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REVIEW

Disulfide bond formation and redox regulation in the Golgi apparatus

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Formation of disulfide bonds in secreted and cell-surface proteins involves numerous enzymes and chaperones abundant in the endoplasmic reticulum (ER), the first and main site for disulfide bonding in the secretory pathway. Although the Golgi apparatus is the major station after the ER, little is known about thiol-based redox activity in this compartment. QSOX1 and its paralog QSOX2 are the only known Golgi-resident enzymes catalyzing disulfide bonding. The localization of disulfide catalysts in an organelle downstream of the ER in the secretory pathway has long been puzzling. Recently, it has emerged that QSOX1 regulates particular glycosyltransferases, thereby influencing a central activity of the Golgi. Surprisingly, a few important disulfide-mediated multimerization events occurring in the Golgi were found to be independent of QSOX1. These multimerization events depend, however, on the low pH of the Golgi lumen and secretory granules. We compare and contrast disulfide-mediated multimerization in the ER *vs.* the Golgi to illustrate the variety of mechanisms controlling covalent supramolecular assembly of secreted proteins.

Keywords: disulfide bonds; glycosyltransferases; Golgi; mucins; QSOX1; redox regulation; secretory pathway; sialic acid; von Willebrand factor

The endoplasmic reticulum (ER) is the main site for disulfide-bond formation in secretory and cell-surface proteins [1] (Fig. 1). Upon co-translational insertion of nascent polypeptide chains into the ER lumen, cysteine amino acids are exposed to enzymes with redox-active disulfide bonds, to the glutathione redox buffer, and to small-molecule oxidants [2,3]. The ER promotes correct disulfide-bond formation in client proteins with the aid of feedback regulatory mechanisms that maintain the appropriate thiol/disulfide balance under a range of physiological and stress conditions [4–7]. The luminal environment of the ER must support oxidation of correct cysteine pairs but must also contain a sufficient concentration of reducing agents to prevent excessive kinetic trapping of misfolded proteins with improperly paired cysteines. The large family of protein disulfide isomerase (PDI) enzymes [8], which undergo dithiol-disulfide exchange with substrate proteins, are key contributors to the oxidative environment promoting protein folding within the ER. Other major players are enzymes that transfer electrons from cysteine pairs in their active sites to oxygen or other electron acceptors, often *via* bound cofactors, to generate disulfides [1]. Newly generated disulfides in these enzymes are then passed by dithiol-disulfide exchange to PDI proteins and on to oxidatively folding substrates [1].

Disulfide-bond quality and completeness are monitored at the exit from the ER. The protein primarily

Abbreviations
ECM, extracellular matrix; ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; IgM, immunoglobulin M; KO, knock out; PDI, protein disulfide isomerase; QSOX1, quiescin sulfhydryl oxidase 1; QSOX2, quiescin sulfhydryl oxidase 2; STs, sialyltransferases; VWF, von Willebrand factor.

responsible for disulfide quality control is ERp44, a PDI family protein that contains a single cysteine in its active site rather than the CXXC redox-active disulfide typical of the PDI family [9]. Selenoprotein F is another potential quality-control factor for secreted disulfide-rich proteins, but its mechanism of action is still unknown [10]. ERp44 functions by making mixed disulfides with cysteines that are either unpaired, mispaired, or transiently paired in functional intermediates. ERp44 has three major tasks: to help retain or retrieve certain ER-resident proteins, to prevent escape of incompletely oxidized client proteins from the ER, and to aid in the assembly of disulfide-bonded secretory complexes. Some of these tasks rely on the pH and zinc ion gradients along the secretory pathway, which induce a conformational change in ERp44 as it transits from the ER to the Golgi, exposing client-interacting surfaces as well as the cysteine in the active site [11]. This conformational change also enables the carboxy-terminal tail of ERp44 to interact with KDEL receptor, thereby allowing retrieval of ERp44 and its bound clients from the Golgi to the ER [12]. This mechanism suggests that, unless the quality-control system becomes overwhelmed or is bypassed, proteins with incompletely or improperly oxidized cysteines would not reach the Golgi apparatus or other downstream compartments of the secretory pathway.

The Golgi, in turn, is almost devoid of catalysts of disulfide-bond formation and rearrangement (Fig. 1). Though PDI proteins and other oxidoreductases typically localized to the ER are secreted from cells in certain cases [13,14] and may hence traverse the Golgi in the process, they do not accumulate in the Golgi.

Quiescin sulfhydryl oxidase 1 (QSOX1) and its less abundant paralog QSOX2 are the only sulfhydryl oxidases or oxidoreductases known to be specifically localized to the Golgi in animal cells [15,16]. This review will focus on QSOX1, which has been studied structurally [17–19], mechanistically [19,20], and functionally [21]. QSOX2, in contrast, is largely uncharacterized but, like QSOX1 [22–25], has been reported to be associated with cancer progression [26–28]. Golgi localization of QSOX1 was seen in multiple mammalian cell types [15] and in the unicellular parasite *Trypanosoma brucei* [29], suggesting a conserved and widespread function in this compartment. Correspondingly, QSOX1 has no detectable

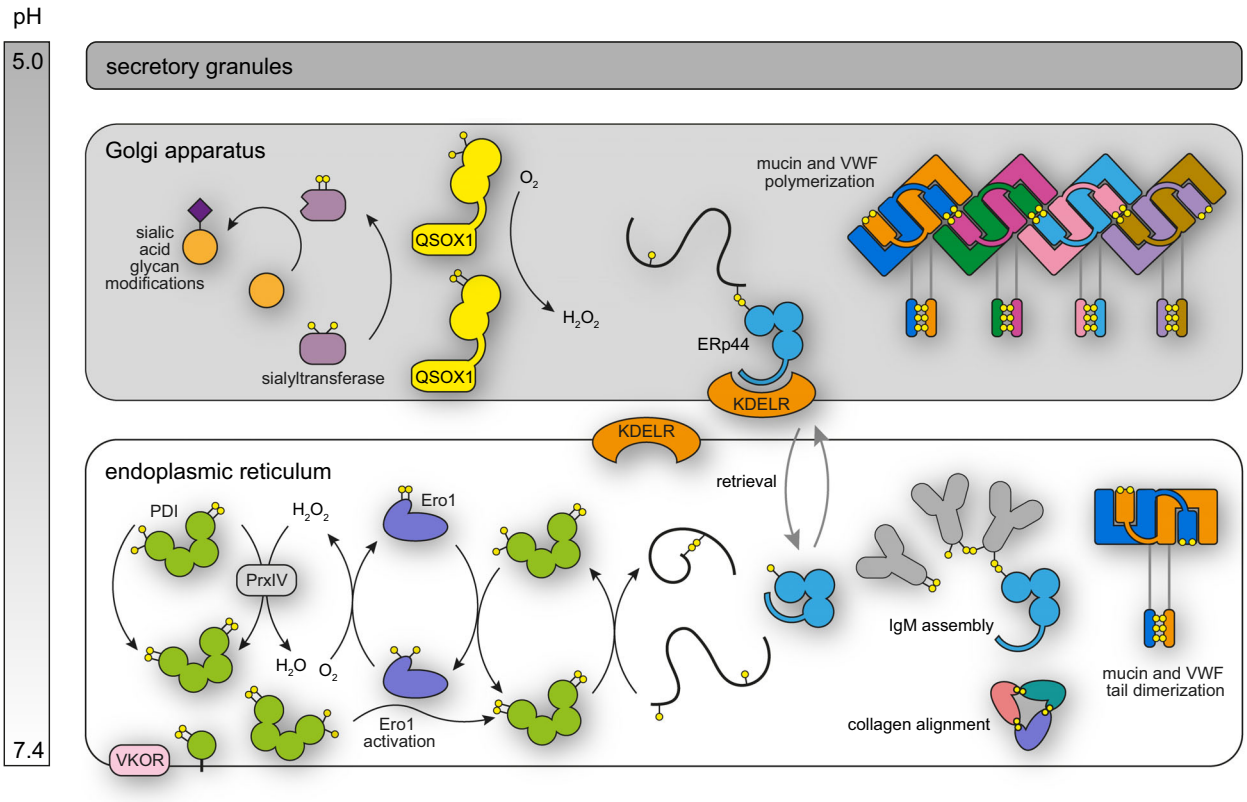

Fig. 1. Overview of disulfide bonding in the secretory pathway. Cysteine amino acids are represented by yellow balls. QSOX1, quiescin sulfhydryl oxidase 1; KDELR, KDEL receptor; VKOR, vitamin K epoxide reductase; ERO1, endoplasmic reticulum oxidoreductin 1; PrxIV, peroxiredoxin IV.

impact on oxidative protein folding in the ER [30]. Mammalian QSOX1 has two main splice variants, one with and one without a transmembrane region [23]. The transmembrane region is not required for Golgi localization, as recombinant QSOX1 segments containing only the globular, soluble domains accumulate in the Golgi when transfected into cultured cells [31]. In addition to its Golgi localization, QSOX1 is secreted in certain scenarios. For example, QSOX1 expression is highly upregulated in quiescent fibroblasts [32], and the enzyme is secreted from these cells [15,33]. QSOX1 is also secreted into blood [24,34], mucus [35], and other body fluids [36]. Mass spectrometry of QSOX1 purified from conditioned media of fibroblasts revealed that the splice variant with a transmembrane region is secreted along with a shorter form [15], indicating proteolytic cleavage as a means to release the ectodomain from the membrane. It is not yet clear whether secretion of QSOX1 is due to high expression saturating the Golgi retention mechanism or to a specific mechanism that overrides retention.

The extensive support system for oxidative protein folding in the ER, together with the ERp44-based quality control mechanism and the paucity of disulfide catalysts in the Golgi, emphasize the importance of disulfide-bond formation in the early secretory pathway. The dominance of the ER in oxidative protein folding raises the question of why it is beneficial for disulfide bonding to be largely complete prior to the trafficking of disulfide bond-containing proteins to the Golgi. One likely reason is that restricting disulfide-bond formation to the neutral pH of the ER lumen, which favors reactive thiolates, is more efficient than allowing the process to drift into the Golgi, where the lower pH increases protonation of cysteines, making them less reactive in oxidation and isomerization reactions. An additional, speculative reason relates to the function of the Golgi apparatus in providing secreted metalloenzymes with their metal cofactors [37–39]. A high concentration of cysteine thiol groups, such as that found in the ER, might result in non-specific chelation of copper and zinc. Instead, transporters in the Golgi membrane import these metals into a compartment with fewer unpaired cysteines, minimizing unproductive side reactions.

Another puzzle that arises from the canonical completion of oxidative protein folding and assembly in the ER is why the Golgi requires a dedicated "stand-alone" catalyst of disulfide bond formation, namely QSOX1. What are the functional benefits and beneficiaries of disulfide formation in the Golgi? It seems unlikely that the role of QSOX1 is merely to oxidize secretory substrates that have slipped through quality control in the ER. Moreover, we are not aware of any evidence that points to a role for QSOX1 in supporting mixed disulfide formation between ERp44 and target proteins for Golgi retrieval. Until recently, direct substrates that depend on QSOX1 for their activities were not known, although QSOX1 was speculated to be involved in specific disulfide-mediated protein assembly in the Golgi [40,41]. What features characterize true, physiological substrates of QSOX1? Why is disulfide-mediated protein complex formation in the Golgi so rare, and what distinguishes such assembly processes that do occur in the Golgi from those that occur in the ER? Recent progress in understanding the biological function of QSOX1 and the mechanisms of disulfide-bonded protein complex assembly help shed light on these questions.

QSOX1 catalyzes disulfide bond formation in the Golgi and extracellular environments

QSOX1 is a stand-alone enzyme in multiple senses. First, the lack of other enzymes that catalyze disulfide bonding in the Golgi (aside from its paralog QSOX2) is in sharp contrast to the numerous, diverse factors engaged in disulfide formation in the ER [1] (Fig. 1). In addition, QSOX1 performs, alone, activities that are typically carried out in the ER by multiple, separate proteins. In the ER, enzymes such as Endoplasmic reticulum oxidoreductin 1 (Ero1 in yeast; Ero1-like protein alpha and Ero1-like protein beta paralogs in humans) generate disulfides *de novo* by transferring electrons to small-molecule electron acceptors [42,43]. Disulfides formed within Ero1 are then transferred to PDI family proteins, which in turn transfer them to substrates. QSOX1 can carry out the roles of both Ero1 and PDI because it has two functional modules [18,20,44]. Like Ero1, the carboxy-terminal module of QSOX1 contains a flavin adenine dinucleotide (FAD) cofactor used to transfer electrons from a pair of FAD-proximal cysteines to molecular oxygen, generating a disulfide and a molecule of hydrogen peroxide [43]. However, the QSOX1 FAD-binding domain has a different helical topology than that of Ero1 [45], consisting of an ERV fold [46]. The amino-terminal module of QSOX1 resembles the first two thioredoxin-fold domains of PDI and contains another redox-active disulfide, which is reduced by substrate thiols and regenerated by dithiol-disulfide exchange with the FAD-proximal disulfide in the carboxy-terminal module [18,47,48] (Fig. 2). Switching of the PDI-like module between intra- and intermolecular interactions requires conformational changes, which are enabled by

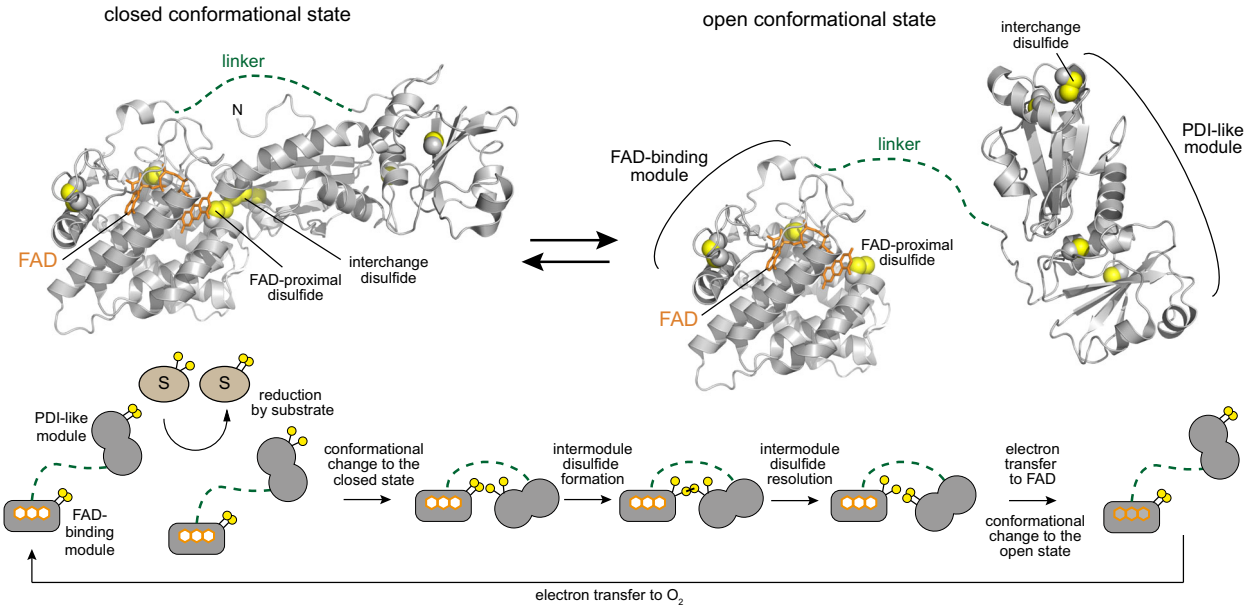

Fig. 2. Structure and catalytic cycle of QSOX1. The FAD cofactor is shown as orange sticks and cysteine side chains as spheres with yellow sulfurs. The closed state of QSOX1 (PDB ID 4P2L) juxtaposes the substrate-interacting (interchange) disulfide with the FAD-proximal disulfide. The open state structure in this image was generated by arbitrarily re-positioning the PDI-like module relative to the FAD-binding module. In the illustration of the QSOX1 mechanism below, the transient intermodule disulfide is emphasized with a short, black line connecting the cysteine sulfurs. "S" denotes a substrate. The FAD is represented as three fused hexagons with a white interior when in the oxidized state and a gray interior when reduced. Some of the events diagrammed as stepwise may occur concomitantly.

the flexible linker connecting the two modules [18]. In its full cycle, QSOX1 independently catalyzes electron transfer from substrate proteins *via* multiple redox centers to molecular oxygen.

The identity of the substrate proteins on which QSOX1 acts has been a long-standing question. QSOX1 oxidizes a range of reduced proteins *in vitro* [49,50], including some that are localized to cell types and environments expressing high levels of QSOX1 [51]. Nevertheless, the conclusion that any given protein is a physiological substrate of QSOX1 requires a demonstration that cysteines that are normally disulfide bonded in the protein remain reduced, or the function of the protein in cells or in organisms is compromised, in the absence of QSOX1 activity. Such evidence has until recently been lacking.

Physiological pathways affected by QSOX1

Several physiological functions have been identified for QSOX1 by focusing on cell types that express high levels of the enzyme. Quiescent fibroblasts produce large amounts of QSOX1 and secrete it into the extracellular milieu. Fibroblast entry into quiescence is also characterized by a massive increase in extracellular matrix (ECM) production [52]. As expected from its co-expression with ECM precursors [32], QSOX1 contributes to ECM organization in cultured cells [15], in tumors [53], and in certain normal tissues (unpublished observations). In the absence of QSOX1, fibroblast ECM is mechanically less resilient, but somewhat paradoxically, also less penetrable by tumor cells [15]. This apparent paradox is resolved by considering the importance of cross-linked, stiff ECM for tumor cell migration [54]. In addition to increased expression in cancer-associated fibroblasts [53,55], QSOX1 has been observed to be upregulated in tumor cells [22,56]. Tumor-cell QSOX1 has been proposed to activate matrix metalloproteases, leading to increased cell invasion into matrix [57,58]. The contribution of QSOX1 to ECM assembly *vs.* ECM degradation may be cell-type and context-dependent, and the mechanisms by which the enzyme contributes to each process are currently unknown.

Goblet cells of the intestinal mucosa are another cell type producing high levels of QSOX1. Like typical fibroblasts, goblet cells are terminally differentiated cells that specialize in the secretion of large network-forming proteins. The product of goblet cell secretion, however, is mucus rather than ECM. Intestinal mucus was found to be unstable and poorly functional when

produced in mice lacking QSOX1, such that these animals were hypersensitive to induced colitis [21]. As described below, no evidence was found that QSOX1 introduces disulfide bonds *in vivo* into the major glycoproteins that make up mucus. *In vitro*, QSOX1 oxidizes an intestinal mucus-associated lectin protein, ZG16 [51], but it is not yet known whether ZG16 is a physiological substrate of QSOX1 *in vivo* or how the ZG16 disulfide might contribute to mucus properties [59]. In contrast, QSOX1 was found to introduce disulfides into certain Golgi glycosyltransferases in the intestinal epithelium, with clear physiological consequences [21]. Specifically, the sialyltransferases (STs) St6gal1 and St3gal1, enzymes that introduce sialic acid into glycoproteins and glycolipids, were excessively reduced, and thus presumably inactive [60–62], in QSOX1 knockout (KO) mice [21]. Inactivity of the reduced STs was supported by the observation that colon mucosal tissue of QSOX1 KO mice had a much lower content of sialylated glycans than normal colon [21]. QSOX1 was seen to oxidize rapidly a regulatory disulfide bond adjacent to the active site of the St6gal1 human ortholog [21]. Together, these observations establish that QSOX1 activity influences the levels of sialic acid decoration of glycans in intestinal tissue, but the biochemical or biophysical mechanism by which under-sialylation destabilizes colonic mucus remains to be determined.

Interestingly, the main activity of QSOX1 in goblet cells appears to be carried out in the Golgi, whereas the activity of QSOX1 in the ECM can be accomplished extracellularly. The importance of intracellular QSOX1 activity in the mucosa was demonstrated by the presence of unpaired sialyltransferase cysteines in colon epithelial cell extracts of QSOX1 KO mice [21]. As evidence for extracellular QSOX1 activity, ECM formation in fibroblast cultures lacking QSOX1 was rescued by supplying the enzyme to the culture supernatants [15]. Moreover, extracellular inhibition of QSOX1 during construction of a fibroblast monolayer interferes with the adhesion and migration of cancer cells subsequently added in co-culture [15]. Last, intraperitoneal administration of QSOX1 inhibitory antibodies affected tumor ECM organization in a murine model for breast cancer [53], but inhibitory antibody administration by the same route did not recapitulate the QSOX1 KO phenotypes of intestinal mucus instability and poor protective function [21]. The explanation for the latter observation could be either that Golgi-localized QSOX1 is inaccessible to antibodies or that the antibodies did not reach the intestinal mucosa. In either case, these observations show that the various physiological activities of QSOX1 are distinguishable conceptually and pharmacologically.

Other physiological processes affected by QSOX1 include proper maintenance of heart tissue, as mice lacking QSOX1 exhibited cardiomyopathy along with altered cardiac calcium homeostasis and chronic unfolded protein response [63]. Another possibly related observation is that QSOX1 supports the vascular response to mechanical injury [64]. Interestingly, in a human genome-wide association study, a potential link was noted between rare coding sequence variants of QSOX1 and hypertension [65]. It is not known whether these phenomena reflect QSOX1 function in ECM assembly or represent a distinct activity specific to heart and vascular tissue nor whether Golgi-localized or secreted QSOX1 is involved. However, QSOX1 supplied extracellularly was found to promote vascular smooth muscle cell migration in culture [64].

Disulfide-mediated complex formation in the Golgi

In parallel with the limited number of Golgi-localized enzymes that catalyze disulfide bond formation are a limited number of disulfide-mediated protein assembly events that occur in the Golgi. The best-established of these is the disulfide-mediated polymerization of the large glycoproteins known as gel-forming mucins, which are the major macromolecular constituents of mucus, and von Willebrand factor (VWF), which is a blood protein involved in hemostasis. Despite the different sites in the body in which these glycoproteins function and their different physical properties and biological roles, VWF and gel-forming mucins (hereafter referred to as mucins) are highly conserved in the regions responsible for disulfide-mediated polymerization [66] (Fig. 3A). Therefore, their polymerization mechanisms can be described interchangeably, and progress in understanding each of these molecules has informed research on the other [40,66–70].

von Willebrand factor and mucins polymerize in two steps, the first occurring in the ER and the second beginning in the Golgi. In the first step, VWF and mucins undergo disulfide-mediated dimerization near their carboxy termini in the ER (Fig. 3A) [71,72], aided by PDI [73]. After the formation of the three intermolecular disulfide bonds that connect the carboxy-terminal "tails," the glycoproteins then face an engineering challenge: how to form additional disulfide bonds linking their amino termini to produce long head-to-head, tail-to-tail polymers rather than disulfide-bonded dimers linked by both termini (Fig. 3B). Before describing the solution to this problem, we will briefly review the evidence that the

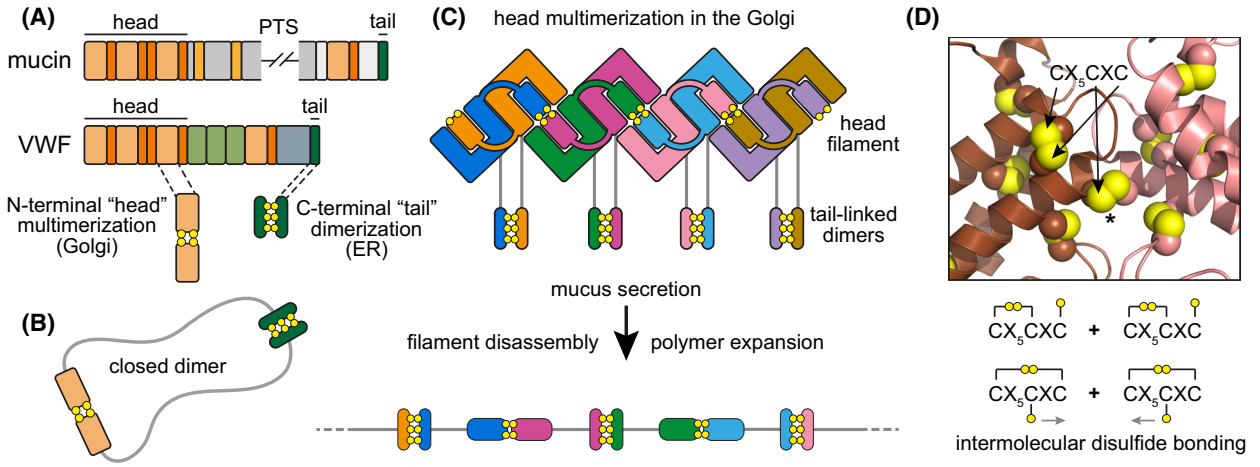

Fig. 3. Schematic diagrams of mucin and VWF organization and assembly. (A) Domain organization of mucins and VWF, emphasizing the similar "head" and "tail" regions, which engage in disulfide bonding in the Golgi or the ER, respectively. "PTS" stands for proline, serine, and threonine, which are common amino acids in the large, heavily O-glycosylated middle portion of mucins, which has been deleted for compactness. Disulfides are represented as paired yellow spheres. (B) Illustration of a possible side product of disulfide formation between mucin heads and tails. Polymerization depends on avoiding closed dimers. (C) Illustration of self-assembly of mucin head regions into filaments upon exposure to the low pH of the Golgi. Filament formation juxtaposes the multimerization domains of the heads and promotes intermolecular disulfide bonding. Domain swapping ensures that heads belonging to the same tail-linked dimers do not become linked. Upon secretion, the filament disassembles, and mucin polymers expand to form the mucus hydrogel. (D) Mucin 2 (MUC2; PDB ID 6RBF) structure in the region of the CX_5CXC motif and illustration of the thiol-disulfide rearrangement that exposes the middle cysteine for intermolecular disulfide bonding. The brown- and salmon-colored molecules are two copies of the MUC2 head linked by the intermolecular disulfide near the middle of the panel (*).

second step of polymerization indeed occurs in the Golgi. Pulse-chase experiments showed that VWF polymerization and processing of high mannose to complex carbohydrates occur together and after a delay that represents transport from the ER to the Golgi [74]. Further support was provided by the observation that the addition of ammonium chloride to cultured endothelial cells, to make the Golgi less acidic, blocked VWF polymerization [75]. VWF dimers purified from supernatants of ammonium chloride-treated cells could be induced to form disulfide-linked polymers *in vitro* by incubation at *trans*-Golgi pH, i.e., pH ~ 5.8 [76].

The participation of the Golgi in mucin and VWF assembly is the key to solving the problem of how to generate polymers rather than closed dimers. The observation that lowering the pH promotes disulfide-mediated polymerization seems counterintuitive, as protonation of cysteine thiols is expected to slow redox chemistry. It can be explained, however, by an indirect role for low pH in bringing together the proper protein domains to position cysteines for disulfide bonding. High-resolution analysis of low-pH assembly intermediates of mucins and VWF amino-terminal regions [66,69,70,77], together with extensive low-resolution studies of the domain organization of VWF [78], led to a model in which the amino termini of the tail-linked dimers interact non-covalently in such a way that they are prevented from disulfide bonding with one another. Instead, the low pH of the Golgi lumen induces filamentation of the amino-terminal regions, which juxtaposes the relevant cysteines from separate dimers. This mechanism ensures that the second step of disulfide-mediated polymerization, formation of two intermolecular disulfide bonds between the two juxtaposed amino termini, occurs between rather than within tail-linked dimers [66] (Fig. 3C). This elegant synergism of cell and structural biology produces head-to-head, tail-to-tail polymers in a regulated and organized manner.

While supramolecular assembly solves the problem of how to juxtapose the correct cysteines, it does not explain how cysteines are oxidized to form disulfides during mucin and VWF polymerization in the Golgi. Before relevant structural information on these glycoproteins was available, the observation that the VWF amino acid sequence contains a CXXC motif led to the hypothesis that isomerase activity of this motif promoted intermolecular disulfide bonding of the amino termini [76,79]. However, there is no evidence that this motif is redox-active, and the failure of polymerization upon mutation of the motif was likely an indirect effect of undermining protein folding or

stability. In the structures of VWF and mucins, the cysteines of the CXXC motif were found to be buried and disulfide bonded to two other cysteines in the fold [66,70], making it likely that they are conserved for structural purposes and that the redox-resonant cysteine spacing is functionally insignificant. Instead, another set of closely spaced cysteines, in a CX_5CXC motif, appears to be involved in a thiol-disulfide rearrangement that promotes intermolecular disulfide bond formation [40]. The current working hypothesis is that the first two cysteines in this motif are disulfide-bonded to one another in the ER, and the third is buried and unpaired [40]. To promote filamentation in the Golgi, the buried, third cysteine is thought to perform a nucleophilic attack on the original disulfide, forming a new disulfide with the first cysteine and liberating the second cysteine to form one of the intermolecular disulfides seen in the high-resolution mucin and VWF amino-terminal region structures (Fig. 3D) [66,68–70]. Such a mechanism could prevent premature disulfide bonding of the amino termini in the ER, prior to the alignment of the glycoprotein heads for correct cysteine pairing in the Golgi.

The disulfide rearrangement described above for the amino-terminal regions of mucins and VWF involves the transfer of electrons from one cysteine to another but no net oxidation. In contrast, the final formation of the two intermolecular disulfide bonds are oxidation reactions that require an electron acceptor. As QSOX1 accepts electrons from cysteine pairs and delivers them to molecular oxygen, and the enzyme is also found in the right cell types and subcellular localization, QSOX1 was an obvious candidate for catalyzing or facilitating this reaction. Surprisingly, VWF and lung mucin polymerization occurred to wild-type levels in QSOX1 knockout mice [21]. This finding shows that QSOX1 is not required as the direct catalyst of disulfide-mediated polymerization in the Golgi, nor is it needed indirectly for establishing the redox conditions in the Golgi that support polymerization. The identity of the electron acceptor for mucin and VWF intermolecular disulfide bonding in the Golgi is still unknown, but small thiol-containing compounds such as cysteine or glutathione may be involved [70].

Comparison of disulfide-mediated protein complex formation in the Golgi and in the ER

In addition to mucins and VWF, other large, secreted protein complexes are supported by intermolecular disulfide bonds. In contrast to mucins and VWF, however, most other disulfide-mediated complex assembly processes do not occur in the Golgi. One may ask what is different about disulfide bonding of quaternary structures such that some take place in the ER and others require participation of downstream compartments. Formation of fibrillar collagens is a notable example of a disulfide-mediated assembly process that occurs in the ER. Collagen I contains the characteristic proline- and glycine-rich region that forms collagen triple-helices, appended at each terminus by globular N-propeptide and C-propeptide domains. The C-propeptide domain aids in collagen trimer assembly by establishing the correct alignment of the strands in the triple helix, as well as by determining the allowed stoichiometries of collagen chain types (e.g., two copies of the C-Proα1(I) and one copy of C-Proα2(I) to form the standard type I collagen) [80]. Assembly of C-propeptide trimers depends on the presence of calcium, which is bound at a crucial site next to an intermolecular disulfide bond [81,82] (Fig. 4), such that calcium is necessary for covalent multimerization [80]. As the calcium concentration is high in the ER, the intermolecular disulfide bonding of collagens can occur in this compartment. Disulfides are important in mediating the alignment and assembly of collagens, but the formation of closed homo- or heterotrimers does not require the stepwise assembly occurring in multiple organelles seen for mucins and VWF.

Like VWF and mucins, assembly of penta- or hexameric immunoglobulin M (IgM) is a multistep process, but like collagens, it appears to be completed prior to the Golgi. Multimeric IgM antibodies are produced early in infection, before affinity maturation refines the humoral immune response. The low affinity of a single antibody "monomer" (μ_2L_2) is compensated in IgM by increased avidity of the assembled multimeric antibody. The heavy chain of secreted IgM is terminated with a conserved tailpiece that forms

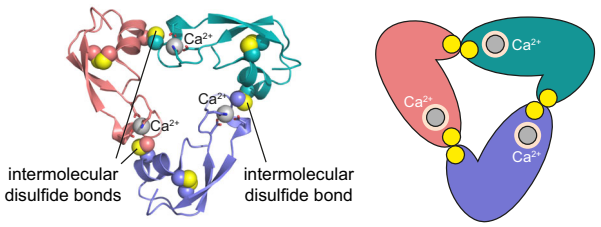

Fig. 4. Disulfide-mediated trimerization of collagen. A segment of the procollagen I homotrimeric C-propeptide domain, based on PDB ID 5K31, is shown as a ribbon diagram (left) and a schematic illustration (right). Calcium-binding side chains are shown as sticks in the left image; the acidic pockets formed by these side chains are represented by pink circles surrounding the gray calcium ions (Ca^{2+}) in the right image. Disulfides are represented by yellow spheres.

intermonomer disulfide bonds [83–85]. However, these intermonomer bonds do not form in the first step of intracellular assembly. Instead, the tailpiece cysteines initially engage in a temporary intramonomer disulfide (Fig. 5A). To promote the formation of the intermonomer bonds in the second step of assembly, ERp44 attacks one of the disulfide-bonded tailpiece cysteines, forming a covalent intermediate of monomeric IgM and ERp44. The other, displaced tailpiece cysteine is concomitantly freed to engage another IgM monomer, or ERp44-IgM monomer complex, propagating assembly toward the complete IgM multimer [86]. In the case of pentameric IgM, disulfide-mediated multimerization also includes a heteromeric, and strikingly asymmetric, interaction with a protein known as the J chain (Fig. 5B). Most of the intermonomer disulfides at the carboxy termini of the tailpieces were not resolved in a cryo-EM structure of pentameric IgM assembled with the J chain [85]. The cysteines involved are on flexible segments of the polypeptide, outside a β-sandwich formed by two sheets of parallel β-strands contributed by the 10 tailpieces in the assembly (Fig. 5B). The flexibility of the cysteine-containing region may facilitate participation of ERp44 in the assembly process, as the ERp44 active site would not be able to access juxtaposed cysteines in folded, packed domains. This situation contrasts with the head regions of mucins and VWF, which form intermolecular disulfide bonds using relatively well-folded domains closely juxtaposed to one other [67–70,77].

Additional differences can be noted between IgM and mucin multimerization. In contrast to mucins, in which the wrong head-to-head disulfides must be avoided to achieve polymerization (Fig. 3B), IgM intramonomer disulfides were shown to be essential intermediates on the pathway toward multimers [86]. Consequently, there is no need in IgM assembly to mask the tailpiece cysteines such that they do not form disulfides with one another in the monomer. The ERp44-mediated process of IgM assembly can thus be conducted in the ER, and the quality-control role of ERp44 likely ensures that partially assembled intermediates do not progress along the secretory pathway.

Golgi thiol redox-active enzymes in animal cells *vs.* yeast

This review focuses on disulfide formation and redox regulation in the Golgi of mammalian cells. Many insights into cysteine-mediated redox processes occurring in other organelles, such as the ER and mitochondria, have initially been obtained from research in yeast [87–89] and subsequently extended to mammals [90,91]. However, unicellular organisms are less informative regarding Golgi function in higher organisms. Yeast do not produce the complex extracellular matrix, mucins, hemostasis factors, and components of intercellular communication characteristic of multicellular animals. These proteins are heavily reliant on Golgi activities for their essential posttranslational modifications. Yeast lack QSOX enzymes and ERp44, and they perform a restricted set of O-glycosylation reactions. In contrast, yeast possess certain Golgi redox-active enzymes apparently lacking in animals. Glutaredoxins (Grxs) are enzymes that reduce disulfide bonds in client proteins and are typically re-reduced by glutathione. They may also catalyze the addition or removal of glutathione from protein thiols. Grxs are

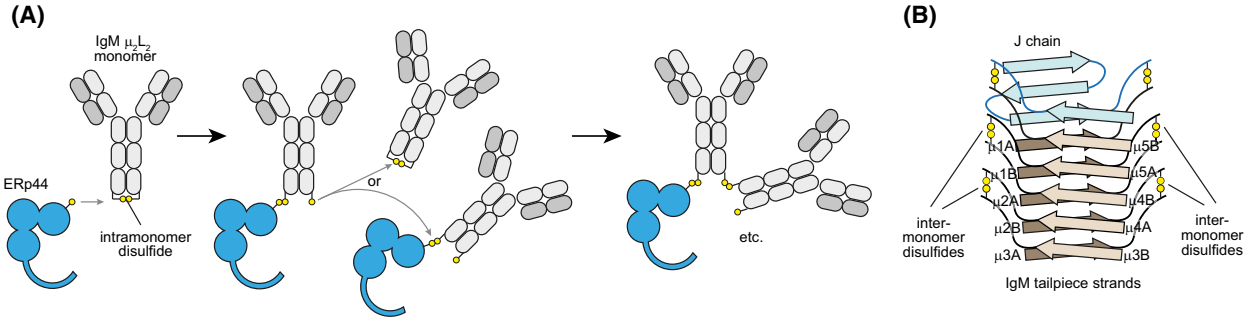

Fig. 5. Schematic diagram of IgM disulfide mediated assembly. (A) An IgM μ_2L_2 monomer (the Y-shaped structure) first assembles with a disulfide bond between the two heavy (μ) chains (intramonomer disulfide). ERp44 forms a mixed disulfide with one of the cysteines in this disulfide, freeing the second cysteine to attack another disulfide-bonded monomer or an ERp44-IgM monomer complex. A continuation of this process produces the complete disulfide-bonded penta- or hexameric IgM complex. (B) Cartoon representation of the central portion of the final product of IgM intermonomer disulfide bonding (tan and brown) in the presence of the J chain (light blue), based on structure PDB ID 6KXS. Ten distinct tailpieces contribute to the complex, two of which engage in disulfide bonds with the J chain. Some of the intermonomer disulfides do not appear in the original structure but are drawn here for completeness.

found in a variety of subcellular locations in both yeast and animals, but, to our knowledge, none have been identified in the Golgi of animal cells. In contrast, the glutaredoxins Grx6 and Grx7, which contain monothiol active sites (CXXS), are found in the *cis*-Golgi of *Saccharomyces cerevisiae* [92], with Grx6 also present in the ER [93]. Grx6 was shown to protect yeast from oxidative stress [94] and to be involved in cellular calcium homeostasis [95]. The function of Grx7 is less clear, but its loss exacerbates the sensitivity of cells lacking Grx6 to heat stress [92]. Overall, it appears that, despite major overlaps in the functions of the Golgi apparatus in yeast and animals, the organelle has substantially diverged to accommodate distinct cellular environments, stresses, and secretomes.

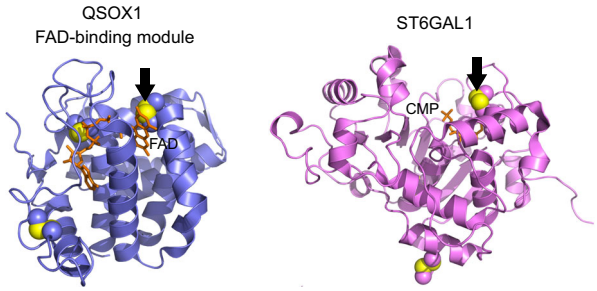

Fig. 6. The FAD-binding module of QSOX1. The cartoons show the FAD-binding module of QSOX1 (PDB ID 3LLK) and the human ST6GAL1 protein in complex with cytidine 5′-monophosphate (CMP) (PDB ID 4JS2). The cysteines that engage with the QSOX1 PDI-like module (not shown) are indicated by arrows. In both cases, these cysteines are exposed on the surface of a helical domain.

Conclusions and perspectives

The identification of the first physiological QSOX1 substrates in the Golgi, together with the demonstration that certain proteins undergoing oxidative supramolecular assembly in the Golgi are not QSOX1 substrates, suggest hallmarks of biologically relevant QSOX1 targets. Unfolded model proteins can be oxidized by QSOX1 *in vitro* [49] but are not likely to be relevant QSOX1 substrates because they are absent from the Golgi and extracellular fluids. Folded proteins with relatively buried juxtaposed cysteines, such as supramolecular assemblies of mucins or VWF, were also found not to be QSOX1 substrates, consistent with steric exclusion of QSOX1 from the interface [49,96]. It should be noted that the QSOX1 PDI-like module interacts alternately with substrate proteins and with the QSOX1 FAD-binding module during catalysis (Fig. 2). Therefore, one might hypothesize that QSOX1 substrates would somehow resemble the QSOX1 FAD-binding module. Interestingly, the ST6GAL1 sialyltransferase cysteines that are oxidized rapidly by QSOX1 [21] are exposed on a surface of the enzyme with some general similarities to the positioning of the redox-active cysteines in the QSOX1 FAD-binding module (Fig. 6). The question as to whether any specific similarities exist between QSOX1 inter-module interactions (Fig. 2) and the interactions of QSOX1 with its substrates can be addressed in the future by determining the structures of trapped covalent complexes between QSOX1 and STs or other yet-unknown *bona fide* substrates.

In addition to understanding what makes a protein a physiological substrate for QSOX1, there is the question of how such substrates would avoid oxidation prior to arriving at the Golgi. The Golgi is substantially more oxidizing than the ER, as indicted by intraluminal redox probes [62,97]. Therefore, QSOX1 substrates may simply pass through the ER reduced and inactive. However, to bypass ERp44 or for other reasons, more sophisticated mechanisms that enable ER exit may also come into play. For example, STs and their partner glycosyltransferases have been reported to undergo quaternary structural rearrangements upon transit to the Golgi [98], which may indicate a more complex process for exposure of regulatory cysteines and oxidative activation in the Golgi. Disulfide bonding and activation in the Golgi may be functionally important for these enzymes to prevent premature sialic-acid capping, allowing glycans to become extended by other glycosyltransferases prior to receiving their terminal sugars.

The link between Golgi QSOX1-mediated redox regulation and sialic-acid addition to glycans [21] is a significant recent discovery. A function for QSOX1 in regulating glycan elaboration in the Golgi explains its unique intracellular localization and distinguishes it from the oxidative protein folding machinery of the ER. Connecting QSOX1-mediated disulfide-bond formation with glycan elaboration further emphasizes the generality and centrality of glycosylation in Golgi function. In contrast, the contribution of disulfide bonding in the Golgi to protein complex assembly appears to be limited to specific cases, i.e., mucins and VWF, and to proceed in the absence of catalysis by QSOX1 [21]. The full implications of positioning QSOX1 on the sialylation pathway remain to be explored. It has been observed that perturbing Rab11, a regulator of endocytic recycling, alters the distribution of particular STs among Golgi and post-Golgi compartments, selectively affecting addition of sialic acid to glycans in certain linkages [99]. Though speculative, it is possible that

altered sialyltransferase localization enhances or diminishes the co-localization with QSOX1 and thus the extent of sialyltransferase oxidative activation. STs are active not only in the colonic mucosa, and sialylation has a role in diverse physiological and pathological processes such as the immune response, pathogen-host recognition, and cancer [100–102]. It will therefore be important to explore whether QSOX1, either through its expression levels or its access to oxygen, contributes to regulating sialic-acid modification of glycoproteins in the contexts listed above.

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