Division of labor between oxidoreductases:
TMX1 preferentially acts upon transmembrane polypeptides

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Running Title: TMX1 selectivity for transmembrane proteins

The endoplasmic reticulum (ER) is the site of maturation for secretory and membrane proteins in eukaryotic cells. The lumen of the mammalian ER contains more than 20 members of the protein disulfide isomerase (PDI) superfamily that ensure formation of the correct set of intra- and inter-molecular disulfide bonds as crucial, rate-limiting reactions of the protein folding process. Components of the PDI superfamily may also facilitate dislocation of misfolded polypeptides across the ER membrane for ER-associated degradation (ERAD). The reasons for the high redundancy of PDI family members and the substrate features required for preferential engagement of one, or the other, are poorly understood. Here we show that TMX1, one of the few transmembrane members of the family, forms functional complexes with the ER lectin calnexin (CNX) and preferentially intervenes during maturation of cysteine-containing membrane-associated proteins, while ignoring the same cysteine-containing ectodomains if not anchored at the ER membrane. As such, TMX1 is the first example of a topology-specific client protein redox catalyst in living cells.

Introduction
The mammalian ER contains 23 members of the PDI family (Tannous et al, 2015). These are characterized by the presence of one or more thioredoxin (Trx)-like domains that may contain an active site with a Cys-Xxx-Xxx-Cys (CXXC) consensus sequence. Enzymatically active PDIs catalyze formation, reduction and isomerization of intra- or inter-molecular covalent bonds between luminal cysteine residues of newly synthesized polypeptides entering the secretory pathway, conferring structural stability to the native proteome (Appenzeller-Herzog & Ellgaard, 2008; Bulleid, 2012; Ellgaard & Ruddock, 2005; Oka & Bulleid, 2013). The reason for the high number

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of PDIs in the ER and their individual roles in protein biogenesis remains unclear (Bulleid, 2012). Certainly, individual members of the PDI family show some substrate preference. For example, ERp57 forms functional complexes with CNX and calreticulin (CRT) (Frickel et al, 2002; Jessop et al, 2007; Oliver et al, 1997; Pollock et al, 2004; Zapun et al, 1998). CNX and CRT are ER lectins that bind newly synthesized polypeptides displaying mono-glucosylated N-linked oligosaccharides (Hammond et al, 1994; Hebert et al, 1995). ERp57 acts upon their ligands i.e., viral glycoproteins (Molinari & Helenius, 1999; Solda et al, 2006) or endogenous glycoproteins sharing common structural domains (Jessop et al, 2007) thereby promoting the formation of native inter- and intra-molecular disulfide bonds. In contrast, P5 targets BiP-bound proteins (Jessop et al, 2009) and PDI, ERp72 and ERdj5 facilitate dislocation of misfolded proteins or toxin subunits from the ER lumen into the cytosol (Forster et al, 2006; Majoul et al, 1997; Molinari et al, 2002; Tsai et al, 2001).

The majority of the PDIs are soluble luminal proteins characterized by a KDEL-like retention signal and transcriptional up-regulation in response to activation of the unfolded protein response (UPR) (Anelli et al, 2002; Chichiarelli et al, 2007; Cunnea et al, 2003; Galligan & Petersen, 2012; Lee et al, 2003; Roy & Lee, 1999; Tasanen et al, 1992). Five members of the family, TMX1-5, are anchored at the ER membrane. Though no information is available for TMX5, for TMX1-4 it has been reported that they are not induced by cellular stresses (Haugstetter et al, 2005; Koritzinsky et al, 2010b; Matsuo et al, 2009; Sugiura et al, 2010).

Here we focus on TMX1, which is highly expressed in kidney, liver, placenta and lungs (Matsuo et al, 2001). The presence of a proline residue at position 2 of the TMX1 catalytic site (CPAC, residues 56-59) hints at a possible role of TMX1 as an ER reductase (Hatahet & Ruddock, 2009). Consistent with such a role, TMX1 facilitates cell intoxication by ricin and abrin, which requires a reductive step promoting dislocation of the catalytic subunit to the cell cytosol (Pasetto et al, 2012) and reduces insulin disulfides in vitro (Matsuo et al, 2001). Deletion of the TMX1 gene results in susceptibility to liver damage in mice (Matsuo et al, 2013). At the cellular level, TMX1 deletion has no phenotype, thus indicating the activation of compensatory mechanisms (our unpublished results and (Matsuo et al, 2013)). In this study, we report on a mass spectrometry analysis with a trapping mutant version of TMX1 expressed in mammalian cultured cells that reveals the selective association of TMX1 with a series of endogenous membrane-bound client proteins. A systematic analysis performed with membrane-bound and soluble model polypeptides confirmed the exquisite preference of TMX1 for membrane-bound substrates and identifies TMX1 as the first example of topology-specific PDI transiently associating both with folding-competent and folding-defective membrane-bound polypeptides.
Results

TMX1 preferentially associates with membrane-bound substrates

During the reaction cycles leading to disulfide bond formation, disassembly or re-arrangement, active site cysteines of PDIs transiently form short-lived mixed disulfides with surface-exposed cysteine residues of proteins expressed in the ER lumen (Huppa & Ploegh, 1998; Molinari & Helenius, 1999). The replacement of the resolving carboxy-terminal cysteine residue in the PDIs catalytic site efficiently traps mixed disulfides in the reductive pathway (Fig. 1A) (Hatahet & Ruddock, 2009). Thus, PDI trapping mutants have been used to identify endogenous substrates of select ER-resident oxidoreductases such as ERp57, PDI, P5, ERp18, ERp72, ERp46 and ERdj5 (Dick & Cresswell, 2002; Jessop et al, 2007; Jessop et al, 2009; Oka et al, 2013; Schulman et al, 2010).

To identify endogenous substrates of TMX1, we expressed the trapping mutant TMX1_{C/A} (Fig. 1B) in mouse embryonic fibroblasts (MEF) and performed a mass spectrometry analysis of the cellular proteins co-immunoisolated with the ectopically expressed reductase. In contrast to the analyses performed for other PDIs that revealed both soluble and membrane-bound proteins as endogenous substrates (Dick & Cresswell, 2002; Jessop et al, 2007; Jessop et al, 2009; Oka et al, 2013; Schulman et al, 2010), TMX1_{C/A} selectively trapped a series of cysteine-containing membrane proteins (Table 1). To determine whether the selective immunoisolation of membrane proteins was symptomatic of the intrinsic specificity of TMX1 for membrane-bound substrates, we made use of a series of model polypeptides characterized by the presence of a cysteine-containing ectodomain tethered or non-tethered at the ER membrane (Fig. 2A).

Firstly, MEF were mock-transfected (EV for empty vector, Fig. 2B, lane 1), transfected with a plasmid for expression of β1-tagged TMX1_{C/A} (lane 2), or with plasmids for expression of TMX1_{C/A} and the folding-competent membrane-bound BACE501 (lane 3), the folding-competent and soluble BACE501Δ (lane 4) (Solda et al, 2007), the folding-defective membrane-bound BACE457 (lane 5), or the folding-defective soluble BACE457Δ (lane 6) (Molinari et al, 2002). Cells were detergent-solubilized and the ectopically expressed, HA-tagged BACE variants were immunoisolated from post-nuclear supernatants. The immunocomplexes were separated in SDS-PAGE and transferred to PVDF membranes. The presence of the BACE variants was revealed by western blot (WB) with anti-HA antibodies (Fig. 2B, upper panel). The association of TMX1_{C/A} was assessed, in the same PVDF membrane, upon decoration of the membrane with anti-TMX1 antibodies (Fig. 2B, lower panel).

The membrane-bound BACE501 is separated in two forms (G is the Golgi, mature, EndoH-resistant form and E is the ER, immature, EndoH-sensitive form of the protein, Fig. 2B, lane 3) (Solda et al,
In the cell lysate, the soluble BACE501Δ is only present in the E form as the G form is rapidly released in the extracellular medium (Solda et al., 2007). BACE457 and BACE457Δ are only present in their E form since they are ER-retained, folding-defective polypeptides (Molinari et al., 2002). The experiment reveals that TMX1_{C/A} associates with the membrane-bound versions of BACE (i.e., BACE501 and BACE457, Fig. 2B, lower panel, lanes 3 and 5). In contrast, TMX1_{C/A} is not found in immunocomplexes containing the soluble variants of BACE (i.e., BACE501Δ and BACE457Δ, Fig. 2B, lower panel, lanes 4 and 6, respectively). The selectivity of TMX1 for membrane-bound polypeptides was confirmed upon immunoisolation from the cell lysates of the ectopically expressed β1-tagged TMX1_{C/A} (Fig. 2C, lower panel) that shows the abundant coprecipitation of BACE501 and BACE457 (upper panel, lanes 3 and 5). Confirming the specificity of this essay, of the G and the E forms of BACE501, only the latter, which is in the ER, is in the TMX1_{C/A}-containing immunocomplexes (Fig. 2C, lane 3). The association with both BACE501 and BACE457 shows that TMX1 does not discriminate between folding-competent and folding-defective polypeptides.

To further confirm the preference of TMX1 for association with membrane-bound proteins, we assessed association of the TMX1 trapping mutant with the soluble protein A1AT (Fig. 2A and (Perlmutter, 2011)) and of two variants of A1AT that were artificially tethered at the ER membrane with two different membrane anchors (Fig. 2A, A1AT-BACE and A1AT-CD3δ). Consistent with a preferential association of TMX1 with membrane-bound polypeptides, TMX1_{C/A} associated with A1AT only when tethered at the membrane (Figs. 2D and 2E, lanes 1 vs. 2). Interestingly, and in contrast with the case of BACE proteins, the A1AT ectodomain contains a single cysteine residue. Hence, there are no intramolecular disulfides to be attacked by TMX1. However, there is published evidence that the A1AT cysteine undergoes various reversible modifications such as S-nitrosylation, S-glutathionylation, sulfenic acid formation, S-cysteinylolation and oxidation, which induces polymerization on the A1AT cysteine (Alam et al., 2011; Glaser et al., 1982; Grek et al., 2012; Griffiths et al., 2002; Miyamoto et al., 2000; Tyagi & Simon, 1992). Each of these modifications could be attacked by the substrate trap mutant of TMX1 to form a mixed disulfide.

**TMX1 establishes mixed disulfides with newly synthesized membrane-bound BACE501**

An active involvement of TMX1 in determining the fate of the associating proteins should involve formation of mixed disulfides as reaction intermediates (Fig. 1A). To directly assess this, we performed a pulse and chase experiment. MEF were transfected with two empty vectors (Figs. 3A-3D, EV, lane 1), with expression vectors for BACE501, BACE501Δ, TMX1 or TMX1_{C/A} and an empty vector (lanes 2-5), or with expression vectors for BACE501 and TMX1 (lane 6), BACE501 and TMX1_{C/A} (lane 7), BACE501Δ and TMX1 (lane 8) or BACE501Δ and TMX1_{C/A} (lane 9).
Transfected cells were pulsed with $^{35}$S-methionine and cysteine for 13 min and chased for 10 min. At this time of chase, the newly synthesized BACE501 variants are still folding in the ER as confirmed by the EndoH-sensitivity of their oligosaccharides (SFig. 1A) (Solda et al, 2007). BACE (Figs. 3A-3B) or TMX1 variants (Figs. 3C-3D) were immunoisolated from detergent extracts and complexes were analyzed in non-reducing (Figs. 3A and 3C) and reducing SDS-PAGE (Figs. 3B and 3D).

In non-reducing gels, BACE501 and BACE501Δ expressed alone (Fig. 3A, lanes 2 and 3) or in combination with TMX1 (lanes 6-9) are separated in a series of radiolabeled bands corresponding to various oxidation forms (Figs. 3A and SFig. 1B, fully oxidized, fOx; partially oxidized, pOx, for BACE501 and BACE501Δ respectively). The bands with faster electrophoretic mobility correspond to BACE forms with lower hydrodynamic radius due to the presence of intramolecular disulfide bonds (fOx in Fig. 3A and SFig. 1B) that relapse into the reduced (Red) BACE501 or BACE501Δ forms in the reducing gel (Figs. 3B, 3D and SFig. 1B) (Braakman et al, 1992).

In cells where the membrane-bound BACE501 was co-transfected with the trapping mutant TMX1C/A, separation of the BACE immunoisolates under non-reducing conditions revealed an abundant labeled polypeptide band with an apparent MW of about 90 kDa (MD, Fig. 3A, lane 7). Sample reduction dissociated the radiolabeled 90 kDa polypeptide in its components, i.e., BACE501 with an apparent MW of about 60 kDa and a radiolabeled polypeptide with an apparent MW of about 35 kDa (Fig. 3B, lane 7). Both the 90 kDa polypeptide (Fig. 3A, lane 7) and the 35 kDa polypeptide (TMX1, Fig. 3B, lane 7) are much less abundant when BACE501 is co-transfected with the wild type form of TMX1, which is unable to stabilize the mixed disulfide with the substrate. This led us to conclude that the 90 kDa polypeptide is a mixed disulfide (MD, Fig. 3A, lane 7) containing BACE501 and TMX1C/A, which is dissociated under reducing conditions releasing the radiolabeled TMX1 polypeptide of 35 kDa (Fig. 3B, lane 7). Consistent with this hypothesis, mixed disulfides were significantly more abundant when the BACE501 was co-expressed with the trapping mutant compared to the wild type version of TMX1 (Fig. 3C, lanes 7 vs. 6). Moreover, both the MD in the non-reducing gel (Fig. 3A, lane 9) and the 35 kDa polypeptide in the reducing gel were virtually absent when the trapping mutant of TMX1 was co-transfected with the soluble BACE501Δ (Fig. 3B, lane 9) that does not associate with TMX1 (Figs. 2B-2C). Again, MD were separated in their BACE501 and TMX1 constituents in the reducing gel (Fig. 3C, lane 7). This confirms that the TMX1 trapping mutant stabilizes the otherwise short-lived MD intermediate of the BACE501 redox reaction. Cells expressing TMX1C/A and BACE501, also contained larger Disulfide Bonded Complexes (DBC) of more than 200 kDa (Figs. 3A and 3C, lanes 7).
Reduction of the functional complexes containing BACE501 and TMX1\textsubscript{C/A} revealed a radiolabeled polypeptide with an apparent MW of 97 kDa in Figs. 3B and 3D, lanes 7. This polypeptide was identified by WB and by specific immunoprecipitation as the lectin chaperone CNX. The increased presence of CNX in the BACE and in the TMX1\textsubscript{C/A} immunoisolates from cells over-expressing BACE501 (lane 7 in Figs 3B and 3D, respectively) compared to cells that are not over-expressing BACE501 (lanes 5 and 9), lead us to propose that CNX is a component of TMX1 functional complexes, which are assembled or stabilized in the presence of TMX1 substrates.

**BACE501:TMX1:CNX complexes**

To assess whether CNX forms functional complexes with TMX1, we examined the consequences of cell exposure to castanospermine (CST), a glucose analog that prevents association of newly synthesized proteins with CNX (Hammond et al, 1994). MEFs were mock-transfected (EV, Figs. 4A-4B, lanes 1-2), co-transfected with EV and a plasmid for expression of TMX1\textsubscript{C/A} (lanes 3-4), co-transfected with EV and a plasmid for expression of BACE501 (lanes 5-6) or with plasmids for expression of TMX1\textsubscript{C/A} and BACE501 (lanes 7-8). Before solubilization and immunoisolation of ectopic BACE501 (Fig. 4A) or of endogenous CNX with the associated polypeptides (Fig. 4B), cells were incubated for 10h in absence (-) or in presence (+) of CST to clear the CNX chaperone system from endogenous substrates (Figs. 4A-4B, lanes 2 and 4) or from endogenous substrates and ectopically expressed BACE501 (lanes 6 and 8). In the absence of CST, CNX strongly interacted with TMX1\textsubscript{C/A}, which is a non-glycosylated protein (Fig. 4B, lower panel, lanes 3 and 7). This association was substantially reduced upon CST treatment (lower panel, lanes 4 and 8; TMX1 is not glycosylated, thus excluding the possibility of its direct, glycan-lectin association with CNX). Thus, inhibition of *endogenous* (Fig. 4B, lane 4) and *endogenous + ectopic* (lane 8) substrates access to the CNX chaperone system substantially reduces the fraction of TMX1 co-precipitated (i.e., participating in a functional complex) with CNX. Altogether, the data in Figs. 3-4 show that CNX and TMX1 may form a functional complex, which is stabilized by client substrates. This conclusion is supported by the hampered association of TMX1 with CNX in cells with reduced protein synthesis (Figs. 4C, lane 4 and 4D, lane 3), or with defective N-glycosylation upon exposure to tunicamycin (Figs. 4C, lane 5 and 4D, lane 4).

**Characterization of TMX1\textsubscript{C/A}:BACE501 mixed disulfides by WB**

To further confirm the selective involvement of TMX1 in mixed disulfides with membrane-bound clients, BACE501 was expressed alone (Fig. 5A, lanes 1-2), with TMX1 (lanes 3-4) or with TMX1\textsubscript{C/A} (lanes 5-6). After immunoisolation of the HA-tagged bait, the immunocomplexes were separated in SDS-PAGE under non-reducing (NR, Fig. 5A, lanes 1, 4, 5) and reducing conditions.
(R, lanes 2, 3, 6). Proteins were then transferred to a PVDF membrane. BACE501 (Figs. 5A, lanes 1-6) or TMX1 (lanes 7-12) were revealed with HA- or TMX1-specific antibodies.

Confirming the data obtained with radiolabeled proteins (Fig. 3), the MD with the apparent MW of 90 kDa were only seen upon separation under non-reducing conditions of immunoisolates of cells co-transfected with BACE501 and TMX1_{CA} (Fig. 5A, lane 5 for the BACE501 component of the MD and lane 11 for the TMX1 component). The undetectable level of immunoreactivity in cells expressing the wild type form of TMX1 (Fig. 5A, lanes 3-4 and 9-10) confirms that TMX1_{CA} stabilizes the otherwise short-lived reaction intermediate. Disassembly upon sample reduction revealed BACE501 and TMX1 as the constituents of the mixed disulfides (Fig. 5A, lanes 6 and 12, respectively). The absence of mixed disulfides when the same experiment was performed with the soluble BACE501_{Δ} variant further supported the selectivity of TMX1 for membrane-bound substrates (Fig. 5B).

**TMX1 and BACE501 maturation**

Mixed disulfides are short-lived intermediates formed during the productive interaction between an oxidoreductase and its substrates (Huppa & Ploegh, 1998; Molinari & Helenius, 1999). Their stabilization upon mutation of the resolving cysteine residue in the oxidoreductase’s catalytic site is expected to delay substrate release from the ER. As for all glycoproteins, release of BACE501 from the ER can be monitored by the modification of protein-bound oligosaccharides that occurs during transit in the Golgi compartment, which reduces the electrophoretic mobility of the polypeptide chain and confers resistance to EndoH cleavage (Rothman et al, 1984). To determine whether the co-expression of the TMX1 trapping mutant delays the attainment of the BACE501’s EndoH-resistant status, MEFs expressing only BACE501, BACE501 and TMX1 or BACE501 and TMX1_{CA} were pulse labeled and chased for 10 (Fig. 6A, lanes 1, 3 and 5) or for 90 min (lanes 2, 4 and 6). After 10 min of chase, radiolabeled BACE501 expressed alone (Fig. 6B, lanes 1-2), co-expressed with TMX1 (lanes 5-6), or with TMX1_{CA} (lanes 9-10) is sensitive to EndoH cleavage. This is consistent with the ER localization of the newly synthesized polypeptide (Solda et al, 2007). The analysis after 90 min of chase showed that when expressed alone or with the wild type form of TMX1, more than 85% of radiolabeled BACE501 displayed EndoH-resistant oligosaccharides (Figs. 6B, lanes 3-4 and 7-8, respectively and 6C). This is consistent with the efficient export to the Golgi of this protein (Solda et al, 2007). The stabilization of the TMX1:BACE501 mixed disulfides upon co-expression of TMX1_{CA} dramatically reduced the fraction of EndoH-resistant BACE501 to less than 40% of the radiolabeled protein (Figs. 6B, lanes 11-12 and 6C). In contrast, and consistent with the selectivity of TMX1 for membrane-bound polypeptides (Figs. 2-4), the secretion of BACE501_{Δ} (Figs. 6D, panel on the right and 6E) was unaffected by the co-expression of TMX1_{CA}.
It is of interest that only the co-expression of the TMX1 trapping mutant substantially reduced attainment of EndoH-resistant oligosaccharide as a measure of delayed BACE501 secretion (Fig. 6, Fig. 7A, lanes 3-4 and 7B). ERdj5C/A did associate with BACE501 but only marginally delayed secretion (by 10-15%, Fig. 7A, lanes 5-6 and 7B). BACE501 co-expression with ERp57C/A (Fig. 7A, lanes 7-8 and 7B), ERp72C/A (lanes 9-10 and 7B), PDIc/A (lanes 11-12 and 7B) or P5c/A (lanes 13-14 and 7B) (Ellgaard & Ruddock, 2005; Jessop et al, 2009; Oka et al, 2013; Rutkevich et al, 2010) had no consequences. Notably, and in contrast with TMX1 (Figs. 6D-6E), ERdj5 also associated with BACE501Δ, thereby weakly reducing (by 20%) the secretion of this soluble model protein (SFigs. 2A, lanes 5-6 and 11-12, 2B and 3).

Discussion

The mammalian ER contains 23 members of the PDI superfamily marked by the presence of one or more thioredoxin-like domains (Ellgaard & Ruddock, 2005; Galligan & Petersen, 2012; Kozlov et al, 2010b; Tannous et al, 2015). Apart from this common feature, PDIs display different active site compositions, enzymatic properties, domains arrangement, interacting partners, sub-compartmental and tissue distribution (Bulleid, 2012; Galligan & Petersen, 2012). This high degree of divergence encourages the study of the individual PDI proteins because it suggests that they might have peculiar substrate specificity and/or might participate in distinct oxidative, reductive or isomerase pathways. Although informative, studies with purified enzymes fail to recapitulate the complex environment of the ER where molecular crowding, participation in supramolecular complexes, variable redox conditions in different compartmental microdomains may substantially affect the action of individual PDIs. Studies performed in living cells, where substrate specificity has been determined by using trapping mutants that substantially retard resolution of the mixed disulfide formed as intermediate during the folding or unfolding process, reveal a certain degree of redundancy (i.e., different PDIs may engage the same substrate in mixed disulfides) (Bulleid, 2012). The high redundancy of the PDI system is also highlighted by the hardly detectable phenotypes in cell lines derived from knockout mice where surrogate PDIs can functionally replace PDIs that have been deleted (e.g., ERp72 efficiently replaces ERp57 in assisting maturation of model glycoproteins (Solda et al, 2006) and other examples have been reported (Appenzeller-Herzog & Ellgaard, 2008; Kang et al, 2009; Rutkevich et al, 2010; Zhang et al, 2009)).

The studies performed in living cells, however, also underscore the preference of PDIs for specific substrates or for specific classes of substrates (e.g., ERp57 for glycoproteins entering the CNX/CRT cycle or ERp18 for proteins forming interchain disulfides as select examples (Frickel et al, 2002;
Our finding that the membrane-bound member of the PDI family TMX1 shows selectivity for transmembrane polypeptides and virtually ignores the same cysteine containing ectodomains when not tethered at the ER membrane regardless for their capacity to eventually attain the native structure represents, to our knowledge, the first example of topology-determined substrate selection of a PDI family member. The proline residue at position 2 of the catalytic site, the capacity to reduce insulin disulfides in vitro and the role in translocation of catalytic toxin subunits across the ER membrane (Hatahet & Ruddock, 2009; Matsuo et al, 2001; Pasetto et al, 2012) support a role of TMX1 as an ER reductase and infer at a possible involvement of TMX1 in ERAD, where reduction of inter- and intra-molecular disulfide bonds is a crucial step for misfolded protein retrotranslocation across the ER membrane (Hebert et al, 2010).

Notably, it has previously been shown that access of folding polypeptides to the CNX chaperone system leads to assembly/stabilization of functional complexes between CNX and the oxidoreductase ERp57 (Ellgaard & Frickel, 2003; Frickel et al, 2002; Jessop et al, 2007) or between CNX and the peptidyl-prolyl isomerase CypB (Kozlov et al, 2010a). Here we show that inhibition of endogenous or ectopic transmembrane protein association with CNX leads to disassembly/destabilization of functional complexes between CNX and TMX1 (Fig. 4B, lower panel, lane 3 vs. 4 for endogenous proteins and lane 7 vs. 8 for ectopic BACE501). On the other hand, sequestration of substrates in mixed disulfides with the trapping mutant version of TMX1 stabilizes the complexes between CNX and substrates (Fig. 4B, middle panel, lane 5 vs. 7). The fact that TMX1 is not glycosylated supports the conclusion that the membrane-bound oxidoreductase TMX1 participates in functional complexes with the membrane-bound lectin CNX. Having PDI members with different topologies possibly determining their substrate selection is reminiscent of the lectin chaperone system where the membrane-bound CNX and the soluble CRT show different substrate specificity to broaden the range of newly synthesized gene products that can be assisted early after synthesis in the ER (Danilczyk et al, 2000; Hebert et al, 1997; Molinari et al, 2004; Wada et al, 1995). Notably, topology-determined preferences have also been reported for the supramolecular complexes that regulate delivery at and dislocation across the ER membrane of misfolded polypeptides to be degraded into the cytosol, where soluble misfolded polypeptides show strict requirement of members of the HRD1 complex (Bernasconi et al, 2010; Ninagawa et al, 2011). Thus, cumulating evidence reveal that substrate topology is a key factor in determining engagement of folding, quality control and degradation pathways to insure production of the cellular proteome in appropriate quantity and quality.

Experimental Procedures
Antibodies, expression plasmids and inhibitors
Antibodies to TMX1 and HA were from Sigma-Aldrich, antibody to V5 from Invitrogen. The rabbit polyclonal antisera 855 and 809 (used to recognize the membrane-bound and the soluble BACE variants, respectively) were kind gift of P. Paganetti. Genes encoding for the HA-tagged TMX1 and TMX1<sub>C/A</sub> were subcloned in pCDNA3. β1-tagged TMX1 and TMX1<sub>C/A</sub> variants have been created by replacing the HA tag with an EFRH epitope, which is recognized by a monoclonal β1 antibody (Paganetti et al, 2005). Plasmids encoding for the membrane-bound and soluble BACE are described in (Molinari et al, 2002). V5-tagged ERdj5<sub>C/A</sub>, ERp57<sub>C/A</sub>, ERp72<sub>C/A</sub>, PDIC<sub>C/A</sub> and P5<sub>C/A</sub> expressing vectors are described in (Jessop et al, 2009; Oka et al, 2013). Tunicamycin, cycloheximide and CST (Sigma-Aldrich) have been used at final concentrations of 5 μg/ml, 50μg/ml and 1mM, respectively.

Cell lines, transient transfection
MEF were cultured in DMEM supplemented with 10% FBS. Cells grown on 3.5/6 cm culture dishes were transfected with 3 μg/6 μg of total plasmid DNA, using the jetPrime® reagent (Polyplus transfection). Experiments were performed 17 h after transfection.

Cell lysis, western blots
Cells were washed with phosphate buffered saline (PBS) containing 20 mM N-ethylmaleimide (NEM) for 1 min and then lysed with 2% CHAPS (Anatrace) in HEPES-buffered saline, pH 6.8, supplemented with 20 mM NEM and protease inhibitors for 20 min on ice. Post nuclear supernatants were collected by centrifugation at 10,000 g for 10 min. Samples were denatured and reduced in dithiothreitol (DTT)-containing sample buffer for 10 min at 65 °C and separated by SDS-PAGE. Proteins were transferred to PVDF membranes with the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with 10% (w/v) non-fat dry milk (Bio-Rad) and stained with the above mentioned primary antibodies and HRP-conjugated secondary antibodies. Membranes were developed using the Luminata<sup>TM</sup> Forte ECL detection system (Millipore), signals were detected with the ImageQuant LAS 4000 system in the Standard acquisition mode (GE Healthcare Life Sciences) and bands were quantified using the Multi Gauge Analysis tool (Fujifilm). For each antigen, the linearity of the detected signal range was ensured with appropriate loading controls.

Metabolic labeling, immunoprecipitations and EndoH treatment
Cells were pulse labeled with 0.1 mCi [<sup>35</sup>S]-methionine/cysteine and chased in DMEM supplemented with 5 mM unlabeled methionine and cysteine. Cells were lysed as described before and PNS and extracellular medium were collected by centrifugation at 10,000 g for 10 min, pre-cleared with protein A beads (Sigma, 1:10 w/v swollen in PBS) for 1 h at 4° C. Immunoprecipitation was performed with protein A beads and specific antibody overnight at 4° C.
After extensive washing of the immunoprecipitates with 0.5% CHAPS, beads were resuspended in sample buffer without (NR, non-reducing conditions) or with DTT (R, reducing conditions) and denatured for 10 min at 65 °C. Samples were subjected to SDS-PAGE. After exposure of the gels to autoradiography films (GE Healthcare, Fuji), films were scanned with the Typhoon™ FLA 9500 (Software Version 1.0). Bands were quantified using the ImageQuant software (Molecular Dynamics, GE Healthcare). For EndoH (New England Biolabs) treatment, immunoisolated proteins were split in two aliquots and incubated in the presence or absence of 5 mU of EndoH for 2h at 37°C. Samples were then analyzed by reducing SDS-PAGE.

**Mass Spectrometry**

Confluent MEF transfected with an empty vector or transfected with HA-tagged TMX1\textsubscript{C/A} were rinsed with PBS, 20 mM NEM. Cells were lysed with 2% CHAPS (Anatrace) in HEPES-buffered saline, pH 6.8, supplemented with 20 mM NEM and protease inhibitors for 20 min on ice. Immunoisolates were washed three times with lysis buffer. MS analysis was performed at the Protein Analysis Facility, University of Lausanne, Switzerland.

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Figure 1. TMX1 trapping mutant to stabilize TMX1-substrates complexes. A, Mechanism of reduction of disulfide bonds catalyzed by members of the PDI family. The formation of a mixed disulfide by the nucleophilic attack of the N-terminal active site cysteine on a substrate disulfide bond is shown. The resulting mixed disulfide can be resolved upon nucleophilic attack of the enzyme C-terminal active site cysteine on the mixed disulfide. B, TMX1 and TMX1_{C/A} constructs. The replacement of the resolving cysteine (amino acid 59 of the TMX1 sequence) with an alanine residue substantially stabilizes mixed disulfides between TMX1 and its substrates. SS, signal sequence, Trx, thioredoxin-like domain, TM, transmembrane domain. The sequence of the active site of TMX1 and TMX1_{C/A} and the position of the active site residues are shown.
Figure 2. TMX1 preferentially associates with membrane-bound substrates. A. Model polypeptides used in this study. B. MEF transfected with empty vectors (EV, lane 1), β1-tagged TMX1_{C/A} (lane 2) or TMX1_{C/A} in combination with HA-tagged BACE501, BACE501Δ, BACE457 or BACE457Δ (lanes 3-6, respectively). The HA-tagged model substrates were immunoisolated from cell lysates. Upper panel, WB with an anti-HA antibody to reveal the model substrates; lower
panel, WB with a TMX1-specific antibody to reveal TMX1\(_{C/A}\). C, Same as B, but TMX1\(_{C/A}\) was immunoisolated from the same cell lysates with an anti-β1 antibody to verify the presence of the model proteins (upper panel) in the immunocomplexes. D, Same as B-C for the soluble HA-tagged A1AT and the membrane-anchored A1AT-BACE (lanes 1 and 2, respectively). E, Same as D for the soluble HA-tagged A1AT and the membrane-anchored A1AT-CD3δ (lanes 1 and 2, respectively). G, mature Golgi form of BACE proteins; E, immature ER form; D, de-glycosylated form; *, antibody heavy chain recognized by the secondary antibody in WB.
Figure 3. TMX1 selectively establishes mixed disulfides with membrane-bound BACE501. A, MEF were transfected with empty vector (EV), BACE501, BACE501Δ, HA-tagged TMX1 or TMX1_{CA} (lanes 1-5), BACE501 in combination with HA-tagged TMX1 or TMX1_{CA} (lanes 6-7), BACE501Δ in combination with HA-tagged TMX1 or TMX1_{CA} (lanes 8-9). ^{35}S-methionine and -cysteine radiolabeled model substrates were immunoisolated from cell lysates with anti-BACE
antibodies. The immunocomplexes were separated under non-reducing conditions. B, Same as A but immunocomplexes were separated under reducing conditions. C, Same as A for complexes immunoisolated with anti-HA. D, Same as C, analysis under reducing conditions. pOx<sub>501</sub>, partially oxidized BACE501; pOx<sub>501,Δ</sub>, partially oxidized BACE501Δ; fOx<sub>501,Δ</sub>, fully oxidized BACE501Δ; fOx<sub>501</sub>, fully oxidized BACE501; Red<sub>501</sub>, reduced BACE501; Red<sub>501,Δ</sub>, reduced BACE501Δ; DBC, disulfide-bonded complexes; MD, mixed disulfides; CNX, calnexin.
Figure 4. Client-mediated association between TMX1 and CNX. A, MEF were co-transfected with an empty vector (EV, lanes 1-2), an empty vector and HA-tagged TMX1C/A (3-4), an empty vector and BACE501 (5-6) or with BACE501 and HA-tagged TMX1C/A (7-8). Cells were incubated for 10h in absence (-) or in presence (+) of CST [1mM]. The expression level of BACE501 was checked upon WB of the immunoisolated ectopic protein. B, Same as A, but endogenous CNX with associated proteins was immunoisolated from cell lysates. Immunocomplexes were analyzed under reducing conditions. Ectopically expressed BACE501 and TMX1C/A were revealed with an anti-BACE and an anti-HA antibody, respectively. C, Same as A, in cells treated with CST, cycloheximide (Chx) or tunicamycin (Tun) for 3 h. D, Same as B for cells treated with CST, Chx or Tun. G, mature Golgi form of BACE501; E, immature ER form; D, de-glycosylated form.
Figure 5. Characterization of TMX1<sub>C/A</sub>-BACE501 mixed disulfides by WB. A, MEF were co-transfected with BACE501 and an empty vector (EV, lanes 1-2), TMX1 (3-4) or TMX1<sub>C/A</sub> (5-6). BACE501 was immunoisolated from cell lysates and immunocomplexes were analyzed under non-reducing (NR) or reducing (R) conditions. B, Same as A, for BACE501Δ. red, reduced BACE; ox, oxidized BACE; DBC, disulfide-bonded complexes; MD, mixed disulfides.
Figure 6. Co-expression of TMX1<sub>C/A</sub> selectively delays BACE501 maturation. A, MEF were co-transfected with BACE501 and an empty vector (EV, lanes 1-2), TMX1 (3-4) or TMX1<sub>C/A</sub> (5-6). Transfected cells were pulsed with <sup>35</sup>S-methionine and -cysteine for 13 min and chased for 10 or 90 min. Ectopically expressed BACE501 was immunoisolated from cell lysates with an anti-BACE antibody. B, MEF were co-transfected with BACE501 and an empty vector (lanes 1-4), TMX1 (5-8) or TMX1<sub>C/A</sub> (9-12). The maturation of immunoisolated radiolabeled BACE501 (i.e., the attainment of EndoH-resistant oligosaccharides upon arrival in the Golgi complex) was monitored after 10 and 90 min chase. C, Quantification of the EndoH-resistant fraction of BACE501 after 90 min chase. The error bars show standard deviations of four independent experiments. D, MEF were co-transfected with BACE501Δ and an empty vector (EV, lanes 1-2), TMX1 (3-4) or TMX1<sub>C/A</sub> (5-6). The panel on the left shows the disappearance of immunoisolated BACE501Δ from the cell lysates (intracellular) that correlates with its secretion in the extracellular media (right panel, Secreted). E, Quantification of secreted BACE501Δ after 90 min chase. The error bars show standard deviation of three independent experiments. Significance analyzed by paired t-test (n.s.= not significant; *= p<0.05; **= p<0.01; ***= p<0.001). G, mature Golgi form of BACE501; E, immature ER form.
Figure 7. Trapping mutants of several PDI members and BACE501 maturation. A, MEF were co-transfected with BACE501 in combination with an empty vector (EV, lanes 1-2), TMX1_C/A (3-4), ERdj5_C/A (5-6), ERp57_C/A (7-8), ERp72_C/A (9-10), PD1/C_A (11-12) or P5_C/A (13-14). Ectopically expressed BACE501 was immunoisolated from cell lysates with an anti-BACE antibody. The maturation of radiolabeled BACE501 was monitored as in Fig. 6B. B, Quantification of the EndoH-resistant fraction of BACE501 after 90 min chase. The error bars show standard deviations of three independent experiments. Significance analyzed by paired t-test (n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001). G, mature Golgi form of BACE501; E, immature ER form.
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Table 1. Endogenous substrates of TMX1 identified by MS analysis.