Protein degradation in saccharomyces cerevisiae: studying connections between degradation, N-glycosylation and protein complexes using mass spectrometric tools

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PROTEIN DEGRADATION IN SACCHAROMYCES CEREVISIAE

Studying connections between degradation, N-glycosylation and protein complexes using mass spectrometric tools

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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Summary

Protein degradation is crucial for many cellular processes, e.g. signal transduction, cell cycle regulation, and cell differentiation. Consequently, it fulfills several tasks: It eliminates misfolded proteins, regulates amounts of cell cycle proteins, and might even ensure correct ratios for the subunits of multi-protein complexes. The aim of this thesis was to analyze protein degradation in *Saccharomyces cerevisiae* and its connection to several cellular processes, such as asparagine-linked glycosylation (N-glycosylation) and protein complex assembly. To this end we developed and used mass spectrometric methods.

The first chapter gives an overview of protein degradation in the yeast endoplasmic reticulum (ER). This process is termed ER-associated degradation (ERAD) and eliminates misfolded, mutated, and unassembled proteins from the ER. It is part of the stress response that ensures ER homeostasis. Furthermore, the fundamentals of N-glycosylation, the role of the oligosaccharyl transferase complex (OST), and dynamics of protein complexes are explained. In a last part, an overview of different mass spectrometry methods and fragmentation techniques is given.

In the second chapter we coupled a selected reaction monitoring (SRM) method to stable isotope labelling (SILAC) to measure protein degradation of medium- to low-abundant yeast proteins. We focused on protein complexes located in the ER membrane, especially the OST complex. Using our SILAC-SRM method we discovered that, while constitutive levels of ERAD were low, excess complex subunits were degraded. Furthermore, using a SILAC approach we were able to derive an assembly model for the OST complex and to propose a potential evolutionary role for protein degradation.

Our studies of the OST complex brought about important findings on complex dynamics. We discovered that overexpressed subunits did not disturb complex assembly while deletion of
essential subunits destabilized the complex. Chapter 3 focused on extending those findings and investigating if the same principles applied also for other protein complexes. To this end we analyzed datasets from several global overexpression and deletion studies for enrichment in complex subunits. Our results were in agreement with the basic principles discovered for yeast OST. In the same chapter, we further extended our studies of OST, confirming that the stt3-7 mutation affected complex formation and presenting an approach to determine which subunits mainly stabilized each other.

In the fourth chapter we assessed if a defect in lipid-linked oligosaccharide biosynthesis (Δalg3), or in the unfolded protein response (Δire1) or elevated temperatures would lead to global changes in protein levels. Decreased protein levels might indicated an increase in degradation rates. However, protein levels remained largely constant under the conditions tested, indicating that protein degradation was not increased globally. Furthermore, we conducted a pilot study to determine if a data-independent mass spectrometry approach could be implemented for future degradation rate measurements.

Chapter 5 presents the PTM MarkerFinder software. This software relies on the detection of low-mass HCD-fragments to pinpoint MS/MS spectra of post-translationally modified peptides. Manual annotation of such spectra is often requested when publishing post-translational modification studies. PTM MarkerFinder not only facilitates retrieval of promising spectra, but also their annotation.

The thesis is concluded by Chapter 6 which summarizes the results gathered from the different projects, discusses the conclusions drawn and provides an outlook on potential research directions.
Zusammenfassung


gering war, dass aber überschüssige Komplex-Untereinheiten abgebaut wurden. Ausserdem wendeten wir einen SILAC-Ansatz an mit dem es uns gelang ein Modell für die Assemblierung des OST-Komplexes aufzustellen und eine mögliche evolutionäre Rolle für Proteinabbau aufzudecken.


Im fünften Kapitel stellen wir die PTM MarkerFinder Software vor. Dieses Programm erkennt MS/MS Spektren von posttranslational modifizierten Peptiden anhand von Fragmenten mit kleiner Masse die bei HCD-Fragmentierung entstehen. Manuelle Annotierung solcher Spektren ist oft nötig um die Ergebnisse globaler Massenspektrometrie-Studien von posttranslationalen Modifikationen veröffentlichen zu können. PTM MarkerFinder erleichtert nicht nur das Auffinden vielversprechender Spektren, sondern auch deren Annotierung.

Die Dissertation endet mit Kapitel 6 das die Resultate der verschiedenen Projekte zusammenfasst. Ausserdem werden die Schlussfolgerungen diskutiert und es wir ein Überblick gegeben über mögliche zukünftige Forschungsschwerpunkte.
Chapter 1:

Introduction
1. Oligosaccharyl transferase and N-glycosylation

1.1. N-glycosylation: Overview and biological importance

Asparagine-linked glycosylation (N-glycosylation) is a conserved and one of the most frequent post-translational modifications on proteins and is involved in a host of biological processes (Apweiler et al., 1999). In the model organism Saccharomyces cerevisiae N-glycosylation proceeds via several stages. In a first phase, asparagine-linked glycosylation (ALG) gene products assemble an oligosaccharide from nucleotide activated sugar residues such as UDP-GlcNAc and GDP-Mannose (GDP-Man) (Figure 1). The resulting lipid linked oligosaccharides (LLO) are anchored into the ER membrane by dolicholpyrophosphate and locate to the cytoplasmic side of the ER. Subsequently, the Man5GlcNAc2 precursor is flipped to the ER lumen, possibly by Rft1p (Helenius et al., 2002). LLO assembly is completed within the organelle by ALG glycosyltransferase using Dol-P-Man and Dol-P-Glc as substrates. In a final step, the oligosaccharyl transferase (OST) enzyme complex transfers Glc3Man9GlcNAc2 glycan to asparagine (N) residues that are part of N-X-S/T motives of proteins. Around two thirds of all proteins contain N-X-S/T motives (where X can be all amino acids except proline) but not all of them are glycosylated (Apweiler et al., 1999). One reason is that only secreted proteins passing through the ER come in contact with OST and can be N-glycosylated. But also in well documented glycoproteins, only two thirds of glycosites are occupied. Future studies on the OST complex will hopefully explain the underlying mechanism.

Oligosaccharyl transferase can be positioned in close proximity to the Sec61 translocon channel that associates with ribosomes and imports freshly synthesized proteins to the ER (Pfeffer et al., 2014). This is in agreement with the fact that most N-glycosylation takes place co-translationally before folding (Ben-Dor et al., 2004; Chen et al., 1995; Rothman and Lodish, 1977). Therefore, N-glycosylation can influence the folding, structure, stability, and solubility of proteins (Gauss et al., 2011; Imperiali and O'Connor, 1999; Jakob et al., 1998; Kundra and Kornfeld, 1999; O'Connor and Imperiali, 1996; Spear and Ng, 2005; Wormald and Dwek, 1999). Moreover, N-glycosylation can also affect oligomerisation, localization, and activity of proteins (Qi et al., 2014; Rajagopalan et al., 2010; Watson et al., 1999). These diverse functions on the protein-level are reflected in the importance of N-glycosylation for many cellular functions: Glycosylation is involved in ER-quality control of protein folding, the rigidity of organism cell walls, cell adhesion, defense against pathogens, and immune recognition of viruses among other processes (Bleuler-Martinez et al., 2011; Denobel et al., 1990; Ferris et al., 2014; Scott et al., 2012; Wei et al., 2003).
As a consequence, glycosylation defects lead to disease and impaired fitness in yeast, plants, and animals (Ohtsubo and Marth, 2006; Schulz et al., 2009). In humans, these conditions are termed congenital disorders of glycosylation (CDG) and the different diseases are classified into two groups, depending if the impairment occurs before (type 1) or after glycan transfer by OST (type 2). Alterations in LLO assembly and in OST subunits fall into the first category. Regarding OST, only defects in the human Ost3p, Ost6p, Wbp1p, and Swp1p homologs (N33/Tusc3 and IAP, OST48, and ribophorin II, respectively) have been observed (Garshasbi et al., 2008; Jones et al., 2012; Molinari et al., 2008; Vleugels et al., 2009). Also mutations in glycan biosynthesis genes (e.g. in the ALG genes or RFT1) can lead to CDGs (Cylwik et al., 2013; Haeuptle et al., 2008). Severe, pleiotropic morphogenic and metabolic defects, as well as mental retardation and impaired development are common manifestations of most CDGs, underlining the importance of N-glycoproteins in the development of the brain and other organs (Freeze et al., 2012).
1.2. Oligosaccharyl transferase

1.2.1. Overview and composition of oligosaccharyl transferases

The oligosaccharyl transferase is the central enzyme of N-glycosylation and transfers glycans from LLOs to asparagine residues that are part of N-X-S/T motives on proteins. N-glycosylation is preserved among all domains of life. Thus, many organisms ranging from archa to humans have enzymes fulfilling the oligosaccharyl transferase functions. However, already among different eukaryotes, these oligosaccharyl transferase enzymes vary greatly in structure, in the glycans they transfer, and in their composition (Kelleher et al., 2007; Kelleher and Gilmore, 2006; Samuelson et al., 2005). Oligosaccharyl transferases consisting of only an Stt3p homolog are present in some protozoans such as the pathogen *Leishmania major*. In turn, *L. major* contains four Stt3p isoforms termed LmSTT3A, LmSTT3B, LmSTT3C, and LmSTT3D which might be active at different stages during development. LmSTT3D has a relaxed LLO specificity and is able to also transfer Man5-7GlcNAc2 as well as full length Glc3Man9GlcNAc2 glycans when expressed in yeast (Nasab et al., 2008).

The OST enzyme from *S. cerevisiae*, on the other hand, has a more complex architecture. It is an eight-subunit enzyme complex located in the ER membrane and preferentially transfers Glc3Man9GlcNAc2 glycans (Figure 2). Components include Ost1p, Ost2p, Ost3p, Ost4p, Ost5p, Ost6p, Swp1p, Wbp1p, and Stt3p (Knauer and Lehle, 1999). Ost3p and Ost6p are mutually exclusive and thus lead to two different forms of OST in yeast (Spirig et al., 2005). Ost1p, Ost2p, Swp1p, Wbp1p, and Stt3p are essential proteins and their deletion is lethal. Deletion of Ost3p, Ost4p, Ost5p, or Ost6p, on the other hand, causes a growth phenotype but cells will still be viable.

Figure 2. The oligosaccharyl transferase complex.

OST is located in the ER-lumen and transfers glycans onto asparagines of proteins that are transported into the ER by the Sec61 translocon. The complex consists of eight subunits including Ost1p, Ost5p, Stt3p, Ost4p, Swp1p, Wbp1p, Ost2p and the mutually exclusive Ost3p and Ost6p which have been reported to form three subcomplexes, depicted in yellow, red, and green.
Deletion of an essential OST subunit in yeast can be complemented by expressing LmSTT3D. LmSTT3D expressed in yeast cells glycosylates yeast N-X-S/T sequons without incorporating into the yeast OST complex (Nasab et al., 2008). Thus, cells with a non-functional yeast OST are viable in the presence of LmSTT3D protein. This raises the question why an eight-subunit complex evolved if a single-subunit OST can compensate its functions. An enzyme complex might allow a higher concentration of substrates at the catalytic center through the action of additional subunits compared to single subunit oligosaccharyl transferases. Moreover, additional subunits might allow for a more subtle regulation of the OST and different complexes can glycosylate different proteins or might regulate the selection of specific LLO substrates (Kelleher et al., 2007; Schulz et al., 2009).

### 1.2.2. Functions of the OST components

This section focuses on the functional characterization of components of the yeast OST. Only few OST subunits have been functionally characterized: Stt3p is the catalytic subunit that attaches glycans to amides on asparagine residues. Despite their low sequence identity, STT3 proteins of different organisms have similar predicted structures. This indicates that also structural motives involved in enzyme functionality might be conserved (Jaffee and Imperiali, 2011; Lizak et al., 2013; Lizak et al., 2011). Recently, the formation of a twisted amide has been proposed as the catalytic mechanism of a bacterial Stt3p homolog (Lizak et al., 2013). It is likely that a similar mechanism is used by the eukaryotic enzyme.

The Ost3p and Ost6p proteins have been proposed to function as oxidoreductases that delay folding and thus facilitate glycan attachment in yeast and in humans (Jamaluddin et al., 2011; Mohorko et al., 2014; Schulz et al., 2009). The OST subpopulations containing either Ost3p or Ost6p associated with either the Sec61p or the Ssh1p translocons (Yan and Lennarz, 2005). Probably due to differences in their peptide binding grooves or in consequence of their association with different translocons, the Ost3p and Ost6p complexes glycosylated different protein sites (Jamaluddin et al., 2011; Schulz and Aebi, 2009). Ost3p and Ost6p have been shown to associate with Stt3p (Spirig et al., 2005; Spirig et al., 1997).

For all other OST subunits the function is less clear. Ost4p consists only of a short membrane stalk with no discernible catalytic domain (Gayen and Kang, 2011). It might therefore mainly have a structural function by anchoring other components into the complex (Dumax-Vorzet et al., 2013). Also Ost5p does not contain large luminal domains and its function might mainly be as structural component. For Ost1p, studies suggested that its mammalian homolog ribophorin...
I associated with ribosomes and facilitated delivery of proteins to the catalytic center of OST (Wilson and High, 2007; Wilson et al., 2008; Yu et al., 1990). Deletion of mammalian Wbp1p (OST48), Ost2p (DAD1), and Swp1p (ribophorin II) homologs caused destabilization of the OST complex and a subcomplex of these three proteins might contain a regulatory LLO binding site (Kelleher et al., 2007; Pathak et al., 1995).

1.2.3. Subunit structures and OST complex architecture

Molecular structures of the OST subunits can give insight into their functions (Fetrow et al., 2001). However, such structures are only available for Ost4p and the soluble domains of Ost6p, Ost3p, and of Stt3p from yeast (Gayen and Kang, 2011; Huang et al., 2012; Mohorko et al., 2014; Schulz et al., 2009). In addition, the crystal structures of three single-subunit glycosyl transferases, comprising the soluble domains of archeal AglB and of PglB from C. jejuni, as well as full length PglB from C. lari, are known. Despite their rather low sequence homology with yeast Stt3p, they can serve as promising templates for structural modelling of Stt3p (Igura et al., 2008; Lizak et al., 2011; Maita et al., 2010; Matsumoto et al., 2013).

Apart from the structures of the single subunits, also the architecture of the whole OST complex is of interest. It has been reported that Ost1p-Ost5p, Wbp1p-Ost2p-Swp1p, and Stt3p-Ost4p-Ost3p/Ost6p form three subcomplexes in yeast (Karaoglu et al., 1997; Kelleher and Gilmore, 2006; Reiss et al., 1997; Silberstein et al., 1995; Spirig et al., 2005; Spirig et al., 1997; te Heesen et al., 1993). Despite several efforts, the interaction sites between subunits have not been conclusively determined. Two low resolution structures have been acquired for yeast and canine oligosaccharyl transferases (Li et al., 2008; Pfeffer et al., 2014). The mammalian OST was shown to closely localize with the Sec translocon and the ribosome, however, none of the two studies provided detailed insights into catalysis mechanism or subunit connectivity. Neither of the structures confirmed earlier reports that the OST complex consisted of two octamers (Chavan et al., 2006). Therefore, we assume that in one molecule of the complex one copy of each subunit is present (Karaoglu et al., 1997).
2. Protein complexes

2.1. Regulation of subunit stoichiometry

*S. cerevisiae* has been reported to encode several thousand protein complexes (Sun et al., 2009). Assembling proteins in a complex has benefits such as increasing substrate concentrations at the catalytic center through the function of accessory, substrate-binding subunits or close proximity of all enzymatic reactions. Furthermore, by assembling subunits into an enzyme complex, enzyme activity will most likely remain the same even if one subunit is overexpressed. Despite their cellular importance, information on complex dynamics is relatively scarce.

Complex subunits are produced as individual proteins that are folded and finally are assembled into a hetero- or homomeric complex. How does the cell make sure that subunits are present in the concentrations reflecting their ratios in the assembled complex? Yeast does not have a feedback mechanism to control levels of most proteins (Springer et al., 2010). Therefore, the component levels are either adjusted through degradation, or they are already expressed at the correct levels. Recently, this question has been addressed in *E. coli*, yeast and mammalian cells (Li et al., 2014; Schwanhausser et al., 2011). For all three organisms, it was shown that levels of most proteins were mainly regulated by protein synthesis. I.e. in *E. coli* and yeast, synthesis rates for different subunits largely reflected their stoichiometries in the complex.

2.2. Principles of subunit stability and complex assembly

The complex components need to be assembled into the final complex. Adopting an unstructured assembly process where subunits bind to each other in a random order would be inefficient since it would give rise to diverse subpopulations of partially assembled complexes that might be deleterious to the cell. For example they might engage with the substrate but might not be able to complete the enzymatic reaction and therefore compete with the full complex. The situation would become particularly critical if, through genetic imbalance, one subunit was overexpressed, increasing the amount of partially assembled complexes and reducing the amount of the complete complex. Therefore, cells need to optimize the assembly pathway to favor formation of the complete complexes over partially assembled complexes. An obvious way to shift reaction equilibria towards the complete complex is to stabilize the final assembly product while intermediates are less stable and degraded.
Based on findings from *E. coli* (cytochrome b03, divisome), yeast, and plants, Daley reviewed membrane protein complex assembly in 2008 and discussed which general principles it follows (Daley, 2008). In summary, assembly is an ordered process where subunits enter the complex in a defined sequence. Unassembled subunits are in general unstable and assembly proceeds via subcomplexes until formation of the complete complex confers maximum stability. Often, the assembly process is coordinated by chaperones, e.g. for the yeast F1 F0-ATP synthase.

Several biological examples confirm the hypothesis that unassembled subunits are degraded and that incorporation into the complex confers stability to its components. One example for this principle comes from the yeast mating type factors: Heterodimerization of MATα2 with MATα1 buries degradation signals and stabilizes the two proteins that would be degraded when present on their own (Johnson et al., 1998). Likewise, the yeast Vph1p protein that is part of the V-ATPase is degraded if not assembled into the complex in the ER (Hill and Cooper, 2000). Another example from yeast is Ndc10p, a component of the kinetochore, whose ndc10-2 variant is not incorporated into the complex and therefore is rapidly degraded in an ubiquitin-independent manner (Kopski and Huffaker, 1997). Also in *E. coli* unassembled subunits are degraded, e.g. the ribosomal protein L10 if it is present in excess (Petersen, 1990). Further examples are complex components that are degraded by the FtsH protease when unassembled, including the SecY subunit of protein translocase and a subunit of the F0 ATPase (Akiyama et al., 1996; Kihara et al., 1995). The same principle applies also to plant and mammalian cells (Berthold et al., 1995; Bonifacino et al., 1990; Bruce and Malkin, 1991; Leto et al., 1985; Lippincott-Schwartz et al., 1988; Ostergsetzer and Adam, 1997; Schmidt and Mishkind, 1983). However, for some complexes subunits are stable even if not incorporated into the complex, including subunits of the photosystem complexes in the thylakoid lumen of pea chloroplasts (Hashimoto et al., 1996).

In summary, cells have mechanisms to distinguish between assembled and unassembled subunits and to degrade the latter. A common degradation signal seem to be hydrophobic domains that are buried when subunits are incorporated in the complex but are exposed in unassembled subunit monomers. The first studies reporting degradation of unassembled T-cell receptor components in rodent cells further led to the discovery of ER-associated degradation (Bonifacino et al., 1990; Lippincott-Schwartz et al., 1988; Needham and Brodsky, 2013).
3. Protein degradation: Biological processes and analytical tools

3.1. ER-associated degradation (ERAD)

3.1.1. Role of ERAD and basic processes during protein degradation

Ribosomes translate mRNA to proteins, some of which are co-translationally transported into the ER through the SEC translocon. Approximately one third of the total proteome passes through the ER (Huh et al., 2003). In the ER, some proteins immediately interact with oligosaccharyl transferase and are glycosylated co-translationally. The attached hydrophilic glycan chains can help folding and stabilize a protein. Molecular chaperones further influence the equilibrium of the folding reaction and help proteins reach their correctly folded conformation. Subsequently, some proteins are assembled into complexes or post-translationally modified. Despite the assistance of chaperones the folding process or incorporation into a complex might be unsuccessful. Therefore, protein conformations are closely monitored by ER-associated degradation (ERAD), a quality control system that will eliminate misfolded proteins (Christianson and Ye, 2014; Thibault and Ng, 2012). The importance of such a quality control system is confirmed by diseases resulting from accumulation of misfolded proteins such as Parkinson and Alzheimer (Kaneko et al., 2012). However, it is important to only target terminally misfolded proteins for ERAD since also degradation of a protein can be harmful (Guerriero and Brodsky, 2012).

The ERAD machinery of yeast and mammals share many homologous components and the basic processes in ERAD are the same (Hirsch et al., 2009). Terminally misfolded proteins are recognized by the ERAD machinery and exported to the cytosolic face of the ER where they are ubiquitinated, targeting them for degradation by the proteasome (Figure 3). Thus, the four stages of ERAD are 1. recognition of misfolded proteins, 2. retrotranslocation to the cytoplasm, 3. ubiquitination, and 4. proteasomal degradation.
Figure 3. Main steps in ERAD.
Proteins are transported into the ER via the Sec61 translocon. Chaperone and other proteins will assist their folding. Misfolded proteins are recognized and translocated to the cytosolic side of the ER where they are ubiquitinated and thus marked for degradation. The Cdc48 ATPase binds the degradation substrate and transports it to the proteasome where it is degraded. Modified from (Bagola et al., 2011).

3.1.2. Recognition of misfolded proteins

Both, accumulation of misfolded proteins, as well as aberrant degradation of native proteins are harmful. Therefore, the ERAD-L, ERAD-M, and ERAD-C systems have evolved to distinguish between native and misfolded proteins with defects in their luminal (L), membrane (M), or cytosolic (C) parts (Figure 4A). However, since lesions in one part of the protein can result in changes in the whole structure the three pathways also have common substrates. Moreover, the structural differences between native and misfolded proteins are sometimes subtle. Therefore, recognition of misfolded proteins can be challenging.

The mechanism for substrate recognition is best understood for ERAD-L of glycoproteins (Figure 4B) (Gauss et al., 2011; Thibault and Ng, 2012). After entry into the ER, OST attaches a Glc3Man9GlcNAc2 to N-X-S/T sequons on proteins. Subsequently, Gls1p and Gls2p remove the glucose residues and Mns1p further trims one mannose, yielding Man8GlcNAc2. Meanwhile the proteins are folding to attain their native conformation. Several chaperones, including the protein disulfide isomerase Pdi1p and Kar2p, assist this process. Ideally, folding is successful and a native protein carrying Man8GlcNAc2 exits the ER. However, folding is not always successful and misfolded glycoproteins need to be recognized and targeted to ERAD. This task is solved by several luminal proteins. While most Pdi1p is monomeric and assists protein folding, a fraction of it forms a complex with the mannosidase Htm1p (Gauss et al., 2011). Upon binding of misfolded proteins to this complex, Htm1p will process the
Man8GlcNAc2 glycan to Man7GlcNAc2. The lectin Yos9p recognizes the terminal α1-6 mannose and in combination with Hrd3p that detects misfolded stretches on the protein, targets the protein for ubiquitination by Hrd1p (Quan et al., 2008).

In ERAD-C, proteins with defects in domains in the cytosol are recognized by Doa10p or by cytosolic chaperones interacting with it, such as Hsp40p and Hsp70p (Nakatsukasa et al., 2008). ERAD-M does not require additional proteins apart from the ubiquitin ligase for substrate recognition. Its substrates directly interact with transmembrane domains (TM) of the Hrd1p ubiquitin ligase which might detect misfolded substrate TM domains exposing hydrophilic residues to the lipid membrane (Sato et al., 2009).

Figure 4: ERAD-L, ERAD-M, and ERAD-C in *S. cerevisiae.*

A) The Hrd1p and Doa10p E3 ubiquitin ligases form two ligase complexes containing diverse components and responsible for processing proteins with lesions in different domains. The Doa10p complex recognizes proteins with cytosolic defects (ERAD-C), while the Hrd1p complex ubiquitinates proteins misfolded in their luminal (ERAD-L) or membrane domains (ERAD-M), modified from (Ruggiano et al., 2014); B) Recognition of misfolded glycoproteins during ERAD-L. N-glycans are processed to GN2Man8 by glucosidases and Mns1p. The misfolded protein is bound by a complex of the Pdi1p chaperone and Htm1p mannosidase which cleaves the N-glycan to GN2Man7. The Man7 signal is recognized by the Yos9p lectin that targets the misfolded protein to Hrd1p-mediated ubiquitination followed by degradation, modified from (Gauss et al., 2011).
3.1.3. Ubiquitin ligase complexes

Once a protein has been recognized as ERAD substrate, it is ubiquitinated at the cytosolic side of the ER. Protein ubiquitination is a step-wise process where E1 enzymes first activate ubiquitin, followed by transfer of ubiquitin to proteins by ubiquitin-conjugating enzymes (E2) and the ubiquitin ligases (E3). The different sets of misfolded proteins from ERAD-L, ERAD-M, and ERAD-C are ubiquitinated by the Hrd1p or Doa10p ubiquitin ligases (Figure 4A).

The two E3 ubiquitin ligases are organized in two separate complexes (Bays et al., 2001; Swanson et al., 2001) (Figure 4A). The Doa10p complex includes Ubc6p and the Hrd1p associates with Ubc1p, both of which are E2 ubiquitin conjugating enzymes. The Hrd1p complex further consists of the lectin Yos9p and Hrd3p that bind to glycan degradation signals or potentially to misfolded stretches of protein, respectively (Gauss et al., 2006; Quan et al., 2008). Moreover, Usa1p associates with Hrd1p and acts as a scaffold that brings Der1p close to the ubiquitin ligase (Horn et al., 2009). Der1p itself is involved in ERAD-L by threading proteins into the ER membrane and targeting them towards Hrd1p for ubiquitination (Mehnert et al., 2014).

Both ubiquitin ligases are further associated with the E2 enzyme Ubc7p and its membrane anchor Cue1p. Additionally, Ubx2p and the Cdc48 AAA ATPase, which transports substrates to the proteasome for degradation, are present in both complexes (Neuber et al., 2005).

3.1.4. Retrotranslocation and processes leading to protein degradation

Ubiquitination is not just a signal for degradation, but also for retrotranslocation since polyubiquitin chains are recognized by the Cdc48 complex. The retrotranslocation process is energy-dependent and probably requires unfolding of the protein. Energy is provided by the Cdc48p AAA ATPase in yeast (Ye et al., 2001). Cdc48p oligomerizes and forms a complex with Npl4p and Ufd1p. The route of retrotranslocation is still unknown and it has been suggested that proteins left the ER through a yet unknown channel. It has been proposed that the Sec61p translocon which transports proteins into the ER could be used in the reverse direction for ER exit of misfolded proteins (Hampton and Sommer, 2012). Alternatively, Der1p or the Hrd1p and Doa10p ubiquitin ligases themselves have been proposed as candidates for retrotranslocation channels. Furthermore, entirely different mechanisms, such as export via lipid droplets have been suggested. It cannot be excluded that different modes of retrotranslocation transport different proteins, just as different mechanisms exist for their recognition.
Once retrotranslocation of the ubiquitinated protein to the cytosol is completed, it is polyubiquitinated and deglycosylated by Png1p which generates free oligosaccharides (Thibault and Ng, 2012). Subsequently, Rad23p delivers the protein to the 26S proteasome for degradation.

3.2. Analysing protein degradation

Studies toward the end of the 1980s revealed a degradation system for proteins passing through the secretory pathway and led to the discovery of the ERAD machinery (Lippincott-Schwartz et al., 1988; Needham and Brodsky, 2013). For a long time, ERAD was studied using a limited number of fast-degraded, misfolded proteins. Many of them were model yeast proteins with mutations or partial deletions such as CPY*, PrA*, sec61-2, and Pdr5*. For example PrA* results from an N-terminal deletion in the Pra1p protease (Finger et al., 1993; Spear and Ng, 2005). While wild type PrA is glycosylated in the ER followed by transfer to the vacuole, PrA* is misfolded and degraded by ERAD. Examples of the few known endogenous wild type proteins that are ERAD substrates are HMG-CoA reductase 2 (Hmg2p) and Erg2p, both enzymes involved in sterol biosynthesis (Jaenicke et al., 2011; Jo and DeBose-Boyd, 2010).

Figure 5: Pulse-chase methods to measure protein degradation.

A) Scheme of a radioactive pulse chase. Proteins in cells are labelled radioactively followed by a chase in cold medium where the radioactive signal decreases due to degradation of radioactive proteins, modified from (Geva-Zatorsky et al., 2012); B) Results for a radioactive pulse-chase with the misfolded protein variant Pra*, modified from (Spear and Ng, 2005).

Protein degradation rates were usually assessed using a pulse-chase setup: cellular proteins were radioactively labelled (e.g. with 35S methionine), chased with non-radioactive medium for different time points, immunoprecipitated and analyzed by gel-based methods (Figures 5A, 5B). Alternatively, protein synthesis was inhibited by cycloheximide and decreasing protein levels were visualized by Western blot after SDS-PAGE. The disadvantage of both methods is
that throughput was limited since only a single protein was monitored per experiment. Besides, sometimes proteins were tagged or cellular metabolism was altered by addition of cycloheximid. Both methods might alter protein behavior. Recently, the toolbox for protein degradation analysis has been extended. MS identification of proteins co-immunoprecipitating with known ERAD components was used to identify the members of the degradation network (Christianson et al., 2012; Nakatsukasa et al., 2014; Tyler et al., 2012). Furthermore, stable isotope labelling of amino acids (SILAC) coupled to shotgun mass spectrometry has been used to identify endogenous substrates whose levels are increased in the absence of ERAD (Foresti et al., 2013). Still the number of endogenous ERAD substrates remains small, making it questionable if ERAD is active under standard culture conditions. However, while these methods helped to complete the understanding of ERAD, they do not provide protein degradation rates. To increase the throughput of protein degradation rate measurements shotgun mass spectrometry methods were coupled to stable isotope labelling pulse-chase setups (Hinkson and Elias, 2011). Such an experimental setup allowed measurements of half-lives of hundreds to thousands of proteins in a single experiment in unicellular, as well as multicellular organisms (Doherty et al., 2005; Helbig et al., 2011; Pratt et al., 2002; Schwanzausser et al., 2011; Toyama et al., 2013). However, due to its low sensitivity, low-abundant proteins were not captured by the approach.

4. Mass spectrometry analysis of proteins

Mass spectrometry (MS) is an analytical method that allows determining the molecular weight of compounds. Every mass spectrometer includes an ion source, a mass analyzer and a detector. For each part of the instrument different types of devices are available, and their combination allows a number of different applications. Originally, MS was developed to analyze small molecules, e.g. to assess purity of pharmaceutic chemicals or to assess pesticide contamination. The development of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) allowed to analyze macromolecules such as proteins and led to the emergence of proteomics (Fenn et al., 1989).

4.1. Shotgun mass spectrometry for proteomics

In proteomics, mass spectrometry is often used to identify the protein content of a sample using a process named data-dependent or shotgun MS. A standard shotgun proteomics workflow includes extraction of proteins and digestion into peptides using trypsin protease (bottom-up proteomics). The peptides are separated via reverse-phase liquid chromatography
(LC) and ionized by ESI. Voltage is applied at the ion source causing the liquid sample to disperse into fine droplets containing charged molecules. Once the ions are in gas phase, they enter the vacuum of the mass analyzer where they are moved by an electric field and are resolved according to their mass-to-charge (m/z) ratio (Steen and Mann, 2004). Finally, ions are detected, resulting in MS spectra acquired over the whole gradient and containing the m/z of intact peptides. These spectra are also called MS or MS1 spectra.

In a data-dependent workflow the most intense peaks present in the MS spectra are selected (normally 5 to 50). These ions are sequentially isolated and fragmented, e.g. by collision with Helium gas, a process termed collision-induced dissociation (CID). The fragment ions of a single peptide are recorded in a spectrum defined as MS/MS or MS2 spectrum. To identify the peptides and proteins contained in the sample, the MS/MS spectra are then searched against a database containing the theoretical spectra of the whole proteome of interest. Shotgun proteomics experiments systematically enable the identification of hundreds to thousands of proteins in a single experiment, while requiring only minimal prior knowledge about the sample. As an example, 4000 yeast proteins were recently identified in a one hour LC-MS/MS analysis (Hebert et al., 2014). The drawback is that the acquisition speed of mass spectrometers allows only for a limited number of MS1 precursors to be selected for fragmentation, leaving many peptides unidentified (Michalski et al., 2011). Moreover, the acquisition of MS/MS spectra is somewhat stochastic, and slight fluctuations in peak intensities can cause different peptides to be identified even in replicate MS acquisitions of the same sample. Besides, sensitivity is lower compared to targeted mass spectrometry methods (Picotti et al., 2009). Nonetheless, shotgun MS is firmly established in the proteomics field and has helped study proteomes to new depths.

4.2. Targeted mass spectrometry: Selected reaction monitoring

Not always the whole content of a sample needs to be characterized. In cases where only a few, specific analytes need to be quantified methods targeting only these compounds are better suited than shotgun MS. Mass spectrometry methods where a limited number of selected compounds are monitored are termed "targeted " and are mainly performed on triple quadrupole instruments. Selected reaction monitoring (SRM) is a targeted MS approach that has been applied to proteomics during recent years (Picotti et al., 2009). Using this method, only selected precursor masses (peptides) and product ions (fragments) are monitored, thus increasing sensitivity. The list of targeted precursors and fragment pairs, termed "transition list", is entered into the instrument software (Figure 6A). During an SRM experiment, the first quadrupole (Q1) acts as
a mass filter selecting only the first precursor mass on the transition list and transferring it to the second quadrupole (Q2) where it is subjected to fragmentation (Figure 6B) (Lange et al., 2008). The fragments enter the third quadrupole (Q3) which acts as an additional mass filter, allowing only the first product ion in the transition list to pass to the detector. One by one all transitions eluting from the chromatographic column are acquired. Finally, the data for all fragments of a peptide are assembled in a peak (Figure 6A).

Figure 6: Selected reaction monitoring.
A) Transition lists containing precursor and fragment pairs are entered into the MS instrument software, peptide and fragment intensities are measured and results are visualized as peaks of coeluting fragments (modified from Surinova et al., 2013). B) Peptide mixtures enter the first quadrupole (Q1) that lets a selected precursor m/z pass to the second quadrupole (Q2) where it is fragmented. Fragments are transferred to the third quadrupole (Q3) where a selected fragment is passed to the detector and its signal is registered. Taken from (Huttenhain et al., 2009).

The advantages of SRM over shotgun proteomic approaches are high sensitivity and a large dynamic range, that allow to measure previously undetectable proteins in an unfractionated sample (Picotti et al., 2009). Since analytes of interest are fragmented regardless of their intensity, SRM is especially suitable when defined sets of proteins need to be monitored or for the detection of low-abundant peptides that might be missed with shotgun MS (Picotti and Aebersold, 2012). However, being a targeted technique, SRM requires prior knowledge which proteins are of interest. This has the disadvantage that the peptide transitions have to be developed prior to analysis. Nevertheless, protocols have been developed for the high-throughput generation of transition lists and an increasing number of SRM assays is available in databases (Picotti et al., 2013; Picotti et al., 2008; Picotti et al., 2010; Soste and Picotti, 2013). In order to ensure the success of an SRM experiment, it is important to select suitable precursors and fragments for the analysis, i.e. peptides unique to the protein of interest (proteotypic) that ionize well. In case two proteins with a large sequence similarity need to be analyzed, the selection of proteotypic peptides that fulfill these criteria can be challenging.
4.3. Data-independent MS methods

Shotgun MS as well as SRM have been largely used in proteomics during recent years and have proved useful for a host of applications ranging from biomarker discovery and validation to analysis of post-translational modifications. Nonetheless, each approach has some drawbacks. The limitation of shotgun MS (data-dependent analysis, DDA) is that only a limited number of co-eluting precursors (normally the 5 to 50 most intense) is sequenced during each MS cycle while all other peptides remain unidentified (Michalski et al., 2011). This leads to an identification bias towards more abundant proteins and an under-sampling of medium- to low-intensity peptides and hampers the reproducibility of identifications. Furthermore, DDA has a narrow dynamic range which is important for quantitative studies. SRM on the other hand, has a large dynamic range and is more sensitive but also less flexible since it requires prior knowledge of target proteins. Further drawbacks include labor intense optimization of transitions and instrument parameters, as well as detection of false-positive signals due to the low resolution and mass accuracy of quadrupole mass analyzers.

Recently, data-independent (DIA) MS methods such as SWATH, MS\textsubscript{E} and MS(all) emerged, combining features of targeted and shotgun methods (Chapman et al., 2013; Law and Lim, 2013). The common feature of all DIA methods is that not a single precursor peak is selected and sequenced, but larger mass windows, or swaths, are sent for fragmentation (Figure 7A). This avoids the undersampling typical for shotgun methods and allows to obtain fragmentation spectra for all peptides present in the sample, rather than just a snapshot as in targeted methods. For peptide identification the product ion spectra can then be searched against theoretical spectra or can be mined using SRM-like traces. The detected fragments can subsequently be arranged in SRM-like peak groups (Figures 8A, 8B). Thus, after post-processing of data DIA approaches potentially provide both, fragment spectra to identify peptides and fragment peak groups for quantification.
DIA experiments are conducted on hybrid mass spectrometers and the product ion spectra are generally acquired in a high resolution mass analyzer (e.g. a time-of-flight (TOF) or an orbitrap). SWATH in particular was developed on a triple-TOF instrument (Andrews et al., 2011; Gillet et al., 2012). During SWATH acquisition, no MS1 spectrum is acquired but all ions in a 25 Th mass window (“swaths”) are isolated by the quadrupole and sent to the collision cell for fragmentation. Fragments are subsequently analyzed in the TOF analyzer. Thus, for each mass window the instrument records retention time (RT), m/z of fragments and fragment signal intensities. Data analysis is challenging because the resulting spectra contain fragments from several precursors and also because the resolution of TOF analyzers is lower than for orbitraps. Sophisticated software, such as Skyline, Spectronaut or OpenSWATH, is required to group the fragments into SRM-like peaks based on their retention time and the precursor mass window (MacLean et al., 2010; Rost et al., 2014). The end result is an SRM-like experiment where a much larger number of precursors are monitored compared to targeted methods. The development of the SWATH method was only possible due to advances in instrument sequencing speed. With the triple-TOF being able to sequence the whole mass range from 400-1200 Th in 3 seconds, 10 data points could be acquired for a peptide peak eluting over 30 seconds. Slower sequencing speed would lead to less data points, a decreased signal-to-noise ratio and peak group resolution. The linear dynamic range of SWATH is four orders of magnitude and the limit of detection lies in the attomole range which means its sensitivity is lower than SRM (Gillet et al., 2012).
One drawback of SWATH is that the data processing workflow requires a spectral library. Up to now it is not possible to do conventional database searches using SWATH data since peptide fragments cannot be attributed to their precursor masses. Despite this drawback, SWATH has not only been applied to identify proteins, but also to quantify post-translational modifications, to analyze protein interactions, and its suitability for the validation of biomarkers has been assessed (Collins et al., 2013; Liu et al., 2014; Liu et al., 2013a; Liu et al., 2013b). Technical progress and development of new instruments will further drive the DIA approaches and increase the number of identified proteins. This also includes developments in separation techniques such as LC-MS, but also coupling of ion mobility to instruments which offers an orthogonal dimension of ion separation.

In conclusion, like DDA, DIA methods strive to identify all sample content and, as opposed to SRM, provide mass spectra for the quantification of peptides from the sample without prior knowledge. Furthermore, the DIA data structures and processing workflow allow retrospective data mining. In summary, DIA covers disadvantages of DDA and SRM and is an extension of the proteomic toolbox.

4.4 Fragmentation techniques and post-translational modifications

Apart from the different acquisition techniques, shotgun vs. targeted vs. data-independent etc., also several mass spectrometry fragmentation techniques exist. A hybrid instrument like the LTQ Orbitrap mass spectrometer for example can perform different types of fragmentation: (I) Collision-induced dissociation (CID) is used to fragment peptides in the ion trap (Louris et al., 1987). Peptides collide with gas molecules, typically helium, and fragment due to the vibrational energy transferred during these collisions. Though widely used, the drawback of CID in an ion trap is the low-mass cutoff common to all ion trap instruments which does not allow to monitor small m/z fragments. Furthermore, mass accuracy and resolution are low. To alleviate these drawbacks, (II) higher energy C-trap dissociation (HCD) was developed for LTQ Orbitrap instruments (Olsen et al., 2007). During HCD, fragmentation takes place in a dedicated octopole collision cell, allowing fragments to be analyzed in the Orbitrap. This results not only in higher mass accuracy and resolution, but allows also to track small fragment ions. (III) Electron-transfer dissociation (ETD) is another technique that can be used to fragment peptides in an LTQ Orbitrap instrument (McAlister et al., 2007; Syka et al., 2004). During ETD electrons are transferred to peptides, thus inducing the fragmentation. The advantage of this method is that the peptide backbone is fragmented while post-translational modifications (PTMs) are retained (Figure 8). Therefore it is especially suitable for PTM site localization (Singh et al., 2012).
Figure 8: ETD and CID fragmentation fragment peptides at different positions.
CID fragmentation will mainly produce fragment ions containing parts of N-glycans and will lead to loss of phosphate modifications while the peptide backbone remains largely intact (blue arrows). ETD on the other hand will cleave at the peptide backbone (black triangles) and leave the PTMs intact which makes it especially suitable for PTM localization. Modified from J. Kemsley, Analyzing Protein Drugs, 2009.

These three fragmentation methods are complementary and will yield different fragments for the same peptide and can be used in combination with each other (Frese et al., 2011; Olsen and Mann, 2013). For example when analyzing N-glycopeptides, CID and HCD will mainly fragment the glycan, which is useful for analyzing the glycan-composition (Cao et al., 2014; Singh et al., 2012). ETD, on the other hand will yield peptide fragments while leaving the glycan modification intact, thus providing useful information for the localization of the PTM. Thus, complementary data can be obtained through the use of several fragmentation techniques, which helps to complete the picture.

A special feature of HCD fragmentation is that, unlike CID, it has no low-mass cutoff, making it possible to analyze low-mass ions (Olsen et al., 2007). Many PTMs produce low-mass signature ions that can be detected after HCD fragmentation, e.g. acetylation, methylation, or also glycosylation that produces for example marker ions with m/z 204, corresponding to a single HexNAc (Huddleston et al., 1993). These marker ions can be used to identify spectra of peptides with the corresponding PTM. For example, an HCD-triggered ETD approach has been used to identify N-glycopeptides (Singh et al., 2012). Basically, an HCD spectrum was acquired for a peptide, and screened for marker ions. Only if they were detected, an ETD spectrum of the same peptide was acquired, ensuring optimal use of instrument time to acquire the desired peptide population.
References:


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Chapter 2:

*Analysis of protein degradation in yeast dissects events during complex assembly of oligosaccharyl transferase*

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**Contributions:**

Setting up SILAC-SRM method, including sample preparation and data analysis workflows  
Construction of strains  
Planning and execution of experiments  
Preparation of samples for mass spectrometry  
SRM and shotgun MS measurements  
Data analysis  
Writing the manuscript
Summary

Protein degradation is an essential factor for cellular homeostasis. We developed a sensitive method to examine protein degradation rates in *Saccharomyces cerevisiae* by coupling a pulsed-SILAC approach to selected reaction monitoring (SRM) mass spectrometry. For most proteins we found low degradation levels in exponential-phase wild type cells. The majority of proteins were stable and were turned over by cell division rather than degradation. However, in cells where subunits of the oligosaccharyl transferase (OST) complex were overexpressed or deleted, components present in excess over their binding partners were degraded. This altered subunit stability allowed us to develop a model for OST complex assembly. On a global scale, protein degradation in the ER is relevant when gene dosage is altered, as demonstrated further by degradation of subunits in heterozygous diploid cells. Thus alleviating fitness defects due to abnormal gene copy numbers might be an important function of protein degradation.
Highlights:

- SILAC-SRM is a sensitive method to measure protein turnover.

- Levels of degradation are low for most yeast proteins.

- Protein degradation eliminates complex subunits present in imbalanced stoichiometry.

- OST assembly proceeds via several stable subcomplexes.

- Deletion but not overexpression of subunits destabilizes oligosaccharyl transferase
Introduction:
Protein turnover is a process driven by loss of old and synthesis of new protein. This replacement of the protein pool can be important for cellular processes such as the regulation of cell division (Lecker et al., 2006). Furthermore, the interplay between transcription, translation, and protein degradation regulates protein abundances (Gygi et al., 1999; Vogel and Marcotte, 2012). Homeostasis is of particular importance for complex components and degradation might be a means to equilibrate the system.

In the yeast *Saccharomyces cerevisiae*, protein complexes fulfill many cellular functions, such as the SEC translocon transporting proteins into the endoplasmic reticulum (ER) or the protein-mannosyl transferase (PMT) attaching O-mannose to proteins. The oligosaccharyl transferase (OST) complex, is the central enzyme of asparagine-linked (N-) glycosylation and attaches glycans to N-X-S/T sequons on proteins in the ER lumen (Breitling and Aebi, 2013). This multi-subunit complex consists of eight proteins including the two mutually exclusive thioreductases Ost3p and Ost6p. Information on OST assembly or degradation dynamics remains scarce but based on biochemical and genetics data, assembly pathways based on stable subcomplexes have been suggested (Kelleher and Gilmore, 2006; Schwarz et al., 2005). Analysis of other complexes showed that subunits stabilize each other during assembly and that proteins enter the complex in a defined sequence (Asher et al., 2006; Daley, 2008; Johnson et al., 1998).

ER-associated degradation (ERAD) accounts for the main part of protein degradation from the ER (Thibault and Ng, 2012). This pathway recognizes misfolded proteins at the ER and redirects them to the cytosol for proteasomal degradation (Ruggiano et al., 2014). In yeast, the key components are two E3 ubiquitin ligase complexes, the HRD1 and DOA10 complexes, which recognize misfolded proteins and ubiquitinate substrates. ERAD has mainly been studied by analyzing mutated proteins that are quickly degraded, like PrA* or CPY* in yeast (Finger et al., 1993; Xie et al., 2009). In general, such studies use a pulse-chase setup to distinguish old from newly synthesized protein coupled to SDS-PAGE-based methods for detection. Large-scale studies were impractical since only one protein could be monitored per experiment. To increase throughput, a pulse-chase approach based on stable amino acid isotopes labeling in culture (SILAC) was coupled to shotgun mass spectrometry (MS) (Pratt et al., 2002). This approach made it possible to determine degradation rates for hundreds of proteins in a single experiment. However, despite the progress in MS technologies and increasing sensitivity of mass spectrometers, low-abundant proteins remained difficult to detect. Adapting these high-throughput methods for the analysis of low-abundant proteins requires a more sensitive MS technique such as selected reaction monitoring (SRM). This targeted MS method quantifies only selected peptides and their fragments in triple quadrupole mass spectrometers (Lange et
SRM is characterized by a high sensitivity, large dynamic range, and consistent data acquisition, avoiding missing data points typical for shotgun MS experiments (Picotti et al., 2009; Soste and Picotti, 2013). This makes it especially suitable for the analysis of low-abundant proteins and for studies where selected target proteins need to be monitored, for example all subunits of a protein complex (Picotti and Aebersold, 2012).

We implemented a sensitive SILAC-SRM method to analyze 25 target proteins with different cellular abundances. This revealed low levels of ERAD for proteins in wild type cells. Moreover, we studied OST assembly and how the complex reacted to a change in subunit abundance. Based on our findings we propose a sequence of events for OST complex assembly while gaining insights into dynamics of protein complexes in general.
**Results:**

Developing and validating a sensitive SILAC-SRM method to measure protein degradation

The aim of this study was to analyze the turnover of ER membrane protein complexes in *Saccharomyces cerevisiae* with a special focus on OST. In addition, we analyzed the SEC translocon, PMT complexes, ER chaperones (Kar2p, Pdi1p), known ERAD substrates (PrA, Pdr5p), and cytoplasmic proteins (Rpl5p, Rps1ap, Ilv5p) as slowly degrading controls (Belle et al., 2006; Pratt et al., 2002; Schwanhausser et al., 2011).

Attempts to monitor all proteins of interest by shotgun MS were unsuccessful because of the low sensitivity of this acquisition method. Therefore, we coupled a SILAC pulse-chase with SRM acquisition (SILAC-SRM) (Figure 1A). In short, proteins were labelled by culturing the cells in shake flasks with medium containing heavy isotopes of arginine (R6) and lysine (K8). Subsequently, cells were transferred to medium with light isotopes of arginine and lysine (R0, K0). At different time points, cells were collected, lysed, membranes and luminal fractions were collected, and proteins were solubilized and digested with LysC and trypsin. At each time point ratios of heavy to light peptides were recorded by SRM on a QTRAP mass spectrometer (Table S5). The rate of protein loss (kLOSS) was calculated by linear curve fitting. Dilution rates (kDIL) were subtracted from kLOSS to yield degradation rates (kDEG) (Larrabee et al., 1980).

To test whether we could use the SILAC-SRM method to determine protein half-lives accurately, we used the ERAD model substrates PrA*-aB and PrA*-Ab, misfolded variants of the PrA glycoprotein (Spear and Ng, 2005). Of the two N-glycosites of the PrA* protein, only the first is occupied in PrA*-Ab and only the second in PrA*-aB, influencing the rate by which they are degraded via ERAD (slow and fast, respectively). Cells deficient in PrA were transformed with PrA*-aB or PrA*-Ab expression plasmids. SRM-traces and degradation rates showed that PrA*-Ab was quickly degraded, while PrA*-aB was more stable (Figures 1B, 1C). These values were in agreement with the respective half-lives reported in the literature (Spear and Ng, 2005).
Figure 1: Validation of the SILAC-SRM method with PrA.

(A) Schematic representation of the SILAC-SRM method. After labelling yeast proteins with heavy arginine (R6) and lysine (K8), cells were transferred to light medium. Cells were sampled at different timepoints and proteins prepared for mass spectrometry. Changing heavy to light ratios for peptides were detected by SRM on an ABSCIEX QTRAP5500 instrument. Loss rates (kLOSS) were calculated by linear curve fitting and dilution rates (kDIL) were subtracted yielding degradation rates (kDEG). (B) SRM traces for peptide FDGILGLGDTISVDK of PrA in cells expressing PrA*-aB (SMA822) or PrA*-Ab (SMA823) versions of the protein. (C) kDEG values for PrA in cells expressing PrA*-aB (SMA822) or PrA*-Ab (SMA823) versions of the protein. Degradation rates were calculated as described in (A). Error bars show standard deviation in 3 biological replicates. Significant differences in average kDEG were determined with a t-test. Values below the dashed line indicate a half-life > 8 cell divisions (= 12 h). *** = p-value ≤ 0.001. (D) kDEG values for PrA in wildtype (SMA1648) or Δhrd1Δdoa10 cells (SMA1649) containing a PrA expression plasmid or vector controls (SMA1574, SMA1584). Degradation rates were calculated as described in (A). Error bars represent standard deviation in 3 biological replicates. Values below the dashed line indicate a half-life > 8 cell divisions (= 12 h).
Moreover, we overexpressed PrA in wild type cells and in ERAD-deficient Δhrd1Δdoa10 cells. When we measured degradation rates of PrA, no significant differences were detected between wild type and mutated cells. In agreement with previous reports, PrA had a half-life over 12 h (or >8 doubling times) (Figure 1D) (Finger et al., 1993). Thus, within the time that a given amount of PrA was reduced by a factor two via degradation, cell division diluted the same amount by a factor of $2^6 = 256$ (Figure S1B). Since the contribution of degradation to protein turnover was minimal in such a case, we subsequently classified proteins with half-lives >8 doubling times, including PrA, as "stable". From the results of these experiments we concluded that the SILAC-SRM method was a sensitive and reliable method for measuring turnover of proteins and yielded accurate half-lives compared to previously used methods. Its sensitivity and consistency makes SRM especially suitable to analyze medium- to low-abundant proteins (potentially as low as 50 copies/cell) and subunits of protein complexes (Picotti et al., 2009).

Exponentially growing yeast cells show low levels of protein degradation

Next, we examined if and to what extent the ERAD system eliminated the 25 target proteins (Figure 2A). We conducted pulse-chase experiments in wild type and Δhrd1Δdoa10 cells, isolated membrane and vesicular content fractions, and extracted and digested proteins. We then analyzed protein turnover for 25 target proteins by SRM (Figure 2A). Almost all proteins were stable in wild type cells (Figures 2B, S1A). Besides, we did not find significant differences to the degradation rates for most proteins observed in Δhrd1Δdoa10 cells. Only Pmt2p showed a significantly different half-life in the two cell types ($t_{1/2}$ of 17 h vs. 42 h). Thus, most proteins were stable in exponentially growing cells.

To analyze if the low level of degradation was a general feature of yeast proteins, we monitored the turnover of proteins from all cellular fractions. To this end, we prepared whole cell extracts from pulse-chase labelled wild type cells by glass bead lysis in detergent. The extracts were processed for mass spectrometry and were analyzed by shotgun MS. Half-lives were obtained for 188 proteins from cytosol, mitochondria, nucleus, Golgi, ER, cell wall, and the vacuole. Most of the detected proteins were not degraded under the culturing conditions used in this study (Figures 2C, 2D). Pdi1p, Kar2p and Rps1ap which we had monitored with the SILAC-SRM method before were again stable. Only eight proteins showed half-lives <12 hours. This group included proteins that cope with oxidative damage (Sod1p, Ahp1p), as well as a known fast degrading ribosomal protein (Rps31p), and a protein involved in ergosterol synthesis.
(Erg11p), a pathway that contains several enzymes whose amount is regulated by degradation (Figures 2C, 2D) (Hampton and Rine, 1994; Helbig et al., 2011; Jaenicke et al., 2011). From these experiments, we concluded that most proteins were stable in exponentially growing yeast.

Figure 2: Low levels of protein degradation in exponentially growing yeast cells.

(A) Schematic representation of target protein localisation and function. Proteins are synthesized by ribosomes and enter the ER through the SEC translocon. They are glycosylated by OST (N-glycans) or the PMT complexes (O-glycans). Folding is assisted by chaperones Pdi1p and Kar2p. Further target proteins are vacuolar protease PrA, plasma membrane transporter Pdr5p and mitochondrial protein Ilv5p. OST subunits are coloured in green, yellow or orange according to subcomplexes proposed in literature (Kelleher and Gilmore, 2006).

(B) kDEG values for 25 target proteins in wild type (SMA673) or $\Delta hrld1 \Delta doa10$ cells (SMA1566). Degradation rates were calculated as described in Fig. 2A. Error bars represent standard deviation in 3 biological replicates. Significant differences in average kDEG in the two strains were determined with a t-test. Values below the dashed line indicates a half-life $> 8$ cell divisions ($= 12$ h). * = p-value $\leq 0.05$.

(C) Protein half-lives in whole cell extracts. Whole cell extracts of wildtype were prepared, proteins were digested for MS and measured by shotgun LC-MSMS on an LTQ Orbitrap Velos and quantified with MaxQuant. Results of 3 biological replicates are shown.
Overexpressed OST subunits do not affect complex activity and are degraded with protein-dependent rates

In a next step we altered protein levels and analyzed the effect of single subunit overexpression on the stability of the OST complex. Wild type and \( \Delta \text{hrd1} \Delta \text{doa10} \) cells were transformed with a high-copy number plasmid carrying an expression copy of \( \text{STT3} \). First, we analyzed steady-state levels of OST components by SDS-PAGE and immunoblot (Figure 3A). As expected, Stt3p was present in higher amounts in cells containing the overexpression plasmid as compared to the control cells while protein levels for all other subunits remained the same. Analysis of the glycoprotein CPY revealed that glycosylation was not altered upon \( \text{STT3} \) overexpression. We repeated this experiment for all other OST subunits with the same results: overexpression of one subunit did not affect the protein levels of the other subunits (data not shown). We concluded that the OST complex remained intact and functional in the presence of overexpressed subunits.

We then measured degradation rates of overexpressed Stt3p in wild type and \( \Delta \text{hrd1} \Delta \text{doa10} \) cells. Membrane fractions were isolated, proteins processed for MS and peptides quantified by SRM. Stt3p was stable when present at normal levels (Figure 3B). In contrast, overexpressed Stt3p was degraded with higher rates in wild type, as well as \( \Delta \text{hrd1} \Delta \text{doa10} \) cells. Its half-life was significantly higher in \( \Delta \text{hrd1} \Delta \text{doa10} \) (4.4 h) compared to wild type cells (1.3 h), demonstrating that degradation of Stt3p depends on a functional ERAD system. All other OST subunits remained stable. Like Stt3p, overexpressed Ost1p, Ost2p, and Swp1p were degraded in wild type cells (Figure 3C). Interestingly, degradation rates were significantly different for each protein with half-lives of 3.9 h (Ost1p), 0.9 h (Ost2p), and 1.5 h (Swp1p). Subunits that were not overexpressed, on the other hand, remained stable. Our data implied that the fully assembled OST complex remained undisturbed while excess subunits were unstable and degraded with protein-specific rates by the ERAD machinery. However, it is important to note that overexpression of a protein is not sufficient for protein degradation as shown for overexpressed PrA (Figure 1D).
Overexpressing OST subunits results in increased degradation.

(A) Immunoblot analysis of Stt3p overexpression. SDS extracts were prepared from wildtype (SMA1578) or \( \Delta \text{hrd1} \Delta \text{doa10} \) cells (SMA1588) containing a \( \text{STT3} \) expression plasmid and vector controls (SMA1574, SMA1584). Equal amounts of protein were separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose membranes followed by detection with ECL reagent. (B) \( k_{DEG} \) values for OST subunits in wildtype (SMA1578) or \( \Delta \text{hrd1} \Delta \text{doa10} \) cells (SMA1588) containing an \( \text{STT3} \) expression plasmid and vector controls (SMA1574, SMA1584). Degradation rates were calculated as described in Fig. 2A. Error bars represent standard deviation in 3 biological replicates. Average \( k_{DEG} \) that were significantly different from wild type controls, or different between wild type and \( \Delta \text{hrd1} \Delta \text{doa10} \) Stt3p overexpressing cells were determined with a t-test. Values below the dashed line indicate a half-life > 8 cell divisions (= 12 h). *** = p-value ≤ 0.001. (C) \( k_{DEG} \) values for overexpressed Swp1p (SMA1576), Ost2p (SMA1577), Ost1p (SMA1578), and Stt3p (SMA1582) in wildtype background. Degradation rates were calculated as described in Fig. 2A. Error bars represent standard deviation in 3 biological replicates. Values below the dashed line indicate a half-life > 8 cell divisions (= 12 h).

Deletions in essential subunits destabilize the OST complex

Our results so far suggested that integration of OST subunits into the complex prevented their degradation. In a next step, we analyzed the effect of single subunit deletions on OST component degradation. To study the effect of a deletion of an essential OST subunit, we took advantage of the finding that a plasmid-borne STT3D protein from \textit{Leishmania major} (LmSTT3D) can functionally replace endogenous OST (Figures 4A, 4C) (Nasab et al., 2008). LmSTT3D is a single-subunit oligosaccharyl transferase that glycosylates most proteins when expressed in yeast cells but does not incorporate into the yeast OST complex. In the presence of LmSTT3D, even cells containing a deletion in one of the OST subunits remained fully viable.
First, wild type, \( \Delta \text{stt3} \), and \( \Delta \text{wbp1} \) cells carrying the LmSTT3D plasmid were analyzed by SDS-PAGE and immunoblot (Figure 4B). Deletion of Stt3p and Wbp1p had different effects: both resulted in a decrease of Ost3p and Ost6p levels, but deleting \( WBP1 \) also decreased Swp1p levels. While CPY was completely glycosylated in \( \Delta \text{stt3} \) cells, the Wbp1p subunit was hypoglycosylated, indicating that LmSTT3D fully glycosylated many but not all yeast glycoproteins (Figure 4B).

**Figure 4**: Deletion of essential OST subunits affects stability of other complex components.

(A) Schematic representation of LmSTT3D compensating N-glycosylation in \( \Delta \text{wbp1} \) cells. LmSTT3D glycosylates yeast proteins and ensures cell viability in the absence of a functional yeast OST.
Figure 4: continued.

(B) Immunoblot analysis of OST deletions. SDS extracts were prepared from cells carrying a LmSTT3D expression plasmid and containing all OST subunits (SMA1603) or deletions in STT3 (SMA1604) or WBP1 (SMA1605). Equal amounts of protein were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes followed by detection with ECL reagent. Ost3p signal is indicated by an arrow to distinguish it from unspecific signal. (C) $k_{DEG}$ values for OST subunits in wildtype (SMA1603), or in cells deleted in STT3 (SMA1604) or WBP1 (SMA1605) and carrying a LmSTT3D expression plasmid are shown. Degradation rates were calculated as described in Fig. 2A. Error bars represent standard deviation in 3 biological replicates. Average $k_{DEG}$ that were significantly different from wild type controls were determined with a t-test. The dashed line marks half-lifes > 8 cell divisions. * = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001.

In a subsequent experiment, we measured protein degradation by SILAC-SRM in LmSTT3D-complemented wild type, Δstt3 and Δwbp1 cells. Deletion of both, STT3 and WBP1 resulted in degradation of several OST subunits but did not affect the PMT and SEC complexes (Figure 4C, data not shown). In Δstt3 cells Ost3p ($t_{1/2} = 2.4$ h), Ost6p (3.0 h), and Ost5p (3.8 h) were degraded rapidly, and also degradation rates of Ost1p ($t_{1/2} = 20.2$ h), and Wbp1p (11.7 h) were significantly higher than in wild type cells. In Δwbp1 cells Swp1p ($t_{1/2} = 1.0$ h), Ost2p (1.2 h), Ost3p (2.1 h), and Ost6p (3.0 h) were degraded. Stt3p ($t_{1/2} = 4.4$ h) and Ost5p (8.5 h) were also disposed but with slower rates. We concluded that complex integration prevented subunit degradation and absence of subunits rendered the complex unstable.

**Protein abundance in cells with deletion in one subunit elucidates OST complex assembly**

Our results are in line with published data that subunits of membrane protein complexes stabilize each other and assemble in a sequential manner via stable intermediates (Daley, 2008). Therefore, we conducted SILAC experiments with cells deleted in one of the nine OST subunits to monitor steady state levels of proteins (Figures 5A, 5B). Pellets of equal weight of wild type cells grown in medium with heavy arginine and lysine isotopes and of cells with a deletion in one OST subunit grown in medium with light isotopes were pooled. Cells were lysed, membranes collected, proteins prepared for MS, and peptides measured by SRM. Apart from the OST components we also monitored the behavior of the SEC translocon and PMT subunits, as well as the plasma membrane glycoprotein Pdr5p. With exception of Pdr5p, most of these proteins showed minor alterations. We concluded that differences reflect the activity of endogenous OST and the LmSTT3D enzyme.
Figure 5: Differential effect of subunit deletion on OST component stability.

(A) Schematic representation for SILAC analysis of cells with an OST subunit deletion. LmSTT3D-complemented wild type and cells with a subunit deletion (Δost4) were grown in medium containing heavy (R6, K8, WT) or light amino acids (R0, K0, Δost4). Equal amounts of cells were pooled, proteins were extracted and peptides were prepared for mass spectrometry. Light to heavy ratios for all OST peptides were measured by SRM on an ABSCIEX QTRAP 5500. Subunit abundance relative to wildtype was calculated. Values were normalized to the average of all non-OST proteins. Decreased amounts of a subunit indicated destabilisation by the Δost4 deletion. (B) Amounts of OST components relative to wild type in cells deleted in a single subunit and containing an LmSTT3D expression plasmid were analyzed as described in Fig. 5A. The values were normalized by dividing the average of all non-OST proteins (SEC, PMT components, Pdr5p). The deleted subunit is indicated by a black bar. Error bars represent the 95% confidence interval of the average of 3 biological replicates.
We observed a response for the OST subunits that was specific for each deletion: A deletion in \textit{WBP1}, \textit{SWP1}, or \textit{OST2} reduced the levels of all three proteins indicating that the three subunits stabilized each other (Figure 5B). In addition, Wbp1p, Swp1p, and Ost2p were present in similar amounts relative to each other in \textit{\Delta ost1} (83-88\%) and \textit{\Delta stt3} cells (86-92\%).

We found a similar scenario for \textit{OST1}, \textit{STT3}, and \textit{OST5}: a deletion in \textit{OST1} reduced levels of Stt3p, and Ost5p to <20\% of wild type levels. Deletion of \textit{STT3} decreased the protein levels of Ost1p and Ost5p to 68\%, while a deletion in \textit{OST5} did not affect the levels of any other subunits. Moreover, the three subunits Ost1p, Ost5p, and Stt3p were present in similar amounts relative to each other in \textit{\Delta wbp1} (65-83\%), \textit{\Delta swp1} (72-93\%), and \textit{\Delta ost2} cells (73-86\%). Again, this indicated mutual stabilization of these three subunits. Interestingly, Ost1p had been stable in pulse-chase experiments with \textit{\Delta wbp1} cells but its levels were reduced in SILAC experiments with the same cells. Therefore, determining relative protein levels might be more sensitive and could detect small differences that are not visible in pulse-chase experiments.

In \textit{\Delta ost4} cells only levels of Ost3p and Ost6p were reduced, in agreement with previous findings that Ost4p was necessary for anchoring Ost3p or Ost6p into the OST complex (Spirig et al., 2005). Deletions in \textit{OST3}, and \textit{OST6} did not affect the levels of any of the other subunits while levels of Ost3p and Ost6p were reduced upon deletion of any of the other subunits except for Ost5p (Figure 5B). This indicated that Ost3p and Ost6p entered the complex only after all the other subunits had been assembled.

Our findings suggested that Wbp1p, Swp1p, and Ost2p on one hand, and Ost1p, Ost5p, and Stt3p on the other hand, formed two subcomplexes that might act as intermediates during complex assembly. Ost4p entered the complex after the two subcomplexes and anchored Ost3p or Ost6p into the OST complex (Spirig et al., 2005; Spirig et al., 1997).

**Protein degradation eliminates excess complex subunits in diploid cells**

To eliminate the effect of altered N-glycosylation we verified our results by the analysis of heterozygous deletions in diploid yeast cells. Since yeast cells do not have a feedback mechanism to control protein levels, deletion of one gene copy in diploid cells decreases the protein level by 50\% (Li et al., 2014; Springer et al., 2010).

We compared protein levels in diploid cells with a heterozygous deletion in \textit{OST1} (\textit{\Delta ost1/OST1}) or \textit{WBP1} (\textit{\Delta wbp1/WBP1}) to diploid wild type cells. Heterozygous diploid and diploid wild type
cells were cultured in medium containing light arginine and lysine isotopes or heavy arginine and lysine isotopes. We harvested cells in exponential phase, pooled equal cell weights and isolated membranes as described above. Proteins were extracted and digested, and peptides were quantified using SRM.

Figure 6: Gene dosage of OST components affects subunit stability in diploid yeast cells. Wild type cells and cells with a heterozygous subunit deletion (Δost1/OST1, Δwbp1/WBP1) were grown in medium containing light amino acids (R0, K0). In parallel, wild type cells were grown in medium containing heavy amino acids heavy (R6, K8). Equal amounts of cells grown in heavy and light medium were pooled, proteins were extracted and peptides were prepared for mass spectrometry. Light to heavy ratios for all OST peptides were measured by SRM on an ABSCIEX QTRAP 5500. Subunit abundance relative to wildtype was calculated and normalized to the ribosomal protein Rpl5p. Subunit levels relative to wild type cells grown in heavy medium are displayed for Δost1/OST1 cells (A), and Δwbp1/WBP1 cells (B). Averages of 3 biological replicates are shown. Error bars indicate the 95% confidence interval.

Heterozygous deletions of OST1 and WBP1 reduced the levels of the respective protein to around 50% relative to wild type cells (Figure 6). In addition, deletion of OST1 decreased protein levels of Ost5p, Stt3p, Ost3p, and Ost6p while Wbp1p, Swp1p, and Ost2p remained stable. Deletion of WBP1 reduced the amounts of all other OST subunits. The pattern of subunit degradation was the same as the one observed in haploid cells with a single subunit deletion and containing the complementing LmSTT3D protein. We concluded that protein degradation is essential for subunit homeostasis of protein complexes, in particular in heterozygous cells.
**Discussion:**

In this study, we combined pulsed SILAC and SRM mass spectrometry to measure degradation rates of yeast proteins in exponentially growing yeast cultures using standard laboratory flasks (Figure 1A). The advantage of this method is that many proteins can be analyzed in the same sample and with assays for the whole yeast proteome at hand the scale of future studies can be largely extended (Picotti et al., 2013). Employing the highly sensitive SRM acquisition method furthermore allowed to record degradation rates of intermediate to low-abundant proteins that are not detectable by pulsed-SILAC shotgun MS approaches, potentially down to 50 copies per cell (Picotti et al., 2009). Moreover, the consistent peak acquisition of SRM is especially beneficial in pulse-chase experiments where a defined set of peptides is monitored over several time points since it avoids missing data points due to varying peak intensities that are typical for data-dependent MS experiments. In summary, we proved that the SILAC-SRM approach was a sensitive and reliable method for measuring degradation rates of medium- to low-abundant proteins and of protein complexes.

Using this SILAC-SRM setup, we measured protein half-lives of yeast proteins ranging from around 20 min up to many hours. However, most proteins in wild type yeast cells had very low degradation rates and half-lives >12 hours (Figures 2A, 2B, 2C, S1A). Only a small number of proteins showed half-lives <12 hours. Our findings are in agreement with previous results obtained in yeast (Futcher et al., 1999; Xie et al., 2009). Two studies using pulsed SILAC coupled to shotgun MS reported average or median half-lives of 32 h and 11 h for yeast protein from chemostat cultures grown under nutrient limitation (Helbig et al., 2011; Pratt et al., 2002). Low protein degradation levels or long median half-lives (ranging from 46 - 204 h) were also found for other species, e.g. *E. coli*, mouse fibroblasts and brain, and human adenocarcinoma (Doherty et al., 2009; Larrabee et al., 1980; Li et al., 2014; Price et al., 2010; Schwanhausser et al., 2011). This absence of protein degradation implies that protein abundance is mainly regulated via synthesis. Indeed two recent studies showed that protein abundance is mainly controlled at the level of translation in *E. coli* and mammalian cells, minimizing the need for degradation of excess protein (Li et al., 2014; Schwanhausser et al., 2011). At the same time, the apparent absence of degradation implies that complex subunits were synthesized in correct ratios. This is confirmed by the fact that synthesis rates correlated to subunit stoichiometry for complexes in *E. coli* and *S. cerevisiae* (Li et al., 2014).

But if most cellular proteins are stable and general turnover is very low, what is the function of ERAD in a normally growing yeast cell? We showed that ERAD regulates some proteins, and maintains ER homeostasis by degrading misfolded proteins and excess complex subunits. Our
findings suggest that ERAD adjusts levels of proteins that are unstable as a result of stoichiometric imbalance and prevents their aggregation. Thus, we suggest that protein degradation becomes important in case of gene duplication, loss of homozygosity and other genetic events that alter gene homeostasis. In yeast, large-scale gene duplications, such as aneuploidy or chromosomal rearrangements, promote adaptation to changing environments and diversification of species (Chang et al., 2013; Magadum et al., 2013). However, the resulting stoichiometric imbalance of functionally and physically interacting proteins and complex subunits impairs cellular growth (Torres et al., 2010; Torres et al., 2007; Torres et al., 2008). This imbalance further leads to increased protein aggregation in aneuploid cells (Oromendia et al., 2012). In addition, there is evidence that protein degradation is crucial under these conditions, e.g. aneuploid cells are sensitive to proteasome-inhibitors and growth is improved by mutations that increase degradation (Torres et al., 2010; Torres et al., 2007). Moreover, many complex subunits are not overexpressed in disomic cells despite increased transcription, rather their levels are adjusted by degradation (Dephoure et al., 2014; Torres et al., 2010; Torres et al., 2007). Large-scale gene duplications play a major role in generating novel gene functions during evolution. Thus, by maintaining protein homeostasis in the face of such genetic rearrangements, protein degradation is essential for fixing novel genes.

A major focus of our study was the assembly of the OST complex. We found that overexpressed OST subunits were unstable and were degraded by ERAD with protein-specific rates (Figures 3B, 3C). Protein-specific interactions with the ERAD machinery can explain the different degradation rates. Deleting essential OST subunits, on the other hand, destabilized the complex and also resulted in subunit degradation (Figure 4C). This suggests that in the absence of their binding partners, subunits might be thermodynamically instable or expose degradation signals targeting them for ERAD. We therefore suggest that complex destabilization by subunit deletion but not overexpression applies to many yeast protein complexes. Such "cooperative stability" has been described for several heteromeric and homomeric complexes in yeast and other organisms (Asher et al., 2006; Daley, 2008; Johnson et al., 1998). In agreement with this hypothesis, overexpressed single subunits of protein complexes were degraded in aneuploid yeast cells (Dephoure et al., 2014). This model also implies that protein complex function is robust in the face of large-scale gene duplications since overexpression of a single subunit does not increase activity and simultaneous duplication of all subunit loci is unlikely. Therefore, by organizing enzymes in complexes the cell ensures constant activity of key processes, such as N-glycosylation or protein synthesis.
Figure 7: Model for OST subcomplexes and complex assembly.

Model for OST complex assembly. Wbp1p, Ost3p, and Swp1p form subcomplex 1. Ost1p is stabilized by Ost5p and forms subcomplex 2 with Stt3p. Together with Ost4p the two subcomplexes form subcomplex 3. Ost4p then anchors Ost3p or Ost6p into the final complex. Subunits are coloured in green, yellow, or orange according to (Kelleher and Gilmore, 2006). Graphical arrangements do not reflect actual interaction sites between subunits.

We quantified subunit amounts in cells deleted in a OST subunit relative to wild type and deduced a sequence of events for the assembly of the OST complex (Figures 6B, 7): at the beginning, Wbp1p, Swp1p, and Ost2p form a subcomplex; likewise, Ost1p and Ost5p bind to each other and form another subcomplex together with Stt3p. In a next step these two subcomplexes interact to form the next assembly intermediate. In a next step, Ost4p binds to Stt3p and anchors Ost3p or Ost6p into the OST complex (Spirig et al., 2005; Spirig et al., 1997). This model extends on previous studies on OST architecture and contradicts a proposed Stt3p-Ost4p-Ost3p/Ost6p subcomplex (Karaoglu et al., 1997; Nasab et al., 2008; Reiss et al., 1997; Schwarz et al., 2005; Spirig et al., 2005; Spirig et al., 1997; te Heesen et al., 1993). Based on our results, the observed subcomplexes are assembly intermediates that have lower stability compared to fully assembled OST but confer stability to subunit monomers and are processed to the fully assembled complex. Apart from their enzymatic properties, an essential function of the OST subunits is to contribute to complex stability. This has to be taken into account when reverse genetic tools are used for functional studies.
**Experimental Procedures:**

**Strain construction**

Lists of plasmids, primers, and strains and further information are given in Tables S1, S2, and S4 in the Supplemental Experimental Procedures section. Standard techniques and media were used for handling *Saccharomyces cerevisiae* (Guthrie, 1991). For transformations of yeast cells we used lithium acetate methods (Gietz and Schiestl, 2007a, b). Genes were deleted via homologous recombination of resistance cassettes and via the Cre-loxP system (Guldener et al., 1996; Wach et al., 1994). To create heterozygous diploid cells SMA1593 (BY4742, Δarg4::0, MATa) was mated to wildtype, Δwbp1, or Δost1 cells complemented with *LmSTT3D* (Herskowitz and Jensen, 1991). Counter-selection of the pLmSTT3D plasmid yielded strains SMA1620 (WT), SMA1628 (Δwbp1/WBP1), SMA1621 (Δost1/OST1).

**Immunoblot analysis:**

Exponential cells were lysed with glass beads in sample buffer containing 2% SDS. Proteins were loaded on 10% SDS-PAGE gels, transferred to nitrocellulose membranes (Whatman) and hybridised with antibodies (Table S3). Signals were visualized using ECL solutions (GE Healthcare, Amersham, U.K.).

**Pulsed SILAC experiment:**

Yeast cells auxotrophic for arginine and lysine were grown in shake flasks in SD medium containing 20 mg/l heavy 13C6 arginine (R6, Cambridge Isotope Lab, Andover, U.S.A.) and 13C6-15N2 lysine isotopes (K8, Sigma Aldrich, Buchs, Switzerland). Upon reaching OD600 1 cells were transferred to medium containing light 12C6 arginine (R0) and 12C6-14N2 lysine (K0) isotopes, diluted 2-fold, and chased for maximum one doubling time. Fifty OD of cells were sampled at three time points.

**Isolation of ER proteins for mass spectrometry:**

Cells were subjected to glass bead lysis without detergent. Membranes were collected, washed with Na2CO3, high salt, and Tris-HCl, solubilized in 4% SDS buffer, and digested using a FASP protocol adapted from Wisniewski et al. (for details see Supplemental Experimental Procedures) (Wisniewski et al., 2009). Samples were desalted using C18 ZipTips (Millipore, Zug, Switzerland). Proteins in the Na2CO3 wash were collected by TCA-Acetone precipitation and digested as described.
SRM assay development:
Transitions for proteotypic target peptides were extracted from a yeast spectral library, from previous shotgun measurements or were calculated using Skyline (MacLean et al., 2010; Picotti et al., 2013). Retention times were determined through unscheduled SRM-runs or transferred from previous shotgun experiments. The precursor peak was identified by coelution of transitions, and a dotproduct of > 0.7 for spectral library peptides (Picotti et al., 2013). For non-library peptides, transitions were validated using synthetic peptides (SpikeTides, JPT, Berlin, Germany). 4 – 5 transitions were selected for subsequent SRM measurements (Table S5).

SRM mass spectrometry:
For each sample 2 - 8 µl containing iRT peptides (Biognosys, Zurich, Switzerland) were analyzed by SRM on a QTRAP 5500 (AB Sciex, Zug Switzerland). Heavy and light peptide peaks were integrated in Skyline (MacLean et al., 2010).

Calculating degradation rates
The loss rate of old protein from the cell (kLOSS) was calculated from the time course of isotopic ratios according to Larrabee et al. (Larrabee et al., 1980). kDIL ( = kLOSS of stable protein Rpl5p) was subtracted to yield degradation rates kDEG and half-lives were calculated as t1/2 = LN(2) / kDEG.

Preparation of whole cell extracts and mass spectrometry analysis:
Fifty OD cells were lysed with glass beads in lysis buffer (100 mM Tris-HCl, pH 7.6, 50 mM DTT, 4% SDS). Soluble material was incubated 5 min at 95°C. After elimination of unsoluble material, DNA was sheared using an Ultrasonic processor (Hielscher Ultrasound technologies, Teltow, Germany, 10 min, amplitude 65%, 0.5 cycle). Two hundred micrograms of protein were diluted with 400 µl UA buffer (8 M urea in 0.1M Tris-HCl, pH 8.5) and prepared for mass spectrometry as described before. Peptides were separated by liquid chromatography with an acetonitrile gradient of 3 to 35% acetonitrile at flow rate 250 nl/min. Runs were acquired using an LTQ Orbitrap Velos (Thermo Scientific, Rockland, U.S.A.) and a data-dependent Top-20 shotgun MS/MS method. Peptides were quantified using MaxQuant and an in-house yeast database (Cox and Mann, 2008). Only unambiguous proteins with a PEP score < 0.01 for which H/L ratios were recorded in all samples in the protein groups output were retained, resulting in an FDR of 0. Degradation rates were calculated as described.
**SILAC pooling experiment for OST assembly and for experiments with diploid cells**

Equal weights of wild type cells grown in heavy and mutant cells in light medium were pooled together (Figure 6A). Light and heavy precursor signals were measured as described above. L/H ratios were calculated for each peptide. The amount of OST subunits relative to wild type was calculated from the average L/H over all peptides of a protein. Values were normalized by dividing by the ribosomal control protein Rpl5p or by the average L/H of all non-OST proteins (SEC, PMT proteins, and Pdr5p) of each replicates. Average L/H values over 3 biological replicates and their 95% confidence interval was displayed.

More detailed experimental procedures, as well as tables containing strains, plasmids, antibodies, and primers can be found in the Supplemental Experimental Procedures.
Author Contributions:

S.M., R.G., and M.A. designed the general approach and planned the experiments. S.M. executed all experiments. A.W. supported the LC-MS applications and aided in the MS method set-up. A.F., C.O., and N.S. were involved in developing the pulse-chase setup or parts of the SRM method and strain construction. R.G. assisted with the SILAC-pooling experiments. E.N. and K.P. constructed some strains used in this study. S.M., R.G. and M.A. wrote the manuscript.
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References:


Addition to Supplemental Items:

Supplemental Experimental Procedures:

Media
We used standard media for yeast cultures (Guthrie & Fink, 1991). Synthetic media were devoid of appropriate amino acids to ensure plasmid maintenance. Media for SILAC experiments are described below.

PrA* containing strains
A SILAC-compatible wild type strain SMA673 (SS328, Δarg4::natMX) was constructed by deleting the ARG4 gene using a natMX cassette amplified with primers Fwd Arg4_pAG36 and Rev Arg4_pAG36 from plasmid pAG36 (courtesy of Y. Y. Fan, Tables S1, S2, S4). A BY4742 Δarg4::natMX strain (SMA674) was constructed the same way. SMA820 (SS328, Δarg4::natMX, ΔprA::hygBMX) had been constructed using primers CO047 and CO048 to delete the PrA gene in SMA673. SMA820 was then transformed with plasmids pKK127 (PrA*-aB) and pKK129 (PrA*-Ab) using the lithium acetate method or a slightly modified version thereof to yield SMA822 and SMA823, respectively (Gietz and Schiestl, 2007a, b).

Construction of an ERAD-deficient strain:
Primers Δdoa10::MX4_for, Δdoa10::MX4_rev and Δhrd1::MX4_for, Δhrd1::MX4_rev were used to amplify kanMX4 or hygBMX4 cassettes with ends homologous to flanking regions of DOA10 or HRD1 from plasmids pFA6-kanMX4 and pAG32, respectively (Tables S1, S2). Strain SMA673 was transformed with the purified PCR products using the lithium acetate method or a slightly modified version thereof (Table S4). Deletions were verified by PCR, yielding strain SMA1566 (SS328, Δarg4::natMX Δhrd1::hygBMX Δdoa10::kanMX).

PrA overexpressing strains:
The SaIl-BamHI fragment containing the PrA gene from PEP4-pRS316 was inserted into YEp352 digested with the same enzymes to obtain pPrA (Table 1). Strains SMA673 (SS328, Δarg4::natMX) and SMA1566 (SS328, Δarg4::natMX Δhrd1::hygBMX Δdoa10::kanMX) were transformed with pPrA or the empty vector (YEp352) to yield strains SMA1648 (SS328, Δarg4::natMX, pPrA), SMA1649 (SS328, Δarg4::natMX Δhrd1::hygBMX Δdoa10::kanMX, pPrA), SMA1574 (SS328, Δarg4::natMX, YEp352), and SMA1584 (SS328, Δarg4::natMX Δhrd1::hygBMX Δdoa10::kanMX, YEp352), respectively (Table S4). The lithium acetate
method or a slightly modified version thereof was used for transformation and transformants were selected on SD-URA (Gietz and Schiestl, 2007a, b).

**OST subunit overexpressing strains:**

Using the lithium acetate procedure or a slightly modified version thereof, strains SMA673 (SS328, \(\Delta arg4::natMX\)) and SMA1566 (SS328, \(\Delta arg4::natMX\ \Delta hrd1::hygBMX\ \Delta doa10::kanMX\)) were transformed with plasmids pOST1, pOST2, pOST3, pOST4, pOST5, pOST6, pSWP1, pWBP1, and pSTT3 (Tables S1, S4). Transformants were selected on SD-URA, yielding strains SMA1575 - 1592 and SMA1594.

**LmSTT3D-LEU and LmSTT3D-URA plasmids and deletion of OST subunits:**

The pRS425 (LEU) plasmid containing the *Leishmania major* STT3D ORF under the GPD-promoter has been published before and was called pLmSTT3D-LEU in this study (Nasab et al., 2008). A high-copy plasmid containing the *Leishmania major* STT3D ORF under the GPD-promoter and with a URA selection marker was constructed by inserting GPD-LmSTT3D-cyc1 from pRS426-GPD-LmSTT3D (Parsaie Nasab et al., 2013) into SacI-, EcoRV-digested YEp352. The plasmid was called pLmSTT3D-URA in this study.

The ARG4 gene was deleted in strain BY4742 via the Cre-loxP system (Gueldener et al., 2002; Guldener et al., 1996): the \(loxP-kanMX-loxP\) cassette was amplified from plasmid pUG6 using primers A1, A2, and was transformed into BY4742 using the lithium acetate method or a slightly modified version thereof (Tables 1, 2, 4). \(\Delta arg4::loxP-kanMX-loxP\) cells were selected on YPD + G418. After transformation with pSH47 the kanMX cassette was excised by inducing Cre recombinase in YPGal (1% yeast extract, 2% bacteriological peptone, 2% galactose) for 2 h and subsequently plating cells on YPD and replica-plating on YPD + G418. After loss of the kanMX cassette had been verified by PCR, cells that had lost the pSH47 plasmid were selected on 5-FOA plates. This procedure yielded strain SMA1753 (BY4742 \(\Delta arg4::0\)) which was transformed with pLmSTT3D-URA or pLmSTT3D-LEU to yield strains SMA1603 (BY4742 \(\Delta arg4::0 + pLmSTT3D-URA\)) and SMA1651 (BY4742 \(\Delta arg4::0 + pLmSTT3D-LEU\)), respectively.

The *Kluyveromyces lactis* LEU2 cassette was amplified from plasmid pUG73 with corresponding primers Kl-Leu2-ostX-Fw/Rev to produce DNA products with ends homologous to up- and downstream fractions of the targeted OST subunits (Tables S1, S2) (Gueldener et al., 2002). SMA1753 (BY4742 \(\Delta arg4::0\)) or SMA1603 (BY4742 \(\Delta arg4::0 + pLmSTT3D-URA\)) cells were transformed with PCR products using the lithium acetate method or a slightly modified version thereof and OST genes were deleted via homologous recombination. If the pLmSTT3D-URA plasmid had not been present in the strain before, it was introduced using
the lithium acetate method or a slightly modified version thereof (Gietz and Schiestl, 2007a, b). This procedure yielded strains SMA1604 - 1609.

**Heterozygous diploid strain construction**

To create heterozygous diploid strains containing $\Delta ostx/OSTx$ alleles in the BY4742 background, strain SMA674 (BY4742, $\Delta arg4::natMX$, $MAT\alpha$) was transformed with pME665 (Table S1) carrying the HO-endonuclease under a galactose promoter using the lithium acetate method or a slightly modified version thereof (Gietz and Schiestl, 2007b). Transformants were cultured in SD-URA until OD$_{600}$ 1, and 5 OD of washed cells were transferred to 5 ml YPGal medium, inducing HO-endonuclease to recombine the mating type locus. After 4 h, dilutions of cells were plated on YPD to suppress HO-endonuclease expression. Clones were picked and the pME665 plasmid was counter-selected on 5FOA plates. The mating type of the clones was tested by assessing halos on a lawn of XBH8-2C and RC634 cells and a $MAT\alpha$ clone (SMA1593, BY4742, $MAT\alpha \Delta arg4::0$) was selected for further strain construction.

SMA1593 was mated to strains SMA1603 (WT + pLmSTT3D-URA), YG2073 ($\Delta ost1$ + pLmSTT3D-LEU), and SMA1605 ($\Delta wbp1$ + pLmSTT3D-URA). Diploid clones were selected on SD-URA + NAT or SD-LEU + NAT plates and the pLmSTT3D-LEU and pLmSTT3D-URA plasmids were counter-selected on YPD or 5FOA, respectively. This yielded strains SMA1620 (WT), SMA1628 ($\Delta wbp1/WBP1$), SMA1621 ($\Delta ost1/OST1$).

**Immunoblot analysis:**

Cultures were grown in appropriate dropout media until reaching an OD$_{600}$ of 1. Cells were lysed with glass beads in sample buffer (2% SDS, 62.5 mM Tris/HCl pH 6.8, 10% glycerol, 6 M urea, 5% $\beta$-mercaptoethanol, 0.02% bromophenol blue, 1 X complete protease inhibitor cocktail (Roche), 5 mM PMSF, 25 mM EDTA) by vortexing for 15 min at 4°C. Prior to adding $\beta$-mercaptoethanol, bromophenol blue, and EDTA the protein concentration of samples 10-fold diluted in 50 mM Tris pH 7.5, 1 % SDS, 1 % Triton-X100 was measured by Pierce BCA (Thermo Scientific, Rockland, U.S.A.). Proteins were dissolved for 20 min at 37°C. Equal amounts of protein contained in 10 - 15 $\mu$l of sample were loaded on 10% PAGE gels. Proteins were blotted onto nitrocellulose membranes. After blocking with 10% milk in PBS-0.1% Tween, membranes were hybridized with primary antibodies (Table 3), washed and hybridized with appropriate secondary antibodies (anti-Rabbit, sc-2004, or anti-mouse IgG, sc-2005, Santa Cruz, Table S3). Bands were visualized with Amersham ECL detection solution (GE Healthcare, Amersham, U.K.) on radiofilms (Super RX, Fuji Medical X-Ray Film, Tokyo, Japan).
**Pulsed SILAC experiment:**
Yeast cells auxotrophic for arginine and lysine were grown in shake flasks in appropriate dropout media deficient in arginine and lysine but supplemented with 20 mg/l heavy $^{13}$C$_6$ arginine (R6, Cambridge Isotope Lab, Andover, U.S.A.) and $^{13}$C$_6$-$^{15}$N$_2$ lysine isotopes (K8, Sigma Aldrich, Buchs, Switzerland) until all proteins were heavily labelled and the culture volume was sufficiently expanded. Culture-to-flask ratios were always 1:5 and saturation past exponential phase was avoided by dilution into fresh medium. 200 ml pulse-cultures were inoculated with the exponential precultures and were grown for at least 2 doubling times until they reached OD$_{600}$ 1. At the start of the chase, cells were collected on a 0.22 $\mu$m filter and washed with 400 ml of appropriate dropout medium without arginine nor lysine, and resuspended in prewarmed medium containing light $^{12}$C$_6$ arginine (R0) and $^{12}$C$_6$-$^{14}$N$_2$ lysine (K0) isotopes to ca. OD$_{600}$ 0.5. Cells were chased for maximum one doubling time until they reached OD$_{600}$ 1 with all newly synthesized proteins incorporating light arginine and lysine. At three timepoints (0 min, 15 min, 60 min for wild-type-like growth or 0 min, 30 min, 120 min for slow growing strains) throughout this 1 - 2 hours period 50 OD of cells were sampled, frozen with liquid nitrogen and stored at - 80°C.

**Isolation of ER proteins for mass spectrometry:**
Fifty OD of cells were processed to isolate target proteins. Cells were washed in 5 - 10 ml of lysis buffer (2 M NaCl, 10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, Roche complete 1 X) and were lysed with glass beads and vortexing for up to 15 min at 4°C. Beads were washed with lysis buffer several times and unbroken cells were pelleted 5 min at 1000 g. Membranes were pelleted by centrifuging the collected supernatant 20 min at 16 000 g and 4°C. Membranes were prepared with a protocol adapted from Wisniewski et al. (Nielsen et al., 2005; Wisniewski et al., 2009). In short, membrane vesicle content was released by resuspending membranes in 1 ml of 0.1 M Na$_2$CO$_3$, 1 mM EDTA, pH 11.3 using an Eppendorf douncer and a sonication bath, and incubating at 4°C for 30 min. After pelleting membranes 20 min at 16 000 g and 4°C, proteins in half of the Na$_2$CO$_3$-supernatant were precipitated with 0.11 volumes tricholoro-acetic acid for 5 min on ice. Na$_2$CO$_3$ protein pellets were washed two times with acetone and solubilized as described below. In the meantime the membrane pellet was resuspended in 1 ml 5 M urea, 100 mM NaCl, 10 mM HEPES, pH 7.4, and 1 mM EDTA using an Eppendorf douncer to eliminate membrane-associated proteins. Membranes were again pelleted and washed two times in 1 ml 0.1 M Tris/Cl, pH 7.6. Finally, membranes were collected for 20 min at 16 000 g and 4°C. All samples were solubilized in 100 - 200 µl of 4% SDS, 50 mM DTT, 0.1 M Tris/Cl, pH 7.6 by repeatedly heating (90°C, 1 min), vortexing and
sonicating the sample at room temperature. Unsolubilised material was pelleted 20 min at 16,000 g and discarded. Samples were frozen with liquid nitrogen and stored at -80°C.

The whole sample volume containing solubilized proteins were processed using a filter- assisted sample preparation (FASP) protocol adapted from Wisniewski et al. (Wisniewski et al., 2009). Samples were thawed, sonicated, diluted at least 20-fold with UA (8 M urea in 0.1 M Tris/HCl, pH 8.5), and loaded onto 30 kDa cutoff Amicon spin filters (Millipore, Zug, Switzerland) at 14,000 g for 15 min. After washing with 0.4 ml UA proteins were alkylated in 0.2 ml 50 mM iodoacetamide for 30 min in the dark. Peptides were washed three times with 0.4 ml UB (8 M urea in 0.1M Tris/HCl, pH 8.0) and resuspended in 60 ml UB. Proteins were digested with 2 μg of endoproteinase Lys-C (Wako Pure Chemical Ind., Osaka, Japan) overnight, diluted with 400 μl of 40 mM NaHCO₃, and again digested with 2 μg trypsin for 4 hours at 37°C. Peptides were eluted for 20 min at 14,000 g. Filters were washed twice with 50 μl NaCl 0.5 M and eluates were pooled. Peptide concentration was measured as absorption at 280 nm using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, U.S.A.). Samples were frozen with liquid nitrogen and stored at -80°C. Five micrograms of protein were brought to 3% acetonitrile and pH 2-3 with trifluoro-acetic acid (TFA). Samples were desalted using C18 ZipTips (Millipore, Zug, Switzerland). In short, after wetting the ZipTip with acetonitrile it was equilibrated in 3% ACN, 0.1% TFA. Peptides were bound to the resin and washed with 3% ACN, 0.1% TFA prior to eluting two times with 50% ACN, 0.1% TFA. The procedure was repeated and eluted peptides were dried in a SpeedVac and dissolved in 20 μl 3% ACN, 0.1% FA by sonication.

**SRM assay development:**

Proteotypic peptides were selected for 26 target proteins using BLAST and PeptideAtlas and SRM assays were set up. The majority of transitions (for 66 out of 83 target peptides) were extracted from SRM-atlas using the yeast spectral library build “201207_QQQ_lib.tgz” described by Picotti et al., Nature 2013 (for notes how to download the spectral library, see supplementary notes from Picotti et al., 2013 ). For peptides not present in the spectral library but previously detected in LTQ Orbitrap runs, we used ions from QTRAP or LTQ Orbitrap runs or theoretical transitions possibly with an m/z higher than the precursor to ensure selectivity. Retention times were determined through unscheduled SRM-runs, or were transferred from earlier LTQ Orbitrap runs using Biognosys iRT peptides and linear extrapolation.

Up to 10 transitions per precursor were analyzed. The precursor peak was identified by coelution of the transitions, by corresponding peptide abundances and relative fragment intensities in light and heavy precursors in SILAC samples, and, depending on availability, by retention time and/or by comparison to the spectral library (dotproduct > 0.7) (Picotti et al., 2013). The 4 – 5 transitions showing most intense signal, highest selectivity and least
background or interference were selected for the final method. For 43 peptides, including all peptides not present in the spectral library, transitions were validated using synthetic peptides (SpikeTides, JPT, Berlin, Germany) either in solvent or spiked into a yeast sample heavy labelled with Arg6 and Lys8.

The complete set of transitions was split into two methods of which one contained assays for peptides found mainly in the membrane fraction and the other one contained transitions for peptides in the sodium carbonate fraction (Table S5).

**SRM mass spectrometry:**
Retention time iRT peptides (Biognosys, Zurich, Switzerland) were added to all samples in ratios of 1:20 – 1:40. This allowed us to transfer peptide retention times between different samples, instruments and LC-gradients using linear regression in an Excel spreadsheet. 2 - 8 μl of sample were loaded for mass spectrometry measurements.

Samples were injected onto self-packed column (15 cm x 75 μm, OD: 375 μm; beads: Magic C18 AQ, 3 μm, 200 A) coupled to an Eksigent nanoLC-Ultra 1D plus (ABSciex, Zug Switzerland). Samples were separated using a binary solvent system with a flow rate of 500 nl/min and eluted using a gradient from 3% B to 35% B over 60 minutes (A: 0.1 % FA, B: 100% ACN, 0.1% FA). Targeted SRM analysis was performed on a Qtrap 5500 (AB Sciex, Zug Switzerland). SRM Transitions are listed in Table S5. Unscheduled methods were measured with 30 ms dwell time, a maximum cycle time of 3 s was used for both unscheduled and unscheduled methods and a declustering potential value of 100, collision cell exit potential of 13 and entrance potential 10.

Results from scheduled SRM runs were exported to Skyline and peaks were integrated in the software (MacLean et al., 2010). Changing heavy to light ratios were recorded over time due to old heavy protein being lost from the cell and new light protein being synthesized. The measured heavy to light ratios were exported and degradation rates were calculated as described below.

**Calculating degradation rates:**
The loss rate of old protein from the cell, $k_{LOSS}$, was calculated according to Larrabee et al. (Larrabee et al., 1980; Li, 2010). This method is mathematically equivalent to exponential curve fitting through $H/(H+L)$ values that has been used in other studies (Helbig et al., 2011; Pratt et al., 2002), i.e. will yield the same degradation rates. Detailed formula for calculations can be found in the Supplemental Experimental Procedures. In short, we plotted the LN(light/heavy+1) value of each peptide over time and determined $k_{LOSS}$ by linear curve fitting as the slope of the curve. The curve fit, as well as the subsequent calculations were either executed by a self-written Python script or done using the SigmaPlot 12.2 software and Microsoft Excel. Values
for all peptides of a protein were averaged to yield $k_{\text{LOSS}}$ for a protein. Finally, $k_{\text{DIL}}$, defined as $k_{\text{LOSS}}$ of the stable protein Rpl5p, was subtracted from the $k_{\text{LOSS}}$ values to yield degradation rates $k_{\text{DEG}}$ and half-lives were calculated using the equation $t_{1/2} = \ln(2) / k_{\text{DEG}}$. We arbitrarily classified proteins with half-lives longer than eight cell divisions (i.e. > 12 hours for wild type cells) as stable since in the same time the protein would be diluted by cell division 256 times.

**Formula for calculating degradation rates:**
Degradation rates were calculated according to Larrabee et al. (Larrabee et al., 1980). In short, the total amount of protein in a cell at a timepoint $t$ $A_{\text{tot},t}$ consists of the amount of old protein $A_{\text{old},t}$ (heavy labelled) and new protein $A_{\text{new},t}$ (light) and increases with the growth rate $k_{\text{DIL}}$ in an exponential fashion. This yields equation 1.

$$A_{\text{tot},t} = A_0 e^{k_{\text{DIL}} t} = A_{\text{old},t} + A_{\text{new},t} \quad (1)$$

Old protein is exponentially degraded with the degradation rate $k_{\text{DEG}}$, leading do equation 2.

$$A_{\text{old},t} = A_0 e^{-k_{\text{DEG}} t} \quad (2)$$

Inserting equation 2 into 1 and reformulating we get equation 3.

$$A_{\text{new},t} = A_0 (e^{k_{\text{DIL}} t} - e^{-k_{\text{DEG}} t}) \quad (3)$$

Dividing $A_{\text{new},t}$ by $A_{\text{old},t}$ or equation 3 by equation 2 and reformulating the leads to equation 4.

$$\frac{A_{\text{new},t}}{A_{\text{old},t}} = \frac{e^{k_{\text{DIL}} t} - e^{-k_{\text{DEG}} t}}{e^{-k_{\text{DEG}} t}} = k_{\text{DIL}} + k_{\text{DEG}} = k_{\text{LOSS}} \quad (4)$$

Based on this equation, plotting $\ln(A_{\text{new},t}/A_{\text{old},t} + 1)$ or in other words $\ln(\text{Light/Heavy} + 1)$ for a peptide over time will yield a line with the slope $k_{\text{LOSS}}$, which is the rate by which the amount of old, heavy protein in a cell decreases. Since dilution by cell division and degradation both diminish old protein, the dilution rate $k_{\text{DIL}}$ needs to be subtracted from the $k_{\text{LOSS}}$ values to get the degradation rate $k_{\text{DEG}}$ with the dimension $h^{-1}$ or $\text{min}^{-1}$. Half-lives were calculated via equation 5.
\[ \frac{1}{t_{1/2}} = \frac{\ln(2)}{k_{\text{DEG}}} = \frac{0.693}{k_{\text{DEG}}} \quad (5) \] 

We experimented with different methods to measure the dilution rate \( k_{\text{DIL}} \). Setting \( k_{\text{DIL}} \) equal to the growth rate led to underestimation of degradation rates, probably due to technical variation and mathematical constraints (Claydon and Beynon, 2012), to the use of a different measuring method and to amino acid recycling (Cambridge et al., 2011). Therefore, we decided to set \( k_{\text{DIL}} \) equal to the loss rate of the Rpl5p protein which thus defined our zero-degradation cutoff. We argue that this was valid since Rpl5p has been shown in the past to be stable (Lam et al., 2007; Schwanhausser et al., 2011).

**Preparation of whole cell extracts and mass spectrometry analysis:**

For whole cell extracts, 50 OD of cells were washed in 10 ml and 0.5 ml Tris-HCl 100 mM pH 7.6 + 1 X protease inhibitor cocktail, EDTA-free (Roche). Cells were lysed with glass beads and vortexing for 15 min at room temperature in solubilisation buffer (100 mM Tris-HCl, pH 7.6, 50 mM DTT, 4% SDS). Unsolubilised cells were pelleted for 5 min at 1000 g. Proteins in the supernatant were solubilized by incubating 5 min at 95°C. Unsolubilised material was eliminated by centrifuging 1 min at 16 000 g. DNA was sheared by sonicating 10 min with amplitude 65% and 0.5 cycle on an UTR200 Ultrasonic processor (Hielscher Ultrasound technologies, Teltow, Germany). Unsoluble material was pelleted for 5 min at 16 000 g and the protein concentration of the supernatant was measured with Qubit (Invitrogen), Quant-IT. For whole cell extracts, 200 \( \mu \)g protein were diluted with 400 \( \mu \)l UA buffer and prepared for mass spectrometry using the FASP and ZipTip protocols described above. Biognosys retention time peptides were added to samples in a 1:40 ratio. Four microlitres of sample were injected using the same liquid chromatography setup as for SRM measurements, except that flow rate was 250 nl/min. Runs were acquired using an LTQ Orbitrap Velos (Thermo Scientific, Rockland, U.S.A.) running a standard LC-MS/MS program of 1 survey (MS) scan followed by 20 dependent scans (MS/MS) looped throughout the run.

Spectra were searched for peptide identifications using MaxQuant version 1.2.7.4., the Andromeda search software, and an in-house yeast database based on the SwissProt reference proteome and containing contaminants and reversed sequences (Cox and Mann, 2008; Cox et al., 2011). Search parameters were Arg6, Lys8 as labels, trypsin as enzyme, and cysteine carbamidomethylation as constant and methionine oxidation as variable modification. Protein false-discovery-rate (FDR) was set to 0.05 and minimal peptide length to 5 amino acids. The protein groups output of the MaxQuant search was processed manually. Only proteins for which H/L ratios had been detected for all strains, replicates and time points were retained. Proteins with a PEP score < 0.01 were discarded, resulting in an FDR of 0.
Furthermore, protein group hits containing more than one protein were discarded and L/H values were calculated for the remaining proteins. Degradation rates were calculated for all proteins whose L/H ratios increased over time using an in-house Python script as described.

**SILAC experiments for OST assembly and for experiments with diploid cells:**
Wild type cells were cultured in medium containing heavy amino acids while the mutant strains carrying deletions in one of the OST subunits were grown in light amino acid medium (Fig. 5A). All haploid cells with a single subunit deletion carried the LmSTT3D plasmid to compensate glycosylation defects. After complete labelling of cellular protein, equal amounts of cells (normalized by weight) for wild type and deletion mutant were pooled together, proteins were isolated, peptides were prepared for mass spectrometry using FASP and were analyzed by SRM as described. From the heavy and light peptide signals we calculated the amount of each subunit in the deletion mutant relative to the wild type. More precisely, L/H values were log2-transformed before calculating the average L/H ratio over all peptides for each protein. Log2-transformed values were normalized. For haploid cells this was done by dividing by the average log2(L/H) of all non-OST proteins (SEC, PMT components and Pdr5p) of the same replicates. For diploid cells we divided by log2(L/H) of the ribosomal control protein Rpl5p. Then the average log2(L/H) over all three biological replicates was calculated for each protein. The resulting ratios were back-transformed to linear space, yielding normalized average L/H ratios for each protein. The 95% confidence interval for the average log2(L/H) values of three replicates was calculated, values were back-transformed to linear space and displayed.
**Figure S1: Low levels of protein degradation in exponential cells. Related to Figure 2.**

(A) SRM trace of peptide LSDFLHVSSGSDEK from Ost1p in wildtype and \(\Delta erad\) cells. (B) Scheme comparing effect of degradation and cell division on a protein with half-life of 12 h. After 12 h half of the original protein will be degraded. In the same time, a wildtype cell will divide eight times, diluting the original protein two-fold at each division and resulting in a dilution factor of 256 after 12 h.
Table S1. Plasmids used in this study. Related to Experimental Procedures.

Vectors created and/or used in this study.

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<tr>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
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<td>Primer</td>
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### Table S3. Antibodies. Related to Experimental Procedures.

Antibodies used in this study.

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<th>Production species</th>
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<th>Dilution</th>
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<td>αCPY (S. cerevisiae)</td>
<td>mouse</td>
<td>commercial, Invitrogen, mouse IgG1 clone 10A5</td>
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<td>αPdi1p (S. cerevisiae)</td>
<td>rabbit</td>
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<td>αHXK (S. cerevisiae)</td>
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<td>αOst1p (S. cerevisiae)</td>
<td>rabbit</td>
<td>gift from R. Gilmore, University of Massachusetts, USA</td>
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<tr>
<td>αOst3p (S. cerevisiae)</td>
<td>rabbit</td>
<td>own laboratory, M. Aebi, ETH Zurich, Switzerland, produced by Simon Kuster, used in (Spirig et al., 2005)</td>
<td>1:1000</td>
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<tr>
<td>αOst6p (S. cerevisiae)</td>
<td>rabbit</td>
<td>gift from R. Knauer &amp; L. Lehle, University of Regensburg, Germany</td>
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<td>own laboratory, M. Aebi, ETH Zurich, Switzerland, used in (Silberstein et al., 1995)</td>
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<td>(te Heesen et al., 1991)</td>
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### Table S4. Yeast strains. Related to Experimental Procedures.

Yeast strains used in this study.

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<td>Vijayraghavan et al., 1989</td>
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<td>BY4742</td>
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<td>(Chan and Otte, 1982)</td>
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<tr>
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<td>(Schenk et al., 2001)</td>
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<td>(Nasab et al., 2008)</td>
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For further details, please refer to Picotti et al., 2013.
<p>| Protein | SwissProt Name | Peptide | change | precursor/ fragment ion | mass light | mass heavy | collision energy light | collision energy heavy | fraction | Source of transitions | validated with synthetic peptides in this study | transitions validated with synthetic strains | RT in nmol/10 g fr (Ran. 20131012_0500_HanRF18_R1_r2) | RT in nmol/10 g fr (Ran. 20131012_0500_HanRX2_VF1_N42 C2D_R1_30mm_Aux) |
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| Ost1p   | OST1_YEAST    | NLSQWGQGLI9K | 1+ fragment | y9 | 941.377 | 949.923 | memb | Picotti 2013 | Y | absent in both 1, increase during GST7 overexpression absent in both 1, increase | | | |
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| Ost1p   | LTFSYR        | 673.334 | 679.385 | memb | this study | Y | | | | | | |
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| Ost2p   | OST2_YEAST    | RARFQAPIR    | 1+ fragment | b3 | 800.470 | 806.471 | memb | this study | Y | absent in both 2, increase during GST7 overexpression absent in both 2, increase | | | |
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Supplemental References:


Li, Q. (2010). Advances in protein turnover analysis at the global level and biological insights. Mass spectrometry reviews 29, 717-736.


Chapter 3:

Overexpression and deletion: analysis of genetic tools
in view of protein complex homeostasis
**Introduction:**

Balancing protein abundance is crucial for many cellular processes and might be optimized during evolution (Dekel and Alon, 2005; Seidman and Seidman, 2002). It has been shown that both overexpression and deletion of certain genes can lead to phenotypes in yeast cells (Deutschbauer et al., 2005; Sopko et al., 2006). Deutschbauer et al. studied the effect of heterozygous deletions (1 allele deletions) in diploid cells and showed that some deletions were haploinsufficient and impaired cellular fitness. The mechanism for haploinsufficiency has been debated and two hypotheses have been proposed: (I) the insufficiency hypothesis suggests that defects arise because too little protein is produced in a heterozygote cell and the absolute amount required for normal growth is not met (Deutschbauer et al., 2005). (II) The balance hypothesis proposes that phenotypes are caused rather by altered relative amount of protein compared to its interacting partners (e.g. complex components) (Papp et al., 2003). According to the balance hypothesis, not only gene deletions but also protein overexpression would lead to a phenotype for protein complexes, while this is not likely according to the insufficiency hypothesis. In agreement with the insufficiency hypothesis, overexpression of a complex component can even rescue a defect in another subunit. This is termed dosage suppression and has been used to pinpoint interacting proteins (Silberstein et al., 1995; te Heesen et al., 1993). In a global yeast screen 525 interactions showed a dosage suppression phenotype in protein complex interactions (Magtanong et al., 2011). In summary, the levels of complex components are dependent on each other and thus influence complex stability. Thus, the effect of altered subunit stoichiometry on protein levels might also help to reveal complex subunits.

In this chapter we focused on the importance of protein abundance for protein complex components. Thus, we analyzed a mutation in the catalytic subunit which diminished complex incorporation and determined which proteins stabilized specific OST subunits. Furthermore, we conducted enrichment analyses of datasets from global genetic studies and proposed general principles for complex stability when subunit stoichiometry is altered.
Results

A point mutation in Stt3p destabilizes the OST complex

In a genetic screen Zufferey et al. discovered a point mutation in the yeast STT3 gene (Zufferey et al., 1995). An exchange of nucleotide 1654 from T to C caused a conversion of Ser552 in the C-terminal hydrophilic domain to proline. This mutation was termed stt3-7 and caused a temperature sensitive phenotype, including destabilization of Stt3p and hypoglycosylation that were lethal at 37°C (Spirig et al., 1997). Deletion of UBC6, MNS1, or HTM1 leading to decreased ERAD activity rescued the cells from lethality at high temperatures (Jakob et al., 2001). Besides, overexpression of Ost3p or Ost4p, subunits that normally interact with Stt3p in the OST complex, stabilized mutant Stt3p (Spirig et al., 1997). This indicated that decreased degradation of mutant Stt3p or overexpression of interacting subunits shifted the assembly equilibrium towards increased incorporation into the OST complex. In summary, these findings suggested that the stt3-7 mutation did not affect catalytic residues but that the phenotype was due to destabilization and reduced incorporation into the complex followed by degradation.

To analyze dynamics of OST components in stt3-7 cells we constructed cells containing the stt3-7 mutation in a SILAC compatible lys2-801 Δarg background. In short, wild type (SS328, lys2-801, Δarg4::natMX4) and YG0535 (stt3-7) cells were mated, followed by sporulation of diploid cells and tetrad dissection (Guthrie, 1991; Spirig et al., 1997). Tetrads with two offspring showing normal colony size (termed b1, b2) and two showing a growth defect on YPD at 23°C (termed s1, s2) were selected. Cells of all four strains were cultured in YPD at 23°C and proteins were analyzed by immunoblot after SDS-PAGE (Figure 1A). Signals of lower molecular weight were detected in s1 and s2 cells for the glycoproteins Ost1p, Wbp1p, CPY and also Stt3p, indicating hypoglycosylation of those proteins. In addition, signal intensity of Ost3p was decreased in s1, and s2 cells. Stt3p abundance was difficult to determine because of the diffuse signal. We concluded that even at a temperature as low as 23°C the point mutation affected complex assembly, and as a consequence destabilized Ost3p and produced a hypoglycosylation phenotype.

We further wanted to analyze if the phenotype of stt3-7 cells (i.e. hypoglycosylation and reduced abundance of OST subunits) was more pronounced at 30°C. If this was the case, stt3-7 cells might not be viable at 30°C. To ensure viability at higher temperatures, we introduced a high-copy plasmid carrying the Leishmania major STT3D (LmSTT3D) gene into wild type and stt3-7 cells. This one-subunit oligosaccharyl transferase operates independently of the yeast OST and is able to glycosylate yeast proteins, thus preventing hypoglycosylation-
dependent lethality of the stt3-7 mutation at higher temperatures. We analyzed proteins from wild type and stt3-7 cells containing empty vector or plasmid-borne LmSTT3D cultured at 30°C in minimal medium (Figure 1B). Immunoblot results were similar to those obtained at 23°C. While protein levels of most subunit levels remained constant, levels of Stt3p, Ost3p, and Ost6p clearly decreased in the presence of the stt3-7 mutation. In agreement with earlier studies this suggested that Stt3p was destabilized and that this in turn led to degradation of its interactors Ost3p and Ost6p (Jakob et al., 2001). LmSTT3D compensated hypoglycosylation of CPY, Ost1p, and Stt3p, however not of Wbp1p, indicating that not all yeast proteins were LmSTT3D-substrates and that OST activity was decreased.

Figure 1: Analysis of the temperature sensitive stt3-7 point mutation.
A) Cells from all four offspring of a stt3-7/STT3 tetrad and their parents, wild type (SS328, lys201, Δarg4::natMX) and YG0535 (stt3-7), were analyzed by immunoblot. Cells were grown in YPD at 23°C, lysed with glass beads and detergent, and equal amounts of protein were analyzed. Two offspring had shown normal colony size (b1, b2, b = big) and two showed a growth defect on YPD at 23°C (s1, s2, s = small). Strain s1 (SMA1640) was chosen for further strain construction. B) Wild type and stt3-7 cells were transformed with a high-copy plasmid expressing LmStt3D to compensate hypoglycosylation phenotypes. Cells were grown in SD - URA at 30°C and analyzed by immunoblot.
**Wbp1p is one of the main stabilizers of Swp1p and Ost2p but not Stt3p**

We had shown previously that overexpressed OST subunits were unstable and were degraded (Chapter 2, Figure 3). Based on that we put forward the hypothesis that the degradation rate of a subunit would be maximal if the protein was overexpressed since all stabilizing subunits would be present only in lower amounts. Thus, a subunit degraded with the same maximal rate in cells deleted in another OST component, would indicate that the component had been the main stabilizer of the subunit. Lower than maximal degradation rates, on the other hand, would result from stabilization of the subunit by other complex partners. This would reveal which subunits stabilized each other.

![Comparison of degradation rates for OST subunits during overexpression and in Δwbp1 cells.](image)

*Figure 2: Comparison of degradation rates for OST subunits during overexpression and in Δwbp1 cells.*

Degradation rates were measured for OST subunits in SILAC-SRM pulse-chase experiments. Values from three biological replicates are displayed. Error bars correspond to the standard deviation. Degradation rates are depicted for OST components in wild type cells overexpressing Ost2p, Swp1p, or Stt3p, and in cells with a Δwbp1 deletion. Bars for Ost2p, Swp1p, and Stt3p are marked with an arrow in the respective overexpression strains and in the Δwbp1 cells.

We measured degradation rates and calculated half-lives of OST subunits in wild type, Δstt3, and Δwbp1 cells using a SILAC-SRM (selected reaction monitoring) method. Cells were grown in medium containing heavy amino acids ($^{13}$C$_6$ arginine, $^{13}$C$_6$$^{15}$N$_2$ lysine) until all proteins were heavy-labelled. Then cells were transferred to light medium ($^{12}$C$_6$ arginine, $^{12}$C$_6$$^{14}$N$_2$ lysine). Samples were taken at different time points, proteins were extracted and digested, and heavy and light peptides were measured by SRM. Degradation rates and subsequently half-lives
were calculated from the changing H/L ratios. We compared the half-lives of Swp1p, Ost2p, and Stt3p in \( \Delta \text{wbp1} \) deletion cells to their half-lives during individual overexpression (Figure 2, Table 1). Half-lives of Ost2p (k_{DEG}: 0.009 vs. 0.013 min^{-1}, half-life: 1.2 vs. 0.9 h) were not significantly different in both types of cells (t-test, p-value 0.081). Stt3p on the other hand was degraded significantly more slowly (p-value 0.001) in \( \Delta \text{wbp1} \) cells compared to Stt3p overexpressing cells (k_{DEG}: 0.003 vs. 0.009 min^{-1}, half-life 4.4 vs. 1.3 h). For Swp1p (k_{DEG}: 0.012 vs. 0.007 min^{-1}, half-life: 1.0 vs. 1.5 h) the half-lives were in a similar range in both cell types. However, they were significantly lower (p-value 0.004) in \( \Delta \text{wbp1} \) cells, indicating faster degradation. This result is unexpected since we assumed degradation of Swp1p would be maximal in Swp1p-overexpressing cells. It is possible that overexpression of Swp1p led to protein aggregation, thereby reducing the role of degradation. In summary, our results suggested that Wbp1p acted as a main stabilizing protein and binding partner for Swp1p and Ost2p, while Stt3p was also stabilized by interactions with other proteins.

Table 1: Half-lives of OST subunits in overexpression and deletion experiments.

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<td>Ost2p</td>
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*average values +/- standard deviation of 3 biological replicates

Analyses of yeast libraries uncover global principles of complex dynamics

Assembly of membrane protein complexes is guided by a few principles. One of them is degradation of non-assembled subunits and their stabilization by their complex partners (Daley, 2008). We confirmed that this applied to the OST complex in our previous experiments and showed that excess subunits were degraded (Chapter 2, Figure 3). Thus, overexpression of OST components had no effect on complex assembly and activity, but deletion potentially disrupted the OST complex (Chapter 2, Figures 3 & 4). We hypothesized this to be a general
principle in yeast complexes and analyzed if results from global overexpression or deletion studies were in line with this hypothesis.

**Figure 3:** Overexpression of complex subunits is less detrimental than overexpression of a single protein. According to our hypothesis, overexpression of a single protein increased enzyme abundance and thus enzyme activity and cells might display a phenotype. Overexpressing a single complex subunit on the other hand would not lead to increased enzyme activity since this would be conveyed only by the complete complex and because unassembled subunits would be degraded. Therefore, overexpression of a complex subunit would not lead to a discernible phenotype in cells.

Based on our data for OST we hypothesized that complexes were less sensitive to excess subunits. Superfluous unassembled subunits would be degraded and thus would not disturb complex assembly. According to our hypothesis, excess of one subunit would not lead to an increase of complex activity while an increase in a single protein not requiring complex partners for activity might lead to an imbalance in the cell (Figure 3). Thus, a list of viable protein-overexpression strains should contain complex subunits in the same or even higher frequency as compared to their occurrence in the genome. We prepared a list of the yeast strains present in the MOBY2 overexpression library (overexpression of 4547 yeast genes on 2-micron plasmids), mapped the primary SGD identifiers for 4495 genes using YeastMine (www.yeastgenome.org) and analyzed enrichment of GO terms using AMIGO2 (Ashburner et al., 2000; Magtanong et al., 2011). In agreement with our hypothesis we found enrichment factors of 1.2 and 1.1 for the terms "macromolecular complex" and "protein complex" in the MOBY2 overexpression library, respectively (Tables 2, 3) (Magtanong et al., 2011).
Table 2: Analysis of macromolecular complex gene enrichment in a yeast overexpression library.
Gene lists were prepared from entries in the MOBY2 overexpression library (Magtanong et al., 2011). Primary SGD gene IDs were analyzed for enrichment of GO Term "macromolecular complex" using AMIGO2. EF = (% of proteins with GO term in study dataset) / (% of proteins with GO term in proteome).

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Table 3: Analysis of protein complex gene enrichment in yeast overexpression or deletion libraries.
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Along the same line, we hypothesized that overexpression of subunits would also produce less fitness-reducing phenotypes. Therefore, protein complexes would be underrepresented in datasets of slow-growing cells from different global overexpression datasets (3 studies) (Tables 4, 5) (Osterberg et al., 2006; Sopko et al., 2006; Yoshikawa et al., 2011). Lists containing the slow-growing strains from all studies were prepared and analyzed with AMIGO2 as described above. The GO terms "macromolecular complex" and "protein complex" were either significantly underrepresented (EF 0.3 - 0.8, p-value < 0.05) in these datasets or no significant change was detected. Different results in the three datasets might be explained by different levels of overexpression or, alternatively, by different methods used to pinpoint slow growth and resulting in different sets of strains: Sopko et al. relied on colony size on solid medium, while Osterberg et al. and Yoshikawa et al. used growth rate in liquid cultures to determine slow growth. Our results are in line with the hypothesis that overexpression of subunits is generally not detrimental for yeast protein complexes.
Table 4: Analysis of macromolecular complex gene enrichment in strains with impaired fitness for three overexpression datasets.
Gene lists were prepared from fitness-impaired strains in global deletion or overexpression study datasets. The lists were analyzed for enrichment of "macromolecular complex" as described in Table 2.

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Table 5: Analysis of protein complex gene enrichment in strains with impaired fitness for three overexpression datasets.
Gene lists were prepared from fitness-impaired strains in global overexpression study datasets. The lists were analyzed for enrichment of "protein complex" as described in Table 2.

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<td>187</td>
<td>0.78</td>
<td>0.8</td>
</tr>
</tbody>
</table>

We have shown in previous experiments that deletion of a subunit had the potential to destabilize other subunits in the protein complex (Figure 4; Chapter 2, Figure 4). Thus, deletion in a single complex subunit could result in lethality, even if the deleted subunit itself did not have an essential function. Therefore, the probability of a deleterious effect would be higher when a complex component was deleted as compared to non-complex protein. As a consequence, we expected deletions in complex subunits to be overrepresented in lists of lethal mutations or slow growing strains.
Figure 4: Deletion of complex subunits has more potential to be lethal than deletion of a single protein.

According to our hypothesis, deletion of a single protein would abolish its function and would be lethal only if the protein was essential. Deletion of a single subunit would result in reduced levels also of the other subunits that it stabilized (here 4 subunits). Lethality would ensue if any of the five proteins were essential. Therefore compared to the single protein the risk for lethality is multiplied by the number of subunits destabilized by the deletion.

In a first step, we analyzed a list of 1100 essential genes (COMP-SET-ESS of the Euroscarf deletion library, http://web.uni-frankfurt.de/fb15/mikro/euroscarf/complete.html) whose deletion abolished growth of haploid yeast cells in YPD at 30°C. We investigated enrichment of protein complex subunits using AMIGO 2 (http://beta.geneontology.org, (Ashburner et al., 2000)). The GO terms "macromolecular complex" and "protein complex" were both significantly enriched by a factor of 2.1 (Tables 6, 7).

Table 6: Analysis of macromolecular complex gene enrichment in a yeast deletion library.

<table>
<thead>
<tr>
<th>Source</th>
<th>Total mapped genes</th>
<th># Macromolecular complex (GO:0032991)</th>
<th>p-value</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background Genome</td>
<td>6442</td>
<td>2072</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>EUROSCARF essential genes</td>
<td>1086</td>
<td>726</td>
<td>1.70E-117</td>
<td>2.1</td>
</tr>
</tbody>
</table>
Table 7: Analysis of protein complex gene enrichment in a yeast deletion library.

Gene lists were analyzed for enrichment as described for Table 6, except that GO Term "protein complex" was used.

<table>
<thead>
<tr>
<th>Source</th>
<th>Total mapped genes</th>
<th># Protein complex (GO:0043234)</th>
<th>p-value</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background Genome</td>
<td>6442</td>
<td>1500</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>EUROSCARF essential genes</td>
<td>1086</td>
<td>531</td>
<td>6.34E-72</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Since not all deletions would be lethal, we hypothesized that deletions in complex subunits would also be more prone to induce fitness-reducing phenotypes compared to overexpression. Therefore, we analyzed complex enrichment in slow-growing strains from global deletion datasets (4 studies) (Tables 8, 9) (Breslow et al., 2008; Deutschbauer et al., 2005; Yoshikawa et al., 2011). The GO complex terms showed enrichment factors > 1 and in most cases values were significant.

Table 8: Analysis of macromolecular complex gene enrichment in strains with impaired fitness for 4 deletion datasets.

Gene lists were prepared from fitness-impaired strains in global deletion study datasets. The lists were analyzed for enrichment of "macromolecular complex" as described in Table 6.

<table>
<thead>
<tr>
<th>Source</th>
<th>Type</th>
<th>Total mapped genes</th>
<th># Macromol. complex (GO:0032991)</th>
<th>p-value</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background Genome</td>
<td>NA</td>
<td>6442</td>
<td>2072</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>(Yoshikawa et al., 2011)</td>
<td>deletion (haploid)</td>
<td>591</td>
<td>287</td>
<td>2.64E-13</td>
<td>1.5</td>
</tr>
<tr>
<td>(Deutschbauer et al., 2005)</td>
<td>deletion (YPD, homozygous diploid)</td>
<td>1981</td>
<td>1238</td>
<td>8.55E-165</td>
<td>1.9</td>
</tr>
<tr>
<td>(Deutschbauer et al., 2005)</td>
<td>deletion (minimal medium, homozygous diploid)</td>
<td>2034</td>
<td>1192</td>
<td>7.62E-129</td>
<td>1.8</td>
</tr>
<tr>
<td>(Breslow et al., 2008)</td>
<td>deletion (haploid)</td>
<td>1410</td>
<td>550</td>
<td>9.53E-05</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Table 9: Analysis of protein complex gene enrichment in strains with impaired fitness for 4 deletion datasets.

Gene lists were prepared from fitness-impaired strains in global deletion study datasets. The lists were analyzed for enrichment of "protein complex" as described in Table 6.

<table>
<thead>
<tr>
<th>Source</th>
<th>Type</th>
<th>Total mapped genes</th>
<th># Protein complex (GO:0043234)</th>
<th>p-value</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background Genome</td>
<td>NA</td>
<td>6442</td>
<td>1500</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>(Yoshikawa et al., 2011)</td>
<td>deletion (haploid)</td>
<td>591</td>
<td>179</td>
<td>0.15, n.s.</td>
<td>1.3</td>
</tr>
<tr>
<td>(Deutschbauer et al., 2005)</td>
<td>deletion (YPD, homozygous diploid)</td>
<td>1981</td>
<td>849</td>
<td>5.87E-79</td>
<td>1.8</td>
</tr>
<tr>
<td>(Deutschbauer et al., 2005)</td>
<td>deletion (minimal medium, homozygous diploid)</td>
<td>2034</td>
<td>855</td>
<td>1.19E-74</td>
<td>1.8</td>
</tr>
<tr>
<td>(Breslow et al., 2008)</td>
<td>deletion (haploid)</td>
<td>1410</td>
<td>386</td>
<td>0.53, n.s.</td>
<td>1.2</td>
</tr>
</tbody>
</table>

In conclusion, the results from the enrichment analyses were in line with our hypothesis that deletion of complex subunits was generally detrimental for yeast cells due to destabilization of the complex, while excess subunits were degraded and therefore did not affect the complex. However, a bias for complex subunits to be essential would also explain why a cell would be able to cope with an excess of the protein while its absence would be lethal. Therefore, different scenarios might explain our observations.
Discussion:

The stt3-7 point mutation leads to destabilization of OST

The stt3-7 point mutation was first discovered by Zufferey et al. as being lethal in combination with a wbp1-2 mutation. It was further characterized by Spirig et al. and Jakob et al. (Jakob et al., 2001; Spirig et al., 1997; Zufferey et al., 1995). In agreement with these studies we confirmed that stt3-7 cells had reduced Stt3p levels at higher temperatures (Figure 1). As a consequence also protein levels of Ost3p, a subunit that is stabilized by Stt3p, were lower. Overexpression of Ost4p or Ost3p had been shown to partially rescue the hypoglycosylation phenotype of stt3-7 cells (Spirig et al., 1997). In addition, reducing ERAD stabilized both, Stt3p and the OST complex in stt3-7 cells (Jakob et al., 2001). The fact that Stt3p activity was rescued by protein stabilization indicated that the Ser552 residues was involved in complex assembly rather than in catalysis (Spirig et al., 1997). Stt3p residues involved in catalysis in yeast include N61 (proposed homolog to D56 from PglB) and E350 (proposed homolog to E319 in PglB) that bind to the asparagine residue (Lizak et al., 2011). Together with R159 (proposed homolog to R147 in PglB) and a DXE motif (D166, N167, E168) these residues further coordinate metal-ions. Additionally, the WWDYGY motif (W516 - 520) is responsible for peptide-binding. For S552, on the other hand, no catalytic or substrate-binding role has been reported. Nonetheless, residue S552 was conserved between yeast and higher eukaryotes and thus may influence complex formation in several organisms (Yan and Lennarz, 2002; Zufferey et al., 1995). Analyses of further point mutations might give insights into the importance of this region.

Taken together, our results and previous findings suggest that the S552P mutation leads to a conformational change limiting Stt3ps interactions with other complex components and targeting it for degradation. Increased amounts of Ost4p and Ost3p probably reconstituted Stt3p incorporation into the OST complex, and thus restored its Stt3p activity. Such "dosage suppression" where overexpression of one protein rescues a defect in an interacting protein has been shown for a number of proteins in yeast (Magtanong et al., 2011). The underlying mechanism was most likely a shift of the assembly reaction equilibrium towards Stt3p incorporation. We suggest that the S552P mutation leads to a weaker binding of Stt3p to its complex partners. Increased molecular movement at higher temperatures further weakens this bond and leads to less incorporation of mutated Stt3p into the OST complex. This scenario would also explain the temperature sensitive phenotype of stt3-7 cells. Pulse-chase experiments of the OST subunits in stt3-7 cells, as well as complex composition analysis by
blue native PAGE would give further insights into the exact dynamics of the stt3-7 dependent phenotype.

**Weighting the stabilizing effect of an OST subunit through comparison of degradation rates from overexpression and deletion experiments**

In earlier experiments we showed that OST subunits relied on each other for stabilization (Chapter 2, Figure 5). More precisely, we proposed that subunits were unstable on their own and would be degraded, unless they were incorporated into the OST complex where they were stabilized by their binding partners. Therefore, we hypothesized that the degradation rate for a subunit would be maximal when it was not stabilized by any of its complex partners. This is the case when a subunit is overexpressed and the other OST subunits are only present in lower amounts. Also deletion of a complex component can destabilize OST subunits. Comparing the degradation rate of the destabilized subunit to its maximum degradation rate allows to weight the stabilizing effect of the deleted complex component.

We compared degradation rates and half-lives of Ost2p, Swp1p, and Stt3p both in overexpression and \( \Delta \text{wbp1} \) deletion strains (Figure 2, Table 1). We found that Wbp1p was the main stabilizer of Ost2p and Swp1p, and that its deletion completely destabilized Ost2p and Swp1p. This was in agreement with previous studies suggesting Wbp1p, Swp1p, and Ost2p formed a subcomplex (Fu et al., 1997; Karaoglu et al., 1997; Kelleher and Gilmore, 1997, 2006; Knauer and Lehle, 1994; Nasab et al., 2008; Sanjay et al., 1998; Silberstein et al., 1995; te Heesen et al., 1993). Besides, deletion of \( WBP1 \) also destabilized Stt3p but to a lower extent (Table 1). This corroborated our previous speculations that Stt3p was mainly stabilized by Ost1p (Chapter 2). In conclusion we showed that degradation rates could be used to weight the stabilizing effect of a subunit on other complex components.

**Mining of global yeast data to compare phenotypes from overexpression or deletion of complex subunits**

Protein abundance has been shown to depend on the number of gene copies for most proteins in yeast (Springer et al., 2010). Thus deletion of one allele of a gene in diploid yeast cells led to a 50% reduction in protein levels. The fact that most heterozygous diploid cells do not show any growth defect indicates that proteins are produced at twice their required level in diploid cells (Deutschbauer et al., 2005; Springer et al., 2010). However, some heterozygous deletions
result in haploinsufficiency, i.e. produce a negative growth phenotype. Many haploinsufficient deletions are in genes encoding subunits of protein complexes (77% vs. 20% in the genome) (Deutschbauer et al., 2005). Examples are components of the spindle pole body, the exosome, the ribosome, the CCT folding chaperone, RNA polymerase II, and the translation initiation factor. The underlying mechanism might either be protein levels that are too low to meet cellular needs (insufficiency hypothesis) or imbalanced ratios of interacting proteins (balance hypothesis) (Deutschbauer et al., 2005; Papp et al., 2003). The fact that overexpression of most genes did not have deleterious effects even if up to 50 copies were present in yeast cells speaks against the balance hypothesis (Sopko et al., 2006; Yoshikawa et al., 2011). Furthermore, overexpression of complex subunits might even compensate defects in another complex component in a mechanism termed dosage suppression (Magtanong et al., 2011; Silberstein et al., 1995; te Heesen et al., 1993).

Information is scarce regarding protein complex dynamics and complex behavior when subunit levels are altered. Based on our previous experiments with the OST complex (Chapter 2, Figures 3 & 4) we set up the hypothesis that protein complexes are destabilized by deletion but not by overexpression of subunits since excess subunits are degraded. To investigate this we mined strain libraries and data from global studies for the occurrence of protein complexes. We found that complex subunits were overrepresented among growth-impairing gene deletions but not overexpression in yeast (Tables 2 - 9). Our findings are in line with the proposed hypothesis. One of the datasets we analyzed came from a study by Yoshikawa et al. where several thousand strains from overexpression and deletion libraries had been analyzed in parallel (Yoshikawa et al., 2011). They found that many proteins whose deletion caused a growth phenotype, while their overexpression did not, encoded complex components. Examples for protein complexes are the ribosome, the THO/TREX complexes, components of the respiratory chain and the elongator complex.

While enrichment analyses of most genetic datasets support our hypothesis, results for some studies differ, e.g. some datasets show a significant enrichment of complexes while others do not. The reasons for this might be that each study analyzed a different number and set of strains. Moreover, diverse methods were used to assess growth phenotypes, ranging from measuring colony sizes on agar plates to assessing growth rates in different liquid media or conducting fitness competition experiments. These methods have different sensitivities for pinpointing growth phenotypes, such that deletion of the same gene might be classified as deleterious in one study but not in another. This effect is exacerbated by the use of different statistics tools, as well as significance and fold-change cutoffs. However, most of the enrichment analyses support our hypothesis that overexpression of complex subunits do not disturb complex assembly while deletion destabilizes the complex.
Single protein studies corroborating this statement include our findings from the OST membrane protein complex (Chapter 2). Moreover, the phenomenon of unassembled subunits being eliminated while incorporation into the complex confers stability is also known for other complexes from yeast (mating type factors, V-ATPase, kinetochore), *E. coli*, plants and animals (Berthold et al., 1995; Bonifacino et al., 1990; Bruce and Malkin, 1991; Hill and Cooper, 2000; Johnson et al., 1998; Kopski and Huffaker, 1997; Leto et al., 1985; Lippincott-Schwartz et al., 1988; Ostersetzer and Adam, 1997; Schmidt and Mishkind, 1983). Further studies are required to determine the global principles of complex dynamics in yeast and other organisms.
Material and Methods

Immunoblot analysis
Yeast cells were collected at OD_{600} of 1 and were lysed with glass beads in sample buffer (2% SDS, 62.5 mM Tris/HCl pH 6.8, 10% glycerol, 6 M urea, 5% β-mercaptoethanol, 0.02% bromophenol blue, 1 X complete protease inhibitor cocktail (Roche), 5 mM PMSF, 25 mM EDTA). Protein concentrations of 10-fold diluted samples were measured (Pierce BCA, Thermo Scientific, Rockland, U.S.A) and β-mercaptoethanol, bromophenol blue, and EDTA were added. Proteins were dissolved for 20 min at 37°C. Equal amounts of protein were loaded on 10% PAGE gels, transferred to nitrocellulose and hybridised with antibodies. We visualized signals using ECL solutions (GE Healthcare, Amersham, U.K.).

stt3-7 strain construction
Strains SMA673 (SS328, MATα, ade2-101 his3Δ200 lys2-801 ura3-52 Δarg4::natMX4) and YG0535 (SS328, MATα, ade2-101 leu2 ura3-52 his3Δ1200 lys2-801 stt3-7) were mated (Spirig et al., 1997). Cells were streaked as thin layers on top of each other on YPD agar plates and incubated at 23°C for 1 d. Mated cells were then streaked twice on SD/MSG\(^1\)-LEU-ARG + NAT (100 μg/ml) to select for diploid cells. For sporulation, the diploid cells were spread in a thin layer on GNA agar plates (5% glucose, 3% nutrient broth, 1% yeast extract, 2.5% agar) and incubated for one day at 23°C. 10 X amino acid mix (0.4 g adenine, 0.2g arginine, 0.2 g histidine, 1.0 g leucine, 0.3 g lysine, 0.4 g uracil in 1 l ddH2O) was used to prepare sporulation plates (1% potassium acetate, 0.1% yeast extract, 2.5% washed agar, 0.05% glucose, 1 X amino acid mix). After one day pre-incubation on GNA agar, diploid cells were streaked in thick patches on the sporulation plates and incubated at 23°C for two weeks until tetrads (10 - 15% of population) were visible under the microscope. Tetrads were dissected using a tetrad dissector (Singer Instruments MSM). A loopfull of cells were dissolved in 45 μl sterile ddH2O, 5 μl Glusulase was added, and the spore saculus was digested for 5 min at 37°C. Sorbitol was added to a final concentration of 0.2 M and 20 μl of cell suspension were applied to a YPD plate for tetrad dissection. Tetrads were subsequently incubated at 23°C. A tetrad consisting of two normally growing and two slow-growing spores was selected. Spores were streaked on agar plates to ensure the presence of all auxotrophic markers and the stt3-7 mutation was confirmed by sequencing, yielding strain SMA1640 (SS328, MATα, ade2-101 his3Δ200 lys2-801 ura3-52 Δarg4::natMX4). SMA1596 (SS328, MATα, stt3-7 ade2-101 his3Δ200 ura3-

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\(^1\) MSG = monosodium glutamate
lys2-801 \( \Delta \)arg4::natMX4 + pAX309 (pLmSTT3D)) was constructed by transforming SMA1640 with a high-copy plasmid containing \( LmSTT3D \).

**SILAC-SRM pulse-chase experiments**

Yeast cells were grown in standard shake flasks in media deficient in arginine and lysine but supplemented with 20 mg/l heavy \( ^{13}\text{C}_6 \) arginine (R6, Cambridge Isotope Lab, Andover, U.S.A.) and \( ^{13}\text{C}_6-^{15}\text{N}_2 \) lysine isotopes (K8, Sigma Aldrich, Buchs, Switzerland). At OD\(_{600}\) 1 cells were transferred to medium containing light \( ^{12}\text{C}_6 \) arginine (R0) and \( ^{12}\text{C}_6-^{14}\text{N}_2 \) lysine (K0) isotopes. Fifty OD of cells were collected at three timepoints (0 min, 15 min, 60 min for wild-type-like growth or 0 min, 30 min, 120 min for slow growing strains).

Cells were lysed with glass beads. Membranes were pelleted by centrifuging (20 min, 16 000 g), and washed with sodium carbonate (0.1 M Na\(_2\)CO\(_3\), 1 mM EDTA, pH 11.3), urea (5 M urea, 100 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EDTA) and Tris-HCl (0.1 M Tris-HCl, pH 7.6). Membrane proteins were solubilized in 100 - 200 \( \mu \)l of 4% SDS, 50 mM DTT, 0.1 M Tris/HCl, pH 7.6 by repeatedly heating (90 °C, 1 min), vortexing and sonicating. Solubilized proteins were digested using a filter-assisted sample preparation (FASP) protocol adapted from Wisniewski et al. (Wisniewski et al., 2009). Thirty kilodalton cutoff spin filters (Amicon, Millipore, Zug, Switzerland) were used together with Lys-C and trypsin proteases. After elution, peptides were desalted using C18 ZipTips (Millipore, Zug, Switzerland).

Retention time iRT peptides (Biognosys, Zurich, Switzerland) were added to the samples. Peptides were separated on 15 cm frit column (ID: 75 \( \mu \)m, OD: 375 \( \mu \)m) packed with C18 material (beads: Magic C18 AQ, 3 \( \mu \)m, 200 A) using a 3 to 35% acetonitrile gradient and a flow rate of 500 nl/min (Eksigent nanoLC-Ultra 1D plus, ABSciex, Zug Switzerland). Ions were analyzed by SRM on a QTRAP 5500 (AB Sciex, Zug Switzerland). Peaks were integrated in Skyline and degradation rates were calculated according to Larrabee et al. (Larrabee et al., 1980; Li, 2010; MacLean et al., 2010). In short, we calculated the loss rate of old protein (\( k_{\text{LOSS}} \)) from the light to heavy ratios of the peptide peaks and subtracted \( k_{\text{DIL}} \) ( = \( k_{\text{LOSS}} \) of Rpl5p) which yielded degradation rates \( k_{\text{DEG}} \). Half-lives were calculated by dividing LN(2) by the degradation rate.

**Analysis of yeast deletion and overexpression datasets**

**Overexpression:**

To obtain a set of genes whose overexpression was non-lethal we analyzed the strain list in the MOBY2 overexpression library (Magtanong et al., 2011). We also analyzed complex gene enrichment in slow-growing strains from global overexpression datasets (3 studies) (Osterberg
et al., 2006; Sopko et al., 2006; Yoshikawa et al., 2011). For details of analysis see paragraph below.

Deletions:
We analyzed the heterozygous diploid part of the Euroscarf deletion library containing 1100 essential genes that abolished yeast growth on YPD at 30°C (COMP-SET-ESS on the EUROSCARF website). Furthermore, complex gene enrichment in slow-growing strains from global deletion datasets (4 studies) was analyzed (Breslow et al., 2008; Deutschbauer et al., 2005; Yoshikawa et al., 2011). For details of analysis see paragraph below.

Analysis:
Primary SGD gene IDs were retrieved from www.yeastgenome.org. AMIGO2 version 2.0.0-rc1 (http://beta.geneontology.org, Ashburner et al., 2000) based on PANTHER was used to obtain the GO terms associated with the SGD IDs (Mi et al., 2013). AMIGO2 returned a p-value for enrichment of the GO terms "macromolecular complex" and "protein complex" in the cellular component class. We further compared the enrichment of the two GO terms to their frequency in the complete yeast proteome by calculating the enrichment factor (EF) as EF = (% of proteins with GO term in study dataset) / (% of proteins with GO term in proteome).
References


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Chapter 4:

*Influence of altered glycan-assembly and unfolded protein response on global protein levels: data-dependent and data-independent mass spectrometry approaches*

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**Contributions:**
- Construction of strains
- Planning and execution of SILAC experiments
- Supervision of students
- Preparation of samples for mass spectrometry
- LTQ Orbitrap measurements
- Data analysis
Introduction:

Assembly of the lipid-linked oligosaccharide (LLO) precursor is a vital part of yeast N-glycosylation. It is mediated by Asparagine-linked glycosylation (ALG) genes at the cytosolic and luminal sides of the ER (Breitling and Aebi, 2013). The ALG genes encode glycosyltransferases that assemble a dolichol-pyrophosphate glycan precursor from nucleotide activated or lipid linked monosaccharides (UDP-GlcNAc, GDP-Man, Dol-P-Man, Dol-P-Glc) (Figure 1). Each glycosyltransferase accepts only a specific building block and glycan acceptor substrate making glycan assembly an ordered process (Burda et al., 1999). During early synthesis steps, the glycan precursor is anchored at the cytosolic side of the ER membrane. As soon as a GlcNAc2Man5 precursor has been built up, it is enzymatically flipped into the ER lumen where it is further extended (Helenius et al., 2002). Finally, the fully assembled GlcNac2Man9Glc3 glycan is transferred onto proteins by the oligosaccharyl transferase complex.

Figure 1: Assembly of the lipid-linked oligosaccharide (LLO) during yeast N-glycosylation. Modified from (Breitling and Aebi, 2013).

Defects in ALG genes result in truncated glycan precursors (Frank and Aebi, 2005; Reiss et al., 1996; Runge and Robbins, 1986). The yeast OST prefers to transfer the fully assembled GlcNAc2Man9Glc3 glycan onto proteins. Therefore, a defect in ALG genes not only gives rise to glycoproteins with smaller glycan chains, but also leads to lower glycosylation efficiency and will leave some glycosites unoccupied (Burda et al., 1999; Huffaker and Robbins, 1983;
Karaoglu et al., 2001; Kelleher et al., 2007). For example, the ALG3 gene encodes a trans-membrane anchored mannosyl transferase catalyzing the first luminal step of LLO assembly (Aebi et al., 1996; Sharma et al., 2001). It attaches an α-1,3 mannos from Dol-P-Man on the α-1,6 mannose residue of the GlcNAc2Man5 sugar, yielding GlcNAc2Man6 (Figure 1 & 2). A Δalg3 deletion therefore causes an accumulation of GlcNAc2Man5 LLOs and as a consequence shorter N-glycan chains and hypoglycosylation of some proteins (Bailey et al., 2012; Huffaker and Robbins, 1983). On a physiological scale, ALG3 deficiency leads to severe psychomotor retardation and other developmental defects in humans (Korner et al., 1999). It is thus apparent that correctly assembled Glc3Man9GlcNAc2 N-glycans are crucial for survival and homeostasis of organisms.

N-glycans also play an important role in ERAD which protects the ER from stress by targeting misfolded or unstable proteins for degradation. The recognition of defective glycoproteins and their elimination largely depends on the composition of their N-glycans: In concert with Pdi1p, which binds to misfolded proteins, the Htm1p mannosidase cleaves one mannose residue off the Man8 glycans of misfolded proteins (Clerc et al., 2009; Gauss et al., 2011). The resulting Man7 N-glycans act as degradation signal and are recognized by the ERAD machinery during ERAD-L. More specifically, the free α-1,6 mannose is bound by the lectin Yos9p which targets the protein to Hrd1p-mediated degradation (Clerc et al., 2009; Denic et al., 2006). An alteration in the glycan signal could result in global changes of glycoprotein degradation. For example, a Δalg3 deletion in yeast cells leads to accumulation of Man5 LLO precursor and to Man5-glycans on proteins (Figure 2) (Huffaker and Robbins, 1983). All Man5 glycans present a free α-1,6 mannose that can be recognized by Yos9p, although with a lower efficiency compared to the usual Man7 signal (Jakob et al., 1998; Szathmary et al., 2005). Thus, levels of glycoprotein degradation might be increased in Δalg3 cells since the degradation signal would be present on all glycoproteins. Besides, the limited transfer of shorter glycan chains could destabilize proteins due to unoccupied glycosites.

ERAD is not the only pathway that protects the ER from folding stress but works in concert with the unfolded protein response (UPR) (Wu et al., 2014). The UPR is activated by misfolded proteins and induces target genes that enhance the folding capacity of the ER. In the budding yeast, this pathway relies on Ire1p that senses folding stress either by directly binding misfolded proteins or by release of the Kar2p chaperone from its luminal domain. It oligomerizes, autophosphorylates and activates the transcription factor Hac1p which in turn regulates expression of UPR target genes. Target genes include chaperones, such as Kar2p and Pdi1p, the ERAD machinery and lipid synthesis (Thibault et al., 2011). The UPR regulates multiple functions of the ER and defects cause diseases in humans, such as Parkinson’s
disease, Huntington’s disease and diabetes (Hetz et al., 2013). In yeast, on the other hand, a \( \Delta \text{ire1} \) deletion is not lethal at the normal growth temperature (30°C). However, \( \Delta \text{ire1} \) cells are not viable at 37°C if they carry a second mutation that abolishes ERAD (Travers et al., 2000). This suggests that either UPR or ERAD are required for growth at higher temperatures. To analyze the importance of ERAD under heat stress, protein degradation rates would need to be measured.

Figure 2: Glycan processing and quality control in the ER.

The GlcNAc2Man5 LLO precursor is assembled at the cytosolic side of the ER and flipped into the lumen where Alg3p attaches an \( \alpha \)-1,3 mannose to the B-branch. The resulting Man6 glycan is further elongated by ALG genes and is finally transferred to an asparagine residue of a protein by the oligosaccharyl transferase complex. Subsequently, glucosidases I and II and Mns1p trim the sugar to a Man8 structure. In case a protein is misfolded, Htm1p will cleave off an additional mannose, yielding a Man7 glycan that acts as degradation signal. The free \( \alpha \)-1,6 mannose is recognized by the lectin Yos9p and the protein is targeted to ERAD. Deletion of the \( \text{ALG3} \) gene therefore results in a Man5 glycan with a free \( \alpha \)-1,6 mannose that can also bind to Yos9p albeit with lower efficiency.
Global protein degradation has been studied in a variety of organisms. In a number of studies a pulsed-SILAC approach has been coupled to shotgun mass spectrometry to measure degradation rates at the proteome level (Helbig et al., 2011; Pratt et al., 2002; Schwanhausser et al., 2011). Although the use of data-dependent approaches allowed to obtain degradation rates of hundreds of proteins, these cover only a fraction of the complete proteome. Many proteins might have been missed because of the typical disadvantages associated with shotgun proteomics experiments and the somewhat stochastic acquisition of MS/MS spectra (Michalski et al., 2011). As described in chapter 1, in recent years a number of data-independent mass spectrometry approaches (DIA) emerged. These methods fragment larger mass windows instead of single peaks, thus providing MS/MS information for every precursor and avoiding the under-sampling of shotgun methods. SWATH MS is a DIA approach where "swaths" of 25 Th are fragmented on a triple-TOF instrument, resulting in product ion spectra containing fragments from more than one peptide (Gillet et al., 2012). For peptide identification and quantification, the product ion spectra are mined using a library of SRM-traces and data are displayed as SRM-like peak groups. The advantage of SWATH over shotgun MS is the avoidance of under-sampling, its larger dynamic range and high sensitivity (Gillet et al., 2012). The drawback is that data processing is challenging and is dependent on the quality of the previously generated spectral libraries.

In this study we analyzed how protein levels were affected by alterations in N-glycan structures, defective UPR, and heat stress using steady-state SILAC and shotgun mass spectrometry. Furthermore, we conducted a pilot study using a data-independent MS approach that will help implementing SWATH MS for measuring global protein degradation rates in the future.
Results:

Influence of glycan signal and unfolded-protein response on global protein levels

We had discovered previously that most proteins in exponentially growing wild type cells were stable (Chapter 2, Figure 2). Only a handful of endogenous proteins, as well as proteins with folding defects or destabilized complex components were degraded (Chapter 2, Figures 2, 3). Here, we wanted to assess if ERAD was more active under different conditions. For example, a Δalg3 deletion leads to the presence of a free α-1,6 mannose degradation signal on glycoproteins and to hypoglycosylation of proteins (Figure 2). This might destabilize a large number of proteins. On another note, it had been reported that ERAD-deficiency in combination with a Δire1 deletion was lethal at 37°C, suggesting that ERAD was active at higher temperatures and in absence of UPR and vital to decrease the load of misfolded proteins (Friedlander et al., 2000; Travers et al., 2000). These findings prompted us to compare protein levels in wild type, Δalg3 and Δire1 cells at 30°C and 37°C. It has been reported that for most proteins, yeast cells have no feedback mechanism and these low protein levels would not induce increased protein synthesis (Springer et al., 2010). Therefore, we expected increased degradation rates to decrease the cellular abundance of proteins. As a consequence, increased protein degradation rates in Δalg3 or Δire1 cells might result in reduced levels of some proteins compared to wild type cells where degradation levels are low (Chapter 2, Fig. 2).

We used SILAC combined with shotgun mass spectrometry to measure protein levels in Δalg3 and Δire1 cells grown at 30°C or 37°C in light medium (R₀, K₀) relative to wild type cells from heavy-medium cultures (R₆, K₈) at 30°C. Additionally, a control (wild type, light medium, 30°C) was prepared. Equal cellular weights from light and heavy cultures were pooled, followed by cell lysis and membrane isolation to increase the percentage of glycoproteins in the sample. Proteins were then identified and quantified using an LTQ Orbitrap Velos instrument and the MaxQuant software. Setting the probability for a random protein identification (PEP score cutoff) to < 0.001, we identified 933 proteins with an FDR of 0.2%.
Figure 3: Protein levels in wild type, Δalg3, and Δire1 cells compared to wild type.

Protein levels were measured using SILAC and shotgun mass spectrometry. Protein amounts relative to wild type levels (heavy medium, 30°C) were assessed for wild type (A, n=3), Δalg3 (B, n=2), and Δire1 cells grown at 30°C (C, n=3) or 37°C (D, n=3). A PEP score cut-off < 0.001 (-log10[PEP] = 3) is indicated by a red line. Fold-changes of 50% and 200% of wild type (heavy) levels are indicated by grey dashed lines (log2[L/H] = +/-1, respectively). Boxplots indicating mean L/H ratios of the four experiments and outliers from 0 to 2 are displayed in E). Ratios > 2 are not shown.

For each combination of strains (mutant vs. wild type) heavy to light ratios for roughly 500 proteins were quantified throughout all replicates (Figure 3). Protein amounts below 50% or over 200% of wild type (heavy) amounts were regarded as reduced or increased, respectively. As expected, no proteins fulfilled these criteria in the control experiment (wild type cells grown in light versus wild type cells grown in heavy medium, Figure 3A). Although protein abundances slightly differed from wild type in Δalg3 cells, only two protein groups showed lower protein levels compared to wild type (Figure 3B). These contained ribosomal proteins Rpl31A/Bp and the glycerol-phosphatase Gpp1p (RHR2), both unglycosylated proteins (Table 1). L/H ratios showed a slightly larger spread compared to wild type, indicating deviations from wild type levels (Figure 3E). In Δire1 cells grown at 30°C, only HIS3 was increased relative to wild type due to the Δire1::HIS3 deletion (Figure 3C, Table 2). Growth of the same cells at 37°C led to
a decrease in two ribosomal protein groups (Figure 3D, Table 3). The HIS3 marker was also increased in \( \Delta \text{ire1} \) cells grown at 37° C along with several heat-shock proteins and chaperones (e.g. Kar2p) or other proteins promoting protein folding (e.g. Cpr6p) or transport into the Golgi (Yet1p). Levels of a ribosomal protein group and of the transcriptional regulator Wtm1p were increased too. Other proteins whose levels were increase included enzymes involved in glucose metabolism (Glk1p, Hxkap, Eno1p, Dak1p, Ald4p, Tps1p). Protein abundances showed a larger spread compared to the other experiments, indicating deviations from wild type cells (Figure 3E).

In summary, analyzing \( \Delta \text{alg3} \) and \( \Delta \text{ire1} \) (at 30°C and 37°C) cells led to the identification of only 3 protein groups present in decreased levels compared to wild type cells. It could be an indication that they were degraded in these cells.

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<th>L/H</th>
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Table 3: Protein groups present in increased or decreased levels (-1 < \log_2(L/H) > 1) in \textit{Aire1} cells grown at 37°C (L) compared to wild type cells (H).

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**Analyzing yeast proteins using SWATH MS**

Previous studies used a combination of pulsed-SILAC and shotgun MS to measure degradation rates of eukaryotic proteomes (Helbig et al., 2011; Schwanhausser et al., 2011). Because of the advantages of data-independent approaches, we conducted a pilot study to determine how many proteins could be identified in our yeast samples using SWATH MS and,
more in general, to evaluate if SWATH MS could be useful to measure protein degradation rates globally in the future.

**Figure 4: SWATH MS of yeast proteins.**

A) Scheme of the sample preparation procedure: After cell lysis membrane vesicles were collected by centrifugation. Vesicles were opened and linearized by incubation in Na₂CO₃. Membranes were pelleted and luminal proteins in the supernatant (Na₂CO₃ fraction) were collected by TCA/acetone precipitation. Proteins were solubilized, digested and analyzed by SWATH MS. A single biological replicate was analyzed with two technical replicates for each fraction. B) Numbers of proteins identified in both technical replicates of each fraction and the overlap between fractions are displayed. C) Peptide counts in membrane and Na₂CO₃ fractions. Non-redundant peptides detected in both technical replicates of membrane and Na₂CO₃ fractions were counted. Average numbers were listed for detected subunits of OST, the SEC translocon, the PMT complexes, as well as ER chaperones Pdi1p and Kar2p, ribosomal proteins Rpl5p and Rps1ap, vacuolar proteins Pra1p and CPY, and plasma membrane protein Pdr5p. Shading in dark green and pink indicate fractions with higher or lower peptide counts for a protein, respectively. Shading in light green indicates similar or equal peptide counts in both fractions.

Yeast cells grown in minimal medium were lysed and membrane vesicles were collected (Figure 4A). Vesicle content was released through incubation in Na₂CO₃ at high pH and both, vesicle content and vesicle membranes, were collected separately (Nielsen et al., 2005). Proteins from the two fractions (membrane and Na₂CO₃) were extracted, digested for mass spectrometry using a FASP protocol and analyzed by SWATH-MS on a triple-TOF instrument (Andrews et al., 2011; Wisniewski et al., 2010). Results from two technical replicates for both fractions of the same sample are shown in Figure 4B. 1139 and 1145 proteins were identified throughout both replicates for membrane and Na₂CO₃ fractions, respectively. The overlap between fractions, i.e. the number of proteins identified in both of them, was substantial (902 proteins, Figure 4B). To determine if the protein content of the two fractions differed
nonetheless, we compared the peptide counts of several proteins for each fraction, including subunits of the OST complex, the SEC translocon, the PMT complexes, the ER chaperones Pdi1p and Kar2p, as well as vacuolar proteins CPY and Pra1p, the plasma membrane protein Pdr5p and two ribosomal proteins (Rpl5p, Rps1ap) (Figure 4C). More specifically, we calculated the average number of non-redundant peptides identified in both technical replicates of each fraction. Membrane proteins were clearly enriched in the membrane fraction, while for some soluble proteins more peptides were detected in the luminal (Na₂CO₃) fraction. In conclusion, we have shown that the SWATH MS pipeline had been implemented and could be applied to yeast samples. Further steps would include testing a pipeline for quantification of proteins in SILAC samples and determining how many proteins can be identified before finally starting pulse-chase experiments.
Discussion:

We compared protein levels in wild type, \( \Delta alg3 \), and \( \Delta ire1 \) cells by measuring protein amounts relative to wild type. Around 500 proteins were quantified in all replicates of each sample and this number would probably increase if the sample was further fractionated. We expected increased protein degradation rates to result in decreased levels for some proteins in our SILAC experiments. However, care should be taken with this approach since we are not able to distinguish the cause of the lower protein levels which could either be degradation or repression of protein synthesis. Furthermore, we are not able to exclude that increased degradation rates and simultaneously increased protein synthesis compensate each other and produce protein levels comparable to wild type. Nonetheless, our SILAC experiments give a first global overview of protein levels and might allow conclusions regarding protein degradation.

Surprisingly, only three protein groups were present in decreased levels (log2\(L/H\) < -1) in any of the mutant cells. Results for the \( \Delta alg3 \) cells indicate that the glycan signal itself is not sufficient to target a protein for degradation. Indeed, it had been reported that the degradation signal was normally bipartite, consisting of the Man7 glycan presenting a free \( \alpha\)-1,6 mannose to Yos9p and a misfolded stretch of protein recognized by Hrd3p (Gauss et al., 2006; Jakob et al., 1998; Xie et al., 2009). In conclusion, a Man7 glycan acts as an additional security checkpoint ensuring that the protein went through several folding attempts before being delivered to ERAD but neither the free \( \alpha\)-1,6 mannose, nor truncated Man5 sugar chains or hypoglycosylation destabilize proteins globally in wild type cells. In a next step, it could be analyzed if a \( \Delta alg3 \) deletion had a bigger effect in \( \Delta ire1 \) cells since they are more sensitive to tunicamycin-induced glycosylation defects compared to wild type cells (Friedlander et al., 2000).

It had been shown that a double deletion in \( \Delta ire1 \) and an ERAD component was lethal at 37°C (Friedlander et al., 2000; Travers et al., 2000). Therefore, we expected ERAD to be highly active in \( \Delta ire1 \) cells grown at 37°C. Surprisingly, we only observed a decreased abundance, for two protein groups (Table 3, Figure 3). There are two explanations for this result. Either the stress response in yeast cells is more complex than previously thought or our experimental setup was not able to pinpoint proteins with increased degradation rates, e.g. because protein abundances were increased in response to heat shock and ERAD readjusted them to wild type levels. On the other hand, 25 proteins were found to be increased in heat stressed \( \Delta ire1 \) cells (Table 3). Several of them were chaperones or other proteins that assist protein folding and
thus alleviate the amount of misfolded proteins under stress conditions. Apart from that, many heat-shock proteins were upregulated indicating activation of the cytosolic heat-shock response (Verghese et al., 2012). Proteins include the HSP70 family proteins Ssa1p, Ssa2p, the Hsp90 chaperone Sti1p that interacts with them, and the Hsp104p disaggregase that cooperates with Ssa1p. Additionally, the Sse1p ATPase and its paralog Sse2p and the small heat shock protein Hsp42p were upregulated. Moreover, also levels of the co-chaperone Aha1p that binds Hsp82, as well as the Hsp90 chaperones Hsp82p and Hsc82p were increased. Hsc82p levels have been reported to increase two- to threefold upon heat-shock, which is in agreement with our results (Borkovich et al., 1989). The heat-shock response represses transcription and translation. Although it mainly influences cytoplasmic processes it also has an effect on the ER folding capacity by upregulating Kar2p which is itself an HSP70 family protein (Hou et al., 2014). Besides, proteins involved in glucose metabolism were induced in \( \Delta \text{ire1} \) cells at high temperature. For example the Tps1p protein is involved in the conversion of glucose to trehalose. The cellular levels of trehalose correlate with survival of severe heat shock (Verghese et al., 2012).

In summary, abundance of only few proteins changed in mutant cells relative to wild type cells grown at 30°C. It has to be kept in mind that our SILAC experiment will only reveal proteins that have altered abundances relative to wild type. Therefore, proteins that were degraded also in wild type cells would not show any change in abundance. However, based on our previous results these are few and we therefore expected to capture most of the degraded proteins in mutant cells (Chapter 2, Figure 2). Another possibility would be that instead of protein abundances remaining constant, heat-stress actually increased protein production in \( \Delta \text{ire1} \) cells and that ERAD was vital to lower protein levels back to wild type amounts. Pulse-chase experiments would be necessary to calculate degradation rates of proteins and to distinguish between the two scenarios.

So far, we have no evidence for a global increase of protein degradation in \( \Delta \text{alg3} \) or \( \Delta \text{ire1} \) cells at 30°C or 37°C. It is possible that under different growth conditions protein degradation levels are higher. These could include e.g. in cells with excess chromosomes to correct overexpression of proteins, during growth on ethanol or under nutrient limitation, or during DTT-induced redox stress (Liu and Chaloupka, 1983; Lopez and Gancedo, 1979; Travers et al., 2000). Furthermore, ERAD could be active globally in non-exponential phase cultures, during shifts from one growth condition to another, or possibly in case defects in lipid synthesis destabilized membrane proteins (Bakalkin et al., 1976).
Using steady-state SILAC coupled to shotgun MS we were not able to calculate degradation rates for the measured proteins. This would have helped to distinguish between stable proteins and protein amounts compensating faster degradation. Therefore, a pulsed SILAC approach to globally measure degradation rates of the yeast proteome would be necessary to complete this study. Ideally, all 6000 yeast proteins would be monitored. However, a large number of peptides are not identified in shotgun MS experiments because only the most intense peaks are fragmented (Michalski et al., 2011). Due to fluctuations in the number and intensities of co-eluting peptides, data-dependent MS/MS sequencing can result in varying peptide identifications between replicates. The inconsistent peak picking is especially disadvantageous in pulsed SILAC experiments because the same protein needs to be detected throughout several time points and replicates to produce good quality results. The complexity of SILAC samples further complicates the matter by doubling the number of co-eluting peptide peaks.

In data-independent MS approaches all peaks are fragmented thus avoiding problems arising from inconsistent precursor selection. We therefore implemented a data-independent MS approach called SWATH MS to identify and quantify proteins in our yeast samples. We separated our yeast extracts into a membrane and a Na$_2$CO$_3$ fraction containing luminal proteins. For both fractions 1100 proteins were identified with an overlap of 900 proteins. Nonetheless, quantification showed that the protein content differed between the two fractions and that membrane proteins were enriched in the former (Figure 4C). The increased complexity of SILAC samples and the varying protein content in biological replicates will probably lead to a lower number of proteins being quantified in pulse-chase experiments. Additional fractionation on the other hand could balance this and would increase the number of identifications. A study of the yeast proteome measured degradation rates for 641 proteins (10% of the yeast proteome) despite separating the samples with strong-cation exchange into 40 fractions (Helbig et al., 2011). We would hope to achieve similar numbers with less fractions using SWATH MS. Of course, these predictions would need to be tested in a pulsed-SILAC study. Prior to that, an automated data analysis pipeline would need to be implemented that allows the quantification of proteins in SILAC samples and subsequent calculation of degradation rates.
**Material and Methods**

**SILAC experiments: Sample preparation**

Wild type (SMA673: SS328, *MATα*, ade2-101 his3Δ200 lys2-801 ura3-52 Δarg4::natMX), Δalg3 (SMA1601: SS328, *MATα*, ade2-101 his3Δ200 lys2-801 ura3-52 Δarg4::natMX, Δalg3::HIS), and Δire1 (YG1399: SS328, *MATα*, ade2-101 his3Δ200 ura3-52 lys2-801 Δire1::HIS3) were grown in minimal medium containing 20 mg/l light arginine (¹²C₆) and lysine (¹²C₆¹⁴N₂) isotopes. In parallel, wild type cultures in medium containing heavy arginine (¹³C₆) and lysine (¹³C₆¹⁵N₂) isotopes were prepared. Growth conditions for cultures were 30°C or 37°C and 180 rpm. 25 OD of cells were harvested at OD₆₀₀ 1 (or 4 x 25 OD for wild type grown in heavy medium). Cells were washed in a lysis buffer (2 M NaCl, 10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 1 X Complete protease inhibitor (Roche)). Equal amounts of cells (normalized by weight) for grown in light medium were pooled with wild type cells grown in heavy medium.

The cellular proteome was fractionated and proteins were extracted and based on previous protocols (Nielsen et al., 2005; Wisniewski et al., 2009). In short, cells were lysed with glass beads in lysis buffer. The lysate was collected and membranes were pelleted for 20 min at 4°C and 16 000 g. The membrane pellet was washed twice in 1 ml 0.1 M Tris-HCl, pH 7.6 and membranes were again collected in a high speed spin. Proteins were solubilized in 100 µl solubilisation buffer (4% SDS, 50 mM DTT, 0.1 M Tris-HCl, pH 7.6) using a douncer and repeated heating at 90°C for 1 min, sonication and vortexing. Unsolubilized material was eliminated (16 000 g, 20 min) and the supernatant was frozen with liquid nitrogen and stored at -80°C.

Proteins were digested using a filter-assisted sample preparation (FASP) protocol (Nielsen et al., 2005; Wisniewski et al., 2010): Thawed samples were diluted 20-fold in UA buffer (8 M urea, 0.1 M Tris-HCl, pH 8.5) and volumes were loaded on 30 kDa cut-off spin filters (Amicon) for 15 min at 14 000 g. Filters were washed with 400 µl UA and proteins were alkylated using 200 µl iodoacetamide 50 mM for 30 min. This was followed by several washing steps with 400 ml UB buffer (8 M urea, 0.1 M Tris-HCl, pH 8.0). Proteins were first digested overnight in UB buffer with 1 µg Lys-C endoproteinase (Wako Chemicals) at room temperature. Subsequently, 400 µl NaHCO₃ 40 mM and 1 µg trypsin (Promega) were added and samples were incubated at 37°C for 4 h. Peptides were eluted from the filter by spinning at 14 000 g for 20 min and
washing two times with 50 μl NaCl 0.5 M. Protein concentration was measured by NanoDrop as A280.

Prior to mass spectrometry measurements, 5 μg of peptides were desalted using C18 ZipTips (Waters). Peptides were dried in a SpeedVac and dissolved in 20 μl 3% ACN, 0.1% formic acid.

**SILAC experiments: MS-measurements and data analysis**

Four microliters of each sample were measured by LC-MS/MS. Chromatographic separation of peptides was performed on a NanoLC-2Dplus HPLC system (Eksigent, Dublin, CA) using a self-made column (75 μm x 150 mm) packed with C-18 material (AQ 3 μm Bischoff GmbH, Leonberg, Germany). A gradient from 3 to 35% acetonitrile at flow rate 250 nl/min was applied. Data were acquired on an LTQ Orbitrap Velos (Thermo Scientific, Rockland, U.S.A.) using a standard data dependent top-20 method (1 survey scan, 20 dependent scans). Peptides were identified using MaxQuant version 1.2.7.4., Andromeda, and an in-house yeast database containing common contaminants and reversed sequences (Cox and Mann, 2008; Cox et al., 2011). Search parameters defined multiplicity (2, Arg6, Lys8), protease (trypsin, 1 missed cleavage), constant (Cys carbamidomethylation), and variable modifications (methionine oxidation, (NQ) deamidation). Protein false-discovery-rate (FDR) was set to 0.05 and peptide length 5 amino acids.

We manually processed the protein groups output from MaxQuant. I.e. proteins not detected in all replicates for a strain were discarded as well as contaminants and reverse hits. We calculate L/H ratios for all proteins from MaxQuant's normalized H/L values. Values were log2-transformed before calculating the average L/H ratio over all replicates for each protein. The resulting ratios were back-transformed to linear space, yielding normalized average L/H ratios for each protein, and were used to create volcano plots. PEP scores < 1E-299 were listed as equal to zero by MaxQuant. To enable a log-scale in the Volcano plot, we set these values equal to 1E-300. We calculated -log10(PEP) and log2(L/H) and depicted those values for all protein groups. Only proteins with PEP < 0.001 (-log10(PEP) > 3) and a fold-change > 2 (-1 > log2(L/H) > 1) were considered as regulated.
SWATH MS pilot study: Sample preparation

Wild type (SMA673: SS328, MAT\(\alpha\), ade2-101 his3Δ200 lys2-801 ura3-52 Δarg4::natMX) cells were grown in minimal medium containing 20 mg/l light arginine (\(^{12}\text{C}_6\)) and lysine (\(^{12}\text{C}_6\text{N}_2\)) isotopes. Fifty OD of cells were harvested at OD\(_{600}\) 1 and prepared for mass spectrometry measurements as described before but with slight alterations: membrane pellets were incubated in 1 ml Na\(_2\)CO\(_3\) 0.1 M, EDTA 1 mM, pH 11.3 for 30 min at 4°C to release vesicle content. Subsequently, membranes were pelleted and solubilized as described (membrane fraction). Meanwhile, luminal proteins in the supernatant were precipitated with 0.1 Vol. trichloro-acetic acid (TCA) and acetone and were later solubilized (Na\(_2\)CO\(_3\) fraction).

Proteins were digested using a FASP protocol as described with the following changes: proteins were digested in 30 \(\mu\)l UB buffer with Lys-C, followed by addition of 200 \(\mu\)l NH\(_4\)CO\(_3\) 50 mM and digestion by trypsin. Both proteases were added in the following amounts: 0.5 \(\mu\)g for membrane samples, 1 \(\mu\)g for Na\(_2\)CO\(_3\) samples. Peptides were eluted from the filter, including two washes with 50 \(\mu\)l NH\(_4\)CO\(_3\) 50 mM.

The samples (around 300 \(\mu\)g peptides according to A280 measurements) were desalted using SepPak C18 columns (Finisterre SPE C18, Teknokroma). Peptides were diluted to 0.25 \(\mu\)g/\(\mu\)l and iRT peptides (Biognosys) were added in a 1:40 ratio.

SWATH MS pilot study: LC-MS set up

Tryptic digests were analyzed on a 5600 TripleTof™ mass spectrometer (ABSciex, Concord, Ontario, Canada). Chromatographic separation of peptides was performed on a NanoLC-2Dplus HPLC system (Eksigent, Dublin, CA) using a self-made column (75 \(\mu\)m x 150 mm) packed with C-18 material (AQ 3 \(\mu\)m, Bischoff GmbH, Leonberg, Germany). Peptides were loaded on the column from a cooled (4°C) Eksigent autosampler and separated with a linear gradient of acetonitrile/water, containing 0.1% formic acid, at a flow rate of 300 nl/min. A gradient from 5 to 35% acetonitrile in 180 minutes was used. In SWATH-MS, a set of 35 overlapping windows (1 amu overlap) was constructed covering the mass range 385 to 1250 amu and using an isolation width of 25 amu. The collision energy for each window was determined based on the appropriate collision energy for a 2+ ion centered in the respective window with a spread of +/- 15 eV. An accumulation time of 100 ms was used for each fragment ion scan and for the survey scans acquired at the beginning of each cycle.
Targeted data extraction of the SWATH data was performed by the Spectronaut software from Biognosys (Schlieren, Switzerland). Spectronaut processes the SWATH data using a targeting analysis strategy similar to that of the SRM analysis tool mProphet (Reiter et al., 2011). In addition to mProphet, Spectronaut uses retention time prediction based on iRT (Escher et al., 2012), the m/z dimension in the SWATH MS data, mass accuracy and isotopic distribution of fragment ions. False discovery rates are determined using SWATH-MS adapted decoy model similar to the model used by mProphet (Reiter et al., 2011).
References


Chapter 5: Technical Brief

PTM MarkerFinder, a software tool to detect and validate spectra from peptides carrying post-translational modifications

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Contributions:
Setup of protocol for glycopeptide enrichment
Preparation of yeast sample, enriched in glycopeptides
Contributions to writing and revising the manuscript
Abstract

Mass spectrometry (MS) analysis of peptides carrying post translational modifications (PTMs) is challenging due to the instability of some modifications during MS analysis. However, glycopeptides as well as acetylated, methylated and other modified peptides release specific fragment ions during CID (Collision Induced Dissociation) and HCD (Higher-Energy Collisional Dissociation) fragmentation. These fragment ions can be used to validate the presence of the PTM on the peptide.

Here we present PTM MarkerFinder, a software tool that takes advantage of such marker ions. PTM MarkerFinder screens the MS/MS spectra in the output of a database search (i.e. Mascot) for marker ions specific for selected PTMs. Moreover, it reports and annotates the HCD and the corresponding Electron Transfer Dissociation (ETD) spectrum (when present), and summarizes information on the type, number, and ratios of marker ions found in the data set.

In the present work, a sample containing enriched N-Acetylhexosamine (HexNAc) glycopeptides from yeast has been analyzed by liquid chromatography-mass spectrometry on an LTQ Orbitrap Velos using both HCD and ETD fragmentation techniques. The identification result (Mascot .dat file) was submitted as input to PTM MarkerFinder and screened for HexNAc oxonium ions. The software output has been used for high-throughput validation of the identification results.
**Technical Brief**

The assignment of stable post-translational modifications (PTM) by liquid chromatography-mass spectrometry (LC-MS/MS) analysis is well established. However, the characterization of peptides carrying labile modifications still remains challenging, due to the instability of these PTMs during MS fragmentation.

For example, Collision Induced Dissociation (CID) spectra of phosphorylated peptides are characterized by intense peaks corresponding to the neutral loss of the phosphate group. Acetylated and methylated residues release distinctive immonium ions (m/z 98.0964 for monomethylated lysine, m/z 126.0913 and 143.1179 for acetylated lysine) and unique neutral fragments (MH+ 59.0735 for tri-methylated lysine) during CID fragmentation, making it possible to distinguish between acetylated and tri-methlyated lysine [1].

N- and O-linked glycans are another class of modifications that form characteristic low-mass product ions during CID, Higher-Energy Collisional Dissociation (HCD) or Pulsed Q Collision Induced Dissociation (PQD) [2]. Some of these ions are oxonium ions corresponding to monosaccharides, for example N-acetylhexosamine (HexNAc+, m/z 204.0866), hexose (Hex+, m/z 163.0601), and N-acetylneuraminic acid (NeuAc+, m/z 292.1032). In addition, glycopeptide spectra contain signals resulting from internal fragmentation of HexNAc (m/z 186.0761, 168.0655, 144.0655, 138.0550, 126.0550) or Hex (m/z 145.0495, 127.0390, 115.0390, 109.0284) [3]. MS/MS fragmentation of N- and O-linked glycopeptides may also lead to the formation of marker ions containing more than one monosaccharide unit, for example 325.1135 (Hex-Hex+), and 366.1400 (Hex-HexNAc+).

Peptides with labile modifications have been fragmented using different techniques, and their characteristic fragmentation patterns have been used to identify, validate and localize the modification site. Methods based on additional activation of modified peptides after neutral loss of the modification (MS3, multistage activation) or on alternative fragmentations (Electron Capture Dissociation (ECD) and Electron Transfer Dissociation (ETD) have been proposed and successfully applied to sequence modified peptides [4].

A common MS-based approach takes advantage of the glycan-specific markers: first, HCD or PQD spectra are acquired and screened for these characteristic oxonium ions to detect potentially modified peptides. The precursors are then fragmented by ETD for peptide sequencing and
modification site localization. This approach has been successfully applied to the identification and characterization of glycopeptides [3,5-7].

HCD and ETD fragmentations can be performed on every peptide in a single run, in a two stage approach (a discovery LC-MS/MS run for the detection of putative glycosylated peptides, followed by a targeted ETD LC-MS/MS analysis [3]), or in a more elegant combination of fragmentation techniques, named HCD product ion-triggered ETD (HCD-PD-ETD) [3, 6-7]. In the latter, ETD spectra are acquired only when the oxonium ions of interest are detected in the HCD MS/MS spectra, reducing the instrument duty cycle, while improving the sensitivity and simplifying the data analysis. Potential pitfalls of this approach include a faulty triggering of the ETD event, the requirement of a-priori knowledge of the triggering ions, and different peptide assignments from HCD and ETD spectra.

In any case, mass spectrometric data from glycoproteomic experiments often contain hundreds of glycopeptide fragmentation spectra, which need to be validated. Although manual validation of all individual spectra in such experiments is obviously not feasible, annotated spectra of the modified peptides are required for publication and reviewing purposes.

Software tools to screen large data sets for potentially modified peptides and validate the assignment of peptide sequences are necessary. PTM MarkerFinder is a tool that selects spectra of interest and greatly facilitates the visualization and quality assessment of the assignment of modifications. Briefly, the PTM MarkerFinder software searches a-posteriori for any type of product ion(s) appearing in the HCD or PQD spectra, reports and annotates the corresponding ETD spectrum (when present) and summarizes information on the type, number, and ratios of product ions found in the dataset. An overview of the data analysis workflow using PTM MarkerFinder software is shown in Figure 1.

In the described approach spectra of the sample of interest are acquired by LC-MS/MS on a LTQ-Orbitrap Velos instrument using a combination of HCD/ETD or PQD/ETD fragmentation techniques (Figure 1a). Since the only pre-requisite for PTM MarkerFinder is the presence of marker product ions, the input LC-MS/MS dataset could consist only of HCD or PQD. The use of CID fragmentation on LTQ-Orbitrap instruments is not suggested, because of the low mass cut-off limitation inherent to all ion traps.
Figure 1: Overview of the PTM MarkerFinder workflow: (a) the samples containing modified peptides are analyzed by LC-MS/MS analysis using HCD/PQD fragmentation technique (with or without additional ETD fragmentation). Spectra are converted into mgf files, the database searches are performed (i.e. Mascot) and the outputs are exported (i.e. as .dat file); (b) PTM MarkerFinder screens the spectra for user defined marker ions, extracts the spectra of putative PTM (pPTM) peptides and produces graphical and text outputs.

For every sample, the protein identification is performed using search engines such as Mascot (Matrix Science), one of the leading protein database interrogation software for MS data. In case the expected modification is present in the database, it can be selected for database searches (i.e. HexNAc, Hex, ...).

PTM MarkerFinder (Figure 1b) then screens the MS/MS spectra in the output file (i.e. Mascot .dat file) for marker ions specific for selected PTMs. The software tool is a function of the R package protViz that requires an R-object containing the mass spectrometric measurement, the database search results, and a list of marker ions (http://cran.r-project.org/web/packages/protViz/index.html, version higher than 0.1.25) [8]. The R-object can be created from any search engine output; however, only the perl script for the conversion of Mascot .dat file is included in protViz (‘mascotDat2RData.pl’). The PTM MarkerFinder function iterates over each MS/MS spectrum of the mass spectrometric measurement and searches for the marker
ions specified by the user (default window is 20 ppm). Since the .dat file does not provide information on the type of fragmentation technique applied, PTM MarkerFinder considers the tandem mass spectrum containing the marker ions as HCD scan. Spectra containing at least two product ions and with the sum of the marker ion intensities representing at least 2% of the total ion intensities are annotated with the corresponding ion series (b, y) using the protViz:peakplot method and are subsequently called putative PTM (pPTM) spectra. Afterwards, the function screens the succeeding scans for the corresponding ETD scan acquired from the same peptide ion and plots its peptide sequence assignment (c, z, and y ions). Note that the PTM MarkerFinder expects the ETD scan immediately after the HCD scan. If the MS acquisition changes the method has to be adapted accordingly. The final PTM MarkerFinder output contains information about the presence and the intensity of marker ions in pPTM spectra and peptide sequence assignments (Figures 2 and 3).

We tested the PTM MarkerFinder workflow on a sample containing enriched glycopeptides from yeast. A detail description of the procedure can be found in the supplementary material. In short, membrane proteins were extracted from log phase yeast cells and digested using the filter aided sample preparation protocol (FASP) by Wisniewski et al. [9]. Peptides were mixed with Concanavalin A (ConA) lectin and loaded onto 30 kDa cut-off filter units according to the protocols by Zielinska et al. [10]. Unglycosylated peptides were washed away while ConA-bound glycopeptides were retained on the filter. Finally, they were released with endoglycosidase H, which cleaves N-linked mannose-rich oligosaccharides from glycopeptides, generating a truncated sugar molecule with one HexNAc residue remaining on the asparagine. This procedure resulted in the enrichment of HexNAc-glycopeptides. The sample was analyzed by LC-MS/MS on an LTQ-Orbitrap Velos (Thermo Scientific) instrument using a combination of HCD and ETD fragmentation techniques. The raw file was converted to a Mascot Generic Format file (mgf) using Proteowizard (http://proteowizard.sourceforge.net/) and the HCD spectra were deconvoluted using H-score [11]. Protein identification was performed using Mascot v2.3 and the resulting .dat file was submitted to PTM MarkerFinder to screen for HexNAc specific marker ion (as described above).

The output provides the following information: (i) plots summarizing the distribution of marker ions in the pPTM spectra during the whole measurement (Figure 2); (ii) plots reporting the peptide sequence assignment and the marker ion information for each pPTM spectrum (Figure 3); (iii)
.csv table containing the list of marker ions found in each pPTM spectrum; (iv) reduced mgf file, containing the m/z values and the intensities of the pPTM spectra.

Figure 2: Example of PTM MarkerFinder output from a search for N-HexNAc modifications on a single Mascot output .dat file: (a) overview of the marker ion intensities of the HCD pPTM spectra; (b) pie chart for each pPTM spectrum showing the contribution of single marker ions to the total marker ion intensity. In both (a) and (b) only few pPTM spectra are here reported, and the scan 4752 is highlighted; (c) box plots of marker ion intensities from all pPTM spectra (the arrows indicates the scan 4752); (d) summed marker ion intensities for each pPTM spectrum.

The plots (i) summarize the number and distribution of putative modified peptides in the analyzed LC-MS run and facilitate a pre-screening of the data quality. In Figure 2a, an overview of the marker ion intensities in the pPTM HCD spectra is given. This allows the detection of scans containing several marker ions (i.e. scan 4752, with 6 HexNAc oxonium ions). Figures 2b and 2c display a pie chart for each pPTM spectrum representing the contribution of single marker ions to the total marker ion intensity, and the summed marker ion intensities for each pPTM spectrum, respectively. From Figure 2c, we understand that scan 4752 shows higher marker ion intensities compared to other pPTM spectra. The box plot in Figure 2d shows the marker ion intensities from all pPTM spectra. The reproducibility in the intensity of the HexNAc signature ions can be observed. By using this output, 74 pPTM HCD spectra have been extracted from the 4083 HCD
spectra present in the .dat file. The efficiency of PTM MarkerFinder in extracting spectra of putative glycopeptides and in discarding false-positives has been validated by manual comparison to the XIC (extracted ion chromatogram) of the frequently observed ion 204.0866 (supplementary Figure S1). Screening with less stringent settings (intensity and number of marker ions) increased the number of detected pPTM spectra (supplementary Table S1), but the added pPTM spectra did not contain any additional Mascot glycopeptide identifications. This shows that the filter settings are crucial to discard false-positives, that the number of HexNAc glycopeptides in the sample was rather low and that with our initial settings we were able to pinpoint all glycopeptide spectra.

Figure 3: For every putative PTM spectrum the following plots are provided: (a) HCD spectrum, with marker ions highlighted (circles) and peptide sequence assignment; (b) marker ion mass deviation (ppm); (c) marker ion intensities; (d) pie chart showing the contribution of summed marker ion intensities to the total fragment intensity; (e) pie-chart showing the contribution of single marker ions to the total marker ion intensity; (f) ETD spectrum (plotted only if present and assigned).

In addition to the annotated spectra, PTM MarkerFinder reports the identified peptide sequence, the type and localization of the modification, the Mascot identification score, the scan and the
query number. When no significant sequence assignment of the \textit{pPTM} HCD \textit{spectrum} is achieved, the uninterpreted spectrum is shown. Corresponding unassigned ETD spectra are not plotted. An example of PTM MarkerFinder output \textit{(ii)} for a HexNAc modified peptide is reported in Figure 3. The annotated HCD and ETD spectra are displayed in Figure 3a and Figure 3f, respectively (scan 4752 and 4753).

PTM MarkerFinder highlights the identified marker ions (Figure 3a (circles) and Figure 3c) and shows their mass deviation (ppm) (Figure 3b). Moreover, it calculates the relative intensity ratios of such ions and represents them as pie charts (Figure 3d, 3e), allowing a quick visual validation of the Mascot results. As an example: if Mascot identifies a HexNAc(N) modification, but the HCD spectrum does not contain any oxonium ion characteristic for this modification, the Mascot assignment can be considered as false. On the other hand, spectra showing a clear HexNAc(N) fragment ion pattern that do not give any significant Mascot output could be considered for manual interpretation. In the example shown, we can see at a glance that both HCD and ETD identified the same peptide sequence and modification site.

The combination of all the information reported in Figure 2 and 3 greatly helped the validation of spectra from glycopeptides and the identification of additional spectra from glycopeptides that remained unassigned during protein database searches. In total, PTM MarkerFinder allowed the validation of 24 peptide sequences by both HCD and ETD spectra. The localization of an additional 13 glycosites was validated only by ETD (9) or HCD (4). The remaining \textit{pPTMs} HCD \textit{spectra} could not be confidently assigned to any peptide sequence neither by HCD nor by ETD fragmentation, but since they contain 3 to 6 marker ions, they most probably represent glycopeptides.

In addition to the plots \textit{(i)} and \textit{(ii)}, PTM MarkerFinder returns a summary table (.csv) containing the following column attributes: "scans", "m/z", "markerIonMZ", "markerIonIntensity", "markerIonMzError", "markerIonPpmError", and "query" which can be used for statistics \textit{(iii)}. The reduced mgf file \textit{(iv)} contains only the \textit{pPTM} HCD \textit{spectra} and their corresponding ETD scans, allowing to re-run Mascot searches using different parameters while decreasing dramatically the required computational time. The complete dataset is available in the supplementary material.

Since PTM MarkerFinder is able to search a-posteriori for any type of product ion(s), it can be used on datasets containing any type of modification producing marker ions upon HCD or PQD.
MS/MS fragmentation. To test this, a tryptic digest of yeast proteins was analyzed by LC-MS/MS using HCD fragmentation. The spectra were searched for glycan signatures (such as HexNAc (N) and Hex (S,T)), and methyl (K,R), dimethyl (K,R) and acetyl (K) modifications. Despite the low specificity of some marker ions, PTM MarkerFinder allowed the validation of 45 modified peptides. All the results are reported in the supplementary material (table S2). As a conclusion, our software can be applied to validate not only various types of glycosylations, but also completely unrelated protein modifications.
Acknowledgements

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References


**Material and Methods**

*Protein extraction and digestion*

Log phase yeast cells were lysed and membranes were collected. Membrane proteins were extracted using the filter aided sample preparation protocol (FASP) by Wisniewski et al. [1]. In short, the membrane pellet was washed several times and solubilized in buffer containing 4% SDS. A total of 400 µg of protein were diluted in urea buffer and loaded onto Vivacon 30 kDa filter units. Proteins were first digested overnight with Lys-C (enzyme/protein ratio 1:100, w/w) in buffer containing 8 M urea. The solution was then diluted with NaHCO3 to > 2 M urea and trypsin was added to the peptides (1:100, w/w). After four hours peptides were eluted from the filters by centrifugation.

For whole yeast lysate analysis, log phase yeast cells were lysed in buffer containing 8M Urea. A total of 50 µg of protein was diluted with NaHCO3 to 2 M urea and digested overnight with trypsin (in a 1:50 (w/w) enzyme/protein ratio).

*Glycopeptide enrichment*

Glycopeptides were enriched based on protocols by Zielinska et al. and Breidenbach et al. [2, 3]. In short, peptides were mixed with Concanavalin A (ConA) lectin and loaded onto 30 kDa cut-off filter units. Unglycosylated peptides were washed away while ConA-bound glycopeptides were retained on the filter and finally released with endoglycosidase H. The peptide mixture was then desalted using ZipTip C18 (Millipore) and dried before mass spectrometry analysis.

*Mass Spectrometry analysis*

Samples were analyzed on a LTQ-Orbitrap Velos ETD mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) coupled to an Eksigent-Nano-HPLC system (Eksigent Technologies, Dublin (CA), USA). Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% acetonitrile for channel B. Peptides were loaded on a self-made tip column (75 µm × 80 mm) packed with reverse phase C18 material (AQ, 3 µm 200 Å, Bischoff GmbH, Leonberg, Germany) and eluted with a flow rate of 250 nl per min by a gradient from 3 to 35% of B in 60min.
For the analysis of enriched glycopeptides, the acquisition method consisted of an orbitrap full MS scan followed by double-play data-dependent HCD MS/MS and ion trap ETD MS/MS on the top 10 signals above a threshold of 1000. For the analysis of the whole yeast lysate, the data-dependent acquisition consisted of HCD MS/MS on the top 10 signals above a threshold of 1000. Full-scan MS spectra (300–2000 m/z) were acquired with a resolution of 60000 at 400 m/z after accumulation to a target value of 1x10⁶. The AGC values for MS/MS analysis were set to 1x10⁴ for ETD (ion trap detection, 100ms injection time) and to 1x10⁵ for HCD (200ms injection time) respectively. The HCD normalized collision energy was set to 40%, enabling the stepped collision energy (width 15%, 3 steps), and detecting the ions at a resolution of 7500 at 400m/z. The ETD anion target value was set to 1e5 and the activation time to 100 ms. Charge-state dependent ETD reaction times were enabled, setting a reference value of 100 ms for doubly charged peptides. A supplemental activation with 25% normalized collision energy was always applied. The isolation width was set to 2 amu and 4 amu for HCD and ETD experiments, respectively.

Charge state screening was enabled and singly charged ions were rejected. Precursor masses already selected for MS/MS were excluded for further selection for 45 s and the exclusion window was set to 10 ppm. The size of the exclusion list was set to a maximum of 500 entries.

Database search
MS and MS/MS spectra were converted into Mascot generic format (mgf) using Proteowizard (http://proteowizard.sourceforge.net/), and the HCD spectra were further deconvoluted using H-score [4].

The mgfs were searched against a yeast database (Saccharomyces cerevisiae (strain ATCC 204508 / S288c) downloaded from Uniprot in February 2011) containing reversed decoy sequences and the common contaminants, using a local Mascot Server 2.3 instance (Matrix Science). The peptide tolerance was defined to 10 ppm and the MS/MS tolerance to 0.6 Da. Carbamydomethylation of cysteine was set as fixed modification, while HexNAc (N) and oxidation of methionine were selected as variable modification. The HexNAc (N) modifications were defined in two different ways: one more suitable for ETD spectra (mass shift of 203.0794) and one more suitable for the HCD spectra (the marker masses at 204.0871, 186.0761, 168.0655, 144.0655, 138.0550 and 126.0550 were ignored, and two neutral losses equal to 203.0793 and 221.0899 were considered).
For the analysis of the whole yeast lysate, the HCD mgf file was searched for the following variable modifications: HexNAc (N), Hex (S,T), methyl (K,R), dimethyl (K,R), acetyl (K) modifications.

**PTM MarkerFinder parameters**

Different filters were used for the PTM MarkerFinder analysis. In particular, for the analysis of enriched glycopeptides: HexNAc marker ions (m/z 204.0866, 186.0761, 168.0655, 144.0655, 138.0550, 126.0550), minimum of 2 marker ions, minimum of 2% relative marker ions intensity. For validation purposes, different minimum numbers of marker ions (1, 2, 3) and minimum relative marker ion intensities (0.01, 1, 2, 5 %) were used (Table S1).

The whole yeast digest dat files were screened for: arginine methylation (m/z 143.1291, 115.0866, 112.0869, 74.0713, 70.0651; 3 marker ions; 2%), arginine dimethylation (m/z 157.1448, 115.0866, 112.0869, 88.0869, 71.0604; 2 marker ions; 2%), lysine methylation (m/z 115.123, 98.0964, 84.0808; 2 marker ions; 2%), lysine dimethylation (m/z 129.1386, 112.1121, 84.0808; 2 marker ions; 2%), lysine acetylation (m/z 143.1179, 126.0913, 84.0808; 2 marker ions; 5%), N-acetylhexosamine (HexNAc, m/z 204.0866, 186.0761, 168.0655, 144.0655, 138.0550, 126.0550; 3 marker ions; 1% ), hexose (Hex, m/z 163.0601, 145.0495, 127.0390, 115.0390, 109.0284; 2 marker ions; 1%) and for glycan signature ions (m/z 366.1400 (Hex-HexNAc⁺), 325.1135 (Hex-Hex⁺), 274.0927 (NeuAc⁺-H₂O), 204.0866 (HexNAc⁺), 163.0601 (Hex⁺); 3 marker ions; 1%). A mass deviation of 20 ppm was allowed for the analyses.

**References**


**Tables**

**Table S1:** Results of PTM MarkerFinder analysis of a Mascot output file (.dat) containing HCD and ETD spectra from a sample enriched in HexNAc glycopeptides. By using lower intensity thresholds (min relative % ions) and a lower number of marker ions (min # Marker ions), different numbers of putative PTM spectra (pPTM spectra) can be detected. The number of pPTM HCD and ETD spectra that Mascot could identify as glycopeptides are reported.

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**Table S2:** Results of PTM MarkerFinder screening of HCD spectra from a tryptic digest of yeast proteins analysed by LC-MS/MS. The spectra have been searched for HexNAc (N), Hex (S,T), methyl (K,R), dimethyl (K,R) and acetyl (K) using Mascot. For every modification, a different minimum number of required marker ions (min # Marker ions) and marker ion intensity threshold (min relative % ions) were set. The number of putative PTM spectra (pPTM spectra) are reported. The number of pPTM spectra that Mascot could identify with low (score <20, Exp. value >0.05, "low conf.") or high (score >20, Exp. value <0.05, "high conf.") confidence are shown. The values were selected arbitrarily as threshold for correct peptide sequence identification. The list of marker ions is defined as in reference [1] (Matthiesen et al., J. Proteome Res. 2005, 4(6):2338-47.), except for the modifications HexNAc and Hex (described in the manuscript) and "glycan signatures" (m/z 366.1400 (Hex-HexNAc+), 325.1135 (Hex-Hex+), 274.0927 (NeuAc+-H2O), 204.0866 (HexNAc+), 163.0601 (Hex+)

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Chapter 6:

Conclusions and future perspectives
Conclusions and future perspectives

In the scope of my thesis I analyzed protein degradation in the yeast *Saccharomyces cerevisiae*. A special focus was placed on the degradation dynamics of protein complexes in the ER and their subunits by ERAD. Moreover, the assembly of oligosaccharyl transferase (OST) was studied and a model for this process was proposed. Another focus was placed on N-glycosylation, a process which is catalyzed by OST, and how it influenced protein degradation. Lastly, we looked at how this post-translational modification is studied using mass spectrometry.

In chapter 2 a SILAC-SRM method was developed to study protein degradation and the oligosaccharyl transferase enzyme. As opposed to previous studies, we analyzed cells grown in standard culture flasks (Helbig et al., 2011; Pratt et al., 2002). This facilitated handling of the cultures and allowed us to correlate our findings more easily to results from exponentially growing cells. Moreover, this setup allowed to monitor medium- to low-abundant proteins that would be missed with a shotgun MS approach. Furthermore, in contrast to previous approaches, such as radioactive pulse-chase or cycloheximide chase experiments, we were able to measure degradation rates for several proteins in a single experiment.

In agreement with previous studies we found that degradation levels were low for most native proteins in exponentially growing yeast, indicating that yeast protein translation and folding are efficient and result in few misfolded proteins (Halvorson, 1958b; Losson et al., 1978). On the other hand, ERAD was induced for single misfolded proteins, such as Pra*, correcting excess amounts of complex subunits and eliminating destabilized complex components.

Apart from eliminating single misfolded proteins, it remains unclear under which conditions degradation becomes globally important in yeast. Previous studies reported that: (I) protein degradation was crucial for yeast growing under natural conditions where nutrient sources were scarce (Halvorson, 1958a; Lopez and Gancedo, 1979), (II) degradation rates increased when yeast was grown on ethanol as carbon source (Liu and Chaloupka, 1983), and (III) protein degradation may be more prominent in growth phases that do not allow the rapid turnover of cellular material as in early exponential growth phase (Bakalkin et al., 1976). Furthermore, degradation might be important while shifting from one condition to another (e.g. low to high temperature, rich to minimal medium) rather than during steady state. Besides, in nature yeast cells may be exposed to environmental stress conditions where misfolding is more prominent and degradation of misfolded proteins becomes vital. The sensitivity and the reproducibility of the SILAC-SRM method make it ideal for many types of protein degradation.
studies. However, it relies on the use of minimal media and uptake of amino acids that might not be efficient in some conditions such as stationary phase. This makes it difficult to measure protein degradation under more natural conditions. Furthermore, the mathematical calculations are based on the assumption that yeast grows exponentially and that protein levels are constant. In case of stationary growth or perturbation, the calculations would have to be adjusted. It would be possible, though, to expose wild type and ERAD-deficient cells to a natural environment and to assess if the absence of ERAD has a negative effect on fitness of the cells. After all it has been shown that small amounts of misfolded proteins can lead to a slight fitness defect (Geiler-Samerotte et al., 2011).

Another area where ERAD activity might be vital is in aneuploid yeast cells, i.e. cells with abnormal numbers of chromosomes. These cells overexpress not only one protein but almost all proteins in a duplicated region of the chromosome, causing a fitness defect or even lethality. Protein degradation pathways such as ERAD might alleviate the phenotype by degrading the excess protein (Torres et al., 2010). In this function protein degradation pathways might play an important role in evolution: During gene duplications levels of a gene product are doubled. If protein degradation lowers the protein abundance again to wild type levels, it avoids fitness impairing phenotypes. Thus, one of the gene copies can freely mutate until it adopts a fitness-enhancing function, e.g. by interacting with different binding partners, that also stabilizes it. In summary, future experiments could focus on comparing protein degradation globally in different yeast media and could analyze its importance for fitness of aneuploid yeast cells.

In another experiment we showed that the SILAC-SRM method can be used to assess the stability of protein complexes. In particular, we investigated the dynamics of the OST complex showing that the complex tolerated excess subunits while a loss of essential subunits led to destabilization. This raises the question if these principles apply also to other membrane protein complexes. Furthermore, it paves the way for mutational studies to unravel the function of the individual subunits. Not much is known about the function of the individual subunits except that Ost3p and Ost6p act as thioreductases, and that Stt3p is the catalytic subunit (Huang et al., 2012; Schulz et al., 2009; Yan and Lennarz, 2002). It has furthermore been proposed that Wbp1p, Ost2p, and Swp1p might be involved in allosteric regulation by binding to the LLO and that Ost1p, and Ost5p are responsible for delivering the peptide to the catalytic site of the enzyme (Kelleher et al., 2007; Pathak et al., 1995; Wilson and High, 2007; Wilson et al., 2008; Yu et al., 1990). Recent research revealed the structure of the bacterial STT3 protein PglB from Campylobacter lari and subsequent studies have identified a potential catalytic mechanism (Lizak et al., 2013; Lizak et al., 2011). Furthermore, conserved residues were identified in the STT3 proteins of several different species, among them also some of the
catalytic PglB motives (Jaffee and Imperiali, 2011). This data now allows to select residues and to modify them. The effects of the modifications can be checked using the SILAC-SRM method to distinguish between hits causing catalytic inactivity from hits causing complex disruption.

Finally, in chapter 2 we analyzed OST complex assembly. We complemented our results with data from previous protein and genetic studies to propose an assembly model (Karaoglu et al., 1997; Kelleher and Gilmore, 2006; Nasab et al., 2008; Reiss et al., 1997; Silberstein et al., 1995; Spirig et al., 2005; Spirig et al., 1997; te Heesen et al., 1993). We propose that Wbp1p, Swp1p, and Ost2p form one subcomplex and Ost1p, Ost5p, and Stt3p a second one. During assembly first the two subcomplexes form and bind to each other, before Ost4p anchors Ost3p or Ost6p into the complex. This model needs to be verified using different experimental techniques, e.g. blue-native PAGE or crosslinking studies in wild type and OST subunit deletion cells (Schagger and von Jagow, 1991) (Guerrero et al., 2006; Yan et al., 2003). Blue-native PAGE would allow to observe subcomplexes by immunoblot. Determining the percentage of each subcomplex in the cell or pairing blue native PAGE with a pulse-chase setup would allow to calculate how quickly the subcomplex are incorporated into the complete OST. Results from such studies may be valuable to attain the goal of obtaining a crystal structure for the OST complex. Structural studies have so far only been conducted on Ost4p, and on the soluble domains of Ost6p and Stt3p (Gayen and Kang, 2011; Huang et al., 2012; Mohorko et al., 2014; Schulz et al., 2009). Structures for the yeast and the canine OST complexes have been reported, albeit only at low-resolution (Li et al., 2008a; Pfeffer et al., 2014). To simplify crystallization, it would be possible to begin with OST enzymes from lower organisms containing less subunits or to start with one of the subcomplexes, e.g. Wbp1p-Ost2p-Swp1p isolated from Δost1 cells, though they are less stable compared to completely assembled OST (Kelleher and Gilmore, 2006).

Prior to crystallization, other means could be used to collect more structural information about the organization of the OST complex. In recent years, many mass spectrometry methods for structural studies became more refined and adapted for the study of protein complexes (Snijder and Heck, 2014). Among them hydrogen/deuterium exchange (HDX) coupled to mass spectrometry could be used to assess solvent accessibility of complex regions (Macakova et al., 2013; Rodriguez et al., 2014; Suchanova and Tuma, 2008; Zhang et al., 2013). Alternatively, another MS technique used for complex studies is ion mobility separation, which separates proteins by structure, coupled to native mass spectrometry to learn about the connectivity of protein complexes (Bereszczak et al., 2012; van Duijn et al., 2012). Finally, another popular method to analyze complex architecture is cross-linking coupled to mass
spectrometry (e.g. formaldehyde, glutaraldehyde), an approach already used in the past for OST subunits (Yan et al., 2003). For all of the cited structural-MS techniques it would be beneficial, if not necessary, to work with purified complex instead of complex samples.

In a next stage our method could be applied to study dynamics of other protein complexes. Our experiments analyzing whole cell extract already revealed interesting dynamics for ribosomal proteins, some of which were very stable, while others were degraded more quickly. As opposed to the OST subunits that are synthesized at the correct stoichiometry and are stable once complex assembly is completed, the stability of ribosomal proteins differs largely (Helbig et al., 2011). This suggests that some subunits are exchanged faster in the complex than others. Apart from these interesting dynamics ribosomal proteins are soluble, high abundant, and ubiquitous in the cell, which makes them an ideal target for further study.

Moreover, it would be interesting to conduct a more large-scale study and to monitor a large number of protein complexes in yeast to find out if the dynamics we observed are characteristic for OST or apply to membrane complexes in general. For intermediate to low-abundant proteins like the OST subunits, the sensitive SRM approach was required. However, due to progress in instruments and MS methods it should be possible to monitor more abundant proteins using shotgun MS in the future (Hebert et al., 2014; Kelstrup et al., 2012). Otherwise, large scale SRM experiments could be conducted since now SRM transitions for many yeast proteins are available and deposited in spectral libraries (Picotti et al., 2013; Soste and Picotti, 2013).

In chapter 3, we analyzed the dynamics of protein complexes. We showed that the stt3-7 mutation in S552 of the Stt3p subunit interfered with complex formation, corroborating earlier results that the mutation did not affect catalysis (Jakob et al., 2001; Spirig et al., 1997). Furthermore, we postulated that unassembled OST subunits were degraded with maximal rates. Comparing these rates to degradation rates in cells with partial complexes, allowed us to determine subunits that stabilized each other. Moreover, we had seen in chapter 2 that overexpressed subunits did not affect activity of the OST complex and were degraded, while deletions in essential genes destabilized the complex. To determine if this principle was also valid for other protein complexes we analyzed several datasets from global overexpression and deletion studies for enrichment of complex subunits. In agreement with our hypothesis deletions in slow growing strains were enriched for complex subunit genes. Furthermore, overexpressed genes leading to slow growth were not enriched in complex subunits. Direct biological studies would be necessary to confirm that these principles apply to a large number of protein complexes. E.g. overexpression and deletion experiments with many complexes
would need conducted and complex integrity would need to be analyzed by measuring
degradation rates or relative subunit abundances. Studies in aneuploid yeast cells with either
a gain or a loss of chromosomal regions could address this.

In chapter 4, global protein levels were analyzed in \( \Delta \text{alg}3 \) and \( \Delta \text{ire}1 \) mutant cells at normal
and elevated temperatures. The abundance of only few proteins changed as compared to wild
type cells. This indicates either that an altered glycan signal, the absence of UPR, or elevated
temperature do not destabilize the proteome or that it cannot be detected using steady-state
SILAC. Conditions where a larger fraction of the proteome would be destabilized could include
nutrient starvation as discussed above (Bakalkin et al., 1976; Halvorson, 1958a, b; Liu and
Chaloupka, 1983). Alternatively, cells with a defect in ergosterol biosynthesis with altered lipid
membrane thickness and composition could be analyzed. It is expected that membrane
proteins would be destabilized under these conditions, because of transmembrane domains
not being properly buried within the phospholipid bilayer. A potential handle to alter membrane
composition would for example be to inhibit ERAD of Hmg2p (= HMG-CoA reductase 2) which
is responsible for the central step of sterol biosynthesis. This would lead to increased cellular
abundance of the protein. Overexpression of Hmg2p has been reported to alter ER morphology
and could possibly lead to lead to higher turnover of membrane proteins (Federovitch et al.,
2008).

In the same project we completed a pilot study aiming to measure our yeast samples by
SWATH MS. The goal of future studies would be to couple pulsed SILAC to SWATH MS
measurements for half-life measurements of the complete yeast proteome. Several other DIA
strategies have emerged recently and time will tell which of the workflows will be more widely
applied. Developments in data processing and analysis software will be crucial for the
establishment of any of the methods due to the large amounts of complex data structures
generated by these approaches. Technological progress on MS instruments will further drive
development of DIA methods. In the meantime, progress on instrument platforms continues
and new instruments have allowed coverage of almost complete proteomes by data-
dependent approaches (Hebert et al., 2014; Kelstrup et al., 2012).

Chapter 5 presented the PTM MarkerFinder software (Nanni et al., 2013). This software uses
low-mass HCD fragments (marker ions) to select peptide-spectra containing specific PTMs.
Moreover, it annotates retrieved HCD and ETD spectra using the peptide identification from a
database search. Thus, it greatly facilitates manual validation and annotation of modified
peptide spectra.
The PTM MarkerFinder tool can be used for validating the spectra when making an N-glycosite inventory for any organism. Such a glycosite inventory would be useful e.g. to analyse biomarkers for cancers (Boersema et al., 2013; Deeb et al., 2014; Huttenhain et al., 2013) and has already been acquired for several model organisms (Zielinska et al., 2012; Zielinska et al., 2010). In general, these studies enriched glycopeptides and subsequently marked glycosites by cleaving off N-glycans with PNGase F which resulted in a +1 Da mass shift of the glycosylated asparagine. However, this reaction is notorious for yielding false-positive identifications due to spontaneous deamidation of asparagine residues that will lead to the same mass shift (Hao et al., 2011). A way to alleviate this problem is to conduct the PNGase F reaction in presence of heavy water (H_2^{18}O) which will lead to incorporation of the heavy oxygen molecule, thus leading to a bigger mass shift of +3 Da and allowing to distinguish it from spontaneous deamidation occurring before or after enzymatic treatment (Li et al., 2008b). However, the method is still error-prone (Palmisano et al., 2012). Enriching glycopeptides, cleaving sugars to a single N-HexNAc residue with Endoglycosidase H, and subsequent MS analysis and processing with PTM MarkerFinder could give a higher confidence in peptide identifications and modification site localization with a lower rate of false-positives compared to PNGase F-based workflows.

Besides, since PTM MarkerFinder allows the user to specify marker ions for any PTM, it could also be useful for studies that need to identify and validate several PTMs, e.g. studying the interplay between different modifications.

In summary, the presented works have hopefully contributed towards a better understanding of protein turnover and its connection to N-glycosylation, as well as oligosaccharyl transferase complex dynamics.
References:


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