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Current Opinion in Cell Biology

Finding the will and the way of ERAD substrate retrotranslocation

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ER-associated degradation (ERAD) is a mechanism by which numerous ER-localized proteins are targeted for cytosolic degradation by the ubiquitin–proteasome system. A surprising and still-cryptic requirement of this process is the energy dependent *retrotranslocation* of both lumenal and membrane-embedded ER proteins into the cytosol for ongoing ubiquitination and proteasomal destruction. The current understanding, results, and open questions are discussed below for this intriguing and critical process of ERAD.

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Current Opinion in Cell Biology 2012, **24**:460–466

This review comes from a themed issue on **Membranes and organelles**

Edited by **Weimin Zhong** and **Maho Niwa**

For a complete overview see the Issue and the Editorial

Available online 30th July 2012

0955-0674/$ – see front matter, Published by Elsevier Ltd.

http://dx.doi.org/10.1016/j.ceb.2012.05.010

ERAD and retrotranslocation

ER (endoplasmic reticulum)-associated protein degradation (ERAD) is a general term for the proteolytic pathways that degrade numerous ER proteins, including both luminal and integral membrane substrates [1,2]. (We hesitate to use the term 'clients' for the substrates, since except for some members of Wall Street, most operations do not function to destroy their clients...). ERAD is primarily involved in protein quality control, since most ERAD substrates are damaged or misfolded proteins. ERAD pathways involve the ubiquitin–proteasome pathway to execute degradation, marking the substrates by covalent addition of multiubiquitin chains, leading to recognition and destruction by the 26S proteasome [3•–6•] ERAD starts with recognition of ER-localized substrates, ubiquitination by the integral membrane E3 ligases, and subsequent delivery to and destruction by the proteasome. The ERAD ubiquitination machinery, the proteasome, and the various adaptors that catalyze ERAD all lurk in the cytosol. This cytological fact leads to one the most interesting, and still perplexing, aspects of ERAD pathways: the requirement for movement of ER substrates from their starting positions inside the ER lumen, or embedded in the ER membrane, to their site of cytoplasmic destruction. This process of substrate removal from the ER to the cytosol is known as 'retrotranslocation' or 'dislocation'. The mechanism of retrotranslocation remains one of the most interesting still unanswered questions in ERAD and cellular biochemistry. This review will describe the current thinking and work on this intriguing and important process.

Retrotranslocation of all classes of ERAD substrates

Retrotranslocation was discovered in the Wolf group's pioneering studies of *DER* genes responsible for ER degradation of the prototype ERAD substrate CPY*. The normally vacuole-bound CPY* is retained and degraded in the ER by virtue of a point mutation that renders it unfoldable. The reasonable model that the entirely lumenal CPY* was degraded within the ER was shattered by the stunning discovery that *DER2* encoded the cytoplasmic ubiquitin E2 known as Ubc7 [7••], indicating that lumenal CPY* was being tagged and degraded by the cytoplasmic ubiquitin–proteasome system [8]. This unlikely idea has been borne out for numerous misfolded ER proteins [9–13], making retrotranslocation a signal feature of luminal ERAD, or ERAD-L, substrates.

A second large class of ERAD substrates includes single-spanning and multi-spanning integral membrane proteins, with segments in the lumen and the cytosol. Usually, these are misfolded variants of membrane proteins, or orphaned subunits that lack a binding partner [14••]. In some cases normal proteins are degraded by ERAD pathways as a mode of physiological regulation, such as the sterol synthetic enzyme HMG-CoA reductase (HMGR) [15,16]. Integral membrane 'ERAD-M' substrates, like their more introverted lumenal cousins, are ubiquitinated by the cytosolic machinery and degraded completely by the proteasome; they too are subject to retrotranslocation required for processive degradation by the cytoplasmic proteasome [17–20]. The retrotranslocation of ERAD-M substrates from the ER membrane has been observed *in vivo* and *in vitro* [21•,22,23•], including direct demonstration of relocation of the full-length 8-spanning yeast HMGR to the cytosol during reconstituted ERAD [23•].

Thus, understanding ERAD of both lumenal and integral membrane substrates demands resolution of the mechanisms at play in retrotranslocation. This intriguing problem includes considerations of enzymology, membrane

biology, biophysics, and biochemistry; making it a problem of great interest to diverse investigators.

Retrotranslocation employs the Cdc48/p97 AAA-ATPase

The 'will' or the energy requirements for retrotranslocation are unknown. However, current thinking is that removal of ER proteins might be expected to require cellular energy. For luminal substrates, if ER exit is analogous to ER entry, then complete unfolding of the substrate would be required, although this requirement is not clear. Experiments using strongly folded luminal domains such as DHFR appended to ERAD substrates to test the requirement for unfolding have provided diverse results [24,25]. Retrotranslocation of membrane proteins is also expected to require energy, since the process requires removal of membrane spans from their secure thermodynamic roosts in the lipid bilayer, and extraction of lumenal portions that can be large and folded. Whatever the exit mechanism(s), a unifying feature of retrotranslocation is the requirement for the AAA ATPase (AAA = ATPases Associated with diverse cellular Activities) known as Cdc48 in budding yeast, or p97 in mammals [21•,26•–30•]. Cdc48/p97 forms a hexamer, and usually operates with proteins Npl4 and Ufd1 in a heteromeric complex that functions in numerous cellular processes [31,32]. The ERAD requirement for the Cdc48/Npl4/Ufd1 complex is highly conserved, as are many of the ERAD components from ligase to protease. The presence of six-ATP sites in the assembled complex may reflect an extreme energy requirement for this process, or the need for a complex allosteric mechanism to convert ATP energy into substrate extraction. The Cdc48/Npl4/Ufd1 complex binds multiubiquitin chains, and has been implicated in energy-dependent movement of multiubiquitinated proteins from non-covalent complexes in a wide variety of circumstances [33,34], of which ERAD retrotranslocation appears be the best-studied case. With this in mind, it is possible to imagine Cdc48 either mediating the actual 'pulling' of substrates across the membrane, or alternatively, mediating removal of ubiquitinated substrates bound to the ERAD complex after retrotranslocation. An intriguing question surrounding Cdc48/p97 is whether this complex functions in the same manner with ERAD-L and ERAD-M substrates.

While we know that Cdc48/p97 provides the energy for retrotranslocation, exactly how Cdc48/p97 harbors ATP energy to extract a protein from the ER is not clear. Studies on the solved structure of p97 will certainly help reveal the mechanism [35–37]. It will also be interesting to see if this generally used action of Cdc48 can be reconstituted biochemically, or studied with single-molecule approaches. It is a still-open question if Cdc48/p97 is the sole provider of ATP energy in the dislocation process. The proteasome is similarly studded with AAA ATPase proteins, and has been implicated directly in at least some versions of retrotranslocation [38,39•,40]. Thus, it may be that the chemical energy for retrotranslocation has contributors that operate alongside the Cdc48/p97 complex. In addition, a role for chaperones has been demonstrated in ERAD of some proteins with large cytoplasmic domains (called 'ERAD-C' substrates) [22,41,42], allowing the possibility that this distinct class of ATPases may also participate in some types of ER removal.

Way-ing in on egress: how do ERAD substrates leave the ER?

The old saying goes, ''where there's a will, there's a way'' and ERAD is no exception: if Cdc48/p97 provides the energetic 'will', then the way would be the actual route across and out of the ER membrane. This aspect of the ERAD remains unclear despite much activity from many groups. Because the usual way to get polypeptide into the ER is to employ a protein channel, it is reasonable to imagine that this is also the way proteins exit the ER. There have been a variety of candidates suggested and tested as channels for ERAD substrate removal. However, it is important to remember that as reasonable as this idea seems, there is no direct evidence for a channel (yet). In addition, it is possible that the exit mechanisms for luminal and membrane proteins are not identical. More recently, some alternative ideas have been suggested, and all models currently await definitive resolution by future experiments.

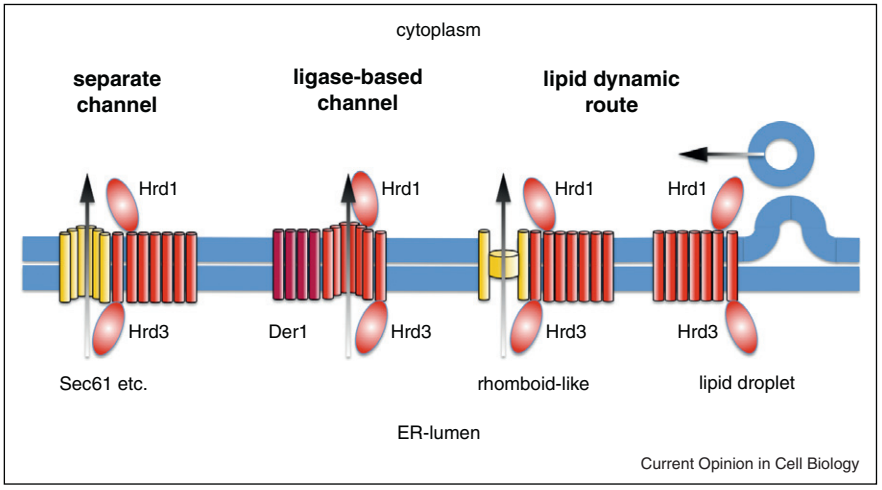
Sec61 anterograde channel as an exit route

In the quest to find the 'ERAD channel', an early and reasonable suggestion was that the same channel used for ER entry, Sec61 and its partner subunits, was also employed for the similar task of moving polypeptides the other direction. Consistent with this idea, small peptides have been observed to exit the ER by this route [43], and mammalian Sec61α has been reported to associate with some ERAD substrates [44], proteasomes [45], and the HRD ERAD ligase complex [46,47]. Unfortunately, because the *SEC61* gene is essential, the definitive test of sec61Δ null mutant cannot be done, unless an extragenic suppressor can be found. Studies of partial loss of function mutants have provided mixed results. Some experiments show strong dependent of ERAD-L on Sec61 [48,49]. Similarly, membrane proteins with a Deg1 ERAD signal show Sec61 dependence for the degradation [50,51], which has been interpreted as both evidence for Sec61-mediated retrotranslocation [50], or Sec61-mediated alteration of substrate orientation [51]. In other ERAD assays, there was no apparent role for Sec61 [23•,39•,52]. So the jury remains out on a role for Sec61 in retrotranslocation (Figure 1).

Derlin: an alDERnative channel for retrotranslocation

A second channel candidate arose from proteomic studies of the viral US11 complex that orchestrates the extremely efficient degradation of single-spanning MHC-I molecules.

Figure 1



The figure depicts different ideas for routes of ERAD substrate retrotranslocation, as discussed in the text. They include independent channels, such as Sec61 or a yet-unknown component, components of the ER ligase complexes such as derlins or the intergral membrane ligases themselves (Hrd1 shown), or lipid dynamic mechanisms such as rhomboid-like activities of derlins, or use of the lipid droplet pathway. These are not necessarily mutually exclusive routes.

They revealed a family of ER-localized candidate channel proteins known as derlins [53••,54••], homologous to the very first ERAD gene ever identified, DER1, which is the yeast version of this family [55]. This connection between the mammalian and yeast studies implies a broadly conserved role for these factors in ERAD. Derlins are all small multispanning membrane proteins, as might be expected of a channel component. In yeast, the der1Δ null mutation has a profound deficiency in lumenal substrate ERAD (ERAD-L). This defect occurs upstream of ubiquitination, as would be expected for loss of the luminal exit route. Elegant *in vitro* studies in which ATP-dependent dislocation of a luminal ERAD substrate from mammalian microsomes similarly implicates derlin by use of inhibitory antibodies, but no role for Sec61 [39•]. In yeast, ERAD-M substrates do not typically depend on Der1 for their degradation. For example, neither degradation, nor reconstituted *in vitro* retrotranslocation of Hmg2 requires Der1, its homologue Dfm1, nor Sec61 [23•,52]. Taken together, these diverse studies indicate that while derlins are clearly involved in ERAD, their function may be multifaceted, including dislocation of some, but certainly not all substrates. Importantly, the Der1 structure is predicted to be only 4 transmembrane spans, precluding it from acting alone as a channel. However, it may form part of a channel with other components of ERAD complexes.

ERAD E3 ligases as channel components

A third class of pore candidates is suggested from ERAD E3 ligase topology. The two principal ERAD E3 ligases, Hrd1 and Doa10 both have large multispanning membrane domains (Hrd1: 6 spans, Doa10: 14 spans [56,57]). Furthermore, the Hrd1 transmembrane domain has a high proportion of hydrophilic residues, as might be expected to line a peptide-transporting pore [58••]. These features have led to the appealing idea that in addition to ubiquitination, these E3s might provide a retrotranslocation pore, or provide a component of a pore. In addition, Cdc48 clearly associates with Hrd1 in a RING-domain (and Ubx2)-dependent manner, appropriate for a ligase that simultaneously mediates ER dislocation [59,60]. It is clear that at sufficient levels, Hrd1 can mediate ERAD in the absence of a variety of ERAD factors including Der1, Hrd3, and Usa1, all transmembrane ERAD components; also consistent with Hrd1 mediating both ubiquitination and retrotranslocation [58••,61,62••]. In addition, ERAD component Usa1 promotes Hrd1 multimerization, which could assist in creating a pore of appropriate size [63•]. In a technical tour-de-force, Carvalho *et al.* developed the '21st amino acid' programming approach to generate *in vivo* versions of Hrd1 with substituted photocrosslinking amino acids to query the ability of individual Hrd1 residues to engage ERAD-L substrates [62••,64]. By this approach, the classic substrate CPY* undergoes crosslinking with a number of inter-bilayer residues of the Hrd1 transmembrane domain, consistent with a role in transport across the bilayer. However, it is also clear that the Hrd1 transmembrane region participates in recognition of some substrates [58••]. Thus the crosslinking results could also be interpreted as indications of this role. Nevertheless, it is the case that Hrd1 has intimate contact with luminal substrates. Further use of this assay will undoubtedly by important in determining the role of Hrd1 in substrate transport from the ER.

One of the problems with testing Hrd1 for a separate role in retrotranslocation is that substrate ubiquitination is

required for the process, thus negating the possibility of removing Hrd1 channel function without altering the also-required ubiquitination. To separately examine possible Hrd1 channel activity, Sato and co-workers devised a 'self-destructive' ERAD substrate that self-ubiquitinates independently of full-length Hrd1. This was accomplished by appending the catalytic C-terminal, cytosolic, RING-H2 domain of Hrd1 to the highly stable transmembrane domain of the Hmg1 isozyme of yeast HMGR, thus creating the Hmg1–Hrd1 fusion protein. Hmg1–Hrd1 is ER localized, and undergoes remarkably rapid self-catalyzed ubiquitination and proteasome dependent degradation [23•]. Hmg1–Hrd1 ERAD is dependent on its built-in RING domain, the correct E2 (Ubc7) and the Cdc48 AAA ATPase, indicating that at least by these criteria it is a bone fide ERAD substrate. When tested *in vitro*, the Hmg1–Hrd1 fusion undergoes complete retrotranslocation, such that the full-length protein can be recovered from the supernatant upon enzymatic removal of ubiquitin chains. Surprisingly, *in vivo* Hmg1–Hrd1 degradation and *in vitro* Hmg1–Hrd1 retrotranslocation were observed in a *hrd1Δdoa10Δ* double null strain, indicating that neither of the 'potential channel domains' were needed for retrotranslocation of this substrate. Thus, the importance of each E3 ligase transmembrane domain as a channel component remains to be discerned, despite the obvious appeal of this notion from a structural standpoint. Whether this result applies to all substrates remains an open question, but it may indicate that the routes out of the ER are different for luminal and integral membrane substrates.

Towards an integrated picture of retrotranslocation

The collection of often ambiguous results described above may be providing clues about features of retrotranslocation, and resolution of those issues will be important steps in understanding the mechanism of this transport pathway. It may be that the lone still-undiscovered channel is a 'small' genetic target, and thus still awaiting discovery by assiduious genetic means. Another possibility for the lack of consensus on a channel is that we are dealing with the 'Escape from New York' scenario: there are many ways out of the great metropolis, and egress is determined by route availability: if the Holland Tunnel is closed, one can leave by the Lincoln Tunnel; if both are closed, then its up to the George Washington Bridge. Put less allegorically, it could by that there are redundant channel-based routes out of the ER, any of which will allow retrotranslocation. Perhaps that is why no ERAD mutants besides those in the CDC48 complex have been found that are deficient in all modes of retrotranslocation. In that case, definitive results could come from reconstitution of retrotranslocation with purified components, or location of route-specific substrates, or the correct multiple mutant studies to clarify the route(s) of retrotranslocation. Alternatively, creation of a substrate that is 'frozen' during retrotranslocation, such as has been done to study Sec61 or mitochondrial import, could reveal specific routes in the face of *in vivo* redundancy. If however, the channel is actually mediating movement of partially or fully folded proteins, as has been suggested [24], this approach might be difficult to develop. Another line of inquiry that may reveal retrotranslocation routes are the studies on numerous toxins that appear to exit the ER lumen in the course of their nasty cytosolic business. It appears that often components of the ERAD machinery are employed, despite the ability of many of these agents to escape degradation [65,66]. These specialized proteins may be able to show us routes of exit also employed by mainstream ERAD substrates.

Lipid dynamics in retrotranslocation

Alternatively, it may be that there are no retrotranslocation channels, and that a totally novel route is employed to remove ERAD substrates to the cytosol. It has been suggested that the machinery that forms lipid droplets, which are derived from the ER outer leaflet and end up on the cytosol, might provide a non-channel mechanism for dislocation, at least for membrane proteins [67]. However, it is clear that at least in yeast the loss of lipid droplet formation by removal of the appropriate lipid synthetic enzymes has no effect on ERAD-L or ERAD-M [68]. Conversely, it is true that mammalian lipid droplets are far more complex entities than those in yeast [69], and it has been observed that at least one retrotranslocated ERAD substrate, mammalian HMGR, does appear to be associated with a lipid droplet-like compartment after ER extraction [70]. Thus it may be that the connection to this novel cellular body is real but more subtle than simply providing an escape pod for departing proteins. The ongoing, heroic proteomic analyses of the mammalian ERAD network(s) will be an important testing ground for these possible connections [71].

The possibility of membrane dynamics distinct from simple pore action being involved in retrotranslocation is suggested from some very recent studies exploring the role of derlin 'rhomboid' homology. Rhomboid is one of the proteases involved in regulated intermembrane proteolysis (RIP) that involves cleavage of membrane anchored substrates at specific sites within the bilayer. Thus, the rhomboid enzyme function may include perturbation of the juxtasubstrate bilayer as part of its mechanism. Mammalian derlin has homology to the rhomboid family, but is missing the critical residues for proteolysis. Consistent with this idea, retrotranslocation of mammalian ERAD substrate alpha1-antitrypsin requires the expected p97-binding N-terminus, but also the rhomboid-homologous residues of derlin-1. Alteration of these derlin rhomboid sequences has effects on AAT degradation distinct from loss of p97 binding [72••]. This leads to the intriguing idea that the membrane-invasive or perturbing functions of rhomboid proteins may be harnessed to facilitate ERAD retrotranslocation. This could

mean that retrotranslocation employs membrane alteration as part of membrane crossing, fueled by ATP hydrolysis.

Remarkably, it has very recently been shown that a bone fide rhomboid protease variant with a UBL-related ubiquitin binding motif, distinct from the Derlins, is required for ERAD of some membrane proteins, and this role in ERAD requires its intramembrane protease function [73••]. Thus it may be that rhomboids, like their poetic name implies, could have been involved in multiple facets of the ERAD process.

There are many open questions that must be answered to gain a full understanding of the mechanism(s) at play in ER substrate dislocation. Continued genetic analysis and the eventual reconstitution of this process with fully purified components will get us to these insights, and they promise to be interesting, surprising and relevant to multiple aspects of cellular function.

Acknowledgements

We (RYH and TS) wish to thank members of our research groups past and present for discussions, data, and down-home fun. In addition, we dedicate this article to the memory of Steve Jobs, to whom nearly every biological scientist owes a debt of gratitude for powerful, friendly and whimsical computing tools of all stripe. RYH is supported by NIH grant DK051996, and by an ARRA supplement to that award. TS is generously supported by the Deutsche Forschungsgemeinschaft.

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See annotation in Ref. [72••]