Components and mechanisms of cytoplasmic protein quality control and elimination of regulatory enzymes

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

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Tag der mündlichen Prüfung: 24. Mai 2011

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2011

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Stuttgart, 12. Januar 2011

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Abbreviations

Å	Ångström
AAA	ATPases associated with diverse cellular activities
ADP	Adenosine 5’-diphosphate
ALS	Amyotrophic lateral sclerosis
APC	Anaphase promoting complex
ATP	Adenosine 5’-triphosphate
AZC	L-azetidine-2-carboxylic acid
BRR	Basic rich region
C-terminal	Carboxy-terminal
CP	Core particle
Cvt	Cytosol to vacuole targeting
DNA	Desoxyribonucleic acid
DUB	Deubiquitylating enzyme
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
ERAC	ER-associated compartment
ERAD	ER-associated protein degradation
ERQD	ER quality control and associated protein degradation
Fig	Figure
g	Grams
GFP	Green fluorescent protein
GID	Glucose induced degradation deficient
HA	Hemagglutinin
HECT	Homologous to the E6-AP carboxyl terminus
Hsp	Heat shock protein
HSR	Heat shock response
IPOD	Insoluble protein deposit
JUNQ	Juxtanuclear quality control compartment

kDa	Kilodalton
l	Litre
mRNA	Messenger RNA
N-terminal	Amino-terminal
NAT	N-terminal acetyltransferase
NBD	Nucleotide-binding domain
NEF	Nucleotide exchange factor
NMR	Nuclear magnetic resonance
Ntn	N-terminal nucleophile
ODC	Ornithine decarboxylase
OST	Oligosaccharyl transferase
PCNA	Proliferating cell nuclear antigen
PDI	Protein disulfide isomerase
PGK	3-phosphoglycerate kinase
RING	Really interesting new gene
RNA	Ribonucleic acid
S	Svedberg
S. cerevisiae	*Saccharomyces cerevisiae*
SBD	Substrate-binding domain
SCF complex	Skp, Cullin, F-box containing complex
SUMO	Small ubiquitin-like modifier
TAP	Tandem affinity purification
TM	Transmembrane
TOP	Thimet oligopeptidase
tRNA	Transfer RNA
TS	Temperature sensitive
UBA	Ubiquitin-associated
UBD	Ubiquitin-binding domain
UBL	Ubiquitin-like
UBX	Ubiquitin regulatory X

UDP	Uridine diphosphate
UFD	Ubiquitin fusion degradation
UGGT	UDP-glucose:glycoprotein glucosyl transferase
UIM	Ubiquitin-interacting motif
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
VHL	Von Hippel-Lindau tumor suppressor
WT	Wild-type

Abstract

Relatively little is known about cytoplasmic protein quality control in eukaryotic cells. After proteins have been translated on ribosomes, they have to achieve their native conformation, get to their place of action and be assembled into protein complexes when indicated. Errors in the protein sequence caused by DNA mutations, mistakes during transcription or translation, as well as folding disorders caused by chemical or physical stress can impair the proper functionality of the cell and evoke diseases. Therefore, it is the task of the cellular protein quality control system to assist proteins while folding into their native conformation, to unfold misfolded proteins and to refold them. Finally, irreversibly misfolded proteins have to be transferred for degradation to the proteolytic systems of the cell, the 26S proteasome or the vacuole (lysosome).

The components that are involved in the control of protein folding and in the transfer of misfolded cytoplasmic proteins to the proteolytic systems have been poorly investigated. In this work, novel components of the cytoplasmic quality control system have been discovered by studying mutated variants of carboxypeptidase Y (CPY*), a vacuolar enzyme, which due to deletion of its signal sequence cannot be imported into the endoplasmic reticulum (ER) for further transfer into the vacuole and therefore is permanently located to the cytoplasm of the budding yeast *Saccharomyces cerevisiae*. Studies investigating ΔssCPY* (signal sequence deleted CPY*), ΔssCG* (ΔssCPY* carrying a C-terminal GFP tag) and the corresponding wild-type enzyme ΔssCPY showed that for proteasomal degradation of these substrates the cytoplasmic chaperone Hsp70 (Heat shock protein) Ssa1, the Hsp40 co-chaperone Ydj1 and the ubiquitin-conjugating enzymes (E2) Ubc4 and Ubc5 are necessary. It could be shown that Ssa1 and Ydj1 are involved in the resolubilization of precipitated ΔssCG*, in keeping ΔssCG* in solution and in the transport of ubiquitylated ΔssCG* to the 26S proteasome.

The following study searched for further factors of the cytoplasmic quality control, especially a ubiquitin ligase (E3), which is capable of targeting misfolded cytoplasmic proteins for proteasomal degradation. Yeast mutants were isolated in a genetic screen, which are able to stabilize the fusion protein ΔssCL*myc (ΔssCPY* C-terminally fused to myc-tagged 3-isopropylmalate dehydrogenase (*LEU2*myc)) and are therefore able to grow on media lacking leucine. This led to the discovery of the E3 Ubr1. Subsequent investigations revealed that the proteasomal degradation of ΔssCL*myc is strongly dependent on Ubr1 and that the misfolded substrate

physically interacts with this E3. Furthermore, it could be shown that for degradation of ΔssCL*myc and ΔssCG* the Hsp110s Sse1 and Sse2 are necessary, probably functioning as nucleotide exchange factors for Ssa1.

Besides the degradation of finally misfolded cytoplasmic proteins, the eukaryotic cell utilizes its proteolytic systems to eliminate regulatory enzymes upon changes in the cellular environment. After switching cells from non-fermentable to fermentable media, a key regulatory enzyme in the gluconeogenesis pathway, fructose-1,6-bisphosphatase (FBPase), is ubiquitylated by the Gid-E3 complex and then degraded by the ubiquitin proteasome system (UPS) to allow switching from gluconeogenesis to glycolysis. In a further study we found that for degradation of ubiquitylated FBPase procession by the AAA-ATPase Cdc48 and its co-factors Ufd1 and Npl4 is necessary. This is the first time that for degradation of a native substrate by the UPS a dependency on the Cdc48-Ufd1-Npl4 complex could be shown. In addition, it could be shown that the ubiquitin receptor proteins Dsk2 and Rad23 are also necessary for the proteasomal degradation of FBPase.

Before a ubiquitylated substrate of the 26S proteasome is degraded, its ubiquitin chains are cleaved off. The ubiquitin-specific protease Ubp14 cleaves these free chains to single ubiquitin molecules. Cells deleted in *UBP14* accumulate ubiquitin chains, which leads to impairment of the UPS dependent protein degradation. In a further study we demonstrated that inhibition of proteasomal degradation by deletion of *UBP14* does not occur in the degradation process of all substrates tested. While *e.g.* UPS dependent degradation of the gluconeogenic enzyme FBPase is impaired in Δ*ubp14* strains, degradation of ΔssCG* is only slightly reduced and degradation of a misfolded substrate of the ER, CPY*HA is not at all affected. This finding suggests that there are several substrate specific pathways to proteasomal degradation, which can be defined by a varying dependency on Ubp14.

Zusammenfassung

Über die Proteinqualitätskontrolle im Zytoplasma von eukaryontischen Zellen ist vergleichsweise wenig bekannt. Nachdem Proteine an den Ribosomen translatiert wurden, müssen sie sich in ihre native Konformation falten, an ihren Wirkungsort gelangen und gegebenenfalls als Untereinheit eines Proteinkomplexes in einem solchen eingebaut werden. Fehler in der Proteinsequenz, verursacht durch

Mutationen der DNA, Fehler bei der Transkription oder Translation, sowie durch chemischen und physikalischen Stress auftretende Faltungsstörungen der Proteine, können die korrekte Funktionsweise der Zelle stören und Krankheiten hervorrufen. Aufgabe der Proteinqualitätskontrolle der Zelle ist es daher, Proteinen bei der Faltung in ihre natürliche Konformation zu helfen, fehlgefaltete Proteine zu entfalten und wieder von neuem zu falten. Endgültig fehlgefaltete Proteine müssen den proteolytischen Systemen der Zelle, 26S Proteasom oder Vakuole (Lysosom), zum Abbau zugeführt werden. Über die Komponenten, welche im Zytoplasma an der Durchführung und Kontrolle der korrekten Faltung, sowie an der Übergabe an die proteolytischen Systeme beteiligt sind, ist relativ wenig bekannt. Mittels Studien an mutierten Varianten des vakuolären Enzyms Carboxypeptidase Y (CPY*), die aufgrund genetischer Entfernung ihrer Signalsequenzen nicht in das Endoplasmatische Retikulum (ER) zum Weitertransport in die Vakuole importiert werden können, und daher permanent im Zytoplasma der Knospungs-Hefe *Saccharomyces cerevisiae* verbleiben, wurden neue Komponenten der zytoplasmatischen Qualitätskontrolle entdeckt. Durch Studien an ΔssCPY* (signalsequenzdeletierte CPY*), ΔssCG* (ΔssCPY* mit C-terminalem GFP), sowie am entsprechenden Wildtypenzym ΔssCPY konnte gezeigt werden, dass für den proteasomalen Abbau das zytoplasmatische Hsp70 (Hitzeschockprotein) Ssa1, das Hsp40 Ko-Chaperon Ydj1, sowie die ubiquitin-konjugierenden Enzyme (E2) Ubc4 und Ubc5 notwendig sind. Es konnte gezeigt werden, dass Ssa1 und Ydj1 an der Wiederauflösung von ausgefallenem ΔssCG*, an dem Prozess es in Lösung zu halten und am Transport von ubiquitinyliertem ΔssCG* zum 26S Proteasom beteiligt sind.

In einer anschließenden Studie wurde nach weiteren Faktoren der zytoplasmatischen Qualitätskontrolle gesucht, insbesondere nach einer Ubiquitinligase (E3), welche in der Lage ist, fehlgefaltete zytoplasmatische Proteine durch spezifische Ubiquitinylierung dem proteasomalen Abbau zu übergeben. Dafür wurden in einem genetischen Screen Hefemutanten isoliert, welche das Fusionsprotein ΔssCL*myc (ΔssCPY* mit C-terminaler Myc getaggter 3-Isopropylmalatdehydrogenase (*LEU2myc*)) stabilisieren und dadurch auf Medium ohne Leucin wachsen können. Dabei wurde das E3 Ubr1 gefunden. Durch anschließende Untersuchungen konnte gezeigt werden, dass der proteasomale Abbau von ΔssCL*myc stark von Ubr1 abhängig ist, sowie der Befund erhoben werden, dass das fehlgefaltete Substrat mit diesem E3 physikalisch interagiert. Ferner konnte gezeigt werden, dass für den

Abbau von ΔssCL*myc und ΔssCG* die Hsp110 Proteine Sse1 und Sse2, wahrscheinlich in ihrer Funktion als Nukleotidaustauschfaktoren für Ssa1, notwendig sind.

Neben endgültig fehlgefalteten zytoplasmatischen Proteinen, entfernt das Ubiquitin Proteasom System der eukaryontischen Zelle auch regulatorische Enzyme bei sich verändernden Umweltbedingungen. Werden Zellen von einem nicht-fermentierbarem auf fermentierbares Medium gewechselt, wird ein Schlüsselenzym der Gluconeogenese, Fructose-1,6-bisphosphatase (FBPase), durch den Gid-E3-Komplex ubiquitinyliert und dann durch das Ubiquitin Proteasom System (UPS) abgebaut, um von Gluconeogenese auf Glycolyse umzuschalten. In einer weiteren Studie konnte gezeigt werden, dass für den Abbau von ubiquitinylierter FBPase die Weiterverarbeitung durch die AAA-ATPase Cdc48 und seine Kofaktoren Ufd1 und Npl4 notwendig ist. Damit konnte zum ersten mal für ein natürliches Substrat des Ubiquitin Proteasom Systems eine Abhängigkeit vom Cdc48-Ufd1-Npl4 Komplex gezeigt werden. Außerdem konnte gezeigt werden, dass die Ubiquitinrezeptorproteine Dsk2 und Rad23 für den proteasomalen Abbau der FBPase notwendig sind.

Vor dem Abbau eines ubiquitinylierten Substrates durch das 26S Proteasom werden die Ubiquitinketten abgeschnitten. Die ubiquitin-spezifische Protease Ubp14 spaltet dann diese freien Ketten zu monomeren Ubiquitineinheiten. In *UBP14* deletierten Hefestämmen akkumulieren Ubiquitinketten, was dazu führt, dass der gesamte UPS abhängige Proteinabbau gestört wird. In einer weiteren Studie konnte gezeigt werden, dass die Hemmung des proteasomalen Abbaus durch Deletion von *UBP14* nicht, wie ursprünglich angenommen, für alle Substrate des UPS gleichermaßen gilt. Während z.B. der UPS abhängige Abbau des gluconeogenetischen Enzyms FBPase in Δ*ubp14* Stämmen gehemmt ist, ist der Abbau von ΔssCG* nur wenig und der von einem fehlgefalteten Substrat des Endoplasmatischen Retikulums (ER), CPY*HA, überhaupt nicht gestört. Dieser Befund deutet darauf hin, dass es verschiedene substratspezifische Wege zum Proteasom gibt, welche sich durch eine variierende Abhängigkeit von Ubp14 beschreiben lassen.

1. Introduction

A hallmark of the eucaryotic cell is its compartmentalization. DNA, the blueprint of life is stored in the nucleus. Certain sections of DNA are transcribed to mRNA. mRNA is transported out of the nucleus into the cytoplasm where it is translated into protein with the help of ribosomes and tRNA. Nascent polypeptides evolving from ribosomes need to fold into their native three-dimensional structures. This can be a problem in the molecularly crowded environment of the cell. Molecular chaperones assist newly synthesized proteins to fold properly and help in the process of multi-protein complex assembly.

In a yeast cell approximately 47% of proteins stay in the cytoplasm (Kumar et al., 2002). Proteins belonging to other compartments, with exception of a few polypeptides of mitochondria and chloroplasts in plants, have to be synthesized in the cytoplasm and subsequently transported to their final destination. Proteins belonging to the nucleus have to be transported back by passing through the nuclear pore complex. Proteins that have to be secreted out of the cell, inserted into the cell membrane, destined for the vacuole, the Golgi complex, or the endoplasmic reticulum (ER) have to enter the secretory pathway.

Protein levels in the cell are tightly regulated. Throughout their life, proteins are facing many threats. Aging, radiation and oxidative stress requires rapid and efficient removal of damaged proteins. Also, the cell cycle demands tight regulation and degradation of regulatory factors. Proteins that have to be finally removed from the cellular environment are usually degraded via the ubiquitin proteasome system. This protein degradation system is tightly regulated and involves many factors that specifically recognize client substrates. Most damaged proteins of the secretory pathway become substrates of the ER-associated protein degradation pathway (ERAD) which is intensively studied and of which many factors are known. Protein quality control and degradation mechanisms of the cytoplasm are much less intensively studied. This work concentrates on the identification of novel factors that assign the ubiquitin proteasome system to specifically recognize misfolded proteins of the cytoplasm.

1.1. Folding of proteins

For the description of protein structure four different levels are generally referred to. The primary structure is specified by the amino acid sequence. The secondary

structure describes the spatial arrangement of the backbone of amino acid residues that are in close proximity to each other. α helices and β strands are the most common elements of the secondary structure (which have been proposed in the fifties of the last century by Linus Pauling and Robert Corey). The tertiary structure describes the completely folded protein by defining the spatial arrangement of amino acid residues that are further away in the sequence and formation of intramolecular disulfide bonds. The quaternary structure describes the spatial distribution of polypeptide chains, which arrange in a protein complex.

In a test tube, folding of most proteins into their native conformation happens spontaneously and follows the interaction of side chains given by its linear amino acid sequence (Anfinsen, 1973). *In vivo*, folding takes place in a molecular crowded environment with up to 300-400 g l^{-1} of protein and other macromolecules (Hartl and Hayer-Hartl, 2009). Interactions between partially folded structures can cause misfolding and aggregation. Aggregation of folding proteins can happen when they expose hydrophobic patches and regions of unstructured polypeptides. In order to achieve proper folding and to prevent aggregation of different polypeptide chains in such an environment, most proteins need the assistance of molecular chaperones. Molecular chaperones are proteins that assist folding and unfolding of their client proteins by transient and in most cases non-covalent binding. Molecular chaperones shield folding intermediates from their environment and by doing so prevent aggregation with other peptides that are also in the process of folding (Dobson, 2003). John Ellis and Sean Hemmingsen originally justified the use of the term "molecular chaperone" by arguing that the traditional role of a human chaperone, if described in biochemical terms, is to prevent improper interactions between potentially complementary surfaces and to disrupt any improper liaisons that may occur (Ellis and Hemmingsen, 1989). Molecular chaperones are found in all kingdoms of life and within all compartments of the cell. One distinguishes between different families of chaperones some of which will be briefly described here.

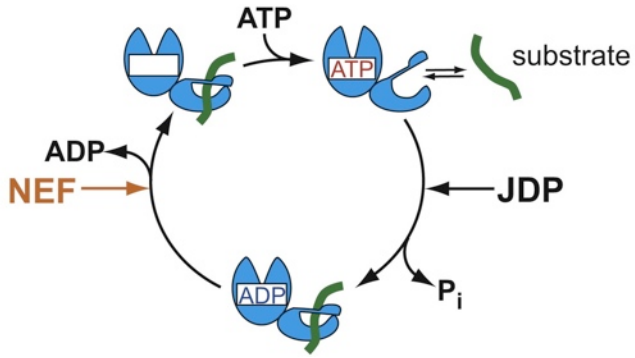
1.1.1. Hsp70 chaperone family

Members of the Hsp70 (Heat shock protein) family are among the best studied chaperones (Mayer and Bukau, 2005). A typical hallmark of Hsp70s is that they are rather unspecific in choosing client proteins which they bind and release in an ATP-dependent cycle (Fig. 1 A). Hsp70s have an N-terminal adenine nucleotide-binding domain, which regulates conformation and thereby accessibility of the C-terminal

peptide-binding domain that can bind to clients exposing hydrophobic patches of 4 to 5 amino acids in length (Fig. 1 B) (Rüdiger et al., 1997). Typical substrates of Hsp70s are unfolded proteins and partially folded intermediates. In the ATP bound state client proteins can bind and dissociate rapidly, while in the ADP bound state affinity between Hsp70s and client proteins is increased. Since ATP hydrolysis of Hsp70s is slow, they need co-factors to accelerate ATP transition to ADP in order to enable capturing of client proteins. Members of the Hsp40 family trigger this ATPase activity. Finally, for dissociation of the bound ADP, nucleotide exchange factors are needed, which typically belong to the Bag or the Hsp110 and Hsp170 families. With the help of their co-factors, Hsp70s are able to accomplish a client protein-chaperone binding and release cycle which is driven by ATP hydrolysis and ADP dissociation (Kampinga and Craig, 2010; Mayer, 2010; Mayer and Bukau, 2005).

It was shown in several studies that in metazoan cells Hsp70s bind to and prevent toxicity of aggregation causing proteins like polyglutamine proteins and α-synuclein, triggering Huntington's disease and Parkinson's disease, respectively (Auluck et al., 2002; Kim et al., 2002; Muchowski et al., 2000). The yeast Hsp70 family member Ssa1 was shown to not only prevent aggregation of client proteins but also to enable proteasomal degradation (McClellan et al., 2005a; Park et al., 2007). Deletion of all Hsp70s of the Ssa subfamily (Ssa1, Ssa2, Ssa3 and Ssa4) is lethal to yeast cells.

A



B

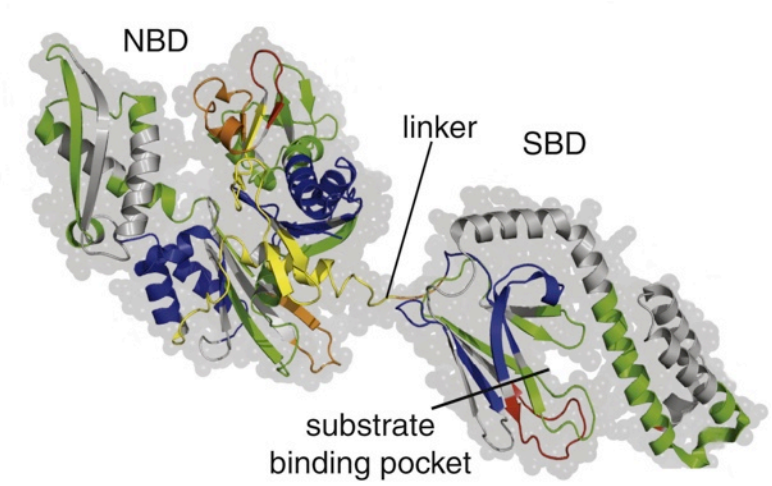


Figure 1. Hsp70's mode of action in protein folding and structure. A. Reaction cycle of Hsp70 (reproduced from Mayer, 2010). In the ATP bound state the substrate rapidly but transiently interacts with the peptide-binding site of Hsp70. An Hsp40/JDP (J domain protein) promotes ATP hydrolysis, which closes the lid over the cleft and stabilizes substrate interaction. NEFs (Nucleotide exchange factors) cause dissociation of ADP and binding of ATP. The substrate dissociates due to the lower client protein affinity of Hsp70 in the ATP bound state. B. Overlay of sphere and secondary structure representation of *E. coli* Hsp70 DnaK determined by NMR residual dipolar coupling (adapted from Mayer, 2010). NBD (Nucleotide-binding domain, SBD (Substrate-binding domain). NBD and SBD are connected by a flexible hydrophobic linker.

1.1.2. Hsp40 chaperone family

All members of the Hsp40 family possess a J domain which was first found in the *E. coli* Hsp40 DnaJ. Therefore, members of the Hsp40 family are often called J proteins. The J domain is important for the interaction with members of the Hsp70 family by

stimulating their ATPase activity (Craig et al., 2006; Kampinga and Craig, 2010). Apart from the J domain, the Hsp40 family is not very homogeneous. Some members like DnaJ or yeast Ydj1 can bind to a broad set of substrates, while others have a more restricted number of interactors that they can target to Hsp70s. Further Hsp40s consist mainly of a J domain like the yeast ER membrane anchored protein Hlj1, which faces to the cytosol and functions by solely recruiting soluble cytosolic Hsp70s (Ssa1, Ssa2) to assist in degradation of misfolded proteins of the ER (Taxis et al., 2003).

1.1.3. Hsp110 chaperone family

The Hsp110 family is characterized as a subclass of the Hsp70 family due to sequence similarity. Members of the family are only found in the eucaryotic cytosol. The yeast homologues of mammalian Hsp110 are Sse1 and Sse2. It was proposed that they can function as "holdases" for unfolded proteins but are not able to perform nucleotide dependent peptide-binding and release cycles like Hsp70s (Polier et al., 2008). Hsp110 chaperones were shown to act on Hsp70s as nucleotide exchange factors (NEFs) directing the exchange of ADP to ATP. This causes release of the bound substrate, which is thereby enabled to acquire its native conformation (Fig. 1 A). The nucleotide release can be also catalyzed by the structurally different protein Bag-1 (Andreasson et al., 2008; Raviol et al., 2006). A similar mechanistic NEF activity on the ER resident Hsp70 Kar2 was recently shown to be performed by the yeast Grp170 protein Lhs1 (Andréasson et al., 2010). Double deletion of *SSE1* and *SSE2* in yeast is lethal, but this phenotype can be compensated by overexpression of other NEFs like Fes1 or the Bag-1 domain of Snl1 (Raviol et al., 2006; Sadlish et al., 2008).

1.1.4. Hsp100 chaperone family

The Hsp100 family of molecular chaperones is a subfamily of the ATPases associated with diverse cellular activities (AAA or AAA+) domain-containing proteins. Hsp100s like the yeast Hsp104 or the *E. coli* ClpB are able to re-solubilize aggregated proteins in cooperation with Hsp70s and Hsp40s (Glover and Lindquist, 1998; Goloubinoff et al., 1999). Hsp70s and Hsp40s present polypeptides to the central pore of the homohexameric Hsp100 complex. Solubilization is then achieved by an ATP driven threading of the substrate’s polypeptide chain through this pore yielding a solubilized protein, which can be refolded again (Haslberger et al., 2010).

1.1.5. Hsp60 chaperone family

Hsp60s, which are also called chaperonins are found in all kingdoms of life. They are composed of two rings that are stacked on each other and possess an inner cavity. Non-native proteins can be captured in the inner cavity for folding into their native conformation in an ATP-dependent process in an encapsulated environment. Two classes of chaperonins are described. Class I chaperonins are found in bacteria, as well as in mitochondria and chloroplasts of eukaryotes. The best described member of class I chaperonins is the *E. coli* GroEL. Its two rings are built up as homo-oligomers with seven subunits per ring. For closure of the inner cavity, class I chaperonins need a co-chaperone (GroES), which blocks access to the inner chamber like a lid.

Class II chaperonins like TRiC/CCT are found in the cytosol of eukaryotes and archaea. In case of archaea the two rings consist of eight subunits of one or two kinds. In case of the eucaryotic TRiC the rings are composed of eight different subunits with different binding properties. This type of chaperonins possesses a built-in lid for encapsulation of the substrate (Horwich et al., 2007; Mayer, 2010).

The main substrates of the eucaryotic chaperonin TRiC are cytosceletal proteins like actin, α- and β-tubulin. But TRiC also seems to play an important role in folding of proteins that are rich in β-sheets and that are subunits of oligomeric complexes like VHL (Von Hippel-Lindau tumor suppressor) of the VHL-elonginBC ubiquitin ligase complex, as well as Cdc20 and Cdh1 of the anaphase promoting complex. TRiC seems to assure proper folding of these subunits in order to prevent premature incorporation into their complexes (Spiess et al., 2004).

1.1.6. Hsp90 chaperone family

The Hsp90s are a highly conserved chaperone family that is found in bacteria and all eukaryotes. They are highly abundant in cells and interact with a significant number of proteins. Like Hsp60s and Hsp70s, Hsp90s can bind to misfolded proteins and prevent their aggregation. However, their main function is considered to be the binding to a diverse set of proteins that are called Hsp90 clients. The molecular basis for specific binding of Hsp90 and its clients is not well understood. Among these clients are transcription factors and protein kinases. Clients need the chaperoning functions of Hsp90s to acquire their native conformations (Pearl and Prodromou, 2006).

Active Hsp90s form dimers. Monomers of Hsp90 consist of an amino-terminal domain with an ATP binding pocket, a middle domain and a carboxy-terminal dimerization domain. The carboxy-terminal domain of eucaryotic Hsp90s is extended by a MEEVD motif, which is important for association with TPR domain containing co-chaperones. An interesting co-chaperone of Hsp90 is Sti1, which is an orthologue of mammalian HOP (Hsp70-Hsp90-organizing protein) and possesses two TPR domains. Via these domains it can bind to Hsp90's MEEVD motif and to Hsp70's C-terminal EEVD motif, thereby connecting these two molecular chaperones and stabilizing the ADP bound open position of Hsp90. This enables transfer of substrates from Hsp70 and Hsp40 to Hsp90 (Chang et al., 1997; Frydman and Höhfeld, 1997; Scheufler et al., 2000).

Like the Hsp70s, Hsp90 chaperones can perform an ATP consuming cycle. In the apo and the ADP bound state conformation is open. Upon ATP binding the Hsp90 acquires a closed conformation by N-terminal dimerization (Hessling et al., 2009; Mayer, 2010; Taipale et al., 2010; Wandinger et al., 2008).

1.1.7. Small Heat Shock Proteins

Small heat shock proteins (sHsps) work ATP independently and function as holdases, *i.e.* by binding to misfolded proteins and thus preventing aggregation. In cells they are often found to form large dynamic oligomers. They are thought to form complexes with aggregation prone proteins upon massive folding stress in order to facilitate refolding by ATP dependent molecular chaperones (Richter et al., 2010).

1.2. Degradation of proteins

Proteolysis is essential to all cells. Accumulation of misfolded or aggregated proteins can lead to severe neurodegenerative diseases like Parkinson's disease, Alzheimer's disease, prion diseases, Huntington's disease and amyotrophic lateral sclerosis (ALS) (Chiti and Dobson, 2006). Proteins that cannot achieve a stable conformation despite chaperone mediated refolding are targeted for degradation.

One distinguishes between lysosomal degradation in animal cells and vacuolar degradation in plant and in fungal cells via autophagocytosis, and degradation via the ubiquitin proteasome system (UPS). The UPS is regarded as the system that rapidly and specifically degrades proteins, while autophagy is supposed to be responsible to degrade long-lived proteins and entire organelles.

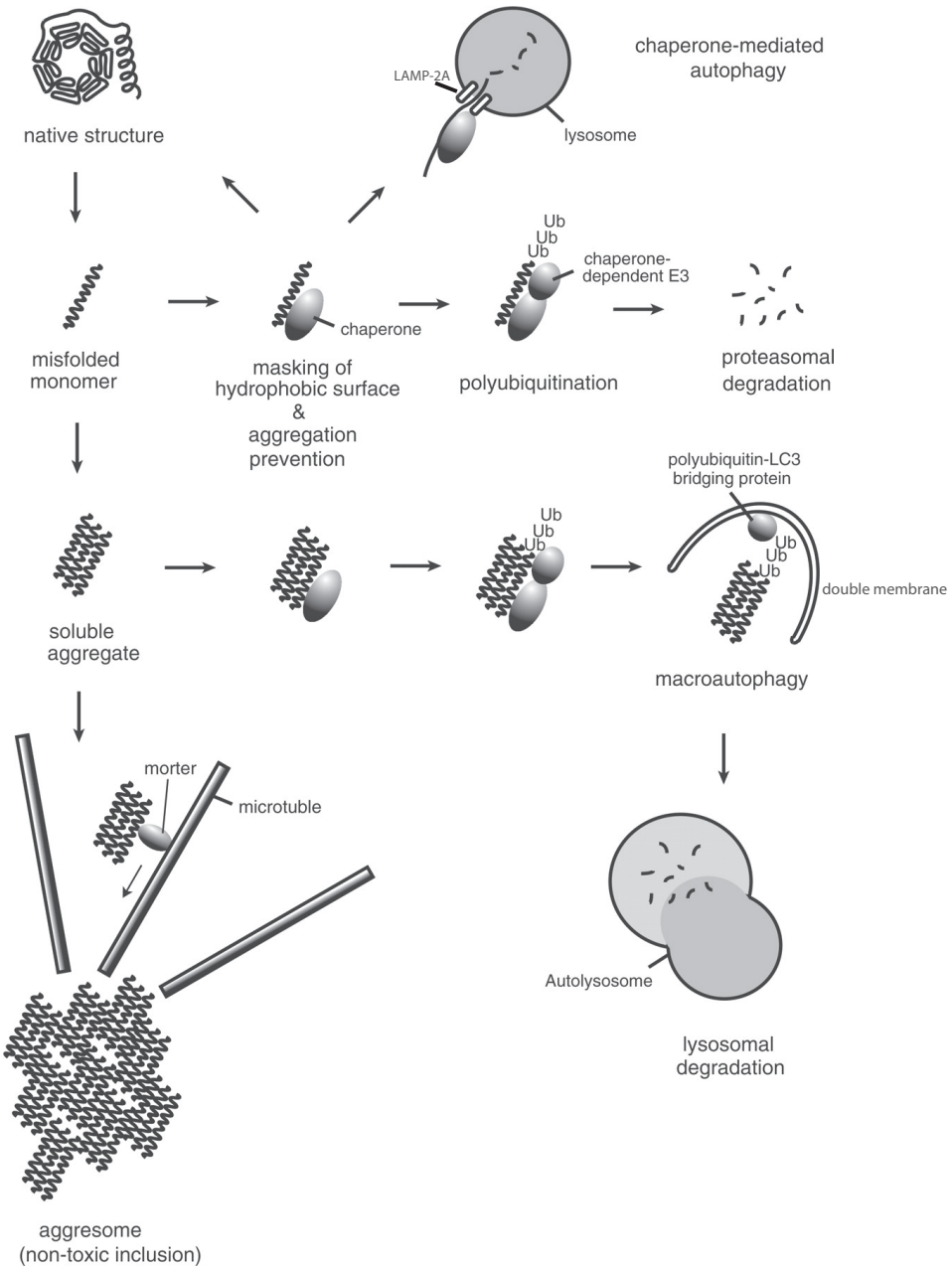


Figure 2. Mechanisms for removal of misfolded and potentially toxic proteins. Molecular chaperones bind to hydrophobic surfaces of misfolded monomeric proteins and prevent aggregation. Chaperones promote triage decision for folding, refolding or degradation in the lysosome (autophagy) or proteasome. Misfolded monomers can form soluble aggregates, which can be targeted by chaperones and autophagy factors to degradation by macroautophagy. Alternatively, soluble and potentially cytotoxic aggregates can be stored in the aggresome (figure adapted from Kubota, 2009).

1.2.1. Lysosomal or vacuolar protein degradation via autophagocytosis and endocytosis

Vacuoles are membrane bound compartments of fungal and plant cells. For the most parts they are functional equivalents of lysosomes in animal cells. The yeast vacuole is defined by a set of resident proteins, an exclusive ionic milieu and membrane lipid composition. A characteristic of vacuoles is their low pH of around 5 which is maintained by the vacuolar H^+-ATPase (V-ATPase) pumping protons from the cytoplasm into the lumen of the vacuole (Li and Kane, 2009). Vacuoles contain many hydrolases, which become only active in an acidic environment. Together with the membrane separation from the cytoplasm, this feature constitutes a protective mechanism against self-digestion. The acidic hydrolases of the vacuole include proteases, lipases, RNAses, glycosidases, phospholipases, phosphatases and sulfatases. Enzymes and structural proteins of the vacuole are translated in the cytosol and transported via various pathways to their point of destination. Most of these proteins make use of the secretory pathway which involves translocation through the ER membrane and vesicular transport to the Golgi. The so-called "CPY pathway" involves vesicular transport through the Golgi apparatus, and from the late Golgi to the multivesicular body to the vacuole (Bowers and Stevens, 2005; Piper et al., 1995). A short cut of this pathway is described by the "ALP pathway", which transports alkaline phosphatase by direct vesiculation from the Golgi to the vacuole (Cowles et al., 1997; Piper et al., 1997; Stepp et al., 1997). A completely different pathway is used by the yeast proteins aminopeptidase I and α-mannosidase. This pathway that is described as the cytosol to vacuole targeting (Cvt) pathway utilizes autophagocytosis factors and is characterized by direct transport from cytosol to vacuole (Harding et al., 1996; Hutchins and Klionsky, 2001).

Substrates of the yeast vacuole can enter via two distinct pathways: Endocytosis or autophagocytosis.

Intracellular substrates of the vacuole or lysosome are transported to the vacuole via autophagy. Three kinds of autophagy are described: chaperone-mediated autophagy, microautophagy and macroautophagy.

In chaperone-mediated autophagy, substrates are recognized and unfolded by the Hsc70-Hsp40-Hip-Hop chaperone complex and directly transported into the lysosome via the LAMP-2A (lysosome-associated membrane protein type2A) (Fig. 2) (Cuervo et al., 2004). Until now there is no equivalent of this process in yeast known.

In microautophagy, small cytosolic components are directly taken up by tubular invaginations of the vacuolar membrane and larger components can be taken up by arm-like protrusions of the vacuolar membrane. This pathway is the least characterized one (Kraft et al., 2009).

In macroautophagy, the cytosolic substrates are enclosed by the autophagosome, a double membrane vesicle (Fig. 2). Substrates can be single proteins, aggregated proteins or even whole organelles like peroxisomes (pexophagy), mitochondria (mitophagy) or ribosomes (ribophagy) (Filimonenko et al., 2010; Kanki and Klionsky, 2010; Kraft et al., 2008; Oku and Sakai, 2010). The formation of the autophagosome occurs at the pre-autophagosomal structure, which is in proximity to the vacuole. The substrates are released together with the inner membrane of the autophagosome into the vacuole by fusion of the autophagosomes' outer membrane with the vacuolar membrane. Inner membrane and substrates are subsequently digested by hydrolases (Klionsky, 2005; Mizushima et al., 2008). In yeast, bulk autophagocytosis of cytosolic components is induced upon starvation and inhibited in a nutrient-rich environment by the Tor kinase (Zaman et al., 2008).

By genetic screening in yeast, approximately 30 genes that are involved in autophagocytosis have been identified, *e.g.* the ubiquitin-like protein Atg8 (Autophagy related) that binds to phosphatidylethanolamine and thus delivers this lipid to the inner membrane of nascent autophagosomes (Suzuki and Ohsumi, 2007).

Endocytosis describes uptake of extracellular compounds by mainly clathrin-coated vesicles, which evolve by invagination of the plasma membrane (Doherty and McMahon, 2009). Vesicles of the endocytic pathway are transported to the early endosome, from where they are transported further to the multi vesicular body and then to the vacuole. Many plasma membrane proteins, which have to be degraded, are sequestered into endocytotic vesicles for being finally degraded inside the vacuole. It has been shown that for efficient internalization of these plasma membrane proteins ubiquitylation is necessary (Hicke and Dunn, 2003).

1.2.2. The ubiquitin proteasome system

For a long time the vacuolar/ lysosomal system was classified as the only proteolytic system in eucaryotic cells. In the seventies and eighties of the last century several studies described high molecular weight particles with potential proteolytic properties and cylindrical shape that turned out to be the proteasome. In parallel, several

studies described the small protein ubiquitin and its role in degradation of proteins. Now it has turned out that the ubiquitin proteasome system (UPS) is the major cytosolic proteolytic system in eucaryotic cells, with pivotal functions in cell cycle control, apoptosis, transcription, signal transduction, protein quality control and many other biological processes (reviewed in Finley, 2009; Wolf and Hilt, 2004).

1.2.2.1. Polyubiquitylation as signal for proteasomal degradation

The 76 amino acids small protein ubiquitin is found in all eucaryotic cells and its sequence is highly conserved. Conjugation of ubiquitin or a ubiquitin chain to intracellular proteins regulates many cellular functions.

Ubiquitin can form an isopeptide bond between its carboxy-terminal glycine residue and an ε-amino group of an internal lysine residue or the amino-terminus of the substrate protein, or an internal lysine residue of another ubiquitin. The formation of this bond is catalyzed by an enzymatic cascade carried out by 3 classes of enzymes. The initial two steps of the cascade are catalyzed by the ubiquitin activating enzyme (E1). First, the C-terminus of the ubiquitin moiety is activated by adenylation. This enables the E1 enzyme in a second step to form a high-energy thioester bond between the ubiquitin and the catalytic cysteine residue. Then the E1 binds to an E2 enzyme and the ubiquitin is transferred by transesterification to a ubiquitin conjugating enzyme (E2). In the next step the ubiquitin conjugated E2 interacts with a ubiquitin ligase (E3). In case of HECT-type (homologous to the E6-AP carboxyl terminus) ubiquitin ligases the ubiquitin is again transferred by transesterification to a cysteine residue of the ligase (Huibregtse et al., 1995; Scheffner et al., 1995). When this ligase binds to a substrate protein, the ubiquitin will be transferred to an internal lysine ε-amino group. In case of RING-type (really interesting new gene) ubiquitin ligases, the ligase interacts with the ubiquitin conjugating enzyme via its RING domain and with the substrate protein via specificity factors (Fig. 3 A). The RING finger domain is able to complex two zinc ions via a series of histidine and cysteine residues (Freemont, 1993, 2000; Pickart, 2001). RING-type ubiquitin ligases do not possess an enzymatic activity for ubiquitylation. They are rather scaffolds for bringing the ubiquitin conjugating enzyme and the substrate in close proximity. The ubiquitin conjugating enzyme then transfers the ubiquitin on an ε-amino group of an internal lysine residue of the substrate (Pickart, 2001).

In the yeast *S. cerevisiae* the essential gene *UBA1* encodes for the only ubiquitin activating enzyme. There are eleven different ubiquitin conjugating enzymes in yeast:

Ubc1 to Ubc8, Ubc10, Ubc11 and Ubc13. They seem to take part in several pathways and often have overlapping functions. Ubc4 and Ubc5 were shown to be involved in the degradation of short-lived proteins or of excess histones (Seufert and Jentsch, 1990; Singh et al., 2009). Ubc1, Ubc6 and Ubc7 are involved in the degradation of misfolded proteins of the ER (ERAD) (Biederer et al., 1996; Hiller et al., 1996) (see 1.2.2.3). Ubc2/ Rad6 is involved in the N-end rule pathway, in the modification of histones and of PCNA, a processivity factor for DNA polymerases (Dohmen et al., 1991; Dover et al., 2002; Hoege et al., 2002).

The number of E3s is much higher than the number of E2s. This reflects their function to provide specificity to the ubiquitylation process by binding to specific sets of protein substrates and ubiquitylate them depending on defined signals.

There are five proteins in yeast containing a HECT domain: Rsp5, Tom1, Hul4, Hul5 and Ufd4. Ubiquitylation activity has been shown for Rsp5, Tom1, Hul5 and Ufd4. For Hul4 proof of this activity still has to be adduced (Singh et al., 2009; Wang et al., 1999; Xie and Varshavsky, 2002). Hul5, which was demonstrated to physically interact with the 26S proteasome was shown to function as a ubiquitin chain elongase (E4) on certain substrates (Crosas et al., 2006; Kohlmann et al., 2008).

Proteins containing a classic RING finger domain or the structurally related U-box domain, which is not able to complex zinc ions, are numerous in yeast. In total there are 48 proteins containing a classic RING domain, a RING finger related domain or U-box domain in yeast (324 in mammals).

The SCF complex (Skp1, Cullin, F-box) (Fig. 3 B) is an example for a complex ubiquitin ligase with a core RING finger protein Hrt1/Rbx1, a cullin Cdc53 and Skp1. The RING finger of Hrt1 recruits the E2 Ubc3. The cullin Cdc53 serves as a scaffold and binds Hrt1 and Skp1. Skp1 is able to bind to proteins containing an F-box. There are different F-box proteins in yeast and mammals that can bind via Skp1 to this core complex. The different F-box proteins function as interchangeable substrate specificity factors. This enables the SCF to ubiquitylate different substrates, while the E3 core complex remains unchanged. Related complexes differ from the SCF complex by usage of other specificity proteins, defined by their SOCS-box or BTB domain. The APC (anaphase promoting complex) belongs to the SCF-complexes as well, but contains more subunits and uses Apc11 instead of Hrt1 as a RING finger protein (Fig. 3 B) (Willems et al., 2004).

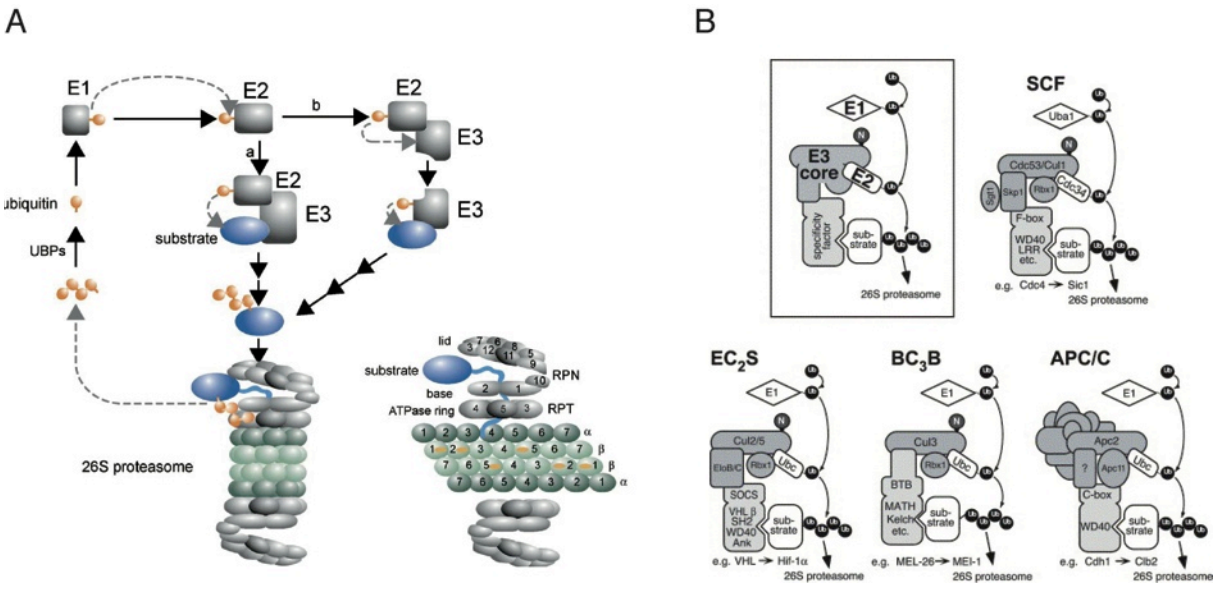


Figure 3. The ubiquitin proteasome pathway and the cullin-RING ligase superfamily. (Figure A is reproduced from Wolf and Hilt, 2004 and Figure B from Willems et al., 2004.)

The so called Gid (Glucose induced degradation deficient) complex is another example of a complex ubiquitin ligase. It is composed of seven Gid proteins (Ho et al., 2002; Krogan et al., 2006; Pitre et al., 2006; Regelmann et al., 2003; Santt et al., 2008). One subunit of the complex, Gid2, contains a degenerated RING finger domain. The Gid complex was shown to trigger proteasomal degradation of Fructose-1,6-bisphosphatase (FBPase) by ubiquitylation. FBPase is a key regulatory enzyme of gluconeogenesis which is completely inactivated and degraded upon switch of cells from non-fermentable to fermentable carbon sources. Six Gid proteins are already present in gluconeogenic cells. Expression of Gid4 occurs upon shift to glucose containing media and leads to proteasomal degradation of FBPase (Santt et al., 2008).

Many physiological processes in the cell are regulated by ubiquitylation. Conjugation of a single ubiquitin to one or several sites of a substrate (monoubiquitylation and multiubiquitylation, respectively) can alter protein localization or activity, and promote or inhibit interactions with other proteins. *E.g.* the monoubiquitylation of a plasma membrane protein can lead to internalization into endocytic vesicles (Mukhopadhyay and Riezman, 2007). The C-terminal glycine residue of a ubiquitin can be linked to one of the seven internal lysine residues of another ubiquitin moiety which causes formation of a ubiquitin chain (polyubiquitylation). Furthermore, the amino terminus of

a linked ubiquitin can be an acceptor for the formation of a linear ubiquitin chain. The type of connection between the ubiquitin moieties of a chain alters structure and function. Lys48- and Lys11-linked ubiquitin chains target substrates to proteasomal degradation. Lys63 chains can target substrates to lysosomal degradation or function as a signal during DNA-repair. Together with linear ubiquitin chains, Lys63 is also involved in activation of NF-κB. Lys6, Lys27, Lys29, Lys33 linked chains as well as branched chains resulting from attachment of two ubiquitin moieties to different Lys residues of one ubiquitin have been detected. However, the functions of these types of ubiquitin chains are still unclear. Increasing evidence emerges demonstrating that E2s have an important role in determining which type of ubiquitin chain is attached to a substrate and thereby influence the fate of the modified protein (Ye and Rape, 2009).

Proteins that are able to interact with ubiquitin or ubiquitylated proteins are prevalent in cells. Several different ubiquitin-binding domains (UBDs) that are different in sequence and structure have evolved. One example is the ubiquitin-interacting motif (UIM), which is found in the yeast protein Rpn10 or in its mammalian orthologue S5a. These proteins exist as a subunit of the 19S cap of the 26S proteasome and in free pools in the cytoplasm. They function in recognition of polyubiquitylated substrates, their delivery to the 26S proteasome and subsequent degradation (Deveraux et al., 1994; Elsasser et al., 2002). A further UIM domain containing receptor is Vps27, which is involved in recognition of monoubiquitylated substrates and endosomal sorting (Bilodeau et al., 2002; Swanson et al., 2003). Another ubiquitin-interacting domain is the ubiquitin-associated domain (UBA) found *e.g.* in the yeast proteins Dsk2 and Rad23. Both function as receptors of ubiquitylated substrates and function in the delivery to the 26S proteasome. They are associated with the 26S proteasome via a ubiquitin-like (UBL) domain. Genetic studies showed that Dsk2, Rad23 and Rpn10 have redundant roles in protein degradation (Elsasser et al., 2004; Matiuhin et al., 2008).

Ubiquitin has a large number of homologous proteins. Ubiquitin-like proteins that can be attached postranslationally to other proteins are referred to as type I ubiquitin-like proteins. Examples are members of the SUMO (Small ubiquitin-like modifier) family (SUMO 1-4 in mammals and Smt3 in yeast) or mammalian NEDD8 and its yeast orthologue Rub1. Conjugation of these proteins to a substrate protein occurs like in the case of ubiquitin via a C-terminal glycine residue.

SUMO chains can be formed by conjugation to an internal Lys15 residue in yeast Smt3. In yeast, conjugation requires Uba2 as an E1, Ubc9 as E2 and one of four E3 ligases (Siz1, Mms21, Cst9 and Nfi1). Sumoylation modulates many processes like transcription, recombination, chromosome segregation and nuclear transport (Dohmen, 2004; Dohmen et al., 1995; Johnson, 2004). In yeast, Rub1 conjugation requires the E1 pair Ula1 and Uba3, and Ubc12 as an E2. The only known substrates are the Cullin-RING ubiquitin ligases, whose activity is stimulated by conjugation of Nedd8/Rub1 (Liakopoulos et al., 1998; Merlet et al., 2009).

Type II ubiquitin-like proteins are proteins that contain a ubiquitin-like homology domain but cannot be conjugated to substrates. Examples are the already mentioned UBL domain containing proteins Dsk2 and Rad23, or proteins containing a UBX (Ubiquitin regulatory X) domain, which folds similar to ubiquitin despite the lack of relevant sequence homology. Proteins containing a UBX domain were shown to interact via this domain and function as regulatory co-factors of the AAA-ATPase Cdc48. Interestingly, many UBX domain containing proteins also possess a UBA domain allowing recruitment of ubiquitylated proteins to Cdc48 (Buchberger, 2002; Schuberth and Buchberger, 2008; Schuberth et al., 2004).

1.2.2.2. The 26S proteasome

Once a substrate is marked with polyubiquitin chains of the K48 or K11 type it is directed to the 26S proteasome where it is unfolded by the 19S cap and threaded into the 20S core complex to be cleaved into peptides ranging from 2 to 30 amino acids in length (Finley, 2009; Goldberg et al., 2002; Heinemeyer et al., 1991; Nussbaum et al., 1998). The 26S proteasome is localized in the cytoplasm and nucleus (Laporte et al., 2008; Russell et al., 1999).

1.2.2.2.1. The 19S regulatory particle

The 19S cap or regulatory particle can be subdivided in base and lid (see Fig. 3 A). The base is composed of a ring of six homologous but not identical AAA-ATPase subunits, which are called Rpt1-Rpt6 (Regulatory particle triple A protein) in yeast. On top of this ring there are the subunits Rpn1 and Rpn2 (Regulatory particle non-ATPase), which are the biggest subunits of the proteasome and function as scaffolds. They are proposed to form a physical link from the site of substrate recruitment to the site of proteolysis (Rosenzweig et al., 2008). Other base subunits are the ubiquitin receptor proteins Rpn10 and Rpn13 (Finley, 2009; Glickman et al., 1998). These two

subunits, together with Rpt5 and the proteasome associated UBA/UBL proteins Dsk2, Rad23 and Ddi1, which dock to the proteasome via their UBL domain, are responsible for targeting ubiquitylated substrates to the proteasome (Wolf and Hilt, 2004). The Rpt subunits are important for the complex formation between the 19S cap and the 20S core. This binding is accomplished by insertion of the C-termini of Rpt subunits into the spaces between the α-subunits of the inner core. In addition, the Rpt subunits allow gate opening to the inner core and thereby activation of the 26S proteasome which is mediated by ATP binding (Rabl et al., 2008; Smith et al., 2007). Furthermore, ATP binding is also necessary for the stability of the 26S proteasome, unfolding of proteasomal substrates and threading of unfolded protein through the pore into the interior chamber for proteolysis (Liu et al., 2006; Rubin et al., 1998).

The lid is composed of the subunits Rpn3, Rpn5 to Rpn9, Rpn11, Rpn12 and Rpn15. Until now only Rpn11 has a known function. It has been shown to possess a deubiquitylating (DUB) activity and functions in removal of ubiquitin chains and single ubiquitin moieties from proteasome substrates prior to degradation (Verma et al., 2002; Yao and Cohen, 2002).

1.2.2.2.2. The 20S proteolytic core particle

The 20S proteolytic core particle (CP) is a barrel shaped stack of four heptameric rings (see Fig. 3 A). The outer rings are composed of α subunits and the inner rings of β subunits (Finley, 2009; Wolf and Hilt, 2004). The core particle has two outer chambers formed by the α and β rings and a central chamber composed by the two β rings. All 14 different eucaryotic core particle subunits possess characteristic insertion segments and defined contact sites between related subunits that cause their unique location within the 20S proteasome. The α subunits have a structural role in proteasome assembly and are supposed to act as gatekeepers. In free 20S subunits the access to the channel is blocked by the N-termini of α subunits. The channel can be opened by a regulatory particle, which is able to rearrange the α subunits (Bajorek and Glickman, 2004). The proteolytic activity of the core particle lies within three of the 7 β subunits of each ring. Subunits β_1 (Pre3), β_2 (Pup1) and β_5 (Pre2) possess a peptidyl-glutamyl-peptide cleaving activity cutting after acidic residues, a trypsin like activity that cuts after basic residues and a chymotrypsin-like activity that cuts after hydrophobic residues, respectively. Since the proteolytic activities are located within the central cavity, they are isolated from the cytoplasm and only unfolded proteins

threaded into the core particle can be degraded. This self-compartmentalization prevents unregulated protein degradation. During the process of proteasome formation, the three proteolytic β subunits remain in their inactive pro-peptide form. Autocatalytic cleavage of the pro-peptide occurs shortly after formation of the core particle, thereby protecting cytoplasmic proteins from unspecific proteolysis. Cleavage reveals an N-terminal threonine that functions as a nucleophil. This type of proteases is therefore referred to as N-terminal nucleophile (Ntn)-hydrolases (Chen and Hochstrasser, 1996; Ditzel et al., 1998; Groll and Huber, 2004; Heinemeyer et al., 1991; Wolf and Hilt, 2004).

1.2.2.2.3. Proteasomal degradation

Ubiquitylated substrates of the 26S proteasome are recognized by ubiquitin binding proteins like the proteasome associated factors Ddi1, Dsk2 and Rad23, or proteasome integral proteins like Rpt5, Rpn10 or Rpn13 (Wolf and Hilt, 2004). Substrates are unfolded and translocated by the Rpt subunits of the base into a narrow channel leading to the core particle (Braun et al., 1999). In addition, the Rpt subunits allow gate opening to the 20S core particle. Before proteolysis, substrates become deubiquitylated by Rpn11. Cleaved off ubiquitin chains are subsequently disassembled to single ubiquitin moieties by the DUB Ubp14 (Amerik and Hochstrasser, 2004; Amerik et al., 1997). The substrate is threaded into the 20S core particle and degraded. Few proteasomal substrates are degraded independently of prior ubiquitylation. One example is the enzyme ornithine decarboxylase (ODC). The C-terminus of ODC mimics a polyubiquitin chain and is able to compete with these chains for binding to and subsequent degradation by the proteasome (Hoyt and Coffino, 2004). Substrates of the 26S proteasome are cleaved into small oligopeptide fragments (Nussbaum et al., 1998). The adaptive immune system of mammalian cells makes use of such peptides. Fragments of 8-10 amino acids in length can bind to major histocompatibility (MHC) class I molecules which are then routed to the cell surface to be presented to cytotoxic T lymphocytes. If the presented peptide is recognized to be “non-self“, the presenting cell is induced to undergo apoptosis (Rock and Goldberg, 1999). However, most fragments are processed further to amino acids by endo- and exopeptidases *e.g.* by the thimet oligopeptidase (TOP) in mammals or the proteinase yscD in yeast (Büchler et al., 1994; Goldberg et al., 2002).

1.2.2.3. ER quality control and associated protein degradation (ERQD)

The best studied protein quality control mechanism in eucaryotic cells is the one of the endoplasmic reticulum (ER). About one third of all cellular proteins are targeted to the secretory pathway. This pathway starts with translocation of the secretory proteins through the Sec61 channel into the ER in a co-translationally or in a post-translationally manner. Glycans of the structure $Glc_3Man_9GlcNAc_2$ are covalently linked to an asparagine residue located within an Asn-X-Ser/Thr glycosylation consensus sequence during import of the protein by the oligosaccharyl transferase (OST) complex. Glycosylation of the protein increases the hydrophilicity and functions as a signal for the folding state. While proteins are being folded by the ER Hsp70 BiP (Kar2 in yeast), the carbohydrate chain is trimmed by glucosidases I and II to $Glc_1Man_9GlcNAc_2$. Substrates with this glycan structure can be bound and further folded by the molecular chaperone activity exhibiting lectins calnexin and calreticulin. In mammalian cells calnexin and calreticulin, together with the UDP-glucose:glycoprotein glucosyl transferase (UGGT), constitute a quality control system called the calnexin calreticulin cycle. When the last outer glucose residue is cleaved off by glucsodiase II, binding to calnexin and calreticulin is abolished. Properly folded proteins can proceed their travel through the secretory pathway. Scanning of not yet folded substrates by UGGT leads to reglucosylation allowing another round of folding assisted by calnexin and calreticulin in mammals. No reglucosylation is possible in yeast cells. If the substrate fails to fold in time, slow acting mammalian ER α-mannosidase or yeast mannosidase Mns1 cleave off a mannose residue from the central B-branch. Thereupon a mannose residue of the C-branch is cleaved off by EDEM (mammals) or Htm1/ Mnl1 (yeast) leading to an α 1-6 bound mannose (see Fig. 1 of chapter 4.5). So there is only a restricted time window for folding until the cleavage of mannose residues and therefore these slow acting mannosidases can be contemplated as timers in protein quality control (Aebi et al., 2010; Buchberger et al., 2010; Helenius and Aebi, 2004). In the next step of ERQD the misfolded glycoprotein and the glycan structure is recognized by the lectins OS-9 or XTP3-B in mammals and Yos9 in yeast. Yos9 binds to Hrd3 of the Hrd E3 ligase complex. OS-9 and XTP3-B were found to interact with SEL1 (orthologue of yeast Hrd3) and HRD1 (orthologue of yeast Hrd1/Der3) (Buschhorn et al., 2004; Christianson et al., 2008; Clerc et al., 2009; Cormier et al., 2009; Quan et al., 2008).

Folding of secretory proteins is supported by folding catalysts of the protein disulfide isomerase (PDI) family. The oxidizing environment of the ER favors the formation of

disulfide bridges. PDIs are able to connect and disconnect disulfide bridges in order to enable refolding and unfolding of terminally misfolded proteins. In addition, PDIs are able to detect hydrophobic patches of misfolded proteins and therefore exhibit chaperone-like activities (Freedman et al., 1994; Primm et al., 1996; Stolz and Wolf, 2010).

Finally misfolded lumenal proteins and membrane proteins of the ER with lesions in their transmembrane domain are unfolded, retrotranslocated and ubiquitylated through a translocation channel to the cytoplasm (Hiller et al., 1996; Plemper et al., 1997; Schäfer and Wolf, 2009).The unfolded protein becomes ubiquitylated by the E3 ligase Hrd1/ Der3 mainly in cooperation with the ubiquitin conjugating enzyme Ubc7. Misfolded membrane proteins with lesions in their cytosolic domains are ubiquitylated by the ubiquitin ligase Doa10. Driving force for retrotranslocation is provided by the AAA-ATPase Cdc48-Ufd1-Npl4 complex. The UBA-UBL proteins Dsk2 and Rad23 are needed for further transport of the polyubiquitylated substrate to the proteasome for degradation (Hirsch et al., 2009; Kostova and Wolf, 2003; Vembar and Brodsky, 2008). More details on the ubiquitylation process mediated by the different ligases in yeast and mammals can be found in the article “Ubiquitylation in the ERAD pathway” (see 4.5) (Eisele et al., 2010).

As a parallel process to the cytoplasmic heat shock response (HSR) (see 1.2.2.5), the unfolded protein response (UPR) constitutes a cellular mechanism to cope with folding stress in the ER and regulates transcription of almost all proteins involved in ERAD. In yeast cells, UPR is induced upon overload of misfolded proteins in the ER. The ER resident transmembrane protein Ire1 senses ER stress, which causes oligomerization of this receptor. Oligomerization activates the exonuclease activity of Ire1 on the cytosolic side, which enables splicing of the *HAC1* (*XBP1* in mammals) pre-mRNA. Maturation of this pre-mRNA enables translation of the Hac1/ XBP1 transcription factor which promotes transcription of specific target genes leading to an increase in translation of molecular chaperones and proteolytic systems and to a reduction of overall protein translation (Ron and Walter, 2007).

1.2.2.4. The mammalian E3 ligase CHIP

CHIP (carboxy terminus of Hsc70-interacting protein) is a co-chaperone of the constitutively expressed Hsp70 chaperone Hsc70, as well as stress inducible Hsp70 and Hsp90 of the cytoplasm and nucleus of mammalian cells (Arndt et al., 2007). The Hsc/Hsp70- or Hsp90-CHIP complex is a good example of how chaperones

recognize misfolded proteins and prevent aggregation by either facilitating folding or degradation. CHIP binds to the C-terminus of Hsc70 and mediates the interaction of a ubiquitin conjugating enzyme (Ubc, E2) and a chaperone bound substrate, which will be subsequently ubiquitylated. CHIP possesses a U-box domain for E2 recruitment and is regulated by BAG domain co-chaperones. Binding of the ubiquitin-like domain possessing protein BAG-1 to CHIP and Hsc70 guides the chaperone complex to the proteasome and thereby triggers proteasomal degradation of the chaperone bound substrate. Binding of the co-chaperone BAG-2 inhibits ubiquitin ligase activity of CHIP by blocking the binding of the corresponding ubiquitin conjugating enzyme. BAG-3 binding to the chaperone complex causes recruitment of the small heat shock protein HspB8 which induces the build-up of a autophagosome by binding to the adaptor protein p62 and finally causes the degradation of the substrate in the lysosome (Kettern et al., 2010). During aging the expression of co-chaperone BAG-1 decreases and BAG-3 increases. This results in a more intensive usage of autophagy in older cells (Gamerdinger et al., 2009).
Substrates of CHIP are Hsc70 controlled proteins, like the ion-channel CFTR, which causes cystic fibrosis when mutated, or the Tau protein, which is found in intracellular protein aggregates of Alzheimer patients (Kettern et al., 2010).
In yeast, no E3 ligase of the cytoplasm has yet been found to interact directly in a similar way with Hsp70s in recognition and targeting for degradation of misfolded proteins like CHIP.

1.2.2.5. Cytoplasmic protein quality control and degradation

Misfolded proteins are a constant threat to the efficiency of cells. Therefore, quality control mechanisms of the cell monitor correct folding, assembly in respective complexes and functionality of proteins. A non-native protein has to be either refolded or degraded by the protein quality control system, a process which is referred to as triage decision (Connell et al., 2001; Wickner et al., 1999). Proteins being recognized by the protein quality control system often have the tendency to form aggregates with other non-native proteins due to the exposure of hydrophobic patches at their surface. Most chaperone families are involved in recognition of misfolded proteins. Members of the Hsp70 family are able to interact with most cellular proteins in different folding states ranging from unfolded to native or aggregated states (Mayer and Bukau, 2005). The interaction was shown to be based on short hydrophobic and basic peptide stretches of client proteins (Rüdiger et al., 1997; Zhu et al., 1996).

Certain Hsp40s, which are able to recognize hydrophobic patches on substrates can recruit Hsp70s for folding by the described ATP-controlled cycle (see 1.1.1) (Fig. 1 A). The role of Hsp70s in prevention of protein aggregation can be due to binding only, or also due to the induction of conformational changes in the aggregation prone proteins (Buchberger et al., 2010; Rodriguez et al., 2008).

Disaggregation can be promoted by Hsp100 family members in cooperation with Hsp70 members (Glover and Lindquist, 1998). The role of the Hsp90 family in protein quality control is unclear. Specificity for aggregation prone proteins may reside within the co-factors of Hsp90 chaperones (Taipale et al., 2010). A function of the Hsp60s is to isolate slow folding proteins, especially beta sheet rich proteins in its inner cavity. Probably only a relatively small set of substrates is recognized by Hsp60s. Examples are proteins that are subunits of oligomers (Horwich et al., 2007), like the model substrate VHL, which cooperates with Hsp70s and Hsp60s for folding, and with Hsp70s and Hsp90s for degradation (McClellan et al., 2005b). Small heat shock proteins (sHSP) play a role in prevention and breakup of aggregates as holdases. Binding of sHSPs to aggregates enables refolding by the Hsp70 and Hsp100 machinery (Richter et al., 2010).

Cells that experience stress by overload of misfolded proteins respond by an increase in repair and degradation of misfolded and damaged proteins. Reduction of general protein translation and increase in molecular chaperones and proteolytic proteins secure containment of protein damage. In reaction to presence of damaged proteins in the cytoplasm and nucleus caused by heat, the heat shock response (HSR) is activated. Upon this stressor the transcription factor Hsf1 is titrated away from Hsp90s and probably also from Hsp70s due to increase of chaperone clients. Free Hsf1 forms a trimer and activates HSR genes after transport to the nucleus by binding to heat shock promoter elements (HSEs) (Akerfelt et al., 2010; Nieto-Sotelo et al., 1990; Sorger, 1990). A similar and probably partially overlapping response called UPR-cyto has been described recently (Metzger and Michaelis, 2009). This response was triggered by overexpression of cytosolic misfolded protein VHL (von Hippel Lindau protein) (McClellan et al., 2005a), while the HSR has not been shown to be caused by misfolded proteins directly. How similar both responses actually are has to be addressed.

Upon recognition of a misfolded protein, triage decision whether a protein is going to be refolded or degraded has to be made. From an energetic point of view refolding is in favor of degradation and subsequent de-novo protein translation since all three

processes are energy consuming. Still, finally misfolded proteins have to be sorted out in order to prevent permanent occupation of chaperones and consequently increased ATP consumption. For selection of proteins that have to be degraded, a direct recognition of features within the substrate by factors of the protein quality control system or, alternatively, a timer model based on the time a chaperone is occupied by a substrate is possible. Mechanisms that are responsible for selection are not yet understood on the molecular level but an increasing number of factors involved in pathway selection are being found and described (Buchberger et al., 2010).

The best understood pathway selection mechanism is described for the mammalian E3 ligase CHIP (see also 1.2.2.4). Pathway selection whether a protein is going to be refolded or degraded is achieved by binding of different nucleotide exchange factors. Binding of BAG-2 to the CHIP-HSP70 or Hsp90-substrate complex promotes protein folding. Binding of BAG-1 and BAG-3 promotes protein degradation via the ubiquitin proteasome system and autophagocytosis, respectively (Arndt et al., 2010; Arndt et al., 2007). Despite the knowledge how different co-factors control pathway selection of CHIP substrates, the molecular mechanism, which constitutes the selection is not yet understood. Binding of CHIP to a chaperone-substrate complex could be caused by specific conformational properties of the complex that is stabilized when binding to a substrate, which is delicate to fold. Alternatively, the lower abundance of CHIP in comparison to Hsp70s could lead to preferred binding of CHIP to Hsp70-substrate complexes that persist for a longer time (Buchberger et al., 2010; Stankiewicz et al., 2010).

Yeast cells do not possess a homologue of the CHIP ligase. Studies making use of misfolded proteins in *S. cerevisiae* revealed cellular components that are involved in the cytoplasmic protein quality control. Expression of VHL (McClellan et al., 2005a) or different species of ER import defective CPY* (mutated carboxypeptidase Y) called ΔssCPY* (Park et al., 2007) (see 2.2 and 4.1) showed the need for cytoplasmic Hsp70s of the Ssa family and the ubiquitin proteasome system for degradation of both substrates.

Several recent studies identified the yeast ubiquitin ligase of the N-end rule pathway, Ubr1 (Bartel et al., 1990) (see 1.2.2.6), to be responsible for degradation of misfolded cytoplasmic protein. In this novel role Ubr1 was first identified in a screen for components involved in the degradation of ΔssCPY*Leu2myc (ΔssCL*myc), a variant of ΔssCPY* which is genetically C-terminally fused to the gene *LEU2* encoding the

enzyme 3-isopropylmalate dehydrogenase. Presence of this enzyme activity enables cells stabilizing this fusion substrate to grow on media lacking leucine (Eisele and Wolf, 2008; Schäfer and Wolf, 2005) (see 2.3 and Fig. 1 A of 4.2). Another study showed that the ligases Ubr1, and to a smaller extent its paralogue Ubr2, are involved in ubiquitylation of several kinds of misfolded cytoplasmic proteins (Nillegoda et al., 2010). Ubr2 is not involved in degradation of N-end rule substrates but was shown to ubiquitylate Rpn4, the transcription factor for proteasome subunits (Wang et al., 2004). Nillegoda and co-workers showed that Ubr1 promotes degradation of the newly synthesized protein Tpk2 when the Hsp90 machinery is blocked with the specific inhibitor geldanamycin. They also showed that over-all protein ubiquitylation is reduced in strains deleted in *UBR1* or *UBR2* when cells are cultivated in the presence of L-azetidine-2-carboxylic acid (AZC), a proline analogue that incorporates competitively with L-proline in proteins and causes increased thermal instability. A similar decrease in ubiquitylation is presented in this study when a temperature sensitive mutant of *SSA1* in a *SSA2* to *SSA4* deletion strain is incubated with AZC showing the importance of the Hsp70 machinery for ubiquitylation of misfolded proteins. Furthermore, they showed that the degradation of heat stressed mature proteins are dependent on Ubr1 and Ubr2.

Two other studies identified the ligases Ubr1 and San1 to be necessary for degradation of ΔssCPY*GFP (ΔssCG*), ΔssPrA-HA, a cytoplasmic version of Proteinase A due to signal sequence deletion, and Δ2GFP, a due to an internal deletion unstable GFP variant, the latter two tagged with a C-terminal HA epitope (Heck et al., 2010; Prasad et al., 2010). San1 is a nuclear ubiquitin ligase which was shown to be required for degradation of mutated nuclear proteins (Gardner et al., 2005). To a certain extent, these three misfolded model substrates seem to be imported into the nucleus where they are recognized and ubiquitylated by San1. The degradation pathway via San1 also depends on the Hsp70 Ssa1 and Hsp110 Sse1. Whether the chaperones are needed for prevention of cytoplasmic aggregation, the transport into the nucleus or presenting of the substrate to San1 still has to be shown (Prasad et al., 2010). A recent study by Gardner and co-workers shows that San1 possesses disordered domains interspersed with ordered and conserved domains within its N- and C-terminal regions. These disordered regions are thought to confer plasticity to the ligase in order to interact with a broad set of differently shaped misfolded substrates (Rosenbaum et al., 2011).

Again, like it is the case for CHIP, the molecular mechanisms underlying the recognition of misfolded cytoplasmic proteins by the ubiquitin ligases Ubr1, Ubr2 and San1 are not yet understood. As for ERAD of soluble lumenal substrates, degradation of all model substrates depends on Hsp70s. The Ssa family of the cytoplasm is used but it is not clear whether the ligases interact directly with the chaperone-substrate complex as it was shown for CHIP (Arndt et al., 2007). Also it is unclear which properties guide the substrate to the nucleus for ubiquitylation via San1 and which properties enable recognition via Ubr1. The question remains, how recognition of misfolded proteins by Ubr1 is mediated. One possibility rests in the detection of the substrate via an internal degron as proposed for the Ubr1 substrate Cup9 (Byrd et al., 1998; Xia et al., 2008b). Another possibility is processing of the N-termini of misfolded proteins to become N-end rule substrates. This could either be achieved by attachment of a destabilizing amino acid to the N-terminus as it was shown for type-1 N-end rule substrates which become N-terminally arginylated (Hu et al., 2008) or by cleavage by an exo- or endopeptidase in front of an N-terminally destabilizing residue as it was shown for the cohesin subunit Scc1 (Rao et al., 2001). N-terminal acetylation could be a mechanism to guide misfolded proteins to degradation via the Doa10 branch of the N-end rule pathway (Hwang et al., 2010a). However, until now this mechanism has not been observed for misfolded proteins. Nevertheless, ubiquitylation by Doa10 has been observed for the Mat-α2 repressor and the fusion protein Ura3p-CL1 (Gilon et al., 1998; Metzger et al., 2008; Ravid et al., 2006; Swanson et al., 2001). Mat-α2 has an internal degron called Deg1. Deg1 forms an amphipathic helix. The hydrophobic residues of this helix are essential for its instability. Deg1 is masked by Mat-a1, a binding partner of Mat-α2. In absence of Mat-a1, Mat-α2 is rapidly degraded. When it is exposed, Deg1 may resemble a misfolded protein since misfolded proteins present hydrophobic patches at their surface which are normally buried within the inside of native proteins (Johnson et al., 1998; Metzger et al., 2008). CL1 is a degron found in a screen for sequences that could target the Ura3 enzyme for ubiquitin proteasome dependent degradation when being C-terminally fused (Gilon et al., 1998). CL1 is predicted to also form an amphipathic helix that depends, like in case of Deg1, on its hydrophobic residues to function as a degron. Degradation of CL1 was shown to depend on several factors of the ERAD machinery like the E2s Ubc6, Ubc7 and its membrane anchor Cue1, the E3 Doa10, Hsp40 co-chaperone Ydj1 and Hsp70 Ssa1, and the Cdc48-Npl4-Ufd1 complex (Metzger et al., 2008; Ravid et al., 2006). Mat-α2 repressor was shown to be

nearly completely N-terminally acetylated in strains deleted in *DOA10* and *UBC4*, which is a ubiquitylation signal for Doa10 (Hwang et al., 2010a). In this study Ura3p-CL1 was not examined. It has to be demonstrated whether both degrons, Deg1 and CL1, in fact behave like misfolded proteins or whether their degradation mediated by Doa10 underlies another not yet defined mechanism in order to assign these pathways to the cytoplasmic quality control.

Another aspect of cytoplasmic protein quality control is how the cell handles aggregates. Defects in recognition and degradation of misfolded proteins lead to the formation of aggregates which may cause neurological disorders and protein folding diseases like Huntington's, Parkinson's and Alzheimer's disease (Chiti and Dobson, 2006; Gregersen et al., 2006; Lin et al., 2008; Powers et al., 2009). A recent study shows that toxicity of an aggregation prone protein is associated with soluble non-native protein oligomers and that large insoluble oligomers can be protective for the cell (Arrasate et al., 2004). In accord with this observation, Frydman and colleagues showed that in yeast and in mammalian cells aggregation prone proteins are sorted depending on their properties into two different inclusions named IPOD (insoluble protein deposit) and JUNQ (juxtanuclear quality control compartment) (Kaganovich et al., 2008). JUNQ formation is observed upon induction of cellular stress. This protein aggregate is localized in proximity to the nucleus and consists of soluble misfolded globular proteins and is in exchange with the cytoplasmic pool. Also proteasomes and ubiquitylated proteins are found there. This subcellular compartment seems to constitute a temporary storage site for misfolded ubiquitylated substrates that accumulate during stress conditions.

In contrast, the subcellular compartment IPOD can also be found in non-stressed cells. Proteins that are targeted to this perivacuolar-localized inclusion are finally aggregated amyloidogenic protein species that are mostly non-diffusing and not ubiquitylated. Subsequently no proteasomes are found there. In accordance with that, deletion of ubiquitin conjugating enzymes Ubc4 and Ubc5 or overexpression of deubiquitylating enzyme Ubp4 results in increased substrate protein accumulation in IPOD. The assignment of the IPOD seems to be the permanent sequestration of non-ubiquitylated amyloidogenic substrates to protect the cell from their potential toxicity. Whether proteins aggregated in the IPOD, which co-localizes with the autophagic marker Atg8, can be degraded by autophagocytosis has to be addressed (Bagola and Sommer, 2008; Kaganovich et al., 2008). How other subcellular compartments, which have been defined in earlier studies like inclusion bodies, the aggresome or

the ERAC (ER associated compartment) are related to IPOD and JUNQ has to be shown (Huyer et al., 2004; Kopito, 2000; Kubota, 2009). The same is true for the Hsp104-containing protein aggregates observed in a study, describing an active transport mechanism in buddying yeast of these aggregated proteins via actin filaments from daughter to mother cells. The transport of the Hsp104-containing protein aggregates from daughter to mother cells establishes age asymmetry by inhibiting inheritance of potentially toxic protein aggregates (Liu et al., 2010).

1.2.2.6. N-end rule pathway and the ubiquitin ligase Ubr1

The N-end rule defines the stability of a protein according to the type of its N-terminal residue (Bachmair et al., 1986; Varshavsky, 1996). This principle is conserved from bacteria to mammals (Mogk et al., 2007). In eucaryotic cells, the N-end rule pathway is dependent on the ubiquitin proteasome system. The N-end rule pathway recognizes several kinds of degradation signals, also called N-degrons. N-degrons are defined by a destabilizing N-terminal residue, an internal lysine residue for attachment of a ubiquitin chain and a conformationally flexible region in vicinity of the N-terminus (Bachmair and Varshavsky, 1989; Chau et al., 1989; Prakash et al., 2004; Tobias et al., 1991; Xia et al., 2008b). The N-end rule classifies N-terminal amino acids as primary, secondary or tertiary destabilizing residues. In yeast and higher eukaryotes, primary destabilizing amino acids are Arg, Lys, His, Leu, Phe, Trp, Tyr and Ile. Secondary destabilizing residues are Asp and Glu. They become arginylated via the arginyl-transferase Ate1 in yeast or mouse, which results in a primary destabilizing N-terminus. This process is inhibited by hemin (Hu et al., 2008). Tertiary destabilizing residues are Asn and Gln, which are transformed into Asp and Glu, respectively, via deamidation catalyzed by yeast or mouse Nta1 enzymes (Hu et al., 2008; Xia et al., 2008b). In mammalian cells, Cys represents another tertiary destabilizing residue, which can become oxidized by nitric oxide to Cys-sulfinate or Cys-sulfonate. This residue becomes arginylated by mammalian ATE1 as well (Gonda et al., 1989; Kwon et al., 2002).

Varshavsky and co-workers discovered the N-end rule pathway when trying to express genetic fusion proteins of ubiquitin and the *E. coli* protein β-galactosidase in yeast. The ubiquitin moiety became rapidly removed by deubiquitylating enzymes (DUBs) and revealed the first amino acid of β-galactosidase after the last residue of ubiquitin. Shuffling of the first amino acid X of the ubiquitin-X-β-galactosidase fusion

protein revealed different half-lives of X-β-galactosidase ranging from less than 3 minutes to more than 20 hours (Bachmair et al., 1986).

Until now only a few native substrates of the N-end rule pathway have been found. Due to the genetic code newly synthesized proteins bear an N-terminal Methionine (fMet in procaryotes), which is a stabilizing residue. Sherman and colleagues showed that Methionine aminopeptidases remove N-terminal Met, however, only if the residue at the second position has a side chain of 1.29 Å or less, *e.g.* Gly, Ser, Ala or Cys. These residues are all stabilizing according to the N-end rule (Giglione et al., 2004; Moerschell et al., 1990). The Sherman rule, allows generation of N-degrons only by cleavage of an exo- or endopeptidase in front of a primary, secondary or tertiary destabilizing residue or by addition of a destabilizing residue to the N-terminus. In principal, a substrate can also be recognized by the N-end rule pathway in trans, *i.e.* by another protein bearing a destabilizing N-terminus that binds to the substrate and conducts the ubiquitylation of an accessible Lys residue on the substrate (Johnson et al., 1990). Examples of native N-end rule substrates are the cohesin subunit Scc1 of yeast and the apoptotic inhibitor DIAP1 of *Drosophila melanogaster*. Scc1 is cleaved by the *ESP1* gene encoded protease separin at the onset of anaphase. This generates an unstable C-terminal fragment of Scc1 that possesses an N-terminal Arg and is therefore rapidly degraded (Rao et al., 2001). DIAP1 is cleaved by caspases generating a tertiary destabilizing Asn at the N-terminus of the C-terminal fragment. Degradation of this fragment was found to be necessary for the anti-apoptotic activity of DIAP1 raising the possibility that pro-apoptotic factors are co-degraded (Ditzel et al., 2003; Varshavsky, 2003).

Components that are able to recognize an N-degron are called N-recognins. The first N-recognin was found in a reticulocyte lysate *in vitro* system (Gonda et al., 1989; Reiss et al., 1988). These proteins turned out to be the E3 proteins, which had been previously shown to directly interact with proteolytic substrates (Hershko et al., 1986). The first N-recognin that was cloned, sequenced and whose functional activity was shown by *in vitro* and *in vivo* analyses is the yeast ubiquitin ligase Ubr1 (Bartel et al., 1990). The 225 kDa protein Ubr1 is the only N-recognin in yeast. Ubr1 possesses three known substrate-binding sites.

The type-1 site is specific for basic N-terminal residues *i.e.* Arg, Lys and His. The type-1 binding site is located within a conserved zinc finger like domain of Ubr1 called UBR box motif, found in many eucaryotic N-recognins (Choi et al., 2010; Tasaki et al., 2005; Tasaki et al., 2009).

The type-2 site is specific for N-terminal bulky hydrophobic residues like Trp, Phe, Tyr, Leu and Ile with side chains that have a radius of gyration larger than 1.54 Å (Levitt, 1976; Moerschell et al., 1990). The type-2 substrate-binding site is located within the so called N-domain or ClpS homology domain of Ubr1 (Dougan et al., 2002; Lupas and Koretke, 2003). ClpS is a prokaryotic N-recognin found in *E. coli* with homologues present in many bacterial species and in plant chloroplasts. It was shown to be a specific adaptor protein of the ClpA/ ClpP protease. ClpS proteins directly interact with type-2 substrates. *E. coli* strains mutated in ClpS were shown to be deficient in degradation of N-end rule substrates (Erbse et al., 2006).

The third binding site of yeast Ubr1 is specific for Cup9, a transcriptional repressor of the peptide transporter Ptr2. Ubr1 targets an internal degron of Cup9 when concentrations of dipeptides are high. Ubr1 senses dipeptides with a basic or bulky hydrophobic N-terminus, which bind to the type-1 or type-2 sites. This causes a conformational change and allosteric activation of Ubr1 which promotes ubiquitylation of Cup9 for degradation (Du et al., 2002; Xia et al., 2008a; Xia et al., 2008b).

The dedicated ubiquitin conjugating enzyme of yeast Ubr1 is Rad6/ Ubc2 (Dohmen et al., 1991). Lack of Rad6/ Ubc2 can be partially compensated by Ubc4 (Byrd et al., 1998). Via the basic rich region (BRR) of yeast Ubr1, a strong physical interaction to the polyacidic C-terminal tail of Rad6/ Ubc2 is established. Deletion in either of these interacting domains decrease the physical interaction of the E2/ E3 pair but does not prevent the degradation of N-end rule substrates. In contrast, mutations in the RING-H2 finger of Ubr1 impair ubiquitylation and degradation of N-end rule substrates, but not the interaction with Rad6/ Ubc2 (Xie and Varshavsky, 1999).

More than 50% of the yeast and more than 80% of the human proteome is N-terminally acetylated. N-terminal acetylation is catalyzed by N-terminal acetyltransferases (NATs) and is found in all kingdoms of life (prokaryotes, archaea and eukaryotes). It was shown to occur in eukaryotes also co-translationally (Arnesen et al., 2009; Polevoda and Sherman, 2002). N-terminal Met, Ala, Val, Ser, Thr, Cys, Gly and Pro were classified as stabilizing residues before (Bachmair and Varshavsky, 1989). In a recent study, Varshavsky and co-workers show that N-terminal acetylation of Met, Ala, Val, Ser, Thr or Cys can be a degradation signal in *S. cerevisiae* (Hwang et al., 2010a). They show that N-terminal Met, Ala, Val, Ser, Thr and Cys preferentially become acetylated if the next amino acid in sequence is a non-basic residue. N-terminal Gly or Pro are only rarely acetylated independently of the second amino acid in the sequence. They show that the known ER and inner nuclear

membrane spanning E3 ligase Doa10 (Deng and Hochstrasser, 2006; Ravid et al., 2006) recognizes acetylated substrates and subjects them for proteasomal degradation. N-terminal Met, Ala, Val, Ser, Thr and Cys can now be considered as secondary destabilizing residues of the eucaryotic N-end rule pathway which must be acetylated to become recognized by the E3 ligase Doa10 as an ^{Ac}N-degron.

When Varshavsky and co-workers discovered the N-end rule pathway they also showed that the N-terminal ubiquitin moiety is hardly cleaved off in case of ubiquitin-Pro-β-galactosidase (Bachmair et al., 1986). This protein is finally degraded by a different ubiquitin proteasome pathway called the ubiquitin fusion degradation (UFD) pathway (Johnson et al., 1995). This pathway recognizing N-terminal ubiquitin is dependent on the HECT type ubiquitin ligase Ufd4 in cooperation with the ubiquitin conjugating enzymes Ubc4 and Ubc5. It was shown that the Mgt1 DNA repair protein is co-targeted for degradation by Ufd4 and Ubr1 (Hwang et al., 2009). A more recent study showed that Ufd4 and Ubr1 physically and functionally interact, and that Ufd4 and Ubr1 enhance the length of polyubiquitin chains of N-end rule and UFD pathway substrates, respectively (Hwang et al., 2010b).

In yeast, deletion of the N-recognin Ubr1 leads to an increased frequency of chromosome loss, due to the stabilization of the Scc1 fragment (Rao et al., 2001), and to decreased peptide import (Du et al., 2002). In mammals, a family of UBR box containing proteins named UBR1 through UBR7 is described. UBR1, UBR2, UBR4 and UBR5 were shown to bind to N-degrons, whereas UBR3, UBR6 and UBR7 do not (Tasaki et al., 2005; Tasaki et al., 2009). Yeast Ubr1 is an orthologue of mammalian UBR1 and UBR2. UBR1$^{-/-}$ knock-out mice have lower amounts of skeletal muscle and show exocrine pancreatic insufficiency (Kwon et al., 2001; Zenker et al., 2005). In an inbred mouse strain, an UBR2 $^{-/-}$ knock-out is lethal for most embryos. In a mixed strain most female UBR2 $^{-/-}$ knock-out mice were shown to still die as embryos, while males are viable but infertile due to apoptosis of the UBR2 $^{-/-}$ spermatocytes (Kwon et al., 2003). Data from this study suggest an essential role of the UBR2 ubiquitin ligase and the N-end rule pathway in male meiosis and spermatogenesis and in female embryogenesis. UBR1$^{-/-}$ UBR2 $^{-/-}$ double knock-out mice die as early embryos. However, rescued fibroblasts still have a low N-end rule activity due to the other N-recognins (Tasaki et al., 2009). In humans, missense and truncating mutations in both UBR1 alleles cause the Johanson-Blizzard syndrome, an autosomal recessive disorder. Patients suffering from this disease show symptoms

like congenital pancreatic insufficiency, malformations such as nasal wing aplasia and often mental retardation (Zenker et al., 2005).

2. Results and discussion

2.1. Scope of this work

In my work I investigated several aspects of protein degradation mediated by the ubiquitin proteasome system (UPS), a machinery central in cellular regulation. The studies elucidated essential aspects of the mechanisms involved in the elimination of misfolded cytoplasmic proteins. In addition, they led to the surprising discovery of a cellular AAA-ATPase functioning in the degradation process of a regulatory enzyme, fructose-1,6-bisphosphatase, of the carbohydrate metabolism. Furthermore my studies lead to the finding that, contrary to published knowledge, non-hydrolyzed ubiquitin chains do not possess a general inhibitory effect on the UPS, but act differently in different UPS pathways.

2.2. The Hsp70 chaperone machinery subjects misfolded proteins to degradation via the ubiquitin-proteasome system

(see also 4.1)

Translocation efficiency of different proteins into the secretory pathway varies from more than 95% to less than 60% (Levine et al., 2005). The resulting mislocalization of proteins to the cytoplasm may lead to defects in yeast and to severe diseases in mammals due to misfolding and aggregation of secretory proteins in the reducing environment of the cytoplasm. To study degradation of mislocalized and misfolded proteins in the cytoplasm, Medicherla et al., (2004) had developed the substrate ΔssCG*, which is derived from the ERAD substrate CPY* and due to deletion of the signal sequence unable to enter the endoplasmic reticulum. They could show that degradation of ΔssCG* does not depend on already known cytosolic components of the ERAD system, like the Cdc48-Ufd1-Npl4 complex, Dsk2 or Rad23 (Medicherla et al., 2004).

For the discovery of new factors that are involved in the UPS dependent degradation of ΔssCG* as a model substrate for misfolded cytoplasmic proteins, cytoplasmic chaperones were tested. The major Hsp70 chaperones of the yeast cytoplasm are the members of the Ssa subfamily. This subfamily consists of four members, Ssa1 to Ssa4, which show high sequence similarity and possess overlapping functions. Therefore, strains deleted in *SSA2* to *SSA4* containing either the wild-type *SSA1* or the temperature sensitive allele *ssa1-45* (Becker et al., 1996) have to be used for testing the effect of Ssa1 on the stability of ΔssCG*.

It could be shown that for degradation of ΔssCG* the Hsp70 Ssa1 and the co-chaperone Ydj1 are essential (published in Park et al., 2007) (see 4.1).

Unfolding of the tightly folded GFP domain by Hsp70 chaperones was uncovered to be a prerequisite for proteasomal degradation of some GFP fusion proteins (Liu et al., 2003). Therefore, also untagged ΔssCPY* was tested, since dependency on Ssa1 for degradation of ΔssCG* could be caused by the GFP tag. Since Levine and colleagues had observed a high frequency of mislocalization of native secretory proteins to the cytoplasm (Levine et al., 2005), it was also interesting to test ΔssCPY, which is identical with wild-type CPY in sequence but due to the missing signal sequence mislocalized to the cytoplasm. This protein is probably also misfolded resulting from disturbed formation of disulphide bridges (Endrizzi et al., 1994).

Both substrates, ΔssCPY* and ΔssCPY showed similar dependency on Ssa1 and Ydj1 as was found for degradation of ΔssCG* (published in Park et al., 2007) (see 4.1). Furthermore, it was shown in this study that no Ssa1 is needed for degradation of GFP-cODC (a 37 amino acid long proteasome targeting sequence of ODC C-terminally fused to GFP) (see 1.2.2.2.3). But when this construct is fused to ΔssCPY* yielding in ΔssCPY*GFP-cODC, dependency on Ssa1 for degradation is reimposed. This demonstrates that Ssa1 is obviously required for targeting the misfolded CPY* domain to the degradation pathway and that the misfolded protein domain dictates the route for protein degradation. By biochemical solubilization assays and fluorescence microscopy it could be shown in this study that Ssa1 and Ydj1 are needed to keep ΔssCG* soluble and are necessary for resolubilization of already precipitated substrate. Furthermore, it could be shown that ΔssCG* is polyubiquitylated independently of Ssa1 and Ydj1. Detection of more polyubiquitylated immunoprecipitated ΔssCG* in *ssa1-45*^{ts} and *ydj1-151*^{ts} strains under non-permissive conditions indicates that in these strains transport of already ubiquitylated protein to the proteasome is restrained.

Frydman and co-workers analyzed factors necessary for degradation of the recombinantly expressed orphan protein VHL (von Hippel-Lindau tumor suppressor) in yeast. They showed that in addition to the Hsp70 Ssa machinery, the Hsp40 co-chaperone Sti1/ Hop, the Hsp90 machinery and the nucleotide exchange factor Sse1 are necessary for degradation of VHL (McClellan et al., 2005a). In contrast, for the degradation of ΔssCPY*, ΔssCPY and ΔssCPY*GFP another Hsp40 co-chaperone, Ydj1, was shown to be required. The Hsp90 machinery and Sse1 had no effect on the degradation of the different ΔssCPY* species (published in Park et al., 2007).

Hampton and co-workers tested the need for Sse1 for degradation of ΔssCG* again and found almost complete stabilization of ΔssCG* in a Δ*sse1* strain (ΔssCG* was named CPY‡-GFP in this study and expressed under the *TDH3* promoter) (Heck et al., 2010). This difference may be explained by the higher level of ΔssCG* when expressed under the strong *TDH3* promoter as compared when expression is done using the relatively weak *PRC1* promoter used by us (published in Park et al., 2007), as well as potentially different levels of the highly homologous Sse2 protein in the different yeast strain backgrounds used. Therefore, I examined the degradation of the ΔssCG* protein expressed in a *SSE2* deletion strain with a temperature sensitive *SSE1* allele. The misfolded substrate ΔssCG* showed strong stabilization under non-permissive conditions (Fig. 4). Remarkably, degradation of ΔssCG* was restored in a Δ*sse1* Δ*sse2* strain overexpressing the BAG-1 like protein Snl1 without its transmembrane domain (see 1.1.3). Restoration of ΔssCG* degradation via overexpression of a BAG-1 like protein suggests that here Sse1 and Sse2 are rather required to function as a nucleotide exchange factor of Ssa1 than as an additional molecular chaperone.

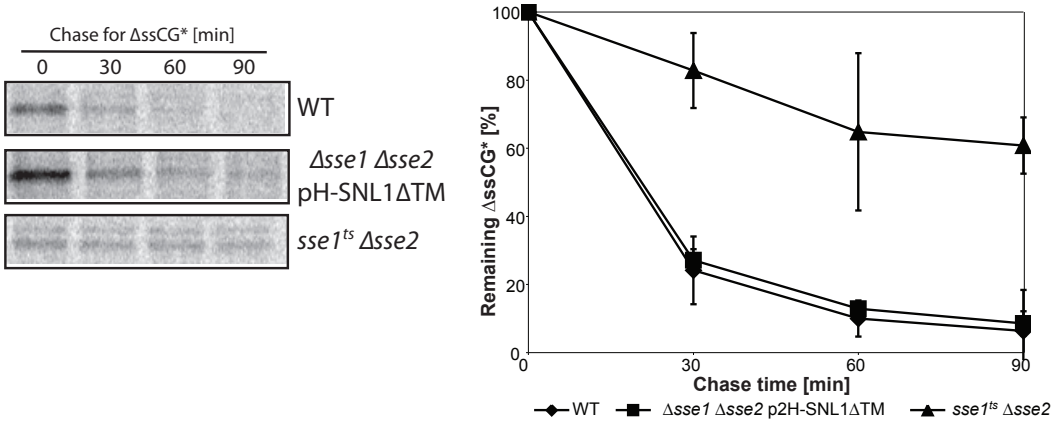


Figure 4. Yeast cytoplasmic Hsp110 of the Sse type is involved in degradation of ΔssCG*. Effect of mutations in the Sse chaperones on degradation of ΔssCG* is neutralized by overexpression of BAG-1 like protein Snl1ΔTM. Pulse-chase analysis of degradation of ΔssCG* was done in wild-type BY4741, *Δsse1 Δsse2* cells with a high-copy plasmid expressing Snl1 deleted in its transmembrane domain (Δ*sse1* Δ*sse2* pH SNL1ΔTM) which rescues the lethal phenotype of the double deletion, and a *sse1ts* Δ*sse2* strain (Strains obtained from B. Bukau, Heidelberg). Cells were grown at 25°C and shifted to 37°C 30 min prior to labeling

with ^{35}S-Methionine. Pulse-chase analysis was performed as described in Park et al., 2007.

In summary, the data demonstrate that the Ssa1-Ydj1 chaperone machinery, in cooperation with Hsp110s of the Sse type, is able to recognize misfolded cytoplasmic proteins and keeps misfolded proteins soluble. Furthermore, this machinery is able to solubilize precipitated proteins and delivers misfolded proteins in a ubiquitylated state to the 26S proteasome.

2.3. E3 ligases involved in the degradation of misfolded cytoplasmic proteins

(see also 4.2)

Growth based screens have been successfully applied for the elucidation of components involved in degradation of misfolded proteins of the ER (Buschhorn et al., 2004; Kohlmann et al., 2008; Medicherla et al., 2004). These screens were based on ERAD substrates like CT* (CPY* fused with the last transmembrane domain of Pdr5) or Sec61-2, both C-terminally fused to Leu2 (3-isopropylmalate dehydrogenase) protein. Background of these screens is the fact that the *leu2* based leucine auxotrophy of the tested strains is neutralized when a mutation is present that leads to stabilization of the different misfolded proteins fused to Leu2. Mutants stabilizing these fusion proteins can be isolated by selecting them for growth on media lacking leucine.

I used a similar approach for discovering the lacking ubiquitin ligase (E3) involved in the degradation of cytoplasmic CPY* species. The genetic sequence encoding ΔssCPY* was fused to the *LEU2* gene and the sequence of a myc tag yielding ΔssCL*myc. In order to elucidate whether this new substrate is comparable to the tested ΔssCPY* species I performed experiments investigating the degradation of this new substrate in strains mutated in chaperones of the Ssa and Sse type. As it was found for signal sequence deleted ΔssCPY*, ΔssCPY and ΔssCG*, degradation of ΔssCL*myc is dependent on the Hsp70 chaperone Ssa1 as well (Fig. 5 A). A similar dependency on the Hsp110 chaperones Sse1 and Sse2 as shown for degradation of ΔssCG* (Fig. 4), could be shown for degradation of ΔssCL*myc. (Fig. 5 B). A strong stabilization of ΔssCL*myc could be observed upon cycloheximide chase analysis in the *sse1^{ts} Δsse2* strain. In the Δ*sse1* Δ*sse2* strain harboring a rescue plasmid overexpressing the BAG-1 like protein Snl1 without its transmembrane domain, degradation of ΔssCL*myc was similar as in the wild-type

strain. Again, as in the case of ΔssCG*, the role of Sse1 and Sse2 seems to be rather that of a nucleotide exchange factor than that of a molecular chaperone observed by the restoration of degradation achieved by overexpression of the BAG-1 like protein in the Δ*sse1* Δ*sse2* strain background (Fig. 5 B) (see also 1.1.3).

Upon transformation of strains lacking known or putative ubiquitin ligases with a plasmid expressing ΔssCL*myc and subsequent growth analysis I discovered the E3 ligase Ubr1 to be an enzyme necessary for degradation of this misfolded cytoplasmic protein (see 1.2.2.6 and 4.2). Pulse-chase analysis showed a strong stabilization of ΔssCL*myc in a Δ*ubr1* strain. This phenotype could be rescued by overexpression of N-terminally FLAG tagged Ubr1 (fUbr1) from a 2*μ* plasmid under the control of an *ADH1* promoter. Overexpression of fUbr1 in the wild-type strain led to accelerated degradation of ΔssCL*myc in comparison to wild-type carrying the corresponding empty vector control (Fig. 2 in 4.2). Previously, physical interactions between a ligase and its substrate had been observed, *e.g.* in the case of the ERAD substrate CPY* and the Hrd/ Der ligase complex (Gauss et al., 2006; Schäfer and Wolf, 2009). Also in case of ΔssCL*myc and Ubr1, a physical interaction of substrate and E3 could be detected by co-immunoprecipitation (Fig. 3 in 4.2) indicating their functional relationship.

Recent studies on different cytoplasmically mislocalized proteins showed that degradation of ΔssCG* (Heck et al., 2010), mislocalized vacuolar Proteinase A (ΔssPrA) and a mutated GFP version (Δ2GFP) (Prasad et al., 2010) depend substantially also on the nuclear ubiquitin ligase San1 (see 1.2.2.5). Truncated versions of cytoplasmic fatty acid synthetase Fas1, of cytoplasmic phosphogluconate dehydrogenase Gnd1, and of Yor296w, an unknown protein that is predicted to reside in the cytosol, were also found to be substrates of both E3‘s, Ubr1 and San1. In case of truncated Gnd1 (tGnd1), dependency on the two ligases varied with the truncated region of the substrate (Heck et al., 2010).

For testing the involvement of the nuclear ligase San1 in the degradation of the different ΔssCPY* species I observed a strong stabilization of cytoplasmic CPY* (ΔssCPY*) and cytoplasmic CPY (ΔssCPY) in a Δ*ubr1* Δ*san1* strain. In a Δ*ubr1* strain ΔssCPY* and ΔssCPY are only slightly more stable than in wild-type cells (Fig. 5 C and D). In contrast, degradation of ΔssCL*myc is not influenced by deletion of *SAN1.* This explains the discovery of only Ubr1 as the E3 for targeting this protein for degradation. ΔssCL*myc shows the strongest stabilization in a Δ*ubr1* strain seen up to now. Double deletion in *UBR1* and *SAN1* does not increase stabilization (Fig. 5 E).

A possible explanation for these varying dependencies on the different ubiquitin ligases Ubr1 and San1 could be due to intrinsic properties or the size of the substrates. Possibly transport of the relatively small proteins ΔssCPY* (~60 kDa), ΔssCPY (~60 kDa), ΔssPrA (~40 kDa) and Δ2GFP (~26 kDa) into the nucleus, the locus of highest proteasomal concentration (Laporte et al., 2008; Russell et al., 1999) is easily accomplished by the cell. Import into the nucleus leads to efficient recognition by the nuclear ligase San1, while the relatively large substrate ΔssCL*myc (~130 kDa) is excluded from transport into the nucleus. Transport of the somewhat smaller substrate ΔssCG* (~86 kDa) into the nucleus could be caused by properties of the GFP tag, which can lead to nuclear localization as was shown for the protein Fbp1-GFP (Barbin, PhD thesis, 2010). See figure 5 G for a current model of the degradation process of different ΔssCPY* species.

Another study showed that Ubr1 and its paralogue Ubr2 function in degradation of misfolded cytoplasmic proteins like *e.g.* newly synthesized Tpk2 in the absence of Hsp90 activity (Nillegoda et al., 2010). Deletion of *UBR2* had no effect on degradation of ΔssCL*myc (Kathrin Deuschle, Diploma thesis, 2010).

Ubr1 was originally found by Varshavsky and co-workers to be the ubiquitin ligase of the N-end rule pathway, which defines the stability of a protein according to the type of its N-terminal residue (Bachmair et al., 1986; Varshavsky, 1996) (see also 1.2.2.6). Despite the finding of Ubr1 to be responsible for degradation of ΔssCL*myc and at least partially of ΔssCG*, it was excluded that these substrates are degraded in an N-end rule specific way (Eisele and Wolf, 2008; Heck et al., 2010). Due to the DNA sequence of all ΔssCPY* species, these proteins start with Met-Ile-Ser at their N-terminus. According to the Sherman rule cleavage of the N-terminal methionine in such a sequence environment is prohibited. This rule allows cleavage of the N-terminal Met residue only if the penultimate N-terminal residue has a radius of gyration of 1.29 Å or less (Moerschell et al., 1990). The Ile residue has a radius of gyration of 1.56 Å and it was shown by Sherman and co-workers that the methionine of a model protein starting with Met-Ile was fully blocked. If this rule is also true for the model substrate ΔssCL*myc it should retain its N-terminal Met, which is a stabilizing residue according to the N-end rule.

Hampton and colleagues showed that N-terminally FLAG tagged tGnd1-GFP is ubiquitylated in an Ubr1 dependent manner. Upon immunoprecipitation of FLAG-tGnd1-GFP with antibodies specific for the FLAG epitope and subsequent detection

of ubiquitylated material with antibodies specific for ubiquitin or GFP it was argued that the N-terminus of this substrate is not altered (Heck et al., 2010).

Surprisingly, in our hands N-terminal tagging of ΔssCL*myc with the V5 epitope had a stabilizing effect on the substrate (Kathrin Deuschle, diploma thesis, 2010). We did not expect this tag to be stabilizing but this result indicates that a modification of the misfolded substrate is necessary for its degradation and that this modification is inhibited by the N-terminal tagging with V5. Like the FLAG tag, the V5 epitope is short in sequence and does probably not contribute to the folding of the protein it is fused to. The V5 tag starts with the amino acid glycine after the amino acid methionine encoded by the start codon, which is rapidly cleaved off by Met aminopeptidases. According to the N-end rule, Gly constitutes a stabilizing N-terminus. Whether this stabilizing N-terminus abrogates Ubr1 dependent degradation of the misfolded protein has to be shown by future experiments. This finding suggests that this N-terminally tagged misfolded substrate can no longer become a substrate of Ubr1 and that untagged ΔssCL*myc must somehow be processed to an N-end rule substrate. In order to test the possibility whether ΔssCL*myc is modified to an N-end rule substrate, point mutants of Ubr1, which were shown to be incapable of degrading either type-1 or type-2 N-end rule substrates (Xia et al., 2008b) were tested for their ability to promote degradation of ΔssCL*myc. The type-1 binding site is located within the UBR box of Ubr1 and has been shown to be specific for basic amino acids (Choi et al., 2010; Tasaki et al., 2009). The type-2 binding site is located within the ClpS homology region of Ubr1 and has been shown to be specific for bulky hydrophobic amino acids (Dougan et al., 2002; Lupas and Koretke, 2003) (see also 1.2.2.6).

Upon pulse-chase analysis, I found strains expressing the type-1 specific point mutation to have a strong stabilizing effect on ΔssCL*myc, comparable to deletion of Ubr1. The type-2 specific point mutation led to a delay in degradation of ΔssCL*myc in comparison to cells expressing functional Ubr1 (Fig. 5 F). These findings suggest that the misfolded substrate is modulated in a fashion that it can be recognized in an N-end rule dependent way. Whether this is actually the case has to be addressed by future experiments. Possible mechanisms involve N-terminal cleavage via aminopeptidases, internal cleavage in front of destabilizing residues by endopeptidases, or N-terminal attachment of destabilizing amino acids for recognition of the substrate by Ubr1. It is also conceivable that the substrate is recognized *in trans,* which was shown to function for degradation of N-end rule substrates like X-βgal (Johnson et al., 1990) (see 1.2.2.6). This would involve an additional factor

being capable of binding to the misfolded protein like a molecular chaperone and possessing a destabilizing N-terminus recognized by Ubr1. Such a factor could then lead to ubiquitylation of the misfolded substrate.

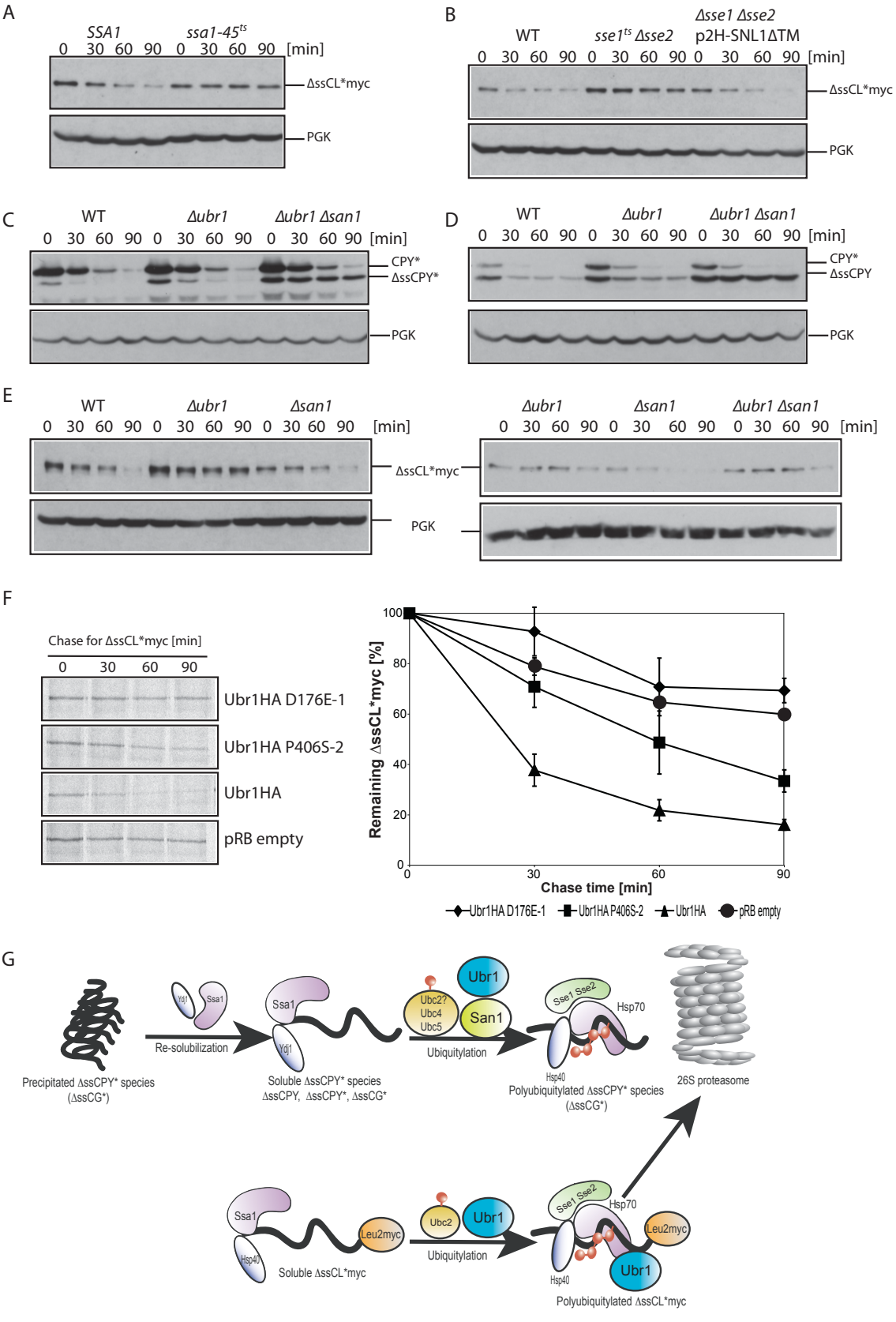


Figure 5. Involvement of the chaperones Ssa1, Sse1 and Sse2 and the ubiquitin ligases Ubr1 and San1 in the degradation of misfolded cytoplasmic proteins.

A. The Hsp70 Ssa1 is required for degradation of ΔssCL*myc. **B.** The Hsp110s Sse1 and Sse2 are required for degradation of ΔssCL*myc. Overexpression of Snl1ΔTM restores degradation. **A and B.** Strains were grown at 25°C and shifted one hour prior to addition of cycloheximide to 37°C. Samples were taken at the indicated time-points and proteins were detected by western-blotting using anti-CPY, and anti-PGK as a loading control. **C and D.** Degradation of ΔssCPY* and ΔssCPY depends on Ubr1 and San1. **E.** Degradation of ΔssCL*myc depends on Ubr1. In a Δ*san1* strain, degradation pattern of this substrate is like in the wild-type. *UBR1 SAN1* double deletion has no additional stabilizing effect in comparison to Δ*ubr1* strain. **C, D, E.** Samples were taken at the indicated time-points after addition of cycloheximide. Proteins were detected by western-blotting using anti-CPY, and anti-PGK as a loading control. **F.** ΔssCL*myc is stabilized in a Δ*ubr1* strain expressing a type-1 N-end rule defective Ubr1 mutant. Pulse-chase analysis of ΔssCL*myc degradation in Δ*ubr1* strains expressing either HA tagged Ubr1 (Ubr1HA), Ubr1 point mutants defective in degradation of type-1 (Ubr1HA D176E-1) or type-2 N-end rule (Ubr1HA P406S-2) substrates, or a Δ*ubr1* strain with an empty vector (pRB empty) as a control.

Cycloheximide and pulse-chase experiments were performed like described in Park et al., 2007. *SSA1* and *ssa1-45^{ts}* strains were described in Taxis et al., 2003, *sse1^{ts}* Δ*sse2*, Δ*sse1* Δ*sse2* p2H-SNL1ΔTM were obtained from B. Bukau, Heidelberg (see Fig. 4), plasmids expressing C-terminal HA tagged Ubr1 as described in Xia et al., 2008b were obtained from A. Varshavsky, Pasadena, USA. **G.** Model of cytoplasmic protein quality control factors involved in the degradation process of ΔssCPY* species.

2.4. The Cdc48-Ufd1-Npl4 complex is central in ubiquitin-proteasome dependent catabolite degradation of fructose-1,6-bisphosphatase

(see also 4.3)

The AAA-ATPase Cdc48 together with its co-factors Npl4 and Ufd1 were shown to be involved in the delivery of ubiquitylated misfolded proteins to the proteasome in ERAD and the UFD pathway, as well as in the UPS dependent activation of certain membrane-bound transcription factors (see also 1.2.2.3 and 1.2.2.6) (Ghislain et al., 1996; Hitchcock et al., 2001; Raasi and Wolf, 2007; Rape et al., 2001). In contrast,

studies by Medicherla et al. and Park et al. showed that the misfolded cytoplasmic proteins ΔssCPY*, ΔssCPY and ΔssCG* are normally degraded in *ufd1* mutants. As a control for proper impairment of the Cdc48-Ufd1-Npl4 machinery in the *ufd1* mutated cells tested, stabilization of the misfolded ERAD protein CPY* could be shown (Medicherla et al., 2004; Park et al., 2007). Since the Cdc48 complex constitutes a central component of some UPS pathways I constructed a new temperature sensitive mutant of *CDC48* in order to test the effect of this AAA-ATPase on the misfolded ΔssCPY* species more directly than by testing only its mutated co-factor Ufd1. However, no stabilizing effect in the *cdc48^{ts}* mutant protein could be observed on the degradation of misfolded cytoplasmic ΔssCPY* species corroborating the previous conclusion.

For checking further cytoplasmic UPS substrates for their dependency on Cdc48 for degradation I tested also a regulatory enzyme of the carbohydrate metabolism, fructose-1,6-bisphospatase (FBPase). FBPase constitutes a key regulatory enzyme of the gluconeogenesis pathway and is a known substrate of the UPS pathway. The FBPase protein is rapidly eliminated from growing cells after the switch from a non-fermentable to a fermentable carbon source. This is due to ubiquitylation of this enzyme by the Gid-E3 complex for degradation by the proteasome (Regelmann et al., 2003; Santt et al., 2008). Surprisingly, when testing the degradation of FBPase in the *cdc48^{ts}* mutant after switching the cells from ethanol containing media to glucose containing media I could observe a strong stabilization of FBPase under non-permissive conditions (see Fig. 1 A, chapter 4.3) (Barbin et al., 2010). FBPase is the first natural cytoplasmic substrate of the UPS that is shown to depend on the Cdc48-Npl4-Ufd1 machinery for its degradation. Following experiments of Lise Barbin led to the model that after polyubiquitylation of FBPase by the Gid-E3 complex, the Cdc48-Ufd1-Npl4 complex processes the polyubiquitylated enzyme. Since FBPase is present in a homotetrameric complex in the cell (Rittenhouse et al., 1986; Rittenhouse et al., 1984) which is bound to the Gid complex already under gluconeogenic conditions (Santt et al., 2008), it is conceivable that the pulling force of the Cdc48-Ufd1-Npl4 complex is needed for dissociation of the single ubiquitylated FBPase subunits from the complex. After isolation of single ubiquitylated FBPase proteins by the Cdc48-Ufd1-Npl4 complex, Dsk2 and Rad23 bind the polyubiquitylated substrate with the help of their UBA domains for further delivery to the 26S proteasome.

2.5. Mutants of the deubiquitylating enzyme Ubp14 decipher pathway diversity of ubiquitin–proteasome linked protein degradation

(see also 4.4)

Polyubiquitylated substrates bind to the 26S proteasome via specific ubiquitin receptors. These receptors can be subunits of the 26S proteasome like Rpt5, Rpn10 and Rpn13, or associated proteins like Dsk2 and Rad23 (Finley, 2009) (see 1.2.2.2.1 and 1.2.2.2.3). Prior to degradation of a ubiquitylated substrate, ubiquitin chains are cleaved off via deubiquitylating enzymes (DUBs) as for instance the proteasomal subunit Rpn11. Subsequently, free ubiquitin chains are recycled to single ubiquitin moieties by the DUB Ubp14 (Amerik and Hochstrasser, 2004). In a previous study by Hochstrasser and co-workers it was shown that deletion of *UBP14* leads to accumulation of free unanchored ubiquitin chains. Stabilization of the N-end rule substrate Leu-β-gal, the UFD-pathway substrate Ub-Pro-β-Gal and the Mat-α2 repressor (see also 1.2.2.6 and 1.2.2.5) was observed. Therefore, free ubiquitin chains, due to competitive binding to ubiquitin receptors, were thought to inhibit degradation of polyubiquitylated substrates by the 26S proteasome in general (Amerik et al., 1997). This hypothesis was taken up and tested using different well characterized substrates of ubiquitin dependent protein degradation of our laboratory. In parallel, a strain deleted in the gene encoding *RPN10* was also tested for checking whether the ubiquitin-interacting motif (UIM) domain containing protein plays a role in the degradation process of the different substrates.

Ubp14 has been found in a screen performed in the lab for factors involved in the degradation of the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase, yeast Fbp1) (Regelmann et al., 2003). Therefore, I tested degradation of FBPase by pulse-chase analysis and could show almost complete stabilization of Fbp1 in Δ*ubp14* cells after switch from ethanol containing labeling media to glucose containing chase media. Deletion of the ubiquitin receptor protein Rpn10 led to a mild delay in degradation of Fbp1. In contrast, degradation of an HA tagged version of the ERAD substrate CPY* (Hiller et al., 1996) was not inhibited at all in Δ*ubp14* or Δ*rpn10* cells. Degradation of the misfolded cytoplasmic protein ΔssCG* (Medicherla et al., 2004; Park et al., 2007) was slightly inhibited in cells deleted in *UBP14* or *RPN10*. Another substrate tested was Deg1-GFP_2. This substrate consists of the Deg1 degradation signal of the transcriptional repressor Matα2 and two C-terminal GFP tags (Lenk and Sommer, 2000) (see also 1.2.2.5). Degradation of this substrate was not inhibited in cells lacking Ubp14 or Rpn10.

These data clearly show that free ubiquitin chains accumulating due to deletion of *UBP14* inhibit degradation of only some substrates and do not inhibit degradation of ubiquitin proteasome substrates in general. We propose that these different substrates of the ubiquitin proteasome system follow different degradation pathways and that these pathways are differently affected by competitive inhibition with free ubiquitin chains (see 4.4) (Eisele et al., 2006).

Many human cancer types are caused by mutations in the gene encoding for the tumor suppressor p53 or by overexpression of its negative regulator Mdm2. Mdm2 is believed to suppress p53 by transcriptional inhibition and by its E3 activity (Itahana et al., 2007). Both p53 and Mdm2, are subject to degradation by the ubiquitin proteasome system. Similar to the different analyzed substrates in yeast (Amerik et al., 1997; Eisele et al., 2006), in mammalian cells p53 and Mdm2 seem to be dependent on different pathways for ubiquitin proteasomal degradation. A recent study demonstrated that silencing of USP5/ isopeptidase T, the mammalian orthologue of yeast Ubp14, increases the stability of p53 (Dayal et al., 2009). In this study the authors demonstrated that a knock-down of USP5 leads to an increase in free ubiquitin chains and to delayed degradation of p53, but did not influence the degradation kinetics of its repressor Mdm2. This results in a higher abundance and transcriptional activity of p53. In contrast, inhibition of the proteasome caused stabilization of p53 and Mdm2, which led to inhibition of p53 by direct binding to Mdm2. The observation that free ubiquitin chains stabilize the tumor suppressor p53, but do not alter rapid degradation of its repressor Mdm2, makes Usp5 a good drug target for cancer treatment (Dayal et al., 2009).

The question remains which factors of the ubiquitin proteasome pathway the free ubiquitin chains bind to and how this affects the different pathways to proteasomal degradation.

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4. Publications

4.1. The cytoplasmic Hsp70 chaperone machinery subjects misfolded and ER import incompetent proteins to degradation via the ubiquitin-proteasome system

Sae-Hun Park, Natalia Bolender, Frederik Eisele, Zlatka Kostova, Junko Takeuchi, Philip Coffino, and Dieter H. Wolf

Molecular Biology of the Cell 18 (2007) 153-165

Link to the original publication in *MBoC Online*:
http://www.molbiolcell.org/cgi/content/full/18/1/153

Molecular Biology of the Cell
Vol. 18, 153–165, January 2007

The Cytoplasmic Hsp70 Chaperone Machinery Subjects Misfolded and Endoplasmic Reticulum Import-incompetent Proteins to Degradation via the Ubiquitin–Proteasome System

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Submitted April 21, 2006; Revised October 13, 2006; Accepted October 17, 2006
Monitoring Editor: Randy Schekman

The mechanism of protein quality control and elimination of misfolded proteins in the cytoplasm is poorly understood. We studied the involvement of cytoplasmic factors required for degradation of two endoplasmic reticulum (ER)-import–defective mutated derivatives of carboxypeptidase yscY (ΔssCPY* and ΔssCPY*-GFP) and also examined the requirements for degradation of the corresponding wild-type enzyme made ER-import incompetent by removal of its signal sequence (ΔssCPY). All these protein species are rapidly degraded via the ubiquitin–proteasome system. Degradation requires the ubiquitin-conjugating enzymes Ubc4p and Ubc5p, the cytoplasmic Hsp70 Ssa chaperone machinery, and the Hsp70 cochaperone Ydj1p. Neither the Hsp90 chaperones nor Hsp104 or the small heat-shock proteins Hsp26 and Hsp42 are involved in the degradation process. Elimination of a GFP fusion (GFP-cODC), containing the C-terminal 37 amino acids of ornithine decarboxylase (cODC) directing this enzyme to the proteasome, is independent of Ssa1p function. Fusion of ΔssCPY* to GFP-cODC to form ΔssCPY*-GFP-cODC reimposes a dependency on the Ssa1p chaperone for degradation. Evidently, the misfolded protein domain dictates the route of protein elimination. These data and our further results give evidence that the Ssa1p-Ydj1p machinery recognizes misfolded protein domains, keeps misfolded proteins soluble, solubilizes precipitated protein material, and escorts and delivers misfolded proteins in the ubiquitinated state to the proteasome for degradation.

INTRODUCTION

Newly synthesized proteins must fold into their native three-dimensional structures and maintain this state throughout their lifetime. Molecular chaperones facilitate the initial folding of proteins to their native form, as well as the assembly of multiprotein complexes. Translocation of proteins into the endoplasmic reticulum (ER) or into mitochondria and their folding also relies on molecular chaperones associated with these cellular compartments (Caplan *et al.*, 1992; Parsell and Lindquist, 1993; Hartl, 1996; Frydman, 2001; Hartl and Hayer-Hartl, 2002; Anken *et al.*, 2005; Mayer and Bukau, 2005). Molecular chaperones are involved not only in the folding of proteins but also in their quality control. This includes recognition of misfolding, prevention of protein aggregation, and facilitation of refolding of partially unfolded proteins due to stresses (Goldberg, 2003; Kleizen and Braakman, 2004). Terminally misfolded proteins have to be recognized and eliminated. This process is essential to all cells. Misfolding leads to the exposure of hydrophobic patches in proteins, which may cause their aggregation in the aqueous cellular environment. This may result in the formation of toxic protein precipitates, which are associated with severe diseases such as Alzheimer's, Parkinson, or Creutzfeldt-Jakob disease in humans or bovine spongiform encephalopathy (BSE) in cattle (Kopito, 2000; Dobson, 2003; Goldberg, 2003; Barral *et al.*, 2004).

Selective protein degradation via the ubiquitin–proteasome system is a major pathway conserved throughout eukaryotic evolution (Hochstrasser, 1996; Varshavsky, 1997; Hershko and Ciechanover, 1998; Wolf and Hilt, 2004). Ubiquitination of proteins is mediated by three consecutive reactions: ubiquitin activation via an E1 enzyme, ubiquitin conjugation via E2 enzymes, and the action of ubiquitin protein ligases, E3's, which mediate the selection of substrate and initiate its ubiquitination. Quality control and degradation of secretory proteins (ERQD) as well as of cytoplasmic proteins is under intensive study (Plemper *et al.*, 1997; Sommer and Wolf, 1997; Brodsky and McCracken, 1999; Kostova and Wolf, 2003; Hirsch *et al.*, 2004; McClellan *et al.*, 2005b; Schafer and Wolf, 2005; Bukau *et al.*, 2006). Cytoplasmic degradation is pertinent not only to proteins native to the cytoplasm, but also to secretory proteins that fail to fold properly. Misfolded secretory proteins are recognized in the ER, prevented from continuing along the secretory pathway, retrotranslocated to the cytoplasmic side of the ER, polyubiquitinated, and delivered to the proteasome for degradation. This mechanism

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–04–0338) on October 25, 2006.

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Table 1. Yeast strains used in this study

Name	Genotype	Source
YWO1	*Matα ura3-52 leu2-3,2-112 his3 Δ200 lys2-801 trp1-1*	Seufert and Jentsch (1990)
YWO23	*Matα ura3-52 leu2-3,2-112 his3 Δ200 lys2-801 trp1-1 Δubc4::HIS3 Δubc5::LEU2*	Seufert and Jentsch (1990)
YPH499Y	*Mata ura3-52 leu2-1 his3Δ200 trp1-63 lys2-801 ade2-101 prc1-1*	Hiller *et al.* (1996)
CMY762Y	*Mata cim3-1 ura3-52 leu2-1 his3 Δ200 prc1-1*	Hiller *et al.* (1996)
W303-1C	*Matα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*	Knop *et al.* (1996)
YPK002	W303-1C Δ*snl1::KAN*^{R}	This study
YPD5	W303-1C Δ*ydj1-2::HIS3 LEU2::ydj1-151*	Taxis *et al.* (2003)
YPD21	*Matα his3-11, 15 leu2-3, 112 ura3-52 trp1-81 lys2 prc1-1 Δssa2::LEU2 Δssa3::TRP1 Δssa4::LYS2*	Taxis *et al.* (2003)
YPD22	YPD21 Δ*ssa2::LEU2 Δssa3::TRP1 Δssa4::LYS2 ssa1-45*	Taxis *et al.* (2003)
YCT397	*Mata leu2-3,112 ura3-52 ade1-100 his4-519 prc1-1*	Jarosch *et al.* (2002)
YCT415	YCT397 *ufd1-1*	Jarosch *et al.* (2002)
W303-1B	*Matα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	Chiang and Schekman (1991)
AGC14	*Matα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 Δhsp26::LEU2 Δhsp42::HygB*^{R}	Cashikar *et al.* (2005)
MHY501	*Matα his3-200 leu2-3,112 ura3-52 lys2-801 trp1-1*	Swanson *et al.* (2001)
MHY1631	MHY501 Δ*ssm4/doa10::HIS3*	Swanson *et al.* (2001)
MHY1669	MHY501 Δ*hrd1/der3::LEU2*	Swanson *et al.* (2001)
MHY1703	MHY501 Δ*hrd1/der3::LEU2 Δssm4/doa10::HIS3*	Swanson *et al.* (2001)
YRH023	W303-1C Δ*hsp104::KAN*^{R}	Taxis *et al.* (2003)
YRH030	W303-1c Δ*sti1-1::HIS3*	Taxis *et al.* (2003)
YRH050	W303-1C Δ*hsc82::KAN*^{R} *hsp82*^{G170D}	Taxis *et al.* (2003)
Y406-C	*Matα ura3-52 leu2-3,112 his3-11,15 lys2 trp1-1 prc1-1*	Deak (1998)
Y420-C	Y406-C Δ*ssb1::LEU2 Δssb2::HIS3*	Deak (1998)
BY4743	*Matα/a his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0*	EUROSCARF
BY474Δsse1	BY4743 Δ*sse1::kanMX4/Δsse1::kanMX4*	EUROSCARF

for delivering misfolded ER proteins to the proteasome makes use of a trimeric AAA-ATPase complex consisting of Cdc48p-Ufd1p-Npl4p and of two UBA-UBL-domain proteins, Dsk2p and Rad23p, which are able to dock to the proteasome (Hartmann-Petersen and Gordon, 2004; Elsasser and Finley, 2005). Such a mechanism ensures that misfolded soluble or membrane bound secretory proteins are not released into the cytoplasm, where aggregation would occur, but are escorted instead in a protein bound form to the proteasome for elimination (Medicherla *et al.*, 2004).

This broadly accepted view was in part inferred from experiments using the misfolded ER-lumenal model substrate CPY* (Finger *et al.*, 1993; Hiller *et al.*, 1996; Schafer and Wolf, 2006). Studies of CPY* processing and degradation have been more recently extended to two of its membrane bound derivatives, CT* and CTG*, carrying the ER lumenal CPY* module, a transmembrane domain (CT*), or, in addition, the green fluorescent protein GFP (CTG*) (Taxis *et al.*, 2003). During our studies on the delivery mechanism of these misfolded ER model substrates to the proteasome, we also studied the degradation requirements of the cytoplasmically located CPY* derivative ΔssCPY*-GFP. This protein lacks a signal sequence directing it to the ER. Thus, in contrast to ER lumen misfolded proteins like CPY*, which makes a round trip from cytoplasm to ER and back, ΔssCPY*-GFP is made and remains in the cytoplasm. ΔssCPY*-GFP was also rapidly degraded via the proteasome but did not require the Cdc48p-Ufd1p-Npl4p AAA-ATPase complex nor the UBA-UBL proteins Dsk2p and Rad23p (Medicherla *et al.*, 2004). This pointed to a completely different recognition and delivery mechanism for this misfolded ER import defective secretory protein. Recently it has been found in mammalian cells that the efficiency of protein compartmentalization into the secretory pathway is far from perfect. Because of inefficient signal sequence recognition, inefficient translocation into the ER, and leaky ribosomal scanning, the efficiency of segregation to the ER was shown to vary considerably (Levine *et al.*, 2005). This raises the question of the fate of these remnant proteins mislocalized to the cytoplasm. It was the aim of this study to unravel the agents that recognize misfolded cytoplasmically located proteins and deliver them to the proteasome.

MATERIALS AND METHODS

Yeast Strains and Plasmids

Yeast strains used in this study are summarized in Table 1. All other methods for yeast manipulation and genetic experiments were carried out using standard methods (Guthrie and Fink, 1991; Ausubel *et al.*, 1992). The *SNL1* gene in *W303–1C* was disrupted by PCR amplification of the Δ*snl1::KAN*^{R} fragment from strain *BY4743* (EUROSCARF, Frankfurt, Germany) using the primer pairs SNL1 5′ Primer (GACGAATATAAGGTCAAAAGCTTCA) and SNL1 3′ Primer (TTTATTTTGGTATGATTTTAGGCGA). Correct integration of the disrupted DNA was confirmed by PCR analysis and Southern blotting. The identity of DNA fragments generated by PCR was verified by sequencing. Detailed cloning strategies are available on request. The plasmid pRS316-ΔssCPY*-GFP is described previously (Medicherla *et al.*, 2004). To generate the plasmid pZK116 expressing ΔssCPY*, the signal sequence was removed from the CPY* allele in plasmid pRS316-CPY* (Kostova and Wolf, 2005) by a QuickChange–based (Stratagene, La Jolla, CA) PCR-mutagenesis approach. The DNA of cytoplasmically localized, N-terminally GFP fused CPY* (ΔssGC*) was cloned in two steps. First, the SphI restriction site of the plasmid was introduced to the end of the CPY promoter in pZK116 by QuickChange (Stratagene)-based PCR mutagenesis, yielding pZK116m. Then the PCR-amplified 0.7-kb GFP DNA fragment prepared from plasmid pRS316-ΔssCPY*-GFP as template was cloned into the SphI restriction site of pZK116m, generating plasmid pRS316-ΔssGFP-CPY*. The PCR-amplified 0.75-kb DNA fragment of GFP_{uv} from p416-P_{ADH}-GFP_{uv} as template (Hoyt *et al.*, 2003) was cloned into pRS316-ΔssCPY*-GFP between the HpaI and EcoRI restriction sites, yielding plasmid pRS316-ΔssCPY*-GFP_{uv}. The PCR-amplified 1.2-kb partial fragment of PRC1 from pYEP13/PRC1, which encodes wild-type CPY was used as template and inserted into pZK116 between the Bsu36I and EcoRI restriction sites, generating pRS316-ΔssCPY. The 3.4-kb ORF, which encodes ΔssCPY*-GFP from plasmid pRS316-ΔssCPY*-GFP was subcloned into the 2μ plasmid pRS426 between the ClaI and EcoRI restriction sites, leading to overexpression of ΔssCPY*-GFP. The PCR-amplified 0.5-kb DNA fragment of GFP_{uv}-cODC or GFP_{uv}-cODC-C441A from p416P_{ADH}-GFP_{uv}425cODC or p416P_{ADH}-GFP_{uv}425cODC-C441A as templates (Hoyt *et al.*, 2003) were cloned

into respective sites of pRS316-ΔssCPY*-GFP$_{uv}$ between the MluI and EcoRI restriction sites, yielding pRS316-ΔssCPY*-GFP$_{uv}$-cODC or pRS316-ΔssCPY*-GFP$_{uv}$-cODC-C441A, respectively.

Antibodies

Polyclonal anti-rabbit CPY (Knop *et al.,* 1993), polyclonal anti-rabbit GFP antibodies (Molecular Probes, Eugene, OR) were used for immunoprecipitation of ΔssCPY*-GFP* and its derivates. Monoclonal anti-mouse CPY (Molecular Probes), polyclonal anti-rabbit GFP antibodies were diluted 1:10,000 for immunodetection. Monoclonal anti-mouse ubiquitin antibody (BabCO, Richmond, CA) was used at 1:2000 dilution for immunodetection.

Pulse-Chase Analysis

Pulse-chase experiments using cells expressing CPY* or CPY fusion proteins, respectively, cell breakage in buffer containing urea, and SDS were performed as described previously (Hiller *et al.,* 1996; Taxis *et al.,* 2003). Temperature-sensitive strains were grown at 25°C and shifted to restrictive temperature for labeling with 200 μCi of ^{35}S-Met at 37°C for 20 min. Cells were chased with excess of unlabeled chase media for the times indicated in the respective figure legends.

Cycloheximide Decay Experiments

Cells were grown in synthetic complete medium. Temperature-sensitive strains were shifted to restrictive temperature of 37°C for 60 min. Cycloheximide was added (0.5 mg/ml), and 2 OD_{600} of cells were taken at the indicated time points. Cell extracts were prepared by alkaline lysis and subjected to SDS-PAGE followed by immunodetection (Hiller *et al.,* 1996; Taxis *et al.,* 2003).

Solubility Assay

Cells expressing ΔssCG* were grown at 30°C and shifted to 37°C for 60 min before assay. Twenty OD_{600} of yeast cells were harvested, washed once with four volumes of 20 mM sodium azide, and resuspended in 1 ml of ice-cold sorbitol lysis buffer (0.7 M sorbitol, 50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 1 μg/ml pepstatin-A). Subsequently, all material was kept on ice, and cells were lysed with glass beads in ice-cold sorbitol lysis buffer. Lysates were precleared by centrifugation at 500 × *g* for 5 min at 4°C. Total protein (T) was precipitated from 400 μl of lysate with TCA (11% final concentration). Total protein (T) was solubilized with 60 μl of urea buffer (40 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 100 mM EDTA, pH 8, 200μg/ml bromophenol blue, 1.5% beta mercaptoethanol). In addition 400 μl of lysate was spun in a Beckman T110 rotor (Fullerton, CA) at 130,000 × *g* for 30 min at 4°C. The supernatant was subjected to TCA precipitation and treated as soluble protein (S). The pellet of the 130,000 × *g* centrifugation step was washed once with sorbitol lysis buffer followed by solubilization with 60 μl of urea buffer as described above. Equal amounts of solubilized protein were analyzed by SDS-PAGE followed by immunoblotting. Immunoblots were analyzed with anti-CPY or anti-PGK. Resolubilization of aggregated ΔssCG* was tested as follows: After temperature shift of cells to 37°C for 1 h, cycloheximide was added to a final concentration of 0.5 mg/ml. Twenty OD_{600} of cells were taken at the indicated time points, and the solubility assay was performed as stated above.

Fluorescence Microscopy

Cells overexpressing ΔssCPY*-GFP or harboring an empty plasmid were grown at 30°C and shifted to 37°C for 60 min before viewing fluorescence in living cells. Cells were collected by centrifugation, washed once, and resuspended in fresh SC medium. The suspension, 2.2 μl, was dropped onto a 76 × 26-mm microscopy slide, covered with a coverslip, and subjected to immediate viewing. Fluorescence microscopy was performed with an Axioplan microscope equipped with a 100× oil-immersion objective (Carl Zeiss, Thornwood, NY) and GFP filter.

*Ubiquitination of ΔssCG**

Fifty OD_{600} of yeast cells overexpressing ΔssCPY*-GFP or harboring an empty plasmid were grown at 25°C and shifted to 37°C for 60 min before analysis. Cells were washed once with ice-cold washing buffer (20 mM sodium azide, 2 mM PMSF, 20 mM NEM) and incubated for 10 min on ice. Cells were resuspended in ice-cold IP buffer (50 mM Tris-HCl, pH 7.5, 190 mM NaCl, 1.25% Triton X-100, 6 mM EDTA, 2 mM PMSF, 20 mM NEM), and 500 μl of 0.5-mm glass beads were added. Cells were lysed by five pulses of 1-min duration in a Mini-bead beater, with cooling on ice between pulses. Lysates were cleared by centrifugation (130,000 × *g*, 30 min at 4°C), immunoprecipitated with anti-GFP, fractionated, and analyzed using anti-ubiquitin or anti-CPY.

RESULTS

Because degradation of the cytoplasmically localized substrate ΔssCPY*-GFP(ΔssCG*) by the proteasome did not require any of the cytoplasmic helper components of the ERAD pathway (Medicherla *et al.,* 2004), we searched for different chaperones that might be involved in its elimination. We reasoned that, as found for misfolded ER proteins, recognition, unfolding, escort, and delivery machineries must exist to deliver misfolded cytoplasmic proteins to the proteasome for degradation. Previous in vivo experiments in yeast had indicated that the Hsp40 cofactor of the Hsp70 chaperone Ssa1, Ydj1p, promotes the degradation of some short-lived and abnormal proteins (Lee *et al.,* 1996), thus suggesting the requirement for Hsp70. We therefore assessed whether the Hsp70 chaperone machinery of the Ssa class had a crucial role in the degradation of ΔssCPY*-GFP (ΔssCG*). We tested the requirement for the Hsp70 Ssa chaperones by comparing the properties of two strains, both of which lack three of the four Ssa proteins (Ssa2p, Ssa3p, Ssa4p). In *ssa1-45^{ts}* cells Ssa1 is present as a temperature-sensitive allele, whereas in isogenic *SSA1* cells the gene is present as a wild-type copy (Becker *et al.,* 1996; Taxis *et al.,* 2003). As can be seen in Figure 1A, degradation of ΔssCG* progresses with a half-life of 20–30 min in *SSA1* cells. Degradation of ΔssCG* is nearly completely abolished in *ssa1-45^{ts}* cells under restrictive conditions. A similar almost complete dependence on Ssa1 for ΔssCG* degradation is observed using antibodies directed against either CPY or GFP for immunoprecipitation (Figure 1A). As expected, degradation of endogenously expressed CPY*, which is retrotranslocated from the ER lumen to the cytoplasm (Hiller *et al.,* 1996), is not affected by the absence of Ssa1p. To test whether the position of the strongly folded GFP domain within ΔssCG* had any effect on the degradation pattern and whether its context influenced the Ssa1p-dependence of degradation, we constructed ΔssGFP-CPY* (ΔssGC*), carrying GFP N-terminally fused to signal sequence deleted CPY*. As can be seen in Figure 1B, ΔssGC* is degraded nearly as rapidly as ΔssCG*, and lack of an active Ssa apparatus blocks degradation of this substrate as well. Also, fusion of a variant of GFP$_{uv}$ that fluoresces more brightly than wild-type GFP at the C-terminus of ΔssCPY* does not affect the half life of ΔssCG* degradation (Figure 1C).

In vitro studies had shown that the 26S proteasome is unable to degrade the GFP moiety of certain fusion proteins, because of its strongly folded structure (Liu *et al.,* 2003). It was therefore possible that Ssa1p was only required for unfolding of the GFP moiety of ΔssCPY*-GFP (ΔssCG*) to allow its degradation by the proteasome in vivo. We constructed a CPY* protein without signal sequence, ΔssCPY*. It is an ER import-incompetent CPY* species that due to mutation (G255R) is misfolded. As previously published for ΔssCPY*-GFP (Medicherla *et al.,* 2004), the signal sequence deletion causes ΔssCPY* to be located in the cytosol (data not shown). This protein is rapidly degraded by the proteasome: elimination of ΔssCPY* is severely disrupted in the proteasome mutant *cim3-1* (Figure 2A). We have previously shown (Medicherla *et al.,* 2004) that the elimination of cytosolic ΔssCPY*-GFP does not require the trimeric Cdc48p-Ufd1p-Npl4p complex. Testing the requirement of this trimeric complex for degradation in *ufd1-1* mutant shows that Cdc48p-Ufd1p-Npl4p is also not involved in the proteasomal elimination process of ΔssCPY* (Figure 2B). As can be seen in Figure 2C, ΔssCPY* is rapidly degraded in *SSA1* but not in *ssa1-45^{ts}* mutant cells under restrictive conditions. These experiments indicate that the Ssa machinery is needed for the degradation of misfolded proteins of the cytoplasm.

For elucidation if proteasomal degradation of the strongly folded GFP domain is indeed independent of Ssa helper proteins we tested the degradation of GFP linked to the

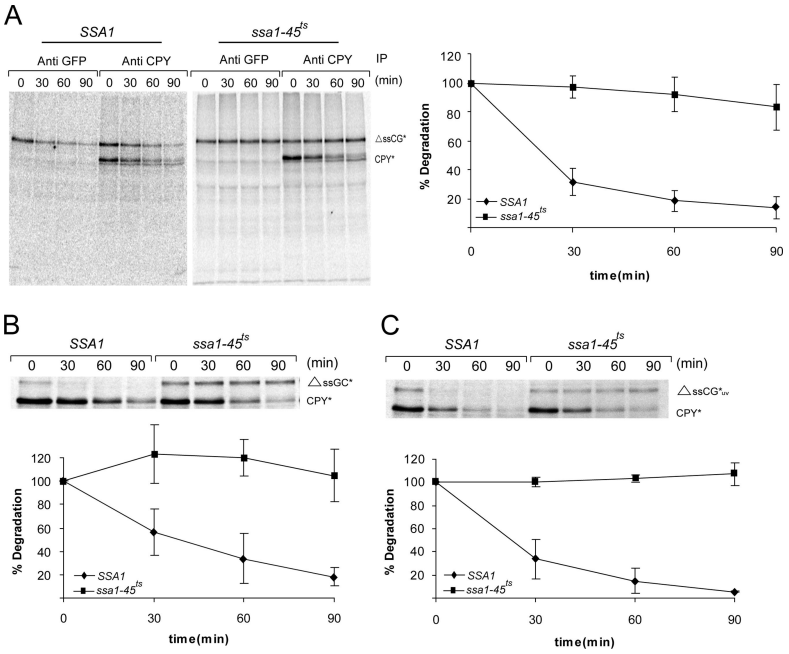

Figure 1. The Hsp70 chaperone machinery of Ssa1p is required for the degradation of cytoplasmically localized misfolded proteins. Pulse-chase analysis was done in *SSA1* and *ssa1-45^{ts}* cells. Cells expressing the substrates were lysed at the indicated times, and proteins were immunoprecipitated with anti CPY (A–C) or anti GFP (A), separated by SDS-PAGE, and analyzed using a PhosphoImager and ImagerQuaNT (Amersham Bioscience). Plotted data represent the mean values of three independent experiments. Substrates: A: ΔssCG*; B: ΔssGC*; C: ΔssCG*$_{uv}$. The ERQD substrate CPY* served as a control.

C-terminal 37 amino acids of mouse ornithine decarboxylase (cODC). This 37 amino acid C-terminal sequence has been shown to be a ubiquitin-independent transferable element, one with the capacity to direct diverse proteins to proteasomal degradation (Hoyt *et al.*, 2003; Zhang *et al.*, 2003, 2004). We tested the Ssa1 dependency of degradation of the fusion proteins GFP$_{uv}$-cODC and GFP-cODC. The GFP-cODC proteins are rapidly degraded, regardless of the Ssa status of the cell turnover is similar in *SSA1* and *ssa1-45^{ts}* mutant cells, whether under permissive or restrictive conditions of incubation (Figure 3, A and B). These experiments indicate that in the cellular environment, there must be means to unfold the GFP domain for degradation that do not depend on the Ssa machinery. Interestingly, degradation of a fusion protein consisting of ΔssCPY* and GFP$_{uv}$-cODC (ΔssCG*-cODC) is again dependent on Ssa1p, as is ΔssCG* (Figure 3C). It has been reported that the C-terminal 37 amino acids of ODC represent a critical signal for rapid ODC degradation and that a mutation of Cys$_{441}$ to Ala$_{441}$ in this sequence causes a significant stabilization of ODC or of proteins to which cODC is attached (Hoyt *et al.*, 2003). However, the Cys$_{441}$ to Ala$_{441}$ mutation in ΔssCG*-cODC-C441A did not lead to stabilization but directed this protein to a form of degradation that relied on the Ssa1 protein (Figure 3D). Obviously, Ssa1p-directed degradation of the ΔssCPY* moiety of the protein dominates over the Ssa1p-independent cODC-directed degradation in the fusion protein.

It has been shown that the import of secretory proteins into the ER can be faulty (Levine *et al.*, 2005). Because the intracellular mislocalization of proteins may lead to severe defects, we were also interested in the question of how wild-type secretory proteins that fail to advance into the ER are handled by the cell's cytosol. We chose mislocalized but otherwise wild-type carboxypeptidase yscY (CPY) for this analysis. Using a multicopy plasmid for expression Blachly-Dyson and Stevens (1987) found ~90% of signal sequence deleted CPY in the cytosol and ~10% in the ER. We constructed a signal sequence deleted CPY (ΔssCPY) and expressed it from a single-copy plasmid. We found ΔssCPY, like ΔssCPY*, to be solely located in the cytosol (data not shown). The fact that in contrast to Blachly-Dyson and Stevens (1987) we did not find a small portion of ΔssCPY in the ER may be due to the different expression conditions. We analyzed the fate of ΔssCPY. The mislocalized and presumably misfolded ΔssCPY is rapidly degraded; its turnover is performed by the proteasome, as evidenced by the stabilization conferred by the proteasomal *cim3-1* mutant (Figure 4A). As is true for the mutated CPY species, degradation of ΔssCPY is independent of the trimeric Cdc48p-Ufd1p-Npl4p complex required for elimination of misfolded ER proteins (Figure 4B). However, elimination of ΔssCPY does require an intact Ssa1 protein (Figure 4C). The fate and chaperone dependence of the cytoplasmically mislocalized wild-type CPY species is similar to that of its mutated counterpart in the cytoplasmic environment. In the reducing environment of the cytoplasm, folding of CPY is likely to be defective due to disturbed formation of disulphide bonds (Endrizzi *et al.*, 1994; Jamsa *et al.*, 1994).

Hsp70 chaperones function in a complex with cochaperones of the Hsp40 family, which modulate the substrate specificity of the Hsp70s (Cheetham and Caplan, 1998; Johnson and Craig, 2001; Rudiger *et al.*, 2001; Fan *et al.*, 2003). In a previous study, we had shown that for degradation of the ERQD substrate CTG* the help of the Hsp40 cochaperones Hdj1p, Cwc23p, and Jid1p is required (Taxis *et al.*, 2003). However, for the degradation of cytoplasmic ΔssCG*, none of these Hsp40 cochaperones are needed (data not shown). In contrast, the Hsp70 cochaperone Ydj1p has a strong influence on degradation of ΔssCG*, as well as ΔssCPY* and ΔssCPY: degradation of all three cytosolic model substrates is considerably slowed in *ydj1-151^{ts}* mutant cells under restrictive conditions (Figure 5, A–C). Ydj1p is not required for any of the ERQD substrates derived from CPY* (Taxis *et al.*, 2003). It can be concluded that the CQD (cytoplasmic quality control and degradation) substrates ΔssCG*, ΔssCPY*, ΔssCPY,

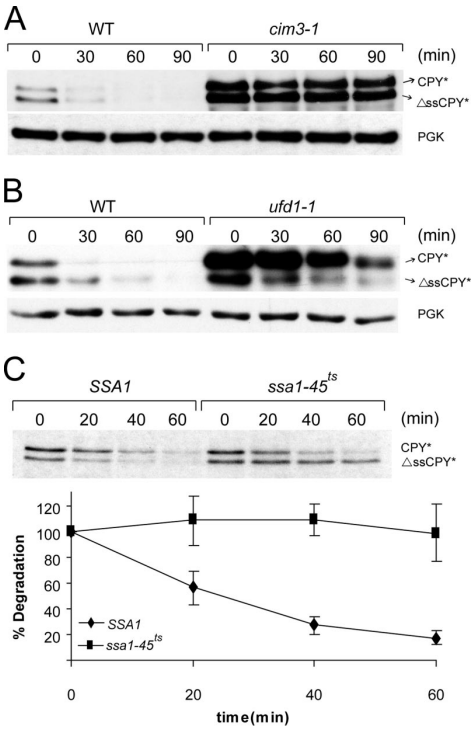

Figure 2. Degradation of misfolded and ER import incompetent CPY* is dependent on the proteasome and Ssa1p but not on the Cdc48-Ufd1-Npl4 complex. Cycloheximide decay experiments were performed in the proteasomal mutant *cim3-1* (A) and in *ufd1-1* cells (B) expressing ΔssCPY*. Cycloheximide was added (t = 0 min), and samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting. Immunoblots were analyzed with anti-CPY and anti-PGK as a loading control. Pulse-chase analysis in *SSA1* and *ssa1-45*^{ts} cells (C) was performed and analyzed as described in the legend to Figure 1. The ERQD substrate CPY* served as a control.

and the ERQD substrate CTG* have different cochaperone requirements.

Another class of Hsp70 chaperones, the Ssb members, are ribosome associated and involved in the folding of newly synthesized polypeptide chains (Pfund *et al.*, 1998, 2001). We tested a strain defective in this chaperone family (Δssb1Δssb2) and found that they are dispensable for degradation of ΔssCG* (Figure 6A). We also tested whether components of the Hsp90 chaperones were involved in degradation of ΔssCG*. The yeast Hsp90 chaperone family consists of two proteins, Hsc82p and Hsp82p. They are associated with the cochaperone Sti1p/HOP, which is also an activator of the Ssa1 proteins (Nathan *et al.*, 1997; Wegele *et al.*, 2003). The Hsp90 chaperones Hsc82p and Hsp82p are not required for degradation of ΔssCG* (Figure 6B). Consequently, the Hsp70/Hsp90 cochaperone Sti1p/HOP has no effect on the degradation of ΔssCG* (Figure 6C). It has been suggested that another major cytoplasmic chaperone, Hsp104, works together with the Hsp70s of the Ssa family and binds in an ATP-dependent manner to the Ssa1p-Ydj1p complex to unfold proteins (Parsell and Lindquist, 1993; Parsell *et al.*, 1994; Glover and Lindquist, 1998; Lum *et al.*, 2004). ER-associated degradation of CTG* requires both Ssa and Hsp104 chaperones (Taxis *et al.*, 2003). However, Hsp104p is not required for elimination of ΔssCG* (Figure 6E). We were further interested in the involvement of the Hsp110 chaperone Sse1p in elimination of ΔssCG*. The protein is a component of the Hsp90 chaperone complex and mediates degradation of misfolded VHL (McClellan *et al.*, 2005a). No function of Sse1p in ΔssCG* degradation can be observed (Figure 6D). Two small heat-shock proteins, Hsp26 and Hsp42 are ubiquitous molecular chaperones that protect yeast cells from a variety of cellular stresses. In vitro they have been found to bind to unfolded proteins to form large cocomplexes and by this prevent their aggregation (Haslbeck *et al.*, 1999, 2004; Cashikar *et al.*, 2005). We tested the involvement of Hsp26 and Hsp42 in degradation of ΔssCG*. As can be seen Figure 6F, degradation of ΔssCG* was not affected by the absence of Hsp26 and Hsp42. Recently, BAG domain proteins were shown to interact with Hsp70 chaperones as a nucleotide exchange factor in the cytosol of higher eukaryotic cells. In mammalian cells, together with the E3 ligase CHIP, they are known to be partners in a degradative Hsp70 complex (Esser *et al.*, 2004). There exists a BAG-1 homologue in yeast, Snl1p, which functionally interacts with Hsp70 chaperones (Sondermann *et al.*, 2001, 2002). However, no alteration of degradation of ΔssCG* is seen in *SNL1* deletion mutant cells (Figure 6G).

We tested whether the Ssa machinery has any function in keeping misfolded ΔssCG* in the soluble state in the cyto-

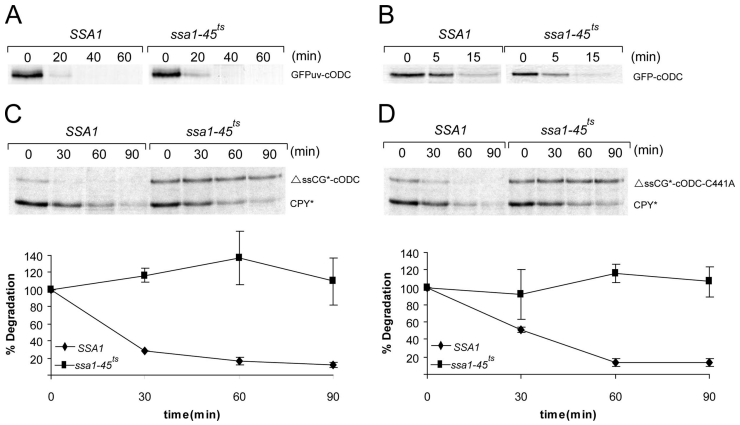

Figure 3. Ubiquitin-independent degradation of GFP-cODC does not require Ssa1p activity, but its fusion to ΔssCPY* makes the process Ssa1p dependent. Pulse-chase analysis was done in *SSA1* and *ssa1-45*^{ts} cells expressing GFP_{uv}-cODC (A), GFP-cODC (B), ΔssCG*-cODC (C), and ΔssCG*-cODC-C441A (D).

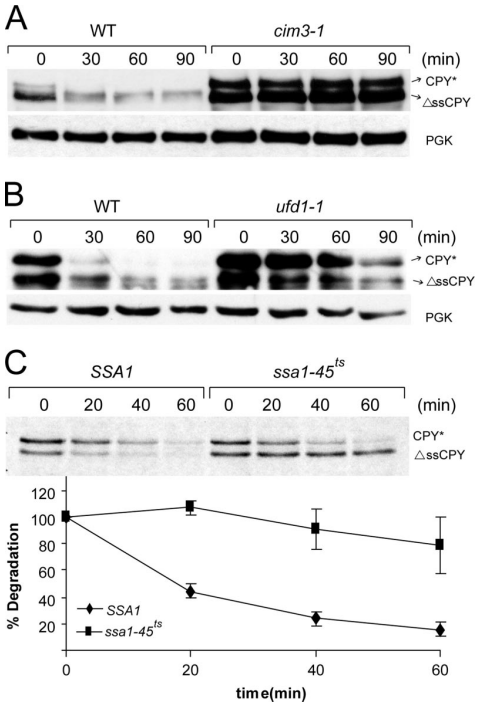

Figure 4. The fate of the cytoplasmically mislocalized wild-type CPY is similar to its mutated counterpart. Cycloheximide decay experiments (A and B) and pulse-chase analysis (C) were performed as described in the legend to Figure 2.

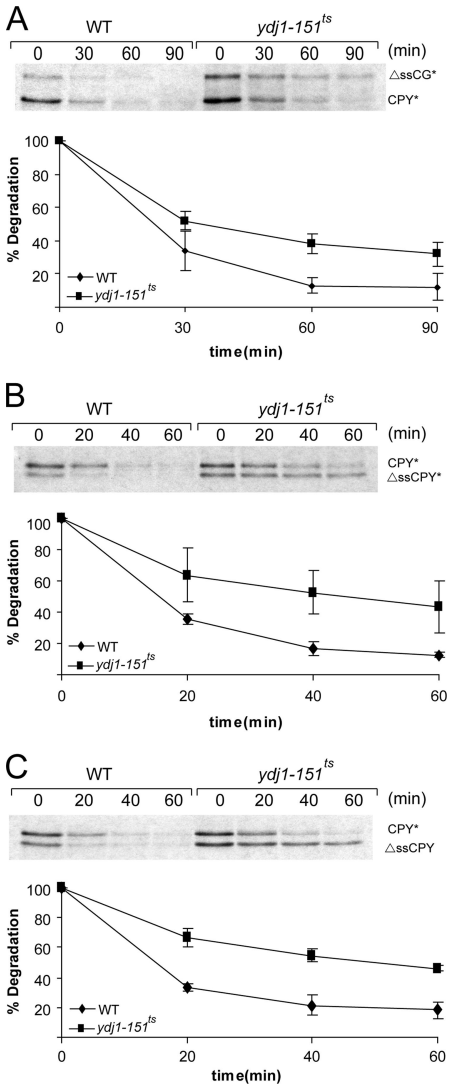

Figure 5. The Hsp70 cochaperone Ydj1p promotes the degradation of cytoplasmically localized misfolded proteins. Pulse-chase analysis was performed in wild-type (WT) and *ydj1-151*^{ts} cells expressing ΔssCG* (A), ΔssCPY* (B), and ΔssCPY (C).

plasm. When testing wild-type cells harboring all four Ssa chaperones (Ssa1p, Ssa2p, Ssa3p, Ssa4p), most of the ΔssCG* protein is found in the soluble state, and this does not change when cells are shifted from 30 to 37°C (Figure 7B). As can be seen in Figure 7A, when *SSA1* cells containing solely Ssa1p are transferred from 30 to 37°C, the ΔssCG* material in the pellet increases, indicating aggregation of the misfolded protein material with increased temperature. The amount of precipitated ΔssCG* in *SSA1* cells varied somewhat in different experiments (data not shown). Apparently, in the absence of Ssa2, Ssa3, and Ssa4 the single Ssa1 species is functioning at or beyond its limits in keeping misfolded protein soluble under heat stress. However, analyzing the amount of soluble and precipitated cellular protein material in vitro may not be fully informative of the solubility properties of ΔssCG*, because in vitro conditions (buffer, salt, protein concentration, etc.) are very different from the cellular environment. We therefore analyzed the solubility of ΔssCG* in the different strains by fluorescence microscopy, thus visualizing the distribution pattern of the GFP moiety of the protein. As can be seen in Figure 6C, no precipitated ΔssCG* material can be seen at 37°C in wild-type cells containing all four Ssa species, regardless of whether ΔssCG* was expressed from a single-copy (data not shown) or multicopy plasmid (Figure 7C). In contrast, at 37°C some punctuated fluorescent dots, indicating precipitated material, are visible in cells containing only Ssa1p, substantiating the in vitro finding. Nevertheless, the misfolded protein is rapidly degraded in *SSA1* cells at 37°C (Figures 1, A–C). A dramatic increase in such precipitated fluorescent material appears under the restrictive conditions of 37°C in the *ssa1-45* and *ydj1-151* mutant cells. Under the restrictive conditions of 37°C in *ssa1-45*^{ts} mutant cells, we see most of the misfolded ΔssCG* material in the pellet (Figure 7A), and degradation is completely blocked (Figures 1, A and C). The behavior of ΔssCG* in the *ydj1-151*^{ts} mutant mirrors the behavior of this substrate in the *ssa1-45*^{ts} mutant. Under permissive conditions a significant fraction of ΔssCG* is soluble, whereas at restrictive conditions a major part of the protein is found in the pellet fraction precipitated in cells (Figures 7, A and B). We have shown that ΔssCG* is nearly completely degraded in *SSA1* cells at 37°C (Figure 1A) despite the fact that under these conditions ΔssCG* partly precipitates (Figure 7A). This indicates that Ssa1p may have the capacity to resolubilize the precipitated material for degradation under the conditions tested. We tested resolubilization of ΔssCG* in *SSA1* and *ssa1-45*^{ts} cells in a cycloheximide decay experiment at 37°C (Figure 7D). As can be

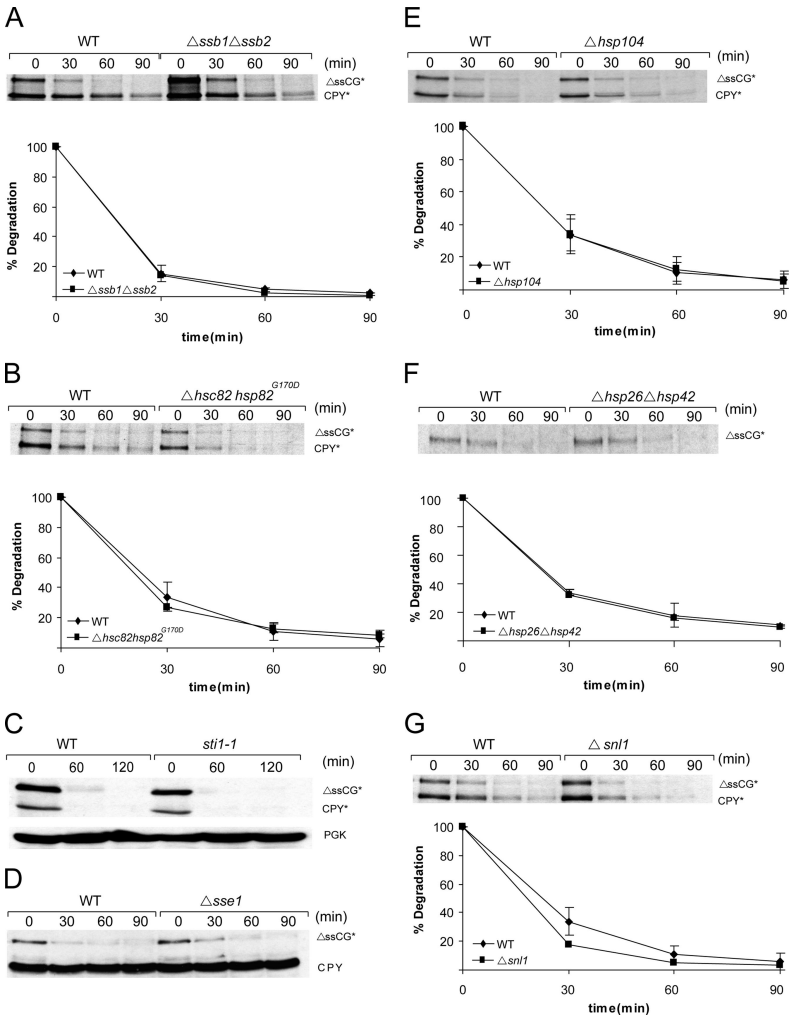

Figure 6. The Hsp70 Ssb class; the Hsp90 complex, Hsp104, Hsp110; small heat shock proteins Hsp26, Hsp42; and the yeast Bag1 homologue, Snl1p, are not involved in the degradation of ΔssCG*. Pulse-chase analysis was done in Δ*ssb1*Δ*ssb2* (A), Δ*hsc82hsp82*^{G170D} (B), Δ*hsp104* (E), Δ*hsp26*Δ*hsp42* (F), and Δ*snl1* (G) cells expressing ΔssCG*, and cycloheximide decay experiments were performed in *sti1-1* (C) and Δ*sse1* (D) cells expressing ΔssCG* as described in the legend to Figure 2. PGK and CPY were served as a loading control.

seen, within 30 min of cycloheximide treatment the amount of ΔssCG* material increases in *SSA1* cells but thereafter nearly completely disappears in the total fraction and in the pellet within 90 min. In *ssa1-45*^{ts} cells the precipitated material persists, whereas GFP-cODC carrying the 37 amino acid targeting sequence of ODC for the proteasome is rapidly degraded by the enzyme (Hoyt *et al.*, 2003 and Figures 3A and 7E). GFP carrying the mutated version of the proteasomal-targeting sequence (GFP-cODC-C441A) is not eliminated by the proteasome (Hoyt *et al.*, 2003). Indeed, the GFP-cODC-C441A protein accumulates in *SSA1* cells (Figure 7E). However, in contrast to ΔssCG* (Figure 7C) the accumulated material does not show any sign of aggregation.

With few exceptions, like ODC and the cyclin-dependent kinase inhibitor p21 (Sheaff *et al.*, 2000; Verma and Deshaies, 2000; Liu *et al.*, 2003; Hoyt and Coffino, 2004), ubiquitination of substrates is required before their elimination via the proteasome (Heinemeyer *et al.*, 1991; Pickart, 2001; Wolf and Hilt, 2004). Several groups have shown that in mammalian cells a CHIP-associated Hsp70 chaperone complex triggers ubiquitination of its protein clients and mediates proteasomal degradation (Connell *et al.*, 2001; Demand *et al.*, 2001; Jiang *et al.*, 2001; Murata *et al.*, 2001). We searched for ubiquitinated ΔssCG* material in mutant and wild-type cells, under the experimental design of Figure 7, and analyzed the soluble fraction of the respective cell extracts. The buffer used for solubilization (Figure 7, sorbitol, or Figure 8, Tris/HCl) did not alter the experimental result (data not shown). Although we find clearly similar amounts of ubiquitinated ΔssCG* in wild-type and mutant cells at 25°C (Figure 8A), conditions that do not induce the mutant character, we see a considerably changed ubiquitin pattern of ΔssCG* material at 37°C, which leads to the expression of the mutant phenotype of *ssa1-45*^{ts} and *ydj1-151*^{ts} cells. Interestingly, considerably more ubiquitinated ΔssCG* can be found in *ssa1-45*^{ts} and *ydj1-151*^{ts} under restrictive conditions compared with WT (*SSA1, SSA2, SSA3, SSA4*) and *SSA1* cells (Figure 8B), despite the fact that the mutant cells show much less soluble ΔssCG* material (Figure 7). This might indicate that ΔssCG* in the *SSA1* and wild-type cells is completely degraded, whereas degradation of the ubiquitinated material is retarded in the mutant cells.

We were also interested in the components of the ubiquitination machinery in the degradation pathway. At present there are 13 ubiquitin-conjugating enzymes known to exist in yeast. As can be seen Figure 9A, deletion of the

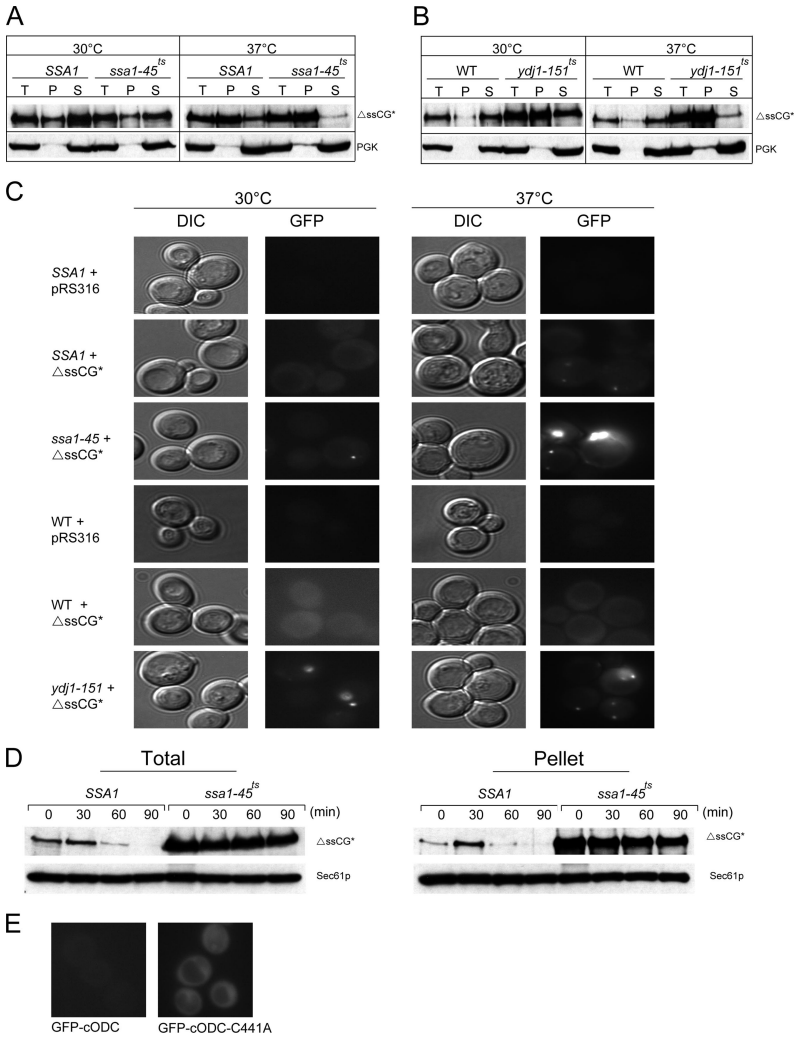

Figure 7. Ssa1p and its cochaperone Ydj1p are required for rescue of aggregated ΔssCG*. Cells expressing ΔssCG* were grown at 30°C and shifted to 37°C for 60 min before the solubility assay. The solubility of ΔssCG* was assessed in *SSA1, ssa1-45^{ts}* (A), wild-type *W303-1C (SSA1, SSA2 ,SSA3, SSA4*), and *ydj1-151^{ts}* strains (B). The same amount of total (T), supernatant (S), and pellet (P) fraction was analyzed via SDS-PAGE and immunoblot. Immunoblots were analyzed with CPY antibody and PGK antibody as a control. The fluorescence of ΔssCG* was analyzed in living cells (C) as described in *Material and Methods.* The cells harboring overexpressed ΔssCG* or an empty plasmid were grown at 30°C and shifted to 37°C for 60 min before analysis. All the cells were visualized by fluorescence microscopy using equal exposure times and conditions. Resolubilization of aggregated ΔssCG* was assessed in *SSA1* and *ssa1-45^{ts}* cells (D). After temperature shift of cells to 37°C for 1 h, cycloheximide was added to a final concentration of 0.5 mg/ml to block further protein synthesis. Twenty OD_{600} of cells were taken at the indicated time points and treated as indicated for the above solubility assay. Immunoblot of Sec61p served as control. Three independent experiments gave similar results. The fluorescence of GFP-cODC and GFP-cODC-C414A were analyzed in *SSA1* cells at 37°C as stated above (E).

ubiquitin-conjugating enzymes Ubc4p and Ubc5p leads to a considerable stabilization of ΔssCG*, indicating involvement of Ubc4p and Ubc5p in the degradation of this misfolded cytoplasmic protein. Because degradation is not completely halted in the *ubc4/ubc5* double deletion mutant, an overlapping E2 activity must be present for ubiquitination of ΔssCG*. In mammalian cells, CHIP has been discovered as an important E3 ligase involved in degradation of proteins in the cytoplasm (Connell *et al.*, 2001; Demand *et al.*, 2001; King *et al.*, 2001; Murata *et al.*, 2001; Cyr *et al.*, 2002; Esser *et al.*, 2004). In yeast cells no CHIP orthologue has been found yet. However, there are a multitude of E3 ligases present in

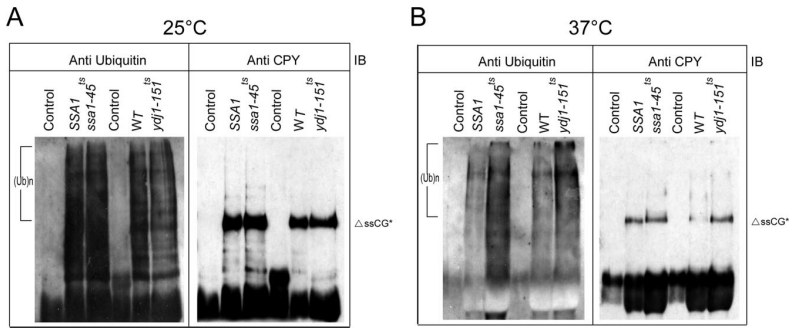

Figure 8. The state of ubiquitinated misfolded proteins in wild-type, *SSA1*, *ssa1-45^{ts}*, and *ydj1-151^{ts}* cells at the different temperature of 25 and 37°C. The cells harboring overexpressed ΔssCG* or an empty plasmid (control) were grown at 25°C (A) and shifted to 37°C (B) for 60 min before analysis. Cell extracts were immunoprecipitated with anti-GFP antibody, separated by SDS-PAGE, followed by immunoblotting, and analyzed with anti-ubiquitin or anti-CPY antibodies.

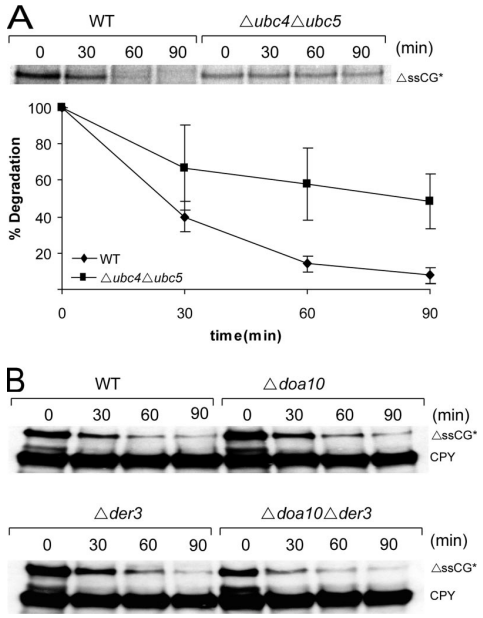

Figure 9. Degradation of ΔssCG* requires the E2 proteins Ubc4p and Ubc5p but not the E3 ligases Doa10p and Der3p. Pulse-chase analysis was done in Δ*ubc4*Δ*ubc5* mutant cells (A) and cycloheximide decay experiments were performed in Δ*doa10*Δ*der3* cells (B) as described in the legend to Figure 2. CPY served as a loading control.

yeast cells. Besides its involvement in degradation of several ERQD substrates, the ER membrane–located E3 ligase Doa10p is required for degradation of Deg1-GFP, a cytoplasmically and nuclear localized substrate (Swanson *et al.,* 2001; Huyer *et al.,* 2004; Ravid *et al.,* 2006). However, degradation of ΔssCG* is independent of the function of the E3 ligase Doa10p (Figure 9B). Degradation of ΔssCG* did also not require the second ER membrane–located E3 ligase Der3/Hrd1p (Figure 9B).

DISCUSSION

Misfolded proteins of the ER are eliminated by proteasomal degradation in the cytosol. After detection, retrotranslocation, and ubiquitination at the cytosolic surface of the ER, they are channelled to the proteasome via the trimeric AAA-ATPase complex Cdc48p-Ufd1p-Npl4p and the UBA-UBL domain proteins Dsk2p and Rad23p (Brodsky and McCracken, 1999; Kostova and Wolf, 2003; Hirsch *et al.,* 2004; Medicherla *et al.,* 2004). Understanding of this mechanism, to a large extent, had been elaborated by using what turned out during time to be a model substrate for studying the ER-associated degradation process, mutated vacuolar carboxypeptidase yscY (CPY*) (Hiller *et al.,* 1996; Schafer and Wolf, 2006). We had shown that degradation of a cytoplasmically localized derivative of CPY* devoid of the signal sequence required for ER import (ΔssCPY*-GFP) did not depend on the Cdc48p-Ufd1p-Npl4p, Dsk2p and Rad23p pathway for proteasomal degradation (Medicherla *et al.,* 2004). It became therefore our aim to understand the mechanism of degradation of misfolded proteins in the cytoplasm.

We therefore sought to determine the components that are required for elimination of ΔssCG* in the cytoplasm. As can be seen in Figure 1, degradation of ΔssCG* requires the Hsp70 chaperone Ssa1p. Recent in vitro experiments had shown that the 26S proteasome is unable to unfold the strongly folded GFP moiety of several fusion proteins tested (Liu *et al.,* 2003). We constructed a signal-sequence–deleted, cytoplasmically localized ΔssCPY* molecule devoid of the GFP domain to inquire if unfolding of that domain is responsible for the Ssa1p requirement. Surprisingly, ΔssCPY* degradation also depended on Ssa1p function (Figure 2C), clearly indicating that this Hsp70 species has a more general function in the degradation of cytoplasmically located misfolded proteins. The present finding that degradation of ΔssCPY* and ΔssCPY also require Ssa1p points to the fact that the role of this chaperone is not limited to unfolding, but serves additional purposes. Degradation of GFP fused to the C-terminal 37 amino acids of ornithine decarboxylase (GFP-cODC) without the aid of Ssa1p implies that the proteasome has other means to unfold GFP (Figure 3, A and B). A C441A mutation in the C-terminal 37-amino acid tail of ODC abolishes degradation of the fusion protein GFP-cODC-C441A (Hoyt *et al.,* 2003). The 37-amino acid stretch of cODC, whether wild type or mutated is not recognized as a misfolded protein domain by the cell (Hoyt *et al.,* 2003), and therefore the fate of GFP-cODC is independent of Ssa1p. Interestingly, fusion of mutated ΔssCPY* to GFP-cODC (ΔssCG*-cODC) reimposes a dependence of the Ssa1 chaperone for degradation (Figure 3C). Also, mutation of cODC does not lead to stabilization of ΔssCG*-cODC-C441A (Figure 3D). Thus Ssa1p seems to function in the recognition of the misfolded ΔssCPY* domain of the fusion protein; its misfolded status dictates the route of elimination.

It has recently been shown that the in vivo efficiency of signal sequence-mediated protein segregation into the secretory pathway varies tremendously, ranging from >95% to <60% in mammalian cells (Levine *et al.,* 2005). Remnant secretory proteins thus find themselves entrapped in the cytoplasm. Because mislocalized proteins may be harmful to the cell, the fate of these proteins is of high interest. The usefulness of mutated CPY variants in defining degradation pathways impelled a test of the fate of wild-type CPY remaining in the cytoplasm. As are ΔssCG* and ΔssCPY*, ER import incompetent wild-type CPY is rapidly degraded by the proteasome (Figure 4A), indicating an altered structure that is recognized by the cytoplasmic proteolysis system. We reason that proper folding of the enzyme is most likely defective because of disturbed formation of disulphide bonds (Endrizzi *et al.,* 1994; Jamsa *et al.,* 1994) in the reducing environment of the cytoplasm, compared with the oxidative environment of the ER in which CPY normally assumes its native and active form. As shown for ΔssCG* (Medicherla *et al.,* 2004), glycosylation of the enzyme is also likely to be absent in the cytoplasm. Thus the cell is easily able to eliminate mislocalized secretory proteins, which cannot fold efficiently in the cytoplasmic environment, in this way avoiding their unwanted presence in the cytoplasm.

All three cytoplasmically localized CPY derivatives, whether mutated (ΔssCG*, ΔssCPY*) or wild-type (ΔssCPY), required the Hsp70 chaperone Ssa1p for elimination. While our work was in progress McClellan *et al.* (2005) reported the requirement of Ssa1p for degradation of misfolded von Hippel Lindau (VHL) tumor suppressor protein in the yeast cytoplasm. We therefore conclude that the need for Ssa1p is likely to be a general feature of degradation of misfolded proteins in the cytoplasm. A crucial role for Hsp70 function in the degradation of different substrates has also been shown in mammalian cells (for review see Esser *et al.,* 2004). The functional requirement of Ssa1p for substrate recogni-

tion does not seem to be limited to ubiquitin-dependent substrates only. It has been reported that overexpression of the molecular chaperones Hsp70 and Hsp40 facilitate degradation of α-synuclein, which is natively disordered and degraded by the proteasome in the absence of ubiquitin modification (Tofaris *et al.,* 2001; Muchowski and Wacker, 2005).

In contrast to degradation of the ERQD substrate CTG*, which, along with Ssa1p, is dependent on the Hsp40 cochaperones Hdj1p, Cwc23p, and Jid1p but not Ydj1p (Taxis *et al.,* 2003), elimination of the CQD substrate ΔssCG* instead depends on the cochaperone Ydj1p and is independent of the other three cochaperones. Degradation of ΔssCPY* and ΔssCPY, too, is dependent on Ydj1p (Figure 5). In their work on the degradation of misfolded VHL tumor suppressor protein in the yeast cytoplasm, McClellan *et al.* (2005) reported the Hsp70 cochaperone Sti1/HOP to be required for degradation of VHL. They also reported the necessity of the Hsp90 chaperone system for elimination of misfolded VHL. In addition, the participation of the Hsp110 chaperone Sse1p was found for degradation of misfolded VHL. Ydj1p was not required for elimination of misfolded VHL (McClellan *et al.,* 2005a). Surprisingly, except for Ssa1p, the requirement of factors required for elimination of the three cytosolic substrates tested in our work differs completely from the factors reported by McClellan *et al.* (2005) for degradation of VHL. Neither the Hsp90 family of chaperones nor the Hsp110 chaperone Sse1p is required for degradation of ΔssCG* (Figure 6, B and D). Although the cochaperone Sti1p/HOP is necessary for degradation of misfolded VHL (McClellan *et al.,* 2005a), this factor is not involved in ΔssCG* degradation (Figure 6C). In contrast, the Hsp40 cochaperone Ydj1p is an important factor in ΔssCG* as well as ΔssCPY* and ΔssCPY elimination (Figure 5). Although McClellan *et al.* (2005) show only a minor portion of insoluble misfolded VHL in cells devoid of the Hsp70 cochaperone Sti1/HOP, the situation concerning ΔssCG* is again different.

In vitro analysis shows that in wild-type cells harboring all four Hsp70 species of the Ssa type (Figure 7B, WT) the majority of ΔssCG* is found in the soluble fraction of cells grown either at 30 or 37°C. As expected, the fluorescence of ΔssCG* is distributed throughout the cytoplasm of these cells in vivo (Figure 7C). In contrast, in vitro analysis at 30°C of *SSA1* or *ssa1-45^{ts}* cells harboring only one functional Ssa-species shows that the insoluble portion of ΔssCG* increases, indicating that one Ssa-species is at its limits in keeping the misfolded protein soluble. At 37°C the insolubility of ΔssCG* increases in *SSA1* cells, and nearly all ΔssCG* material is insoluble in *ssa1-45* cells, which lack Ssa1p activity at this temperature (Figure 7A). Similar results have been observed for ΔssCPY* and ΔssCPY (data not shown). This behavior is reflected in vivo when analyzing the fluorescence of ΔssCG* (Figure 7C). The fact that less aggregated ΔssCG* material is seen in the fluorescence images compared with the solubility assay in vitro may be due to the presence of oligomeric ΔssCG* species in vivo, which under in vitro conditions form insoluble precipitates. It is interesting to note that degradation of ΔssCG* is rapid and nearly complete in *SSA1* cells at 37°C, indicating that the precipitated material is susceptible to degradation (Figure 1). It has been shown that the Hsp70 chaperone machinery is able to remodel and disaggregate protein aggregates in vitro (Zietkiewicz *et al.,* 2006). Here we show that Ssa1 is able to resolubilize precipitated ΔssCG* material in vivo (Figure 7D). We also tested the involvement of Hsp104 and the small heat-shock proteins Hsp26 and Hsp42 in the degradation process of ΔssCG*. Surprisingly none of them exhibited any effect (Figure 6, E and F). Cells defective in the activity of the Hsp40 cochaperone Ydj1p also show increasing amounts of ΔssCG* aggregates (Figure 7, B and C). Degradation of ΔssCG* is not completely blocked in *ydj1-151^{ts}* cells at the nonpermissive temperature of 37°C (Figure 5). The most likely explanation for this behavior is that Ssa1p is active without Ydj1p and that this cochaperone only augments the capacity of Hsp70 chaperone to disaggregate oligomeric and insoluble precipitates. The absence of Ydj1p dependency of misfolded VHL degradation may be due to the fact that this protein remains soluble in the cytoplasm and does not form aggregates (McClellan *et al.,* 2005a). The Hsp40 cochaperones have a conserved J-domain, which is proposed to interact with Hsp70, and have been shown to exhibit a protective function in experimental model protein aggregation (Schaffar *et al.,* 2004; Muchowski and Wacker, 2005; Novoselova *et al.,* 2005). This implies that Ydj1p cannot be only some "specificity factor" for protein recognition, but rather represents an Ssa1p-linked activity enhancer. After substrate solubilization Ssa1p is obviously able to perform the additional tasks of keeping the substrate soluble and delivering it to the proteasome. The discovery that the neuronal Hsc70 cochaperone Hsj1p can act as a neuronal shuttling factor for sorting of chaperone clients to the proteasome supports this idea (Westhoff *et al.,* 2005).

When comparing the protein quality control process in the two major folding compartments of the cells, the cytoplasm and the ER, it is obvious that similar mechanisms operate. As found for the Hsp70 class of Ssa-chaperones in the cytoplasm (Hartl and Hayer-Hartl, 2002; Deuerling and Bukau, 2004), the major Hsp70 protein of the ER, BiP in mammalian cells (Sitia and Braakman, 2003) or Kar2p in yeast, is required for protein folding (Simons *et al.,* 1995). In case folding is not successful, Kar2p is necessary to prevent proteins from aggregation and keep soluble misfolded proteins of the ER in the soluble state (Nishikawa *et al.,* 2001), to finally allow their retrotranslocation into the cytoplasm and degradation by the proteasome (Plemper *et al.,* 1997; Brodsky *et al.,* 1999). These functions of Kar2p are also dependent on cochaperones (Nishikawa *et al.,* 2001). As shown here and elsewhere (McClellan *et al.,* 2005a), Ssa1p together with its cochaperones seems to have parallel functions in the cytoplasm.

Central agents of CQD seem to be the Hsp70 chaperone Ssa1p (Figures 1 and 2 and McClellan *et al.,* 2005), the ubiquitin-conjugating enzymes Ubc4p and Ubc5p (Figure 9A and McClellan *et al.,* 2005), and the proteasome (Figure 2 and McClellan *et al.,* 2005). The ubiquitin protein ligase (E3) that functions in this system remains to be identified. We analyzed a subset of known ubiquitin protein ligases (E3's) Doa10p, Der3p (Figure 9B), Rsp5p, Hul5p, Ufd4p, and the SCF complex (data not shown). None of these ligases is involved in the degradation of the model substrate ΔssCG* in the cytoplasm. This suggests the involvement of a novel E3 in degradation process of the misfolded proteins in the cytoplasm.

Our experiments show that the Hsp90 family of chaperones is not invariably needed for degradation of misfolded proteins (Figure 6B). In the case of degradation of misfolded VHL, Hsp90 action may be uniquely required to generate a specific conformation of this substrate, one that can subsequently be recognized by an ubiquitin ligase involved in quality control. The specific cochaperone required for Ssa1p-dependent ubiquitin–proteasome degradation of misfolded cytoplasmic proteins may depend on the function Ssa1p has to fulfill in this process. Because only the soluble form of ΔssCG* can be degraded by the proteasome, we consider the

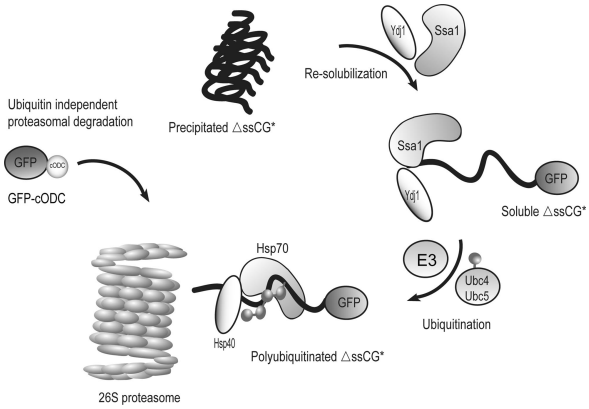

Figure 10. Model of protein quality control in the cytoplasm. See text for details.

polyubiquitinated ΔssCG* material in wild-type and *SSA1* cells at 37°C to be the steady state level of resolubilized and not yet degraded ΔssCG* (Figure 8B). Compared with wild-type and Ssa1p-proficient cells, a considerably greater amount of ubiquitinated soluble ΔssCG* material can be found in *ssa1-45^{ts}* and *ydj1-151^{ts}* cells under these restrictive conditions (Figure 8B), despite the fact that much less soluble ΔssCG* material is present in the mutant cells (Figures 7, A and B). From this one may conclude that ΔssCG* material ubiquitinated before the temperature shift of cells to 37°C may remain undegraded in the ubiquitinated state in the *ssa1-45^{ts}* cells or less well degraded in the *ydj1-151^{ts}* mutant after the temperature shift, because of inactivation of the chaperone proteins. The fact that polyubiquitinated protein material accumulates in *ssa1-45^{ts}* mutant cells at the restrictive temperature of 37°C, despite the presence of an active proteasome (Figure 8B) indicates that Ssa1p may have a function beyond solubilization of precipitated protein material or keeping misfolded proteins soluble. We conclude that Ssa1p is likely to have several functions. Ssa1p can unfold proteins (Taxis *et al.*, 2003), recognize misfolded protein domains (Figure 3), solubilize (and keep soluble) aggregated misfolded proteins (Figures 1A and 7D), and escort and deliver misfolded cytoplasmic proteins to the proteasome for degradation (Figure 10). The finding of an interaction of Ssa1p with the 26S proteasome (Verma *et al.*, 2000; Coffino, P., and Maxwell, R. A., unpublished data) substantiates the validity of this last conclusion.

ACKNOWLEDGMENTS

We thank S. Lindquist, E. A. Craig, J. L. Brodsky, and J. Buchner for yeast strains, plasmids, and reagents. We are grateful to members of Wolf group and especially Dr. A. Schaefer for helpful discussions. This work was supported by grants from the German-Israeli Foundation for Scientific Research and Development (GIF), Jerusalem, the Deutsche Akademische Austauschdienst (DAAD), Bonn, the Fonds der Chemischen Industrie, Frankfurt, the EU Network of Excellence RUBICON, and the Deutsche Forschungsgemeinschaft (DFG), Bonn, to D. H. W. and by grants from the National Institutes of Health No. GM45335 and GM074760 to P. C.

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4.2. Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1

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FEBS Letters 582 (2008) 4143-4146

FEBS Letters 582 (2008) 4143–4146

Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1

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Received 6 October 2008; revised 3 November 2008; accepted 10 November 2008

Available online 27 November 2008

Edited by Noboru Mizushima

Abstract Protein quality control and subsequent elimination of terminally misfolded proteins occurs via the ubiquitin–proteasome system. Tagging of misfolded proteins with ubiquitin for degradation depends on a cascade of reactions involving an ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3). While ubiquitin ligases responsible for targeting misfolded secretory proteins to proteasomal degradation (ERAD) have been uncovered, no such E3 enzymes have been found for elimination of misfolded cytoplasmic proteins in yeast. Here we report on the discovery of Ubr1, the E3 ligase of the N-end rule pathway, to be responsible for targeting misfolded cytosoplasmic protein to proteasomal degradation.

Keywords: Protein quality control; Misfolded protein; Ubiquitin ligase; Ubr1; Proteasome; Protein degradation

1. Introduction

Proper protein folding is essential for cellular well-being and survival. Very sophisticated mechanisms including the action of chaperones help the proteins fold into their native conformation. Stresses like heat, heavy metal ions, oxidation or simply mutations might prevent folding of a protein into its native state. Sensing of the folding process and recognition of misfolded proteins is summarized as a process called protein quality control. Conformational aberrant proteins which in many cases are toxic for the cell have to be eliminated. The importance of protein quality control and degradation of terminally misfolded proteins for cellular well-being is underscored by the many examples of disease, as are for instance Parkinson-, Alzheimer- or Creutzfeldt–Jakob-disease. Protein quality control and degradation has been extensively studied for secretory proteins (ERQD). A multitude of components required for folding, folding control, recognition and delivery of misfolded secretory proteins to the proteolytic system for elimination has been uncovered [1–7]. Recently, advances in our understanding of the quality control of misfolded cytoplasmic proteins and their degradation (CQD) has been published [8,9]. It is a common feature of the protein quality control pathways of the ER and the cytosol, that Hsp70-type chaperones bind and sense misfolded proteins and finally deliver them for degradation by the ubiquitin–proteasome pathway of the cytosol [7,10]. This major proteolytic pathway of all eukaryotic cells requires tagging of the misfolded protein by the 76 amino acid polypeptide ubiquitin, which is brought about by a cascade of reactions catalyzed by an ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3). The tagging reaction ends up in the formation of a polyubiquitin chain at intrinsic lysine residues or the amino terminus of the protein to be degraded. This process finally targets the protein for degradation via the proteasome, a proteolytic nanomachine [11,12]. The concerted action of ubiquitin conjugating enzymes and ubiquitin ligases determines the specificity of the polyubiquitination process of a selected protein. While degradation of misfolded secretory proteins mainly depends on the ubiquitin conjugating enzymes Ubc6 and Ubc7 [3,13,14] misfolded cytoplasmic proteins are targeted by the ubiquitin conjugating enzymes Ubc4 and Ubc5 for degradation [8,9]. The involvement of the ubiquitin ligase in the ubiquitin targeting reaction of misfolded cytoplasmic proteins in yeast cells remained elusive: Even though the ubiquitin ligases Der3/Hrd1 and Doa10 required for polyubiquitination of misfolded secretory proteins carry the specificity for recognition of unfolded protein patches, they do not function in polyubiquitination of the misfolded cytoplasmic proteins tested [9]. In mammalian cells the E3 enzymes CHIP and Parkin have been reported to be responsible for ubiquitination of misfolded or aggregation-prone protein substrates of the cytoplasm (reviewed in [15]). However, no orthologous E3 enzymes have been found in yeast. Here we report on the discovery of the RING-finger ubiquitin ligase Ubr1 as an essential E3-enzyme for delivering misfolded protein of the yeast cytoplasm to proteasomal degradation.

2. Materials and methods

2.1. Yeast strains and plasmids

Media preparation, genetic and molecular biology techniques were carried out using standard methods [16,17]. All experiments were done in the genetic background of *Saccharomyces cerevisiae* strain W303 *prc1-1* (*MATα ade2-1ocre can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 prc1-1*) [18]. The *UBR1* gene was deleted via homologous recombination with a KanMX deletion module [19].

Plasmid pFE15 encoding the cytoplasmic fusion protein ΔssCL*myc (pRS316-P_{PRC1}-*prc1-1Δss*, lacking base pairs 2–57 encoding the signal sequence (ss) and the last 39 base pairs of *PRC1*), *LEU2*-myc_{13} (bps 1813–3453 of CTL*myc encoded by pSK7 [20]) was constructed by PCR amplification of the LEU2-myc_{13} encoding region of pSK7 using the oligonucleotides TCCGCGGCAGTTAACTCTGCCCCTAAGAAGATCGTC and CGACGGTATCGATAAGCTTGCATGC thereby introducing the restriction enzyme sites of HpaI and Hind3. The HpaI and Hind3 digested fragment was ligated with digested plasmid pZK116m (pRS316-P_{PRC1} *prc1-1Δss*) [9].

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0014-5793/$34.00
doi:10.1016/j.febslet.2008.11.015

A high-copy plasmid expressing N-terminally flag tagged *UBR1* from the *ADH1* promoter and a respective control plasmid pRB where the ORF of flagUbr1 had been removed were a kind gift from Du et al. [21].

2.2. Antibodies

For precipitation of ΔssCL*myc and detection in immunoblots monoclonal c-myc antibodies (Santa Cruz, clone 9E10) were used. For precipitation and immunoblots of flag tagged Ubr1 polyclonal flag antibodies (Sigma) were used.

2.3. Pulse chase analysis

Pulse chase experiments using cells expressing ΔssCL*myc were performed as described [22,23].

Briefly, cells were grown in selective media (CM without uracil and leucine) and shifted to starvation media (CM without uracil, leucine and sulfate) for 50 min. Eight OD_{600} of cells were labelled with nine MBq of ^{35}S-Met for 20 min. Cells were chased with unlabelled media containing an excess of non-radioactive methionine. Samples were taken at the time points indicated in the respective figure legends and extracts were prepared.

Diagrams represent data of three independent experiments. Error bars indicate the respective standard error of the mean.

2.4. Immunoprecipitation

One-hundred and fifty OD_{600} of logarithmically grown cells were harvested and washed once in ice-cold destilled water containing 30 mM NaN_3. Cells were resuspended in 2 ml IP buffer (50 mM Tris (pH 7.5), 200 mM NaAcetate, 10% glycerol). Complete inhibitor mix (Roche), 1 mM PMSF, 1 μg/ml each of benzamidin, pepstatin A, and chymostatin were added shortly before use of the buffer. Cells were lysed with glass beads [20]. Lysates were pre-cleared by centrifugation at 500 × *g* for 5 min at 4 °C. Lysates were centrifuged at 100 000 × *g* for 1 h at 4 °C. ΔssCL*myc and flag tagged Ubr1 were immunoprecipitated from the supernatant using 5 μl of anti-myc or 5 μl of anti-flag, respectively, and incubating for 1 h at room temperature. Five milligrams of Protein A sepharose, blocked with 10% BSA, was added for antibody precipitation for an additional hour. After washing with IP buffer the proteins were eluted with 60 μl urea loading buffer (8 M urea, 200 mM Tris/HCL (pH 6.8), 0.1 mM EDTA, 5% (w/v) SDS, 0.03% (w/v) bromophenol blue, 1% β-mercaptoethanol). Fifteen microliters of each sample were used for immunoblot analysis.

3. Results and discussion

For elucidation of ubiquitin ligases involved in the degradation of the cytoplasmic misfolded protein ΔssCL*myc, a derivative of signal sequence deleted mutated carboxypeptidase yscY, we tested yeast strains of the EUROSCARF collection deleted in the genes of proteins predicted to be ubiquitin ligases [24]. We had previously shown that signal sequence deleted carboxypepdidase yscY derivatives locate to the cytoplasm of cells [9]. Plasmids expressing the cytoplasmic misfolded protein ΔssCL*myc (Fig. 1A) were transformed into these strains which are defective in the *LEU2* gene encoding 3-isopropylmalate dehydrogenase.

Strains wild type for ΔssCL*myc degradation are unable to grow on media without leucine because the misfolded protein including the Leu2 moiety is rapidly eliminated, thus being unable to complement the leucine auxotrophy. In contrast, strains defective in a component of the degradation pathway of ΔssCL*myc are able to grow due to stabilization of the Leu2 containing substrate and by this complementing the *LEU2* deficiency [23,25,26].

As can be seen in Fig. 1B a promising candidate of the screen is a strain deleted in the gene of the ubiquitin ligase Ubr1. This strain exhibited strong growth when compared to wild type on

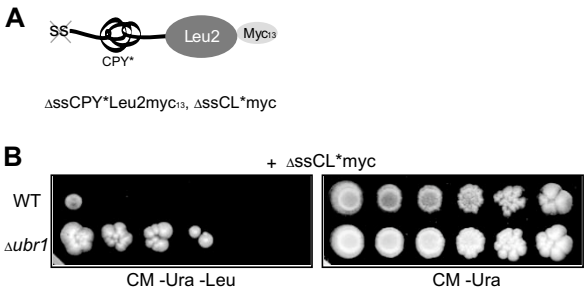

Fig. 1. A strain expressing ΔssCL*myc and deleted in the ubiquitin ligase Ubr1 grows on medium lacking leucine. (A) Schematic drawing of the chimeric protein ΔssCL*myc, consisting of cytoplasmically misfolded CPY* C-terminally fused to Leu2 and a 13myc tag. (B) Growth of a W303 *prc1-1* wild type (WT) strain and a Δ*ubr1* strain, both defective in the *LEU2* and *URA3* genes, harbouring a plasmid with the *URA3* selection marker expressing ΔssCL*myc under the control of the *PRC1* promoter. Cells were spotted in a five fold dilution series on solid CM medium lacking leucine and uracil, or solely uracil, respectively.

medium lacking leucine, indicating stabilization of the substrate.

To elucidate whether degradation of ΔssCL*myc is indeed disturbed in the Δ*ubr1* strain, pulse chase analysis was performed to follow the fate of the substrate.

As can be seen in Fig. 2A and B degradation of ΔssCL*myc is considerably delayed in the Δ*ubr1* mutant. Expression of a flag tagged Ubr1 protein in the Δ*ubr1* deletion strain led to complementation of the degradation defect. As flagUbr1 is expressed from a multi-copy plasmid, degradation kinetics in strains expressing this construct is even faster than in the wild type strain expressing Ubr1 from its chromosomal locus (Fig. 2B). These data indicate that Ubr1 is indeed involved in the degradation process of ΔssCL*myc. As degradation of the substrate is not completely blocked in Δ*ubr1* cells we predict additional ubiquitin ligase activities to be involved in the elimination of misfolded cytoplasmic proteins.

Involvement of Ubr1 in degradation of ΔssCL*myc predicts physical interaction of the E3 ligase with its substrate. This interaction is expected, however, to be rather quick. For a co-immunoprecipitation experiment we transformed a plasmid expressing flag tagged Ubr1 into wild type cells expressing ΔssCL*myc at the same time. When pulling down the substrate using myc antibodies we were able to coprecipitate flag-Ubr1 (Fig. 3, Lane 8). When pulling down flag tagged Ubr1 no substrate was coprecipitated (Lane 10). Obviously, when pulling down the substrate, part of the precipitated ΔssCL*myc molecules are complexed with Ubr1 by this selectively enriching the ligase in this sample. In contrast, when pulling down flagUbr1 the precipitated molecules should contain a multitude of interacting substrates of which ΔssCL*myc is only a minority and therefore cannot be visualized.

We noted that the input levels of ΔssCL*myc are lower when flagUbr1 is expressed in cells (Fig. 3, Lane 2 and 4). This is most likely due the short half-life of ΔssCL*myc in the presence of flagUbr1. Whether the rather low steady state level of flagUbr1 in the presence of substrate is due to degradation has to be explored in future studies.

Ubr1 was shown to be an ubiquitin ligase, which is able to recognize N-end rule substrates. Recognition of these N-end

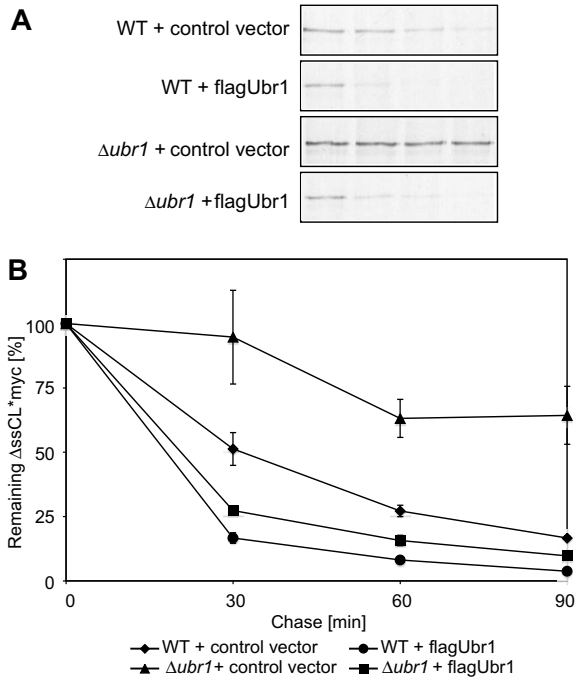

Fig. 2. Deletion of *UBR1* results in prolonged half-life of ΔssCL*myc while over-expression of *UBR1* accelerates its degradation. (A) Pulse chase analysis of WT and Δ*ubr1* cells expressig ΔssCL*myc. Where indicated a flag tagged Ubr1 from a high-copy vector under the control of the *ADH1* promoter or a respective empty control vector was transformed into cells. Cells were harvested at the indicted time points, lysed and subjected to immunoprecipitation with myc antibodies and separated by SDS–PAGE (B). Pulse chase experiments were quantified by using a PhosphorImager and ImageQuaNT. Data represent the mean values of three independent experiments.

rule substrates occurs at two sites, type-1 and type-2. The type-1 site is specific for recognition of basic N-terminal amino acid residues, the type-2 site is responsible for recognition of bulky hydrophobic amino acid residues of proteins. In addition Ubr1 contains a third substrate-binding site, which targets an internal degron of Cup9, a transcriptional repressor of peptide import [27–29]. Removal of the signal sequence from misfolded carboxypeptidase yscY and construction of the ΔssCL*myc substrate resulted in a novel amino terminus starting with Met-Ile-Ser as the first three amino acids. According to the "Sherman-rule" the amino terminal methionine is only cleaved off a polypeptide chain in yeast when it is followed by an amino acid with a radius of gyration of 1.29 Å or less [30]. The second amino acid in ΔssCL*myc is an isoleucine which according to the "Sherman-rule" does not allow cleavage of methionine from the amino terminus of this substrate. As methionine is a stabilizing N-terminal amino acid, ΔssCL*myc cannot be recruited to Ubr1 via the type-1 or type-2 binding sites. ΔssCL*myc might be recruited to Ubr1 by the third binding site which was demonstrated to bind Cup9. Targeting of Cup9 to Ubr1 was shown to be dependent on the binding of cognate dipeptides to the type-1/2 sites of Ubr1 [21,31,32]. Specific binding of Cup9 to Ubr1 can also occur by a chaperone such as yeast EF1A or through macromolecular crowding, conditions which are present in vivo [27]. We have shown that degradation of all tested misfolded ΔssCPY* variants in the cytoplasm require the Hsp70 chaperone Ssa1 [9]. It is therefore most likely that also degradation of ΔssCL*myc is dependent on Ssa1. This chaperone might target ΔssCL*myc to ubiquitination via Ubr1. This process may involve the Cup9 binding site or some other yet unknown site of Ubr1 which may detect the chaperone bound substrate or, alternatively, hydrophobic patches of the substrate. We cannot however completely exclude the generation of a destablizing N-end rule amino terminus on ΔssCL*myc by some unrecognized proteolytic cut. However, our pulse chase experiments do not show the occurrence of a cleaved intermediate product of ΔssCL*myc. Only if a few amino acids were taken off the substrate, this event would escape unrecognized. For the moment we consider this to be rather unlikely.

Acknowledgements: We thank Alex Varshavsky for the plasmid encoding flag tagged Ubr1 and Mario Scazzari for discussions. The work was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn.

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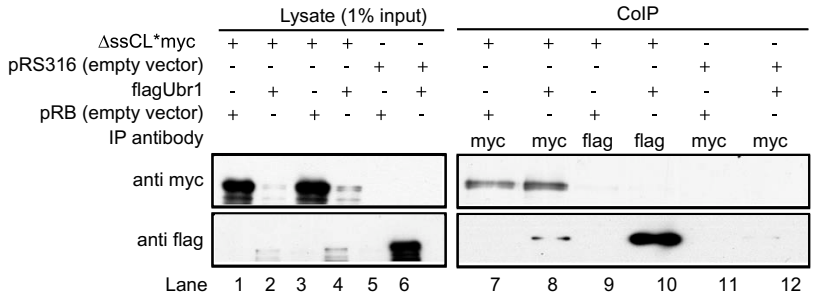

Fig. 3. ΔssCL*myc co-immunoprecipitates with flag tagged Ubr1. Yeast cells expressing ΔssCL*myc and flag tagged Ubr1, or harbouring empty vectors (pRS316 and/or pRB, respectively) were lysed. One percent of total cell extract was analysed by Western blotting using antibodies as indicated. Samples obtained after co-immunoprecipitation (CoIP) were separated by SDS–PAGE and analysed by Western blotting using anti-myc and anti-flag.

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4.3. The Cdc48-Ufd1-Npl4 complex is central in ubiquitin-proteasome triggered catabolite degradation of fructose-1,6-bisphosphatase

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Biochemical and Biophysical Research Communications 394 (2010) 335-341

Biochemical and Biophysical Research Communications 394 (2010) 335–341

Contents lists available at ScienceDirect

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The Cdc48–Ufd1–Npl4 complex is central in ubiquitin–proteasome triggered catabolite degradation of fructose-1,6-bisphosphatase

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ARTICLE INFO

Article history:
Received 12 February 2010
Available online 4 March 2010

Keywords:
Protein degradation
Gluconeogenesis
Fructose-1,6-bisphosphatase
Cdc48 complex
Polyubiquitination
Proteasome

ABSTRACT

The switch from gluconeogenesis to glycolysis in yeast has been shown to require ubiquitin–proteasome dependent elimination of the key enzyme fructose-1,6-bisphosphatase (FBPase). Prior to proteasomal degradation, polyubiquitination of the enzyme occurs via the ubiquitin-conjugating enzymes Ubc1, Ubc4, Ubc5 and Ubc8 in conjunction with a novel multi-subunit ubiquitin ligase, the Gid complex. As an additional machinery required for the catabolite degradation process, we identified the trimeric $Cdc48^{Ufd1-Npl4}$ complex and the ubiquitin receptors Dsk2 and Rad23. We show that this machinery acts between polyubiquitination of FBPase and its degradation by the proteasome.

1. Introduction

Glucose is the preferred carbon and energy source of most organisms but also an important provider of precursor molecules for different anabolic pathways. Glucose consumption via glycolysis and its regeneration via gluconeogenesis are central pathways of carbohydrate metabolism. Regulation of both pathways occurs at three steps catalysed by different reciprocally acting enzymes. In glycolysis, these steps include the phosphorylation of glucose by hexokinase, the phosphorylation of fructose-6-phosphate by phosphofructokinase, and the synthesis of pyruvate and ATP from phosphoenolpyruvate by pyruvate kinase. In gluconeogenesis, these steps are circumvented by glucose-6-phosphatase, fructose-1,6-bisphosphatase (FBPase), pyruvate carboxylase and phosphoenolpyruvate carboxykinase (PEPCK), respectively [1]. Dysregulation of these antagonistic pathways in humans leads to type-2 diabetes [2]. This illustrates the high importance of the regulation of these two pathways.

When cells of the budding yeast *Saccharomyces cerevisiae* are grown on a non-fermentable carbon source (e.g. ethanol), FBPase and other gluconeogenic enzymes are synthesized. Shift of cells to glucose-containing medium leads to a rapid switch from gluconeogenesis to glycolysis. Under these conditions FBPase is quickly regulated at four different steps: (i) repression of the *FBP1* gene, (ii) allosteric inhibition by fructose-2,6-bisphosphate and AMP [3], (iii) enzyme inactivation by phosphorylation [4–7], and finally (iv) degradation [8–10]. The overall inactivation process is called catabolite inactivation [10]. Two different mechanisms were reported for the final proteolytic elimination step [11]. A vacuolar degradation pathway of FBPase was proposed after glucose addition to cells starved for 48 h on acetate [12,13]. In contrast, glucose addition to *S. cerevisiae* cells grown on the natural carbon source ethanol for 16–18 h leads to polyubiquitination and degradation of FBPase via the 26S proteasome [6,11,14–19]. This process is called catabolite degradation [6,11].

A genome wide screen previously identified nine *GID* (glucose induced degradation deficient) genes essential for FBPase degradation which were termed *GID1* to *GID9* [17]. On the basis of biochemical and proteome interaction studies, seven of these nine Gid proteins were discovered to form the so-called Gid complex of 600 kDa [17,19–22]. Gid3 turned out to be the ubiquitin-conjugating enzyme Ubc8, which is centrally involved in the ubiquitination process of FBPase [18]. Gid6/Ubp14 is a general ubiquitin protease which has no specific role in the degradation process of FBPase [23]. The Gid complex represents a novel ubiquitin ligase (E3). Gid4/Vid24 was uncovered as a key regulator of this E3 complex triggering degradation of FBPase and of an additional gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK) by the proteasome [19].

The delivery mechanism of polyubiquitinated FBPase to the 26S proteasome remained an open question. An additional well-known

Abbreviations: FBPase, fructose-1,6-bisphosphatase; Gid, glucose-induced degradation deficient; PEPCK, phosphoenolpyruvate carboxykinase; ER, endoplasmic reticulum; Pgk, 3-phosphoglycerate kinase; WT, wild type.

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doi:10.1016/j.bbrc.2010.03.005

member of the ubiquitin–proteasome machinery is the Cdc48 complex (p97/VCP in higher eukaryotes), a conserved hexameric ring-shaped AAA–ATPase. For instance, Cdc48 is involved in the ER-associated degradation (ERAD) of misfolded proteins, extracting the polyubiquitinated species from the cytosolic side of the ER membrane for delivery to the proteasome for degradation [24–29], in the ubiquitin-fusion-degradation pathway (UFD) [30] and the OLE pathway [31]. An array of cofactors regulates the activity of Cdc48 [24,26–33]. Among these cofactors the heterodimeric Ufd1–Npl4 complex is required for all these segregation purposes [24,26–29,31,32]. Until now, among cytosolic proteins only engineered substrates like Ub-Pro-β-gal have been described to depend on the Cdc48 complex for degradation [30]. Since FBPase is degraded through the ubiquitin–proteasome pathway, we considered it of great importance to test if this natural cytosolic substrate also requires the Cdc48 machinery for elimination.

Here we show that catabolite degradation of FBPase does indeed require the $Cdc48^{Ufd1-Npl4}$ machinery. Also the UBA–UBL domain receptor proteins Dsk2 and Rad23 were found to be required for FBPase elimination. All these protein components act after polyubiquitination of FBPase by the Gid complex. In conjunction with the UBA–UBL receptor proteins Dsk2 and Rad23, the $Cdc48^{Ufd1-Npl4}$ complex probably delivers the enzyme to the proteasome for degradation. Elimination of PEPCK, another gluconeogenic enzyme, was also found to be dependent on the $Cdc48^{Ufd1-Npl4}$ complex.

2. Materials and methods

2.1. Growth conditions, yeast strains and plasmids

Media preparation, genetic and molecular biological techniques were carried out using standard methods [34,35]. The *S. cerevisiae* strains used in this study are summarized in Table 1. Unless otherwise stated, all yeast strains were grown at 30 °C. Pre-cultures were grown during 16 h in YPD medium containing 2% glucose, diluted 1:12.5 into YPD and grown for 6–7 additional hours. Thereafter cells were resuspended in YPEthanol (2%) and grown for 16 h to allow FBPase synthesis. For induction of FBPase degradation, cells were shifted to YPD medium containing 2% glucose. Gene deletions were generated via homologous recombination [36,37]. The strains YSK018, YJMR2 and YLB47 were obtained by transformation of W303-1C and W303-1B, respectively, with a KanMX6 deletion cassette. For construction of the YFE18 (W303-1B *prc1-1 cdc48-T413R*) strain also called *cdc48-ts*, the mutated *CDC48* allele was amplified from the KFY189 strain (*MATa lys2 leu2 ura3 cdc48-8*) obtained from K.U. Fröhlich and integrated into a plasmid which was then sent for sequencing. The insert possessed two point mutations. After pop-in/pop-out [38] in the W303-1C strain with this plasmid and subsequent sequencing, the YFE18 (W303-1B *prc1-1 cdc48-T413R*) strain was found to possess only one out of the two point mutations in the *CDC48-8* allele: threonine 413 was mutated into arginine (T413R). Plasmids pRG6 and pFPase-TAP have been described by Santt et al. [19].

Table 1
Yeast strains used in this study.

Name	Genotype	Reference
BWG1-7a	MATa *leu2-3,112 ura3-52 ade1-100 his4-519 prc1-1*	[55]
PM373	BWG1-7a *ufd1-1*	[55]
W303-1B	MATα *ade2 leu 2-3,112 his3 trp1 ura3*	[12]
W303-1C	W303-1B *prc1-1*	[56]
YAG003	FY23 *ura3-52 leu2Δ1 trp1Δ63 GAL+*	[57]
YAG005	YAG003 *npl4-2*	[57]
YAT2851	W303-1B *dsk2::TRP1*	[58]
YAT2525	W303-1B *rad23::URA3*	[58]
YCR1	W303-1B *prc1-1 ubx2::his5+*$^{S.\ pombe}$	[48]
YCR2	W303-1B *prc1-1 ubx5::his5+*$^{S.\ pombe}$	[48]
YCR4	W303-1B *prc1-1 ubx6::his5+*$^{S.\ pombe}$	[48]
YFE18	W303-1B *prc1-1 cdc48-T413R*	This work
YJMR2	W303-1B *otu1::kanMX*$^{Tn\ 903}$	This work
YLB47	W303-1B *ddi1::kanMX*$^{Tn\ 903}$	This work
YSA10	W303-1B *prc1-1 ubx4::kanMX*$^{Tn\ 903}$	[48]
YSA12	W303-1B *prc1-1 ubx7::kanMX*$^{Tn\ 903}$	[48]
YSA18	W303-1B *prc1-1 ubx3::his5+*$^{S.\ pombe}$	[48]
YSA21	W303-1B *prc1-1 ubx1::his5+*$^{S.\ pombe}$	[48]
YSK018	W303-1B *prc1-1 ufd2::kanMX*$^{Tn\ 903}$	This work

2.2. Western blotting

Experiments were performed as described by Schork et al. [16]. Extracts were prepared via alkaline lysis [39] and finally resuspended in urea buffer (200 mM Tris/HCl pH 6.8, 8 M urea, 5% SDS, 0.1 mM EDTA, 1% 2-mercaptoethanol and 0.05% bromophenol blue). 1.5 OD_{600} cells were used for each sample. The monoclonal ubiquitin antibody (clone P4G7) was obtained from Covance and the FBPase polyclonal antibody was produced by rabbit immunization using purified FBPase–GST [19].

2.3. Polyubiquitination of FBPase

Experiments were performed by growing cells with a plasmid encoding FBPase fused to the tandem affinity purification (TAP)-tag on CM medium without uracil, 2% glucose, and further processed as described in Santt et al. [19].

2.4. Pulse-chase analysis

Experiments were performed as described by Schork et al. [16], using a specific antibody against FBPase. Protein bands were quantified with a PhosphorImager (Molecular Dynamics).

3. Results and discussion

3.1. The $Cdc48^{Ufd1-Npl4}$ complex is required for degradation of polyubiquitinated FBPase

Recently, the Gid complex has been discovered as the ubiquitin ligase essential for ubiquitination and degradation of FBPase. FBPase is bound to the Gid complex for polyubiquitination from where it has to be removed for delivery to the proteasome. In addition, FBPase is a homotetrameric enzyme [40], the subunits of which have to be unfolded and threaded into the chambers of the 20S core part of the proteasome for degradation. Cdc48 has been reported to disassemble protein complexes and segregate proteins from their binding partners at the expense of ATP hydrolysis [24–32,41]. This machinery may thus be expected to have a similar function in the degradation process of FBPase. We therefore tested FBPase degradation in a mutant conditionally defective in Cdc48. Wild type and temperature-sensitive *cdc48-ts* (*cdc48-T413R*) strains were grown overnight in YPEthanol at the permissive temperature of 25 °C. After 16 h of growth and subsequent shift for 1 h to the restrictive temperature of 37 °C, cells were transferred to glucose-containing complete medium and samples were taken at the indicated time points. As can be seen in Fig. 1A, degradation of FBPase is dramatically impaired in the *cdc48-ts* strain. To be able to quantify the effect of the *CDC48* mutation on FBPase degradation, pulse-chase experiments were carried out in the wild type and the temperature-sensitive *cdc48-ts* strain. The half-life time of FBPase was increased about 3-fold in the strain conditionally defective in Cdc48 activity (Fig. 1B).

Recent experiments demonstrated that the Gid complex constitutes the ubiquitin ligase which triggers polyubiquitination of

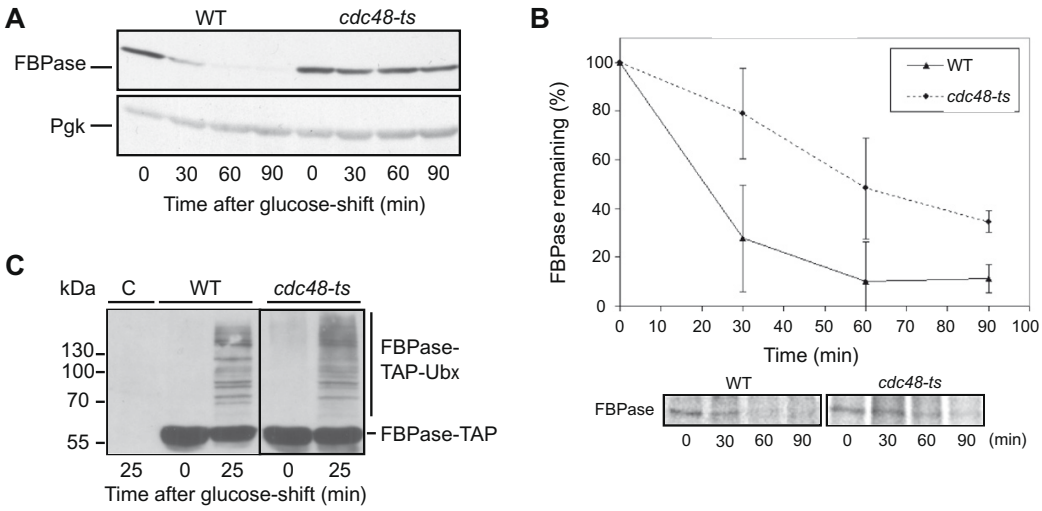

Fig. 1. The Cdc48 complex is required for proteasomal catabolite degradation of polyubiquitinated FBPase. Wild type and mutant cells (*cdc48-ts*) were grown overnight in YPEthanol at the permissive temperature (25 °C). They were then shifted to the restrictive temperature (37 °C) for 1 h and thereafter to YPD medium to trigger FBPase degradation. Samples were taken every 30 min after shift to glucose. (A) Metabolic chase analysis. FBPase was detected via immunoblotting using FBPase antibody. Pgk: 3-phosphoglycerate kinase, loading control. (B) Pulse-chase analysis of FBPase in wild type (WT) (—) and mutant (*cdc48-T413R*) (- - -) cells was carried out as described in Section 2 (mean of three independent experiments, ±confidence interval, $\alpha = 0.05$). (C) FBPase polyubiquitination in wild type and *cdc48* mutated strains. A plasmid expressing a FBPase-TAP fusion protein was transformed into these strains. Samples were taken at the indicated time points, and FBPase was purified using IgG-Sepharose. Polyubiquitination was detected using a monoclonal ubiquitin antibody. C: control; the wild type strain expressing FBPase from a plasmid.

FBPase [19]. To determine at which step of FBPase degradation Cdc48 is required, an *in vivo* ubiquitination assay was performed. Detection of immunoprecipitated FBPase was often complicated by the fact that the enzyme migrates slightly faster than the heavy chains of IgGs on SDS–polyacrylamide gels. We therefore transformed a plasmid expressing a C-terminally TAP-tagged FBPase into wild type and the *cdc48-ts* strain to ensure proper detection of the enzyme and of its polyubiquitinated forms [19]. The TAP-tagged version of FBPase is fully functional and undergoes the identical degradation mechanism as non-tagged FBPase (J. Juretschke, unpublished). Pull-down of FBPase-TAP with IgG-Sepharose and subsequent immunodetection with ubiquitin antibody revealed that a strain expressing the mutant Cdc48 protein behaved like wild type: the mutant was still able to polyubiquitinate FBPase (Fig. 1C). This indicates that the function of the Cdc48 complex for degradation of FBPase becomes essential after polyubiquitination of the enzyme. It suggests that Cdc48 is necessary for the delivery of the polyubiquitinated FBPase subunits to the 26S proteasome. The results reveal a novel function of the Cdc48 complex: besides its role in the delivery of misfolded polyubiquitinated ER-associated proteins [24–29] and of engineered cytosolic substrates [30] to the 26S proteasome for elimination, Cdc48 is involved in the degradation of a natural cytosolic substrate, the gluconeogenic enzyme FBPase.

To exert its function, Cdc48 often requires substrate-recruiting cofactors. Two types of such cofactors have been identified so far, the heterodimeric Ufd1–Npl4 complex and members of the UBX domain protein family (Ubx1 to Ubx7), the founding member being Shp1 (Ubx1) [32,33]. Both of these adaptors, the Ufd1–Npl4 complex and Shp1 (Ubx1) bind ubiquitin–protein conjugates and interact with Cdc48 in a mutually exclusive manner. Recruitment of such cofactors leads to fundamentally distinct cellular functions of Cdc48. While the $Cdc48^{Shp1}$ complex is reported to control homotypic membrane fusion [42], the $Cdc48^{Ufd1-Npl4}$ complex was found in ubiquitin-dependent protein processing and degradation pathways [24–31,43,44]. We therefore tested whether FBPase degradation also requires some of these cofactors. Deletion of Shp1 (Ubx1) had no effect on FBPase degradation (not shown). However, FBPase showed significantly decreased degradation kinetics in *ufd1* (Fig. 2A) and *npl4* mutants (Fig. 2D), respectively. Pulse-chase experiments confirmed these results: the half-life of FBPase was increased about 3-fold in a conditional *ufd1-1* strain and more than 3-fold in a conditional *npl4-2* strain (Fig. 2B and E). As found for the *cdc48-ts* mutant, strains expressing the Npl4 and Ufd1 mutant proteins were still able to polyubiquitinate FBPase *in vivo* (Fig. 2C and F). Thus, the trimeric $Cdc48^{Ufd1-Npl4}$ complex is a central component of the FBPase degradation pathway and acts after polyubiquitination of the enzyme triggered by the Gid complex. Interestingly, FBPase is the first natural cytosolic substrate shown to depend on the $Cdc48^{Ufd1-Npl4}$ machinery for its further elimination by the 26S proteasome.

3.2. Also Ubx4 has some function in FBPase degradation

Only one protomer of the homohexameric AAA–ATPase Cdc48 is occupied by the Ufd1–Npl4 heterodimer [32]. Unoccupied protomers of Cdc48 can bind additional cofactors like members of the Ubx family which are characterized by the presence of a so-called "ubiquitin regulatory X" (UBX) domain [33,45]. It was found that Ubx2 recruits the $Cdc48^{Ufd1-Npl4}$ complex to the ER membrane for degradation of misfolded ER proteins by linking the Cdc48 complex to membrane localised E3 ligases for pulling misfolded ER substrates away from the ER membrane and delivering them to the proteasome [45,46]. Ubx4, Ubx6 and Ubx7 are also known to be involved in ubiquitin-dependent protein degradation [33,47]. Ubx4 has recently been reported to modulate Cdc48 activity and influence degradation of ubiquitinated misfolded proteins of the endoplasmic reticulum [48]. We therefore tested the involvement of these Ubx proteins in FBPase degradation. FBPase degradation was not at all impaired in *ubx2, ubx3, ubx5, ubx6* and *ubx7* knock-out mutants (not shown). However pulse-chase analysis of FBPase in an *ubx4Δ* strain revealed that degradation of the polyubiquitinated enzyme was slowed down

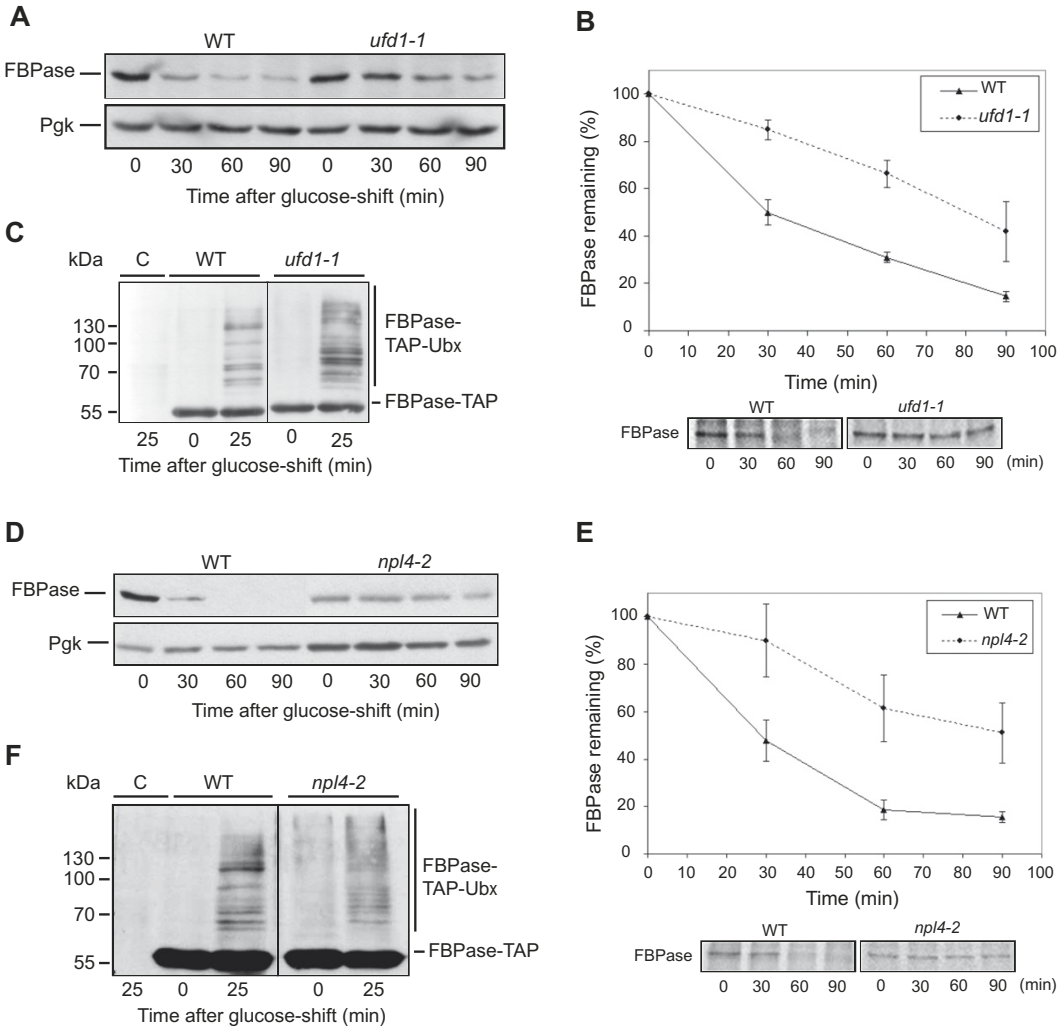

Fig. 2. Ufd1 and Npl4 are required for proteasomal catabolite degradation of polyubiquitinated FBPase. Wild type and their respective mutant (*ufd1-1* and *npl4-2*) cells were grown as described in Fig. 1. (A and D) Metabolic chase analysis (B and E) pulse-chase analysis of FBPase in wild type (WT) (—) and mutant (*ufd1-1* and *npl4-2*) (- - -) cells (C and F) FBPase polyubiquitination assay as described in Fig. 1.

about 1.5-fold (Fig. 3A and B). This suggests a moderate requirement of Ubx4 for FBPase degradation. As proposed in Alberts et al. [48] the function of Ubx4 might consist in facilitating the transport of polyubiquitinated proteins to the proteasome for degradation.

3.3. Involvement of additional cofactors of Cdc48 in catabolite degradation of FBPase

Besides its "segregase" activity, Cdc48 was also shown to control the degree of ubiquitination of bound substrates. This latter activity is brought about by "substrate-processing cofactors" [32]. The E4 enzyme Ufd2 polyubiquitinates substrates, thereby promoting proteasomal degradation. This U-box domain-containing protein binds to the second AAA domain of Cdc48 through the Ufd1–Npl4 complex [43,44]. The deubiquitinating enzyme Otu1 removes ubiquitin modifications from the substrate [44]. We tested the dependency of FBPase degradation on these processing cofactors. FBPase elimination was neither impaired in Ufd2 nor in Otu1 mutant strains (not shown).

3.4. Rad23 and Dsk2 are required for FBPase degradation

Ubiquitinated substrates have been described to be recruited by the ubiquitin-conjugate binding receptor proteins Rad23 and Dsk2 for proteasomal targeting and degradation [32,43,49,50]. These proteins contain ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains and are suggested to shuttle ubiquitinated substrates to the proteasome [43,49]. $Cdc48^{Ufd1-Npl4}$-processed ERAD substrates have been shown to be delivered to the proteasome via Dsk2 and Rad23 [43,49,50]. We therefore tested if polyubiquitinated FBPase follows the same Dsk2–Rad23 escorted pathway. As shown in Fig. 3C and D, degradation of polyubiquitinated FBPase is considerably delayed in *rad23* and *dsk2* knock-out mutants. Deletion of an additional UBA–UBL domain-containing protein, Ddi1, did not show any alteration in the degradation kinetics of FBPase (not shown).

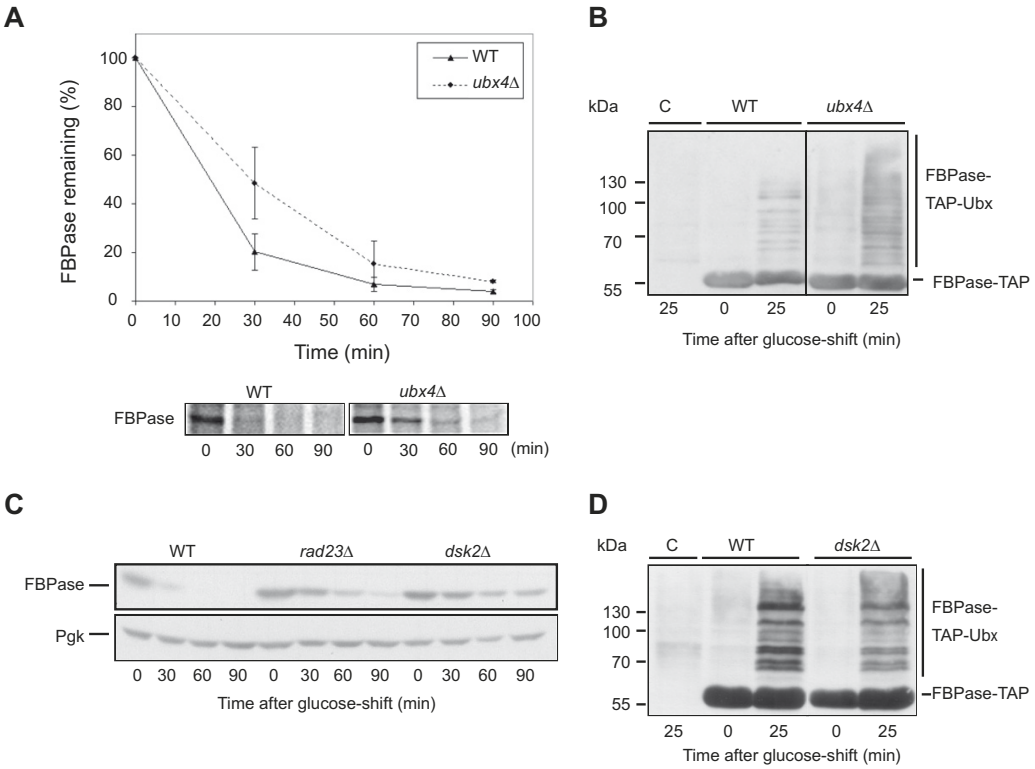

Fig. 3. Deletion of Ubx4 shows partial impairment of proteasomal catabolite degradation of polyubiquitinated FBPase. Additionally FBPase degradation is impaired in strains deleted in *RAD23* and *DSK2*. *ubx4Δ*, *rad23Δ*, *dsk2Δ* and respective wild type cells were grown as described in Fig. 1. (A) Pulse-chase analysis of FBPase in wild type (WT) (—) and mutant (*ubx4Δ*) (- - -) cells. (B and D) FBPase polyubiquitination assays as described in Fig. 1. (C) Metabolic chase analysis as described in Experimental Procedures. FBPase was detected via immunoblotting using FBPase antibody. Pgk: 3-phosphoglycerate kinase, loading control.

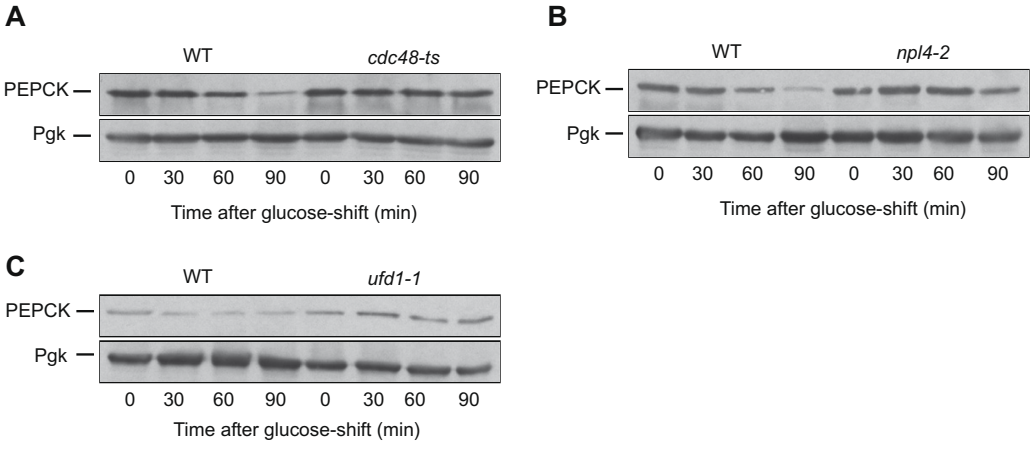

Fig. 4. Catabolite degradation of PEPCK requires the $Cdc48^{Ufd1-Npl4}$ complex. *cdc48-ts* (A), *npl4-2* (B), *ufd1-1* (C) and respective wild type strains were grown overnight in YPEthanol at the permissive temperature (25 °C). They were then shifted to the restrictive temperature (37 °C) for 1 h, and thereafter to YPD medium to trigger PEPCK degradation. Samples were taken every 30 min after shift to glucose. Metabolic chase analysis was done as described in Experimental Procedures and PEPCK was detected via immunoblotting using a PEPCK antibody. Pgk: 3-phosphoglycerate kinase, loading control.

3.5. Catabolite degradation of PEPCK requires the $Cdc48^{Ufd1-Npl4}$ complex

In gluconeogenesis the first step, formation of pyruvate, involves the enzyme pyruvate carboxykinase (PEPCK). Like FBPase, PEPCK synthesis is repressed by glucose, and the enzyme is subjected to catabolite degradation when cells are shifted from gluconeogenic to glycolytic conditions [10,51–53]. As described for FBPase, its degradation depends on the Gid complex and on the proteasome [19]. Also PEPCK is a homotetrameric enzyme

[54].We monitored the requirement of the $Cdc48^{Ufd1-Npl4}$ machinery for PEPCK degradation by shifting cells defective in either Cdc48, Ufd1 or Npl4 from ethanol- to glucose-containing medium. PEPCK degradation was strongly impaired in these mutants, indicating that the $Cdc48^{Ufd1-Npl4}$ complex is also required for catabolite degradation of PEPCK (Fig. 4). The role of the $Cdc48^{Ufd1-Npl4}$ machinery in catabolite degradation is thus not restricted to FBPase but extends to other gluconeogenic enzymes.

Taken together, we suggest the following working model. On a non-fermentable carbon source, FBPase interacts with the Gid ubiquitin ligase complex. After addition of glucose, Gid4 is synthesized and binds to the Gid complex which then polyubiquitinates FBPase [19]. Polyubiquitinated FBPase is then processed by the $Cdc48^{Ufd1-Npl4}$ machinery. Thereafter, with the help of their UBA domain, Rad23 and Dsk2 may bind polyubiquitinated FBPase and further direct it to the proteasome via interaction of their UBL domain with the regulatory 19S complex of the protease machine. A function of the $Cdc48^{Ufd1-Npl4}$ complex might reside in dissociating polyubiquitinated FBPase from the Gid complex, as well as in segregating the FBPase subunits from each other to allow their unfolding by the ATPases of the 19S cap of the proteasome, enabling the subunit chains to enter the 20S core. PEPCK may follow the same route.

Acknowledgments

We thank Sonia Barbosa for invaluable advice in this work. This work was supported by the Deutsche Forschungsgemeinschaft, Bonn.

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4.4. Mutants of the deubiquitinating enzyme Ubp14 decipher pathway diversity of ubiquitin–proteasome linked protein degradation

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Biochemical and Biophysical Research Communications 350 (2006) 329-333

Available online at www.sciencedirect.com
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Mutants of the deubiquitinating enzyme Ubp14 decipher pathway diversity of ubiquitin–proteasome linked protein degradation

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Received 30 August 2006
Available online 18 September 2006

Abstract

Selective proteolysis is an important regulatory mechanism in all cells. In eukaryotes, this process gains specificity by tagging proteins with the small protein ubiquitin. K48 linked polyubiquitin chains of four and more ubiquitin moieties target proteins for hydrolysis by the proteasome. Prior to degradation the polyubiquitin chain is removed from the protein, cleaved into single units, and recycled. The deubiquitinating enzyme Ubp14 is an important catalyst of this process. Mutants of Ubp14 had been shown to accumulate non-cleaved oligo- and polyubiquitin chains, which resulted in inhibition of overall ubiquitin–proteasome linked proteolysis as well as in inhibition of degradation of some known substrates. Here we show that accumulation of ubiquitin chains due to defective Ubp14 does not uniformly lead to inhibition of ubiquitin–proteasome linked protein degradation. Instead, inhibition of degradation depends on the substrate tested. The results indicate the existence of different paths through which proteins enter the proteasome.

Keywords: Ubiquitin; Deubiquitinating enzyme; Ubp14; Proteasome; Protein degradation

Degradation of proteins is an essential process in cellular life. Most of regulated proteolysis is exerted by the proteasome, a multisubunit nanomachine [1]. Tagging of proteins with K48 linked polyubiquitin chains is a major prerequisite for the protein's destination to proteasomal proteolysis. Regulation of transcription, of metabolic enzymes, of cell cycle regulators, generation of antigens, and degradation of protein waste are major tasks of ubiquitin–proteasome linked proteolysis. Polyubiquitination of proteins depends on a ubiquitin activating enzyme (Uba, E1), ubiquitin conjugation enzymes (Ubc's, E2's), and ubiquitin–protein ligases (E3's). This tagging machinery catalyzes the formation of isopeptide bonds between the C-terminus of ubiquitin and a lysine residue of the target protein as well as the binding between the ubiquitin moieties forming the polyubiquitin chain. On lysine-less proteins a peptide bond between ubiquitin and the aminoterminus of the protein may be formed. Specificity for polyubiquitination of a certain protein is generated by the use of specific ubiquitin conjugating enzymes in combination with a ubiquitin–protein ligase (E3). This results in specific ubiquitination pathways [2–5]. The delivery of polyubiquitinated proteins to the proteasome occurs via binding of the polyubiquitin chain to specific adaptors on the 26S proteasome or via receptor proteins, which themselves bind to 26S proteasome subunits [6]. The way of delivery of a certain polyubiquitinated protein to the proteasome may be pathway specific. Ubiquitination of proteins is a reversible process. Deubiquitination is catalyzed by specific proteases called deubiquitinating or DUB enzymes. These proteases hydrolyze the amide bond between Gly76 of ubiquitin and the substrate protein or the amide bond between the ubiquitin moieties of the polyubiquitin chain. Cleavage of the polyubiquitin chains and recycling of monoubiquitin are an essential process [7]. One of the classes of DUB enzymes is the so-called ubiquitin-specific processing protease or UBP class of thiol proteases [7]. Among those the yeast deubiquitinating enzyme Ubp14, the ortholog of mammalian isopeptidaseT, has been

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doi:10.1016/j.bbrc.2006.09.041

shown to disassemble unanchored (“free”) ubiquitin chains in vitro. Correspondingly, deletion of the *UBP14* gene from yeast cells results in a striking accumulation of ubiquitin chains and was shown to lead to severe defects in proteolysis [8]. Specifically tested ubiquitin–proteasome substrates of the N-end rule pathway (Leu-β-gal) or the UFD-pathway (Ub-Pro-β-gal) showed a strong inhibition of degradation in *UBP14* deleted cells. Also degradation of the Matα2 repressor, a sensitive substrate of the ubiquitin–proteasome system, was considerably retarded in cells devoid of Ubp14 [8]. It is proposed that Ubp14 and isopeptidaseT facilitate proteolysis in vivo by preventing unanchored ubiquitin chains from competitively inhibiting polyubiquitin-substrate binding to the 26S proteasome. Here we show that uncleaved ubiquitin chains due to lack of Ubp14 do not affect the degradation of polyubiquitinated proteins in general but inhibit the degradation of only certain proteins and not others by this showing “pathway specificity”.

Materials and methods

Yeast strains and plasmids. Strains BY4743 (MATa/α, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0), BY4743 Δubp14 (Mat a/α, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, YBR058c::kanMX4/YBR058c::kanMX4) and BY4743 Δrpn10 (Mat a/α, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, YHR200w::kanMX4/YHR200w::kanMX4) were taken from the EUROSCARF collection (Frankfurt, Germany).

Triple HA-tagged CPY* was expressed from plasmid pCT42 [9], cytosolic CPY*-GFP (ΔssCPY*-GFP) from plasmid pBM1 [10], and Deg1-GFP_2 from plasmid pUL28 [11].

Antibodies. Monoclonal anti-HA antibody (mouse, Babco) was used for immunoprecipitation of CPY*-HA, polyclonal anti-CPY antibodies (rabbit, Rockland) and anti-GFP antibodies (rabbit, Molecular Probes) for immunoprecipitation of ΔssCPY*-GFP and Deg1-GFP_2, polyclonal anti-FBPase antibodies (rabbit, raised against recombinant FBPase) for immunoprecipitation of FBPase.

Pulse-chase analysis. Pulse-chase experiments with cells expressing CPY* fusion proteins were performed as described previously [12,13]. Pulse-chase experiments with cells expressing the Deg-GFP_2 fusion protein were performed like for CPY* derivatives with the exception that for induction of the CUP1 promoter $CuSO_4$ was added [11]. For pulse-chase analysis of FBPase turnover, cells were grown to an OD_{600} of 1 in complete media with 2% of glucose lacking cysteine and methionine. 10 OD_{600} of cells were collected and transferred to CM media with 2% of ethanol lacking cysteine, methionine, and glucose, and grown for additional 2 h. Cells were labeled with 250 μCi ^{35}S-methionine (Hartmann Analytic, Braunschweig) for 3 h and then transferred to CM with 2% of glucose and 10 mM of methionine. Samples were chased at the time points indicated in Fig. 1. Subsequent procedures were done as described for the CPY* derivatives [12,13].

Results and discussion

The gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) is under strict glucose regulation. When cells growing on a non-fermentable carbon source are transferred to glucose containing media, FBPase is rapidly degraded by the ubiquitin–proteasome system, a process called catabolite degradation [14]. In a genome-wide screen, we had isolated mutants defective in catabolite degradation

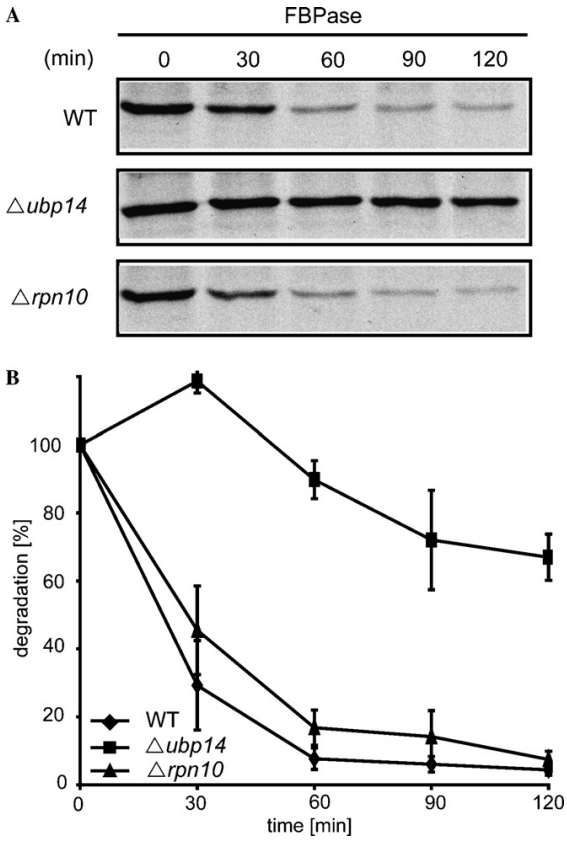

Fig. 1. Lack of the deubiquitinating enzyme Ubp14 inhibits catabolite degradation of fructose-1,6-bisphosphatase. Absence of Rpn10 only mildly affects degradation of FBPase. (A) Pulse-chase analysis with FBPase as substrate was done in wild type (WT), Δ*ubp14*, and Δ*rpn10* cells (all isogenic with wild type) grown on ethanol. After cells were transferred to glucose containing media, cells were lysed at the indicated times, proteins were immunoprecipitated with anti-FBPase antibodies, separated by SDS–PAGE, and analyzed using a PhosphoImager and ImageQuaNT$_{TM}$ (Amersham Bioscience). (B) Plotted data represent mean values of three independent experiments. Error bars indicate standard error of the mean.

of FBPase [15]. Among the deletion mutants, which exhibited a strong stabilization phenotype was a strain deleted in the ORF YBR058c, the wild type gene encoding the deubiquitinating enzyme Ubp14. As the *UBP14* gene was identified amongst seven other genes required for FBPase degradation, which were all named *GID* (for glucose induced degradation deficient) it had received the name *GID6* in addition [15]. While the gene products of the additional *GID*-genes found, formed a complex of about 600 kDa, Ubp14/Gid6p could not be found in this complex indicating a separate function in the degradation process of FBPase (T. Pfirrmann and D.H. Wolf, unpublished). Fig. 1 shows the analysis of FBPase degradation in wild type cells and Δ*ubp14* mutant cells under the catabolite inactivation conditions, supply of glucose to cells grown on a non-fermentable carbon source. As can be seen, degradation of

FBPase is nearly completely inhibited in *UBP14* deleted cells. An inhibitory effect due to Ubp14 deletion on ubiquitin–proteasome degradation and on degradation of some proteins had been shown previously [8] indicating that lack of Ubp14 might block ubiquitin linked proteasomal proteolysis in general. To elucidate this finding in more general terms we tested the degradation of a misfolded ERAD protein, which also depends on the ubiquitin–proteasome system. It has been shown that the ERAD pathway requires retrotranslocation of misfolded proteins out of the ER back to the cytoplasm, where they are polyubiquitinated and degraded by the proteasome [12,16,17]. As standard substrate of ER–associated degradation the disappearance of mutated and by this misfolded carboxypeptidase yscY (CPY*) [12] carboxyterminally linked to the hemagglutinin (HA) tag was analyzed. Surprisingly CPY*-HA was degraded as fast in these mutant cells as in wild type cells (Fig. 2) which is in contrast to FBPase elimination and degradation of all other previously tested substrates [8]. Obviously, the absence of Ubp14 and thus the accumulation of free ubiquitin chains [8] interfered very differently with various ubiquitin–proteasome dependent degradation pathways. CPY* and other ERAD substrates require the trimeric Cdc48-Ufd1-Npl4 complex as well as the UBA-UBL domain containing receptor proteins Dsk2 and Rad23 for proteasomal degradation [10,18].

In contrast to the ERAD substrate CPY* it has been shown that misfolded cytosolic ΔssCPY* derivatives do not require the Cdc48-Ufd1-Npl4 and Dsk2-Rad23 machinery for degradation [10]. As can be seen in Fig. 3 in contrast to the ERAD substrate CPY* degradation of cytosolic ΔssCPY*-GFP (ΔssCG*) is somewhat inhibited in the absence of Ubp14. We tested an additional cytosolic substrate of the ubiquitin–proteasome system, Deg1-GFP_2 [11]. Degradation of Deg1-GFP_2 is not inhibited in cells lacking Ubp14 (Fig. 4). The question remains which features of a ubiquitin–proteasome degradation pathway determine whether degradation of a substrate is inhibited

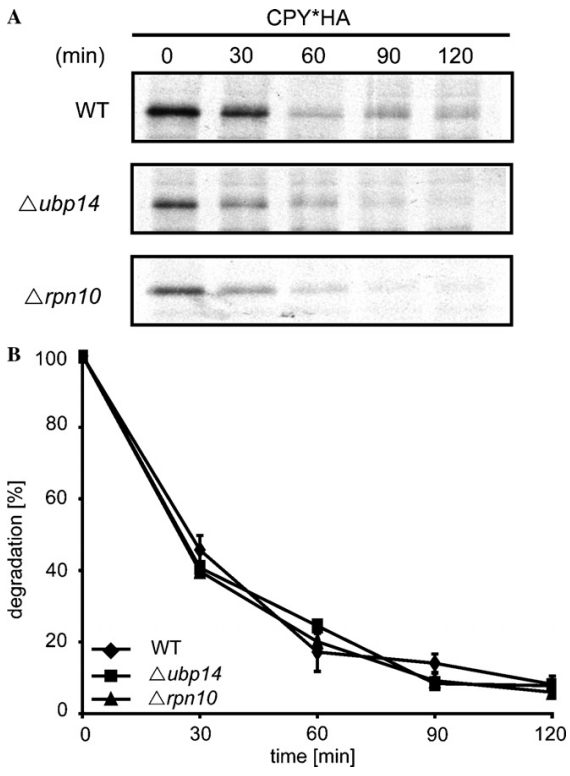

Fig. 2. ER-associated degradation of the ERQD substrate CPY*-HA is not influenced by the absence of Ubp14 or Rpn10. (A) Pulse-chase analysis with wild type (WT), Δ*ubp14*, and Δ*rpn10* cells expressing CPY*-HA was done as indicated in Materials and methods. Cells were lysed at the indicated times, proteins were immunoprecipitated with anti-HA antibody, separated by SDS–PAGE, and analyzed using a PhosphoImager and ImageQuaNT$_{TM}$ (Amersham Bioscience). (B) Plotted data represent mean values of three independent experiments. Error bars indicate standard error of the mean.

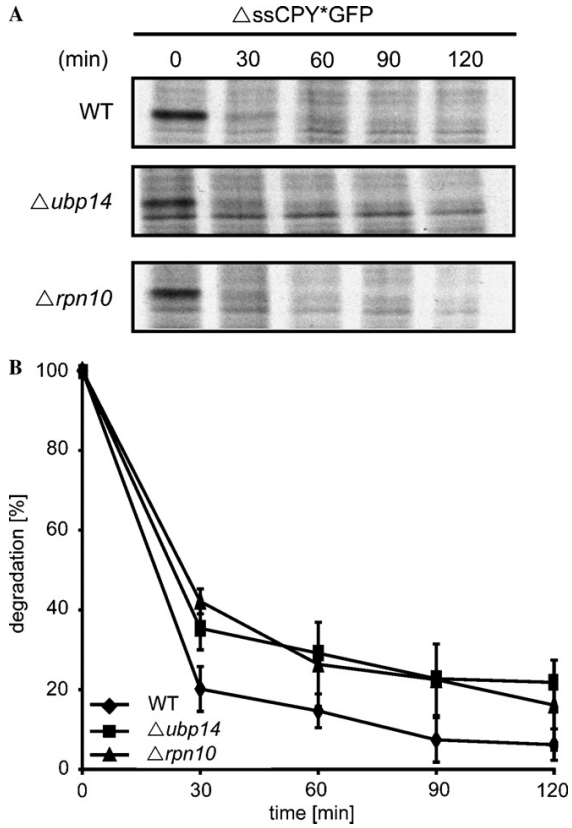

Fig. 3. Degradation of the ER import defective ΔssCPY*-GFP is mildly affected by absence of Ubp14 and Rpn10. (A) Pulse-chase analysis of wild type (WT), Δ*ubp14*, and Δ*rpn10* cells expressing ΔssCPY*-GFP. Cells were lysed at the indicated times, proteins were immunoprecipitated with anti-CPY antibodies, separated by SDS–PAGE, and analyzed using a PhosphoImager and ImageQuaNT$_{TM}$ (Amersham Bioscience). (B) Plotted data represent mean values of three independent experiments. Error bars indicate standard error of the mean.

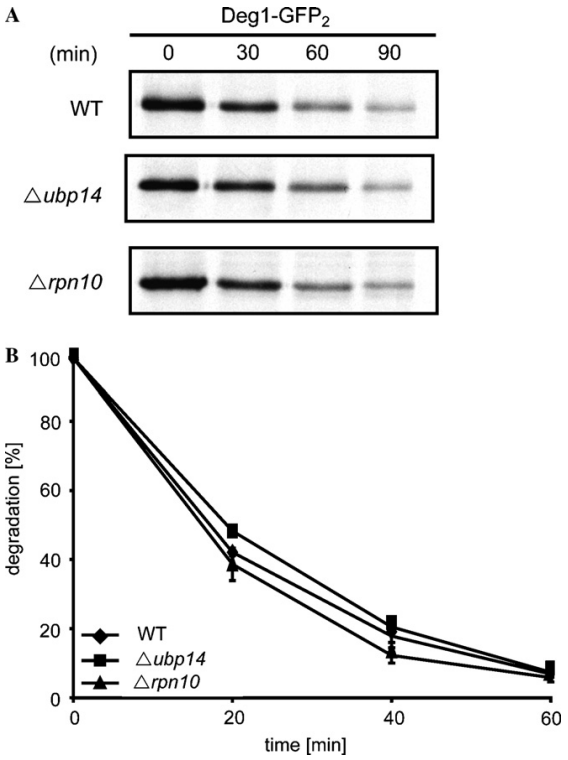

Fig. 4. Degradation of the Deg1-GFP_2 fusion protein is independent of the absence of the deubiquitinating enzyme Ubp14 and Rpn10. (A) Pulse-chase analysis of wild type (WT), Δ*ubp14*, and Δ*rpn10* cells expressing Deg1-GFP_2. Cells were lysed at the indicated times, proteins were immunoprecipitated with anti-GFP antibodies, separated by SDS–PAGE, and analyzed using a PhosphoImager and ImageQuaNT$_{TM}$ (Amersham Bioscience). (B) Plotted data represent mean values of three independent experiments. Error bars indicate standard error of the mean.

in the presence of polyubiquitin chains accumulating in UBP14 deleted cells or not [8].

Interference of non-cleaved polyubiquitin chains with the ubiquitin–proteasome degradation pathway could occur at two different levels, (i) the ubiquitination machinery, especially the ubiquitin conjugating enzymes (Ubc's; E2's) and the ubiquitin–protein ligases (E3's) and/or (ii) the polyubiquitin receptors of the proteasome. One might argue that the affinity of the polyubiquitinated substrate and by this its ability to compete with the polyubiquitin chains accumulating in *UBP14* deleted cells for components of the ubiquitin–proteasome system determines whether inhibition of degradation occurs or not. To carry this idea to an extreme, certain components of the ubiquitin–proteasome system may be easy targets of polyubiquitin chains, others not. These components may be specifically used in different ubiquitin dependent proteasomal degradation pathways. One of the polyubiquitin chain binding receptors is the UIM motif containing 19S cap subunit of the proteasome, Rpn10 [1,19–21]. A deletion of Rpn10 is not lethal to cells [19]. We tested the involvement of Rpn10 in the degradation of our tested substrates (Figs. 1–4). As can be seen only degradation of FBPase and degradation of cytosolic misfolded ΔssCPY*-GFP are somewhat affected in Δ*rpn10* mutants. Thus, Rpn10 plays only a minor role in the degradation of both substrates. Whether it is a target of the inhibitory action of polyubiquitin chains due to failure of Ubp14 activity has to be shown. Clearly, most of the inhibition of FBPase degradation due to absence of Ubp14 activity must have other reasons. Candidates are the ubiquitin conjugating enzyme Ubc8, potently involved in catabolite inactivation of FBPase [22], a yet not characterized E3 ubiquitin ligase or some polyubiquitin chain receptor of the proteasome. From analysis of the ERAD pathway of CPY*-HA which is not at all affected by the absence of Ubp14, one would like to conclude that the ubiquitination machinery consisting of Ubc1, Ubc6, Ubc7, and the ubiquitin ligase Der3/Hrd1 [12,23–25] are not inhibited by the accumulatated ubiquitin chains. Also the ubiquitin chain binding components necessary for this process, consisting of the trimeric Cdc48 complex [18] and the ubiquitin chain receptors Dsk2 and Rad23 [10], do not seem to be inhibited by the presence of the ubiquitin chains left uncleaved in Δ*ubp14* mutants (Fig. 2).

It has been shown that Dsk2 and Rad23 bind size restricted ubiquitin chains of between three and six ubiquitin units [26]. Obviously the polyubiquitin chains accumulating in the Δ*ubp14* mutant are unable to bind Dsk2 and Rad23 in a manner, which would block binding of the ubiquitinated ERAD substrate and thus inhibit ERAD. This is surprising as oligoubiquitin chains of between two and five ubiquitin units accumulate in the Δ*ubp14* mutant [8]. Also degradation of the mainly cytosolically located Deg1-GFP_2 is not inhibited by the presence of the polyubiquitin chains accumulating in Δ*ubp14* mutants (Fig. 4). As in the case of the ERAD substrate CPY*-HA, degradation depends on the ubiquitin conjugating enzymes Ubc6 and Ubc7 [11], which are obviously not targets of polyubiquitin chain inhibition accumulating in Δ*ubp14* cells. Our study clearly shows that accumulation of polyubiquitin chains does not inhibit ubiquitin–proteasome triggered protein degradation uniformly but that this inhibition is pathway specific. This demonstrates again the intricate diversity of ubiquitin–proteasome triggered proteolytic pathways. The identification of the polyubiquitin chain binding targets of the pathways affected is the aim of future experimentation.

Acknowledgments

The authors thank Thomas Sommer for providing the plasmid expressing Deg1-GFP_2, Jeanette Juretschke for anti-FBPase antibodies, and Antje Schäfer for critical reading of the manuscript. The work was supported by a grant of the Deutsche Forschungsgemeinschaft (Bonn, Germany), the Fonds der Chemischen Industrie (Frankfurt, Germany), and the EU network of excellence, RUBICON.

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4.5. Ubiquitylation in the ERAD pathway

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Abstract

Ubiquitylation is a protein modification mechanism, which is found in a multitude of cellular processes like DNA repair and replication, cell signaling, intracellular trafficking and also, very prominently, in selective protein degradation. One specific protein degradation event in the cell concerns the elimination of misfolded proteins to prevent disastrous malfunctioning of cellular pathways. The most complex of these ubiquitylation dependent elimination pathways of misfolded proteins is associated with the endoplasmic reticulum (ER). Proteins, which enter the endoplasmic reticulum for secretion, are folded in this organelle and transported to their site of action. A rigid protein quality control check retains proteins in the endoplasmic reticulum, which fail to fold properly and sends them back to the cytosol for elimination by the proteasome. This requires crossing of the misfolded protein of the endoplasmic reticulum membrane and polyubiquitylation in the cytosol by the ubiquitin-activating, ubiquitin-conjugating and ubiquitin-ligating enzyme machinery.

Ubiquitylation is required for different steps of the ER-associated degradation process (ERAD). It facilitates efficient extraction of the ubiquitylated misfolded proteins from and out of the ER membrane by the Cdc48-Ufd1-Npl4 complex and thereby triggers their retro translocation to the cytosol. In addition, the modification with ubiquitin chains guarantees guidance, recognition and binding of the misfolded proteins to the proteasome in the cytosol for efficient degradation.

About 30% of all cellular proteins are secretory proteins, which enter the endoplasmic reticulum (ER) for further distribution to their site of action. They pass the ER membrane in an unfolded state via a channel, the Sec61 translocon.

Upon entry into the ER the proteins are folded and undergo modifications as are glycosylation and disulfide bridge formation. After reaching their native conformation the proteins are allowed to leave the ER for further transport to their cellular location. The proper folding state of a protein is monitored by quality control systems of the ER, which finally recognize misfolded proteins and retain them in the ER. Subsequently they are retro-translocated out of the ER membrane via a channel comprising in some cases Sec61. After poly-ubiquitylation and removal from the ER membrane the misfolded proteins are guided to the proteasome where they are degraded (Brodsky and McCracken, 1999; Hirsch et al., 2004; Kostova and Wolf, 2003; Meusser et al., 2005; Plemper and Wolf, 1999; Sommer and Wolf, 1997; Vembar and Brodsky, 2008).

Protein folding, quality control in the ER and the ERAD degradation signal

Directly after import of the polypeptide chain into the ER through the Sec61 translocon the Hsp70 chaperone Kar2/BiP (yeast/mammals; Table 1) binds to hydrophobic patches of the protein and the oligosaccharyl transferase (OST) complex links glycans of the structure $Glc_3Man_9GlcNAc_2$ covalently to asparagine residues located within an Asn-X-Ser/Thr motif (N-glycosylation) (Fig. 1). Glycans increase the hydrophilicity of proteins. At the same time the glycans play an important role in the folding process of proteins in the ER lumen (Ellgaard et al., 1999; Helenius and Aebi, 2004). During the Kar2/BiP assisted folding of the polypeptide chain, trimming of the carbohydrate chain occurs. One glucose residue is rapidly removed from the glycan chains by glucosidase I (Gls1) followed by removal of the second glucose residue by glucosidase II (Gls2) (Fig. 1). In mammalian cells the $Glc_1Man_9GlcNAc_2$ carrying protein then associates with ER resident lectin chaperones, the membrane bound calnexin and the soluble calreticulin. Upon release of the folding polypeptide from these chaperones, glucosidase II removes the innermost glucose, generating the $Man_9GlcNAc_2$ structure, which prevents association with both chaperones. Successfully folded proteins are allowed to leave the ER. However, some proteins require more time for folding. For this purpose an UDP-glucose: glycoprotein glucosyltransferase (UGGT) inspects the folding state of the $Man_9GlcNAc_2$ carrying protein and re-glucosylates the terminal mannose of not

yet properly folded proteins. Regeneration of the $Glc_1Man_9GlcNAc_2$ oligosaccharide leads to re-association with calnexin/calreticulin for an additional round of folding. Repeated calnexin-calreticulin cycles with the counteracting actions of UGGT and glucosidase II generates off-phases where the N-glycan is exposed to ER-resident α-1,2-mannosidases. At first trimming of the α-1,2 bounded mannose of the central oligosaccharide branch (B-branch) by the slow acting ER α-mannosidase I (Mns1) occurs (Jakob et al., 1998; Knop et al., 1996b). Subsequently an α-1,2 bounded mannose residue of the C-branch is cleaved off by Htm1/Mnl1 (yeast) or EDEM (mammals) generating an α-1,6 terminal mannose providing the N-glycan degradation signal (Aebi et al., 2009; Clerc et al., 2009; Quan et al., 2008) (Fig. 1).

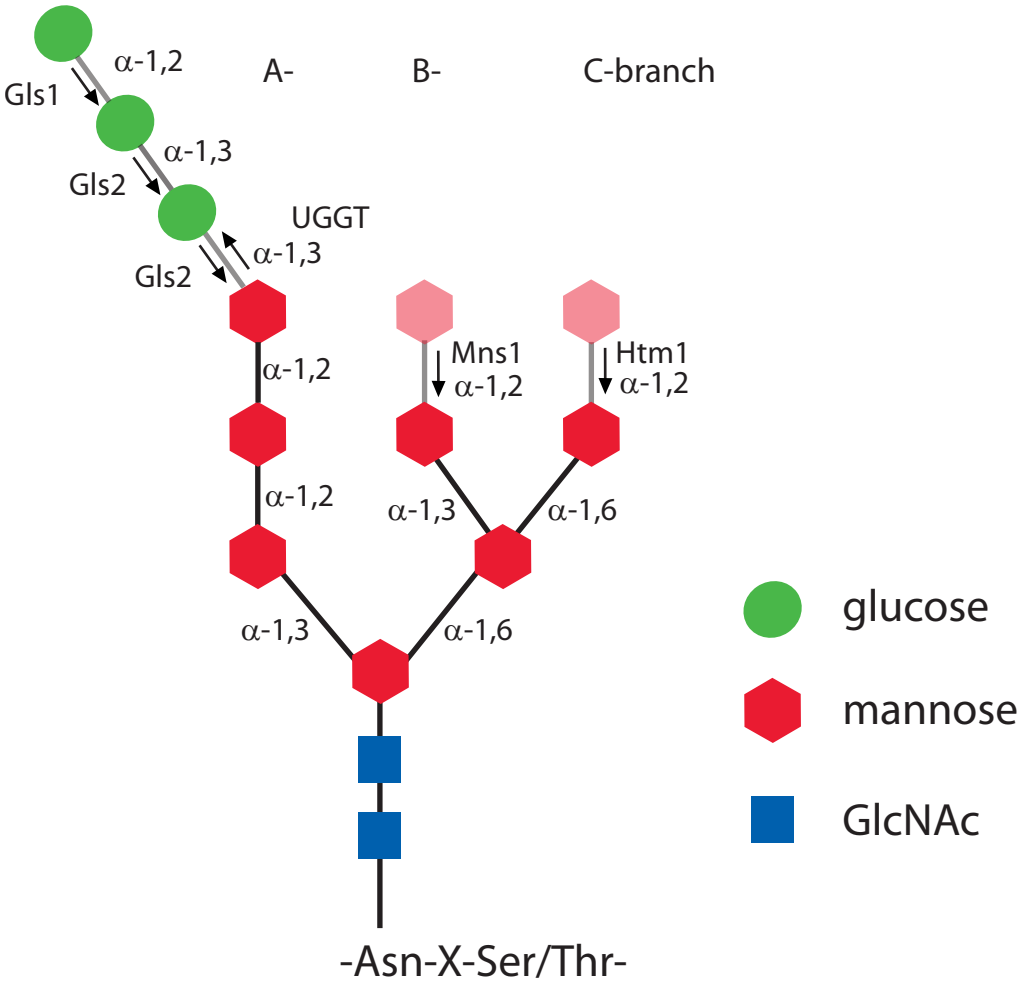

Figure 1. The N-linked core oligosaccharide structure of secretory proteins. Cleavage of the three glucose residues followed by trimming of the terminal mannose residue in the B-branch (indicated in orange) and subsequently the terminal mannose

residue in the C-branch (indicated in orange) offers an α-1,6 linked mannose for recognition of the misfolded protein for elimination.
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In yeast, reglucosylation by UGGT and the calnexin/calreticulin cycle of binding of a folding protein does not exist, leaving only the time frame for protein folding until α-1,2 mannosidase cleaves off the mannose of the central, B-branch followed by removal of a mannose of the C-branch.
In addition, ER localized protein disulfide isomerase (PDI) activity or/and its chaperone function is required for retrotranslocation and degradation of misfolded proteins of the ER (Gillece et al., 1999). Of the five PDI family members in yeast, Pdi1 has been found to form an intermolecular disulfide bounded complex with Htm1/Mnl1 (Clerc et al., 2009; Sakoh-Nakatogawa et al., 2009). Also one of the 19 PDI orthologues of mammals, the DnaJ domain containing oxido-reductase ERdj5 was found to interact with EDEM1, the mammalian orthologue of Htm1/Mnl1. Interestingly the DnaJ domain of ERdj5 contacts the ER lumenal Hsp70 chaperone BiP. Both, ERdj5 reductase activity and interaction between ERdj5, BiP and EDEM are required for efficient degradation of disulfide-bond containing ERAD substrates (Ushioda et al., 2008).
The trimmed mannose glycan signal is interpreted by the glycan binding lectins Yos9 in yeast (Buschhorn et al., 2004; Denic et al., 2006; Gauss et al., 2006) and OS-9 and XTP-3B in mammalian cells (Bernasconi et al., 2008; Christianson et al., 2008; Hosokawa et al., 2009). These proteins have lectin-like domains with homology to the mannose-6-phosphate receptor family. Yeast Yos9 is linked to the Hrd3 protein, a type 1 transmembrane protein with a large lumenal domain, which itself is connected to the Hrd1/Der3 ubiquitin ligase. Mammalian OS-9 and XTP-3B were found in complexes containing SEL1 and the E3 ligase HRD1, the orthologues of yeast Hrd3 and Hrd1/Der3, respectively (Bernasconi et al., 2008; Christianson et al., 2008; Hosokawa et al., 2009; Mueller et al., 2008; Mueller et al., 2006).
Earlier work had shown that the positioning of a carbohydrate chain on the misfolded protein is important for degradation (Kostova and Wolf, 2005; Spear and Ng, 2005). This led to the detection of a bipartite signal for degradation of a misfolded protein:

the trimmed carbohydrate and an exposed hydrophobic amino acid patch close to this carbohydrate chain (Xie et al., 2009). This hydrophobic amino acid patch may be decoded by the Hrd3 (yeast)/ Sel1 (mammals) proteins and/ or the Hsp70 chaperone Kar2/ BiP to initiate the elimination process.

Ubiquitylation and degradation of ER-lumenal substrates: The Hrd-Der ligase complex

The detailed mechanism of ER associated ubiquitin-proteasome dependent degradation of a lumenal misfolded protein was first discovered by virtue of a mutated vacuolar (lysosomal) enzyme of yeast, carboxypeptidase yscY (CPY*) (Hiller et al., 1996). The protein carries a Gly-Arg mutation at a highly conserved site of serine proteases (Finger et al., 1993), is fully imported into the ER lumen, N-glycosylated, discovered as being misfolded, retrograde transported out of the ER, polyubiquitylated and degraded by the proteasome (Hiller et al., 1996; Plemper et al., 1999b) (Fig. 2). Polyubiquitylation occurs to a minor part by the soluble cytosolic ubiquitin-conjugating enzyme Ubc1 and by the ubiquitin-conjugating enzyme Ubc6, a tail anchored ER membrane protein with its active site reaching into the cytosol (Friedlander et al., 2000). The main ubiquitin-conjugating enzyme of the ubiquitylation process of CPY* is represented by the ubiquitin-conjugating enzyme Ubc7 (Hiller et al., 1996). Ubc7 is recruited to the ER membrane by the membrane anchor protein Cue1 which leads to its activation (Biederer et al., 1997).

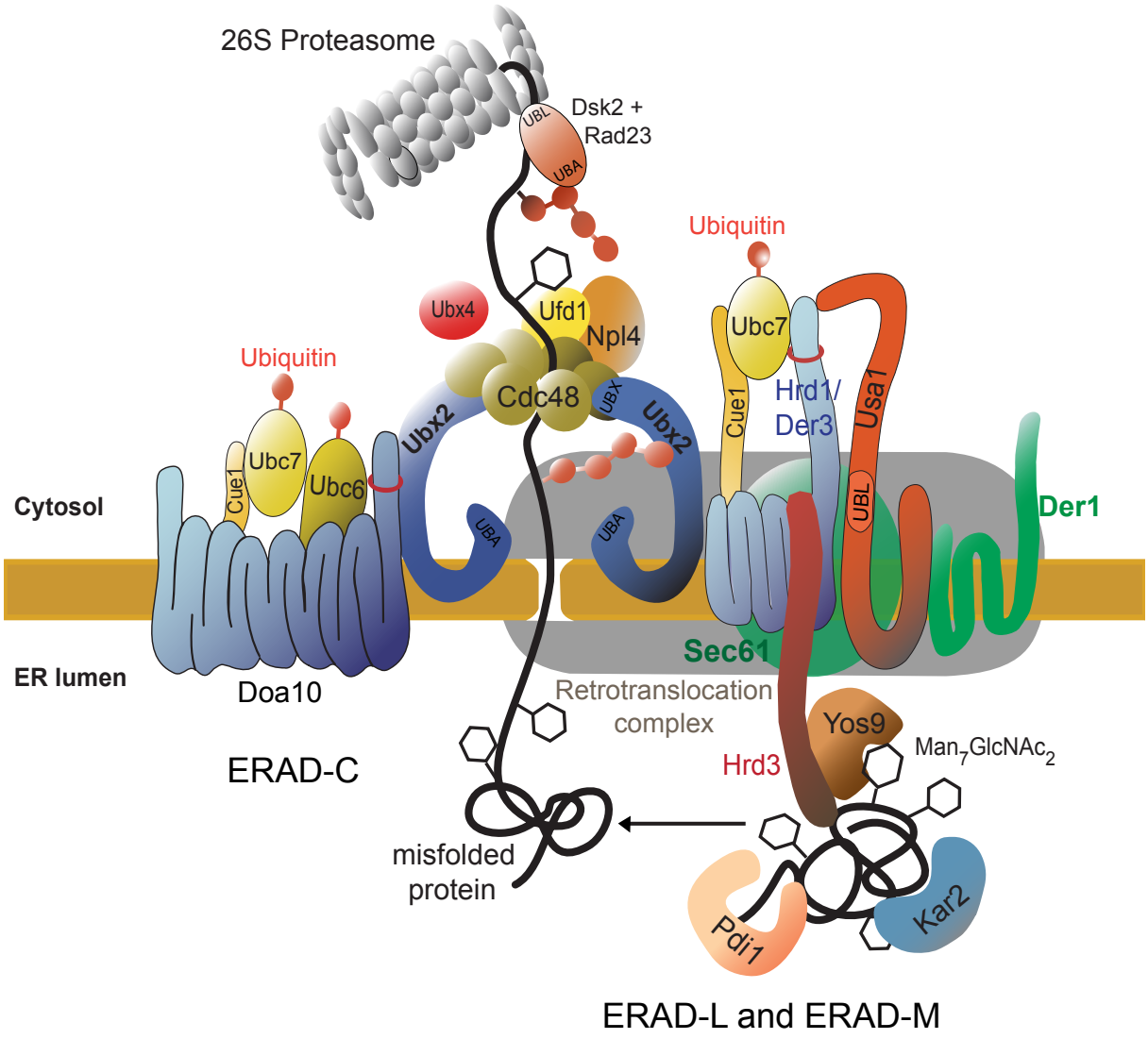

Figure 2. The ubiquitylation machineries of the ER for misfolded secretory proteins. The two ubiquitin ligation machineries of yeast consisting of the Hrd1/Der3 ligase and the Doa10 ligase merge with their polyubiquitylation activity at the AAA-ATPase complex Cdc48-Ufd1-Npl4, which initiates delivery of the polyubiquitylated ERAD substrates to the proteasome.

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The ubiquitin ligase responsible for the polyubiquitylation process of CPY* turned out to be Der3 (Bordallo et al., 1998), a six times the ER membrane spanning protein (Deak and Wolf, 2001). Der3 exposes a RING (Really Interesting New Gene) finger motif into the cytoplasm, which is necessary for its activity (Bays et al., 2001a;

Bordallo and Wolf, 1999; Deak and Wolf, 2001). The same enzyme was also found as Hrd1 in the regulated degradation process of the integral ER membrane protein HMG-CoA reductase (Hampton et al., 1996) (Fig. 2). The Hrd1/Der3 ligase is also involved in the degradation of the misfolded ER membrane protein, Pdr5*, carrying a mutation in its ER lumenal domain (Plemper et al., 1998) as well as in the degradation of Sec61-2 (Bordallo et al., 1998), a mutated translocation channel protein with a defect in an ER membrane segment (Carvalho et al., 2006). Hrd1/Der3 is linked to Hrd3, a type I transmembrane protein composed of a large N-terminal ER lumenal domain, a single transmembrane span and a short C-terminal cytosolic region (Gardner et al., 2000; Plemper et al., 1999a). Together with Yos9 acting as a gatekeeper, Hrd3 is thought to be responsible for handing over mannose trimmed Man_7-$GlcNAc_2$ containing misfolded proteins to the Hrd1/Der3 ligase for polyubiquitylation (Clerc et al., 2009; Denic et al., 2006; Gauss et al., 2006; Quan et al., 2008) (Fig. 2). Interestingly, an Hrd1/Der3- Hrd3 ligase complex without Yos9 was found which might be responsible for the delivery of lumenal, non-glycosylated proteins to degradation (Gauss et al., 2006). Cells lacking Hrd3 cannot degrade CPY*. A *HRD3* deletion leads to rapid digestion of the Hrd1/Der3 ligase. Most interestingly, however, overexpression of the Hrd1/Der3 ligase in the absence of Hrd3 leads to the recovery of CPY* degradation (Plemper et al., 1999a). An Hrd-Der ligase complex of the ER membrane has been defined which is composed of the Hrd1/Der3 ubiquitin ligase with its interaction partner Hrd3, as well as Usa1, which connects the four transmembrane domain protein Der1 with the ligase (Carvalho et al., 2006; Gauss et al., 2006; Hitt and Wolf, 2004; Knop et al., 1996a) (Fig. 2). Usa1 has been described as a double spanning ER membrane protein with cytosolic N- and C-termini. At the N-terminus it possesses a ubiquitin-like (UBL) domain. A recent study uncovered that the C-terminus of Usa1 interacts with Der1, while the N-terminus directly contacts the Hrd1/Der3 ligase at its very C-terminus while the ligase itself stays in contact with Hrd3 and through this also with Yos9 (Horn et al., 2009) (Fig. 2). Another study shows direct interaction of Usa1 to both Hrd3 and Hrd1/Der3 (Kim et al., 2009). Bridging of Der1 to the Hrd1/Der3 ligase via Usa1 is essential for the degradation of ER lumenal misfolded proteins. The N-terminus of Usa1 induces oligomerization of the Hrd1/Der3 ligase, necessary for the degradation of some misfolded ER membrane proteins, but not required for elimination of misfolded ER-lumenal proteins (Horn et al., 2009). Interestingly, the mammalian orthologues of Der1, Derlin-1, Derlin-2 and Derlin-3 are required for efficient proteolysis of both,

soluble and transmembrane ERAD substrates (Lilley and Ploegh, 2004; Oda et al., 2006; Ye et al., 2004; Younger et al., 2006).

Recently the translocon Sec61 has been shown to interact with central components of the Hrd-Der ligase complex indicating its participation in retrotranslocation of misfolded ERAD substrates with a lumenal misfolded domain (Schäfer and Wolf, 2009). This extends the Hrd-Der ligase complex to a retrotranslocation complex (RTC) (Fig. 2): The RTC connects retrotranslocation with polyubiquitylation. The finding of Sec61 biochemically interacting with components of the Hrd-Der ligase complex complements previous genetic studies which assigned a function of the translocon to degradation of ER-lumenal proteins (Plemper et al., 1997; Plemper et al., 1999a; Plemper and Wolf, 1999; Schäfer and Wolf, 2009; Willer et al., 2008).

While the core components of the ligase complex Hrd1/Der3, Hrd3, Usa1 and Der1 are required for degradation of all soluble substrates with an ER lumenal misfolded domain (ERAD-L substrates, see above), both proteins Usa1 and Der1 were found to be dispensable for the elimination of Pdr5*, Sec61-2 and HMG-CoA reductase. All these proteins are characterized as ERAD-M substrates (Carvalho et al., 2006). The polytopic membrane substrate Pdr5* has a misfolded lumenal domain, which may extend into the membrane. Sec61-2 carries most likely a misfolded membrane section and HMG-CoA reductase undergoes intramembrane domain misfolding upon regulation by farnesol (Shearer and Hampton, 2005). The fact that Usa1 was not required for degradation of these membrane substrates as published in Carvalho et al., 2006 (Carvalho et al., 2006) is in contrast to the results of Horn et al., 2009 (Horn et al., 2009). The latter authors attribute the necessity of oligomerization of the Hrd1/Der3 ligase by Usa1 to its potential to degrade membrane substrates. For the recognition of misfolded ERAD-M substrates specific hydrophilic amino acid residues within the multi-membrane spans of the Hrd1/Der3 ubiquitin ligase are required (Sato et al., 2009).

In the cytosol, the homohexameric AAA-ATPase Cdc48p (p97 in mammals) and its substrate recruiting factors Ufd1 and Npl4 provide the driving force for final extraction of polyubiquitylated misfolded proteins from the ER membrane (Bays et al., 2001b; Braun et al., 2002; Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001). Ubx2, an ER membrane protein with two membrane spans enables the binding of the Cdc48 complex to the retrotranslocation complex. Its cytoplasmic N-terminal ubiquitin-associated (UBA) domain is important for binding to ubiquitylated ERAD substrates while a C-terminal UBX (ubiquitin-regulatory X) domain is necessary for

recruiting the Cdc48 complex to the ER membrane.(Neuber et al., 2005; Schuberth et al., 2004) The UBX domain containing protein Ubx4 modulates the Cdc48-Ufd1-Npl4 complex loaded with polyubiquitylated proteins to guarantee its correct function (Alberts et al., 2009). In the cytosol the two UBA-UBL domain ubiquitin receptor proteins Dsk2 and Rad23 are required for further delivery of polyubiquitylated proteins to the proteasome (Medicherla et al., 2004; Richly et al., 2005) (Fig. 2).
A proteasome bound E4 ligase, Hul5 (Crosas et al., 2006) was found to be required for degradation of the ERAD substrate CTL*, a CPY* fusion protein spanning the ER membrane and containing the enzyme 3-isopropylmalate dehydrogenase at the cytoplasmic side of the ER. It may be involved in the extension of the ubiquitin chain of the substrate (Kohlmann et al., 2008).

Ubiquitylation and degradation of ER substrates containing a misfolded cytoplasmic domain: The ubiquitin ligase Doa10

ER proteins carrying cytosolic misfolded domains as degradation signals (ERAD-C substrates in yeast) are degraded by the ER membrane located ubiquitin ligase Doa10 (Carvalho et al., 2006; Huyer et al., 2004; Vashist and Ng, 2004) (Fig. 2). Doa10 (degradation of alpha2) was found in a screen for factors involved in degradation of proteins containing the Deg1 domain of the soluble short-lived transcriptional repressor Matα2 (Swanson et al., 2001). Doa10 is a 151 kDa ER/nuclear envelope protein with 14 transmembrane domains and an N-terminal RING-finger (Kreft et al., 2006). No additional helper proteins of this ubiquitin ligase are known. The enzyme works together with the E2 enzymes Ubc6 and Ubc7. While Ubc6 contains a transmembrane domain and is therefore linked to the ER, the enzyme Ubc7 is recruited to the ER membrane by Cue1 (Biederer et al., 1997) (see previous chapter). The requirement of membrane substrates for polyubiquitylation by Doa10 is often not absolute: In addition to Doa10 also the Hrd1/Der3 ligase is often involved in the degradation process (Gnann et al., 2004; Huyer et al., 2004). The ubiquitylation function of Doa10 is not only limited to ERAD substrates (Swanson et al., 2001; Vashist and Ng, 2004). Also mutated nuclear envelope proteins, soluble nuclear proteins, as well as synthetic cytoplasmic proteins fused to the Deg1 domain (Ravid et al., 2006) or to another degron called CL1 (Metzger et al., 2008) are substrates of Doa10. The ERAD-C pathway using the ubiquitin ligase Doa10 and the ERAD-L and ERAD-M pathways, which make use of the Hrd1/Der3 ubiquitin ligase, merge at the Cdc48-Ufd1-Npl4 complex segregating the polyubiquitylated substrates

from the ER membrane for further delivery to the proteasome (see previous paragraph; Fig. 2).

Mammalian E3s involved in ERAD

Due to the easy amenability to biochemical, genetic and molecular biological methods the yeast *Saccharomyces cerevisiae* has been the model and a pacemaker in the elucidation of the mechanisms of polyubiquitylation in the ERAD pathway. Several E3 ligases being involved in ERAD have been described in mammalian cells but in many cases little is known about their substrates and their reaction mechanism.

Two structural orthologues of the yeast Der3/Hrd1 ligases are known: HRD1 (or Synoviolin) and gp78 (also known as RNF45 or AMFR; Table 1).

HRD1 has been described as an orthologue of yeast Hrd1/Der3 (Nadav et al., 2003). The enzyme is known to function together with the E2 Ube2g2 *in vitro* but no conjugating enzyme working together with HRD1 *in vivo* has been described yet (Kikkert et al., 2004). It is involved in the degradation process of the ERAD substrates TCR-α, CD3-δ (Kikkert et al., 2004), unassembled Ig*μ* chains (Cattaneo et al., 2008) and a non-glycosylated variant of the Igκ light chain (Okuda-Shimizu and Hendershot, 2007). Also cytosolic proteins like serum- and glucocorticoid-induced kinase 1 (Sgk1) (Arteaga et al., 2006) or tumor suppressor gene p53 (Yamasaki et al., 2007) were shown to be ubiquitylated via HRD1.

gp78 was the first E3 ligase found in the ER membrane of mammals (Fang et al., 2001). In comparison to HRD1 it possesses a G2BR (UBE2G2-binding region) that enables the enzyme to recruit the ubiquitin-conjugating enzyme UBE2G2 (Chen et al., 2006). As in the case of HRD1, substrates of gp78 are the unassembled subunits of the T-cell receptor TCR-α and CD3-δ (Chen et al., 2006; Fang et al., 2001). In addition, gp78 seems to be the mammalian E3 that is able to ubiquitylate HMG-CoA reductase in a sterol regulated fashion (Song et al., 2005). Two recent studies showed that HRD1 targets gp78 for ubiquitin-proteasome dependent degradation (Ballar et al., 2009; Shmueli et al., 2009). Fang and co-workers also proposed a role of gp78 in the degradation of the mutant form of cystic fibrosis transmembrane conductance regulator (CFTRΔF508). Silencing of gp78 leads to accumulation of CFTRΔF508 (Ballar et al., 2009).

TEB4 (or MARCH VI) is a mammalian protein with homology to yeast Doa10 (Hassink et al., 2005; Kreft et al., 2006). It is a multi membrane spanning protein of

the ER with a RING finger domain. TEB4 was shown to be able to auto-ubiquitylate with the help of Ube2g2, by this inducing its own degradation (Hassink et al., 2005). A recent study revealed that TEB4 is involved in ubiquitylation of type 2 iodotyronine deiodinase (D2), which is the key thyroid hormone-acivating deiodinase (Zavacki et al., 2009). This enzyme was also shown to be ubiquitylated by a SOCS-box containing ligase called WSB-1 (Dentice et al., 2005), suggesting tissue specific and expression dependent parallel pathways of ubiquitylation.

Trc8 is another ER membrane RING finger containing ubiquitin ligase, which was originally identified as a tumor suppressor associated with hereditary renal cell carcinoma (Gemmill et al., 1998). In addition the enzyme has sterol-sensing capacity (Irisawa et al., 2009). Recently it was shown that the US2 and US11 proteins of human cytomegalovirus trigger Trc8 to ubiquitylate the major histocompatibility complex class I (MHC I) receptor leading to its dislocation and degradation by the 26S proteasome (Stagg et al., 2009). With this mechanism cytomegalovirus misuses the ERAD system and Trc8 to reduce the overall abundance of MHC class I receptors on the cell surface to escape from immune response (Wiertz et al., 1996).

A recent study revealed Rfp2 to be an additional ERAD ligase. The Rfp2 gene is reported to be frequently lost in various malignancies including subtypes of lymphoma, myeloma and several solid tumors making it a tumor suppressor gene candidate. Rfp2 is localized to the ER via a C-terminal transmembrane domain. It contains a RING domain and was shown to ubiquitylate the heterologously expressed proteolytic substrate CD3-δ and showed autoubiquitylation activity *in vitro* (Lerner et al., 2007). Native substrates of this ligase have not yet been described.

In addition, there are E3 ligases involved in ERAD of mammalian cells that are not ER membrane proteins but located in the cytosol. An example is the CHIP ligase which cooperates with membrane-bound RMA1 (RNF5) to target CFTRΔF508 for degradation via ERAD.(Younger et al., 2006). RMA1 was also shown to act upstream of gp78 in ubiquitylation of CFTRΔF508 suggesting that gp78 has an E4-like activity in this process (Morito et al., 2008).

An additional example of such a cytosolic ligase is the two RING finger and cysteine-rich In-Between-RING fingers (IBR) region containing protein Parkin. A mutated version of Parkin is one of the main reasons for hereditary Parkinson's disease. The protein acts as an ubqiuitin ligase for polyubiquitylation of the Parkin-associated endothelin receptor- like receptor (Pael-Receptor) (Imai et al., 2001). This receptor is

polyubiquitylated by HRD1 as well, suggesting that these two ligases, Parkin and HRD1, function in a common pathway (Omura et al., 2006).

Conclusion

The different ubiquitylation systems used by the ER to remove misfolded proteins of the lumen and the membrane reflect the different tasks of recognizing the multitude of misfolded proteins with their many different misfolded domains on different sides of the ER to be able to finally send them to the proteasome. Here our understanding of the recognition processes is still very limited and requires intensive further research.

Acknowledgements

We thank Alexandra Stolz, Elena Martinez Benitez and Konrad Otte for reading of the manuscript. The work of the authors was supported by grants of the Deutsche Forschungsgemeinschaft, Bonn, and the European Network of Excellence RUBICON.

Keywords

Protein quality control

ER-associated degradation (ERAD)

Ubiquitin

Hrd-Der ligase complex

Ubiquitin ligases (E3's)

- Hrd1/Der3
- Doa10
- HRD1
- gp78
- Parkin
- CHIP
- TEB4

Retrotranslocation complex

Sec61 translocon

Chaperones

- Kar2/ BiP
- ERdj5

Cdc48-Npl4-Ufd1 complex

N-glycosylation

Carbohydrate trimming

Lectins

- Yos9
- OS-9
- XTB-3B

Glucosidase

Mannosidase

- Htm1/ Mnl1
- Mns1

ERAD substrates

- CPY*
- Pdr5*
- CFTRΔF508
- MHC class I receptor
- TCR-α
- CD3-δ
- HMG-CoA reductase
- Sgk1
- p53
- Pael-Receptor
- Type 2 iodotyronine deiodinase (D2)
- Ig*μ* chains
- Igκ light chain

Der1

Derlins

Usa1

Table 1. Mammalian orthologues of yeast proteins involved in ERAD.

Yeast	Mammals	References
Kar2	BiP/Grp78	Fewell et al., 2001; Nishikawa et al., 2001; Taxis et al., 2003

Yos9	OS-9, XTP-3B	Bernasconi et al., 2008; Buschhorn et al., 2004; Christianson et al., 2008; Denic et al., 2006; Gauss et al., 2006; Hosokawa et al., 2009
Htm1/ Mnl1	EDEM1, EDEM2, EDEM3	Clerc et al., 2009; Quan et al., 2008; Ruddock and Molinari, 2006
Der1	Derlin-1, Derlin-2, Derlin-3	Hitt and Wolf, 2004; Knop et al., 1996a; Lilley and Ploegh, 2004; Oda et al., 2006; Wang et al., 2006; Ye et al., 2004
Usa1	HERP	Carvalho et al., 2006; Horn et al., 2009
Hrd3	SEL1L	Hampton et al., 1996; Mueller et al., 2008; Mueller et al., 2006
Hrd1/ Der3	HRD1 (Synoviolin), Gp78	Bordallo et al., 1998; Deak and Wolf, 2001; Hampton et al., 1996; Chen et al., 2006; Fang et al., 2001; Kikkert et al., 2004; Nadav et al., 2003
Doa10	TEB4 (MARCH-IV)	Kreft et al., 2006; Swanson et al., 2001
Ubc6	Ube2g1	Hiller et al., 1996; Oh et al., 2006; Tiwari and Weissman, 2001
Ubc7	Ube2g2	Biederer et al., 1997; Chen et al., 2006; Hassink et al., 2005; Hiller et al., 1996; Kikkert et al., 2004
Ubx2/ Sel1	KIAA0887?	Neuber et al., 2005; Schuberth et al., 2004
Ubx4	TUG (ASPCR1/ UBXD9)?	Alberts et al., 2009; Schuberth and Buchberger, 2008
Cdc48	P97/ VCP	Bays et al., 2001b; Braun et al., 2002; Jarosch et al., 2002; Kothe et al., 2005; Rabinovich et al., 2002; Wang et al., 2006; Ye et al., 2001
Ufd1	UFD1	Meyer et al., 2002; Ye et al., 2001, 2003
Npl4	NPL4	Meyer et al., 2002; Ye et al., 2001, 2003
Dsk2	PLIC-1, PLIC-2	Kleijnen et al., 2003; Medicherla et al., 2004

Rad23	hHR23A and B	Chen and Madura, 2006; Medicherla et al., 2004

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5. Acknowledgments

This PhD work would not have been possible without the support of many people. My thanks go to all of them.

I would like to thank Professor Dieter H. Wolf for giving me the opportunity to work in his lab for my doctoral thesis and allowing me to do research in the fascinating ubiquitin proteasome field. I am thankful for many scientific discussions, for his inspirations, encouragements, patients, support and advice. I am also thankful for giving me a lot of freedom, which allowed me to pursue own scientific ideas and to develop creativity and a sense of criticism.

I wish to thank Sae-Hun Park for introducing me to the cytoplasmic quality control system and to my colleagues Mario Scazzari, Ingo Amm, Kathrin Deuschle, Derrick Norell and Corinna Kübler for many discussions and for co-operation in the project.

I am grateful to all current and former members of the lab, especially Antje Schäfer, Alexandra Stolz, Elena Martinez-Benitez, Alexander Becher, Stefanie Besser, Sven Alberts, Olivier Santt, Lise Barbin, Sonia Barbossa, Bernhard Braun, Ruth Menßen-Franz, Regina Philipp, Andrea Spitzner, Dragica Kapucija and Elisabeth Tosta for the fantastic working atmosphere.

I am also thankful to Wolfgang Hilt and Hans Rudolph for many fruitful scientific discussions and advices.

I am grateful to my family especially to my beloved wife Anja for her infinite support and encouragement.

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- Topic: "Protein quality control in the cytoplasm“
- Diploma thesis at the Institute of Biochemistry, University of Stuttgart - 2005
- Topic: ERAD of membrane attached misfolded CPY (CPY*): Importance of the cytosolic tail“
- Student of technical Biology, Diploma degree, University of Stuttgart - 1999-2005
- Student research project in the laboratory of Prof. Dr. Philip Coffino, University of California, San Francisco (UCSF) - Nov 2003 - May 2004
- Topic: Tethering substrate to the 26S proteasome“
- A-Levels ("Abitur"), Jörg-Ratgeb-Gymnasium, Stuttgart - 1998

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- Guerra, L., Guidi, R., Slot, I., Callegari, S., Sompallae, R., Pickett, C. L., Astrom, S., Eisele, F., Wolf, D., Sjogren, C., Masucci, M. G., and Frisan, T. (2011) Bacterial genotoxin triggers FEN1-dependent RhoA activation, cytoskeleton remodeling and cell survival. *J Cell Sci* **124**, 2735-2742
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