Endoplasmic Reticulum Associated Protein Degradation (ERAD): The Function of Dfm1 and Other Novel Components of the Pathway

Von der Fakultät Energie-, Verfahrens- und Biotechnik der
Universität Stuttgart zur Erlangung der Würde eines
Doktors der Naturwissenschaften (Dr. rer. nat.)
genehmigte Abhandlung

Vorgelegt von
Dipl.-Biochem. Alexandra Stolz
aus Nürnberg

Hauptberichter: Prof. Dr. Dieter H. Wolf
Mitberichter: Priv. Doz. Dr. Wolfgang Hilt
Tag der mündlichen Prüfung: 20.12.2011

Institut für Biochemie der Universität Stuttgart
2011

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbst verfasst und dabei keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Stuttgart, den 11.11.11

Alexandra Stolz

Table of Contents

Appendix

1. Mnl2, a novel component of the ER associated protein degradation pathway
2. Yos9, a control protein for misfolded glycosylated and non-glycosylated proteins in ERAD
3. Dfm1 forms distinct complexes with Cdc48 and the ER ubiquitin ligases and is required for ERAD
4. ERAD without canonical ER ubiquitin ligases: A novel role for Ubr1
5. Endoplasmic reticulum associated protein degradation: A chaperone assisted journey to hell
6. Use of CPY* and its derivatives to study protein quality control in various cell compartments
7. Cdc48: a power machine in protein degradation
8. The Cdc48 machine in endoplasmic reticulum associated protein degradation

Abbreviations

AAA	ATPases associated with diverse cellular activities
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
C-terminal	Carboxy-terminal
CP	Core particle
DNA	Desoxyribonucleic acid
DRiPs	Defective ribosomal products
DUB	Deubiquitylating enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
ERAD-C	ERAD-cytosolic
ERAD-L	ERAD-lumenal
ERAD-M	ERAD-membrane
ERQD	ER quality control and associated protein degradation
Fig	Figure
GFP	Green fluorescent protein
HA	Hemagglutinin
HECT	Homologous to the E6-AP carboxyl terminus
Hsp	Heat shock protein
K48	Lysine residue at position 48
kDa	Kilodalton
MDa	Megadalton
mRNA	Messenger RNA
MRH	Mannose 6-phosphate receptor homology
MVB	Multivesicular body
N-linked	Amino-linked
N-terminal	Amino-terminal

NBD	Nucleotide-binding domain
NEF	Nucleotide exchange factor
OST	Oligosaccharyl transferase
PDI	Protein disulfide isomerase
PGK	3-phosphoglycerate kinase
RING	Really interesting new gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Regulatory particle
SRH	Second region of homology
TM	Transmembrane
TS	Temperature sensitive
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
WT	Wild type

Abstract

Proteins, featured with a multitude of enzymatic activities as well as structural and other physiological functions are the main operators in the cell. Proteins are synthesized in the cytosol by ribosomes, which use m-RNA as a template to translate DNA based structural information into an amino acid sequence. During translation many errors occur resulting in so-called defective ribosomal products. In addition, stresses as heat, heavy metal ions and oxygen lead to the formation of partially unfolded and misfolded proteins. In human accumulation of these proteins results in severe diseases as are Alzheimer's disease, Parkinson's disease, Huntington's disease and many others. Therefore quality control systems exist, which recognize unfolded or misfolded proteins and support their folding process. If a protein is unable to reach its native conformation or to refold, the quality control system marks it as terminally misfolded and hands it over to the degradation machinery of the cell. In case of proteins of the secretory pathway this process is called endoplasmic reticulum quality control and associated protein degradation (ERQD). ERQD includes the recognition of the misfolded protein species, the trimming of glycan trees to signal misfolding, retrograde transport out of the ER lumen into the cytosol, ubiquitylation of the misfolded protein and degradation by the proteasome.

The following thesis was engaged in the identification of new components of ERQD and tried to get insights into some mechanistic functions of the involved proteins. The proteins Dfm1, Mnl2 and Ubr1 were found as new components of the endoplasmic reticulum associated protein degradation (ERAD) machinery. Mnl2 was identified as a putative α-1,2-mannosidase. It was shown to be involved in the degradation of the misfolded glycoprotein CPY*. Most probably Mnl2 trims down the glycan trees of ERAD substrates, which are subsequently recognized by the lectin Yos9. Yos9 accelerates the degradation of terminally misfolded glycoproteins which expose these glycan structures. However, Yos9 does not seem to act only on glycosylated proteins but also seems to affect the degradation kinetics of unglycosylated ERAD substrates. In contrast to misfolded glycoproteins Yos9 delays degradation in case of the unglycosylated ERAD substrate CPY*0000. Most likely Yos9 has a chaperone like function in addition to its lectin function and provides more time for refolding of the

misfolded protein. This function is, however, independent of its MRH domain that recognizes glycans.

The other new ERAD component, the polytopic ER membrane localized Dfm1 protein, was found to form distinct complexes with the ligases Hrd1/Der3 and Doa10 as well as with the AAA type ATPase Cdc48. Degradation of different ERAD substrates containing a transmembrane domain was tested for Dfm1 involvement. The degradation and ubiquitylation of the ERAD-C substrate Ste6* was shown to depend on Dfm1. In addition, Dfm1 seems to be involved in a new degradation pathway, which acts independently of the ubiquitin ligases Hrd1/Der3 and Doa10. In the absence of these canonical ER ligases the cytosolic ubiquitin ligase Ubr1 seems to be recruited to maintain degradation of at least some ERAD substrates by the proteasome. Extraction of the misfolded protein species no longer depends on Cdc48 in all cases, but the driving force of other machines, most probably chaperones of the Ssa family of Hsp70 chaperones, were found to be sufficient to keep extraction and degradation of the substrates going.

Zusammenfassung

Proteine, ausgestattet mit einer Vielzahl an enzymatischen Aktivitäten sowie strukturellen und physiologischen Funktionen, sind die eigentlichen Maschinen der Zelle. Sie werden im Cytosol an Ribosomen synthetisiert, die m-RNA als Matrize verwenden um auf DNA basierende Information in eine Aminosäuresequenz zu übersetzen. Während dieses Vorgangs können Fehler auftreten, die zu sogenannten defekten ribosomalen Produkten führen. Streßzustände wie Hitze, Schwermetallbelastung und Oxidation können zusätzlich die Entstehung von teilentfalteten bzw. mißgefalteten Proteinen fördern. Die Zusammenlagerung von Proteinen kann beim Menschen zu schwerwiegenden Erkrankungen führen, wie beispielsweise in der Alzheimer-, der Parkinson- oder der Huntingtonkrankheit. Um dies zu verhindern gibt es eine zelluläre Qualitätskontrolle, die unvollständig gefaltete und fehlgefaltete Proteine erkennt und ihren Faltungsprozeß bzw. ihre Rückfaltung unterstützt. Falls ein Protein trotz Hilfe seine native Konformation nicht erreichen kann, wird es als definitiv fehlgefaltet markiert und an die Degradationsmaschinerie der Zelle weitergeleitet. Im Falle von Proteinen des sekretorischen Weges wird dieser Prozeß als endoplasmatische Reticulum assoziierte Qualitätskontrolle und Degradation (ERQD) bezeichnet. Die ERQD beinhaltet das Erkennen fehlgefalteter Proteine, die Verkürzung von Kohlenhydratstrukturen auf Glykoproteinen um Fehlfaltung zu signalisieren, den retrograden Transport der Proteine aus dem ER in das Zytosol, ihre Ubiquitylierung und den proteasomalen Abbau der fehlgefalteten Proteine.

Die nachfolgende Dissertation beschäftigt sich mit der Identifizierung neuer Komponenten der ER assoziierten Degradation und deren Funktionsweise. Das ER Membranprotein Dfm1, die putative α-1,2-Mannosidase Mnl2 und die Ubiquitinligase Ubr1 konnten als neue Komponenten identifiziert werden. Es kann vermutet werden, dass Mnl2 mit seiner anzunehmenden Mannosidaseaktivität die Kohlenhydratstrukturen fehlgefalteter Glykoproteine verkürzt. Diese werden anschließend von dem Lektin Yos9 mit seiner MRH Domäne erkannt und dem Proteinabbau zugeführt. Interessanterweise scheint Yos9 auch an dem ERQD Prozeß unglykosylierter Proteine beteiligt zu sein. Hier hat die Anwesenheit von Yos9 jedoch den gegenteiligen Effekt. Während es den Abbau von fehlgefalteten

Glykoproteinen beschleunigt, verzögert es den Abbau von nicht glykosilierten Proteinen, hier getestet an dem Substrat CPY*0000. Anscheinend besitzt Yos9 eine Chaperon-ähnliche Eigenschaft, die nicht glykosylierten Proteinen ein längeres Zeitfenster für den Faltungsprozeß zur Verfügung stellt. Diese Funktion von Yos9 ist nicht von der MRH Domäne des Proteins abhängig.

Die neu identifizierte ERAD Komponente Dfm1 wurde in distinkten Komplexen mit den ER Ligasen Hrd1/Der3 und Doa10 sowie mit der AAA ATPase Cdc48 gefunden. Ihr Einfluß auf den Abbau verschiedener ERAD Substrate mit Transmembrandomänen wurde untersucht. Im Falle des ERAD-C Substrates Ste6* konnte eine Beteiligung von Dfm1 an der Ubiquitylierung und der Degradation dieses fehlgefalteten Proteins festgestellt werden. Es scheint, dass Dfm1 auch an einem neuen, von den bekannten ER Ubiquitinligasen Hrd1/Der3 und Doa10 unabhängigen Abbauweg beteiligt ist. In Abwesenheit der beiden einzig bekannten ER Ubiquitinligasen, Hrd1/Der3 und Doa10, wird der partielle proteasomale Abbau von zwei getesteten ERAD Substraten von der cytosolischen Ubiquitinligase Ubr1 aufrecht erhalten. Die Extraktion der fehlgefalteten Proteine aus der ER Membran ist in diesem neu identifizierten Abbauweg nicht mehr zwingend auf die Aktivität von Cdc48 angewiesen. Die Antriebskraft cytosolischer Chaperone, speziell die der Ssa Familie, scheint ausreichend zu sein, um den Transport des getesteten ERAD Substrates CTG* durch bzw. von der Membran weg zu ermöglichen.

1. Introduction

Life on earth can be divided into three kingdoms: Prokaryotes, eukaryotes and archae. Yeast – like human – belongs to the kingdom of eukaryotes. The protozoan yeast shows compartmentalization into organelles as are the nucleus, the endoplasmic reticulum (ER), the golgi apparatus, mitochondria, the vacuole and peroxisomes. There are several advantages in using yeast as an eukaryotic model organism in research. One of them lies in its genome, which is relatively small, completely sequenced [1, 2] and amenable to several tools as genetics and molecular biology for its manipulation [3, 4]. Basic eukaryotic cell functions as well as metabolic and disease related pathways are therefore often studied first in yeast due to ethical concerns with animal research and easy handling of this model organism. Results obtaind are later assigned to the human system. In this thesis the yeast *Saccharomyces cerevisiae* was used as a model organism to study different steps within the quality control system of misfolded proteins of the secretory pathway.

1.1 Protein synthesis and degradation

Proteins, featured with a multitude of enzymatic activities as well as structural and other physiological functions are the main operators in the cell. They are synthesized in the cytosol by ribosomes, which use m-RNA as a template to translate DNA based structural information into an amino acid sequence. During translation many errors occur resulting in so-called defective ribosomal products (DRiPs) which are rapidly degraded via the main degradation machinery of the cell – the proteasome. It is thought that over 30% of newly synthesized proteins undergo this rapid degradation. This is reflected in the high proteasome abundance of about 1% of the total protein pool [5]. In addition, stresses such as heat, heavy metal ions and oxygen lead to the formation of misfolded proteins [6-10]. In human accumulation of misfolded proteins results in severe diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and many others [11-14].

1.2 The secretory pathway

Many proteins carry signal sequences that guide them during or after synthesis to their site of action. About one third of all proteins enter the secretory pathway (for review: [15]). As a first step proteins of the secretory pathway are transported from the cytosol into the ER through the import channel Sec61 [16-19], where they are folded and further delivered to their site of action. Depending on the protein, this transport across the ER membrane occurs simultaneously with the translation event or after the protein has been synthesized in a process called co-translational and post-translational import, respectively [16, 20]. During protein synthesis all parts of the protein are temporarily exposed to the environment including hydrophobic regions that are buried within the protein structure after the successful folding process. Without any help, this could lead to the aggregation of newly synthesized proteins, impair protein functionality and harbor the risk of cell damage [21]. To avoid these problems a machinery consisting of different kinds of chaperones exists.

1.3 The chaperone equipment

As mentioned above, folding mistakes and trapped intermediates during protein synthesis caused by genetic mutations or cellular stresses can occur. The cell allocates chaperones to sustain cell functionality and to promote protein folding in the crowded cellular environment (for review see appendix 5 [7]). Chaperones are able to keep misfolded or partially unfolded proteins soluble to prevent or re-solubilize aggregates [22, 23] by recognizing the folding state of a protein and constantly accompanying the folding process. Thereby partially unfolded proteins gain a longer time window to reach their native conformation and misfolded proteins get the chance to refold. In the past, chaperones had been thought to be solely responsible for the folding process of newly synthesized proteins or the refolding of proteins that had been partially unfolded by stresses, e.g. heat ("heat shock proteins") [8, 24, 25]. Nowadays chaperones are known to accompany proteins in almost all periods of their life.

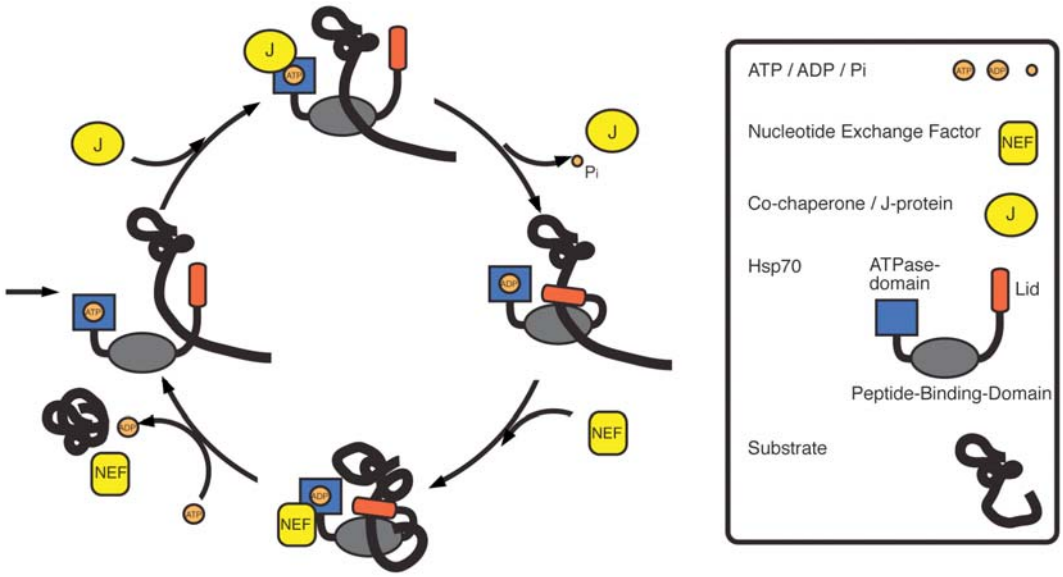


Figure 1: The folding cycle of Hsp70 chaperones

Hsp70s in an ATP bound state exhibit low affinity for their substrates and cycles of substrate association and dissociation occurs fast. Interaction of an appropriate J-protein induces the ATPase activity of the Hsp70. During ATP hydrolysis the tertiary structure of the Hsp 70 changes, causing a „closing" of the C-terminal lid. This tightly attaches substrates to the chaperone. Subsequently a nucleotide exchange factor initiates the exchange of ADP against ATP, resulting in the opening of the lid and the release of the substrate (taken from appendix 5).

Chaperones represent a protein class of great variety. Besides the variation in size, from about 20 kDa to over 100 kDa, they differ in the need for co-factors and ATP and are subdivided into several families. One well established chaperone family, which is highly consered among all organisms, is the family of Hsp70 chaperones (heat shock protein of about 70 kDa). They act in protein folding, in preventing protein aggregation and in membrane translocation [26-28]. Hsp70 members are ATP consuming chaperones, which are assisted by Hsp40 co-chaperones (J-proteins) and nucleotide exchange factors. While acting on their substrates, Hsp70 chaperones run through a cycle of substrate binding events, ATP hydrolysis and release of the substrate (Fig. 1) [7, 29]. Well-known members of this chaperone family in yeast are the members of the Ssa family residing in the cytosol and Kar2 (BiP in mammals), which is located to the ER lumen.

One additional family of chaperones, which is central to this work, are lectin-like chaperones. Proteins of the secretory pathway are often modified with N-linked carbohydrates after their entry into the ER [30, 31]. Lectins are able to bind glycan structures of a protein with their mannosidase like domain or a mannosidase receptor

homology domain [32-34]. Dependent on the glycan structure lectins facilitate folding, ER retention of folding intermediates and identification of terminally misfolded proteins. Binding to some non-glycosylated misfolded proteins suggests chaperone-like activity of this protein family (appendix 2) [35, 36]. Besides carbohydrate modification, proteins of the secretory pathway often contain inter- and intramolecular disulfide bonds formed by cysteine residues, which strengthen the protein structure or connect different subunits of a protein complex. Protein disulfide isomerases (PDIs) assist in their formation and reduce mismatched disulfide bonds [37-40]. All oxidoreductases contain a characteristic CXXC motif, however, the number of CXXC motifs and the precise sequence varies among family members.

Despite the variety of chaperones acting on misfolded proteins not all misfolded species can be refolded. Terminally misfolded proteins have to be recognized and specifically channeled to proteolysis. Failure of the protein quality control system consisting of chaperones, protein disulfide isomerases and lectins finally leads to the formation of protein aggregates that in turn can cause severe diseases like Alzheimers' -, Parkinsons' - or Creutzfeldt–Jakob disease [11, 41-43]. In many cases terminally misfolded proteins are degraded by the ubiquitin-proteasome-system (UPS).

1.4 The ubiquitin system

Besides glycosylation, phosphorylation and acetylation, proteins can be covalently modified with ubiquitin and ubiquitin like proteins [44, 45]. Ubiquitin, which is highly conserved in all eukaryotes, is an essential protein of 76 amino acids and terminates with a diglycine motif. In yeast there are four genes coding for ubiquitin. Three of them are fused with the coding sequence of a ribosomal subunit. The fourth ubiquitin gene contains five ubiquitin repeats in a head-to-tail arrangement [46]. After translation ubiquitin is cleaved off the fused ribosomal subunit and the ubiquitin repeat is cut into single ubiquitin moieties. In order to ubiquitylate proteins ubiquitin has to be activated at its C-terminus by an ubiquitin activating enzyme, called E1 (Fig. 2). Activation consumes ATP and occurs through the formation of a thiolester bond between the E1 and the C-terminal glycine of the ubiquitin moiety. After activation, ubiquitin is

transferred onto an ubiquitin conjugating enzyme (E2), again via formation of a thiolester bond. For transfer onto a lysine residue or the N-terminus of the target protein an ubiquitin ligase (E3) is required. Transfer results in generation of a peptide bond between the C-terminus of the ubiquitin and a lysine residue or the N-terminus of the target protein [47-49]. Two families of ubiquitin ligases exist: The so-called RING (really interesting new gene) ligases bind the E2 and the target at the same time, thereby enabling transfer of the ubiquitin from the E2 onto the target protein. In contrast, the HECT (homologous to the E6-AP carboxyl terminus) ligases first receive the ubiquitin from the E2, again forming a thiolester bond and then bind to the target for direct ubiquitylation. Besides mono-ubiquitylation this system is able to built up ubiquitin chains by cycling through this reaction. From the second round of ubiquitylation, ubiquitin is transferred onto one of the seven lysine residues within the previous ubiquitin. Ubiquitin can also be fused to the N-terminus of another ubiquitin moiety. Therefore, different kinds of ubiquitin chains can be build up, which are thought to serve as recognition signals in different pathways [50-52]. Recent findings point to the possibility that not only lysine residues but also serine, threonine and cysteine residues of the target protein can be ubiquitylated, thereby extending the variety of ubiquitylation [53]. One major task of the ubiquitylation machinery is to provide a recognition motif for proteasomal degradation [49]. For substrate recognition by the proteasome the major linkage within the ubiquitin chain is the K48 linkage [50, 54].

1.5 The proteasome

The proteasome is a huge cytosolic proteinase complex consisting of about 33 subunits in yeast *Saccharomyces cerevisiae* with a total size of around 2.5 MDa [55-57]. It is composed of a cylindrical central 20S core particle (CP), which contains the proteolytic activity of the proteasome and two regulatory 19S particles (RP), which can bind to either axial end of the CP and regulate proteasomal activity (Fig. 2). Ubiquitylated proteasomal substrates have to be recognized first by subunits of the RP, unfolded and transported into the CP, which can only occur after the CP has been opened by the RP. Prior to their entry into the CP substrates are deubiquitylated

and unfolded to be able to pass the pore into the CP and avoid unnecessary degradation of ubiquitin. Four stacked rings, each containing seven subunits, form the CP (Fig. 2). While the external rings contain seven α subunits, the two inner rings are each formed by seven β subunits. The two inner rings contain three distinct proteolytic activities, each represented twice: Chymotryptic activity, tryptic activity and post-acidic activity cleaving after hydrophobic, basic and acidic residues, respectively. The generated oligopeptides, consisting of three to thirty amino acids, are subsequently converted into free amino acids by other peptidases. The proteolytic activity of the proteasome can be partially blocked by specific inhibitors of the proteasome like MG132 [58, 59]. Proteolysis, in principle, is an exergonic reaction. However, protein degradation in the cell is highly energy consuming in order to reach specificity in this process and avoid degradation of functional and needed proteins. Therefore, substrates are unable to enter the core particle before the RP binds.

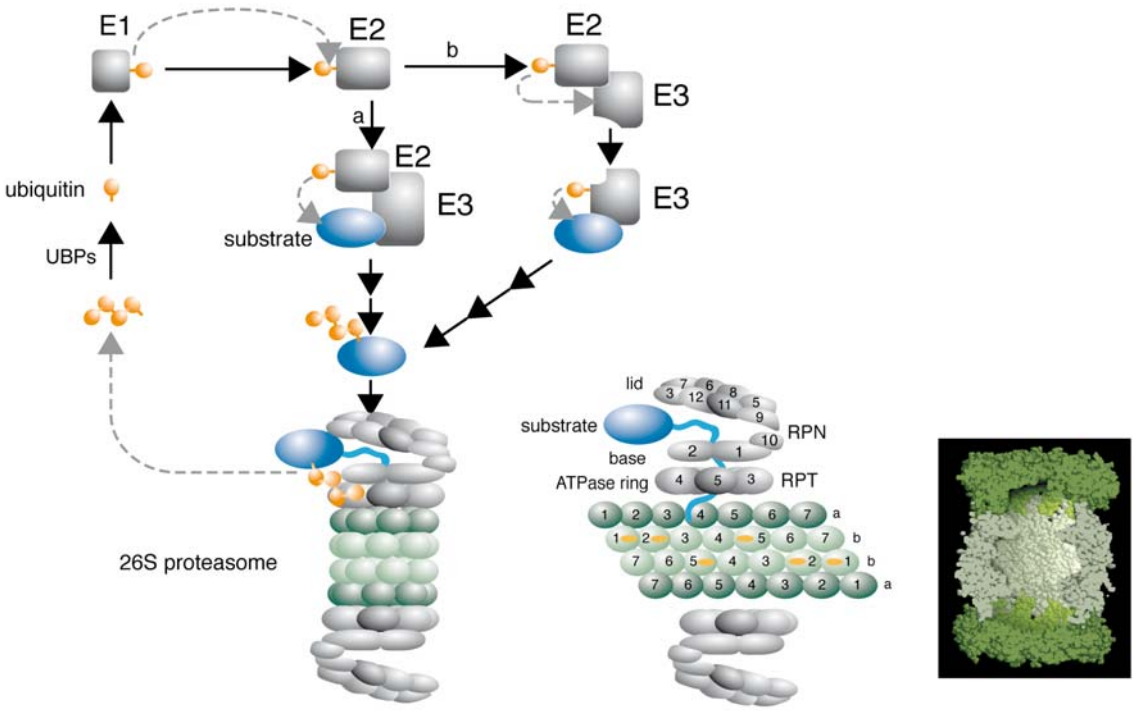


Figure 2: The ubiquitin-proteasome-system

Tagging of proteasomal substrates with ubiquitin is achieved by the coordinated action of E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases. The 19S cap of the 26S proteasome, which can be dissociated into a lid and a base component, recognizes polyubiquitylated substrates. 19S caps are composed of 11–12 Rpn and six Rpt (ATPase) subunits. Substrates are finally degraded by the proteolytic activity of the 20S proteasome core, which is provided by three pairs of different subunits residing in the inner chamber of the proteasome cylinder (modified from Wolf and Hilt, 2004 [55]).

The RP consists of a base and a lid linked together by the subunit Rpn10, which is an ubiquitin chain receptor [60, 61]. The RP base contains the subunits Rpn1, Rpn2 and Rpn13, which is also reported to be an ubiquitin receptor [62], as well as six ATPases called Rpt1 to Rpt6, which are thought to possess protein unfolding activity [57]. The lid of the RP is formed by subunits Rpn3 to Rpn9, Rpn11, Rpn12 and Rpn15.

After recognition the ubiquitin chain is removed from the substrates by deubiquitinases (DUBs). The subunit Rpn11 is known to carry a MPN domain that contains a metallo-protease-like deubiquitylating activity, which removes ubiquitin chains from proteasomal substrates before they enter the core particle [63, 64]. Another deubiquitylating enzyme is Ubp6, which can connect to the proteasome via the subunit Rpn1 [65]. Its antagonist, the E4 HECT ligase Hul5, is recruited by Rpn2 [66]. All together, this makes the base of the lid very important for proteasomal regulation.

For degradation to occur substrates need so-called „loose ends“ with a minimal length to be fed into the proteasome. A length of about 20 to 25 amino acids is necessary to enter the proteasome [67]. Loose ends are most probably generated by ATP consuming factors as are the AAA type ATPase Cdc48 and Hsp70 chaperones [11, 68].

1.6 The AAA type ATPase Cdc48

A major player in protein degradation is the essential AAA type ATPase Cdc48, which is highly conserved among all organisms [69-71]. While it was long thought that Cdc48 is only involved in protein degradation by the UPS, recent reports unraveled a function of Cdc48 in several selective autophagy pathways [72-74]. Besides protein degradation, Cdc48 is involved in a multitude of cellular processes including transcriptional and metabolic regulation, DNA damage response, chromatin remodeling, cell cycle progression and cell death [12, 75-79].

Cdc48 is a homohexameric complex composed of six protomers arranged in a ring around a central pore. Each protomer contains four major domains: a flexible N-terminal domain, two conserved AAA domains (D1 and D2) and a disordered C-terminal domain (Fig. 3).

Each AAA domain itself contains a Walker A and a Walker B motif necessary for nucleotide binding and hydrolysis, respectively. The precise mechanistic function of Cdc48 is not yet clear but combined nucleotide binding and hydrolysis result in global conformational changes, which are essential for Cdc48 activity as a segregase [80-82]. Substrates of Cdc48 are thought to be recognized by substrate-recruiting factors, which mainly bind to the N-terminal domain of Cdc48 [83, 84]. Recognition often includes ubiquitylation of the substrate, however, Cdc48 was recently found to act also in assumed ubiquitin independent pathways [85]. The fate of a substrate is governed by substrate-processing factors of Cdc48. A well established Cdc48 complex consisting of Cdc48, Npl4 and Ufd1 acts in endoplasmic reticulum associated protein degradation, a pathway that delivers misfolded proteins of the secretory pathway to proteasomal degradation (summarized in appendix 7 [11] and 8 [69]).

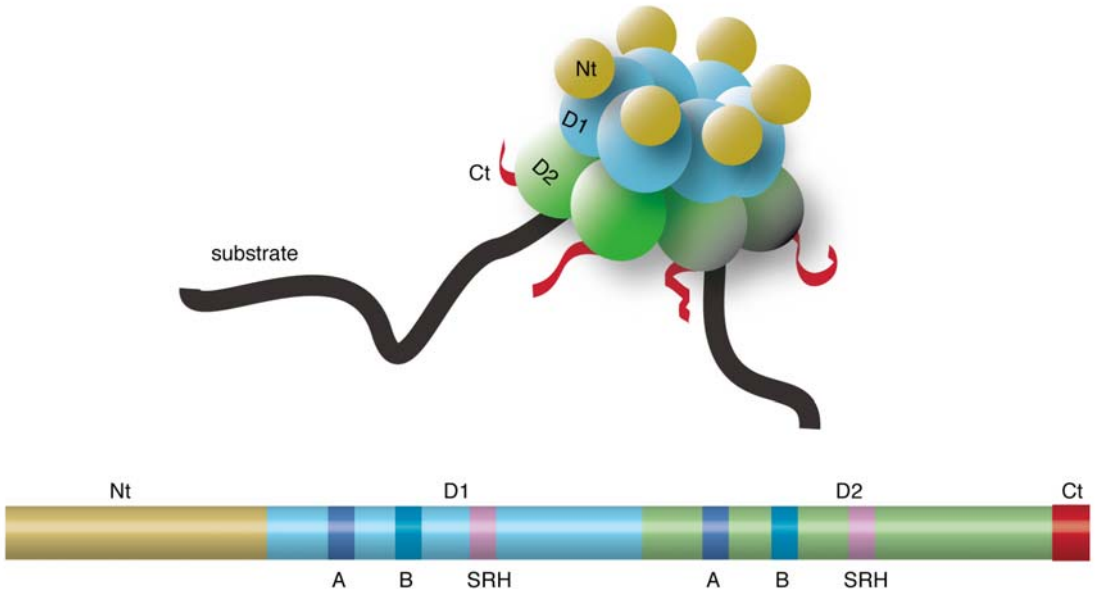


Figure 3:Topology of the hexameric Cdc48/p97 complex and its domain architecture

The important domain structures of Cdc48 of the yeast Saccharomyces cerevisiae and homo sapiens are shown. The N-terminus (Nt) and the unstructured C-terminus (Ct) of Cdc48 are indicated. The N-terminus of the motor protein is followed by the D1 and D2 domains with their Walker A and Walker B motifs as well as the second region of homology 1 (SRH; modified from appendix 8).

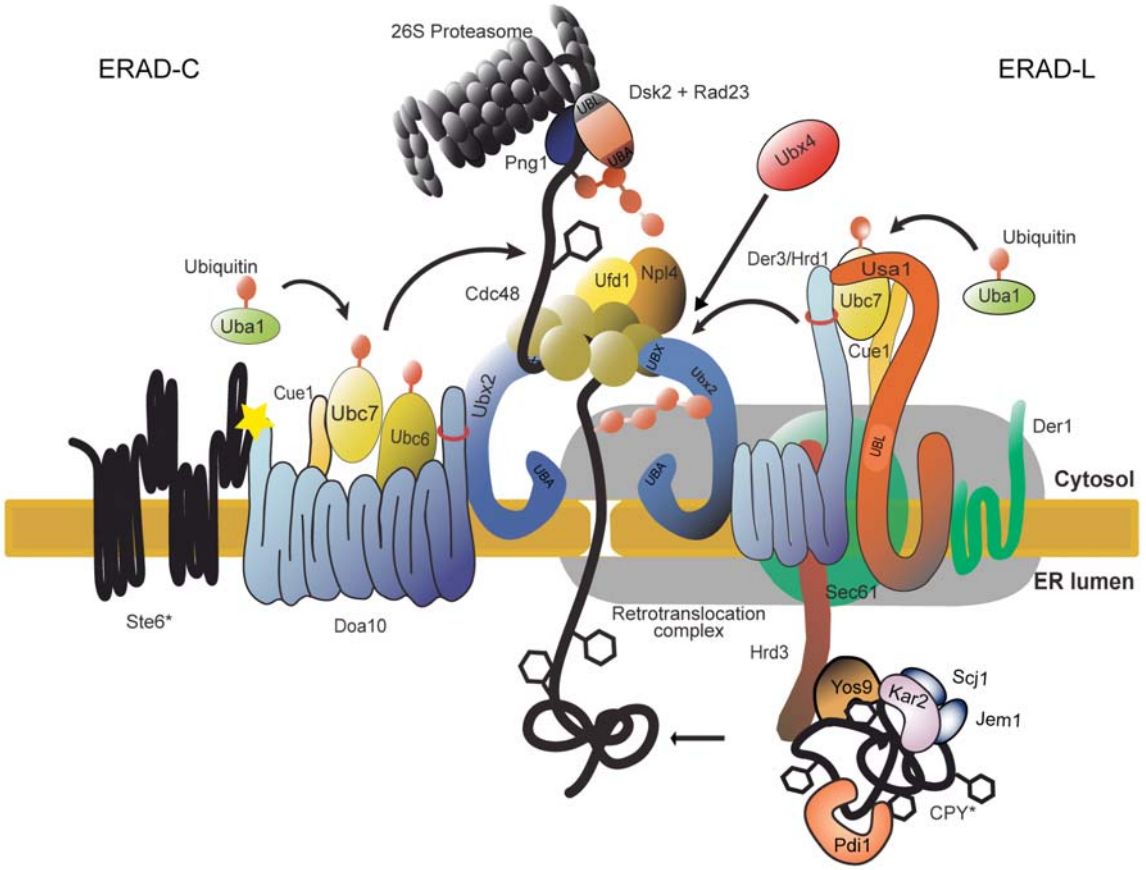


Figure 4: Cdc48 and cofactors as part of the endoplasmic reticulum associated protein degradation (ERAD) machinery

Misfolded proteins carrying the misfolded domain in the lumen of the ER (ERAD-L substrates) or in the cytoplasm (ERAD-C substrates) are recognized, retrogradely transported across the ER membrane into the cytosol, polyubiquitylated and degraded by the proteasome. The ERAD-L and ERAD-C pathways use two different polytopic membrane-localized ubiquitin ligases, Hrd1/Der3 and Doa10, respectively. Whereas Hrd1/Der3 is embedded into a variety of ER membrane proteins of, in many cases, not yet precisely known function, Doa10 is localized in a much simpler environment. Both pathways, ERAD-L and ERAD-C, merge at the Cdc48 motor, complexed with Ufd1 and Npl4 as cofactors and recruited to the ER membrane by Ubx2. According to the present literature, the Cdc48–Ufd1–Npl4 complex is thought to pull or segregate the substrates from the ER membrane and finally deliver them to the proteasome for degradation (modified from appendix 7).

1.7 ER associated protein degradation

As described above misfolded proteins have the potential to impair cellular functions by the formation of aggregates and interference with functional proteins. To avoid such situations, quality control systems and degradation processes are available to recognize misfolded proteins or trapped folding intermediates and provide these proteins to proteolysis [7, 86-88]. For proteins of the secretory pathway the major elimination systems are vacuolar (lysosomal) degradation and ER associated protein degradation (ERAD), which results in proteasomal degradation of the misfolded proteins (Fig. 4). The ERAD process includes a quality control system in the ER that recognizes misfolded protein species. They are subsequently retrotranslocated from

the ER lumen back to the cytosol, ubiquitylated at the cytosolic side of the ER membrane and targeted to the proteasome [89-91].

Recognition of misfolded proteins occurs via exposed hydrophobic patches and – in case of glycoproteins – via certain glycan structures [92]. Until now, three major ERAD pathways have been identified: ERAD-L, ERAD-M and ERAD-C [93, 94]. The letters L, M and C stand for lumenal, membrane and cytosolic, respectively, and refer to the localization of the misfolded domain within an ERAD substrate (Fig. 5). Depending on this localization, different components of the ERAD machinery are necessary to mediate efficient degradation.

There are two canonical ubiquitin ligases embedded in the ER membrane: Hrd1/Der3 and Doa10. While substrate ubiquitylation in the ERAD-C pathway mainly depends on the ligase activity of Doa10 [95, 96], ERAD-M and ERAD-L substrates are preferentially ubiquitylated by the ligase Hrd1/Der3 [69, 97-100]. Other factors known to act in ERAD-C are the E2 enzymes Ubc6 and Ubc7 [69, 101-103]. While Ubc6 is anchored to the ER membrane, Ubc7 recruitment depends on the membrane protein Cue1 [104]. The ERAD-C pathway is supposed to be more complex. Several factors of the quality control system within the ER lumen have to recognize the substrate as being misfolded. This includes the membrane anchored Hrd3 protein, the essential Hsp70 chaperone Kar2 with its co-factors Scj1 and Jem1, the protein disulfide isomerase Pdi1, which also contains a chaperone-like activity, and the lectin Yos9 [105]. After retrotranslocation Hrd1/Der3, which is in complex with the single transmembrane spanning protein Hrd3 [98, 106], ubiquitylates the substrate. Hrd3 on one hand is necessary to stabilize Hrd1/Der3 and on the other hand is also thought to interact with unfolded or misfolded proteins by its ER luminal domain. Other factors important for efficient degradation of ERAD-L substrates are the membrane proteins Usa1 and Der1 [94, 107-109]. While Usa1 was shown to mediate oligomerisation of the Hrd/Der complex and be the linker between Der1 and the Hrd/Der complex, the precise function of Der1 still remains unknown.

As previously mentioned, ubiquitylation occurs at the cytosolic side of the ER membrane. The mechanism of how the misfolded substrates are retrotranslocated through the ER membrane prior to ubiquitylation is not yet understood. Several membrane proteins had been suggested to form a channel, including Doa10, Hrd1/Der3, Hrd3, Usa1, Der1 as well as the import channel protein Sec61 [110-112].

However, only hints but no clear evidence for the participation of one of these proteins in the formation of a retrotranslocation channel have been found so far.
All ERAD pathways merge on the outer face of the ER at the cytosolic Cdc48 complex consisting of Cdc48 and its co-factors Ufd1 and Npl4 (see above; appendix 7 and 8). The AAA type ATPase Cdc48 is thought to provide the energy that is necessary to pull substrates out and away from the ER membrane. Degradation of some ERAD substrates also depends on the activity of the cytosolic Hsp70 Ssa1 [27]. Ubiquitylated substrates are then delivered to the proteasome by the shuttle factors Dsk2 and Rad23 [113-115] (summarized in appendix 5 [7]).

1.8 Model substrates of ERAD

Several model substrates have been designed to study the ERAD pathways. A well-known ERAD-C substrate is the truncated version of the α-factor transporter, Ste6*, missing the last 42 amino acids of the wild type protein (Fig. 5) [116, 117]. The point mutation Q1249X results in a premature stop codon and a misfolded C-terminal domain on the cytosolic side of the ER membrane. A well established ERAD-L substrate is the mutated version of carboxypeptidase yscY (CPY*) [118]. Wild type CPY is located to the vacuole, while the mutated version carrying the point mutation G255R near the active site of the enzyme is retained in the ER lumen due to its misfolded structure and rapidly degraded. The CPY* moiety fused to a transmembrane domain of Pdr5 led to the model substrate CT*. Additional fusion of a cytosolic GFP- and Leu2 moiety resulted in the model substrates CTG* and CTL*, respectively (Fig. 5; appendix 6) [113, 119]. CPY* is glycosylated at four sites within the protein. Mutation of each glycosylation site resulted in the unglycosylated CPY*0000 protein [120]. The removal of the signal sequence (ss) that guides the CPY* moiety to the ER lumen after synthesis, resulted in nonglycosylated, cytosolic ΔssCPY*. This CPY* version is no longer degraded by the ERAD machinery, but still ubiquitylated and provided to proteasomal degradation by a cytosolic machinery [22, 121]. Recognition and ubiquitylation of ΔssCPY* occurs by the cytosolic ubiquitin ligase Ubr1. Ubr1 was first found to function in the N-end rule pathway [122-124]. However, Ubr1 was recently found to be also involved in the degradation of misfolded cytosolic proteins like

ΔssCPY* [121, 125]. While Ubr1 acts in concert with the E2 Ubc2 in the case of N-end rule substrates, degradation of misfolded cytosolic proteins require also the activity of Ubc4 and Ubc5.

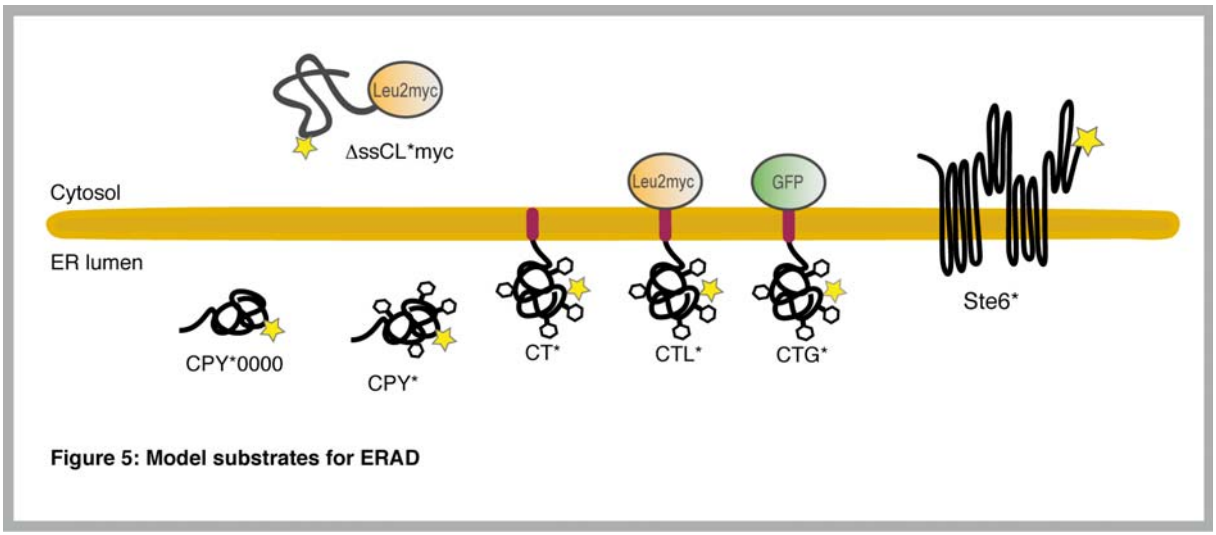


Figure 5: Model substrates for ERAD

2. Results and discussion

2.1 Mnl2, a putative mannosidase of ERAD

In cooperation with Elena Martinez Benitez early steps in the recognition of ERAD substrates have been analyzed. First, the trimming process of the glycan structures carried by misfolded glycoproteins was studied. Proteins of the secretory pathway to be glycosylated are modified with a core glycan during their entry into the ER [92]. A glycan with the structure Glc_3-Man_9-$GlcNAc_2$ (Fig. 6) is transferred onto an asparagine residue of the protein by the oligosaccharyl transferase (OST) complex within a suitable glycosylation site with the consensus sequence Asp-X-Ser/Thr (X= no Pro). A trimmed N-glycosyl structure, which is recognized by the lectin Yos9 has turned out to be an important attribute of misfolded proteins [87, 126, 127]. During the trimming process of the glycan, glucosidase I and II first remove the three glycan residues from the core glycan of proteins during their folding into their native conformation [128, 129]. Properly folded proteins carrying a Man_9-$GlcNAc_2$ structure are able to leave the ER and reach their final destination. If a protein has not succeeded in proper folding until the glucosidases have removed the three glucose residues, the

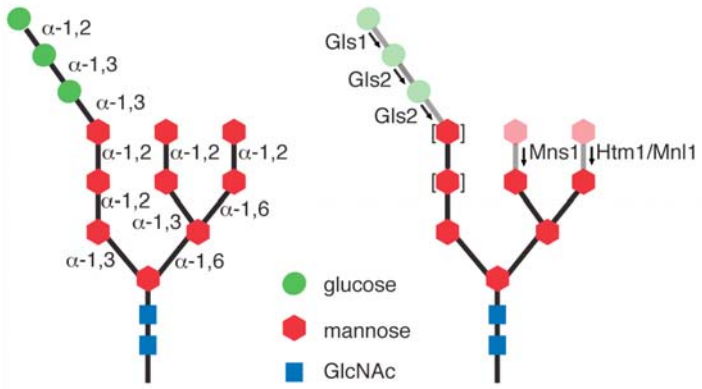


Figure 6: During the folding process of glycoproteins in the ER N-linked glycan structures are processed

In yeast the three terminal glucose residues of the initial Glc_3-Man_9-$GlcNAc_2$ structure are removed by glucosidase I (Gls1) and glucosidase II (Gls2). Prolonged ER lumenal retention of the protein due to incompleted folding recruits α-1,2-mannosidase (Mns1) and α-mannosidase like protein (Htm1/Mnl1) to remove two mannose residues resulting in a Man_7-$GlcNAc_2$ glycan structure. This structure, containing a terminal α-1,6-linked mannose, is an attribute of misfolded glycoproteins. In mammalian cells the orthologues of Htm1/Mnl1, EDEM1-3 are able to trim oligosaccharides to Man_6-$GlcNAc_2$ or Man_5-$GlcNAc_2$ glycan structures (modified from appendix 5).

slow acting mannosidase I (Mns1) removes a mannose residue from the B branch of the glycan (Fig. 6) [128, 130]. This process was suggested to serve as a ‚timer', giving proteins a certain time window for folding. The glycan tree of misfolded proteins is further trimmed within the ER lumen by the mannosidase like protein Mnl1/Htm1 to finally yield a structure containing a α-1,6-linked mannose residue in the C-branch of the carbohydrate [131, 132].

In the beginning of this thesis it was still under discussion, which glycan tree is most effective for recognition of the substrate by the lectin Yos9 for subsequent delivery to the UPS. Several glycan structures down to Man_5 structures had been identified on ERAD substrates, but Mnl1/Htm1 and Mns1 were found to trim down the glycan to a Man_7 structure only [131, 132]. Via database search a putative α-1,2-mannosidase encoded by ORF YLR057W, renamed as Mnl2, was identified and analyzed for its involvement in ERAD of glycosylated CPY* [133]. Single deletion of *MNL2* had no influence on the degradation kinetics of CPY* (Fig. 7A). However, *MNL1 MNL2* double deleted cells exhibited an additive effect on CPY* stabilization as compared to the single *MNL1* deletion (Fig. 7B). A deletion of *MNL2* together with *MNL1* did not affect degradation of an unglycosylated CPY* species, indicating that Mnl2 does indeed work on the glycan part of CPY* (Fig. 7C). These findings propose Mnl2 as a new ERAD component with possible mannosidase activity and point to overlapping functions of Mnl1 and Mnl2 in the carbohydrate trimming process. Mnl2 could serve as a backup for Mnl1 in the case of an ER overload with misfolded proteins due to heat shock or other stress conditions. In addition Mnl2 could be the mannosidase

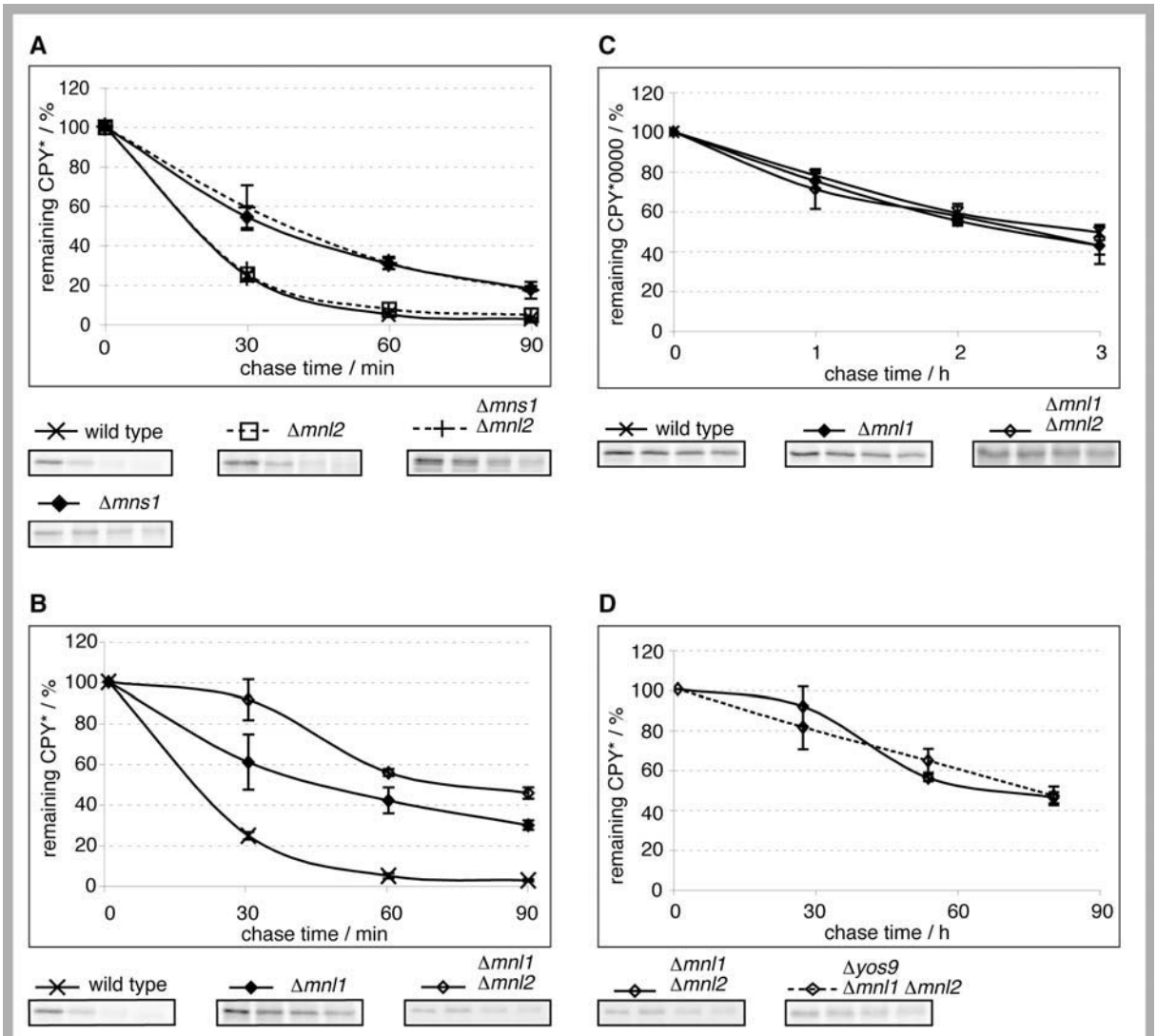


Figure 7: Mnl2 is involved in the degradation of CPY*

(**A**): Mnl2 does not show an effect on its own or in combination with Mns1, in the degradation of the glycosylated ERAD substrate CPY*. (**B**) Mnl2 plays a role in glyco-protein ERAD in combination with Mnl1 since their absence decreases the speed of CPY* turnover. Endo-genously expressed CPY* turnover was followed by pulse chase analysis of the different strains The pulse chase analyses data represent the means of 3 to 4 independent experiments ± the interval of confidence calculated with an $\alpha = 0.05$. (**C**) Mnl1 and Mnl2 do not influence the turnover of the unglycosylated ERAD substrate CPY*0000. (**D**) Yos9 cannot recognize the glycan structure of the substrate as degradation signal if mannosidases are absent. Endogenously expressed CPY* turnover was followed by pulse chase analysis of the different strains The pulse- chase analyses data represent the means of 3 to 4 independent experiments ± the interval of confidence calculated with an a = 0.05. (modified from appendix 1).

that trims the glycan tree down to the Man_5 structure. This glycan structure showed the strongest affinity for the lectin Yos9, which acts as a gatekeeper for delivery of misfolded proteins to the cytosol [35, 132]. Further biochemical studies have to be performed to test this hypothesis.

Published in appendix 1 [133].

2.2 Yos9 is involved in the ERAD process of unglycosylated CPY*

Trimmed glycan trees of misfolded proteins are recognized by the lectin Yos9 and further transmitted to the ubiquitylation machinery for subsequent degradation [35, 134-136]. Yos9 contains a so-called MRH domain, which was shown to interact with glycans [137, 138]. However, when the need of ERAD components for the degradation of the unglycosylated ERAD substrate CPY*0000 [36, 120] - a CPY* version with mutated glycosylation sites (Fig. 5) - was analyzed, a function of Yos9 in the degradation of this unglycosylated ERAD substrate was also found. While glycosylated CPY* is stabilized in cells lacking Yos9, degradation of unglycosylated CPY*0000 was enhanced in *YOS9* deleted cells compared to wild type (Fig. 8). Most probably Yos9 can bind unglycosylated proteins and prevent their rapid degradation, giving them more time to fold. This stabilizing function of Yos9 is independent of its MRH domain. Cells carrying a mutation of the MRH domain showed the same degradation pattern as wild type cells concerning CPY*0000 (Fig. 8) [35]. It is yet not clear, if this represents a general mechanism for unglycosylated proteins to allocate more time for proteins to fold before they are delivered to the degradation machinery. If so, this would expand the timer model that up to now only includes glycosylated proteins and strengthen the importance of Yos9 as a gatekeeper of the ER in general.
Published in appendix 2 [36].

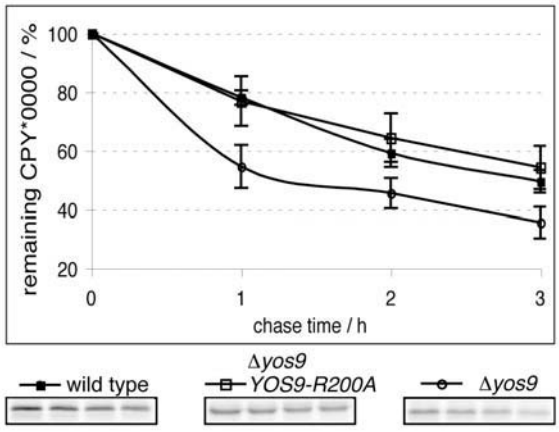


Figure 8: Yos9 stabilizes the ERAD substrate CPY*0000

The degradation efficiency of CPY*0000 in *YOS9* deleted cells and cells expressing Yos9 carrying a mutation in the MRH domain. Wild type and Δ*yos9* strains were transformed with CPY*0000 or CPY*0000 and Yos9R200A-Flag expression plasmids, respectively. Data represent the means of four (wild type), six (*YOS9R200A*) and seven (Δ*yos9*) independent experiments and interval of confidence with an α=0.05 for the error bars is calculated (modified from appendix 2).

2.3 Dfm1, a new component of ERAD

Dfm1 is the only known homologue of Der1, an important component of the ERAD-L pathway (Fig. 4), and located at the ER membrane. Like Der1 it contains four transmembrane domains with its C- and N-terminus facing the cytosol. The C-terminus of Dfm1 is elongated compared to Der1 and carries two SHP boxes, known to interact with Cdc48. No precise function of Dfm1 in ERAD or other processes was known until the start of this thesis [108, 139].

2.3.1 Dfm1 is necessary for cell homeostasis

To test a possible participation of Dfm1 in the degradation of ERAD substrates, cells deleted in *DFM1* were transformed with a high copy plasmid coding for the ERAD-C substrate Ste6* [117]. Surprisingly transformation rates were very poor and many transformants did not express the protein. Those who did express the protein showed a growth defect compared to wild type cells and cells lacking the main E3 ligase for Ste6*, Doa10 (Fig. 9A). Further studies on the influence of the overexpression of Ste6* on cells deleted in *DFM1* were done in cooperation with Konrad Otte [140].
Growth tests and FACS analyses revealed, that overexpression of Ste6* leads to a growth defect due to severe cell death in cells lacking Dfm1 (Fig. 9A-C). This could be due to an overload of the ER membrane with the misfolded membrane protein Ste6*. However cells lacking the E3 ligases Doa10 and Hrd1/Der3 resulting in comparable stabilization of Ste6* (see below) did not show this characteristics. More interestingly, additional deletion of *HRD1* or *DOA10* in a Δ*dfm1* background rescued the phenotype. A possible explanation for this phenomenon could be the differences in the activation of the unfolded protein response (UPR): Deletion of *DFM1* alone does not activate the UPR, while deletion of *HRD1* and *DOA10* does [139, 141, 142]. Activation of the UPR upregulates the expression of a multitude of genes. This includes a large number of chaperones, which could help the cell to handle the overload of the ER with Ste6*.
Cell death of *DFM1* deleted cells were accompanied by a strong increase of the production of reactive oxygen species (ROS; Fig. 9D,E), indicating programmed cell

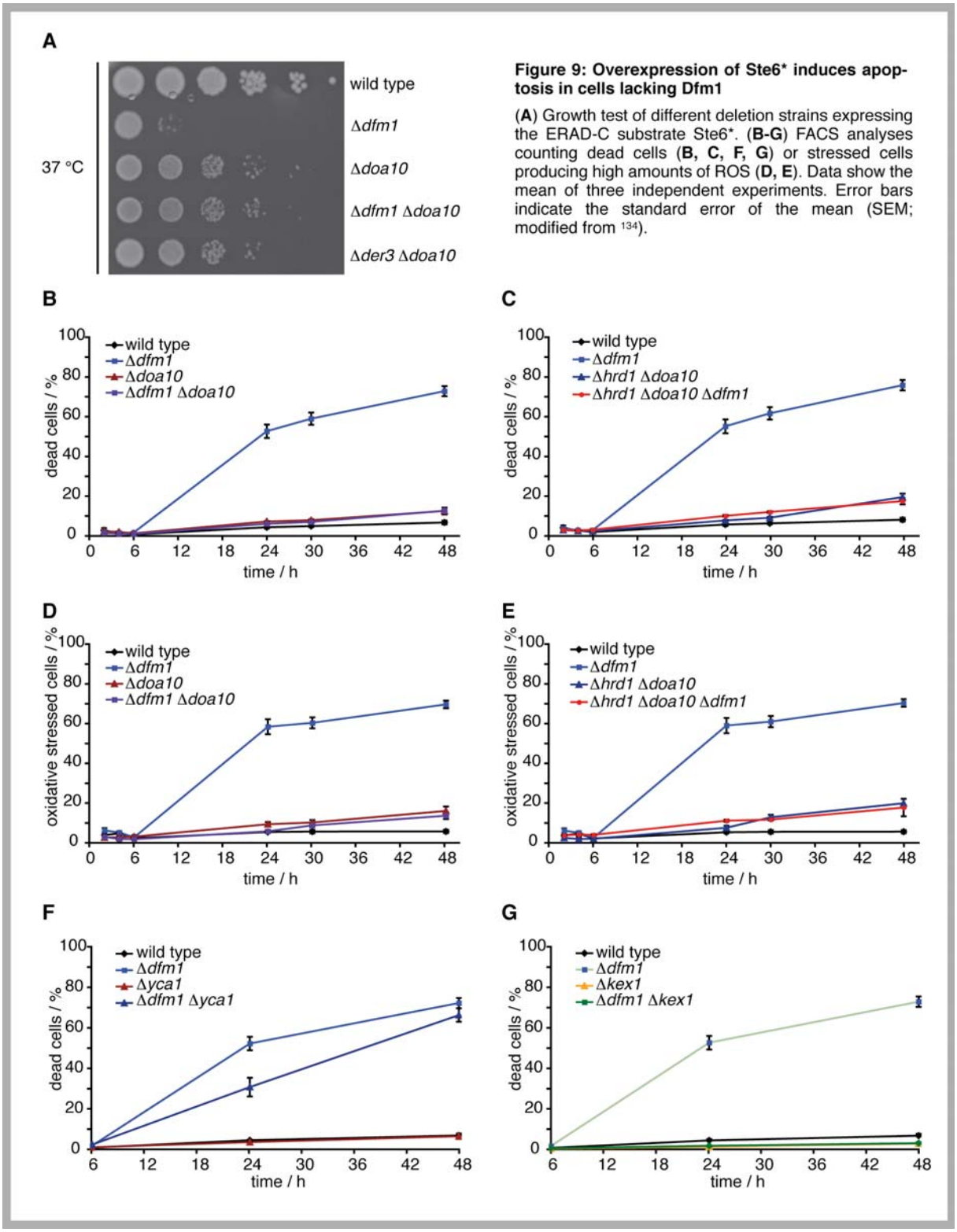


Figure 9: Overexpression of Ste6* induces apoptosis in cells lacking Dfm1

(**A**) Growth test of different deletion strains expressing the ERAD-C substrate Ste6*. (**B-G**) FACS analyses counting dead cells (**B, C, F, G**) or stressed cells producing high amounts of ROS (**D, E**). Data show the mean of three independent experiments. Error bars indicate the standard error of the mean (SEM; modified from [134]).

death (also called apoptosis) of cells (for review see: [143-145]). To test this hypothesis genes coding for the key enzymes *KEX1* and *YCA1*, triggering different apoptotic pathways [146-149], were deleted in a Δ*dfm1* background. If one of the enzymes should be involved in Δ*dfm1* mediated apoptosis, additional deletion of the coding region for Yca1 or Kex1 should rescue the apoptotic phenotype from the *DFM1* deletion. While

additional deletion of *YCA1* only delayed cell death (Fig. 9F), *KEX1* deletion fully rescued the apoptotic *DFM1* deletion phenotype (Fig. 9G). This indicates the activation of an apoptotic pathway due to overexpression of Ste6* in the absence of Dfm1, which is dependent on Kex1. Apoptosis can most probably be circumvented by activation of the UPR, however, this hypothesis remains to be confirmed. It is not yet clear which function Dfm1 plays in this process. However, it is already known that Cdc48 plays a role in apoptosis and the possibility exists that Dfm1 with its SHP domains functions as a recruitment factor for Cdc48 in this process [73].

2.3.2 Dfm1 function in ERAD

To test whether Dfm1 is a component of ERAD and therefore interacts with other components of the ERAD pathway a peptide antibody was raised against Dfm1 and immunoprecipitation experiments with solubilized membrane protein complexes were performed.

Dfm1 and its homologue Der1 interact via Usa1

The first candidate for a possible interaction with Dfm1 was its homologue Der1. It was under discussion that these two membrane proteins could build a retrotranslocation channel for misfolded proteins. In cells with genomically HA tagged Der1 a clear interaction between Dfm1 and the fully functional Der1-HA [109] fusion protein was found by precipitating Dfm1 with Dfm1 antibodies (Fig. 10A lane 4). The reverse experiment, using HA antibodies for Der1-HA precipitation, confirmed the interaction between the two homologues Der1 and Dfm1 (Fig. 10B lane 4). No unspecific binding was found for Der1-HA and Dfm1, respectively (Fig. 10A lane 5; 10B lane 3). Der1 is known to be part of the Hrd/Der ligase complex and to interact with Hrd1/Der3 via Usa1 [107]. When precipitating Dfm1, Hrd1/Der3 could be found in the precipitate in addition to Der1. To test whether Dfm1 interacts with the Hrd1/Der3 ligase like Der1 through the linker protein Usa1, the same experiment was performed in a strain deleted in *USA1*. While interaction between Dfm1 and the ligase Hrd1/Der3 remained stable, interaction to Der1 was lost (Fig. 10A lane 6). It is

concluded that Dfm1 and Der1 interact indirectly via Usa1. Dfm1 may interact directly with Hrd1/Der3 or by a yet unknown linker protein. The identified Dfm1 complex also contains Hrd3 (Fig. 10C), which was previously shown to stabilize Hrd1/Der3 [106, 111]. Taken together, theses results show the existence of a membrane complex consisting of the Hrd/Der ligase complex, Dfm1, Usa1 and Der1.
Published in appendix 3 [150].

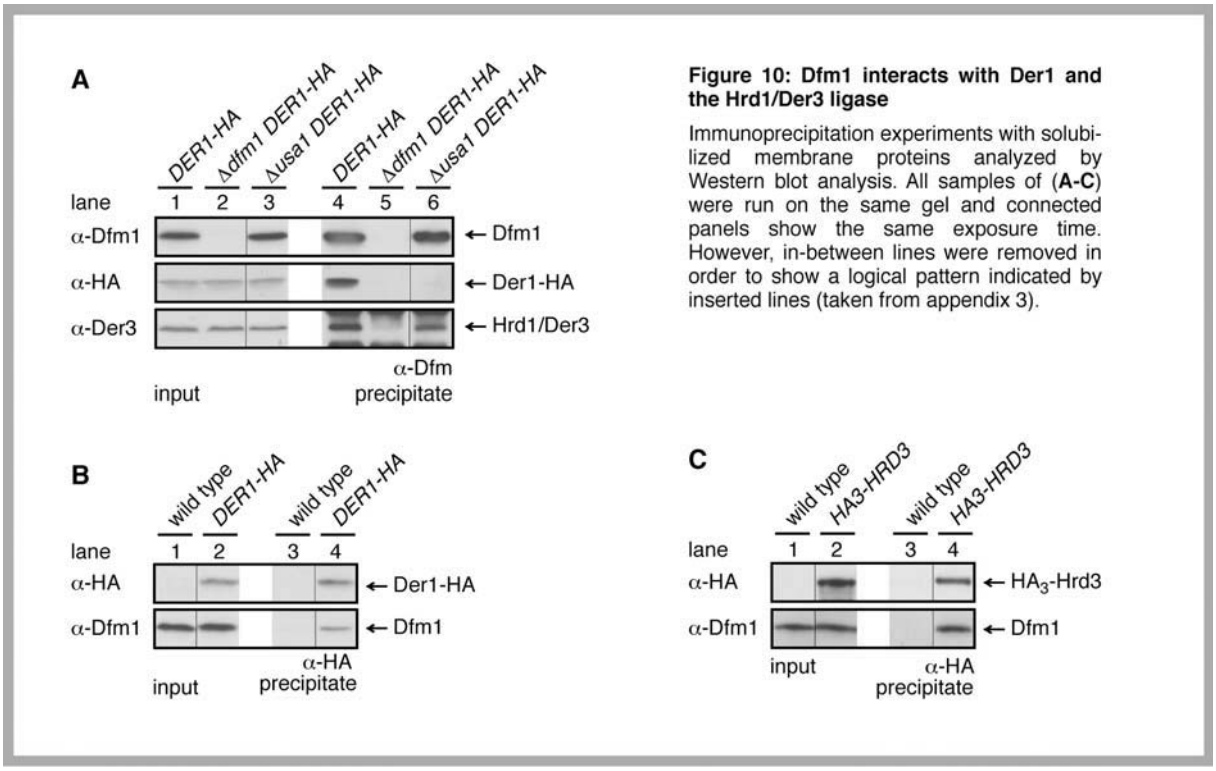


Figure 10: Dfm1 interacts with Der1 and the Hrd1/Der3 ligase

Immunoprecipitation experiments with solubilized membrane proteins analyzed by Western blot analysis. All samples of (**A-C**) were run on the same gel and connected panels show the same exposure time. However, in-between lines were removed in order to show a logical pattern indicated by inserted lines (taken from appendix 3).

Dfm1 interacts with Cdc48 and Hrd1/Der3 independent of the Cdc48 anchor protein Ubx2

Dfm1 carries two so-called SHP boxes at its C-terminus facing the cytosolic side of the ER membrane [108]. These SHP boxes were shown to be necessary for an interaction between Dfm1-HA_3 and the AAA type ATPase Cdc48 [139]. When testing the stability of the Dfm1-HA_3 fusion protein a destabilization compared to the wild type protein was observed (Fig. 11). The Dfm1-HA_3 fusion protein was stabilized in an ERAD deficient strain deleted in *USA1*, indicating its degradation through the ERAD pathway (Fig. 11). It was yet to be determined, if the reported interaction between Dfm1-HA_3 and Cdc48 was only due to the degradation of Dfm1-HA_3 through the ERAD pathway. By performing immunoprecipitation analysis with Dfm1

antibodies interaction between Cdc48 and wild type Dfm1 could be shown (Fig. 12 lane 4). No unspecific binding of Cdc48 with Dfm1 antibodies could be detected (Fig. 12 lane 6) verifying that Cdc48 also interacts with wild type Dfm1.

Ubx2 is the membrane protein anchoring the Cdc48 complex to the ER membrane embedded ligases Hrd1/Der3 and Doa10, thereby ensuring efficient degradation of ERAD substrates (Fig. 4). In the absence of Ubx2 neither the ligase Hrd1/Der3 nor the ligase Doa10 can be found in complex with Cdc48 [151, 152]. It was interesting to see, whether Ubx2 links Dfm1 to the Hrd1/Der3 ligase. However, in the absence of Ubx2 Dfm1 still interacts with both proteins, Cdc48 and Hrd1/Der3 (Fig. 12 lane 5). Assuming that loss of Ubx2 does not lead to irregular decomposition of the Cdc48-Hrd1/Der3 complex, this finding indicates two independent Dfm1 complexes containing Hrd1/Der3 and Cdc48, respectively.

Published in appendix 3 [150].

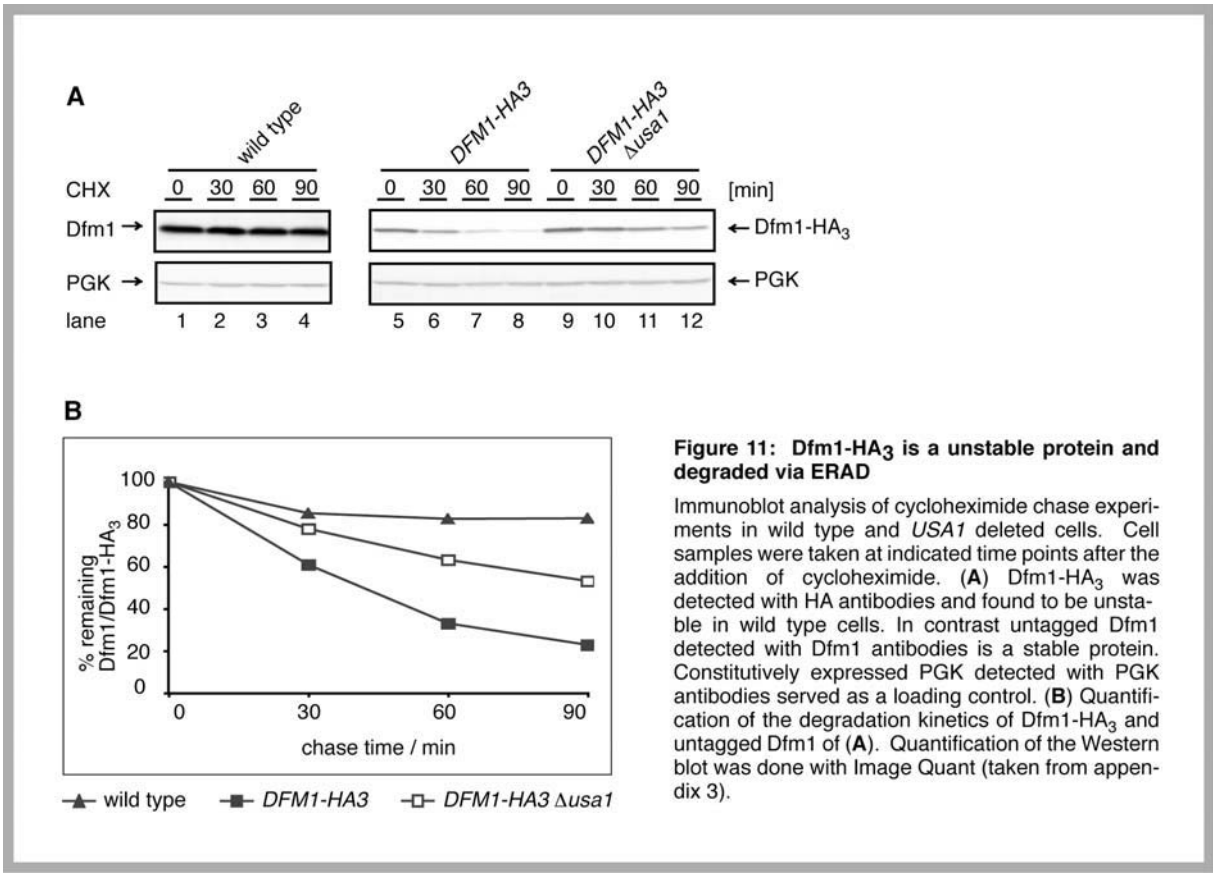


Figure 11: Dfm1-HA$_3$ is a unstable protein and degraded via ERAD

Immunoblot analysis of cycloheximide chase experiments in wild type and *USA1* deleted cells. Cell samples were taken at indicated time points after the addition of cycloheximide. (**A**) Dfm1-HA$_3$ was detected with HA antibodies and found to be unstable in wild type cells. In contrast untagged Dfm1 detected with Dfm1 antibodies is a stable protein. Constitutively expressed PGK detected with PGK antibodies served as a loading control. (**B**) Quantification of the degradation kinetics of Dfm1-HA$_3$ and untagged Dfm1 of (**A**). Quantification of the Western blot was done with Image Quant (taken from appendix 3).

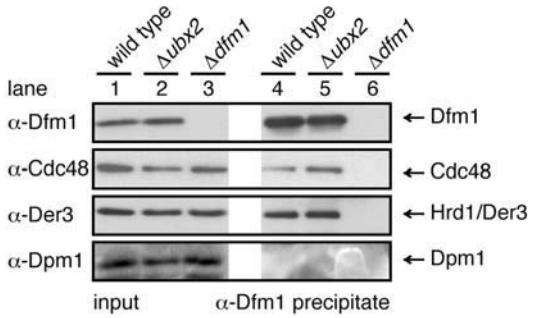


Figure 12: Wild type Dfm1 interacts with Cdc48 and Hrd1/Der3 in the absence of Ubx2

Immunoprecipitation experiment of indicated strains analyzed by Western blot analysis. The input fraction contained solubilized membrane proteins. Dfm1 and its interaction partners were precipitated with Dfm1 antibodies. Dpm1 served as a negative control to show specificity (modified from appendix 3).

Dfm1 interacts with the ligase Doa10

In the next step, a possible interaction between Dfm1 and Doa10, the main E3 ligase of the ERAD-C pathway, should be tested. When precipitating Dfm1 with Dfm1 antibodies, Doa10 was found to co-precipitate with Dfm1 (Fig. 13B lane 5). Also the reverse experiment using myc antibodies to precipitate myc tagged Doa10 confirmed interaction between Dfm1 and Doa10 (Fig. 13A lane 9). The experiment was repeated in a strain missing the Cdc48 recruiting factor Ubx2. As expected, Cdc48 did no longer co-precipitate with Doa10 in the absence of Ubx2 (Fig. 13C lane 6). However, interaction between Dfm1 and Doa10 remained stable (Fig. 13B lane 6; Fig. 13C lane 6). This result shows that Dfm1 does not interact indirectly with Doa10 via Ubx2 or via Cdc48 with its six possible binding sites but forms a Cdc48 independent complex with Doa10 instead.

Published in appendix 3 [150].

*Loss of Dfm1 leads to the stabilization of the ERAD-C substrate Ste6**

After identifying Dfm1 to be part of the ERAD machinery a function of Dfm1 in the degradation process of misfolded proteins was tested. As Dfm1 had been already shown to have no role in the degradation of ER lumenal CPY* [108], different ERAD substrates with a transmembrane domain were chosen. While deletion of *DFM1* showed no effect on the degradation of Pdr5* and CTL*myc, Ste6* was considerably stabilized (Fig. 14A,B). Also CTG* seems to be somewhat stabilized, however, the difference between wild type and *DFM1* deleted cells was much more pronounced in the case of Ste6*. To quantify the stabilization of the ERAD-C substrate Ste6* pulse

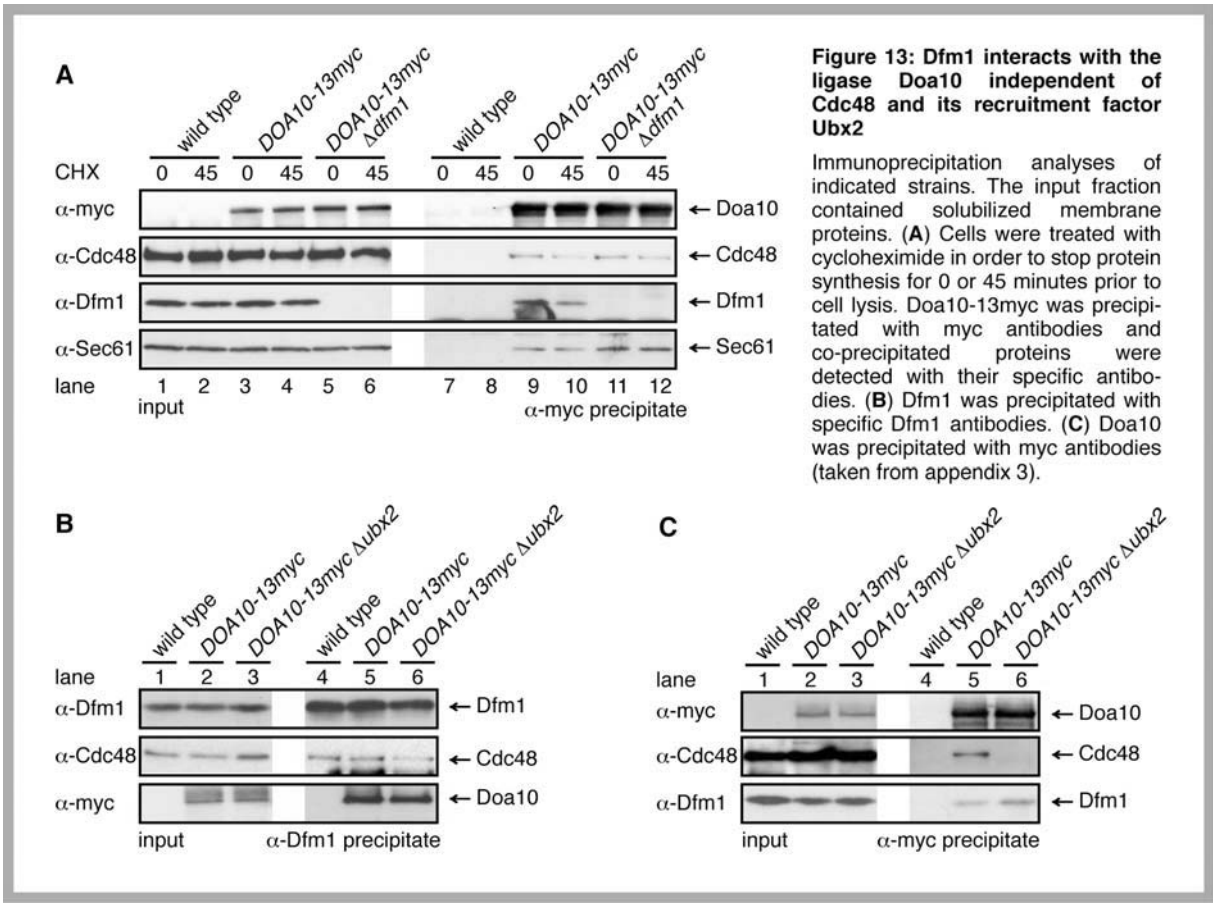


Figure 13: Dfm1 interacts with the ligase Doa10 independent of Cdc48 and its recruitment factor Ubx2

Immunoprecipitation analyses of indicated strains. The input fraction contained solubilized membrane proteins. (**A**) Cells were treated with cycloheximide in order to stop protein synthesis for 0 or 45 minutes prior to cell lysis. Doa10-13myc was precipitated with myc antibodies and co-precipitated proteins were detected with their specific antibodies. (**B**) Dfm1 was precipitated with specific Dfm1 antibodies. (**C**) Doa10 was precipitated with myc antibodies (taken from appendix 3).

chase analyses were performed. Even though Ste6* is defined as an ERAD-C substrate, which is mainly degraded via the E3 ligase Doa10, Hrd1/Der3 can partially take over the ubiquitylation function of Doa10. Therefore, *DOA10 HRD1* double deleted cells were used as a control for an ERAD deficient strain. Deletion of *DFM1* leads to approximately the same stabilization of Ste6* as the deletion of the genes coding for the ER ligases Doa10 and Hrd1/Der3 (Fig. 14C). Interestingly, even though deletion of *DFM1* or the coding sequences of the two ER ligases Doa10 and Hrd1/Der3 showed stabilization of the misfolded protein, this stabilization was only partial.

Published in appendix 3 [150].

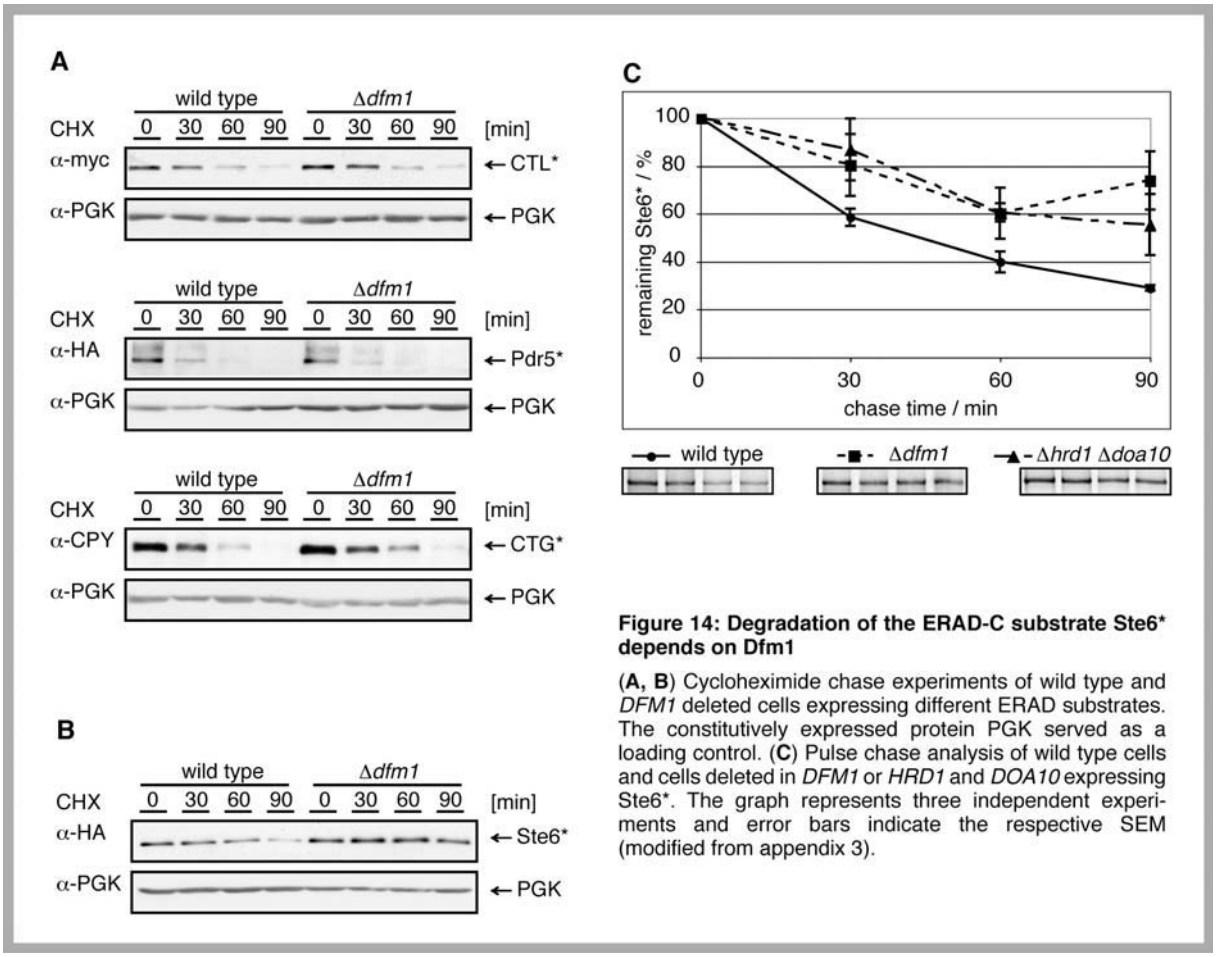


Figure 14: Degradation of the ERAD-C substrate Ste6* depends on Dfm1

(**A, B**) Cycloheximide chase experiments of wild type and *DFM1* deleted cells expressing different ERAD substrates. The constitutively expressed protein PGK served as a loading control. (**C**) Pulse chase analysis of wild type cells and cells deleted in *DFM1* or *HRD1* and *DOA10* expressing Ste6*. The graph represents three independent experiments and error bars indicate the respective SEM (modified from appendix 3).

2.3.3 A novel role for the ubiquitin ligase Ubr1 in ERAD

This topic was done in cooperation with Stefanie Besser.

Ste6 is still ubiquitylated in the absence of the two ER ligases Hrd1/Der3 and Doa10*

It was still unclear if the remaining Ste6* degradation in cells deficient in the two membrane embedded ubiquitin ligases Hrd1/Der3 and Doa10 was of vacuolar or proteasomal origin. To address this question an ubiquitylation essay of Ste6* was performed. The misfolded protein was precipitated under denaturing conditions with HA antibodies. The presence of NEM prevented deubiquitylation of the substrate. As can be seen, overall ubiquitylation of the input fraction did not differ between the deletion strains used and wild type cells (Fig. 15 lane 1-6). While strong ubiquitylation of Ste6* could be detected in wild type cells only weak unspecific signals appeared in the negative control (Fig. 15 compare lane 7 and 8) demonstrating the specificity of

the pull down. In coincidence with the stabilization of Ste6* in pulse chase analysis (Fig. 14C), cells deleted in *DFM1* showed a strong reduction in the ubiquitylation of Ste6* compared to wild type cells (Fig. 15 lane 10,11). Additional deletion of *DOA10* increased ubiquitylation of Ste6* compared to singly *DFM1* deleted cells (Fig. 15 lane 9). Even though unexpected, this might be explained by the activation of the unfolded protein response (UPR) and subsequent higher ubiquitylation of Ste6* by the Hrd1/Der3 ligase in *DFM1 DOA10* double deleted cells. While deletion of *DFM1* alone does not activate the UPR, deletion of most of the other known ERAD components like Der1, Hrd1/Der3 and Doa10 does [139, 141, 142]. In *DOA10 HRD1* double deleted cells, which are completely blocked in the canonical ERAD pathways, Ste6* clearly was still ubiquitylated (Fig. 15 lane 12). Together with the finding of only partial stabilization of Ste6* in *DOA10 HRD1* double deleted cells (Fig. 14C) this suggests the existence of an additional, ubiquitin dependent degradation pathway independent of Hrd1/Der3 and Doa10.

Manuscript submitted and in the review process: appendix 4 [153].

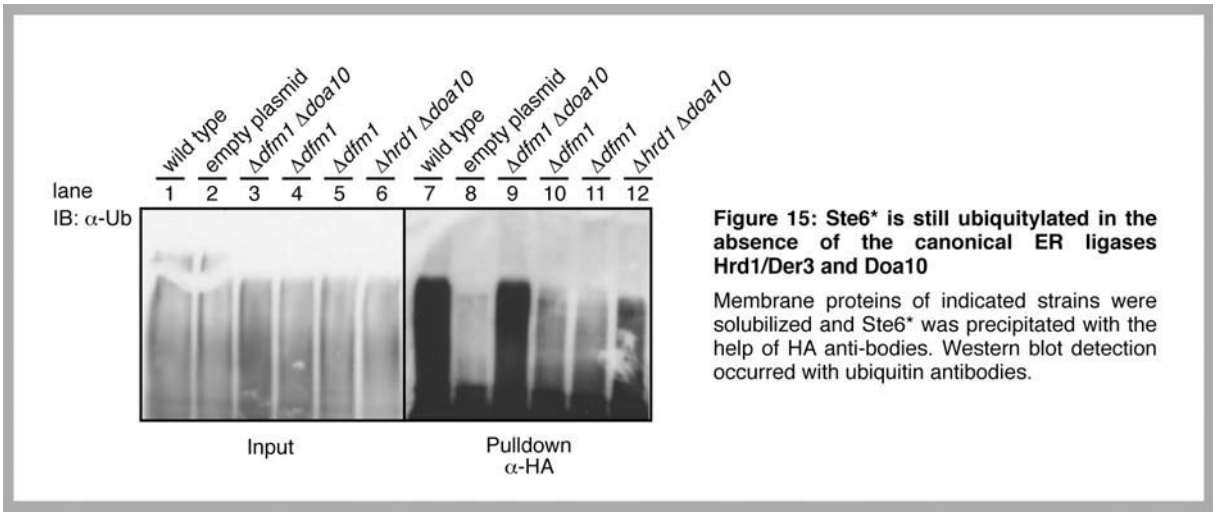


Figure 15: Ste6* is still ubiquitylated in the absence of the canonical ER ligases Hrd1/Der3 and Doa10

Membrane proteins of indicated strains were solubilized and Ste6* was precipitated with the help of HA anti-bodies. Western blot detection occurred with ubiquitin antibodies.

Dfm1 participates in a Doa10 Hrd1/Der3 independent ERAD pathway

Ubiquitylation of Ste6* in *DOA10 HRD1* double deleted cells seemed even stronger than in *DFM1* single deleted cells (Fig. 15 lane 10-12), suggesting that Dfm1 participates in the hypothesized Hrd1/Der3 and Doa10 independent degradation pathway. To address this question the degradation of Ste6* was quantified in *DFM1 DOA10 HRD1* triple deleted cells. The additional deletion of *DFM1* led to further

stabilization of the substrate compared to cells only deleted in *DOA10* and *HRD1* (Fig. 18D). This finding validates the presence of a yet unidentified degradation pathway.

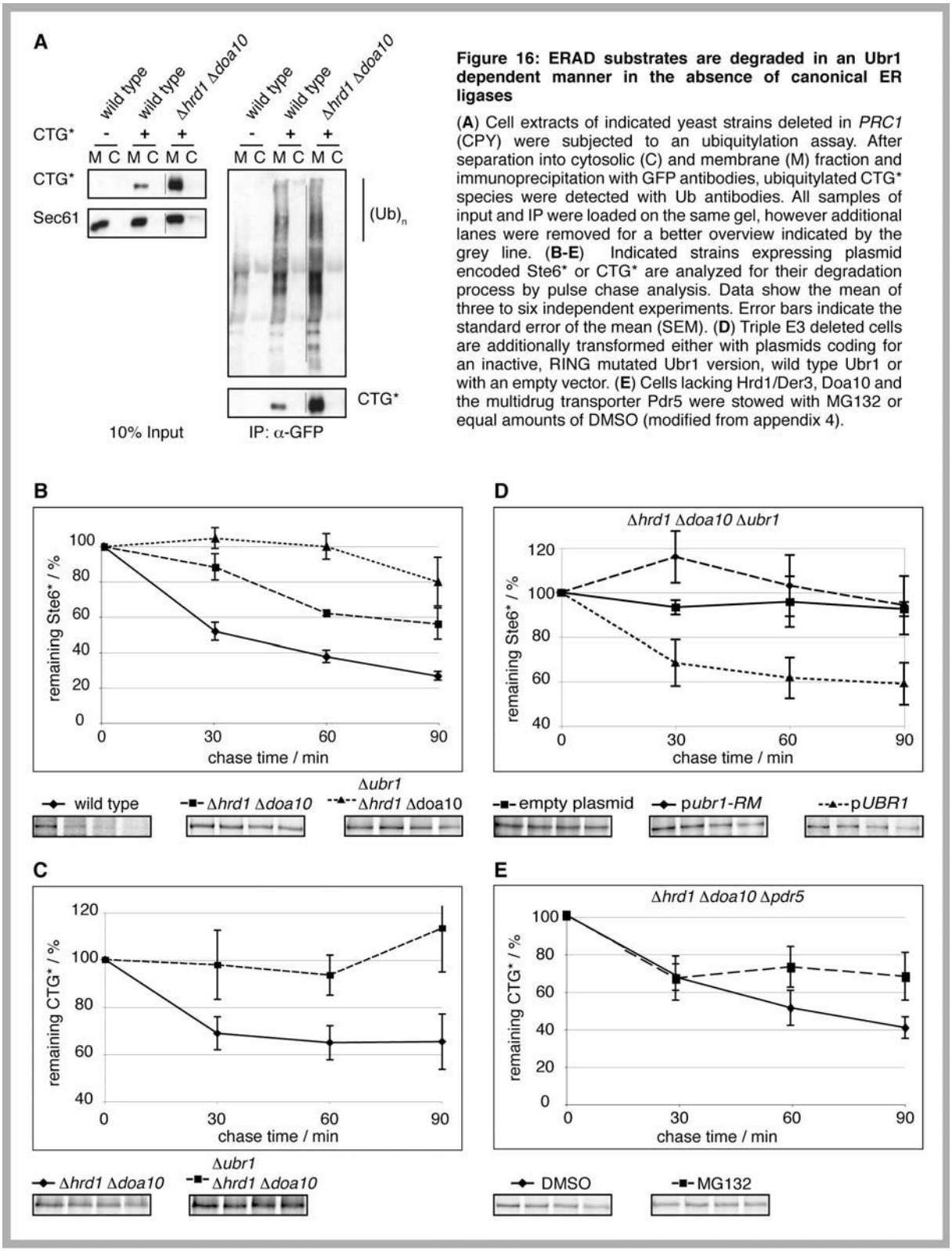


Figure 16: ERAD substrates are degraded in an Ubr1 dependent manner in the absence of canonical ER ligases

(**A**) Cell extracts of indicated yeast strains deleted in *PRC1* (CPY) were subjected to an ubiquitylation assay. After separation into cytosolic (C) and membrane (M) fraction and immunoprecipitation with GFP antibodies, ubiquitylated CTG* species were detected with Ub antibodies. All samples of input and IP were loaded on the same gel, however additional lanes were removed for a better overview indicated by the grey line. (**B-E**) Indicated strains expressing plasmid encoded Ste6* or CTG* are analyzed for their degradation process by pulse chase analysis. Data show the mean of three to six independent experiments. Error bars indicate the standard error of the mean (SEM). (**D**) Triple E3 deleted cells are additionally transformed either with plasmids coding for an inactive, RING mutated Ubr1 version, wild type Ubr1 or with an empty vector. (**E**) Cells lacking Hrd1/Der3, Doa10 and the multidrug transporter Pdr5 were stowed with MG132 or equal amounts of DMSO (modified from appendix 4).

As CTG* seemed to be stabilized in *DFM1* deleted cells to some extent (Fig. 14A) an ubiquitylation assay of CTG* was performed. Like Ste6*, CTG* was still ubiquitylated in the absence of both canonical ERAD ligases (Fig. 16A) supporting the assumption that also CTG* undergoes Hrd1/Der3 and Doa10 independent degradation. To test if this additional degradation pathway is due to proteasomal degradation, pulse chase analyses of CTG* in the absence of Hrd1/Der3 and Doa10 under proteasomal inhibition through MG132 were performed. In order to prolong the presence of MG132 in the cells, the gene coding for Pdr5, a multi drug transporter of the plasma membrane, was deleted [154]. Since MG132 was dissolved in DMSO, cells were treated either with MG132 or an equivalent volume of DMSO as a negative control. Indeed the substrate CTG* was stabilized in the presence of MG132 in *HRD1 DOA10 PDR5* deleted cells compared to cells treated only with DMSO (Fig. 16E). This finding suggests to the existence of an Hrd1/Der3 and Doa10 independent ERAD pathway finally resulting in proteasomal degradation.

Besides Hrd1/Der3 and Doa10 there are no other known E3 ubiquitin ligases in the ER membrane. This raised the question, which E3 ligase is necessary for the proteasomal degradation of CTG* in the absence of Hrd1/Der3 and Doa10. Under these conditions ERAD substrates may leave the overcrowded ER towards the Golgi and be degraded at the Golgi membrane in a process similar to ERAD. A candidate for such a hypothetical process is Tul1, an E3 RING ligase located in the Golgi membrane necessary for the multivesicular body (MVB) sorting pathway [155]. Another possibility includes cytosolic E3 ligases that might be recruited to the ER membrane in the absence of Hrd1/Der3 and Doa10. Besides others, candidates in this case would be Rsp5, known to participate in protein trafficking [156], and Ubr1, which was recently shown to be involved in the proteasomal degradation of misfolded cytosolic proteins [121, 125]. As Tul1 and Rsp5 are mainly involved in degradation processes resulting in vacuolar degradation, the best candidate in this case seemed to be Ubr1. Indeed additional deletion of *UBR1* in a *Δhrd1 Δdoa10* background led to 100% stabilization of CTG* (Fig. 16C). Equivalent results were obtained using Ste6* as a

substrate (Fig. 16B). These findings implicate a new ERAD pathway dependent on Ubr1. More specifically, this ERAD pathway seems to serve as a backup system for Doa10 and Hrd1/Der3 dependent degradation as single deletion of *UBR1* had no visible effect on the degradation of CTG* (Fig. 17).

Manuscript submitted and in the review process: appendix 4 [153].

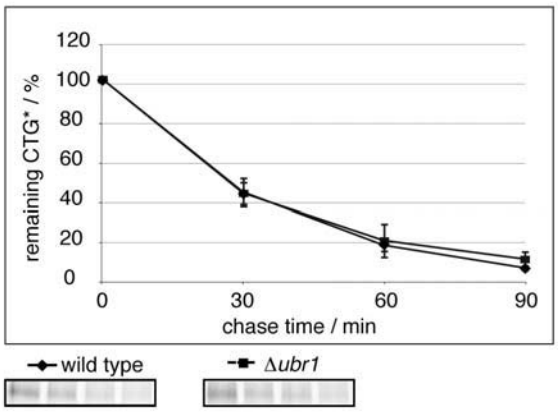


Figure 17: Degradation of CTG* occurs independently of Ubr1 in Hrd1/Der3 and Doa10 wild type cells

Wild type and *UBR1* deleted cells expressing plasmid encoded CTG* are analyzed for their degradation process by pulse chase analysis. Data show the mean of three independent experiments. Error bars indicate the standard error of the mean (SEM; modified from appendix 4).

Ubiquitylation activity of Ubr1 is necessary for the degradation of Ste6 in HRD1 DOA10 deleted cells*

It should be tested if the ubiquitylation activity based on the RING domain of Ubr1 is necessary for its effect on Ste6* and CTG* degradation in *HRD1 DOA10* double deleted cells or if also RING mutated Ubr1 is sufficient to support degradation. Cells deleted in *DOA10, HRD1* and *UBR1* were transformed with plasmids carrying either active Ubr1 or inactive RING mutated Ubr1 or with an empty plasmid. Monitoring the degradation pattern of Ste6* in pulse chase analysis showed, that RING mutated Ubr1 (Ubr1-RM) was not able to complement the *UBR1* deletion (Fig. 16D). In contrast active Ubr1 was able to complement the *UBR1* deletion completely. This indicates, that Ubr1 acts as an ubiquitin ligase on Ste6* to trigger proteasomal degradation of this ERAD substrate in the absence of the canonical ER ligases Hrd1/Der3 and Doa10.

Ste6* and CTG* belong to different classes of ERAD substrates. CTG* carries a misfolded CPY* moiety in the ER lumen and therefore belongs to the class of ERAD-L substrates (see 1.7; 1.8). Ste6* is an ERAD-C substrate with a misfolded domain that faces the cytosol (see 1.7; 1.8). Details and possible differences of the Ubr1

dependent degradation pathway of the two ERAD substrates Ste6* and CTG* were investigated. Cdc48 and Dfm1 were found to be required to different extents. Ste6* degradation in the absence of Hrd1/Der3 and Doa10 was dependent on Dfm1 and Cdc48 (Fig. 18B, D). This led to the hypothesis that Dfm1 is needed to recruit Cdc48 to the substrate via its SHP boxes. In contrast, CTG* degradation was not impaired by the loss of Cdc48 and only slightly affected by the loss of Dfm1 (Fig. 18A, C). Therefore Ste6* but not CTG* seems to be extracted from the ER membrane with the help of the segregase activity of Cdc48.

To test whether Dfm1 functions in the recruitment of Cdc48 in the novel Ubr1 dependent ERAD pathway, co-immunoprecipitation experiments were performed. Ste6* was found to specifically interact with Ubr1, Dfm1 and Cdc48 (Fig. 18E). However, the interaction between Ste6* and Cdc48 remained stable in the absence of Dfm1. This finding excludes the possibility that Dfm1 functions as a Cdc48 recruitment factor in the novel Ubr1 dependent ERAD pathway of Ste6*.

Manuscript submitted and in the review process: appendix 4 [153].

Involvement of Ssa1 in the novel Ubr1 dependent ERAD pathway

After the finding that CTG* is degraded independently of Cdc48 in the novel Ubr1 dependent ERAD pathway the question arose, which machine would be able to take over Cdc48 functions. The cytosolic Hsp70 Ssa1 was tested for its function to provide the energy to pull CTG* out and away from the ER membrane. Ssa1 is an essential protein in the absence of its family members Ssa2 to Ssa4. Indeed CTG* degradation was strongly dependent on the activity of Ssa1 (Fig. 19A). Interestingly, also the degradation of Ste6* was dependent on Ssa1 (Fig. 19B; Manuscript submitted and in the review process: appendix 4 [153]).

It is unclear, how Ssa1 functions in this pathway on a molecular level. An ubiquitylation assay with *SSA1* wild type and temperature sensitive *ssa1-45* mutant cells was performed. Therefore cells were grown at permissive temperature (25 °C) and subsequently shifted to the restrictive temperature (37 °C) for several hours.

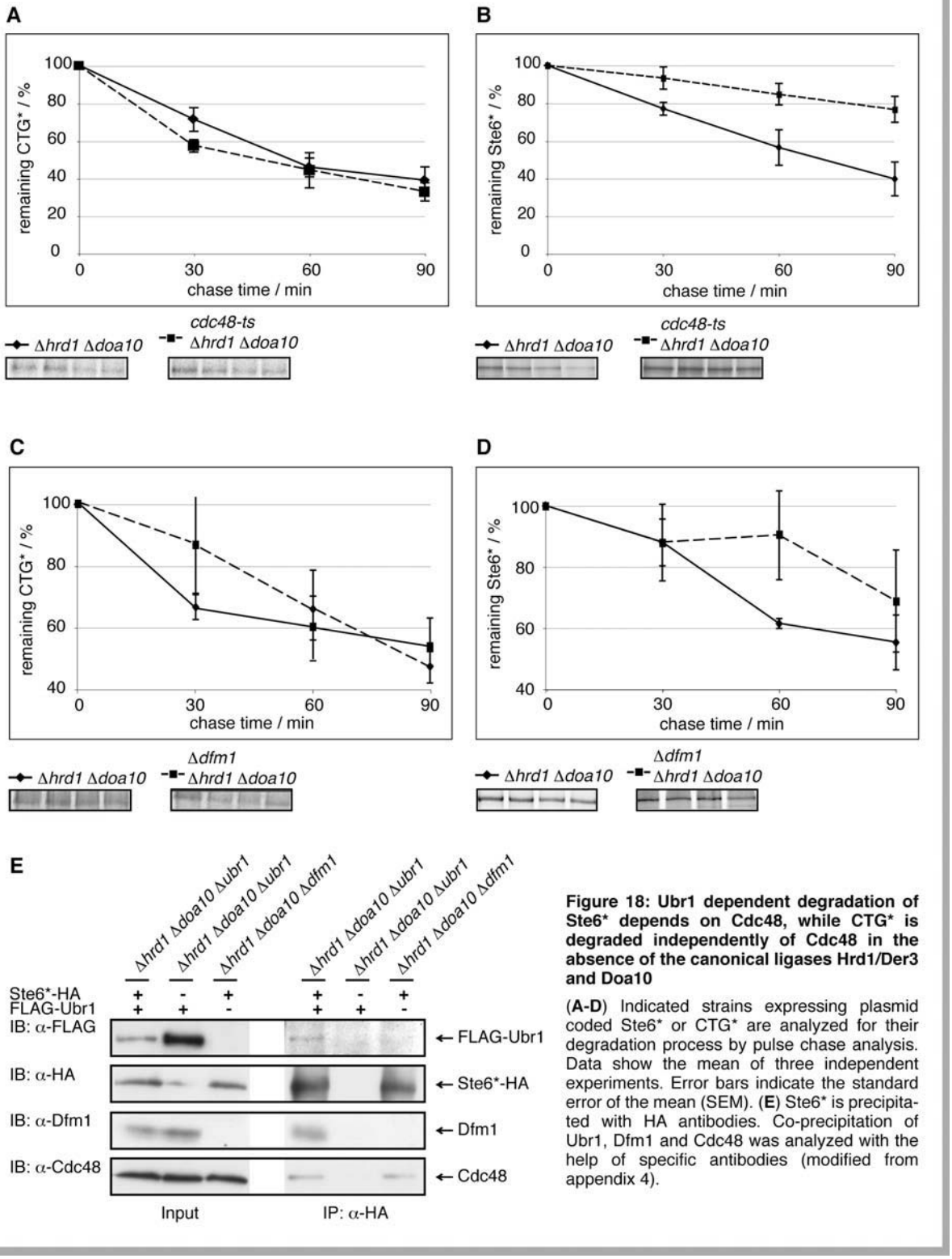


Figure 18: Ubr1 dependent degradation of Ste6* depends on Cdc48, while CTG* is degraded independently of Cdc48 in the absence of the canonical ligases Hrd1/Der3 and Doa10

(**A-D**) Indicated strains expressing plasmid coded Ste6* or CTG* are analyzed for their degradation process by pulse chase analysis. Data show the mean of three independent experiments. Error bars indicate the standard error of the mean (SEM). (**E**) Ste6* is precipitated with HA antibodies. Co-precipitation of Ubr1, Dfm1 and Cdc48 was analyzed with the help of specific antibodies (modified from appendix 4).

Ubiquitylated CTG* species of high molecular weight were found to accumulate between stacking gel and separation gel in *SSA1* wild type cells (Fig. 19C). In the *ssa1-45* background only some amount of these species could be detected after a 6 h shift at 37 °C. These species may be not yet degraded multi-ubiquitylated CTG* proteins built up during cell growth at 25°C or species that are stucked within the ER membrane due to insufficient transport out of the ER. These findings suggest a function of Ssa1 prior to ubiquitylation. One might suggest a ratcheting mechanism as has already been shown for Kar2, the Hsp70 of the ER lumen [157]. In such a model, Ssa1 would bind to the substrate directly at the cytosolic side of the ER membrane and enable its extraction. In this case Ssa1 most likely would act before substrate ubiquitylation by Ubr1.

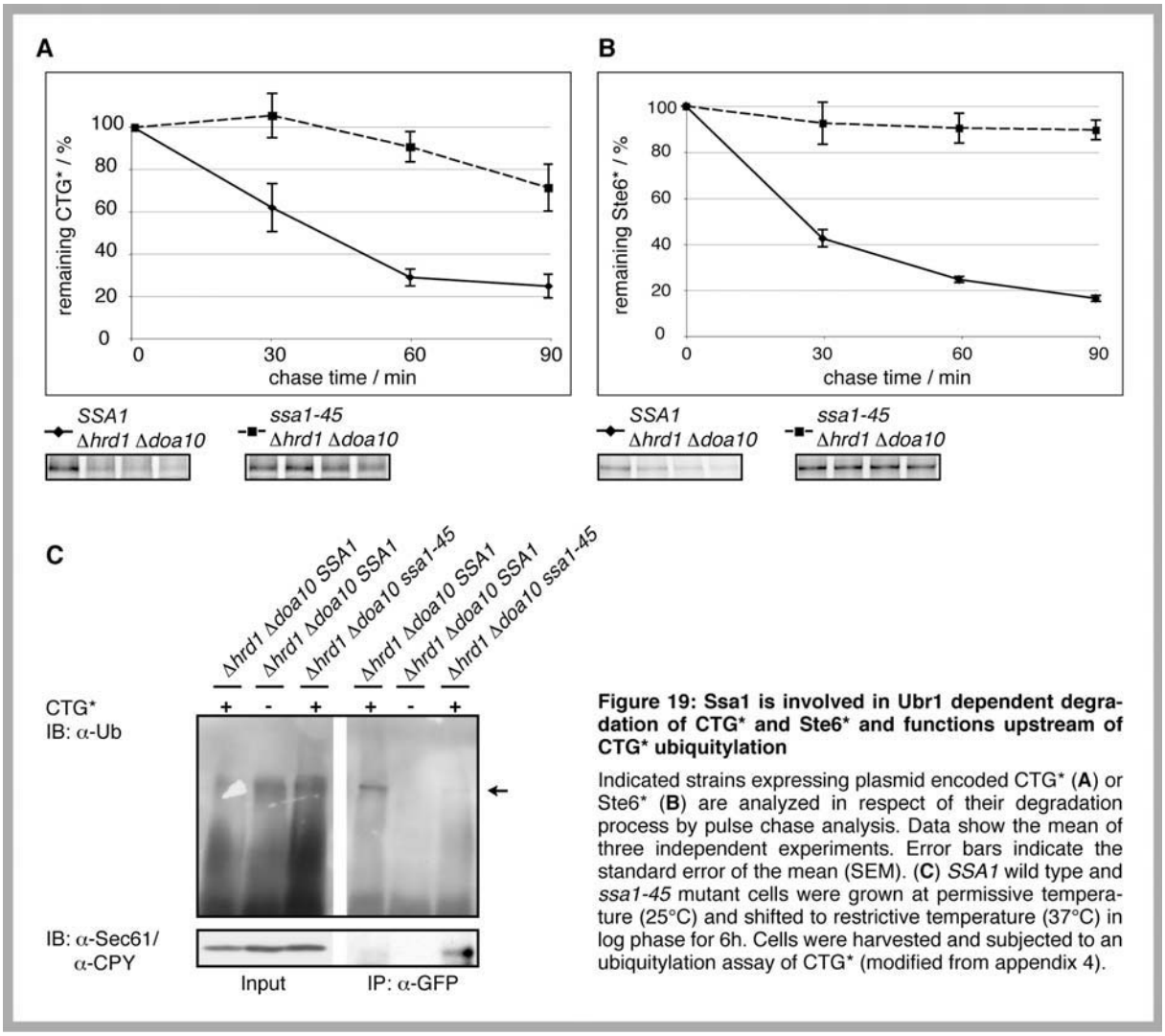


Figure 19: Ssa1 is involved in Ubr1 dependent degradation of CTG* and Ste6* and functions upstream of CTG* ubiquitylation

Indicated strains expressing plasmid encoded CTG* (**A**) or Ste6* (**B**) are analyzed in respect of their degradation process by pulse chase analysis. Data show the mean of three independent experiments. Error bars indicate the standard error of the mean (SEM). (**C**) *SSA1* wild type and *ssa1-45* mutant cells were grown at permissive temperature (25°C) and shifted to restrictive temperature (37°C) in log phase for 6h. Cells were harvested and subjected to an ubiquitylation assay of CTG* (modified from appendix 4).

3. The current view of ERAD

The findings summarized in this thesis enlarge our present picture of the ERAD system considerably (Fig. 20). Dfm1 and Mnl2 have to be included as new components of the ERAD machinery. Dfm1 forms distinct complexes with the ligases Hrd1/Der3 and Doa10 as well as with the AAA type ATPase Cdc48 [1]. In addition, Dfm1 is involved in the degradation and ubiquitylation of the ERAD-C substrate Ste6*, however, its precise mechanistic function remains presently still elusive.

Mnl2 is most probably an α-1,2-mannosidase involved in the trimming process of glycans exposed on soluble misfolded glycoproteins or domains of misfolded membrane proteins facing the ER lumen [2]. These trimmed glycan structures are subsequently recognized by Yos9. However, Yos9 does not seem to act only on glycosylated proteins but seems also to affect the degradation kinetics of unglycosylated ERAD substrates [3]. While degradation of glycosylated substrates is delayed in the absence of Yos9, degradation of unglycosylated CPY*0000 is enhanced in *YOS9* deleted cells. Most likely Yos9 prolongs the time for refolding of a misfolded unglycosylated protein.

In the absence of the canonical ER ligases Hrd1/Der3 and Doa10 the cytosolic ubiquitin ligase Ubr1 seems to be recruited to maintain degradation of at least some ERAD substrates by the proteasome as a backup system [4]. Extraction of the misfolded protein species no longer depends on Cdc48 in all cases, but the driving force of Hsp70 chaperones of the Ssa family seems to be sufficient to keep extraction and degradation of the ERAD substrates going.

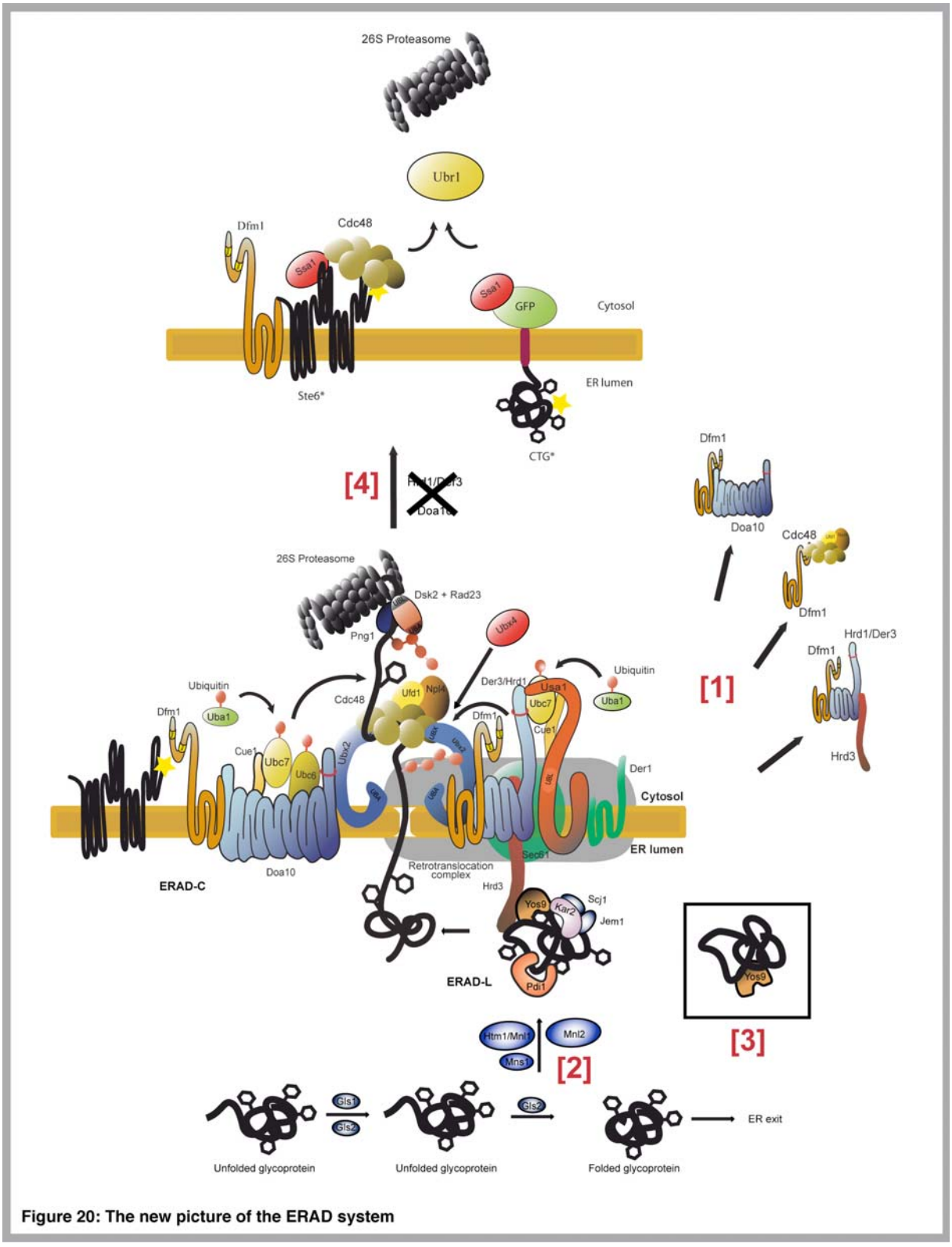


Figure 20: The new picture of the ERAD system

4. Bibliography

1. Goffeau, A. *et al.* Life with 6000 genes. *Science* **274**, 546, 563-547 (1996).
2. Hieter, P., Bassett, D.E., Jr. & Valle, D. The yeast genome--a common currency. *Nat Genet* **13**, 253-255 (1996).
3. Orr-Weaver, T.L., Szostak, J.W. & Rothstein, R.J. Yeast transformation: a model system for the study of recombination. *Proc Natl Acad Sci U S A* **78**, 6354-6358 (1981).
4. Sikorski, R.S. & Hieter, P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**, 19-27 (1989).
5. Schubert, U. *et al.* Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404**, 770-774 (2000).
6. Welihinda, A.A., Tirasophon, W. & Kaufman, R.J. The cellular response to protein misfolding in the endoplasmic reticulum. *Gene Expr* **7**, 293-300 (1999).
7. Stolz, A. & Wolf, D.H. Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell. *Biochim Biophys Acta* **1803**, 694-705 (2010).
8. Morimoto, R.I., Kline, M.P., Bimston, D.N. & Cotto, J.J. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Essays Biochem* **32**, 17-29 (1997).
9. Dobson, C.M. Protein folding and misfolding. *Nature* **426**, 884-890 (2003).
10. Goldberg, A.L. Protein degradation and protection against misfolded or damaged proteins. *Nature* **426**, 895-899 (2003).
11. Stolz, A., Hilt, W., Buchberger, A. & Wolf, D.H. Cdc48: a power machine in protein degradation. *Trends Biochem Sci* **36**, 515-523 (2011).
12. Koike, M. *et al.* Valosin-containing protein (VCP) in novel feedback machinery between abnormal protein accumulation and transcriptional suppression. *J Biol Chem* **285**, 21736-21749 (2010).
13. Watts, G.D. *et al.* Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nat Genet* **36**, 377-381 (2004).
14. Rutishauser, J. & Spiess, M. Endoplasmic reticulum storage diseases. *Swiss Med Wkly* **132**, 211-222 (2002).
15. Vazquez-Martinez, R. *et al.* Revisiting the regulated secretory pathway: From frogs to human. *Gen Comp Endocrinol* (2011).
16. Rapoport, T.A. Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. *Nature* **450**, 663-669 (2007).
17. Deshaies, R.J., Sanders, S.L., Feldheim, D.A. & Schekman, R. Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. *Nature* **349**, 806-808 (1991).
18. Johnson, A.E. & van Waes, M.A. The translocon: a dynamic gateway at the ER membrane. *Annu Rev Cell Dev Biol* **15**, 799-842 (1999).
19. Gething, M.J. Role and regulation of the ER chaperone BiP. *Semin Cell Dev Biol* **10**, 465-472 (1999).
20. Luirink, J. & Sinning, I. SRP-mediated protein targeting: structure and function revisited. *Biochim Biophys Acta* **1694**, 17-35 (2004).
21. Tyedmers, J., Mogk, A. & Bukau, B. Cellular strategies for controlling protein aggregation. *Nat Rev Mol Cell Biol* **11**, 777-788 (2010).

22. Park, S.H. *et al.* The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin-proteasome system. *Mol Biol Cell* **18**, 153-165 (2007).
23. Sun, Y. & MacRae, T.H. Small heat shock proteins: molecular structure and chaperone function. *Cell Mol Life Sci* **62**, 2460-2476 (2005).
24. Ellis, R.J. & Hemmingsen, S.M. Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. *Trends Biochem Sci* **14**, 339-342 (1989).
25. Hartl, F.U. & Hayer-Hartl, M. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**, 1852-1858 (2002).
26. Flaherty, K.M., DeLuca-Flaherty, C. & McKay, D.B. Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature* **346**, 623-628 (1990).
27. Bukau, B. & Horwich, A.L. The Hsp70 and Hsp60 chaperone machines. *Cell* **92**, 351-366 (1998).
28. Zhu, X. *et al.* Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**, 1606-1614 (1996).
29. Sousa, R. & Lafer, E.M. Keep the traffic moving: mechanism of the Hsp70 motor. *Traffic* **7**, 1596-1603 (2006).
30. Yan, Q. & Lennarz, W.J. Oligosaccharyltransferase: a complex multisubunit enzyme of the endoplasmic reticulum. *Biochem Biophys Res Commun* **266**, 684-689 (1999).
31. Parodi, A.J. Protein glucosylation and its role in protein folding. *Annu Rev Biochem* **69**, 69-93 (2000).
32. Lipari, F. & Herscovics, A. Calcium binding to the class I alpha-1,2-mannosidase from Saccharomyces cerevisiae occurs outside the EF hand motif. *Biochemistry* **38**, 1111-1118 (1999).
33. Vallee, F. *et al.* Crystal structure of a class I alpha1,2-mannosidase involved in N-glycan processing and endoplasmic reticulum quality control. *EMBO J* **19**, 581-588 (2000).
34. Munro, S. The MRH domain suggests a shared ancestry for the mannose 6-phosphate receptors and other N-glycan-recognising proteins. *Curr Biol* **11**, R499-501 (2001).
35. Bhamidipati, A., Denic, V., Quan, E.M. & Weissman, J.S. Exploration of the topological requirements of ERAD identifies Yos9p as a lectin sensor of misfolded glycoproteins in the ER lumen. *Mol Cell* **19**, 741-751 (2005).
36. Martinez Benitez, E., Stolz, A. & Wolf, D.H. Yos9, a control protein for misfolded glycosylated and non-glycosylated proteins in ERAD. *FEBS Lett* **585**, 3015-3019 (2011).
37. Tu, B.P. & Weissman, J.S. Oxidative protein folding in eukaryotes: mechanisms and consequences. *J Cell Biol* **164**, 341-346 (2004).
38. Appenzeller-Herzog, C. & Ellgaard, L. The human PDI family: versatility packed into a single fold. *Biochim Biophys Acta* **1783**, 535-548 (2008).
39. Freedman, R.B., Hirst, T.R. & Tuite, M.F. Protein disulphide isomerase: building bridges in protein folding. *Trends Biochem Sci* **19**, 331-336 (1994).
40. Primm, T.P., Walker, K.W. & Gilbert, H.F. Facilitated protein aggregation. Effects of calcium on the chaperone and anti-chaperone activity of protein disulfide-isomerase. *J Biol Chem* **271**, 33664-33669 (1996).
41. Kakizuka, A. Roles of VCP in human neurodegenerative disorders. *Biochem Soc Trans* **36**, 105-108 (2008).
42. Johnson, J.O. *et al.* Exome sequencing reveals VCP mutations as a cause of familial ALS. *Neuron* **68**, 857-864 (2010).

43. Riederer, B.M., Leuba, G., Vernay, A. & Riederer, I.M. The role of the ubiquitin proteasome system in Alzheimer's disease. *Exp Biol Med (Maywood)* **236**, 268-276 (2011).
44. Wolf, D.H. The ubiquitin clan: A protein family essential for life. *FEBS Lett* **585**, 2769-2771 (2011).
45. Pickart, C.M. Ubiquitin in chains. *Trends Biochem Sci* **25**, 544-548 (2000).
46. Ozkaynak, E., Finley, D., Solomon, M.J. & Varshavsky, A. The yeast ubiquitin genes: a family of natural gene fusions. *EMBO J* **6**, 1429-1439 (1987).
47. Hershko, A. Ubiquitin: roles in protein modification and breakdown. *Cell* **34**, 11-12 (1983).
48. Kornitzer, D. & Ciechanover, A. Modes of regulation of ubiquitin-mediated protein degradation. *J Cell Physiol* **182**, 1-11 (2000).
49. Finley, D. *et al.* Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol Cell Biol* **14**, 5501-5509 (1994).
50. Ikeda, F. & Dikic, I. Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO Rep* **9**, 536-542 (2008).
51. Kim, H.T. *et al.* Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. *J Biol Chem* **282**, 17375-17386 (2007).
52. Peng, J. *et al.* A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* **21**, 921-926 (2003).
53. Wang, X., Herr, R.A. & Hansen, T.H. Ubiquitination of Substrates by Esterification. *Traffic* (2011).
54. Chau, V. *et al.* A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243**, 1576-1583 (1989).
55. Wolf, D.H. & Hilt, W. The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. *Biochim Biophys Acta* **1695**, 19-31 (2004).
56. Tomko, R.J., Jr. & Hochstrasser, M. Order of the proteasomal ATPases and eukaryotic proteasome assembly. *Cell Biochem Biophys* **60**, 13-20 (2011).
57. Hanna, J. & Finley, D. A proteasome for all occasions. *FEBS Lett* **581**, 2854-2861 (2007).
58. Rock, K.L. *et al.* Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**, 761-771 (1994).
59. Jensen, T.J. *et al.* Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* **83**, 129-135 (1995).
60. Isasa, M. *et al.* Monoubiquitination of RPN10 regulates substrate recruitment to the proteasome. *Mol Cell* **38**, 733-745 (2010).
61. Rubin, D.M. *et al.* ATPase and ubiquitin-binding proteins of the yeast proteasome. *Mol Biol Rep* **24**, 17-26 (1997).
62. Husnjak, K. *et al.* Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature* **453**, 481-488 (2008).
63. Verma, R. *et al.* Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* **298**, 611-615 (2002).
64. Yao, T. & Cohen, R.E. A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* **419**, 403-407 (2002).
65. Hanna, J. *et al.* Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation. *Cell* **127**, 99-111 (2006).
66. Crosas, B. *et al.* Ubiquitin chains are remodeled at the proteasome by opposing ubiquitin ligase and deubiquitinating activities. *Cell* **127**, 1401-1413 (2006).

67. Verhoef, L.G. *et al.* Minimal length requirement for proteasomal degradation of ubiquitin-dependent substrates. *FASEB J* **23**, 123-133 (2009).
68. Beskow, A. *et al.* A conserved unfoldase activity for the p97 AAA-ATPase in proteasomal degradation. *J Mol Biol* **394**, 732-746 (2009).
69. Wolf, D.H. & Stolz, A. The Cdc48 machine in endoplasmic reticulum associated protein degradation. *Biochim Biophys Acta* (2011).
70. Moir, D., Stewart, S.E., Osmond, B.C. & Botstein, D. Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics* **100**, 547-563 (1982).
71. Frohlich, K.U. *et al.* Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. *J Cell Biol* **114**, 443-453 (1991).
72. Jentsch, S. & Rumpf, S. Cdc48 (p97): a "molecular gearbox" in the ubiquitin pathway? *Trends Biochem Sci* **32**, 6-11 (2007).
73. Braun, R.J. & Zischka, H. Mechanisms of Cdc48/VCP-mediated cell death: from yeast apoptosis to human disease. *Biochim Biophys Acta* **1783**, 1418-1435 (2008).
74. Raasi, S. & Wolf, D.H. Ubiquitin receptors and ERAD: a network of pathways to the proteasome. *Semin Cell Dev Biol* **18**, 780-791 (2007).
75. Barbin, L., Eisele, F., Santt, O. & Wolf, D.H. The Cdc48-Ufd1-Npl4 complex is central in ubiquitin-proteasome triggered catabolite degradation of fructose-1,6-bisphosphatase. *Biochem Biophys Res Commun* **394**, 335-341 (2010).
76. Meyer, H. & Popp, O. Role(s) of Cdc48/p97 in mitosis. *Biochem Soc Trans* **36**, 126-130 (2008).
77. Deichsel, A., Mouysset, J. & Hoppe, T. The ubiquitin-selective chaperone CDC-48/p97, a new player in DNA replication. *Cell Cycle* **8**, 185-190 (2009).
78. Wilcox, A.J. & Laney, J.D. A ubiquitin-selective AAA-ATPase mediates transcriptional switching by remodelling a repressor-promoter DNA complex. *Nat Cell Biol* **11**, 1481-1486 (2009).
79. Verma, R., Oania, R., Fang, R., Smith, G.T. & Deshaies, R.J. Cdc48/p97 mediates UV-dependent turnover of RNA Pol II. *Mol Cell* **41**, 82-92 (2011).
80. Pye, V.E. *et al.* Going through the motions: the ATPase cycle of p97. *J Struct Biol* **156**, 12-28 (2006).
81. Briggs, L.C. *et al.* Analysis of nucleotide binding to P97 reveals the properties of a tandem AAA hexameric ATPase. *J Biol Chem* **283**, 13745-13752 (2008).
82. Davies, J.M., Brunger, A.T. & Weis, W.I. Improved structures of full-length p97, an AAA ATPase: implications for mechanisms of nucleotide-dependent conformational change. *Structure* **16**, 715-726 (2008).
83. Buchberger, A. Control of ubiquitin conjugation by cdc48 and its cofactors. *Subcell Biochem* **54**, 17-30 (2010).
84. Madsen, L., Seeger, M., Semple, C.A. & Hartmann-Petersen, R. New ATPase regulators--p97 goes to the PUB. *Int J Biochem Cell Biol* **41**, 2380-2388 (2009).
85. Krick, R. *et al.* Cdc48/p97 and Shp1/p47 regulate autophagosome biogenesis in concert with ubiquitin-like Atg8. *J Cell Biol* **190**, 965-973 (2010).
86. Pearse, B.R. & Hebert, D.N. Lectin chaperones help direct the maturation of glycoproteins in the endoplasmic reticulum. *Biochim Biophys Acta* **1803**, 684-693 (2010).
87. Aebi, M., Bernasconi, R., Clerc, S. & Molinari, M. N-glycan structures: recognition and processing in the ER. *Trends Biochem Sci* **35**, 74-82 (2010).

88. Kostova, Z. & Wolf, D.H. For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. *EMBO J* **22**, 2309-2317 (2003).
89. Sommer, T. & Wolf, D.H. Endoplasmic reticulum degradation: reverse protein flow of no return. *FASEB J* **11**, 1227-1233 (1997).
90. Brodsky, J.L. & McCracken, A.A. ER protein quality control and proteasome-mediated protein degradation. *Semin Cell Dev Biol* **10**, 507-513 (1999).
91. Hiller, M.M., Finger, A., Schweiger, M. & Wolf, D.H. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* **273**, 1725-1728 (1996).
92. Helenius, A. & Aebi, M. Roles of N-linked glycans in the endoplasmic reticulum. *Annu Rev Biochem* **73**, 1019-1049 (2004).
93. Vashist, S. & Ng, D.T. Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *J Cell Biol* **165**, 41-52 (2004).
94. Carvalho, P., Goder, V. & Rapoport, T.A. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* **126**, 361-373 (2006).
95. Kreft, S.G., Wang, L. & Hochstrasser, M. Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). *J Biol Chem* **281**, 4646-4653 (2006).
96. Swanson, R., Locher, M. & Hochstrasser, M. A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. *Genes Dev* **15**, 2660-2674 (2001).
97. Bordallo, J., Plemper, R.K., Finger, A. & Wolf, D.H. Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* **9**, 209-222 (1998).
98. Hampton, R.Y., Gardner, R.G. & Rine, J. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* **7**, 2029-2044 (1996).
99. Bays, N.W., Gardner, R.G., Seelig, L.P., Joazeiro, C.A. & Hampton, R.Y. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nat Cell Biol* **3**, 24-29 (2001).
100. Deak, P.M. & Wolf, D.H. Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. *J Biol Chem* **276**, 10663-10669 (2001).
101. Lenk, U. *et al.* A role for mammalian Ubc6 homologues in ER-associated protein degradation. *J Cell Sci* **115**, 3007-3014 (2002).
102. Sommer, T. & Jentsch, S. A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. *Nature* **365**, 176-179 (1993).
103. Vassal, A., Boulet, A., Decoster, E. & Faye, G. QRI8, a novel ubiquitin-conjugating enzyme in Saccharomyces cerevisiae. *Biochim Biophys Acta* **1132**, 211-213 (1992).
104. Biederer, T., Volkwein, C. & Sommer, T. Role of Cue1p in ubiquitination and degradation at the ER surface. *Science* **278**, 1806-1809 (1997).
105. Eisele, F., Schafer, A. & Wolf, D.H. Ubiquitylation in the ERAD Pathway. *Subcell Biochem* **54**, 136-148 (2010).
106. Gardner, R.G. *et al.* Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. *J Cell Biol* **151**, 69-82 (2000).
107. Horn, S.C. *et al.* Usa1 functions as a scaffold of the HRD-ubiquitin ligase. *Mol Cell* **36**, 782-793 (2009).
108. Hitt, R. & Wolf, D.H. Der1p, a protein required for degradation of malfolded soluble proteins of the endoplasmic reticulum: topology and Der1-like proteins. *FEMS Yeast Res* **4**, 721-729 (2004).

109. Knop, M., Finger, A., Braun, T., Hellmuth, K. & Wolf, D.H. Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *EMBO J* **15**, 753-763 (1996).
110. Plemper, R.K., Bohmler, S., Bordallo, J., Sommer, T. & Wolf, D.H. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature* **388**, 891-895 (1997).
111. Plemper, R.K. *et al.* Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. *J Cell Sci* **112 (Pt 22)**, 4123-4134 (1999).
112. Schafer, A. & Wolf, D.H. Sec61p is part of the endoplasmic reticulum-associated degradation machinery. *EMBO J* **28**, 2874-2884 (2009).
113. Medicherla, B., Kostova, Z., Schaefer, A. & Wolf, D.H. A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep* **5**, 692-697 (2004).
114. Funakoshi, M., Sasaki, T., Nishimoto, T. & Kobayashi, H. Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome. *Proc Natl Acad Sci U S A* **99**, 745-750 (2002).
115. Rao, H. & Sastry, A. Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. *J Biol Chem* **277**, 11691-11695 (2002).
116. Loayza, D., Tam, A., Schmidt, W.K. & Michaelis, S. Ste6p mutants defective in exit from the endoplasmic reticulum (ER) reveal aspects of an ER quality control pathway in Saccharomyces cerevisiae. *Mol Biol Cell* **9**, 2767-2784 (1998).
117. Huyer, G. *et al.* Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. *J Biol Chem* **279**, 38369-38378 (2004).
118. Finger, A., Knop, M. & Wolf, D.H. Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast. *Eur J Biochem* **218**, 565-574 (1993).
119. Taxis, C. *et al.* Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. *J Biol Chem* **278**, 35903-35913 (2003).
120. Kostova, Z. & Wolf, D.H. Importance of carbohydrate positioning in the recognition of mutated CPY for ER-associated degradation. *J Cell Sci* **118**, 1485-1492 (2005).
121. Eisele, F. & Wolf, D.H. Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1. *FEBS Lett* **582**, 4143-4146 (2008).
122. Varshavsky, A. Recent studies of the ubiquitin system and the N-end rule pathway. *Harvey Lect* **96**, 93-116 (2000).
123. Varshavsky, A. The N-end rule pathway of protein degradation. *Genes Cells* **2**, 13-28 (1997).
124. Bartel, B., Wunning, I. & Varshavsky, A. The recognition component of the N-end rule pathway. *EMBO J* **9**, 3179-3189 (1990).
125. Heck, J.W., Cheung, S.K. & Hampton, R.Y. Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1. *Proc Natl Acad Sci U S A* **107**, 1106-1111 (2010).
126. Helenius, A., Trombetta, E.S. & Hebert, D.N. Calnexin, calreticulin and the folding of glycoproteins. *Trends Cell Biol* **7**, 193-200 (1997).
127. Zapun, A., Jakob, C.A., Thomas, D.Y. & Bergeron, J.J. Protein folding in a specialized compartment: the endoplasmic reticulum. *Structure* **7**, R173-182 (1999).

128. Jakob, C.A., Burda, P., Roth, J. & Aebi, M. Degradation of misfolded endoplasmic reticulum glycoproteins in Saccharomyces cerevisiae is determined by a specific oligosaccharide structure. *J Cell Biol* **142**, 1223-1233 (1998).
129. Hitt, R. & Wolf, D.H. DER7, encoding alpha-glucosidase I is essential for degradation of malfolded glycoproteins of the endoplasmic reticulum. *FEMS Yeast Res* **4**, 815-820 (2004).
130. Knop, M., Hauser, N. & Wolf, D.H. N-Glycosylation affects endoplasmic reticulum degradation of a mutated derivative of carboxypeptidase yscY in yeast. *Yeast* **12**, 1229-1238 (1996).
131. Clerc, S. *et al.* Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum. *J Cell Biol* **184**, 159-172 (2009).
132. Quan, E.M. *et al.* Defining the glycan destruction signal for endoplasmic reticulum-associated degradation. *Mol Cell* **32**, 870-877 (2008).
133. Martinez Benitez, E., Stolz, A., Becher, A. & Wolf, D.H. Mnl2, a novel component of the ER associated protein degradation pathway. *Biochem Biophys Res Commun* (2011).
134. Buschhorn, B.A., Kostova, Z., Medicherla, B. & Wolf, D.H. A genome-wide screen identifies Yos9p as essential for ER-associated degradation of glycoproteins. *FEBS Lett* **577**, 422-426 (2004).
135. Xie, W., Kanehara, K., Sayeed, A. & Ng, D.T. Intrinsic conformational determinants signal protein misfolding to the Hrd1/Htm1 endoplasmic reticulum-associated degradation system. *Mol Biol Cell* **20**, 3317-3329 (2009).
136. Gauss, R., Jarosch, E., Sommer, T. & Hirsch, C. A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery. *Nat Cell Biol* **8**, 849-854 (2006).
137. Mikami, K. *et al.* The sugar-binding ability of human OS-9 and its involvement in ER-associated degradation. *Glycobiology* **20**, 310-321 (2010).
138. Hosokawa, N., Kamiya, Y. & Kato, K. The role of MRH domain-containing lectins in ERAD. *Glycobiology* **20**, 651-660 (2010).
139. Sato, B.K. & Hampton, R.Y. Yeast Derlin Dfm1 interacts with Cdc48 and functions in ER homeostasis. *Yeast* **23**, 1053-1064 (2006).
140. Otte, K.B. Das Fehlen des ER-Membranproteins Dfm1 führt zu Kex1 Protease vermittelter Apoptose. (2010).
141. Travers, K.J. *et al.* Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101**, 249-258 (2000).
142. Friedlander, R., Jarosch, E., Urban, J., Volkwein, C. & Sommer, T. A regulatory link between ER-associated protein degradation and the unfolded-protein response. *Nat Cell Biol* **2**, 379-384 (2000).
143. Abdelwahid, E. *et al.* Mitochondrial involvement in cell death of non-mammalian eukaryotes. *Biochim Biophys Acta* **1813**, 597-607 (2011).
144. Lushchak, V.I. Oxidative stress in yeast. *Biochemistry (Mosc)* **75**, 281-296 (2010).
145. Carmona-Gutierrez, D. *et al.* Apoptosis in yeast: triggers, pathways, subroutines. *Cell Death Differ* **17**, 763-773 (2010).
146. Madeo, F. *et al.* Caspase-dependent and caspase-independent cell death pathways in yeast. *Biochem Biophys Res Commun* **382**, 227-231 (2009).
147. Mazzoni, C. & Falcone, C. Caspase-dependent apoptosis in yeast. *Biochim Biophys Acta* **1783**, 1320-1327 (2008).
148. Madeo, F. *et al.* A caspase-related protease regulates apoptosis in yeast. *Mol Cell* **9**, 911-917 (2002).

149. Hauptmann, P. & Lehle, L. Kex1 protease is involved in yeast cell death induced by defective N-glycosylation, acetic acid, and chronological aging. *J Biol Chem* **283**, 19151-19163 (2008).
150. Stolz, A., Schweizer, R.S., Schafer, A. & Wolf, D.H. Dfm1 forms distinct complexes with Cdc48 and the ER ubiquitin ligases and is required for ERAD. *Traffic* **11**, 1363-1369 (2010).
151. Neuber, O., Jarosch, E., Volkwein, C., Walter, J. & Sommer, T. Ubx2 links the Cdc48 complex to ER-associated protein degradation. *Nat Cell Biol* **7**, 993-998 (2005).
152. Schuberth, C. & Buchberger, A. Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. *Nat Cell Biol* **7**, 999-1006 (2005).
153. Stolz, A., Besser, S., Hottmann, H. & Wolf, D.H. ERAD without canonical ER ubiquitin ligases: A novel role for Ubr1. *submitted* (2011).
154. Collins, G.A., Gomez, T.A., Deshaies, R.J. & Tansey, W.P. Combined chemical and genetic approach to inhibit proteolysis by the proteasome. *Yeast* **27**, 965-974 (2010).
155. Reggiori, F. & Pelham, H.R. A transmembrane ubiquitin ligase required to sort membrane proteins into multivesicular bodies. *Nat Cell Biol* **4**, 117-123 (2002).
156. Belgareh-Touze, N. *et al.* Versatile role of the yeast ubiquitin ligase Rsp5p in intracellular trafficking. *Biochem Soc Trans* **36**, 791-796 (2008).
157. Matlack, K.E., Misselwitz, B., Plath, K. & Rapoport, T.A. BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane. *Cell* **97**, 553-564 (1999).

5. Danksagung

Ich möchte mich ganz herzlich bei meinem Betreuer Herrn Professor Dieter H. Wolf für die Möglichkeit bedanken, dieses interessante Themengebiet für meine Promotion nutzen zu können. Neben den vielen wissenschaftlichen Diskussionen hat er mir stets die Möglichkeit und die Freiheit gegeben, eigene Gedanken und Ziele in meiner Forschung zu verfolgen. Ebenfalls danken möchte ich Frau Dr. Antje Schäfer, die mich in mein Themengebiet eingeführt und mit mir viele Ideen für den Start meiner Arbeit entwickelt hat.

Dr. Wolfgang Hilt, Dr. Hans Rudolph, Bernhard Braun, Olivier Santt und Elena Martinez Benitez haben mit mir in kreativen Kaffeepausen interessante Aspekte meiner wissenschaftlichen Ergebnisse diskutiert. Die Versorgung mit Tee, Karotten und aufmunternden Zusprüchen von Elisabeth Tosta haben oft hochwillkommene Stärkung gebracht.

Elena Martinez Benitez, Stefanie Besser und Konrad Otte möchte ich für die fruchtbare Zusammenarbeit danken. Ohne sie wäre es mir nicht möglich gewesen an so vielen verschiedenen Bereichen des ERAD zu forschen.

Für die angenehme Arbeitsatmosphäre möchte ich allen aktuellen und ehemaligen Labormitgliedern danken! Hierzu gehören unter anderem auch Sven Alberts, Ingo Amm, Lise Barbin, Sonia Barbossa, Alexander Becher, Stefanie Besser, Frederik Eisele, Anja Eisele, Heike Hottmann, Dragica Kapucija, Ruth Menßen-Franz, Derrik Norell, Sae-Hun Park, Regina Philipp, Olivier Santt, Mario Scazzari, Andrea Spitzner und Marion Weberruss.

Appendix 1

Mnl2, a novel component of the ER associated protein degradation pathway

Elena Martinez Benitez, Alexandra Stolz, Alexander Becher and Dieter H. Wolf
BBRC 2011 Oct 28;414(3):528-32

Biochemical and Biophysical Research Communications 414 (2011) 528–532

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mnl2, a novel component of the ER associated protein degradation pathway

Elena Martinez Benitez, Alexandra Stolz, Alexander Becher, Dieter H. Wolf *

Institut für Biochemie der Universität Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany

ARTICLE INFO

Article history:
Received 12 September 2011
Available online 24 September 2011

Keywords:
Protein quality control
CPY*
ERAD
Mannosidase
N-glycosylation

ABSTRACT

In eukaryotes, membrane and soluble proteins of the secretory pathway enter the endoplasmic reticulum (ER) after synthesis in an unfolded state. Directly after entry, most proteins are modified with glycans at suitable glycosylation sites and start to fold. A protein that cannot fold properly will be degraded in a process called ER associated degradation (ERAD). Failures in ERAD, either by loss of function or by premature degradation of proteins, are a cause of severe diseases. Therefore, the search for novel ERAD components to gain better insight in this process is of high importance. Carbohydrate trimming is a relevant process in ER quality control. In this work a novel putative yeast mannosidase encoded by the open reading frame YLR057W was identified and named Mnl2. Deletion of *MNL2* diminished the degradation efficiency of misfolded CPY* in the absence of the cognate mannosidase Mnl1, indicating a specific role in ERAD.

1. Introduction

The proteins of a cell are constantly checked for their folding status. Inadvertent folding mistakes during synthesis or misfolding of proteins because of mutations or stresses as heat, heavy metal ions and oxidation have to be discovered and the terminally misfolded proteins have to be eliminated [1,2]. Failure to do so leads to severe folding diseases, including Alzheimer's disease, Parkinson's disease or Creutzfeldt–Jakob disease [3].

About one third of the proteome follows the secretory pathway, which covers the endomembrane compartments of the endoplasmic reticulum (ER), the Golgi apparatus, secretory vesicles and the plasma membrane. Proteins of the secretory pathway are co- or posttranslationally transferred into the lumen of the ER where they are folded and modified [4,5]. Modifications of the imported proteins in the ER comprise glycosylation, disulfide bond formation, multisubunit assembly and specific proteolytic cleavages [6]. During entry into the ER, a core glycan tree containing three glucose residues (Glc), nine mannose residues (Man) and two N-acetylglucosamine residues (GlcNAc), $Glc_3Man_9(GlcNAc)_2$, is transferred onto proteins designed for N-glycosylation [7–9]. Transfer of the glycan occurs via the oligosaccharyl transferase (OST) complex on an asparagine residue of the protein within the consensus sequence Asn-X-Ser/Thr (where X is any amino acid except proline) [10]. The structure of glycan trees in the ER has been shown to reveal the folding status of the protein [8,11].

The major mechanism responsible for eliminating misfolded secretory proteins is the ubiquitin proteasome system. For this purpose misfolded proteins have to be retrotranslocated out of the ER, polyubiquitylated and delivered to the proteasome. This process is called ER-associated degradation (ERAD) [12–17]. Prior to retrotranslocation a quality control (QC) system of the ER has to differentiate between terminally misfolded proteins and folding intermediates. In the case of glycoproteins the QC process, additional to chaperones, involves the action of glucosidases, mannosidases, lectins and lectin like proteins [11,18–20]. Lectin-like chaperones are responsible for controlling folding intermediates, folded proteins and terminally misfolded proteins [11,21–23]. Their decision will finally allow the glycosylated proteins to be either delivered via the secretory pathway to their site of action or to be eliminated. In yeast, N-linked glycan trimming starts right after glycosylation of the imported protein, a process named the "timer model" [8,11,24]. As a first step, glucosidase 1 (Gls1) removes the terminal α1,2 linked glucose on the A branch of the $Glc_3Man_9(GlcNAc)_2$ structure. Glucosidase 2 (Gls2) then cleaves off the additional two glucose residues [25]. In higher eukaryotes, but not in the yeast *Saccharomyces cerevisiae*, reglucosylation by one glucose residue can occur by the enzyme UDP glucose: glycoprotein glucosyltransferase (UGGT), allowing the folding intermediate to reassociate with calnexin/calreticulin for additional folding time. In general, thereafter mannosidase 1 (Mns1) specifically removes one mannose residue from the B branch of the glycan, generating the Man8 structure. Mns1 is a slow acting enzyme; it gives the protein time to fold [26]. If the folding is

Abbreviations: AAA, ATPase associated with a variety of cellular activities; CM, complete minimal medium; CPY, carpoxipeptidase Y; EDEM, ER degradation enhancing α-mannosidase-like protein; ER, endoplasmic reticulum; ERAD, ER associated degradation; PCR, polymerase chain reaction; PGK, 3-phospholglcerate kinase; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

* Corresponding author. Fax: +49 711 68564392.
E-mail address: dieter.wolf@ibc.uni-stuttgart.de (D.H. Wolf).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.09.100

not completed in the time given or the protein is not capable to achieve its native structure, a mannose residue on the C branch will be cleaved off by the mannosidase like protein 1 (Mnl1), generating a Man7 structure. Both proteins are glycoside hydrolase family 47 (GH47) members with an α1,2 mannosidase activity. The trimming event of the outermost α1,2-linked mannose of the C branch of the glycan generates an α1,6 mannose linkage which is recognized by the lectin Yos9 and thus marks the protein for degradation [21,22,27–29]. The absence of Yos9 leads to a partial block of the degradation of glycosylated ER proteins [30]. This recognition of an α1,6 linked mannose residue in the Man7 structure occurs via the mannose 6-phosphate receptor homology (MRH) domain of Yos9, disruption of which impairs degradation of the glycosylated misfolded substrate CPY* of the ER [29,31–33]. Yos9 has also been shown to recognize Man6 and Man5 structures carrying the α1,6 mannose linkage whereby the Man5 structure exhibited the highest affinity towards Yos9 *in vitro* [21]. The occurrence of Man5 structures in ERAD proteins of mammalian cells [34] and the fact that Yos9 actually has highest affinity for Man5 structures containing an α1,6 linked mannose linkage in the C branch led us to search for additional GH47 members able to interfere with ERAD of N-glycosylated proteins.

2. Materials and methods

2.1. Yeast strains and plasmids

Molecular biological and genetic techniques were carried out using standard methods [35]. *S. cerevisiae* strains used in this study are based on YWO0343 strain (MATa, *ade2-1*, *can1-100*, *his3-11*, *leu2-3*, *trp1-1*, *ura3-1*, *prc1-1*) and the gene deletion method according to Guldener [36,37] (Supp. table 1). The yeast strains YWO1779, YWO1780, YWO1781 and YWO1782, where the gene *MNL2* was deleted, were obtained from the yeast strains YWO0343, YWO0566, YWO0823 and YWO1477 respectively. They were generated by homologous recombination of these strains and the PCR amplified fragment from the pUG27 plasmid and the primer set GTGTTAACTAGAAAAAGGCCGCCACTACTCTATAAGCAAAACCTTCAGCTGAAGCTTCGTACGC and CTATATGTATGTATGTATGTGCGTACGATTTTTCTAACGTTAACTGCATAGGCCACTAGTGGATCTG. Cells were plated on CM-His solid media for selection of clones. The correct integration of the disruption cassette was confirmed by PCR and Southern blotting. The strain YWO1786, where the gene *MNL2* was deleted and all marker genes from previous gene disruptions were popped out, was obtained from the yeast strain YWO1782 expressing the Cre recombinase from the plasmid pSH63. The strain YWO1790 were the gene *YOS9* was deleted, was obtained from the yeast strain YWO1786 by homologous recombination with the PCR amplified fragment from the pUG27 plasmid and the primer set agatcttcacatatatcgttatcatccccttttcttccctgtttcacagctgaagcttcgtacgc and gcaaaacgtgaaaaaaaaaaattaaaagtttatactcctccttgtgcataggccactagtggatctg. Cells were plated on CM-His solid media for selection of clones. The correct integration of the disruption cassette was confirmed by PCR and Souther blotting. Table S1 list the yeast strains with the corresponding genotype and source.

The plasmid pMnl2-13myc was constructed by PCR amplification of the 4.8 kb *MNL2-13myc* fragment from the strain YWO1946 with the primer set AGATTTCGGTACCGCGCAAGTGTTAAC and GACTGGGCGGCCGCATACCCATTGGCTGTG and ligation with a T4 DNA ligase (fermentas) into the Kpn1-Not1 sites of the pYes2 vector (invitrogen).

2.2. Cell fractionation

Wild type cells transformed with pMnl2-13myc or the empty vector were grown at 30 °C in selective media containing 2% galactose to logarithmic phase. Two hundred ODs of cells were harvested, washed with NaN_3, resuspended in sorbitol buffer (0.7 M sorbitol, 50 mM Tris–HCl, protease inhibitors (Fermentas)) and lysed by adding glass beads and vortexing 10 min at 4 °C. After a preclearing step (500 g, 5 min), 1 ml of the lysate was centrifuged at 13,000*g* for 10 min. Eight hundred μl of the supernatant were then subjected to ultracentrifugation at 100,000*g* for 1 h. After addition of SDS loading buffer samples were subjected to

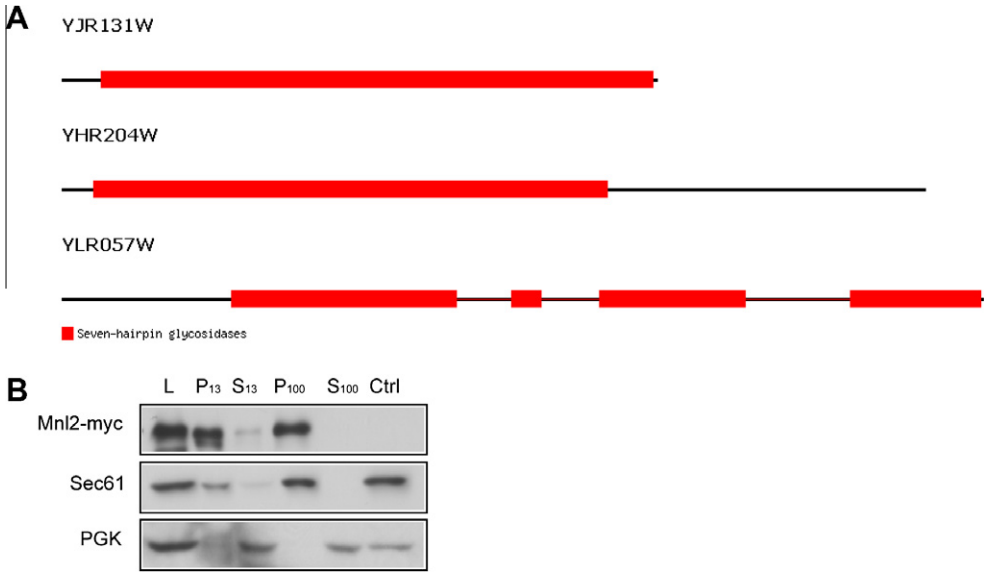


Fig. 1. (A) Proposed seven-hairpin glycosidases superfamily assignments to *Saccharomyces cerevisiae* SGD. The glycoside hydrolase family 47 comprises enzymes with only one known activity; α1-2 mannosidase. The three proteins with similar domain architecture (highlighted with red bars) were the result of the search applied to *S. cerevisiae* in the SUPERFAMILY database, a database of structural and functional annotation for all proteins and genomes. The ORFs YJR131W, YHR204W and YLR057W encode the proteins Mns1, Mnl1 and Mnl2 respectively. (B) ER localization of the putative mannosidase Mnl2: whole cell lysate expressing Mnl2-13myc (L), ER pellet fraction (P13), transport vesicles/Golgi complex/endosomal membranes (P100), cytoplasmic soluble proteins (S100) and wild type whole cell lysate transformed with an emply vector (Ctrl). The Sec61 ER membrane protein and the cytosolic PGK protein were used as localization controls.

SDS–PAGE and Western blotting. Immunodetection of the proteins was done with monoclonal α-myc (Sigma), monoclonal α-PGK (Molecular Probes), polyclonal α-Sec61 (T. Sommer) and ECL Western blotting substrate (Pierce).

2.3. Pulse-chase analysis and immunoprecipitation

Cells were grown at 30 °C to logarithmic phase in 20 ml of YPD. For each strain 40 ODs of cells were taken. They were washed five times with 1 ml starvation media (0,67% (w/v) yeast nitrogen base w/o amino acids, 2% glucose, 0.012% (w/v) L-alanine, L-Isoleucine, L-Leucine, L-arginine, L-Lysine, L-Aspartate, L-Cysteine, L-Phenylalanine, L-Glutamate, L-Threonine, L-Glutamine, L-Tryptophan, L-Glycine, L-Valine, L-Proline, L-Histidine, L-Serine, L-Tyrosine, L-Asparagine, myo-inositol, p-aminobenzoic acid, pH5,6) and resuspended in 1 ml starvation media in 50 ml tubes. Cells underwent starvation for 50 min at 30 °C. Then 20 μl of methionine labeled with the radioactive isotope ^{35}S (10 μCi/μl) was added and cultures were incubated 20 min under the same conditions. During this time all newly synthesized proteins integrate radioactive methionine into their sequence. After the 20 min, 1 ml of chase media (Starvation plus 0.2% BSA, 0.6% L-Methionine was added and samples (450 μl) were collected at different time points in microtubes containing 500 μl of 200 mM NaN_3. Samples were kept on ice or stored at −20 °C. The tubes with thawed cells were centrifuged for 15 min at 13,000 rpm and the supernatant was discarded. The pellet was resuspended in 100 μl of BB1 buffer (50 mM Tris/HCl pH 7.5, 6 M urea, 1 mM EDTA, 1% (w/v) SDS) and 67 μl of glass beads added. Samples were vortexed five times for 1 min with a minute interruption on ice. Then, cell extracts were resuspended in 1 ml IP buffer (50 mM Tris/HCl pH 7.5, 190 mM NaCl 1.25% TritonX-100(v/v), 6 mM EDTA) and spun down for 15 min at 14,000 rpm. Next, 900 μl of the supernatant was transferred to a microtube containing 3 μl of polyclonal α-CPY antibody (Rockland) for immunoprecipitation. Immunoprecipitation took place during 90 min at room temperature, followed by the addition of 80 μl of 7% Protein A Sepharose suspension (GE healthcare). During 90 min incubation the antibodies bind to the Sepharose. After five washings with IP buffer (2000 rpm) all non bound proteins to the antibody and Sepharose are eliminated. In the final centrifugation step the IP buffer is removed completely and 60 μl SDS loading buffer are added. Samples are subjected to SDS–PAGE and the gel is dried at 60 °C on a whatman paper with the aid of a vacuum. Whatman papers with the samples are fixed in a cassette and exposed to a phosphor screen. The screen is analyzed using a PhosphoImager scanner and ImageQuaNT™ software. In each time point of experiments the mean and interval of confidence with an $\alpha = 0.05$ for the error bars is calculated.

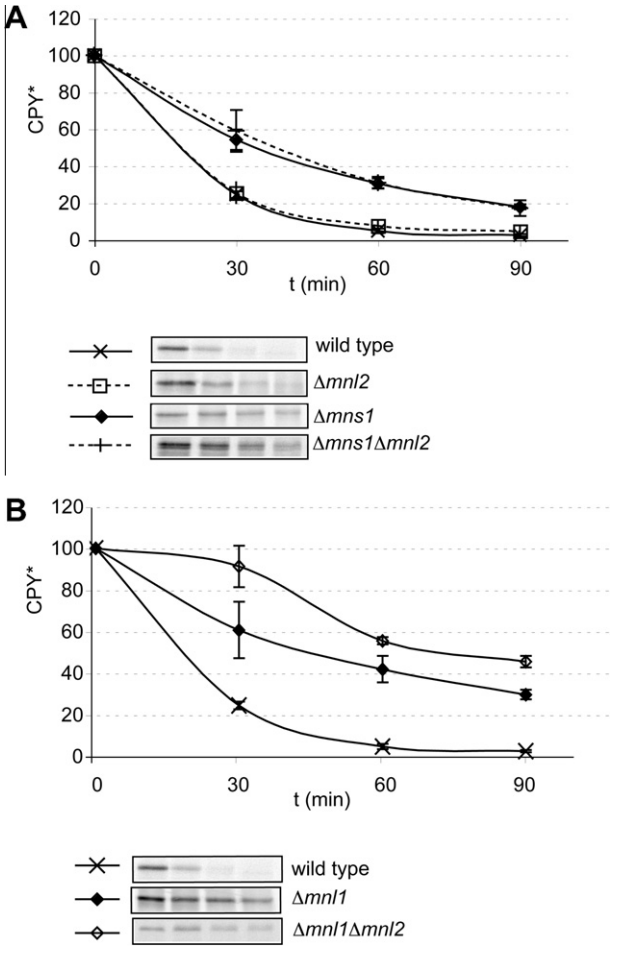


Fig. 2. (A): Mnl2 does not show an effect on its own or in combination with Mns1, in the degradation of the glycosylated ERAD substrate CPY*. (B) Mnl2 plays a role in glycoprotein ERAD in combination with Mnl1 since their absence decreases the speed of CPY* turnover. Endogenously expressed CPY* turnover was followed by pulse chase analysis of the different strains The pulse-chase analyses data represent the means of 3 (*Δmns1, Δmnl1, Δmnl2, Δmns1 Δmnl2* and *Δmnl1 Δmnl2*) to 4 (wild type and *Δmns1*) independent experiments ± the interval of confidence calculated with an $\alpha = 0.05$.

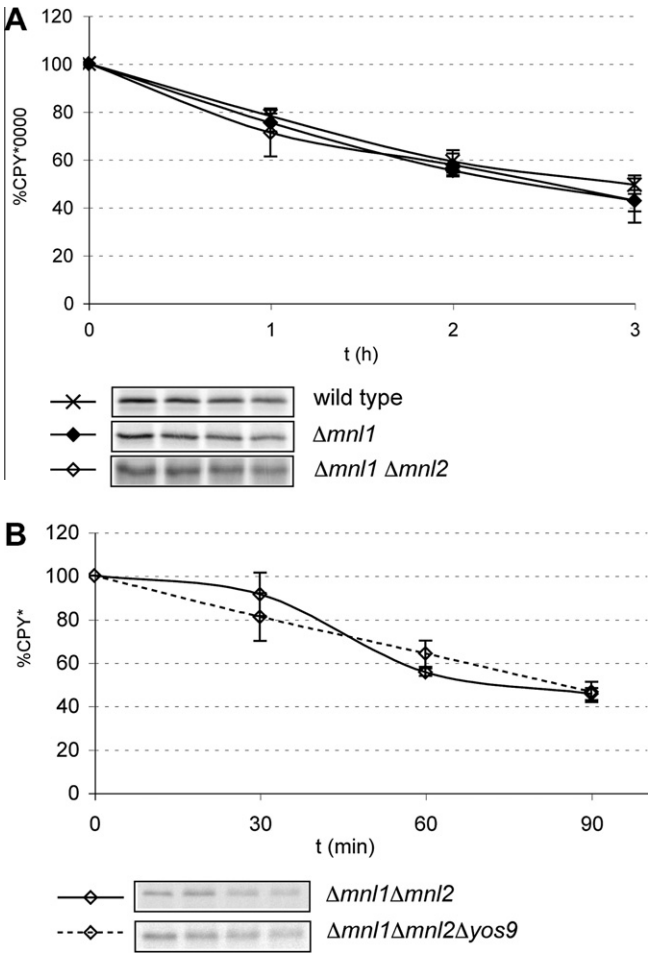


Fig. 3. (A) Mnl1 and Mnl2 do not influence the turnover of the unglycosylated ERAD substrate CPY*0000. (B) Yos9 cannot recognize the glycan structure of the substrate as degradation signal if mannosidases are absent. Endogenously expressed CPY* turnover was followed by pulse chase analysis of the different strains The pulse-chase analyses data represent the means of 3 (*Δmnl1 Δmnl2 (B)* and *Δmnl1 Δmnl2 Δyos9*) to 4 (wild type, *Δmnl1,* and *Δmnl1 Δmnl2 (A)*) independent experiments ± the interval of confidence calculated with an $\alpha = 0.05$.

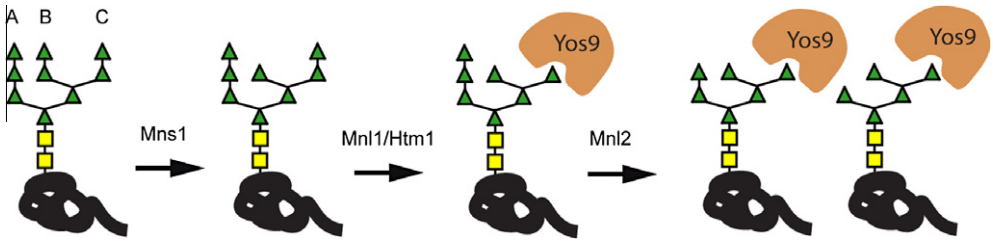


Fig. 4. Proposed model for Mnl2 activity.

3. Results and discussion

3.1. Involvement of Mnl2 in ERAD

Searching the *S. cerevisiae* Genome Database (http://www.yeastgenome.org/) and SUPERFAMILY Database (http://www.supfam.cs.bris.ac.uk/SUPERFAMILY/index.html) for proteins that contained domains or motifs in common with the GH47 family members Mns1 and Mnl1 [38], led to the discovery of a putative mannosidase protein encoded by the open reading frame YLR057 W (Fig. 1A). We renamed the open reading frame YLR057 W as Mnl2 for mannosidase like protein 2. Glycan trees linked to proteins become a degradation signal when the time that was given to fold is insufficient or if the protein is terminally misfolded, resulting in the recognition by the lectin Yos9 and subsequent delivery to the degradation machinery [11,29,39]. As Mns1 and Mnl1 play a crucial role in the quality control of misfolded glycosylated proteins of the ER, we hypothesized that Mnl2, due to its predicted similarities to Mns1 and Mnl1 as a GH47 family member would be part of the glycan trimming process and by this be involved in ERAD. Such an enzyme would be expected to localize to the ER. Indeed a localization experiment locates Mnl2 in the pellet fractions which also contain the ER import channel Sec61 (Fig. 1B).

For analysis of the impact of Mnl2 on ERAD we used the standard ERAD substrate CPY* [12,40]. We first checked whether a *MNL2* deletion had an effect on the degradation profile of endogenous CPY*. As can be seen in Fig. 2A this was not the case: CPY* was degraded as efficiently in the *Δmnl2* mutant as in wild type cells. To exclude redundancy of Mnl2 with the established mannosidases Mns1 and Mnl1, all combinations of deletions of genes encoding these mannosidases were generated and analyzed in pulse-chase experiments. The deletion of *MNS1* in a *Δmnl2* background was dominant: double deleted cells showed the same stabilization of CPY* as cells solely deleted in *MNS1*, showing an approximately 20% stabilization after 90 min (Fig. 2A). This result can be interpreted as Mnl2 either being without function in ERAD or exert its activity in the same pathway as Mns1.

Since Mns1 and Mnl1 act sequentially in the same pathway, trimming a mannose residue in the B branch (Mns1) and a mannose residue in the C branch (Mnl1) of the glycan, the deletion of *MNL2* was analyzed in the background of a *MNL1* deletion. Interestingly, a stronger stabilization of CPY* was detected in this double mutant as compared to the single *Δmnl1* (Fig. 2B) deletion strain or the double *Δmns1 Δmnl1* deletion strain (Supp. Fig. 1A). This result proves that Mnl2 plays a role in CPY* degradation. In addition, degradation of CPY* was tested in the triple deletion *Δmns1 Δmnl1 Δmnl2* strain. Again, a similar stabilization of CPY* was visible in this strain as compared to the double deletion *Δmnl1 Δmnl2* strain, supporting the idea that Mnl1 and Mnl2 act in the same pathway (Supp. Fig. 2B).

Proof that Mnl2 acts on the glycan part of CPY* and not on its protein part was given by experiments using the unglycosylated ERAD substrate CPY*0000 [41,42]. Degradation of CPY*0000 was not affected by the absence of Mnl2 or Mnl1 (Fig. 3A). This excludes Mnl2 as being a chaperone acting on the protein part of CPY*.

The Mnl2 dependent stabilization of CPY* led to the idea that Mnl1 and Mnl2 either act sequentially or in parallel in the glycan trimming process, generating different glycan structures and by this different degradation signals. The presence of Man6 and Man5 structures in processed glycan chains had been shown [21–23], but no responsible enzyme for their generation had been found so far. Also, the most effective binding to the lectin Yos9 had been found for the Man5 structure exposing not only the α1,6 mannose linkage in the C branch but also α1,3 mannose linkages in the A and B branch [21]. The GH 47 members only carry α1,2 mannosidase activity. Thus our data implies that Mnl2 is the enzyme that generates Man6 and Man5 glycan structures by cleaving the α1,2 linkages in the A chain of the N-linked glycan. Also, Mnl2 could partly play the role of Mnl1 in its absence. However, Mnl1 seems to be able to compensate the loss of Mnl2 (Fig. 2A).

To prove the hypothesis that Mnl2 cooperates with Mnl1 generating the glycan degradation signal recognized by Yos9 best, we generated yeast strains where *YOS9* was deleted in cells devoid of Mnl1 and Mnl2. Turnover of CPY* was examined in this strain (Fig. 3B and Supp. Fig. 2). When comparing CPY* degradation in the mannosidase double mutant and the triple mutant lacking Yos9 in addition there was no stronger stabilization of CPY* visible. Obviously, the *MNL1 MNL2* double deletions did not expose a glycan signal which could be recognized by Yos9 as degradation signal. Therefore it did not matter whether Yos9 was present or not. Our data indicate that the α1,2 mannosidase Mnl1 together with the putative α1,2 mannosidase Mnl2 are sufficient to fully generate the binding signal for the lectin Yos9 to trigger fast degradation of the protein as depicted in the model (Fig. 4). Future studies analyzing the carbohydrate structures in the different mutants will have to prove the model.

Funding

This work was supported by the Marie Curie UbiRegulators Research Training Network and the Rubicon Network of Excellence of the European Commission as well as a grant from the Deutsche Forschungsgemeinschaft, Bonn.

Acknowledgments

We thank T. Sommer for the gift of the α-Sec61 antibody. The help of all members of the Wolf lab with discussions and in the preparation of the manuscript is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.100.

References

[1] C.M. Dobson, Protein folding and misfolding, Nature 426 (2003) 884–890.
[2] A.L. Goldberg, Protein degradation and protection against misfolded or damaged proteins, Nature 426 (2003) 895–899.
[3] A. Ciechanover, A.L. Schwartz, The ubiquitin system: pathogenesis of human diseases and drug targeting, Biochimica et Biophysica Acta 1695 (2004) 3–17.
[4] T.A. Rapoport, Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes, Nature 450 (2007) 663–669.
[5] I. Braakman, N.J. Bulleid, Protein folding and modification in the Mammalian endoplasmic reticulum, Annual Review of Biochemistry 80 (2011) 71–99.
[6] H. Lodish, A. Berk, C.A. Kaiser, M. Krieger, M.P. Scott, A. Bretscher, H. Ploegh, P. Matsudaira, Molecular Cell Biology, W. H. Freeman, New York, 2007.
[7] Y. Yoshida, A novel role for N-glycans in the ERAD system, Journal of Biochemistry 134 (2003) 183–190.
[8] A. Helenius, M. Aebi, Roles of N-linked glycans in the endoplasmic reticulum, Annual Review of Biochemistry 73 (2004) 1019–1049.
[9] A. Varki, R.D. Cummings, J.D. Esko, H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler, Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY), 2009.
[10] I.M. Nilsson, G. von Heijne, Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane, The Journal of Biological Chemistry 268 (1993) 5798–5801.
[11] M. Aebi, R. Bernasconi, S. Clerc, M. Molinari, N-glycan structures: recognition and processing in the ER, Trends in Biochemical Sciences 35 (2010) 74–82.
[12] M.M. Hiller, A. Finger, M. Schweiger, D.H. Wolf, ER degradation of a misfolded luminal protein by the cytosolic ubiquitin–proteasome pathway, Science 273 (1996) 1725–1728.
[13] B. Meusser, C. Hirsch, E. Jarosch, T. Sommer, ERAD: the long road to destruction, Nature Cell Biology 7 (2005) 766–772.
[14] T. Sommer, D.H. Wolf, Endoplasmic reticulum degradation: reverse protein flow of no return, The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology 11 (1997) 1227–1233.
[15] Z. Kostova, D.H. Wolf, For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin–proteasome connection, The EMBO Journal 22 (2003) 2309–2317.
[16] S.S. Vembar, J.L. Brodsky, One step at a time: endoplasmic reticulum-associated degradation, Nature Reviews. Molecular Cell Biology 9 (2008) 944–957.
[17] A. Stolz, D.H. Wolf, Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell, Biochimica et Biophysica Acta (BBA) – Molecular Cell Research 1803 (2010) 694–705.
[18] R.G. Spiro, Role of N-linked polymannose oligosaccharides in targeting glycoproteins for endoplasmic reticulum-associated degradation, Cellular and Molecular Life Sciences: CMLS 61 (2004) 1025–1041.
[19] B.R. Pearse, D.N. Hebert, Lectin chaperones help direct the maturation of glycoproteins in the endoplasmic reticulum, Biochimica et Biophysica Acta 1803 (2010) 684–693.
[20] W. Xie, D.T. Ng, ERAD substrate recognition in budding yeast, Seminars in Cell & Developmental Biology 21 (2010) 533–539.
[21] E.M. Quan, Y. Kamiya, D. Kamiya, V. Denic, J. Weibezahn, K. Kato, J.S. Weissman, Defining the glycan destruction signal for endoplasmic reticulum-associated degradation, Molecular Cell 32 (2008) 870–877.
[22] S. Clerc, C. Hirsch, D.M. Oggier, P. Deprez, C. Jakob, T. Sommer, M. Aebi, Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum, The Journal of Cell Biology 184 (2009) 159–172.
[23] R. Gauss, K. Kanehara, P. Carvalho, D.T. Ng, M. Aebi, A complex of pdi1p and the mannosidase htm1p initiates clearance of unfolded glycoproteins from the endoplasmic reticulum, Molecular Cell 42 (2011) 782–793.
[24] N. Hosokawa, Y. Kamiya, K. Kato, The role of MRH domain-containing lectins in ERAD, Glycobiology 20 (2010) 651–660.
[25] R.G. Spiro, Glucose residues as key determinants in the biosynthesis and quality control of glycoproteins with N-linked oligosaccharides, The Journal of Biological Chemistry 275 (2000) 35657–35660.
[26] A. Herscovics, Importance of glycosidases in mammalian glycoprotein biosynthesis, Biochimica et Biophysica Acta 1473 (1999) 96–107.
[27] N. Hosokawa, Y. Kamiya, D. Kamiya, K. Kato, K. Nagata, Human OS-9, a lectin required for glycoprotein endoplasmic reticulum-associated degradation, recognizes mannose-trimmed N-glycans, The Journal of Biological Chemistry 284 (2009) 17061–17068.
[28] Y. Oda, N. Hosokawa, I. Wada, K. Nagata, EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin, Science 299 (2003) 1394–1397.
[29] A. Bhamidipati, V. Denic, E.M. Quan, J.S. Weissman, Exploration of the topological requirements of ERAD identifies Yos9p as a lectin sensor of misfolded glycoproteins in the ER lumen, Molecular Cell 19 (2005) 741–751.
[30] B.A. Buschhorn, Z. Kostova, B. Medicherla, D.H. Wolf, A genome-wide screen identifies Yos9p as essential for ER-associated degradation of glycoproteins, FEBS Letters 577 (2004) 422–426.
[31] K. Kanehara, S. Kawaguchi, D.T. Ng, The EDEM and Yos9p families of lectin-like ERAD factors, Seminars in Cell & Developmental Biology 18 (2007) 743–750.
[32] J.C. Christianson, T.A. Shaler, R.E. Tyler, R.R. Kopito, OS-9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD, Nature Cell Biology 10 (2008) 272–282.
[33] S. Munro, The MRH domain suggests a shared ancestry for the mannose 6-phosphate receptors and other N-glycan-recognising proteins, Current Biology: CB 11 (2001) R499–R501.
[34] T. Satoh, Y. Chen, D. Hu, S. Hanashima, K. Yamamoto, Y. Yamaguchi, Structural basis for oligosaccharide recognition of misfolded glycoproteins by OS-9 in ER-associated degradation, Molecular Cell 40 (2010) 905–916.
[35] C. Guthrie, G.R. Fink, Guide to Yeast Genetics and Molecular Biology, Academic Press, San Diego, 1991.
[36] U. Guldener, S. Heck, T. Fielder, J. Beinhauer, J.H. Hegemann, A new efficient gene disruption cassette for repeated use in budding yeast, Nucleic Acids Research 24 (1996) 2519–2524.
[37] U. Gueldener, J. Heinisch, G.J. Koehler, D. Voss, J.H. Hegemann, A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast, Nucleic Acids Research 30 (2002) e23.
[38] J. Gough, K. Karplus, R. Hughey, C. Chothia, Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure, Journal of Molecular Biology 313 (2001) 903–919.
[39] R. Gauss, E. Jarosch, T. Sommer, C. Hirsch, A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery, Nature Cell Biology 8 (2006) 849–854.
[40] A. Finger, M. Knop, D.H. Wolf, Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast, European Journal of Biochemistry 218 (1993) 565–574.
[41] Z. Kostova, D.H. Wolf, Importance of carbohydrate positioning in the recognition of mutated CPY for ER-associated degradation, Journal of Cell Science 118 (2005) 1485–1492.
[42] E. Martinez Benitez, A. Stolz, D.H. Wolf, Yos9, a control protein for misfolded glycosylated and non-glycosylated proteins in ERAD, FEBS Letter 585 (2011) 3015–3019.

Appendix 2

Yos9, a control protein for misfolded glycosylated and non-glycosylated proteins in ERAD

Elena Martinez Benitez, Alexandra Stolz and Dieter H. Wolf
FEBS Letters 585 (2011) 3015–3019

FEBS Letters 585 (2011) 3015–3019

FEBS Letters

journal homepage: www.FEBSLetters.org

Yos9, a control protein for misfolded glycosylated and non-glycosylated proteins in ERAD

Elena Martinez Benitez, Alexandra Stolz, Dieter H. Wolf*

Institut für Biochemie der Universität Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany

ARTICLE INFO

Article history:
Received 28 June 2011
Revised 8 August 2011
Accepted 16 August 2011
Available online 23 August 2011

Edited by Felix Wieland

Keywords:
ER associated degradation
N-glycosylation
Misfolded protein
Lectin Yos9
CPY*0000
MRH domain

ABSTRACT

The endoplasmic reticulum (ER) is responsible for folding and delivery of secretory proteins to their site of action. One major modification proteins undergo in this organelle is N-glycosylation. Proteins that cannot fold properly will be directed to a process known as endoplasmic reticulum associated degradation (ERAD). Processing of N-glycans generates a signal for ERAD. The lectin Yos9 recognizes the N-glycan signal of misfolded proteins and acts as a gatekeeper for the delivery of these substrates to the cytoplasm for degradation. Presence of Yos9 accelerates degradation of the glycosylated model ERAD substrate CPY*. Here we show that Yos9 has also a control function in degradation of the unglycosylated ERAD substrate CPY*0000. It decelerates its degradation rate.

1. Introduction

The endoplasmic reticulum (ER) is the entry site of proteins into the secretory pathway of eukaryotic cells. More than one quarter of the eukaryotic proteome passes the ER membrane and folds to their native conformation in this compartment. Over 5% of the cell's proteome is responsible for a functional ER (Saccharomyces Genome Database). During folding these secretory proteins can undergo three kinds of modifications: glycosylation, disulfide bond formation and specific proteolytic cleavage [1]. An optimal chaperone equipment in the ER assists folding of the heavy protein load [2]. Properly folded proteins are finally sorted to their different sites of action, which include the lysosome (vacuole), the plasma membrane and the exterior of the cell [1].

However, due to statistic errors, mutations, heat, heavy metals or oxidative stress folding errors occur resulting in misfolded proteins that impair cellular protein homeostasis [2–4]. Therefore the ER must detect permanently misfolded proteins, prevent them from secretion and deliver them to the endoplasmic reticulum associated degradation (ERAD) machinery for degradation. Upon import into the ER most nascent proteins are N-glycosylated with a $Glc_3Man_9GlcNAc_2$ carbohydrate chain [5,6]. During protein folding the three glucose residues are removed. In *Saccharomyces cerevisiae* if a protein is not properly folded by then, the slow-acting mannosidase I (Mns1) cleaves a mannose from the B branch of the glycan tree linked to the protein. Subsequently the lectin Mnl1/Htm1, which has mannosidase activity, cleaves an additional mannose residue from the C branch yielding a $Man_7GlcNAc_2$ structure with an exposed α1,6 linkage [7–10]. If the misfolded protein has a lesion located in the ER lumen it is recruited to the Hrd/Der ligase complex via binding to Hrd3 [11]. The lectin Yos9, an integral subunit of the Hrd/Der ligase complex, recognizes the α1,6 linked mannose of the $Man_7GlcNAc$ chain of the glycosylated proteins via its MRH domain and targets them to the ERAD machinery [7,8,12–14]. Substrates are retro-translocated across the ER membrane, polyubiquitylated by the E3 enzyme Der3/Hrd1, extracted from the membrane by the AAA-ATPase machinery Cdc48-Ufd1-Npl4, and delivered to the proteasome via the ubiquitin receptors Dsk2 and Rad23 for degradation [15–19].

Major attention has been given to the degradation of misfolded glycosylated proteins of the ER [9,10,20,21]. As the ER processes non-glycosylated proteins, which might also undergo misfolding, here we investigated their fate in ERAD. For this purpose, we chose a non-glycosylated version of misfolded carboxypeptidase Y (CPY*0000) [20].

2. Materials and methods

2.1. Yeast strains and plasmids

Molecular biological and genetic techniques were carried out using standard methods [22]. *S. cerevisiae* strains used in this study are based on YWO0636 (MATa, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3*,

* Corresponding author.
E-mail address: dieter.wolf@ibc.uni-stuttgart.de (D.H. Wolf).

0014-5793/$36.00
doi:10.1016/j.febslet.2011.08.021

Table 1
Strains used in this study.

Strain	Genotype	Source
YWO0340	W303 MATalpha, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1Δ::hisG*, *pep4Δ::HIS3*, *prb1Δ::hisG*	K. Kuchler
YWO0361	WCG MATa, *his3-11 15*, *leu2-3 112*, *ura3*, *prc1-1*	Knop et al. 1996 [30]
YWO0364	WCG MATa, *his3-11 15*, *leu2-3 112*, *ura3*, *prc1-1*, *pre1-1*, *pre4-1*	Hilt et al. 1993 [28]
YWO0636	W303 MATalpha, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1Δ::LEU2*	Plemper et al. 1999 [41]
YWO0820	W303 MATa, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc Δ::LEU2*, *mnl/htm1Δ::kanMX6*	Kostova et al. 2005 [20]
YWO1154	W303 MATa, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1Δ ::LEU2*, *yos9Δ::HIS3MX6*	Buschhorn et al. 2004 [12]
YWO1268	W303 MATa, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1Δ::LEU2*, *usa1Δ::kanMX6*	H. Hoshida
YWO1408	W303 MATalpha, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1Δ::LEU2*, *der3Δ::HIS3*	A. Schäfer
YWO1526	W303 MATa, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1Δ::LEU2*, *doa10Δ::kanMX6*	This study
YWO1643	W303 MATa, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1Δ::LEU2*, *der1Δ::His5+*	S. Besser
YWO1792	W303 MATa, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1Δ::LEU2*, *der1Δ*, *yos9Δ::HIS3MX6*	A. Becher
YWO1793	W303 MATa, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1Δ::LEU2*, *usa1Δ*, *yos9Δ::HIS3MX6*	A. Becher

Table 2
Plasmids used in this study.

Plasmid	Characteristics	Backbone	Source
PWO0604	CPY*0000, *PRC1* promoter and terminator	pRS316	Kostova et al. 2005 [20]
PWO0612	CPY*, *PRC1* promoter and terminator	pRS316	Buschhorn et al.2004 [12]
pUG27	Deltion vector		Güldener et al. 1996 [23]
PWO1114	Yos9R200A-Flag	pRS314	O. Fischer

112, *trp1-1*, *ura3-1*, *prc1Δ::LEU2*) and YWO0361 (WCG MATa, *his3-11 15, leu2-3 112, ura3, prc1-1*), Strains YWO1792 and YWO1973 were generated from strains YWO1643 and YWO1268 respectively, according to Güldener et al. after pop out of the auxotrophic markers and using plasmid pFA6a-His3MX6 and primers EMB021 and EMB022 for the deletion of the *YOS9* gene [23]. Construction of the glycosylation mutant CPY*0000 was described previously [20]. Tables 1–3 list the yeast strains, plasmids and primers used.

2.2. Pulse-chase analysis and immunoprecipitation

Cells were grown at 30 °C to logarithmic phase in 20 ml of selective media. For each strain 40 ODs of cells were taken. They were washed 5 times with 1 ml starvation media (0.67% (w/v) yeast nitrogen base w/o amino acids, 2% glucose, 0.012% (w/v) L-alanine, L-isoleucine, L-leucine, L-arginine, L-lysine, L-aspartate, L-cysteine, L-phenylalanine, L-glutamate, L-threonine, L-glutamine, L-tryptophan, L-glycine, L-valine, L-proline, L-histidine, L-serine, L-tyrosine, L-asparagine, myo-inositol, *p*-aminobenzoic acid, pH 5.6) and resuspended in 1 ml starvation media in 50 ml tubes. Cells underwent starvation for 50 min at 30 °C. Then 20 μl of methionine labeled with the radioactive isotope ^{35}S (10 μCi/μl) was added and cultures were incubated 20 min under the same conditions. During this time all newly synthesized proteins integrate radioactive methionine into their sequence. After these 20 min, 1 ml of chase media (starvation media plus 0.2% BSA, 0.6% non-labeled L-methionine) was added and samples (450 μl) were collected at different time points (see figures) in microtubes containing 500 μl of 20 mM NaN_3. Samples were kept on ice or stored at −20 °C. The tubes with thawed cells were centrifuged for 15 min at 13 000 rpm and the supernatant was discarded. The pellet was resuspended in 100 μl of BB1 buffer (50 mM Tris/HCl pH 7.5, 6 M urea, 1 mM EDTA, 1% (w/v) SDS) and 67 μl of glass beads added. Samples were vortexed 5 times for 1 min with a minute interruption on ice. Then, cell extracts were resuspended in 1 ml IP buffer (50 mM Tris/HCl pH 7.5, 190 mM NaCl 1.25% TritonX-100 (v/v), 6 mM EDTA) and spun down for 15 min at 14 000 rpm. Next, 900 μl of the supernatant was transferred to a microtube containing 3 μl of polyclonal CPY antibody (Rockland) for immunoprecipitation. Immunoprecipitation took place during 90 min at room temperature, followed by the addition of 80 μl of 7% Protein A sepharose suspension (GE healthcare). During 90 min incubation the antibodies bind to the sepharose. After 5 washings with IP buffer (2000 rpm) all non-bound proteins to the antibody and sepharose are eliminated. In the final centrifugation step the IP buffer is removed completely and 60 μl SDS loading buffer are added. Samples are subjected to SDS–PAGE (7.5% polyacrylamide gel) and thereafter the gel is dried at 60 °C on a Whatman paper with the aid of a vacuum. Whatman papers containing the sample gels are fixed in a cassette and exposed to a phosphor screen. The screen is analyzed using a PhosphoImager scanner and ImageQuaNT™ software. In each time point of the experiments the mean ± interval of confidence with an $\alpha = 0.05$ for the error bars is calculated.

Table 3
Primers.

EMB021	agatcttcacatatatcgttatcatccccttttcttccctgtttcacagctgaagcttcgtacgc
EMB022	gcaaaacgtgaaaaaaaaaaattaaaagtttatactcctccttgtgcataggccactagtggatctg

3. Results and discussion

It had been shown that the four N-carbohydrates on CPY* are not equal in their capability to act as signals for ERAD. The most C-terminal of the four glycans in CPY* is required and sufficient to trigger efficient degradation. However, the unglycosylated version of CPY* (CPY*0000) is still degraded but at a considerably reduced rate [20].

We wanted to elucidate the degradation mechanism of this misfolded unglycosylated CPY* protein. To rule out autophagy we checked the degradation profile of CPY*0000 by pulse-chase experiments in cells with defective vacuolar function. The vacuole is equipped with two major endopeptidases, proteinase yscA (gene *PEP4*/*PRA1*) an aspartyl protease, and proteinase yscB (gene *PRB1*), a serine protease. When both proteinases are absent the proteolytic vacuolar function is severely compromised [24,25]. When testing CPY*0000 degradation in *Δpep4Δprb1* double mutants the same CPY*0000 degradation profile was visible as in wild

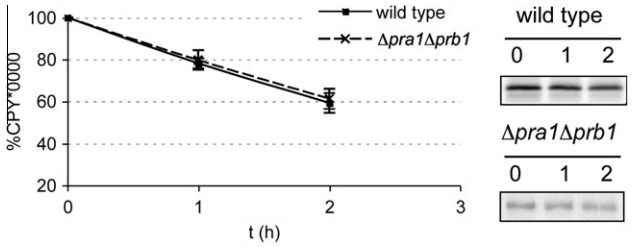


Fig. 1. The vacuole is not required for CPY*0000 degradation. Cells with defective vacuolar function (*Δpep4/pra1Δprb1*) were transformed with a CPY*0000 expression plasmid. The pulse-chase data represent the means of four independent experiments and interval of confidence with an $\alpha = 0.05$ for the error bars is calculated.

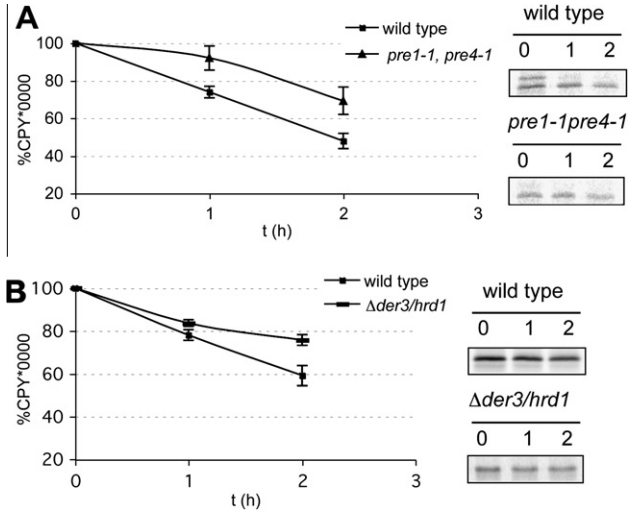


Fig. 2. CPY*0000 is an ERAD-L substrate. (A) Cells with defective proteasomes (*pre1-1, pre4-1*) were transformed with a CPY*0000 expression plasmid. (B) *DER3/HRD1* deleted cells were transformed with a CPY*0000 expression plasmid showing a stabilization of the substrate. The pulse-chase data represent the means of 3 (wild type and proteasome mutant) to four (wild type and *Δder3/hrd1*) independent experiments and interval of confidence with an $\alpha = 0.05$ for the error bars is calculated.

type cells proving that the vacuole is not the locus of CPY*0000 degradation (Fig. 1).

To confirm whether CPY*0000 is a *bona fide* ERAD substrate we tested the involvement of the main components of the ubiquitin proteasome system (UPS) in its elimination. We followed CPY*0000 degradation in cells expressing defective proteasomes, as well as cells deleted in different E3 ubiquitin ligases [26–28]. When testing degradation of CPY*0000 in mutants with defects in the Pre1 and the Pre4 subunits of the proteasome a considerable stabilization of the misfolded protein as compared to wild type cells was apparent (Fig. 2A). Thus, the proteasome is the proteolytic machinery for CPY*0000 elimination. Ubiquitylation of glycosylated CPY*, a prerequisite for degradation, is carried out by the E3 ligase Der3/Hrd1. We therefore tested if this enzyme was involved in elimination of non-glycosylated CPY*0000. As can be seen in Fig. 2B degradation of the misfolded non-glycosylated enzyme is indeed slowed down in *Δder3/Δhrd1* mutant cells, indicating its requirement for the elimination process. The second E3 ligase of the ER, Doa10 [29] was also tested, with no effect in CPY*0000 turnover (data not shown). Clearly, CPY*0000 is an ERAD-L substrate.

We therefore tested if also the ER membrane protein Der1, required for elimination of soluble ERAD-L substrates and Usa1, which links Der1 to the Der3/Hrd1 ligase, are part of the degradation process [30–35]. Fig. 3A shows that deletion of any of these

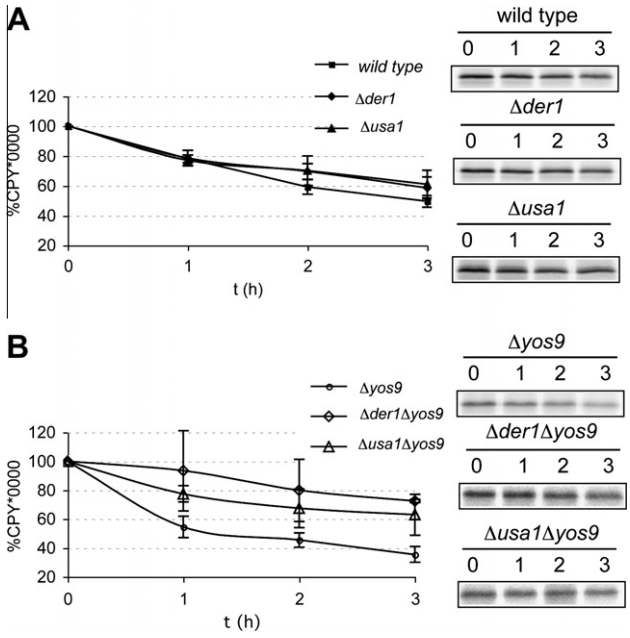


Fig. 3. Degradation efficiency of CPY*0000 in *DER1* and *USA1* deleted cells. All indicated strains were transformed with a CPY*0000 expression plasmid. (A) CPY*0000 degradation in wild type, *Δder1* and *Δusa1* strains was followed by pulse-chase experiments. (B) CPY*0000 degradation in *Δyos9* (control), *Δder1Δyos9 and Δusa1Δyos9* strains. The pulse-chase data represent the means of two (*Δder1Δyos9 and Δusa1Δyos9*), four (wild type and *Δusa1*) and seven (*Δder1* strain and *Δyos9* control) independent experiments and interval of confidence with an $\alpha = 0.05$ for the error bars is calculated.

proteins leads to a similar, but rather marginal reduction in the degradation kinetics of CPY*0000.

Even though at first glance we did not expect any effect on the degradation of non-glycosylated CPY*0000 in mutants carrying deletions in the lectin-like proteins Mnl1/Htm1 and Yos9 which interact with the glycan residues of glycosylated CPY* [7,12,14,36], we nevertheless tested the degradation behavior of CPY*0000 in such mutant strains. As expected, deletion of *MNL1/HTM1* did not lead to any alteration of the degradation kinetics of CPY*0000 (Fig. 4A). Unexpectedly, deletion of the *YOS9* gene led to a considerably enhanced elimination kinetics of CPY*0000 (Fig. 4B). Obviously, Yos9 does not only play a role in recognition in the ERAD of glycosylated substrates, but also of non-glycosylated substrates. While Yos9 promotes degradation of glycosylated substrates (Supplementary Fig. 1) [12,14], it seems to have an opposite function in degradation of non-glycosylated CPY*0000 by slowing down its degradation. The finding that absence of Yos9 accelerates degradation of CPY*0000 led us to repeat the experiments on the influence of the ERAD-L components Der1 and Usa1 in a *YOS9* deletion strain. The degradation kinetics of CPY*0000 is considerably reduced in *DER1* and *USA1* deleted strains when Yos9 is absent (Fig. 3B). This clearly shows the need of Der1 and Usa1 for degradation of non-glycosylated CPY*0000.

We tested if the mannose-6-phosphate receptor homology domain (MRH) of Yos9, which is responsible for recognition of glycosylated substrates, is recognizing non-glycosylated CPY*0000 [37,38]. This is not the case: The degradation kinetics of CPY*0000 is similar in wild type and in Yos9 mutant cells bearing a mutation in the MRH domain (R200A), which abrogates its lectin function (Fig. 4B). An interaction between the MRH domain mutated Yos9 protein and CPY*0000 had previously been shown [14]. This correlates with our data and suggests a checkpoint function of Yos9 for unglycosylated substrates.

This work shows that the non-glycosylated substrate CPY*0000 is a *bona fide* ERAD-L substrate. Recently, this has also been shown

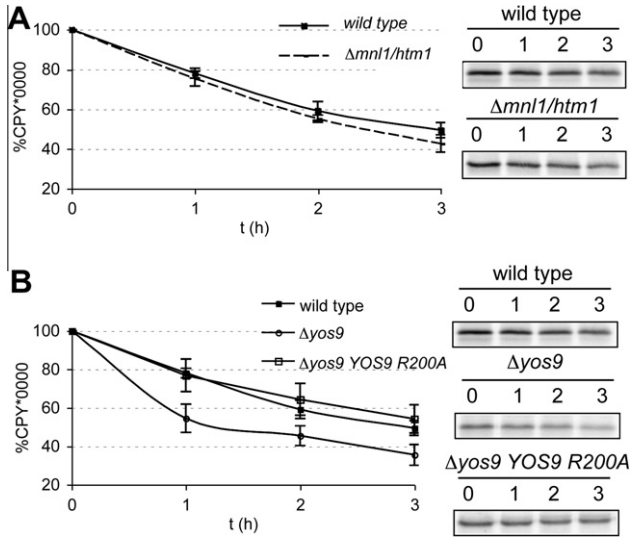


Fig. 4. The degradation efficiency of CPY*0000 in *MNL1*/*HTM1* deleted cells, *YOS9* deleted cells and cells expressing Yos9 carrying a mutation in the MRH domain. (A) *MNL1*/*HTM1* deleted cells were transformed with a CPY*0000 expression plasmid. Pulse-chase analyses were performed. (B) Wild type and *Δyos9* trains were transformed with CPY*0000 or CPY*0000 and Yos9R200A-Flag expression plasmids respectively and pulse-chase analyses were performed. Data represent the means of four (wild type and *Δmnl1*/*htm1*), six (Yos9R200A) and seven (*Δyos9*) independent experiments and interval of confidence with an $\alpha = 0.05$ for the error bars is calculated.

to be the case for a mutant unglycosylated version of proteinase yscA [39,40]. Efficient degradation of CPY*0000 depends on the Hrd/Der complex. While the presence of the lectin Yos9 in the complex promotes degradation of glycosylated misfolded proteins, we could show here that it hinders degradation of unglycosylated CPY*0000. Degradation kinetics of CPY*0000 is not affected by the R200A mutation in the MRH domain of Yos9 which abrogates its lectin function, confirming a second function for this ERAD component. We hypothesize that Yos9 has a checkpoint function for non-glycosylated misfolded proteins. This could be part of a timer for unglycosylated substrates: Yos9 may bind any misfolded protein via a hydrophobic patch and after checking the glycan structure by the MRH domain, it delivers the glycosylated misfolded proteins to the ubiquitin-proteasome system, while non-glycosylated proteins remain bound until they are properly folded or, when unsuccessful, are slowly delivered to the degradation machinery. Without doubt, Yos9 plays a tuning role in ERAD, independent of the glycosylation state of the substrate.

4. Note added in proof

While this paper was under review the following paper has been published: Yos9p assists in the degradation of certain non-glycosylated proteins from the endoplasmic reticulum. Jaenicke L A, Brendebach H, Selbach M and Hirsch C. (2011) Mol Biol Cell. Jul 7. Epub ahead of print.

Acknowledgments

We thank J. Weissman for the gift of the original *Yos9R200A* plasmid. We are grateful to A. Becher, S. Besser, O. Fischer, H. Hoshida and A. Schäfer for the generation of strains and plasmid. The help of I. Amm, B. Braun, F. Eisele, R. Menssen, M. Scazzari with discussions and in the preparation of the manuscript is gratefully acknowledged. This work was supported by the UbiRegulators Research Training Network (Marie Curie Actions, the European Commission) and the Rubicon Network of Excellence (European Commission) as well as a grant from the Deutsche Forschungsgemeinschaft, Bonn.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.08.021.

References

[1] Berg, J.M. (2007) Biochemistry, W, H. Freeman and Company, New York.
[2] Braakman, I. and Bulleid, N.J. (2010) Protein Folding and Modification in the Mammalian Endoplasmic Reticulum. Annu Rev Biochem 80, 71–99.
[3] Goldberg, A.L. (2003) Protein degradation and protection against misfolded or damaged proteins. Nature 426, 895–899.
[4] Dobson, C.M. (2003) Protein folding and misfolding. Nature 426, 884–890.
[5] Helenius, A. and Aebi, M. (2004) Roles of N-linked glycans in the endoplasmic reticulum. Annu Rev Biochem 73, 1019–1049.
[6] Varki, A., Cummings, R.D., Esko, J.D., Freeze, H., Stanley, P., Bertozzi, C.R., Hart, G.W. and Etzler, M.E. (2009) Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).
[7] Quan, E.M., Kamiya, Y., Kamiya, D., Denic, V., Weibezahn, J., Kato, K. and Weissman, J.S. (2008) Defining the glycan destruction signal for endoplasmic reticulum-associated degradation. Mol Cell 32, 870–877.
[8] Clerc, S., Hirsch, C., Oggier, D.M., Deprez, P., Jakob, C., Sommer, T. and Aebi, M. (2009) Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum. J Cell Biol 184, 159–172.
[9] Aebi, M., Bernasconi, R., Clerc, S. and Molinari, M. (2010) N-glycan structures: recognition and processing in the ER. Trends Biochem Sci 35, 74–82.
[10] Pearse, B.R. and Hebert, D.N. (2010) Lectin chaperones help direct the maturation of glycoproteins in the endoplasmic reticulum. Biochim Biophys Acta 1803, 684–693.
[11] Gauss, R., Jarosch, E., Sommer, T. and Hirsch, C. (2006) A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery. Nat Cell Biol 8, 849–854.
[12] Buschhorn, B.A., Kostova, Z., Medicherla, B. and Wolf, D.H. (2004) A genome-wide screen identifies Yos9p as essential for ER-associated degradation of glycoproteins. FEBS Lett 577, 422–426.
[13] Xie, W., Kanehara, K., Sayeed, A. and Ng, D.T. (2009) Intrinsic conformational determinants signal protein misfolding to the Hrd1/Htm1 endoplasmic reticulum-associated degradation system. Mol Biol Cell 20, 3317–3329.
[14] Bhamidipati, A., Denic, V., Quan, E.M. and Weissman, J.S. (2005) Exploration of the topological requirements of ERAD identifies Yos9p as a lectin sensor of misfolded glycoproteins in the ER lumen. Mol Cell 19, 741–751.
[15] Raasi, S. and Wolf, D.H. (2007) Ubiquitin receptors and ERAD: a network of pathways to the proteasome. Semin Cell Dev Biol 18, 780–791.
[16] Vembar, S.S. and Brodsky, J.L. (2008) One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol 9, 944–957.
[17] Meusser, B., Hirsch, C., Jarosch, E. and Sommer, T. (2005) ERAD: the long road to destruction. Nat Cell Biol 7, 766–772.
[18] Stolz, A. and Wolf, D.H. (2010) Endoplasmic reticulum associated protein degradation: A chaperone assisted journey to hell. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1803, 694–705.
[19] Buchberger, A., Bukau, B. and Sommer, T. (2010) Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. Mol Cell 40, 238–252.
[20] Kostova, Z. and Wolf, D.H. (2005) Importance of carbohydrate positioning in the recognition of mutated CPY for ER-associated degradation. J Cell Sci 118, 1485–1492.
[21] Spear, E.D. and Ng, D.T. (2005) Single, context-specific glycans can target misfolded glycoproteins for ER-associated degradation. J Cell Biol 169, 73–82, doi:10.1083/jcb.2004,11,136.
[22] C. Guthrie; G. Fink *Guide to Yeast Genetics and Molecular and Cell Biology*. Methods of enzymology, Vol. 350 and 351, 2001, Academic Press.
[23] Güldener, U., Heck, S., Fielder, T., Beinhauer, J. and Hegemann, J.H. (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 24, 2519–2524.
[24] Teichert, U., Mechler, B., Muller, H. and Wolf, D.H. (1989) Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival. J Biol Chem 264, 16037–16045.
[25] Rupp, S. and Wolf, D.H. (1995) Biogenesis of the yeast vacuole (lysosome). The use of active-site mutants of proteinase yscA to determine the necessity of the enzyme for vacuolar proteinase maturation and proteinase yscB stability. Eur J Biochem/FEBS 231, 115–125.
[26] Bordallo, J., Plemper, R.K., Finger, A. and Wolf, D.H. (1998) Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol Biol Cell 9, 209–222.
[27] Heinemeyer, W., Kleinschmidt, J.A., Saidowsky, J., Escher, C. and Wolf, D.H. (1991) Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. Embo J 10, 555–562.

[28] Hilt, W., Enenkel, C., Gruhler, A., Singer, T. and Wolf, D.H. (1993) The PRE4 gene codes for a subunit of the yeast proteasome necessary for peptidylglutamyl-peptide-hydrolyzing activity. Mutations link the proteasome to stress- and ubiquitin-dependent proteolysis. J Biol Chem 268, 3479–3486.
[29] Swanson, R., Locher, M. and Hochstrasser, M. (2001) A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes Dev 15, 2660–2674.
[30] Knop, M., Finger, A., Braun, T., Hellmuth, K. and Wolf, D.H. (1996) Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. Embo J 15, 753–763.
[31] Hitt, R. and Wolf, D.H. (2004) Der1p, a protein required for degradation of malfolded soluble proteins of the endoplasmic reticulum: topology and Der1-like proteins. FEMS Yeast Res 4, 721–729.
[32] Carvalho, P., Goder, V. and Rapoport, T.A. (2006) Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell 126, 361–373.
[33] Kim, I., Li, Y., Muniz, P. and Rao, H. (2009) Usa1 protein facilitates substrate ubiquitylation through two separate domains. PLoS One 4, e7604.
[34] Carroll, S.M. and Hampton, R.Y. (2010) Usa1p is required for optimal function and regulation of the Hrd1p endoplasmic reticulum-associated degradation ubiquitin ligase. J Biol Chem 285, 5146–5156.
[35] Horn, S.C., Hanna, J., Hirsch, C., Volkwein, C., Schutz, A., Heinemann, U., Sommer, T. and Jarosch, E. (2009) Usa1 functions as a scaffold of the HRD-ubiquitin ligase. Mol Cell 36, 782–793.
[36] Xie, W. and Ng, D.T. (2010) ERAD substrate recognition in budding yeast. Semin Cell Dev Biol 21, 533–539.
[37] Mikami, K., Yamaguchi, D., Tateno, H., Hu, D., Qin, S.Y., Kawasaki, N., Yamada, M., Matsumoto, N., Hirabayashi, J., Ito, Y., et al. (2010) The sugar-binding ability of human OS-9 and its involvement in ER-associated degradation. Glycobiology 20, 310–321.
[38] Hosokawa, N., Kamiya, Y. and Kato, K. (2010) The role of MRH domain-containing lectins in ERAD. Glycobiology 20, 651–660.
[39] Finger, A., Knop, M. and Wolf, D.H. (1993) Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast. Eur J Biochem 218, 565–574, doi:10.1111/j.1432-1033.1993.tb18410.x.
[40] Kanehara, K., Xie, W. and Ng, D.T. (2010) Modularity of the Hrd1 ERAD complex underlies its diverse client range. J Cell Biol 188, 707–716.
[41] Plemper, R.K., Bordallo, J., Deak, P.M., Taxis, C., Hitt, R. and Wolf, D.H. (1999) Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. J Cell Sci 112 (Pt 22), 4123–4134.

Appendix 3

Dfm1 Forms Distinct Complexes with Cdc48 and the ER Ubiquitin Ligases and Is Required for ERAD

Alexandra Stolz, Regina S. Schweizer, Antje Schäfer and Dieter H. Wolf
Traffic 2010; 11: 1363–1369

Traffic 2010; **11:** 1363–1369

doi:10.1111/j.1600-0854.2010.01093.x

Dfm1 Forms Distinct Complexes with Cdc48 and the ER Ubiquitin Ligases and Is Required for ERAD

Alexandra Stolz[1], Regina S. Schweizer[2], Antje Schäfer[3,#] and Dieter H. Wolf[1,*,#]

[1]Institut für Biochemie, Universität Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany
[2]Max-Planck-Institut für medizinische Forschung, Jahnstraße 29, 69120 Heidelberg, Germany
[3]Rudolf Virchow Zentrum für Experimentelle Biomedizin, Universität Würzburg, Josef-Schneider-Str. 2, D-97080 Würzburg, Germany
[#]senior authors
**Corresponding author: Dieter H. Wolf, dieter.wolf@ibc.uni-stuttgart.de*

Proteins imported into the endoplasmic reticulum (ER) are scanned for their folding status. Those that do not reach their native conformation are degraded via the ubiquitin-proteasome system. This process is called ER-associated degradation (ERAD). Der1 is known to be one of the components required for efficient degradation of soluble ERAD substrates like CPY* (mutated carboxypeptidase yscY). A homologue of Der1 exists, named Dfm1. No function of Dfm1 has been discovered, although a C-terminally hemagglutinin $(HA)_3$-tagged Dfm1 protein has been shown to interact with the ERAD machinery. In our studies, we found Dfm1-HA_3 to be an ERAD substrate and therefore not suitable for functional studies of Dfm1 in ERAD. We found cellular, non-tagged Dfm1 to be a stable protein. We identified Dfm1 to be part of complexes which contain the ERAD-L ligase Hrd1/Der3 and Der1 as well as the ERAD-C ligase Doa10. In addition, ERAD of Ste6*-HA_3 was strongly dependent on Dfm1. Interestingly, Dfm1 forms a complex with the AAA-ATPase Cdc48 in a strain lacking the Cdc48 membrane-recruiting component Ubx2. This complex does not contain the ubiquitin ligases Hrd1/Der3 and Doa10. The existence of such a complex might point to an additional function of Dfm1 independent from ERAD.

Key words: Cdc48, Dfm1, Doa10, ERAD, Hrd1/Der3, Ste6*

Received 12 April 2010, revised and accepted for publication 22 June 2010, uncorrected manuscript published online 23 June 2010, published online 21 July 2010

Secretory proteins of eukaryotic cells enter the endoplasmic reticulum (ER) for further distribution to their site of action. They pass the ER membrane via the Sec61 channel. Upon entry into the ER membrane or the ER lumen, the proteins are folded and undergo modifications, which optimize their biological activity. The proteins can leave the ER for delivery to their site of action only after proper folding. Proteins unable to fold in a reasonable time frame are retained in the ER by the quality control system, and retrograde transported out of the ER into the cytoplasm, polyubiquitylated and degraded by the proteasome (1–4). Crossing of misfolded protein through the ER membrane into the cytosol requires the retrotranslocation complexes (RTCs) in the ER membrane, which allow retrograde passage of the protein and give it directionality towards its final destination, the cytoplasmic proteasome.

Depending on the localization of the misfolded domain, different subsets of components and enzymes are required for recognition, translocation and polyubiquitylation of endoplasmic reticulum-associated degradation (ERAD) substrates. Proteins with misfolded lumenal domains (ERAD-L substrates) require the 6 transmembrane-spanning ubiquitin ligase Hrd1/Der3, whereas membrane proteins carrying a misfolded domain in the cytosol (ERAD-C substrates) require the 14 transmembrane-spanning protein Doa10 for recognition and polyubiquitylation (5–10). However, the requirement of Doa10 for polyubiquitylation of ERAD-C substrates is not absolute. Also, the Hrd1/Der3 ligase can partly take over polyubiquitylation (11,12). Both pathways, ERAD-L and ERAD-C, merge at the Cdc48-Ufd1-Npl4 machinery, a central complex pulling the ubiquitylated proteins off the ER (13–17) and giving them direction towards the proteasome for degradation. Cdc48 is linked to the ligases Hrd1/Der3 and Doa10 via the membrane protein Ubx2. In the absence of Ubx2, the ER ligases completely lose their interaction with Cdc48. However, Cdc48 to a certain amount is still anchored to the ER membrane by a yet unknown factor (18,19). The translocation channel Sec61 has been shown to be part of the ERAD-L machinery (20–24). In addition, the ER membrane protein Der1, which has 4 transmembrane spanning domains, is essential for degradation of misfolded ER-lumenal proteins (25,26). Der1 is linked to the Hrd1/Der3 ligase via the membrane protein Usa1 (27,28). Sec61 (23) as well as Der1 and its mammalian orthologues (derlins) are under discussion to be part of the retrotranslocation channel (29,30).

A Der1-like protein, Dfm1, has been recently discovered in yeast. Like Der1, it contains four transmembrane domains and cytosolic N- and C-termini. The C-terminal tail of Dfm1 is considerably elongated and contains the repeated amino acid pattern FxGxGQRn (SHP box), which is characteristic for Cdc48-binding proteins (26). Dfm1 is an unfolded protein response (UPR)-upregulated protein (31). Recent studies identified Dfm1 as being part of complexes involved in ERAD and proposed a function of Dfm1 in ER homeostasis. No involvement of Dfm1 in the elimination of misfolded protein was found (32,33). Parts of these studies have been performed with a Dfm1-HA_3

fusion protein, which we found to be an ERAD substrate. We therefore re-evaluated the previous findings and were able to show that endogenous Dfm1 is a stable protein. It interacts with components of the ERAD-L and ERAD-C pathways. We furthermore show that Dfm1 takes part in the elimination of the membrane substrate Ste6*.

Results

C-terminal HA_3-tagged Dfm1 is unstable, while endogenous Dfm1 is a stable protein

To further unravel the cellular role of Dfm1, we first used a triple HA-tagged version of the protein as had been introduced by Sato and Hampton (2006), Goder et al. (2008) and Schuberth and Buchberger (2005) (18,32,33). When checking the stability of Dfm1-HA_3 via cycloheximide chase analysis, we surprisingly discovered that the tagged version of Dfm1 is rapidly degraded (Figure 1A,B). Measuring the fate of Dfm1-HA_3 in a mutant missing Usa1, which was shown to link Der1 to the ligase Hrd1/Der3 (27,28), we found a considerable stabilization of Dfm1-HA_3. This raised the question if Dfm1 is a naturally unstable protein or if the C-terminal HA_3 tag confers instability to the protein. We therefore raised antibodies in rabbits against a C-terminal peptide of Dfm1 (see *Materials and Methods*) to visualize the endogenous Dfm1 protein. Interestingly, when following the fate of wild-type Dfm1 in cycloheximide chase analysis, it turned out that the protein was stable (Figure 1A,B). In fact, C-terminal tagging of Dfm1 induces its instability, mirroring the behaviour of its homologue Der1. Also here, a C-terminal HA_3 tag leads to loss of protein function (25). As all the previous interaction studies had been performed with tagged Dfm1 (18,32,33), the possibility existed that the interaction with ERAD components found was due to tagged Dfm1 being a substrate of the ERAD machinery. We therefore performed interaction studies of intrinsic, wild-type Dfm1 with components of the ERAD system using Dfm1 antibodies for precipitation.

Dfm1 interacts with Der1 and the Hrd/Der ligase

To determine if Dfm1 interacts with its homologue Der1, cells expressing the single HA-tagged functional and fully stable Der1-HA protein [(25) and our unpublished results] were harvested and membranes were separated. Subsequent solubilization of the membrane proteins was done with the mild detergent digitonin to prevent membrane complexes from decomposition. When precipitating Dfm1 with Dfm1 antibodies, we were able to detect Der1-HA in the pulldown (Figure 2A, lane 4). Conversely, we were able to pulldown Dfm1 together with Der1 when using HA antibodies (Figure 2B, lane 4). No corresponding signals appeared in the control experiments (Figure 2B, lane 3). Obviously, Der1 and Dfm1 are part of the same complex. To further specify the interaction between Der1 and Dfm1, we tested whether they interact directly with each other or not. We therefore precipitated Dfm1 and searched for coprecipitation of Der1-HA in the presence and absence of the linker protein Usa1. In cells expressing Usa1, we found Dfm1 coprecipitating with Der1-HA and the Hrd1/Der3 ligase (Figure 2A, lane 4). While the interaction between Dfm1 and Hrd1/Der3 remained in the absence of Usa1, Der1-HA did not coprecipitate with Dfm1 any longer (Figure 2A, lane 6). No unspecific binding of Hrd1/Der3 and Der1-HA with Dfm1 antibodies could be detected (Figure 2A, lane 5). Based on these observations, a direct interaction between Dfm1 and Der1 can be excluded. By using the completely stable N-terminally

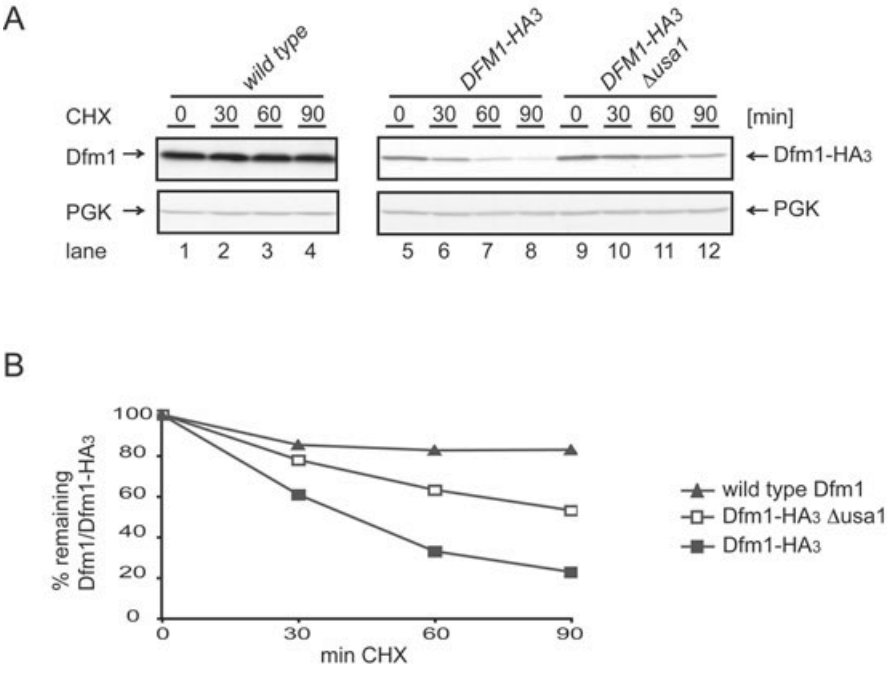


Figure 1: Dfm1-HA_3 is an unstable protein and degraded via ERAD. Immunoblot analysis of cycloheximide chase experiments in wild-type and *USA1*-deleted cells. Cell samples were taken at indicated time-points after the addition of cycloheximide. A) Dfm1-HA_3 was detected with HA antibodies and found to be unstable in wild-type cells. In contrast, untagged Dfm1 detected with Dfm1 antibodies was stable. Constitutively expressed PGK (phosphoglycerate kinase) detected with PGK antibodies served as a loading control. B) Quantification of the degradation kinetics of Dfm1-HA_3 and untagged Dfm1 of (A). Quantification of the western blot was done with ImageQuant.

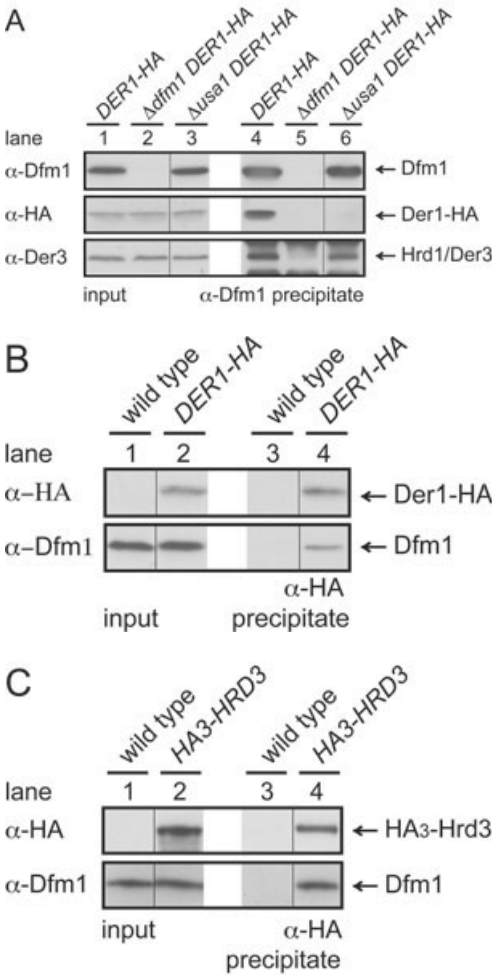


Figure 2: Dfm1 interacts with the Hrd/Der ligase complex and with Der1 in a Usa1-dependent manner. Immunoprecipitation experiments were analysed by western blot analysis. Possible interaction partners were detected using specific antibodies. The input fraction contained solubilized membrane proteins. A) C-terminal HA-tagged Der1 expressed in wild-type cells or cells deleted in *DFM1* or *USA1*. Dfm1 was precipitated by the use of Dfm1 antibodies. B) Extracts of wild-type cells and cells expressing C-terminal HA-tagged Der1 were treated with HA antibodies for precipitation of Der1. C) Extracts of wild-type and *HRD3*-deleted cells expressing plasmid-encoded, triple HA-tagged Hrd3 were treated with HA antibodies to precipitate Hrd3.

triple HA-tagged Hrd3 (our unpublished data and (34)), we were also able to coimmunoprecipitate Dfm1 together with Hrd3 (Figure 2C). Hrd3 is known to form the Hrd/Der ligase complex with Hrd1/Der3 (28,35–37). This proves the existence of a complex in the ER membrane composed of Dfm1, the Hrd/Der ligase, Usa1 and Der1.

Dfm1 interacts with Cdc48 and Hrd1/Der3 independent of the anchor protein Ubx2

Dfm1 carries two sequences on its C-terminus, which were shown to also exist in the Cdc48-binding proteins

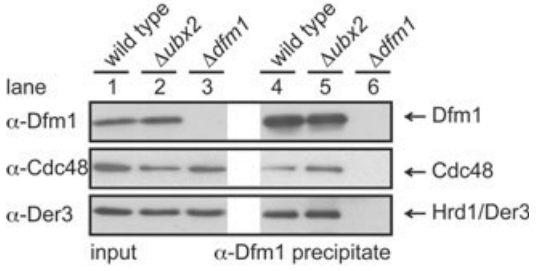


Figure 3: Dfm1 interacts with Cdc48 and Hrd1/Der3 in the absence of Ubx2. Immunoprecipitation experiment of indicated strains analysed by western blot analysis. The input fraction contained solubilized membrane proteins. Dfm1 and interaction partners were precipitated with Dfm1 antibodies.

Shp1 and Ufd1 (26). These so-called SHP boxes were shown to mediate the interaction between Dfm1-HA_3 and Cdc48 (32). To evaluate whether this interaction only occurs with unstable Dfm1-HA_3 or also with stable endogenous Dfm1, we immunoprecipitated Dfm1 and searched for Cdc48. As can be seen in Figure 3 (lane 4), functional Dfm1 is able to coprecipitate Cdc48. Ubx2 is a membrane protein anchoring the Cdc48 complex to the ER membrane and linking it to the Hrd/Der ligase complex (18,19). We were interested to see whether the interaction of Dfm1 with Cdc48 is dependent on the Cdc48 anchor Ubx2 or not. We therefore analysed a pulldown experiment of Dfm1 for coprecipitation with Cdc48 as well as with Hrd1/Der3 in a *UBX2*-deleted strain. As can be seen in Figure 3 (lane 5), Cdc48 as well as the ligase Hrd1/Der3 are bound to Dfm1 despite the absence of Ubx2.

Dfm1 interacts with the ligase Doa10 and forms a distinct complex with Cdc48

Cdc48 is part of all three defined ERAD pathways: ERAD-L, ERAD-C (8) as well as ERAD-M (27). To address whether or not Dfm1 interacts with the ligase Doa10, which is mainly involved in the ERAD-C pathway, we used a strain carrying a genomically integrated 13myc Doa10 fusion (38). After precipitating Doa10 with myc antibodies, we analysed immunoblots for coprecipitation of Dfm1. Indeed, Dfm1 coprecipitates with Doa10 (Figure 4A, lanes 9 and 10). This specific interaction was also visible when precipitation was done with Dfm1 antibodies (Figure 4B, lanes 5 and 6). These experiments uncover Doa10 as a novel interaction partner of Dfm1. As does the Hrd1/Der3 ligase, Doa10 interacts with Cdc48 via Ubx2 (18,19). This interaction remained intact also in the absence of Dfm1 (Figure 4A, lane 11). We then analysed the interaction between Dfm1 and Doa10 in a Δ*ubx2* background. When precipitating Doa10 the interaction with Cdc48 disappeared in cells lacking the Cdc48 recruiting factor Ubx2, while the interaction between Doa10 and Dfm1 remained (Figure 4C, lane 6). However, when we precipitated Dfm1 instead of Doa10 in the Δ*ubx2* strain, we found, in addition to Doa10, Cdc48 coprecipitated with

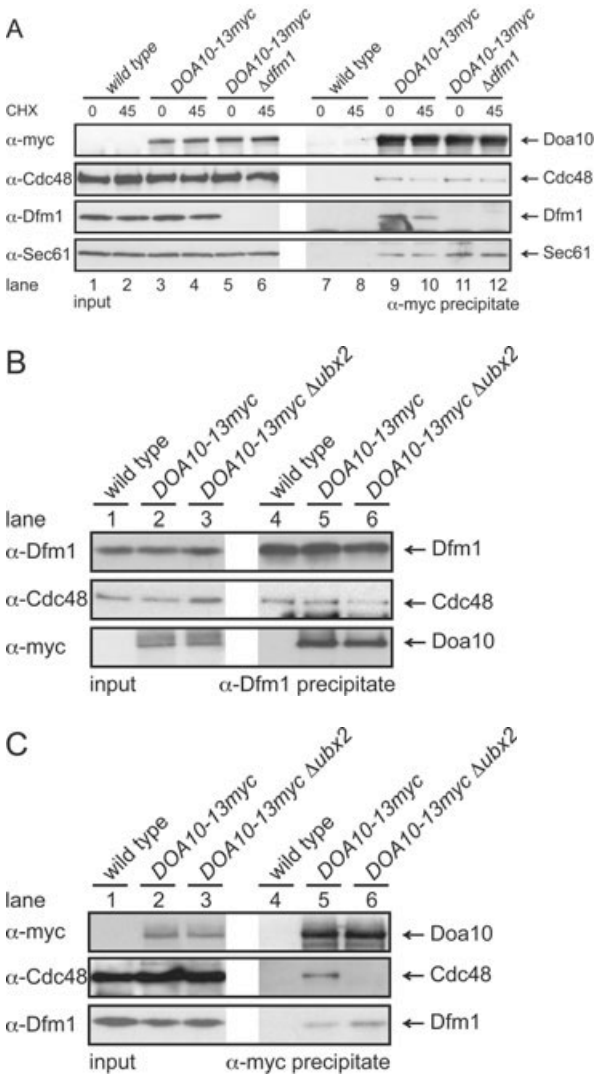


Figure 4: Doa10 loses its interaction with Cdc48 in the absence of Ubx2, while the interaction with Dfm1 remains. Immunoprecipitation analyses of indicated strains. The input fraction contained solubilized membrane proteins. A) Cells were treated with cycloheximide to stop protein synthesis for 0 or 45 min prior to cell lysis. Doa10-13myc was precipitated with myc antibodies and coprecipitated proteins were detected with their specific antibodies. B) Dfm1 was precipitated with specific Dfm1 antibodies. Coprecipitated Cdc48 was detected with its specific antibody, Doa10 was detected with myc antibodies. C) Doa10 was precipitated with myc antibodies. Coprecipitated Cdc48 and Dfm1 were detected with their specific antibodies.

Dfm1 (Figure 4B, lane 6). This uncovers the interaction between Cdc48 and Dfm1 independently of Ubx2 and the ER ligase. In the absence of Ubx2, a higher amount of Cdc48 seemed to be coprecipitate with Dfm1 (Figure 3). We quantified the amount of membrane-bound Cdc48 material of cells deficient in Ubx2, Dfm1 or both proteins. In the absence of Ubx2, the amount of Cdc48 seemed to be unchanged when compared with wild-type cells. In contrast, there was a slight decrease in Cdc48 when Dfm1 was missing. This decrease was, however, quite pronounced when both proteins, Ubx2 and Dfm1, were absent (our unpublished data).

Doa10 and the translocon Sec61 are part of the same complex

It was interesting to note that precipitates of Doa10 also contained the translocon protein Sec61 (Figure 4A, lanes 9–12). Obviously, the protein-conducting channel Sec61 also contacts the ERAD-C machinery as had been found previously for the ERAD-L machinery and the glycosylated substrate CPY* (23).

Dfm1 is necessary for the efficient degradation of the membrane substrate Ste6-HA$_3$*

To elucidate a possible function of Dfm1 in ERAD, we analysed the half-life of different ERAD substrates in wild-type and *DFM1*-deleted cells treated with cycloheximide (Figure 5A,B). As previously found, the absence of Dfm1 did not influence degradation of lumenal CPY* (not shown) (26). Also, no significant effect on the degradation of the two membrane substrates CTL*myc (39) and HA-Pdr5* (40) was visible in the absence of Dfm1 (Figure 5A). Lack of Dfm1 did somewhat affect degradation of the membrane substrate CTG* (41) (Figure 5A). However, a very strong stabilization of the ERAD-C substrate Ste6* (42) was visible in *DFM1*-deleted cells (Figure 5B). Deletion of *DFM1* led to a similar stabilization in pulse-chase analysis as the deletion of both ubiquitin ligases, Hrd1/Der3 and Doa10 (Figure 5C), which are essential for Ste6* polyubiquitylation (11).

Discussion

Der1 (25) is the founding member of the so-called mammalian Derlins of the ER membrane, which are discussed as parts of the retrotranslocation channel for transport of misfolded ER proteins back to the cytosol for proteasomal degradation (29,30). The recent finding of a Der1 homologue, Dfm1, raised the question of its possible function in the ERAD process. First, experiments using the misfolded proteins CPY* and Sec61-2 could not show any affect of the absence of Dfm1 on the degradation of these ERAD substrates (26). Earlier studies of Sato and Hampton (2006) did not reveal a function for Dfm1 in ERAD. Genetic and overexpression studies led to the proposal that Dfm1 functions in ER homeostasis (32). However, studies with tagged Dfm1-HA$_3$ or Dfm1-CA proteins uncovered some components of the Hrd/Der machinery as interaction partners indicating a function of Dfm1 in ERAD (33).

Our study shows that a C-terminally triple HA-tagged Dfm1 protein of the ER membrane used previously for functional studies of the Dfm1 protein (18,32,33) is rapidly degraded through the ERAD pathway (Figure 1A,B). Although the misfolded domain of Dfm1-HA$_3$ should reside in the cytoplasm, classifying it as a possible ERAD-C substrate,

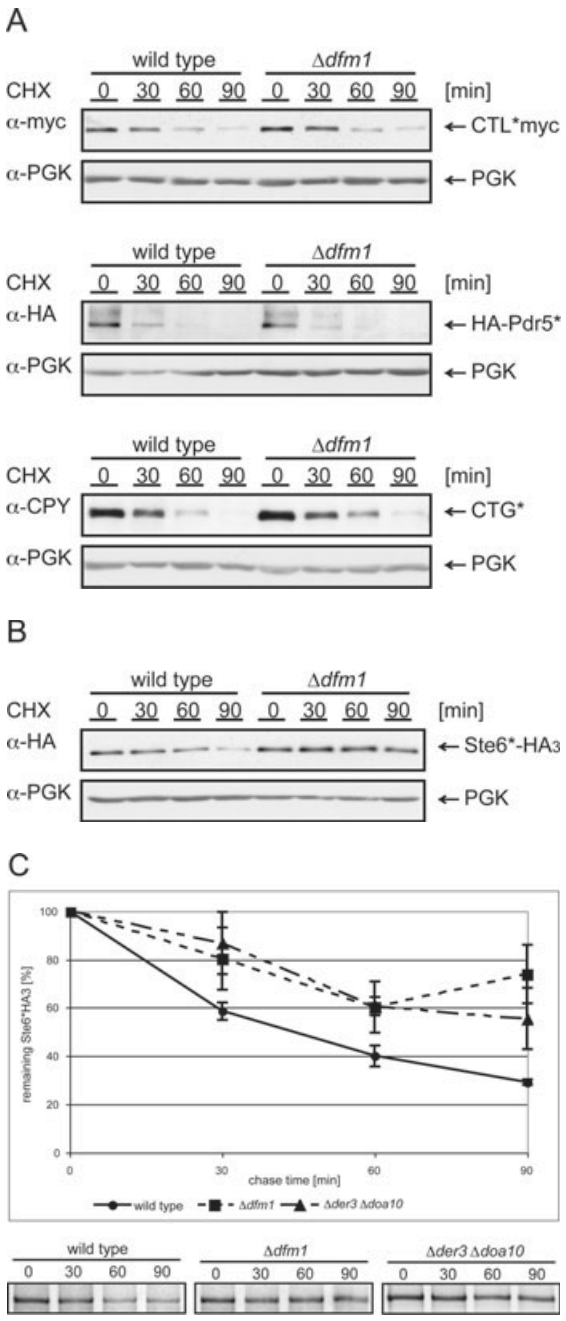


Figure 5: Degradation of the ERAD-C substrate Ste6*-HA$_3$ depends on Dfm1. A and B) Immunoblot analysis of cycloheximide chase experiments of wild-type and *DFM1*-deleted cells. Cell samples were taken at indicated time-points after the addition of cycloheximide. The ERAD substrates CTL*myc, HA-Pdr5* and CTG* were plasmid encoded and detected with myc antibodies, HA antibodies and CPY antibodies, respectively. The ERAD substrate Ste6*-HA$_3$ was detected with HA antibodies. The constitutively expressed protein PGK was detected with PGK antibodies and served as a loading control. C) Pulse-chase analysis of wild-type cells and cells deleted in *DFM1* or *HRD1/DER3* and *DOA10* expressing Ste6*-HA$_3$ from a plasmid. The graph represents three independent experiments and error bars indicate the respective SE.

degradation of Dfm1-HA$_3$ is partly dependent on Usa1, known to be a component of the ERAD-L/M pathway (27). A reason for this behaviour could be the result of an extensive disorder of the structure of Dfm1 caused by the triple haemagglutinin (HA) tag, which extends into the membrane region of the protein. Usa1 is known to be an important component of the degradation machinery of ERAD-M substrates (28). The fact that Dfm1-HA$_3$ is an ERAD substrate makes results from experiments questionable that are designed to elucidate a function of Dfm1 in ERAD with the use of tagged Dfm1 constructs.

We therefore raised an antibody against a peptide of the C-terminal tail of Dfm1 and found untagged, endogenous Dfm1 to be a stable protein (Figure 1A,B). For this reason, we re-evaluated the previous interaction studies to gain insight into Dfm1 function.

We were able to show that untagged Dfm1 interacts with the Hrd/Der ligase complex and the functional Dfm1 homologue Der1-HA (Figure 2A,B). These findings are in contrast to a previous study where the interaction between the nearly inactive triple HA-tagged Der1 species (25) and a calmodulin peptide-tagged Dfm1 protein (Dfm1-CA) was excluded (33). However, the interaction with Der1 was only visible when the linker protein Usa1 was present, which bridges Der1 to the Hrd1/Der3 ligase. In the absence of Usa1, the complex comprising Der1 and Dfm1 decomposes, while the contact of Dfm1 with the Hrd1/Der3 ligase remains stable (Figure 2A, lane 6).

We also discovered an interaction of Dfm1 with the ubiquitin ligase Doa10, which is mainly involved in the ERAD-C pathway (Figure 4). In addition, we observed a stabilization of the ERAD substrate Ste6* in a strain deleted for *DFM1* (Figure 5B,C). The degradation of Ste6*-HA$_3$ depends on both ER ligases, Doa10 and Hrd1/Der3 (11), of which Dfm1 is an interaction partner (Figures 3 and 4). Loss of Dfm1 leads to a comparable stabilization of Ste6*-HA$_3$ as is caused by the deletion of both ligases (Figure 5C). All these findings point to a functional role of Dfm1 in the ERAD pathway. As ERAD-C substrates are often dependent on both E3 ligases, Doa10 and Hrd1/Der3, to variable extents, the interaction of Dfm1 with Hrd1/Der3 might suit the purpose of guiding these substrates also to this ligase (11,12).

Schuberth and Buchberger (18) as well as Neuber et al. (19) have shown that Hrd1/Der3 looses its interaction with Cdc48 in the absence of Ubx2. Interestingly, Dfm1 is able to interact with Cdc48 despite the absence of the Cdc48 linker Ubx2 in the ER membrane (Figures 3 and 4B). Thus, this interaction is Hrd1/Der3- as well as Doa10-independent and most likely because of the conserved FxGxGQRn sequences in Dfm1, which were also found in proteins interacting with Cdc48, as are for instance Shp1/Ubx1 and Ufd1. Also genetic studies point to an interaction of Dfm1 with Cdc48 (32). Taking all results into account, at least three different Dfm1 complexes

must exist in the absence of Ubx2: one complex consisting of Dfm1 and Hrd1/Der3, one Dfm1–Doa10 complex and another complex consisting of Dfm1 and Cdc48. The ability of Dfm1 to bind Cdc48 might account for the finding of Schuberth and Buchberger (18) that a certain portion of Cdc48 remains bound to the ER in the absence of Ubx2. The Dfm1–Cdc48 complex, which does not comprise the ubiquitin ligases Hrd1/Der3 and Doa10, might have a function different from ERAD. The findings of a genetic interaction of *DFM1* and *UFE1*, encoding a t-SNARE, as well as the described physical interaction of Dfm1 with Shp1/Ubx1 and Ubx7, which have not yet been found in ERAD, might reflect such a possibility (32,33).

Materials and Methods

Yeast strains and plasmids

Media preparation, genetic and molecular biology techniques were carried out using standard methods (43,44). The strains *DER1-HA* (YWO 773), Δ*dfm1* (YWO 713) and Δ*hrd3* have been described previously (23,25,26). Crossing of strain YWO 785 (26) with wild-type (YWO 343) and strain YWO 713, respectively, and subsequent tetrad analysis led to the strains *DFM1-HA3* (YWO 1169) and Δ*dfm1 DER1-HA* (YWO 1465). The strains Δ*usa1* (YWO 1266) and *USA1-myc* (YWO 1273) were constructed using the method given by Güldener et al. (45). Crossing these strains with strain YWO 1169 and strain YWO 773, respectively, and subsequent tetrad analysis resulted in strain Δ*usa1 DFM1-HA3* (YWO 1294) and strain Δ*usa1 DER1-HA* (YWO 1486). The strain *DOA10-13myc* (KNY 111) was provided by J. F. Brodsky (38). Crossing this strain with strain YWO 713 and strain YWO 1191 (46) with subsequent tetrad analysis led to the strains *DOA10-13myc* Δ*dfm1* (YWO 1222) and *DOA10*-13myc Δ*ubx2* (YWO 1523).

The plasmids carrying the coding sequences of *ste6-166-HA*$_3$ (42) and *HA*$_3$*-HRD3* (23) were described previously.

Antibodies

Immunoprecipitation (IP) experiments were performed using monoclonal HA antibodies (clone 16B12; Covance), monoclonal c-Myc antibodies (clone 9E10; Santa Cruz Biotechnology) or polyclonal Dfm1 antibodies generated against the peptide SQRETRTFSGRGQR (aa 277-290). For the detection of Hrd1/Der3, specific polyclonal antibodies were used (9). Cdc48 and Sec61 antibodies have been provided by Thomas Sommer (MDC Berlin).

Cycloheximide chase analysis and immunoprecipitation experiments

Cycloheximide chase analysis and immunoprecipitation experiments were performed as described before (23,39,41).

Pulse-chase analysis

Composition of media and growth conditions were described previously (39). Cell samples of 30 optical density (OD) were harvested at OD_{600} 0.8–1.2, washed 3× with starvation medium and resuspended in 1 mL starvation medium. After 50-min starvation, 700 μCi of ^{35}S-labelled methionine was added to label cells for 20 min; 2.5 mL of chase medium was added and the culture was incubated for additional 30 min. Cell samples of 6 OD were taken at indicated time-points. Metabolism was stopped by incubation with 20 mM NaN_3. Cells were lysed by vortexing (5 min) with glass beads (0.4–0.6 mm) in 100 μL extraction buffer [50 mM Tris/HCl pH 7.5; 0.5 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0; 0.1 mM phenylmethylsulphonyl fluoride (PMSF)]. After addition of 1 mL extraction buffer, the suspension (without glass beads) was transferred into a new tube and membranes were isolated by centrifugation at 16 000 × ***g***. Membrane proteins were dissolved by incubation with 100 μL solubilization buffer (30 min; 50 mM Tris/HCl pH 7.5; 1% SDS) and 1 mL IP buffer (1 h; 165 mM NaCl; 5.5 mM EDTA pH 8.0; 0.1 mM PMSF). Unsolubilized proteins were removed by centrifugation at 16 000 × ***g***. IP, detection and quantification were performed as described previously (39).

Acknowledgments

We thank J. Brodsky, H. Hoshida, S. Michaelis and T. Sommer for providing strains, plasmids and antibodies. This work was funded by the EU Network of Excellence RUBICON, the Deutsche Forschungsgemeinschaft and the Landes-graduiertenförderung Baden-Württemberg.

References

1. Hiller MM, Finger A, Schweiger M, Wolf DH. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. Science 1996;273:1725–1728.
2. Kostova Z, Wolf DH. For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. EMBO J 2003;22:2309–2317.
3. Meusser B, Hirsch C, Jarosch E, Sommer T. ERAD: the long road to destruction. Nat Cell Biol 2005;7:766–772.
4. Vembar SS, Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol 2008;9:944–957.
5. Bays NW, Gardner RG, Seelig LP, Joazeiro CA, Hampton RY. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nat Cell Biol 2001;3:24–29.
6. Swanson R, Locher M, Hochstrasser M. A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes Dev 2001;15:2660–2674.
7. Kreft SG, Wang L, Hochstrasser M. Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). J Biol Chem 2006;281:4646–4653.
8. Vashist S, Ng DT. Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. J Cell Biol 2004;165:41–52.
9. Bordallo J, Plemper RK, Finger A, Wolf DH. Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol Biol Cell 1998;9:209–222.
10. Deak PM, Wolf DH. Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. J Biol Chem 2001;276:10663–10669.
11. Huyer G, Piluek WF, Fansler Z, Kreft SG, Hochstrasser M, Brodsky JL, Michaelis S. Distinct machinery is required in *Saccharomyces cerevisiae* for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. J Biol Chem 2004;279:38369–38378.
12. Gnann A, Riordan JR, Wolf DH. Cystic fibrosis transmembrane conductance regulator degradation depends on the lectins Htm1p/EDEM and the Cdc48 protein complex in yeast. Mol Biol Cell 2004;15:4125–4135.
13. Raasi S, Wolf DH. Ubiquitin receptors and ERAD: a network of pathways to the proteasome. Semin Cell Dev Biol 2007;18:780–791.
14. Braun S, Matuschewski K, Rape M, Thoms S, Jentsch S. Role of the ubiquitin-selective CDC48(UFD1/NPL4) chaperone (segregase) in ERAD of OLE1 and other substrates. EMBO J 2002;21:615–621.
15. Ye Y, Meyer HH, Rapoport TA. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature 2001;414:652–656.
16. Jarosch E, Taxis C, Volkwein C, Bordallo J, Finley D, Wolf DH, Sommer T. Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nat Cell Biol 2002;4:134–139.
17. Rabinovich E, Kerem A, Frohlich KU, Diamant N, Bar-Nun S. AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. Mol Cell Biol 2002;22:626–634.

18. Schuberth C, Buchberger A. Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. Nat Cell Biol 2005;7: 999–1006.
19. Neuber O, Jarosch E, Volkwein C, Walter J, Sommer T. Ubx2 links the Cdc48 complex to ER-associated protein degradation. Nat Cell Biol 2005;7:993–998.
20. Plemper RK, Bordallo J, Deak PM, Taxis C, Hitt R, Wolf DH. Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. J Cell Sci 1999;112:4123–4134.
21. Willer M, Forte GM, Stirling CJ. Sec61p is required for ERAD-L: genetic dissection of the translocation and ERAD-L functions of Sec61P using novel derivatives of CPY. J Biol Chem 2008;283:33883–33888.
22. Pilon M, Schekman R, Romisch K. Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. EMBO J 1997;16:4540–4548.
23. Schafer A, Wolf DH. Sec61p is part of the endoplasmic reticulum-associated degradation machinery. EMBO J 2009;28: 2874–2884.
24. Plemper RK, Böhmler S, Bordallo J, Sommer T, Wolf DH. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature 1997;388:891–895.
25. Knop M, Finger A, Braun T, Hellmuth K, Wolf DH. Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. EMBO J 1996;15:753–763.
26. Hitt R, Wolf DH. Der1p, a protein required for degradation of malfolded soluble proteins of the endoplasmic reticulum: topology and Der1-like proteins. FEMS Yeast Res 2004;4:721–729.
27. Carvalho P, Goder V, Rapoport TA. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell 2006;126:361–373.
28. Horn SC, Hanna J, Hirsch C, Volkwein C, Schutz A, Heinemann U, Sommer T, Jarosch E. Usa1 functions as a scaffold of the HRD-ubiquitin ligase. Mol Cell 2009;36:782–793.
29. Lilley BN, Ploegh HL. A membrane protein required for dislocation of misfolded proteins from the ER. Nature 2004;429:834–840.
30. Ye Y, Shibata Y, Yun C, Ron D, Rapoport TA. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature 2004;429:841–847.
31. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 2000;101:249–258.
32. Sato BK, Hampton RY. Yeast Derlin Dfm1 interacts with Cdc48 and functions in ER homeostasis. Yeast 2006;23:1053–1064.
33. Goder V, Carvalho P, Rapoport TA. The ER-associated degradation component Der1p and its homolog Dfm1p are contained in complexes with distinct cofactors of the ATPase Cdc48p. FEBS Lett 2008;582:1575–1580.
34. Saito Y, Yamanushi T, Oka T, Nakano A. Identification of SEC12, SED4, truncated SEC16, and EKS1/HRD3 as multicopy suppressors of ts mutants of Sar1 GTPase. J Biochem 1999;125:130–137.
35. Gardner RG, Swarbrick GM, Bays NW, Cronin SR, Wilhovsky S, Seelig L, Kim C, Hampton RY. Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. J Cell Biol 2000;151:69–82.
36. Gauss R, Sommer T, Jarosch E. The Hrd1p ligase complex forms a linchpin between ER-lumenal substrate selection and Cdc48p recruitment. EMBO J 2006;25:1827–1835.
37. Denic V, Quan EM, Weissman JS. A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. Cell 2006;126:349–359.
38. Nakatsukasa K, Huyer G, Michaelis S, Brodsky JL. Dissecting the ER-associated degradation of a misfolded polytopic membrane protein. Cell 2008;132:101–112.
39. Kohlmann S, Schafer A, Wolf DH. Ubiquitin ligase Hul5 is required for fragment-specific substrate degradation in endoplasmic reticulum-associated degradation. J Biol Chem 2008;283:16374–16383.
40. Plemper RK, Egner R, Kuchler K, Wolf DH. Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. J Biol Chem 1998;273:32848–32856.
41. Taxis C, Hitt R, Park SH, Deak PM, Kostova Z, Wolf DH. Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. J Biol Chem 2003;278:35903–35913.
42. Loayza D, Tam A, Schmidt WK, Michaelis S. Ste6p mutants defective in exit from the endoplasmic reticulum (ER) reveal aspects of an ER quality control pathway in *Saccharomyces cerevisiae*. Mol Biol Cell 1998;9:2767–2784.
43. Guthrie C, Fink GR (eds). Guide to Yeast Genetics and Molecular Biology in Methods in Enzymology, Academic Press. San Diego: 1991.
44. Ausubel FM, Kingston RE, Seidman FG, Struhl K, Moore DD, Brent R, Smith FA. Current Protocols in Molecular Biology. New York: Greene; 1992.
45. Güldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 1996;24:2519–2524.
46. Alberts SM, Sonntag C, Schafer A, Wolf DH. Ubx4 modulates cdc48 activity and influences degradation of misfolded proteins of the endoplasmic reticulum. J Biol Chem 2009;284:16082–16089.

Appendix 4

ERAD without canonical ER ubiquitin ligases: A novel role for Ubr1

Alexandra Stolz, Stefanie Besser, Heike Hottmann and Dieter H. Wolf
manuscript in review process (Nat. Cell Biol.)

ERAD without canonical ER ubiquitin ligases: A novel role for Ubr1

Alexandra Stolz[(1)], Stefanie Besser[(1)], Heike Hottmann and Dieter H. Wolf

Institut für Biochemie, Universität Stuttgart

Pfaffenwaldring 55, 70565 Stuttgart, Germany

[(1)]: These authors contributed equally to the study.

To whom correspondence should be addressed:

Dieter H. Wolf

Institut für Biochemie, Universität Stuttgart

Pfaffenwaldring 55, 70565 Stuttgart, Germany

dieter.wolf@ibc.uni-stuttgart.de

Phone: +49 711 68564390

Fax: +49 711 68564392

Statistic errors, mutations and different stresses lead to the formation of misfolded proteins. In humans the accumulation of misfolded proteins results in deadly diseases as are Alzheimer's disease, Parkinson's disease and many others [1, 2]. Elimination systems for misfolded proteins, including endoplasmic reticulum (ER) associated protein degradation (ERAD), are therefore essential for cell survival. Permanently misfolded proteins of the secretory pathway are recognized in the ER and retrotranslocated back to the cytosol. Subsequent polyubiquitylation by ER membrane localized ubiquitin ligases enables their segregation from the ER membrane by the AAA type ATPase Cdc48 leading to proteasomal degradation [3-5]. The model eukaryote *Saccharomyces cerevisiae* contains two canonical ER membrane localized ubiquitin ligases, Hrd1/Der3 and Doa10, involved in elimination of most ERAD substrates [3-5]. The ERAD-L substrate CTG* and the ERAD-C substrate Ste6*, are still partially degraded in the absence of both ligases. Here we report that the cytosolic ubiquitin ligase Ubr1 with the help of the Ssa Hsp70 family serves as a back up to maintain proteasomal degradation of ERAD substrates in the absence of the two ER ligases. As a unique case thus far CTG* is degraded independently of the Cdc48 machinery in this novel pathway.

The eukaryotic model organism yeast has turned out to be an excellent tool for the elucidation of ERAD. After discovery of the basic principles of ubiquitin triggered ERAD [6], many missing mechanistic gaps have been filled by then step-by-step giving a more complete picture of the process today. The ubiquitin ligases of the ER membrane have been discovered to be central for the ERAD process. The yeast *Saccharomyces cerevisiae* contains two ER ligases located in the membrane, which have been found to be responsible for directing most yet known ERAD

substrates to proteasomal degradation: Hrd1/Der3, the orthologue of mammalian HRD1 and gp78, and Doa10 sharing homology with human TEB4 [7-13]. Both ligases have been proposed to be involved in the formation of an ER membrane embedded channel for the retrotranslocation process of misfolded protein species [11, 14]. Proteins exposing a misfolded domain in the ER lumen (ERAD-L substrates) or the ER membrane (ERAD-M substrates) are preferential targets of Hrd1/Der3, whereas membrane proteins exposing a misfolded domain in the cytosol (ERAD-C substrates) are mainly targets of Doa10 [15-17].

Previous studies on a variety of ERAD substrates had revealed that in many cases deletion of one of the two canonical ER membrane embedded ubiquitin ligases, Hrd1/Der3 and Doa10, did not lead to complete block of degradation of the substrate tested. Even in the few cases, where the fate of a substrate was analyzed in strains missing both ligases, no complete cessation of degradation could be observed [16, 18]. We therefore reasoned that a back up system might exist, which is responsible for the final clean up of misfolded ER proteins. We selected two membrane substrates, the ERAD-L substrate CTG* and the ERAD-C substrate Ste6*, which have previously been used as model substrates for studies on ERAD. Ste6* is a transmembrane protein exposing its truncated and misfolded C-terminal domain into the cytosol [16]. CTG* consists of a misfolded CPY* domain with four glycans in the lumen of the ER, a transmembrane domain and the green fluorescent protein on the cytosolic face of the ER [19]. According to our assumption of an existing back up system, both substrates were still ubiquitylated in the absence of Hrd1/Der3 and Doa10 (Fig. 1a, S1) and degraded (Fig. 1b and c). We and others recently showed the involvement of the cytosolic E3 ubiquitin ligase Ubr1, previously found to be the enzyme polyubiquitylating N-end rule substrates [20], in the elimination of cytoplasmically located misfolded CPY* fusion proteins [21, 22]. The participation of Ubr1 in the cytoplasmic clean up machinery of misfolded proteins

prompted us to ask if the enzyme could also take over a function in ERAD when the two canonical ER ligases Hrd1/Der3 and Doa10 are absent.

While single deletion of *UBR1* had no effect on the degradation of CTG* (Fig. S2), degradation of CTG* and Ste6* was completely blocked when the cytosolic ligase was deleted in addition to *HRD1* and *DOA10* (Fig. 1b and c). As tested for Ste6*, this can be traced back to the activity of Ubr1: Introduction of plasmid encoded, catalytically inactive Ubr1 (Ubr1 RING mutant) could not complement the *UBR1* deletion, whereas re-introduction of wild type Ubr1 could rescue the degradation defect (Fig. 1d). Clearly, Ubr1 can take over when the two canonical ER ligases are absent and sustain proteasomal degradation of the ERAD substrate (Fig. 1e). To ensure that the involvement of Ubr1 is not attributed to a mislocalization of CTG* to the cytosol due to the deletion of *HRD1* and *DOA10*, glycosylation of the CPY* moiety was tested. As expected, CTG* was still glycosylated in *HRD1 DOA10* double deleted cells, proving proper insertion into the ER membrane (Fig. S3). Clearly, Ubr1 has a back up function when the canonical ER membrane localized E3 ligases are missing. Obviously, the substrates can be retro-translocated from the ER to the cytoplasmic degradation system without the two polytopic ER ligases Hrd1/Der3 and Doa10.

Where tested, all ERAD pathways converge at the cytosolic side of the ER membrane and degradation is dependent on the activity of the essential AAA type ATPase Cdc48. Cdc48 in complex with the receptor proteins Ufd1 and Npl4 is thought to segregate the ERAD substrates from the ER membrane and deliver them to the proteasome [3-5, 23, 24]. We tested the involvement of Cdc48 in the degradation of CTG* and Ste6* in the absence of the two canonical ER ligases Hrd1/Der3 and Doa10 using a mutant conditionally defective in Cdc48 activity [25]. When

analyzing wild type cells and cells expressing the temperature sensitive Cdc48 mutant under restrictive conditions the degradation kinetics of the ERAD-L substrate CTG* did not differ (Fig. 2a). This is the first time a membrane embedded ERAD substrate was found to be degraded independently of the Cdc48 machinery. This is in contrast to the ERAD-C substrate Ste6*: Its degradation is still strongly dependent on a functional Cdc48 complex in the absence of Hrd1/Der3 and Doa10 (Fig. 2b).

We had recently discovered, that the Der1 homologue Dfm1 is required for degradation of Ste6* [26]. Dfm1 is known to carry two SHP-boxes at the cytosolic side of the ER membrane, which are able to interact with Cdc48 [27]. This prompted us to investigate the function of Dfm1 in the novel Ubr1 catalyzed ERAD pathway. CTG* degradation was not dependent on Dfm1 (Fig. 2c), while Ste6* was stabilized in the absence of Dfm1 (Fig. 2d). A possible function of Dfm1 in Ubr1 dependent ERAD of Ste6* could reside in the recruitment of Cdc48 to the substrate. To test this model we performed an interaction study where we precipitated Ste6* in the presence and absence of Dfm1. In *HRD1 DOA10* deleted cells, Ste6* specifically interacts with Ubr1, Dfm1 and Cdc48 (Fig. 2e). However, the interaction between Ste6* and Cdc48 remained stable in the absence of Dfm1 (Fig. 2e) excluding the possibility that Dfm1 functions as a Cdc48 recruiting factor in Ubr1 dependent ERAD.

Due to the finding that proteasomal CTG* degradation in the absence of the canonical ER ligases Hrd1/Der3 and Doa10 occurs independently of Cdc48, the question arose which machine provides the energy to extract the protein from the ER membrane. In many cases proteasomal degradation of cytosolic proteins is aided by the action of the Ssa family of Hsp70 chaperones [28, 29]. This chaperone family contains four members, Ssa1 to Ssa4, exhibiting overlapping activities [5]. We therefore tested if this Hsp70 class is involved in degradation of CTG* in cells lacking the

canonical ER ligases Hrd1/Der3 and Doa10. For these studies we used a mutant devoid of the three Hsp70 chaperones Ssa2, Ssa3 as well as Ssa4 but containing a temperature sensitive allele of *SSA1* (*ssa1-45*) [19]. Under restrictive conditions CTG* is rapidly degraded in Ssa1 wild type cells while degradation of the protein is considerably slowed down in *ssa1-45* mutant cells (Fig. 3a). Interestingly, also degradation of Ste6* requires the activity of the Hsp70 Ssa1 (Fig. 3b).
As a model one might propose that the cytoplasmic Hsp70 acts as a molecular ratchet pulling the misfolded proteins out and away from the ER membrane. Such a mechanism has been shown for the Hsp70 of the ER, Kar2, during the import of proteins into the ER lumen [30].

The novel Ubr1 and Ssa1 dependent but partly Cdc48 independent cytosolic back up system for degradation of ERAD-L and ERAD-C substrates discovered here demonstrates the enormous plasticity and flexibility of the ERAD system of cells. Crucial questions remain to be answered: (i) How do the substrates cross the membrane without the ER membrane located ubiquitin ligases? (ii) Why is the Cdc48 machine required for degradation of one substrate (Ste6*) but not the other (CTG*)? (iii) How does the Hsp70 Ssa1 actually work in this process? Is it in addition to a pulling function also required for ubiquitylation of the substrates as is known for catabolite degradation of fructose-1,6-bisphosphatase [31]? Is it involved in some additional delivery process of the substrate to the proteasome? These are some questions which guide the way of future research.

Note: Supplementary Information is available.

ACKNOWLEDGEMENTS

We thank Thomas Sommer (MDC Berlin) and Alexander Varshavsky (Caltech Pasadena) for providing antibodies and plasmids. Janina Preissler and Konrad Otte helped with the generation of strains. This work was supported by the Deutsche Forschungsgemeinschaft (DFG, Bonn), the EU network of excellence RUBICON and the Landesgraduiertenförderung Baden-Württemberg.

AUTHOR CONTRIBUTIONS

Dieter H. Wolf was the supervisor of this work. Heike Hottmann helped with experimental procedures. Stefanie Besser performed the experiments resulting in Fig. 1A, 1C, 2A and 2C, Alexandra Stolz the experiments resulting in Fig. 1A, 1B, 1E, 2B, 2D and 3A,B. Dieter H. Wolf and Alexandra Stolz jointly wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

FIGURE LEGENDS

Figure 1 ERAD substrates are degraded in an Ubr1 dependent manner in the absence of cononical ER ligases

(**a**) Cell extracts of indicated yeast strains deleted in *PRC1* were subjected to an ubiquitylation assay. After seperation into cytosolic (C) and membrane (M) fraction and immunopricipitation with GFP antibodies, ubiquitylated CTG* species were detected with Ub antibodies. All samples of input and IP were loaded on the same gel, however additional lanes were removed for a better overview indicated by grey lines. (**b-e**) Indicated strains expressing plasmid encoded Ste6* or

CTG* are analyzed for their degradation process by pulse chase analysis. Data show the mean of three to six independent experiments. Error bars indicate the standard error of the mean (SEM). (**d**) Tripple E3 deleted cells (YWO 1944) are additionally transformed either with plasmids encoding for an inactive, RING mutated Ubr1 version (◆), wildtype Ubr1 (▲) or with an empty vector (■). (**e**) Cells lacking Hrd1/Der3, Doa10 and the multidrug transporter Pdr5 were stowed with MG132 (■) or equal amounts of DMSO (◆). MG132 partially blocks proteasomal degradation.

Figure 2 Ubr1 dependent degradation of Ste6* depends on Cdc48, while CTG* is degraded independently of Cdc48 in the absence of the canonical ligases Hrd1/Der3 and Doa10

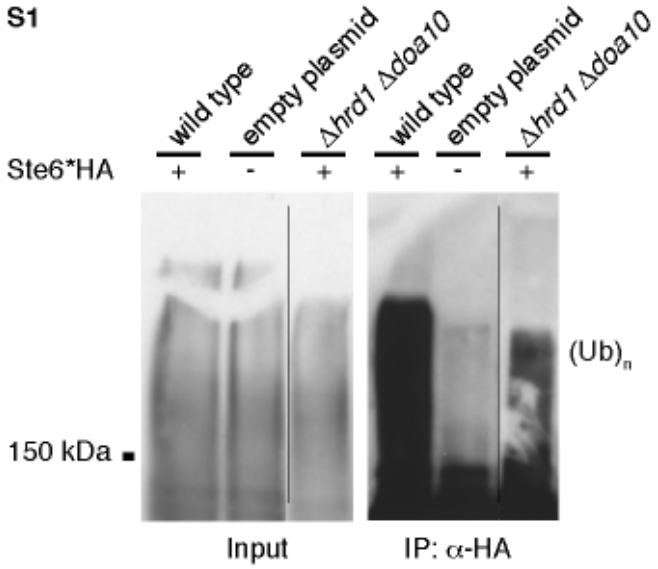
(**a-d**) Indicated strains expressing plasmid encoded Ste6* or CTG* are analyzed for their degradation process by pulse chase analysis. Data show the mean of three independent experiments. Error bars indicate the standard error of the mean (SEM). (**e**) Ste6* is precipitated with HA antibodies. Co-precipition of Ubr1, Dfm1 and Cdc48 was analysed with the help of specific antibodies.

Figure 3 Ssa1 is involved in Ubr1 dependent degradation of CTG* and Ste6*

Indicated strains expressing plasmid encoded CTG* (**a**) or Ste6* (**b**) are analyzed in respect of their degradation process by pulse chase analysis. Data show the mean of three independent experiments. Error bars indicate the standard error of the mean (SEM).

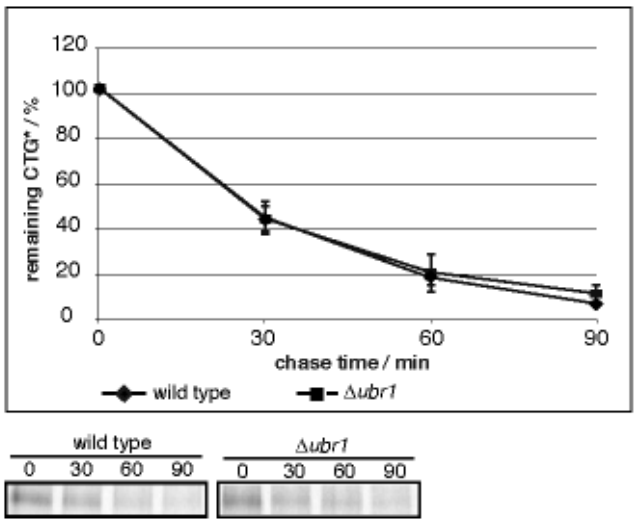
Supporting Online Material

Supplementary figures



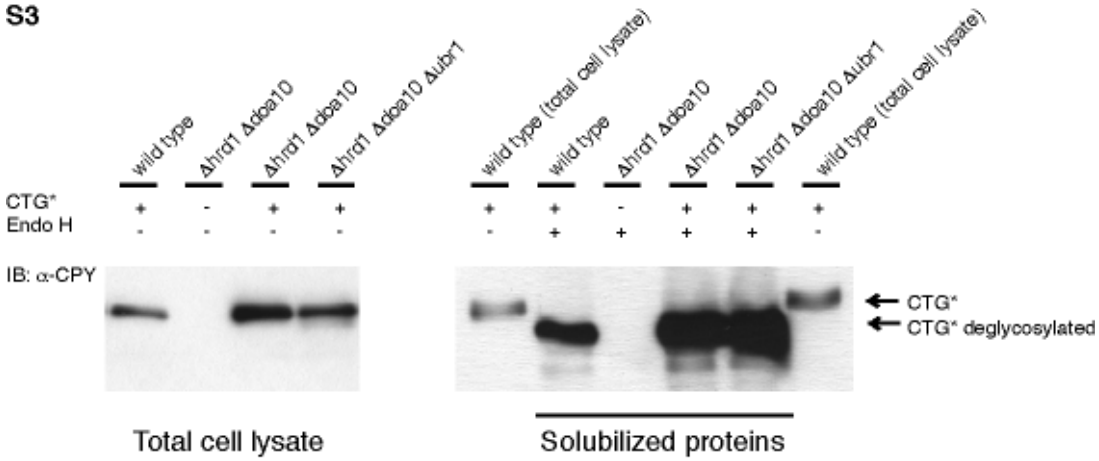
The ERAD substrate Ste6*is still ubiquitylated in the absence of Hrd1/Der3 and Doa10
Cell extracts of indicated yeast strains were subjected to an ubiquitylation assay. Ubiquitylated species of Ste6* in the membrane fraction were detected with ubiquitin antibodies. All samples of the input and the IP were loaded on the same gel, however additional lanes were removed for a better overview indicated by grey lines.

S2



CTG* is degraded independently of Ubr1 in Hrd1/Der3 and Doa10 wild type cells
Wild type (YWO 636) and *UBR1* deleted cells expressing plasmid encoded CTG* are analyzed for their degradation process by pulse chase analysis. Data show the mean of three independent experiments. Error bars indicate the standard error of the mean (SEM).

S3



CTG* is properly inserted into the ER membrane and glycosylated in the absence of Hrd1/Der3 and Doa10
Cells of indicated strains were lysed and total cell lysate was seperated into cytosol and membrane fraction. After removal of the cytosolic fraction, membrane proteins were solubilized with the help of digitonine and treated with EndoH to remove attached glycan trees.

Table S1 Yeast strains (W303 background) used in this study

Name	Genotype	Reference
YWO 343	prc1-1	ref. as wild type
YWO 636/1580	prc1Δ::LEU2	ref. as wild type
YWO 713	prc1-1 dfm1Δ::HIS3MX6	Described in Stolz et al., 2010 [26]
YWO 1428	prc1-1 ubr1Δ::HIS5^{+}	Described in Eisele et al., 2008 [21]
YWO 1527	prc1-1 hrd1Δ::HIS3 doa10Δ::kanMX	Described in Stolz et al., 2010 [26]
YWO 1528	prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::kanMX	this study
YWO 1648	prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::kanMX dfm1Δ::HIS3MX6	this study
YWO 1731	prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::kanMX ubr1Δ::HIS5^{+}	this study
YWO 1741	prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::kanMX pdr5Δ::TRP1	this study
YWO 1803	prc1Δ::kanMX hrd1Δ::HIS5^{+} doa10Δ::LEU2 cdc48^{T413R} (ts)	this study
YWO 1944	prc1-1 hrd1Δ::HIS3 dao10Δ::loxP ubr1Δ::loxP	this study
YWO 1956	prc1Δ::LEU2 hrd1Δ::loxP doa10Δ::NAT ssa2Δ::loxP ssa3Δ1−1126::loxP ssa4Δ::loxP	this study
YWO 1957	prc1Δ::LEU2 hrd1Δ::loxP doa10Δ::NAT ssa1-45 (ts) ssa2Δ::loxP ssa3Δ1−1126::loxP ssa4Δ::loxP	this study

Table S2 Plasmids used in this study

	promoter	insert	other	Reference
pMA1 / PWO 0621	PRC1	CTG*	URA3; CEN	described in Taxis et al., 2003 [19]
pSM1911	PGK	ste6-166::HA	URA3; 2µ	described in Huyer et al., 2004 [16]
pNTFLAG-UBR1	ADH1	FLAG-UBR1	LEU	described in Xia and Varshavsky, 1999 [20]
pNTFLAG-UBR1-MR1	ADH1	FLAG-UBR1-RM	LEU	described in Xie and Varshavsky, 1999 [20]

METHODS

Yeast Strains and plasmids

Media preparation, genetic and molecular biology techniques were carried out using standard methods.

Yeast strains used in this study contain the genetic background *ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1ocre can1-100*. The strains YWO 343 and YWO 636, which contain a mutated and a deleted allele of *PRC1*, respectively, are referred as wild type. Deletion strains were made by homologues recombination [32]. All strains and plasmids used in this study are summarized in the supplemented table 1 and 2, respectively.

Antibodies

CPY (rabbit; Rockland; 200-401-135) and GFP (rabbit; Molecular Probes; A6455) antibodies were used to precipitate CTG* in pulse chase experiments and ubiquitylation assays, respectively. Ste6*HA was precipitated with the help of HA 16B12 antibodies (mouse; Covance; MMS-101P). Thomas Sommer (MDC Berlin) provided Sec61 and Cdc48 antibodies. FLAG-Ubr1, ubiquitin and CTG* were detected on immunoblots with FLAG (mouse; SIGMA; F3165), Ub P4G7 (mouse; Covance; MMS-258R) and CPY (mouse; Molecular Probes; A6428) antibodies, respectively. Production of the Dfm1 peptide antibody was described previously [26].

Ubiquitylation assay

100 OD of cells in logarithmic growth phase were harvested and washed with cold water. After centrifugation cells were resuspended in ice-cold sodium azide (20 mM sodium azide; 1 mM phenylmethanesulfonyl fluoride (PMSF); 20 mM N-ethylmaleimide (NEM)) and icubated on ice

for 10 min to stop ATP dependent metabolic pathways. Subsequently, cells were washed with and resuspended in 0.3 ml and 0.5 ml sorbitol buffer (0.7 M sorbitol; 50 mM Tris/HCl pH 7.5; 1 mM PMSF; 1 µg/µl Pepstatin A; 20 mM NEM), respectively. After addition of 0.3 ml glass beads (0.4 – 0.6 mm) cells were lysed by 10 min of vortexing at 8 °C. 1ml sorbitol buffer was added, samples were mixed and centrifuged (10 min; 500 xg; 4 °C) to remove large cell fragments and unbroken cells. The supernatant was subjected to ultracentrifugation (35 min; 100,000 xg; 4 °C) to separate cytosolic from membrane fractions. The supernatant of the ultracentrifugation containing the cytosolic protein pool was stowed with 600 µl buffer C (50 mM Tris/HCl pH 7.5; 1 % TritonX-100; 5 mM EDTA; 165 mM NaCl; 0.1 % SDS; 2 µg/µl Aprotinin; 1x complete inhibitor mix (Roche)) and indicated antibodies. The pellet containing the membrane fractions were washed with 1 ml sorbitol buffer, suspension was centrifuged (20 min; 100,000 xg; 4° C) and the supernatant discarded. Membranes were resuspended in 150 µl solubilization buffer (50 mM Tris/HCl pH 7.5; 1 % SDS) and incubated on a rotating incubator for 45 min on 8 °C. After addition of 1,350 µl IP buffer (165 mM NaCl; 50 mM Tris/HCl pH 7.5; 1.1 % TritonX-100; 5.5 mM EDTA; 1x complete inhibitor mix (Roche); 2 µg/µl Aprotinin) and another 15 min incubation, insolubilized components were removed by ultracentrifugation (35 min; 100,000 xg; 4 °C). Indicated antibodies were added to the supernatant, containing solubilized membrane proteins, and samples were incubated for 1 h. 80 µl of blocked (10 % BSA), 6.25 % proteinA sepharose were added and precipitation took place over night. Precipitates were washed thrice with IP buffer and proteins were removed from the sepharose by addition of 50 µl urea buffer (200 mM Tris/HCl pH 6.8; 8 M urea; 0.1 M EDTA; 5 % SDS (w/v); 0.05 % bromphenol blue; 1 % 2-mercaptoethanol).

Pulse chase analysis

For pulse chase analysis of the substrate CTG* 10 OD of cells in logarithmic growth phase were harvested and washed thrice with labelling media (0.17 % yeast nitrogen base w/o ammonium sulphate and w/o amino acids; 0.1 % D-glucose; 0.002 % adenine; 0.002 % uracil; 0.002 %L-tryptophan; 0.002 % L-histidine; 0.003 % L-arginine; 0.003 % L-tyrosine; 0.003 % L-lysine; 0.003 % L-leucine; 0.005 % L-glutamic acid; 0.005 % L-aspartic acid; 0.015 % L-valine; 0.02 % L-threonine; 0.04 % L-serine). Cells were resuspended in 1 ml labelling media and incubated for 50 min at 30 °C except for temperature sensitive strains, which were incubated at 25 °C. Cells were labelled for 30 min with 200 µCi ^{35}S-methionine at 30 °C. Temperature sensitive strains were shifted to 37 °C at this step. In case of proteasomal inhibition MG132 solved in DMSO (end concentration 150 µM) or an equivalent amount of pure DMSO were added at this time point and subsequently every 30 min. After labelling 1 ml chase media (labelling media plus 2 mg/ml BSA and 6 mg/ml methionine) was added and 2.5 OD cells were harvested in 50 µl ice-cold 110 % trichloroacetic acid at indicated time points. Cells were washed with ice-cold acetone and subsequently dried at 60 °C for 30 sec. 100 µl glass beads (∅ 0.4 – 0.6 mm) and 100 µl BB1 buffer (50 mM Tris/HCl pH 7.5; 6 M urea; 1 % SDS; 1 mM ethylenediaminetetraacetic acid (EDTA)) were added and lysis occurred via alternately rounds of 1 min voretexing and 1 min incubation at 95 °C for six times. 1 ml IP buffer (50 mM Tris/HCl pH 7.5; 190 mM NaCl; 1.25 % TritonX-100 (v/v); 6 mM EDTA) and complete Inhibitor Cocktail (Roche) were added and insoluble properties were removed by centrifugation (V_{max}; 15 min). 950 µl of the supernatant were incubated with CPY antibodies (rabbit; Rockland) for 1 h, afterwards 80 µl of 6.25 % proteinA were added and precipitation took place over night at 4 °C. Precipitates were washed thrice with IP buffer. Proteins were released by addition of 50 µl urea buffer (200 mM Tris/HCl pH 6.8; 8 M urea; 0.1 M EDTA; 5 % SDS (w/v); 0.05 % bromphenol blue; 1 % 2-

mercaptoethanol). Samples were analyzed after SDS PAGE with the help of Phosphoimager and ImageQuaNT (Molecular Dynamics).

Pulse chase analysis for the substrate Ste6* have been described earlier [26]. In case of temperature sensitive strains, cells were grown at 25 °C and shifted to 37 °C one hour before the first sample was taken.

Immunoprecipitation

Immunoprecipitation of Ste6*HA was performed as described earlier in Kohlmann et al., 2008 [33], except for using 4µl of HA antibodies instead of myc antibodies for precipitation.

References:

1. Rutishauser, J. & Spiess, M. Endoplasmic reticulum storage diseases. *Swiss Med Wkly* **132**, 211-222 (2002).
2. Aridor, M. & Hannan, L.A. Traffic jams II: an update of diseases of intracellular transport. *Traffic* **3**, 781-790 (2002).
3. Vembar, S.S. & Brodsky, J.L. One step at a time: endoplasmic reticulum-associated degradation. *Nat Rev Mol Cell Biol* **9**, 944-957 (2008).
4. Buchberger, A., Bukau, B. & Sommer, T. Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. *Mol Cell* **40**, 238-252 (2010).
5. Stolz, A. & Wolf, D.H. Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell. *Biochim Biophys Acta* **1803**, 694-705 (2010).
6. Hiller, M.M., Finger, A., Schweiger, M. & Wolf, D.H. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* **273**, 1725-1728 (1996).
7. Hampton, R.Y., Gardner, R.G. & Rine, J. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* **7**, 2029-2044 (1996).
8. Bordallo, J., Plemper, R.K., Finger, A. & Wolf, D.H. Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* **9**, 209-222 (1998).
9. Bays, N.W., Gardner, R.G., Seelig, L.P., Joazeiro, C.A. & Hampton, R.Y. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nat Cell Biol* **3**, 24-29 (2001).
10. Deak, P.M. & Wolf, D.H. Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. *J Biol Chem* **276**, 10663-10669 (2001).
11. Swanson, R., Locher, M. & Hochstrasser, M. A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. *Genes Dev* **15**, 2660-2674 (2001).
12. Kreft, S.G., Wang, L. & Hochstrasser, M. Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). *J Biol Chem* **281**, 4646-4653 (2006).
13. Kostova, Z., Tsai, Y.C. & Weissman, A.M. Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. *Semin Cell Dev Biol* **18**, 770-779 (2007).
14. Carvalho, P., Stanley, A.M. & Rapoport, T.A. Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. *Cell* **143**, 579-591 (2010).
15. Vashist, S. & Ng, D.T. Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *J Cell Biol* **165**, 41-52 (2004).
16. Huyer, G. *et al.* Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. *J Biol Chem* **279**, 38369-38378 (2004).
17. Carvalho, P., Goder, V. & Rapoport, T.A. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* **126**, 361-373 (2006).
18. Gnann, A., Riordan, J.R. & Wolf, D.H. Cystic fibrosis transmembrane conductance regulator degradation depends on the lectins Htm1p/EDEM and the Cdc48 protein complex in yeast. *Mol Biol Cell* **15**, 4125-4135 (2004).

19. Taxis, C. *et al.* Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. *J Biol Chem* **278**, 35903-35913 (2003).
20. Xia, Z. *et al.* Substrate-binding sites of UBR1, the ubiquitin ligase of the N-end rule pathway. *J Biol Chem* **283**, 24011-24028 (2008).
21. Eisele, F. & Wolf, D.H. Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1. *FEBS Lett* **582**, 4143-4146 (2008).
22. Heck, J.W., Cheung, S.K. & Hampton, R.Y. Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1. *Proc Natl Acad Sci U S A* **107**, 1106-1111 (2010).
23. Ye, Y., Meyer, H.H. & Rapoport, T.A. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* **414**, 652-656 (2001).
24. Jarosch, E. *et al.* Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat Cell Biol* **4**, 134-139 (2002).
25. Barbin, L., Eisele, F., Santt, O. & Wolf, D.H. The Cdc48-Ufd1-Npl4 complex is central in ubiquitin-proteasome triggered catabolite degradation of fructose-1,6-bisphosphatase. *Biochem Biophys Res Commun* **394**, 335-341 (2010).
26. Stolz, A., Schweizer, R.S., Schafer, A. & Wolf, D.H. Dfm1 forms distinct complexes with Cdc48 and the ER ubiquitin ligases and is required for ERAD. *Traffic* **11**, 1363-1369 (2010).
27. Sato, B.K. & Hampton, R.Y. Yeast Derlin Dfm1 interacts with Cdc48 and functions in ER homeostasis. *Yeast* **23**, 1053-1064 (2006).
28. McClellan, A.J., Scott, M.D. & Frydman, J. Folding and quality control of the VHL tumor suppressor proceed through distinct chaperone pathways. *Cell* **121**, 739-748 (2005).
29. Park, S.H. *et al.* The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin-proteasome system. *Mol Biol Cell* **18**, 153-165 (2007).
30. Matlack, K.E., Misselwitz, B., Plath, K. & Rapoport, T.A. BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane. *Cell* **97**, 553-564 (1999).
31. Juretschke, J., Menssen, R., Sickmann, A. & Wolf, D.H. The Hsp70 chaperone Ssa1 is essential for catabolite induced degradation of the gluconeogenic enzyme fructose-1,6-bisphosphatase. *Biochem Biophys Res Commun* **397**, 447-452 (2010).
32. Gueldener, U., Heinisch, J., Koehler, G.J., Voss, D. & Hegemann, J.H. A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res* **30**, e23 (2002).
33. Kohlmann, S., Schafer, A. & Wolf, D.H. Ubiquitin ligase Hul5 is required for fragment-specific substrate degradation in endoplasmic reticulum-associated degradation. *J Biol Chem* **283**, 16374-16383 (2008).

Figure 1

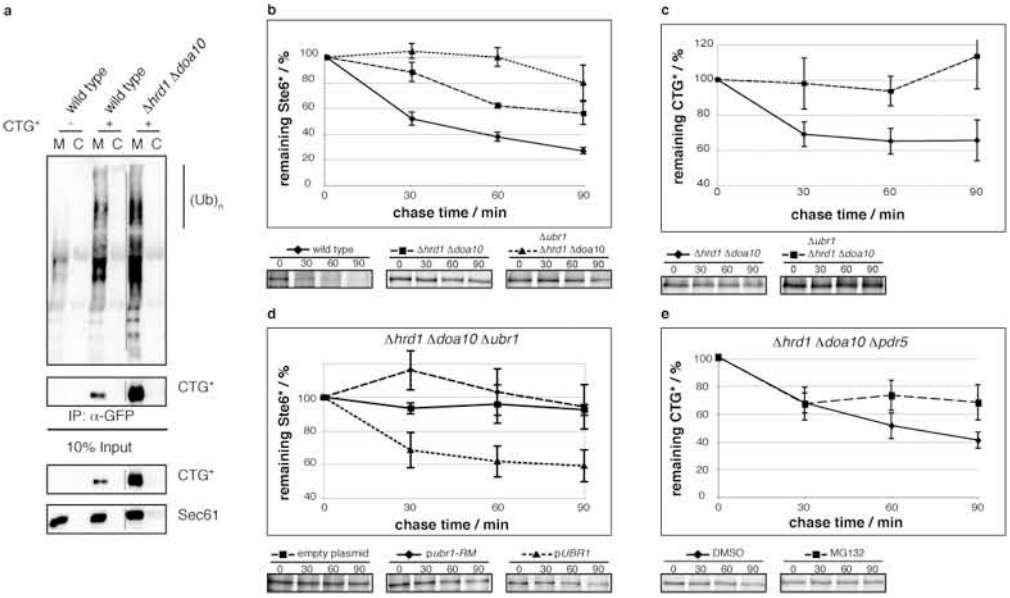
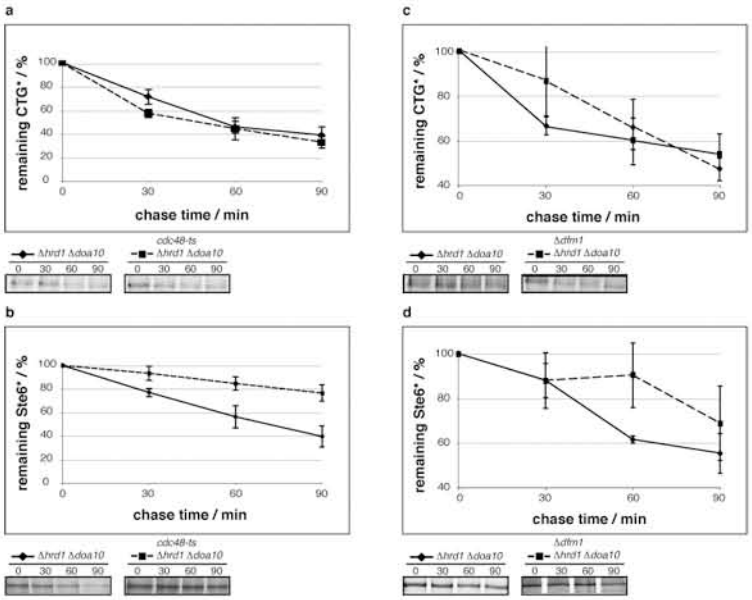


Figure 2



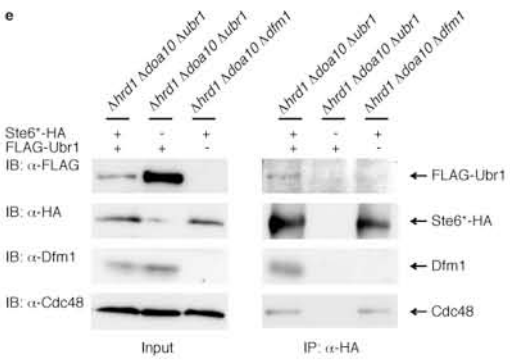
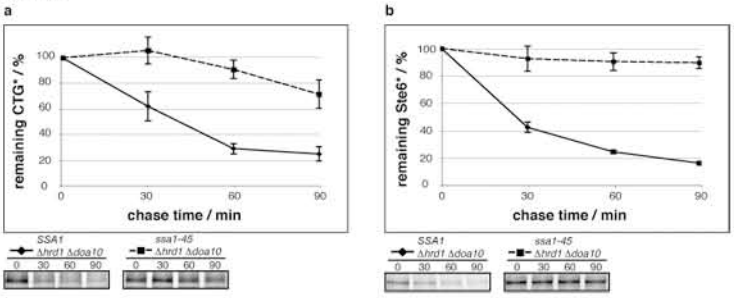


Figure 3


a
remaining CTG* / %
100
80
60
40
20
0
0
30
60
90
chase time / min
SSA1
Δhrd1 Δdoa10
ssa1-45
Δhrd1 Δdoa10
0 30 60 90
0 30 60 90
b
remaining Ste6* / %
100
80
60
40
20
0
0
30
60
90
chase time / min
SSA1
Δhrd1 Δdoa10
ssa1-45
Δhrd1 Δdoa10
0 30 60 90
0 30 60 90

Appendix 5

Endoplasmic reticulum associated protein degradation: A chaperone assisted journey to hell

Alexandra Stolz and Dieter H. Wolf
Biochimica et Biophysica Acta 1803 (2010) 694–705

Biochimica et Biophysica Acta 1803 (2010) 694–705

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Endoplasmic reticulum associated protein degradation: A chaperone assisted journey to hell

Alexandra Stolz, Dieter H. Wolf *

Institut für Biochemie, Universität Stuttgart, Germany

ARTICLE INFO

Article history:
Received 19 November 2009
Received in revised form 11 February 2010
Accepted 18 February 2010
Available online 25 February 2010

Keywords:
Chaperone
ERAD
Lectin
Protein disulfide isomerase
Hsp70
Hsp40

ABSTRACT

Recognition and elimination of misfolded proteins are essential cellular processes. More than thirty percent of the cellular proteins are proteins of the secretory pathway. They fold in the lumen or membrane of the endoplasmic reticulum from where they are sorted to their site of action. The folding process, as well as any refolding after cell stress, depends on chaperone activity. In case proteins are unable to acquire their native conformation, chaperones with different substrate specificity and activity guide them to elimination. For most misfolded proteins of the endoplasmic reticulum this requires retro-translocation to the cytosol and polyubiquitylation of the misfolded protein by an endoplasmic reticulum associated machinery. Thereafter ubiquitylated proteins are guided to the proteasome for degradation. This review summarizes our up to date knowledge of chaperone classes and chaperone function in endoplasmic reticulum associated degradation of protein waste.

Life depends on the proper function of every protein in a cell. The function relies on the three dimensional conformation, which the amino acid chain of the protein acquires during and after its synthesis by the ribosome. Reaching the state of an energy minimum in the landscape of folding intermediates seems rather easy in diluted physiological solutions. However, conditions in cells are quite different. The cellular environment is crowded with a multitude of macromolecules. Folding mistakes and trapped intermediates caused by genetic mutations or cellular stresses occur constantly. To minimize these mistakes, helper proteins – chaperones – bind and accompany the folding event. The cell possesses a highly active quality control system that recognizes the folding state of proteins. The system scans the folding process, detects terminally misfolded proteins and specifically eliminates them by proteolysis. Failure of the protein quality control system finally leads to the formation of protein aggregates that in turn can cause severe diseases like Alzheimer-, Parkinson- or Creutzfeldt–Jakob disease. Chaperones have been thought to be solely responsible for the folding process of newly synthesized proteins or the refolding of proteins that had been partially unfolded by stresses, e.g. heat ("heat shock proteins") [1–3]. Nowadays chaperones are known to accompany proteins in almost all periods of their life.

This review focuses on chaperones that accompany the folding and degradation process of proteins of the secretory pathway. Proteins of the secretory pathway represent around one third of the proteins synthesized in eukaryotic cells. They integrate into the membrane of the endoplasmic reticulum (ER) or pass this membrane barrier to reach the ER lumen, where they are folded and modified. Import of proteins into the ER can occur in a ribosome-coupled fashion (co-translationally) or in an uncoupled fashion (post-translationally). Upon folding failures chaperones keep proteins soluble and guide them to the ubiquitin proteasome system (UPS), the major cellular protein elimination machinery in eukaryotic cells [4–7]. As the degradation machinery of misfolded ER proteins has turned out to be the UPS, located in the cytosol [8,9], it was obvious that these false ER-imported proteins have to be recognized and retro-translocated across the ER membrane back into the cytosol, where they are polyubiquitylated and degraded [9–11]. The ER associated degradation (ERAD) machinery retro-translocates the misfolded proteins by a yet not well-defined mechanism. Subsequently, they are modified with polyubiquitin chains and by this designated for proteasomal degradation. For misfolded ER lumenal proteins the retro-translocation complex (RTC) comprises the Hrd-Der ubiquitin ligase complex of the ER membrane, connected to the Derlin Der1 and the Sec61 translocon. On the cytoplasmic side of the ER a Cdc48-Ufd1-Npl4 segregation machinery, linked to the RTC, delivers the polyubiquitylated proteins to the proteasome for degradation. Misfolded membrane proteins with lesions in the cytosol find their way to the proteasome via the trimeric Cdc48 complex after polyubiquitylation by the ER membrane integrated ubiquitin ligase

* Corresponding author.
E-mail address: dieter.wolf@ibc.uni-stuttgart.de (D.H. Wolf).

0167-4889/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2010.02.005

Doa10 (Fig. 1) [12–16]. Overload of misfolded proteins leads to the induction of the unfolded protein response (UPR), a mechanism, which upregulates proteins necessary for dealing with this unfavorable situation. These proteins include chaperones and components of the ERAD machinery [17,18]. In the case of such an overload, misfolded proteins may aggregate. Chaperones show the capacity to re-solubilize these aggregates [5,19,20]. Thus, they not only "chaperone" protein folding but also act as "scavengers" for intracellular protein waste. Polyubiquitylation prior to proteasomal degradation is not required for elimination of a mutated secretory polypeptide, pro-α-factor [21]. It is still not clear what distinguishes this misfolded polypeptide from other misfolded ER proteins analyzed, which all require a fully active polyubiquitylation system for degradation. Chaperones have been found in all folding compartments of the eukaryotic cell: the cytoplasm, the endoplasmic reticulum and mitochondria. In addition, the nucleus and peroxisomes are sites of chaperone activity [22].

1. The cellular chaperone equipment

Chaperones belong to a protein class of great variety. They show remarkable differences concerning their mechanistic behaviour, the need of co-factors and ATP. Chaperone sizes start around 25 kDa, but can reach over 100 kDa. This chapter introduces the different chaperone families and describes their modes of action.

1.1. The Hsp70 family

Hsp70s are most probably the best-studied class of chaperones. They have a molecular mass of approximately 70 kDa and are highly

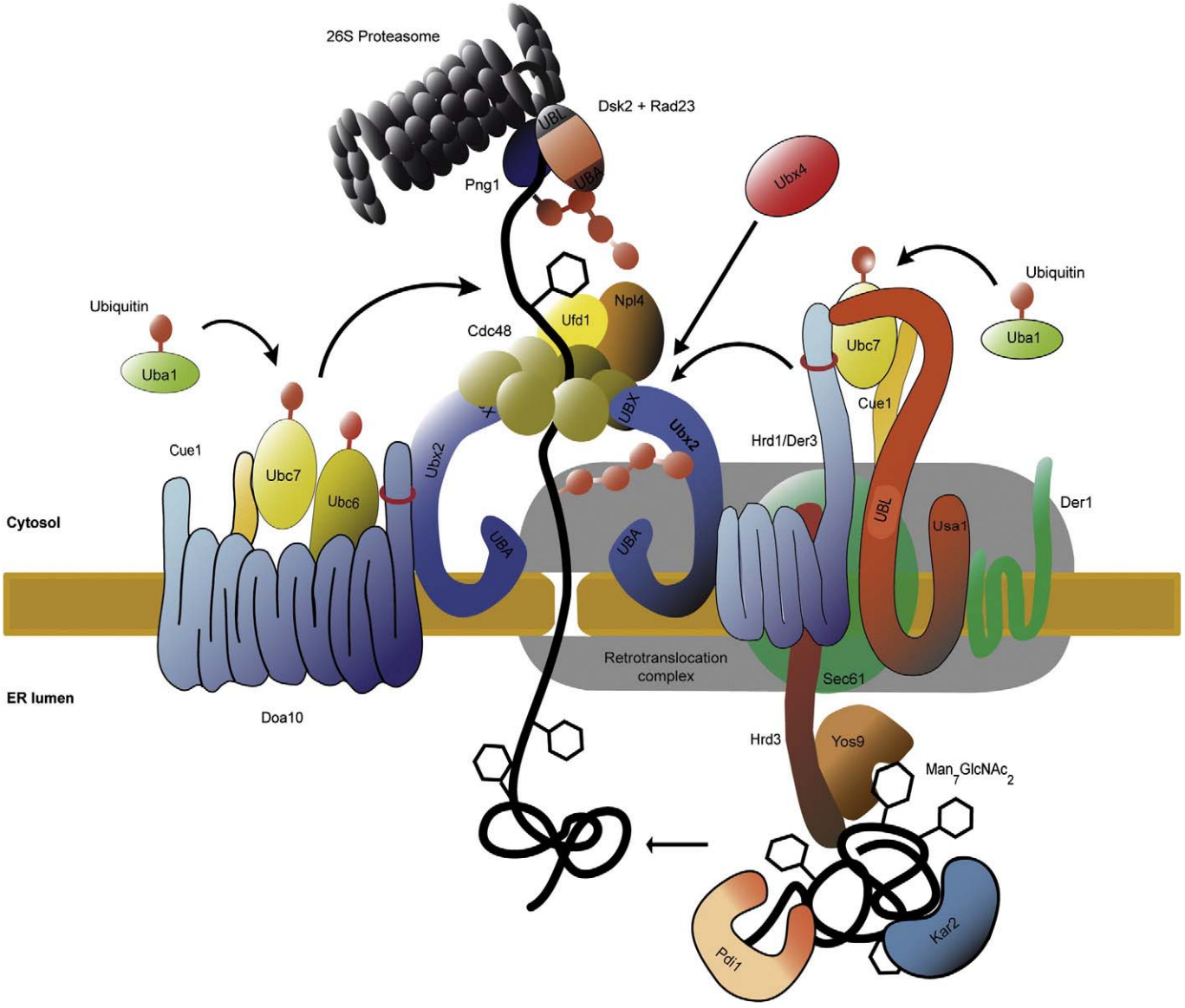


Fig. 1. The ERAD machinery for delivery, ubiquitylation and degradation of misfolded ERAD-L and ERAD-C proteins. ERAD-L and ERAD-C substrates can be distinguished by their requirement of two different ER machineries. After carbohydrate trimming (Fig. 3), Pdi1 action and binding to Hrd3 as well as Yos9 scanning, soluble ERAD-L substrates are delivered to a retro-translocation complex (RTC) composed of different ER membrane proteins including Sec61 [89], Hrd3 itself, Usa1, Der1 and the ubiquitin ligase Hrd1/Der3. During retro-translocation the misfolded proteins are polyubiquitylated on the cytosolic side of the ER via the Hrd1/Der3 ligase [12,13,16]. The following steps are similar for ERAD-L and ERAD-C: the trimeric AAA-ATPase complex composed of Cdc48-Ufd1-Npl4, which is linked to the ER membrane by Ubx2 [12,13,16,183,184] pulls the polyubiquitylated protein from the ER membrane and, via the ubiquitin receptors Dsk2 and Rad23 [185] delivers the polyubiquitylated protein to the proteasome for degradation. Ubx4 may release the Cdc48 complex from the ER membrane [186], Png1 [187] cleaves off the carbohydrate chains prior to degradation. For ERAD-C substrates the RTC is in part less well-defined but simpler: after or during retro-translocation from the ER membrane the ERAD-C substrates are polyubiquitylated by virtue of a second ubiquitin ligase, Doa10. The pathway merges with the ERAD-L pathway at the Cdc48-Ufd1-Npl4 machinery.

conserved among all organisms. Members of this family act e.g. in protein folding, in preventing protein aggregation and in membrane translocation. Classical chaperone activity of Hsp70s is ATP dependent and includes cycles of protein binding and ATP hydrolysis, affected by co-chaperones (Hsp40s) and nucleotide exchange factors (NEFs) (Fig. 2). Hsp70s typically contain three domains: an N-terminal ATPase domain, a central peptide binding domain and a C-terminal lid [23–26]. In an ATP bound state Hsp70s show low affinity for their substrates and cycles of substrate association and dissociation are fast. During ATP hydrolysis the tertiary structure changes and a 'closing' of the C-terminal lid is stimulated. Thereby substrates get tightly attached to the chaperone [25,27–29]. In the ADP bound state, the affinity of the peptide binding domain for substrates strongly increases [30,31]. Due to a subsequent nucleotide exchange of ADP and ATP the lid opens and substrates are released. Rates of ATP hydrolysis and nucleotide exchange are very low, but can be stimulated by binding of the substrate, co-chaperones and NEFs, respectively [30,32–35]. However, the precise mechanism of exchange events could not be ascertained till now.

1.2. The Hsp40 family

Hsp40s, acting as co-chaperones, induce the ATPase activity of Hsp70s. All Hsp40s contain a J-domain and are therefore often referred to as J-proteins. J-domains were named after a conserved motif in the *E. coli* protein DnaJ [36]. Hsp40s have a conserved HPD tripeptide in the J-domain that is necessary for the stimulation of Hsp70 ATPase activity [37–39]. One classification divides members of the Hsp40 family into three subgroups [40]. While Type III Hsp40s just contain the J-domain, additional domains were found in Hsp40s of type I and II. Type I Hsp40s, like DnaJ, possess the J-domain and a glycine/phenylalanine-rich domain (G/F) that seems to function as a spacer and is required for efficient stimulation. In addition they contain a cysteine-rich Zn^{2+} binding domain (CR) which functions as a peptide binding region of non-native substrates [41,42]. Type II Hsp40s lack this CR-domain but contain the G/F-domain. Even though Hsp40s and Hsp70s mainly bind the same types of peptides, there is specificity for some peptides. It is not yet clear whether Hsp70s replace Hsp40s at the substrate, or whether there exists a 'handover' mechanism between them, which would postulate different substrate binding sites and simultaneous binding of the Hsp40 and Hsp70 to the substrate. The second mechanism indicates active substrate guidance by Hsp40s to their Hsp70 partner [43].

1.3. Nucleotide exchange factors

Nucleotide exchange factors help to promote Hsp70 triggered folding cycles by accelerating the exchange of ADP against ATP. NEFs do not feature a special domain but are highly diverse in structure and mechanism. Bacterial GrpE, the first identified NEF, forms dimers, interacts with the ATPase domain of the Hsp70 homologue DnaK and can stimulate the exchange of both, ADP and ATP [44,45]. The BAG domain, which was found in BAG-1 (*B*cl-2 *a*ssociated athanogene; mammals) and Snl1 (yeast), shows a different mechanism of stimulation. BAG-1 discriminates between ATP and ADP exchange and the efficiency of nucleotide exchange is dependent on Hsp70/BAG-1 stoichiometry [46]. Another unique mechanism was found for HspBP1 (*H*sp *B*inding *P*rotein 1). This mechanism does not comprise the conserved conformational change in the ATPase domain caused by the binding of GrpE or BAG-1 [47,48]. Quite recently Hsp70 (Lhs1) and Hsp70 related Hsp110 chaperones were shown to act as NEFs on other Hsp70s [49–51]. These examples make clear, how diverse the mechanisms of nucleotide exchange can be and thus how many types of regulation and specification are possible.

1.4. The Hsp90 family

Hsp90 chaperone activity is linked to ATP hydrolysis. Therefore, these chaperones contain an ATP binding domain in the N-terminal region of the protein [52]. A single ATP binding domain is not sufficient for ATP binding but requires transient dimerization of two N-terminal domains. A helical motif was identified in the C-terminal region of Hsp90, which enable dimerization [53,54]. Substrate binding is attributed to a large hydrophobic patch in the middle region of Hsp90 [55]. Prodromou et al. [54] proposed a molecular 'clamp' mechanism. Clients are bound to the middle region of C-terminally dimerized Hsp90s. ATP binding to the N-terminal ATP binding domains induces closing of a 'lid' above the clients and

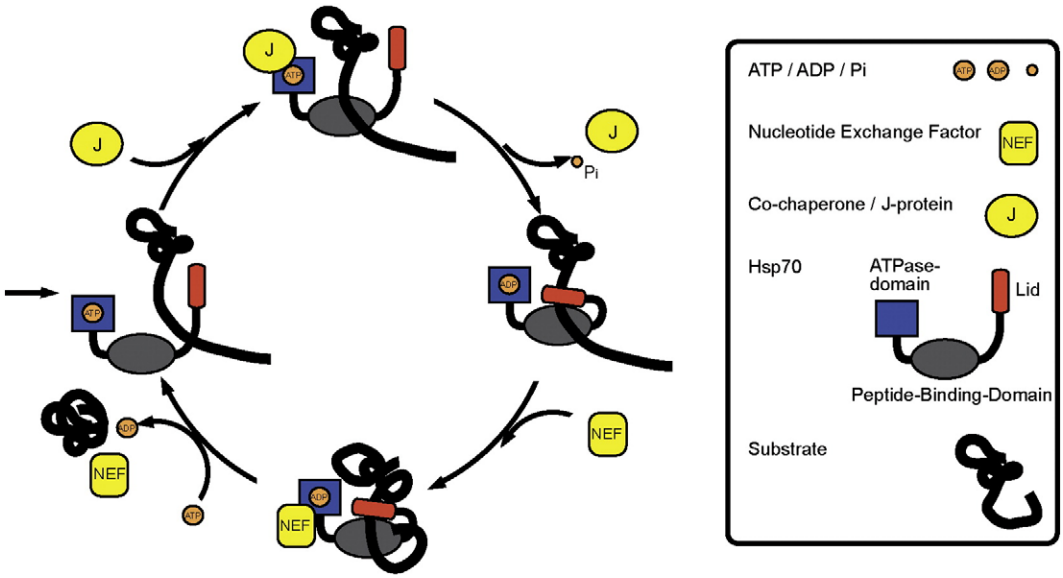


Fig. 2. The folding cycle of Hsp70 chaperones. Hsp70s in an ATP bound state exhibit low affinity for their substrates and cycles of substrate association and dissociation occur fast. Interaction of an appropriate J-protein induces the ATPase activity of the Hsp70. Some Hsp40s are able to actively deliver substrates to their Hsp70 partner. During ATP hydrolysis the tertiary structure of the Hsp 70 changes, causing a 'closing' of the C-terminal lid. By this substrates get tightly attached to the chaperone. Subsequently a nucleotide exchange factor initiates the exchange of ADP against ATP, resulting in the opening of the lid and the release of the substrate. Please note that this figure is a schematic representation of a working model. It is not yet clear in which precise order substrates and co-factors bind and leave the Hsp70.

ATPase activity. After ATP hydrolysis ADP dissociates and the lid opens again. However, ATPase activity is low and regulated via co-chaperones including Hsp70s and Hsp40s [52,56–60]. As for Hsp70s, interaction partners influence whether Hsp90s promote folding or proteolytic degradation of clients.

1.5. Small heat shock proteins

Small heat shock proteins (sHsp) represent a class of chaperones, which bind substrates rather unspecifically and prevent proteins from irreversible aggregation. Clients are partially denatured proteins in an unstable state [61,62], which are bound until general cell conditions are suitable for proper folding or final degradation. Chaperone activity is considered to be energy-independent, however substrate affinity binding sites are potentially affected by ATP binding [63]. sHsp-substrate interaction is quite stable and disassembly may require interaction of ATP dependent chaperones like Hsp70s or Hsp90s, which facilitate substrate refolding [64,65]. Small heat shock proteins contain a conserved α-crystallin domain [66–68] and are enriched in β-strands. Crystallization revealed sHsps to form dimers and oligomers. Dynamic disassembly of these complexes seems to be required for effective chaperon function [69]; for review see [20].

1.6. Thiol oxidoreductases

Thiol oxidoreductases contain a characteristic CXXC motif that is located in the active site of the enzyme and are referred to as members of the PDI (*p*rotein *d*isulfide *i*somerase) family. They take part in disulfide bond formation, protein folding and ERAD [70,71]. The precise sequence and number of the CXXC motifs and protein structures varies among family members. Because the environment in the ER is highly optimized for oxidative protein folding, PDI family members are mainly ER localized. Five members in yeast and over 19 in human have been identified so far. At least some of them were shown to possess chaperone-like functions [72–74]. Except for single species, oxidative activities of the PDI family members are very similar [75,76]. It is not yet clear if and how substrate specificity is achieved.

1.7. Lectin-like chaperones

Lectin-like chaperones of the ER predominantly bind substrates with N-glycan modification. Dependent on the glycan structure they either facilitate efficient folding and ER retention of immature glycoproteins or identify aberrant proteins, prevent aggregation and facilitate destruction [77–79]. Members of this class contain a sugar binding domain like a mannosidase-like domain or a mannosidase receptor homology (MRH) domain [80–82]. However, they are able to bind at least some non-glycosylated ERAD substrates, too, suggesting a chaperone-like activity [77,83].

2. Chaperone function in ERAD

With respect to the localization of the misfolded domain in a protein two major pathways have been defined, ERAD-L and ERAD-C, which are used when the misfolded protein domain resides in the ER lumen and the cytosol, respectively [84]. A third, not yet precisely defined ERAD-M (membrane) pathway was proposed [85]. It was a surprising discovery in the beginning of ERAD research, that misfolded, fully glycosylated ER lumenal proteins are finally eliminated in the cytoplasm [9]. Specific misfolded substrates have been discovered or designed as useful tools for the elucidation of how ERAD in general and ERAD-L specifically works. The mutated vacuolar/lysosomal yeast enzyme carboxypeptidase yscY (CPY*) has become a model for an ER lumenal misfolded protein. The enzyme is mutated two amino acids distant from the active site serine (G255R). This glycine residue is highly conserved among all serine peptidases and the mutation leads to misfolding and loss of activity [86]. CPY* is imported into the ER lumen, fully glycosylated, recognized as being misfolded, retrograde transported out of the ER in glycosylated form, fully ubiquitylated and degraded by the proteasome [9].

2.1. Kar2 keeps proteins soluble during import into the ER

During post-translational import of CPY* into the ER via the Sec61 translocation channel, the protein is N-glycosylated at its four glycosylation sites. The spatial gap between the point of modification by the oligosaccharyl transferase (OST) complex and the Sec61 import channel was identified to be 15 amino acid residues [87]. Glycosylation at an engineered fifth glycosylation site at the very C-terminus of CPY* proved complete import of the misfolded protein into the ER lumen [88,89]. The first chaperone that contacts post-translationally imported proteins like CPY* in yeast is the Hsp70 Kar2, the orthologue of mammalian BiP. Kar2 binds short hydrophobic patches of incoming proteins and is tethered to the ER membrane by virtue of Sec63, an integral membrane partner of Sec61. In a concerted action of Kar2 (BiP) and the J-domain of Sec63, nascent proteins are imported. In this process Kar2 (BiP) works as a Brownian ratchet. Interaction with Kar2 prevents the polypeptide chain to slide back through the Sec61 channel into the cytosol, by this enabling only forward movement [90]. In general Kar2 (BiP) supports folding of post- and co-translationally imported proteins and keeps them soluble. At a certain stage along the folding process the quality control system marks proteins like CPY* as misfolded. At this point Kar2 (BiP) becomes an important component of the machinery that directs misfolded substrates towards the degradation pathway. Mutations in *KAR2* significantly slow down CPY* degradation [91,92]. In addition, degradation of the mutated pro-α-factor was shown to be Kar2 (BiP) dependent [70,93]. Failure to assemble with the immunoglobulin (Ig) heavy chain makes the Ig light chain an ERAD substrate. Dissociation of BiP from unassembled Ig light chains correlates with the degradation kinetics of the substrate, indicating the importance of this ER lumenal Hsp70 chaperone for the ERAD process [94].

2.2. J-proteins enhancing the ATPase activity of Kar2 vary in protein import and ERAD

In addition to the ER membrane embedded Sec63, two well studied J-proteins, Jem1 and Scj1, reside in the ER lumen and enhance the ATPase activity of Kar2. While Sec63 was shown to exhibit a rather minor role during ERAD of CPY* [91,92], Jem1 and Scj1 seem to act as redundant enhancers of the ATPase activity of Kar2. Deletion of both Hsp40s resulted in prolonged degradation and led to aggregation of the soluble ERAD substrate CPY* [91,95]. Another J-protein called Erj5 was recently identified. Deletion of *ERJ5* was found to increase growth defects associated with deletion of *JEM1* or *SCJ1*. Cells with a triple deletion (*Δjem1Δscj1Δerj5*) show a constitutive induction of the unfolded protein response (UPR) and increased levels of *KAR2* mRNA, compared to cells deleted only in *SCJ1* and *JEM1*. This points to an Erj5 requirement for the maintenance of an optimal folding environment in the ER [96]. However, direct involvement of Erj5 in ERAD has not been observed yet.

2.3. Kar2 and its NEF Lhs1 show coupled ATPase cycles

Lhs1 (*l*umenal *h*sp *s*eventy) could be identified in the ER lumen and is conserved in higher eukaryotic cells (human: Grp170) [97]. Deletion of *LHS1* is not essential but shows synthetic lethality with *KAR2* mutations or deletion of *IRE1*, the kinase triggering the UPR [98]. The protein acts as a nucleotide exchange factor on Kar2 and together

with an appropriate J-protein increases Kar2 ATPase activity up to 30-fold [50]. The structure of Lhs1 contains a nucleotide binding domain (NBD), which is essential for NEF activity. Besides NEF activity, Lhs1 was shown to act as a holdase and to prevent protein aggregation in vitro, independent of its nucleotide binding domain [49]. ATPase cycles of Kar2 and Lhs1 seem to be coupled for efficient chaperone function. In addition to Lhs1, Sil1 ("suppressor of the Δ*ire1* Δ*lhs1* double mutant number 1") has been identified as a nucleotide exchange factor of Kar2 (BiP). Sil1 is a GrpE-like non-essential protein, which shows some functional redundancy with Lhs1. Lack of both proteins causes synthetic lethality [99]. However, function of Lhs1 and Sil1 is not completely redundant. *LHS1* mutated cells are defective in protein translocation into the ER whereas cells containing a *SIL1* mutation are not [50,100].

2.4. Mutation of the chaperone Rot1 leads to rapid protein degradation via ERAD

Rot1 was identified as a chaperone responsible for folding of nascent proteins. It is an ER localized glycosylated type I membrane protein [101,102]. Initially, the essential gene *ROT1* was reported to encode a protein that is required for 1,6-β-glucan synthesis [103,104]. From the current point of view this finding might be attributed to a secondary effect. The viable mutant *rot1-2* triggers UPR and shows synthetic lethality with *kar2-1* indicating a partially redundant function. Rot1 and Kar2 interact in vivo [102]. In vitro Rot1 is able to prevent protein aggregation and specifically interacts with nascent proteins. In *rot1-2* mutated cells several proteins are unable to reach their native conformation. As a consequence they are rapidly degraded via ERAD [105]. Based on this finding general participation of Rot1 in ERAD can be excluded. However, involvement in the degradation of specific proteins is still possible.

2.5. The glycan structure of glycoproteins reflects their folding status

Upon entrance of secretory proteins into the ER, two modifications are introduced during the folding process: carbohydrates are added and disulfide bonds between cysteine residues are formed. As mentioned above, most proteins become N-glycosylated upon their entry into the ER. A glycan consisting of Glc_3-Man_9-$GlcNAc_2$ is linked to asparagine residues within the consensus sequence Asn-X-Ser/Thr of the imported proteins. A trimmed N-glycosyl structure (Fig. 3) has turned out to be an important attribute of misfolded proteins. It acts as an accelerator of degradation. While the three terminal glucose residues are cleaved off by two glucosidases, glucosidase I and II, the protein folds into its native structure. After cleavage of the third glucose residue, properly folded proteins are ready for travelling to the Golgi apparatus for further delivery to their final destination [106]. However, if the last glucose residue is cleaved off before the protein has folded properly, mannose units are removed from the carbohydrate structure in order to mark it as misfolded [107,108].

2.6. The calnexin/calreticulin cycle promotes protein folding

In higher eukaryotic cells chaperones are part of a cycle for efficient protein folding [106]. After the removal of the two terminal glucose residues from the glycan structure, the molecular chaperones calnexin and calreticulin interact with the Glc_1-Man_9-$GlcNAc_2$ charged protein and promote its folding [109] (Fig. 4). After dissociation, the remaining glucose residue can be removed by glucosidase II and properly folded proteins are able to exit the ER. In the case of incomplete folding, the folding sensor UDP-glucose/glycoprotein glucosyltransferase (UGGT) reloads proteins with a terminal glucose and thereby initiates another cycle of folding [110–112]. Proteins that do not fold within several times of chaperone cycling are released from the cycle due to mannosidase action and become determined to ERAD [107,108,113–116] (Figs. 3 and 4).

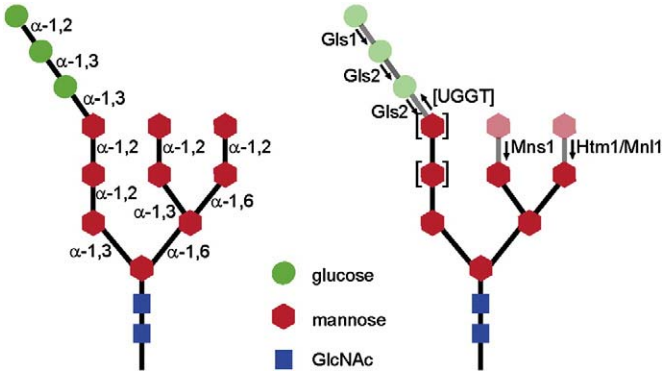


Fig. 3. During the folding process of glycoproteins in the ER N-linked glycan structures are processed. In yeast the three terminal glucose residues of the initial Glc_3-Man_9-$GlcNAc_2$ structure are removed by glucosidase I (Gls1) and glucosidase II (Gls2). Prolonged ER lumenal retention of the protein due to not yet completed folding recruits an α-1,2-mannosidase (Mns1) and an α-mannosidase-like protein (Htm1/Mnl1) to remove two mannose residues resulting in a Man_7-$GlcNAc_2$ glycan structure. This structure, containing a terminal 1,6-linked mannose, is an attribute of misfolded glycoproteins. In mammalian cells the orthologues of Htm1/Mnl1, EDEM1-3 are able to trim oligosaccharides to Man_6-$GlcNAc_2$ or Man_5-$GlcNAc_2$ glycan structures.

In *Saccharomyces cerevisiae* only the calnexin/calreticulin orthologue Cne1 has been found. An UGGT othologue remained elusive [117,118]. Cne1 exhibits efficient chaperone activity for glycosylated and non-glycosylated substrates [118,119]. It was found to participate in the quality control of some under-glycosylated variants of CPY*. Here the mannosidase Htm1/Mnl1 and Cne1 play antagonistic roles in targeting the substrate for degradation: loss of Cne1 accelerates degradation, loss of Htm1/Mnl1 stabilizes the substrate [120]. A crosstalk of Cne1 with Kar2 (BiP) and Pdi1 (*p*rotein *d*isulfide *i*somerase) under heat shock conditions was discovered [93,121,122]. Cne1 was shown to interact with the Pdi1 homologue Mpd1, enhancing its reductive activity, while at the same time the chaperone activity of Cne1 is inhibited [123]. These interactions are conserved in higher eukaryotes. In mammalian cells an interaction between PDI family members and calnexin could be shown [124]. Due to the relatively slow action of α-mannosidase I (Mns1) and Htm1/Mnl1, a time window for the folding of ER-imported glycoproteins is kept open. The mannose timer hypothesis was put forward to exemplify this behaviour [125]. However, not all ERAD substrates are glycosylated. Thus, there has to be an additional mechanism, which distinguishes unglycosylated proteins in the process of folding from terminally misfolded proteins.

2.7. α-Mannosidases mark glycoproteins as misfolded

In yeast the α-1,2-mannosidase Mns1 removes a mannose residue of the middle branch of the Man_9-$GlcNAc_2$ carbohydrate tree, resulting in a terminal 1,3-linked mannose (Fig. 3). This leads to an acceleration of ERAD of N-glycosylated substrates [126]. Lectin-like proteins sharing a mannosidase-homology domain (MHD) had been discovered in the ER lumen of yeast and mammalian cells: Htm1/Mnl1 and EDEM (*ER D*egradation *E*nhancing α-*M*annosidase-like protein) [127–129]. They considerably enhance the degradation of N-glycosylated misfolded proteins. While the MHD domain of Htm1/Mnl1 is necessary for substrate binding in yeast, mammalian EDEM1 was shown to bind misfolded proteins independently of the MHD domain and therefore independently of their glycan structure. The MHD domain of EDEM1 was required for the interaction with the ubiquitin ligase adaptor protein SEL1, the equivalent of the yeast Hrd3 protein [77]. Recently, Htm1/Mnl1 and the EDEM proteins have

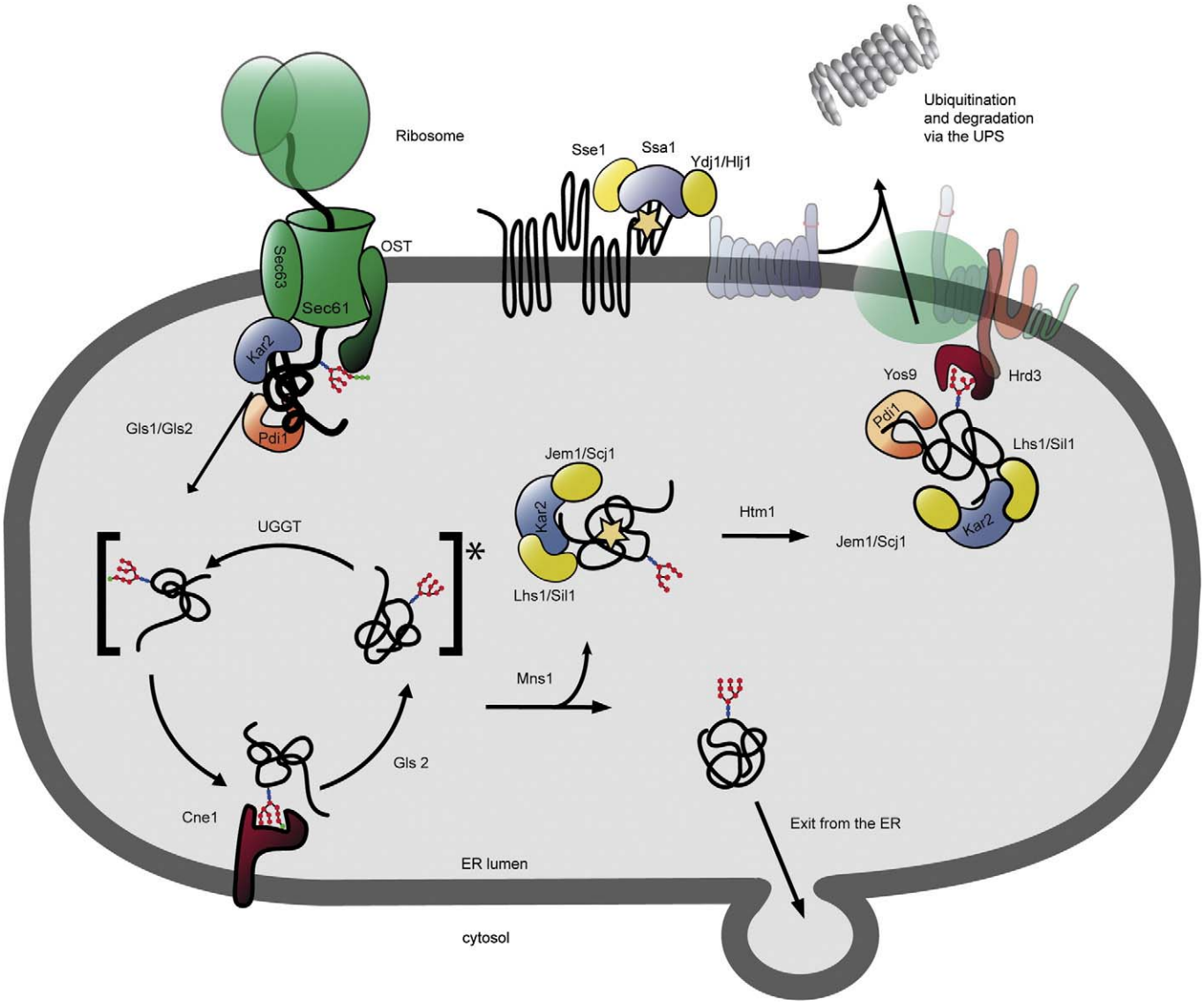


Fig. 4. ER import, folding cycle and quality control of a secretory protein. Nascent ER lumenal proteins enter the ER via the Sec61 translocon. The oligosaccharyl transferase (OST) complex charges the protein with a core oligosaccharide (Glc_3-Man_9-$GlcNAc_2$) at appropriate consensus sequences. Together with its co-chaperone Sec63 the Hsp70 Kar2 drives import of post-translationally imported proteins and in general promotes the folding of the imported proteins. Protein disulfide isomerase (Pdi1) facilitates the formation of intramolecular disulfide bonds. Yeast Cne1, an orthologue of mammalian calnexin, may support Kar2 in the folding process. In mammalian cells (denoted by asterisk) calnexin and UDP-glucose/glycoprotein glucosyltransferase (UGGT) support a folding cycle. Turns of deglucosylation and reloading of the glycan structure with a single glucose residue provide further time for the folding process. Properly folded proteins exit from the ER and travel to the Golgi apparatus for further distribution to their final secretory destination. Terminally misfolded proteins become substrates of the α-mannosidases Mns1 and Htm1/Mnl1, which further trim the glycan structures (Fig. 3). By this the protein becomes marked as being misfolded and is recognized by the lectin Yos9. Prior to retro-translocation into the cytoplasm Pdi1 reduces disulfide bonds present in a protein. In the case of ER membrane proteins exposing misfolded cytosolic domains, the cytosolic Hsp70 machinery, in yeast consisting of the Hsp70 Ssa1 and its co-factors Sse1, Ydj1 and Hlj1, is necessary for efficient protein degradation via the UPS.

been classified as mannosidases. Htm1/Mnl1 cleaves off a mannose residue from the Man_8-$GlcNAc_2$ structure generating a Man_7 structure with an α-1,6-mannose linkage [130,131] (Fig. 3). Interestingly, not all carbohydrate chains of a protein are equivalent in their potential to signal misfolding. A misfolded protein structure around at least one of the glycan chains is equally important for recognition [120,132,133]. In mammalian cells the EDEM proteins, similar to yeast Mns1 and Htm1/Mnl1, cause mannose trimming, resulting in the formation of Man_6 and Man_5 structures [134–137].

2.8. PDI and its orthologues interact with ERAD components and reduce disulfide bonds for retro-translocation

The formation of disulfide bonds within proteins is dependent on a family of enzymes called protein disulfide isomerases (PDI). This ER localized class of enzymes with thiol oxido-reductase activity also exhibits chaperone-like activity [138,139]. The best-known member is PDI (Pdi1 in yeast), which is essential and conserved in all eukaryotic cells [140]. Four additional members of the PDI family are found in yeast: Mpd1 [141], Mpd2 [142], Eug1 [143] and Eps1 [144]. Even though they are not essential like Pdi1, they can suppress inviability of a *Δpdi1* mutant if overexpressed. In human 19 orthologues have been identified so far [72]. The oxidative activities within the human enzymes are not necessarily different [75]. However, their specificity with respect to their chaperone-like activity may differ for particular protein substrates [73,74,124,144,145]. In yeast chaperone function of Pdi1 is required for the retro-translocation of the thiol-free misfolded polypeptide mutated pro-α-factor. In contrast, the enzymatic activity of Pdi1 is necessary for the export of disulfide bond containing CPY* to the cytosol, indicating the requirement of disulfide

bond reduction for retro-translocation [146]. The ER membrane located Eps1 specifically binds a mutant form of the plasma membrane ATPase Pma1, Pma1D378N, but not the wild type enzyme. Eps1 prevents escape of the mutant protein from the ER membrane and facilitates its degradation [144]. In recent studies Pdi1 was shown to form a stable (>60 min) intermolecular disulfide bond with the lectin-like chaperone Htm1/Mnl1 [131,147]. It is possible that this interaction facilitates Pdi1 recruitment to ERAD substrates with the aim to reduce disulfide bonds prior to transport out of the ER. The PDI orthologue of mammals, ERdj5, a J-domain containing oxidoreductase with the highest reducing equilibrium constant found so far, interacts with EDEM1, the mammalian orthologue of Htm1/Mnl1. At the same time ERdj5 interacts with BiP via its His-Pro-Asp containing J-domain. The degradation of two ERAD substrates containing disulfide bonds was dependent on ERdj5. ERdj5 reductase activity and interaction between ERdj5, BiP and EDEM1 were required for efficient degradation [76].

Based on the experiments of Ushioda et al. [76] the following sequence of events can be hypothesized: EDEM, ERdj5 and BiP have distinct but concerted roles in the degradation of misfolded proteins. After the release of terminally misfolded proteins from the calnexin–calreticulin cycle, EDEM further trims the Man_8-$GlcNAc_2$ structure. Concomitantly the disulfide bonds of the substrate are reduced by ERdj5 to allow retrograde translocation into the cytosol. At the same time ERdj5 activates the ATPase activity of BiP via its J-domain. The ADP-form of BiP in turn strongly binds the substrate and holds it in a retro-translocation competent form until it gets transferred to the RTC.

2.9. Yos9 releases misfolded proteins for retrograde transport and subsequent ubiquitylation

A crucial protein factor in the delivery pathway of misfolded lumenal proteins out of the ER to the cytosol is Yos9 and its mammalian orthologue OS-9. Yos9 is a lectin-like protein, which shares a mannosidase receptor homology (MRH) domain with members of the mannose-6-phosphate receptor (MPR) family [82,148]. The protein carries a HDEL ER retention signal at its C-terminus. Analyses with CPY* and CPY* derivatives proved Yos9 to be required for the degradation of N-glycosylated substrates [83,149–151]. The function of its MRH domain was shown to reside in glycan structure dependent substrate binding [83,151]. Bhamidipati et al. [83] demonstrated also an interaction between Yos9 and the unglycosylated ERAD-L substrate CPY*0000. However, accelerated degradation of CPY* depends on recognition of its glycan structure by Yos9 [149]. Recent studies in yeast strongly support the requirement of glycan dependent interaction between Yos9 and lumenal ERAD substrates for efficient degradation [78,130,131]. Yos9 has been proposed to scan proteins for their mannose-trimmed glycan structure and by this acting as a gatekeeper. It is thought that only terminally misfolded proteins carrying a glycan with a terminal 1,6-linked mannose are allowed to leave the ER for proteasomal degradation [78,83,130,152]. One important interaction partner of Yos9 is Hrd3, a type I transmembrane protein, which together with Hrd1/Der3 is part of an ER membrane embedded ubiquitin ligase complex. Hrd3 is able to bind misfolded proteins independently of Yos9 [78]. Most likely, only the scanning function is left to Yos9. Thus, a Hrd3–Yos9 complex recognizes a bipartite signal in the substrate protein, hydrophobic protein patches and the properly mannose-trimmed glycan. The Yos9 MRH domain is not necessarily required for Yos9 Hrd3 interaction. Gauss et al. [78] found evidence for an additional ligase complex without Yos9. It is likely that this complex is specific for the recognition and cytosolic delivery of non-glycosylated and therefore Yos9 independent misfolded lumenal ERAD substrates. Yos9 has two human orthologues, XTP3-B and OS-9. These lectin-like proteins contain MRH domains that are required for interaction with the human Hrd3 orthologue SEL1 but not with the substrate [153,154]. In addition, OS-9 associates with the ER chaperone GRP94, which together with SEL1 and the HRD1 ligase is required for degradation of the ERAD substrate mutant α1-antitrypsin. Both proteins, XTP3-B and OS-9 are components of distinct, partially redundant quality control pathways [153].

*2.10. A model for the degradation of the ERAD-L substrate CPY**

CPY* remains bound to the Hsp70 Kar2 and its Hsp40 co-factors Jem1 and Scj1 during import, glycosylation and the formation of disulfide bonds within the molecule. After unsuccessful attempts to fold properly and removal of the three glucose residues of the N-linked carbohydrate structure, the α-mannosidases Mns1 and Htm1/Mnl1 process the glycan chains of the protein. At the same time Htm1/Mnl1 bound Pdi1 reduces the disulfide bonds. CPY* and the Kar2–Jem1–Scj1 complex bind to Hrd3, which is part of the ER membrane located RTC. Yos9, bound to Hrd3, recognizes the processed carbohydrate structure at position 4 of the protein, which is surrounded by exposed hydrophobic residues. CPY* is then delivered to the RTC, which contains Sec61. The RTC relocates CPY* from the ER lumen to the cytoplasm where it is polyubiquitylated and delivered to the proteasome for degradation (Figs.1 and 4).

3. The cytosolic face of ERAD

The degradation of misfolded ER membrane proteins requires a different set of chaperones as compared to ER lumenal proteins, due to the exposure of protein domains to the cytosol. Model substrates with cytosolic domains studied in yeast comprise the fusion protein CTG* that contains the lumenal misfolded CPY* domain and a transmembrane domain fused to the green fluorescent protein (GFP) on the cytoplasmic side of the ER [155]. Others are: the mutated ATP binding cassette transporter Ste6*, in its wildtype form exporting the mating pheromone a-factor through the plasma membrane of cells [156,157], a mutated form of the plasma membrane ATPase Pma1 [158] and the mutated yeast ABC transporter Pdr5* [159]. Furthermore, mammalian apolipoprotein B (apoB) [160] and cystic fibrosis transmembrane conductance regulator (CFTR) expressed in yeast are intensely studied ERAD substrates [161–163]. In mammalian cells the best-studied ERAD substrate is a mutant form of the CFTR channel, CFTRΔF508. The protein carries a deletion of phenylalanine at position 508 and causes the disease cystic fibrosis in humans [164,165]. The prominent role of the cytosolic Hsp70 machinery in the degradation of membrane proteins becomes visible by analyzing these latter substrates, exposing large misfolded domains or hydrophobic patches to the cytosol (ERAD-C substrates). ERAD-C either fully or in part requires the ubiquitin ligase Doa10 for polyubiquitylation (Fig. 1). In some instances the Hrd1/Der3 ligase has some overlapping specificity with Doa10 [166]. The degradation pathway of ERAD-L and ERAD-C substrates merges at the Cdc48-Ufd1-Npl4 machinery, which further delivers the misfolded polyubiquitylated proteins to the proteasome for degradation (Fig. 1).

3.1. The Hsp70 Ssa1 is required for the degradation of ERAD-C substrates

Degradation of Ste6*, Pma1D378S as well as yeast expressed CFTR and apoB relies on the Hsp70 family member Ssa1 [156,158,163,167]. Most likely, the additional cytosolic Hsp70s of the Ssa family (Ssa2, Ssa3, Ssa4), which share 85–99% amino acid identity with Ssa1, have overlapping activities in the elimination process of these proteins. Given the large lipophilic N-terminal domains of apoB that reside in the ER, it is not surprising that the degradation of yeast expressed apoB in addition requires the ER lumenal Kar2 (BiP) [160]. Kar2 (BiP) is not required for the degradation of the polytopic membrane proteins Ste6*, yeast expressed CFTR and the mutated yeast ABC

transporter Pdr5* [156,159,163]. Obviously, these substrates do not contain large unfolded domains in the ER lumen. Both ER membrane localized cytosolic Hsp40 chaperones Ydj1 and Hlj1 have been found to be important for ERAD of Ste6* and yeast expressed CFTR [156,161] (Fig. 4). In authentic surrounding – mammalian cells – the degradation of CFTR and CFTRΔF508 is dependent on the chaperone pair Hsp70–Hsp40 (Hsc70–Hdj2) [168].

3.2. The ERAD-L substrate CTG* needs cytosolic chaperones for efficient degradation

In contrast to soluble CPY* and membrane bound CT* elimination of CTG* is dependent on the cytosolic Hsp70 chaperone Ssa1 [155]. This requirement was unexpected and must be due to the tightly folded GFP moiety of CTG*. Among all tested cytosolic Hsp40 species, only the DnaJ-family member Hlj1 had a noticeable modulating effect on the degradation of CTG*. Interestingly, Ydj1, which like Hjl1 is localized at the ER membrane, the J-domain facing the cytosol, is not involved in CTG* degradation. In contrast, membrane proteins that expose misfolded domains into the cytosol do need Ydj1 for efficient degradation (see above). Hsp104, known to act in concert with Hsp70 and Hsp40 in reactivation of proteins [169], is to some extent involved in the degradation of CTG*. The two Hsp90 family members Hsc82 and Hsp82 are not active in the degradation process of this protein [155].

3.3. The diverse effects of the nucleotide exchange factor Sse1

One nucleotide exchange factor (NEF) of Ssa1 was shown to be important for ERAD: Sse1. Sse1 is a homologue of Hsp110, a divergent relative of Hsp70 chaperones. It was found to act very efficiently on Ssa1 [51,170–172]. Like for Lhs1, a NEF of Kar2 (BiP) in the ER lumen of yeast, a chaperone-like activity was proposed for Sse1. Studies on the ERAD substrate apoB expressed in yeast revealed a stabilizing effect of Sse1 on this substrate [160,173]. In particular, Sse1 stabilizes apoB in an ATP dependent manner and thus acts contrary to Ssa1, which facilitates degradation of apoB [167,174]. The stabilizing effect of Sse1 for apoB matches the reported interaction of Sse1 and Ssa1 with nascent proteins [175]. Substrate specificity turns the balance of Sse1 activity: Sse1 facilitates degradation (Ste6*), prevents degradation (apoB) or does not show any influence on the degradation process at all (CFTR) [160,161]. A recent study specifies some important differences between Hsp110 and Hsp70. For instance peptide binding to Hsp70s stimulates ATP hydrolysis, however, it does not in the case of Sse1. In addition, distinct peptide preferences have been shown for Sse1 [176].

3.4. Hsp90 is involved in the ERAD-C pathway

Analyses of the Hsp90 machinery Hsc82 and Hsp82 revealed functions that are dependent on the tested substrate: analysis of CFTR degradation in a Hsp90 mutant strain revealed acceleration of CFTR disappearance, indicating that these chaperones preserve CFTR in a folded state [161]. Assistance in folding by Hsp90 has also been found for CFTR in mammalian cells [177]. In contrast, Hsp90 facilitates ERAD of apoB [167]. Obviously, some substrates utilize Hsp90 for protection and folding, while others engage Hsp90 en route to degradation. In some cases Hsp90 is involved in both events. It first helps to fold and, if this cannot successfully be accomplished, targets the substrate for degradation [178].

3.5. Small heat shock proteins facilitate re-solubilization of protein aggregates

Small heat shock proteins prevent irreversible protein aggregation, for instance after heat shock. Two sHsps, Hsp26 and Hsp42, have been identified in the yeast *S. cerevisiae*. While Hsp42 seems to be a general chaperone, which is permanently active under all conditions, Hsp26 is activated upon heat shock temperatures. Above 37 °C it changes its structure by dissociating from an oligomeric into a dimeric state [179–182]. Deletion of both sHsps leads to full stabilization of the yeast expressed ERAD substrate CFTRΔF508. The sHsps seem to work in a redundant fashion as single mutations showed only a minor stabilization of CFTRΔF508. Quantification revealed the same amount of polyubiquitylated CFTRΔF508 in wild type and *Δhsp26Δhsp42* mutant cells, indicating that the sHsps act downstream of the ubiquitylation of the substrate [162]. Degradation of Ste6*, a topologically comparable ERAD-C substrate, was not influenced by deletion of both sHsps [162].

What can be extracted from the studies on chaperone requirement for ERAD? It has become clear in recent years, that chaperones play a central role in the elimination process of proteins that are either damaged, terminally misfolded or do not fold in a certain time frame. In addition, orphan proteins, which cannot assemble with their interaction partners to form native complexes, are targets for chaperones. All these species have in common, that they expose hydrophobic amino acid patches at their surface, which constitute the interaction motifs of chaperones. Usually, this interaction keeps unfolded proteins soluble and helps to acquire a native structure. The clearest picture is presently available for the chaperones of the Hsp70 family members with their Hsp40 co-chaperones. In case, a protein exposes large hydrophobic areas in the ER lumen the Kar2/BiP machinery takes over the binding. Exposure of large hydrophobic patches in the cytosol leads to the recruitment of the cytosolic Hsp70 machinery, in yeast the Ssa family. Many questions remain. When do chaperones "decide" that proteins are terminally misfolded or remain an orphan? Is it an extended time window, kept open by chaperones or chaperone complexes that allow the ubiquitylation machinery to bind and polyubiquitinate the protein? Or are additional factors recruited, which finally signal the decision for entering the degradation pathway? What is the exact function and mechanism of the many different chaperones, which either stabilize or target a protein for degradation? How do chaperone family members, protein disulfide isomerases, lectins and other factors interact and finally hand over a misfolded protein to the RTC and the ubiquitylation machinery for ERAD? Many more experiments have to be performed in the future to allow a detailed understanding of how the chaperone machinery assists a misfolded protein's journey to hell.

Acknowledgements

We thank Wolfgang Hilt, Frederik Eisele and Elena Martinez Benitez for critically reading of the manuscript. The work of the authors was funded by the Deutsche Forschungsgemeinschaft, the EU network of excellence RUBICON and the LGF Baden-Württemberg.

References

[1] R.J. Ellis, S.M. Hemmingsen, Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures, Trends Biochem. Sci. 14 (1989) 339–342.
[2] F.U. Hartl, M. Hayer-Hartl, Molecular chaperones in the cytosol: from nascent chain to folded protein, Science 295 (2002) 1852–1858.
[3] R.I. Morimoto, M.P. Kline, D.N. Bimston, J.J. Cotto, The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones, Essays Biochem. 32 (1997) 17–29.
[4] C. Esser, S. Alberti, J. Hohfeld, Cooperation of molecular chaperones with the ubiquitin/proteasome system, Biochim. Biophys. Acta 1695 (2004) 171–188.
[5] S.H. Park, N. Bolender, F. Eisele, Z. Kostova, J. Takeuchi, P. Coffino, D.H. Wolf, The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin–proteasome system, Mol. Biol. Cell 18 (2007) 153–165.
[6] A.J. McClellan, M.D. Scott, J. Frydman, Folding and quality control of the VHL tumor suppressor proceed through distinct chaperone pathways, Cell 121 (2005) 739–748.
[7] A.J. McClellan, S. Tam, D. Kaganovich, J. Frydman, Protein quality control: chaperones culling corrupt conformations, Nat. Cell Biol. 7 (2005) 736–741.

[8] T. Sommer, S. Jentsch, A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum, Nature 365 (1993) 176–179.
[9] M.M. Hiller, A. Finger, M. Schweiger, D.H. Wolf, ER degradation of a misfolded luminal protein by the cytosolic ubiquitin–proteasome pathway, Science 273 (1996) 1725–1728.
[10] C.E. Shamu, C.M. Story, T.A. Rapoport, H.L. Ploegh, The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate, J. Cell Biol. 147 (1999) 45–58.
[11] E.J. Wiertz, T.R. Jones, L. Sun, M. Bogyo, H.J. Geuze, H.L. Ploegh, The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol, Cell 84 (1996) 769–779.
[12] Z. Kostova, D.H. Wolf, For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin–proteasome connection, EMBO J. 22 (2003) 2309–2317.
[13] S.S. Vembar, J.L. Brodsky, One step at a time: endoplasmic reticulum-associated degradation, Nat. Rev. Mol. Cell Biol. 9 (2008) 944–957.
[14] T. Sommer, D.H. Wolf, Endoplasmic reticulum degradation: reverse protein flow of no return, FASEB J. 11 (1997) 1227–1233.
[15] R.K. Plemper, D.H. Wolf, Retrograde protein translocation: ERADication of secretory proteins in health and disease, Trends Biochem. Sci. 24 (1999) 266–270.
[16] C. Hirsch, R. Gauss, S.C. Horn, O. Neuber, T. Sommer, The ubiquitylation machinery of the endoplasmic reticulum, Nature 458 (2009) 453–460.
[17] K.J. Travers, C.K. Patil, L. Wodicka, D.J. Lockhart, J.S. Weissman, P. Walter, Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation, Cell 101 (2000) 249–258.
[18] R. Friedländer, E. Jarosch, J. Urban, C. Volkwein, T. Sommer, A regulatory link between ER-associated protein degradation and the unfolded-protein response, Nat. Cell Biol. 2 (2000) 379–384.
[19] D.A. Parsell, A.S. Kowal, M.A. Singer, S. Lindquist, Protein disaggregation mediated by heat-shock protein Hsp104, Nature 372 (1994) 475–478.
[20] Y. Sun, T.H. MacRae, Small heat shock proteins: molecular structure and chaperone function, Cell. Mol. Life Sci. 62 (2005) 2460–2476.
[21] E.D. Werner, J.L. Brodsky, A.A. McCracken, Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 13797–13801.
[22] J. Hageman, M.J. Vos, M.A. van Waarde, H.H. Kampinga, Comparison of intra-organellar chaperone capacity for dealing with stress-induced protein unfolding, J. Biol. Chem. 282 (2007) 34334–34345.
[23] K.M. Flaherty, C. DeLuca-Flaherty, D.B. McKay, Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein, Nature 346 (1990) 623–628.
[24] R. Sousa, E.M. Lafer, Keep the traffic moving: mechanism of the Hsp70 motor, Traffic 7 (2006) 1596–1603.
[25] X. Zhu, X. Zhao, W.F. Burkholder, A. Gragerov, C.M. Ogata, M.E. Gottesman, W.A. Hendrickson, Structural analysis of substrate binding by the molecular chaperone DnaK, Science 272 (1996) 1606–1614.
[26] B. Bukau, A.L. Horwich, The Hsp70 and Hsp60 chaperone machines, Cell 92 (1998) 351–366.
[27] G.C. Flynn, T.G. Chappell, J.E. Rothman, Peptide binding and release by proteins implicated as catalysts of protein assembly, Science 245 (1989) 385–390.
[28] R. Jordan, R. McMacken, Modulation of the ATPase activity of the molecular chaperone DnaK by peptides and the DnaJ and GrpE heat shock proteins, J. Biol. Chem. 270 (1995) 4563–4569.
[29] P. D'Silva, J. Marszalek, E.A. Craig, An essential connection: link between Hsp70's domains at last, Mol. Cell 20 (2005) 493–494.
[30] J.S. McCarty, A. Buchberger, J. Reinstein, B. Bukau, The role of ATP in the functional cycle of the DnaK chaperone system, J. Mol. Biol. 249 (1995) 126–137.
[31] R. Russell, A. Wali Karzai, A.F. Mehl, R. McMacken, DnaJ dramatically stimulates ATP hydrolysis by DnaK: insight into targeting of Hsp70 proteins to polypeptide substrates, Biochemistry 38 (1999) 4165–4176.
[32] S. Alberti, C. Esser, J. Hohfeld, BAG-1—a nucleotide exchange factor of Hsc70 with multiple cellular functions, Cell Stress Chaperones 8 (2003) 225–231.
[33] J.H. Ha, D.B. McKay, ATPase kinetics of recombinant bovine 70 kDa heat shock cognate protein and its amino-terminal ATPase domain, Biochemistry 33 (1994) 14625–14635.
[34] C. Harrison, GrpE, a nucleotide exchange factor for DnaK, Cell Stress Chaperones 8 (2003) 218–224.
[35] H. Theyssen, H.P. Schuster, L. Packschies, B. Bukau, J. Reinstein, The second step of ATP binding to DnaK induces peptide release, J. Mol. Biol. 263 (1996) 657–670.
[36] C.P. Georgopoulos, A. Lundquist-Heil, J. Yochem, M. Feiss, Identification of the *E. coli* dnaJ gene product, Mol. Gen. Genet. 178 (1980) 583–588.
[37] Z. Lu, D.M. Cyr, The conserved carboxyl terminus and zinc finger-like domain of the co-chaperone Ydj1 assist Hsp70 in protein folding, J. Biol. Chem. 273 (1998) 5970–5978.
[38] A.A. Michels, B. Kanon, O. Bensaude, H.H. Kampinga, Heat shock protein (Hsp) 40 mutants inhibit Hsp70 in mammalian cells, J. Biol. Chem. 274 (1999) 36757–36763.
[39] D. Wall, M. Zylicz, C. Georgopoulos, The NH2-terminal 108 amino acids of the *Escherichia coli* DnaJ protein stimulate the ATPase activity of DnaK and are sufficient for lambda replication, J. Biol. Chem. 269 (1994) 5446–5451.
[40] M.E. Cheetham, A.J. Caplan, Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function, Cell Stress Chaperones 3 (1998) 28–36.
[41] B. Banecki, K. Liberek, D. Wall, A. Wawrzynow, C. Georgopoulos, E. Bertoli, F. Tanfani, M. Zylicz, Structure–function analysis of the zinc finger region of the DnaJ molecular chaperone, J. Biol. Chem. 271 (1996) 14840–14848.
[42] A. Szabo, R. Korszun, F.U. Hartl, J. Flanagan, A zinc finger-like domain of the molecular chaperone DnaJ is involved in binding to denatured protein substrates, EMBO J. 15 (1996) 408–417.
[43] S. Rüdiger, J. Schneider-Mergener, B. Bukau, Its substrate specificity characterizes the DnaJ co-chaperone as a scanning factor for the DnaK chaperone, EMBO J. 20 (2001) 1042–1050.
[44] C.J. Harrison, M. Hayer-Hartl, M. Di Liberto, F. Hartl, J. Kuriyan, Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK, Science 276 (1997) 431–435.
[45] H.J. Schonfeld, D. Schmidt, H. Schroder, B. Bukau, The DnaK chaperone system of *Escherichia coli*: quaternary structures and interactions of the DnaK and GrpE components, J. Biol. Chem. 270 (1995) 2183–2189.
[46] C.S. Gassler, T. Wiederkehr, D. Brehmer, B. Bukau, M.P. Mayer, Bag-1M accelerates nucleotide release for human Hsc70 and Hsp70 and can act concentration-dependent as positive and negative cofactor, J. Biol. Chem. 276 (2001) 32538–32544.
[47] D.A. Raynes, V. Guerriero Jr., Inhibition of Hsp70 ATPase activity and protein renaturation by a novel Hsp70-binding protein, J. Biol. Chem. 273 (1998) 32883–32888.
[48] Y. Shomura, Z. Dragovic, H.C. Chang, N. Tzvetkov, J.C. Young, J.L. Brodsky, V. Guerriero, F.U. Hartl, A. Bracher, Regulation of Hsp70 function by HspBP1: structural analysis reveals an alternate mechanism for Hsp70 nucleotide exchange, Mol. Cell 17 (2005) 367–379.
[49] J. de Keyzer, G.J. Steel, S.J. Hale, D. Humphreys, C.J. Stirling, Nucleotide binding by LHS1P is essential for its nucleotide exchange activity and for function in vivo, J. Biol. Chem. 284 (2009) 31564–31571.
[50] G.J. Steel, D.M. Fullerton, J.R. Tyson, C.J. Stirling, Coordinated activation of Hsp70 chaperones, Science 303 (2004) 98–101.
[51] Z. Dragovic, S.A. Broadley, Y. Shomura, A. Bracher, F.U. Hartl, Molecular chaperones of the Hsp110 family act as nucleotide exchange factors of Hsp70s, EMBO J. 25 (2006) 2519–2528.
[52] W.M. Obermann, H. Sondermann, A.A. Russo, N.P. Pavletich, F.U. Hartl, In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis, J. Cell Biol. 143 (1998) 901–910.
[53] P. Meyer, C. Prodromou, B. Hu, C. Vaughan, S.M. Roe, B. Panaretou, P.W. Piper, L.H. Pearl, Structural and functional analysis of the middle segment of hsp90: implications for ATP hydrolysis and client protein and cochaperone interactions, Mol. Cell 11 (2003) 647–658.
[54] C. Prodromou, B. Panaretou, S. Chohan, G. Siligardi, R. O'Brien, J.E. Ladbury, S.M. Roe, P.W. Piper, L.H. Pearl, The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains, EMBO J. 19 (2000) 4383–4392.
[55] S.F. Harris, A.K. Shiau, D.A. Agard, The crystal structure of the carboxy-terminal dimerization domain of htpG, the *Escherichia coli* Hsp90, reveals a potential substrate binding site, Structure 12 (2004) 1087–1097.
[56] J.P. Grenert, B.D. Johnson, D.O. Toft, The importance of ATP binding and hydrolysis by hsp90 in formation and function of protein heterocomplexes, J. Biol. Chem. 274 (1999) 17525–17533.
[57] B. Panaretou, C. Prodromou, S.M. Roe, R. O'Brien, J.E. Ladbury, P.W. Piper, L.H. Pearl, ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo, EMBO J. 17 (1998) 4829–4836.
[58] M. Mickler, M. Hessling, C. Ratzke, J. Buchner, T. Hugel, The large conformational changes of Hsp90 are only weakly coupled to ATP hydrolysis, Nat. Struct. Mol. Biol. 16 (2009) 281–286.
[59] M. Hessling, K. Richter, J. Buchner, Dissection of the ATP-induced conformational cycle of the molecular chaperone Hsp90, Nat. Struct. Mol. Biol. 16 (2009) 287–293.
[60] S.K. Wandinger, K. Richter, J. Buchner, The Hsp90 chaperone machinery, J. Biol. Chem. 283 (2008) 18473–18477.
[61] J.A. Carver, R.A. Lindner, C. Lyon, D. Canet, H. Hernandez, C.M. Dobson, C. Redfield, The interaction of the molecular chaperone alpha-crystallin with unfolding alpha-lactalbumin: a structural and kinetic spectroscopic study, J. Mol. Biol. 318 (2002) 815–827.
[62] R.A. Lindner, T.M. Treweek, J.A. Carver, The molecular chaperone alpha-crystallin is in kinetic competition with aggregation to stabilize a monomeric molten-globule form of alpha-lactalbumin, Biochem. J. 354 (2001) 79–87.
[63] A. Biswas, K.P. Das, Role of ATP on the interaction of alpha-crystallin with its substrates and its implications for the molecular chaperone function, J. Biol. Chem. 279 (2004) 42648–42657.
[64] E. Basha, G.J. Lee, L.A. Breci, A.C. Hausrath, N.R. Buan, K.C. Giese, E. Vierling, The identity of proteins associated with a small heat shock protein during heat stress in vivo indicates that these chaperones protect a wide range of cellular functions, J. Biol. Chem. 279 (2004) 7566–7575.
[65] K.L. Friedrich, K.C. Giese, N.R. Buan, E. Vierling, Interactions between small heat shock protein subunits and substrate in small heat shock protein–substrate complexes, J. Biol. Chem. 279 (2004) 1080–1089.
[66] B.K. Derham, M.A. van Boekel, P.J. Muchowski, J.I. Clark, J. Horwitz, H.W. Hepburne-Scott, W.W. de Jong, M.J. Crabbe, J.J. Harding, Chaperone function of mutant versions of alpha A- and alpha B-crystallin prepared to pinpoint chaperone binding sites, Eur. J. Biochem. 268 (2001) 713–721.
[67] N. Lentze, S. Studer, F. Narberhaus, Structural and functional defects caused by point mutations in the alpha-crystallin domain of a bacterial alpha-heat shock protein, J. Mol. Biol. 328 (2003) 927–937.
[68] P.J. Muchowski, G.J. Wu, J.J. Liang, E.T. Adman, J.I. Clark, Site-directed mutations within the core "alpha-crystallin" domain of the small heat-shock protein, human alphaB-crystallin, decrease molecular chaperone functions, J. Mol. Biol. 289 (1999) 397–411.

[69] P.L. Wintrode, K.L. Friedrich, E. Vierling, J.B. Smith, D.L. Smith, Solution structure and dynamics of a heat shock protein assembly probed by hydrogen exchange and mass spectrometry, Biochemistry 42 (2003) 10667–10673.
[70] S. Nishikawa, J.L. Brodsky, K. Nakatsukasa, Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD), J. Biochem. 137 (2005) 551–555.
[71] B.P. Tu, J.S. Weissman, Oxidative protein folding in eukaryotes: mechanisms and consequences, J. Cell Biol. 164 (2004) 341–346.
[72] C. Appenzeller-Herzog, L. Ellgaard, The human PDI family: versatility packed into a single fold, Biochim. Biophys. Acta 1783 (2008) 535–548.
[73] H. Cai, C.C. Wang, C.L. Tsou, Chaperone-like activity of protein disulfide isomerase in the refolding of a protein with no disulfide bonds, J. Biol. Chem. 269 (1994) 24550–24552.
[74] J.L. Song, C.C. Wang, Chaperone-like activity of protein disulfide-isomerase in the refolding of rhodanese, Eur. J. Biochem. 231 (1995) 312–316.
[75] H.I. Alanen, K.E. Salo, A. Pirneskoski, L.W. Ruddock, pH dependence of the peptide thiol-disulfide oxidase activity of six members of the human protein disulfide isomerase family, Antioxid. Redox Signal. 8 (2006) 283–291.
[76] R. Ushioda, J. Hoseki, K. Araki, G. Jansen, D.Y. Thomas, K. Nagata, ERdj5 is required as a disulfide reductase for degradation of misfolded proteins in the ER, Science 321 (2008) 569–572.
[77] J.H. Cormier, T. Tamura, J.C. Sunryd, D.N. Hebert, EDEM1 recognition and delivery of misfolded proteins to the SEL1L-containing ERAD complex, Mol. Cell 34 (2009) 627–633.
[78] R. Gauss, E. Jarosch, T. Sommer, C. Hirsch, A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery, Nat. Cell Biol. 8 (2006) 849–854.
[79] S. Svedine, T. Wang, R. Halaban, D.N. Hebert, Carbohydrates act as sorting determinants in ER-associated degradation of tyrosinase, J. Cell Sci. 117 (2004) 2937–2949.
[80] F. Lipari, A. Herscovics, Calcium binding to the class I alpha-1, 2-mannosidase from *Saccharomyces cerevisiae* occurs outside the EF hand motif, Biochemistry 38 (1999) 1111–1118.
[81] F. Vallee, F. Lipari, P. Yip, B. Sleno, A. Herscovics, P.L. Howell, Crystal structure of a class I alpha1, 2-mannosidase involved in N-glycan processing and endoplasmic reticulum quality control, EMBO J. 19 (2000) 581–588.
[82] S. Munro, The MRH domain suggests a shared ancestry for the mannose 6-phosphate receptors and other N-glycan-recognising proteins, Curr. Biol. 11 (2001) R499–R501.
[83] A. Bhamidipati, V. Denic, E.M. Quan, J.S. Weissman, Exploration of the topological requirements of ERAD identifies Yos9p as a lectin sensor of misfolded glycoproteins in the ER lumen, Mol. Cell 19 (2005) 741–751.
[84] S. Vashist, D.T. Ng, Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control, J. Cell Biol. 165 (2004) 41–52.
[85] P. Carvalho, V. Goder, T.A. Rapoport, Distinct ubiquitin–ligase complexes define convergent pathways for the degradation of ER proteins, Cell 126 (2006) 361–373.
[86] A. Finger, M. Knop, D.H. Wolf, Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast, Eur. J. Biochem. 218 (1993) 565–574.
[87] I.M. Nilsson, G. von Heijne, Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane, J. Biol. Chem. 268 (1993) 5798–5801.
[88] R.K. Plemper, P.M. Deak, R.T. Otto, D.H. Wolf, Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase yscY species, FEBS Lett. 443 (1999) 241–245.
[89] A. Schäfer, D.H. Wolf, Sec61p is part of the endoplasmic reticulum-associated degradation machinery, EMBO J. 28 (2009) 2874–2884.
[90] K.E. Matlack, B. Misselwitz, K. Plath, T.A. Rapoport, BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane, Cell 97 (1999) 553–564.
[91] S. Nishikawa, S.W. Fewell, Y. Kato, J.L. Brodsky, T. Endo, Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation, J. Cell Biol. 153 (2001) 1061–1070.
[92] R.K. Plemper, S. Böhmler, J. Bordallo, T. Sommer, D.H. Wolf, Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation, Nature 388 (1997) 891–895.
[93] J.L. Brodsky, E.D. Werner, M.E. Dubas, J.L. Goeckeler, K.B. Kruse, A.A. McCracken, The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct, J. Biol. Chem. 274 (1999) 3453–3460.
[94] M.R. Knittler, S. Dirks, I.G. Haas, Molecular chaperones involved in protein degradation in the endoplasmic reticulum: quantitative interaction of the heat shock cognate protein BiP with partially folded immunoglobulin light chains that are degraded in the endoplasmic reticulum, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1764–1768.
[95] S. Silberstein, G. Schlenstedt, P.A. Silver, R. Gilmore, A role for the DnaJ homologue Scj1p in protein folding in the yeast endoplasmic reticulum, J. Cell Biol. 143 (1998) 921–933.
[96] M. Carla Fama, D. Raden, N. Zacchi, D.R. Lemos, A.S. Robinson, S. Silberstein, The *Saccharomyces cerevisiae* YFR041C/ERJ5 gene encoding a type I membrane protein with a J domain is required to preserve the folding capacity of the endoplasmic reticulum, Biochim. Biophys. Acta 1773 (2007) 232–242.
[97] A. Weitzmann, J. Volkmer, R. Zimmermann, The nucleotide exchange factor activity of Grp170 may explain the non-lethal phenotype of loss of Sil1 function in man and mouse, FEBS Lett. 580 (2006) 5237–5240.
[98] R.A. Craven, M. Egerton, C.J. Stirling, A novel Hsp70 of the yeast ER lumen is required for the efficient translocation of a number of protein precursors, EMBO J. 15 (1996) 2640–2650.
[99] J.R. Tyson, C.J. Stirling, LHS1 and SIL1 provide a lumenal function that is essential for protein translocation into the endoplasmic reticulum, EMBO J. 19 (2000) 6440–6452.
[100] M. Kabani, J.M. Beckerich, C. Gaillardin, Sls1p stimulates Sec63p-mediated activation of Kar2p in a conformation-dependent manner in the yeast endoplasmic reticulum, Mol. Cell. Biol. 20 (2000) 6923–6934.
[101] M. Angeles Juanes, J. Carlos Igual, M. Carmen Bano, Membrane topology and post-translational modification of the *Saccharomyces cerevisiae* essential protein Rot1, Yeast 25 (2008) 93–106.
[102] M. Takeuchi, Y. Kimata, A. Hirata, M. Oka, K. Kohno, *Saccharomyces cerevisiae* Rot1p is an ER-localized membrane protein that may function with BiP/Kar2p in protein folding, J. Biochem. 139 (2006) 597–605.
[103] M. Bickle, P.A. Delley, A. Schmidt, M.N. Hall, Cell wall integrity modulates RHO1 activity via the exchange factor ROM2, EMBO J. 17 (1998) 2235–2245.
[104] K. Machi, M. Azuma, K. Igarashi, T. Matsumoto, H. Fukuda, A. Kondo, H. Ooshima, Rot1p of *Saccharomyces cerevisiae* is a putative membrane protein required for normal levels of the cell wall 1, 6-beta-glucan, Microbiology 150 (2004) 3163–3173.
[105] M. Takeuchi, Y. Kimata, K. Kohno, *Saccharomyces cerevisiae* Rot1 is an essential molecular chaperone in the endoplasmic reticulum, Mol. Biol. Cell 19 (2008) 3514–3525.
[106] A. Helenius, How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum, Mol. Biol. Cell 5 (1994) 253–265.
[107] M. Aebi, R. Bernasconi, S. Clerc, M. Molinari, N-glycan structures: recognition and processing in the ER, Trends Biochem. Sci. 35 (2010) 74–82.
[108] A. Helenius, M. Aebi, Roles of N-linked glycans in the endoplasmic reticulum, Annu. Rev. Biochem. 73 (2004) 1019–1049.
[109] C. Hammond, I. Braakman, A. Helenius, Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 913–917.
[110] J.J. Caramelo, O.A. Castro, L.G. Alonso, G. De Prat-Gay, A.J. Parodi, UDP-Glc: glycoprotein glucosyltransferase recognizes structured and solvent accessible hydrophobic patches in molten globule-like folding intermediates, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 86–91.
[111] S.C. Taylor, P. Thibault, D.C. Tessier, J.J. Bergeron, D.Y. Thomas, Glycopeptide specificity of the secretory protein folding sensor UDP-glucose glycoprotein: glucosyltransferase, EMBO Rep. 4 (2003) 405–411.
[112] C. Ritter, K. Quirin, M. Kowarik, A. Helenius, Minor folding defects trigger local modification of glycoproteins by the ER folding sensor GT, EMBO J. 24 (2005) 1730–1738.
[113] Y. Oda, N. Hosokawa, I. Wada, K. Nagata, EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin, Science 299 (2003) 1394–1397.
[114] J. Chillaron, C. Adan, I.G. Haas, Mannosidase action, independent of glucose trimming, is essential for proteasome-mediated degradation of unassembled glycosylated Ig light chains, Biol. Chem. 381 (2000) 1155–1164.
[115] C. Fagioli, R. Sitia, Glycoprotein quality control in the endoplasmic reticulum. Mannose trimming by endoplasmic reticulum mannosidase I times the proteasomal degradation of unassembled immunoglobulin subunits, J. Biol. Chem. 276 (2001) 12885–12892.
[116] M. Molinari, V. Calanca, C. Galli, P. Lucca, P. Paganetti, Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle, Science 299 (2003) 1397–1400.
[117] F. Parlati, M. Dominguez, J.J. Bergeron, D.Y. Thomas, *Saccharomyces cerevisiae* CNE1 encodes an endoplasmic reticulum (ER) membrane protein with sequence similarity to calnexin and calreticulin and functions as a constituent of the ER quality control apparatus, J. Biol. Chem. 270 (1995) 244–253.
[118] X. Xu, K. Kanbara, H. Azakami, A. Kato, Expression and characterization of *Saccharomyces cerevisiae* Cne1p, a calnexin homologue, J. Biochem. 135 (2004) 615–618.
[119] X. Xu, H. Azakami, A. Kato, P-domain and lectin site are involved in the chaperone function of *Saccharomyces cerevisiae* calnexin homologue, FEBS Lett. 570 (2004) 155–160.
[120] Z. Kostova, D.H. Wolf, Importance of carbohydrate positioning in the recognition of mutated CPY for ER-associated degradation, J. Cell Sci. 118 (2005) 1485–1492.
[121] H. Zhang, J. He, Y. Ji, A. Kato, Y. Song, The effect of calnexin deletion on the expression level of PDI in *Saccharomyces cerevisiae* under heat stress conditions, Cell. Mol. Biol. Lett. 13 (2008) 38–48.
[122] H. Zhang, B. Hu, Y. Ji, A. Kato, Y. Song, The effect of calnexin deletion on the expression level of binding protein (BiP) under heat stress conditions in *Saccharomyces cerevisiae*, Cell. Mol. Biol. Lett. 13 (2008) 621–631.
[123] T. Kimura, Y. Hosoda, Y. Sato, Y. Kitamura, T. Ikeda, T. Horibe, M. Kikuchi, Interactions among yeast protein-disulfide isomerase proteins and endoplasmic reticulum chaperone proteins influence their activities, J. Biol. Chem. 280 (2005) 31438–31441.
[124] J.D. Oliver, H.L. Roderick, D.H. Llewellyn, S. High, ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin, Mol. Biol. Cell 10 (1999) 2573–2582.
[125] A. Helenius, E.S. Trombetta, D.N. Hebert, J.F. Simons, Calnexin, calreticulin and the folding of glycoproteins, Trends Cell Biol. 7 (1997) 193–200.
[126] M. Knop, N. Hauser, D.H. Wolf, N-Glycosylation affects endoplasmic reticulum degradation of a mutated derivative of carboxypeptidase yscY in yeast, Yeast 12 (1996) 1229–1238.

[127] N. Hosokawa, I. Wada, K. Hasegawa, T. Yorihuzi, L.O. Tremblay, A. Herscovics, K. Nagata, A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation, EMBO Rep. 2 (2001) 415–422.
[128] C.A. Jakob, D. Bodmer, U. Spirig, P. Battig, A. Marcil, D. Dignard, J.J. Bergeron, D.Y. Thomas, M. Aebi, Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast, EMBO Rep. 2 (2001) 423–430.
[129] K. Nakatsukasa, S. Nishikawa, N. Hosokawa, K. Nagata, T. Endo, Mnl1p, an alpha-mannosidase-like protein in yeast *Saccharomyces cerevisiae*, is required for endoplasmic reticulum-associated degradation of glycoproteins, J. Biol. Chem. 276 (2001) 8635–8638.
[130] E.M. Quan, Y. Kamiya, D. Kamiya, V. Denic, J. Weibezahn, K. Kato, J.S. Weissman, Defining the glycan destruction signal for endoplasmic reticulum-associated degradation, Mol. Cell 32 (2008) 870–877.
[131] S. Clerc, C. Hirsch, D.M. Oggier, P. Deprez, C. Jakob, T. Sommer, M. Aebi, Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum, J. Cell Biol. 184 (2009) 159–172.
[132] E.D. Spear, D.T. Ng, Single, context-specific glycans can target misfolded glycoproteins for ER-associated degradation, J. Cell Biol. 169 (2005) 73–82.
[133] W. Xie, K. Kanehara, A. Sayeed, D.T. Ng, Intrinsic conformational determinants signal protein misfolding to the Hrd1/Htm1 endoplasmic reticulum-associated degradation system, Mol. Biol. Cell 20 (2009) 3317–3329.
[134] F. Foulquier, S. Duvet, A. Klein, A.M. Mir, F. Chirat, R. Cacan, Endoplasmic reticulum-associated degradation of glycoproteins bearing Man5GlcNAc2 and Man9GlcNAc2 species in the MI8-5 CHO cell line, Eur. J. Biochem. 271 (2004) 398–404.
[135] Z. Frenkel, W. Gregory, S. Kornfeld, G.Z. Lederkremer, Endoplasmic reticulum-associated degradation of mammalian glycoproteins involves sugar chain trimming to Man6-5GlcNAc2, J. Biol. Chem. 278 (2003) 34119–34124.
[136] N. Hosokawa, L.O. Tremblay, Z. You, A. Herscovics, I. Wada, K. Nagata, Enhancement of endoplasmic reticulum (ER) degradation of misfolded Null Hong Kong alpha1-antitrypsin by human ER mannosidase I, J. Biol. Chem. 278 (2003) 26287–26294.
[137] C. Kitzmuller, A. Caprini, S.E. Moore, J.P. Frenoy, E. Schwaiger, O. Kellermann, N.E. Ivessa, M. Ermonval, Processing of N-linked glycans during endoplasmic-reticulum-associated degradation of a short-lived variant of ribophorin I, Biochem. J. 376 (2003) 687–696.
[138] R.B. Freedman, T.R. Hirst, M.F. Tuite, Protein disulphide isomerase: building bridges in protein folding, Trends Biochem. Sci. 19 (1994) 331–336.
[139] T.P. Primm, K.W. Walker, H.F. Gilbert, Facilitated protein aggregation. Effects of calcium on the chaperone and anti-chaperone activity of protein disulfide-isomerase, J. Biol. Chem. 271 (1996) 33664–33669.
[140] R. Farquhar, N. Honey, S.J. Murant, P. Bossier, L. Schultz, D. Montgomery, R.W. Ellis, R.B. Freedman, M.F. Tuite, Protein disulfide isomerase is essential for viability in *Saccharomyces cerevisiae*, Gene 108 (1991) 81–89.
[141] H. Tachikawa, Y. Takeuchi, W. Funahashi, T. Miura, X.D. Gao, D. Fujimoto, T. Mizunaga, K. Onodera, Isolation and characterization of a yeast gene, MPD1, the overexpression of which suppresses inviability caused by protein disulfide isomerase depletion, FEBS Lett. 369 (1995) 212–216.
[142] H. Tachikawa, W. Funahashi, Y. Takeuchi, H. Nakanishi, R. Nishihara, S. Katoh, X.D. Gao, T. Mizunaga, D. Fujimoto, Overproduction of Mpd2p suppresses the lethality of protein disulfide isomerase depletion in a CXXC sequence dependent manner, Biochem. Biophys. Res. Commun. 239 (1997) 710–714.
[143] C. Tachibana, T.H. Stevens, The yeast EUG1 gene encodes an endoplasmic reticulum protein that is functionally related to protein disulfide isomerase, Mol. Cell. Biol. 12 (1992) 4601–4611.
[144] Q. Wang, A. Chang, Eps1, a novel PDI-related protein involved in ER quality control in yeast, EMBO J. 18 (1999) 5972–5982.
[145] Q. Wang, A. Chang, Substrate recognition in ER-associated degradation mediated by Eps1, a member of the protein disulfide isomerase family, EMBO J. 22 (2003) 3792–3802.
[146] P. Gillece, J.M. Luz, W.J. Lennarz, F.J. de La Cruz, K. Römisch, Export of a cysteine-free misfolded secretory protein from the endoplasmic reticulum for degradation requires interaction with protein disulfide isomerase, J. Cell Biol. 147 (1999) 1443–1456.
[147] M. Sakoh-Nakatogawa, S. Nishikawa, T. Endo, Roles of protein-disulfide isomerase-mediated disulfide bond formation of yeast Mnl1p in endoplasmic reticulum-associated degradation, J. Biol. Chem. 284 (2009) 11815–11825.
[148] Y. Kimura, M. Nakazawa, M. Yamada, Cloning and characterization of three isoforms of OS-9 cDNA and expression of the OS-9 gene in various human tumor cell lines, J. Biochem. (Tokyo) 123 (1998) 876–882.
[149] B. Buschhorn, Z. Kostova, B. Medicherla, D.H. Wolf, A genome wide screen identifies Yos9p as a new lectin essential for ER-associated degradation (ERAD) of glycoproteins, FEBS Lett. 577 (2004) 422–426.
[150] W. Kim, E.D. Spear, D.T. Ng, Yos9p detects and targets misfolded glycoproteins for ER-associated degradation, Mol. Cell 19 (2005) 753–764.
[151] R. Szathmary, R. Bielmann, M. Nita-Lazar, P. Burda, C.A. Jakob, Yos9 protein is essential for degradation of misfolded glycoproteins and may function as lectin in ERAD, Mol. Cell 19 (2005) 765–775.
[152] V. Denic, E.M. Quan, J.S. Weissman, A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation, Cell 126 (2006) 349–359.
[153] J.C. Christianson, T.A. Shaler, R.E. Tyler, R.R. Kopito, OS-9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD, Nat. Cell Biol. 10 (2008) 272–282.
[154] B. Müller, B.N. Lilley, H.L. Ploegh, SEL1L, the homologue of yeast Hrd3p, is involved in protein dislocation from the mammalian ER, J. Cell Biol. 175 (2006) 261–270.
[155] C. Taxis, R. Hitt, S.H. Park, P.M. Deak, Z. Kostova, D.H. Wolf, Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD, J. Biol. Chem. 278 (2003) 35903–35913.
[156] G. Huyer, W.F. Piluek, Z. Fansler, S.G. Kreft, M. Hochstrasser, J.L. Brodsky, S. Michaelis, Distinct machinery is required in *Saccharomyces cerevisiae* for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein, J. Biol. Chem. 279 (2004) 38369–38378.
[157] K. Nakatsukasa, G. Huyer, S. Michaelis, J.L. Brodsky, Dissecting the ER-associated degradation of a misfolded polytopic membrane protein, Cell 132 (2008) 101–112.
[158] S. Han, Y. Liu, A. Chang, Cytoplasmic Hsp70 promotes ubiquitination for endoplasmic reticulum-associated degradation of a misfolded mutant of the yeast plasma membrane ATPase, PMA1, J. Biol. Chem. 282 (2007) 26140–26149.
[159] R.K. Plemper, R. Egner, K. Kuchler, D.H. Wolf, Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome, J. Biol. Chem. 273 (1998) 32848–32856.
[160] S.L. Hrizo, V. Gusarova, D.M. Habiel, J.L. Goeckeler, E.A. Fisher, J.L. Brodsky, The Hsp110 molecular chaperone stabilizes apolipoprotein B from endoplasmic reticulum-associated degradation (ERAD), J. Biol. Chem. 282 (2007) 32665–32675.
[161] R.T. Youker, P. Walsh, T. Beilharz, T. Lithgow, J.L. Brodsky, Distinct roles for the Hsp40 and Hsp90 molecular chaperones during cystic fibrosis transmembrane conductance regulator degradation in yeast, Mol. Biol. Cell 15 (2004) 4787–4797.
[162] A. Ahner, K. Nakatsukasa, H. Zhang, R.A. Frizzell, J.L. Brodsky, Small heat-shock proteins select deltaF508-CFTR for endoplasmic reticulum-associated degradation, Mol. Biol. Cell 18 (2007) 806–814.
[163] Y. Zhang, G. Nijbroek, M.L. Sullivan, A.A. McCracken, S.C. Watkins, S. Michaelis, J.L. Brodsky, Hsp70 molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast, Mol. Biol. Cell 12 (2001) 1303–1314.
[164] T.J. Jensen, M.A. Loo, S. Pind, D.B. Williams, A.L. Goldberg, J.R. Riordan, Multiple proteolytic systems, including the proteasome, contribute to CFTR processing, Cell 83 (1995) 129–135.
[165] C.L. Ward, S. Omura, R.R. Kopito, Degradation of CFTR by the ubiquitin–proteasome pathway, Cell 83 (1995) 121–127.
[166] A. Gnann, J.R. Riordan, D.H. Wolf, Cystic fibrosis transmembrane conductance regulator degradation depends on the lectins Htm1p/EDEM and the Cdc48 protein complex in yeast, Mol. Biol. Cell 15 (2004) 4125–4135.
[167] V. Gusarova, A.J. Caplan, J.L. Brodsky, E.A. Fisher, Apoprotein B degradation is promoted by the molecular chaperones hsp90 and hsp70, J. Biol. Chem. 276 (2001) 24891–24900.
[168] G.C. Meacham, Z. Lu, S. King, E. Sorscher, A. Tousson, D.M. Cyr, The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis, EMBO J. 18 (1999) 1492–1505.
[169] J.R. Glover, S. Lindquist, Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins, Cell 94 (1998) 73–82.
[170] H. Raviol, H. Sadlish, F. Rodriguez, M.P. Mayer, B. Bukau, Chaperone network in the yeast cytosol: Hsp110 is revealed as an Hsp70 nucleotide exchange factor, EMBO J. 25 (2006) 2510–2518.
[171] L. Shaner, H. Wegele, J. Buchner, K.A. Morano, The yeast Hsp110 Sse1 functionally interacts with the Hsp70 chaperones Ssa and Ssb, J. Biol. Chem. 280 (2005) 41262–41269.
[172] J.P. Schuermann, J. Jiang, J. Cuellar, O. Llorca, L. Wang, L.E. Gimenez, S. Jin, A.B. Taylor, B. Demeler, K.A. Morano, P.J. Hart, J.M. Valpuesta, E.M. Lafer, R. Sousa, Structure of the Hsp110:Hsc70 nucleotide exchange machine, Mol. Cell 31 (2008) 232–243.
[173] H. Sadlish, H. Rampelt, J. Shorter, R.D. Wegrzyn, C. Andreasson, S. Lindquist, B. Bukau, Hsp110 chaperones regulate prion formation and propagation in *S. cerevisiae* by two discrete activities, PLoS One 3 (2008) e1763.
[174] E.A. Fisher, M. Zhou, D.M. Mitchell, X. Wu, S. Omura, H. Wang, A.L. Goldberg, H.N. Ginsberg, The degradation of apolipoprotein B100 is mediated by the ubiquitin–proteasome pathway and involves heat shock protein 70, J. Biol. Chem. 272 (1997) 20427–20434.
[175] V. Albanese, A.Y. Yam, J. Baughman, C. Parnot, J. Frydman, Systems analyses reveal two chaperone networks with distinct functions in eukaryotic cells, Cell 124 (2006) 75–88.
[176] J.L. Goeckeler, A.P. Petruso, J. Aguirre, C.C. Clement, G. Chiosis, J.L. Brodsky, The yeast Hsp110, Sse1p, exhibits high-affinity peptide binding, FEBS Lett. 582 (2008) 2393–2396.
[177] M.A. Loo, T.J. Jensen, L. Cui, Y. Hou, X.B. Chang, J.R. Riordan, Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome, EMBO J. 17 (1998) 6879–6887.
[178] C. Schneider, L. Sepp-Lorenzino, E. Nimmesgern, O. Ouerfelli, S. Danishefsky, N. Rosen, F.U. Hartl, Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 14536–14541.
[179] R.M. Ferreira, L.R. de Andrade, M.B. Dutra, M.F. de Souza, V.M. Flosi Paschoalin, J.T. Silva, Purification and characterization of the chaperone-like Hsp26 from *Saccharomyces cerevisiae*, Protein Expr. Purif. 47 (2006) 384–392.
[180] M. Haslbeck, N. Braun, T. Stromer, B. Richter, N. Model, S. Weinkauf, J. Buchner, Hsp42 is the general small heat shock protein in the cytosol of *Saccharomyces cerevisiae*, EMBO J. 23 (2004) 638–649.

[181] M. Haslbeck, S. Walke, T. Stromer, M. Ehrnsperger, H.E. White, S. Chen, H.R. Saibil, J. Buchner, Hsp26: a temperature-regulated chaperone, EMBO J. 18 (1999) 6744–6751.
[182] H.E. White, E.V. Orlova, S. Chen, L. Wang, A. Ignatiou, B. Gowen, T. Stromer, T.M. Franzmann, M. Haslbeck, J. Buchner, H.R. Saibil, Multiple distinct assemblies reveal conformational flexibility in the small heat shock protein Hsp26, Structure 14 (2006) 1197–1204.
[183] C. Schuberth, A. Buchberger, Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation, Nat. Cell Biol. 7 (2005) 999–1006.
[184] O. Neuber, E. Jarosch, C. Volkwein, J. Walter, T. Sommer, Ubx2 links the Cdc48 complex to ER-associated protein degradation, Nat. Cell Biol. 7 (2005) 993–998.
[185] B. Medicherla, Z. Kostova, A. Schäfer, D.H. Wolf, A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation, EMBO Rep. 5 (2004) 692–697.
[186] S.M. Alberts, C. Sonntag, A. Schäfer, D.H. Wolf, Ubx4 modulates cdc48 activity and influences degradation of misfolded proteins of the endoplasmic reticulum, J. Biol. Chem. 284 (2009) 16082–16089.
[187] C. Hirsch, D. Blom, H.L. Ploegh, A role for N-glycanase in the cytosolic turnover of glycoproteins, EMBO J. 22 (2003) 1036–1046.

Appendix 6

Use of CPY* and Its Derivatives to Study Protein Quality Control in Various Cell Compartments

Alexandra Stolz and Dieter H. Wolf
Methods in Molecular Biology ahead of print

Chapter 35

Use of CPY* and its derivatives to study protein quality control in various cell compartments

Alexandra Stolz and Dieter H. Wolf

Institut für Biochemie, Universität Stuttgart

Address for correspondence:

Institut für Biochemie, Universität Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany

Telephone: (+49)-711-685-64390

Fax: (+49)-711-685-64392

E-mail: dieter.wolf@ibc.uni-stuttgart.de

Summary

Mutated derivatives of carboxypeptidase yscY (CPY) are potent substrates to study protein quality control and protein degradation in different cell compartments. Depending on the subcellular compartment of interest, the design of the model substrate used has to be adapted. Here we describe derivatives of CPY* used in genetic screens based on a sensitive growth test in order to identify new components of the protein quality control system and different degradation pathways.

Keywords: genetic screen, degradation, CPY*, protein quality control, ubiquitin proteasome system

1. Introduction

When in 1975 a mutant of the vacuolar enzyme carboxypeptidase yscY (CPY/ gene *PRC1*) of yeast was isolated to study the function of this peptidase, the importance of the mutant protein carrying the *prc1-1* mutation in seminal discoveries in the field of protein quality control was unforseeable ***(1)***. The discovery of ubiquitin-triggered protein degradation in cells and finally, the discovery of the enzyme complex, which *in vivo* degrades ubiquitylated proteins, the proteasome ***(2)***, were prerequisites for the beginning to understand how misfolded proteins are recognized by a cell and subsequently eliminated ***(3-5)***.

Elimination of secretory proteins came into focus because a variety of unassembled membrane receptor proteins were found to disappear before leaving the ER membrane ***(6, 7)***. At the time an ER localized proteolytic system was made responsible for this disappearence ***(6)***. Also, one cause of the very prominent heriditary disease cystic fibrosis was shown to

reside in a mutation, ΔF508, of the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel of the plasma membrane ***(8)***. Studies on CFTRΔF508 and a mutated Sec61 translocation channel of the ER membrane gave indication that the cytosolic ubiquitin-proteasome system was involved in the elimination of these ER membrane proteins ***(9-11)***.

Fig.1

The finding that the *prc1-1* mutant protein of yeast carboxypeptidase yscY, named CPY*, did not reach its final destination, the vacuole (lysosome), but was retained in the ER instead from where it was eliminated ***(12)***, opened the field to study the elimination mechanism of ER proteins via genetics, molecular biology and biochemistry (Fig. 1). A mutation in a highly conserved site of all serine peptidases, two amino acids away from the active site serine, Gly255Arg in carboxypeptidase yscY, renders this protein misfolded and it is rapidly degraded with a half life of 15 to 20 min ***(12)***. A standard genetic screen for mutants defective in degradation of CPY* at the ER led to the isolation of six so-called *der* mutants ***(13)***, which quickly uncovered the basic elimination mechanism of a fully glycosylated and completely imported misfolded soluble protein from the ER ***(14)***(re-published in ***(15)***: CPY* was imported into the lumen of the ER, fully glycosylated, retrograde transported back into the cytosol, polyubiquitylated and degraded by the proteasome (Fig. 2). The isolated *der* mutants uncovered in addition Ubc7 (Der2), which together with Ubc6 is the major ubiquitin conjugating enzyme of CPY* ***(14)*** and Der3 (also called Hrd1 for its function in regultated degradation of HMGCoA reductase ***(16)***, the six-transmembrane spanning RING-ubiquitin ligase (Fig. 2) ***(17, 18)***. In addition, Der1, a four transmembrane spanning protein of yet unknown function ***(13, 19, 20)***, suggested to play a role in the retrotranslocation process, was

discovered (Fig. 2). These studies also uncovered a link between misfolded protein in the ER and the unfolded protein response ***(13)***. The fact, that trimming of the N-carbohydrate in the ER lumen is an important degradation determinant, was discovered by using an α-1,2-mannosidase I deletion mutant ***(19)*** (Fig. 2). Further elaboration of this topic showed that undisturbed degradation of CPY* required in addition rapid removal of the three terminal glucose residues of the carbohydrate chain by glucosidases I and II prior to α-1,2-mannosidase action ***(20, 21)***. As α-1,2-mannosidase is a Ca^{2+} dependent enzyme, not surprisingly, the *DER5* gene encoding the Ca^{2+} pump Pmr1, was found to be required for CPY* degradation ***(22)***. In addition, the lectin Htm1/Mnl1, later discovered as having an additional α-1,2-mannosidase activity and generating the $Man_7GlcNAc_2$ oligosaccharide on CPY*, was found to be a necessary component for rapid removal of the misfolded enzyme (Fig. 2) ***(23-25)***. A timer model was proposed which allows the protein to fold until the slow acting mannosidases Mns1 and Htm1/Mnl1 get their hand on the carbohydrate chain to trim it down to $Man_7GlcNAc_2$, containing a crucial α-1,6 linked mannose residue ***(23)***. The discovery of Yos9 on the basis of a genome-wide screen and its elucidation as a lectin ***(26)*** binding the trimmed carbohydrate on CPY* led to the proposal that it acts as a "gatekeeper" component allowing only misfolded ER proteins carrying the Man_7 structure to leave the ER for degradation (Fig. 2) ***(23, 24, 27-29)***. The use of CPY* for a closer look into the requirement of distinct carbohydrate chains for elimination uncovered, that from the four carbohydrate chains of the enzyme only the chain linked to Asn368 is necessary and sufficient for degradation ***(30, 31)***. The discovery of a similar signal in misfolded proteinase yscA (PrA*; ***(12)***) supported the generality of the mechanism ***(31)***. Using CPY* as the model, a carbohydrate adjacent peptide segment required for degradation was found, indicating a bipartite signalling mechanism ***(32)***. The study of Kostova and Wolf ***(30)*** showed that there

must be a second elimination mechanism working, which is able to dispose also non-glycosylated misfolded proteins of the ER lumen, however, with a slower rate.

First hand recognition of not properly folded proteins in the ER lumen based on the exposure of a disordered protein structure became obvious when the dependence of CPY* degradation on the ER luminal Hsp70 chaperone Kar2 (BiP) ***(33, 34)*** and its J-domain partners Jem1 and Scj1 ***(35)*** was discovered. On the basis of CPY* recruitment a Hrd1/Der3 complex of the ER membrane was biochemically defined, which consists of the ubiquitin ligase Hrd1/Der3 and Hrd3, a single membrane spanning protein, which is required for Hrd1/Der3 ligase stability (Fig. 2) ***(36, 37)***. Hrd1/Der3 degradation in the absence of Hrd3 also occurs via the ERAD system. Most interestingly, rapid disappearence of Hrd1/Der3 in *ΔHRD3* cells is completely blocked by a conditional mutation in the translocon protein Sec61, indicating a close interaction of the Hrd1/Der3-Hrd3 complex and Sec61 (***(36)***, see below). Hrd3 acts as a docking factor for misfolded proteins ***(38-40)***. In the ER lumen this core complex recruits the lectin Yos9 which itself interacts with Kar2 ***(40)***. Hrd3p, Yos9 and Kar2 act as a "gatekeeper" device allowing only misfolded proteins to be retro-translocated to the cytosol for ubiquitin-triggered proteasomal degradation (Fig. 2) ***(40)***.

The composition and assembly of the retro-translocation complex RTC for CPY* in the ER membrane is under intensive investigation. Core part is the ubiquitin ligase complex Hrd1/Der3 associated with the substrate receptor Hrd3 (***(36)***; see above). A novel ER membrane protein, Usa1, was found ***(39)*** which links the earlier found four transmembrane spanning Der1 protein ***(19, 20)*** to the Hrd1/Der3 ligase complex ***(41)***. On the basis of being polytopic membrane proteins, Der1 as well as the Hrd1/Der3 ligase have been proposed to be part of the retro-translocation channel. As expected for an E3 ligase, Hrd1/Der3 interacts with a misfolded CPY* derivative ***(41-43)***. Very early in ERAD research genetic studies gave indication that the protein translocation channel Sec61 may be also involved in retro-

translocation of CPY* ***(33, 36)***. Recent genetic studies using a different experimental set up corroborated the previous assumption ***(44)***. Indeed, biochemical studies were able to show interaction of CPY*, the Hrd/Der ligase complex and in addition Sec61. It is therefore likely, that the RTC passing CPY* from the ER lumen to the cytosol is composed of the Hrd/Der ligase complex and Sec61, whereby Sec61 represents a very dynamic component of this assembly of not yet precisely defined role ***(45)*** (Fig. 2).

Fig. 2

CPY* also unraveled the further pathway of misfolded ER protein after retro-translocation into the cytosol ***(14)***. Ubc7, recruited to the ER membrane via the membrane anchor Cue1 ***(46)***, turned out to be the major ubiquitin conjugating enzyme, which together with the RING-finger ubiquitin ligase Hrd1/Der3 conjugates a polyubiquitin chain onto CPY* and prevents its slipping back into the ER lumen ***(17, 18, 47)***. Further delivery to the proteasome is carried out by the AAA-ATPase Cdc48 complexed with the adaptor proteins Ufd1 and Npl4 ***(48-50)***, which is recruited to the ER membrane by the Ubx2 protein ***(51, 52)***. Action of the Cdc48-Ufd1-Npl4 complex is modulated by the cofactor Ubx4 ***(53)***. The ubiquitin receptors Dsk2 and Rad23 are in addition prominently involved in the substrate delivery process ***(54)*** (Fig. 2).

For elucidation of mechanisms of ERAD of single membrane spanning proteins containing the misfolded protein domain in the ER lumen, a spectrum of substrate fusions was constructed, all containing the luminal CPY* moiety and the last transmembrane domain of the multidrug resistance transporter Pdr5. The three substrates designed were CT*, containing the transmembrane domain of Pdr5* and CPY*, CTG* harboring in addition the domain of the green fluorescent protein (GFP) in the cytosol and CTL* (or CTL*myc), having the Leu2 protein (3-isopropylmalate dehydrogenase) domain instead of GFP in the cytosol ***(54-56)*** (Fig. 1). Experiments following degradation of these fusion proteins uncovered a certain

variability of components required for their elimination: In contrast to soluble, luminal CPY*, CT* and CTG* do not require the ER located Hsp70, Kar2 (BiP), for degradation. Furthermore CTG* is the only substrate of the tested ones which depends on the cytosolic Hsp70 chaperones of the Ssa family for elimination ***(56)***. A comparison between the two substrates with large cytosolic domains, CTG* and CTL*myc, shows that only CTL*myc required the proteasome associated ubiquitin ligase Hul5 for complete degradation: in its absence only the CPY* domain is degraded while the transmembrane domain fused to the Leu2 domain remains stable ***(55)***. These studies demonstrate the high flexibility and plasticity of the ERAD system.

CPY* and fusions with GFP as well as with Leu2 did not only lead to breakthroughs in the discovery of the ERAD system, but also in the protein quality control and elimination of misfolded proteins of the cytoplasm ***(57)***. Deletion of the signal sequence of CPY* or CPY*-GFP, preventing import of these proteins into the ER, yielded in localization of these misfolded species in the cellular cytosol. Following their fate unravelled crucial components of cytoplasmic protein quality control. The major chaperone pair required for solubilization of precipitated misfolded protein and its guidance to the degradation machinery, the proteasome, turned out to be Ssa1-Ydj1 of the cytosolic Hsp70-Hsp40 chaperone machinery. As ubiquitin conjugating enzymes, Ubc4 and Ubc5 were found ***(57)***. A genome-wide screen for the cytoplasmic ubiquitin ligase responsible for polyubiquitylation uncovered Ubr1 ***(58)*** (Fig. 3). Depending on the ΔssCPY* fusion protein used for search of ubiquitin ligases of cytoplasmic misfolded protein, the nuclear E3 ligase San1 was discovered in addition ***(59)***. As found for ERAD, one may expect a high degree of flexibility and plasticity also in the cytosolic proteostasis network.

Fig. 3

2. Materials

2.1 Equipment

1. 96well microtiter plates (VWR, Darmstadt, Germany)
2. square (120x120 mm) petri dishes (Greiner Bio-one, Frickenhausen, Germany)
3. 30°C incubator (with and without shaker)
4. 42°C incubator
5. autoclave
6. round toothpicks (VWR, Darmstadt, Germany)
7. (multichannel) pipettes, tips and other standard laboratory tools and materials
8. 48 (6x8) or 96 (8x12) pin replicator: the replicator is needed to transfer cells and cell solution. To save time (in step 7 of the protocol) disposable plastic replicators for cell transfer are available (Genetix GmbH, Dornbach, Germany).
To transfer drops of cell suspensions as well as for step 3 replicators made out of steel are preferred. So-called slot pin replicators and cleaning solutions are commercially available (V&P scientific, San Diego, USA; general information about all kind of pins are available on the homepage of V&P under 'pin tools'). If a workshop is available at your place you may get a much cheaper, less volume defined, self-made replicator. Pins (stainless steel) should have a diameter of 3 mm with slight concave surface at the end. Drops transferred with such a replicator should have a volume of approximately 5 µl.

2.2 Solutions

Prepare all solutions with autoclaved deionized water. Chemicals should be ultra pure and adequate for cell biology. Reagents and solutions can be stored at room temperature unless indicated otherwise. Liquid media and plates should be stored in the dark. Plasmid suspension

should be stored at -20°C. Solutions have to be sterile and mixing should occur under sterile conditions.

1. 90 % (w/v) PEG 600 (Polyethylenglycol): Dissolve 90 g of PEG 600 in water (final volume 100 ml). Autoclave solution for 20 min at 121 °C.

2. 4 M lithium acetate (LiOAc): Dissolve 26,4 g of LiOAc (MG 65,99 g/mol) in water (final volume 100 ml). Autoclave solution for 20 min at 121 °C.

3. 1M dithiothreitol (DTT): Dissolve 1,54 g in water (final volume 10 ml). Filter sterilize (bottle top filter; 0,2 µm pore size). Store in aliquots at -20 °C.

4. Transformation solution for one 96 well microtiter plate: Mix 1,5 ml 90 % PEG 600 with 250 µl 4 M LiOAc and 250 µl 1 M DTT. Add 500 µl of plasmid suspension (*see* **Note 4**). Prepare fresh on the day of use.

2.3 Media

1. YPD: liquid media contains 1 % (w/v) yeast extract, 2 % (w/v) Bacto™ Peptone and 2 % (w/v) D-glucose. Mix and adjust pH (5.5) with NaOH and HCl. Solid YPD media contains in addition 2 % Bacto™ Agar. Autoclave for 20 min at 121 °C.

2. CM media: liquid media contains 0,67 % (w/v) Yeast nitrogen base w/o amino acids, 2 % (w/v) D-glucose and 0,0117 % (w/v) of L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, L-glycine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine, L-myo-inositol and aminobenzoic acid. Mix and adjust pH (5.5) with NaOH and HCl. Autoclave for 20 min at 121 °C. Depending on the selection media add sterile 0.3 mM L-adenine hemisulfate, 0.3 mM L-histidine monohydrate, 1,0 mM L-lysine, 0,4 mM L-tryptophane and 1,7 mM L-leucine (omit in CM –ura –leu). Solid CM media contains in addition 2% Bacto™ Agar. (*see* **Notes 5** and **6**).

2.4 Yeast strains

For this screen one available yeast mutant library is the EUROSCARF collection containing over 5000 mutant strains each deleted in a single gene (University of Frankfurt, Germany; *see* **Notes 2** and **3**). The collection is available as a haploid (*MATa* or *MATα*) and diploid homozygous or heterozygous (including essential genes) strain collection. The diploid heterozygous collection contains over 6000 strains including deletions of essential genes. To perform the screen with a subsequent first analysis one should plan a time frame of about 4-6 months.

Cells carrying a *LEU2* deletion are unable to grow on media lacking leucine. However, if the substrates CTL* or ΔssCL* are present in these cells, leucine can be synthesized due to the Leu2 domain and cells are able to grow. This growth is suppressed by the degradation of the substrates due to efficient protein quality control and associated protein degradation systems. Strains with defects in protein quality control and protein degradation are delayed in these processes and thereby the presence of Leu2 in the cell is prolonged in time allowing growth of cells on media lacking leucine.

3. Method

Carry out all procedures at room temperature unless otherwise specified. Use sterile materials and solutions.

1. Choose the substrate (ΔssCL*myc in pRS316 vector, *see* **Note 1**) and adequate promoter. Strength of the promoter has to be adapted to the substrate's half-life and its subcellular location. Perform a pilot test prior to the screen using negative (wild type) and positive (proteasomal mutant) control strains. Transformed with the chosen substrate, wild type cells

should be unable to grow on media lacking uracil (-ura) and leucine (-leu) while the proteasomal mutant should grow due to retarded degradation of the substrate. Both strains should be able to grow on CM –ura media. In the case of CTL* the leaky non-induced Gal4 promoter allows sufficient expression, while degradation of ΔssCL* in the cytosol appears to be much faster. Therefore the stronger CPY promoter is more suitable for this screen (*see* **Note 7**). To start the main screen, prepare YPD plates in square (120x120 mm) petri dishes and label them according to the EUROSCARF strain collection (*see* **Notes 2** and **8**). The collection contains more than 5000 strains. It is therefore reasonable to subdivide the screen into units. 5-6 microtiter plates per round are doable. Transfer yeast strains from 96 well microtiter glycerol stock onto YPD plates with the help of a replicator.

2. Make sure to efficiently clean and sterilize the replicator in between transfers to avoid mixing and contaminating strains (*see* **Notes 9** and **10**).

3. Incubate plates for 2-3 days at 30 °C until colonies have formed but individual strains did not grow into one another.

4. Provide 25 µl of transformation solution to each well of a 96 well microtiter plate.

5. Transfer cells from YPD plates into the transformation solution in the 96 well microtiter plate with the help of a replicator. Release cells from the pins by gently shaking the replicator while dipping it into the transformation solution.

6. When not using disposable replicators, make sure to efficiently clean and sterilize the replicator in between the single transfers to avoid mixing and contaminating strains (*see* **Notes 9** and **10**).

7. Incubate for 1 h at 42 °C.

8. Selection of the transformants occurs at 30 °C on selection media: CM -ura. Two possibilities exist for transformant selection: 1) Use mulitichannel pipette or replicator to transfer a drop of each transformation on plates with selection media (CM-ura, *see* **Note 6** and

11). 2) (Preferred; *see* **Note 12**) Transfer total volume of the transformation with a pipette onto selection plate (*see* **Note 6**). The rate of transformation is about 80%.

9. Incubate at 30°C until colonies have formed (*see* **Note 13**).

Fig. 4

10. Deletion strains that were not transformed have to be transformed again individually e.g. using the standard heat shock lithium acetate method ***(60)*** or the procedure has to be repeated in a second 96 well approach together with outstanding transformations (*see* **Note 14**).

11. Analyze transformants for growth on media lacking uracil (growth control) and media lacking leucine and uracil. This can be done by frogging or streaking of cells on solid media.

12. Streaking: This method is faster and saves material. Take a sterile toothpick of cells and streak them out as illustrated in Fig. 5A. Use one side of a toothpick to make the first streak then turn toothpick for the second one. First and second streak should be thick and contain a relatively high amount of cells (find proper amount of cells in the pilot test of wild type and proteasomal mutant – step 1). Take a new toothpick for the third streak. Each strain has to be streaked on CM –ura and CM –ura –leu (*see* **Note 15**).

13. Frogging: This method is more time consuming but allows identification of only milder growth effects. Resuspend cells from the plate in 500 µl 1.2 M sorbitol. Measure OD_{600} of the cell suspension and harvest 4 OD of cells. Resuspend cells in 200 µl 1.2 M Sorbitol and transfer into microtiter plates. Prepare 1:10 dilution series of the cell suspension and transfer a drop of each well with the help of a replicator onto plates containing CM –ura and plates containing CM –ura –leu (Fig. 5B; *see* **Notes 9** and **11**).

14. Incubate plates at 30 °C until colonies have formed (*see* **Note 13**).

Fig. 5

15. Identify strains that show improved growth on CM-ura –leu compared to wild type (*see* **Note 16**).

16. To confirm candidates and sort out false positives perform more specific analyses e.g. cycloheximide or pulse chase analyses (*see* **Note 17**).

4. Notes

1. In principle every protein of interest fused to a Leu2 domain can be used as a substrate if major characteristics are not altered or suppressed by the fusion. Please note that efficient growth is only supported if the Leu2 domain is located in the cytosol, or the chimeric protein stays long enough in the cytosol prior to delivery to its site of action.

2. For specific qustions more specific mutant collections can be used in order to minimize time and effort. E.g. collection of potential ubiquitin ligases (E3s) ***(59)***. Basic requirement is a leucine deficiency of the host cell due to mutations in the *LEU2* gene.

3. The EUROSCARF library is delivered on YPD agar in 96well microtiter plates. For continuous use, it is advised to prepare stocks in similar multiwell plates containing YPD and 15% glycerol for storage at -80°C. Growth of some mutant strains is very poor. One may have to cultivate them separately on YPD plates before storage to get sufficient cell material.

4. Concentration of DNA is recommended to be higher than 5 µg/µL. For efficient plasmid isolation one can make a maxi prep ***(61)***. Omit the use of RNAse. Remaining RNA compensates for the absence of single stranded DNA as a carrier thereby increasing transformation efficiency and reducing the cost of the experiment.

5. For efficiency prepare 2x CM media without D-glucose, split, autoclave and store in the dark. Prepare and autoclave separately 40% D-glucose, 4% Bacto™ agar and stock solutions of the complement amino acids (except L-tryptophan). Tryptophan is light and heat sensitive and therefore shouldn't be autoclaved but filter sterilized instead for preperation of the stock solution. Boil agar in the microwave until cords disappear. Add same amount of 2x CM media, glucose (1/20 Vol) and complementary amino acids from stock solutions.

6. Level the surface of plates during pouring. Otherwise drops will drain and mix.

7. Media containing only 1 % glucose should be used in order to induce the CPY promoter.

8. Each plate of the EUROSCARF collection contains some empty wells thereby exhibiting a specific pattern. Hence labeling errors can be easily noticed and corrected.

9. For best time efficiency use several stamps. Clean first in water bath, dab water away with sterile velvet cloth and subsequently dip pins in 70% ethanol and flame. Let the replicator cool down. Change water and ethanol regularly.

10. If you have stored strains with poor growth separately, you can now streak them on the plate where they originally belonged according to the position of the deletion in the genome. There is enough space at the bottom of the plates if you arrange your stamping properly.

11. Resuspend cells before transfer by gently mixing the cell suspension with the replicator. Check efficiency and consistency of the transport of liquid on the replicator or on the plate after transfer. If you have problems to receive liquid drops on the pins of the replicator, try to withdraw the replicator faster from the cell suspension.

12. Method one is faster, but the drops tend to consolidate if too much volume was used. However, use of too little volume causes low transformation efficiency. Method 2 takes longer, but transformation efficiency is much higher.

13. Cells on CM -ura need to grow about 2-3 days and on CM -ura –leu about 5-6 days. Scan plates with interesting growth effects for comparison at the end of the screen.

14. You can transform strains for the second time in one of the empty wells together with one of the successional batches (*see* **Note 8**).

15. Your streaking may vary with your daily condition and mood. Therefore always include a positive (proteasomal mutant) and negative wild type control with every batch streaked.

16. Strains that show a general growth defect (see CM –ura control plates) may be positive candidates even though they do not show much elevated growth than wild type on CM –ura –leu plates.

17. The myc tag is not necessary for the screen. However it allows to identify remaining C-terminal degradation fragments during degradation process immunologically ***(55)***.

Acknowledgements

We thank Frederik Eisele, Mario Scazzari and Hisashi Hoshida for essential help and Hans Rudolph and Elena Martinez Benitez for critical reading. The work was supported by the EU network of excellence RUBICON, the Deutsche Forschungsgemeinschaft (Bonn, Germany) and the Fonds der Chemischen Industrie (Frankfurt, Germany).

References:

1. Wolf DH, Fink GR (1975) Proteinase C (carboxypeptidase Y) mutant of yeast. J Bacteriol **123**:1150-1156.
2. Heinemeyer W, Kleinschmidt JA, Saidowsky J et al (1991) Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. EMBO J **10**:555-562.
3. Wolf DH, Hilt W (2004) The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. Biochim Biophys Acta **1695**:19-31.
4. Hershko A, Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem **67**:425-479.
5. Varshavsky A (1997) The ubiquitin system. Trends Biochem Sci **22**:383-387.
6. Klausner RD, Sitia R (1990) Protein degradation in the endoplasmic reticulum. Cell **62**:611-614.
7. Bonifacino JS, Lippincott-Schwartz J (1991) Degradation of proteins within the endoplasmic reticulum. Curr Opin Cell Biol **3**:592-600.

8. Cheng SH, Gregory RJ, Marshall J et al (1990) Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell **63**:827-834.
9. Jensen TJ, Loo MA, Pind S et al (1995) Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. Cell **83**:129-135.
10. Ward CL, Omura S, Kopito RR (1995) Degradation of CFTR by the ubiquitin-proteasome pathway. Cell **83**:121-127.
11. Sommer T, Jentsch S (1993) A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. Nature **365**:176-179.
12. Finger A, Knop M, Wolf DH (1993) Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast. Eur J Biochem **218**:565-574.
13. Knop M, Finger A, Braun T et al (1996) Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. EMBO J **15**:753-763.
14. Hiller MM, Finger A, Schweiger M, Wolf DH (1996) ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. Science **273**:1725-1728.
15. Linder P, Shore D, Hall MN. (2006) Landmark Papers in Yeast Biology, in *Cold Spring Harbor Laboratory Press*, pp 273-274.
16. Hampton RY, Gardner RG, Rine J (1996) Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Mol Biol Cell **7**:2029-2044.
17. Bordallo J, Plemper RK, Finger A, Wolf DH (1998) Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol Biol Cell **9**:209-222.
18. Deak PM, Wolf DH (2001) Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. J Biol Chem **276**:10663-10669.
19. Knop M, Hauser N, Wolf DH (1996) N-Glycosylation affects endoplasmic reticulum degradation of a mutated derivative of carboxypeptidase yscY in yeast. Yeast **12**:1229-1238.
20. Hitt R, Wolf DH (2004) Der1p, a protein required for degradation of malfolded soluble proteins of the endoplasmic reticulum: topology and Der1-like proteins. FEMS Yeast Res **4**:721-729.
21. Jakob CA, Burda P, Roth J, Aebi M (1998) Degradation of misfolded endoplasmic reticulum glycoproteins in Saccharomyces cerevisiae is determined by a specific oligosaccharide structure. J Cell Biol **142**:1223-1233.
22. Durr G, Strayle J, Plemper R et al (1998) The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca2+ and Mn2+ required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. Mol Biol Cell **9**:1149-1162.
23. Clerc S, Hirsch C, Oggier DM et al (2009) Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum. J Cell Biol **184**:159-172.
24. Quan EM, Kamiya Y, Kamiya D et al (2008) Defining the glycan destruction signal for endoplasmic reticulum-associated degradation. Mol Cell **32**:870-877.
25. Jakob CA, Bodmer D, Spirig U et al (2001) Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast. EMBO Rep **2**:423-430.
26. Buschhorn BA, Kostova Z, Medicherla B, Wolf DH (2004) A genome-wide screen identifies Yos9p as essential for ER-associated degradation of glycoproteins. FEBS Lett **577**:422-426.

27. Bhamidipati A, Denic V, Quan EM, Weissman JS (2005) Exploration of the topological requirements of ERAD identifies Yos9p as a lectin sensor of misfolded glycoproteins in the ER lumen. Mol Cell **19**:741-751.
28. Kim W, Spear ED, Ng DT (2005) Yos9p detects and targets misfolded glycoproteins for ER-associated degradation. Mol Cell **19**:753-764.
29. Szathmary R, Bielmann R, Nita-Lazar M et al (2005) Yos9 protein is essential for degradation of misfolded glycoproteins and may function as lectin in ERAD. Mol Cell **19**:765-775.
30. Kostova Z, Wolf DH (2005) Importance of carbohydrate positioning in the recognition of mutated CPY for ER-associated degradation. J Cell Sci **118**:1485-1492.
31. Spear ED, Ng DT (2005) Single, context-specific glycans can target misfolded glycoproteins for ER-associated degradation. J Cell Biol **169**:73-82.
32. Xie W, Kanehara K, Sayeed A, Ng DT (2009) Intrinsic conformational determinants signal protein misfolding to the Hrd1/Htm1 endoplasmic reticulum-associated degradation system. Mol Biol Cell **20**:3317-3329.
33. Plemper RK, Bohmler S, Bordallo J et al (1997) Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature **388**:891-895.
34. Brodsky JL, Werner ED, Dubas ME et al (1999) The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct. J Biol Chem **274**:3453-3460.
35. Nishikawa SI, Fewell SW, Kato Y et al (2001) Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. J Cell Biol **153**:1061-1070.
36. Plemper RK, Bordallo J, Deak PM et al (1999) Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. J Cell Sci **112 (Pt 22)**:4123-4134.
37. Gardner RG, Swarbrick GM, Bays NW et al (2000) Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. J Cell Biol **151**:69-82.
38. Gauss R, Sommer T, Jarosch E (2006) The Hrd1p ligase complex forms a linchpin between ER-lumenal substrate selection and Cdc48p recruitment. EMBO J **25**:1827-1835.
39. Carvalho P, Goder V, Rapoport TA (2006) Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell **126**:361-373.
40. Denic V, Quan EM, Weissman JS (2006) A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. Cell **126**:349-359.
41. Horn SC, Hanna J, Hirsch C et al (2009) Usa1 functions as a scaffold of the HRD-ubiquitin ligase. Mol Cell **36**:782-793.
42. Carvalho P, Stanley AM, Rapoport TA (2010) Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. Cell **143**:579-591.
43. Mehnert M, Sommer T, Jarosch E (2010) ERAD ubiquitin ligases: multifunctional tools for protein quality control and waste disposal in the endoplasmic reticulum. Bioessays **32**:905-913.
44. Willer M, Forte GM, Stirling CJ (2008) Sec61p is required for ERAD-L: genetic dissection of the translocation and ERAD-L functions of Sec61P using novel derivatives of CPY. J Biol Chem **283**:33883-33888.
45. Schafer A, Wolf DH (2009) Sec61p is part of the endoplasmic reticulum-associated degradation machinery. EMBO J **28**:2874-2884.

46. Biederer T, Volkwein C, Sommer T (1997) Role of Cue1p in ubiquitination and degradation at the ER surface. Science **278**:1806-1809.
47. Bordallo J, Wolf DH (1999) A RING-H2 finger motif is essential for the function of Der3/Hrd1 in endoplasmic reticulum associated protein degradation in the yeast Saccharomyces cerevisiae. FEBS Lett **448**:244-248.
48. Jarosch E, Taxis C, Volkwein C et al (2002) Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nat Cell Biol **4**:134-139.
49. Ye Y, Meyer HH, Rapoport TA (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature **414**:652-656.
50. Rabinovich E, Kerem A, Frohlich KU et al (2002) AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. Mol Cell Biol **22**:626-634.
51. Neuber O, Jarosch E, Volkwein C et al (2005) Ubx2 links the Cdc48 complex to ER-associated protein degradation. Nat Cell Biol **7**:993-998.
52. Schuberth C, Buchberger A (2005) Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. Nat Cell Biol **7**:999-1006.
53. Alberts SM, Sonntag C, Schafer A, Wolf DH (2009) Ubx4 modulates cdc48 activity and influences degradation of misfolded proteins of the endoplasmic reticulum. J Biol Chem **284**:16082-16089.
54. Medicherla B, Kostova Z, Schaefer A, Wolf DH (2004) A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. EMBO Rep **5**:692-697.
55. Kohlmann S, Schafer A, Wolf DH (2008) Ubiquitin ligase Hul5 is required for fragment-specific substrate degradation in endoplasmic reticulum-associated degradation. J Biol Chem **283**:16374-16383.
56. Taxis C, Hitt R, Park SH et al (2003) Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. J Biol Chem **278**:35903-35913.
57. Park SH, Bolender N, Eisele F et al (2007) The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin-proteasome system. Mol Biol Cell **18**:153-165.
58. Eisele F, Wolf DH (2008) Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1. FEBS Lett **582**:4143-4146.
59. Heck JW, Cheung SK, Hampton RY (2010) Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1. Proc Natl Acad Sci U S A **107**:1106-1111.
60. Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol **350**:87-96.
61. Heilig JS, Elbing KL, Brent R (2001) Large-scale preparation of plasmid DNA. Curr Protoc Mol Biol **Chapter 1**:Unit1 7.

Figures and Legends

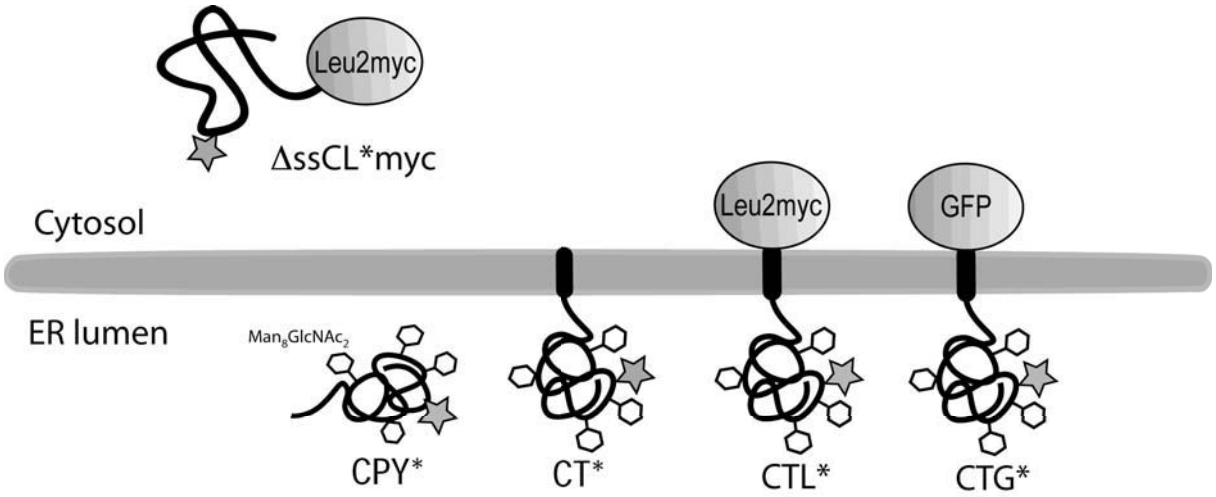


Figure 1: CPY* and its derivatives. Several derivatives of misfolded CPY* carrying the mutation G255R have been developed. In order to study ER membrane proteins, CPY* was fused to a transmembrane domain resulting in the substrate CT* ***(56)***. Expanding the substrate CT* with additional cytosolic protein domains like GFP (green fluorescent protein) or Leu2 (3-isopropylmalate dehydrogenase), resulting in CTG* and CTL*, respectively, broadened the possibilities of the studies ***(54, 56)***. To allow analysis of cytosolic substrates the signal sequence (ss) of CPY* was removed. Like CPY*, ΔssCPY* can be fused to several proteins like GFP or Leu2.

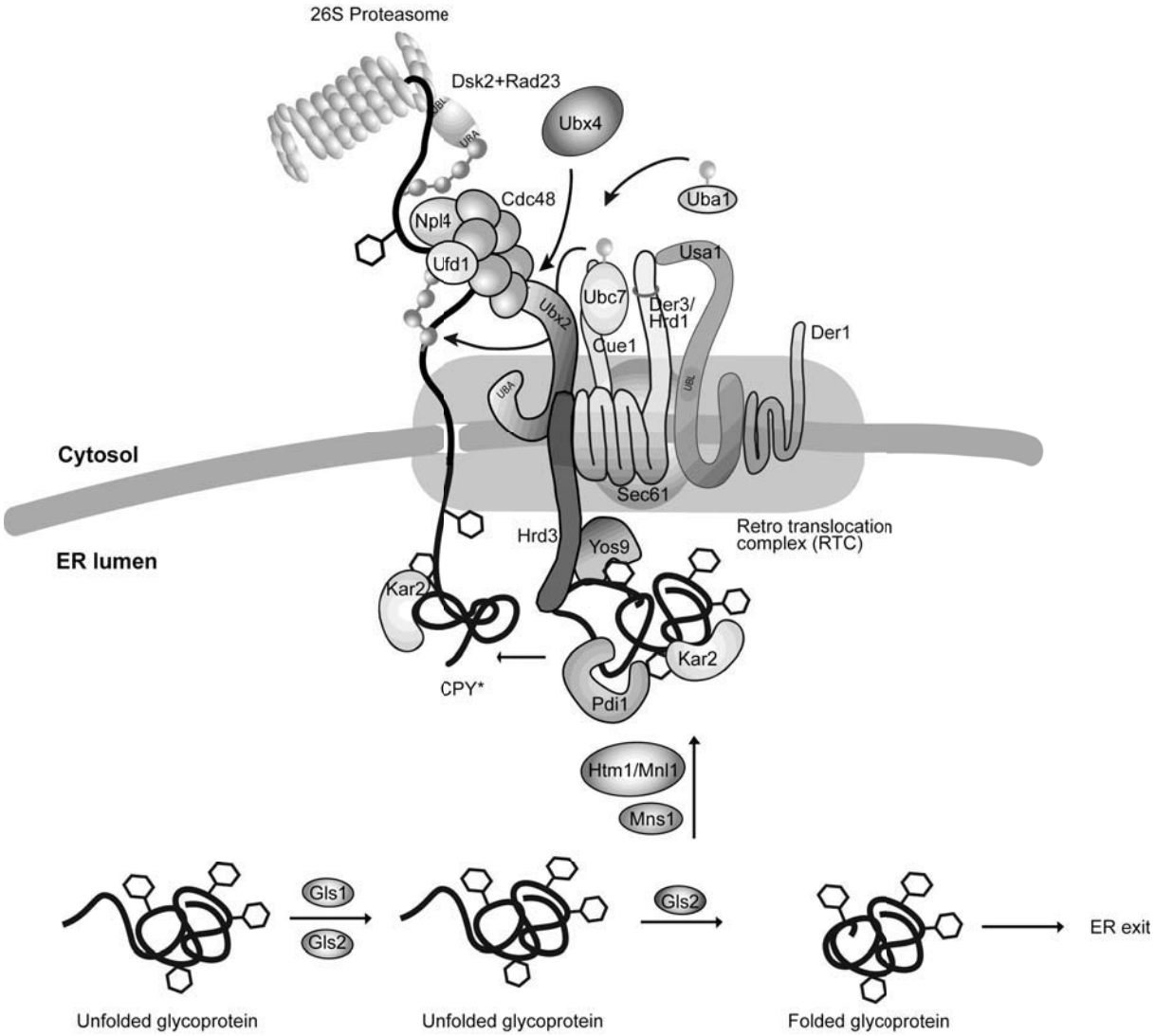


Figure 2: Schematic illustration of the ERAD system responsible for recognition and degradation of CPY*.

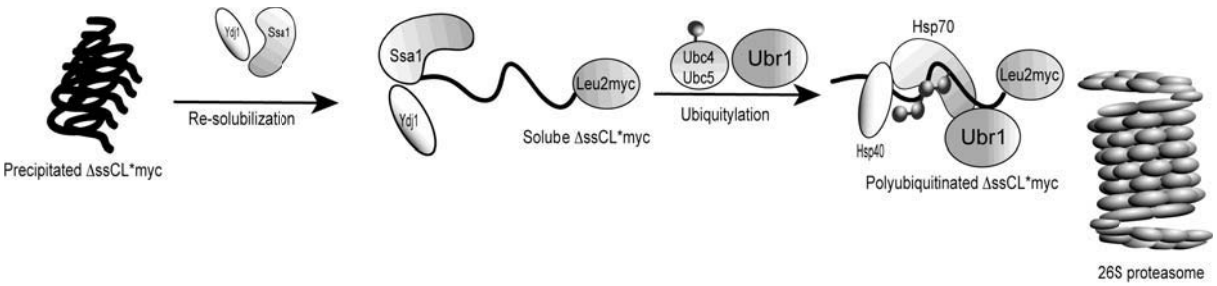


Figure 3: Schematic illustration of the cytocolic system responsible for recognition and degradation of ΔssCL*myc.

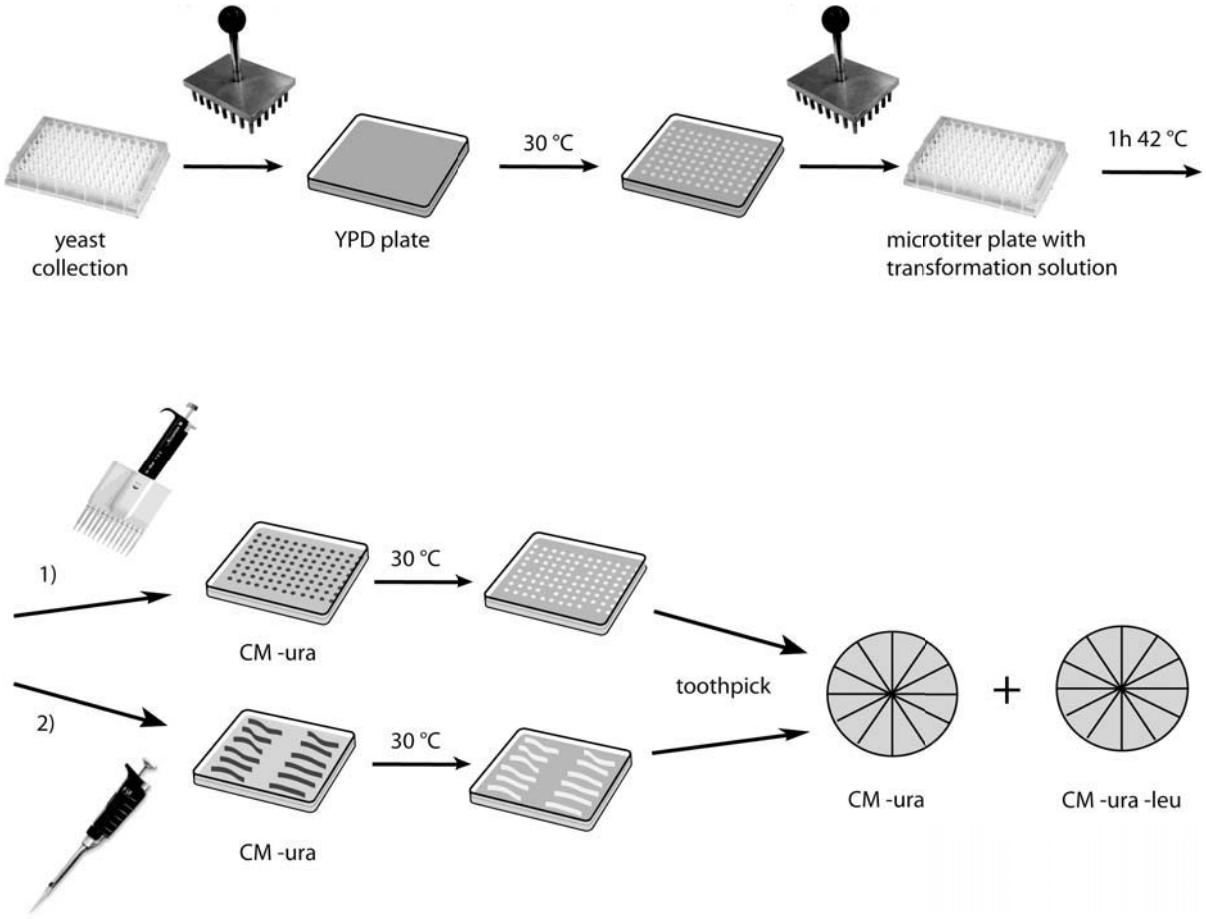
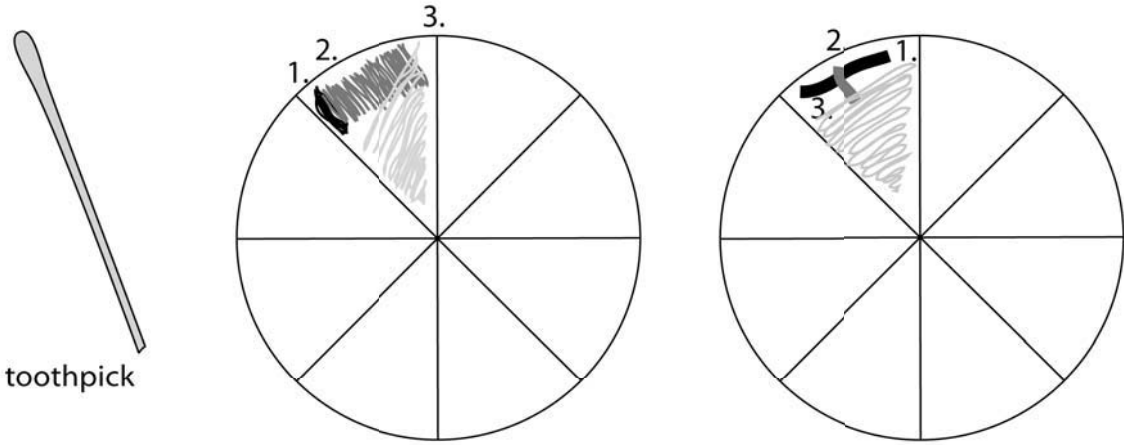
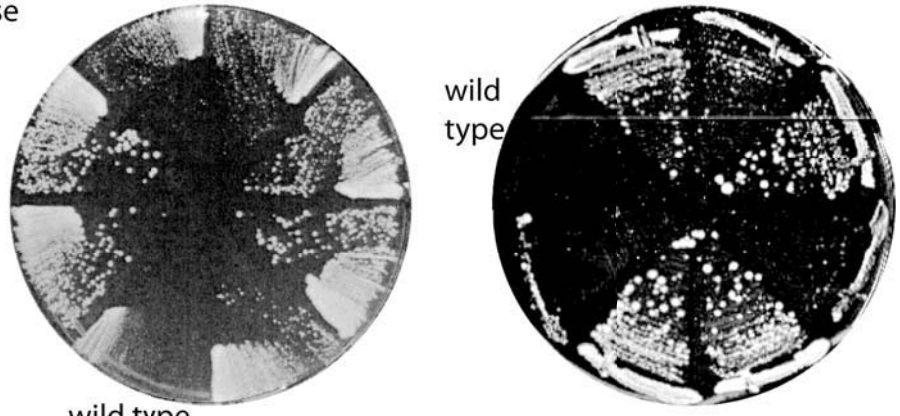


Figure 4: Schematic illustration of the genetic screen. Cells of a yeast mutant collection are transferred into microtiter plates and transformed with a plasmid carrying the sequence of the substrate of interest. Transformants of mutant strains are subsequently compared with wild type cells for growth differences.

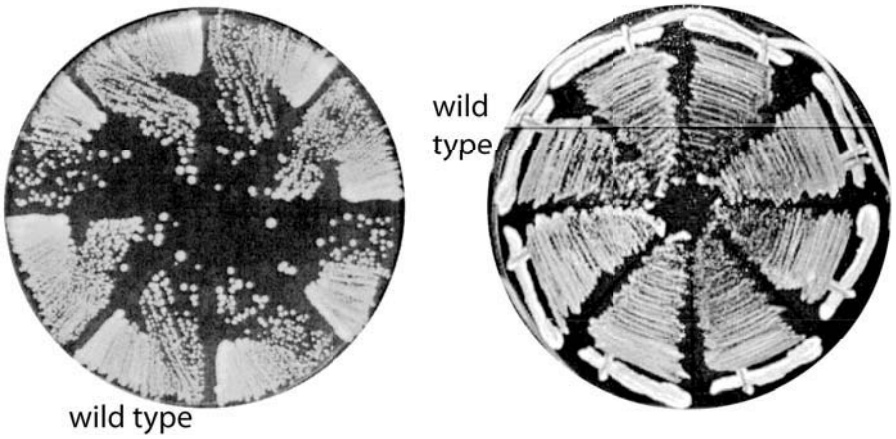
A



CM -ura -leu 1 % Glucose



CM -ura 1 % Glucose (control)



B

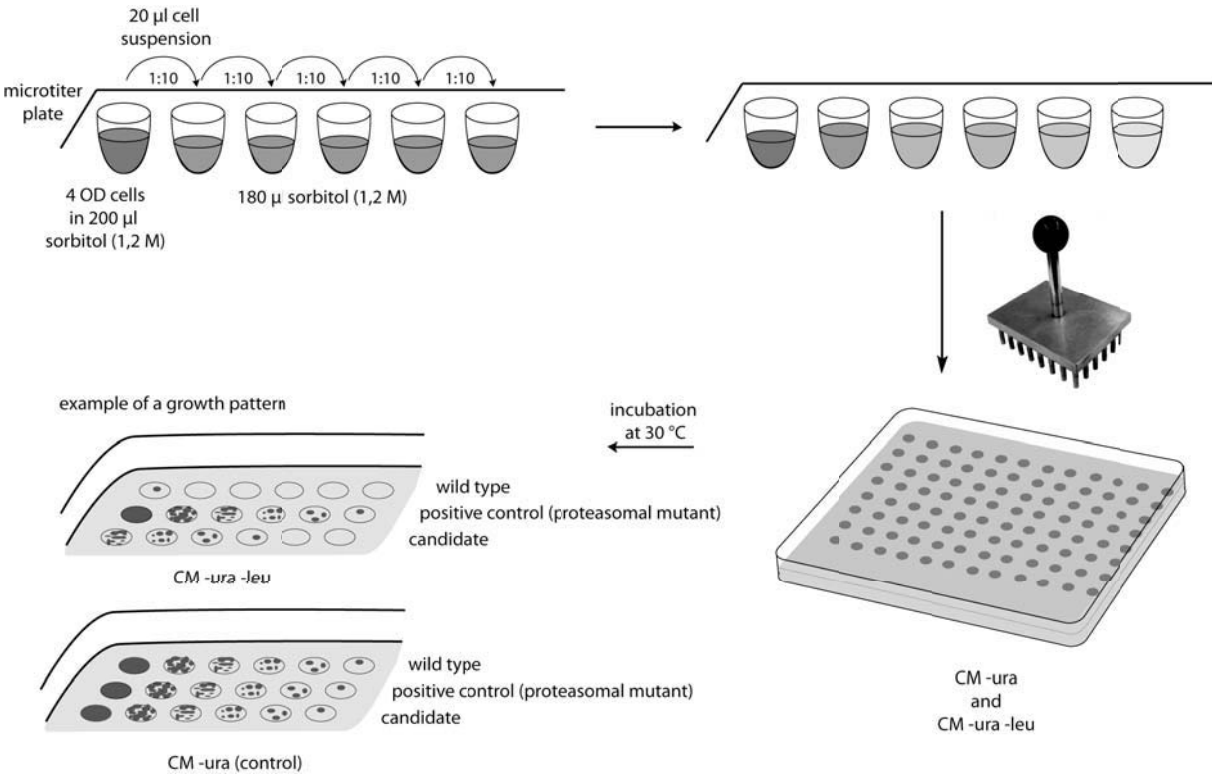


Figure 5: A) Cartoon and examples illustrating two techniques for streaking out cells to single colonies (M. Scazzari 2007; Diploma Thesis; University of Stuttgart). B) Schematic illustration of the preperation of a cell dilution series and subsequent transfer of cell suspension onto selection plates.

Appendix 7

Cdc48: a power machine in protein degradation

Alexandra Stolz, Wolfgang Hilt, Alexander Buchberger and Dieter H. Wolf
Trends Biochem Sci. 2011 Oct;36(10):515-23

Review

Cdc48: a power machine in protein degradation

Alexandra Stolz[1], Wolfgang Hilt[1], Alexander Buchberger[2] and Dieter H. Wolf[1]

[1] Institute for Biochemistry, University of Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany
[2] Department of Biochemistry, Biocenter, University of Würzburg, Am Hubland, 97074 Würzburg, Germany

Cdc48 is an essential, highly prominent ATP driven machine in eukaryotic cells. Physiological function of Cdc48 has been found in a multitude of cellular processes, for instance cell cycle progression, homotypic membrane fusion, chromatin remodeling, transcriptional and metabolic regulation, and many others. The molecular function of Cdc48 is arguably best understood in endoplasmic reticulum-associated protein degradation by the ubiquitin proteasome system. In this review, we summarize the general characteristics of Cdc48/p97 and the most recent results on the molecular function of Cdc48 in some of the above processes, which were found to finally end in proteolysis-connected pathways, either involving the proteasome or autophagocytosis-mediated lysosomal degradation.

Cdc48 function

Even though peptide bond hydrolysis in proteins is a chemically exergonic reaction, which does not require energy, intracellular protein degradation is highly energy dependent. This is mainly due to the requirement for selectivity and precise regulation of the degradation process as well as for protein transport and unfolding. A major player in eukaryotic protein degradation is the essential, highly conserved ATPase Cdc48 (p97/VCP in mammals, TER94 in the fly and CDC-48 in nematodes). Cdc48 (cell division cycle), which belongs to the family of ATPases associated with various cellular activities (AAA), forms a homohexameric, ring-shaped complex. With approximately 1% of the cytosolic protein [1], Cdc48 is highly abundant in mammalian cells. The *CDC48* gene was first identified in a yeast screen for conditional mutations affecting the cell cycle [2]. In 1991, the gene was sequenced and the encoded protein of 92 kDa was identified [3]. Studies in the years thereafter unraveled a central function of Cdc48 in ubiquitin-dependent proteolysis, and in 2007 it was dubbed a 'molecular gearbox in the ubiquitin pathway' [4]. Until recently, little was known about Cdc48 functions at the molecular level outside the ubiquitin proteasome system (UPS). In addition to its well-known molecular function in protein quality control in the process of endoplasmic reticulum associated protein degradation (ERAD) [5,6], the participation of Cdc48 in a variety of other cellular processes has been revealed only recently. These include transcriptional and metabolic regulation, DNA damage response, chromatin remodeling, selective autophagy, cell cycle progression and cell death [7–12]. In many cases it has still to be elucidated if the reported function of Cdc48 is directly linked to protein degradation processes. Also, in its long-known involvement in membrane fusion no molecular mechanism of Cdc48 has been unraveled [9]. Concerning new molecular functions, it has been found that Cdc48 specifically controls transcription through the turnover of RNA Pol II upon UV irradiation or high protein accumulation [12,13] and is required for metabolic regulation in glucose controlled catabolite degradation [7]. Under stress conditions, damaged proteins are not instantly degraded but stored in aggresomes. This enables the cell to focus on essential tasks. Aggresomes are resolubilized and eliminated when conditions allow. Both processes, aggresome formation and aggresome elimination, are Cdc48-dependent [14–16].

In general, protein degradation is executed by the UPS or by autophagy, which finally results in lysosomal (vacuolar) degradation. Impairment in basic protein degradation processes is the reason for many human diseases such as Parkinson's disease, Alzheimer's disease and Huntington's disease, and many others [13]. Although Cdc48 function is well established in the UPS [4–6], recent reports unraveled a function of Cdc48 in several selective autophagy pathways, with and without involvement of ubiquitin [17,18]. When degradation is the task, obviously Cdc48 guides proteins either into the UPS or into autophagy [14]. This decision enables the cell to dispose of an unwanted or dysfunctional protein by at least one of the two degradation pathways.

This review describes the basic characteristics of Cdc48 and summarizes recent findings of Cdc48 functions as well as the identification of new cofactors involved in protein degradation. Cited studies in this review were done in a variety of organisms, regardless we will use the term 'Cdc48' for this AAA ATPase throughout.

The Cdc48 machinery

In the following sections we will describe the structure of the Cdc48 complex and its modifications, substrate handling by the complex as well as the cofactors with their Cdc48 interaction motifs.

Cdc48 structure

Cdc48 is a homohexameric complex composed of six protomers arranged in a ring around a central pore (Figure 1). Each protomer contains four domains: a mobile N-terminal domain, two conserved AAA domains called D1 and D2, and

Corresponding author: Wolf, D.H. (dieter.wolf@ibc.uni-stuttgart.de).

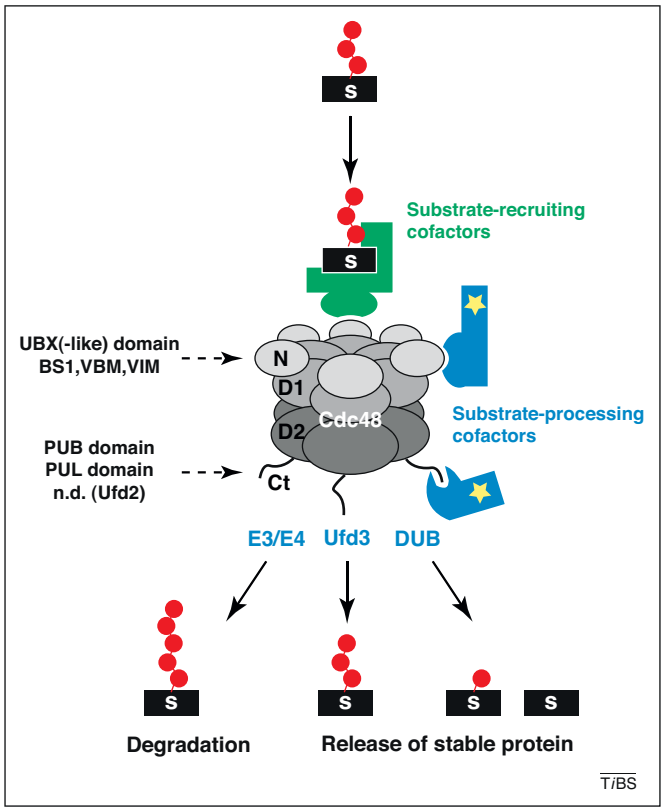


Figure 1. Substrate turnover by Cdc48 and its cofactors. The Cdc48 hexamer is shown in the center, with Cdc48 domain organization (N, D1, D2; Ct: carboxy terminus) and binding sites for cofactor interaction modules indicated at the left. n.d.: the Cdc48 binding site of Ufd2 is unknown. Substrate proteins (S) marked by ubiquitin (red circles) are recognized by substrate-recruiting cofactors (green) and segregated from protein complexes, lipid membranes, or chromatin by Cdc48 (not shown). Substrate-processing cofactors (blue) catalyze substrate polyubiquitylation, which targets it for degradation by the 26S proteasome (E3/E4; left); prevent polyubiquitylation (Ufd3; middle); or catalyze deubiquitylation (DUBs; right). The latter two possibilities result in the release of stable substrates representing Cdc48 function in non-proteolytic pathways, not discussed in this review. Modified from [5].

a presumably disordered C-terminal tail (Figure 2a). The six D1 and D2 domains of oligomeric Cdc48 form two stacked rings. Assembly of the hexameric complex seems to be sustained by the D1 domains and does not require nucleotide [19,20]. Each AAA domain contains the conserved signature nucleotide binding (Walker A) and hydrolysis (Walker B) motifs, as well as a second region of homology (SRH) necessary for efficient hydrolysis. The arginine residue of the D1 SRH motif protrudes into the nucleotide binding pocket of the adjacent protomer thereby restricting the pocket size. This could be the reason for the preferential binding of ADP to D1. ATP hydrolysis induces cooperative structural changes within the six protomers. However, it is still under discussion whether the arginine residues of D2 – also referred to as the arginine collar – or the linker between D1 and D2 facilitate cooperativity between the protomers during nucleotide binding, hydrolysis and subsequent global conformational changes [19,21,22].

Cdc48 contains a total of 12 ATPase active sites. D1 domains show only weak ATPase activity under physiological conditions (37 °C) leaving the bulk of activity to the D2 domains [22]. However, under heat shock conditions ATPase activity of Cdc48 is elevated and D1 AAA domains develop strong ATPase activity with a maximum at 60 °C [19]. During hydrolysis Cdc48 undergoes pronounced conformational changes, which result in open and closed configurations of the D1 and D2 rings [19]. The precise correlation between conformation and nucleotide state is controversial. It is clear, however, that conformational changes within the different domains occur and that they are necessary for Cdc48 function [23]. For instance, locking D1 in the ATP bound state causes inhibition of Cdc48 function and cell death [24].

Substrate handling

The mechanism of Cdc48 activity on ubiquitylated substrates for proteasomal degradation or Atg8 (autophagy related) modified phosphatidylethanolamine (PE) is still poorly understood. Even though the mechanism of Cdc48 action in different pathways seems to be different, it might be similar. Whether substrates are completely threaded through the central pore of Cdc48 or merely loop into it awaits experimental verification [25]. The D1 ring is relatively narrow with six histidine residues constricting the central pore, whereas the D2 ring is wider and lined with 12 arginine residues [26]. Recent reports came to the conclusion that substrates are unlikely to pass the D1 pore [26]. It is still possible, however, that they enter the D2 pore and exit between the D1 ring and D2 ring. In light of the high thermodynamic stability of ubiquitin itself, this model would imply a deubiquitylation of the substrate prior to its entry into the D2 pore and reubiquitylation after exit to facilitate efficient recognition of the substrate by downstream factors. There are known cofactors of Cdc48 that possess the corresponding activities such as the deubiquitylating enzyme Otu1 (ovarian tumor) and the ubiquitin ligase Ufd2 (ubiquitin fusion degradation). Ufd2 was reported to function exclusively as an E4, which elongates existing ubiquitin chains, but could contain yet uncharacterized E3 activity [4]. A second prediction of this model would be that either the substrate has to be entirely unfolded to pass through the D2 pore or, less likely, that the Cdc48 oligomer opens for substrate release. It is also possible that in some cases complete unfolding of a substrate by Cdc48 is required, whereas in others segregation of only small parts of the protein is sufficient.

Indeed, a recent study supports a model favoring the requirement of only partial substrate unfolding prior to proteasomal degradation. Using a substrate of the proteasomal ubiquitin fusion degradation (UFD) pathway, it was shown [27] that a linear C-terminal fusion with a 20 amino acid tag of mostly random coil structure bypasses the need of Cdc48 for proteasomal degradation. Apparently, one function of Cdc48 in the UFD pathway is to provide an appropriate initiation site for proteasomal degradation by producing an unstructured 'loose end' [27,28]. Many folded proteins have their termini buried within the native structure, making remodeling of that structure and exposition of unstructured termini necessary for accessibility to the proteasome. As the proteasome is also able to degrade proteins lacking accessible N-and C-termini but exposing internal disordered regions [29,30], Cdc48 might also provide such internally unstructured regions. The arginine collar lining the D2 pore could be responsible for an unfolding activity as the 12 arginine residues of the collar have been proposed to resemble a denaturing equivalent of approximately 8 M

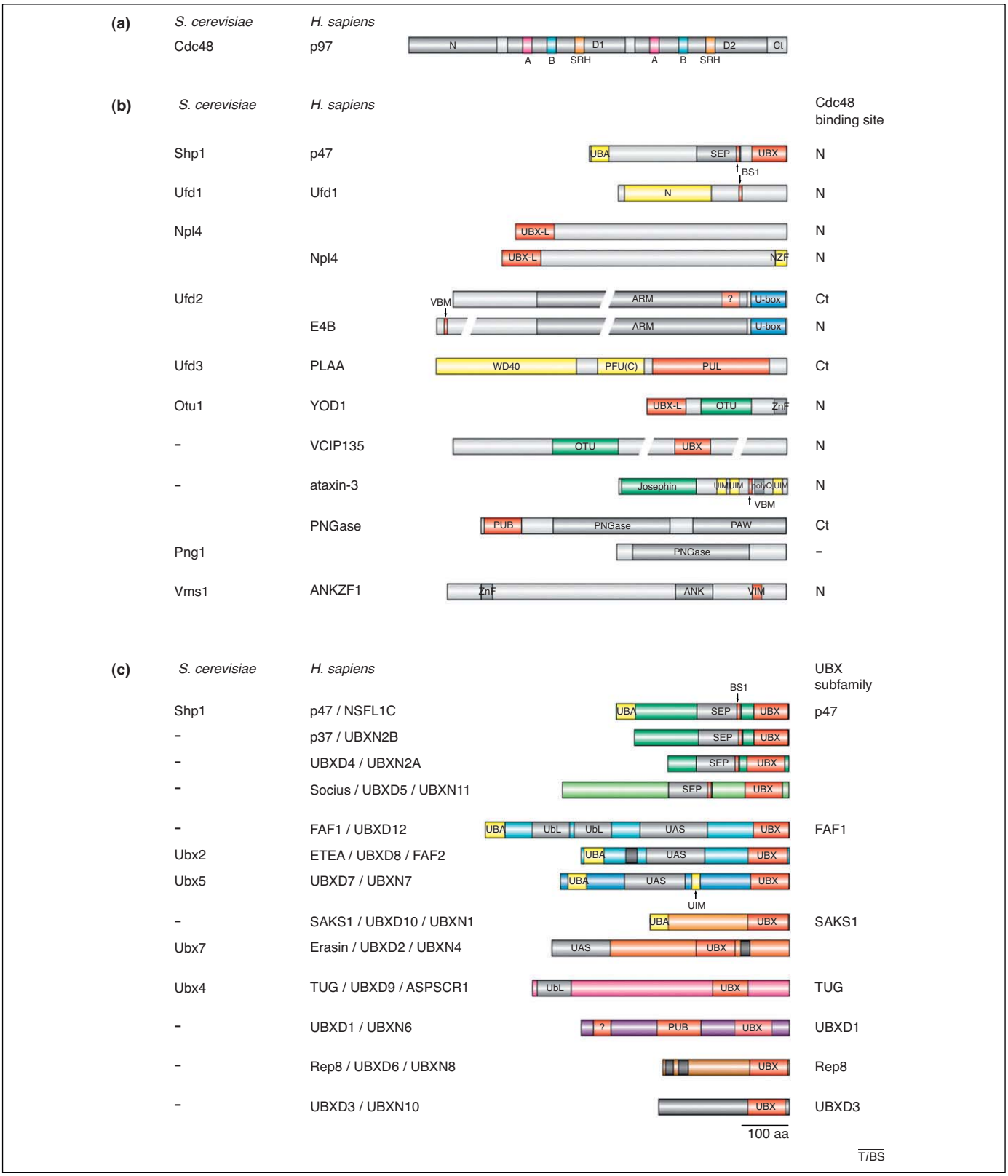


Figure 2. **Domain architecture of Cdc48 and important cofactors**. Protein names of the Baker's yeast and human homologs are given (right), no homolog was identified in *Saccharomyces cerevisiae*. **(a)** Cdc48. Indicated are the structural domains (N, D1, D2) and the unstructured carboxy-terminal tail (Ct), as well as the Walker A and B motifs and the second region of homology (SRH) of the D1 and D2 domains. **(b)** Representative cofactors are discussed in the text. Shp1, Ufd1 and Npl4 are substrate-recruiting, the others are substrate-processing cofactors. The binding site on Cdc48 is indicated at the right (N, N domain; Ct: carboxy-terminal tail). Cdc48 binding modules including the UBX and UBX-like (UBX-L) domains, the PUL domain, the PUB domain, the linear binding site 1 (BS1), VCP binding motif (VBM) and VCP interaction motif (VIM) are shown in red. The exact position and nature of the Cdc48 binding motif in Ufd2 are unknown (red box labeled with question mark). Ubiquitin binding modules including the UBA, N, NZF, WD40 and PFU(C) domains and the UIM motif are shown in yellow. The U-box ubiquitin ligase domain of Ufd2 and human E4B is in blue, whereas the OTU and Josephin deubiquitylating domains are in green. Further domains of interest are shown in dark gray. polyQ, polyglutamine stretch. **(c)** The UBX protein family. Shown are representative members of the subfamilies indicated at the right. Cdc48 and ubiquitin binding modules are indicated in red and yellow, respectively. Transmembrane and membrane insertion regions are indicated by black, unlabeled bars. Other defined domains are in gray. Sequence homology outside defined domains is indicated by similar colors. For human UBX proteins, alternative names are listed, with the official gene name last.

guanidine. Thus, the arginine collar together with a pair of phenylalanine and tryptophane residues within the flexible loop between D1 and D2 might provide the environment and a driving force to unfold substrates [26].

Cofactors and binding motifs

Cellular functions of Cdc48 crucially depend on an arsenal of regulatory cofactors (Figure 2b, c). Whereas a substrate-recruiting factor defines pathways in which Cdc48 acts, simultaneous binding of a substrate-processing factor defines the destination a protein takes within a pathway, for example whether a substrate is processed or degraded. Most cofactors interact with Cdc48 by virtue of a relatively small number of binding modules. These include the N domain interacting UBX domain, UBX-like domain, SHP box (also called Binding Site 1), VCP binding motif (VBM) and VCP interacting motif (VIM), as well as the PUB and PUL domains that bind to the C-terminal tail of Cdc48 [5,31]. It is important to note that the Cdc48 binding sites for some of these modules overlap with each other, thereby allowing specific exclusion of two competing cofactors. Examples for this are Doa1/Ufd3 (degradation of alpha), an interaction partner of the ubiquitin protease Ubp3 (ubiquitin-specific protease) [18], and the E4-ubiquitin ligase Ufd2. Both proteins exclude each other due to overlapping binding regions within Cdc48, thereby avoiding unnecessary rounds of ubiquitylation and deubiquitylation occurring on one and the same Cdc48 complex [32,33]. Many different cofactors of Cdc48 have been identified to date and their number is still growing (Figure 2,b c) [5].

Post-translational modifications of Cdc48

The C-terminal tail of Cdc48 can be modified by phosphorylation and acetylation. These modifications influence Cdc48 ATPase activity, its localization and its binding to partners [34]. Phosphorylation of a conserved tyrosine residue at the C-terminus inhibits the interaction with cofactors that bind via a PUB or PUL domain, including Doa1/Ufd3 and PNGase [35,36]. The C-terminus is not the only target of post-translational modification, however, D1 and D2 also contain potential phosphorylation sites [37,38]. Even though the precise functions of all proposed modifications have not been unraveled to date, it seems plausible that by inducing structural changes they influence either ATPase activity, freeze an existing Cdc48 conformation or regulate the binding of cofactors.

One well-documented example is the phosphorylation and acetylation of human Cdc48 in a motif containing the amino acid sequence STK. These modifications are induced by overexpression of polyQ proteins and mediate nuclear translocation of modified Cdc48 followed by deacetylation of core histones [13]. Even though yeast Cdc48 does not contain the same STK motif, one could speculate that nuclear translocation of Cdc48, dependent on such an equivalent modification, also occurs in the case of degradation of the large subunit of RNA Pol II upon UV irradiation [12].

Cdc48 functions in protein degradation

In this section, we will discuss the most recent findings of Cdc48 function in different proteasomal degradation and autophagy pathways.

Endoplasmic reticulum-associated protein degradation (ERAD)

The arguably best understood function of Cdc48 on the molecular level is its role in ERAD (Figure 3). Cdc48 in complex with Ufd1 and Npl4 (nuclear protein localization) is recruited to the ER membrane via the integral membrane protein Ubx2 (ubiquitin regulatory x) [39,40]. There, Cdc48–Ufd1–Npl4 binds ubiquitylated ERAD substrates, which are subsequently retrotranslocated and segregated from the ER membrane or their binding partners. Additional downstream factors (e.g. Dsk2, Rad23) help to guide substrates to the proteasome where they are degraded [4,6]. Based on landmark studies in yeast and mammalian cells, the retrotranslocation function of Cdc48 in ERAD had initially been exclusively assigned to the trimeric Cdc48–Ufd1–Npl4 complex. From recent studies in mammalian cells, however, it became evident that ERAD could consist of several subpathways relying on distinct Cdc48 complexes. An analysis of the degradation of major histocompatibility complex class I heavy chain (MHC class I HC) induced by the US2 protein of human cytomegalovirus (HCMV) revealed a Cdc48 pathway independent of Ufd1 [41]. Intriguingly, this is in contrast to the pathway triggered by the HCMV protein US11, which induces the prototypical Cdc48–Ufd1–Npl4 dependent degradation of MHC class I HC. Although the US11-dependent ERAD pathway is mediated by the canonical E3 ligase HRD1 (HMG-CoA reductase degradation) and the ER membrane protein Derlin-1, the US2-dependent branch uses the E3 ligase TRC8 (patched related protein translocated in renal cancer), which is not linked to Derlin-1 [41–43]. Based on the idea that Npl4 is only capable of binding Cdc48 in the presence of Ufd1 [42], it was presumed that the US2-dependent branch is also independent of Npl4 [41]. In another study, Ufd1 was shown to be also dispensable for degradation of substrates of the ERAD E3 ligase gp78 [44]. In this pathway, however, Cdc48-mediated degradation was clearly dependent on Npl4 [44], indicating that Cdc48 might be able to interact with both cofactors independently. Indeed, Npl4 has a weak Cdc48 interaction site, which can be activated by a small region within Ufd1 [42]. It remains to be seen which other cofactor can take over this activation of Npl4 to form a Cdc48–Npl4 complex in the case of degradation of Ufd1-independent gp78 substrates [44].

Independently from each other, two groups recently found the yet uncharacterized Ydr049 protein [45], renamed VCP/Cdc48-associated mitochondrial stress-responsive protein 1, Vms1, to participate in protein degradation [46,47]. Vms1 and Ufd1 are soluble proteins that interact with Cdc48, but seem to exclude each other from simultaneous binding [46]. Co-immunoprecipitation experiments demonstrated interaction between Vms1 and Cdc48 in the cytosol and in highly enriched ER fractions. Consistent with Cdc48 function in ERAD, Vms1 is involved in degradation of the well-known ERAD substrate cystic fibrosis transmembrane conductance regulator (CFTR) expressed in yeast [47]. An additional Cdc48 cofactor involved in ERAD is Ubx4 [48]. Vms1, similar to Ubx4, seems to act after the ubiquitylation of misfolded proteins in the release of the ubiquitylated species from the

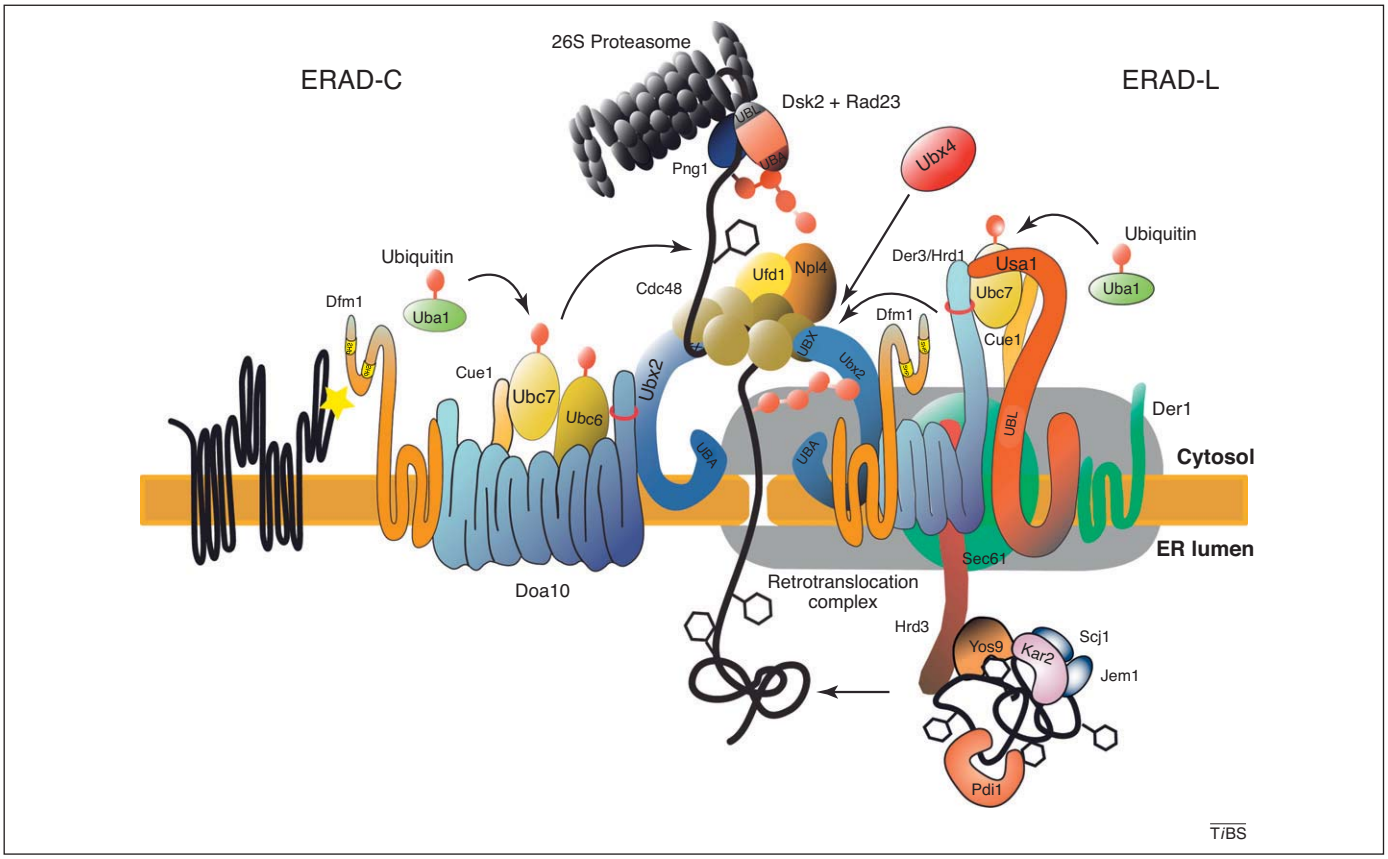


Figure 3. Cdc48 and cofactors as part of the endoplasmic reticulum-associated protein degradation (ERAD) machinery. Misfolded proteins carrying the misfolded domain in the lumen of the ER (ERAD-L substrates) or in the cytoplasm (ERAD-C substrates) are recognized, retrogradely transported across the ER membrane into the cytosol, polyubiquitylated and degraded by the proteasome. The ERAD-L and ERAD-C pathways use two different polytopic membrane-localized ubiquitin ligases, Hrd1/Der3 and Doa10, respectively. Whereas Hrd1/Der3 is embedded into a variety of ER membrane proteins of, in many cases, not yet precisely known function, Doa10 is localized in a much simpler environment. Both pathways, ERAD-L and ERAD-C, merge at the Cdc48 motor, complexed with Ufd1 and Npl4 as cofactors and recruited to the ER membrane by Ubx2. According to the present literature, the Cdc48–Ufd1–Npl4 complex is thought to pull or segregate the substrates from the ER membrane and finally deliver them to the proteasome for degradation. For details, see [71,72].

Cdc48 complex. Double deletion of *UBX4* and *VMS1* showed an additive effect in stabilizing the lumenal ERAD substrate CPY [47,48].

Mitochondrial protein degradation

Vms1 was first found to be required for mitochondrial stress response. Exposure of cells to rapamycin or hydrogen peroxide induces translocation of Vms1 and Cdc48 to the mitochondrial membrane where they are involved in the degradation of selected mitochondrial proteins via the UPS. It was shown that Npl4, but not Ufd1, is part of this specific Cdc48 complex [46]. The Cdc48–Vms1–Npl4 complex triggers degradation of the yeast mitofusin Fzo1 (fuzzy onions homolog), which is targeted for proteasomal degradation via the SCF^{Mdm30} ubiquitin ligase (Skp1/Cul1/F-box protein) [49]. Human orthologs of Fzo1 – MFN1 and MFN2 (mitofusin) – were shown to be direct targets of the E3 ligase Parkin and to be degraded via the UPS in a Cdc48-dependent manner [50]. The bona fide human ortholog of Vms1, ANKZF1, could be responsible for recruiting Cdc48 in the mammalian process. It has been hypothesized that the Parkin and Cdc48 controlled degradation of mitofusins inhibits the fusion of damaged and healthy mitochondria, thereby facilitating the elimination of terminally defective mitochondria by autophagy [50]. The degradation of mitofusins and even some intramitochondrial proteins via the UPS [51,52] could suggest that a Cdc48-dependent mitochondrial retrotranslocation system exists that is analogous to the one in ERAD [6,46].

Cytoplasmic and nuclear protein degradation

The first involvement of Cdc48 in cytoplasmic protein degradation was demonstrated for Ubi-Pro-β-galactosidase, a synthetic substrate of the UFD pathway [53]. A role of the Cdc48–Ufd1–Npl4 complex in degradation of native cytoplasmic proteins was only recently found in regulation of carbohydrate metabolism in yeast: two key enzymes of the gluconeogenic pathway, fructose-1,6-bisphophatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK), are degraded by the UPS in a process called catabolite inactivation upon glucose signaling [7]. The function of the Cdc48 complex in this process could reside in the removal of the ubiquitylated enzymes from the Gid complex (glucose-induced degradation deficient), a multisubunit ubiquitin ligase targeting FBPase and PEPCK, in the separation of the ubiquitylated subunits of the homotetrameric enzymes and/or in creating 'loose ends' to feed the subunits into the proteasome for degradation.

In the nucleus, UV-induced proteasomal turnover of chromatin-bound Rpb1 (RNA polymerase B), the largest subunit of RNA Pol II, was found to depend on the Cdc48–Ufd1–Npl4 complex as well as the cofactors Ubx4 and

Ubx5. It is thought that the Cdc48 machinery and the proteasome are recruited to ubiquitylated Rpb1 stalled at DNA lesion sites, mediating selective extraction of Rpb1 from chromatin-bound Pol II holoenzyme. Here, the function of the Cdc48 complex could also reside in protein disassembly and unfolding [12]. A segregase function of Cdc48 has previously been shown for extraction of the Aurora B kinase from chromatin [54].

Macroautophagy and piecemeal microautophagy of the nucleus

It was believed for a long time that Cdc48 is exclusively involved in protein degradation via the UPS. It has recently become clear, however, that Cdc48 also functions in several selective vacuolar degradation pathways, including macroautophagy, piecemeal microautophagy of the nucleus (PMS) and ribophagy. The substrate recruiting factor for Cdc48 in macroautophagy and PMS in yeast was identified as the UBX domain containing protein Shp1/Ubx1 (suppressor of high-copy PP1), whereas no involvement of other known Cdc48 cofactors in macroautophagy and PMS was found [17]. Even though it was Cdc48-dependent, macroautophagy was shown to be independent of the proteasome. Consistent with a Cdc48 function independent of the UPS, Shp1 excludes simultaneous binding of the substrate recruiting factor Ufd1, which is involved in ERAD and other UPS processes [6,39] (see above). Of special interest for the Cdc48 function in macroautophagy is the fact that the target of the Cdc48 complex appears to be the Atg8–PE conjugate crucial for autophagosome maturation. Shp1 seems to act as an antagonist of Ufd1 by interacting with Atg8, a ubiquitin-fold protein that is essential for initial steps of autophagy [55]. Interestingly, the Shp1–Atg8 interaction was shown to depend on a FK motif in the 24 amino acid N-terminal helical domain of Atg8, which is absent in ubiquitin.

Ribophagy

In the case of nutrient limitation, mature ribosomes are degraded to assure cell survival in a process named ribophagy [56]. Ribophagy is a selective autophagy pathway, which results in vacuolar degradation of the large ribosomal subunit. It is one example of ubiquitin-dependent but proteasome-independent protein degradation involving Cdc48 [18]. Partners of Cdc48 involved in this pathway are Ubp3 and Bre5 (brefeldin A sensitivity), which build up a deubiquitylating enzyme complex. Deletion of *UBP3* and *BRE5* results in increased ubiquitylation of several ribosomal subunits and subsequent stabilization of the 60S ribosome. This suggests a direct regulation of this pathway via ubiquitylation. Furthermore, Cdc48 seems to directly interact with the ubiquitin binding cofactor Doa1/Ufd3 in this pathway. A possible involvement of Shp1 in ribophagy and the precise mechanism of Cdc48 action in this pathway should be analyzed.

Mitophagy

Damaged mitochondria are ultimately eliminated through an autophagic pathway called mitophagy [57]. In addition to the Cdc48-dependent degradation of mitofusins by the UPS (see above), an involvement of Cdc48 in the elimination of damaged mitochondria by mitophagy seems plausible [50], suggesting a central role of Cdc48 in the control of mitochondrial integrity.

Cdc48 involvement in human disease

Many human diseases are related to impaired protein degradation including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS) and inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia (IBMPFD). IBMPFD is caused by mutations within Cdc48 [58] that are linked to the formation of intracellular inclusions consisting of aggregated proteins, most notably TDP-43 [59,60]. Although protein aggregation was initially believed to be the consequence of impaired Cdc48 function in the UPS, it is now clear that mutant Cdc48 causes defects in autophagy leading to the accumulation of aggregates [14,61–63]. On the molecular level, the disease-causing mutations are localized at the interface between the D1 and N-terminal domains and the linker connecting them. Consistently, mutant Cdc48 proteins exhibit moderately elevated ATPase activity and significant conformational alterations of the N-terminal domain [64–67]. Importantly, these structural alterations cause an imbalance in cofactor binding as well as accumulation of ubiquitylated proteins associated with Cdc48 [14,64,67,68]. However, the Cdc48 cofactor(s) and substrates crucial for IBMPFD pathogenesis still remain elusive.

Recently, Cdc48 mutations have also been identified in familial ALS, another proteinopathy involving TDP-43 aggregation [69], suggesting an unexpected common pathogenesis mechanism for IBMPFD and ALS.

Concluding remarks

In the future, classical research on Cdc48 and cofactors could be complemented by top-down approaches, for example network analysis using entire interaction datasets including high-throughput results. To date, 137 direct physical interactors and 137 genetic neighbors make Cdc48 a hub in both the protein–protein interaction network and the genetic interaction network of yeast. Only 1.8% of the yeast proteins possess more physical interactors and merely 4.6% of proteins have more genetic partners than Cdc48. Both datasets harbor significant percentages of automatized and therefore unbiased high-throughput data (47% in the case of physical interactions, 82% in the case of genetic interactions). Interestingly, closer analysis of Cdc48 interactors uncovers a remarkably small overlap between physical and genetic Cdc48 partners; only 7.3% of the physical interactors also exhibit a genetic link to Cdc48 (Figure 4a). In light of the fact that analysis of the genetic interaction network has reached 75% coverage [70], this result is significant. It indicates a low functional redundancy between the different complexes made up from Cdc48 and its interactors. Thus, the majority of Cdc48 derived systems are expected to fulfill highly specific cellular tasks.

Inspection of the physical interaction network of established Cdc48 cofactors including the connections among these proteins leads to another interesting result

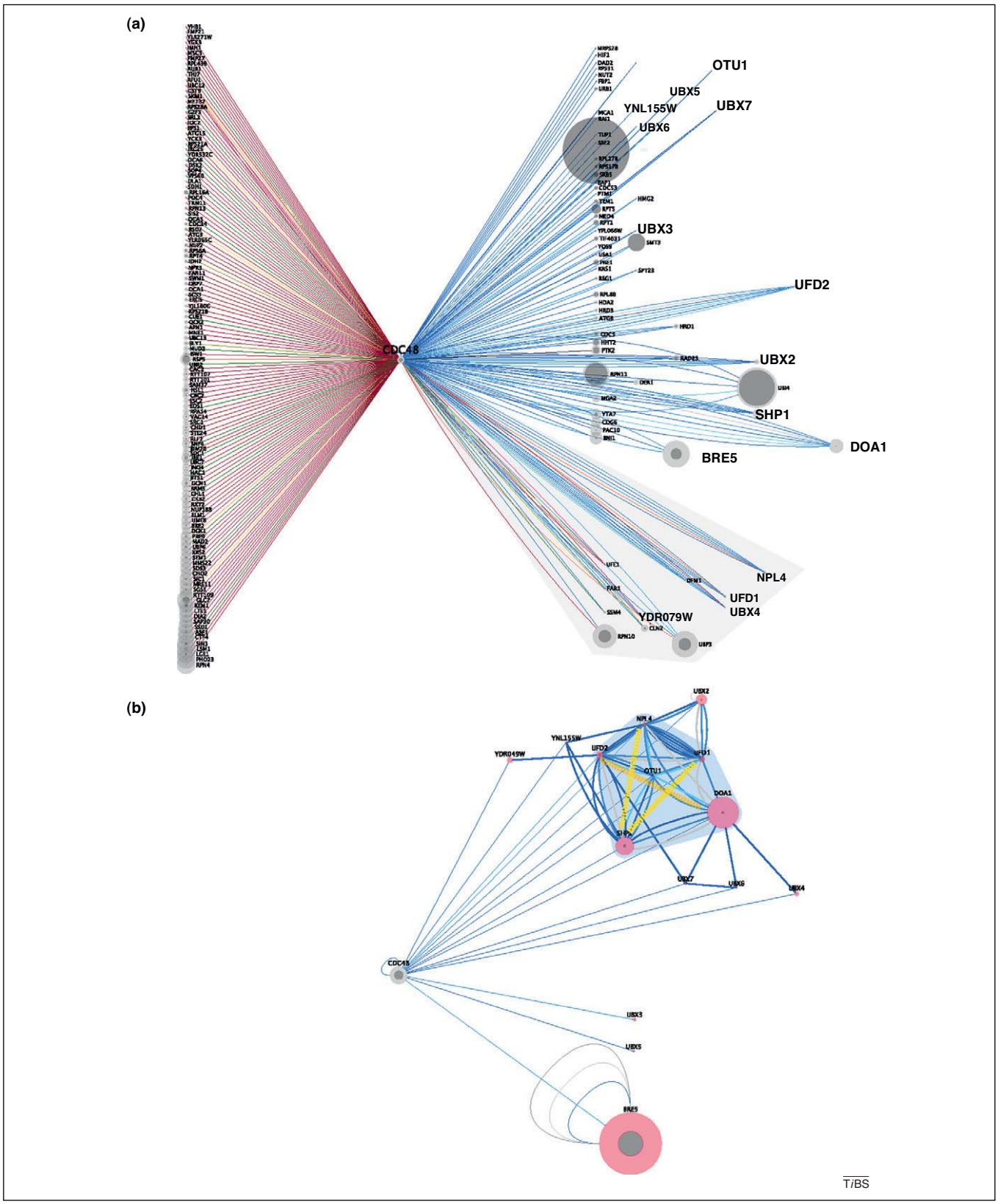


Figure 4. (a) Physical and genetic neighbors of Cdc48. Proteins exhibiting genetic interactions with Cdc48 are lined up on the left side (edges printed in yellow, red or green depending on the type of functional interaction). Proteins showing physical interaction with Cdc48 (blue marked edges) are placed on the upper right side. Proteins with mixed interactions (physical and genetic) are positioned on the lower right side (underlined by a gray field). Within these three cohorts, proteins are sorted from top to bottom according to their genetic interaction degree (total number of genetic edges) as a measure for their general importance for yeast cell function. Physical and mixed interactors are in addition positioned from left to right according to increasing numbers of physical connections with Cdc48. **(b)** Physical interaction network of Cdc48 and cofactors. The network shows established Cdc48 cofactors including the interactions among these proteins. The network nodes are positioned with the aim to cluster members showing high connectivity as well as to avoid crossovers of edges. A core cluster consisting of highly interconnected proteins indicating a set of Cdc48 derived subcomplexes is underlined by a blue area. Dashed yellow lines mark proteins within the cluster, which appear to exclude each other. The graphs represent the current Cdc48 network data and were produced especially for this review using Cytoscape and features of the conjunction analysis plug-in (W. Hilt, personal communication). Node core sizes reflect the physical degree of a node (number of total physical edges), and node border sizes reflect the genetic degree (number of total genetic edges).

(Figure 4b). The cofactors might be classified into two types: (i) 'lone wolf' modifiers, which as single moieties mediate modification of Cdc48 for specific tasks; and (ii) 'herd' proteins, which seem to operate in a concerted manner and are arranged in a highly diverse set of versatile Cdc48 subcomplexes. Here, the DUB Otu1 appears to act as a major core unit. Remarkably, within the set of subcomplexes certain combinations of cofactors appear to be forbidden (Ufd2 excludes Doa1, Shp1 excludes Npl4 and Ufd1, and vice versa), confirming direct experimental evidence. The future awaits better resolution of the remarkably complex Cdc48-derived systems. This task will require both sophisticated hypothesis-driven research and extended systematic high-throughput data, for example data obtained at different conditions and cellular stages, as well as improved methods for the interpretation of mass data.

Consistent with the notion of Cdc48 being a cellular hub, recent experiments concerning protein degradation indeed show that Cdc48 is not only central for the UPS but also for pathways that direct proteins to the vacuole. Here, one function of Cdc48 could reside in protein-linked delipidation of membranes to allow autophagy [17]. It is completely unknown how Cdc48 triggers the many cellular processes it is involved in, such as homotypic membrane fusion [9] or cell cycle regulation. Many of them might finally end in protein degradation via the UPS. This is only speculation, however, and the hard scientific research to elucidate all mechanistic aspects of this powerful machine and its sophisticated regulation still lies ahead of us.

Note added in proof

A recent study on mammalian ERAD indicates the possibility that substrate handling by Cdc48 might involve deubiquitylation of the misfolded protein, which would permit its subsequent threading through the narrow central pore of Cdc48. Following a supposed reubiquitylation event, the ERAD substrate would be targeted to the proteasome [73].

Acknowledgments

The author's research was supported by the Deutsche Forschungsgemeinschaft, Bonn and the EU Network of Excellence RUBICON. We apologize for not being able to cite the work of all authors who contributed to the reviewed topic, due to space constraints.

References

1 Wang, Q. *et al.* (2004) Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions. *J. Struct. Biol.* 146, 44–57
2 Moir, D. *et al.* (1982) Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics* 100, 547–563
3 Frohlich, K.U. *et al.* (1991) Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. *J. Cell Biol.* 114, 443–453
4 Jentsch, S. and Rumpf, S. (2007) Cdc48 (p97): a "molecular gearbox" in the ubiquitin pathway? *Trends Biochem. Sci.* 32, 6–11
5 Buchberger, A. (2010) Control of ubiquitin conjugation by cdc48 and its cofactors. *Subcell. Biochem.* 54, 17–30
6 Raasi, S. and Wolf, D.H. (2007) Ubiquitin receptors and ERAD: a network of pathways to the proteasome. *Semin. Cell Dev. Biol.* 18, 780–791
7 Barbin, L. *et al.* (2010) The Cdc48–Ufd1–Npl4 complex is central in ubiquitin-proteasome triggered catabolite degradation of fructose-1,6-bisphosphatase. *Biochem. Biophys. Res. Commun.* 394, 335–341
8 Braun, R.J. and Zischka, H. (2008) Mechanisms of Cdc48/VCP-mediated cell death: from yeast apoptosis to human disease. *Biochim. Biophys. Acta* 1783, 1418–1435
9 Meyer, H. and Popp, O. (2008) Role(s) of Cdc48/p97 in mitosis. *Biochem. Soc. Trans.* 36, 126–130
10 Deichsel, A. *et al.* (2009) The ubiquitin-selective chaperone CDC-48/p97, a new player in DNA replication. *Cell Cycle* 8, 185–190
11 Wilcox, A.J. and Laney, J.D. (2009) A ubiquitin-selective AAA-ATPase mediates transcriptional switching by remodelling a repressor-promoter DNA complex. *Nat. Cell Biol.* 11, 1481–1486
12 Verma, R. *et al.* (2011) Cdc48/p97 mediates UV-dependent turnover of RNA Pol II. *Mol. Cell* 41, 82–92
13 Koike, M. *et al.* (2010) Valosin-containing protein (VCP) in novel feedback machinery between abnormal protein accumulation and transcriptional suppression. *J. Biol. Chem.* 285, 21736–21749
14 Ju, J.S. and Weihl, C.C. (2010) Inclusion body myopathy, Paget's disease of the bone and fronto-temporal dementia: a disorder of autophagy. *Hum. Mol. Genet.* 19, R38–R45
15 Wang, Y. *et al.* (2009) Abnormal proteins can form aggresome in yeast: aggresome-targeting signals and components of the machinery. *FASEB J.* 23, 451–463
16 Ju, J.S. *et al.* (2008) Impaired protein aggregate handling and clearance underlie the pathogenesis of p97/VCP-associated disease. *J. Biol. Chem.* 283, 30289–30299
17 Krick, R. *et al.* (2010) Cdc48/p97 and Shp1/p47 regulate autophagosome biogenesis in concert with ubiquitin-like Atg8. *J. Cell Biol.* 190, 965–973
18 Ossareh-Nazari, B. *et al.* (2010) Cdc48 and Ufd3, new partners of the ubiquitin protease Ubp3, are required for ribophagy. *EMBO Rep.* 11, 548–554
19 Pye, V.E. *et al.* (2006) Going through the motions: the ATPase cycle of p97. *J. Struct. Biol.* 156, 12–28
20 Brunger, A.T. and DeLaBarre, B. (2003) NSF and p97/VCP: similar at first, different at last. *FEBS Lett.* 555, 126–133
21 Briggs, L.C. *et al.* (2008) Analysis of nucleotide binding to P97 reveals the properties of a tandem AAA hexameric ATPase. *J. Biol. Chem.* 283, 13745–13752
22 Davies, J.M. *et al.* (2008) Improved structures of full-length p97, an AAA ATPase: implications for mechanisms of nucleotide-dependent conformational change. *Structure* 16, 715–726
23 DeLaBarre, B. and Brunger, A.T. (2005) Nucleotide dependent motion and mechanism of action of p97/VCP. *J. Mol. Biol.* 347, 437–452
24 Esaki, M. and Ogura, T. (2010) ATP-bound form of the D1 AAA domain inhibits an essential function of Cdc48p/p97. *Biochem. Cell Biol.* 88, 109–117
25 Halawani, D. and Latterich, M. (2006) p97: the cell's molecular purgatory? *Mol. Cell* 22, 713–717
26 DeLaBarre, B. *et al.* (2006) Central pore residues mediate the p97/VCP activity required for ERAD. *Mol. Cell* 22, 451–462
27 Beskow, A. *et al.* (2009) A conserved unfoldase activity for the p97 AAA-ATPase in proteasomal degradation. *J. Mol. Biol.* 394, 732–746
28 Verhoef, L.G. *et al.* (2009) Minimal length requirement for proteasomal degradation of ubiquitin-dependent substrates. *FASEB J.* 23, 123–133
29 Liu, C.W. *et al.* (2003) Endoproteolytic activity of the proteasome. *Science* 299, 408–411
30 Schrader, E.K. *et al.* (2009) Targeting proteins for degradation. *Nat. Chem. Biol.* 5, 815–822
31 Madsen, L. *et al.* (2009) New ATPase regulators – p97 goes to the PUB. *Int. J. Biochem. Cell Biol.* 41, 2380–2388
32 Nishimasu, R. *et al.* (2010) Crystal structure of a PFU-PUL domain pair of *Saccharomyces cerevisiae* Doa1/Ufd3. *Kobe J. Med. Sci.* 56, E125–E139
33 Rumpf, S. and Jentsch, S. (2006) Functional division of substrate processing cofactors of the ubiquitin-selective Cdc48 chaperone. *Mol. Cell* 21, 261–269
34 Ewens, C.A. *et al.* (2010) Structural and functional implications of phosphorylation and acetylation in the regulation of the AAA+ protein p97. *Biochem. Cell Biol.* 88, 41–48

35 Li, G. *et al.* (2008) Tyrosine phosphorylation of ATPase p97 regulates its activity during ERAD. *Biochem. Biophys. Res. Commun.* 375, 247–251
36 Mullally, J.E. *et al.* (2006) Doa1 is a Cdc48 adapter that possesses a novel ubiquitin binding domain. *Mol. Cell. Biol.* 26, 822–830
37 Mori-Konya, C. *et al.* (2009) p97/valosin-containing protein (VCP) is highly modulated by phosphorylation and acetylation. *Genes Cells* 14, 483–497
38 Vandermoere, F. *et al.* (2006) The valosin-containing protein (VCP) is a target of Akt signaling required for cell survival. *J. Biol. Chem.* 281, 14307–14313
39 Schuberth, C. and Buchberger, A. (2005) Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation. *Nat. Cell Biol.* 7, 999–1006
40 Neuber, O. *et al.* (2005) Ubx2 links the Cdc48 complex to ER-associated protein degradation. *Nat. Cell Biol.* 7, 993–998
41 Soetandyo, N. and Ye, Y. (2010) The p97 ATPase dislocates MHC class I heavy chain in US2-expressing cells via a Ufd1-Npl4-independent mechanism. *J. Biol. Chem.* 285, 32352–32359
42 Bruderer, R.M. *et al.* (2004) The AAA ATPase p97/VCP interacts with its alternative co-factors, Ufd1-Npl4 and p47, through a common bipartite binding mechanism. *J. Biol. Chem.* 279, 49609–49616
43 Stagg, H.R. *et al.* (2009) The TRC8 E3 ligase ubiquitinates MHC class I molecules before dislocation from the ER. *J. Cell Biol.* 186, 685–692
44 Ballar, P. *et al.* (2011) Different p97/VCP complexes function in retrotranslocation step of mammalian ER-associated degradation (ERAD). *Int. J. Biochem. Cell Biol.* 43, 613–621
45 Krogan, N.J. *et al.* (2006) Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* 440, 637–643
46 Heo, J.M. *et al.* (2010) A stress-responsive system for mitochondrial protein degradation. *Mol. Cell* 40, 465–480
47 Tran, J.R. *et al.* (2011) A Cdc48p-associated factor modulates ER associated degradation, cell stress, and ubiquitinated protein homeostasis. *J. Biol. Chem.* 286, 5744–5755
48 Alberts, S.M. *et al.* (2009) Ubx4 modulates cdc48 activity and influences degradation of misfolded proteins of the endoplasmic reticulum. *J. Biol. Chem.* 284, 16082–16089
49 Cohen, M.M. *et al.* (2008) Ubiquitin-proteasome-dependent degradation of a mitofusin, a critical regulator of mitochondrial fusion. *Mol. Biol. Cell* 19, 2457–2464
50 Tanaka, A. *et al.* (2010) Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J. Cell Biol.* 191, 1367–1380
51 Azzu, V. and Brand, M.D. (2010) Degradation of an intramitochondrial protein by the cytosolic proteasome. *J. Cell Sci.* 123, 578–585
52 Azzu, V. *et al.* (2010) Rapid turnover of mitochondrial uncoupling protein 3. *Biochem. J.* 426, 13–17
53 Ghislain, M. *et al.* (1996) Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. *EMBO J.* 15, 4884–4899
54 Ramadan, K. *et al.* (2007) Cdc48/p97 promotes reformation of the nucleus by extracting the kinase Aurora B from chromatin. *Nature* 450, 1258–1262
55 Kiel, J.A. (2010) Autophagy in unicellular eukaryotes. *Philos. Trans. R. Soc. Lond. B: Biol. Sci.* 365, 819–830
56 Kraft, C. *et al.* (2008) Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. *Nat. Cell Biol.* 10, 602–610
57 Kim, I. *et al.* (2007) Selective degradation of mitochondria by mitophagy. *Arch. Biochem. Biophys.* 462, 245–253
58 Watts, G.D. *et al.* (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nat. Genet.* 36, 377–381
59 Neumann, M. *et al.* (2007) TDP-43-positive white matter pathology in frontotemporal lobar degeneration with ubiquitin-positive inclusions. *J. Neuropathol. Exp. Neurol.* 66, 177–183
60 Weihl, C.C. *et al.* (2008) TDP-43 accumulation in inclusion body myopathy muscle suggests a common pathogenic mechanism with frontotemporal dementia. *J. Neurol. Neurosurg. Psychiatry* 79, 1186–1189
61 Ju, J.S. *et al.* (2009) Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. *J. Cell Biol.* 187, 875–888
62 Vesa, J. *et al.* (2009) Valosin containing protein associated inclusion body myopathy, abnormal vacuolization, autophagy and cell fusion in myoblasts. *Neuromuscul. Disord.* 19, 766–772
63 Tresse, E. *et al.* (2010) VCP/p97 is essential for maturation of ubiquitin-containing autophagosomes and this function is impaired by mutations that cause IBMPFD. *Autophagy* 6, 217–227
64 Fernandez-Saiz, V. and Buchberger, A. (2010) Imbalances in p97 co-factor interactions in human proteinopathy. *EMBO Rep.* 11, 479–485
65 Tang, W.K. *et al.* (2010) A novel ATP-dependent conformation in p97 N-D1 fragment revealed by crystal structures of disease-related mutants. *EMBO J.* 29, 2217–2229
66 Halawani, D. *et al.* (2009) Hereditary inclusion body myopathy-linked p97/VCP mutations in the NH2 domain and the D1 ring modulate p97/VCP ATPase activity and D2 ring conformation. *Mol. Cell. Biol.* 29, 4484–4494
67 Manno, A. *et al.* (2010) Enhanced ATPase activities as a primary defect of mutant valosin-containing proteins that cause inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia. *Genes Cells* 15, 911–922
68 Kakizuka, A. (2008) Roles of VCP in human neurodegenerative disorders. *Biochem. Soc. Trans.* 36, 105–108
69 Johnson, J.O. *et al.* (2010) Exome sequencing reveals VCP mutations as a cause of familial ALS. *Neuron* 68, 857–864
70 Costanzo, M. *et al.* (2010) The genetic landscape of a cell. *Science* 327, 425–431
71 Stolz, A. and Wolf, D.H. (2010) Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell. *Biochim. Biophys. Acta* 1803, 694–705
72 Stolz, A. *et al.* (2010) Dfm1 forms distinct complexes with Cdc48 and the ER ubiquitin ligases and is required for ERAD. *Traffic* 11, 1363–1369
73 Ernst, R. (2011) Enzymatic Blockade of the Ubiquitin-Proteasome Pathway. *PLos Biol.* 8, e1000605

Appendix 8

The Cdc48 machine in endoplasmic reticulum associated protein degradation

Dieter H. Wolf and Alexandra Stolz
Biochimica et Biophysica Acta, article in press

Biochimica et Biophysica Acta xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

The Cdc48 machine in endoplasmic reticulum associated protein degradation ☆

Dieter H. Wolf *, Alexandra Stolz

Institut für Biochemie, Universität Stuttgart, Paffenwaldring 55, 70569 Stuttgart, Germany

ARTICLE INFO

Article history:
Received 8 July 2011
Received in revised form 1 September 2011
Accepted 2 September 2011
Available online xxxx

Keywords:
AAA-type ATPase
Cdc48
p97/VCP
Cdc48–Ufd1–Npl4 complex
ER-associated protein degradation (ERAD)
Ubiquitin–proteasome-system (UPS)

ABSTRACT

The AAA-type ATPase Cdc48 (named p97/VCP in mammals) is a molecular machine in all eukaryotic cells that transforms ATP hydrolysis into mechanic power to unfold and pull proteins against physical forces, which make up a protein's structure and hold it in place. From the many cellular processes, Cdc48 is involved in, its function in endoplasmic reticulum associated protein degradation (ERAD) is understood best. This quality control process for proteins of the secretory pathway scans protein folding and discovers misfolded proteins in the endoplasmic reticulum (ER), the organelle, destined for folding of these proteins and their further delivery to their site of action. Misfolded lumenal and membrane proteins of the ER are detected by chaperones and lectins and retro-translocated out of the ER for degradation. Here the Cdc48 machinery, recruited to the ER membrane, takes over. After polyubiquitylation of the protein substrate, Cdc48 together with its dimeric co-factor complex Ufd1–Npl4 pulls the misfolded protein out and away from the ER membrane and delivers it to down-stream components for degradation by a cytosolic proteinase machine, the proteasome. The known details of the Cdc48–Ufd1–Npl4 motor complex triggered process are subject of this review article. This article is part of a Special Issue entitled: AAA ATPases: Structure and function.

1. Introduction

Proper function of the workhorses of a cell, the proteins, is essential for all organisms. Statistic folding errors during synthesis or folding mistakes induced upon stresses like heat, oxidation or heavy metal ions require a functional protein quality control machinery, which recognizes misfolded proteins and delivers them to an elimination system [1,2]. Impairment of the quality control and elimination system leads to severe diseases in humans as are Alzheimer's-, Parkinson's-, Huntington's-, Creutzfeldt–Jakob- and many other diseases [3,4]. About 30% of proteins of a eukaryotic cell are proteins of the secretory pathway, which function in the ER, the Golgi apparatus, the lysosome, the cell membrane or the exterior of the cell. The ER contains the protein folding factory for these proteins [5–7]. After import into the ER in an unfolded state, it is a specialty of proteins of the secretory pathway to acquire certain modifications during the folding process, as is the formation of disulfide bonds and N-glycosylation. The status of protein folding is also efficiently scanned in this organelle on the level of exposed hydrophobic regions and glycan modification. An array of chaperones, co-chaperones, oxido-reductases, glycan chain modifying enzymes and lectins is constantly in a dynamic action to fold and scan a protein for proper folding [8–11]. After having passed the quality control, a protein is either released for further transport through the secretory pathway to its site of action or, when incorrect, is retained in the ER and retrograde transported across the ER membrane back into the cytosol, where it is rapidly degraded by the ubiquitin–proteasome-system [8,12–17]. It is between retrograde transport to the cytoplasm and degradation of the misfolded substrates by the proteasome where an ATP consuming machine, the Cdc48–Ufd1–Npl4 complex, acts as a motor required for substrate delivery to the proteasome. Cdc48 (yeast) was found as p97/VCP in mammals, TER94 in fly and CDC-48 in nematodes. However, we will use the term Cdc48 throughout this review article, irrespective of the organism used in the cited articles.

2. Import of proteins into the ER, folding and quality control

Proteins of the secretory pathway are imported into the ER in an either co-translational or post-translational fashion. The membrane translocation occurs via Sec61, a component of a large multiprotein complex providing the channel for protein entry [7,18]. Global folding of the protein occurs right upon its entry into the ER lumen. At the same time disulfide bonds are formed and sugar chains are added. Major players of the folding process are chaperones and their co-factors, protein disulfide isomerases, the oligosaccharyltransferase (OST) complex, carbohydrate trimming enzymes and lectins [5–11,19–21]. Proteins unable to reach their final conformation are mainly degraded by a process named ERAD.

The eukaryotic model organism yeast *Saccharomyces cerevisiae* has been a pacemaker in studies on ERAD [12–17]. According to the location of the misfolded protein domain relative to the ER, three types of

☆ This article is part of a Special Issue entitled: AAA ATPases: Structure and function.

* Corresponding author at: Institut für Biochemie, Universität Stuttgart, Pfaffenwaldring 55, D-70569 Stuttgart, Germany. Tel.: +49 711 685 64390; fax: +49 711 685 64392.
E-mail address: dieter.wolf@ibc.uni-stuttgart.de (D.H. Wolf).

0167-4889/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2011.09.002

ERAD substrates have been defined. (i) ERAD-L substrates carrying their misfolded domain in the lumen of the ER, (ii) ERAD-M substrates having a misfolded domain in the ER membrane and (iii) ERAD-C substrates exposing a misfolded domain into the cytoplasm [22,23].

In yeast, two polytopic RING-finger type ER membrane embedded ubiquitin ligases, Hrd1/Der3 [24–27] and Doa10 [28,29] are required for polyubiquitylation of these substrates to render them to proteasomal proteolysis. While ERAD-L and ERAD-M substrates are targets of the ubiquitin ligase Hrd1/Der3, ERAD-C substrates are mainly targets of Doa10. The mammalian equivalents of the yeast ligase Hrd1/Der3 are HRD1 and gp78. TEB4 (MARCH-IV) is the equivalent of yeast Doa10 [30,31]. However, the clear definition of two ERAD recognition pathways was only possible for yeast up to now (Fig. 1).

Substrates are not exclusively dependent on either E3 ligase system, Hrd1/Der3 or Doa10. Both pathways exhibit some plasticity: A Doa10 substrate can for instance also in part be a target of the Hrd1/Der3 ligase [32,33]. As compared to yeast, the mammalian repertoire of ER-associated E3 ligases has significantly expanded [34–36] making a high plasticity in the use of different recognition pathways of misfolded ERAD substrates likely. Even the participation of a cytosolic E3 in ERAD is possible [34,35,37].

When a misfolded protein of the ER is discovered as such, either because it has been retrograde transported back to the ER surface or because in its membrane location it presents a misfolded domain to the cytosolic environment, it is recognized by the respective E3 ubiquitin ligase [38] and polyubiquitylated. In yeast the major ubiquitin conjugating enzymes working together with the two E3 ligases are Ubc7 and Ubc6 [12–17]. A third ubiquitin conjugating enzyme, Ubc1, has only been shown to work together with Hrd1/Der3. Ubc7 is recruited to the ER membrane via the membrane protein Cue1 [34,39,40], while Ubc6 is tail-anchored in the ER membrane. The mammalian orthologues of Ubc6 and Ubc7 are Ube2j1 as well as Ube2j2 and Ube2g1 as well as Ube2g2, respectively [34].

Prior to ubiquitylation, the different ERAD substrates have to be recognized and excluded from folding intermediates and properly

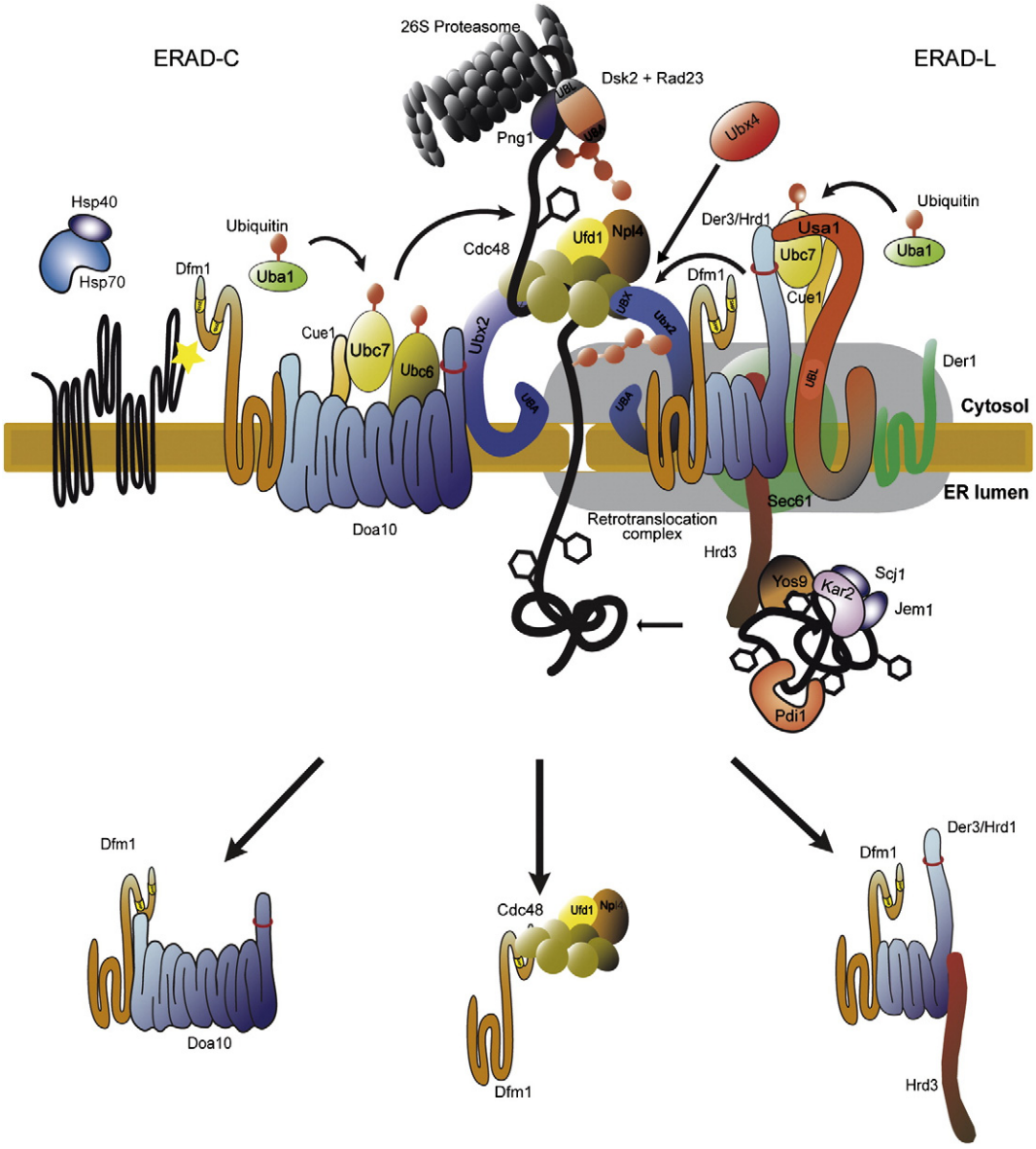


Fig. 1. Cdc48 in ERAD of yeast. Misfolded proteins in the ER lumen (ERAD-L recognition pathway) and in the ER membrane, the misfolded domain facing the cytosol (ERAD-C recognition pathway), are discovered and polyubiquitylated at the outer surface of the ER by two polytopic membrane localized ubiquitin ligases, Hrd1/Der3 and Doa10, respectively. At this stage the ternary Cdc48–Ufd1–Npl4 motor complex takes over, makes contact with the misfolded protein and prepares it for delivery to the proteasome for degradation. Besides Ubx2, recruiting the Cdc48 complex to the ER membrane and by this bringing the complex into contact with Hrd1/Der3 and Doa10, the Der1 homologue Dfm1 is able to bind Cdcc48 via its SHP domain. For details, see text.

Please cite this article as: D.H. Wolf, A. Stolz, The Cdc48 machine in endoplasmic reticulum associated protein degradation, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbamcr.2011.09.002

folded proteins. As compared to ERAD-C, the recognition pathway of ERAD-L substrates is rather complicated. A standard substrate of this pathway is CPY*, a mutated vacuolar (lysosomal) peptidase of yeast, which has led to the discovery of the major principles of ERAD [41–43]. After post-translational import into the ER, the mutated protein is N-glycosylated at four sites. In general, folding in the ER is controlled by chaperones detecting hydrophobic patches on the surface of a protein accompanied by a trimming process of the N-linked Glc_3–Man_9–$GlcNAc_2$ carbohydrate [9,11,44–48]. Specifically, CPY* is contacted by the Hsp70 chaperone of the ER, Kar2, which is linked to its Hsp40 cofactors Jem1 and Scj1 [49–51] (Fig. 1). During folding the three glucose residues at the A-branch of the carbohydrate chain [11] are cleaved off [52,53]. After cleavage of the first two glucose residues the protein is a substrate of the mammalian calnexin/calreticulin chaperone system, which is able to keep on folding of the protein moiety until the third glucose residue is cleaved off [8,54–56]. Subsequently, two α-mannosidases, Mns1 [52,57] and Htm1/Mnl1 [58,59], remove two defined mannose residues, creating an α-1,3 mannose linkage at the B-Branch and an α-1,6 mannose linkage at the C-branch of the carbohydrate chain, respectively [11].The moment the mannosidase residues are cleaved off, the protein is determined for elimination [9,11,60–62]. When a protein is not properly folded upon removal of the third glucose residue in the first round, in contrast to yeast, in mammalian cells an UDP-glucose: glycoprotein glucosyltransferase prolongs the folding cycle of a protein by adding a glucose residue onto the yet not mannose processed glycan [62–64].

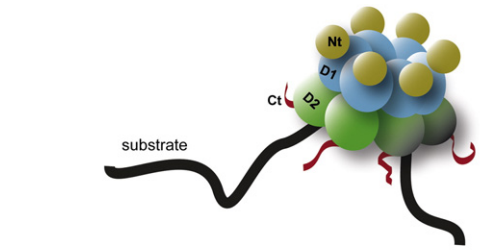
The α-1,6 mannose linkage created by Htm1/Mnl1, which is recognized by the lectin Yos9, represents a signal to remove the protein [65]. The C-terminus of Htm1/Mnl1 is able to interact with protein disulfide isomerase Pdi1, which has been previously shown to recognize terminally misfolded proteins and target them to retro-translocation [58,66]. Recognition of the misfolded protein and the reduction of disulfide bonds might here be the major tasks of Pdi1. Yos9 contacts an ER membrane anchored chaperone, Hrd3, which is able to recognize non-folded protein patches. They together deliver the misfolded protein in a yet unknown fashion to a channel, which allows movement of the misfolded protein across the membrane to the cytoplasmic side of the ER with concomitant ubiquitylation by the Hrd1/Der3 ligase. A similar mechanism with some variations is proposed for the mammalian Yos9 orthologues, OS-9 and XTB3-B, which connect to the mammalian ubiquitin ligase machinery HRD1-SEL1L, the orthologous machinery of yeast Hrd1/Der3-Hrd3 [67–72]. In mammalian cells a machinery consisting of the Pdi1 orthologue ERdj5, the Htm1/Mnl1 mannosidase orthologue EDEM1 in conjunction with the chaperone BiP prepares misfolded protein for retro-translocation [73,74]. In yeast, the channel through which misfolded proteins pass, could be part of a retrotranslocation complex, which consists of the six transmembrane spanning ER ligase Hrd1/Der3, the Hrd3 chaperone and the linker protein Usa1, which recruits a four transmembrane protein, Der1, to the assembly. Also the translocon protein Sec61 is thought to be part of this membrane assembly and the retro-translocation process [49,75–78]. Even though there are many speculations about the nature of the retrotranslocation channel [79–81], there is no proof of its identity so far.

The second ERAD recognition pathway, ERAD-C, is characterized by the discovery of membrane proteins retained in the ER, which expose their misfolded domains to the cytoplasm. The polytopic ER-membrane localized ubiquitin ligase, Doa10 in yeast, TEB4 (MARCH-IV) in mammalian cells, is here the major player [28,29,34,39]. Recognition of the misfolded domain in the cytoplasm occurs via Hsp70-Hsp40 chaperones. In yeast the Hsp70 Ssa-family and the Hsp40's Ydj1 and Hlj1 are prominent helpers [8,32].

Once the misfolded proteins are polyubiquitylated, on the outer surface of the ER membrane, the pathways merge at an ATP driven molecular machine, the AAAtype-ATPase Cdc48 (p97/VCP in mammals). In yeast, the machine is recruited to the ERAD ligases by the membrane anchor protein Ubx2 [82,83]. The functional equivalents of yeast Ubx2 in mammalian cells are UBXD2 and UBXD8 [84,85]. The mammalian ligases HRD1 and gp78, in contrast to the yeast equivalent Hrd1/Der3, possess Cdc48 binding motifs by themselves being also able to recruit the ATPase machine to the ER membrane [86,87].

3. The Cdc48 machine

The Cdc48 gene was first identified in a genetic screen in yeast when searching for conditional mutations affecting the cell cycle (cdc: cell division cycle) [88]. Sequencing of the gene uncovered a protein of 92 kDa molecular mass [89]. The protein has been found to function in a multitude of cellular processes [90,91], however, the best understood is its function in ERAD. Cdc48 forms a homohexameric complex composed of six protomers. Each subunit is composed of an N-terminal domain followed by two conserved AAA domains (D1 and D2) and an unstructured C-terminus (Fig. 2). The overall architecture of the Cdc48 complex can be viewed as two stacked hexameric rings, which are formed by the D1 and D2 domains of the 806 amino-acids containing protein [92–95]. Assembly of the homohexameric Cdc48 complex seems to depend on the D1 domains. Nucleotide is not required for the assembly process [94,95]. The two AAA domains contain the conserved nucleotide binding (Walker A) and hydrolysis (Walker B) motifs. They also contain a second region of homology (SRH), which is required for efficient ATP hydrolysis. ADP is preferentially bound to D1. D2 interacts with different nucleotides. It is the function of Cdc48 to perform mechanical work, which is fueled by ATP hydrolysis. This ATP hydrolysis causes global conformational changes of the complex, which result in open and closed conformations of the D1 and D2 rings [94,96,97]. The Cdc48 hexamer contains 12 ATPase active sites. Due to the low ATPase activity of the D1 domains, the bulk of ATPase activity is exerted by the D2 domains [97]. However, this changes under heat shock conditions under which also the D1 domains gain strong ATPase activity. The conformational changes of the Cdc48 complex are transmitted to the proteins bound. However, it is still quite unclear how Cdc48 works mechanistically. The substrate may either be threaded through the central pore of the Cdc48 complex or, alternatively, loop into this pore and leave it again [98]. The D1 ring is relatively narrow. The D2 ring, however, is wider and contains twelve arginine residues. This arginine "collar" resembles the calculated protein denaturing power of about 8 M guanidine. The arginine collar together with a pair of phenylalanine and tryptophane residues just next to the denaturation collar might be responsible for protein unfolding [99]. Some studies consider it unlikely that ubiquitylated proteins as are ERAD substrates



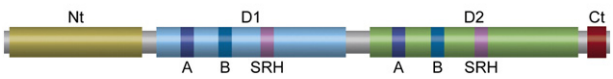


Fig. 2. Topology of the hexameric Cdc48/p97 complex and its domain architecture. The important domain structures of Cdc48 of the yeast *Saccharomyces cerevisiae* and homo sapiens are shown. The N-terminus (Nt) and the unstructured C-terminus (Ct) of Cdc48 are indicated. The N-terminus of the motor protein is followed by the D1 and D2 domains with their Walker A and Walker B motifs as well as the second region of homology 1 (SRH).

Please cite this article as: D.H. Wolf, A. Stolz, The Cdc48 machine in endoplasmic reticulum associated protein degradation, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbamcr.2011.09.002

pass the D1 pore [99]. It would still be possible that they enter the D2 pore and leave between the D1 and D2 ring. As ubiquitin is a thermodynamically stable protein this model would predict de-ubiquitylation prior to entry and re-ubiquitylation after exit of the substrate. Actually, Cdc48 cofactors able to perform this work are present, for instance in yeast in form of the de-ubiquitylating enzymes Otu1 and Ufd3 and the E4 ubiquitin chain elongating ligase Ufd2, which may contain a yet uncharacterized E3 ubiquitin ligase activity [100,101]. Indeed, the degradation of some ERAD substrates in yeast [36,102] and mammals [103] requires these activities. However, not all ERAD substrates of yeast tested so far require Otu1 or Ufd2 activity. A recent study on mammalian ERAD gave support for the idea, that substrates, after de-ubiquitylation, might be threaded through the narrow pore of Cdc48 and subsequently re-ubiquitylated for proteasomal targeting [104]. Future experiments must clarify the mechanism of how Cdc48 pulls the ubiquitylated misfolded proteins away from the ER membrane prior to delivery to the proteasome. Ataxin-3, a Cdc48 associated de-ubiquitylating enzyme, mutated in type-3 spinocerebellar ataxia, has also been shown to be involved in efficient elimination of misfolded ER protein [105,106]. It is speculated that ataxin-3 acts as an ubiquitin chain editing enzyme, promoting Cdc48 associated de-ubiquitylation to facilitate transfer of misfolded proteins from Cdc48 to the proteasome [105]. Ataxin-3 has also been suggested to negatively regulate retrograde protein translocation in ERAD [106,107]. Obviously, the precise role of ataxin-3 in ERAD has still to be elucidated.

The cellular functions of the Cdc48 complex and thus its function in ERAD are critically dependent on a multitude of regulatory cofactors (Fig. 3). Topologically they can be grouped into N-domain binding or C-domain binding [107–109]. Functionally they can also be grouped into two classes: substrate-recruiting- and substrate-processing cofactors [100,107]. A substrate recruiting cofactor dictates the pathway in which Cdc48 acts (i.e. ERAD). Binding of a substrate processing cofactor defines the fate, which the bound substrate is destined to (in the case of ERAD, degradation). The most important substrate recruiting cofactors known so far are p47 and the Ufd1-Npl4 heterodimer [107–109]. Binding of these two cofactors is mutually exclusive. P47 has been found to link Cdc48 to homotypic membrane fusion [110], while Ufd1–Npl4 was discovered to direct Cdc48 to ER-associated degradation of misfolded proteins [14,16,17,101,107].

4. Cdc48 in ERAD

The involvement of Cdc48 and its cofactor dimer Ufd1-Npl4 in ERAD was detected first in yeast using genetic and biochemical techniques [111–115]. Using CPY* as a substrate [41] as well as ERAD-prone MHC class I heavy – or immunoglobulin chains resulted in the observation that a defective Cdc48–Ufd1–Npl4 complex fails to remove the misfolded proteins from the ER and prevents their proteasomal degradation [111–113,116]. This led to the proposal that the Cdc48–Ufd1–Npl4 complex is a "ratcheting machine" [101] unwinding misfolded proteins away from the ER membrane or a "segregase" segregating proteolysis-prone molecules form the ER membrane [114]. This motor work is exerted via ATP driven domain motions, which generate the mechanical force to "extract" and retro-translocate the respective misfolded protein from the ER membrane [100,101,107,114]. As found in budding yeast, the Cdc48 complex in conjunction with the Ufd1–Npl4 heterodimeric cofactor is recruited to the ERAD machinery with the help of the UBX domain protein Ubx2 [82,83].

Recently, a homologue of the four transmembrane spanning Der1 protein [117], the founding member of the mammalian Derlins, was found in yeast and named Dfm1 (Der1 family member1) [118]. Besides forming complexes with both ER ligases, Hrd1 and Doa10, Dfm1 forms a complex with Cdc48 on its own via its SHP boxes [119,120]. Dfm1 is required for ERAD of certain substrates [119]. However the function of the Dfm1–Cdc48 complex in this process is not known yet.

It is also not yet fully established, if Cdc48 first interacts with a non-modified peptide stretch of the retro-translocated misfolded protein with subsequent ubiquitylation taking place to prevent back-sliding into the ER membrane [121,122]. Extracted ubiquitylated substrate proteins are further delivered to ubiquitin receptors

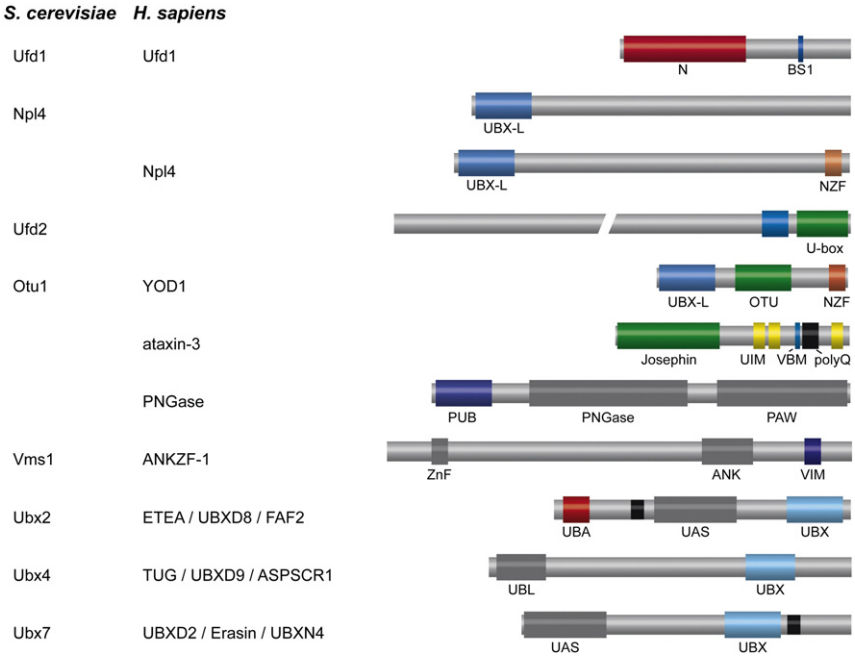


Fig. 3. Domain structure of substrate recruiting and substrate processing cofactors of Cdc48 reqired for ERAD. Cdc48 binding motifs are labeled in blue, while ubiquitin binding motifs are shown in red and yellow. Ligase and deubiquitylating domains are colored in green. All other domains are shown in dark gray. Transmembrane domains are indicated in black. Ufd1 and Npl4 represent the canonical substrate recruiting cofactors in ERAD which bind to the Nt domain of Cdc48. The UBX-like (UBX-L) and the linear binding site 1 (BS1) modules for binding Cdc48 are indicated. Substrate processing cofactors of Cdc48 involved in ERAD are Ufd2, binding via an unknown domain to the Ct domain of Cdc48, Otu1 (YOD1) binding the N-terminus (Nt) of Cdc48 via the UBX-L domain, ataxin-3 binding the Nt of Cdc48 via its VBM domain, Vms1 binding the Nt of Cdc48 via its VIM domain and PNGase binding the C-terminus of Cdc48 via its PUB domain. The UBX domain substrate processing cofactors listed, all bind to the Nt domain of Cdc48 with their UBX domain. Further details see in the text.

Please cite this article as: D.H. Wolf, A. Stolz, The Cdc48 machine in endoplasmic reticulum associated protein degradation, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbamcr.2011.09.002

(e.g. Dsk2, Rad23), which connect to the proteasome and guide the substrates to degradation [102,123]. Cdc48 and its cofactors Ufd1 and Npl4 from mammals contain intrinsic ubiquitin binding activity [101]. Npl4 from yeast is devoid of this activity due to the absence of a putative Zn-finger motif (NZF) present in mammalian Npl4 [124] (Fig. 3). As expected, mutations in all three components of the ternary Cdc48–Ufd1–Npl4 complex abrogate ERAD [111,112]. Only one Ufd1–Npl4 heterodimer binds to one Cdc48 hexamer, whereby Npl4 interacts with Cdc48 via its UBX-related UBD domain and Ufd1 binds Cdc48 via a BS1 domain [125]. This leaves further Ubx binding domains unoccupied on the hexameric Cdc48 complex [126] and with this, space is available for the association with additional UBX proteins to modify function and activity of the ternary Cdc48–Ufd1–Npl4 complex. Besides Ubx2, which recruits the Cdc48–Ufd1–Npl4 complex to the ER membrane (see above), Ubx4 has been shown to bind Cdc48, modulating the activity of the complex [127]: Ubx4 bound Cdc48 does not contain Ubx2. Furthermore, absence of Ubx4 leads to a massive accumulation of ubiquitylated proteins and a disturbed ERAD process [127]. Possibly, release of the poly-ubiquitylated material from the Cdc48–Ufd1–Npl4 complex is disturbed when Ubx4 is absent. Recently a yet uncharacterized yeast protein, Ydr49 and renamed VCP/Cdc48-associated mitochondrial stress responsive protein, Vms1, has been shown to interact with Cdc48 [128–130]. Vms1, like Ufd1, is a soluble protein but it excludes Ufd1 from simultaneous binding to Cdc48 [129]. Vms1 was shown to be involved in degradation of yeast expressed cystic fibrosis trans-membrane conductance regulator [130]. As Ubx4, Vms1 seems to have a function after the ubiquitylation reaction of ERAD substrates in the release of these ubiquitylated species from the Cdc48 complex. Interestingly, a genetic interaction between the *UBX4* and *VMS1* genes was observed and a double deletion exhibited an additive effect in stabilizing the standard misfolded luminal protein CPY* [130]. Recent studies in mammalian cells indicate that the retro-translocation function of Cdc48 is not exclusively linked to the ternary Cdc48–Ufd1Npl4–complex. ERAD may consist of several sub-pathways, which are driven by distinct Cdc48 complexes. A study on the cytomegalovirus (HCMV) induced degradation of major histocompatibility complex class I heavy chain (MHC class I HC) by the viral protein US2 [131] revealed the pathway to be independent of Ufd1 [132]. In contrast, the ERAD pathway of the HCMV protein US11 driven degradation of MHC class I HC [133] is dependent on the canonical ternary Cdc48–Ufd1–Npl4 complex. Both pathways also rely on different E3 ligases. While US11 triggered degradation of MHC-class I HC is initiated by the HRD1 ligase in conjunction with the Der1-like protein Derlin-1, the US2 driven degradation of MHC class I HC depends on the TRC8 ligase without the participation of Derlin-1 [134]. The two pathways also differ in their requirement for the mammalian Ubx2 orthologue UBXD8: This adaptor, recruiting Cdc48 to the ER membrane is only required for viral US11 driven, HRD1 dependent degradation of MHC class I HC [84]. Based on the idea that Npl4 can only bind to Cdc48 when Ufd1 is present [125], it was assumed that the Ufd1–Npl4 heterodimer as such is not involved in Cdc48 triggered retro-translocation of MHC class I HC upon action of the viral US2 protein. However, a recent study indicates that Npl4 by itself can work together with Cdc48 without the need of Ufd1 [135]. When comparing the tested ERAD pathways on the basis of the E3 ligases involved, the studies suggest that HRD1 mediated ERAD requires the well established ternary Cdc48–Ufd1–Npl4 complex (as does HMC class I HC degradation driven by the viral US11 protein, see above), while gp78 mediated ERAD requires only the Cdc48–Npl4 dimer [135]. This gives indication that Cdc48 is able to interact with both cofactors, Ufd1 and Npl4, independently. It has actually been shown that Npl4 has a weak Cdc48 interaction site, which can be activated by a small region in Ufd1 [125]. At the moment it is unclear, which other factor can take over this activating function of Ufd1 to allow formation of a dimeric Cdc48–Npl4 complex and trigger Ufd1 independent degradation of gp78 substrates [135].

ERAD of the misfolded N-glycosylated protein, CPY* (see above), led to the surprising observation that the protein is retro-translocated across the ER membrane into the cytosol in its fully glycosylated form [41]. The carbohydrate chain of such N-glycosylated misfolded proteins is cleaved off in the cytosol prior to proteasomal degradation via a peptide: N-glycanase (PNGase) [131,136]. In yeast the enzyme was found to associate with the proteasome via the ubiquitin receptor protein Rad23 [137], which is thought to take over the ubiquitylated proteins from Cdc48 to deliver them to the proteasome [123]. In contrast to yeast Png1, mammalian PNGase contains an N-terminally localized PUB (peptide: N-glycanase UBA or UBX containing protein) domain [138–140]. As shown in mice, the PUB domain links the enzyme to the ten C-terminal amino acids of Cdc48 [141]. The penultimate tyrosine residue of Cdc48 (Tyr805 in mouse Cdc48) can be phosphorylated. This phosphorylation abolishes binding of the PUB domain to the Cdc48 complex, leading to failure of the AAA-ATPase machine to bind PNGase. In vitro and in vivo studies indicate Src kinase to be responsible for phosphorylation of Tyr805 of Cdc48. In case of one ERAD substrate, TCR-α-GFP, inhibition of degradation was shown due to this phosphorylation event. These studies suggest that one way to regulate degradation of glycosylated misfolded proteins in mammalian ERAD rests in this phosphorylation step of Cdc48 [141,142]. It is, however, unclear which function the inhibition of ERAD at the stage of deglycosylation on the Cdc48 machine might have.

5. Conclusions and perspectives

Cdc48 has turned out to be an ATP driven machine in eukaryotes, which functions in many cellular processes. They range from cell cycle progression (the process of its original discovery) chromatin remodeling, DNA damage response, transcriptional and metabolic regulation, homotypic membrane fusion, selective autophagy to ERAD and cell death [90,100,143–146]. As expected from a central cellular motor, its presence is essential for life and mutations lead to severe diseases [90,146]. The only up to now well documented molecular mechanism of Cdc48 rests in its ratcheting or segregase function in proteolytic processes of which its involvement in ERAD is the best documented. However, also here the exact mechanistic details of this machine still await elucidation. What happens right after appearance of a piece of a misfolded ER luminal protein on the surface of the ER membrane in the ERAD-L pathway? Does the Cdc48–Ufd1–Npl4 complex first bind an unfolded patch of the protein to allow subsequent polyubiquitylation by the ER ligase and to prevent back-sliding of the molecule into the ER lumen [121]? At what point does the Cdc48–Ufd1–Npl4 machine recognize an ER membrane bound misfolded protein of the ERAD-C pathway to be removed? How does ATP hydrolysis alter the structure of the machine to allow mechanic movement of the protein chain? Does this mechanic movement actually occur by driving a chain through the narrow pore of Cdc48 formed by the D1 and D2 rings (whereby prior to passage through the pore deubiquitylation is required and after passage reubiquitylation of the chain must take place) or does unfolding and movement of the protein chain only occur in a hole formed by the D2 ring? Is for different ERAD substrates a different processing of the polyubiquitin chain required for which Cdc48 serves as a platform? How does the Cdc48-complex hand over the polyubiquitylated proteins to the downstream components for final proteasomal degradation? How do the many cofactors of Cdc48 support the function of the machine? Also, the molecular mechanism of Cdc48 in most of the cellular processes it is involved in, is completely unclear. Does its main function also reside here in protein delivery to the ubiquitin proteasome system for degradation? It is obvious that this molecular power machine will keep scientists busy for many years to come.

Please cite this article as: D.H. Wolf, A. Stolz, The Cdc48 machine in endoplasmic reticulum associated protein degradation, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbamcr.2011.09.002

Acknowledgements

The authors thank Elisabeth Tosta for the help with the preparation of this manuscript and Alexander Buchberger for figure templates published in [147]. Work of the authors was supported by a grant from the Deutsche Forschungsgemeinschaft (Bonn, Germany) and the European Network of Excellence, RUBICON.

References

[1] C.M. Dobson, Protein folding and misfolding, Nature 426 (6968) (2003) 884–890.
[2] A.L. Goldberg, Protein degradation and protection against misfolded or damaged proteins, Nature 426 (6968) (2003) 895–899.
[3] M. Aridor, L.A. Hannan, Traffic jam: a compendium of human diseases that affect intracellular transport processes, Traffic 1 (11) (2000) 836–851.
[4] J. Rutishauser, M. Spiess, Endoplasmic reticulum storage diseases, Swiss Med. Wkly. 132 (17–18) (2002) 211–222.
[5] E. van Anken, I. Braakman, Versatility of the endoplasmic reticulum protein folding factory, Crit. Rev. Biochem. Mol. Biol. 40 (4) (2005) 191–228.
[6] N.G. Haigh, A.E. Johnson, Protein sorting at the membrane of the endoplasmic reticulum, in: R.E. Dalbey, G. von Heijne (Eds.), Protein Targeting, Transport and Translocation, Academic Press, London-New York, 2002, pp. 74–106.
[7] T.A. Rapoport, Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes, Nature 450 (7170) (2007) 663–669.
[8] A. Stolz, D.H. Wolf, Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell, Biochim. Biophys. Acta 1803 (6) (2010) 694–705.
[9] B.R. Pearse, D.N. Hebert, Lectin chaperones help direct the maturation of glycoproteins in the endoplasmic reticulum, Biochim. Biophys. Acta 1803 (6) (2010) 684–693.
[10] A. Helenius, M. Aebi, Roles of N-linked glycans in the endoplasmic reticulum, Annu. Rev. Biochem. 73 (2004) 1019–1049.
[11] M. Aebi, R. Bernasconi, S. Clerc, et al., N-glycan structures: recognition and processing in the ER, Trends Biochem. Sci. 35 (2) (2010) 74–82.
[12] T. Sommer, D.H. Wolf, Endoplasmic reticulum degradation: reverse protein flow of no return, FASEB J. 11 (14) (1997) 1227–1233.
[13] J.L. Brodsky, A.A. McCracken, ER protein quality control and proteasome-mediated protein degradation, Semin. Cell Dev. Biol. 10 (5) (1999) 507–513.
[14] Z. Kostova, D.H. Wolf, For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin–proteasome connection, EMBO J. 22 (10) (2003) 2309–2317.
[15] R.K. Plemper, D.H. Wolf, Retrograde protein translocation: ERADication of secretory proteins in health and disease, Trends Biochem. Sci. 24 (7) (1999) 266–270.
[16] B. Meusser, C. Hirsch, E. Jarosch, et al., ERAD: the long road to destruction, Nat. Cell Biol. 7 (8) (2005) 766–772.
[17] S.S. Vembar, J.L. Brodsky, One step at a time: endoplasmic reticulum-associated degradation, Nat. Rev. Mol. Cell Biol. 9 (12) (2008) 944–957.
[18] N.G. Haigh, A.E. Johnson, Protein sorting at the membrane of the endoplasmic reticulum, Protein Targeting, Transport and Translocation, Academic Press, 2002, pp. 74–106.
[19] R.B. Freedman, T.R. Hirst, M.F. Tuite, Protein disulphide isomerase: building bridges in protein folding, Trends Biochem. Sci. 19 (8) (1994) 331–336.
[20] B.P. Tu, J.S. Weissman, Oxidative protein folding in eukaryotes: mechanisms and consequences, J. Cell Biol. 164 (3) (2004) 341–346.
[21] C. Appenzeller-Herzog, L. Ellgaard, The human PDI family: versatility packed into a single fold, Biochim. Biophys. Acta 1783 (4) (2008) 535–548.
[22] S. Vashist, D.T. Ng, Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control, J. Cell Biol. 165 (1) (2004) 41–52.
[23] P. Carvalho, V. Goder, T.A. Rapoport, Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins, Cell 126 (2) (2006) 361–373.
[24] J. Bordallo, R.K. Plemper, A. Finger, et al., Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins, Mol. Biol. Cell 9 (1) (1998) 209–222.
[25] R.Y. Hampton, R.G. Gardner, J. Rine, Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3- methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein, Mol. Biol. Cell 7 (12) (1996) 2029–2044.
[26] N.W. Bays, R.G. Gardner, L.P. Seelig, et al., Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation, Nat. Cell Biol. 3 (1) (2001) 24–29.
[27] P.M. Deak, D.H. Wolf, Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation, J. Biol. Chem. 276 (14) (2001) 10663–10669.
[28] R. Swanson, M. Locher, M. Hochstrasser, A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation, Genes Dev. 15 (20) (2001) 2660–2674.
[29] S.G. Kreft, L. Wang, M. Hochstrasser, Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI), J. Biol. Chem. 281 (8) (2006) 4646–4653.
[30] Z. Kostova, Y.C. Tsai, A.M. Weissman, Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation, Semin. Cell Dev. Biol. 18 (6) (2007) 770–779.
[31] F. Eisele, A. Schafer, D.H. Wolf, Ubiquitylation in the ERAD pathway, Subcell. Biochem. 54 (2010) 136–148.
[32] G. Huyer, W.F. Piluek, Z. Fansler, et al., Distinct machinery is required in *Saccharomyces cerevisiae* for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein, J. Biol. Chem. 279 (37) (2004) 38369–38378.
[33] A. Gnann, J.R. Riordan, D.H. Wolf, Cystic fibrosis transmembrane conductance regulator degradation depends on the lectins Htm1p/EDEM and the Cdc48 protein complex in yeast, Mol. Biol. Cell 15 (9) (2004) 4125–4135.
[34] Z. Kostova, Y.C. Tsai, A.M. Weissman, Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation, Semin. Cell Dev. Biol. 18 (6) (2007) 770–779.
[35] J.M. Younger, L. Chen, H.Y. Ren, et al., Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator, Cell 126 (3) (2006) 571–582.
[36] K. Nakatsukasa, G. Huyer, S. Michaelis, et al., Dissecting the ER-associated degradation of a misfolded polytopic membrane protein, Cell 132 (1) (2008) 101–112.
[37] T. Omura, M. Kaneko, Y. Okuma, et al., A ubiquitin ligase HRD1 promotes the degradation of Pael receptor, a substrate of Parkin, J. Neurochem. 99 (6) (2006) 1456–1469.
[38] B.K. Sato, D. Schulz, P.H. Do, et al., Misfolded membrane proteins are specifically recognized by the transmembrane domain of the Hrd1p ubiquitin ligase, Mol. Cell 34 (2) (2009) 212–222.
[39] F. Eisele, A. Schafer, D.H. Wolf, Ubiquitylation in the ERAD pathway, Subcell. Biochem. 54 (2010) 136–148.
[40] T. Biederer, C. Volkwein, T. Sommer, Role of Cue1p in ubiquitination and degradation at the ER surface, Science 278 (5344) (1997) 1806–1809.
[41] M.M. Hiller, A. Finger, M. Schweiger, et al., ER degradation of a misfolded luminal protein by the cytosolic ubiquitin–proteasome pathway, Science 273 (5282) (1996) 1725–1728.
[42] D.H. Wolf, A. Schäfer, CPY* and the power of yeast genetics in the elucidation of quality control and associated protein degradation of the endoplasmic reticulum, Curr. Top. Microbiol. Immunol. 300 (2006) 41–56.
[43] A. Stolz, D.H. Wolf, Use of CPY* and its derivatives to study protein quality control in various cell compartments. Meth. Mol. Biol. (in press).
[44] A. Helenius, E.S. Trombetta, D.N. Hebert, et al., Calnexin, calreticulin and the folding of glycoproteins, Trends Cell Biol. 7 (5) (1997) 193–200.
[45] T.A. Rapoport, B. Jungnickel, U. Kutay, Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes, Annu. Rev. Biochem. 65 (1996) 271–303.
[46] A. Zapun, C.A. Jakob, D.Y. Thomas, et al., Protein folding in a specialized compartment: the endoplasmic reticulum, Structure 7 (8) (1999) R173–R182.
[47] C. Hammond, A. Helenius, Quality control in the secretory pathway, Curr. Opin. Cell Biol. 7 (4) (1995) 523–529.
[48] L. Ellgaard, M. Molinari, A. Helenius, Setting the standards: quality control in the secretory pathway, Science 286 (5446) (1999) 1882–1888.
[49] R.K. Plemper, S. Böhmler, J. Bordallo, et al., Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation, Nature 388 (6645) (1997) 891–895.
[50] J.L. Brodsky, E.D. Werner, M.E. Dubas, et al., The requirement for molecular chaperones during endoplasmic reticulum- associated protein degradation demonstrates that protein export and import are mechanistically distinct, J. Biol. Chem. 274 (6) (1999) 3453–3460.
[51] S. Nishikawa, S.W. Fewell, Y. Kato, et al., Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation, J. Cell Biol. 153 (5) (2001) 1061–1070.
[52] C.A. Jakob, P. Burda, J. Roth, et al., Degradation of misfolded endoplasmic reticulum glycoproteins in *Saccharomyces cerevisiae* is determined by a specific oligosaccharide structure, J. Cell Biol. 142 (5) (1998) 1223–1233.
[53] R. Hitt, D.H. Wolf, DER7, encoding alpha-glucosidase I is essential for degradation of malfolded glycoproteins of the endoplasmic reticulum, FEMS Yeast Res. 4 (8) (2004) 815–820.
[54] B.R. Pearse, D.N. Hebert, Lectin chaperones help direct the maturation of glycoproteins in the endoplasmic reticulum, Biochim. Biophys. Acta 1803 (6) (2010) 684–693.
[55] M. Aebi, R. Bernasconi, S. Clerc, et al., N-glycan structures: recognition and processing in the ER, Trends Biochem. Sci. 35 (2) (2010) 74–82.
[56] C. Hammond, I. Braakman, A. Helenius, Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control, Proc. Natl. Acad. Sci. U. S. A. 91 (3) (1994) 913–917.
[57] M. Knop, N. Hauser, D.H. Wolf, N-Glycosylation affects endoplasmic reticulum degradation of a mutated derivative of carboxypeptidase yscY in yeast, Yeast 12 (12) (1996) 1229–1238.
[58] S. Clerc, C. Hirsch, D.M. Oggier, et al., Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum, J. Cell Biol. 184 (1) (2009) 159–172.
[59] E.M. Quan, Y. Kamiya, D. Kamiya, et al., Defining the glycan destruction signal for endoplasmic reticulum-associated degradation, Mol. Cell 32 (6) (2008) 870–877.
[60] J. Chillaron, C. Adan, I.G. Haas, Mannosidase action, independent of glucose trimming, is essential for proteasome-mediated degradation of unassembled glycosylated Ig light chains, Biol. Chem. 381 (12) (2000) 1155–1164.
[61] Y. Oda, N. Hosokawa, I. Wada, et al., EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin, Science 299 (5611) (2003) 1394–1397.
[62] C. Fagioli, R. Sitia, Glycoprotein quality control in the endoplasmic reticulum. Mannose trimming by endoplasmic reticulum mannosidase I times the

Please cite this article as: D.H. Wolf, A. Stolz, The Cdc48 machine in endoplasmic reticulum associated protein degradation, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbamcr.2011.09.002

proteasomal degradation of unassembled immunoglobulin subunits, J. Biol. Chem. 276 (16) (2001) 12885–12892.
[63] S.C. Taylor, P. Thibault, D.C. Tessier, et al., Glycopeptide specificity of the secretory protein folding sensor UDP-glucose glycoprotein:glucosyltransferase, EMBO Rep. 4 (4) (2003) 405–411.
[64] C. Ritter, K. Quirin, M. Kowarik, et al., Minor folding defects trigger local modification of glycoproteins by the ER folding sensor GT, EMBO J. 24 (9) (2005) 1730–1738.
[65] A. Bhamidipati, V. Denic, E.M. Quan, et al., Exploration of the topological requirements of ERAD identifies Yos9p as a lectin sensor of misfolded glycoproteins in the ER lumen, Mol. Cell 19 (6) (2005) 741–751.
[66] P. Gillece, J.M. Luz, W.J. Lennarz, et al., Export of a cysteine-free misfolded secretory protein from the endoplasmic reticulum for degradation requires interaction with protein disulfide isomerase, J. Cell Biol. 147 (7) (1999) 1443–1456.
[67] R. Gauss, E. Jarosch, T. Sommer, et al., A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery, Nat. Cell Biol. 8 (8) (2006) 849–854.
[68] R. Bernasconi, T. Pertel, J. Luban, et al., A dual task for the Xbp1-responsive OS-9 variants in the mammalian endoplasmic reticulum: inhibiting secretion of misfolded protein conformers and enhancing their disposal, J. Biol. Chem. 283 (24) (2008) 16446–16454.
[69] J.C. Christianson, T.A. Shaler, R.E. Tyler, et al., OS-9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD, Nat. Cell Biol. 10 (3) (2008) 272–282.
[70] T. Satoh, Y. Chen, D. Hu, et al., Structural basis for oligosaccharide recognition of misfolded glycoproteins by OS-9 in ER-associated degradation, Mol. Cell 40 (6) (2010) 905–916.
[71] N. Hosokawa, Y. Kamiya, D. Kamiya, et al., Human OS-9, a lectin required for glycoprotein endoplasmic reticulum-associated degradation, recognizes mannose-trimmed N-glycans, J. Biol. Chem. 284 (25) (2009) 17061–17068.
[72] R. Gauss, T. Sommer, E. Jarosch, The Hrd1p ligase complex forms a linchpin between ER-lumenal substrate selection and Cdc48p recruitment, EMBO J. 25 (9) (2006) 1827–1835.
[73] R. Ushioda, J. Hoseki, K. Araki, et al., ERdj5 is required as a disulfide reductase for degradation of misfolded proteins in the ER, Science 321 (5888) (2008) 569–572.
[74] R. Bernasconi, M. Molinari, ERAD and ERAD tuning: disposal of cargo and of ERAD regulators from the mammalian ER, Curr. Opin. Cell Biol. 23 (2) (2011) 176–183.
[75] R.K. Plemper, J. Bordallo, P.M. Deak, et al., Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation, J. Cell Sci. 112 (Pt 22) (1999) 4123–4134.
[76] M. Willer, G.M. Forte, C.J. Stirling, Sec61p is required for ERAD-L: genetic dissection of the translocation and ERAD-L functions of Sec61P using novel derivatives of CPY, J. Biol. Chem. 283 (49) (2008) 33883–33888.
[77] A. Schäfer, D.H. Wolf, Sec61p is part of the endoplasmic reticulum-associated degradation machinery, EMBO J. 28 (19) (2009) 2874–2884.
[78] D.C. Scott, R. Schekman, Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins, J. Cell Biol. 181 (7) (2008) 1095–1105.
[79] Y. Ye, Y. Shibata, C. Yun, et al., A membrane protein complex mediates retrotranslocation from the ER lumen into the cytosol, Nature 429 (6994) (2004) 841–847.
[80] B.N. Lilley, H.L. Ploegh, A membrane protein required for dislocation of misfolded proteins from the ER, Nature 429 (6994) (2004) 834–840.
[81] P. Carvalho, A.M. Stanley, T.A. Rapoport, Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p, Cell 143 (4) (2010) 579–591.
[82] O. Neuber, E. Jarosch, C. Volkwein, et al., Ubx2 links the Cdc48 complex to ER-associated protein degradation, Nat. Cell Biol. 7 (10) (2005) 993–998.
[83] C. Schuberth, A. Buchberger, Membrane-bound Ubx2 recruits Cdc48 to ubiquitin ligases and their substrates to ensure efficient ER-associated protein degradation, Nat. Cell Biol. 7 (10) (2005) 999–1006.
[84] B. Mueller, E.J. Klemm, E. Spooner, et al., SEL1L nucleates a protein complex required for dislocation of misfolded glycoproteins, Proc. Natl. Acad. Sci. U. S. A. 105 (34) (2008) 12325–12330.
[85] J. Liang, C. Yin, H. Doong, et al., Characterization of erasin (UBXD2): a new ER protein that promotes ER-associated protein degradation, J. Cell Sci. 119 (Pt 19) (2006) 4011–4024.
[86] P. Ballar, S. Fang, Regulation of ER-associated degradation via p97/VCP-interacting motif, Biochem. Soc. Trans. 36 (Pt 5) (2008) 818–822.
[87] G. Morreale, L. Conforti, J. Coadwell, et al., Evolutionary divergence of valosin-containing protein/cell division cycle protein 48 binding interactions among endoplasmic reticulum-associated degradation proteins, FEBS J. 276 (5) (2009) 1208–1220.
[88] D. Moir, S.E. Stewart, B.C. Osmond, et al., Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies, Genetics 100 (4) (1982) 547–563.
[89] K.U. Fröhlich, H.W. Fries, M. Rudiger, et al., Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression, J. Cell Biol. 114 (3) (1991) 443–453.
[90] A. Stolz, W. Hilt, A. Buchberger, et al., Cdc48: a power machine in protein degradation, Trends Biochem. Sci. (2011) [Epub ahead of print].
[91] Y. Ye, Diverse functions with a common regulator: ubiquitin takes command of an AAA ATPase, J. Struct. Biol. 156 (1) (2006) 29–40.
[92] B. DeLaBarre, A.T. Brunger, Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains, Nat. Struct. Biol. 10 (10) (2003) 856–863.
[93] X. Zhang, A. Shaw, P.A. Bates, et al., Structure of the AAA ATPase p97, Mol. Cell 6 (6) (2000) 1473–1484.
[94] V.E. Pye, I. Dreveny, L.C. Briggs, et al., Going through the motions: the ATPase cycle of p97, J. Struct. Biol. 156 (1) (2006) 12–28.
[95] A.T. Brunger, B. DeLaBarre, NSF and p97/VCP: similar at first, different at last, FEBS Lett. 555 (1) (2003) 126–133.
[96] L.C. Briggs, G.S. Baldwin, N. Miyata, et al., Analysis of nucleotide binding to P97 reveals the properties of a tandem AAA hexameric ATPase, J. Biol. Chem. 283 (20) (2008) 13745–13752.
[97] J.M. Davies, A.T. Brunger, W.I. Weis, Improved structures of full-length p97, an AAA ATPase: implications for mechanisms of nucleotide-dependent conformational change, Structure 16 (5) (2008) 715–726.
[98] D. Halawani, M. Latterich, p97: the cell's molecular purgatory? Mol. Cell 22 (6) (2006) 713–717.
[99] B. DeLaBarre, J.C. Christianson, R.R. Kopito, et al., Central pore residues mediate the p97/VCP activity required for ERAD, Mol. Cell 22 (4) (2006) 451–462.
[100] S. Jentsch, S. Rumpf, Cdc48 (p97): a "molecular gearbox" in the ubiquitin pathway? Trends Biochem. Sci. 32 (1) (2007) 6–11.
[101] S. Raasi, D.H. Wolf, Ubiquitin receptors and ERAD: a network of pathways to the proteasome, Semin. Cell Dev. Biol. 18 (6) (2007) 780–791.
[102] H. Richly, M. Rape, S. Braun, et al., A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting, Cell 120 (1) (2005) 73–84.
[103] R. Ernst, B. Mueller, H.L. Ploegh, et al., The otubain YOD1 is a deubiquitinating enzyme that associates with p97 to facilitate protein dislocation from the ER, Mol. Cell 36 (1) (2009) 28–38.
[104] R. Ernst, J.H. Claessen, B. Mueller, et al., Enzymatic blockade of the ubiquitin–proteasome pathway, PLoS Biol. 8 (3) (2011) e1000605.
[105] Q. Wang, L. Li, Y. Ye, Regulation of retrotranslocation by p97-associated deubiquitinating enzyme ataxin-3, J. Cell Biol. 174 (7) (2006) 963–971.
[106] X. Zhong, R.N. Pittman, Ataxin-3 binds VCP/p97 and regulates retrotranslocation of ERAD substrates, Hum. Mol. Genet. 15 (16) (2006) 2409–2420.
[107] A. Buchberger, Control of ubiquitin conjugation by cdc48 and its cofactors, Subcell. Biochem. 54 (2010) 17–30.
[108] C. Schuberth, A. Buchberger, UBX domain proteins: major regulators of the AAA ATPase Cdc48/p97, Cell. Mol. Life Sci. 65 (15) (2008) 2360–2371.
[109] H.O. Yeung, P. Kloppsteck, H. Niwa, et al., Insights into adaptor binding to the AAA protein p97, Biochem. Soc. Trans. 36 (Pt 1) (2008) 62–67.
[110] H.H. Meyer, Golgi reassembly after mitosis: the AAA family meets the ubiquitin family, Biochim. Biophys. Acta 1744 (2) (2005) 108–119.
[111] Y. Ye, H.H. Meyer, T.A. Rapoport, The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol, Nature 414 (6864) (2001) 652–656.
[112] E. Jarosch, C. Taxis, C. Volkwein, et al., Protein dislocation from the ER requires polyubiquitination and the AAA- ATPase Cdc48, Nat. Cell Biol. 4 (2) (2002) 134–139.
[113] E. Rabinovich, A. Kerem, K.U. Frohlich, et al., AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation, Mol. Cell. Biol. 22 (2) (2002) 626–634.
[114] S. Braun, K. Matuschewski, M. Rape, et al., Role of the ubiquitin-selective CDC48 (UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates, EMBO J. 21 (4) (2002) 615–621.
[115] N.W. Bays, S.K. Wilhovsky, A. Goradia, et al., HRD4/NPL4 is required for the proteasomal processing of ubiquitinated er proteins, Mol. Biol. Cell 12 (12) (2001) 4114–4128.
[116] Y. Elkabetz, I. Shapira, E. Rabinovich, et al., Distinct steps in dislocation of luminal endoplasmic reticulum-associated degradation substrates: roles of endoplasmic reticulum-bound p97/Cdc48p and proteasome, J. Biol. Chem. 279 (6) (2004) 3980–3989.
[117] M. Knop, A. Finger, T. Braun, et al., Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast, EMBO J. 15 (4) (1996) 753–763.
[118] R. Hitt, D.H. Wolf, Der1p, a protein required for degradation of malfolded soluble proteins of the endoplasmic reticulum: topology and Der1-like proteins, FEMS Yeast Res. 4 (7) (2004) 721–729.
[119] A. Stolz, R.S. Schweizer, A. Schafer, et al., Dfm1 forms distinct complexes with Cdc48 and the ER ubiquitin ligases and is required for ERAD, Traffic 11 (10) (2010) 1363–1369.
[120] B.K. Sato, R.Y. Hampton, Yeast Derlin Dfm1 interacts with Cdc48 and functions in ER homeostasis, Yeast 23 (14–15) (2006) 1053–1064.
[121] Y. Ye, H.H. Meyer, T.A. Rapoport, Function of the p97–Ufd1–Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains, J. Cell Biol. 162 (1) (2003) 71–84.
[122] S. Thoms, Cdc48 can distinguish between native and non-native proteins in the absence of cofactors, FEBS Lett. 520 (1–3) (2002) 107–110.
[123] B. Medicherla, Z. Kostova, A. Schaefer, et al., A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation, EMBO Rep. 5 (7) (2004) 692–697.
[124] H.H. Meyer, Y. Wang, G. Warren, Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1–Npl4, EMBO J. 21 (21) (2002) 5645–5652.
[125] R.M. Bruderer, C. Brasseur, H.H. Meyer, The AAA ATPase p97/VCP interacts with its alternative co-factors, Ufd1-Npl4 and p47, through a common bipartite binding mechanism, J. Biol. Chem. 279 (48) (2004) 49609–49616.
[126] V.E. Pye, F. Beuron, C.A. Keetch, et al., Structural insights into the p97–Ufd1–Npl4 complex, Proc. Natl. Acad. Sci. U. S. A. 104 (2) (2007) 467–472.
[127] S.M. Alberts, C. Sonntag, A. Schafer, et al., Ubx4 modulates cdc48 activity and influences degradation of misfolded proteins of the endoplasmic reticulum, J. Biol. Chem. 284 (24) (2009) 16082–16089.
[128] N.J. Krogan, G. Cagney, H. Yu, et al., Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*, Nature 440 (7084) (2006) 637–643.

Please cite this article as: D.H. Wolf, A. Stolz, The Cdc48 machine in endoplasmic reticulum associated protein degradation, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbamcr.2011.09.002

[129] J.M. Heo, N. Livnat-Levanon, E.B. Taylor, et al., A stress-responsive system for mitochondrial protein degradation, Mol. Cell 40 (3) (2010) 465–480.
[130] J.R. Tran, L.R. Tomsic, J.L. Brodsky, A Cdc48p-associated factor modulates endoplasmic reticulum-associated degradation, cell stress, and ubiquitinated protein homeostasis, J. Biol. Chem. 286 (7) (2011) 5744–5755.
[131] E.J. Wiertz, D. Tortorella, M. Bogyo, et al., Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction, Nature 384 (6608) (1996) 432–438.
[132] N. Soetandyo, Y. Ye, The p97 ATPase dislocates MHC class I heavy chain in US2-expressing cells via a Ufd1–Npl4–independent mechanism, J. Biol. Chem. 285 (42) (2010) 32352–32359.
[133] E.J. Wiertz, T.R. Jones, L. Sun, et al., The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol, Cell 84 (5) (1996) 769–779.
[134] H.R. Stagg, M. Thomas, D. van den Boomen, et al., The TRC8 E3 ligase ubiquitinates MHC class I molecules before dislocation from the ER, J. Cell Biol. 186 (5) (2009) 685–692.
[135] P. Ballar, A. Pabuccuoglu, F.A. Kose, Different p97/VCP complexes function in retrotranslocation step of mammalian ER-associated degradation (ERAD), Int. J. Biochem. Cell Biol. 43 (4) (2011) 613–621.
[136] T. Suzuki, H. Park, N.M. Hollingsworth, et al., PNG1, a yeast gene encoding a highly conserved peptide:N-glycanase, J. Cell Biol. 149 (5) (2000) 1039–1052.
[137] T. Suzuki, H. Park, M.A. Kwofie, et al., Rad23 provides a link between the Png1 deglycosylating enzyme and the 26S proteasome in yeast, J. Biol. Chem. 276 (2001) 21601–21607.
[138] T. Suzuki, H. Park, E.A. Till, et al., The PUB domain: a putative protein–protein interaction domain implicated in the ubiquitin-proteasome pathway, Biochem. Biophys. Res. Commun. 287 (5) (2001) 1083–1087.
[139] G. Li, G. Zhao, X. Zhou, et al., The AAA ATPase p97 links peptide N-glycanase to the endoplasmic reticulum-associated E3 ligase autocrine motility factor receptor, Proc. Natl. Acad. Sci. U. S. A. 103 (22) (2006) 8348–8353.
[140] M.D. Allen, A. Buchberger, M. Bycroft, The PUB domain functions as a p97 binding module in human peptide N-glycanase, J. Biol. Chem. 281 (35) (2006) 25502–25508.
[141] G. Zhao, X. Zhou, L. Wang, et al., Studies on peptide:N-glycanase-p97 interaction suggest that p97 phosphorylation modulates endoplasmic reticulum-associated degradation, Proc. Natl. Acad. Sci. U. S. A. 104 (21) (2007) 8785–8790.
[142] G. Li, G. Zhao, H. Schindelin, et al., Tyrosine phosphorylation of ATPase p97 regulates its activity during ERAD, Biochem. Biophys. Res. Commun. 375 (2) (2008) 247–251.
[143] S. Bar-Nun, The role of p97/Cdc48p in endoplasmic reticulum-associated degradation: from the immune system to yeast, Curr. Top. Microbiol. Immunol. 300 (2005) 95–125.
[144] Y. Ye, Diverse functions with a common regulator: ubiquitin takes command of an AAA ATPase, J. Struct. Biol. 156 (1) (2006) 29–40.
[145] H. Meyer, O. Popp, Role(s) of Cdc48/p97 in mitosis, Biochem. Soc. Trans. 36 (Pt 1) (2008) 126–130.
[146] E. Chapman, A.N. Fry, M. Kang, The complexities of p97 function in health and disease, Mol. Biosyst. 7 (3) (2011) 700–710.
[147] A. Buchberger, Control of ubiquitin conjugation by cdc48 and its cofactors, Subcell. Biochem. 54 (2010) 17–30.

Please cite this article as: D.H. Wolf, A. Stolz, The Cdc48 machine in endoplasmic reticulum associated protein degradation, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbamcr.2011.09.002