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Focus Quality Control

Protein quality control in the early secretory pathway

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Eukaryotic cells are able to discriminate between native and non-native polypeptides, selectively transporting the former to their final destinations. Secretory proteins are scrutinized at the endoplasmic reticulum (ER)–Golgi interface. Recent findings reveal novel features of the underlying molecular mechanisms, with several chaperone networks cooperating in assisting the maturation of complex proteins and being selectively induced to match changing synthetic demands. 'Public' and 'private' chaperones, some of which enriched in specializes subregions, operate for most or selected substrates, respectively. Moreover, sequential checkpoints are distributed along the early secretory pathway, allowing efficiency and fidelity in protein secretion.

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Introduction

As the per-capita income increases in Western societies, the quality of the products that appear in the market is becoming more important than their quantity. Depending on high quality and innovative design, industries employ abundant personnel and devices to ensure a stringent control of their products, the quality of which must fulfill strict pre-determined standards. This key activity is usually referred to as 'quality control' (QC). At the same time, the need for innovation makes it very hard to dictate a fixed set of standards and rules. Moreover, selling more (high quality) products remains a common goal of any commercial activity. Italian parents often say to their hasty offspring 'Presto e bene raro avviene' (Fast and good is a rare combination). How can our factories contradict this rather wise saying? And, of more interest for *The EMBO Journal* readership, how do cells cope with somewhat similar problems? In this essay, we analyze the mechanisms that cells employ to couple abundant synthesis and high quality for secretory proteins.

After synthesis, proteins must rapidly fold to perform their biological activities. Folding takes place in three main subcellular compartments, cytosol, endoplasmic reticulum (ER) and mitochondria. Each organelle is equipped with a specific set of chaperones and folding enzymes optimized to work in the local conditions. In all cases, the final outcome must be a native molecule devoid of errors. Moreover, structural maturation must be completed within a rather short time frame. In the crowded environment of the cell, unfolded proteins are a danger as they may aggregate and become toxic. In viable cells, extensive aggregation is prevented by several proteolytic systems that rapidly dispose of aberrant or damaged polypeptides (see Goldberg, Liberek, Haas, this issue).

A considerable fraction of the proteome consists of molecules that are destined to the extracellular space (Chen *et al*, 2005): these are either secreted by the cell or inserted in membranes, to act as ligands and receptors, respectively. Proteins destined to the extracellular space are synthesized on ER-bound ribosomes, and are cotranslationally translocated into the ER lumen where they attain their native conformation, before being transported to the Golgi and downstream compartments (Figure 1). Secreted and membrane proteins are the main devices of intercellular communications. The fidelity of ligand–receptor interactions requires that both molecules attain the very conformations that allow signals to be properly transmitted and understood. Protein folding in the secretory pathway must therefore be controlled in the tightest way.

Protein QC in the secretory compartment

In the late 1980s, work on oligomeric viral proteins (Kreis and Lodish, 1986; Boulay *et al*, 1988; Gething and Sambrook, 1989), the T-cell receptor (Bonifacino *et al*, 1989; Sancho *et al*, 1989) and immunoglobulins (Bole *et al*, 1986; Sitia *et al*, 1987; Hendershot and Kearney, 1988) revealed that assembly is a requisite for transport to the Golgi apparatus and onwards along the secretory route. Klausner (1989) referred to this phenomenon as 'Architectural editing'; the term 'ER quality control' (Hurtley and Helenius, 1989; Hurtley *et al*, 1989) eventually stuck to indicating the processes of conformation-dependent molecular sorting of secretory proteins. Until then, the lysosome was considered the site where secretory molecules are degraded. Since proteins retained in the ER cannot reach downstream lysosomes, the question arose as to how aberrant proteins are degraded (Klausner and Sitia, 1990).

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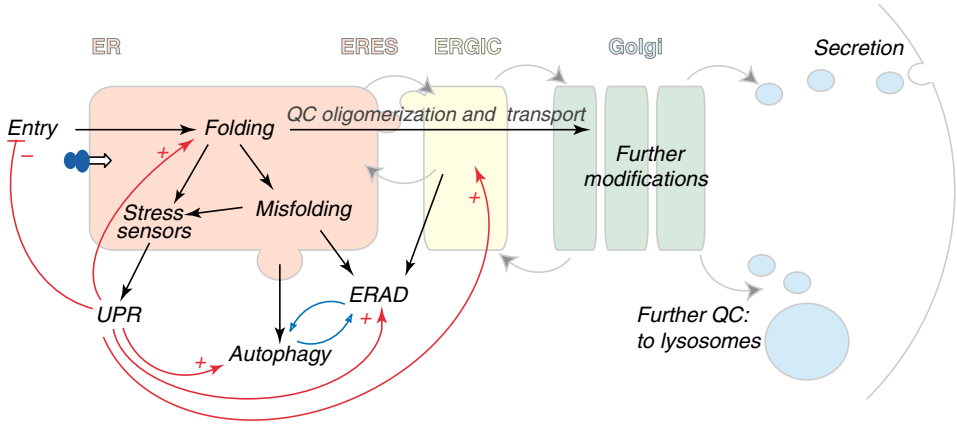

Figure 1 The early secretory pathway. Proteins destined to the extracellular space or to organelles of the secretory route are synthesized by ER-bound ribosomes and cotranslationally translocated (entry) into the ER. Here they attain their native structure (folding), under strict QC scrutiny. Only properly folded and assembled proteins can reach the Golgi, where they are further modified, to be transported to the extracellular space or to lysosomes. Gray arrows indicate the direction of vesicles moving among different compartments; dark arrows indicate the pathways followed by cargoes in the early secretory pathway; red lines show homeostatic control pathways (+ stimulatory, − inhibitory). Misfolded proteins are recognized, retained and eventually routed to degradation by ERAD or autophagy (which are likely reciprocally regulated, as indicated by the blue arrows). Some misfolded soluble ERAD substrates are transported to ERGIC or *cis*-Golgi before retrotranslocation and degradation. Too high load for the folding machinery or the accumulation of misfolded proteins activate resident ER stress sensors, which elicit the UPR. ER stress can selectively inhibit protein entry into the ER, and increase the capacity of folding and degradation (via ERAD and autophagy). The UPR induces also molecules acting downstream of the ER.

In the mid 1990s, studies on CFTR and MHC class I (Michalek *et al*, 1993; Ward *et al*, 1995; Wiertz *et al*, 1996) revealed that proteins that fail to fold or assemble are eventually retrotranslocated (or dislocated) across the ER membrane for degradation by cytosolic proteasomes. The players, mechanisms and physiopathologic implications of this process (ER-associated degradation, ERAD) remain a hot topic in molecular cell biology (Yoshida, 2007). During their lifetime, cells must integrate all the different reactions schematized in Figure 1, and adapt them to face possible changes in the quality and quantity of secretory proteins they produce during differentiation. As colocalized signals can dictate assembly, retention and degradation of membrane and soluble cargo proteins (Bonifacino *et al*, 1990; Fra *et al*, 1993), a competition between ER export and degradation can explain homeostatic control.

An interesting mathematical model has been recently introduced, which considers protein folding in the ER (ERAF, ER-assisted folding), ERAD and ER export as single biochemical parameters (see Wiseman *et al*, 2007 and references therein). Despite the limits imposed by the simplification, this approach leads to some interesting and testable predictions: export efficiency of a particular cargo protein depends on the activity of the ERAF, ERAD and export systems, which in turn are influenced by the proteome expressed by the cell. This partially simplified model could be further expanded and tested to integrate new emerging evidence. Recent data highlight a spatial subdivision of the early secretory compartment that seems particularly suited for the biogenesis of complex, multimeric proteins. Both parallel and sequential QC pathways coexist in cells, some common to all polypeptides, others specific for particularly demanding proteins. This diversity likely evolved to cope with the myriads of polypeptides that our cells produce, often in exuberant amounts.

Protein QC (Box 1) is intimately linked to the processes of folding (Ellgaard and Helenius, 2003; Sitia and Braakman, 2003). Both rely on chaperones and devoted resident enzymes. QC serves different roles: (i) it prevents the deployment of aberrant protein conformers, ensuring that only native proteins proceed along the secretory pathway; (ii) it retains precursors in an environment suitable for their maturation; (iii) it increases their local concentration to favor assembly and polymerization; (iv) it reduces the risks of proteotoxicity by inhibiting aggregation and degrading terminally misfolded proteins; (v) it maintains homeostasis in the early secretory pathway; (vi) it is involved in the developmental regulation of protein secretion (IgM, adiponectin, see below) and (vii) it is important for storing proteins for regulated secretion. In certain plants, in fact, ER retention/accumulation is utilized to store abundant proteins during seed formation (Larkins *et al*, 1993; Jolliffe *et al*, 2005; Vicente-Carbajosa and Carbonero, 2005).

Box 1 The logics of QC

1. Preventing the deployment of aberrant protein conformers
2. Retaining precursor proteins in an environment suitable for their maturation
3. Favoring correct assembly by increasing subunit concentration
4. Reducing the risks of proteotoxicity by inhibiting aggregation and degrading terminally misfolded proteins
5. Maintaining homeostasis in the early secretory pathway
6. Developmental regulation of protein secretion (IgM, adiponectin)
7. Storing proteins for regulated secretion (plants, adipocytes)

QC, quality control.

Protein folding in the ER

Upon cotranslational translocation, nascent secretory proteins enter the crowded environment of the ER lumen and soon begin folding into more stable, lower energy, conformation(s) (Dobson, 2004). While the basic principles governing folding are common to other cellular compart-

ments (Anfinsen and Scheraga, 1975; Dobson, 2004), the ER is unique in sustaining a set of covalent modifications, which include removal of the signal sequences, disulfide bond formation, *N*-glycosylation and GPI addition. A plethora of enzymes and assistants are found in the early secretory pathway, which catalyze each step (Box 2). How is their synthesis regulated so as to have the right balance in different cell types? How are they functionally interconnected? How are the different steps executed in the right order? How are unfolded proteins recognized (Box 3)?

Owing to the fact that *N*-glycosylation is unique to secretory proteins, the folding and QC of glycoproteins have been analyzed in great detail and can be used as a prototypic example of labor organization in the ER protein factory. As in

Box 3 Monitoring non-native structure

1. Exposure of hydrophobic patches
2. Presence of immature glycans
3. Exposure of reactive thiols

Box 2 Workers in the secretory protein factory (an incomplete list)

(A) 'Public' chaperones and enzymes

Class	Name	Localization	Function
Chaperones	BiP/GRP78	ER	Folding assistant/unfolding Regulation of IRE1, PERK and ATF6 in ER signaling Translocon gating and regulation
	GRP94	ER	Folding assistant
	ORP150	ER	Folding assistant, hypoxia
	HERP	ER membrane	ERAD
	SEL1L	ER membrane	ERAD
Co-chaperones	Sil1/BAP	ER	ATP exchange factor
	ERdjs	ER	BiP cofactors
Lectins	CNX	ER membrane	Folding
	CRT	ER soluble	Folding
	ERGIC-53	ERGIC	Transport F5, F8, CatZ, CatC, IgM polymers
	VIPL	ER	Transport
	VIP-36	*Cis*-Golgi	Transport
	EDEM1, 2, 3	ER subregion	ERAD
	OS9	ER membrane	ERAD
	Erlectin/XTP3-B	ER membrane	ERAD
Enzymes redox	Ero1α	ER + ERGIC	Oxidase
	Ero1β	ER	Oxidase
	PDI	ER	Oxidase, isomerase, reductase Subunit of prolyl 4-hydroxylase Subunit of microsomal triacylglycerol transfer protein
	ERp57	ER	Isomerase, oxidase?
	ERp72	ER	Unclear
	ERp44	ERGIC-*cis*-Golgi	Thiol-mediated retention/IP3R1 regulation
Proline isomerases	PPIases	ER	Proline isomerization
	Cyclophilins	ER, mitochondria, nucleus, cytosol	Proline isomerization
Sugar processing	Glucosidase I	ER	
	Glucosidase II	ER	
	ER Man I	ER	
	ER Man II	ER	
	UGGT	ER	Folding sensor
	Man IA, IB, IC	Golgi	

(B) 'Private' tissue- or substrate-specific factors

Name	Tissue expression	Substrates/function
Hsp47	Fibroblasts	Collagen biosynthesis/chaperone
PDIp	Exocrine pancreas	Zymogens/oxidoreductase
PDILT	Sertoli cells in testis	Calmegin, Δ-somatostatin/ chaperone
Egasyn	Ubiquitous	β-Glucuronidase/chaperone
Invariant chain	APC	MHC class II assembly and transport
Tapasin	Ubiquitous	MHC class I assembly
SCAP-RAP Boca/Mesd	Ubiquitous	LDL receptor assembly and transport

APC, antigen-presenting cell; CNX, calnexin; CRT, calreticulin; ER Man, ER α1,2-mannosidase; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERGIC, ER–Golgi intermediate compartment; LDL, low-density lipoprotein; MHC, major histocompatibility complex; UGGT, UDP-glucose glycoprotein glucosyltransferase.

all assembly lines, transport must follow the execution of a given step. The time allocated to the latter, however, must be precisely controlled in order to allow efficiency and prevent jams along the line. The sequential modifications of the oligosaccharides provide an elegant solution to dictate and time the manufacture of cargo glycoproteins.

N-glycosylation involves binding of a preformed oligosaccharide (Glc3Man9GlcNAc2) to asparagine side chains in the sequence NXS/T, where X is any amino acid other than proline (Khalkhall and Marshall, 1975). The sugar moieties are then progressively trimmed by resident enzymes of the secretory pathway. Soon after synthesis, glucosidases I and II sequentially remove the three glucose moieties from the A branch of the oligosaccharide moieties (Figure 2). UDP-glucose glycoprotein glucosyltransferase (UGGT) adds back a glucose residue to *N*-glycans positioned near regions of disorders (Taylor *et al*, 2004). Therefore, UGGT acts as a folding sensor and produces monoglucosylated proteins (Glc1Man9GlcNAc2) that can interact with calnexin (CNX) or calreticulin (CRT), two ER chaperones with lectin activity (Waisman *et al*, 1985; Ahluwalia *et al*, 1992; reviewed in Williams, 2006). CNX and CRT retain misfolded substrates in the ER, prevent their aggregation and promote oxidative folding via interactions with ERp57 (Ellgaard *et al*, 2001; Schrag *et al*, 2001; Frickel *et al*, 2002; Russell *et al*, 2004). By removing the terminal glucose, glucosidase II dissociates the substrate from CNX/CRT for a novel round of inspection by UGGT.

How do terminally misfolded proteins escape the cycle? Glycan processing again comes into action, because removing the terminal mannose moieties inhibits glucose re-addition. Mannose trimming hence acts as a timer, discriminating between junior proteins (which should be given the time to fold) and senior ones, which should be either secreted or sent to degradation. Many proteins with mannosidase activity reside in the early secretory apparatus (e.g., ER α1,2-mannosidase I (ER Man I), EDEM1 and 3, Golgi Man IA, IB, IC, ER Man II). Man I inhibitors (e.g., kifunensine), which prevent removal of the terminal B-branch mannose, stabilize ERAD glycoprotein substrates, but do not prevent secretion of native species. Overexpression of ER Man I and EDEMs accelerates degradation (Hosokawa *et al*, 2006 and references therein). ER–Golgi intermediate compartment-53 (ERGIC-53) (a protein transporter

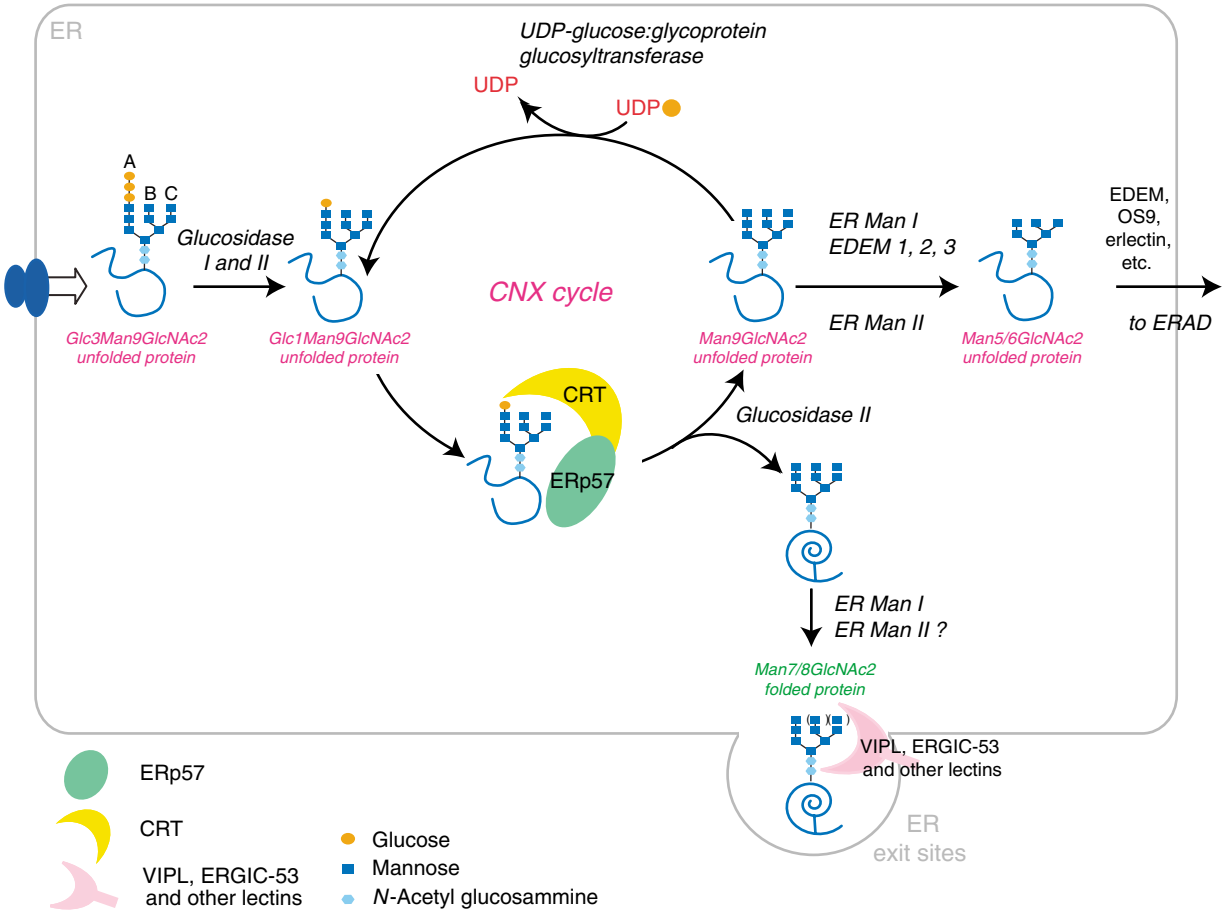

Figure 2 The CNX/CRT cycle. After transfer of the preformed core oligosaccharide (Glc3Man9GlcNAc2) onto nascent proteins, glucosidase I and II sequentially remove the two terminal glucoses from the A branch. The monoglucosylated Glc1Man9GlcNAc2 unfolded protein can now interact with the lectin chaperones CNX and CRT. In association with the oxidoreductase ERp57, CNX and CRT prevent aggregation and facilitate glycoprotein folding. Removal of the glucose by glucosidase II (Man9GlcNAc2) interrupts the interaction of the protein with CNX/CRT. If the protein has attained its native structure, it can now proceed along the secretory pathway by bulk flow or by interaction with specific lectin transporters such as ERGIC-53 or VIPL. If unfolding persists, the glycoprotein is recognized by UGGT1, which places a single glucose back onto the A branch, causing the protein to enter the CNX/CRT cycle again. Mannose trimming causes exit from the CNX/CRT cycle. Misfolded proteins can be recognized by specific lectins (EDEMs, OS9, etc) and targeted to degradation.

with lectin activity, cycling between the ER and the ERGIC, see below) and possibly other L-type lectins (e.g., VIPL, VIP36; Kamiya *et al*, 2007) bind high-mannose cargoes, facilitating their forward transport. Further mannose trimming in the ER may favor degradation, possibly also because reducing the hydrodynamic volume of substrate glycoproteins could facilitate their retrotranslocation. It will be of great interest to determine the precise binding specificities and fate of the various intermediates in glycan processing (Helenius and Aebi, 2001).

Another well-characterized folding pathway is based on Binding Protein (BiP, also called GRP78), an abundant chaperone of the hsp70 family, which serves also a key regulatory role in ER signalling (Bertolotti *et al*, 2000). BiP was first isolated as a protein associating with unassembled Ig-H chains (Haas and Wabl, 1983). It consists of an N-terminal ATPase domain and a C-terminal domain with affinity for hydrophobic patches (Flynn *et al*, 1991; Blond-Elguindi *et al*, 1993). The affinity for substrates depends on ATP binding at the N-terminal domain. When ATP is hydrolyzed to ADP, a conformational change occurs, which determines substrate release. Thus, substrates can undergo cycles of BiP binding and release, depending on ATP hydrolysis (Gething, 1999). Owing to the weak BiP ATPase activity, hsp40-like co-chaperones containing J domains (ERdj) play a key regulatory role. Five ERdj proteins have been isolated so far (Shen *et al*, 2002; Cunnea *et al*, 2003; Kroczynska *et al*, 2004; Shen and Hendershot, 2005). One of them, ERdj5, also displays oxidoreductase acitivity, possibly linking BiP-dependent folding/unfolding and disulfide bond formation, isomerization or reduction (Nagata *et al*, personal communication).

Very rarely glycoproteins are found to bind simultaneously to BiP and CNX or CRT. Therefore, it seems that a given glycoprotein enters first either the BiP or the CNX/CRT pathway (Figure 3). The initial choice is dictated by the localization of the *N*-glycans: the closer these are to the N-terminus of the nascent protein, the higher the tendency to use CNX as a chaperone system (Molinari and Helenius, 2000). If the first attempts to fold fail, the protein can shift to the alternative pathway. Altogether, these data imply that sites of conjunction exist in which the substrate can jump from one pathway to another. In principle, however, it should be possible for large multi-domain proteins to engage with both. It would be of interest to determine whether ER sub-regions exist that are enriched in either pathway.

Supplementary Table 1S shows the phenotypes of cells, mice and patients in which individual chaperones, enzymes or sensor molecules are insufficient or absent altogether. $BiP^{-/-}$, $ERp57^{-/-}$, $CNX^{-/-}$ and $CRT^{-/-}$ mice show embryonic or perinatal lethality, but their phenotypes vary considerably: $CRT^{-/-}$ mice have severe problems in cardiac development, while large myelinated fibers in peripheral nerves are the main targets in $CNX^{-/-}$ animals (Mesaeli *et al*, 1999; Denzel *et al*, 2002; Garbi *et al*, 2006). BiP is essential also for survival of cells in culture, in agreement with its role in regulating translocation and ER signalling. In contrast, $CRT^{-/-}$ and $CNX^{-/-}$ cells are viable, and their phenotypes surprisingly mild, suggesting redundancy in substrate recognition by the two chaperones.

Oxidative folding

In terms of ionic composition and redox potential, the ER is similar to the extracellular space, providing an ideal folding place/test bench for proteins destined to the external world. Although certainly important, the higher ratio between oxidized and reduced glutathione (GSSG/GSH) in the ER, compared with the cytosol (Hwang *et al*, 1992), is not enough to guarantee efficient oxidative folding. Indeed, for many

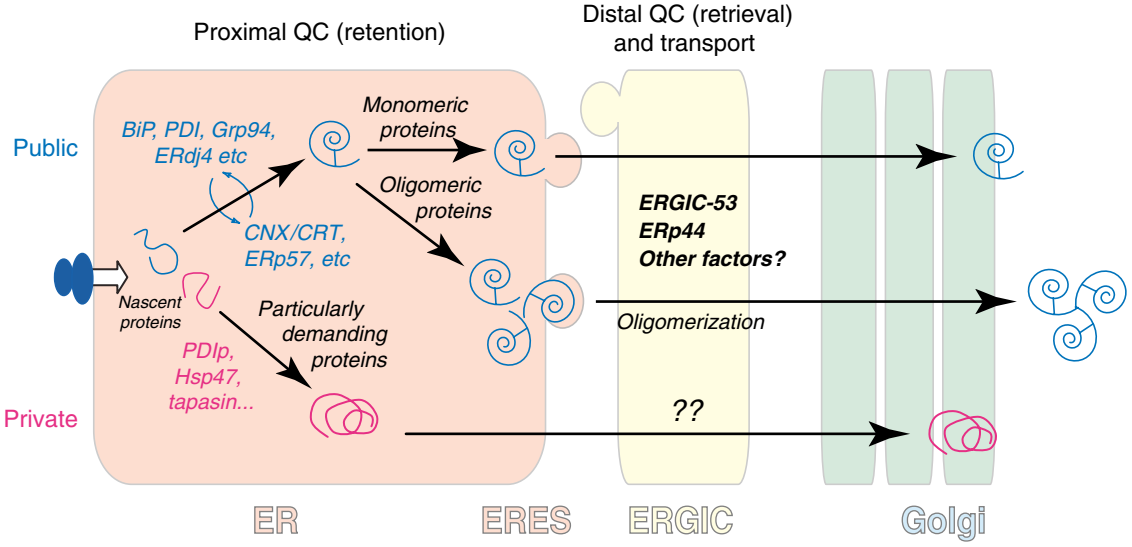

Figure 3 QC in the early secretory pathway. Chaperones and folding assistants can be grouped in different classes according to their specificity and subcellular localization. The majority of secretory proteins utilize *public* chaperones: some initially go with BiP, PDI and their partners, others enter the CNX/CRT cycle, the choice depending on the location of the *N*-glycans. Certain proteins that are produced in large amounts, or are intrinsically difficult to fold, are assisted by specific (*private*) chaperones and enzymes (see also Box 2). In addition, QC can occur in sequential steps. After a *proximal* QC, certain substrates (generally multimeric proteins) seem to undergo also *distal* QC checkpoints in ERGIC and *cis*-Golgi. This model could mediate cargo concentration and selective export of oligomerized species, thus coupling fidelity and efficiency in the secretory protein factory. While proximal QC can rely on simple retention, the distal checkpoints likely imply substrate retrieval to the ER, either for further attempts to fold, or for retrotranslocation and degradation.

proteins to fold correctly, disulfide bond isomerization, and sometimes also reduction (Jansens *et al*, 2002), is needed. A hyper-oxidizing environment in the ER lumen may hence inhibit folding of proteins with multiple disulfides, and promote aggregation (Molteni *et al*, 2004). Therefore, oxidative folding relies primarily on protein–protein interchange relays. The main pathway involves disulfide transfer from PDI or PDI-like proteins to nascent cargoes. PDI consists of four thioredoxin (trx) domains: the two lateral domains (a and a′) are endowed with oxidoreductase activity, while the two central ones, b and b′, provide a hydrophobic surface suited to bind and present nascent proteins to the active sites in a and a′. This overall structure is likely important for the redox-dependent chaperone function of PDI (Wilkinson and Gilbert, 2004; Forster *et al*, 2006a), particularly with terminally misfolded proteins, which must be reduced before dislocation to the cytosol for proteasomal degradation (Gillece *et al*, 1999; Fagioli *et al*, 2001; Tsai *et al*, 2001, 2002; Molinari *et al*, 2002).

After transferring a disulfide bond to nascent proteins, PDI is re-oxidized by members of the Ero1 flavoprotein family (Frand and Kaiser, 1998; Pollard *et al*, 1998; Cabibbo *et al*, 2000; Pagani *et al*, 2000; Mezghrani *et al*, 2001). *In vitro*, yeast Ero1p can use molecular oxygen as terminal electron acceptor, in a reaction that produces hydrogen peroxide in stoichiometric amounts to the disulfides formed (Tu and Weissman, 2002; Gross *et al*, 2006). Studies are ongoing in several laboratories to determine whether H_2O_2 is generated in living cells as a byproduct of oxidative folding, because this could serve signalling purposes. However, at least in yeast, disulfide bond formation can proceed in anaerobic conditions (Gross *et al*, 2006), suggesting that alternative electron acceptors exist.

Over the last years, many other ER-resident PDI-like oxidoreductases have been characterized in mammalian cells. The precise role(s) of these molecules, as well as the mechanisms controlling their redox state and activity, remain to be clarified (Ellgaard and Ruddock, 2005). Some of them, for example, PDIp (Desilva *et al*, 1996) and PDILT (van Lith *et al*, 2005; van Lith *et al*, 2007), are selectively expressed in pancreas and testis, respectively, and hence belong to the growing group of substrate- or tissue-specific ('private') chaperones, including Hsp47, etc. (Box 2B).

Disulfide bond formation is crucial in the folding and QC of secretory proteins. Since they increase the stability of the native conformation, their absence, or even worse, their mispairing, generally produces severely misfolded species. Furthermore, an exposed cysteine in the proper amino acid context is sufficient to cause retention and degradation of otherwise transport competent intermediates (Fra *et al*, 1993; Guenzi *et al*, 1994), likely because the reactivity of thiol groups favors the formation of mixed disulfides with PDI, ERp44 and other resident proteins (Reddy *et al*, 1996; Anelli *et al*, 2003, 2007). The thiol-dependent retention mechanisms, originally described in the developmental control of IgM (Sitia *et al*, 1990), have recently been shown to control also adiponectin secretion (Wang *et al*, 2007).

Bulk flow, retention, retrieval and selective export

Since the discovery of the KDEL motif as a means to localize soluble proteins in the ER (Munro and Pelham, 1987), the problem arose as to how these extremely abundant residents rarely saturate the KDEL receptors. A possible answer lies in the discovery of supra-molecular complexes comprising different chaperones (BiP, GRP94, ERdj3 and UGGT, but no CRT; Meunier *et al*, 2002; Gilchrist *et al*, 2006). Because of their different diffusibility, these complexes could be excluded from forward moving vesicles, and form a matrix to retain folding intermediates in a suitable environment for their maturation. The presence of UGGT in these complexes may be important for shifting misfolded substrates to the CNX–CRT pathway.

Unless retained by interactions with resident proteins, a protein could exit from the ER by bulk flow (Wieland *et al*, 1987). However, many proteins are actively transported out of the ER by interaction with specialized export machineries (see Gurkan *et al*, 2006 for a review). Export from the ER occurs at ER exit sites (ERES; Mezzacasa and Helenius, 2002), where budding of COPII-coated vesicle takes place. It is now evident that transportable cargoes contribute to the formation of ERES- and COPII-coated vesicles (Forster *et al*, 2006b). Moreover, in exocrine pancreatic cells, the ER–Golgi interface is where different secretory proteins reach their highest intracellular concentration (Martinez-Menarguez *et al*, 1999; Oprins *et al*, 2001), which could have important consequences for the biogenesis of oligomeric proteins (see below).

Specific transporter molecules mediate the exit from the ER of certain glycoproteins, concentrating them into forward transport vesicles (see Hauri *et al*, 2002; Lee *et al*, 2004 for reviews). In mammalian cells, one of the best characterized is ERGIC-53, a hexameric transmembrane lectin (Schindler *et al*, 1993) that derives its name from being particularly abundant in the ERGIC. ERGIC-53 is described to capture high-mannose glycoproteins in the ER, and release them in the ERGIC in a Ca^{2+}- and pH-dependent manner (Appenzeller-Herzog *et al*, 2004). Mutations in ERGIC-53 (also known as LMAN1) or in MCFD2, a gene encoding a small soluble protein that associates with ERGIC-53 (Zhang *et al*, 2003, 2005; Baines and Zhang, 2007), are responsible for most cases of combined deficiency of coagulation factors V and VIII (F5F8D), a recessive bleeding disorder characterized by decreased serum levels of both clotting factors (Nichols *et al*, 1998; Neerman-Arbez *et al*, 1999). The rather limited phenotype of patients who lack functional ERGIC-53 suggests that other lectins serve redundant functions in controlling glycoprotein traffic (e.g., VIP36 (Fiedler *et al*, 1994), VIPL (Neve *et al*, 2003), ERGL (Yerushalmi *et al*, 2001; see Hauri *et al*, 2002 for a review)). The specificity of these lectins has recently been analyzed (Kamiya *et al*, 2007): VIPL and VIP36 interact preferentially with glycans carrying, on their A branch, three mannoses but no terminal glucoses, (see also Fullekrug *et al*, 1999). Unexpectedly, ERGIC-53 displays low-affinity and broad-specificity interactions with high-mannose oligosaccharides also when monoglucosylated at the A branch. Moreover, while VIPL and ERGIC-53 bind better at pH 7, (as found in the ER), VIP36 has an optimum at pH 6.5 (as in the Golgi). From these data, it has been suggested that VIPL binds de-glucosylated cargoes exiting the CNX/CRT cycle, protecting them from de-mannosylation and hence degradation. The cargo is then passed to ERGIC-53, perhaps owing to its hexameric structure, and exported toward the Golgi. In the *cis*-Golgi, VIP36 may play additional roles in glycoprotein QC and transport.

Sequential QC checkpoints along the early secretory pathway

For many ER-synthesized proteins, attaining the native structure is a long endeavor, requiring several hours or more. This generally reflects the sequential execution of multiple steps, often including oligomerization. In a factory, engineers would most likely design a sequential assembly line, in which each step is carefully executed and monitored. Evidence in favor of such a physical compartmentalization within the early secretory pathway of mammalian cells is accumulating.

ERGIC-53, ERp44 and IgM polymerization

IgM polymers are planar, multimeric proteins with an overall hexameric shape, secreted as either $[(\mu_2L_2)_5{-}J]$ or $(\mu_2L_2)_6$. Individual subunits are linked by disulfide bonds involving Cys575 in the C-terminal tailpiece of secretory $\mu(\mu_s)$ chains. Cys575 acts also as a retention and degradation signal for unpolymerized subunits (Fra *et al*, 1993). The first assembly step (μ_2L_2 formation) is fast and efficient in both B and plasma cells: its fidelity is mainly checked by BiP (see Hendershot and Sitia, 2005 and references therein). In contrast, polymerization is slow and occurs only in plasma cells. Recent studies suggest that a post-ER QC mechanism plays a key role in the sequential assembly of IgM polymers. An unexpected finding was that ERp44, a soluble member of the trx family equipped with an RDEL localization motif (Anelli *et al*, 2002), accumulates in the ERGIC and *cis*-Golgi (Gilchrist *et al*, 2006; Anelli *et al*, 2007; Wang *et al*, 2007). ERp44 mediates the thiol-dependent retention of μ_2L_2, μL and other unpolymerized IgM subunits that have already passed the BiP-dependent checkpoints (Anelli *et al*, 2003, 2007). ERp44 localization partly depends on interactions with ERGIC-53, which also binds IgM subunits (Mattioli *et al*, 2006). As a hexameric membrane-embedded lectin, ERGIC-53 may provide a platform for IgM polymerization (Anelli *et al*, 2007). In the *cis*-Golgi, ERp44 could capture unpolymerized IgM subunits and retrieve them via RDEL-dependent mechanisms. In view of its ability of binding Ero1 (Otsu *et al*, 2006), ERp44 could also provide oxidative power to the polymerization machinery. In this scenario, the compartmentalization of assembly and polymerization in the early secretory pathway of professional antibody secreting cells may couple QC and transport, thus achieving high production capacity.

ERp44, Ero1α and adiponectin oligomerization

Another example of sequential QC comes from adiponectin, a plasma protein secreted by adipocytes (Scherer *et al*, 1995; Chandran *et al*, 2003), low levels of which associate with diabetes and cardiovascular diseases (Hotta *et al*, 2000; Phillips *et al*, 2003; Shetty *et al*, 2004). Plasma adiponectin is composed of trimers, hexamers and higher molecular weight oligomers, the latter being more active in preventing diseases (Tonelli *et al*, 2004; Bobbert *et al*, 2005; Lara-Castro *et al*, 2006). ERp44 was shown to retain folded adiponectin trimers, forming mixed disulfides with Cys39 in one subunit (Qiang *et al*, 2007; Wang *et al*, 2007). Ero1α, whose synthesis is regulated by hypoxia, SIRT1 and PPARγ, sequesters ERp44 and favors secretion of adiponectin oligomers (Wang *et al*, 2007). As in the case of IgM, therefore, folded adiponectin intermediates are retained by thiol-dependent mechanisms. ERp44-bound adiponectin may provide a reservoir of molecules easily mobilized for rapid release. The wide distribution of ERp44 in secretory cells (see www.hpr.se) and its high evolutionary conservation in worms and insects imply a wider role for ERp44 in the early secretory compartment, including the coupled control of Ca^{2+} and redox homeostasis and signalling (Higo *et al*, 2005).

Oligomeric membrane proteins

Different mechanisms exist for ER retrieval of membrane proteins (reviewed in Lee *et al*, 2004). Yeast Rer1p, a Golgi transmembrane protein, binds and mediates the retrieval of the ER-localized proteins Sec12p, Sec63p, Sec71p via their transmembrane domains (Sato *et al*, 1995, 1996, 1997, 2001). Interestingly, yeast Rer1p has also a role as quality controller for the iron transporter subunit Fet3p, retrieving it back in the ER by interactions with the TMD, unless the subunit is correctly assembled with its partner Ftr1p (Sato *et al*, 2004). The mammalian homologue of Rer1p localizes in the Golgi (Fullekrug *et al*, 1997). RER1 binds unassembled nicastrin and regulates transport of the γ-secretase complex (Spasic *et al*, 2007). On the contrary, the thermosensitive mutant tsO45 VSV-G protein can no longer be retained once it reaches the ERGIC or Golgi (Mezzacasa and Helenius, 2002), impying that the machineries recognizing mutant tsO45 VSV-G protein are not present downstream of the ER.

Therefore, QC occurs in multiple stations of the early secretory compartments, each station likely recognizing particular features in cargo molecules.

Multiple ways for ERADicating proteins from the early secretory pathway

Because of mutations and/or lacking of cofactors, partner proteins or prosthetic groups, some proteins cannot reach their native conformation and hence must be degraded. Substrates vary widely in size, structure and topology, and multiple pathways are active in cells to handle them. However, some common features exist. Aberrant proteins must be first recognized. As anticipated above, mannose trimming allows discrimination between unfolded and terminally misfolded glycoproteins (Helenius and Aebi, 2001); the latter are recognized by the concerted action of α1/α2 mannosidase-like proteins like Htm1p/Mnl1p in yeast (EDEM1, 2, 3 in mammalian cells) (Molinari *et al*, 2003; Oda *et al*, 2003; Mast *et al*, 2005; Olivari *et al*, 2005; Hirao *et al*, 2006), yeast Yos9p (Buschhorn *et al*, 2004) and mammalian OS-9 and erlectin/XTP3-B. The latter two mammalian lectins interact with SEL1L (J Christianson and R Kopito, personal communication), a membrane-bound molecule involved in ERAD (Mueller *et al*, 2006) and capable of binding the E3 ligase HRD1 (Neuber *et al*, 2005), providing a possible link between substrate recognition, translocation and ubiquitination. Much less is known on how terminally misfolded proteins that lack *N*-glycans are targeted to destruction.

Once recognized, aberrant proteins likely undergo partial unfolding and reduction (Tortorella *et al*, 1998; Fagioli *et al*, 2001) before retrotranslocation. Both BiP and PDI have been implicated in this process (Nishikawa *et al*, 2001; Tsai *et al*, 2001; Molinari *et al*, 2002). Certain exogenous toxins utilize ERAD pathways to reach the cytosol. Since their folding occurs in the producer cell (generally a bacterium or a

plant), toxins provide powerful tools to analyze the interactions occurring during retrotranslocation independently from the ones occurring during folding. Using these models, reduced PDI was shown to bind and unfold cholera toxin before dislocation. Upon Ero1-dependent oxidation, PDI releases dislocation-competent A subunits, thus acting as a redox-dependent chaperone (Tsai *et al*, 2001, 2002; Tsai and Rapoport, 2002).

ERdj5 might play an important role in the dislocation of certain substrates that form interchain disulfide bonds. Both its oxidoreductase and DnaJ activities are essential, suggesting that ERdj5 could couple reduction and BiP-mediated unfolding (K Nagata, personal communication).

Dislocation is the least understood step in ERAD. Besides Sec61 (Gillece *et al*, 2000; Jarosch *et al*, 2002; Clemons *et al*, 2004; Ng *et al*, 2007), additional transmembrane proteins (yeast Der1p and its homologues Derlin1, 2 and 3 in mammalian cells) have been implicated. It remains to be seen whether derlins directly mediate dislocation, or act by recruiting additional essential components such as the AAA-ATPase p97, which provides part of the energy necessary for this step (Ye *et al*, 2001, 2004; Rabinovich *et al*, 2002; Oda *et al*, 2006). It has been recently suggested that also lipid-based mechanisms mediate the transport of large substrates across the ER membrane, as observed for the entry in the cell of certain non enveloped viruses.

Once they emerge into the cytosol, ERAD substrates are ubiquitinated by E2–E3 complexes generally associated with the ER membrane (yeast E3 ligase Doa10p or Hrd1p and E2 ubiquitin conjugating enzyme Ubc7p-Cue1p (Carvalho *et al*, 2006; Denic *et al*, 2006). Then, an *N*-glycanase removes the oligosaccharide moieties from glycoproteins (Hirsch *et al*, 2003, 2004). Proteasome inhibitors generally impair dislocation (Chillaron and Haas, 2000; Mancini *et al*, 2000), implying that the above steps (dislocation, ubiquitination, proteolysis) are tightly coupled. The association of proteasomes with the ER membranes (Kalies *et al*, 2005) may be important in coupling substrate extraction and degradation.

Experiments in yeast and mammalian cells have recently shown that molecules involved in ERAD form multiprotein complexes specialized in the handling of topological classes of proteins (Vashist and Ng, 2004; Lilley and Ploegh, 2005; Carvalho *et al*, 2006). In yeast, the ERAD-L pathway recognizes substrates with misfolded ER luminal domains (on both soluble and membrane proteins). It needs the cytosolic factor Cdc48p (p97), the E3 ubiquitin ligase Hrd1p (HRD1 in mammalian cells) and its transmembrane cofactor Hrd3p (SEL1L), the putative channel component Der1p (Derlin1), the luminal lectins Yos9p (OS9 and XTP3-B) and Htm1p (EDEMs). SEL1L binds substrate proteins via its luminal domain, possibly favoring their inspection by OS9, EDEM and other molecules capable of discriminating between native and non-native species. Proteins with defects in their transmembrane regions utilize instead the ERAD-M pathway. In yeast, ERAD-M substrates require Cdc48p, Hrd1p and Der1p, but not Yos9 and Hrd3p. Finally, ERAD-C takes care of transmembrane proteins with misfolded cytosolic domains. These substrates appear to be handled in a way similar to ERAD-M substrates, but in yeast they use the E3 ubiquitin ligase Doa10p instead of Hrd1p.

In both yeast and mammalian cells, certain ERAD substrates are stabilized when ER–Golgi transport is blocked, while others are disposed of efficiently. An Hrd1p-independent pathway (HIP) has been described in yeast that implies substrate transport through the Golgi (Haynes *et al*, 2002). In mammalian cells, Golgi α1,2-mannosidases were shown to accelerate the degradation of a terminally misfolded α1-antitrypsin variant primarily localized in the ER, suggesting that mannose trimming in distal compartments contributes to ERAD (Hosokawa *et al*, 2007). Altogether, these findings underscore the existence of sequential checkpoints along the early secretory compartments, finalized to optimize protein folding and QC.

Autophagy

In addition to ERAD, cells can dispose of aberrant proteins accumulating in the ER by autophagy. In yeast, autophagy was shown to remodel the ER after its expansion during the unfolded protein response (UPR) (ER-phagy; Bernales *et al*, 2007). Additional links are emerging between the two pathways. On the one hand, blocking ERAD stimulates autophagy via signalling pathways that involve elements of the UPR- and Ire1-dependent JNK phosphorylation (Yorimitsu *et al*, 2006; Hosokawa *et al*, 2007; Kouroku *et al*, 2007; Yorimitsu and Klionsky, 2007). On the other, since EDEM is constitutively degraded by autophagic pathways (M Molinari, personal communication), inhibition of the latter stimulates glycoprotein dislocation and degradation. An intriguing possibility is that EDEM, which concentrates in defined ER subregions (Zuber *et al*, 2007), targets some glycoproteins to autophagic degradation. Also ER Man I is rapidly degraded by non-proteasomal pathways in hepatoma cells (Wu *et al*, 2007b). It will be of interest to identify additional short-lived ER-resident proteins, as they could include molecules that integrate distinct proteolytic pathways and hence, ER homeostasis.

Adapting the factory to new demands: ER stress and signalling

The accumulation of misfolded proteins in the ER lumen elicits a multidimensional signalling cascade finalized to relieve ER stress. The UPR activates several mechanisms to handle the increase of unfolded proteins (Ma and Hendershot, 2004; Bernales *et al*, 2006; Ron and Walter, 2007): (i) decreased protein translation, (ii) increased transcription of genes enhancing protein folding (ER-resident chaperones and folding enzymes) and ERAD, (iii) decreased entry of proteins into the ER (Kang *et al*, 2006; Orsi *et al*, 2006) and (iv) selective degradation of certain mRNAs encoding secretory proteins (Hollien and Weissman, 2006). If these measures are not sufficient for eliminating misfolded proteins from the ER, apoptotic pathways are activated (reviewed in Szegezdi *et al*, 2006). The UPR serves a key role also during the development of professional secretory cells. A challenging problem, with important medical implications, is what turns an adaptive response (finalized to increase the ER folding capacity, as in the case in professional secretory cells) into a mal-adaptive response that causes cell death (Lin *et al*, 2007; Rutkowski and Kaufman, 2007; Wu *et al*, 2007a). On the one hand, the latter can be viewed as an organismal defence mechanism against cells producing potentially harmful polypeptides. On the other,

it is increasingly clear that apoptosis caused by chronic ER stress underlies many diseases, such as diabetes, and that preventing apoptotic pathways retards disease progression (Ozcan *et al*, 2004, 2006).

QC and disease

Disturbances in the QC mechanisms are the cause of many diseases (Supplementary Table 1S; Otsu and Sitia, 2007). Diseases can arise because of mutations in cargo proteins as well as in folding, transport or signalling molecules. Clearly, different therapeutic strategies have to be envisaged in each of the above classes (Wiseman *et al*, 2007).

The most frequent mutation in cystic fibrosis mutations does not preclude chloride transport by CFTR: it is its absence from the plasma membrane that causes a loss of function. In such cases, therapy should aim at weakening QC. Instead, gain of function often arises from defective degradation. Serpinopathies are ER storage disorders in which transport-incompetent mutants form large polymers in the ER (Lomas, 2005). Not only the intrinsic tendency of a protein to polymerize, but also the interactions it establishes in the secretory pathway determine the extent and site of condensation (Mattioli *et al*, 2006). Accumulation in post-ER compartments has been described for mutant pro-insulin in the Akita diabetes model (Zuber *et al*, 2004; Fan *et al*, 2007) and vasopressin V2 receptor in nephrogenic diabetes insipidus (Oueslati *et al*, 2007), in line with the existence of sequential checkpoints. It remains to be seen whether these unusual localizations reflect accumulation in a specialized early secretory pathway subregion, the so-called 'quality control compartment' originally described for ASGPR H2a mutants (Kamhi-Nesher *et al*, 2001; Kondratyev *et al*, 2007).

The phenotypes of ERQC disorders generally involve tissues specialized in secretion, such as exocrine and endocrine glands, osteoblasts, plasma cells. Pancreatic β-cells are particularly sensitive, likely because the circadian oscillations in insulin biosynthesis require stringent translational control. Accordingly, the absence or insufficiency of ER stress sensors, particularly PERK and downstream elements, are responsible for many cases of diabetes (see Supplementary Table 1S). The absence of CHOP (a UPR factor involved in apoptosis) delays β-cell destruction and hyperglycemia. Type II diabetes can also ensue from environmental factors (e.g., a high-fat diet). The JNK-dependent phosphorylation of IRS1 inhibits insulin signalling, thus creating an exaggerated demand on β-cells, which in turn leads to stress and apoptosis (Ozcan *et al*, 2004). Confirming the role of ER stress, systemic administration of tauro-urso desoxy cholic acid (an analog of which is particularly abundant in the bile of bears, a common prescription in traditional Chinese medicine) leads to normalization of hyperglycemia, restoration of systemic insulin sensitivity and enhancement of insulin action in a murine model of type II diabetes, the ob/ob mice (Ozcan *et al*, 2006).

Concluding remarks

The subdivision of the early secretory pathway into distinct functional regions provides an efficient and dynamic factory capable of handling myriads of polypeptides, while maintaining stringent homeostatic control. The various elements that regulate folding and QC of the secretory proteome need to work in tight synergy and change their relative abundance (and qualitative composition) when facing a particular synthetic task. Signalling pathways are being identified that regulate the efficiency of ER folding, export and degradation so as to adapt to the changing demands of the ER protein factory during differentiation. Clearly, a better understanding of these pathways is bound to improve dramatically our capability to deal with many severe chronic diseases.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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