Ubiquitin-proteasome dependent catabolite degradation of fructose-1,6-bisphosphatase: localization and involvement of novel components

vorgelegt von

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

Stuttgart, den 2. November 2009

Lise BARBIN
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Part I

Abbreviations
## Abbreviations

<table>
<thead>
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<th>Definition</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>APS</td>
<td>Ammoniumpersulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine Triphosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>bzw.</td>
<td>beziehungsweise</td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5'-cyclo-Adenosine-monophosphate</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>dNTP</td>
<td>Desoxynucleosidetriphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
</tr>
<tr>
<td>DUBs</td>
<td>DeUbiquiting Binding enzymes</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemoluminescence</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraaceticacid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBPase</td>
<td>Fructose-1,6-bisphosphatase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GID</td>
<td>Glucose Induced degradation Deficient</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin A</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to the E6-AP Carboxyl Terminus</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilobasepairs</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>NE</td>
<td>Nuclear envelope</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>OD$_{600}$</td>
<td>Optical density at 600 nm</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PGK</td>
<td>3-Phosphoglycerate kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazin-N,N-bis(2-ethanolsulfonic acid)</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
</tr>
<tr>
<td>RNAse</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Polyoxyethylenesorbitan-Monolaurate</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UBPs</td>
<td>Ubiquitin specific processing protease</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>VID</td>
<td>Vacuole Induced Degradation</td>
</tr>
<tr>
<td>v/v, w/v</td>
<td>Volume per volume, weight per volume</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Abstract

In the yeast *Saccharomyces cerevisiae*, when cells are grown on a non-fermentable carbon source like ethanol, the key regulatory gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) is synthesized. Addition of glucose to the medium leads to a quick change from gluconeogenesis to glycolysis. FBPase is then rapidly inactivated by phosphorylation, polyubiquitinated and degraded by the 26S proteasome. Prior to proteasomal degradation, polyubiquitination of the enzyme occurs via the ubiquitin-conjugating enzymes Ubc1, Ubc4, Ubc5 and Ubc8 in conjunction with a novel multi-subunit ubiquitin ligase, the Gid (Glucose Induced degradation Deficient) complex. Upon glucose addition to cells, the regulatory subunit Gid4/Vid24 is synthesized, binds to the Gid-E3 complex and triggers FBPase ubiquitination and subsequent degradation.

Gid1 and Gid8, two members of the Gid complex, were found to possess a CRA (CT11-RanBPM) domain of unknown function. Mutations inserted in this domain of Gid8 led to a notable impairment in FBPase degradation and polyubiquitination. However interactions between the mutated Gid8 and FBPase or interactions between the mutated Gid8 and the Gid complex still remained.

Further work focused on the determination of the localization of most Gid proteins by indirect immunofluorescence. These experiments confirmed the cytosolic and nuclear localization of the proteins Gid1 and Gid8 described by Huh *et al*. Gid2 could not be localized by indirect immunofluorescence. However a nuclear localization sequence (NLS), a usual hint of the localization of proteins in the nucleus of eukaryotes, was discovered in the protein. This NLS sequence was fused to GFP and shown to actively transport the recombinant protein into the nucleus, confirming its efficiency as a nuclear localization sequence. A deletion of the NLS sequence was then carried out in Gid2, but the mutated protein was not stable in a non-fermentable carbon source; thus no further study on the influence of this NLS on FBPase degradation was performed.

To determine the subcellular localization of FBPase, the enzyme was C-terminally marked with several tags. However FBPase localization was dependent on the tag used. FBPase-TAP was the only fusion protein degraded like the untagged FBPase. Using indirect immunofluorescence microscopy, the site of FBPase-TAP degradation was determined to be cytosolic.
Finally, as an additional machinery required for the catabolite degradation process, we identified the trimeric Cdc48\(^{Ufd1-Npl4}\) complex. This AAA-ATPase complex acts between polyubiquitination of FBPase and its degradation by the proteasome. Furthermore, the amount of FBPase polyubiquitination, and the degradation of FBPase are strongly impaired in mutants of Ufd3, a substrate-processing cofactor of Cdc48. Interestingly Cdc48 interacts with the Gid8 subunit of the Gid complex. Finally Rad23 and Dsk2, two ubiquitin-binding proteins reported to escort polyubiquitinated substrates to the proteasome, were shown to be necessary for FBPase degradation. All these results suggest that polyubiquitinated FBPase may be recruited by the Cdc48\(^{Ufd1-Npl4}\) machinery for its delivery to the 26S proteasome. Elimination of an additional gluconeogenic enzyme, phosphoenolpyruvate carboxykinase, was also shown to be dependent on the Cdc48\(^{Ufd1-Npl4}\) complex.
Zusammenfassung

In der Hefe Saccharomyces cerevisiae wird das regulatorische glukoneogenetische Schlüssel-enzym Fructose-1,6-bisphosphatase (FBPase) synthetisiert, wenn Zellen in Medien mit nicht fermentierbarer Kohlenstoffquelle wie Ethanol wachsen. Der Wechsel auf glukosehaltiges Medium führt zu einem schnellen Umschalten von der Glukoneogenese zur Glykolyse. FBPase wird dabei sehr schnell durch Phosphorylierung inaktiviert, polyubiquitiert und durch das 26S Proteasom abgebaut. Die Polyubiquitinierung der FBPase wird von den ubiquitin-konjugierenden Enzymen Ubc1, Ubc4, Ubc5 und Ubc8 und der neuen Gid (Glucose Induced degradation Deficient) Ubiquitin Ligase durchgeführt. Nach Glukosegabe zu glukoneogenetischen Zellen wird die regulatorische Untereinheit Gid4 synthetisiert. Sie bindet an den Gid-E3 Komplex und steuert die Polyubiquitinierung und den Abbau der FBPase.

Gid1 und Gid8, zwei Proteine des Gid Komplexes, enthalten eine CRA (CT11-RanBPM) Domäne mit unbekannter Funktion. Ziel dieser Arbeit war es die Bedeutung dieser Domäne näher zu untersuchen. Mutationen der Domäne von Gid8 zeigten einen signifikanten Einfluß auf die Degradationsgeschwindigkeit und Polyubiquitinierung der FBPase, jedoch konnte kein Unterschied hinsichtlich der Interaktionen zum Gid Komplex bzw. zur FBPase festgestellt werden.


Zur Bestimmung der subzelluläre Lokalisierung von FBPase wurde das Enzym C-terminal mit verschiedenen Epitopen markiert. Es stellte sich jedoch heraus, dass die FBPase Degradation/Lokalisation von den unterschiedlichen Tag’s beeinflusst wurde. Lediglich FBPase-TAP wurde gleich schnell wie die Wilt Typ Form abgebaut. Dieses Epitop-markierte Protein wurde
mittels indirekter Immunfluoreszenz cytosolisch localisiert.

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Part II

Introduction
Chapter 1

The proteolytic system: the degradation of intracellular proteins

Degradation of proteins in cells is as essential as their synthesis. Degradation is for instance required for down-regulation of enzymes or transcription factors, for elimination of misfolded proteins, for supplying amino acids for new protein synthesis. There are two major intracellular devices in which these proteins are broken down: vacuoles and proteasomes. The adjustment of those two processes rules the survival of the cell which has to constantly adapt to changing environmental conditions.

The assembly of new proteins requires a source of amino acids, which are either synthesized or created through degradation of proteins in the cell. Lots of cellular proteins are constantly degraded and recycled. The turnover of proteins, i.e. the degradation and resynthesis of proteins, constantly occurs in cells. Although some proteins are very stable, a lot of them have a short life time, especially those which are playing an important role in the regulation of the metabolism (Berg et al., 2003). A modification in the rate of those proteins can rapidly change the flux in metabolic pathways.

Moreover, cells have detection and elimination mechanisms of misfolded proteins. A significant part of newly synthesized proteins appears to be defective because of translation errors or errors in their folding (Hilt and Wolf, 1995). Intracellular proteolysis plays a crucial role in the maintenance of proper protein folding, but also in the cell cycle, signal transduction, gene expression, development antigen processing and other cellular processes (von Mikecz, 2006).

Through evolution two proteolytic pathways have emerged. On the one hand, non specific
proteases are engulfed in a special organelle, the lysosome or vacuole into which proteins to be degraded are delivered via vesicular transport. On the other hand, specific proteins are first recognized by a recognition system, marked and then degraded through a highly specific proteinase complex called proteasome (Hilt and Wolf, 2004).

1.1 The vacuole/lysosome system

The vacuole of the yeast *Saccharomyces cerevisiae* corresponds to the lysosome of higher eukaryotic cells. It is a large membrane-surrounded compartment that contains digestive enzymes (acidic hydrolases) to digest macromolecules. At pH 4.8-6, the interior of the vacuoles is more acidic than the cytosol (pH 7.0). The lysosome’s single membrane stabilizes the low pH by pumping protons (H\(^{+}\)) from the cytosol to the vacuolar lumen by the use of ATP. The vacuolar membrane protects the cytosol and the rest of the cell from the degradative enzymes located in the lysosome. Lysosomal enzymes, to a large degree, are not very active at a neutral pH, they need the acidic environment of the lysosome to function. This seems to constitute a protection mechanism against autodigestion of the cell, in case digestive enzymes pass into the cytoplasm. Lysosomes are the ”stomach” of the cell and serve for the degradation of cellular components thanks to around 40 different types of hydrolases able to degrade all types of macromolecules.

Lysosomes are important degradative organelles particularly under stress conditions. Most of the proteolysis of cytosolic proteins that occurs in lysosomes is relatively non specific (Knop et al., 1993). In yeast, around 85 % of total protein degradation occurs in the vacuole under stress conditions and around 40 % under normal growth conditions (Teichert et al., 1989). Delivery of intracellular proteins into the vacuole/lysosome occurs via autophagocytosis (Mizushima and Klionsky, 2007). Vacuoles serve a variety of different functions in addition: capturing food materials or unwanted structural debris surrounding the cell, sequestering materials that might be toxic to the cell, maintaining fluid balance within the cell, exporting unwanted substances from the cell, or even determining relative cell size (Schwenke, 1991). Besides proteins and other macromolecules like polysaccharides or lipids, whole cell organelles like mitochondria and peroxysomes are also degraded through autophagocytosis or endocytosis (Klionsky, 1997). While most long-lived proteins are degraded in the vacuole, the selective degradation of short-lived proteins is carried out by the proteasome (Thumm and Wolf, 1998).
1.2 The proteasome

Proteasomes are large multi-subunit protease complexes (Hilt and Wolf, 1995, 1996, 2004; Wolf and Hilt, 2004) which selectively degrade intracellular proteins. They are located in the cytoplasm and in the nucleus of all eukaryotic cells. The majority of intracellular proteins are proteolyzed by the proteasomes. In most cultured mammalian cells, these account for 80-90 % of protein breakdown (von Mikecz, 2006). Proteasomes have essential roles to play in controlling cellular processes such as metabolism and the cell cycle through signal-mediated proteolysis of key enzymes and regulatory proteins. They operate in the stress response (Heinemeyer et al., 1991; Hilt et al., 1993), degrade metabolic enzymes like fructose-1,6-bisphosphatase (Schork et al., 1995, 1994a,b), remove abnormal proteins and function in the immune response by generating antigenic peptides (Hilt and Wolf, 1996).

There are two types of proteasomes: the proteolytic 20S core complex with a molecular mass of 700 kDa and the 26S proteasome with 1700 kDa.

1.2.1 The 20S proteasome: structure and proteolytic activities

The 20S proteasome has been found in all eukaryotes, from yeast to human (Hilt and Wolf, 1995, 1996). Its structure and proteolytic activities have been discovered through X-ray analysis and electron microscopy (Baumeister et al., 1988; Hilt and Wolf, 2000; Wolf and Hilt, 2004). The 20S proteasome consists of 2 rings of 7 α subunits and 2 rings of 7 β subunits, stacked in the order α7β7β7α7 as heptameric rings (Figure 1.1 A). The barrel of the 20S proteasome is about 15 nm long and 11.5 nm wide. The α7β7β7α7 architecture leads to the formation of three chambers in the 20S particle: the catalytic chamber of about 84 nm$^3$ formed by the β rings and the two antechambers formed by one α and one β ring of about 59 nm$^3$. The size of the antechambers indicates that they can accommodate a considerable amount of protein substrates or degradation products (Figure 1.1 B). The active sites in the catalytic chamber can only be reached by two axial pores of about 2 nm formed by the α subunits on each end of the cylindrical barrel. This pore dimension ensures that only unfolded polypeptides (single strands or double stranded hairpin loops) can enter the catalytic center of the proteasome. In addition, access to the channel is gated: access to the interior is only possible after rearrangement of the N-terminal HO helices of the α subunits.

The α subunits have a structural role while the β subunits have a catalytic function. The
20 The proteolytic system: the degradation of intracellular proteins

Figure 1.1: **Structure of the 20S core complex.** (A) The 20S proteasome is composed of a stack of four rings composed of seven subunits each. The two outer rings are made up of seven different α subunits (left graph, view from the top), whereas the two central rings are composed of seven different β subunits (right graph, view from the center). The active sites reside within the central chamber (shadow ring) of the 20S proteasome at subunits β1/Pre3, β2/Pup1 and β5/Pre2 (marked by circles). (B) The α7β7β7α7 architecture leads to the formation of three chambers in the 20S particle: the catalytic chamber of about 84 nm³ formed by the β rings, and the two antechambers formed by one α and one β ring of about 59 nm³. The size of the antechambers indicates that they can accommodate a considerable amount of protein substrates or degradation products.
20S proteasome is a protease with its active sites sequestered in the interior of the hollow, barrel-shaped complex formed by these stacked rings. The catalytic activity of the 20S proteasome is mainly run by the three active $\beta$ subunits residing within the central chamber: $\beta_5$ (in yeast Pre2) harboring the chymotrypsin-like activity (the most important one), $\beta_2$ (in yeast Pup1) providing the trypsin-like activity and $\beta_1$ (in yeast Pre3) carrying the acidic (peptidyl-glutamyl-peptide splitting) activity (the least important one) (Hilt and Wolf, 2004).

### 1.2.2 The 26S proteasome: structure

Contrary to the 20S proteasome the 26S proteasome digests ubiquitinylated proteins by using ATP. This multi-subunit protease of 1700 kDa saves ubiquitin which is recycled. The 26S proteasome is a complex of two components: the 20S proteasome, the catalytic subunit, and a 19S regulatory subunit. Attached to both ends of the 20S core particle are two of the so-called 19S cap complexes or regulatory subunits.

The 19S regulatory cap complexes recognize multi-ubiquitinated proteins and provide a passageway for threading unfolded proteins into the proteasome core complex. The 19S cap can be split into two different subcomplexes, base and lid (Figure 1.2). Both subcomplexes are linked to each other via the subunit Rpn10 (Rpn, regulatory particle non-ATPase). The base is composed of a ring of six different ATPase subunits of the AAA-type (Rpt1 to Rpt6; Rpt, regulatory particle triple A protein) which dock onto the $\alpha$ rings on both ends of the 20S core and two additional non-ATPase subunits, Rpn1 and Rpn2. The lid is built up from eight different subunits, Rpn3 and Rpn5 to Rpn11 (Hilt and Wolf, 2004).

The 19S regulatory particle performs a set of functions: (1) it has to recognize and bind selectively the protein substrates prone to degradation; (2) these substrates have to be unfolded; (3) the ubiquitin chains have to be cleaved off the polyubiquitinated proteins; (4) the gates formed by the $\alpha$ subunits on each side of the 20S proteasome have to be opened; and (5) the unfolded substrates have to be driven into the proteolytic chamber of the 20S cylinder for degradation (Hilt and Wolf, 2004). The ATPase subunits would function as reverse chaperones that carry out ATP-dependent unfolding of proteins and their translocation into the proteolytic cavity of the proteasome. Isopeptidases in the 19S cap disassemble ubiquitin chains that can then be re-used. Ubiquitin binding and isopeptidase activities would be assigned to non-ATPase subunits (Deveraux et al., 1994; Lam et al., 1997). Most of the specific genes of
The proteolytic system: the degradation of intracellular proteins

Figure 1.2: **Structure of the 26S proteasome.** Polyubiquitinated substrates are recognized by the 19S cap of the 26S proteasome, which can be dissociated into a lid and a base component. 19S caps are composed of 11-12 Rpn and six Rpt (ATPase) subunits. Substrates are finally degraded by the proteolytic activity of the 20S proteasome core which is provided by three pairs of different $\beta$ subunits residing in the inner chamber of the proteasome cylinder.

In cells the life time of proteins changes a lot. While long-lived proteins have a half-life time of 200 hours, short-lived proteins are degraded within a few minutes or even seconds (Rechsteiner, 1987). The criteria which determine the half-life of a protein are still not well known. One of these signals is the presence of certain amino terminal residues which is known as the "N-end rule" (Bachmair et al., 1986). Sequence comparison of short-lived proteins revealed that most of them bear a degradation signal known as PEST sequence: polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) target proteins for rapid destruction (Rogers et al., 1986). Sequences termed "destruction boxes" form another degradation signal, having a big influence on the stability of cyclins, regulatory proteins of the cell cycle (Glotzer et al., 1991).

The ubiquitin-proteasome system is responsible for the degradation of most short-lived
proteins. Short-lived proteins are typically key regulatory proteins. Very importantly, also abnormal proteins which are malfolded are prone to rapid degradation. Short-lived proteins, designed for regulatory functions in the cell, bear signals for their degradation by the ubiquitin-proteasome-system. Misfolded proteins are most likely recognized by exposed hydrophobic patches.

One of the most important tags linked to proteins which are discovered for degradation is ubiquitin. Ubiquitin is a small protein composed of 76 amino acids. This protein is found only in eukaryotic organisms and is not found in either eubacteria or archaebacteria. Among eukaryotes, ubiquitin is highly conserved (Berg et al., 2003). For example, human and yeast ubiquitin share 96% sequence identity. This strong sequence conservation suggests that the vast majority of amino acids that make up ubiquitin are essential, since apparently any mutations that have occured over evolutionary history have been removed by natural selection. Ubiquitin is a heat-stable protein that folds up into a compact globular structure. It is found both as free monomer and covalently bound to itself and other proteins.

Ubiquitin controls protein turnover in a cell by closely regulating the degradation of selected proteins. Ubiquitin tagging of a protein is an ATP-dependent process and serves mostly as a tag that marks proteins for degradation. The degradation itself is carried out by the proteasome. Briefly, if a protein is to be degraded, it must be first conjugated with ubiquitin, then recognized and shuttled to the proteasome for degradation.

Three types of enzymes are required for the ubiquitin-proteasome pathway: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin-protein ligases (E3). First ubiquitin is activated with the aid of ATP through the formation of an energy-rich thiolester bond between the C-terminal glycine residue of ubiquitin (G76) and a cysteine residue of the E1 enzyme (in *S. cerevisiae* Uba1p) (McGrath et al., 1991). The activated ubiquitin is then transferred to the cysteine residue of an ubiquitin-conjugating E2 enzyme (in yeast: Ubc1p-Ubc8p, Ubc10p, Ubc11p and Ubc13p). In the following step an isopeptide bond is formed between ubiquitin and the ε-amino group of a lysine residue of the target protein. Further additions to internal lysine residues of ubiquitin extend the modification up to the formation of polyubiquitin chains which are required for the targeting of many proteasome substrates to the enzyme (Chau et al., 1989). Finally substrate specificity mostly requires an additional enzyme named E3 (ubiquitin ligase) which recognizes a motif in the substrate
protein and targets it for ubiquitinylation. It has been suggested that such motifs are called "degrons". By binding to the specific degradation signal of the target protein and to the E2 enzyme, a ternary E2-E3-substrate complex is formed. Ubiquitin is transferred to the target protein by an E3 enzyme either directly or in combination with an E2 enzyme.

Monoubiquitinylation is not sufficient as a degradation signal by the 26S proteasome. Four ubiquitins (Ub₄) linked through lysine Lys⁴⁸ appear to constitute the minimal structure that is effective in directing degradation (Thrower et al., 2000). However, substrates are most commonly marked by covalent attachment of multiple copies of ubiquitin (Pickart, 2001). Polyubiquitin chains may be of considerable length. Such chains confer to polyubiquitylated substrates the capacity to be recognized at sub-micromolar concentrations. Polyubiquitin chains can direct proteins to the proteasome or, if detached from substrates, can act as competitive inhibitors of the degradation of polyubiquitylated substrates (Amerik et al., 1997; Thrower et al., 2000). However ubiquitylation does not always lead to proteolysis. Non-degradative functions of ubiquitin have emerged in recent years, including the regulation of protein location, protein function and protein-protein interactions. Monoubiquitylation regulates for example the activity of proteins located at the plasma membrane (von Mikecz, 2006).

Ubiquitin ligases feature one of two structural elements: they are of the HECT domain or RING domain type (Jackson et al., 2000). In one catalytic class, a HECT ("Homologous to the E6-AP Carboxyl Terminus") domain transfers ubiquitin directly to substrate bound to a non-catalytic domain. It is a 350 residue region that contains a strictly conserved cysteine residue around 35 residues upstream of the C-terminus. This residue acts as a site of thiolester formation with ubiquitin (Huibregtse et al., 1995).

Members of the other catalytic class use a RING ("Really Interesting New Gene") finger domain to facilitate ubiquitylation. It is a protein sequence with numerous histidine and cysteine residues and two zinc ions coordinated in between. The RING-finger is a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, and is probably involved in mediating protein-protein interactions. There are two different variants, the C3HC4-type and a C3H2C3-type. They are clearly related despite the different cysteine/histidine pattern. The latter type is sometimes referred to as "RING-H2 finger". Several 3D-structures for RING-fingers are known. The 3D-structure of the zinc ligation system is unique to the RING domain and is referred to as the "cross-brace" motif. The spacing of the cysteines in such a domain is
C-X(2)-C-X(9 to 39)-C-X(1 to 3)-H-X(2 to 3)-C-X(4 to 48)-C-X(2)-C-X(2) (X: any kind of residues).

More recently however, U-box proteins containing a U-box domain of about 70 amino acids have been identified as a new type of E3. They are conserved from yeasts to humans. Recent experiments have pointed out another class of enzymes involved in ubiquitin conjugation called E4 enzymes. These E4 enzymes take part in the formation and extension of the ubiquitin chain (Koegl et al., 1999).

The delivery of polyubiquitinated proteins to the proteasome occurs via binding of the polyubiquitin chain to specific adaptors on the 26S proteasome or via receptor proteins which themselves bind to 26S proteasome subunits (Elsasser and Finley, 2005).

After being channeled via this central passageway into the degradation chamber of the proteasome, proteins are degraded into peptides and finally released. Proteasomes cleave their substrates to 3 to 20 residue peptides which are further degraded into single amino acids by downstream endo- and amino-peptidases (von Mikecz, 2006).

Like most biochemical reactions, ubiquitinylation is a reversible process. Deubiquitylation is catalysed by specialised proteases called deubiquitinating or DUB enzymes (Wilkinson and Hochstrasser, 1997). These proteases hydrolyze the amide bond between Gly of ubiquitin and the substrate protein or the amide bond between the ubiquitin moieties of the polyubiquitin chain. One of the two known classes of DUB enzymes is the so-called ubiquitin-specific processing protease or UBP class. All members of this class contain several short consensus sequences, the Cys and His boxes, that are likely to form part of the active site, and to be critical for catalytic activity (Baker et al., 1992). The disassembly of free polyubiquitin chains by yeast Ubp14 modulates for example rates of protein degradation by the proteasome (Amerik et al., 1997). Un cleaved ubiquitin chains due to the lack of Ubp14 were recently showed not to affect the degradation of polyubiquitinated proteins in general but inhibit the degradation of only certain proteins and not others, by this showing “pathway specificity” (Eisele et al., 2006).

Figure 1.3 summarizes the ubiquitin-proteasome pathway.
Figure 1.3: **The ubiquitinylation pathway.** Free ubiquitin (Ub) is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl-terminus of ubiquitin. Ubiquitin is transferred to one of a number of different E2s. E2s associate with E3s which may have a substrate already bound. For HECT domain E3s (pathway b), ubiquitin is next transferred to the active site cysteine of the HECT domain followed by transfer to the substrate or to the substrate-bound multibiquitin chain. For RING E3s (pathway a), current evidence indicates that ubiquitin is transferred directly from E2 to the substrate. (Wolf and Hilt, 2004)
1.2.4 The nuclear ubiquitin-proteasome system

Proteasomes are found in the cytoplasm and in the nucleus of the cell. Whereas mammalian proteasomes were reported to be equally distributed all over the cyto- and nucleoplasm, in yeast about 80% of proteasomal complexes were found to reside within the nucleus and the nuclear envelope (NE) (McDonald and Byers, 1997; Russell et al., 1999). Nuclear localization of proteasomes in higher eukaryotic cells was indeed reported to greatly depend on cell type, cell density and growth conditions. The relative proportion of nuclear versus cytoplasmic proteasomes could vary between 17% and 50% (von Mikecz, 2006). A significant accumulation of active core and regulatory complexes of the yeast 26S proteasome was however shown mainly in the nuclear envelope-endoplasmic reticulum (NE-ER) network, and this was shown to be independent of the cell cycle. Moreover a major part of proteasomal proteolysis was shown to take place in the NE-ER network in yeast (Enenkel et al., 1998), suggesting that in yeast the subcellular distribution of proteasomes would implicate a major location of protein degradation in the NE-ER network.

Several subunits of the 20S proteasome have nuclear localization signals and tyrosine phosphorylation of these may play a role in nucleocytoplasmic transfer of proteasomes (von Mikecz, 2006). In yeast, 20S proteasomes and the 19S regulator seem to be imported into the nucleus as inactive precursor complexes by karyopherins α β (Wendler et al., 2004; Lehmann et al., 2002) indicating the nucleus/ER to be the major subcellular compartment of both proteasomal proteolysis and proteasome biogenesis.

In contrast to nuclear import there is no experimental evidence for export of proteasomes or proteasomal subunits from the cell nucleus to the cytoplasm. These might be therefore unidirectional from the cytoplasm to the nucleus during interphase, and the cytoplasmic and nuclear proteasome pools could then reequilibrate during mitosis (von Mikecz, 2006). A maturation pathway, independent of nucleocytoplasmic transport, would also be conceivable for cytoplasmic and ER-associated 20S proteasomes. A sorting mechanism may exist by which proteasome precursor complexes are allowed to be matured outside the nucleus. In mammals, posttranslational modifications were proposed to determine the destination of proteasomes to the cytoplasm or nucleoplasm (Tanaka et al., 1990).

The nucleus also contains other components of the ubiquitin-proteasome system, such as ubiquitin, the regulatory subunit PA28, the 19S regulatory complex, the proteasome activator...
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Blm10, the ubiquitin-specific protease HAUSP and E1-E3 enzymes. These may be regulated by changes in their intracellular distribution. Up to now, the nuclear UPS (ubiquitin-proteasome system) has been reported to have a role in DNA repair but also in gene expression (Muratani and Tansey, 2003).

Proteasomes are generally known to serve as a quality-control system that rapidly eliminates 30% of the proteins that are misfolded or damaged and would interfere with normal cell function, if allowed to accumulate (Schubert et al., 2000; von Mikecz, 2006). One well-known quality-control system has been described at the endoplasmic reticulum membrane (ERAD) (see subsection 3.4.3 page 41). In this regard, Gardner et al. have further identified a nuclear quality-control system in yeast, involving the ubiquitin ligase San1 (Gardner et al., 2005). A dysregulation of this tuned balance of ubiquitylation and proteasome-dependent protein degradation of nuclear proteins leads to the development of diseases such as polyQ disorders and other neurodegenerative conditions (von Mikecz, 2006).
Chapter 2

Catabolite inactivation and degradation of the enzyme fructose-1,6-bisphosphatase

2.1 Fructose-1,6-bisphosphatase in gluconeogenesis

All living organisms have to solve the same problems: how to find in their environment the required elements to grow, maintain their cellular structure, and above all how to produce the required energy for life. Therefore most organisms degrade organic molecules found in their environment and metabolize them to produce energy. One of the major steps bringing a great part of energy directly usable by cells is the metabolism of glucose via glycolysis (from greek glyk- ”sweet” and lysis ”dissolution”). Glycolysis is probably one of the first metabolic pathways developed during evolution. Almost all living cells possess the enzymes necessary to transform glucose to pyruvate. Some variations exist in each organism, but reactions are the same in animals, plants, fungi and a great number of bacteria (Berg et al., 2003). Even more importantly glucose provides building blocks required for the life of a cell. Among those are dihydroxyacetonephosphate for the generation of lipids and fat, ribose for nucleotide synthesis or pyruvate for replenishing the citric acid cycle with oxalacetate. This central role of glucose in metabolism requires its permanent presence.

Organisms always have to face constant environmental changes like for example changes
in the supply of nutrients. Because of the central role of glucose in metabolism, the cell has to ensure a permanent presence of this sugar. This is guaranteed by the anabolic pathway of gluconeogenesis, a reaction chain which in most parts reverses the glycolytic reactions.

Metabolism, a complex system of anabolic and catabolite reactions, is tightly regulated (Berg et al., 2003). Availability of substrates is an important criterium that influences the regulation of the metabolism. The amount of enzymes varies according to their synthesis and degradation rates. Besides their allosteric interactions some enzymes are controlled by feedback inhibition, reversible covalent modification (for example phosphorylation at a serine, tyrosine or threonine residue) or by proteolytic activation. As a result of signal transduction, cyclic AMP and calcium ions can bind to specific proteins, thereby yielding eventually enzyme activation or repression (Berg et al., 2003).

In carbohydrate metabolism in yeast gluconeogenesis and glycolysis are under reciprocal regulation, so that one pathway does not take place while the other is active. Gluconeogenesis refers to the synthesis of glucose from non-glucidic precursors, the so-called non-fermentable carbon sources, and is a sort of reverse pathway of glycolysis with the exception that for thermodynamic and regulatory reasons three enzymes (fructose-1,6-bisphosphatase, FBPase; phosphoenolpyruvate carboxykinase, PepCK and cytoplasmic malate dehydrogenase) of crucial steps of glycolysis and gluconeogenesis differ. In glycolysis, phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate is catalysed by phosphofructokinase. The reverse reaction providing fructose-6-phosphate from fructose-1,6-bisphosphate in gluconeogenesis is carried out by fructose-1,6-bisphosphatase. Most importantly, by using two different enzymes for phosphorylation and dephosphorylation, the cell has the ability to tightly regulate both pathways.

### 2.2 Catabolite inactivation of fructose-1,6-bisphosphatase

When yeast cells are grown on a non-fermentable carbon source like ethanol, fructose-1,6-bisphosphatase is synthesized and has a half-life of around 90 hours (Funayama et al., 1980). Shifting these cells subsequently to a glucose-containing medium leads to a rapid switch from gluconeogenesis to glycolysis and fructose-1,6-bisphosphatase is then rapidly inactivated.
2.3 Catabolite degradation of fructose-1,6-bisphosphatase

(Mazón et al., 1982; Funayama et al., 1980; Müller and Holzer, 1981; Schork et al., 1995; Hämmerle et al., 1998). This rapid inactivation occurs at two different levels: first at a pre-transcriptional level, i.e. a glucose-dependent transcriptional repression of enzyme synthesis; second at a post-transcriptional level, where the activity of the enzymes is stopped through phosphorylation at serine 11. This phosphorylation of FBPase is reversible for 5-10 minutes and leads to a loss of enzyme activity of around 50 % through a shift of the pH-optimum to pH 8.8. Finally, once inactivated, the enzyme is degraded with a half-life of around 20-30 minutes. Such a process is called catabolite inactivation and catabolite degradation and is one of the main steps of the gluconeogenesis/glycolysis shift (Gancedo, 1971; Holzer, 1976). At the same time, complete inactivation of FBPase avoids a "futile cycle" of ATP consumption.

2.3 Catabolite degradation of fructose-1,6-bisphosphatase

Until now the mechanism and the site of the catabolite degradation of FBPase in yeast has been controversial (Schork et al., 1994a,b). Two FBPase degradation pathways have been proposed: the one studied in our laboratory depends on the cytosolic ubiquitin-proteasome machinery; the other studied in the laboratory of H.L. Chiang, Hershey, USA, depends on vacuolar proteolysis (Schork et al., 1994b).

The model described by our laboratory proposes an ubiquitin-proteasome degradation pathway of FBPase. The breakdown of FBPase depends on polyubiquitination and the activity of the cytosolic 26S proteasome. This linkage of polyubiquitin chains marks this protein for degradation via the cytoplasmic- and nuclear-located proteolytic nanocompartment, the proteasome. It has been shown by our laboratory that FBPase is polyubiquitinated by the ubiquitin-conjugating enzymes Ubc1p, Ubc4p, Ubc5p and Ubc8p (Schork et al., 1995; Schüle et al., 2000). By overexpression of a mutant form of ubiquitin (exchange of Lys48 against Arginine), the polyubiquitination of FBPase is affected and leads to a decrease in the proteolysis rate of the enzyme (Schork et al., 1995). In mutants with defects in the 19S-"caps" or in the active proteolytic site of the 20S proteasome, the half-life time of FBPase significantly increases.
Recently a gene-deletion library of yeast has been screened for stabilization of FBPase upon glucose-shift. Nine novel \textit{GID} genes required for catabolite degradation of FBPase have been identified (Schüle et al., 2000; Regelmann et al., 2003): \textit{GID1/VID30}, \textit{GID2}, \textit{GID3} encoding Ubc8p, \textit{GID4/VID24}, \textit{GID5/VID28}, \textit{GID6}, \textit{GID7}, \textit{GID8} and \textit{GID9}. Seven of those Gid proteins, Gid1, Gid2, Gid4, Gid5, Gid7, Gid8 and Gid9, were shown to form a complex which binds FBPase. One of the subunits, Gid2/Rmd5, contains a degenerated RING finger domain. In an \textit{in vitro} assay, heterologous expression of GST-Gid2 led to polyubiquitination of proteins. In addition, a mutation in the degenerated RING domain of Gid2/Rmd5 abolishes fructose-1,6-bisphosphatase polyubiquitination and elimination \textit{in vivo}. Six Gid proteins are present in gluconeogenic cells. A seventh protein, Gid4/Vid24, appears upon glucose addition to gluconeogenic cells and is afterwards eliminated. Forcing abnormal expression of Gid4/Vid24 in gluconeogenic cells leads to fructose-1,6-bisphosphatase degradation, suggesting that Gid4/Vid24 initiates fructose-1,6-bisphosphatase polyubiquitination by the Gid complex and its subsequent elimination by the proteasome. An additional gluconeogenic enzyme, phosphoenolpyruvate carboxykinase, has also been shown to be subject to Gid complex dependent degradation. The Gid complex is thus a new type of ubiquitin ligase involved in carbohydrate metabolism. The subunit Gid4/Vid24 of the Gid complex has been identified as a major regulator of this E3 (Santt et al., 2008; Santt, 2009).

In the other model described by Hui-Ling Chiang’s group, FBPase is targeted from the cytosol via small vesicles with a diameter of 30-40 nm to the vacuole for degradation (Chiang et al., 1996). This vacuolar mechanism is observed in an \textit{in vitro} system and is dependent on so-called Vid-proteins (Vacuolar Import and Degradation), among them Vid24/Gid4, Vid30/Gid1 and Vid28/Gid5 (Hoffman and Chiang, 1996; Chiang and Chiang, 1998; Brown et al., 2001, 2002). Vid24 would play a critical role in delivering FBPase from the vesicles to the vacuole for degradation (Chiang and Chiang, 1998). Until now, these results were not reproducible in our hands. Different inactivation protocols may explain the different results. Vacuolar FBPase degradation was obtained by starving yeast cells more than 48 hours in media containing acetate. In proteasome-dependent degradation of FBPase, yeast cells were grown overnight in ethanol-containing medium before glucose addition. Recently however, the Chiang group was able to corroborate our findings of ubiquitin-proteasome triggered catabolite degradation of FBPase (Hung et al., 2004).
Chapter 3

The Cdc48 complex

3.1 Structure of the Cdc48 complex

Cdc48, also known as p97 or VCP in mammals, belongs to the family of AAA-ATPases (ATPases associated with various cellular activities). This is a highly conserved, essential, chaperone-related protein of eukaryotic cells which has nowadays attracted attention because of its numerous roles in cellular functions, from ubiquitin-dependent protein degradation and processing to fusion of homotypic membranes, nuclear envelope reassembly or cell cycle progression. Although primarily identified for its requirement for progression through cell division cycle (as its yeast name indicates), Cdc48 was discovered to be linked to the ubiquitin pathway, being necessary for the degradation of some ubiquitinated proteins. Although Cdc48/p97 is able to bind to non-ubiquitylated proteins, it appears to act primarily on ubiquitylated substrates in vivo.

Cdc48 is a ring-shaped complex of six identical subunits that consist of two consecutive ATPase domains called D1 and D2, and an amino-terminal N-domain, which is mainly responsible for cofactor and substrate binding (Figure 3.1 page 34, (Zhang et al., 2000; Rouiller et al., 2002; Beuron et al., 2003; Davies et al., 2005)).

Most functions of Cdc48 seem to be directly linked to the ability of the protein to bind (oligo)ubiquitinated proteins and to segregate them from their binding partners or to extract them from protein complexes. This ”segregase” activity is mediated by the ATPase activity of Cdc48 which translates ATP hydrolysis into mechanical force able to move and partially rotate the outside rim of the ring-shaped enzyme. Through its N-terminal domain Cdc48 may directly
Figure 3.1: **The Cdc48 homohexameric complex.** Each subunit of Cdc48 possesses an N-domain and two consecutive AAA ATPase domains, D1 and D2. The substrate-recruiting cofactor Ufd1-Npl4 and the deubiquitination enzyme Otu1 bind to the N-domain. The polyubiquitination enzyme Ufd2 binds to the D2 domain.
bind ubiquitinated substrates. But Cdc48 mostly requires "substrate-recruiting cofactors" to indirectly bind ubiquitinated substrates. These "substrate-recruiting cofactors" indeed possess some ubiquitin-binding domains and would interact with Cdc48 by its N-domain (Jentsch and Rumpf, 2007). Besides its "segregase" activity Cdc48 was also shown to control the degree of ubiquitination of the bound substrates. This latter activity brought about by "substrate-processing cofactors" that either promote polyubiquitination, inhibit polyubiquitination or even deubiquitinate the bound ubiquitinated substrate, led to the proposal that Cdc48 might function similarly to a gearbox in a car and might control protein fate using different types of cofactors.

3.2 Different kinds of cofactors required for the activity of Cdc48

The cofactors of Cdc48 can be grouped into two classes: the substrate-recruiting cofactors and the substrate-processing cofactors.

3.2.1 Substrate-recruiting cofactors of Cdc48

Although able to directly interact with unfolded proteins and with ubiquitin in vitro, in vivo Cdc48 requires substrate-recruiting cofactors to exert its function. "Substrate-recruiting cofactors" include on the one hand the heterodimeric Ufd1-Npl4 complex, on the other hand the Ubx-protein family (Ubx1 to Ubx7), the most famous one being Shp1 (or Ubx1) (Jentsch and Rumpf, 2007).

The Ufd1-Npl4 complex

Ufd1 possesses a N-domain which resembles the N-domain of Cdc48. Like Ubx proteins, the Npl4 subunit of the Ufd1-Npl4 heterodimeric complex possesses an amino-terminal domain that interacts with the N-domain of Cdc48 but that miss the R...FPR motif of UBX. Such domains are classified as "UBX-like" domains.
The Ubx-protein family

Ubx proteins possess a so-called UBX domain that binds to the N-terminal domain of Cdc48, and a UBA (ubiquitin-associated) domain that binds to the ubiquitylated substrate. They thus act as adaptors for Cdc48 that regulate interactions with ubiquitylated substrates (Schuberth and Buchberger, 2008). Until now 7 Ubx proteins are known, Ubx1 to Ubx7.

The UBX domain of about 80 amino acid residues turns out to be a close structural homologue of ubiquitin itself. However the carboxy-terminal diglycine motif found in ubiquitin is absent in the UBX domain, indicating that UBX domains are not covalently bound to target proteins in an ubiquitin-like manner. Moreover structure-based alignments of some UBX domains revealed a highly conserved R...FPR surface patch which is absent from ubiquitin and ubiquitin-like proteins. This conserved surface patch found to be the major binding site of the UBX domain binds to a hydrophobic pocket between the two subdomains of the Cdc48 N-domain.

Substrate-recruiting cofactors typically associate with Cdc48 through the N-domain of the AAA-ATPase. Not all substrate-recruiting cofactors however associate with Cdc48 in a mutually exclusive manner: for example, Cdc48 can bind to Ufd1-Npl4 complex and Ubx2 simultaneously (Jentsch and Rumpf, 2007).

Substrate-recruiting cofactors can also be subdivided into major substrate-recruiting cofactors like Shp1/Ubx1 or the Ufd1-Npl4 complex, and proteins acting as coadaptors like Ubx2 for the Cdc48^{Ufd1-Npl4} complex in ERAD. The importance of major substrate-recruiting cofactors lies in the separation of fundamentally distinct cellular functions of Cdc48. While Cdc48^{Shp1} complex controls the fusion of homotypic membranes, the Cdc48^{Ufd1-Npl4} complex is required for ubiquitin-dependent protein degradation as well as for reformation of nuclear envelope double-membrane structure after mitosis. Such distinct functions of Cdc48 are possible through steric hindrance, as stable binding of Ufd1-Npl4 to any two N domains would leave no space for the binding of Shp1. Besides the steric hindrance, Ufd1-Npl4 and Shp1 would induce different conformational changes of Cdc48 that might prevent the binding of the respective other major substrate-recruiting cofactor to Cdc48 (Schuberth and Buchberger, 2008).
3.2 Different kinds of cofactors required for the activity of Cdc48

3.2.2 Substrate-processing cofactors of Cdc48

Some "substrate-processing cofactors" directly influence the degree of ubiquitination of substrates whereas others modify the bound substrates in another way.

Mono- or oligo-ubiquitinated substrates are recruited to the Cdc48 hexameric complex (Figure 3.2 page 37).

![Figure 3.2: The gearbox function of Cdc48](image)

Mono- or oligo-ubiquitinated substrates (brown; ubiquitin, red) are recruited to the Cdc48 (p97) gearbox (gray). In the position "forward" (F) the E4 enzyme Ufd2 polyubiquitinates the substrate, thereby promoting proteasomal degradation. In "neutral" (N), the WD-40 protein Ufd3 competes with Ufd2 for Cdc48 binding, thereby preventing further ubiquitination of the substrate by Ufd2. In the position "reverse" (R), the deubiquitination enzyme Otu1 removes the ubiquitin modification of the substrate. Substrates released from Cdc48 through the "N" and "R" positions of the gearbox are either mono- (oligo-)ubiquitinated or unmodified and thus metabolically stable. (Jentsch and Rumpf, 2007)

The E4 enzyme Ufd2 polyubiquitinates the substrate, thereby promoting proteasomal
degradation. This U-box domain-containing protein binds to the second AAA domain of Cdc48 through the Ufd1-Npl4 complex. Besides promoting degradation of oligoubiquitylated Cdc48\(^{Ufd1-Npl4}\) substrates, Ufd2 also provides a physical link to the 26S proteasome via the proteasomal receptors Rad23 and Dsk2 forming a ternary complex (Richly et al., 2005).

The WD-40 protein Ufd3 competes with Ufd2 for Cdc48 binding, thereby preventing further polyubiquitination of the substrate by Ufd2: indeed, both factors utilize the same docking site on Cdc48 (Rumpf and Jentsch, 2006). Recently, Mullally et al. (2006) found that Ufd3/Doa1 possessed a novel ubiquitin binding domain named the PFU (PLAA family ubiquitin binding domain) domain. Interestingly in an \(ufd3\Delta\) strain a depletion of mono- and polyubiquitin was observed (Ghislain et al., 1996). Ufd3 would recruit Cdc48 to ubiquitin through its PUL C-terminal domain while its PFU domain would bind ubiquitinated substrates, and this without requirement of ATP (Mullally et al., 2006).

The deubiquitination enzyme Otu1 removes the ubiquitin modification of the substrate. Interestingly Cdc48 can bind Otu1 and Ufd3 simultaneously, making a cooperation of both inhibitory mechanisms possible. Otu1 like Ubx proteins and the Npl4 subunit of the Ufd1-Npl4 heterodimeric complex, also possesses a so-called "UBX-like" domain. Otu1 was found to bind polyubiquitin chain analogs more tightly than monoubiquitin, and to preferentially hydrolyze longer polyubiquitin chains with Lys\(^{48}\) linkages (Messick et al., 2008).

The Cdc48 complex may thus play a key role in the ubiquitin pathway as a central regulator, switching on either multiubiquitylation and degradation of bound substrates or catalysing their stabilisation and/or deubiquitylation (Rumpf and Jentsch, 2006).

### 3.3 Delivery of ubiquitylated substrates from Cdc48 to the proteasome

Once a multiubiquitin chain is attached to a substrate, it must be shielded from deubiquitylating enzymes for the 26S proteasome to recognize it. Some Ub-binding proteins likely exist to deliver ubiquitylated substrates to the proteasome. Dsk2 and Rad23 are two candidates, belonging to the family of proteins that preferentially binds ubiquitylated substrates and multi-Ub chains through a motif called the ubiquitin-associated domain (UBA), probably through the help of the substrate-processing cofactor Ufd2 (Richly et al., 2005). Rad23 contains a
Ub-like domain (UBL) at its N-terminus responsible for its interaction with the proteasome and two ubiquitin-associated domains (UBA), whereas Dsk2 has a UBL but only one UBA domain.

Besides Rad23 and Dsk2, Ddi1 was also reported as UBL-UBA ubiquitin receptors that mediate proteasomal degradation of ubiquitylated cargoes. As reported by Saeki et al., Ddi1 has the capacity to bind polyubiquitinated substrates, although to a lesser extent than Rad23 and Dsk2, and has a weaker affinity toward the 26S proteasome than Rad23 and Dsk2 (Saeki et al., 2002). Like Dsk2, Ddi1 has only one UBA domain, whereas Rad23 has two. Interestingly, Rad23 forms heterodimers with Ddi1 or Dsk2, and Ddi1 binding to Rad23 is mediated by interactions between their UBL and UBA domains (Rao and Sastry, 2002). On the one hand, Rad23 and Dsk2 homodimerize through their UBA domains, the other UBA domain of Rad23 binding to the polyubiquitylated substrate and the two UBL domains of Rad23 and Dsk2 binding to the proteasome. On the other hand, Ddi1 requires neither the UBA nor the UBL domains for dimerization. Moreover K48-linked tetraubiquitin can interact simultaneously with the UBA domains of Rad23 and Ddi1, although the Rad23-Ddi1 heterodimer has to undergo dissociation before tetraubiquitin binding (Kang et al., 2006). The UBL-UBA ubiquitin receptors are thus thought to prevent unnecessary ubiquitin-chain elongation or deubiquitination during the transit of substrates to the proteasome (Kang et al., 2006).

Two subunits of the proteasome are able to bind polyubiquitinated substrates, Rpn10 and Rpt5. The precise function of Rpt5, a member of the ATPase ring in the 19S base, in the recognition process is not known yet (Wolf and Hilt, 2004).

Rad23 was recently found to stimulate the interaction between ubiquitinated proteins and the proteasome, maybe through Rpn10, a proteasome-associated multi-Ub chain binding protein. However, because Rad23 can interact with the proteasome in rpn10Δ cells, and because Rpn10 is not essential for the degradation of many proteins, it is likely that Rpn10 does not serve as the major recognition subunit of polyubiquitinated substrates, and that Rad23 can bind multiple subunits in the proteasome (Chen and Madura, 2002).

If Rad23 does not stimulate the interaction between ubiquitinated substrates and the proteasome through the Rpn10 subunit, it may do it through the Rpn1 subunit of the proteasome. The UBL domain of yeast proteins was indeed referred to bind Rpn1 (Elsasser et al., 2004; Saeki et al., 2002). Rpn1 which directly binds to Rad23, was also found to directly compete
with Ufd2 for Rad23-association. The UBL domain of Rad23 would then have to be freed from Ufd2 to further bind to the Rpn1 proteasomal subunit (Kim et al., 2004).

Quite recently, a new ubiquitin receptor for the proteasome, Rpn13, has been discovered. Interestingly, it is unrelated to Rpn10 and the 3 UBL/UBA proteins, and differs dramatically from other proteasomal ubiquitin receptors. First, Rpn13 is docked into the proteasome through a surface that, contrary to Rpn10, is in close spatial proximity to its ubiquitin-binding region. Second, it is not so well equipped than UBL/UBA proteins to capture ubiquitinated substrates. Third, in striking contrast to Rad23, Dsk2 and Ddi1, Rpn13 promotes chain deubiquitination. Fourth, it binds definitely better to monoubiquitin and diubiquitin than other ubiquitin receptors associated with proteasome-mediated degradation (Koraljka et al., 2008).

### 3.4 Diverse functions of Cdc48

#### 3.4.1 The UFD pathway

Most knowledge of the function of Cdc48 and its cofactors derives from studies of different cellular pathways. Several of the components involved in these pathways have been initially identified by genetic and biochemical dissection of the so-called UFD pathway. This "ubiquitin-fusion degradation" pathway mediates the degradation of short-lived synthetic linear ubiquitin-fusion proteins by the proteasome. Among those "UFD proteins" are Cdc48 itself, the substrate-recruiting cofactors Ufd1 and Npl4, and the substrate-processing cofactors Ufd2 and Ufd3 (Ghislain et al., 1996; Koepl et al., 1999). Other proteins like Ufd4 and Ufd5 have also been identified (Johnson et al., 1995). Substrates like Ub-P-βgal or Ub$^{V76-V}$-βgal were fusion proteins bearing a "non-removable” N-terminal ubiquitin (Ub) moiety. The fusion’s Ub was shown as a degradation signal (Johnson et al., 1992), therefore leading to short-lived synthetic proteins in vivo. It has to be noted that at least the initial steps of the UFD pathway are distinct from those of the N-end rule pathway: mutational elimination of N-recognin, the recognition component of the latter pathway, abolishes the degradation of N-end rule substrates but does not impair the degradation of Ub fusions such as Ub$^{V76-V}$-βgal (Johnson et al., 1992).
3.4 Diverse functions of Cdc48

3.4.2 The OLE pathway

The OLE pathway controls in yeast the synthesis of unsaturated fatty acids. Ole1, an integral membrane-bound protein, provides yeast cells with unsaturated fatty acids which are crucial for membrane fluidity and essential for viability. Transcription of OLE1 is driven by Spt23. Spt23 first needs to homodimerize through its IPT domain to be further processed. After SPT23 processing, the processed p90 remains tightly associated with a membrane-bound p120 partner molecule. The Cdc48\(Ufd1-Npl4\) complex, an ubiquitin-specific chaperone, is only then needed to disassemble the homodimer Spt23 and remove p90 from its unprocessed partner (Rape et al., 2001). Away from the ER, Spt23 p90 can enter the nucleus and activate OLE1. After activation, p90 is degraded in the nucleus, also through Cdc48, plus the proteins Ufd2 and Rad23 (Jentsch and Rumpf, 2007). More precisely, Spt23 p90 breakdown would be mediated by two alternative pathways. One pathway depends on Ufd2-catalysed p90 multiubiquitylation (in collaboration with E1, E2 and the E3 ligase Rsp5), followed by Rad23/Dsk2 binding and subsequent proteasomal degradation. The alternative route seems to depend on Rsp5 as well, yet without a requirement for Ufd2, and it involves Rpn10 (Richly et al., 2005).

3.4.3 The ERAD pathway

ERAD is an ubiquitin-proteasome dependent degradation process of misfolded ER luminal and membrane proteins (CPY* substrates) (Kostova and Wolf, 2003; Hiller et al., 1996) and of proteins of the ER (the OLE1 protein for example) for regulatory purposes. In this pathway Cdc48 is necessary to retrotranslocate substrates to the cytosol or extract luminal and ER-membrane-bound substrates from the ER membrane (Rabinovich et al., 2002; Ye et al., 2001; Jarosch et al., 2002; Bays et al., 2001; Braun et al., 2002). Ubiquitylated ERAD substrates, after being recognized by specific E2s and E3s, are directly translocated from a channel containing the SEC61 translocon into the proteasome by the joint forces of 2 types of hexameric AAA-ATPases, the Cdc48 chaperone and the 6 Rpt subunits of the 19S proteasome, and finally degraded (Braun et al., 2002).

Two types of ERAD pathways are known: the ERAD-C and the ERAD-L pathways. These pathways differ in respect to the site of the misfolded lesion. Membrane and soluble proteins with luminal lesions are targeted to the ERAD-L pathway, whereas membrane proteins with misfolded cytoplasmic domains use the ERAD-C pathway. The ERAD-C pathway defined by
the Doa10 complex requires the Doa10 ER-associated ligase, an E2 complex (Ubc7 anchored to the membrane by the factor Cue1/Ubx4, and Ubc6) and the Cdc48 complex (the AAA-ATPase Cdc48p, its cofactors Ufd1 and Npl4, and its membrane anchor protein Ubx2) (Figure 3.3). The ERAD-L pathway defined by the Hrd-Der complex is more complicated. In this case, the E3 ligase is the Hrd1/Der3-Hrd3 complex, Hrd3 being an ERAD protein with a large ER luminal domain (Figure 3.3). The Ubc7-Cue1/Ubx4 E2 complex and the Cdc48 complex, found in the ERAD-C pathway, are also found in the ERAD-L pathway. Very recently, Ubx4 was found to modulate Cdc48 activity and to influence degradation of misfolded proteins of the endoplasmic reticulum (Alberts et al., 2009). However, additional factors are unique to the Hrd1/Der3-Hrd3 complex: Der1, Yos9, Kar2 and Usa1. Der1 is a small protein that spans the ER membrane four times. Usa1 composed of a large cytosolic domain and two transmembrane segments, constitutes a linker protein between Der1 and Hrd1 (Carvalho et al., 2006). Yos9 is a ER-luminal lectin-like protein that specifically binds misfolded glycoproteins in ERAD. It may function as a substrate receptor able to discriminate between folded and misfolded proteins due to differently processed protein linked carbohydrate (Buschhorn et al., 2004). The ER-luminal Hsp70 chaperone Kar2 (also known as BiP) binds substrates and keeps them soluble for ERAD (Plemper and Wolf, 1999). It associates with Yos9 independently of its peptide binding activity. All three proteins Kar2, Yos9 and Hrd3 can bind misfolded proteins independently.

Like in the OLE1 pathway the downstream-acting factors Ufd2, Rad23 and Dsk2 also contribute to ERAD pathways (Richly et al., 2005; Medicherla et al., 2004). This machinery involves Ufd2, the Ufd1-Npl4 heterodimer, Rad23 or Dsk2, but also Ubx2. Ubx2 is an ERAD specific cofactor that possesses two transmembrane domains and binds not only to Cdc48 and ubiquitinated substrates but also to the dedicated E3 ubiquitin ligases.
Figure 3.3: **Two distinct membrane protein complexes define ERAD pathways.** (Top) The Doa10p complex of the ERAD-C pathway. (Bottom) The Hrd1p complex of the ERAD-L pathway. Yos9 recognizes sugar modifications on misfolded proteins. In both panels, substrates are represented in gray with lesion sites marked by stars. The figure is an adaptation of the models presented in Carvalho *et al.*, (2006) and Denic *et al.*, (2006).
Chapter 4

Objectives

Fructose-1,6-bisphosphatase is a key enzyme of gluconeogenesis. Upon shift of cells to glycolytic conditions, the enzyme is rapidly regulated at different levels: FBPase inhibition by fructose-2,6-bisphosphate and AMP, inactivation of the enzyme upon phosphorylation and a last irreversible step, FBPase ubiquitination and further degradation by the 26S proteasome. This tight regulation of FBPase thus prevents a futile cycle of ATP hydrolysis. The so-called catabolite inactivation and degradation of fructose-1,6-bisphosphatase in *Saccharomyces cerevisiae* represents an appropriate model system to study metabolic regulation of the carbohydrate metabolism and the selective degradation of proteins.

Through a genome wide screen for components involved in the catabolite degradation of FBPase, nine *GID* (Glucose Induced degradation Deficient) genes were identified. Among these 9 Gid proteins, seven (Gid1, Gid2, Gid4, Gid5, Gid7, Gid8 and Gid9) form a Gid complex recently discovered as a novel ubiquitin ligase the activity of which is carried out by the subunit Gid2. Gid4, the only Gid protein not present under gluconeogenic conditions, was pointed out to be the switch for FBPase polyubiquitination and further degradation.

Some of these Gid proteins have been reported to be localised in the cytosol and the nucleus of cells. Moreover mammalian muscle and liver FBPases were described to be located in the cell nucleus as well, suggesting a putative nuclear degradation of yeast FBPase. To clarify the subcellular localisations of the Gid proteins, of FBPase and of its degradation, biochemical cell fractionation experiments and fluorescence microscopy analyses had to be carried out.

Extensive sequence analysis unravelled the existence of a nuclear localisation sequence (NLS) in Gid2 and of a CRA domain of unknown function in Gid1 and Gid8. Further work
thus consisted on one hand in the study of this NLS sequence and in its role in FBPase degradation, on the other hand in the determination of the function of this CRA domain in the protein Gid8. Deletion of the NLS sequence in Gid2 and point mutations in the CRA domain of Gid8 had thus to be done. Additional co-immunoprecipitation experiments between the Gid proteins, and between FBPase and the CRA-mutated Gid8, fluorescence microscopy studies as well as ubiquitination assays should shed light onto the function of these domains in polyubiquitination and degradation process of fructose-1,6-bisphosphatase.

Another part of this work was devoted to the clarification of the transport mechanism of FBPase to the 26S proteasome through the identification of novel FBPase interaction partners like members of the Cdc48\textsuperscript{Ufd1−Npl4} machinery. The exact function of this complex in the proteasomal FBPase degradation pathway had to be further elucidated. Pulse chase analyses, ubiquitination assays and co-immunoprecipitation experiments should help to do so.
Part III

Results
Chapter 1

Studies on the Gid complex

In a recent genome wide screen, several genes were discovered to take part in FBPase degradation. In addition to the previously identified \textit{GID1}/\textit{VID30}, \textit{GID2}/\textit{RMD5} and \textit{GID3} genes, six other so-called \textit{GID} (\textit{Glucose} \textit{Induced} degradation \textit{Deficient}) genes termed \textit{GID4} to \textit{GID9} have been discovered. The deletion of one of these genes is enough to lead to a notably slower degradation of FBPase by the 26S proteasome (Regelmann et al., 2003). Gid3/Ubc8 works as an ubiquitin-conjugating enzyme (Schüle et al., 2000) while Gid6/Ubp14 was characterized as a deubiquitinating enzyme (Amerik et al., 1997). Recently the Gid complex was discovered as an ubiquitin ligase (E3) which activity is provided by the Gid2/Rmd5 subunit (Santt et al., 2008). However functions of other Gid proteins in FBPase degradation still remains unknown. It was thus crucial to further investigate these components by focusing on specific domains present in these Gid proteins and on their localization in the cell.

1.1 The CRA domain of Gid8

Recently, a human homologue of the yeast Gid complex was found and called the human CTLH complex. RanBPM, Muskelin, p48EMLP and p44CTLH, proteins building this 20S large complex, were found to have LisH/CTLH motifs which are present in proteins involved in microtubule dynamics, cell migration, nucleokinesis and chromosome segregation (Umeda et al., 2003). Interestingly some notable similarities were revealed between RanBPM, p44CTLH, ARMC8α and p48EMLP, and Gid1/Vid30, Gid2, Gid5/Vid28 and Gid9 respectively (Kobayashi et al., 2007). Besides CTLH and LisH domains common to Gid1 and Gid8
proteins (Figure 1.1), one more shared domain, also of unknown function, is the CRA domain discovered from the SMART computer program.

<table>
<thead>
<tr>
<th>Alternative name</th>
<th>MW (kDa)</th>
<th>Proposed function</th>
<th>H. sapiens orthologues, GenBank identification</th>
<th>Common domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gid1 Vid30</td>
<td>108</td>
<td>Vacular import and degradation</td>
<td>RANBP10, RANBP9 a NM_020850, NM_005493</td>
<td>SPRY, LisH, CTLH, CRA</td>
</tr>
<tr>
<td>Gid2 Rmd5</td>
<td>49</td>
<td>/</td>
<td>RMND5A a, RMND5B NM_022780, NM_022762</td>
<td>LisH, CTLH, RING</td>
</tr>
<tr>
<td>Gid4 Vid24</td>
<td>41</td>
<td>Vacular import and degradation</td>
<td>C17orf39 NM_024052</td>
<td>/</td>
</tr>
<tr>
<td>Gid5 Vid28</td>
<td>105</td>
<td>Vacular import and degradation</td>
<td>ARMC8 a NM_213654</td>
<td>ARM</td>
</tr>
<tr>
<td>Gid7 Moh2</td>
<td>85</td>
<td>/</td>
<td>Muskelin b NM_013225</td>
<td>LisH, CTLH, WD40 or Kelch repeat c (Muskelin)</td>
</tr>
<tr>
<td>Gid8 Dcr1</td>
<td>52</td>
<td>Involved in START completion</td>
<td>C20orf11/Twa1 b NM_017896</td>
<td>LisH, CTLH, CRA</td>
</tr>
<tr>
<td>Gid9 Fyl10</td>
<td>60</td>
<td>Function required for yeast viability on toxin exposure</td>
<td>MAEA a BC001225</td>
<td>LisH, CTLH</td>
</tr>
</tbody>
</table>

Figure 1.1: Overview of the Gid proteins and their counterparts in H. sapiens. The table summarizes the common features in Gid proteins and their human counterparts. aOrthologues identified in a CTLH complex by Kobayashi et al. (Kobayashi et al., 2007); bOrthologues found to interact together by Umeda et al. (Umeda et al., 2003). cMuskelin bears CTLH and Kelch domains, which makes its overall structure similar to Gid7. Other functions and names, SGD, www.yeastgenome.org

This CRA domain is found in the RanBPM protein. Most RanBP (Ran-binding) proteins were reported to have a role in the nuclear pore complex machinery and to possess a so-called Ran binding domain. The closely related proteins, RanBPM and RanBP10, are however distinct RanBP family members in that they appear to have no effect on nuclear trafficking and do not interact with Ran in vivo. Unlike most other identified Ran binding proteins, RanBPM does not contain the consensus Ran-binding domain but a CRA domain of unknown function. Nevertheless, this protein possesses many characteristics of a scaffolding protein,
1.1 The CRA domain of Gid8

including protein-interaction motifs, a cytoskeletal-binding domain, and multiple canonical
docking sites for signaling intermediates (Denti et al., 2004).

To determine the most conserved amino acids, an alignment of the CRA domain found
in Gid1 and Gid8, and other CRA domains of different human proteins was done using the
Clustalv computer program. Amino acids, either completely conserved or with same chemical
properties, were sorted using different colours. Amino acids 255 and 256 in Gid8 seemed
particularly conserved. Point mutations were thus inserted into the CRA domain of Gid8
(section 3.1 page 123) using the pRS316-GID8HA plasmid (section 3.1 page 123). Two leucines,
L255 and L256 of Gid8, were changed into serine and glutamic acid, which have other chemical
properties than leucine (Figure 1.2).

1.1.1 Verification of the stability of the Gid8*-HA3 protein

Mutations inserted into the CRA domain of Gid8 may lead to a change of conformation of the
molecule and to an instability of the protein. It was thus important to check the stability of
the Gid8*-HA3 protein. Gid8 is known to be present and stable on a non-fermentable carbon
source before any shift of cells to a glucose-rich medium (Santt et al., 2008). If the mutations
in the GID8 gene were critical for the stability of Gid8-HA3, a degradation of Gid8*-HA3
should be observed when treating cells with the translation inhibitor cycloheximide on an
ethanol-containing medium. The YML2 (W303-1B gid8Δ) strain was transformed with the
pRS316-GID8HA and pRS316-GID8*HA plasmids respectively. Cells were then treated with
cycloheximide on ethanol-medium as described in subsubsection 5.2.4 page 139 and samples
were taken 0, 30, 60 and 90 minutes after treatment. As expected, the signal of Gid8-HA3 was
stable after treating cells with cycloheximide. Gid8*-HA3 showed the same behaviour as wild
type Gid8-HA3. The mutated Gid8 is thus stable on ethanol medium (Figure 1.3).

1.1.2 Influence of the mutations inserted into the CRA domain
of Gid8 on FBPase catabolite degradation

As the Gid8*-HA3 protein was stable on ethanol medium, it was interesting to look at FBPase
catabolite degradation in case of a CRA-mutated Gid8. The YML2 strain was transformed
with the pRS316-GID8HA and pRS316-GID8*HA plasmids. After 16 hours growth at 30°C on
2 % EtOH-CM-Ura medium, cells were shifted to 2 % glucose-CM-Ura medium and samples
Figure 1.2: Alignment of the CRA domains found in the Gid1 and Gid8 yeast proteins, and other CRA domains of human proteins. Amino acids, either completely conserved or with comparable chemical properties, were sorted using different colours. Amino acids 255 and 256 in Gid8 seemed particularly conserved.
1.1 The CRA domain of Gid8

Figure 1.3: Stability of the Gid8*HA<sub>3</sub> protein on ethanol medium. The YML2 (W303-1B gid8<sup>Δ</sup>) strain was transformed with the pRS316-GID8HA and pRS316-GID8*HA plasmids. After 16 hours growth at 30°C on 2% EtOH-CM-Ura medium, 100 µg/mL cycloheximide was added to cell cultures and samples were taken 0, 30, 60 and 90 minutes after treatment with cycloheximide. Gid8-HA<sub>3</sub> and Gid8*-HA<sub>3</sub> were detected via immunoblotting using the mouse HA antibody. PGK: 3-phosphoglycerate kinase, loading control.

were taken 0, 30, 60 and 90 minutes after glucose-shift. As expected, in the YML2 (W303-1B gid8<sup>Δ</sup>) strain transformed with the pRS316-GID8HA plasmid, FBPase was degraded after shifting cells from ethanol- to glucose-containing medium, whereas in the YML2 strain expressing Gid8*-HA<sub>3</sub>, FBPase was stabilised (Figure 1.4). As a conclusion, leucines 255 and 256 in Gid8 play an important role in FBPase degradation.

1.1.3 Interaction studies of the CRA-mutated Gid8 with the Gid complex and FBPase

Leucines 255 and 256 are amino acids that are conserved in Gid8 along evolution. Their mutation was shown to lead to a strong impairment of FBPase catabolite degradation. However it still remains unanswered, which important role these leucines have in FBPase degradation: change of conformations of Gid8, no formation of the Gid complex, no more interaction of Gid8 with FBPase and/or with other members of the Gid complex. Using co-immunoprecipitation experiments, interactions between the CRA-mutated Gid8 and FBPase, and the CRA-mutated
Figure 1.4: **Mutations in the CRA domain of Gid8 lead to a stabilisation of FBPase after shift of cells from ethanol to glucose medium.** The YML2 (W303-1B gid8Δ) strain was transformed with the pRS316-GID8HA and pRS316-GID8*HA plasmids. After 16 hours growth at 30°C on 2 % EtOH-CM-Ura medium, cells were shifted to 2 % glucose-CM-Ura medium and samples were taken 0, 30, 60 and 90 minutes after glucose-shift. FBPase, Gid8-HA₃ and Gid8*-HA₃ were detected via immunoblotting using the FBPase and the mouse HA antibodies respectively. PGK: 3-phosphoglycerate kinase, loading control.

Gid8 and the Gid complex were studied. This work was done in cooperation with Julia Reuther during her "Studienarbeit".

To study interactions with the Gid complex, the protein Gid1 which seems to be part of the core complex was used (Pitre et al., 2006). To look for interactions between the mutated Gid8 and the Gid complex, plasmids pRS316-GID8HA and pRS316-GID8*HA were transformed into the strain GID1myc-gid8Δ and cells were grown overnight on ethanol-containing medium without uracil. Samples were then withdrawn at the indicated time points and Gid1-myc₁₃ and Gid8-HA₃ were precipitated using MYC and HA antibodies respectively (Figure 1.5). The precipitates were monitored by immunoblotting with MYC- and HA-antibodies. Figure 1.5 shows that Gid8-HA₃ and Gid8*-HA₃ strongly coimmunoprecipitate with Gid1-myc₁₃ in ethanol medium at the time point "0" and after 25 minutes of glucose addition to cells. Only a slight signal of Gid8-HA₃ is seen in a non-tagged Gid1 strain, demonstrating a weak unspecific binding of Gid8-HA₃. As a conclusion the mutations inserted in the CRA domain of Gid8 do not impair interactions of the protein Gid8 with Gid1, and thus probably do not affect binding within the whole Gid complex.

To study interactions of the CRA-mutated Gid8 with FBPase, plasmids pRS316-GID8HA
Figure 1.5: The CRA-mutated Gid8 interacts with Gid1. Plasmids pRS316-GID8HA and pRS316-GID8*HA were transformed into the strain GID1myc-gid8Δ and cells were grown overnight on ethanol-containing medium without uracil. Samples were withdrawn 0 and 25 minutes after glucose treatment and proteins were extracted. Coimmunoprecipitation was then performed with either a rat HA antibody (A) or a mouse MYC antibody (B). Protein immunoblots were finally carried out with mouse HA and MYC antibodies. The HA₃-tagged Gid8 chromosomal YSA1 (Gid1myc13 respectively) strain grown on YPEtOH medium provides a control for unspecific binding of Gid8-HA₃ (Gid1-myc₁₃ respectively) to protein A-sepharose and MYC antibody (HA antibody respectively). (YE, extracts; IP, immunoprecipitations)
and pRS316-GID8*HA were transformed into the YML2 (gid8Δ) strain. The strain fbp1Δ is unable to grow on non fermentable carbon source and was therefore grown on glucose-containing medium only. The YBB1 (Gid6-HA3) strain was grown overnight on ethanol-containing complete medium, while the YML2 strain transformed with plasmids pRS316-GID8HA and pRS316-GID8*HA respectively was grown overnight on ethanol-containing medium without uracil. Samples were then withdrawn at the indicated time points and FBPase was precipitated using specific FBPase antibody (Figure 1.6). Figure 1.6 shows that FBPase strongly coimmunoprecipitates with Gid8-HA3 and Gid8*-HA3 in ethanol medium at the time point "0" and after 25 minutes of glucose addition to cells. No interaction between FBPase and Gid6-HA3/Ubp14-HA3 could be observed, demonstrating that no unspecific interaction occurs between FBPase and the HA-tag. Thus, the mutations inserted in the CRA domain of Gid8 do not impair interactions between FBPase and the protein Gid8.

1.1.4 Influence of the mutations inserted in the CRA domain of Gid8 on FBPase ubiquitination

If the mutations in the CRA domain of Gid8 do not impair interactions between Gid8 and the Gid complex, and between Gid8 and FBPase, they may however influence polyubiquitination of FBPase after addition of glucose to cells. Polyubiquitination of FBPase was thus tested in a strain mutated in the CRA domain of Gid8.

Immunoprecipitation of FBPase was often complicated, since the enzyme only slightly faster migrates on SDS-polyacrylamide gels than heavy chains of IgGs. A TAP-tagged version of the protein, usually used to purify proteins, was then used for this experiment (Puig et al., 2001). Because it bears a protein A sequence, the TAP-tag binds tightly to an IgG matrix. Besides when fused to an otherwise poorly detectable protein, it easily facilitates protein detection. This specificity allowed us to perform IgG-Sepharose pull-downs of FBPase. Therefore, a plasmid expressing a C-terminally TAP-tagged FBPase was transformed in the YSA1 (GID8-HA3) and YJMR1 (GID8*-HA3) strains to ensure a right detection of the enzyme and of its polyubiquitinated forms. Pull-down with IgG-Sepharose and subsequent immunodetection with an ubiquitin antibody revealed that a strain expressing the mutant Gid8 protein was unable to polyubiquitinate FBPase in vivo (Figure 1.7). The CRA domain of Gid8 is thus essential for FBPase polyubiquitination and degradation.
Figure 1.6: **FBPase binds to the CRA-mutated Gid8.** Plasmids pRS316-GID8HA and pRS316-GID8*HA were transformed into the YML2 \((gid8\Delta)\) strain. The strain \(fbp1\Delta\) is unable to grow on non fermentable carbon source and was therefore grown on glucose-containing medium only. The YBB1 \((Gid6-HA_3)\) strain was grown overnight on ethanol-containing complete medium, while the YML2 strain transformed with plasmids pRS316-GID8HA and pRS316-GID8*HA respectively was grown overnight on ethanol-containing medium without uracil. Samples were then withdrawn at the indicated time points and FBPase was precipitated using specific FBPase antibody. Protein immunoblots were then carried out with HA and FBPase antibodies.
Figure 1.7: **FBPase ubiquitination in a strain expressing the CRA-mutated Gid8 protein.** A plasmid expressing an FBPase-TAP fusion protein was transformed into the YSA1 (GID8-HA3) and YJMR1 (GID8*-HA3) strains. Samples were taken at the indicated time points, and FBPase was purified using IgG-Sepharose. Polyubiquitination was detected using a monoclonal ubiquitin antibody. C: control; the YSA1 (GID8-HA3) strain expressing wild type FBPase from a plasmid.
1.2 Localization of the Gid complex

As Gid proteins are essential for FBPase degradation and some direct interactions between Gid proteins and FBPase were recently discovered (Santt et al., 2008), it was interesting to investigate the site of actions of some of these proteins. In a proteome wide localization study, GFP fusions of GID1, GID3, GID4, GID5, GID6, GID7, GID8 and GID9 encoded proteins were described to be localized in the cytoplasm and in the nuclei of cells (Huh et al., 2003). We thus focused on subcellular localizations of these proteins in the nuclear and cytosolic compartments of yeast cells.

1.2.1 Gid2 possesses a nuclear localization sequence (NLS)

Proteins, transported from the cytosol to the nucleus of cells, usually require the classical nuclear import machinery. The classical nuclear import pathway transports large cargo proteins from the cytosol to the nucleus using a signal-mediated system. These cargos are carried from the cytoplasm to the nucleus by nuclear transport receptors called karyopherins α and β. The α subunit recognizes the cargo through a nuclear localization sequence NLS, while the β subunit adapts the cargo complex to the nuclear pore and moves it into the nucleus.

Classic NLS are characterized by short stretches of basic amino acid residues. Two types of NLSs are known: monopartite (K R/K X R/K) and bipartite (K R X10-12 K R X K) (where X is any amino acid). A bipartite nuclear localization sequence (NLS) was detected in Gid2 using the web based algorithm Predict NLS (http://cubic.bioc.columbia.edu/predictNLS/) (Letunic et al., 2004). The amino acid sequence of the Gid2 protein is shown with the putative NLS sequence highlighted in red (Figure 1.8).

If this protein sequence of Gid2 functions as NLSs in vivo, it should direct a non-nuclear protein such as GFP into the nucleus of living yeast cells. This putative NLS sequence of Gid2 was thus expressed as a fusion protein with GFP and the Strep-tag II (S) by induction with galactose. The nuclear localization sequence (K R K REL I I E K L S K S Q R Q W D H S V K K Q I K97) of Gid2 was inserted by annealed oligonucleotides in the pRpt2NLSG vector.

As positive and negative controls, GFP was fused to the wild type NLS and mutant NLS of the monopartite SV40 T antigen respectively (Wendler et al., 2004). Both positive pNLSG and negative pmNLSG control plasmids (Wendler et al., 2004) as well as the pGID2NLSG plasmid were transformed into yeast cells using the W303-1B strain. Fusion proteins were
Amino acid sequence of Gid2. A NLS sequence has been detected using the web based algorithm Predict NLS (http://cubic.bioc.columbia.edu/predictNLS/). The putative NLS sequence is highlighted in red.

then expressed by incubating cells for a minimum of 4 hours at 30°C in a galactose-containing medium (2 % galactose). Each sample was then further prepared as described in subsubsection 7.8.1 page 157.

The SV40 NLS fusion protein clearly localized in the cytosol and accumulated inside the nucleus; the mutant SV40 NLS was only localised in the cytosol and did not accumulate inside the nucleus (Figure 1.9). The potential NLS within the N-terminal region of Gid2 directed GFPS into the nucleus with high efficiency, comparable with wild type SV40 NLS. Gid2 is thus shown to harbor a functional NLS.

1.2.2 The NLS-deleted Gid2 protein is not stable

The nuclear localization sequence of Gid2 had previously been found to be functional by itself and able to transport a non-nuclear protein like GFP into the nucleus of cells. However the question whether this sequence is functional in the protein Gid2, still needs to be answered. The protein Gid2 may harbor such a spatial conformation that its NLS cannot be recognized anymore by karyopherins involved in the nuclear import pathway. This nuclear localization sequence was thus deleted by mutagenesis in the pRS316-GID2 plasmid (Barbin, 2005) using Clontech protocol ”Transformer Site-directed mutagenesis kit user manual” (subsection 6.8 page 144). Swa1 was used as a selection enzyme and Pme1 as a restriction enzyme. The proteins Gid2 and Gid2∆NLS were tagged with HA3 and checked for their stability on ethanol-medium by treating cells with cycloheximide (subsubsection 5.2.4 page 139). The Gid2-HA3 protein expressed from a plasmid displays a similar behaviour as the chromosomal HA3-tagged
Figure 1.9: **Localization of the NLS-GFPS fusion protein.** The putative NLS sequence of Gid2 was expressed as a GFPS (GFP with the Strep-tag II) fusion protein. The pNLSG plasmid was taken as a positive control and the pmNLSG plasmid as a negative control (Wendler et al., 2004). Each fusion protein was overexpressed through induction of strains for 4 hours at 30°C with 2 % galactose and observed through the microscope (subsubsection 7.8.1 page 157). Hoechst 33342: DNA dye, nuclear marker.
protein: it is functional and stable on ethanol medium (results not shown). Contrary to Gid2-HA3, Gid2ΔNLS-HA3 is however unstable on ethanol-containing medium (Figure 1.10). The presence of this NLS sequence is thus crucial for the stability of the protein Gid2.

Figure 1.10: The NLS-deleted Gid2 is not stable on an ethanol-containing medium. The YTS1 (W303-1B-gid2Δ) strain was transformed with the pRS316, pRS316-GID2HA and pRS316-GID2ΔNLSHA plasmids. After 16 hours growth at 25°C on 2 % EtOH-CM-Ura medium, 100 µg/mL cycloheximide was added to cell cultures and samples were taken 0, 1 and 2 hours after treatment of cells with cycloheximide. Gid2-HA3 and Gid2ΔNLS-HA3 were detected via immunoblotting using the mouse HA antibody. PGK: 3-phosphoglycerate kinase, loading control.

Gid2p is known to be essential for FBPase degradation when cells are shifted from ethanol-to glucose-containing medium. Since Gid2 lacking its NLS sequence was discovered not to be stable on ethanol-medium, no FBPase degradation could be tested. Moreover no localization of Gid2 could have been done: Gid2-HA3 was not detectable through indirect immunofluorescence microscopy.

1.2.3 Subcellular localization of Gid1 and Gid8

Wild type subcellular localization of Gid1 and Gid8

In a proteome wide localization study, GFP fusions of GID1, GID3, GID4, GID5, GID6, GID7, GID8 and GID9 encoded proteins were described to be localized in the cytoplasm and in the nuclei of cells (Huh et al., 2003). To confirm these data from Huh et al., biochemical and immunofluorescence microscopy methods were used.

Subcellular localization of most Gid proteins was first done biochemically by purification
of yeast nuclei through differential centrifugation followed by a Ficoll gradient (section 7.9 page 158). However, nuclear fractions could not be properly separated from the vacuolar ones. A biochemical subcellular localization of these proteins thus remained unsuccessful.

For studies through direct fluorescence microscopy, GFP fusions of Gid1, Gid2, Gid7 and Gid8 were checked for their ability to degrade FBPase normally by switching cells from an ethanol- to a glucose-containing medium. GFP fusions of Gid1 and Gid7 were unable to trigger degradation of FBPase. The GFP fusion of GID8 encoded protein was functional since it was able to degrade FBPase, but no GFP signal of the fusion protein could be detected through direct fluorescence (Pfirrmann, 2006). N- and C-terminal fusions of the protein Gid2 to the green fluorescent protein (GFP) were also done as described in Gauss et al. (2005) and Longtine et al. (1998) respectively, but remained unsuccessful as well.

Subcellular localization of Gid proteins was finally done with indirect immunofluorescence. HA3-tagged Gid1 (Figure 1.11 A) and Gid8 (Figure 1.11 B) proteins expressed from a plasmid were both localized in the cytosol and accumulated into the nucleus in gluconeogenic (0 min after shift of cells from ethanol- to glucose-containing medium) and glycolytic (30 min and 60 min after glucose-shift) conditions. No signal of the proteins was seen in the vacuole. These data thus support localization studies done by Huh et al. Indirect immunofluorescence of HA3-tagged Gid2 and Gid7 was however not successful.

Subcellular localization of Gid1 and Gid8 in a gid2 deletion strain

Gid proteins were shown to interact together forming a complex, Gid2 was discovered to possess a nuclear localization sequence, and Gid1 and Gid8 were found to be localized in the cytosol and to accumulate in the nucleus of yeast cells. Taken together, these data suggest that Gid2 may transport the Gid complex into the nucleus. It was then interesting to look at the localization of Gid1 and Gid8 when Gid2 was missing, expecting only a cytosolic localization of Gid1 and Gid8 with no nuclear accumulation. Interestingly in a gid2 deletion strain, Gid1-HA3 and Gid8-HA3 still showed a cytosolic and nuclear wild type localization (results not shown). Thus Gid2 does not have any influence and then any role on the nuclear localization of Gid1 and Gid8.
Studies on the Gid complex

Figure 1.11: **Intracellular localization of Gid1 and Gid8 in yeast cells through indirect immunofluorescence microscopy.** The YML1 (W303-1B gid1Δ) and YML2 (W303-1B gid8Δ) strains were transformed with the pJR15 and pRS316-GID8HA plasmids respectively. After 16h growth on 2% EtOH-CM-Ura medium, 2% glucose was added to culture to induce FBPase degradation. Samples were taken 0, 30 and 60 minutes after glucose-shift and prepared for indirect immunofluorescence. As negative control (Neg), a sample of the YML1 and YML2 strains respectively grown on ethanol medium was used. Cells were then examined under a Zeiss microscope. Gid1-HA3 (A) and Gid8-HA3 (B) were detected by the mouse HA antibody and visualized by Alexa Fluor 488-conjugated goat mouse IgG. DAPI: DNA dyer, nuclear marker.
1.2 Localization of the Gid complex

Localization of the CRA-mutated Gid8

As described previously, Gid8 possesses a CRA domain which is usually found in the protein RanBPM, a Ran-binding scaffolding protein differentiating from other Ran-binding proteins which do have a role in the nuclear pore machinery. To determine if this domain has a role in the nuclear localization of Gid8, the localization of a HA$_3$-tagged CRA-mutated Gid8 protein expressed from a plasmid was done by indirect immunofluorescence. The CRA-mutated Gid8 localized in the cytosol and accumulated into the nucleus like the wild type (results not shown). As a conclusion, the mutations inserted in the CRA domain of Gid8 do not have any influence on the localization of Gid8. The CRA domain of Gid8 probably does not play any role in the nuclear pore complex machinery, thus supporting its homology with RanBPM.

1.2.4 Instability of Gid1 and Gid8 in $gid8\Delta$ and $gid1\Delta$ strains respectively

Localization studies of Gid1 and Gid8 in $gid8\Delta$ and $gid1\Delta$ strains respectively led to the discovery of a notable decrease of protein signals by indirect immunofluorescence microscopy. Such observations may be due to a change in regulation of the expression of Gid proteins, or the degradation of one Gid component when another key Gid protein is missing. The stability of Gid1, Gid2 and Gid8 was thus investigated in each $gid$ deletion strain. Since these proteins are usually stable on ethanol medium, next experiments were processed in this medium (work done in cooperation with Katja Latendorf).

As expected, Gid1-HA$_3$ is stable on ethanol medium and the type of expression of Gid1-HA$_3$ in the YML1 (W303-1B-$gid1\Delta$) strain transformed with the pJR15 Gid1-HA$_3$ expression plasmid, seems to be comparable to the one in the YJR12 Gid1-HA$_3$ chromosomal strain. As described in Figure 1.12 A and B, Gid1-HA$_3$ is stable on ethanol medium in a $gid2\Delta$ strain but not in a $gid8\Delta$ strain.

In the same way, Gid8-HA$_3$ was shown to be stable on ethanol medium in a $gid2\Delta$ strain but not in a $gid1\Delta$ strain (Figure 1.13).

Gid2-HA$_3$ however was discovered not to be stable on ethanol-containing medium, neither in a $gid1\Delta$ strain nor in a $gid8\Delta$ strain (Figure 1.14).
Figure 1.12: Gid1-HA$_3$ is stable on ethanol medium in a gid2$\Delta$ strain but not in a gid8$\Delta$ strain. Cycloheximide chase analysis. (A) The YJR12 (W303-1B-GID1-HA$_3$), YML1 (W303-1B-gid1$\Delta$) and YLB25 (gid1$\Delta$gid8$\Delta$) strains, (B) the YJR12 (W303-1B-GID1-HA$_3$), YML1 (W303-1B-gid1$\Delta$) and YLB23 (gid1$\Delta$gid2$\Delta$) strains, were transformed with the pJR15 Gid1-HA$_3$ expression plasmid and inoculated in glucose medium. After 16 hours growth at 30°C on 2 % EtOH-CM-Ura medium, 100 $\mu$g/mL cycloheximide was added to cell cultures. Samples were taken every 30 minutes after treatment with cycloheximide and finally analysed by performing a SDS-gel electrophoresis with subsequent Western blotting. Gid1-HA$_3$ was detected via immunoblotting using the mouse HA antibody. Cpy: Carboxy-peptidase Y; loading control; PGK: 3-phosphoglycerate kinase, loading control.
1.2 Localization of the Gid complex

Figure 1.13: Gid8-HA<sub>3</sub> is stable on ethanol medium in a gid<sub>2</sub>Δ strain but not in a gid<sub>1</sub>Δ strain. Cycloheximide chase analysis. (A) The YSA1 (W303-1B-GID8-HA<sub>3</sub>), YML2 (W303-1B-gid8Δ) and YLB25 (gid<sub>1</sub>Δgid8Δ) strains, (B) the YSA1 (W303-1B-GID8-HA<sub>3</sub>), YML2 (W303-1B-gid8Δ) and YLB24 (gid8Δgid2Δ) strains, were transformed with the pRS316-GID8HA Gid8-HA<sub>3</sub> expression plasmid and grown as described in Figure 1.12.
Gid2-HA$_3$ is not stable on ethanol medium in a gid1$\Delta$ and in a gid8$\Delta$ strain. Cycloheximide chase analysis. (A) The YTS3 (W303-1B-GID2-HA$_3$), YTS1 (W303-1B-gid2$\Delta$) and YLB23 (gid1$\Delta$ gid2$\Delta$) strains, (B) the YTS3 (W303-1B-GID2-HA$_3$), YTS1 (W303-1B-gid2$\Delta$) and YLB24 (gid8$\Delta$ gid2$\Delta$) strains, were transformed with the pRS316-GID2HA Gid2-HA$_3$ expression plasmid and and grown as described in Figure 1.12.
Chapter 2

Studies on catabolite degradation of FBPase

2.1 FBPase degradation is dependent on the Cdc48 machinery

2.1.1 Involvement of the Cdc48\(^{Ufd1-Npl4}\) complex in the degradation of polyubiquitinated FBPase

Recently, the Gid complex has been discovered to act as an ubiquitin ligase and to be essential for ubiquitination and degradation of yeast fructose-1,6-bisphosphatase. However the mechanism used to deliver FBPase to the proteasome remains unknown. FBPase is a homotetrameric enzyme (Rittenhouse et al., 1984, 1986) and may be split into monomers to be further recognized and degraded by the proteasome. Cdc48, reported to disassemble protein complexes and segregate proteins from their binding partners through the hydrolysis of ATP, may thus be expected to have such a function in FBPase degradation. This hypothesis is supported by the fact that some cytosolic but artificial substrates like Ub-P-βGal, have been reported to require the Cdc48 machinery for their further degradation (Johnson et al., 1995).

We thus tested FBPase degradation in a \(cde48\) mutant. Strains W303-1B \(prc1-1\) and \(cdc48-ts\) were grown overnight in YPEthanol at the permissive temperature (25°C). The next day, after a shift of one hour to the restrictive temperature (37°C), cells were transferred to YPD and
samples were taken at the indicated time points. As can be seen in Figure 2.1 A, degradation of FBPase was dramatically impaired in the $\textit{cdc48}$ mutant strain. To be able to consistently quantify the effect of such mutations in $\textit{CDC48}$ on FBPase degradation, pulse-chase experiments, where radioactively labelled FBPase was immunoprecipitated (section 7.6 page 155), was carried out. The half-life time of FBPase was increased by about three-fold in a strain defective in Cdc48 activity (Figure 2.1 B).

Recent discoveries demonstrated that Gid2 confers a ubiquitin ligase activity to the Gid complex. To determine at which step of the FBPase degradation Cdc48 is required, an $\textit{in vivo}$ ubiquitination assay was performed (section 7.7 page 156). Immunoprecipitation of FBPase was often complicated, by the fact that the enzyme migrates only slightly faster on SDS-polyacrylamide gels than IgGs. The method described by (Santt et al., 2008) was therefore used for these polyubiquitination assays. A plasmid expressing a C-terminally TAP-tagged FBPase was transformed in W303-1B $\textit{prc1-1}$ (WT) and $\textit{cdc48-ts}$ strains to ensure a proper detection of the enzyme and of its polyubiquitinated forms. Pull-down with IgG-Sepharose and subsequent immunodetection with an ubiquitin antibody revealed that a strain expressing the mutant Cdc48 protein was still able to polyubiquitinate FBPase (Figure 2.1 C). This result suggested that Cdc48 is not required for FBPase ubiquitination but for subsequent processing of polyubiquitinated FBPase.

To exert its function, Cdc48 requires substrate-recruiting cofactors. Two types of such cofactors have been identified so far, the heterodimeric Ufd1-Npl4 complex and members of the UBX domain protein family (Ubx1 to Ubx7), the founding member being Shp1 (or Ubx1) (Jentsch and Rumpf, 2007). Both of these adaptors bind ubiquitin-protein conjugates and interact with Cdc48. Recruitment of such cofactors results in fundamentally distinct cellular functions of Cdc48. While the Cdc48$^{\text{Shp1}}$ complex is reported to control the fusion of homotypic membranes (Kondo et al., 1997), ubiquitin-dependent protein degradation pathways require the Cdc48$^{Ufd1−Npl4}$ complex (Braun et al., 2002; Rape et al., 2001; Ghislain et al., 1996; Richly et al., 2005; Rumpf and Jentsch, 2006; Raasi and Wolf, 2007). It was thus interesting to test whether FBPase degradation would also require some of these cofactors. FBPase was indeed dramatically slower degraded in a $\textit{npl4}$ (Figure 2.2 A) and $\textit{ufd1}$ (Figure 2.3 A) mutant strain. Pulse-chase experiments confirmed these results: the half-life of FBPase was increased by more than three-fold in an $\textit{npl4-2}$ strain and about three-fold in an $\textit{ufd1-1}$ strain (Figures 2.2 B and 2.3 B). Strains expressing the Npl4 and Ufd1 mutant proteins were still able to polyubiquitinate
2.1 FBPase degradation is dependent on the Cdc48 machinery

Figure 2.1: The Cdc48 complex is involved in proteasomal catabolite degradation of FBPase after polyubiquitination of the enzyme. W303-1B \textit{prc1-1} (WT) and \textit{cdc48-ts} strains were grown overnight in YPEthanol at the permissive temperature (25°C). They were then shifted to the restrictive temperature (37°C) for one hour and thereafter to YPD medium to trigger FBPase degradation. Samples were taken every 30 minutes after shift to glucose. (A) Metabolic chase analysis. FBPase was detected via immunoblotting using a FBPase antibody. PGK: 3-phosphoglycerate kinase, loading control. (B) Pulse chase analysis of FBPase in strains W303-1B \textit{prc1-1} (WT) and \textit{cdc48-ts} was carried out as described in Materials and methods (means of three independent experiments, \pm confidence interval, \(\alpha = 0.05\)). (C) FBPase polyubiquitination in a \textit{cdc48} mutated strain. A plasmid expressing an FBPase-TAP fusion protein was transformed into the W303-1B \textit{prc1-1} (WT) and \textit{cdc48-ts} strains. Samples were taken at the indicated time points and FBPase was purified using IgG-Sepharose. Polyubiquitination was detected using a monoclonal ubiquitin antibody. C: control; the W303-1B \textit{prc1-1} strain expressing wild type FBPase from a plasmid.
FBPase *in vivo* (Figures 2.2 C and 2.3 C). Thus, the Cdc48$^{Uf1-Npl4}$ complex is important for FBPase degradation, and its action on FBPase occurs after FBPase polyubiquitination by the Gid complex. Interestingly, FBPase is the first natural cytosolic substrate shown to depend on the Cdc48$^{Uf1-Npl4}$ complex for its further degradation by the 26S proteasome.

2.1.2 Ubx4 plays some role in the degradation of FBPase

Only one protomer of the homohexameric AAA ATPase Cdc48 is occupied by the Ufd1-Npl4 heterodimeric complex. Unoccupied protomers of Cdc48 can thus bind additional cofactors, like members of the UBX domain protein family, which are characterized by the presence of a so-called "ubiquitin regulatory X" (UBX) domain. This domain, the only sequence motif shared by all seven Ubx proteins, was recently reported to be a general binding module for Cdc48 (Schuberth et al., 2004). The best studied UBX protein, Shp1 (or Ubx1), is well known for its function in membrane fusion. In the ERAD machinery, Ubx2 recruits the Cdc48$^{Uf1-Npl4}$ complex to the ER membrane, thus allowing the Cdc48 complex to bind
2.1 FBPsase degradation is dependent on the Cdc48 machinery

Figure 2.3: Ufd1 is involved in proteasomal catabolite degradation of FBPsase after polyubiquitination of the enzyme. BWG1-7a (WT) and PM373 (ufd1-1) strains were grown as described in Figure 2.1. (A) Metabolic chase analysis. (B) Pulse chase analysis of FBPsase in strains BWG1-7a (WT) and PM373 (ufd1-1). (C) FBPsase polyubiquitination in a ufd1 mutated strain.
E3 ligases and misfolded luminal substrates (Schuberth and Buchberger, 2005). Ubx1, Ubx4, Ubx6 and Ubx7 are also involved in ubiquitin-dependent protein degradation (Schuberth et al., 2004; Decottignies et al., 2004), but their function is still not well characterized. Ubx4 has recently been reported to modulate Cdc48 activity and influence degradation of misfolded proteins of the endoplasmic reticulum (Alberts et al., 2009). It was therefore interesting to test the involvement of these UBX proteins in FBPase degradation. FBPase degradation was not impaired in ubx1, ubx2, ubx3, ubx5, ubx6 and ubx7 deletion strains (Figures 2.4 and 2.5). However from catabolic chase and pulse-chase analysis, the degradation of FBPase was determined to be 1.5-fold slowed down in an ubx4Δ strain (Figure 2.6 A-B), thus suggesting some involvement of Ubx4 in FBPase degradation. FBPase appeared to be more polyubiquitinated 25 minutes after shift of cells from a non-fermentable to a fermentable carbon source (Figure 2.6 C). The Ubx4 protein thus represents an additional substrate-recruiting cofactor which is involved in degradation of polyubiquitinated FBPase.

2.1.3 Involvement of additional cofactors of Cdc48 in the degradation of FBPase

Besides its “segregase” activity, Cdc48 was also shown to control the degree of ubiquitination of the bound substrates. This latter activity is brought about by ”substrate-processing cofactors” that either promote polyubiquitination, inhibit polyubiquitination or even deubiquitinate the bound ubiquitinated substrate. The E4 enzyme Ufd2 polyubiquitinates the substrate, thereby promoting proteasomal degradation. This U-box domain-containing protein binds to the second AAA domain of Cdc48 through the Ufd1-Npl4 complex (Richly et al., 2005). The WD-40 protein Ufd3 competes with Ufd2 for Cdc48 binding thereby preventing further polyubiquitination of the substrate by Ufd2. Indeed both factors utilize the same docking site on Cdc48 (Rumpf and Jentsch, 2006). Finally the deubiquitinating enzyme Otu1 removes the ubiquitin modification from the substrate. Interestingly Cdc48 can bind Otu1 and Ufd3 simultaneously, making a cooperation of both inhibitory mechanisms possible (Rumpf and Jentsch, 2006). The dependency of FBPase degradation on such processing cofactors was investigated.

Interestingly FBPase degradation is not impaired in Ufd2 and Otu1 mutant strains (Figure 2.7 A-B page 76). However in an ufd3 mutant the degradation of FBPase is dramatically slowed down (Figure 2.8 A). The half-life of fructose-1,6-bisphosphatase was indeed determined to be
2.1 FBPase degradation is dependent on the Cdc48 machinery

Figure 2.4: FBPase degradation is not impaired in \textit{ubx1}, \textit{ubx3}, \textit{ubx5}, \textit{ubx6} and \textit{ubx7} deletion strains. Metabolic chase analysis. After overnight growth in YPEthanol, strains W303-1B \textit{prc1-1} (WT), YSA21 (\textit{ubx1Δ}), YSA18 (\textit{ubx3Δ}), YCR2 (\textit{ubx5Δ}), YCR4 (\textit{ubx6Δ}) and YSA12 (\textit{ubx7Δ}) were shifted to YPD and samples were taken every 30 minutes after glucose-shift. FBPase was detected via immunoblotting using a FBPase antibody. PGK: 3-phosphoglycerate kinase, loading control.
Figure 2.5: Ubx2 is not involved in proteasomal catabolite degradation of FBPase. W303-1B pcr1-1 (WT) and YCR1 (ubx2Δ) strains were grown as described in Figure 2.1. (A) Metabolic chase analysis. (B) Pulse chase analysis of FBPase in strains W303-1B pcr1-1 (WT) and YCR1 (ubx2Δ).
2.1 FBPase degradation is dependent on the Cdc48 machinery.

Figure 2.6: Ubx4 somewhat impairs proteasomal catabolite degradation of FBPase and is required after polyubiquitination of the enzyme. W303-1B prc1-1 (WT) and YSA10 (ubx4Δ) strains were grown as described in Figure 2.1. (A) Metabolic chase analysis. (B) Pulse chase analysis of FBPase in strains W303-1B prc1-1 (WT) and YSA10 (ubx4Δ). (C) FBPase polyubiquitination in a ubx4 deleted strain.
more than 3-fold longer in a *ufd3* mutant strain as compared to wild type (Figure 2.8 B). Additionally a striking impairment of FBPase polyubiquitination was observed in the *ufd3-1* temperature-sensitive strain (Figure 2.8 C). However, because the *doa1Δ/ufd3Δ* mutant is depleted for free ubiquitin, this effect could be of indirect nature (Ghislain et al., 1996; Johnson et al., 1995).

Figure 2.7: Neither the ubiquitin ligase Ufd2 nor the deubiquitinating enzyme Otu1 are required for FBPase catabolite degradation by the proteasome. (A-B) Metabolic chase analysis. After overnight growth in YPEthanol, strains W303-1B *prc1-1* (WT), YSK018 (*ufd2Δ*) and YJMR2 (*otu1Δ*) were shifted to YPD and samples were taken every 30 minutes after glucose-shift. FBPase was detected via immunoblotting using a FBPase antibody. PGK: 3-phosphoglycerate kinase, loading control.

### 2.1.4 Cdc48 interacts with the Gid complex

The Gid complex acts as an ubiquitin ligase, necessary for FBPase polyubiquitination. Here we show that the Cdc48<sup>Ufd1−Npl4</sup> complex acts after polyubiquitination of FBPase by the Gid complex. From these data, one can hypothesize that Cdc48 is a link between the Gid complex and the 26S proteasome. It was therefore interesting to see if the Cdc48 complex binds to the Gid complex. We therefore tested whether the Gid complex coimmunoprecipitates with Cdc48. The W303-1B (wild type), YBB1 (GID6-HA3), YSA1 (GID8-HA3) and YSA29 (NPL4-HA3)
Figure 2.8: Ufd3 is necessary for FBPase degradation and polyubiquitination. BWG1-7a (WT) and PM164 (ufd3-1) strains were grown as described in Figure 2.1. (A) Metabolic chase analysis. (B) Pulse chase analysis of FBPase in strains BWG1-7a (WT) and PM164 (ufd3-1). (C) FBPase polyubiquitination in a ufd3 mutated strain.

strains were grown in ethanol medium and shifted to glucose medium. Samples were withdrawn at the indicated time points and Cdc48 was precipitated using a specific Cdc48-antibody (Figure 2.9 A). The immunoprecipitates were then monitored via immunoblotting with Cdc48- and HA-antibodies. Figure 2.9 A shows that Cdc48, as expected, strongly interacts with Npl4-HA\textsubscript{3} and with Gid8-HA\textsubscript{3} in ethanol grown cells at the time point "0" and 25 minutes after addition of glucose to cells. No interaction between Cdc48 and Gid6-HA\textsubscript{3}/Ubp14-HA\textsubscript{3} could be observed, demonstrating that no unspecific interaction occurs between Cdc48 and the HA-tag.

2.1.5 FBPase degradation is impaired in mutants of Rad23 and Dsk2

Oligoubiquitylated substrates have been described to be first recognized by the substrate-recruiting cofactors of Cdc48, in certain cases relocated onto the E4 enzyme (Ufd2) for multiquitination and subsequently handed over to additional ubiquitin-conjugate binding proteins, Rad23 and Dsk2, for proteosomal targeting and degradation (Richly et al., 2005; Medicherla et al., 2004). These latter proteins, suggested to shuttle ubiquitinated substrates to the pro-
Figure 2.9: **Cdc48 binds to the Gid complex.** Strains expressing HA-tagged Gid (Gid8 and Gid6/Ubp14) and Npl4 proteins were grown 16 hours on YPEtOH. Samples were harvested 0 and 25 min after glucose treatment, and proteins were extracted. Co-immunoprecipitation was performed with Cdc48 antibody. The non-interacting Gid6/Ubp14 protein serves as a control. (YE: yeast extract; IP: immunoprecipitations; *: Cross-reaction).
teasome, contain ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains. There are three UBL-UBA containing proteins in budding yeast: Ddi1, Dsk2 and Rad23. Polyubiquitinated fructose-1,6-bisphosphatase, after being processed by the Cdc48\(^{Ufd1-Npl4}\) complex, has to be delivered to the 26S proteasome for its further degradation and may thus require the ubiquitin-binding proteins Rad23, Dsk2 and/or Ddi1.

As seen in Figure 2.10 A, FBPase degradation is delayed in rad23 and dsk2 deletion strains. An add1\(\Delta\) strain displayed wild type FBPase degradation kinetics (Figure 2.11). As expected, a strain expressing the mutant Dsk2 protein was still able to polyubiquitinate FBPase (Figure 2.10 B). This result thus shows that Dsk2 is not required for FBPase polyubiquitination and probably acts, as expected as a receptor, after FBPase polyubiquitination by the Gid complex.

2.1.6 Catabolite degradation of PEPCK requires the Cdc48-Ufd1-Npl4 complex

The last reaction step in glycolysis which leads to the formation of pyruvate and ATP is catalysed by pyruvate kinase. In gluconeogenesis this step is circumvented by pyruvate carboxylase and pyruvate carboxykinase (PEPCK). Like FBPase, PEPCK synthesis is repressed by glucose and PEPCK is subjected to catabolite degradation when cells are shifted from gluconeogenic to glycolytic conditions (Holzer, 1976; Müller and Holzer, 1981; Mercado and Gancedo, 1992; Yin et al., 2000). As described for FBPase, the degradation of PEPCK was reported to be dependent on the Gid complex (Santt et al., 2008). Since efficient FBPase degradation requires the Cdc48 complex, this may be also the case for PEPCK degradation. PEPCK degradation was thus monitored in mutants of the Cdc48 machinery after shifting cells from ethanol- to glucose-containing medium. Interestingly, PEPCK degradation was strongly impaired in mutants of Cdc48, Ufd1 and Npl4, indicating that the Cdc48\(^{Ufd1-Npl4}\) complex is required for catabolite degradation of PEPCK (Figure 2.12). This suggests that the Cdc48\(^{Ufd1-Npl4}\) machinery, like the Gid complex, plays a general role in the catabolic degradation of gluconeogenic enzymes.
Figure 2.10: FBPase degradation is slightly impaired in rad23 and dsk2 deleted strains. (A) After overnight growth in YPEthanol, strains W303-1B (WT), YAT2525 (rad23Δ) and YAT2851 (dsk2Δ) were shifted to YPD and samples were taken every 30 minutes after glucose-shift. FBPase was detected via immunoblotting using an FBPase antibody. PGK: 3-phosphoglycerate kinase, loading control. (B) FBPase polyubiquitination in a dsk2 mutated strain. A plasmid expressing an FBPase-TAP fusion protein was transformed into the W303-1B (WT) and YAT2851 (dsk2Δ) strains. Samples were taken at the indicated time points and FBPase was pulled down using IgG-Sepharose. Polyubiquitination was detected using monoclonal ubiquitin antibody. C: control; a plasmid expressing a wild-type FBPase was transformed into the W303-1B strain.
2.1 FBPase degradation is dependent on the Cdc48 machinery

Figure 2.11: **Ddi1 is not involved in FBPase catabolite degradation.** Metabolic chase analysis. After overnight growth in YPEthanol, strains W303-1B (WT) and YLB47 (ddi1Δ) were shifted to YPD and samples were taken every 30 minutes after glucose-shift. FBPase was detected via immunoblotting using an FBPase antibody. PGK: 3-phosphoglycerate kinase, loading control.

![FBPase and PGK levels over time](image)

Figure 2.12: **Catabolite degradation of PEPCK requires the Cdc48Ufd1−Npl4 complex.** W303-1B prc1-1 (WT) and cdc48-ts (A), YAG003 (WT) and YAG005 (npl4-2) (B) and BWG1-7a (WT) and PM373 (ufd1-1) (C) strains were grown overnight in YPEthanol at the permissive temperature (25°C). They were then shifted to the restrictive temperature (37°C) for one hour and thereafter to YPD medium to trigger PEPCK degradation. Samples were taken every 30 minutes after shift to glucose. PEPCK was detected via immunoblotting using an PEPCK antibody. PGK: 3-phosphoglycerate kinase, loading control.

![PEPCK and PGK levels over time](image)
2.2 Subcellular localization of FBPase degradation

Gid proteins were shown to constitute the E3 ligase for FBPase degradation and direct interactions between Gid proteins and FBPase have been discovered (Santt et al., 2008). Moreover results obtained from fluorescence microscopy experiments indicated a cytosolic localization of Gid1 and Gid8 and an accumulation in the nucleus. Finally Gid2 was found to possess a nuclear localization sequence. From all these data, the question arose whether FBPase might be degraded in the nucleus of cells. This hypothesis is supported by some recent publications (Gizak et al., 2005; Yáñez et al., 2003), which suggest a localization of mammalian FBPase in the nuclei of cells after supplementation with glucose or insulin. Moreover yeast FBPase was found to interact with the karyopherin α Srp1 and the karyopherin β Kap95 from the nuclear pore complex (Ito et al., 2001). Interactions were also discovered between Gid4 and Gid7 with Nup116, which itself interacts with Kap95, an importin β which is used in the classical nuclear transport machinery.

To further investigate a possible nuclear localization of FBPase, FBPase was C-terminally marked with several tags. However FBPase degradation and localization was dependent on the tag used (section 2.3 page 88). FBPase-TAP was the only fusion protein degraded like the untagged FBPase. Thus, experiments were done with a C-terminal TAP fusion of FBPase (Pfirrmann, 2006). In the resulted strain, FBPase-TAP was active since cells could grow on ethanol-containing medium. The strain was tested for its ability to degrade FBPase-TAP and was indeed shown to display wild type FBPase degradation kinetics (Figure 2.13 B).

FBPase degradation is known to be dependent on the 26S proteasome and on the Gid complex. Before investigating the localization of FBPase-TAP in the cell, it was necessary to check if FBPase-TAP degradation was also dependent on the Gid complex and on the proteasome.

2.2.1 FBPase-TAP degradation is dependent on the proteasome and on the Gid complex

The dependence of FBPase-TAP degradation on the proteasome was tested. Proteasome proteolysis was inhibited by the inhibitor MG132 in the W303-FBPase-TAP strain which carried a PDR5 gene deletion to prevent the export of the inhibitor by this ABC transporter
(Balzi et al., 1994; Bissinger and Kuchler, 1994). As expected in the W303-FBPase-TAP strain treated with the solvent, FBPase-TAP was degraded after shift of cells from an ethanol- to a glucose-containing medium, whereas in the same strain treated with MG132, FBPase-TAP was stabilized (Figure 2.13 A). Thus like FBPase degradation, FBPase-TAP degradation depends on the proteasome.

Also the dependence of FBPase-TAP degradation on Gid proteins (Gid1, Gid2, Gid3, Gid5, Gid6, Gid8 and Gid9) was tested. While FBPase-TAP was normally degraded after shift to glucose, a stabilisation of the protein could be observed in each gid deletion strain (Figure 2.13 B). Like FBPase degradation, FBPase-TAP degradation is dependent on the Gid complex, on the ubiquitin-conjugating enzyme Gid3 and on the deubiquitinating enzyme Gid6.

2.2.2 FBPase-TAP is localized and degraded in the cytoplasm of yeast cells

FBPase-TAP was further used for localization studies of the fusion protein by indirect immunofluorescence. FBPase-TAP was seen to be localized throughout the cell but not in the vacuole before and after shift of cells from an ethanol- to a glucose-containing medium (Figure 2.14). No accumulation of the protein was seen in the nucleus. Since FBPase-TAP is degraded after addition of glucose to an ethanol-containing medium, shifting cells from ethanol to glucose-medium led to a decrease of the fluorescence signal of FBPase-TAP.

When the experiment was repeated under conditions when the proteasome is inhibited to prevent degradation of FBPase-TAP, no change in the localization pattern was visible, clearly indicating cytoplasmic localization of FBPase-TAP (Figure 2.15).

An additional way to enlighten the site of FBPase degradation was to look if FBPase degradation is dependent on some essential key nucleoporins of the nuclear pore complex. Supposing that it would be imported into the nucleus for a further degradation, FBPase would be likely imported through the nuclear import pathway and thus expected to bind the essential importin α Srp1 and maybe another nucleoporin Nup116. It was therefore interesting to check the degradation of FBPase in the Nup116 and Srp1 mutated strains.

As seen in Figures 2.16 and 2.17, in the W303-1B strain FBPase was degraded after shift of cells from ethanol- to glucose-containing medium, while in the gid1 deletion strain FBPase was stabilized. In the nucleoporins-mutated strains, FBPase was seen to be degraded like in
Figure 2.13: Degradation of FBPase-TAP is dependent on the proteasome and on the Gid complex. (A) W303-1B expressing FBPase-TAP was deleted for \( PDR5 \). The YLB33 (W303-FBPaseTAP-\( \Delta \)pdr5) strain was grown for 16h on YPEthanol and shifted to YPD. Proteasome involvement in FBPase-TAP degradation was analysed in a \( \Delta \)pdr5 strain using the proteasome inhibitor MG132. Samples were harvested 0, 30 and 120 minutes after shift to glucose, prepared for SDS and Western blot, and FBPase was detected via immunoblotting using the FBPase antibody. -: solvent, +: 100\( \mu \)M MG132. (B) Deletions of some \( GID \) genes (\( GID1, GID2, GID3, GID5, GID6, GID8 \) and \( GID9 \)) were done in the W303-FBPase-TAP strain. After overnight growth in ethanol-containing medium, the YLB26, YLB27, YLB28, YLB29, YLB30, YLB31 and YLB32 (\( \text{gid1, gid2, gid3, gid5, gid6, gid8 and gid9 respectively deletion} \)) strains were shifted to glucose-containing medium to trigger FBPase degradation. Samples were then taken 0 and 120 minutes after shift to glucose, prepared for SDS and western blot and FBPase-TAP was detected via immunoblotting using the FBPase antibody. PGK: 3-phosphoglycerate kinase, loading control.
Figure 2.14: **Wild type localization of FBPase-TAP.** The W303-1B and W303-FBPase-TAP strains were grown to induce FBPase degradation. After addition of glucose and intake of 0, 60 and 120 min samples, cells were prepared for indirect immunofluorescence (subsection 7.8.2 page 157) and examined under a Zeiss microscope using Nomarski, DAPI and UV fluorescence lamp. As negative control (Neg), the W303-1B expressing a non-tagged FBPase was used. FBPase-TAP was detected using the rabbit TAP antibody (1:500) and visualized by goat rabbit 594 IgG (1:500). DAPI: DNA dye, nuclear marker.
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Studies on catabolite degradation of FBPase

Figure 2.15: Localization of FBPase-TAP in a proteasome-inhibited strain. The YLB34 (W303-pdr5Δ) and YLB33 (W303-FBPaseTAP-pdr5Δ) strains were grown to induce FBPase degradation, treated with MG132 (+) for 30 minutes and shifted from an ethanol- to a glucose-containing medium. After intake of samples 0, 30 and 120 minutes after glucose-shift, cells were prepared for indirect immunofluorescence and examined under a Zeiss microscope. As negative controls for proteasome-inhibition (-), the same strains were treated with the solvent used for MG132 (ethanol). FBPase-TAP was detected by the rabbit TAP antibody (1:500) and visualized by goat rabbit 594 IgG (1:500). DAPI: DNA dyer, nuclear marker.
2.2 Subcellular localization of FBPase degradation

a wild type strain. These mutations in the importin α Srp1 and in the nucleoporin Nup116 do not seem to influence FBPase degradation, further confirming a cytosolic degradation of FBPase.

Figure 2.16: FBPase degradation does not depend on the nucleoporin Srp1 of the classical nuclear import pathway. After overnight growth in ethanol-containing medium, the W303-1B, *srp1-31* and *srp1-49* strains were shifted for one hour at 37°C. 2 % glucose was then added to cultures to trigger FBPase degradation and cells were let incubate at 37°C. Samples were taken 0, 30, 60 and 90 minutes after shift to glucose. After alcaline lysis, samples were processed for SDS and western blot. PGK: 3-phosphoglycerate kinase, loading control.

Figure 2.17: FBPase degradation does not depend on the nucleoporin Nup116 from the classical nuclear import pathway. After overnight growth in ethanol-containing medium, the W303-1B and *nup116-5* strains were grown and prepared as described in Figure 2.16. PGK: 3-phosphoglycerate kinase, loading control.
2.3 FBPase-GFP and FBPase-HA\textsubscript{3} fusion proteins escape catabolite degradation becoming subject of another degradation pathway

2.3.1 FBPase-GFP is not degraded after shift of cells from ethanol to glucose medium

In contrast to TAP-tagged FBPase, tagging of the enzyme with GFP or HA\textsubscript{3} led to a completely different behaviour of the enzyme. The protein FBPase-GFP was active since cells could grow on ethanol medium, but failed to be degraded upon glucose addition to cells (Figure 2.18).

![Figure 2.18: Functionality and expression of the FBPase-GFP fusion protein.](image)

The BY4743 wild type strain and the YLB01 strain were first grown on YPD medium. After a 16-hour growth on ethanol medium, cells were transferred to glucose medium and samples were taken before addition of glucose and 30, 60, 90 and 120 minutes after shift to glucose. *: cross-reaction; PGK: 3-phosphoglycerate kinase, loading control.

Interestingly direct fluorescence revealed a cytosolic as well as a nuclear localization of FBPase-GFP before and after shift of cells from ethanol to glucose medium. No fluorescence signal was found in the vacuole (Barbin, 2005).
2.3.2 **FBPase-HA$_3$** is not stable on a non-fermentable carbon source. Its degradation is dependent on the proteasome but not on the Gid complex.

The protein FBPase-GFP was active but not degraded after shift of cells from ethanol to glucose medium. The GFP fusion of FBPase was therefore not further used. The second tagged version of FBPase was a HA$_3$ fusion of FBPase.

At first sight the new HA$_3$-tagged FBPase seemed to behave like wild type FBPase since it displays wild type FBPase degradation kinetics (results not shown). However the chromosomal HA$_3$-tagged FBPase strain was pointed out to grow notably slower than the wild type strain. An explanation for such a behaviour may be an instability of the protein already on ethanol medium. Indeed, FBPase-HA$_3$ was degraded after treatment of cells with the translation inhibitor cycloheximide on an ethanol-containing medium (Figure 2.19 A). FBPase-HA$_3$ is thus not stable on ethanol medium. It has nevertheless to be pointed out that the protein FBPase-HA$_3$ was easily detectable after immunodetection with a HA antibody, but a lot harder even not at all with a FBPase antibody (from Entian and J. Juretschke respectively).

Because of this instability, it was interesting to know if the degradation of FBPase-HA$_3$ already occurring on ethanol was, like wild type FBPase, dependent on the Gid complex and on the proteasome. As observed in Figure 2.19 B, FBPase-HA$_3$ degradation on ethanol medium does depend on the proteasome since degradation was inhibited in proteasome mutants.

From a previous work, Gid4/Vid24, necessary for FBPase degradation, was shown to be absent in ethanol growing cells and to appear early after glucose addition to the medium (Santt et al., 2008). Since FBPase-HA$_3$ is degraded already on ethanol medium, its degradation on a non fermentable carbon source is not dependent on Gid4. As shown in Figure 2.19 C, after cycloheximide treatment of the *gid1*, *gid2* and *gid3* deletion strains, FBPase-HA$_3$ was degraded. FBPase-HA$_3$ degradation on ethanol medium is thus not dependent on the Gid complex.
Figure 2.19: **FBPase-HA**₃ is not stable on a non-fermentable carbon source. Its degradation is dependent on the proteasome but not on the Gid complex. (A-B-C) Cycloheximide chase analysis. The WCG4, YLB08 (WCG4-FBPase-HA₃), YLB10 (WCG4 pre1-1 pre2-2-FBPaseHA₃), YLB43 (WCG4-FBPase-HA₃-gid1Δ), YLB16 (WCG4-FBPase-HA₃-gid2Δ) and YLB12 (WCG4-FBPase-HA₃-gid3Δ) strains were grown on YPD medium. After 16 hours growth at 25°C on YPEtOH, 100 µg/mL cycloheximide was added to cell cultures and samples were taken at the indicated time points after treatment with cycloheximide. PGK: 3-phosphoglycerate kinase, loading control.
2.3.3 Determination of E2s and E3s involved in degradation of FBPase-HA

FBPase-HA was previously shown to be unstable on ethanol medium and in this way differs from wild type FBPase. The degradation of FBPase-HA on a non-fermentable carbon source was moreover discovered to depend on the proteasome. Most substrates degraded by the proteasome are ubiquitinated and for their ubiquitination, follow the ubiquitin-proteasome pathway which requires the activity of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), ubiquitin ligases (E3) and potentially E4s (subsection 1.2.3 page 22). It was thus interesting to determine which E1, E2 and E3 enzymes are required for degradation of FBPase-HA.

As described previously, degradation of FBPase-HA on ethanol medium is not dependent on the Gid complex. Gid3, a subunit of the Gid complex, was discovered to act as an ubiquitin-conjugating enzyme, E2 (Schüle et al., 2000). FBPase-HA is still degraded on ethanol medium in a gid3Δ strain, after treating cells with cycloheximide (Figure 2.19 C). FBPase-HA degradation is thus not dependent on the ubiquitin-conjugating enzyme Gid3. Moreover, since the Gid complex was recently discovered by our lab to act as an E3 (Santt et al., 2008), FBPase-HA is not degraded by this novel ubiquitin ligase that constitutes the Gid complex.

A possible reason for the degradation of HA-tagged FBPase on ethanol medium is a mal-folding of this protein. As described later FBPase-HA, like FBPase-GFP, is localized in the nucleus of cells (see next subsection). Therefore two E3 candidates for FBPase-HA degradation would be Doa10 and San1. Doa10 is an ER-associated ligase and works in the ERAD pathway in cooperation with the ubiquitin-conjugating enzymes Ubc7 and Ubc6 (Hampton, 2002). This yeast transmembrane ubiquitin ligase, also known as Ssm4, resides in the endoplasmic reticulum (ER), but it attaches ubiquitin to soluble proteins that concentrate in the nucleus. Localization of Doa10 to the inner nuclear membrane is necessary for nuclear substrate degradation (Deng and Hochstrasser, 2006). San1 is a nuclear E3 ligase and a key enzyme in nuclear PQC (protein quality control) pathway which works together with two E2s, Ubc1 and Cdc34. San1 is suggested to act as the last line of proteolytic defense against the deleterious accumulation of aberrant proteins in the nucleus (Gardner et al., 2005).
If FBPase-HA₃ degradation is dependent on San1 or Doa10 on ethanol medium, treating cells with the translation inhibitor cycloheximide on an ethanol-containing medium in a san1 or doa10-deletion strain should lead to a stabilisation of FBPase-HA₃ levels. As observed in Figure 2.20, in the san1 and doa10 deletion strains FBPase-HA₃ was degraded like in the wild type after cycloheximide treatment. FBPase-HA₃ degradation on ethanol medium is thus not dependent on the two E3s, San1 and Doa10.

Figure 2.20: FBPase-HA₃ degradation on ethanol medium is not dependent on the two E3s, San1 and Doa10. The YLB08 (WCG-FBPase-HA3), YLB37 (WCG-FBPase-HA3-san1Δ) and YLB38 (WCG-FBPase-HA3-doa10Δ) strains were grown on YPD medium. After 16 hours growth at 25°C on YPEtOH, 100 µg/ml cycloheximide was added to cell cultures and samples were taken 0, 30 and 120 minutes after treatment with cycloheximide.

2.3.4 FBPase-HA₃ is localized and degraded in the nucleus of yeast cells

The localization of the protein FBPase-HA₃ was done by indirect immunofluorescence in the YLB08 (WCG-FBP1HA3) strain. As seen in Figure 2.21, FBPase-HA₃ was mainly located in the cytosol and some was found in the nucleus after 10 minutes glucose-shift. Up to 10 minutes glucose-shift, the signal was considerably decreased, since FBPase-HA₃ was degraded. 30 minutes after glucose-shift, FBPase-HA₃ signal was too weak to be able to conclude on the localization of FBPase-HA₃. No signal of FBPase-HA₃ was seen in the vacuole.

To better localize FBPase-HA₃ degradation, FBPase-HA₃ was localized through indirect immunofluorescence in the YLB10 proteasome-mutated WCG-pre1-1-pre2-2-FBP1HA3 strain.
Figure 2.21: Localization of FBPase-HA$_3$ in the WCG4-FBP1HA3 strain through indirect immunofluorescence. The WCG and YLB09 strains were grown in an ethanol-containing medium. 2% glucose was then added to the cultures and samples were taken after 0, 10 and 30 minutes glucose-shift. Cells were then prepared for indirect immunofluorescence and examined under a Zeiss microscope using Nomarski, DAPI and UV fluorescence lamp. As negative control the WCG 0min was used. For the detection of FBPase-HA$_3$ protein, the mouse HA antibody was used as primary antibody and mouse Alexa fluor 488 antibody as secondary antibody.
In such a strain, FBPase-HA$_3$ should be notably slower degraded and then let the possibility to determine the localization of FBPase-HA$_3$ and of its degradation in the yeast cell. Figure 2.22 shows that FBPase-HA$_3$ mainly localized in the nucleus and a bit in the cytosol both before and after 10, 30 and 60 minutes glucose-shift. A stronger signal in the nucleus was seen 10 minutes after glucose-shift, but after 30 minutes shift to glucose, the signal of FBPase-HA$_3$ was decreased and still seen in the nucleus and the cytosol. After 60 minutes glucose-shift, the signal of FBPase-HA$_3$ was considerably diminished, since FBPase-HA$_3$ is degraded even if notably slower than in the wild type YLB08 strain. No signal of FBPase-HA$_3$ was seen in the vacuole and a very slight signal was localized in the cytosol. From these results, it can be proposed that FBPase-HA$_3$ is imported into the nucleus of cells and further degraded in this compartment by the proteasome.
2.3 FBPase-GFP and FBPase-HA<sub>3</sub>, another degradation pathway

Figure 2.22: Localization of FBPase-HA<sub>3</sub> in the WCG4-pre1-1-pre2-2-FBP1HA<sub>3</sub> strain through indirect immunofluorescence. The WCG4-pre1-1-pre2-2 and YLB10 strains were grown in ethanol-containing medium and prepared as described in Figure 2.21.
Part IV

Discussion
When *Saccharomyces cerevisiae* cells are grown on a non-fermentable carbon source (e.g. ethanol or acetate), fructose-1,6-bisphosphatase (FBPase) is synthesized. Under these conditions, FBPase has a half-life time of 90 hours. The addition of glucose to the medium however leads to a quick change from gluconeogenesis to glycolysis, and thus to a rapid inactivation of FBPase (Schork et al., 1995, 1994a; Holzer, 1976; Gancedo, 1971). Catabolite inactivation encompasses repression of the *FBP1* gene, decrease of the enzyme activity by phosphorylation, and allosteric inhibition by fructose-2,6-bisphosphate and AMP (Mazón et al., 1982; von Herrath and Holzer, 1988). After inactivation, FBPase is polyubiquitinated and, within 20-30 minutes, rapidly degraded by the 26S proteasome (Schork et al., 1994a,b, 1995; Hämmerle et al., 1998; Holzer, 1989). FBPase polyubiquitination occurs via the ubiquitin-conjugating enzymes Ubc1, Ubc4, Ubc5 and Ubc8/Gid3 in conjunction with a novel ubiquitin ligase called the Gid (glucose induced degradation deficient) complex (Santt et al., 2008; Regelmann et al., 2003). This 600 kDa Gid complex is composed of seven subunits (Gid1, Gid2, Gid4, Gid5, Gid7, Gid8 and Gid9) (Ho et al., 2002; Pitre et al., 2006; Krogan et al., 2006) and binds FBPase already under gluconeogenic conditions. After addition of glucose to the environment, subunit Gid4 is synthesized, interacts with the Gid-E3 and triggers FBPase polyubiquitination and degradation by the 26S proteasome (Santt et al., 2008).

A better understanding of FBPase proteasomal catabolite degradation in the budding yeast *Saccharomyces cerevisiae* encompasses the study of the mechanism of FBPase proteasomal degradation and thus the study of the Gid and other proteins involved in this pathway, but also the identification of the subcellular localization of FBPase degradation by the 26S proteasome.

**Subcellular localization of the degradation of fructose-1,6-bisphosphatase in yeast**

Until now the localization of FBPase degradation has been controversial. Catabolite degradation of FBPase has originally been described to take place in the vacuole of the cell (Holzer, 1976; Molano and Gancedo, 1974). However the invagination of bulk proteins into the vacuole is a relatively unspecific and slow process (Thumm and Wolf, 1998). Further studies in the laboratory of D. H. Wolf revealed that the degradation process of FBPase was dependent on the ubiquitin-proteasome machinery (Schork et al., 1995, 1994a,b). However, publications from the research group of H.L. Chiang favoured a vacuolar degradation of FBPase. In this
pathway, FBPase is described to be engulfed into vesicles in the cytoplasm and transported to the vacuole where it is degraded by unspecific vacuolar proteases (Chiang and Schekman, 1991; Huang and Chiang, 1997). In the laboratory of H.L. Chiang, cells are starved for 48 hours or more on acetate, a poor carbon source. Such long starvation periods have been shown to induce the unspecific bulk process of autophagocytosis (Thumm, 2000). In our lab cells were grown overnight in ethanol-containing media before glucose addition. A recent publication finally demonstrated that degradation of the gluconeogenic enzyme fructose-1,6-bisphosphatase was mediated by two distinct proteolytic pathways and signalling events: FBPase was degraded outside the vacuole (most likely in the proteasome) when glucose was added to cells that were grown in low glucose media containing ethanol for a short period of time. By contrast, cells grown in the same low glucose media or acetate for a longer period of time degraded FBPase in the vacuole in response to glucose (Hung et al., 2004).

To better understand the degradation mechanism of FBPase by the 26S proteasome, the subcellular localization of FBPase and of its degradation in yeast was investigated. Several publications suggested a nuclear localization of FBPase. In mammals a nuclear localization of muscle and liver FBPase was reported (Gizak et al., 2005; Yáñez et al., 2003, 2004) and this localization was described to be modulated by metabolic conditions. Addition of glucose, insulin and dihydroxyacetone triggered the translocation of liver FBPase from the cytosol to the nucleus of hepatocytes (Yáñez et al., 2004). Moreover all known muscle FBPases (human, rabbit, rat and mouse) possess a characteristic KKKKG sequence in their primary structure, which may act as a nuclear localization sequence (NLS) targeting the protein to the nucleus (Gizak and Dzugaj, 2003). Whereas Gizak and Dzugaj discussed that rat liver FBPase does not contain such a specific canonical nuclear localization sequence compared to the muscle isoform, Yáñez et al. suggested that the liver FBPase has other classical NLSs (PXKRKXX) allowing the translocation of FBPase into the nuclei (Yáñez et al., 2003). Finally yeast FBPase was found to interact with the karyopherin α Srp1 and the karyopherin β Kap95 from the nuclear pore complex (Ito et al., 2001). Interactions were also discovered between the yeast E3 subunits Gid4 and Gid7 (two subunits of the Gid-E3 complex) with Nup116, a nucleoporin which itself interacts with Kap95 in the classical nuclear transport machinery.

An easy way to enlighten the site of FBPase degradation was to determine whether the degradation of fructose-1,6-bisphosphatase requires essential key nucleoporins of the nuclear
pore complex. Proteins imported into the nucleus are indeed usually dependent on some key components of the classical nuclear import pathway like the importin α Srp1 and the nucleoporin Nup116. In nucleoporin mutants FBPase however still displayed a wild type degradation (Figures 2.16 and 2.17), suggesting no specific role of these nucleoporins in FBPase proteasomal degradation.

FBPase localization was further investigated by indirect immunofluorescence microscopy using a C-terminal TAP fusion of FBPase, the only functional tagged version of FBPase (section 2.3 page 88). FBPase-TAP was seen to be localized throughout the cell except in the vacuole before and after shift of cells from an ethanol- to a glucose-containing medium (Figure 2.14). No accumulation of the protein was seen in the nucleus, even when FBPase-TAP degradation was slowed down by inhibiting the proteasome (Figure 2.15). Thus FBPase located and was degraded in the cytoplasm but not in the nucleus of yeast cells. Interestingly under our growth conditions (growth of cells in ethanol-containing medium before glucose addition), FBPase was never seen in the yeast vacuole or in a vesicular compartment by indirect immunofluorescence microscopy, underlining a cytoplasmic localization of the catabolite degradation process (Schüle et al., 2000). As a conclusion, when yeast cells are grown overnight on ethanol-containing medium as a derepression medium, FBPase is degraded through the ubiquitin-proteasome pathway and the localization of its catabolite degradation is neither vacuolar, nor nuclear, but cytosolic. This proteasomal catabolite degradation of FBPase in the yeast cytosol and more precisely the inactivation of the enzyme by the Gid complex, is supported by a cytoplasmic localization of most Gid proteins, as shown for Gid1 and Gid8 from indirect immunofluorescence microscopy experiments (Figure 1.11 A, B) and as described by Huh et al. (2003).

In a proteome wide localization study, GFP fusions of GID1, GID3, GID4, GID5, GID6, GID7, GID8 and GID9 encoded proteins were described to be localized in the cytoplasm but also in the nucleus of cells (Huh et al., 2003). Further work thus focused on the subcellular nuclear localization of these proteins. Localization of some Gid proteins could be determined by fluorescence microscopy. These studies confirmed previous data on the cytoplasmic but also on the nuclear localization of Gid1 and Gid8 (Figure 1.11 A, B). The other Gid proteins could not be further localized by this method. A biochemical method was therefore applied, isolating yeast nuclei by differential Ficoll gradient cell fractionation. However the nuclear
fractions could not be properly separated from vacuolar fractions, by this not permitting to give any successful conclusions on the localization of the Gid proteins. Although no localization of Gid2 could be done, Gid2 was nevertheless found to possess a nuclear localization sequence (NLS): a nuclear localization of Gid2 is thus suspected. A deleted NLS should fail to support the import of the protein into the nucleus. Unfortunately this hypothesis could not be tested since this NLS sequence, even though it was shown to facilitate the import of GFP into the nucleus (Figure 1.9), is necessary for the stability of Gid2 (Figure 1.10).

Since all Gid proteins are part of the same complex (Santt et al., 2008) and since Gid2 has a nuclear localization sequence, Gid2 may be suspected to transport the Gid complex into the nucleus. However Gid1 and Gid8 still localized in the cytoplasm but also in the nucleus of cells in a gid2Δ strain (results not shown). Thus Gid2 is likely to have no role in the nuclear localization of Gid1 and Gid8.

Bioinformatics studies predict the interaction of some Gid proteins with histone acetylation factors (Wolfgang Hilt, unpublished results). This suggests a nuclear localization of the Gid complex, and suggests additional functions of Gid proteins, especially in transcription.

For subcellular localization of FBPase degradation, FBPase was C-terminally fused with several tags. FBPase-TAP was the only fusion protein degraded like the untagged FBPase. Other tagged versions of FBPase were not functional. FBPase-GFP was active as cells could grow on ethanol medium, but was not degraded anymore when cells were shifted from an ethanol- to a glucose-containing medium (Figure 2.18). This behaviour is probably due to the GFP moiety linked to FBPase which cannot be degraded by the proteasome (Liu et al., 2003). An additional tagged form of FBPase, a HA3 fusion, was also active, but discovered to be unstable already on a non-fermentable carbon source (Figure 2.18 A). Interestingly, its degradation on ethanol medium was shown to depend on the proteasome but not on the Gid complex (Figure 2.18 B-C). Both FBPase-GFP and FBPase-HA3 were localized in the cytoplasm and in the nucleus of cells, differing from wild type FBPase (Figures 2.21 and 2.22).

Most substrates degraded by the proteasome are ubiquitinated via an ubiquitin-activating enzyme E1, an ubiquitin-conjugating enzyme E2, an ubiquitin ligase E3 and potentially an E4. In contrast to wild type FBPase, degradation of FBPase-HA3 was not dependent on Gid3/Ubc8, on Gid6, a yeast deubiquitinating enzyme and on the Gid ubiquitin ligase complex.
Two other E3 candidates for FBPase-HA\textsubscript{3} degradation were the Doa10 and San1 ubiquitin ligases which are required for the degradation of nuclear substrates (Gardner et al., 2005; Deng and Hochstrasser, 2006). FBPase-HA\textsubscript{3} degradation on ethanol medium was thus tested in \textit{san1\Delta} and \textit{doa10\Delta} strains. However it did not depend on these two nuclear E3s (Figure 2.20). Recently Ubr1, the E3 ligase of the N-end rule pathway, has been shown to be responsible for targeting misfolded cytosplasmic protein to proteasomal degradation (Eisele and Wolf, 2008). FBPase-HA\textsubscript{3} may require this Ubr1 ubiquitin ligase for its further degradation, but may also resort to E1, E2 and E3 enzymes which have still to be discovered.

While a number of enzymes require ubiquitination for their further proteasomal degradation, certain proteins, inherently unstable, have been described to undergo ”default” degradation by the 20S proteasome without being ubiquitinated. Indeed some unstructured regions present in proteins may facilitate and serve as a signal for degradation ”by default” by the core 20S proteasomes (Asher et al., 2006). FBPase-HA\textsubscript{3} may not require ubiquitination for its degradation by the 26S proteasome as well. It would therefore be interesting to perform ubiquitination assays to determine the state of ubiquitination of the protein FBPase-HA\textsubscript{3}.

**Mechanism of the ubiquitin-proteasome dependent catabolite degradation of fructose-1,6-bisphosphatase in yeast**

Recently a human orthologue of the yeast Gid complex was found and called the human CTLH complex. Indeed RanBPM, Muskelin, p48EMLP and p44CTLH, proteins building a 20S large complex renamed as the large CTLH complex, were found to have LisH/CTLH motifs which are present in proteins involved in microtubule dynamics, cell migration, nucleokinesis and chromosome segregation. These functions overlap with functions suggested for the RanGTPase cycle (Umeda et al., 2003). Interestingly some notable similarities were revealed between RanBPM, p44CTLH, ARMC8\textalpha, and p48EMLP and Gid1/Vid30, Gid2, Gid5/Vid28, and Gid9 respectively (Kobayashi et al., 2007). Besides CTLH and LisH domains common to Gid1 and Gid8 proteins, one more shared domain also of unknown function is the CRA domain, a domain found in the Ran binding proteins RanBPM and RanBP10. The closely related proteins, RanBPM and RanBP10, are distinct RanBP family members in that they appear to have no effect on nuclear trafficking and do not interact with Ran \textit{in vivo}. Unlike most other identified Ran binding proteins, RanBPM does not contain the consensus Ran-binding
domain responsible for their role in the nuclear pore complex machinery. Its precise role still
remains unknown but RanBPM seems to act more as a scaffolding protein possessing many
characteristics of this kind of proteins, including protein-interaction motifs, a cytoskeletal-
binding domain and multiple canonical docking sites for signaling intermediates (Murrin and
Talbot, 2007; Denti et al., 2004). For further investigation on the function(s) of this CRA
domain, an alignment of the CRA sequences from different species was done to determine
amino acids conserved through evolution (Figure 1.2). In yeast this CRA domain is only found
in two proteins, Gid1 and Gid8. Mutations of two highly conserved amino acids in Gid8 were
introduced and shown to be sufficient to stop FBPase degradation when cells were shifted from
a non-fermentable to a fermentable carbon source (Figure 1.4). Since the mutated Gid8 showed
a wild type localization in the cytosol and in the nucleus of cells (results not shown), the CRA
domain is likely not involved in a “targeted” nuclear localization of Gid8. Interestingly the
CRA-mutated Gid8 still binds FBPase (Figure 1.6) and the putative core complex subunit
Gid1 of the Gid complex (Figure 1.5), but however fails to ubiquitinate FBPase (Figure 1.7).
This suggests that the Gid complex is probably still built together but that its molecular
conformation is modified, thus leading to a loss of its ubiquitin ligase activity.

Interestingly when the CTLH domain is deleted in the Gid1 protein, Gid1 still binds to
Gid2 and Gid8 but not anymore to Gid7, suggesting that the CTLH domain is necessary for
binding of Gid1 to Gid7. When the LisH domain is deleted in Gid1, Gid1 binds to Gid7 but
not to Gid2 and Gid8, indicating that the LisH domain is required for binding of Gid1 to Gid2
and Gid8. The SPRY domain was shown not to be necessary for binding of Gid1 to Gid2,
Gid7 and Gid8. Moreover as observed for the CRA-mutated Gid8, FBPase ubiquitination is
strongly impaired in mutants of Gid1 in which either the SPRY, the CTLH or the LisH domain
was deleted, suggesting again that if the complex is still built together, it probably does not
have the same molecular conformation and thus loses its ubiquitin ligase activity (Schreiner,
2009).

While looking for protein-protein interactions in protein complexes in glucose-grown cells,
Krogan et al. (2006) and Pitre et al. (2006) identified a complex composed of all Gid proteins
except Gid7, which was termed the Vid30 complex (Vid30c). They however discovered addi-
tional proteins of unknown function, YDL176W and YDR255C. Pitre et al. (2006) proposed
that the Vid30 complex may consist of a core and a surrounding complex. Gid1, Gid8 and
Gid5 together with the yet unknown protein YDL176W would build up the core complex.
Gid4, Gid9 and Gid2 seemed to only interact with Gid1 and Gid5 but not with each other, suggesting that they would belong to the surrounding complex of Vid30c. Moreover the deletion of a member of the core component was observed to interfere with the formation of the Vid30 complex, whereas deletion of a member of the surrounding complex had little effect on the formation of the Vid30 complex (Pitre et al., 2006). In my work studies of the Gid complex focused on the proteins Gid1, Gid2 and Gid8, three putative components of the core complex, and on their stability on a non-fermentable carbon source. Gid1 was found to be stable on ethanol-containing medium in a gid2Δ strain but not in a gid8Δ strain (Figure 1.12). In the same way, Gid8 was stable on ethanol-containing medium in a gid2Δ strain but not in a gid1Δ strain (Figure 1.13). Gid2 was shown not to be stable in a gid1Δ strain and in a gid8Δ strain (Figure 1.14). All these observations suggest that when one of the proteins of the core complex is missing, the structure of this core complex and probably of the Gid complex in general will break down. This probably leads to degradation of each subunit of the complex. These results rather support the model proposed by Pitre et al. (2006), with a core complex composed of minimum two proteins Gid1 and Gid8, and a secondary complex to which Gid2 would belong. To further underline this hypothesis, it would be necessary to test the stability of Gid5, on ethanol medium, a putative additional component of the core of the Gid complex.

Further work focused on a detailed elucidation of FBPase degradation pathway itself. FBPase was previously described to be polyubiquitinated and further degraded by the 26S proteasome. One major step still remained to be clarified: how is yeast FBPase, once polyubiquitinated by the Gid complex, delivered to the 26S proteasome. Ritthenhouse and Noda et al. found that FBPase is a tetramer constituted of subunits of 40 kDa (Rittenhouse et al., 1984, 1986; Noda et al., 1984). This FBPase homotetramer must be dissociated into monomers before the single polypeptides can be degraded. A common chaperone reported to disassemble protein complexes and segregate proteins from their binding partners is the Cdc48 complex, a member of the AAA ATPase family of molecular chaperones (Jentsch and Rumpf, 2007; Beuron et al., 2006). Like other members of this family, Cdc48 exists as a hexamer and hydrolyses ATP to perform its diverse cellular functions. These functions include several ubiquitin-dependent processes like the endoplasmic reticulum-associated degradation (ERAD) (Kostova and Wolf, 2003; Ahner and Brodsky, 2004), membrane fusion, transcription factor activation (Rape et al.,
spindle disassembly and even organization of the nucleus after division of metazoan cells (Ramadan et al., 2007). Cdc48 might thus be an active factor in FBPase catabolite degradation as well. Catabolic chase and pulse chase analysis showed that FBPase degradation was indeed impaired in a Cdc48 mutated strain (Figure 2.1 A and B), whereas FBPase polyubiquitination was not (Figure 2.1 C). This result indicates that the Cdc48 complex is essential for degradation of fructose-1,6-bisphosphatase and acts after polyubiquitination of the enzyme by the Gid complex. Furthermore fructose-1,6-bisphosphatase is the first natural cytoplasmic substrate that requires the Cdc48 complex for its degradation.

\textit{In vivo} Cdc48 requires substrate-recruiting cofactors to exert its function: the Ufd1-Npl4 heterodimer and proteins of the Ubx family. The separation of fundamentally distinct cellular Cdc48 functions essentially resides in the recruitment of these cofactors. Mutants of Npl4 were shown to display a strong defect in FBPase catabolite degradation (Figure 2.2 A-B). The same result was observed in mutants of Ufd1 (Figure 2.3 A-B). This was actually not astonishing since the binding of Ufd1 to Cdc48 is a prerequisite for the interaction of Cdc48 with Npl4 (Bruderer et al., 2004). Obviously, it is the Cdc48\textsuperscript{Ufd1-Npl4} complex which is required for FBPase degradation. This trimeric complex has in many cases been identified to have protein-protein and membrane-protein segregating activity (Ye et al., 2001; Jarosch et al., 2002; Braun et al., 2002; Bays et al., 2001; Kostova and Wolf, 2003; Ghislain et al., 1996; Rape et al., 2001). As expected from the above mentioned result, a Cdc48\textsuperscript{Shp1/Ubx1} complex, which is required for homotypic membrane fusion (Kondo et al., 1997), has no function in catabolite degradation of FBPase. With the exception of Ubx4, deletions of members of the Ubx family did not show any impairment of FBPase degradation (Figures 2.4 and 2.5). Ubx4 seems to have some influence on the degradation process (Figure 2.6 A-B). In ER-associated protein degradation the function of Ubx4 has been proposed to reside in the delivery of the ubiquitinated substrates bound to the trimeric Cdc48\textsuperscript{Ufd1-Npl4} complex to downstream components and the proteasome (Alberts et al., 2009). Ubx4 might have a similar function in catabolite degradation of FBPase. Interestingly, as observed in a Cdc48 mutant, FBPase was shown to be still polyubiquitinated in npl4, ufd1 and ubx4 mutants (Figures 2.2 C, 2.3 C and 2.6 C), indicating that the whole Cdc48\textsuperscript{Ufd1-Npl4} machinery acts at a FBPase post-ubiquitination step upstream of the 26S proteasome.

Cdc48 not only functions as a segregase but together with cofactors also controls the degree of ubiquitination of the bound substrates. This activity is brought about by so-called substrate-
processing cofactors which include the ubiquitin ligase (E4) Ufd2, the deubiquitinating enzyme Otu1 and the protein Ufd3/Doa1 (Jentsch and Rumpf, 2007). Neither Ufd2 nor Otu1 are involved in FBPase catabolite degradation (Figure 2.7 A and B). On the other hand, a ufd3 mutant displays a striking impairment of amounts of FBPase ubiquitination, and of FBPase degradation (Figure 2.8). This result might however be an indirect effect since the loss of Ufd3 in yeast has been reported to result in the depletion of cellular ubiquitin (Ghislain et al., 1996; Rumpf and Jentsch, 2006; Mullally et al., 2006).

FBPase has been shown to require the Gid complex for its polyubiquitination, but not the Cdc48 complex, suggesting that the latter acts after polyubiquitination of FBPase but upstream of the 26S proteasome. Cdc48 may bind to the Gid complex and pulls the polyubiquitinated FBPase away from the ubiquitin ligase. Here it has been shown that Cdc48 co-immunoprecipitates with the subunit Gid8 of the Gid complex (Figure 2.9 A). Since Gid8 is part of the Gid complex (Krogan et al., 2006; Pitre et al., 2006; Ho et al., 2002), this result indicates that Cdc48 and the Gid complex interact. From these results it may be suspected that the Cdc48Ufd1Npl4 complex pulls FBPase away from the Gid complex and escorts it to the proteasome. To confirm this hypothesis, it would be interesting to study interactions between FBPase and the Cdc48 complex itself.

Ubiquitin (Ub) regulates important cellular processes through covalent attachment to its substrates. The fate of a substrate depends on the number of ubiquitin moieties conjugated, as well as the topology of the polyubiquitin chains formed. A major function of ubiquitin action is to regulate the in vivo half-life of its targets. Once a multi-ubiquitin chain is attached to a substrate, it must be shielded from deubiquitinating enzymes for recognition by the 26S proteasome. Some Ub-binding proteins have been shown to deliver ubiquitinated substrates to the proteasome. Dsk2 and Rad23 belong to the family of proteins which preferentially bind ubiquitinated substrates and polyubiquitin chains through an ubiquitin-associated (UBA) motif (Medicherla et al., 2004). The protein Ddi1 was also reported as a UBL-UBA ubiquitin receptor that mediates proteasomal degradation of ubiquitinated cargoes. Besides the Cdc48Ufd1Npl4 complex, efficient FBPase degradation was shown to require the Dsk2 and Rad23 proteins (Figure 2.10 A), but not Ddi1 (Figure 2.11). Dsk2 and Rad23 might thus shuttle polyubiquitinated FBPase from Cdc48 to the proteasome, as already suggested for other substrates in diverse studies (Chen and Madura, 2002; Rao and Sastry, 2002).

The requirement of Rad23 and Dsk2 has often been reported to be linked to the Cdc48-
Ufd1-Npl4 machinery but also to the Ufd2 E4 enzyme (Richly et al., 2005). Ufd2 has been proposed to polyubiquitinate and rearrange the length of the ubiquitin chain bound to the substrate so that it can further be recognized by Rad23 and Dsk2 (Raasi et al., 2004; Richly et al., 2005). Since the Ufd2 protein is not required for degradation of FBPase, it is likely that the length of the ubiquitin chain bound to FBPase does not require to be reprocessed for a putative recognition by Rad23 and Dsk2. These latter proteins may thus bind to Cdc48 either directly or indirectly through the Gid complex.

Another gluconeogenic enzyme, PEPCK, which has also been shown to be composed of four identical subunits (Tortora et al., 1985; Stucka et al., 1988), is also subject to Gid complex-dependent proteasomal catabolite degradation (Santt et al., 2008). As shown in Figure 2.12, the degradation of PEPCK is notably slower in cdc48, npl4 and ufd1 mutated strains compared to the wild type strain. The role of the Cdc48Ufd1-Npl4 machinery in catabolite degradation is thus not restricted to FBPase but extends to other gluconeogenic enzymes.

Taken together, these data suggest the following working model (Figure 23). On a non-fermentable carbon source, the FBPase tetramer is bound to a Gid-Cdc48Ufd1-Npl4 “supercomplex”. After addition of glucose, Gid4 is synthesized and binds to the Gid complex which can then polyubiquitinate FBPase. Polyubiquitinated FBPase is then segregated away from the Gid complex and dissociated by the Cdc48Ufd1-Npl4 complex, followed by binding of the polyubiquitinated subunits to Rad23 and Dsk2, which direct them to the proteasome where they are finally degraded. Further work is however required to fully address the role of these proteins in the catabolite degradation of FBPase.
Figure 23: **FBPase requires the Cdc48^{Ufd1-Npl4} machinery for its degradation.**

On a non-fermentable carbon source, the FBPase tetramer binds to the Gid- and the Cdc48^{Ufd1-Npl4} complexes. After addition of glucose, Gid4 is synthesized and binds to the Gid complex which can then act as an ubiquitin ligase. Polyubiquitinated FBPase then associates with the Cdc48^{Ufd1-Npl4} complex, which is proposed to pull FBPase away from the Gid complex, dissociate the enzyme into monomers, and probably together with the ubiquitin-binding proteins Rad23 and Dsk2, ultimately direct the polyubiquitinated subunits to the proteasome where they are degraded.
Part V

Materials and methods
Chapter 1

Material

1.1 Chemicals, materials and their suppliers

<table>
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<th>Supplier</th>
<th>Chemicals, materials and their suppliers</th>
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<tr>
<td>Amersham Biosciences</td>
<td>ECL\textsuperscript{TM}-System, Hyperfilm\textsuperscript{TM} ECL, Protein A Sepharose\textsuperscript{TM} CL-4B, Glutathion Sepharose\textsuperscript{TM} 4B, [(\alpha)-35S]-L-methionine</td>
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<td>Calbiochem</td>
<td>MG132</td>
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<td>Difco</td>
<td>Bacto-agar, Bacto-peptone, Bacto-yeast extract</td>
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<td>Fermentas</td>
<td>Miniprep Kit, Page ruler prestained protein ladder plus</td>
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<td>Fluka</td>
<td>Formaldehyde</td>
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<td>Jena Bioscience</td>
<td>Nourseothrycine</td>
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<td>MWG-Biotech</td>
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<td>Peqlab</td>
<td>Gel-extraction kit</td>
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### 1.2 Laboratory equipment

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<th>Company</th>
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</tr>
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<tr>
<td>Beckman Coulter, USA</td>
<td>Table ultracentrifuge Optima™ TLX</td>
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<tr>
<td>Biorad</td>
<td>Acrylamide electrophoresis apparatus, DNA-gel electrophoresis chamber, Dry blot apparatus, Protein gel electrophoresis apparatus</td>
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<td>Eppendorf, Germany</td>
<td>Centrifuge 5804 R</td>
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<td>Fröbel, Germany</td>
<td>Semi-dryer blotting apparatus</td>
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<tr>
<td>Heidolph Instruments, Schwabach</td>
<td>Overhead rotator REAX2, Overhead shaker REAX 2</td>
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<tr>
<td>Heraeus, Germany</td>
<td>Ultracentrifuge Sorvall SS-34 and GS-3 rotor</td>
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<tr>
<td>IKA-Labortechnik, Germany</td>
<td>Magnetic stirrer Ikamag® REO, Vortexer</td>
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<td>Kontron, Germany</td>
<td>Ultracentrifuge Centrifon T-124,</td>
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<td>Ultracentrifuge Kontron-Instruments,</td>
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<td>Apparatus</td>
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<tr>
<td>Kühner, Switzerland</td>
<td>Shakers</td>
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<td>Mettler, Giessen, Switzerland</td>
<td>Analyse weighing machines, Balance AE 163, Balance PM460</td>
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<td>Millipore, Germany</td>
<td>Ion exchanger Milli-Q Plus</td>
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<td>Molecular Dynamics, Sunnyvale, USA</td>
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<td>Pall corporation, USA</td>
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<td>Protec Medizintechnik, Oberstenfeld</td>
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<td>Roth, Germany</td>
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<td>Snijders Scientific, Tilburg, NL</td>
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<td>Schott, Germany</td>
<td>pH-Meter CG 832</td>
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<td>Scientific Industries, Bohemia, USA</td>
<td>Overhead shaker Roto Shake Genie</td>
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<td>Stratagene, Germany</td>
<td>PCR machine Robocycler® Gradient 40</td>
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<td>Zeiss, Germany</td>
<td>Microscope</td>
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Chapter 2

Protein biochemistry

2.1 Antibodies

2.1.1 Antibodies used for indirect immunofluorescence

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<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
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<td>Mouse anti-HA, monoclonal, clone 16B12</td>
<td>1:100</td>
<td>Roche Applied Science</td>
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<tr>
<td>Rabbit anti-TAP antibody, polyclonal</td>
<td>1:500</td>
<td>Open Biosystems</td>
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<tr>
<td>cy3-conjugated AffiniPure Fab fragment goat anti-mouse IgG (H+L)</td>
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<td>1:500</td>
<td>Invitrogen</td>
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<td>Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) highly cross-adsorbed</td>
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<td>Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) highly cross-adsorbed</td>
<td>1:500</td>
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Table 2.1: Antibodies used for indirect immunofluorescence
### 2.1.2 Antibodies used for Western blot, coIP, pulse-chase

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<tr>
<th>Antibody</th>
<th>Dilution for Western blot</th>
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<tr>
<td>Mouse anti-HA, monoclonal, clone 16B12</td>
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<td>Mouse anti-Phosphoglycerate (PGK), monoclonal</td>
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<td>Molecular Probes, Leiden</td>
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<td>Mouse anti-Nop1, monoclonal</td>
<td>1:10,000</td>
<td>Abcam</td>
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<tr>
<td>Rabbit anti-fructose-1,6-bisphosphatase (FBPase), polyclonal</td>
<td>1:5,000</td>
<td>J. Juretschke, University of Stuttgart</td>
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<tr>
<td>Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L)</td>
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<td>Rat anti-HA, monoclonal, clone 3F10</td>
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<td>Roche Applied Science</td>
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<td>Mouse anti-rabbit, monoclonal</td>
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<td>Goat anti-rabbit IgG (H+C) HRPO conjugated</td>
<td>1:5,000</td>
<td>Caltag Laboratories</td>
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<tr>
<td>Mouse anti-carboxypeptidase Y, monoclonal</td>
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<td>Dianova; Hamburg</td>
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<td>Rabbit anti-Cdc48, polyclonal</td>
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<td>Rabbit anti-GFP, polyclonal</td>
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<td>Mouse anti-ubiquitin, monoclonal, clone P4G7</td>
<td>1:500</td>
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Table 2.2: Antibodies used for Western blot, coIP, pulse-chase
2.2 Strains

2.2.1 Escherichia coli

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<td>DH5α</td>
<td>F'/o80dlacZΔ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk-, mk+) deoR recA1 endA1 hsdR17(rk-, mk+) phoA supE44 lambda-thi-1 gyrA96 relA1/F’ proAB+ lac lqZΔM15 Tn10(tetr)</td>
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| E. coli BMH 71-18 mutS | E. coliB, thi supE (lac proAB) [mutS:Tn10] [F’ proA+B+ laclqZ M15] | Clontech            |

Table 2.3: Escherichia coli strains

2.2.2 S. cerevisiae

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<thead>
<tr>
<th>Source</th>
<th>Genotype</th>
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<tr>
<td>BWG1-7a</td>
<td>MATα leu2-3,112 ura3-52 ade1-100 his4-519 prc1-1</td>
<td>(Johnson et al., 1995)</td>
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<tr>
<td>BY4743</td>
<td>MATα/α his3Δ 1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0</td>
<td>EUROSCARF</td>
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<td>cdc48-ts</td>
<td>W303 prc1-1 cdc48-T413R-ts</td>
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<tr>
<td>fbp1Δ gid8Δ</td>
<td>W303-BKO gid8Δ::KANMX6</td>
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Table 2.4: Saccharomyces cerevisiae strains used in this work
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<td>BWG1-7a ufd3-1</td>
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<td>srp1-31</td>
<td>W303-1B srp1-31</td>
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Table 2.5: *Saccharomyces cerevisiae* strains used in this work (2)
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<th>Source</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YCR2</td>
<td>W303 prc1-1 ubx5Δ::HIS5</td>
<td>(Alberts et al., 2009)</td>
</tr>
<tr>
<td>YCR4</td>
<td>W303 prc1-1 ubx6Δ::HIS5</td>
<td>(Alberts et al., 2009)</td>
</tr>
<tr>
<td>YJMR1</td>
<td>W303-1B GID8-L255S-L256G::HA3::URA3</td>
<td>This work</td>
</tr>
<tr>
<td>YJMR2</td>
<td>W303-1B otu1Δ::KANMX6</td>
<td>This work</td>
</tr>
<tr>
<td>YJR12</td>
<td>W303-1B GID1::HA3::HIS5&lt;sup&gt;S.p.&lt;/sup&gt;</td>
<td>(Regelmann et al., 2003)</td>
</tr>
<tr>
<td>YLB01</td>
<td>BY Matα fbp1::GFP::HIS3MX6</td>
<td>(Barbin, 2005)</td>
</tr>
<tr>
<td>YLB03</td>
<td>W303-1B fbp1::HA3::HIS3</td>
<td>This work</td>
</tr>
<tr>
<td>YLB08</td>
<td>WCG4 fbp1::HA3::HIS3</td>
<td>This work</td>
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<tr>
<td>YLB09</td>
<td>WCG pre1-1 fbp1::HA3::HIS3</td>
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<td>YLB10</td>
<td>WCG4 pre1-1 pre2-2</td>
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<td>fbp1::HA3::HIS3</td>
<td></td>
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<tr>
<td>YLB11</td>
<td>WCG4 gid2Δ::KANMX6</td>
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<td>YLB12</td>
<td>YLB08 gid3Δ::KANMX6</td>
<td>This work</td>
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<td>YLB13</td>
<td>YLB08 gid5Δ::KANMX6</td>
<td>This work</td>
</tr>
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<td>YLB14</td>
<td>YLB08 gid9Δ::KANMX6</td>
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<td>YLB15</td>
<td>WCG4 gid3Δ::KANMX6</td>
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<td>YLB16</td>
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</tr>
<tr>
<td>YLB17</td>
<td>WCG pre3-6 fbp1::HA3::HIS3</td>
<td>This work</td>
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<tr>
<td>YLB18</td>
<td>WCG4 gid1Δ::KANMX6</td>
<td>This work</td>
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<tr>
<td>YLB19</td>
<td>WCG4 gid8Δ::KANMX6</td>
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<td>YLB22</td>
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</tr>
<tr>
<td>YLB23</td>
<td>YML1 gid2Δ::NatNAT2</td>
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<tr>
<td>YLB24</td>
<td>YML2 gid2Δ::NatNAT2</td>
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<tr>
<td>YLB25</td>
<td>YML2 gid1Δ::NatNAT2</td>
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Table 2.6: *Saccharomyces cerevisiae* strains used in this work (3)
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<tbody>
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<td>YLB26</td>
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<td>This work</td>
</tr>
<tr>
<td>YLB27</td>
<td>W303-1B FBP1::TAP::TRP1\textsuperscript{K,I.} gid2\Delta::KANMX</td>
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</tr>
<tr>
<td>YLB28</td>
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<tr>
<td>YLB29</td>
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<tr>
<td>YLB31</td>
<td>W303-1B FBP1::TAP::TRP1\textsuperscript{K,I.} gid8\Delta::KANMX</td>
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<tr>
<td>YLB32</td>
<td>W303-1B FBP1::TAP::TRP1\textsuperscript{K,I.} gid9\Delta::KANMX</td>
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</tr>
<tr>
<td>YLB33</td>
<td>W303-1B FBP1::TAP::TRP1\textsuperscript{K,I.} pdr5\Delta::KANMX</td>
<td>This work</td>
</tr>
<tr>
<td>YLB34</td>
<td>W303-1B pdr5\Delta::KANMX6</td>
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<tr>
<td>YLB35</td>
<td>WCG4 san1\Delta::KANMX6</td>
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<tr>
<td>YLB36</td>
<td>WCG4 doa10\Delta::KANMX6</td>
<td>This work</td>
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<td>YLB37</td>
<td>YLB08 san1\Delta::KANMX6</td>
<td>This work</td>
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<td>YLB38</td>
<td>YLB08 doa10\Delta::KANMX6</td>
<td>This work</td>
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<td>YLB39</td>
<td>W303-1B san1\Delta::KANMX6</td>
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<td>YLB40</td>
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<tr>
<td>YLB41</td>
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<td>YLB08 gid1\Delta::KANMX6</td>
<td>This work</td>
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<tr>
<td>YLB44</td>
<td>YLB08 gid8\Delta::KANMX6</td>
<td>This work</td>
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<td>YLB45</td>
<td>YLB08 gid7\Delta::KANMX6</td>
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<td>(Lehmann, 2008)</td>
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<td>YML2</td>
<td>W303-1B gid8\Delta::KANMX6</td>
<td>(Lehmann, 2008)</td>
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<td>YOS1</td>
<td>W303-1B MYC9::GID4</td>
<td>(Santtt et al., 2008)</td>
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<tr>
<td>YSA1</td>
<td>W303-1B GID8::HA3::HIS3\textsuperscript{S,p.}</td>
<td>(Pfirrmann, 2006)</td>
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Table 2.7: *Saccharomyces cerevisiae* strains used in this work (4)
Table 2.8: *Saccharomyces cerevisiae* strains used in this work (5)

<table>
<thead>
<tr>
<th>Source</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>YSA10</td>
<td>W303 prc1-1 ubx4Δ::KANMX6</td>
<td>(Alberts et al., 2009)</td>
</tr>
<tr>
<td>YSA12</td>
<td>W303 prc1-1 ubx7Δ::KANMX6</td>
<td>(Alberts et al., 2009)</td>
</tr>
<tr>
<td>YSA18</td>
<td>W303 prc1-1 ubx3Δ::HIS5</td>
<td>(Alberts et al., 2009)</td>
</tr>
<tr>
<td>YSA21</td>
<td>W303 prc1-1 ubx1Δ::HIS5</td>
<td>(Alberts et al., 2009)</td>
</tr>
<tr>
<td>YSA29</td>
<td>W303 prc1-1 NPL4::HA3::HIS3MX6</td>
<td>(Alberts et al., 2009)</td>
</tr>
<tr>
<td>YSK018</td>
<td>W303 prc1-1 ufd2Δ::KANMX6</td>
<td>Sonja Kohlmann-Dieter</td>
</tr>
<tr>
<td>YTP10</td>
<td>W303-1B GID7::HA3::HIS3*S.p.</td>
<td>(Pfirrmann, 2006)</td>
</tr>
<tr>
<td>YTS1</td>
<td>W303-1B gid2Δ::KANMX6</td>
<td>(Schüle et al., 2000)</td>
</tr>
<tr>
<td>YTS3</td>
<td>W303-1B GID2::HA3::HIS5*S.p.</td>
<td>(Schüle et al., 2000)</td>
</tr>
<tr>
<td>YTS4</td>
<td>W303-1B GID3::HA3::HIS5*S.p.</td>
<td>(Schüle et al., 2000)</td>
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</tbody>
</table>

**Construction of strains**

For construction of the CRA-mutated Gid8 chromosomal strain YJMR1, the pJMR1 integration plasmid (subsection 3.1 page 124) was digested with the enzyme BseRI and transformed into the W303-1B strain. Clones were selected on CM-URA plates. The integration of the point mutations in the *GID8* gene was further checked by sequencing.

For construction of the double deletion YLB23 (*gid1Δgid2Δ*) and YLB24 (*gid8Δgid2Δ*) strains, *GID2* was deleted in the single deletion YML1 (W303-1B-*gid1Δ*) and YML2 (W303-1B-*gid8Δ*) strains by chromosomal integration of a 400 bps PCR fragment. The integration product was amplified by PCR from the pFA6a-natNT2 plasmid using primers P47 and P48 and transformed in the YML1 and YML2 strains. For construction of the double deletion YLB25 (*gid1Δgid8Δ*) strain, *GID1* was deleted in the single deletion YML2 (W303-1B-*gid8Δ*) strain by chromosomal integration of a 400 bps PCR fragment. The integration product was amplified by PCR from the pFA6a-natNT2 plasmid using primers P51 and P52 and transformed in the YML2 strain. Transformed cells were then streaked out on YPD + nourseothrycine (100 µg/mL) plates. Colonies obtained were controlled by PCR using primers P19 and P50. This work was done together with Nadja Graf (Graf, 2007).
For construction of the YLB33 and YLB34 *PDR5* deletion strains (W303-FBPaseTAP-\(pdr5\Delta\) and W303-\(pdr5\Delta\) respectively), a 1.7 kbps KANMX6 integration cassette was amplified by PCR from the pUG6 plasmid using primers P27 and P28 (Gueldener et al., 2002). The cassette possessed the kanamycin resistance gene and homologous regions to the 50 bps before the start codon and after the stop codon of the *PDR5* gene respectively. The integration cassette was then transformed into the W303-FBPaseTAP strain and cells were streaked out on YPD + geneticine (300 \(\mu\)g/mL) plates. Colonies obtained were controlled by PCR using primers P29 and P30.

The construction of the YLB26, YLB27, YLB28, YLB29, YLB30, YLB31 and YLB32 (*gid1, gid2, gid3, gid5, gid6, gid8 and gid9* respectively deletions in W303-FBPaseTAP) strains was done as described previously for the YLB33 strain using primers P5 to P18 (Gueldener et al., 2002). Clones obtained after selection on YPD + geneticine plates were controlled by PCR using primers P19-P25 and P26.

For construction of the YLB03 (W303-FBPase-HA3), YLB08 (WCG4-FBPase-HA3) and YLB10 (WCG4 pre1-1 pre2-2-FBPaseHA3) strains, a 1742 bps HA3-HIS5 fragment was amplified from the pFA6a-3HA-His3MX6 plasmid (Longtine et al., 1998) by PCR (subsection 6.11 page 148) using primers P3 and P4 and an annealing temperature of 52°C. The integration cassette was then transformed into the W303-1B and WCG4 yeast strains and cells were streaked out on CM-HIS plates. The correct integration of the cassette was verified by southern blot or control PCR.

The construction of the *GID* deletions in the YLB08 (WCG-FBPase-HA3) (YLB12-14, YLB16, YLB43-45) strain was done like the *GID* deletions in the W303-FBPaseTAP strain described previously. This work was done in cooperation with Nadja Graf (Graf, 2007).

The construction of the *SAN1* (YLB35, YLB37, YLB39) and *DOA10* (YLB36, YLB38, YLB40) deletion strains was done as described in Gueldener et al. (2002) using primers P57 to P60 and the pUG6 plasmid.

The *ddi1\Δ* strain was done as described previously for deletions of *GID* genes in the W303-FBPaseTAP strain, using primers P55 and P56.

For construction of the Gid1myc *gid8\Δ* and *fbp1\Δ* *gid8\Δ* strains, *GID8* was deleted in the Gid1myc13 and W303-BKO (*fbp1\Δ*) strains by chromosomal integration of a PCR product amplified from the pUG6 plasmid using primers P15 and P16. Transformed cells were selected on YPD + geneticine plates. Control PCR was done using primers P24 and P26.
Chapter 3

Molecular biology

3.1 Plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBS1479</td>
<td>Plasmid to generate PCR integration modules for C-terminal TAP fusion proteins, contains the TRP1 marker from Kluyveromyces lactis</td>
<td>(Puig et al., 2001)</td>
</tr>
<tr>
<td>pFA6a-3HA-KANMX6</td>
<td>Plasmid to generate PCR integration modules for C-terminal triple HA fusion proteins. KANMX6 gene of S. pombe, Triple HA (Hemaglutinin) epitope from influenza virus, bla gene, multiple cloning site, pSP72 derivative</td>
<td>(Longtine et al., 1998)</td>
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</tbody>
</table>

Table 3.1: Plasmids used in this work
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<thead>
<tr>
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<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFA6a-3HA-His3MX6</td>
<td>Plasmid to generate PCR integration modules for C-terminal triple HA fusion proteins. <em>HIS3</em> gene of <em>S. pombe</em>, Triple HA (Hemaglutinin) epitope from influenza virus, bla gene, multiple cloning site, pSP72 derivative</td>
<td>(Longtine et al., 1998)</td>
</tr>
<tr>
<td>pFA6a-GFP(S65T)-</td>
<td>Plasmid to generate PCR integration modules for C-terminal GFP fusion proteins. <em>HIS3</em> gene of <em>S. pombe</em>, GFP, bla gene, multiple cloning site, pSP72 derivative</td>
<td>(Longtine et al., 1998)</td>
</tr>
<tr>
<td>His3MX6</td>
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<td></td>
</tr>
<tr>
<td>pFA6a-natNT2</td>
<td>Plasmid useful as template for PCR to create gene deletions. Contains the <em>nat</em> gene from <em>Streptomyces noursei</em> encoding nourseothricin N-acetyl-transferase and confers resistance to the antibiotic nourseothricin of transformed yeasts</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>pGEM-T easy</td>
<td>Plasmid used to improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. Contains T7 and SP6 RNA Polymerase promoters flanking a multiple cloning site (MCS) within the a-peptide coding region of the enzyme b-galactosidase, and the origin of replication of the filamentous phage f1</td>
<td>Promega</td>
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Table 3.2: Plasmids used in this work (2)
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<tbody>
<tr>
<td>pGid2NLSG</td>
<td>2µ URA3, GAL:Gid2NLS-GFP-S (pYES)</td>
<td>This work</td>
</tr>
<tr>
<td>pJMR1</td>
<td>Based on the pRS306 plasmid, \textit{GID8-L255S-L256G-HA3} fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pJR15</td>
<td>ARS-CEN, URA3, GID1-HA3::HIS5\textsubscript{S.\textit{Pombe}}</td>
<td>(Regelmann, 2005)</td>
</tr>
<tr>
<td>pNLSG</td>
<td>2µ URA3, GAL:SV40NLS-GFP-S (pYES)</td>
<td>(Wendler et al., 2004)</td>
</tr>
<tr>
<td>pmNLSG</td>
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<td>(Wendler et al., 2004)</td>
</tr>
<tr>
<td>pRpt2NLSG</td>
<td>2µ URA3, GAL:Rpt2NLS-GFP-S (pYES)</td>
<td>(Wendler et al., 2004)</td>
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<tr>
<td>pRS306</td>
<td>YI-type integrating shuttle vector based on the pBluescript-vector, URA3 f1 ori (NaeI) T7 promoter, lacZ'/MCS, T3 promoter, pMB1 ori, bla</td>
<td>(Sikorski and Hieter, 1989)</td>
</tr>
<tr>
<td>pRS316</td>
<td>Based on the pBluescript-vector, URA3 f1 ori (NaeI) T7 promoter, lacZ'/MCS, T3 promoter, pMB1 ori, bla, CEN6, ARSH4</td>
<td>(Sikorski and Hieter, 1989)</td>
</tr>
<tr>
<td>pRS316-GID2</td>
<td>Based on the pBluescript-vector, URA3 f1 ori (NaeI) T7 promoter, lacZ'/MCS, T3 promoter, pMB1 ori, bla, CEN6, ARSH4, \textit{GID2} fragment</td>
<td>(Barbin, 2005)</td>
</tr>
<tr>
<td>pRS316-GID2ΔNLS</td>
<td>Based on the pBluescript-vector, URA3 f1 ori (NaeI) T7 promoter, lacZ'/MCS, T3 promoter, pMB1 ori, bla, CEN6, ARSH4, \textit{GID2ΔNLS} fragment</td>
<td>(Barbin, 2005)</td>
</tr>
<tr>
<td>pRS316-GID2HA</td>
<td>ARS-CEN, URA3, GID2-HA3::HIS3MX6</td>
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Table 3.3: Plasmids used in this work (3)
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<tbody>
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<td>pRS316-GID2ΔNLSHA</td>
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<td>This work</td>
</tr>
<tr>
<td>pRS316-GID8HA</td>
<td>ARS-CEN, URA3, GID8-HA3::HIS3MX6</td>
<td>This work</td>
</tr>
<tr>
<td>pRS316-GID8*HA</td>
<td>ARS-CEN, URA3, GID8*-HA3::HIS3MX6, in CRA domain of Gid8p, L_{255}L_{256} changed into S_{255}G_{256}</td>
<td>This work</td>
</tr>
<tr>
<td>pUG6</td>
<td>Plasmid to generate a KAN gene disruption cassette. loxP-gene marker-loxP disruption cassette,</td>
<td>(Gueldener et al., 2002)</td>
</tr>
<tr>
<td>YCP50</td>
<td>ARS-CEN, URA3</td>
<td>(Rose et al., 1987)</td>
</tr>
</tbody>
</table>

Table 3.4: Plasmids used in this work (4)

**Construction of the pRS316-GID8HA plasmid**

The construction of the $GID8HA_3$ expression vector was done in cooperation with Nadja Graf (Graf, 2007). A 2.7 kbps fragment composed of the $GID8HA_3$ gene with the $GID8$ promotor, the $ADH1$ terminator and the $HIS3$ marker gene, was amplified by PCR from chromosomal DNA of the YSA1 strain using primers P41 and P42. One primer was chosen in the sequence of the promotor of $GID8$ and the XbaI cutting site, while the other one had a sequence homologous to the sequence of the terminator of $ADH1$ and to the KpnI enzyme sequence. The PCR product and the pRS316 plasmid DNA were digested with both XbaI and KpnI enzymes and fragments were purified from agarose gel. Ligation (subsection 6.5 page 142) was then done and the ligation product was transformed into *E. coli* DH5α competent cells (subsection 6.7 page 143). Clones obtained were controlled by digestion and by sequencing. The protein Gid8-HA$_3$ was further controlled to be functional.

**Insertion of mutations in the CRA domain of Gid8**

The insertion of the two mutations was done by the amplification of a 1.8 kbps fragment (Figure 3.1). Two fragments (1.3 kbps and 500 bps), each of which containing the two point
Mutations and a new cutting site DrIII, were first separately amplified by PCR using primers P43 to P46. Both separate fragments were then used as template for the amplification of the 1.8 kbps final fragment by PCR using primers P43 and P46. Primers P43 and P46 were designed to insert the SalI and EcoRI enzymes cutting sites. The insertion of point mutations in the final PCR product was controlled by digestion with the newly inserted DrIII enzyme sequence. The desired amplicon as well as the pRS316-GID8HA plasmid were digested with SalI and EcoRI enzymes, fragments obtained were then purified from agarose gel and used for further ligation (subsection 6.5 page 142) and transformation in E. coli DH5α competent cells (subsection 6.7 page 143). Clones obtained were finally controlled for integration of point mutations in the GID8 gene by digestion with DrIII and by sequencing. The new plasmid was named pRS316-GID8*HA.

**Construction of the pJMR1 plasmid**

The construction of the GID8*HA3 integration plasmid was done in cooperation with Julia Reuther during her "Studienarbeit". The plasmids pRS316-GID8*HA (digestion: 15 μL plasmid, 2 μL enzyme, 10 μL enzyme buffer, 73 μL H2O) and pRS306 (digestion: 7 μL plasmid, 2 μL enzyme, 5 μL enzyme buffer, 36 μL H2O) were first digested with the restriction enzyme KpnI, then with XhoI (digestion: 2 μL enzyme, 88 μL of KpnI digestion sample, 10 μL enzyme buffer). After gel extraction, the GID8*-HA fragment obtained from digestion of the pRS316-GID8*HA plasmid was inserted into the linearised digested pRS306 plasmid through ligation (subsection 6.5 page 142). The ligation product was then transformed into E. coli DH5α competent cells (subsection 6.7 page 143). Clones obtained were finally controlled for integration of mutations into the GID8 gene by digestion and by sequencing.

**Construction of the NLS-GFPS fusion protein**

The pGid2NLSG plasmid, further used to express the NLS-GFPS fusion protein, was done by ligation of the NLS-amplified PCR fragment with HindIII and KpnI cutting sites in the HindIII-KpnI digested pRpt2NLSG plasmid (Wendler et al., 2004). Transcription was under control of the GAL1 promotor and the CYC1 terminator (Figure 3.2). For synthesis of the pGid2NLSG plasmid, the NLS sequence of Gid2 (71KRKRELIEKLSKSQRQWDHSVKKQIK37) was inserted by annealed oligonucleotides.
Figure 3.1: Insertion of the two mutations in \textit{GID8}. The insertion of the two mutations was done by the amplification of a 1.8 kbps fragment. Two fragments (1.3 kbps and 500 bps), each of which containing the two point mutations and a new cutting site DraIII, were first separately amplified by PCR using primers P43 to P46 (steps 1 and 2). Both separate fragments were then used as template for the amplification of the 1.8 kbps final fragment by PCR using primers P43 and P46 (step 3). Primers P43 and P46 were designed to insert the SaiI and EcoRI enzymes cutting sites. The insertion of point mutations in the final PCR product was controlled by digestion with the newly inserted DraIII enzyme sequence. The desired amplicon as well as the pRS316-GID8HA plasmid were digested with SaiI and EcoRI enzymes, fragments obtained were then purified from agarose gel and used for further ligation.
Figure 3.2: **Fusion of the NLS sequence of Gid2p to GFPS.** Putative NLS sequence of Gid2p was expressed as a GFPS fusion protein.

Oligonucleotides P31 and P32, whose sequence is the sequence of the potential NLS of *GID2* with HindIII and KpnI cutting sites and ATG as a start codon, were synthesized and phosphorylated by the firm BioTez Berlin. They were then freshly prepared. 50 µL of each oligomer (with a concentration of 75 ng/µL) were mixed together with 10 µL 1 M NaCl. The sample was incubated at 95°C for 10 minutes and put on ice. Hybridization of primers in the annealing fraction was potentially tested by separating a part of the hybridization sample on a 5% polyacrylamid gel. 50 mL 5% polyacrylamid gel were prepared mixing 8.3 mL 30% acrylamid solution with 5 mL 10x TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0) and the corresponding quantity of water. Polymerisation was processed adding 500 µL 10% APS and 33 µL TEMED.

The pRpt2NLSG vector used for cloning, was freshly transformed in DH5α competent *E. coli* cells, isolated and controlled before use. Then a 400 µL digestion of this plasmid was performed mixing 40 µL plasmid DNA, 4 µL of each enzyme (KpnI and HindIII), 40 µL 10x respective buffer and 3 µL BSA. The digestion sample was incubated for 2 to 4 hours at 37°C. 15 µL digestion product was tested on an agarose gel to check linearisation of the plasmid. The whole digestion sample was then loaded on an agarose gel, cut out of the gel, extracted using the QIAGEN protocol and eluted in 30 µL elution buffer. 3 µL of the eluted digested plasmid DNA were checked on an agarose gel before ligation.

Ligation was done with a ratio vector:oligomers mix of 1:1, 1:3, 1:5 and 1:10, and as described in subsection 6.5 page 142. Ligations were then transformed in DH5α *E. coli* competent cells (subsection 6.7.2 page 144).

The different clones were finally checked for fusion of the NLS sequence to GFPS through PCR using primers P33 and P34. Primers were done so that one primer’s sequence was
homologous to the beginning of the NLS sequence and the other primer’s sequence homologous to the end of the GFPS sequence. Clones able to amplify a 900 bps NLS-GFPS fragment were finally sent for sequencing.

**Construction of the pRS316-GID2HA and pRS316-GID2ΔNLSHA plasmids**

Construction of the pRS316-GID2HA and pRS316-GID2ΔNLSHA plasmids (Figure 3.3) was done by the insertion of a 1527 bps and 1474 bps fragment respectively in the EcoRI/SalI digested pRS316-GID2 and pRS316-GID2ΔNLS plasmid respectively. The insert was done from two PCR products able to recombine at a specific sequence. The first fragment (1170 bps for GID2 and 1117 bps for GID2ΔNLS) was amplified from the pRS316-GID2 and pRS316-GID2ΔNLS plasmids using primers P37 and P38. The forward primer (P37) had a sequence homologous to a sequence in the GID2 gene and containing the EcoRI cutting site. The reverse primer (P38) had a sequence homologous to the sequence of the GID2 gene just before but without the stop codon. The second 357 bps fragment was obtained by PCR using the pFA6a-3HA-His3MX6 plasmid and primers P39 and P40. The forward primer (P39) was chosen to have a part of its sequence homologous to the sequence of the GID2 gene just before but without the stop codon, and another part homologous to the sequence of HA3. The reverse primer (P40) was designed to have a sequence homologous to the sequence of the terminator of ADH1 contained in the pFA6a-3HA-His3MX6 plasmid, and to the sequence of the SalI cutting site. The two PCR products were then purified with the Kit from Qiagen, and 2 to 5 µL of each PCR product were mixed and amplified by PCR again using primers P37 and P40. Finally each end PCR product (1527 bps for GID2HA3, 1474 bps for GID2ΔNLSHA3) was subcloned into the pGEM-T easy plasmid. Both pGEM-Teasy-GID2HA3 / pGEM-TeasyGID2ΔNLSHA3 and pRS316-GID2 plasmids were digested with EcoRI and SalI enzymes. The 1527/1474 bps fragment got from the digestion of the pGEM-Teasy-GID2HA3/pGEM-TeasyGID2ΔNLSHA3 plasmid with EcoRI and SalI, and the digested pRS316-GID2 plasmid were loaded on an agarose gel and extracted from the gel. The 1527/1474 bps fragment was then ligated into the pRS316-GID2 digested plasmid as described in subsection 6.5 page 142. The ligation product was transformed into *E. coli* DH5α competent cells. Clones obtained were controlled by digestion and by sequencing.
Figure 3.3: **Tagging of Gid2p and Gid2ΔNLSp with HA$_3$.** Construction of the pRS316-GID2HA and pRS316-GID2ΔNLSHA plasmids was done by the insertion of a 1527 bps and 1474 bps fragment respectively in the EcoRI/SalI digested pRS316-GID2 and pRS316-GID2ΔNLS plasmid respectively. The insert was done from two PCR products able to recombine at a specific sequence. The first fragment (1170 bps for *GID2* and 1117 bps for *GID2ΔNLS*) was amplified from the pRS316-GID2 and pRS316-GID2ΔNLS plasmids using primers P37 and P38. The second 357 bps fragment was obtained by PCR using the pFA6a-3HA-His3MX6 plasmid and primers P39 and P40. After purification, each end PCR product (1527 bps for *GID2HA3*, 1474 bps for *GID2ΔNLSHA3*) was subcloned into the pGEM-T easy plasmid. The 1527/1474 bps fragment got from the digestion of the pGEM-Teasy-GID2HA3/pGEM-TeasyGID2ΔNLSHA3 plasmid with EcoRI and SalI, and the EcoRI/SalI digested pRS316-GID2 plasmid were loaded on an agarose gel and extracted from the gel. The 1527/1474 bps fragment was then ligated into the pRS316-GID2 digested plasmid as described in subsection 6.5 page 142. The ligation product was transformed into *E. coli* DH5α competent cells. Clones obtained were controlled by digestion and by sequencing.
### 3.2 Oligonucleotides

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<td>P2</td>
<td>FBP1insiderev</td>
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<td>P3</td>
<td>FBP1-HA3-HISfor</td>
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<td>FBP1-HA3-HISrev</td>
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<td>Gid1 forward</td>
<td>5’-AGT ACG TTA AAG CCA AGC GTC GAA TTT CAG CAT AAT TAA GAG GAA CAG CTG AAG CTT CGT ACG C-3’</td>
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Table 3.5: Oligonucleotides used in this work
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Table 3.6: Oligonucleotides used in this work (2)
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<td>Gid3 Kontrolle</td>
<td>5'-AAA ACG AAT AAG AGG GTG TCT TT G-3'</td>
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<td>Gid5 Kontrolle</td>
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<td>Gid7 Kontrolle</td>
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<td>Gid8 Kontrolle</td>
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<td>P27</td>
<td>pdr5delfor</td>
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Table 3.8: Oligonucleotides used in this work (4)
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Table 3.10: Oligonucleotides used in this work (6)

### 3.3 Enzymes

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Chapter 4

Media, buffers, solutions

4.1 Media

Standard media are used for *E. coli* and *S. cerevisiae* cultures (Ausubel et al., 1992; Sambrook et al., 1989; Guthrie and Fink, 1991). The Agar plates were prepared by supplementing the corresponding media with 2 % Bacto-Agar. Each derepression medium was prepared with distilled water (MILLIPORE H\textsubscript{2}O), adjusted with the desired pH using hydrochloric acid or natrium hydroxide, and autoclaved for sterilization. Finally aminoacids like L-histidine, L-leucine, L-lysine, L-tryptophane, and nucleotide bases supplements were added to synthetic media.

4.1.1 *S. cerevisiae* yeast cultures

**Rich medium:** YPD medium (Yeast Peptone Dextrose, glucose-complete medium), pH5.5 : 1.0 % (w/v) yeast extract; 2.0 % (w/v) Bacto\textsuperscript{TM} peptone; 2.0 % (w/v) D-Glucose; 20 mg/L adenine

**Synthetic complete medium CM:** 0.13 % (w/v) aminoacids mix (drop-out powder for CM medium); 0.67 % (w/v) yeast nitrogen base without aminoacids; 2.0 % (w/v) D-Glucose

**Drop-out mix for CM medium:** 1.0 g alanine, 1.0 g asparagine, 1.0 g cysteine, 1.0 g glutamine, 1.0 g glycine, 1.0 g isoleucine, 1.0 g proline, 1.0 g phenylalanine, 2.0 g aspartate,
2.0 g glutamate, 0.4 g arginine, 0.4 g methionine, 4.0 g threonine, 0.6 g tyrosine, 3.0 g valine, 7.5 g serine.

**Ethanol composed starvation medium (YPEtOH, pH 5.5 or CMEtOH):** For the induction of gluconeogenesis, glucose in YP or CM media was replaced by 2.0 % (v/v) ethanol.

**YPD Agar:** 2.0 % (w/v) Bacto\textsuperscript{TM} Agar is added to YPD-Medium.

**Aminoacid stocks:** 0.1 M adenine (500x); 0.1 M histidine (500x); 0.1 M lysine (500x); 0.1 M tryptophane (500x); 0.1 M uracile (500x). Adenine, histidine, lysine and uracile were autoclaved and tryptophane was steril filtered.

### 4.1.2 *E. coli* cultures

**LB-Medium:** 0.5 % (w/v) yeast extract; 1.0 % (w/v) Bacto\textsuperscript{TM} Tryptone; 85 mM NaCl; 75 mg/L Ampicillin

**LBAmp-Medium:** For culture of transformed *E. coli*, 75 mg/L Ampicillin are added to LB-medium.

**LBAmp Agar:** 2.0 % (w/v) Bacto\textsuperscript{TM} Agar is added to LBAmp-Medium.

**Transformation medium (SOC Medium, pH 7.4):** 0.5 % (w/v) yeast extract; 2.0 % (w/v) Bacto\textsuperscript{TM} Tryptone; 0.4 % (w/v) D-Glucose; 10 mM NaCl; 10 mM MgCl\textsubscript{2}; 10 mM MgSO\textsubscript{4}; 2.5 mM KCl

### 4.2 Buffers and solutions

Only the currently used buffers and solutions were given below. The other buffers and solutions used for particular experiments were detailed in the corresponding chapter.

**DNA-sample buffer:** 50 mM EDTA; 50 % (v/v) glycerine; 0.1 % (w/v) bromophenolblue; 0.25 % (w/v) xylene cyanol
4.2 Buffers and solutions

**Urea buffer:** 40 mM Tris HCl, pH 6.8; 8 M urea; 5 % (w/v) SDS; 0.1 M EDTA; 0.05 % (w/v) bromophenolblue

**Protein-sample buffer:** 1 % (v/v) β-ME (β-mercaptoethanol) were added to urea buffer.

**TAE (Tris Acetate with EDTA) buffer:** 40 mM Tris/Acetate pH 7.5; 2 mM EDTA

**TBS-T (Tris Buffered Saline with Tween-20) buffer:** 137 mM NaCl; 0.1 % (v/v) Tween-20; 20 mM Tris/HCl pH 7.6

**TE (Tris with EDTA) buffer:** 10 mM Tris/HCl pH 7.5; 1 mM EDTA

**2x Lämmli buffer:** 139 mM SDS; 100 mM Tris, pH 6.8; 5 mM EDTA pH 8.0; 9.90 % (v/v) glycerol; 0.02 % (w/v) bromophenol; 2.50 % (v/v) β-mercaptoethanol
Chapter 5

Growth conditions

*S. cerevisiae* grows well at 30°C with good oxygenation. Liquid cultures are therefore grown in Erlenmeyer flask which volume is 5-times bigger than the culture volume. Cells are incubated on a shaker at the speed of 220 rpm at 30°C for yeast cultures and at 37°C for *E. coli* cultures. Growth of cells is determined measuring the optical density of a tenth-diluted culture at the wave length of 600 nm. One $OD_{600}$ of 1.0 corresponds to roughly $2 \times 10^7$ cells per mL.

5.1 Permanent cultures

Permanent cultures were streaked out with cells on Agar plates or liquid cultures in sterile glycerine with an end concentration of 15 % (v/v) in yeasts and 30 % (v/v) in *E. coli* respectively, and stored at -80°C.

5.2 Growth conditions of cells

5.2.1 Growth conditions of *E. coli* cultures

*E. coli* strains are grown in LB-media at 37°C in liquid culture or on LB Agar plates. When needed, ampicillin is used as a selective marker and added to the media to a concentration of 50-100 µg/mL.
5.2 Growth conditions of cells

5.2.2 Growth conditions of yeast cultures

Cell cultures on Agar plates were incubated for 2 to 3 days at 30°C. For liquid cultures cells from Agar plates were taken and inoculated in 2 mL preculture. They were incubated for 1 or 2 days at 30°C on a shaker. Then main cultures were made from the liquid precultures.

5.2.3 Induction and inactivation of FBPase

When the cells are grown in a non-fermentable carbon source medium like ethanol, there is induction of the expression of the enzyme fructose-1,6-bisphosphatase and of other gluconeogenic enzymes.

To study the catabolite degradation, cells were first inoculated in 2 mL glucose rich medium (YPD) and incubated over night at 30°C. A main culture was then done: 400 μL of preculture were taken and put in 5 mL glucose rich medium (YPD). After incubating for 6 hours at 30°C, 600 μL of the main culture were taken and put in 10 mL ethanol rich medium (YPEtOH). Cells were let incubate for 16 hours at 30°C. Thereafter, 1.5 OD were taken as "zero sample" and 8*1.5 OD cells were incubated in 8 mL glucose rich medium at 30°C to induce catabolic degradation. Other 1.5 OD samples were taken after 0, 30, 60, 90 and 120 min glucose-shift. Each sample was then centrifuged for 1 min at 13,000 rpm, washed with 1 mL H₂O and frozen at -20°C until alcaline lysis or SDS electrophoresis.

5.2.4 Treatment of yeast cells with cycloheximide

All experiments done in this work with cycloheximide were done on ethanol medium. Cells were first grown in a glucose-rich medium, then shifted on an ethanol-containing medium to induce expression of FBPase. After overnight growth, cycloheximide was added to cell culture from a 10 mg/mL stock solution (in DMSO) to an end concentration of 100 μg/mL. After addition of cycloheximide, samples were taken 0 min, 30 min, 60 min, 90 min and 120 min after cycloheximide shift. Each sample was then centrifuged for 1 min at 13,000 rpm and washed with 1 mL H₂O and frozen at -20°C until the alcaline lysis or SDS electrophoresis.
5.2.5 Treatment of yeast cells with the proteasome inhibitor MG132

A 30 mM stock solution (in ethanol or DMSO) of MG132 was prepared and conserved at -20°C. To treat yeast cells with the proteasome inhibitor MG132, MG132 was given to cell culture from a 30 mM stock solution to an end concentration of 100 µM. As a negative control, the same volume of solvent (ethanol or DMSO) as MG132 added volume was parallelly added to another sample of the same culture. Samples were incubated for 30 minutes at 30°C. The shift from an ethanol- to a glucose-containing medium could then be processed. MG132 and the solvent (ethanol or DMSO) respectively were thus given together with glucose to each sample and added every 30 minutes. Each sample was centrifuged for 1 min at 13,000 rpm, washed with 1 mL H₂O and frozen at -20°C until the alcaline lysis or SDS electrophoresis.
Chapter 6

DNA manipulation techniques

6.1 Isolation of plasmid-DNA from *E. coli*

Plasmid isolation (Minipreparations or Midipreparations depending on the quantity of cells) was done according to the basic protocol of QIAprep Spin Miniprep or Midiprep Kit from Qiagen. All the solutions and columns used for lysis of cells and purification of plasmids were from the supplier Qiagen.

6.2 DNA gel extraction

DNA-gel samples were cut out of the electrophoresis gel. DNA was then extracted from the gel by using the basic protocol of QIAquick gel extraction kit from Qiagen. Solutions and columns used for extraction and purification were directly obtained from the supplier Qiagen. DNA fragments were then digested and ligated into plasmids of interest.

6.3 Digestion of DNA with restriction enzymes

For a 20 µL digestion, 3 to 5 µL plasmid-DNA was digested using 0.5 µL of enzyme(s) and 1 µL of the respective digestion buffers each incubation buffer. The sample was incubated for two hours at 37°C if not otherwise stated.
6.4 Agarose gel electrophoresis

Materials

- TAE-buffer (see subsection "Buffers and solutions")
- Agarose Gel (1 %): 1.5 g Agarose in 150 mL 1 x TAE buffer.
- DNA Loading Dye (5x): 50 % (v/v) glycerol (diluted in 10x TAE); 2 mg/mL bromophenol blue.

DNA fragments were separated on a 1 % Agarose gel by electrophoresis. Gels were prepared melting the agarose in a microwave oven and pouring the gel in an appropriate electrophoresis chamber. 6 µL of ethidium bromide (10 mg/mL) were added to the agarose. Once the gel was cold, it was covered with 1x TAE-buffer, loaded and a voltage of 120 V was applied for 30 min. A 1 kb DNA length standard (NEB, Frankfurt) was used. After running electrophoresis, the gel was lightened under UV-light (302 nm) and documented with a videoprinter from MWG Biotech.

6.5 Ligation of DNA fragments

The digested plasmid and the insert were put in a 20 µL sample in a proportion of 1:5 respectively. After adding 4 µL 5x Ligase Buffer and 1 µL T4-DNA-Ligase, steril water was added up to 20 µL and the ligation was carried out over night at 16°C. To control ligation, two other samples were prepared where no insert fragment was given. One control sample checking the digestion of the plasmid was prepared mixing digested plasmid DNA, T4 ligase buffer and sterile water. The other control sample checking the religation of the plasmid was done together with digested plasmid DNA, T4 DNA ligase, T4 ligase buffer, sterile water. The next day 1 µL T4-DNA-Ligase was added again and the sample was incubated at room temperature for 4 hours. It was then directly transformed in E. coli (subsection 6.7 page 143).
6.6 Preparation of heat-shock competent DH5α E. coli cells

**TFb1:** 10 mM MES; 100 mM RbCl; 10 mM CaCl$_2$; 50 mM MnCl$_2$. Adjust at pH=5.8 with 0.2 M acetic acid. Autoclave the solution and conserve at 4°C.

**TFb2:** 10 mM MOPS; 10 mM RbCl; 75 mM CaCl$_2$; 15 % (w/v) glycerol. Adjust at pH=6.5 with KOH. Autoclave the solution and conserve at 4°C.

**Preparation of bacteria:** DH5α cells were incubated over night at 37°C on LB plates. Four colonies were taken with a toothpick, inoculated in 5 mL LB medium in 10 mL falcon tubes and incubated for around 2 hours at 37°C until obtaining an OD of 0.3 at 550 nm. 2 mL of preculture were put in 40 mL prewarmed LB medium and the sample was then incubated until obtaining an OD of 0.45-0.49 at 550 nm.

**Treatment of bacteria treatment to render them chemically competent:** All experiments were done in a cold room or on ice. Cells were put for 15 min on ice to stop them growing and transferred in 50 mL steril falcon tube. They were centrifuged for 15 min at 2500 rpm (Centrifuge 5804 R, Eppendorf) in a precooled centrifuge at 4°C. The supernatant was withdrawn and tubes were dried. The pellet was then resuspended in 2 mL TFb1 by gently shaking, and filled up to a final volume of 16 mL with TFb1. It was incubated for 15 min on ice, centrifuged for 15 min at 4°C at 2500 rpm (Centrifuge 5804 R, Eppendorf). The supernatant was withdrawn, the tubes were dried and the pellet was resuspended in 1.6 mL TFb2 by gently shaking. It was incubating for 15 min on ice. 100 µL competent cells were then aliquoted in microcentrifuge tubes and put in a box at -80°C.

6.7 Transformation methods in E. coli

There are two ways of introducing DNA into E. coli cells.
6.7.1 Electroporation

Electroporation consists in a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by externally applied electrical field. It is another way to make holes in cells, by briefly shocking them with an electric field of 100-200V. Then plasmid DNA can enter the cell through these holes. Natural membrane-repair mechanisms will close these holes afterwards. Several hundred Volts across a distance of several millimeters are typically used in this process. Afterwards, the cells have to be handled carefully until they have had a chance to divide, producing new cells that contain reproduced plasmid. Thereafter is the protocol used for the experiments.

Before proceeding to electroporation, a dialyse may be useful so that DNA is free of ions. Then 10 µL DNA were added in 100 µL E. coli competent cells. Cells were transferred in an electroporation cuvette. Before proceeding to electroporation, the conditions had to be set up. During this time, cells were put on ice. The cuvette was then placed into the electroporation chamber. A voltage of 2.3 V was applied for one minute. Cells were resuspended in 1 mL SOC medium and transferred from the cuvette to a microcentrifuge tube. The samples were let at 37°C for one hour. Finally they were streaked out on LBAmp plates and put at 37°C.

6.7.2 Heat-shock transformation

For this type of transformation, heat-shock competent DH5α E. coli cells have to be used. First DH5α E. coli cells were prepared (for details on the protocol of preparation of heat-shock competent DH5α E. coli cells, confer to the subsection 6.6 page 143). Then 5 µL DNA were added to 50 µL DH5α E. coli and the samples were put on ice for one hour. To operate heat-shock, E. coli cells were exposed for 2 min at 42°C and resuspended in 300 µL SOC medium. The samples were then incubated for one hour at 37°C and centrifuged at 3000-4000 rpm. The supernatant was removed and bacteria were streaked out on a LBAmp plate.

6.8 In vitro site-directed mutagenesis

Mutagenesis in genes is done according to the Clontech protocol ”Transformer Site-directed mutagenesis kit user manual”. In addition to the mutagenic primer that introduces the desired mutation, it employs an additional selection primer containing a mutation in the recognition
sequence for a unique restriction enzyme site. The two primers simultaneously anneal to one strand of the denatured double-stranded plasmid under conditions favoring the formation of hybrids between the primers and the DNA template. After standard DNA elongation, ligation and a primary selection by restriction digest, the mixture of mutated and unmutated plasmids is transformed into a mutS E. coli strain defective in mismatch repair. Transformants are pooled and plasmid DNA is prepared from the mixed bacterial population. The isolated DNA is then subjected to a second selective restriction enzyme digestion. Since the mutated DNA lacks the restriction enzyme recognition site, it is resistant to digestion. The parental DNA however is sensitive to digestion and will be linearized. A final transformation with the selectively digested DNA results in highly efficient and specific recovery of the desired mutated plasmid (Figure 6.1 page 146).

For deletion of the NLS sequence of GID2 in pRS316-GID2, Swa1 was used as a selection enzyme and Pme1 as a restriction enzyme.

**Phosphorylation of primers:** Primers were phosphorylated as described: 1 µL primer, 2 µL PNK (=Polynucleotide kinase) buffer, 2 µL 10 mM ATP, 1 µL T4 Polynucleotide kinase were mixed together and the volume was completed with ddH2O up to 20 µL. The samples were incubated at 37°C for one hour, then at 65°C for 10 minutes.

**Denaturation of plasmid DNA and annealing of primers to the DNA template:** The primer-plasmid annealing reaction was set up in a 0.5 mL microcentrifuge tube as described: 2 µL plasmid (300 ng), 2 µL 10x annealing buffer, 2 µL selection primer (50 ng/µL) and 2 µL mutagenic primer (50 ng/µL) were mixed together and the volume was completed with ddH2O up to 20 µL. Sample was incubated at 100°C for 6.5 minutes and finally put on ice for 5 minutes.

**Synthesis of the mutant DNA strand:** To the primer-plasmid annealing reaction 3 µL 10x synthesis buffer, 1 µL T4-DNA polymerase, 1 µL T4-DNA ligase and 1 µL 100 mM ATP were added. The volume was completed with ddH2O up to 30 µL. Sample was then incubated at 37°C for one hour and a half.

**Primary selection by restriction digestion:** To the 30 µL samples, 1 µL SwaI selection enzyme, 4 µL of the corresponded buffer were added and the volume was completed with
Figure 6.1: Strategy for generating specific base changes using the Transformer Site-Directed Mutagenesis Kit from Clontech.

1. Denature dsDNA

2. Re-anneal with Primers

3. Synthesize second strand with T4 DNA Polymerase and seal gaps with T4 DNA Ligase; primary digestion with selection restriction enzyme.

4. Transform *mutS E. coli* (FIRST TRANSFORMATION)

5. Isolate DNA from transformant pool

6. Secondary digestion with selection enzyme

7. Transform *E. coli* (SECOND (FINAL) TRANSFORMATION)

8. Isolate DNA from individual transformants to confirm presence of desired mutation
First transformation: The mutated strand was transformed in mutS (repair-deficient) E. coli. 7 µL of plasmid-primer DNA solution were added to 100 µL competent BMH71-18 mutS E. coli cells and incubated on ice for 20 minutes. Sample was transferred to 42°C for 90 seconds. 500 µL SOC medium was added and the sample was incubated for one hour at 37°C. Finally 4 mL LBAmp medium were added. The culture was incubated at 37°C overnight.

Selection of the mutant plasmid: The mixed plasmid pool was isolated using the basic protocol of QIAprep Spin Miniprep Kit from Qiagen. Three digestion samples were prepared. For sample A which corresponds to the digestion of the parental plasmid 10 µL isolated plasmid, 0.5 µL Swa selection enzyme, 2 µL of the corresponded buffer were mixed together and the volume was completed with ddH2O up to 20 µL. For sample B which controls the effective digestion of the mutated plasmid 4 µL isolated plasmid, 0.5 µL Pme1 restriction enzyme, 1 µL of the corresponded buffer and 1 µL BSA were mixed together and the volume was completed with ddH2O up to 10 µL. For sample C which is a non-digestion control 4 µL isolated plasmid, 1 µL of the Pme1 restriction enzyme buffer and 1 µL BSA were mixed together and the volume was completed with ddH2O up to 10 µL. Samples were incubated at 37°C for 2 hours. 8 µL of sample A and all of the samples B and C were loaded on an agarose gel.

Final transformation: Finally 5 µL sample A were transformed in DH5α competent cells using heat-shock transformation method.

Control digestion with the Pme1 restriction enzyme: Colonies grown on LBAmp plates were inoculated in 5 mL LBAmp medium overnight, plasmid DNA was isolated and a control digestion with the Pme1 restriction enzyme was performed. Clones 6.5A1 and 6.5A4 were sent for sequencing to check the deletion.

6.9 Plasmid DNA sequencing

For plasmid DNA sequencing two minipreparations of the plasmid DNA were prepared and eluted in 30 µL 70°C ddH2O. 75 µL (2.5-times the volume of water used for elution) of 100
% ethanol and 7.5 µL 10 % (v/v) 3 M NaOAc were added to precipitated DNA. The sample was let at room temperature for 30 minutes and centrifuged for 30 min at 13,000 rpm. The supernatant was withdrawn away by siphoning and let dry at room temperature. Sequencing was then done by the firm MWG Biotech.

### 6.10 Isolation of chromosomal DNA from yeast

5 mL of a YPD-overnight-culture was centrifuged at 3000 rpm for 4 min, washed with 1 mL water and pelleted in an Eppendorf tube. For lysis of cells and DNA extraction, the pellet was resuspended in 200 µL breaking buffer (2 % (w/v) Triton X-100, 1 % w/v) SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0), 200 µL phenol-chloroform-isoamylalcohol mix (phenol, chloroform, isoamylalcohol) and 300 µL glass pearls. Cells were vortexed five times, in between they were put on ice. Then 200 µL water were added and lysate was centrifuged for 10 min at 13,000 rpm. The upper aqueous phase (around 300 µL) was collected and precipitated with 1 mL 100 % cold ethanol for 20 min at -80°C. It was centrifuged for 10 min at 13,000 rpm at 4°C. DNA was resuspended with 400 µL water. After addition of 3 µL RNAse stock solution (10 mg/mL), the sample was incubated for 5 min at 37°C. DNA was precipitated again for 15 min at -80°C with 10 µL 5 M NH₄Ac and 1 mL 100 % (v/v) cold ethanol, centrifuged for 5-10 min at 13,000 rpm at 4°C and washed with 1 mL 70 % (v/v) ethanol, dried and resuspended in 25 µL water.

### 6.11 Polymerase Chain Reaction (PCR)

PCR allows an amplification of DNA fragments. For this experiment two oligonucleotides or "primers" which hybridize with the end of the sequence that has to be amplified, are required as well as a heat-stable DNA polymerase and the four desoxyribonucleosidetriphosphates. The in vitro amplification of specific parts of double plasmid DNA was conducted using a Robocycler Gradient 40 PCR-cycler (Stratagene). Therefore, a 50 µL reaction mix was prepared in 200 µL thin wall Eppendorf tubes. This reaction mix consisted of 1 µL dNTP mix, 1 µL of each oligonucleotide primer from either the 5' or the 3' end of the fragment to be amplified, 2 µL template DNA, 1 µL polymerase and 5 µL of the appropriate buffer. The whole mix was filled up to 50 µL with sterile ddH₂O.
To amplify DNA the thermocycler changed between three different temperatures. The annealing temperature of the primer to the template depends on the composition of bases in the primer and was calculated before programming the cycle conditions, according to the following formula:

\[ T = 4 \times (dGTP's + dCTP's) + 2 \times (dATP's + dTTP's) - 4 \, ^\circ\text{C} \]

First the temperature was held at 95°C to denature the double stranded DNA. Then the actual amplification process was initiated by repeating the following temperature steps 30 times. DNA was first denatured at a temperature of 95°C for 1 min; the annealing step was carried out at a temperature between 50-60°C for 1 min according to the formula above. The amplification step takes place at a temperature of 72°C for 2-4 minutes depending on the length of the product and on the polymerase used. To complete the extension the temperature is kept at 72°C for 7 minutes.

Finally 10 µL of the PCR product was run on a 1 % agarose gel to check the amplification of the expected fragment.
<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Repetition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturing</td>
<td>95</td>
<td>5 min</td>
<td>1x</td>
</tr>
<tr>
<td>Denaturing</td>
<td>95</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>use formula</td>
<td>1 min</td>
<td>30x</td>
</tr>
<tr>
<td>Amplification</td>
<td>72</td>
<td>3 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>7 min</td>
<td>1x</td>
</tr>
</tbody>
</table>

Table 6.2: Program for amplification DNA via PCR

6.12 Transformation of yeast cells according to the LiOAc method

An overnight culture of yeast cells was grown in 100 mL YPD medium until obtaining 0.3 OD/mL or 1-2.10^7 cells/mL. 50 mL of the culture was then taken and let centrifuge at 4000 rpm for 5 min. Cells were then washed twice with 1x TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA), resuspended in 5 mL 1x lithium acetate LiOAc (0.1 M LiAc) and let incubate for one hour at room temperature. After 5 min centrifugation at 4000 rpm cells were resuspended with 1x LiOAc. To 300 µL of the suspension, 4 µL carrier DNA, 700 µL 50 % (w/v) PEG and DNA (50 µL digestion cassette or 5 µL plasmid DNA) were added in a sterile eppendorf tube. The sample was then let incubate for one hour at room temperature. After addition of 100 µL DMSO cells sustained heat-shock by incubating at 42°C for 5 min. Cells were finally directly streaked out on plates if no antibiotic is used. In case of the use of an antibiotic (for example genetinice or nourseothrycine), 5 mL YPD medium was added to samples using a 15 mL falcon tube and cells were let incubate for three additional hours at 30°C. Cells were centrifuged at 4000 rpm for 5 min, the supernatant was discarded until having around 600 µL. Cells were finally resuspended and streaked out on 2 YPD plates with the corresponding antibioticum (for genetinice, 300 µg/mL per plate). After 3 days-growth at 30°C replica of plates were done on new YPD + antibioticum plates.
Chapter 7

Biochemistry and cell biology techniques

All the methods described in the following section are processed in sterile conditions (sterile solutions and containers).

7.1 Alkaline lysis of yeast cells

1.5 OD yeast cells pellet was resuspended in 1 mL ddH$_2$O and rapidly vortexed. 150 µL NaOH/β-ME (1.85 M NaOH, 7.5 % (v/v) β-ME) were added to the sample which was then mixed and put for 10 minutes on ice. To precipitate proteins 150 µL 50 % (w/v) TCA were added. The sample was mixed again, put for 10 minutes on ice, centrifuged for 10 minutes at 13,000 rpm. The supernatant was then taken away, the pellet washed with acetone, centrifuged for 5 minutes at 13,000 rpm, dried for 30 minutes at 37°C, later resuspended in 50 µL urea buffer and boiled for 10 minutes at 90°C. The sample was finally centrifuged at 13,000 rpm for 1 minute and a SDS-PAGE was performed.
7.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Glycine-SDS-PAGE is used to separate protein samples with proteins bigger than 10 kDa in size. Here are the components of resolving gel and stacking gel.

### 7.2.1 Resolving gel

<table>
<thead>
<tr>
<th>Component</th>
<th>7.5 %</th>
<th>10 %</th>
<th>12 %</th>
<th>15 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH$_2$O</td>
<td>4.85 mL</td>
<td>4.02 mL</td>
<td>3.35 mL</td>
<td>2.35 mL</td>
</tr>
<tr>
<td>1.5 M TRIS (pH 8.8)</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
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<tr>
<td>10 % SDS</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>30 % N,N-Bisacrylamide</td>
<td>2.5 mL</td>
<td>3.33 mL</td>
<td>4.0 mL</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>TEMED$^1$</td>
<td>5 µL</td>
<td>5 µL</td>
<td>5 µL</td>
<td>5 µL</td>
</tr>
<tr>
<td>10 % APS$^2$</td>
<td>50 µL</td>
<td>50 µL</td>
<td>50 µL</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

Table 7.1: Resolving gel

### 7.2.2 Stacking gel

<table>
<thead>
<tr>
<th>Component</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH$_2$O</td>
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</tr>
<tr>
<td>0.5 M TRIS (pH 6.8)</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 µL</td>
</tr>
<tr>
<td>30 % N,N-Bisacrylamide</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µL</td>
</tr>
<tr>
<td>10 % APS</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

Table 7.2: Stacking gel
7.3 Western Blotting

**Running buffer:** 1x SDS-solution prepared from a 10x stock SDS-solution (30.4 g/L TRIS; 144.0 g/L Glycine; 10.0 g/L SDS).

To separate proteins a SDS gel electrophoresis is performed. The surface of the resolving gel was covered with 200 µL of isobutanol after pouring. As soon as the resolving gel was polymerized the isobutanol was rinsed off with ddH$_2$O and the stacking gel was poured. Before the setting of the gel a comb was put in the stacking gel and removed after setting. Samples prepared were loaded on the gel as well as a protein marker. Electrophoresis was then carried out with 800 mL 1x SDS running buffer and a voltage of 150 V for around one hour. The proteins could thus be separated according to their molecular weight using a BIO-RAD PROTEAN 2 apparatus. After electrophoresis the gel was used for a western blot.

7.3 Western Blotting

**Blotting buffer:** 11 mM Tris; 192 mM Glycin; 16 % (v/v) MeOH

Western Blotting permits to detect very little quantities of a determinated protein. It was processed just after a gel electrophoresis. The separated proteins were transferred on a nitrocellulose membrane using a semi-dry blot apparatus. For each polyacrylamid gel 6 pieces of a Whatman filter paper and one nitrocellulose membrane were used, they were dropped into Blotting buffer and put to build a "sandwich": 3 pieces of a Whatman filter paper, the polyacrylamid gel, the nitrocellulose membrane, 3 pieces of a Whatman filter paper. A current of 0.07 mA per membrane was applied for 1.5 hour. After the Western Blotting the membrane was put in 10 % (w/v) milk blocking solution over night at 4°C or for one hour at room temperature. An immunological detection was then processed.

7.4 Immunological detection

After blocking the membrane was finally washed twice with 1x TBS-T for 10 minutes. It was incubated for one hour at room temperature with the first antibody directed against the antigen which has to be detected. Then the excess of antibody was washed away three times with 1x TBS-T solution for 10 minutes. The secondary antibody directed against the first one was poured over the membrane and incubation was carried out for one hour at
room temperature. The membrane was washed with 1x TBS-T (once for 10 minutes, three times for 5 minutes). It was then overlayed with 600 µL ECL solution and incubated for one minute. Membrane was then put in a cassette and overlayed with an ECL film (ECL<sup>TM</sup>-system, Amersham Biosciences, Freiburg).

The membrane could be used several times (four-five times). After a first use the membrane was to be stripped: it had to be treated with 10 % (v/v) acetic acid to wash the antibodies away, then the membrane was washed 1x TBS-T buffer until 10 % (v/v) acetic acid was completely gone.

### 7.5 Co-immunoprecipitation

For immunoprecipitation experiments cells were cultivated as described in subsection 5.2.3 page 139 and samples were taken at the indicated time points. 30 OD cells were harvested, washed once with 10 mL cold sterile water, once with 1 mL cold sterile water + protease inhibitors (1x complete<sup>TM</sup>, 1 mM PMSF, 1x protease inhibitor mix from a 100x stock solution described in subsection 7.9 page 158), and resuspended in 600 µL PBS buffer pH 7.4 + previously described protease inhibitors. Cells were lysed at 4°C with glass beads for 20 minutes on a multi-vortexer and centrifuged for 10 minutes at 4°C and 13,000 rpm. After centrifugation 500 µL of the supernatant was transferred to a new test tube. The corresponding antibody (3 µL FBPase antibody, 5 µL rat anti-HA antibody, 5 µL mouse anti-MYC antibody, 3 µL rabbit anti-Cdc48 antibody) was added and samples were gently agitated end over end for 2 hours at 4°C. Immunoprecipitates were collected by adding 50 µL of 5 % (w/v) protein A-sepharose CL-4B (GE-Healthcare, Little Chalfont, United Kingdom) and further incubated for 1.5 hours at room temperature. Sepharose beads were then centrifuged and washed five times with ice-cold PBS buffer (+ 0.5 % Triton). Proteins were released from sepharose by boiling in 50 µL of urea buffer (1 mL urea buffer + 100 µL 1 M Tris pH 8.0 + 10 µL β-mercaptoethanol). Before addition of the antibody 20 µL sample were taken, added to 30 µL urea buffer (1 mL urea buffer + 100 µL 1 M Tris pH 8.0 + 10 µL β-mercaptoethanol) and further used as controls for yeast extracts.

**PBS buffer pH 7.4:** 137 mM NaCl; 1.25 g/L Na<sub>2</sub>HPO<sub>4</sub>; 0.35 g/L NaH<sub>2</sub>PO<sub>4</sub>;
7.6 Pulse chase experiment

For pulse chase experiments cells were inoculated overnight in a CM-medium + all supplements and 2 % glucose. The next afternoon the optical density was measured and cells were shifted to a CM-medium without methionine and without cysteine, and with 2 % glucose with 0.02 OD/mL and let incubate overnight at 30°C (or 25°C for mutant strains). The next day, the OD should be about 3.0 to 5.0 OD/mL. 20 OD cells were harvested, washed once with 4 mL steril water, resuspended in 4 mL CM-medium without methionine and without cysteine and 2 % ethanol (use of 50 mL Falcon tubes) and let incubate for 2.5 hours at 30°C (or 25°C for mutant strains) in a water bath (previously heated at the respective temperature). Thereafter everything was done in the isotopic laboratory. After these 2.5 hours incubation, in case of mutant strains, samples were shifted to the 37°C restrictive temperature for an additional hour. 25 µL 25S-methionine were then added to each culture and cells were let incubate for 3.5 further hours at 30°C (or 37°C for mutant strains). After incubation cells were harvested and resuspended in 5.5 mL chase medium (CM-medium with all supplements, 10 mM methionine and 2 % glucose). 1 mL sample was taken for each time point, pipetted into 1.5 mL Eppendorf tubes with screwing caps previously filled with 100 µL 110 % TCA, and freeze at -80°C. The next day immunoprecipitation was performed also in the isotopic laboratory. Samples were centrifuged for 15 minutes at 14,000 rpm, the supernatant was pipetted away, the pellet washed with 100 µL acetone and resuspended in 100 µL BB1 buffer. Around 100 µL glass beads were added for lysis of cells. Samples were then vortexed in the Vibrax apparatus three times for 5 minutes and in between put for 1 minute at 95°C. Before centrifuging for 15 minutes at 14,000 rpm 900 µL IP buffer with inhibitors were added to the samples. After the centrifugation step the supernatant was carefully pipetted with long white tips and transferred into new 1.5 mL Eppendorf tubes. 3 µL of anti-FBPase antibody was added to each tube and samples were gently agitated end over end for 2 hours at room temperature. Immunoprecipitates were collected by adding 50 µL of 5 % (w/v) protein A-sepharose CL-4B (GE-Healthcare, Little Chalfont, United Kingdom) and further incubated for one hour at room temperature. Sepharose beads were then centrifuged and washed three times with 1 mL IP buffer. Proteins were released from sepharose by boiling in 50 µL of urea buffer (1 mL urea buffer + 100 µL 1 M Tris pH 8.0 + 10 µL β-mercaptoethanol).

Finally, samples were cooked for 10 minutes at 95°C and centrifuged for one minute at
14,000 rpm. 15 µL were loaded on a 10% SDS-gel. After running the gel was put on Whatman paper and let dry for 1.5 hours on the gel dryer. The screen was put on the gel for 2 to 3 days and quantified using the programs Phosphoimager and Imagequant.

**BB1 buffer:** 50 mM Tris; 6 M urea; 1% SDS; 1 mM EDTA, pH 7.5 (HCl)

**IP buffer:** 50 mM Tris; 1.25% Triton X-100; 190 mM NaCl; 6 mM EDTA pH 7.5 (HCl)

**IP buffer + inhibitors:** IP buffer with 1x protease inhibitor mix (stock solution: 100x protease inhibitor mix, see subsection 7.9 page 158), 1 mM PMSF and 1x complete$^TM$ (from 25x complete$^TM$ stock)

### 7.7 Polyubiquitination assay

Polyubiquitination was assessed by growing cells containing the FBPase-TAP encoding plasmid on CM medium without uracil, 2% glucose to OD$_{600}$ 3-4. After harvesting (5 min, 500x g), cells were resuspended in the same medium containing 2% ethanol and let grow during 6 hours to allow derepression of FBPase. 50 OD$_{600}$ yeasts were harvested before and 25 minutes after addition of 2% glucose. After washing in 1 mL water containing 20 mM NEM, 20 mM NaN$_3$ and 1 mM PMSF, cells were pelleted at 500x g for 4 min at 4°C and resuspended in 600 µL ice-cold PBS buffer containing protease inhibitors and lysed at 4°C with glass beads (300 µL; 0.4 to 0.6 mm in diameter) during 20 minutes. The supernatant was kept and transferred into new Eppendorf tubes. FBPase-TAP was then pulled down using 80 µL of 50% (v/v) IgG-Sepharose beads. After 3 hours incubation at room temperature, beads were washed four times with PBS added with 150 mM NaCl and 0.5% (v/v) Triton X-100. Beads were resuspended in 50 µL urea buffer, boiled for 5 min at 95°C and used for immunoblotting with a monoclonal anti-ubiquitin antibody (clone P4G7, Covance).
7.8 Fluorescence microscopy techniques

7.8.1 Direct fluorescence microscopy

Cells were inoculated in 5ml YPD medium and incubated at 30°C over night. 100 µL of preculture were taken and put in 10 mL YPD and then incubated for 6 hours at 30°C under shaking. 1.2 mL of the previous culture were taken and transferred in 10 mL SD-CA medium (0.17 % yeast nitrogen base w/o aminoacids and ammoniumsulfate, 0.5 % ammonium sulfate, 0.5 % casaminoacids and 0.002 % supplements) containing 2 % EtOH as a non-fermentable carbon source and incubated for 16 hours at 30°C under shaking. For fluorescence microscopy 1.5 OD₆₀₀ were taken. A glucose shift (subsection 5.2.3 page 139) was then performed for some samples. Samples without glucose shift were centrifuged at 4,000 rpm, resuspended in 1 mL SD-CA-EtOH medium. 1 µL 2 mM Hoechst 33342 was added and the samples were incubated for 15 min at 30°C under agitation. For samples after 30, 60, 90 and 120 glucose shift, 20 OD₆₀₀ of cells were taken, centrifuged at 4,000 rpm and resuspended in 20 mL SD-CA-Glc medium. 10 µL 2 mM Hoechst 33342 was added and samples were incubated for 15 min at 30°C under agitation. After 0, 30, 60, 90 and 120 min, 3 µL of solution was put on a microscope slide and examined at a wave length $\lambda_{\text{excitation}}$ = 355 nm for Hoechst 33342 and at a wave length $\lambda_{\text{excitation}}$ = 395 nm for GFP.

7.8.2 Indirect immunofluorescence

Yeast cells were grown to a density of about 1.0 OD and 3 OD of yeast cells culture were taken out. 37 % formaldehyde was then added to cultures to an 4.5 % end concentration and samples were let to incubate for at least two hours at room temperature to fix cells. Samples were then centrifuged for 5 minutes at 2,000 rpm, washed 3 times with 1.0 mL buffer I. The pellet was resuspended in 1.0 mL buffer I and 2 µL $\beta$-mercaptoethanol and 20 µL of zymolyase 100T (4 mg/mL) were added. Spheroplasting was performed at 30°C for 45 minutes and samples were centrifuged for 5 minutes at 2,000 rpm. The pellet was washed twice with 1.0 mL buffer I, resuspended in 500 µL buffer I and stored at 4°C.

Parallely microscopy slides were prepared. 20 µL poly-L-lysine (1 mg/mL) were put on each well, let incubate for 10 minutes, aspirated and let dry. Wells were then washed 5 times

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3Hoechst 33342 Trihydrochloridtrihydrat was used to dye DNA in living cells.
with 20 µL steril water and dried. 30 µL of fixed cells were adsorbed to the slide for 15 minutes, aspirated, covered with 20 µL PBS + BSA (5 mg/mL) and incubated for 30 minutes to one hour. They were then covered with 20 µL primary antibody and incubated overnight in a humid chamber. Cells were later rinsed 5 times with PBS + BSA (5 mg/mL), covered with 20 µL secondary fluorescent antibody and incubated for 2 hours in a dark humid chamber. Wells were washed 4 times with PBS + BSA and 3 times with PBS. Cells were then covered with one drop of glycerin 90 % containing DAPI (final concentration of 10 ng/mL). Coverslips were sealed to slide with nailpolish. Cells were finally viewed with a Zeiss fluorescence microscope ($\lambda_{excitation} = 552$ nm and $\lambda_{emission} = 570$ nm for cy3; $\lambda_{excitation} = 365$ nm and $\lambda_{emission} = 420$ nm for DAPI) or stored at 4°C in a dark humid chamber.

1 M KPi buffer pH 6.5: 68.1 % 1 M $KH_2PO_4$; 31.9 % 1 M $K_2HPO_4$

Buffer I, sterile filtered: 100 mM KPi buffer pH 6.5; 1.2 M sorbitol

PBS for immunofluorescence (10x stock): 530 mM $Na_2HPO_4$; 130 mM $NaH_2PO_4$; 750 mM NaCl; For use, autoclave and dilute the stock solution 1:10 with sterile water.

7.9 Nuclei isolation by Ficoll gradient using differential centrifugation

In this protocol from "Current Protocols" (Ausübel et al., 1992) cells are first enzymatically converted to spheroplasts which are then lysed in the presence of Ficoll. A support protocol for isolating intact nuclei by differential centrifugation is then presented. Each time cells were grown in a flask which volume was five times the culture volume.

7.9.1 Spheroplast preparation and lysis

Materials: YPD Medium; YPETOH Medium; zymolyase buffer, room temperature and ice-cold (50 mM Tris.HCl, pH 7.5; 10 mM $MgCl_2$; 1 M Sorbitol; 1 mM or 30 mM DTT); zymolyase 100T (Seikagaku Corp., Tokyo, Japan); 1M Sorbitol; Sorvall SS-34 rotor; Sorvall GS-3 rotor; Kontron Instruments A6.9 rotor; Kontron Instruments A6.14 rotor; Kontron Instruments A8.24 rotor.
Cells were taken from a YPD plate with a tooth pick and inoculated in 5 mL YPD Medium. After two days growth at 30°C 5 mL were taken and added to 50 mL fresh YPD medium; cells were let grow for six hours at 30°C under agitation. 5 mL of this suspension were transferred to 600 mL YPEtOH. Mid-log phase cells were harvested by centrifugation 5 min at 1500x g (Sorvall GS-3 or Kontron Instruments A6.14 rotor at around 3000 rpm), 4°C, in preweighed centrifuge bottles.

Wet weight (in grams) of yeast cells in the pellet was determined by calculating the weight increase over that of the preweighed bottle. This was approximately equal to the packed cell volume (in milliliters) and for all subsequent steps would be considered 1 volume (1 vol). Cells were resuspended in 4 vol. ice-cold water and immediately centrifuged for 5 minutes at 1500x g (Eppendorf Centrifuge 5804R at 3500 rpm), 4°C. Supernatant was discarded. Cells were resuspended with 1 vol zymolyase buffer containing 30 mM DTT and incubated for 15 min at room temperature. This step facilitated subsequent zymolyase treatment and spheroplast lysis by breaking disulfide bonds. The samples were centrifuged again for 5 minutes at 1500x g, 4°C and resuspended in 3 vol zymolyase buffer. 2 mg (200 U) zymolyase 100T per mL of original packed cell volume were added and the samples were incubated for 40 min at 30°C under agitation at around 50 rpm. To determine if lysis has been completed OD of cells was measured using a spectrophotometer diluting the samples 1:10 in water. If spheroplasting was not completed, incubation was carried on. Spheroplasts were then centrifuged for five min at 1500x g, 4°C. Supernatant was carefully discarded, spheroplasts were washed by gently resuspending the pellet in 2 vol ice-cold zymolyase buffer and centrifuging 5 min at 1500x g, 4°C. This step was repeated two more times.

At this step a Bradford test (subsection 7.10 page 160) was performed to determine the concentration of total proteins for each sample (0 min and 30 min after glucose shift). When the concentrations of total proteins differed a lot between the two samples, the same concentrations were taken for both samples thus eliminating the errors due to loss of material.

7.9.2 Nuclei preparation by differential centrifugation

Nuclear protein extracts were prepared by osmotically lysing spheroplasts in the presence of Ficoll which preserved nuclear structure and prevented proteins from leaking out of the nucleus, followed by differential centrifugation.
**Ficoll buffer ice-cold:** 18% (w/v) Ficoll-400; 10 mM Tris.HCl, pH 7.5; 20 mM KCl; 5 mM \(MgCl_2\); 3 mM DTT; 1 mM EDTA; 1x protease inhibitor mix (diluted from a 100x protease inhibitor mix: 100 \(\mu\)g/mL chymostatin, 200 \(\mu\)g/mL aprotinin, 100 \(\mu\)g/mL peptatin A, 720 \(\mu\)g/mL E-64, 10 mM benzamidine); 1 mM PMSF

Cells were resuspended in 0.5 vol zymolyase buffer. At this step a Bradford test (subsection 7.10 page 160) was performed to determine the concentration of total proteins and 100 \(\mu\)g proteins were taken as a sample of the total lysate. Cells were then slowly dropped into a beaker containing 25 mL ice-cold Ficoll buffer with continuous stirring at 4°C in a cold room. The suspension was transferred to 25 mL glass centrifuge tubes and centrifuged for 5 min at 3000x g (Sorvall SS-34 or Kontron Instruments A8.24 at 5000 rpm), 4°C, to pellet cell debris and unlysed spheroplasts. This step was repeated twice. The supernatant was transferred to new 25 mL glass centrifuge tubes and centrifuged for 20 min at 20,000x g (Sorvall SS-34 or Kontron Instruments A8.24 at 13,000 rpm), 4°C. The supernatant was discarded. The nuclei containing pellet was then resuspended in around 5 mL Ficoll buffer. After performing a Bradford test samples of the supernatant and pellet containing 100 \(\mu\)g proteins were taken. Urea buffer was finally added to each sample to an end volume of 100 \(\mu\)L and 10 \(\mu\)L sample were then loaded on a 10% SDS gel before proceeding to a western blot.

### 7.10 Determination of proteins concentration: Bradford test

The Bradford reagent can be used to determine the concentration of proteins in solution. The procedure is based on the formation of a complex between the dye, Coomassie Blue G250 and proteins. The reactant red in a free state becomes blue when binding to proteins and allows very sensitive protein concentration assay. This protein-dye complex causes a shift in the absorption maximum of Coomassie Blue G250 from 465 to 595 nm. The amount of absorption is proportional to the proteins that are present in the sample. To prepare Bradford solution 0.01% (w/v) Coomassie Brilliant Blue G250 was dissolved over night in 2% (w/v) 95% ethanol. 10% (v/v) 85% phosphoric acid was added to the solution which was completed with \(ddH_2O\). For calibration a BSA (Bovine Serum Albumin-solution with a protein concentration
of 1000 µg/mL) was prepared and dilutions with concentrations of 10, 20, 40, 80, 100, 120, 150, 175 and 200 µg/mL were made. 100 µL of each dilution solution were mixed in 900 µL of Bradford solution, incubated for 10 min at room temperature and absorbance was measured at 595 nm. For calibration, solvent used had to be the same as the one of the solution from which the protein concentration had to be determined. The calibration curve Absorbance = f(concentration) was drown. The linear part was used to determine the sample protein concentration. After mixing 100 µL sample with 900 µL Bradford solution, absorbance was measured and protein concentration was inferred from the calibration curve.
Part VI

Bibliography


