The Yeast GID Complex, a Novel Ubiquitin Ligase (E3) Involved in the Regulation of Carbohydrate Metabolism

Olivier Santt,^{*,†} Thorsten Pfirrmann,^{*,†‡} Bernhard Braun,^{*} Jeannette Juretschke,^{*} Philipp Kimmig,^{*} Hartmut Scheel,[§] Kay Hofmann,[§] Michael Thumm,^{*,||} and Dieter H. Wolf^{*}

^{*}Institut für Biochemie, Universität Stuttgart, 70569 Stuttgart, Germany; and [§]Miltenyi Biotec GmbH, 50829 Köln, Germany

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Glucose-dependent regulation of carbon metabolism is a subject of intensive studies. We have previously shown that the switch from gluconeogenesis to glycolysis is associated with ubiquitin-proteasome linked elimination of the key enzyme fructose-1,6-bisphosphatase. Seven glucose induced degradation deficient (Gid)-proteins found previously in a genomic screen were shown to form a complex that binds FBPase. One of the subunits, Gid2/Rmd5, contains a degenerated RING finger domain. In an in vitro assay, heterologous expression of GST-Gid2 leads to polyubiquitination of proteins. In addition, we show that a mutation in the degenerated RING domain of Gid2/Rmd5 abolishes fructose-1,6-bisphosphatase polyubiquitination and elimination in vivo. Six Gid proteins are present in gluconeogenic cells. A seventh protein, Gid4/Vid24, occurs upon glucose addition to gluconeogenic cells and is afterwards eliminated. Forcing abnormal expression of Gid4/Vid24 in gluconeogenic cells leads to fructose-1,6-bisphosphatase degradation. This suggests that Gid4/Vid24 initiates fructose-1,6-bisphosphatase polyubiquitination by the Gid complex and its subsequent elimination by the proteasome. We also show that an additional gluconeogenic enzyme, phosphoenolpyruvate carboxykinase, is subject to Gid complex-dependent degradation. Our study uncovers a new type of ubiquitin ligase complex composed of novel subunits involved in carbohydrate metabolism and identifies Gid4/Vid24 as a major regulator of this E3.

INTRODUCTION

Glucose is the main carbon source of many cells, providing energy and building blocks for a variety of essential cellular components. Glucose also has a pivotal role in cellular regulation. Its consumption via glycolysis and its regeneration via gluconeogenesis are central pathways of carbohydrate metabolism in many organisms. Regulation of both pathways occurs at three steps catalyzed by different reciprocally acting enzymes. In glycolysis, these steps include the phosphorylation of glucose by hexokinase, the phosphorylation of fructose-6-phosphate by phosphofructokinase, and the synthesis of pyruvate and ATP from phosphoenolpyruvate by pyruvate kinase. In gluconeogenesis, these steps are circumvented by pyruvate carboxylase and phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase

(FBPase), and glucose-6-phosphatase. Dysregulation of these antagonistic pathways in humans leads to type 2 diabetes (Wahren and Ekberg, 2007).

In the yeast *Saccharomyces cerevisiae*, major regulation events occur when cells previously grown in a glucose-deprived medium are supplied with this sugar. These events include gene expression and repression, alterations in the stability of certain mRNAs, and posttranslational modification of many gene products (Gancedo, 1998; Vaulont *et al.*, 2000). FBPase is expressed when yeast cells are growing in media without a fermentable carbon source (growth on ethanol [EtOH] or acetate). After glucose addition, catabolite inactivation and degradation of FBPase occur (Gancedo, 1971; Holzer, 1976; Marcus *et al.*, 1988; Hämmerle *et al.*, 1998; Wolf, 2004). Catabolite inactivation encompasses repression of the *FBP1* gene, decrease of the enzyme activity upon phosphorylation, and allosteric inhibition by fructose-2,6-bisphosphate and AMP (Mazon *et al.*, 1982; von Herrath and Holzer, 1988). Subsequently, FBPase undergoes rapid degradation (Holzer, 1989). Similar mechanisms are described for PEPCK, cytosolic malate dehydrogenase, and isocitrate lyase (Holzer, 1976; Hämmerle *et al.*, 1998). Degradation of FBPase blocks gluconeogenesis and prevents an otherwise ongoing futile cycle of ATP hydrolysis (Schork *et al.*, 1994a,b, 1995; Wolf, 2004).

In addition to the previously identified *GID1/VID30*, *GID2/RMD5*, and *GID3* genes, a genome-wide screen for FBPase stabilization upon glucose shift allowed the discovery of six further so-called glucose induced degradation deficient (*GID*) genes termed *GID4* to *GID9* (Regelmann *et al.*, 2003). Among those genes, *GID3* and *GID6* were shown to encode the ubiquitin-conjugating enzyme Ubc8 and the

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[†] These authors contributed equally to this work.

Present addresses: [†] University of Stockholm, Wenner-Gren Institute, Svante Arrheniusväg 16-18, 10691 Stockholm, Sweden; ^{||} Institut für Biochemie und Molekulare Zellbiologie, Universität Göttingen, Heinrich-Dueker-Weg 12, 37073 Göttingen, Germany.

Address correspondence to: Dieter H. Wolf (dieter.wolf@ibc.uni-stuttgart.de).

Abbreviations used: CTLH, C-terminal to LisH motif; FBPase, fructose-1,6-bisphosphatase; GST, Glutathione transferase; LisH, Lissencephaly type-1-like homology motif; ORF, Open reading frame; PEPCK, phospho-enol-pyruvate carboxykinase.

deubiquitinating enzyme Ubp14 (Schüle *et al.*, 2000; Regelmann *et al.*, 2003). Gid1, Gid4, and Gid5 had previously been identified as Vid30, Vid24, and Vid28, respectively. They were implicated in a glucose-induced vesicular targeting of FBPase for vacuolar degradation observed when yeast cells were starved >48 h in media containing acetate as a nonfermentable carbon source (Hoffman and Chiang, 1996; Chiang and Chiang, 1998; Hung *et al.*, 2004). Proteasome-dependent degradation of FBPase was obtained using different conditions: yeast cells were grown overnight in ethanol-containing media before glucose addition (Schüle *et al.*, 2000; Regelmann *et al.*, 2003; Hung *et al.*, 2004). Detailed characterization of Gid2/Rmd5 demonstrated that it is not present as a monomeric protein within the cell. In a glycerol step gradient, it sediments at ~600 kDa, suggesting that it is part of a soluble protein complex called Gid complex (Regelmann *et al.*, 2003). Proteomic interaction studies demonstrated that seven of the Gid proteins belong to one and the same complex (Ho *et al.*, 2002; Krogan *et al.*, 2006; Pitre *et al.*, 2006). A similar complex, constituted from protein homologues to the Gid proteins, has been found in mammals, in which one of its subunits has been shown to be involved in proteasome-dependent degradation of α -catenin (Kobayashi *et al.*, 2007; Suzuki *et al.*, 2008). Except for Gid3/Ubc8, which is not integral to the complex, and Gid2/Rmd5, no function in proteasome-dependent catabolite degradation of FBPase has been described for the newly discovered Gid proteins (Regelmann *et al.*, 2003).

Here, we further characterize the contribution of Gid2/Rmd5 to proteasomal degradation of FBPase, and we show that it confers E3 activity to the Gid complex. In addition, we show that degradation of PEPCCK is dependent on Gid2/Rmd5. Moreover, we uncover Gid4/Vid24 as a key regulator of FBPase degradation by the proteasome.

MATERIALS AND METHODS

Yeast Strains and Media

Previously described standard methods were used for media preparation and genetic and molecular biological techniques (Guthrie and Fink, 1991; Ausubel *et al.*, 1992). The *S. cerevisiae* strains used in these studies are summarized in Supplemental Table 1. Unless otherwise stated, all yeast strains were grown at 30°C. Precultures were grown 16 h in YPD medium containing 2% glucose, diluted 1:12.5 into YPD, and grown for additional 6–7 h. Thereafter, cells were resuspended in YPethanol (2%) and grown for 16 h to allow FBPase synthesis. For induction of FBPase degradation, cells were shifted onto YPD medium containing 2% glucose.

Construction of Epitope-tagged Gid Proteins and Plasmids

Gid1/Vid30, Gid5/Vid28, Gid6/Ubp14, Gid7, Gid8, and Gid9/Fyv10 were C-terminally labeled with a 3xHA-tag. Strain YJR12 (*GID1-HA₃*) was constructed by chromosomal integration of a 1.6-kb polymerase chain reaction (PCR) fragment consisting of a 3xHA-tag and a *Schizosaccharomyces pombe* HIS5 marker (Cottarel, 1995) into W303-1B cells. The PCR fragment was generated using oligonucleotides YJR12fwd, YJR12rev and plasmid p3xHA-HIS5 (S. Munro, Cambridge, United Kingdom) as template. Strains YTP10 (*GID7-HA₃*), YTP11 (*GID5-HA₃*), YTP12 (*GID9-HA₃*), YSA1 (*GID8-HA₃*), and YBB1 (*GID6-HA₃*) were generated by chromosomal integration of a 1.75-kb PCR fragment. Plasmid pFA6a-3HA-His3MX6 was described previously (Longtine *et al.*, 1998). Gid4/Vid24 was N-terminally Myc₃ tagged according to Gauss *et al.* (2005). For URA3 marker rescue with pSH63, instead of 1% raffinose and 1% galactose, the medium contained 2% galactose. The integration cassette was amplified from pOM22 (Gauss *et al.*, 2005) by using primer YOS1fwd and YOS1rev and transformed into strain W303-1B. Correct integration was confirmed by Southern blot analysis or PCR. *GID2-HA₃* construction is described in Regelmann *et al.* (2003). The pOS2 plasmid was constructed by insertion of a *Myc₃-GID4* fragment in a StuI/SbfI-digested pCM184 plasmid (Euroscarf, Frankfurt, Germany). All oligonucleotides used are listed in Supplemental Table 2. The construction of FBPase C-terminal TAP fusion was conducted as described previously based on the homologous recombination of a PCR product at a specific gene locus on the chromosome (Puig *et al.*, 2001). The plasmid expressing the FBPase-TAP fusion protein was

obtained using a gap-repair strategy. Plasmid pRG6 (de la Guerra *et al.*, 1988), which contains the *FBP1* gene and its genomic flanking regions was digested with NcoI. The plasmid, lacking the 800 base pair NcoI fragment was then transformed in a yeast strain expressing a chromosomally C-terminally tagged FBPase. Cells able to survive on complete minimal (CM) media lacking uracil were selected, plasmid rescue was performed, and the obtained plasmids were analyzed for the presence of an FBPase-TAP coding sequence, under the native FBPase promoter.

To construct the plasmid pFBPase, a genomic fragment encompassing the *FBP1* gene together with 1000 base pairs of its upstream and 200 base pairs of its downstream sequences was amplified by PCR with primers pFBPase-fwd and pFBPase-rev (Supplemental Table 2) and inserted into a SpeI/ClaI-digested pRS316 plasmid (Sikorski and Hieter, 1989). The resulting plasmid was verified by enzymatic restriction and sequencing. The plasmid-expressed FBPase is functional and undergoes degradation as the chromosomally expressed enzyme.

Mutation of the Degenerated RING Domain of GID2/RMD5

A point mutation in the conserved Cys residue 379 of Gid2/Rmd5 was performed using the Transformer site-directed mutagenesis kit (Clontech, Mountain View, CA). The template plasmid was generated by digesting a YCP50 plasmid (Rose *et al.*, 1987) using BamHI and SalI. The resulting fragment, bearing the *GID2/RMD5* ORF with its endogenous promoter and terminator regions was inserted in pRS316. Oligonucleotides are listed in Supplemental Table 2. The mutated *GID2/RMD5* was integrated in pRS306 digested with BamHI and SalI. Genomic integration was carried out by transforming the resulting plasmid in YTS3 yeast cells. Chromosomal DNA of colonies that lost the ability to grow on 5-fluorouracil containing medium was extracted, and the *GID2/RMD5* gene was sequenced.

Western Blotting

Western blotting was performed as described in Schork *et al.* (1995). Extracts were prepared via alkaline lysis (Yaffe and Schatz, 1984) and finally resuspended in urea buffer (200 mM Tris-HCl pH 6.8, 8 M urea, 5% SDS, 0.1 mM EDTA, 1% 2-mercaptoethanol, and 0.05% bromophenol blue). We used 3 OD₆₀₀ of cells for each sample. Antibodies used were obtained from BABCO (Richmond, CA) (hemagglutinin [HA], clone 16B12) and Calbiochem (San Diego, CA) (Myc, clone 9E10); FBPase polyclonal antibody was obtained from K. D. Entian (Goethe Universität, Frankfurt, Germany) or was produced by rabbit immunization using a purified FBPase-glutathione transferase (GST).

Immunoprecipitation

For immunoprecipitations (IPs) cells were cultivated as described above for FBPase turnover assays and samples were withdrawn at the indicated time points. Cells (30 OD₆₀₀) were harvested, washed with water, and resuspended in 600 μ l of phosphate-buffered saline (PBS) buffer pH 7.4 (137 mM NaCl, 1.25g/l Na₂HPO₄, and 0.35g/l NaH₂PO₄) containing protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany; 1.1 mM phenylmethylsulfonyl fluoride [PMSF]; 1 μ g/ml each of antipain, pepstatin A, chymostatin, and leupeptin) and lysed at 4°C with glass beads for 20 min. After centrifugation, 500 μ l of the supernatant was transferred to a new test tube. FBPase antibody was added, and the samples were gently agitated end over end for 2 h at 4°C. Immunoprecipitates were collected by adding 50 μ l of 5% (wt/vol) protein A-Sepharose CL-4B (GE Healthcare, Little Chalfont, United Kingdom) and further incubated for 1.5 h. For IP, the Sepharose beads were centrifuged and washed five times with ice-cold PBS buffer. Proteins were released from Sepharose by boiling in 50 μ l of urea buffer.

Glycerol Density Gradient Fractionation

Yeast cells were grown for 16 h in YPethanol. For analysis in gluconeogenic cells, 50 OD₆₀₀ of cells were harvested; for analysis in glycolytic cells, cells were shifted on YPD for 20 min before 50 OD₆₀₀ of cells were harvested. Cells were resuspended in 600 μ l of 0.1 M KH₂PO₄ pH 7.0, in the presence of Complete, 1.1 mM PMSF, and protease inhibitors; lysed with glass beads (20 min; 4°C); and centrifuged at 10,000 $\times$ g for 15 min. Then, 200 μ l aliquots of the resulting cell extracts were layered on top of a glycerol step gradient [450 μ l each of 50%, 40%, 30%, 20 and 10% of glycerol in 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) buffer pH 6.8] and centrifuged at 55,000 rpm and 15°C for 4 h in a TLS-55 rotor. Thereafter, 200 μ l fractions were collected, precipitated with trichloroacetic acid (10%), solubilized in urea buffer, and analyzed by Western blotting.

Polyubiquitination of FBPase

Polyubiquitination was assessed either by growing the cells containing the FBPase-tandem affinity purification (TAP)-tag encoding plasmid on CM medium without uracil, 2% glucose or growing the *GID2-HA₃* and *GID2C379S-HA₃* strains on YPD to OD₆₀₀ 3–4. After harvesting (500 $\times$ g; 5 min), cells were resuspended in the same medium containing 2% ethanol and left to grow for 6 h to allow derepression of FBPase. Then, 50 OD₆₀₀ of yeast were harvested

before and 25 min after addition of 2% glucose. After washing in 1 ml of water containing 20 mM N-ethylmaleimide, 20 mM Na₂S₂O₃, and 1 mM PMSF, cells were pelleted at 500 × g for 4 min at 4°C and resuspended in 600 µl of ice-cold PBS buffer containing protease inhibitors and lysed at 4°C with glass beads (300 µl; 0.4–0.6 mm in diameter) during 20 min. IP of FBPase was performed as described above, and the pellet was washed five times with PBS buffer containing 0.2% Triton X-100. Alternatively, FBPase-TAP was pulled down using 80 µl of 50% (vol/vol) immunoglobulin G (IgG)-Sephadex beads. After 3 h of incubation at room temperature, beads were washed four times with PBS added with 150 mM NaCl and 1% (vol/vol) Triton X-100. In both cases, beads were resuspended in 50 µl of urea buffer, boiled for 5 min at 95°C and used for immunoblotting with monoclonal anti-ubiquitin antibody (clone P4G7; Covance Research Products, Princeton, NJ).

In Vitro Polyubiquitination Assay

A GST-fusion protein of Gid2/Rmd5, and, as a positive control, the mammalian RING protein gp78 (309–643-amino acid fragment) were expressed in the *Escherichia coli* BL21 strain. Bacteria were grown to OD₆₀₀ 0.8–1 in 2× yeast tryptone containing 100 µg/ml ampicillin, and expression of the extrinsic protein was induced at 16°C with 0.5 mM isopropyl β-D-thiogalactoside during 6–12 h. Cells were harvested, resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM dithiothreitol (DTT), 2 mM PMSF, and 1% Triton-X 100) and lysed with a French press. A typical 20 µl ubiquitination reaction was carried out by adding 0.25 µg of E1 (yeast), 0.6 µg of UbcH5b, 10 µg of HA-ubiquitin, 1 µl of energy regeneration solution (all BostonBiochem, Cambridge, MA), 2 µl of 10× ubiquitin reaction buffer (500 mM Tris-HCl, pH 7.5, 500 mM NaCl, 100 mM MgCl₂, 10 mM DTT, and 250 µM ZnCl₂), and 8 µl of Gid2/Rmd5 or gp78 cell lysate. The reactions were incubated at 30°C for 2 h, and ubiquitination of proteins was monitored by Western blotting using monoclonal anti-HA antibody.

Gid4/Vid24 Expression in Gluconeogenic Cells

gid4Δ/vid24Δ yeast cells were transformed with the pOS2 plasmid encoding Gid4/Vid24 under control of the Tet^R promoter or the corresponding control vector. After 16 h of growth on CM-Trp media containing 2% glucose and 40 µg/ml doxycycline, cells were transferred into the same medium lacking cysteine and methionine for 16-h growth. Five OD₆₀₀ of yeast were pelleted washed once with distilled water and resuspended in 2% EtOH-CM-Trp medium without cysteine and methionine, but containing 40 µg/ml doxycycline. After 3 h of incubation; radiolabeling was achieved by addition of 9.25 MBq of [³⁵S]methionine and a further 2 h of incubation time (pulse). Consecutively, cells were centrifuged, washed with water and resuspended in 6.5 ml of CM-Trp-EtOH (2%) containing 10 mM nonradioactive methionine (chase). Samples were harvested every hour during 3 h. After FBPase immunoprecipitation and 10% SDS-polyacrylamide gel electrophoresis, gels were overlaid with Phosphor screen and scanned with Storm 860 (GE Healthcare) for protein quantification.

Degradation of Gid4/Vid24

To monitor Gid4/Vid24 degradation, cells were grown as described above for FBPase turnover assays. After 16 h of growth on YPethanol (2%); cells were shifted onto YPD for 30 min to induce *GID4/VID24* expression. Cells were then shifted onto fresh YPD with 100 µg/ml cycloheximide. For the proteasome inhibition experiment, along with cycloheximide treatment, 60 µM MG-132 were added 30 min ahead of harvesting. This treatment was repeated every 30 min to ensure a proper proteasome inhibition. To monitor Gid4/Vid24 stability; 1.5 OD₆₀₀ of cells were taken at each time point and analyzed by immunoblotting.

Sequence Analysis

Sequence database searches were carried out with a nonredundant data set assembled from current releases of Uniprot and GenPept (Benton, 1990; Bairoch and Apweiler, 1997). Multiple alignments were calculated by MUSCLE (Edgar, 2004), using excised domains instead of the entire protein sequences. Generalized profile construction (Bucher *et al.*, 1996), and searches were run locally using the pftools package, version 2.1. Generalized profiles were constructed using the BLOSUM45 substitution matrix (Henikoff and Henikoff, 1992) and default penalties of 2.1 for gap opening and 0.2 for gap extension. Profile matches were analyzed for statistical significance by means of the score distribution of a randomized database (Hofmann, 2000). Only sequence matches that were detected with a probability *p* lower than 0.01 were included in subsequent rounds of iterative profile refinement.

RESULTS

Expression Profiles and Sedimentation Pattern of Gid Proteins

In a genomic screen, the previously discovered *GID1/VID30*, *GID2/RMD5*, and *GID3/UBC8* genes were identified along

with six additional *GID* genes to be necessary for glucose-induced FBPase degradation (Schork *et al.*, 1995; Hämmerle *et al.*, 1998; Schüle *et al.*, 2000; Regelman *et al.*, 2003). Further characterization of Gid2/Rmd5 suggested that it is part of a complex of at least 600 kDa (Regelman *et al.*, 2003). Mass spectrometry analyses of proteins interacting with either Gid7 or Gid1/Vid30 uncovered the Gid proteins as subunits of the same complex (Ho *et al.*, 2002; Krogan *et al.*, 2006; Pitre *et al.*, 2006).

To be able to detect the Gid proteins immunologically, their genes were chromosomally tagged with either 3xHA or 9xMYC coding sequences. The resulting strains were tested for their ability to degrade FBPase. Except for cells expressing the Gid9-HA₃ protein, all strains displayed wild-type FBPase degradation kinetics (data not shown). The expression of the Gid proteins was monitored in ethanol-growing cells and up to 120 min after shift to glucose-containing media. Besides Gid2/Rmd5 (Regelman *et al.*, 2003), five additional Gid proteins (Gid1/Vid30, Gid5/Vid28, Gid7, Gid8, and Gid9/Fyv10) were already present in ethanol-grown (gluconeogenic conditions) cells, and their levels remained constant over 120 min after shift to glucose (Figure 1A). An additional Gid protein, Gid4/Vid24 was undetectable when cells were grown in ethanol-containing medium. However, as soon as cells were transferred to glucose-containing medium, the Gid4/Vid24 protein rapidly occurred, its levels culminating ~30 min after shift. Thereafter, Gid4/Vid24 levels decreased in parallel with those of FBPase (Figure 1A). Gid4/Vid24 synthesis upon glucose addition to cells growing on a nonfermentable carbon source had already been described, whereas no subsequent degradation of the protein had been observed under the inactivation conditions used (Chiang and Chiang, 1998).

Using glycerol step gradient analysis, Regelman *et al.* (2003) found that Gid2/Rmd5 exists in a complex of ~600 kDa. As shown in Regelman *et al.* (2003) for the Gid2/Rmd5 protein, the other six Gid proteins (Gid1/Vid30, Gid4/Vid24, Gid5/Vid28, Gid7, Gid8, and Gid9/Fyv10) also sediment at high molecular mass (data not shown). Indeed, mass spectrometry analyses identified a complex in which the seven Gid proteins reside (Ho *et al.*, 2002; Krogan *et al.*, 2006; Pitre *et al.*, 2006). All these analyses were performed in glucose-containing media. Because Gid4/Vid24 is absent under gluconeogenic conditions, we tested its requirement for the formation of a high molecular mass Gid complex. Glycerol step gradients were performed to observe the sedimentation patterns of Gid1-HA₃ and Gid7-HA₃ in gluconeogenic conditions (Gid4/Vid24 absent; Figure 1A), and 20 min after shift to glucose, where Gid4/Vid24 is present (Figure 1A). As shown in Figure 1B, no significant differences in the sedimentation patterns of Gid1-HA₃ (116 kDa) and Gid7-HA₃ (92 kDa) before (*T* = 0) and 20 min (*T* = 20) after glucose shift could be seen: both proteins sediment with molecular masses in the range of 600 kDa, as shown by their cofractionation with the 600 kDa aminopeptidase I homodimer complex (Ape1). Although it cannot be completely excluded that the binding partners within the Gid-complex vary between ethanol- and glucose-grown cells, this experiment suggests that the tested Gid proteins are already present in a complex when cells grow on a nonfermentable carbon source and do not dissociate to their respective monomers when Gid4/Vid24 is absent from the cell.

Gid1/Vid30 Interacts with FBPase

A direct involvement of the Gid complex in FBPase degradation requires binding of FBPase to Gid proteins. We therefore examined whether Gid1-HA₃, as an example,

Figure 1. Gid complex subunits are present in gluconeogenic cells. (A) Strains expressing chromosomally tagged Gid proteins with the HA or Myc epitope were grown 16 h in YPethanol (E) and thereafter treated with 2% glucose. Samples were taken at the indicated time points and processed as described in *Material and Methods*. Proteins were visualized by immunoblotting. *, cross reaction; WT, wild type, control for cross-reaction. (B) Strains bearing a chromosomally expressed HA-tagged Gid protein (Gid1/Vid30 and Gid7) were grown 16 h in YPethanol. Samples were harvested before (T = 0) or 20 min after shift to glucose (T = 20), and proteins were separated on a glycerol step gradient. Ten fractions were collected, and proteins were visualized by immunoblotting (Pgk, 3-phosphoglycerate kinase; Ape1, aminopeptidase I).

coimmunoprecipitates with FBPase. We used a *GID6-HA₃* strain as a control for the interaction of FBPase with the Gid complex. *GID6* was shown previously to encode Ubp14 deubiquitinating enzyme, its deletion prevents the cleavage of polyubiquitin chains released from proteasome substrates into single ubiquitin moieties. This deletion leads to competition between uncleaved polyubiquitin chains and polyubiquitinated proteins for the proteasome (Amerik *et al.*, 1997), which has been shown to impair proteasomal degradation of FBPase (Eisele *et al.*, 2006). Thus, the effect of *GID6* deletion on FBPase degradation is unspecific. Moreover, Gid6/Ubp14 was not detected as being part of the Gid complex (Ho *et al.*, 2002; Krogan *et al.*, 2006; Pitre *et al.*, 2006). YJJ9 (*GID1-HA₃*, *fbp1Δ*) and YBB1 (*GID6-HA₃*) strains were transformed with a plasmid expressing a wild-type FBPase (pFBPase) or pRS316 as a vector control. YJJ9 strain transformed with pFBPase and YBB1 strain transformed with pRS316 were grown in YPethanol and shifted to YPD. The YJJ9

strain transformed with pRS316, for which the endogenous FBPase is not replaced by a plasmid-encoded enzyme, is unable to grow on nonfermentable carbon sources and was therefore grown on YPD only. Samples were withdrawn at the indicated time points, and FBPase was precipitated using specific anti-FBPase antibodies (Figure 2). The precipitates were monitored by immunoblotting with FBPase and HA-antibodies. Figure 2 shows that FBPase strongly coimmunoprecipitates with Gid1-HA₃ in YPethanol at the time point "0" and after 20 min of glucose addition to cells. A similar result was obtained when a strain expressing FBPase chromosomally was used for the immunoprecipitation (data not shown). No interaction between FBPase and Gid6-HA₃/Ubp14-HA₃ could be observed, demonstrating that no unspecific interaction occurs between FBPase and the HA-tag. Moreover, Gid1-HA₃ was not immunoprecipitated when no FBPase was present, establishing that the anti-FBPase antibody does not bind unspecifically to Gid1-HA₃.

Figure 2. FBPase binds to Gid1/Vid30. Strains expressing HA-tagged Gid proteins (Gid1/Vid30 and Gid6/Ubp14) and transformed with a plasmid expressing a wild-type FBPase (pFBPase) or its corresponding vector control (pRS316) were grown 16 h on YPethanol. Samples were harvested 0, 20, and 90 min after glucose treatment, and proteins were extracted. Coimmunoprecipitation was performed with FBPase antibody. Protein immunoblot was carried out with FBPase and HA antibodies. The noninteracting Gid6/Ubp14 protein serves as a control (YE, extracts; IP, immunoprecipitations). The *FBP1* deletion mutant transformed with the vector control was grown on glucose and provides a control for the specificity of the anti-FBPase antibody.

Gid2/Rmd5 Confers Ubiquitin Ligase (E3) Activity

Gid2/Rmd5 contains a Lissencephaly type-1-like homology motif (LisH), a C-terminal to LisH motif (CTLH), and a degenerated RING finger domain, an organization shared with its human orthologues (Figure 3, A and C). Many ubiquitin ligases contain a RING-finger domain bearing eight Cys/His coordination sites to complex Zn^{2+} ions necessary for activity (Lorick *et al.*, 1999; Fang and Weissman, 2004). A complete cysteine and histidine pattern in a RING domain is not necessarily critical for the E3 function, as the U-box domain family shows. Although the U-box domain still adopts the same structure as the RING domain, it is an extreme example of degeneration of zinc-coordinating residues, where none of them are retained (Ohi *et al.*, 2003). In Gid2/Rmd5 the degenerated RING domain is characterized by an incomplete series of Zn^{2+} ion-coordinating residues compared with the classic RING finger (Figure 3B, compare top and bottom alignments). The canonical RING domain encompasses eight residues coordinating two Zn cations. The first Zn^{2+} ion is coordinated by the first, second, fifth, and sixth residue; the second by the remaining residues three, four, seven, and eight (Lorick *et al.*, 1999; Fang and Weissman, 2004). In Gid2/Rmd5 besides the first cysteine, the four residues coordinating the second Zn^{2+} ion are conserved (Figure 3B), which strongly suggests that one ion is retained in this degenerated RING domain. This prompted us to suspect that Gid2/Rmd5 may bear an ubiquitin ligase activity. The ubiquitin system is present in all eukaryotes but is not found among bacteria. A heterologous expression of a GST-Gid2 fusion protein in *E. coli* led to polyubiquitination of proteins in the bacterial lysate when E1 and E2 enzymes, ATP and HA-ubiquitin were added (Figure 4A). When only the GST protein was expressed, no polyubiquitination could be detected (Figure 4A). We further determined the contribution of the Gid2/Rmd5 degenerated RING finger to FBPase polyubiquitination in vivo by mutating the conserved cysteine residue at position 379 of the putative Gid2/Rmd5 RING domain to serine. The HA-tagged version of the mutated gene was introduced into the yeast genome. As can be seen in Figure 4B, degradation of FBPase

is dramatically impaired in cells carrying the *GID2C379S* mutation. The strain expressing the mutant protein is unable to polyubiquitinate FBPase in vivo (Figure 4C). We conclude that Gid2/Rmd5 bears an E3 activity necessary for degradation of FBPase.

Role of Gid4/Vid24 in the FBPase Catabolite Degradation

Gid4/Vid24 is necessary for FBPase degradation (Regelmann *et al.*, 2003). The protein is absent in ethanol-growing cells and occurs early after glucose addition to the medium (Figure 1A). Thus, blocking de novo protein synthesis is likely to prevent Gid4/Vid24 appearance and subsequent FBPase breakdown by the proteasome. To test this hypothesis, cells were treated with the translation inhibitor cycloheximide when they were shifted from gluconeogenic to glycolytic conditions. Indeed, cells that underwent cycloheximide treatment were unable to express Gid4/Vid24 and therefore to degrade FBPase (Figure 5A). Because Gid3/Ubc8 and other Gid proteins, except Gid4/Vid24, are present in ethanol-growing cells (Figure 1, Regelmann *et al.*, 2003), this result suggests that Gid4/Vid24 might trigger FBPase degradation. If Gid4/Vid24 were a switch, initiation of FBPase elimination should take place when Gid4/Vid24 is expressed in ethanol-growing cells. *FBP1* gene expression is repressed when cells grow on a glucose-containing medium; on the contrary, when grown on a nonfermentable carbon source, yeast cells readily express FBPase (Zaragoza and Gancedo, 2001). Thus, a pulse-chase experiment, where FBPase was radioactively labeled, has been carried out to monitor FBPase degradation when Gid4/Vid24 expression is forced in ethanol-growing cells. Indeed, expression of Gid4/Vid24 in YPethanol growing cells lowers the half-life of FBPase by approximately threefold (Figure 5B). Although one cannot exclude that additional modifications are necessary to signal FBPase degradation, this result strongly suggests that Gid4/Vid24 is the only protein whose de novo synthesis is required to trigger FBPase elimination.

The fact that Gid4/Vid24 is a molecular switch triggering FBPase degradation even in gluconeogenic conditions led us to ask whether the Gid2/Rmd5-dependent polyubiquitination of FBPase would depend on Gid4/Vid24. The TAP tag allows to purify proteins (Puig *et al.*, 2001). Because it bears a protein A sequence, it is able to strongly bind the IgG constant fragment. This property allowed us to use the tag to perform IgG-Sepharose pull-downs. Moreover, when fused to an otherwise poorly detectable protein, it easily facilitates protein detection. Immunoprecipitation of FBPase was often complicated by the fact that the enzyme migrates only slightly faster on SDS-polyacrylamide gels than IgGs. Therefore, a plasmid expressing a catalytically active C-terminally TAP-tagged FBPase was transformed in W303 (WT) and *gid4Δ/vid24Δ* strains to ensure a good detection of the enzyme and its polyubiquitinated forms. Pull-down with IgG-Sepharose and subsequent detection with an anti-ubiquitin antibody revealed that Gid4/Vid24 is indeed necessary for FBPase polyubiquitination (Figure 5C). Together, these results suggest that Gid4/Vid24 is a switch allowing the polyubiquitination of FBPase before its degradation by the proteasome.

Elimination of Gid4/Vid24 Depends on the Ubiquitin-Proteasome System and the Gid Complex

As shown in Figure 1A, Myc₉-Gid4 levels increase with time when cells are shifted from YPethanol to YPD. After reaching a maximum at ~30 min after shift to glucose, the Myc₉-Gid4 signal diminishes in parallel with FBPase. To test whether this diminution is dependent on the ubiquitin-

Figure 3. Domain structures of Gid proteins. (A) Structure similarities between the Gid2/Rmd5 (*S. cerevisiae*) and RMND5A/B (*Homo sapiens*) proteins. (B) Alignment of the RING domains found in the Gid2/Rmd5 protein family and classic RING-finger proteins. Positions with invariant or conservatively replaced residues in at least 50% of the sequences are printed on black or gray background, respectively (not all sequences analyzed are shown). The leftmost column describes the sequence name and contains the species abbreviation: YEAST, *S. cerevisiae*;

conjugating enzyme Ubc8, components of the Gid complex, and the proteasome, we deleted *GID2/RMD5*, *UBC8*, and, for proteasome inhibition by the proteasome inhibitor MG-132, *PDR5* in a strain expressing Myc₉-Gid4. *PDR5* encodes an ATP-binding cassette transporter, which prevents accumulation of hydrophobic compounds in the yeast cytosol (Balzi *et al.*, 1994; Bissinger and Kuchler, 1994). Its deletion is thus necessary to prevent inhibitor efflux from the cell. After overnight growth in ethanol-containing medium, the tested strains were shifted to glucose-containing medium for 30 min to allow Myc₉-Gid4 expression before cycloheximide treatment. Samples were collected at the indicated time points after cycloheximide treatment. Figure 6 shows that, similar to elimination of FBPase, degradation of Gid4/Vid24 depends on Ubc8 (E2) and the proteasome. Interestingly, degradation of Gid4/Vid24 also depends on components of the Gid complex because deletion of *GID2/RMD5* stalls its degradation (Figure 6). Elimination of Myc₉-Gid4 is not due to the Myc-tag as native, endogenous Gid4/Vid24 shows identical behavior (Josupeit and Wolf, unpublished).

Catabolite Degradation of PEPCK Requires Subunits of the Gid Complex

The highly exergonic last reaction step in glycolysis, the formation of pyruvate and ATP by pyruvate kinase is circumvented in gluconeogenesis by the action of pyruvate carboxylase and PEPCK. As for FBPase, PEPCK synthesis is repressed by glucose and subjected to catabolite degradation when cells are shifted from gluconeogenic to glycolytic conditions (Holzer, 1976; Muller *et al.*, 1981; Mercado and Gancedo, 1992; Yin *et al.*, 2000). This allowed us to monitor the degradation of PEPCK after shift of cells from YPethanol to YPD. Interestingly, deletions of *GID2/RMD5* or *GID4/VID24* stall degradation of PEPCK, indicating the requirement of the Gid complex for its catabolite degradation (Figure 7). This suggests that the Gid complex plays a general role in catabolite degradation.

DISCUSSION

FBPase is essential for yeast growth on nonfermentable carbon sources such as acetate or ethanol. When a fermentable carbon source becomes available, the *FBP1* gene expression is repressed and the enzyme is inactivated by allosteric inhibition before being degraded (Holzer, 1976; von Herrath and Holzer, 1988). The site of degradation (vacuole or proteasome) has been subject to controversy. Now, it seems rather clear that the type of gluconeogenic conditions influence the degradation pathway (Schork *et al.*, 1994b; Chiang

Figure 3 (cont). POMBE, *S. pombe*; CANAL, *Candida albicans*; HUMAN, *H. sapiens*; XENLA, *Xenopus laevis*; DROME, *Drosophila melanogaster*; CAEEL, *Caenorhabditis elegans*; TETNG, *Tetraodon nigroviridis*. Larger insertions are not shown; the number of omitted residues is indicated in parentheses. Asterisks above the Gid2/Rmd5 family alignment correspond to positions homologous to zinc coordinating residues in classic RING finger domains (red). The mutation introduced in the Gid2/Rmd5 degenerated RING domain is highlighted (C379S, red). Above the classic RING-finger proteins alignment, residues of Cbl in contact with UbcH7 are marked by # (Zheng *et al.*, 2000). (C) Overview of the Gid proteins and their counterparts in *H. sapiens*. The table summarizes the common features in Gid proteins and their human counterparts. ^aOrthologues identified in a CTLH complex by Kobayashi *et al.* (2007); ^bOrthologues found to interact together by Umeda *et al.* (2003). ^cMuskelin bears CTLH and Kelch domains, which makes its overall structure similar to Gid7. Other functions and names, SGD, www.yeastgenome.org.

Figure 4. Gid2/Rmd5 shows E3 activity. (A) Gid2/Rmd5 ubiquitinates protein in vitro. Lysates of *E. coli* expressing either Gid2/Rmd5 or the mammalian RING-finger protein gp78 as a positive control and GST as a negative control were incubated with E1, E2 (UbcH5b), ATP and HA-ubiquitin for 2 h at 30°C. To assess the specificity of the reaction, same lysates were incubated without E1 or E2. Polyubiquitination was detected using monoclonal HA antibody. (B) *GID2-HA3* and its mutated C379S counterpart-expressing cells were grown 16 h in YPethanol at 25°C and shifted to YPD. Samples were taken at indicated time points and FBPase degradation was monitored by immunoblotting. Pgk, 3-phosphoglycerate kinase, loading control. (C) Point mutation in the Gid2/Rmd5 degenerated RING domain abolishes FBPase polyubiquitination. Chromosomally tagged *GID2-HA3*, which shows a wild-type phenotype, and a strain bearing the C379S point mutation in a *GID2-HA3* strain 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 was detected using monoclonal ubiquitin antibody. *fbp1Δ*: FBPase deletion. Presence of FBPase in the immunoprecipitates was controlled by immunoblotting with FBPase antibody. *, cross-reaction.

and Chiang, 1998; Hämmerle *et al.*, 1998; Hung *et al.*, 2004). Vacuolar import and degradation of FBPase occurs when cells are grown 2–3 d on an acetate-containing medium. Such conditions trigger autophagy, which is not likely to be relieved by the sole addition of glucose to the medium. Thus, one may speculate that yeast cells use the pre-existing

Figure 5. Gid4/Vid24 promotes FBPAse degradation. (A) De novo protein synthesis is necessary for FBPAse degradation and Myc₉-Gid4 expression. Wild-type cells were grown 16 h in YPethanol (E) and shifted to YPD with (+CHX) or without (–CHX) cycloheximide (100 μg/ml). FBPAse and Myc₉-Gid4 levels were monitored at indicated times via immunoblotting with anti-FBPAse or anti-Myc antibodies, respectively. (B) Gid4/Vid24 expression triggers FBPAse degradation. Myc₉-GID4 was cloned under a Tet^R promoter and expressed in cells growing on YPethanol. Pulse-chase analysis of FBPAse in cells bearing either the Myc₉-GID4 expressing plasmid (♦) or the respective vector control (■) was carried out (means of three independent experiments, ± confidence interval, $\alpha = 0.05$). Inset, immunoblot showing the steady-state levels of Myc₉-Gid4 in glucose-inactivated cells (30 min, YOS1, G) and in ethanol grown cells (pOS1, E). (C) Gid4/Vid24 is necessary for FBPAse-TAP polyubiquitination. A plasmid expressing a FBPAse-TAP fusion protein was transformed into W303 (WT) and *gid4Δ/vid24Δ* strains. Samples were taken at indicated time points, and FBPAse was pulled-down using IgG-Sepharose. Polyubiquitination was detected using monoclonal ubiquitin antibody.

degradation machinery to specifically degrade FBPAse when growth conditions favor this autophagic process. On the contrary, overnight growth on ethanol, a nonfermentable but natural carbon source for *S. cerevisiae* triggers FBPAse degradation by the proteasome. A genome-wide screen revealed nine genes whose deletion impaired proteasomal-dependent FBPAse degradation upon shift to glucose-containing medium (Regelmann *et al.*, 2003). Among these genes, Gid1/Vid30, Gid4/Vid24, Gid5/Vid28 were first implicated in a vacuolar-dependent FBPAse degradation (Chiang and Chiang, 1998). Moreover, *GID3* and *GID6* genes were shown to encode Ubc8 and Ubp14, respectively (Schüle *et al.*, 2000; Regelmann *et al.*, 2003). It was demonstrated that Gid2/Rmd5 belongs to a soluble complex of ~600 kDa (Regelmann *et al.*, 2003). Also, Gid1/Vid30, Gid4/Vid24, Gid5/Vid28, Gid7, Gid8, and Gid9/Fyv10 belong to a soluble complex (data not shown). Systematic interaction studies identified those Gid proteins as units of the same complex (Ho *et al.*, 2002; Krogan *et al.*, 2006; Pitre *et al.*, 2006). Epitope tagging allowed us to monitor the expression of the Gid proteins. Interestingly, Gid4/Vid24 is the only Gid protein that cannot be detected in ethanol-growing cells. This led us to suspect that Gid4/Vid24 might be an activator of the Gid

complex. In glycerol step gradient analysis, no differences between the sedimentation patterns of Gid1-HA₃ and Gid7-HA₃ before (Gid4/Vid24 absent) and 20 min after shift to glucose (Gid4/Vid24 present) could be revealed (Figure 1B). Although it cannot be excluded that the binding partners of Gid1/Vid30 or Gid7 differ between gluconeogenic and glycolytic conditions, both proteins remain in a complex even when Gid4/Vid24 is absent, suggesting that this Gid protein has no influence on the assembly of monomeric Gid proteins to a higher molecular mass complex. The Gid proteins are involved in the proteasomal degradation of FBPAse. Whether the Gid complex is part of an upstream signaling pathway or whether it is directly involved in FBPAse polyubiquitination and degradation remained to be clarified. We show that Gid1/Vid30 interacts with FBPAse. As Gid1/Vid30 is part of the Gid complex (Ho *et al.*, 2002; Krogan *et al.*, 2006; Pitre *et al.*, 2006), this result indicates that FBPAse and the Gid complex interact.

A *GID2/RMD5* deletion prevents polyubiquitination of FBPAse (Regelmann *et al.*, 2003). Alignments of the Gid2/Rmd5 protein sequence with known RING-finger E3s revealed that it bears a so-called degenerated RING-finger where Zn²⁺ coordination residues are missing (Figure 3B).

Figure 6. Gid4/Vid24 degradation depends on Gid2/Rmd5, Ubc8 and the proteasome. W303-1B expressing Mycg-Gid4 was deleted for *UBC8*, *GID2/RMD5*, or *PDR5* (*ubc8Δ*, *gid2Δ*, *pdr5Δ*, respectively). Strains were grown 16 h on YPethanol, shifted to YPD, and expression of Gid4/Vid24 was allowed to proceed during 30 min. Thereafter, cells were treated with cycloheximide and samples were harvested at the indicated times. Proteins were visualized via immunoblotting. Proteasome involvement in Gid4/Vid24 degradation was analyzed in a *pdr5Δ* strain using the proteasome inhibitor MG-132.

Although the U-box domain family lacks all Zn^{2+} coordinating residues, it still polyubiquitinates proteins (Hatakeyama *et al.*, 2001; Ohi *et al.*, 2003). This prompted us to test whether this would also be the case for Gid2/Rmd5. Indeed, Gid2/Rmd5 polyubiquitinates proteins in vitro. Moreover, destroying a remaining Zn^{2+} coordination site in Gid2/Rmd5 leads to a failure to polyubiquitinate FBPAse in vivo (Figure 4). These results imply that the Gid complex is an ubiquitin ligase (E3), whose activity is provided by the Gid2/Rmd5 subunit (Figure 8).

We further characterized the function of the Gid4/Vid24 subunit within the new E3 complex. Gid4/Vid24 had previously been implicated in targeting FBPAse from vesicles to the vacuole and was shown to occur only upon glucose addition to acetate-starved cells where it remained stable (Chiang and Chiang, 1998). We here show that also in cells growing on ethanol, a natural carbon source of yeast, Gid4/Vid24 occurs upon glucose shift (Figure 1A). Because Gid4/Vid24 becomes detectable as fast as 5 min after shift of cells from ethanol to glucose, it is unlikely that *GID4/VID24* gene expression depends on transcription followed by translation. Indeed, *GID4/VID24* mRNA levels do not vary signif-

Figure 7. *GID* deletions impair PEPCCK degradation. Wild type, *gid2Δ/rmd5Δ*, or *gid4Δ/vid24Δ* cells were grown 16 h on YPethanol, and thereafter shifted onto YPD. Cells were harvested every hour, and extracts were prepared. Proteins were visualized via immunoblotting. Pgk, 3-phosphoglycerate kinase, loading control.

Figure 8. The Gid complex, a novel ubiquitin ligase (E3) required for the degradation of the key gluconeogenic enzyme fructose-1,6-bisphosphatase. The Gid complex binds to FBPAse, when *S. cerevisiae* cells are growing on an ethanol-containing medium. On shift of cells to glucose, Gid4/Vid24 occurs and activates the complex, which then polyubiquitinates FBPAse before its degradation by the proteasome. Gid4/Vid24 is itself degraded by the proteasome.

icantly when cells grow on these two different media (DeRisi *et al.*, 1997). Thus, the regulation of *GID4/VID24* expression might be carried out at the posttranscriptional level. Hung *et al.* (2004) confirmed our data that Gid4/Vid24 partakes in proteasome-dependent FBPAse degradation. The different expression patterns of Gid4/Vid24 observed during the vacuolar degradation process of FBPAse in which it remains stable (Chiang and Chiang, 1998) and the proteasomal degradation of the enzyme in which Gid4/Vid24 is degraded might reflect the different requirements of Gid4/Vid24 in these processes. Because Gid4/Vid24 has no influence on sedimentation patterns of two Gid proteins known to be part of the Gid complex (Figure 1B), it seems more likely that Gid4/Vid24 activates the Gid complex rather than stabiliz-

ing it. As shown for acetate-starved cells (Chiang and Chiang, 1998), cycloheximide treatment of cells grown overnight on an ethanol-containing medium prevents Gid4/Vid24 expression on glucose (Figure 5A).

Our result suggests that Gid4/Vid24 might be a switch activating the Gid complex to degrade FBPAse. Indeed, forced expression of Gid4/Vid24 under conditions where FBPAse is actively expressed and stable, triggered its degradation (Figure 5B). Moreover, lack of Gid4/Vid24 inhibits polyubiquitination of an FBPAse-TAP fusion protein (Figure 5C). Together, these results suggest that Gid4/Vid24 activates the Gid complex by promoting Gid2/Rmd5-dependent FBPAse polyubiquitination. The mechanism of Gid complex activation by Gid4/Vid24 remains to be elucidated. Because FBPAse interacts with the Gid complex already in ethanol-grown cells (Figure 2), Gid4/Vid24 is not likely to recruit FBPAse to the complex. Gid4/Vid24 binds to the Gid complex (Ho *et al.*, 2002; Krogan *et al.*, 2006; Pitre *et al.*, 2006); thus, one hypothesis is that it alters the complex conformation to activate it (Figure 8).

Gid4/Vid24 is degraded during elimination of FBPAse by the proteasome (Figure 1A). Its degradation depends on the ubiquitin conjugating enzyme Ubc8 and the E3 component Gid2/Rmd5 (Figure 6). Moreover, degradation of Gid4/Vid24 requires the proteasome (Figures 6, 8). Obviously, Gid4/Vid24 undergoes a regulatory loop exerted by components of the Gid complex: it might limit the activity of the Gid complex to a certain type of substrates like FBPAse or prepare cells for new growth on nonfermentable carbon sources.

PEPCK, another gluconeogenic enzyme, is also subject to catabolite degradation (Holzer, 1976; Muller *et al.*, 1981). As Figure 7 shows, it is stabilized in *GID2/RMD5* and *GID4/VID24* deleted cells. Thus, Gid complex-dependent degradation is not restricted to FBPAse but plays a more general role within the regulation of carbohydrate metabolism.

Gid protein homologues were also found to form a complex in mammals (Kobayashi *et al.*, 2007). Although no function for this CTLH complex has been described, one subunit has been implicated in proteasomal degradation of α -catenin (Suzuki *et al.*, 2008). This suggests that the CTLH complex, like the Gid complex, might also bear E3 activity.

In conclusion (Figure 8), our study shows that the Gid complex is a new ubiquitin ligase with novel types of subunits involved in catabolite degradation of gluconeogenic enzymes in yeast. We also identify Vid24/Gid4 as an important regulator of its ubiquitin ligase activity.

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REFERENCES

- Amerik, A., Swaminathan, S., Krantz, B. A., Wilkinson, K. D., and Hochstrasser, M. (1997). In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. *EMBO J.* 16, 4826–4838.
- Ausubel, F. M., Kingston, R. E., Seidman, F. G., Struhl, K., Moore, D. D., Brent, R., and Smith, F. A. (1992). *Current Protocols in Molecular Biology*, New York: Greene.
- Bairoch, A., and Apweiler, R. (1997). The SWISS-PROT protein sequence data bank and its supplement TrEMBL. *Nucleic Acids Res.* 25, 31–36.

- Balzi, E., Wang, M., Leterme, S., Van Dyck, L., and Goffeau, A. (1994). PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. *J. Biol. Chem.* 269, 2206–2214.
- Benton, D. (1990). Recent changes in the GenBank On-line Service. *Nucleic Acids Res.* 18, 1517–1520.
- Bissinger, P. H., and Kuchler, K. (1994). Molecular cloning and expression of the *Saccharomyces cerevisiae* STS1 gene product. A yeast ABC transporter conferring mycotoxin resistance. *J. Biol. Chem.* 269, 4180–4186.
- Bucher, P., Karplus, K., Moeri, N., and Hofmann, K. (1996). A flexible motif search technique based on generalized profiles. *Comput. Chem.* 20, 3–23.
- Chiang, M. C., and Chiang, H. L. (1998). Vid24p, a novel protein localized to the fructose-1, 6-bisphosphatase-containing vesicles, regulates targeting of fructose-1,6-bisphosphatase from the vesicles to the vacuole for degradation. *J. Cell Biol.* 140, 1347–1356.
- Cottarel, G. (1995). The *Saccharomyces cerevisiae* *HIS3* and *LYS2* genes complement the *Schizosaccharomyces pombe* *his5-303* and *lys1-131* mutations, respectively: new selectable markers and new multi-purpose multicopy shuttle vectors, pSP3 and pSP4. *Curr. Genet.* 28, 380–383.
- de la Guerra, R., Valdes-Hevia, M. D., and Gancedo, J. M. (1988). Regulation of yeast fructose-1,6-bisphosphatase in strains containing multicopy plasmids coding for this enzyme. *FEBS Lett.* 242, 149–152.
- DeRisi, J. L., Iyer, V. R., and Brown, P. O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 278, 680–686.
- Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797.
- Eisele, F., Braun, B., Pfirrmann, T., and Wolf, D. H. (2006). Mutants of the deubiquitinating enzyme Ubp14 decipher pathway diversity of ubiquitin-proteasome linked protein degradation. *Biochem. Biophys. Res. Commun.* 350, 329–333.
- Fang, S., and Weissman, A. M. (2004). A field guide to ubiquitylation. *Cell Mol. Life Sci.* 61, 1546–1561.
- Gancedo, C. (1971). Inactivation of fructose-1,6-diphosphatase by glucose in yeast. *J. Bacteriol.* 107, 401–405.
- Gancedo, J. M. (1998). Yeast carbon catabolite repression. *Microbiol. Mol. Biol. Rev.* 62, 334–361.
- Gauss, R., Trautwein, M., Sommer, T., and Spang, A. (2005). New modules for the repeated internal and N-terminal epitope tagging of genes in *Saccharomyces cerevisiae*. *Yeast* 22, 1–12.
- Guthrie, C., and Fink, G. R. (1991). Guide to yeast genetics and molecular biology. *Methods Enzymol.* 194, 21–37.
- Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K. I. (2001). U box proteins as a new family of ubiquitin-protein ligases. *J. Biol. Chem.* 276, 33111–33120.
- Henikoff, S., and Henikoff, J. G. (1992). Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. USA* 89, 10915–10919.
- Ho, Y. *et al.* (2002). Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 415, 180–183.
- Hoffman, M., and Chiang, H. L. (1996). Isolation of degradation-deficient mutants defective in the targeting of fructose-1,6-bisphosphatase into the vacuole for degradation in *Saccharomyces cerevisiae*. *Genetics* 143, 1555–1566.
- Hofmann, K. (2000). Sensitive protein comparisons with profiles and hidden Markov models. *Brief. Bioinform.* 1, 167–178.
- Holzer, H. (1976). Catabolite inactivation in yeast. *Trends Biochem. Sci.* 1, 178–181.
- Holzer, H. (1989). Proteolytic catabolite inactivation in *Saccharomyces cerevisiae*. *Revis. Biol. Cellular* 21, 305–319.
- Hung, G. C., Brown, C. R., Wolfe, A. B., Liu, J., and Chiang, H. L. (2004). Degradation of the gluconeogenic enzymes fructose-1,6-bisphosphatase and malate dehydrogenase is mediated by distinct proteolytic pathways and signaling events. *J. Biol. Chem.* 279, 49138–49150.
- Hämmerle, M., Bauer, J., Rose, M., Szallies, A., Thumm, M., Dusterhus, S., Mecke, D., Entian, K. D., and Wolf, D. H. (1998). Proteins of newly isolated mutants and the amino-terminal proline are essential for ubiquitin-proteasome-catalyzed catabolite degradation of fructose-1,6-bisphosphatase of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273, 25000–25005.
- Kobayashi, N., Yang, J., Ueda, A., Suzuki, T., Tomaru, K., Takeno, M., Okuda, K., and Ishigatsubo, Y. (2007). RanBPM, Muskelein, p48EMLP, p44CTLH, and the armadillo-repeat proteins ARMC8alpha and ARMC8beta are components of the CTLH complex. *Gene* 396, 236–247.
- Krogan, N. J. *et al.* (2006). Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* 440, 637–643.

- Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl. Acad. Sci. USA* 96, 11364–11369.
- Marcus, F., Rittenhouse, J., Moberly, L., Edelstein, I., Hiller, E., and Rogers, D. T. (1988). Yeast (*Saccharomyces cerevisiae*) fructose-1,6-bisphosphatase. Properties of phospho and dephospho forms and of two mutants in which serine 11 has been changed by site-directed mutagenesis. *J. Biol. Chem.* 263, 6058–6062.
- Mazon, M. J., Gancedo, J. M., and Gancedo, C. (1982). Inactivation of yeast fructose-1,6-bisphosphatase. In vivo phosphorylation of the enzyme. *J. Biol. Chem.* 257, 1128–1130.
- Mercado, J. J., and Gancedo, J. M. (1992). Regulatory regions in the yeast FBP1 and PCK1 genes. *FEBS Lett.* 311, 110–114.
- Muller, M., Muller, H., and Holzer, H. (1981). Immunochemical studies on catabolite inactivation of phosphoenolpyruvate carboxykinase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 256, 723–727.
- Ohi, M. D., Vander Kooi, C. W., Rosenberg, J. A., Chazin, W. J., and Gould, K. L. (2003). Structural insights into the U-box, a domain associated with multi-ubiquitination. *Nat. Struct. Biol.* 10, 250–255.
- Pitre, S. *et al.* (2006). PIPE: a protein-protein interaction prediction engine based on the re-occurring short polypeptide sequences between known interacting protein pairs. *BMC Bioinformatics* 7, 365.
- Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* 24, 218–229.
- Regelmann, J., Schüle, T., Josupeit, F. S., Horak, J., Rose, M., Entian, K. D., Thumm, M., and Wolf, D. H. (2003). Catabolite degradation of fructose-1,6-bisphosphatase in the yeast *Saccharomyces cerevisiae*: a genome-wide screen identifies eight novel GID genes and indicates the existence of two degradation pathways. *Mol. Biol. Cell* 14, 1652–1663.
- Rose, M. D., Novick, P., Thomas, J. H., Botstein, D., and Fink, G. R. (1987). A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* 60, 237–243.
- Schork, S., Bee, G., Thumm, M., and Wolf, D. H. (1994a). Catabolite inactivation of fructose-1,6-bisphosphatase in yeast is mediated by the proteasome. *FEBS Lett.* 349, 270–274.
- Schork, S., Bee, G., Thumm, M., and Wolf, D. H. (1994b). Site of catabolite inactivation. *Nature* 369, 283–284.
- Schork, S. M., Thumm, M., and Wolf, D. H. (1995). Catabolite inactivation of fructose-1,6-bisphosphatase of *Saccharomyces cerevisiae*. Degradation occurs via the ubiquitin pathway. *J. Biol. Chem.* 270, 26446–26450.
- Schüle, T., Rose, M., Entian, K. D., Thumm, M., and Wolf, D. H. (2000). Ubc8p functions in catabolite degradation of fructose-1,6-bisphosphatase in yeast. *EMBO J.* 19, 2161–2167.
- Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19–27.
- Suzuki, T., Ueda, A., Kobayashi, N., Yang, J., Tomaru, K., Yamamoto, M., Takeno, M., and Ishigatsubo, Y. (2008). Proteasome-dependent degradation of alpha-catenin is regulated by interaction with ARMC8alpha. *Biochem. J.* 411, 581–591.
- Umeda, M., Nishitani, H., and Nishimoto, T. (2003). A novel nuclear protein, Twa1, and Muskelein comprise a complex with RanBPM. *Gene* 303, 47–54.
- Vaulont, S., Vasseur-Cognet, M., and Kahn, A. (2000). Glucose regulation of gene transcription. *J. Biol. Chem.* 275, 31555–31558.
- von Herrath, M., and Holzer, H. (1988). Sensitivity of fructose-1,6-bisphosphatase from yeast, liver and skeletal muscle to fructose-2,6-bisphosphate and 5'-adenosine monophosphate. *Z. Lebensm. Unters. Forsch.* 186, 427–430.
- Wahren, J., and Ekberg, K. (2007). Splanchnic Regulation of Glucose Production. *Annu. Rev. Nutr.* 27, 329–345.
- Wolf, D. H. (2004). From lysosome to proteasome: the power of yeast in the dissection of proteinase function in cellular regulation and waste disposal. *Cell Mol. Life Sci.* 61, 1601–1614.
- Yaffe, M. P., and Schatz, G. (1984). Two nuclear mutations that block mitochondrial protein import in yeast. *Proc. Natl. Acad. Sci. USA* 81, 4819–4823.
- Yin, Z., Hatton, L., and Brown, A. J. (2000). Differential post-transcriptional regulation of yeast mRNAs in response to high and low glucose concentrations. *Mol. Microbiol.* 35, 553–565.
- Zaragoza, O., and Gancedo, J. M. (2001). Elements from the cAMP signaling pathway are involved in the control of expression of the yeast gluconeogenic gene FBP1. *FEBS Lett.* 506, 262–266.
- Zheng, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* 102, 533–539.