A genomic screen in *Saccharomyces cerevisiae* identifies multiple new gene products essential for protein quality control of the endoplasmic reticulum and degradation: The role of Dsk2p, Rad23p and Yos9p.

Von der Fakultät
Geo- und Biowissenschaften der Universität Stuttgart
zur Erlangung der Würde eines
Doktors der Naturwissenschaften (Dr. rer. nat.)
genehmigte Abhandlung

vorgelegt von
Bala Subrahmanyam Medicherla
aus Anakapalli, Indien

Hauptberichter : Prof. Dr. Dieter H. Wolf
Mitberichter : Prof. Dr. Peter Scheurich

Tag der mündlichen Prüfung: 13-12-2004

Institut für Biochemie der Universität Stuttgart
2004

Index

Abbreviations

A_{600}	Absorbance at 600nm
A	Ampere
AAA	ATPases associated with various cellular activities
ATP	Adenosine triphosphate
bp	Basepairs
CM	Critical minimum medium
CPY	Carboxypeptidase ysc Y
CPY*	mutated Carboxypeptidase Y (prc1-1/ CPY^{G255R})
CT*	mutated Carboxypeptidase Y-Transmembrane domain
CTG*	mutated Carboxypeptidase Y-Transmembrane domain-GFP
DER	degradation in the ER
DH_20	Distilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation
5-FOA	5-Fluoroorotic acid
g	gram
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
HECT	Homologous to the E6-AP carboxyl terminus
hr	hour
HRPO	horseradish peroxidase
IP	Immunoprecipitation
KDa	Kilodalton
M	Molar
Mg	Milligram
mM	Millimolar
min	minute
ml	Milliliter

OD	optical Density
ORF	open reading frame
OS-9	osteosarcoma-9
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulphonyl fluoride
RING	really interesting new gene
RT	room temperature (25°C)
SDS	Sodium dodecylsulphate
sec	second
TCA	Trichloroacetic acid
UBA	ubiquitin associated domain
UBC	ubiquitin conjugating enzyme
UBL	ubiquitin like domain
UFD	ubiquitin fusion degradation
UPR	unfolded protein response
V	Volt
WT	wild type
YOS9	Yeast OS-9
YPD	Yeast peptone dextrose

Zusammenfassung

Das endoplasmatische Retikulum (ER) ist das Kompartiment in eukaryontischen Zellen, in dem Proteine des sekretorischen Systems ihre native Konformation erhalten. Für diesen Prozess enthält das ER ein effizientes System aus faltungsunterstützenden und kontrollierenden Proteinen. Sekretorische Proteine, die ihre endgültige native Konformation nicht erlangen können, werden durch ein Qualitätskontrollsystem im ER erkannt, in das Cytosol zurücktransportiert und dort über das Ubiquitin-Proteasom-System abgebaut. Dieser Prozess wird auch als ER-assoziierte Degradation (ERAD) bezeichnet.

Viele molekulare Mechanismen und die daran beteiligten Komponenten der Proteinqualitätskontrolle und des ERAD sind noch unbekannt. Daher war ein Ziel der vorliegenden Arbeit, neue Komponenten dieser Prozesse zu identifizieren und ihre mögliche Funktion in der Qualitätskontrolle und im ERAD zu analysieren. Zu diesem Zweck wurde eine EUROSCARF Stammsammlung (Universität Frankfurt) durchsucht (genomischer Screen), die 5000 verschiedene Mutantenstämme der Hefe *Saccharomyces cerevisiae* enthält. Zur Identifizierung der Mutanten in dieser Sammlung, die einen Defekt in der Proteinqualitätskontrolle oder im ERAD aufweisen, wurde ein sensitiver Wachstumstest etabliert. Es wurde ein Plasmid erstellt, das zur Expression eines ERAD Substrates führt und aus einem ER lumenalen, fehlgefalteten Glycoprotein CPY* in Fusion mit einer Transmembrandomäne und einem cytosolisch orientierten Leu2 Protein (Isopropyl-Malatdehydrogenase) besteht. Das resultierende Fusionsprotein CTL* wurde unter Kontrolle des *GAL4*-Promotors gestellt, der zu einer geringen Expression führt und den Screen erst ermöglichte. Das für CTL* kodierende Plasmid wurde in alle 5000 verschiedenen *leu2*-auxotrophen Mutantenstämme transformiert und der Wachstumsphänotyp auf Leucin defizienten Medien untersucht. Nur wenn CTL* stabil war und nicht abgebaut wurde, war der transformierte Mutantenstamm in der Lage, auf Leucin defizienten Medien zu wachsen. Mit diesem Wachstumstest konnten zum einen Mutanten identifiziert werden, deren deletierte Gene für bereits bekannte ERAD Komponenten kodieren. Dies unterstreicht die Sensitivität und Spezifität dieses Wachstumstests. Zum anderen konnten zusätzlich 25 Mutanten identifiziert werden, die ein reproduzierbares Wachstum auf Leucin defizienten Medien aufwiesen. Für die zugehörigen 25 Gene und deren Genprodukte war bisher noch keine Funktion im ERAD bekannt. Unter diesen neu gefundenen möglichen ERAD Komponenten wurden die mit dem Proteasom interagierenden Proteine Dsk2p und Rad23p hinsichtlich ihrer Funktion im ERAD untersucht. Durch metabolische Markierung mittels radioaktivem

Methionin in *Pulse-Chase* Analysen konnte eine Stabilisierung der ERAD Substrate CPY*HA und CTG* in der Doppeldeletionsmutante *Δdsk2Δrad23* gezeigt und damit eine Beteiligung von Dsk2p und Rad23p am ERAD belegt werden. Des weiteren weist die Akkumulation polyubiquitinylierter Proteine in der cytosolischen Fraktion des Stammes *Δdsk2Δrad23* nach subzellulärer Fraktionierung auf einen Defekt im Transport zum Proteasom hin. Diese Daten führten zu dem Modell, in dem Dsk2p und Rad23p als Adaptoren für den Transfer fehlgefalteter, polyubiquitinylierter Proteine vom trimeren Cdc48-Komplex zum Proteasom fungieren. Die Ergebnisse zeigen außerdem, dass der Transfer fehlgefalteter Protein im Cytosol ein gezielter, ununterbrochener Prozess ist, der die Bildung unlöslicher Proteinaggregate in der Zelle verhindert.

Des Weiteren wurde die Funktion des ER lumenalen Proteins Yos9p charakterisiert, das ebenfalls in dem beschriebenen Screen identifiziert werden konnte. Die Untersuchungen zur Degradation der ERAD-Substrate CPY*HA und CTG* in der Deletionsmutante *Δyos9* zeigten eine deutliche Stabilisierung dieser fehlgefalteten Glykoproteine, während die Deletion von *YOS9* keinen Einfluss auf den Abbau des nicht glycosylierten Substrates Sec61-2p hat. Yos9p besitzt somit wahrscheinlich eine Lektin-ähnliche, zuckerbindende Funktion innerhalb der Qualitätskontrolle im ER speziell für sekretorische, fehlgefaltete Glycoproteine.

In dem in dieser Arbeit durchgeführten genomischen Screen konnten neben den Proteinen Dsk2p, Rad23p und Yos9p, deren Funktion in der ER Qualitätskontrolle untersucht wurde, weitere 22 möglicherweise am ERAD Prozess beteiligten Komponenten identifiziert werden. Die Analysen zur Beteiligung dieser 22 Kandidaten an der ER-Qualitätskontrolle könnten das Verständnis der dem ERAD zugrundeliegenden molekularen Mechanismen deutlich erweitern.

Summary

The endoplasmic reticulum (ER) is characterized by the presence of a highly efficient quality control system, which recognizes malfolded proteins, and inhibits their further transport to the secretory pathway. These proteins are retrotranslocated back into cytosol where they are polyubiquitinated and degraded by the 26S proteasome. This process is known as ER-associated degradation or ERAD. Failure of this process results in inactive proteins forming insoluble aggregates, which ultimately lead to cellular malfunction and unhealthy states.

To gain a deeper insight into the molecular mechanisms of protein quality control and ER-associated degradation, a genome wide screen using the EUROSCARF yeast library, consisting of about 5, 000 *Saccharomyces cerevisiae* strains each deleted for a single non-essential gene was undertaken. Such a screen is possible as cells tolerate a defect in this process as long as the unfolded protein response is intact. As cell growth is one of the most sensitive indicators of alterations in cell physiology due to mutations, a growth phenotype test to identify new mutants in quality control and ERAD was devised. Previously the membrane-localized ERAD substrate CTG* has been described, which consists of an ER-lumenal malfolded CPY* domain connected to the green fluorescent protein (GFP) in the cytoplasm via a transmembrane domain. To screen for new mutants the cytoplasmic GFP moiety was replaced with the Leu2 protein (3-isopropylmalate-dehydrogenase). This new construct called CTL* was placed under the control of the weak *GAL4* promoter. Consequently strains with *leu2* auxotrophy, but otherwise, wild type for ERAD are unable to grow in the media lacking leucine. Only when ERAD is defective, CTL* is stabilized and able to complement the *leu2* deficiency. The low expression of CTL* then allows for sharp growth differences to be easily observed. Advantage of this growth phenotype was taken of to screen the 5, 000 individual deletion mutants of the EUROSCARF yeast library expressing CTL*. Strains defective in most of the known ERAD components resulted in growth in the absence of leucine, highlighting the reliability of the selection procedure. Apart from the genes known to be defective in ER quality control, we identified a reproducible growth phenotype of approximately 25 mutants. The role of the proteins defined by the mutated genes in ERAD was not described earlier.

Out of the new genes identified in the screen, the function of twoproteasome interacting proteins Dsk2p and Rad23p were characterized as adapters for transferring the ERAD substrates from the trimeric Cdc48 complex to the 26S proteasome. Degradation of two well characterized ERAD substrates, soluble CPY*HA and membrane-anchored CTG* is

significantly delayed in a Δ*dsk2*Δ*rad23* double mutant, indicating that these proteins directly participate in ERAD. Microsomal extracts from a Δ*dsk2*Δ*rad23* strain showed the accumulation of large amounts of polyubiquitinated CPY*HA in the cytosol as a consequence of failed delivery to the proteasome, and are in agreement with a role for Dsk2p and Rad23p downstream of the Cdc48-Ufd1-Npl4p complex. It is believed that, Dsk2p and Rad23p transfers, malfolded substrates of the ER from the AAA-ATPase Cdc48 complex to the proteasome by this maintaining an uninterrupted path from the ER membrane to the proteasome, thus preventing the formation of insoluble protein aggregates in the cytoplasm. The studies further more show that the function of Dsk2p and Rad23p in transferring multiubiquitinated substrates to the 26S proteasome is pathway specific and depends on the presence of the trimeric AAA-ATPase Cdc48 complex.

In addition the function of an ER lumenal glycoprotein, Yos9p was identified as a lectin or lectin like protein specifically involved in the degradation of misfolded glycoproteins, but not for the degradation of misfolded non glycosylated ERAD substrates. Yos9p posses a mannose-6-phosphate receptor homology (MRH) domain and degradation of model glycoprotein ERAD substrates CPY*HA and CTG* is significantly delayed in a Δ*yos9* strain. The Δ*yos9* strain has no influence in the turnover of non-glycoprotein ERAD substrate, Sec61-2p, suggesting a lectin like activity for Yos9p in the quality control of glycoproteins in the secretory pathway. The genetic screen also resulted in the identification of many additional proteins whose function with regard to ER quality control and degradation can be explored in future.

Introduction

Quality control and endoplasmic reticulum

Eukaryotic cells are characterized by the presence of highly efficient quality control systems, which ensure the delivery of only structurally intact and biologically active macromolecules to their site of action. In cells there are quality control systems for every particular step that leads to the synthesis of DNA, RNA, and protein molecules (Ellgaard, L. and Helenius, A. 2003). As a result, the number of accumulated errors in macromolecules that are ultimately present in cells is rather low. For proteins proof reading occurs at the level of transcription, translation, folding and assembly. To pass the quality control check points at the protein level a protein must typically have reached a correctly folded conformation. This is the so-called native conformation that corresponds to the energetically most favourable state. In the case of proteins with several sub-units, proper oligomeric assembly is usually necessary. If the folding and maturation process fails, proteins are not targeted to there final destination in the cell and are eventually destroyed.

The endoplasmic reticulum (ER) is the site of entry of proteins into the secretory pathway. It supplies the ER, Golgi apparatus, plasma membrane, lysosome (vacuole) and cellular exterior with the protein equipment necessary for proper cellular function. In this pathway proteins first pass the membrane of the endoplasmic reticulum (ER) through a translocation chanel termed translocon, in an unfolded state (Rapoport et al., 1996). Assembly into the native conformation occurs as a next step. The ER provides an environment that is optimized for protein folding and maturation. In the ER several cotransloational and posttranslational modifications takes place that do not occur in the cytosol. These are signal peptide cleavage, disulphide bond formation N-linked glycosylation and glycosylphosphatidylinositol (GPI) anchor addition. These covalent changes are essential for proper protein folding. Presence of high concentrations of chaperones and folding enzymes in ER lumen will assist in proper folding of the proteins. In order to ensure the transfer of properly folded proteins only, the ER is characterized by the presence of a highly efficient quality control system which discriminates between properly folded proteins and terminally misfolded proteins as well as unassembled protein subunits (Ellegard et al., 1999). The misfolded polypeptides and orphan subunits are subsequently removed from the ER, most probably by retrotranslocation through the components of same translocon used for translocation (Plemper et al., 1997) and degraded by the cytosolic 26S proteasome after polyubiquitination by a process known as ER-

associated degradation or simply ERAD. (Sommer and Wolf, 1997). A detailed knowledge of this process is thus of great importance not only for our understanding of this basic cellular mechanism but also for the development of new strategies to treat a diverse set of diseases which have their basis in ERAD as are for instance cystic fibrosis, neurological diseases immune responses, etc. (Plemper and Wolf, 1999).

Folding, recognition of misfolded proteins and UPR

In yeast and mammals proteins of the secretory pathway are synthesized in the cytoplasm and are transported into the ER either by the cotranlational translocation and post translational translocation where by the cotranslational translocation process dominates in mammals. In ER lumen, proteins are properly folded, assembled into multi subunit complexes and covalently modified by a large array of ER-resident chaperones and enzymes before they enter into the further compartments of the secretory pathway (Haigh and Johnson, 2002). The ER quality control system recognizes the misfolded proteins and inhibits further transport into the secretory pathway. The molecular chaperones and folding sensors that are used in quality control are abundant in the ER. They include Kar2p (Bip in mammals), calnexin, calreticulin, thiol-disulfide oxidoreductases protein disulfide isomerase (PDI), signal peptidase, oligosaccaryl-transferase complex (OST). Signal peptidase cleaves the signal peptide from the newly translocated proteins and OST plays an essential role in N-glycosylation. (Ellegard et al., 1999) PDI participates in the disulfide bond formation. Kar2p is the Hsp70 chaperone in the ER lumen, which recognizes hydrophobic patches on the protein and plays a crucial role in folding (Plempler et al., 1997). Kar2p is also involved in acting as a seal of the protein translocation channel from the lumenal side of the ER (Haigh and Johnson, 2002). The importance of proper folding of proteins before ER exit is exemplified by the existence of the unfolded protein response (UPR) (Sidrauski et al., 2002). The concentration of unfolded proteins in ER is sensed by Ire1p a transmembrane kinase localized to the ER/Nuclear envelope, which interacts with Kar2p through its lumenal domain (Liu et al., 2000). Both, unfolded proteins and Ire2 will compete for bind to Kar2. Accumulate of unfolded proteins in the ER leads to the depletion of free Kar2p which in turn leads to the dimerization of Ire1p, a conformational change that transmits a signal across the membrane and activates the cytoplasmic kinase activity. The kinase induces a non-canonical splicing of *HAC1* mRNA, allowing synthesis of the Hac1p transcription factor, which up regulates genes containing a UPR response element. This cascade of events leads to an increase in the levels of proteins

required for folding and quality control (Travers et al., 2000, Sidrauski et al., 2002). UPR and ERAD are inter connected. Overexpression of unfolded proteins due to the absence of a component required for ERAD induces UPR (Knop et al., 1996) which, in turn, upregulates the overall level of components of the ERAD machinery (Friedländer et al., 2000, Travers et al., 2000). Under normal conditions, cells are able to cope with the amount of naturally present unfolded proteins and do not require the induction of the UPR. (Kostova and Wolf, 2003). When the level of misfolded proteins rises to the critical level, the UPR becomes essential. In yeast, loss of function of components of both ERAD and UPR is lethal (Friedländer at al., 2000, Travers et al., 2000).

Quality control of glycoproteins

Glycosylation plays a crucial role in the proper folding and quality control of the glycoproteins. The oligosaccharyltransfarese (OST) is associated with the translocon complex and is involved in cotranslational transfer of the core oligosaccharide Glc3Man9GlcNAc2 to the Asn-X-Ser/Thr consensus sequence of the proteins. (Helinius and Aebi, 2004). During the process of folding the two terminal glucose residues are cleaved by glucosidase I and glucosidase II. The protein containing the resulting Glc1Man9GlcNac2 structure interacts with clanexin, calreticulin and ERp57, a member of the PDI family, which specifically interacts with calnexin and calreticulin and functions as a disulfide isomerase on monoglucosylated glycoproteins. (Kostova and Wolf, 2003) Association of glycoproteins with calnexin and calreticulin involves a binding and release cycle driven by the opposing actions of two soluble ER enzymes glucosidase II and UDP glucose: glycoprotein glucosyltransferase the former removes a glucose residue leading to a mannose9 structure and the later adds a glucose residue which finally leads to binding of the protein to calnexin and calreticulin again (Helinius and Aebi, 2004). In this cycle UDP glucose: glycoprotein glucosyltransferase serves as the folding sensor. *In vivo* and *in vitro* studies have shown that this remarkable enzyme only reglucosylates incompletely folded glycoprotein that is glucose addition depends on the folding status of the protein. This induces new round of clanexin/calreticulin binding, which prevents the escape of not properly folded glycoproteins from the ER. If this cycle persists, a slow acting membrane bound ER enzyme α-1,2 mannosidase, cleaves the α-1,2 - linked mannose of the middle branch generating a glycan with the structure Man8GlcNac2. Direct recognition of Man8GlcNac2 by a specific lectin or attenuated release of the reglucosylated

form (Glc1 Man8GlcNac2) from calnexin are events, which determine the delivery of malfolded glycoproteins to the elimination machinery (Cabral et al., 2001).

In yeast the UDP- glucose: glycoprotein glucosyl transferase and clanexin and calreticulin cycle are absent, but the basic machinery for carbohydrate trimming and recognition of malfolded glycoproteins is present which includes glucosiadase I and II and α-1,2 mannosidase (Knop et al., 1996b, Jakob et al., 1998, Hitt and Wolf, 2004b). Thus, carbohydrate trimming by glucosidases I and II, and later by α-1,2 mannosidase, is currently believed to be the timer for folding or, if unsuccessful, for degradation (Kostova and Wolf, 2003). Recently the vast area of ERAD was divided into 2 broad fields: glycoprotein ERAD and non-glycoprotein ERAD as both glycoproteins and non-glycoproteins differs in components required for degradation. (Carbal et al., 2001). A typical model substrate which is used extensively for the study of ER quality control and ERAD in *Saccharomyces cerevisiae* is a mutated vacuolar enzyme, carboxypeptidase yscY commonly known as CPY*. CPY*, a glycoprotein, carries a gly255-arg mutation which concerns a highly conserved position in all serine proteases (Finger et al., 1993). Ste6p, proteinase yscA*, Pdr5* are also well-studied ERAD glycoprotein substrates in yeast. Unassembled MHC class I heavy chain, CFTR, mutant thyroglobulin and unassembled T cell receptor subunits are typical ERAD glycoprotein model substrates in mammals (Cabral et al., 2001). Sec61-2p a mutated form of Sec61 translocon and prepro-αfactor are typical non-glycoprotein model substrates for ER degradation in yeast (Biederer et al., 1996, McCracken and Brodsky, 1996). Insulin is example for a nonglycoprotein ER degradation substrate in mammals.

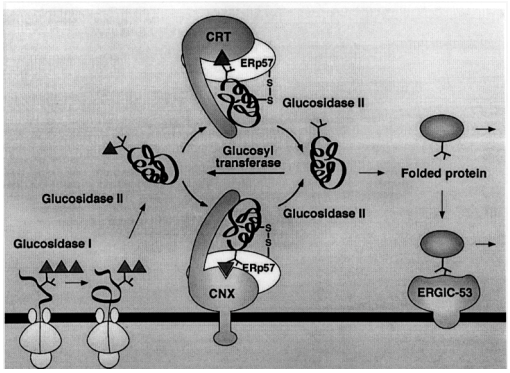

Fig 1: Calnexin Calreticulin cycle for the folding of glycoproteins
(Figure reproduced from Helinius and Aebi. 2004)

Misfolded glycoproteins specifically require the presence of additional quality control components for degradation. α-1,2 mannosidase is essential for the degradation of misfolded

glycoproteins as it is required for the formation of the Man8GlcNac2 structure, which will be liberated from the calnexin/calretuculin cycle and targeted towards the elimination machinery. (Jacob et al., 1998, Tokunaga et al., 2000). α-1,2 mannosidase requires the presence of calcium for its activity. So the ER calcium pumps Pmr1p and Cod1p are essential for the proper functioning of α-1,2 mannosidase. Deletion of *PMR1* and *COD1* inhibits the degradation of misfolded glycoproteins. (Dürr et al., 1998, Vashist et al., 2002). Alg12p, a protein essential for the synthesis of N-glycans is also required for the degradation of misfolded glycoproteins, as this protein is essential for the formation of typical Man8GlcNac2 structure (Jakob et al., 1998). Man8GlcNac2 is supposed to bind to lectins, which specifically recognize the misfolded glycoproteins. Recently an ER residing lectin Htm1p/Mnl1p was found to be involved in the turnover of CPY*. Htm1p/Mnl1p in yeast and its mouse homolog EDEM are believed to bind to Man8GlcNac2 structure and provide the link between recognition and targeting for degradation (Hosokokawa et al., 2001, Jacob et al., 2001, Molinari et al., 2003, Oda et al., 2003). Htm1p/Mnl1p is also required for the degradation of Pdr5*, a mutated form of the ATP-binding cassette transporter Pdr5 (plemper et al., 1998), Stt3-7p, a mutant subunit of the OST complex (Jacob et al., 2001) and mammalian CFTR expressed in yeast (Gnann et al., 2004). Degradation of Sec61-2p a non-glycosylated ERAD substrate is independent of Htm1p/Mnl1p (Jacob et al., 2001). Thus the lectin is believed to bind to misfolded glycoproteins, inhibits further transport through the secretory pathway and brings them to the retrotranslocation channel. Even though much is known about the components required for the recognition of misfolded glycoproteins, the factors known to be essential for the degradation of misfolded nonglycosylated proteins are not known till now. It is yet to be determined whether the non-glycoslyated proteins require the presence of any special factors like Htm1p/Mnl1p for recognition and subsequent degradation.

Retrotranslocation

After the proteins are recognized as misfolded by the quality control system in the ER, they are ultimately degraded. The site of degradation of misfolded ER proteins was an issue of debate for a long time. Previously it was thought that some unknown proteases in the ER are responsible for the degradation of misfolded ER proteins (Bonifacino and Klausner, 1994). However the presence of proteases in ER was hard to reconcile with it's primary function, the folding and assembly of proteins. Involvement of lysosome/vacuole in the degradation of misfolded ER proteins was also suggested, as these compartments in cells are mostly

associated with protein degradation. But following the fate of the CPY* it was found that this protein never reached the vacuole but was retained in the ER and rapidly degraded (Hiller et al., 1996, Knop et al., 1996). Further studies uncovered that degradation of CPY* is dependent on the ubiquitin conjugating enzymes Ubc6p and Ubc7p, and the 26S proteasome (Hiller et al., 1996). The cytoplasmic localization of the ubiquitin conjugating enzymes and the proteasome together with the finding of signal sequence cleaved glycosylated and ubiquitinated CPY* on the cytoplasmic face of the ER membrane led to the conclusion that retrograde transport of the mutated enzyme species from the lumen of the ER back to the cytoplasm had occurred (Hiller et al., 1996). The involvement of cytosolic components and the proteasome in degradation, implying the necessity of retrograde transport was also found for a mutated pro-α-factor pheromone and a mutated form of α1-proteinase inhibitor (Werner et al., 1996).

Using CPY* as substrate it was found that yeast mutants carrying a mutation in the major component of the translocon of the ER membrane Sec61, were defective in retrograde transport as degradation of CPY* was considerably slowed down under conditions at which protein import into the ER lumen was unaffected (Plemper et a l., 1997). *In vitro* experiments using a mutated pro-α-factor also pointed to participation of Sec61 in retrograde transport (Pilon et al., 1997). Coimmunoprecipitation studies indicated that sec61β was associated with cytomegalovirus induced degradation of MHC class I heavy chains, with wild type and mutant forms of CFTR and with a mutant form of ribophorin during there dislocation from the ER (Kostova and Wolf, 2002). These studies support the notion that the Sec61 import channel may also be part of the export channel. It is very likely that the retrotranslocation channel differs in its composition from the import channel. The Sec61 translocon is composed of 3 different subunits Sec61p, Sbh1p, Sss1p (Sec61α, Sec61β, Sec61γ in mammals). The presence of two subsets of translocon can easily explain how this complicated two-way traffic across the membrane is regulated. Genetic analysis of *sec61* mutants also resulted in identification of three *sec61* mutant alleles, which are fully proficient in protein translocation into the ER but defective in the elimination of misfolded ER proteins. Detailed analysis of these mutants reveals that a fourth luminal loop and third transmembrane domain of Sec61p markedly influence the dislocation (Zhou and Schekman, 1999). But these findings couldn't be confirmed yet biochemically. Possible components could also be Der1p, a ER membrane protein of unknown function (Knop et al., 1996), Kar2p, PDI, the lectin Htm1p, and Hrd3p, an ER membrane protein which functions together with the ubiquitin-protein ligase Der3p (Gardner et al., 2001, Deak and Wolf. 2001), and AAA-ATPase Cdc48 complex (Jarosch et

al., 2002). Given the cooperativity between targeting, dislocation and degradation, it is very likely that these components do not act independently of each other. Proteins targeted for ER-associated degradation are completely translocated into the ER lumen before retrotranslocation. This was proven by the use of a CPY* species containing a newly introduced glycosylation site at the very C terminus. As glycosylation occurs at a distance of about 14 amino acids away from the inner face of the ER membrane (Nilsson and von Hein, 1993) this experiment showed that indeed the entire CPY* chain was fully imported into the ER lumen prior to its recognition by the ER quality control system (Plumper et al., 1999b).

Recent studies suggest that proteins can be retrotranslocated in a folded state. This idea is proposed from the results of an experiment in which a fusion protein with a GFP and a MHC class I heavy chain (EGFP-HCI) domain was shown to be retronslocated without loosing fluorescence indicating no unfolding of the EGFP domain (Fiebiger et al., 2002). Further studies also showed that a fusion protein with dihydrofolate reductase (DHFR) and MHC class I heavy chain (DHFR-HCI) is also retrotranslocated without the unfolding of DHFR domain (Tirosh et al., 2003). The inner diameter of the translocon pore is estimated to fluctuate between 15°A, in it inactive state and 40-60A° during protein translocation (Johnson and Van Weas, 1999, Haigh and Johnson, 2002). Therefore it is possible that in its active state the translocon may accommodate a completely folded molecule. However dislocation might also take place through a channel completely different from the Sec61p channel. Additional biochemical proof is essential to support these ideas.

Even though it has shown that mutations in Sec61 will inhibit the degradation of ER lumenal proteins like CPY* and membrane proteins like MHC class I heavy chain and Pdr5* suggesting it's role in retrotranslocation, ER degradation of Ubc6p a short lived ER membrane protein is independent of Sec61 (Walter et al., 2001). Ubc6p is thought to be inserted into the ER membrane via its tail, independent of Sec61 translocation pore. It is thought that this protein is also extracted from the ER membrane in the same way, independent of Sec61p for degradation by proteasome.

Recent studies in mammalian cells revealed that the human homologue of Der1p, Derlin-1 shows interaction with p97 (Cdc48p in yeast) and is essential for the degradation of MHC class I heavy chain molecules (Ye et al., 2004, Lilley and Ploegh, 2004). The authors also propose that Derlin can also act as a major component of the retrotranslocon for a specific subset of ER proteins for degradation. However further experiments are essential to prove this hypothesis. This over all view of the retrotranlocation process, as it appears today is

insufficient and further experiments are essential to shed light into this crucial biological phenomenon.

Polyubiquitination of misfolded ER proteins

Following retrotranlocation from ER, nearly all misfolded proteins are polyubiquitinated prior to degradation. One exception never the less is a mutated pro-α-factor (Werner et al., 1996), which, although not ubiquitylated, is still targeted to the proteasome. Genetic and biochemical studies have characterized the ubiquitin machinery in yeast in great detail. Ubiquitin is a highly conserved protein of 76 amino acids present in all eukaryotes from yeast to mammals. Marking of a protein with ubiquitin chain includes its degradation by the proteasome. Monoubiquitination is used as a signal for vesicular transport to the vacuole/lysosome. The formation of ubiquitin conjugates on proteins requires the successive action of 3 classes of enzymes: the E1 or ubiquitin activating enzyme, E2 or ubiquitin-conjugating enzymes (Ubc) and E3 or ubiquitin protein ligases. (Sommer, 2000). The first step in the ubiquitin conjugation cascade is the activation of ubiquitin by the E1, E1 hydrolyses the ATP to first adenylate the C-terminal glycine of ubiquitin and then link it to the side chain of it's central cysteine residue, yielding an high energy E1-Ub thioester, free AMP and pyrophosphate. The 114 Kda E1 enzyme of yeast is encoded by the essential gene *UBA1* (McGrath et al., 1991). Following activation, one of the several E2 enzymes receives activated ubiquitin from the E1 forming a thioester and in turn transfers the activated ubiquitin moiety to a cysteine residue on E3 to form another thio ester bond. This type of transfer has been shown in the case of HECT family of E3s, where as in RING family of E3s activated ubiquitin is directly transferred from E2 to the E3 bound substrate protein. The first moiety of ubiquitin is transferred to an E-NH2 group of lysine of the protein substrate to generate an isopeptide bond. In successive reactions, a polyubiquitin chain is synthesized by processive transfer of additional activated ubiquitin moieties to the lysine 48 residue of the previously conjugated ubiquitin molecule (Sommer, 2000).

After activation of ubiquitin by the E1 the E2s that take part in ERAD are Ubc1p, Ubc6p, and Ubc7p. Ubc7p is a soluble cytoplasmic E2 recruited to the ER membrane via Cue1p (Biederer et al., 1997), Ubc6p is a tail anchored ER membrane protein which is attached to ER membrane by it's single C terminally located hydrophobic domain (Walter et al., 1997). Ubc1p, a cytoplasmic located E2, is also shown to be involved in the ubiquitination of malfolded ER proteins (Friedländer et al., 2000). Ubc7p is the major E2 of ERAD. Absence

of this protein leads to the accumulation of model ERAD substrates like CPY* due to failure in ubiquitination (Hiller et al., 1996). Cue1p recruits Ubc7p to the ER membrane. In the absence of Cue1p, unassembled and thus cytosolically localized Ubc7p is unable to participate in ER degradation and in the turnover of soluble non-ER proteins (Biederer et al., 1997). Ubc6p is a short-lived tail anchored ER membrane protein and it undergoes ERAD (Walter et al., 2001). Ubc6p is also essential for the turnover of misfolded ER proteins (Hiller et al., 1996). Recently it was also found that Ubc1p a protein which is up regulated during UPR along with Ubc7p and Ubc6p is also essential for the degradation of CPY* (Friedländer. 2000). The main E3, which is involved in ERAD, is Der3p/Hrd1p. In the absence of Der3p CPY* degradation is abolished (Bordallo et al., 1998). Der3p is an ER membrane protein, which spans the membrane six times. The amino terminus and the carboxyl terminus containing the RING finger domain face the cytoplasm (Deak and Wolf, 2001). The function of Der3p as E3 is dependent on it's RING-H2 domain, Deletion of the RING-H2 domain or exchange of a single cysteine residue at position 399 against serine in the Der3p completely abolishes degradation of CPY* and Pdr5* indicating the essentiality of this domain (Bordallo and Wolf, 1999). A RING-H2 finger domain is defined by the position and distance between six cysteines and two histidines and is able to bind two zinc atoms RING-H2 finger is also essential for the interaction of Der3p with Ubc7p and C399S mutant of RING-H2 finger is defective in binding to Ubc7p. This finding shows that the RING-H2 domain of the ligase is crucial for recruitment of the E2 Ubc7p (Deak and Wolf, 2001). Many ubiquitin-protein ligases tend to self-ubiquitinate *in vitro* in the absence of other substrate proteins (Lorick et al., 1999) and Der3p RING-H2 finger also shows *in vitro* self-ubiquitination. Both the interaction of Der3p with Ubc7p in a RING-H2 finger dependent manner and self-ubiquitination *in vitro* confirms the fact that Der3p/Hrd1p is a major E3 involved in ERAD (Deak and Wolf, 2001). RING-H2 domain of Der3p also shows interaction with the ER membrane protein Hrd3p allowing Der3p stability by Hrd3p dependent control of the Der3p RING-H2 domain activity (Gardner at al., 2000). In the absence of Hrd3p, Der3p is highly unstable and looses its activity, underlining the importance of Hrd3p in controlling the function of Der3p.

Apart from Der3p/Hrd1p an additional E3, Doa10p was found also to be involved in the degradation of misfolded and short-lived ER proteins. Doa10p is an ER membrane protein with a RING-HC domain. It was identified in a screen for mutants involved in the degradation of the Matα2 repressor. Doa10p is also involved in the degradation of the tail-anchored short-lived ER membrane protein Ubc6p via its action as an E3 (Swanson et al., 2001). Doa10p

along with Der3p is also involved in the degradation of Pdr5* and CFTR expressed in yeast, two polytopic membrane proteins (Gnann et al., 2004). Rsp5p a HECT domain E3 ligase is also believed to be involved in the degradation of overexpressed CPY*. When CPY* is overexpressed in yeast, Der3p/Hrd1p is no more involved in it's degradation and Rsp5p acts as E3 for the proteasomal degradation under these conditions. (Haynes et al., 2002). This finding is controversial because other reports show that degradation of overexpressed CPY* is still dependent on Der3p (Friedländer et al., 2000).

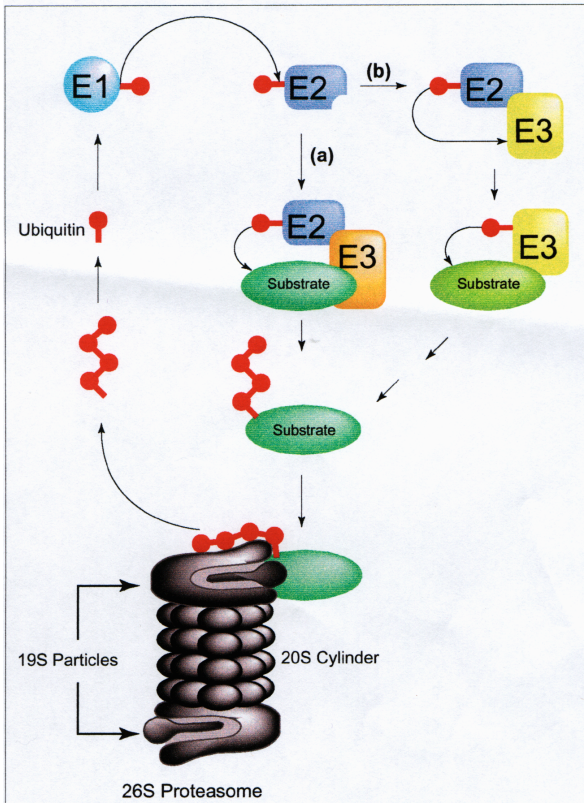

FIG.2: Ubiquitin proteasome system in yeast
(Figure reproduced from Plemper and Wolf, 1999)

Role of the AAA ATPase Cdc48 complex in dislocation of ER proteins

After polyubiquitination the misfolded ER proteins are delivered to the 26S proteasome for degradation. Progressive polyubiquitination may serve as a ratcheting mechanism in moving the polypeptide from the retrotranslocation channel into the cytoplasm. Here the long bulky polyubiquitin chains prevent the polypeptide from slipping back into the ER (Kostova and Wolf, 2003). Consistent with this idea are the findings that a hypo-ubiquitylated CPY* fails to be completely transported into the cytosol (Jarosch et al., 2002). These findings suggest that

polyubiquitinated proteins of ER may require some factors, which are essential for dislocation of ERAD substrates. Recent data from various groups suggest that AAA ATPase proteins of the 26S proteasome are not directly involved in substrate export. Instead a related AAA ATPase Cdc48 complex (p97 in mammals) is required for ERAD upstream of the proteasome. Mutants of Cdc48/p97 and it's associated partners Ufd1 and Npl4 are defective in the degradation of ERAD substrates like CPY*, HMGCoA reductase, in yeast and MHC class I heavy chain in mammals (Ye et al., 2001, Brun et al., 2002, Jarosch et al., 2002, Rabinovich et al., 2002). Previously the AAA ATPase Cdc48 had been implicated in a wider variety of cellular processes like cell cycle, and homotypic membrane fusion. In a complex with two other proteins Ufd1p and Npl4p, its function in the ERAD was discovered. *UFD1* was initially identified as a gene essential for the degradation of the ubiquitin fusion degradation (UFD) substrate Ub-Pro-β-Gal, but was not essential for the degradation of N-end rule substrates. *NPL4* is an essential gene that was identified in a selection for mutants defective in nuclear import. Recent experiments show that *cdc48* mutants are defective in turnover of UFD substrates at a step after ubiquitination (Ghislain et al., 1996). VCP, the mammalian orthologue of Cdc48 copurifies with several polyubiquitinated proteins, when tagged versions of VCP are affinity purified from whole cell lysates. This copurification of polyubiquitinated proteins is inhibited by the addition of increasing amounts of tetra-ubiquitin chains (Dai and Li, 2001). VCP preferentially binds tetra-ubiquitin in an ATP dependent manner, with little affinity for mono, di and tri ubiquitin molecules *in vitro* (Dai and Li, 2001). This preference is especially interesting as tetra-ubiquitin is apparently the preferential unit in recognition of polyubiquitin chains, and thus would be consistent with the idea that Cdc48 is a polyubiquitin chain binding protein (Thrower et al., 2000).

As mutants of the AAA ATPase Cdc48/p97 are defective in the turnover of various ERAD substrates in yeast and mammals, this indicates that it is a polyubiquitin binding protein for ERAD substrates, too (Ye et al., 2001, Bays et al., 2001, Jarosch et al., 2002). Cdc48 exists as a homo-hexameric ring complex, which undergoes strong conformational changes upon ATP hydrolysis (Rouiller et al., 2000, Zhang et al., 2000), critical for substrate dislocation from the ER. In the absence of the Cdc48 complex, plyubiquitinated substrates accumulate at the ER membrane, suggesting that they are essential for the dislocation of ubiquitinated substrates from ER (Jarosch, et al., 2002). There is convincing evidence that the Cdc48 complex can separate a tightly associated protein at the ER membrane, a processed dimer of the Spt23 transcription factor, by this, releasing active transcription factors into the nucleus (Rape et al., 2001). The emerging picture is the following: After polyubiquitination and partial dislocation

of the substrate from the retrotranslocon, the ER associated Cdc48-Ufd1p-Npl4p complex binds the polyubiquitylated substrate in an ATP dependent manner, pulls it away from the ER membrane and hands it over to the proteasome for degradation. This present data confirm that the proteasome acts after the release of polyubiquitylated substrates from the ER membrane.

Glycosylated proteins of the ER are retrotranslocated into the cytoplasm in a glycosylated state (Hiller at al., 1996, Wiertz et al., 1996). The identification of N-glycosylated intermediates in the cytoplasm upon inhibition of the proteasome alludes to the activity of cytoplasmic N-glycanase prior to proteasomal degradation. A highly conserved cytoplasmic N-glycanase (Png1p) has been identified in yeast. *PNG1* is not an essential gene but it's product has a weak affect on the degradation of CPY* (Suzuki et al., 2000). Rad23, a protein that interacts with the proteasome via it's UBL domain escorts Png1p to the proteasome where it is thought to act in a complex to efficiently deglycosylate the substrate prior to degradation (Suzuki et al., 2001). After dislocation from the ER membrane it is not yet clear whether the substrates are delivered directly from the Cdc48 complex to the proteasome or by the help of proteins that can identify polyubiquitinated proteins and deliver them to proteasome. Recently two polyubiquitin chain-binding proteins Dsk2p, Rad23p have been identified (Wilkinson et al., 2001, Chen and Madura 2002, Funakoshi et al., 2002, Hartmann-Petersen et al., 2003). Dsk2p and Rad23p are not 19S cap subunits of proteasome but posses an N-terminal ubiquitin like domain (UBL), which binds to a specific site on the 19S cap, and a C-terminal ubiquitin-associated domain(s) (UBA), capable of binding polyubiquitin chains (Wilkinson et al., 2001, Rao and Sastry, 2002, Hartmann-Petersen et al, 2003). These characteristics suggest that substrates destined for degradation can bind to the Dsk2p or Rad23p UBA domain through the polyubiquitin chain and, consequently, can be delivered to the proteasome by means of the UBL-19S cap interaction. However there is controversy about assigning this role to the Dsk2p and Rad23p. Although degradation of UFD substrates depends on these two proteins (Chen and Madura, 2002, Rao and Sastry, 2002), in some *in vitro* studies an inhibitory effect of Rad23p on proteasome activity has been noted (Raasi and Pickart, 2003).

The 26S proteasome: The proteolytic machinery

ERAD Proteins are finally degraded by the 26S proteasome, which binds the polyubiquitinated substrates, unfolds and degrades them (Heinemeyer et al., 1991, Hilt and Wolf, 1996, Wolf and Hilt, 2004). The eukaryotic proteasome is complex structure, which consists of the 20S core and the 19S regulatory particle. The 20S core is a cylinder composed of four stacked rings, each containing seven different α or β subunits with an over all α7β7α7 geometry. The three different active sites are located inside the cylindrical core within the β-subunit rings (Hilt and Wolf, 1996, Groll et al., 1997, Baumeister et al., 1998, Wolf and Hilt, 2004). N-terminal stretches of the external α-subunits regulate the entry of substrate into the proteolytic core (Groll et al., 2000). The 19S cap is involved in recognition, binding and unfolding of ubiquitylated proteins, and in the regulation of the opening of the 20S core. It is composed of 17 different subunits, functionally divided two parts base and the lid. The base consists of a ring of six ATPases (Rpt1-Rpt6), which dock onto the α-rings of the 20S core, and three non-ATP subunits (Rpn1, Rpn2, Rpn10). The specific function of the ATPase subunits in binding and unfolding are slowly emerging (Braun et al., 1999). Rpt5 binds ubiquitylated substrates (Lam et al., 2002). Rpt2 is believed to control both substrate entry and product release from the 20S channel (Köhler et al., 2001). Rpn1 interacts with Rad23 and Dsk2, two proteins harbouring ubiquitin-like domains (UBL) and capable of binding and delivering ubiquitylated cargo to the proteasome

.

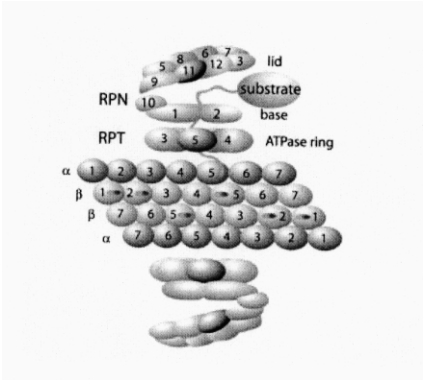

Fig.3: The 26S proteasome (from Kostova and Wolf, 2003)

Recent findings suggest that Rpn10 contribute to the binding of ubiquitin chains as well (Elsässer et al., 2002). The lid is composed of eight subunits: Rpn3, Rpn5 to Rpn9, Rpn11 and Rpn12. Rpn11 contains a highly conserved metallo isopeptidase motif and this activity is

necessary for deubiquitylation, and proteasomal proteolysis of substrates. It is currently believed that Rpn11 de-ubiquitylates the substrate after it has been threaded into the 20S channel, thereby resulting in an irreversible commitment to proteolysis. Failure to de-ubiquitylate probably causes a sterical block of further insertion of substrate into the proteolytic core (Varma et al., 2002, Yao and Cohen, 2002). Following release from the substrate, the polyubiquitin chain is hydrolyzed into single ubiquitin moieties, which can take part in a new round of protein degradation.

Soluble and membrane bound ERAD substrates differ in quality control components

Both the soluble and membrane bound ERAD substrates differ in the components required for quality control to certain extent. An ER membrane protein of unknown function, Der1p, and the major Hsp70 chaperone of ER lumen Kar2p, are essential only for the degradation of soluble ERAD substrates like CPY* but not required for the degradation of type I membrane ERAD substrates like CTG* composed of the CPY* moiety, a transmembrane domain followed by a tightly folded GFP molecule, and Sec61-2p (Taxis et al., 2003, Hitt and Wolf, 2004). DnaJ like proteins Scj1p and Jem1p (Nishikawa et al., 2001) interact with Kar2p and they are also essential only for the degradation of soluble proteins but not for membrane proteins. CFTR expressed in yeast requires the presence of the cytosolic chaperone Hsp70 for its degradation (Zhang et al., 2001). CTG* also requires the presence of Hsp70, Hsp40 and to a certain extent Hsp104 for it's degradation (Taxis et al., 2003). These cytoplasmic chaperones have no role in the degradation of soluble ERAD substrates. One may, therefore, speculate that the tighter a tightly folded cytoplasmic domain of a misfolded ER protein is, the stronger is the requirement for cytoplasmic chaperone activity for degradation. In addition, a mutant form of the yeast membrane ATPase Pma1p requires the presence of a membrane protein, Eps1p, a PDI family member protein for degradation. However, Eps1p is not required for degradation of soluble proteins like CPY* (Wang and Chang, 1999).

Regulated degradation of ER proteins

The quality control system of the ER, which is involved in the recognition and degradation of misfolded proteins, is also involved in the regulated degradation of ER resident enzymes. The best-studied example in yeast is a key enzyme in the sterol biosynthetic pathway, HMGCoA-reductase. Signals from the mevalonate pathway are presumed to lead to conformational

changes, which channel the protein into the ER quality control pathway (Hampton, 2002). Elimination of this enzyme requires all of the major components of the ER degradation machinery. ER quality control is also involved in the regulated expression of Ole1p (Δ-9 fatty acid desaturase) as misregulation of Ole1p is toxic to yeast cells (Braun et al., 2002).

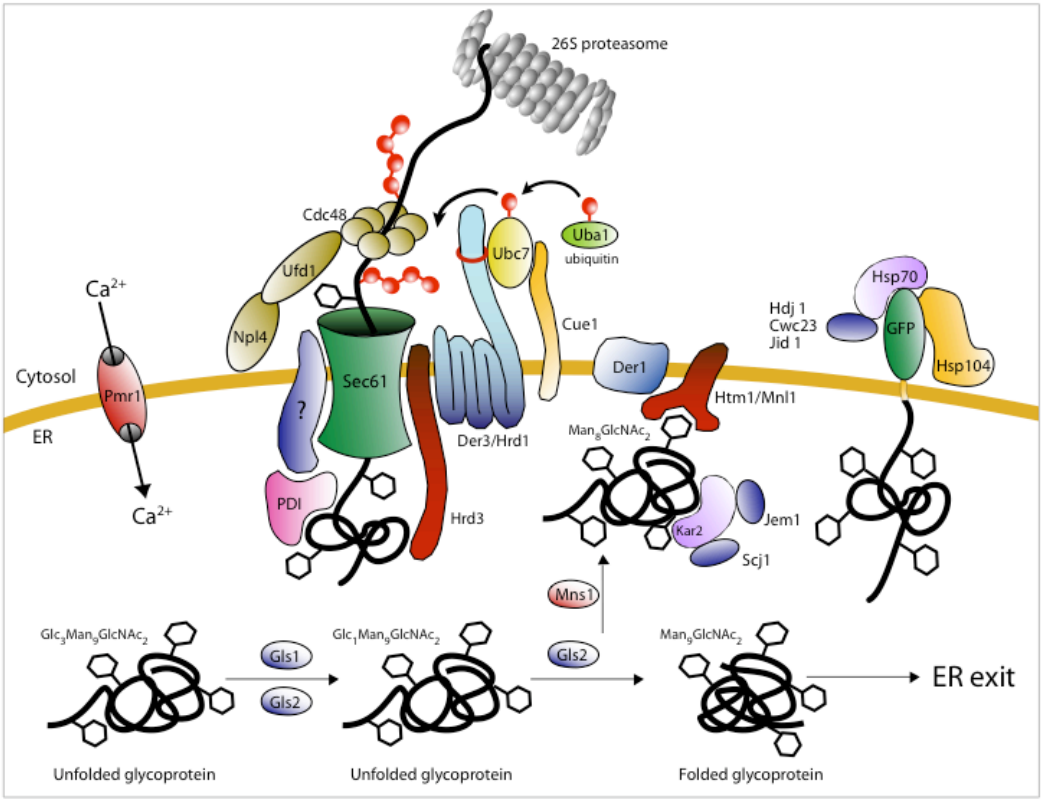

FIG: 4 Model of ER protein quality control system in yeast with all known components
(Figure reproduced from Kostova and Wolf. 2003)

ER quality control in health and disease

Knowledge of ER quality control is not only important for understanding the basic cellular physiology but it is also essential for understanding the reasons behind various physiological disorders in mammals. Human cytomegalovirus (HCMV) encodes two ER resident transmembrane proteins US2 and US11, that target MHC class I molecules to protesomal degradation. Each of these proteins can interact with heavy chains of MHC I molecules

mediating their extraction from the ER membrane. MHC class I is also polyubiquitinated and its elimination uses all the components involved in ER protein quality control. ER quality control is also involved in genetic disorders like cystic fibrosis and lung emphysema. (Plemper and Wolf, 2000). ER quality control is also involved in neurodegenerative diseases like Wilson disease, prion diseases like BSE, scrapie, or CJD (Plemper and Wolf, 2000).

Aim of the work

In order to gain deeper insight into the molecular mechanisms of protein quality control and ER-associated degradation new components involved in this vital physiological process in the cell should be uncovered and analyzed therefore a genome wide screen should be undertaken, using the EUROSCARF yeast library, consisting of about 5, 000 *Saccharomyces cerevisiae* strains, each deleted for a single non essential gene. A test had to be developed which is based on a growth phenotype to identify new mutants involved in ERAD. For this purpose a chimeric protein CTL* (CPY*-transmembrane domian-Leu2p) had to be constructed as a model substrate. The reasoning behind this screen rests in the fact that when transformed with CTL*, wild type strains are unable to grow in media lacking leucine because of an intact ERAD system and therefore facilitates degradation of CTL*, while mutant strains are able to grow because of a defective ERAD system, unable to degrade CTL* and by this complementing the *leu2* auxotrophy of the respective mutant strain. Mutants should be selected, complementivity gene had to be identified and the function of the respective protein in quality control or ERAD should be uncovered.

Materials and Methods

Media, Buffers, Solutions

YPD
(Used for growing yeast in liquid culture)

Yeast extract..... 4g

Bacto peptone..... 8g

D-glucose..... 8g

DH_2O..... 400ml

pH 5.5, autoclaved

YP+ agar
(used for growing yeast in solid media)

a)

Yeast extract..... 12.4g

Bacto peptone..... 6.2

DH_2O..... 300ml

pH 5.5, autoclaved

b)

Agar..... 12g

DH_2O..... 300ml

autoclaved

Before plating, mix autoclaved YP and agar and, add 2% D-glucose (autoclaved) to it.

Critical Minimum (CM) medium
Yeast nitrogen base..... 4.15g

Droup out mix..... 1.24g

DH_2O..... 300ml

pH 5.6, autoclaved

For growing yeast on liquid CM medium

Mix 300ml CM medium + 300ml of autoclaved DH_2O and add 30mM adenine, 20mM uracil, 100mM leucine 60mM histidine 100mM lysine 40mM tryptophan, 2%D-glucose (always

omit the required amino acid or nucleotide, when you are selecting the yeast for that particular marker)

For growing yeast on solid CM medium

Agar..... 12g

DH_2O..... 300ml

autoclaved

Before plating mix 300ml of CM medium +300ml of agar and add 30mM adenine 20mMuracil, 100mM leucine, 60mM histidine 100mM lysine 40mM tryptophan, 2%D-glucose (always omit the required amino acid or nucleotide when you are selecting the yeast for that particular marker).

Sporulation medium

CH_3COOK.....10g
Bacto-yeast extract.....1g
D-glucose.....0.5g
Bacto-agar.....20g
DH_2O.....to 1000ml
autoclaved

LB

(used for growing *E.coli* in liquid media)

Bacto tryptone peptone.....4g

Yeast extract.....2g

Sodium chloride.....2g

DH_2O.....400ml

pH7.5, autoclaved

2x LB

(used for growing *E.coli* in solid medium)

a)

Bacto trypton peptone.....6.2g

Yeast extract.....3.1g

Sodium chloride.....3.1g

DH2O..... 300ml

pH7.5, autoclaved

b)

Agar.....12g

DH_2O.....300ml

autoclaved

Before plating mix 300ml of 2x LB and 300ml of agar

SOC

Bacto trypton.....2g

Yeast extract.....0.5g

D-glucose.....0.4g

NaCl.....2mM

Kcl.....0.25mM

$MgCl_2$.....1mM

$MgSO_4$.....1mM

DH_2O.....100ml

pH7.4, autoclaved

Buffers and solutions for chemically competent *E.coli*

TC buffer

$CaCl_2$.....75mM

TRIS-HCl pH 7.5.....10mM

DH_2O.....to 100ml

autoclaved

TC: glycerol buffer

TC buffer and Glycerol were mixed in the ratio of 85:15(V/V)

autoclaved

Other solutions

$MgCl_2$.....100mM

autoclaved

Buffers and solutions for DNA gels

50X TAE buffer (Tris – acetate- EDTA)

TRIS base.....242gm

CH_3COOH.....57.1ml

0.5M EDTA pH 8.....100ml

DH_2O.....to 1000ml

DNA loading buffer

Glycerol.....30%

BPB.....0.25%

DH_2O.....to 100%

Gel composition

1x TAE+ 1% or 0.8% agarose (W/V)+0.00005g/l ethidium bromide

Running buffer

1X TAE buffer

1kb DNA ladder

1 kb ladder.....10µl

λ buffer.....10µl

1X TE.....80µl

DNA loading dye.....20µl

Buffers and solutions for SDS-PAGE

SDS PAGE loading buffer

TRIS-Hcl pH 6.8.....40mM

Urea.....8M

SDS.....5%

EDTA pH 8.....100mM

Bromophenol blue.....40mg

DH_2O.....200ml

Add 1.5% of beta mercapto-ethanol freshly to the above buffer before use

4X Stacking buffer
TRIS-Hcl pH 6.8.....0.5M

SDS.....0.4%

DH_2O.....250ml

4X Separating buffer
TRIS-Hcl pH 8.8.....1.5M

SDS.....0.4%

DH_2O.....250ml

Gel composition
Table 1: composition for SDS-PAGE gels

% of gel	Acrylamide (40%) stock	DH_2O	4X separating buffer
7.5%	3ml	6ml	3ml
8%	3.2ml	5.77ml	3ml
9%	3.6ml	5,37ml	3ml
10%	4ml	4.97ml	3ml
12%	4.8ml	4.17ml	3ml

Mixture was polymerized by 60µl of 10%APS and 6µl of TEMED

Stacking gel
40% acrylamide: bisacrylamide mix.....0.65ml

4X stacking buffer.....1.25ml

DH_2O.....3.05ml

10%APS.....10 l

TEMED.....5 l

Running buffer
TRIS-Hcl.....3.03g

Glycine.....14.4g

SDS.....1g

DH_2O.....to 100ml

Buffers and solutions for western blotting

5X Western transfer buffer
TRIS.....6.5g

Glycine.....72g

DH_2O.....1000ml

While blotting 62.5ml of 5X blotting buffer was mixed with 50ml of methanol and 200ml of DH_2O.

10X PBST
Na_2HPO_4.....80mM

NaH_2PO_4.....20mM

NaCl.....100mM

Tween 20.....10ml

DH_2O.....to 1000ml

pH 7.5

Blocking buffer
1X PBST+10%(W/V) non-fat dry milk powder

Washing buffer
1XPBST

Ponceau S stain
Acetic acid.....1%

Ponceau S Stain.....0.5%

DH_2O.....to 100%

Stripping buffer
TRIS-HCl pH 6.7.....62.5mM

SDS.....2%

ß- Mercapto ethanol.....100mM

DH_2O.....to 500ml

Buffers and solutions for pulse chase

Breaking buffer (BBI)

TRIS-HCl pH7.5.....50mM

Urea.....6M

SDS.....1%

EDTA pH 8.....1mM

DH_2O.....100ml

IP buffer

TRIS-HCl pH7.5.....50mM

NaCl.....190mM

TritonX-100.....1.25%(V/V)

EDTA pH 8.....6mM

DH_2O.....to 200ml

Labeling medium

Yeast nitrogen base w/o ammonium sulfate and w/o amino acids.....1.7g

D-glucose..... 1g

Adenine.....20mg

Uracil.....20mg

Tryptohan.....20mg

Histidine.....20mg

Arginine.....30mg

Tyrosine.....30mg

Lysine.....30mg

Leucine.....30mg

Phenylalanine.....50mg

Glutamic acid.....100mg

Aspartic acid.....100mg

Valine.....150mg

Threonine.....200mg

Serine.....400mg

While preparing omit the auxotrophic marker for respective plasmid selection

pH 6, sterile filtered

NOTE: *For labeling Δdsk2Δrad23 and corresponding WT, and single Δdsk2 and Δrad23 deletions normal CM medium (W/o methionine) was used as labeling medium because Δdsk2Δrad23 strain can not take isotope in the normal labeling medium.*

Chase medium
Same as labeling Medium but add

Methionine.....6mg/ml

BSA.....2mg/ml

NOTE: *for Δdsk2Δrad23 and it's corresponding wild type and single deletions normal CM medium w/o methionine with 2% glucose and supplemented with 6mg/ml of methionine and 2mg/ml of BSA is used as chase medium*

Buffers and solutions for preparing yeast spheroplasts

Tris buffer
TRIS-Sulphate pH9.4.....100mM

DTT.....20mM

DH_2O.....to 100ml

Sorbitol buffer
Sorbitol.....1M

Sodium phosphate pH 7.4.....50mM

DH_2O.....to 100ml

Lysis buffer (PS200)

Sorbitol.....200mM

Pipes pH 6.8.....20mM

$MgCl_2$.....5mM

PMSF.....1mM

KSCN (prevent aggregates formation).....1M

Urea (prevents aggregates formation).....2.5M

Protease inhibitor cocktail EDTA free.....1 tablet

DH_2O.....to 100ml

Buffers and solutions for Endo H treatment

Breaking buffer
Same as described in buffers for pulse chase

IP buffer
Same as described in buffers for pulse chase

Wash buffer
Potassium phosphate 50mM

SDS.....0.02%

DH_2O.....to 50ml

Endo H buffer
Wash buffer+0.7% beta mercapto ethanol

Buffers and solutions for detection of ubiquitinated proteins

Washing buffer
Sodium azide.....20mM

PMSF.....0.1mM

NEM.....20mM

(both PMSF and NEM are to be added freshly)

Sorbitol buffer
Sorbitol.....700mm

TRIS-HCl pH 7.5.....50mM

PMSF.....1mM

NEM.....20mM

(both PMSF and NEM are to be added freshly)

IP buffer
Same as described in buffers for pulse chase

Pellet solubilization buffer
SDS.....1%

TRIS-HCl pH7.5.....50mM

DH2O.....to 100µl

Buffers and solutions for yeast transformation

10XTE buffer
TRIS-HCl pH 7.5.....100mM

EDTA pH 8.....10mM

DH_2O.....to 100ml

LiTE buffer
10XTE.....10ml

CH_3COOLi.....100mM

DH_2O.....to 100ml

PEG-LiTE
10XTE.....10ml

CH_3COOLi.....100mM

50%PEG 3350.....80ml

ss DNA buffer

Tris.....10mM

NaCl.....10mM

EDTA pH 8.....1mM

DH_2O.....to 10ml

pH 8

10mg/ml of Herring Sperm DNA is dissolved in this buffer

Buffers and solutions for isolating yeast genomic DNA

Lysis buffer
NaCl.....100mM

SDS.....1%

Tris pH 8.....10mM

EDTA pH 8.....1mM

Triton X 100.....2%

DH_2O.....to 5ml

10XTE
Same as described in buffers and solutions for yeast transformation

TRIS-NaCl buffer
TRIS-HCl pH7.5.....10mM

NaCl.....15mM

DH_2O.....to 5ml

10mg/ml of RNAase is dissolved in this buffer freshly

Other solutions

Phenol, chloroform, isoamylalchol (25:24:1)

100% ethanol extra pure

4M ammonium acetate

Buffers and solutions for isolating plasmid DNA from *E.coli*

STET buffer
Sucrose.....8g

Triton X 100.....5%

TRIS-HCl pH7.5.....25mM

EDTA pH 8.....50mM

10X DNA loading dye.....1ml

DH_2O.....to100ml

Other Solutions
Phenol, chloroform, isoamylalchol (25:24:1)

Plasmids used in this study

(Protein are expressed under the control of own promoters unless and until specifically mentioned)

Table 2

Plasmid	Insert	Source
pNEB193	*E. coli* plasmid	New England Biolabs
pUC19	*E. coli* plasmid	New England Biolabs

pRS313	Yeast and *E. coli* shuttle centromeric plasmid with *HIS3* marker	Guthrie, C et al., 1991
pRS315	Yeast and *E. coli* shuttle centromeric plasmid with *LEU2* marker	Guthrie, C et al., 1991
pRS316	Yeast and *E. coli* shuttle centromeric plasmid with *URA3* marker	Guthrie, C et al., 1991
pRS406	Yeast and *E. coli* shuttle integrative plasmid with *URA3* marker	Guthrie, C et al., 1991
psec61-2	pRS406 expressing *sec61-2*	Biederer et al., 1996
pCTG* with *TDH3* promoter	pRS 316 expressing CPY* and transmembrane domain followed by GFP	Taxis et al., 2003
pCTG* with *CPY* promoter	pRS 316 expressing CPY* and transmembrane domain followed by GFP	Taxis et al., 2003 (subcloning under CPY promoter by Z. Kostova)
pΔssCPY*-GFP	pRS316 expressing a cytosolic version of CPY*-GFP	This study (Cloned by Z. Kostova)
pΔSSCPY*	pRS316 expressing cytosolic CPY* (without signal sequence)	This study (Cloned by Z. Kostova)
pDeg1-GFP	pRS416 expressing expressing Deg1-GFP-GFP under the control of CUP promoter	Lenk et al., 2000
pDeg1-1-URA3	YCplac22 Expressing Deg1-1-URA3	Swanson et al., 2001
pCPY*-HA in pRS 316	pRS316 expressing CPY*HA under the control of *TDH3* promoter	Taxis et al., 2002

pCPY*-HA in pRS 313	pRS313 expressing CPY*HA under the control of *TDH3* promoter	Taxis et al., 2002
pCPY*	pRS316 expressing CPY*	Knop et al., 1996b (subcloning in pRS316 by R. Hitt)
pCT*-URA3	pRS315 expressing CT*-Ura3p under the control of *TDH3* promoter	This study
pCT*-Linker-URA3	pRS315 expressing CT*- followed by a linker of 110 amino acids and Ura3p	This study
pCT*-Deg1-1-URA3	pRS315 expressing CT*- followed by Deg1-1 and Ura3p under the control of *TDH3* promoter	This study
pSec61-2-URA3	pRS315 expressing a Sec61-2-Ura3p fusion	This study
pSec61-2-Deg1-1-URA3	pRS315 expressing Sec61-2 followed by Deg1-1 and Ura3p	This study
pCTL* on *TDH3* promoter	pRS316 expressing CT* followed by LEU2 under the control of *TDH3* promoter	This study
pCTL* on *CPY* promoter	pRS316 expressing CT* followed by LEU2 under the control of *CPY* promoter	This study
pCTL* on *PDR5* promoter	pRS316 expressing CT* followed by LEU2 under the control of *PDR5* promoter	This study
pCTL* on *GAL4* promoter	pRS316 expressing CT* followed by LEU2 under the control of *GAL4* promoter	This study

pSec61-2-LEU2	pRS316 expressing a Sec61-2 followed by Leu2p	This study
pSec61-2-LEU2 on *GAL4* promoter	pRS316 expressing Sec61-2 followed by Leu2p under the control of *GAL4* promoter	This study

Bacterial strains

For the amplification of plasmid DNA in *E. coli* DH5α, a mutant strain for DNAases is used.

***Saccharomyces cerevisiae* strains used in this study**

Entire EUROSCARF yeast gene deletion library comprising of 5, 000 viable single deletions of yeast is used in this study, other strains used in this study are listed in the table below, which includes yeast strains from EUROSCARF deletion library also when they are used in experiments other then growth phenotype test for genetic screen.

Table 3

Strain	**Genotype**	**Source**
BY4743WT	*MATα/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0*	EUROSCARF
BY4743Δ*der3*	*MATα/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, der3Δ::kanMX4/ der3Δ::kanMX4*	EUROSCARF
BY4743Δ*dsk2*	*MATα/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, der3Δ::kanMX4/ der3Δ::kanMX4*	EUROSCARF
BY4743Δ*rad23*	*MATα/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, rad23Δ::kanMX4/ rad23Δ::kanMX4*	EUROSCARF
BY4743Δ*yos9*	*MATα/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, yos9Δ::kanMX4/ yos9Δ::kanMX4*	EUROSCARF
BY4743Δ*rpn10*	*MATα/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, rpn10Δ::kanMX4, rpn10Δ::kanMX4*	EUROSCARF

BY4743Δ*rad9*	*MATα/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, rad9Δ::kanMX4, rad9Δ::kanMX4*	EUROSCARF
BY4743Δ*mad2*	*MATα/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, mad2Δ::kanMX4, mad2Δ::kanMX4*	EUROSCARF
BY4743Δ*mad3*	*MATα/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, mad3Δ::kanMX4, mad3Δ::kanMX4*	EUROSCARF
MY3589	*MATa, ura3-52, leu, ade2*	Biggins, et al., 1996
MY3588	*MATa, ura3-52, leu2, his3, dsk2Δ::LEU2*	Biggins, et al., 1996
MY3592	*MATα, ura3-52, leu2, his3Δ200, ade2, dsk2Δ::LEU2, rad23Δ*	Biggins, et al., 1996
MY3587F	*MATα, ura3-52, leu2, his3, ade2, rad23Δ*	Rao et al., 2002
YPH499Y	*MATa, ura3-52, leu2Δ1, his3Δ200, trp1Δ63, lys2-801, ade2-101, prc1-1*	Hiller, et al., 1996
CMY762Y	*MATa, ura3-52, leu2Δ1, his3Δ200, cim3-1, prc1-1*	Hiller, et al., 1996
YCT397	*MATa, ura3-52, leu2-3, 112, his4-519, ade1-100, prc1-1*	Taxis, et al., 2003
YCT415	*MATa, ura3-52, leu2-3, 112, his4-519, ade1-100, prc1-1, ufd1-1*	Taxis, et al., 2003
YWO470	*W303-1C, Matα, ura3-52, leu2Δ1, his3Δ200, trp1Δ63, lys2-801, ade2-101, prc1-1, sec61-2*	Plemper et al., 1997
YBB5	*W303-1C, Matα, ura3-52, leu2Δ1, his3Δ200, trp1Δ63, lys2-801, ade2-101, prc1-1, sec61-2, yos9Δ::KanMX4*	B. Buschhorn
DCY1180	*MATa, bar1Δ*	Clarke, et al., 2001
DCY1283	*MATa, bar1Δ, rad23Δ::KAN^R, ddi1Δ::KAN^R*	Clarke, et al., 2001
DCY1264	*MATa, bar1Δ, rad23Δ::KAN^R*	Clarke, et al., 2001
DCY1258	*MATa, bar1Δ, ddi1Δ::KAN^R*	Clarke, et al.,

EGY128	*MATa ura3, leu2, his3, trp1*	Kim, et al., 2004
YHR83	*MATa, ura3, leu2, his3, trp1, Δrad23::KAN^R*	Kim, et al., 2004
YHR84	*MATa, ura3, lue2, trp1, Δrad23::KAN^R, Δufd2::HIS3*	Kim, et al., 2004

Molecular Biology Methods

Isolation of plasmid DNA

Plasmid DNA was isolated using the Qiaprep Spin miniprep kit (Qiagen, Santa Clarita, CA, USA and PEQ lab GmbH, Germany). The plasmid isolation from the bacterial cells was performed according to the manufacturer's protocol and purity was evaluated by agarose gel electrophoresis.

DNA agarose gel electrophoresis

The analytical and preparative separation of DNA molecules of different sizes was carried out on agarose gels cast in horizontal flabed chamber (Bio-RAD). Agarose was dissolved in 1XTAE buffer to a final concentration of 0.8% to 3% (W/V) depending upon necessity, by boiling in a microwave, before casting 0.0005g/1 of ethidium bromide was added to gel to visualize the DNA bands. 1XTAE buffer was used for running the gels, the DNA samples mixed with 4μl of DNA loading buffer and run along with the molecular weight marker at 110V, until the blue dye reaches the end of the gel. The DNA bands were visualized by ethidium bromide fluorescence and photographed.

Purification of DNA

PCR products were purified by using the QUIGEN PCR purification kit, and from the agarose gels using the QUIAEX DNA purification kit, The DNA purification was carried as per the protocol supplied by the manufacturer.

Digestion of DNA with restriction endo nucleases

The reaction for the individual restriction enzymes was based on the information of manufacturers protocol. All restriction enzymes used in this study are from the Roche and

New England Biolabs (NEB); the digestion reactions were performed in water with a total volume of 20µl. Using adequate concentration of DNA, 2µl of reaction buffer, 10mg/ml BSA (for certain enzymes only) and 1 to 10 U restriction enzyme were used. Reactions were carried out at 37°c to 75°C (as per manufacturer's protocol) for 1 to 3 hours (as per manufacturer's advise).

Oligonucleotides

All oligo nucleotides used in this study were synthesized by MWG Biotechnilogy Inc.

Polymerase Chain Reaction

All the PCR reactions used in the study are carried out in robocycler´s from Stratagene. Denaturation, annealing and extension were carried out at specific temperatures for certain time period depending on the T_m value of DNA and the size of the final product. In most cases the reaction were carried out in DH_2O with a total volume of 50µl and 1pmol/µl concentration of primers were used, yeast genomic DNA and Plasmid DNA were mostly used in this study as templates for the PCR reaction.

Ligation of DNA fragments

DNA ligase catalyzes the formation of phosphodiester bonds between 5`phosphate and 3´hydroxyl group of double stranded DNA. To enhance the ligation efficiency different vector to insert ratios (1:2 or 1: 3 or 1:4) and ATP were used. A typical protocol was as follows

Vector 2µl
Insert.....6µl
10X ligation buffer.....1.5µl
ATP.....0.66mM
T4 DNA ligase (500U/µl) 1µl
Autoclaved DH_2O.....to 15µl
Reaction mixture was incubated for ligation at 16°C overnight.

Preparation of chemically competent *E.coli* cells

Chemically competent DH5α were prepared by following way, Logarithmically growing 50 ml of *E.coli* (A_{600} 0.4-0.5) were harvested at 3000 rpm and washed once with ice cold 50ml of 100mM $MgCl_2$ at 4°C, the pellet was resuspended in 30ml of ice cold TC buffer and leave cells in TC buffer at 4°C for 30 min, pellet the cells as described above and resuspend the pellet in 2ml of ice cold TC: glycerol buffer, aliquot 100µl of samples in sterile eppendorfs and store them at –80°C.

Transformation of chemically competent *E.coli* cells by heat shock method

Chemically competent DH5α *E. coli* cells were thawed on ice and 100µl of cells were mixed with 5µl-15µl of ligation mixture or 2µl of plasmid DNA and incubated on ice for 30 minutes. After a heat shock at 42°C for 80-90 seconds, followed by incubation on ice for 20 minutes. Later the reaction mixture was added to 1ml of SOC medium and incubated at 37°C for 45-60 minutes. 200- 300µl were spread on LB_{amp} plates and incubated overnight at 37°C.

Transformation of yeast

Yeast transformation were done by lithium acetate method as follows, logarithmically growing yeast cells (A_{600} 0.4-0.6) of 20ml were harvested at 3000rpm and the pellet was washed twice with equal volume of autoclaved sterile DH_2O and once with equal volume of LiTE buffer, later the pellet was suspended in 500µl of LiTE buffer and cells were incubated at 30°C for 30 min, 5.5µl of ssDNA and the plasmid DNA were added to 100µl of competent yeast cells and incubated at 30°C for 30 min, later 1ml of PEG-LiTE was added to the cells and the mixture is vortexed gently and the cells were incubated at 30°C for 45 min followed by heat shock at 42°C for 17min, later the cells were harvested at 2000rpm for 1 min and the PEG solution is completely removed from the cells and the cells were suspended in 100µl of sterile DH_2O and spread on CM plates lacking the particular amino acid or nucleotide and incubated at 30°C for 3 days.

Transformation of Yeast by Multi well Transformation Assay

Yeast strains from the EUROSCARF deletion bank were transformed in multi well plates for genomic screen in this study by using the protocol described below.

1) Yeast strains were inoculated in 600µl of YPD in a multi well transformation plate of 24 wells and grown overnight at 30°C

2) Yeast culture mostly at stationary phase next day were diluted by adding 80µl of o/n Culture to 600µl of YPD in multi well transformation plate and grown at 30°C for 4 hours (after this period most of the strains are at A_{600} 0.4-0.6)
3) Yeast cells were harvested at 3000rpm for 10 min and the pellet was washed twice with 600µl autoclaved DH_2O and the pellet was resuspended in 80µl of LiTE buffer and cells were incubated at 30°C for 30min, later 10µl of ssDNA and plasmid DNA were added to each strain and incubated at 30°C for 30 min.
4) 300µl of PEG-LiTE was added and incubated again at 30°C for 45min, later 50µl of DMSO was added to each well and heat shock was done at 42°C for 17min (heat shock is done in a water bath by keeping the para film firm around the 24 well plates such that water will not enter inside) later PEG and other buffers were removed by spinning the cells at 3000rpm for 10 min.
5) Add 600µl of respective CM medium (CM medium w/o uracil in this study) and grow them by shaking at 30°C for more then 16 hours.
6) Plate the cells (plate them as drops by using the multi channel pipette, mostly 2 or 3 drops will work) on selection media allow the drops to dry under sterile bench and incubate the plates at 30°C for 3 days.
7) Multi well transformation plates can also be incubated at 30°C after keeping drops for 2 days and if colonies fail to emerge on plates, then streaking cells from well by loop or a tooth pick may help some times.

Generation of constructs

CTL*

CTL* with *TDH3* promoter

pRS316 expressing CTL* with *TDH3* promoter was cloned in a Stepwise process: the *LEU2* gene was PCR-amplified using the primers 5´lPac (GGATCCTTAAGTCTGCCCCTAAGAAG) and 3´l Sph (GTACAGGCATGCATAAATGTAGATTG) and pRS315 as template, the 1.1 KB fragment was cloned into pNEB193 between PacI and SphI restriction sites .The 2.5kb CT* fragment obtained by digesting pMA1 with KpnI and PacI was cloned into the respective sites of

pNEB193-LEU2, yielding pNEB193-CTL*. CTL* was then transferred into pRS316 as a 3.7kb HindIII fragment, giving rise to pRS316-tdh3-CTL*.

CTL* with *CPY* promoter

CTL* with *CPY* promoter was cloned as follows: the 1.8kb fragment obtained by digestion of pRS316-tdh3-CTL* with BglII and HindIII sites (includes 3`end of CT* followed by *LEU2* gene), similarly pRS316 expressing CPY* was also digested with BglII and HindIII sites to remove the 3´end of CPY* gene, later the 1.8kb digested fragment which consists of 3´end of CT* followed by *LEU2* gene was cloned into respective sites of pRS316 CPY*, yielding pRS316-CPY-CTL*.

CTL* with *GAL4* promoter

The TDH3 promoter of pRS316-tdh3-CTL* was replaced by *GAL4* promoter as follows: the GAL4 promoter was PCR-amplified using the primers 5´GAL4CTLNotI (AAATATGCGGCCGAGGACCCTGACGGCGA) and 3´GAL4CTL (GTAAACTGGTGAATGCTTTCATCTTTCAGGCTTGCTTC) the later being partially complementary to both GAL4 and the 5´end of prc1-1 and genomic DNA was used as template. A 500bp N terminal fragment of CPY* was PCR amplified with the primer set 5`CTLGAL4 (GAAGCAAGCCTCCTGAAAGATGAAAGCATTCACCAGTTTAC) having partial complementarity both to CPY* and 3`end of *GAL4* promoter, and 3`CTLBsu36I (GAAGTTATAGACATCCTTACC) and pRS316 expressing CPY* was used as template, 2 PCR products were used as overlapping templates in a third PCR to generate a final promGAL4: prc1-1 fusion fragment, which was cloned to NotI-Bsu36I-digested pRS316-tdh3-CTL*, generating pRS316-gal4-CTL*.

Sec61-2-Leu2p

Sec61-2-Leu2p on *SEC61* promoter

PRS 316 expressing Sec61-2-Lue2p was cloned in a Step wise process: the *sec61-2* genes was PCR-amplified by using the primers 5´Sec61XbaI (CCTCCAACCGTGTTCTAGACTT) and 3´Sec61ura3 (CCTTATATGTAGCTTTCGACATGATCATCAAATCAGAAAATCCTGG) the later being partially complementary to both *sec61-2* and 5énd of ura3 and pRS406 expressing *sec61-2* was used as template, *URA3* gene was PCR amplified with the primer set 5´sec61ura3 (CCAGGATTTTCTGATTTGATGATCATGTCGAAAGCTACATATAAGG)

having partial complementarity both to *URA3* and 3´end of sec61-2 and 3úra3SacI (GGTTCTGGCGAGCTCTTGGATAGTTCC) and genomic DNA was used as template, two PCR products were used as overlapping templates in a third PCR to generate a final sec61-2-ura3 fragment between XbaI and SacI sites. pRS406 expressing *sec61-2* was digested with XbaI and SacI and 2.5kb fragment of sec61-2-ura3 was cloned into XbaI-SacI digested pRS406-sec61-2 to generate pRS406-sec61-2-ura3, *URA3* was replaced with *LEU2* by a PCR reaction as follows: 1.1 kb of *LEU2* gene was PCR amplified by using the primer set 5´leusec61BclI (GCACTGATCATTATGTCTGCCCCTAAGAAG) 3´leusec61Sacx1 (GGTGGCGAGCTCATAAATGTATGTAGATTG) both the PCR product and the pRS406-sec61-2-ura3 were digested with XbaI and SacI and the *LEU2* gene was cloned into XbaI-SacI digested pRS406-sec61-2-ura3 backbone to generate pRS406-sec61-2-leu2 2.6kb fragment of sec61-2-leu2 from pRS406-sec61-2-leu2 was cloned into pRS316 at the HindIII and SacI sites to generate pRS316-sec61-2-leu2.

Sec61-2-Leu2p on *GAL4* promoter

The *SEC61* promoter of pRS316-sec61-2-leu2 was replaced by *GAL4* promoter as follows: the *GAL4* promoter was PCR-amplified by using primer set 5´sec61-leu2gal4HindIII (GCACCCAAGCTTGGAGGACCCTGACGGCGA) 3´sec61-leu2gal4XbaI (AAACAAGTCTAGAACACGGTTGGAGGACATCTTTCAGGAGGCTTGCTT) and genomic DNA was used as template, both pRS316-sec61-2-leu2 and the *GAL4* prompter PCR product were digested with HindIII and XbaI enzymes, and the *GAL4* promoter was cloned into HindIII-XbaI digested pRS316-sec61-2-leu2 to generate pRS316-GAL4-sec61-2-leu2.

Protein Biochemistry

Preparation of samples for SDS-PAGE

Yeast cultures of required amount (2-4 OD) were harvested at 14, 000rpm for 2 min, later the pellet was resuspended in 1ml of DH_2O and to it freshly prepared 150µl of NaOH and β-me mix was added (925µl of 2M NaOH and 75µl of13.3M β-me) and cells were kept on ice for 10 min with a brief vortex of 10 sec for every 3 min, later 150µl of 50% TCA (trichloroacetic acid) was added to the samples and they were kept on ice for 10 min with a brief vortex of 10 sec for every 3 min as described above, later the samples were centrifuged at 14, 000rpm in table top centrifuge for 10min and the supernatant was completely removed, resususpend the

pellet in 100µl of SDS-PAGE loading buffer (if the samples are yellow due to the traces of TCA add 2µl of 2M tris base to make them blue) and vortexed in a multivortexer at max speed for 20 min at 37°C.

SDS-PAGE

The separation of proteins according to their molecular weight was performed on polyacrylamide gels with a polyacrylmide concentration of 7 to 12%; 0.75-1.5mm thickness and a separating distance of 7.3cm were used. Gels were casted using Bio-Rad gel casting slabs and stands, 5cm height of resolving gel followed by 3cm height of stacking gel were casted, samples were run along with the standard protein marker from See Blue at 107V, 25mA through the stacking gel and at 150V, 40mA through the resolving gel.
Western Blotting.

Protein Transfer

Proteins were transferred onto nitrocellulose membrane using the LTF- laborlechnik blot chamber by semi- dry blotting method. The western blot sandwich was prepared using 3 layers of Whatman paper (7.5cm X 10.5cm) soaked in blotting buffer followed by the SDS polyacrylamide gel rinsed with blotting buffer following nitrocellulose membrane (8cm X 6cm) soaked in blotting buffer followed by 3 layers of Whatman paper again, Protein transfer was carried out by applying a weight on the blotting chamber and at 75-80mA/gel and 6V for 70min.

Immunodetection

After electrotransfer the blot was incubated in blocking solution (1XPBST + 10%nonfat milk powder) for 1-2 hours at RT or for o/n at 4°C to inhibit non-specific binding of antibodies.
After washing the gel with (3X10min) washing buffer (1XPBST), the gel was incubated with Required concentration of primary antibody (for concentration of individual antibody see antibodies section) for 1hr at RT or for o/n at 4°C (for anti-Sec61, anti-ubiquitin), followed by washing the gel with wash buffer (5X10min) to remove unbound antibody, then incubated in horseradish peroxidase (POD) coupled secondary antibody in 1XPBST (at 1:10,000 dilution) for 60 min. the blot was washed again with 1XPBST buffer (5X10min). Detection was performed by incubating the blot for 1 min in a solution (ECL from Amersham Biosciences) containing a specific substrate for POD, it's enzymatic activity triggers a luminescence reaction which was detected by exposure against the high performance chemiluminescence

film (Amersham Biosciences) for 20sec to 30min, depending on the intensity of signal and type of antibody used.

Stripping and reprobing of membranes

Stripping was done by the addition of 20ml of stripping buffer to membranes and by incubating them at 65°C for 25min, later the nitrocellulose membrane was washed with 1XPBST buffer (7X10 min) until the smell of ß-mercaptoethanol is gone, followed by blocking and immunodetection with desired antibody was done as described above.

Antibodies

Polyclonal anti-CPY was used for immunoprecipitation of CPY* and CTG*(Finger et al., 1993). Monoclonal anti-CPY and monoclonal anti-GFP (Molecular Probes) and polyclonal anti-Sec61 (gift from T. sommer) and polyclonal anti-Kar2 (gift from R. Schekman) were diluted at 1:10,000 for immunoblotting. Monoclonal anti-HA (Covance) was used at 1:10,000 dilutions for immunoblotting and 1:1,000 dilutions for immunoprecipitation. Monoclonal anti-GFP (Molecular Probes) was used at 1: 200 dilution for immunoprecipitation. Monoclonal anti-ubiquitin antibody (Covance) was used at 1:1,000 dilutions for immunoblotting. HRPO conjugated goat anti-mouse (Dianova) and HRPO conjugated rabbit anti-mouse (Sigma) were used as secondary antibodies at 1:10,000 dilution for immunoblotting.

Cycloheximide decay analysis

Cells were grown at 30°C to logarithmic phase (A_{600}1-1.3) in synthetic complete medium and the temperature sensitive strain *cim3-1* was grown at 25°C and then shifted to restrictive temperature (30°C) for 1 hour. Deg1-GFP expressed under the control of CUP promoter, was induced for 1 hour by addition of 100µM $CuSO_4$ 10-15 OD (A_{600}) of cells were collected and harvested at 3, 000rpm for 3 min the pellet was suspended in 2.5ml of fresh medium and 0.25mgml^{-1} of freshly prepared Cycloheximide was added to inhibit protein synthesis. 2-2.5 (A_{600}) of cells were taken at indicated time points, and to them equal volume of 30mM NaN_3 was added and cells were frozen at –80°C. Later preparation of protein samples for SDS-PAGE, Western blotting and Immunodetection were carried as described above.

Pulse-chase analysis and Immunoprecipitation

Pulse chase

Cells were grown at 30°C to logarithmic phase (A_{600} 0.8-1.3) and 10 OD of cells (A_{600}) were collected and harvested at 3, 000rpm for 3 min, later the cells were washed 3 times with 1ml of the labeling media and the pellet was resuspended in 1ml of the labeling media at 30°C for 50 min. (**NOTE:** *for Δdsk2Δrad23 and it's corresponding wild type and single deletions, normal CM medium with 2% of glucose and w/o methionine was used as labeling medium*), later add 20-25µCi of S^{35} methionine (from Amersham Biosciences) and incubated for 20 min (**NOTE:** *for Δdsk2Δrad23 and it's corresponding wild type and single deletions, labeling with S^{35} was done for 60 min*), then stop labeling by adding 1 ml of pre warmed chase medium **(NOTE:** *for Δdsk2Δrad23 and it's corresponding wild type and single deletions normal CM medium w/o methionine with 2% glucose and supplemented with 6mg/ml of methionine and 2mg/ml of BSA is used as chase medium)*/ and mix, then take 2OD of cells at indicated time points in screw capped eppendorfs containing 50µl of 110% TCA (W/V) and freeze the cells at –80°C. thaw the samples at 37°C and remove the supernatant by spinning the cells at 13,000rpm for 8 min and later wash the cells with 1ml of ice cold acetone and remove acetone after centrifugating the samples at 13, 000rpm for 6 min, add 100µl of BBI buffer and 75µl of acid washed glass beads to the pellet and do lyse the cells by incubating at 95°C for 2 min and vortexing them in multi vortexer at max speed for 2 min , repeat this cycle (keeping the cells cells at 95°C, vortexing in multi vortexer) for 5 times.

Immunoprecipitation

Add 1ml of IP buffer (supplemented with 0.5%of Boehringer inhibitor cocktail mix) to cells after lysis and vortex them well and spin them for 15min at 13, 000rpm, take 950µl of the cell lysate into a new safe-lock eppendorf and add respective antibody for IP (2µl of monoclonal anti HA, 3.5µl of polyclonal anti-CPY, 5µl of polyclonal anti-GFP) and IP was done for 1 hr at RT or overnight at 4°C by rotating the samples, later add 80µl of Protein A-Sepharose from Amersham Biosciences (7% of Protein A Sepharose dissolved in IP buffer w/o triton) to the samples and rotate them for 1-2 hr at RT, later spin the samples at 3, 000rpm for 30sec and remove the IP buffer, wash the Sepharose pellet with 1ml of IP buffer for 3 times and proteins were denatured by addition of 45µl of Urea buffer with 1.5% of DTT and samples were incubated at 95°C for 5min.

SDS PAGE, gel drying and quantification of signals

Samples were run on 8%SDS gels as described above and later the gel was dried on the gel dryer for 90min at 65°C on a whatman filter paper. The dried gel was placed in a cassette with the BioMax enhancer (From Molecular Dynamics) for 2-3 days. Later the signals on the BioMax enhancer were developed by using the Phospho Imager (from Molecular Dynamics). The signals were quantified by using the Image Quant™ programme, V1.1 (from Molecular Dynamics).

Endo H treatment

3 OD of cells (A_{600}) were harvested at 3, 000 rpm for 3 min and later Cell lysis, immunoprecipitation were done as described above in pulse chase, the sepharose beads were later washed with 1ml of wash buffer and later the sepharose beads were incubated in 25µl of Endo H buffer with 1µl of Endo H enzyme (New England Biolabs), always include a control with out the addition of Endo H enzyme and reaction was carried out by incubating the samples at 37°C for120min, later 7µl of urea buffer (with 1.5%of DTT) and 2µl of DNA loading dye (to give blue colour to samples)were added and samples were heated at 95°C for 5min, later SDS-PAGE electrophoresis, western blotting immuno detection were done as described above.

Preparation of yeast spheroplasts and protease protection assay

Preparation of yeast spheroplasts

40 OD of logarithmically growing cells (A_{600} 0.8-1.2) were harvested at 3, 000 rpm for 3 min, later the pellet was resuspended in 4ml of tris buffer and cells were incubated in shaking water bath at 30°C for 20min, later the tris buffer buffer was removed by spinning the cells at 3000rpm for 3 min and 4ml of sorbitol buffer and 40µl of oxalyticase enzyme were added (from 5mg/ml stock solution) and spheroplasting was done for 30 min at 30°C in a shaking water bath, speheroplasting efficiency can be verified in microscope , later the buffer was removed by spinning the cells at 3, 000rpm for 3 min, resuspend the cells in 2ml of ice cold PS 200 buffer and spheroplasting was done in cold homogenizer at 4°C, cell debris can be removed by slow speed centrifugation (3, 000rpm for 5 min at 4°C) .

Protease protection

The spherplasts prepared as described above were divided into 3 equal fractions

a) Mix equal amount of spheroplast and ice cold PS200 lysis buffer
b) Mix equal amount of spheroplast and ice cold PS200 lysis buffer and 0.1mg/ml of trypsin or proteinase K
c) Mix equal amount of spheroplast and ice cold PS200 lysis buffer and 0.1mg/ml of trypsin or proteinase k and 1% of triton

The reaction was carried out in ice at 4°C for 30 min and the reaction was stopped by addition of 30mM PMSF to above samples and they were separated into microsome pellet and cytosolic supernatant fraction by ultracentrifugation at 55, 000 rpm for 30 min at 4°C and supernatant was carefully separated from pellet, proteins in the supernatant fractions were precipitated by addition of 1/10 volume of 50% ice cold TCA and cells were kept on ice for 10 min, later the samples were spinned at 13, 000 rpm for 10 min, supernatant was removed and the pellet was suspended in 40µl urea buffer with 1.5% of DTT and samples were vortexed in a multi vortexer at 30°C for 15 min, and to the microsomal pellet fractions obtained from ultracentrifugation, urea buffer with 1.5% of DTT was added directly to it and the samples were vortexed in a multi vortexer at 30°C for 15 min. Later SDS- PAGE electrophoresis, Western blotting and immuno detection were done as described above.

Detection of ubiquitinated proteins in membranes

Microsomes were prepared either by breaking the cells with glass beads or by spherplasting, by glass beads following procedure is used 50OD (A_{600}) of logarithmically growing yeast cells (A_{600}1-1.3) were harvested at 3, 000 rpm for 3 min and later the pellet was washed with 5ml of washing buffer (20mMNaN_3 supplemented with 0.1mM PMSF and 20mMNEM) once, later the pellet was washed in 2ml of sorbitol buffer and pelleted by spinning cells at 3, 000 rpm for 3 min, later the pellet is resuspended in 0.5ml of sorbitol buffer and cells were lysed by addition of 2/3 volume of glass beads and vortexing in multi vortexer at 4°C for 30 sec and later samples were kept on ice for 30sec, this cycle (vortexing for 30 sec and keeping on ice for 30sec) was repeated for 5 times and 1ml of sorbitol buffer was added to samples and they are mixed well, later cell debris and glass beads were removed by slow centrifugation (3, 000 rpm for 5 min), microsomes can be prepared also by spheroplasting as described above but the buffers were supplemented with 20mMNEM to prevent deubiquitination . Yeast lyastes prepared either by glass beads or by sheroplasting were separated into microsomal

pellet or the soluble cytoplasmic fractions by ultracentrifugation at 65, 000rpm for 30min later the IP buffer (0.1% SDS, 50mM TRIS HCl pH7.5, 1%Triton X 100, 5mM EDTA, 150mMNaCl) was added to supernatant and the pellet was solubulized in 100µl of pellet solubilization buffer and 900µl of IP buffer (165mM NaCl, 50mM TRIS-HCl pH 7.5, 1.1% Triton X 100, 5.5mM EDTA) were added and the samples were centrifuged at 13, 000rpm for 10 min to remove the insoluble material , respective antibody for IP was added in both pellet and supernatant fractions (5µl of polyclonal anti- CPY antibody was used in this study) and IP was done o/n for 4°C, later 80µl of Protein-A-Sepharose(7% of Protein-A-Sepharose dissolved in IP buffer w/o triton) was added to samples and rotated for 2hr, later beads were precipitated by spinning samples at 2, 000rpm for 30sec, later beads were washed with 1ml of IP buffer for 5 times and the proteins were denatured by the addition of urea buffer and samples were heated at 65°C for 10 min, 8% SDS-PAGE gels were used and proteins were ran long enough to get large smear of ubiquitin,. Western blotting and protein transfer were done as described above. Nitrocellulose membranes were autoclaved for 20 min after blotting to enhance the signal. Immunodetection was done as described above.

RESULTS

A genome-wide screen in *Saccharomyces cerevisiae* was undertaken to search for new components involved in ER- protein quality control and degradation. The genome-wide screen was done by using the EUROSCARF yeast deletion library consisting of about 5, 000 *Saccharomyces cerevisiae* strains (Regelmann et al., 2002), each deleted for a single, non-essential gene. The yeast deletion strains are diploid and auxotrophic for *his3*, *leu*2 and *ura3*. Since cell growth is known to be one of the most sensitive indicators of alterations in cell physiology, it was desirable to devise a sensitive growth test to identify new mutants in the quality control and ERAD. Such a screen is possible because deletion of one of the components necessary for quality control and ERAD is not lethal to yeast, as long as the UPR is fully intact. Only deletion of components necessary for both, ERAD and UPR, is lethal in yeast. (Friedländer et al, 2000, Travers et al., 2000). It is known that yeast cells expressing a mutated version of the Sec61p translocon, Sec61-2p, are not viable at elevated temperatures (37°C) because Sec61-2p becomes an ERAD substrate at this temperature (Biederer et al., 1996). Viability under these conditions is restored only in the absence of key ERAD components, like Der3/Hrd1p, UBC6p, and UBC7p (Biederer et al., 1996, Bordallo et al., 1998). On the basis of this growth phenotype of yeast strains defective in ERAD, a screen to identify new components involved in quality control and ERAD for both, misfolded gylcoproteins and nonglycoproteins was devised. ERAD substrates used were CPY* and Sec61-2p, since glycoproteins and nonglycoproteins differ in the requirements of components essential for protein quality control (Cabral et al., 2001).

Genomic screen by using CTU* and Sec61-2-Ura3p as model substrates

Previously the membrane localized ERAD substrate CTG*, which consists of an ER-lumenal malfolded CPY* domain connected to the green fluorescent protein (GFP) in the cytoplasm via a transmembrane domain (Taxis et al., 2003) had been described. The EUROSCARF yeast deletion strains are auxotrophic to *ura3, his3 and lue2* markers. In order to screen for mutants the cytoplasmic GFP moiety in CTG* was replaced with the Ura3 protein (orotidine 5 phosphate decarboxylase) to generate CTU*. In addition the Ura3 protein was fused the C-terminus of Sec61-2p to generate Sec61-2-Ura3p. The rational behind this approach was that strains with *ura3* auxotrophy but otherwise, wild type for ERAD should be unable to grow in media lacking uracil, because the fusion protein is degraded. Only when ERAD is defective

CTU* and Sec61-2-URA3p are stabilized and able to complement the Ura3p deficiency. However when yeast strains were transformed with plasmids expressing CTU* and Sec61-2-Ura3p no growth was observed in strain deleted for a well-known ERAD component, the ubiquitin protein ligase Der3p. Growth analysis using 5FOA (5-fluoro-orotic acid) also showed that the Ura3 protein is not functional, even though both CTU* and Sec61-2-Ura3p are expressed and can be detected in immunoblots. DNA sequence analysis also did not reveal any mutations. The reasons for the non-functionality of Ura3p are not known.

CTU* with linker

As Ura3p is not functional in CTU* and Sec61-2-Ura3p we assumed that this might probably be due to failure in proper folding of the Ura3p domain as Ura3p could be very near to the ER membrane in the fusion protein. In order to facilitate proper folding of the Ura3p domain in CTU* we introduced 118 amino acids from the Suc2 fragment in between CT* and Ura3p. But when yeast strains are transformed with a plasmid containing CTU* with linker also no growth was observed in a strain lacking Der3p. Growth analysis using 5FOA(5-fluoro-orotic acid) also showed that Ura3p is not functional. CTU* with linker is expressed and can be detected in a immunoblot. DNA sequence analysis also did not reveal any mutations, reasons for the non-functionality of Ura3p are not known.

Genomic screen by using CT*- Deg1-1- Ura3p and Sec61-2 Deg1-1-Ura3p as model substrates

As Ura3p is not functional, even in the presence of a linker, it was intended to introduce Deg1-1p in between CT* and Ura3p. Deg1 encodes for the MATα degradation sequence, which makes the repressor, a substrate of the ubiquitin proteasome system. Recently a genetic screen was reported which used Deg1-Ura3p construct to identify new genes involved in MATα repressor degradation (Swanson et al., 2001). As we assumed that the non functionality of the Ura3p domain is probably due to the CT* sequence we rationalized that by introducing the protein sequence of Deg1 which was already successfully used for a genetic screen in connection with the *URA3* marker, we tried to restore the functionality of the Ura3p domain by introducing Deg1-1p, a mutated and non degradable version of Deg1p, in between CT* and Ura3p and Sec61-2p and Ura3p. This way the degradation signal in the fusion protein is from the CT* and Sec61-2p respectively not from the Deg1-1p. But when yeast strains were transformed with plasmids expressing the CT* - Deg1-1-Ura3p or Sec61-2-

Deg1-1- Ura3p again no growth was observed in strains deleted in the *DER3* gene. Growth analysis using 5FOA (5-fluoro-orotic acid) also showed that Ura3p is not functional. Both CT*-Deg1-1-Ura3p and Sec61-2- Deg1-1- Ura3p were expressed and could be detected in an immunoblot. DNA sequence analysis also did not reveal any mutations, reasons for the non-functionality of Ura3p are not known. One explanation might be, that the close location of the Ura3p domain in CTU* and Sec61-2-Ura3p with or without linkers to the ER membrane disturbs its functionality.

Genomic screen by using CTL* and Sec61-2 –Lue2p as model substrates

Due to the repeated failure of Ura3p fusion constructs for the genetic screens, we decided to fuse another marker protein Leu2p (3-isopropylmalate- dehydrogenase), to CT* and Sec61-2p (Fig. 5). As yeast strains can sustain growth even in the minimal presence of amino acids a promoter had to be selected for expression of the fusion constructs, which allowed only minimal synthesis of the fusion proteins.

In order to obtain sharp growth phenotype differences between the wild type and mutants with defective ERAD, we expressed the fusion proteins under the control of weak *GAL4* promoter. *GAL4* is a regulatory gene that is transcribed at an extremely low level as it lacks standard TATA consensus sequences, (Griggs et al., 1993) *GAL4* expression is still repressed (four to seven fold) in the presence of glucose. When yeast strains are transformed with *GAL4* controlled CTL* and *GAL4* controlled Sec61-2- Leu2p sharp growth differences are observed between wild type and mutant strains for ERAD. Strains with *leu2* auxotrophy but otherwise wild type for ERAD can not grow in media lacking leucine. Only when ERAD is defective CTL* and Sec61-2-Leu2p are stabilized and able to complement the Leu2p deficiency. The low expression of proteins under the control of *GAL4* promoter then allows for sharp growth differences to be easily observed (Fig. 6 A and B). Desired growth phenotype differences in yeast strains transformed with *GAL4* controlled Sec61-2-Leu2p are obtained at 38°C, the temperature at which Sec61-2p becomes an ERAD substrate.

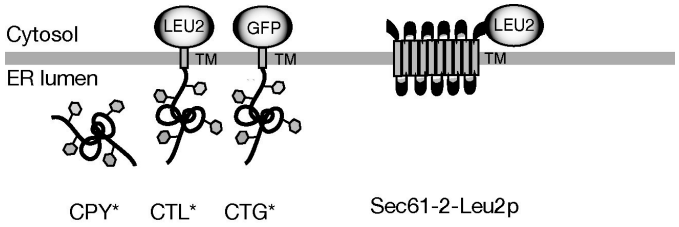

Fig. 5 Schematic representation of the ERAD substrates CPY*, CTL*, CTG*, Sec61-2-Leu2p

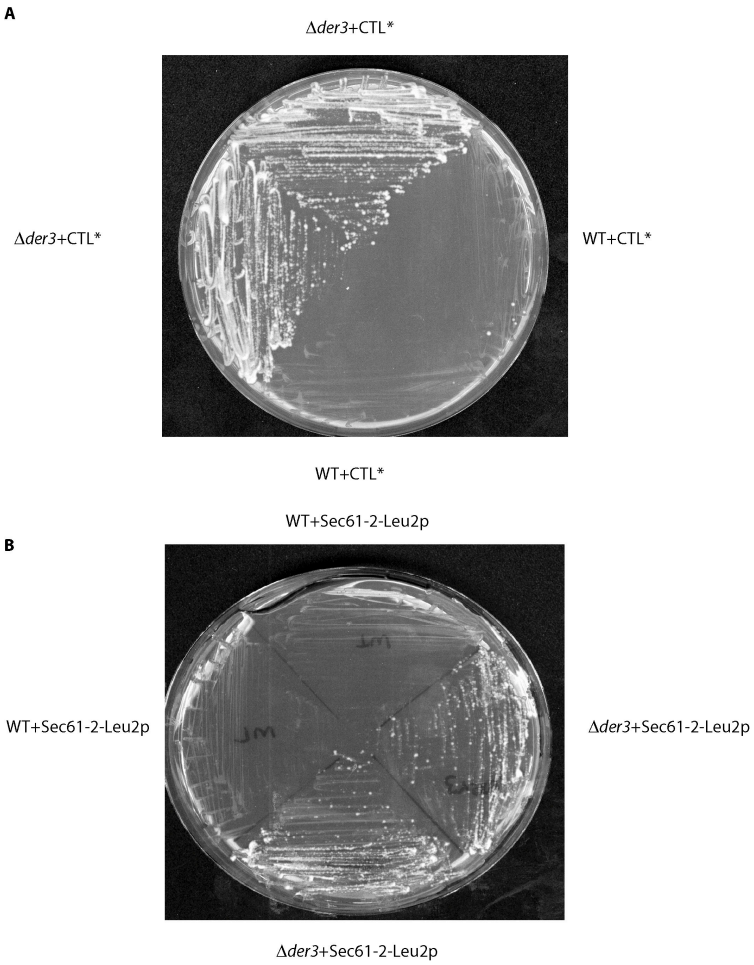

Fig. 6 CTL* and Sec61-2-Leu2p can complement the leucine deficiency in ERAD mutants. The BY4743 wild type (WT) strain expressing CTL*(A) or Sec61-2-Leu2p (B) fails to grow on SC medium lacking leucine, whereas a strain deleted in the well known ERAD component Der3p permits growth. Growth test in case of Sec61-2-Leu2p was done at 38°C.

By exploring such strong growth phenotype differences between WT and yeast strains with defective ERAD, approximately 5, 000 individual deletion mutants of the EUROSCARF yeast library, were screened by using *GAL4* controlled CTL* as model substrate. The Genetic screen was highly reliable and sensitive; the reliability of the screen was documented by the

fact that many of the mutants carrying deletions in genes already known to be required for quality control and ERAD were found.

Table: 4

ORF name	Gene name	Function
YOL013C	*HRD1/DER3*	Ubiquitin-protein ligase
YLR207W	*HRD3*	Der3p interacting protein
YHR204W	*HTM1/MNL1*	ER-lumenal lectin
YNR030W	*ECM39/ALG12*	N-glycan synthesis
YJR131W	*MNS1*	ER α-mannosidase I
YMR 022W	*UBC7*	Ubiquitin-Conjugating enzyme
YEL031W	*SPF1/COD1*	ER calcium pump

Table: 4 Genes found to be already known for their function in quality control and ERAD.

Apart from these mutants deletions sharp growth differences were also observed when plasmid expressing CTL* was transformed in strains with mutated versions of the AAA-ATPase Cdc48 complex (*cdc48-1*, *ufd1-1, npl4-1)* when compared with corresponding wild types.

Apart from the genes known to be required for ER-associated degradation found in the screen, CTL* also complemented the leucine auxotrophy in yeast strains deleted with *UMP1*, Ump1p is essential for the maturation of 20S proteasome core (Ramos et al., 1998) and in the absence of Ump1p 20S proteasome core is not properly assembled resulting in degradation defects of various proteasomal substrates. Finding of the Ump1p in the screen directly points to the necessity of the proteasome for CTL* degradation.

Both CTL* and Sec61-2-Leu2p can clearly differentiate the components essential for glycoproteins and nonglycoproteins quality control by growth phenotype tests. All the components essential for the degradation of glycoproteins like Htm1p, Alg12p, Cod1p, Mns1p are identified in the screen only when CTL* a glycoprotein was used as screening substrate but not when Sec61-2-Lue2p a nonglycoprotein ERAD substrate was used (un published results of S. Dieter).

The screening test can also uncover differences in the requirement of soluble and membrane bound ERAD substrates. Der1p which is essential only for the degradation of soluble proteins but not membrane proteins (Taxis et al., 2003) was not identified in the screen. Similarly Jem1p a component essential for the degradation of soluble proteins was also not identified in the screen. An exception was Scj1p a component, which is not supposed to be involved in the degradation of membrane proteins (Nishikawa et al., 2001), is identified in the screen. Hsp104p which is essential for the degradation of membrane substrates with strongly folded cytoplasmic domains like CTG* (Taxis et al., 2003) was also not identified in the screen.

New proteins identified in the screen

A The following deletion strains (Table 5) whose role with regard to ERAD was not described earlier showed a strong and reproducible growth phenotype when transformed with CTL*.

Table: 5

ORF name	**Gene name**	**Function**
YMR276W	*DSK2*	Spindle pole body duplication and proteolysis of UFD substrates
YDR057W	*YOS9*	ER-Golgi transport of GPI anchored proteins
YIL039W	Not characterized	Not characterized
YJL030W	*MAD2*	Component of the spindle-assembly checkpoint complex
YJL013C	*MAD3*	Component of the spindle-assembly checkpoint complex
YOL054W	*PSH1*	Zn finger protein of unknown function
YGR159C	*NSR1*	Apoptosis and ER-Golgi traffic
YDR281C	*PHM6*	Not known
YGL141W	*HUL5*	HECT-domain ubiquitin-protein ligase
YHL022C	*SPO11*	Meiotic recombination and

		sporulation
YPL253C	*VIK1*	Probably involved in the ER membrane biogenesis
YDR320C	*SWA2*	Required for clatherin coat formation and a probable co-chaperone activity along with cytosolic Hsp70 in clatherin coat disassembly, possess a DnaJ domain
YDR496C	*PUF6*	Not known
YDR504C	Not characterized	Not characterized
YML094W	*GIM5*	Proper folding of tubilin, shows chaperone and unfolded protein binding properties
YDR179W-a	Not characterized	Not characterized
YNR075W	*COS10*	Not known, protein is localized in the ER
YMR214W	*SCJ1*	DnaJ protein, shows interaction with Kar2p and essential for the degradation of soluble ERAD substrates

Table 5: genes identified in genomic screen (shows reproducible growth phenotype)

B The following deletion strains (Table 6), whose role with regard to ERAD was not described earlier showed weak or non reproducible growth phenotype when transformed with CTL*.

Table: 6

ORF name	**Gene name**	**Function**
YEL037C	*RAD23*	Nucleotide excision repair and proteolysis of UFD substrates

YGR135W	*PRE9*	20S core particle of the proteasome
YGL244W	*RTF1*	Transcription cofactor activity
YPL072W	*UBP16*	Protein deubiquitination
YAL055W	*PEX22*	Not known
YPR071W	Not characterized	Not characterized
YIL090W	Not characterized	Not characterized

Table6: Deletion strains showing non reproducible growth phenotype when transformed with CTL*

Role of Dsk2p and Rad23p in ERAD

Out of the new genes we identified in the screen *DSK2* and *RAD23* were selected and further extensive studies on the role of the encoded UBA and UBL domain containing proteins Dsk2p and Rad23p in ER protein quality control and degradation were done.

CTL* can complement the leucine auxotrophy in Δ*dsk2* and Δ*rad23* strains

CTL* can complement the leucine auxotrophy in Δ*dsk2 and* Δ*rad2*3 strains, suggesting the involvement of these gene products in ER quality control and degradation (Fig.7), as described earlier a *dsk2* deletion strain showed a strong and reproducible growth phenotype whereas a *rad23* deletion showed only a weak growth phenotype. Δ*der3* was used a positive control.

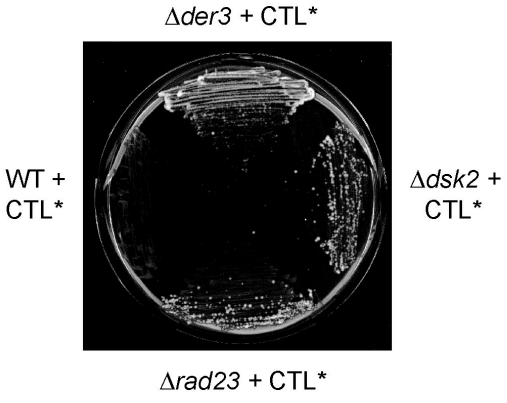

FIG. 7 DSK2 and RAD23 are found in a screen for new ERAD components The BY4743 wild type (WT) strain expressing CTL* fails to grow on SC medium lacking leucine, whereas a strain deleted in the well known ERAD component Der3p, used as a positive control, permits growth. The deletion strains Δ*dsk2* and Δ*rad23* expressing CTL*, complement the leucine auxotrophy of the strain.

Dsk2p and Rad23p are involved in the turnover of both soluble and membrane bound ERAD substrates.

To verify that these two proteins are genuine components of the ER degradation machinery, degradation of two well known ERAD substrates, soluble CPY* (Hiller et al., 1996) and membrane anchored CTG* (Taxis et al., 2003) were analyzed in a set of strains deleted in either one or both genes. Pulse chase analysis showed a strong stabilization of CPY*HA and CTG* in the Δ*dsk2*Δ*rad23* double mutant, indicating that these proteins participate directly in ERAD (Fig. 8). CPY*HA and CTG* exhibited about 10% stabilization in Δ*dsk2* and Δ*rad23* single mutants, and 40% and 45% stabilization, respectively, in the Δ*dsk2*Δ*rad23* double mutant with respect to wild type cells. (Fig.8)

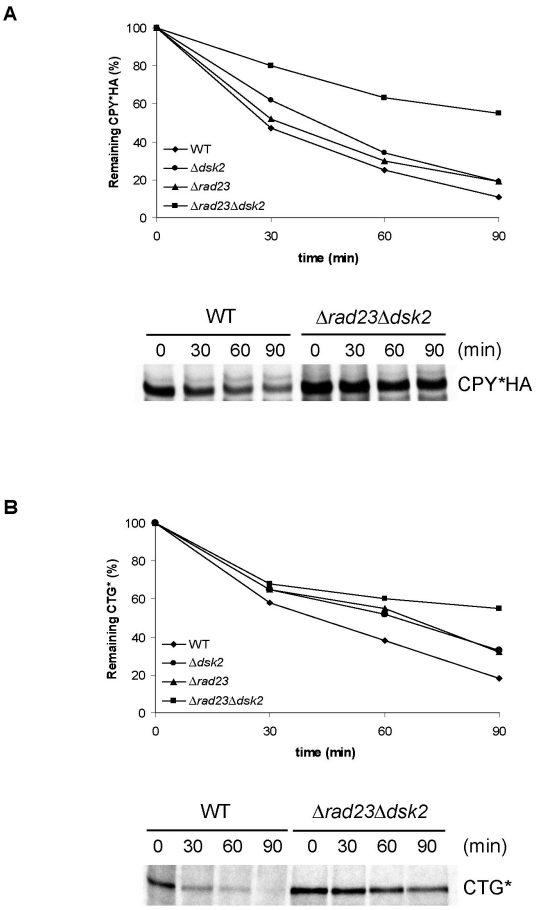

Fig. 8 Mutants deleted in DSK2 and RAD23 are defective in the degradation of the ERAD substrates CPY*HA and CTG*. (A, B) Yeast strains expressing CPY*HA or CTG* were metabolically labelled, chased for the indicated times and immunoprecipitated using anti-HA or anti-CPY antibodies, respectively. Δ*dsk2* and Δ*rad23* single and double deletion mutants show synergistic effects in ERAD. Degradation of CPY*HA (A) and CTG* (B) is significantly delayed in a Δ*dsk2*Δ*rad23* strain. Data represent the mean values of three (CPY*HA) and two (CTG*) independent experiments.

Dsk2p and Rad23p act prior to proteasomal degradation

To elucidate the step at which Dsk2p and Rad23p may be involved, the localization of ubiquitinated protein material in WT and Δ*dsk2*Δ*rad23* strains was examined. Fractionation of microsomes from WT and Δ*dsk2*Δ*rad23* revealed that in wild type cells a considerable

portion of ubiquitinated CPY*HA was retained in the pellet, with only a minor amount present in the soluble fraction. In wild type cells ubiquitinated material released by the trimeric Cdc48 complex into the supernatant (Jarosch, et al., 2002) is rapidly degraded by the proteasome (Fig. 9 lanes 1 and 2). In comparison, a considerably larger ubiquitinated CPY*HA protein fraction was detected in the soluble fraction of the Δ*dsk2*Δ*rad23* double mutant (Fig. 9 lane4, compare lanes 4 and 2). These findings indicate that ubiquitinated CPY*HA accumulates in the cytosol in the Δ*dsk2*Δ*rad23* as a consequence of failed delivery to the proteasome.

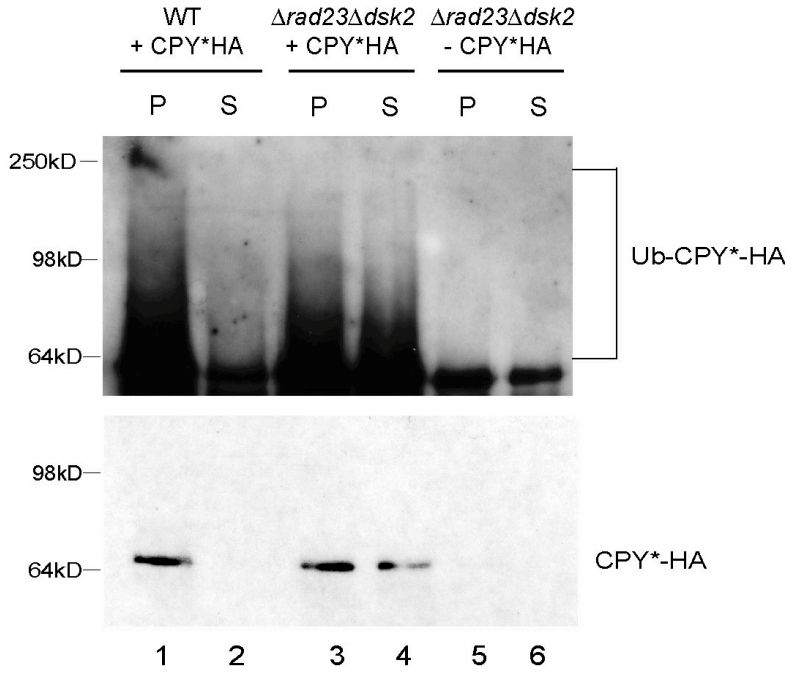

Fig. 9 Polyubiquitinated CPY*HA accumulates in the cytosol of the Δdsk2Δrad23 mutant. Localization of ubiquitinated CPY*HA was investigated in respectively transformed wild type (WT) and Δ*dsk2*Δ*rad23* double mutant strains. Microsomal extracts were prepared and separated into membrane associated (P) and cytosolic (S) fractions. CPY*HA was precipitated with anti-CPY antibodies and analysed by immunoblotting with anti-ubiquitin antibodies. The pellet and supernatant fractions from non-transformed strain Δ*dsk2*Δ*rad23* are included to reveal the background activity of the ubiquitin antibody. Immunoblotting with HA-antibody shows localization of CPY*HA in each fraction.

Pathway specificity of Dsk2p and Rad23p

We also investigated whether Dsk2p and Rad23p are general components of the targeting and degradation machinery of ubiquitinated proteins. We constructed ΔssCPY*-GFP, which lacks the CPY signal sequence but carries the same mutated motif as the ER localized CPY* and CTG* as a representative soluble proteasomal substrate of the cytoplasm.

ΔssCPY*-GFP is located in the cytosol

Cell fractionation and protease protection experiments of cell extracts show indeed that ΔssCPY*-GFP is detectable in the supernatant fraction and susceptible to trypsin digestion. Furthermore, deglycosylation experiments verified that ΔssCPY*-GFP is not glycosylated (Fig.10 A and B). These experiments confirm that ΔssCPY*-GFP resides in the cytoplasm.

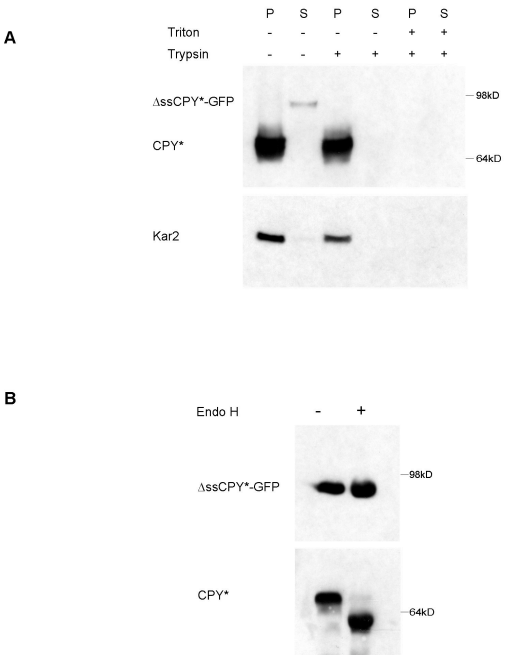

Fig. 10 ΔssCPY*-GFP is located in the cytosol. (A) Spheroplasts from strain YCT397 transformed with ΔssCPY*-GFP and chromosomally expressing CPY* were isolated and fractioned into microsomal pellet (P) and supernatant (S). Both fractions were treated with trypsin and triton as indicated. The ER lumenal Kar2p was used as a control for fractionation and protease protection experimentation accuracy. (B) ΔssCPY*-GFP is not glycosylated. ΔssCPY*-GFP and, as a control, glycosylated CPY* were immunoprecipated using CPY antibodies from YCT397 expressing both proteins, subjected to endoglycosidase H (Endo H) treatment and immunoblotted. ΔssCPY*-GFP was detected with anti-GFP, CPY* was detected with anti-CPY antibodies.

ΔssCPY*-GFP is a substrate of 26S proteasome

Cycloheximide decay analysis performed in WT and in a mutant (*cim3-1*) defective in one of the 19Scap AAA-ATPase subunits (Rpt6p) (Hiller et al., 1996) revealed that ΔssCPY*-GFP is rapidly degraded in wild type cells, whereas degradation is abolished in the *cim3-1* strain Fig11. A), suggesting that ΔssCPY*-GFP is a target of 26S proteasome.

Degradation of ΔssCPY*-GFP is independent of Dsk2p and Rad23p

Cycloheximide decay analysis performed in WT and Δ*dsk2*Δ*rad23* double deletion cells revealed that degradation of ΔssCPY*-GFP is independent of Δ*dsk2*Δ*rad23*. (Fig. 11B)

Degradation of ΔssCPY*-GFP is independent Ufd1p

Cycloheximide decay analysis performed in WT and mutant *ufd1-1* cells revealed that degradation of ΔssCPY*-GFP is independent of Ufd1p, a component of the Cdc48-Ufd1-Npl4p complex required for ERAD. Taken together these experiments show, that Dsk2p and Rad23p are not general components of the ubiquitin-proteasome dependent degradation of proteins, but are pathway specific. (Fig. 11C)

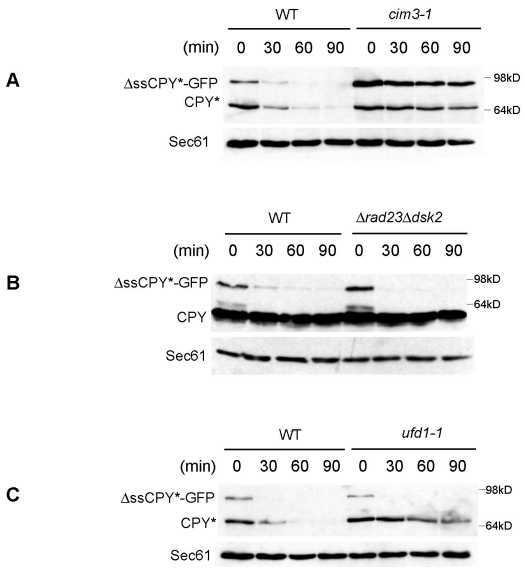

Fig. 11 Degradation of cytosolic CPY*-GFP is independent of Dsk2p and Rad23p. (A) Cycloheximide decay experiments performed in wild type (WT) and proteasomal mutant *cim3-1* strains expressing both CPY* and ΔssCPY*-GFP show that ΔssCPY*-GFP is a substrate of the 26S proteasome. (B) Cycloheximide decay experiments performed in WT and Δ*dsk2*Δ*rad23* strains expressing CPY and ΔssCPY*-GFP indicate that Dsk2p and Rad23p have no role in the degradation of cytosolic ΔssCPY*-GFP. (C) Degradation of ΔssCPY*-GFP is independent of Ufd1p. ER lumenal CPY* conversely, is stabilized in the *ufd1-1* mutant. Sec61p was used as a control for protein loading.

Degradation of ΔssCPY*-GFP is independent of Rpn10p and Rad9p

Rpn10p is a protein of 19S regulatory complex of the proteasome, and Rad9p was previously shown to be involved in the degradation of Ho endonuclease. Role of these 2 proteins in the turnover of the cytoplasmic misfolded protein and proteasomeal substrate, ΔssCPY*-GFP was analysed. Pulse chase analysis reveals that degradation of ΔssCPY*-GFP is independent of Rpn10p and Rad9p. (Fig.12) ΔssCPY*-GFP is degraded rapidly in both wild type and Δ*rpn10* and Δ*rad9* deletion strains with almost identical kinetics.

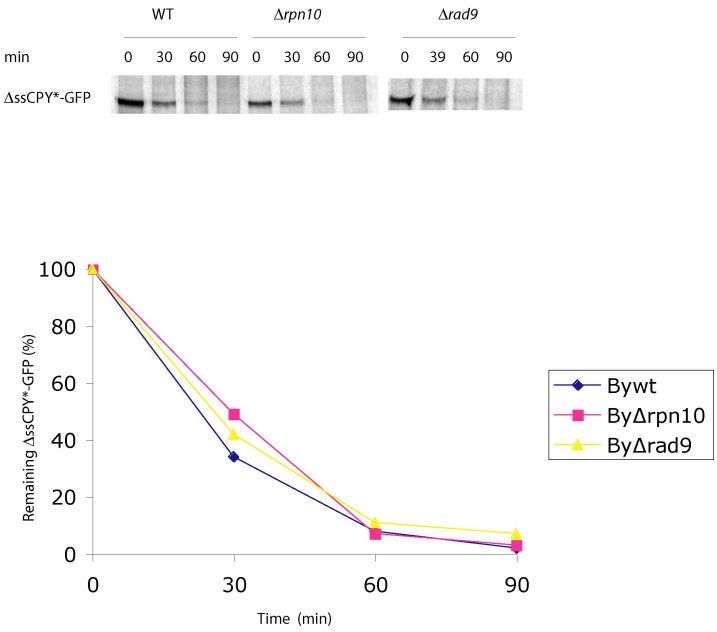

Fig.12 Degradation of ΔssCPY*-GFP is independent of Rpn10p and Rad9p. Yeast strains expressing ΔssCPY*-GFP are metabolically labelled and chased for the indicated time points Immunoprecipitation is done using anti-CPY antibodies. ΔssCPY*-GFP is degraded rapidly in WT and Δ*rpn10* and Δ*rad9* strains indicating Rpn10p and Rad9p have no role in the turn over of ΔssCPY*-GFP.

Role of Ddi1p along with Rad23p in the turnover of ERAD substrates

As Ddi1p is a UBA domain containing protein like Dsk2p and Rad23p, it was logical to test its possible involvement in ERAD. Unlike Dsk2p and Rad23p Ddi1p lacks a proteasome interacting UBL domain (Clarke et al., 2001). Pulse chase analysis in wild type, Δ*rad23*, Δ*ddi1* and Δ*rad23*Δ*ddi1* double mutants revealed that CPY*HA is degraded normally in WT and Δ*ddi1* mutants. 7% of CPY*HA is stabilized in Δ*rad23* when compared to WT. No

additional stabilization is observed in a Δ*rad23*Δ*ddi1* double deletion mutant (Fig.13) indicating that Ddi1p has no effect in the degradation of CPY*HA.

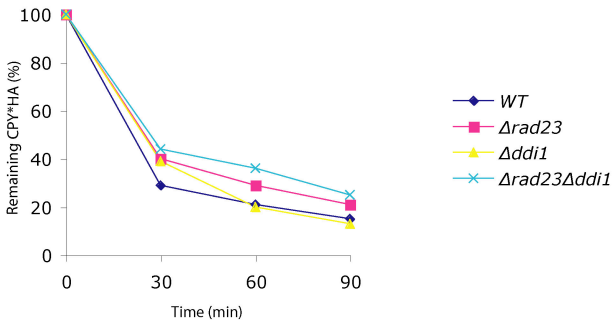

Fig.13 Ddi1p has no role in the degradation of ERAD substrates. Yeast strains expressing CPY*HA are metabolically labelled, chased for the indicated times and immunoprecipitated using anti-HA antibodies. A Δ*ddi1* mutant does not show altered degradation of CPY*HA nor does a Δ*rad23*Δ*ddi1* strain show any synergistic effect in the degradation of CPY*HA. CPY*HA is degraded with similar kinetics in Δ*rad23* and Δ*rad23*Δ*ddi1* strains.

Role of Ufd2p along with Rad23p in the turnover of ERAD substrates

Recent studies showed that the UBL domain of Rad23p interacts also with Ufd2p (Kim et al., 2004). Pulse chase analysis in wild type, Δ*ufd2*, and Δ*rad23*Δ*ufd2* cells reveals that CPY*HA is degraded normally in WT and Δ*ufd2* cells. No additional stabilization is observed in a Δ*rad23*Δ*ufd2* double deletion indicating that Ufd2p has no additive affect in the degradation of CPY*HA (not shown).

Role of Eps1p in the turnover of CTG*

Previously Eps1p had been shown to be involved in the ER quality control of a membrane protein, a mutant form of the yeast membrane ATPase Pma1p (Wang and Chang, 1999). Cycloheximide decay analysis revealed that Eps1p has no role in the turnover of the membrane protein CTG* (not shown).

Role of Yos9p in ER quality control

Out of the new genes, which were identified in the screen, was *YOS9* encoding the mannose-6-phosphate receptor homology domain protein Yos9p. Yos9p is a glycoprotein anchored to the ER membrane and it was previously shown to be involved in the ER-Golgi transport of GPI anchored proteins. (Friedmann et al., 2002) Its possible involvement in ER quality control was followed in more detail.

CTL* but not Sec61-2-Leu2p can supplement the leucine auxotrophy in Δyos9 strain

Performing a growth test it appeared that one of the strains expressing CTL* and capable of growth in the absence of exogenous leucine supplementation carried a deletion in YDR057W, encoding the Yos9 protein. Strains deleted in well-known ERAD component as Der3p or misfolded glycoprotein-binding lectin Htm1p (Jacob et al., 2001) were used as positive controls (Fig. 14A). CTL* is a derivative of CTG*, a N-glycosylated protein carrying four carbohydrate chains. . To investigate whether Yos9p is generally involved in quality control of misfolded proteins or it is essential only for ER- associated degradation of glycosylated proteins, growth of *Δyos9* cells expressing Leu2p fused to the malfolded non-glycosylated ER protein Sec61-2p was examined in addition (Fig. 14A). *Δyos9* cells, as well as cells defective in the lectin Htm1p/Mnl1p expressing Sec61-2-Leu2p are unable to grow at 38°C (Fig. 14B), the restrictive temperature that induces Sec61-2p unfolding. This indicates that Sec61-2-Leu2p is degraded in Δ*yos9* and Δ*htm1*/Δ*mnl1* cells as in wild type.

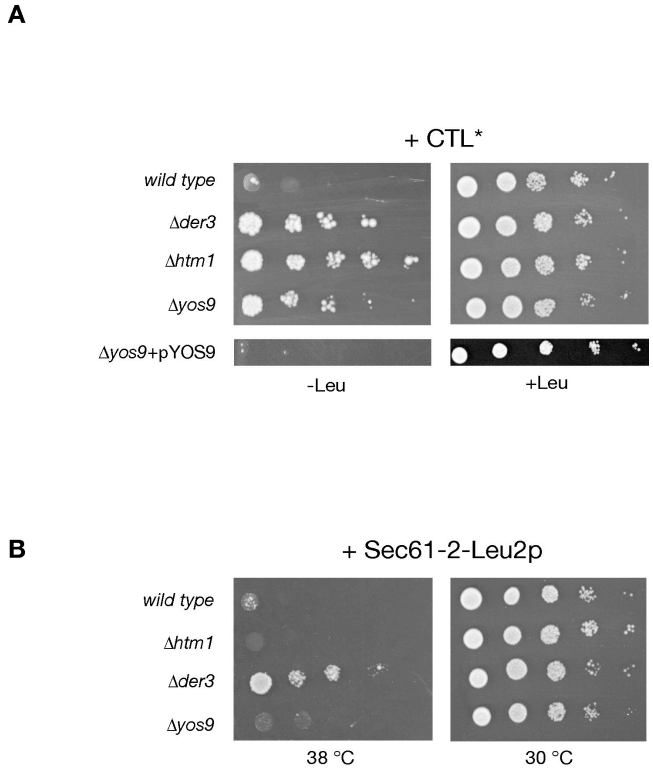

FIG. 14 Yos9p is a putative ERAD component involved in the quality control of glycoproteins.

(**A**) Isogenic wild type and mutant cells were plated in serial dilutions on CM medium with and without leucine. Plates were incubated for 2-4 days. The leucine auxotrophic WT strain expressing CTL* cannot grow in the absence of leucine. However, the leucine deficiency is complemented by CTL* in the ERAD defective strains *Δder3* and *Δhtm1*, and in a strain carrying a deletion of *YOS9*. Wild type phenotype (no growth) is observed when Yos9p is expressed from plasmid pYOS9 in *Δyos9* cells. **B:** Wild type and mutant cells expressing the conditional non-glycosylated ERAD substrate Sec61-2-Leu2p were plated as described in (**A**) and incubated at 30°C (control) and 38°C for 4-5 days. Lethality due to leucine deficiency is complemented only in the Δ*der3* strain, indicating that Yos9p, just like Htm1p, does not participate in the degradation of non-glycosylated substrates.

Yos9p is involved in the turnover of both soluble and membrane bound glycoprotein ERAD substrates.

To verify that Yos9p is a true component of glycoprotein ER degradation machinery, we analysed degradation of two well-known glycoprotein ERAD substrates soluble CPY*HA and membrane anchored CTG*. Pulse chase analysis showed a strong stabilization of CPY*HA and CTG* in the Δ*yos9* mutant (Fig. 15 A and B), indicating that this protein participate directly in ERAD. CPY*HA and CTG* exhibited about 50% stabilization in Δ*yos9* cells with respect to wild-type cells.

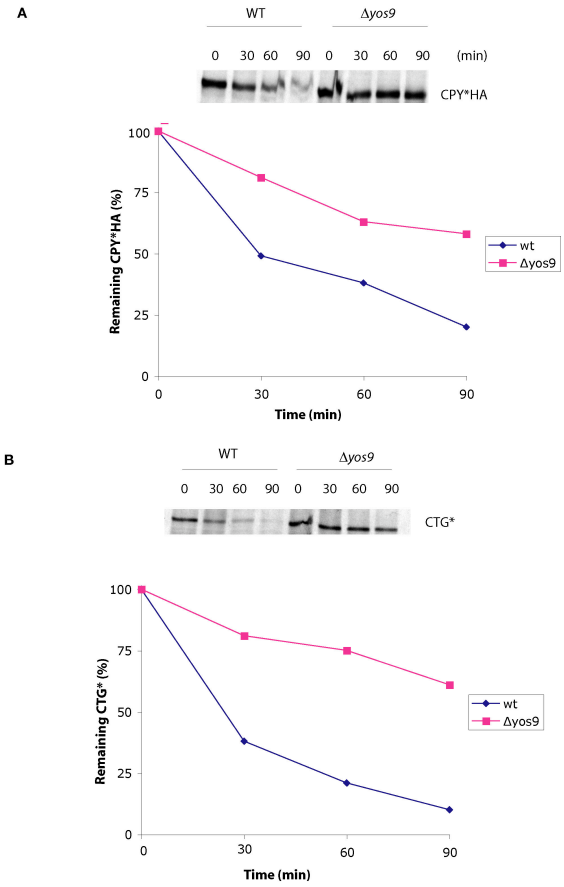

Fig. 15 Mutants deleted in YOS9 are defective in the degradation of the ERAD substrates CPY*HA and CTG*. (A, B) yeast strains expressing CPY*HA and CTG* were metabolically labelled, chased for the indicated times, immunoprecipitated using anti-HA or anti-CPY antibodies, respectively. Degradation of CPY*HA (A) and CTG* (B) is significantly delayed in a Δ*yos9* strain. Data represent the mean value of two independent experiments.

Yos9p is not involved in the turnover of non-glycosylated ERAD substrate Sec61-2p

To verify that Yos9p is not a component of the ER degradation machinery of nonglycosylated proteins, the degradation of a well-known nonglycoprotein ERAD substrate Sec61-2p was analysed. Cycloheximide decay analysis showed Sec61-2p is rapidly degraded in both WT and Δ*yos9* cells, where as Sec61-2p degradation is significantly delayed in Δ*der3* strain (Fig. 16), a E3 known to be essential for the degradation of Sec61-2p.

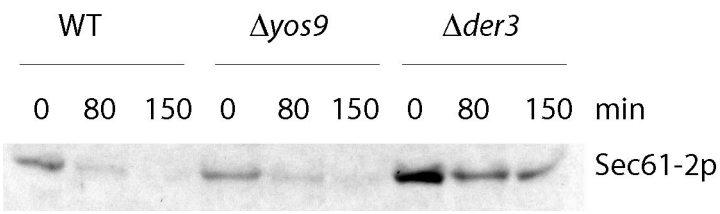

Fig. 16 Degradation of Sec61-2p is independent of Yos9p. Cycloheximide decay analysis performed in Wild type (WT) and Δ*yos9* expressing Sec61-2p show that Yos9p is not essential for the degradation of Sec61-2p, and Δ*der3* strain expressing Sec61-2p was used as a positive control.

These experiments confirm that Yos9p is a lectin or lectin like protein essential for the quality control and ERAD of glycoproteins only.

Round up experiments concerning Yos9p were done in collaboration with Bettina A Buschhorn and Zlatka Kostova as published in Buschhorn, B. A., * Kostova, Z. *, Medicherla, B. * and Wolf, D. H. (2004) A genome-wide screen identifies Yos9p as essential for ER-associated degradation of glycoproteins. *FEBS lett.*, **577**, 422-426.

(* These authors in alphabetical order, contributed equally to this work.)

Discussion

To gain deeper insight into the molecular mechanisms of protein quality control and ER-associated degradation a genome-wide screen using the yeast EUROSCARF deletion library, containing about 5, 000 *Saccharomyces cerevisiae* strains each deleted for a single non essential gene was performed. As cell growth is one of the most sensitive indicators of alterations in cell physiology due to mutations, we devised a growth phenotype test to identify mutants with defective ER quality control by using fusion proteins CTL* and Sec61-2-Leu2p, expressed under the control of the weak *GAL4* promoter. Low expression helps in observing sharp growth differences easily. As both the glycoprotein and nonglycoprotein substrates differ in the requirement of components essential for quality control we constructed both glycoprotein (CTL*) and non- glycoprotein (Sec61-2-Leu2p) model substrates to screen for mutants defective in ER quality control and degradation. In this study we used the glycoprotein model substrate CTL* to screen the yeast genomic library consisting of all viable deletions. CTL* consists of CPY* in the ER lumen, connected to the Leu2 protein (3-isopropylmalate-dehydrogenase) in the cytoplasm via a transmembrane domain .Due to recognition of malfolded CPY* in the ER lumen, the hybrid CTL* protein is retrotranslocated to the cytosol and readily degraded in wild type cells resulting in a no growth phenotype in leucine auxotropic cells incubated in leucine-deficient growth medium. In contrast, leucine deficient cells can grow on media lacking leucine when a component of the ER quality control or degradation system is missing: impaired degradation of CTL* results in complementation of the leucine deficiency (Fig. 6). By exploiting this growth phenotype behaviour the entire yeast deletion library was screened. Most of the ERAD components known to date, as well as a number of new potential ERAD players were recovered from this screen. Leu2p fused to the non- glycosylated ER protein Sec61-2p, Sec61-2-Leu2p, which unfolds at the restrictive temperature of 38°C (Biederer et al., 1996) was also used in the growth phenotype test to identify new components involved in non glycoprotein quality control and degradation.

The genomic screen using the growth phenotype test described is highly reliable and sensitive. Most of the known ERAD components resulted in growth in the absence of the amino acid leucine (Table 2), CTL* also complemented the leucine deficiency in a strain deleted of Ump1p a non-essential protein aiding the assembly of the 20S proteasome core (Ramos et al., 1998). In contrast, we failed to observe any growth phenotype in strain deleted in Pep4p the vacuolar acidic protease, suggesting that the fusion protein CTL* is a substrate

of the 26S proteasome. Components like Der1p which are essential only for the degradation of soluble substrates (Taxis et al., 2003) were not identified in the screen when the membrane substrates CTL* and Sec61-2-Leu2p were used as substrates. Components like Alg12p, Htm1p, Mns1p, Cod1p which are essential only for the degradation of glycoprotein ERAD substrates were identified only when CTL* a glycoprotein was used as a model substrate but not when Sec61-2-Leu2p a non glycoprotein ERAD substrate was used. All these facts confirm the sensitivity and reliability of the genetic screen devised.

Among the new genes we identified in the mutant screen are *DSK2* and *RAD23*. Strong and reproducible growth phenotype was observed in the strain carrying the *DSK2* deletion and a weaker not always reproducible growth phenotype was observed in the strain carrying the *RAD23* deletion (Fig. 7). A detailed study was carried out to elucidate the role of Dsk2p and Rad23p in ER quality control and degradation. Further, the pathway specificity of these proteins in targeting ubiquitinated substrates to the proteasome was tested.

Dsk2p was first identified as a protein essential for spindle pole body duplication in yeast (Biggins et al., 1996) and Rad23p was first identified as a protein essential for nucleotide excision repair in yeast. (Watkins et al., 1993). *Saccharomyces cerevisiae* Rad23p is a 42Kda protein with a N-terminal ubiquitin like (UBL) domain and two ubiquitin associated (UBA) domains one at the C terminus and the other in the middle of the protein. Apart from this Rad23p contains an XPC domain, which mediates its role in nucleotide excision repair. Dsk2p shows little or no significant sequence similarity to Rad23p apart from its UBL and UBA domains. However the functions of both proteins in the cell seem to over lap (Biggins et al., 1996, Wilkinson et al., 2001)

The ubiquitin associated UBA domain is a small domain of about 40 residues that was initially identified in E2s, E3s, and other proteins linked to ubiquitination. Consequently ubiquitin binding was proposed to be a general function of UBA domains. This function has recently been well established for the UBA domains of several *Saccharomyces cerevisiae* proteins including the Rad23 Dsk2 and Ddi1 proteins. (Wilkinson et al., 2001, Funakoshi et al., 2001, Rao and Sastry, 2002). The specificity of UBA domains with respect to ubiquitin chain length and linkage type is still under debate. *In vitro* studies showed that affinity for tetra ubiquitin is higher than for mono and di ubiquitin. (Rao and Sastry, 2002). Both *in vivo* and *in vitro* studies showed that UBA domains could bind to Lys29 and Lys48 linked ubiquituin chains (Rao and Sastry, 2002)

Protein domains with similarity to ubiquitin are known as ubiquitin like (UBL) domains. The three dimensional structure of UBL is very similar to that of ubiquitin and an inframe fusion

of ubiquitin can functionally replace the UBL domain of Rad23p (Buchberger, A., 2002). An emerging general property of UBL domains is their ability to bind to the 26S proteasome. *In vitro* assays from *S. pombe* and *S. cerevisiae* give two different results. In *S. pombe* UBL domains were shown to interact with the ubiquitin interacting motif (UIM) of the Rpn10p subunit of the 19S regulatory complex of the proteasome (Wilkinson et al., 2001). *In vitro* experiments performed in *S. cerevisiae* gave a different result: UBL domains of Rad23p and Dsk2p were shown to interact with Leucine rich repeats (LRR) of the Rpn1p subunit of the proteasome (Elsässer et al., 2001), Irrespective of the subunit with which the UBL domain interacts, it is well established that UBL domains interact with the 26S proteasome.

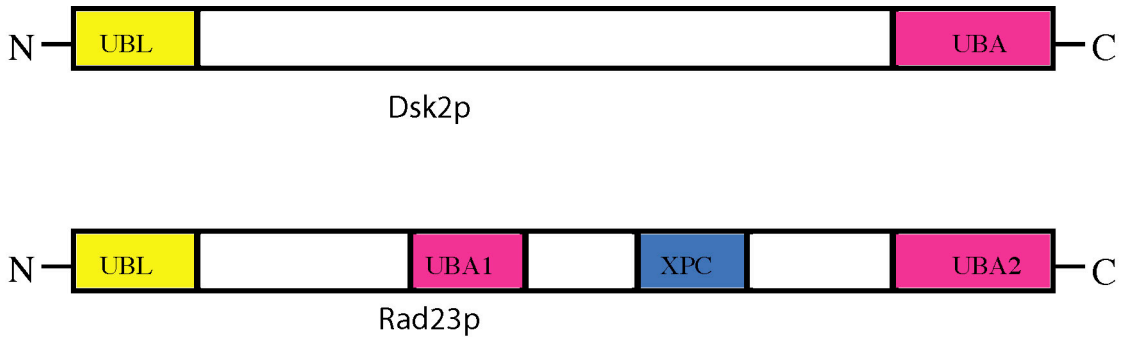

Fig. 17 Domains of Dsk2p and Rad23p

Dsk2p and Rad23p were previously found to be involved in the turnover of the UFD (ubiquitin fusion degradation) substrates Ub-Pro-β-gal and Ub-^{V76}-β-gal (Chen and Madura, 2002, Rao and Sastry, 2002). Degradation of UFD substrates is significantly delayed in the absence of these proteins. Due to their ability to bind to polyubiquitinated substrates and the proteasome, one can expect that these proteins can act as shuttling factors or adapters in transferring polyubiquitinated substrates to proteasome (Chen and Madura, 2002, Rao and Sastry, 2002). Over expression of Dsk2p and Rad23p leads to accumulation of ubiquitinated substrates and over expression of Dsk2p is also toxic to cells (Biggins et al., 1996). Both, the UBA and UBL domains are essential for the functioning of the Dsk2p and Rad23p. Dsk2p and Rad23p with out UBA and UBL domains cannot perform their functions in spindle pole body duplication, DNA repair and protein degradation. (Li and Madura, 2002, Rao and Sastry, 2002). A Δ*dsk2*Δ*rad23* strain is sick, shows temperature sensitivity and a very slow progression from G2-to-M phases of the cell cycle. It also shows sensitivity to canavanine, suggesting severe defects of proteolysis in the Δ*dsk2*Δ*rad23* double deletion mutant. (Biggins

et al., 1996, Li and Madura, 2002, Rao and Sastry, 2002). Based on these findings and their ability to show interaction with both ployubiquitinated substrates and the 26S proteasome, it can be postulated that these proteins are supposed to be involved in targeting ubiquitinated substrates to the 26S proteasome by acting as adapters or shuttling factors. On other hand this conclusion is disputed as in some *in vitro* studies an inhibitory effect of Rad23p on proteasome activity has been noted. (Raasi and Pickart, 2003).

We observed a strong growth phenotype in strain expressing CTL* and carrying the *DSK2* and, to a lesser extent, the *RAD23* deletion. It was further verified whether these two proteins are essential for ERAD or not by analysing the degradation of two well known ERAD substrates, soluble CPY* and membrane anchored CTG* in a set of isogenic strains deleted in either one or both genes. Both cycloheximide and pulse chase analysis showed a significant delay in the degradation of CPY*HA and CTG* in the Δ*dsk2*Δ*rad23* double mutant indicating that these two proteins participate directly in ERAD. CPY*HA and CTG* showed about 10% stabilization in Δ*dsk2* and Δ*rad23* single mutants, and 40 and 45% stabilization, respectively, in the Δ*dsk2*Δ*rad23* double mutant with respect to wild type cells (Fig. 8). The additive affect shows that Dsk2p and Rad23p work together in ERAD, in agreement with reports implicating DSK2p and Rad23p in the proteasomal degradation of the artificial substrates Ub-Pro-β-gal and Ub-^{V76}-β-gal (Chen and Madura, 2002, Rao and Sastry, 2002) via the UFD pathway.

After establishing that Dsk2p and Rad23p are involved in ERAD it was interesting to determine at what step in ERAD Dsk2p and Rad23p are involved. Previously fractionation and analysis of microsomes derived from wild type and *ufd1-1* (a mutant allele of Ufd1p, a component of the AAA-ATPase Cdc48p-Ufd1p-Npl4p complex (Ye et al., 2001, Jarosch et al., 2002) showed that a considerable portion of ubiquitinated CPY* is retained in the pellet fraction, with only a minor amount present in the soluble fraction. In wild-type cells the ubiquitinated material released by the trimeric Cdc48 complex into the supernatant is rapidly degraded by proteasomes. In *ufd1-1* cells there is no cytoplasmic ubiquitinated protein product visible owing to a defective trimeric Cdc48 complex (Jarosch et al., 2002). To elucidate the step at which Dsk2p and Rad23p is involved in ERAD the localization of ubiquitinated material was examined by isolating microsomes from wild type and Δ*dsk2*Δ*rad23* strains. As shown previously we, too, observed little ubiuitinated CPY*HA in the cytoplasm of wild-type cells due to its degradation via the proteasome. In contrast the accumulation of ubiquitinated CPY*HA in the soluble fraction of Δ*dsk2*Δ*rad23* strain was observed (Fig. 9). These findings imply that ubiquitinated CPY*HA is accumulating in the cytoplasm due to failed delivery to the proteasome. One may therefore propose that Dsk2p

and Rad23p act as adapters in transferring the ubiquitinated material from the AAA-ATPase Cdc48 complex to the 26S proteasome.

Previous studies had shown that Dsk2p and Rad23p are involved in the turnover of the UFD substrates (Chen and Madura, 2002, Rao and Sastry, 2002). In this study it is shown that Dsk2p and Rad23p are involved in ERAD. It was interesting to further determine whether these proteins in general act as adapters by transferring the ubiquitinated substrates to the 26S proteasome or whether they show any pathway specificity in targeting substrates for proteasomal degradation. In order to determine this we constructed ΔssCPY*-GFP which carries the same mutated motif as the ER localized CPY* and CTG* but resides in the cytoplasm due to the lack of the signal sequence. It was confirmed that the substrate ΔssCPY*-GFP indeed resides in the cytoplasm. Protease sensitivity and deglycosylation experiments showed that ΔssCPY*-GFP is prone to trypsin digestion and is not glycosylated (Fig. 10 A and B). The newly constructed protein is a substrate of the 26S proteasome: cycloheximde decay analysis showed that ΔssCPY*-GFP is rapidly degraded in wild-type cells, whereas degradation is completely abolished in a mutant (*cim3-1*) (Fig. 11, A) defective in one of the 19Scap AAA-ATPase subunits (Rpt6p) (Hiller et al., 1996). Cycloheximde decay analysis showed that ΔssCPY*-GFP was degraded rapidly in both wild type and Δ*dsk2*Δ*rad23* double deletion mutant underscoring the pathway specific requirement for Dsk2p and Rad23p in ERAD but not cytoplasmic protein degradation (Fig. 11, B). Interestingly both, the UFD and ERAD pathways that require the presence of Dsk2p and Rad23p for function, are also dependent on the Cdc48-Ufd1-Npl4p complex (Johnson et al., 1995, Jarosch et al., 2002). In fact, Ufd1p, the central subunit of the trimeric Cdc48 complex was first identified as a component of the UFD pathway (Johnson et al., 1995). Therefore it was tested whether Ufd1p is necessary for the degradation of the cytoplasmic ΔssCPY*-GFP. Consistent with the fact that Dsk2p and Rad23p are not involved, no difference in the degradation of ΔssCPY*-GFP in wild type and *ufd1-1* cells was observed (Fig. 11, C), degradation of a fusion protein consisting of the Deg1 degradation signal of the MATα2 repressor protein and GFP, Deg1-GFP (Lenk and Sommer, 2000) was also independent of both Ufd1p as well of Dsk2p and Rad23p. On the basis of these findings, one may propose that degradation pathways that require the AAA-ATPase complex Cdc48-Ufd1p-Npl4p may also depend on Dsk2p and Rad23p, a concept that will require further exploration. In this scenario, substrates liberated from the trimeric Cdc48 complex are passed to Dsk2p and /or Rad23p, which, in turn, transfer them to the proteasome for degradation. One may hypothesize that in this way, malfolded substrates of the ER are handed over to the

proteasome following an uninterrupted path from the ER membrane, thus preventing the formation of insoluble protein aggregates in the cytoplasm. We also propose that, contrary to previous interpretations that Dsk2p and Rad23p are essential for proteasomal targeting of ubiquitinated substrates in general or they act as inhibitors in these same processes, the action of Dsk2p and Rad23p is not all or none, but pathway specific and finely tuned.

Ddi1p is also a UBA domain containing protein in *S. cerevisiae* along with Dsk2p and Rad23p. But unlike Dsk2p and Rad23p, it lacks the proteasomal interacting UBL domain (Clarke et al., 2001). Pulse chase analysis of CPY*HA in wild type, Δ*rad23*, Δ*ddi1* and Δ*rad23*Δ*ddi1* cells revealed that the Δ*ddi1* strain has no effect and the Δ*rad23*Δ*ddi1* strain has no additive effect in the degradation of CPY*HA (Fig. 13). This could be probably due to the lack of proteasome interacting UBL domain in Ddi1p. Till now Ddi1p was also not reported to be involved in the degradation of UFD substrates. Due to the lack of UBL domain, Ddi1p may not act as an adapter/ shuttling factor in targeting ubiquitinated proteins for proteasomal degradation.

Mutant form of the yeast membrane ATPase Pma1p requires the presence of a membrane protein Eps1p, a PDI family member protein for ER-degradation. However, Eps1p is not required for ERAD of soluble proteins like CPY* (Wang and Chang, 1999). We studied whether Eps1p is involved in the ERAD of the membrane protein CTG* (Taxis et al., 2003). Cycoheximide decay analysis showed that CTG* is degraded normally in Δ*eps1* cells, suggesting that membrane proteins differ in the components required for ERAD.

Previous studies showed that Ufd2p has no role in the degradation of CPY* (Ye et al., 2001). Recently Ufd2p was shown to interact with the UBL domain of Rad23p (Kim et al., 2004), pulse chase analyses of CPY*HA revealed that a Δ*ufd2*Δ*rad23* deletion strain does not show an additive affect in the degradation of CPY*HA when compared with a Δ*rad23* strain.

In order to identify components involved in the degradation of the newly designed proteasomal cytosolic substrate, ΔssCPY*-GFP, the turnover of ΔssCPY*-GFP was examined in wild type and Δ*rpn10* cells, defective in a ubiquitin binding domain containing subunit of the 26S proteasome. Pulse chase analysis showed that ΔssCPY*-GFP is degraded with almost similar kinetics in wild type and Δ*rpn10* cells. We also determined whether Rad9p which was previously shown to be involved in the ubiquitin dependent protein degradation of Ho endonuclease (Kaplun et al., 2000) is involved in the turnover of ΔssCPY*-GFP. Pulse chase analysis showed that ΔssCPY*-GFP is degraded rapidly in Δ*rad9* cells also (Fig. 12, A and B). Till now the components which recognises the malfolded cytosolic ΔssCPY*-GFP for proteasomal degradation are not known. It will be interesting to identify the components

involved in the quality control of this non ERAD protein, in order to show the differences, in the components, required for the quality control and proteasomal degradation of ERAD and non ERAD substrates.

Among the strains expressing CTL* and capable of growth in the absence of exogenous leucine supplementation was a strain which carried a deletion in the ORF YDR057W, encoding the Yos9 protein. CTL* is a derivative of CTG*, a N-glycosylated protein carrying four carbohydrate chains (Taxis et al., 2003). The Yos9p deletion strain expressing Sec61-2-Leu2p failed to grow in media lacking leucine suggesting that Yos9p is essential only for the quality control and degradation of glycoproteins but not for non-glycosylated proteins (Fig, 14, A and B).

Yos9p is a lumenal, membrane associated ER protein with homology to human OS-9, which is found in all tissues and amplified in osteosarcomas. (Su et al., 1996, Kimura et al., 1998). Yos9p was characterized by the presence of mannose–6-phosphate receptor homology domain of the unknown function. (Munro, S. 2001, Schrag et al., 2003,).

The highly conserved rat and mouse homolog is a peripheral ER protein exposed to the cytoplasm and associates transiently with the metalloendoproteinase merpin β, possibly mediating its ER-to-Golgi transport. (Litovchick et al., 2002). Yeast OS-9 (Yos9p) possesses a carboxy-terminal HDEL ER retention motif and localization studies showed that Yos9p is a lumenal glycoprotein tightly associated with ER membrane (Friedmann et al., 2002). To date, only study directly addressing Yos9p function has been undertaken in yeast in relation to ER-to-Golgi transport of GPI-anchored proteins. Yos9p can be immunoprecipated with GPI-anchored Gas1p and Mkc7p. The maturation rate of Gas1p was found to be directly proportional to the level of Yos9p present, suggesting a direct involvement of Yos9p in GPI-anchored protein processing (Friedmann et al., 2002).

To substantiate the finding that Yos9p is a component of quality control system for malfolded glycoproteins, pulse-chase experiments with soluble CPY*HA and membrane bound CTG* were performed. Degradation of CPY*HA and CTG* is significantly delayed in Δ*yos9* cells (about 50% when compared with wild-type cells) (Fig. 15, A and B). However, there was no observable difference in the turnover of Sec61-2p between Δ*yos9* and wild type cells (Fig. 16), suggesting the direct involvement of Yos9p in the quality control and degradation of glycoproteins.

In this study, we show that Yos9p is necessary for efficient degradation of the glycoslyted ERAD substrates CPY*HA and CTG*, but plays no role in the degradation of unglycoslyted

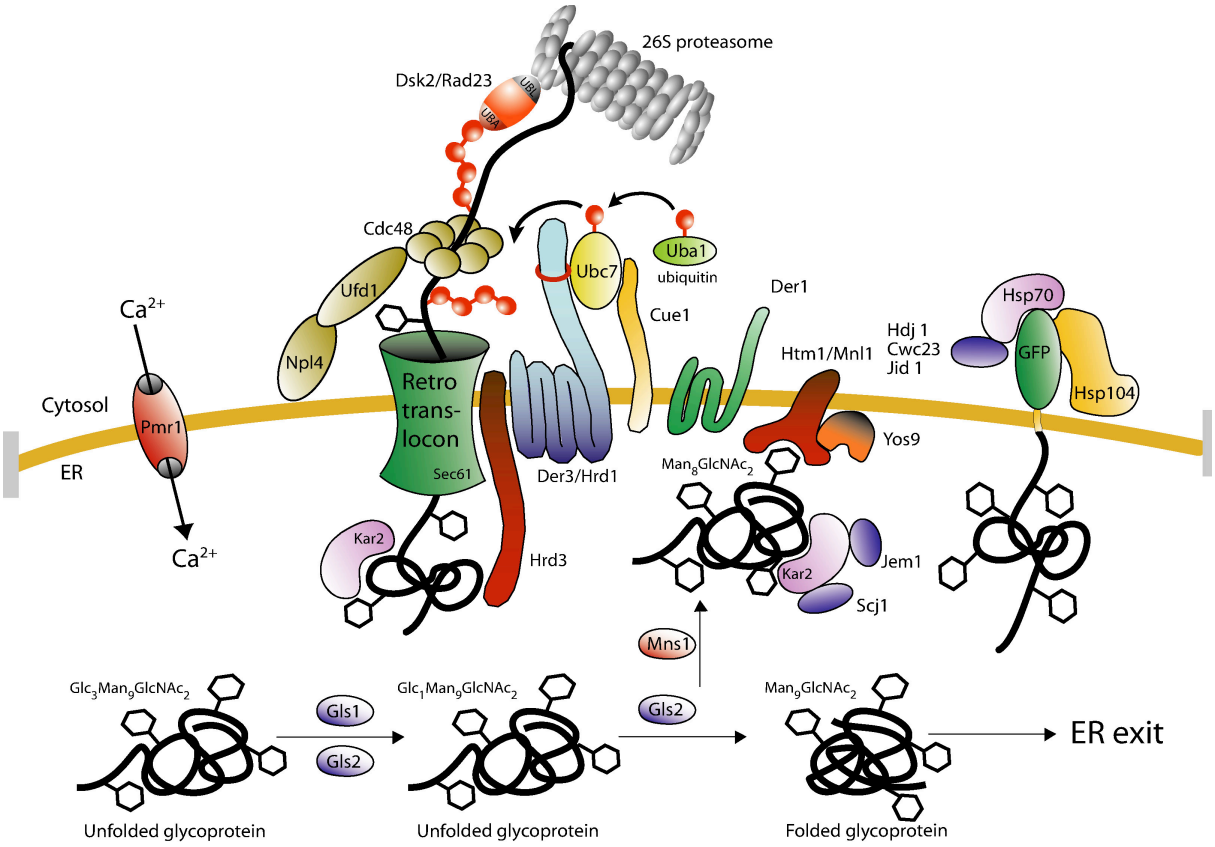

Fig. 18 Model of protein quality control system in yeast with newly identified components: Dsk2p, Rad23p, and Yos9p

Sec61-2p. Like human OS-9, yeast Yos9p contains a mannose-6-phosphate receptor homology (MRH) domain, which may play a general role in the N-glycan recognition (Munro, S. 2001). A similar domain is found in the β-subunits *of H. sapiens*, *S. pombe* and *S. cerevisiae* glucosidase II, an enzyme required for ER quality control (Helenius, A and Aebi, M. 2004). One may assume that Yos9p is a lectin with a general role in the ER quality control of misfolded N- glycoproteins. In fact, like Htm1p (Jakob et al., 2001) Yos9p is also not required for the transport of properly folded glycoproteins such as CPY and invertase, nor does it's absence lead to the induction of UPR (Friedmann et al., 2002)

To date with the exception of calnexin/calreticulin, the only other ER-lectin directly associated with ER quality control is Htm1p/Mnl1p (Jakob et al., 2001). The still undefined involvement of Yos9p in GPI anchor processing does not exclude its participation in ER quality control of malfolded proteins. It is plausible that Yos9p may be a dual function ER

–quality control component, like calnexin and PDI. (Ellgard and Helenius, 2003), with a general lectin-chaperon role for glycosylated malfolded substrates and more direct role in the processing or even quality control of GPI-anchored proteins. Disposal of proteins that fail to become GPI-anchored either due to the presence of faulty GPI-anchoring signal or due to problems in GPI-anchor assembly seem to occur via intracellular cytoplasmic degradation with ERAD features (Wilbourn et al., 1998, Ali et al., 2000). Proteins destined to acquire a GPI-anchor may appear as malfolded to the ER quality control machinery till they become attached to the GPI-anchor. Yos9p may be the component involved in quality control of GPI-anchor addition. Recognition and function may lie within the glycans (either on Yos9p or the substrate or both) of the MRH domain or a, still unknown determinant. It could also be speculated that Yos9p is a part of the delivery process of the glycoslylated substrate to the retrotranslocation machinery.

It will be interesting to see if Yos9p shows interaction with Htm1p/Mnl1p and if its action is dependent on Htm1p/Mnl1p or not, if they recognise different glycan based determinants, and at what stage of core glycan structure Yos9p binds to misfolded glycoproteins. Studies addressing all these aspects will broaden our understanding about glycoprotein quality control and degradation.

Apart from Dsk2p, Rad23p and Yos9p whose function in ER quality control and degradation was characterized in this study to a certain extent, the genomic screen also resulted in the identification of many other interesting proteins whose function with respect to ERAD was not known before. The genomic screen also resulted in the identification of proteins like Hul5p which was characterized as a of HECT domain ubiquitin ligase, Nsr1p whose deletion results in defects of the ER-Golgi traffic and apoptosis in yeast, Mad2p and Mad3p which were shown to be involved in the duplication of spindle pole bodies. It is interesting to note that proteins like Cdc48p and Dsk2p which plays a crucial role in ERAD, were also first identified as components essential for spindle pole body duplication, suggesting the involvement of common components in both processes to some extent. The genomic screen also identified, Psh1p which is characterized by the presence of a Zn finger domain as in Der3p, Swa2p which possesses Hsp70 like chaperone activity in the formation of the vesicles for ER-Golgi transport and many other proteins about which very little or nothing is known. It will be very interesting to identify the functions of these proteins in ER quality control and degradation. It will also be interesting to compare the proteins which were identified in the screen by using the glycoprotein CTL* with the proteins which are going to be identified by using the non-glycoprotein Sec61-2-Leu2p to understand the differences in the requirements

of both, misfolded glycoproteins and misfolded non glycosylated proteins in quality control and ERAD. In depth analysis of the new proteins identified in the screen by using both glycoprotein and non-glycoprotein ERAD substrates will help us to gain a lot of knowledge and better understanding about these vital physiological processes by which living cells get rid of the junk proteins. A detailed knowledge of these processes is of great importance not only for our understanding of this basic cellular mechanism but also for the development of new strategies to treat a diverse set of diseases.

References

Ali, B. R., Claxton, S. and Field, M. C. (2000) Export of a misprocessed GPI-anchored protein from the endoplasmic reticulum *in vitro* in an ATP- and cytosol- dependent manner. *FEBS lett.*, **483**, 32-36.

Baumeister, W., Walz, J., Zhul, F. and Seemuller, E. (1998) The proteasome: paradigm of a self-compartmentalizing protease. *Cell*, **92**, 367-380.

Biederer, T., Volkwein, C. and Sommer, T. (1996) Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin-proteasome pathway. *EMBO J.*, **15**, 2069-2076.

Biggins, S., Ivanovska, I. and Rose, M. D. Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing centre. *J. Cell Biol.*, **133**, 1331-1346.

Braun, B. C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P.M., Finley, D., and Schmidt, M. (1999) The base of proteasome regulatory particle exhibits chaperone like activity. *Nat. Cell. Biol.*, **1**, 221-226.

Braun, S., Mattuschewski, K., Rape, M., Thomas, S. and Jentsch, S. (2002). Role of ubiquitin selective CDC48 $^{UFD1/NPL4}$ chaperone (segregase) in ERAD of OLE1 and other substrates. *EMBO J.*, **21**, 615-621.

Biederer, T., Volkwein, C. and Sommer, T. (1997) Role of Cue1p in ubiquitylation and degradation at the ER surface. *Science*, **278**, 1806-1809.

Bonifacino, J. S., and Klausner, R.D. (1994) Degradation of proteins retained in the endoplasmic reticulum. *Modern cell Biol.*, **9**, 209-222.

Bordallo, J., Plemper, R. K., Finger, A. and Wolf, D. H. (1998) Der3/Hrd1p is required for endoplasmic reticulum – associated degradation of misfolded lumenal and integral membrane proteins. *Mol. Biol. Cell*, **9**, 209-222.

Bordallo, J. and Wolf, D. H. (1999) A RING-H2 finger motif is essential for the function of Der3/Hrd1p in endoplasmic reticulum associated degradation in the yeast Scaacharomyces cerevisiae. *FEBS lett.*, **448**, 244-248.

Buchberger, A. (2002) From UBA to UBX: new words in the ubiquitin vocabulary. *Trends in Cell Bio.*, **12**, 216-221.

Cabral, C. M., Liu, Y. and Sifers, R. N. (2001) Dissecting glycoprotein quality control in the secretory pathway. *Trends Biochem. Sci.*, **26**, 619-624.

Chen, L., Madura, K. (2002) Rad23 promotes the targeting of proteolytic substrates to the proteasome. *Mol. Cell. Biol.*, **22**, 4902-4913.

Clarke, D. J., Mondesert, G., Segal, M., Bertolaet, B. L., Jensen, S., Wolff, M., Henze, M. and Reed, S. I. (2001) Dosage suppressors of pds1 implicate ubiquitin-associated domains in checkpoint control. *Mol. Cell. Biol.*, **21**, 1997-2007.

Dai, R. M. and Li, C. C. (2001) Valosin containing protein is a multi ubiquitin chain targeting factor required in ubiquitin-proteasome degradation. *Nat.Cell Biol.*, **3**, 740-744.

Deak, P. M. and Wolf, D. H. (2001) Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. *J. Biol. Chem.*, **276**, 10663-10669.

Dürr, J., Strayle, J., Plemper, R. K., Elbs, S., Klee, S. K., Catty, P., Wolf, D. H. and Rudolph, H. K. (1998) The medial- Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca2+ and Mn2+ required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. *Mol. Biol. Cell*, **9**, 1149-1162.

Ellgaard, L and Helenius, A. (2003) Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol.*, **43**, 181-191.

Ellgaard, L, Molinari, M, Helenius, A. (1999) Setting the standards: quality control in the secretory pathway. *Science*, **286**, 1882-1888.

Elsässer, S., Gali, R. R., Schwickart, M., Larsen, C. N., Leggett, D. S., Müller, B., Feng, M. T., Tubing, F., Dittmar, G. A. and Finley, D. (2002) proteasome subunit Rpn1 binds ubiquitin like protein domains. *Nat. Cell. Biol.*, **4**, 725-730.

Feibiger, E., Strocy, C., Ploegh, H.L. and Tortorella, D. (2002) visualization of the ER-cytosol dislocation reaction of a type I membrane protein. *EMBO J.*, **21**, 1041-1053.

Finger, A., Knop, M. and Wolf, D. H. (1993) Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment in yeast. *Eur. J. Biochem.*, **218**, 565-574.

Friedländer, R., Jarosch, E., Urban, J., Volkwein, C. and Sommer, T. (2000) A regulatory link between ER-associated protein degradation and the unfolded -protein response. *Nat. Cell. Biol.*, **2**, 379-384.

Friedmann, E., Salzberg, Y., Weinberger, A., Shaltiel, S. and Gerst, J. E. (2002) *Yos9*, the putative yeast homolog of a gene amplified in osteosarcomas, is involved in the endoplasmic reticulum (ER)-Golgi transport of GPI-anchored proteins. *J. Biol. Chem.*, **277**, 35274-35281.

Funakoshi, M., Sasaki, T, Nishimoto, T. and Kobayashi, H. (2002) Budding yeast Dsk2p is a polyubiquitin- binding protein that can interact with the proteasome. *Proc. Natl. Acad. Sci., USA*, **99**, 745-750.

Gardner, R. G., Swarbrick, G. M., Bays, N.W., Cronin, S.R., Wilhovsky, S., Seelig, L., Kim, C., and Hampton, R. Y. (2000) Endoplasmic reticulum degradation requires lumen to cytosol signalling. Transmembrane control of Hrd1p by Hrd3p. *J. Cell Biol.*, **151**, 69-82.

Glick, B. S. (2002) The secretory pathway. In Dalbey, R. E. and von Heijne, G. (eds), Protein *targeting, transport and translocation.* Academic press, London, UK, 358-376.

Griggs, D. W. and Johnson, M. (1993) Promoter elements determining weak expression of the *GAL4* regulatory gene of *Saccharomyces cerevisiae*. *Mol. Cell Biol.*, **13**, 4999-5009.

Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D. and Huber, R. (1997) Structure of the 20S proteasome from yeast at 2.4A° resolution. *Nature*, **386**, 463-471.

Groll, M., Bajorek, M., Kohler, A., Moroder, L., Rubin, D. M., Huber, R., Glickman, M. H. and Finley, D. (2000) A gated channel into the proteasome core particle. *Nat. Struct. Biol.*, **7**, 1062-1067.

Gnann, A., Riordan, J. R., and Wolf, D. H. (2004) CFTR degradation depends on the lectins Htm1p/EDEM and the Cdc48 protein in yeast. *Mol. Biol. Cell*, **15**, 4125-4135.

Gurthrie, C. and Fink, G. R. (1991) *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Academic press, **194**, San Diego, CA, USA.

Haigh, N. G. and Johnson, A. E. (2002) Protein sorting at the membranes of the endoplasmic reticulum. In Dalbey, R. E. and von Heijne, G. (eds), Protein *targeting, transport and translocation*. Academic press, London, UK, 74-106.

Hampton, R. Y. (2002) Proteolysis and sterol regulation. *Annu. Rev. Cell. Dev. Biol.*, **18**, 345-378.

Hartmann-Petersen, R., Seeger, M. and Gordon, C. (2003) Transferring substrates to the 26S proteasome. *Trends. Biochem. Sci.*, **28**, 26-31.

Haynes, C. M., Caldwell, S. and Cooper, A. A. (2002) An HRD/DER independent quality control mechanism involves Rsp5p dependent ubiquitylation and ER-Golgi transport. *J. Cell Biol.*, **158**, 91-101.

Heinemeyer, W., Kleinschimdt, J. A., Saidowsky, J., Escher, C. and Wolf, D. H. (1991) Proteinase yscE, the yeast proteasome/multicatalytic multifunctional proteinase: mutants unravel it's function in stress induced proteolysis and uncover it's necessity for cell survival. *EMBO J.*, **10**, 555-562.

Helenius, A. and Aebi, M. (2004) Role of N-linked glycans in the endoplasmic reticulum. *Annu. Rev. Biochem.*, **73**, 1019-1049.

Hiller, M. M., Finger, A., Schweiger, M. and Wolf, D. H. (1996) ER degradation of a misfolded lumenal protein by the cytosolic ubiquitin-proteasome pathway. *Science*, **273**, 1725-1728.

Hitt, R. and Wolf, D. H. (2004) Der1p a protein required for degradation of malfolded soluble proteins of the endoplasmic reticulum: topology and Der1 like proteins. *FEMS Yeast Res.*, **4**, 721-729.

Hitt, R. and Wolf, D. H. (2004b) *DER7*, encoding alpha-glucosidase I is essential for degradation of malfolded glycoproteins of the endoplasmic reticulum. *FEMS Yeast Res.*, **4**, 815-820.

Hilt, W. and Wolf, D. H. (1996) Proteasomes: destruction as a programme. *Trends Biochem. Sci.*, **21**, 96-102.

Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L. O., Herscovics, A. and Nagata, K. (2001) A novel ER α-mannosidase like protein accelerates ER-associated degradation. *EMBO Rep.*, **2**, 415-422.

Jakob, C. A., Burda, P., Roth, J. and Aebi, M. (1998) Degradation of misfolded endoplasmic reticulum glycoproteins in *Saccharomyces cerevisiae* is determined by a specific oligosaccharide structure. *J. Cell Biol.*, **142**, 1223-1233.

Jakob, C.A., Bodmer, D., Spirig, U., Battig, P., Marcil, A., Dignard, D., Bergeron, J.J., Thomas, D. Y. and Aebi, M. (2001) Htm1p a mannosidase–like protein, involved in glycoprotein degradation in yeast. *EMBO Rep.*, **2**, 423-430.

Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H. and Sommer, T. (2002) Protein dislocation from the ER requires polyubiquitylation and the AAA-ATPase Cdc48. *Nat. Cell. Biol.*, **4**, 134-139.

Johnson, A. E. and Van Waes, M. A. (1999) The translocon: a dynamic gateway at the ER membrane. *Annu. Rev. Cell. Dev. Biol.*, **15**, 799-842.

Johnson, E. S., Ma, P. C., Ota, I. M. and Varshavsky (1995) A proteolytic pathway that recognises ubiquitin as degradation signal. *J. Biol. Chem.*, **270**, 17442-17456.

Kaplun, L., Ivanstiv, Y., Kornitzer, D. and Raveh, D. (2000) Functions of the DNA damage response pathway target Ho endonuclease of yeast for degradation via the ubiquitin-26S proteasome pathway. *Proc. Natl. Acad. Sci.*, **97**, 10077-10082.

Kim, I., Mi, K. and Rao, H. (2004) Multiple interactions of RAD23 suggest a mechanism for ubiquitylated substrate delivery important in proteolysis. *Mol. Biol. Cell.* **15**, 3357-3365.

Kimura, Y., Nakazawa, M., and Yamada, M. (1998) Cloning and characterization of three isoforms of OS-9 cDNA and expression of the OS-9 gene in various human tumour cell lines. *J. Biochem. (Tokyo)* **123**, 876-882.

Knop, M., Finger, A., Braun, T., Hellmuth, K. and Wolf, D. H. (1996a) Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *EMBO J.*, **15**, 753-763.

Knop, M., Hauser, N. and Wolf, D. H. (1996b) N-Glycosylation affects endoplasmic reticulum degradation of a mutated derivative of carboxypeptidase yscY in yeast. *Yeast*, **12**, 1229-1238.

Kostova, Z. and Wolf, D. H. (2002) Protein quality control in the export pathway: the endoplasmic reticulum and its cytoplasmic proteasome connection. In Dalbey, R. E. and von Heijne, G. (eds). *Protein targeting, transport and translocation.* Academic press, London, UK, 180-213.

Kostova, Z. and Wolf, D. H. (2003) For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. *EMBO J.*, **22**, 2309-2317.

Köhler, A., Cascio, P., Leggett, D. S., Woo, K. M., Goldberg, A. L. and Finley, D. (2001) The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and conducts both substrate and product release. *Mol. Cell*, **7**, 1143-1152.

Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L. and Pickart, C. M. (2002) A proteasomal ATPase subunit recognises the polyubiquitin degradation signal. *Nature*, **416**, 763-767.

Lilley, B. N. and Ploegh, H. L. (2004) A membrane protein required for dislocation of misfolded ER proteins from the ER. *Nature*, **430**, 264-271.

Litovchick, L., Friedmann, E. and Shaltiel, S. (2002) A selective interaction between OS-9 and the carboxyl-terminal tail of merpin beta. *J. Biol. Chem.*, **277**, 34413-34423.

Liu, C. Y., Schroder, M. and Kaufman, R. J. (2000) Ligand independent dimerization activates the stress response kinases IRE1 and PERK in the lumen of the endoplasmic reticulum. *J. Biol. Chem.*, **275**, 24881-24885.

Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S. and Weissman, A. M. (1999) RING finger mediates ubiquitin- conjugating enzyme (E2)- dependent ubiquitination. *Proc. Natl. Acad. Sci.*, **96**, 11364-11369.

McCraacken, A. A. and Brodsky, J. L. (1996) Assembly of ER-associated protein degradation *in vitro*: dependence of cytosol, clanexin and ATP. *J. Cell Biol.*, **132**, 291-298.

McGrath, J. P., Jentsch, S., Varshavsky, A. (1991) *UBA1* an essential yeast gene encoding ubiquitin activating enzyme. *EMBO J.*, **10**, 227-236.

Molinari, M., Calanca, V., Galli, C., Lucca, P., Paganetti, P. (2003) Role of EDEM in the release of misfolded glycoprotein from calnexin cycle. *Science*, **299**, 1397-1340.

Munro, S. (2001) The MRH domain suggests a shared ancestry for the mannose 6-phosphate receptors and other N-glycan-recognising proteins. *Curr. Biol.*, **11**, 499-501.

Nilsson, I. M. and von Heijne, G. Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J. Biol. Chem.*, **268**, 5798-5801.

Nishikawa, S. I., Fewell, S. W., Kato, Y., Brodsky, J. L. and Endo. T (2001) molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. *J. Cell Biol.*, **153**, 1061-1070.

Oda, Y., Hosokawa, N., Wada, I. and Nagata, K. (2003) EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. *Science*, **299**, 1394-1397.

Pilon, M., Schekman, R. and Romisch, K. (1997) Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *EMBO J.*, **16**, 4540-4548.

Plemper, R. K., Bohmler, S., Bordallo, J., Sommer, T. and Wolf, D.H. (1997) Mutant analysis links the translocon and Bip to retrograde protein transport for ER degradation. *Nature*, **388**, 891-895.

Plemper, R. K., Egner, R., Kuchler, K. and Wolf D. H. (1998) Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. *J. Biol. Chem.*, **273**, 32848-32856.

Plemper, R.K. and Wolf, D. H. (1999) Retrograde protein translocation: ERADication of secretory proteins in health and disease. *Trends Biochem. Sci.* **24**, 266-270.

Plemper, R. K., Deak, P. M., Otto, R. T. and Wolf, D. H. (1999b) Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase ysc Y species. *FEBS Lett.*, **443**, 241-245.

Plemper, R. K. and Wolf, D.H. (2000) *Function of the proteasome in the protein quality control process of the endoplasmic reticulum*. In Hilt, W. and Wolf, D. H., *Proteasomes: The world of regulatory proteolysi*s, Landes Bioscience, Texas, USA, pp 332-341.

Rapoport, T. A., Jungnickel, B, and Kutay, U. (1996) Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.* **65**, 271-303.

Raasi, S. and Pickart, C. M. (2003) Rad23 ubiquitin associated domains (UBA) inhibit 26S proteasome-catalyzed proteolysis by sequestering lysine 48 linked polyubiquitin chains. *J. Biol. Chem.*, **278**, 8951-8959.

Rabinoviche, E., Kerem, A., Frohlich, K. U., Diamant, N. and Bar-Nun, S. (2002) AAA ATPase p97/Vdv48p a cytosolic chaperone required for endoplasmic reticulum protein degradation. *Mol. Cell. Biol.*, **22**, 626-634.

Ramos, P.C., Hockendorff, J., Johnson, E. S., Varshavsky, A. and Dohmen, R. J. (1998) Ump1p is required for the proper maturation of the 20S proteasome and become its substrate upon completion of the assembly. *Cell*, **92**, 489-499.

Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H. and Jentsch, S. (2001) Mobilization of processed, membrane –tethered SPT23 transcription factor by CDC48 $^{UFD1/NPL4}$, a ubiquitin-selective chaperone. *Cell*, **107**, 667-677.

Rao, H. and Sastry, A. (2002) Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. *J. Biol. Chem.*, **277**, 11691-11695.

Regelmann, J., Schule, T., Josupeit, F. S., Horak, J., Rose, M., Thumm, M. and Wolf. D. H. (2003) Catabolite degradation of fructose -1,6-bisphosphatase in the yeast *Saccharomyces cerevisiae*: a genome wide screen identifies eight novel GID genes and indicates the existence of two degradation pathways. *Mol. Biol. Cell.*, **14**, 1652-1663.

Rouiller, I., Butel, V. M., Latterich, M., Milligan, R. A. and Wilson-Kaubalek, E. M. (2000) A major conformational change in p97 AAA ATPase upon ATP binding. *Mol. Cell*, **6**, 1485-1490.

Scharg, J., Procopio, D. O., Cygler, M., Thomas, D. Y. and Bergeron, J. J. (2003) Lectin control of protein folding and sorting in the secretory pathway. *Trends Biochem. Sci.*, **28**, 49-57.

Sidrauski, C., Brickner, J. H. and Walter, P. (2002) The unfolded protein response. In Dalbey, R. E. and von Heijne, G. (eds), *Protein targeting, transport and translocation*. Academic press, London, UK, 151-179.

Sommer, T and Wolf, D. H. (1997) Endoplasmic reticulum degradation: reverse protein flow of no return. *FASEB J*., **11**, 1227-1223.

Sommer, T. (2000) The *ubiquitin system in yeast* In Hilt, W. and Wolf, D. H., *Proteasomes: The world of regulatory proteolysi*s, Landes Bioscience, Texas, USA, pp204-211.

Su, Z. A., Hutter, C. M., Trent, J. M. and Meltzer, P. S. (1996) Complete sequence analysis of a gene (OS-9) ubiquitously expressed in human tissues and amplified in sarcomas. *Mol. Carcinog*., **15**, 270-275.

Suzuki, T., Park, H., Hollingsworth, N. M., Sternglannz, R. and Lennarz, W. J. (2000) *PNG1*, a yeast gene encoding a highly conserved peptide: N-glycanase. *J. Cell Biol.*, **149**, 1039-1052.

Suzuki, T., Park, H., Kwofie, M. A. and Lennarz, W. J. (2001) Rad23 provides a link between the Png1 deglycosylating enzyme and the 26S proteasome in yeast. *J. Biol. Chem.*, **276**, 21601-21607.

Swanson, R., Locher, M. and Hochstrasser, M. (2001) A conserved ubiquitin ligase of the nuclear envelope /endoplasmic reticulum that functions in both ER-associated and Matα2 repressor degradation. *Genes Dev*., **15**, 2660-2674.

Taxis, C., Vogel, F. and Wolf, D. H. (2002) ER-Golgi traffic is a prerequisite for efficient ER degradation. *Mol. Biol. Cell*, **13**, 1806-1818.

Taxis, C., Hitt, R., Park, S- H., Deak P. M., Kostova, Z. and Wolf, D. H. (2003) Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. *J. Biol. Chem.*, **278**, 35903-35913.

Thrower, J. S., Hoffman, J. L., Rechsteiner, M, and Pickart, C. M. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J*., **19**, 84-102.

Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weismann, J. S. and Walter, P. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell*, **101**, 249-258.

Triosh, B., Furman, M. H., Tortorella, D. and Ploegh, H. L. (2003) Protein unfolding is not a prerequisite of endoplasmic reticulum- to-cytosol dislocation. *J. Biol. Chem.*, **278**, 6664-6672.

Vashist, S., Frank, C. G., Jakob, C. A. and Ng D. T. (2002) Two distinctly localized P-type ATP-ases collaborate to maintain organelle homeostasis required for glycoprotein processing and quality control. *Mol. Biol. Cell*, **13**, 3955-3966.

Verma, R., Aravind, L., Oania, R., Mcdonald, W. H., Yates, I. J., Koonin, E. V. and Deshaies, R. J. (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome, *Science*, **298**, 611-615.

Walter, J., Urban, J., Volkwein, C. and Sommer, T. (2001) Sec61p independent degradation of the tail-anchored ER membrane protein Ubc6p. *EMBO J.*, **20**, 3124-3131.

Wang, Q. and Chang, A. (1999) Eps1, a novel PDI-related protein involved in ER quality control in yeast. *EMBO J.*, **18**, 5972-5982.

Watkins, J. F., Sung, P., Prakash, L. and Prakash, S. (1993) The *Saccharomyces cerevisiae* DNA repair gene *RAD23* encodes a nuclear protein containing a ubiquitin like domain required for biological function. *Mol. Cell Biol.*, **13**, 7757-7765.

Werner, E. D., Brodsky, J. J., and McCracken, A. A. (1996) Proteasome dependent endoplasmic reticulum-associated protein degradation an unconventional route to a familiar fate. *Proc. Natl. Acad. Sci. USA*, **93**, 1397-13801.

Wiertz, E. J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A. and Ploegh, H. L. (1996) Sec61- mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature*, **384**, 432-438.

Wilbourn, B., Nesbeth, D. N., Wainwright, L. J. and Fied, M. C. (1998) Proteasome and thiol involvement in quality control of glycosyl phosphatidyl inositol anchor addition *Biochem J*., **332**, 111-118.

Wilkinson, C. R., Seeger, M., Hartmann-Petersen, R., Stone, M., Wallace, M., Semple, C. and Gordon, C. (2001) Proteins containing the UBA domains are able to bind to multi-.ubiquitin chains. *Nat. Cell. Biol.* **3**, 939-943.

Wolf, D. H. and Hilt, W. (2004) The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. *BBA*, **1695**, 19-31.

Yao, T. and Cohen, R. E. (2002) A cryptic protease couples deubiquitylation and degradation by the proteasome. *Nature*, **419**, 403-407.

Ye, Y., Meyer, H. H. and Rapoport, T. A. (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature*, **414**, 652-656.

Ye, Y., Shibata, Y., Yun, C., Ron, D. and Rapoport, T. A. (2004) A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature*, **429**, 841-847.

Zhang, X., Shaw, A., Bates, P. A., Newman, R. H., Gowen, B., Orlova, E., Gorman, M. A., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., Van Heel, M. and Freemont, P. S. (2000) Structure of the AAA ATPase p97, *Mol. Cell*, **6**, 1473-1484.

Zhang, X., Nijbroeck, G., Sullivan, M. L. McCracken, A. A., Watkins, S. C., Michaelis, S. and Brodsky, J. L. (2001) Hsp70 molecular chaperone facilitates endoplasmic reticulum –associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. *Mol. Biol. Cell*, **12**, 1303-1314.

Zhou, M. and Schekman, R. (1999) The engagement of Sec61p in ER dislocation process. *Mol. Cell*, **4**, 925-934.

Curriculum Vitae

BALA SUBRAHMANYAM MEDICHERLA

PERSONAL INFORMATION

NAME	Bala Subrahmanyam Medicherla
NATIONALITY	Indian
SEX	Male
DATE OF BIRTH	15-08-1977
PLACE OF BIRTH	Anakapalli, India

EDUCATION

B.Sc Andhra University, Visakhapatnam, India, March 1998

M.Sc Department of Biochemistry, University of Hyderabad, Hyderabad, India, May 2000

(Ph.D). Institute of Biochemistry, University of Stuttgart, Stuttgart, Germany (To be finished by December 2004)

RESEARCH EXPERIENCE

2001- present **Graduate Student, Institute of Biochemistry, University of Stuttgart, Stuttgart, Germany**

Identified and studied various new components involved in the ER-associated degradation of *S.cerevisiae* under the supervision of Prof. Dieter H Wolf.

Ph.D. Thesis: A genomic screen in *Saccharomyces cerevisiae* identifies multiple new gene products essential for protein quality control of the endoplasmic reticulum and degradation: The role of Dsk2p, Rad23p and Yos9p

TEACHING EXPERIENCE

2001-2003 Autumn Teaching Assistant for Graduate level Biochemistry laboratory courses, University of Stuttgart, Stuttgart, Germany.

1998-2000 spring Teaching Assistant for UnderGraduate level Biochemistry laboratory courses, University of Hyderabad, Hyderabad, India.

AWARDS & HONORS

1996-1998 University Grants Commission, India, fellowship in B.Sc.

1998-2000 Department of Science and Technology, India, fellowship for meritorious students in M.Sc.

2001- present Fellowship from the University of Stuttgart.

RESEARCH EXPERTISE

Genetic and molecular biological manipulation of *Saccharomyces cervisiae*
Molecular biological techniques
Expression of epitope tagged eukaryotic proteins in *E. coli* and *S. cervisiae*
Immunoprecepitation, SDS-PAGE and immunoblot analysis of proteins
Preparation of yeast microsomes and determining the ER localization of proteins
Detection of Ubiquitinated proteins in various cellular compartments
Determination of the ER-associated degradation of proteins by pulse chase and Cycloheximide decay analysis
Genome wide mutant screen in *S. cervisiae* by multi well transformation assay

PUBLICATIONS

Medicherla, B., Kostova, Z., Schaefer, A. and Wolf, D. H. (2004) A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Rep*., **5**, 692-697.
Buschhorn, B. A*., Kostova, Z*., **Medicherla B*.** and Wolf, D. H. (2004) A genome-wide screen identifies Yos9p as essential for ER-associated degradation of glycoproteins. *FEBS lett*., **577**, 422-426.
(* *These authors in alphabetical order, contributed equally to this work)*

RESEARCH PRESENTATIONS

A Genomic screen identifies multiple new gene products involved in ER-associated Degradation (ERAD). Annual conference of the *European Life Scientist Organization* (ELSO) Dresden, Germany, September 20-23, 2003.

Two proteasome interacting proteins Dsk2p and Rad23p are essential components of ER-associated degradation. *Yeast, an Eukaryotic Model Organism to Elucidate Central Questions in Cell Biology* Hirschegg, Austria, September 26-28, 2003.

POSTER PRESENTATION

Medicherla, B., Kostova, Z., Schaefer, A. and Wolf, D. H. A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *FASEB Summer Research Conferences: Ubiquitin and cellular regulation*. Saxtons River, Vermont, USA, June26-July1, 2004.

•

Acknowledgments

This thesis would not have come to form were it not for my interaction with several other individuals, and I am happily in their debt.

I would like to express my sincere thanks and appreciation to my supervisor Prof. Dieter H Wolf. I could not have imagined having a better advisor and mentor for my PhD, without his common sense, knowledge, perceptiveness, patience I would never have finished. I am proud to have been able to work with you.

I am grateful to Dr. Zlatka Kostova for patiently introducing me to the basic concepts of Molecular Biology and Biochemistry, her helpful comments and suggestions were of great use.

I am extremely thankful to the effort and time of Fotini Bratsika, while introducing the multi well transformation technique.

It's indeed a wonderful opportunity to work with my colleagues, Li Xiao, Sae-Hun Park, Andreas Gnann, Dr. Antje Schaefer, Cecile Alberola, and Simone Guthörlein. I am thankful to all of them for creating a wonderful atmosphere in the lab and for helping me almost everyday, whether it is with science or personal life. I miss you all a lot.

I am thankful to Joachium Schmidt for his help with the German language or with searching a room in Stuttgart; I am also grateful to Harish Karnam and Rui Estiveira for patiently listening to my joy or sorrow.

I am grateful to Ms. Huth and Ms. Tosta for taking care of all my official work during my stay and for spending their valuable time to my private work. I am also thankful to the help of Dr. Rudolph and Dr. Hilt (with computers) and Dr. Heinemeyer (with iso lab) and to all the members of Institute of Biochemisty, University of Stuttgart.

Last but not the least I would like to thank my Parents and Sister for their love, support, encouragement and advise.

Erklärung

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

Stuttgart,

Balasubrahmanyam Medicherla