Gid4p, The Regulatory Unit Of A Novel E3 Ubiquitin Ligase Involved In Carbohydrate Metabolism In Yeast.

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Vorgelegt von Dipl.-Biol. Olivier Santt
aus Frankreich

Hauptberichter: Prof. Dr. Dieter H. Wolf
Mitberichter: Priv. Doz. Dr. Wolfgang Hilt

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Institut für Biochemie der Universität Stuttgart
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Contents

I Introduction **1**

1 Carbohydrate metabolism. **2**

1.1 Glycolysis. . . . 3

1.2 Gluconeogenesis. . . . 3

2 Carbohydrate-dependent regulation of metabolism. **6**

3 The regulation of fructose-1,6-bisphosphatase. **15**

4 Protein degradation. **20**

4.1 The lysosome/vacuole system. . . . 20

4.2 The ubiquitin-proteasome pathway. . . . 21

4.2.1 Tagging a protein for degradation: ubiquitin. . . . 22

4.2.2 The proteasome. . . . 25

4.2.2.1 The 20 s core complex. . . . 25

4.2.2.2 The 19 S regulatory complex. . . . 27

5 Scope of this work. **29**

II Materials and methods. **31**

6 Plasmids, strains and Oligonucleotides. **32**

6.1 Plasmids. . . . 32

6.2 Strains. . . . 34

6.2.1 Yeast strains. . . . 34

6.2.2 *E. coli* strains. . . . 36

6.3 Oligonucleotides. 36

7 Media, chemicals, antibodies and apparatus. 40

7.1 Media. 40

7.1.1 Yeast media. 40

7.1.1.1 *E. coli* media. 41

7.2 Chemicals, kits, enzymes and antibodies. 41

7.2.1 Chemicals material, and enzymes. 41

7.2.1.1 Chemicals and material 41

7.2.1.2 Enzymes 43

7.2.1.3 Kits. 44

7.2.1.4 Antibody solutions. 44

7.3 Laboratory equipment. 45

8 Cell cultures. 47

8.1 Yeast cell cultures. 47

8.2 *E. coli* cell cultures. 48

9 Molecular Biological Methods. 49

9.1 DNA isolation. 49

9.1.1 Phenol / Chloroform DNA extraction and sodium acetate precipitation. . 49

9.1.1.1 Chromosomal DNA isolation. 50

9.1.1.2 Plasmid DNA isolation. 50

9.2 PCR method. 50

9.3 Nucleic acid electrophoresis. 51

9.4 DNA restriction. 52

9.5 Plasmid DNA dephosphorylation. 52

9.6 Ligation. 52

9.7 Transformation. 53

9.7.1 In yeast cells. 53

9.7.2 In *E. coli* cells. 54

9.7.2.1 Preparation of competent *E. coli* cells. 54

9.7.2.2 Transformation. 54
9.8 DNA sequencing. 55

10 Cell Biology and Biochemistry Methods. 56
10.1 Proteins extraction. 56
10.1.1 Alkaline lysis. 56
10.1.2 Non-denaturing glass beads lysis. 57
10.1.3 Denaturing glass beads lysis. 57
10.2 SDS-PAGE. 57
10.3 Protein detection. 58
10.3.1 Coomassie staining. 59
10.3.2 Immunoblotting. 59
10.3.2.1 Protein transfer onto nitrocellulose or PVDF membranes. . . . 59
10.3.2.2 Immunodetection of transferred proteins. 59
10.4 Glycerol step gradient. 60
10.5 Immunoprecipitation. 61
10.6 Pulse-chase analysis. 61
10.7 Ubiquitination assay. 61

III Results. 63

11 Degradation of FBPase. 64
11.1 Ubiquitin ligases (E3) involved in the degradation of FBPase. 65
11.2 Gid co-factors necessary for FBPase degradation. 67
11.3 The degenerated RING domain of Gid2 is necessary for FBPase polyubiquitination. 68

12 The Vid24p/Gid4p protein. 71
12.1 Gid4p is necessary for FBPase and PEPCK degradation. 73
12.2 Amino-terminal tagging of *GID4*. 73
12.3 Expression of the Vid24p/Gid4p protein. 78
12.3.1 Influence of glucose on *GID4* expression. 78
12.3.2 Influence of other carbon sources on *GID4* expression. 82

12.4 Gid4p triggers FBPase degradation. 85
12.4.1 *De novo* protein synthesis is necessary for FBPase degradation. 85
12.4.2 Gid4p is a molecular switch necessary for FBPase degradation to take place. 86
12.4.3 Gid1p, Gid4p and Gid7p are required for FBPase polyubiquitination. . . 90
12.4.4 Gid4p does not influence the sedimentation profiles of Gid1p-HA_3 and Gid7-HA_3 in a glycerol step gradient. 92
12.5 Gid4p degradation. 93
12.5.1 Construction of gene deletions in YOS1. 93
12.5.2 Gid2 is necessary for Gid4p degradation. 96
12.5.3 Ubc8 is necessary for Gid4p degradation. 97
12.5.4 The proteasome is necessary for the degradation of Gid4p. 98

IV Discussion. 99

Bibliography 109

V Addendum. 131

13 Localisation of known ubiquitin ligases in the yeast *Saccharomyces cerevisiae*. 132

14 Abbreviations used in this work. 145

15 *Curriculum Vitae*. 149

16 Erklärung. 153

17 Note on published work. 154

Abstract

The nutrient glucose is a major component influencing gene expression in the yeast *Saccharomyces cerevisiae*. Hence, cells grown on a nonfermentable carbon source undergo important metabolic changes when a fermentable sugar – *e. g.* glucose – is added to the media. These metabolic changes are well exemplified by the fate of the gluconeogenic enzymes, whose expressions are repressed and their activities inhibited when yeast cells are treated with glucose. This process is termed catabolite repression when it refers to the repression of gene expression or catabolite inhibition when the focus is set on the enzymatic activities. The key gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) as long been a subject of catabolite inactivation studies. Once glucose is conveyed to cells grown on a nonfermentable carbon source, the FBPase gene is repressed and the enzyme is inhibited in a two-step mechanism where i) FBPase activity is inhibited by phosphorylation and by inhibitors such as fructose-2,6-bisphosphate or adenosine monophosphate (AMP) and ii) the enzyme is degraded.

FBPase degradation has been found to depend on either the vacuole or the proteasome. Apparently, the starving conditions of the budding yeasts prior to the glucose treatment influence which system will be used, the cytosolic degradation via the proteasome or the vacuolar degradation which resembles an autophagic process. In our laboratory, the focus was set on the proteasomal degradation of FBPase. A genome-wide screen identified nine *GID* (glucose induced degradation deficient) genes whose deletion blocks the breakdown of FBPase when cells are shifted from an ethanol- (nonfermentable) to a glucose-containing medium. Among those genes, *GID3* and *GID6* were found to be identical to *UBC8* and *UBP14*, respectively. Ubc8p is an ubiquitin conjugating enzyme, and is the most important one in FBPase polyubiquitination. On the other hand, *UBP14* deletion has only a minor effect on FBPase degradation, this effect is supposed to be a consequence of a competition of uncleaved polyubiquitin chains resulting from the absence of Ubp14p and substrate-bound polyubiquitin chains for the proteasome. The seven remaining *GID* genes were further characterised and were found to belong to the same complex, termed the GID complex. Interestingly, some of these *GID* genes are also involved in the vacuolar degradation process of FBPase.

Gid2p/Rmd5p, Gid1p/Vid30p and Gid7p are necessary for polyubiquitination of FBPase. As no ubiquitin ligase could be involved in the degradation of FBPase, it was postulated that the GID complex might have an ubiquitin ligase activity. The discovery of a "degenerated" RING domain in Gid2p substantiated this hypothesis. This is further confirmed by the failure of a strain bearing a RING-mutated GID2 gene to polyubiquitinate FBPase upon shift of the cells to glucose. This study was focused on the Gid4p subunit of the Gid complex, which, like other Gid proteins, is necessary for the degradation of FBPase, but also of PEPCK. N-terminal tagging of Gid4p with the Myc_9 epitope allowed to detect Gid4p and confirmed that among Gid proteins, Gid4p is particular. It is the only Gid complex subunit absent in ethanol-grown cells. Indeed, Gid4p becomes detectable shortly after the growth medium has been replenished with glucose or other fermentable sugars. Cycloheximide treatment confirmed that *de novo* biosynthesis of Gid4p was necessary for the degradation of FBPase. Taken together, this raised the possibility that Gid4p is a switch, triggering FBPase degradation upon shift of the cells to glucose-containing medium.

The forced expression of Gid4p in gluconeogenetic cells resulted in a shorter half-life of FBPase under those conditions, which let hypothesise that Gid4p constitutes the molecular signal for FBPase degradation. This is further confirmed by the fact that, together with Gid1p and Gid7p, Gid4p is essential for FBPase polyubiquitination. How Gid4p activates the Gid complex is still an open question as glycerol step gradients performed with lysates from gluconeogenetic yeasts or glycolytic yeasts do not reveal any difference in the distribution of Gid1-HA_3 and Gid7-HA_3. This result has also been confirmed by performing the same experiments with a *GID4* deletion.

After 30 minutes on glucose, Gid4p levels reach a maximum and thereafter begin to decrease. As other ubiquitin ligase subunits are degraded by the protein complex they belong to, it was of interest to verify whether this is also the case for Gid4p. Indeed, deletion of *GID2* or *UBC8* lead to a stabilisation of Gid4p. Moreover, this degradation occurs via the 20 S proteasome as its inhibition by MG132 results in a Gid4p stabilisation.

Taken together, this work highlights the necessity of the degenerated RING domain of Gid2p for in vivo polyubiquitination of FBPase. Furthermore, results presented here shed light on the function of Gid4p within the Gid complex and show that it is a major regulator of the ubiquitin

ligase activity of the complex.

Abstract

Der Nährstoff Glucose ist ein wichtiger Signalgeber der Genexpression in der Hefe *Saccharomyces cerevisiae*. Zellen, die auf einer nicht -fermentierbaren Kohlensoffquelle wachsen, sind erheblichen Stoffwechseländerungen ausgesetzt, sobald ein fermentierbarer Zucker, wie z.B. Glucose, dem Medium zugesetzt wird. Diese Stoffwechseländerungen können beispielhaft am Schicksal der gluconeogenetischen Enzyme veranschaulicht werden, deren Expression durch Glucosegabe unterdrückt und deren Aktivität herabgesetzt wird. Hierbei bezeichnet man die Unterdrückung der Genexpression als Katabolitrepression. Ist der Fokus auf die Enzymaktivität gerichtet, spricht man von Katabolitinhibition. Schon seit langem ist das Schlüsselenzym der Gluconeogenese, die Fructose-1,6-bisphosphatase (FBPase), Gegenstand intensiver Untersuchungen zur Katabolitinaktivierung. Wird Glucose zu auf nicht-fermentierbaren Kohlenstoffquellen kultivierten Zellen hinzugefügt, führt dies zu einem Stopp der Genexpression der FBPase und zum Verlust der Enzymfunktion: mittels Phosphorylierung sowie durch die Inhibitoren Fructose-2,6-bisphosphat oder Adenosinmonophosphat (AMP) wird die Enzymaktivität gehemmt. Im Anschluß daran folgt der Abbau des Proteins.

Bislang wurden zwei mögliche FBPase Degradationswege beschrieben, zum einen über die Vakuole, ein der Autophagie ähnliches Ereignis, zum anderen selektiv durch das Proteasom. Welcher der beiden Prozesse letztendlich greift, hängt offensichtlich von den Hungerbedingungen der Hefezellen vor Glucosegabe ab. In unserem Labor steht der proteasomale Abbau im Fokus der wissenschaftlichen Bemühungen. In ein genomweiten Screen wurden neun *GID* (glucose induced degradation deficient) Gene identifiziert, deren Nullmutationen zur Stabilisierung von FBPase führen, nachdem die Zellen von nicht-fermentierbarem Medium auf glucosehaltiges Medium transferiert wurden. Zwei dieser Gene, *GID3* und *GID6*, konnten als die schon bekannten Gene *UBC8* und *UBP14* identifiziert werden. Ubc8p gehört zur Familie der Ubiquitin konjungierenden Enzyme und ist bezüglich der Polyubiquitinierung der FBPase besonders wichtig. Dagegen hat die UBP14 Deletion nur einen geringfügigen Einfluß auf den FBPase-Abbau. Der beobachtete Effekt beruht auf der Anreicherung freier Ubiquitinketten in eines *UBP14* Mutante, die in direkte Konkurrenz mit den substratgebundenen Ubiquitinketten um die Ub-Bindestellen am Proteasom treten. Die sieben verbleibenden GID Gene wurden

ebenfalls untersucht und wurden einem gemeinsamen Komplex zugehörig gefunden, dem GID Komplex. Interessanterweise sind einige der GID Gene auch im vakuolären Abbauprozess der FBPase involviert.

Gid2p/Rmd5p, Gid1p/Vid30p und Gid7p sind für die Polyubiquitinierung der FBPase unabdingbar. Da keine bekannte Ubiquitin-Ligase am Abbau der FBPase beteiligt zu sein scheint, wurde vermutet, das diese Aufgabe möglicherweise dem GID Komplex zukommt. Die Entdeckung einer „degenerierten" RING Domäne in Gid2p untermauerte diese Hypothese. Zusätzlich konnte gezeigt werden, dass durch Mutation der RING Domäne die Polyubiquitinierung der FBPase nach Glucosegabe verhindert werden kann.

Im Fokus dieser Arbeit steht die GID Komplex Untereinheit Gid4p, die wie die anderen Gid Proteine für den FBPase Abbau notwendig ist, allerdings auch einen Einfluß auf die Degradation von Phosphoenolpyruvatcarboxykinase (PEPCK) zu haben scheint. Die N-terminale Markierung von Gid4p mit dem Myc_9 Epitop erlaubte ihren gezielten und spezifischen Nachweis. Als einzige GID Komplex Untereinheit ist Gid4p in Ethanol gewachsenen Zellen nicht exprimiert. Tatsächlich ist Gid4p erst kurz nach Zugabe von Glucose oder einem anderen fermentierbaren Zucker zum Wachstumsmediums nachweisbar. Behandlung der Zellen mit Cycloheximid bestätigte, dass die *de novo* Biosynthese von Gid4p für den FBPase Abbau notwendig ist. All diese Befunde bestätigen die Annahme, dass Gid4p ein molekularer Schalter ist, der den Abbau der FBPase einleitet, nachdem die Zellen auf glucosehaltiges Medium transferiert wurden.

Wird Gid4p artifiziell in gluconeogenetischen Zellen exprimiert, resultiert dies in einer verkürzten Halbwertszeit der FBPase, was wiederum nahe legt, dass Gid4p das molekulare Signal zum FBPase Abbau darstellt. Außerdem konnte gezeigt werden, dass Gid4p, wie auch Gid1p und Gid7p, für die Polyubiquitinierung der FBPase essentiell ist. Wie Gid4p den GID Komplex aktiviert bleibt noch zu klären; ein mit gluconeogenetischen und glycolytischen Hefezellenlysaten durchgeführter Glycerolgradient konnte keine Unterschiede in der Masseverteilung von Gid1-HA_3 und Gid7-HA_3 aufdecken. Auch mit einer einer GID4 Deletion konnte in diesem Experiment kein Unterschied in der Masseverteilung des Komplexes festgestellt werden.

Gid4p erreicht seine höchste Expressions nach 30 Minuten auf Glucose und nimmt danach langsam ab. Da verschiedene Untereinheiten von Ubiquitin-Ligasen in Abhängigkeit ihres eige-

nen Proteinkomplexes abgebaut werden, wirft das die Frage auf, ob dieser Befund auch für Gid4p zutrifft. Tatsächlich führen die Nullmutationen von *UBC8* und *GID2* zur Stabilisierung von Gid4p. Des weiteren wird Gid4p vom Proteasom abgebaut, da die Blockade des 26 S Proteasoms durch den Inhibitor MG132 ebenfalls in einer Stabilisierung von Gid4p resultiert.

Diese Arbeit zeigt die Notwendigkeit der degenerierten RING Domäne in Gid2p für die in vivo Polyubiquitinierung der FBPase. Weiterhin decken die gezeigten Ergebnisse die Funktion von Gid4p innerhalb des GID Komplexes auf und verdeutlichen, dass Gid4p den hauptsächlichen Regulator der Ubiquitin-Ligaseaktivität darstellt.

Part I

Introduction

Chapter 1

Carbohydrate metabolism.

In nature, an unicellular organism is subject to drastic and very rapid changes in its environment. Therefore, such an organism must be able to rapidly adapt its metabolism with respect to its surroundings.

The carbon source is of the utmost importance to the living organisms. It provides them with building blocks for diverse components such as nucleic-, fatty- and amino-acids. Moreover, cells use carbon sources to derive the necessary energy to sustain biosynthetic processes (Berg et al., 2002). Glucose is the principal carbohydrate used by many cells. Among crown eukaryotes, this is even the only one in normally fed brain cells, and it is the sole one for blood red cells, bone marrow, renal medulla and peripheral nerves (Berg et al., 2002; Wahren and Ekberg, 2007). Hence, glucose homoeostasis in mammals is very important for survival over long fasting periods. Moreover, disturbance of the glucose sensing / signalling in mammals leads to severe metabolic defects like diabetes mellitus type II (Klover and Mooney, 2004).

Thus, carbohydrate metabolism is under intense investigations, as unravelling its different aspects might shed light on the signal pathways and the adaptation strategies involved when cells encounter a change in sugar availability. This might as well help curing metabolic diseases which have high prevalence in Western countries (Klover and Mooney, 2004).

The quantities and the qualities of carbohydrates and, more generally, nutrients are determining for organisms. While cells part of a multicellular organism adapt to different hormonal signals or react to their micro-environment, protozoans have to adapt very rapidly to changes in their neighbourhood.

1.1 Glycolysis.

Toward the end of the XIXth century, many discoveries paved the way to the development of modern biochemistry. Emil Fischer synthesised different sugars which he used to demonstrate that yeast was not only able to fermentate glucose but a host of saccharides (Barnett and Lichtenthaler, 2001). Moreover, he showed that only the D-components of racemic sugar mixtures were used by yeast cells for fermentation (for a review, see Barnett and Lichtenthaler, 2001), suggesting that the as yet not defined enzymes would function according to a "key-lock" principle. Almost as important; if not more, was the demonstration that fermentation was possible in a cell-free yeast lysate by Eduard and Hans Buchner (Barnett and Lichtenthaler, 2001; Berg et al., 2002). This allowed the further exploration of glycolysis as a major catabolic pathway.

Glycolysis is a nearly universal process, whose apparition was probably anterior to the use of oxygen by cells during cellular respiration. Hence, it is used universally by anaerobic an aerobic organisms. During glycolysis, one glucose molecule is transformed into pyruvate, thereby producing a net gain of two ATP molecules (Berg et al., 2002). The overall process can be divided into three stages, where glucose is first phosphorylated to be sequestered within the cell, interconverted into fructose-1,6-bisphosphate, split – after isomerisation – into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate which is eventually oxidised into pyruvate. This oxidation reaction eventually leads to the production of the ATP molecules (Berg et al., 2002). During the course of this transformation, NAD^+ when no oxygen is present, is reduced into NADH. To regenerate NAD^+, several organisms will use diverse strategies, either producing lactate as in mammals or ethanol as in *Saccharomyces cerevisiae* (Berg et al., 2002). Glycolysis is a central metabolic process in the cell as it does not only produces energy in form of ATP but also delivers building blocks to various biosynthetic pathways (Berg et al., 2002). Thus, when glucose is limiting in the environment, cells have to adapt to produce these building blocks or their precursors. This is achieved by the gluconeogenesis pathway.

1.2 Gluconeogenesis.

Gluconeogenesis can be defined as the process of producing glucose from non-carbohydrates precursors (Berg et al., 2002). Yet, it is not *stricto sensu* the reciprocal pathway to glycolysis. Most

of the enzymes involved in glycolysis are partaking in gluconeogenesis, but several reactions need different enzymes to proceed in the gluconeogenic direction: these are the phosphorylations of glucose to glucose-6-phosphate, fructose-6-phosphate to fructose-1,6-bisphosphate and the dephosphorylation of phosphoenolpyruvate to pyruvate. During glycolysis, these reactions are catalysed by the hexokinase, the phosphofructokinase and the pyruvate kinase enzymes, respectively (Rawn, 1989; Berg et al., 2002). These reactions are thermodynamically possible in only one direction, which makes it necessary for the cell to use another set of enzymes when it has to carry out gluconeogenesis. Also, divergent regulation of both pathways is only possible when at least one enzyme differs in the two antagonistic pathways. The first set of reactions specific for gluconeogenesis concerns the export of pyruvate out of the mitochondria. While pyruvate can enter the mitochondrial matrix through a pyruvate antiporter, which has not yet been firmly identified (Hildyard and Halestrap, 2003 and Saccharomyces Genome Database), it has to be transformed into malate to be transported from the mitochondria to the cytosol (Berg et al., 2002). Pyruvate carboxylase catalyses the carboxylation of pyruvate into oxaloacetate which is then reduced into malate by the mitochondrial malate dehydrogenase. After malate is transported out of the mitochondria, it is oxidised in oxaloacetate by the cytoplasmic malate dehydrogenase. Oxaloacetate is then converted into phosphoenolpyruvate by the phosphoenolpyruvate carboxykinase (PEPCK). The next irreversible reaction in the course of gluconeogenesis is driven by the fructose-1,6-bisphosphatase (FBPase) which catalyses the dephosphorylation of fructose-1,6-bisphosphate into fructose-6-phosphate. Interestingly, cytosolic malate dehydrogenase, PEPCK and FBPase were shown to interact together in yeast (Gibson and McAlister-Henn, 2003) which could result in "substrate channelling" and optimise the speed of gluconeogenesis (Miles et al., 1999). The last reaction of gluconeogenesis is a dephosphorylation of glucose-6-phosphate into glucose. It is catalysed by the glucose phosphatase enzyme. In most tissues of metazoans, this later step is not taken, as glucose-6-phosphate is used to form glycogen. However, in the case of the liver, and marginally the kidneys, gluconeogenesis proceeds until free glucose is formed and released into the blood stream. The dephosphorylation of glucose-6-phosphate does not take place in the cytoplasm but in the lumen of the endoplasmic reticulum, where glucose-6-phosphate is transported. Glucose is then shuttled back into the cytoplasm by a transporter akin to the one found in the plasma membrane (Berg et al., 2002). To

date, no glucose phosphatase has been identified in Saccharomyces cerevisiae, gluconeogenesis in that organism does therefore not seem to go beyond the production of glucose-6-phosphate.

Chapter 2

Carbohydrate-dependent regulation of metabolism.

As glucose is the most important sugar for many organisms or cell types, sensing its concentration in the media or the micro-environment is important for the cell to regulate its metabolism. Therefore, glucose itself acts as a primary messenger that binds to receptors and activates several signalling cascades. This is especially true for the yeast species that use glucose as the main fuel (Rolland et al., 2002). The brewer's yeast *Saccharomyces cerevisiae* has long been used as a model organism to study sugar metabolism (Barnett and Lichtenthaler, 2001; Barnett, 2003). It has been as well considered as a model to study its regulation (Barnett and Entian, 2005). Addition of glucose to yeast cells growing in a medium containing a nonfermentable carbon source results in dramatic changes. Those changes reside eventually in the repression of genes whose products are necessary for the use of alternative carbon sources and respiration while genes whose expression is necessary for fermentation are induced (Gancedo, 1998; Rolland et al., 2002; Santangelo, 2006).

Glycolysis and gluconeogenesis are both highly exergonic in normal conditions. Thus, these pathways have to be regulated to prevent their simultaneous occurrence within a cell (Berg et al., 2002). To ensure a rapid shift from oxidative phosphorylation to fermentation, the gluconeogenic enzymes undergo allosteric inhibition and covalent modifications followed by proteolysis (Holzer and Purwin, 1986; Kalisz et al., 1987; Holzer, 1989). As an example, the key gluconeogenic enzyme FBPase is inhibited by AMP and fructose-2,6-bisphosphate while phosphofructokinase, the enzyme catalysing the reciprocal reaction, is activated by those compounds

(Berg et al., 2002; Rolland et al., 2002). Inhibition of phosphoenolpyruvate carboxykinase and fructose-1,6-bisphophatase is necessary to provide a competitive advantage when cells grow on media containing high glucose concentration, by preventing futile cycles of ATP (Navas and Gancedo, 1996; Berg et al., 2002). In *Saccharomyces carlbergensis*, the simultaneous run of glycolysis and gluconeogenesis has an even more pronounced effect, as it leads to a severe loss in survival capability of the yeasts (van de Poll et al., 1974).

Different eukaryote species use specific systems to sense glucose in the micro-environment. In yeast, it is supposed that there are three different glucose sensing systems. Each of them having heterogeneous effects, or influencing diverse set of genes (Rolland et al., 2001b, 2002; Belinchón and Gancedo, 2007; Belinchón and Gancedo, 2007). The Hxk2p-Mig1p pathway is mainly associated with glucose-dependent repression effects, due to the huge set of genes repressed, it is also known as the "main glucose repression pathway" (Klein et al., 1998). The Snf3p-Rgt2p pathway, on the contrary, is associated with glucose-dependent expression (or derepression) effects. The role of the cAMP pathway does not seem to be that clear: it has a broad spectrum of downstream effectors and therefore may affect expression of genes in both directions.

As glucose enters the cell, it is rapidly phosphorylated by three sugar kinases: glucokinase 1 (Glk1p), hexokinase 1 (Hxk1p) and hexokinase 2 (Hxk2p). This phosphorylation is thought to prevent glucose to diffuse passively out of the cell (Berg et al., 2002). Moreover, besides their role in the glycolytic process, the function of the hexokinases has been investigated in glucose signalling, although with different outcomes regarding FBPase degradation (Horak et al., 2002; Hung et al., 2004; Belinchón and Gancedo, 2007). *HXK2* was the first gene identified as a glucose sensor (Rolland et al., 2001b, 2002). As shown with 2-deoxy-glucose, no further metabolization seems to be needed to trigger a glucose phosphorylation-dependent response (Rolland et al., 2002). The three hexose kinases seem to have diverse functions: while all three were implicated in the short-term response to glucose, only Hxk2p exerts its effects on a long-term period. Hxk2p is involved mainly in glucose-dependent repression of gene expression (Rolland et al., 2001a, 2002). Moreover, it seems that the three glucose kinases have different expression pattern regarding the yeast growth conditions. *HXK1* and *GLK1* were shown to be expressed when cells were grown on nonfermentable carbon sources or galactose and absent

when cells were cultivated on highly fermentable carbon sources. On the contrary, levels of *HXK2* mRNA were basal during respiratory growth, but strongly induced after shift to glucose (Herrero et al., 1995). Interestingly, the Hxk2p is also found in the nucleus of yeast cells, where it interacts with transcriptional repressors, such as Mig1p, which control glucose-repressed genes expression (Rolland et al., 2001b, 2002; Moreno et al., 2005). As opposed to Mig1p, The role of Hxk2p in the main glucose repression pathway is poorly understood. Mig1p is a DNA-binding factor and directs the repressors Tup1p and Ssn6p to the promoters of the genes to be repressed upon glucose shift (Klein et al., 1998; Santangelo, 2006). Hxk2p has been shown to be partially located to the nucleus upon glucose shift. This nuclear localisation is necessary for the glucose-dependent repression of genes like *SUC2* which encodes invertase (Herrero et al., 1998; Randez-Gil et al., 1998). It has been demonstrated to interact with Mig1p both *in vivo* and *in vitro* (Ahuatzi et al., 2004). Moreover, Hxk2p is recruited to glucose-repressible promoters by Mig1p (Ahuatzi et al., 2004; Moreno et al., 2005). The relationships between Mig1p and Hxk2p are not restricted to an interaction and a partial co-localisation after glucose treatment: while the quantity of Mig1p within the cell regulates the proportion of Hxk2p being located in the nucleus (Ahuatzi et al., 2004), Hxk2p itself determines the localisation of Mig1p (Moreno et al., 2005; Ahuatzi et al., 2007). Mig1p subcellular localisation depends on its phosphorylation state. If not phosphorylated, Mig1p is localised within the nucleus. When glucose is removed from the growth media, Mig1p becomes phosphorylated in a Snf1-dependent manner; which eventually leads to its export from the nucleus (Gancedo, 1998; Treitel et al., 1998; Santangelo, 2006). Interestingly, whereas an *HXK2* deletion mutant abolishes the Mig1p nuclear localisation upon glucose shift, in a *SNF1 / HXK2* double deletion mutant, as well as in a *SNF1* deletion, Mig1p is always located in the nucleus, suggesting that Hxk2p might exert its effects upon Mig1p indirectly (Tomás-Cobos and Sanz, 2002; Moreno et al., 2005; Ahuatzi et al., 2007). It has been proposed that, on high glucose concentrations, Hxk2p might negatively regulate the activity of the Snf1 kinase complex towards Mig1p, thereby maintaining the glucose-repression of the genes involved in utilisation of alternative carbon sources (Ahuatzi et al., 2007). Another player in that pathway is the type 1 protein phosphatase (PP1), which antagonises Snf1 (Rolland et al., 2002). When cells are shifted to a glucose-containing media, the Reg1p-Glc7p phosphatase dephosphorylates Mig1p, which leads to its translocation into the nucleus, and the subsequent

repression of genes driven by a promoter bound by Mig1p. Interestingly, PP1 has not only a promoting effect on Mig1p repressive functions, it also regulates the activity of the Snf1 kinase complex (composed of a α subunit, Snf1p; a γ subunit, Snf4p; and a β subunit, either Gal83p, Sip1p or Sip2p) as well (Sanz et al., 2000). Snf1p has itself a regulatory and a catalytic domain. At a basal state, the Snf1p catalytic domain is bound to its regulatory domain which results in Snf1p auto-inhibition (Rolland et al., 2002). When glucose is scarce, Snf1p is phosphorylated on threonine 210 in its activation loop by either Pak1p / Sak1p, Elm1p, or Tos3p (Nath et al., 2003; Sutherland et al., 2003; Elbing et al., 2006). This phosphorylation is not sufficient for Snf1p activation: binding of Snf4p to the Snf1p regulatory domain, which stabilises the complex in an open, active conformation is also required (Jiang and Carlson, 1996; McCartney and Schmidt, 2001). Moreover, the Gal83p subunit of the kinase complex targets the previously cytoplasm-located Snf1p to the nucleus (Vincent et al., 2001). After glucose limitation, the Reg1p regulatory subunit of the Reg1p-Glc7p form of PP1 interacts with the phosphorylated Snf1p and is itself rapidly phosphorylated. This interaction and phosphorylation are necessary to promote the return of an active Snf1p kinase complex to an inactive, auto-inhibited one, possibly by Glc7p-dependent dephosphorylation of the Snf1 kinase complex (Sanz et al., 2000). Overall, the "main glucose repression pathway" is a triad composed of an activator (PP1), a repressor (the Snf1p kinase complex) and an effector (Mig1p and possibly other DNA-binding factors). Once glucose is added, Mig1p is activated by PP1, which also represses the inactivator Snf1p kinase complex. Moreover, hexokinase 2 has been suggested to inhibit the Snf1p kinase complex at the nuclear level and thereby sustain the glucose dependent repression on a long-term basis (Moreno et al., 2005).

Addition of glucose to culture media leads to a spike in cAMP (Holzer and Purwin, 1986; Wilson and Roach, 2002). The cAMP pathway has been shown to have an influence over many, if not all, genes whose expression (either induction or repression) depends on glucose; among them *FBP1* (Zaragoza and Gancedo, 2001; Rolland et al., 2002; Wang et al., 2004; Santangelo, 2006). *RAS1* and *RAS2* encode small GTPases that activate adenylyl cyclase upon glucose stimulation. Single deletion mutants grow well on glucose containing media, while a double deletion in *RAS1* and *RAS2* arrests in G_1. As nutrient-deprived cells also arrest in G_1, this is suggestive of a role of the Ras proteins in nutrient sensing. A *RAS2* allele , $ras2^{318S}$, in a strain

deleted for *RAS1*, has been shown to abolish the cAMP spike observed after shift of the cells from a nonfermentable carbon source to a glucose containing media. Moreover, resumption of growth in cells bearing this allele was delayed compared to wild-type yeasts (Jiang et al., 1998). As growth on either nonfermentable or fermentable carbon sources is not affected, this suggests that the cAMP spike observed, and the subsequent activation of the protein kinase A (PKA) holoenzyme is necessary for an efficient transition from gluconeogenesis to glycolysis, but has no impact on growth itself (Jiang et al., 1998). Activation of the Ras pathway seems to be dependent on transport of glucose into the cell and its subsequent phosphorylation by the sugar kinases Hxk1p, Hxk2p and Glk1p (Colombo et al., 2004). Although the exact mechanism by which it operates is not known, this phosphorylation is thought to relieve the inhibition exerted over Ras2p by Ira1p and Ira2p proteins. After this inhibition relief, Ras2p can interact with and activate the adenylyl cyclase (Colombo et al., 2004).

Besides the small G-proteins, the cAMP second messenger is often associated with an upstream G-Protein Coupled Receptor (GPCR) in mammals (Berg et al., 2002). This holds true for yeast as well (Rolland et al., 2001b, 2002; Santangelo, 2006). Here, the signalling pathway obeys to the same principles as in higher eukaryotes. Several genes have been implicated in glucose GPCR-dependent signalling in yeast: *GPR1* encodes a seven transmembrane receptor, whose ligand is supposed to be glucose (Lemaire et al., 2004), while *GPA2* encodes the Gα subunit of a heterotrimeric G-protein (Colombo et al., 1998). After ligand binding, Gpa2p is thought to bind to and stimulate Cyr1p, the yeast adenylyl cyclase, thereby triggering the production of cAMP which in turns activates PKA. In *Saccharomyces cerevisiae*, the Ras2p and the Gpa2p pathways both exert their effects through PKA (Jiang et al., 1998; Wang et al., 2004). The yeast PKA is a holoenzyme of the products of four different genes, three of them encoding the catalytic subunits (*TPK1*, *TPK2*, *TPK3*) and a regulatory subunit (*BCY1*). The holoenzyme is a heterotetramer, constituted by two catalytic subunits and two regulatory subunits. The cAMP spike observed seems to be necessary to relieve the inhibition exerted by Bcy1p over the catalytic subunits (Santangelo, 2006). Understanding which proteins are the targets of the PKA is complicated by the fact that the different catalytic isoenzymes are not functionally redundant, eventually activating the transcription of diverse readouts that might have opposite effects (Robertson et al., 2000). Nevertheless, the cAMP-PKA pathway has been

shown to activate Rap1p, a transcription activator that triggers the expression of genes involved in the biogenesis of ribosomes and of genes involved in glycolysis. Moreover, this pathway leads to an inhibition of Msn2p and Msn4p transcription factors, thereby lowering or inhibiting the expression of STRE-dependent genes (in agreement with the observation that a *gpa2*Δ strain resists better to heat shock has shown by Colombo et al.) and activates the Snf1p kinase complex which is implicated in a host of carbon source regulation events and a member of the "glucose main repression pathway" (see above and Santangelo, 2006).

The two PKA-dependent pathways are responsible for ninety percent of the remodelling of gene expression upon glucose shift; but, interestingly enough, there seem to be an overlapping, cAMP-independent signalling pathway for some of those genes that are regulated by Ras2p and Gpa2p (Wang et al., 2004). Interestingly, Gpa2p was reported to be able to activate the PKA pathway in a cAMP-independent manner (Peeters et al., 2006, 2007). This could account for the cAMP-independent effects observed by Wang et al. (2004).

The third system is formed by the two hexoses transporter homologues Snf3p and Rgt2p, which have been implicated in expression regulation of the hexose transporters themselves (Ozcan and Johnston, 1995; Rolland et al., 2002). These proteins are only 26 % to 30 % identical to the other members of the hexose transporter family (Ozcan and Johnston, 1999). Both sensors are composed of two functional domains: twelve transmembrane regions and a long cytoplasmic tail at their C-termini (Marshall-Carlson et al., 1990). This C-terminal cytoplasmic tail is much shorter in the other members of the hexose transporters family. Therefore, it was postulated that these C-terminal tails should mediate the signal transduction from the sensors (see below, Ozcan and Johnston, 1999). Although they share high identity (60 % identity, 72 % similarity), Snf3p and Rgt2p have been shown to be involved in the expression of different hexose transporter genes. Snf3p is expressed at its highest level when glucose is present in low concentration and thereby thought to sense glucose when it is scarce in the media. Indeed, hexose transporters genes like *HXT2*, *HXT3* and *HXT4*, normally induced on low glucose concentration, cannot be expressed when *SNF3* is deleted (Ozcan and Johnston, 1995). On the other hand, Rgt2p is constituvely expressed, and necessary for full induction of *HXT1*, which codes for a low-affinity glucose transporter (Ozcan and Johnston, 1999). Hence, *HXT1* expression raises with glucose concentration in the media, with an maximum at 4 % of glucose (Ozcan and Johnston, 1995).

The C-terminal tails of both sensors were demonstrated to be necessary and sufficient for glucose signal transduction: deletion of the Snf3p C-terminal tail led to a defect in expression of *HXT2* and *HXT4*. On the other hand, when fused to the C-terminus of a hexose transporter, this tail was able to complement the expression defects seen in *rgt2* and *snf3* deletion mutants (Ozcan et al., 1998). Snf3p and Rgt2p were found to bind to the yeast casein kinases Yck1p and Yck2p and to the Rgt1p co-repressors Mth1p and Std1p (Moriya and Johnston, 2004). Upon glucose stimulation, the casein kinases are activated and phosphorylate Mth1p and Std1p which then become substrates of the ubiquitin protein ligase SCF^{Grr1}. Polyubiquitination of these proteins results in their degradation by the 26 S proteasome (4.2). Interestingly, however, the main pathway involved in the relief of the inhibition exerted by Rgt1p on the *HXT* genes expression is the PKA pathway. Phosphorylation of the N-terminus of Rgt1p by all three isoforms of PKA results in the binding of this N-terminal, DNA-binding part with a middle segment of Rgt1p. Eventually, this abolishes the binding of the Rgt1p transcription factor to the DNA and thereby allows the expression of the *HXT* genes when cells are replenished with glucose (Kim and Johnston, 2006). When cells are grown on a nonfermentable carbon source, Mth1p binds to Rgt1p and prevents this intramolecular interaction to happen (Polish et al., 2005).

Another system is involved in signalling of nutrient availability. This system is composed of two different kinase complexes: TORC1 and TORC2. Although some subunits are shared, they both show different properties as the TORC1 complex is sensitive to rapamycin while the TORC2 complex is not (Rohde et al., 2008). Both complexes share common readouts which complexifies the study of their respective effects. Tor signalling have been implicated in the glucose-dependent induction of glycolytic enzymes and ribosomal proteins upon shift of the yeast cells onto a glucose-containing medium (Santangelo, 2006). In particular, carbon starvation leads to dephosphorylation and inactivation of the newly identified AGC kinase Sch9p, a substrate of TORC1 (Urban et al., 2007; Rohde et al., 2008). Indeed, rapamycin treatment led to a delay in FBPase degradation upon shift of the yeasts to glucose. This delay was not observed in a *TOR1-1* mutant, insensitive to rapamycin. Altogether, this is suggestive for a role of the TORC1 complex in the general carbon catabolite repression / inactivation (Regelmann, 2005). This hypothesis has been later confirmed in the case of amino acids permeases (Peter et al., 2006). Moreover, the Tor pathway, together with PKA and glucose transport account

for the vast majority of the changes in gene expression observed when cells are replenished with nutrients (Slattery et al., 2008). In spite of these results, this pathway remains poorly understood, particularly with regards to glucose signalling, although it is thought that, rather than sensing the presence of fermentable carbon source, Tor1 / Tor2 sense the energy state of the cell (Liao et al., 2008).

Therefore, several pathways are intricated to sense glucose and allow the expression of the hexose transporters genes (Fig. 2.1). The interconnections between these different pathways involved in glucose sensing highlight the importance of a tight regulation of the carbohydrate metabolism. The Snf1p-Mig1p repression pathway, for example, regulates the Snf3p-Rgt2p sensing system by repressing *SNF3* and *MTH1* and the hexose transporters when glucose is abundant in the media (Klein et al., 1998; Kaniak et al., 2004). Also, the Snf3p-Rgt2p induction pathway induces the transcription of *MIG2*, which encodes a Mig1p functionally redundant protein (Kaniak et al., 2004).

As depicted in that section, the regulation of gene expression with respect to the availability of the carbon source has been extensively studied. However, the events leading to catabolite inhibition (or catabolite degradation) only begin to be better understood. Fructose-1,6-bisphosphatase has long been known as a subject of such catabolite inhibition / degradation (Holzer, 1976). Thus, studying the regulation of the activity of this enzyme might shed light on this part of the regulation of the metabolism of a cell when its environment is changing.

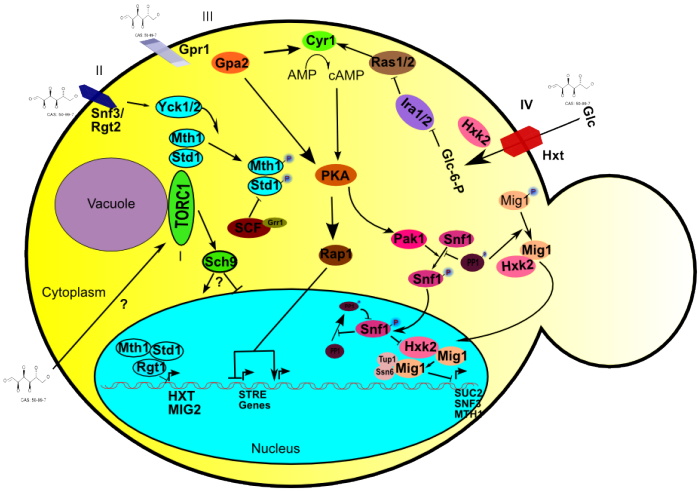

Figure 2.1: **The glucose signalisation pathway in *Saccharomyces cerevisiae*.** This figure describes the signalisation pathways outlined in Chapter 2. The four main pathways are described. I: The TORC1 complex is activated when cells are replenished with fresh medium, its upstream and downstream effectors are unknown. II: The glucose sensors Snf3 and Rgt2 activate the yeast casein kinases which phosphorylate Mth1 and Std1. Once phosphorylated, these proteins become substrates of the SCF^{Grr1} ubiquitin ligase and are subsequently degraded by the proteasome. This degradation relieves the inhibition exerted by Rgt1 on genes repressed in cells are grown on nonfermentable carbon sources (*e.g.*: *HXT* genes). III: Glucose binds to the seven-transmembrane receptor Gpr1 this triggers the activation of the protein G α subunit Gpa2 which subsequently binds to and activates the adenylate cyclase Cyr1. The following activation of PKA leads to activation of Rap1 and other transcription factors. Gpa2 can also directly activate PKA. IV: Entry of glucose into the cell and its subsequent phosphorylation. Phosphorylated glucose inhibits Ira1 and Ira2, thereby allowing the small GTPases Ras1 and Ras2 to activate the adenylate cyclase. On the other hand, Hxk2 binds to Mig1 and translocates to the nucleus where it inhibits the phosphorylation of Mig1 together with the PP1 phosphatase. Mig1 participates in the inhibition of several genes like *SUC2*, *SNF3* and *MTH1*. The complexes (PKA, PP1, Snf1) are detailed in Chapter 2, of the three kinases phosphorylating Snf1, only Pak1 is shown. Glc: glucose; Glc-6-P: glucose-6-phosphate; P: phosphate.

Chapter 3

The regulation of fructose-1,6-bisphosphatase.

As mentioned before (1.1); the third reaction of glycolysis, the phosphorylation of fructose-6-phosphate into fructose-1,6-bisphosphate, catalysed by phosphofructokinase (PFK), is irreversible. During gluconeogenesis, fructose-1,6-bisphosphatase catalyses the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate (Berg et al., 2002). Both reactions are exergonic, hence they can take place at the same time. If glycolysis and gluconeogenesis would run simultaneously, this would lead to a futile cycle of ATP consumption which could be deleterious to the cell (van de Poll et al., 1974; Holzer, 1976). Therefore, a tight regulatory control is necessary to ensure that these metabolic pathways do not take place concomitantly.

The genes encoding the gluconeogenic enzymes FBPase and PEPCK are repressed by few amounts of glucose (Gancedo, 1998; Yin et al., 2000). In addition to the repression of the *FBP1* gene, the enzymatic activity of its product has been shown to be controlled by short-term and long-term inactivation events (Gancedo, 1971; Lenz and Holzer, 1980). Interestingly, the short-term inactivation (as measured three minutes after shift to glucose) is reversible, which led to the assumption that this inactivation would rely on modifications of the enzyme (Lenz and Holzer, 1980). On the other and, the long-term inactivation (observed after one hour shift on glucose) has been shown to be irreversible: restoration of the FBPase activity required *de novo* protein synthesis (Gancedo, 1971). The late inactivation event was suggested to depend on selective proteolysis of FBPase (Gancedo, 1971). Indeed, proteolysis of FBPase in *Saccharomyces cerevisiae* has been later confirmed (Molano and Gancedo, 1974; Holzer and

Purwin, 1986; Holzer, 1989). Thus, the decrease in FBPase activity observed in yeast is due to a two-step phenomenon, the first step being driven by inhibition of the enzyme while the second step relies on selective proteolysis of FBPase.

The reversible inhibition of fructose-1,6-bisphosphatase has been documented has being the result of allosteric changes triggered by AMP (Taketa and Pogell, 1965) and / or fructose-2,6-bisphosphate (Pilkis et al., 1981; Lederer et al., 1981; Pontremoli et al., 1982). Interestingly, these two molecules are widespread inhibitors has they both inhibit FBPase in eukaryotic cells. Moreover, although the *E. coli* FBPase is insensitive to fructose-2,6-bisphosphate, it is highly sensitive to the intracellular concentration of AMP (Hines et al., 2007b).

PFK and fructose-1,6-bisphosphatase (FBPase) are controlled reciprocally, with activators of one enzyme being the repressors of the other (Berg et al., 2002). During glycolysis, fructose-6-phosphate is not only phosphorylated into fructose-1,6-bisphosphate but into fructose-2,6-bisphosphate as well. This latter reaction is catalysed in humans by the phosphofructokinase-2 (PFK-II) enzyme. Fructose-2,6-bisphosphate, albeit being a metabolically inactive compound, is important: it activates phosphofructokinase and inhibits FBPase. In mammals, when the cells have to perform gluconeogenesis, PFK-II is inhibited through phosphorylation by PKA. As a result, the quantity of fructose-2,6-bisphosphate within the cell is lowered, eventually leading to a diminished – if not completely abolished – activity of PFK, while the inhibition upon FBPase is relieved (Rawn, 1989; Berg et al., 2002). In *S. cerevisiae*, fructose-2,6-bisphosphate is a potent inhibitor of FBPase as well (Lederer et al., 1981; von Herrath and Holzer, 1988). Upon shift of yeasts grown on nonfermentable carbon source to a fermentable carbon source, the fructose-2,6-bisphosphate concentration in the cell increases rapidly. After one minute of shift, the intracellular concentration of fructose-2,6-bisphosphate in *S. cerevisiae* is sufficient to inhibit FBPase (Lederer et al., 1981). A recent study demonstrated that Pfk27, which fulfils the same role in *Saccharomyces cerevisiae* as PFK-II in humans, is phosphorylated and degraded via the ubiquitin-proteasome pathway following glucose removal from the media (Benanti et al., 2007). Failure to phosphorylate Pfk27 in a Snf1-dependent manner leads to its stabilisation and, as a consequence, yeast cells cannot grow on nonfermentable carbon sources (Benanti et al., 2007). This growth defect might be due to ongoing presence of fructose-2,6-bisphosphate which would inhibit FBPase and therefore gluconeogenesis. Pohlig and Holzer (1985) showed that fructose-

2,6-bisphosphate increases the rate of FBPase phosphorylation by a cAMP-dependent kinase. Importantly, a control experiment performed by those authors demonstrated that fructose-2,6-bisphosphate had no influence of the phosphorylation of histone by the same kinase, an indication that fructose-2,6-bisphosphate interacts with FBPase rather than with the kinase (Pohlig and Holzer, 1985). Several studies were undertaken to investigate the inhibition mechanism of fructose-2,6-bisphosphate over FBPase. Most of them were carried out using porcine liver FBPase which bears 48 % identity and 69 % homology with yeast FBPase. As the mammalian liver FBPase and the baker's yeast FBPase are regulated in the same way (von Herrath and Holzer, 1988), it is possible to infer the inhibition mechanism of FBPase in yeast from what has been observed in higher eukaryotes. In rat liver, fructose-2,6-bisphosphate inhibits FBPase competitively with respect to fructose-1,6-bisphosphate (Pilkis et al., 1981). Fructose-2,6-bisphosphate has been shown to bind to the active site of the mammalian enzyme (Liang et al., 1992). This binding results in a 13° rotation of a subunit pair from the active R-state, and the active site loops are disengaged, which eventually leads to inhibition of the FBPase activity (Hines et al., 2007a).

5'-adenosine monophosphate (AMP) is another inhibitor of FBPase (Taketa and Pogell, 1965). AMP and fructose-2,6-bisphosphate do not bind on the same sites on the FBPase. The nearest binding site of AMP is located 28 Å away from the active site (Ke et al., 1990). The inhibition mechanism exerted by AMP is therefore not competitive. Indeed, biochemical experiments have shown long ago that the rat liver FBPase is allosterically inhibited by AMP (Taketa and Pogell, 1965). As for binding of fructose-2,6-bisphosphate, binding of AMP results in a transition of the quaternary structure of the enzyme from an active R-state to an inactive T-state (Ke et al., 1990; Choe et al., 2000).

As outlined in chapter 2, when cells growing on a nonfermentable carbon source are resupplied with glucose, the cAMP pathway is activated, which in turn activates PKA. Yeast FBPase has been shown to be phosphorylated *in vitro* on its Serine 11 residue (Rittenhouse et al., 1986), this phosphorylation occurs *in vivo* has well, in a PKA-dependent manner (Rittenhouse et al., 1987). The activity of a phosphorylated FBPase is optimal at a more alkaline pH than the cytosolic pH. Therefore, once the FBPase is phosphorylated, its enzymatic activity decreases in the cell. Fructose-2,6-bisphosphate and AMP were reported to accelerate FBPase phosphory-

lation (Pohlig and Holzer, 1985). However, this latter observation is in discrepancy with what has been observed by Marcus et al. and Gancedo et al., who both published an increased sensitivity of the phosphorylated FBPase towards its inhibitors (Gancedo et al., 1982; Marcus et al., 1988). These results may be reconciled in a feedforward model were the phosphorylation of FBPase would render it more sensitive to its inhibitors; those, in turn, would make the enzyme more readily phosphorylatable. Such a phenomenon would lead to a rapid inactivation of the enzyme upon shift to a fermentable carbon source.

Although 60 % inactivation of FBPase is observed when cells are shifted from a nonfermentable carbon source containing medium to a glucose containing medium (Lenz and Holzer, 1980), the inhibitors mentioned above do not achieve a complete inactivation of FBPase nor cannot they ascertain that this inactivation is irreversible. The latter is accomplished by the proteolytic degradation of FBPase which has been suggested to take place first by Gancedo in 1971 and was later confirmed (Molano and Gancedo, 1974). The precise location of the degradation of FBPase was later a subject of controversy. It was first suggested to depend on vacuolar enzymes and thus was predicted to occur in the vacuole (Funaguma et al., 1985). It late first suggested to occur outside the vacuole (Schafer et al., 1987). Mutants defective in the proteinases of the vacuole gave indeed clear evidence that FBPase degradation occurs outside of the vacuole (Wolf and Ehmann, 1979; Zubenko and Jones, 1979; Mechler and Wolf, 1981). Surprisingly, thereafter Chiang and Schekman published a vacuolar degradation of the enzyme . However, degradation of FBPase was soon shown to depend on a cytoplasmic system for its degradation (Gamo et al., 1994; Schork et al., 1994b,a). The ubiquitin-proteasome system was found to trigger the degradation of FBPase upon shift of yeast cells from an ethanol- to a glucose-containing medium (Schork et al., 1994b,a, 1995; Hämmerle et al., 1998; Schüle et al., 2000; Regelmann et al., 2003). Eventually, the two different degradation pathways, vacuolar and proteasomal, were suggested to occur (Hung et al., 2004). However, different starvation conditions before the glucose shift seem to be responsible for the two different degradation pathways of FBPase: FBPase of cells grown two to three days on an acetate-containing medium seems to be channelled to the vacuolar pathway while FBPase of cells which underwent only an overnight starvation period on ethanol-containing medium was degraded though the proteasomal pathway (Schork et al., 1995; Hämmerle et al., 1998; Josupeit, 2003; Regelmann et al.,

2003; Hung et al., 2004; Pfirrmann, 2006). Most likely, the cytosolic proteins such as FBPase are degraded by both the ubiquitin-proteasome system and the lysosome-vacuole system in eukaryotes. Under proliferating growth conditions, the former accounts for 80 % of the overall protein turn-over, while the latter becomes predominant under starvation conditions (Teichert et al., 1989; Egner et al., 1993; Abeliovich and Klionsky, 2001). It is therefore reasonable to postulate that the vacuolar-dependent degradation of FBPase observed by Chiang and coworkers reflects more a phenomenon akin to autophagy, although with some differences (see below: 4.1).

Chapter 4

Protein degradation.

Two systems participate in protein breakdown in eukaryotes: the lysosome / vacuole system and the ubiquitin-proteasome system. As FBPase has been reported to be substrate of the both systems, it is of interest to review them.

4.1 The lysosome/vacuole system.

The vacuole is a homoeostatic organelle, involved in both short-term and long-term adaptation to the availability of nutrients in the yeast's surroundings. It also responds to changes in the constraints from the environment such as the osmotic pressure and is a reserve of small molecules like ions, purines and amino acids. The precise nature of these molecules and their relative proportion vary between yeast species (Schwencke, 1991). Due to the fact that it contains several proteases it was identified as the major site of proteolysis before the discovery of the proteasome. Unlike the proteasome, however, the vacuole is able to degrade not only proteins, but organelles such as mitochondria and peroxysomes as well (Klionsky, 1997). The specificity of this degradation system seems to only be ensured by the vacuolar membrane (Schwencke, 1991). Thus, it was thought that specific proteolysis of cytosolic proteins would rely on a specific import of the proteins to be degraded into the vacuole (Schwencke, 1991).

The main role of the vacuole as a proteolytic machinery arises when cells are faced with a lack of nutrients in their environment. Then the cells carry out a process named autophagy, in which cytosolic proteins are unspecifically engulfed into double layered membrane vesicles, transported to and degraded in the vacuole (Klionsky and Emr, 2000; Abeliovich and Klionsky,

2001; Nair and Klionsky, 2005). In some instances, cells can specifically degrade peroxysomes upon shift to glucose (Klionsky, 1997). Such a specific uptake has also been documented for proteins, like the aminopeptidase 1 dodecamer complex. Interestingly, while autophagy occurs when cells undergo nutrient starvation, the aminopeptidase 1 import occurs in cells growing on a rich medium. However, autophagy and aminopeptidase 1 import pathways share some components (Harding et al., 1996; Kim et al., 1997; Scott et al., 1997; Klionsky, 1998, 1997). Ribosomes have also recently been found to be degraded by a so-called "ribophagy" pathway (Kraft et al., 2008). The observations of the Chiang group suggest that a selective autophagic mechanism might occur for gluconeogenic enzymes when cells are shifted to a glucose-containing medium after being grown two to three days on the poor carbon source acetate (Chiang and Schekman, 1991; Huang and Chiang, 1997; Chiang and Chiang, 1998; Hung et al., 2004). These conditions might trigger autophagy (Egner et al., 1993). As is the case for pexophagy, glucose may trigger a specific import of the FBPase into so-called vid vesicles prior to its delivery to the vacuole (Chiang and Schekman, 1991; Chiang et al., 1996). Thus, it occurs as if fermentable sugars were able to trigger a shift from a non-specific to a specific autophagocytosis. A binding of FBPase to the vacuole was proposed to be specific, although no FBPase receptor on the yeast vacuole membrane has been identified to date (Ko, 2000).

4.2 The ubiquitin-proteasome pathway.

For a long time, the only proteolytic machinery known in eukaryotes was the lysosome / vacuole system. Although there was evidence of an energy-consuming proteolytic process outside this system, no components could be found before the late eighties. At that time, several studies converged to designate a barrel-shaped protein complex of 10×15 nanometres as the effector of the non-lysosomal proteolysis observed. This particle showed ATP-independent peptidase activity and was eventually named the proteasome (Wolf, 2000; Wolf and Hilt, 2004). Further studies allowed better understanding of the functioning of such a complex, which has been showing growing complexity as new discoveries were achieved. It was demonstrated that this proteasome is integral to a larger complex, later named the 26 S proteasome, whose proteolytic activity is ATP-dependent (Wolf, 2000; Wolf and Hilt, 2004). This complex appeared to bear specific proteolytic activities: only certain proteins were processed by the enzymatic complex

while others remained untouched (DeMartino and Slaughter, 1999). Such a system requires a recognition machinery, in order to allow the substrates to be specifically processed. This is provided by ubiquitin, a small, 76 amino acids containing protein found in all eukaryotes and highly conserved among them(Hershko, 1998). This protein is bound to substrates via an isopeptide linkage after a consecutive three-step enzymatic reaction binding the C-terminal glycine of ubiquitin to either the α-amino group of the N-terminal amino acid or to the ϵ-amino group of an internal lysine residue of the target protein. It was later shown that this binding of ubiquitin to other proteins would mark them for destruction by the proteasome (Heinemeyer et al., 1991).

Under normal growth conditions, the ubiquitin proteasome system accounts for 80 % of the protein turnover within *Saccharomyces cerevisiae* cells. Since its discovery this system has been implicated in many cellular processes such as progress through the cell cycle, disposal of malfolded proteins, apoptosis, antigen presentation, signal transduction and gene expression (Wolf and Hilt, 2004). The proteasome is thus an essential component of the eukaryotic cell.

The ubiquitin proteasome system can be divided into two discrete parts: first a tagging system that marks proteins for degradation; second, a proteolytic machinery that recognises these proteins and processes them into peptides. In addition, parts of the ubiquitin proteasome system have also been implicated in either non-degradative processes within the cell (Fig. 4.1).

4.2.1 Tagging a protein for degradation: ubiquitin.

Proteins to be degraded must be sorted out from the proteins that should not undergo degradation. While in bacteria this is achieved by specific amino-acid sequences in the protein itself (Zwickl et al., 2000), in eukaryotes a more complex scheme arises. Several short-lived proteins carry so-called destruction boxes, as for instance PEST sequences (Rogers et al., 1986), or bear a destabilising N-terminal amino acid (Varshavsky, 1997; Dohmen, 2000). Some sequences need to be modified before becoming active as a destruction signal. Eventually, all these proteins become a target for ubiquitination and degradation either by the proteasome or the vacuole in the case of membrane proteins like the α factor receptor (Roth and Davis, 1996; Roth et al., 1998; Roth and Davis, 2000)or the galactose permease (Horak and Wolf, 2001; Horak et al., 2002; Horak, 2003). Thus, in eukaryotes, a two-step process is necessary for the degradation

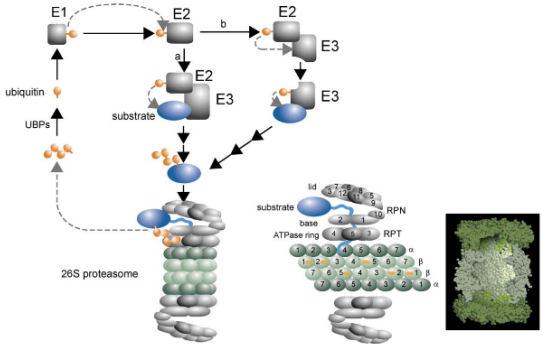

Figure 4.1: **The ubiquitin-proteasome system.** This figure describes the tagging of a substrate by the ubiquitin system before its processing by the proteasome. First ubiquitin is adenylylated and forms a thio-ester bond a cysteine of the active site of the ubiquitin activating enzyme (E1). Second, this high-energy bond is transferred onto a cysteine of the active site of an ubiquitin conjugating enzyme (E2). Thereafter, either the E2 binds to a RING-type ubiquitin ligase complex, or E3 (complex E2-E3, left) or to a HECT-type ubiquitin ligase (E3, right). In the first case, the ubiquitin ligase serves as a scaffold and bring the E2 and the substrate in close vicinity. In the second case, the ubiquitin bound to the E2 enzyme is transferred to the HECT-type ligase which then ubiquitinates the substrate. These steps are repeated until the polyubiquitin chain is long enough to direct the substrate to the proteasome where it is processed. This figure is taken from Wolf and Hilt (2004).

of some cytosolic proteins. This process would involve first a recognition of the protein to be degraded through its primary degradation signal, second the marking of this protein by one or more ubiquitin moieties which, depending on their topology, direct it for degradation by the proteasome (Dohmen, 2000).

Ubiquitin conjugation to a substrate is a three-step process, catalysed sequentially by three different enzymes (Glickman and Ciechanover, 2002). First ubiquitin is adenylylated before being conjugated to an active cysteine of a ubiquitin-activating enzyme (Uba, E1) thus forming a high-energy thio-ester bond between the E1 and the carboxy-terminal glycine of ubiquitin. In yeast, only one Uba enzyme is known, Uba1p, which was found to be essential (McGrath et al., 1991). Second, the ubiquitin moiety is carried over by a transesterification reaction to a ubiquitin-conjugating enzyme (Ubc, E2). Again, a high energy thio-ester bond is formed between the cysteine of the active site of the enzyme and the C-terminal glycine of ubiquitin (Fig 4.1). In yeast, there exist 13 ubiquitin-conjugating enzymes, of which eleven where shown to catalyse ubiquitin conjugation: Ubc1 to Ubc8 and Ubc10, 11 and 13 (Glickman and Ciechanover, 2002). The last step in ubiquitin conjugation to a protein involves the ubiquitin

ligases or E3s. The number of E3s found exceeds by far the number of E2s in *S. cerevisiae*, this difference is even more pronounced in higher eukaryotes (Glickman and Ciechanover, 2002). Indeed, E3s constitute the proteins that bring specificity to the pathway. While E2s seem to be involved in general pathways [e.g. Ubc4 and Ubc5 were found to partake in the degradation of short-lived proteins, Ubc6 and Ubc7 were shown to be more specifically involved in ERAD (Biederer et al., 1996; Hiller et al., 1996)], E3s have a more substrate-oriented specificity: they will bind to and ubiquitinate specific proteins, depending on definite signals (Glickman and Ciechanover, 2002). Therefore, two proteins can be substrates of the same E2 but of different E3s.

Ubiquitin ligases can be divided into two groups: the HECT ligases are analogous to the E2s in that the ubiquitin moiety is carried over to a cysteine of the active site of the ligase, again forming a thio-ester bond. When the protein to be ubiquitinated binds to this type of ligases, the ubiquitin moiety is then transferred to one of its internal lysine ϵ-amino groups. Thus, the HECT ligases bear an enzymatic activity *per se*. The RING-type ligases, on the other hand, show a more complex pattern and can be divided in several subgroups. Nevertheless, in contrast to what is known concerning the HECT-type ligases, they do not have an enzymatic activity regarding ubiquitination. Rather, they seem to play a scaffolding role by bringing the substrate protein and the E2 in close vicinity. The E2 then transfers its ubiquitin to a lysine ϵ-amino group of the substrate (Fig. 4.1 andWillems et al., 2004).

A single ubiquitin moiety is not sufficient to direct a protein for degradation by the proteasome (DeMartino and Slaughter, 1999). However, in the case of membrane proteins, it is sufficient to promote their internalisation and transport to the lysosome / vacuole (Horak and Wolf, 2001; Soetens et al., 2001; Horak et al., 2002). At least four ubiquitin moieties are necessary for a protein to become a proteasomal substrate (Thrower et al., 2000). However, the overall topology of the ubiquitin chain is important as well: on ubiquitin itself, isopeptides bonds have been found to occur at the seven lysine residues of ubiquitin, namely: Lys-6, Lys-11, Lys-27, Lys-29, Lys-33, Lys-48 and Lys-63 (Arnason and Ellison, 1994; Ikeda and Dikic, 2008; Li and Ye, 2008). Lys-29, and Lys-48 branched polyubiquitin chains seem to dispatch a protein for proteasomal degradation, while the Lys-63 branched polyubiquitin chains are implicated in DNA repair (Moldovan et al., 2007; Ikeda and Dikic, 2008). The role of the other polyubiquitin

chains is not well known. However, it has been shown that some of these chains are implicated in ubiquitin-dependent signalling events (Ikeda and Dikic, 2008). In some cases, ubiquitination of a substrate is done by a ubiquitin ligase while the ubiquitin chain elongation depends on additional enzymatic activity (Koegl et al., 1999; Hoppe, 2005). Proteins bearing this activity have a less defined primary structure than the E1, E2 and E3 enzymes engaged upstream in the process and a lot is still unknown concerning their regulation and the way they function (Hoppe, 2005).

4.2.2 The proteasome.

After being earmarked by K 48 polyubiquitin chains, proteins are directed to the 26 S proteasome, a multicatalytic protease which cleaves these proteins into peptides ranging from 3 to 30 amino acids in length. The 26 S proteasome consists of two main subcomplexes: the 20 S core complex and the 19 S regulatory complex.

4.2.2.1 The 20 s core complex.

The 20 S core proteasome is a barrel-shaped complex build up by two closely related subtypes of subunits, α and β, which form heptameric rings stacked together. The two outer rings constitute of α subunits while the β subunits build up the two inner rings. In eukaryotes, seven different α and β subunits are found, respectively. They build a 28 subunit-containing particle with a C2-symmetry. Thus, the stoichiometry of the complex is $\alpha_7\beta_7\beta_7\alpha_7$ (Schmidtke et al., 1996; Hilt and Wolf, 1996; Schmidt and Kloetzel, 1997; Schmidt et al., 1997; DeMartino and Slaughter, 1999; Wolf and Hilt, 2004). The lumen of this barrel-shaped particle contains three cavities. The two outer cavities are found between one α ring and one β ring, the remaining central cavity is built up by the two β rings. The proteolytic activity of the eukaryotic proteasome is provided by three subunits of each β-ring. Importantly, these activities reside in the central cavity of the 20 S core particle, thereby insulating them from the rest of the cytoplasm. The α subunits seem to play only a structural role: during proteasome formation they are supposed to spontaneously form α rings, which have been proposed to provide a scaffold for the β subunits. In addition to their structural role in the proteasome formation, the α subunits act as "gatekeepers". They exhibit an additional N-terminal extension which closes the channel formed

within the α rings, preventing any peptide to enter the free 20 S proteasome (Bajorek and Glickman, 2004; Wolf and Hilt, 2004). When the regulatory particle (19 S) binds to the 20 S core complex, the channel is opened and the substrates can gain access to the active sites. The Rtp2p ATPase subunit of the 19 S regulatory particle has been shown to play a crucial role in this process (Bajorek and Glickman, 2004).

The archaeal α subunit is able to spontaneously form α rings when expressed in *E. coli*. On the contrary, the archaeal β subunit need an α ring to form rings. Thus the α ring is thought to be a scaffold, providing a frame for the β ring to form. However, the formation of the 20 S proteasome is thought to be simpler in archaea compared to eukaryotes, as the former have only one type of α and one type of β subunit. In eukaryotes, each subunit has to interact with its correct partner; and false interactions must be prevented. Eukaryotic α subunits can form rings when expressed in *E. coli*. However, this also results in the formation of double α rings, never observed *in vivo*. Indeed, several studies highlighted the need for short-lived, specialised chaperones. These chaperones are important to ensure a correct formation of the proteasome: at the α ring formation [Poc1 and Poc2, (Le Tallec et al., 2007)], at the β ring [Poc3 and Poc4, (Le Tallec et al., 2007)] or at the binding of the two pro-holo-proteasome halves [Ump1, (Ramos et al., 1998)].

Importantly, during the proteasome formation process, the three active β subunits remain with their propeptide uncleaved. The autocatalytic cleavage of the N-terminal propeptides occurs shortly after the formation of the 20 S proteasome. This system prevents the exposure of the active threonine to the cytoplasm which could lead to unspecific proteolysis. On the other hand, it protects the active residue against N_α-acetylation and thereby safeguards the proteasome activity (Murata, 2006).

The 20 S proteasome is able to degrade oligopeptides; an activity enhanced *in vitro* by SDS or heating. This led to the discovery of "gating" at the proteasomal α rings (Bajorek and Glickman, 2004). However, folded proteins cannot be degraded by the 20 S core particle. This is achieved by the binding of the 20 S core particle to a 19 S "cap" or regulatory particle, hereby forming the 26 S proteasome which carries one 19 S particle at each end of the 20 S core.

4.2.2.2 The 19 S regulatory complex.

The three different peptidase activities of the 20 S proteasome are ATP-independent. Regulation of the free core particles occurs only by virtue of the steric hindrance exerted by the N-terminal α-helices of the α-type subunits as mentionned above (4.2.2.1). However, hydrolysis of ATP is necessary for protein degradation *in vivo* (Wolf, 2000; Wolf and Hilt, 2004), which suggests that some proteasome functions *in vivo* require an ATP cleavage. Indeed, the discovery of the 19 S particle, also termed the regulatory particle provided an explanation for this ATP requirement. The yeast 19 S regulatory particle contains at least 20 subunits and is itself subdivided into two subcomplexes: the base and the lid (Glickman et al., 2000). Two types of subunits form the yeast regulatory particle: the Rpt (Regulatory particle triple A protein) and the Rpn (Regulatory particle non-ATPase) subunits. The base binds to the α rings of the 20 S core particle. It is constituted of six homologous but not identical AAA-ATPases subunits (Rpt1 to Rpt6) and two non-ATPases subunits (Rpn1 and Rpn2). The lid subcomplex is exclusively composed of Rpn subunits (Rpn3, -5, -6, -7, -8, -9, -11, -12 and -13) (Glickman et al., 2000). Although the ATPase subunits are highly homologous (roughly 40 % identity), they are thought to play different roles as similar mutations result in distinct phenotypes (Glickman and Ciechanover, 2002). The two remaining non-ATPase subunits found in the base of the 19 S regulatory particle are supposed to serve a structural role, by bridging the AAA-ATPases to the α-rings of the core particle (Rosenzweig et al., 2008). Interestingly, the yeast Rpn1 protein is able to bind ubiquitin-like domains (Elsasser et al., 2002).

The 20 S core particle is unable to process folded proteins, thus, a likely role for the AAA-ATPases found in the 19 S regulatory particle is to unfold the proteins in an ATP-dependent manner and subsequently thread them through the central channel of the core particle (Glickman and Ciechanover, 2002). Moreover, as mentionned above, one ATPase subunit has been involved in the gating of the 20 S core particle (Kohler et al., 2001; Bajorek and Glickman, 2004). Hydrolysis of ATP is not only required by the 26 S proteasome to fulfil its function, it is also thought to be necessary for its formation (Armon et al., 1990; Hoffman and Rechsteiner, 1997).

The Rpn10 subunit was found to stabilise the interactions between the two regulatory subcomplexes (Glickman et al., 2000; Glickman and Ciechanover, 2002). In *rpn10*Δ deletion

mutant, the 19 S regulatory particle looses its lid but the base still interacts with the 20 S core particle. This proteasome subcomplex shows an enhanced peptidase activity compared to the core particle alone (Glickman et al., 2000). However, the ability of the proteasome to degrade ubiquitinated proteins requires an intact 19 S particle (Glickman et al., 2000). Moreover, the Rpn10 subunit, as well as Rpn1 has been found to bind ubiquitin or ubiquitin-like proteins (Glickman and Ciechanover, 2002). Recently, Rpn13 has also been found to bind ubiquitin through its pleckstrin-homology domains (Husnjak et al., 2008; Schreiner et al., 2008), confirming a previous prediction that the lid binds the polyubiquinated proteins and processes them before the AAA-ATPases of the base unfold them and thread them into the lumen of the 20 S particle. Indeed, at least one subunit, Rpn11 has been shown to deubiquitinate proteins prior to their degradation by the proteasome (Verma et al., 2002; Guterman and Glickman, 2004).

Chapter 5

Scope of this work.

The gluconeogenic key enzyme fructose-1,6-bisphosphatase has long been shown to be degraded upon shift of cells from a nonfermentable to a fermentable carbon source. This process is called catabolite inactivation (Holzer, 1976), later termed catabolic degradation (Hilt, 2004). Although it was suspected at the time that this degradation would take place in the vacuole, later, non-vacuolar proteolysis has been also suggested to occur (Schafer et al., 1987). This latter assumption has meanwhile been substantiated by the discovery of the involvement of the proteasome in this process. Vacuolar degradation of FBPase has been proposed to proceed under some conditions which likely favour autophagocytosis (Huang and Chiang, 1997; Chiang and Chiang, 1998; Shieh and Chiang, 1998). This process is under ongoing investigation by the group of Hui-Ling Chiang group (Brown et al., 2000, 2001; Shieh et al., 2001; Brown et al., 2002, 2003). However, studies carried out in this laboratory unravelled a novel proteasome dependent route for FBPase degradation (Schork et al., 1994b, 1995). The Wolf laboratory further demonstrated that some components of the ubiquitin system are involved in this catabolite degradation pathway (Schork et al., 1995; Schüle et al., 2000). During this work, eight novel additional genes (termed *GID1* to *GID9*) were found to be necessary for the proteasome dependent degradation process of FBPase (Regelmann et al., 2003; Josupeit, 2003; Regelmann, 2005). Among the newly found gene products, the proteins Gid1p, Gid4p and Gid5p had previously been identified as components of the vacuolar degradation pathway and are also known as Vid30p, Vid24p and Vid28p, respectively. Except for Gid3p, which had previous by discovered as the ubiquitin conjugating enzyme Ubc8p and Gid6 / Ubp14p, the Gid proteins were found to co-fractionate (Regelmann et al., 2003; Regelmann, 2005) and to inter-

act with each other (Ho et al., 2002; Pfirrmann, 2006; Pitre et al., 2006; Krogan et al., 2006). Therefore it was proposed that they build up a complex. We termed this complex, consisting of Gid1, Gid2, Gid4, Gid5, Gid7, Gid8 and Gid9, the Gid complex. This complex is required for the proteasome catalysed degradation of FBPase. All the identified Gid proteins, with the exception of Gid4p, show the same expression pattern (Pfirrmann, 2006; Santt et al., 2008). Gid4p becomes detectable only when cells are shifted from a nonfermentable to a fermentable carbon source (Chiang and Chiang, 1998; Josupeit, 2003; Santt et al., 2008). This peculiar property among the Gid proteins made it interesting to study the function of this Gid-protein in the context of the proteasomal-dependent catabolic degradation of FBPase.

Part II

Materials and methods.

Chapter 6

Plasmids, strains and Oligonucleotides.

6.1 Plasmids.

This section describes the plasmids used or constructed during this work. All plasmids listed in table 6.1 bear a ß-lactamase gene (Amp^R) for selection of bacteria on ampicilline containing media.

Table 6.1: **Plasmids used in this work. Their features and origin are described.**

Name	Features	Origin
pOS2	Plasmid carrying the Myc_9-*GID4* ORF. Generated from pGEM-T-easy.	This work
pOM12	Plasmid to generate PCR integration module for N-terminal tagging. Bearing the 6x HA tag and the K.l *URA3* auxotrophic marker.	Gauss et al. (2005)

pOM22	Plasmid to generate PCR integration module for N-terminal tagging. Bearing the 9x Myc tag and the K.l *URA3* auxotrophic marker.	Gauss et al. (2005)
pOM42	Plasmid to generate PCR integration module for N-terminal tagging. Bearing the GFP tag and the K.l *URA3* auxotrophic marker.	Gauss et al. (2005)
pSH62	Plasmid for expression of cre recombinase under the GAL1 promoter. Bears the *HIS3* auxotrophic marker.	Güldener et al. (1996)
pUG6	Plasmid to generate PCR integration module to generate deletion cassette. Bearing the KanMX6 resistance marker surrounded by LoxP cassettes.	Güldener et al. (1996)
pFA6a-KanMX6	Plasmid to generate PCR integration module to generate deletion cassette. Bearing the KanMX6 resistance marker.	Longtine et al. (1998)
pCM182	Plasmid for ORF expression under a tetracycline-dependent promoter. Bears Tta activator under CMV promoter, 2 tetO boxes. Auxotrophic marker: *TRP1*.	Garí et al. (1997)

pCM184	Plasmid for ORF expression under a tetracycline-dependent promoter. Bears Tta activator under CMV promoter, 7 tetO boxes. Auxotrophic marker: *TRP1*.	Garí et al. (1997)
pOS1	Plasmid for expression of *GID4* under a constitutive promoter, generated from pCM184 plasmid.	This work
pGEM-T-Easy	Plasmid for subcloning of PCR products. Bears Amp^R gene for bacterial transformants selection, LacZ for integration control by blue / white screen and SP6 and T7 RNA polymerases binding region for *in vitro* expression.	Promega, Madison, USA

6.2 Strains.

Different strains (*i.e.* with different genetic backgrounds) were used in this work depending on the experiments that had to be conducted. This section describes the yeast and *E. coli* strains used in this work.

6.2.1 Yeast strains.

The table 6.2 describes the yeast strains that were constructed and / or used during this work.

Name	Genotype	Origin
W303-1B	Matα *ade2 leu2-3, 112 his3 trp1 ura3*	H.-L. Chiang
W303-1BKO	W303-1B *fbp1*Δ::LEU2	H.-L. Chiang
YFJ1	W303-1B *gid4*Δ::KAN^R	F. Josupeit
YBB1(W303-1B-GID6HA$_3$)	MATα *ade2 leu2-3,112 his3 trp1 ura3* GID6-HA3::HIS5 (*S.p*)	B. Braun
YJR12 (W303-1B GID1-HA$_3$)	MATα *ade2 leu2-3,112 his3 trp1 ura3* GID1-HA3::HIS5 (S. p)	J. Regelmann
YOS1	MATα *ade2 leu2-3,112 his3 trp1 ura3* MYC$_9$-GID4	This work
YOS1-*gid2*Δ	YOS1 *gid2*Δ::KAN^R	This work
YOS1-*pdr5*Δ	YOS1 *pdr5*Δ::KAN^R	This work
YOS1-*ubc8*Δ	YOS1 *ubc8*Δ::KAN^R	This work
GID7-HA$_3$-*gid4*Δ	YTP10 *gid4*Δ::KAN^R	This work
YTS3	W303-1B GID2-HA$_3$	T. Schüle
YTS4	W303-1B UBC8-HA$_3$	T. Schüle
YTP10	MATα *ade2 leu2-3,112 his3 trp1 ura3* GID6-HA$_3$::HIS5 (*S.p*)	T. Pfirrmann
YTP12	MATα *ade2 leu2-3,112 his3 trp1 ura3* GID9-HA$_3$::HIS5 (*S.p*)	T. Pfirrmann
HKY36	MATa *ura 3-52 leu2*Δ*1 his3*Δ*200*	H. Krebber
HKY77	MATa *rpb1-1 ura trp his*	R. A. Young, provided by H. Krebber
FBPase-TAP	MATα *ade2 leu2-3 112 his3 trp1 ura3* FBP1-TAP::TRP1(*K.l*)	T. Pfirrmann
MY3589	MATa *ura3-52, leu, ade2*	Biggins et al. (1996)
MY3592	MATa *ura3-52, leu2, his3*Δ*200, ade2, dsk2*Δ::LEU2, *rad23*Δ	Biggins et al. (1996)
YBB2 (GID2C379SHA$_3$)	W303-1B GID2C379S-HA$_3$::HIS5(*S. p*)	B. Braun
BY4743 (also known as Y20000)	Mata/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0	EUROSCARF
Y34633	BY4743, *CUL3*::KanMX4	EUROSCARF
Y31376	BY4743, *CUL8*::KanMX4	EUROSCARF
Y34077	BY4743, *SAN1*::KanMX4	EUROSCARF

Table 6.2: ***Saccharomyces cerevisiae* strains used during this work.**

6.2.2 *E. coli* strains.

Two *E. coli* strains were used in this work: DH5α and BL21, which is better suited for recombinant proteins expression and purification because it lacks proteases. The genotypes of those strains are described below:

- *DH5α:* F- ϕ80d *lac Z* ΔM15 (argF-lacZYA) U169 end A1recA1 *hsd* R17(rk-, mk+) deo R *thi*- 1 *sup*E44 λ- gyrA96 relA1Δ, from Stratagene (La Jolla, USA).
- *BL21 (DE3):* F- *dcm ompT hsd*S(rB- mB-) *gal* λ(DE3), from Stratagene (La Jolla, USA).

6.3 Oligonucleotides.

This section describes the various oligonucleotides used in this work for polymerase chain reaction (9.2).

Table 6.3: **Oligonucleotides used in this work.** Their sequences, usage and origin are mentionned.

Name	Sequence	Purpose	Sources
StuI-mycgid4F	5'-aaggcctatgtgcaggtcgacaac-3'	*GID4* cloning	this work
SbfI-mycgid4R	5'-aaacctgcaggtcaagcaaactcaaaagaac-3'	*GID4* cloning	this work
del-gid4F	5'-catttgtggcgtcttgtgcatgacacca acacatatcgc aagcttgagtccagctgaagcttcgtacgc-3'	*GID4* deletion	this work
del-gid4R	5'-aaagttagtgaagagaaaagggtatg caggtaaaaac gaatatatcacacagcataggcc actagtggatctg-3'	*GID4* deletion	this work

delgid4test1F	5'-aattctaatgtacatgatga-3'	*GID4* deletion control	this work
delgid4test1R	5'-tctaatacctggaatgct-3'	*GID4* deletion control	this work
TS3	5'-taatgaaacagtagctctaaaagaag gatcgagacag atgttcagctgaagcttcgtacgc-3'	*UBC8* deletion	(Schüle, 2000)
TS4	5'-acatacatatatatatatatatatatg tgtgtgctcga aaagcataggccactagtggatctg-3'	*UBC8* deletion	(Schüle, 2000)
ubc8contF	5'-cacagacgcgttgaattgtc-3'	*UBC8* deletion control	This work
ubc8contR	5'-tcaagtgagaaatcaccatgag-3'	*UBC8* deletion control	This work
Gid2delF	5'-taagtgtgtcttcaagagagatgcagc actgagtaggg aaccaagaaacgcagctgaagcttcgtacgc-3'	*GID2* deletion	This work
Gid2delR	5'-ttatcgcttccaataaaaaaaaaaaaa aaaaacctatg caaaaatttcaggcataggccacta gtggatctg-3'	*GID2* deletion	This work
gid2delcontF	5'-aggtctagagatctgtttagct-3'	*GID2* deletion control	This work

gid2delcontR	5'-ttattcattcgtgattgcgc-3'	*GID2* deletion control	This work
pdr5 Fwd	5'-agacccttttaagttttcgtatccgctcg ttcgaaagact ttagacaaaacagctgaagcttcgtacgc-3'	*PDR5* deletion	this work
pdr5 Rev	5'-aaaaagtccatcttggtaagtttcttttc ttaaccaaatt caaaattctagcataggccactagtggatctg-3'	*PDR5* deletion	this work
pdr5delcontF	5'-tttactaagactccggtgagtg-3'	*PDR5* deletion control	this work
pdr5delcontR	5'-ctcgtccaacatcaatacaacct-3'	*PDR5* deletion control	this work
n-term-gid4F	5'-gtggcgtcttgtgcatgacaccaacac atatcgcaagc ttgagtcatgtgcaggtcgacaaccttaat-3'	*GID4* N-terminal tagging	this work
n-term-gid4R	5'-agctttgggtttctccgctacactgtcta ccttaggattat tgatgcggccgcataggccact-3'	*GID4* N-terminal tagging	this work
promgid4F	5'-ccggccgagctctggcgggctgctcg gttc-3'	*GID4* N-terminal tagging control	this work

tergid4R	5'-tcccccgggaaaacatcgttttatagatatag-3'	*GID4* N-terminal tagging control	this work
184-myc	5'-acttctatagacacgcaaacac-3'	Sequencing of MYC_9-*GID4* ligations in pCM184	This work

All oligonucleotides were synthesised by either MWG Eurofins (Martinsried, Germany) or Biomers (Ulm, Germany).

Chapter 7

Media, chemicals, antibodies and apparatus.

This section describes the various solutions and the chemicals used. The buffer compositions are detailed for each experiment in the respective sections.

7.1 Media.

All media for the cultivation of *S. cerevisiae* and *E. coli* strains were prepared using double deionised water (ddH_2O) prepared with the Ion exchanger Milli-Q Academics. The pH was adjusted with NaOH or HCl and media got sterilised by autoclaving at 121°C for 20 min. When solid media was used, 2 % (w / v) agar was added to the liquid media. The solution was then poured in Petri dishes and dried.

7.1.1 Yeast media.

Table 7.1 gives an overview of the media used in this work for *S. cerevisiae* cultures. CM media are selections media were the clones of interest are screened for their ability to grow on a medium lacking an auxotrophic marker. In our case, six auxotrophic markers are used: uracil (URA), tryptophane (TRP), histidine (HIS), leucine (LEU), adenine (ADE) and lysine (LYS). The one used for the selection (*i.e.* the one missing in the medium) is noted after a minus (-) sign.

Name	Composition
YPD medium	1 % (w / v) yeast extract, 2 % (w / v) Bacto Peptone, 2 % (w / v) D-glucose, pH 5.5
YPEtOH medium	1 % (w / v) yeast extract, 2 % (w / v) Bacto Peptone, 2 % (v / v) Ethanol p.a., pH 5.5
CM medium	0.669 % (w / v) Yeast nitrogen base w / o amino acids, 0.0117 % (w / v) L-alanine, L-arginine, L-asparagine, L-asparatic acid, L-cysteine , L-glutamine, L- glutamic acid, L-glycine L-isoleucine, L-methionine, L-phenylalanine, L-prolin, L-serine, L-threonine, L- tyrosine, L-valin, myo-inositol, p-amino benzoic acid; pH 5.6 Depending whether cells have to be grown on ethanol or glucose, 2 % (v / v) ethanol or 2 % (w / v) D-glucose were added, respectively
Pulse-chase growth medium	Like CM medium but without L-methionine
Pulse medium	Like CM medium but without L-methionine and 2 % (v / v) ethanol instead of D-glucose
Chase medium	Like CM medium but without L-methionine and 2 % (v / v) ethanol instead of D-glucose

Table 7.1: **Overview of the media used for yeasts cells cultures.**

7.1.1.1 *E. coli* media.

Table 7.2 gives an overview of the media used in this work for *E. coli* cultures.

7.2 Chemicals, kits, enzymes and antibodies.

7.2.1 Chemicals material, and enzymes.

7.2.1.1 Chemicals and material

- Acros organics, Geel, Belgium: acetic acid, DMSO
- BioRad, Hercules, USA: protein marker (Precision Plus Protein™ Standards, All Blue)
- Calbiochem, San Diego, USA: MG-132, rapamycin
- Difco, Detroit, USA: Bacto-Agar, Bacto-Peptone, Bacto-Tryptone, Yeast extract, Yeast nitrogen base w/o amino acids
- Dr Gross, Sauerlach, Germany: skimmed milk

Name	Composition
LB (Luria Broth) medium	0.5 % (w / v) Bacto Yeast Extract LB Medium (Luria Broth), 1 % (w / v) Bacto Tryptone, 0.5 % (w / v) Sodium chloride; pH 7.5
LB-Amp medium	For selection of plasmid-bearing cells, ampicilline is added at an end concentration of 100 µg / ml
SOC medium	2 % (w / v) Bacto Tryptone, 0.5 % (w / v) yeast extract, 0.4 % (w / v) D-glucose, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.5 mM KCl; pH 7.4
YTA medium	1.6 % (w / v) Bacto Tryptone, 1.0 % (w / v) yeast extract, 0.5 % NaCl; 100 µg / ml ampicilline; pH 7.0

Table 7.2: **Overview of the media used used for *E. coli* cells cultures.**

- Fermentas, St. Leon-Rot, Germany: protein size marker (PageRuler^{TM}Precision Plus)
- Fisher Scientific, Loughborough, UK: acetone, chloroform
- GE Healthcare, Munich, Germany: [$\alpha -^{35} S$]-L-methionin, glutathion Sepharose^{TM} 4B, Hybond-N-Nylon membrane, Hyperfilm^{TM} ECL^{TM}, protein A Sepharose CL-4B
- Genaxxon, Biberach, Germany: ampicilline, dNTPs, geneticine
- Merck, Darmstadt, Germany: Pefabloc, TEMED
- Millipore, Eschborn, Germany: polyvinyliden difluoride (PVDF) membranes (Immobilon-P)
- Pall Corporation, Pensacola, USA: nitrocellulose membrane BioTrace^{TM} NT
- Prolabo, Fontenay-sous-bois, France: ethanol, methanol
- Promega, Madison, USA: Hering sperm DNA
- Roche Applied Science, Mannheim, Germany: dNTPs, DNA standard (1 kb DNA-ladder), Complete^{TM} protease inhibitor tablets,
- Roth, Karlsruhe, Germany: 30 % (w / v) acrylamide / bis-acrylamide solution (Rotiphorese 30), agarose NEEO, ammonium acetate, ammonium peroxodisulphate, chloroform, dithiothreitol, DMF, D-glucose, DNA standard (1 kb DNA-ladder), isopropanol, L-glycine, mag-

nesium chloride, magnesium sulfate, ß-mercapto-ethanol, potassium acetate, potassium chloride, potassium dihydrogen phosphate, potassium hydrogen phosphate, sodium acetate, sodium chloride, sodium hydroxide, sodium dihydrogen phosphate, sodium hydrogen phosphate, phenol (water saturated, Roti-phenol), PMSF, D-saccharose, TCA, Tris, Triton X-100, urea, X-Gal

- Riedel-De Haën, Seelze, Germany: bromophenol blue, isobutanol, glycerol, HCl, orthophosphoric acid, sulphuric acid
- Sartorius AG, Göttingen, Germany: glass beads
- Schleicher and Schüll, Dassel, Germany: blotting paper GB001, GB002 and GB003
- Serva, Heidelberg, Germany: Coomassie Brilliant Blue R250
- Sigma-Aldrich, Taufkirchen, Germany: amino acids, adenine, p-amidobenzoic acid, antipaine, benzamidine, bovine serum albumin, cycloheximide, chymostatine, EDTA, ethidium bromide, D-galactose, HEPES, IPTG, leupeptine, lithium acetate, sodium citrate, PEG 4000, pepstatine A, Ponceau S, sodium azide, sodium dodecyl sulfate (SDS), D-sorbitol, tween 20, uracil

7.2.1.2 Enzymes

- Enzogenetics, Corvallis, USA: Oxalyticase
- Genaxxon, Biberach, Germany: Taq DNA polymerase and buffer system
- Invitrogen, Carlsbad, USA: T4-DNA-Ligase and ligation buffer
- New England Biolabs, Beverly, USA: Vent-DNA-Polymerase, alkaline phosphatase (CIP), BSA, restriction enzymes and appropriate buffer systems
- Roche, Mannheim, Germany: Taq DNA polymerase and buffer system, T4-DNA-Ligase, lysozyme, Klenow enzyme, RNase A, restriction enzymes and appropriate buffer systems
- Fermentas, St. Leon-Rot, Germany: restriction enzymes and appropriate buffer systems
- Seikagaku Kyogo, Tokyo, Japan: Zymolyase 100-T

Antibodies	Dilution used in Western-blot	Suppliers
Goat anti-mouse IgG, HRPO conjugated	1 / 5000	Dianova, Hamburg, Germany
Goat anti-rabbit IgG, HRPO conjugated	1 / 5000, 1 % (w / v) skimmed milk	Medac, Hamburg, Germany
Anti-Ape1, polyclonal, rabbit	1 / 5000	Klionsky et al., 1992
Anti-CPY, monoclonal, mouse	1 / 10000	Molecular Probes, Leiden, Netherlands
Anti-Fas, polyclonal, rabbit	1 / 10000	Egner et al. 1993
Anti-FBPase, polyclonal, rabbit	1 / 5000	K.D. Entian, Frankfurt, Germany
Anti-GST, polyclonal, rabbit	1 / 5000 in 2 % (w / v) skimmed milk	T. Lang
Anti-HA, monoclonal, clone 16B12, mouse	1 / 5000 in 1 % (w / v) skimmed milk	Babco, Richmond, USA
Anti-Pgk, monoclonal, mouse	1 : 10000	Molecular Probes, Leiden, Netherlands
Anti-myc, monoclonal, clone 9E10, mouse	1 : 5000	Sigma-Aldrich, Taufkirchen, Germany
Anti-Ubiquitin, monoclonal, clone P4D1	1 : 2500	Covance Research Products, Inc., Berkeley, USA

Table 7.3: **Antibody solutions, their dilutions and their suppliers.**

- Stratagene, La Jolla, USA: Pfu UltraTM high-fidelity DNA-polymerase

7.2.1.3 Kits.

The kits were used for plasmidic DNA isolations (Qiaprep Spin Miniprep Kit) and agarose gel extraction of DNA (Quiaquick Gel Extraction Kit), both from Qiagen, Hilden, Germany. For immunoblotting development, the PIERCE ECL Western blotting substrate was used (Pierce, Rockford, USA).

7.2.1.4 Antibody solutions.

All antibodies used in this work were diluted in TBS-T (20 mM Tris / HCl, pH 7.6; 137 mM NaCl; 0.1 % Tween-20). In table 7.3, type of antibodies used and their respective dilutions are described.

7.3 Laboratory equipment.

- Agarose gel electrophoresis apparatus, Bio-Rad, Hercules, USA
- Balance AE 163, Mettler, Giessen, Switzerland
- Balance PM 460, Mettler, Giessen, Switzerland
- Biofuges fresco and pico, Heraeus, Hanau, Germany
- Centrifuge 5417 C and 5804 R Eppendorf, Hamburg, Germany
- Centrifuge Centrikon T-124 Kontron Instruments, Neufarn, Germany
- Centrifuge Sorvall RC 5B, Kendro, Osterode, Germany
- Centrifuge Z320K, Eppendorf, Hamburg, Germany
- Disruptor Genie SI-D256, Scientific Industries, Bohemia, USA
- Film developer machine Optimax, Protec Medizintechnik, Oberstenfeld, Germany
- Gel dryer, Fröbel Labortechnik, Lindau, Germany
- Heating block Test tube Thermostat TCR100, Roth, Karlsruhe, Germany
- Incubator B6200, Heraeus, Hanau, Germany
- Ion exchanger Milli-Q Academics, Millipore, Eschborn, Germany
- Multi vortexer IKA-VIBRAX VXR, Staufen i. Br., Germany
- Overhead rotator REAX2, Heidolph Instruments, Schwabach, Germany
- Overhead shaker 34528, Snijders Scientific, Tilburg, USA
- Overhead shaker REAX 2, Heidolph Instruments, Schwabach, Germany
- Overhead shaker Roto Shake Genie, Scientific Industries, Bohemia, USA
- PCR-Machine Robocycler Gradient 40, Stratagene, La Jolla, USA
- pH-Meter CG 832, Schott, Hofheim, Germany

- PhosphoImager Storm860 GE Healthcare, Munich, Germany
- Pipettes (2-1000 µl), Gilson, Middleton, USA
- Power supply units Model 200 / 2.0 and Power-Pac 300, Bio-Rad, Hercules, USA
- Protein electrophoresis apparatus Protean II, BioRad, Hercules, USA
- Semidry blot chamber, ITF Labortechnik, Wasserburg, Germany
- Shakers, various sizes, A. Kühner AG, Birsfelden, Switzerland
- Spectrophotometer Novaspec II, GE Healthcare, Uppsala, Sweden
- Stirrer lkamag REO IKA-Labortechnik, Staufen i. Br., Germany
- Supersonic Sonificator, Sonic Power Branson, Danbury, USA
- Thermomixer 5437, Eppendorf, Hamburg, Germany
- Ultracentrifuge Optima^{TM} TLX, Beckman, Palo Alto, USA
- Video printer for agarose gel pictures MWG Biotech, München, Germany
- Vortexer Vibrofix VF 1 and VF 2 IKA-Labortechnik, Staufen i. Br., Germany

Chapter 8

Cell cultures.

This section refers to the protocols regularly used during this work to cultivate either yeast or *E. coli* cells. For special experiments, the growth conditions are described in the respective sections.

8.1 Yeast cell cultures.

Solid cultures were carried out on media containing 2 % Bacto^{TM} agar (7.1.1). Cells were either streaked out from 15 % glycerol stock cultures kept at - 80°C or taken from another agar plate and let grown two to three days at 30°C.

For liquid cultures, cells were taken from either YPD (7.1.1) or a selection media (see 7.1.1 for a list of those) agar plate and inoculated in 2 ml of the corresponding liquid media (7.1.1) and grown overnight at 30°C, 250 rpm. Then, the suspension was diluted 1 / 20 in fresh glucose-containing media and cells were further let grown for 6 hours at 30°C, 250 rpm. To allow the expression of the gluconeogenic enzymes, the cells were then shifted to an ethanol-containing media for 16 hours at 30°C, 250 rpm. OD_{600} was then measured and cells were shifted back to glucose-containing media to trigger the catabolite inactivation / degradation of the gluconeogenic enzymes.

8.2 *E. coli* cell cultures.

Solid cultures were carried out on media containing 2 % Bacto^{TM} agar (7.1.1.1). Cells were either streaked out from 60 % glycerol stock cultures kept at - 80°C or from another agar plate and let grown overnight at 37°C.

For liquid cultures, *E. coli* colonies were picked from either a LB or LB-Amp agar plate (see 7.1.1.1) and grew overnight in 2 ml liquid LB or LB-Amp, at 37°C, 180 rpm.

Chapter 9

Molecular Biological Methods.

This section will describe the techniques used in this work to manipulate DNA.

9.1 DNA isolation.

Here are described the two major DNA isolation methods used in this work, depending on whether yeast chromosomal or bacterial plasmidic DNA was needed in the consecutive experiments.

9.1.1 Phenol / Chloroform DNA extraction and sodium acetate precipitation.

In order to get a clean, usable DNA, it has to be separated from the proteins and the lipids present in all cells. This is achieved by doing a phenol / Chloroform extraction, where the lysate, or the DNA solution is mixed with one volume of phenol / chloroform / isoamylic alcohol (25 : 24 : 1) solution. The mix is then vortexed during 4 min and centrifuged 15 min at room temperature; 13,000 rpm. The upper phase is transferred to a new Eppendorf vial, and 3 M sodium acetate (NaOAc, pH 5.3) is added up $\frac{1}{10}^{\text{th}}$ of the solution volume. Cold (- 20°C) 100 % ethanol is then added to the mix up to 2.5 of the initial solution volume. Samples were kept at - 20°C during 30 min and centrifuged 15 min, 13,000 rpm. Supernatant was soaked away and pellet let dried at 37°C before being resuspended in 50 µl distilled water.

9.1.1.1 Chromosomal DNA isolation.

Yeast cells of interest were cultivated overnight at 30°C in 2 ml of corresponding culture media (7.1.1). 1.5 ml of the grown cells were then harvested, centrifuged at 13,000 rpm during one minute and the pellet was washed with 0.5 ml of ddH_2O. Cells were resuspended in 200 µl of breaking buffer (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0), to which 200 µl glass beads and 200 µl of phenol / chloroform solution were added. DNA extraction and precipitation was carried out as described in section 9.1.1. After resuspension of the DNA, it was treated with 75 µg / ml RNase A, 5 min at 37°C to degrade the yeast RNA. DNA was the precipitated again using 3 M sodium acetate (9.1.1), and resuspended in ddH_2O.

9.1.1.2 Plasmid DNA isolation.

Plasmids were amplified in transformed *Escherichia coli* cells grown as described in 8.2 and extracted using the Qiaprep Miniprep Kit^{tm}, according to the manufacturer's instructions.

9.2 PCR method.

The PCR, standing for "Polymerase Chain Reaction" allows the amplification of low-abundant DNA fragments and thereby facilitates DNA manipulations. This consist of the amplification of a DNA fragment lying between two synthetic oligonucleotides also called "primers" (see 6.3). These primers anneal on precise, complementary regions of the DNA to be amplified and are extended at their 3'-hydroxyl group by the DNA polymerase. This DNA polymerase being heat stable, allows several denaturations (carried out at 94°C), renaturations or annealing of the oligonucleotides to the template DNA (depending on the primer's Tm) and extensions (at the DNA polymerase optimal temperature, *i.e.* 72°C) steps. During this work, PCR is typically set up as described in table 9.1.

The *in vitro* reaction was conducted in Robocycler Gradient 40 PCR machine (Stratagene, La Jolla, USA) using a specific temperature program. The latter, concerning the annealing step, is determined using the melting temperature of the oligonucleotides (Tm), following, for oligonucleotides less than 20 bases long, such a formula: $Tm = 4(G + C) + 2(A + T)$. For longer primers, another formula was used: $Tm = 81.5 + 16.6 \log[K^+] + 0.41(GC\%) - (675/N)$.

Reagent	Volume (µl)	Concentration / Activity
DNA polymerase	0.5 - 1	0.1 / 1 U
Reaction buffer	5	10 X
dNTP	1	25 mM
Oligonucleotide 1	0.5	1 µM
Oligonucleotide 2	0.5	1 µM
DNA template	1 - 2	0.2 - 0.4 µg / µl
ddH_2O	qs 50	

Table 9.1: **Reagents and their volumes used in standard PCR reactions.**

This step was usually lasting 45 s to 1 min. A typical PCR programme comprises a 5 min initial denaturation at 94°C followed by 45 s to 1 min denaturations steps during the cycles. The elongation is carried out at 72°C on the basis of 1 min elongation for 1,000 bp. To ensure a complete extension, a last step at 72°C was conducted during 7 min. 30 cycles of PCR were sufficient to yield a suitable quantity of the DNA fragment of interest. The efficiency of the reaction was controlled using an agarose gel electrophoresis (9.3).

9.3 Nucleic acid electrophoresis.

DNA fragments can be readily detected using an agarose gel electrophoresis coupled to an ethidium-bromide staining. DNA samples were mixed to 10 X DNA-loading buffer (50 mM Tris / HCl pH 7.6, 0.25 % (w / v) bromophenol blue, 0.25 % xylene cyanol, 60 % (v / v) glycerol). To estimate the size of the DNA fragments, a 1 kb DNA ladder was used (Carl Roth Laboratories, Karlsruhe, Germany). Agarose was used at a concentration ranging from 0.8 to 1.2 % (w / v) in TAE (40 mM Tris / acetic acid, pH 7.5; 1 mM EDTA). The agarose solution was heated in a microwave oven before being poured in a casting chamber. 0.5 µg / µl of ethidium-bromide was added to the gel. The hardened gel was then transferred to an electrophoresis chamber, covered with TAE buffer and DNA samples were loaded on the gel. Electrophoresis was carried out during 30 to 45 min at 120 V. The gel was then laid over a UV lamp to detect the DNA bands. Depending on whether an analytic or a preparative gel was run; UV light was set at 100 % or 70 %, respectively. In the case of a preparative electrophoresis, slices containing the DNA of interest were cut out of the gel and underwent gel extraction using the Gel Extraction Kit from Qiagen used according to the manufacturer's instructions.

Reagents	Volumes (µl)	Quantity / Concentration / Activity
DNA	depending on DNA concentration	2.5 - 5 µg
Reaction buffer	2	10 X
Restriction enzyme	1	5 - 10 U
ddH_2O	qs 20	

Table 9.2: **Typical DNA digestion carried out in this work.** Reagents, their volumes and concentration / activity are listed.

9.4 DNA restriction.

DNA endonucleases type II, also called restriction enzymes, cut DNA at precise recognition sites, which are always palindromes. Different restriction enzymes recognise different restriction sites, thereby allowing specific fragments to be yielded after digestion. This makes the use of restriction enzymes necessary in molecular biology as a cloning tool. Most digestion reactions were set up as described in table 9.2. Enzymes were purchased either from Roche Applied Science (Mannheim, Germany) or New England Biolabs (Ipswich, USA). In case of double digestions, both enzymes were chosen according to their compatibility regarding the buffer system. To control digestions, a DNA electrophoresis was conducted (9.3), in case of a preparative digestion, the DNA fragment of interested was cut out of the gel, extracted (9.3) and further processed.

9.5 Plasmid DNA dephosphorylation.

To ensure that no spontaneous re-ligation of digested plasmids could occur, those were dephosphorylated in their 5' position immediately after digestion. To the digestion reaction, 5 µl of 10 X CIP (Calf Intestinal Phosphatase) buffer, 1 µl of CIP and 24 µl of distilled water were added. After 30 min incubation at 37°C, DNA was extracted using phenol / chloroform and precipitated with sodium acetate 3 M (9.1.1). DNA pellet was then resuspended in 50 µl distilled water.

9.6 Ligation.

The enzyme T4-ligase (Roche Applied Science) was used to insert DNA fragments in linearised and dephosphorylated plasmidic DNA. T4 ligase catalyses a phosphodiester bond between the

vector and the insert. This reaction was carried out using an insert / plasmid molar ratio of 3 / 1 to 6 / 1 in a reaction volume of 20 µl. The crude concentration ratio was determined by running 3 µl of each sample on a 1 % agarose gel. DNA was mixed with 1 µl of T4 ligase (Roche Applied Science), 2 µl of the appending buffer system (10 X ligation buffer) and was filled up to 20 µl with distilled water. A first ligation step was conducted during 4 hours at room temperature, then 1 µl of T4 DNA ligase was added to the reaction mixture. The reaction was run overnight at 16°C to ensure a proper ligation. Samples were afterwards transformed into *E. coli* (9.7.2).

9.7 Transformation.

9.7.1 In yeast cells.

To prepare competent yeast cells, a colony from the selected yeast strain was picked and grown in 5 ml YPD-medium at 30°C. The OD_{600} of the overnight culture was measured and diluted in 10 ml of YPD to get an OD_{600} of 0.2. After approximately 3 h of incubation at 30°C, cells were transferred into 15 ml tubes and spun for 3 min in an Eppendorf Z320K bench-top centrifuge at a speed of 3000 rpm. The pelleted cells were then washed with sterile ddH_2O, then with Li-Sorb buffer (10 mM Tris / HCl, pH 7.5; 1 mM EDTA; 0.1 M LiOAc; 1 M Sorbitol) and were finally resuspended in 500 µl Li-Sorb buffer. 50 µl per transformation were aliquoted in sterile 1.5 ml reaction tubes and incubated at 30°C for 30 min. To each aliquot 5 µl of herring sperm DNA, 5 - 20 µl of the DNA of interest and 300 µl of 40 % PEG solution (10 mM Tris / HCl, pH 7.5; 1 mM EDTA; 0.1 M LiOAc; 40 % (w / v) PEG) were added and cells were incubated for an additional 30 min at 30°C. Before first use, herring sperm DNA was denatured for 5 min at 95°C and kept on ice during 5 min afterwards. The heat shock transformation was then carried out by keeping the aliquots for 20 min at 42°C. Then cells were pelleted by centrifugation for 3 min at 3000 rpm. The supernatant was decanted and the remaining cells were resuspended in 250 µl of sterile water. These cells were plated out on selective media and positive clones grew after 2 - 4 days incubation at 30°C.

9.7.2 In *E. coli* cells.

9.7.2.1 Preparation of competent *E. coli* cells.

Before being transformed, *E. coli* cells have to be competent i.e. treated in a way that renders them porous to exogenic DNA. This sub-section describes the method used in this work for that purpose.

Cells were taken from an overnight culture (8.2) and inoculated in 5 ml LB medium (7.1.1.1), after two hours incubation at 37°C, 2 ml of that pre-culture were transferred to 40 ml of pre-warmed LB medium. Cells were grown till they reached an OD_{600} of 0.45 - 0.5. Growth was then stopped by placing the cultures 15 min on ice. Cells were then centrifuged 2,500 rpm at 4°C during 15 min. Pellet was resuspended in 2 ml of Tfb1 (MES 10 mM; $RbCl_2$, 100 mM; $CaCl_2$, 10 mM; $MnCl_2$, 50 mM; pH 5.8, with CH_3COOH), after Tfb1 volume being brought to 16 ml, cells were incubated 15 min on ice and centrifuged again at 2,500 rpm, 4°C during 15 min. Pellet was resuspended in 1.6 ml of Tfb2 (MOPS, 10 mM; $RbCl_2$, 10 mM; $CaCl_2$, 75 mM; glycerol, 15 % (v / v); pH 6.5 with KOH) and incubated for additional 15 min on ice. The resulting suspension was then aliquoted in sterile Eppendorf vials and competent bacteria were kept at - 80°C.

9.7.2.2 Transformation.

As for yeast cell transformation, *E. coli* transformation was carried out using a heat shock method. Competent cells (9.7.2.1) were thawed on ice and 5 µl of the plasmid of interest were mixed with 50 µl of competent cells. After 45 - 60 min incubation on ice, the bacteria underwent a heat shock during 2 min at 42°C. Cells were then kept on ice during 5 min before being resuspended in 1 ml of SOC medium (7.1.1.1). After one hour growth at 37°C, cells were spun down, resuspended in 250 µl of SOC or sterile ddH_2O and platted on LB-Amp agar (7.1.1.1). The plates were then kept at 37°C overnight.

For a so called "Blue-White" screen, 100 µl of X-Gal (20 mg X-Gal / ml DMF) and 40 µl of a 100 mM IPTG stock were stroke out on an LB-Amp plate and dried on the sterile bench for 30 min. Plasmids containing a multiple cloning site in the LACZ gene (e.g. pRS series) were then plated out on LB-Amp agar plates. Clones carrying a plasmid with an intact LACZ gene were able to metabolise X-Gal and turned blue. However clones carrying an insert in the multi cloning site (MCS) were not able to metabolise X-Gal and therefore stayed white.

9.8 DNA sequencing.

To sequence certain DNA regions on a plasmid the plasmid DNA was isolated according to section 9.1.1.2. Conventionally, the quantity of plasmid DNA from two QIAprep Miniprep extractions was eluted with 50 µl of ddH_2O and pooled in a 1.5 ml reaction tube. The amount of DNA should then be between 1 µg and2 µg. Then the complete DNA was precipitated by adding 250 µl of ethanol (100 %) and 35 µl of 3 M sodium acetate (NaOAc, pH 5.3) to the pooled 100 µl DNA solutions. Samples were kept at room temperature for 30 min and the precipitated DNA was then spun down for 15 min at 13,000 rpm. After carefully removing the supernatant the pellet was dried for 30 - 60 min and samples were sent for sequencing. Sequencing was conducted at MWG-Biotech, Ebersberg, Germany and primers for sequencing were either ordered or 10 µl were sent at a concentration of 10 pmol / µl.

Chapter 10

Cell Biology and Biochemistry Methods.

10.1 Proteins extraction.

Depending on the analysis to be carried out, proteins were extracted using different methods. Here is a description of the methods used regularly during the course of this work.

10.1.1 Alkaline lysis.

Yeast cells cultures (grown as described in 8.1) with an OD_{600} from three to five (except when otherwise stated in the respective figure's legend), were harvested, centrifuged during 1 min at 13,000 rpm. The pellet was resuspended in 1 ml water, to which 150 µl of the lysis solution was added (1,85 M NaOH, 7,5 % (v / v) ß-mercaptoethanol) to the suspension. After short mixing, this was incubated ten minutes at room temperature (RT), then proteins were precipitated for ten minutes at RT using 10 % (v / v) TCA (final concentration). Eventually, the precipitate was centrifuged 10 min at 13,000 rpm before being washed with acetone and dried at 37°C for 30 min. The pellet was resuspended in either Laemmli buffer (200 mM Tris / HCl, pH 6.8; 5 % (w / v) SDS; 20 % (v / v) glycerol; 0.05 % (w / v) bromophenolblue, 1 % (v / v) ß-mercaptoethanol) or urea buffer (8 M urea, 140 mM Tris / HCl pH 6.8, 0.1 mM EDTA, 5 % (w / v) SDS, 0.05 % (w / v) bromophenolblue, 1 % (v / v) ß-mercaptoethanol).

Before being loaded on a SDS-gel, samples were shook during 30 min on a multi-vortex (IKA-vibrax), heated at 68°C for 5 min and centrifuged for 1 min at 13,000 rpm.

10.1.2 Non-denaturing glass beads lysis.

15 to 30 OD_{600} of cells obtained with standard growth protocol (8.1) were harvested (3000 rpm at 4°C, 5 min). Pellet was resuspended in 1 ml ice-cold water containing protease inhibitors (1 µg / ml each of chymostatine, leupeptine, antipaine and pepstatine), 2 mM PMSF, 1 X of Complete™ (from a 25 X stock solution in water) and 20 mM of NaN_3 and frozen at - 80°C before further processing. Cells were then centrifuged at 4°C, 13,000 rpm for 10 min and resuspended in 500 µl ice-cold PBS (137 mM NaCl, 1.25 g / l Na_2HPO_4, 0.35 g / l NaH_2PO_4) containing protease inhibitors as described before, 2 mM PMSF and 1 X of Complete™ (from a 25 X stock solution in PBS). 100 µl of glass beads (Ø 0.4 - 0.6 mm, Sartorius) were added and cells were lysed at 4°C by vortexing (Disruptor Genie, Bohemia, USA) during 25 min Cell lysate was then cleared off by centrifugation at 4°C, 13,000 rpm for 10 min 450 µl of the supernatant were then transferred to a new Eppendorf vial.

10.1.3 Denaturing glass beads lysis.

Cells harvested and kept at - 80°C were thawed (1 to 2 minutes at 95°C) and centrifuged 15 min at 13,000 rpm. The pellet was washed with 100 µl of acetone and resuspended in 100 µl of BB1 buffer (50 mM Tris / HCl pH 7.5, 6 M urea, 1 mM EDTA, 1 % (w / v) SDS). After 100 µl of glass beads were added, the samples were vortexed 3 times three minutes, with 1 min heating at 95°C in between. After 2 min on ice, 900 µl of IP buffer were added [50 mM Tris / HCl pH 7.5, 190 mM NaCl, 1.25 % (w / v) Triton X-100, 6 mM EDTA, 1 mM PMSF, Complete™ inhibitor cocktail (25 X)] and mixed with the sample. To avoid contamination with cell debris and glass beads, the samples were then spun down 10 min at 13,000 rpm and 850 µl of supernatant were transferred to a new 1.5 ml Eppendorf vial.

10.2 SDS-PAGE.

SDS-PAGE is a common method to separate proteins according to their molecular weight. Here, this was carried out as described by Laemmli Laemmli (1970). Table 10.1 shows the components and volumes used to set up typical polyacrylamide gels. Sodium dodecyl sulfate (SDS) is an anionic detergent which denatures secondary structures and tertiary structures which do not

Components	7.5 %	10 %	12 %	15 %	Stacking gel
ddH_2O	4.85 ml	4.01 ml	3.35 ml	2.34 ml	6.1 ml
1.5 M Tris pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	
0.5 M Tris pH 6.8					2.5 ml
10 % SDS (w / v)	100 µl	100 µl	100 µl	100 µl	100 µl
30 % Acrylamide solution (w / v)	2.5 ml	3.33 ml	4.0 ml	5.0 ml	1.3 ml
10 % APS (w / v)	100 µl	100 µl	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl	10 µl	10 µl

Table 10.1: **Components and volumes used in a SDS polyacrylamide gel setup.**

rely on the formation of disulphide bonds. Moreover, it charges proteins negatively, the extent of that charge being dependant on the mass of a particular protein. Therefore, proteins can be separated using an electric field and a polyacrylamide gel according solely to their molecular mass, the reticulation of the gel allowing this separation.

Typically, SDS-PAGE system consisted of two glass plates separated by 1 mm thick spacers. This created a chamber that was filled with a resolving gel ranging from 7.5 % to 15 % in polyacrylamide concentration depending on the experiment carried out and the resolution to be achieved during the electrophoresis. This gel was covered with a 4 % stacking gel in which the loading lanes were formed. Resolving and stacking gels were freshly prepared and poured between the glass plates after the addition of APS and TEMED. First, about 5 ml of resolving gel was cast and layered over with ethanol. Once this gel was polymerised, ethanol was removed and the stacking gel was poured. A ten slots comb was added before the setting of the gel.

To perform the electrophoresis, a Bio-Rad Protean II chamber was filled with 800 ml of SDS-buffer (25 mM Tris, 200 mM glycine, 0.1 % (w / v) SDS). The gel was set in this chamber and 10 µl to 20 µl of protein samples and 5 µl of protein molecular mass standards were separated at a voltage of 150 V during 60 to 90 min. Proteins from the gel were then revealed by using either Coomassie staining (10.3.1) or immunoblotting (10.3.2).

10.3 Protein detection.

Several methods were used in this work to detect proteins after a SDS electrophoresis, depending on whether the detection had to be specific or not. For a non-specific revelation of protein, Coomassie staining was generally used while immunoblotting was the method of choice when specific detection had to be carried out.

10.3.1 Coomassie staining.

After SDS-PAGE (10.2), the gel was laid in a Coomassie blue solution (0.1 % (w / v) Coomassie Brilliant Blue G250, 50 % (v / v) methanol, 10 % (v / v) acetic acid) and rocked at room temperature for at least one hour. The gel was then washed several times with a destaining solution (40 % (v / v) methanol, 10 % (v / v) acetic acid) to remove the background staining. Once a satisfying staining pattern was reached, the gel was eventually laid over a Whatman paper 3M and vacuum dried at 80°C.

10.3.2 Immunoblotting.

This method has been described by Towbin et al. (1992). This consists of a protein transfer from the polyacrylamide gel (10.2) to a nitrocellulose (Pall Corporation, Pensacola, USA) or PVDF (Immobilon ™, Millipore, Bedford, USA) membrane to allow the subsequent detection of particular proteins using antibodies raised against their epitopes. Immunoblotting (or Western-blotting) can be either "wet" or "semi-dry" depending on the analysis to be carried out.

10.3.2.1 Protein transfer onto nitrocellulose or PVDF membranes.

The semi-dry method has been the only one used in this work, therefore, it will be the only one described. A "sandwich" made up by two layers of three Whatman papers (GB002) encompassing a SDS-gel (10.2) and a membrane was placed on the anode of the semi-dry blotting apparatus; with the membrane being on the side of the anode. Before the building of that "sandwich", the whatman papers and the membrane were soaked in 1 X transfer buffer (150 mM L-Glycine, 20 mM Tris, 20 % (v / v) methanol) . After removing excess buffer and air bubbles within the "sandwich", the cathode lid was set onto it. Proteins were transferred to the membrane for 90 min at 70 mA per gel.

10.3.2.2 Immunodetection of transferred proteins.

After the proteins have been transferred to the membrane (10.3.2.1), the membranes were blocked with 5 % (w / v) skimmed-milk in TBS-T (20 mM Tris / HCl, pH 7.6; 137 mM NaCl; 0.1 % Tween-20) for either one hour at room temperature or overnight at 4°C. The membrane was then washed once with TBS-T and incubated with the primary antibody solution during

one hour at room temperature. After washing away the primary antibody three times ten minutes with TBS-T, the membrane was incubated a further hour with the secondary antibody (HRPO conjugated antibody) solution and washed again with TBS-T as described. It was then placed on a glass plate, overlaid with 600 µl Pierce ECL Western blotting substrate (1 : 1 mix of detection reagent 1 and detection reagent 2). For detection, The membrane was then transferred into a developing cassette and an ECL film (GE Healthcare, Uppsala, Sweden) was exposed to the membrane from 30 s to 45 min before being developed.

If needed, the membrane was stripped with 10 % (v / v) acetic acid, blocked again and another antibody used to detect another protein. Antibody solutions and the concentration thereof are detailed in 7.2.1.4.

10.4 Glycerol step gradient.

This method derives from the one described by Kim et al. (1997). After 16 hours growth on YPEthanol, cells were shifted to glucose for either 0 or 20 min. At each time point, 30 OD_{600} of cells were harvested, centrifuged for 3 min at 3,000 rpm, 4°C. After washing with ice-cold water, the pellet was resuspended in 520 µl ice-cold potassium-phosphate buffer (0.1 M KH_2PO_4 pH 7,0). After adding 280 µl of Complete^{TM} (25 X in phosphate buffer), 35 µl of PMSF (200 mM in DMSO), protease inhibitors (1 µg / ml each of chymostatine, leupeptine, antipaine and pepstatine) and 100 µl glass beads, cells were lysed by vortexing (30 min at 4°C). After removing cell debris (10 min at 13,000 rpm, 4°C), 200 µl of the samples were layered over a glycerol step gradient (450 µl of each 50 %, 40 %, 30 %, 20 % and 10 % glycerol in 20 mM PIPES buffer, pH 6,8) and centrifuged for 4 hours at 55,000 rpm and 15°C in a TLS 55 rotor (Beckman Instruments). Subsequently, 250 µl fractions were collected starting from the top of the centrifuge tube. Proteins contained in those fractions were precipitated 10 min at room temperature with 10 % (v / v) TCA (final concentration). Precipitates were centrifuged at 13,000 rpm during 10 min and washed with acetone. After air drying, pellets were resuspended in 50 µl urea buffer (see 10.1.1) and separated using SDS-PAGE (10.2). Proteins in the different fractions were revealed by immunoblotting using specific antibodies (see 10.3.2).

10.5 Immunoprecipitation.

For immunoprecipitation, cells were cultivated as described (8.1) and underwent a native glass beads lysis (10.1.2). Anti-FBPase (3 µl) antibody was added to the samples. After two hours of gentle agitation at room temperature, 60 µl of a 5 % (w / v) protein A Sepharose™ CL-4B slurry (GE Healthcare, Sweden) were added and the samples were further incubated for 1 - 1.5 hour. The protein A Sepharose - IgGs complexes were spun down for 5 min at 2,000 rpm and then washed five times with PBS. The protein A Sepharose pellet was eventually resuspended in 40 µl of urea buffer (see 10.1.1 for composition). To release the bound proteins from the protein A Sepharose beads, the samples were boiled 5 min at 97°C and centrifuged at 13,000 rpm to pellet the beads. Ten to fifteen microliters of samples were then loaded on a SDS-gel (10.2).

10.6 Pulse-chase analysis.

GID4 deleted cells were transformed with the pOS1 plasmid (6.1) and its corresponding control vector. Cells were first grown overnight on CM-Trp media containing 2 % glucose and 40 µg / ml of doxycycline. Then, they were transferred to a CM-Trp media without Cysteine and Methionine, but containing 2 % (v / v) glucose, 40 µg / ml doxycycline and were further grown overnight. 5 OD_{600} of cells were then centrifuged, pellet was washed once with distilled water and resuspended in 2 % EtOH-CM-Trp without Cysteine and Methionine, containing 40 µl / ml doxycycline (pulse medium). After 3 hours growth; 9.25 MBq of ^{35}S-methionine were added to the cells which were grown for a further 2 hours. Cells were subsequently centrifuged, washed with water and resuspended in 6.5 ml of 2 % Ethanol-containing CM-Trp with 10 mM Methionine, but lacking any doxycycline (chase medium). Samples were taken every hour during 3 hours. After immunoprecipitation with FBPase antibody (10.5) and SDS-PAGE (10.2), gels were overlaid with GE Healthcare Phosphor screen and scanned with Storm 860 (GE Healthcare, Uppsala, Sweden).

10.7 Ubiquitination assay.

To test the polyubiquitination of FBPase in WT, $\triangle$*gid1*, $\triangle$*gid4* and $\triangle$*gid7* strains, cells were grown overnight in YPEtOH to an OD_{600} of 3 to 4. For negative control, a W303-1B strain

deleted for *FBP1* was used. After harvesting (5 min at 500 × g), cells were resuspended in YPD. 50 OD_{600} cells were taken before and 25 min after shift to YPD. Cells were harvested and resuspended in 1 ml water containing 20 mM NEM, 20 mM NaN_3, 1 mM PMSF) and stored at - 80°C. After thawing, cells were pelleted at 500 × g for 4 min at 4°C and resuspended in 600 µl PBS buffer containing protease inhibitors (Complete™, Roche Diagnostics; 1.1 mM PMSF; 1 µg / ml each of antipaine, pepstatine A, chymostatine, leupeptine, Pefabloc, 20 mM NEM) and lysed at 4°C with glass beads (300 µl; 0.4 to 0.6 mm in diameter) for 20 min on a multivortexer. Immunoprecipitation of FBPase was performed as described before (10.5) and the pellet was washed five times with PBS buffer containing 0.2 % Triton X-100. Beads were resuspended in 50 µl urea buffer, boiled for 5 min at 95 °C and used for immunoblotting with anti-ubiquitin antibody from Babco (clone P4G7, monoclonal, mouse).

Alternatively, a plasmid expressing FBPase-TAP was used. In this case, cells carrying the FBPase-TAP expressing plasmid were grown overnight on CM-URA containing 2 % ethanol at 30°C. 50 OD_{600} of these cells were then harvested prior to or 25 min after a shift to a CM-URA containing 2 % glucose. Cells were lysed as described above and FBPase-TAP was pulled down using 60 µl of 50 µl IgG-Sepharose during two hours at room temperature. Beads were washed as described above and resuspended in 50 µl urea buffer. Immunoblotting was carried out as in described (10.3.2).

Part III

Results.

Chapter 11

Degradation of FBPase.

When yeast cells grown overnight on a nonfermentable carbon source like ethanol are shifted onto a glucose containing medium, the key gluconeogenic enzyme FBPase is degraded (Molano and Gancedo, 1974). This breakdown depends on polyubiquitination of FBPase and its subsequent degradation by the proteasome (Schork et al., 1994b,a, 1995; Schork, 1995). Indeed, an at the time suspected ubiquitin conjugating enzyme, Ubc8p, has been found to be necessary for the polyubiquitination of FBPase to occur (Schüle et al., 2000). Later, a genome-wide screen identified seven so-called Gid proteins (glucose induced degradation deficient), of which Gid1p, Gid2p, Gid7p have been found to be necessary for this polyubiquitination as well Regelmann et al. 2003; Regelmann 2005; Pfirrmann 2006. Therefore it was of great interest to address the function of the Gid complex in the degradation of FBPase by the ubiquitin-proteasome system. The ubiquitin-proteasome system consists of at least least four components (4.2), three of them have already been identified as playing a crucial role in the proteasome-driven FBPase degradation. These are the ubiquitin activating enzyme (E1) Uba1, which is unique in *Saccharomyces cerevisiae*, the ubiquitin conjugating enzymes Ubc1, Ubc4, Ubc5 and Ubc8, and the proteasome itself (McGrath et al., 1991; Schork et al., 1995; Schüle et al., 2000). However, the nature of the ubiquitin protein ligase (E3) required for the degradation of FBPase was still an open question. It was also unknown whether the Gid-complex, as an effector of FBPase breakdown, requires co-factors for its function.

11.1 Ubiquitin ligases (E3) involved in the degradation of FBPase.

Previous work had already ruled out the participation of several ubiquitin ligases or candidate ubiquitin ligases as components in the FBPase degradation process (Josupeit, 2003). Pfirrmann (2006) suggested that Gid1p, Gid7p and Gid8p are located within the nucleus of the yeast cell. This raised the question whether the putative ubiquitin ligase involved in the polyubiquitination of FBPase would be nuclear. A search among two databases, the "Yeast GFP Fusion Localisation Database" (`http://yeastgfp.ucsf.edu/index.php`, Huh et al., 2003) and the "TRIPLES database" (`http://ygac.med.yale.edu/triples/default.htm`, Kumar et al., 2000, 2002) revealed that several proteins already tested by Josupeit were also located, at least partially, within the nucleus (Table 13.1 in chapter 13). None of those putative E3s were demonstrated to have a clear effect on FBPase degradation (Josupeit, 2003). Thus, other nuclear ubiquitin ligases might play a role in proteasome-dependent FBPase degradation. Among the identified nuclear ubiquitin ligases, Met30p, a F-box protein, was identified as part of the nuclear SCF complex, as were Cdc53p and Skp1p. *MET30* deletion was not tested for FBPase stabilisation as it is nonviable. In 2004, a new nuclear ubiquitin protein ligase was discovered: San1p (Dasgupta et al., 2004). This E3 was involved in protein quality control in the nucleus where it polyubiquitinates mutant proteins prior to their degradation via the proteasome (Gardner et al., 2005). It was therefore of interest to test whether this ubiquitin ligase is the trigger of the polyubiquitination of FBPase. Wild Type and *san1*Δ strains were grown overnight on YPEtOH before being shifted to YPD. Cells were harvested and steady state level of FBPase at different time points after shift to YPD was analysed in both strains. They show an equivalent FBPase degradation pattern (Fig. 11.1). Thus, the ubiquitin ligase San1p is not involved in the proteasome-dependent degradation of FBPase.

Ubiquitin ligases can either be a single protein or a protein complex (4.2). This prompted the testing of newly identified E3 ubiquitin ligase subunits for their involvement in the glucose-dependent FBPase degradation. Cul3p has similarity with Cdc53p and was implicated in the ubiquitin-dependent degradation process of the RNA polymerase II subunit Rpo21p (Lammer et al., 1998; Michel et al., 2003; Ribar et al., 2007). Cul8p is a subunit of the Roc1p dependent

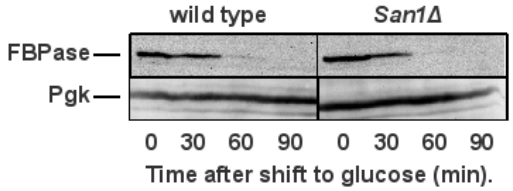

Figure 11.1: **Effect of *SAN1* deletion on FBPase degradation.** Wild type or a strain lacking the *SAN1* gene were grown 16 hours in ethanol-containing media before being shifted to glucose-containing media. 4 OD_{600} of cells were harvested at indicated time points and lysed using the alkaline lysis method. Proteins were separated using a 10 % SDS-PAGE followed by Western-blotting. FBPase antibody and Pgk antibodies were used to detect FBPase or the phosphoglycerate kinase (Pgk), respectively.

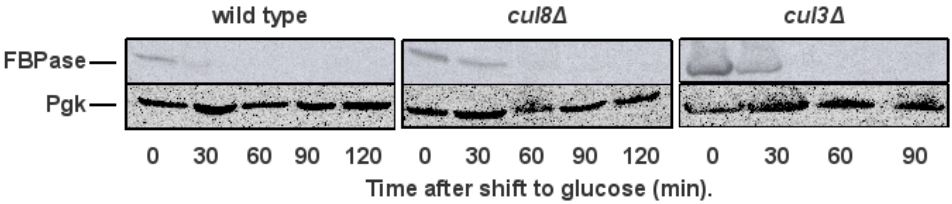

Figure 11.2: **Effect of a *CUL3* and a *CUL8* deletion on FBPase degradation.** Wild type or strains lacking *CUL3* or *CUL8* genes were grown 16 hours in ethanol-containing media before being shifted to glucose-containing media. 4 OD_{600} of cells were harvested at indicated time points and lysed using the alkaline lysis method. Proteins were separated using a 10 % SDS-PAGE followed by Western-blotting. FBPase antibody and Pgk antibodies were used to detect FBPase or Pgk, respectively.

ubiquitin ligase and is involved in Mms22p-dependent DNA repair (Michel et al., 2003; Laplaza et al., 2004; Baldwin et al., 2005). As no other function was known for these proteins, their involvement in FBPase degradation was tested. Cells were grown overnight on YPEtOH before being shifted to YPD. Four OD_{600} of cells were taken at 30 minutes intervals to monitor the steady state level of FBPase in either Wild Type or *cul3*Δ and *cul8*Δ strains at different time points. As seen in figure 11.2, no influence of either *CUL3* or *CUL8* deletions was detected: the degradation of FBPase proceeded in both strains as in the wild type strain. This excludes any role for those gene products in proteasome-dependent catabolite degradation of FBPase.

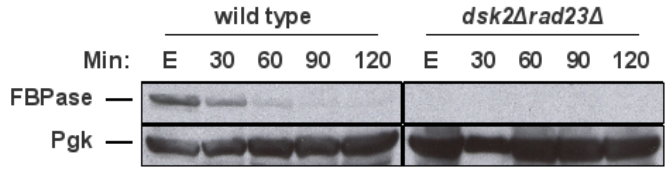

Figure 11.3: **FBPase degradation in a *dsk2*Δ*rad23*Δ double deletion strain**. Cells were grown overnight on YPD and then shifted to YPEtOH for six hours. Four OD_{600} of yeasts were then harvested before ("E") and after shift to glucose at indicated time points. Samples were processed as described in 10.1.1, specific proteins were stained using either FBPase antibody or Pgk antibody. FBPase: fructose-1,6-bisphosphatase; Pgk: phosphoglycerate kinase, loading control.

11.2 Gid co-factors necessary for FBPase degradation.

In addition to the E1-E2-E3 pathway described in 4.2, the degradation of some proteins may need other factors that can either help to unfold the proteasomal substrates or carry them to the proteasome. The former is exemplified by Cdc48p, a AAA ATPase that helps pulling soluble ERAD substrates such as CPY* away from the endoplasmic reticulum membrane prior to its degradation (Kostova and Wolf, 2003). The latter consists of protein bearing a UBA, a UBL domain or both. Among them, Dsk2p and Rad23p have been shown to be involved in ER-associated degradation of CPY* (Medicherla et al., 2004). Single deletions of either *DSK2* or *RAD23* showed no effect on FBPase degradation (Josupeit, 2003). This result could not exclude a redundancy of these genes in the FBPase degradation process. To test this hypothesis, a double deletion mutant was used to monitor FBPase degradation. MY3589 (wild type) and MY3592 (*dsk2*Δ*rad23*Δ) strains were inoculated in YPD for six hours growth at 30°C before being shifted to YPEtOH for overnight growth at the same temperature. It appeared that the double mutant, while growing on a glucose containing media, was not able to do so on an ethanol containing media (data not shown). When grown a shorter time on ethanol-containing medium, enough cells could be harvested to monitor FBPase degradation. Surprisingly, the FBPase antibody did not uncover any FBPase signal in the double deletion strain, while in the wild type strain, FBPase degradation proceeds normally (fig. 11.3). Both facts that *dsk2*Δ*rad23*Δ cells were unable to grow on a nonfermentable carbon source and that no FBPase signal could be seen in this double mutant prompted to a verify FBPase expression after a shift of yeast cells from a glucose- to an ethanol-containing medium. In the double

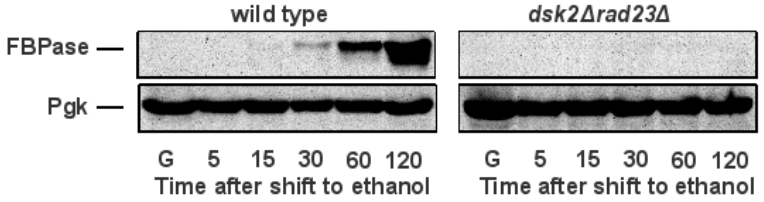

Figure 11.4: **FBPase expression in a *dsk2*Δ*rad23*Δ double deletion strain.** Cells were grown overnight on YPD and then shifted to YPEtOH. Four OD_{600} of cells were harvested before shift to YPEtOH ("G") and at indicated time points thereafter. Samples were processed as described in 10.1.1, specific proteins were stained using either FBPase antibody or Pgk antibody. FBPase: fructose-1,6-bisphosphatase; Pgk: phosphoglycerate kinase, loading control.

deletion mutant, no FBPase could be seen after a two hours shift of cells to YPEtOH whereas in the wild type a weak FBPase signal could be detected already 15 minutes after the shift to ethanol. The strength of this signal rose as a function of time in ethanol containing medium (fig. 11.4). Obviously, expression of FBPase is impaired in a strain where *DSK2* and *RAD23* are deleted. This explains the failure of such a strain to grow on a nonfermentable carbon source. This result is interesting as it might indicate the need of both, Dsk2p and Rad23p, for expression of *FBP1*, thereby suggesting a broader role of the ubiquitin-proteasome system regarding FBPase: it could be involved in both synthesis and degradation of this protein. Moreover, the fact that FBPase is not expressed in a *dsk2*Δ*rad23*Δ does not exclude a role for both of these proteins in FBPase degradation. Clearly, more experiments are needed to characterise the contribution of Dsk2p and Rad23p on FBPase expression.

11.3 The degenerated RING domain of Gid2 is necessary for FBPase polyubiquitination.

As no ubiquitin ligases were discovered to act together with the Gid complex for the polyubiquitination of FBPase and its subsequent degradation by the proteasome, it was postulated that the Gid complex itself might have an ubiquitin ligase activity. Gid2p was already shown to be necessary for both FBPase polyubiquitination and degradation Josupeit 2003; Regelmann et al. 2003; Regelmann 2005. A search through the SMART Database (`http://smart.embl-heidelberg.de/`) identified a so-called degenerated RING domain at

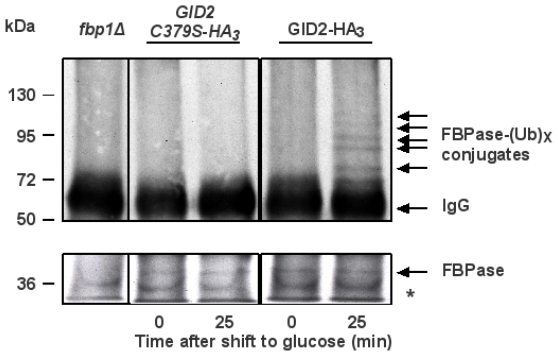

Figure 11.5: **Point mutation in the degenerated RING domain of Gid2 abolishes FBPase polyubiquitination.** A *GID2*-HA3 carrying strain, a *gid2*Δ, and a strain bearing the *GID2* C379S point mutation were grown 16 h in YPEthanol at 25°C and shifted to YPD. Samples were taken at indicated time points and FBPase was immunoprecipitated. Polyubiquitination of FBPase was detected after SDS-PAGE and Western-blot using monoclonal anti-ubiquitin antibody. *fbp1*Δ: FBPase deletion strain. *: cross-reaction. Presence of FBPase in the immunoprecipitates was controlled by immunoblotting with FBPase antibody (data not shown).

the C-terminus of Gid2p. As many ubiquitin ligases rely on RING or RING-related domains for their activity (4.2), this led to the assumption that Gid2p might bear an ubiquitin ligase activity. To test this hypothesis, a point mutation of the degenerated RING domain protein was constructed (Pfirrmann, 2006). In classical RING domains, eight Zn^{2+} ion coordinating residues define two Zn^{2+} fingers that are thought to bind the ubiquitin conjugating enzyme. In the degenerated RING domain of Gid2p, three out of the eight Zn^{2+} coordinating residues found in classical RING domains are missing (Pfirrmann, 2006; Santt et al., 2008). These residues all lie within the first Zn^{2+} finger, where only one coordinating residue is conserved., Thus the first Zn^{2+} finger is assumed to be absent from Gid2p. The point mutation introduced by Pfirrmann (2006) removes a residue necessary to coordinate the second Zn^{2+} ion, and thus might destroy the degenerated RING domain, thereby abolishing its function. The strain carrying the point mutated Gid2p, in which cysteine 379 is changed into a serine, was unable to degrade FBPase (Santt et al., 2008). To test whether this Gid2p mutant was impaired in FBPase polyubiquitination, cells carrying a HA-tagged point mutated Gid2p and their wild type counterpart were inoculated, grown overnight in YPEtOH, and 50 OD_{600} of cells were harvested at time points 0 or 25 minutes after shift of the cells to glucose. Proteins were extracted as described

(10.1.3) and FBPase was immunoprecipitated (10.5). After Western-blotting, the membrane was autoclaved, blocked and probed with monoclonal ubiquitin antibody to uncover any polyubiquitination or lack of polyubiquitination. As seen in figure 11.5, while the strain bearing a C-terminally tagged Gid2 displays a ubiquitin ladder – reminiscent of polyubiquitination – after 25 minutes of shift of the cells to glucose, no such signal is visible in the strain carrying the mutated Gid2p (fig. 11.5). Thus, the point mutation in the degenerated RING domain of Gid2p abolishes its ability to polyubiquitinate FBPase *in vivo* (fig. 11.5). Although this result requires further confirmation, it argues in favour of an ubiquitin ligase activity of Gid2p.

Chapter 12

The Vid24p/Gid4p protein.

Vid24p/Gid4p has been established to be necessary both for vacuolar and proteasomal FBPase degradation (Chiang and Chiang, 1998; Regelmann et al., 2003; Hung et al., 2004). It was proposed that its expression pattern differs from that of the other Gid proteins: whereas the latter seem to have stable steady states within the cell, Vid24p/Gid4p seem to be only expressed upon glucose shift (Chiang and Chiang, 1998; Josupeit, 2003; Pfirrmann, 2006). This particular expression pattern prompted a study of the role of Vid24p/Gid4p within the Gid complex.

Vid24p/Gid4p is a protein with a molecular mass of 41.25 kDa, present in the relatively high copy number of 6,650 molecules per cell (Ghaemmaghami et al., 2003). Its localisation seem to be both nuclear and cytosolic (Chiang and Chiang, 1998; Huh et al., 2003). It had been first identified as being necessary for the vacuolar degradation of FBPase, where it is supposed to promote the fusion of so-called Vid vesicles, containing FBPase, with the vacuole (Chiang and Chiang, 1998). It is also part of the proteasomal degradation pathway of FBPase, as its deletion stabilises FBPase under conditions where FBPase is degraded by this cytoplasmic and nuclear proteolytic machinery (Josupeit, 2003; Regelmann et al., 2003; Hung et al., 2004; Regelmann, 2005). Although the role of Vid24p/Gid4p has been somewhat characterised in the vacuolar degradation of FBPase, its function in the proteasomal degradation had remained unclear. Analysis of Gid4p using the Pfam database (`http://pfam.sanger.ac.uk/`, Finn et al., 2006; Mistry and Finn, 2007) reveals that it contains two PfamB domains. Domain PB010916 is found within 13 protein sequences belonging to 11 different species, while domain PB008050 is shared by 16 different proteins from 12 different species (fig. 12.1). Those domains are widespread among eukaryotes, being present from yeast to human, including at least two plants species.

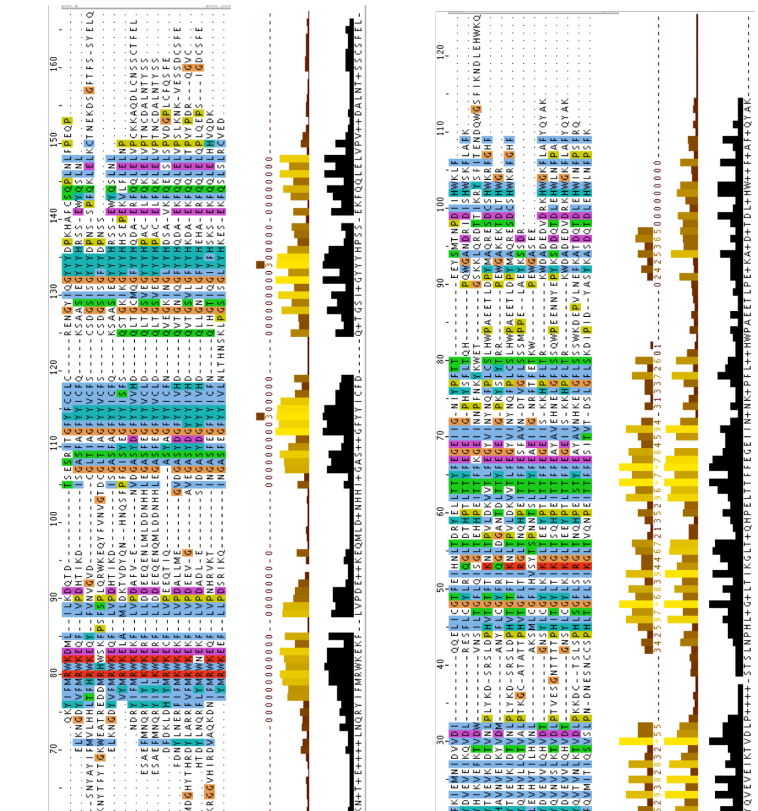

Figure 12.1: **Alignment of PfamB domains found in Gid4p and other protein sequences. A:** domain PB010916. **B:** domain PB008050. Downward is shown the name of the protein and the species of origin in the alignment. Species are: SCHPO: *Schizosaccharomyces pombe*; MOUSE: *Mus musculus*; ORYSA: *Orysa sativa*; ARATH: *Arabidopsis thaliana*; HUMAN: *Homo sapiens*; CANGA: *Candida glabrata* ; YEAST: *Saccharomyces cerevisiae*; YARLI: *Yarrowia lipolytica*; ASHGO: *Ashbya gossypii*; KLULA: *Kluyveromyces lactis*; NEUCR: *Neurospora crassa*; DEBHA: *Debaryomyces hansenii*; ENCCU: *Encephalitozoon cuniculi.* The alignment was performed with clustalw (`http://www.ebi.ac.uk/Tools/clustalw/`) and visualised using Jalview.

Interestingly, the overall architecture found in Gid4p, namely the location of domain PB010916 N-terminal of domain PB008050, is observed in several other proteins. The fact that both the domain architecture and the domains themselves are evolutionarily conserved among eukaryotes suggests that they bear an important function. Therefore, studying Gid4p might also shed light on the putative functions of its homologues.

12.1 Gid4p is necessary for FBPase and PEPCK degradation.

The expression of the gene encoding FBPase is inhibited and therefore no new FBPase protein is produced when cells grown on a nonfermentable carbon source like ethanol are treated with 2 % glucose (Gancedo, 1998). It is thus possible to monitor the catabolite degradation of FBPase once the cells are handled with glucose.

To verify that a *GID4* deletion leads to a disturbed catabolic degradation of FBPase in ethanol grown cells treated with 2 % glucose. Wild type and *gid4*Δ in the genetic background of strain W303 (Josupeit, 2003) were grown overnight in YPEtOH and shifted to YPD (8.1). Cells were harvested at different time points before and after glucose treatment. Samples were prepared according to 10.1.1 and loaded onto a 10 % SDS gel. FBPase steady state levels were monitored on Western-blots using a FBPase antibody.

Deletion of *GID4* leads to a stabilisation of FBPase and PEPCK, as shown both by Western-blotting (FBPase, fig. 12.2A) and cycloheximide chase (PEPCK, fig. 12.2B, C). This not only confirms previously observed results (Josupeit, 2003; Regelmann et al., 2003; Regelmann, 2005), but also suggests that Gid4p, and hence the Gid complex, might play a central role in the regulation of carbohydrate metabolism in yeast.

12.2 Amino-terminal tagging of *GID4*.

Following Gid4p during different growth conditions in cellular extracts requires antibodies directed against the protein. For preparation of polyclonal antibodies from rabbits, pure protein has to be available. However, the expression and purification of a plasmid-encoded GST-Gid4 fusion protein in the bacterium *Escherichia coli* was difficult as the protein aggregated and

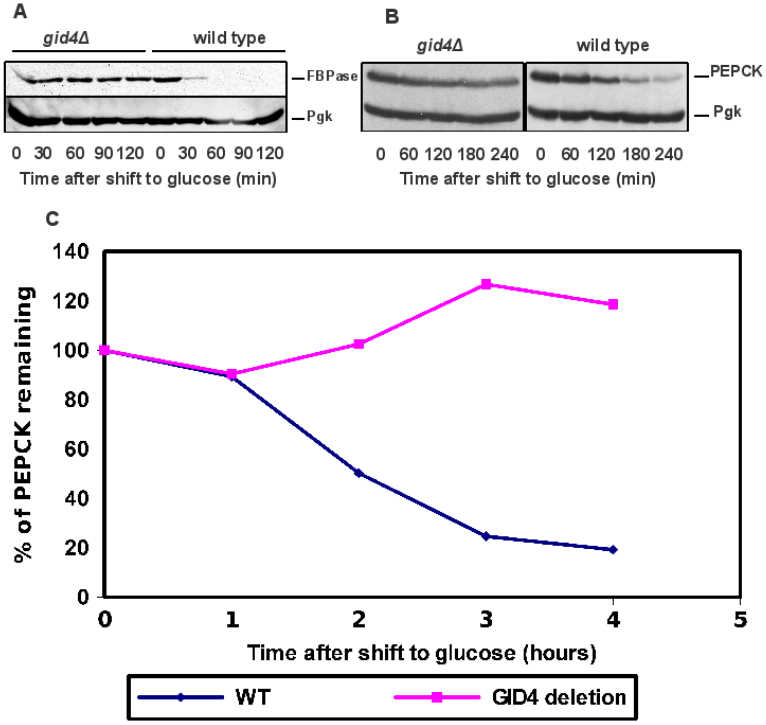

Figure 12.2: **Gid4p is necessary for degradation of FBPase and PEPCK.** Wild type and *GID4* deleted cells were grown overnight on YPD and then shifted to YPEtOH overnight. Four OD_{600} of cells were harvested before shift to glucose ("0") and at indicated time points thereafter. Samples were processed as described in section 10.1.1, specific proteins were stained using either FBPase, PEPCK or Pgk antibody. (A): Steady-state levels of FBPase monitored by Western-blotting. (B): Cycloheximide chase to check for PEPCK degradation. (C): Quantification of the cycloheximide chase shown in (B). Quantification was carried out as follows: PEPCK signal at each time points was normalised with the level of the corresponding Pgk signal: ($PEPCK_{signal}/Pgk_{signal}$), the mean of two independent experiments was calculated and 100 % was set at time point "0". PEPCK and Pgk signals were extracted using the ImageJ software (`http://rsb.info.nih.gov/ij/index.html`). FBPase: fructose-1,6-bisphosphatase; PEPCK: Phosphoenol pyruvate carboxykinase. Pgk: phosphoglycerate kinase, loading control, WT: wild type.

could not be brought into solution (data not shown and Josupeit, 2003). Therefore, the epitope tagging of Gid4p was seen as an alternative.

However, C-terminal tagging of the Gid4 protein led to its inactivation: although tagging according to Longtine et al. yielded a detectable Gid4p-HA_3 fusion protein, FBPase was stabilised in such a strain (Josupeit, 2003). Therefore, introduction of a tag had to be tried either

internally or N-terminally. Here, an amino-terminal tagging was performed. The existence of HA_3-tagged *GID* genes in the W303 strain background led to the use of the same background for the *GID4* N-terminal tagging (Schüle et al., 2000; Regelmann et al., 2003; Regelmann, 2005; Pfirrmann, 2006). To ensure that the tagged protein remains under its native promoter, the selection marker has to be removed once the chromosomal integration is successful. This is achieved with the help of the Cre-Lox system where the Cre recombinase recognises two LoxP cassettes flanking the auxotrophic marker and performs an homologous recombination between those cassettes. Thus, the marker can be removed from the yeast genome, leaving one LoxP cassette behind. Clones can be then tested for the loss of the ability to grow on a medium lacking this auxotrophic marker. Figure 12.3 describes the principle of the N-terminal tagging method used in this work, which derives from the work of Gauss et al. (2005). Briefly, the tagging cassette is amplified from a plasmid (pOM12 for HA_6 tagging, pOM22 for Myc_9 tagging and pOM42 for GFP tagging, 6.1), with regions of homology to the gene of interest brought about by the oligonucleotides used for this amplification (see 6.3). After a heat shock transformation of yeasts cells (9.7.1), transformants were screened according to their capability to grow on a selection medium lacking uracil. Only transformation carried out with the cassette amplified from pOM22 plasmid was successful. Several clones were then chosen, inoculated in the liquid selection medium and grown overnight. Cells were transformed with a plasmid bearing the Cre recombinase gene driven by the *GAL1* promoter (pSH62, 6.1) and transformants were again selected on a media lacking uracil (selecting for the chromosomal integration) and histidine (selecting for the pSH62 plasmid). Expression of the Cre recombinase was then induced in several clones by transferring the transformants on a medium containing 2 % galactose. After four hours, cells were plated on a YPD petri dish and 35 single colonies were screened for the lack of ability to grow on a medium without uracil. Nineteen positive clones were obtained. To be further used, those clones had to be tested for the expression of the tagged protein, its functionality and the correct integration of the tag within the genome. As tags on Gid4p had already shown to impair its function when they were located at its C-terminus, it was important to check whether the N-terminally tagged Gid4p retained its biological function. This was performed by the means of a Western-blotting were both expression of Myc_9-Gid4p and degradation of FBPase upon glucose shift could be assessed for several clones.

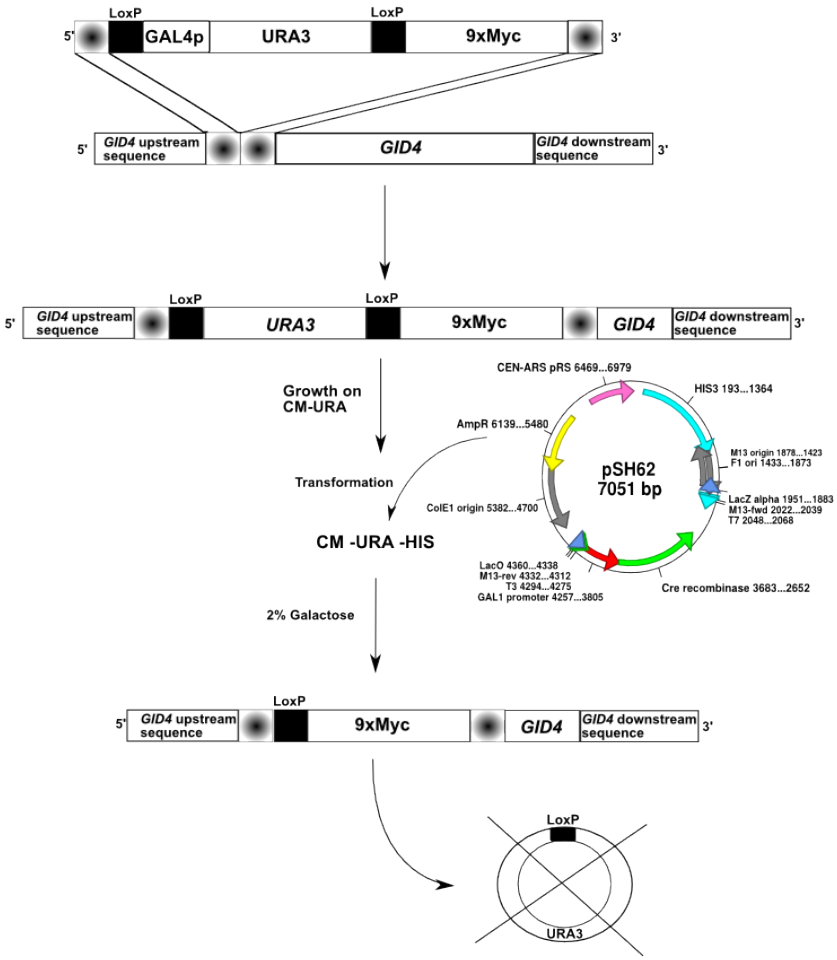

Figure 12.3: **The principle of N-terminal tagging of *GID4* in S. cerevisiae strain W303-1B**. A 1891 bp PCR fragment containing the *GAL4* promoter and the *URA3* auxotrophic marker flanked by two LoxP cassettes was inserted 5' of GID4 by homologous recombination. Recombinants were then transformed with a pSH62 plasmid bearing the Cre recombinase under a *GAL1* promoter. After expression of the recombinase, strains were checked for the loss of ability to grow on a medium lacking uracil.

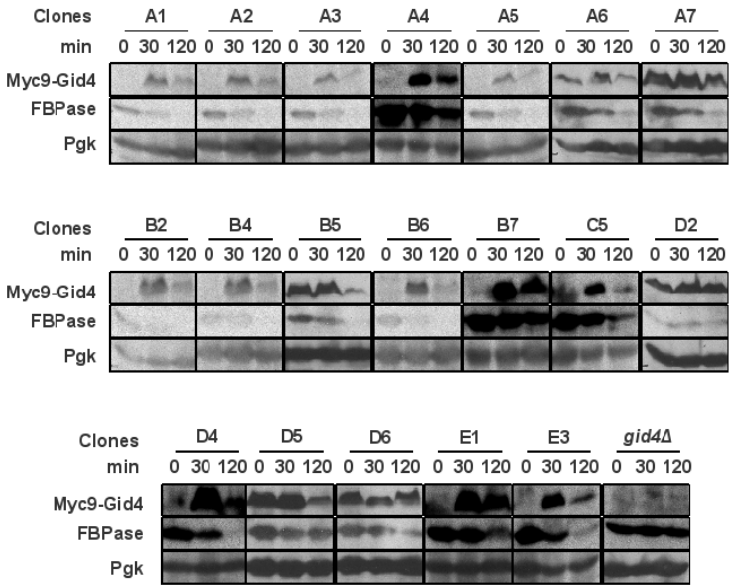

Figure 12.4: **Screening for Myc$_9$-Gid4p expression and complementation for FBPase degradation.** The Nineteen clones that lost the ability to grow on CM-URA medium were isolated and tested for the expression of Myc$_9$-Gid4p as well as their capacity to perform FBPase degradation upon glucose shift. Indicated clones were inoculated on YPD, then passed over to YPEtOH for overnight growth. 4 OD_{600} of yeast cells were harvested before ("0"), 30 and 120 minutes after glucose shift. Cells underwent alkaline lysis and samples were subjected to SDS-PAGE followed by Western-blotting. Anti-Myc antibody, anti-FBPase antibody and anti-Pgk antibody were used to reveal Myc$_9$-Gid4p, FBPase and Pgk, respectively.

All the tested clones displayed a Myc$_9$-Gid4p expression, although with somewhat different patterns: in some clones, Gid4p was not detectable when cells were grown on ethanol containing media (fig. 12.4, see A1, A2, A3, A4, A5, B2, B4, B6, C5, D4, E1 and E3); in other clones, Gid4p was already present in ethanol-grown cells (fig. 12.4, see A6, A7, B5, D2, D5 and D6). FBPase degradation was monitored in the same clones. Again, different patterns arose: while in several clones FBPase was degraded in a wild-type fashion (fig. 12.4, see A1, A2, A3, A5, A6, A7, B2, B4, B5, B6, D4, D6 and E3); FBPase was stabilised in other clones, albeit to different extents (fig. 12.4, see clones A4, B7, C5, D2, D5 and E1). As criterion to select the clones of interest, the facts that Vid24p/Gid4p is upregulated when cells are shifted from a nonfermentable carbon source to glucose, and that wild-type FBPase degradation is not disturbed by the tagged Gid4 protein, was used (Chiang and Chiang, 1998; Josupeit, 2003;

Kaniak et al., 2004). After confirmation of the integration of the tag by PCR using the primers promgid4F and tergid4R (6.3, data not shown), the clone D4 (fig. 12.5) was renamed YOS1 and used for further experiments.

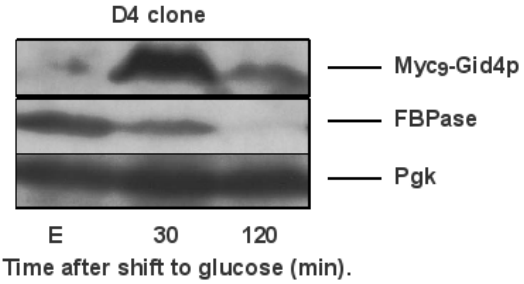

Figure 12.5: **Expression pattern of the D4 clone.** D4 clone was inoculated on YPD, then passed over YPEtOH for overnight growth. 4 OD_{600} of yeast cells were harvested before ("E"), 30 and 120 minutes after glucose shift. Cells underwent alkaline lysis and samples were subjected to SDS-PAGE followed by Western-blotting. Anti-Myc antibody, anti-FBPase antibody and anti-Pgk antibody were used to reveal Myc_9-Gid4p, FBPase and Pgk, respectively.

12.3 Expression of the Vid24p/Gid4p protein.

12.3.1 Influence of glucose on *GID4* expression.

Vid24p/Gid4p is upregulated when cells are shifted from a nonfermentable to a fermentable carbon source (Chiang and Chiang, 1998; Josupeit, 2003; Kaniak et al., 2004). The antibody used in the laboratory was not stable and yielded, even when purified, various cross-reactions (Josupeit, 2003; Pfirrmann, 2006). Therefore, it was of interest to confirm the upregulation of Gid4p in glucose grown cells with the tagged version of Gid4p. Indeed, Gid4p is undetectable when cells are grown on ethanol (fig. 12.6). Gid4p seems to be present during logarithmic growth of yeasts on YPD, although at a very low steady state level (fig. 12.6, Log). It is undetectable after overnight growth on YPEtOH (E). Interestingly, upregulation of Gid4p requires less than five minutes of shift of cells to glucose to be present in rather high amounts. The steady state level of Gid4p within the cell raises dramatically till 30 minutes after shift of the cells onto glucose, thereafter the Myc signal diminishes (fig. 12.6). Interestingly, this decrease parallels the decrease observed for FBPase.

The fact that Gid4p is observed already five minutes after cells have been transferred onto

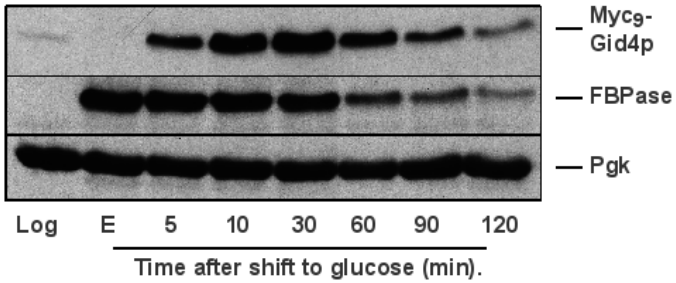

Figure 12.6: **Expression of Gid4p/Vid24p.** Cells were grown overnight in YPD medium. 4 OD_{600} were harvested ("Log"). Cells were then diluted $\frac{1}{20}^{e}$ in YPD and grown for six hours before being transferred to YPEtOH for overnight growth. Four OD_{600} of cells were harvested before ("E") and at indicated time points after shift to glucose. Yeast cells underwent alkaline lysis and protein were resuspended in urea-buffer. Samples were loaded on a 10 % SDS gel and semi-dry Western-blot was performed. Proteins were detected by either monoclonal Myc antibody, monoclonal Pgk and polyclonal FBPase antibodies.

glucose containing medium suggests that either the protein is always produced but unstable in gluconeogenic cells. Another possibility is that the *GID4* mRNA; whose steady-state level is roughly constant during diauxic shift (DeRisi et al., 1997), is already present in those cells. Therefore, it was of interest to test whether any inhibition of the transcription could lead to a block in FBPase degradation and / or *GID4* gene product appearance. Using 1,10-phenanthroline, a compound known to inhibit transcription, should allow to determine whether *GID4* mRNA has to be *de novo* transcribed or whether the regulation of the expression of *GID4* occurs at the post-translational level. To ensure that 1,10-phenanthroline is not expelled from the cells, a strain deleted for *PDR5* was used (see 12.5.1). Yeasts cells were grown as described (8.1) and treated with 100 µg / ml 1,10-phenanthroline before (time point "0"), and after shift to glucose. As shown by figure 12.7, the treatment does not impair Gid4p expression.. Moreover, albeit to a slower extent, FBPase degradation still takes place in the samples taken from cells treated with 1,10-phenanthroline. 1,10-phenanthroline has been shown to have the same effect on total mRNA transcription as a thermosensitive mutant of the RNA polymerase II subunit *RPB1* (Grigull et al., 2004). *RPB1* is a subunit of the RNA polymerase II, also known as the B220 subunit. Its thermosensitive (*ts*) mutant *rpb1-1* permitted to identified this subunit as a major component of the RNA polymerase II (Nonet et al., 1987). This conditional mutant, when shifted from permissive (24°C) to restrictive (36°C) temperature produces a rapid block in

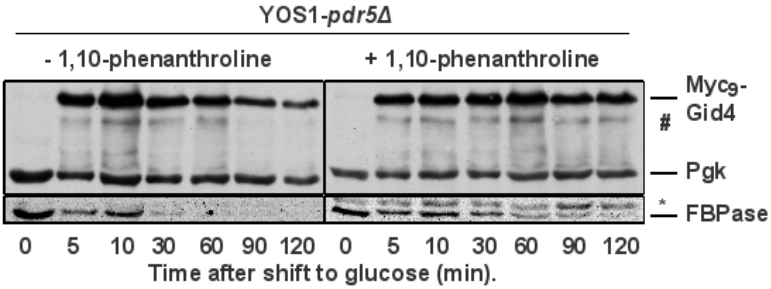

Figure 12.7: **Gid4p expression is not inhibited by 1,10-phenanthroline an inhibitor of mRNA synthesis.** Cells were grown overnight in YPEtOH media, treated with 100 µg / ml of 1,10-phenanthroline or, for control, the corresponding volume of solvent. 4 OD_{600} were harvested before (time point "0") and after glucose shift at indicated time points. Yeast cells were subjected to alkaline lysis and proteins were resuspended in urea-buffer. Samples were loaded on a 10 % SDS gel and semi-dry Western-blot analysis was performed. Proteins were detected by either monoclonal Myc antibody, monoclonal Pgk and polyclonal FBPase antibodies. #: degradation product of Gid4p; *: cross-reaction.

mRNA synthesis, occurring between five and 15 minutes upshift. As all three RNA polymerases are metabolically stable once assembled, this suggests that this mutant leads to a functional defect rather than to a defect in RNA polymerase II complex assembly (Nonet et al., 1987). Those characteristics made it a perfect tool to study the importance of *de novo* transcription requirements for FBPase degradation upon shift to glucose. Therefore, a *rpb1* thermosensitive mutant was used to confirm this result, sparing the side effects that may be associated with the use of a metal chelator such as 1,10-phenanthroline. HKY36 (wild type) and HKY77 (*rpb1-1*) strains were inoculated and grown at 24°C during six hours on YPD before being transferred to YPEtOH medium and grown overnight at 24°C. Thereafter, cells were transferred to a 37°C pre-warmed YPD medium and four OD_{600} were taken every 30 minutes of incubation. Samples were processed as described (10.1.1) and Western-blotting was performed. FBPase degradation in the wild type and the *rpb1-1* temperature sensitive mutant were comparable (fig. 12.8). This result, together with the fact that 1,10-phenanthroline does not impair neither Gid4p expression nor FBPase catabolite degradation (fig. 12.7) suggests that no transcription is needed upon glucose shift for catabolite degradation of FBPase to proceed normally. When cells are growing on a nonfermentable carbon source, the *GID4* mRNA might be already present in amounts sufficient to produce enough protein to trigger FBPase degradation after a shift to a

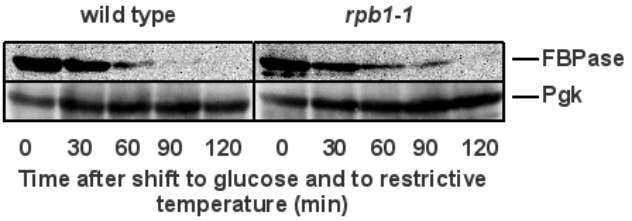

Figure 12.8: **Block of transcription does not impair FBPase degradation.** HKY36 (Wild Type) and HKY77 (*rpb1-1*) strains were inoculated and grown at 24°C during six hours on YPD before being transferred to YPEtOH media and grown overnight at 24°C. Then, cells were transferred to a 37°C pre-warmed YPD medium and maintained at 37°C for the duration of the experiment. Four OD_{600} of cells were taken at indicated time points. Samples were processed as indicated in 10.1.1 and were subjected to a 10 % SDS-PAGE followed by Western-blotting. FBPase and Pgk were detected using their specific antibodies.

fermentable carbon source. Another possibility is that Gid4p, although synthesised when cells are growing on nonfermentable carbon source, is highly unstable under these conditions and becomes relatively more stable after the cells change from the gluconeogenic to the glycolytic state. This is also suggested by the fact that steady-state levels from a repressible promoter-driven Myc_9-Gid4p expressed in cells grown overnight on ethanol-containing medium are lower when compared to those obtained from cells that have been shifted to a glucose-containing media for 30 minutes (fig. 12.9, see below).

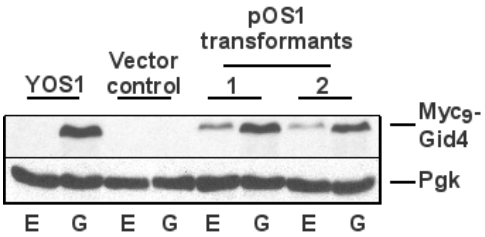

Figure 12.9: **Steady-state levels of P_{tet}-driven Myc_9-Gid4p in gluconeogenic and glycolytic cells.** W303-1B cells were transformed with the pOS1 plasmid as described above (9.7). YOS1 (control) and two transformants were then grown overnight in either YPEtOH (YOS1) or CM-Trp-EtOH (Vector control, pOS1 transformants 1 and 2). Cultures were then split in two halves. One half was immediately harvested while the second half was transferred on YPD (YOS1) or CM-Trp-Glc (Vector control, pOS1 transformants 1 and 2). For each part, five OD_{600} of cells were harvested. Samples were processed as described in 10.1.1, loaded onto a 10 % SDS-acrylamide gel and subjected to electrophoresis followed by Western-blotting. Myc_9-Gid4p and Pgk were detected using specific antibodies. "E": gluconeogenic cells; "G": glycolytic cells.

12.3.2 Influence of other carbon sources on *GID4* expression.

Pfirrmann (2006) used a *GAL1* promoter-driven recombinant *GID7-FLAG* to study the interactions between the Gid-complex subunits. Surprisingly, he was able to co-immunoprecipitates a Myc_9-tagged Gid4p and a FLAG-tagged Gid7p even before the cells were shifted to a glucose-containing medium. However, to express the FLAG-tagged Gid7p, galactose must be added to the media two hours before the shift to glucose. An explanation of the interaction observed would be that not only glucose, but other sugars might trigger the expression of Gid4p as well. To test this hypothesis, YOS1 cells were grown overnight in minimal medium containing ethanol before being shifted to a sugar-containing medium.

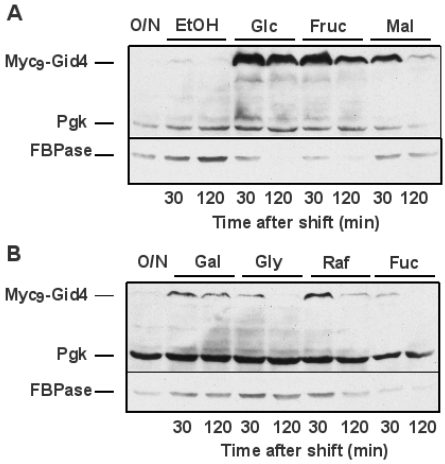

Figure 12.10: **Gid4p expression is triggered by different types of sugars or other carbon sources.** (A): Gid4p expression in cells treated with ethanol (EtOH), glucose (Glc), fructose (Fruc), maltose (Mal), galactose (Gal), maltose (Mal) and raffinose. (B): Gid4p expression in cells treated with galactose (Gal), glycerol (Gly), raffinose (Raf) and fucose (Fuc). YOS1 cells were grown 16 hours in CM medium containing all complements and ethanol as carbon and shifted to a medium containing 2 % of the aforementioned carbon sources. 4 OD_{600} of cells were harvested at the indicated time points after the beginning of the sugar treatment. Cells were subjected to alkaline lysis and proteins were separated by a 10 % SDS-PAGE followed by Western-blotting. Proteins were stained using their specific antibodies.

As shown in figure 12.10, treatment with different carbon sources produces an expression of Gid4p. However, the extent of this expression depends on the carbon source provided. Glucose and fructose produce an equivalent response, while other sugars, like fucose yield a very weak Gid4p expression. Notably, glycerol, although not referred as a fermentable carbon

source, produces a weak Gid4p expression as well. Interestingly, when cells were still growing on YPEtOH, either glucose (data not shown) or galactose could produce a Gid4p response (fig. 12.11A), thereby explaining the result obtained by Pfirrmann (2006).

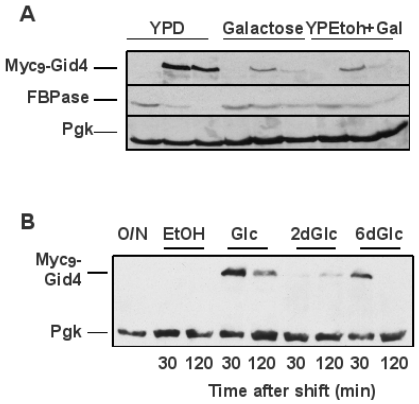

Figure 12.11: **Gid4p expression and FBPase degradation triggered by different sugars and sugar analogues.** (A): Gid4p expression in cells treated by either glucose (YPD), galactose, or 2 % ethanol added with 2 % galactose (YPEtOH+Gal). YOS1 cells were grown 16 hours in YPEtOH and shifted to a medium containing 2 % of the aforementioned sugars. (B): Gid4p expression in cells treated with two glucose analogues: Here, YOS1 cells were grown overnight in a minimal complete (CM) medium and shifted to either ethanol (EtOH), glucose (Glc), 2-deoxy-glucose (2dGlc) and 6-deoxy-glucose (6dGlc). 4 OD_{600} of cells were harvested at the indicated time points after the beginning of the sugar treatment. Cells underwent alkaline lysis and proteins were separated by a 10 % SDS-PAGE followed by Western-blotting. Proteins were stained using their specific antibodies.

Furthermore, this suggests that ethanol does not act as a repressor for Gid4p expression but rather that easily fermentable sugars are positive signals for Gid4p expression. Interestingly, although some sugars were producing a Gid4p expression, they failed to yield a significant change in FBPase steady-state levels after two hours induction, as shown for maltose or glycerol (fig. 12.10). Although it cannot be excluded that FBPase is degraded under these conditions, its biosynthesis might still occur, which could explain the stability observed in the steady-state level.

It was proposed by several authors (Klein et al., 1998; Rolland et al., 2001b; Colombo et al., 2004; Santangelo, 2006; Belinchón and Gancedo, 2007) that metabolism of glucose might be necessary to trigger a response such as catabolite inactivation or degradation. The availability of glucose analogues that lack a hydroxyl group at either position 2 (2-deoxy-glucose) or 6 (6-

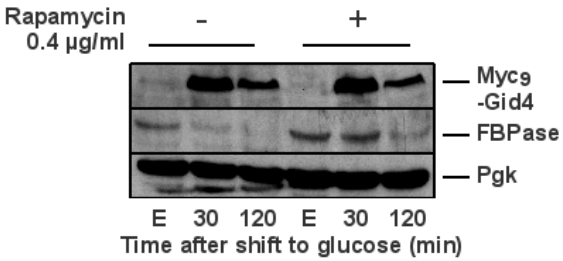

Figure 12.12: **Gid4p expression in rapamycin-treated cells.** Cells were grown overnight in YPEtOH and treated with DMSO (carrier) or rapamycin (0.4 µg / ml) ten minutes prior the shift to glucose. Four OD_{600} of cells were harvested before ("E") and at indicated time points after shift to glucose. Samples were processed as described (10.1.1) and were subjected to SDS-PAGE followed by semi-dry Western-blotting. Myc_9-Gid4p, FBPase and Pgk (loading control) were detected using their specific antibodies.

deoxy-glucose), can help unravelling the involvement of glucose metabolism in Gid4p expression. While 2-deoxy-glucose can be phosphorylated, it cannot be isomerised into fructose-6-phosphate which impairs its further metabolization. Lacking the 6-hydroxyl group, 6-deoxy-glucose cannot be phosphorylated by the different glucose kinases. These two different analogues could thus help to differentiate between the need of glucose phosphorylation or further metabolization. Figure 12.11B shows that treatment of cells with 2-deoxy-glucose blocks expression of Gid4p while treatment with 6-deoxy-glucose leads to a weak expression of the protein. Furthermore, while 6-deoxy-glucose treatment does not have any effect on FBPase degradation, 2-deoxy-glucose treatment stabilises FBPase (not shown).

When cells face changes in their environment, they adapt by modifying their gene expression according to the stimuli present in their neighbourhood. Several pathways are implicated in glucose signalling and hence FBPase degradation. Among them, disruption of the PKA or the TOR pathways lead to an impaired FBPase elimination (Regelmann, 2005; Pfirrmann, 2006). On the contrary, in a *SNF1* deletion mutant the steady-state level of FBPase was lower when compared to its wild type counterpart. These facts raised the question whether the observed stabilisation of FBPase in rapamycin-treated cells was due to a lack of Gid4p expression. Cells were grown overnight in YPEtOH and treated with the macrolide rapamycin for ten minutes before the shift to glucose. Four OD_{600} of cells were harvested before, 30 and 120 minutes after the shift to glucose and samples were processed as described (10.1.1). After SDS-PAGE

and semi-dry Western-blotting, the steady state level of Gid4p and FBPase were analysed. As shown in figure 12.12, rapamycin treatment hinders FBPase degradation, which confirms previously observed results (Regelmann, 2005). Surprisingly though, Gid4p expression does not seem to be influenced in those conditions. The TOR 1 kinase complex, although participating in the onset of FBPase degradation, does not seem to exert its effect through the expression of Gid4p.

12.4 Gid4p triggers FBPase degradation.

12.4.1 *De novo* protein synthesis is necessary for FBPase degradation.

During growth on YPEtOH, no Gid4p can be detected in *Saccharomyces cerevisiae* (fig. 12.6). Moreover, Gid4p has been shown to be necessary for FBPase degradation (fig. 12.2 and Josupeit, 2003). Cycloheximide, which blocks the translational elongation during protein synthesis by inhibiting the peptidyl transferase activity of the eukaryotic ribosome (Berg et al., 2002), has also been shown to prevent FBPase polyubiquitination (Schork, 1995). Gid4p *de novo* synthesis might therefore be necessary for catabolite degradation of FBPase to proceed. Indeed, cells treated with 100 µg / ml cycloheximide at the time of glucose shift are unable to degrade FBPase (fig. 12.13A, B). Hence, the same treatment prevents Gid4p to appear (fig. 12.13B). Thus, FBPase catabolite degradation needs an efficient translational elongation to proceed normally. The necessity of *de novo* protein synthesis is further underlined by the absence of Gid4p in cycloheximide-treated cells. Although this result does not exclude an expression of Gid4p on ethanol-containing media, it suggests that either the mRNA of *GID4* is present, but not translated, in the yeasts cells prior to the glucose shift or that the protein is less stable in gluconeogenic cells as it is in glycolytic cells (12.3).

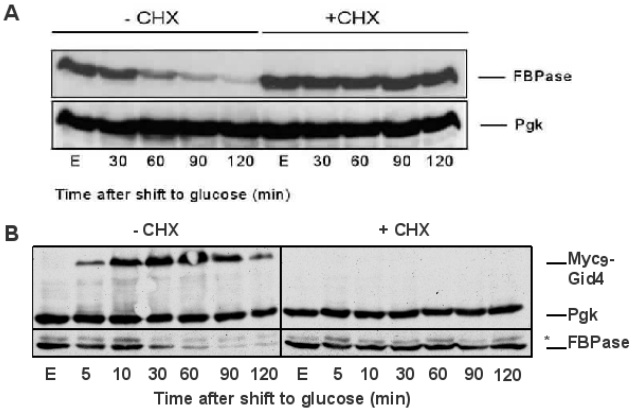

Figure 12.13: **FBPase degradation and Gid4p expression depend on *de novo* protein synthesis.** Cells were grown overnight on YPD and then shifted overnight to YPEtOH. Cells were treated with cycloheximide (100 µg / ml final concentration), thereafter four OD_{600} of cells were harvested before shift to glucose ("E") and at indicated time points after shift to glucose. Samples were processed as described in 10.1.1, specific proteins were stained using either FBPase antibody, Myc or Pgk antibodies. (A): Steady-state levels of FBPase monitored by Western-blotting. (B): Steady-state levels of Myc_9-Gid4p. E: YPEtOH; FBPase: fructose-1,6-bisphosphatase. Pgk: phosphoglycerate kinase, loading control.

12.4.2 Gid4p is a molecular switch necessary for FBPase degradation to take place.

Gid4p is the only Gid protein which becomes detectable upon shift to glucose, all the other Gid proteins are already expressed in gluconeogenic cells (Pfirrmann, 2006). Prevention of its appearance leads to a failure to degrade FBPase (12.4.1). Thus, it is reasonable to hypothesise that Gid4p might be a switch triggering FBPase catabolite degradation of FBPase. If this would be the case, expression of Gid4p in gluconeogenic cells should result in FBPase degradation. To test this, a plasmid expressing Myc_9-Gid4p under the Tet^R promoter was constructed (fig. 12.14). The *MYC_9-GID4* ORF was amplified using primers StuI-mycgid4F and SbfI-mycgid4R (table 6.3) and subcloned into the pGEM-T-easy plasmid (table 6.1). After the correct integration of the insert in pGEM-T-easy had been tested and confirmed, the fragment of interest was excised from pGEM-T-easy using the restriction enzymes StuI and SbfI and inserted into the pCM184 plasmid (see table 6.1 and Garí et al., 1997). The correct insertion was tested by restriction with the enzyme AlwNI and sequencing using the primer 184-Myc (table 6.3).

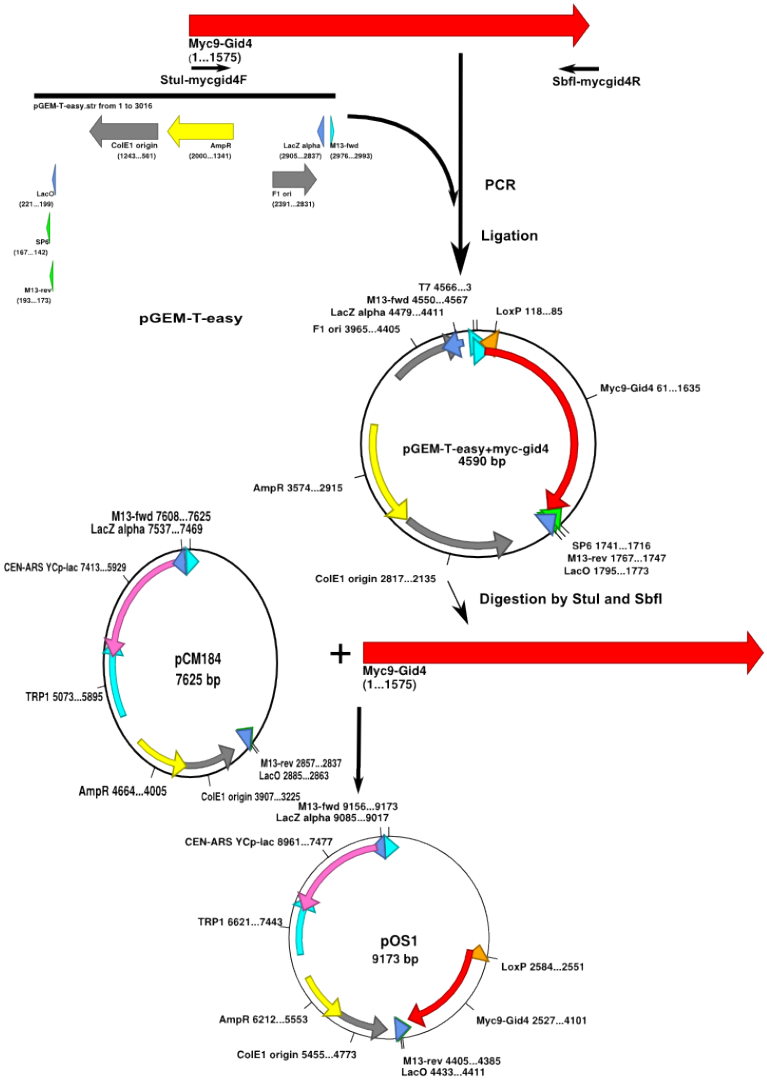

Figure 12.14: **Cloning of *MYC*$_9$*-GID4* under the Tet^R promoter.** First, *MYC*$_9$*-GID4* open reading frame from the chromosomally tagged strain YOS1 (12.2) was amplified by PCR using the oligonucleotides StuI-mycgid4F and SbfI-mycgid4R (table 6.3). The PCR product was then subcloned in the pGEM-T-easy plasmid. Once correct integration of the insert was verified, the *MYC*$_9$*-GID4* DNA fragment was excised from the plasmid using the restriction enzymes StuI and SbfI and insert in pCM184 (table 6.1) which eventually yielded a plasmid bearing the *MYC*$_9$*-GID4* ORF under the control of the Tet^R promoter (pOS1 plasmid).

A constitutive expression of Myc_9-Gid4p that would trigger FBPase degradation already in ethanol-grown cells might result in an impaired growth of those cells in ethanol-containing media. Thus, *gid4*Δ strains were transformed with plasmid pOS1 whose construction is described above (see also table 6.1). Strains bearing either a chromosomally expressed *MYC*$_9$-*GID4*, a *FBP1* deletion, a plasmid-expressed *MYC*$_9$-*GID4* or a vector control were plated on petri dishes with CM-Trp or CM+6C (containing all auxotrophic marker) with either 2 % glucose, 2 % ethanol or 2 % ethanol added with doxycycline (40 µg / ml). Cells were grown four days at 30°C, and a picture of the petri dishes was taken.

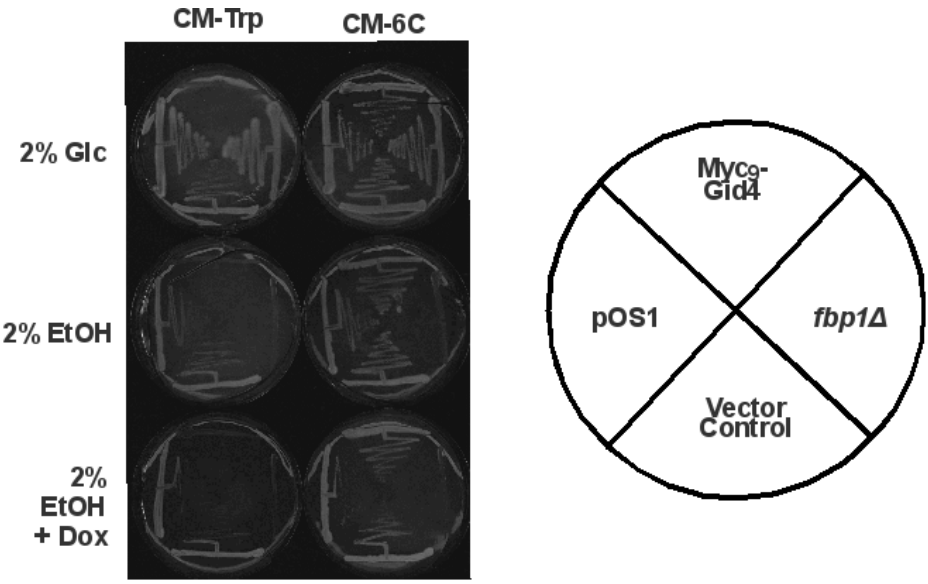

Figure 12.15: **Growth of gluconeogenic cells expressing *GID4*.** A *GID4* deletion strain was transformed with either pOS1 or pCM184 (vector control) and selected transformants were plated on CM-Trp (lacking Trp) or CM-6C (containing all the supplements) with 2 % glucose (2 % Glc) or 2 % ethanol (2 % EtOH) treated or not with doxycycline (40 µg / ml). As controls, YOS1 and *fbp1*Δ strains were also plated. After four days at 30°C, growth was monitored.

Surprisingly, cells constituvely expressing *GID4* were able to grow on ethanol as cells bearing the corresponding vector only (fig. 12.15). One may assume that in those cells, FBPase might not be degraded or, although degradation does occur, a sufficient amount of *FBP1* transcription still takes place. A lack of *MYC*$_9$-*GID4* expression can be excluded as figures 12.9 and 12.16 show that the P*tet* promoter leads to an expression of the recombinant protein.

Figure 12.16: **Expression control of *MYC*$_9$-*GID4* under the tet^R promoter.** Cells were inoculated in CM-Trp-Glc and grown overnight at 30°C. They were thereafter transferred to fresh CM-Trp-Glc and let grown without doxycycline during six hours at 30°C. Subsequently, cells were transferred to either CM-Trp-Glc with or without 40 µg / ml of doxycycline or CM-Trp-EtOH with or without 40 µg / ml doxycycline and grown overnight at 30°C (8.1). Four OD_{600} of cells were taken and samples were processed as described (10.1.1). After 10 % SDS-PAGE and subsequent transfer onto nitrocellulose membrane, FBPase and Myc$_9$-Gid4p were visualised with specific antibodies.

Thus, the fact that no growth defect is observed in the pOS1 transformants cannot be due to a lack of *MYC*$_9$-*GID4* expression. As seen in figure 12.16, FBPase signals on YPEtOH seem to be equivalent, whether the cells are treated with 40 µg / ml doxycycline or not. On the other hand, on YPD, inhibition of *MYC*$_9$-*GID4* expression by doxycycline results in a higher FBPase signal compared to the one observed when the recombinant *GID4* is expressed, confirming that the plasmid expressed Myc$_9$-Gid4p is functional. Overall, although confirming the efficient expression of the recombinant Gid4p, the results shown in figures 12.9 and 12.16 do not allow to discriminate between an inefficient FBPase degradation on YPEtOH or an ongoing synthesis of FBPase under these conditions.

To assess whether ectopic expression of *GID4* triggers a degradation of FBPase already in gluconeogenic cells, the FBPase present in the cell has to be marked to distinguish it from the newly synthesised enzyme. For this purpose a pulse-chase analysis was carried out. Cells were treated with methionine containing a ^{35}S isotope (pulse medium, 10.6) to radioactively label the proteins. After two hours of labelling, the pulse medium was removed and replaced by the chase medium containing non-radioactive methionine (10.6). This allows the monitoring of radioactive proteins solely: the newly synthesised, non-radioactive proteins cannot be detected by autoradiography. An immunoprecipitation ensures that the signal observed is specific (10.6). In cells bearing a *GID4* deletion transformed with pOS1 or the corresponding vector control (6.1), a pulse-chase analysis was performed as described above (10.6). Expression of less than physiological amounts of Gid4p (compare YOS1, "G" and pOS1, "E", figure 12.17B) led to a

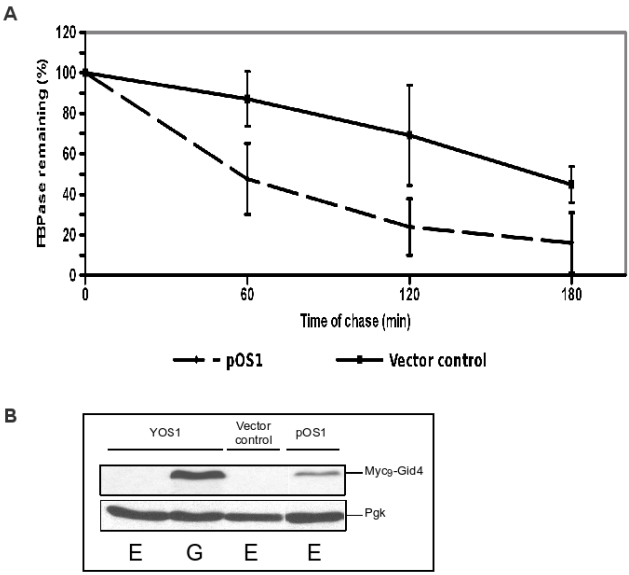

Figure 12.17: **Expression of *GID4* in gluconeogenic cells triggers FBPase degradation.** *MYC_9-GID4* was cloned under a Tet^R promoter and expressed in cells growing on YPEthanol. (A): Pulse-chase analysis of FBPase in cells bearing either the Myc_9-Gid4p expressing plasmid ("pOS1") or the respective vector control was carried out (means of three independent experiments, ± confidence interval, α=0.05). (B): Immunoblot showing the steady state levels of Myc9–Gid4p in glucose inactivated cells (30 min, YOS1, G) and in ethanol grown cells (pOS1, E).

decrease in FBPase half-life by roughly three fold. Thus Gid4p expression, in the presence of all other Gid proteins, is necessary and sufficient to trigger FBPase degradation, even when the cells are growing on an ethanol-containing media.

12.4.3 Gid1p, Gid4p and Gid7p are required for FBPase polyubiquitination.

Pfirrmann (2006) demonstrated that Gid1p and Gid7p are necessary for FBPase polyubiquitination. Gid2p as well was shown to be an essential component of FBPase poly-ubiquitination (11.3 and Regelmann et al., 2003; Regelmann, 2005). The peculiar characteristics of Gid4p, particularly the fact that it is sufficient to trigger FBPase degradation when expressed in ethanol-growing cells (12.4.2) led to test whether Gid4p is also necessary for FBPase polyu-

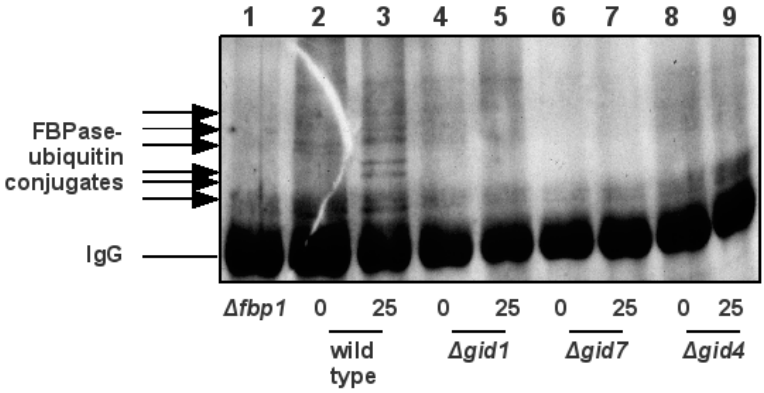

Figure 12.18: **Gid1p, Gid4p and Gid7p are necessary for FBPase polyubiquitination.** Wild type, *gid1*Δ, *gid7*Δ and *gid4*Δ strains were grown 16 h in YPEthanol at 30°C and shifted to YPD. Samples were taken at indicated time points and FBPase was immunoprecipitated. Polyubiquitination was detected using monoclonal ubiquitin antibody. Δ*fbp1*: FBPase deletion. Presence of FBPase in the immunoprecipitates was controlled by immunoblotting with FBPase antibody (data not shown).

biquitination. Deletion mutants of *GID1*, *GID4* and *GID7* and their wild type counterpart were inoculated, grown overnight in YPEtOH. 50 OD_{600} of cells were harvested at time points 0 or 25 minutes after glucose shift. Proteins were extracted and FBPase was immunoprecipitated as described (10.5). After Western-blotting, the nitrocellulose membrane was autoclaved, blocked and probed with monoclonal ubiquitin antibody to visualise any polyubiquitination of FBPase. Figure 12.18 shows the result of the ubiquitination experiment described above. A strain deleted in the *FBP1* gene serves as a negative control. No FBPase from this strain can be immunoprecipitated and therefore, no ubiquitination is visible (lane 1). In a W303 wild type strain (WT), no FBPase ubiquitination can be seen in cells growing on YPEtOH (lane 2), while 25 minutes after the shift of cells to glucose, polyubiquitination of FBPase is readily visible (lane 3). No polyubiquitination of FBPase can be seen when *GID1*, *GID7* and *GID4* are deleted (lanes 4 to 9). This confirms the results previously observed for mutants deleted in *GID1* and *GID7* genes (Pfirrmann, 2006) and shows that Gid4p is necessary for FBPase polyubiquitination as well. Thus, not only the RING bearing subunit (Gid2p) of the Gid complex is required for the polyubiquitination of FBPase (11.3), but other Gid proteins as well. As the FBPase signal is masked by the IgG signal of the immunoprecipitates (fig. 12.18), the ubiquitination experiment was repeated using a FBPase-TAP encoding plasmid to confirm the

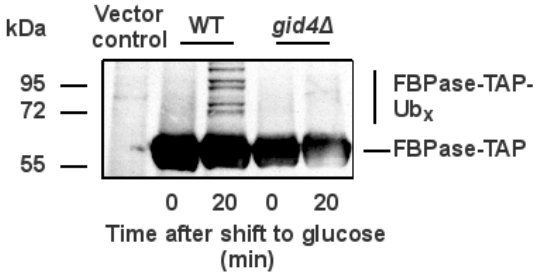

Figure 12.19: **Gid4p is necessary for FBPase-TAP polyubiquitination.** A plasmid expressing a FBPase-TAP fusion protein was transformed into W303 (WT) and *gid4* Δ strains. Samples were taken at indicated time points and FBPase was pulled down using IgG-Sepharose. Polyubiquitination of FBPase was assessed using monoclonal ubiquitin antibody. The vector control consists of a plasmid-expressed FBPase without any tag. It allows to control for the specificity of the pull down.

involvement of Gid4p in FBPase polyubiquitination. Tandem affinity purification represents an easy method to purify proteins using a two-step affinity chromatography (Puig et al., 2001). The TAP-tag bears a protein A domain which strongly binds to IgGs. This property was used to perform an IgG-Sepharose pull-down of a C-terminally TAP-tagged FBPase and which allowed a better detection of the protein of interest. Figure 12.19 shows that a FBPase-TAP fusion protein is indeed polyubiquitinated when Gid4p is present, while it is not when the Gid4 protein is not expressed in cells. This confirms the results observed in figure 12.18 on the requirement of Gid4p for polyubiquitination of FBPase. Although a TAP-tagged FBPase was not used in *GID1* and *GID7* deletions, the fact that the results regarding the need of Gid4p for polyubiquitination of FBPase are consistent with either experimental design allows to infer that it is also the case for Gid1p and Gid7p.

12.4.4 Gid4p does not influence the sedimentation profiles of Gid1p-HA$_3$ and Gid7-HA$_3$ in a glycerol step gradient.

All the Gid proteins, except Gid4p, are readily detectable in gluconeogenic cells. Moreover, an ectopic expression of Gid4p in such cells leads to a destabilisation of FBPase (12.4.2). This suggests that some Gid complex, except for Gid4p, might already be present in gluconeogenic cells, Gid4p being an activator of this complex. Another possibility is that Gid4p is required for the formation of a fully functional Gid complex from the single Gid proteins. To discriminate

between these two possibilities, a glycerol step gradient was carried out. It had already been shown that Gid2p sediments at a molecular mass of about 600 kDa, although none of the Gid subunits have such a high molecular mass (Regelmann et al., 2003; Regelmann, 2005; Pfirrmann, 2006). Thus, glycerol step gradients (10.4) performed with extracts of gluconeogenic cells may provide information as to whether some Gid subunits are present in a complex or are present only as monomers in ethanol-grown cells. As shown in figure 12.20A and B, HA_3-tagged versions of Gid1p and Gid7p distribute equally along the gradient, either in extracts of gluconeogenic (T=0 min, Gid4p absent) or in extracts of glycolytic cells (T=20 min), when Gid4p is present. Interestingly, in a *GID4* deletion, Gid7-HA_3 shows the same distribution pattern as in its non deleted counterpart (compare fig. 12.20B and C). Although it cannot be excluded that the binding partners of Gid1p or Gid7p differ between gluconeogenic and glycolytic conditions, both proteins remain in a complex even when Gid4p is absent, suggesting that this Gid protein has no influence on the assembly of monomeric Gid proteins to a higher molecular mass complex.

12.5 Gid4p degradation.

As figure 12.6 shows, Gid4p appears at last five minutes after the cells are shifted from ethanol- to glucose-containing media. The steady state levels of Gid4p raise till 30 minutes after glucose treatment of cells. thereafter, Gid4p steady state levels decrease again, in parallel with the disappearance of FBPase. This is a further difference between Gid4p and the other Gid proteins as the latter (except Gid3p) remain stable for at least 120 minutes in cells shifted to glucose. Here, the degradation requirements of Gid4p will be studied.

12.5.1 Construction of gene deletions in YOS1.

As Gid4p degradation seems to parallel FBPase degradation, it was of interest to assess whether Gid4p might require the same apparatus as FBPase to be degraded. Josupeit (2003) had tested the influence of the Gid proteins on Gid4p degradation after shift of cells to glucose with an antibody directed against the native protein. As this antibody showed too many cross-reactions in the laboratory and, in addition, is not stable (Josupeit, 2003; Pfirrmann, 2006). Therefore, to be able to visualise the Gid4 protein, the strain YOS1, bearing a Myc_9-tagged Gid4p was used, in which other *GID* genes were deleted. Genes of particular interest are *GID3*, which

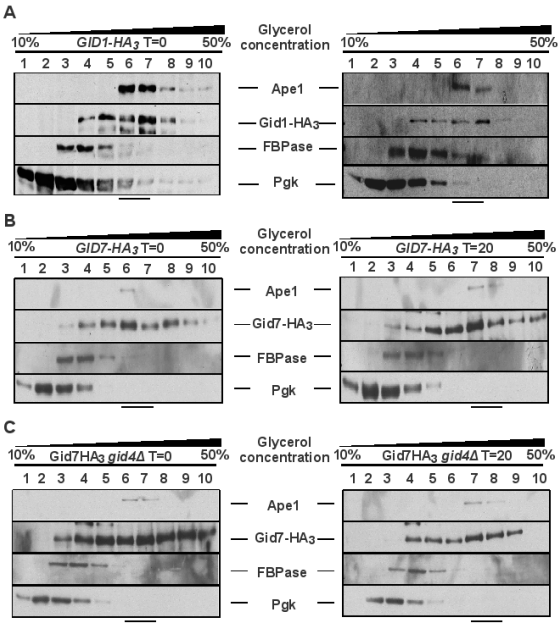

Figure 12.20: **Distribution of Gid1p-HA$_3$ and Gid7-HA$_3$ in a glycerol step density gradient.** Strains expressing Gid1-HA$_3$ or Gid7-HA$_3$ in either a wild type background or a *GID4* deletion background were grown in YPEthanol. Proteins were separated by centrifugation in a glycerol step gradient before (T=0) or 20 minutes after shift of the strains to YPD (T=20). Ten fractions were collected and proteins were visualised by immunoblotting (Pgk, 3- phosphoglycerate kinase (41 kDa); Ape1 (600 kDa), aminopeptidase I). A: distribution of Gid1-HA$_3$ in a wild type background; B: distribution of Gid7-HA$_3$ in a wild type background; C: distribution of Gid7-HA$_3$ in a *gid4*Δ background. Proteins were detected using their specific antibodies.

encodes the ubiquitin conjugating enzyme Ubc8p and *GID2*, which encodes the Gid complex subunit bearing a degenerated RING-finger domain. Therefore, these two genes were deleted in the aforementioned strain to test whether they are required for Gid4p degradation. The proteasome function can selectively be inhibited by compounds like MG132 (Lee and Goldberg, 1996). However, MG132 is hydrophobic and therefore can be readily expelled from the yeast cell by the ABC transporter Pdr5p (Balzi et al., 1994; Bissinger and Kuchler, 1994). Thus, in order to assess the role of the proteasome in Gid4p degradation, it was also necessary to delete the *PDR5* gene in the strain expressing a Myc$_9$-tagged version of Gid4p. Gene deletions were carried out according to Güldener et al. (1996). The KanMX4 cassette from pUG6 was amplified by PCR using oligonucleotides bearing 5' and 3' homology regions (table 6.3) to the

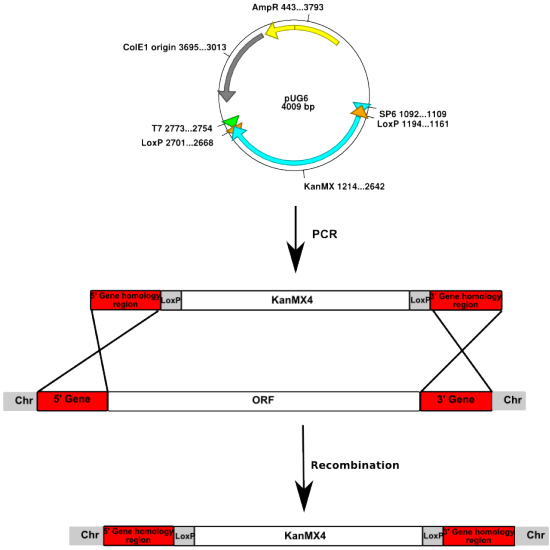

Figure 12.21: **Deletion of *GID2*, *UBC8* and *PDR5* genes.** The KanMX4 cassette was amplified by oligonucleotides bearing homology region to *GID2*, *UBC8* or *PDR5* open reading frames, respectively (oligonucleotides used are summarised in table 6.3). The homology region was 50 bp for each primer. After amplification, the cassette was transformed in yeast strain YOS1 (9.7.1). Clones able to grow on 300 µg / ml geneticin integrated successfully the cassette into their genome. Correct integration was verified by PCR (data not shown). Chr: chromosomal DNA; ORF: Open Reading Frame.

gene to be deleted. This permits a recombination event to occur, whereby the gene that has to be removed is replaced by the KanMX4 cassette. This cassette, in turns, is necessary to screen for the deletion as it provides the cells which performed a recombination event with resistance to geneticin. The YOS1 strain was then transformed with the so-called deletion cassette (9.7.1). Success of the recombination was tested by the ability of the clones to grow on a media containing geneticin (300 µg / ml). The correct replacement of the gene to be deleted by the KanMX4 cassette was assessed by PCR using gene-specific control oligonucleotides (table

6.3, data not shown). Figure 12.21 shows the principle of the gene deletion carried out in this work. Using specific oligonucleotides, *GID2*, *UBC8* and *PDR5* open reading frames were replaced by the KanMX4 cassette.

12.5.2 Gid2 is necessary for Gid4p degradation.

Gid2p was shown to be involved in polyubiquitination of FBPase *in vivo* (11.3).

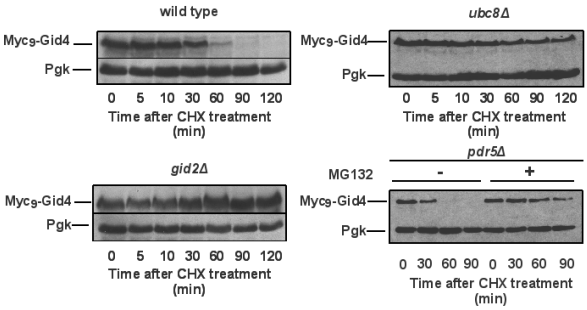

Figure 12.22: **Gid4p degradation depends on Gid2p, Ubc8p and the proteasome.** W303-1B expressing Myc_9-Gid4p was deleted for *UBC8*, *GID2*, or *PDR5* (*ubc8*Δ, *gid2*Δ , *pdr5*Δ respectively). Strains were grown 16 hours on YPEthanol, shifted to YPD, and expression of Gid4p was allowed to proceed during 30 min. Thereafter cells were treated with 100 µg / ml cycloheximide and samples were harvested at the indicated time points. Proteins were visualised via immunoblotting with anti-Myc or anti-Pgk monoclonal antibodies. Proteasome involvement in Gid4 degradation was analysed in a *pdr5*Δ strain using the proteasome inhibitor MG-132 (60 µM final concentration).

Furthermore, we recently demonstrated that the Gid complex is an ubiquitin ligase involved in FBPase polyubiquitination and degradation (Santt et al., 2008). The SCF complex is also a ubiquitin ligase involved in many processes within the cell. Its specificity is brought about by F-box proteins (Kipreos and Pagano, 2000; Willems et al., 2004), which bind both to the ubiquitin ligase complex and the substrate protein. Interestingly, some F-box proteins, like Cdc4p, Grr1p and Met30p are also substrates of the complex they belong to: they become polyubiquitinated prior to their degradation by the proteasome (Kipreos and Pagano, 2000). It was therefore of interest to test whether the Gid complex itself would be necessary for the degradation of Gid4p. As Gid2p as been demonstrated to bear a functional degenerated RING domain, its involvement in the degradation of Gid4p was tested. To that end, the gene encoding the Gid2 protein has been deleted in the strain YOS1 according to the method described above

(12.5.1). After the YOS1-*gid2*Δ deletion mutants have been controlled by PCR for the correct integration of the deletion cassette (data not shown), the strains were further used to test the requirement of Gid2p for Gid4p degradation. After overnight growth in ethanol-containing media (YPEtOH), cells were shifted to glucose during 30 minutes to allow Gid4p expression to proceed, thereafter, a cycloheximide chase experiment was performed to block Gid4p *de novo* synthesis and monitor the levels of the pre-existing Gid4p protein in the cell extracts. Samples were then harvested and treated as described (10.1.1) and proteins were separated by SDS-PAGE (10.2). Myc_9-Gid4p and Pgk (loading control) were uncovered by immunoblotting using specific antibodies. Figure 12.22 shows that a *GID2* deletion mutant fails to degrade Gid4p. Thus, Gid2p is necessary for Gid4p degradation to proceed normally, which confirms the result observed previously (Josupeit, 2003).

12.5.3 Ubc8 is necessary for Gid4p degradation.

Among several ubiquitin conjugating enzymes involved in FBPase degradation, Ubc8p is the most important (Schüle et al., 2000). As the Gid complex itself is involved in the degradation of FBPase (12.5.2), it was of interest to test whether the same E2 involved in the degradation of FBPase is also involved in Gid4p breakdown. Therefore, the strain YOS1 has been deleted for *UBC8* according to the method described above (12.5.1). After the YOS1-*ubc8*Δ deletion mutants had been controlled for the correct integration of the deletion cassette by PCR (data not shown), the strains were further used to test the need for Ubc8p concerning the Gid4p degradation. After overnight growth in ethanol-containing media (YPEtOH), cells were shifted to glucose during 30 minutes to allow Gid4p expression to proceed, thereafter, cells were treated with cycloheximide to block Gid4p *de novo* synthesis and monitor the levels of the pre-existing Gid4p protein in the cell extracts. Samples were harvested and treated as described (10.1.1) and proteins were separated by SDS-PAGE (10.2). Myc_9-Gid4p and Pgk (loading control) were visualised by immunoblotting using specific antibodies. Figure 12.22 shows that a *UBC8* deletion mutant fails to degrade Gid4p. This result is a further confirmation of the result observed by Josupeit (2003). Together with the previously observed results (Josupeit, 2003; Regelmann, 2005), this strongly suggests that several Gid proteins are implicated in the degradation of Gid4p in glycolytic cells.

12.5.4 The proteasome is necessary for the degradation of Gid4p.

Gid4p is supposed to be a membrane-bound, cytosolic protein (Chiang and Chiang, 1998). On YPD, as shown in figure 12.6, Gid4p is a relatively short-lived protein, which makes it a good candidate for proteasomal degradation in yeast (Wolf, 2000). To assess the need for proteasomal activity in a given process, one can either use several proteasomal thermosensitive mutants that impair the 20 S or the 19 S functions of the enzyme or use proteasome inhibitors such as MG-132 or lactacystin. MG-132 is a potent inhibitor of the 26S proteasome which is often used to block proteasome activity in mammalian cell lines (Wolf, 2000). In yeast, use of proteasome inhibitors is complicated by the fact that none of the compound is maintained in the cell. This constraint can be circumvented by the use of strains with increased membrane permeability, *i.e.* strains that are either defective in ergosterol biosynthesis (*erg6*Δ strains) or strains where the inhibitor efflux is impaired (*pdr5*Δ strains). The efflux activity of Pdr5p is involved in multidrug resistance of a broad range of organic compounds (Balzi et al., 1994; Bissinger and Kuchler, 1994). Its deletion ensures an efficient uptake of MG-132 into yeast cells. Consequently, a *PDR5* deletion was carried out in the strain YOS1 according to the method described above (12.5.1) to assess the requirement for the proteasome in Gid4p degradation. The YOS1-*pdr5*Δ deletion mutants were tested by PCR for correct integration of the deletion cassette (data not shown). After overnight growth in ethanol-containing media (YPEtOH), cells were shifted to glucose and pretreated with 60 µM of the proteasome inhibitor MG-132 for 30 minutes to allow Gid4p expression to proceed. Thereafter, cells were treated with cycloheximide to block Gid4p *de novo* synthesis and monitor the levels of the pre-existing Gid4p protein in the cell extracts. MG-132 inhibition is reversible, thus the inhibitor treatment was repeated every 30 minutes until the final time point of the test to ensure a proper proteasome inhibition over the test period. Cell samples were then harvested and treated as described (10.1.1) and proteins were separated by SDS-PAGE (10.2). Myc_9-Gid4p and Pgk (loading control) were visualised by immunoblotting using specific antibodies. Figure 12.22 shows that a *PDR5* deletion mutant treated with MG-132 fails to degrade Gid4p (*"pdr5*Δ" panel). This result suggests that Gid4p degradation occurring after 30 minutes of shift of cells to glucose containing medium is proteasome-dependent.

Part IV

Discussion.

FBPase degradation upon shift of *Saccharomyces cerevisiae* cells from a medium containing a nonfermentable carbon source to a medium with a fermentable carbon source has been documented since the middle of the seventies of the last century (Molano and Gancedo, 1974; Holzer, 1976). However, the precise mechanism by which this degradation occurs has remained elusive for a long time. Two main processes have been identified as potential candidates for the degradation of FBPase: a vacuolar and a proteasomal degradation pathway (Chiang and Schekman, 1991; Chiang et al., 1996; Chiang and Chiang, 1998; Schüle et al., 2000; Schork et al., 1994b, 1995; Hämmerle et al., 1998). In our laboratory, no vacuolar degradation could be observed under the conditions used by the Chiang group (Josupeit, 2003). Hence, work has been carried out to better characterise the proteasomal degradation of FBPase. FBPase appears to be polyubiquitinated upon shift of the cells to glucose. Moreover, mutants in either the 19 S cap or active site subunits of the 20 S core particle have been shown to stall FBPase degradation at the restrictive temperature (Schork et al., 1994b, 1995; Hämmerle et al., 1998; Schüle et al., 2000). A further step towards the characterisation of the proteolytic process was the identification of nine so-called *GID* genes (GID stands for glucose induced degradation deficient) which are all necessary for FBPase degradation. Among these genes, one had previously been identified as coding for the ubiquitin conjugating enzyme Ubc8p (Gid3p) (Schüle et al., 2000; Josupeit, 2003; Regelmann et al., 2003; Regelmann, 2005), while *GID6* had been found to encode the deubiquitinating enzyme Ubp14p (Regelmann et al., 2003; Regelmann, 2005). Most interestingly, several *GID* genes had also previously been found to be identical to *VID* genes, which had been implicated in the vacuolar dependent degradation pathway of FBPase (Chiang and Chiang, 1998; Brown et al., 2002; Regelmann et al., 2003; Regelmann, 2005): *GID1*, *GID4* and *GID5* had already been described as *VID30*, *VID24* and *VID28*, respectively. A MALDI-TOF analysis demonstrated that all Gid proteins (with the exception of Gid3p and Gid6p) belong to the same complex (Ho et al., 2002). Moreover, glycerol step gradient experiments had suggested that Gid2p is part of a complex of 600 kDa, termed the Gid complex (Regelmann et al., 2003). The existence of such a complex was later confirmed by TAP purification and algorithm-based analysis of several datasets (Krogan et al., 2006; Pitre et al., 2006). However, the precise role of the Gid complex within the frame of the proteasomal degradation process of FBPase still remained obscure.

Three subunits of the Gid complex had been tested and shown to be necessary for FBPase polyubiquitination: Gid2p, Gid1p and Gid7p (Regelmann et al., 2003; Pfirrmann, 2006). Moreover Gid3p/Ubc8p, whose ubiquitin conjugating activity was not linked to any other known ubiquitin-dependent pathway in yeast, was shown to be the most important ubiquitin conjugating enzyme in FBPase degradation (Schüle et al., 2000). As no ubiquitin ligases had been found to be involved in the polyubiquitination event of FBPase(Josupeit, 2003), it was tempting to assume that the Gid complex might be the long sought ubiquitin ligase. However, some Gid proteins had been suggested to be located within the yeast nucleus (Pfirrmann, 2006). Thus, E3s located in the yeast nucleus had to be tested for their participation in the degradation of FBPase. The already known E3s were screened for their localisation using two databanks and the data were compared with the results obtained by Josupeit (2003). No E3 located within the nucleus could be found to have any influence on FBPase degradation (see Addendum, 13.1, for a localisation of these E3s and their effect on FBPase). However, a newly found nuclear ubiquitin ligase, San1p, had been implicated in the quality control of nuclear proteins (Dasgupta et al., 2004). A deletion mutant of *SAN1* did not result in any stabilisation of FBPase upon shift of cells from an ethanol- to a glucose-containing medium, by this excluding it as a player in FBPase degradation (Fig. 11.1). Other subunits of ubiquitin ligases complexes were tested with the same negative outcome (Fig. 11.2). Other proteins often implicated in the polyubiquitination and subsequent degradation of proteins are proteins bearing a UBL (ubiquitin like)and / or a UBA (ubiquitin affinity) domain. Among them, Dsk2p and Rad23p were shown to be involved in the ERAD process, where they are necessary for processing malfolded proteins like CPY* (Medicherla et al., 2004). Their single deletions did not show any effect on FBPase degradation (Josupeit, 2003). However, the double deletion mutants (*dsk2*Δ*rad23*Δ) failed to grow on YPEthanol in an overnight culture. After six hours on ethanol, no FBPase could be detected in the double mutant, suggesting that FBPase is not synthesised in this strain (Fig. 11.3). Indeed, when glucose-grown cells were shifted to ethanol-containing medium, no FBPase signal appeared in the double mutant, while this signal is readily detectable after 30 minutes of growth on ethanol in the corresponding wild type (Fig. 11.4). Josupeit (2003) demonstrated that neither of the single deletion mutants shows an impairment of the expression of FBPase. Together with the results presented here, this suggests that Rad23p and Dsk2p are redundant

for the expression of the *FBP1* gene. The ubiquitin-proteasome system has been implicated in gene expression regulation by several means like chromatin remodelling or activation of transcription factors (Conaway et al., 2002). This result is a further hint of the association of the ubiquitin proteasome system and transcription, which is likewise supported by the known involvement of the *RAD23* gene product in transcription-coupled DNA repair (Mueller and Smerdon, 1996). However, these results do not allow to exclude any role for the Dsk2p-Rad23p doublet in FBPase degradation: independently of their role in FBPase expression, these two proteins might be necessary for the breakdown of FBPase. The observations of Josupeit (2003) suggest that, if the latter were true, then Dsk2p and Rad23p would display some redundancy in that pathway. Overall, no known ubiquitin ligase could be identified has being the effector of FBPase polyubiquitination upon shift of the *S. cerevisiae* cells to a glucose containing medium.

As Gid1p/Vid30p, Gid2p/Rmd5p and Gid7p were later reported to be necessary for the polyubiquitination of FBPase (Schüle, 2000; Regelmann et al., 2003; Regelmann, 2005; Pfirrmann, 2006), the Gid complex was inferred to be the long sought ubiquitin ligase. Among the E3 ubiquitin ligases, two types can be distinguished. One is defined as the HECT-type ubiquitin ligases, which transfer a ubiquitin moiety bound to the cysteine of their active site onto the substrate. The other type, the RING ligases – carrying a RING (really interesting new gene) domain – consists of one or more proteins which bind to the substrate and connect it with the ubiquitin conjugating enzyme (4.2). Sequence analysis revealed that Gid2p/Rmd5p bears a strong homology with the RING domain, with five Zn^{2+} coordinating residues conserved out of eight. Moreover all residues forming the second zinc finger (residues three, four, seven and eight) are all conserved (Santt et al., 2008). Albeit no Zn^{2+} coordinating residues are conserved in the U-box family of proteins, these proteins still retain an ability to polyubiquitinate proteins (Ohi et al., 2003). Gid2p/Rmd5p has been demonstrated to be necessary for FBPase polyubiquitination (Regelmann et al., 2003; Regelmann, 2005). Moreover, it is able to polyubiquitinate proteins *in vitro* (Santt et al., 2008). To test whether Gid2p/Rmd5p bears a RING-type ubiquitin ligase activity, the cysteine residue 379 of its RING finger domain was changed into a serine (Pfirrmann, 2006; Santt et al., 2008). The mutated Gid2 fails to polyubiquitinate and degrade FBPase *in vivo* (Fig. 11.5 and Santt et al., 2008). This allows the conclusion that the Gid complex is the ubiquitin ligase (E3) involved in polyubiquitination of FBPase and that its

activity is provided by the Gid2p/Rmd5p subunit.

No antibody is available for any of the Gid proteins. Thus HA_3- (Pfirrmann, 2006) or Myc_9-epitope tagging (this work) of these proteins was necessary to allow their immunological detection. Gid4p/Vid24p proved to be more complicated to tag as a C-terminal HA_3 tag led to its inactivation (Josupeit, 2003). However, an amino-terminal tagging with the Myc_9 epitope preserved the physiological function of Gid4p/Vid24p (12.2). Gid4p/Vid24p is necessary for the degradation of FBPase and PEPCK, another gluconeogenic enzyme involved upstream of FBPase in gluconeogenesis (Fig. 12.2 and Josupeit, 2003). This suggests that Gid4p/Vid24p (and most probably the entire Gid complex) has a general role in the regulatory switch from gluconeogenesis to glycolysis (see below).

Most of the Gid proteins were shown to be already detectable in ethanol-grown cells, with levels remaining stable over two hours after shift of the cells to a glucose-containing media (Pfirrmann, 2006). Gid4p/Vid24p is the exception: it is undetectable in cells growing on a nonfermentable carbon source and becomes readily visible at last five minutes after shift of the cells to glucose (Fig. 12.6). Notably, the levels of the protein decrease with time after the glucose shift. However, Gid4p levels still remain detectable, as Gid4p/Vid24p is noticeable after overnight growth on a glucose-containing medium (Fig. 12.6). These characteristics are suggestive of a regulatory role for Gid4p/Vid24p in the frame of the Gid complex. This is further underlined by the fact that Gid1p/Vid30p and Gid7p distributions along a glycerol step gradient do not change significantly, regardless of the presence of Gid4p/Vid24p in the cells (Fig. 12.20). Although one cannot exclude that the Gid complex subunits studied may interact with other partners on ethanol- and on glucose-containing media, these results suggest that the expressed Gid proteins are already present in a complex in ethanol-grown cells and that they do not dissociate into their respective monomers when Gid4p/Vid24p is absent from cells. Thus Gid4p has no influence on the assembly of monomeric Gid proteins into a higher molecular mass complex. As Gid4p/Vid24p is necessary for fructose-1,6-bisphosphatase degradation (Fig. 12.2) and polyubiquitination (Figs. 12.18, 12.19), it was thought that this protein might carry FBPase to the Gid complex, thereby triggering its polyubiquitination. However, Gid1p/Vid30p interacts with FBPase already in ethanol growing cells (Pfirrmann, 2006; Santt et al., 2008) and, as mentionned above, the interactions in which Gid1p/Vid30p and Gid7p

are engaged are not disrupted when Gid4p/Vid24p is absent from the cells. Together with the fact that cycloheximide prevents Gid4p/Vid24p appearance and FBPase degradation (Fig. 12.13), these results suggest that Gid4p/Vid24p, rather than bridging the FBPase to the Gid complex, might be an activator of the latter. This is indeed the case, as ectopic expression of Gid4p/Vid24p is sufficient to trigger FBPase degradation in ethanol growing cells (Fig. 12.17). How Gid4p/Vid24p exerts its effect on the Gid complex is not clear. A possibility is that it might alter the conformation of the complex and thereby activates it, for example by bringing the substrate binding and the E2 binding subunits in close vicinity. As an alternative, the transient high number of Gid4p/Vid24p proteins within the cell might help to displace a yet unknown inhibitor from the Gid complex. Gid4p might as well trigger the degradation of this putative inhibitor. Apart from *YDL176w*, no other proteins were shown by Krogan et al. (2006) and Pitre et al. (2006) to interact with the Gid complex. However, the experiments carried out by these authors were performed in glucose-grown cells only. Thus, an inhibitor of the Gid complex, most likely present only when cells are grown on a nonfermentable carbon source might have escaped detection in these studies. Therefore, a purification of the Gid complex from gluconeogenic cells is necessary to determine whether such an inhibitor exists and is finally removed from the complex by Gid4p.

After shift of yeasts cells from an ethanol-containing to a glucose-containing medium, Gid4p/Vid24p appears in as little as five minutes (Fig. 12.6). The average of translation rate in eukaryotic cells is about 20 nucleotides per second, whereas the average of polypeptide elongation is of about two amino acids per one second (Alberts et al., 2002). The *GID4* ORF consists of 1089 base pairs and together with the Myc_9 tag it amount to 1575 base pairs. Therefore this ORF is transcribed in about than 1 min 20 s. Its translation takes roughly four minutes and twenty seconds. Moreover, in eukaryotes cells, transcription occurs in the nucleus whereas translation takes place within the cytosol. Thus, the messenger RNA has to be exported to the cytosol in order to be processed and translated into protein. This export is carried out with an average time of 20 minutes (Lewin, 1997). Thus, the fast emergence of Gid4p after shifting cells to glucose is incompatible with an expression pattern of less than five minutes. Inhibition of yeast RNA polymerase II or the use of a thermosensitive mutant of the enzyme support this observation, as this inhibition neither prevent Gid4p to become detectable nor

does it preclude FBPase degradation (Figs. 12.7 and 12.8). This suggests that *GID4* mRNA is present in the yeast cells prior to their shift from a gluconeogenic to a glycolytic metabolism. Indeed, *GID4* mRNA levels were not reported to vary significantly in yeast during the shift from glycolytic to gluconeogenic growth conditions (DeRisi et al., 1997). Interestingly, when *GID4* is placed under a Tet^R promoter and expressed from a plasmid, the steady state levels of the protein vary depending on whether the growth medium allows fermentation or not (Fig. 12.9). The untranslated regions of the mRNAs from the endogenous and the plasmid-encoded *GID4* are most probably different, which could account for the fact that Gid4p is detectable in ethanol-grown cells when it is encoded from a plasmid, whereas it is not seen when it is chromosomally expressed. Moreover, the difference in the steady state levels observed suggests that Gid4p is less stable in ethanol-grown cells as compared to glucose-grown cells (Fig. 12.9). Yeasts seem thus to have two systems to ensure that Gid4p is not expressed under gluconeogenic conditions. First the mRNA seem to be translated only once the shift to a fermentable carbon source occurs. Second, the protein is unstable under the metabolic conditions (gluconeogenesis) where it should not be present. Such a tight control is important to ensure that while the cells are growing on a nonfermentable carbon source, the FBPase is not degraded too rapidly and that gluconeogenesis can proceed normally. The fact that the mRNA of *GID4* might remain untranslated and stable in ethanol-grown cells, on the other hand, ensures a rapid expression of Gid4p and degradation of FBPase once enough glucose is present in the medium to sustain a glycolytic metabolism. Saccharomyces cerevisiae growing "in the wild" face ongoing changes in nutrient availability, particularly regarding glucose. Therefore, this system is likely to allow a rapid switch from one metabolic state to the other. During this work, cells grown overnight in ethanol containing medium were shifted to a 2 % glucose-containing medium to induce FBPase degradation (8.1). Yeasts cells are able to sense the glucose concentration present in their environment (Rolland et al., 2001b, 2002; Santangelo, 2006). The onset of FBPase degradation might consequently occur only when the concentration of glucose is sufficient to sustain a relatively long fermentation period. On the other hand, when glucose concentration is low, the enzymatic activity of FBPase might be inhibited by other means (see chapter 3). This hypothesis remains to be tested by shifting the yeasts cells onto media containing different glucose concentrations and monitor the FBPase degradation in those cells. Likewise, as Gid4p is the

molecular switch triggering FBPase degradation, its expression might depend on the glucose concentrations as well.

Treatment of the cells with rapamycin, which inhibits the Tor kinase complex 1 (TORC1) and thereby mimics autophagy (Abeliovich and Klionsky, 2001), led to a slower degradation of FBPase, albeit with no changes concerning the Gid4p expression profile (Fig. 12.12). The group of H.L. Chiang reported that, depending on growth conditions used prior to the shift to glucose-containing medium, FBPase takes different degradation routes (Hung et al., 2004). In cells starved three days on a medium containing acetate as a carbon source, FBPase degradation occurs through import of the protein into vesicles which then fuse to the vacuole, a process recalling autophagy (Chiang and Schekman, 1991; Chiang et al., 1996; Huang and Chiang, 1997; Chiang and Chiang, 1998). On the other hand, cells grown overnight in YPEthanol degrade FBPase through the ubiquitin-proteasome system once they are shifted onto YPD (Schork et al., 1994b,a, 1995; Hämmerle et al., 1998; Schüle et al., 2000; Regelmann et al., 2003; Santt et al., 2008). Why FBPase is degraded by two different routes is not yet clear. However, one can postulate that these two pathways correspond to two different physiological conditions of the cell. In the observed vacuolar degradation of FBPase, the conditions used by the Chiang group are likely to favour autophagy. Selective autophagy has already been observed for peroxysomes upon shift of yeasts to glucose-containing media (Hutchins et al., 1999) and has been suggested to occur for single proteins as well. Thus, the phenomenon observed by the group of H.L. Chiang might reflect a new kind of selective autophagy, with new gene products involved besides the Atg proteins. In more favourable conditions, where the yeasts grow on a more natural carbon source as is ethanol, the FBPase is degraded through the ubiquitin proteasome pathway, the Gid complex being the E3 involved in this process. Although an upregulation of Gid4p/Vid24p was already observed in the vacuolar degradation process of FBPase (Chiang and Chiang, 1998), under these conditions, no subsequent disappearance of Gid4p was observed after shift to glucose. On the contrary, in the proteasomal degradation process of FBPase, Gid4p appears in as little as five minutes after shift of the cells from ethanol to glucose, and its levels diminish from 30 minutes upshift. These discrepancies might reflect the different requirements of Gid4p in the two processes. Gid4p/Vid24p behaviour under conditions when proteasomal degradation of FBPase occurs is reminiscent of what is observed with certain subunits of the SCF complex.

Several F-box proteins of this complex undergo polyubiquitination and degradation by the same complex they are part of (Kipreos and Pagano, 2000; Willems et al., 2004). Indeed, Gid4p degradation, like FBPase degradation, is dependent on Ubc8p and Gid2p/Rmd5p (Fig. 12.22). This degradation is also dependent on the proteasome, as treatment with MG132 stalls Gid4p/Vid24p degradation (Fig. 12.22). Thus, the Gid complex undergoes a regulatory loop where it degrades its own activator, Gid4p/Vid24p. This might limit the activity of the Gid complex to a specific subset of substrates, like FBPase, PEPCK (12.1), or cytosolic malate dehydrogenase (Gibson and McAlister-Henn, 2003; Hung et al., 2004) or prepare the cells to a renewed growth on a nonfermentable carbon source.

An interesting point is the overlap of proteins observed between vacuolar and proteasomal degradation of FBPase: three proteins are implicated in both pathways: Gid1p/Vid30p, Gid4p/Vid24p and Gid5p/Vid28p (Pfirrmann, 2006; Santt et al., 2008). The Gid complex has been shown to be the effector of FBPase polyubiquitination (11.3 and Santt et al., 2008), however, the precise roles of several Gid proteins remain unclear. For example, Gid1p/Vid30p is a member of the so-called "core complex" identified by Pitre et al. (2006) and it has also been shown to be phosphorylated (Regelmann, 2005). Although the physiological relevance of this phosphorylation is not clear, Gid1p/Vid30p might be a part or the endpoint of a signal transduction pathway which might be activated in response to changes in the yeasts' environment. Supportive of this hypothesis is the fact that Gid1p/Vid30p, Gid2p/Rmd5p and Gid5p/Vid28p have been implicated in the internalisation and vacuolar degradation of the hexose transporter Hxt7p upon rapamycin treatment (Snowdon et al., 2007). Furthermore, Gid1p/Vid30p is implicated in nitrogen starvation signalling (van der Merwe et al., 2001). Thus, not only the gluconeogenic enzymes like cytosolic malate dehydrogenase (Hung et al., 2004), FBPase (Fig. 12.2) and PEPCK (Fig. 12.2 and Santt et al., 2008) are under the influence of the Gid complex, other metabolic pathways, like the amino acids synthesis pathways, which depend on both the availability of carbon and nitrogen in the medium are likely to be influenced by the Gid complex as well.

In conclusion, the work presented here shed light on the function of the Gid complex in the catabolite degradation of FBPase. Of particular interest is Gid4p/Vid24p, the unique Gid protein which is synthesised *de novo* upon shift from a nonfermentable to a fermentable

carbon source. Although this protein does not seem to have any influence on the stability of the complex, it is required by the Gid complex to be able to polyubiquitinate FBPase. Furthermore, it has also been shown to be involved in the catabolite degradation process of PEPCK, another gluconeogenic enzyme. Blocking Gid4p synthesis inhibits FBPase degradation (Fig. 12.13). On the contrary, forcing the expression of even limited amounts of Gid4p leads to a drop in the half-life of FBPase in ethanol-containing medium (Fig. 12.17). Although one cannot exclude other events in signalling FBPase degradation, this leads to the assumption that Gid4p/Vid24p is the switch triggering this breakdown through the proteasome. Gid4p/Vid24p has also been identified as a component of the vacuolar degradation pathway of FBPase, where it is supposed to promote the fusion of the "Vid vesicles" with the vacuole (Chiang and Chiang, 1998). Indeed, Gid4p/Vid24p has been found by the same group to interact with the coatamer surrounding these "Vid vesicles" (Brown et al., 2008). In both the proteasomal and the vacuolar processes, Gid4p/Vid24p is upregulated, although the half-life of Gid4p/Vid24p in the vacuolar degradation pathway of FBPase seem to extend by far the half-life of Gid4p/Vid24p observed in the proteasomal degradation pathway. In the conditions used here, Gid4p/Vid24p breakdown is dependent on the Gid complex, the E3 linking the polyubiquitination of FBPase to its proteasomal degradation. However, a basal level of Gid4p/Vid24p can be observed in yeast cells in logarithmic growth on glucose. Thus, while Gid4p/Vid24p degradation might limit the activity of the Gid complex, Gid4p/Vid24p might as well play a "gate keeper" role, preventing any gluconeogenic or – why not – respiratory metabolism to take place during logarithmic growth on a fermentable carbon source.

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Part V

Addendum.

Chapter 13

Localisation of known ubiquitin ligases in the yeast *Saccharomyces cerevisiae.*

Table 13.1: **Localisation of putative E3s tested by Josupeit.** Localisation data was retrieved from to two databases, the TRIPLES database and the GFP database from UCSF (see text in 11.1 for references and web sites). Description column shows description of each genes as found in SGD database (http://www.yeastgenome.org/. Deg column shows the effect of the gene deletion on FBPase degradation; +: FBPase is degraded in a Wild Type fashion; -: FBPase is stabilised; n.d.: not determined. GFP and TRIPLES columns show the localisation data retrieved from GFP or TRIPLES databases, respectively. n.d.: not determined; C: cytoplasmic; C(p): cytoplasmic, punctuated; N: nuclear; C/N: nuclear and cytoplasmic; n: nucleolar; ER: endoplasmic reticulum; V-M: vacuolar membrane; E: endosomes; P: peroxysomes; C-P: cell periphery.

Nb	Gene	ORF name	Description	Deg	GFP	TRIPLES
1	*HUL 4*	YJR036c	Protein with similarity to HECT domain E3 ubiquitin-protein ligases, not essential for viability	+	n.d.	n.d.

2	*HUL 5*	YGL141w	Protein with similarity to HECT domain E3 ubiquitin-protein ligases, not essential for viability	+	C/N	n.d.
3	*RSP 5*	YER125w	Ubiquitin-protein ligase involved in ubiquitin-mediated protein degradation; functions in multivesicular body sorting, heat shock response and ubiquitylation of arrested RNAPII; contains a HECT (homologous to E6-AP carboxy terminus) domain	+	n.d.	n.d.
4	*TOM 1*	YDR457w	E3 ubiquitin ligase of the HECT-domain class; has a role in mRNA export from the nucleus and may regulate transcriptional coactivators	+	n	n
5	*UFD 4*	YKL010c	Ubiquitin-protein ligase (E3) that interacts with Rpt4p and Rpt6p, two subunits of the 19S particle of the 26S proteasome; cytoplasmic E3 involved in the degradation of ubiquitin fusion proteins	+	C/N	n.d.

6	*APC 11*	YDL008w	Catalytic core subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C), which is a ubiquitin-protein ligase required for degradation of anaphase inhibitors, including mitotic cyclins, during the metaphase/anaphase transition	n.d.	V-M	n.d.
7	*DER 3*	YOL013c	Ubiquitin-protein ligase required for endoplasmic reticulum-associated degradation (ERAD) of misfolded proteins; genetically linked to the unfolded protein response (UPR); regulated through association with Hrd3p; contains an H2 ring finger	+	ER	n.d.
8	*FAR 1*	YJL157c	Cyclin-dependent kinase inhibitor that mediates cell cycle arrest in response to pheromone; also forms a complex with Cdc24p, Ste4p, and Ste18p that may specify the direction of polarised growth during mating; potential Cdc28p substrate	+	N	n.d.

9	*PEP 3*	YLR148w	Component of CORVET tethering complex; vacuolar peripheral membrane protein that promotes vesicular docking/fusion reactions in conjunction with SNARE proteins, required for vacuolar biogenesis	n.d.	E	n.d.
10	*PEP 5*	YMR231w	Component of CORVET tethering complex; peripheral vacuolar membrane protein required for protein trafficking and vacuole biogenesis; interacts with Pep7p	n.d.	E	n.d.
11	*STE 5*	YDR103w	Pheromone-response scaffold protein; binds kinases Ste11p, Ste7p, and Fus3p to form a MAPK cascade complex that interacts with the plasma membrane, via a PH (pleckstrin homology) and PM/NLS domain, and with Ste4p-Ste18p, during signalling	n.d.	C/N	n.d.

12	*UBR 1*	YGR184c	Ubiquitin-protein ligase (E3) that interacts with Rad6p/Ubc2p to ubiquitinate substrates of the N-end rule pathway; binds to the Rpn2p, Rpt1p, and Rpt6p proteins of the 19S particle of the 26S proteasome	n.d.	n.d.	n.d.
13	*UBR 2*	YLR024c	Cytoplasmic ubiquitin-protein ligase (E3)	+	C	n.d.
14	*VPS 8*	YAL002w	Membrane-associated protein that interacts with Vps21p to facilitate soluble vacuolar protein localisation; component of the CORVET complex; required for localisation and trafficking of the CPY sorting receptor; contains RING finger motif	+	E	n.d.
15		YHL010c		+	n.d.	n.d.
16	*DMA 1*	YHR115c	Protein involved in regulating spindle position and orientation, functionally redundant with Dma2p	+	n.d.	C

17	*TUL 1*	YKL034w	Golgi-localised RING-finger ubiquitin ligase (E3), involved in ubiquitinating and sorting membrane proteins that contain polar transmembrane domains to multivesicular bodies for delivery to the vacuole for quality control purposes	+	n.d.	n.d.
18	*DMA 2*	YNL116w	Protein involved in regulating spindle position and orientation, functionally redundant with Dma1p	+	C	n.d.
19	*ASR 1*	YPR093c	Protein involved in a putative alcohol-responsive signalling pathway; accumulates in the nucleus under alcohol stress; contains a RING/PHD finger domain	+	n.d.	n.d.
20	*SLX 1*	YBR228w	Subunit of a complex, with Slx4p, that hydrolyses 5' branches from duplex DNA in response to stalled or converging replication forks; function overlaps with that of Sgs1p-Top3p	+	n.d.	n.d.

21	*BRE 1*	YDL074c	E3 ubiquitin ligase for Rad6p, required for the ubiquitination of histone H2B, recruitment of Rad6p to promoter chromatin and subsequent methylation of histone H3 (on K4 and K79), contains RING finger domain	+	N	n.d.
22	*PEX 10*	YDR265w	C3HC4-type RING-finger peroxisomal membrane peroxin required for peroxisomal matrix protein import, interacts with Pex12p, links ubiquitin-conjugating Pex4p to import machinery	+	P	n.d.
23	*RAD 5*	YLR032w	Single-stranded DNA-dependent ATPase, involved in postreplication repair; contains RING finger domain	+	C/N	n.d.
24	*RAD 16*	YBR114w	Protein that recognises and binds damaged DNA in an ATP-dependent manner (with Rad7p) during nucleotide excision repair; subunit of Nucleotide Excision Repair Factor 4 (NEF4) and the Elongin-Cullin-Socs (ECS) ligase complex	+	C/N	n.d.

25	*RAD 18*	YCR066w	Protein involved in postreplication repair; binds single-stranded DNA and has single-stranded DNA dependent ATPase activity; forms heterodimer with Rad6p; contains RING-finger motif	+	n.d.	n.d.
26	*RIS1*	YOR191w	Member of the SWI/SNF family of DNA-dependent ATPases, plays a role in antagonising silencing during mating-type switching, contains an N-terminal domain that interacts with Sir4p and a C-terminal SNF2 domain	+	N	n.d.
27	*SLX 8*	YER116c	Subunit of the Slx5p-Slx8p substrate-specific ubiquitin ligase complex; stimulated by prior attachment of SUMO to the substrate	n.d.	C/N	n.d.
28		YKR017c		+	n.d.	n.d.
29	*AIR2*	YDL175c	RING finger protein that interacts with the arginine methyltransferase Hmt1p; may regulate methylation of Npl3p, which modulates Npl3p function in mRNA processing and export; has similarity to Air1p	+	N	n.d.
30		YDR266c		+	C	n.d.

31	*AIR1*	YIL079c	RING finger protein that interacts with the arginine methyltransferase Hmt1p to regulate methylation of Npl3p, which modulates Npl3p function in mRNA processing and export; has similarity to Air2p	+	C/N	n.d.
32		YLR247c	Putative helicase	n.d.	N	n.d.
33	*CWC 24*	YLR323c	Essential protein, component of a complex containing Cef1p	n.d.	N	n.d.
34	*MAG 2*	YLR427w	Cytoplasmic protein of unknown function predicted to encode a DNA-3-methyladenine glycosidase II that catalyses the hydrolysis of alkylated DNA	+	C	n.d.
35	*PSH 1*	YOL054w	Nuclear protein, putative RNA polymerase II elongation factor; isolated as Pob3p/Spt16p-binding protein	+	N	n.d.
36	*ECM 32*	YER176w	DNA dependent ATPase/DNA helicase belonging to the Dna2p- and Nam7p-like family of helicases that is involved in modulating translation termination; interacts with the translation termination factors, localised to polysomes	n.d.	C	n.d.

37	*FAB 1*	YFR019w	1-phosphatidylinositol-3-phosphate 5-kinase; vacuolar membrane kinase that generates phosphatidylinositol (3,5)P2, which is involved in vacuolar sorting and homoeostasis	n.d.	V-M	C(p)
38	*HEX 3*	YDL013w	Subunit of the Slx5p-Slx8p substrate-specific ubiquitin ligase complex; stimulated by prior attachment of SUMO to the substrate	-	n.d.	C
39	*NAM 7*	YMR080c	ATP-dependent RNA helicase of the SFI superfamily, required for nonsense mediated mRNA decay and for efficient translation termination at nonsense codons; involved in telomere maintenance	+	C(p)	C
40	*PEP 7*	YDR323c	Multivalent adaptor protein that facilitates vesicle-mediated vacuolar protein sorting by ensuring high-fidelity vesicle docking and fusion, which are essential for targeting of vesicles to the endosome; required for vacuole inheritance	+	E	n.d.

41	*PIB2*	YGL023c	Protein binding phosphatidylinositol 3-phosphate, involved in telomere-proximal repression of gene expression; similar to Fab1p and Vps2p	+	V-M	n.d.
42		YDR034w-b		n.d.	C-P	n.d.
43	*ITT1*	YML068w	Protein that modulates the efficiency of translation termination, interacts with translation release factors eRF1 (Sup45) and eRF3 (Sup35) in vitro, contains a zinc finger domain characteristic of the TRIAD class of proteins	+	n.d.	n.d.
44	*TFB 3*	YDR460w	Subunit of TFIIH and nucleotide excision repair factor 3 complexes, involved in transcription initiation, required for nucleotide excision repair; RING finger protein similar to mammalian CAK and TFIIH subunit	n.d.	N	n.d.

45	*VPS 27*	YNR006w	Endosomal protein that forms a complex with Hse1p; required for recycling Golgi proteins, forming lumenal membranes and sorting ubiquitinated proteins destined for degradation; has Ubiquitin Interaction Motifs which bind ubiquitin (Ubi4p)	+	E	n.d.
46	*PEX 12*	YMR026c	C3HC4-type RING-finger peroxisomal membrane peroxin required for peroxisome biogenesis and peroxisomal matrix protein import; forms translocation subcomplex with Pex2p and Pex10p	+	P	n.d.
47	*PEX 2*	YJL210w	RING-finger peroxin, peroxisomal membrane protein with a C-terminal zinc-binding RING domain, forms translocation subcomplex with Pex10p and Pex12p which functions in peroxisomal matrix protein import	+	P	n.d.

48	*SSM 4*	YIL030c	Ubiquitin-protein ligase of the ER/nuclear envelope, required for degradation of Alpha2 and other proteins containing a Deg1p degradation signal; ssm4 mutation suppresses mRNA instability caused by an rna14 mutation	+	ER	n.d.
49	*GRR 1*	YJR090c	F-box protein component of the SCF ubiquitin-ligase complex; involved in carbon catabolite repression, glucose-dependent divalent cation transport, high-affinity glucose transport, morphogenesis, and sulfite detoxification	-/+	n.d.	n.d.

Chapter 14

Abbreviations used in this work.

Amp: Ampicillin.

AMP: Adenosine 5'-monophosphate.

ATP: Adenosine 5'-triphosphate.

APS: Ammonium Persulfate.

BSA: Bovine serum albumin.

ddH_2O: distilled water.

cAMP: cyclic adenosine monophosphate.

CIP: Calf intestinal phosphatase.

CM: Synthetic medium.

DMF: Dimethyl formamide.

DMSO: Dimethyl sulfoxide.

DNA: Desoxyribonucleic Acid.

dNTP: desonucleotide triphosphate (N= Adenosine, Guanosine, Cytidine, Thymidine).

ECL: Enhanced chemiluminescence.

E. coli: *Escherichia coli.*

EDTA: Ethylene Diamine Tetraacetic Acid.

ERAD: Endoplamic reticulum associated degradation.

EtOH: Ethanol.

Fas: Fatty acid synthase.

FBPase: Fructose-1,6-bisphosphatase.

Glc: Glucose.

HRPO: Horse radish peroxidase.

IgG: Immunoglobulin G.

IP: immunoprecipitation.

IPTG: Isopropyl-ß-D-thiogalactopyranoside.

LB: Luria Broth medium.

LiOAc: Lithium acetate.

MES: 4-Morpholineethanesulfonic acid.

mRNA: messenger RNA.

NaOAc: Sodium acetate.

NEM: N-ethyl maleimide.

OD_{600}: Absorbance (Optical Density) measured at $\lambda = 600$ nanometres.

ORF: Open reading frame.

PBS: Phosphate buffer saline.

PCR: Polymerase chain reaction.

PEG: Polyethylene glycol.

PIPES: 1,4-piperazinediethanesulfonic acid.

PMSF: Phenylmethylsulphonyl fluoride.

PVDF: Polyvinylidene fluoride.

RING: Really interesting new gene.

RNA: Ribonucleic acid.

rpm: rotations per minute.

RT: room temperature.

S. cerevisiae: Saccharomyces cerevisiae.

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis.

TAE: Tris acetic acid acid EDTA.

TAP: Tandem affinity purification.

TBS: Tris buffer saline.

TBS-T: Tris buffer saline -tween 20.

TCA: trichloracetic acid.

TEMED: N,N,N',N'-Tetramethyl-1-,2-diaminomethane.

Tm: Melting temperature.

UBA: Ubiquitin associated domain.

UBL: Ubiquitin like domain.

UV: ultraviolet.

v/v: volume per volume.

w/v: weight per volume.

X-gal: 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

YPD: Yeast complete medium containing 2 % glucose.

YPEtOH: Yeast complete medium containing 2 % ethanol.

Chapter 15

Curriculum Vitae.

Olivier SANTT

Waldburgstraße 85

D-70563 STUTTGART

GERMANY

Phone:

- home: + 49 711 971 28 40
- work: + 49 711 685 643 82

e-mail: olivier.santt@ibc.uni-stuttgart.de

Personal Details

Date of birth: 11/01/1975

Nationality: French

Languages: French (native), English, German

Education

- 2002-2008: Thesis, Institute of Biochemistry, University of Stuttgart, Stuttgart, Germany
- 2000: Harvard Mediterranean school of Public health and epidemiology. Courses in clinical epidemiology, genetic epidemiology and categorical data analysis

- 1999: Diploma (DEA) in cell physiology and molecular genetics, Auvergne University, Clermont-Ferrand, France
- 1998: Master (maîtrise) in cell biology and physiology, Blaise-Pascal University, Clermont-Ferrand, France.
- 1997: Bachelor in cell biology and physiology, Blaise-Pascal University, Clermont-Ferrand, France.
- 1995: DUT (Technical biology diploma), Applied biology, biological and biochemical analyses, University Institute of Technology, Claude-Bernard University, Lyon, France.
- 1993: Baccalauréat; Biology and Mathematics.

Experience:

- 2000-2002: - Genetic epidemiological study: assessing genetic interaction of GSTM1 null genotype and CYP2D6 poor metabolizers alleles in the onset of sporadic Parkinson´s Disease in a French population.
- - Molecular biology study: assessing radiation-dependent expression of BRCA1 gene at the molecular oncology laboratory, Jean Perrin Anti-Cancer Center, Clermont-Ferrand, France
- 1999: Diploma thesis (9 months): Study of N9-benzylguanine-benzamidine-induced mechanisms of specific cell cytotoxicity against melanoma cell-line. INSERM unit 484, Clermont-Ferrand, France.
- 1998: Internship (3 months): 20S proteasome, purification and characterisation from rat liver. Diabetes Forschungzentrum, Düsseldorf, Germany.
- 1995: Internship (1 month): Setting up dosing-method of platelet selenium using Electrothermic Atomic Absorption Spectrometry. Edouard Herriot University Hospital, Lyon, France

Publications:

- Olivier Santt*, Thorsten Pfirrmann*, Bernhard Braun, Jeannette Juretschle, Philipp Kimmig, Hartmut Scheel, Kay Hofmann, Michael Thumm, Dieter H. Wolf, (2008); The yeast Gid complex, a novel ubiquitin ligase (E3) involved in the regulation of carbohydrate metabolism. Mol Biol. Cell, Aug 2008; 19: 3323 – 3333. DOI: 10.1091/mbc.E08-03-0328. (*: equal contributors).

- Santt O, Baranova H., Albuisson E., Bignon Y-J., Lucotte G., (2004). Interaction between GSTM1-null and CYP2D6-deficient alleles in the pathogenesis of Parkinson´s disease. European Journal of Neurology. 11:247-51.

- Vidal V., D'Incan C., Santt O., Laplace-Mariez V ., Delgado-Viscogliosi P., Baud V., Deval C., Baranova H. , Champagnac S, Mazel C., Hill D., Albuisson E., Ferrara M., Pradeyrol C., Bignon Y-J., (2000). Les biopuces en Auvergne: aspects techniques et bioinformatiques. Bulletin du Cancer volume 87, p. 411.

Recent scientific communications:

- Poster, (title: Regulation of gluconeogenesis in yeast: a ubiquitin-proteasome catalyzed event). "Ubiquitin and Ubiquitin-like modifiers in cellular regulation", EMBO conferences, 22-26 September 2007, Riva del Garda, Italy.

- Talk: Regulation of gluconeogenesis in yeast: A ubiquitin-proteasome catalyzed event. Ubiquitin-Proteasome System Workshop, Berlin Zeuthen, 2007.

- Poster, (title: Regulation of gluconeogenesis in yeast: a ubiquitin-proteasome catalyzed event). XXIIIrd International Conference on Yeast Genetics and Molecular Biology, 1-6 July 2007, Melbourne, Australia.

- Poster, (title: Catabolite degradation of fructose-1,6-bisphosphatase in the yeast Saccharomyces cerevisiae: studies on the function of a multi-unit protein complex containing Gid proteins). Second International Symposium of the SFB 495: "Topology and Dynamics of Signaling Processes", October 4-6, 2004, Stuttgart, Germany (contributing author) .

- Poster, (title: New components of the ubiquitin-proteasome dependent catabolite degradation of fructose 1,6 biphosphatase in the yeast Saccharomyces cerevisiae).1st joint-

meeting of the SFB 604 and 495. Freudenstadt-Lauterbach, 6. - 8. November 2003 (contributing author).

- Poster, (title: Human DNA microarrays for genetic cartography of breast tumors). Lab Chips and microarrays for biotechnical, applications, Zürich, 22-24 January 2001 (contributing author).

Chapter 16

Erklärung.

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

Stuttgart, den 30-04-2009

Chapter 17

Note on published work.

Part of the work presented here has been published in the following article: Olivier Santt*, Thorsten Pfirrmann*, Bernhard Braun, Jeannette Juretschle, Philipp Kimmig, Hartmut Scheel, Kay Hofmann, Michael Thumm, Dieter H. Wolf, (2008); The yeast Gid complex, a novel ubiquitin ligase (E3) involved in the regulation of carbohydrate metabolism. Mol Biol. Cell, Aug 2008; 19: 3323 – 3333. DOI: 10.1091/mbc.E08-03-0328. (*: equal contributors).