Quality Control in the Secretory Pathway

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The intracellular fate of secretory immunoglobulin M and its µs heavy chain is altered during differentiation of B lymphocytes. We have shown that sIgM is efficiently secreted from plasma cells but is retained and rapidly degraded from B cells [1-3]. The degradation mechanism of retained proteins was elucidated by studies on the ΔF508 of the cystic fibrosis transmembrane conductance regulator [4,5]. It became evident that proteins that are retained in the endoplasmic reticulum are tagged by ubiquitin and eliminated by the 26S proteasome. This pathway, known as the ER-associated degradation or ERAD, is a quality control mechanism, because it eliminates misfolded proteins and orphan subunits of oligomeric proteins. In addition, the ERAD pathway is responsible for the destruction of unwanted proteins as the µs in B cells. We have shown that µs is a *bona fide* lumenal ERAD substrate. In B cells, µs is tagged by ubiquitin and degraded by the proteasome [2,6].

We are studying *cis*-acting motifs that act as retention signals and degrons and searching for *trans*-acting cellular factors that play a role in conferring retention and degradation. The best-characterized *cis*-acting motif is the µstpCys, the carboxy-terminal tailpiece of the µs that distinguishes this secretory heavy chain from the membrane µm form. It has been shown that µstpCys, comprising the unique 20 residues of the µs and its penultimate Cys575, are necessary for the retention of µs in B cells [7]. As shown in Figure 1, the µstpCys is also sufficient to confer ER retention onto reporter proteins, when fused to thyroid peroxidase (TPO) from which the transmembrane segment was deleted [Figure 1A] or to yellow fluorescent protein which is led to the secretory pathway by hen lysozyme signal sequence (ssYFP) [Figure 1B]. Clearly, when the penultimate Cys is replaced by Ser, the TPOµstpSer is secreted to the medium [Figure 1A] and the ssYFPµstpSer reaches the Golgi [Figure 1B]. Moreover, this µstpCys-conferred ER retention is not restricted to B cells, as this phenomenon takes place in the non-lymphoid COS-7 or HEK293 cells [Figure 1].

The µstpCys acts as a 'degron', as it confers degradation onto TPO [Figure 1 A,C and D] and ssYFP [Figure 1E]. Moreover, the µstpCys-conferred degradation is proteasomal, as TPOµstpCys [Figure 1D] as well as ssYFPµstpCys [Figure 1E] are stabilized in the presence of proteasome inhibitors. Again, this degradation occurs in the non-lymphoid HEK293 cells and interestingly, when ssYFPµstpCys is expressed in the 38C B cells, its degradation rate resembles the turnover of the endogenous µs heavy chain [Figure 1E]. Acting as a degron, the µstpCys most likely recruits the ubiquitination machinery to the otherwise stable reporter proteins. Nonetheless, the ubiquitin tag is not necessarily conjugated to the µstpCys, because degradation of the TPOµstpCys is indifferent to K-to-R substitution of the only Lys in the µstp [Figure 2A]. The half-life of 174 minutes measured for the wild-type chimera remains 178 minutes for the KR mutant [Figure 2C]. Hence, it indicates that either the ubiquitin is always conjugated to other Lys residues located in the reporter protein, or the ubiquitin conjugation is a promiscuous process that allows additional Lys residues to take over if the preferred K is mutated. Moreover, Asn563 in the µstp is a glycosylation site that in the case of TPOµstpCys is the most C-terminal of an additional four putative glycosylation sites. N-glycans play an important role in ER quality control and in ERAD [8,9] and it has been shown for the yeast ERAD substrate carboxypeptidase Y* that the most C-terminal of four N-glycans is necessary and sufficient to direct this protein to degradation [10,11]. However, the N-to-Q substitution in the TPOµstpCys not only does not hamper degradation, but in fact accelerates the turnover of the NQ chimera [Figure 2B], shortening the half-life from 174 to 112 minutes [Figure 2C].

In our search for *trans*-acting factors that participate in ERAD, we used the µs heavy chain as a bait and co-precipitated it with p97 [12]. The connection of this cytosolic AAA-ATPase to ERAD was suggested from the co-immunoprecipitation of p97 with the rapidly degraded µs in B cells but not with the same µs in plasma cells in which this heavy chain is a stable, secreted protein [12]. To unequivocally show the involvement of p97/Cdc48p in ERAD, we used yeast strains that harbor conditional mutants of Cdc48p, the p97 ortholog, as p97 is an essential protein [12]. Several groups have shown that p97/Cdc48p is an essential ERAD component [13-16]. Here we show that in mammalian cells, expression of excess p97 does not affect the turnover of ssYFPµstpCys [Figure 2D], suggesting that this abundant protein is not limiting. However, expression of equimolar amounts of the K524A p97 mutant stabilizes the ssYFPµstpCys [Figure 2D], suggesting that the ATP binding is essential for the function of this AAA-ATPase in ERAD.

sIgM = secretory immunoglobulin M
ER = endoplasmic reticulum
TPO = thyroid peroxidase

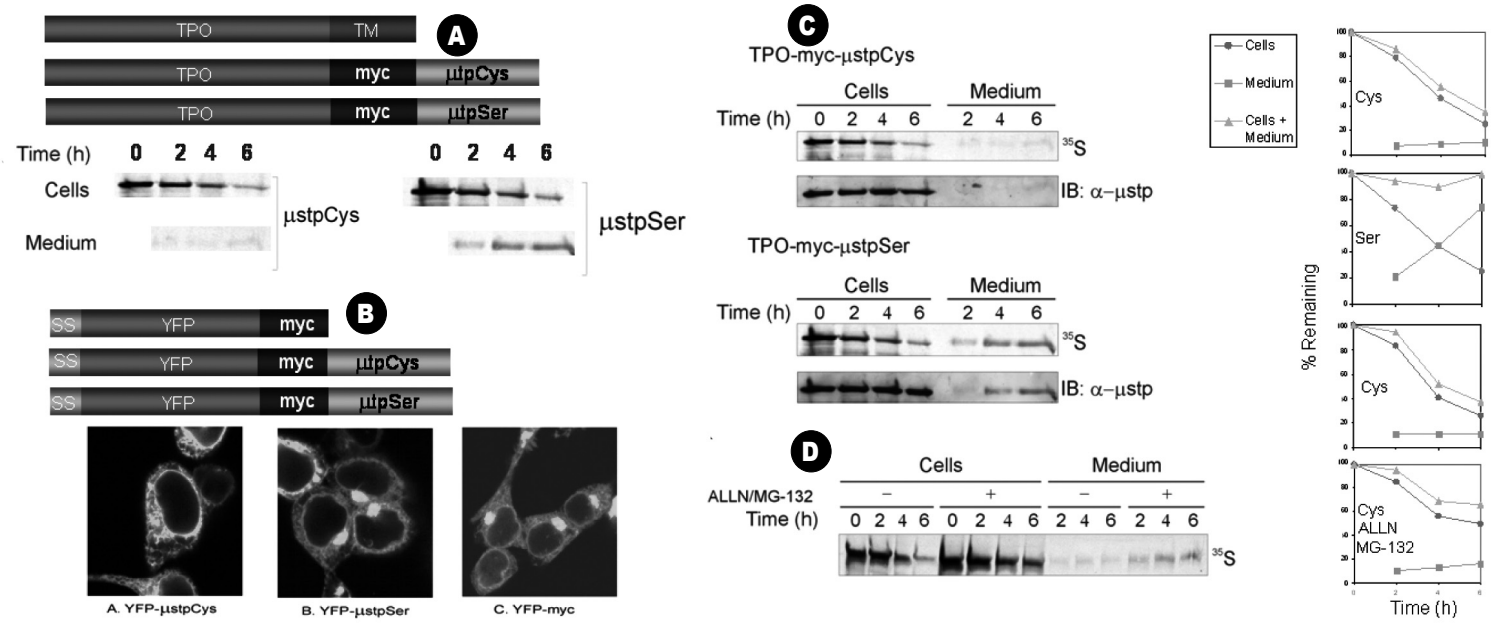

Figure 1. The µstpCys acts as a retention signal and as a 'degron' HEK293 were transfected with vectors encoding myc-tagged TPO-µstpCys or TPO-µstpSer (schematic constructs are presented) **[A,C]**, TPO-µstpCys **[D]**, myc-tagged ssYFP, ssYFP-µstpCys or ssYFP-µstpSer (schematic constructs are presented) **[B]**, or ssYFP-µstpCys **[E, right panel]**. A vector expressing ssYFP-µstpCys was also introduced to 38C B cells **[E, left panel]**. Cells were pulse-labeled with S^{35}-Met/Cys and chased for the indicated time without **[A,C]** or with (+) or without (-) ALLN and MG-132 **[D,E]**. Cells and medium were separated, and substrates were immunoprecipitated (IP) by an anti-myc antibody **[A,C,D]**, or from cells only by anti-myc **[E, right panel]** or by a combination of anti-myc and anti-µ antibodies **[E, left panel]**. Immunoprecipitates were resolved by SDS-PAGE, electroblotted and blots were exposed to autoradiography (^{35}S) **[A,C,D,E]** and then probed (IB) with an anti-µstp antibody (α-µstp) **[C, lower panels]**. For TPO-µstpCys/Ser, the remaining amounts – cells (circles), medium (squares) or their sum (triangles) – were calculated from densitometry as the percentage of the level at the beginning of chase (100%) and presented in graphs **[C,D]**. Alternatively, YFP was followed by confocal fluorescent microscopy **[B]**.

Being a cytosolic ATPase, we hypothesized that p97/Cdc48p provides the driving force for dislocating ERAD substrates from the ER back to the cytosol, where the ubiquitin-proteasome system resides. This notion has been supported by activation of the unfolded protein response in the *cdc*48 mutant at the non-permissive temperature [12]. Subsequently, we dissected the dislocation of the lumenal ERAD substrate CPY* into two consecutive steps – passage across the ER membrane and release to the cytosol – and showed that Cdc48p is essential for the passage [17]. The possibility that p97/Cdc48p provides the driving force for dislocation is in agreement with the view that AAA-ATPases are molecular machines that generate mechanical force by undergoing conformational changes upon nucleotide binding [18].

Additional AAA-ATPases that could play a role in ERAD are located at the base of the 19S regulatory particle of the proteasome. Known in yeast as Rpt subunits, we have taken advantage of strains in which a single Rpt is mutated at the conserved ATP binding K [19]. K-to-R substitution has been introduced to Rpt3, Rpt4, Rpt5 and Rpt6; K-to-S substitution has been introduced to Rpt1; K-to-R substitution accompanied by a second site S-to-F substitution has been introduced to Rpt2. It appears that from the six 19S Rpt subunits, Rpt2p and Rpt4p are the only AAA-ATPases required for ERAD of either the lumenal substrate CPY*-HA or the membrane substrate 6myc-Hmg2p [Figure 3A]. However, striking functional differences between Rpt2p and Rpt4p are revealed by monitoring the proteasome activity by zymography and visualizing the proteasome in non-denaturing PAGE upon incubation with the fluorogenic substrate Suc-LLVY-AMC [19]. The Rpt2RF-containing proteasome is inactive in this *in vitro* assay, probably due to gated access to the proteolytic chamber within the catalytic particle [19]. Conversely, the Rpt4R-containing proteasome is structurally stable and proteolytically active in this *in vitro* assay [Figure 3B]. This suggests that while Rpt2p is involved in the degradation of every proteasomal substrate, Rpt4p could preferentially participate in the degradation of ERAD substrates.

A step that is unique to ERAD substrates is their dislocation; we therefore speculate that Rpt4p contributes to dislocation. Indeed, cell fractionation and protease protection assay show that the dislocation of the lumenal ERAD substrate CPY*-HA is hampered in the *rpt*4R strain but not in the *rpt*2RF strain [Figure 4 A and B]. Using cell fractionation and protease protection assay,

CPY* = carboxypeptidase Y*

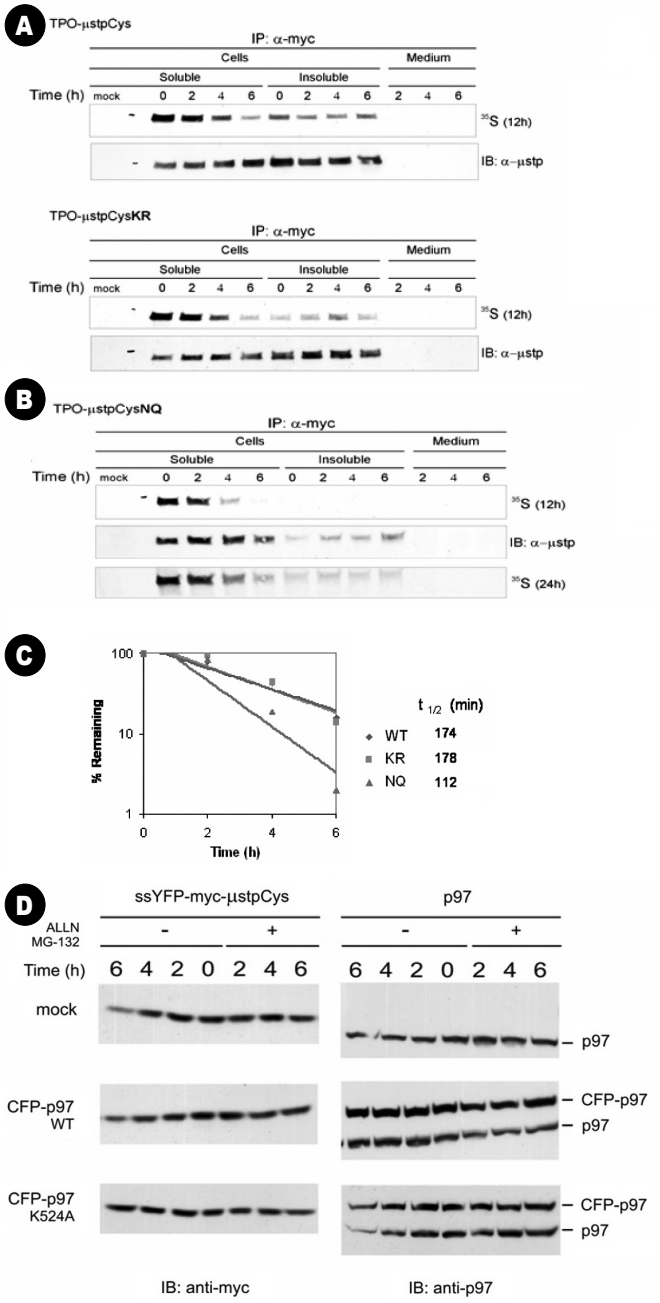

Figure 2. The μstpCys is not ubiquitinated, its N-glycosylation decelerates degradation, and expression of p97(K524A) stabilizes ssYFPμstpCys.
HEK293 were transfected with an empty vector (mock), with vectors encoding myc-tagged TPO-μstpCys (WT) or TPO-μstpCysKR in which the only K in the μstp was replaced by R **[A]**, TPO-μstpCysNQ in which the only N in the μstp was replaced by Q **[B]**, or combinations of myc-tagged ssYFPμstpCys and vectors encoding CFP-p97 wild-type (WT) or the K524A mutant **[D]**. Cells were pulse-labeled with S^{35}-Met/Cys and chased for the indicated time. Cells and medium were separated, cells were lysed and substrates were immunoprecipitated (IP) by an anti-myc antibody. Immunoprecipitates were resolved by SDS-PAGE, electroblotted and blots were exposed to autoradiography (^{35}S) **[A,B]** and then probed (IB) with an anti-μstp antibody (α-μstp) **[A,B]**. The remaining amounts of WT TPO-μstpCys (romboids), TPO-μstpCysKR (squares) or TPO-μstpCysNQ (triangles) were determined from densitometry as the percentage of the level at the beginning of chase (100%), plotted on a semi-logarithmic graph **[C],** and half-life values were calculated. Alternatively, at the indicated time after cycloheximide addition, degradation of ssYFPμstpCys was monitored by immunoblotting with an anti-myc antibody **[D, left panels]** and blots were re-probed with an anti-p97 antibody **[D, right panels]**. When indicated, the proteasome inhibitors ALLN and MG-132 were also added.

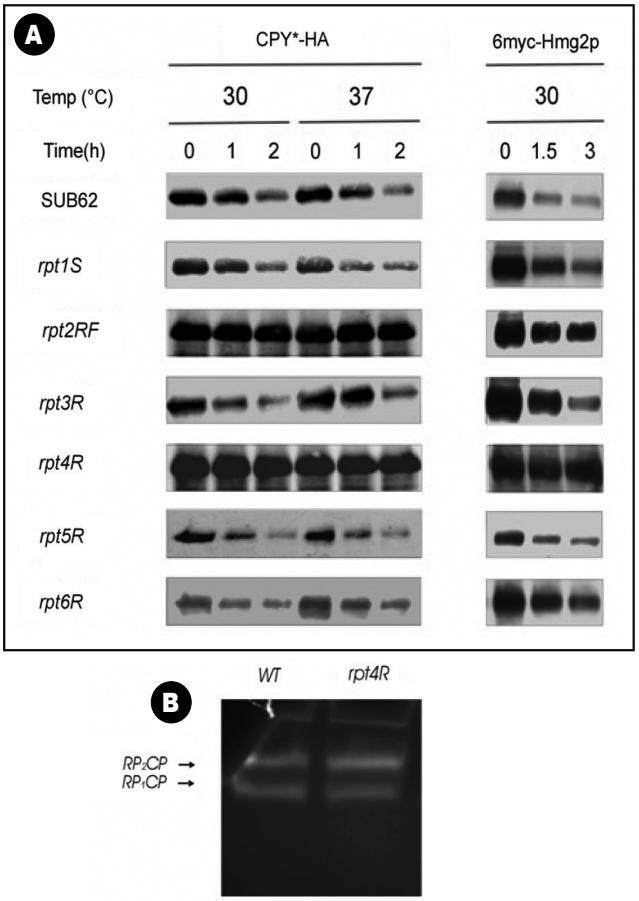

Figure 3. *rpt* mutants – stability, *in vitro* proteolytic activity and *in vivo* turnover of lumenal and membrane ERAD substrates.
[A] Degradation of CPY*-HA (left panels) and 6myc-Hmg2p (right panels) was followed in strains SUB62 (wild-type RPT), *rpt1S*, *rpt2RF*, *rpt3R*, *rpt4R*, *rpt5R* and *rpt6R* (*rpt* mutants). Following pre-incubation for 2 hours at the indicated temperatures, cycloheximide (150 μg/ml) was added and cells (2 OD_{600}) were collected at the indicated time points. Cells were dissolved and total cellular proteins were resolved by reducing SDS-PAGE and electroblotted onto nitrocellulose. Blots were probed with anti-HA (left panels) or anti-myc (right panels) mouse antibodies followed by HRP-conjugated anti-mouse IgG, and the HRP was visualized by ECL.
[B] Post-nuclear supernatant (PNS) was prepared from SUB62 (WT) and *rpt*4R mutant strain by disrupting yeast cells with glass beads in ice-cold Tris-HCl buffer. PNS was cleared by centrifugation (14,000 rpm, 10 minutes, 4°C) and equal amounts of protein were loaded onto non-denaturing PAGE supplemented with 0.5 mM ATP. Proteasome bands were visualized by peptidase activity assay using the fluorogenic substrate Suc-LLVY-AMC. The slower migrating band represents the doubly capped proteasome (RP_2CP) and the faster migrating band the singly capped proteasome (RP_1CP).

we show that CPY*-HA is similarly trapped within the ER lumen in either yeast strain expressing the temperature-sensitive *cdc*48 at restrictive temperature [17] or the *rpt*4R strain [Figure 4 A and B], suggesting that Cdc48p and Rpt4 participate in the passage of CPY*-HA across the ER membrane. Hence, we have hypothesized that excess Cdc48p would be able to suppress the ERAD defects in the *rpt*4R strain. Indeed, over-expression of Cdc48p from a plasmid to 145% of its initial level accelerates the degradation of CPY*-HA in the *rpt*4R strain by about fivefold [Figure 4C]. While the half-life of CPY*-HA in the *rpt*4R mutant was 17 times longer than that in the wild-type strain, extending from 26 minutes to

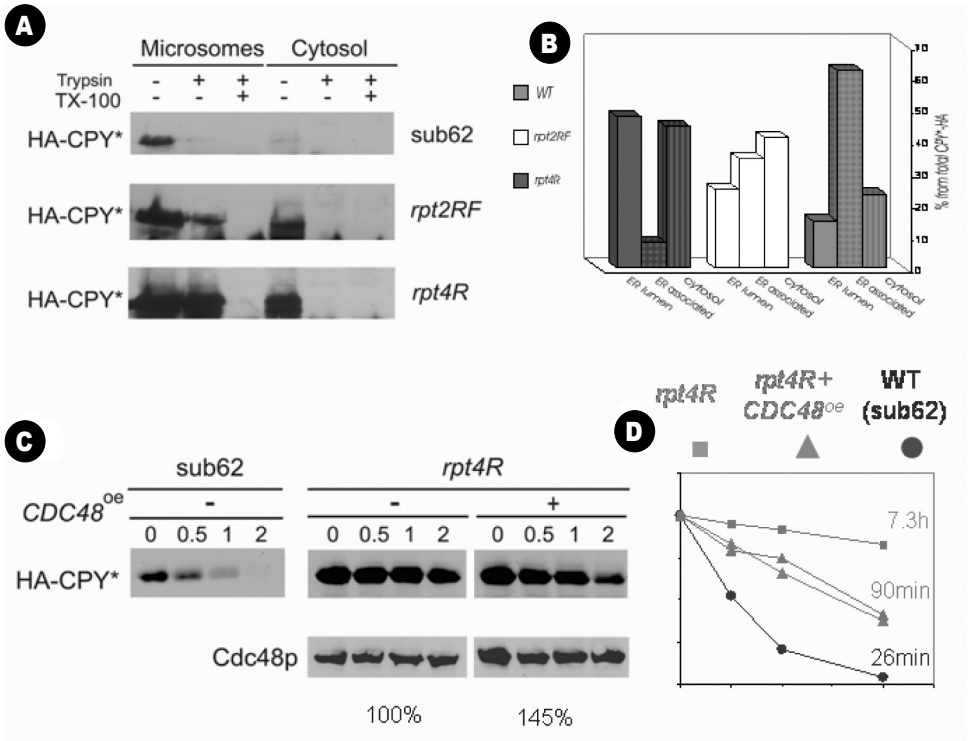

Figure 4. Rpt4 is involved in dislocation of CPY*-HA and over-expression of Cdc48p suppresses ERAD deficiency in *rpt*4R.

[A] Spheroplaste from SUB62 (WT strain) (upper panel), *rpt*2RF (middle panel) or *rpt*4R (lower panel) expressing CPY*-HA were disrupted in sorbitol buffer, and post-nuclear supernatants (PNS) were treated with (+) or without (-) trypsin together (+) or without (-) Triton-X100. After trypsin inactivation, samples were dissolved, CPY*-HA was immunoprecipitated with a mouse anti-HA antibody, resolved by SDS-PAGE, immunoblotted and blots were probed with anti-HA.

[B] Blots were quantified by densitometry and the diagrams represent the distribution of CPY*-HA in the ER lumen (microsomes treated with trypsin), at the cytosolic face of the ER membrane (subtracting microsomes treated with trypsin from untreated microsomes) or in the cytosol (untreated cytosol), as percent of total CPY*-HA (sum of untreated microsomes and untreated cytosol).

[C] A plasmid for expression of CPY*-HA together (+) or without (-) a plasmid for expression of Cdc48p was introduced into SUB62 (WT) or *rpt*4R strains. Degradation of CPY*-HA was followed by addition of cycloheximide (150 µg/ml) and samples (2 OD_{600}) were collected at the indicated time points. Cells were dissolved and total cellular proteins were resolved by reducing SDS-PAGE and electroblotted onto nitrocellulose. Blots were probed with a rabbit anti-Cdc48 antibody (lower panel) followed by HRP-conjugated anti-rabbit IgG, and re-probed with a mouse anti-HA antibody (upper panel) followed by HRP-conjugated anti-mouse IgG, and HRP was visualized by ECL. Relative amounts of Cdc48p were quantified by densitometry.

[D] Blots were quantified by densitometry and the plot represents the decay of CPY*-HA as remaining protein calculated as a percentage of its level at the time of cycloheximide addition (100%). Half-life values are presented for WT (circles), *rpt*4R (squares) and *rpt*4R+CDC48 (triangles).

7.3 hours, increased expression of Cdc48p results in half-life shortening to only 85–95 minutes [Figure 4D].

What could be the inter-relations between Cdc48p and Rpt4p with respect to the ERAD-specific dislocation process? In principle, Cdc48p and Rpt4p may operate in parallel, performing similar but independent functions. The parallel model is corroborated by the binding of the proteasome as well as of p97/Cdc48p to the ER membrane, because mechanistically, association with the ER membrane is anticipated to facilitate the function of AAA-ATPases in pulling substrates out of the ER. Although p97/Cdc48p and the proteasome are largely free in the cytosol, their small ER-bound subpopulations might be the one relevant to ERAD, as suggested by the exclusive interaction of the lumenal ERAD substrate µs with these small ER-bound subpopulations [17]. Moreover, it has been reported that binding of proteasomes to ER membranes is salt-sensitive, ATP-dependent, and mediated by the high affinity binding of the Rpt-containing base of the 19S regulatory particle [20].

Alternatively, Rpt4p and Cdc48p may act sequentially, where Cdc48p binds ERAD substrates and delivers them to the 26S proteasome in a fashion that depends on the ATPase activity of Rpt4p. Indeed, ATP hydrolysis has been shown to modulate the association of many proteins with the 26S proteasome [21]. In this model, the proteasome binding to the ER membrane is irrelevant to its role in ERAD, whereas the binding of Cdc48p to the ER membrane is obligatory. Several lines of evidence support the notion that the p97/Cdc48p that operates in ERAD is ER-bound. First, the lumenal ERAD substrate µs pulls down only the small subpopulation of the ER-bound p97 [17]. Second, p97 is bound to the ER membrane by a membrane protein complex containing VCP-interacting membrane protein and Derlin-1, the mammalian homologue of Der1 and Dfm1 and the putative dislocation pore protein [22]. Third, the UBX domain protein Ubx2/Sel1, an integral ER membrane protein, is required for ERAD in yeast because it recruits Cdc48p to the ER and together they interact with Der1 and Dfm1 [23,24]. Finally, deletion of the PKC1 gene impairs ERAD and causes accumulation of CPY* in the ER lumen, most probably due to Cdc48p mislocalization [25].

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