Endoplasmic Reticulum redox pathways: in sickness and in health

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Abbreviations:

EMC	ER membrane protein complex
ER	Endoplasmic reticulum
ERAD	ER associated degradation
Ero1-PDI	Endoplasmic Reticulum Oxidoreductase-Protein Disulfide Isomerase
GSH	reduced glutathione
GSSG	oxidised glutathione
ORPs	oxysterol-binding protein related proteins
SNO	S-nitrosylation
SRP	signal recognition particle
UPR	unfolded protein response

Abstract

The Endoplasmic Reticulum (ER) is the major site for secretory protein production in eukaryotic cells and like an efficient factory, it has the capacity to expand or contract its output depending on the demand for its services. A primary function of the ER is to co-ordinate the quality control of proteins as they enter this folding factory at the base of the secretory pathway. Reduction-oxidation (redox) reactions have an important role to play in the quality control process, through the provision of disulfide bonds and by maintaining a favourable redox environment for oxidative protein folding. The ER is also a major contributor to calcium homeostasis and is a key site for lipid biosynthesis, two processes that additionally impact upon, and are influenced by, redox in the ER compartment.

Introduction

The old English television series “till death us do part” and its sequel “in sickness and in health” look rather dated on screen these days and the programmes can make for uncomfortable viewing. The three central characters, bigoted Alf, his long-suffering wife Else, and their daughter Rita are frequently at odds with each other, but they still maintain a deep bond of kinship that helps them through the flux of their everyday lives. In cell biology, the relationship between the ER and the surrounding environment is similar: the hard-working ER compartment may have reactive redox chemistry, but is inextricably tethered to and must work with a family of organelles for the benefit of cellular union. Functional homeostasis depends on positive and negative feedback to and from all the partners. With this theme in mind, the review will discuss how redox homeostasis is communicated within and beyond the ER, how redox pathways help the ER achieve its main task of oxidative protein folding, and how we can gain insights into ER redox biology when things go wrong in sickness and in health.

Targeting of proteins to the ER

The discovery of the secretory pathway and the organisation of molecular targeting and transport in eukaryotic cells won the Nobel prize for Palade in 1974, Blobel in 1999 and Rothman, Schekman and Suedhof in 2013 (see [1] and references therein). Nevertheless, there is still much to be discovered about the compartmentalisation and co-ordination of major biological processes within organelles, and in particular the ER. Proteins targeted to the ER may be destined to reside in the lumen itself, or they may be transported elsewhere in the secretory pathway, including the Golgi apparatus, the lysosomal-endosomal system, the

plasma membrane or the growing body of autophagy-related compartments [2]. The majority of proteins entering the ER are directed to the organelle by a signal sequence and are co-translationally threaded through the Sec61 translocon as they emerge from the ribosome [3] (Figure 1, step 1). Although an N-terminal signal peptide has long been established as the primary route for targeting a protein to the ER, it has recently become apparent that signal peptide recognition by the signal recognition particle (SRP) is not the only molecular mechanism. Proteins can be sent to the ER lumen by at least three different, overlapping pathways: the SRP-Sec61 translocon pathway (dependent on N-terminal signal sequence recognition), the GET pathway for tail anchored proteins (dependent on C-terminal transmembrane-domain recognition), and the SND-Sec61 pathway, which uses a (likely) ribosome associated Snd1 protein and two ER localised Sec61-interacting transmembrane Snd2/3 proteins to target a broad range of substrates to the ER [4, 5]. The SND pathway was identified quite recently using an elegant genetic localisation screen in *S. cerevisiae*, together with a proximity-specific ribosomal profiling approach to identify ER-specific SND clients. It will be interesting to see how the SND system operates in higher eukaryotes and whether the import process is responsive to changes in ER load and redox conditions.

Quality control of nascent ER proteins

When a translocated protein enters the ER, it emerges in a reduced state. On exposure to the oxidising environment of the ER, proteins start to fold and acquire post-translational modifications. N-glycosylation is the most common modification for proteins with an appropriate NXS/T acceptor site for the glycan (Figure 1 step 2). It is well established that the bulky $GlcNAc_2Man_9Glu_3$ sugar tree of an N-glycoprotein is used as a handle to monitor a glycoproteins folded status, first through glucose trimming by glucosidases I/II and folding by the sugar-loving calnexin/calreticulin chaperones, then by removal of the terminal mannose by α1,2 mannosidase (reviewed in [6]) (Figure 1, steps 6 and 7). This leaves behind an export competent glycoprotein with a $GlcNAc_2Man_8$ sugar tree that can be further modified (e.g. with addition of sialic acid) in the Golgi apparatus. Despite seminal work by Helenius, Parodi and many others to establish the molecular details of the calnexin cycle for sugar-bearing protein quality control (reviewed in [7] and [8]), it remained a mystery how sugar-free proteins were assessed for their road-worthiness. Recently, part of the answer has been provided by Davis Ng's group, who have discovered a new quality control system for soluble non-glycosylated clients in *S. cerevisiae* [9]. This novel proteostatic effector is composed of a complex of two conserved ER resident proteins: Slp1, a single ER membrane spanning SUN-like protein, and Emp65, its multimembrane-spanning partner. Cells that lack Slp1-Emp65 degrade ~20-30% of their newly synthesised proteins. Pulse-chase and binding studies suggest that Slp1-Emp65 forms a complex that protects nascent proteins by shielding them from the destructive forces of the ER, giving them enough time to complete their folding cycle. It will be fascinating to decipher the function of the equivalent system in higher eukaryotes and to establish the molecular details of client handover to downstream quality control operatives (Figure 1, step 8). Slp1 was reported previously to recruit Mps3 (an *S. cerevisiae* SUN protein) to the nuclear envelope [10], so it will be exciting to determine how SUN/KASH domain-containing proteins contribute to both ER and nuclear envelope lumen quality control. The system is likely to be under redox regulation, as both Slp1 and Emp65 have solvent exposed cysteines, and it has been shown that intermolecular disulfide bonds mediate the interaction between the perinuclear space localised SUN1/2 proteins and the KASH2 peptide-domain of outer nuclear membrane-spanning nesprins [11, 12]. In this latter context, the KASH-SUN disulfide bond acts as a sensor for force transmission between the nuclear envelope and the cytoskeleton.

Disulfide bond formation and redox control

Disulfide bond formation is a necessary post-translational modification for many glycosylated and non-glycosylated secretory pathway proteins. Disulfides occur between the -SH groups of two cysteine residues and involve the concomitant loss of electrons. Hence redox reactions lie at the very heart of ER quality control. Redox pathways in the ER manifest themselves at four levels: first, there are protein oxidoreductases and folding enzymes that mediate disulfide bond formation through electron exchange with nascent client proteins, as they begin to fold; second, an overlapping set of oxidoreductases and isomerases help to untangle (directly isomerise) misfolded or misoxidised proteins that may accumulate due to high secretory demand, or inefficient, off-pathway misfolding; third, reductases may process terminally misoxidised proteins in preparation for their degradation, and fourth, there are small molecular weight thiols and other redox-active compounds, such as glutathione, that act as redox "buffers" and control the redox equilibrium of the ER [13, 14].

The Ero1-PDI (Endoplasmic Reticulum Oxidoreductase-Protein Disulfide Isomerase) pathway is the major route for disulfide bond formation in the ER [15] (Figure 1, step 3). Of the two mammalian Ero1 proteins,

Ero1α is expressed in all cell types and is upregulated by both ER stress and hypoxia, whereas Ero1β is inducible by the unfolded protein response (UPR) and is enriched in selected secretory cells, chiefly in the pancreas [16-18]. Ero1 proteins receive an oxidising equivalent from molecular oxygen via the oxidation of $FADH_2$ to FAD and in turn generate peroxide from the two-electron reduction of oxygen. The newly acquired disulfide bond is passed from Ero1 to PDI through an internal shuttle system of disulfides, which are regulated to ensure that the system is switched on by reducing equivalents (namely unfolded or newly translocated ER proteins). PDI, a thioredoxin-domain containing protein with an abb'xa' domain arrangement, donates the acquired disulfide bond to a range of clients through a highly labile disulfide in the PWCGHC motif of the a/a' domains. The domain arrangement of PDI is depicted in Figure 2. Various NMR and crystal structures of PDI domains and PDI/Ero proteins have been solved in impressive detail (reviewed by [19]), but co-crystals of PDI and Ero locked in their molecular embrace remain to be published. The provision of disulfide bonds generates peroxide, which is recycled in higher eukaryotes (or used *de novo*) by PRDX4 to create a further disulfide bond for PDI [20-22] (Figure 1, step 5). Two ER-localised glutathione peroxidases (GPX7 and GPX8) are also capable of generating disulfide bonds from peroxide *in vitro*, and may contribute to the control of disulfide bond formation and calcium fluxes *in vivo* [23-25] (Figure 1, step 4).

The flow of electrons from active site cysteine residues in Ero1 to PDI in the ER is chiefly determined by both kinetics and the thermodynamic redox potential of the system (discussed in detail by Hatahet and Ruddock [26]). Redox potentials in biological systems are relative measurements; proteins with a high reduction potential tend to be poor reductants and proteins with low reduction potentials tend to be poor oxidants. The yeast and mammalian Ero-PDI pathways are set up slightly differently, but in mammals, the redox potential of PDI active site disulfides is ~ -160-180 mV. The Ero1α non-catalytic cysteines, with which PDI communicates, have a reduction potential of -280mV. The high reduction potential of the PDI active site disulfides limits the ability of Ero1α to oxidise PDI and ensures that PDI is only ever partially oxidised in the ER, enabling it to respond to fluctuations in oxidative protein folding [27].

One might expect that such an important quality control pathway for disulfide bond formation would be essential for life. This turns out not to be the case for multicellular organisms. Although loss of the *ERO1* gene in *S. cerevisiae* is fatal [28, 29], *D. melanogaster* flies that lack their Ero1L gene have an unusual Notch ER retention phenotype attributed to the failure to secrete Notch in the absence of Ero1L [30] and mice that lack Ero1a, Ero1b and Prdx4 are viable [31]. These triple knockout animals have mild diabetes, arising from a requirement for Ero1β in insulin folding in the pancreas, and a notable dysregulation of collagen quality control. Procollagen production is diminished 5-fold and collagen fails to be properly hydroxylated in these animals. The mice also suffer from scurvy as a result of ascorbic acid depletion. In humans, there are no known disease-causing mutations in either Ero-encoding gene, but interestingly a Y393C mutation in the PDI gene (P4HB) results in Cole-Carpenter syndrome, a very rare skeletal disorder in which patients suffer from fragility and fracturing of the bones, premature ossification of the skull, and other disrupted facial features [32, 33]. Why should a mutation in P4HB result in such a specific phenotype? The answer is not yet known, but the issue may not necessarily be with collagen production, since patient fibroblasts produce type I collagen at a normal rate. The Cole-Carpenter PDI Y393C mutation results in the gain of a reactive cysteine, close to the redox active CGHC motif, which may alter the redox poise of PDI. It has also been reported that ER stress is enhanced in Cole-Carpenter patients, so the explanation for skeletal disease could be a "gain of dysfunction" that sensitises particular cells, like osteoblasts, that need to export complex secreted cargoes.

Overall, it seems that the Ero1-PDI pathway in higher eukaryotes, whilst being the go-to route for general disulfide bond formation, has redundancy. Given the mild phenotype of Ero1 deficient animals, perhaps the critical importance of the Ero1-PDI pathway is in controlling the redox poise of the ER, rather than disulfide bond formation *per se*. In support of this idea, changes in the redox poise by nitrosative stress lead to PDI dysfunction in sporadic neurodegenerative diseases. The PDI a and a' domains can become S-nitrosylated (SNO) at their active site cysteines, resulting in a loss of functional redox activity without ablating global oxidative folding. Patients suffering from Alzheimer's or Parkinson's disease have elevated SNO-PDI levels, which correlate with an increase in UPR activation, protein misfolding and ER dysfunction [34]. Although PDI does become S-nitrosylated in these two diseases, cause and effect remains to be demonstrated fully. A number of other ER stress sensors, such as Ire1α and PERK, can also become SNO modified, at least in cell line models [35], and it is not known to what extent other members of the PDI family become functionally altered by this modification. Indeed, Ire1α can be sulfenylated at a cysteine within it kinase activation loop, initiating an Nrf2 driven antioxidant response, suggesting a redox-based route for the control of cytoplasmic, as well as ER localised, stress responses [36].

Glutathione and redox modifications

The chief redox buffer in the ER with which the Ero-PDI pathway communicates is glutathione, which exists in equilibrium as both a reduced (GSH) and oxidised (GSSG) form (see [26] for a comprehensive review). Glutathione concentrations are ~5 mM in the cytoplasm and ~10 mM in the ER. Glutathione is a tripeptide comprising glutamate, cysteine and glycine, with an unorthodox gamma peptide bond that covalently links the cysteine amine group to the carboxyl group of the glutamate side chain. Glutathione is synthesised by γglutamylcysteine synthetase (rate limiting) and glutathione synthetase in the cytoplasm, where glutathione reductase can reduce GSSG back to GSH using NADPH as an electron donor [37]. Recently, it has been shown that NADPH is also required to reduce non-native disulfides in mis-oxidised ER proteins through cytosolic thioredoxin reductase 1 (TrxR1) [38]. How the reducing equivalents are transferred to the ER from the cytosol is not yet known [39], but in the periplasm of prokaryotes such as *E. coli*, the membrane-spanning DsbD protein can transfer electrons from NADPH (via cytosolic TrxA) to the disulfide isomerase DsbC [40]. It is tempting to speculate that an as-yet unidentified ER membrane spanning redox protein does the same job in eukaryotes. *E. coli* can also directly transport GSH from the cytoplasm to the periplasm through an ABC transporter, CydD/CydC [41]. No stand-alone GSH (or GSSG) transporter has been identified for the ER membrane; however, recent evidence in yeast has suggested that the translocon (Sec61p) may be able to multitask and act as a conduit for GSH as well as newly synthesised proteins [42]. Using a yeast genetics approach, Ponsero *et. al.* have suggested that the Sec61 translocon, aided and abetted by the ER resident hsp70 chaperone Kar2p (BiP/grp78 in higher eukaryotes) and Ero1p, can transport GSH into the ER. This model is attractive because it couples redox requirements to demand from translated proteins. However, the evidence for GSH transport by the translocon in the yeast system is indirect. The direct functional transport of GSH by Sec61 has not been demonstrated and it remains to be seen whether the findings hold for higher eukaryotes, where the existence of alternative, dedicated glutathione transporters cannot be ruled out. Thanks to GSH transport by Sec61 and/or other mechanisms, the redox poise of the ER is kept more oxidising that the cytosol, and facilitates the formation of disulfide bonds.

Given that Ero1 proteins are flavoenzymes, it is likely that the FAD:$FADH_2$ ratio is also important for setting the redox poise of the ER, but how FAD accesses the ER remains mysterious. A satisfying possibility would be a dedicated ER localised quinone/hydroquinone transporter. Indeed, flavin transporters have been found at the eukaryotic plasma membrane and bacterial periplasm; and in *S. cerevisiae*, potential ER localised flavin importers (FLC1 and FLC2) have been identified, from a screen of heme uptake mutants. Tagged FLC1 and 2 proteins localise to the ER, based on fractionation and microscopy studies, and *flc1Δflc2Δ* strains have compromised oxidative protein folding and N-glycosylation of the model substrate carboxypeptidase Y [43]. Alternatively, FAD could be transported through the Sec61 translocon (in a similar manner to that proposed above for glutathione), or delivered by retrograde transport from an upstream secretory compartment. It may take another inventive genetic screen or an unexpected disease mutation to confirm the long-sought control point for setting the lumenal FAD concentration in higher eukaryotes.

Beyond the ER: reaching out to the plasma membrane and mitochondria

Classically, the ER has been considered as a staging post for the transport of proteins through the secretory pathway, chiefly via the Golgi apparatus, but modern cell biology and advanced, high resolution imaging techniques have revealed that the ER interacts with a variety of other organelles and membrane systems. ER plasma membrane contact sites, for example, are important for membrane organisation and can help mediate non-vesicular calcium transport and adjust plasma membrane lipid composition through the activities of lipid transfer proteins such as the oxysterol-binding protein related proteins (ORPs) [44]. At the plasma membrane, perturbation of cystine import (through the system x_c^- transport machinery) by a small molecule called erastin can lead to glutathione depletion, compromising the function of the cytosolic glutathione peroxidase GPX4 [45, 46]. GPX4 is a phospholipid peroxidase that limits the build-up of toxic lipid hydroperoxides, by converting them into benign lipid alcohols. In the absence of GPX4, or when GPX4 is compromised, toxic lipid accumulation induces ferroptosis, an iron-dependent form of pathological cell death caused by lethal lipid peroxidation that has also been linked to neurodegenerative diseases such as Alzheimer's, Huntington's or Parkinson's disease. Given the close control that the ER exerts on glutathione levels and the still relatively unclear mechanistic link between ER chaperones and neurodegenerative disease, it is notable that erastin also induces an ER stress response. Redox and cytosolic stresses can trigger other eIF2α kinases than PERK, and it is noteworthy that erastin triggers the eIF2α-ATF4 branch of the UPR, without touching the Ire1-directed splicing of XBP1 [47]. Murine embryonic fibroblast cells lacking the ATF4 transcription factor exhibit an iron-dependent death phenotype, requiring supplementation with

reductants for viability. These cells become protected from death when given the iron chelator desferoxamine [48]. These data suggests that ATF4 may help set the threshold for ferroptosis as well as promoting resistance to oxidative stress in general. NRF2, a transcription factor that can dimerise with ATF4, facilitates resistance to ferroptosis by controlling the expression of iron-responsive genes including ferritin and feroportin, together with genes required for GSH synthesis.

It is tempting to speculate that glutathione peroxidases, in addition to GPX4, may be involved in phospholipid detoxification of the ER leaflet under normal circumstances, especially as mechanical perturbation of the ER lipid bilayer can induce a UPR in the absence of unfolded ER proteins [49]. Knockdown of subunit 2 of the ER membrane protein complex (EMC), a protein implicated in ER associated degradation [50] and the insertion of transmembrane domains into the ER membrane [51], suppresses erastin-induced ferroptosis [52]. Further exploration of the link between ER-cytosolic redox conditions, the EMC and cell fate decisions may be warranted, given the function of the EMC in ER quality control. In yeast, the EMC complex is also involved in mitochondrial tethering, and may facilitate the non-vesicular transfer of phospholipids from the ER to mitochondria [53]. The ER is well known to associate closely with mitochondria, where exchange of ions such as calcium and lipids can occur (reviewed in [54, 55]). Recent evidence suggests that ER-mitochondrial contact sites are quite diverse and have a number of other key roles. For example, these contact sites are the birthplace of autophagosomes in mammalian cells. Periods of starvation initiate the process by inducing ATG14 and ATG5 recruitment to mitochondrial contact sites by an ER resident SNARE, syntaxin 17 [56]. Immunologists have recently turned their attention ER-mitochondrial contact sites too, having appreciated that metabolic reprogramming can control the activation of T lymphocytes and influence their development into either regulatory or cytotoxic/helper effector subtypes [57]. Bantug *et. al.* have shown that memory CD8+ T cells use mitochondria-ER contact sites as a hub to drive rapid glycolytic remodelling, facilitating the increase in demand for glucose oxidation after IFNγ stimulation in response to an infection [58]. The ER acts as a scaffold in this set-up: it positions mTORC2-AKT signalling so that pAKT can phosphorylate and inhibit GSK3β. The inactivation of GSKβ by phosphorylation permits hexokinase to interact with the channel protein VDAC at the mitochondrial outer membrane. VDAC is then free to transport pyruvate into the mitochondria to feed the TCA cycle. The ER-mitochondrial contact sites in CD8+ T cells appear to be required for respiration and pyruvate-dependent cytotoxic T cell responses, but not for glycolysis *per se* in this model.

ER-mitochondrial contact sites are enriched in Ero1α under oxidising conditions, which may help regulate calcium flux through inositol 1,4,5-trisphosphate receptors at the associated membranes [59]. These contact sites may also shape the outcome of disease in oncology, where it has been proposed that the ER-localised oxidoreductase TMX1 can also control calcium dynamics at the mitochondrial interface. Cancer cell lines with a low level of TMX1 have reduced calcium transfer to mitochondria; this may give the cancer cell an advantage, as it decreases the production of ATP in the mitochondria and disfavours apoptosis [60]. *In vivo*, however, the situation might not be so clear-cut. A number of cancer tissues, including colorectal cancer, melanoma and head and neck cancer, have high expression of TMX1 whereas prostate, ovarian and renal cancer (amongst others) have low TMX1 expression (observations from the Human Protein Atlas; https://www.proteinatlas.org/tissue). It will be of interest to determine whether the relationship between the ER and mitochondria, and the calcium exchange mechanisms at play, differ in these distinct cancer types.

ER chaperones and redox in disease

As hinted above, ER chaperones and folding enzymes are frequently upregulated in disease, particularly in conditions where proteins misfold or aggregate in the secretory pathway [61]. This makes sense, because unfolded proteins in the ER trigger the UPR by titrating BiP/grp78 away from the UPR sensors Ire1α, PERK and ATF6, allowing them to become activated and to induce ER chaperone expression in an attempt to alleviate the overload of unfolded client proteins (covered elsewhere in this special edition). High levels of ER chaperones such as BiP are therefore a signal that the cell is attempting to correct intracellular indigestion caused by an engorged ER. BiP/grp78 is a classical Hsp70 chaperone, using a hydrophobic patch to interact with exposed hydrophobic domains on client proteins and shield them from aggregation during an ATP-driven folding cycle, in partnership with a nucleotide exchange factor. However, recent studies have shown that in *S. cerevisiae*, Kar2p (the yeast homolog of BiP) engages with a nucleotide exchange factor, Sil1p, in an unexpected way - to sense oxidising (as opposed to reducing) conditions in the ER [62]. This is achieved via a previously under-appreciated cysteine residue at position 63 in Kar2p, which becomes oxidised. Sil1p has a redox active C52-C57 cysteine pair that reverses the oxidised C63 residue in Kar2p, restoring BiP to its steady-state, non-stressed, reduced form. The Kar2p-Sil1p system also appears to be regulated by the N-oligosaccharyl transferase component OST3, since N-glycosylation of Sil1p decreases when cells

experience reductive stress [63]. Recent structural studies have shown that human equivalent of Sil1p (also called BAP) is a conformational regulator that promotes the release of both substrate and ADP from BiP, positioning it at a late stage in the BiP chaperone cycle [64]. Gaining a detailed understanding of SIL1/BAP and its relationship with BiP is important, because mutations in the human SIL1 gene can result in Marinesco-Sjögren syndrome, a rare autosomal recessive ataxia that presents with mental retardation, muscular and skeletal abnormalities [65]. It remains unclear how SIL1 mutations cause Marinesco-Sjögren syndrome, but it will be informative to determine whether compromising SIL1 activity as an ER reductant, or diminishing its function as a NEF for BiP, is critical for disease progression.

The key role of calcium in ER health and disease has been extensively reviewed elsewhere and will not be dealt with further here [66]. However, potent metal oxidants such as copper, zinc and magnesium and their binding proteins are being increasingly implicated in ER dysfunction. One example is Wilson's disease, an autosomal recessive condition that results in liver damage and, in 50% of patients, neuropsychiatic symptoms. Wilson's is caused by mutations in the copper-transporting ATPase ATP7B. In normal circumstances, ATP7B is transported from the ER to the plasma membrane, where it expels copper from liver hepatocyte cells into the bile [67]. In Wilson's disease, the ATP7B H1069Q mutant protein does not reach the copper exit sites at the cell surface and is instead retained in the ER. The result is a cellular double whammy: copper accumulates to toxic levels, and the ER-retained ATP7B mutant protein induces a damaging ER stress response, initiating JNK kinase signalling to trigger its own destruction by ER associated degradation (ERAD). It is hoped that inhibiting or down regulating JNK, together with assistance from chemical chaperone "correctors", might help patients with the H1069Q mutation – if the ATP7B H1069Q protein can be rescued from the ER and sent to the plasma membrane, it has sufficient activity to excrete copper at manageable levels [68].

Selenoproteins in the secretory pathway

ER resident selenoproteins contribute to the redox environment of the lumen, because selenocysteine (Sec; or U in one letter code) is even more oxidising than cysteine. However, the impact of Sec is difficult to measure directly because of the unusual way that it is incorporated into the selenoprotein. Sec is encoded by UGA, which is normally a stop codon. A special tRNA, tRNASec, reads UGA in the context of a cis-acting selenocysteine insertion sequence in the 3' UTR of the mRNA. The tRNASec is first charged with serine by seryl-tRNA ligase, the serine residue is then converted to a selenocysteine-residue by selenocysteine synthase and finally, the dedicated eukaryotic elongation factor mSelB/eEFSec binds to the tRNASec in a multi-protein complex at the ribosome [69]. Consequently, selenoprotein studies often have to make do with cysteine-substituted selenoproteins, which will underestimate the oxidising power of the system. There are up to seven mammalian selenoproteins that are localised to the ER/secretory pathway, of which four will be considered briefly here: SELENOF (Sep15), SELENOS (SelS/VIMP), SELENOP (SelP) and SELENON (SelN) [70, 71]. SELENOF is a widely expressed 15 kD ER selenoprotein with a quality control function [72]. SELENOF is retained in the ER through interaction with the folding sensor UDP-glucose:Glycoprotein Glucosyltransferase (GT) via its cysteine rich domain [73]. GT interacts with the innermost N-acetylglucosamine of misfolded glycoproteins, which should normally be buried, and monoglucosylates them, facilitating their return to the calnexin/calreticulin cycle. Based on the phenotype of SELENOF deficient mice, which have an increase (rather than decrease) in non-functional circulating antibodies, the protein has been suggested to act as gatekeeper of ER-to-Golgi transport for a subset of secretory glycoproteins [74].

Structural data suggest that SELENOF is an ER disulfide (or sulfide-selenide) isomerase/oxidoreductase and it is estimated to have a redox potential of -225 mV, consistent with that of a reductase [72]. However, since the exact redox function of SELENOF is not known, it remains to be established whether native, selenium-loaded SELENOF is regulated by other electron donors (e.g. PDI) or electron acceptors (e.g. Ero1α) or works alongside them to drive disulphide bond reduction reactions. SELENOS operates downstream of SELENOF, as a component of the ERAD machinery [75] and has an overall antioxidant capacity, at least *in vitro.* The precise function of the SELENOS selenium moiety in ERAD is unknown, but one possibility is that it is required for the unfolding of mis-oxidised proteins prior to their retrotranslocation from the ER to the cytosol.

The SELENOP selenoprotein has up to 10 Sec residues per protein, and is the major source, and a likely carrier, of selenocysteine in the body, with a role in antioxidant defence [76]. SELENOP is folded in the ER, requires glycosylation and possesses both sulfide-selenide and disulfide bonds. Only one of the SELENOP Sec residues is in a CxxU motif. Two other Sec-Cys bonds have been proposed for the C terminus (of the rat

protein) and two disulfides have been detected by mass spectrometry between C149–C167 and C153–C156. How spurious selenide-sulfide (S-Se) bonds are corrected or avoided (between the 10 SELENOP Sec residues and the 16 Cys residues) during folding of SELENOP in the ER is not known, and poses a thought-provoking question for redox-dependent protein quality control. Of the secretory pathway selenoproteins, SELENON is perhaps the most puzzling because of its association with a form of muscular dystrophy called SEPN1-related muscular dystrophy. Patients with this disease have axial muscle weakness, scoliosis and spinal rigidity with variable skeletal muscle pathology. Curiously, SELENON is a widely expressed selenoprotein but is associated quite specifically with myotubule formation [77]. The reason why SELENON mutations give such a restricted phenotype is unclear, but may be due to dysregulation of calcium homeostasis by SELENON, increased sensitivity of myotubes to oxidative/nitrosative stress, or induction of the ER stress response, similar to that suggested for the disruptive role of PDI in neurodegenerative/Cole-Carpenter diseases, and ATP7B in Wilson's disease. Predicting and understanding how such disparate mutations can cause unusual disease phenotypes remain major challenges for the field.

Despite much knowledge of the molecular details of ER quality control, disulfide bond formation and redox homeostasis, the picture remains incomplete. One major gap in the field concerns bridging "scales" – we understand the biochemistry and cell biology of ER redox proteins quite well, but this knowledge is not always easy to translate to more complex systems. Hence we can be taken by surprise by unexpected animal model phenotypes and curious human disease associations. The next steps forward in understanding ER redox biology at different scales and levels of complexity may well be driven by on-going technological developments – the advent and now widespread use of CRISPR-Cas9 gene editing, for example, provides an opportunity to study the effects of bespoke "knock–out" or point mutated "knock-in" mutants of redox proteins with altered redox poise in a range of experimental animal, tissue-specific or 3D culture models. The development of super-resolution imaging, coupled with brighter and less perturbing redox reporters, has taken scientists beyond the diffraction limit for both organelles such as the ER and individual protein complexes; and single-cell biochemistry, employing microfluidic lysis and single-molecule pull down techniques, will facilitate higher resolution and differentiation of redox-related molecular dynamics. Unlike the old television series "in sickness and in health", the ER "in sickness and in health" is an on-going and engaging serial, with many twists, turns and new plot developments in wait ahead.

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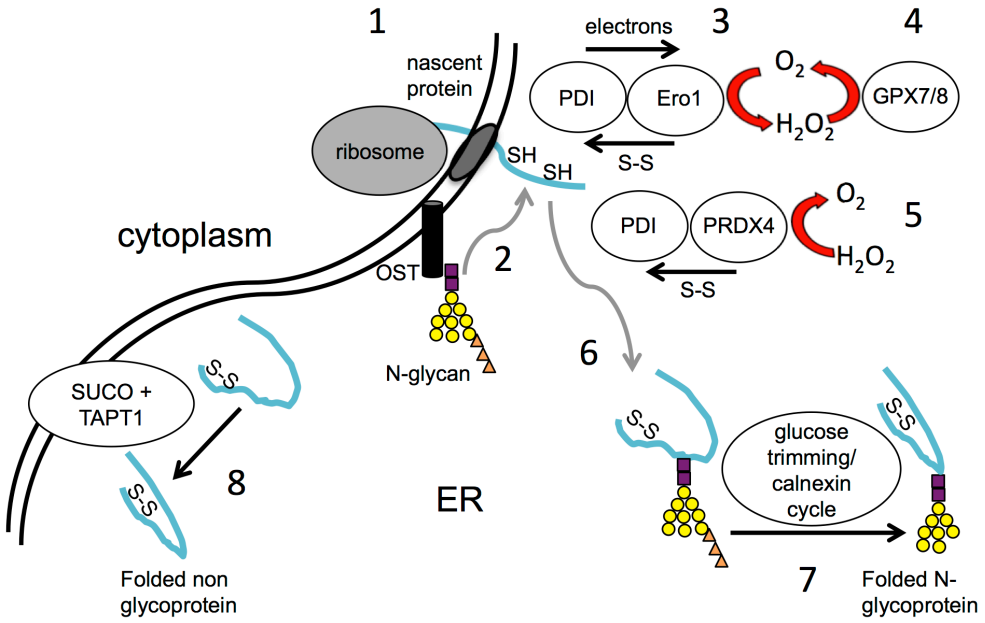

Figure 1. 1: N-glycoproteins are translated by the ribosome and translocated into the ER by the translocon (or SND pathway). 2: OST adds the $GlcNac_2Man_9Glu_3$ sugar to the glycoprotein. 3: Ero1/PDI catalyse the formation of disulfide bonds (S-S) from oxygen. 4: GPx7/8 may reoxidise generated peroxide. 5: PRDX4 utilises peroxide to oxidise PDI. 6: the oxidatively folding immature glycoprotein is handed over to the calnexin cycle. 7: The N-glycoprotein is productively folded. 8: Non-glycoproteins are quality controlled by SUCO and TAPT1 (proven only for the Slp1-Emp65 homologues in *S. cerevisiae* to date). ERAD pathways for misfolded/misoxidised proteins are not shown for simplicity. GlcNAc (purple), mannose (yellow), glucose (orange), folding protein (blue).

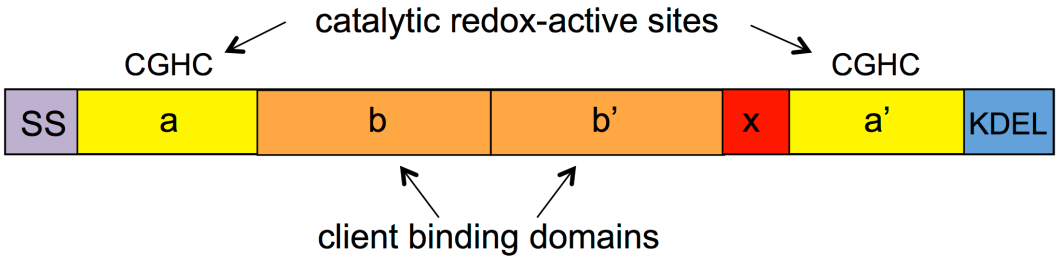

Figure 2. The domain arrangement of the PDI protein. PDI is targeted to the ER by an N-terminal signal sequence (SS, light purple) that is removed from the mature protein. PDI is retained in the ER by a C-terminal KDEL sequence (blue). The a and a' domains are thioredoxin-like catalytic domains with redox active CGHC motifs (yellow). The b and b' domains chiefly interact with client proteins (orange). A flexible linker domain is donated by x (red).