ER-associated protein degradation (ERAD): An unexpected function of Yos9 and the discovery of Mnl2, a new component of the pathway

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

Stuttgart, den 20.07.2011

Elena Martínez Benítez

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Abbreviations

AAA	ATPase associated with a variety of cellular activities
Ac	Acetate
Amp	Ampicillin
AMP	Adenosine monophosphate
APS	Ammonium persulphate
ATP	Adenosine triphosphate
ATPase	Adenosintriphospatase
BSA	Bovine serum albumine
cAMP	Cyclic adenosine monophosphate
CHX	Cycloheximide
CM	Complete minimal medium
CPY	Carboxypeptidase yscY (Gen *PRC1*)
CPY*	Mutated CPY (allele *prc1-1*)
CPY*0000	Unglycosylated CPY*
ddH2O	Double deionised water
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
DTT	Dithiotheitol
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
ERAD	ER associated degradation
Fig	Figure
g	Gram
h	Hour

HA	Haemaglutinin
Hect	Homologous to E6-AP c-terminous
HRP	Horse radish peroxidase
IB	Immunoblot
IgG	Immunoglobulin G
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
Kb	Kilobase pair
l	Liter
LB	Luria Brooth
M	Molar
min	Minute
NEM	N-ethiylmaleimide
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline-Tween20
PCR	Polymerase chain reaction
PEG	Polyethileneglycol
PGK	3-Phosphoglycerate kinase
PMSF	Phenylmethylsulphonylfluoride
RING	Really interesting new gene
rpm	Revolutions per minute
RT	*Room temperature*
S.*cerevisiae*	Saccharomyces cerevisiae
SDS	Sodium dodecylsulphate
T4 ligase	Bacteriophage T4 ligase
TAE	Tris acetate EDTA
TCA	Trichloroacetic acid
TE	Tris EDTA

TEMED	Tetramethylethydiamine
Tris	Tris (hydroxymethyl)aminomethane
TritonX-100	Akylphenylpolyethylenglycol
Tween 20	Polyoxyethylensorbitolmonolaurate
Ub	Ubiquitin
UBA	Ubiquitin associated domain
UBL	Ubiquitin like domain
UV	Ultraviolet
V	Volts
v/v	Volume/volume
w/v	Weight/volume
WT	Wild type
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Zusammenfassung

In eukaryotischen Zellen werden Membranproteine und lösliche Proteine des sekretorischen Weges an dem rauhen Endoplasmatischen Reticulum (ER) synthetisiert. Proteine, die sich im Folgenden nicht korrekt falten, werden in einem Prozess abgebaut, der als ER assoziierte Degradation (ERAD) bezeichnet wird. Störungen des ERAD-Prozesses - dazu zählen leichte Beeinträchtigungen des Abbaus, ein vollständiger Abbaustopp von Substraten sowie auch ein vorzeitiger Abbau - führen beim Menschen zu einer Vielzahl von Erkrankungen.

Im Jahre 1989 konnte ein Gen identifiziert werden, dessen Mutation für die Erkrankung Mukoviszidose verantwortlich ist. Dieses Gen codiert für das Protein CFTR (engl. für ‚cystic fibrosis transmembrane conductance regulator'), welches einen Chloridkanal in bestimmten Zelltypen ausbildet. Die Mutation ΔF508, die bei 80% der Erkrankungen auftritt, führt zu einer sehr kurzen Lebenserwartung und zu schwerwiegenden körperlichen Symptomen bei einem Erkrankten. Die Konsequenz der Mutation ist ein vollständiger Abbau des Proteins CFTRΔF508. Bereits von dem Wildtyp-CFTR Protein erreichen lediglich 25% der exprimierten Proteine den eigentlichen Wirkungsort, die Plasmamembran.

Die Aufklärung der Vorgänge im ERAD, im Besonderen des Unterschieds zwischen dem Abbau von Wildtyp-CFTR und mutiertem CFTR, ist für die Entwicklung einer effektiven Behandlungsmethode der Mukoviszidose von großer Bedeutung. Es wurden bereits verschiedene Komponenten identifiziert, die im ERAD von CFTR eine Rolle spielen. Der erste Teil dieser Arbeit beinhaltet eine systematische Analyse des Abbaus von humanem CFTR in Hefe (humanes Wildtyp-CFTR verhält sich in Hefe wie CFTRΔF508 in humanen Zellen). Alle hier untersuchten Komponenten zeigten keinen bzw. lediglich einen geringen Einfluss auf die Degradation von CFTR.

Prizipiell erkennt die Qualitätskontrolle des ER fehlgefaltete Proteine anhand von zwei Faktoren, der Exposition hydrophober Bereiche und einer modifizierten Glycanstruktur. Die Mehrzahl der Proteine, die das ER durchlaufen, wird mittels N-Glykosylierung modifiziert. Während des Faltungsprozesses von Glycoproteinen trimmen verschiedene Enzyme die Glycane der Proteine. Abhängig von der Faltungsdauer können dabei Glycanstrukturen entstehen, die von dem Lectin Yos9 erkannt werden und als Degradationssignal dienen. Über die ER assoziierte Degradation unglycosylierter Proteine ist bisher nur wenig bekannt. Der zweite Teil dieser Arbeit befasst sich mit

Befunden, die den Unterschied zwischen glykosylierten und unglykosylierten ERAD-Substraten aufzeigen.

Um den Degradationsprozess unglykosylierter Proteine zu spezifizieren, wurden ERAD defiziente Hefestämme auf ihre Funktionalität getestet, diese Substratklasse abzubauen. Dabei stellte sich heraus, dass auch die unglykosyllierte CPY*0000 dem Abbauweg folgt, dem auch die glykosylierte CPY* unterliegt (ERAD-L). Überraschend war allerdigns, dass das Lectin Yos9, das für die Erkennung von fehlgefalteten, glycosylierter Proteinen notwendig ist, auch Einfluss auf die Degradation von unglykosylierter CPY* (CPY*0000) hat. Es konnte gezeigt werden, dass Yos9 den Abbau von glykosylierten Proteinen begünstigt, während der Abbau von CPY*0000 durch Yos9 verzögert wird.

Es werden kontinuierlich neue Komponenten des ERAD gesucht. In dieser Arbeit konnte eine mögliche Mannosidase identifiziert werden, die hier als Mnl2 (engl. für ‚mannosidase like protein 2') benannt wurde. Mnl2 führt zu einem beschleunigten CPY* Abbau. Der Einfluss der *MNL2* Deletion auf die Degradation von CPY* wird besonders deutlich, wenn zuvor bereits *MNL1* deletiert wurde. Die Substratdegradation ist dann beeinträchtigt, weil die Glycanstruktur des ERAD-Substrats nicht länger als Abbausignal dient. In diesem letzen Teil der Arbeit wird

Mnl2 als neue Komponente der ER Qualitätskontrolle vorgestellt, die die Glykanmodifizierung beeinflusst.

Abstract

In eukaryotes, membrane and soluble secretory proteins are synthesized at the rough endoplasmic reticulum (ER). 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 cause a range of severe diseases in humans.

In 1989 the gene responsible for the human disease cystic fibrosis (CF), cystic fibrosis transmembrane conductance regulator (CFTR), encoding a chloride channel was found. The mutation ΔF508 present in 80% of the patients provokes the most severe symptoms and shortest life expectancy. The disease is a consequence of the accelerated degradation of the protein CFTRΔF508, which never reaches its site of action. Moreover, wild type CFTR has a 25% success in reaching its final destination.

Is of great value to understand the ERAD of CFTR and CFTRΔF508 in order to understand the disease. Many components involved in ERAD of these substrates had been discovered. The first part of this work involves a systematic study of human CFTR degradation in yeast (the turnover of CFTR in yeast cells behaves like CFTRΔF508 in

human cells). All components examined were found not to be required for CFTR ERAD or had a very mild effect.

The ER protein quality control recognizes misfolded proteins in two ways: via exposure of hydrophobic patches on the surface of a protein and modification of its glycan structure. The majority of the proteins that enter the ER are N-glycosylated. During folding of a protein, several enzymes trim these glycan trees generating a degradation signal, which is recognized by the lectin Yos9. There is little known about proteins that enter the ER but are not glycosylated. The second part of the work refers to findings that shed light onto the differences between glycosylated and non-glycosylated substrates in ERAD.

To define the ERAD pathway for non-glycosylated proteins, ERAD deficient mutants were checked in their capacity to deliver a non-glycosylated protein for elimination. It is shown that unglycosylated CPY* (CPY*0000) is degraded by the same pathway as is glycosylated CPY* (ERAD-L). However, the Yos9 protein, known to be the recognition component of glycosylated misfolded proteins in ERAD, is shown in this work to have a tuning role in the ERAD of unglycosylated CPY*0000. Yos9 promotes degradation of glycosylated substrates while it hinders degradation of unglycosylated CPY*0000.

Additional ERAD components are still to be discovered. In this work a putative mannosidase was found. This protein was named mannosidase like protein 2 (Mnl2). Mnl2 accelerates CPY* degradation. The effect of the deletion of *MNL2* is most notable when its homologue *MNL1/HTM1* is absent. Substrate degradation in the deletion strain is affected because the glycan structure on the ERAD substrate is no longer a degradation signal. This section of the work introduces a novel ER quality control component involved in glycan trimming.

1.Introduction

1.1.Saccharomyces cerevisiae as cell model

The budding yeast *Saccharomyces cerevisiae* (from now on yeast) is a unicellular, eukaryotic organism that can exist in either haploid or diploid form. Mating types of haploids are a and α (Strathern, et al. 1981; Broach 1991; Guthrie and Fink 1991). It is non pathogenic and is easy to handle. The doubling time of haploids is approximately 90 minutes and one cell can have 20 to 30 scars resulting from the budding process. Haploid yeast has its DNA organized in 16 chromosomes with over 6000 open reading frames (ORF) that encode about 6000 proteins. For over 30 years DNA transformation in yeast has made possible gene cloning and genetic engineering techniques(Hinnen, et al. 1978). The complete genome of yeast is known since 1996 (Dujon 1996; Goffeau, et al. 1996). The phenotypes observed after disruption of yeast genes have shed light onto the function of many proteins *in vivo*(Rose and Broach 1991). These particular circumstances make yeast a highly regarded eukaryotic experimental organism. (Broach 1991; Guthrie and Fink 1991)(*Saccharomyces* Genome Database)

1.2.The secretory pathway

It is vital for cells to synthesize faultless proteins. Proteins are biomolecules with great responsibility in the life of an organism due to their structural, catalytic, transport, regulatory, signaling and motor functions in cells. Most proteins are synthesized on free ribosomes in the cytoplasm (Becker, et al. 2009) Depending on their initial amino acid sequence they will be sorted into several pathways (Fig. 1.1).

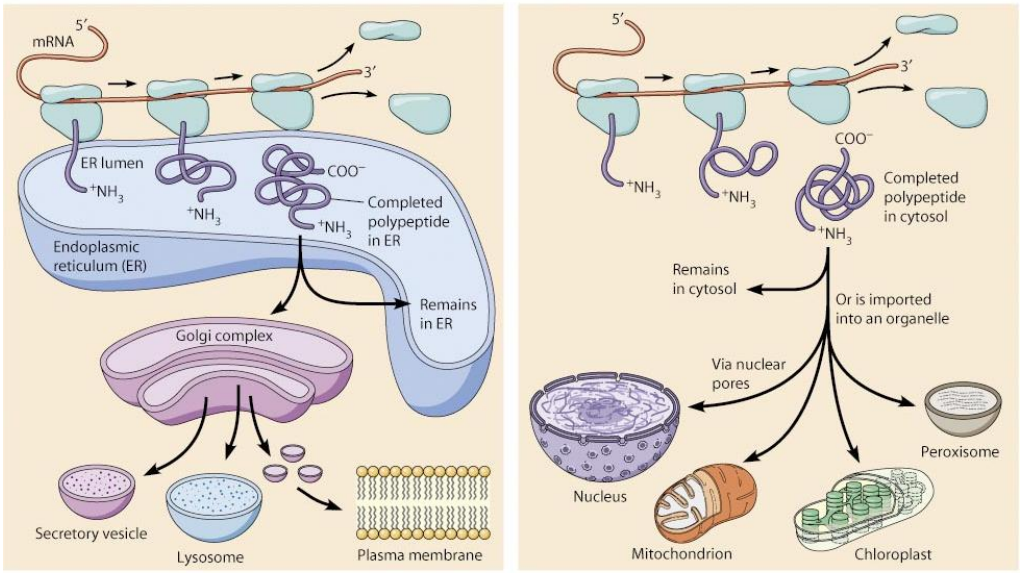

Figure 1.1: Protein sorting. (Left panel) The secretory pathway: proteins destined for the endomembrane system or for export from the cell. (Right panel) The cytosolic pathway: proteins destined for the cytosol or import into the nucleus, mitochondria, chloroplasts or peroxisomes (Becker, et al. 2009).

The proteins with a signal peptide for the endoplasmic reticulum (ER), approximately one third of the proteome, follow the secretory pathway (Fig. 1.1).

The secretory pathway covers the endomembrane compartments of the ER, Golgi apparatus, secretory vesicles and the plasma membrane. In yeast, the peptide chain is mainly post-translationally transferred into the lumen of the ER through the Sec61 translocon, but a certain percentage of proteins is also transported co-translationally (Deshaies, et al. 1991; Luirink and Sinning 2004; Rapoport 2007). Before proteins exit the ER they can undergo four principal modifications: glycosylation, disulfide bond formation, multisubunit assembly and specific proteolytic cleavages (Lodish, et al. 2007). N-linked glycan trees are transferred onto an asparagine as a preformed precursor containing three glucoses (Glc), nine mannoses (Man) and two N-acetylglucosamine (GlcNAc) molecules, written as $Glc_3Man_9(GlcNAc)_2$ (Yoshida 2003; Helenius and Aebi 2004; Varki, et al. 2009).

Initially, glycan trees are linked to dolichol molecules which are embedded in the ER membrane. Proteins with an asparagine residue within the consensus sequence Asn-X-Ser/Thr (where X is any amino acid except proline)

are candidates to be glycosylated by the oligosaccharyl transferase (OST) complex (Nilsson and von Heijne 1993).

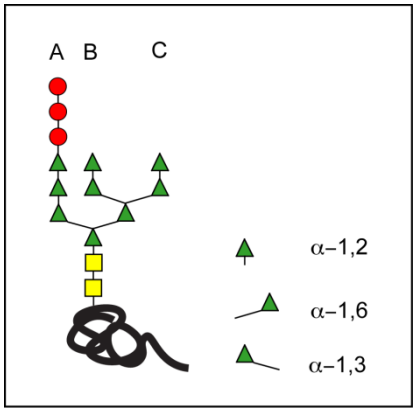

Figure 1.2: The glycan tree. Glycan trees consist of three glucoses (in red), nine mannoses (in green) and two N-acetylglucosamines (in yellow) and can be bound to asparagine residues of proteins in the consensus sequence N-X-S/T were X is any amino acid except proline.

Glycans in the ER reveal the folding status of the proteins (Helenius and Aebi 2004; Aebi, et al. 2010). Disulfide bonds are only formed in the ER owing to its reductive environment (Freedman, et al. 1994; Primm, et al. 1996). The reaction between a pair of cysteine residues is catalyzed by members of the protein disulfide isomerase (PDI) family. During folding proteins depend on chaperone activity. In general Kar2, an Hsp70 family member, binds after translocation and keeps proteins soluble (Plemper, et al. 1997; Matlack, et al. 1999; Nishikawa, et al. 2005). Lectin-like chaperones are responsible for controlling folding intermediates, folded proteins and terminally misfolded proteins (Quan, et al. 2008; Clerc, et al. 2009;

Aebi, et al. 2010). Their decision will allow the proteins to either be delivered to their site of action through the secretory pathway or to be eliminated.

1.3.Degradation of proteins in eukaryotes

Cells have the possibility to degrade proteins in two different fashions depending on their needs: via the vacuole or via the proteasome.

Vacuoles (lysosomes) are organelles that contain digestive enzymes (hydrolases) that work at pH 4.5 to break down waste materials and cellular debris. For protein degradation by the vacuole there are two pathways operating: endocytosis and autophagy. Proteins of the plasma membrane, after fulfilling their function are incorporated into vesicles and degraded via endocytosis in the vacuole (lysosome) (Mukhopadhyay and Riezman 2007). Autophagy is a regulated process where the bulk of long-lived cytosolic proteins are taken up into double layered vesicles which then fuse with the vacuole (Yorimitsu and Klionsky 2005a). A more specific process of this kind is ribophagy and mitophagy(Kiel 2010).

Proteasomes are part of a major mechanism by which cells regulate the concentration of particular proteins

specifically and degrade misfolded proteins (Heinemeyer, et al. 1991; Hiller, et al. 1996; Meusser, et al. 2005; Salomons, et al. 2010). The degradation process yields peptides of about three to thirty amino acids long, which in mammalian cells are used for immunodetection or generally, be further degraded into amino acids and used for synthesizing new proteins (Lodish, et al. 2007). Proteins that follow the secretory pathway but cannot fold to their native structure are first retained in the ER,then retrotranslocated to the cytosol and degraded by the proteasome in a process called ER associated degradation ERAD (Sommer and Wolf 1997; Plemper and Wolf 1999; Kostova and Wolf 2003; Meusser, et al. 2005).

1.4.Quality control in the endoplasmic reticulum

Terminally misfolded proteins are recognized by the quality control system of the ER. This is a mayor protective mechanism in cells (Spiro 2004; Aebi, et al. 2010; Pearse and Hebert 2010). Since most of the proteins of the secretory pathway are glycosylated very few studies deal with non glycosylated substrates (Kanehara, et al. 2010). Folding and degradation of glycoproteins in the ER are regulated by molecular chaperones and enzymes recruited

by specific oligosaccharide structures (Aebi, et al. 2010). Glycosylated ERAD substrates such as CPY* and PrA* have their glycan structure and adjacent unfolded peptide segments as degradation signals (Quan, et al. 2008; Clerc, et al. 2009; Xie and Ng 2010)(Quan 2008, Clerc 2009, xie 2009). Nevertheless, observations on non glycosylated variants of PrA* show that the substrate is retained in the ER by the QC system and degraded by ERAD (Kanehara, et al. 2010).

In yeast, N-linked glycan trimming starts right after glycosylation in what is known as the Timer model in quality control (Helenius and Aebi 2004; Hosokawa, et al. 2010). First, on the A branch of the glycan glucosidase 1 (Gls1) removes the terminal α1,2 linked glucose of the triglucosyl sequence (Fig. 1.2). Glucosidase 2 (Gls2) cleaves off the additional two glucoses which has been attributed to two distinct active sites on the enzyme (Spiro 2000). Thereafter mannosidase 1 (Mns1) acts specifically to remove one mannose residue from the B branch generating the Man8 structure (Tremblay and Herscovics 1999)(Fig. 1.3). Mns1 is a slow enzyme; it gives time to the protein to fold (Herscovics 1999). If the folding process is not fast enough in the time given or the protein is not capable to achieve its native structure, a

mannose on the C branch will be cleaved off by mannosidase like protein 1 (Mnl1/Htm1) generating a Man7 structure. Trimming of the outermost α1,2-linked mannose on the C branch of the glycan generates a degradation signal for ERAD, which is recognized by the lectin Yos9 (Oda, et al. 2003; Quan, et al. 2008; Hosokawa, et al. 2009).

Yos9 was found in a genetic screen to identify components of ERAD (Buschhorn, et al. 2004). Yos9 contains a mannose 6-phosphate receptor homology (MRH) domain and a HDEL peptide at its C terminus that retains it in the ER lumen. Disruption of the MRH domain abrogates degradation of the glycosylated substrate CPY*.

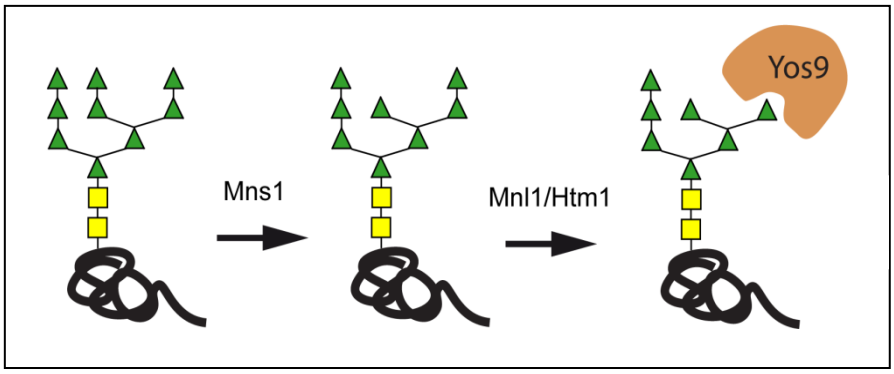

Figure 1.3: Mannosidase trimming in the ER. Mns1 and Mnl1/Htm1 are α1,2 mannosidases that act on different mannoses on the glycan tree generating the glycan structure recognized by Yos9 that triggers ERAD of the substrate.

This suggests that Yos9 acts by detecting glycan structures through its MRH domain (Bhamidipati, et al. 2005; Kanehara, et al. 2007; Christianson, et al. 2008). In

a recent study, Yos9 has also been shown to recognize Man6 and Man5 structures. The enhanced affinity of Yos9 for the Man5 structures shown could provide a mechanism for preferentially degrading a subset of potential substrates (Quan, et al. 2008).

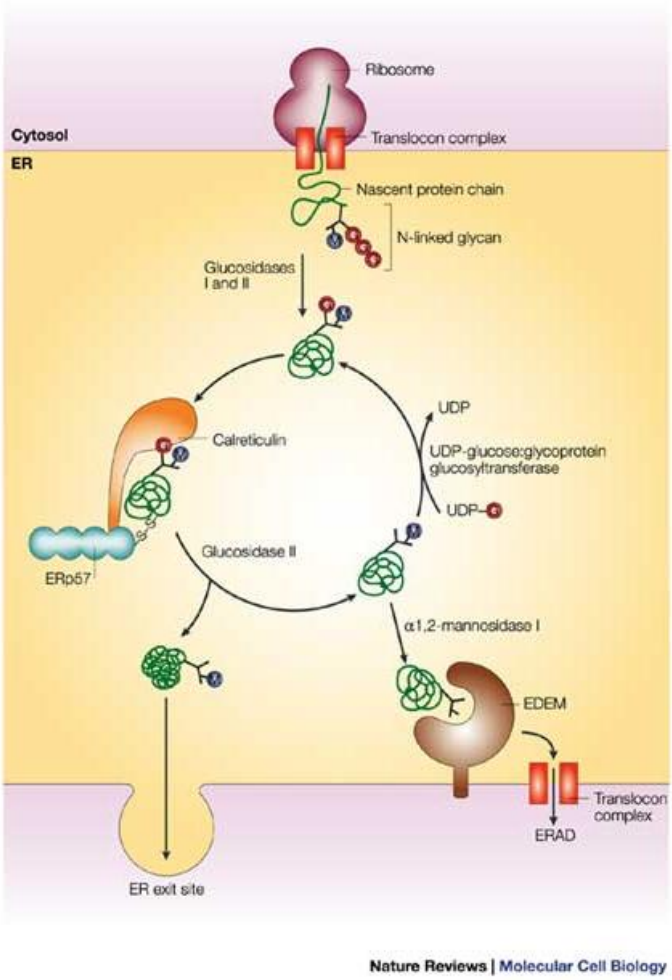

Figure 1.4: Chaperone function of calreticulin. CTR assists in the folding of glycoproteins in the ER. CRT interacts with the glycosylated protein until it is folded correctly. Upon release, the correctly folded glycoproteins can exit the ER. Misfolded proteins can be re-glycosylated, giving them another chance to interact with CRT and fold into the correct three-dimensional structure and exit the ER. Permanently misfolded proteins are processed and degraded by the proteasome.(Ellgaard and Helenius 2003)

Higher eukaryotes have an extra step in QC that allows protein intermediates several rounds of folding. After removal of the first two glucoses, proteins are bound by the homolog chaperones calnexin and calreticulin (Hammond, et al. 1994). Calnexin is membrane-anchored, whereas calreticulin is luminal.

After removal of the last glucose from the N-glycan, UDP-glucose:glycoprotein glucosyltransferase (UGGT) acts as folding sensor (Caramelo, et al. 2003). UGGT probes the folded state of the substrate by interacting with both the glycan structure and hydrophobic patches of the protein intermediate.

In case a protein is not properly folded UGGT reglucosylates the N-glycan, thereby allowing the folding intermediate to reassociate with calnexin or reticulin for an additional folding cycle. The cycles of deglucosylation and reglucosylation continue until the protein is correctly folded or targeted for degradation by the lectins EDEM1/3 (orthologs of Mnl1/Htm1) which generate a Man7 structure (Molinari, et al. 2003).

In this case the glycan structure is recognized by OS-9 (mammalian ortholog of Yos9) (Kanehara, et al. 2007; Satoh, et al. 2010). Recently, the structure of this interaction has been discovered. It includes a WW motif in the MRH

domain and the α1,6-linked trisaccharide Man(B)-Man(4')-Man(3) of the glycan structure.

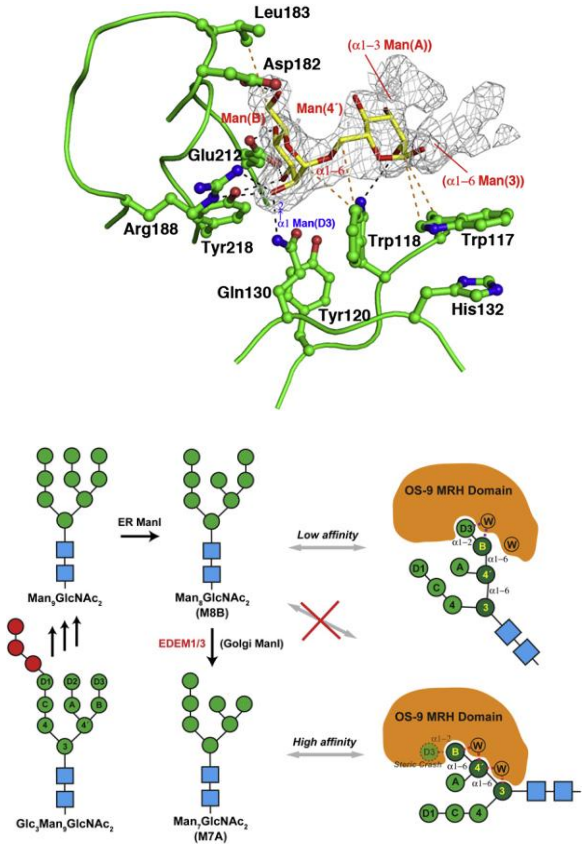

Figure 1.5: OS-9 interaction with the glycan signal and mode of action. (A) OS-9MRH/Manα1,6Manα1,6Man interaction at the WW motif as model based on the solution data from NMR analyses. Oligosaccharide residues are shown as yellow stick models, residues of OS-9MRH involved in binding the ligand are shown as ball-and-stick models. (B) Model for OS-9 Recognition of oligosaccharides on ERAD substrates.(Satoh, et al. 2010)

1.4.1.Endoplasmic reticulum associated degradation

ERAD takes place in four steps: recruitment and recognition of the substrate, retrotranslocation to the cytosol, ubiquitylation and guidance to the proteasome (Hiller, et al. 1996; Kostova and Wolf 2003; Meusser, et al. 2005).

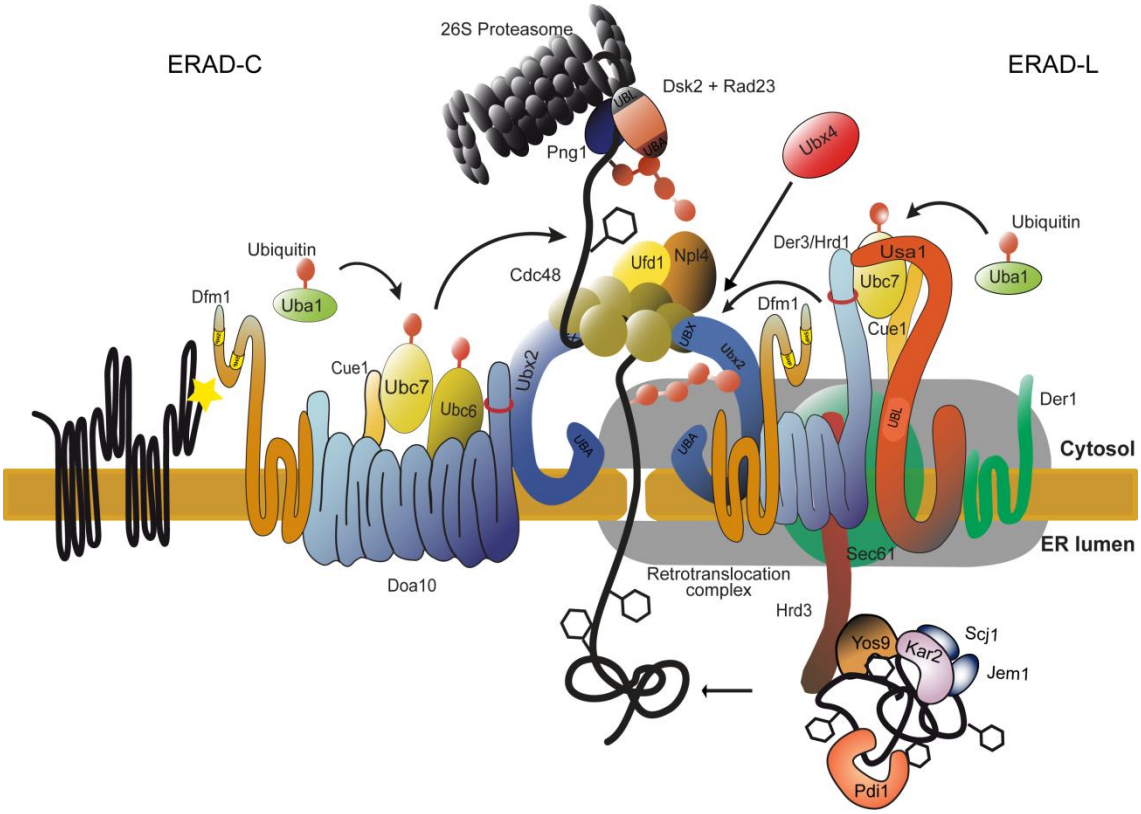

Figure 1.5: The ERAD pathway. Recruitment and recognition of the substrate, retrotranslocation to the cytosol, ubiquitylation and guidance to the proteasome for degradation of misfolded proteins. (Stolz and Wolf 2010)

The ERAD pathway has several branches of recognition. Substrates with lesions in a luminal domain follow the

ERAD-L (luminal) recognition pathway and require a set of ER luminal proteins as well as the E3 ubiquitin ligase Der3/Hrd1. Substrates with lesions in a cytosolic domain follow the ERAD-C recognition pathway and require the E3 ubiquitin ligase Doa10 and (Kostova and Wolf 2003; Vembar and Brodsky 2008). Both pathways converge at the motor protein complex Cdc48 on the cytosolic side of the ER as depicted in figure 1.5.

Recognition and recruitment of substrates to the ERAD machinery has been largely studied especially for luminal glycosylated substrates (Finger, et al. 1993; Hampton, et al. 1996; Hiller, et al. 1996; Kostova and Wolf 2005). After trimming by the mannosidases, glycosylated substrates bind to the ER embedded Hrd3 component of the HRD/DER complex which is composed of Der3/Hrd1 ligase, Hrd3, Usa1, Der1 and Yos9. Then, the lectin Yos9 scans the glycan modifications on the substrate and delivers it to the retrotranslocation machinery (Denic, et al. 2006; Gauss, et al. 2006).

Retrotranslocation is not well understood but seems to also involve the Sec61 complex (Schafer and Wolf 2009). Substrates are retrotranslocated in an unfolded state to the cytosolic face of the ER membrane where the AAA ATPase complex Cdc48 together with the co-factors Npl4 and Ufd2

provide the energy to extract the substrates from the ER. The Cdc48 complex is bound to the ER membrane via the ER embedded protein Ubx2 by the UBX domain. A UBA domain in Ubx2 binds ubiquitin (Neuber, et al. 2005; Schuberth and Buchberger 2005).

Ubiquitylation takes place while the substrate is being extracted. Ubiquitin is a small protein (76 amino acid) that contains 7 lysine residues. Lysine 48 ubiquitin chains on substrates marks them for destruction (Pickart 1997; Varshavsky 2006; Zhang, et al. 2011). Ubiquitin is highly conserved among eukaryotes. Human and yeast ubiquitin share 96% sequence identity. Ubiquitylation is an enzymatic process that takes place in tree steps. First, ubiquitin is activated in a process requiring ATP as an energy source resulting in a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1 cysteine sulfhydryl group. In the second step there is a transfer of ubiquitin from E1 to the active site cysteine of an E2 ubiquitin conjugating enzyme via a trans(thio)esterification reaction. E3 ubiquitin ligases in the final step create an isopeptide bond between a lysine of the substrate and the C-terminal glycine of ubiquitin (Ciechanover, et al. 1984; Varshavsky 2006; Lodish, et al. 2007; Hershko 2009). If a lysine is not available, ubiquitin can be linked to the N-

terminus of the protein (Ciechanover and Ben-Saadon 2004; Wang, et al. 2007).Der3/Hrd1 and Doa10 are the only known E3 ubiquitin ligases in the ER of yeast (Kostova and Wolf 2003; Carvalho, et al. 2006; Kostova, et al. 2007; Eisele, et al. 2010). Both have a RING (Really interesting new gene) domain. RING ligases bind to E2 ubiquitin conjugating enzymes that transfer ubiquitin to the substrate, since they cannot transfer ubiquitin to the substrate directly themselves. Other type of E3 ubiquitin ligases are the HECT (Homologous to E6-AP Carboxyl Terminus) ligases. HECT ligases need E2 ubiquitin conjugating enzymes to be loaded with an ubiquitin molecule. After transferring ubiquitin from the E2 to the E3, the HECT E3 ubiquitin ligase catalyzes the ubiquitylation reaction of the substrate (Wolf and Hilt 2004; Kostova, et al. 2007).

Guidance of the ubiquitylated substrate to the proteasome is aided by receptor proteins containing UBA domains (that bind ubiquitin) and UBL domains (that bind to the proteasome). Dsk2 and Rad23 are ubiquitin receptors that contain UBA and UBL domains and escort substrates to the proteasome (Medicherla, et al. 2004; Raasi and Wolf 2007).

Ubiquitylated substrates are finally degraded by the proteasome. The proteasome is a 2 MDa protease complex

consisting of 28 subunits (Wolf and Hilt 2004) . Proteolytic activity is localized in the interior of the 20S core. Six ATPases of the 19S lid use ATP to unfold the substrate and shift it to the interior where degradation takes place (Groll, et al. 1997). In this process ubiquitin is not degraded but cleaved off from the substrate by deubiquitylating enzymes (DUBs) prior to degradation and is recycled (Fig 1.7) (Wilkinson 2009).

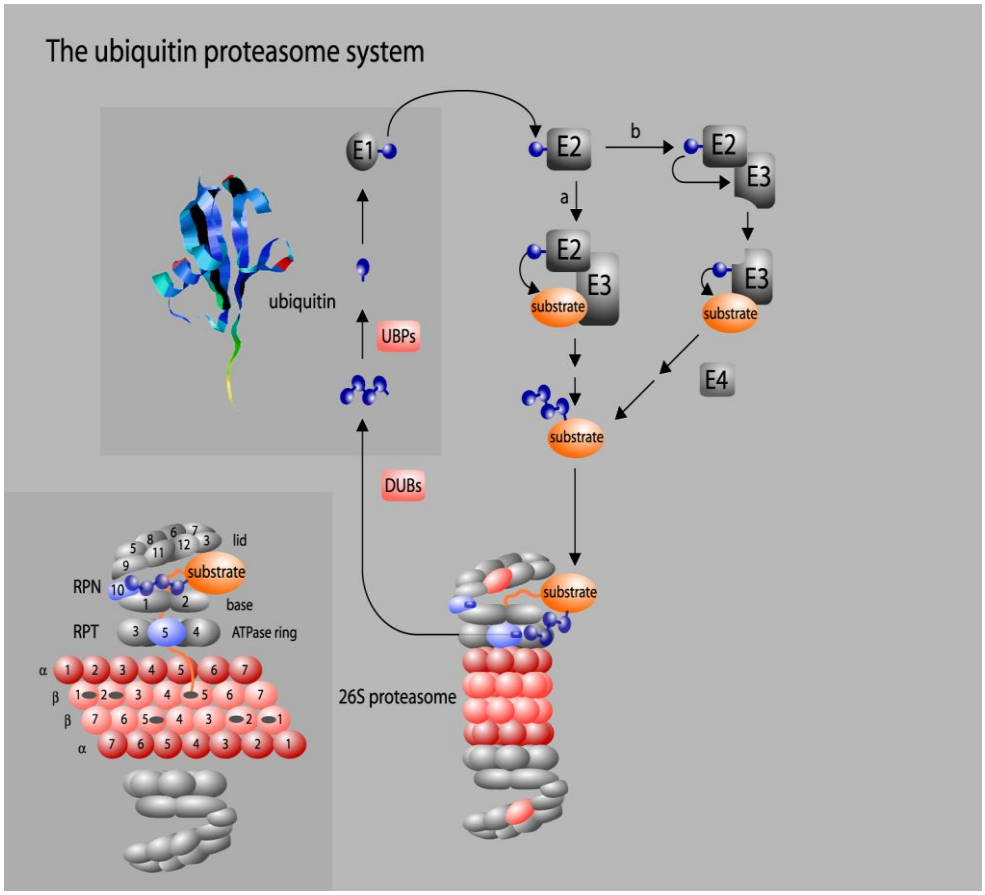

Figure 1.7: The Ubiquitin-Proteasome system. (a) RING E3 ubiquitin ligase ubiquitylation cascade. (b) HECT E3 uquiquitin ligase ubiquitylation cascade. The lid of the proteasome is coloured in grey and the core in red.(Wolf and Hilt 2004)

1.5.Model substrates

1.5.1.Cystic fibrosis transmembrane conductance regulator (CFTR)

CFTR is an ion channel of the plasma membrane that allows Cl^- ions to leave the cell. Mutations in this channel cause the disease cystic fibrosis (CF) (Buchwald, et al. 1989). The folding process of human CFTR is very inefficient and only 25% of the channels successfully produced are transported to the plasma membrane. The remainder is not able to fold and is degraded in an ubiquitin dependent manner by the proteasome. In the case of the mutant protein CFTRΔF508 almost 100% of the translation product is degraded by the proteasome (Ward and Kopito 1994; Jensen, et al. 1995). Human wild type CFTR expressed in yeast behaves like mutant CFTRΔF508 in human cells and is 100% degraded by the proteasome (Kiser, et al. 2001).

ERAD of CFTR is explored in yeast provided that in a future step it could be corroborated in human cell lines.

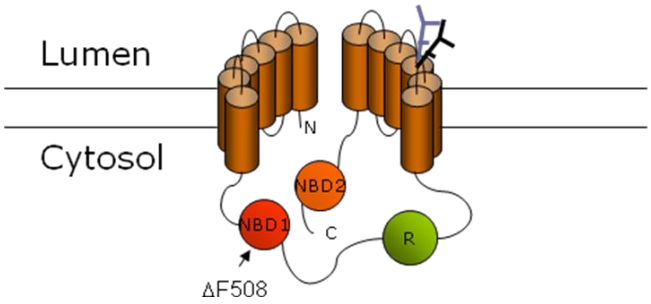

Figure 1.8: CFTR. CFTR has 2 membrane spanning domains, 2 nucleotide binding domains (NBD) and a highly hydrophobic large regulatory domain (R) with multiple phosphorylation sites (unique in ABC transporters). It is glycosylated twice. Mutant CFTRΔF508 has its lesion in the first NBD (Original from A. Gnann).

1.5.2.Carboxypeptidase Y mutants

Carboxypeptidase Y (CPY) is a vacuolar serine protease of yeast encoded in the gen *PRC1.* The allele *prc1-1* carries a point mutation resulting in the exchange of a glycine residue to an arginine residue in the position 255 of the protein. This misfolded protein is named CPY* (Fig 1.9) (Wolf and Fink 1975; Finger, et al. 1993). CPY* is found to be terminally misfolded in the ER and is degraded in an ERAD-L dependent manner (Hiller, et al. 1996). Since then CPY* has been used as a model substrate to study ERAD (Wolf and Schafer 2005).

Figure 1.9: The ERAD-L substrate CPY*. The four glycosylation sites on CPY* are numbered according to their positions in the mature enzyme. G255R is the site of the CPY* mutation.

CPY*, as wild type CPY, has four glycan residues. The substrate CPY*0000 has been mutated in its glycosylation sites in order to abrogate glycosylation by the OST complex at all sites and thereby obtain an unglycosylated version of CPY* (Kostova and Wolf 2005).

1.6.Aim of this work

ERAD has been thoroughly studied, but there are still many details that need to be better understood.

A first goal of the work had been the hope to discover new ERAD components needed for CFTR turnover. However, since no fruitful outcome on ERAD of CFTR could be reached, I explored other possibilities in the ERAD field.

What became the main purpose of this work was to address the question: What are the differences in degradation between glycosylated and non glycosylated substrates in ERAD? Which degradation pathway does non-glycosylated CPY* follow? Also, it has been found that the

glycan degradation signal that triggers ERAD varies between Man7 to Man5 glycan structures. Are there additional mannosidases besides Mns1 and Mnl1/Htm1 necessary to process the glycan on misfolded proteins?

2.Materials and methods

2.1.Materials

2.1.1.Saccharomyces cerevisiae strains

Strain	Genotype	Source
YWO0340	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, Δprc1::hisG, Δpep4/pra1::HIS3, Δprb1::hisG*	K. Kuchler
YWO0343	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1*	(Knop, et al. 1996)
YWO0361	WCG4 Mat alpha, *ura3, his3-11,15, leu2-3,112 prc1-1*	(Hiller, et al. 1996)
YWO0364	WCG4 Mat alpha, *ura3, his3-11,15, leu2-3,112 prc1-1, pre1-1, pre4-1*	(Hiller, et al. 1996)
YWO0566	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmns1::URA3*	(Kostova and Wolf 2003)
YWO0636	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, Δprc1::LEU2*	(Plemper, et al. 1999b)
YWO0820	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, Δprc1::LEU2, Δmnl1/htm1::KanMX6*	(Kostova and Wolf 2005)
YWO0823	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmnl1::KanMX6*	(Kostova and Wolf 2005)
YWO0830	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100*	(Chiang and Schekman 1991)

Strain	**Genotype**	**Source**
YWO0831	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100*	(Chiang and Schekman 1991)
YWO0855	MY Mat alpha, *ura3-52, leu2, his3Δ200, ade2*	(Rose, et al. 1981)
YWO0857	MY Mat alpha, *ura3-52, leu2, his3Δ200, ade2, Δrad23, Δdsk2::LEU2*	(Biggins, et al. 1996)
YWO1011	BWG1-7a Mat a, *ura3-52, leu2-3,112, his4-519, ade1-100, prc1-1*	(Johnson, et al. 1995)
YWO1019	BWG1-7a Mat a, *ura3-52, leu2-3,112, his4-519, ade1-100, prc1-1, ufd3-1*	(Johnson, et al. 1995)
YWO1088	MHY501 Mat alpha, *ura3-52, leu2-3,112, his3Δ200, lys2-801, trp1-1*	(Swanson, et al. 2001)
YWO1092	MHY501 Mat alpha, *ura3-52, leu2-3,112, his3Δ200, lys2-801, trp1-1, Δdoa10::HIS3, Δhrd1::LEU2*	(Swanson, et al. 2001)
YWO1154	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, Δprc1::LEU2, Δyos9::HIS3MX6*	(Buschhorn, et al. 2004)
YWO1164	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δufd2::KanMX*	(Kohlmann, et al. 2008)
YWO1168	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, doa10::KanMX*	A. Stolz
YWO1268	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δusa::KanMX6*	H. Hoshida
YWO1306	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, Δprc1::LEU2, Δubp6::KanMX*	(Kohlmann, et al. 2008)

Strain	**Genotype**	**Source**
YWO1320	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, Δprc1::LEU2, Δhul5::HIS3*	(Kohlmann, et al. 2008)
YWO1408	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, Δprc1::LEU2, Δder3/hrd1::HIS3*	L.Xiao
YWO1477	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmns1::URA3, Δmnl1::KanMX6+B33*	O. Fisher
YWO1526	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, Δprc1::LEU2, doa10::KanMX*	A. Stolz
YWO1580	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, Δprc1::hisG*	K. Kuchler
YWO1643	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δder1::his5+*	S. Besser
YWO1779	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmnl2::his5+*	This study
YWO1780	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmns1::URA3, Δmnl2::his5+*	This study
YWO1781	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmnl1::KanMX6, Δmnl2::his5+*	This study
YWO1782	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmns1::URA3, Δmnl1::KanMX6, Δmnl2::his5+*	This study
YWO1783	W303 Mat alpha, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmnl2*	This study

Strain	**Genotype**	**Source**
YWO1784	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmns1, Δmnl2*	This study
YWO1785	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmnl1, Δmnl2*	This study
YWO1786	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmns1, Δmnl1, Δmnl2*	This study
YWO1787	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmnl2*	This study
YWO1788	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmns1, Δmnl2, Δyos9::his5+*	This study
YWO1789	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmnl1, Δmnl2, Δyos9::his5+*	This study
YWO1790	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δmns1, Δmnl1, Δmnl2, Δyos9::his5+*	This study
YWO1791	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δder1*	This study
YWO1792	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δder1, Δyos9::his5+*	This study
YWO1793	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, Δusa::KanMX6, Δyos9::his5+*	This study
YWO1794	MHY501 Mat alpha, *ura3-52, leu2-3,112, his3Δ200, lys2-801, trp1-1, Δdoa10::HIS3, Δhrd1::LEU2, Δpep4::KanMX*	This study

Strain	Genotype	Source
YWO1945	W303 Mat a, *ura3-1, his3-11,15, leu2-3,112 trp1-1, ade2-1ocre can1-100, prc1-1, doa10::KanMX, Δyos9::HIS3MX6*	This study

2.1.1.Escherichia coli strains

For amplification and cloning of plasmids the DH5α *E.coli* strain was used (Hanahan 1983).

Strain	Genotype
DH5α	F'/endA1 hsdR17(rk-mk+) supE44 thi-1 recA1 gyrA (Nalr) s80dlacZM15

2.1.2.Plasmids

Plasmid	Characteristics	Source
pRS314	CEN *TRP1* vector for yeast and *E. coli*	(Sikorski and Hieter 1989)
pRS316	CEN *URA3* vector for yeast and *E. coli*	(Sikorski and Hieter 1989)
pFA6a13myc-His3MX6	*E. coli p*lasmid for myc tagging	(Longtine, et al. 1998)
pUG6	*E. coli p*lasmid for gen deletion	(Guldener, et al. 1996)
pUG27	*E. coli p*lasmid for gen deletion	(Guldener, et al. 1996)

pSH63	Cre recombinase expression plasmid	(Gueldener, et al. 2002)
pYes2	2µ URA3 vector for yeast	Invitrogen
PWO0612	pRS316-CPY* under *PRC1* promotor	R. Hitt
PWO0604	pRS316-CPY*0000 under *PRC1* promotor	(Kostova and Wolf 2005)
PWO1113	pRS314-Yos9-Flag	O. Fischer
PWO1114	pRS314-Yos9R200A-Flag	O. Fischer

2.1.3. Primers

Primer	**Sequence 5'→3'**	**Source**
5' dis Pep4	GTGACCTAGTATTTAATCCAAATAAAATTCAAACAAAAACCAA AACTAACATGCAGCTGAAGCTTCGTACGC	This study
3' dis Pep4	GTCTAGGTGATCACCGGATACGCTCTCTAGATGGCAGAAAAGG ATAGGGCGGAGAAGTAAGAAAAGTTTAGC	This study
Dis Pep4 checkA	GTAATTCGCTGCTATTTA	This study
Dis Pep4 checkB	GGAGTACCCAAAGTAATG	This study
Dis Pep4 checkC	TGCTGAAATTGGGGCCAA	This study
Dis Pep4 checkD	GCTACCCGCATATAATGACA	This study
EMB002	CCCTGGACAAACACATCTTAACTCAAGGAGGGCACATAATCAA AAAGAAAcggatccccgggttaattaa	This study
EMB003	TGTACCTATATGTATGTATGTATGTGCGTACGATTTTTCTAAC GTTAACTgaattcgagctcgtttaaac	This study
EMB004	AAGATTTCGGTGAAGCGC	This study

Primer	**Sequence 5'→3'**	**Source**
EMB005	GCGAGTGCCATAGTAGTC	This study
EMB006	CTTGGCGGCATTCTAGG	This study
EMB007	ATCACCACAACGACACTTAA	This study
EMB008	ggatgtatgggctaaatg	This study
EMB009	cctcgacatcatctgccc	This study
EMB010	GTGTTAACTAGAAAAAGGCCGCCACTACTCTATAAGCAAAACC TTcagctgaagcttcgtacgc	This study
EMB011	CTATATGTATGTATGTATGTGCGTACGATTTTTCTAACGTTAA CTgcataggccactagtggatctg	This study
EMB012	TAACCGGGCTGTTCTTTG	This study
EMB013	TGTTTGGGCTTGATCGAG	This study
EMB014	GGCTGGTCAAGGAAGATC	This study
EMB015	CCGCGATTAAATTCCAACAT	J. Juretschke
EMB016	CGACAGCAGTATAGCGACCA	J. Juretschke
EMB017	catttagcccatacatcc	This study
EMB018	gggcagatgatgtcgagg	This study
EMB021	AGATCTTCACATATATCGTTATCATCCCCTTTCTTCCCTGTTT CAcagctgaagcttcgtacgc	This study
EMB022	GCAAAACGTGAAAAAAAAAAATTAAAAGTTTATACTCCTCCTT GTgcataggccactagtggatctg	This study
EMB023	cagtgcttccatccttattct	This study
EMB024	gctttggatgctcagttac	This study
EMB025	GACGTCCAGATGATCCATG	This study
EMB026	TTTGCATACGTGCGCTG	This study
EMB035	CCAAGCCGGATTTTGG	This study
EMB036	ACCGCAGAAATTCGGTC	This study
EMB037	CCCTGGACAAACACATCTTAACTCAAGGAGGGCACATAATCAA AAAGAAAagggaacaaaagctgg	This study
EMB038	TGTACCTATATGTATGTATGTATGTGCGTACGATTTTTCTAAC	This study

Primer	Sequence 5'→3'	Source
	GTTAACTtatagggcgaattgg	
EMB039	AGATTTCGGTACCGCGCAAGTGTTAAC	This study
EMB040	GACTGGgcggccgcATACCCATTGGCTGTG	This study
EMB041	GTTTATCAGATTGTCACAG	This study
EMB042	GGCGTGTTTCAGCGAC	This study
EMB043	TCGGTAAAACCGGCC	This study
EMB047	ATTAGAAGCCGCCGAGCG	This study
5′ CLM1	cccaaaccaggttatgag	F. Eisele
5′ CLM2	ggacagaaacttcaactt	F. Eisele
5′ CLM3	tttgagagtcttcaatgg	F. Eisele
5′ CLM4	CCTAGTTAAGAACCCAAC	F. Eisele
5′ CLM5	CTCAGAAGAAGACTTGAA	F. Eisele

2.1.4.Antibodies

Antibody	Dilution and use	Source
Rabbit α CPY	3:300 IP	Rockland
Mouse α myc (9E10)	1:5000 IB	Santa Cruz Biotechnology
Mouse α CPY	1:10000 IB	Molecular Probes
Mouse α HA (16B12)	1:2000 IB	Covance
mouse α CFTR (M3A7)	1:1000 IB	Upstate
mouse α PGK	1:10000 IB	Molecular Probes
HRPO conjugated goat α mouse IgG	1:15000 IB	Jackson ImmunoResearch
HRPO conjugated goat α rabbit IgG	1:10000 IB	Sigma
Rabbit α Flag	1:5000 IB	Sigma

2.1.5.Kits, enzymes, chemicals and media

Supplier	**Reagents**
Acros organics	acetic acid, DMSO
Amersham	CDP-Star™ Detection reagent Hyperfilm ECL™ Hybond-N-Nylon membrane
Bioline	Velocity™ DNA polymerase
Calbiochem	MG132
Difco	Yeast extract, Yeast nitrogen base w/o amino acids, Yeast nitrogen base w/o amino acids and ammnonium sulfate, Bacto-Agar, Bacto-Peptone, Bacto-Tryptone
Dr Gross	skimmed milk
Enzogenetics	Oxalyticase
Fermentas	GeneJET™ Plasmid Miniprep Kit Restriction enzymes T4 DNA ligase Proteoblock (protease inhibitor) PageRuler™ prestained Protein Ladder Plus
Finnenzymes	Phusion™ DNA polymerase
Fisher Scientific	acetone, chloroform
Genaxxon	Oligonucleotides, Taq DNA polymerase
Hartmann Analytic GmbH	^{35}S methionine
Kodak	Autoradiography Film Biomax MR
MACHEREY-NAGEL	NucleoSpin® Extract II
Merck	TEMED
Millipore	polyvinyliden difluoride (PVDF) membranes

Supplier	**Reagents**
New England Biolabs	Restriction enzymes T4 DNA ligase
Pierce	ECL Western Blotting Substrate
Prolabo	ethanol, methanol
Roche	Compleat inhibitor cocktail, Hering sperm DNA
Roth	1kb DNA ladder, Rotiphoerese30, agarose, ammonium acetate, ammonium, peroxodisulphate, chloroform, dithiothretiol, dimethylformamide, isopropanol, magnesium chloride, magnesium sulphate, β-mercaptoethanol, potassium acetate, potasiumchloride, potassium dihydrogen phosphate, potassium hydrogen phosphate, sodium acetate, sodium chloride, sodium hydroxide, sodium dihydrogen phosphate, sodium hydrogen phosphate,Roti-phenol, phenylmethanesulfonylfluoride, trichloroacetic acid, Tris, TritonX-100,X-Gal, L-sorbitol, urea
Sartorius AG	Glass beads
Schleicher and Schüll	Blotting paper GB001, BG002 and GB003
Seigagaku kyogo	Zymolyase 100-T
Serva	Coomassie Brilliant Blue R250
Sigma-Aldrich	amino acids, ethidium bromide, ethylenediaminetetraacetic acid, polyethylene glycol 3350, Ponceau S, sodium dodecylsulphate, Tween-20, BSA
Riedel-De Haën	glycerol

All media used for yeast or E. coli cultures were prepared with double deionised water. The pH was adjusted either with NaOH or HCl. Media was autoclaved at 120°C for

20 minutes for sterilization. To prepare solid media 2%(W/V) sterile agar was added to the liquid media.

2.1.5.1.Yeast media

All yeast strains were grown in yeast complete medium containing 2% glucose (YPD) or complete minimal medium (CM) media. To select clones of interest depending on auxotrofphic markers CM media were supplemented with uracil, L-tryptophan, L-histidine, L-leucine, adenine and L-lysine.

YPD	10 g/L BactoYeast 20 g7L BactoPeptone 20 g/L D-glucose pH 5,5
CM	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-Methionine, 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

Presporulation	3 g/L Peptone 8 g/L Yeast extract 20 g/L Potassium acetate
Sporulation	20 g/L potassium acetate 1 g/L yeast extract
Pulse chase: Starvation	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
Pulse chase: Chase	Starvation plus 0,2% BSA 0,6% L-Methionine

2.1.5.2. *E.coli* media

E. coli was grown in LB, SOB or SOC media. To select for ampicillin resistant transformants carrying plasmid with the gene Amp^r as selection marker, 0,1mg/ml ampicillin was added to the media respectively.

LB	1% Bacto trypto-peptone 0,5% Yeast extract 0,5% NaCl pH 7,5
SOB	2% Bacto trypto-peptone 0,5% Yeast extract 0,05% NaCl 10 mM MgCl2 pH 7,0
SOC	2% Bacto trypto-peptone 0,5% Yeast extract 0,05% NaCl 10 mM MgCl2 20 mM glucose pH 7,0

2.1.6.Laboratory equipment

- Agarose gel electrophoresis apparatus and protein electrophoresis aparatus Protean II and III, Bio-Rad.
- Balance AE163, Mettler
- Biofuges fresco and pico, incubator B6200, Heraeus
- Centrifuge 5417C, 5804R and Z320K, Eppendorf
- Centrifuge centrikkon T-124, Kontron Instruments
- Centrifuse Sorvall RC5B, Kendro
- Film developer machine Optimax, Proec Medizintechnik
- Heating block Thermostat TCR100, Roth

- Ion exchanger milli-Q Academics, Millipore
- Multi vortexer IKA-VIBRAX VXR, Staufen i. Br.
- Overhead rotator REAX2, Heidolph Instruments
- Overhead shaker 34528, Snijders Scientific
- PCR thermocycler Robocycler Gradient 40, Stratagene
- pHmeter CG832, Schott
- Phosphorimager Storm860 and Spectrophotometer Novaspec II, GE Healthcare
- Micropipettes, Gilson
- Ultracentrifuge Optima™ TLX, Beckman
- Tetrad dissection needles, Singer instruments.

2.2.Methods

2.2.1.S. cerevisiae and E.coli growth

All procedures involving cell cultures were done under sterile conditions. Quantification of the optical density of cell suspensions was done with a spectrophotometer at room temperature and with a wave length of 600 nm.

2.2.1.1.*E.coli* cell cultures

E. coli plasmid containing cells taken from -80°C permanent cultures were streaked out on LB/amp solid media and incubated at 37°C for 1 day. Newly transformed *E. coli* cells were plated in LB/amp solid media and grown overnight at 37°C. In both cases, for isolation of plasmid DNA a single colony was inoculated in 5 ml LB/amp liquid media and grown overnight at 37°C on a shaker at 160 rpm.

2.2.1.2.Preparation of competent DH5α *E.coli* cells

E. coli cells were streaked out on LB plates and incubated overnight at 37°C. One single colony from the plate was then inoculated into 50 ml of LB liquid medium and cells were grow at 37°C shaking overnight. 250 ml of SOB medium was inoculated at a dilution of 1/50 with the preculture. Cells were grown to an O.D. of 0,5 (λ=600nm). The cells were recovered by centrifugation at 3000 r.p.m. for 15 minutes, washed with cold TfbI buffer (2/5 of the original culture volume), centrifuged for 5 minutes at 3000 r.p.m. and resuspended in cold TfbII buffer (1/25 of the original culture volume). The suspension was incubated on ice for 15 minutes. Finally, 250 μl aliquots of the cell

suspension were frozen in a MeOH/dryice bath and stored at -80° (Inoue, et al. 1990).

TbfI:	AcOK 30 mM $RbCl_2$ 100 mM $CaCl_2$ 10 mM $MnCl_2$ 50 mM Glycerol 15% (v/v) pH 5,8 Sterilized.
TbfII:	MOPS 10 mM $CaCl_2$ 75 mM $RbCl_2$ 10 mM pH 6,5 Sterilized.

2.2.1.3.Yeast cell cultures

Yeast cells taken from -80°C permanent cultures were streaked out on YPD solid media and incubated at 30°C for 3 days. For precultures, a single colony was inoculated in 5 ml YPD liquid media and grown for 1 or 2 days at 30°C on a shaker at 160 rpm. Main cultures were inoculated in fresh media and grown to a logarithmic phase at 30°C unless otherwise specificated.

2.2.1.4.Yeast growth test

Growth tests were done in serial dilutions to assess the growth rate of cells. Precultures of the strains to be compared were grown in liquid media and the optical density measured. All strains were then resuspended in sterile water to the same concentration (approximately 5 OD/ml). Four to 5 serial 1:10 dilutions were prepared. The dilutions were then plated in the solid media with a metal stapler. The Petri dishes were kept at 30°C between 2 to 5 days until colonies appeared.

For cell selection after a mutation procedure, colonies were streaked out on selective media and let grow at 30°C until colonies appeared.

2.2.2.Molecular biology

2.2.2.1.Calculation of *E. coli* cell competence

The competence of the *E. coli* cells was quantified by transformation of 100 µl of competent cells with 1 µl pUC18 plasmid with a concentration 0,1 ng/µl. After plating 100 µl of the 1 ml transformation suspension in LB/amp media, and overnight incubation at 37°C, the number of single

colonies was counted and the colony formal units per μg of DNA calculated.

2.2.2.2.E. coli heat shock transformation

Cell and plasmid suspensions were thawed on ice. One μl of the plasmid suspension was pipetted into the cell suspension and shook gently. The cells were subjected to heat shock by placing the tube for 45 seconds on a 42°C heatblock and then placed on ice for 2 minutes. One ml of SOC medium was pipetted into the mixture and incubated for 1 hour on a shaker at 37°C. Finally, a certain volume of the suspensions was plated on selective LB media.

2.2.2.3.Plasmid amplification and mini-prep

DH5α E. coli cells were transformed with plasmids following the heat shock transformation protocol and plated on LB/amp medium. Then a single colony was inoculated into a 5 ml LB liquid media with 0,1 mg/ml ampicilin and incubated overnight with shaking at 37°C. On the next day, the tubes were spun down for 8 minutes at 4000 r.p.m. and the supernatant was discarded. For the isolation of plasmid

DNA a miniprep kit was used following the instructions of the supplier.

2.2.2.4.Generation of strains by homologous recombination

Deletions of the genes *PEP4/PRA1, MNL2* and *YOS9* were achieved as described by Güldener (Guldener, et al. 1996; Gueldener, et al. 2002). For the genotype see section 2.1.1.

The strain YWO1794 was obtained by homologous recombination of the yeast strain YWO1092 with the PCR amplified fragment from the pUG6 plasmid and the primer set 5' dis Pep4 and 3' dis Pep4. Cells were plated on YPD/kan solid media for the selection of clones. The correct integration of the disruption cassette was confirmed by PCR (primers: Dis Pep4 checkA, Dis Pep4 checkB, Dis Pep4 checkC, Dis Pep4 checkD, EMB008 and EMB009) and Southern blotting with the Kan^R probe obtained from the PCR amplification product from the pUG6 sequence with primers EMB015 and EMB016.

The 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 EMB010 and EMB011. Cells were plated on CM-His solid media for selection of clones. The correct integration of the disruption cassette was confirmed by PCR (primers: EMB004, EMB005, EMB006, EMB007, EMB008 and EMB009) and Southern blotting with the *His5+* probe obtained from the PCR amplification product from the pUG27 sequence with primers EMB017 and EMB018.

The strains YWO1783, YWO1784, YWO1785 and YWO1786, where the gene *MNL2* was deleted and all marker genes from previous gene disruptions were popped out, were obtained from the yeast strains YWO1779, YWO1780, YWO1781 and YWO1782 respectively. The strain YWO1791, where the gene *DER1* was deleted and the marker gene from its disruption was popped out, was obtained from the yeast strains YWO1643. One or more *loxP-markergene-loxP* cassettes were popped out by transforming the latter strains with the expression plasmid pSH63 that encondes the Cre recombinase protein. The result is a single *loxP* site at the deleted gene locus.

The strains YWO1787, YWO1788, YWO1789, YWO1790 and YWO1792, where the gene *YOS9* was deleted, were obtained from the yeast strains YWO1783, YWO1784, YWO1785, YWO1786

and YWO1791 respectively. They were generated by homologous recombination of these strains and the PCR amplified fragment from the pUG27 plasmid and the primer set EMB021 and EMB022. Cells were plated on CM-His solid media for selection of clones. The correct integration of the disruption cassette was confirmed by PCR (primers: EMB023, EMB024, EMB035, EMB036, EMB008 and EMB009) and Southern blotting with the *His5+* probe obtained from the PCR amplification of the pUG27 sequence with primers EMB017 and EMB018.

2.2.2.5.Generation of strains by mating

The yeast strain YWO1945 was obtained by mating of the strains YWO1168 and YWO1154 with opposite mating types. The strains were mixed on a presporulation plate and incubated overnight at 30°C. A toothpick tip full of cells was then transferred to a sporulation plate and incubated at 25°C for 5 days. For tetrad analysis a toothpick tip full of cells was resuspended in 200 µl of sorbitol buffer (1,2 M sorbitol, 50 mM Tris/HCl pH 7,5 in sterile water), 4 µl of 5mg/ml oxalyticase and incubated 25 minutes at 30°C. Then 1ml of sterile water was added. Tetrads were separated on YPD plates using a dissection needle and a micromanipulator

assembled on a light microscope. Plates were incubated at 30°C and when colonies arouse, they were replica-plated onto YPD/kan and CM-His solid media. Colonies were checked for mating type and *PRC1* expression (Guthrie and Fink 1991).

2.2.2.6.Transformation of *S. cerevisiae* cells

A yeast single colony was inoculated in 5ml of YPD and grown overnight at 30°C. Inoculation with precultures into 10 ml of fresh YPD to an optical density of about 1 OD/ml was done and grown until approximately 2,5 OD/ml at 30°C. Cells were then centrifuged 5 minutes at 3500 rpm at room temperature and washed first with 10 ml and then 1ml of sterile water. Next, cells were centrifuged 1 minute at 3000 rpm at room temperature and resuspended in 1.5 ml TE/AcOLi, and again centrifuged 1 minute at 3000 rpm. Finally, the cells were resuspend in TE/AcOLi/PEG to a final concentration of 2 OD/50 µl. Aliquots of 50 µl competent yeast cells were prepared and stored at -80°C.

For transformation a mix of 50 µl of cells with 1 µl of plasmid, 5 µl ssDNA (5’ at 95°C and then ice) and 300 µl of PEG was mixed gently and then incubated 30 minutes at 30°C on a rotor. Heat shock was done in a heating block at

42°C for 15 minutes. Then 800 µl of sterile water was added and the suspension centrifuged for 1 minute at 3000 rpm. Cells were resuspended gently in 200 µl of 1M sorbitol and plated in selection media. Plates were incubated at 30°C until colonies emerged (Guldener, et al. 1996).

2.2.3.Protein and DNA biochemistry

2.2.3.1.Preparation of yeast cell lysates

2.2.3.1.1.TCA precipitation

Proteins were precipitated from a yeast culture aliquot by the addition of 6% (w/v) final concentration of trichloroacetic acid (TCA) and kept at -80°C for 30 minutes. After a 20 minutes centrifugation at 13,000 rpm, pellets were washed with 1 ml –20°C cold acetone. Finally the pellets were dried and stored at -20°C.

2.2.3.1.2.Denaturing glass-bead lysis

Cells (10 ODs) were collected on 500 µl of 200mM NaN_3 containing tubes placed on ice. The tubes were centrifuged for 15 minutes at 13000 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 minute with a minute interruption on ice (for CFTR samples) or 1 minute interruption at 95°C (for CPY derivatives). Then 900 µl of IP buffer was added.

2.2.3.2.SDS-PAGE

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was used to separate proteins. A solution of acrylamide and bisacrylamide was polymerized and the 'pore size' was determined by the concentration of acrylamide. Polymerization of acrylamide and bisacrylamide monomers was induced by ammonium persulfate (APS), which spontaneously decomposes to form free radicals. TEMED, a free radical stabilizer, was included to promote polymerization. Sodium dodecyl sulfate (SDS) is an amphipathic detergent. It has an anionic headgroup and a lipophilic tail. It binds non-covalently to proteins, with a stoichiometry of around one SDS molecule per two amino acids. SDS causes proteins to denature and disassociate from each other. It also confers negative charge. In the presence of SDS, the intrinsic charge of a protein is

masked. During SDS PAGE, all proteins migrate towards the anode. SDS-treated proteins have very similar charge-to-mass ratios, and similar shapes. When using SDS-PAGE, the rate of migration of SDS-treated proteins can be effectively determined and the molecular mass can be calculated. The gels were 1 mm or 1,5 mm thick and consisted of a 4% acrylamide stacking gel with the loading wells for the samples and a resolving gel of different acrylamide percentage (7,5%, 10%) depending on the proteins to be separated. Polymerized gels were transferred to the electrophoresis chamber and overlaid with SDS-running buffer (25 mM Tris, 190 mM glycin, 0,1% SDS). The samples were mixed with loading buffer containing 2,5% β mercaptoethanol, heated for 5 minutes at 95°C (when analyzing CPY variants) or 60°C (when analyzing CFTR) and spun down for 5 minutes at 12000 r.p.m. Two µl of protein molecular mass standard and proteins were separated at 110 V. After electrophoresis, the gels containing the proteins were either dried, stained with Coomassie Brilliant Blue (0,25% (w/v) Commassie BB, 7,5% (v/v) acetic acid, 50% (v/v) methanol) or the proteins transferred onto a nitrocellulose membrane for detection by immunoblotting.

Components	7,5% gel	10% gel	Stacking gel
dd H2O (ml)	4	2,8	2,6
1,5M Tris pH 8,8	2	2	
1,5M Tris pH 6,8			1
Acrylamide/bisacrylamide Solution (37,5:1) (ml)	2	3,2	0,4
10% SDS (ml)	32	32	14
10% (w/v) APS (ml)	32	32	14
TEMED (ml)	3,2	3,2	5

	loading buffers		
	Laemmli 5X	Urea	SDS
SDS	10% w/v	5% w/v	01,% w/v
Glycerol	20% w/v		200mM
Tris-HCl, pH 6.8	0.2 M	40 mM	
Bromophenolblue	0,2% w/v	0.05% w/v	0.05% w/v
Urea		8 M	

2.2.3.3.Western blotting

During Western blotting proteins separated by SDS-PAGE were transferred from the gel onto a nitrocellulose membrane. A sandwich consisting of a sponge, a Whatman paper, a nitrocellulose membrane, the SDS gel, a Whatman paper and a sponge was prepared in a tank with blotting buffer (150 mM glycine, 20 mM Tris, 20% methanol). The sandwich was

transferred into the blotting chamber and the proteins were transferred for 1,5 hours at constant intensity of 300A. After transfer, the membrane was incubated for 5 minutes in Ponceau S solution (5% acetic acid, 0,2% Ponceau S) and washed with H_2O to detect transfer of proteins. The membrane was then dried and stored at room temperature or blocked (see below) for immunodetection.

2.2.3.4.Immnunodetection

For the immunodetection of proteins, the nitrocellulose membrane was blocked (either with 3% BSA or 5% milk in PBS-T) for 1 hour at room temperature. Then the membrane was incubated with the primary antibody diluted in PBS-T (20mM Tris/HCl, pH7,6; 137 mM NaCl; 0,1% Tween20) buffer for 1 hour. The washing of unbound antibodies was carried out 3 times for 10 minutes with fresh PBS-T buffer. Then the membrane was incubated for 1 hour with the secondary antibody coupled to horseradish peroxidase (HRP) diluted in PBS-T buffer. Thereafter, the membrane was washed 3 times in PBS-T buffer for 5 minutes. Finally, the membrane was overlayed with 400 µl of ECL solution for a minute and transfered to a developing cassette. An ECL film was exposed to the membrane and then developed.

The detection of other proteins from the same membrane was possible after stripping the membrane with 10% acetic acid for 8 minutes followed by thorough rinsing with ddH_2O.

2.2.3.5.Chromosomal DNA isolation from yeast

Five ml of an YPD overnight culture were centrifuged at 3000 rpm for 4 minutes, washed with 1 ml of water and pelleted in a microtube. Cells were lysed by resuspending the pellet in 200 µl of breaking buffer (2% (w/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8,0), 200 µl phenol-chloroform-isoamylalcohol mix and 300 µl of glass beads. Cells were vortexed for 1 minute followed by 1 minute of chilling on ice for 5 times. Then 200 µl of water were added and the tube centrifuged for 10 minutes at 13000 rpm at 4°C. DNA was resuspended in 400 µl of water, 3 µl or RNAse (10mg/ml) added and tubes incubated for 5 minutes at 37°C. DNA was precipitated incubating the tubes for 30 minutes at -80°C with 10 µl of 5 M NH_4Ac and 1 ml cold ethanol. Finally, the tubes were centrifuged for 10 minutes at 13000 rpm at 4°C, washed once with 1 ml 70% (v/v) ethanol, dried and resuspended in 25 µl of sterile water.

2.2.3.6.Agarose gel electrophoresis

DNA fragments were separated on a 1% agarose gel (5 g agarose, 5 µl of 10 mg/ml ethidium bromide in 50 ml of TAE buffer) by electrophoresis. The purpose of the electrophoresis was to check band sizes of digested DNA, to isolate a particular band from the gel or preparation of samples for Southern blotting. Ethidium bromide binds strongly to DNA by intercalating between the bases and is fluorescent showing that it absorbs invisible UV light and transmits the energy as visible orange light. The DNA samples were run on gels at 120 V for 30 minutes. Then the gel was exposed to UV light and pictures were taken.

2.2.3.7.Southern blotting

The Southern blot is used to verify the presence or absence of a specific nucleotide sequence of DNA. After isolation of chromosomal DNA, DNA is digested with specific restriction enzymes. The DNA fragments are loaded onto an agarose gel and separated by electrophoresis. The DNA is then transferred from the gel to a nylon membrane. Then the alkaline phosphatase-conjugated probe is added. The probe only binds complementary DNA fragments. To detect the

position of the probe the membrane is exposed to a film and developed.

The DNA sequence to be checked was digested with adequate restriction enzymes, which were chosen with the aid of the program APE, yielding fragments between 1 and 5 Kbp. First 3 to 5 µl of isolated DNA were completely digested with restriction enzymes following the suggested protocol of the provider, at 37°C overnight. To ensure the complete digestion of the sample, addition of 0,5 µl fresh enzyme on the second day and incubation for 3h at 37°C was done. DNA sample buffer was added to the mixture and samples subjected to agarose electrophoresis for 60 minutes. A picture was taken for the record. The DNA was transferred to a nylon membrane. The membrane was equilibrated in 2X SSC (0,3 M NaCl, 0,03 M sodium citrate, pH 7) buffer for 15 minutes and placed on the blotting chamber. The chamber was covered with a plastic lining leaving the center of the membrane, where the DNA is bound, uncovered. The agarose gel was placed on top of the membrane and 40 mbar vacuum applied. The gel was covered with 25 ml of denaturing buffer (1,5 M NaCl, 0,5 M NaOH) for 10 minutes. The excess of buffer was removed and 25 ml of neutralizing buffer (0,5 M Tris/HCl, 3 M NaCl, pH 7) were added for 10 minutes. Then the buffer was exchanged to

20X SSC buffer (3 M NaCl, 0,3 M sodium citrate, pH 7) and DNA transfer accomplished in 60 minutes. DNA was covalently linked to the membrane by exposing it to UV light for 5 minutes. All steps were done at room temperature.

The PCR generated probe was labeled with a thermostable alkaline phosphatase using a kit. The probe was first denatured to yield single-stranded DNA. Via a cross-linker DNA was covalently coupled to the enzyme as suggested by the provider. The hybridizing buffer was prepared as described by the provider and 30 ml were preheated to 55°C in a glass tube. The nylon membrane was inserted into the tube and preincubated for 30 minutes under rotation. Then 32 µl of the probe were pipetted into the hybridizing buffer and the membrane was incubated overnight with the probe. Next, the membrane was washed 3 times for 5 minutes with 50 ml of secondary wash buffer (0,05 M Tris, 0,1 M NaCl, 2 mM $MgCl_2$, pH 10). The membrane was then covered with 3 ml of CDP star (alkaline phosphatase-based chemiluminescent detection) solution and placed in a film cassette. An ECL film was exposed to the membrane and signals observed after developing the film.

2.2.3.8.Cycloheximide decay experiments

Cells were grown at 30°C (unless otherwise indicated) to logarithmic phase (approximately 5 OD/ml) in 20ml of selective media or YPD. Then they were washed with fresh media and 50 ODs of cells were resuspended in 2ml fresh media with 150 μl of 10mg/ml cycloheximide. Samples (450 μl) were collected at different time points in microtubes containing 500 μl of 200 mM NaN_3 and kept on ice or stored at -20°C. Cell extracts were prepared by TCA precipitation or glass-bead lysis and finally subjected to SDS-PAGE, Western blotting and immunodetection.

2.2.3.9.Pulse-chase experiments

Cells were grown at 30°C to logarithmic phase (approximately 5 OD/ml) in 20ml of selective media or YPD. For each strain 10 ODs of cells were taken. They were washed 5 times with 1 ml starvation media and resuspended in 1 ml starvation media in 50 ml tubes. Cells underwent starvation for 50 minutes 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 minutes under the same

conditions. During this time all newly synthesized proteins integrate radioactive methionine into their sequence. After the 20 minutes, 1 ml of chase media, containing a high concentration of non-radioactive methionine (2mg/ml) 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. Cells were lysed with glass beads. 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) with protease inhibitors and spun down for 15 minutes at 14000 rpm. Next, 900 µl of the supernatant was transferred to a microtube containing 3 µl of polyclonal α-CPY antibody for immunoprecipitation. Immunoprecipitation took place during 90 minutes at room temperature, followed by the addition of 80 µl of 7% Protein A sepharose suspension as suggested by the supplier. During 90 minute 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 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.

3.Results

- **Part I**

3.1.Degradation studies on the human ERAD substrate CFTR in yeast

Cystic fibrosis (CF) is the most common life threatening inherited disease among Caucasians (Griesenbach, et al. 1999). CFTR is an ion channel of the plasma membrane that allows the flow of Cl^- ions out of the cell. CFTR is integrated into the ER membrane with 12 transmembrane domains. Proteins of the quality control system of the ER may recognize CFTR as being malfolded and may therefore guide it to the ERAD degradation machinery. CFTR is retrotranslocated, ubiquitinated and pulled out of the ER into the cytosol, where it is finally degraded by the proteasome.

The first link between a CFTR mutant that causes CF and ERAD was found in 1995 (Jensen, et al. 1995; Ward, et al. 1995) with the proteasomal dependent degradation of the chloride channel. At this time, however, the delivery mechanism of the protein to the proteasome remained completely unclear. Only after discovery of the

retrotranslocation of a soluble misfolded protein out of the ER for degradation, the mechanism became clear (Hiller, et al. 1996). Also, the close evolutionary relationship of all members of the ABC transporter superfamily was an excellent starting point to further improve the knowledge on the molecular cause of CF. As CFTR, a Pdr5 mutant protein of yeast with a luminal lesion called Pdr5* was found to be an ERAD substrate. Efficient degradation of Pdr5* was dependent on the E3 ubiquitin ligase Der3/Hrd1, the engaging component Hrd3 and the E2 ubiquitin conjugating enzymes Ubc6 and Ubc7 but not on the ER luminal chaperone Kar2 (Plemper, et al. 1998). Moreover, the CFTR gene was cloned into a yeast expression vector and pulse chase experiments revealed a turnover rate similar to that of CFTR in mammalian cells (Kiser, et al. 2001). All components of yeast ERAD needed for Pdr5* degradation were also found to be required for CFTR elimination (Kiser, et al. 2001; Sullivan, et al. 2003).

A. Gnann (Gnann, et al. 2004), a former member of the group of Prof. Dr. Wolf, searched for ERAD components required for CFTR degradation by performing pulse chase experiments of CFTR in yeast strains where the genes *DER3/HRD1* and *DOA10* were deleted, genes encoding for the ERAD-L and ERAD-M ubiquitin E3 ligase Der3/Hrd1 and the

ERAD-C E3 ubiquitin ligase Doa10, respectively. CFTR was degraded with the efficiency of a wild type cell. Only when the experiment was done in a strain with both E3 ligases absent, there was 75% stabilization of the substrate after 90 minutes of chase. He also studied how CFTR was extracted from the ER membrane and discovered that the AAA ATPase Cdc48 was required for this process. In addition, he found that the lectin with mannosidase activity, Mnl1/Htm1, which generates the glycan structure that triggers degradation, was a necessary component of ERAD of CFTR. Moreover, when he coexpressed the human homolog of Mnl1/Htm1, EDEM1 and CFTR in a *MNl1/HTM1* deletion strain, degradation was recovered.

Because in no case complete stabilization of CFTR was found in yeast one could imagine that there might be either a yet unknown ubiquitin E3 ligase to be found or human chaperones that if co-expressed would yield a higher stabilization of the substrate.

In addition, more complex combinations of ERAD components present in mammalian cells were found for different folding status of CFTR. For instance the Hsc70-CHIP-UBCH5 combination is required for CFTRΔF508 mutant protein degradation, whereas EDEM is only required for degradation of wild type CFTR (Farinha and Amaral 2005).

Later, an ER membrane-associated ubiquitin ligase complex was identified which contains the E3 RMA1, the E2 UBC6e, and Derlin-1 cooperating with the cytosolic Hsc70/CHIP E3 complex to triage CFTR and CFTRΔF508. Thus, the RMA1 and CHIP E3 ubiquitin ligases act sequentially in ER membrane and cytosol to monitor the folding status of CFTR and CFTRΔF508 (Younger, et al. 2006).

3.1.1.Systematic studies of CFTR degradation

Probably more information on CFTR turnover could be unraveled. At this point there were two ways to explore the issue: to test known ERAD components and to test potential ERAD components found in recent screens in yeast.

A systematic analysis of all yet known ERAD components would bring information on which components are needed and which are dispensable and by this define a degradation pathway for CFTR that could later be confirmed in human cell lines. In addition, novel screens would not only contribute to ERAD of CFTR but also to the discovery of new components in the ERAD pathway in general.

In the screens, two ERAD substrates with different defects were used: CTL* and Sec61-2L. Both substrates represent protein fusions with the Leu2 protein

(isopropylmalate dehydrogenase). CTL* has a CPY* moiety fused to a transmembrane domain from Pdr5 and the Leu2 moiety. Sec61-2L is a truncated version of Sec61 fused to Leu2. The tests are based on the leucine auxotrophic requirement of cells, carrying a *LEU2* deletion, for growth. In a cell with a deleted gene encoding for an ERAD component, the substrate is not degraded and the cell can live in media lacking leucine because the Leu2 protein fo the substratefusion can complement the *LEU2* deficiecy. The single gene deletion yeast collection from Euroscarf was used for testing. In figure 3.1 is the outcome of an ERAD component candidate found in recent screens by the Wolf Group depicted (Medicherla, et al. 2004; Kohlmann, et al. 2008).

The goal of this part of my thesis was to try to elucidate which known ERAD components were required for CFTR degradation and to examine new candidate ERAD components found in the recent screens. To accomplish this plan I followed CFTR turn over in cells treated with cycloheximide. Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis, blocking translational elongation. Yeast strains with and without the deletion of the candidate component's gene were transformed with the expression plasmid pCFTR-HA

(Zhang, et al. 2002b). In all degradation studies endogenous PGK, a stable protein under these conditions, was used as loading control. The strain expressing the empty vector was used as antibody specificity control.

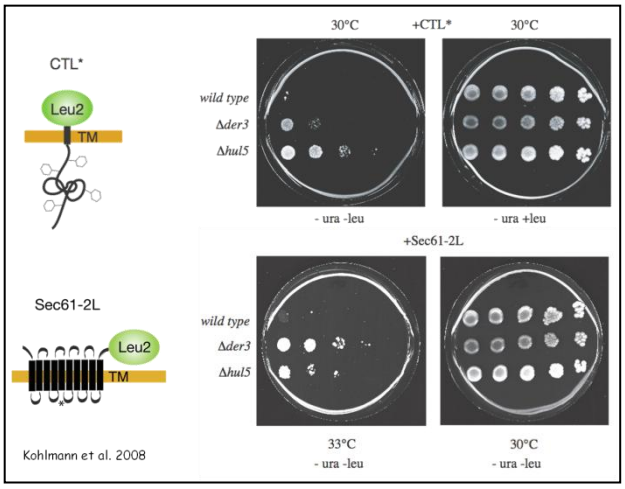

Figure 3.1: Growth test of strains with W303 genetic background to confirm that the deletion of *HUL5* stabilizes CTL* and Sec61-2L and therefore allows cell growth. All cell types were transformed with the corresponding expression plasmids, plated in serial dilution and growth until colonies emerged. Growth is recovered when cells cannot degrade the expressed ERAD substrate indicating that the deleted gene in this cell type encodes for a protein involved in ERAD (Kohlmann, et al. 2008).

3.1.1.1.UPS components of the cytosol

To examine the influence of the E4 ligase Hul5 on CFTR degradation, cycloheximide chases were performed, followed by immunodetection. Hul5, an E4 ubiquitin elongating enzyme (containing a Hect domain) transfers polyubiquitin chains to ubiquitylated substrates and is located on the

proteasome (Crosas, et al. 2006; Kohlmann, et al. 2008), the experiments showed a very mild stabilization of the CFTR protein in a *HUL5* deletion mutant(Fig. 3.2).

The next candidate examined was Ubp6. Ubp6 is an ubiquitin specific protease situated on the base of the 26S proteasome releasing free ubiquitin from branched polyubiquitin chains. Ubp6 is known to act in opposition to the polyubiquitin elongation activity of Hul5 (Crosas, et al. 2006). The experiment shows that there is no effect of *UBP6* deletion on CFTR. Degradation takes place as in wild type cells (Fig. 3.3).

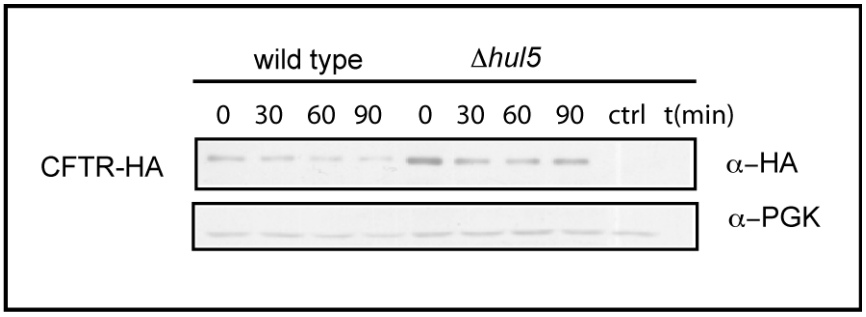

Figure 3.2: Influence Hul5 in the degradation of CFTR. Wild type and *HUL5* deleted cells were transformed with the expression plasmid pCFTR-HA. Protein expression was stopped using cycloheximide and samples were taken at different incubation times after addition. Proteins were immunodetected with antibodies against HA and PGK. Endogenous PGK expression was used as loading control and the empty expression vector as antibody specificity control.

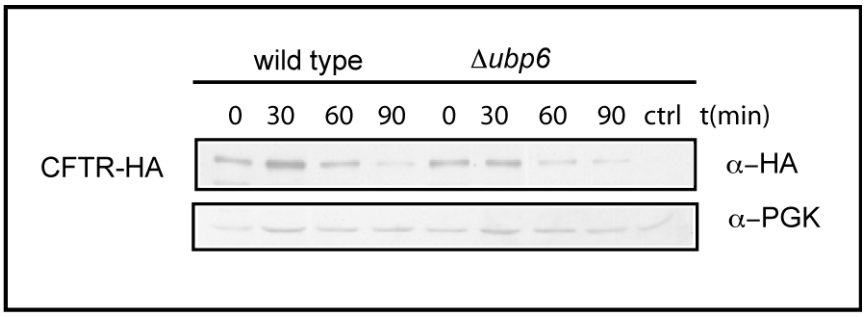

Figure 3.3: Ubp6 is not required for degradation of CFTR. Wild type and *UBP6* deleted cells were transformed with the expression plasmid pCFTR-HA. Protein expression was stopped using cycloheximide and were samples taken at different incubation times after addition. Proteins were immunodetected with antibodies against HA and PGK. Endogenous PGK expression was used as loading control and the empty expression vector as antibody specificity control.

The possibility of another E4 ubiquitin elongating factor acting in CFTR degradation could not be excluded. Ufd2 is a cofactor of the Cdc48 complex and has similar characteristics as Hul5 (Koegl, et al. 1999). When testing a deletion mutant of *UFD2*, after 90 minutes of chase, the amount of CFTR was practically the same in wild type and the mutant cells. One can conclude that Ufd2 had no detectable effect in CFTR turnover (Fig. 3.4).

The binding sites for Ufd2 and Ufd3 on Cdc48 overlap and depend critically on the conserved residue Y834, but are not identical (Ghislain, et al. 1996; Koegl, et al. 1999). As Cdc48 is required for CFTR degradation and the absence of Ufd2 had no evident effect, there is the possibility that the Cdc48 complex has a different cofactor assembly. Ufd3 function is linked to the maintenance of

ubiquitin homeostasis, but the mechanism by which it accomplishes this is unclear (Ghislain, et al. 1996). In an *ufd3-1* point mutant the levels of ubiquitin are diminished. The amount of CFTR in the Ufd3 mutant is higher than in wild type cells during the chase (Fig. 3.4). This observation could be due to the effect of Ufd3 on the ubiquitin pool. Nevertheless the degradation rate of CFTR seems to be the same in wild type and the *ufd3-1* mutant strains.

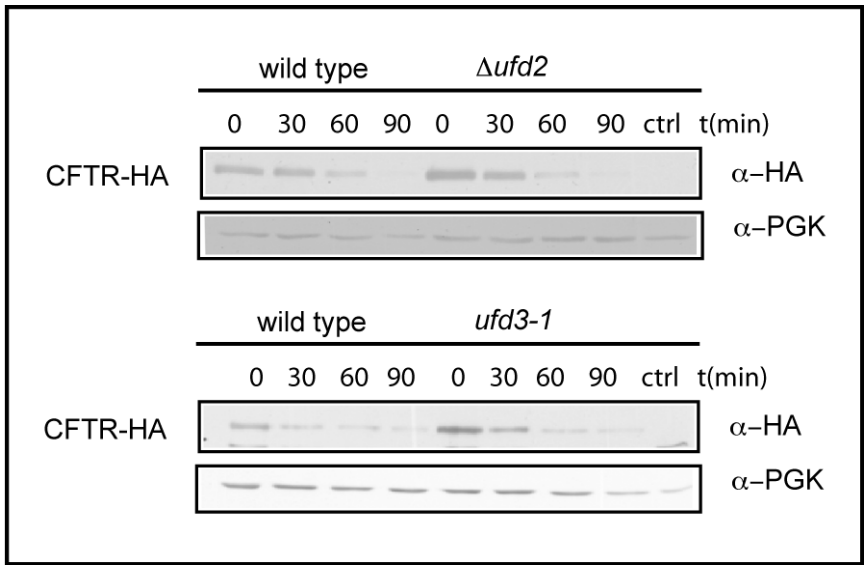

Figure 3.4: The Cdc48 co-factors Ufd2 and Ufd3 influence in CFTR degradation. Wilde type, *UFD2* and *UFD3* deleted cells were transformed with the expression plasmid pCFTR-HA. Protein expression was stopped using cycloheximide and samples were taken at 0, 30, 60 and 90 minutes after addition. Proteins were immunodetected with antibodies against HA and PGK. Endogenous PGK expression was used as loading control and the empty expression vector as antibody specificity control.

This set of results suggests that the action of the two known ubiquitin E3 ligases and the elongation factor Hul5

is sufficient to target CFTR for proteasomal degradation. However, it cannot be completely ruled out, that CFTR does not need extra ubiquitin chain processing for degradation.

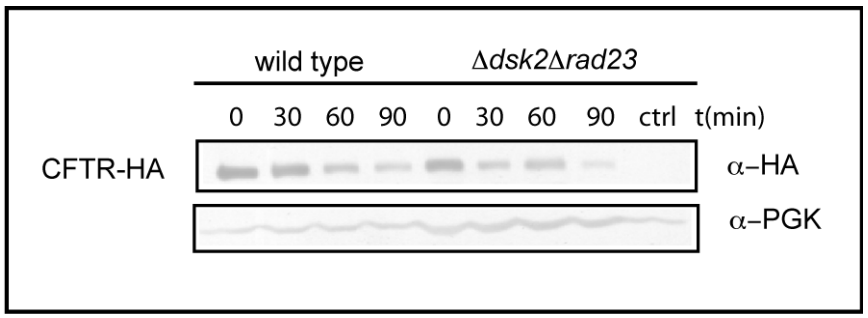

Figure 3.5: CFTR does not need Dsk2 and Rad23 to reach the proteasome. Wilde type, *DSK2 RAD23* double deleted cells were transformed with the expression plasmid pCFTR-HA. Protein expression was stopped using cycloheximide and samples were taken at 0, 30, 60 and 90 minutes after addition. Proteins were immunodetected with antibodies against HA and PGK. Endogenous PGK expression was used as loading control and the empty expression vector as antibody specificity control.

The next question to address was whether CFTR turnover relied on components that bring together ubiquitylated ERAD substrates and the proteasome. Dsk2 and Rad23 are two proteins that contain UBL and UBA domains and have been shown to act as ubiquitin receptors. UBL domains bind to the proteasome while UBA domains bind to ubiquitin chains linked to substrates (Raasi and Wolf 2007). The effect of these proteins is additive, therefore degradation of substrates is stronger delayed in double deletion mutants compared to single deletion mutants (Medicherla, et al. 2004). A change in the degradation profile of CFTR in the

double deletion mutant was not observable, leading to the conclusion that Dsk2 and Rad23 are not required for CFTR degradation (Fig 3.5).

3.1.1.2.Observations on possible vacuolar degradation of CFTR

At the point where no tested components of the cytosolic ERAD machinery had a strong influence on CFTR degradation, it was considered to disrupt the degradation function of the vacuole to see if this degradative organelle was also somehow involved in CFTR elimination. It had previously been shown that after 90 minutes of chase in the double deletion mutant *Δdoa10 Δder3* approximately 30% of CFTR was still degraded (Gnann, et al. 2004). Pep4/Pra1 is a vacuolar aspartyl protease required for the posttranslational precursor maturation of other vacuolar proteinases (Teichert, et al. 1989). A strain where the gene *PEP4/PRA1* was deleted in the *Δdoa10Δder3* background was generated. Comparing the double and triple deletion mutant strains, no additional stabilization of the substrate was observed (Fig. 3.6 please note that the 60 and 90 min bands in the wild type strain chase are not as wide as the bands in double and triple mutant strains

chases). This is a hint that misfolded CFTR is not dependent on vacuolar degradation which is in agreement with findings in human cell lines (Ward and Kopito 1994). In addition, since no 100% stabilization of the substrate is found in double E3 ligase deletion mutants, a third E3 ubiquitin ligase may also be involved in CFTR elimination.

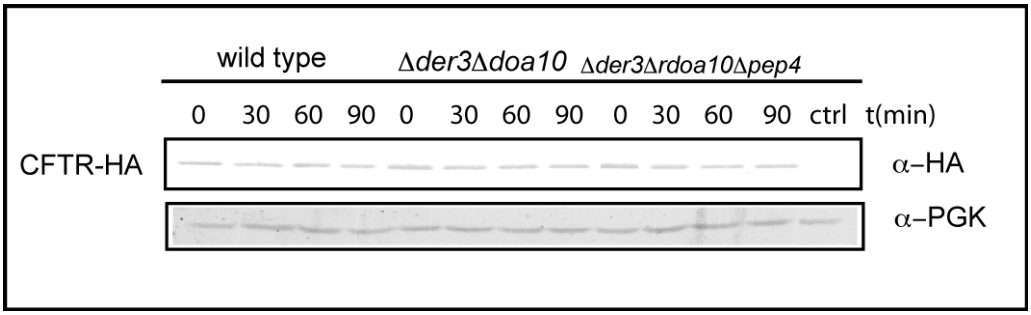

Figure 3.6: CFTR is most likely not degraded in the vacuole. Wild type, *DER3 DOA10 and DER3 DOA10 PEP4/PRA1* deleted cells were transformed with the expression plasmid pCFTR-HA. Protein expression was stopped using cycloheximide and samples were taken at 0, 30, 60 and 90 minutes after addition. Proteins were immunodetected with antibodies against HA and PGK. Endogenous PGK expression was used as loading control and the empty expression vector as antibody specificity control.

3.1.1.3.Quality control of CFTR folding in the ER

Misfolded proteins have a bipartite signal for degradation. One is the exposure of the amino acid sequence and the other is the glycan structure that they may have (Kanehara, et al. 2010). While proteins are folding, the glycan structures that they acquire as they enter the ER

are processed. Terminally misfolded proteins carry a processed glycan structure with 7 mannoses, a signal which is recognized by Yos9, an ER quality-control lectin and integral subunit of the Hrd/Der ligase complex (Gauss, et al. 2006; Aebi, et al. 2010; Yoshida and Tanaka 2010). The Hrd/Der ligase complex comprises the E3 ubiquitin ligase Der3/Hrd1 among other components. The next approach was to examine the influence of the two glycans linked to the second transmembrane domain of the CFTR chloride channel. As Mnl1/Htm1 was shown to be required for the glycan processing (Gnann, et al. 2004), it would thus be coherent if Yos9 recognizes this processed glycan of CFTR. In order to avoid the influence of the Doa10 E3 ubiquitin ligase, which can be reached without substrate recognition by Yos9, the gene *YOS9* was deleted in a *Δdoa10* background. In this case, ubiquitylation would depend solely on Der3/Hrd1 and the HRD complex. CFTR expression in the double deleted strain was slightly diminished (Fig. 3.7). This effect is controversial, as other substrates show slower degradation in the absence of Yos9. The effect of the single deletion of the gene *DOA10* in CFTR degradation could bring more information on the influence of Yos9.

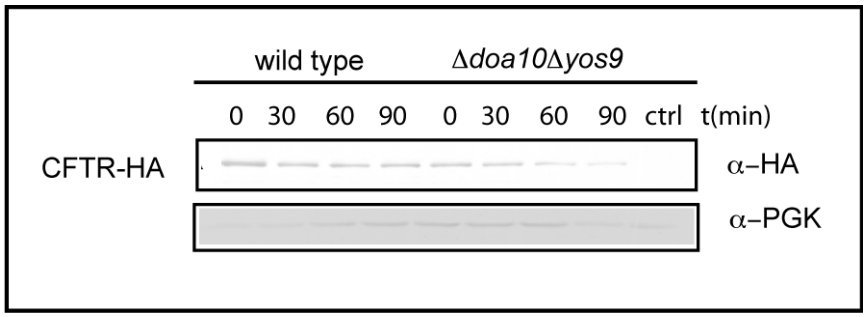

Figure 3.7: The double deletion of *DOA10* and *YOS9* slightly accelerates CFTR degradation. Wilde type *and DOA10 YOS9* deleted cells were transformed with the expression plasmid pCFTR-HA. Protein expression was stopped using cycloheximide and samples were taken at 0, 30, 60 and 90 minutes after addition. Proteins were immunodetected with antibodies against HA and PGK. Endogenous PGK expression was used as loading control and the empty expression vector as antibody specificity control.

3.1.1.4.Conclusion

Many components of ERAD promote CFTR degradation. Here, the idea of completing the set of components and describe the pathway for ERAD of CFTR was pursued. After examining the influence of several proteins, the absence of Hul5 was found to have a modest effect on CFTR degradation (Fig.3.2). According to the fact that ERAD of CFTR is very efficient (Zhang, et al. 2002a), Hul5 would add ubiquitin chains to ubiquitylated CFTR and by this speed up its degradation.

The lysosome (vacuole in yeast) had been shown to play a role only in degradation of well folded CFTR that had been functioning in the plasma membrane as chloride channel in human cells . Strains with disturbed vacuolar function

did not show a visible effect on CFTR degradation in yeast (Fig. 3.6). On one hand this agrees with the findings in human cells. On the other hand, one must take into account that very small differences are not detectable in cycloheximide chases and therefore it cannot be completely ruled out that the vacuole might have a slight effect on CFTR degradation.

A function of the lectin Yos9 was anticipated in the turnover of CFTR. Yos9 recognizes Man7 glycan structures processed by Mnl1/Htm1 on misfolded proteins in the ER and delivers them to destruction. The mannosidase Mnl1/Htm1 promotes degradation of CFTR in yeast and the deletion of *MNL1/HTM1* can be healed if EDEM1 (human homologue of Mnl1/Htm1) is coexpressed with CFTR(Gnann, et al. 2004). When tested, the deletion of *YOS9* showed faster degradation (Fig. 3.7). Thus, contrary of what it was expected, Yos9 slows down CFTR degradation. An explanation for this could be that the misfolding of the peptide sequence is faster recognizable than the processed glycan structure. In the absence of Yos9, the recognition process is accelerated and the substrate faster degraded.

As a general observation, a more sensitive technique to quantify changes in the amount of CFTR is needed, for example radio labeling pulse-chase experiments. This type

of experiment was tried and abandoned after several trials since no suitable conditions for satisfactory results were found. Furthermore, it would be interesting to test whether human HUWE1 and Smurf2 could rescue the *Δhul5* phenotype in yeast. Human HUWE1 and Smurf2 are Hect E3 ubiquitin ligases found to have certain homology to Hul5 (Protein Data Bank, PDB, http://www.wwpdb.org/). The homology analysis was performed by comparing the Hul5 yeast protein sequence, against protein sequences in The Research Collaboratory for Structural Bioinformatics (RCSB) (http://home.rcsb.org/), using the Smith-Waterman analysis program. However, no studies have been done in human cell lines with respect to CFTR. In the case of Yos9, co-expressing human OS-9 would show if this lectin is needed and whether they have similar efficiencies to deliver CFTR to the degradation machinery. The final proof to get to know if HUWE1, Smurf2 or OS-9 are involved in CFTRΔF508 degradation should be assayed in human cell lines.

Because of handling complexity reasons of the human CFTR protein in yeast cells and the unsatisfactory previous results I decided to explore ERAD from another point of view.

- **Part II**

3.2.Non-glycosylated and glycosylated substrates in ERAD

3.2.1.Degradation studies on non-glycosylated CPY*0000

It has 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 sufficient to trigger efficient degradation (Kostova and Wolf 2005). However, the unglycosylated version of CPY* (CPY*0000) is still degraded but at a considerably reduced rate. The goal of this project was to elucidate the degradation mechanism of this misfolded unglycosylated CPY* protein.

3.2.1.1.The vacuole is not required for CPY*0000 degradation.

To rule out autophagy as the pathway of degradation(Yorimitsu and Klionsky 2005b), the degradation profile of CPY*0000 was observed 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. When tested, CPY*0000 turnover in *Δpep4/pra1 Δprb1* double mutants appeared to be the same as in wild type cells proving that the vacuole is not the locus of CPY*0000 degradation (Fig. 3.8).

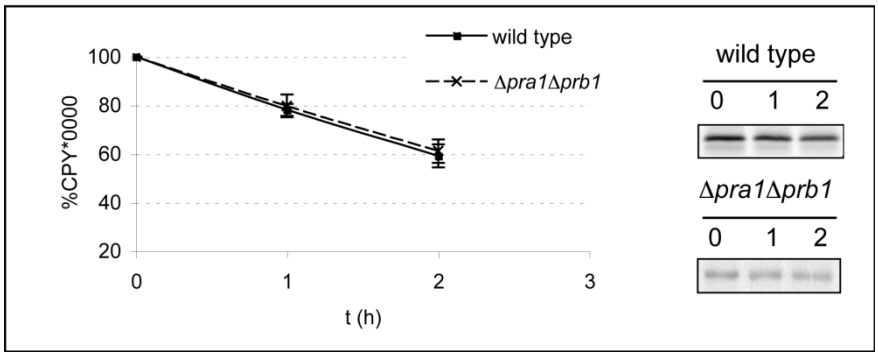

Figure 3.8: The vacuole is not required for CPY*0000 degradation. Cells with defective vacuolar function were transformed with a CPY*0000 expression plasmid. Data represent the mean of 4 independent experiments ±S.D with a p<0,05.

3.2.1.2.CPY*0000 is an ERAD-L substrate.

To confirm whether CPY*0000 is a *bona fide* ERAD substrate the involvement of the main components of the ubiquitin proteasome system (UPS) were tested in its elimination. The degradation of CPY*0000 was followed in cells expressing defective proteasomes (Hilt, et al. 1993), as well as cells deleted in genes encoding for different E3

ubiquitin ligases.

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 was observed. Thus, the proteasome is the proteolytic machinery for CPY*0000 elimination (Fig. 3.9A).

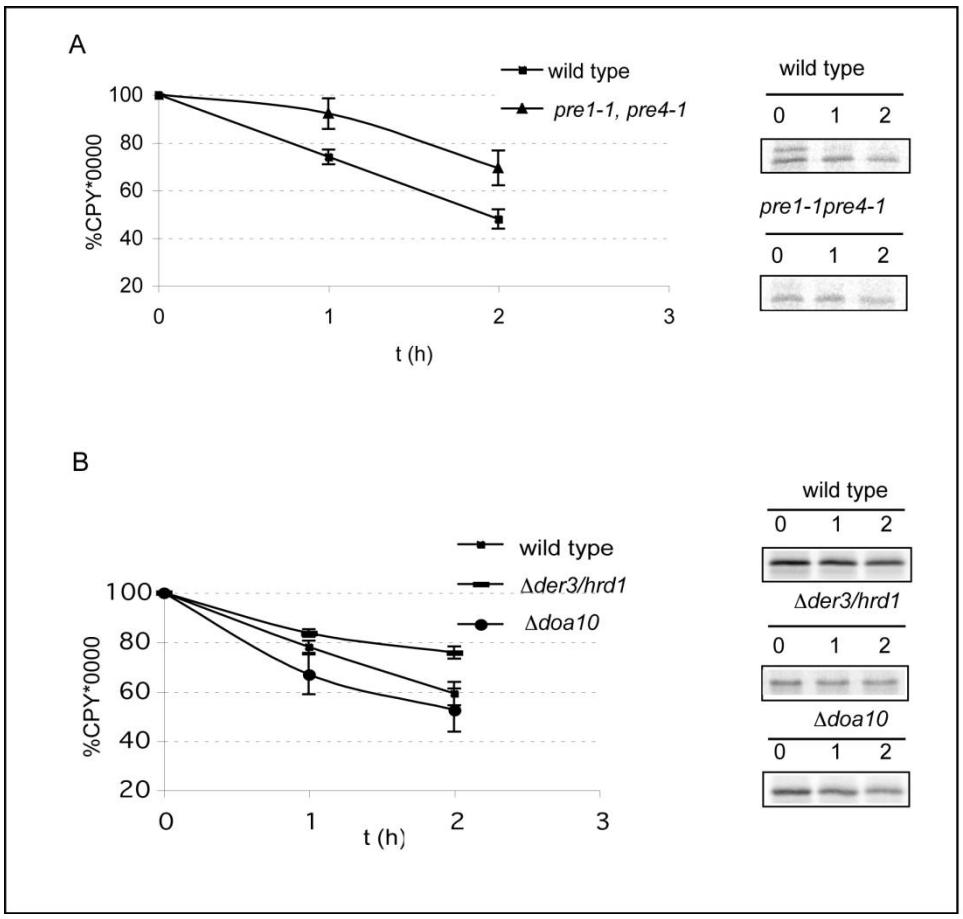

Figure 3.9: CPY*0000 is an ERAD-L substrate. (A) Cells with defective proteasomes (*pre1-1, pre4-1*) were transformed with a CPY*0000 expression plasmid. (B) *DER3/HRD1* deleted cells and *DOA10* deleted cells were transformed with a CPY*0000 expression plasmid showing a stabilization of the substrate only in the *DER3/HRD1* mutant. Data represent the mean of 3 (wild type and proteasome mutant) as well as 4 (wild type, *Δder3/hrd1* and *Δdoa10*) independent experiments ±S.D with a $p<0,05$.

Ubiquitylation of glycosylated CPY* is carried out by the E3 ligase Der3/Hrd1 (Bordallo, et al. 1998). Therefore, the involvement of this enzyme in polyubiquitylation of non-glycosylated CPY*0000 was tested. As can be seen in Fig. 3.9B, degradation of the misfolded non-glycosylated CPY*0000 is slowed down in *DER3/HRD1* deleted cells, indicating that the ligase is required for elimination.

Involvement of the second E3 ligase of the ER, Doa10 was also tested, however with no effect in CPY*0000 turn over (Figure 3.9). Clearly, CPY*0000 is an ERAD-L substrate.

3.2.1.3.ERAD-L requirements for CPY*0000

3.2.1.3.1.Lectins of the ER and quality control of CPY*0000

A priori it was not expected that the degradation of non-glycosylated CPY*0000 relied on the lectin-like proteins Mnl1/Htm1 and Yos9. These proteins interact with the glycan residues of glycosylated CPY* (Aebi, et al. 2010). To confirm this consideration the degradation behavior of CPY*0000 was tested in mutant strains where these genes were deleted.

3.2.1.3.1.1.The mannosidase Mnl1/Htm1 has no influence on CPY*0000 degradation

The Mnl1/Htm1 trimming function of α1,2 mannoses is the only found so far (Quan, et al. 2008; Clerc, et al. 2009). As expected, the degradation of CPY*0000 in *MNL1/HTM1* deleted cells remained as wild type (Fig. 3.10). The mannosidase cannot exert its function on a substrate with no glycans.

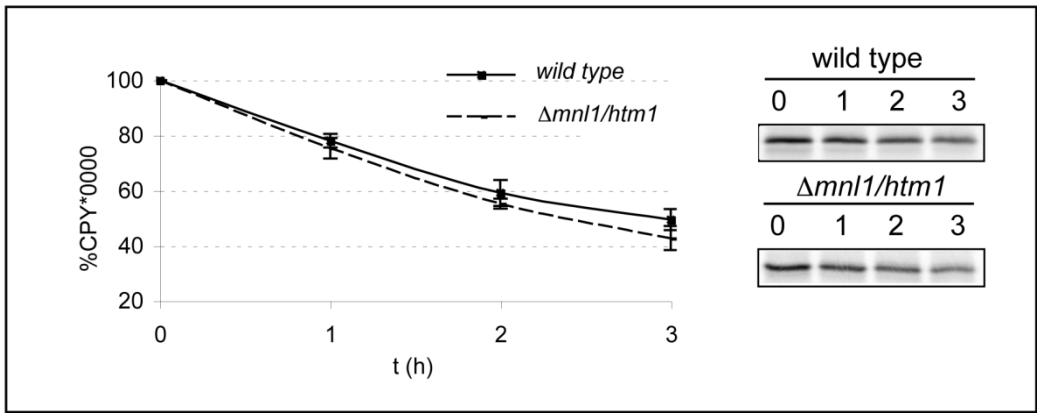

Figure 3.10: CPY*0000 degradation is not influenced by the absence of the mannosidase Mnl1/Htm1. *MNL1/HTM1* deleted cells were transformed with a CPY*0000 expression plasmid. Quantitative pulse-chase analyses were performed. Data represent the mean of 4 independent experiments ±S.D with a $p<0,05$.

3.2.1.3.1.2.The degradation efficiency of CPY*0000 in *YOS9* deleted cells is increased and is independent of the MRH domain.

Yos9 plays a crucial role in recognition in the ERAD of glycosylated substrates. It is thought to be the gatekeeper for delivery of processed glycosylated substrates to degradation (Buschhorn, et al. 2004; Bhamidipati, et al. 2005; Quan, et al. 2008). Surprisingly, the degradation efficiency of CPY*0000 in *YOS9* deleted cells was enhanced (Fig. 3.12). This indicates that Yos9 prevents degradation of unglycosylated CPY*0000, whereas for glycosylated CPY* it promotes degradation.

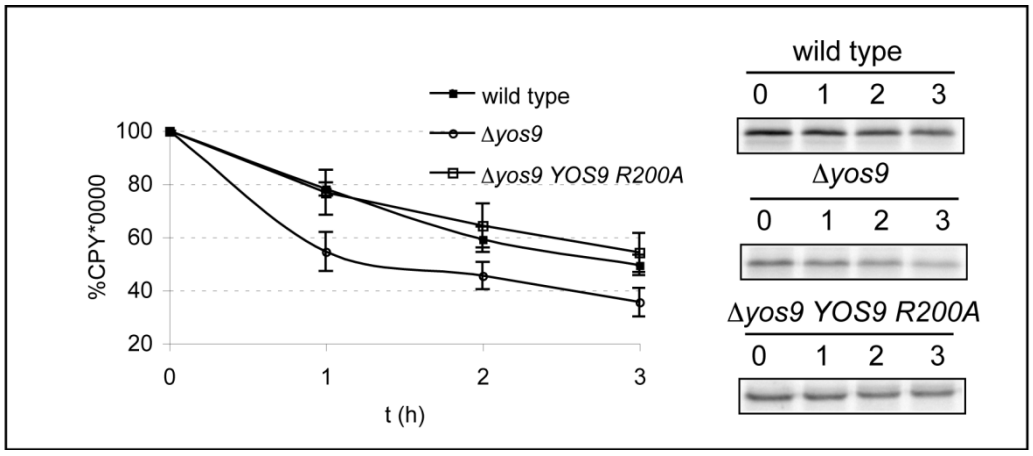

Figure 3.11: The degradation efficiency of CPY*0000 in *YOS9* deleted cells is increased and is independent of the MRH domain. Wild type and *Δyos9* trains were transformed with CPY*0000 or CPY*0000 and Yos9R200A-Flag expression plasmids respectively and pulse-chase analyses performed. Data represent the mean of 4 (wild type), 6 (Yos9R200A) and 7 (*Δyos9*) independent experiments ±S.D with a $p<0,05$.

At this point, it was obvious that Yos9 could also be a recognition component of the ER quality control for terminally misfolded non-glycosylated proteins. Yos9 recognizes glycosylated substrates that carry a Man_7 glycan structures via the Mannose 6-phosphate receptor homology domain(MRH) (Hosokawa, et al. 2010). Therefore it was checked, if the MRH domain of Yos9 was responsible for the fact that the presence of Yos9 diminishes CPY*0000 degradations. Pulse-chase experiments were done comparing the effects of the expression of wild type Yos9, the MRH mutant Yos9 (R200A) as well as the absence of Yos9 on CPY*0000 degradation. The results show that Yos9 recognizes CPY*0000 independently of its MRH domain (Fig. 3.11). It had recently been shown that Yos9 mutated the MRH domain still physically interacts with CPY*0000 (Bhamidipati, et al. 2005; Denic, et al. 2006). Taken together, this indicates that the glycan-recognition domain of Yos9 is not involved in ERAD of CPY*0000. This correlates with our data and suggests a checkpoint function of Yos9 for unglycosylated substrates in addition to the gatekeeper function of Yos9 for glycosylated substrates.

3.2.1.3.2.The degradation efficiency of CPY*0000 decreases in *DER1* and *USA1* deleted cells

Next, it was examined if the ER membrane protein Der1, required for elimination of soluble ERAD-L substrates and Usa1, which links Der1 to the Hrd1/Der3 ligase, are part of the degradation machinery of CPY*0000 (Hitt and Wolf 2004; Horn, et al. 2009). Here it is shown how the deletion of these genes leads to a similar, but rather marginal reduction in the degradation kinetics of CPY*0000 (Fig. 3.12A).

The finding that absence of Yos9 accelerates degradation of CPY*0000 led to a repetition of the experiments on the influence of the ERAD-L components Der1 and Usa1 in a *YOS9* deletion background.

The degradation kinetics of CPY*0000 is considerably reduced in *DER1* and *USA1* deleted strains when *YOS9* is absent (Fig. 3.12B). This clearly shows the need of Der1 and Usa1 for the degradation of non-glycosylated CPY*0000

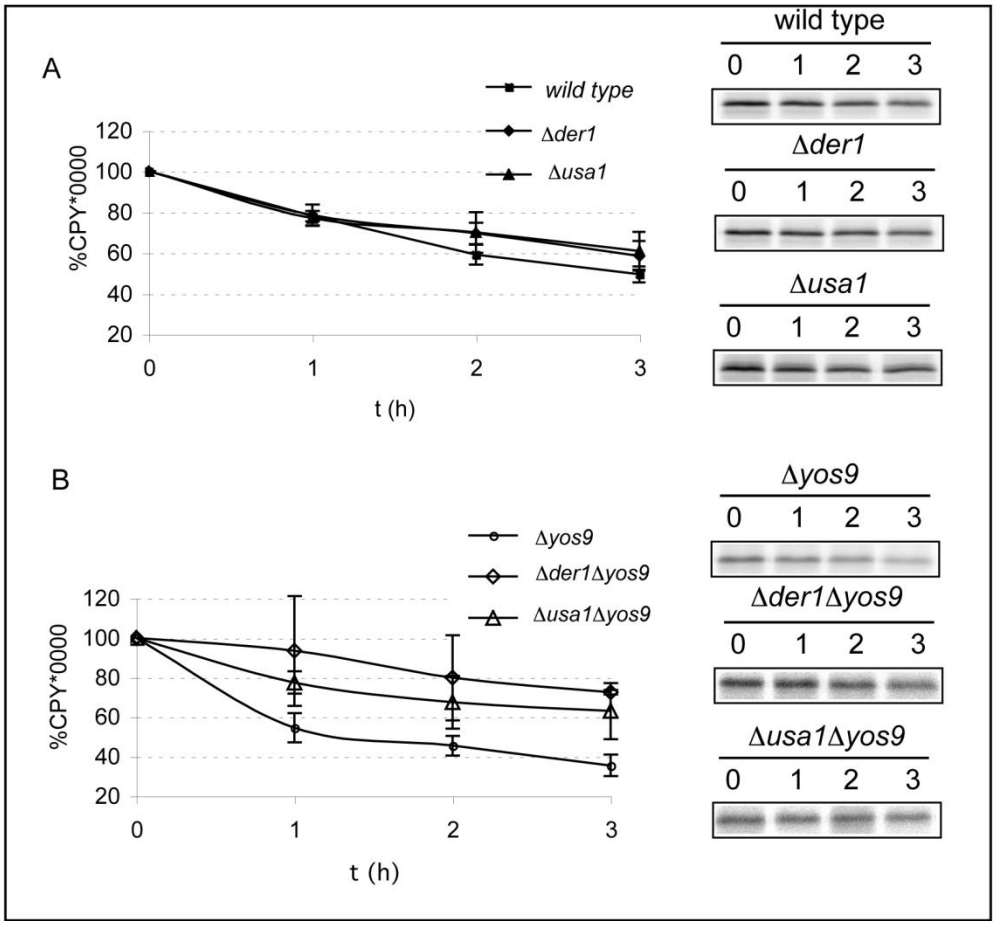

Figure 3.12: The degradation efficiency of CPY*0000 decreases in *DER1* and *USA1* deleted cells. All indicated strains were transformed with a CPY*0000 expression plasmid. (A) CPY*0000 degradation in wild type, *Δder1* and *Δusa1* strains. (B) CPY*0000 degradation in *Δyos9* (control), *Δder1Δyos9 and Δusa1 Δyos9* strains. Data represent the mean of 2 (*Δder1Δyos9 and Δusa1Δyos9*), 4 (wild type and *Δusa1*) and 7 (*Δder1* strain and *Δyos9* control) independent experiments ±S.D with a p<0,05.

3.2.2.Mnl2, a novel component of the ER quality control of misfolded glycoproteins.

The putative protein with the open reading frame YLR057W was identified in the *Saccharomyces cerevisiae* Genome Database (SGD)(http://www.yeastgenome.org/) and SUPERFAMILY Database (http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html) (Gough, et al. 2001) in a search for proteins that contained domains or motifs in common with Mannosidase 1 (Mns1) and Mannosidase-like protein 1 (Mnl1/Htm1) (Fig. 1). Therefore, we renamed YLR057W as Mnl2 for mannosidase like protein 2 (Fig. 3.13). Mnl2, as Mns1 and Mnl1/Htm1, is predicted to have the α/α toroid fold of the seven-hairpin glycosidases superfamily. This structure comprises up to seven α-hairpins arranged in closed circular array. Glycoside hydrolase family 47 comprises enzymes with only one known activity; α-mannosidase (SCOPE database, http://scop.mrc-lmb.cam.ac.uk). These enzymes hydrolyze terminal 1,2-α-mannose residues in the Man9 glycan structure in a calcium-dependent manner. The mannose residues are trimmed away to produce, first, Man8 and then Man5 glycan structures (http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html).

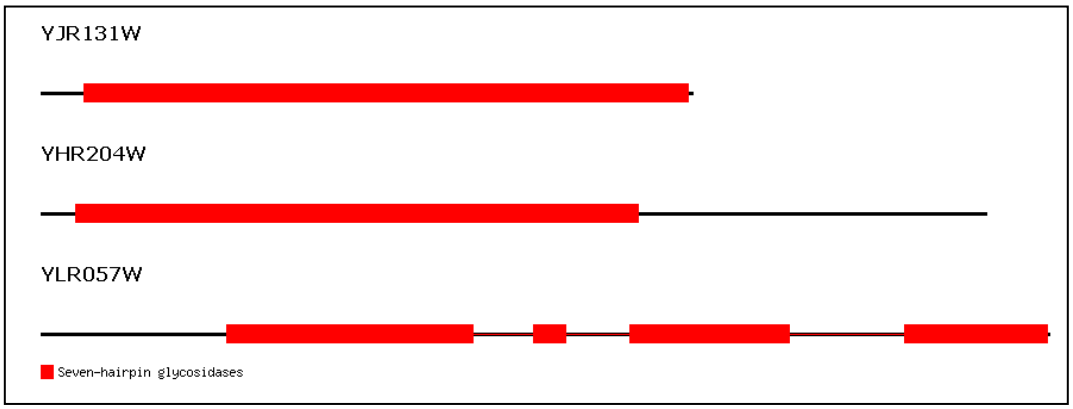

Figure 3.13: Proposed Seven-hairpin glycosidases superfamily assignments to *Saccharomyces cerevisiae* SGD. The Glycoside hydrolase family 47 comprises enzymes with only one known activity; α1-2 mannosidase activity. Only three proteins with similar domain architecture (highlighted in red bars) were found in the *S. cerevisiae* search submitted to the SUPERFAMILY database, a database of structural and functional annotation for all proteins and genomes. The ORFs YJR131W, YHR204W and YLR057W correspond to the proteins Mns1, Mnl1/Htm1 and Mnl2.

In mammalian cells, trimming of the glycan tree of misfolded glycosylated proteins in the ER is done down to Man7, Man6 and Man5 structures. These structures are recognized as degradation signals by OS-9 (Satoh, et al. 2010). In yeast, Mns1 and Mnl1/Htm1 play a crucial role in the quality control of misfolded proteins in the ER by generating the Man7 structure that targets a protein for degradation (Fig 3.14)(Aebi, et al. 2010). In yeast Man6 and Man5 glycan structures bound to proteins have been found but no enzyme has been identified to be responsible for the generation of them (Quan, et al. 2008). The

hypothesis that Mnl2 is part of the glycan trimming process that would affect ERAD had to be proven.

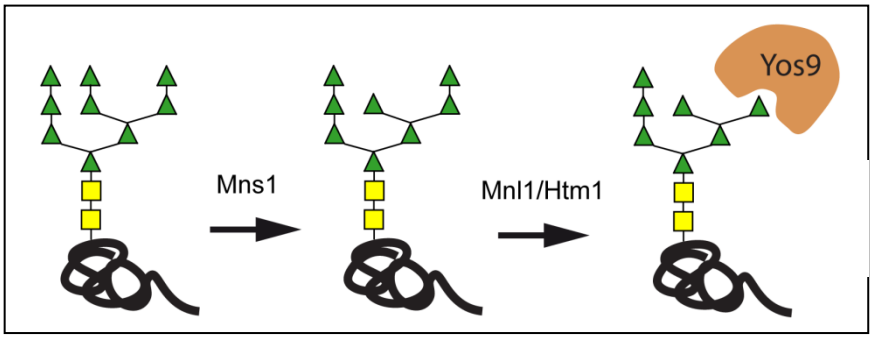

Figure 3.14: The mannosidase cascade. ER mannosidase I (Mns1) cleaves the middle (B branch) α1,2-linked mannose to generate the $Man_8GlcNAc_2$ glycan structure, common among folded proteins leaving the ER. Finally, Mnl1/Htm1 cleaves the terminal mannose residue from the C-branch to yield the terminal α1,6-mannose residue as the Yos9 ligand.

3.2.2.1. Involvement of Mnl2 in ERAD

First, it was checked whether a *MNL2* deletion had an effect on the degradation profile of CPY*. This was not the case: CPY* was degraded as efficiently in *Δmnl2* mutant cells as in wild type cells (Fig. 3.16). Nevertheless, all combinations of deleted genes encoding mannosidases were generated and tested. The deletion of *MNS1* in a *Δmnl2 background* had the same effect as the *MNS1* deletion alone with approximately 20% stabilization of CPY* after 90 minutes (Fig. 3.15).

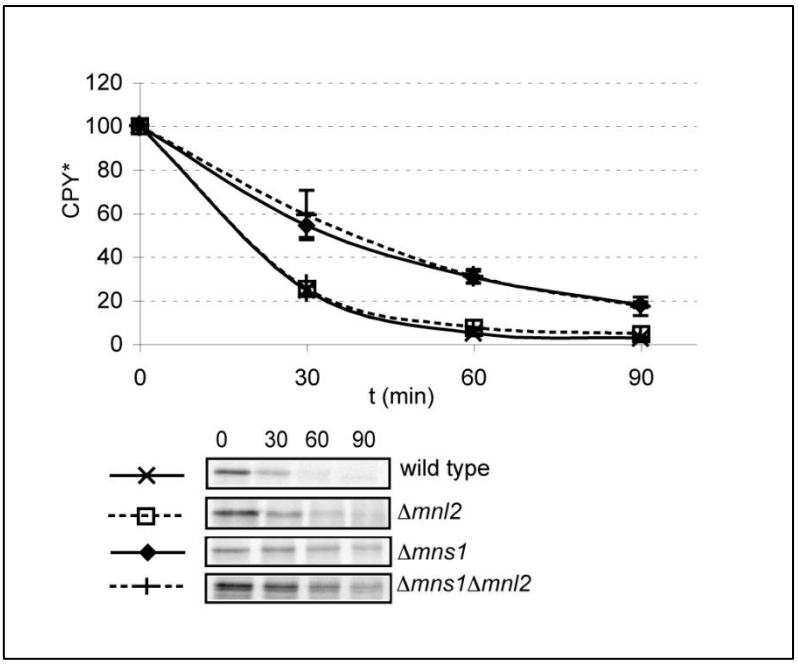

Figure 3.15: Deletion of *MNL2* does not show an effect on its own or in combination with a deletion of *MNS1*. Endogenously expressed CPY* turnover was followed by pulse chase analysis of the different strains. Data represent the mean of 3 (*Δmnl2* and *Δmns1 Δmnl2*) to 4 (wild type and *Δmns1*) independent experiments ±S.D with a $p<0,05$.

This indicates that either Mnl2 has no function in ERAD or that it works in the same pathway as Mns1.

Since Mns1 and Mnl1/Htm1 act sequentially in the same pathway, trimming a mannose in the B branch (Mns1) and a mannose in the C branch (Mnl1/Htm1) (Fig. 1.2) (Clerc, et al. 2009), the deletion of *MNL1/HTM1* in the *Δmnl2 background* was analyzed. A considerably stronger stabilization of CPY* was detected in the *Δmns1 Δmnl2* double mutants as compared to the single *MNL1/HTM1* deletion. This result clearly proves that Mnl2 has an effect in the CPY* degradation process. In addition, the

triple mannosidase deletion strain Δ*mns1* Δ*mnl1/htm1* Δ*mnl2* was tested and the degradation kinetics of CPY* observed.

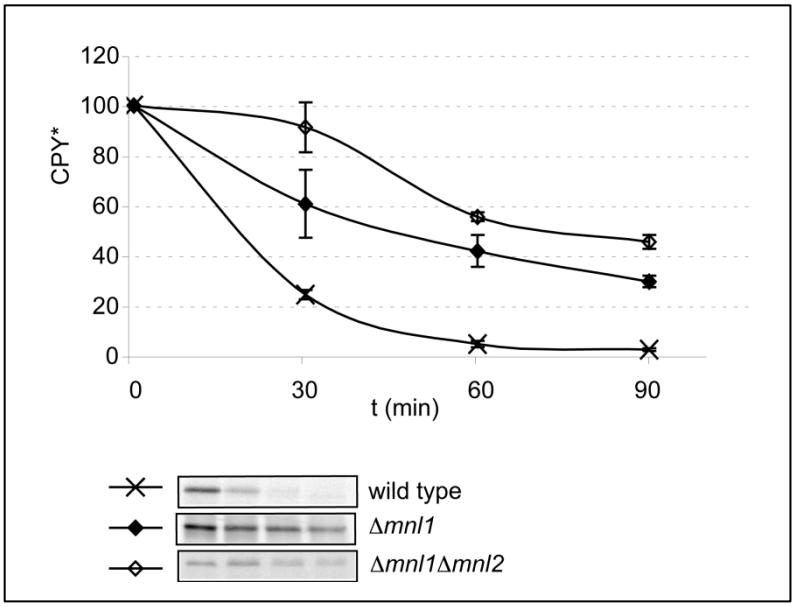

Figure 3.16: Mnl2 plays a role in glycoprotein quality control in the ER. Endogenously expressed CPY* turnover was followed by pulse chase analysis of the different strains. Data represent the means of 3 (Δ*mnl1/htm1 and Δmnl1/htm1 Δmnl2*) and 4 (wild type) independent experiments ±S.D with a p<0,05.

No statistically stronger stabilization of CPY* in the double deletion strain Δ*mns1*Δ*mnl1/htm1* could be seen (Fig. 3.17).

This stabilization of the substrate upon deletion of *MNL2* led to the idea that Mnl1/Htm1 and Mnl2 may act sequentially or in parallel generating different degradation signals. These signals would have different efficiencies to qualify a protein for degradation as suggested (Quan, et al. 2008; Pearse and Hebert 2010). It may also be possible that Mnl2 could partially play the

role of Mnl1/Htm1 in its absence. These experiments confirm the hypothesis that Mnl2 plays a role in glycoprotein ERAD.

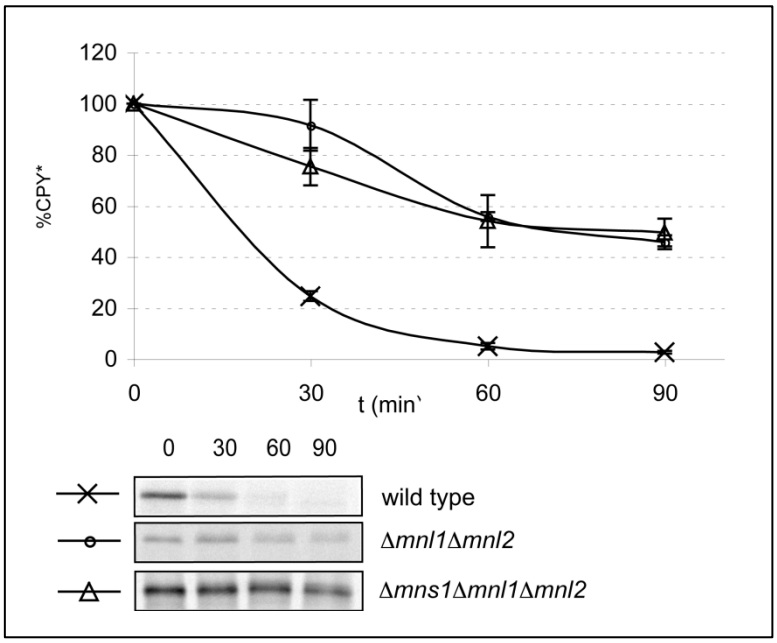

Figure 3.17: Mnl2 plays a role in glycoprotein quality control in the ER. Endogenously expressed CPY* turnover was followed by pulse chase analysis of the different strains. Data represent the means of 3 (*Δmnl1/htm1 Δmnl2) 7 and Δmns1 Δmnl1/htm1 Δmnl2*) and 4 (wild type) independent experiments ±S.D with a $p<0,05$.

3.2.2.2.Mnl2 is involved in glycan processing

The ER lectin Yos9 is shown to recognize Man_7 glycan structures linked to misfolded proteins and act downstream from the mannosidases Mns1 and Mnl1/Htm1 (Aebi, et al. 2010; Pearse and Hebert 2010). The next step was to analyze the relationship between Mnl2 and Yos9. In the absence of Mnl1/Htm1 and Mnl2, the glycan structure that substrates present to Yos9 has no α1,6 mannose linkage exposed and is

therefore not a degradation signal. Thus it cannot trigger recruitment of the glycosylated protein to the ERAD machinery. If this is true, Yos9 cannot act and its absence will not affect the degradation of CPY* when the glycan has not been processed.

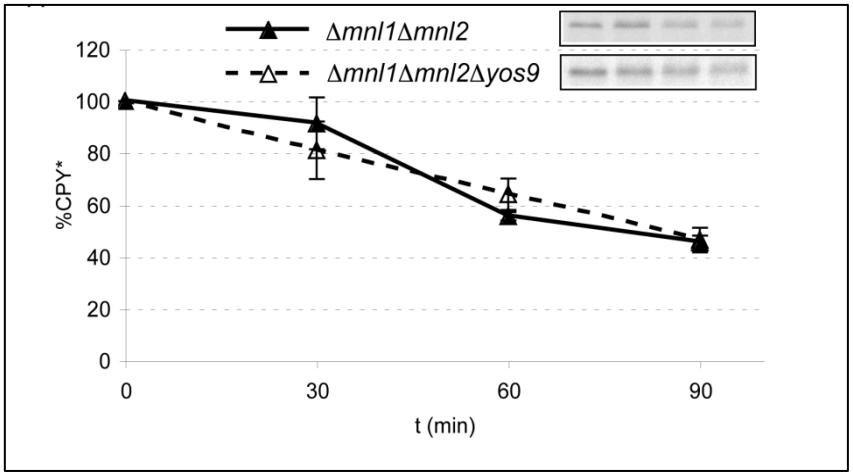

Figure 3.18: Yos9 cannot recognize the glycan structure of the substrate if mannosidases are absent. Endogenously expressed CPY* turnover was followed by pulse chase analysis of the different strains. Data represent the means of 3 independent experiments ±S.D with a $p<0,05$.

Therefore the triple deletion strain Δ*mnl1/htm1* Δ*mnl2* Δ*yos9* was generated. As expected, no higher stabilization of CPY* was reached in the Δ*mnl1/htm1* Δ*mnl2* Δ*yos9* deletion mutant comparing to the mutant strain that contained YOS9 (Fig. 3.18).

Clearly, when the recognition signal cannot be produced due to the absence of the mannosidases Mnl1/Htm1 and Mnl2, lack of Yos9 cannot enhance stabilization of CPY*.

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 Degradation of CPY*0000 was not affected by the absence of Mnl1 or Mnl2 (Fig. 3.19). This excludes Mnl2 as being a chaperone acting on the protein part of CPY*.

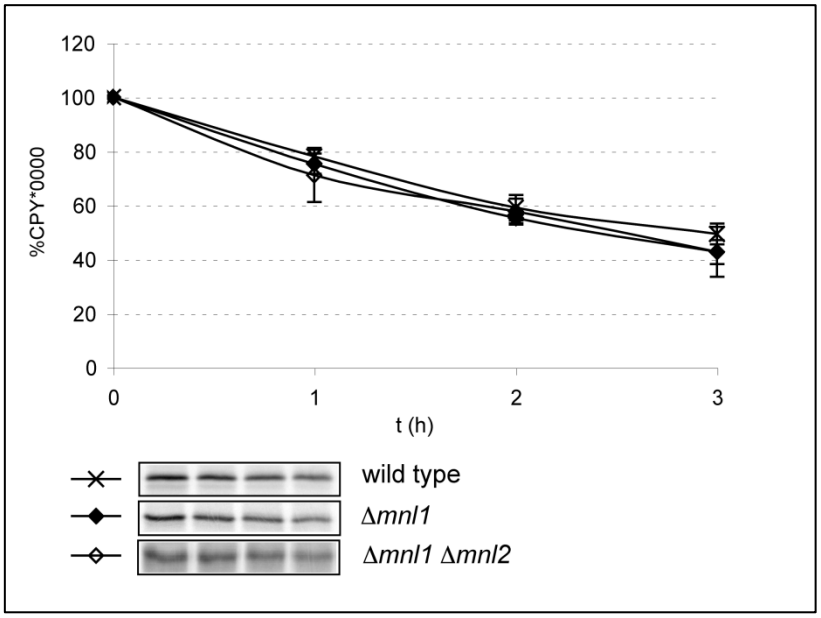

Figure 3.19: Unglycosylated CPY*0000 is not affected by the absence of mannosidases. Cells were transformed with a CPY*0000 expression plasmid. Data represent the means of 3 independent experiments ±S.D with a $p<0,05$.

4.Discussion

The main part of this thesis is concerned with the questions of how non-glycosylated and glycosylated substrates are recognized and degraded by ERAD, described in the Part II of the results. The CFTR project described in the Part I of the results was left as an alternative project. Finally, I made a break in the continuity of the CFTR project, as the successful results of the Glycan project needed fulltime attention.

4.1.New function of the lectin Yos9

4.1.1.CPY*0000 is an ERAD-L substrate

This work validates the non-glycosylated misfolded protein CPY*0000 as a *bona fide* ERAD-L substrate. Until now hypoglycosylated variants of CPY* were thought not to be recognized by ERAD (Denic, et al. 2006; Hirsch, et al. 2009). The fact that CPY*0000 directly interacted with the ERAD components Hrd3 and Yos9 and indirectly interacted with Der1 and Usa1 (Denic, et al. 2006) led to the idea that this may not be the case and gave the impulse to prove this hypothesis.

To exclude other possible degradation pathways than ERAD CPY*0000 turnover was examined in a mutant strain with a compromised vacuolar degradation function (Fig. 3.8). Wild type cells and vacuolar proteinase mutant cells degraded CPY*0000 with same efficiency. Thus the vacuole is not the site of degradation of CPY*0000 and thus, not due to autophagy.

If CPY*0000 were an ERAD substrate, the proteasome is the machine that would degrade the misfolded protein. To confirm this possibility CPY*0000 turnover was analyzed in a proteasomal mutant strain. Null proteasome cells are not viable. Therefore, proteasomal mutants which have a residual catalytic activity that allows the cells to survive were used. The comparison between mutant and wild type cells confirmed the proteasome as the site of destruction of CPY*0000 (Fig 3.9A).

In yeast, Der3/Hrd1 and Doa10 are the only E3 ubiquitin ligases of the ER known so far. Both share several ERAD partners but they are ligases involved in different ERAD pathways. Der3/Hrd1 clients have lesions in the lumen of the ER (ERAD-L), while Doa10 clients have lesions in the cytosol region (ERAD-C and) (Vembar and Brodsky 2008). According to this, it was expected that CPY*0000, a luminal substrate, would require Der3/Hrd1 for ubiquitylation, like

CPY* (Bordallo, et al. 1998). Both E3 ligases were tested for their involvement in degradation. Whereas Doa10 showed no influence on CPY*0000 degradation (Figure 3.10), there was an obvious stabilization of the substrate in cells deprived of Der3/Hrd1 (Fig. 3.9B).

To this end one can conclude that CPY*0000 is an ERAD-L substrate because its efficient degradation depends on the proteasome and the E3 ubiquitin ligase Der3/Hrd1.

4.1.2.ERAD-L requirements for CPY*0000

Consequently, other components of the ERAD-L pathway were checked. The lectins of the ER that generate the glycan degradation signal and recognize it are Mnl1/Htm1 and Yos9, respectively. To prove that the unglycosylated substrate CPY*0000 does not require these ER lectins, mutants devoid of their activities were examined in their capacity to degrade CPY*0000.

As predicted, the deletion of the *MNL1/HTM1* gene had no effect on CPY*0000 degradation (Fig. 3.10). The mannosidase cannot apply its function if the substrate does not have a glycan chain.

The lectin Yos9 was up to now believed to have only a gatekeeper function in ERAD of glycosylated substrates.

Folding intermediates as well as misfolded proteins have a high affinity to Hrd3. After binding to Hrd3, glycoproteins are scanned by Yos9, which is directly interacting with Hrd3 (Gauss, et al. 2006). If they expose the α-1,6 mannose linkage on the C branch, the glycan degradation signal, they will be recognized by Yos9 and delivered to degradation (Quan, et al. 2008; Clerc, et al. 2009; Hosokawa, et al. 2010). Intriguingly, Hrd3 and Yos9 have been found to directly interact with CPY*0000 (Denic, et al. 2006). In addition, the affinity of Hrd3 to CPY*0000 is higher than to other hypoglycosylated variants of CPY* (Gauss, et al. 2006). This is thought to be due to the increasing hydrophobicity with decreasing number of glycosylations of the substrate. Hydrophobic patches are a sign of misfolded proteins and therefore Hrd3 would bind faster to unglycosylated proteins.

To verify that Yos9 was not involved in the degradation of CPY*0000, wild type cells and *YOS9* deleted cells were compared. Surprisingly, the degradation efficiency of the substrate was enhanced in the *YOS9* deleted cells (Fig. 3.11). Evidently, Yos9 does not only interact with CPY*0000 but also holds onto it, slowing down its delivery to the degradation machinery.

It has been shown that the MRH domain in Yos9 is

responsible for scanning glycans on glycoproteins (Hosokawa, et al. 2010; Mikami, et al. 2010; Satoh, et al. 2010). Additionally, the mutation R200A in *YOS9* has been shown not to be required for binding of CPY*0000 to Yos9. The hypothesis that abolishing MRH function by the mutation R200A in Yos9 would have the same effect as wild type Yos9 on the degradation of CPY*0000 was explored. Indeed, abolishing the MRH function in Yos9 by the mutation R200A in cells, led to the same degradation kinetics of CPY*0000 as in Yos9 wild type cells (Fig. 3.11).

Combining these results the following model is suggested: Yos9 may bind any misfolded protein. After a quick check by the MRH domain it delivers the properly processed 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. In the absence of Yos9, the unglycosylated substrate is not retained by the protein and is quickly available for degradation. The MRH domain of Yos9 which provides the gate keeping function for glycosubstrates is not required for CPY*0000, due to the absence of a glycan, confirming a second function for this ERAD component. Yos9 plays a tuning role in ERAD, delivering glycosylated substrates to

ERAD in an MRH dependent manner and delivering non glycosylated CPY*0000 to ERAD in an MRH independent manner.

Finally, the influence of Der1 and Usa1 on CPY*0000 was observed. The fact that they indirectly interacted with CPY*0000 (cite) and that they are required for ERAD-L substrates, already gave a hint that they could affect CPY*0000 degradation. *DER1* and *USA1* deleted cells were examined and they showed a mild but visible effect in delaying CPY*0000 degradation kinetics (Fig. 3.12A). For clarity, the same experiments were performed in cells devoid of Yos9 (Fig. 3.12B). In this case the same conclusion could be drawn, Der1 and Usa1 are necessary to degrade CPY*0000 and this is consistent with previous analysis of ERAD-L substrates (Hitt and Wolf 2004; Carroll and Hampton 2010).

The effect of the component Hrd3 was not tested. It is known that deletions of the *HRD3* gene provoke the destabilization of Der3/Hrd1 (Plemper, et al. 1999a)and Yos9 (Gauss, et al. 2006). Overexpression of Der3/Hrd1 and Yos9 in the *HRD3* deletion strain would have made a very artificial environment.

4.2.Mnl2 is a novel putative α-1,2 mannosidase of the ER

Quality control of glycosylated proteins in the ER begins with the attachment of glycan to an asparagine of the newly synthesized protein by the OST complex in the moment of translocation into the ER lumen (Helenius and Aebi 2004; Buchberger, et al. 2010; Xie and Ng 2010). This glycan is processed to a degradation signal carrying an α-1,6 mannose linkage when the protein is misfolded or when the time that was given to the protein to fold was insufficient. This results in the recognition of the protein by the lectin Yos9 followed by its delivery for degradation (Aebi, et al. 2010). In this work, some aspects of the quality control mechanism were extended with the finding of the novel component Mnl2. The putative protein with the open reading frame YLR057W was found in the SGD and SUPERFAMILY Database in a search for proteins that contained domains or motifs in common with Mannosidase 1 (Mns1) and Mannosidase-like protein 1 (Mnl1/Htm1). Therefore, we renamed YLR057W as Mnl2 for Mannosidase like protein 2, for its similarities to Mnl1/Htm1 and analogy to the three human EDEMs (all having α-1,2 mannosidase activity).

4.2.1.Mnl2 is involved in ERAD

Due to its predicted structural and functional similarities to the α-1,2 mannosidases Mns1 and Mnl1/Htm1, Mnl2 was examined in comparison to those mannosidases (SGD, Superfamily database). The absence of Mnl2 had its most pronounced effects on CPY* degradation in the absence of the mannosidase Mnl1/Htm1 (Fig 3.15). The Man8 structure generated by Mns1 is not a degradation signal and proteins with such a signal are allowed to exit the ER. Nevertheless, formation of this structure is a prerequisite for further glycan processing if the protein does not properly fold. In the presence of Mnl1/Htm1, the absence of Mnl2 has no effect. One possibility for this phenotype rests in the fact that Mnl2 can only bind when Mnl1 has created the α-1,6 linkage. This would explain why the double deletion of the genes *MNS1* and *MNL2* does not enhance CPY* stabilization over the *MNS1* single mutant. It is known that Mnl1/Htm1 generates the glycan signal for degradation by cleaving an α-1,2 mannnose linkage in the C branch of the glycan generating an α-1,6 mannose linkage. When cells are devoid of both Mnls the degradation of the glycosylated ERAD substrate CPY* is delayed to a greater extent than in

MNL1/HTM1 single deletion mutants. This suggests that Mnl1/Htm1 and Mnl2 work closely together in the generation of the glycan degradation signal. The presence of Man6 and Man5 structures has been shown in several studies but no enzyme responsible for their generation has been proposed yet. These data suggest that Mnl2 could represent the protein that generates Man6 and Man5 glycan structures by processing the additional α-1,2 mannose linkages.

To prove the hypothesis that Mnl2 cooperates in the generation of the glycan degradation signal, *YOS9* was deleted in the yeast strains where two or three mannosidases were deleted.

Turnover of CPY* was examined in cells devoid of Mns1, Mnl1/Htm1 and Mnl2 or devoid of Mnl1/Htm1 and Mnl2. The turnover of CPY* was compared in cells with de additional deletion of *YOS9*. The glycan structure that substrates present in the absence of mannosidases is not a glycan degradation signal and therefore does not trigger degradation in the presence of Yos9 (Fig. 3.18). Moreover cells that lack Yos9, in addition to Mnl1/Htm1 and Mnl2, do not stabilize the substrate further. The same degradation pattern of CPY* as in the mannosidase mutants alone is obtained. This confirms that the mannosidases act before the glycan recognition lectin Yos9. In addition,

degradation of CPY*0000 is not affected by the absence of Mnl1/Htm1 or Mnl2 (Fig. 3.19). This excludes Mnl2 as being a chaperone acting on the protein part of CPY*.

The observations of the experiments suggest that Mnl1/Htm1 and Mnl2 generate the glycan degradation signal. Mnl2 may partially play the role of Mnl1/Htm1 in its absence, while Mnl1/Htm1 fully compensates the loss Mnl2. Whether Mnl1/Htm1 and Mnl2 act sequentially or in parallel cannot be determined so far.

Wild type cells can efficiently degrade glycosylated ERAD substrates owing to the collaborative functions of Mnl1/Htm1 and Mnl2 in generating a degradation signal. Mnl1/Htm1 generates the α-1,6 signal by cleaving the α-1,2 linkage on the C branch. This was measured Clerc et al. and Quan et al. (Quan, et al. 2008; Clerc, et al. 2009). Mnl2 has the chance to further cleave the two residual α-1,2 linkages to generate a Man5 structure which shows stronger binding to Yos9 in vitro (Quan, et al. 2008) The lack of Mnl2 shows reduced degradation only in the absence of Mnl1/Htm. A cell deleted in *MNL1/HTM1* and *MNL2* can no longer generate a degradation signal and in consequence the substrate is stabilized.

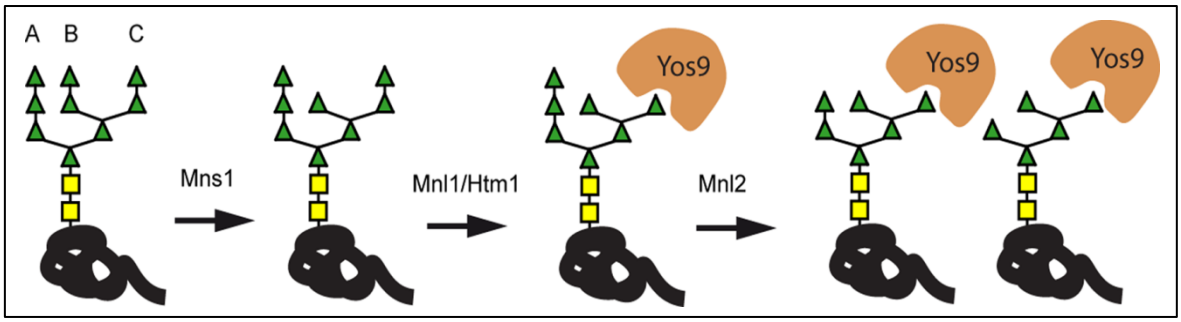

Figure 4.1: Proposed model for Mnl2 activity.

4.3. Prospect

Future experiments have to proof the glycan structure in cells generated by Mnl2 and have to analyze the reactivity and structure of the enzyme.

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Highschool graduation and university access examination.

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Functions of Ubiquitin and Ubiquitin-Related Protein-Protein Modifications in Cell Biology.

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The Ubiquitin-Proteasome System in health and disease.

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Advances in Molecular Biology.

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History of science: scientific revolutions.

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Analytical separation techniques: Liquid chromatography.

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Internship at the Universitat de Barcelona.

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Scientific-technical Services. Separating techniques service.

Lab technician

Elemental organic analysis

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Lab of clinical biochemistry.

Lab technician

Atomic absorption spectrophotometry. Immunoassays

February 2005- May 2005

Internship at the Universitat de Barcelona.

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Elemental organic analysis

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Group of Proteomics and Protein Chemistry.

Research support lab technician.

Solid phase peptide synthesis, peptide-protein conjugation, amino acid analysis, HPLC, GC, MALDI-TOF.

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Publications:

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