate release from BiP during processing of the samples. BiP was immunoisolated from cell lysates with the associated BACEs at the end of the indicated chase times. The proteins were separated in reducing SDS-PAGE. BACEs co-immunoisolated with BiP after a 5 min chase is considered the initial amount (100%). BACEs for BACEs1. The labeled BACEs0 immunoisolated from cell lysates as for Fig. 3.2, was separated in non-reducing gel to monitor formation of disulfide-bonded (CHAPS-soluble) aggregates (DBA). BACEs for BACEs1. BACEs for BACEs4. The CHAPS-insoluble material was solubilized in 1% SDS, was immunoisolated with the BACEs-specific antibody and was separated in reducing SDS-PAGE.

3.2.5 An inverse correlation between extent of BACEs glycosylation and formation of detergent-soluble and -insoluble aggregates

N-glycosylation determines the fate of the associated polypeptide chain in many different ways (Molinari, 2007). The highly hydrophilic N-glycans enhance solubility of intermediates of the folding process and allow entry of nascent chains into the calnexin chaperone system. Significantly, separation of the glycosylation mutants in non-reducing gels (Figs. 3.4E-3.4H) revealed that the extent of glycosylation inversely
correlated with the propensity of a given model polypeptide to form aberrant, high molecular weight disulfide-bonded aggregates (DBA).

For BACEs0, only a fraction of the labeled protein entered the running gel and was separated in few intermediates of the oxidative BACEs0 folding program (FI for Folding Intermediates, Fig. 3.4E). A substantial amount of the labeled protein progressively entered in complexes that remained at the interface between stacking and running gel when the samples were separated under non-reducing conditions (Fig. 3.4E, DBA for Disulfide-Bonded Aggregates). After a 240 min of chase, the vast majority of labeled BACEs0 was in DBA (Fig. 3.4E). Under reducing conditions, the BACEs0-containing, high molecular weight complexes were disassembled confirming that they were disulfide-bonded aggregates (Fig. 3.2A). Formation of disulfide-bonded aggregates is symptom of extensive protein misfolding and explains both the long intracellular persistence of BACEs0 (Fig. 3.2A, intracellular) as well as the low secretion efficiency (Fig. 3.2A, secreted and Figs. 3.2F-3.2G). Only a minor fraction of labeled BACEs1 and s2 was trapped in disulfide-bonded aggregates (DBA in Figs. 3.4F and 3.4G, respectively), whereas aggregates were virtually absent in the case of BACEs4 (Fig. 3.4H, DBA), consistent with the higher folding efficiency and secretion yield of the glycosylated variants of BACEs.

Solubilization of cells with CHAPS allows the separation of a soluble fraction, from which proteins are immunoisolated with specific antibodies as done for the figures shown so far, and of a detergent-insoluble fraction. CHAPS-insoluble proteinaceous aggregates were dissolved by boiling the pellet of the CHAPS solubilization in 1% SDS. Subsequent immunoisolations with the BACEs-specific antibody were performed upon sample dilution with 10 volumes of 1% Triton X-100 to visualize the fraction of labeled, extensively misfolded, insoluble BACEs. Consistent with the data shown in Figs. 3.4E-3.4H, a substantial amount of BACEs0 (20% of the synthesized protein) remained as insoluble material throughout the chase (Fig. 3.4I, lanes 1-2). A fraction of the labeled glycosylated variants BACEs1 and BACEs3 was detergent-insoluble immediately after synthesis (Fig. 3.4I, lanes 3 and 5, respectively). At longer chase times, however, the entire population of glycosylated BACEs was soluble in CHAPS (Fig. 3.4I, lanes 4 and 6).
To summarize, the extent of BACEs glycosylation directly correlates with rate and efficiency of secretion and inversely with the propensity of the model protein to form detergent-soluble and -insoluble aggregates in association with BiP.

3.2.6 Blocking substrate-release from calnexin differently affects secretion of BACEs glycosylation mutants

Most, if not all, newly synthesized glycoproteins expressed in the mammalian ER attain their native structure within the calnexin chaperone system. Substrate association with calnexin is mediated by mono-glucosylated N-glycans transiently displayed by nascent and newly synthesized polypeptides (Hammond et al., 1994). The association signal is generated by the co-ordinated action of two ER-resident glycanases, the α-glucosidase I and the α-glucosidase II. They sequentially remove two of the three glucose residues from the polypeptide-bound oligosaccharide branch A. Upon substrate release from calnexin, removal of the third glucose residue by α-glucosidase II is required to prevent immediate substrate re-association with calnexin. Calnexin interactions with glycopolypeptides are normally investigated by co-immunoprecipitation (Hammond et al., 1994). This is a reliable assay for multi-glycosylated polypeptides, but interactions of mono- or di-glycosylated polypeptides with calnexin are often lost during processing of the samples possibly generating false negatives. To circumvent this technical issue, we coupled conventional co-immunoprecipitation with a second assay in which we monitored variations in polypeptide secretion under conditions in which substrate association with calnexin progressed normally, but release was inhibited. To this end, cells expressing the five BACEs glycosylation mutants were exposed to the α-glucosidase inhibitor N-butyl-deoxynojirimycin (bDNJ) few minutes after the pulse with radioactivity. Post-translational addition of bDNJ does not affect the removal of the two outermost glucose residues, which allows entry of nascent polypeptides into the calnexin chaperone system. Rather, it substantially inhibits the glucosidase II-operated de-glucosylation of labeled glycoproteins, which is required for their efficient release from calnexin (Hebert et al., 1995). We anticipated that this treatment would substantially decrease secretion of those mutants that associate with calnexin during maturation. Cells were pulsed with radioactivity as described above and were chased for 5 min to allow BACEs association with calnexin. Incubation was prolonged to 120
min in the absence (- in Fig. 3.5) or in the presence of bDNJ to inhibit substrate release from the chaperone (+ in Fig. 3.5).

Immunooisolation of calnexin from detergent-lysates of cells expressing BACEs0 revealed transient association of several labeled polypeptides. None of them had the electrophoretic mobility of ectopically expressed BACEs0 (an arrow (X) shows the calculated mobility of BACEs0, Fig. 3.5A, lane 1). This was expected because N-glycans are required for substrate association with calnexin (Ou et al., 1993). Most of the labeled polypeptides co-precipitating with calnexin disappeared after 120 min of chase consistent with their maturation resulting in dissociation from the lectin chaperone (lane 2). Addition of 1 mM bDNJ after a 5 min chase inhibited substrate release from calnexin as shown by the significant amount of labeled polypeptides still co-precipitated with the chaperone after additional 120 min of chasing (lane 3).

Analysis of intracellular (Fig. 3.5B, lanes 1-2) and of secreted BACEs0 (Fig. 3.5B, lane 4) confirmed the disappearance of the labeled polypeptide from cells and the secretion of about 4% of the labeled protein after 120 min of chase as shown in Fig. 3.2. bDNJ-treatment did not modify the intracellular content (Fig. 3.5B, lane 3) and the amount of secreted BACEs0 (lane 5) as expected for a non-glycosylated polypeptide that does not associate with calnexin.

Similarly to the non-glycosylated variant, the mono-glycosylated BACEs1 did not co-precipitate with calnexin (Fig. 3.5C). In this case, however, lack of co-precipitation must be ascribed to a disassembly of the BACEs1:calnexin complex during processing of the samples, as explained above. Analysis of the polypeptide fate, in fact, revealed that inhibition of substrates release from calnexin caused intracellular accumulation of labeled BACEs1 (compare lane 3 vs. lane 2 in Fig. 3.5D) and a corresponding reduction of the polypeptide secretion by half from almost 30% of the total protein in untreated cells (Fig. 3.5D, lane 4 and Fig. 3.2B, lane 10) to about 15% in bDNJ treated cells after 120 min (Fig. 3.5D, lane 5). The case of BACEs1 is relevant because there is some confusion in the literature on whether the single oligosaccharide displayed on mono-glycosylated polypeptides is or is not efficiently de-glucosylated by the α-glucosidase II to generate the mono-glucosylated trimming intermediate required to associate with calnexin (Deprez et al., 2005; Totani et al., 2006; Wilkinson et al., 2006). Our data show that despite the technical difficulty to co-
precipitate BACEs1 with calnexin (Fig. 3.5C), this protein actually recruits the calnexin chaperone system where it remains trapped when de-glucosylation is inhibited (Fig. 3.5D). Thus, \( \alpha \)-glucosidase II efficiently generates mono-glucosylated trimming intermediate in vivo even in proteins displaying a single N-glycan.

The efficient action of the ER-resident \( \alpha \)-glucosidase II on mono-glycosylated polypeptides was confirmed in another cell type, namely Chinese hamster ovary cells (CHO). CHO lack a Golgi-resident endo-\( \alpha \)-D-mannosidase that de-mannosylates polypeptides released from the ER with terminal glucose on the oligosaccharide branch A (Hiraizumi et al., 1993). In these cells, only if de-glucosylation in the ER progresses normally oligosaccharides are processed by Golgi enzymes to complex structures that cannot be cleaved by EndoH (Hiraizumi et al., 1993; Lubas and Spiro, 1987; Moore and Spiro, 1990; Rothman et al., 1984). Analysis of the BACEs1 secreted from CHO cells, confirmed that this mono-glycosylated protein had attained an EndoH-resistant status. In fact, the electrophoretic mobility of the mock-treated (Fig. 3.5E, lane 1) and of the EndoH-treated polypeptide (lane 2) was the same. As a control, glycans displayed on secreted BACEs1 remained EndoH sensitive, and the labeled polypeptide showed faster electrophoretic mobility upon EndoH-treatment, (compare lanes 3 and 4 in Fig. 3.5E), when ER-glucosidase activity was inhibited by cell incubation with bDNJ. Thus, mono-glycosylated polypeptides are efficiently de-glucosylated by \( \alpha \)-glucosidases and enter in the calnexin chaperone system in vivo, even though substrate association with calnexin cannot directly be monitored by co-precipitation.
Figure 3.5 - Consequences of inhibition of substrate release from calnexin on secretion of enzymatically active BACEs

A Calnexin (with associated substrates) was immunoisolated from cell lysates after a 5 min chase (lane 1), after 120 min of chase (lane 2) or after a 5 min chase followed by 115 min chase in the presence of 1 mM bDNJ to inhibit substrate release from calnexin. B Same as A but BACEs0 was immunoisolated from the cell lysates (lanes 1-3) or from the cell culture media (lanes 4-5). C Same as A for cells expressing BACEs1. D Same as B for cells expressing BACEs1. E BACEs1 was expressed in CHO cells. Secreted BACEs1 was immunoisolated from the cell culture media and was mock-treated (lane 1) or treated with EndoH (lane 2). Lanes 3 and 4 show the same treatment for the protein expressed in CHO cells incubated during starvation, pulse and chase with bDNJ. F Same as A for cells expressing BACEs2. G Same as B for cells expressing BACEs2. H Same as A for cells expressing BACEs3. I Same as B for cells expressing BACEs3. J Same as A for cells expressing BACEs4. K Same as B for cells expressing BACEs4. L Fold-decrease in secretion upon inhibition of substrate release from calnexin (average of at least three independent experiments).
Efficient co-precipitation with calnexin was observed for di-glycosylated BACEs2 (Fig. 3.5F), showing that two N-glycans are sufficient for preserving the substrate chaperone complex during isolation. Analysis of the cell extracts and of the secreted material confirmed that inhibition of substrate release from calnexin resulted in intracellular retention (Fig. 3.5G, compare lane 3 vs. 2) and impaired secretion of BACEs2 (lane 5 vs. 4). Similarly, bDNJ treatment inhibited both release from calnexin and secretion of BACEs3 (Figs. 3.5H-3.5I) and BACEs4 (Figs. 3.5J-3.5K). Thus, analysis of the intracellular protein retention and of the secretion in the extracellular media revealed that all variants displaying at least one N-glycan entered the calnexin chaperone system. Inhibition of substrate release from calnexin variably affected secretion of the individual mutants (Fig. 3.5L, quantifications of three independent experiments).

### 3.2.7 Inhibiting substrate association with calnexin differently affects secretion of BACEs glycosylation mutants

Next, we determined for each glycosylation variant the importance of folding in the calnexin chaperone system to attain a transport-competent conformation. To this end, cells were incubated in the presence of 1 mM bDNJ during starvation, pulse and chase to preserve all N-glycans in the tri-glucosylated form that prevents substrate association with calnexin (Hammond et al., 1994). As expected, secretion of BACEs0, which is not glycosylated and does not associate with calnexin, was not affected by cell exposure to bDNJ (Fig. 3.6A). All other glycosylation mutants showed a significant reduction of secretion efficiency in cells treated with the specific α-glucosidase inhibitor (Figs. 3.6B-3.6E and quantifications of three independent experiments in Fig. 3.6F). Notably, secretion of BACEs1, which cannot be co-immunoisolated with calnexin (Fig. 3.5C), was also substantially reduced when access into the calnexin chaperone system was inhibited (Fig. 3.6B).

Some confusion does exist on this issue in the available literature (Deprez et al., 2005; Totani et al., 2006; Wilkinson et al., 2006). Our data clearly show that polypeptides displaying a single N-glycan are efficiently processed by ER α-glucosidases and attain a transport-competent conformation in association with calnexin. Surprisingly, inactivation of the calnexin cycle affects more dramatically the secretion of BACEs1 (5-fold reduction) and of BACEs2 (6.5-fold reduction) than the secretion of the more extensively glycosylated variants BACEs3 (3-fold reduction)
and BACEs4 (3.7-fold reduction) (Fig. 3.6F). These data show that the presence of 3 and 4 N-glycans has, per se, a positive effect on folding even when access to the lectin chaperone system is inhibited. By substantially increasing the hydrophilic surface, extensive glycosylation certainly reduces the propensity to enter in hydrophobic contacts with other unfolded chains that would irreversibly deviate the newly synthesized polypeptide into off pathways of the folding program.

Figure 3.6 - Consequences of inhibition of substrate association with calnexin on BACEs secretion
A Secretion of BACEs0 was compared in mock-treated vs. bDNJ-treated cells. B Same as A for BACEs1. C Same as A for BACEs2. D Same as A for BACEs3. E Same as A for BACEs4. F Fold-decrease in secretion upon inhibition of substrate association with calnexin (average of at least three independent experiments).
3.2.8 Secretion efficiency of the BACEs variants is not dependent on N-glycans position

Next, we determined whether the position of the N-glycan affected secretion efficiency and association with calnexin. To this end, the four mono-glycosylated variants (BACEs1) displaying the single N-glycan at the four possible glycosylation sites were expressed in transfected cells. Fig. 3.7 shows that secretion efficiency for all four radiolabeled BACEs1 mutants after 120 min chase was about 25% (Fig. 3.7, lanes 1,4,7,10 and Fig. 3.2). To confirm association of all the four variants with calnexin, we expressed them in the presence of the glucosidases inhibitor bDNJ. The inhibitor was added either during starvation, radiolabeling and chase to prevent association with calnexin (Fig. 3.7, lanes 2,5,8,11 and Fig. 3.6B) or after 5 min of chase to block substrates in association with the lectin chaperone (Fig. 3.7, lanes 3,6,9,12 and Fig. 3.5D). Treatment with bDNJ affected similarly the four mono-glycosylated mutants. These results show that for BACEs1 secretion efficiency and exploitation of the calnexin cycle depend on the number and not on the position of the N-linked glycan.

3.2.9 Proteasome inhibition increases secretion of active BACEs mutants

Secretion efficiency is a peculiar property of every polypeptide. The cystic fibrosis transmembrane conductance regulator (CFTR) is a paradigmatic example of a protein with very low secretion efficiency. More than 70% of the newly synthesized protein is deviated into the ER-associated degradation pathway, it is extracted from
the ER membrane and it is degraded by cytosolic proteasomes (Kopito, 1999). In the ER lumen, machineries that regulate protein folding and machineries that interrupt folding programs and deviate non-native polypeptides into the ERAD pathway compete for newly synthesized polypeptides (Molinari, 2007). This is an important aspect because several human conformational diseases are caused by missense mutations that may not affect function but rather delay proper folding to such an extent to favor the disposal of the mutant protein before attainment of the native conformation. Also, competition between polypeptide folding and degradation may substantially reduce the yield of production of recombinant proteins of industrial or clinical interest.

Secretion efficiency for the BACEs proteins analyzed in this study ranged from the 60% for the normally glycosylated polypeptide, to less than 5% for the non-glycosylated variant. The secretion deficient glycosylation mutants were retained intracellularly and/or degraded.

To assess whether productivity was improved by inhibition of ERAD, secretion of the 5 BACEs variants was compared in cells mock-treated or treated during the chase with PS-341 (Bortezomib), a specific inhibitor of the proteasome, which is used as therapeutic agent for relapsed multiple myeloma (Adams and Kauffman, 2004).

Cells were incubated for 2 hours and 30 minutes without (- in Figs. 3.8A-3.8E) or with PS-341 (+ in Figs. 3.8A-3.8E). Cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with anti-ubiquitin antibodies to confirm proteasome inhibition and accumulation of poly-ubiquitylated polypeptides (poly-ub, Figs. 3.8A-3.8E). The proteins in the extracellular media were also separated electrophoretically and transferred on PVDF membranes. These membranes were probed with the antibody to BACE to visualize the BACEs variants secreted from mock-treated cells (-) and from cells incubated with PS-341 (+).
Figure 3.8 - Consequences of proteasome inhibition on BACEs secretion
A Cells were mock-treated (-) or were incubated for 150 minutes with 9 µM PS-341 (+). At the end of the incubation, cell lysates were separated in reducing SDS-PAGE and proteins were blotted on PVDF. The membranes were decorated with antibodies to ubiquitin to check accumulation of poly-ubiquitylated chains as an indication of proteasomal inactivation (upper gel, poly-ub). The cell culture media were separated in reducing SDS-PAGE and proteins were blotted on PVDF. The membranes were decorated with the antibody to BACEs (100% is the amount of BACEs secreted from untreated cells in 150 min). The increase in secreted protein and in enzymatic activity recovered from the cell culture media is plotted.

B Same as A for BACEs1.
C Same as A for BACEs2.
D Same as A for BACEs3.
E Same as A for BACEs4.
F Cells were immunolabeled and chased for 4 hours without (-) or with (+) 50 µM MG132. The labeled BACEs variants were immunoisolated from cell culture media and separated in reducing SDS-PAGE. Numbers above the labeled bands show the percentage of protein secreted from mock-treated and from MG132-treated cells (in bold, 100% is the initial amount of intracellular BACEs, as in Fig. 3.2). Percentages of increase in secretion for individual glycosylation mutants are shown below the gel.
For BACEs0, inhibition of the proteasome enhanced secretion by a two-folds (Fig. 3.8A). For all other variants, enhancement of secretion was much more modest and ranged between the 15 and the 30% (Figs. 3.8B-3.8E, Secreted protein, quantifications are an average of three independent experiments). Importantly, for all proteins, the enhancement in polypeptide secretion resulted in a corresponding increase in the enzymatic activity recovered from the cell culture media as determined by spectrofluorometric quantification of the cleavage of a fluorogenic ALEXA-conjugated substrate peptide (Methods). The enhancement of protein secretion upon inhibition of the proteasomal activity was confirmed with another experimental approach and by using a different inhibitor of the proteasome. To this end, cells were pulsed with radioactivity for 10 min. After radioactivity washout, incubation was prolonged for 4 hr in the presence of MG132. At the end of the chase, the labeled BACEs variants secreted in the cell culture media were immunoisolated with the specific antibody and were separated in SDS-PAGE (Fig. 3.8F). Quantifications confirmed the data shown in Figs 3.8A-3.8E showing a doubling of the BACEs0 secretion and an increase of 15-40% of secretion of the glycosylated form of the polypeptide. In this case as well, the increase in secretion corresponded to an equivalent increase in the enzymatic activity recovered from the cell culture media. Hence, at least in the case of BACEs, inhibition of protein disposal resulted in an increase of the yield of enzymatically active protein indirectly showing that under normal conditions a variable fraction of BACEs glycosylation mutants is degraded before attainment of the native structure.
3.3 Discussion

We have performed a thorough analysis of the maturation in mammalian cells of a model protein as a function of the number of glycan modifications present on the polypeptide backbone. The data show that the number of glycans displayed on BACEs correlated directly with rate and efficiency of polypeptide folding and secretion (Figs. 3.2-3.4) and that one N-linked glycan was sufficient to recruit the calnexin chaperone system (Figs. 3.5-3.6). This latter finding merits a specific comment. It is well known that substrate association with calnexin is very weak (Kapoor et al., 2003). A direct and transient interaction can reliably be monitored by co-immunoprecipitation of multi-glycosylated substrates with the lectin chaperone, while for mono- and even for di-glycosylated polypeptides association might be lost during sample processing. Moreover, in isolated microsomes, the ER α-glucosidase II can only very inefficiently generate the mono-glucosylated trimming intermediate of the N-glycan processing reaction that allows association of a mono-glycosylated arrested chain with calnexin (Deprez et al., 2005). How and if mono-glycosylated polypeptides make use of the calnexin chaperone system is therefore unclear (Deprez et al., 2005; Totani et al., 2006; Wilkinson et al., 2006). By coupling conventional co-immunoprecipitation with two assays in which we monitored variations in polypeptide secretion under conditions in which substrate association with or release from calnexin were inhibited, our data convincingly show that mono-glycosylated polypeptides gain access to the calnexin system in living cells. The variants with 1 and 2 N-glycans actually suffered much more than the variants with 3 and 4 N-glycans upon inactivation of the calnexin chaperone system (Fig. 3.6). Thus, hyper-glycosylation per se enhances the protein folding process, even in the absence of lectin assistance, possibly by preventing entry in off pathways of the polypeptide’s folding program. Consistently, the number of glycans correlated inversely with the propensity of the polypeptide chain to enter in detergent-soluble or -insoluble aggregates (Fig. 3.4).

Stepwise addition of N-glycans also correlated with a progressive increase in the rate of substrate release from BiP (Fig. 3.4). This finding implies that not only chaperone selection (Molinari and Helenius, 2000), but also substrate release from specific chaperone machineries could be dictated by competition with other chaperones.
willing to act on the newly synthesized chain. In the specific case, we propose that the intervention of sugar processing enzymes (α-glucosidases) and lectins (e.g. calnexin) may contribute to BiP displacement from newly synthesized polypeptides. Finally, manipulation of the folding vs. ERAD capacity of cells expressing ectopic proteins offers interesting opportunity to enhance productivity of functional polypeptides (Fig. 3.8). For example, proteasome inhibition increased the yield of active BACEs harvested from the cell culture media. It should be noted that, even a modest increase of the fraction of a mutated protein terminating the folding program as an active entity may considerably reduce the course of loss-of-function disorders caused by premature polypeptide disposal (Aridor, 2007). A 20-30% increase in the yield of active recombinant proteins may also offer a significant economic interest for industrial production of biomolecules (Baldi et al., 2007).
3.4 Materials and Methods

Expression plasmids, antibodies, and transfections

pRK7-based plasmid expressing the soluble form of wild-type BACE was generated by PCR with appropriate primers with consequent deletion of 48 amino acids at the C-terminal. Removal of the different N-glycosylation sites was performed by site directed mutagenesis: codon AAC (asparagine coding) was substituted with codon CAA (glutamine coding). Anti-ubiquitin antibody was from DAKO, anti-human BiP from Stressgen, HRP-conjugated anti-mouse and anti-rabbit from Amersham Biosciences. HEK293 and CHO cells were grown in DMEM and MEMα, respectively, supplemented with 10% FBS. Cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer.

Radiolabeling and immunoprecipitations

Eighteen hours post-transfection, cells were starved for 15 min in Met/Cys free medium, pulsed for 10 min with 50 μCi [³⁵S]Met/Cys in 1 ml starvation medium/dish and chased for the indicated times with DMEM supplemented with 5 mM cold Met/Cys. Extracellular medium was centrifuged (10 min at 10000g) before immunooisolation of BACEs. PNS were prepared by solubilization of cells in 800 μl/dish ice-cold 2% CHAPS in Hepes buffered saline (HBS), pH6.8 containing 20 mM N-ethylmaleimide, protease inhibitors and 10 U/dish of apyrase for BiP immunoprecipitations. CHAPS-insoluble material was separated by 10 min centrifugation at 10000g. CHAPS-insoluble material was solubilized by boiling in 1% SDS and subsequent addition of 10 volumes of 1% Triton X-100. Immunoprecipitations were performed by adding Protein A beads ((Sigma), 1:10w/v swollen in HBS) and the selected antibody to extracellular medium or cell extracts. Incubations were 1-4 hours at 4°C. The immunoprecipitates were extensively washed, 3 times, with 0.5% CHAPS in HBS and resuspended in sample buffer for SDS-PAGE. Relevant bands were quantified by ImageQuant software (Molecular Dynamics). Gels were also exposed to BioMax (Kodak) films and scanned with an AGFA scanner.
**EndoH treatment**
Secreted BACEs1 was immunoisolated and denatured in 0.5% SDS, 40 mM DTT and incubated for 2 hr with recombinant EndoH (New England BioLabs) at 37°C.

**Immunoblotting**
Forty hours after transfection cells were grown in absence/presence of 9 µM PS-341 (kind gift of R. Sitia, Milan) for 150 min. The extracellular media were collected, separated in reducing SDS-PAGE and transferred onto PVDF membranes. Membranes were decorated with a monoclonal anti-BACEs or a polyclonal anti-ubiquitin (1:1000). Secondary antibodies were HRP-conjugated anti-mouse IgG (1:5000) and HRP-conjugated anti-rabbit IgG (1:5000). Detection and protein amount analysis were performed with the ECL-Plus detection system (Amersham GE Healthcare) and with the LAS4000 software.

**BACE activity assay**
Extracellular medium (10 µl) was directly incubated for 30 min in a reaction buffer (20 mM acetic acid pH4.5, 0.01% CHAPS, 0.01% TOP BLOCK) containing 1 µMfin ALEXA-substrate. Activity was measured in CORNING 96 well microplates using the SPECTRAmax GEMINI XPS spectrofluorometer (Molecular Devices). The increase in fluorescence upon substrate hydrolysis was measured (every 50 sec) with the SoftMax Pro software. Relative activities represent the slope of the straight line obtained by plotting the increase in fluorescence over time.
Persistent Glycoprotein Misfolding Activates the Glucosidase II/UGT1-Driven Calnexin Cycle to Delay Aggregation and Loss of Folding Competence

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Authorship

This chapter is shared work with Maurizio Molinari, Carmela Galli, Stacey M. Arnold and Randal J. Kaufman. It has been published in \textit{Molecular Cell} (Molinari et al., 2005). To avoid splitting the work into incoherent fragments, it is presented in its entirety here.

My contributions to this work are the carrying out of the investigations on persistent misfolding status of tsO45 G protein at the non-permissive temperature (Fig. 4.3A-4.3B), on its association to calnexin (Fig. 4.3F) and on expression of the protein in cells with reduced intralumenal level of BiP (Fig. 4.3I-4.3J). I was involved in preparation of the manuscript to some extent.
The UDP-glucose:glycoprotein glucosyltransferase (UGT) is a central player of glycoprotein quality control in the endoplasmic reticulum (ER). UGT re-glucosylation of non-native glycopolypeptides prevents their release from the calnexin cycle and secretion. Here we compared the fate of a glycoprotein with a reversible, temperature-dependent folding defect in cells with and without UGT1. Upon persistent misfolding, tsO45 G was slowly released from calnexin and entered a second level of retention-based ER quality control by forming BiP/GRP78-associated disulfide-bonded aggregates. This correlated with loss in the ability to correct misfolding. Deletion of UGT1 did not affect the stringency of ER quality control. Rather, it accelerated release from calnexin and transfer to the second ER quality control level, but it did so after an unexpectedly long lag, showing that cycling in the calnexin chaperone system is not frenetic as claimed by existing models and is fully activated only upon persistent glycoprotein misfolding.
4.1 Introduction

The ER is fully equipped to assist maturation of proteins destined for the extracellular space, for the cellular plasma membrane and for endo/exocytic compartments. Only native proteins are released from the ER. Folding intermediates, orphan subunits of oligomeric complexes and misfolded polypeptides are retained in the ER because they display structural features that elicit binding to ER-resident molecular chaperones. ER retention and eventual disposal of non-native polypeptides rely on a complex series of events named ER quality control whose mechanisms are better understood for N-glycosylated proteins (Ellgaard et al., 1999; Hebert et al., 2005). N-glycosylation is a frequent covalent modification of proteins expressed in the ER. It occurs by addition of pre-assembled core glycans (N-acetylglucosamine$_2$-mannose$_9$-glucose$_3$) to nascent polypeptides emerging in the ER lumen (Li et al., 1978). Rapid trimming of the 2 outermost glucose residues prepares protein-bound N-glycans for association with the ER lectins calnexin and calreticulin. The calnexin/calreticulin-associated oxidoreductase ERp57 facilitates protein folding by catalyzing formation of intra- and intermolecular disulfide bonds, a rate-limiting step in the folding process. Release from calnexin/calreticulin is followed by glucosidase II cleavage of the innermost glucose residue. Native polypeptides transit the ER but, as an essential part of the protein quality control in the secretory pathway, non-native polypeptides are tagged for re-association with calnexin/calreticulin, thus for ER retention, by re-glucosylation on N-glycans mediated by UGT (for reviews (Ellgaard et al., 1999; Helenius and Aebi, 2004; Kleizen and Braakman, 2004; Parodi, 2000; Schrag et al., 2003; Spiro, 2000; Trombetta, 2003)). The mammalian genome contains two UGT homologues, UGT1 and UGT2 (Arnold et al., 2000), but only the former displays re-glucosylating activity in vitro (Arnold et al., 2000; Arnold and Kaufman, 2003).

The tsO45 variant of the vesicular stomatitis virus (VSV) G protein is a model protein to investigate ER quality control. It is characterized by a point mutation in the ectodomain that renders its folding temperature-dependent (Gallione and Rose, 1985). At the permissive temperature (32°C), tsO45 G accomplishes efficient folding and trimerization before transport to the plasma membrane. At the non-permissive temperature (39°C), tsO45 G is folding-defective and remains trapped in the ER.
(Bergmann et al., 1981; Lafay, 1974). The folding-defect is reversible upon shift to the permissive temperature at 32°C (Balch et al., 1986).

Current models establish that glucose residues persist on misfolded glycoproteins (Suh et al., 1989) and that calnexin-dependent ER retention consists of continuous and rapid glucosidase II/UGT-operated de-/re-glucosylation reactions protracted as long as glycoproteins remain un/misfolded. The turn-over rate of the terminal glucose is between 5 to 10 minutes (Van Leeuwen and Kearse, 1997; Wada et al., 1997). Deletion of the re-glucosylation activity by knockout of the UGT gene is thus expected to cause rapid release of misfolded glycoproteins from the calnexin cycle with unpredictable consequences on ER quality control. We have generated cells that lack UGT1, the ER folding sensor for glycopolypeptides, in order to re-examine the mechanisms ensuring glycoprotein quality control and to verify the validity of existing models.

In this paper, we show that in wt cells, persistently misfolded tsO45 G protein was eventually released from calnexin (first retention-based quality control level) and entered disulfide-bonded aggregates associated with BiP/GRP78 (second retention-based quality control level). The transfer from the first to the second level of ER quality control correlated with an inability to correct the tsO45 G protein folding-defect upon shift to the permissive temperature. Deletion of UGT1 abolished re-glucosylation of misfolded tsO45 G protein, thus inactivating its cycling in the calnexin chaperone system. It did not cause, however, release of non-native G from the ER. Rather, it resulted in faster loss of folding competence upon formation of BiP-associated disulfide-bonded aggregates. UGT1 deletion did also not result in the expected rapid release of misfolded G protein from calnexin. Actually, acceleration of misfolded G protein release from calnexin in cells without UGT1 became evident only after an unexpected long lag period. Thus, the first release from calnexin that initiates the cycling of misfolded glycopolypeptides in the calnexin chaperone system requires persistent glycoprotein misfolding.
4.2 Results

4.2.1 Deletion of UGT1 prevents re-glucosylation of misfolded tsO45 G

To better define the mechanisms of protein quality control in the mammalian ER we compared the fate of a model glycoprotein with a reversible, temperature-dependent folding defect (the tsO45 G protein of VSV) in wt mouse embryonic fibroblasts (MEFs) and in MEFs lacking the ER folding sensor UGT1 (ugt1−/− MEFs, Fig. 4.1).

Figure 4.1 - Generation and characterization of ugt1−/− mouse embryonic fibroblasts

A The mUGT1 genomic sequence is comprised of 41 exons (boxed in exaggerated size, proportionally, for visibility). Exons 34-41 encompass the catalytic domain of the protein. B The mUGT1 sequence was disrupted after Exon 13 by insertion of a secretory gene trap vector that contains an Engrailed 2 intronic splice acceptor site (En2). The translation product from the gene-targeted allele comprises a truncated mUGT1 (ending at amino acid residue 459 of 1551 total) fused to a CD4 transmembrane domain/βgal, neomycin phosphotransferase reporter fusion protein (β-geo). Arrows A-C represent primers used for amplification of wild-type (A and C) or knockout-specific (A and B) products from genomic DNA for genotyping analysis. C Representative genotyping. Primers A, C amplify only the wild-type allele to produce an 899 bp species, where primers A,B amplify only the deletion allele to produce an 1123 bp species. D Metabolically labeled wt and ugt1−/− MEFs were pulse-labeled for 30 min with 50 µCi [35S]methionine and [35S]cysteine and chased for 1 hr. Detergent lysates were supplemented with a UGT1-specific antibody to isolate labeled UGT1. ugt1−/− cells do not synthesize UGT1. M is a labeled protein marker for SDS-PAGE.
Of the two UGT gene products in mammalian cells, only UGT1 displays re-glucosylating activity in vitro (Arnold et al., 2000; Arnold and Kaufman, 2003). A gene trap vector was previously used to obtain murine embryonic stem cells with targeted deletion of UGT1 (Mitchell et al., 2001) (Figs. 4.1A-4.1C). Although most homozygous ugt1<sup>−/−</sup> mice were embryonic lethal at E13, the phenotype was variable with some mice surviving until birth. MEFs derived from viable ugt1<sup>−/−</sup> embryos propagated well, did not exhibit any significant morphological change, but did not express UGT1 protein (data not shown and Fig. 4.1D).

We first verified that UGT1-deletion prevented re-glucosylation of misfolded tsO45 G protein. To this end, wt and ugt1<sup>−/−</sup> MEFs infected with VSV were pulse-labeled for 10 min and chased for 2 hr at 10°C. At 10°C, tsO45 G protein folding proceeds to the native structure, the protein is de-glucosylated and released from calnexin (Mezzacasa and Helenius, 2002), but it remains in the ER because release of cargo-loaded vesicles from ER exit sites is inhibited at this temperature (Saraste and Kuismanen, 1984; Tartakoff, 1986). One plate was lysed at the end of the chase at 10°C to preserve de-glucosylated N-glycans on G protein (lane 1 for wt and lane 5 for ugt1<sup>−/−</sup> MEFs in Fig. 4.2B). Upon shift to the non-permissive temperature, the tsO45 G protein rapidly misfolds and it is rapidly re-glucosylated (Mezzacasa and Helenius, 2002). To compare G protein re-glucosylation in wt and ugt1<sup>−/−</sup> MEFs the other plates were therefore further incubated for 10, 30 or 60 min at 39°C. The G protein was immunoisolated from cell lysates and separated in reducing SDS polyacrylamide gels (SDS-PAGE). The shift from 10° to 39°C caused rapid re-glucosylation of misfolded G protein only in wt MEFs. Accordingly, the apparent molecular weight (MW) of the G protein expressed in wt MEFs slightly increased between the 0 and the 10-30-60 min chase at 39°C (labeled G protein migrates above the thin line in Fig. 4.2B, lanes 2-4 and Fig. 4.2C, lanes 2-4). In ugt1<sup>−/−</sup> cells, the MW of the G protein did not change (lanes 5-8 in Figs. 4.2B-4.2C). The increase in the G protein MW caused by protein re-glucosylation in wt MEFs became more evident (compare lane 9 with lanes 10-12) upon treatment with jack bean α-mannosidase (JBαM). This exoglycosidase only removes terminal mannose residues from N-linked oligosaccharides (Hammond et al., 1994) and Fig. 4.2A), thus exaggerating MW differences between non-glucosylated (Fig. 4.2B, lane 9 (wt MEFs) and lanes 13-16 (ugt1<sup>−/−</sup> MEFs)) and glucosylated G protein (Fig. 4.2B, lanes 10-12). The absence of re-glucosylation activity in ugt1<sup>−/−</sup> MEFs was also observed using an in vitro UGT activity assay with
misfolded thyroglobulin as a glucose acceptor (data not shown, (Arnold et al., 2000)). Therefore, deletion of UGT1 prevented re-glucosylation of misfolded tsO45 G protein, thus confirming in vitro data showing that only UGT1 displays re-glucosylating activity (Arnold et al., 2000; Arnold and Kaufman, 2003).

Figure 4.2 - UGT1 deletion prevents re-glucosylation of misfolded G protein but does not affect ER retention of misfolded tsO45 G protein

A Protein-bound N-glycans. Triangles, circles and squares represent glucose, mannose and N-acetylglucosamine residues, respectively. JBαM only removes terminal mannose residues.

B (+ cst) wt ugt1−/− wt ugt1−/− 120° 10°C 39°C 120°

C (- cst) 10°C 39°C

D wt 32°C 39°C

wt ugt1−/− 10’ 30’ 60’ 10’ 30’ 60’

E wt ugt1−/− wt ugt1−/− wt ugt1−/−

102030061020300610203006

Non-reducing Anti-VSV Reducing Reducing

Figure 4.2 - UGT1 deletion prevents re-glucosylation of misfolded G protein but does not affect ER retention of misfolded tsO45 G protein

A Protein-bound N-glycans. Triangles, circles and squares represent glucose, mannose and N-acetylglucosamine residues, respectively. JBαM only removes terminal mannose residues. B tsO45 VSV infected cells were pulse-labeled for 10 min and transferred for 2 hr at 10°C to allow G protein de-glucosylation. Only the misfolded G protein expressed in wt MEF is re-glucosylated during the chase at 39°C and changes the electrophoretic mobility, thus migrating above the dotted line that shows the position in the gel of non-glucosylated G. To exclude that lack of changes in MW are caused by an hypothetical hyperactivity of glucosidase II in ugt1−/− MEFs, castanospermine (cst) was added during the chase at 39°. C Same as B but without castanospermine during the chase. D In both cell lines the tsO45 G protein showed normal maturation at 32°C as monitored by acquisition of EndoH-resistant N-glycans (left panel). At 39°C, misfolded G protein was efficiently retained in the wt and in the ugt1−/− ER as shown by persistence of EndoH-sensitive N-glycans (right panel). E UGT1 deletion did not inhibit folding and transport of tsO45 G protein at the permissive temperature, as confirmed by the increase in the G protein MW due to N-glycans processing in the Golgi and by immunoprecipitation with a native conformation-specific monoclonal antibody (114).
4.2.2 UGT1 deletion does not cause release of misfolded tsO45 G from the ER

By re-glucosylating N-glycans of non-native glycopolypeptides prematurely released from calnexin, UGT1 prolongs their calnexin-mediated retention in the ER. We therefore next determined if UGT1-deletion caused inappropriate release from the ER of non-native tsO45 G. To this end, we compared efficiency of ER retention for the misfolded tsO45 G protein expressed at the non-permissive temperature in wt and ugt1−/− MEFs. Four hours after infection with VSV, cells were metabolically labeled with [35S]methionine and cysteine and were chased in non-radioactive media for up to 60 min at 32°C (Fig. 4.2D, left panel) or at 39°C (right panel). The tsO45 G protein was immunoisolated from cell lysates and incubated with endoglycosidase H (EndoH). EndoH cleaves N-glycans of ER-localized glycopolypeptides, but fails to cleave complex glycans generated by Golgi enzymes after release of the native protein from the ER (Rothman et al., 1984).

After a 10 min chase at 32°C, the VSV G protein is still undergoing folding in the ER (Hammond and Helenius, 1994). Consistently, and in both wt and ugt1−/− MEFs, EndoH efficiently cleaved the G protein’s N-glycans, thus increasing G protein mobility in SDS-PAGE (Fig. 4.2D, 32°C, 10 min). After 30 and 60 min of chase at 32°C, N-glycans eventually became EndoH-resistant, in both cell lines, confirming export of the native G protein from both the wt and the UGT1-deleted ER (Fig. 4.2D, 32°C, 30 and 60 min). Appropriate maturation of the tsO45 G protein was also confirmed by acquisition, in both cell lines, of the native B2 epitope recognized by the I14 monoclonal antibody (Fig. 4.2E). At the non-permissive temperature, the tsO45 G protein was efficiently retained in the ER of wt and ugt1−/− MEFs as shown by the persistence of EndoH-sensitive N-glycans (Fig. 4.2D, 39°C). Thus, UGT1-deletion abolished re-glucosylation of misfolded G but did not compromise its ER retention.

4.2.3 Two phases in the retention-based ER quality control

It was previously reported that non-native glycoproteins are subjected to rapid de-/re-glucosylations, with the turn-over rate of the terminal glucose in the range of 5-10 min (Van Leeuwen and Kearse, 1997; Wada et al., 1997), thus implying that a frenetic substrate cycling of immature and/or folding-defective polypeptides in the calnexin/calreticulin chaperone system exists to facilitate acquisition of native structure and/or ER retention of non-native glycopolypeptides. Based on these previously published data, we hypothesized that rapid release of misfolded tsO45 G
protein from calnexin should occur in cells lacking the re-glucosylation activity by UGT1. As UGT1 deletion does not affect tightness of ER quality control, we wondered how these rapidly-released misfolded glycopolypeptides were retained in the ER and if in ugt1−/− MEFs an alternative ER retention mechanism was activated de novo or if a pre-existing one was exploited.

4.2.4 Progressive conversion of misfolded tsO45 G into covalent aggregates

To analyze quality control, we first characterized the sequence of events upon expression of persistently misfolded tsO45 G protein in wt MEFs. After metabolic labeling, cells were chased for up to 120 min at 39ºC (Figs. 4.3A-4.3H). The labeled misfolded G protein was present in two species that were clearly distinguished in non-reducing gels (Fig. 4.3A). The first species entered the non-reducing gels (MG for Misfolded G in Fig. 4.3A) and had faster electrophoretic mobility than reduced G protein (RG in Fig. 4.3A). MG therefore consisted of a heterogeneous population of partially and fully oxidized non-native G protein. The second species remained at the top of the nonreducing gel and entered the gel only upon reduction showing that it consisted of intermolecular disulfide-bonded G protein (DBA for disulfide-bonded aggregates in Fig. 4.3A). After a 10 min chase at the non-permissive temperature most of the labeled G protein was in the MG form. MG progressively disappeared with time (Fig. 4.3A, Non-reducing). Notably, the total amount of labeled G did not decrease (Fig. 4.3A, Reducing). Thus, MG was not degraded. Rather, it was converted into DBA, the disulfide-bonded form of misfolded tsO45 G whose amount consistently increased during the chase (Fig. 4.3A).

In ugt1−/− MEFs the conversion of MG into DBA occurred initially slightly faster than in wt MEFs (Figs. 4.3B-4.3C). After the 60 min chase, the fraction of MG over the total amount of labeled G protein was still 60% in wt MEFs vs. 50% in ugt1−/− MEFs (Fig. 4.3C). In the following hour of chase, a significant fraction of labeled G protein remained protected from covalent aggregation in wt cells (Figs. 4.3A and 4.3C), whereas in cells lacking UGT1 the conversion of labeled G protein into covalent aggregates was virtually complete (Figs. 4.3B-4.3C). Although there was some small variation in the percentages among individual experiments, all experiments were characterized by this abrupt conversion of MG into DBA when incubation at the non-permissive temperature was protracted beyond 1 hr in ugt1−/− cells. UGT1 activity therefore delayed entry of misfolded G protein in covalent aggregates.
4.2.5 Association with calnexin protects misfolded tsO45 G protein from covalent aggregation

Analysis of chaperone interaction revealed that only the MG species (and not the DBA) was associated with calnexin in cells with and without UGT1 (Figs. 4.3D-4.3E). In wt MEFs, calnexin slowly released MG (Figs. 4.3D and 4.3F). In ugt1−/− MEFs, release of MG occurred with similar kinetics for about 1 hr, but accelerated then significantly between the 60 and the 120 min chase compared to wt MEFs (Figs. 4.3E and 4.3F). In both cell lines, the kinetics of MG release from calnexin (Fig. 4.3F) correlated with the kinetics of conversion of MG into DBA (Fig. 4.3C). Thus, association with calnexin protected misfolded G from premature formation of covalent aggregates. Significantly, consequences of UGT1-deletion only emerged clearly when misfolding of the G protein was protracted beyond the 60 min chase (Figs. 4.3C and 4.3F). This showed that UGT1 deletion only affected the fate of persistently misfolded G, hence that cycling of misfolded G in the calnexin chaperone system was fully activated only if misfolding persisted for more than 60 min.

4.2.6 BiP associates with disulfide-bonded tsO45 G protein

In both cell lines, BiP was only associated with the more extensively misfolded, disulfide-bonded form of non-native G (DBA in the non-reducing gels, Figs. 4.3G-4.3H). The timing of calnexin and BiP interactions was also different. Where calnexin associated earlier and slowly released the MG form (Figs. 4.3D-4.3F), BiP association increased with time (Figs. 4.3G-4.3H) consistent with the time-dependent worsening of the G protein misfolded state (Figs. 4.3A-4.3C).
Figure 4.3 - Progression of tsO45 G misfolding correlates with transfer from calnexin to BiP and formation of disulfide-bonded aggregates

A The status of G protein retained for increasing chase times in the ER of wt MEFs at 39°C was analyzed in non-reducing and reducing gels (all experiments in this figure were performed at 39°C). MG is misfolded, oxidized G protein separated in the non-reducing gel. DBA is more extensively misfolded G protein in disulfide-bonded aggregates. Upon reduction, MG and DBA migrate as a single polypeptide species (RG for reduced G). B Same as A for ugt1−/− MEFs. C Quantification of the kinetics of MG conversion into DBA in wt and ugt1−/− MEFs. For each time point at least three independent experiments were analyzed, error bars represent standard deviations. D Association of labeled G protein in the MG form with calnexin in wt MEFs. E Same as D for ugt1−/− MEFs. F Kinetics of MG release from calnexin in cells with and without UGT1, error bars represent standard deviations. G Same as D for BiP. H Same as E for BiP. I Reduction of the intralumenal level of BiP has been determined by immunoblot of 3 μg of wt and A6B total cell extracts. The PVDF membrane was decorated with antibodies to BiP (upper panel) and to calnexin, as a loading control (LC, lower panel). J wt and A6B cells were infected with tsO45 VSV, pulse-labeled for 10 min at 39°C and chased for 10, 30, 60 or 120 min at 39°C. As shown in the wt and in the ugt1−/− MEFs, also in these cell lines MG is progressively converted in DBA during the chase. The tsO45 G protein maintained EndoH-sensitive glycans in both cell lines during the 120 min of chase at the non-permissive temperature.
4.2.7 BiP down-regulation does not cause release of misfolded tsO45 G from the ER

Our findings support the idea that there is a first phase of ER retention of misfolded tsO45 G protein in association with calnexin and a second phase that consists of formation of high molecular weight complexes containing disulfide-bonded G in association with the abundant molecular chaperone BiP. It is known that aggregates formation and association with ER resident chaperones contribute to the efficient retention of non-native polypeptides in the ER (reviewed in (Ellgaard et al., 1999)).

We next determined if BiP down-regulation resulted in release of misfolded tsO45 G protein from the ER.

BiP is a master regulator of ER function. It maintains the permeability barrier of the ER during protein translocation, it is involved in protein folding and assembly, it targets misfolded proteins for retrograde translocation into the cytosol, it contributes to storage of calcium, and senses conditions of stress thereby activating the mammalian unfolded protein response (for a recent review (Hendershot, 2004)). BiP down-regulation is challenging and our attempts to transiently down-regulate BiP expression in HeLa, in HEK293 and in MEFs using RNA interference were unsuccessful. We therefore made use of a Chinese hamster ovary (CHO) cell line (A6B) with reduced BiP content (20-30% compared to the wt level (Fig. 4.3I)) obtained by stable transfection of a BiP antisense mRNA expression vector (Dorner et al., 1988). Although cells with a reduced content in BiP are very unstable because of the many critical roles of BiP in the ER, our experiments clearly showed that retention of misfolded tsO45 G protein was unaffected. In fact, after 120 min incubation at the non-permissive temperature both in the wt CHO and in the A6B CHO cells the labeled tsO45 G protein had maintained EndoH-sensitive N-glycans (Fig. 4.3J). Evidently, the residual level of BiP in the “antisense” cells was enough to retain the misfolded G protein maintaining tightness of ER quality control. Alternatively, covalent aggregation of misfolded G protein contributed, with the association of the ER-retained chaperone BiP, in ER retention of the misfolded G protein.

4.2.8 UGT1 deletion affects thermoreversibility of the tsO45 G folding-defect

The folding-defect of tsO45 G protein is thermoreversible. We next determined how persistence of the misfolded state affected the capacity of misfolded tsO45 G protein
to re-acquire a transport-competent structure. VSV-infected cells were therefore metabolically labeled and chased for 10, 30, 60 or 120 min at 39°C before shift to 32°C. We reasoned that consequences of UGT1 deletion on thermoreversibility of the misfolding could be monitored by taking advantage of the fact that during the chase at the permissive temperature, the fraction of G protein that had maintained folding-competence in cells with and without UGT1 will exit the ER and will acquire EndoH-resistant N-glycans.

Figure 4.4 - Prolonged folding competence of the tsO45 G protein requires UGT1

A VSV infected wt and ugt1−/− MEFs were kept for increasing times at the non-permissive temperature and then transferred to 32°C to allow maturation of the folding-competent fraction of tsO45 G protein (as measured by the presence of EndoH-resistant N-glycans after 30 min of chase at the permissive temperature). B Quantification of the fraction of G protein with EndoH-resistant N-glycans upon shift to the permissive temperature. C Recovery of G protein from misfolding upon 120 min incubation at the non-permissive temperature and 0, 30, 60 min, respectively, at the permissive temperature.

In wt cells incubation for 10 min at 39°C did not significantly reduce the refolding-competence of misfolded G protein because most of it acquired EndoH-resistant N-glycans upon return to the permissive temperature (Fig. 4.4A, lane 2). Longer exposures at 39°C gradually decreased the re-folding ability as shown by a gradual decrease in the fraction of G protein harboring EndoH-resistant N-glycans (Figs. 4.4A, lanes 4, 6 and 8 and 4.4B). Longer incubation at 32°C only slightly increased the fraction of G protein acquiring a transport-competent architecture (Fig. 4.4C).
In UGT1-deleted cells, the ability to refold the G protein was more rapidly lost (Figs. 4.4A-4.4C) and there was a direct correlation between the abrupt acceleration of exclusion from the calnexin cycle (Fig. 4.3F), the abrupt acceleration of MG to DBA conversion (Fig. 4.3C) and the premature loss of re-folding competence (Fig. 4.4B). Consistent with an important role of calnexin in protecting associated glycopolypeptides from terminal misfolding and/or premature disposal ((Cabral et al., 2002; Molinari et al., 2002) and references therein), the formation of covalent aggregates signaling the loss of G protein’s re-folding capacity was substantially accelerated upon inhibition of the substrate association with calnexin obtained upon cell exposure to castanospermine, a specific ER α-glucosidases inhibitor. Under these experimental conditions, the newly synthesized tsO45 G protein expressed at the non-permissive temperature rapidly entered disulfide-bonded complexes (Fig. 4.5A) that, similarly to those formed in untreated cells after release from calnexin, were associated with BiP (Fig. 4.5B).

**Figure 4.5 - Cell exposure to castanospermine accelerates formation of disulfide-bonded aggregates containing terminally misfolded G protein at the non-permissive temperature**

A. Inhibition of calnexin-binding accelerates formation of tsO45 G protein-containing disulfide-bonded aggregates. Note that at the permissive temperature (32°C, in bold) treatment with castanospermine does not cause formation of G protein containing aggregates.

B. Upon castanospermine-treatment disulfide-bonded aggregates containing the tsO45 G protein are associated with BiP. Note that a significant fraction of the MG form of misfolded tsO45 G is also associated with BiP when association with calnexin is inhibited.
4.3 Discussion

Analysis of a temperature-dependent protein-folding defect in *wt* cells and in cells lacking UGT1, the ER folding sensor for newly synthesized glycopolypeptides, revealed several important principles of protein quality control in the mammalian ER. Although two homologues of UGT exist in the mammalian genome, UGT1 deletion was sufficient to abolish re-glucosylation of misfolded tsO45 G *in vivo*. Since re-glucosylation is instrumental to prevent release of misfolded glycopolypeptides from the calnexin cycle and the terminal glucose is thought to rapidly turn over (t₁/₂=5-10 min, (Van Leeuwen and Kearse, 1997; Wada et al., 1997)), a fast exclusion of the misfolded G protein from the calnexin cycle was expected in cells lacking UGT1 with unpredictable consequences on quality control. Instead, the phenotype of UGT1-deleted cells was surprisingly mild. Firstly, UGT1 activity proved dispensable for ER retention of folding-incompetent glycopolypeptides and secondly, UGT1-deletion did not result in rapid release of our model folding-defective glycopolypeptide from the calnexin cycle.

ER retention of non-native glycopolypeptides was maintained even upon UGT1-deletion because, as we show in this study, calnexin is only involved in a first phase of retention-based ER quality control. In *wt* cells, the terminally misfolded tsO45 G was in fact eventually released from the calnexin cycle to sequentially enter a second phase of retention-based quality control consisting in the formation of disulfide-bonded aggregates associated with BiP and characterized by complete loss of refolding capacity. UGT1-deletion accelerated the transfer of folding-defective G protein from the first (calnexin-mediated), to the second (covalent aggregation-mediated and BiP-mediated) retention-based quality control level. As it has been recently shown that UGT1 only re-glucosylates nearly-native folding intermediates (Caramelo et al., 2004), it is possible that while cycling off/on calnexin, misfolded G eventually acquires a terminally misfolded architecture that cannot elicit UGT1 activity and thus escapes the cycle. Alternatively or concomitantly, during the off phase, the misfolded glycopolypeptides eventually become substrates of ER-resident mannosidases and lectins that actively extract them from the calnexin cycle and deviate them in the disposal pathway leading to dislocation into the cytosol for proteasome-mediated degradation (Hebert et al., 2005). Both scenarios would
represent a safety-valve to prevent futile cycling of folding-incompetent polypeptides in the calnexin chaperone system.

Somewhat at odds with existing models predicting frenetic cycling of misfolded glycopolypeptides in the calnexin chaperone system, significant differences between wt and ugt1<sup>−/−</sup> MEFs in the release of misfolded tsO45 G from calnexin only emerged upon persistent G protein misfolding. Substrate cycling in the calnexin chaperone system is elicited after a first release from calnexin followed by trimming of the terminal glucose by the glucosidase II. Only then UGT1 activity becomes essential to drive the re-association of the non-native polypeptide with calnexin (Fig. 4.6).

![Figure 4.6 - Retention-based quality control in the ER.](image)

Glucose trimming by ER α-glucosidase I and II starts co-translationally and promotes association of nascent chains with calnexin (Cnx) and calreticulin (Chen et al., 1995) (step 1). Misfolded VSV G protein is eventually released from Cnx in both wt and ugt1<sup>−/−</sup> MEFs (step 2). Release from calnexin exposes the terminal glucose residue to glucosidase II cleavage (step 3). For about 60 min, no significant acceleration of tsO45 G protein release from calnexin was observed in cells lacking UGT1, thus, step 2 and/or step 3 must be slow processes. Cycling in the calnexin chaperone system initiates only upon UGT1 mediated re-glucosylation (step 4) and re-association with calnexin for additional folding attempts (step 5). This happens only upon persistent glycoprotein misfolding. Misfolded G protein eventually acquires an extensively unstructured architecture that is not recognized by UGT1 (Caramelo et al., 2004) and exits the calnexin cycle (step 6). At least for some folding-defective glycopolypeptides, extraction from the calnexin cycle may require the intervention of ER α-mannosidase(s) and/or EDEM variants (Hosokawa et al., 2001; Mast et al., 2005; Olivari et al., 2005).

The finding reported here that for about 60 min the re-glucosylation activity of UGT1 was dispensable for the calnexin-mediated phase of ER retention shows that the first release of misfolded G from calnexin occurs only upon persistent misfolding and that
cycling of misfolded glycopolypeptides in the calnexin chaperone system is activated very late as if repeated releases from dynamic constrictions caused by calnexin binding would only be exploited as a very last attempt to eventually fold defective polypeptides (Fig. 4.6).

It is interesting to note that during productive protein folding, BiP normally associates prior to calnexin (Hammond and Helenius, 1994; Molinari and Helenius, 2000). The factors that determine the hierarchy of chaperone interventions during productive versus non-productive folding pathways still remain to be established. Calnexin seems to be invariably involved in assisting productive protein folding, maintaining (re)folding capacity (this work), and in protecting folding-defective glycopolypeptides from premature degradation. Conversely, BiP associates with and promotes folding of newly synthesized polypeptides entering the secretory pathway at the level of the Sec61 translocon, but it becomes a marker for terminal misfolding when it captures non-native polypeptides released from calnexin ((Cabral et al., 2002; Molinari et al., 2002) and Fig. 4.6). The existence of chaperone-complexes in the ER has been demonstrated ((Meunier et al., 2002) and references therein). Enclosure of BiP in distinct functional complexes with specific ER oxidoreductases and/or specific DnaJ homologues may regulate its involvement in one or the other pathway determining the fate of polypeptides expressed in the ER.
4.4 Materials and Methods

Derivation of ugt1−/− MEFs

The murine embryonic stem (ES) cell line that harbors a disruption in the mUGT1 gene was generated by Bay Genomics (Mitchell et al., 2001). The mUGT1 genomic sequence was disrupted by insertion of a secretory gene trap vector, pGT0TMpfs, containing a fusion of the Type II transmembrane domain of CD4 to the amino terminus of a βgal, neomycin phosphotransferase reporter (β-geo). Flanking vector components directed C-terminal fusion of β-geo to Exon 13 of mUGT1 (upstream Engrailed 2 splice acceptor, En2) and truncation of the translation product before Exon 14 (downstream SV40 polyadenylation signal). The resultant ES cell line, RST539, was identified, following an initial selection with G418, by a screen for perinuclear βgal expression. Standard culture and blastocyst injection techniques were used to produce chimeric mice (Joyner, 1993). Heterozygous germ line-targeted SV129/J mice were crossed with C57Bl/6 mice. MEFs were prepared from F2 13 day embryos as described (Hogan et al., 1994).

Genotyping was performed with the following primer sequences (letters correspond to those used in Fig. 4.1B): (A) ACA CAT GTG AGA GGT CGG TTG G, (B) GGC TTC ACT GAG TCT CTG GCA TCT, and (C) GTC ACA GAA TGG CGG CTA CT. Primers A and C were mUGT1 sequence-specific, while B was specific to the pGT0TMpfs gene trap vector. Use of a short extension time in the PCR cycling prevented the appearance of an 11,940 bp product (primers A and C) from sequence containing the gene trap vector insertion.

Cells infection, metabolic labeling, preparation of cell extracts, immunoprecipitation

tsO45 VSV was propagated and used as in (Gallione and Rose, 1985). MEFs were incubated for 30 min in methionine/cysteine-free medium, pulse labeled for 10 min with [35S]Met/Cys in 1 ml Met/Cys-free medium/dish and chased for the times indicated in the figures with DMEM supplemented with 5 mM unlabelled Met/Cys. Postnuclear supernatants were prepared by solubilization of cells in 800 µl/dish ice-cold 2% CHAPS in Hapes buffer saline (HBS), pH6.8 containing 20 mM ice-cold N-ethylmaleimide, protease inhibitors and 10 U/dish of apyrase for BiP immunoprecipitations. Postnuclear supernatants were prepared by 10 min
centrifugation at 10000g and analyzed by reducing/nonreducing SDS-PAGE. Immunoprecipitations were performed by adding protein A beads ((Sigma), 1:10 w/v swollen in HBS) and the selected antibody to the cell extracts. Anti-BiP was from Stressgen; anti-Calnexin, anti-UGT, anti-VSV were kind gifts of A. Helenius, A. Parodi and J.C. Perriard, respectively. Incubations were 1-4 hr at 4ºC. The immunoprecipitates were extensively washed 3 times with 0.5% CHAPS in HBS and resuspended in sample buffer for SDS-PAGE. Relevant bands were quantified by ImageQuant software (Molecular Dynamics). At least three independent experiments were analyzed. For N-glycan removal, immunoisolated G protein was incubated for 1 hr at 37ºC with 1 mU of EndoH (Roche Molecular Biochemicals). JBαM-treatment was as described in (Molinari et al., 2003).

**Jack bean α-mannosidase treatment**

tsO45 G protein was immunoisolated and washed as described above. Incubation with jack bean α-mannosidase was carried out over night in 50 mM sodium citrate (pH4.5).
Concluding Remarks and Perspectives

Glycoprotein folding and retention-based quality control

About 90% of the proteins that are translocated into the ER are likely to be N-glycosylated (Apweiler et al., 1999). As shown in this thesis with the systematic study of BACEs glycosylation mutants, N-glycosylation has clear positive effects on protein maturation. N-glycans increase solubility of nascent chains, inhibit aggregation events, recruit a dedicated chaperone system and are likely to enhance, *per se*, folding and secretion of enzymatically active BACEs mutants. In fact, secretion efficiency of BACEs variants decreases if access to the calnexin system is inhibited, but polypeptides displaying 3 and 4 N-glycans suffer less than polypeptides displaying only 1 or 2 N-glycans if association with calnexin is inhibited. The calnexin and the BiP chaperone systems act sequentially to assist polypeptide folding, but also to insure ER-retention of misfolded polypeptides. For polypeptide folding, calnexin associates with nascent chains only if these display N-glycans close to their N-terminus. Otherwise, nascent chains bind first to BiP and are transferred into the lectin chaperone system only subsequently, to complete the maturation program (Molinari and Helenius, 2000). If folding fails, terminally misfolded polypeptides are handed off from the calnexin into the BiP chaperone system and are eventually degraded (Molinari et al., 2002).

Persistent misfolding of glycoproteins activates substrate cycling in the calnexin system initiated by the α-glucosidase II that removes the innermost glucose residue displayed on N-glycans of glycopolypeptides released from calnexin. The folding sensor UGT1 only re-glucosylates pseudo-native glycoproteins (Caramelo et al., 2003). Both native glycoproteins and terminally misfolded polypeptides are not re-glucosylated by UGT1. For the former, this allows transport through the secretory pathway at the final intra- or extra-cellular destination; for the latter, this initiates a series of events eventually concluded with the translocation of the terminally misfolded polypeptide in the cytosol for proteasomal degradation.
In *Arabidopsis thaliana*, mutation of the gene encoding the UGT1-homolog allows a structurally imperfect but functional brassinosteroid receptor mutant to reach the plasma membrane (Jin et al., 2007). In mammalian cells, the calnexin chaperone system and the BiP chaperone system act sequentially to efficiently retain misfolded conformers. As a consequence, deletion of UGT1 does not affect the stringency of ER retention for a misfolded polypeptide (the tsO45 G protein). It only accelerates entry of misfolded cargo in the BiP chaperone system (Molinari et al., 2005) and their degradation (unpublished data).

Retention-based ER quality control inhibits secretion of misfolded conformers, independent on the function of the aberrant polypeptide. It may therefore happen that functional polypeptide chains are not transported at their site of activity because they display structural defects that elicit chaperone association. Paradoxically therefore, the tightly regulated ER quality control operating in mammalian cells might be a disadvantage in conformational diseases, inhibiting ER export of biologically active mutant proteins that carry minimal structural perturbations.

**The competition between conformational maturation and ERAD**

Cargo proteins that acquire the native conformation are quickly released from the ER. Therefore, the majority of the newly synthesized cargo present in the ER lumen is non-native, thus accessible to the ERAD machinery that operates in the ER lumen to interrupt futile folding attempts. Especially for slow folders, it is conceivable that a fraction of the newly synthesized chains becomes substrate for the ERAD machinery before the native, transport-competent structure is attained. Thus, acceleration of conformational maturation with pharmacologic or chemical chaperones (see below) or reduction of the ERAD activity may offer interesting opportunities to improve production of native proteins.

In the study of BACEs glycosylation mutants, proteasome inhibition with PS-341 or MG132 significantly improves secretion of the non-glycosylated BACEs variant, which is secreted with the slowest kinetics and the lowest efficiency under normal conditions. It has much less effect on secretion of the glycosylated forms of the same polypeptide. Possibly, attainment of the native structure for the glycosylated forms of BACEs is sufficiently fast to avoid premature destruction of folding intermediates. In fact, even cell exposure to inhibitors of α1,2-mannosidases, another class of
substances that potently delay protein disposal from the ER, did not increase the yield of glycosylated BACEs secreted from cells.

ERAD inhibition was shown to rescue a fraction of the ΔF508-cystic fibrosis transmembrane regulator (the chloride channel with the most common mutation in cystic fibrosis patients) that could reach the cell surface (Aridor and Hannan, 2002) and has proven effective, in some cases, to enhance protein production and secretion. Other approaches could rely on controlled down-regulation of proteins involved in polypeptide disposal, for example E3 ligases.

Protein maturation and quality control in the ER are highly regulated in mammalian cells. Manipulation of the relative activities of folding versus degradation capacity in the ER environment may increase cellular productivity, at least for select cargo proteins. This may offer important advantages, for example, in the production of recombinant proteins to be used in the industry and in the clinics.

**Production of recombinant proteins**

*Protein engineering.* Exploitation of the calnexin/calreticulin cycle by the influenza virus hemagglutinin was shown to substantially increase the folding efficiency by inhibiting aggregation, preventing premature oxidation and oligomerization, and by suppressing degradation of incompletely folded glycopolypeptides (Hebert et al., 1996). It is likely that several other endogenous and exogenous glycopolypeptides profit of similar advantages by making use of this glycoprotein-dedicated chaperone system. Certainly, glycosylation per se offers great advantage. The results obtained by the analysis of the BACEs glycosylation mutants reveal the direct correlation between N-glycan number and secretion efficiency. We observed a 8-fold enhancement in secretion efficiency between the non-glycosylated BACEs0 and the mono-glycosylated BACEs1 and a 12-fold enhancement between BACEs0 and BACEs4.

The importance of the glycosylation status for industrial protein production is shown in the case of chymosin ectopically expressed in the filamentous fungus *Aspergillus niger*. The yield of recombinant chymosin was doubled by improving glycosylation efficiency of a poorly used glycosylation site upon appropriate modification of the consensus sequence for N-glycosylation (van den Brink et al., 2006) and was increased about 10-times by introducing a new glycosylation site (Berka et al., 1991). Thus, addition of N-glycans can improve productivity, however, this should be tested
case by case because sequence modifications could result in structural perturbations and/or in loss of activity.

Other approaches to optimize production of recombinant proteins of industrial and clinical interest should be considered. For example, to enhance secretion of recombinant proteins in the highly secreting filamentous fungi (in particular *Aspergillus*), the highly expressed endogenous glucoamylase gene can be fused with the heterologous gene. Glucoamylase acts here as carrier molecule to optimize production of the recombinant proteins-of-interest (Gouka et al., 1997; Ward et al., 1997; Ward et al., 1995).

**Cell engineering.** The activity of metabolic and apoptotic pathways essential for cell homeostasis can be modulated to improve the production of a protein-of-interest (Fussenegger and Bailey, 1998). For example, cell lines overexpressing cyclin E were generated to overcome the requirement of growth factors, thus eliminating the need of serum and other animal-derived proteins in the cell culture, a priority for biopharmaceutical manufacturing (Zang et al., 1995). Moreover, apoptosis induced by overexpression of recombinant proteins might be contrasted with medium additives such as the antioxidant vitamin E (Ramakrishnan and Catravas, 1992) or apoptosis inhibitors (Bump et al., 1995). Arrest of the cell cycle in the G1 phase was also reported to increase productivity of hybridoma cells expressing monoclonal antibodies and was achieved with starvation or DNA synthesis inhibitors such as thymidine (al-Rubeai et al., 1992; Suzuki and Ollis, 1990).

Inducible expression systems have also been developed to regulate the expression of recombinant proteins that may be toxic (Weber and Fussenegger, 2007). Moreover, recombinase-based approaches allow precise integration of transgenes-of-interest into predefined integration sites (Wirth et al., 2007).

Target for cell engineering aiming to increase glycoproteins production could be the manipulation of glycan biosynthesis. As an example, in the fungus *Trichoderma reesii*, expression of the *Saccharomyces cerevisiae* mannosylphosphodolichol synthase significantly increased cellulases secretion up to 7-fold-higher concentration (Kruszewska et al., 1999). Northern blot revealed no increase in transcripts level,
suggesting that post-transcriptional processes are responsible for the increase in cellulases production in *T. reesii*.

Finally, a last example for cell engineering aiming at improving protein production is the reduction of protease activities, a manipulation of other degradation machineries than the proteasome. Strains of *Aspergillus awamori* with deletion of the gene encoding the major protease, aspergillopepsin A, were generated to express high amount of the protein-of-interest (Berka et al., 1990).

**Intervention on conformational diseases**

Conformational diseases (e.g. cystic fibrosis, Alzheimer’s disease, Prion disorders) are devastating human disorders associated with protein misfolding and aggregation. Most conformational diseases are caused by genetic or environmental factors that destabilize the protein involved in the pathology or impair the clearance mechanisms of misfolded aggregates.

Emerging approaches for the therapeutic treatment of conformational diseases are based on the use of the so-called chemical and pharmacological chaperones.

*Chemical chaperones* consist in low-molecular-weight compounds that enhance maturation and stabilization of folding-defective proteins by modifying the ER environment or by binding the polypeptide chains. Despite some limitation such as the low specificity for target proteins and the high dosage required, some chemical chaperones have been used in animal models of human diseases and in the clinics. As an example, the ER stress alleviating 4-phenylbutyric acid (PBA) was approved by the US Food and Drug Administration for clinical use as inducer of CFTR activity in cystic fibrosis patients (Zeitlin et al., 2002). PBA was recently shown to induce interaction of ERAD chaperones (GRP94, GRP78, GRP58) with the immature form of CFTR in ER. The finding that HSP70 and HSC70 associate with the mature form of CFTR at the cell surface shows that HSP70 family proteins could be target for therapeutic intervention (Singh et al., 2008). PBA was also shown to alleviate the disease state for α1-antitrypsin deficiency in animal models (Burrows et al., 2000) but it was unfortunately ineffective in preliminary clinical trials (Teckman, 2004).

*Pharmacological chaperones* have specific intracellular targets. They are ligands, inhibitors, agonists or antagonists that stabilize pseudo-native conformations or act
as folding accelerators by specifically binding intermediates of the folding program of mutated target polypeptides (Bernier et al., 2004; Fan, 2008).

Examples of active-site specific chaperones (e.g. enzyme inhibitors) are found in the therapeutic intervention of lysosomal storage diseases (e.g. Fabry, Gaucher, Tay-Sachs disease). These disorders are caused by mutations that impair maturation and transport of lysosomal enzymes. The deficiency in α-galactosidase A generates accumulation of undegraded glycosphingolipids in the compartment, leading to the Fabry disease. Active-site specific chaperones, like 1-deoxygalactonojirimycin (Fan et al., 1999), have been rapidly developed into clinical evaluation against this disorder. Active-site specific chaperones can be rationally designed, as structural information is generally available for enzymes active-sites.

With the same principle of intervention on conformational diseases, pharmacological chaperones could be used to investigate the molecular mechanisms of protein folding. In a follow-up study, maturation and secretion efficiency of the BACEs glycosylation mutants could be investigated with the use of the available BACE inhibitors.

*Intrabodies* are other promising therapeutic agents against conformational diseases. They are antibodies expressed intracellularly that can be directed to a specific target antigen present in various subcellular locations. They combine conformation specificity and high antigen-binding affinity. They have been used as a biotechnological tool to modulate the functions of a wide range of target antigens at the post-translational level (Lo et al., 2008). They can prevent amyloidogenic proteins aggregation and/or deviate them from the potential aggregation site to other subcellular locations (Cardinale and Biocca, 2008). As an example, the production of the Alzheimer’s disease related Aβ peptide was shown to be decreased in cultured cells with the expression of single chain intrabodies that bind to the β-secretase cleavage site of amyloid precursor protein (Paganetti et al., 2005).
The general, long-term aim of our studies is the detailed characterization of the function and regulation of the machineries ensuring protein folding, quality control and disposal in the mammalian ER. We believe that these analyses are of high importance because a correct protein biogenesis, an appropriate control of proteins transiting through the secretory pathway and the capacity to efficiently remove folding-defective chains from the protein folding compartment are essential to maintain the integrity of the protein factory and the homeostasis of cells, tissues and organs. The capacity to control and intervene in the kinetic competition of polypeptide folding versus degradation in the ER lumen will offer approaches to enhance the fraction of newly synthesized chains being secreted from cells, a valuable concept for biotechnology. It will also offer therapeutic treatments to alleviate the several human hereditary diseases characterized by unwanted degradation of intermediates of a folding program that has been slowed by mutations.
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Appendix

Curriculum Vitae

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Education

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