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From lysosome to proteasome: the power of yeast in the dissection of proteinase function in cellular regulation and waste disposal

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Abstract. The yeast *Saccharomyces cerevisiae* has turned out to be an invaluable tool in the molecular biological sciences for elucidating the housekeeping functions of eukaryotic cells. Due to its easy amenability to biochemical, genetic, molecular biological and cell biological experimentation, including genomics and proteomics, yeast has become one of the most frequently used eukaryotic model organisms. One of the fields where studies in yeast have a truly pacemaking character is cellular control by proteolysis. The function of vacuolar (lysosomal) proteolysis was elucidated. The in vivo role of ubiquitin and its relation to the proteasome was uncovered. This research led to an avalanche of studies in many different eukaryotic systems, including mammals, and provided us with surprising new insights in cellular control in health and disease.

Key words. Yeast; ubiquitin; proteasome; vacuole/lysosome; catabolite degradation; ERAD; ER-quality control.

Introduction: From lysosome to proteasome

In 1942 Schönheimer [1] established the concept of the 'dynamic state of body constituents'. This concept introduced the instability of proteins in cells and their constant resynthesis. At the time this proposal did not receive much attention because scientists were too much occupied by the idea that cells were stable entities and machines which dealt economically with their energy. These facts would inhibit constant degradation of macromolecules, which required a huge amount of energy for resynthesis. About 2 decades later, deDuve and collaborators discovered the lysosome in eukaryotic cells [2–5]. Biochemical analysis identified this organelle as a membraneous sac filled with a multitude of hydrolytic enzymes, including a wide spectrum of protein-degrading enzymes [6, 7]. The lysosome was accepted as the gut of the eukaryotic cell, designed to degrade no longer necessary proteins. Protein uptake into lysosomes was shown to occur via autophagocytosis, a non-selective bulk process [7–9]. There was great skepticum at the time that proteins could be degraded selectively. However, this issue came gradually into focus when the first measurements of turnover of some enzymes in mammalian cells under different nutrient conditions were performed [10], and when it became obvious that structurally deformed proteins were eliminated [11, 12]. However, a function of proteolysis in general and the system involved remained obscure. From today's point of view, two developments, rather unrecognized at the time, ignited the present burst of research on proteolysis as one of the main regulatory tools of eukarytic cells: the discovery of ubiquitin [13, 14] and the use of yeast genetics in proteolysis research [15–19]. Inactivation of specific enzymes of the gluconeogenetic pathway was observed very early in yeast [20]. Biochemical experiments strongly indicated that lysosomal (vacuolar) proteinases were responsible for this event [21, 22]. Since proteinases are capable of degrading proteins to different extents and in different time frames in vitro depending on the tightness of a protein structure, biochemical in vitro experiments are not suitable in the search of the biological involvement of a certain proteinase in a cellular process. Isolation of mutants in various lysosomal (vacuolar) proteinases quickly showed the correctness of this prediction: none of these vacuolar enzymes were specifically involved in the degradation of the gluconeogenetic enzymes measured [17] (see below). However, the availability of vacuolar

(lysosome) proteinase defective yeast strains suddenly opened the possibility to answer the following questions: (i) What is the function of vacuolar proteolysis? (ii) How do proteins reach the vacuole for proteolysis to occur? (iii) Is the vacuole the only locus of proteinases or do proteinases different from the vacuolar (lysosomal) enzymes exist and what is their function?

The yeast vacuole (lysosome) contains two endopeptidases, proteinase yscA, an acidic proteinase, and proteinase yscB, a serine proteinase, which together degrade proteins down to peptides. Two carboxypeptidases – carboxypeptidase yscY and carboxypeptidase yscS – as well as a dipeptidylaminopeptidase V and two aminopeptidases, aminopeptidase yscI and aminopeptidase yscCo, also residing in the vacuole, further degrade the generated peptides into single amino acids [23]. Under vegetative growth conditions protein turnover rate in cells amount to ~1% per hour. Mutants defective in the two vacuolar proteinases yscA and yscB exhibit a 40% reduced turnover rate under these conditions. This deficiency, however, is perfectly well tolerated by cells, when nutrients are not limiting [24]. Under nutrient starvation (nitrogen deprivation and acetate as energy source) the activity of the two vacuolar proteinases increases severalfold [23], and protein turnover rate increases 3.5-fold as compared to actively growing cells. Deficiency of the two endopeptidases reduces the turnover rate by 85%, leading to severe physiological defects. The differentiation process during sporulation is dramatically impaired and cells finally die [24]. Thus, vacuolar proteolysis is essential for degradation of the cell's own protein to provide amino acids, amino nitrogen and carbon backbones for anabolic reactions under conditions of nutrient deprivation. Under these conditions proteinase yscA and yscB-deficient cells accumulate vesicles in the vacuolar lumen [25, 26], identified as autophagosomes [26]. This observation explained the pathway by which proteins entered the vacuole and opened the field to genetic analysis for elucidation of the autophagic pathway at the molecular level [27, 28] (for review see [29]). Autophagic uptake of proteins is a bulk process which does not allow selective regulation of individual proteins [30]. It was, thus, not surprising that a thorough biochemical screen in vacuolar proteinase-deficient mutants using chromogenic peptide substrates uncovered a multitude of new peptidolytic enzymes of which an enzyme called proteinase yscE turned out to be most prominent [31, 32]. Since the discovery of ubiquitin the proteolytic mechanism linking ubiquitin to ATP-dependent selective proteolysis has been thoroughly studied in vitro [13, 14, 33, 34]. The 76-amino acid polypeptide ubiquitin is bound in an isopeptide linkage via its C-terminal glycine to the N-terminus or to an ε-amino group of internal lysine residues of targets proteins. The reaction sequence starts with an energy-requiring activation step whereby the carboxy-terminal glycine residue of ubiquitin is attached to a cysteine residue of a ubiquitin activating enzyme (E1, Uba), forming a thioester bond. Subsequently ubiquitin is transferred to a cysteine of one of the multiple ubiquitin conjugating enzymes (E2, Ubc), followed by its transfer to an amino group (N-terminus or intrinsic lysines) of the substrate. This step requires ubiquitin-protein ligases (E3, Ubl). Subsequent substrate degradation requires polyubiquitination: additional ubiquitin molecules are attached to lysine residues, usually Lys48, in ubiquitins already bound to substrate [35–37] (fig. 1). Our knowledge about the in vivo function of the ubiquitin pathway and the multitude of different ubiquitin conjugating enzymes (E2) and ubiquitin-protein ligases (E3) came mainly from genetic and molecular biological studies in yeast. The mode of recognition of different N-termini in an artificially constructed fusion protein by the ubiquitin system was elucidated, thus defining the N-end rule pathway [38]. The first set of ubiquitin activating, conjugating and ligating enzymes was dissected genetically [39–43]. Furthermore the polyubiquitin gene and the family of ubiquitin gene fusions to ribosomal proteins was discovered, which explained how the cell produces enough ubiquitin at any given time [44]. The necessity for the ubiquitin system in the elimination of a transcription factor was discovered [45].

However, the proteinase that finally degrades the ubiquitinated proteins remained an enigma. An ATP-dependent high molecular mass proteinase had been identified and purified which degraded ubiquitinated lysozyme in vitro [46, 47]. Genetic studies on the yeast proteinase yscE [48], thereafter called the proteasome [49], finally uncovered the in vivo function of this enzyme in the degradation of ubiquitinated proteins and identified it as the proteinase of the ubiquitin degradation pathway [50] (fig. 1). For proteasome structure and the mechanism of proteasomal proteolysis, see the review by W. Heinemeyer et al. and M. Bajorek and M. Glickman of this issue. Since this discovery, the use of yeast proteasome mutants and, later, proteasome active site inhibitors in mammalian cells has revolutionized our understanding of the function of proteolysis in selective regulation of proteins and its role in cell function. We know now that the ubiquitin proteasome system, localized in the cytoplasm and nucleus of eukaryotic cells, is vital for cellular life under all circumstances and that a malfunctioning of this machinery leads to disease and cell death. Initial experiments in yeast uncovered its role in cell cycle control by degrading cylins, a class of proteins which are responsible for controlling Cdc28 kinase activity during progression of the cell cycle and its halt at G1/S and G2/M checkpoints [51, 52] (see review by W. Hilt of this issue). Also the role of the proteasome in transcriptional control was first elucidated in yeast. The MATα2 repressor, which governs yeast cell mating type, is rapidly turned over via the proteasome [53].

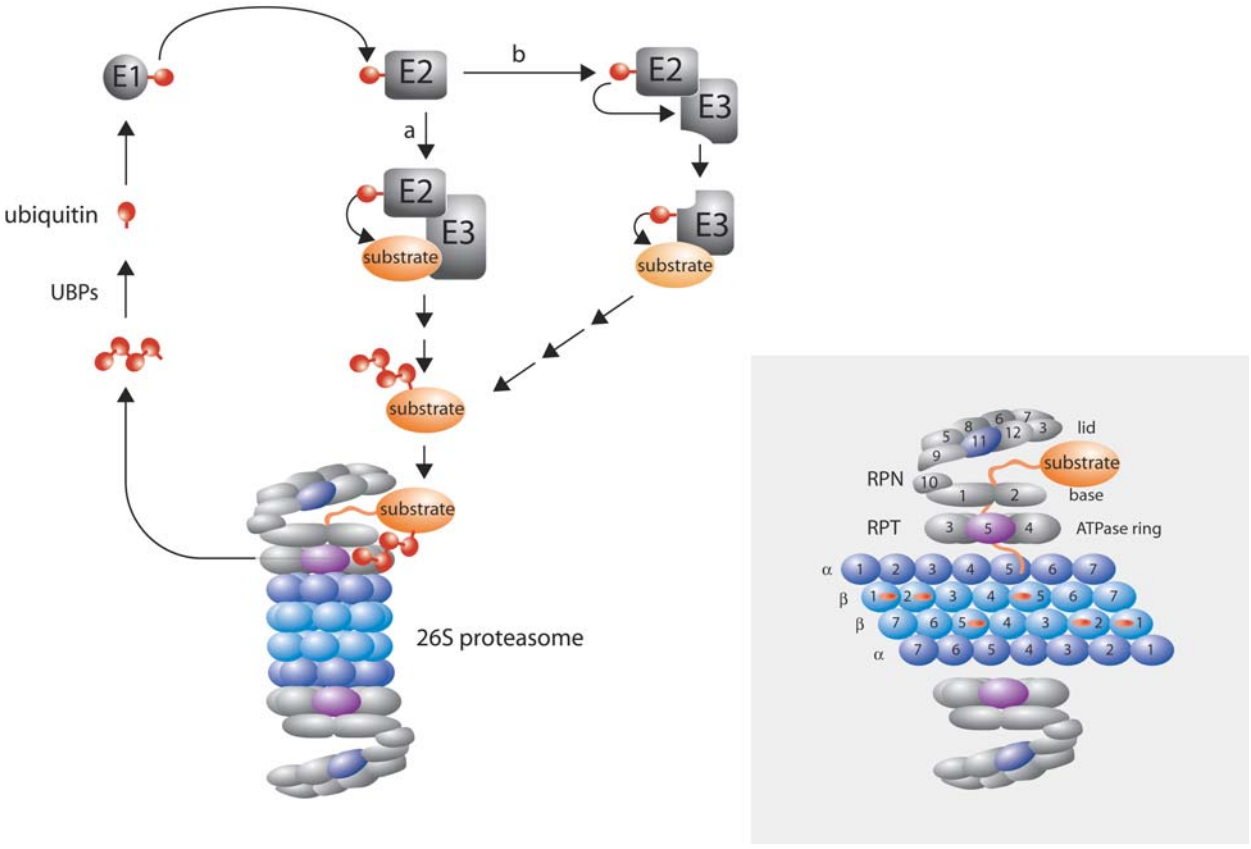

Figure 1. The pathway of ubiquitin-linked degradation of proteins and the 26S proteasome. After linkage of a polyubiquitin chain onto the protein to be degraded, its recognition occurs at the 19S cap of the 26S proteasome, which is composed of 12Rpn and 6Rpt (ATPase) subunits. Degradation is brought about by the proteolytic activity of a pair of three different β-subunits each located in the cylinder of the 20S $\alpha 7 \beta 7 \beta 7 \alpha 7$ core unit of the 26S proteasome (inset after [88]).

Control of cell metabolism: fructose-1,6-bisphosphatase

The discovery of the involvement of the proteasome in regulation of the gluconeogenetic enzyme fructose-1,6-bisphosphatase (FBPase), a key enzyme of carbon metabolism, in yeast [54, 55] led the way in recognizing the central role of this proteolytic machine in regulating the cellular metabolism. Fructose-1,6-bisphosphatase, a regulatory enzyme in gluconeogenesis, is active when cells grow on poor carbon sources and have to synthesize glucose de novo. Addition of glucose to the growth medium leads to repression of FBPase synthesis and to inactivation of the enzyme, thus preventing a futile cycle of ATP degradation between ongoing glycolysis and gluconeogenesis. Prior to the discovery of the proteasome, vacuolar proteinases were believed to be responsible for the elimination of FBPase in the presence of glucose [20, 22]. However, mutants defective in vacuolar proteinases did not exhibit an altered catabolite degradation of the enzyme [24, 56, 57]. The availability of such mutants quickly led to the discovery of the proteasome and the ubiquitin system as key players in the catabolite degradation of FBPase [54, 55, 58, 59]. Glucose-triggered action of the ubiquitin conjugating enzymes Ubc1, Ubc4/Ubc5 and Ubc8 as well as polyubiquitination of FBPase results in proteasomal degradation with a half-life of about 30 min (fig. 2). Glucose-induced phosphorylation of the enzyme precedes degradation, and the phospho-enzyme is threefold more sensitive to AMP and fructose-2,6-bisphosphate inhibition [60]. However, phosphorylation is not a prerequisite for degradation to occur [61]. In contrast to phosphorylation, ubiquitin-proteasome-catalyzed degradation requires synthesis of one or several new proteins upon glucose signaling. In addition, the amino-terminal proline of FBPase is absolutely necessary for the ubiquitination of the enzyme, which is a prerequisite for proteasomal degradation [61]. Growth of cells on ethanol and subsequent glucose addition leads to execution of the catabolite degradation pathway by the ubiquitin proteasome system [54, 55, 59, 61] (fig. 2). A long starvation period of cells on acetate with subsequent glucose addition seems to trigger uptake of FBPase into vesicles and vacuolar proteolysis

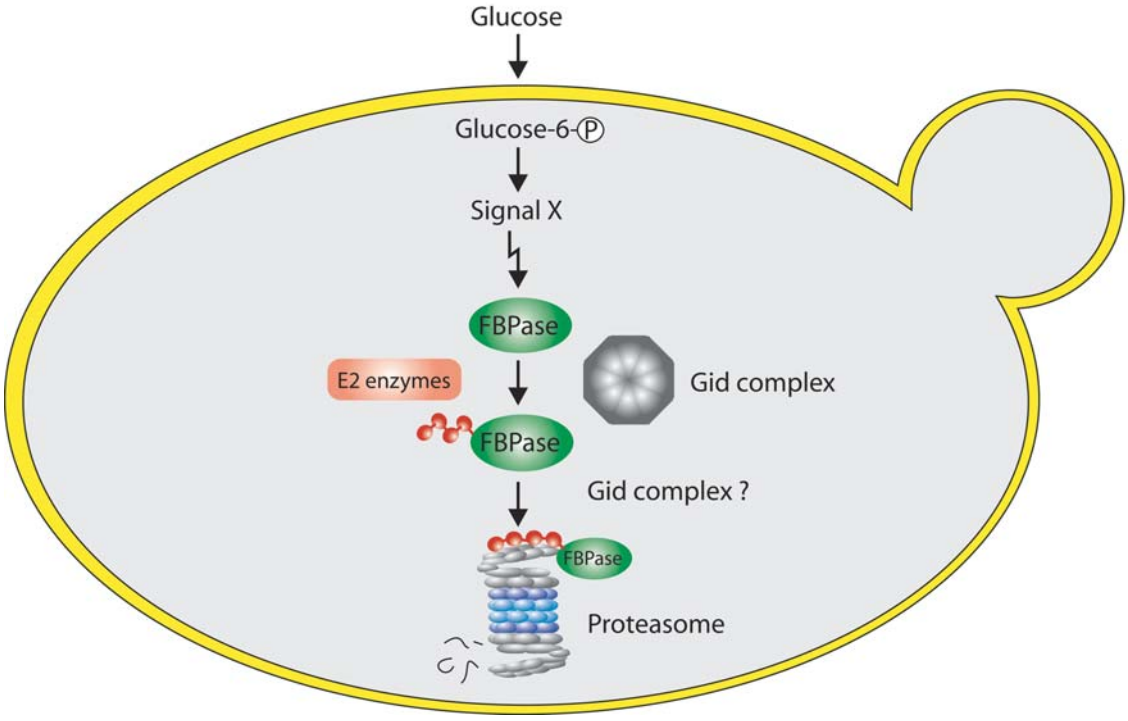

Figure 2. Catabolite degradation of the gluconeogenetic key enzyme fructose-1,6-bisphosphatase (FBPase). Addition of glucose to glucose-deprived cells triggers a yet unknown signal, which induces ubiquitin-proteasome-dependent degradation of the enzyme.

[62–65]. Recently, eight new genes (*GID1*, *GID2* and *GID4* to *GID9*) required for ubiquitin-proteasome-triggered catabolite degradation of FBPase have been identified [66] (fig. 2). One of these gene products, Gid2p, is required for ubiquitination of FBPase, while Gid6p turned out to be identical to Ubp14p, a deubiquitinating enzyme required for disassembly of free polyubiquitin chains [67, 68]. Interestingly, three of the newly discovered *GID* genes (*GID1/VID30, GID*4/*VID24* and *GID5/VID28*) are identical with genes described to be involved in vesicular uptake of FBPase after long acetate starvation [62, 64]. This finding seems to indicate functional overlap of these three proteins in both degradation pathways [66]. Studies have determined that Vid24p/Gid4p is synthesized newly when glucose is added to derepressed cells [64]. Interestingly, Gid2p, bearing a molecular mass of 49 kDa, sediments as a 600-kDa cytosolic protein in density gradient fractionations, indicating that it is part of a large complex ('Gid-complex'). It is likely that this Gid-complex includes most of the new Gid proteins [66] (fig. 2). It is a challenging task to uncover the function of the Gid-complex in the ubiquitin-proteasome-triggered catabolite degradation of FBPase and other gluconeogenic enzymes.

Degradation of protein waste

Degradation of intracellular protein waste was thought to occur solely in the lysosome (vacuole) of eukaryotic cells. However, genetic studies in yeast also uncovered the involvement of the ubiquitin-proteasome pathway in this event. Canavanine incorporation into proteins induces unfolding and renders these proteins susceptible to proteolysis via the proteasome [50]. Also, the discovery of the degradation pathway for malfolded soluble secretory proteins of the endoplasmic reticulum originated mainly from genetic studies in yeast. With the purpose of understanding vacuolar function, a mutant defective in the activity of carboxypeptidase yscY was created [15]. Sequence analysis uncovered that the protein had acquired a non-conservative mutation in a highly sensitive region of all serine proteases: the conserved glycine residue 255, two amino acids upstream of the active site serine, had been converted to an arginine [69]. The mutated carboxypeptidase yscY, for simplicity named CPY*, is completely misfolded [69]. The mutant enzyme was no longer transported to its normal destination, the vacuole, and surprisingly, even malfolded, it was not delivered to the cellular gut, the vacuole, where non-selective proteolysis occurs. Instead CPY* remained in the endoplasmic reticulum (ER) from where it disappeared rapidly [69]. CPY* and a second protein, a mutated pro-α-factor pheromone which failed to be glycosylated, led

the way to uncover the degradation pathway of malfolded secretory proteins [70–72]. Many surprises accompanied the discovery of this degradation pathway.

The secretory pathway, protein folding and quality control

Carboxypeptidase yscY and the mating pheromone α-factor are secretory proteins. This class of proteins is synthesized in the cytoplasm of cells, and the proteins are subsequently transported to the lysosome/vacuole (carboxypeptidas yscY), to the plasma membrane and the exterior of cells (α-factor) or they are components of the secretory machinery itself, the ER and the Golgi apparatus. The ER is the organelle into which secretory proteins are transferred and is responsible for their proper folding, assembly and modification before release into the secretory pathway [73, 74]. Transport across the ER membrane is achieved by an aqueous channel, the Sec61 translocon, which consists of three different subunits in yeast: Sec61p, Sbh1p and Sss1p. The proteins traverse the channel in an unfolded state, and a multitude of accessory proteins inside the ER are responsible for core glycosylation, disulfide-bond formation and proper folding of the emerging protein [74, 75]. The most prominent are signal peptidase, which cleaves off the signal peptide from newly translocated proteins; oligosaccaryl transferase, which transfers the core carbohydrate tree onto asparagine residues of proteins (N-glycosylation); protein disulfide isomerase, which participates in disulfide formation; and a variety of chaperones, which assist protein folding in different ways. The Hsp70 chaperone Kar2p (BiP in mammalian cells), which recognizes hydrophobic protein stretches, and calnexin, which binds the carbohydrate moieties of glycoproteins, are two well-characterized examples. Only when properly folded and modified is a protein allowed to leave the ER for the journey to its site of action [76]. The importance of proper protein folding in the ER before delivery to the secretory compartment is underlined by the existence of the unfolded protein response (UPR), a process that controls the concentration of the auxiliary folding proteins [77, 78]. Ire1p, an ER/nuclear envelope-localized transmembrane kinase/endonuclease senses the concentration of unfolded proteins in the ER via its ER lumenal domain. Sensing occurs via a competition between the Ire1p lumenal domain and free unfolded proteins for binding to Kar2p (BiP), the major chaperone of the ER. Depletion of free Kar2p (BiP) due to binding to increasing amounts of unfolded proteins causes dimerization of Ire1p by this, transducing the signal across the membrane and activating the kinase activity. The kinase/endonuclease induces a non-canonical splicing of the *HAC1* mRNA, which allows synthesis of the Hac1p transcription factor. Hac1p, in turn, upregulates genes containing the UPR response element and leads to an increase in the concentration of folding proteins in the ER as well as of proteins required for protein quality control in this organelle [78–80].

In 1890 William Russell observed peculiar intracellular structures in mammalian cells, now called Russell bodies. More recently these structures were determined to be dilated ER cisternae containing aggregated immunoglobulins [81, 82]. The prion diseases of sheep (scrapie), cattle (bovine spongioform encephalopathy, BSE), human (Creutzfeldt-Jakob disease, CFD) or Parkinson disease, all caused by protein precipitate deposition, had long been of mysterious origin [83]. On the other hand, there are human diseases such as cystic fibrosis and lung emphysema which were shown to be caused by the rapid disappearance of crucial proteins, like the cystic fibrosis transmembrane conductance regulator and α-1-antitrypsin, respectively [84–87]. Only recently has it become possible to shed light on the molecular mechanisms underlying these diseases. This breakthrough is in great part due to the discovery of the degradation process of mutated malfolded ER proteins in yeast and the elegant use of the genetics of this organism [70, 83, 88–90]. The function of proteins depends on their precise three-dimensional conformation. This requires an intact primary amino acid sequence and an uninterrupted, competent folding and assembly process. Mutations leading to an abnormal structure or an erroneous folding process may lead to inactive proteins and, if not properly dealt with, to protein aggregates. To minimize folding mistakes, nature has established a highly active chaperone system which assists the folding of proteins to prevent dead-end conformations, causing inactive proteins and in many cases insoluble aggregates [82]. A folding machinery of this kind is especially active in the ER, which nearly all secretory proteins enter in an unfolded state. Protein stretches that leave the translocon are immediately occupied by chaperones sheltering hydrophobic surfaces from forming intra- or intermolecular contacts. The most active accessory folding proteins are the Hsp70 chaperone Kar2p (BiP), the carbohydrate binding, lectin-like chaperones calnexin and calreticulin, and the enzymes involved in disulfide bond formation; protein disulfide isomerase (PDI), the oxidoreductase Erp57 and other enzymes connected with disulfide bond formation. However, even the chaperone-assisted folding process cannot guarantee 100% perfection nor can it be perfect when trying to fold a mutated protein. Therefore, a quality control system has evolved to detect proteins unable to fold properly and to eliminate them. The ER represents the major quality control checkpoint known up to now for secretory proteins [76, 91].

When following the fate of CPY*, it was found that the mutated enzyme is completely transported into the ER lumen, fully glycosylated but never transported to the vac-

uole [92]. It is retained in the ER and, surprisingly, destroyed by the cytoplasmic proteasome [71, 93]. CPY* is translocated across the ER membrane, and four core oligosaccharides composed of $Glc_3Man_9GlcNAc_2$ are added co-translationally in an N-glycosidic bond to the side chains of asparagine residues that are part of the Asn-X-Ser/Thr consensus sequence. The immediate folding process is supported by several chaperones and PDI. The function of N-glycans in the retention and elimination of the protein could be traced in detail via mutant analysis and appears very similar to the mechanism in mammalian cells [91]. Glucosidases I and II cut the outermost three glucose residues, and α-1,2 mannosidase removes $mannose_9$ from the middle branch of the carbohydrate and is necessary for the elimination of CPY* [94, 95]. The trimming process by the two glucosidases and the α-1,2 mannosidase to $Man_8GlcNAc_2$ is thought to set the timer for folding and, if unsuccessful, for elimination of the protein. The recently discovered lectin-like protein Htm1/Mnl1p is believed to bind the trimmed $Man_8GlcNAc_2$ carbohydrate and deliver the glycoprotein to the elimination machinery [96, 97]. The similar requirement for Htm1p/Mnl1p during the degradation of other malfolded glycoproteins with different topologies, such as Pdr5*, a mutated form of the ATP-binding cassette transporter Pdr5 [98], and Stt3-7p a mutated subunit of the oligosaccharyl-transferase complex [97], but not of mutant Sec61-2p, a non-glycoprotein [99], underscores the necessity of this lectin during the recognition process of carbohydrate containing malfolded proteins [97]. A homologous lectin, EDEM, has been identified in mammalian cells, and it is believed to serve a similar function [100]. It is not known whether recognition of malfolded glycoproteins relies solely on recognition of the modified carbohydrate or if, in addition, recognition of a protein motif(s) is required. In addition, the recognition mechanism of malfolded glycoproteins leaves the discovery mechanism of malfolded non-glycoproteins completely open. In this case recognition of exposed, distinct protein stretches on the misfolded protein by chaperone-like molecules seems to be an obvious solution. Interestingly, the lectin calnexin, necessary for carbohydrate binding in proteins of mammalian cells, in yeast, is required for the elimination of a non-glycosylated mutant protein, carbohydrate deficient pro-α-factor [72]. The major Hsp70 chaperone of the ER, BiP (Kar2p in yeast), is known for its vital role in binding hydrophobic protein stretches to allow post-translational protein import into the ER and for protein folding and assembly in the ER [74, 101]. Therefore, a general function for Kar2p in protein quality control was quite plausible. It is, indeed, required for the degradation of soluble mutated pro-α-factor and CPY* in yeast [89, 102, 103]. However, Kar2p function is dispensable when CPY* is anchored to the ER membrane. Thus, converting the same malfolded protein, CPY*, from a soluble to a membrane-bound protein abolishes Kar2p (BiP) requirement for degradation [102]. Degradation of other membrane proteins was also found to be independent of Kar2p [98]. This indicates that Kar2p (BiP) and its DnaJ-like partners Jem1p and Scj1p [104] do not have a general function in the protein quality control process. Rather, Kar2p action is limited to soluble proteins. One function of Kar2p on malfolded proteins that have to be eliminated from the ER seems to reside in its ability to remain bound to these proteins after their unsuccessful folding trials and in this way keeping them in a soluble form. Scj1p and Jem1p may be triggering the release of Kar2p from substrates to make the subsequent elimination process possible. BiP and its partners could also deliver soluble substrates to some machinery combining recognition with elimination [104]. BiP has been implicated in sealing the Sec61 protein translocation channel from the ER lumen [74, 101, 105]. If, additionally, BiP (Kar2p) had a function as gatekeeper for the retrograde transport of malfolded proteins out of the ER (see below), then this role would be restricted to soluble proteins [102]. The requirement of Ca^{2+} ions in the ER-associated degradation of CPY* may in part be directly related to their action on Kar2p (BiP) [106]. Protein disulfide isomerase (PDI), an oxidoreductase involved in disulfide formation in secretory proteins [107], has also been implicated in elimination of malfolded proteins from the ER. However, its function has not yet been narrowed down to a single principle: while elimination of CPY* [108] requires the enzymatic activity of PDI, elimination of the mutated, non-glycosylated and cysteine-free pro-α-factor requires binding of PDI without its enzymatic function, suggesting a chaperone-like activity of PDI in this case [108]. In a third scenario, PDI has been found to function as a redox-driven chaperone in the unfolding of the A1 chain of cholera toxin in the ER lumen prior to its delivery into the cytoplasm of the cell. In the reduced state PDI binds and unfolds the substrate, while in its oxidized state it releases it [109]. Since a PDI mutated in the active site cysteines is not defective in elimination of the mutated pro-α-factor from the ER [108], the redox-driven chaperone mechanism cannot be a unifying principle of PDI action in ER-protein quality control. The requirement of Eps1p, an ER membrane-localized family member of PDI, in the elimination of a mutant form of the yeast membrane ATPase Pma1p [110], however, underlines the general necessity of this class of proteins as part of the protein quality control machinery in the ER.

The presence of unspecific proteinases in the ER, a compartment into which proteins are imported in an unfolded state, and where they have to undergo a time-consuming folding process, was difficult to understand. Rather, delivery of the malfolded proteins to the lysosome/vacuole, the degradative compartment of the cell, seemed more plausible. The discovery of the cytoplasmic ubiquitin-

proteasome system in the degradation of mutated ER-membrane-associated proteins hinted at a different possibility [84, 85, 111]. The realization that a mutated and malfolded soluble yeast vacuolar peptidase, carboxypeptidase yscY*, containing all the features for delivery into the vacuole, was retained in the ER and degraded via the cytoplasmic ubiquitin-proteasome system [71] or that a mutated soluble secretory protein, unglycosylated pro-α-factor, was degraded by the proteasome [112], provided the breakthrough necessary to establish the idea that retrograde transport of malfolded proteins into the cytoplasm had to occur for proteasomal degradation to be possible. This finding was unexpected and violated the dogma that proteins, once imported into the ER, were trapped in the secretory pathway and were unable to return to the cytoplasm [113]. Indication about the identity of the channel in the ER membrane through which malfolded proteins were returned back into the cytoplasm came from genetic studies: mutants defective in the Sec61 translocon showed a significantly retarded degradation kinetics of CPY* [103], of mutated pro-α-factor [114] and of a mutated polytopic membrane protein, Pdr5* [98]. Interestingly, a number of studies in mammalian cells uncovered a biochemical association of the glycosylated and dislocated protein substrates tested with the Sec61β subunit [115–117]. These findings were crucial for the establishment of the notion that the Sec61 translocon protein may also be part of the export channel. In the case of CPY*, we know that the imported protein leaves the translocon completely on the lumenal side of the ER [92]. Therefore, retargeting the protein to the Sec61 channel is necessary for retrotranslocation into the cytoplasm. Little is known about this process. Components that could be involved in retargeting soluble proteins to the retrotranslocation channel are Der1p, an ER membrane protein of unknown function [93], Kar2p (BiP), PDI [108, 109] and Hrd3p, an ER membrane protein connected to the ubiquitination machinery via the ubiquitin-protein-ligase Der3p/Hrd1p [118–120]. Hrd3p has also been shown to be involved in the degradation of membrane proteins [98, 121]. For glycosylated proteins another component of the retargeting machinery could be Htm1/Mnl1p, the $Man_8GlcNAc_2$-binding lectin [96, 97]. For none of these proteins has such a function been traced yet, however. It is most likely that the retrotranslocation channel differs in its composition from the import channel. A genetic interaction found between Hrd3p and Sec61p might be indicative of such a complex [120, P. Deak, unpublished]. This would easily explain the two-way protein traffic across the ER membrane: it is achieved by two subsets of translocons. Interestingly, CPY* is retrotranslocated in a glycosylated state [71, 122].

An exception to the rule of being transported back to the cytoplasm via a Sec61 translocon channel is the rapidly turned over, ER-membrane-located ubiquitin conjugating enzyme Ubc6 of yeast [123]. This seems plausible as Ubc6p is a tail-anchored protein which inserts post-translationally into the ER membrane, independent of Sec61p [124, 125]. Obviously, its removal from the ER is also independent of the retrotranslocon.

The elimination pathway

After retrotranslocation from the ER, CPY*, mutated pro-α-factor and the other malfolded and orphan proteins tested end up in the proteolytic machinery of the proteasome [70, 83, 88–90]. Before proteolysis can take place, a variety of different events have to occur which move and guide the malfolded protein to the proteasome. Polyubiquitination is the most frequently used step to target a protein for proteasomal degradation [35–37]. It is thus not surprising that CPY* and nearly all other malfolded proteins of the ER were found to be polyubiquitinated prior to degradation [83, 88]. One exception to this rule is mutated pro-α-factor [112]. It is not yet clear how this molecule is targeted to the proteasome. Genetic studies in yeast identified the ubiquitination machinery and localized it to the cytoplasmic side of the ER membrane. Ubc6p, an ER membrane tail anchored E2 [111], and Ubc7p, a soluble cytoplasmic E2 which is recruited to the ER membrane via an adapter protein Cue1p [126], are the major ubiquitin conjugating enzymes found up to now in the ubiquitination process [71, 98, 99]. Recently, Ubc1p was identified as an additional E2 involved in ubiquitin conjugation onto CPY* [127]. The ubiquitin-protein ligase (E3) cooperating with and binding the cytoplasmic E2 enzymes Ubc1p and Ubc7p was found to be the RING-H2 finger containing polytopic ER-membrane protein Der3/Hrd1p [119, 121, 128–130]. Activity of the Der3/Hrd1p ubiquitin ligase depends on an interacting ER-membrane protein, Hrd3p [121]. Hrd3p is believed to play a role in signaling events between the ER lumen and the cytoplasmic ubiquitination machinery [118–120]. It is necessary for degradation of malfolded soluble and polytopic membrane proteins alike. Doa10/Ssm4p has been identified as an additional ER-membrane-bound ubiquitin-protein ligase in yeast. Besides its involvement in the ubiquitination of the Deg1 degradation domain of the transcriptional repressor MATα2, it has been shown to be involved in the degradation of the tail-anchored ER-membrane protein Ubc6p [131]. Interestingly, the ubiquitin-protein ligase Der3/Hrd1p and its interaction partner Hrd3p do not participate in the elimination of Ubc6p. Also, elimination of malfolded or orphan membrane proteins such as the vacuolar membrane ATPase subunit Vph1p [132, 133] do not depend on the function of the E3-complex Der3/Hrd1p-Hrd3p. It is still an open question whether Doa10/Ssm4p can fill this function. The

mammalian orthologues of the yeast ER ubiquitination machinery have been found [134–138].

ER degradation is a protein sorting process which requires directionality from the ER lumen or membrane to the proteasome. Once the protein has reached the ER membrane and has been threaded into the retrotranslocon, a mechanism has to be applied which directs the protein to the proteasome without liberating it into the cytosol in a free form. It has been shown that lack of polyubiquitination of secretory waste proteins not only prevents degradation by the proteasome [71, 126] but also leads to failure of being transported into the cytoplasm: they remain in the ER [71, 126, 128, 139, 140]. This points to the necessity of polyubiquitination for the retrotranslocation step. Modification of the retrotranslocated protein may occur when the amino terminus or the first lysine residue becomes accessible to the ubiquitination machinery [14, 141]. Polyubiquitination could serve as a mechanism which prevents the polypeptide slipping back into the ER. Consistent with such an idea is the fact that a hypo-ubiquitinated CPY*, due to lack of the ubiquitin conjugating enzymes Ubc1p and Ubc7p, fails to be completely transported to the cytosol [142]. Recent experiments showed that a member of the AAA family of ATPases, Cdc48, (p97) together with two partner proteins, Ufd1p and Npl4p, is also crucial for extraction of CPY* and a variety of other substrate proteins from the ER membrane [142–145]. In the absence of the trimeric Cdc48 complex polyubiquitinated substrates accumulate at the ER membrane, indicating its action after polyubiquitination of the substrate. Cdc48/p97 forms a double-barrel structure of two homo-hexameric rings which undergo strong conformational changes upon ATP hydrolysis [146, 147]. It has been reported that Cdc48/p97 binds ubiquitinated proteins and ubiquitin-free polypeptide stretches [148–150]. Polyubiquitin chain binding by the Ufd1 unit of the trimeric complex is considered also to be crucial for retrotranslocation [148]. The Cdc48/p97 complex has furthermore been found to associate with ER membranes [151]. ATP hydrolysis by Cdc48p has been shown to be critical for substrate dislocation from the ER [143, 148]. It has also been shown that the Cdc48-Ufd1-Npl4 complex is able to separate a tightly associated protein at the ER membrane, a processed dimer of the Spt23 transcription factor, by this releasing active transcription factor molecules into the cytosol [152]. Taking these data together, the following picture emerges: After polyubiquitination and (partial) dislocation of the substrate molecule from the retrotranslocation channel, the ubiquitinated substrate protein is bound by the ER-associated Cdc48-Ufd1p-Npl4 machinery. Strokes of ATP hydrolysis then induce conformational changes in the Cdc48 complex, which pull the polyubiquitinated substrate away from the ER membrane. Successive rounds of substrate binding and conformational changes of Cdc48 finally completely remove the substrate from the ER. In mutants defective in either the catalytic activity of the proteasome or one of the ATPases of the 19S cap, CPY* accumulates to a large extent in the cytoplasm [142]. This indicates that the proteasome acts after removal of the ubiquitinated substrate from the ER membrane. In a few cases the proteasome itself has been implicated in dislocation of membrane-bound proteins [123, 153]. As in these instances an involvement of Cdc48p in ER-membrane removal of the respective substrate has not been tested, it is not clear whether there exist different modes of ER-membrane extraction of proteins. Recently, a genome-wide screen identified the two cytosolic ubiquitin domain proteins Dsk2p and Rad23p in degradation of soluble and membrane-bound substrate proteins of the ER [B. Medicherla, Z. Kostova. A. Schaefer and D. H. Wolf, unpublished]. In mutants defective in both proteins, the substrate measured, CPY*, accumulates polyubiquitinated in the cytoplasm. As it has been shown that the UBA domain of both proteins can interact with polyubiquitinated proteins while the respective UBL domains can bind to the 19S cap of the protesome [154, 155], it is most likely that both proteins are shuttling factors handing the polyubiquitinated misfolded ER proteins over to the proteasome. This way the malfolded proteins of the ER lumen and membrane which contain large stretches of exposed hydrophobic sequences are prevented from forming insoluble aggregates in the cytosol [B. Medicherla, Z. Kostova A. Schaefer and D. H. Wolf, unpublished] (fig. 3).

A multitude of genetic and biochemical studies show that the 26S proteasome is the end point of the journey of the ERAD substrates [71, 83–85, 98, 99, 112, 115, 117]. Glycosylated CPY* is retrotranslocated into the cytoplasm in its glycosylated state [71, 92]. An N-glycanase (Png1p) has been identified in yeast which also exists in a highly conserved manner in mammalian cells. *PNG1* is not an essential gene, but its product is required for efficient degradation of CPY* [122]. Rad23 was found to escort Png1p to the proteasome where it is thought to act in the complex to efficiently de-N-glycosylate the arriving proteins prior to degradation [156].

So far, besides the Cdc48-Ufd1-Npl4 complex no other cytoplasmic chaperone component has been found to be necessary for degradation of malfolded soluble ER-lumenal proteins. In contrast, malfolded ER-membrane proteins containing folded cytoplasmic protein domains require additional chaperone activity for efficient degradation to occur. Elimination of the cystic fibrosis transmembrane conductance regulator (CFTR) expressed in yeast was dependent on the presence of Hsp70 chaperone activity [157]. Construction of modular substrate chimeras containing (i) the malfolded CPY* domain in the ER lumen with a membrane anchor (CT*) or (ii) CT* with the tightly folded cytosolic GFP domain added (CTG*) gave additional information about cytosolic

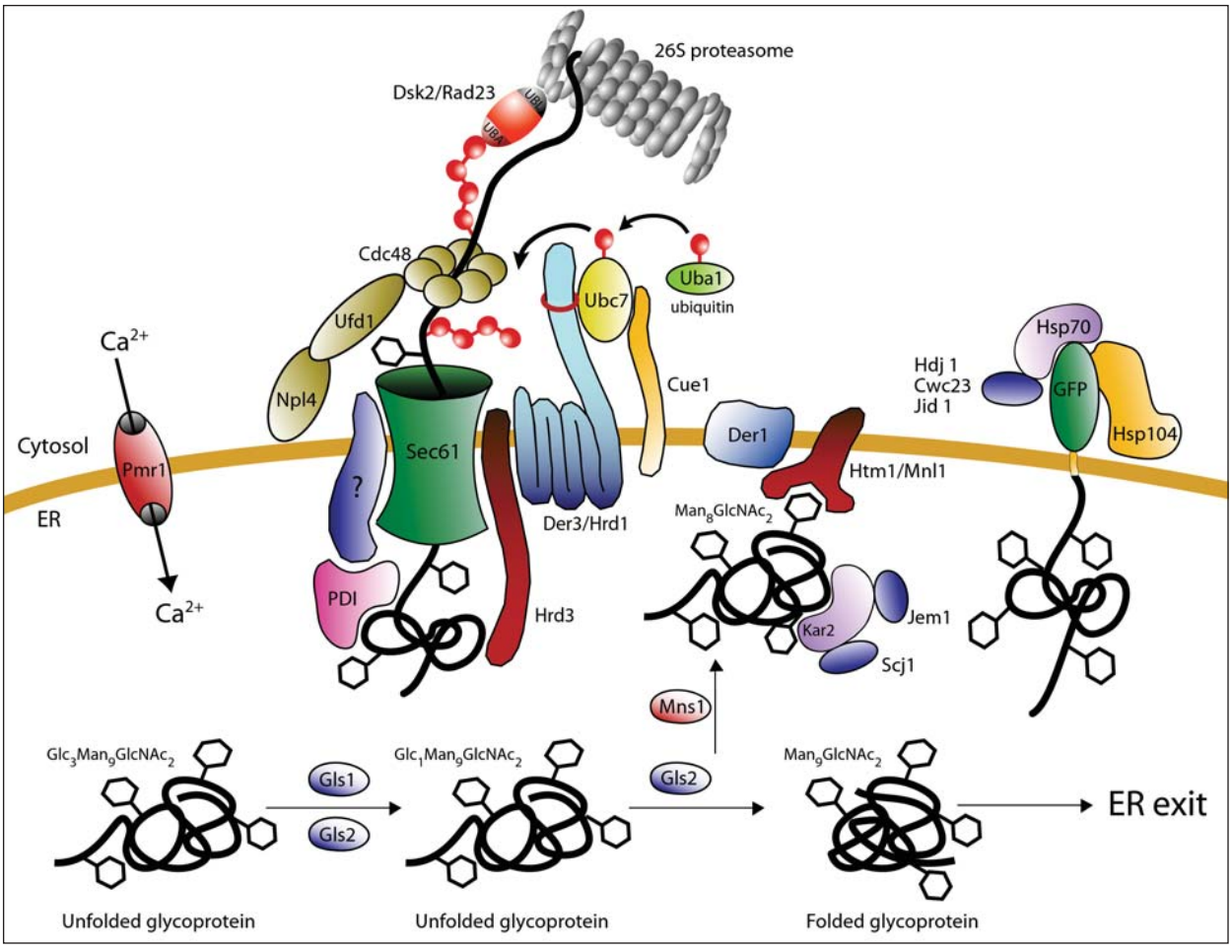

Figure 3. Protein quality control and elimination of misfolded proteins of the endoplasmic reticulum. See text (modified after [88]).

chaperone requirements for efficient degradation to occur. While soluble CPY* and membrane-anchored CPY* (CT*) did not require additional cytoplasmic chaperones besides the Cdc48 complex, degradation of CPY* anchored in the ER membrane and carrying the tightly folded GFP domain (CTG*) was dependent on Hsp70 and Hsp40 chaperone activity. In addition the Hsp100 chaperone family member Hsp104 exhibited some effect in the degradation process [102]. One may therefore assume that tightly folded cytoplasmic domains of malfolded ER membrane proteins require cytoplasmic chaperone activity for degradation (fig. 3).

An increase of unfolded proteins in the ER induces the unfolded protein response (UPR) [78, 158–160]. The UPR is directly linked to ER degradation. The accumulation of unfolded proteins due to absence of a component of the ER degradation machinery induces the unfolded protein response [93]. Likewise, overexpression of malfolded proteins leads to induction of UPR. Under these conditions, the level of components of the ER degradation machinery is significantly enhanced [80, 127]. Under normal growth conditions the cell is able to deal with the amount of unfolded proteins and does not require the induction of UPR. However, when increased levels of malfolded proteins accumulate, the UPR becomes crucial. Loss of function of components of both systems is lethal [80, 127, 161].

Interestingly, the protein quality control machinery of the ER is also involved in regulation of enzymes of the ER. The best-studied example is the key enzyme of sterol biosynthesis, 3-hydroxy-3-methyglutaryl CoA (HMG-CoA) reductase. In yeast, two HMG-CoA reductases designated 1 and 2 exist which are located in the ER membrane. While HMG-CoA reductase1 is a relatively stable protein, HMG-CoA reductase 2 is rapidly degraded. Farnesyl pyrophosphate is the primary signal for HMG-CoA reductase 2 degradation. Elimination of the enzyme requires the major components of the ER degradation machinery used also for elimination of malfolded proteins such as CPY*, Der3/Hrd1p, Hrd3p, Ubc7p, Cdc48-Ufd1-Npl4 and the 26S proteasome [118, 121, 130, 162, 163]. Signals from the mevalonate pathway lead to conformational changes which channel the protein into the ER quality control pathway [164, 165].

It has recently been shown that efficient ER degradation of soluble malfolded proteins requires efficient ER-Golgi traffic [166–168]. As an explanation for this phenomenon, the necessity of transport of the malfolded proteins from the ER to the Golgi for modification or ER-Golgi cycling of an as yet unknown factor as a prerequisite for efficient degradation has been given [166, 167]. This explanation was extended when in addition to the necessity for ER-Golgi transport, overexpression of CPY* and other malfolded proteins was found to require the Golgi-associated E3 Rsp5 for proteasomal degradation instead of the ubiquitin-protein-ligase complex Der3/Hrd1p-Hrd3p [169]. Other authors, however, found degradation of overexpressed CPY* still dependent on Der3/Hrd1p [127]. The experiments of Taxis et al. (2002) may lead to a different interpretation of the necessity of efficient ER-Golgi traffic for ERAD: These studies propose that efficient ER degradation simply requires an intact ER with all its components, which is based on unimpaired ER-Golgi traffic. Further experimentation will be required to solve this somewhat controversial issue.

The final fate of the malfolded ER proteins is their degradation by the proteasome. The proteasome cleaves its cellular substrates into fragments of between 3 and 25 amino acids, the maximum fraction being around 7–8 amino acids in size. This feature of the proteasome is essential for the generation of MHC class I antigens in mammalian cells [170]. However, non-processed peptides can be highly toxic to cells. Therefore, rapid degradation of the generated peptides has to occur. In yeast, an oligopeptidase, proteinase yscD, the equivalent of mammalian thimet oligopeptidase, has been discovered as an enzyme to take over this job [171].

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