Jure Piškur · Concetta Compagno
Editors

Molecular Mechanisms in Yeast Carbon Metabolism

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Springer

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ISBN 978-3-642-55012-6 ISBN 978-3-642-55013-3 (eBook)
DOI 10.1007/978-3-642-55013-3
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014938986

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The authors dedicate this book to those closest to them: Judita, Beppe, Natalia, Jan and Jure Jr.

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Chapter 1
Introduction to Carbon Metabolism in Yeast

Concetta Compagno, Sofia Dashko and Jure Piškur

1.1 A Brief History of Yeast Carbon Metabolism

Yeast fermentation of different plant carbohydrate sources, like grape must, is one of the oldest human technologies and its origins date back to the Neolithic period. These fermentations were initially spontaneous and their chemical, physiological, or microbiological background were not understood until the late eighteenth century. The first scientific studies were presented by Antoine Lavoisier in 1789, and he was the first who proposed the chemical changes, conversion of sugar into alcohol and CO_2, occurring during wine fermentation. In 1836–1837 Charles Cagniard-Latour, Friedrich Kützing, and Theodor Schwann independently found that alcoholic fermentation was carried out by living organisms, the sugar fungus. Starting in the late 1850s Louis Pasteur carried out physiological studies; he also clearly demonstrated the role of yeast in alcoholic fermentation, and determined the quantitative differences between aerobic and anaerobic conversion of sugar. Under Pasteur's influence, Emil Christian Hansen isolated the first pure yeast cultures and used them in malt-based fermentation, thereby starting a revolution in brewing industry (reviewed in Barnet 1998, 2003a).

In 1897, Eduard Buchner carried out fermentation by cell-free extracts and opened the way to determine the main biochemical steps. Further studies of yeast and muscle revealed the pathway of glycolysis and demonstrated that under anaerobic conditions pyruvate in yeast was converted into ethanol, whereas muscle

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_1, © Springer-Verlag Berlin Heidelberg 2014

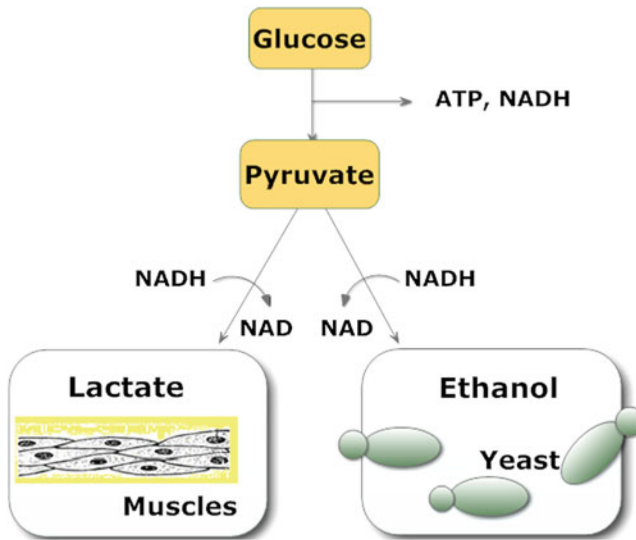

Fig. 1.1 Generation of lactate and ethanol. Sugar is in general degraded to pyruvate and later to CO_2, releasing the stored chemical energy. However, if the availability of oxygen is low, then pyruvate can be reduced either to lactate (in the muscle cell) or to ethanol (in the yeast cell). In this way only little ATP is generated but the NAD/NADH balance is preserved

converted it to lactate (Fig. 1.1). Soon afterward, it became recognized that glycolysis was a universal biochemical pathway found in all organisms and yeast one of the central model organisms to understand this crucial pathway. In the first half of the twentieth century, the discovery of phosphorylated compounds, phosphate bond energy, NAD, and NADH, added important details to the understanding of the energy and redox aspects of the glycolytic and fermentation pathways (reviewed in Barnett 2003b, c).

From the mid-twentieth century, yeast became one of the central model organisms to study the role of genes behind different physiological and biochemical traits. Availability of the first mutants opened a new window to understand the molecular background of alcohol fermentation. For example, in the 1950s and 1960s a lot of focus was on respiration deficient mitochondrial mutants, the so-called *petites* (for review see Piskur 1994).

Over the last few decades, gene sequences have enabled us to study and understand regulatory mechanisms determining the quantitative outcome of sugar conversion into ethanol. In 1996, *Saccharomyces cerevisiae* became the first eukaryote to have its genome fully sequenced (Goffeau et al. 1996). This represented the beginning of the genomics and post-genomic era (for review see Piskur and Langkjaer 2004), which provided new in silico-based approaches and tools, such as comparative genomics, transcriptomics, proteomics, and metabolomics. These global approaches can now provide further insight into the regulatory networks operating at various levels in the yeast carbon metabolism.

1.2 Yeast Biodiversity

In 2011, almost 99,000 fungal species were described, and more recent data from high-throughput sequencing approaches supported an estimate of over 3.5 million species; thus, a majority of fungi still needs to be described (McLaughlin et al. 2009). While a majority of fungi live as multicellular organisms, several lineages have independently adopted a unicellular life mode, the so-called yeast form.

In nature, yeasts are the predominant group of organisms involved in breakdown of simple carbohydrates, especially in the autumn when fruits ripen. Yeasts also include important industrial organisms, pathogens, and popular laboratory organisms that serve as general models to understand the eukaryotic cell. For decades *S. cerevisiae*, baker's yeast, has been one of the best characterized organisms from the genetics, biochemistry, and physiology points of view, and the first eukaryote with a sequenced genome. In analogy with fungi in general, several hundred yeast species have been described so far, but these represent only a small fraction of yeast biodiversity on our planet (Kurtzman et al. 2011).

Within Ascomycota two yeast lineages are well-studied, one is called *Saccharomycetes* (*Hemiascomycetes*) and includes *S. cerevisiae*, and the other is *Schizosaccharomycetes* and includes the fission yeast *Schizosaccharomyces pombe* (Fig. 1.2) The two groups, separated more than 500 million years ago, have independently developed yeast life-forms (Medina et al. 2011). Several yeasts can also be found among *Basidiomycota*.

The *Hemiascomycetes* clade includes several interesting yeast groups, like the pathogenic *Candida* group (including *Candida albicans*), the wine yeasts *Dekkera*/*Brettanomyces*, the methylotrophic yeasts *Komagataella* (including *Komagataella*/*Pichia pastoris*, and the *Saccharomycotina* group (Kurtzman et al. 2011).

Many of the industrially interesting yeasts belong to *Saccharomycotina,* and this family covers over 200 million years of the yeast evolutionary history; and includes six post-whole genome duplication (post-WGD) genera: *Saccharomyces*, *Kazachstania*, *Naumovia*, *Nakseomyces*, *Tetrapisispora,* and *Vanderwaltozyma*; and six non-WGD genera: *Zygosaccharomyces*, *Zygotorulaspora*, *Torulaspora*, *Lachancea*, *Kluyveromyces,* and *Eremothecium* (Kurtzman and Robnett 2003; Casaregola et al. 2011). A rough phylogenetic relationship among a few *Hemiascomycetes* yeasts, thoroughly presented in later chapters, is shown in Fig. 1.2.

1.3 Sugar Uptake

Yeast needs a supply of energy for growth and maintenance of the biological order in the cell. This energy comes from the chemical energy stored in food molecules, such as carbohydrates. Before sugars, such as glucose and fructose, can be utilized in the cell, they need to be transported through the plasma membrane. Monohexose uptake in yeast is mainly mediated by hexose transporters (*HXT*). In *S. cerevisiae*

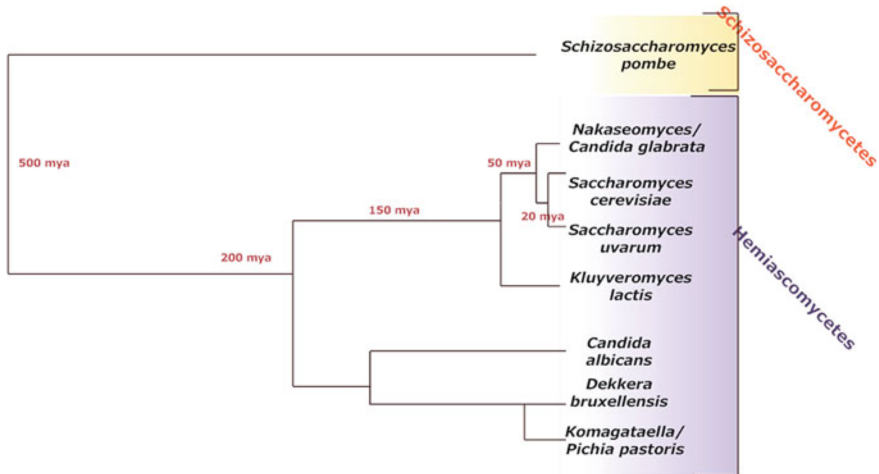

Fig. 1.2 Phylogenetic relationship among some studied yeasts. Note that some of the shown yeast lineages separated from each other many million years ago and have therefore accumulated several molecular and physiological changes regarding their carbon metabolism

all monohexose transport takes place by facilitated diffusion. The *HXT* family in this yeast has 20 members, identified as *HXT1-17*, *GAL2*, *SNF3,* and *RGT2,* and they differ in their affinity toward glucose.

Snf3p and Rgt2p act as sensors of extracellular glucose, in particular, Snf3p senses low glucose levels, and Rgt2p high glucose levels (Özcan and Johnoston 1999). In this way *S. cerevisiae* can detect the availability of glucose in the environment and respond by expressing the appropriate transporters. This is due to the combined action of different regulatory mechanisms, including transcriptional regulation of some *HXT* genes and inactivation of Hxt transporters in response to extracellular glucose (Sabina and Johnston 2009). The high-affinity transporters are most useful when glucose is scarce, and the corresponding genes are repressed by high glucose levels. The low-affinity glucose transporter Hxt1 is, on the contrary, expressed when glucose is abundant (Diderich et al. 1999; Kaniak et al. 2004). This controlled expression can then allow *S. cerevisiae* to fine tune sugar uptake in response to the substrate availability.

Also, the uptake of amino acids and nucleotide bases is relatively well-studied in several yeast models. The ability to use different amino acids, purines, and pyrimidines varies among different yeasts (Kurtzman et al. 2011). On the other hand, the uptake of free fatty acids has not yet been fully characterized in yeasts (Casal et al. 2008; see also further chapters).

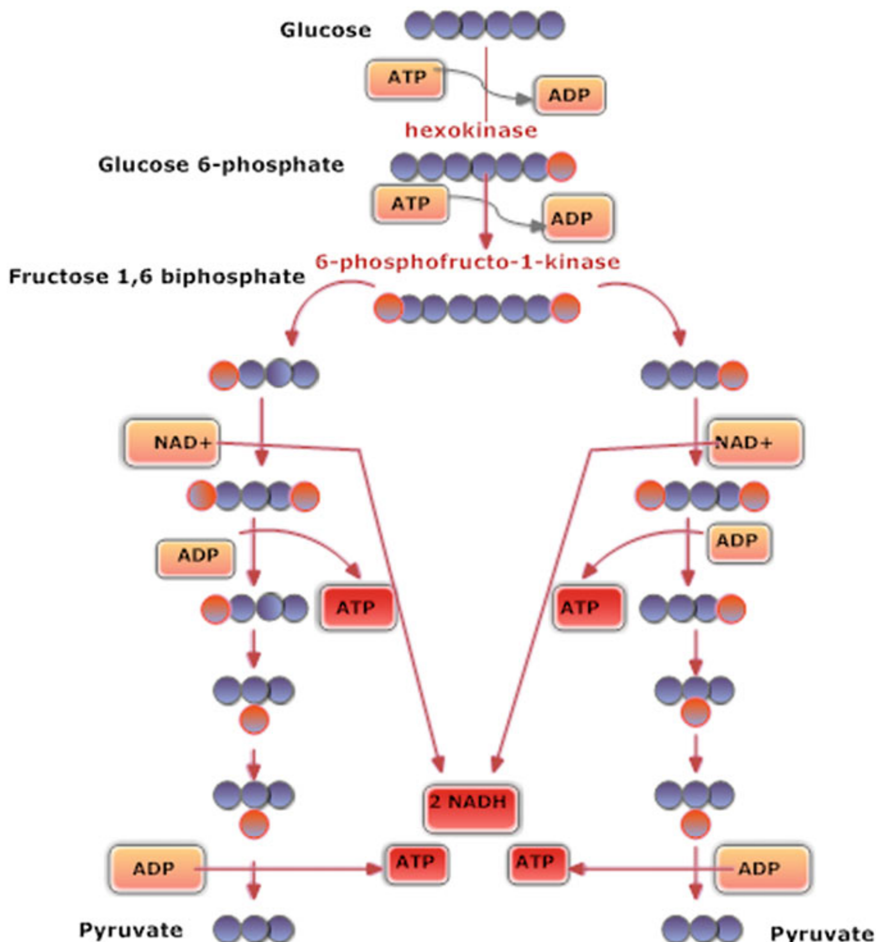

Fig. 1.3 Glycolytic pathway degrades glucose to pyruvate. Glycolysis is a several-step degradation pathway found in many bacteria and almost all eukaryotes. Carbon molecules are shown as *blue circles*, and phosphates as *red circles*. Only the most central intermediates and enzymes are presented. The final products obtained from one molecule of glucose are two molecules of pyruvate, two reduced NADH molecules, and two ATP molecules

1.4 Glycolysis and Fermentation

Sugars, which are preferable yeast food, must be broken down into smaller molecules to become a source of energy and building blocks for the synthesis of other molecules. The major initial process for oxidizing sugars, glycolysis, is a series of ten reactions breaking down a glucose molecule into two molecules of pyruvate. During glycolysis, the cell produces ATP and NADH, without the involvement of molecular oxygen (Fig. 1.3). In the first step, glucose is activated by hydrolysis of

one ATP molecule to generate glucose 6-phosphate. In *S. cerevisiae*, this reaction is catalyzed by three hexokinases (EC 2.7.1.1) encoded by *HXK1*, *HXK2,* and *GLK1*. Hxk2p plays also an important role in the regulation of glucose metabolism, being involved in the mechanism of glucose repression (Klein et al. 1998; Palaez et al. 2010). Later, another ATP molecule is used to generate a central intermediate fructose 1,6-biphosphate, which may play one of the central roles in the regulation of the carbon flow in the cell. The key enzyme catalyzing the irreversible generation of fructose 1,6-biphosphate is 6-phosphofructo-1-kinase (EC 2.7.1.11) and is encoded by *PFK1* and *PFK2*. Studies on the regulation of glycolytic genes indicate that glucose strongly induces the expression of *PFK1* and *PFK2* (Moore et al. 1991). On the other hand, phosphofructokinase activity is inhibited by ATP and citrate and activated by other adenine nucleotides and fructose-2,6-bisphosphate (F2,6P).

Fructose 1,6-biphosphate is converted into two three-carbon products, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. These two intermediates can be reversibly interconverted. The two three-carbon molecules are oxidized in several steps and electrons removed by NAD resulting in two NADH molecules (from one glucose molecule). The reduced equivalents need to be later re-oxidized in the respiratory chain or during the fermentation pathways to keep the redox balance. Four ATP molecules are also generated during the last glycolytic steps resulting in the final production of two ATP molecules per molecule of glucose. The final glycolysis products are also two molecules of pyruvate. In a majority of organisms, pyruvate is transported into the mitochondria, where it is converted into acetyl CoA and CO_2, and the former later completely oxidized in the presence of molecular O_2 into CO_2 and H_2O. The conversion of pyruvate into acetyl CoA is catalyzed by the pyruvate dehydrogenase complex in the mitochondria. However, in many yeasts, cytoplasmic pyruvate enters into the alcoholic fermentation pathway (Fig. 1.4). Yeasts, depending on conditions, can use sugars by fermentation and/or by respiration (Flores et al. 2000). Since respiration of sugars is energetically more favorable than fermentation, most organisms use fermentation only when respiration is impaired, for example when oxygen availability decreases. However, in several yeast species, like *S. cerevisiae*, the metabolic destiny of pyruvate formed at a high rate is largely switched from respiration to fermentation even when oxygen is abundant. In other words, *S. cerevisiae* may ferment sugars also under aerobic conditions, showing the so-called “Crabtree positive” phenotype (for review see Pronk et al. 1996; Piskur et al. 2006).

The first step in the production of ethanol from pyruvate is the cytosolic decarboxylation to acetaldehyde and CO_2 by the enzyme pyruvate decarboxylase (EC 4.1.1.1). In *S. cerevisiae* three genes encode this enzymatic activity, *PDC1*, *PDC5,* and *PDC6*. *PDC2* encodes a positive regulator of the transcription of *PDC1* and *PDC5* (Hohmann and Cederberg 1990; Hohmann 1993). Acetaldehyde is then reduced to ethanol by the activity of alcohol dehydrogenase (EC 1.1.1.1), leading to the cytosolic re-oxidation of glycolytic NADH. In *S. cerevisiae* five genes encode alcohol dehydrogenases involved in ethanol metabolism (Thomson et al. 2005). Four of these enzymes, Adh1p, Adh3p, Adh4p, and Adh5p, preferentially reduce

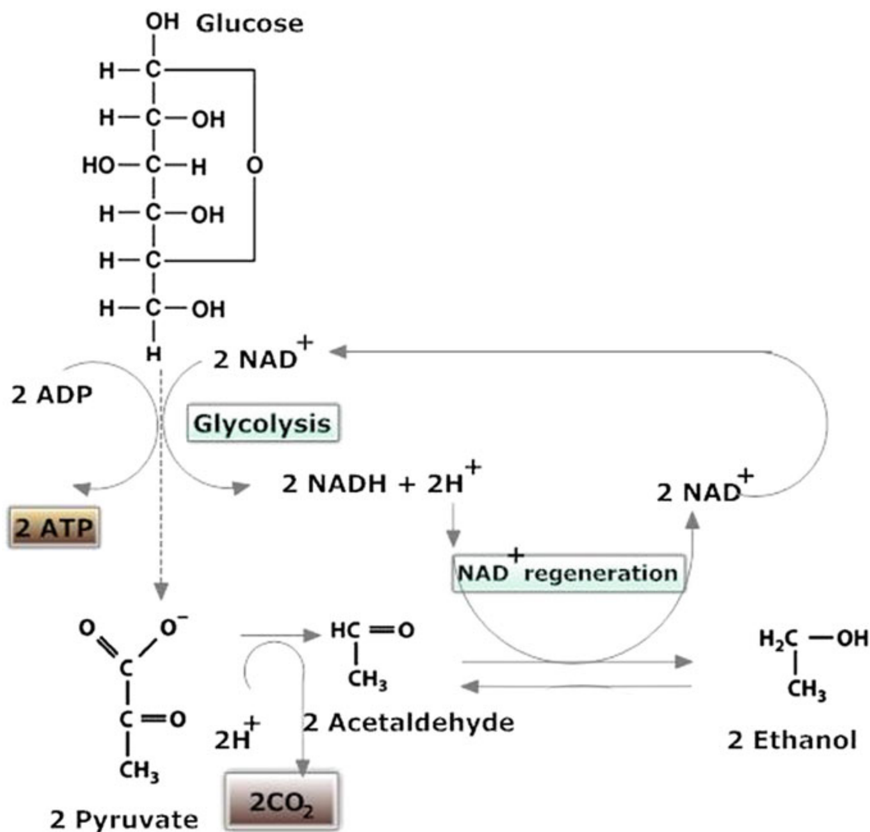

Fig. 1.4 Alcohol fermentation pathway. NADH generated in the glycolytic pathway is re-oxidized to NAD by the help of acetaldehyde reduction to ethanol. The regeneration of NAD thus results in redox balance of the glycolytic and fermentation pathways. The conversion of acetaldehyde into ethanol is reversible and catalyzed by alcohol dehydrogenases

acetaldehyde to ethanol during glucose fermentation, whereas Adh2p preferentially catalyzes the reverse reaction of oxidizing ethanol to acetaldehyde. As described elsewhere in this book, the alcohol fermentation pathway is crucial for production of bio-ethanol and alcoholic beverages.

In addition to this route, pyruvate can also be converted into acetyl CoA at the cytoplasmic level by "pyruvate dehydrogenase bypass." This pathway involves the conversion of acetaldehyde into acetic acid, which is then converted into acetyl CoA. The involved enzymes are an NADP-dependent acetaldehyde dehydrogenase isoform (Ald6p) and an acetyl CoA synthetase (van den Berg and Steensma 1995; Saint-Prix et al. 2004). This bypass route has in *S. cerevisiae* been demonstrated to be essential for growth on glucose, because it is the only source of cytoplasmic acetyl CoA and thus necessary for lipid biosynthesis (van den Berg and Steensma 1995).

1.5 Respiration Part I: Krebs Cycle and Fatty Acid Beta-Oxidation

In eukaryotes, if oxygen is present, the pyruvate produced during glycolysis is transported from cytoplasm into the mitochondria. There, it is decarboxylated and oxidized by a complex of three enzymes, called pyruvate dehydrogenase. In yeast, the decarboxylation reaction is catalyzed by the E1 alpha subunit (EC 1.2.4.1), encoded by the *PDA1* gene. The products are acetyl CoA, NADH, and CO_2. *S. cerevisiae* can also use fatty acids as carbon and energy sources. In this case a remarkable proliferation of peroxisomes is observed, in which fatty acid beta-oxidation occurs (Hiltunen et al. 2003). Fatty acids are broken down by a cycle of reactions, which remove two carbons at a time from their carboxyl end, generating acetyl CoA (Trotter 2001). The main export route of this compound is through the peroxisomal isoform of citrate synthase Cit2p (see also below), and citrate is then able to leave the peroxisomal compartment. Export of acetyl CoA is also accomplished through its conjugation to carnitine by the carnitine acetyl transferase Cat2p, which is localized in both peroxisomes and mitochondria. This pathway is only possible when yeast cells are grown in rich media that contain carnitine, which otherwise cannot be synthesized by the yeast cell. Acetyl CoA is a central intermediate where the sugar and fatty acid degradation pathways meet (Fig. 1.5). The acetyl CoA molecules still store a majority of useful energy and this gets first released in the subsequent degradation cycles.

The citric acid cycle, also known as the tricarboxylic (TCA) acid cycle or the Krebs cycle, accounts for a majority of the total oxidation of carbon compounds in most cells. The Krebs cycle is also an important provider for several substrate molecules, which are crucial in de novo biosynthesis of several amino acids and other essential cellular compounds. Acetyl CoA reacts with oxaloacetate in a reaction catalyzed by citrate synthase (EC 2.3.3.1) to produce citrate. Three genes encoding citrate synthases have been identified in *S. cerevisiae*: *CIT1* and *CIT3* encoding mitochondrial enzymes, and *CIT2* encoding the peroxisomal isoenzyme. In the next step citrate is converted into isocitrate by aconitase (Jia et al. 1997).

The genes corresponding to several of the Krebs cycle and fatty acid catabolism enzymes have been well characterized (Ciriacy 1977; Huynen et al. 1999; Black and DiRusso 2007). *IDH1* and *IDH2* encode the two subunits of the NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41), which transforms isocitrate in 2-oxoglutarate. Another mitochondrial NADP-specific isocitrate dehydrogenase catalyzes the oxidation of isocitrate to 2-oxoglutarate, encoded by *IDP1*, but other isoforms exist; a cytosolic one is encoded by *IDP2* and a peroxisomal one by *IDP3*. The mitochondrial 2-oxoglutarate dehydrogenase complex (EC 1.2.4.2) catalyzes the oxidative decarboxylation of 2-oxoglutarate to form succinyl-CoA. Succinate dehydrogenase (EC 1.3.5.1), composed of four subunits (Sdh1p, Sdh2p, Sdh3p, Sdh4p), couples the oxidation of succinate to the transfer of electrons to ubiquinone as part of the TCA cycle and the mitochondrial respiratory chain. FAD binding to Sdh1p is required for the assembly of the succinate dehydrogenase

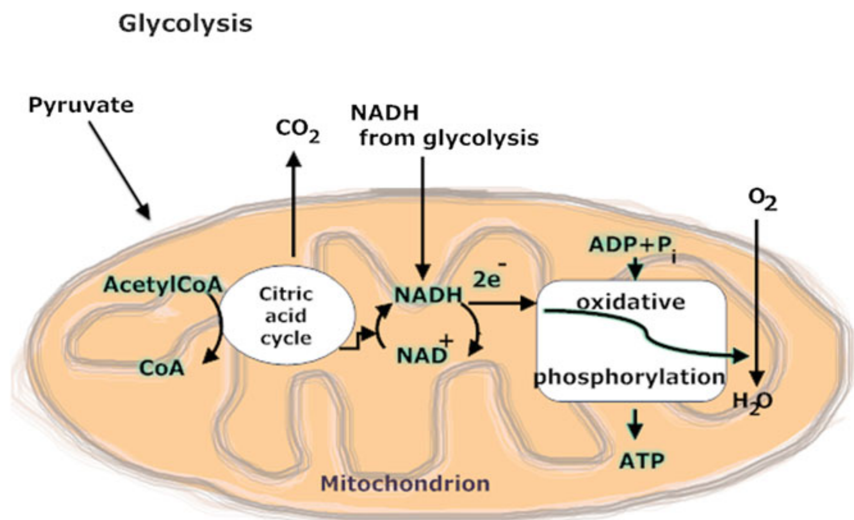

Fig. 1.5 The respiratory part of the sugar catabolism. If oxygen is available as the terminal acceptor of sugar electrons, pyruvate enters mitochondria and is converted into acetyl CoA. During the citric acid cycle CO_2 and reduced equivalents, such as NADH, are generated. NADH is re-oxidized during a cascade of reactions within the respiratory chain, a proton gradient is generated, and oxygen becomes the final acceptor of the electrons and is reduced to water. The proton gradient drives the synthesis of ATP

subunits. The fumarate molecule is then converted by fumarase (EC: 4.2.1.2) into L-malic acid in the TCA cycle; its cytosolic and mitochondrial distribution is determined by the N-terminal targeting sequence, protein conformation, and status of glyoxylate shunt. Mitochondrial malate dehydrogenase (EC 1.1.1.37), encoded by *MDH1* gene, catalyzes interconversion of malate and oxaloacetate. The cytoplasmic form (encoded by *MDH2*) and the peroxisomal one (encoded by *MDH3*) catalyze interconversion of malate and oxaloacetate in the glyoxylate cycle, during growth on two-carbon compounds as well as on fatty acids (described below).

1.6 Respiration Part II: Respiratory Chain and ATP Synthase

The major end products of acetyl CoA degradation through TCA cycle are CO_2 and high-energy electrons stored in NADH. During the respiration part, the NADH electrons are passed to a membrane-bound electron-transport cascade. The respiratory chain consists of several complexes: NADH dehydrogenase complex, cytochrome b-c_1 complex, and cytochrome oxidase complex (Fig. 1.5). In combination with oxygen, which is the terminal acceptor of electrons, proton gradient and H_2O are generated. It is interesting to point out that the genes coding for the

respiratory chain elements can be found in the nuclear as well as in the mitochondrial genome. One of the first studied classes of yeast mutants, called *petites*, were respiratory deficient strains with mitochondrial DNA, which had lost the coding ability for the respiratory chain and/or ATP synthase (reviewed in Piskur 1994). The cytochrome bc1 complex (also known as ubiquinol:cytochrome c oxidoreductase, ubiquinol:ferricytochrome c oxidoreductase, and respiratory complex III) (EC 1.10.2.2) is a highly conserved enzyme of the mitochondrial respiratory chain (Smith et al. 2012). In *S. cerevisiae* it consists of three catalytic subunits, Cobp, Rip1p, and Cyt1p, plus seven additional subunits: Cor1p, Qcr2p, Qcr6p, Qcr7p, Qcr8p, Qcr9p, and Qcr10p.

The nuclear *CYC1* and *CYC7* genes and the corresponding products, iso-1-cytochrome c and iso-2-cytochrome c are among the most thoroughly studied gene-protein systems (Sherman 1990) and pioneered the development of yeast molecular genetics. The cytochrome c oxidase complex (EC 1.9.3.1) catalyzes the terminal step in the electron transport chain involved in cellular respiration (Soto et al. 2012). This multisubunit enzyme of the mitochondrial inner membrane, also known as Complex IV, is composed of three core subunits encoded by the mitochondrial genome (Cox1p, Cox2p, and Cox3p) and eight additional subunits encoded by nuclear genes (Cox4p, Cox5Ap or Cox5Bp, Cox6p, Cox7p, Cox8p, Cox9p, Cox12p, and Cox13p).

The electron transport, through the established proton gradient, drives the synthesis of the majority of ATP (Fig. 1.5). The generation of ATP is catalyzed by F_1F_0 ATP synthase, which is a large, evolutionarily conserved enzyme complex required for ATP synthesis (EC 3.6.3.14) (Stuart 2008).

The efficiency of proton pumping by respiratory chain and ATP synthesis is represented by the P/O ratio (ATP formed per electron pair transferred to oxygen). Several independent yeast lineages, including *S. cerevisiae*, do not have Complex I. As a result, its effective P/O ratio is low, close to 1, and the complete glucose oxidation probably only yields 16 ATP (Bakker et al. 2001). In contrast, in mammals the complete oxidation of a glucose molecule can in theory produce about 30 molecules of ATP. On the other hand, during the glycolytic and fermentation pathways only 2 ATP molecules can be produced. In some yeast species alternative oxidases that transfer electrons from cytochrome c to molecular oxygen without proton translocation have been described (Veiga et al. 2003).

1.7 Glycolysis Reversed: Gluconeogenesis and Glyoxylate Cycle

Metabolism consists of catabolic and anabolic pathways. In the previous sections we mainly focused on the catabolic or degradation aspects. Gluconeogenesis is the process whereby glucose and other sugars are synthesized from other precursors, enabling yeast cells to grow on ethanol, glycerol, or peptone (amino acids).

In short, this pathway provides hexose building blocks when the yeast food consists of only C2 and C3 compounds (non-fermentable carbon sources). Reactions of gluconeogenesis mediate the conversion of pyruvate into glucose, in a sense the opposite of glycolysis. Overall, the gluconeogenic reactions convert two molecules of pyruvate into a molecule of glucose, with the expenditure of six high-energy phosphate bonds, four from ATP and two from GTP. While the two pathways, glycolysis and gluconeogenesis, have several reactions in common, they are not the exact reverse of each other. As the glycolytic enzymes 6-phosphofructo-1-kinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) only function in the forward direction, the gluconeogenesis pathway replaces those steps with the enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.49, encoded by *PCK1*), generating phosphoenolpyruvate from oxaloacetate, and with the enzyme fructose-1,6-bisphosphatase (EC 3.1.3.11, encoded by *FBP1*) to generate fructose-6-phosphate.

Intermediates from the TCA cycle are removed for biosynthesis, and the cycle needs to be replenished. The enzyme pyruvate carboxylase (EC 6.4.1.1, encoded by *PYC1* and *PYC2*) generates oxaloacetate from pyruvate. The glyoxylate cycle comprises many of the same reactions as the Krebs cycle, but intermediates and enzymes are located in the yeast peroxisome and cytoplasm, and decarboxylation enzyme activities are missing. Thus, this cycle allows cells to utilize simple carbon compounds as an energy and carbon source when glucose is absent. Two-carbon substrates, such as acetate or ethanol, can enter as acetyl CoA and are converted into four-carbon compounds and later to other essential compounds (Strijbis and Distel 2010). This cycle can also be a provider of NADH for de novo biosynthetic reactions and helps to balance the redox potential in the cell. Enzymes activities specific for this cycle are isocitrate lyase (EC 4.1.3.1 encoded by *ICL1*) and malate synthase (EC: 2.3.3.9 encoded by *MLS1*).

1.8 Aerobic and Anaerobic Yeasts

The availability of oxygen varies among different niches. One of the main problems an organism faces under anaerobic conditions is the lack of the final electron acceptor in the respiratory chain. This reduces or completely eliminates the activity of Krebs cycle, respiratory chain, and mitochondrial ATP synthases. As a response to hypoxic and anaerobic conditions, organisms have developed several processes to optimize the utilization of oxygen and even reduce the dependence on the presence of oxygen. According to the dependence on oxygen during the life cycle, yeasts are classified as: (i) obligate aerobes displaying exclusively respiratory metabolism, (ii) facultative fermentatives (or facultative anaerobes), displaying both respiratory and fermentative metabolism, and (iii) obligate fermentatives (or obligate anaerobes) (Merico et al. 2007).

The ability of yeasts to grow under oxygen-limited conditions seems to be strictly dependent on the ability to perform alcoholic fermentation. In other words, enough ATP should be generated during glycolysis to support the yeast growth,

and NADH generated during glycolysis gets re-oxidized. The yeast also needs to develop resistance to larger quantities of the final fermentation product, ethanol, as well as any other fermentation products. Apart from the energy and NADH/NAD redox problems, under anaerobic conditions, yeast must also find a way to run various reactions independent of the respiratory chain and a normal Krebs cycle. In other words, substrates (intermediates) for de novo reactions, for example for the amino acid pathways (synthesis), need to originate from a modified metabolic network. One of the alternative providers is the glyoxylate cycle. However, as detailed elsewhere in this book, under anaerobic growth, some mitochondrial Krebs cycle enzymes must be active, at least to generate 2-oxoglutarate.

Anaerobically growing *S. cerevisiae* cells contain only a few large, branched mitochondria (Visser et al. 1990). Under these conditions the mitochondria do not play a role in free-energy metabolism. However, important assimilatory reactions still take place in the mitochondria, generating NADH, which then needs to be re-oxidized (Nissen et al. 1997). During anaerobic growth in *S. cerevisiae* the Krebs cycle operates as two branches, but there is no flux from 2-oxoglutarate to fumarate (Nissen et al. 1997). Anaplerotic pathways and the glyoxylate cycle provide intermediates for anabolic reactions. Glycerol production is the primary redox sink for the re-oxidation of NADH, under anaerobic conditions (van Dijken and Scheffers 1986). The mitochondrial form of alcohol dehydrogenase Adh3p is essential to couple the reoxidation of mitochondrial NADH and glycerol formation (Bakker et al. 2000).

On the other hand, some yeast compounds, like unsaturated fatty acids and sterols, cannot be synthesized in the cell under anaerobiosis and must originate from the medium or from previous aerobic growth. Also, the ability to translocate ATP generated in the cytoplasm into the mitochondria, for biosynthetic purposes, should be well developed to survive in the absence of oxygen. In *S. cerevisiae*, the presence of the mitochondrial ATP transporters, encoded by *AAC2* and *AAC3*, is essential for growth under anaerobic conditions (Sabova et al. 1993; Betina et al. 1995).

1.9 Physiological Aspects: Crabtree Effect

One of the most prominent features of the baker's yeast *S. cerevisiae* is its ability to rapidly convert sugars into ethanol and carbon dioxide at both anaerobic and aerobic conditions. When oxygen is absent, acetaldehyde is the final electron acceptor and gets converted into ethanol under purely fermentative growth. Under aerobic conditions, respiration is possible with oxygen as the final electron acceptor, but *S. cerevisiae* still exhibits alcoholic fermentation until the sugar/glucose reaches a low level (Fig. 1.6). This phenomenon is called the Crabtree effect (De Deken 1966) and the yeasts expressing this trait called Crabtree-positive yeasts. In contrast, "Crabtree-negative" yeasts lack fermentative products, and under aerobic conditions, biomass and carbon dioxide are the sole products.

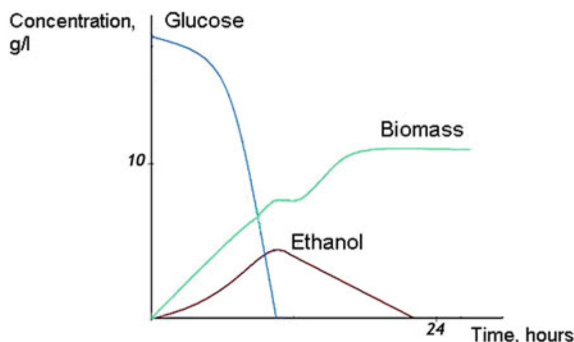

Fig. 1.6 Batch culture of a Crabtree-positive yeast. The yeast was grown under aerobic conditions in a defined minimal medium and the disappearance of glucose and the appearance of biomass and ethanol were followed. When glucose disappeared, a characteristic diauxic growth occurs, due to the utilization of ethanol, previously generated from glucose, as the main carbon source. This represents a switch between fermentative and respiratory metabolism

However, it is possible to obtain pure respiratory utilization of glucose by *S. cerevisiae* under aerobic conditions if the glucose concentration is kept very low in the medium, e.g., by using a glucose-limited continuous culture operating below a certain strain-specific threshold value (called "critical" dilution rate) or by using fed-batch cultivations (Postma et al. 1989). Briefly, glucose is sensed by the yeast cell, and this signal is transmitted further to diminish the respiratory activities (see further chapters). This glucose repression phenomenon involves different signal transduction pathways activated by extracellular and intracellular levels of glucose and related metabolites and/or their fluxes through the key glycolytic enzymes (reviewed in Klein et al. 1998; Johnston 1999; Westergaard et al. 2007). In other words, the complexity of glucose repression regulatory networks is still far from being completely understood. Some of the regulatory activities operate at the transcriptional regulation level and some may operate directly on the involved enzymes and their regulators.

There does not seem to be a clear consensus about the definition of the Crabtree effect, and different physiological and molecular approaches have been used as the background for the current definitions (von Meyenburg 1969; Barford and Hall 1981; Kappeli 1986; Alexander and Jeffries 1990). We define the long-term Crabtree effect as aerobic alcoholic fermentation under steady-state conditions at high growth rates. When *S. cerevisiae* is cultivated in a glucose-limited chemostat, the long-term effect appears when the dilution rate (or in other words: the glucose uptake rate) exceeds the strain-specific threshold value. The same effect is observed also when yeast cells are cultivated in glucose-based conditions, e.g., batch cultivations. The molecular background for the long-term Crabtree effect seems to be a limited respiratory capacity due to the repression of the corresponding respiration associated genes (Postma et al. 1989). On the other hand, we define the short-term Crabtree effect as the immediate appearance of aerobic alcoholic fermentation upon

addition of excess sugar to sugar-limited and respiratory cultures. This effect has also been explained as an overflow in the sugar metabolism and could be associated directly with the biochemical properties of the respiration-associated enzymes and their regulators (Petrik et al. 1983; Postma et al. 1989; Pronk et al. 1996). In addition, it could depend on immediate repression of some key genes involved in respiration. However, it is still unclear if the regulatory molecular mechanisms operating during the long-term and short-term Crabtree effect are indeed different from each other. A very interesting aspect is the evolutionary background for the development of these regulatory mechanisms (Piskur et al. 2006).

The oxygen availability during sugar metabolism can also determine other kinds of effects. The Pasteur effect has been defined as the inhibition of fermentative metabolism by oxygen, but in *S. cerevisiae* this phenomenon is observable only at low glycolytic fluxes (Pronk et al. 1996). In some yeast species the absence of oxygen impairs the utilization of particular disaccharides, although one or both of the monosaccharide components can be used anaerobically by fermentation, the so-called "Kluyver effect" (Fukuhara 2003). This characteristic seems to be determined mainly by the activity of sugar carriers (Goffrini et al. 2002). The inhibition of fermentation of glucose as well as of other sugars in the absence of oxygen has been described as the Custer effect. This effect has been found in some *Brettanomyces/Dekkera* and *Candida* species, and proposed to be due to a redox imbalance (van Dijken and Scheffers 1986), as confirmed in a recent study (Galafassi et al. 2013).

1.10 Regulatory Aspects

In *S. cerevisiae*, the main elements of the glucose sensing systems have been studied from many years, and apparently the main involved elements have been characterized (Fig. 1.7). Snf3p and Rgt2p have been shown to likely act as receptors that sense external glucose concentration, Snf3p for low and Rgt2p for high levels of glucose (Özcan and Johnoston 1999). The signal is then transduced to Rgt1p, a transcription factor for glucose-regulated genes. The signal that causes the repressing capacity of Rgt1p is lost, allowing derepression of the appropriate *HXT* genes and enabling glucose transport. Another sugar signaling mechanism has been described, the Gpr1-Gpa2 system, which is linked to the Ras/cAMP-PKA cascade. This system mediates many effects produced by glucose, as stimulating glycolysis, inhibiting gluconeogenesis, regulating carbohydrate metabolism, as well as cell cycle progression, stress response, and ribosomal biogenesis (Verstrepen et al. 2004). Transcription activators Rap1/Gcr1/Gcr2 and Sfp1 are known to stimulate the transcription of glycolytic and ribosomal protein genes in response to the glucose presence (Shore 1994; Clifton and Fraenkel 1981; Marion et al. 2004).

The presence of glucose is also known to trigger the repression of hundreds of genes. The key elements are: (i) transcription repressors Mig1, Mig2, and Mig3; (ii) protein kinase Snf1; and (iii) protein phosphatase Glc7-Reg1. A drop in level

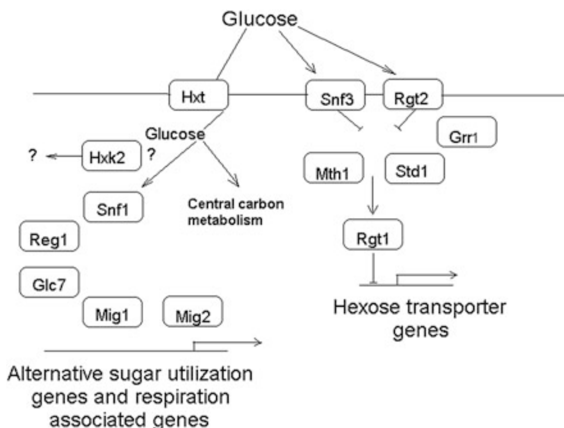

Fig. 1.7 Regulatory aspects of glucose utilization. Some of the main players in yeast glucose sensing and signal transduction are shown. Some of these elements can act as activators or repressors of the terminal genes involved in glucose and other sugars uptake and metabolism

of glucose activates the Snf1 kinase activity, causing phosphorylation of Mig1p, and promotes the Mig1p to leave the nucleus. This results in de-repression of glucose-repressed genes. The sensor of this pathway is not yet known, but it is believed to involve Hxk2p, which also acts in the nucleus as a regulator of the transcription of several Mig-regulated genes (through interaction with Mig1 and Snf1 protein kinase). Under high glucose conditions, in fact, it has been found that nuclear Hxk2p stabilises the repressor complex blocking the phosphorylation of Mig1 by Snf1 kinase (Ahuatzi et al. 2007). Recent developments have provided two important observations: the cross-talk between the different pathways involved in carbon source metabolism and the induction/repression duality of some involved transcription factors, like Mig1, Rgt1, and others (Westergaard et al. 2007; Santangelo 2006; see also further chapters).

An important regulatory system is the Hap2/3/4/5p complex, which activates transcription of genes encoding respiratory chain components and enzymes of the TCA cycle in the absence of easily fermentable carbon sources such as glucose (Schuller 2003). The utilization of non-fermentable substrates requires also the induction/derepression of gluconeogenesis and glyoxylate enzymes, which occurs by carbon source-responsive element (CSRE) binding factor Cat8p (Schuller 2003). *HAP4* and *CAT8* are both glucose repressed genes.

The availability of oxygen is another environmental factor that widely affects cell metabolism. Functional analyses indicate that the transcription factors Rox1p and Upc2p predominate in the regulation of carbon metabolism, lipid metabolism, and cell wall maintenance (Kwast et al. 2002; see also further chapters).

1.11 Evolution of Ethanol Fermentation

A majority of ascomycotic fungi under aerobic conditions convert sugar-based substrates into CO_2. However, at least three groups, including budding and fission yeasts, have apparently independently evolved the metabolic ability to produce

ethanol in the presence of oxygen and excess of glucose (reviewed in Rozpedowska et al. 2011; Rhind et al. 2011). This metabolic invention (Crabtree effect), represents in nature a possible tool to outcompete other microbes. For example when fruits ripen, microbial communities start a fierce competition for the freely available sugars. Yeasts from the *Saccharomyces* clade, which are good ethanol producers and also ethanol-resistant and facultative anaerobes, soon become the predominant microbes in these niches. Both groups of ethanol-producing budding yeast, including *S. cerevisiae* and *Dekkera bruxellensis*, can also efficiently catabolize ethanol, and therefore their corresponding life style has been named as "make-accumulate-consume (ethanol)" strategy (Thomson et al. 2005; Piskur et al. 2006; Rozpedowska et al. 2011). On the other hand, the third Crabtree positive group, including the fission yeast *Sch. pombe*, only poorly metabolizes ethanol (Fig. 1.2).

The onset of yeast genomics (Goffeau et al. 1996) has provided a tool to reconstruct several molecular events, which have reshaped the budding yeasts during their evolutionary history (reviewed in Dujon 2010). Several molecular events have left a clear fingerprint in the modern genomes, while the origin of more complex traits, like the Crabtree effect, is often not easy to determine using only a genome analysis approach. Until recently, very few yeast species have been studied for their carbon metabolism (Merico et al. 2007). In a recent work, over 40 different *Saccharomycetales* yeasts have been studied for the presence of long-term Crabtree effect and it has been found that this effect originated after the split of the *Saccharomyces-Lachancea* and *Kluyveromyces-Eremothecium* lineages, approximately 125 million years ago, prior to the whole genome duplication (WGD) event, and after the loss of the respiratory complex I (Hagman et al. 2013). The origin of modern plants with fruits, more than 125 million years ago, brought to microbial communities a new larger and increasingly abundant source of food based on simple sugars. On the other hand, ancient yeasts could hardly produce the same amount of new biomass as bacteria during the same time interval, and could therefore be outcompeted. We speculate that slower growth rate could in principle be counteracted by production of compounds that could inhibit the growth rate of bacteria, like ethanol and acetate. However, the initial molecular mechanisms that promoted the evolution of the new "lifestyle" and rewiring of the carbon metabolism are so far not identified.

References

Alexander MA, Jeffries TW (1990) Respiratory efficiency and metabolize partitioning as regulatory phenomena in yeasts. Enz Microbiol Technol 12:2–19

Ahuatzi D, Riera A, Pelaez R, Herrero P, Moreno F (2007) Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. J Biol Chem 282:4485–4493

Bakker BM, Bro C, Kötter P, Luttik MAH, van Dijken JP, Pronk JT (2000) The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in *Saccharomyces cerevisiae*. J Bacteriol 182:4730–4737

Bakker BM, Overkamp KM, van Maris AJA, Kotter P, Luttik MAH, van Dijken JP, Pronk JT (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Micr Rev 25:15–37

Barford JP, Hall RJ (1981) A mathematical model for the aerobic growth of *Saccharomyces cerevisiae* with a saturated respiratory capacity. Biotechnol Bioeng 23:1735–1762

Barnett JA (1998) A history of research on yeast 1: Work by chemists and biologists 1789–1850. Yeast 14:1439–1451

Barnett JA (2003a) Beginnings of microbiology and biochemistry: the contribution of yeast research. Microbiology 149:557–567

Barnett JA (2003b) A history of research on yeasts 5: the fermentation pathway. Yeast 20:509–543

Barnett JA (2003c) A history of research on yeasts 6: the main respiratory pathway. Yeast 20:1015–1044

Betina S, Garurnikova G, Haviernik P, Sabova L, Kolarov J (1995) Expression of the Aac2 gene encoding the major mitochondrial ADP/ATP translocator in *Saccharomyces cerevisiae* is controlled at the transcriptional level by oxygen, heme and HAP2 factor. Eur J Biochem 229:651–657

Black PN, Di Russo CC (2007) Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. Biochim Biophys Acta 1771:286–298

Casal M, Paiva S, Queirós O, Soares-Silva I (2008) Transport of carboxylic acids in yeasts. FEMS Microbiol Rev 32:974–994

Casaregola S, Weiss S, Morel G (2011) New perspectives in hemiascomycetous yeast taxonomy. C R Biol. 334:590–598

Ciriacy M (1977) Isolation and characterization of yeast mutants defective in intermediary carbon metabolism and in carbon catabolite derepression. Mol Gen Genet 154:213–220

Clifton D, Fraenkel DG (1981) The gcr (glycolysis regulation) mutation of *Saccharomyces cerevisiae*. J Biol Chem 256:13074–13078

Diderich JA, Schepper M, van Hoek P, Luttik MAH, van Dijken JP, Pronk JT, Klaassen P, Boelens HFM, Texteira de Mattos MJ, van Dam K, Kruckeberg AL (1999) Glucose uptake kinetics and transcription of *HXT* genes in chemostat cultures of *Saccharomyces cerevisiae*. J Biol Chem 274:15350–15359

De Deken RH (1966) The Crabtree effect: a regulatory system in yeast. J Gen Microbiol 44:149–156

Dujon B (2010) Yeast evolutionary genomics. Nature Rev Genet 11:512–524

Flores CL, Rodríguez C, Petit T, Gancedo C (2000) Carbohydrate and energy-yielding metabolism in non-conventional yeasts. FEMS Microbiol Rev 24:507–529

Fukuhara H (2003) The Kluyver effect revisited. FEMS Yeast Res 3:327–331

Galafassi S, Capusoni C, Moktaduzzaman M, Compagno C (2013) Utilization of nitrate abolishes the ''Custers effect'' in *Dekkera bruxellensis* and determines a different pattern of fermentation products. J Ind Microbiol Biotechnol 40:297–303

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B et al (1996) Life with 6000 genes. Science 274:547–563

Goffrini P, Ferrero I, Donnoni C (2002) Respiration-dependent utilization of sugars in yeasts: a determinant role for sugar transporters. J Bacteriol 184:427–432

Hagman A, Säll T, Compagno C, Piskur J (2013) Yeast "make-accumulate-consume" life strategy evolved as a multi-step process that predates the whole genome duplication. PLoS ONE 8:e68734

Hiltunen JK, Mursula AM, Rottensteiner H, Wierenga RK, Kastaniotis AJ, Gurvitz A (2003) The biochemistry of peroxysomal beta-oxidation in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 27:35–64

Hohmann S, Cederberg H (1990) Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. Eur J Biochem 188:615–621

Hohmann S (1993) Characterization of *PDC2*, a gene necessary for high-level expression of pyruvate decarbolylase structural genes in *Saccharomyces cerevisiae*. Mol Gen Genet 241:657–666

Huynen MA, Dandekar T, Bork P (1999) Variation and evolution of the citric-acid cycle: a genomic perspective. Trends Microbiol 7:281–291

Johnston M (1999) Feasting, fasting and fermenting: glucose sensing in yeast and other cells. Trends Gen 15:29–33

Jia YK, Bécam AM, Herbert CJ (1997) The *CIT3* gene of *Saccharomyces cerevisiae* encodes a second mitochondrial isoform of citrate synthase. Mol Microbiol 24:53–69

Kaniak A, Xue D, Macool J, Kim JH, Johnoston M (2004) Regulatory network connecting two glucose signal transduction pathways in *Saccharomyces cerevisiae*. Euk Cell 3:221–231

Kappeli O (1986) Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts. Adv Microbiol Physiol 28:181–209

Klein CJL, Olsson L, Nielsen J (1998) Glucose control in *Saccharomyces cerevisiae*: the role of *MIG1* in metabolic functions. Microbiology 144:13–24

Kurtzman CP, Robnett CJ (2003) Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses. FEMS Yeast Res 3:417–432

Kurtzman CP, Fell JW, Boekhout T (2011) The yeasts, a taxanomic study, 5th edn. Elsevier, London

Kwast KE, Lai L-C, Menda N, James DT III, Aref S, Burke PV (2002) Genomic analysis of anaerobically induced genes in *Saccharomyces cerevisiae*: functional role of Rox1 and other factors in mediating the anoxic response. J Bacteriol 184:250–265

Marion RM, Regev A, Segal E, Barash Y, Koller D, Friedman N, O'Shea EK (2004) Sfp1p is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression. Proc Natl Acad Sci USA 101:14315–14322

McLaughlin DJ, Hibbett DS, Lutzoni F, Spatafora JW, Vilgalys R (2009) The search for the fungal tree of life. Trends Microbiol 17:488–497

Medina EM, Jones GW, Fitzpatrick DA (2011) Reconstructing the fungal tree of life using phylogenomics and a preliminary investigation of the distribution of yeast prion-like proteins in the fungal kingdom. J Mol Evol 73:116–133

Merico A, Sulo P, Piskur J, Compagno C (2007) Fermentative lifestyle in yeasts belonging to the *Saccharomyces* complex. FEBS J 274:976–989

Moore PA, Sagliocco FA, Wood RM, Brown AJ (1991) Yeast glycolytic mRNAs are differentially regulated. Mol Cell Biol 1:5330–5337

Nissen TL, Schulze U, Nielsen J, Villadsen J (1997) Flux distribution in anaerobic, glucose-limited continuous cultutres of *Saccharomyces cerevisiae*. Microbiology 143:203–218

Özcan S, Johnoston M (1999) Function and regulation of yeast hexose transporters. Microbiol Mol Biol 63:554–569

Pelaez R, Herrero P, Moreno F (2010) Functional domains of yeast hexokinase 2. Biochem J 432:181–190

Petrik M, Kappeli O, Fiechter A (1983) An expanded concept for the glucose effect in the yeast *Saccharomyces cerevisiae*: involvement of short- and long-term regulation. J Gen Microbiol 129:43–49

Piskur J (1994) Inheritance of the yeast mitochondrial genome. Plasmid 31:229–241

Piskur J, Langkjaer RB (2004) Yeast genome sequencing: the power of comparative genomics. Mol Microbiol 53:381–389

Piskur J, Rozpedowska E, Polakova S, Merico A, Compagno C (2006) How did *Saccharomyces cerevisiae* evolve to become a good brewer? Trends Gen 22:183–186

Postma E, Verduyn C, Scheffers WA, van Dijken JP (1989) Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Env Microbiol 55:468–477

Pronk JT, Steensma HY, van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12:1607–1633

Rhind N, Chen Z, Yassour M, Thompson DA, Haas BJ et al (2011) Comparative functional genomics of the fission yeasts. Science 332:930–936
Rozpędowska E, Hellborg L, Ishchuk OP, Orhan F, Galafassi S, Merico A, Woolfit M, Compagno C, Piskur J (2011) Parallel evolution of the make-accumulate-consume strategy in *Saccharomyces* and *Dekkera* yeasts. Nat Commun 2:302
Sabina J, Johnoston M (2009) Asymmetric signal transduction through paralogs that comprise a genetic switch for sugar sensing in *Saccharomyces cerevisiae*. J Biol Chem 284:29635–29642
Sabova L, Zeman I, Supek F, Koralov J (1993) Transcriptional control of the *AAC3* gene encoding mitochondrial ADP/ATP translocator in *Saccharomyces cerevisiae* by oxygen, heme and ROX1 factor. Eur J Biochem 213:547–553
Saint-Prix F, Bonquist L, Dequin S (2004) Functional analysis of the *ALD* gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the NADP-dependent Ald6p and Ald5p isoforms play a major role in acetate formation. Microbiology 150:2209–2220
Santangelo GM (2006) Glucose signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 70:253–282
Schuller H-J (2003) Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. Curr Genet 43:139–160
Sherman F (1990) Studies of yeast cytochrome c: how and why they started and why they continued. Genetics 125:9–12
Shore D (1994) RAP1: a protean regulator in yeast. Trends Genet 10:408–412
Smith PM, Fox JL, Winge DR (2012) Biogenesis of the cytochrome bc(1) complex and role of assembly factors. Biochim Biophys Acta 1817:872–882
Soto IC, Fontanesi F, Liu J, Barrientos A (2012) Biogenesis and assembly of eukaryotic cytochrome c oxidase catalytic core A. Biochim Biophys Acta 1817:883–897
Strijbis K, Distel B (2010) Intracellular acetyl unit transport in fungal carbon metabolism. Eukaryot Cell 9:1809–1815
Stuart RA (2008) Supercomplex organization of the oxidative phosphorylation enzymes in yeast mitochondria. J Bioenerg Biomembr 40:411–417
Thomson JM, Gaucher EA, Burgan MF, De Kee DW, Li T, Aris JP, Benner SA (2005) Resurrecting ancestral alcohol dehydrogenases from yeast. Nat Genet 37:630–635
Trotter PJ (2001) The genetics of fatty acid metabolism in *Saccharomyces cerevisiae*. Annu Rev Nutr 21:97–119
van den Berg MA, Steensma HY (1995) *ACS2*, a *Saccharomyces cerevisiae* gene encoding acetyl-cenzyme A synthetase, essential for growth on glucose. Eur J Biochem 231:704–713
van Dijken JP, Scheffers WA (1986) Redox balances in the metabolism of sugars by yeast. FEMS Micr Rev 32:199–224
Veiga A, Arrabaca JD, Loureiro-Dias MC (2003) Cyanide-resistant respiration, a very frequent metabolic pathway in yeasts. FEMS Yeast Res 3:239–245
Visser W, van der Baan AA, Batenburg-van der Vegte W, Scheffers WA, Kramer R, van Dijken JP (1990) Involvement of mitochondria in the assimilatory metabolism of Saccharomyces cerevisiae. Microbiology 140:3039–3046
von Meyenburg HK (1969) Energetics of the budding cycle of *Saccharomyces cerevisiae* during glucose limited aerobic growth. Arch Mikrobiol 66:289–303
Verstrepen KJ, Iserentant d, Malcorps P, Derdelinckx G, van Dijck P, Winderickx J, Pretorius IS, Thevelein JM, Delvaux FR (2004) Glucose and sucrose: hazardous fast-food for industrial yeast? Trends Biotechnol 22:531–537
Westergaard SL, Oliveira AP, Bro C, Olsson L, Nielsen J (2007) A system biology approach to study glucose repression in the yeast *Saccharomyces cerevisiae*. Biotechnol Bioeng 96:134–145

Chapter 2
Glucose Sensing and Signal Transduction in *Saccharomyces cerevisiae*

Ken Peeters and Johan M. Thevelein

Abstract Cells of the yeast *Saccharomyces cerevisiae* have an exquisite preference for high concentrations of glucose compared to other sugars or carbon sources. The likely explanation is that glucose is the best fermentable sugar, i.e., the sugar that allows the yeast to accumulate most rapidly high levels of ethanol, which are strongly inhibitory to competing microorganisms. To accomplish rapid fermentation of glucose, *S. cerevisiae* has evolved multiple glucose sensing and signaling pathways, which stimulate both fermentation and rapid cell proliferation. The latter is important for rapid fermentation in order to recycle the ATP generated in glycolysis to ADP. Downregulation of respiration to maximize ethanol production is accomplished by the main glucose repression pathway, in which the Snf1 protein kinase is a central regulator. It is inactivated by dephosphorylation upon glucose addition, and its reactivation upon glucose exhaustion is essential for induction of genes sustaining respiration, gluconeogenesis, and the catabolism of alternative carbon sources. Stimulation of fermentation and growth is mainly exerted by the protein kinase A pathway, which senses glucose with an extracellular and intracellular sensing mechanism that activates protein kinase A in a concerted manner through stimulation of cAMP synthesis. Sensing of other nutrients by plasma membrane transceptors integrates with this glucose-sensing mechanism to maintain high protein kinase A activity throughout fermentative growth. Induction of appropriate glucose transporters during fermentative growth is controlled by plasma membrane transporter-like proteins, which function as glucose sensors. Although detailed knowledge has been gained on the molecular mechanisms involved in glucose signaling, multiple important questions still remain.

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_2, © Springer-Verlag Berlin Heidelberg 2014

Keywords Glucose transporters · Glucose sensors · Ras · cAMP · PKA · Gpr1 · GPCR · Glucose repression · Snf1 · Mig1

2.1 Introduction

Glucose is the preferred source of energy and building blocks for the yeast *Saccharomyces cerevisiae*. It is mainly metabolized by fermentation and also sustains the fastest growth rate in spite of producing much less ATP per mole glucose than respiration. Other sources of carbon and energy, like glycerol, ethanol, and acetate, are respired and sustain much slower growth rates. Some sugars, like galactose, are slowly fermented and partially respired. The preference of *S. cerevisiae* for glucose and related rapidly fermented sugars, like fructose and mannose, is manifested by the multiple regulatory pathways triggered by these sugars, which all have as main goal to stimulate both fermentation and cell proliferation (Rolland et al. 2001, 2002; Santangelo 2006). Regulation occurs at different levels: allosteric, post-translational, and transcriptional. We can distinguish different regulatory pathways, which have been elucidated in great detail during many years of focussed research. These pathways are connected to each other and to signaling pathways for other nutrients, but at this moment we understand much less about these interconnections than about the components and regulation within the pathways.

2.2 The Snf3/Rgt2 Glucose Sensors for Induction of *HXT* Glucose Transporter Expression

Glucose is transported into yeast cells by an extensive set of glucose transporters, which function as facilitated diffusion carriers and are encoded by the *HXT* genes (Ozcan and Johnston 1999; Boles and Hollenberg 1997; Bisson et al. 1993). These carriers have different affinities and catalytic activities, and their expression is adjusted according to the glucose concentration in the medium (Ozcan and Johnston 1995). The Snf3-Rgt2 regulatory pathway plays a major role in this control (Fig. 2.1). It was a breakthrough in the glucose-sensing field when two plasma membrane proteins were discovered with high sequence similarity to glucose carriers, which were unable to transport glucose and instead functioned as glucose sensors (Ozcan et al. 1996; Bisson et al. 1987). Snf3 has a high affinity while Rgt2 has a low affinity for extracellular glucose. Snf3 is required for expression of *HXT2* and *HXT4* in the presence of low levels of glucose, but not for induction of *HXT1* by high glucose levels (Bisson et al. 1987; Ozcan and Johnston 1999). In contrast, Rgt2 seemed to exert the opposite effect by playing a vital role

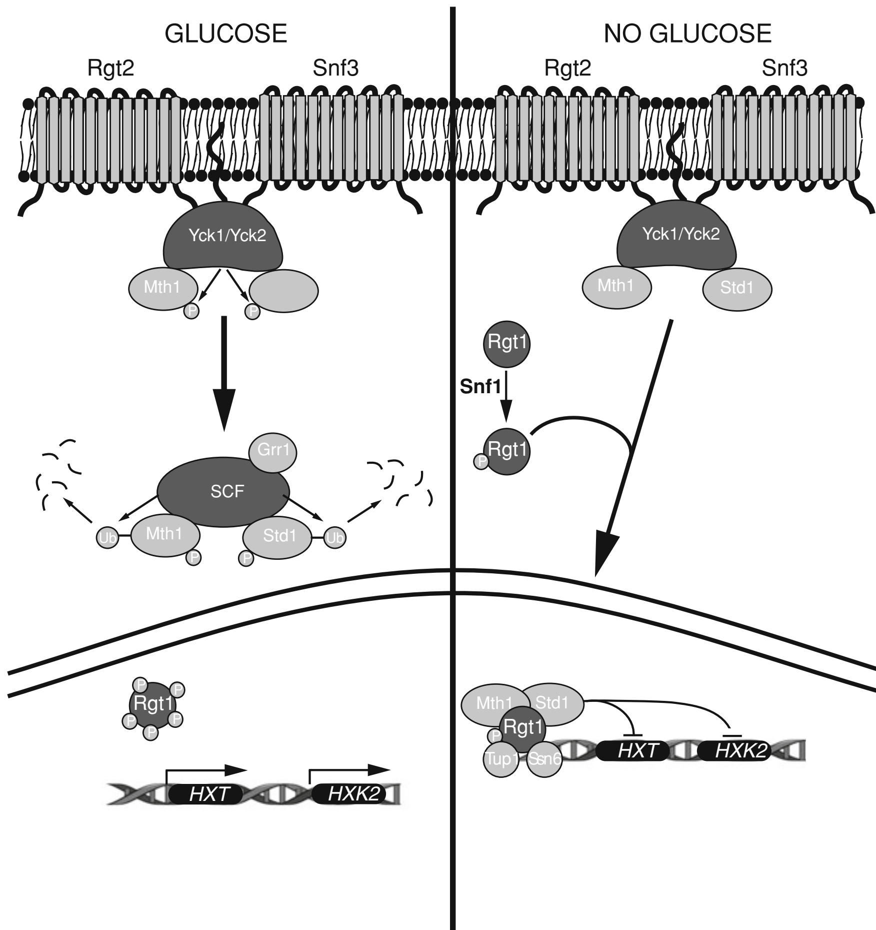

Fig. 2.1 The Rgt network. When glucose binds to the glucose receptors Snf3 and Rgt2, the corepressors Std1 and Mth1 are recruited to the plasma membrane to be phosphorylated by the Yck kinases. SCF^{Grr1} targets phosphorylated Std1 and Mth1 to the ubiquitin conjugating complex for degradation by the proteasome. Rgt1 becomes hyperphosphorylated by PKA when glucose is present and is in this form not able to repress the *HXT* and *HXK2* genes. When glucose becomes limited or is absent, Rgt1 becomes phosphorylated directly or indirectly by Snf1 and is recruited to the nucleus together with Mth1 and Std1. Together with the transcriptional corepressor complex Ssn6-Tup1, the Rgt1/Mth1/Std1 complex represses the transcription of the *HXT* and *HXK2* genes

in the induction of *HXT1* expression by high levels of glucose (Ozcan et al. 1996; Ozcan and Johnston 1999).

Both Snf3 and Rgt2 consist of two functional parts, a transmembrane-spanning part, which binds glucose, and a large cytosolic extension that is involved in triggering an intracellular signal to the downstream machinery (Fig. 2.1) (Marshall-Carlson et al. 1990; Moriya and Johnston 2004). The precise mechanism

of how the two glucose sensors generate the glucose signal and transduce it to the intracellular machinery is not fully understood. It appears to include the phosphorylation of two signal transduction proteins, Mth1 and Std1, via Yck kinases (Robinson et al. 1992; Moriya and Johnston 2004; Babu et al. 2002; Ozcan and Johnston 1999). Yck1 and its paralog Yck2 are anchored in the plasma membrane via palmitate chains. They are activated through interaction with Snf3 and Rgt2, when these sense glucose (Babu et al. 2002; Johnston and Kim 2005; Moriya and Johnston 2004). The large C-terminal domain of the glucose sensor, Snf3 or Rgt2, facilitates interaction with the Yck kinases as well as their substrates, Mth1 and Std1 (Coons et al. 1997; Dlugai et al. 2001; Moriya and Johnston 2004). Once activated, Yck1 and Yck2 inactivate Std1 and Mth1 by phosphorylation. The latter two proteins function as inhibitors of glucose-induced *HXT* gene expression in the absence of glucose (Johnston and Kim 2005; Moriya and Johnston 2004). The phosphorylation of Std1 and Mth1 will cause their ubiquitination and subsequent degradation by the proteasome, which occurs through the SCF-Grr1 complex. The F-box protein Grr1 has two protein interaction domains that are essential for its function. The F-box motif interacts with Skp1, a subunit of the SCF complex. It is preceded by a leucine rich repeat domain that is necessary for substrate recruitment (Kishi et al. 1998).

Grr1 is required for glucose regulation of the transcription factor Rgt1 (Ozcan and Johnston 1999). Rgt1 exerts a repressor role on glucose-induced genes and recruits the transcriptional co-repressor complex Ssn6-Tup1. Together with Rgt1, it will condense chromatin into a repressive conformation (Edmondson et al. 1996). This process occurs at the promoters of the *HXT* genes and causes repression of their transcription in the absence of glucose. Transcriptional repression by Rgt1 also requires Mth1, which causes a conformational change that allows Rgt1 to bind to its recognition sites in DNA (Polish et al. 2005). In addition, the presence of Std1 is also required although it does not regulate Rgt1 binding capacity (Lakshmanan et al. 2003). Under conditions of high glucose, Rgt1 is hyperphosphorylated and this process requires Snf3 and Rgt2 as glucose sensors. It converts Rgt1 from a repressor into an activator although the latter function does not act through direct DNA binding (Kim et al. 2003; Mosley et al. 2003).

The Snf3-Rgt2 signaling pathway is connected to other glucose signaling pathways. It was discovered that Rgt1 can act as a repressor of *HXK2* expression. Hxk2 is the most active hexokinase isoenzyme and is required for glucose repression through the main glucose repression pathway, also called “catabolite repression pathway” (Palomino et al. 2005). The Snf1 protein kinase, a central component of the main glucose repression pathway, seems to be involved in this process possibly through phosphorylation of Rgt1, which is essential for *HXK2* repression (Palomino et al. 2006). The latter study also showed that Tpk3 relieves *HXK2* repression by hyperphosphorylation of Rgt1. Tpk3 is one of the isoenzymes functioning as catalytic subunits of protein kinase A (PKA), which is the mediator of the cAMP glucose signaling pathway. Recent work has shown that Mth1 regulates the interaction between the Rgt1 repressor and the Ssn6-Tup1 co-repressor complex by modulating PKA-dependent phosphorylation of Rgt1 (Roy et al.

2013). Much more, however, has to be learned about the precise interactions of the Snf3-Rgt2 signaling pathway with other glucose signaling pathways.

2.3 Glucose Signaling Through the cAMP-PKA Pathway

2.3.1 Physiological Role of the cAMP-PKA Pathway in Nutrient Signaling

The PKA protein kinase affects a wide variety of processes in yeast, supporting its crucial role as a main cellular regulator. It is involved in control of metabolic pathways, like glycolysis and gluconeogenesis, in control of growth, proliferation, and aging of the cells, in reserve carbohydrate accumulation, stress tolerance, in developmental pathways, like pseudohyphal differentiation, invasive growth and sporulation, and multiple other pathways and processes (Santangelo 2006; Smets et al. 2010; Thevelein et al. 2000; Thevelein and de Winde 1999). The general role of PKA is stimulation of fermentative growth and inhibition of stationary-phase characteristics and other processes, like sporulation, which depend on respiration. Investigation of PKA targets in different growth conditions has revealed a striking correlation with the nutrient composition of the medium. Conditions supporting rapid fermentative growth, i.e., the presence of glucose or another rapidly fermented sugar, and a complete growth medium, are always associated with a status of the PKA targets indicating high activity of PKA in vivo. On the other hand, conditions supporting slow, respiratory growth, i.e., the presence of a nonfermentable carbon source, like glycerol, ethanol or acetate, or stationary phase conditions after carbon source depletion, are always associated with a status of the PKA targets indicating low activity of PKA in vivo. Up to this point, this correlation suggests that glucose and other fermentable sugars are activators of PKA in vivo. This has led to the concept that PKA is part of a glucose signaling pathway, which has been confirmed by the discovery of a complex glucose-sensing network controlling the level of cAMP, the second messenger that controls the activity of PKA.

Subsequently, however, a novel level of PKA regulation has been discovered following the awareness that starvation of yeast cells on a glucose-containing medium for any other single essential nutrient downregulated the PKA targets in a manner consistent with the presence of low PKA activity in vivo (Thevelein et al. 2005; Thevelein and de Winde 1999). Hence, fermentable sugar was clearly not the sole determinant for high PKA activity. Observations linking PKA targets, like trehalose and glycogen content, to conditions of starvation for specific essential nutrients on a glucose-containing medium, were already made long before the glucose-sensing role of the PKA pathway had become clear (Lillie and Pringle 1980). Subsequently, the role of other nutrients in regulating PKA activity was clearly demonstrated by experiments showing rapid activation of PKA targets after

addition of nitrogen sources, phosphate, and sulfate, to appropriately starved cells on a glucose-containing medium (Hirimburegama et al. 1992; Thevelein and Beullens 1985; Thevelein 1984a). Hence, this previous work has revealed that maintenance of high PKA activity in yeast cells requires the combination of a rapidly fermented sugar and a complete growth medium, which led to the concept of a "fermentable–growth-medium" (FGM)-induced pathway for activation of PKA in vivo.

Further research on the FGM pathway led to the discovery of multiple nutrient transporters/receptors or "transceptors," acting as sensors for activation of PKA by the other nutrients, and apparently without using cAMP as a second messenger (Kriel et al. 2011; Thevelein and Voordeckers 2009; Thevelein et al. 2005, 2008; Holsbeeks et al. 2004). Hence, in the case of the PKA pathway, integration of glucose sensing with sensing of other nutrients is very well established although the mechanistic details of the integration are not well understood.

2.3.2 Glucose Activation of cAMP Synthesis and PKA

The dramatic effects of glucose addition to yeast cells on PKA targets, like trehalose and trehalase, led already in 1974 to the discovery of the "glucose-induced cAMP signal," a conspicuous and drastic, but very transient spike in the cAMP level that happens in the first 1–3 min after addition of glucose to respiring yeast cells (either growing or stationary-phase cells) (van der Plaat 1974). This cAMP signal activates PKA, which then phosphorylates target proteins like trehalase. This enzyme shows a conspicuous and rapid increase in activity, as measured in cell extracts, following the cAMP signal. Initially, nonspecific mechanisms were evaluated as possible triggers for the glucose-induced cAMP signal (Mazon et al. 1982; Purwin et al. 1982; Thevelein 1984a; Tortora et al. 1982). The observation that intracellular acidification caused a strong and persistent accumulation of cAMP in yeast cells (Purwin et al. 1986; Thevelein 1991; Caspani et al. 1985), while glucose addition triggered a rapid, transient drop in the intracellular pH, led to the suggestion that the cAMP signal was caused by the glucose-induced transient intracellular acidification. This explanation as well as other suggestions of transient plasma membrane depolarization or increases in ATP, the substrate of adenylate cyclase, were contradicted by a variety of experimental approaches (Thevelein et al. 1987a, b).

Like previously established for mammalian PKA, yeast PKA is also a hetero-tetrameric protein consisting of two catalytic and two regulatory subunits. The catalytic subunits are encoded by *TPK1*, *TPK2,* and *TPK3*, while the regulatory subunits are encoded by *BCY1* (Toda et al. 1987a, b). Binding of the second messenger cAMP to the regulatory subunit Bcy1 causes its dissociation from the Tpk1-3 catalytic subunits, resulting in activation of PKA (Kuret et al. 1988) (Fig. 2.2). The three catalytic subunits have redundant functions for some phenotypes and specific functions for other phenotypes. For instance, any *TPK* gene

can sustain viability of the cells while the absence of all three is lethal (Thevelein and de Winde 1999). On the other hand, pseudohyphal growth induction is stimulated by Tpk2 but counteracted by Tpk3 (Robertson and Fink 1998), while mitochondrial biogenesis is specifically stimulated by Tpk3 (Chevtzoff et al. 2010).

Synthesis of the second messenger molecule cAMP from ATP is catalyzed by adenylate cyclase, which is encoded by *CYR1*/*CDC35* (Kataoka et al. 1985; Matsumoto et al. 1982). The activity of adenylate cyclase is controlled in yeast by two distinct G-protein systems, the Ras1,2 proteins (Toda et al. 1985; Broek et al. 1985) and Gpa2, a homolog of the Gα subunit of the heterotrimeric G-proteins (Nakafuku et al. 1988; Lorenz and Heitman 1997; Kubler et al. 1997) (Fig. 2.2). This led to the discovery that these G-protein systems are involved in intracellular and extracellular glucose sensing, respectively (Rolland et al. 2000).

2.3.3 The Ras Proteins and Their Role in Intracellular Glucose Sensing

Discovery of the yeast Ras proteins The yeast Ras proteins were discovered based on sequence similarity with the mammalian RAS oncogenes (Kataoka et al. 1984; Powers et al. 1984; Tatchell et al. 1984). The purpose was to use yeast as a model system to identify the elusive physiological function of the mammalian *RAS* gene products. Deletion of both *RAS* genes in yeast was lethal because it caused cell cycle arrest in G_1 and entrance into stationary phase, similar to cells starved for nutrients. A specific category of temperature-sensitive cell cycle mutants (*cdc* mutants), including the *cdc35* mutant, also arrested at the restrictive temperature at the same point in the cell cycle (Hayles and Nurse 1986). This suggested that the function of these gene products was related to that of Ras. Cloning of *CYR1*/*CDC35* revealed that it encodes adenylate cyclase (Kataoka et al. 1985; Matsumoto et al. 1982), and subsequent work showed that the yeast Ras proteins act as essential G-proteins for yeast adenylate cyclase (Toda et al. 1985). This work formed the basis for the further elucidation of the cAMP-PKA pathway in yeast, but it failed to deliver originally expected insight on two points. First, in mammalian cells, the Ras proteins do not act on adenylate cyclase (Beckner et al. 1985) and the yeast work therefore failed to help identify the mammalian Ras target. Second, in spite of many efforts no upstream activators of Cdc25 could be found, which would have pointed to the physiological signal being transmitted by the Ras proteins in yeast. Hence, the original goal of using yeast as a model system to understand the physiological function of the mammalian Ras proteins as signal transmission proteins and thus to shed light on their oncogenic mechanism was not fulfilled.

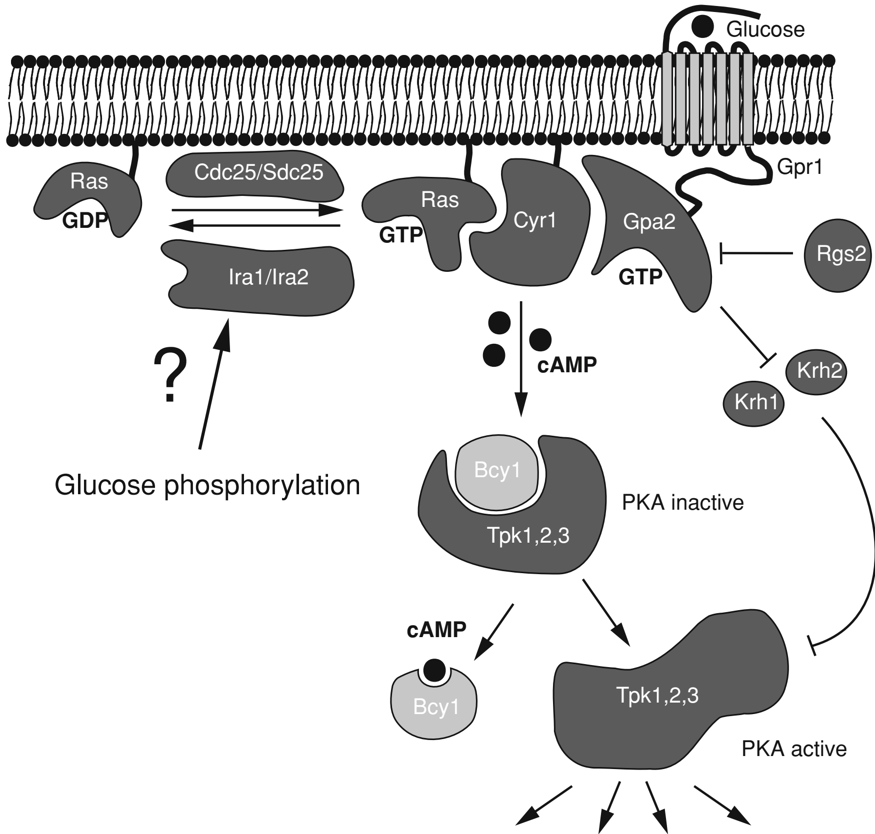

Fig. 2.2 The cAMP-PKA pathway in *S. cerevisiae*. Glucose activates PKA via two different pathways. When glucose is transported and phosphorylated, it activates the Ras proteins by increasing their GTP/GDP loading state. The mechanism involved is not known. Active Ras will consequently activate Cyr1, the adenylate cyclase of yeast. Cyr1 catalyzes the synthesis of cAMP from ATP. This second messenger is able to bind to the regulatory subunit of PKA, Bcy1, thereby dissociating it from the catalytic subunits, Tpk1, Tpk2, and Tpk3. These are then able to phosphorylate downstream targets and regulate in this way protein activity and gene expression. Extracellular glucose can also activate PKA through the glucose-sensing G-protein-coupled receptor Gpr1. This receptor triggers activation of the G-protein Gpa2, of which the intrinsic GTPase activity is stimulated by Rgs2. Active Gpa2 in turn activates Cyr1 with the generation of cAMP as a consequence. Gpa2 can also inhibit the Krh proteins, thereby, activating PKA through the adenylate cyclase bypass pathway

Ras and its regulatory proteins In spite of this, detailed analysis of the Ras proteins and their direct, physical regulators in yeast revealed strong conservation with the system in mammalian cells. The yeast Ras1 and Ras2 proteins share more than 70 % amino acid similarity and approximately 90 % similarity in their 180 N-terminal residues (Powers et al. 1984; Kataoka et al. 1984), and these 180 amino acids are also highly conserved in the human Ras proteins. The Ras proteins

are monomeric GTPases whose activity depends on GDP/GTP exchange and GTP hydrolysis (Broach and Deschenes 1990). The activity of monomeric GTPases is displayed as a binary switch. When GTP is bound, the Ras proteins are activated and stimulate cAMP synthesis by activating Cyr1/adenylate cyclase (Matsumoto et al. 1982). Conversely, when Ras-bound GTP is hydrolysed to GDP by the intrinsic Ras GTPase activity, it switches to the inactivated state. Mammalian Ras oncogene products usually contain mutations that render the protein constitutively active, for instance by reducing the intrinsic GTPase activity. A major example is Ras^{val12}, in which $glycine^{12}$ is converted into a valine residue. The corresponding mutation was engineered into the yeast Ras2 protein, which resulted in the $Ras2^{val19}$ protein, which is also constitutively active in yeast (Broek et al. 1985). It causes higher cAMP levels and PKA activity, which is detrimental to the cells when they grow on non- or poorly fermentable carbon sources or enter into stationary phase. Originally, the failure of this mutant to arrest properly at the start site in the G_1 phase of the cell cycle upon nitrogen starvation was ascribed to its oncogenic character, causing defective cell cycle control, but was later attributed to its inability to complete the cell cycle because of deficient internal amino acid stores (Markwardt et al. 1995).

Ras activity is modulated by stimulation of guanine nucleotide exchange and stimulation of the intrinsic GTPase activity (Fig. 2.2). Cloning of the *CDC25* gene by complementation of another temperature-sensitive mutant that arrested at the restrictive temperature like nutrient-starved cells, showed that it encodes an essential guanine nucleotide exchange factor (GEF) of Ras (Broek et al. 1987; Camonis et al. 1986; Jones et al. 1991). Later work also identified a homolog of *CDC25*, *SDC25*, but this gene contains an inactivating nonsense mutation in the S288c background causing *CDC25* to be essential (Boy-Marcotte et al. 1996; Damak et al. 1991). In the W303 lab strain, deletion of *CDC25* is not lethal under growth conditions in which *SDC25* is expressed (Folch-Mallol et al. 2004; Boy-Marcotte et al. 1996). These GEF proteins only bind and thereby stabilize the open nucleotide-free state of Ras (Lai et al. 1993; Haney and Broach 1994). Because the cytosolic concentration of GTP is higher than that of GDP in well-energized cells, nucleotide-free Ras will be loaded preferentially with GTP when it binds a new nucleotide, leading to activation of Ras. GTP enters Ras together with one molecule of Mg^{2+}, which creates a GTP-Mg^{2+} complex that will close the Ras protein and stabilize its active conformation (Pai et al. 1990; Farnsworth and Feig 1991). The C-terminus of Cdc25 includes the catalytic domain and a membrane localization signal, while the N-terminus contains an SH3 domain that regulates Ras interaction with adenylate cyclase (Daniel 1986; Garreau et al. 1996; Mintzer and Field 1999). The C-terminus of Cdc25 shows very high sequence similarity with the human Ras GEF factor hSos1. The catalytic part of hSos1 is referred to as the Cdc25 domain (Boguski and McCormick 1993).

Inactivation of the Ras proteins occurs through their intrinsic GTPase activity. However, without aid this reaction is very slow, and therefore it is stimulated by two GTPase activating proteins (GAPs), Ira1 and Ira2 (Tanaka et al. 1990). These proteins stick an arginine finger into the catalytic site of Ras, which decreases the

activation energy for hydrolysis of the γ-phosphate from GTP (Kotting et al. 2008). Ira1 and Ira2 are among the largest proteins present in yeast (3,093 and 3,080 amino acids, respectively) and, in addition to their GTPase activating function, they show further regulatory functions (Tanaka et al. 1990). Ira1, for instance, was found to interact with Cyr1 and seems to be necessary for its membrane localization (Mitts et al. 1991). Tfs1 was found to inhibit Ira2, but not Ira1 (Chautard et al. 2004). Deletion of Ira1 or Ira2 can suppress lethality caused by deletion of *CDC25*, just like the presence of a constitutively active allele of Ras. This is consistent with higher activity of Ras in *ira1* and *ira2* deletion strains (Tanaka et al. 1990).

The essential character of Cyr1/Cdc35/adenylate cyclase as well as its regulators Ras1 and Ras2, or Cdc25 and Sdc25, for cell viability in all tested genetic backgrounds, indicates that a critical concentration of cAMP is essential for cell growth in yeast and more specifically for progression over the START site in the G_1 phase of the cell cycle and prevention of precocious entrance into the stationary phase G_0 (Boy-Marcotte et al. 1998; Broach and Deschenes 1990; Ptacek et al. 2005; Smith et al. 1998; Thevelein 1994). Since nutrient starvation also prevents progression through G_1 and forces cells into G_0, this suggested that the Cdc25-Ras-adenylate cyclase system responds to nutrient availability (Thevelein 1994; Thevelein et al. 2000). The precise connection between glucose and cAMP, however, was not revealed in cell cycle studies but rather by research on glucose regulation of storage carbohydrate metabolism (Thevelein 1991; Thevelein and de Winde 1999). Whether there is a mechanistic connection between the availability of all the other nutrients, besides glucose and related rapidly fermented sugars, and cAMP synthesis remains unclear up to today. In this respect, it is important to realize that a critical level of PKA activity may be required for growth rather than a critical concentration of cAMP *per se*. In the presence of a basal level of cAMP, other regulators, such as the kelch repeat proteins Krh1 and Krh2, may modulate PKA activity (Peeters et al. 2007).

Another protein involved in activation of Cyr1/adenylate cyclase by Ras is Srv2 (Fedor-Chaiken et al. 1990; Field et al. 1990). It is bound to Cyr1 (and therefore also called CAP or cyclase-associated protein) and also binds to actin, which facilitates the interaction between Cyr1 and Ras. Its main task, however, appears to be in the regulation of the actin skeleton in yeast, although there is also evidence that modulation of the actin cytoskeleton can cause hyperactivation of Ras (Gourlay and Ayscough 2006).

Glucose activation of Ras and its role in glucose activation of cAMP synthesis Investigation of the glucose-induced cAMP signal in different mutants in yeast glycolysis revealed that glucose phosphorylation was essential for the glucose-induced cAMP signal (Beullens et al. 1988). This suggested that the trigger for this process was an intracellular event originating in intracellular glucose catabolism. Subsequently, evidence was provided that the Ras proteins were involved in mediating the glucose-induced cAMP signal, which indicated for the first time a connection between glucose sensing and Ras (Mbonyi et al. 1988). Combined with the previous finding, it suggested that Ras is activated by one or more factors

generated in glucose catabolism. Other evidence for involvement of Ras in glucose-induced cAMP signaling has later been provided. Ras is anchored in the plasma membrane via palmitoylation and farnesylation of the two cysteine residues at positions 318 and 319, respectively. Membrane targeting of Ras is not required for maintenance of a basal level of cAMP and thus for sustaining viability, but is required for rapid glucose-induced cAMP signaling (Bhattacharya et al. 1995). Evidence for involvement of Cdc25 and especially its C-terminus in glucose-induced cAMP signaling was also reported, strengthening the evidence for a role of the Ras proteins as signal transducers in glucose-induced cAMP signaling (van Aelst et al. 1990, 1991). In the absence of glucose, Cdc25 is also located at the plasma membrane, and adenylate cyclase, although not an intrinsic membrane protein in yeast, also associates with the plasma membrane. This configuration of Cdc25, Ras, and adenylate cyclase at the plasma membrane appears to be important for rapid glucose-induced cAMP signaling and its loss may play a role in the rapid decrease of the cAMP level after the initial surge. The increase in cAMP activates PKA, which hyperphosphorylates Cdc25 resulting in its translocation to the cytosol and reduction of its ability to activate Ras (Gross et al. 1992; Dong and Bai 2011; Jian et al. 2010).

Direct measurement of the GTP/GDP loading state on Ras after addition of glucose, however, failed to reveal any increase in GTP, as opposed to intracellular acidification, which triggered a rapid and huge increase in Ras-GTP (Colombo et al. 1998). For technical reasons, these experiments required overexpression of Ras, and subsequent work, using a more sensitive assay for Ras-GTP based on the interaction of mammalian Ras with the Ras-binding domain of Raf, revealed that the overexpression of Ras, possibly through a feedback inhibition mechanism, prevented detection of the glucose-induced increase in the Ras-GTP level (Colombo et al. 2004). In the same work, it was shown that glucose activation of Ras requires glucose phosphorylation, again linking glucose catabolism with activation of Ras. How glucose catabolism causes activation of Ras is still not clear today.

The establishment of Ras activation by glucose catabolism in yeast brings us back to the original aim of the studies of Ras in yeast. The purpose was to understand the physiological role of the oncogenic Ras protein in mammalian cells with a goal of finding an explanation for its role in induction of cancer. The absence of the Ras—adenylate cyclase connection in mammalian cells (Beckner et al. 1985) suggested that yeast Ras had a different function compared to mammalian Ras and that yeast, therefore, was not a good model system to learn about Ras functionality, which made the interest in the yeast Ras system by mammalian researchers fade away. However, cancer cells and yeast cells present a striking similarity in the related so-called Warburg and Crabtree effects (Diaz-Ruiz et al. 2011). As opposed to other eukaryotic cells, cancer cells and (in the presence of a high concentration of fermentable sugar) yeast cells favor fermentation over respiration in the presence of oxygen and also show the most rapid proliferation when fermenting in spite of the fact that fermentation delivers much less ATP compared to respiration. Whether the high glycolytic flux in cancer cells is a consequence or

a cause of the cancerous state has been a matter of much debate and is still not clear (Upadhyay et al. 2013). In this respect, the connection between glucose catabolism and activation of the oncogenic Ras protein in yeast might still serve as a valuable model system to understand the Warburg effect in cancer cells and to make a distinction between high fermentation activity as a consequence or a cause of cancer.

2.3.4 The Gpr1-Gpa2 GPCR System and Its Role in Extracellular Glucose Sensing

The observation that the Ras proteins were not activated after glucose addition in cells overexpressing Ras stimulated the search for an alternative G-protein involved in glucose-induced cAMP signaling. This led to the discovery of a G-protein-coupled receptor (GPCR) system that senses extracellular glucose and is dependent on the intracellular glucose-sensing system that activates Ras for stimulation of adenylate cyclase and cAMP signaling (Thevelein and de Winde 1999).

The GPCR system is composed of the receptor, Gpr1, and its Gα protein Gpa2 (Fig. 2.2). Gpr1 has the typical structure of a GPCR with seven transmembrane domains but little sequence similarity to other GPCR families (Kraakman et al. 1999; Xue et al. 1998; Yun et al. 1997). Together with its homologues in other fungi, it represents a separate subfamily in the large GPCR superfamily (Graul and Sadee 2001). Glucose and sucrose, but not fructose, mannose, galactose, or other sugars, act as ligands of the Gpr1 receptor, with sucrose having much higher affinity (±1 mM) compared to glucose (±20 mM) (Lemaire et al. 2004). The sugar specificity of Gpr1 indicates that fructose- and mannose-induced cAMP signaling are exclusively mediated by the intracellular sugar catabolism-dependent activation of Ras. The glucose sensitivity fits with the concentrations of glucose that cause full stimulation of fermentative growth in yeast, while the high sensitivity for sucrose suggests that detection of low sucrose concentrations may be important for survival in the natural habitat of yeast (Van de Velde and Thevelein 2008). Deletion of Gpr1 is not lethal and causes delayed activation of the cAMP-PKA signaling pathway upon addition of glucose (Kraakman et al. 1999). Whereas extracellular glucose signaling through the Gpr1–Gpa2 system is entirely dependent on intracellular activation of Ras by glucose catabolism, the opposite is not true, and therefore glucose still causes stimulation of the cAMP-PKA pathway in the absence of Gpr1 or Gpa2 (Rolland et al. 2000). A constitutively active allele of Ras2 also causes a stronger effect on gene expression controlled by the cAMP-PKA pathway compared to a constitutively active allele of Gpa2 (Wang et al. 2004) consistent with the Ras system having a more dominant effect on adenylate cyclase than the Gpr1–Gpa2 GPCR system. Gpr1 was discovered in two independent ways. The C-terminus of Gpr1 was isolated in two hybrid screens with Gpa2, and a mutant with delayed glucose-induced stimulation of PKA targets

turned out to have a nonsense mutation in Gpr1 (Kraakman et al. 1999; Xue et al. 1998).

Gpa2 is a member of the Gα family of heterotrimeric G-proteins (Nakafuku et al. 1988; Kubler et al. 1997). It was the first member of this family that does not function in association with a classical Gβ and Gγ subunit (Peeters et al. 2007; Hoffman 2007). Deletion of Gpa2 is not lethal; it delays glucose-induced stimulation of the cAMP-PKA pathway and affects other PKA-dependent phenotypes like pseudohyphal growth (Nakafuku et al. 1988; Kubler et al. 1997; Colombo et al. 1998). In general, deletion of Gpa2 seems to cause stronger phenotypic effects than deletion of Gpr1, which may hint to additional regulation at the level of Gpa2. The intrinsic GTPase activity of Gpa2 is stimulated by the *RGS2* gene product, which thus acts as an inhibitor of signaling (Versele et al. 1999). Gpa2 is anchored in the plasma membrane via myristoylation and palmitoylation of its N-terminus (Harashima and Heitman 2005).

The observation that Gpa2 functions without classical β and γ subunits has led to intensive research and also much debate concerning possible alternative Gβ and Gγ proteins. Initially, the kelch repeat proteins, Krh1 and Krh2, were proposed as alternative Gβ subunits (and called Gpb2 and Gpb1) and Gpg1 was proposed to be the γ subunit of Gpa2. Krh1 and Krh2 have a seven-kelch repeat structure, which results in a conformation very similar to the seven-WD-40 repeat structure of Gβ proteins, and physically binds to Gpa2 (Harashima and Heitman 2002, 2005). This initial suggestion was contradicted by later, more extensive work (Hoffman 2007; Niranjan et al. 2007). Krh1 and Krh2 do not interact with Gpa2 in a way that would be expected from a genuine Gβ replacement subunit. Deletion of Krh1 and Krh2 causes a high PKA phenotype, but this is apparently not due to relief of inhibition on Gpa2, as would be expected for a genuine Gβ protein. Krh1 and Krh2 directly interact with the catalytic subunits of PKA, Tpk1-3, and stimulate their interaction with the regulatory subunit, Bcy1, causing a higher cAMP level to be required for their dissociation. Krh1 and Krh2 promote the phosphorylation of the Bcy1 regulatory subunit of PKA and this produces a form of Bcy1 that is more stable and more effective as an inhibitor catalytic subunits (Budhwar et al. 2010). Hence, inactivation of Krh1 and Krh2 causes higher PKA activity in the presence of the same cAMP concentration. Gpa2 appears to inhibit Krh1 and Krh2, creating a bypass pathway for activation of adenylate cyclase, directly from the Gα protein Gpa2 to PKA (Batlle et al. 2003; Lu and Hirsch 2005; Peeters et al. 2006; Niranjan et al. 2007). Krh1 and Krh2 were also shown to function as regulators of the Ras GAPs, Ira1, and Ira2, either by stabilizing the proteins (Harashima et al. 2006) or target them for degradation (Phan et al. 2010). Asc1, another protein with seven-WD-40 repeats that binds most tightly to the GDP-loaded Gpa2 protein, has also been proposed as an alternative Gβ subunit (Zeller et al. 2007). There remain many questions concerning the precise role of Krh1 and Krh2 and the two G-protein signaling modules, Ras and Gpa2, in the control of cAMP synthesis and PKA activity in yeast.

2.3.5 Downstream Targets of PKA

Negative feedback regulation of PKA on cAMP synthesis Yeast strains with reduced PKA activity display huge increases in the basal cAMP level (Nikawa et al. 1987). This suggested that PKA downregulates cAMP synthesis by negative feedback regulation. This phenomenon also explains why the glucose-induced cAMP increase is very short-lived and actually occurs as a sharp cAMP signal. The extent of the glucose-induced cAMP signal is inversely correlated with the activity of PKA, and strains with attenuated PKA activity display large glucose-induced cAMP increases (Mbonyi et al. 1990). In a strain with elevated PKA activity, the cAMP signal is completely suppressed. This explains the seemingly contradictory finding that in a yeast strain devoid of the two cAMP phosphodiesterases the glucose-induced cAMP signal is virtually absent, rather than strongly enhanced (Ma et al. 1999). In spite of many efforts, the main target of the negative feedback regulation still remains elusive. Several targets have been proposed, including Ras and Cdc25. Mutagenesis of Ser^{214} to alanine ($Ras2^{S214A}$) caused phenotypes consistent with higher activity of the cAMP-PKA pathway and also resulted in a higher basal level of cAMP and stronger glucose-induced cAMP signaling (Xiaojia and Jian 2010). However, the increase in the basal cAMP level was very limited compared to the huge cAMP increases in the Tpk-attenuated strains, indicating that phosphorylation of Ras cannot be the main target of the negative feedback regulation. As previously mentioned, glucose-induced hyperphosphorylation of Cdc25 resulting in its translocation from the plasma membrane to the cytosol and hence, reduced ability to activate Ras, may also form part of the negative feedback regulation mechanism (Gross et al. 1992; Dong and Bai 2011; Jian et al. 2010). Moreover, it has been shown that the Ras2 guanine nucleotide exchange activity of Cdc25 in vitro is inhibited by phosphorylation, due to downregulation of the association between Cdc25 and GTP-bound Ras2 (Dong and Bai 2011; Jian et al. 2010). Based on these data, it was suggested that PKA causes negative feedback regulation on cAMP synthesis through phosphorylation of Cdc25 (Jian et al. 2010). Putative phosphorylation sites in Cdc25 have been eliminated, and multiple truncations of the protein were made with various effects on the basal cAMP level or on glucose-induced cAMP signaling, but in all cases these changes were limited and never even approached the huge increase in cAMP as observed in Tpk-attenuated strains (Schomerus et al. 1990; van Aelst et al. 1990, 1991).

The low-affinity cAMP phosphodiesterase, Pde1, was shown to have a specific function in downregulating glucose-induced cAMP signaling, whereas the high-affinity cAMP phosphodiesterase, Pde2, controls the basal cAMP level in the cell. Pde1 is a target of PKA, and inactivation of its PKA phosphorylation site, Ser252, caused a higher glucose-induced cAMP signal (Ma et al. 1999). Pde2 is also regulated by PKA (Hu et al. 2010). The half-life of Pde2 seems to be increased in strains growing on glucose or strains with a high PKA phenotype. Pde2 localization in these strains is mainly in the nucleus. In contrast, in derepressed cells or strains with an attenuated PKA phenotype, Pde2 protein levels are lower and it is

distributed over the nucleus and cytoplasm. Neither mutagenesis of the PKA phosphorylation site in Pde1 nor mutagenesis of any other putative target of PKA negative feedback regulation has resulted in a strain with equally high cAMP hyperaccumulation as in a tpk-attenuated strain. This seems to indicate that the main target of PKA negative feedback regulation has not been identified yet or that there are multiple parallel targets.

Post-translational targets of PKA in storage carbohydrate metabolism and glycolysis The first cellular target of the cAMP-PKA pathway identified was storage carbohydrate metabolism. Yeast has two storage carbohydrates, glycogen and trehalose, of which the second also serves as a stress protectant sugar. Trehalose appears most important for long-term survival in stationary phase cells and likely also in ascospores since these are devoid of glycogen (Thevelein 1984b). Addition of glucose to derepressed yeast cells, i.e., cells growing on a nonfermentable carbon source, glucose-starved stationary phase cells or ascospores, causes rapid mobilization of trehalose and glycogen, which is mediated by activation of the PKA pathway. Neutral trehalase was probably the first PKA target identified in yeast. It is within a few minutes activated after glucose addition to glucose-deprived cells (van der Plaat 1974), which is due to phosphorylation by PKA on several sites of the enzyme and binding of 14-3-3 proteins to the phosphorylated sites (Schepers et al. 2012; App and Holzer 1989). Mutants with reduced or constitutively enhanced activation of PKA show similarly reduced or constitutively elevated trehalase activity (Hirimburegama et al. 1992; Durnez et al. 1994; Giots et al. 2003; Mbonyi et al. 1990; Thevelein and Beullens 1985; Van Nuland et al. 2006). Glycogen synthase is downregulated by phosphorylation, while glycogen phosphorylase is activated by phosphorylation. Although it is well established that PKA activity in vivo is inversely correlated with the glycogen level and that both enzymes are phosphorylated by PKA in vitro, the precise contribution of direct phosphorylation by PKA of these enzymes is not very clear (Francois and Hers 1988; Hardy and Roach 1993; Francois and Parrou 2001; Wilson et al. 2010).

A second well-characterized target activated by PKA is 6-phosphofructo-2-kinase, which synthesizes fructose-2,6-bisphosphate, an allosteric activator of phosphofructokinase 1 and allosteric inhibitor of fructose-1,6-bisphosphatase (Dihazi et al. 2003; Noda et al. 1984). Fructose-1,6-bisphosphatase is also directly inactivated through phosphorylation by PKA (Pohlig and Holzer 1985). Through these mechanisms, activation of PKA stimulates glycolysis and fermentation, while it inhibits gluconeogenesis. Additional stimulation of glycolysis occurs through phosphorylation of pyruvate kinase (Cytrynska et al. 2001; Portela et al. 2006). This fits with the conclusion that fermentatively growing cells have high PKA activity while respiratively growing cells have low PKA activity.

Transcription factors as direct and indirect targets of PKA PKA has dramatic effects on the expression of a wide variety of genes involved in energy metabolism, cell cycle progression, stress response, ribosomal biogenesis and accumulation of the storage carbohydrate glycogen, and the storage and stress protectant sugar trehalose (Boy-Marcotte et al. 1998; Broach and Deschenes 1990; Ptacek

et al. 2005; Smith et al. 1998; Thevelein 1994). Ninety percentage of the transcriptional remodeling of the cell in response to glucose is mediated via the G-proteins Ras1, Ras2, and Gpa2, which act in a redundant manner through activation of the cAMP-PKA pathway (Wang et al. 2004).

Since PKA activity is high in cells growing on glucose or other rapidly fermented sugars, i.e., glucose-repressed cells, and it is low in cells growing on nonfermentable carbon sources or glucose-starved, i.e., glucose-derepressed cells, there has initially been confusion between the function of the main glucose repression pathway and the PKA pathway in repression of transcription. Initially, the genes regulated by both pathways appeared to be very similar. The distinction between the two sets of transcription targets, however, can be made based on the fact that the main glucose repression pathway is only regulated by glucose or related rapidly fermented sugars, whereas the PKA pathway is also regulated by all other essential nutrients. Hence, when yeast cells are starved on a glucose-containing medium for another essential nutrient, e.g., nitrogen or phosphate, the main glucose repression pathway will remain active and the cells glucose repressed as long as there is a sufficient level of glucose in the medium. The PKA pathway, on the other hand, will be downregulated when the cells enter stationary phase and its target genes therefore will either no longer be repressed or induced. This does not preclude that the expression of some genes, like *GSY2*, encoding glycogen synthase, is regulated both by the main glucose repression and the PKA pathway (Wilson et al. 2010).

PKA controls the transcription factors Msn2, Msn4, and Gis1 by direct phosphorylation but also through control of protein kinases Rim15 and Yak1. Msn2 and Msn4 mediate the induction of a set of stress responsive genes, which contain so-called STRE elements (STress Response Element) in their promoters (Boy-Marcotte et al. 1998; Estruch and Carlson 1993; Martinez-Pastor et al. 1996; Schmitt and McEntee 1996; Smith et al. 1998). The STRE element consists of a pentameric core of CCCCT (Wieser et al. 1991). Glucose-induced activation of PKA triggers phosphorylation of Msn2 and Msn4, which blocks their translocation toward the nucleus and in this way inhibits targeted gene expression. As a result, high PKA activity counteracts the stress response and thus prevents establishment of high stress tolerance in yeast cells (Gorner et al. 1998, 2002). Deletion of both Msn2 and Msn4 suppresses the lethality caused by inactivation of the cAMP-PKA pathway, e.g., it can rescue a *tpk-null* strain or a *ras1*Δ *ras2*Δ strain (Smith et al. 1998), which reflects the importance of Msn2/Msn4-dependent targets for control of cell proliferation.

The Gis1 transcription factor supports expression of another set of genes through the PDS (Post-Diauxic Shift) element T(T/A)AGGGAT in their promoter (Pedruzzi et al. 2000; Zhang et al. 2009). These genes are expressed during the diauxic shift, and their regulation is not dependent on Msn2 or on Msn4 (Boy-Marcotte et al. 1998). However, most genes containing the PDS consensus sequence also contain one or more STRE consensus sequences.

The Rim15 and Yak1 protein kinases are positive effectors of gene expression and regulate the activity of the transcription factors Msn2, Msn4, and Gis1

(Garrett and Broach 1989; Garrett et al. 1991; Reinders et al. 1998). Rim15 is a glucose-repressible protein kinase (Vidan and Mitchell 1997) that is inhibited by PKA via direct phosphorylation. The deletion of *RIM15* can also suppress the lethality caused by the loss of PKA activity (Reinders et al. 1998). This protein acts as an activator of STRE-controlled gene expression during entry into stationary phase (G_0). The induction of genes during the diauxic shift via Rim15 is almost entirely mediated via the Msn2, Msn4, and Gis1 transcription factors (Cameroni et al. 2004; Pedruzzi et al. 2000; Reinders et al. 1998). Yak1 and PKA have an antagonistic effect on cell cycle progression through G_1 (Garrett and Broach 1989; Garrett et al. 1991). Expression of the protein kinase Yak1 is controlled in a Msn2/Msn4-dependent manner (Smith et al. 1998). The deletion of *YAK1* rescues lethal PKA deletion, i.e., it renders a *tpk-null* strain viable (Garrett and Broach 1989) and the activation of Yak1 is directly counteracted by PKA phosphorylation (Lee et al. 2008). Yak1 in turn can activate Msn2 by direct phosphorylation and in this way provides a positive feedback loop upon glucose limitation (Lee et al. 2008). Nuclear localization of Yak1 is promoted by glucose availability, while glucose limitation causes phosphorylation of Pop2, a substrate of the Yak1 protein kinase, and a regulator of transcription of many genes (Moriya et al. 2001). In addition, upon glucose starvation, Bcy1 is phosphorylated by Yak1 and restricted to the cytoplasm (Griffioen et al. 2001; Werner-Washburne et al. 1991). PKA thus counteracts stationary phase and stress response-related gene expression in at least two ways, by phosphorylation of the transcription factors and by phosphorylation of protein kinases required for proper activity of the same transcription factors.

PKA also plays a role in the transcriptional induction of genes upon addition of glucose. This has been investigated most intensively for the glucose-induced upshift in expression of the ribosomal protein genes (Herruer et al. 1987; Griffioen et al. 1994; Kraakman et al. 1993). In general, expression of ribosomal protein genes is strongly correlated with the growth rate of the cells. The glucose-induced upshift was claimed not to involve cAMP signaling. PKA was shown to promote expression of the ribosomal protein genes through the transcription factor Sfp1. Under optimal growth conditions, Sfp1 is localized in the nucleus, bound to the promoters of ribosomal protein genes, and helps promote ribosomal protein gene expression. When glucose gets depleted, Sfp1 is released from ribosomal protein gene promoters and leaves the nucleus, resulting in downregulation of ribosomal protein gene expression (Marion et al. 2004).

Although it has been known for a long time that inactivation of the Ras-cAMP-PKA pathway causes arrest in the G_1 phase of the cell cycle and permanent entry into G_0, the underlying mechanism is not well understood. Recent work has shown that Whi3, a negative regulator of the G_1 cyclins, is inhibited through phosphorylation by PKA on Ser^{568}. Phosphorylation of Whi3 by PKA leads to decreased interaction with *CLN3* G_1 cyclin mRNA and is required for the promotion of G_1/S progression, implicating Whi3 in PKA regulation of cell cycle control (Mizunuma et al. 2013).

2.3.6 The PKA-Related Protein Kinase Sch9

The Sch9 protein kinase was originally discovered as a multicopy suppressor of lethality caused by inactivation of the cAMP-PKA pathway, i.e., as a suppressor of a *cdc25*^{ts} strain (Toda et al. 1988). Although much new information on Sch9 has been obtained since then, including evidence for requirement of Sch9 in different nutrient signaling processes (Zaman et al. 2008), its precise role in nutrient signaling remains enigmatic. Sch9 is a serine/threonine kinase and is part of the AGC kinase family (including protein kinase A, G and C). The sequence of Sch9 shows high similarity with other AGC protein kinases like Tpk1, 2, and 3 (Toda et al. 1988). Overexpression of *SCH9* also suppresses other lethal PKA mutations like the *tpk* triple deletion strain, *cyr1*Δ or *ras1*Δ *ras2*Δ. This is probably due to the fact that Sch9 regulates a similar set of genes as the Ras-cAMP-PKA pathway (Jorgensen et al. 2002). For example, overexpression of *SCH9* induces expression of ribosomal protein genes and represses genes involved in carboxylic acid metabolism (Zaman et al. 2008). Sch9 affects the PKA pathway since its deletion causes increased PKA activity in derepressed cells (Crauwels et al. 1997), which is probably mediated by controlling the localization and phosphorylation of Bcy1, the regulatory subunit of PKA. In repressed cells, Bcy1 is almost entirely localized in the nucleus. However, when yeast is grown on nonfermentable carbon sources, Bcy1 is observed both in the nucleus and in the cytoplasm (Griffioen et al. 2000, 2001). Deletion of *SCH9* causes constitutive nuclear localization of Bcy1, even in cells growing on glycerol (Zhang et al. 2011; Zhang and Gao 2012). Also the feedback regulation of Cdc25 by PKA phosphorylation seems to be controlled by Sch9 (Zhang et al. 2011). Although these studies provided evidence for direct involvement of Sch9 in control of PKA, other studies indicated that PKA and Sch9 also work in parallel, with either the same or different effects on specific phenotypes (Roosen et al. 2005). Sch9 also directly phosphorylates Rim15, which causes its inhibition by preventing its nuclear accumulation. Proper entrance into G_0 requires release of both PKA-mediated inhibition of its protein kinase activity and Sch9-mediated inhibition of its nuclear accumulation (Pedruzzi et al. 2003; Wanke et al. 2008).

Sch9 itself is a phosphoprotein, and its phosphorylation state is dramatically decreased upon carbon, nitrogen, and phosphate starvation. It has been shown that the rapamycin-sensitive, nutrient-responsive TORC1 (target of rapamycin complex 1) protein kinase causes activation of Sch9 by direct phosphorylation of its C-terminal part when nutrients are available (Urban et al. 2007). This activation leads to enhanced expression of ribosomal protein genes, stimulates ribosome biogenesis and translation initiation, and prevents entry into the G_0 phase (Urban et al. 2007; Huber et al. 2009, 2011). Sch9 is also phosphorylated and activated by the Snf1 protein kinase complex (Lu et al. 2011). Also the Pkh1 and Pkh2 protein kinases, which are involved in nutrient and stress signaling, are able to phosphorylate Sch9 (Roelants et al. 2004). Most likely, there are also other yet unknown kinases involved in the phosphorylation and regulation of Sch9.

2.4 The Main Glucose Repression Pathway

Another major regulator of cellular homeostasis in yeast carbon metabolism is the main glucose repression pathway. This pathway is responsible for the downregulation of respiration and the utilization of alternative sugars in the presence of glucose or related fermentable sugars, like fructose and mannose. In a typical aerobic yeast culture on glucose, the yeast will first grow rapidly by fermentation on the glucose, a phase in which respiration is repressed and ethanol accumulated. In this phase, the main glucose repression pathway is active and the cells are said to be glucose repressed. When the glucose concentration drops to a low level, the cells show a transient growth arrest, called diauxic shift, during which the enzymes for respiration and utilization of ethanol are being derepressed. Subsequently, the derepressed cells start to consume the ethanol utilizing respiration. In this phase, they grow much more slowly than during the first fermentation phase. When the ethanol is depleted, the cells enter stationary phase and remain derepressed. In this phase, they utilize storage carbohydrates (trehalose and glycogen) with respiration.

The Snf1 protein kinase is a major player in the main glucose repression pathway. It is an ortholog of the AMPK kinase family in mammalian cells. Snf1 acts in the sensing of glucose limitation (less than ±20 mM) and allows the cells to grow on less-preferred sugars, like sucrose and galactose, and on nonfermentable carbon sources, like ethanol and glycerol (Hedbacker and Carlson 2008; Zaman et al. 2008). Snf1 stands for "Sucrose Non Fermenting," a name allocated to the *snf1* mutant strain since it was unable to ferment sucrose but still able to ferment glucose (Carlson et al. 1981). The *snf1* mutant showed a defect in the expression of *SUC2*, which encodes invertase, an enzyme that catalyzes the conversion of sucrose into glucose and fructose (Neigeborn and Carlson 1984). The *snf4* mutant had the same phenotype and was also discovered in a screen for genes affecting the regulation of *SUC2* gene expression (Neigeborn and Carlson 1984). Subsequent work showed that Snf1 is part of a serine/threonine protein kinase complex with a heterotrimeric structure: it contains one catalytic α subunit (encoded by *SNF1*), one of three β subunits (encoded by *SIP1*, *SIP2,* and *GAL83*), and one regulatory γ subunit (encoded by *SNF4*) (Celenza and Carlson 1984, 1986).

The Snf1 protein kinase complex is regulated in different ways (Fig. 2.3). Activation of Snf1 occurs upon glucose limitation through phosphorylation by upstream protein kinases, release of autoinhibition by Snf4, and through control of its subcellular localization, which is regulated by the β subunits (Celenza et al. 1989; Celenza and Carlson 1989; Jiang and Carlson 1996; Leech et al. 2003). Three protein kinases with related kinase domains, Sak1, Elm1, and Tos3, activate Snf1 by phosphorylation of Thr^{210}. These kinases display high similarity and exert overlapping functions, so that abolishment of Snf1 activity in vivo is only observed in the triple mutant (Hong et al. 2003; Sutherland et al. 2003). The three upstream protein kinases are not affected by a drop in the external glucose level (Rubenstein et al. 2008), and glucose sensing for downregulation of Snf1 must therefore be

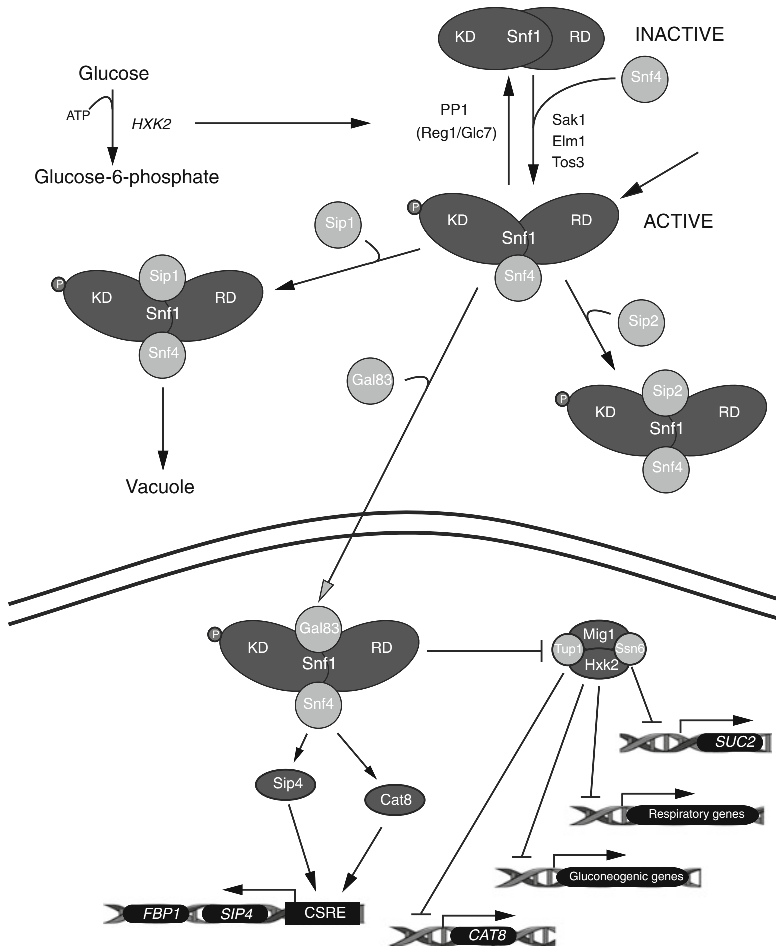

Fig. 2.3 The main glucose repression pathway in *S. cerevisiae*. In the inactive state, the regulatory domain (RD) of Snf1 covers the kinase domain of the catalytic domain (KD) thereby autoinhibiting it. In the absence of glucose, Snf4 can counteract the inhibition thereby opening up the complex. This open complex is phosphorylated by the redundant kinases Sak1, Elm1, and Tos3. The open phosphorylated Snf1/Snf4 complex is the active state and phosphorylates downstream targets. Upon glucose addition, the Snf1 complex is dephosphorylated by the Protein Phosphatase 1 (PP1) catalytic subunit Glc7, as controlled by its regulatory subunit Reg1. Glucose phosphorylation, possibly through activation of PKA, is probably responsible for PP1 activation. Active Snf1 complex is localized by its β subunits (Sip1, Sip2, and Gal83). Sip1 localizes the Snf1 complex toward the vacuole, Sip2 keeps the Snf1 complex in the cytoplasm, and Gal83 (the most abundant β) translocates the Snf1 complex toward the nucleus. In the nucleus, Snf1 phosphorylates Mig1, thereby inhibiting its repression of many target genes. Snf1 also phosphorylates the transcription factors Sip4 and Cat8 causing their activation

mediated by another mechanism. The activity of Snf1 is downregulated by dephosphorylation, mediated by Protein Phosphatase 1 (PP1). The catalytic subunit of this enzyme is encoded by *GLC7*. It has multiple regulatory subunits that target the catalytic domain to specific substrates, of which the Reg1 regulatory subunit plays a role in the downregulation of Snf1 and thus in control of the main glucose repression pathway (Feng et al. 1991; Tu and Carlson 1995; Tu et al. 1996). In a *reg1*Δ mutant, Snf1 is constitutively phosphorylated and active (McCartney and Schmidt 2001).

The control of Snf1 activity via phosphorylation/dephosphorylation is tightly connected with a second way of regulation, which is mediated by Snf4, the γ subunit of the Snf1 complex. Interaction between Snf1 and Snf4 is regulated by glucose availability. When glucose levels are low, Snf1 is phosphorylated on Thr^{210} and is then able to interact with Snf4. This leads to an open and active conformation of the complex, and thereby releases the autoinhibition caused by the regulatory domain of Snf1 (Celenza and Carlson 1989; Jiang and Carlson 1996; Estruch et al. 1992; Ludin et al. 1998). The active Snf1 kinase complex phosphorylates Reg1, thereby stabilizing the interaction between Snf1 and Reg1-Glc7 (Sanz et al. 2000). Upon glucose addition, Glc7 dephosphorylates Reg1 and subsequently dephosphorylates Snf1, causing its inactivation. The dephosphorylation of Reg1 by Glc7 seems to require Hxk2 activity (Sanz et al. 2000). Deletion of *HXK2* leads to an Snf1 kinase complex that is trapped in the active conformation. The *hxk2* mutant lacks glucose repression, and overexpression of *REG1* suppresses this defect (Sanz et al. 2000). The dephosphorylation of the Snf1 complex seems to stimulate its conversion from an open, active conformation to a closed, inactive autoinhibitory conformation (Ludin et al. 1998). The autoinhibitory state of the complex is thus restored by the dephosphorylation of Snf1 by Glc7. New evidence has shown that Reg1 can also bind to Snf1 independently of Glc7 (Elbing et al. 2006), and binding of Reg1 to Snf1 seems to use the same site in Reg1 as binding of Glc7 to Reg1 (Tabba et al. 2010), suggesting competition between the binding of Glc7 and Snf1 with Reg1.

Recent studies have identified Sit4 as a second phosphatase involved in the deactivation of Snf1 by dephosphorylation (Ruiz et al. 2011). The intracellular ADP concentration is also involved in the regulation of Snf1. Increased concentrations of ADP protect Snf1 from dephosphorylation by binding to Snf4 (Chandrashekarappa et al. 2011; Mayer et al. 2011). This contrasts with regulation of its mammalian homolog, which is protected from dephosphorylation by both high AMP and ADP levels (Davies et al. 1995; Xiao et al. 2011).

How glucose is sensed for regulation of the main glucose repression pathway has remained enigmatic in spite of the many detailed studies of this pathway. Also the discovery of the three upstream kinases of Snf1 did not bring an answer to this question, since they do not appear to be regulated by glucose availability (Rubenstein et al. 2008). All evidence, on the other hand, points to regulation of Snf1 dephosphorylation by glucose availability. Recent work may finally have brought an answer to this question. It revealed that addition of glucose to derepressed yeast cells triggers a rapid increase in the intrinsic activity of the PP1

protein phosphatase and that this activation depends on the regulatory subunits Reg1 and Shp1. Deletion of Shp1 also caused strong derepression of the invertase gene *SUC2*. Rapid glucose-induced activation of PP1 was dependent on activation of the PKA pathway (Castermans et al. 2012). There has been other evidence for interaction between the PKA pathway and the main glucose repression pathway. The deletion of *IRA1*, *IRA2,* or *BCY1*, which causes constitutive activation of the PKA pathway, causes reduced activation of the Snf1 kinase complex and suppresses the slow-growth phenotype of a *reg1* mutant. Conversely, downregulation of the PKA pathway by deletion of *GPR1* caused elevated Snf1 kinase activation (Barrett et al. 2012).

Finally, the activity of the Snf1 complex is also regulated by control of its intracellular localization as a function of glucose availability. When glucose concentrations are high, Snf1 and the three β subunits reside in the cytosol. Upon glucose limitation, the different β subunits direct the Snf1 kinase complex to different locations within the cell. Gal83 is the most abundant β subunit and is involved in the translocation of active Snf1 toward the nucleus (Vincent et al. 2001; Hedbacker et al. 2004a). Sip1 is involved in localization toward the vacuolar membrane, but in glucose-grown cells the maintenance of the cytosolic Sip1 localization is dependent on PKA activity (Hedbacker et al. 2004b). Sip2 is required to keep the Snf1 kinase complex in the cytoplasm (Vincent et al. 2001).

The activation of the Snf1 kinase complex has multiple functions. The complex can be translocated in a Gal83-mediated way toward the nucleus to affect the expression of a set of genes involved in the metabolism of alternative carbon sources, gluconeogenesis, respiration, transport, and meiosis (Hedbacker and Carlson 2008; Schuller 2003; Zaman et al. 2009). This set of genes is only small compared with the much more extensive changes in gene expression triggered by the Ras-cAMP-PKA pathway. In addition, a large part of the genes repressed after inactivation of Snf1 is also repressed by activation of the Ras-cAMP-PKA pathway (Zaman et al. 2009). This reflects the possible cooperation of PKA with the Snf1 kinase complex at least under certain conditions in affecting a similar set of cellular functions (Thompson-Jaeger et al. 1991; Hubbard et al. 1992).

Mig1 is the main transcription factor downstream in the glucose repression pathway (Nehlin et al. 1991; Nehlin and Ronne 1990). It is involved in glucose repression of at least 90 different genes, mostly required for the metabolism of alternative carbon sources (Klein et al. 1998; Lutfiyya et al. 1998). Snf1 phosphorylates the Mig1 transcriptional repressor and thereby promotes its nuclear export, causing derepression of Mig1-controlled genes. Mig1 also recruits the transcriptional co-repressor complex Ssn6-Tup1 (Treitel and Carlson 1995). Hxk2 is translocated toward the nucleus in a Mig1-dependent way and is part of the Mig1 repressor complex (Ahuatzi et al. 2007). For interaction between Mig1 and Hxk2, the serine at position 311 of Mig1 seems to be important. This site is the major Snf1 phosphorylation site and promotes nuclear export of Mig1 after phosphorylation. Hxk2 binds to this site thereby inhibiting Snf1-dependent phosphorylation of Mig1 (Ahuatzi et al. 2007).

Snf1 also positively regulates the transcriptional activators Cat8 and Sip4 (Lesage et al. 1996; Rahner et al. 1999; Hiesinger et al. 2001). These two transcriptional activators bind specifically to carbon source responsive elements (CSRE) under glucose-limiting conditions (Vincent and Carlson 1998). When activated, they induce the expression of genes involved in gluconeogenesis, respiration, and the glyoxylate cycle (Santangelo 2006). *SIP4* has a CSRE element in its promoter and is expressed upon activation of Cat8 by Snf1 phosphorylation (Vincent and Carlson 1998). The expression of *CAT8* in turn, is repressed by Mig1 (Hedges et al. 1995; Randez-Gil et al. 1997). Besides regulating gene transcription, Snf1 also regulates through phosphorylation proteins involved in fatty acid metabolism, carbohydrate storage, and transport (Hedbacker and Carlson 2008). For instance, Snf1 phosphorylates and inactivates acetyl-CoA carboxylase (Acc1). This results in blocked fatty acid biosynthesis under glucose-limiting conditions (Woods et al. 1994).

2.5 Conclusions

The exquisite preference of the yeast *S. cerevisiae* for glucose as carbon source is reflected in the multiple, sophisticated mechanisms that it has developed to detect the presence of glucose and to adjust various cellular functions accordingly. Two types of plasma membrane glucose sensors have been discovered first in *S. cerevisiae*: transporter homologues, which have developed into nontransporting glucose sensors, and a glucose-sensing GPCR. The concerted action of extracellular and intracellular glucose sensing has also been demonstrated and elucidated for the first time in *S. cerevisiae*. The Snf1 protein kinase has been discovered in *S. cerevisiae* as a central element of a glucose signaling pathway and has served as a model for investigation of the related AMP-activated kinase in other organisms. Elucidation of the enigmatic role of Ras in yeast glucose signaling may have important consequences for understanding aberrant glucose metabolism in tumor cells. We predict that glucose regulation of major protein phosphatases will reveal many novel and important aspects about glucose signaling and its interplay with other signal transduction pathways and mechanisms of cellular regulation.

Acknowledgments Original research performed in our laboratory was supported by a predoctoral fellowship to KP from the Agency for Innovation by Science and Technology (IWT-Flanders) and by grants from the Fund for Scientific Research—Flanders, Interuniversity Attraction Poles Network P6/14 and P7/40, the Research Fund of the KU Leuven (Concerted Research Actions) and the Hercules Foundation (Flanders) to JMT.

References

Ahuatzi D, Riera A, Pelaez R, Herrero P, Moreno F (2007) Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. J Biol Chem 282(7):4485–4493. doi:10.1074/jbc.M606854200 M606854200 [pii]

App H, Holzer H (1989) Purification and characterization of neutral trehalase from the yeast ABYS1 mutant. J Biol Chem 264(29):17583–17588

Babu P, Bryan JD, Panek HR, Jordan SL, Forbrich BM, Kelley SC, Colvin RT, Robinson LC (2002) Plasma membrane localization of the Yck2p yeast casein kinase 1 isoform requires the C-terminal extension and secretory pathway function. J Cell Sci 115(Pt 24):4957–4968

Barrett L, Orlova M, Maziarz M, Kuchin S (2012) Protein kinase A contributes to the negative control of Snf1 protein kinase in *Saccharomyces cerevisiae*. Eukaryot Cell 11(2):119–128. doi:10.1128/EC.05061-11 EC.05061-11 [pii]

Batlle M, Lu A, Green DA, Xue Y, Hirsch JP (2003) Krh1p and Krh2p act downstream of the Gpa2p G(alpha) subunit to negatively regulate haploid invasive growth. J Cell Sci 116(Pt 4):701–710

Beckner SK, Hattori S, Shih TY (1985) The *ras* oncogene product p21 is not a regulatory component of adenylate cyclase. Nature 317(6032):71–72

Beullens M, Mbonyi K, Geerts L, Gladines D, Detremerie K, Jans AW, Thevelein JM (1988) Studies on the mechanism of the glucose-induced cAMP signal in glycolysis and glucose repression mutants of the yeast *Saccharomyces cerevisiae*. Eur J Biochem 172(1):227–231

Bhattacharya S, Chen L, Broach JR, Powers S (1995) Ras membrane targeting is essential for glucose signaling but not for viability in yeast. Proc Natl Acad Sci USA 92(7):2984–2988

Bisson LF, Coons DM, Kruckeberg AL, Lewis DA (1993) Yeast sugar transporters. Crit Rev Biochem Mol Biol 28(4):259–308. doi:10.3109/10409239309078437

Bisson LF, Neigeborn L, Carlson M, Fraenkel DG (1987) The SNF3 gene is required for high-affinity glucose transport in *Saccharomyces cerevisiae*. J Bacteriol 169(4):1656–1662

Boguski MS, McCormick F (1993) Proteins regulating Ras and its relatives. Nature 366(6456):643–654. doi:10.1038/366643a0

Boles E, Hollenberg CP (1997) The molecular genetics of hexose transport in yeasts. FEMS Microbiol Rev 21(1):85–111 S0168-6445(97)00052-1 [pii]

Boy-Marcotte E, Ikonomi P, Jacquet M (1996) *SDC25*, a dispensable Ras guanine nucleotide exchange factor of *Saccharomyces cerevisiae* differs from *CDC25* by its regulation. Mol Biol Cell 7(4):529–539

Boy-Marcotte E, Perrot M, Bussereau F, Boucherie H, Jacquet M (1998) Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*. J Bacteriol 180(5):1044–1052

Broach JR, Deschenes RJ (1990) The function of ras genes in *Saccharomyces cerevisiae*. Adv Cancer Res 54:79–139

Broek D, Samiy N, Fasano O, Fujiyama A, Tamanoi F, Northup J, Wigler M (1985) Differential activation of yeast adenylate cyclase by wild-type and mutant RAS proteins. Cell 41(3):763–769 S0092-8674(85)80057-X [pii]

Broek D, Toda T, Michaeli T, Levin L, Birchmeier C, Zoller M, Powers S, Wigler M (1987) The *S. cerevisiae CDC25* gene product regulates the RAS/adenylate cyclase pathway. Cell 48(5):789–799 0092-8674(87)90076-6 [pii]

Budhwar R, Lu A, Hirsch JP (2010) Nutrient control of yeast PKA activity involves opposing effects on phosphorylation of the Bcy1 regulatory subunit. Mol Biol Cell 21(21):3749–3758. doi:10.1091/mbc.E10-05-0388

Cameroni E, Hulo N, Roosen J, Winderickx J, De Virgilio C (2004) The novel yeast PAS kinase Rim 15 orchestrates G0-associated antioxidant defense mechanisms. Cell Cycle 3(4):462–468 786 [pii]

Camonis JH, Kalekine M, Gondre B, Garreau H, Boy-Marcotte E, Jacquet M (1986) Characterization, cloning and sequence analysis of the *CDC25* gene which controls the cyclic AMP level of *Saccharomyces cerevisiae*. EMBO J 5(2):375–380

Carlson M, Osmond BC, Botstein D (1981) Mutants of yeast defective in sucrose utilization. Genetics 98(1):25–40

Caspani G, Tortora P, Hanozet GM, Guerritore A (1985) Glucose-stimulated cAMP increase may be mediated by intracellular acidification in *Saccharomyces cerevisiae*. FEBS Lett 186(1):75–79

Castermans D, Somers I, Kriel J, Louwet W, Wera S, Versele M, Janssens V, Thevelein JM (2012) Glucose-induced posttranslational activation of protein phosphatases PP2A and PP1 in yeast. Cell Res 22(6):1058–1077. doi:10.1038/cr.2012.20 cr201220 [pii]

Celenza JL, Carlson M (1984) Cloning and genetic mapping of SNF1, a gene required for expression of glucose-repressible genes in *Saccharomyces cerevisiae*. Mol Cell Biol 4(1):49–53

Celenza JL, Carlson M (1986) A yeast gene that is essential for release from glucose repression encodes a protein kinase. Science 233(4769):1175–1180

Celenza JL, Carlson M (1989) Mutational analysis of the *Saccharomyces cerevisiae* SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. Mol Cell Biol 9(11):5034–5044

Celenza JL, Eng FJ, Carlson M (1989) Molecular analysis of the SNF4 gene of *Saccharomyces cerevisiae*: evidence for physical association of the SNF4 protein with the SNF1 protein kinase. Mol Cell Biol 9(11):5045–5054

Chandrashekarappa DG, McCartney RR, Schmidt MC (2011) Subunit and domain requirements for adenylate-mediated protection of Snf1 kinase activation loop from dephosphorylation. J Biol Chem 286(52):44532–44541. doi:10.1074/jbc.M111.315895 M111.315895 [pii]

Chautard H, Jacquet M, Schoentgen F, Bureaud N, Benedetti H (2004) Tfs1p, a member of the PEBP family, inhibits the Ira2p but not the Ira1p Ras GTPase-activating protein in *Saccharomyces cerevisiae*. Eukaryot Cell 3(2):459–470

Chevtzoff C, Yoboue ED, Galinier A, Casteilla L, Daignan-Fornier B, Rigoulet M, Devin A (2010) Reactive oxygen species-mediated regulation of mitochondrial biogenesis in the yeast *Saccharomyces cerevisiae*. J Biol Chem 285(3):1733–1742. doi:10.1074/jbc.M109.019570

Colombo S, Ma P, Cauwenberg L, Winderickx J, Crauwels M, Teunissen A, Nauwelaers D, de Winde JH, Gorwa MF, Colavizza D, Thevelein JM (1998) Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. EMBO J 17(12):3326–3341. doi:10.1093/emboj/17.12.3326

Colombo S, Ronchetti D, Thevelein JM, Winderickx J, Martegani E (2004) Activation state of the Ras2 protein and glucose-induced signaling in *Saccharomyces cerevisiae*. J Biol Chem 279(45):46715–46722. doi:10.1074/jbc.M405136200 [pii]

Coons DM, Vagnoli P, Bisson LF (1997) The C-terminal domain of Snf3p is sufficient to complement the growth defect of snf3 null mutations in *Saccharomyces cerevisiae*: SNF3 functions in glucose recognition. Yeast 13(1):9–20. doi:10.1002/(SICI)1097-0061(199701)13:1<9:AID-YEA51>3.0.CO;2-U [pii] 10.1002/(SICI)1097-0061(199701)13:1 < 9:AID-YEA51 > 3.0.CO;2-U

Crauwels M, Donaton MC, Pernambuco MB, Winderickx J, de Winde JH, Thevelein JM (1997) The Sch9 protein kinase in the yeast *Saccharomyces cerevisiae* controls cAPK activity and is required for nitrogen activation of the fermentable-growth-medium-induced (FGM) pathway. Microbiology 143(Pt 8):2627–2637

Cytrynska M, Frajnt M, Jakubowicz T (2001) *Saccharomyces cerevisiae* pyruvate kinase Pyk1 is PKA phosphorylation substrate in vitro. FEMS Microbiol Lett 203(2):223–227

Damak F, Boy-Marcotte E, Le-Roscouet D, Guilbaud R, Jacquet M (1991) *SDC25*, a *CDC25*-like gene which contains a RAS-activating domain and is a dispensable gene of *Saccharomyces cerevisiae*. Mol Cell Biol 11(1):202–212

Daniel JH (1986) The CDC25 "Start" gene of *Saccharomyces cerevisiae*: sequencing of the active C-terminal fragment and regional homologies with rhodopsin and cytochrome P450. Curr Genet 10(12):879–885

Davies SP, Helps NR, Cohen PT, Hardie DG (1995) 5′-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. FEBS Lett 377(3):421–425. doi:10.1016/0014-5793(95)01368-7 0014-5793(95)01368-7 [pii]

Diaz-Ruiz R, Rigoulet M, Devin A (2011) The Warburg and Crabtree effects: on the origin of cancer cell energy metabolism and of yeast glucose repression. Biochim Biophys Acta 1807(6):568–576. doi:10.1016/j.bbabio.2010.08.010

Dihazi H, Kessler R, Eschrich K (2003) Glucose-induced stimulation of the Ras-cAMP pathway in yeast leads to multiple phosphorylations and activation of 6-phosphofructo-2-kinase. Biochemistry 42(20):6275–6282. doi:10.1021/bi034167r

Dlugai S, Hippler S, Wieczorke R, Boles E (2001) Glucose-dependent and -independent signalling functions of the yeast glucose sensor Snf3. FEBS Lett 505(3):389–392 S0014-5793(01)02854-X [pii]

Dong J, Bai X (2011) The membrane localization of Ras2p and the association between Cdc25p and Ras2-GTP are regulated by protein kinase A (PKA) in the yeast *Saccharomyces cerevisiae*. FEBS Lett 585(8):1127–1134. doi:10.1016/j.febslet.2011.03.057 S0014-5793(11)00223-7 [pii]

Durnez P, Pernambuco MB, Oris E, Arguelles JC, Mergelsberg H, Thevelein JM (1994) Activation of trehalase during growth induction by nitrogen sources in the yeast *Saccharomyces cerevisiae* depends on the free catalytic subunits of cAMP-dependent protein kinase, but not on functional Ras proteins. Yeast 10(8):1049–1064. doi:10.1002/yea.320100807

Edmondson DG, Smith MM, Roth SY (1996) Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. Genes Dev 10(10):1247–1259

Elbing K, McCartney RR, Schmidt MC (2006) Purification and characterization of the three Snf1-activating kinases of *Saccharomyces cerevisiae*. Biochem J 393(Pt 3):797–805. doi:10.1042/BJ20051213 BJ20051213 [pii]

Estruch F, Carlson M (1993) Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. Mol Cell Biol 13(7):3872–3881

Estruch F, Treitel MA, Yang X, Carlson M (1992) N-terminal mutations modulate yeast SNF1 protein kinase function. Genetics 132(3):639–650

Farnsworth CL, Feig LA (1991) Dominant inhibitory mutations in the Mg(2 +)-binding site of RasH prevent its activation by GTP. Mol Cell Biol 11(10):4822–4829

Fedor-Chaiken M, Deschenes RJ, Broach JR (1990) *SRV2*, a gene required for RAS activation of adenylate cyclase in yeast. Cell 61(2):329–340

Feng ZH, Wilson SE, Peng ZY, Schlender KK, Reimann EM, Trumbly RJ (1991) The yeast GLC7 gene required for glycogen accumulation encodes a type 1 protein phosphatase. J Biol Chem 266(35):23796–23801

Field J, Vojtek A, Ballester R, Bolger G, Colicelli J, Ferguson K, Gerst J, Kataoka T, Michaeli T, Powers S et al (1990) Cloning and characterization of *CAP*, the *S. cerevisiae* gene encoding the 70 kd adenylyl cyclase-associated protein. Cell 61(2):319–327. doi:10.1099/mic.0.27144-0150/9/2865 [pii]

Folch-Mallol JL, Martinez LM, Casas SJ, Yang R, Martinez-Anaya C, Lopez L, Hernandez A, Nieto-Sotelo J (2004) New roles for CDC25 in growth control, galactose regulation and cellular differentiation in *Saccharomyces cerevisiae*. Microbiology 150(Pt 9):2865–2879. doi:10.1099/mic.0.27144-0150/9/2865 [pii]

Francois J, Hers HG (1988) The control of glycogen metabolism in yeast. 2. A kinetic study of the two forms of glycogen synthase and of glycogen phosphorylase and an investigation of their interconversion in a cell-free extract. Eur J Biochem 174(3):561–567

Francois J, Parrou JL (2001) Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25(1):125–145

Garreau H, Geymonat M, Renault G, Jacquet M (1996) Membrane-anchoring domains of Cdc25p, a *Saccharomyces cerevisiae* ras exchange factor. Biol Cell 86(2–3):93–102

Garrett S, Broach J (1989) Loss of Ras activity in *Saccharomyces cerevisiae* is suppressed by disruptions of a new kinase gene, YAKI, whose product may act downstream of the cAMP-dependent protein kinase. Genes Dev 3(9):1336–1348

Garrett S, Menold MM, Broach JR (1991) The *Saccharomyces cerevisiae YAK1* gene encodes a protein kinase that is induced by arrest early in the cell cycle. Mol Cell Biol 11(8):4045–4052

Giots F, Donaton MC, Thevelein JM (2003) Inorganic phosphate is sensed by specific phosphate carriers and acts in concert with glucose as a nutrient signal for activation of the protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol Microbiol 47(4):1163–1181

Gorner W, Durchschlag E, Martinez-Pastor MT, Estruch F, Ammerer G, Hamilton B, Ruis H, Schuller C (1998) Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev 12(4):586–597

Gorner W, Durchschlag E, Wolf J, Brown EL, Ammerer G, Ruis H, Schuller C (2002) Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. EMBO J 21(1–2):135–144. doi:10.1093/emboj/21.1.135

Gourlay CW, Ayscough KR (2006) Actin-induced hyperactivation of the Ras signaling pathway leads to apoptosis in *Saccharomyces cerevisiae*. Mol Cell Biol 26(17):6487–6501. doi:10.1128/MCB.00117-06

Graul RC, Sadee W (2001) Evolutionary relationships among G protein-coupled receptors using a clustered database approach. AAPS PharmSci 3(2):E12

Griffioen G, Anghileri P, Imre E, Baroni MD, Ruis H (2000) Nutritional control of nucleocytoplasmic localization of cAMP-dependent protein kinase catalytic and regulatory subunits in *Saccharomyces cerevisiae*. J Biol Chem 275(2):1449–1456

Griffioen G, Branduardi P, Ballarini A, Anghileri P, Norbeck J, Baroni MD, Ruis H (2001) Nucleocytoplasmic distribution of budding yeast protein kinase A regulatory subunit Bcy1 requires Zds1 and is regulated by Yak1-dependent phosphorylation of its targeting domain. Mol Cell Biol 21(2):511–523. doi:10.1128/MCB.21.2.511-523.2001

Griffioen G, Mager WH, Planta RJ (1994) Nutritional upshift response of ribosomal protein gene transcription in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 123(1–2):137–144

Gross E, Goldberg D, Levitzki A (1992) Phosphorylation of the S. cerevisiae Cdc25 in response to glucose results in its dissociation from Ras. Nature 360(6406):762–765. doi:10.1038/360762a0

Haney SA, Broach JR (1994) Cdc25p, the guanine nucleotide exchange factor for the Ras proteins of *Saccharomyces cerevisiae*, promotes exchange by stabilizing Ras in a nucleotide-free state. J Biol Chem 269(24):16541–16548

Harashima T, Anderson S, Yates JR 3rd, Heitman J (2006) The kelch proteins Gpb1 and Gpb2 inhibit Ras activity via association with the yeast RasGAP neurofibromin homologs Ira1 and Ira2. Mol Cell 22(6):819–830. doi:10.1016/j.molcel.2006.05.011 S1097-2765(06)00324-8 [pii]

Harashima T, Heitman J (2002) The Galpha protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic Gbeta subunits. Mol Cell 10(1):163–173 S1097276502005695 [pii]

Harashima T, Heitman J (2005) Galpha subunit Gpa2 recruits kelch repeat subunits that inhibit receptor-G protein coupling during cAMP-induced dimorphic transitions in *Saccharomyces cerevisiae*. Mol Biol Cell 16(10):4557–4571. doi:10.1091/mbc.E05-05-0403 E05-05-0403 [pii]

Hardy TA, Roach PJ (1993) Control of yeast glycogen synthase-2 by COOH-terminal phosphorylation. J Biol Chem 268(32):23799–23805

Hayles J, Nurse P (1986) Cell cycle regulation in yeast. J Cell Sci Suppl 4:155–170

Hedbacker K, Carlson M (2008) SNF1/AMPK pathways in yeast. Front Biosci 13:2408–2420 2854 [pii]

Hedbacker K, Hong SP, Carlson M (2004a) Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. Mol Cell Biol 24(18):8255–8263. doi:10.1128/MCB.24.18.8255-8263.2004 24/18/8255 [pii]

Hedbacker K, Townley R, Carlson M (2004b) Cyclic AMP-dependent protein kinase regulates the subcellular localization of Snf1-Sip1 protein kinase. Mol Cell Biol 24(5):1836–1843

Hedges D, Proft M, Entian KD (1995) CAT8, a new zinc cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol 15(4):1915–1922

Herruer MH, Mager WH, Woudt LP, Nieuwint RT, Wassenaar GM, Groeneveld P, Planta RJ (1987) Transcriptional control of yeast ribosomal protein synthesis during carbon-source upshift. Nucleic Acids Res 15(24):10133–10144

Hiesinger M, Roth S, Meissner E, Schuller HJ (2001) Contribution of Cat8 and Sip4 to the transcriptional activation of yeast gluconeogenic genes by carbon source-responsive elements. Curr Genet 39(2):68–76

Hirimburegama K, Durnez P, Keleman J, Oris E, Vergauwen R, Mergelsberg H, Thevelein JM (1992) Nutrient-induced activation of trehalase in nutrient-starved cells of the yeast *Saccharomyces cerevisiae*: cAMP is not involved as second messenger. J Gen Microbiol 138(10):2035–2043

Hoffman CS (2007) Propping up our knowledge of G protein signaling pathways: diverse functions of putative noncanonical G beta subunits in fungi. Sci STKE 370:pe3

Holsbeeks I, Lagatie O, Van Nuland A, Van de Velde S, Thevelein JM (2004) The eukaryotic plasma membrane as a nutrient-sensing device. Trends Biochem Sci 29(10):556–564. doi:10.1016/j.tibs.2004.08.010S0968-0004(04)00211-7 [pii]

Hong SP, Leiper FC, Woods A, Carling D, Carlson M (2003) Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. Proc Natl Acad Sci USA 100(15):8839–8843. doi:10.1073/pnas.15331361001533136100 [pii]

Hu Y, Liu E, Bai X, Zhang A (2010) The localization and concentration of the PDE2-encoded high-affinity cAMP phosphodiesterase is regulated by cAMP-dependent protein kinase A in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 10(2):177–187. doi:10.1111/j.1567-1364.2009.00598.x FYR598 [pii]

Hubbard EJ, Yang XL, Carlson M (1992) Relationship of the cAMP-dependent protein kinase pathway to the SNF1 protein kinase and invertase expression in *Saccharomyces cerevisiae*. Genetics 130(1):71–80

Huber A, Bodenmiller B, Uotila A, Stahl M, Wanka S, Gerrits B, Aebersold R, Loewith R (2009) Characterization of the rapamycin-sensitive phosphoproteome reveals that Sch9 is a central coordinator of protein synthesis. Genes Dev 23(16):1929–1943. doi:10.1101/gad.532109 23/16/1929 [pii]

Huber A, French SL, Tekotte H, Yerlikaya S, Stahl M, Perepelkina MP, Tyers M, Rougemont J, Beyer AL, Loewith R (2011) Sch9 regulates ribosome biogenesis via Stb3, Dot6 and Tod6 and the histone deacetylase complex RPD3L. EMBO J 30(15):3052–3064. doi:10.1038/emboj.2011.221 emboj2011221 [pii]

Jian D, Aili Z, Xiaojia B, Huansheng Z, Yun H (2010) Feedback regulation of Ras2 guanine nucleotide exchange factor (Ras2-GEF) activity of Cdc25p by Cdc25p phosphorylation in the yeast *Saccharomyces cerevisiae*. FEBS Lett 584(23):4745–4750. doi:10.1016/j.febslet.2010.11.006 S0014-5793(10)00900-2 [pii]

Jiang R, Carlson M (1996) Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. Genes Dev 10(24):3105–3115

Johnston M, Kim JH (2005) Glucose as a hormone: receptor-mediated glucose sensing in the yeast *Saccharomyces cerevisiae*. Biochem Soc Trans 33(Pt 1):247–252. doi:10.1042/BST0330247 BST0330247 [pii]

Jones S, Vignais ML, Broach JR (1991) The CDC25 protein of *Saccharomyces cerevisiae* promotes exchange of guanine nucleotides bound to ras. Mol Cell Biol 11(5):2641–2646

Jorgensen P, Nishikawa JL, Breitkreutz BJ, Tyers M (2002) Systematic identification of pathways that couple cell growth and division in yeast. Science 297(5580):395–400. doi:10.1126/science.10708501070850 [pii]

Kataoka T, Broek D, Wigler M (1985) DNA sequence and characterization of the *S. cerevisiae* gene encoding adenylate cyclase. Cell 43(2 Pt 1):493–505 0092-8674(85)90179-5 [pii]

Kataoka T, Powers S, McGill C, Fasano O, Strathern J, Broach J, Wigler M (1984) Genetic analysis of yeast RAS1 and RAS2 genes. Cell 37(2):437–445 0092-8674(84)90374-X [pii]

Kim JH, Polish J, Johnston M (2003) Specificity and regulation of DNA binding by the yeast glucose transporter gene repressor Rgt1. Mol Cell Biol 23(15):5208–5216

Kishi T, Seno T, Yamao F (1998) Grr1 functions in the ubiquitin pathway in *Saccharomyces cerevisiae* through association with Skp1. Mol Gen Genet 257(2):143–148

Klein CJ, Olsson L, Nielsen J (1998) Glucose control in *Saccharomyces cerevisiae*: the role of Mig1 in metabolic functions. Microbiology 144(Pt 1):13–24

Kotting C, Kallenbach A, Suveyzdis Y, Wittinghofer A, Gerwert K (2008) The GAP arginine finger movement into the catalytic site of Ras increases the activation entropy. Proc Natl Acad Sci USA 105(17):6260–6265. doi:10.1073/pnas.0712095105 0712095105 [pii]

Kraakman L, Lemaire K, Ma P, Teunissen AW, Donaton MC, Van Dijck P, Winderickx J, de Winde JH, Thevelein JM (1999) A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. Mol Microbiol 32(5):1002–1012

Kraakman LS, Griffioen G, Zerp S, Groeneveld P, Thevelein JM, Mager WH, Planta RJ (1993) Growth-related expression of ribosomal protein genes in *Saccharomyces cerevisiae*. Mol Gen Genet 239(1–2):196–204

Kriel J, Haesendonckx S, Rubio-Texeira M, Van Zeebroeck G, Thevelein JM (2011) From transporter to transceptor: signaling from transporters provokes re-evaluation of complex trafficking and regulatory controls: endocytic internalization and intracellular trafficking of nutrient transceptors may, at least in part, be governed by their signaling function. BioEssays 33(11):870–879. doi:10.1002/bies.201100100

Kubler E, Mosch HU, Rupp S, Lisanti MP (1997) Gpa2p, a G-protein alpha-subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. J Biol Chem 272(33):20321–20323

Kuret J, Johnson KE, Nicolette C, Zoller MJ (1988) Mutagenesis of the regulatory subunit of yeast cAMP-dependent protein kinase. Isolation of site-directed mutants with altered binding affinity for catalytic subunit. J Biol Chem 263(19):9149–9154

Lai CC, Boguski M, Broek D, Powers S (1993) Influence of guanine nucleotides on complex formation between Ras and CDC25 proteins. Mol Cell Biol 13(3):1345–1352

Lakshmanan J, Mosley AL, Ozcan S (2003) Repression of transcription by Rgt1 in the absence of glucose requires Std1 and Mth1. Curr Genet 44(1):19–25. doi:10.1007/s00294-003-0423-2

Lee P, Cho BR, Joo HS, Hahn JS (2008) Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. Mol Microbiol 70(4):882–895. doi:10.1111/j.1365-2958.2008.06450.x MMI6450 [pii]

Leech A, Nath N, McCartney RR, Schmidt MC (2003) Isolation of mutations in the catalytic domain of the snf1 kinase that render its activity independent of the snf4 subunit. Eukaryot Cell 2(2):265–273

Lemaire K, Van de Velde S, Van Dijck P, Thevelein JM (2004) Glucose and sucrose act as agonist and mannose as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast *Saccharomyces cerevisiae*. Mol Cell 16(2):293–299. doi:10.1016/j.molcel.2004.10.004 S1097276504006100 [pii]

Lesage P, Yang X, Carlson M (1996) Yeast SNF1 protein kinase interacts with SIP4, a C6 zinc cluster transcriptional activator: a new role for SNF1 in the glucose response. Mol Cell Biol 16(5):1921–1928

Lillie SH, Pringle JR (1980) Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. J Bacteriol 143(3):1384–1394

Lorenz MC, Heitman J (1997) Yeast pseudohyphal growth is regulated by GPA2, a G protein alpha homolog. EMBO J 16(23):7008–7018. doi:10.1093/emboj/16.23.7008

Lu A, Hirsch JP (2005) Cyclic AMP-independent regulation of protein kinase A substrate phosphorylation by Kelch repeat proteins. Eukaryot Cell 4(11):1794–1800. doi:10.1128/EC.4.11.1794-1800.2005 4/11/1794 [pii]

Lu JY, Lin YY, Sheu JC, Wu JT, Lee FJ, Chen Y, Lin MI, Chiang FT, Tai TY, Berger SL, Zhao Y, Tsai KS, Zhu H, Chuang LM, Boeke JD (2011) Acetylation of yeast AMPK controls intrinsic aging independently of caloric restriction. Cell 146(6):969–979. doi:10.1016/j.cell.2011.07.044 S0092-8674(11)00888-9 [pii]

Ludin K, Jiang R, Carlson M (1998) Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 95(11):6245–6250

Lutfiyya LL, Iyer VR, DeRisi J, DeVit MJ, Brown PO, Johnston M (1998) Characterization of three related glucose repressors and genes they regulate in *Saccharomyces cerevisiae*. Genetics 150(4):1377–1391

Ma P, Wera S, Van Dijck P, Thevelein JM (1999) The *PDE1*-encoded low-affinity phosphodiesterase in the yeast *Saccharomyces cerevisiae* has a specific function in controlling agonist-induced cAMP signaling. Mol Biol Cell 10(1):91–104

Marion RM, Regev A, Segal E, Barash Y, Koller D, Friedman N, O'Shea EK (2004) Sfp1 is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression. Proc Natl Acad Sci USA 101(40):14315–14322. doi:10.1073/pnas.0405353101

Markwardt DD, Garrett JM, Eberhardy S, Heideman W (1995) Activation of the Ras/cyclic AMP pathway in the yeast *Saccharomyces cerevisiae* does not prevent G1 arrest in response to nitrogen starvation. J Bacteriol 177(23):6761–6765

Marshall-Carlson L, Celenza JL, Laurent BC, Carlson M (1990) Mutational analysis of the SNF3 glucose transporter of *Saccharomyces cerevisiae*. Mol Cell Biol 10(3):1105–1115

Martinez-Pastor MT, Marchler G, Schuller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The *Saccharomyces cerevisiae zinc* finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J 15(9):2227–2235

Matsumoto K, Uno I, Oshima Y, Ishikawa T (1982) Isolation and characterization of yeast mutants deficient in adenylate cyclase and cAMP-dependent protein kinase. Proc Natl Acad Sci USA 79(7):2355–2359

Mayer FV, Heath R, Underwood E, Sanders MJ, Carmena D, McCartney RR, Leiper FC, Xiao B, Jing C, Walker PA, Haire LF, Ogrodowicz R, Martin SR, Schmidt MC, Gamblin SJ, Carling D (2011) ADP regulates SNF1, the *Saccharomyces cerevisiae* homolog of AMP-activated protein kinase. Cell Metab 14(5):707–714. doi:10.1016/j.cmet.2011.09.009 S1550-4131(11)00355-X [pii]

Mazon MJ, Gancedo JM, Gancedo C (1982) Phosphorylation and inactivation of yeast fructose-bisphosphatase in vivo by glucose and by proton ionophores. A possible role for cAMP. Eur J Biochem 127(3):605–608

Mbonyi K, Beullens M, Detremerie K, Geerts L, Thevelein JM (1988) Requirement of one functional *RAS* gene and inability of an oncogenic *ras* variant to mediate the glucose-induced cyclic AMP signal in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol 8(8):3051–3057

Mbonyi K, van Aelst L, Arguelles JC, Jans AW, Thevelein JM (1990) Glucose-induced hyperaccumulation of cyclic AMP and defective glucose repression in yeast strains with reduced activity of cyclic AMP-dependent protein kinase. Mol Cell Biol 10(9):4518–4523

McCartney RR, Schmidt MC (2001) Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. J Biol Chem 276(39):36460–36466. doi:10.1074/jbc.M104418200M104418200 [pii]

Mintzer KA, Field J (1999) The SH3 domain of the *S. cerevisiae* Cdc25p binds adenylyl cyclase and facilitates Ras regulation of cAMP signalling. Cell Signal 11(2):127–135 S0898-6568(98)00044-8 [pii]

Mitts MR, Bradshaw-Rouse J, Heideman W (1991) Interactions between adenylate cyclase and the yeast GTPase-activating protein IRA1. Mol Cell Biol 11(9):4591–4598

Mizunuma M, Tsubakiyama R, Ogawa T, Shitamukai A, Kobayashi Y, Inai T, Kume K, Hirata D (2013) Ras/cAMP-dependent protein kinase (PKA) regulates multiple aspects of cellular events by phosphorylating the Whi3 cell cycle regulator in budding yeast. J Biol Chem 288(15):10558–10566. doi:10.1074/jbc.M112.402214

Moriya H, Johnston M (2004) Glucose sensing and signaling in *Saccharomyces cerevisiae* through the Rgt2 glucose sensor and casein kinase I. Proc Natl Acad Sci USA 101(6): 1572–1577. doi:10.1073/pnas.03059011010305901101 [pii]

Moriya H, Shimizu-Yoshida Y, Omori A, Iwashita S, Katoh M, Sakai A (2001) Yak1p, a DYRK family kinase, translocates to the nucleus and phosphorylates yeast Pop2p in response to a glucose signal. Genes Dev 15(10):1217–1228. doi:10.1101/gad.884001

Mosley AL, Lakshmanan J, Aryal BK, Ozcan S (2003) Glucose-mediated phosphorylation converts the transcription factor Rgt1 from a repressor to an activator. J Biol Chem 278(12):10322–10327. doi:10.1074/jbc.M212802200M212802200 [pii]

Nakafuku M, Obara T, Kaibuchi K, Miyajima I, Miyajima A, Itoh H, Nakamura S, Arai K, Matsumoto K, Kaziro Y (1988) Isolation of a second yeast *Saccharomyces cerevisiae* gene (*GPA2*) coding for guanine nucleotide-binding regulatory protein: studies on its structure and possible functions. Proc Natl Acad Sci USA 85(5):1374–1378

Nehlin JO, Carlberg M, Ronne H (1991) Control of yeast *GAL* genes by MIG1 repressor: a transcriptional cascade in the glucose response. EMBO J 10(11):3373–3377

Nehlin JO, Ronne H (1990) Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. EMBO J 9(9):2891–2898

Neigeborn L, Carlson M (1984) Genes affecting the regulation of SUC2 gene expression by glucose repression in *Saccharomyces cerevisiae*. Genetics 108(4):845–858

Nikawa J, Cameron S, Toda T, Ferguson KM, Wigler M (1987) Rigorous feedback control of cAMP levels in *Saccharomyces cerevisiae*. Genes Dev 1(9):931–937

Niranjan T, Guo X, Victor J, Lu A, Hirsch JP (2007) Kelch repeat protein interacts with the yeast Galpha subunit Gpa2p at a site that couples receptor binding to guanine nucleotide exchange. J Biol Chem 282(33):24231–24238. doi:10.1074/jbc.M702595200

Noda T, Hoffschulte H, Holzer H (1984) Characterization of fructose 1,6-bisphosphatase from bakers' yeast. J Biol Chem 259(11):7191–7197

Ozcan S, Dover J, Rosenwald AG, Wolfl S, Johnston M (1996) Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proc Natl Acad Sci USA 93(22):12428–12432

Ozcan S, Johnston M (1995) Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. Mol Cell Biol 15(3): 1564–1572

Ozcan S, Johnston M (1999) Function and regulation of yeast hexose transporters. Microbiol Mol Biol Rev 63(3):554–569

Pai EF, Krengel U, Petsko GA, Goody RS, Kabsch W, Wittinghofer A (1990) Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis. EMBO J 9(8):2351–2359

Palomino A, Herrero P, Moreno F (2005) Rgt1, a glucose sensing transcription factor, is required for transcriptional repression of the HXK2 gene in *Saccharomyces cerevisiae*. Biochem J 388(Pt 2):697–703. doi:10.1042/BJ20050160 BJ20050160 [pii]

Palomino A, Herrero P, Moreno F (2006) Tpk3 and Snf1 protein kinases regulate Rgt1 association with *Saccharomyces cerevisiae* HXK2 promoter. Nucleic Acids Res 34(5): 1427–1438. doi:10.1093/nar/gkl028 34/5/1427 [pii]

Pedruzzi I, Burckert N, Egger P, De Virgilio C (2000) *Saccharomyces cerevisiae* Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1. EMBO J 19(11):2569–2579. doi:10.1093/emboj/19.11.2569

Pedruzzi I, Dubouloz F, Cameroni E, Wanke V, Roosen J, Winderickx J, De Virgilio C (2003) TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into G0. Mol Cell 12(6):1607–1613 S1097276503004854 [pii]

Peeters T, Louwet W, Gelade R, Nauwelaers D, Thevelein JM, Versele M (2006) Kelch-repeat proteins interacting with the Galpha protein Gpa2 bypass adenylate cyclase for direct regulation of protein kinase A in yeast. Proc Natl Acad Sci USA 103(35):13034–13039. doi:10.1073/pnas.0509644103 0509644103 [pii]

Peeters T, Versele M, Thevelein JM (2007) Directly from Galpha to protein kinase A: the kelch repeat protein bypass of adenylate cyclase. Trends Biochem Sci 32(12):547–554. doi:10.1016/j.tibs.2007.09.011 S0968-0004(07)00262-9 [pii]

Phan VT, Ding VW, Li F, Chalkley RJ, Burlingame A, McCormick F (2010) The RasGAP proteins Ira2 and neurofibromin are negatively regulated by Gpb1 in yeast and ETEA in humans. Mol Cell Biol 30(9):2264–2279. doi:10.1128/MCB.01450-08

Pohlig G, Holzer H (1985) Phosphorylation and inactivation of yeast fructose-1,6-bisphosphatase by cyclic AMP-dependent protein kinase from yeast. J Biol Chem 260(25):13818–13823

Polish JA, Kim JH, Johnston M (2005) How the Rgt1 transcription factor of *Saccharomyces cerevisiae* is regulated by glucose. Genetics 169(2):583–594. doi:10.1534/genetics.104.034512 genetics.104.034512 [pii]

Portela P, Moreno S, Rossi S (2006) Characterization of yeast pyruvate kinase 1 as a protein kinase A substrate, and specificity of the phosphorylation site sequence in the whole protein. Biochem J 396(1):117–126. doi:10.1042/BJ20051642

Powers S, Kataoka T, Fasano O, Goldfarb M, Strathern J, Broach J, Wigler M (1984) Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian ras proteins. Cell 36(3):607–612 0092-8674(84)90340-4 [pii]

Ptacek J, Devgan G, Michaud G, Zhu H, Zhu X, Fasolo J, Guo H, Jona G, Breitkreutz A, Sopko R, McCartney RR, Schmidt MC, Rachidi N, Lee SJ, Mah AS, Meng L, Stark MJ, Stern DF, De Virgilio C, Tyers M, Andrews B, Gerstein M, Schweitzer B, Predki PF, Snyder M (2005) Global analysis of protein phosphorylation in yeast. Nature 438(7068):679–684. doi:10.1038/nature04187 nature04187 [pii]

Purwin C, Leidig F, Holzer H (1982) Cyclic AMP-dependent phosphorylation of fructose-1,6-bisphosphatase in yeast. Biochem Biophys Res Commun 107(4):1482–1489 S0006-291X(82)80166-6 [pii]

Purwin C, Nicolay K, Scheffers WA, Holzer H (1986) Mechanism of control of adenylate cyclase activity in yeast by fermentable sugars and carbonyl cyanide m-chlorophenylhydrazone. J Biol Chem 261(19):8744–8749

Rahner A, Hiesinger M, Schuller HJ (1999) Deregulation of gluconeogenic structural genes by variants of the transcriptional activator Cat8p of the yeast *Saccharomyces cerevisiae*. Mol Microbiol 34(1):146–156 mmi1588 [pii]

Randez-Gil F, Bojunga N, Proft M, Entian KD (1997) Glucose derepression of gluconeogenic enzymes in *Saccharomyces cerevisiae* correlates with phosphorylation of the gene activator Cat8p. Mol Cell Biol 17(5):2502–2510

Reinders A, Burckert N, Boller T, Wiemken A, De Virgilio C (1998) *Saccharomyces cerevisiae* cAMP-dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase. Genes Dev 12(18):2943–2955

Robertson LS, Fink GR (1998) The three yeast A kinases have specific signaling functions in pseudohyphal growth. Proc Natl Acad Sci USA 95(23):13783–13787

Robinson LC, Hubbard EJ, Graves PR, DePaoli-Roach AA, Roach PJ, Kung C, Haas DW, Hagedorn CH, Goebl M, Culbertson MR et al (1992) Yeast casein kinase I homologues: an essential gene pair. Proc Natl Acad Sci USA 89(1):28–32

Roelants FM, Torrance PD, Thorner J (2004) Differential roles of PDK1- and PDK2-phosphorylation sites in the yeast AGC kinases Ypk1, Pkc1 and Sch9. Microbiology 150(Pt 10):3289–3304. doi:10.1099/mic.0.27286-0 150/10/3289 [pii]

Rolland F, De Winde JH, Lemaire K, Boles E, Thevelein JM, Winderickx J (2000) Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. Mol Microbiol 38(2):348–358 mmi2125 [pii]

Rolland F, Winderickx J, Thevelein JM (2001) Glucose-sensing mechanisms in eukaryotic cells. Trends Biochem Sci 26(5):310–317

Rolland F, Winderickx J, Thevelein JM (2002) Glucose-sensing and -signalling mechanisms in yeast. FEMS Yeast Res 2(2):183–201

Roosen J, Engelen K, Marchal K, Mathys J, Griffioen G, Cameroni E, Thevelein JM, De Virgilio C, De Moor B, Winderickx J (2005) PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability. Mol Microbiol 55(3):862–880. doi:10.1111/j.1365-2958.2004.04429.x MMI4429 [pii]

Roy A, Shin YJ, Cho KH, Kim JH (2013) Mth1 regulates the interaction between the Rgt1 repressor and the Ssn6-Tup1 corepressor complex by modulating PKA-dependent phosphorylation of Rgt1. Mol Biol Cell 24(9):1493–1503. doi:10.1091/mbc.E13-01-0047

Rubenstein EM, McCartney RR, Zhang C, Shokat KM, Shirra MK, Arndt KM, Schmidt MC (2008) Access denied: Snf1 activation loop phosphorylation is controlled by availability of the phosphorylated threonine 210 to the PP1 phosphatase. J Biol Chem 283(1):222–230. doi:10.1074/jbc.M707957200 M707957200 [pii]

Ruiz A, Xu X, Carlson M (2011) Roles of two protein phosphatases, Reg1-Glc7 and Sit4, and glycogen synthesis in regulation of SNF1 protein kinase. Proc Natl Acad Sci USA 108(16):6349–6354. doi:10.1073/pnas.1102758108 1102758108 [pii]

Santangelo GM (2006) Glucose signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 70(1):253–282. doi:10.1128/MMBR.70.1.253-282.2006 70/1/253 [pii]

Sanz P, Alms GR, Haystead TA, Carlson M (2000) Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. Mol Cell Biol 20(4):1321–1328

Schepers W, Van Zeebroeck G, Pinkse M, Verhaert P, Thevelein JM (2012) In vivo phosphorylation of Ser21 and Ser83 during nutrient-induced activation of the yeast protein kinase A (PKA) target trehalase. J Biol Chem 287(53):44130–44142. doi:10.1074/jbc.M112.421503

Schmitt AP, McEntee K (1996) Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 93(12):5777–5782

Schomerus C, Munder T, Kuntzel H (1990) Site-directed mutagenesis of the *Saccharomyces cerevisiae CDC25* gene: effects on mitotic growth and cAMP signalling. Mol Gen Genet 223(3):426–432

Schuller HJ (2003) Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. Curr Genet 43(3):139–160. doi:10.1007/s00294-003-0381-8

Smets B, Ghillebert R, De Snijder P, Binda M, Swinnen E, De Virgilio C, Winderickx J (2010) Life in the midst of scarcity: adaptations to nutrient availability in *Saccharomyces cerevisiae*. Curr Genet 56(1):1–32. doi:10.1007/s00294-009-0287-1

Smith A, Ward MP, Garrett S (1998) Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. EMBO J 17(13):3556–3564. doi:10.1093/emboj/17.13.3556

Sutherland CM, Hawley SA, McCartney RR, Leech A, Stark MJ, Schmidt MC, Hardie DG (2003) Elm1p is one of three upstream kinases for the *Saccharomyces cerevisiae* SNF1 complex. Curr Biol 13(15):1299–1305 S0960982203004597 [pii]

Tabba S, Mangat S, McCartney R, Schmidt MC (2010) PP1 phosphatase-binding motif in Reg1 protein of *Saccharomyces cerevisiae* is required for interaction with both the PP1 phosphatase Glc7 and the Snf1 protein kinase. Cell Signal 22(7):1013–1021. doi:10.1016/j.cellsig.2010.02.003 S0898-6568(10)00056-2 [pii]

Tanaka K, Nakafuku M, Satoh T, Marshall MS, Gibbs JB, Matsumoto K, Kaziro Y, Toh-e A (1990) *S. cerevisiae* genes *IRA1* and *IRA2* encode proteins that may be functionally equivalent to mammalian ras GTPase activating protein. Cell 60(5):803–807 0092-8674(90)90094-U [pii]

Tatchell K, Chaleff DT, DeFeo-Jones D, Scolnick EM (1984) Requirement of either of a pair of ras-related genes of *Saccharomyces cerevisiae* for spore viability. Nature 309(5968):523–527

Thevelein JM (1984a) Cyclic-AMP content and trehalase activation in vegetative cells and ascospores of yeast. Arch Microbiol 138(1):64–67

Thevelein JM (1984b) Regulation of trehalose mobilization in fungi. Microbiol Rev 48(1):42–59

Thevelein JM (1991) Fermentable sugars and intracellular acidification as specific activators of the RAS-adenylate cyclase signalling pathway in yeast: the relationship to nutrient-induced cell cycle control. Mol Microbiol 5(6):1301–1307

Thevelein JM (1994) Signal transduction in yeast. Yeast 10(13):1753–1790

Thevelein JM, Beullens M (1985) Cyclic AMP and the stimulation of trehalase activity in the yeast *Saccharomyces cerevisiae* by carbon sources, nitrogen sources and inhibitors of protein synthesis. J Gen Microbiol 131(12):3199–3209

Thevelein JM, Beullens M, Honshoven F, Hoebeeck G, Detremerie K, den Hollander JA, Jans AWH (1987a) Regulation of the cAMP level in the yeast *Saccharomyces cerevisiae*: intracellular pH and the effect of membrane depolarizing compounds. J Gen Microbiol 133:2191–2196

Thevelein JM, Beullens M, Honshoven F, Hoebeeck G, Detremerie K, Griewel B, den Hollander JA, Jans AWH (1987b) Regulation of the cAMP level in the yeast *Saccharomyces cerevisiae*: the glucose-induced cAMP signal is not mediated by a transient drop in the intracellular pH. J Gen Microbiol 133:2197–2205

Thevelein JM, Bonini BM, Castermans D, Haesendonckx S, Kriel J, Louwet W, Thayumanavan P, Popova Y, Rubio-Texeira M, Schepers W, Vandormael P, Van Zeebroeck G, Verhaert P, Versele M, Voordeckers K (2008) Novel mechanisms in nutrient activation of the yeast protein kinase A pathway. Acta Microbiol Immunol Hung 55(2):75–89. doi:10.1556/AMicr.55.2008.2.1

Thevelein JM, Cauwenberg L, Colombo S, De Winde JH, Donation M, Dumortier F, Kraakman L, Lemaire K, Ma P, Nauwelaers D, Rolland F, Teunissen A, Van Dijck P, Versele M, Wera S, Winderickx J (2000) Nutrient-induced signal transduction through the protein kinase A pathway and its role in the control of metabolism, stress resistance, and growth in yeast. Enzyme Microb Technol 26(9–10):819–825 S0141022900001770 [pii]

Thevelein JM, de Winde JH (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol Microbiol 33(5):904–918 mmi1538 [pii]

Thevelein JM, Gelade R, Holsbeeks I, Lagatie O, Popova Y, Rolland F, Stolz F, Van de Velde S, Van Dijck P, Vandormael P, Van Nuland A, Van Roey K, Van Zeebroeck G, Yan B (2005) Nutrient sensing systems for rapid activation of the protein kinase A pathway in yeast. Biochem Soc Trans 33(Pt 1):253–256. doi:10.1042/BST0330253 BST0330253 [pii]

Thevelein JM, Voordeckers K (2009) Functioning and evolutionary significance of nutrient transceptors. Mol Biol Evol 26(11):2407–2414. doi:10.1093/molbev/msp168 msp168 [pii]

Thompson-Jaeger S, Francois J, Gaughran JP, Tatchell K (1991) Deletion of SNF1 affects the nutrient response of yeast and resembles mutations which activate the adenylate cyclase pathway. Genetics 129(3):697–706

Toda T, Cameron S, Sass P, Wigler M (1988) *SCH9*, a gene of *Saccharomyces cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. Genes Dev 2(5):517–527

Toda T, Cameron S, Sass P, Zoller M, Scott JD, McMullen B, Hurwitz M, Krebs EG, Wigler M (1987a) Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. Mol Cell Biol 7(4):1371–1377

Toda T, Cameron S, Sass P, Zoller M, Wigler M (1987b) Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. Cell 50(2):277–287 0092-8674(87)90223-6 [pii]

Toda T, Uno I, Ishikawa T, Powers S, Kataoka T, Broek D, Cameron S, Broach J, Matsumoto K, Wigler M (1985) In yeast, RAS proteins are controlling elements of adenylate cyclase. Cell 40(1):27–36 0092-8674(85)90305-8 [pii]

Tortora P, Burlini N, Hanozet GM, Guerritore A (1982) Effect of caffeine on glucose-induced inactivation of gluconeogenetic enzymes in *Saccharomyces cerevisiae*. A possible role of cyclic AMP. Eur J Biochem 126(3):617–622

Treitel MA, Carlson M (1995) Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc Natl Acad Sci USA 92(8):3132–3136

Tu J, Carlson M (1995) REG1 binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae*. EMBO J 14(23):5939–5946

Tu J, Song W, Carlson M (1996) Protein phosphatase type 1 interacts with proteins required for meiosis and other cellular processes in *Saccharomyces cerevisiae*. Mol Cell Biol 16(8):4199–4206

Upadhyay M, Samal J, Kandpal M, Singh OV, Vivekanandan P (2013) The Warburg effect: insights from the past decade. Pharmacol Ther 137(3):318–330. doi:10.1016/j.pharmthera.2012.11.003

Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, Deloche O, Wanke V, Anrather D, Ammerer G, Riezman H, Broach JR, De Virgilio C, Hall MN, Loewith R (2007) Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae*. Mol Cell 26(5):663–674. doi:10.1016/j.molcel.2007.04.020 S1097-2765(07)00256-0 [pii]

van Aelst L, Boy-Marcotte E, Camonis JH, Thevelein JM, Jacquet M (1990) The C-terminal part of the *CDC25* gene product plays a key role in signal transduction in the glucose-induced modulation of cAMP level in *Saccharomyces cerevisiae*. Eur J Biochem 193(3):675–680

van Aelst L, Jans AW, Thevelein JM (1991) Involvement of the *CDC25* gene product in the signal transmission pathway of the glucose-induced RAS-mediated cAMP signal in the yeast *Saccharomyces cerevisiae*. J Gen Microbiol 137(2):341–349

Van de Velde S, Thevelein JM (2008) Cyclic AMP-protein kinase A and Snf1 signaling mechanisms underlie the superior potency of sucrose for induction of filamentation in *Saccharomyces cerevisiae*. Eukaryot Cell 7(2):286–293. doi:10.1128/EC.00276-07

van der Plaat JB (1974) Cyclic 3′,5′—adenosine monophosphate stimulates trehalose degradation in bakers' yeast. Biochem Biophys Res Commun 56:580–587

Van Nuland A, Vandormael P, Donaton M, Alenquer M, Lourenco A, Quintino E, Versele M, Thevelein JM (2006) Ammonium permease-based sensing mechanism for rapid ammonium activation of the protein kinase A pathway in yeast. Mol Microbiol 59(5):1485–1505. doi:10.1111/j.1365-2958.2005.05043.x

Versele M, de Winde JH, Thevelein JM (1999) A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. EMBO J 18(20):5577–5591. doi:10.1093/emboj/18.20.5577

Vidan S, Mitchell AP (1997) Stimulation of yeast meiotic gene expression by the glucose-repressible protein kinase Rim15p. Mol Cell Biol 17(5):2688–2697

Vincent O, Carlson M (1998) Sip4, a Snf1 kinase-dependent transcriptional activator, binds to the carbon source-responsive element of gluconeogenic genes. EMBO J 17(23):7002–7008. doi:10.1093/emboj/17.23.7002

Vincent O, Kuchin S, Hong SP, Townley R, Vyas VK, Carlson M (2001) Interaction of the Srb10 kinase with Sip4, a transcriptional activator of gluconeogenic genes in *Saccharomyces cerevisiae*. Mol Cell Biol 21(17):5790–5796

Wang Y, Pierce M, Schneper L, Guldal CG, Zhang X, Tavazoie S, Broach JR (2004) Ras and Gpa2 mediate one branch of a redundant glucose signaling pathway in yeast. PLoS Biol 2(5):E128. doi:10.1371/journal.pbio.0020128

Wanke V, Cameroni E, Uotila A, Piccolis M, Urban J, Loewith R, De Virgilio C (2008) Caffeine extends yeast lifespan by targeting TORC1. Mol Microbiol 69(1):277–285. doi:10.1111/j.1365-2958.2008.06292.x MMI6292 [pii]

Werner-Washburne M, Brown D, Braun E (1991) Bcy1, the regulatory subunit of cAMP-dependent protein kinase in yeast, is differentially modified in response to the physiological status of the cell. J Biol Chem 266(29):19704–19709

Wieser R, Adam G, Wagner A, Schuller C, Marchler G, Ruis H, Krawiec Z, Bilinski T (1991) Heat shock factor-independent heat control of transcription of the CTT1 gene encoding the cytosolic catalase T of *Saccharomyces cerevisiae*. J Biol Chem 266(19):12406–12411

Wilson WA, Roach PJ, Montero M, Baroja-Fernandez E, Munoz FJ, Eydallin G, Viale AM, Pozueta-Romero J (2010) Regulation of glycogen metabolism in yeast and bacteria. FEMS Microbiol Rev 34(6):952–985. doi:10.1111/j.1574-6976.2010.00220.x

Woods A, Munday MR, Scott J, Yang X, Carlson M, Carling D (1994) Yeast SNF1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase in vivo. J Biol Chem 269(30):19509–19515

Xiao B, Sanders MJ, Underwood E, Heath R, Mayer FV, Carmena D, Jing C, Walker PA, Eccleston JF, Haire LF, Saiu P, Howell SA, Aasland R, Martin SR, Carling D, Gamblin SJ (2011) Structure of mammalian AMPK and its regulation by ADP. Nature 472(7342): 230–233. doi:10.1038/nature09932 nature09932 [pii]

Xiaojia B, Jian D (2010) Serine214 of Ras2p plays a role in the feedback regulation of the Ras-cAMP pathway in the yeast *Saccharomyces cerevisiae*. FEBS Lett 584(11):2333–2338. doi:10.1016/j.febslet.2010.04.011

Xue Y, Batlle M, Hirsch JP (1998) GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Galpha subunit and functions in a Ras-independent pathway. EMBO J 17(7):1996–2007. doi:10.1093/emboj/17.7.1996

Yun CW, Tamaki H, Nakayama R, Yamamoto K, Kumagai H (1997) G-protein coupled receptor from yeast *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 240(2):287–292. doi:10.1006/bbrc.1997.7649 S0006-291X(97)97649-X [pii]

Zaman S, Lippman SI, Schneper L, Slonim N, Broach JR (2009) Glucose regulates transcription in yeast through a network of signaling pathways. Mol Syst Biol 5:245. doi:10.1038/msb.2009.2 msb20092 [pii]

Zaman S, Lippman SI, Zhao X, Broach JR (2008) How *Saccharomyces* responds to nutrients. Annu Rev Genet 42:27–81. doi:10.1146/annurev.genet.41.110306.130206

Zeller CE, Parnell SC, Dohlman HG (2007) The RACK1 ortholog Asc1 functions as a G-protein beta subunit coupled to glucose responsiveness in yeast. J Biol Chem 282(34):25168–25176. doi:10.1074/jbc.M702569200 M702569200 [pii]

Zhang A, Gao W (2012) Mechanisms of protein kinase Sch9 regulating Bcy1 in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 331(1):10–16. doi:10.1111/j.1574-6968.2012.02552.x

Zhang A, Shen Y, Gao W, Dong J (2011) Role of Sch9 in regulating Ras-cAMP signal pathway in *Saccharomyces cerevisiae*. FEBS Lett 585(19):3026–3032. doi:10.1016/j.febslet.2011.08.023 S0014-5793(11)00625-9 [pii]

Zhang N, Wu J, Oliver SG (2009) Gis1 is required for transcriptional reprogramming of carbon metabolism and the stress response during transition into stationary phase in yeast. Microbiology 155(Pt 5):1690–1698. doi:10.1099/mic.0.026377-0

Chapter 3
Anaerobic Carbon Metabolism of *Saccharomyces cerevisiae*

Paula Jouhten and Merja Penttilä

3.1 Introduction

The yeast *Saccharomyces cerevisiae* is a facultative anaerobic organism able to grow in the absence of oxygen. Oxygenation is one of the major costs in biotechnical production processes, and the anaerobic performance of *S. cerevisiae* is thus attractive in the development of low-cost bioprocesses. Understanding of the carbon metabolism of *S. cerevisiae* in the lack of oxygen is crucial also for the optimization of oxygenated large-scale processes. Transient oxygen-depleted conditions and oxygen gradients commonly appear in large bioreactors and in high-cell density cultures due to imperfect mixing. In addition of being an industrial production organism, *S. cerevisiae* is an attractive model organism for studying the cell physiology and regulation under conditions of different energetic challenges such as anaerobiosis. The observations and understanding can be translated to higher eukaryotes since many of the regulatory mechanisms are conserved within Eukaryota. It is also fascinating that there is a similarity between the anaerobic organization of the carbon metabolism of *S. cerevisiae* and the 'low ATP yield-high rate' energy metabolism, which is a regulatory choice and competitive advantage behind the behaviour of not only *S. cerevisiae* but also for example cancer cells. In this review, the response of the carbon metabolism of *S. cerevisiae* to the lack of oxygen will be discussed in the light of comprehensive data on multiple levels of cell function.

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_3, © Springer-Verlag Berlin Heidelberg 2014

3.1.1 Many Factors Provide Competence for Anaerobic Growth

Lack of oxygen is both a relief and a challenge to the cells. While being a strong threat due to the oxidative damage it may provoke (Jamieson 1998), oxygen is essential for energy generation through respiration, which is highly efficient. Oxygen acts as the final electron acceptor in the respirative ATP generation. The electrons of the redox cofactor NADH are transferred to oxygen in the mitochondrial electron transfer chain, and the proton pumping enzymes in the chain generate a proton-motive force across the mitochondrial inner membrane. The proton-motive force rotates the ATP synthase enzyme, which transforms the energy of the rotation into the chemical bonds of ATP. In the absence of oxygen energy can be loaded into ATP only through substrate level phosphorylations. *S. cerevisiae* is able to efficiently remodel its carbon metabolism to produce ATP with an adequate speed also under anaerobiosis to fuel the biosynthesis. In particular, the glycolytic and fermentative pathways respond to oxygen depletion with an immediate increase in the flux and concomitant high rate of ATP generation in glucose fermentation to ethanol. In addition, in the lack of oxygen as an acceptor of the electrons from NADH, NAD^+ must be regenerated by alternative means to maintain the redox balance within the cell and its compartments. *S. cerevisiae* maintains the redox balance under anaerobic conditions by generation of by products, mainly ethanol and glycerol. The high tolerance of *S. cerevisiae* against the by-products further supports anaerobic growth. Even the metabolite and ion transport are altered in the absence of an active electron transfer chain creating a proton gradient across the mitochondrial membrane in anaerobic conditions (Visser et al. 1990). The transport of molecules and ions from cytosol to mitochondria or vice versa requires simultaneous proton translocation and thus is affected by anaerobic conditions.

Oxygen is essential not only for the aerobic respiration but also for the biosynthesis of biomass constituents like haem, unsaturated fatty acids and sterols (Rosenfeld and Beauvoit 1998). To fuel the anabolic needs of continuous growth under anaerobiosis, *S. cerevisiae* must import from outside the cell unsaturated fatty acids and ergosterol, which provide essential functional properties for the cell membrane (Jacquier and Schneiter 2012; Sinensky 1974). The ratio of unsaturated to saturated fatty acids in the membrane determines the membrane fluidity (Sinensky 1974). Both the membrane and the cell wall adapt to anaerobiosis to meet the challenges caused by the condition and the altered import of extracellular compounds (Abramova et al. 2001; Kwast et al. 2002), i.e. uptake of sterols is activated in the absence of oxygen (Jacquier and Schneiter 2012). The capability to take up sterols has been suggested to provide competence for anaerobic growth. The capability for anaerobic growth emerged in evolution after the split of *S. cerevisiae* and *Kluyveromyces* lineages (Hagman et al. 2013; Møller et al. 2001). *Kluyveromyces lactis* which is unable to grow under anaerobiosis lacks a few of the corresponding genes that are functioning in anaerobiosis in *S. cerevisiae*

(Snoek and Steensma 2006), including genes involved in sterol uptake. Consequently, the inability to import sterols was suggested as one of the possible reasons why *K. lactis* requires oxygen for growth.

De novo nucleotide synthesis includes an essential dihydroorotate dehydrogenase (EC 1.3.3.1) reaction which is an oxygen utilizing reaction for instance in *Pichia stipitis* (*Scheffersomyces stipitis*), which is incapable of continuous growth when oxygen is lacking (Shi and Jeffries 1998). On the contrary, the *S. cerevisiae* dihydroorotate dehydrogenase is known to be independent of the respiratory chain and active also under anaerobic conditions (Nagy et al. 1992; Gojkovic et al. 2004). The dihydroorotate dehydrogenase of *S. cerevisiae* has specificity for alternative electron acceptors such as fumarate (Nagy et al. 1992). When introduced into *P. stipitis*, continuous anaerobic growth was enabled (Shi and Jeffries 1998). A transfer of a gene encoding dihydroorotate dehydrogenase, independent of respiratory activity, from bacteria to *Saccharomyces* yeasts has been proposed (Gojkovic et al. 2004). Moreover, it is not fully known, how *S. cerevisiae* copes with the absence of haem synthesis under anaerobic conditions and is able to support continuous growth (Kwast et al. 2002). It was suggested that recycling of haem bound to proteins could be involved but this would not be sustainable during continuous growth, and alternative solutions must exist.

3.1.2 Models Allow for Simulations of Anaerobic Performance

The genome-scale metabolic models currently allow for simulations of oxygen-dependent and anaerobic metabolism of *S. cerevisiae*. The first consensus genome-scale metabolic network reconstruction of *S. cerevisiae* was created in 2008 (Herrgård et al. 2008). In 2010, the model was refined in pathway connectivity and thereby turned into a computable form allowing constraint-based analyses (Dobson et al. 2010). Recently, the model was revised in the lipid metabolism, in particular in the reactions involved in sphingolipid metabolism (Heavner et al. 2012), and in oxidative phosphorylation and other oxygen-dependent and anaerobic metabolic reactions (Jouhten et al. 2012). After the revision, the metabolic behaviour of *S. cerevisiae* culture upon sudden depletion of oxygen and subsequent adaptation to anaerobiosis was successfully simulated with dynamic flux balance analysis (dFBA) at genome-scale (Jouhten et al. 2012).

3.1.3 Haem and Sterols as Sensors of Anaerobiosis

A delicate transcriptional regulatory system of *S. cerevisiae* utilises haem and sterols in sensing the lack of oxygen. The transcriptional regulation of *S. cerevisiae* in response to anaerobic conditions has been extensively studied (ter Linde et al. 1999;

Tai et al. 2005; Lai et al. 2006; Kwast et al. 2002; Rintala et al. 2009, 2011; Wiebe et al. 2008). The main mediator of oxygen-responsive transcriptional regulation is Hap1p which acts both as an activator and a repressor depending on the presence of haem (Hon et al. 2005). Under aerobic conditions, when haem is present, Hap1p activates genes involved, e.g. in respiration and oxidative stress (Becerra et al. 2002; Zhang and Guarente 1994). It also activates *ROX1* encoding a repressor of anaerobic genes (Lowry and Zitomer 1984; ter Linde and Steensma 2002). Rox1p together with Mot3p, which also is at least partly under the regulation of Hap1p, synergistically repress anaerobic genes (Sertil et al. 2003). Under conditions where oxygen and haem are lacking, Hap1p represses genes involved in the biosynthesis of ergosterol (Hickman and Winston 2007). The transcription factor complex Hap2/3/4/5p, in which the activator subunit is Hap4p, is responsive to respiratory carbon sources and haem (Forsburg and Guarente 1989). Under respiratory conditions Hap2/3/4/5p triggers the expression of genes involved in respirative metabolism, which are down-regulated under anaerobiosis. However, it is interesting that despite the absence of oxygen *S. cerevisiae* fails to fully down-regulate oxidative phosphorylation when growing anaerobically on the five carbon sugar xylose, which is a non-natural carbon source for *S. cerevisiae* (Runquist et al. 2009). The most likely reason is the cofactor imbalance caused in the engineering of the xylose utilization pathway using xylose reductase (XR) and xylitol dehydrogenase (XDH) (Toivari et al. 2001; Runquist et al. 2009). The observation suggests that induction of the respiratory pathway in *S. cerevisiae* is not solely dependent on oxygen.

A large fraction of the anaerobic genes, including genes involved in the import of sterols, possess a promoter binding site shared by the transcriptional activators Upc2p/Ecm22p (Kwast et al. 2002). Upc2/Ecm22p mediated regulation is dependent on the presence of sterols (Davies and Rine 2006).

3.2 Remodelling of Carbon Metabolism in the Absence of Oxygen

Under anaerobic conditions the widely used *S. cerevisiae* strain CEN.PK is able to grow with maximal specific growth rate as high as 0.30 h^{-1} on minimal medium (Verduyn et al. 1992), using glucose as the sole carbon source, at 30 °C, pH 5, given that essential ergosterol and unsaturated fatty acids are provided (supplementation with 10 mg l^{-1} of ergosterol and 420 mg l^{-1} of Tween-80) (van Hoek et al. 2000). The rate is not much lower than the observed maximum specific growth rate of 0.41 h^{-1} of the same strain under aerobic conditions in the same medium with glucose as the sole carbon source, at 30 °C, pH 5. Thus, the reorganization of the carbon metabolism of *S. cerevisiae* is efficient—in terms of retaining the specific growth rate—when the cells encounter anaerobic conditions. Observations on the anaerobic reorganization and regulation of the central pathways of carbon metabolism of *S. cerevisiae* are reviewed below. The reviewed

data provide a wide view across the multiple levels of cell function during anaerobic adaptations.

3.2.1 High Glycolytic Flux Supports Anaerobic Growth

Under conditions where oxygen is lacking, ATP generation occurs in substrate-level phosphorylations mainly in glycolysis (Fig. 3.1). When one molecule of glucose is fermented to two molecules of ethanol, the yield of ATP molecules is two (Verduyn et al. 1990b), whereas a complete oxidation of a mole of glucose through aerobic respiration, in addition to the glycolytic ATP generation, would produce approximately 16 moles of ATP (assuming an apparent P/O ratio of 1 in the oxidative phosphorylation), two moles of which is consumed in glycolysis (Verduyn et al. 1991). Consequently, the glycolytic flux must adapt for a substantially higher speed in anaerobic conditions in order to not severely limit growth by the availability of energy. *S. cerevisiae* is able to meet this challenge and increase the glycolytic rate correspondingly. In anaerobic glucose-limited continuous cultures at a low growth rate, the glycolytic flux of *S. cerevisiae* has been observed to be 7.5 times higher than under otherwise same but fully aerobic conditions (Jouhten et al. 2008) (Fig. 3.2). Most of the glycolytic proteins are also significantly more abundant under anaerobiosis than under aerobic conditions (de Groot et al. 2007; Bruckmann et al. 2009; Rintala et al. 2009), and the enzyme activities have been shown to be higher in the absence of oxygen (Daran-Lapujade et al. 2004; van Hoek et al. 2000). The glycolytic enzymes are very abundant proteins in general in the proteome of *S. cerevisiae* making approximately 8 % of the total abundance under aerobic conditions (van Hoek et al. 2000). In the absence of oxygen, the relative abundance of glycolytic proteins increases to 21 %, which most likely corresponds to a substantial fraction of the capacity of the translational machinery (de Groot et al. 2007). The protein abundances of glycolytic enzymes in *S. cerevisiae* have been observed to be post-transcriptionally regulated in response to anaerobiosis (de Groot et al. 2007; Bruckmann et al. 2009; Rintala et al. 2009). However, the regulation of glycolytic flux has turned out to be even more complex than the regulation of the protein levels. Early attempts to increase the glycolytic flux by single, double (Schaaff et al. 1989) and multiple (Hauf et al. 2000; Smits et al. 2000) overexpressions of glycolytic enzymes did not succeed. Smits et al. (2000) observed substantially increased enzyme levels as a result of multiple overexpressions of lower glycolytic enzymes and yet in batch cultivations the engineered strain behaved as the control strain showing no sign of an increased glycolytic rate. In later studies, no direct dependences between protein levels or the transcript abundances and flux have been observed (Daran-Lapujade et al. 2004, 2007). Accordingly, the expression of genes encoding hexose transporters are not upregulated under anaerobic conditions, despite the substantially increased glucose uptake rate compared to aerobic conditions (Rintala et al. 2008). The decrease in the activity of the transporters having

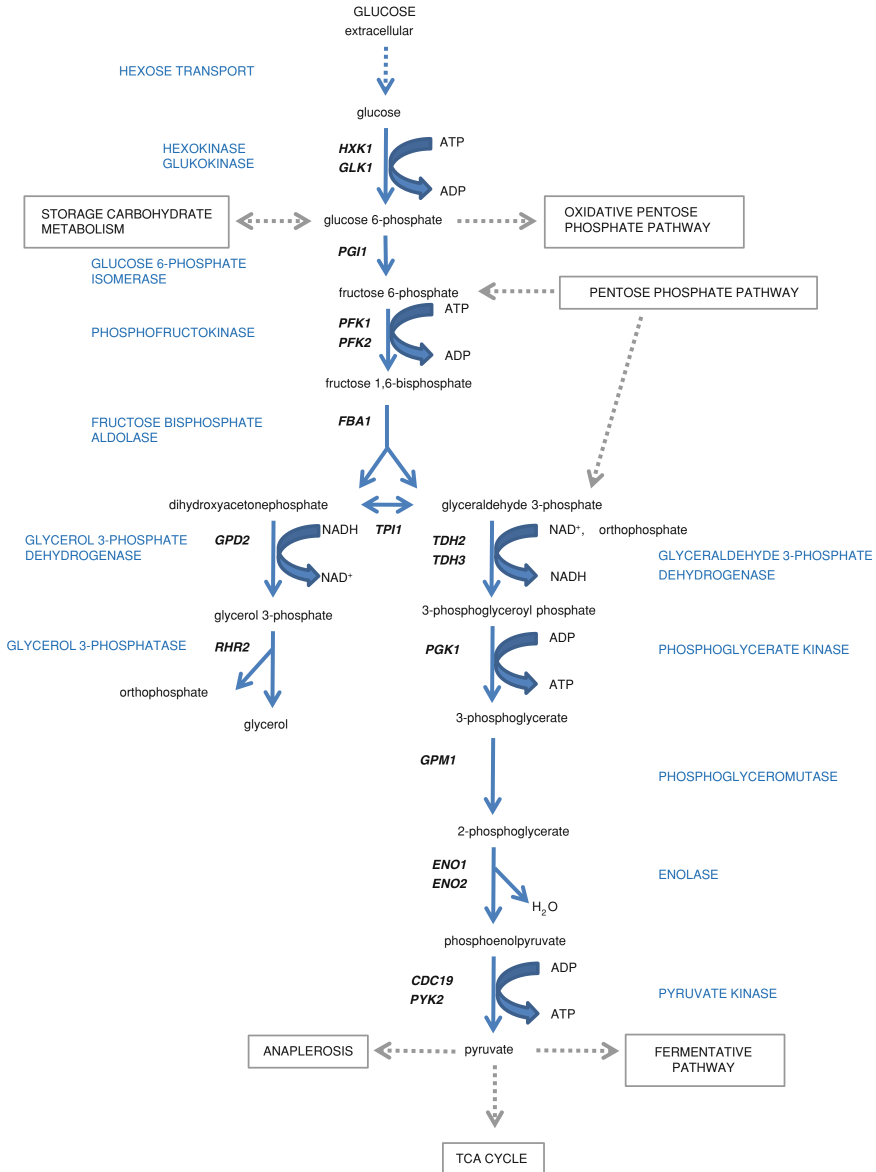

Fig. 3.1 Glycolytic pathway of *S. cerevisiae* under anaerobic conditions. Glycolytic pathway from glucose to pyruvate and diverging glycerol synthesis route are shown with the enzymes catalyzing the reactions (in *light blue*) and relevant genes encoding the enzymes (in *black*) in *S. cerevisiae* under anaerobic growth conditions

lower affinity and the consequent relative increase in the high affinity transport have been suggested to accomplish the increased specific uptake flux in the absence of oxygen.

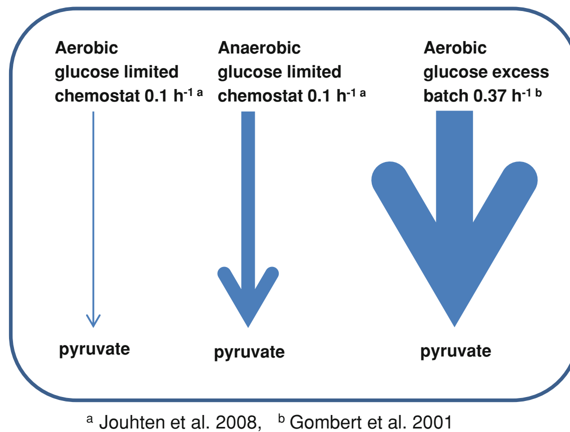

Fig. 3.2 Illustration of the capacity of glycolytic flux of *S. cerevisiae*. Magnitudes of the glycolytic flux of *S. cerevisiae* under anaerobic glucose-limited and aerobic glucose-excess conditions relative to the magnitude of the glycolytic flux under aerobic glucose-limited conditions visualised as the thickness of the arrows. Glycolytic flux has been observed to be 7.5 times higher under anaerobic than aerobic conditions in glucose-limited continuous cultures at specific growth rate of 0.1 h^{-1} (Jouhten et al. 2008). Under glucose-excess conditions in batch cultures in cells growing at maximum specific growth rate of 0.37 h^{-1} a 20.3 times higher glycolytic flux has been observed than in aerobic glucose-limited continuous cultures at specific growth rate of 0.1 h^{-1} (Gombert et al. 2001)

The glycolytic metabolite pools that are interdependent with the flux through the mechanistic kinetic activities of the glycolytic enzymes, adjust to enable the high glycolytic rate. Metabolites affect the reaction rates as substrates, products and allosteric effectors mediating activation or inhibition. The concentrations of upper glycolytic intermediates (glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate) and lower glycolytic pyruvate (Fig. 3.1) are higher under anaerobic conditions than in aerobic conditions whereas the lower glycolytic metabolites (2-phosphoglycerate+3-phosphoglycerate, phosphoenolpyruvate) (Fig. 3.1) are less abundant in the absence oxygen than under aerobic conditions (Wiebe et al. 2008). Allosteric enzyme regulation creates couplings also between metabolic pathway reactions which are not directly coupled such as between reactions of the upper and lower parts of glycolysis. The lower glycolytic enzyme pyruvate kinase (Fig. 3.1) is activated by the anaerobically abundant upper glycolytic metabolite fructose 1,6-bisphosphate (Murcott et al. 1992), and the lower glycolytic metabolite phosphoenolpyruvate inhibits the upper glycolytic enzyme triose phosphate isomerase (Grüning et al. 2011). Thus, the low anaerobic pool of phosphoenol-pyruvate (Wiebe et al. 2008) supports a high flux through triose phosphate isom-erase and the high anaerobic concentration of fructose 1,6-bisphosphate supports the high rate of conversion of phosphoenolpyruvate to pyruvate. Further, the upper glycolytic enzyme phosphofructokinase is activated by fructose 2,6-bisphosphate and the products AMP and ADP, and is inhibited by ATP, which have global effects

in the metabolism. The low-affinity effects of ATP, ADP and AMP on the activities of those glycolytic enzymes for which they are neither substrates nor products were recently investigated by Mensonides et al. (2013). They found that all those enzymes were affected by the nucleotides, slightly or substantially. For example phosphoglucose isomerase activity was severely inhibited by ATP.

S. cerevisiae makes a regulatory choice of 'low ATP yield-high rate' energy metabolism under conditions of high glucose abundance even in presence of oxygen. This choice is called the Crabtree effect, which is thought to give *S. cerevisiae* a capability to out-compete other consumers of glucose in the environment. *S. cerevisiae* consumes glucose fast, makes and accumulates ethanol which becomes toxic for competitors, and then after the depletion of glucose it consumes the ethanol. It produces ethanol rather than gains high yields of energy and biomass which respirative metabolism under aerobic conditions would allow for. High glycolytic flux observed under anaerobiosis is characteristic also for the metabolism of *S. cerevisiae* in high-glucose conditions. Hagman et al. (2013) has recently showed that the emergence of the 'make-accumulate-consume' strategy of *S. cerevisiae* followed the anaerobic capability in yeast evolution. The high capacity of the glycolytic pathway which is important for the anaerobic growth was one of the factors enabling also the aerobic alcoholic fermentation as a regulatory strategy. The relative magnitude of the high glycolytic flux under excess-glucose conditions (Gombert et al. 2001) in comparison to the glycolytic flux under aerobic and anaerobic glucose-limited conditions is shown in Fig. 3.2.

The glycolytic pathway is not directly or primarily regulated by the lack of oxygen, which can be learned from the observations made when anaerobic xylose metabolism in engineered *S. cerevisiae* strains has been investigated. Xylose is a five carbon sugar abundant in hemicellulose but not naturally metabolised by *S. cerevisiae*. During anaerobic xylose metabolism in *S. cerevisiae* strains with engineered heterologous xylose utilization pathway, the glycolytic flux is low and gluconeogenetic enzymes catalysing the reverse activity of glycolysis are expressed in contrast to being repressed under anaerobic conditions on glucose (Runquist et al. 2009). In accordance with the low glycolytic flux, the pools of the lower glycolytic metabolites glyceraldehyde 3-phosphate and phosphoenolpyruvate are high whereas the level of fructose 6-phosphate was low during anaerobic xylose metabolism (Toivari et al. 2001; Klimacek et al. 2010). Klimacek et al. (2010) further found that the low concentration of fructose 6-phosphate limited the activity of the phosphofructokinase reaction. Even higher levels of anaerobically accumulated phosphoenolpyruvate were found in a *S. cerevisiae* strain harbouring xylose isomerase (XI) reaction for xylose metabolism than in the xylose reductase (XR)/xylitol dehydrogenase (XDH) reaction pair containing strain in response to switch from glucose to xylose utilization (Bergdahl et al. 2012). XI harbouring strain had lower xylose uptake rate, which implies lower glycolytic flux, than in the XR/XDH harbouring strain. Accordingly, an overexpression of xylulokinase encoding *XKS1* in a *S. cerevisiae* strain harbouring the XR/XDH pathway for xylose utilization increased the uptake rate of xylose which resulted in higher

anaerobic concentrations of the metabolites of the upper glycolysis, glucose 6-phosphate and fructose 6-phosphate and higher rate of ethanol production than in the control strain (Toivari et al. 2001).

S. cerevisiae maintains an overcapacity in its glycolytic and fermentative pathways, which is mobilised in need of energy (van Hoek et al. 1998). Such a need of energy, drop of ATP levels, occurs for example when cells in respirative metabolic state are suddenly exposed to excess glucose (Rizzi et al. 1997; Visser et al. 2004; van den Brink et al. 2008b). The glycolytic flux is indeed readily increased in response to both sudden depletion of oxygen and a sudden appearance of excess glucose. The glycolytic rate increased almost eight times when fully respirative cells of *S. cerevisiae* growing continuously at 0.1 h^{-1} were suddenly exposed to anaerobiosis and let to adapt to an anaerobic steady state (Jouhten et al. 2012). Similarly, an eight-fold increase in the glycolytic flux occurred, when *S. cerevisiae* cells growing under aerobic conditions were suddenly exposed to fermentative conditions of both anaerobiosis and excess glucose (van den Brink et al. 2008b). The immediate flux increase within the initial 45 min occurred on the level of metabolic regulation without any increases in the capacities of the enzymes (van den Brink et al. 2008b). The upper glycolytic metabolite concentrations (glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate) increased whereas the lower glycolytic phosphoenolpyruvate concentration decreased and pyruvate concentration increased to support the increase in flux. An equal response was observed by Wiebe et al. (2008) when *S. cerevisiae* cells grown in glucose-limited aerobic chemostat cultures were switched to anaerobic conditions. Further, the response was generally independent of the level of aerobiosis, the level of oxygen in the chemostat inlet gas before the switch to anaerobiosis. The response of glycolytic intermediates to a pulse of glucose is similar (Visser et al. 2004; Wu et al. 2006). Wu et al. (2006) observed fast dynamics of glycolytic intermediates when *S. cerevisiae* cells growing under aerobic glucose-limited conditions were exposed sudden pulse of glucose. The lower glycolytic metabolites (2-phosphoglycerate+3-phosphoglycerate, phosphoenolpyruvate) responded within 10 s whereas the upper glycolytic metabolites (glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate) reached their highest levels within approximately 1 min. The capacities (in vitro measured V_{max}) of most of the glycolytic enzymes increased, and hierarchical regulation, including transcriptional, post-transcriptional, translational, and post-translational regulation, accounted for further flux increase of the majority of glycolytic enzymes only later, after 45 min of the shift of aerobically grown glucose-derepressed cells of *S. cerevisiae* into fully fermentative conditions of anaerobiosis and glucose excess (van den Brink et al. 2008b). In contrast, hexokinase, phosphofructokinase and phosphoglycerate kinase (Fig. 3.1) were regulated solely at metabolic level in *S. cerevisiae* during the 2 h after the shift into the fully fermentative conditions. Yet unknown effectors of phosphofructokinase are expected to exist since van den Brink et al. (2008a) found that it was not possible to fit the in vivo flux data obtained as a response to the shift to fully fermentative conditions with a kinetic equation taking into account the known metabolic regulators of

phosphofructokinase. After 2 h of the perturbation, the glycolytic flux had increased 13-fold, and van den Brink et al. (2008b) could conclude that the enzyme capacity increases had only marginally contributed to the substantial increase in the glycolytic rate.

In addition to metabolic regulation, post-translational modifications such as protein phosphorylation, enable fast, often reversible, responses in metabolism in sudden changes of conditions. The prevalence of phosphorylation as a post-translational modification in *S. cerevisiae* was shown by Breitkreutz et al. (2010) who mapped genome-wide kinase and phosphatase interactions into a dense network (Breitkreutz et al. 2010). The phosphorylation mediated control of in vivo activity of reactions in the central carbon metabolism of *S. cerevisiae* was recently investigated with a novel phosphoproteomics approach by Oliveira et al. (2012). It was known prior to their investigation that phosphorylation plays a functional role in 17 enzymes of the central carbon metabolism of *S. cerevisiae*. Among those are glycolytic enzymes encoded by *HXK2* (phosphorylation causing change in intracellular localizational), *PYK1/CDC19* (enzyme activation) and *FBP1* (enzyme inhibition). When comparing cells growing aerobically or anaerobically on glucose Oliveira et al. (2012) observed changes in phosphoenzyme abundances of five glycolytic enzymes encoded by *HXK2*, *PFK2*, *FBA1*, *TDH1/2/3*, and *GPM1*. Phosphofructokinase encoded by *PFK2*, was found to be inhibited by phosphorylation at amino acid position S163 and the amount of non-phosphorylated enzyme correlated with the glycolytic flux over four common culture conditions including anaerobic growth conditions (Oliveira et al. 2012). Thus, if the phosphofructokinase reaction was rate controlling, the rate of glycolytic flux could be quickly modulated with dynamic phosphorylation or dephosphorylation of the enzyme.

3.2.2 The Relative Pentose Phosphate Pathway Flux is Lower Under Anaerobic Than Aerobic Conditions

The in vivo activity of the pentose phosphate pathway (PPP) of *S. cerevisiae*, relative to the glycolytic flux, is lower under anaerobic than aerobic conditions, which has been confirmed with ^{13}C-labelling in chemostat cultivations at a dilution rate of 0.1 h^{-1} (Fiaux et al. 2003; Jouhten et al. 2008). Pentose phosphate pathway contributed to less than 10 % of the phosphoenolpyruvate pool under anaerobic conditions whereas under fully aerobic conditions the contribution of pentose phosphate pathway to the phosphoenolpyruvate pool was over 30 % at maximum (Table 3.1). Interestingly, the *ZWF1* gene encoding glucose 6-phosphate dehydrogenase in the entry point of the oxidative branch of PPP (Nogae and Johnston 1990) has an elevated expression under anaerobic conditions at low growth rates on glucose (Rintala et al. 2009). Runquist et al. (2009) found it even more upregulated under anaerobic conditions on xylose. *S. cerevisiae* with an engineered xylose pathway of XR and XDH enzymes requires NADPH for the XR

Table 3.1 Metabolic flux ratios of *S. cerevisiae* under aerobic and anaerobic conditions

		Fraction of PEP from PPP (ub) Percentage of total pool	±	Fraction of OAA_{mit} from PEP Percentage of total pool	±
Chemostat 0.1 h^{-1} [a]	Aerobic	32	10	31	2
Chemostat 0.1 h^{-1} [a]	Anaerobic	4	5	100	2
Chemostat 0.1 h^{-1} [b]	Aerobic	40	8	31	2
Chemostat 0.1 h^{-1} [b]	Anaerobic	8	5	98	2
Batch max growth rate[c]	Aerobic	4	nd	76	4
Batch max growth rate[c]	Anaerobic	4	nd	92–100	nd

Upper bound (ub) for a fraction of phosphoenolpyruvate (PEP) originating from pentose phosphate pathway (PPP) and a fraction of mitochondrial oxaloacetate (OAA_{mit}) originating from phosphoenolpyruvate (PEP), i.e. relative rate of anaplerosis of *S. cerevisiae* under aerobic and anaerobic conditions in different culture modes of glucose-limited chemostat cultivations and glucose-excess batch cultivations. A relative anaplerotic flux of 100 %, replenishment of intermediates drawn into biosynthesis, indicates that there is no respiratory activity in the TCA cycle

[a] Jouhten et al. 2008

[b] Fiaux et al. 2003

[c] Maaheimo et al. 2001, *PEP* phosphoenolpyruvate, *PPP* pentose phosphate pathway, *ub* upper bound, OAA_{mit} mitochondrial oxaloacetate

reaction of xylose dissimilation. The oxidative branch of PPP is the main source of reducing power NADPH, also under anaerobic conditions (Fig. 3.3). In vivo activity of the NADPH generating oxidative branch of PPP has been proposed to be affected by the cellular $NADPH/NADP^{+}$ ratio and $MgATP^{2-}$ pool, which regulate the activity of glucose 6-phosphate dehydrogenase (Fig. 3.1) (Llobell et al. 1988; Vaseghi et al. 1999). NADPH is utilised mostly in biosynthesis, i.e. in the reduction steps in lipid and amino acid biosyntheses. Accordingly, a dependency of the relative in vivo pentose phosphate pathway flux on the growth rate and biomass yield has been observed in *S. cerevisiae* growing at different constant rates ranging from below the critical dilution rate, above which aerobic alcoholic fermentation occurs, to higher dilution rates (Frick and Wittmann 2005). This dependency has been observed also in different yeast species (Blank et al. 2005). Under aerobic conditions, NADPH is utilised also as a redox buffer in the defence against oxidative damage under aerobic conditions (Minard and Mc Alister-Henn 1999). The transcription factor Stb5p, which regulates the transcription of the genes encoding the enzymes of the NADPH producing oxidative branch and most of the other genes involved in the pentose phosphate pathway (Larochelle et al. 2006), responses to oxidative stress (Cadière et al. 2010). Stb5p is also required for normal growth under aerobic conditions, while anaerobic growth was not found impaired by the deletion of *STB5* (Cadière et al. 2010).

A low relative in vivo activity of PPP has been observed also in aerobic batch cultivations of *S. cerevisiae* during exponential maximal growth rate by ^{13}C-labelling experiments (Maaheimo et al. 2001) (Table 3.1). Thus, the low in vivo activity of PPP relative to the glycolytic flux is associated with a high glycolytic flux during fermentative metabolism irrespective of the oxygenation conditions. The dependency of the in vivo activity of PPP on the biomass yield (Frick and Wittmann 2005) is not contradictory to this statement since the higher the proportion of the fermentative metabolism the lower is the biomass yield.

3.2.3 Roles of the Isoenzymes of Fermentative Pathway Depend on the Availability of Oxygen

At the pyruvate branching point, the glycolytic flux is distributed into three main directions via pyruvate dehydrogenase, pyruvate carboxylase and pyruvate decarboxylase (Fig. 3.4). The fermentative pathway of *S. cerevisiae* starts at the pyruvate decarboxylase reaction producing acetaldehyde. Acetaldehyde has two alternative destinations via either an alcohol dehydrogenase reaction to ethanol or via acetaldehyde dehydrogenase reaction to acetate. The pathway possesses fermentative capacity (van Hoek et al. 1998) together with the glycolytic pathway, which enable a rapid onset of a substantial ethanol production in response to altered conditions (van den Brink et al. 2008b). When respirative cells of *S. cerevisiae* were exposed to a pulse of glucose, Wu et al. (2006) observed an

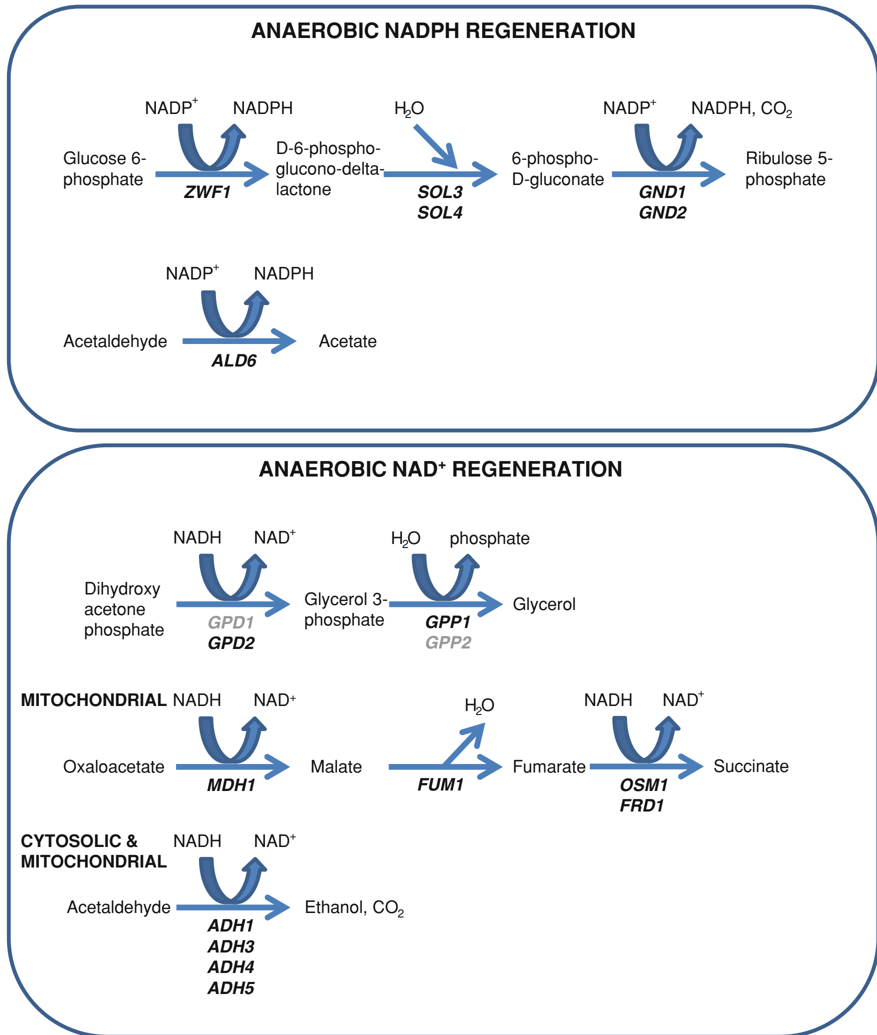

Fig. 3.3 Illustration of redox cofactor regeneration of *S. cerevisiae* under anaerobic conditions. The main reactions responsible for the regeneration of NADPH and NAD^+ redox cofactors in *S. cerevisiae* under anaerobic conditions. The genes encoding the main enzymes are shown in *black* and the minor contributors in *grey*. NADPH regeneration occurs mainly in cytosol whereas for the regeneration of mitochondrial NAD^+ *S. cerevisiae* can utilise also the reductive branch of TCA cycle or alcohol dehydrogenase reaction

onset of substantial ethanol accumulation as fast as in 50 s. van den Brink et al. (2008a) in turn reported an ethanol production rate of 19.6 ± 1.4 mmol/g^{-1} CDW h^{-1} 2 h after a fully respiratory culture of *S. cerevisiae* producing no ethanol had been switched to conditions of excess glucose and anaerobiosis. *S. cerevisiae*

possesses five isoenzymes of alcohol dehydrogenase encoded by separate genes (Russel et al. 1983; Young and Pilgrim 1985). The *ADH2* encoded isoform is mainly involved in the reverse reaction of the utilization of ethanol under respirative conditions (Russel et al. 1983) and it is transcriptionally downregulated on high glucose concentrations (Gancedo 1998). *ADH2* is also downregulated under anaerobic conditions (Wiebe et al. 2008; ter Linde et al. 1999; Piper et al. 2002). The abundance of Adh2p has also been found to be lower under anaerobiosis than in aerobic conditions (Rintala et al. 2009). Accordingly, the expression of *ADH2* has been observed to be rapidly downregulated in correlation with the cessation of respirative metabolism in a sudden depletion of oxygen (Jouhten et al. 2012). Decrease in the expression of *ADH2* was observed within 12 min of the switch of gas flow from air to nitrogen, and correspondingly the expression of *PDC1* encoding pyruvate decarboxylase responded within 12 min (Wiebe et al. 2008). In contrast, *ADH1* is more highly expressed under anaerobic conditions than fully aerobic conditions (Wiebe et al. 2008; ter Linde et al. 1999). Nevertheless, Adh1p has been found to be the only isoform of alcohol dehydrogenase capable of efficient conversion of acetaldehyde into ethanol during growth on glucose independent of oxygen availability (de Smidt et al. 2012). The *ADH1* and *ADH2* encoded isoforms of alcohol dehydrogenase are cytosolic whereas the *ADH3* encoded isoenzyme has a mitochondrial localization and is involved in an anaerobic ethanol—acetaldehyde redox shuttle between mitochondria and cytosol (Bakker et al. 2000) (Fig. 3.3). Shuttling mechanisms are required since the redox cofactors cannot directly pass the mitochondrial membrane and under anaerobic conditions the surplus of NADH which is produced in mitochondria cannot be oxidised in the respiratory chain, but need to be transferred into cytosol.

The major isoforms of acetaldehyde dehydrogenase producing acetate under anaerobic conditions are encoded by *ALD6* and *ALD5*, which are cytosolic and mitochondrial, respectively (Saint-Prix et al. 2004) (Fig. 3.4). The acetaldehyde dehydrogenase reaction catalysed by *ALD6* encoded isoform is an alternative source of cytosolic NADPH, in addition to the main source, PPP (Grabowska and Chelstowska 2003). The *IDP2* encoded cytosolic isocitrate dehydrogenase is able to compensate the loss of both PPP and acetaldehyde dehydrogenase as cytosolic NADPH sources but only in aerated conditions since the enzyme is active only in the respirative metabolic state (Minard and McAlister-Henn 2005). Thus, *ALD6* encoded acetaldehyde dehydrogenase is presumably an essential alternative for NADPH production in PPP under anaerobic conditions (Saint-Prix et al. 2004) (Fig. 3.2). *ALD4* encodes a mitochondrial isoform of acetaldehyde dehydrogenase which is NAD^+ specific (Saint-Prix et al. 2004). The activity of the *ALD4* encoded isoform has been found to be strain and medium dependent under anaerobic conditions. Both *ALD6* and *ALD4* encoded acetaldehyde dehydrogenases have lower mRNA and protein abundances under anaerobic than aerobic conditions, in a set up where *S. cerevisiae* CEN.PK113-1A was cultured in chemostats on minimal medium having glucose as the sole carbon source, and where no acetate secretion was detected (Rintala et al. 2009). The other two isoforms of

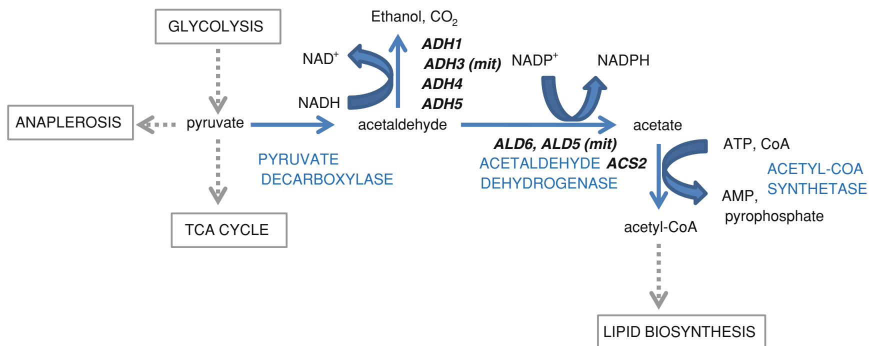

Fig. 3.4 Fermentative pathway and acetyl-CoA synthesis of *S. cerevisiae* under anaerobic conditions. Fermentative pathway branching from pyruvate and the acetyl-CoA synthesis reaction are shown with the enzymes catalysing the reactions (in *light blue*) and the relevant genes encoding the enzymes (in *black*) in *S. cerevisiae* under anaerobic growth conditions

acetaldehyde dehydrogenase do not contribute to acetate production on glucose (Saint-Prix et al. 2004).

3.2.4 Synthesis of Acetyl-CoA is Regulated in Response to the Absence of Oxygen

In *S. cerevisiae* acetate can be converted into acetyl-CoA via acetyl-CoA synthetase reaction which is ATP consuming (Fig. 3.4). There are two isoforms of acetyl-CoA synthetases in *S. cerevisiae* encoded by *ACS1* and *ACS2* (van den Berg et al. 1996). The isoforms have distinct expression depending on the availability of oxygen and whether the metabolism is respirative or fermentative. The *ACS1*-encoded isoform is induced under respirative metabolism whereas the *ACS2*-encoded isoform is constitutively expressed independent of the metabolic state (van den Berg and Steensma 1995). Thus, *ACS2*-encoded enzyme is also the anaerobically active isoform (van den Berg et al. 1996). Consistently, Wiebe et al. (2008) observed that the expression level of *ACS1* was very low in an anaerobic chemostat culture while *ACS2* was highly expressed (Wiebe et al. 2008). The *ACS1*-encoded aerobic isoform has been shown to have a dual distribution between cytosol and peroxisomes (Chen et al. 2012), but Acs1p has also been observed in the mitochondrial proteome (Sickmann et al. 2003) and localised additionally into nucleus based on a GFP signal (Huh et al. 2003). The anaerobic isoenzyme encoded by *ACS2* is active in cytosol (van den Berg et al. 1996). Acetyl-CoA requires transport systems to move between compartments since the membranes of intracellular organelles are impermeable for acetyl-CoA (van Roermund et al. 1995). There are two possible transport systems for the transport of acetyl-CoA across the mitochondrial membrane in *S. cerevisiae*, carnitine shuttle and glyoxylate shunt. However, it has been stated that exogenous

carnitine is required for the carnitine shuttle to be active since *S. cerevisiae* does not synthesise carnitine (Lange 2002; Swiegers et al. 2001; van Roermund et al. 1999). In addition, carnitine acetyltransferase activity that is required for the carnitine shuttle transporting acetyl-CoA across the mitochondrial membrane, has not been detected in *S. cerevisiae* grown in anaerobic chemostats at 0.1 h^{-1} (Nissen et al. 1997). The second option, glyoxylate shunt is involved in respirative metabolism and is repressed on glucose (Duntze et al. 1969). Further, no in vivo activity of the glyoxylate cycle was observed in ^{13}C-labelling experiments on glucose under anaerobic conditions (Jouhten et al. 2008). Thus, under anaerobic conditions and during aerobic growth on glucose the pyruvate dehydrogenase bypass and the Acs2p catalysed acetyl-CoA synthetase reaction form an essential route to the generation of cytosolic acetyl-CoA, which is required for lipid biosynthesis (Flikweert et al. 1996).

3.2.5 Glycerol Production as a Redox Sink

The pathway from glucose to ethanol is redox-neutral. Two NADH units are formed when one glucose unit is metabolised in glycolysis. The NADH molecules are oxidised by alcohol dehydrogenase when two molecules of ethanol are produced (from the two pyruvates formed of one molecule of glucose) (Fig. 3.2). However, a fraction of glucose is directed to biosynthesis and a net generation of NADH occurs in assimilatory reactions. Under aerobic conditions the anabolic NADH is oxidised by the external or internal NADH dehydrogenases which shuttle the electrons into the mitochondrial electron transfer chain (Bakker et al. 2001; Rigoulet et al. 2004). The electrons of cytosolic NADH can enter the mitochondria and the electron transfer chain via the external NADH dehydrogenases and the glycerol 3-phosphate shuttle. If oxygen availability is limiting *S. cerevisiae* uses oxygen preferentially to oxidise the anabolic NADH (Weusthuis et al. 1994; Franzen 2003). Glycerol production is triggered when there is not enough oxygen to accept all the electrons required to regenerate NAD^+ (see Figs. 3.1 and 3.2). The ATP requirement for glycerol production could be important for this regulation. In the glycerol production pathway, the glycolytic intermediate dihydroxyacetone phosphate is first reduced to glycerol 3-phosphate. A transient increase in glycerol 3-phosphate concentration has been observed as a response to a sudden switch of *S. cerevisiae* into fully fermentative conditions (anaerobiosis and glucose excess) (van den Brink et al. 2008b). The accumulation of glycerol 3-phosphate has been suggested to generally imply an increased intracellular $NADH/NAD^+$ ratio (Påhlman et al. 2001). The conversion of dihydroxyacetonephosphate into glycerol 3-phosphate is catalysed by glycerol 3-phosphate dehydrogenase which exists in two isoforms in *S. cerevisiae* encoded by *GPD1* (Albertyn et al. 1994) and *GPD2* (Eriksson et al. 1995), respectively. Both *GPD1* and *GPD2* encoded isoenzymes are NAD^+ specific. Glycerol formation is associated not only with fermentative metabolism but also with osmoregulation in *S. cerevisiae* (Ansell et al. 1997). The two isoenzymes of glycerol 3-phosphate

dehydrogenase have distinct roles, the *GPD2* encoded isoform being the one induced in the absence of oxygen. De Groot et al. (2007) observed the effect of transcriptional regulation of *GPD1* and *GPD2* at a protein level. Gpd2p had a higher abundance under anaerobic conditions than aerobic conditions whereas Gpd1p had a slightly lower anaerobic than aerobic abundance. In addition, the growth of a *gpd2* deletion mutant is impaired under anaerobic conditions whereas *gpd1* deletion does not affect growth in the absence of oxygen (Björkqvist et al. 1997). The double deletion mutant of *gpd1 gpd2* is unable to grow without oxygen or a supplementation of NADH-oxidizing agent in the medium (Ansell et al. 1997). While the transcriptional regulation of glycerol 3-phosphate dehydrogenases is seen at protein level, additional post-translational regulation of the activity is possible. Indeed, at least the activity of the isoform of glycerol 3-phosphate dehydrogenase encoded by *GPD1* is inhibited by phosphorylation in *S. cerevisiae* (Oliveira et al. 2012). Only a deletion of all four phosphosites in the enzyme abolished this inhibition. Glycerol 3-phosphate is converted to glycerol by glycerol 3-phosphate phosphatases encoded by *GPP1* and *GPP2* (Norbeck et al. 1996). The glycerol 3-phosphate phosphatase encoding genes are also regulated differentially under anaerobiosis. The *GPP1* gene is induced under anaerobic conditions whereas *GPP2* mRNA is present at a lower abundance under anaerobic than aerobic conditions (Påhlman et al. 2001). *GPP1* induction has been observed to correlate with an increase in the amount of protein (de Groot et al. 2007). A substantially higher abundance of Gpp1p has been observed under conditions where oxygen is lacking than under aerobiosis.

Glycerol secretion is performed by the *FPS1* encoded plasma membrane channel protein (Luyten et al. 1995). *FPS1* is upregulated in the absence of oxygen (ter Linde et al. 1999). Glycerol secretion in *S. cerevisiae* occurs in stoichiometric relation to the anabolic NADH generation under anaerobic conditions. The theoretical amount of assimilatory NADH formed is 11 mmol g biomass^{-1} at a growth rate 0.1 h^{-1} (Verduyn et al. 1990a), which is consistent with an experimentally observed glycerol production rate (Wiebe et al. 2008). However, when *S. cerevisiae* was simultaneously exposed to sudden oxygen depletion and glucose excess, glycerol formation transiently exceeded the rate expected on the basis of anabolic NADH generation (van den Brink et al. 2008b).

3.2.6 Anaerobic TCA Cycle Operates in a Branched Mode

Under anaerobic conditions the TCA cycle of *S. cerevisiae* operates as a branched pathway of oxidative and reductive branches (Nissen et al. 1997; Maaheimo et al. 2001; Fiaux et al. 2003; Camarasa et al. 2003). The cycle is interrupted at succinate dehydrogenase which is not active under anaerobiosis (Camarasa et al. 2003). The oxidative branch has an important anabolic function in the production of the biosynthetic precursor 2-oxoglutarate while the reductive branch may contribute to redox balancing. Under aerobic glucose-limited conditions the

relative anaplerotic flux of *S. cerevisiae*, which replenishes the TCA cycle intermediates drawn for biosynthesis, is about 30 % whereas under anaerobic conditions there is no respiratory activity and the relative anaplerotic flux is 100 % (Jouhten et al. 2008; Maaheimo et al. 2001) (Table 3.1). Even under aerobic conditions in the presence of glucose excess, the relative anaplerotic flux to the TCA cycle is high (Table 3.1), which indicates of a low respiratory versus biosynthetic activity of the TCA cycle (Maaheimo et al. 2001). Under aerobic conditions, the TCA cycle flux of *S. cerevisiae* has been observed to show inversely correlated activity with the specific glucose uptake rate (Blank and Sauer 2004). When the glucose uptake rates are high, the cyclic TCA cycle flux is low or becomes completely interrupted even in the presence of oxygen (Maaheimo et al. 2001). The metabolites of the TCA cycle (citrate, succinate, fumarate, malate) are more abundant in the absence of oxygen than under aerobic conditions (Wiebe et al. 2008; Villas-Boas et al. 2005). Higher anaerobic concentrations have been observed independent of the simultaneous glucose repression. The importance of the anabolic function of mitochondria even in the absence of aerobic respiration has been recognised for long time (Visser et al. 1994). Interestingly, most of the enzyme complexes of oxidative phosphorylation are present under anaerobiosis, though in lower abundances than under normoxic conditions (Helbig et al. 2009).

After the mitochondrial conversion of pyruvate into acetyl-CoA by pyruvate dehydrogenase, acetyl-CoA enters the oxidative branch of the TCA cycle which is a chain of reactions producing 2-oxoglutarate from acetyl-CoA and oxaloacetate. While oxygen is available and the respiratory pathway is active, most of the TCA cycle enzymes, including enzymes of oxidative branch, are under transcriptional regulation of the haem dependent Hap2/3/4/5p complex. Retrograde signalling between mitochondria and the nucleus is triggered in dysfunction or absence of the aerobic respiration in mitochondria in order to align nitrogen and carbon metabolisms (Butow and Avadhani 2004). The expression of enzymes in the oxidative branch of the TCA cycle, i.e. citrate synthase, aconitase and isocitrate dehydrogenase, encoded by *CIT1*, *ACO1* and *IDH1/2*, respectively, switch from Hap2/3/4/5p dependent regulation to regulation by the retrograde transcription factors (Liu and Butow 1999). The retrograde regulation is mediated by the transcription factors Rtg1p, Rtg2p and Rtg3p. Retrograde regulation of the oxidative branch of the TCA cycle has been proposed to ensure sufficient production of L-glutamate when the respiratory activity is low or absent. The proteins of the oxidative branch (Cit1p, Aco1p, Aco2p, Idh1p, Idh2p) have been shown to increase or have unchanged expression in the absence of oxygen compared to aerobic conditions (de Groot et al. 2007). On the contrary, Lsc2p, Sdh2p and Kgd2p belonging to the enzyme complexes involved in the conversion of 2-oxoglutarate to succinate were less abundant under anaerobic than aerobic conditions. In earlier studies, the activities of the corresponding enzymes, 2-oxoglutarate dehydrogenase, isocitrate dehydrogenase and succinate dehydrogenase, have been observed to be low or absent under anaerobiosis (Machado et al. 1975; Camarasa et al. 2003). Interestingly, defects either in the 2-oxoglutarate dehydrogenase complex or aconitase in the oxidative branch of TCA cycle have been observed to induce expression of

anaerobic genes and down-regulation of aerobic genes, similarly as occurs when haem dependent regulation is triggered (McCammon et al. 2003). This suggests a role of the oxidative branch in the sensing of the status of respirative metabolism.

During anaerobic growth malate dehydrogenase and fumarate reductase of the reductive branch of the TCA cycle can contribute to the regeneration of mitochondrial pool of NAD^+ together with mitochondrial alcohol dehydrogenase (Nissen et al. 1997) (Fig. 3.2). Cytoplasmic and mitochondrial fumarate reductases are encoded by *FRD1* and *OSM1*, respectively. While fumarate reductase is a major contributor to the anaerobic production of succinate, a substantial production of succinate by 2-oxoglutarate dehydrogenase has been observed also under anaerobic conditions when the nitrogen source is glutamate (Camarasa et al. 2003). On the other hand, a double deletion mutant of *frd1 osm1* is unable to grow under anaerobiosis (Camarasa et al. 2007). Camarasa et al. (2007) proposed that fumarate reductase activity is essential for the reoxidation of $FADH_2$ in the absence of oxygen. The cytosolic fumarate reductase is the main isoform under anaerobiosis but can be partly replaced by the mitochondrial enzyme (Camarasa et al. 2007). Replacement is possible since flavin co-factors can be transferred across the mitochondrial membrane by the carrier Flx1p (Bafunno et al. 2004; Tzagoloff et al. 1996). Recently, Liu et al. (2013) proposed that the reoxidation of $FADH_2$ by fumarate reductase is required for protein folding under anaerobiosis since FAD in the endoplasmic reticulum acts as an electron acceptor for the protein folding associated electrons. Fum1p (fumarase) and Frd1p of the reductive branch of TCA have been observed in higher abundances in conditions lacking oxygen compared to aerobic conditions (de Groot et al. 2007). In addition to the Fum1p and Frd1p protein amounts, the operation of the reductive branch under anaerobic conditions is further supported by an upregulated protein abundance of pyruvate carboxylase (Pyc1p) which compensates the anabolic loss from the TCA cycle by production of oxaloacetate. Nevertheless, results by Oura et al. (1980) in their early study implicated that the flux of the reductive branch of TCA cycle of *S. cerevisiae* is substantially lower than the flux in the oxidative branch under anaerobic conditions. Expression of *MAE1* encoding a malic enzyme has been shown to be induced under anaerobic conditions (Boles et al. 1998) and higher protein abundance of Mae1p has been observed in the absence of oxygen than under aerobic conditions (de Groot et al. 2007). Malic enzyme may contribute to the provision of NADPH in mitochondria (Boles et al. 1998).

The in vivo activity of the TCA cycle in *S. cerevisiae*, closely coupled to aerobic respiration, has been found to be less robust against deletions of transcription factors than other parts of the central carbon metabolism (Fendt et al. 2010). Interestingly, single transcription factor deletions can interrupt the in vivo activity of the TCA cycle and aerobic metabolism whereas fermentative metabolism is more robust, even though several regulators have assigned target genes encoding the TCA cycle enzymes in *S. cerevisiae*, the haem dependent Hap2/3/4/5p complex being among them. Fendt et al. (2010) identified Gcn4p as a novel upstream regulator of the Hap2/3/4/5p complex. Gcn4p was previously shown to

be a major regulator of amino acid biosynthesis (Hinnebusch and Fink 1983; Natarajan et al. 2001) with many of its target genes upregulated under anaerobiosis (ter Linde et al. 1999; Piper et al. 2002; Lai et al. 2006). In 2008 Beckhouse et al. noted that Gcn4p activity is essential for anaerobic adaptation. *Gcn4* deletion mutants exhibit an extended lag-phase of growth in sudden depletion of oxygen but remain viable (Tsoi et al. 2009). Tsoi et al. (2009) further observed that during adaptation to anaerobiosis *S. cerevisiae* has an increased L-serine demand. This occurs most likely to supply the cell wall with serine-rich mannoproteins Dan1-4p and Tir1-4p that are expressed under anaerobic conditions. To fulfil the increased L-serine demand, C1-metabolism is activated as an alternative synthesis route of L-serine. However, in the adapted anaerobic state, the fraction of L-serine originating from the C1-pool is not higher than under fully aerobic conditions (Jouhten et al. 2008), supporting only a transient requirement of the activation of C1-metabolism for L-serine synthesis at the exposure to anaerobic conditions.

3.3 Conclusions

The central carbon metabolism distributes major fluxes in cells by merging the catabolic pathways and providing precursors for anabolic pathways. Also energy generation occurs in the central metabolism. The yeast *S. cerevisiae* is superior in its adaptability to anaerobiosis. In response to anaerobic conditions, it increases the glycolytic rate substantially and remodels the central carbon metabolism extensively, as is evident from the literature reviewed above. Systems biology approaches have provided a vast amount of data on the anaerobic adaptation of cellular components at different functional levels and also on the time-dependent progress of the adaptation. Also quantitative data is abundant. Yet, the picture isn't complete. The remodelling of metabolism occurs as a flow of events, some of which are directly triggered by the depletion of oxygen, whereas others are mediated by intracellular regulatory interactions and feedback systems. A flow chart describing the order and relative importance of the cellular events for the adaptability of *S. cerevisiae* is still sketchy. An ability to target metabolic engineering to specific events occurring through regulatory interactions would open new prospects for the exploitation of the strength of the anaerobic performance of *S. cerevisiae* in industrial processes, e.g. in conversion of biomass sugars into valuable biochemicals. The remodelling of carbon metabolism of *S. cerevisiae* in response to the absence of oxygen offers also an ideal model case for the investigations of 'low ATP yield-high rate' energy metabolism and the importance of components and interactions involved in the adaptation to such a mode.

References

Abramova N, Sertil O, Mehta S et al (2001) Reciprocal regulation of anaerobic and aerobic cell wall mannoprotein gene expression in *Saccharomyces cerevisiae*. J Bacteriol 183:2881–2887

Albertyn J, Hohmann S, Thevelein JM et al (1994) GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol Cell Biol 14:4135–4144

Ansell R, Granath K, Hohmann S et al (1997) The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. EMBO J16:2179–2187

Bafunno V, Giancaspero TA, Brizio C et al (2004) Riboflavinuptake and FAD synthesis in *Saccharomyces cerevisiae* mitochondria: involvement of the Flx1p carrier in FAD export. J Biol Chem 279:95–102

Bakker B, Bro C, Kötter P et al (2000) The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in *Saccharomyces cerevisiae*. J Bacteriol 182:4730–4737

Bakker B, Overkamp K, van Maris A et al (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol 25:15–37

Becerra M, Lombardía-Ferreira LJ, Hauser NC et al (2002) The yeast transcriptome in aerobic and hypoxic conditions: effects of hap1, rox1, rox3 and srb10 deletions. Mol Microbiol 43(3):545–555

Bergdahl B, Heer D, Sauer U et al (2012) Dynamic metabolomics differentiates between carbon and energy starvation in recombinant *Saccharomyces cerevisiae* fermenting xylose. Biotechnol Biofuels 5:34

Björkqvist S, Ansell R, Adler L et al (1997) Physiological response to anaerobicity of glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisae*. Appl Environ Microbiol 63:128–132

Blank LM, Sauer U (2004) TCA cycle activity in *Saccharomyces cerevisiae* is a function of the environmentally determined specific growth and glucose uptake rates. Microbiology 150:1085–1093

Blank LM, Lehmbeck F, Sauer U (2005) Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. FEMS Yeast Res 5(6–7):545–558

Boles E, de Jong-Gubbels P, Pronk JT (1998) Identification and characterization of MAE1, the *Saccharomyces cerevisiae* structural gene encoding mitochondrial malic enzyme. J Bacteriol 180:2875–2882

Breitkreutz A, Choi H, Sharom JR et al (2010) A global protein kinase and phosphatase interaction network in yeast. Science 328(5981):1043–1046

Bruckmann A, Hensbergen PJ, Balog CI et al (2009) Proteome analysis of aerobically and anaerobically grown *Saccharomyces cerevisiae* cells. J Proteomics 71(6):662–669

Butow RA, Avadhani NG (2004) Mitochondrial signaling: the retrograde response. Mol Cell 14(1):1–15

Cadière A, Galeote V, Dequin S (2010) The *Saccharomyces cerevisiae* zinc factor protein Stb5p is required as a basal regulator of the pentose phosphate pathway. FEMS Yeast Res 10(7):819–827

Camarasa C, Grivet JP, Dequin S (2003) Investigation by 13C-NMR and tricarboxylic acid (TCA) deletion mutant analysis of pathways for succinate formation in *Saccharomyces cerevisiae* during anaerobic fermentation. Microbiology 149:2669–2678

Camarasa C, Faucet V, Dequin S (2007) Role in anaerobiosis of the isoenzymes for *Saccharomyces cerevisiae* fumarate reductase encoded by OSM1 and FRDS1. Yeast 24(5):391–401

Chen Y, Siewers V, Nielsen J (2012) Profiling of cytosolic and peroxisomal acetyl-CoA metabolism in *Saccharomyces cerevisiae*. PLoS ONE 7(8):e42475

Daran-Lapujade P, Jansen ML, Daran JM et al (2004) Role of transcriptional regulation in controlling fluxes in central carbon metabolism of *Saccharomyces cerevisiae*. A chemostat culture study. J Biol Chem 279(10):9125–9138

Daran-Lapujade P, Rossell S, van Gulik WM et al (2007) The fluxes through glycolytic enzymes in *Saccharomyces cerevisiae* are predominantly regulated at posttranscriptional levels. Proc Natl Acad Sci U S A 104(40):15753–15758

Davies BS, Rine J (2006) A role for sterol levels in oxygen sensing in *Saccharomyces cerevisiae*. Genetics 174(1):191–201

de Groot MJ, Daran-Lapujade P, van Breukelen B et al (2007) Quantitative proteomics and transcriptomics of anaerobic and aerobic yeast cultures reveals post-transcriptional regulation of key cellular processes. Microbiology 153:3864–3878

de Smidt O, du Preez JC, Albertyn J (2012) Molecular and physiological aspects of alcohol dehydrogenases in the ethanol metabolism of *Saccharomyces cerevisiae*. FEMS Yeast Res 12(1):33–47

Dobson PD, Smallbone K, Jameson D et al (2010) Further developments towards a genome-scale metabolic model of yeast. BMC Syst Biol 4:145

Duntze W, Neumann D, Gancedo JM et al (1969) Studies on the regulation and localization of the glyoxylate cycle enzymes in *Saccharomyces cerevisiae*. Eur J Biochem 10(1):83–89

Eriksson P, André L, Ansell R et al (1995) Cloning and characterization of GPD2, a second gene encoding sn-glycerol 3-phosphate dehydrogenase (NAD+) in *Saccharomyces cerevisiae*, and its comparison with GPD1 Mol. Microbiol 17:95–107

Fendt SM, Oliveira AP, Christen S (2010) Unraveling condition-dependent networks of transcription factors that control metabolic pathway activity in yeast. Mol Syst Biol 6:432

Fiaux J, Ìakar PZ, Sonderegger M et al (2003) Metabolic-Flux profiling of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*. Eukaryot Cell 2:170–180

Flikweert MT, van Der Zanden L, Janssen WM et al (1996) Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. Yeast 12(3):247–257

Forsburg SL, Guarente L (1989) Identification and characterization of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer. Genes Dev 3:1166–1178

Franzén CJ (2003) Metabolic flux analysis of RQ-controlled microaerobic ethanol production by *Saccharomyces cerevisiae*. Yeast 20(2):117–132

Frick O, Wittmann C (2005) Characterization of the metabolic shift between oxidative and fermentative growth in *Saccharomyces cerevisiae* by comparative 13C flux analysis. Microb Cell Fact 4:30–46

Gancedo JM (1998) Yeast carbon catabolite repression. Microbiol Mol Biol 62:334–361

Gojković Z, Knecht W, Zameitat E et al (2004) Horizontal gene transfer promoted evolution of the ability to propagate under anaerobic conditions in yeasts. Mol Genet Genomics 271(4):387–393

Gombert AK, Moreira dos Santos M, Christensen B et al (2001) Network identification and flux quantification in the central metabolism of Saccharomyces cerevisiae under different conditions of glucose repression. J Bacteriol 183(4):1441–1451

Grabowska D, Chelstowska A (2003) The ALD6 gene product is indispensable for providing NADPH in yeast cells lacking glucose-6-phosphate dehydrogenase activity. J Biol Chem 278:13984–13988

Grüning NM, Rinnerthaler M, Bluemlein K, Mülleder M, Wamelink MM, Lehrach H, Jakobs C, Breitenbach M, Ralser M (2011) Pyruvate kinase triggers a metabolic feedback loop that controls redox metabolism in respiring cells. Cell Metab 14(3):415–427

Hagman A, Säll T, Compagno C et al (2013) Yeast "Make-Accumulate-Consume" life strategy evolved as a multi-step process that predates the whole genome duplication. PLoS ONE 8(7):e68734

Hauf J, Zimmermann FK, Müller S (2000) Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*. Enzyme Microb Technol 26(9–10):688–698

Heavner BD, Smallbone K, Barker B, Mendes P, Walker LP (2012) Yeast 5—an expanded reconstruction of the *Saccharomyces cerevisiae* metabolic network. BMC Syst Biol 6:55

Helbig AO, de Groot MJ, van Gestel RA et al (2009) A three-way proteomics strategy allows differential analysis of yeast mitochondrial membrane protein complexes under anaerobic and aerobic conditions. Proteomics 9(20):4787–4798

Herrgård MJ, Swainston N, Dobson P et al (2008) A consensus yeast metabolic network reconstruction obtained from a community approach to systems biology. Nat Biotechnol 26(10):1155–1160

Hickman MJ, Winston F (2007) Heme levels switch the function of Hap1 of *Saccharomyces cerevisiae* between transcriptional activator and transcriptional repressor. Mol Cell Biol 27(21):7414–7424

Hinnebusch AG, Fink GR (1983) Positive regulation in the general amino acid control of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A. 80(17):5374–8

Hon T, Lee HC, Hu Z et al (2005) The heme activator protein Hap1 represses transcription by a heme-independent mechanism in Saccharomyces cerevisiae. Genetics 169(3):1343–1352

Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW et al (2003) Global analysis of protein localization in budding yeast. Nature 425:686–691

Jacquier N, Schneiter R (2012) Mechanisms of sterol uptake and transport in yeast. J Steroid Biochem Mol Biol 129(1–2):70–78

Jamieson DJ (1998) Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast 14:1511–1527

Jouhten P, Rintala E, Huuskonen A et al (2008) Oxygen dependence of metabolic fluxes and energy generation of *Saccharomyces cerevisiae* CEN.PK113-1A. BMC Syst Biol 2:60

Jouhten P, Wiebe M, Penttilä M (2012) Dynamic flux balance analysis of the metabolism of *Saccharomyces cerevisiae* during the shift from fully respirative or respirofermentative metabolic states to anaerobiosis. FEBS J 279(18):3338–3354

Klimacek M, Krahulec S, Sauer U et al (2010) Limitations in xylose-fermenting *Saccharomyces cerevisiae*, made evident through comprehensive metabolite profiling and thermodynamic analysis. Appl Environ Microbiol 76:7566–7574

Kwast KE, Lai L-C, Menda N et al (2002) Genomic analyses of anaerobically induced genes in *Saccharomyces cerevisiae*: functional roles of Rox1 and other factors in mediating the anoxic response. J Bacteriol 184:250–265

Lai LC, Kosorukoff AL, Burke PV et al (2006) Metabolic-state-dependent remodeling of the transcriptome in response to anoxia and subsequent reoxygenation in *Saccharomyces cerevisiae*. Eukaryot Cell 5:1468–1489

Lange HC (2002) Quantitative physiology of saccharomyces cerevisiae using metabolic network analysis. Ph.D. thesis, Technical University Delft, The Netherlands

Larochelle M, Drouin S, Robert F et al (2006) Oxidative stress-activated zinc cluster protein Stb5 has dual activator/ repressor functions required for pentose phosphate pathway regulation and NADPH production. Mol Cell Biol 26:6690–6701

Liu Z, Butow RA (1999) A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. Mol Cell Biol 19:6720–6728

Liu Z, Österlund T, Hou J et al (2013) Anaerobic α-amylase production and secretion with fumarate as the final electron acceptor in *Saccharomyces cerevisiae*. Appl Environ Microbiol 79(9):2962–2967

Llobell A, Lopez-Ruiz A, Peinado J et al (1988) Glutathione reductase directly mediates the stimulation of yeast glucose-6-phosphate dehydrogenase by GSSG. Biochem J 249:293–296

Lowry CV, Zitomer RS (1984) Oxygen regulation of anaerobic and aerobic genes mediated by a common factor in yeast. Proc Natl Acad Sci 81:6129–6133

Luyten K, Albertyn J, Skibbe WF et al (1995) Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. EMBO J 14:1360–1371

Maaheimo H, Fiaux J, Cakar ZP et al (2001) Central carbon metabolism of *Saccharomyces cerevisiae* explored by biosynthetic fractional (13)C labeling of common amino acids. Eur J Biochem 268(8):2464–2479

Machado A, Nuñez de Castro I, Mayor F (1975) Isocitrate dehydrogenases and oxoglutarate dehydrogenase activities of baker's yeast grown in a variety of hypoxic conditions. Mol Cell Biochem 6(2):93–100

McCammon MT, Epstein CB, Przybyla-Zawislak B et al (2003) Global transcription analysis of krebs tricarboxylic acid cycle mutants reveals an alternating pattern of gene expression and effects on hypoxic and oxidative genes. Mol Biol Cell 14:958–972

Mensonides FIC, Bakker BM, Cremazy F (2013) A new regulatory principle for in vivo biochemistry: Pleiotropic low affinity regulation by the adenine nucleotides—Illustrated for the glycolytic enzymes of *Saccharomyces cerevisiae*. FEBS Lett 587(2860):2867

Minard KI, McAlister-Henn L (1999) Dependence of peroxisomal β-Oxidation of cytosolic sources of NADPH. J Biol Chem 274:3402–3406

Minard KI, Mc Alister-Henn L (2005) Sources of NADPH in yeast vary with carbon source. J Biol Chem 280(48):39890–39896

Møller K, Olsson L, Piskur J (2001) Ability for anaerobic growth is not sufficient for development of the petite phenotype in *Saccharomyces kluyveri*. J Bacteriol 183(8):2485–2489

Murcott TH, Gutfreund H, Muirhead H (1992) The cooperative binding of fructose-1,6-bisphosphate to yeast pyruvate kinase. EMBO J 11(11):3811–3814

Nagy M, Lacroute F, Thomas D (1992) Divergent evolution of pyrimidine biosynthesis between anaerobic and aerobic yeasts. Proc Natl Acad Sci 89(19):8966–8970

Natarajan K, Meyer MR, Jackson BM et al (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol Cell Biol 21(13):4347–4368

Nissen TL, Schulze U, Nielsen J et al (1997) Flux distributions in anaerobic, glucose-limited continuous cultures of *Saccharomyces cerevisiae*. Microbiology 143:203–218

Nogae I, Johnston M (1990) Isolation and characterization of the ZWF1 gene of *Saccharomyces cerevisiae*, encoding glucose-6-phosphate dehydrogenase. Gene 96(2):161–169

Norbeck J, Pahlman AK, Akhtar N et al (1996) Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from *Saccharomyces cerevisiae*. J Biol Chem 271:13875–13881

Oliveira AP, Ludwig C, Picotti P et al (2012) Regulation of yeast central metabolism by enzyme phosphorylation. Mol Syst Biol 8:623

Oura E, Haarasilta S, Londesborough J (1980) Carbon dioxide fixation by baker's yeast in a variety of growth conditions. J Gen Microbiol 118:51–58

Pahlman AK, Granath K, Ansell R et al (2001) The yeast glycerol 3-phosphatases gpp1p and gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress. J Biol Chem 276:3555–3563

Piper MDW, Daran-Lapujade P, Bro C et al (2002) Reproducibility of oligonucleotide microarray transcriptome analyses. J Biol Chem 277:37001–37008

Rigoulet M, Aguilaniu H, Avéret N et al (2004) Organization and regulation of the cytosolic NADH metabolism in the yeast *Saccharomyces cerevisiae*. Mol Cell Biochem 256–257(1–2):73–81

Rintala E, Wiebe MG, Tamminen A et al (2008) Transcription of hexose transporters of Saccharomyces cerevisiae is affected by change in oxygen provision. BMC Microbiol 8:53

Rintala E, Toivari M, Pitkänen JP et al (2009) Low oxygen levels as a trigger for enhancement of respiratory metabolism in Saccharomyces cerevisiae. BMC Genom 10:461

Rintala E, Jouhten P, Toivari M et al (2011) Transcriptional responses of *Saccharomyces cerevisiae* to shift from respiratory and respirofermentative to fully fermentative metabolism. OMICS Biology 15:461–476

Rizzi M, Baltes M, Theobald U et al (1997) *In vivo* analysis of metabolic dynamics in *Saccharomyces cerevisiae* II Mathematical model. Biotechnol Bioeng 55:592–608

Rosenfeld E, Beauvoit B (1998) Role of the non-respiratory pathways in the utilization of molecular oxygen by Saccharomyces cerevisiae. Yeast 20:1115–1144

Runquist D, Hahn-Hägerdal B, Bettiga M (2009) Increased expression of the oxidative pentose phosphate pathway and gluconeogenesis in anaerobically growing xylose-utilizing *Saccharomyces cerevisiae*. Microb Cell Fact 8:49

Russell DW, Smith M, Williamson VM et al (1983) Nucleotide sequence of the yeast alcohol dehydrogenase II gene. J Biol Chem 258(4):2674–2682

Saint-Prix F, Bonquist L, Dequin S (2004) Functional analysis of the ALD gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the NADP 1-dependent Ald6p and Ald5p isoforms play a major role in acetate formation. Microbiology 150:2209–2220

Schaaff I, Heinisch J, Zimmermann FK (1989) Overproduction of glycolytic enzymes in yeast. Yeast 5:285–290

Sertil O, Kapoor R, Cohen BD et al (2003) Synergistic repression of anaerobic genes by Mot3 and Rox1 in *Saccharomyces cerevisiae*. Nucleic Acids Res 31:5831–5837

Shi NQ, Jeffries TW (1998) Anaerobic growth and improved fermentation of *Pichia stipitis* bearing a URA1 gene from *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 50(3):339–345

Sickmann A, Reinders J, Wagner Y et al (2003) The proteome of *Saccharomyces cerevisiae* mitochondria. PNAS 100:13207–13212

Sinensky M (1974) Homeoviscous adaptation a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc Natl Acad Sci 71:522–525

Smits P, Hauf J, Müller S et al (2000) Simultaneous overexpression of enzymes of the lower part of glycolysis can enhance the fermentative capacity of *Saccharomyces cerevisiae*. Yeast 16(14):1325–1334

Snoek IS, Steensma HY (2006) Why does *Kluyveromyces lactis* not grow under anaerobic conditions? Comparison of essential anaerobic genes of *Saccharomyces cerevisiae* with the *Kluyveromyces lactis* genome. FEMS Yeast Res 6(3):393–403

Swiegers J, Dippenaar N, Pretorius I et al (2001) Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: three carnitine acetyltranferases are essential in a carnitine dependent strain. Yeast 18:585–595

Tai SL, Boer VM, Daran-Lapujade P, Walsh MC et al (2005) Two-dimensional transcriptome analysis in chemostat cultures combinatorial effects of oxygen availability and macronutrient limitation in *Saccharomyces cerevisiae*. J Biol Chem 280:437–447

ter Linde JJ, Liang H, Davis RW et al (1999) Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*. J Bacteriol 181:7409–7413

ter Linde JJ, Steensma HY (2002) A microarray-assisted screen for potential Hap1 and Rox1 target genes in *Saccharomyces cerevisiae*. Yeast 19:825–840

Toivari MH, Aristidou A, Ruohonen L et al (2001) Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae*: importance of xylulokinase (XKS1) and oxygen availability. Metab Eng 3:236–349

Tsoi BM, Beckhouse AG, Gelling CL et al (2009) Essential role of one-carbon metabolism and Gcn4p and Bas1p transcriptional regulators during adaptation to anaerobic growth of *Saccharomyces cerevisiae*. J Biol Chem 284(17):11205–11215

Tzagoloff A, Jang J, Glerum DM et al (1996) FLX1 codes for a carrier protein involved in maintaining a proper balance of flavin nucleotides in yeast mitochondria. J Biol Chem 271:7392–7397

van den Berg MA, de Jong-Gubbels P, Kortland CJ et al (1996) The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. J Biol Chem 271:28953–28959

van den Berg MA, Steensma HY (1995) ACS2, a *Saccharomyces cerevisiae* gene encoding acetyl-coenzyme a synthetase, essential for growth on glucose. Eur J Biochem 231:704–713

van den Brink J, Daran-Lapujade P, Pronk JT et al (2008a) New insights into the *Saccharomyces cerevisiae* fermentation switch: dynamic transcriptional response to anaerobicity and glucose-excess. BMC Genom 9:100

van den Brink J, Canelas AB, van Gulik WM et al (2008b) Dynamics of glycolytic regulation during adaptation of *Saccharomyces cerevisiae* to fermentative metabolism. Appl Environ Microbiol 74(18):5710–5723

van Hoek P, van Dijken JP, Pronk JT (1998) Effect of specific growth rate on fermentative capacity of baker's yeast. Appl Environ Microbiol 64(11):4226–4233

van Hoek P, van Dijken JP, Pronk JT (2000) Regulation of fermentative capacity and levels of glycolytic enzymes in chemostat cultures of *Saccharomyces cerevisiae*. Enzyme Microb Technol 26:724–736

van Roermund CWT, Elgersma Y, Singh N et al (1995) The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. EMBO J 14:3480–3486

van Roermund C, Hettema E, van der Berg M et al (1999) Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in *Saccharomyces cerevisiae* and identification of a plasma membrane carnitine transporter, Agp2p. EMBO J 18:5843–5852

Vaseghi S, Baumeister A, Rizzi M et al (1999) In vivo Dynamics of the Pentose Phosphate Pathway in *Saccharomyces cerevisiae*. Metab Eng 1:128–140

Verduyn C, Postma E, Scheffers WA et al (1990a) Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J Gen Microbiol 136:395–403

Verduyn C, Postma E, Scheffers WA et al (1990b) Energetics of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J Gen Microbiol 136:405–412

Verduyn C, Stouthamer AH, Scheffers WA et al (1991) A theoretical evaluation of growth yields of yeasts. Antonie Van Leeuwenhoek 59:49–63

Verduyn C, Postma E, Scheffers WA et al (1992) Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8(7):501–517

Villas-Bôas SG, Moxley JF, Akesson M et al (2005) High-throughput metabolic state analysis: the missing link in integrated functional genomics of yeasts. Biochem J 388:669–677

Visser D, van Zuylen GA, van Dam JC et al (2004) Analysis of *in vivo* kinetics of glycolysis in aerobic *Saccharomyces cerevisiae* by application of glucose and ethanol pulses. Biotechnol Bioeng 88(2):157–167

Visser W, Baan AA van der, Batenburg-van der Vegte W et al (1994) Involvement of mitochondria in the assimilatory metabolism of anaerobic Saccharomyces cerevisiae cultures. Microbiology 140:3039–3046

Visser W, Scheffers A, Batenburg-van der Vegte WH et al (1990) Oxygen requirements of yeasts. Appl Environ Microbiol 56(12):3785–3792

Weusthuis RA, Visser W, Pronk JT et al (1994) Effects of oxygen limitation on sugar metabolism in yeasts—a continuous-culture study of the Kluyver effect. Microbiology 140:703–715

Wiebe MG, Rintala E, Tamminen A et al (2008) Central carbon metabolism of *Saccharomyces cerevisiae* in anaerobic, oxygen-limited and fully aerobic steady-state conditions and following a shift to anaerobic conditions. FEMS Yeast Res 8(1):140–154

Wu L, van Dam J, Schipper D et al (2006) Short-term metabolome dynamics and carbon, electron, and ATP balances in chemostat-grown *Saccharomyces cerevisiae* CEN.PK 113-7D following a glucose pulse. Appl Environ Microbiol 72(5):3566–3577

Young ET, Pilgrim D (1985) Isolation and DNA sequence of ADH3, a nuclear gene encoding the mitochondrial isozyme of alcohol dehydrogenase in *Saccharomyces cerevisiae*. Mol Cell Biol 5(11):3024–3034

Zhang L, Guarente L (1994) HAP1 is nuclear but is bound to a cellular factor in the absence of heme. J Biol Chem 269:14643–14647

Chapter 4
Systems Biology: Developments and Applications

Rahul Kumar, Petri-Jaan Lahtvee and Jens Nielsen

General systems theory provides the conceptual framework for systems-level analysis in science and underlines the fact that general systems principles are common in all fields of science (Bertalanffy 1950). Systems theory vision for biological analysis began in the 1960s but took off only after the technological breakthroughs in high-throughput analysis of living cells in the 1990s (Mesarovic 1968; Kitano 2002). The developments in molecular biology, high-throughput technologies, and computation precede the acceptance of systems biology as a new scientific discipline (Box 4.1), where the use of mathematical models is closely integrated with experimental research. Thus, systems biology relies on systems theory concepts and is applicable to both fundamental studies of cellular biology and applied research such as metabolic engineering (Fig. 4.1) (Nielsen and Olsson 2002).

Availability of the whole genome sequence of the yeast *Saccharomyces cerevisiae* followed by the development of DNA microarrays provided the opportunity to observe and investigate the environmental perturbations and subsequent phenotypic changes at the systems level (Goffeau et al. 1996; Lashkari et al. 1997). However, the ease of high throughput data generation clearly illustrated the biological complexity (Weng 1999; Csete and Doyle 2002; DeRisi 1997). The genome scale reconstruction of the *S. cerevisiae* metabolic network was a first attempt to provide a framework for data integration, in silico assessment of the metabolic capabilities, and analysis of phenotypic functions (Förster et al. 2003; Famili et al. 2003; Herrgård et al. 2008). High-throughput technology developments for metabolome, fluxome, and proteome quantification further aided in the comprehensive understanding at the systems level through global integration of such information into genome scale models (Kell et al. 2005; Karr et al. 2012; Picotti et al. 2013; Sauer 2006; Osterlund et al. 2013).

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_4, © Springer-Verlag Berlin Heidelberg 2014

Box 4.1 Key Technological Developments

Timeline	Milestones
1953	Structure of DNA (Watson and Crick, Cold Spring Harb Symp Quant Biol, 1953)
1970s	Recombinant technologies 2D-PAGE for protein measurements (Wein, Anal Biochem, 1969) Enzyme-linked immunosorbent assay (Engvall and Perlmann, Immunochem, 1971)
1980s	DNA sequencing (Sanger et al., Proc Natl Acad Sci, 1977)
1985–1989	Development of soft ionizaion techniques for MS analysis (MALDI and ESI; Karas, Anal Chem, 1985; Fenn, Science, 1989)
1986	First FBA model (Fell and Small, Biochem J, 1986)
1987	PCR (Mullis and Faloona, Meth. Enzymol. 1987)
1990	BLAST- Basic Local Alignment Search Tool (Altschul et al., J. Mol. Biol. 1990)
1995	First sequenced genome (Fleischmann, Science, 1995) Metabolic flux analysis (van Gulik and Heijnen, Biotechnol Bioeng, 1995) KEGG—Kyoto Encyclopedia of Genes and Genomes (Kanehisa, Trends Genet, 1997)
1996	Pyrosequencing (Ronaghi et al., Anal Biochem, 1996)
1997	First complete genome DNA microarray (Lashkari et al., Proc Natl Acad Sci, 1997)
1998	RNA interference technology (Fire et al., Letters to Nature, 1998)
2001	SBML—Systems Biology Markup Language (SBML) (Hucka et al., 2001) First Genome Scale Model (GSM) (Edwards et al., Nat Biotechnol, 2001) Synthetic Genetic Array (SGA) analysis (Tong et al., Science, 2001)
2002	Launch of UCSC Genome Browser
2004	METLIN database (Smith et al., Ther Drug Monit, 2005)
2005	Second generation sequencing (Shendure et al., Science, 2005; Margulies et al., Nature, 2005)
2006	Orbitrap mass spectrometer (Makarov et al., Anal Chem, 2006)
2007	Quantitative shotgun proteomics
2008	RNA-seq (Ryan et al., Bio Techniques, 2008)
2009	Third generation sequencing (SMRT; Eid et al., Science, 2009) Ribosome profiling (Ingolia et al., Science, 2009)
2010	Global scale analysis of posttranslational modifications (Bodenmiller et al., Science Signaling, 2010)
2013	Complete map of yeast proteome (Picotti et al., Nature, 2013)

Understanding of such basic mechanisms as sensing of the environment; transport of the nutrients; metabolism of carbon sources to provide precursor metabolites and their conversion into cellular building blocks and macromolecular components; product formation to generate Gibbs free energy and biomass is of the critical importance for the efficient utilization of *S. cerevisiae* in the biotechnological applications as well as for the elucidation of the mechanistic details of the homologous eukaryotic processes, which may provide the targets for therapeutic interventions. This chapter focuses on the current understanding of the carbon metabolism in *S. cerevisiae* from the systems-level perspective in particular glucose and galactose, and highlights the need for an integrative analysis approach for elucidating the underlying molecular mechanisms.

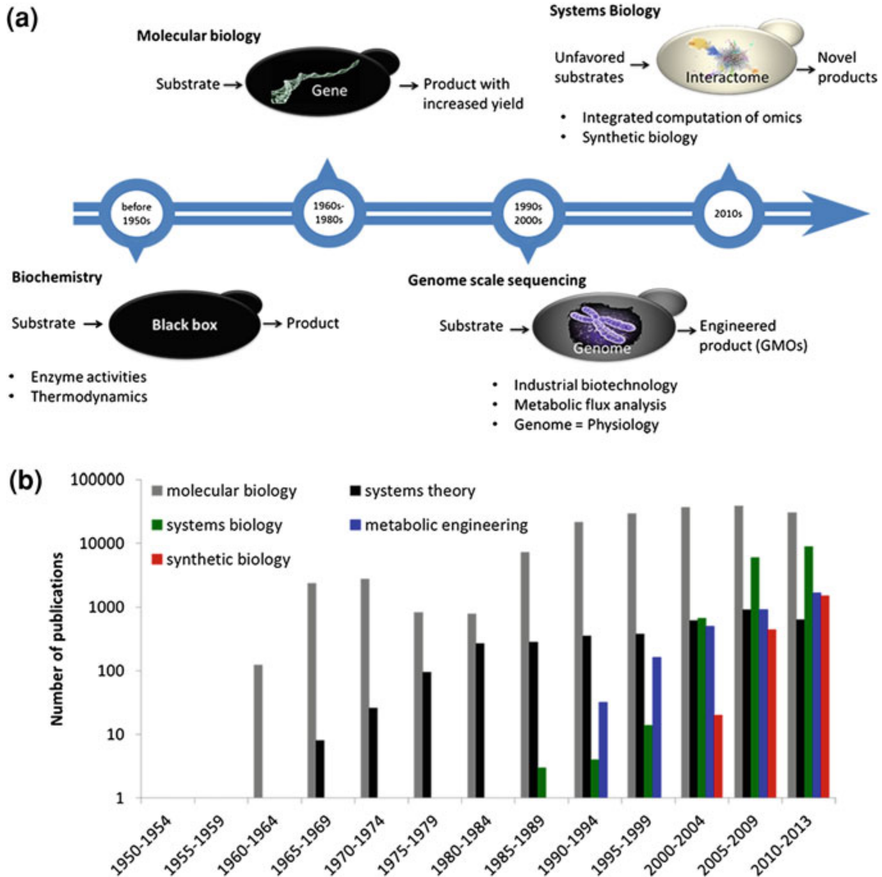

Fig. 4.1 Toward holistic understanding of biological systems: **a** An overview of the scientific progress from the "*black box*" model to the mechanistic details at molecular level that may help to explain phenotypes. Beginning from the determination of the DNA structure, key innovations (see Box 4.1) facilitate the development of new scientific disciplines such as molecular biology, metabolic engineering, systems biology, and synthetic biology. These disciplines allow to understand the dynamic interactions of the genetic material with the physical and chemical environment which potentially determines the unique phenotype of each organism and this understanding can be used for biotechnological or pharmaceutical applications. **b** Timeline of the developments of new scientific fields highlights the inherent interdisciplinary nature of the scientific progress. Chart is based on time-dependent PubMed search of key words as of July 25, 2013

4.1 Yeast Carbon Metabolism: Progress

Evolution has increased the complexity in biological systems as simple life forms have evolved into more advanced organisms. However, the common guiding principles of substrate consumption, the energy production, and biomass formation in the central carbon metabolism are highly conserved. The central carbon

metabolism provides all precursor metabolites required for biosynthesis of macromolecules such as proteins, DNA, RNA, lipids, and carbohydrates as well as it provides the Gibbs free energy and redox power required for cell growth. Despite the high degree of conservation in these pathways, their regulation varies widely among different organisms. Central carbon metabolism consists of sequential enzymatic reactions arranged to derive energy from the carbon sources such as sugars, and has possibly evolved based on the optimality principle where metabolism represents thermodynamically the most favorable walk between the carbon sources and precursor metabolites (Noor et al. 2010; Fell 2010; Hatzimanikatis et al. 2005). The energetic efficiency of the central carbon metabolism is likely to be one of the reasons for its conservation, which allows the breakdown of carbohydrate monomers to be sensed, transported, and metabolized through various pathways. Although, individual pathways or systems are often investigated with exhausting details, obtaining a holistic view of metabolism and understanding global regulatory principles are still in infancy. Mechanistic approaches to understand the metabolism as a series of reactions precede the current approach of systems-level analysis where metabolism consists of complex and functional biological networks (Mesarovic 1968; Wolkenhauer 2001). Sugars are the favored carbon sources for *S. cerevisiae* where the metabolism has preferentially evolved for glucose consumption leading to the repressed utilization of other carbon sources in its presence (Carlson 1999). In glucose rich environment, energy for the production of precursor metabolites becomes available via substrate level phosphorylation in glycolysis where ethanol is one of the main products. In the presence of oxygen, *S. cerevisiae* consumes ethanol after the depletion of glucose in the environment; and this phenomenon is known as the diauxic shift which is essentially a shift from fermentative to respiratory metabolism. However, in glucose-limited aerobic continuous cultures (generally referred to as chemostat cultures), it is possible to keep the glucose concentration sufficiently low to prevent glucose repression and hereby enable respiro-fermentative metabolism of glucose. The first microarray experiment in *S. cerevisiae* studied the diauxic shift to obtain temporal changes in the gene expression as metabolism switched from fermentation to respiration (DeRisi 1997). This was followed by the investigation of the transcriptional switch in response to the reduction or loss of the respiratory function (Liu and Butow 1999).

S. cerevisiae has evolved to have glucose and fructose as its preferred carbon sources, but it can consume various other sugars such as sucrose, mannose, and galactose. Availability of the genome sequence and microarray provides the opportunity to explore the question of adaptation in a new environment by cultivating it under selective pressure and analyzing the transcriptional and genome-wide changes that may occur as subsequent generations get accustomed to the new environment through the process of adaptive evolution. Such studies have led to the identification that *S. cerevisiae* responds to environmental shifts including exposure to less-preferred carbon sources with a remarkable variety of responses, including transcriptional regulation of specific mRNAs (Ferea et al. 1999; Gasch et al. 2000; Kuhn et al. 2001; Hong et al. 2011). Integrative analysis of the genome

sequence, the metabolic network, and the transcriptional response has revealed the underlying transcriptional regulatory networks which map the regulator-gene interactions among the potential pathways that *S. cerevisiae* can use to regulate the global gene expression much in the same fashion as maps of metabolic networks describe the potential pathways that may be used by a cell to accomplish metabolic processes (Lee et al. 2002; Ihmels et al. 2004). The yeast *S. cerevisiae* senses glucose through multiple signal transduction pathways. Two of these pathways are connected in a regulatory network that serves to integrate the different glucose signals operating in these pathways. First, the Snf1 kinase dependent Mig1 pathway enforced glucose repression and, second, the Rgt1 pathway that involves induction of the hexose transporter genes, *HXT*, by cell surface sensors affecting the Rgt1 transcription factor (Kaniak et al. 2004). Flux analysis indicates that the respiratory metabolism is dependent on the tricarboxylic acid cycle (TCA) activity which in *S. cerevisiae* is a function of the environmentally determined specific growth rate and glucose uptake rates (Blank and Sauer 2004). Flux analysis combined with transcriptome analysis of aerobically grown glucose-limited steady state chemostat cultures indicates that the transcripts involved in the glyoxylate cycle and gluconeogenesis showed a good correlation with in vivo fluxes, while no such correlation exists for other important pathways such as pentose-phosphate pathway, TCA cycle, and, specially, glycolysis. In this cultivation condition, fluxes are controlled to a large extent via posttranscriptional mechanisms which highlight the limitations of solely using transcriptome analysis in order to identify global regulation of the central carbon metabolism (Daran-Lapujade et al. 2004; Feder and Walser 2005).

Despite this limitation much has, however, been learned from transcriptome analysis. In particular, the homeostatic adjustment and metabolic remodeling that occurs in glucose-limited chemostat cultures despite the theoretical possibility of a switch to fully aerobic metabolism of glucose; homeostatic mechanisms enforce metabolic adjustment as if fermentation of the glucose is the preferred option until the glucose is entirely consumed (Brauer et al. 2005). Application of genome scale models and metabolism driven treatment of the transcriptome data have assisted systems-level analysis and revealed a close interaction and crosstalk between the two pathways responsible for glucose repression (Westergaard et al. 2007; Förster et al. 2003; Patil and Nielsen 2005). These studies also highlight the importance of not only transcriptome analysis, but the need for quantitative information about the proteome and metabolome to understand the carbon metabolism in *S. cerevisiae* (Kolkman et al. 2006; Kresnowati et al. 2006). Large-scale multi-layered data necessitate reconstruction of genome scale models and the integrated analysis of regulatory and metabolic networks to reveal novel regulatory mechanism and further improvements to the model through experimental validation (Herrgård et al. 2006; Hu et al. 2007). Adopting this integrated approach reconstruction of the yeast Snf1 kinase regulatory network revealed its role as a global energy regulator in yeast (Usaite et al. 2009). In another approach conditional mutation in combination with transcriptome analysis revealed that glucose regulates transcription in yeast through a network of signaling pathways and growth is decided by both sensing and

import of glucose (Zaman et al. 2009; Youk and van Oudenaarden 2009). Systematic quantification of the metabolic fluxes in 119 transcription factor deletion mutants in *S. cerevisiae* revealed that while most knockout deletions did not affect fluxes, a total of 23 transcription factors mediate 42 condition-dependent interactions that control almost exclusively the cellular decision between respiration and fermentation. This approach clearly demonstrates the importance of identifying and quantifying the role of regulatory effectors in altering cellular functions, while also emphasizing that the flux distribution in the central carbon metabolism is tightly controlled and therefore difficult to perturb. This is explained by the fact that perturbations in individual enzyme capacity leading to alteration of one network constituent can be efficiently buffered by converse alteration by other network constituents, a system that has evolved to ensure metabolic homeostasis at varying environmental conditions and in response to mutations appearing in central carbon metabolism enzymes (Fendt et al. 2010a, b). Recent advances in proteomics have revealed that the yeast central carbon metabolism is to a large extent regulated by enzyme phosphorylation (Oliveira et al. 2012), but the full quantitative effect of this type of regulation has still not been studied.

In conclusion, systems-level analysis facilitates the progress on understanding such fundamental aspects as diauxic shift by revealing that multiple events are temporally organized to affect transition from fermentation to respiration and changes in metabolism in response to changes in glucose concentration (Zampar et al. 2013; Geistlinger et al. 2013). In the following two sections, we will focus on specific aspects of glucose and galactose metabolism where combined top-down and bottom-up experimental systems biology approaches provide insights for better understanding of the regulatory mechanisms (Fig. 4.2).

4.2 Molecular Mechanisms in Glucose Metabolism

Ethanol and carbon dioxide are the two main products of the yeast metabolism when glucose is in excess. Production of these compounds is also the main reason why yeast is extensively used in the alcohol and food industry. However, there is an increasing interest to use yeast as a cell factory for the production of various biochemicals, recombinant proteins, biofuels, etc. In those cases ethanol and carbon dioxide represent an important carbon loss which drives carbon away from the desired product. Hence, understanding the molecular mechanism of the formation of these products is essential for the successful redistribution of the fluxes toward the desired pathways and products.

Pyruvate is the branch point intermediate between respiratory dissimilation of sugars and alcoholic fermentation (Pronk et al. 1996). Isolation and characterization of the pyruvate decarboxylase (*PDC*) show the critical role this enzyme plays in the decarboxylation of pyruvate to acetaldehyde and in supplying the cytosolic acetyl-CoA pool (Schmitt and Zimmermann 1982; Hohmann and Cederberg 1990; Pronk et al. 1996). A complete knockout strain without *PDC* genes reveals the

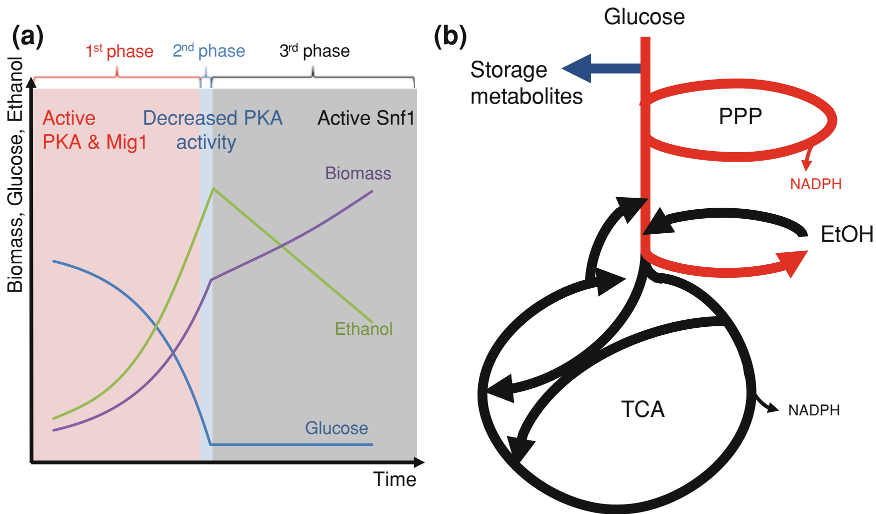

Fig. 4.2 **a** Typical time profile of the diauxic shift. In the 1st phase, there is consumption of glucose with co-current formation of ethanol and biomass. In the 2nd phase, there is transition, and in the 3rd phase, there is consumption of ethanol and further growth of the biomass. Activity of key protein kinases is indicated in the different phases. **b** Overview of carbon flows in the 1st and 3rd phases of the diauxic shift. In the 1st phase, there is ethanol production with very little TCA cycle activity. In the 3rd phase, there is ethanol uptake and respiration with an active TCA cycle

indispensable role of this enzyme for growth of *S. cerevisiae* on glucose and indicates that the mitochondrial pyruvate dehydrogenase (PDH) complex cannot function as the sole source of acetyl-CoA during the growth on glucose (Flikweert et al. 1996). Two different approaches result in the partial growth recovery of *PDC* negative *S. cerevisiae* strains on glucose as the only carbon source. First, the overexpression of *GLY1* gene which encodes threonine aldolase and catalyzes the cleavage of threonine to glycine and acetaldehyde that can be converted to acetyl-CoA. Second, the *PDC* negative strain subjected to directed evolution in the batch and, independently, in glucose-limited continuous cultures where acetate concentration in in-flow feed was gradually reduced (van Maris et al. 2003, 2004). Molecular mechanisms of underlying the glucose-tolerant phenotype remain elusive in these studies; transcriptome analysis shows an increase in glucose-repressible genes relative to the isogenic wild type in nitrogen-limited chemostat cultures with excess glucose (van Maris et al. 2004). Understanding glucose signaling mechanisms appears to be critical for elucidating molecular mechanisms that result in glucose sensitivity of *PDC* negative strain of *S. cerevisiae*. Genetic analysis identifies that glucose signaling is mediated, partially, through the interactions of Std1, Mth1, Snf3, and Rgt2 (Schmidt et al. 1999). Glucose reacts via the F-box protein Grr1 to promote the degradation of Mth1 which leads to phosphorylation and dissociation of Rgt1 from the *HXT* promoters, thereby activating *HXT* gene expression (Flick et al. 2003; Moriya and Johnston 2004). Genome scale analysis of

adaptively evolved *PDC* negative strain identifies a 225 bp in-frame internal deletion in *MTH1*. This internal deletion results in the loss of a phosphorylation site and, hypothetically, increases protein stability (Oud et al. 2012a). Reverse engineering of this phenotype into the nonevolved *PDC* negative strain allows, albeit slow, growth on glucose as sole carbon source. Stable Mth1 in *PDC* negative strain reduces glucose uptake that likely prevents intracellular accumulation of pyruvate and/or redox problems, while releasing the glucose repression (Oud et al. 2012). Although we are still far from recovering the wild-type growth profile for the *PDC* negative phenotype in *S. cerevisiae*, the combination of systems biology tools such as directed evolution, genome scale analysis, and reverse engineering suggest a plausible mechanism and solution to the glucose sensitivity of this strain that may allow it to grow on glucose (Fig. 4.3).

4.3 Molecular Mechanisms in Galactose Metabolism

One of the rationales to understand the underlying molecular mechanisms is the potential opportunity to perturb the metabolism for various applications. These perturbations should be able to redirect the metabolic flux toward the desired pathway, however, due to rigid control of the fluxes through inherently complex molecular mechanisms, it is a difficult goal (Ostergaard et al. 2000). Overexpression of seven glycolytic enzymes in *S. cerevisiae* show that transcriptional perturbations do not necessarily result in the flux change in the central carbon metabolism, partially due to such factors as saturating levels of enzyme concentrations and post-translational modifications (Hauf et al. 2000). A similar conclusion was attained for the Leloir pathway that is responsible for metabolism of galactose. Overexpression of either the individual enzymes or combination of these did not result in improved galactose uptake (de Jongh et al. 2008). On the contrary the galactose utilization was reduced, and this was shown to be due to accumulation of pathway intermediates (de Jongh et al. 2008). However, by perturbing the *GAL* gene regulatory network through the elimination of three known regulators of the *GAL* system, *GAL6*, *GAL80*, and *MIG1*, it was possible to obtain a 41 % increase in flux through the galactose utilization pathway compared with the wild type strain. Improved galactose consumption of the Gal mutants increased the respiro-fermentative metabolism where ethanol production rate linearly correlates with glycolytic flux (Ostergaard et al. 2000). Transcriptome analysis further shows the role of phosphoglucomutase (*PGM2*), and it is shown that overexpression of *PGM2* results in an increased galactose uptake rate by 70 % compared to the one of the reference strain. This strongly suggests that *PGM2* plays a key role in controlling the flux through the Leloir pathways, probably due to increased conversion of glucuose-1-phosphate to glucose-6-phosphate (Bro et al. 2005). However, the molecular mechanism of this very significant enhancement in the glycolytic flux through the galactose metabolism indicates that increased

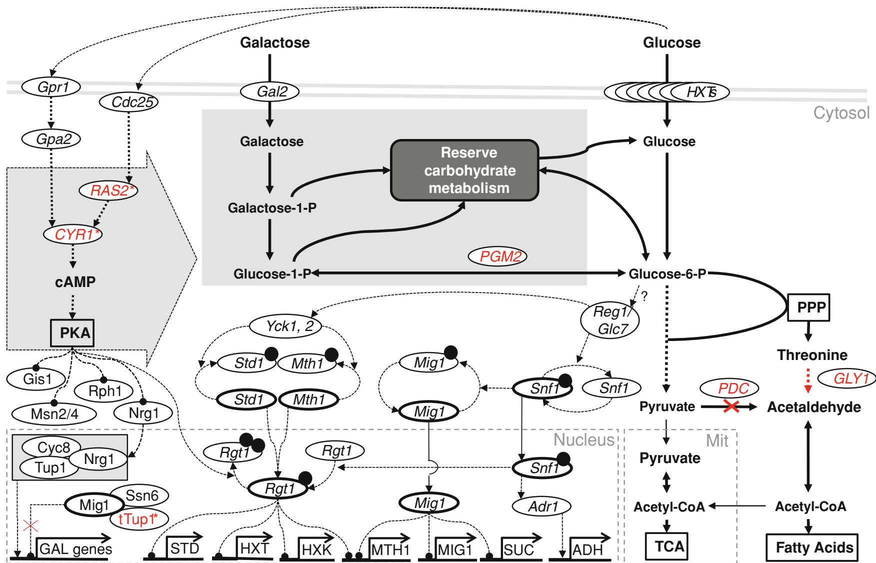

Fig. 4.3 Glucose and galactose metabolism in *S. cerevisiae*: Glucose causes carbon catabolite repression, where other carbon sources cannot be consumed in the presence of glucose. Systems-level analysis has suggested mechanisms for some of the key metabolic regulation related problems in *S. cerevisiae* such as partial growth of a *pdcΔ* strain on glucose and improved galactose uptake mechanism. A *PDC* knockout strain of *S. cerevisiae* is unable to grow solely on hexoses due to potential pyruvate toxicity and lack of cytosolic acetyl-CoA that is required for fatty acid synthesis. First, overexpression of threonine aldolase Gly1, which catalyzes threonine cleavage to glycine and acetaldehyde, was identified to partially restore the growth of *pdcΔ* on glucose. Second, systems-level analysis of adaptively evolved *pdcΔ* strain showed an internal mutation in *MTH1* that results in the reduced glucose uptake rate in *pdcΔ* strain and thereby potentially reducing the pyruvate toxicity and allowing it to grow on glucose albeit slowly as compared to the wild type *S. cerevisiae.* Galactose metabolism overlaps with glucose metabolism except for the upper part of glycolysis. Galactose uptake is inhibited in the presence of glucose and remains lower as compared to glucose even if there is no glucose available, potentially through the canonical regulation of sugar consumption pathways via the Ras/PKA signaling mechanism. Mutations in *RAS2* or *CYR1* result in decreased Ras/PKA pathway activity and corresponding increase in galactose uptake. The overexpression of *PGM2* or expression of truncated *Tup1* (tTup1*) also increases galactose uptake as truncated Tup1 cannot inhibit the expression of galactose pathway genes. Dashed lines with arrows represent activation; dashed lines with circles inhibition; bold lines metabolic conversion; dotted bold line several combined reactions; thin lines relocation; bold circles active form of a gene; black filled circles phosphorylation; pathways are pictured in bold boxes; Mit—mitochondrion

phosphoglucomutase (*PGM1*) activity alleviates the galactose growth defect associated with elevated levels of Ras signaling in *S. cerevisiae* (Howard et al. 2006). Investigation of the Ras-pathway indicates its dual role on galactose metabolism through indirect interaction with a nucleotide exchange factor Cdc25p and intracellular energy status. This interaction is an important factor for the metabolic adaptation upon change in its environment such as a switch between

glucose-galactose or galactose-glucose (Mirisola et al. 2007; van den Brink et al. 2009). Integrated systems-level analysis further clarifies role of the Ras signaling pathway in galactose metabolism with the identification of point mutations in *RAS2* in adaptively evolved strains with increased galactose uptake rate and validation of these mutations in the wild type strain (Hong et al. 2011). A genome-wide perturbation approach led to the identification of *TUP1*, a small nuclear RNA, as a regulatory target for the improved galactose fermentation and inverse metabolic engineering of truncated *TUP1* results in 250 % higher galactose consumption rate and ethanol productivity compared to the control strain (Lee et al. 2011). In conclusion, molecular mechanisms underlying galactose metabolism show the significance and importance of the systems biology approach where basic understanding of regulation of the central carbon metabolism can lead to biotechnological breakthroughs.

4.4 Perspective

Systems biology progress is the result of conceptual leaps based on several technological developments in the past decades. In the last decade, we have moved from genome-centered viewpoint to a systems-level thinking where metabolic control of subjected perturbations spreads across multiple regulatory layers. Next generation of technological breakthrough in genomics, transcriptomics, proteomics, metabolomics, single cell analysis, and computing should facilitate the development of new paradigms that can help to advance our understanding of the molecular mechanisms for designing microbial cell factories as well as therapeutics interventions for personalized medicine.

These kinds of developments necessitate the multidisciplinary studies where dynamic data can be analyzed and modeled using static or dynamic modeling tools. Dynamic data allow identification and monitoring of metabolic switch points in detail and give a comprehensive overview of metabolic response to perturbations. To get the systems-level understanding, used metabolic models should be able to integrate various data including extracellular fluxes, transcriptional regulation, energetic constraints, and posttranslational modifications. Here, absolute quantitative data represent an invaluable source that can be used as an input for metabolic models. Static, constraint-based models can be used to describe dynamic data and analyze the interactions. However, these models lack the predictive possibilities present in dynamic models. However, dynamic models are used for describing smaller subsystems as dynamic information about, e.g., enzyme activities for the whole genome scale network is currently missing. Recently, steps toward this direction have been made for the minimal microorganisms and it could be expected that similar models will be constructed for higher organisms where compartmentalization and lack of information about transportation and regulation pose additional obstacles.

High-throughput data generation provides holistic understanding of the biological complexity which can be used for such nontrivial tasks as strain improvement but challenges remain in mapping networks and perturbing those in space and time (Stephanopoulos et al. 2004; Lehner et al. 2005). Some of the approaches are already resulting in the interaction mapping of such regulators as Snf1 and TORC1, which control glucose and nitrogen assimilation in *S. cerevisiae* and developments in the proteomics may provide posttranslational and epigenetic regulatory information than is currently available (Zhang et al. 2011; Oliveira et al. 2012). And for one of the simplest microbes, *Mycoplasma genitalium*, computational model is able to predict phenotype from genotype which is a significant progress from 1960s when first systems biology model showed cardiac action and pacemaker potentials based on the Hodgkin-Huxley equations. Such methods combined with information on membrane transport and cellular compartmentalization are useful for revealing novel molecular mechanism based on network properties in eukaryotes (Nobel 1960; Karr et al. 2012; Esvelt and Wang 2013; Agren et al. 2013). Molecular mechanisms can also be tested in vitro and synthetic organelles and cells may provide the future insights into the question of how biology works (Jewett et al. 2013).

Acknowledgments We acknowledge financial support from UNICELLSYS and European Research Council.

References

Agren R et al (2013) The RAVEN toolbox and its use for generating a genome-scale metabolic model for Penicillium chrysogenum. PLoS Comput Biol 9(3):e1002980

Bertalanffy LV (1950) An outline of general systems theory. Br J Philos Sci 1(2):134–165

Blank LM, Sauer U (2004) TCA cycle activity in *Saccharomyces cerevisiae* is a function of the environmentally determined specific growth and glucose uptake rates. Microbiology 150(4):1085–1093

Brauer MJ et al (2005) Homeostatic adjustment and metabolic remodeling in glucose-limited yeast cultures. Mol Biol Cell 16:2503–2517

Van den Brink J et al (2009) Energetic limits to metabolic flexibility: responses of *Saccharomyces cerevisiae* to glucose-galactose transitions. Microbiol (Reading, Engl), 155 (Pt 4):1340–1350

Bro C et al (2005) Improvement of galactose uptake in *Saccharomyces cerevisiae* through overexpression of phosphoglucomutase: example of transcript analysis as a tool in inverse metabolic engineering. Appl Environ Microbiol 71(11):6465–6472

Carlson M (1999) Glucose repression in yeast. Curr Opin Microbiol 2:202–207

Csete ME, Doyle JC (2002) Reverse engineering of biological complexity. Sci (NY) 295(5560):1664–1669

Daran-Lapujade P et al (2004) Role of transcriptional regulation in controlling fluxes in central carbon metabolism of *Saccharomyces cerevisiae*. A chemostat culture study. J Biol Chem 279(10):9125–9138

DeRisi JL (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278(5338):680–686

Esvelt KM, Wang HH (2013) Genome-scale engineering for systems and synthetic biology. Mol Syst Biol 9(641):1–17

Famili I et al (2003) Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res 13:244–253

Feder ME, Walser J-C (2005) The biological limitations of transcriptomics in elucidating stress and stress responses. J Evol Biol 18(4):901–910

Fell DA (2010). Evolution of central carbon metabolism. Mol Cell 39(5):663–664

Fendt S-M, Buescher JM et al (2010a) Tradeoff between enzyme and metabolite efficiency maintains metabolic homeostasis upon perturbations in enzyme capacity. Mol Syst Biol 6(356):356

Fendt S-M, Oliveira AP et al (2010b) Unraveling condition-dependent networks of transcription factors that control metabolic pathway activity in yeast. Mol Syst Biol 6(432):432

Ferea TL et al (1999) Systematic changes in gene expression patterns following adaptive evolution in yeast. Proc Natl Acad Sci USA 96:9721–9726

Flick KM et al (2003) Grr1-dependent Inactivation of Mth1 mediates Glucose-induced dissociation of Rgt1 from HXT gene promoters. Mol Biol Cell 14:3230–3241

Flikweert MT et al (1996) Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. Yeast 12:247–257

Förster J et al (2003) Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res 13(2):244–253

Gasch AP et al (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11(12):4241–4257

Geistlinger L, Csaba G, Dirmeier S, Küffner R, Zimmer R (2013) A comprehensive gene regulatory network for the diauxic shift in Saccharomyces cerevisiae. Nucleic Acid Res 41(18):8452–8463

Goffeau A et al (1996) Life with 6000 genes. Science 274(5287):546–567

Hatzimanikatis V et al (2005) Exploring the diversity of complex metabolic networks. Bioinform (Oxf, Engl) 21(8):1603–1609

Hauf J, Zimmermann FK, Müller S (2000) Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*. Enzym Microb Technol 26:688–698

Herrgård MJ et al (2008) A consensus yeast metabolic network reconstruction obtained from a community approach to systems biology. Nat Biotechnol 26(10):1155–1160

Herrgård MJ et al (2006) Integrated analysis of regulatory and metabolic networks reveals novel regulatory mechanisms in *Saccharomyces cerevisiae*. Genome Res 16(5):627–635

Hohmann S, Cederberg H (1990) Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes PDC1 and PDC5. Eur J Biochem 188:615–621

Hong K-K et al (2011) Unravelling evolutionary strategies of yeast for improving galactose utilization through integrated systems level analysis. Proc Natl Acad Sci USA 108(29): 12179–12184

Howard SC, Deminoff SJ, Herman PK (2006) Increased phosphoglucomutase activity suppresses the galactose growth defect associated with elevated levels of Ras signaling in *S. cerevisiae*. Curr Genet 49(1):1–6

Hu Z, Killion PJ, Iyer VR (2007) Genetic reconstruction of a functional transcriptional regulatory network. Nat Genet 39(5):683–687

Ihmels J, Levy R, Barkai N (2004) Principles of transcriptional control in the metabolic network of *Saccharomyces cerevisiae*. Nat Biotechnol 22(1):86–92

Jewett MC et al (2013) In vitro integration of ribosomal RNA synthesis, ribosome assembly, and translation. Mol Syst Biol 9(678):1–8

De Jongh WA et al (2008) The roles of galactitol, galactose-1-phosphate, and phosphoglucomutase in galactose-induced toxicity in *Saccharomyces cerevisiae*. Biotechnol Bioeng 101(2):317–326

Kaniak A et al (2004) Regulatory network connecting two glucose signal transduction pathways in *Saccharomyces cerevisiae*. Eukaryot Cell 3(1):221–231

Karr JR et al (2012) A whole-cell computational model predicts phenotype from genotype. Cell 150(2):389–401

Kell DB et al (2005) Metabolic footprinting and systems biology: the medium is the message. Nat Rev Microbiol 3(7):557–565

Kitano H (2002) Computational systems biology. Nature 420:206–210

Kolkman A et al (2006) Proteome analysis of yeast response to various nutrient limitations. Mol Syst Biol 2:2006.0026

Kresnowati MTAP et al (2006) When transcriptome meets metabolome: fast cellular responses of yeast to sudden relief of glucose limitation. Mol Syst Biol 2:49

Kuhn KM et al (2001) Global and specific translational regulation in the genomic response of *Saccharomyces cerevisiae* to a rapid transfer from a fermentable to a nonfermentable carbon source. Mol Cell Biol 21(3):916–927

Lashkari DA et al (1997) Yeast microarrays for genome wide parallel genetic and gene expression analysis. Proc Natl Acad Sci USA 94:13057–13062

Lee K-S et al (2011) Improved galactose fermentation of *Saccharomyces cerevisiae* through inverse metabolic engineering. Biotechnol Bioeng 108(3):621–631

Lee TI et al (2002) Transcriptional regulatory networks in *Saccharomyces cerevisiae*. Science 298(October):799–804

Lehner B, Tischler J, Fraser AG (2005) Systems biology: where it is at in 2005. Genome Biol 6(8):338

Liu Z, Butow RA (1999) A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. Mol Cell Biol 19(10):6720–6728

Maris AJA Van et al (2004) Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. Appl Environ Microbiol 70(1):159–166

Maris AJA Van et al (2003) Overproduction of threonine aldolase circumvents the biosynthetic role of pyruvate decarboxylase in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 69(4):2094–2099

Mesarovic MD (1968) Systems theory and biology- view of a theoretician. In: Mesarovi MD (ed) Systems theory and biology. Springer, New York, pp 59–87

Mirisola MG, Gallo A, De Leo G (2007) Ras-pathway has a dual role in yeast galactose metabolism. FEBS Lett 581(10):2009–2016

Moriya H, Johnston M (2004) Glucose sensing and signaling in *Saccharomyces cerevisiae* through the Rgt2 glucose sensor and casein kinase I. Proc Natl Acad Sci USA 101(6): 1572–1577

Nielsen J, Olsson L (2002) An expanded role for microbial physiology in metabolic engineering and functional genomics: moving towards systems biology 1. FEMS Yeast Res 2:175–181

Nobel D (1960) Cardiac action and pacemaker potentials based on Hodgkin-huxley equations. Nature 5(188):495–497

Noor E et al (2010) Central carbon metabolism as a minimal biochemical walk between precursors for biomass and energy. Mol Cell 39(5):809–820

Oliveira AP et al (2012) Regulation of yeast central metabolism by enzyme phosphorylation. Mol Syst Biol 8(623):623

Ostergaard S et al (2000) Increasing galactose consumption by *Saccharomyces cerevisiae* through metabolic engineering of the GAL gene regulatory network. Nat Biotechnol 18(12): 1283–1286

Osterlund T et al (2013) Mapping condition-dependent regulation of metabolism in yeast through genome-scale modeling. BMC Syst Biol 7(1):36

Oud B, Flores C-L et al (2012) An internal deletion in MTH1 enables growth on glucose of pyruvate-decarboxylase negative, non-fermentative *Saccharomyces cerevisiae*. Microb Cell Fact 11:131

Oud B, van Maris AJ A et al (2012a) Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast. FEMS Yeast Res 12(2):183–196

Patil KR, Nielsen J (2005) Uncovering transcriptional regulation of metabolism by using metabolic network topology. Proc Natl Acad Sci USA 102(8):2685–2689

Picotti P et al (2013) A complete mass-spectrometric map of the yeast proteome applied to quantitative trait analysis. Nature 494(7436):266–270

Pronk JT, Steensmays HY, van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12:1607–1633

Sauer U (2006) Metabolic networks in motion: 13C-based flux analysis. Mol Syst Biol 2:62

Schmidt MC et al (1999) Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in *Saccharomyces cerevisiae*. Mol Cell Biol 19(7):4561–4571

Schmitt HD, Zimmermann FK (1982) Genetic analysis of the pyruvate decarboxylase reaction in yeast glycolysis. J Bacteriol 151(3):1146–1152

Stephanopoulos G, Alper H, Moxley J (2004) Exploiting biological complexity for strain improvement through systems biology. Nat Biotechnol 22(10):1261–1267

Usaite R et al (2009) Reconstruction of the yeast Snf1 kinase regulatory network reveals its role as a global energy regulator. Mol Syst Biol 5(319):319

Weng G (1999) Complexity in biological signaling systems. Science 284(5411):92–96

Westergaard SL et al (2007) A systems biology approach to study glucose repression in the yeast *Saccharomyces cerevisiae*. Biotechnol Bioeng 96(1):134–145

Wolkenhauer O (2001) Systems biology: the reincarnation of systems theory applied in biology? Briefings in Bioinf 2(3):258–270

Youk H, van Oudenaarden A (2009) Growth landscape formed by perception and import of glucose in yeast. Nature 462(7275):875–879

Zaman S et al (2009) Glucose regulates transcription in yeast through a network of signaling pathways. Mol Syst Biol 5(245):245

Zampar GG et al (2013) Temporal system-level organization of the switch from glycolytic to gluconeogenic operation in yeast. Mol Syst Biol 9(651):651

Zhang J et al (2011) Mapping the interaction of Snf1 with TORC1 in *Saccharomyces cerevisiae*. Mol Syst Biol 7(545):545

Chapter 5
Comparative Genomics and Evolutionary Genetics of Yeast Carbon Metabolism

Zhenguo Lin and Wen-Hsiung Li

Abstract Yeasts display highly diversified physiological characteristics. The most distinct physiological character in many yeast species is their special sugar metabolism. In particular, the baker's yeast *Sacharomyces cerevisiae* and its relatives predominantly ferment sugars into ethanol even in the presence of oxygen, which is known as Crabtree effect or aerobic fermentation. It has been postulated that this unusual carbohydrate metabolism provides these yeasts selective advantages in sugar-rich environments. However, it has long been a mystery as to genetically how these yeasts evolved a predominantly fermentative lifestyle. The rapid accumulation of genomic, transcriptomic, and epigenetic data in many yeast species in recent years has greatly increased our understanding of the genetic basis and molecular mechanism for the diversified sugar metabolisms among yeasts. In this chapter, we provide a review of recent comparative genomics and evolutionary studies related to the metabolisms of glucose and galactose, whose metabolic pathways have been extensively studied in yeasts. A series of studies suggested that the evolution of aerobic fermentation involved many different factors, including increases in copy numbers of genes involved in glucose transport, glycolysis and ethanol production; sequence divergence; and transcriptional reprogramming of genes involved in mitochondrial functions through changes of *cis*-regulatory elements and promoter structures. It has also been found that the different abilities among yeasts to use galactose is strongly correlated with the presence of the galactose pathway genes in their genomes. These studies revealed that the adaptation of yeasts to specific niches has greatly shaped the genomic content and the regulatory program.

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_5, © Springer-Verlag Berlin Heidelberg 2014

5.1 Introduction

Although yeasts are single cell organisms, they have evolved highly diversified physiological characters, especially in carbohydrate metabolism (Barnett et al. 2000). The monosaccharide glucose is the basic carbohydrate unit of cellular metabolism and is the most important carbohydrate source of cellular energy. In the presence of oxygen, most eukaryotic species fully degrade glucose into CO_2 and H_2O through the respiration pathway for the maximum energy yield. However, many yeast species, including *Saccharomyces cerevisiae* and its close relatives, have evolved a remarkable ability to predominantly ferment sugars even under aerobic conditions to produce ethanol. This characteristic is called aerobic fermentation or Crabtree effect (De Deken 1966). These fermentative yeasts are able to tolerate a high concentration of extracellular ethanol and to utilize ethanol as the carbon source after depletion of sugars. This fermentative lifestyle was termed the "make–accumulate–consume" strategy, which in natural habitats enables *Saccharomyces* yeasts to outcompete other microorganisms (Piskur et al. 2006). How *S. cerevisiae* evolved to be a good fermenter has become a subject of intense research in the last decade.

Thanks to the availability of genomic sequences in many yeast species and the abundant high-throughput transcriptomic and epigenetic data, recent studies have greatly advanced our knowledge about the yeast carbon metabolism. As one of the most intensely studied eukaryotic model organisms, *S. cerevisiae* is the first eukaryotic species to have a completely sequenced genome (Goffeau et al. 1996). The second completely sequenced yeast genome came 6 years later from the fission yeast *Schizosaccharomyces pombe*, which diverged from *S. cerevisiae* probably more than 300 million years ago (Wood et al. 2002). With advances in sequencing technology, the number of completely sequenced genomes has been increasing rapidly. To date, the complete genomes of at least 50 different yeast species have been registered at Genbank. In addition, the genomes of 33 different wild and domestic strains of *S. cerevisiae* have also been sequenced (Engel and Cherry 2013). Compared to other eukaryotes, yeasts have streamlined genomes ranging from 9 to 20 megabases in haploid, containing 4,700–6,500 protein-coding genes (Dujon 2010). In addition to the genomic data, the first eukaryotic genome-wide gene expression data was completed in *S. cerevisiae* by microarray technology in 1997 (DeRisi et al. 1997). Since then, large amounts of transcriptomic data have been generated in many yeast species by micro-array, tiling-array, and next-generation sequencing technologies (Ferea et al. 1999; Ihmels et al. 2002; Yuan et al. 2005; Miura et al. 2006; Field et al. 2008; Tsankov et al. 2010). Furthermore, genome-wide epigenetic data and protein-protein interaction data have also been accumulated in several yeast species (Ferea et al. 1999; Ihmels et al. 2002; Yuan et al. 2005; Miura et al. 2006; Field et al. 2008, Tsankov et al. 2010). These various types of data and the availability of powerful bioinformatics tools for data analyses paved the way for comparative genomic and transcriptomic studies and for elucidating the genetic basis underlying the evolution of phenotypic

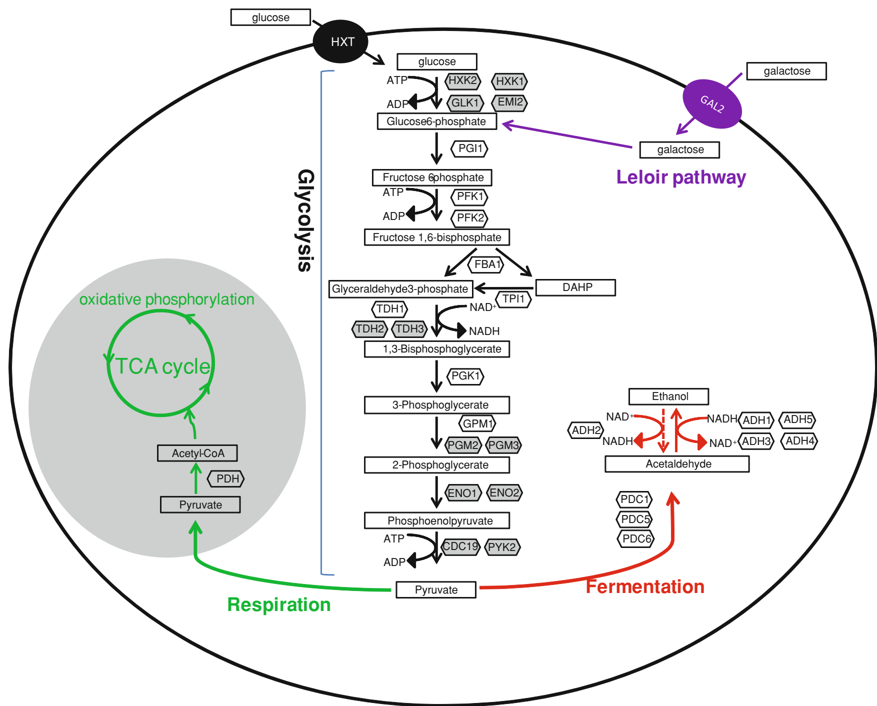

Fig. 5.1 Schematic illustration of major carbohydrate metabolic pathways in *S. cerevisiae*: glycolysis, alcohol fermentation, the TCA cycle, and the galactose Leloir pathway. Enzymes catalyzing each reaction are denoted by gene names in hexagons. Hexagons shaded in *gray* indicate paralogous enzymes derived from the whole genome duplication

traits. Many comparative genomics and evolutionary genetics studies relevant to yeast carbohydrate metabolism focused on the glucose and galactose pathways. This chapter will provide a brief overview of recent advances in our understanding of the genetic basis of yeast glucose and galactose metabolisms.

5.2 Copy Number Variation and Sequence Divergence of Genes Involved in Glucose Metabolism

5.2.1 Copy Number Variation of Hexose Transporter Genes

Glucose does not freely permeate cellular membranes, so the first step in glucose metabolism is to transport glucose across cellular membranes, which requires the aid of transporters (Fig. 5.1). In *S. cerevisiae*, glucose uptake is carried out by a large group of hexose transporters (Hxts) (Boles and Hollenberg 1997; Ozcan and

Johnston 1999). The hexose transporters belong to a superfamily of monosaccharide facilitators that are highly conserved in eukaryotes (Reifenberger et al. 1995). Twenty *HXT* genes have been identified in *S. cerevisiae*: *HXT1–HXT17* encode 17 glucose transporters, *GAL2* encodes a galactose transporter, and *SNF3* and *RGT2* encode two glucose sensors (Boles and Hollenberg 1997).

Phylogenetic analyses of the *HXT* genes from 23 completely sequenced fungal genomes revealed that the 20 *S. cerevisiae HXT* genes were separated into two groups prior to the divergence of major fungal groups (Lin and Li 2011b). According to functional characterizations of these *HXT* genes in *S. cerevisiae*, the two groups play distinct roles in glucose metabolism. One group comprises *S. cerevisiae SNF3* and *RGT2*, which encode sensors that recognize the concentration of extracellular glucose for the induction of *HXT* expression (Boles and Hollenberg 1997; Ozcan and Johnston 1999); this group is called the *Sensor* subfamily (Lin and Li 2011b). All of the yeast species examined, except for the fission yeast *Sch. pombe*, have 1–4 *Sensor* genes and most species contain two copies (Table 5.1), suggesting that the number of *Sensor* genes remain largely constant during the evolution of yeast species. *S. cerevisiae SNF3* and *RGT2* appear to be derived from the whole genome duplication (WGD) event that occurred about 100 million years ago (mya) (Wolfe and Shields 1997; Kellis et al. 2004; Lin and Li 2011b). The *Sensor* gene is absent from the genome of *Sch. pombe*, probably because *Sch. pombe* primarily detects glucose via a cAMP-signaling pathway (Hoffman 2005).

The remaining 18 *S. cerevisiae HXT* genes (*HXT1–17* and *GAL2*) form the other *HXT* group. Because the products of these genes are directly involved in transporting glucose or galactose across cellular membrane, this group was named the *Transporter* subfamily (Lin and Li 2011b). In contrast to the *Sensor* group, the number of *Transporter* genes varies substantially among yeast species (Table 5.1). Evolutionary analyses showed that the *Transporter* genes originated from a single gene in the common ancestor of hemiascomycete (Saccharomycotina) yeasts. The copy number of *Transporter* genes has continually increased during the evolution of the *S. cerevisiae* lineage, starting from a single copy in *Yarrowia lipolytica*, which is most distantly related to *S. cerevisiae* in hemiascomycetes, to 2–8 copies in *Kluyveromyces lactis* and *Lachancea kluyveri* (*Saccharomyces kluyveri*), and to 18–19 copies in the *Saccharomyces sensu stricto* species (including *S. cerevisiae*, *S. paradoxus* and *S. bayanus*) (Table 5.1). All post-WGD species examined contained at least 10 *Transporter* genes except for *Vanderwaltozyma polyspora* (*Kluyveromyces polysporus*), a species most distantly related to *S. cerevisiae* among the post-WGD yeasts (Kurtzman and Robnett 2003), which contains only 5 *Transporter* genes.

Some studies proposed that the WGD event was a major source for the expansion of *Transporter* genes. As the first study that proposed WGD in the ancestor of *S. cerevisiae*, Wolfe and Shields noticed that sugar transporters are among the few gene families that are enriched with WGD gene pairs (Wolfe and Shields 1997). After examining the hexose transporter genes in 7 hemiascomycete yeasts, Conant and Wolfe (2007) found that all of the post-WGD species have at least twice as

Table 5.1 Copy number variations of the *Sensor* and *Transporter* subfamily genes in the HXT gene family

Phylum/Class	Species	Sensors	Transporters
Hemiascomycetes	*Saccharomyces cerevisiae*[*]	2	18
	Saccharomyces paradoxus[*]	2	19
	Saccharomyces mikatae[*]	2	19
	Saccharomyces bayanus[*]	2	18
	Naumovozyma castellii[*]	2	16
	Candida glabrata[*]	2	10
	Vanderwaltozyma polyspora[†]	1	5
	Zygosaccharomyces rouxii[†]	1	4
	Lachancea thermotolerans[†]	4	5
	Lachancea kluyveri[†]	2	7
	Lachancea waltii[†]	2	8
	Kluyveromyces lactis	1	2
	Eremothecium gossypii	1	4
	Candida albicans	2	4
	Debaryomyces hansenii	2	3
	Scheffersomyces stipitis	2	4
	Yarrowia lipolytica	2	1
Dothideomycetes	*Phaeosphaeria nodorum*	1	3
Sordariomycetes	*Gibberella zeae*	1	3
	Magnaporthe grisea	2	1
Schizosaccharomycetes	*Schizosaccharomyces pombe*[*]	0	8
Eurotiomycetes	*Aspergillus fumigatus*	3	3
Basidiomycota	*Ustilago maydis*	2	1

Species name underlined are post-WGD yeasts. The data of gene copy number were retrieved from (Lin and Li 2011b)

[*] Crabtree-positive species

[†] Medium Crabtree effect species (van der Sluis et al. 2000; Moller et al. 2002; Christen and Sauer 2011; Hagman et al. 2013)

many hexose transporter genes as the three pre-WGD species, indicating the impact of WGD. However, based on phylogenetic analysis and gene syntenic structures from 23 species, Lin and Li showed that only two pairs of *Transporters* genes were produced by WGD and most duplicate pairs had become lost immediately after the WGD (Lin and Li 2011b). Most of the *Transporters* genes present in the extant *S. cerevisiae* genome were generated by tandem duplication or can be attributed to the dynamic pattern of telomeric regions where 8 *Transporter* genes are located (Lin and Li 2011b). The *Transporter* genes were also expanded from a single copy to eight copies (*GTH1-GTH8*) in the *Sch. pombe* lineage. Similar to *S. cerevisiae*, *Sch. pombe* is capable of aerobic fermentation in the presence of excess sugars (Alexander and Jeffries 1990). However, *Sch. pombe* did not experience a WGD, suggesting that other mechanisms rather than WGD led to its increase in the number of *Transporter* genes. Four of the eight *Transporter* gene in *Sch. pombe* are tandemly arrayed on chromosome III, indicating that they were produced by a series of tandem duplication events (Lin and Li 2011b).

Past studies have suggested that glucose uptake is the major rate-limiting step in glycolysis and largely controls glucose metabolism activities (Gancedo and Serrano 1989; Diderich et al. 1999; Ye et al. 1999; Pritchard and Kell 2002; Elbing et al. 2004; Otterstedt et al. 2004; Conant and Wolfe 2007). Otterstedt et al. found that an *S. cerevisiae* strain with very limited capacity to transport hexoses switched to respiration in the presence of oxygen (Otterstedt et al. 2004). Replacing the *S. cerevisiae HXT1–17* genes by a chimera *HXT* gene decreased its ethanol production or even caused a switch to fully respiratory metabolism due to reduced glucose consumption rates (Elbing et al. 2004; Otterstedt et al. 2004). Moreover, when yeast cells were grown under glucose limitation, spontaneous duplication of hexose transporters was observed (Brown et al. 1998). The significant expansion of *Transporter* genes had independently occurred in both *Saccharomyces* and *Schizosaccharomyces* lineages, which in parallel evolved aerobic fermentation (Lin and Li 2011b). Furthermore, there is a significant positive correlation between the number of *Transporter* genes and efficiency to produce ethanol from glucose (Lin and Li 2011b). In cells that are operating near their maximal glucose uptake rates, an increase in *Transporter* genes confers a selective advantage in glucose-rich environments to support higher growth rates (Brown et al. 1998). These lines of evidence support the view that the expansion of *Transporter* genes had facilitated the evolution of aerobic fermentation in the two different lineages.

5.2.2 Copy Number Variation of Genes Involved in Glycolysis

A glucose molecule is converted into two molecules of pyruvate through a series of reactions in glycolysis, which is believed to be among the oldest biochemical pathways and is highly conserved in prokaryotes and eukaryotes. Several studies have shown that the occurrence of WGD in the hemiascomycete lineage has had profound impacts on the enzyme dosages involved in glycolysis. Soon after the WGD, there was a period of rapid losses of duplicate genes (Scannell et al. 2006). Most duplicate genes produced by the WGD have been lost in the post-WGD species, and less than 10 % of WGD gene pairs have remained in the genome of *S. cerevisiae* (Wolfe and Shields 1997; Kellis et al. 2004; Thomson et al. 2005; Conant and Wolfe 2007). Conant and Wolfe (2007) found that there are six WGD duplicate pairs of genes that have been maintained in the five out of the ten reactions of glycolysis in *S. cerevisiae* (Fig. 5.1). The retained WGD pairs are not the same among post-WGD species. In general, each post-WGD species has preserved 5–6 WGD pairs, but only one duplicate pair (*GLK1* and *EMI2*) is retained in all pot-WGD species examined (Table 5.2). As only 551 WGD duplicate pairs were preserved in *S. cerevisiae*, it is unlikely that the glycolysis genes were preserved in duplicates at the same frequency as the remainder of the genome (Conant and Wolfe 2007).

Table 5.2 Number of glycolytic gene paralogs in hemiascomycete yeasts

SCE Gene 1	SCE Gene 2	SMI	SBA	CGL	KAF	KNA	NCA	TBL	VPO	ZRO	KLA	EGO	LKL	LTH	LWA
HXK1	HXK2	2	2	2	1	2	2	2	1	1	1	1	1	1	1
GLK1	EMI2	2	2	2	2	2	2	2	2	1	1	1	1	1	1
PGI1		1	1	1	1	1	1	1	1	1	1	1	1	1	1
PFK1		1	1	1	1	1	1	1	1	1	1	1	1	1	1
PFK2		1	1	2	2	2	1	1	2	1	1	1	1	1	1
FBA1		1	1	1	1	1	1	2	2	1	1	1	1	1	1
TPI1		1	1	1	1	1	1	1	1	1	1	1	1	1	1
TDH1		1	1	0	0	0	0	0	0	0	0	0	0	0	0
TDH2	TDH3	2	2	1	2	1	2	2	1	1	1	0	1	1	1
PGK1		1	1	1	2	2	2	1	1	1	1	1	1	1	1
GPM1		1	1	1	1	1	1	1	1	1	1	1	1	1	1
GPM2	GPM3	2	2	1	1	1	1	1	1	1	1	1	1	1	1
ENO1	ENO2	2	2	2	2	1	2	2	2	1	1	1	1	1	1
CDC19	PYK2	2	2	2	1	2	2	1	2	1	1	1	1	1	1

Species with the name underlined are post-WGD yeasts. The data were retrieved from yeast Gene Order Browser (Byrne and Wolfe 2005). SCE *Saccharomyces cerevisiae*; SMI *S. mikatae*; SBA *S. bayanus*; CGL *Candida glabrata*; KAF *Kazachstania africana*; KNA *Kazachstania naganishii*; NCA *Naumovozyma castellii*; TBL *Tetrapisispora blattae*; VPO *Vanderwaltozyma polyspora*; ZRO *Zygosaccharomyces rouxii*; KLA *Kluyveromyces lactis*; EGO *Eremothecium gossypii*; LKL *Lachancea kluyveri*; LTH *L. thermotolerans*; LWA *L. waltii*

The significant higher survival rate of WGD pairs in glycolysis than other pathways might have increased the relative levels of glycolytic enzymes. Papp et al. proposed that the retention of gene duplicates is better explained by selection for high enzymatic flux (Papp et al. 2004). A simulation of the effect of increased concentration of enzymes on glycolytic flux revealed that, when the concentration of glycolytic enzyme increases from 65 to 100 %, the end product of glycolysis pyruvate is increased by 17 % (Conant and Wolfe 2007). This observation is consistent with several studies proposing that the WGD event enhanced *S. cerevisiae*'s ability to metabolize glucose (Wolfe and Shields 1997; Wolfe 2004; Liti and Louis 2005). Pyruvate is the branching point between respiration and fermentation (Fig. 5.1). Pyruvate decarboxylase (Pdc) and pyruvate dehydrogenase (Pdh) compete for pyruvate, and the destiny of pyruvate depends on the consequence of substrate competition. Due to the different inherent kinetics between the two enzyme complexes, increasing the pyruvate concentration increases relative flux through Pdc and thus the fermentation pathway (Conant and Wolfe 2007). Therefore, more pyruvate molecules are directed to the fermentation pathway in post-WGD species as a result of increased glycolytic flux. Another reason for increased pyruvate rerouting to the fermentation pathway is probably the limited availability of mitochondria. Unlike fermentation which occurs in the cytosol, respiration takes place in mitochondria. The increased glycolytic flux might exceed the capacity of mitochondria due to the limitation in the number and size of mitochondria (Pronk et al. 1996). Indeed, it appears that during aerobic respiration, yeast mitochondria are larger and closer to the cell membrane than during anaerobic growth, possibly because this location is more efficient for oxygen uptake (Hoffmann and Avers 1973; Jensen et al. 2000). The WGD did not increase the number of mitochondria or make mitochondria larger, so the increased glycolytic flux might have exceeded the respiratory capacity of mitochondria. Furthermore, unlike the high retention rate of WGD pairs in glycolysis pathway genes, the WGD pairs were preserved in only two out of nine reactions in the TCA cycle in *S. cerevisiae* (Conant and Wolfe 2007).

A survey of over 40 yeast species both with and without the WGD indicates that the presence of the Crabtree effect is strongly associated with yeasts with the WGD (Merico et al. 2007). Another study also found a general, though weak, trend for higher rates of ethanol production in post-WGD yeasts than in pre-WGD yeasts (Blank et al. 2005). These studies suggested that the WGD event played a significant role in the adaptation of *S. cerevisiae* toward aerobic fermentation (Wolfe and Shields 1997; Kellis et al. 2004; Thomson et al. 2005; Conant and Wolfe 2007). However, not all aerobic fermentative species have experienced WGD. For example, *Dekkera bruxellensis*, which is a pre-WGD species separated from the *Saccharomyces* lineage more than 200 mya, also efficiently makes, accumulates and consumes ethanol (Rozpedowska et al. 2011). In addition, the fission yeast *Sch. pombe*, which also predominantly assimilates glucose through the fermentation pathway, diverged from the hemiascomycete lineage about 300 mya and has not experienced WGD (Wood et al. 2002). Based on the analysis of the Crabtree

effect for over 40 species in 12 genera of hemiascomycete yeasts, Hagman et al. found that many pre-WGD species demonstrate an intermediate level of Crabtree effect (Hagman et al. 2013). They argued that the evolution of Crabtree effect is gradual process or at least a two-step "invention". The progressive evolution of aerobic fermentation coincides with gradual duplication of hexose transporter genes (Lin and Li 2011b). The WGD event and regulatory rewiring of respiration-related genes, which occurred at different time points, have further strengthened the Crabtree effect in the lineages of *S. cerevisiae* (Hagman et al. 2013). Therefore, WGD might have facilitated the evolution of aerobic fermentation, but apparently it is not a prerequisite factor.

5.2.3 Copy Number Variation of Genes Involved in Fermentation

In aerobic fermentative species, most pyruvate molecules remain in cytosol and are converted into acetaldehyde by pyruvate decarboxylase (Pdc). The *S. cerevisiae* genome contains three copies of the *Pdc* encoding gene (*PDC1*, *PDC5* and *PDC6*), though *PDC1* encodes the major enzyme in this reaction and is highly expressed in rich medium. The evolutionary history of *PDC* genes in hemiascomycetes suggests that the three *PDC* genes were generated by two consecutive duplication events in the common ancestor of the *sensu stricto* species (Fig. 5.2a). After duplications, loss of *PDC* genes have been detected in some *sensu stricto* species. For example, only *PDC1* is present in the genome of *S. mikatae* (Fig. 5.2a), and loss of *PDC6* genes was observed in *S. kudriavzevii* (Scannell et al. 2011). However, comparing with other *sensu stricto* species, no significant difference in ability of ethanol production was detected in *S. mikatae* (Hagman et al. 2013). In general, 2–3 copies of *PDC* genes are present in most aerobic fermentative species. The number of *PDC* genes in the respiratory species varies from 1 to 4 copies. For example, only a single *PDC* gene is found in *K. lactis* (Bianchi et al. 1996), while three copies are found in *Debaryomyces hansenii* (Fig. 5.2a). Therefore, there is no significant increase in the number of *PDC* genes in fermentative species, in agreement with a previous finding that the number of *PDC* genes is not correlated with the intensity of alcoholic fermentation (Moller et al. 2004).

The second and the last step of reaction in the fermentation pathway is converting acetaldehyde into ethanol and recycling the NADH generated during glycolysis (Fig. 5.1). In most species, this reaction is catalyzed by alcohol dehydrogenase (*Adh*). In *S. cerevisiae*, there are five genes that encode *Adh*, *ADH1–ADH5*. *Adh1p* is the major enzyme responsible for converting acetaldehyde into ethanol, while Adh2p catalyzes the reverse reaction to consume ethanol (Leskovac et al. 2002). *ADH1* and *ADH2* were derived from a duplication event prior to the divergence of *sensu stricto* yeast species (Thomson et al. 2005). Thomson et al. (2005) have reconstructed the last common ancestor of *Adh1p* and *Adh2p*, which is

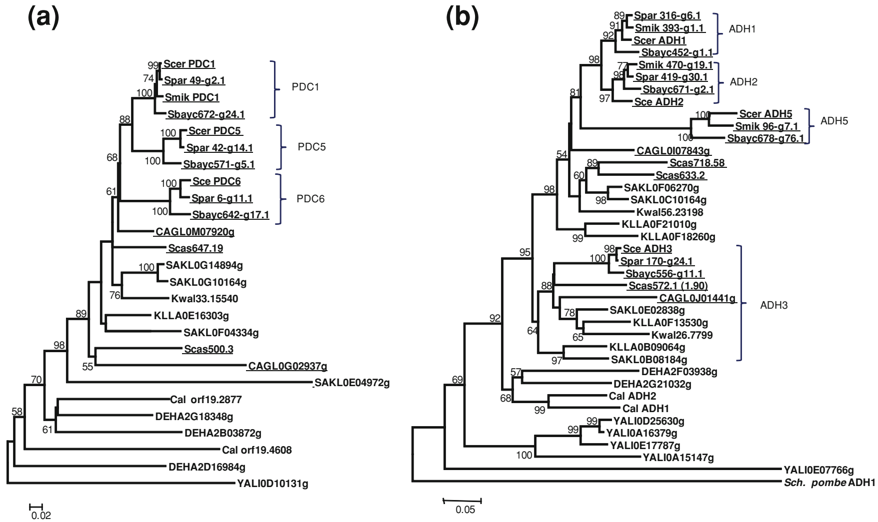

Fig. 5.2 The evolutionary history and copy number variations of the PDC and ADH gene families in hemiascomycete yeasts. **a** Phylogenetic tree of the PDC gene family. **b** Phylogenetic tree of the ADH gene family. The three PDC genes (*PDC1*, *PDC5* and *PDC6*) and the three ADH genes (*ADH1*, *ADH2* and *ADH5*) in *S. cerevisiae* were produced by two consecutive duplications prior to divergence of the *Senso stricto* group. The *S. cerevisiae ADH4* is a distant relative to the other four ADH genes and was not included in the tree. Both phylogenetic trees were constructed using the Neighbor-Joining (NJ) method with 1,000 bootstrap replicates. The evolutionary distances were computed using the JTT matrix-based method. Species names are abbreviated as follows: Scer: *Saccharomyces cerevisiae*; Spar: *S. paradoxous*; Smik: *S. mikatae*; Sbay: *S. bayanus*; CAGL: *Candida glabrata*; Scas: *S. castellii* (*Naumovozyma castellii*); KLLA: *Kluyveromyces lactis*; SAKL: *S. kluyveri* (*Lachancea kluyveri*); Kwal: *K. waltii* (*Lachancea waltii*); Cal: *C. albicans*; DEHA: *Debaryomyces hansenii*; YALI: *Yarrowia lipolytica*

called *AdhA*. The *AdhA* protein has a high Michaelis constant K_M for ethanol, which is similar to *Adh1p*, suggesting that *AdhA* was optimized to make ethanol (Thomson et al. 2005). Therefore, *Adh1p* maintains the ancestral function for making ethanol, while *Adh2p* has evolved a much lower K_M for ethanol, which is optimized for consuming ethanol. Because many microorganisms cannot grow in a high concentration of ethanol, accumulating ethanol may help yeasts to outcompete their competitors for fruit resources (Piskur et al. 2006). Thus, the birth of *ADH2* might have enabled yeasts to tolerate a higher concentration of ethanol produced from fermentation by efficiently consuming ethanol after depletion of sugars (Thomson et al. 2005). By including more hemiascomycete species, we show here (Fig. 5.2b) that the gene duplication event that produced *ADH1* and *ADH2* occurred after the split between the common ancestor of *sensu stricto* yeast species and other post-WGD yeasts including *Candida glabrata* and *N. castelli*. Because *C. glabrata* and *N. castelli* are both Crabtree-positive species (Merico

et al. 2007), it means that *ADH2* is not necessary for the Crabtree effect, suggesting that the birth of *ADH2* might have increased ethanol tolerance, but it was probably not essential for aerobic fermentation.

5.2.4 Elevated Evolutionary Rates and Biased Codon Usage of Respiration-Related Genes

In view of the fact that post-WGD yeasts predominantly assimilate glucose through the fermentation pathway, the role of mitochondria in generating energy for cellular growth appears to be weakened (Merico et al. 2007). Kellis et al. (2004) noticed that some WGD gene pairs have accelerated evolutionary rate at nucleotide level, but not at amino acid level. For example, the pyruvate kinase genes *CDC19* and *PYK2* were produced by WGD, and *PYK2* shows a three-fold acceleration in substitution rate at degenerate third-codon positions (Kellis et al. 2004). Jiang et al. (2008) calculated the rate of non-synonymous substitution (d_N) for 2,603 one-to-one orthologous genes, including 296 nuclear genes for mitochondrial proteins, among six post-WGD yeast species and three closely related pre-WGD yeast species. They found that the rates of sequence divergence of mitochondrial genes are very similar within the post-WGD species and within the pre-WGD species. However, the average evolutionary distance for mitochondrial genes for the post-WGD species pairs is about 13 % higher than that for the pre-WGD species pairs (Jiang et al. 2008), supporting the view that genes involved in mitochondrial functions have experienced relaxation of functional constraints in post-WGD yeasts.

It was postulated that to ensure efficient and accurate translation, highly expressed genes tend to have strong codon usage bias (Ikemura 1981, 1982). The codon usage bias can be reduced if the gene product undergoes a reduction in functional constraint (Akashi 1997; Gu et al. 2005). Therefore, if mitochondrial energy production became less important to the post-WGD species, a decreased codon usage bias is expected for the mitochondrial genes of these species. The codon usage bias for six genes encoding the electron transport chain cytochrome-c (CYC) from five yeasts was found to be stronger in aerobic respiration species than in fermentative species (Freire-Picos et al. 1994). The difference in codon usage of *CYC* genes was correlated with their difference in mRNA level between the two types of yeasts (Freire-Picos et al. 1994). Jiang et al. also found that mitochondrial genes displayed significantly stronger codon usage bias than non-mitochondrial genes in all three studied pre-WGD species. In contrast, there was no significant difference in codon usage bias between mitochondrial and non-mitochondrial genes for all the studied post-WGD species (except for *V. polyspora*). Therefore, the accelerated evolution of mitochondrial function genes in post-WGD yeasts at the nucleotide level appears to reflect a relaxation in selection on the codon usage.

5.3 Comparative Studies of Gene Regulation in Carbohydrate Metabolism

5.3.1 Differential Expression of Genes Involved in Carbohydrate Metabolism Among Yeasts

The major difference between the two glucose metabolism styles depends on how pyruvate is degraded. In respiratory yeasts, most pyruvate enters mitochondria and is completely degraded to CO_2 and H_2O under aerobic conditions, while in fermentative species, most pyruvate remains in the cytosol and is converted into ethanol and CO_2. Because the respiration-related genes have been retained in both respiratory and fermentative species, it means that the evolution of aerobic fermentation required modifications of the regulation of respiration-related genes. Differential expression of genes involved in glucose metabolism between fermentative and respiratory species has been observed in several studies of individual genes. The respiration-related genes, such as *CYC1*, *QCR7,* and *QCR8*, are highly expressed in the presence of oxygen in the Crabtree-negative species *K. lactis* (Freire-Picos et al. 1995; Mulder et al. 1995). In contrast, although the expression of glycolysis and fermentation-related genes is induced in *S. cerevisiae* under growth on glucose, the expression of respiration-related genes is repressed (Holland and Holland 1978; Schmitt et al. 1983; Forsburg and Guarente 1989; DeRisi et al. 1997; Carlson 1999).

The global modification of regulatory control of respiration-related genes has been confirmed by recent studies based on large sets of genome-wide gene expression data from yeasts. Significant expression differences in genes related to carbohydrate metabolism and respiratory functions have been detected by heterologous DNA arrays between *S. cerevisiae* and *K. lactis* growing in a sugar-rich medium (Becerra et al. 2004). Ihmels et al. (2005) compared datasets of 1,000 and 198 published genome-wide expression profiles between *S. cerevisiae* and the human pathogen *Candida albicans* (Ihmels et al. 2002). *C. albicans*, which diverged from the *S. cerevisiae* lineage approximately 100–300 million years ago, is predominantly Crabtree negative. Because the large number of cytosolic ribosomal proteins (CRP) genes are coherently expressed under different conditions and show a strong correlation with cell growth (Mager and Planta 1991; Gasch et al. 2000), they can be used as a good proxy to evaluate the expression profiles for different sets of genes. Ihmels et al. found that genes coding for mitochondrial ribosomal proteins (MRP) and CRP display a strongly correlated expression pattern in *C. albicans*, but this correlation is lost in the fermentative yeast *S. cerevisiae* (Ihmels et al. 2005). Instead, the expression of the 72 MRP genes in *S. cerevisiae* exhibits a distinct correlation with that of genes induced in response to environmental stress conditions. Because the Crabtree positive yeasts evolved from respiratory yeasts, the authors concluded that the regulation of MRP genes in

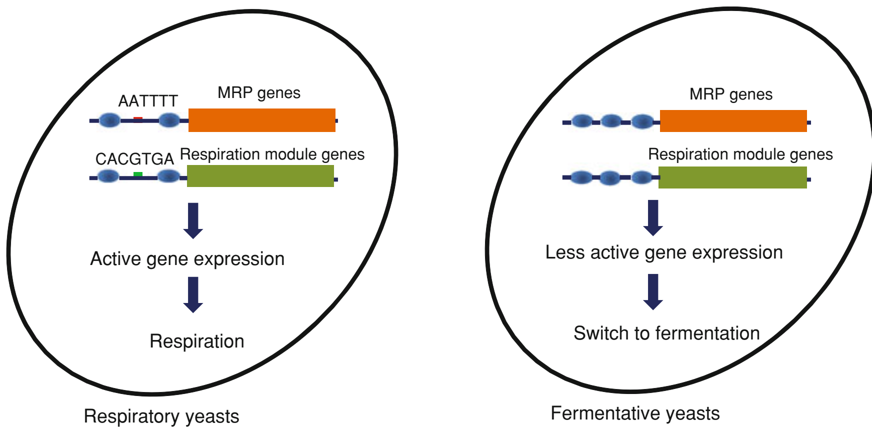

Fig. 5.3 A schematic illustration of the genetic basis underlying the regulatory rewiring of respiration-related genes in fermentative yeasts. In respiratory yeasts, the expression of MRP genes is activated by a transcription factor through binding to the AATTTT motif. The Respiration module genes, such as CYC1, ATP4, QCR7, and QCR and QCR8, are activated by Cbf1p through its binding to the CACGTGA motif. Both groups of genes appear to have nucleosome-depleted type of promoters. The AATTTT and Cbf1 motifs have been lost and the promoter became nucleosome-occupied in the fermentative species. As a consequence of the changes in promoter chromatin structure and *cis*-regulatory elements, the respiration-related genes are not actively expressed, so that most pyruvate is directed to the fermentation pathway in the Crabtree-positive species

S. cerevisiae has been rewired during the evolution of aerobic fermentation (Ihmels et al. 2005).

Field et al. (2009) reanalyzed the same sets of gene expression data using the Gene Ontology (GO) groups as units and calculated their expression correlation with CRP genes in both *S. cerevisiae* and *C. albicans* (Field et al. 2009). Based on the gene expression correlation with CRP genes, the authors identified 13 GO groups of genes (called "category III" genes) that are differentially expressed between the two species. The "category III" set includes 157 and 146 genes in *S. cerevisiae* and *C. albicans*, respectively. The *S. cerevisiae* "category III" genes include 34 MRP genes as well as genes related to cellular respiration and mitochondrial functions, such as the TCA cycle and oxidative phosphorylation. Similar to the MRPgenes, a high expression correlation between "category III" genes and CRP genes was observed in *C. albicans* but not in *S. cerevisiae* (Field et al. 2009). Therefore, in addition to the MRP genes, the transcriptional regulation of other respiration-related genes has also been reprogrammed during the evolution of *S. cerevisiae* (Fig. 5.3).

A recent study measured the genome-wide gene expression levels growing under the same rich medium in 12 completely sequenced yeasts using tiling arrays (Tsankov et al. 2010). The 12 yeasts include six aerobic fermentative species and six respiratory species, offering an ideal opportunity to identify the genes that have highest significant expression differences between the two types of yeasts. Using

the 82 transcriptional modules (Ihmels et al. 2002) as units, Lin et al. (2013) compared the difference in gene expression level for each module between the two types of yeasts. They found that the modules that include the MRP genes showed only the sixth largest expression difference among the 82 modules based on the two-sample Kolmogorov-Smirnov test. In contrast, a module (Module 5) that includes genes involved in mitochondrial energy generation and phosphorylation oxidation has the largest expression difference between the two types of species. For convenience, Module 5 is thereafter called the Respiration module. *S. cerevisiae* respiration module includes 49 genes, 23 of which overlap with the "category III" genes identified by Field et al. (2009). Thus, the genes associated with mitochondrial energy production, instead of MRP genes, have experienced the most significant changes in gene expression levels during the evolution of aerobic fermentation (Lin et al. 2013).

The fission yeast *Sch. pombe* independently evolved the Crabtree effect. So, it was interesting to see if gene expression reprogramming had also occurred during the evolution of *Sch. pombe*. Lin and Li (2011a) conducted pairwise comparisons for the $\sim$1,000 sets of genome-wide gene expression profiles in *Sch. pombe*, and gene expression data in *S. cerevisiae* and *C. albicans*. They found that the two fermentative species *S. cerevisiae* and *Sch. pombe* are more similar to each other on the genome-wide gene expression patterns than to the respiratory yeast *C. albicans*, although *S. cerevisiae* is evolutionarily closer to *C. albicans*. Lin and Li identified a group of genes that are differentially expressed between *Sch. pombe* and *C. albicans* and most of them are involved in mitochondrial respiration process. In summary, similar to what happened in the *S. cerevisiae* lineage, the evolution of aerobic fermentation in the *Sch. pombe* lineage was also associated with regulatory rewiring of genes involved in the mitochondrial respiration process (Lin and Li 2011a).

5.3.2 Genetic Basis for Gene Expression Reprogramming

Although there are some discrepancies about what genes have experienced regulation reprogramming among studies (Ihmels et al. 2005; Field et al. 2009; Lin et al. 2013), there is a general agreement that all these genes are involved in respiration-related processes. However, with respect to the genetic basis underlying these gene expression divergences, these studies have reached different conclusions. Ihmels et al. found a sequence motif "AATTTT" significantly overrepresented in the promoters of the MRP genes in *C. albicans*, but not in their orthologous genes in *S. cerevisiae* (Ihmels et al. 2005). The AATTTT motif was proposed to be involved in the regulation of rRNA processing genes in *S. cerevisiae*, but the protein binding to this motif remains to be identified (Tavazoie and Church 1998). Therefore, Ihmels et al. concluded that the loss of the AATTTT sequence was associated with global regulatory reprogramming of MRP genes in the *S. cerevisiae* lineage, and contributed to its evolution of aerobic fermentation (Ihmels et al. 2005). The loss of AATTTT

motif was also observed in other species that independently evolved aerobic fermentative ability. As mentioned above, aerobic fermentation evolved independently in the pre-WGD hemiascomycete yeast *D. bruxellensis* (Rozpedowska et al. 2011). Similar to what was observed in *S. cerevisiae*, the AATTTT motif did not exhibit any positional conservation in the promoters of the MRP genes in *D. bruxellensis* (Rozpedowska et al. 2011). The authors suggested that the AATTTT element underwent independent massive losses in the promoter of MRP genes in both *S. cerevisiae* and *D. bruxellensis* (Rozpedowska et al. 2011).

It is worth noting that only intermediate numbers of the AATTTT motif in the promoters of MRP genes were observed in the post-WGD yeast *V. polyspora* (Fekete et al. 2007; Chen et al. 2008; Jiang et al. 2008). Moreover, there are several other genetic and physiological characteristics in *V. polyspora* that are more similar to aerobic respiratory yeasts than to the rest of post-WGD yeasts. For example, only five hexose transporter genes are present in *V. polyspora*, compared to 10–19 copies in other post-WGD species (Lin and Li 2011b). *V. polyspora* also shows a different pattern of mitochondrial gene codon usage bias from the other post-WGD species (Fekete et al. 2007; Chen et al. 2008; Jiang et al. 2008). In addition, other post-WGD species are petite positive, the ability to tolerate the loss of mtDNA, whereas *V. polyspora* is petite negative (Fekete et al. 2007; Chen et al. 2008; Jiang et al. 2008). *V. polyspora* was thus speculated as an intermediate fermentative species that cannot carry out efficient aerobic fermentation (Fekete et al. 2007; Chen et al. 2008; Jiang et al. 2008). Surprisingly, a recent study revealed that *V. polyspora* actually has high fermentation efficiency (Hagman et al. 2013). Hagman et al. suggested that the evolution of aerobic fermentation is a progressive process which involves multiple genetic modifications that gradually remodel the yeast carbon metabolism. It is likely that *V. polyspora* represents an intermediate lineage where some traits are still in transition.

On the other hand, Lin et al. (2013) found that in respiratory yeasts, a motif with the core consensus sequence CACGTGA is prevalent in the promoters of Respiration module genes, but present at a much lower frequency in the promoters of their orthologous genes in the aerobic fermentation species (Fig. 5.3). This motif matches that of Cbf1p binding sites in *S. cerevisiae*; Cbf1p is a transcription factor that regulates chromatin modification (Cai and Davis 1990). The homolog of *S. cerevisiae CBF1* has been characterized in *K. lactis* and the Cbf1p proteins from the two species are functionally interchangeable (Mulder et al. 1994). However, despite the functional conservation of *CBF1*, unlike in *S. cerevisiae*, inactivation of the *CBF1* gene in *K. lactis* is not viable, indicating that the normal function of *CBF1* is essential for *K. lactis* (Mulder et al. 1994). Lin et al. (2013) suggested that Cbf1p is a general activator for the respiration-related genes in respiratory yeasts and the massive losses of the Cbf1 motif in fermentative species have led to the reduced expression of respiration-related genes.

In addition to changes in *cis*-regulatory elements, it was suggested that the distinct nucleosome organizations in the promoters of respiration-related genes between the aerobic respiration and the fermentation yeasts are partly responsible for their expression divergences (Field et al. 2009; Tsankov et al. 2010). In

eukaryotes, DNA is repetitively wrapped around nucleosomes. The presence of nucleosome may hinder the direct interaction between a transcription factor and its binding sites and may, therefore, obstruct the transcriptional initiation of a gene. In *S. cerevisiae*, the binding sites of transcription factors are highly enriched in the nucleosome depleted region of promoters (Lin et al. 2010). Several studies have found that genes with different expression profiles are associated with distinct nucleosome occupancy patterns in the promoter regions (Tirosh and Barkai 2008; Jiang and Pugh 2009). The promoters of constantly expressed genes usually contain a nucleosome-depleted region where most transcription factor-binding sites are located (Yuan et al. 2005; Lee et al. 2007). In contrast, conditionally expressed genes, such as stress-response genes, are associated with nucleosome-occupied promoters (Tirosh and Barkai 2008). Field et al. (2009) compared the promoter nucleosome occupancy patterns among 12 hemiascomycete yeasts and found that the promoters of respiration-related genes tend to be more depleted of nucleosomes in respiratory yeasts than that in aerobic fermentative species. They concluded that in aerobic fermentation yeasts, respiration-related gene promoters have evolved from the nucleosome-depleted type to the nucleosome-occupied type and that this change has contributed to regulatory reprogramming of respiration-related genes and the evolution of aerobic fermentation in the hemiascomycete lineage (Field et al. 2009).

However, it is not clear whether the nucleosome reorganization was the leading or a minor cause for the evolution of aerobic fermentation (Tirosh et al. 2010). Comparative studies of nucleosome occupancy between *S. cerevisiae* and its close relative *S. paradoxus* showed that genes that are associated with diverged nucleosome positions are not more likely to diverge in expression and genes that are differentially expressed are not more likely to diverge in nucleosome positioning (Tirosh et al. 2010). Consistent with Tiroshi et al.'s observation, Lin and Li (2011a) found that changes in nucleosome organization were not coupled with the expression reprogramming of respiration-related genes in *Sch. pombe*. Specifically, although the expression regulation of the respiration-related genes in *Sch. pombe* has been reprogrammed during the evolution of aerobic fermentation, their promoter nucleosome organization remains depleted as in aerobic respiration species (Lin and Li 2011a).

In addition, a recent study suggested that changes in the length of the 5′ untranslated region (5′UTR) were linked to the gene expression divergence of respiration-related genes in *S. cerevisiae* (Lin and Li 2012). The length of 5′UTR varies considerably among the genes in a genome, ranging from a few base pairs to several thousand base pairs (Pesole et al. 2001; Mignone et al. 2002; Nagalakshmi et al. 2008). It has been noticed that genes with different functions show distinct 5′UTR lengths in vertebrates and yeasts (Kozak 1987; Hurowitz and Brown 2003; David et al. 2006; Nagalakshmi et al. 2008; Bruno et al. 2010). In general, genes with a long 5′UTR, such as those involved in development or meiosis, are generally highly and finely regulated, whereas genes with a reduced need for regulation, such as housekeeping genes and the ribosomal subunit genes, usually have shorter 5′UTRs. Lin and Li (2012) examined the association between UTR lengths

and the patterns of gene expression across various conditions in *S. cerevisiae* and *C. albicans* (Berman and Sudbery 2002; Sudbery et al. 2004). They calculated the expression correlation of all eligible GO group genes with CRP genes and found that such correlations are negatively associated with their 5′UTR lengths in both species (Lin and Li 2012). It suggests that genes with a longer 5′UTR tend to have higher expression plasticity under different conditions. Among a few GO groups with the largest increase in 5′UTR length in *S. cerevisiae* and the largest decrease in this gene expression correlation, most of these genes are involved in mitochondrial respiration process. It is well known that the expression of mitochondrial respiration genes has been reprogrammed in *S. cerevisiae* during the evolution of aerobic fermentation. These results suggest that the elongation of 5′UTR might have contributed to this global gene regulation modification process. As it is not clear how changes in 5′UTR length affect gene expression plasticity, it was speculated that an increase in 5′ UTR length may affect the nucleosome occupancy patterns in promoters (Lin and Li 2012).

5.4 Comparative Genomics of Galactose Metabolism

Although glucose is the preferred carbohydrate in yeasts, most yeasts are able to use galactose well. Galactose itself cannot be directly used for glycolysis. As shown in Fig. 5.1, after galactose is transported into cells by galactose permease Gal2p, it needs to be converted into glucose-6-phosphate to enter the glycolysis pathway. The pathway that catalyzes this conversion is also called the Leloir pathway (Johnston 1987; Bhat and Murthy 2001). In *S. cerevisiae*, the Leloir pathway is composed of five enzymes: galactose mutarotase, UDP-galactose-4-epimerase Gal10p, galactokinase Gal1p, galactose-1-phosphate uridyl transferase Gal7p, phosphoglucomutase Pgm1 and Pgm2. The genes encoding the Leloir pathway enzymes (often called the *GAL* genes) are tightly controlled at the transcriptional level in yeasts (Sellick et al. 2008). In the presence of glucose, the *GAL* genes are repressed. The repression is released when glucose is absent, and rapid and high-level activation of the *GAL* genes is triggered by three other proteins, Gal4p, Gal80p, and Gal3p (Sellick et al. 2008). The *GAL* genes are broadly distributed in all eukaryotes, bacteria, and archaea.

A number of yeast species lack the ability to use galactose (Naumov et al. 2000). Hittinger et al. examined the genomic data for seven species that can use galactose and four that cannot. The phylogeny of the 11 yeast species suggests that galactose utilization was present in their common ancestor and the loss of galactose utilization in the four species were due to at least three parallel losses (Hittinger et al. 2004). The seven *GAL* genes (*GAL1*, *GAL2*, *GAL3*, *GAL4*, *GAL7*, *GAL10* and *GAL80*) are present in all species that can use galactose. In contrast, these *GAL* genes are absent in the four species lacking the galactose utilization ability, suggesting the degeneration of the entire pathway (Hittinger et al. 2004).

Remarkably, different galactose utilization abilities due to presence/absence of *GAL* genes are also observed between different strains in a species. The Japanese strains of *Saccharomyces kudriavzevii*, a close relative of *S. cerevisiae*, lack the galactose utilization ability. All the seven *GAL* genes in the Japanese strains of *S. kudriavzevii* were heavily degenerated and became pseudogenes soon after the split between the *S. kudriavzevii* and *S. cerevisiae lineages* (Hittinger et al. 2004). However, the *S. kudriavzevii* strains isolated from Portugal were found to be capable of utilizing galactose because of the presence of six functional *GAL* genes in their genomes (the *GAL3* gene is absent) (Hittinger et al. 2010). Because none of the functional *GAL* genes in the Portuguese strains appeared to be acquired from other species, the polymorphisms in these *GAL* genes may have co-existed throughout the evolutionary history of *S. kudriavzevii*, providing a classic example of balancing selection on the multi-loci gene network.

In summary, these lines of evidence revealed a tight correlation between the ability to use galactose and the presence of the *GAL* genes in the genome. This strong correlation of genomic content and galactose utilization probably reflects the adaptation of yeasts to their own niches in which the galactose content varies substantially (Gross and Acosta 1991). The independent losses of all *GAL* pathway genes in multiple lineages were probably facilitated by the clustered organization of these genes. In most species, the *GAL* genes are not clustered. Slot and Rokas found that the genes encoding three major enzymes of the Leloir pathway (*GAL1*, *GAL7,* and *GAL10*) have independently become clustered in four different fungal lineages by different mechanisms (Slot and Rokas 2010). Moreover, a significant higher rate of *GAL* pathway gene loss in the species with clustered *GALs* than in those without clustered *GAL* genes was observed, suggesting that the adaptation of fungal species to different environments by gain or loss of galactose utilization ability could be facilitated by the clustering of *GAL* genes (Slot and Rokas 2010). However, even though the structural members and arrangement of *GAL* gene cluster are conserved among different yeast lineages, their regulatory circuits have been rewired in the Hemiascomycete lineage (Martchenko et al. 2007). In *S. cerevisiae*, the *GAL* genes are activated by Gal4p and repressed by Mig1p through binding to the Gal4 or Mig1 binding sites in their promoters (Lohr et al. 1995). Interestingly, the Gal4 and Mig1 binding sites are absent from the *C. albicans GAL* genes clusters (Martchenko et al. 2007). In contrast, it is found that the expression of *GAL* genes in *C. albicans* is activated by Cph1p, a homolog of *S. cerevisiae* transcription factor Ste12p (Martchenko et al. 2007). By comparing the promoter sequences of *GAL* genes in 11 yeast species, the author proposed that Gal4p and Mig1p were recruited to co-regulate *GAL* genes with Cph1p prior to WGD. The Cph1 binding sites were eventually lost during evolution of *S. cerevisiae* lineage, resulting in the switch of the control *GAL* gene from Chp1p to Gal4p and Mig1p (Martchenko et al. 2007).

As discussed above, the WGD event was considered to have a strong impact on the glucose metabolism. Similarly, it may also have enhanced the ability of galactose utilization. In *S. cerevisiae*, the galactokinase gene *GAL1* and the co-inducer gene *GAL3* are paralogous genes that arose from a single bifunctional

ancestral gene by the WGD (Wolfe and Shields 1997; Hittinger et al. 2004; Kellis et al. 2004). The bifuntional gene is still present in some pre-WGD yeasts, such as *K. lactis*. In *S. cerevisiae*, the expression of *GAL1* is highly induced up to 1,000 fold, in the presence of galactose, while *GAL3* is only induced three–five-fold. The sharply different regulations on the two paralogous genes are believed to be advantageous for galactose utilization. The single bifunctional gene in *K. lactis* may be subject to adaptive conflict at the level of transcriptional regulation. The adaptive conflict appeared to be resolved by the WGD event that produced an extra copy of the *GAL* gene. Specific modifications on the promoters of the *GAL1* and *GAL3* genes allowed them to have totally different ranges of transcription in post-WGD species (Hittinger and Carroll 2007).

5.5 Conclusions and Prospects

Different yeasts show highly distinct preferences in carbohydrate metabolism. Recent comparative genomics and bioinformatics studies revealed that different glucose and galactose metabolisms were associated with changes in genomic content and regulatory landscape. While the evolution of galactose utilization was mainly due to gain or loss of *GAL* genes and changes in *cis*-regulatory elements, the evolution of different glucose metabolisms in yeasts was influenced by many different factors including changes in gene copy number, *cis*-regulatory elements, promoter chromatin structure, and 5′UTR length. The expansion of glucose transporter genes and the high retention rate of WGD gene pairs in glycolytic enzymes have increased the glycolytic flux in the aerobic fermentative species. In addition, the switch from the respiration pathway to the fermentation pathway also required regulatory rewiring of genes involved in mitochondrial functions, so that most pyruvate is directed to the fermentation pathway. However, it would only make sense if the ancestral yeasts had already evolved the active and highly efficient fermentation pathway under aerobic conditions prior to global repression of mitochondrial function, or the yeast cells would suffer selective disadvantages due to shortage of energy and reduced growth rate. Most studies so far focused on the glycolysis and mitochondrial respiration pathways. It is not known if the genes involved in fermentation reactions have been activated in the presence of oxygen or the enzyme activities have been greatly improved at an early evolutionary stage of aerobic fermentation. Further work will be needed to study what evolutionary changes in the fermentation pathway have triggered the switch from the respiratory to the fermentative style.

Acknowledgments We thank Ken Wolfe and Chris Hittinger for their valuable comments.

References

Akashi H (1997) Codon bias evolution in *Drosophila*. Population genetics of mutation-selection drift. Gene 205:269–278

Alexander MA, Jeffries TW (1990) Respiratory efficiency and metabolite partitioning as regulatory phenomena in yeasts. Enzyme and Microb Technol 12:2–19

Barnett JA, Payne RW, Yarrow D (2000) In yeasts: characteristics and identification, 3rd (edn). Cambridge University Press, Cambridge

Becerra M, Tarrio N, Gonzalez-Siso MI, Cerdan ME (2004) Genome-wide analysis of *Kluyveromyces lactis* in wild-type and rag2 mutant strains. Genome 47:970–978

Bhat PJ, Murthy TV (2001) Transcriptional control of the GAL/MEL regulon of yeast *Saccharomyces cerevisiae*: mechanism of galactose-mediated signal transduction. Mol Microbiol 40:1059–1066

Bianchi MM, Tizzani L, Destruelle M, Frontali L, Wesolowski-Louvel M (1996) The 'petite-negative' yeast *Kluyveromyces lactis* has a single gene expressing pyruvate decarboxylase activity. Mol Microbiol 19:27–36

Blank LM, Lehmbeck F, Sauer U (2005) Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. FEMS Yeast Res 5:545–558

Boles E, Hollenberg CP (1997) The molecular genetics of hexose transport in yeasts. FEMS Microbiol Rev 21:85–111

Brown CJ, Todd KM, Rosenzweig RF (1998) Multiple duplications of yeast hexose transport genes in response to selection in a glucose-limited environment. Mol Biol Evol 15:931–942

Bruno VM, Wang Z, Marjani SL, Euskirchen GM, Martin J, Sherlock G et al (2010) Comprehensive annotation of the transcriptome of the human fungal pathogen *Candida albicans* using RNA-seq. Genome Res 20:1451–1458

Byrne KP, Wolfe KH (2005) The yeast gene order browser: combining curated homology and syntenic context reveals gene fate in polyploid species. Genome Res 15:1456–1461

Cai M, Davis RW (1990) Yeast centromere binding protein CBF1, of the helix-loop-helix protein family, is required for chromosome stability and methionine prototrophy. Cell 61:437–446

Carlson M (1999) Glucose repression in yeast. Curr Opin Microbiol 2:202–207

Chen H, Xu L, Gu Z (2008) Regulation dynamics of WGD genes during yeast metabolic oscillation. Mol Biol Evol 25:2513–2516

Christen S, Sauer U (2011) Intracellular characterization of aerobic glucose metabolism in seven yeast species by 13C flux analysis and metabolomics. FEMS Yeast Res 11:263–272

Conant GC, Wolfe KH (2007) Increased glycolytic flux as an outcome of whole-genome duplication in yeast. Mol Syst Biol 3:129

David L, Huber W, Granovskaia M, Toedling J, Palm CJ, Bofkin L et al (2006) A high-resolution map of transcription in the yeast genome. Proc Natl Acad Sci U S A 103:5320–5325

De Deken RH (1966) The Crabtree effect: a regulatory system in yeast. J Gen Microbiol 44:149–156

DeRisi JL, Iyer VR, Brown PO (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278:680–686

Diderich JA, Teusink B, Valkier J, Anjos J, Spencer-Martins I, van Dam K et al (1999) Strategies to determine the extent of control exerted by glucose transport on glycolytic flux in the yeast *Saccharomyces bayanus*. Microbiology 145(Pt 12):3447–3454

Dujon B (2010) Yeast evolutionary genomics. Nat Rev Genet 11:512–524

Elbing K, Larsson C, Bill RM, Albers E, Snoep JL, Boles E et al (2004) Role of hexose transport in control of glycolytic flux in *Saccharomyces cerevisiae*. Appl Environ Microbiol 70:5323–5330

Engel SR, Cherry JM (2013) The new modern era of yeast genomics: community sequencing and the resulting annotation of multiple *Saccharomyces cerevisiae* strains at the Saccharomyces genome database. Database (Oxford) 2013, bat012

Fekete V, Cierna M, Polakova S, Piskur J, Sulo P (2007) Transition of the ability to generate petites in the *Saccharomyces/Kluyveromyces* complex. FEMS Yeast Res 7:1237–1247
Ferea TL, Botstein D, Brown PO, Rosenzweig RF (1999) Systematic changes in gene expression patterns following adaptive evolution in yeast. Proc Natl Acad Sci U S A 96:9721–9726
Field Y, Fondufe-Mittendorf Y, Moore IK, Mieczkowski P, Kaplan N, Lubling Y et al (2009) Gene expression divergence in yeast is coupled to evolution of DNA-encoded nucleosome organization. Nat Genet 41:438–445
Field Y, Kaplan N, Fondufe-Mittendorf Y, Moore IK, Sharon E, Lubling Y et al (2008) Distinct modes of regulation by chromatin encoded through nucleosome positioning signals. PLoS Comput Biol 4:e1000216
Forsburg SL, Guarente L (1989) Communication between mitochondria and the nucleus in regulation of cytochrome genes in the yeast *Saccharomyces cerevisiae*. Annu Rev Cell Biol 5:153–180
Freire-Picos MA, Gonzalez-Siso MI, Rodriguez-Belmonte E, Rodriguez-Torres AM, Ramil E, Cerdan ME (1994) Codon usage in *Kluyveromyces lactis* and in yeast cytochrome c-encoding genes. Gene 139:43–49
Freire-Picos MA, Hollenberg CP, Breunig KD, Cerdan ME (1995) Regulation of cytochrome c expression in the aerobic respiratory yeast *Kluyveromyces lactis*. FEBS Lett 360:39–42
Gancedo C, Serrano R (1989) In energy-yielding metabolism, 2nd (edn). Academic Press, London, England, vol. 3
Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G et al (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11:4241–4257
Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H et al (1996) Life with 6000 genes. Science 274(546):63–67
Gross KC, Acosta PB (1991) Fruits and vegetables are a source of galactose: implications in planning the diets of patients with galactosaemia. J Inherit Metab Dis 14:253–258
Gu Z, David L, Petrov D, Jones T, Davis RW, Steinmetz LM (2005) Elevated evolutionary rates in the laboratory strain of *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 102:1092–1097
Hagman A, Sall T, Compagno C, Piskur J (2013) Yeast "make-accumulate-consume" life strategy evolved as a multi-step process that predates the whole genome duplication. PLoS ONE 8:e68734
Hittinger CT, Carroll SB (2007) Gene duplication and the adaptive evolution of a classic genetic switch. Nature 449:677–681
Hittinger CT, Goncalves P, Sampaio JP, Dover J, Johnston M, Rokas A (2010) Remarkably ancient balanced polymorphisms in a multi-locus gene network. Nature 464:54–58
Hittinger CT, Rokas A, Carroll SB (2004) Parallel inactivation of multiple GAL pathway genes and ecological diversification in yeasts. Proc Natl Acad Sci U S A 101:14144–14149
Hoffman CS (2005) Glucose sensing via the protein kinase a pathway in *Schizosaccharomyces pombe*. Biochem Soc Trans 33:257–260
Hoffmann HP, Avers CJ (1973) Mitochondrion of yeast: ultrastructural evidence for one giant, branched organelle per cell. Sci 181(4101):749–751
Holland MJ, Holland JP (1978) Isolation and identification of yeast messenger ribonucleic acids coding for enolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase. Biochemistry (Mosc) 17:4900–4907
Hurowitz EH, Brown PO (2003) Genome-wide analysis of mRNA lengths in *Saccharomyces cerevisiae*. Genome Biol 5:R2
Ihmels J, Bergmann S, Gerami-Nejad M, Yanai I, McClellan M, Berman J et al (2005) Rewiring of the yeast transcriptional network through the evolution of motif usage. Science 309:938–940
Ihmels J, Friedlander G, Bergmann S, Sarig O, Ziv Y, Barkai N (2002) Revealing modular organization in the yeast transcriptional network. Nat Genet 31:370–377

Ikemura T (1981) Correlation between the abundance of Escherichia coli transfer RNAs and the occurrence of the respective codons in its protein genes: a proposal for a synonymous codon choice that is optimal for the E. coli translational system. J Mol Biol 151:389–409

Ikemura T (1982) Correlation between the abundance of yeast transfer RNAs and the occurrence of the respective codons in protein genes. Differences in synonymous codon choice patterns of yeast and Escherichia coli with reference to the abundance of isoaccepting transfer RNAs. J Mol Biol 158:573–597

Jensen RE et al (2000) Yeast mitochondrial dynamics: fusion, division, segregation, and shape. Microsc Res Tech 51(6):573–583

Jiang C, Pugh BF (2009) Nucleosome positioning and gene regulation: advances through genomics. Nat Rev Genet 10:161–172

Jiang H, Guan W, Pinney D, Wang W, Gu Z (2008) Relaxation of yeast mitochondrial functions after whole-genome duplication. Genome Res 18:1466–1471

Johnston M (1987) A model fungal gene regulatory mechanism: the GAL genes of *Saccharomyces cerevisiae*. Microbiol Rev 51:458–476

Kellis M, Birren BW, Lander ES (2004) Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. Nature 428:617–624

Kozak M (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 15:8125–8148

Kurtzman CP, Robnett CJ (2003) Phylogenetic relationships among yeasts of the 'Saccharomyces complex' determined from multigene sequence analyses. FEMS Yeast Res 3:417–432

Lee W, Tillo D, Bray N, Morse RH, Davis RW, Hughes TR et al (2007) A high-resolution atlas of nucleosome occupancy in yeast. Nat Genet 39:1235–1244

Leskovac V, Trivic S, Pericin D (2002) The three zinc-containing alcohol dehydrogenases from baker's yeast, *Saccharomyces cerevisiae*. FEMS Yeast Res 2:481–494

Lin Z, Li WH (2012) Evolution of 5′ untranslated region length and gene expression reprogramming in yeasts. Mol Biol Evol 29:81–89

Lin Z, Li WH (2011a) The evolution of aerobic fermentation in *Schizosaccharomyces pombe* was associated with regulatory reprogramming but not nucleosome reorganization. Mol Biol Evol 28:1407–1413

Lin Z, Li WH (2011b) Expansion of hexose transporter genes was associated with the evolution of aerobic fermentation in yeasts. Mol Biol Evol 28:131–142

Lin Z, Wang TY, Tsai BS, Wu FT, Yu FJ, Tseng YJ et al (2013) Identifying cis-regulatory changes involved in the evolution of aerobic fermentation in yeasts. Genome Biol Evol 5(6):1065–1078

Lin Z, Wu WS, Liang H, Woo Y, Li WH (2010) The spatial distribution of cis regulatory elements in yeast promoters and its implications for transcriptional regulation. BMC Genom 11:581

Liti G, Louis EJ (2005) Yeast evolution and comparative genomics. Annu Rev Microbiol 59:135–153

Lohr D, Venkov P, Zlatanova J (1995) Transcriptional regulation in the yeast GAL gene family: a complex genetic network. FASEB J 9:777–787

Mager WH, Planta RJ (1991) Coordinate expression of ribosomal protein genes in yeast as a function of cellular growth rate. Mol Cell Biochem 104:181–187

Martchenko M, Levitin A, Hogues H, Nantel A, Whiteway M (2007) Transcriptional rewiring of fungal galactose-metabolism circuitry. Curr Biol 17:1007–1013

Merico A, Sulo P, Piskur J, Compagno C (2007) Fermentative lifestyle in yeasts belonging to the Saccharomyces complex. FEBS J 274:976–989

Mignone F, Gissi C, Liuni S, Pesole G (2002) Untranslated regions of mRNAs. Genome Biol 3, REVIEWS0004

Miura F, Kawaguchi N, Sese J, Toyoda A, Hattori M, Morishita S et al (2006) A large-scale full-length cDNA analysis to explore the budding yeast transcriptome. Proc Natl Acad Sci U S A 103:17846–17851

Moller K, Christensen B, Forster J, Piskur J, Nielsen J, Olsson L (2002) Aerobic glucose metabolism of *Saccharomyces kluyveri*: growth, metabolite production, and quantification of metabolic fluxes. Biotechnol Bioeng 77:186–193

Moller K, Langkjaer RB, Nielsen J, Piskur J, Olsson L (2004) Pyruvate decarboxylases from the petite-negative yeast *Saccharomyces kluyveri*. Mol Genet Genomics 270:558–568

Mulder W, Scholten IH, Grivell LA (1995) Distinct transcriptional regulation of a gene coding for a mitochondrial protein in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* despite similar promoter structures. Mol Microbiol 17:813–824

Mulder W, Winkler AA, Scholten IH, Zonneveld BJ, de Winde JH, Yde Steensma H et al (1994) Centromere promoter factors (CPF1) of the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* are functionally exchangeable, despite low overall homology. Curr Genet 26(3):198–207

Nagalakshmi U, Wang Z, Waern K, Shou C, Raha D, Gerstein M et al (2008) The transcriptional landscape of the yeast genome defined by RNA sequencing. Science 320:1344–1349

Naumov GI, James SA, Naumova ES, Louis EJ, Roberts IN (2000) Three new species in the *Saccharomyces sensu stricto* complex: *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*. Int J Syst Evol Microbiol 50(Pt 5):1931–1942

Otterstedt K, Larsson C, Bill RM, Stahlberg A, Boles E, Hohmann S et al (2004) Switching the mode of metabolism in the yeast *Saccharomyces cerevisiae*. EMBO Rep 5:532–537

Ozcan S, Johnston M (1999) Function and regulation of yeast hexose transporters. Microbiol Mol Biol Rev 63:554–569

Papp B, Pal C, Hurst LD (2004) Metabolic network analysis of the causes and evolution of enzyme dispensability in yeast. Nature 429:661–664

Pesole G, Mignone F, Gissi C, Grillo G, Licciulli F, Liuni S (2001) Structural and functional features of eukaryotic mRNA untranslated regions. Gene 276:73–81

Piskur J, Rozpedowska E, Polakova S, Merico A, Compagno C (2006) How did Saccharomyces evolve to become a good brewer? Trends Genet 22:183–186

Pritchard L, Kell DB (2002) Schemes of flux control in a model of *Saccharomyces cerevisiae* glycolysis. Eur J Biochem 269:3894–3904

Pronk JT, Yde Steensma H, Van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12:1607–1633

Reifenberger E, Freidel K, Ciriacy M (1995) Identification of novel HXT genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux. Mol Microbiol 16:157–167

Rozpedowska E, Hellborg L, Ishchuk OP, Orhan F, Galafassi S, Merico A et al (2011) Parallel evolution of the make-accumulate-consume strategy in Saccharomyces and Dekkera yeasts. Nat Commun 2:302

Scannell DR, Byrne KP, Gordon JL, Wong S, Wolfe KH (2006) Multiple rounds of speciation associated with reciprocal gene loss in polyploid yeasts. Nature 440:341–345

Scannell DR, Zill OA, Rokas A, Payen C, Dunham MJ, Eisen MB et al. (2011) The awesome power of yeast evolutionary genetics: new genome sequences and strain resources for the *Saccharomyces sensu stricto* genus. G3 (Bethesda) 1, 11–25

Schmitt HD, Ciriacy M, Zimmermann FK (1983) The synthesis of yeast pyruvate decarboxylase is regulated by large variations in the messenger RNA level. Mol Gen Genet 192:247–252

Sellick CA, Campbell RN, Reece RJ (2008) Galactose metabolism in yeast-structure and regulation of the leloir pathway enzymes and the genes encoding them. Int Rev Cell Mol Biol 269:111–150

Slot JC, Rokas A (2010) Multiple GAL pathway gene clusters evolved independently and by different mechanisms in fungi. Proc Natl Acad Sci U S A 107:10136–10141

Tavazoie S, Church GM (1998) Quantitative whole-genome analysis of DNA-protein interactions by in vivo methylase protection in E. coli. Nat Biotechnol 16:566–571

Thomson JM, Gaucher EA, Burgan MF, De Kee DW, Li T, Aris JP et al (2005) Resurrecting ancestral alcohol dehydrogenases from yeast. Nat Genet 37:630–635

Tirosh I, Barkai N (2008) Two strategies for gene regulation by promoter nucleosomes. Genome Res 18:1084–1091
Tirosh I, Sigal N, Barkai N (2010) Divergence of nucleosome positioning between two closely related yeast species: genetic basis and functional consequences. Mol Syst Biol 6:365
Tsankov AM, Thompson DA, Socha A, Regev A, Rando OJ (2010) The role of nucleosome positioning in the evolution of gene regulation. PLoS Biol 8:e1000414
van der Sluis C, Wolken WA, Giuseppin ML, Tramper J, Wijffels RH (2000) Effect of threonine, cystathionine, and the branched-chain amino acids on the metabolism of *Zygosaccharomyces rouxii**. Enzyme Microb Technol 26:292–300
Wolfe K (2004) Evolutionary genomics: yeasts accelerate beyond BLAST. Curr Biol 14: R392–R394
Wolfe KH, Shields DC (1997) Molecular evidence for an ancient duplication of the entire yeast genome. Nature 387:708–713
Wood V, Gwilliam R, Rajandream MA, Lyne M, Lyne R, Stewart A et al (2002) The genome sequence of *Schizosaccharomyces pombe*. Nature 415:871–880
Ye L, Kruckeberg AL, Berden JA, van Dam K (1999) Growth and glucose repression are controlled by glucose transport in *Saccharomyces cerevisiae* cells containing only one glucose transporter. J Bacteriol 181:4673–4675
Yuan GC, Liu YJ, Dion MF, Slack MD, Wu LF, Altschuler SJ et al (2005) Genome-scale identification of nucleosome positions in S. cerevisiae. Science 309:626–630

Chapter 6
Similarities and Differences Between Cancer and Yeast Carbohydrate Metabolism

Matic Legiša

Abstract At first glance, there is a high degree of similarity between the metabolisms of human cancer and yeast. Tumors consume larger amounts of glucose compared to normal tissues with most being converted into lactate and excreted, despite the abundant oxygen (Warburg effect). Similarly, yeast cells growing at high specific growth rates accumulate ethanol under aerobic conditions (Crabtree effect). However, advances in our knowledge during the last decade, particularly regarding cancer metabolism, have revealed some details that suggest differences between carbohydrate metabolisms in these two cell types. Although primary metabolism is fairly conservative between all living organisms, some differences have arisen through the course of evolution between the yeast and human cancer cells that were mostly impelled by the different environments these cells proliferate. In this chapter, the most important similarities and differences between cancer and yeast metabolism are outlined and discussed. Despite these differences, the yeast *Saccharomyces cerevisiae* is still a useful model for understanding many aspects of the cancer primary metabolism.

Abbreviations

MFS	Major facilitator superfamily
GLUT	Glucose transporter
HXT	Hexose transporter
HK	Hexokinase
PFK	6-Phosphofructo-1-kinase
TPI	Triose-phosphate isomerase
PK (human) PYK (yeast)	Pyruvate kinase
PDH	Pyruvate dehydrogenase complex
PDC	Pyruvate decarboxylase
PC	Pyruvate carboxylase

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_6, © Springer-Verlag Berlin Heidelberg 2014

LDH	Lactate dehydrogenase
ALD	Aldehyde dehydrogenase
c-Myc	Oncogenic transcription factor
HIF-α	Hypoxia inducible factor
TIGAR	TP53-inducible glycolysis and apoptosis regulator
TCA cycle	Tri-carboxylic acid cycle
ETC	Electron transport chain
OXPHOS	Oxidative phosphorylation
ROS	Reactive oxygen species

6.1 Introduction

The baker's or budding yeast *Saccharomyces cerevisiae* is most likely the best-studied organism and is often used as a model organism to study primary metabolism in other biological systems, including cancer (Guaragnella et al. 2014). As a single-cell organism, yeast is small and easy to cultivate, and its shortest generation time is less than 2 h (Lord and Wheals 1981). As a eukaryote, *S. cerevisiae* has a complex internal cell structure similar to that of animals and plants, but noncoding DNA regions are less common in *S. cerevisiae* than in higher eukaryotes. The genome of baker's yeast was also the first eukaryotic genome to be completely sequenced and publicly released in 1996 (Goffeau et al. 1996), and the genomic data has been updated regularly since then. Last but not least, yeast is an extremely important commercial microorganism; owing to its role in bioethanol production, yeast is the most widely used microbial cell factory.

In the past, *S. cerevisiae* has served as an excellent organism to study the metabolism of carbohydrates; this work began during the time of Louis Pasteur. These early studies have been nicely reviewed by Barnett and Entian (2005). Although the central carbon metabolism seems to be roughly similar in all eukaryotic organisms, there are differences that are particularly evident in the steps involved in pyruvate metabolism. Specifically, one feature of primary metabolism in yeast cells is their ability to excrete ethanol and carbon dioxide under anaerobic conditions. The phenomenon, known as the Pasteur effect, is characterized by suppression of alcoholic fermentation in the presence of oxygen in slowly growing *S. cerevisiae* cells (Pronk et al. 1996). It is believed to be caused by higher affinity (V_{max}/K_m) of the respiratory (aerobic) system for pyruvate via pyruvate dehydrogenase (PDH), than that of the fermentative (anaerobic) route via pyruvate decarboxylase (PDC) (Lagunas 1986). Baker's yeast cells can also excrete ethanol under aerobic conditions. A similar effect was first described in cancer cells by Crabtree (1929), who observed a modest (12 %) reduction in respiration after adding glucose to a solution of tumor tissue that was undergoing an abnormally high amount of aerobic glycolysis. In yeast cells, the phenomenon is most likely

triggered by an "insufficient capacity of respiratory routes of pyruvate dissimilation" (Postma et al. 1989), which starts ethanol excretion—and is named after the author who first described a similar event in cancer cells as a "Crabtree effect."

Indeed, at first glance, there appears to be some similarity between the metabolisms of cancer and yeast. Rapidly growing human cancers are characterized by the consumption of more glucose than normal tissues, with most of this glucose being converted into lactate and excreted, despite an abundance of oxygen. The phenomenon was first described in 1925 (Warburg 1925) and is known as the Warburg effect. Similarly, yeast cells growing at high specific growth rates accumulate ethanol under aerobic conditions (the Crabtree effect), but are there in fact similar or identical underlying metabolic processes in these organisms?

6.2 External Factors That Determine Growth

For a better understanding of the metabolism of cancer and yeast, the environmental growth conditions of both types of cells should be described first. Tumors develop and grow in multicellular metazoic organisms; under these conditions, the cells are more or less constantly supplied with nutrients. However, the growth and proliferation of normal human cells is under the strict control of specific growth factors to preserve the function and shape of the body. On the other hand, one of the hallmarks of cancer cells is their ability to sustain proliferative signaling (Hanahan and Weinberg 2011); in other words, they do not need growth factors to induce growth and duplication, and their growth is thus unrestricted. In contrast, yeast cells must survive in the environment as unicellular organisms where they are exposed to huge fluctuations in nutrient availability. Their metabolic machinery must sense an adequate supply of nutrients and rapidly adjust to such changes. When nutrients are abundant, yeast cells synthesize cellular building blocks to reproduce as quickly as possible, and when supplies of carbon or nitrogen sources are limited, their metabolism must go idle. Through the course of evolution, environmental conditions favored selection for yeast cells with higher specific growth rates and shorter doubling times (Hagman et al. 2013), but the specific growth rates of human cells are significantly slower (Friberg and Mattson 1997).

S. cerevisiae can duplicate within 1–2 h (Lord and Wheals 1981), but the actual doubling times depend on the availability of nutrients and the fastest specific growth rates can only be reached under optimal growth conditions. Human cells grow significantly slower. Rapidly growing tumors, such as testicular carcinomas, pediatric tumors, and some mesenchymal tumors, have doubling times measured in days, but cancers from the breast, prostate, and colon are frequently slow-growing, displaying doubling times measured in months or years (Friberg and Mattson 1997). Cancer, as a typical genetic disease, is initiated by the accumulation of mutations during the process of tumorigenesis. Recent comprehensive sequencing has revealed the genomic landscapes of common forms of human cancer. The studies have discovered approximately 140 genes that can promote or

drive tumorigenesis when altered by intragenic mutations. A typical tumor contains 2–8 of these "driver" mutations that provide a selective growth advantage for cancer cells, and the remaining mutations are known as passengers and confer no selective advantage (Vogelstein et al. 2013). It has been estimated that each "driver" mutation has a surprisingly small contribution to the overall selective growth advantage in a cell, of the order of a 0.4 % increase in the difference between the birth and death of a cell (Bozic et al. 2010). However, some somatic mutations in human tumors cause the constitutive activation of signaling circuits that are normally triggered by activated growth factor receptors. Therefore, it seems that cancer cells do not gain a significantly faster growth rate during tumorigenesis but that they can proliferate with a rate similar to that of normal human embryonic cells.

The key oncogenic signaling pathway that affects metabolism in cancer cells is the PI3K/Akt/mTOR pathway. This pathway becomes constitutively active after specific oncogenes are mutated and enhances many of the metabolic activities that enable fast proliferation in a growth factor independent way (DeBerardinis et al. 2008). It is important to realize that each individual tumor is distinct with respect to its genetic alternations (Vogelstein et al. 2013); this suggests that mutations in different components of the PI3K/Akt/mTOR signaling pathway can lead to the constitutive activation. An active PI3K/Akt/mTOR pathway has a stimulatory impact on cancer biosynthesis at three distinct levels: First, it stimulates cells to increase the expression of nutrient transporters in the membrane, promoting a more rapid uptake of glucose, amino acids, and other nutrients (Roos et al. 2007). Second, Akt kinase upgrades glycolysis by increasing the activity of the c-Myc and HIF-1α transcription factors that promote the expression of a majority of the glycolytic genes (Yeung et al. 2008; Elstrom et al. 2004). Finally, activation of this pathway enhances the biosynthesis of cellular building blocks. For example, mTOR is involved in the regulation of protein translation initiation (Gingras et al. 2001).

In yeast, no mutations or growth factors are needed to affect primary metabolism, but minor changes in nutrient availability can have significant consequences. The most profound effect can be observed by adding glucose to glucose-starved cells in a well-aerated system. Glucose is a highly desired carbon and energy source for yeasts and can be rapidly oxidized under both aerobic and anaerobic conditions. Within seconds after glucose enters the cells, it is rapidly metabolized by the accelerated glycolysis and ultimately causes a saturation of the respiratory pathway. As a consequence of metabolic blockage, ethanol starts to be excreted out of the cells (a short-term Crabtree effect) (Van Urk et al. 1990). A similar long-term Crabtree effect can be observed under growth conditions that support the fast growth of yeast cells, where again the respiratory metabolism has insufficient capacity to deal with the underlying catabolic reactions, especially pyruvate dissimilation (Pronk et al. 1996). Glucose also acts as a signal molecule. The stimulus that is generated by the glucose is transmitted through the cell to specific targets by the Ras/cAMP/PKA nutrient signaling pathway (Thevelein and de Winde 1999).

Glucose triggers the activation of cellular growth by regulating the expression of several glucose transporters and glycolytic genes (DeRisi 1997), induces the mobilization of storage compounds (Verstrepen et al. 2004), and causes the diminution of cellular stress-resistance (Marchler et al. 1993). There is another important signaling pathway controlled by glucose in *S. cerevisiae* that tailors primary metabolism: the glucose repression pathway that, after transmitting a signal through the cascade of specific kinases, represses the expression of the genes involved in breakdown of alternative carbon sources and genes involved in gluconeogenesis (Thevelein and de Winde 1999). However, it is important to realize that glucose signaling pathways are only induced when the glucose or sucrose concentration exceeds a threshold of 20–40 mM (Meneses et al. 2002). In fact, nutritional glucose acts like a hormone in *S. cerevisiae* (Verstrepen et al. 2004) and exhibits a role similar to that of growth factors in mammalian cells.

6.3 Primary Metabolism

For both mammalian and yeast cells, glucose is the preferred carbon and energy source. Glucose can enter the cells by a number of specific transmembrane carriers that are expressed under specific circumstances; the nature and function of transporters often defines the overall cellular metabolism because the first and often limiting step of glucose metabolism is its transport across the plasma membrane.

6.3.1 Sugar Transporters

A total of 14 glucose transporters (GLUT) are expressed in various human tissues and cell types, and their physiological roles have been recently extensively reviewed (Mueckler and Thorens 2013). In a wide variety of tumors, GLUT1 overexpression has been reported, and an increase in GLUT1 is likely to be an essential step in tumor progression (Younes et al. 1996). HIF-1α (hypoxia inducible factor) was reported to be involved in overexpression of GLUT1; this overexpression enables fast-growing cancer cells to acquire energy by harnessing glycolysis, even under hypoxic conditions (Keith and Simon 2007).

S. cerevisiae has 20 genes that encode proteins similar to glucose (hexose) transporters. These hexose transporters (HXT) proteins belong to the major facilitator superfamily (MFS) of transporters (Pao et al. 1998). *S. cerevisiae* has the largest number of MFS transporters of any organism studied so far. MFS proteins transport their substrates by passive, energy-independent facilitated diffusion, with glucose moving down its concentration gradient (Pao et al. 1998).

In general, two uptake systems have been described in *S. cerevisiae*: a constitutive, low affinity system (high K_m, 15–20 mM), and a glucose-repressed high-affinity system (low K_m, 1–2 mM). It now seems clear that the low- and high-affinity

GLUT activities represent the sum of several transporters rather than being the result of individual transporters. The presence of multiple HXTs with different affinities for glucose in baker's yeast is not surprising, given the fact that it grows well in a broad range of glucose concentration (from a few μM to 2 M) (Ozcan and Johnston 1999).

Therefore, it could be stated that glucose transport, both in cancer and yeast cells, does not represent any obstacle for the substantial fueling of the glycolytic flux.

6.3.2 Glycolytic Flux

Glycolysis is central to primary metabolism and is normally tightly regulated by three allosteric enzymes: hexokinase (HK), 6-phosphofructo-1-kinase (PKF1), and pyruvate kinase (PK), which catalyze individual irreversible steps.

6.3.2.1 Hexokinase

Hexokinase is the first regulatory step of glycolysis and is normally feedback-inhibited by its own product, glucose-6-phosphate. In human cells, four different HK isoenzymes can be found, and one, HK2, is predominant in cancer cells (Chen and Russo 2012). HK2 specifically binds to the mitochondrial outer membrane facing the cytosol (Mathupala et al. 2009), where it has preferential access to newly synthesized ATP for the phosphorylation of glucose; additionally, HK2 is not sensitive to inhibition by its own product, glucose-6-phosphate (Mathupala et al. 2009).

S. cerevisiae possess three different HK isoenzymes (HK I, II, and glucokinase) that are not inhibited by their own product (Muratsubaki and Katsume 1979). While HKI and glucokinase are involved predominantly in the glycolytic flux under aerobic conditions, HKII prevails during fermentation where it phosphorylates glucose (Muratsubaki and Katsume 1979) and regulates glucose repression (Gancedo 1998).

6.3.2.2 6-Phosphofructo-1-Kinase

The most complex control over glycolytic flux is attributed to (PFK1), which surmounts the regulatory roles of the other two allosteric enzymes. PFK1 catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, using MgATP as a phosphoryl donor (Dunaway 1983). PFK1 is stimulated by fructose-2,6-bisphosphate (F-2,6-BP), ADP/AMP and ammonium ions, whereas citrate and ATP act as strong inhibitors (Dunaway 1983).

During the course of evolution, eukaryotic PFK1 enzymes have developed by duplication, tandem fusion, and divergence of the catalytic and effector binding sites of a prokaryotic ancestor (Poorman et al. 1984). However, the strict

conservation between the active site residues in the N-terminal segment of the eukaryotic enzyme and those of bacterial PFKs suggests that the active site of eukaryotic PFK1 is located only in the N-terminal portion (Poorman et al. 1984). In contrast, the allosteric ligand-binding sites that developed during evolution by mutations in the C-terminus enable fine-tuning of the regulatory enzyme by the elevated levels of specific downstream metabolites. One of the allosteric ligands is citrate, which acts as a potent inhibitor of all the mammalian PFK1 isoforms (Usenik and Legiša 2010).

In mammalian genomes, three different PFK1 genes are present and are differentially expressed in individual tissues. In human tissues, their protein products have the following molecular masses: muscle type (PFK-M), 85,051 Da (Yamasaki et al. 1991); liver type (PFK-L) 84,917 Da (Elson et al. 1990); and platelet type (PFK-P), 85,596 Da (Eto et al. 1994).

All three isoenzymes are strongly inhibited by citrate, with IC_{50} values of 0.08, 0.13, and 0.18 mM for brain (platelet), muscle, and liver PFK1, respectively (Vora et al. 1985). All the human PFK1 isoforms are also reported to be intensely inhibited by ATP concentrations higher than 0.05 mM, yet F-2,6-BP can antagonize the negative effects of ATP to some extent (Dunaway 1983).

In cancer cells, the activity of the PFK1 enzymes is upregulated by the loss of p53 function, which results in the downregulation of the TIGAR protein, which in turn acts as a fructose-2,6-bisphosphatase (Bensaad et al. 2006). Consequently, the level of F-2,6-BP remains high in tumors and acts as a strong positive stimulus.

The expression of PFK1 genes is enhanced in cancer cells due to the increased activity of the HIF-1α transcription factor, which, in combination with c-Myc, enhances the synthesis of the majority of glycolytic enzymes (Huang 2008). Increased amounts of the wild-type enzymes consequently result in increased specific activities. However, the glycolytic flux in eukaryotic organisms is tightly controlled by allosteric enzymes that maintain their regulation by feedback inhibition despite the elevated activities of intermediary enzymes. Among other effectors, citrate has been reported to play a vital role in suppressing the enzymatic activity of PFK1. Analyses of the variations in allosteric binding sites between different eukaryotic organisms revealed that stronger inhibition of PFK1 enzymes by citrate has developed during evolution, enabling better control over glucose consumption in the slow-growing somatic cells of higher metazoans (Usenik and Legiša 2010). Therefore, one is forced to conclude that important modifications to the kinetics of regulatory enzymes must also be involved in the metabolic changes that occur during the transformation of normal mammalian cells into cancer cells.

Recently, it has been reported that the human 85 kDa native protein PFK of muscular type (PFK-M) is subjected to posttranslational modification (Šmerc et al. 2011). Proteolytic cleavage of the C-terminal portion of PFK-M led to an active, shorter 47 kDa fragment that was insensitive to citrate and ATP inhibition. More importantly, only the short 47 kDa fragment but not the native 85 kDa PFK-M was detected in tumorigenic cell lines, including B16-F10 mouse melanoma cells, HeLa carcinoma cells and two lymphomas, the rat Nb2-11 line and the human TF-1 line. Similar fragments were also detected in tumor tissue that developed in

mice after the subcutaneous infection with tumorigenic B16-F10 cells. The insertion of modified truncated human *pfk*M genes also stimulated glucose consumption and lactate excretion in stable transfectants of nontumorigenic human HEK cells, suggesting an important role of these shorter PFK1 fragments in enhancing the glycolytic flux (Šmerc et al. 2011). Thus, posttranslational modification of the PFK-M enzyme might be the pivotal factor of deregulated glycolytic flux in tumors that, in combination with altered signaling mechanisms, essentially supports the fast proliferation of cancer cells.

In contrast to the cancer PFK1 enzymes, the *S. cerevisiae* enzyme is constitutively expressed under the control of two promoters that are not controlled by regulatory elements (Heinisch et al. 1991). However, the expression of some other glycolytic genes (e.g., *pgk, eno*2, *pyk*, *pdc*) is induced by glucose (Chambers et al. 1995). The RAP1 DNA binding protein was found to be involved in transcriptional control; moreover, the transcription activation function of RAP1 can be increased as a result of phosphorylation by cAMP-dependent protein kinase (PKA), triggered by the presence of glucose (Klein and Struhl 1994). The kinetic measurements of yeast PFK1 displayed a less pronounced inhibition of the enzyme by citrate (apparent Ki = 3.5 mM) (Yoshino and Murakami 1982) than was observed in cancer cells. It seems that the most important control of the key glycolytic enzyme is mediated by the fructose-2,6-bisphosphate (F-2,6BP), which is the most potent activator of eukaryotic PFK1 enzymes and an inhibitor of fructose-1,6-bisphosphatase. F-2,6-BP is synthesized by 6-phosphofructo-2-kinase (PFK2), which was shown to be phosphorylated and activated by increased extracellular glucose concentrations. The Ras-cAMP signaling pathway has been reported to mediate phosphorylation and activation of PFK2 (Dihazi et al. 2003). Another enzyme, fructose-2,6-bisphosphatase (FBPase-2) affects the levels of F-2,6-BP in yeast cells; FBPase-2 is not needed to sustain an adequate glycolytic flux under fermentative conditions, but rather maintains the homeostasis of metabolite concentrations (Müller et al. 1997). Interestingly, 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase are two separate enzymes in *S. cerevisiae* (Kretschmer et al. 1987); this is not the case in mammalian cells, where a single homodimeric bifunctional enzyme PFK2/FBPase-2 is responsible for both the synthesis and degradation of F-2,6-BP (Rider et al. 2004).

6.3.2.3 Pyruvate Kinase

Pyruvate kinase, another regulatory enzyme of the glycolytic pathway, catalyzes the conversion of phospho-enol-pyruvate (PEP) and ADP into ATP and pyruvate. Humans have two PK genes (*PKLR* and *PKM2*) and four tissue-specific isoenzymes (L, R, M1, and M2). PK-L and PK-R are expressed from alternative PKLR promoters in liver and erythroid cells, respectively. PK-M1 and PK-M2 are formed after alternative splicing of PKM2 transcripts. PK-M1 can be found in skeletal muscle, heart, and brain, whereas PK-M2 is predominantly expressed in the fetus and rapidly proliferating cells, including cancer cells (Netzker et al. 1992). PK-M2

can adopt two different quaternary structures: a highly active tetramer favoring the formation of pyruvate and ATP, and a less active dimer, which predominates in tumor cells (Mazurek et al. 2005). PK-M2 is allosterically activated by fructose-1,6-bisphosphate (FBP), which was found to have a significantly higher concentration in cancer cells than in normal human cells (Lu et al. 2010). Indeed, FBP can trigger the re-association of PK-M2 into a tetramer (Mazurek et al. 2005). However, PK-M2 can also be negatively regulated by binding to phosphotyrosine-containing proteins. But again, a concentration of FBP above 20 μM is able to compete for binding of recombinant PK-M2 to phosphotyrosine peptides (Christofk et al. 2008). Because FBP can reach very high levels in tumor cells, a possible predominance of tetrameric form of PK-M2 under physiological conditions and not the dimeric form can be assumed in cancer cells during aerobic glycolysis. However, further research will be needed in the future to clarify this issue.

In yeast cells, initially only one gene (*Pyk*1) encoding the PK was discovered (Burke et al. 1983). The biochemical properties of yeast PYK1 protein suggest that it plays a central regulatory role in carbon metabolism during the transition between glycolysis and gluconeogenesis, when sugars are abundant. Specifically, the enzyme is positively regulated by FBP, which is present at high levels intracellularly during glycolysis and at low levels after the depletion of sugars from the medium. After the completion of the yeast genome sequencing project, a new open reading frame on chromosome XV was found with high degree of similarity to the *Pyk*1 sequence (Purnelle and Goffeau 1996). More detailed analyses of the *Pyk*2 gene revealed that its expression is subject to glucose repression while its enzymatic activity is insensitive to FBP activation. It seems that the PYK2 may be the predominant enzyme under the conditions of very low glycolytic flux (Boles et al. 1997).

6.3.3 Pyruvate Metabolism

In cancer cells, glycolysis significantly outpaces the maximal rate of pyruvate oxidation, so the cells must eliminate pyruvate by converting it into lactate. Using ^{13}C-nuclear magnetic resonance spectroscopy measurements to study glioblastoma cell metabolism under aerobic glycolysis, it has been shown that up to 90 % of glucose has been converted into lactate and alanine, leaving a moderate amount of pyruvate to enter the mitochondrial matrix (DeBerardinis et al. 2007). The conversion of pyruvate into lactate involves the enzyme lactate dehydrogenase (LDH), whose synthesis is enhanced by the c-Myc and HIF-1α transcription factors in the cells with constitutively active PI3K/Akt/mTOR signaling pathway (Shim et al. 1997). HIF-1α also promotes the expression of pyruvate dehydrogenase kinase 1 (PDK1) to inhibit pyruvate dehydrogenase (PDH) activity (Kim et al. 2006), which further contributes to the diminished mitochondrial metabolism of glucose. On the other hand, the significance of another enzyme that may be responsible for the entry of pyruvate into the TCA cycle—pyruvate carboxylase (PC) was found to be negligible in glioblastoma cells (DeBerardinis et al. 2007).

Lactate accumulation in tumors, similar to ethanol excretion by yeast cells, is the result of a dissimilative event where glycolytic NADH is reoxidized to NAD^+ while pyruvate and acetaldehyde, respectively, function as terminal electron acceptors. Lactate formation and excretion is, therefore necessary for the cells with enhanced glycolysis to maintain NADH/NAD^+ redox balance. Tests with LDH-null tumor cell lines showed severely decreased tumorigenesis, indicating that LDH plays a prominent role in tumor proliferation and progression (Fantin et al. 2006). Because it is a weak acid, lactate proves to be toxic when accumulated in the extracellular matrix but it may be consumed by some nontumorigenic cells in tumor tissue as a carbon source. Tumor-associated fibroblasts express high levels of proteins involved in lactate absorption (MCT1/MCT2) and lactate oxidation (LDH1) and reduced levels of proteins involved in glucose transport (GLUT1) (Koukourakis et al. 2006). It seems that cancer cells and nontumorigenic cells have harmoniously collaborating metabolisms that enable the survival of the tumor tissue. Extracellular lactate was found also to inhibit the differentiation of monocytes to dendritic cells and to inactivate the cytokine release from dendritic and cytotoxic T cells (Hirschhaeuser et al. 2011), the key players in anti-tumoral immune response. The conceptual progress in the study of the immune response to cancer development in the human body has recently added the ability of tumors to evade immune destruction to the list of hallmarks of cancer (Hanahan and Weinberg 2011).

In the yeast *S. cerevisiae*, pyruvate metabolism and the regulation of fluxes at the pyruvate branch point have been extensively studied in the past and were reviewed by Pronk et al. (1996). There are three enzymes that play crucial roles in the assimilation or dissimilation of pyruvate: pyruvate dehydrogenase complex (PDH), which enables the direct decarboxylation of pyruvate to acetaldehyde; (PDC), which catalyzes the decarboxylation of pyruvate to acetaldehyde; and (PC), which promotes the formation of oxaloacetate by the anaplerotic carboxylation of pyruvate. Several authors have proposed that the intracellular concentration of pyruvate itself is an important factor in the regulation of fermentative and respiratory dissimilation. That is, the K_m value of the PDH complex is lower than that of PDC (Boiteux and Hess 1970; Kresze and Ronft 1981). However, PDH as a mitochondrial and PDC as a cytosolic enzyme are located in different subcellular compartments and are therefore unable to evenly compete for pyruvate (Van Urk et al. 1989). According to Holzer's model, PDC is largely bypassed at low intracellular pyruvate concentrations, enabling the respiratory dissimilation of pyruvate by the PDH complex during aerobic conditions, accompanied by low specific growth rates (Holzer 1961). In contrast, high intracellular concentrations of pyruvate enable higher PDC activity, triggering alcoholic fermentation under anaerobic conditions and during aerobiosis at high specific growth rates (a long-term Crabtree effect) (Pronk et al. 1996). The PDC enzyme produces acetaldehyde which can be either reduced to ethanol or oxidized to acetate. The K_m of the acetaldehyde dehydrogenase for acetaldehyde is 100-fold lower than that of ethanol dehydrogenase, suggesting that acetate is formed under conditions where no ethanol is produced. Acetate is then transformed into acetyl-CoA by acetyl-CoA synthetase, which enters the mitochondria to bypass the PDH pathway. At slightly

higher specific growth rates, acetate accumulates extracellularly as a result of insufficient activity of acetyl-CoA synthetase, which is required for the complete oxidation of acetate, and ultimately, ethanol formation results from an insufficient activity of acetaldehyde dehydrogenase (Postma et al. 1989). PC in yeast cells appears to be predominantly regulated by the concentrations of substrate and effectors, rather than by changes in enzyme synthesis. An increase in the cytosolic pyruvate concentration, accompanied by an increased growth rate, primarily increases the flux through PDC (K_m 5 mM), but the PC activity is also accelerated (K_m 0.8 mM) (Ruiz-Amil et al. 1965). Therefore, the anaplerotic rate of oxaloacetate formation must increase linearly with the growth rate. The increased flux through PDC may be a direct consequence of the requirement for an increased rate of oxaloacetate formation by PC (Pronk et al. 1996).

6.3.4 Tri-Carboxylic Acid Cycle

The major route of entry of pyruvate into the TCA cycle of glioblastoma cells is through PDH, while the role of PC is negligible (DeBerardinis et al. 2007). The active TCA cycle can be detected by ^{13}C-NMR spectroscopy analysis but it is characterized by an efflux of substrates for use in biosynthetic pathways, particularly fatty acid synthesis. Mitochondrial citrate functions as a precursor for fatty acid synthesis that must be first exported out of mitochondria and subsequently processed by citrate lyase to acetyl-CoA, which is channeled into lipid production. Spectroscopic measurements show that approximately 60 % of the lipogenic acetyl-CoA pool is derived from glucose, while the rest is believed to be formed from glutamine (DeBerardinis et al. 2007). The consumption of citrate for lipid synthesis results in a net loss of oxaloacetate (OAA), the acceptor for pyruvate derived acetyl-CoA, which must be regenerated to maintain the integrity of the TCA cycle. Metabolic flux analyses have revealed that glutamine, the most abundant amino acid in the mammalian blood serum, is used by rapidly growing cancer cells to generate the pool of α-ketoglutarate, which can be metabolized through the TCA cycle to generate OAA. In fact, glutamine is an important source of energy for proliferating cancer cells (DeBerardinis et al. 2008). However, the majority of glutamine (approximately 60 %) is converted into lactate and alanine, two molecules that are largely excreted from the cells as waste (DeBerardinis et al. 2007).

In yeasts, the TCA cycle enzymes are fully functional during growth on nonfermentable carbon sources, and the addition of glucose to the medium represses the activities of some mitochondrial enzymes. Isocitrate lyase, an enzyme of the glyoxylate shunt, is inactivated by phosphorylation mediated by cAMP-dependent protein kinase (PKA) that is triggered by a transient peak in cAMP concentration after sensing glucose or sucrose (Ordiz et al. 1996). Similarly, the inactivation of the cytosolic malate dehydrogenase isoenzyme has been shown to be induced by phosphorylation after a glucose pulse, but no specific kinase has been identified

(Minard and McAlister-Henn 1994). No reports are available in the literature on the changes in the activities of other TCA cycle enzymes in *S. cerevisiae*, suggesting a poor regulation of these enzymes by environmental factors.

6.3.5 Balancing the NADH/NADPH Ratio

In tumors, rapidly proliferating cells demand a stable supply of ATP and reducing power in the form of NADPH. When there is enough ATP formed by substrate-level phosphorylation during accelerated glycolysis, NADPH may become the limiting factor of growth. However, during glutaminolysis, malate must be oxidized to pyruvate by $NADP^{+}$-specific malate dehydrogenase (malic enzyme) in the cytosol; this enzyme enables the robust production of NADPH, the reducing power urgently needed as the electron donor primarily for fatty acid synthesis (Vander Heiden et al. 2009). Cancer cells are particularly sensitive to glutamine withdrawal because the glutamine depletion was found to trigger apoptosis after the drop in TCA cycle intermediates (Yuneva et al. 2007). In tumors, glutaminolysis is driven by the c-Myc oncogene, and the genes involved in glutamine metabolism appear to be under both the direct and indirect control of the c-Myc transcription factor (Wise et al. 2008; Gao et al. 2009).

In the yeast *S. cerevisiae*, acetate formation plays a significant role in NADPH/NADH balance. During rapid aerobic growth of cells, a notable amount of acetic acid is produced in addition to ethanol. The acid continues to accumulate after glucose exhaustion and ethanol oxidation. At higher specific growth rates (higher dilution rates in a chemostat), acid production is considerable, notably in the phase of ethanol consumption, when the alcohol is almost quantitatively converted to acetic acid (Dijken and Scheffers 1986). It is important to realize that acetic acid production results in the formation of NADH or NADPH by the isoforms of acetaldehyde dehydrogenase. Thus, acetic acid production by the NADH dependent mitochondrial isoform of acetaldehyde dehydrogenase (ALD4p) would indicate a limitation in another NADH-producing pathway, such as the TCA cycle, providing that the respiratory capacity to reoxidize the formed NADH is not saturated. Acetic acid production by the NADPH-dependent cytosolic isoenzyme (ALD6p) indicates a limited NADPH production, such as by the pentose phosphate pathway (Ferreira et al. 2004).

6.4 Respiration

Over 80 years ago, Otto Warburg hypothesized that in addition to deregulated glycolysis, mitochondrial respiration is irreversibly damaged in cancer (Warburg 1956). Indeed, it was later confirmed that the mitochondrial number and oxidative phosphorylation (OXPHOS) are downregulated in most cancers (Modica-Napolitano et al. 2007). The main reason for OXPHOS disfunction seems to be the mutations that

occur at different levels. For example, the mitochondrial OXPHOS enzyme succinate dehydrogenase (SDHD, complex II) is shown to be mutated in paragangliomas and pheochromocytomas (Baysal et al. 2000). Complex II and IV activities decrease in some hepatoma cell lines (Sun and Cederbaum 1980), and mitochondrial DNA mutations have been increasingly identified in some colorectal tumors (Polyak et al. 1998). However, it was proposed that injury to OXPHOS induces a mitochondrial checkpoint response, which regulates reversible epigenetic modification (such as DNA methylation) and irreversible genetic changes in the nuclear genome (Chandra and Singh 2011). No reports could be found in the literature on the allosteric inhibition of respiratory complexes in cancer cells that would downregulate OXPHOS and therefore contribute to the overflow of lactate.

On the other hand, in Crabtree-positive yeasts (FBP), an intermediate of glycolysis, was found to inhibit the respiration of isolated mitochondria. More precisely, FBP in physiological concentrations decreased the activity of mitochondrial complexes III and IV (Díaz-Ruiz et al. 2008). The importance of this finding can be supported by the fact that no such phenomenon could be detected in Crabtree-negative yeasts.

6.5 Ros

High specific growth rates supported by nutrient excess in the environment can cause a nutrient stress both in metazoans and unicellular organisms. Under growth conditions with an unlimited supply of nutrients, intracellular intermediates are elevated. When the breakdown of metabolites in the TCA cycle exceeds the capacity of the electron transport chain (ETC) of OXPHOS to assimilate the resulting electrons, an increased production of reactive oxygen species (ROS) is detected, including H_2O_2, superoxide anion, and hydroxyl radical (Wellen and Thompson 2010). Therefore, an elevated $NADH/NAD^+$ ratio is the major factor leading to increased mitochondrial production of ROS. ROS cause wide-ranging damage to macromolecules, resulting in genetic degeneration and physiological disfunction, and eventually lead to cell death (Gutteridge 1993).

In cancer cells, the stalling of the ETC due to mutated OXPHOS genes can cause a buildup of electrons and enhanced production of superoxide (Brandon et al. 2006). Mitochondrial ROS production increases with oncogene-induced metabolic stress (the Warburg effect), and substantial evidence indicates that elevated ROS levels can further promote tumorigenesis (Halliwell 2007). Highly reactive ROS can, therefore, promote cancer by increasing DNA mutations, regulating signaling and transcription, and promoting inflammation (Wellen and Thompson 2010).

In yeasts, similar nutrient stress may occur when nutrients are in excess, and concomitant ROS overproduction can even trigger apoptosis (Madeo et al. 1999). Interestingly, Crabtree-positive yeasts can reach higher specific glucose consumption rates than Crabtree-negative yeasts, while growth rate is similar among

both types of yeasts (Hagman et al. 2013). Therefore, it is tempting to speculate that ethanol overflow by Crabtree-positive yeasts may be a mechanism to prevent metabolic stress and excessive ROS formation. It seems that two mechanisms are needed to efficiently prevent ROS formation in Crabtree-positive yeasts: (i) pyruvate metabolism toward the ethanol production and (ii) inhibition of respiratory Complex III by elevated levels of FBP that are characteristic of rapid growth forcing the cells to reoxidize mitochondrial NADH at the level of ethanol dehydrogenase (Díaz-Ruiz et al. 2008). Recently, another mechanism of controlling redox metabolism in yeast was described with pyruvate kinase (PYK) as a key player. A shift from fermentative to oxidative metabolism caused a decrease in PYK activity that prevented the increase in ROS formation. This adaptation was attributable to accumulation of the PYK substrate phosphor-enol-pyruvate that acted as a feedback inhibitor of glycolytic enzyme triose-phosphate-isomeraze (TPI). TPI inhibition stimulated the pentose phosphate pathway, increased antioxidative metabolism, and prevented ROS accumulation (Grüning et al. 2011).

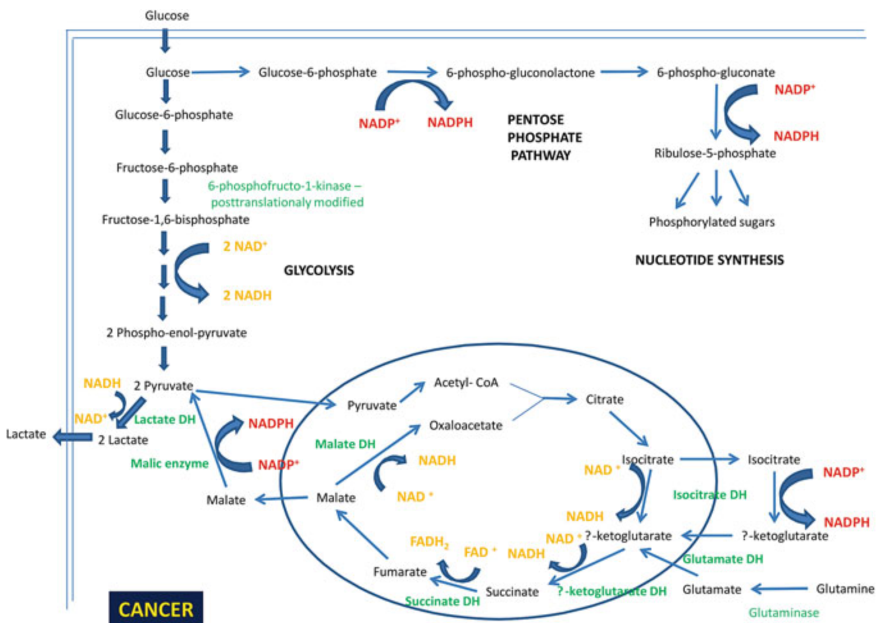
6.6 Conclusion

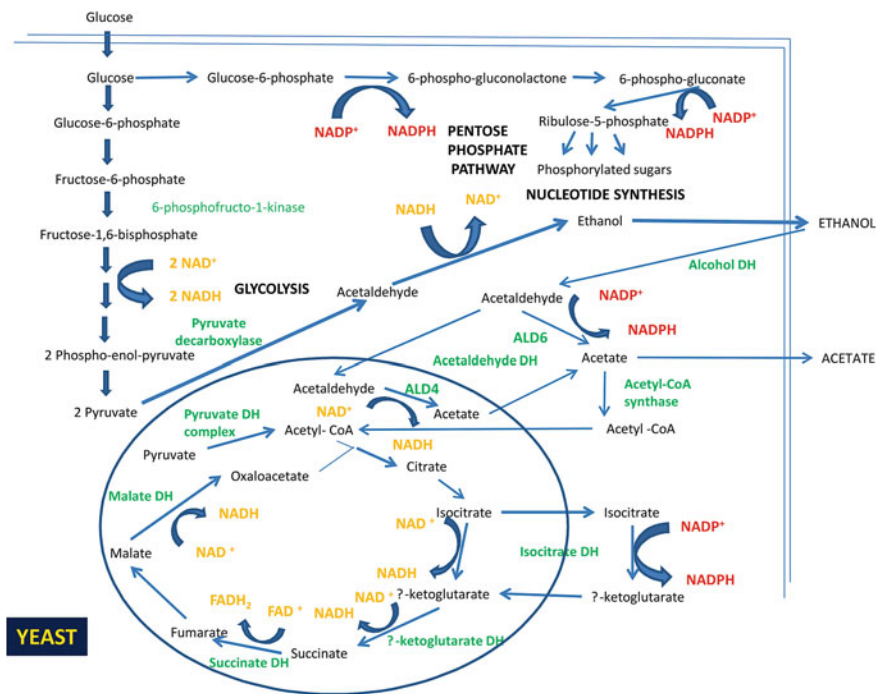
Although yeasts and humans both have eukaryotic cellular structures, there are huge differences between them. Although the catabolic part of the metabolism appears to be similar, there are obvious differences in the organization of their genomes. While the genome of haploid *S. cerevisiae* contains approximately 12 million base pairs and 6,275 genes (Goffeau et al. 1996), the genome of haploid human cells consists of 3 billion base pairs, and the diploid genome found in somatic cells has twice the DNA content. It is estimated that there are approximately 21,000 protein-coding genes in human cells, and it has recently been confirmed that approximately 80 % of the human genome serves a specific biochemical purpose (Pennisi 2012). In other words, the human genome in somatic and cancer cells is approximately 500 times larger than the yeast genome. Such complex organization of the human genome, together with the more complex physiology of human cells, especially at the level of signal transduction, must be reflected in the slower growth of human cells with respect to yeast cells. Moreover, because each tumor is distinct with respect to its genetic alternations (Vogelstein et al. 2013), different tumor cells may display different metabolic features. For instance, some cell lines exhibit enhanced glycolytic flux in the presence of oxygen, convert the majority of consumed glucose into lactate (glioblastoma cells) (DeBerardinis et al. 2007), and show reduction of OXPHOS. In contrast, studies on different types of cancer cells have revealed contradictory modifications with upregulated OXPHOS components and larger dependence of cancer cells on oxidative energy production (Jose et al. 2011).

As all catabolic reactions within the cells must be balanced with anabolic reactions, it is difficult to compare the metabolism of two organisms with different specific growth rates, such as human cancers and yeast cells. However, there are

also numerous similarities between these two types of cells. In general, tumor and yeast cells show accelerated metabolic flux through glycolysis under aerobic conditions; a major portion of ATP is formed by substrate-level phosphorylation at the expense of OXPHOS in both cell types, the NADH/NAD^+ ratio in the cells is maintained by dissimilative production of lactate or ethanol, and the NADPH/NADH balance is sustained by glutaminolysis or by acetate formation. All of these common features enable fast proliferation on the one hand and survival of the cells on the other in both cancer and yeast cells. However, the underlying mechanisms for these features may differ between the various cancer cell lines and yeast cells that have been outlined in this chapter. It is important to realize that primary metabolism is fairly well-conserved between all living organisms but that numerous variations have developed during the course of evolution that are characteristic to individual organisms or cell types.

Schematic





This schematic shows the vital similarities and differences between Warburg effect in cancer cells and Crabtree effect in yeasts. In both cell types, metabolic flux through glycolysis is enhanced (bold arrows) and both cell types excrete a primary metabolite lactate and ethanol, respectively. Enhanced glycolysis is characterized by overproduction of NADH which is partly reoxidized by lactate and ethanol formation but NADH/NADPH ratio cannot be sufficiently maintained by NADPH production through the pentose phosphate shunt. Additional reactions are needed in both cell types for efficient reducing power formation, however, different mechanisms are functional in each cell types. In cancer cells glutaminolysis enables NADPH formation by the activity of cytosolic malic enzyme and in yeasts mitochondrial NADP$^+$-dependent acetaldehyde dehydrogenase isoenzyme (ALD6) plays an important role in balancing the NADH/NADPH ratio.

References

Barnett JA, Entian K-D (2005). A history of research on yeasts 9: regulation of sugar metabolism. Yeast (Chichester, Engl), 22(11):835–894

Baysal BE et al (2000) Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. Sci (NY) 287(5454):848–851

Bensaad K et al (2006) TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 126(1):107–120

Boiteux A, Hess B (1970) Allosteric properties of yeast pyruvate decarboxylase. FEBS Lett 9(5):293–296

Boles E et al (1997) Characterization of a glucose-repressed pyruvate kinase (Pyk2p) in *Saccharomyces cerevisiae* that is catalytically insensitive to fructose-1,6-bisphosphate. J Bacteriol 179(9):2987–2993

Bozic I et al (2010) Accumulation of driver and passenger mutations during tumor progression. Proc Natl Acad Sci USA 107(43):18545–18550

Brandon M, Baldi P, Wallace DC (2006) Mitochondrial mutations in cancer. Oncogene 25(34):4647–4662

Burke RL, Tekamp-Olson P, Najarian R (1983) The isolation, characterization, and sequence of the pyruvate kinase gene of *Saccharomyces cerevisiae*. J Biol Chem 258(4):2193–2201

Chambers A, Graham IR, Packham EA (1995) Control of glycolytic gene expression in the budding yeast (*Saccharomyces cerevisiae*). Curr Genet 29:1–9

Chandra D, Singh KK (2011) Genetic insights into OXPHOS defect and its role in cancer. Biochim Biophys Acta 1807(6):620–625

Chen J-Q, Russo J (2012) Dysregulation of glucose transport, glycolysis, TCA cycle and glutaminolysis by oncogenes and tumor suppressors in cancer cells. Biochim Biophys Acta 1826(2):370–384

Christofk HR et al (2008) The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 452(7184):230–233. http://www.ncbi.nlm.nih.gov/pubmed/18337823 Accessed 22 May 2013

Crabtree HG (1929) Observations on the carbohydrate metabolism of tumours. Biochem J 23(3):536–545

DeBerardinis RJ et al (2007) Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci USA 104(49):19345–19350

DeBerardinis RJ et al (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 7(1):11–20

DeRisi JL (1997) Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale. Science 278(5338):680–686

Díaz-Ruiz R et al (2008) Mitochondrial oxidative phosphorylation is regulated by fructose 1, 6-bisphosphate. A possible role in crabtree effect induction? J Biol Chem 283(40):26948–26955

Dihazi H, Kessler R, Eschrich K (2003) Glucose-induced stimulation of the Ras-cAMP pathway in yeast leads to multiple phosphorylations and activation of 6-phosphofructo-2-kinase. Biochemistry 42(20):6275–6282

Dijken JP, Scheffers WA (1986) Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol Lett 32(3–4):199–224

Dunaway GA (1983) A review of animal phosphofructokinase isozymes with an emphasis on their physiological role. Mol Cell Biochem 52(1):75–91

Elson A et al (1990) The structure of the human liver-type phosphofructokinase gene. Genomics 7(1):47–56

Elstrom RL et al (2004) Akt stimulates aerobic glycolysis in cancer cells. Cancer Research 64:3892–3899

Eto K et al (1994) Cloning of a complete protein-coding sequence of human platelet-type phosphofructokinase isozyme from pancreatic islet. Biochem Biophys Res Commun 198(3):990–998

Fantin VR, St-Pierre J, Leder P (2006) Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 9(6):425–434

Ferreira BS et al (2004) Recombinant *Saccharomyces cerevisiae* strain triggers acetate production to fuel biosynthetic pathways. J Biotechnol 109(1–2):159–167

Friberg S, Mattson S (1997) On the growth rates of human malignant tumors: implications for medical decision making. J Surg Oncol 65(4):284–297

Gancedo JM (1998) Yeast carbon catabolite repression. Microbiol Mol Biol Rev:MMBR 62(2):334–361

Gao P et al (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature 458(7239):762–765
Gingras AC, Raught B, Sonenberg N (2001) Regulation of translation initiation by FRAP/mTOR. Genes Dev 15(7):807–826
Goffeau A et al (1996) Life with 6000 genes. Sci (NY) 274(5287):546, 563–567
Grüning N-M et al (2011) Pyruvate kinase triggers a metabolic feedback loop that controls redox metabolism in respiring cells. Cell Metab 14(3):415–427
Guaragnella N et al (2014) The expanding role of yeast in cancer research and diagnosis: insights into the function of the oncosuppressors p53 and BRCA1/2. FEMS Yeast Res14(1):2–16
Gutteridge JM, (1993) Free radicals in disease processes: a compilation of cause and consequence. Free Radical Res Commun 19(3):141–58. http://www.ncbi.nlm.nih.gov/pubmed/8244084 Accessed June 6, 2013
Hagman A et al (2013) Yeast make-accumulate-consume life strategy evolved as a multi-step process that predates the whole genome duplication (Fairhead C (ed)). PLoS ONE 8(7): e68734
Halliwell B (2007) Oxidative stress and cancer: have we moved forward? Biochem J 401(1):1–11
Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674
Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Sci (NY) 324(5930):1029–33
Heinisch J, Vogelsang K, Hollenberg CP (1991) Transcriptional control of yeast phosphofructokinase gene expression. FEBS Lett 289(1):77–82
Hirschhaeuser F, Sattler UGA, Mueller-Klieser W (2011) Lactate: a metabolic key player in cancer. Cancer Res 71(22):6921–6925
Holzer H (1961) Regulation of carbohydrate metabolism by enzyme competition. Cold Spring Harb Symp Quant Biol 26:277–288
Huang LE (2008) Carrot and stick: HIF-alpha engages c-Myc in hypoxic adaptation. Cell Death Differ 15(4):672–677
Jose C, Bellance N, Rossignol R (2011) Choosing between glycolysis and oxidative phosphorylation: a tumor's dilemma? Biochim Biophys Acta 1807(6):552–561
Keith B, Simon MC (2007) Hypoxia-inducible factors, stem cells, and cancer. Cell 129(3): 465–472
Kim J et al (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 3(3):177–185
Klein C, Struhl K (1994) Protein kinase a mediates growth-regulated expression of yeast ribosomal protein genes by modulating RAP1 transcriptional activity. Mol Cell Biol 14(3):1920–1928
Koukourakis MI et al (2006) Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. Cancer Res 66(2):632–637
Kresze G-B, Ronft H (1981) Pyruvate dehydrogenase complex from baker's yeast. 2. molecular structure, dissociation, and implications for the origin of mitochondria. Eur J Biochem 119(3):581–587
Kretschmer M et al (1987) Fructose-2,6-bisphosphatase and 6-phosphofructo-2-kinase are separable in yeast. Biochem J 246(3):755–759
Lagunas R (1986). Misconceptions about the energy metabolism of *Saccharomyces cerevisiae*. Yeast (Chichester, Engl) 2(4):221–228
Lord PG, Wheals AE (1981) Variability in individual cell cycles of *Saccharomyces cerevisiae*. J Cell Sci 50:361–376
Lu X et al (2010) Metabolomic changes accompanying transformation and acquisition of metastatic potential in a syngeneic mouse mammary tumor model. J Biol Chem 285(13): 9317–9321
Madeo F et al (1999) Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 145(4):757–767
Marchler G et al (1993) A *Saccharomyces cerevisiae* UAS element controlled by protein kinase a activates transcription in response to a variety of stress conditions. EMBO J 12(5):1997–2003

Mathupala SP, Ko YH, Pedersen PL (2009) Hexokinase-2 bound to mitochondria: cancer's stygian link to the warburg effect and a pivotal target for effective therapy. Semin Cancer Biol 19(1):17–24

Mazurek S et al (2005) Pyruvate kinase type M2 and its role in tumor growth and spreading. Semin Cancer Biol 15(4):300–308

Meneses FJ, Henschke PA, Jiranek V (2002) A survey of industrial strains of *Saccharomyces cerevisiae* reveals numerous altered patterns of maltose and sucrose utilization. J Inst Brew 108(3):310–321

Minard KI, McAlister-Henn L (1994) Glucose-induced phosphorylation of the MDH2 isozyme of malate dehydrogenase in *Saccharomyces cerevisiae*. Arch Biochem Biophys 315(2):302–309

Modica-Napolitano JS, Kulawiec M, Singh KK (2007) Mitochondria and human cancer. Curr Mol Med 7(1):121–131. http://www.ncbi.nlm.nih.gov/pubmed/17311537 Accessed 10 June 2013

Mueckler M, Thorens B (2013) The SLC2 (GLUT) family of membrane transporters. Mol Aspects Med 34(2–3):121–138

Müller S, Zimmermann FK, Boles E (1997) Mutant studies of phosphofructo-2-kinases do not reveal an essential role of fructose-2,6-bisphosphate in the regulation of carbon fluxes in yeast cells. Microbiol (Reading, Engl), 143:3055–61

Muratsubaki H, Katsume T (1979) Distribution of hexokinase isoenzymes depending on a carbon source in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 86(4):1030–1036

Netzker R et al (1992) Cell cycle-associated expression of M2-type isozyme of pyruvate kinase in proliferating rat thymocytes. J biol Chem 267(9):6421–64214. http://www.ncbi.nlm.nih.gov/pubmed/1556146 Accessed 7 June 2013

Ordiz I et al (1996) Glucose-induced inactivation of isocitrate lyase in *Saccharomyces cerevisiae* is mediated by the cAMP-dependent protein kinase catalytic subunits Tpk 1 and Tpk2. FEBS Lett 385(1–2):43–46

Ozcan S, Johnston M (1999) Function and regulation of yeast hexose transporters. Microbiol Mol Biol Rev: MMBR 63(3):554–569

Pao SS, Paulsen IT, Saier MH (1998) Major facilitator superfamily. Microbiol Mol Biol Rev: MMBR 62(1):1–34

Pennisi E (2012) Genomics. ENCODE project writes eulogy for junk DNA. Sci (NY) 337(6099):1159–1161

Polyak K et al (1998) Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet 20(3):291–293

Poorman RA et al (1984) Evolution of phosphofructokinase–gene duplication and creation of new effector sites. Nature 309(5967):467–469

Postma E et al (1989) Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 55(2):468–477

Pronk JT, Yde Steensma H, Van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast (Chichester, Engl), 12(16):1607–1633. http://www.ncbi.nlm.nih.gov/pubmed/9123965 Accessed 29 May 2013

Purnelle B, Goffeau A (1996) Nucleotide sequence analysis of a 40 kb segment on the right arm of yeast chromosome XV reveals 18 open reading frames including a new pyruvate kinase and three homologues to chromosome I genes. Yeast (Chichester, Engl) 12(14):1475–1481

Rider MH et al (2004) 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. Biochem J 381(Pt 3):561–579

Roos S et al (2007) Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. J Physiol 582(Pt 1):449–459

Ruiz-Amil M et al (1965) Properties and function of yeast pyruvate carboxylase. J Biol Chem 240(9):3485–3492

Shim H et al (1997) c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci USA 94(13):6658–6663

Sun AS, Cederbaum AI (1980) Oxidoreductase activities in normal rat liver, tumor-bearing rat liver, and hepatoma HC-252. Cancer Res 40(12):4677–4681

Šmerc A, Sodja E, Legiša M (2011) Posttranslational modification of 6-phosphofructo-1-kinase as an important feature of cancer metabolism. PLoS ONE 6(5):e19645

Thevelein JM, de Winde JH (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase a pathway in the yeast *Saccharomyces cerevisiae*. Mol Microbiol 33(5):904–918. http://www.ncbi.nlm.nih.gov/pubmed/10476026

Van Urk H et al (1989) Localization and kinetics of pyruvate-metabolizing enzymes in relation to aerobic alcoholic fermentation in *Saccharomyces cerevisiae* CBS 8066 and Candida utilis CBS 621. Biochim Biophys Acta 992(1):78–86

Van Urk H et al (1990) Transient-state analysis of metabolic fluxes in crabtree-positive and crabtree-negative yeasts. Appl Environ Microbiol 56(1):281–287

Usenik A, Legiša M (2010) Evolution of allosteric citrate binding sites on 6-phosphofructo-1-kinase. PLoS ONE 5(11):e15447

Verstrepen KJ et al (2004) Glucose and sucrose: hazardous fast-food for industrial yeast? Trends Biotechnol 22(10):531–537

Vogelstein B et al (2013) Cancer genome landscapes. Sci (NY) 339(6127):1546–1558

Vora S, Oskam R, Staal GE (1985) Isoenzymes of phosphofructokinase in the rat. Demonstration of the three non-identical subunits by biochemical, immunochemical and kinetic studies. Biochem J 229(2):333–341

Warburg O (1925) Über den Stoffwechsel der Carcinomzelle. Klinische Wochenschrift 4(12):534–536

Warburg O (1956) On the origin of cancer cells. Sci (NY) 123(3191):309–314

Wellen KE, Thompson CB (2010) Cellular metabolic stress: considering how cells respond to nutrient excess. Mol Cell 40(2):323–332

Wise DR et al (2008) Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proc Natl Acad Sci USA 105(48):18782–18787

Yamasaki T et al (1991) Structure of the entire human muscle phosphofructokinase-encoding gene: a two-promoter system. Gene 104(2):277–282

Yeung SJ, Pan J, Lee M-H (2008) Roles of p53, MYC and HIF-1 in regulating glycolysis—the seventh hallmark of cancer. Cell Mol life Sci: CMLS 65(24):3981–3999

Yoshino M, Murakami K (1982) AMP deaminase as a control system of glycolysis in yeast. Mechanism of the inhibition of glycolysis by fatty acid and citrate. J Biol Chem 257(18):10644–10649

Younes M et al (1996) Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers. Cancer Res 56:1164–1167

Yuneva M et al (2007) Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. J Cell Biol 178(1):93–105

Chapter 7
Carbon Metabolism in Pathogenic Yeasts (Especially *Candida*): The Role of Cell Wall Metabolism in Virulence

Keunsook K. Lee and Carol A. Munro

7.1 Introduction

Fungal pathogens are found in the natural environment and associated with living organisms including humans. The major life-threatening human fungal pathogens are *Cryptococcus*, *Aspergillus*, and *Candida* species (spp.). Among *Candida* spp., *C. albicans* is the most prevalent human pathogen responsible for a range of infections that differ in their severity according to the host's immune status. Unlike *C. neoformans* and *A. fumigatus,* which are found in the environment, *C. albicans* is only found in mammalian hosts. For that reason, *C. albicans* has specifically adapted to assimilate and utilise the available nutrients to grow and colonise diverse niches in the human body such as the skin, oral cavity, gastrointestinal and urogenital tracts (Odds 1988; Barelle et al. 2006; Fleck et al. 2011). These niches provide a variety of unique environments that combine different conditions including pH, temperature, oxygen availability, competition with resident microbiota and available nutrients. Therefore, *C. albicans* has acquired the ability to adapt and grow in diverse microenvironments facilitated by a flexible metabolism.

The frontline of the pathogen–host interaction or contact is the fungal cell wall. Cell walls of fungal pathogens play an important role in (i) protection from harmful environments, (ii) providing physical rigidity to maintain cell shape but also control morphogenesis during different developmental stages of the fungus such as formation of mating projections and appressoria, (iii) host immune recognition and pathogenicity and (iv) maintaining cellular integrity as a robust cell wall is essential for cell growth (Kapteyn et al. 2000; Gow and Hube 2012). Hexose sugars such as glucose, mannose and galactose are used by *C. albicans* primarily to produce energy and to synthesise the cell wall polysaccharides glucans, mannan and chitin. The cell wall comprises about a third of the total cellular biomass and cells have to carefully coordinate the production of new wall material

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*, DOI: 10.1007/978-3-642-55013-3_7, © Springer-Verlag Berlin Heidelberg 2014

to enable cell growth. A number of studies have demonstrated the impact of niche-specific metabolic regulation on cell wall remodelling in *C. albicans*, which leads to alterations in host–pathogen interactions and importantly sensitivity to antifungal agents. For example, during *Candida* infections in a rabbit systemic model, transcriptional profiling revealed alterations in the expression of genes associated with alternative carbon source utilization, glucose assimilation, sugar transporters and cell surface remodelling (Walker et al. 2009). Up-regulation of genes associated with gluconeogenesis and the glyoxylate cycle has been observed in *C. albicans* and *C. glabrata* exposed to either human blood or neutrophils (Fradin et al. 2004; Hube 2006; Fradin and Hube 2006; Fukuda et al. 2013). Fluorescent reporter constructs have revealed that the metabolic status of individual *C. albicans* cells within kidney lesions from a murine infection model can vary with some cells undergoing gluconeogenesis and others glycolysis (Barelle et al. 2006), which suggests carbon starvation is relevant in vivo. Utilisation of an alternative carbon source in vitro like lactate by *C. albicans* results in changes in cell wall architecture, and eventually alteration in the host's immune responses (Ene et al. 2012a, 2013). Taken together, the architecture of the *C. albicans* cell wall is strongly influenced by the cell's metabolic status, which is regulated by nutrient availability within the host's niches. Indeed, this leads to the modification of host–pathogen interactions.

The *C. albicans* cell wall is a complex and dynamic polysaccharide and protein network, which undergoes remodelling of both architecture and composition depending on the available carbon sources, growth phase, developmental stage and in response to various external signals (Ene et al. 2012a, 2013; Chaffin et al. 1998; Bowman and Free 2006). The composition of fungal cell walls varies depending on the fungal species. The *C. albicans* cell wall consists of two major layers, a skeletal inner layer (glucan and chitin) and a fibrillar outer layer (mannoproteins) (Bowman and Free 2006; Klis et al. 2001). The composition of the *C. albicans* cell wall is 1–5 % chitin, 60–65 % glucan, and 35–40 % mannoproteins (per dry wall weight) (Klis et al. 2001; Munro et al. 1998). However, the cell wall composition can be flexibly changed under stress conditions such as exposure to antifungal drugs or in response to certain environments. A number of genes that are involved in cell wall biosynthesis and regulation have been shown to be vital for cell growth, viability and pathogenicity. From a therapeutic aspect, fungal cell wall components, especially chitin and glucan, are absent from mammalian host cells, which highlight the potential of the fungal cell wall as a target for antifungal agents. Accordingly, the fungal cell wall is of significant biological interest in order to understand its contribution to pathogenicity and for the development of novel clinical therapies, vaccines and diagnostics. This chapter focuses on *C. albicans* cell wall biosynthesis, mainly discussing key polysaccharides related to sugar metabolism, and to a lesser extent cell wall proteins, related to fungal pathogenesis.

7.2 Glucose Metabolism and Glucan Synthesis

β-glucans, polymers of D-glucose, represent the major and essential components of the fungal cell wall. *β*(1,3)-linked glucan is the most abundant polymer in the fungal cell wall. Other glucans, such as *β*(1,6)-linked, *β*(1,3)/(1,4)-linked, α(1,3)-linked and α(1,4)-linked, have also been found in cell walls of different fungal spp. (Klis et al. 2001; Latgé 2007; Free 2013). In the *C. albicans* cell wall, *β*-glucans contribute to approximately 60–65 % of total cell wall dry weight with *β*(1,3)glucan and *β*(1,6)glucan representing approximately 70 and 30 %, respectively, of the total cell wall glucan (Klis et al. 2001). *β*-glucan together with chitin represent the key structural components of the *C. albicans* cell wall, and give cells physical strength, rigidity and some flexibility.

β-glucans are formed by polymerisation of UDP-D-glucose monomers linked by *β*-glycosidic bonds, and synthesised by glucan synthases. D-glucose is transported into the cells via glucose transporters (e.g. HGT family in *C. albicans*) (Fan et al. 2002). As shown in Fig. 7.1, UDP-glucose in the cytosol is synthesised by a number of enzymes. Glk1 and Glk4 (glucokinases) and Hxk2 (hexokinase) catalyse the phosphorylation of D-glucose, mannose and galactose, and possibly glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc). The glucose-6-phosphate formed is converted into either fructose-6-phosphate by Pgi1 (glucose-6-phosphate isomerase) or glucose-1-phosphate by Pgm2. Fructose-6-phosphate is consumed as a substrate for glycolysis, gluconeogenesis, and can feed into glucan, mannan and chitin biosynthesis pathways (see below). An UDP-glucose pyrophosphorylase, Ugp1, converts UTP and glucose-1-phosphate into UDP-glucose, which is a direct substrate for glucan and glycogen synthesis. Although, our understanding of these enzymes is superficial in the case of pathogenesis and cell wall biosynthesis in *C. albicans*, some evidence has been gathered recently (Hoehamer et al. 2010). In *S. cerevisiae* defects in the phosphorylation of ScUgp1 results in hypersensitivity to Congo Red and Calcofluor White (CFW) and a weakened cell wall due to decreased glucan content (Smith and Rutter 2007). Exposure of *C. albicans* cells to caspofungin, a *β*(1,3)glucan synthase inhibitor, which reduces cell wall glucan levels, induced Pgm2 and Hxk2 according to MALDI-TOF mass spectroscopy proteomic analysis (Hoehamer et al. 2010). This study links cell wall stress response to carbohydrate metabolism in vitro. Gene expression of *C. albicans GLK1* and *UGP1* is significantly upregulated in oropharyngeal candidiasis compared to in vitro conditions (Fanning et al. 2012). Furthermore, exposure of *C. albicans* to macrophages significantly upregulates expression of genes associated with carbohydrate transport/metabolism/assimilation; *HXT5*, *HGT2*, *HGT12*, *PCK1*, *GLK1*, *GLK4*, *ICL1*, *GAL1*, *INO1* and *NAG1* (see below) (Lorenz et al. 2004; Marcil et al. 2008), indicating that *C. albicans* modulates carbohydrate metabolism in response to in vivo conditions including interactions with host cells, which confer a nutrient-deplete environment. Therefore alterations in the pathway to UDP-glucose synthesis are likely to confer changes in the cell wall and thereby influence host–pathogen interactions.

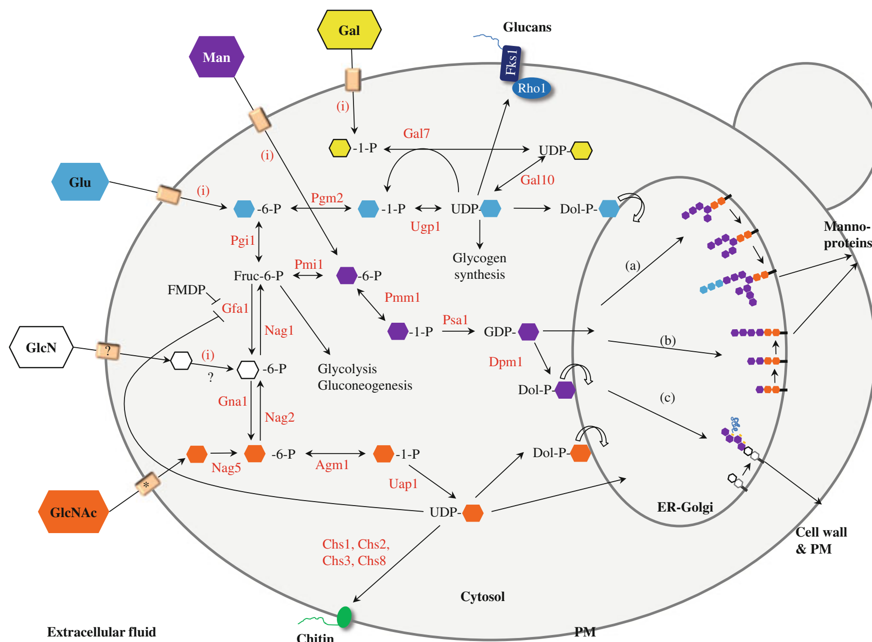

Fig. 7.1 Carbohydrate metabolic pathways involved in *C. albicans* cell wall synthesis. Hexose sugars including glucose (Glu, *blue*), mannose (Man, *purple*), galactose (Gal, *yellow*) and amino sugars such as glucosamine (GlcN, *white*) and *N*-acetylglucosamine (GlcNAc, *orange*) are substrates for cell wall biosynthesis in *C. albicans*. All enzymes, particularly associated with cell wall biosynthesis in the cytosol are named in *red*. Generally hexose sugars can be transported into cells by specific transporters such as the HGT family. There is also a specific GlcNAc transporter, Ngt1 (*) located in the plasma membrane (PM). Unknown proteins (?) may be involved in GlcN transport. (i) Hexokinase (Hxk2) and glucokinases (Glk1 and Glk4) catalyse the phosphorylation of Glu, Man, Gal and GlcN. Gfa1 activity can be inhibited (*dashed lines*) by UDP-GlcNAc (a feedback loop) or FMDP (a glutamine analog with specificity to glucosamine-6-phosphate synthase). Nag3, Nag4 and Nag6 are not shown. In the Endoplasmic reticulum (ER) and Golgi, there are three protein modification pathways; **a** *N*-glycosylation **b** *O*-glycosylation and **c** the synthesis of glycosylinositolphosphatidyl (GPI)-anchors. The substrate for these processes in ER and Golgi are dolichol-phosphate-glucose (Dol-P-Glu), GDP-Man, Dol-P-Man, UDP-GlcNAc and Dol-P-GlcNAc. Post-translationally modified mannoproteins are delivered to the plasma membrane (PM) and the cell wall. The chitin and glucan synthases are integral membrane proteins that bind the UDP-sugar substrates on the cytoplasmic face of the membrane and catalyse their poymerisation with the release of UDP

β(1,3)glucan synthase in *C. albicans* is a complex consisting of alternative catalytic subunits (Fks1/Gsc1, Fks2/Gsl2 and Gsl1) and a regulatory subunit (Rho1) (Qadota et al. 1996; Mio et al. 1997a). Both *FKS1* and *RHO1* in *C. albicans* are essential for cell growth, as attempts to create a null mutant of *FKS1* were unsuccessful (Mio et al. 1997a; Becker et al. 2010). However, partial disruption of *FKS1* resulted in a 50 % reduction of β-glucan levels compared to the control (Mio et al. 1997a). The β(1,3)glucan synthase Fks subunits are the

targets of the echinocandin antifungal drugs (Douglas et al. 1997; Denning 2002). As both *FKS1* and *RHO1* genes are essential for cell viability, they are required for virulence in vivo. Repression of *FKS1* or *RHO1* in *C. albicans* using the regulatable TET-off system resulted in significantly attenuated virulence in a murine model (Becker et al. 2010). Fks1 also plays a role in *C. albicans* biofilm formation (Nett et al. 2007; Sardi et al. 2013). The β(1,3)glucan content in the *C. albicans* biofilm matrix is higher than in the walls of planktonic cells (Nett et al. 2007, 2010). The glucanase gene, *XOG1*, and transglycosidase genes, *BGL2* and *PHR1*, are more highly expressed in *Candida* biofilms compared to planktonic cells (Taff et al. 2012). Deletion of each of these genes resulted in reduced β(1,3)glucan matrix upon biofilm formation. Detection of circulating β-glucan has been utilised for the diagnosis of invasive and bloodstream fungal infections (Ostrosky-Zeichner 2012) with moderate sensitivity and specificity ($\sim$80 %) for candidiasis and aspergillosis (Onishi et al. 2012).

Compared to β(1,3)-glucan synthesis, we have little understanding of β(1,6) glucan synthesis despite this polymer having an important role in covalently tethering the major class of cell wall proteins, the GPI-modified proteins to the wall. So far, several enzymes involved in β(1,6)glucan assembly and organisation have been identified; Kre1, Kre5, Kre6, Kre9, Skn1 and Big1. *C. albicans* strains lacking one of these genes show a significant reduction in β(1,6)glucan levels (Mio et al. 1997b; Lussier et al. 1998; Herrero et al. 2004; Umeyama et al. 2006). Cell wall composition is altered in response to loss of function of β(1,6)glucan-associated genes. For example, deletion of *KRE5* resulted in an approximately 2-fold decrease in mannan, 1.5-fold increase in β(1,3)glucan, and 2-fold increase in chitin content (Herrero et al. 2004), while a *C. albicans big1* mutant had a 2-fold higher chitin content with unchanged β(1,3)glucan levels (Umeyama et al. 2006). *C. albicans KRE6* is an essential gene for cell growth (Mio et al. 1997b). The *C. albicans KRE6/kre6* heterozygous mutant had unchanged β(1,3)glucan levels but high sensitivity to CFW compared to the control, indicating that this mutant had a higher chitin content (Mio et al. 1997b). According to these studies, deletion of *KRE5*, *KRE6* or *BIG1* also leads to defects in hyphal induction by serum and/or virulence in a murine model. Interestingly homozygous disruption of *KRE9* is lethal in cells grown on glucose, but viability can be restored when cells are grown on galactose (Lussier et al. 1998). Also, a *KRE9* conditional mutant generated using the TET-off suppression system showed significantly decreased virulence in a murine model under repression conditions (Becker et al. 2010). It has been reported that derivatives of pyridobenzimidazole inhibit β(1,6)glucan synthesis and their primary target is known to be Kre6 in *S. cerevisiae* (Kitamura et al. 2009). β(1,6)glucan biosynthesis is, therefore, responsible for (i) growth depending on carbon source, (ii) hyphal formation, (iii) cell wall integrity and (iv) virulence, and could be a potential target for antifungal agents (Kitamura et al. 2009). Thus it is important to gain a better understanding of the β(1,6)glucan synthesis pathway.

7.3 Cell Wall Chitin Biosynthesis

Chitin is a linear polymer of β(1,4)-linked *N*-acetylglucosamine (GlcNAc) and is the second most abundant polysaccharide in the environment after cellulose. Although, it is a minor component of *C. albicans* cell walls, it is crucial for cell growth, viability and virulence (Bulawa et al. 1995; Munro et al. 2001; Lenardon et al. 2010). Generally, chitin is thought to be enriched in a region between the plasma membrane and the glucan complex. It is covalently attached to the non-reducing end of β(1,3)glucan (Kollar et al. 1995, 1997), and cross-linked to β(1,6)glucan (Cabib et al. 2007; Cabib 2009). Chitin can be deacetylated to chitosan by chitin deacetylases and in *Cryptococcus neoformans* chitosan is important for virulence (Baker et al. 2011). *C. albicans* has a putative chitin deacetylase Cda2, which has not been fully explored yet.

C. albicans synthesises chitin from UDP-GlcNAc through the chitin biosynthesis pathway (Fig. 7.1). This pathway is regulated by several enzymes that convert substrates including glucose, GlcNAc and GlcN to chitin (Milewski et al. 2006). Synthesis of the chitin building blocks, UDP-GlcNAc, begins with conversion of fructose-6-phosphate to glucosamine-6-phosphate by glucosamine-6-phosphate synthase (Gfa1), which is then acetylated by an acetyltransferase (Gna1) to GlcNAc-6-phosphate. Agm1 (acetylglucosamine phosphomutase) converts GlcNAc-6-phosphate into GlcNAc-1-phosphate. UDP-GlcNAc is produced from UTP and GlcNAc-1-phosphate by the catalytic reaction of UDP-GlcNAc pyrophosphorylase (Uap1). UDP-GlcNAc is used as a substrate by *C. albicans* chitin synthases (Chs1, Chs2, Chs3 and Chs8) to produce chitin chains.

In *S. cerevisiae* some genes (Sc*GFA1*, Sc*GNA1*, Sc*AGM1* and Sc*UAP1*) involved in chitin biosynthesis are essential for cell viability (Milewski et al. 2006). Similarly, *C. albicans GFA1* and *GNA1* genes are essential for growth on glucose (Smith et al. 1996; Mio et al. 2000). *C. albicans gfa1*Δ and *gna1*Δ mutants require GlcNAc to grow, provision of GlcNAc bypasses the essential steps catalysed by Gfa1 and Gna1 in the UDP-GlcNAc pathway. Enzyme activity of Gfa1 is inhibited by UDP-GlcNAc (a negative feedback loop) and N3-(4-methoxyfumaroyl)-(S)-2,3-diaminopropanoic acid (FMDP, a glutamine analogue and a specific inhibitor of GlcN-6-phosphate synthase) (Smith et al. 1996). Becker et al. (2010) have shown that the TET-off suppressed *GFA1* and *GNA1* conditional mutants were severely attenuated in a murine model of candidiasis. Among these enzymes, Gfa1, in particular, has been shown to play a key role in cell wall stress responses, compensatory mechanisms that results in up-regulation of chitin synthesis in response to cell wall defects. Many fungi respond to cell wall stress caused by chemical agents or mutation of cell wall-related genes by increasing cell wall chitin and elevating Gfa1 enzyme activity and mRNA levels (Lagorce et al. 2002; Bulik et al. 2003; Ram et al. 2004; Copping et al. 2005). In *S. cerevisiae*, cell wall-defective mutants such as Sc*fks1*Δ, Sc*mnn9*Δ and Sc*och1*Δ contain a 4- to 5-fold increase in chitin and have up to 7-fold higher activity of Gfa1 (Bulik et al. 2003). Furthermore, those mutants have a significant increase in expression of *CHS3*

(Ram et al. 2004) and elevated Chs3 activity (Bulik et al. 2003). *GFA1* expression levels were threefold upregulated in *C. albicans* exposed to caspofungin (Copping et al. 2005). Likewise, *Aspergillus niger* stimulates chitin biosynthesis in response to CFW (Ram et al. 2004). An*gfaA* gene similar to Sc*GFA1* is also transcriptionally activated when the cell wall integrity of *A. niger* is disrupted by treatment with CFW or caspofungin. Induction of orthologous *gfa* genes has also been found in other fungal spp. such as *Penicillium chrysogenum* and *Fusarium oxysporum* in response to CFW treatment (Ram et al. 2004).

7.4 GlcNAc Metabolism and Signalling

C. albicans is able to utilise amino sugars such as glucosamine (GlcN) and its acetylated form GlcNAc as alternative carbon sources (Singh and Datta 1979; Konopka 2012). These sugars can be readily found in human host niches in particular mucosal membranes, one of the niches colonised by *C. albicans* (Sengupta and Datta 2003). Also, GlcNAc can be produced by bacteria inhabiting the human gastrointestinal tract (Plumbridge 1989) and is a component of bacterial cell wall peptidoglycan.

In *C. albicans,* a cluster of Nag proteins on chromosome 6 are involved in GlcNAc catabolism and chitin biosynthesis (Fig. 7.1). The cluster is comprised of Nag3 and Nag4 (putative hexose-sugar transporters), Nag5/Hxk2 (GlcNAc kinase, EC2.7.1.59), Nag2/Dac1 (GlcNAc-6-P deacetylase, EC3.5.1.25), Nag1 (GlcN-6-P deaminase, EC3.5.99.6) and Nag6 (a cytosolic protein with a putative GTP-binding motif) (Konopka 2012; Natarajan and Datta 1993; Jyothi Kumar et al. 2000; Yamada-Okabe et al. 2001; Yamada-Okabe and Yamada-Okabe 2002). Nag3 and Nag4 are thought to be involved in uptake of exogenous hexoses such as GlcNAc, glucose and galactose (Yamada-Okabe and Yamada-Okabe 2002). Nag3 is a member of the major facilitator superfamily and has 81 % homology to Nag4 at the amino acid level, homologues exist in *S. cerevisiae* (Yamada-Okabe and Yamada-Okabe 2002). In addition to these putative transporters, the first GlcNAc-specific transporter Ngt1 has been characterised in *C. albicans* (Alvarez and Konopka 2007), although it does not belong to the Nag cluster (Fig. 7.1). Deletion of *NGT1* resulted in a reduction in efficiency of GlcNAc uptake. Ngt1-GFP was highly induced in cells grown in medium supplemented with GlcNAc, but not any other sugars including glucose, galactose, fructose, *N*-acetylmannosamine (ManNAc) and GlcN, and repressed by glucose (Alvarez and Konopka 2007). Furthermore, Ngt1-GFP was specifically induced by GlcNAc in vitro, and expressed after phagocytosis by murine macrophages ex vivo. This indicates activation of alternative carbon source metabolism inside macrophages, in agreement with a previous study (Lorenz et al. 2004). The function of Nag6, has not been elucidated. The expression of *NAG6* (as well as *NAG3* and *NAG4*) in *C. albicans* was not induced by GlcNAc, whereas deletion

of *NAG6* (or *NAG3* or *NAG4*) resulted in growth inhibition upon exposure to cycloheximide (Yamada-Okabe and Yamada-Okabe 2002).

In particular, Nag5, Nag2 and Nag1 enzymes in *C. albicans* are involved in the conversion of GlcNAc into Fructose-6-phosphate, the reverse of the Gfa1- and Gna1-catalysed reactions. Nag5 phosphorylates GlcNAc to produce GlcNAc-6-phosphate, which is deacetylated into GlcN-6-phosphate by Nag2, and Nag1 converts GlcN-6-phosphate into fructose-6-phosphate. Compared to *C. albicans*, there are no *S. cerevisiae* homologues of *NAG1*, *NAG2* and *NAG5*, suggesting that GlcNAc catabolism differs in *S. cerevisiae* to *C. albicans* (Wendland et al. 2009). For example, addition of both exogenous GlcN (Walker et al. 2008) and GlcNAc (unpublished) to the growth medium resulted in increased chitin in *C. albicans* cell walls. On the other hand, GlcN (not GlcNAc) stimulated Chs3 activity in *S. cerevisiae*, and increased cell wall chitin content (Bulik et al. 2003). As expected, exogenous GlcNAc does not affect chitin levels in *S. cerevisiae.* Therefore, *C. albicans* differs from *S. cerevisiae* in that exogenous GlcNAc can activate chitin synthesis and *C. albicans* specific genes *NGT1* and the GlcNAc cluster of genes are likely to be involved in this. As well as inducing higher chitin levels mass spectrometry analysis of membrane proteins revealed that a number of cell wall-related proteins (Ecm331, Phr1, Dcw1, Bgl2, Sap9, Rho1 and Pga52) were highly expressed in *C. albicans* cells exposed to GlcNAc (Alvarez and Konopka 2007). This may reflect more global changes in the cell wall in response to exogenous GlcNAc, through Rho1-signalling or by action of carbohydrate-active enzymes such as Phr1.

NAG1, *NAG2* and *NAG5* have been shown to be involved in host interactions and virulence in addition to or as a consequence of cell wall remodelling. For example, deletion of *NAG5* increased sensitivity to the chitin synthase inhibitor Nikkomycin Z (Rao et al. 2013). The *nag5* mutant displayed a reduction in adherence to human buccal epithelial cells and attenuated virulence in a murine model of candidiasis (Yamada-Okabe et al. 2001; Singh et al. 2001). Localisation of Nag5-GFP was mainly observed in the cytosol of *C. albicans* grown with GlcNAc, but no detectable signals were found in *C. albicans* cells grown in a medium containing glucose or serum or Spider medium (Rao et al. 2013). Interestingly, this study also demonstrates that when *C. albicans* cells are grown in a medium containing 5 % ethanol, a non-fermentable carbon, Nag5-GFP was localised to mitochondria. This suggests localisation of Nag5 can be influenced by different carbon sources, and this may influence its cellular function.

GlcNAc is an important signalling molecule, besides inducing *C. albicans* chitin synthesis, GlcNAc also induces white-opaque switching (Huang et al. 2010). *C. albicans* can undergo a phenotypic switch from the normal white cells to opaque cells that have a dramatically increased ability to mate and are altered in interactions with host cells (Soll 2009). The frequency of white-opaque switching was higher when *C. albicans* α/α or a/a cells (homozygous at the mating type locus) were grown on GlcNAc-containing medium compared to glucose-grown cells. Genome-wide expression profiles of opaque cells compared to white cells

showed up-regulation of genes involved in carbohydrate metabolism (*NAG1*, *MLS1*, *MDH1* and *IDP2*), degradation of fatty acid (*POX1*, *FAA2*, *FOX2* and *FOX3*), amino acid permeases (*AGP2*, *CAN3* and *GAP1*) and cell wall proteins (*SCW4*, and *CHT1*) (Lan et al. 2002). Although, we do not have a full understanding of the role of white-opaque switching in pathogenicity opaque cells are able to colonise the skin of a cutaneous infection murine model better than white cells (Huang et al. 2010; Kvaal et al. 1999). *SAP1* encoding a secreted aspartyl proteinase is highly expressed in opaque cells, and its expression impacts on colonisation and adherence to skin in a mouse cutaneous infection model (Kvaal et al. 1999). Fatty acid *β*-oxidation and increased proteinase activity could contribute to better colonisation of the skin by opaque cells as the skin is rich in lipids but lacks free sugars.

GlcNAc also stimulates hypha formation in *C. albicans* (Konopka 2012; Naseem et al. 2011; Martin et al. 2013). Promoter analysis revealed that promoters of *NAG5*, *NAG2* and *NAG1* contain STE12 elements (unpublished). The homologue of Sc*STE12* in *C. albicans* is *CPH1* that encodes a transcription factor involved in the mitogen-activated protein kinase (MAPK) pathway required for mating, virulence, hyphal growth on solid media and filamentous growth in a matrix via Czf1 (Brown et al. 1999; Huang 2012). Cph1 as well as Efg1, a key transcription factor downstream of the cAMP-protein kinase (PKA) (Brown et al. 1999; Stoldt et al. 1997) and the transcriptional repressor Nrg1 contribute to the induction of expression of hyphal-associated genes upon GlcNAc stimulation (Konopka 2012; Naseem et al. 2011; Martin et al. 2013).

7.5 GlcNAc and Galactose Metabolism

Kamthan et al. (2013) recently revisited the biological significance of the link between GlcNAc metabolism and *GAL* gene activation in *C. albicans* (Kamthan et al. 2013). The regulation of galactose metabolism (Leloir metabolism) has been re-wired in *C. albicans* compared to *S. cerevisiae* (Campbell et al. 2008). Gal1 (galactokinase) phosphorylates galactose to produce galactose-1-phosphate, and then Gal7 (galactose-1-phosphate uridyl-transferase) converts this to UDP-galactose. UDP-galactose can also be converted to UDP-glucose by Gal10 (UDP-galactose-4-epimerase) (Fig. 7.1). In *S. cerevisiae* ScGal4 plays a role in regulating Sc*GAL* gene expression, while in *C. albicans* Gal4 has a different role (Martchenko et al. 2007). However, *C. albicans* Gal4 along with Tye7 is a key transcriptional activator that orchestrates control of glycolytic genes (Askew et al. 2009) and cell wall-related genes such as *SUN4* (cell wall glycosidase gene), *PGA52*, *PGA56*, *PGA14*, *PGA29*, *ALS4* and *CWH8* (Martchenko et al. 2007). Deletion of *GAL4* in *C. albicans* resulted in up-regulation of *TYE7* (Martchenko et al. 2007), suggesting compensatory regulation of *TYE7*. Also, both *C. albicans GAL4* and *TYE7* are required for full virulence in a *Galleria mellonella* infection model. Based on promoter analysis, the *GAL* genes in

C. albicans are transcriptionally regulated via Cph1, a transcription factor required for hyphal growth (Martchenko et al. 2007). It has been known for some time that *C. albicans* cells grown in galactose-containing medium were more adherent than glucose-grown cells (Ener and Douglas 1992), which implies changes in cell surface properties. As expected *ALS2* and *ALS4* encoding adhesin-like proteins were 2–3 fold upregulated in *C. albicans* grown on galactose compared to glucose (Martchenko et al. 2007). In this study, genes associated with fatty acid degradation (*POX1-3*, *FOX2*, *FOX3* and *FAA2-2*), gluconeogenesis (*PCK1*) and cell wall functions (*ECM38*, and *RBR2*) were highly expressed in galactose-grown cells. Treatment of *C. albicans* with GlcNAc upregulated expression levels of *GAL1*, *GAL7* and *GAL10*, compared to glucose-grown cells (Kamthan et al. 2013; Gunasekera et al. 2010). Kamthan et al. proposed indirect evidence that *GAL1* induction by GlcNAc could possibly occur via the activation of a secondary pathway. *C. albicans GAL1* and *GAL7* activation by GlcNAc is *GAL10*-dependent. Mass spectrometry analysis of *C. albicans* treated with GlcNAc identified upregulated proteins that included Nag1, Nag2, Gal1 and Gal10 (Kamthan et al. 2012). Interestingly, metabolomic analysis highlighted carbohydrates such as xylulose and D-glucose were only found in glucose-grown *C. albicans* cells, whereas GlcNAc and ManNAc were observed in GlcNAc-grown cells (Kamthan et al. 2012). This suggested changes in metabolite levels between GlcNAc and glucose-grown conditions. Although there is no direct evidence to link galactose metabolism and chitin synthesis via the Nag pathway, deletion of *GAL10* increased the sensitivity of cells to CFW and Congo Red (Singh et al. 2007). Further investigation is required to understand the connection between galactose, GlcNAc metabolism, and cell wall integrity.

7.6 Mannose Metabolism and Glycosylation

The outermost layer of the *C. albicans* cell wall is composed of mannan, a mannose-rich polymer covalently linked to proteins, and represents approximately 40 % of the total cell wall composition (Klis et al. 2001). Mannan is composed of α(1,2)-, α(1,3)-, α(1,6)- and *β*(1,2)-linked mannose monomers (Shibata et al. 2012). *N*-linked mannan is linked to a protein moiety via asparagine and *O*-linked mannan via serine or threonine (Chaffin et al. 1998; Shibata et al. 2012). Both *N*-linked and *O*-linked mannan are important for host immune interactions and virulence (Gow and Hube 2012; Netea et al. 2008). *N*-linked mannan has been described as a comb-like structure comprised of a core glycan and an extensive outer branched structure. The branched outer chain has an α(1,6)-linked mannose backbone and a variety of α(1,2)-, α(1,3)-linked and sometimes *β*(1,2)-linked side chains as well as an acid-labile *β*(1,2)-linked phosphomannan side chain joined to the backbone by a phosphodiester bond. *O*-mannan is a linear chain of

α(1,2)-linked mannose residues, in *C. albicans* this is typically composed of up to five mannose units (Munro et al. 2005).

Mannan is synthesised by a sequence of mannosyltransferase reactions that occur in the ER and Golgi. The mannose-rich glycan is elaborated by the sequential addition of mannose monomers to the growing mannan chain as the glycoprotein it is attached to moves through the secretory pathway. The substrate of the mannosyltransferases is GDP-mannose as well as dolichol-phosphate-mannose (Dol-P-Man) (Fig. 7.1). A number of enzymes are involved in the synthesis of *N*-linked mannan. First the conserved Man8GlcNAc2 glycan core is synthesised in the ER and then joined to a protein bearing a N-X-S/T motif by an oligosaccharyltransferase complex (reviewed by Aebi 2013). Further elaboration of the core *N*-glycan takes place in the Golgi initiated by Och1 with the addition of the first α(1,6)-linked mannose to the core glycan. (Bates et al. 2006). The α(1,6)-linked backbone is extended to contain up to 200 mannose units with side chains composed of α(1,2)-, α(1,3)- and sometimes *β*(1,2)-linked mannose that are added by specific families of Golgi-localised mannosyltransferases. TEM analysis revealed that an *och1*Δ *C. albicans* mutant had severely altered cell wall architecture with a thicker cell wall (glucan and chitin layer) and a barely detectable fibrillar mannoprotein layer (Netea et al. 2006). The *och1* null mutant was avirulent in a murine model of systemic infection (Bates et al. 2006). In addition, stimulation of cytokines such as TNF, INF*γ*, IL-6 and IL-10 was significantly lower in *C. albicans* lacking *OCH1* (Netea et al. 2006). These studies reflect the importance of the outer mannan layer in host interactions. Bmt1–9 are a novel family of *C. albicans* *β*(1,2)-mannosyltransferases that synthesise the *β*(1,2)-linked oligosaccharide side chains, these enzymes are not found in *S. cerevisiae* (Mille et al. 2008). *β*(1,2)-linked oligomannosides have been shown to reduce the stimulation of inflammatory cytokine production by dendritic cells (Ueno et al. 2013). A *C. albicans mnn4* mutant lacking the acid-labile phosphomannan side chain was significantly less phagocytosed by macrophages compared to control cells (McKenzie et al. 2010). Mnn2 is responsible for the addition of the first α(1,2)-linked mannose to the α(1,6)-linked mannose backbone in *C. albicans*. A family of six Mnn2-like enzymes exist and generation of a sextuple mutant lacking the whole family confirmed the important role of this family in generating the outer fibrillar mannan layer, virulence in a mouse model of candidiasis and the stimulation of proinflammatory cytokine production by monocytes (Hall et al. 2013).

The linear *O*-mannan is synthesised by a simpler pathway. The initial α(1,2)-linked mannose is added in the ER to serine or threonine residues of the target protein by the Pmt family of mannosyltransferases that use as their substrate mannose activated with dolichyl phosphate. *C. albicans* has a five-membered Pmt family and disruption of family members in particular Pmt1 results in a broad range of defects commensurate with a damaged wall that influences virulence and host interactions (Prill et al. 2005). The *O*-mannan chain is extended by the α(1,2)-mannosyltransferases Mnt1 and Mnt2 that add the second and third mannose sugars to the chain, respectively (Munro et al. 2005). *O*-linked mannan is involved

in adhesion of *C. albicans* to human buccal epithelial cells, and is required for virulence (Munro et al. 2005; Buurman et al. 1998).

PMR1 encodes a P-type Ca^{2+}/Mn^{2+}-ATPase required for transporting Mn^{2+} into the Golgi. Mn^{2+} is an essential co-factor of Golgi-resident mannosyltransferases and loss of Pmr1 function affects *O*-linked and *N*-linked mannan synthesis (Bates et al. 2005). The *C. albicans pmr1* mutant is hypersensitive to CFW and Congo Red, and is significantly attenuated in virulence in a murine model of systemic candidiasis (Bates et al. 2005). Dectin-2 and Mincle have been shown to recognise α-mannose structures on the *C. albicans* cell surface (Vautier et al. 2012). Galectin-3 has an important role in recognition of β(1,2)-mannose of *N*-linked mannan in *C. albicans* (Netea et al. 2008). Galectin-3, associated with TLR2, also recognises phospholipomannan.

Therefore, defects in mannan biosynthesis alter the properties of cell surface proteins, for example their adhesiveness and results in activation of a compensatory response, which triggers increased chitin levels. Importantly, altered mannosylation and changes in the underlying cell wall architecture, affects the host immune responses and impact on virulence. Recently, mannan has been proposed as a potential target for vaccine development for prevention of *C. albicans* infections, because mannan is highly antigenic (Lipinski et al. 2012).

7.7 *C. albicans* Cell Wall Glycoproteins

There are two major classes of covalently attached cell wall proteins (CWPs) in *C. albicans*; (1) glycosyl-phophatidylinositol (GPI)-modified proteins linked to the wall via β(1,6)glucan, and (2) Pir (Protein with Internal Repeat)-CWPs that are linked directly to β(1,3)glucan (Kapteyn et al. 2000; Klis et al. 2001; Chaffin 2008; Munro and Richard 2012). Pir-CWPs in *C. albicans* are represented by Pir1/Pir2 and Pir3/Pir32. Deletion of either allele of *PIR1* increases sensitivity to CFW and Congo Red (Martínez et al. 2004). *C. albicans pir32*Δ is hyper-filamentous, resistant to SDS, H_2O_2, and NaCl, and hyper-virulent in a murine model of systemic infection (Bahnan et al. 2012). Interestingly, *pir32*Δ shows up-regulation of cell wall chitin deposition. Therefore, the Pir family in *C. albicans* is involved in cell wall integrity and virulence.

GPI-modified proteins are the major CWPs, and have been shown to play a role in morphology, cell wall integrity/organisation, stress responses, host immune responses and virulence (Klis et al. 2009; Shepardson and Cramer 2013). GPI-anchors are post-translationally attached to proteins in the ER, and target proteins to the plasma membrane, some proteins then become translocated to the cell wall, the GPI-anchor is modified and a remnant becomes attached to β(1,6)glucan (Chaffin 2008; Klis et al. 2009; de Groot et al. 2003; Richard and Plaine 2007). The core structure of GPI-anchors consists of GlcN, phosphatidylinositol, ethanolamine-phosphate, and mannose sugars (Mora-Montes et al. 2009; Fujita and Kinoshita 2012).

A number of GPI-modified CWPs are adhesins or adhesin-like proteins including the Als family (Hoyer 2001; Hoyer et al. 2008). Als1 and Als3 have been shown to contribute to virulence as well as cell wall remodelling. *C. albicans als1* null mutant has a significant reduction in adherence to oral mucosa in murine oropharyngeal candidiasis (Kamai et al. 2002). In addition, an *als1*Δ mutant had decreased adherence to human umbilical vein endothelial cells (HUVECs), but not to buccal epithelial cells (BECs) (Zhao et al. 2004). In contrast, the *als3*Δ mutant was less adherent to both HUVECs and BECs. Likewise, adhesion of the *als3*Δ mutant to buccal reconstituted human epithelium was also notably defected (Zhao et al. 2004). Als3 is an important invasin (Phan et al. 2007) as well as being capable of binding ferritin (Almeida et al. 2008). Deletion of *ALS3* resulted in decreased adhesion and less damage to TR146 oral epithelial cell monolayers (Murciano et al. 2012) and production of G-CSF, IL-6 and IL-1α cytokines was significantly reduced in comparison to wild type (Murciano et al. 2012). Als1 and Als3 are promising targets for vaccine development (Spellberg et al. 2008; Liu and Filler 2011).

Some other GPI-anchored proteins play a direct role in cell wall assembly and remodelling (Munro 2013). The Crh family is required for cross-linking chitin and β(1,3)glucan (Cabib et al. 2007; Pardini et al. 2006; Cabib et al. 2008). The Phr family are pH-responsive transglycosidases that modulate β(1,3)glucan (Fonzi 1999). *C. albicans* cells lacking a member of the *CRH* family (*CRH11*, *CRH12* and/or *UTR2*) are sensitive to cell wall disturbing agents such as CFW, Ca^{2+} and Congo Red. A triple mutant lacking the entire family had similar total glucan levels, but reduced alkali-insoluble β(1,3)glucan and increased chitin. Furthermore, *CRH11* is significantly up-regulated in *C. albicans* cells treated with caspofungin (Liu et al. 2005; Bruno et al. 2006). Large numbers of null mutants of predicted GPI-anchored protein genes have been screened to identify genes involved in cell wall integrity and caspofungin sensitivity (Plaine et al. 2008). The *rbt1*Δ and *hwp1*Δ mutants were sensitive to CFW. The *pga31*Δ and *ssr1*Δ mutants were sensitive to both CFW and caspofungin. However, deletion of *PGA62* or *PHR1* resulted in an increase in sensitivity to CFW but a reduction in susceptibility to caspofungin. In this study, cell wall component analysis indicated that both *pga62*Δ and *phr1*Δ mutants had higher chitin content and decreased glucan content. Recently, Pga13 has been characterised in *C. albicans* (Gelis et al. 2012). Deletion of *PGA13* led to an increase in sensitivity to CFW and Congo Red. Also, the *pga13*Δ mutant is resistant to heat shock and 5-fluorocytosine. Thus, several GPI-anchored proteins are important for maintenance and assembly of the cell wall and antifungal susceptibility.

7.8 Dynamic Changes in the *C. albicans* Cell Wall in Response to Nutrient Availability Affect Host–Pathogen Interactions and Virulence

The *C. albicans* cell wall can be dynamically modified depending on growth conditions, morphology, nutrient and oxygen availability, and stress conditions produced by the host. Within the different host niches, *C. albicans* is able to cope with these environmental changes by shifting to alternative metabolic pathways such as glycolysis, gluconeogenesis, and the glyoxlyate cycle to produce energy and substrates for other biological functions (Barelle et al. 2006; Fukuda et al. 2013; Lorenz et al. 2004; Marcil et al. 2008; Rubin-Bejerano et al. 2003; Miramón et al. 2012). Recently, published studies illustrate that an alternative carbon source such as lactate noticeably affects the architecture and properties of the *C. albicans* cell wall, stress responses, host immune recognition and virulence (Ene et al. 2012a, b, 2013). Transmission electronic microscopy (TEM) demonstrated that lactate-grown *C. albicans* cells have much thinner glucan/chitin and mannan layers compared to glucose-grown cells (Ene et al. 2012a). This study also showed that cells grown in the presence of different sugars such as fructose, galactose and oleic acid displayed differential sensitivity to CFW and virulence in a mouse model. For example, lactate-grown cells were hyper-virulent as indicated by the higher kidney burdens and severe weight loss of animals compared to glucose-grown cells. Cells pre-grown in medium supplemented with oleic acid were attenuated in virulence with low fungal kidney burdens and reduced weight loss of mice, in comparison to glucose-grown cells (Ene et al. 2012a). Galactose-grown cells were (partially) more virulent as indicated by higher fungal kidney burdens and greater weight loss of animals (Ene et al. 2012a). As expected, there was a critical impact on the cell wall proteome and secretome of lactate-grown cells (Ene et al. 2012b). In particular, chitinases Cht1 and Cht3, glycosidases Phr1 and Phr2 and some GPI-modified proteins Pga4 and Pga31 were notably induced on lactate-grown cells. Changes on the surface and underlying architecture of the cell wall of lactate-grown cells influence interactions with the host immune cells and cytokine profiles (Ene et al. 2013). Lactate-grown *C. albicans* cells strongly stimulated anti-inflammatory responses by producing the IL-10 cytokine. Moreover, murine macrophages phagocytosed lactate-grown *C. albicans* cells less efficiently, but lactate-grown cells killed more macrophages than glucose-grown cells (Ene et al. 2013). Therefore, availability of different carbon sources results in cell wall remodelling that impacts on stress responses and leads to changes in virulence. Unlike *S. cerevisiae*, the fungal pathogen *C. albicans* has an increased capacity to utilise alternative carbon sources even in the presence of glucose and this should be considered a virulence attribute (Sandai et al. 2012). In summary, the metabolic flexibility of *C. albicans* significantly impacts cell wall properties and so influences host interactions including immune recognition and modulation and pathogenesis.

Analysis of transcriptional profiling from an early stage in the interaction of *C. albicans* with mammalian macrophages highlighted a rapid shift to a starvation mode including increased expression of genes related to gluconeogenesis, fatty acid degradation, and the oxidative stress response and repression of protein translation genes (Lorenz et al. 2004). In later stages of macrophage interactions, *C. albicans* can switch to hyphal growth, escape from the macrophage and glycolysis and protein translation then resume. Similarly, when *C. albicans* cells were exposed to human blood, the expression of gluconeogenic genes such as *PCK1*, *ENO1* and *FBA1* was elevated (Fradin et al. 2004). In this study, they also reported that higher hypha formation was observed in *C. albicans* cells exposed to plasma in comparison to human blood. The hypha-associated gene *HWP1* was transcriptionally more expressed in plasma-exposed *C. albicans* than human blood-exposed cells, whereas *PHR2* was highly expressed in human blood-exposed cells (Fradin et al. 2004). A recent study also demonstrated that the glyoxylate cycle is activated when *C. albicans* is phagocytosed by human neutrophils indicated by increased expression of *ICL1p-GFP* and *MLS1p-GFP* using a GFP reporter controlled by the promoter of each gene (Miramón et al. 2012). *ICL1* is required to survive neutrophil killing. In addition, when exposed to human neutrophils *C. albicans* stimulates up-regulation of amino acid synthesis (Rubin-Bejerano et al. 2003). *GCN4* encoding the transcriptional activator of the general amino acid control response was upregulated upon exposure to human neutrophils (Rubin-Bejerano et al. 2003) as well as when cells are grown in the presence of GlcNAc (Kamthan et al. 2012). Only a couple of genes such as *AGP2* encoding an amino acid permease and *ECM17* encoding an enzyme for sulphur amino acid biosynthesis, are Gcn4-regulated and associated with cell wall regeneration or synthesis. *AGP2* expression is upregulated in *C. albicans* cells treated with caspofungin (Bruno et al. 2006), and regulated during the white-opaque switching (Lan et al. 2002). When phagocytosed by human neutrophils, *C. albicans* also initiates transcriptional activation of antioxidant responses illustrated by up-regulation of superoxide dismutase (*SOD1*), or catalase (*CTA1/CCT1*) (Rubin-Bejerano et al. 2003), and high expression of *CTA1p*-GFP and *SOD5p*-GFP (Miramón et al. 2012). Therefore, exposure of *C. albicans* to the environments generated by host immune cells such as macrophages and neutrophils impacts on metabolism, amino acid starvation, morphology, and stress responses as well as cell wall biosynthesis.

Environmental cues for *C. albicans* yeast-hypha transition are nutrient starvation, accessibility of GlcNAc, CO_2 sensing and changes in pH and temperature (Gow and Hube 2012; Mattia et al. 1982; Sudbery 2011). The response of *C. albicans* to these signals is often accompanied by changes in cell wall composition and architecture, and expression of cell wall proteins, which impacts on the host immune recognition and virulence. *C. albicans* cells grown with serum abundantly induce hyphal-associated proteins such as Als3, Hwp2 and Hyr1 and cell wall remodelling proteins including Phr1 (Heilmann et al. 2011). Hwp2, is a putative GPI-modified cell wall protein, similar to Hwp1, which contributes to hypha formation, invasive growth and virulence (Hayek et al. 2010). A *C. albicans* mutant lacking *HWP1*, encoding a hypha-associated surface protein has a

significant reduction in the stable attachment to human BECs, compared to the control, and is distinctly attenuated in a murine model of systemic infection (Staab et al. 1999). Hwp1 is a substrate for host transglutaminases. *C. albicans HWP1* is also required for biofilm formation (Ene and Bennett 2009), and is associated with cell wall remodelling (Plaine et al. 2008). In addition, it has been shown that some regulators of the yeast-hyphal transition, especially Efg1 (a transcription factors that lies downstream of protein kinase A) and Rim101 (a transcription factor involved in the alkaline pH response), are involved in regulating the expression of cell wall protein genes and in cell wall remodelling (Sohn et al. 2003; Baek et al. 2006; Gregori et al. 2011).

The inner layer of the cell wall of *C. albicans* consists of β-glucan and chitin, which is masked by the outer layer of mannoproteins (Kapteyn et al. 2000; Klis et al. 2001). However, β-glucan becomes exposed preferentially in hyphae compared to yeast in vivo and in vitro. Deletion of *C. albicans EDT1* encoding a key regulator of filamentation resulted in constitutive growth of yeast (Chen et al. 2004). This yeast-locked *edt1*Δ mutant showed less cell surface glucan exposure in comparison to wild-type hyphae and the hypha-locked *nrg1*Δ mutant in vitro (Wheeler et al. 2008). Whereas β-glucan exposure was observed in both hyphae and yeast cells (but preferentially hyphae) during in vivo infection and in response to caspofungin treatment (Wheeler et al. 2008; Wheeler and Fink 2006). Exposure of β-glucan, in particular β(1,3)glucan, stimulates immune responses, which leads to an increase in both pro- and anti-inflammatory responses as indicated by production of cytokines such as TNF-α, IL-6, IL-10 and IFN-γ (Wheeler and Fink 2006; Gow et al. 2007). The well-characterised pattern recognition receptors (PRRs) for β(1,3)-glucan are Dectin-1 as well as TLR2, which are expressed on macrophages, dendritic cells and other myeloid cells (Gow and Hube 2012; Netea et al. 2008; Vautier et al. 2012). A possible PRR for β(1,6)glucan is expressed by neutrophils (Rubin-Bejerano et al. 2007). In this study, human neutrophils efficiently ingested beads coated with β(1,6)glucan extracted from *C. albicans* while ignoring β(1,3)glucan-coated beads.

Chitin is thought to be located in the inner most part of the cell wall, close to the plasma membrane. Mammalian cells are unable to make chitin, but produce chitinases including CHIT-1 and acidic mammalian chitinases (AMCases) (Lee et al. 2008; Lee 2009; Vega and Kalkum 2012). Chitinase activity is thought to be involved in immune responses to fungal infections. Chitin and chitin derivatives such as chitosan can also be pathogen-associated molecular patterns (PAMPs) and stimulate immune responses including allergic inflammation (Lee et al. 2008; Lee 2009). High expression of AMCases is found in the lungs of an allergy animal model. Furthermore, intravenous administration of chitin particles (1–10 μm) into the mouse lung stimulates macrophages and NK cells, and leads to production of cytokines: IL-12, TNFα, IL-18, and INF-γ (Lee 2009). Similarly, chitin microparticles stimulated IL-10 cytokine production in non-inflamed colons in acute and chronic colitis models (Nagatani et al. 2012). The immunological effects of chitin seem to be critically dependent on the size of the chitin-containing particle (Lee 2009). A number of PRRs have been suggested to recognise chitin including the

mannose receptor, Dectin-1, and TLR2 on macrophages (Lee 2009; Vega and Kalkum 2012). Interestingly, Mora-Montes et al. showed that chitin is involved in blocking immune recognition of *C. albicans*, mediated via Dectin-1 (Mora-Montes et al. 2011). When *C. albicans* cells were co-incubated with chitin, production of TNFα, IL-6 and IL-1β cytokines was significantly reduced. Furthermore, *C. albicans* hyphae have 3–5 fold higher chitin content than yeast cells (Munro et al. 1998), and cytokine production of IL-6, TNFα, and IL-1β was stimulated less in hyphae compared to yeast, which may be a result of the increase in chitin content associated with *C. albicans* hyphae (personal communication Liliane Mukaremera and Neil Gow, University of Aberdeen, UK).

An increase in chitin content in the cell wall has been observed as a primary rescue phenomenon when cell wall integrity has been compromised in an attempt to maintain cellular integrity (Walker et al. 2008; Popolo et al. 2001; Munro et al. 2007). Indeed, defects in the cell wall caused by deletion of many cell wall-related genes or exposure to stress reagents, such as CFW and the echinocandins, leads to significant elevation of chitin levels. Walker et al. (2008) have shown that pre-treatment of caspofungin-hypersensitive *C. albicans* cells (*chs3*Δ, *mkc1*Δ and *cna1*Δ) with GlcN resulted in enhanced chitin levels and protected against caspofungin (Walker et al. 2008). High-chitin cells (pre-treated with $CaCl_2$ and CFW) which had 3–4 fold higher chitin were avirulent in a murine model of candidiasis and did not kill the majority of mice despite causing higher fungal kidney burdens compared to cells with normal chitin levels (Lee et al. 2012). These high-chitin cells were resistant to caspofungin in vivo and obtained an amino acid substitution in Fks1. Clinical isolates that have acquired point mutations within the *FKS1* gene have increased chitin levels, a reduced inflammatory response mediated via Dectin-1, and were unable to form hyphae under inducing condition (Ben-Ami et al. 2011; Ben-Ami and Kontoyiannis 2012). Caspofungin treatment of *C. albicans* results in exposure of chitin as well as β(1,3)glucan at the cell surface (Wheeler et al. 2008; Mora-Montes et al. 2011). *C. albicans* treated with sub-MIC of caspofungin stimulated less TNFα, IL-6, IL-1β and IL-10 production by human PBMCs (Mora-Montes et al. 2011). Interestingly, caspofungin-exposed *C. albicans* cells had higher expression of proteins associated with glycolysis and gluconeogenesis (Cdc19, Pdp1, Pgk1, Gnd1, Pgm2, Fba1 and Hxk2), the glyoxylate cycle (Mdh1, Cit1, Idh2 and Aco1) and amino acid biosynthesis (Bat22, Hom2, Gdh3 and Sah1) (Hoehamer et al. 2010). Similar results were observed in a previous study looking at the transcriptional response to caspofungin (Copping et al. 2005). Therefore, in *C. albicans* accessibility of wall polysaccharides at the cell surface, which are key triggers of immune recognition, stimulation and immune evasion, could be affected by antifungal agents, morphology and carbon sources.

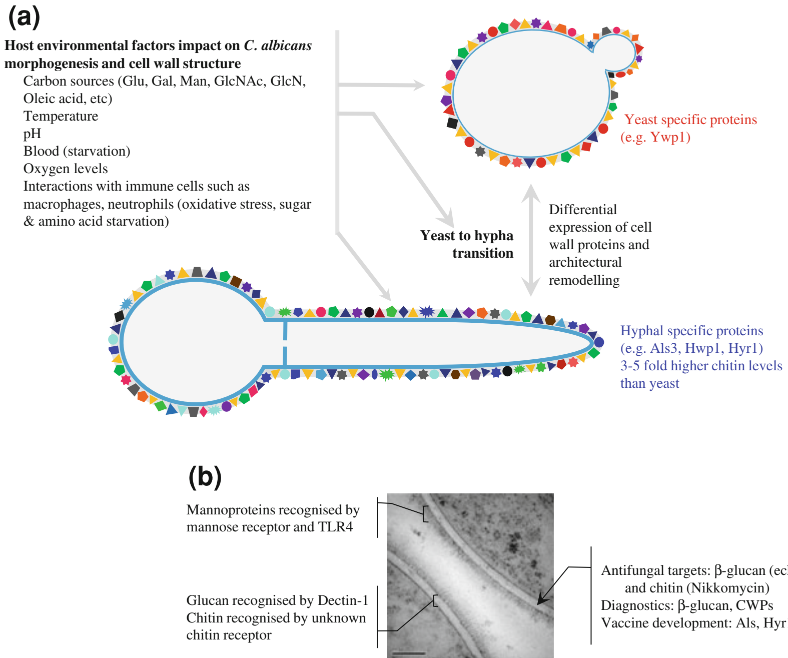

Fig. 7.2 Cell wall remodelling of *C. albicans* in response to host environmental niches. **a** Factors within the host environment such as available nutrients, temperature, pH, oxygen levels and other stresses produced by host immune cells stimulate the yeast to hyphal transition, which leads to dynamic alterations in cell wall architecture and composition, and rearrangement of cell wall proteins. For instance, hyphal-associated proteins including Als3, Hwp1 and Hyr1 are highly expressed on the surface of hyphae in comparison to yeast cells which specifically express Ywp1. Also, hyphal cells contain 3–5 fold higher chitin levels than yeast cells. This cell wall remodelling affects host-pathogen interactions and immune recognition. **b** The figure of a high pressure freezing, freeze-substitution TEM shows *C. albicans* cell wall (scale bar = 200 nm). As cell wall components do not exist in the mammalian host and are specific to the invading pathogen, in this case *C. albicans*, they are unique targets for antifungal agents, diagnostics and vaccine development

7.9 Conclusions and Future Challenges

This chapter describes the influence of metabolism on *C. albicans* cell wall biosynthesis, host interactions and virulence. There are countless enzymes involved in carbon metabolism and cell wall biosynthesis (Fig. 7.1). These enzymes are directly or indirectly responsible for cell viability, cell wall integrity, metabolism, morphology and virulence. All fungi, including *C. albicans,* have to somehow coordinate utilisation of sugars as a source of energy with the production of activated sugars (UDP-glucose, GDP-mannose, UDP-*N*-acetylglucosamine) that are incorporated into the major cell wall polysaccharides (glucan, mannan and chitin) in order to generate the new cell wall required for growth. In addition, fungi can alter the structure and composition of their cell walls in response to external

and internal signals by modulating the production of the cell wall polysaccharides and how they are linked together. The *C. albicans* cell wall can rapidly and dynamically change in response to external signals including oxidative stress produced by macrophages and neutrophils, pH changes depending on host niches, body temperature, amino acid starvation, exposure to antifungal drugs and availability of carbon sources (Fig. 7.2). A better understanding of cell wall remodelling mechanisms in vivo will improve the efficacy of antifungal agents and aid the development of de novo antifungal therapeutics, and rapid, sensitive and accurate diagnostic. Therefore, the complex, dynamic inter-relationship between cell wall synthesis and remodelling, and carbon metabolism is vital for viability and strongly influences host–pathogen interactions and pathogenicity.

References

Aebi M (2013) *N*-linked protein glycosylation in the ER. Biochimica et Biophysica Acta dol 1833(11):2430–2437

Almeida RS, Brunke S, Albrecht A, Thewes S, Laue M, Edwards JE Jr, Filler SG, Hube B (2008) The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. PLoS Pathog 4:e1000217

Alvarez FJ, Konopka JB (2007) Identification of an *N*-acetylglucosamine transporter that mediates hyphal induction in *Candida albicans*. Mol Biol Cell 18:965–975

Askew C, Sellam A, Epp E, Hogues H, Mullick A, Nantel A, Whiteway M (2009) Transcriptional regulation of carbohydrate metabolism in the human pathogen *Candida albicans*. PLoS Pathog 5:e1000612

Baek YU, Martin SJ, Davis DA (2006) Evidence for novel pH-dependent regulation of *Candida albicans* Rim101, a direct transcriptional repressor of the cell wall β-glycosidase Phr2. Eukaryot Cell 5:1550–1559

Bahnan W, Koussa J, Younes S, Rizk MA, Khalil B, Sitt SE, Hanna S, El-Sibai M, Khalaf RA (2012) Deletion of the *Candida albicans PIR32* results in increased virulence, stress response, and upregulation of cell call chitin deposition. Mycopathologia 174:107–119

Baker LG, Specht CA, Lodge JK (2011) Cell wall chitosan is necessary for virulence in the opportunistic pathogen *Cryptococcus neoformans*. Eukaryot Cell 10:1264–1268

Barelle CJ, Priest CL, MacCallum DM, Gow NAR, Odds FC, Brown AJP (2006) Niche-specific regulation of central metabolic pathways in a fungal pathogen. Cell Microbiol 8:961–971

Bates S, Hughes HB, Munro CA, Thomas WP, MacCallum DM, Bertram G, Atrih A, Ferguson MA, Brown AJ, Odds FC, Gow NA (2006) Outer chain *N*-glycans are required for cell wall integrity and virulence of *Candida albicans*. J Biol Chem 281:90–98

Bates S, MacCallum DM, Bertram G, Munro CA, Hughes HB, Buurman ET, Brown AJ, Odds FC, Gow NA (2005) *Candida albicans* Pmr1p, a secretory pathway P-type Ca^{2+}/Mn^{2+}-ATPase, is required for glycosylation and virulence. J Biol Chem 280:23408–23415

Becker JM, Kauffman SJ, Hauser M, Huang L, Lin M, Sillaots S, Jiang B, Xu D, Roemer T (2010) Pathway analysis of *Candida albicans* survival and virulence determinants in a murine infection model. Proc Natl Acad Sci USA 107:22044–22049

Ben-Ami R, Garicia-Effron G, Lewis RE, Gamarra S, Leventakos K, Perlin DS, Kontoyiannis P (2011) Fitness and virulence costs of *Candida albicans FKS1* hot spot mutations associated with echinocandin resistance. J Infec Dis 204:626–635

Ben-Ami R, Kontoyiannis DP (2012) Resistance to echinocandins comes at a cost: the impact of *FKS1* hotspot mutations on *Candida albicans* fitness and virulence. Virulence 3:95–98

Bowman SM, Free SJ (2006) The structure and synthesis of the fungal cell wall. BioEssays 28:799–808

Brown DH Jr, Giusani AD, Chen X, Kumamoto CA (1999) Filamentous growth of *Candida albicans* in response to physical environmental cues and its regulation by the unique *CZF1* gene. Mol Microbiol 34:651–662

Bruno VM, Kalachikov S, Subaran R, Nobile CJ, Kyratsous C, Mitchell AP (2006) Control of the *Candida albicans* cell wall damage response by transcriptional regulator Cas5. PLoS Pathog 2:0204–0210

Bulawa CE, Miller DW, Henry LK, Becker JM (1995) Attenuated virulence of chitin-deficient mutants of *Candida albicans*. Proc Natl Acad Sci USA 92:10570–10574

Bulik DA, Olczak M, Lucero HA, Osmond BC, Robbins PW, Specht CA (2003) Chitin synthesis in *Saccharomyces cerevisiae* in response to supplementation of growth medium with glucosamine and cell wall stress. Eukaryot Cell 2:886–900

Buurman ET, Westwater C, Hube B, Brown AJP, Odds FC, Gow NAR (1998) Molecular analysis of CaMnt1p, a mannosyl transferase important for adhesion and virulence of *Candida albicans*. Proc Natl Acad Sci USA 95:7670–7675

Cabib E (2009) Two novel techniques for determination of polysaccharide cross-links show that Crh1p and Crh2p attach chitin to both β(1-6)- and β(1-3)glucan in the *Saccharomyces cerevisiae* cell wall. Eukaryot Cell 8:1626–1636

Cabib E, Farkas V, Kosík O, Blanco N, Arroyo J, McPhie P (2008) Assembly of the yeast cell wall: Crh1p and Crh2p act as transglycosylases *in vivo* and in *vitro*. J Biol Chem 283:29859–29872

Cabib E, Blanco N, Grau C, Rodríguez-Peña JM, Arroyo J (2007) Crh1p and Crh2p are required for the cross-linking of chitin to β(1-6)glucan in the *Saccharomyces cerevisiae* cell wall. Mol Microbiol 63:921–935

Campbell RN, Leverentz MK, Ryan LA, Reece RJ (2008) Metabolic control of transcription: paradigms and lessons from *Saccharomyces cerevisiae*. Biochem J 414:177–187

Chaffin WL (2008) *Candida albicans* cell wall proteins. Microbiol Mol Biol Rev 72:495–544

Chaffin WL, López-Ribot JL, Casanova M, Gozalbo D, Martínez JP (1998) Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol Mol Biol Rev 62:130–180

Chen H, Fujita M, Feng Q, Clardy J, Fink GR (2004) Tyrosol is a quorum-sensing molecule in *Candida albicans*. Proc Natl Acad Sci USA 101:5048–5052

Copping VMS, Barelle CJ, Hube B, Gow NAR, Brown AJP, Odds FC (2005) Exposure of *Candida albicans* to antifungal agents affects expression of *SAP2* and *SAP9* secreted proteinase genes. J Antimicrob Chemother 55:645–654

de Groot PWJ, Hellingwerf KJ, Klis FM (2003) Genome-wide identification of fungal GPI proteins. Yeast 20:781–796

Denning DW (2002) Echinocandins: a new class of antifungal. J Antimicrob Chemother 49:889–891

Douglas CM, D'Ippolito JA, Shei GJ, Meinz M, Onishi J, Marrinan JA, Li W, Abruzzo GK, Flattery A, Bartizal K, Mitchell A, Kurtz MB (1997) Identification of the *FKS1* gene of *Candida albicans* as the essential target of 1,3-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother 41:2471–2479

Ene IV, Cheng S-, Netea MG, Brown AJP (2013) Growth of *Candida albicans* cells on the physiologically relevant carbon source lactate affects their recognition and phagocytosis by immune cells. Infect Immun 81:238–248

Ene IV, Adya AK, Wehmeier S, Brand AC, Maccallum DM, Gow NAR, Brown AJP (2012a) Host carbon sources modulate cell wall architecture, drug resistance and virulence in a fungal pathogen. Cell Microbiol 14:1319–1335

Ene IV, Heilmann CJ, Sorgo AG, Walker LA, De Koster CG, Munro CA, Klis FM, Brown AJP (2012b) Carbon source-induced reprogramming of the cell wall proteome and secretome modulates the adherence and drug resistance of the fungal pathogen *Candida albicans*. Proteomics 12:3164–3179

Ene IV, Bennett RJ (2009) Hwp1 and related adhesins contribute to both mating and biofilm formation in *Candida albicans*. Eukaryot Cell 8:1909–1913

Ener B, Douglas LJ (1992) Correlation between cell-surface hydrophobicity of *Candida albicans* and adhesion to buccal epithelial cells. FEMS Microbiol Lett 99:37–42

Fan J, Chaturvedi V, Shen S (2002) Identification and phylogenetic analysis of a glucose transporter gene family from the human pathogenic yeast *Candida albicans*. J Mol Evol 55:336–346

Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP (2012) Divergent targets of *Candida albicans* biofilm regulator Bcr1 *in vitro* and *in vivo*. Eukaryot Cell 11:896–904

Fleck CB, Schöbel F, Brock M (2011) Nutrient acquisition by pathogenic fungi: nutrient availability, pathway regulation, and differences in substrate utilization. Int J Med Microbiol 301:400–407

Fonzi WA (1999) *PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of β-1,3- and β-1,6-glucans. J Bacteriol 181:7070–7079

Fradin C, Hube B (2006) Transcriptional profiling of *Candida albicans* in human blood. Microbe 1:76–80

Fradin C, Thewes S, Zakikhany K, Albrecht A, Bader O, Kunze D, Hube B (2004) Transcriptional profiling of *Candida albicans* during infections. Mikologia Lekarska 11:157–163

Free SJ (2013) Fungal cell wall organization and biosynthesis. Adv Genet 81:33–82

Fujita M, Kinoshita T (2012) GPI-anchor remodeling: potential functions of GPI-anchors in intracellular trafficking and membrane dynamics. Biochim Biophys Acta 1821:1050–1058

Fukuda Y, Tsai H-, Myers TG, Bennett JE (2013) Transcriptional profiling of *Candida glabrata* during phagocytosis by neutrophils and in the infected mouse spleen. Infect Immun 81:1325–1333

Gelis S, de Groot PWJ, Castillo L, Moragues M-, Sentandreu R, Gómez M-, Valentín E (2012) Pga13 in *Candida albicans* is localized in the cell wall and influences cell surface properties, morphogenesis and virulence. Fungal Genet Biol 49:322–331

Gow NA, Hube B (2012) Importance of the *Candida albicans* cell wall during commensalism and infection. Curr Opin Microbiol 15:406–412

Gow NA, Netea MG, Munro CA, Ferwerda G, Bates S, Mora-Montes HM, Walker L, Jansen T, Jacobs L, Tsoni V, Brown GD, Odds FC, Van der Meer JW, Brown AJ, Kullberg BJ (2007) Immune recognition of *Candida albicans* beta-glucan by dectin-1. J Infect Dis 196:1565–1571

Gregori C, Glaser W, Frohner IE, Reinoso-Martín C, Rupp S, Schüller C, Kuchler K (2011) Efg1 controls caspofungin-induced cell aggregation of *Candida albicans* through the adhesin Als1. Eukaryot Cell 10:1694–1704

Gunasekera A, Alvarez FJ, Douglas LM, Wang HX, Rosebrock AP, Konopka JB (2010) Identification of *GIG1*, a GlcNAc-induced gene in *Candida albicans* needed for normal sensitivity to the chitin synthase inhibitor nikkomycin Z. Eukaryot Cell 9:1476–1483

Hall RA, Bates S, Lenardon MD, Maccallum DM, Wagener J, Lowman DW, Kruppa MD, Williams DL, Odds FC, Brown AJ, Gow NA (2013) The Mnn2 mannosyltransferase family modulates mannoprotein fibril length, immune recognition and virulence of *Candida albicans*. PLoS Pathog 9:e1003276

Hayek P, Dib L, Yazbeck P, Beyrouthy B, Khalaf RA (2010) Characterization of Hwp2, a *Candida albicans* putative GPI-anchored cell wall protein necessary for invasive growth. Microbiol Res 165:250–258

Heilmann CJ, Sorgo AG, Siliakus AR, Dekker HL, Brul S, Koster CG, de Koning LJ, Klis FM (2011) Hyphal induction in the human fungal pathogen *Candida albicans* reveals a characteristic wall protein profile. Microbiology 157:2297–2307

Herrero AB, Magnelli P, Mansour MK, Levitz SM, Bussey H, Abeijon C (2004) *KRE5* gene null mutant strains of *Candida albicans* are avirulent and have altered cell wall composition and hypha formation properties. Eukaryot Cell 3:1423–1432

Hoehamer CF, Cummings ED, Hilliard GM, Rogers PD (2010) Changes in the proteome of *Candida albicans* in response to azole, polyene, and echinocandin antifungal agents. Antimicrob Agents Chemother 54:1655–1664

Hoyer LL, Green CB, Oh SH, Zhao X (2008) Discovering the secrets of the *Candida albicans* agglutinin-like sequence (*ALS*) gene family—A sticky pursuit. Med Mycol 46:1–15

Hoyer LL (2001) The *ALS* gene family of *Candida albicans*. Trends Microbiol 9:176–180

Huang G (2012) Regulation of phenotypic transitions in the fungal pathogen *Candida albicans*. Virulence 3:6

Huang G, Yi S, Sahni N, Daniels KJ, Srikantha T, Soll DR (2010) *N*-acetylglucosamine induces white to opaque switching, a mating prerequisite in *Candida albicans*. PLoS Pathog 6:e1000806

Hube B (2006) Infection-associated genes of *Candida albicans*. Future Microbiol 1:209–218

Jyothi Kumar M, Jamaluddin MS, Natarajan K, Kaur D, Datta A (2000) The inducible *N*-acetylglucosamine catabolic pathway gene cluster in *Candida albicans*: discrete *N*-acetylglucosamine-inducible factors interact at the promoter of *NAG1*. Proc Natl Acad Sci USA 97:14218–14223

Kamai Y, Kubota M, Kamai Y, Hosokawa T, Fukuoka T, Filler SG (2002) Contribution of *Candida albicans ALS1* to the pathogenesis of experimental oropharyngeal candidiasis. Infect Immun 70:5256–5258

Kamthan M, Kamthan A, Ruhela D, Maiti P, Bhavesh NS, Datta A (2013) Upregulation of galactose metabolic pathway by *N*-acetylglucosamine induced endogenous synthesis of galactose in *Candida albicans*. Fungal Genet Biol 54:15–24

Kamthan M, Mukhopadhyay G, Chakraborty N, Chakraborty S, Datta A (2012) Quantitative proteomics and metabolomics approaches to demonstrate *N*-acetyl-d-glucosamine inducible amino acid deprivation response as morphological switch in *Candida albicans*. Fungal Genet Biol 49:369–378

Kapteyn JC, Hoyer LL, Hecht JE, Muller WH, Andel A, Verkleij AJ, Makarow M, Van Den Ende H, Klis FM (2000) The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. Mol Microbiol 35:601–611

Kitamura A, Someya K, Hata M, Nakajima R, Takemura M (2009) Discovery of a small-molecule inhibitor of β-1,6-glucan synthesis. Antimicrob Agents Chemother 53:670–677

Klis FM, Sosinska GJ, De Groot PWJ, Brul S (2009) Covalently linked cell wall proteins of *Candida albicans* and their role in fitness and virulence. FEMS Yeast Res 9:1013–1028

Klis FM, de Groot P, Hellingwerf K (2001) Molecular organization of the cell wall of *Candida albicans*. Med Mycol 39(Suppl 1):1–8

Kollar R, Petrakova E, Ashwell G, Robbins PW, Cabib E (1995) Architecture of the yeast cell wall. The linkage between chitin and $\beta(1 \rightarrow 3)$-glucan. J Biol Chem 270:1170–1178

Kollár R, Reinhold BB, Petráková E, Yeh HJC, Ashwell G, Drgonová J, Kapteyn JC, Klis FM, Cabib E (1997) Architecture of the yeast cell wall: $\beta(1 \rightarrow 6)$glucan interconnects mannoprotein, $\beta(1 \rightarrow 3)$-glucan, and chitin. J Biol Chem 272:17762–17775

Konopka JB (2012) *N*-acetylglucosamine (GlcNAc) functions in cell signaling. Scientifica (Cairo) 2012:489208

Kvaal C, Lachke SA, Srikantha T, Daniels K, Mccoy J, Soll DR (1999) Misexpression of the opaque-phase-specific gene *PEP1* (*SAP1*) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection. Infect Immun 67:6652–6662

Lagorce A, Berre-Anton VL, Aguilar-Uscanga B, Martin-Yken H, Dagkessamanskaia A, François J (2002) Involvement of *GFA1*, which encodes glutamine-fructose-6-phosphate amidotransferase, in the activation of the chitin synthesis pathway in response to cell-wall defects in *Saccharomyces cerevisiae*. European J Biochem 269:1697–1707

Lan C-, Newport G, Murillo LA, Jones T, Scherer S, Davis RW, Agabian N (2002) Metabolic specialization associated with phenotypic switching in *Candida albicans*. Proc Natl Acad Sci USA 99:14907–14912

Latgé JP (2007) The cell wall: a carbohydrate armour for the fungal cell. Mol Microbiol 66:279–290

Lee CG (2009) Chitin, chitinases and chitinase-like proteins in allergic inflammation and tissue remodeling. Yonsei Med J 50:22–30

Lee CG, Da Silva CA, Lee JY, Hartl D, Elias JA (2008) Chitin regulation of immune responses: an old molecule with new roles. Curr Opin Immunol 20:684–689

Lee KK, MacCallum DM, Jacobsen MD, Walker LA, Odds FC, Gow NAR, Munro CA (2012) Elevated cell wall chitin in *Candida albicans* confers echinocandin resistance *in vivo*. Antimicrob Agents Chemother 56:208–217

Lenardon MD, Munro CA, Gow NAR (2010) Chitin synthesis and fungal pathogenesis. Curr Opin Microbiol 13:416–423

Lipinski T, Wu X, Sadowska J, Kreiter E, Yasui Y, Cheriaparambil S, Rennie R, Bundle DR (2012) A β-mannan trisaccharide conjugate vaccine aids clearance of *Candida albicans* in immunocompromised rabbits. Vaccine 30:6263–6269

Liu TT, Lee REB, Barker KS, Lee RE, Wei L, Homayouni R, Rogers PD (2005) Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. Antimicrob Agents Chemother 49:2226–2236

Liu Y, Filler SG (2011) *Candida albicans* Als3, a multifunctional adhesin and invasin. Eukaryot Cell 10:168–173

Lorenz MC, Bender JA, Fink GR (2004) Transcriptional response of *Candida albicans* upon internalization by macrophages. Eukaryot Cell 3:1076–1087

Lussier M, Sdicu AM, Shahinian S, Bussey H (1998) The *Candida albicans KRE9* gene is required for cell wall β-1,6-glucan synthesis and is essential for growth on glucose. Proc Natl Acad Sci USA 95:9825–9830

Marcil A, Gadoury C, Ash J, Zhang J, Nantel A, Whiteway M (2008) Analysis of *PRA1* and its relationship to *Candida albicans*-macrophage interactions. Infec Immun 76:4345–4358

Martchenko M, Levitin A, Hogues H, Nantel A, Whiteway M (2007) Transcriptional rewiring of fungal galactose-metabolism circuitry. Curr Biol 17:1007–1013

Martin R, Albrecht-Eckardt D, Brunke S, Hube B, Hünniger K, Kurzai O (2013) A core filamentation response network in *Candida albicans* is restricted to eight genes. PLoS ONE 8:e58613

Martínez AI, Castillo L, Garcerá A, Elorza MV, Valentín E, Sentandreu R (2004) Role of Pir1 in the construction of the *Candida albicans* cell wall. Microbiology 150:3151–3161

Mattia E, Carruba G, Angiolella L, Cassone A (1982) Induction of germ tube formation by *N*-acetyl-D-glucosamine in *Candida albicans*: uptake of inducer and germinative response. J Bacteriol 152:555–562

McKenzie CGJ, Koser U, Lewis LE, Bain JM, Mora-Montes HM, Barker RN, Gow NAR, Erwig LP (2010) Contribution of *Candida albicans* cell wall components to recognition by and escape from murine macrophages. Infect Immun 78:1650–1658

Milewski S, Gabriel I, Olchowy J (2006) Enzymes of UDP-GlcNAc biosynthesis in yeast. Yeast 23:1–14

Mille C, Bobrowicz P, Trinel P-, Li H, Maes E, Guerardel Y, Fradin C, Martínez-Esparza M, Davidson RC, Janbon G, Poulain D, Wildt S (2008) Identification of a new family of genes involved in β-1,2- mannosylation of glycans in Pichia pastoris and *Candida albicans*. J Biol Chem 283:9724–9736

Mio T, Kokado M, Arisawa M, Yamada-Okabe H (2000) Reduced virulence of *Candida albicans* mutants lacking the *GNA1* gene encoding glucosamine-6-phosphate acetyltransferase. Microbiology 146:1753–1758

Mio T, Adachi-Shimizu M, Tachibana Y, Tabuchi H, Inoue SB, Yabe T, Yamada-Okabe T, Arisawa M, Watanabe T, Yamada-Okabe H (1997a) Cloning of the *Candida albicans* homolog of *Saccharomyces cerevisiae GSC1/FKS1* and its involvement in β-1,3-glucan synthesis. J Bacteriol 179:4096–4105

Mio T, Yamada-Okabe T, Yabe T, Nakajima T, Arisawa M, Yamada-Okabe H (1997b) Isolation of the *Candida albicans* homologs of *Saccharomyces cerevisiae KRE6* and *SKN1*: expression and physiological function. J Bacteriol 179:2363–2372

Miramón P, Dunker C, Windecker H, Bohovych IM, Brown AJP, Kurzai O, Hube B (2012) Cellular responses of *Candida albicans* to phagocytosis and the extracellular activities of neutrophils are critical to counteract carbohydrate starvation, oxidative and nitrosative stress. PLoS ONE 7:e52850

Mora-Montes HM, Netea MG, Ferwerda G, Lenardon MD, Brown GD, Misura AS, Kullberg BJ, O'Callaghan CA, Sheth CC, Odds FC, Brown AJP, Munro CA, Gow NAR (2011) Recognition and blocking of innate immunity cells by *Candida albicans* chitin. Infect Immun 79:1961–1970

Mora-Montes HM, Ponce-Noyola P, Villagómez-Castro JC, Gow NAR, Flores-Carreón A, López-Romero E (2009) Protein glycosylation in *Candida*. Future Microbiol 4:1167–1183

Munro CA (2013) Chitin and glucan, the yin and yang of the fungal cell wall, implications for antifungal drug discovery and therapy. Adv Appl Microbiol 83:145–172

Munro CA, Richard ML (2012) The cell wall: glycoproteins, remodeling, and regulation. In: Calderone RA, Clancy CJ (eds) *Candida* and Candidiasis, 2nd edn. ASM Press, Washington D.C, pp 197–223

Munro CA, Selvaggini S, de Bruijn I, Walker L, Lenardon MD, Gerssen B, Milne S, Brown AJ, Gow NA (2007) The PKC, HOG and Ca^{2+} signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*. Mol Microbiol 63:1399–1413

Munro CA, Bates S, Buurman ET, Hughes HB, Maccallum DM, Bertram G, Atrih A, Ferguson MA, Bain JM, Brand A, Hamilton S, Westwater C, Thomson LM, Brown AJ, Odds FC, Gow NA (2005) Mnt1p and Mnt2p of *Candida albicans* are partially redundant alpha-1,2-mannosyltransferases that participate in *O*-linked mannosylation and are required for adhesion and virulence. J Biol Chem 280:1051–1060

Munro CA, Winter K, Buchan A, Henry K, Becker JM, Brown AJ, Bulawa CE, Gow NA (2001) Chs1 of *Candida albicans* is an essential chitin synthase required for synthesis of the septum and for cell integrity. Mol Microbiol 39:1414–1426

Munro CA, Schofield DA, Gooday GW, Gow NA (1998) Regulation of chitin synthesis during dimorphic growth of *Candida albicans*. Microbiology 144:391–401

Murciano C, Moyes DL, Runglall M, Tobouti P, Islam A, Hoyer LL, Naglik JR (2012) Evaluation of the role of *Candida albicans* agglutinin-like sequence (*ALS*) proteins in human oral epithelial cell interactions. PLoS ONE 7:e33362

Nagatani K, Wang S, Llado V, Lau CW, Li Z, Mizoguchi A, Nagler CR, Shibata Y, Reinecker HC, Mora JR, Mizoguchi E (2012) Chitin microparticles for the control of intestinal inflammation. Inflamm Bowel Dis 18:1698–1710

Naseem S, Gunasekera A, Araya E, Konopka JB (2011) *N*-acetylglucosamine (GlcNAc) induction of hyphal morphogenesis and transcriptional responses in *Candida albicans* are not dependent on its metabolism. J Biol Chem 286:28671–28680

Natarajan K, Datta A (1993) Molecular cloning and analysis of the *NAG1* cDNA coding for glucosamine-6-phosphate deaminase from *Candida albicans*. J Biol Chem 268:9206–9214

Netea MG, Brown GD, Kullberg BJ, Gow NAR (2008) An integrated model of the recognition of *Candida albicans* by the innate immune system. Nat Rev Microbiol 6:67–78

Netea MG, Gow NAR, Munro CA, Bates S, Collins C, Ferwerda G, Hobson RP, Bertram G, Hughes HB, Jansen T, Jacobs L, Buurman ET, Gijzen K, Williams DL, Torensma R, McKinnon A, MacCallum DM, Odds FC, Van Der Meer JWM, Brown AJP, Kullberg BJ (2006) Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. J Clin Invest 116:1642–1650

Nett J, Lincoln L, Marchillo K, Massey R, Holoyda K, Hoff B, VanHandel M, Andes D (2007) Putative role of beta-1,3 glucans in *Candida albicans* biofilm resistance. Antimicrob Agents Chemother 51:510–520

Nett JE, Crawford K, Marchillo K, Andes DR (2010) Role of Fks1p and matrix glucan in *Candida albicans* biofilm resistance to an echinocandin, pyrimidine, and polyene. Antimicrob Agents Chemother 54:3505–3508

Odds FC (ed) (1988) *Candida and Candidosis* 2nd edn. Bailliere Tindall, London

Onishi A, Sugiyama D, Kogata Y, Saegusa J, Sugimoto T, Kawano S, Morinobu A, Nishimura K, Kumagaia S (2012) Diagnostic accuracy of serum 1,3-ß-D-glucan for Pneumocystis jiroveci pneumonia, invasive candidiasis, and invasive aspergillosis: systematic review and meta-analysis. J Clin Microbiol 50:7–15

Ostrosky-Zeichner L (2012) Invasive mycoses: diagnostic challenges. Am J Med 125:S14–S24

Pardini G, De Groot PWJ, Coste AT, Karababa M, Klis FM, De Koster CG, Sanglard D (2006) The CRH family coding for cell wall glycosylphosphatidylinositol proteins with a predicted transglycosidase domain affects cell wall organization and virulence of *Candida albicans*. J Biol Chem 281:40399–40411

Phan QT, Myers CL, Fu Y, Sheppard DC, Yeaman MR, Welch WH, Ibrahim AS, Edwards JE Jr, Filler SG (2007) Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. PLoS Biol 5:0543–0557

Plaine A, Walker L, Da Costa G, Mora-Montes HM, McKinnon A, Gow NA, Gaillardin C, Munro CA, Richard ML (2008) Functional analysis of *Candida albicans* GPI-anchored proteins: roles in cell wall integrity and caspofungin sensitivity. Fungal Genet Biol 45: 1404–1414

Plumbridge JA (1989) Sequence of the nagBACD operon in Escherichia coli K12 and pattern of transcription within the nag regulon. Mol Microbiol 3:505–515

Popolo L, Gualtieri T, Ragni E (2001) The yeast cell-wall salvage pathway. Med Mycol 39:111–121

Prill SK, Klinkert B, Timpel C, Gale CA, Schroppel K, Ernst JF (2005) PMT family of *Candida albicans*: five protein mannosyltransferase isoforms affect growth, morphogenesis and antifungal resistance. Mol Microbiol 55:546–560

Qadota H, Python CP, Inoue SB, Arisawa M, Anraku Y, Zheng Y, Watanabe T, Levin DE, Ohya Y (1996) Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-β-glucan synthase. Science 272:279–281

Ram AFJ, Arentshorst M, Damveld RA, vanKuyk PA, Klis FM, van den Hondel CAMJJ (2004) The cell wall stress response in *Aspergillus niger* involves increased expression of the glutamine: fructose-6-phosphate amidotransferase-encoding gene (gfaA) and increased deposition of chitin in the cell wall. Microbiology 150:3315–3326

Rao KH, Ghosh S, Natarajan K, Datta A (2013) *N*-acetylglucosamine kinase, *HXK1* is involved in morphogenetic transition and metabolic gene expression in *Candida albicans*. PLoS ONE 8:e53638

Richard ML, Plaine A (2007) Comprehensive analysis of glycosylphosphatidylinositol-anchored proteins in *Candida albicans*. Eukaryot Cell 6:119–133

Rubin-Bejerano I, Abeijon C, Magnelli P, Grisafi P, Fink GR (2007) Phagocytosis by human neutrophils is stimulated by a unique fungal cell wall component. Cell Host Microbe 2:55–67

Rubin-Bejerano I, Fraser I, Grisafi P, Fink GR (2003) Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. Proc Natl Acad Sci USA 100:11007–11012

Sandai D, Yin Z, Selway L, Stead D, Walker J, Leach MD, Bohovych I, Ene IV, Kastora S, Budge S, Munro CA, Odds FC, Gow NA, Brown AJ (2012) The evolutionary rewiring of ubiquitination targets has reprogrammed the regulation of carbon assimilation in the pathogenic yeast *Candida albicans*. MBio 3:e00495–12

Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJS (2013) *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol 62:10–24

Sengupta M, Datta A (2003) Two membrane proteins located in the Nag regulon of *Candida albicans* confer multidrug resistance. Biochem Biophys Res Commun 301:1099–1108

Shepardson KM, Cramer RA (2013) Fungal cell wall dynamics and infection site microenvironments: signal integration and infection outcome. Curr Opin Microbiol S1369–5274(13): 00035. doi:10.1016/j.mib.2013.03.003

Shibata N, Kobayashi H, Suzuki S (2012) Immunochemistry of pathogenic yeast, *Candida* species, focusing on Mannan. Proc Jpn Acad Ser B Phys Biol Sci 88:250–265

Singh B, Datta A (1979) Induction of *N*-acetylglucosamine-catabolic pathway in spheroplasts of *Candida albicans*. Biochem J 178:427–431

Singh P, Ghosh S, Datta A (2001) Attenuation of virulence and changes in morphology in *Candida albicans* by disruption of the *N*-acetylglucosamine catabolic pathway. Infect Immun 69:7898–7903

Singh V, Satheesh SV, Raghavendra ML, Sadhale PP (2007) The key enzyme in galactose metabolism, UDP-galactose-4-epimerase, affects cell-wall integrity and morphology in *Candida albicans* even in the absence of galactose. Fungal Genet Biol 44:563–574

Smith RJ, Milewski S, Brown AJP, Gooday GW (1996) Isolation and characterization of the *GFA1* gene encoding the glutamine: fructose-6-phosphate amidotransferase of *Candida albicans*. J Bacteriol 178:2320–2327

Smith TL, Rutter J (2007) Regulation of glucose partitioning by PAS Kinase and Ugp1 phosphorylation. Mol Cell 26:491–499

Sohn K, Urban C, Brunner H, Rupp S (2003) *EFG1* is a major regulator of cell wall dynamics in *Candida albicans* as revealed by DNA microarrays. Mol Microbiol 47:89–102

Soll DR (2009) Why does *Candida albicans* switch? FEMS Yeast Res 9:973–989

Spellberg B, Ibrahim AS, Lin L, Avanesian V, Fu Y, Lipke P, Otoo H, Ho T, Edwards JE Jr (2008) Antibody titer threshold predicts anti-candidal vaccine efficacy even though the mechanism of protection is induction of cell-mediated immunity. J Infect Dis 197:967–971

Staab JF, Bradway SD, Fidel PL, Sundstrom P (1999) Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. Science 283:1535–1538

Stoldt VR, Sonneborn A, Leuker CE, Ernst JF (1997) Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J 16:1982–1991

Sudbery PE (2011) Growth of *Candida albicans* hyphae. Nat Rev Microbiol 9:737–748

Taff HT, Nett JE, Zarnowski R, Ross KM, Sanchez H, Cain MT, Hamaker J, Mitchell AP, Andes DR (2012) A *Candida* biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. PLoS Pathog 8:e1002848

Ueno K, Okawara A, Yamagoe S, Naka T, Umeyama T, Utena-Abe Y, Tarumoto N, Niimi M, Ohno H, Doe M, Fujiwara N, Kinjo Y, Miyazaki Y (2013) The mannan of *Candida albicans* lacking beta-1,2-linked oligomannosides increases the production of inflammatory cytokines by dendritic cells. Med Mycol 51:385–395

Umeyama T, Kaneko A, Watanabe H, Hirai A, Uehara Y, Niimi M, Azuma M (2006) Deletion of the CaBIG1 gene reduces β-1,6-glucan synthesis, filamentation, adhesion, and virulence in *Candida albicans*. Infect Immun 74:2373–2381

Vautier S, MacCallum DM, Brown GD (2012) C-type lectin receptors and cytokines in fungal immunity. Cytokine 58:89–99

Vega K, Kalkum M (2012) Chitin, chitinase responses, and invasive fungal infections. Int J Microbiol 2012:920459

Walker LA, MacCallum DM, Bertram G, Gow NAR, Odds FC, Brown AJP (2009) Genome-wide analysis of *Candida albicans* gene expression patterns during infection of the mammalian kidney. Fungal Genet Biol 46:210–219

Walker LA, Munro CA, de Bruijn I, Lenardon MD, McKinnon A, Gow NA (2008) Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. PLoS Pathog 4:e1000040

Wendland J, Schaub Y, Walther A (2009) *N*-acetylglucosamine utilization by *Saccharomyces cerevisiae* based on expression of *Candida albicans NAG* genes. Appl Environ Microbiol 75:5840–5845

Wheeler RT, Kombe D, Agarwala SD, Fink GR (2008) Dynamic, morphotype-specific *Candida albicans* β-glucan exposure during infection and drug treatment. PLoS Pathog 4:e1000227

Wheeler RT, Fink GR (2006) A drug-sensitive genetic network masks fungi from the immune system. PLoS Pathog 2:e35

Yamada-Okabe T, Yamada-Okabe H (2002) Characterization of the Ca*NAG3*, Ca*NAG4*, and Ca*NAG6* genes of the pathogenic fungus *Candida albicans*: possible involvement of these genes in the susceptibilities of cytotoxic agents. FEMS Microbiol Lett 212:15–21

Yamada-Okabe T, Sakamori Y, Mio T, Yamada-Okabe H (2001) Identification and characterization of the genes for *N*-acetylglucosamine kinase and *N*-acetylglucosamine-phosphate deacetylase in the pathogenic fungus *Candida albicans*. European J Biochem 268:2498–2505

Zhao X, Oh S-, Cheng G, Green CB, Nuessen JA, Yeater K, Leng RP, Brown AJP, Hoyer LL (2004) *ALS3* and *ALS8* represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. Microbiology 150:2415–2428

Chapter 8
Molecular Mechanisms in Yeast Carbon Metabolism: Lipid Metabolism and Lipidomics

Birgit Ploier, Günther Daum and Uroš Petrovič

Abstract Lipids play several essential roles in the biology and metabolism of eukaryotic cells. In addition to their structural role as constituents of cell membranes, they have been increasingly recognized as dynamic and vital molecules, involved in a variety of cellular processes. Examples are cell signalling, membrane trafficking and influencing the stability of protein complexes in membranes. This chapter provides an overview of lipid classes and metabolic pathways in yeast. Lipid metabolism involves various organelles such as the endoplasmic reticulum (ER), mitochondria, peroxisomes and lipid droplets (LD), which will be highlighted. Specific attention is devoted to examples of recently discovered key players in yeast lipid metabolism, which illustrate our improved understanding of cells as an interconnected biological system. This chapter comprises descriptions of regulatory networks, multifunctional enzymes and lipids that serve as modulators of their own synthesis. The last part of the chapter is dedicated to the increasing numbers of biotechnological processes based on lipid metabolism. Besides the prominent model organism *Saccharomyces cerevisiae*, other predominantly oleaginous yeasts are also included.

Keywords Lipids · Yeast · Fatty acids · Phospholipids · Sterols · Triacylglycerols · Steryl esters · Non-polar lipids · Sphingolipids · Lipid droplets · Peroxisomes · Mitochondria

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*, 169
DOI: 10.1007/978-3-642-55013-3_8, © Springer-Verlag Berlin Heidelberg 2014

Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
CDP	Cytidinediphosphate
CL	Cardiolipin
CoA	Coenzyme A
CTP	Cytidine triphosphate
DAG	Diacylglycerol
DGPP	Diacylglycerol diphosphate
DMAPP	Dimethylallyl diphosphate
ER	Endoplasmic reticulum
ERMES	ER-mitochondria encounter structure
FA	Fatty acids
FIT	Fat storage-inducing transmembrane proteins
FPP	Farnesyl diphosphate
GGPP	Geranylgeranyl diphosphate
GPI	Glycosylphosphatidylinositol
GPP	Geranyl diphosphate
IMM	Inner mitochondrial membrane
IPC	Inositol phosphorylceramide
IPP	Isopentenyl diphosphate
LD	Lipid droplets
MAM	Mitochondria-associated membrane fraction
$M(IP)_2C$	Mannosyl (inositol phosphoryl)$_2$ ceramide
MINOS	Mitochondrial inner membrane organizing system
MIPC	Mannosylinositol phosphorylceramide
Mt	Mitochondria
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Nu	Nucleus
OMM	Outer mitochondrial membrane
PA	Phosphatidic acid
PC	Phosphatidylcholine
PDR	Pleiotropic drug response
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PIP	Phosphatidylinositol phosphate
PL	Phospholipids
PS	Phosphatidylserine
PUFA	Polyunsaturated FA
Px	Peroxisomes
SE	Steryl esters
SPT	Serine palmitoyltransferase complex
TG	Triacylglycerols
TORC2	Target of rapamycin complex 2
UAS_{INO}	Inositol-responsive upstream activating sequence element

8.1 Introduction

The link between central carbon metabolism and lipid synthesis is easily found: acetyl-CoA is the common precursor for all lipid biosynthetic pathways. Membrane biogenesis is, along with amino acid synthesis, one of the major consumers of acetyl-CoA and NADPH—the biosynthesis of one molecule of palmitic acid requires 8 acetyl-CoA and 14 NADPH molecules (Natter and Kohlwein 2012). However, while the understanding of connections between different metabolic routes has already reached an advanced state at the level of metabolites, the identification of regulatory mechanisms is only in its infancy.

Lipids are essential constituents of every living cell. They were long seen as primarily structural components of cellular membranes. However, lipid research over the past decades has shown that they fulfil many more vital functions that are increasingly recognized. Prominent examples are their role as regulators of energy metabolism, cell integrity and membrane-based processes such as endocytosis and vesicular trafficking (Daum et al. 1998; Souza and Pichler 2007). The accepted general definition of lipids is that they are relatively small, hydrophobic or amphiphilic molecules that are classified into eight distinct groups based on their chemical and biochemical properties: fatty acids (FA), glycerolipids, glycerophospholipids, sphingolipids, sterols and sterol derivatives, prenol lipids, glycolipids and polyketides. Altogether, more than 10,000 different lipid structures have been identified (Fahy et al. 2009). In this chapter we focus on the first five most commonly found lipid classes in yeast.

The field of lipid research has attracted more and more interest over the past decades as many lipid-associated disorders such as obesity, type-II-diabetes, insulin resistance and cardiovascular diseases have become increasing health risks in the Western world and recently also in developing countries. As the principles of lipid metabolism are well conserved between all eukaryotes and because of the many advantages of working with yeast, *Saccharomyces cerevisiae* has become a powerful model organism for lipid research. One established approach to dissect the complex network of enzymes and molecular mechanisms responsible for lipid homeostasis is the use of readily available single and multiple deletions mutants. One of the major resources that have enabled systematic studies in this direction is the repertoire of yeast deletion mutants of all non-essential genes, which have helped, in combination with different cultivation conditions, to understand the basics of lipid synthesis, storage and degradation pathways (Winzeler et al. 1999).

Many different cellular compartments are involved in lipid metabolism (Natter et al. 2005). Lipid synthesis takes place mainly in the endoplasmic reticulum (ER) and the Golgi compartment, but also lipid droplets (LD), mitochondria and peroxisomes play influential roles and will therefore be highlighted in this introduction. LD and peroxisomes, especially with respect to their role in lipid metabolism, were recently reviewed (Kohlwein et al. 2012).

LD are generally seen as a storage compartment for the non-polar lipids, triacylglycerols (TG) and steryl esters (SE). They are small spherical organelles of

approximately 400 nm in diameter consisting of a highly hydrophobic core of mainly TG, surrounded by shells of SE which are covered by a phospholipid monolayer with only a few embedded proteins (Athenstaedt et al. 1999a; Czabany et al. 2008). Proteome analysis revealed that these proteins are predominantly enzymes involved in lipid metabolism, for example TG lipases and SE hydrolases (Grillitsch et al. 2011). LD will be further described in the section on non-polar lipids.

Mitochondria are of special interest for lipid research. They provide an independent fatty acid synthesizing system (Tehlivets et al. 2007) and synthesize some phospholipids (Kuchler et al. 1986; Henry et al. 2012), but the majority of lipids are imported. Examples of autonomously formed mitochondrial lipids are phosphatidic acid (PA), cardiolipin (CL) or phosphatidylethanolamine (PE), whereas phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) as well as sterols and sphingolipids have to be imported. Various mechanisms have been proposed for the import of lipids, such as direct membrane contact between the ER and mitochondria via the mitochondria-associated membrane (MAM) fraction, vesicular transport and the involvement of specific lipid binding and transfer proteins (Daum and Vance 1997). Mitochondria are also special regarding their lipid distribution, having an increased amount of CL and PI. The phospholipid CL comprises up to 15 %, which is very high compared to other organelles. Therefore, CL is often referred to as the typical mitochondrial phospholipid (Zinser and Daum 1995). Mitochondria are compartmentalized into four different subcompartments: the outer mitochondrial membrane, the intermembrane space and the inner mitochondrial membrane with its cristae and the matrix. A mitochondrial complex connecting the inner boundary membrane to the cristae membrane was recently identified and termed MINOS (mitochondrial inner membrane organizing system) or MitOS (mitochondrial organizing structure), and shown to be responsible for maintaining mitochondrial morphology (Hoppins et al. 2011; van der Laan et al. 2012; Zerbes et al. 2012). Most interestingly, even among the four subcompartments, lipids are not distributed randomly (Daum 1985). Therefore, intramitochondrial lipid transfer as well as the interorganelle transport of lipids is of outstanding interest. Some recent findings will be reported in the section on novel key players.

Peroxisomes deserve special attention in the description of lipid metabolism. They are spherical organelles with a diameter of about 0.1 μm, consisting of a fine granular matrix with a crystalline core, all surrounded by a single membrane. The protein content of these membranes is typically relatively low, whereas the matrix contains the highest protein concentration in eukaryotic cells with hydrogen peroxide-producing oxidase and catalase as prominent representatives (Kohlwein et al. 2012). Peroxisomes are ubiquitous and are involved in various metabolic pathways, especially detoxification processes and degradation of FA. The latter process, termed β-oxidation, makes FA available as an energy source. In contrast to mammalian cells, where β-oxidation occurs in mitochondria and peroxisomes, yeast β-oxidation takes place exclusively in peroxisomes (Poirier et al. 2006). Prior to degradation, FA have to be activated by one of six specific activators

Faa1p, Faa2p, Faa3p, Faa4p, Fat1p or Fat2p. The uptake of FA into peroxisomes can proceed by different mechanisms. Short and medium-chain length FA are thought to be taken up by diffusion, whereas long chain and very long chain FA require ABC (ATP-binding cassette) transport proteins. In yeast peroxisomes, the two ABC transporters, Pxa1p and Pxa2p, are thought to be responsible for the uptake of FA. These transporters hydrolyze FA-CoA esters prior to their entry into peroxisomes, releasing CoA into the cytoplasm, whereas FA are then re-esterified by a peroxisomal synthetase (van Roermund et al. 2012). Lipid composition of peroxisomes comprises nearly 50 % PC, 23 % PE, 16 % PI together with a remarkably high content of CL (7 %) (Zinser et al. 1991).

Recently, techniques for lipid content analysis have advanced substantially. The prerequisite for lipid analysis is usually lipid extraction into organic solvents, followed by chromatographic separation of lipid species that can then be detected by advanced spectrometric technologies. Currently, lipid research is shifting from basic molecular characterization to a global understanding of dynamic lipid regulation in the cell context. Lipids have been proposed to act as a molecular collective rather than as single molecules, best demonstrated by Guan et al. (2009) who showed that sphingolipids and sterols can interact functionally. In particular, lipidomic approaches and mathematical modelling are promising methods for interpreting lipid metabolism on a global scale (Alvarez-Vasquez et al. 2011; Santos and Riezman 2012). Lipidomics, which involves mapping all lipids of an organism or a cell, is facilitated by sophisticated mass spectrometry techniques combined with state-of-the-art data analysis software (Dennis 2009; Ejsing et al. 2009). The absolute quantification of lipids depends on internal standards which are not always available. Quantitative analysis would be particularly important in finding out how cells adapt their lipid profile to changes in the environment. Specifically, points of regulation could be identified by mathematical modelling, although this approach is still in its infancy. From the experimental point of view, however, mass spectrometry-based shotgun lipidomics has been applied to quantitatively and comprehensively asses the yeast lipidome (Ejsing et al. 2009). This approach was recently used to determine changes in the yeast lipidome under different growth conditions including growth on different carbon sources. Interestingly, different flexibilities (defined as dispersion of a given lipidomic feature across the dataset) were determined for different classes of lipids (Klose et al. 2012). The authors observed marked differences in the lipidome between growth on glucose- and non-glucose-based media.

Another hot topic of lipid research is the investigation of membrane organization by visualizing specific lipids in the cell. Visualization techniques confirmed the view that lipids and proteins are not moving freely within a membrane but that their diffusion is restricted in certain domains called rafts, which are enriched in sphingolipids and sterols (Lingwood and Simons 2010; Eggeling et al. 2009). However, the techniques are challenging, comprising high temporal and spatial super resolution microscopy and are limited by the availability of appropriate probes.

More recently, other yeast genera besides *Saccharomyces* have been attracting interest in lipid research. In particular, oleaginous yeasts, such as *Candida curvata* and *Yarrowia lipolytica*, have been shown to be industrially relevant for the sustainable production of lipids with compositions similar to those of vegetable oils and fats (Beopoulos et al. 2011). *Pichia pastoris*, an industrially highly relevant yeast especially for the expression of heterologous proteins, is another important model organism for lipid-related research, especially in organelle biology studies. Unless indicated otherwise, we will refer to *S. cerevisiae* in this chapter, but particularly in the section on biotechnological aspects other yeasts will also be mentioned.

The aim of this chapter is to provide a fundamental overview of yeast lipid metabolism, but also to point out novel findings and applications of the highly dynamic field of yeast lipid research. For detailed information beyond the scope of this chapter readers will be referred to other recent reviews (Henry et al. 2012; Jacquier and Schneiter 2012; Kohlwein et al. 2012; Natter and Kohlwein 2012; Rajakumari et al. 2008; Santos and Riezman 2012).

8.2 Lipid Classes

Lipids are divided into classes based on their structure and function. The major classes discussed in this chapter are FA, glycerophospholipids, sphingolipids, sterols and the non-polar storage lipids TG and SE. These five classes will be described with special emphasis on a basic understanding of their metabolism and function of their members. Regulatory mechanisms, especially newly identified ones, will be discussed in the section on novel key players.

8.2.1 Fatty Acids

FA are carboxylic acids with long hydrocarbon tails and differ from each other in chain length and degree of saturation. In *S. cerevisiae*, the overall composition of FA is rather simple, the members being mainly of C18:1 (oleate), C16:1 (palmitoleate) and C16:0 (palmitate) followed by C18:0 (stearate) and minor amounts of C14:0 (myristate) and C26:0 (cerotate) (Daum et al. 1998). The composition differs in the different yeast genera. In particular, in the oleaginous yeasts such as *Y. lipolytica,* the FA composition is highly diverse, comprising longer chain lengths and, especially, more double bonds, which makes such organisms useful for the production of nutritionally valuable polyunsaturated fatty acids (PUFA) (Beopoulos et al. 2011) as will be described below.

FA fulfil many different roles in cells. Most importantly, they serve as basic molecules for the biosynthesis of complex membrane and storage lipids (Tehlivets et al. 2007). Other functions include their role as signalling molecules,

transcriptional regulators and post-translational modifiers of proteins (Nadolski and Linder 2007). One prominent example of the latter is the palmitoylation of Ras proteins, but myristate is also often added as lipid moiety (Linder and Deschenes 2004).

FA metabolism in yeast is illustrated in Fig. 8.1. There are three main sources for FA: (i) de novo synthesis (ii) uptake by specific transporters and (iii) catabolism of complex lipids (Tehlivets et al. 2007). A small proportion of FA derives from the catabolism of proteins (Tehlivets et al. 2007). Two independent pathways exist for the biosynthesis of FA, the major cytosolic pathway and the mitochondrial pathway. The former pathway involves mainly three key enzymes, encoded by *ACC1*, *FAS1* and *FAS2* (for review see Henry et al. 2012). Biosynthesis of FA starts with the carboxylation by Acc1p of acetyl-CoA to give malonyl-CoA. Acc1p possesses three different activities: it can act as a biotin carboxylase, as a biotin carboxyl-carrier protein and as a transcarboxylase. It is located on the cytoplasmic surface of the ER, contains one covalently bound biotin molecule and is essential for growth. Malonyl-CoA is metabolized by a series of reactions catalyzed by FA synthases (*FAS* genes) and elongases. *FAS1* and *FAS2* encode two different subunits of the FA synthase complex. The active FAS complex consists of six α-units and six β-units (Chirala et al. 1987). *FAS1* encodes the β-subunit, which comprises four different activities: acetyltransferase, enoyl reductase, dehydratase and malonyl-palmitoyl transferase activities. *FAS2* encodes the α-subunit that displays acyl carrier protein, 3-ketoreductase, 3-ketosynthase and phosphopantetheinyl transferase activities (reviewed by Tehlivets et al. 2007). In yeast, double bonds are introduced by a single acyl-CoA $\Delta 9$ desaturase encoded by *OLE1* (Stukey et al. 1990). Elongation is carried out predominantly by Elo1p, although elongation of very long FA, especially for sphingolipid synthesis, is catalyzed mainly by Elo2p and Elo3p. De novo synthesis of FA takes place mainly in the cytosol, whereas elongation and desaturation reactions are carried out in the ER (Tehlivets et al. 2007).

Imported FA, which can be taken up by diffusion or by transporters, can fully compensate for endogenously synthesized FA. Prerequisite for the uptake of FA is the activation of free FA with coenzyme A, which is carried out by the acyl-CoA synthetases Faa1p, Faa2p, Faa3p, Faa4p and Fat1p. These enzymes are also believed to be involved in the uptake of FA into the cell (reviewed by Black and DiRusso 2007; Henry et al. 2012). While in *S. cerevisiae* machinery for utilization of extracellular complex lipids as energy or carbon source has not been identified, oleaginous yeast species produce extracellular lipases for this purpose. The best studied model for the utilization of hydrophobic substrates such as alkanes, TG and FA is *Y. lipolytica* (reviewed by Fickers et al. 2005). *Y. lipolytica* produces surfactants when grown on lipids as the only carbon source and changes the biophysical and morphological properties of the cell surface to enable adhesion of water insoluble growth substrates. The cells produce both membrane-bound and extracellular lipases, the major one being Lip2p which catalyzes hydrolysis of TG to free FA and glycerol. Free FA are then taken up by a mechanism that is not

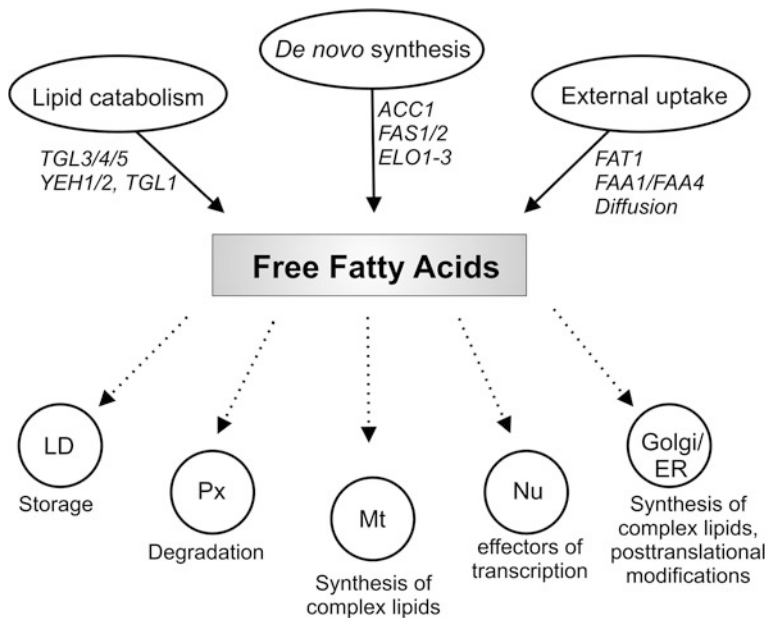

Fig. 8.1 Overview of fatty acid metabolism in yeast. FA derive mainly from three routes: catabolism of storage lipids, de novo synthesis and external uptake. They can be incorporated into storage lipids, degraded by β-oxidation to provide energy or converted into complex lipids like phospholipids or sphingolipids. FA can also act as effectors of transcription. *LD* lipid droplets, *Px* peroxisomes, *Mt* mitochondria, *Nu* nucleus, *ER* endoplasmic reticulum. For details see text

completely understood, activated by specific acyl-CoA synthetases and further metabolized similarly as described below for *S. cerevisiae*.

In general, free FA are metabolized very quickly. Elevated levels of free FA are harmful to cells because they can perturb membrane properties due to changes in fluidity. Thus, FA are either incorporated into complex lipids, i.e. PL or the storage lipids TG and SE, or they are oxidized to provide energy. Regardless of the source of free FA, the prerequisite for further conversion is activation by thioesterification with coenzyme A, which requires the action of acyl-CoA synthetases (*Faa1-4*, *Fat1*) (Black and DiRusso 2007). In mitochondria, the biosynthesis of FA is carried out by a totally different set of enzymes: Hfa1p, the mitochondrial acetyl-CoA carboxylase, catalyzes the production of malonyl-CoA which is then further processed by a different Fas complex (Hiltunen et al. 2010).

As mentioned in the introduction, catabolism of FA in yeast takes place exclusively in peroxisomes. Under standard growth conditions, the abundance of peroxisomes is quite low, but can be increased by FA supplementation to the medium (van Roermund et al. 1995). The classical β-oxidation starts with the oxidation of acyl-CoA to *trans*-2-enoyl-CoA by Fox1p (frequently called Pox1p). This reaction releases hydrogen peroxide, which is detoxified by catalase. The second step is the conversion, by Fox2p, of *trans*-2-enoyl-CoA to 3-ketoacyl-CoA.

This compound is the substrate of Fox3p, a 3-ketoacyl-CoA thiolase, which yields acetyl-CoA and a C2-shortened FA (Einerhand et al. 1991; Hiltunen et al. 1992). The route of FA directed to the site of peroxisomal β-oxidation, either via plasma membrane transport from an exogenous source, or from LD as an endogenous storage compartment, is still not completely understood.

8.2.2 Phospholipids

Phospholipids (PL) are regarded as bulk membrane constituents, since they can form lipid bilayers. They consist of a diacylglycerol backbone and a phosphate group at the *sn*-3 position that is linked to a polar head group. PL can be classified based on their different head groups. The major PL in yeast are PC, which comprises about 45 % of the total phospholipid content, PE, which makes up to 20 %, PI with 15 %, PS accounting for 5 % and CL being present at 2 % (Janssen et al. 2000; Zinser et al. 1991; Schneiter et al. 1999). However, the subcellular distribution of different phospholipids varies quantitatively and by origin. Especially, PS and CL are present just at low amounts in most organelle membranes but are major components of the plasma membrane and the inner mitochondrial membrane, respectively (Zinser and Daum 1995). In general, the lipid composition of membranes is not stochastic but characteristic of each organelle.

In addition to the role of PL as major structural components of cellular membranes, they are involved in a variety of other processes. They provide precursors for the synthesis of membranes, act as reservoirs of second messengers, conduct the lipidation of proteins for membrane association and function as molecular chaperones (reviewed by Carman and Han 2011; Dowhan and Bogdanov 2009; van Meer et al. 2008). PL can also be differentiated according to their shape, which is dictated by their head-to-tail area ratio. PC, PS and phosphatidylglycerol are cylindrically shaped since they display a head group similar to fatty acid chain area. Cylindrical PL are known to favour bilayer structures, while PE and CL, which belong to the group of non-bilayer forming PL, are conically shaped, the result of a smaller head-to-tail area (Cullis et al. 1986).

A key molecule in PL synthesis is PA (Fig. 8.2), which is also an important signalling molecule and regulator of lipid metabolism. PA derives from either glycerol-3-phosphate or dihydroxyacetone phosphate following fatty acyl-CoA dependent acyl transfer. These reactions are catalyzed by the *SCT1*- (*GAT2*) and *GPT2* (*GAT1*)-encoded glycerol-3-phosphate acyltransferases and the *SLC1*- and *ALE1*-encoded lysophospholipid acyltransferases (Athenstaedt and Daum 1997; Athenstaedt et al. 1999b; Chen et al. 2007b; Jain et al. 2007; Riekhof et al. 2007). Dihydroxyacetone phosphate is reduced by Ayr1p, which is present in LD, the ER and the mitochondrial outer membrane. PA is a branch point between the CDP-DAG (cytidinediphosphate- diacylglycerol) pathway and the formation of DAG (Athenstaedt and Daum 1999). In the first case, PA is metabolized to CDP-DAG under the catalytic action of the *CDS1*-encoded CDP-DAG synthase

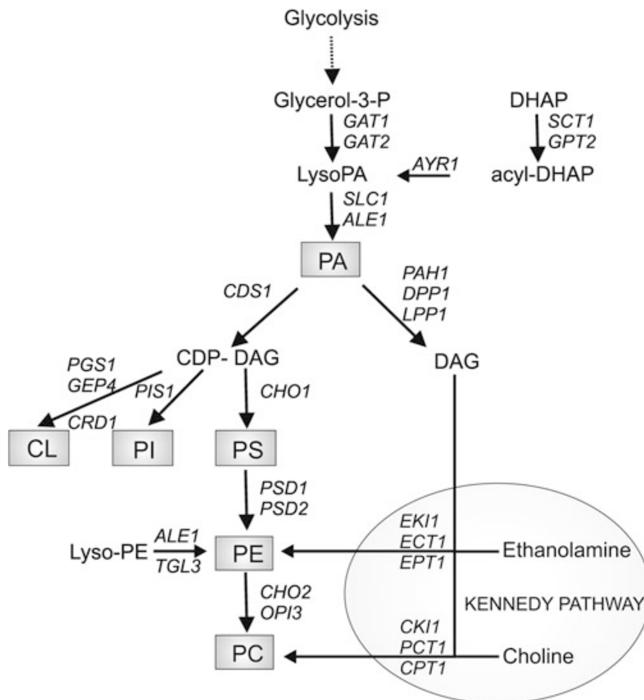

Fig. 8.2 Simplified pathway of phospholipid synthesis in the yeast *S. cerevisiae*. For details see text. *DHAP* dihydroxyacetone phosphate, *PA* phosphatidic acid, *CDP-DAG* cytidinediphosphate diacylglycerol, *DAG* diacylglycerol, *CL* cardiolipin, *PI* phosphatidylinositol, *PS* phosphatidylserine, *PE* phosphatidylethanolamine, *PC* phosphatidylcholine

(Shen et al. 1996). In the second case, the *PAH1*-encoded PA phosphatase forms DAG (Han et al. 2006). CDP-DAG and DAG are both used in the synthesis of PE and PC, but by different pathways. The first biosynthetic route is the CDP-DAG pathway, whereas in the Kennedy pathway DAG is used as a substrate for the conversion (for reviews see Carman and Han 2011; Henry et al. 2012). Both pathways are used in wild-type cells, but the CDP-DAG pathway is the major route for the synthesis of PE and PC when cells are grown in the absence of ethanolamine and choline. It starts with the conversion of CDP-DAG into PS by the ER-localized, *CHO1*-encoded PS synthase. PS is further decarboxylated to PE by two PS decarboxylases, Psd1p and Psd2p. Psd1p is localized to the inner mitochondrial membrane and accounts for the major enzymatic activity, whereas Psd2p is associated with Golgi and vacuolar membranes (Trotter and Voelker 1995; Clancey et al. 1993; Voelker 2003). PE is methylated by Cho2p and Opi3p yielding PC. PE and PC can also be obtained from exogenously supplied lysoPE and lysoPC, which can be acylated by the *ALE1*-encoded lysophospholipid acyltransferase (Riekhof and Voelker 2006; Riekhof et al. 2007). CDP-DAG can also be converted into PI by reaction with inositol catalyzed by Pis1p (Fischl and

Carman 1983). The biosynthesis of CL takes place only in mitochondria, initiated by the transfer of the phosphatidyl moiety of CDP-DAG to glycerol-3-phosphate by Pgs1p and continued by dephosphorylation of phosphatidylglycerophosphate by Gep4p (Chang et al. 1998a; Osman et al. 2010). The *CRD1*-encoded CL synthase finally produces CL (Chang et al. 1998b; Tuller et al. 1998; Jiang et al. 1997).

In the Kennedy pathway, exogenous ethanolamine and choline are transported into the cell by the choline/ethanolamine transporter Hnm1p. They are phosphorylated with ATP by the kinases Eki1p and Cki1p. They are then activated with CTP to form CDP-ethanolamine and CDP-choline, under the action of ethanolaminephosphate cytidylyltransferase Ect1p and cholinephosphate cytidylyltransferase Pct1p (Kennedy and Weiss 1956; Kim et al. 1999; Henry et al. 2012). PE and PC are finally formed by the *sn*-1,2-diacylglycerol ethanolaminephosphotransferase Ept1p and the cholinephosphotransferase Cpt1p catalyzing the reactions of CDP-ethanolamine and CDP-choline with DAG (Hjelmstad and Bell 1992). DAG is provided by dephosphorylation of PA by Pah1p (Fig. 8.2).

The organization of phospholipids within membranes is believed to occur via two principal mechanisms: lateral diffusion within the plane of a membrane and bidirectional, ATP-dependent movement facilitated by flippases. Intercompartmental phospholipid transport mainly occurs via vesicles and monomeric exchange (reviewed by Vehring and Pomorski 2005). These transport mechanisms do not lead to a homogeneous distribution of phospholipids. Moreover, there is increasing evidence that distinct lipid domains exist within certain cellular membranes which are called rafts (London and Brown 2000; Simons and Sampaio 2011).

The majority of PL undergoes rapid turnover and acyl-chain remodelling, which is catalyzed by specific acyltransferases, phospholipases and lipid phosphatases (reviewed in Henry et al. 2012).

8.2.3 Sterols

Sterols are important compounds in eukaryotic cells, serving as both structural and signalling molecules. Due to their rigid structure, they strongly affect membrane fluidity and permeability (Nes et al. 1993). It has been shown that yeast cells are not viable without sterols (Daum et al. 1998). They are often referred to as steroid alcohols that contain cyclopentanoperhydrophenanthrene as parent structure. The main sterol in yeast, and also the final product of sterol biosynthesis in other fungi, is ergosterol. Structural differences from the mammalian counterpart cholesterol are the double bonds between C-7,8 in the ring and C-22 in the side chain and the presence of a methyl group at C-24. The hydroxyl group at the C-3 position is the only hydrophilic component of the molecule which facilitates integration into membranes. The ergosterol biosynthetic pathway is one of the most complex biochemical pathways, comprising nearly 30 different biochemical reactions

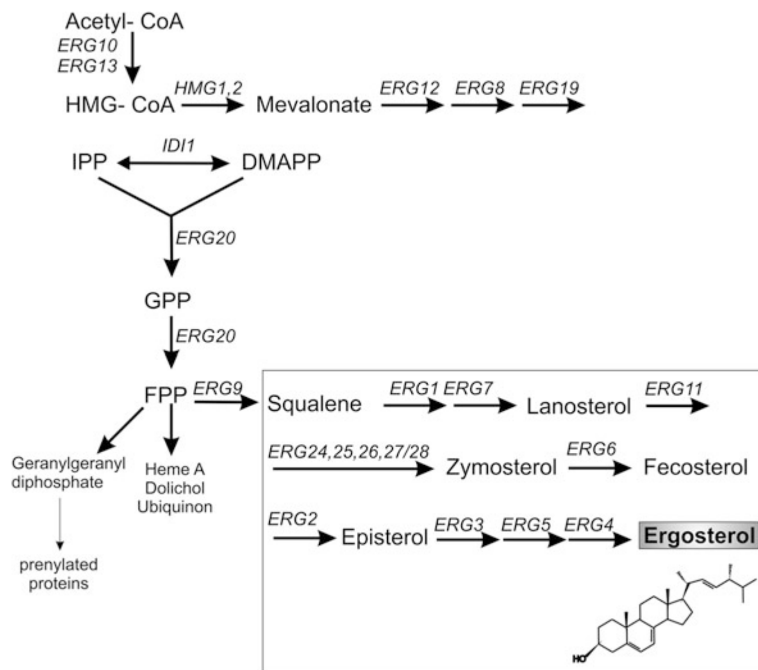

Fig. 8.3 Simplified ergosterol biosynthesis divided into the pre-squalene and post-squalene pathways, the latter being highlighted in the box. Important metabolic intermediates as well as the chemical structure of ergosterol are shown. For details see text. *CoA* Coenzyme A, *HMG* (3S)-3-hydroxy-3-methylglutaryl-CoA, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate

catalyzed by the so-called Erg proteins (for recent reviews see Kristan and Rižner 2012; Kuranda et al. 2010; Pichler 2005). The most important steps are summarized in the following paragraph.

The ergosterol biosynthetic pathway is divided into the pre-squalene and post-squalene pathways, displayed in a much simplified scheme in Fig. 8.3. Most Erg proteins are located to the ER membrane, with the exception of Erg1p, Erg6p and Erg7p, which are localized mainly to LD (Athenstaedt et al. 1999a; Leber et al. 1994, 1998). The first steps of sterol synthesis are similar in fungi, plants and animals starting with the condensation of two acetyl-CoA molecules, catalyzed by Erg10p. This reaction yields acetoacetyl-CoA which reacts with another acetyl-CoA molecule to form (3S)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). In the yeast, this important intermediate is subsequently reduced to mevalonate by HMG-CoA reductases 1 and 2 (*HMG1/2*). This reaction is not only the rate-limiting step of sterol biosynthesis but also one of the major control points, since HMG-CoA reductase shows feedback inhibition by ergosterol (Bard and Downing 1981). Polakowski et al. (1998) showed that overexpression of a truncated version of Hmg1p leads to an increase in early sterol precursors. A cascade of phosphorylations and decarboxylations, also known as the mevalonate pathway, leads to

isopentenyl pyrophosphate (IPP), which is the precursor not only for squalene but also for other isoprenoids (Toth and Huwyler 1996). Isomerization of IPP to dimethylallyl pyrophosphate (DMAPP) and a subsequent head-to-tail condensation reaction of IPP and DMAPP yield geranyl pyrophosphate (GPP). These reactions are catalyzed by Idi1p and Erg20p (Anderson et al. 1989a, b; Chambon et al. 1991). Erg20p also facilitates the formation of farnesyl pyrophosphate (FPP) by adding two IPP units to DMAPP. Finally, coupling of two FPP molecules by Erg9p leads to squalene.

The first step of the post-squalene pathway is epoxidation of squalene by Erg1p. This reaction is followed by a number of complex cyclization events, catalyzed by Erg7p, that form lanosterol, which is the first intermediate with the typical sterol structure. A cascade of demethylations, desaturations and subsequent reduction events (*ERG24–ERG28*) leads to zymosterol. The reaction steps yielding zymosterol are conserved in all eukaryotic cells. It was shown that deletion of genes downstream this biosynthetic sequence leads to sterol auxotrophy, whereas cells depleted of *ERG* genes acting later in the pathway are still viable. The further methylation of zymosterol at the C-24 position by Erg6p yields fecosterol, an intermediate which is unique to yeast and other fungi. Then, Erg2p catalyzes the shift of a double bond to the C-7 position, followed by the introduction of a further double bond at the C-5 position by Erg3p. The last steps of the pathway introducing and removing double bonds (*ERG5*, *ERG4*) yield the end product, ergosterol.

Yeast cells usually synthesize sterols in excess. Since yeast is unable to degrade sterols, mechanisms of detoxification are required to avoid harmful influence on membranes. There are three main mechanisms to maintain sterol homeostasis: (i) esterification of free sterols with FA by Are1p and Are2p and storage in LD (Yang et al. 1996; Yu et al. 1996; Zweytick et al. 2000); (ii) downregulation of sterol biosynthesis; and (iii) sterol acetylation by Aft2p, which enables yeast cells to efficiently secrete excess sterols in the form of sterol acetates into the medium. The latter process is reversible and catalyzed by Say1p (Tiwari et al. 2007; Choudhary and Schneiter 2009). Acetylation of sterols has also been discussed as a possible quality control mechanism. It was suggested that sterols which do not pass a quality control cycle are acetylated and secreted. Recently, the PRY proteins (pathogen-related yeast proteins) that are involved in the secretion of acetylated sterols have been identified (Choudhary and Schneiter 2012).

Since some steps of the sterol biosynthetic pathway require oxygen, yeast becomes strictly sterol auxotroph under anaerobic conditions (Lees et al. 1995). Uptake of external sterols from the exterior is mediated by two ATP-binding cassette transporters, encoded by *AUS1* and *PDR11*, both targets of the transcriptional activator Upc2p (Crowley et al. 1998; Wilcox et al. 2002).

The intracellular sterol concentration is lowest at its place of biosynthesis, the ER and increases along the protein secretory pathway until it reaches its maximum at the plasma membrane (Zinser et al. 1993). The distribution of sterols between different cellular membranes has to be tightly regulated to maintain distinct

membrane properties such as fluidity and thickness. Intracellular sterol transport involves both vesicular and non-vesicular routes but is mainly ATP-dependent. Non-vesicular sterol transport, in addition, requires the action of carrier proteins (for review see Jacquier and Schneiter 2012). Over the last couple of years, specific yeast sterol carrier proteins have been identified, the so-called oxysterol-binding proteins homologues Osh1–7 (reviewed by Schulz and Prinz 2007). Deletion of all seven Osh proteins was found to be lethal and accompanied by a 3.5-fold increase in the cellular level of ergosterol (Schulz and Prinz 2007). Sterols can be either transported to the cell surface or sent to the *trans*-Golgi network where they associate with sphingolipids to form lipid rafts (Mesmin and Maxfield 2009). The exact role of Osh proteins in sterol transport still has to be elucidated. Georgiev et al. (2011) reported that Osh proteins act as sterol sensors and regulate the organization of sterols at the plasma membrane rather than being involved in the transport of sterols between the ER and the plasma membrane. Intracellular sterol trafficking between membranes might also be governed by Arv1p as reported by Tinkelenberg et al. (2000). Mutations of *ARV1* have been shown to render cells which are anaerobically non-viable, depend on sterol esterification and show altered intracellular sterol distribution. The balance of sterol synthesis, uptake, storage and mobilization as well as internal transport is very complex and a hot topic of lipid research.

8.2.4 Non-polar Lipids: TG and SE

TG and SE are storage lipids preserving free FA and sterols in a biologically inert form. All eukaryotic cells store excess FA in specific organelle-like compartments, often referred to as LD, lipid particles or oil bodies, used as energy depots. Yeast cells accumulate only little TG as long as they proliferate but can reach high TG levels in the stationary phase. When required, e.g. during growth or starvation, TG and SE can be mobilized to provide building blocks for membrane biosynthesis. Under these conditions the released FA are channelled into phospholipid biosynthesis (Zanghellini et al. 2008). In yeast, LD are about 400 nm in diameter and consist of a highly hydrophobic core of TG, surrounded by shells of SE and a phospholipid monolayer containing a distinct set of proteins (Czabany et al. 2008; Athenstaedt et al. 2006; Grillitsch et al. 2011; Kohlwein et al. 2012). TG are synthesized by the acyltransferases Dga1p and Lro1p, and SE by the steryl ester synthases Are1p and Are2p. All TG- and SE-synthesizing enzymes are located at the ER. Additionally, Dga1p is also found in LD. The direct precursor for TG is diacylglycerol (DAG), that can derive from different routes: (i) dephosphorylation of de novo synthesized PA, (ii) degradation of PL by phospholipases and (iii) deacylation of TG (see Fig. 8.4) (Henry et al. 2012). For synthesis of TG, DAG is acylated in the *sn*-3 position by Dga1p, Lro1p and with low efficiency by Are1p and Are2p. In *S. cerevisiae*, the acyl-CoA:diacylglycerol acyltransferase Dga1p is the most efficient TG-synthesizing enzyme. Lro1p is an acyl-CoA independent

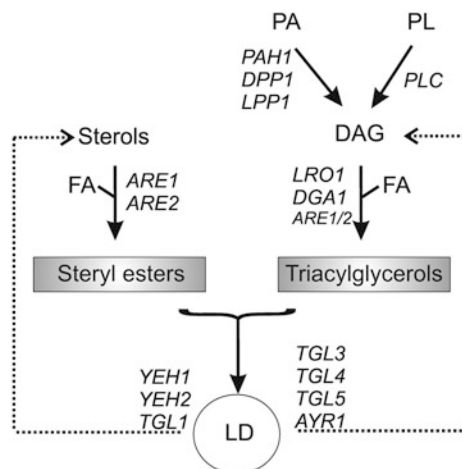

Fig. 8.4 Overview of non-polar lipid metabolism in *S. cerevisiae*. For details see text. *PA* phosphatidic acid, *PL* phospholipids, *DAG* diacylglycerol, *FA* fatty acids, *LD* lipid droplets

enzyme which uses the *sn*-2 acyl group from glycerophospholipids as cosubstrate for the acylation of DAG (Czabany et al. 2007; Rajakumari et al. 2008; Horvath et al. 2011). SE of *S. cerevisiae* are synthesized by the two acyl-CoA:cholesterol acyltransferase (ACAT) related enzymes, Are1p and Are2p (Yang et al. 1996; Yu et al. 1996). Both proteins are located in the ER and harbour multiple transmembrane domains. Are1p and Are2p are 49 % identical in sequence, but have different substrate specificities. Under standard cultivation conditions, Are2p accounts for approximately 70 % of the total SE synthase activity and esterifies preferentially ergosterol. Are1p esterifies mainly sterol intermediates with a slight preference for lanosterol and becomes particularly important under hypoxic conditions (Zweytick et al. 2000). The esterification takes place at the hydroxyl group at the C3-atom with C16:1 as the preferred fatty acid substrate followed by C18:1. Both TG and SE accumulate mainly during the stationary growth phase.

Storage of non-polar lipids would be useless without the possibility to mobilize them as required in order to provide sterols, DAG and FA for membrane synthesis and energy production. TG are mobilized by TG lipases. Currently, four LD-resident TG lipases are known, namely Tgl3p, Tgl4p, Tgl5p and Ayr1p (Athenstaed and Daum 2003, 2005; Ploier et al. 2013). SE are hydrolyzed by the three SE hydrolases Yeh1p, Yeh2p and Tgl1p (Köffel et al. 2005; Müllner et al. 2005), the highest activity being attributed to Yeh2p. The cycle of esterification of free sterols and the hydrolysis of SE are of utmost importance for a balanced level of free ergosterol (Wagner et al. 2009). Yeh1p and Tgl1p are localized to LD, whereas Yeh2p was surprisingly detected in the plasma membrane. The existence of further hydrolytic enzymes is currently under investigation (our own unpublished results). Especially, peroxisomal enzymes might be involved in the mobilization of non-polar lipids (Thoms et al. 2011; Debelyy et al. 2011). TG and SE

have long been viewed as just storage molecules, but this view has changed in recent years. TG in particular appear to be important for various cellular processes and their levels have been found to influence lipotoxicity, iron and phospholipid metabolism and cell cycle progression (Kohlwein 2010).

Non-polar lipid metabolism is inevitably connected to LD biology. Their biogenesis is still a matter of debate and different possible models have been published (Farese and Walther 2009). The most widely accepted model describes its formation at special membrane microdomains in the ER, where non-polar lipids accumulate between the two leaflets of the phospholipid bilayer until the size of the LD reaches a critical dimension (Murphy and Vance 1999; Ploegh 2007). At this stage, LD may bud off forming an independent organelle-like structure. Apart from their classical role as a storage compartment, it has to be noted that LD also participate in many other cellular processes (reviewed by Kohlwein et al. 2012). Connerth et al. (2010) described an indirect role of LD in the maintenance of membrane fluidity under environmental pressure of exogenous FA. Functions of LD unrelated to lipid turnover have also been investigated. As an example, Fei et al. (2009) reported that LD accumulated in yeast mutants with compromised protein glycosylation. The authors discussed a possible role of LD as a temporary safe depot for protein aggregates or incorrectly folded proteins. In recent studies, LD emerged as dynamic organelles through their interaction with the ER (Fei et al. 2009; Jacquier et al. 2011; Wolinski et al. 2011), peroxisomes (Binns et al. 2006), or mitochondria (Pu et al. 2011), and novel factors influencing the biogenesis and dynamics of LD were identified (Adeyo et al. 2011).

8.2.5 Sphingolipids

Sphingolipids are composed of a sphingoid base, a fatty acid and a polar head group. In yeast, the sphingoid base can be dihydrosphingosine or phytosphingosine, linked through an amide bond to a very long chain fatty acid, mostly C26:0, and O-linked to the charged head group inositol. The de novo synthesis of sphingolipids is carried out in the ER starting with the condensation of serine and palmitoyl-CoA (Fig. 8.5). This reaction is catalyzed by the serine palmitoyltransferase complex (*SPT*), which is a heterodimeric complex consisting of two major subunits, Lcb1p and Lcb2p (Nagiec et al. 1994), and one minor subunit, Tsc3p, which is necessary for full enzymatic activity (Gable et al. 2000). The product of this reaction, 3-ketodihydrosphingosine, is rapidly converted to dihydrosphingosine (also named sphinganine) by Tsc10p (Beeler et al. 1998). This product is the first sphingoid base that can be further hydroxylated by Sur2p, yielding a second sphingoid base, phytosphingosine (Grilley et al. 1998). These sphingoid bases can be either acylated to ceramides by gene products of *LIP1*, *LAG1* and *LAC1*, or phosphorylated by the sphingoid kinases encoded by *LCB4* and *LCB5* (Nagiec et al. 1998). Sphingoid base phosphates are further converted by Dpl1p to form fatty aldehydes and ethanolamine phosphates. This is the only

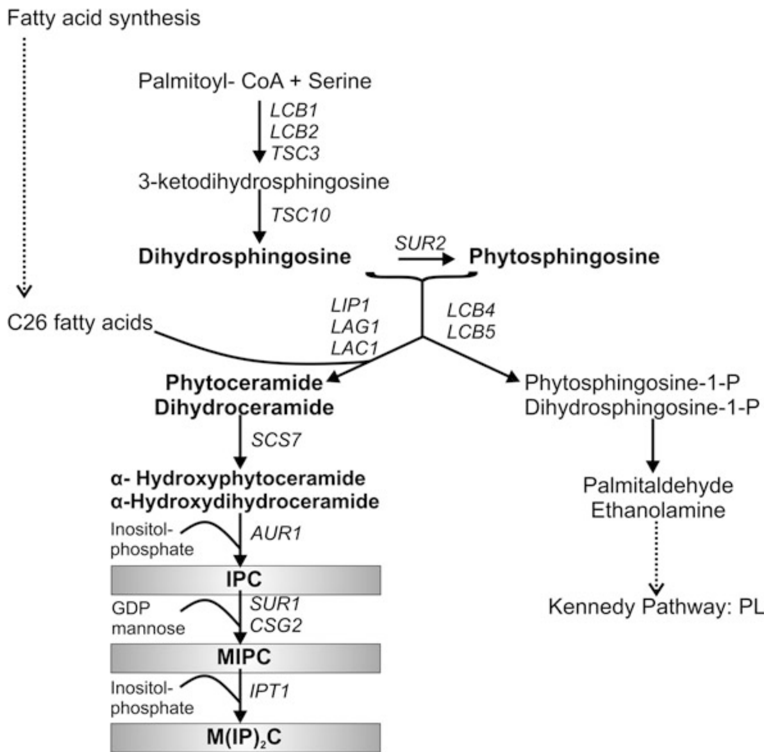

Fig. 8.5 Sphingolipid synthesis in *S. cerevisiae*. For details see text. *IPC* inositol-P-ceramide, *MIPC* mannose-IPC, *M(IP)$_2$C* mannose-(inositol-P)$_2$-ceramide

route by which sphingolipids can exit the pathway and the link of sphingolipid metabolism to the CDP-ethanolamine branch of the Kennedy pathway (Saba et al. 1997; Panwar and Moye-Rowley 2006).

If sphingolipids are not phosphorylated, both sphingoid bases can be N-acylated with C26-CoA by a ceramide synthase. Ceramide synthase comprises an ER membrane protein complex consisting of Lip1p, Lag1p and Lac1p (Schorling et al. 2001; Vallée and Riezman 2005). The two sphingoid bases and ceramides are the first products in the sphingolipid synthetic pathway. Ceramides are N-acylated sphingoid bases lacking additional head groups. They serve as substrates for the formation of complex lipids that may comprise up to 10 % of total membrane lipids. Prior to the formation of the complex sphingolipids, inositol-P-ceramide (IPC), mannose-inositol-P-ceramide (MIPC) and mannose-(inositol-P)$_2$-ceramide [M(IP)$_2$C], ceramides are α-hydroxylated by Scs7p (Haak et al. 1997; Dunn et al. 1998). Aur1p, the inositolphosphorylceramide synthase, attaches a phosphoinositol headgroup to the ceramide-yielding IPC (Nagiec et al. 1997), which is then mannosylated by Csg1p, Csg2p and Csh1p to MIPC. After mannosylation, another

inositol phosphate group is added by Ipt1p forming M(IP)$_2$C (Beeler et al. 1997; Uemura et al. 2003; Dickson et al. 1997).

The key players of sphingolipid catabolism are encoded by *ISC1*, *YPC1* and *YDC1* (Sawai et al. 2000; Mao et al. 2000a, b). Isc1p hydrolyzes the head groups of complex sphingolipids yielding both phyto- and dihydroceramides that can be cleaved reversibly to sphingoid bases. This reaction is catalyzed by the two ceramidases, Ypc1p and Ydc1p.

Although sphingolipids fulfil many important physiological roles (Dickson et al. 2006), little is known about the regulation of cellular sphingolipid levels. Cowart and Obeid (2007) showed that there is no stringent transcriptional regulation of the key enzymes of sphingolipid metabolism. One control mechanism could be phosphorylation of the sphingoid base kinase Lcb4p by interaction of Pho85p with two of its cyclin partners, Pcl1p and Pcl2p, which leads to down-regulation of Lcb4p. This effect is accompanied by a decrease in sphingoid base phosphate levels and a decrease of the cell cycle. Another study showed that ceramide synthase is regulated by casein kinase Cka2p, whose deletion resulted in a 70–75 % reduction of ceramide synthase activity (Kobayashi and Nagiec 2003). Kolaczkowski et al. (2004) found that the promoters of some sphingolipid metabolic enzymes contain a PDR (pleiotropic drug response) element for binding of the transcriptional activators Pdr1p and Pdr3p. Active PDR elements have been found in *LAC1*, *LCB2* and *SUR2*. A central element of sphingolipid regulation appears to be the interplay of Orm proteins (inhibitors of *SPT*) with Ypk1p (a kinase that inactivates Orm1p and Orm2p). These links were discovered recently (Breslow et al. 2010; Roelants et al. 2011; Sun et al. 2000) and will be discussed in the section on novel key players.

Sphingolipids are mainly found in the yeast plasma membrane where they are thought to interact with sterols to form so-called lipid rafts, also described as detergent-resistant membrane domains (Bagnat et al. 2000; Guan et al. 2009; Simons and Sampaio 2011). These domains have been proposed to constitute an important platform for certain membrane proteins, such as Pma1p, Gas1p and Gap1p (Dickson et al. 2006). The physiological role of lipid rafts has been exemplified by mis-localization of Pma1p, plasma membrane proton pump, and Gap1p, a general amino acid permease, in strains with impaired sphingolipid metabolism (Gaigg et al. 2006; Lauwers et al. 2007). The example of mis-localization of Gap1p also illustrates a functional link between sphingolipid and amino acid metabolism. In addition to their structural role, sphingolipids and their metabolites have emerged as important signalling molecules involved in endocytosis, heat stress response and cell cycle regulation (Cowart and Obeid 2007). Additionally, sphingolipids are necessary for the transport of GPI-anchored proteins from the ER to the Golgi (Skrzypek et al. 1997; Horvath et al. 1994). As mentioned above, they also influence the topology, localization, cell surface delivery and stability of important proteins, including the uracil permease Fur4p (Hearn et al. 2003), the plasma membrane ATPase Pma1p (Gaigg et al. 2005) and the vacuolar ATPase (Chung et al. 2003).

8.3 A Selection of Novel Key Players in Yeast Lipid Metabolism

A list of the major yeast lipid synthesizing and degrading enzymes is currently available, but a detailed understanding of lipid homeostasis and regulation of lipid metabolism still awaits clarification. In recent years, several new enzymes involved in lipid metabolism and related mechanisms have been identified. This development shows that the field of lipid research has become broader as links to other cellular processes became evident. To give the reader an impression of the complexity of lipid metabolism and its regulation, a few selected examples of novel insights into yeast lipid homeostasis covering different lipid species will be discussed in the following section.

8.3.1 Regulation of Phospholipid Synthesis: Inositol, PA and Opi1p

Besides acetyl-CoA, inositol is a major link of carbon metabolism to lipid metabolism. It is a carbohydrate synthesized from glucose-6-P in two steps, and is not essential under standard cultivation conditions. Inositol forms the structural component of a number of secondary messenger molecules, the inositol phosphates. In addition to its signalling role, inositol is also an important component of PI and its phosphates (PIPs), and can be regarded as the master regulator of PL biosynthesis. Inositol used for PI synthesis is either synthesized de novo or imported into the cell from the growth medium by inositol transporters encoded by *ITR1* and *ITR2* (Nikawa et al. 1991). The switch between these two possibilities is regulated by PA, which acts as an essential metabolic intermediate and a regulator of phospholipid homeostasis.

The link between inositol and PA is an effector named Opi1p (Loewen et al. 2004). As noted in a previous section, many genes involved in phospholipid biosynthesis carry a *cis*-acting, inositol-sensitive upstream activating sequence (UAS_{INO}) response element (Chen et al. 2007a). All these genes are regulated by the same transcription factors. They are activated by Ino2p and Ino4p, and repressed by Opi1p. The location of Opi1p is the key whether or not it acts as a repressor. In the absence of extracellular inositol, Opi1p is bound to the ER, together with the integral ER membrane protein Scs2p (interaction of an FFAT motif) and PA. With Opi1p in this location, genes involved in inositol synthesis are transcribed. When inositol is added to the medium, PA is consumed by conversion into PI, leading to the translocation of Opi1p to the nucleus where it represses genes carrying the UAS_{INO} element (Carman and Han 2011). This latter process is influenced by pH, because deprotonated PA is a better ligand for Opi1p than protonated PA. The intracellular pH of yeast cells is strongly dependent on the nutritional environment. During glucose starvation, it falls rapidly compromising

the binding between PA and Opi1p. This effect leads to the translocation of Opi1p to the nucleus where it acts as a repressor of phospholipid synthesis (Ktistakis 2010).

The example described above is only one among many other regulatory aspects involved in phospholipid metabolism. As another recent example, Moir et al. (2012) reported that Yft2p and Scs3p, the yeast homologues of the mammalian FIT proteins (fat storage-inducing transmembrane proteins), are required for normal ER membrane biosynthesis. It is suggested that these proteins could be candidates involved in global regulation of phospholipid metabolism. For a more detailed description of phospholipid regulatory networks and interconnections with other pathways, the reader is referred to a recent review (Carman and Han 2011).

8.3.2 Regulation of Sphingolipid Metabolism

Sphingolipid metabolism is regulated by a series of factors, Orm1p, Orm2p, Ypk1p, Slm1p, Slm2p and TORC2. Orm1p and Orm2p are evolutionarily conserved proteins that act as inhibitors of serine:palmitoyl-CoA transferase (*SPT*), encoded by *LCB1* and *LCB2*, which catalyzes the first and rate-limiting step in the de novo synthesis of sphingolipids (Fig. 8.5) (Breslow et al. 2010). Ypk1p is a serine/threonine protein kinase that inactivates Orm1p and Orm2p by phosphorylation in response to compromised sphingolipid synthesis (Roelants et al. 2011; Sun et al. 2000). Slm1p and Slm2p are phosphoinositide-binding proteins that form a complex with each other and are both phosphorylated by the TORC2 complex (Niles and Powers 2012). The interplay of these factors can be regarded as an important control mechanism for sphingolipid homeostasis, because not only do the end products of sphingolipid synthesis but also several intermediates play an essential role for the cell. The feedback loop that controls sphingolipid metabolism can be summarized as follows: Orm1p and Orm2p form a stable complex with SPT when they are dephosphorylated, repressing SPT activity. Upon sphingolipid deficiency, Orm proteins are phosphorylated by Ypk1p, which leads to their relief of SPT. Ypk1p activity is in turn controlled by phosphorylation in a TORC2-dependent manner (Raychaudhuri et al. 2012). The TORC2-dependent phosphorylation of Ypk1p requires the activation of Slm proteins. These proteins appear to sense membrane stress caused by sphingolipid depletion and react by redistribution among different membrane domains. The relocation from eisosomes is caused by the inhibition of sphingolipid synthesis, which is then followed by activation of TORC2-Ypk1 signalling (Berchtold et al. 2012).

8.3.3 Phosphatidate Phosphatase Pah1p, a Switch Point in Glycerolipid Metabolism

PAH1 encodes the enzyme phosphatidate phosphatase which has gained more and more attention, in particular because of its homology to the mammalian lipins 1 and 2, which are involved in several lipid-associated disorders in human physiology (Han et al. 2006; Reue and Brindley 2008; Reue and Dwyer 2009). Pah1p catalyzes dephosphorylation of PA, yielding DAG and P_i, in a Mg^{2+}-dependent manner. Since both the substrate and the product of this reaction are important lipid mediators, Pah1p can be regarded as a central regulator of lipid homeostasis. This enzyme is an important control point deciding whether cells produce storage lipids or phospholipids as membrane constituents (for review see Pascual and Carman 2013). Pah1p is evolutionarily conserved, since genes encoding PAP (phosphatidic acid phosphatase) enzymes have been identified in humans, mice, flies, worms and plants. The influence of Pah1p was best studied in *pah1Δ* yeast deletion strains which were severely affected at several levels of lipid homeostasis. These strains showed defects in the synthesis of TG and PL, elevation in cellular content of PA and decreased levels of DAG and TG (Fakas et al. 2011; Han et al. 2006). Moreover, the amounts of PL, FA and SE were also increased in these mutants. The importance of Pah1p is further underlined by the occurrence in a *pah1Δ* deletion strain of several phenotypic appearances such as slow growth, defects in the biogenesis and morphology of LD, aberrant expansion of the nuclear/ER membranes, FA-induced toxicity and effects in vacuole homeostasis and membrane fusion, as well as in respiratory deficiency (O'Hara et al. 2006; Adeyo et al. 2011; Fakas et al. 2011; Sasser et al. 2012). The increased amount of PL is also typical of a *pah1Δ* mutant, which could be caused by the derepression of UAS_{INO}-containing lipid synthesis genes in response to elevated PA levels (see below) (Carman and Henry 2007; Chirala et al. 1994). Recently, Dgk1p was found to be a cellular counterpart of Pah1p by its regulation of PA homeostasis (Han et al. 2008). Dgk1p is a CTP-dependent DAG kinase that catalyzes the reverse reaction of Pah1p and restores PA levels in a *pah1Δ* mutant. As unbalanced levels of PA and DAG result in many phenotypic consequences, the activity of Pah1p must be fine-tuned to maintain lipid homeostasis and normal cell physiology. Some regulatory mechanisms of Pah1p activity were reported, but all of them are very complex, occurring on different levels (Pascual and Carman 2013). Pah1p expression was found to depend on various physiological conditions such as zinc depletion or different growth phases. Regulation by lipids and nucleotides was identified as another regulatory mechanism since Pah1p activity is stimulated in response to CDP-DAG, PI and CL, whereas it is inhibited by sphingosines, phytosphingosine and sphinganine and the nucleotides ATP and CTP (Wu and Carman 1994, 1996; Wu et al. 1993). Pah1p activity and subcellular distribution are governed by the Nem1p-Spo7p protein phosphatase complex, and several kinases such as Pho85p-Pho80p, Cdc28p-cyclin B, protein kinase A and C as well as casein kinase II can act on Pah1p using phosphorylation/dephosphorylation

mechanisms; however, the fine-tuning of Pah1p still has to be examined (Choi et al. 2011; Siniossoglou et al. 1998). The action of the transmembrane protein phosphatase complex, Nem1p-Spo7p, is responsible for the recruitment of the phosphorylated form of Pah1p from the cytosol to the nuclear/ER membrane. The Nem1p-Spo7p complex dephosphorylates Pah1p, enabling a short aminoterminal amphipathic helix to anchor Pah1p, thus allowing access to its substrate PA (Pascual and Carman 2013).

8.3.4 PS Decarboxylase 1 (Psd1p)

PE belongs to the bulk PL of yeast. It can be synthesized by four different pathways, namely by (i) decarboxylation of PS through Psd1p, (ii) by decarboxylation of PS though Psd2p, (iii) by reacylation of lyso-PE by Ale1p and Tgl3p and (iv) via the CDP-ethanolamine pathway (Henry et al. 2012; Böttinger et al. 2012). These pathways account for different proportions of cellular PE. Horvath et al. (2011) reported that the CDP-ethanolamine pathway preferentially contributes to TG synthesis by providing PE as co-substrate for Lro1p catalyzed TG synthesis, indicating a close interaction between TG and PE synthesis. The main source for PE, however, is the conversion of PS into PE by Psd1p. Psd1p is encoded by a nuclear gene, synthesized on free ribosomes and imported into mitochondria, where protein maturation takes place. This processing occurs in three steps, involving the action of the mitochondrial processing peptidase (MPP), the action of Oct1p (a mitochondrial peptidase that cleaves destabilizing N-terminal residues of a subset of proteins) and autocatalytic cleavage at a highly conserved LGST motif. These processing steps yield the mature form of the enzyme that contains an α-subunit, exposed to the intermembrane space, and a β-subunit anchoring the activated protein to the inner mitochondrial membrane. Correct localization is crucial for full enzymatic activity and also for maintaining lipid homeostasis (Horvath et al. 2012). Deletion of *PSD1* leads to reduced growth on glucose, morphological changes in mitochondria, ethanolamine auxotrophy and an altered pattern of PL (Birner et al. 2001). These observations underline the importance of Psd1p in lipid homeostasis. PE levels were shown to have a tremendous impact, not only on the distribution of other lipids, but also on the function and stability of mitochondrial proteins (Böttinger et al. 2012).

8.3.5 Ups1p, ERMES and Gem1p: Components Affecting Mitochondrial Lipid Transfer

Lipid transfer between and within organelles has been an important issue for several decades but is still under intense investigation. Import of lipids into mitochondria and interaction of mitochondria with the ER are classical examples

for such studies. Recently, identification of new components provided some deeper insight into these problems.

Intramitochondrial lipid transport is important to provide substrates like PA or PS for efficient CL and PE synthesis, respectively, in the inner mitochondrial membrane. Transport of PA between the outer (OMM) and inner mitochondrial membranes (IMM) was found to be mediated by Ups1p, a protein localized to the intermembrane space (Connerth et al. 2012; Tamura et al. 2010). PA is transported in three steps starting with the binding of PA by Ups1p at the surface of the OMM. Ups1p then associates with Mdm35p to be protected against proteases before PA is released at the IMM (Potting et al. 2010). This transport is bidirectional and independent of the acyl-chain composition. Dissociation of Mdm35p from the complex is a prerequisite for PA release and facilitated by the interaction with negatively charged PL like CL. However, a very high concentration of CL prevents the detachment of Ups1p from the acceptor membrane, subsequently impairing the PA flux. This finding indicates that CL is a regulator of its own synthesis (Connerth et al. 2012). Deletion of *UPS1* leads to a decrease in Psd1p levels and causes a reduction of PE. This defect has been explained as Ups1p being responsible not only for PA transport, but also for the import of Psd1p into mitochondria. Moreover, it was shown that Ups1p also mediates the export of PE from the IMM to the OMM and promotes the conversion of PE to PC, which makes Ups1p a central regulator of phospholipid metabolism by influencing lipid traffic (Tamura et al. 2012).

A complex termed ERMES (ER mitochondria encounter structure) that tethers the ER to the OMM has been identified. This complex is composed of the five proteins Mmm1p, Mdm34p, Mdm10p, Mdm12p and Gem1p (Kornmann et al. 2009; Stroud et al. 2011). Gem1p is an OMM GTPase with a C-terminal single transmembrane segment that is exposed to the cytosol (Kornmann et al. 2011; Meisinger et al. 2007). Mmm1p, Mdm34p and Mdm12p each contain an SMP domain (synaptotagmin-like mitochondrial and lipid-binding proteins) that is involved in binding hydrophobic ligands like lipids. This arrangement suggests a possible role for the ERMES complex in lipid transport between the ER and mitochondria (Kopec et al. 2010). A transport route between these two compartments is important because the substrate of the mitochondrial Psd1p, PS, is synthesized in the ER, and PE synthesized by Psd1p in mitochondria is substrate of the ER-localized PC-synthesizing machinery. How lipid transport via ERMES may happen is controversial and still a matter of debate. Kornmann et al. (2009) reported that strains bearing mutations in the ERMES proteins showed phenotypes related to phospholipid metabolism such as decreased CL levels. However, ERMES and Gem1p have been shown not to play a direct role in the transport of PS from the ER to mitochondria. Rather, ERMES fulfils a structural role in maintaining the morphological integrity of mitochondria (Nguyen et al. 2012).

8.3.6 Squalene

Squalene is a polyunsaturated triterpene consisting of six isoprene units. It possesses several beneficial properties, e.g. as antioxidant or emollient, and has therefore become relevant for biotechnological applications (for review see Spanova and Daum 2011). As described above, squalene is an important intermediate of the sterol biosynthetic pathway. Under normal growth conditions, it is rapidly converted and therefore does not accumulate in yeast. However, under certain growth conditions or by genetic manipulations (overexpression of *HMG1/2*, *ERG1* or *ERG6*; deletion of *HEM1*) the amount of squalene can be increased (Polakowski et al. 1998; Jahnke and Klein 1983; Lorenz et al. 1989). Spanova et al. (2010) showed that under squalene-accumulating conditions this lipid is stored in LD. Unexpectedly, accumulation of squalene did not result in lipotoxic effects. In a yeast strain lacking TG and SE, which is unable to synthesize LD, squalene was found mainly in mitochondria and microsomes without causing deleterious effects. Recent reports (Spanova et al. 2012) described functions of squalene as a modulator of membrane properties affecting mainly membrane fluidity. It was shown that ER membranes become more rigid when enriched in squalene, whereas samples of plasma membranes became softer. Unlike sterols, squalene does not necessarily rigidify membranes, but modulates their dynamics in both directions. This effect could depend on the ratio of ergosterol to squalene.

8.3.7 Tgl3p, Tgl4p and Tgl5p: More Than Just Triacylglycerol Lipases?

As described in the section on non-polar lipids, Tgl3p, Tgl4p and Tgl5p are the main TG lipases of the yeast *S. cerevisiae*. Recently, Ayp1p was identified as another TG lipase with minor lipolytic activity (Ploier et al. 2013). They catalyze the cleavage of TG to DAG and FA. However, these enzymes are not only responsible for mobilization of the main storage lipids but also contribute to lipid metabolism as acyltransferases and phospholipases, which makes them novel key players in lipid metabolism (Grillitsch and Daum 2011; Rajakumari and Daum 2010a, b).

In general, lipases are a subclass of hydrolases whose catalytic activity depends on the so-called interfacial activation, which means that they act only at an aqueous/non-aqueous interface (Verger 1997). As all other lipases, the three main TG lipases of yeast, Tgl3p, Tgl4p and Tgl5p, share a common consensus sequence GXSXG, where serine is the essential residue as interaction partner of the catalytic triad aspartic acid, glutamic acid and histidine (Schrag and Cygler 1997). They also contain a patatin domain, named after a plant storage protein that possesses lipid acyl hydrolase activity (Mignery et al. 1988).

Tgl3p was the first yeast TG lipase to be identified and characterized in *S. cerevisiae* (Athenstaedt et al. 1999a; Athenstaedt and Daum 2003). Tgl4p and

Tgl5p, identified some years later, exhibit about 30 % and 26 % similarity with Tgl3p (Athenstaedt and Daum 2005; Kurat et al. 2006). Localization studies have revealed that all three TG lipases are localized to LD, although none of these three lipases show hydrophobic domains (Athenstaedt and Daum 2005; Müllner et al. 2004). In vitro, all three proteins possess lipolytic activity whereas in vivo only Tgl3p and Tgl4p mobilize TG efficiently. This finding was explained by different substrate specificities. It appears that Tgl5p accepts mainly TG-containing cerotic acid (C26:0), a fatty acid of low abundance in yeast, whereas overall effects in vivo on bulk TG hydrolysis were not observed. The main TG lipase in yeast, Tgl3p, was shown to hydrolyze TG as well as DAG, whereas substrate specificity of Tgl4p is restricted to TG (Kurat et al. 2006). A *tgl3Δtgl4Δtgl5Δ* yeast strain lacking all three TG lipases does not reveal any growth defect under standard growth conditions, although mutations in *TGL3* or *TLG4* lead to fat yeast cells that accumulate TG (Athenstaedt and Daum 2005; Kurat et al. 2006). Moreover, deletion of *TGL4* and *TGL5* leads to decreased sporulation efficiency.

Recent characterization of TG lipases has revealed novel functions of these enzymes. Protein sequences of all three TG lipases contain additional sequence motifs besides the conserved GXSXG lipase motif. Tgl3p, Tgl4p and Tgl5p harbour an acyltransferase motif (H-$(X)_4$-D), and Tgl4p was found to have in addition a phospholipase motif (GXGXXG). Further investigations revealed decreased amounts of total PL in a *tgl3Δ* deletion strain and increased amounts of PL in a *TGL3* overexpressing strain (Rajakumari et al. 2010; Rajakumari and Daum 2010a). In vitro enzyme assays showed that both Tgl3p and Tgl5p act as lysophospholipid acyltransferases with different substrate specificities. Tgl3p mainly acylates lysophosphatidylethanolamine, whereas Tgl5p prefers lysophosphatidic acid as a substrate (Rajakumari and Daum 2010a). The lipase activity of Tgl3p acts independently from the acyltransferase activity and vice versa as demonstrated by site-directed mutagenesis, inactivating either one of the two motifs. Interestingly, the sporulation defect in a *tgl3Δtgl5Δ* double mutant was still observed in a strain with mutated lipase motif but not when the acyltransferase activity was abolished.

Besides the conserved lipase motif, Tgl4p contains a (G/A)XGXXG Ca^{2+}-independent phospholipase A_2 domain. Phospholipase activity of Tgl4p was also established in vitro with PC and PE as substrates but not with PA or PS. Additionally, Tgl4p hydrolyzed SE and revealed lysophospholipid acyltransferase activity (Rajakumari and Daum 2010b). Kurat et al. (2009) reported an impact of phosphorylation of Tgl4p activity. They showed that the lipolytic activity of Tgl4p was strongly reduced when phosphorylation sites were mutated, whereas the lysophospholipid acyltransferase activity was not affected (Rajakumari and Daum 2010b). In conclusion, Tgl4p is an excellent example of a multifunctional enzyme involved in yeast lipid metabolism, which does not only hydrolyze TG and SE but also contributes to PL synthesis and membrane remodelling. Recent publications, as well as our own unpublished data, led to the conclusion that besides the currently known TG lipases, Tgl3p, Tgl4p and Tgl5p, also other TG lipases may play a role in the turnover of non-polar lipids (Debelyy et al. 2011; Thoms et al. 2008, 2011).

8.3.8 Phospholipases

Phospholipases cleave different bonds in glycerophospholipid molecules, and their physiological effects are based on the resulting products. Depending on the bond(s) cleaved, phospholipases are divided into groups A_1, A_2, B, C and D (Fig. 8.6). A thoroughly studied example of Plc1p, the canonical yeast phospholipase C, illustrates well how phospholipases activate signalling cascades by generating, in the case of Plc1p, DAG and inositol 1,4,5-triphosphate, both of which exert their intrinsic biological activity as secondary messengers (reviewed in Rebecchi and Pentyala 2000; Strahl and Thorner 2007; York 2006). However, the activity of some phospholipases appears to be restricted to metabolic functions, such as that of Pgc1p, another yeast phospholipase C, which is specific for PG hydrolysis and required for its degradation (Simocková et al. 2008). The third known yeast phospholipase C is encoded by the *ISC1* gene. It accepts phosphosphingolipids (see above) as substrates and generates phytoceramide, a signalling molecule affecting several cellular processes (reviewed in Matmati and Hannun 2008). Two phospholipases D, Spo14p and Fmp30p, have also been described in *S. cerevisiae*. The former hydrolyzes PC to choline and PA and is involved in several cellular processes including growth, secretion and regulation of *INO1* expression (Sreenivas et al. 1998), as well as sporulation (Rudge et al. 1998) and general transcription (García-López et al. 2011). Fmp30p, an IMM protein with sequence similarity to mammalian N-acylethanolamine-specific phospholipases D (Merkel et al. 2005), is also required for CL homeostasis (Kuroda et al. 2011).

Deacylating phospholipases in yeast include phospholipases B, Plb1p, Plb2p, Plb3p, Nte1p and Spo1p; phospholipases A_2, Cld1p, Tgl4p, Per1p and Bst1p; and Yor022cp, a putative phospholipase A_1. A detailed understanding of the biochemical pathways leading to the specific FA composition of PL is important, among other reasons also from the perspective of yeast-based biofuel production (see below). All deacylating phospholipases could, in principle, be involved in acyl-chain remodelling of phospholipids, but a recent study showed that, rather than phospholipases B, it is the PL:DAG acyltransferase Lro1p which provides FA for PL remodelling (Mora et al. 2012). Two other acyltransferases, Psi1p (=Cst26p) and Taz1p, play crucial roles in PL acyl-chain remodelling (reviewed by Henry et al. 2012). Plb1/2/3 proteins, on the other hand, have been proposed to be involved in biosynthesis and, together with the phospholipase D Fmp30p, in signalling through N-acylethanolamines and N-acylphosphatidylethanolamines (Merkel et al. 2005).

A special case among yeast phospholipases is Tgl4p which, as described above, is a multifunctional enzyme with reported triacylglycerol lipase, steryl ester hydrolase and Ca^{2+}-independent phospholipase A_2 activities (Rajakumari and Daum 2010b). Importantly, regulation of the activity of this protein also links lipid metabolism to cell-cycle regulation (Kurat et al. 2009). Multiple cellular processes are also affected by the activity of Per1p and Bst1p, albeit the diversity of their effects stems from the fact that these phospholipases A_2 are active on GPI-protein

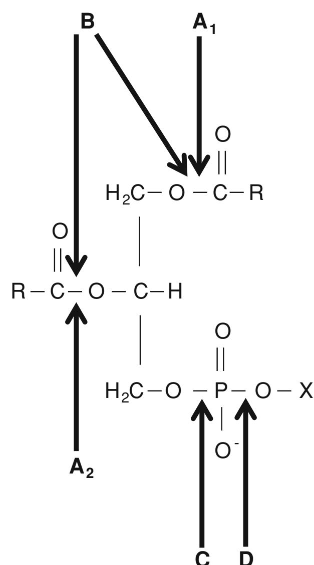

Fig. 8.6 Sites of hydrolytic action of phospholipases A_1, A_2, B, C and D on a model phospholipid molecule

anchors that enable specific localization of the proteins targeted to lipid raft regions of the plasma membrane (Fujita et al. 2006; Tanaka et al. 2004). *SPO1* is a meiosis-induced gene that encodes a phospholipase B with a role in distinct steps of sporulation, exhibiting epistasis with Spo14p phospholipase D, whereas the absence of Spo1p can be partially suppressed by overexpression of *PLB3* gene (Tevzadze et al. 2007). *CLD1* codes for a cardiolipin-specific deacetylase which, together with Taz1p, ensures the biosynthesis of mature CL (Beranek et al. 2009). Also, Nte1p seems to play an interesting role possibly regulating transcription of PL biosynthesis genes through its PC-specific phospholipase A_2 activity and subsequent modulation of Opi1p activity (see above) (Fernández-Murray et al. 2009).

8.3.9 Izh Proteins, Zinc Homeostasis and Regulation by Inositol and Fatty Acids

As described in the previous sections, regulation of inositol biosynthesis is one of the central processes in yeast lipid and general metabolism homeostasis. In this section, we will address the role of Izh (Implicated in Zinc Homeostasis) proteins, yeast homologues of the mammalian adiponectin receptors, which have recently emerged as players enabling the connection between inositol and FA metabolism and zinc homeostasis. Zinc depletion in yeast activates Zap1p transcriptional activator which affects several target genes, among them the *PIS1*-encoded PI synthase and the *DPP1*-encoded DGPP phosphatase. Zn depletion thus causes a

decrease in PE and an increase in PI concentration (Carman and Han 2011; Iwanyshyn et al. 2004). In addition, zinc depletion results in a decreased concentration of PA, thus triggering the release of Opi1p from the ER membrane and its translocation into the nucleus, where it represses expression of *CHO1* gene that encodes PS synthase by binding to and inhibition of the Ino2/4 complex (Carman and Han 2011). There are four genes in yeast, *IZH1, IZH2, IZH3* and *IZH*4 which encode proteins with sequence similarity to adiponectin receptors. In humans, adiponectin receptors mediate the antidiabetic metabolic activity of the polypeptide hormone adiponectin (Kadowaki et al. 2006). The yeast Izh2p has been confirmed as a functional homolog of adiponectin receptors in an experiment where heterologous expression of human adiponectin receptors in yeast functionally complemented Izh2p (Kupchak et al. 2007). *IZH1/2/3/4* genes were implicated to have a role in zinc metabolism after they had been identified in a screening for Zap1p targets, and were confirmed to have zinc-related phenotypes (Lyons et al. 2004). Expressions of *IZH1* and *IZH2* are directly regulated by Zap1p, and the promoters of these genes contain zinc-response elements. In addition, *IZH1, IZH2* and *IZH3* genes are regulated by exogenous FA through Oaf1p/Pip2p transcription factors that bind to oleate-response elements present in their promoters (Lyons et al. 2004). Specifically, *IZH2* expression is highly induced in cells grown in the presence of saturated FA such as myristate, and strains without this gene fail to grow normally in the presence of myristate (Karpichev et al. 2002). Transcriptome analysis of *izh2*Δ cells has revealed that a number of genes encoding proteins involved in FA metabolism and in the phosphate signalling pathway are regulated by Izh2p (Karpichev et al. 2002). Three functions of Izh proteins have been proposed by Lyons et al. (2004): (i) a role in sterol metabolism by which they would influence the permeability of the plasma membrane and consequently zinc homeostasis; (ii) a role as transporters for zinc; and (iii) a role in a zinc-independent signal transduction cascade with Zap1p as downstream target. The above results imply that, at least for Izh2p, the third possibility is the most likely one. Thus, Izh2p is emerging as a central component of a putative feedback regulatory pathway leading from FA to Zap1p activation and finally to inositol and regulation of PL biosynthesis.

8.4 Biotechnological Aspects

Lipids and their expansive roles have become increasingly recognized, resulting in a great demand for industrial high-level production of particular valuable lipid compounds. Lipid metabolism in yeasts as described above has been studied intensively and well described. Since this process is well conserved in eukaryotic cells, yeasts are ideal host systems for the biotechnological production of industrially and pharmaceutically relevant lipid compounds. *S. cerevisiae,* in particular, has been successfully applied for their production. This section describes examples, selected to illustrate the importance of lipid metabolism in biotechnology.

In this section, some of the most important approaches are described. The reader is also referred to recent reviews on these topics (such as Beopoulos et al. 2011; de Jong et al. 2012; Ruenwai et al. 2011; Uemura 2012; Veen and Lang 2004) for more details.

8.4.1 Polyunsaturated Fatty Acids

PUFA are FA with more than 16 carbon atoms in the chain that contain more than one double bond. They have multiple positive effects on human health, such as lowering the risk of heart attacks, cardiovascular diseases and cancer, and they also have major impacts on the development and improvement of retinal and brain function and on the regulation of membrane fluidity (Uemura 2012; Opekarová and Tanner 2003). Since mammals are not able to synthesize essential PUFA such as linoleic acid (C18:2n-6) or the omega-3 and omega-6 PUFA, they must be taken up from the diet. Since natural sources, such as fish oils, are limited, it is highly desirable to produce PUFA from alternative and sustainable sources. One promising option is *S. cerevisiae* or other yeasts. *S. cerevisiae,* in particular, has been shown to have a considerable potential for metabolic engineering approaches to the production of certain metabolites (Ostergaard et al. 2000).

The physiological FA composition of *S. cerevisiae* includes mainly C16:1 and C18:1 as described above in the section on FA. Since *OLE1,* that encodes a Δ9-fatty acid desaturase, is the only endogenous desaturase (Stukey et al. 1990), production of PUFA in *S. cerevisiae* requires the introduction of further desaturase and elongase genes from donor organisms such as *Mucor rouxii*, *Caenorhabditis elegans*, *Arabidopsis thaliana* or *Mortierella alpina* to produce, for example, α-linolenic acid (C18:3n-3), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) (Ruenwai et al. 2011; Uemura 2012). Combinations of multiple desaturases and elongases from various organisms were tried, but since Δ5- and Δ6-fatty acid desaturases can accept both n-3 and n-6 FA, the resulting products mostly depend on the substrate fatty acid added to the medium. Most studies used a large excess of precursor FA, yet the final yield of the PUFA produced was still low and strongly depended on cultivation conditions, such as growth media, temperature and incubation time (Uemura 2012; Misawa 2011). Construction of the complete pathway for the production of C20-PUFA, such as DGLA (dihomogamma linoleic acid) from the endogenous oleic acid, has been described by Yazawa et al. (2007). The authors cloned a Δ12-desaturase gene from *K. lactis*, and a Δ6-desaturase and the elongase *ELO1* genes from rat into *S. cerevisiae*.

One severe limitation of *S. cerevisiae* as a production host for PUFA is the low total lipid content compared to some other yeast genera. One alternative is the use of oleaginous yeasts such as *Y. lipolytica* which are characterized by their ability to accumulate lipids up to 40 % of their biomass (Beopoulos et al. 2011). *Y. lipolytica* has been applied successfully for the production of ω-3 and ω-6 PUFA such as docosahexaenoic acid, eicosapentaenoic acid and γ-linolenic acid. DuPont

de Nemours, for example, genetically engineered *Y. lipolytica* by expressing heterologous desaturases and elongases from organisms like *M. alpina* and *Fusarium moniliforme*, and by genetically inhibiting peroxisomal fatty acid degradation that produces lipids with the highest content of docosahexaenoic and eicosapentaenoic acids available (Xue et al. 2013).

8.4.2 Isoprenoids

Isoprenoids, also referred to as terpenoids, comprise a large group of naturally occurring secondary metabolites built from isoprene units, IPP (isopentenyl diphosphate) and its isomer DMAPP (dimethylallyl diphosphate). Eukaryotes synthesize IPP via the mevalonate pathway as described in the section on sterols. Head-to-tail condensation of IPP and DMAPP yields GPP, which is then converted into FPP by linkage of another molecule IPP. IPP is a branching point between GGPP (geranylgeranyl diphosphate) and the sterol pathway (see Fig. 8.3) (Pichler 2005). GPP and FPP are the precursors of monoterpenoids and sesquiterpenoids, respectively, and GGPP of diterpenes. Typically, two molecules of FPP are condensed to yield squalene, the precursor of sterols and phytoene which can be converted into carotenoids. Steroids will be discussed in the next section while the other isoprenoids are dealt with in this section.

Terpenoids comprise over 40,000 structurally different compounds. They are the largest group of natural products and have valuable properties for medical and industrial usage, especially as constituents of plant oils such as limonene, menthol and citronellol, which are used as flavours and fragrances, in their occurrence of carotenoids and as pharmaceuticals such as taxol (Misawa 2011; Chang and Keasling 2006).

S. cerevisiae does not produce monoterpenoids. Due to industrial requirement of these compounds, however, metabolic engineering approaches have been accomplished to this end (Lee et al. 2009). Herrero et al. (2008), for example, reported a recombinant wine yeast strain of *S. cerevisiae* that expresses the (*S*)-linalool synthase gene from the plant *Clarkia breweri,* and concomitantly overexpresses HMG-CoA reductase, resulting in efficient excretion of linalool reaching concentrations of 77 μg/L.

Sesquiterpenoids comprise the largest group of isoprenoids, and occur in plants and insects as pheromones and defensive agents. Because of their anti-cancer, antitumour and antibiotic properties, they are industrially important compounds (Asadollahi et al. 2010). One prominent example is artemisin, which is an effective anti-malarial drug and has been discussed as an anti-cancer agent (Firestone and Sundar 2009; Chaturvedi et al. 2010).

Several pharmaceuticals belong to the group of diterpenoids, including taxol, which is used as a potent anti-cancer agent (Wani et al. 1971). As the demand for taxol exceeds the amounts which can be isolated from its natural source *Taxus bevifolia*, heterologous production in *S. cerevisiae* by introducing parts of the 19

enzymatic step biosynthetic pathway is one alternative. Engels et al. (2008) described the production of a precursor of taxol, taxa-4(5),11(12)-diene, by expressing *Taxus chinensis* taxadiene synthase and truncated HMG-CoA reductase genes in *S. cerevisiae* together with an archaeal GGPP synthase gene from *Sulfolobus aciocaldarius*. These manipulations resulted in formation of 8.7 mg/L of taxadiene.

Carotenoids, such as *β*-carotene, astaxanthin and lycopene, are also isoprenoids. They are widely distributed as yellow, orange and red natural pigments in all phototrophic plants as well as in some bacteria, algae and fungi. In addition to their important physiological roles as components of the photosynthetic complex, precursors of phytohormones and chromophoric compounds of animals and plants, their anti-oxidative and photoprotective effects were proposed (Fraser and Bramley 2004). These effects are also beneficial for human health and carotenoids attracted attention as nutraceutical agents. Lycopene, for example, which occurs in tomatoes, is thought to prevent cardiovascular disease, UV-light ageing in humans and age-related macular degeneration. Carotenoid biosynthetic pathways have been introduced into *S. cerevisiae* to produce lycopene and *β*-carotene. The engineered strains yielded *β*-carotene at 5.9 mg/g dry cell weight and lycopene ate 7.8 mg/g dry cell weight (Verwaal et al. 2007; Yamano et al. 1994). For a recent review of this topic see Wriessnegger and Pichler (2013).

8.4.3 Steroids

Steroids comprise a large group of compounds with cyclopentanoperhydrophenanthrene as the common basic structure as described in the section on sterols. This group of components are roughly divided into sterols, which are steroid alcohols with a hydroxyl group in the 3-position of the A-ring, steroid hormones, steroid alkaloids and bile acids. Hundreds of distinct steroids are found in plants, animals and fungi, all of them sharing the mutual precursor squalene. They have sex-determining, growth regulating and anti-inflammatory properties and are responsible for membrane fluidity and permeability (Riad et al. 2002). The chemical synthesis is very difficult and extraction from natural sources is low-yielding and unsustainable. Therefore, the production in yeast is an appreciated alternative (Heiderpriem et al. 1992).

Several sterol intermediates are of biotechnological interest and have already found applications in industry (Donova and Egorova 2012). Lanosterol, for example, serves as an emulsifier in cosmetics, zymosterol as a precursor for cholesterol lowering substances and ergosterol itself as provitamin D2 and as a constituent of liposomal steroids used as carriers for drugs. As special pharmaceutical interest, they can serve as valuable precursors for the production of hydrocortisone and other steroid hormones like dehydroepiandosteroine, progesterone, testosterone and estrogens. The natural content of sterols in yeast is, however, too low for commercial applications and several attempts have therefore

been made to increase the total sterol content in this microorganism (Veen et al. 2003). The most successful strategies were the concomitant overexpression of *ERG1* and *ERG11* and a truncated version of *HMG1*, and the overexpression of *ERG4* and *ARE2*. The accumulation of sterols can also be promoted by the addition of ethanol into the cultivation medium by fermentation under nitrogen-limiting conditions (Sajbidor et al. 1995; Shang et al. 2006). For recent reviews of yeast metabolic engineering targeting sterol metabolism see (Wriessnegger and Pichler 2013).

8.4.4 Biofuels

Current transportation fuels are obtained mainly from fossil sources, which are not only limited but also associated with air pollution and global warming. These developments have prompted a desire for a shift from fossil fuels to biofuels. The concept of biofuels relies on the conversion of renewable resources into fuels. It comprises not only first-generation biofuels such as bioethanol and biodiesel, but also advanced biofuels such as alkanes, terpenes, short-chain alcohols and fatty acyl ethyl esters. Compared to bioethanol, the latter compounds promise energy content and combustion properties similar to those of current petroleum-based fuels (de Jong et al. 2012).

The most frequently employed microorganism to produce bioethanol is *S. cerevisiae* since it is able to hydrolyze sucrose from sugar cane into glucose and fructose at concentrations over 100 g/L, which can be converted by fermentation into ethanol. However, the availability of inexpensive fermentable sugars is limited, and re-dedicating farmland for biofuel production causes economic and ethical problems. Another limitation for ethanol as biofuel is the difficulty to distill it from fermentation broth due to its miscibility with water and its corrosive effect to storage and distribution infrastructures. As alternatives, non-food cellulose sources, including wheat straw and forest waste, can be used for the production of biofuels but, since *S. cerevisiae* is unable to convert cellulose or efficiently ferment C-5 sugars (pentoses), metabolic engineering approaches are necessary or the employment of other yeast genera (Madhavan et al. 2012).

Biodiesel is a biodegradable, non-toxic and sulphur-free alternative form of fuel, currently produced mainly by chemical transesterification of vegetable oils. One promising alternative is to use oleaginous yeasts, such as *Y. lipolytica*, *Cryptococcus curvatus* or *Lipomyces starkeyi* to produce lipids using cellulosic sugars as carbon source. These yeasts accumulate lipids at up to 40 % of their biomass, under nutrient-limiting conditions even up to 70 % (Chen et al. 2009). The microbial lipids produced show similar composition and energy values to those of vegetable oils, comprising mainly myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1, n-7), stearic (C18:0), oleic (C18:1, n-9), linoleic (C18:2, n-6) and α- linolenic (C18:3 n-3) acids, and are therefore of great commercial value for the production of sustainable biodiesel which requires C16-C18 FA (Zhao et al.

2011; Yu et al. 2011). However, from an economic point of view, the development of yeasts that produce more than 80 % lipids of their biomass would be necessary. Several engineering strategies have already been published and patented as reviewed by Beopoulos et al. (2011).

8.4.5 Flavour Compounds

Yeast biosynthesis of flavour compounds is important in fermentations of wine, beer and sake. An important group of volatile compounds produced by yeast during fermentation, which include fusel alcohols, monoterpenoids and volatile sulphur compounds, are lipid metabolism-derived acetate esters and medium-chain fatty acid (MCFA) ethyl esters (reviewed by Cordente et al. 2012). These esters are produced intracellularly by acetyl transferases from acetyl-CoA and ethanol or complex alcohols as substrates, or by acyl transferases from MCFA-CoA and ethanol as substrates. Many such esters can pass the plasma membrane and diffuse into the medium. The best studied group from the perspective of biosynthesis pathway are acetate esters. Their synthesis is catalyzed by acetyl transferases I and II, encoded by *ATF1* and *ATF2* genes (Fujii et al. 1994; Nagasawa et al. 1998). *ATF1* has been shown to be localized to LD (Verstrepen et al. 2004). Apart from volatile esters such as ethyl acetate or isoamyl acetate, *ATF1P* and *ATF2P* are also responsible for the formation of less volatile esters which add no flavour characteristics to the fermentation products. A certain amount of acetate esters are produced also in cells deleted of both *AFT* genes, indicating that additional, as yet unknown acetyl transferases may exist in yeast. Ethyl esters are the product of Eeb1p- or Eht1p-catalyzed condensation reaction between acyl-CoA and ethanol (Saerens et al. 2006). These two acyl transferases differ in their specificity towards different length of the substrate molecules and they also possess esterase activity. Similar to acetyl transferases, undiscovered acyl transferases responsible for MCFA ethyl ester biosynthesis are encoded in the yeast genome.

Understanding the physiological regulation of volatile esters biosynthesis is the prerequisite to the engineering of flavour compounds in yeast-fermented beverages. For the synthesis of acetate esters, the main regulatory step is the reaction catalyzed by acetyl transferases, whereas for MCFA ethyl ester formation, the availability of MCFA-CoA substrate is the limiting factor (Saerens et al. 2010). The amount and nature of acetyl esters could therefore be regulated by overexpression of *AFT1* or *AFT2* at different levels, possibly from different strains and therefore with different substrate specificities. The amount of MCFA ethyl esters could be controlled by modifying lipid metabolic pathways, specifically at the level of acetyl-CoA carboxylase whose activity determines the release of MCFAs from the fatty acid synthase complex (Dufour et al. 2003). Alternatively, the level of peroxisomal uptake of MCFAs may be changed, because a specific system exists for the import of this group of FA towards oxidative degradation (van Roermund et al. 2001).

For more details describing the nature and properties of yeast flavour compounds, the reader is referred to recent reviews (Saerens et al. 2010; Sumby et al. 2010; Cordente et al. 2012).

8.5 Conclusions and Perspectives

Over the last few decades, outstanding advancements have been made to identify the major enzymes involved in the pathways of lipid metabolism. Most of them are now known, covering the main cellular routes for synthesis, storage and degradation of lipid compounds. However, some gaps still remain. One intriguing open question is how cells can sense and manage their lipid composition under different environmental conditions. Investigations addressing such lipid sensors might also shed more light on the issue of how the different lipid compositions of different membranes within a single organism can be maintained. The situation gets even more complicated by the fact that enzymes of lipid synthesis are located in close vicinity to each other. To elucidate the topology of these enzymes in detail will be a challenge for the future. Other examples of unsolved problems are metabolic channelling and lipid trafficking that are just beginning to be addressed and understood. Regulation of lipid metabolism is an issue under discussion. It occurs at many different levels, and the cellular lipid composition is not only extremely dependent on growth conditions, such as nutrient availability, growth phase and pH, but also on many transcriptional control mechanisms that have been reported. Thus, the crosstalk between lipid metabolism and other cellular processes, as well as the regulatory network and interconnections of lipid metabolic pathways, will have to be studied in more detail. The elucidation of all these questions will foster the powerful role of yeast as a model organism.

Acknowledgments We thank Prof. Roger Pain and Dr. Karlheinz Grillitsch for carefully reading the manuscript. Research in G.D.'s laboratory was financially supported by the Austrian Science Fund (FWF) project W901 (DK Molecular Enzymology). The work in U.P.'s laboratory was financially supported by the Slovenian Research Agency (ARRS) grants J1̄6507 and P1̄0207.

References

Adeyo O, Horn PJ, Lee S, Binns DD, Chandrahas A, Chapman KD, Goodman JM (2011) The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. J Cell Biol 192:1043–1055

Alvarez-Vasquez F, Riezman H, Hannun YA, Voit EO (2011) Mathematical modeling and validation of the ergosterol pathway in *Saccharomyces cerevisiae*. PLoS ONE 6:e28344

Anderson MS, Muehlbacher M, Street IP, Proffitt J, Poulter CD (1989a) Isopentenyl diphosphate:dimethylallyl diphosphate isomerase. An improved purification of the enzyme and isolation of the gene from *Saccharomyces cerevisiae*. J Biol Chem 264:19169–19175

Anderson MS, Yarger JG, Burck CL, Poulter CD (1989b) Farnesyl diphosphate synthetase. Molecular cloning, sequence, and expression of an essential gene from *Saccharomyces cerevisiae*. J Biol Chem 264:19176–19184

Asadollahi MA, Maury J, Schalk M, Clark A, Nielsen J (2010) Enhancement of farnesyl diphosphate pool as direct precursor of sesquiterpenes through metabolic engineering of the mevalonate pathway in *Saccharomyces cerevisiae*. Biotechnol Bioeng 106:86–96

Athenstaedt K, Daum G (1997) Biosynthesis of phosphatidic acid in lipid particles and endoplasmic reticulum of *Saccharomyces cerevisiae*. J Bacteriol 179:7611–7616

Athenstaedt K, Daum G (1999) Phosphatidic acid, a key intermediate in lipid metabolism. Eur J Biochem 266:1–16

Athenstaedt K, Daum G (2003) *YMR313c/TGL3* encodes a novel triacylglycerol lipase located in lipid particles of *Saccharomyces cerevisiae*. J Biol Chem 278:23317–23323

Athenstaedt K, Daum G (2005) Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast *Saccharomyces cerevisiae* are localized to lipid particles. J Biol Chem 280:37301–37309

Athenstaedt K, Zweytick D, Jandrositz A, Kohlwein SD, Daum G (1999a) Identification and characterization of major lipid particle proteins of the yeast *Saccharomyces cerevisiae*. J Bacteriol 181:6441–6448

Athenstaedt K, Weys S, Paltauf F, Daum G (1999b) Redundant systems of phosphatidic acid biosynthesis via acylation of glycerol-3-phosphate or dihydroxyacetone phosphate in the yeast *Saccharomyces cerevisiae*. J Bacteriol 181:1458–1463

Athenstaedt K, Jolivet P, Boulard C, Zivy M, Negroni L, Nicaud J-M, Chardot T (2006) Lipid particle composition of the yeast *Yarrowia lipolytica* depends on the carbon source. Proteomics 6:1450–1459

Bagnat M, Keränen S, Shevchenko A, Shevchenko A, Simons K (2000) Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. Proc Natl Acad Sci USA 97:3254–3259

Bard M, Downing JF (1981) Genetic and biochemical aspects of yeast sterol regulation involving 3-hydroxy-3-methylglutaryl coenzyme A reductase. J Gen Microbiol 125:415–420

Beeler TJ, Fu D, Rivera J, Monaghan E, Gable K, Dunn TM (1997) *SUR1* (*CSG1/BCL21*), a gene necessary for growth of *Saccharomyces cerevisiae* in the presence of high Ca2+ concentrations at 37 degrees C, is required for mannosylation of inositolphosphorylceramide. Mol Gen Genet 255:570–579

Beeler T, Bacikova D, Gable K, Hopkins L, Johnson C, Slife H, Dunn T (1998) The *Saccharomyces cerevisiae TSC10/YBR265w* gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca^{2+}-sensitive *csg2*Δ mutant. J Biol Chem 273:30688–30694

Beopoulos A, Nicaud J-M, Gaillardin C (2011) An overview of lipid metabolism in yeasts and its impact on biotechnological processes. Appl Microbiol Biotechnol 90:1193–1206

Beranek A, Rechberger G, Knauer H, Wolinski H, Kohlwein SD, Leber R (2009) Identification of a cardiolipin-specific phospholipase encoded by the gene *CLD1* (*YGR110W*) in yeast. J Biol Chem 284:11572–11578

Berchtold D, Piccolis M, Chiaruttini N, Riezman I, Riezman H, Roux A, Walther TC, Loewith R (2012) Plasma membrane stress induces relocalization of Slm proteins and activation of TORC2 to promote sphingolipid synthesis. Nat Cell Biol 14:542–547

Binns D, Januszewski T, Chen Y, Hill J, Markin VS, Zhao Y, Gilpin C, Chapman KD, Anderson RGW, Goodman JM (2006) An intimate collaboration between peroxisomes and lipid bodies. J Cell Biol 173:719–731

Birner R, Bürgermeister M, Schneiter R, Daum G (2001) Roles of phosphatidylethanolamine and of its several biosynthetic pathways in *Saccharomyces cerevisiae*. Mol Biol Cell 12:997–1007

Black PN, DiRusso CC (2007) Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. Biochimica et Biophysica Acta (BBA)-Molecular Cell Biol Lipids 1771:286–298

Böttinger L, Horvath SE, Kleinschroth T, Hunte C, Daum G, Pfanner N, Becker T (2012) Phosphatidylethanolamine and cardiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes. J Mol Biol 423:677–686

Breslow DK, Collins SR, Bodenmiller B, Aebersold R, Simons K, Shevchenko A, Ejsing CS, Weissman JS (2010) Orm family proteins mediate sphingolipid homeostasis. Nature 463:1048–1053

Carman GM, Han G-S (2011) Regulation of phospholipid synthesis in the yeast *Saccharomyces cerevisiae*. Annu Rev Biochem 80:859–883

Carman GM, Henry SA (2007) Phosphatidic acid plays a central role in the transcriptional regulation of glycerophospholipid synthesis in *Saccharomyces cerevisiae*. J Biol Chem 282:37293–37297

Chambon C, Ladeveze V, Servouse M, Blanchard L, Javelot C, Vladescu B, Karst F (1991) Sterol pathway in yeast. Identification and properties of mutant strains defective in mevalonate diphosphate decarboxylase and farnesyl diphosphate synthetase. Lipids 26:633–636

Chang MCY, Keasling JD (2006) Production of isoprenoid pharmaceuticals by engineered microbes. Nat Chem Biol 2:674–681

Chang SC, Heacock PN, Clancey CJ, Dowhan W (1998a) The *PEL1* gene (renamed *PGS1*) encodes the phosphatidylglycero-phosphate synthase of *Saccharomyces cerevisiae*. J Biol Chem 273:9829–9836

Chang SC, Heacock PN, Mileykovskaya E, Voelker DR, Dowhan W (1998b) Isolation and characterization of the gene (*CLS1*) encoding cardiolipin synthase in *Saccharomyces cerevisiae*. J Biol Chem 273:14933–14941

Chaturvedi D, Goswami A, Saikia PP, Barua NC, Rao PG (2010) Artemisinin and its derivatives: a novel class of anti-malarial and anti-cancer agents. Chem Soc Rev 39:435–454

Chen M, Hancock LC, Lopes JM (2007a) Transcriptional regulation of yeast phospholipid biosynthetic genes. Biochim Biophys Acta 1771:310–321

Chen Q, Kazachkov M, Zheng Z, Zou J (2007b) The yeast acylglycerol acyltransferase *LCA1* is a key component of Lands cycle for phosphatidylcholine turnover. FEBS Lett 581:5511–5516

Chen X, Li Z, Zhang X, Hu F, Ryu DDY, Bao J (2009) Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. Appl Biochem Biotechnol 159:591–604

Chirala SS, Kuziora MA, Spector DM, Wakil SJ (1987) Complementation of mutations and nucleotide sequence of FAS1 gene encoding beta subunit of yeast fatty acid synthase. J Biol Chem 262:4231–4240

Chirala SS, Zhong Q, Huang W, al-Feel W (1994) Analysis of *FAS3/ACC* regulatory region of *Saccharomyces cerevisiae*: identification of a functional UASINO and sequences responsible for fatty acid mediated repression. Nucleic Acids Res 22:412–418

Choi H-S, Su W-M, Morgan JM, Han G-S, Xu Z, Karanasios E, Siniossoglou S, Carman GM (2011) Phosphorylation of phosphatidate phosphatase regulates its membrane association and physiological functions in *Saccharomyces cerevisiae*: identification of SER(602), THR(723), AND SER(744) as the sites phosphorylated by *CDC28* (*CDK1*)-encoded cyclin-dependent kinase. J Biol Chem 286:1486–1498

Choudhary V, Schneiter R (2009) Monitoring sterol uptake, acetylation, and export in yeast. Methods Mol Biol 580:221–232

Choudhary V, Schneiter R (2012) Pathogen-Related Yeast (PRY) proteins and members of the CAP superfamily are secreted sterol-binding proteins. Proc Natl Acad Sci USA 109: 16882–16887

Chung J-H, Lester RL, Dickson RC (2003) Sphingolipid requirement for generation of a functional v1 component of the vacuolar ATPase. J Biol Chem 278:28872–28881

Clancey CJ, Chang SC, Dowhan W (1993) Cloning of a gene (*PSD1*) encoding phosphatidylserine decarboxylase from *Saccharomyces cerevisiae* by complementation of an Escherichia coli mutant. J Biol Chem 268:24580–24590

Connerth M, Czabany T, Wagner A, Zellnig G, Leitner E, Steyrer E, Daum G (2010) Oleate inhibits steryl ester synthesis and causes liposensitivity in yeast. J Biol Chem 285: 26832–26841

Connerth M, Tatsuta T, Haag M, Klecker T, Westermann B, Langer T (2012) Intramitochondrial transport of phosphatidic acid in yeast by a lipid transfer protein. Science 338:815–818

Cordente AG, Curtin CD, Varela C, Pretorius IS (2012) Flavour-active wine yeasts. Appl Microbiol Biotechnol 96:601–618

Cowart LA, Obeid LM (2007) Yeast sphingolipids: recent developments in understanding biosynthesis, regulation, and function. Biochim Biophys Acta 1771:421–431

Crowley JH, Leak FW Jr, Shianna KV, Tove S, Parks LW (1998) A mutation in a purported regulatory gene affects control of sterol uptake in *Saccharomyces cerevisiae*. J Bacteriol 180:4177–4183

Cullis PR, Hope MJ, Tilcock CP (1986) Lipid polymorphism and the roles of lipids in membranes. Chem Phys Lipids 40:127–144

Czabany T, Athenstaedt K, Daum G (2007) Synthesis, storage and degradation of neutral lipids in yeast. Biochim Biophys Acta 1771:299–309

Czabany T, Wagner A, Zweytick D, Lohner K, Leitner E, Ingolic E, Daum G (2008) Structural and biochemical properties of lipid particles from the yeast *Saccharomyces cerevisiae*. J Biol Chem 283:17065–17074

Daum G (1985) Lipids of mitochondria. Biochim Biophys Acta 822:1–42

Daum G, Vance JE (1997) Import of lipids into mitochondria. Prog Lipid Res 36:103–130

Daum G, Lees ND, Bard M, Dickson R (1998) Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. Yeast 14:1471–1510

De Jong B, Siewers V, Nielsen J (2012) Systems biology of yeast: enabling technology for development of cell factories for production of advanced biofuels. Curr Opin Biotechnol 23:624–630

Debelyy MO, Thoms S, Connerth M, Daum G, Erdmann R (2011) Involvement of the *Saccharomyces cerevisiae* hydrolase Ldh1p in lipid homeostasis. Eukaryot Cell 10:776–781

Dennis EA (2009) Lipidomics joins the omics evolution. Proc Natl Acad Sci USA 106:2089–2090

Dickson RC, Nagiec EE, Wells GB, Nagiec MM, Lester RL (1997) Synthesis of mannose-(inositol-P)2-ceramide, the major sphingolipid in *Saccharomyces cerevisiae*, requires the *IPT1* (*YDR072c*) gene. J Biol Chem 272:29620–29625

Dickson RC, Sumanasekera C, Lester RL (2006) Functions and metabolism of sphingolipids in *Saccharomyces cerevisiae*. Prog Lipid Res 45:447–465

Donova MV, Egorova OV (2012) Microbial steroid transformations: current state and prospects. Appl Microbiol Biotechnol 94:1423–1447

Dowhan W, Bogdanov M (2009) Lipid-dependent membrane protein topogenesis. Annu Rev Biochem 78:515–540

Dufour J-P, Malcorps P, Silcock P (2003) Control of ester synthesis during brewery fermentation. In: Smart K (ed) Brewing Yeast Fermentation Performance. Blackwell Publishing, Oxford, pp 213–233

Dunn TM, Haak D, Monaghan E, Beeler TJ (1998) Synthesis of monohydroxylated inositol-phosphorylceramide (IPC-C) in *Saccharomyces cerevisiae* requires Scs7p, a protein with both a cytochrome b5-like domain and a hydroxylase/desaturase domain. Yeast 14:311–321

Eggeling C, Ringemann C, Medda R, Schwarzmann G, Sandhoff K, Polyakova S, Belov VN, Hein B, von Middendorff C, Schönle A et al (2009) Direct observation of the nanoscale dynamics of membrane lipids in a living cell. Nature 457:1159–1162

Einerhand AW, Voorn-Brouwer TM, Erdmann R, Kunau WH, Tabak HF (1991) Regulation of transcription of the gene coding for peroxisomal 3-oxoacyl-CoA thiolase of *Saccharomyces cerevisiae*. Eur J Biochem 200:113–122

Ejsing CS, Sampaio JL, Surendranath V, Duchoslav E, Ekroos K, Klemm RW, Simons K, Shevchenko A (2009) Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. Proc Natl Acad Sci USA 106:2136–2141

Engels B, Dahm P, Jennewein S (2008) Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab Eng 10:201–206

Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CRH, Shimizu T, Spener F, van Meer G, Wakelam MJO, Dennis EA (2009) Update of the LIPID MAPS comprehensive classification system for lipids. J Lipid Res 50(Suppl):S9–14

Fakas S, Qiu Y, Dixon JL, Han G-S, Ruggles KV, Garbarino J, Sturley SL, Carman GM (2011) Phosphatidate phosphatase activity plays key role in protection against fatty acid-induced toxicity in yeast. J Biol Chem 286:29074–29085

Farese RV, Walther TC (2009) Lipid Droplets Finally Get a Little R-E-S-P-E-C-T. Cell 139:855–860

Fei W, Wang H, Fu X, Bielby C, Yang H (2009) Conditions of endoplasmic reticulum stress stimulate lipid droplet formation in *Saccharomyces cerevisiae*. Biochem J 424:61–67

Fernández-Murray JP, Gaspard GJ, Jesch SA, McMaster CR (2009) *NTE1*-encoded phosphatidylcholine phospholipase b regulates transcription of phospholipid biosynthetic genes. J Biol Chem 284:36034–36046

Fickers P, Benetti PH, Waché Y, Marty A, Mauersberger S, Smit MS, Nicaud JM (2005) Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications. FEMS Yeast Res 5:527–543

Firestone GL, Sundar SN (2009) Anticancer activities of artemisinin and its bioactive derivatives. Expert Rev Mol Med 11:e32

Fischl AS, Carman GM (1983) Phosphatidylinositol biosynthesis in *Saccharomyces cerevisiae*: purification and properties of microsome-associated phosphatidylinositol synthase. J Bacteriol 154:304–311

Fraser PD, Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res 43:228–265

Fujii T, Nagasawa N, Iwamatsu A, Bogaki T, Tamai Y, Hamachi M (1994) Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene. Appl Environ Microbiol 60:2786–2792

Fujita M, Umemura M, Yoko-o T, Jigami Y (2006) *PER1* is required for GPI-phospholipase A_2 activity and involved in lipid remodeling of GPI-anchored proteins. Mol Biol Cell 17:5253–5264

Gable K, Slife H, Bacikova D, Monaghan E, Dunn TM (2000) Tsc3p is an 80-amino acid protein associated with serine palmitoyltransferase and required for optimal enzyme activity. J Biol Chem 275:7597–7603

Gaigg B, Timischl B, Corbino L, Schneiter R (2005) Synthesis of sphingolipids with very long chain fatty acids but not ergosterol is required for routing of newly synthesized plasma membrane ATPase to the cell surface of yeast. J Biol Chem 280:22515–22522

Gaigg B, Toulmay A, Schneiter R (2006) Very long-chain fatty acid-containing lipids rather than sphingolipids per se are required for raft association and stable surface transport of newly synthesized plasma membrane ATPase in yeast. J Biol Chem 281:34135–34145

García-López MC, Pelechano V, Mirón-García MC, Garrido-Godino AI, García A, Calvo O, Werner M, Pérez-Ortín JE, Navarro F (2011) The conserved foot domain of RNA pol II associates with proteins involved in transcriptional initiation and/or early elongation. Genetics 189:1235–1248

Georgiev AG, Sullivan DP, Kersting MC, Dittman JS, Beh CT, Menon AK (2011) Osh proteins regulate membrane sterol organization but are not required for sterol movement between the ER and PM. Traffic 12:1341–1355

Grilley MM, Stock SD, Dickson RC, Lester RL, Takemoto JY (1998) Syringomycin action gene *SYR2* is essential for sphingolipid 4-hydroxylation in *Saccharomyces cerevisiae*. J Biol Chem 273:11062–11068

Grillitsch K, Daum G (2011) Triacylglycerol lipases of the yeast. Front Biol 6:219–230

Grillitsch K, Connerth M, Köfeler H, Arrey TN, Rietschel B, Wagner B, Karas M, Daum G (2011) Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. Biochim Biophys Acta 1811:1165–1176

Guan XL, Souza CM, Pichler H, Dewhurst G, Schaad O, Kajiwara K, Wakabayashi H, Ivanova T, Castillon GA, Piccolis M et al (2009) Functional Interactions between Sphingolipids and Sterols in Biological Membranes Regulating Cell Physiology. Mol Biol Cell 20:2083–2095

Haak D, Gable K, Beeler T, Dunn T (1997) Hydroxylation of *Saccharomyces cerevisiae* ceramides requires Sur2p and Scs7p. J Biol Chem 272:29704–29710

Han G-S, Wu W-I, Carman GM (2006) The *Saccharomyces cerevisiae* Lipin homolog is a Mg^{2+}-dependent phosphatidate phosphatase enzyme. J Biol Chem 281:9210–9218

Han G-S, O'Hara L, Carman GM, Siniossoglou S (2008) An unconventional diacylglycerol kinase that regulates phospholipid synthesis and nuclear membrane growth. J Biol Chem 283:20433–20442

Hearn JD, Lester RL, Dickson RC (2003) The uracil transporter Fur4p associates with lipid rafts. J Biol Chem 278:3679–3686

Heiderpriem RW, Livant PD, Parish EJ, Barbuch RJ, Broaddus MG, Bard M (1992) A simple method for the isolation of zymosterol from a sterol mutant of *Saccharomyces cerevisiae*. J Steroid Biochem Mol Biol 43:741–743

Henry SA, Kohlwein SD, Carman GM (2012) Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. Genetics 190:317–349

Herrero O, Ramón D, Orejas M (2008) Engineering the *Saccharomyces cerevisiae* isoprenoid pathway for de novo production of aromatic monoterpenes in wine. Metab Eng 10:78–86

Hiltunen JK, Wenzel B, Beyer A, Erdmann R, Fosså A, Kunau WH (1992) Peroxisomal multifunctional beta-oxidation protein of *Saccharomyces cerevisiae*. Molecular analysis of the *FOX2* gene and gene product. J Biol Chem 267:6646–6653

Hiltunen JK, Autio KJ, Schonauer MS, Kursu VAS, Dieckmann CL, Kastaniotis AJ (2010) Mitochondrial fatty acid synthesis and respiration. Biochim Biophys Acta 1797:1195–1202

Hjelmstad RH, Bell RM (1992) Choline- and ethanolaminephosphotransferases from *Saccharomyces cerevisiae*. Meth Enzymol 209:272–279

Hoppins S, Collins SR, Cassidy-Stone A, Hummel E, Devay RM, Lackner LL, Westermann B, Schuldiner M, Weissman JS, Nunnari J (2011) A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. J Cell Biol 195:323–340

Horvath A, Sütterlin C, Manning-Krieg U, Movva NR, Riezman H (1994) Ceramide synthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast. EMBO J 13:3687–3695

Horvath SE, Wagner A, Steyrer E, Daum G (2011) Metabolic link between phosphatidylethanolamine and triacylglycerol metabolism in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1811:1030–1037

Horvath SE, Böttinger L, Vögtle F-N, Wiedemann N, Meisinger C, Becker T, Daum G (2012) Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1. J Biol Chem 287:36744–36755

Iwanyshyn WM, Han G-S, Carman GM (2004) Regulation of phospholipid synthesis in *Saccharomyces cerevisiae* by zinc. J Biol Chem 279:21976–21983

Jacquier N, Schneiter R (2012) Mechanisms of sterol uptake and transport in yeast. J Steroid Biochem Mol Biol 129:70–78

Jacquier N, Choudhary V, Mari M, Toulmay A, Reggiori F, Schneiter R (2011) Lipid droplets are functionally connected to the endoplasmic reticulum in *Saccharomyces cerevisiae*. J Cell Sci 124:2424–2437

Jahnke L, Klein HP (1983) Oxygen requirements for formation and activity of the squalene epoxidase in *Saccharomyces cerevisiae*. J Bacteriol 155:488–492

Jain S, Stanford N, Bhagwat N, Seiler B, Costanzo M, Boone C, Oelkers P (2007) Identification of a novel lysophospholipid acyltransferase in *Saccharomyces cerevisiae*. J Biol Chem 282:30562–30569

Janssen MJ, Koorengevel MC, de Kruijff B, de Kroon AI (2000) The phosphatidylcholine to phosphatidylethanolamine ratio of *Saccharomyces cerevisiae* varies with the growth phase. Yeast 16:641–650

Jiang F, Rizavi HS, Greenberg ML (1997) Cardiolipin is not essential for the growth of *Saccharomyces cerevisiae* on fermentable or non-fermentable carbon sources. Mol Microbiol 26:481–491

Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K (2006) Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J Clin Invest 116:1784–1792

Karpichev IV, Cornivelli L, Small GM (2002) Multiple regulatory roles of a novel *Saccharomyces cerevisiae* protein, encoded by *YOL002c*, in lipid and phosphate metabolism. J Biol Chem 277:19609–19617

Kennedy EP, Weiss SB (1956) The function of cytidine coenzymes in the biosynthesis of phospholipides. J Biol Chem 222:193–214

Kim K, Kim KH, Storey MK, Voelker DR, Carman GM (1999) Isolation and characterization of the *Saccharomyces cerevisiae EKI1* gene encoding ethanolamine kinase. J Biol Chem 274:14857–14866

Klose C, Surma MA, Gerl MJ, Meyenhofer F, Shevchenko A, Simons K (2012) Flexibility of a eukaryotic lipidome–insights from yeast lipidomics. PLoS ONE 7:e35063

Kobayashi SD, Nagiec MM (2003) Ceramide/long-chain base phosphate rheostat in *Saccharomyces cerevisiae*: regulation of ceramide synthesis by Elo3p and Cka2p. Eukaryot Cell 2:284–294

Köffel R, Tiwari R, Falquet L, Schneiter R (2005) The *Saccharomyces cerevisiae YLL012/YEH1*, *YLR020/YEH2*, and *TGL1* genes encode a novel family of membrane-anchored lipases that are required for steryl ester hydrolysis. Mol Cell Biol 25:1655–1668

Kohlwein SD (2010) Triacylglycerol homeostasis: insights from yeast. J Biol Chem 285:15663–15667

Kohlwein SD, Veenhuis M, van der Klei IJ (2012) Lipid droplets and peroxisomes: key players in cellular lipid homeostasis or a matter of fat-store 'em up or burn 'em down. Genetics 193:1–50

Kolaczkowski M, Kolaczkowska A, Gaigg B, Schneiter R, Moye-Rowley WS (2004) Differential regulation of ceramide synthase components *LAC1* and *LAG1* in *Saccharomyces cerevisiae*. Eukaryot Cell 3:880–892

Kopec KO, Alva V, Lupas AN (2010) Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria. Bioinformatics 26:1927–1931

Kornmann B, Currie E, Collins SR, Schuldiner M, Nunnari J, Weissman JS, Walter P (2009) An ER-mitochondria tethering complex revealed by a synthetic biology screen. Science 325:477–481

Kornmann B, Osman C, Walter P (2011) The conserved GTPase Gem1 regulates endoplasmic reticulum-mitochondria connections. Proc Natl Acad Sci USA 108:14151–14156

Kristan K, Rižner TL (2012) Steroid-transforming enzymes in fungi. J Steroid Biochem Mol Biol 129:79–91

Ktistakis NT (2010) Lipid signaling and homeostasis: PA- is better than PA-H, but what about those PIPs? Sci Signal 3:pe46

Kuchler K, Daum G, Paltauf F (1986) Subcellular and submitochondrial localization of phospholipid-synthesizing enzymes in *Saccharomyces cerevisiae*. J Bacteriol 165:901–910

Kupchak BR, Garitaonandia I, Villa NY, Mullen MB, Weaver MG, Regalla LM, Kendall EA, Lyons TJ (2007) Probing the mechanism of *FET3* repression by Izh2p overexpression. Biochim Biophys Acta 1773:1124–1132

Kuranda K, François J, Palamarczyk G (2010) The isoprenoid pathway and transcriptional response to its inhibitors in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 10:14–27

Kurat CF, Natter K, Petschnigg J, Wolinski H, Scheuringer K, Scholz H, Zimmermann R, Leber R, Zechner R, Kohlwein SD (2006) Obese yeast: triglyceride lipolysis is functionally conserved from mammals to yeast. J Biol Chem 281:491–500

Kurat CF, Wolinski H, Petschnigg J, Kaluarachchi S, Andrews B, Natter K, Kohlwein SD (2009) Cdk1/Cdc28-dependent activation of the major triacylglycerol lipase Tgl4 in yeast links lipolysis to cell-cycle progression. Mol Cell 33:53–63

Kuroda T, Tani M, Moriguchi A, Tokunaga S, Higuchi T, Kitada S, Kuge O (2011) *FMP30* is required for the maintenance of a normal cardiolipin level and mitochondrial morphology in the absence of mitochondrial phosphatidylethanolamine synthesis. Mol Microbiol 80:248–265

Lauwers E, Grossmann G, André B (2007) Evidence for coupled biogenesis of yeast Gap1 permease and sphingolipids: essential role in transport activity and normal control by ubiquitination. Mol Biol Cell 18:3068–3080

Leber R, Zinser E, Zellnig G, Paltauf F, Daum G (1994) Characterization of lipid particles of the yeast, *Saccharomyces cerevisiae*. Yeast 10:1421–1428

Leber R, Landl K, Zinser E, Ahorn H, Spök A, Kohlwein SD, Turnowsky F, Daum G (1998) Dual localization of squalene epoxidase, Erg1p, in yeast reflects a relationship between the endoplasmic reticulum and lipid particles. Mol Biol Cell 9:375–386

Lee SY, Kim HU, Park JH, Park JM, Kim TY (2009) Metabolic engineering of microorganisms: general strategies and drug production. Drug Discov Today 14:78–88

Lees ND, Skaggs B, Kirsch DR, Bard M (1995) Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae*–a review. Lipids 30:221–226

Linder ME, Deschenes RJ (2004) Model organisms lead the way to protein palmitoyltransferases. J Cell Sci 117:521–526

Lingwood D, Simons K (2010) Lipid rafts as a membrane-organizing principle. Science 327:46–50

Loewen CJR, Gaspar ML, Jesch SA, Delon C, Ktistakis NT, Henry SA, Levine TP (2004) Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. Science 304:1644–1647

London E, Brown DA (2000) Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). Biochim Biophys Acta 1508:182–195

Lorenz RT, Casey WM, Parks LW (1989) Structural discrimination in the sparking function of sterols in the yeast *Saccharomyces cerevisiae*. J Bacteriol 171:6169–6173

Lyons TJ, Villa NY, Regalla LM, Kupchak BR, Vagstad A, Eide DJ (2004) Metalloregulation of yeast membrane steroid receptor homologs. Proc Natl Acad Sci USA 101:5506–5511

Madhavan A, Srivastava A, Kondo A, Bisaria VS (2012) Bioconversion of lignocellulose-derived sugars to ethanol by engineered *Saccharomyces cerevisiae*. Crit Rev Biotechnol 32:22–48

Mao C, Xu R, Bielawska A, Szulc ZM, Obeid LM (2000a) Cloning and characterization of a *Saccharomyces cerevisiae* alkaline ceramidase with specificity for dihydroceramide. J Biol Chem 275:31369–31378

Mao C, Xu R, Bielawska A, Obeid LM (2000b) Cloning of an alkaline ceramidase from *Saccharomyces cerevisiae*. An enzyme with reverse (CoA-independent) ceramide synthase activity. J Biol Chem 275:6876–6884

Matmati N, Hannun YA (2008) Thematic review series: sphingolipids. *ISC1* (inositol phosphosphingolipid-phospholipase C), the yeast homologue of neutral sphingomyelinases. J Lipid Res 49:922–928

Meisinger C, Pfannschmidt S, Rissler M, Milenkovic D, Becker T, Stojanovski D, Youngman MJ, Jensen RE, Chacinska A, Guiard B et al (2007) The morphology proteins Mdm12/Mmm1 function in the major beta-barrel assembly pathway of mitochondria. EMBO J 26:2229–2239

Merkel O, Schmid PC, Paltauf F, Schmid HHO (2005) Presence and potential signaling function of N-acylethanolamines and their phospholipid precursors in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1734:215–219

Mesmin B, Maxfield FR (2009) Intracellular sterol dynamics. Biochimica et Biophysica Acta (BBA)-Molecular Cell Biol Lipids 1791:636–645

Mignery GA, Pikaard CS, Park WD (1988) Molecular characterization of the patatin multigene family of potato. Gene 62:27–44

Misawa N (2011) Pathway engineering for functional isoprenoids. Curr Opin Biotechnol 22:627–633

Moir RD, Gross DA, Silver DL, Willis IM (2012) *SCS3* and *YFT2* link transcription of phospholipid biosynthetic genes to ER stress and the UPR. PLoS Genet 8:e1002890

Mora G, Scharnewski M, Fulda M (2012) Neutral lipid metabolism influences phospholipid synthesis and deacylation in *Saccharomyces cerevisiae*. PLoS ONE 7:e49269

Müllner H, Zweytick D, Leber R, Turnowsky F, Daum G (2004) Targeting of proteins involved in sterol biosynthesis to lipid particles of the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1663:9–13

Müllner H, Deutsch G, Leitner E, Ingolic E, Daum G (2005) *YEH2*/*YLR020c* encodes a novel steryl ester hydrolase of the yeast *Saccharomyces cerevisiae*. J Biol Chem 280:13321–13328

Murphy DJ, Vance J (1999) Mechanisms of lipid-body formation. Trends Biochem Sci 24:109–115

Nadolski MJ, Linder ME (2007) Protein lipidation. FEBS J 274:5202–5210

Nagasawa N, Bogaki T, Iwamatsu A, Hamachi M, Kumagai C (1998) Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (*ATF2*) from *Saccharomyces cerevisiae* Kyokai No. 7. Biosci Biotechnol Biochem 62:1852–1857

Nagiec MM, Baltisberger JA, Wells GB, Lester RL, Dickson RC (1994) The *LCB2* gene of Saccharomyces and the related LCB1 gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis. Proc Natl Acad Sci USA 91:7899–7902

Nagiec MM, Nagiec EE, Baltisberger JA, Wells GB, Lester RL, Dickson RC (1997) Sphingolipid synthesis as a target for antifungal drugs. Complementation of the inositol phosphorylceramide synthase defect in a mutant strain of *Saccharomyces cerevisiae* by the *AUR1* gene. J Biol Chem 272:9809–9817

Nagiec MM, Skrzypek M, Nagiec EE, Lester RL, Dickson RC (1998) The *LCB4* (*YOR171c*) and *LCB5* (*YLR260w*) genes of *Saccharomyces* encode sphingoid long chain base kinases. J Biol Chem 273:19437–19442

Natter K, Kohlwein SD (2012) Yeast and cancer cells—common principles in lipid metabolism. Biochim Biophys Acta 1831:314–326

Natter K, Leitner P, Faschinger A, Wolinski H, McCraith S, Fields S, Kohlwein SD (2005) The spatial organization of lipid synthesis in the yeast *Saccharomyces cerevisiae* derived from large scale green fluorescent protein tagging and high resolution microscopy. Mol Cell Proteomics 4:662–672

Nes WD, Janssen GG, Crumley FG, Kalinowska M, Akihisa T (1993) The structural requirements of sterols for membrane function in *Saccharomyces cerevisiae*. Arch Biochem Biophys 300:724–733

Nguyen TT, Lewandowska A, Choi J-Y, Markgraf DF, Junker M, Bilgin M, Ejsing CS, Voelker DR, Rapoport TA, Shaw JM (2012) Gem1 and ERMES do not directly affect phosphatidylserine transport from ER to mitochondria or mitochondrial inheritance. Traffic 13:880–890

Nikawa J, Tsukagoshi Y, Yamashita S (1991) Isolation and characterization of two distinct myo-inositol transporter genes of *Saccharomyces cerevisiae*. J Biol Chem 266:11184–11191

Niles BJ, Powers T (2012) Plasma membrane proteins Slm1 and Slm2 mediate activation of the AGC kinase Ypk1 by TORC2 and sphingolipids in *S. cerevisiae*. Cell Cycle 11:3745–3749

O'Hara L, Han G-S, Peak-Chew S, Grimsey N, Carman GM, Siniossoglou S (2006) Control of phospholipid synthesis by phosphorylation of the yeast lipin Pah1p/Smp2p Mg^{2+}-dependent phosphatidate phosphatase. J Biol Chem 281:34537–34548

Opekarová M, Tanner W (2003) Specific lipid requirements of membrane proteins–a putative bottleneck in heterologous expression. Biochim Biophys Acta 1610:11–22

Osman C, Haag M, Wieland FT, Brügger B, Langer T (2010) A mitochondrial phosphatase required for cardiolipin biosynthesis: the PGP phosphatase Gep4. EMBO J 29:1976–1987

Ostergaard S, Olsson L, Nielsen J (2000) Metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 64:34–50

Panwar SL, Moye-Rowley WS (2006) Long chain base tolerance in *Saccharomyces cerevisiae* is induced by retrograde signals from the mitochondria. J Biol Chem 281:6376–6384

Pascual F, Carman GM (2013) Phosphatidate phosphatase, a key regulator of lipid homeostasis. Biochim Biophys Acta 1831:514–522

Pichler H (2005) Modulating membrane properties-sterol cell biology in yeast. In: Daum G (ed) Cell biology and dynamics of yeast lipids, Research Signpost, Trivandrum (Kerala), pp. 179–199

Ploegh HL (2007) A lipid-based model for the creation of an escape hatch from the endoplasmic reticulum. Nature 448:435–438

Ploier B, Scharwey M, Koch B, Schmidt C, Schatte J, Rechberger G et al (2013) Screening for hydrolytic enzymes reveals Ayr1p as a novel triacylglycerol lipase in *Saccharomyces cerevisiae*. J Biol Chem 288:36061–36072

Poirier Y, Antonenkov VD, Glumoff T, Hiltunen JK (2006) Peroxisomal β-oxidation–a metabolic pathway with multiple functions. Biochim Biophys Acta 1763:1413–1426

Polakowski T, Stahl U, Lang C (1998) Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Appl Microbiol Biotechnol 49:66–71

Potting C, Wilmes C, Engmann T, Osman C, Langer T (2010) Regulation of mitochondrial phospholipids by Ups1/PRELI-like proteins depends on proteolysis and Mdm35. EMBO J 29:2888–2898

Pu J, Ha CW, Zhang S, Jung JP, Huh W-K, Liu P (2011) Interactomic study on interaction between lipid droplets and mitochondria. Protein Cell 2:487–496

Rajakumari S, Daum G (2010a) Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions. Mol Biol Cell 21:501–510

Rajakumari S, Daum G (2010b) Multiple functions as lipase, steryl ester hydrolase, phospholipase, and acyltransferase of Tgl4p from the yeast *Saccharomyces cerevisiae*. J Biol Chem 285:15769–15776

Rajakumari S, Grillitsch K, Daum G (2008) Synthesis and turnover of non-polar lipids in yeast. Prog Lipid Res 47:157–171

Rajakumari S, Rajasekharan R, Daum G (2010) Triacylglycerol lipolysis is linked to sphingolipid and phospholipid metabolism of the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1801:1314–1322

Raychaudhuri S, Young BP, Espenshade PJ, Loewen C Jr (2012) Regulation of lipid metabolism: a tale of two yeasts. Curr Opin Cell Biol 24:502–508

Rebecchi MJ, Pentyala SN (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. Physiol Rev 80:1291–1335

Reue K, Brindley DN (2008) Thematic Review Series: Glycerolipids. Multiple roles for lipins/phosphatidate phosphatase enzymes in lipid metabolism. J Lipid Res 49:2493–2503

Reue K, Dwyer JR (2009) Lipin proteins and metabolic homeostasis. J Lipid Res 50(Suppl):S109–S114

Riad M, Mogos M, Thangathurai D, Lumb PD (2002) Steroids. Curr Opin Crit Care 8:281–284

Riekhof WR, Voelker DR (2006) Uptake and utilization of lyso-phosphatidylethanolamine by *Saccharomyces cerevisiae*. J Biol Chem 281:36588–36596

Riekhof WR, Wu J, Jones JL, Voelker DR (2007) Identification and characterization of the major lysophosphatidylethanolamine acyltransferase in *Saccharomyces cerevisiae*. J Biol Chem 282:28344–28352

Roelants FM, Breslow DK, Muir A, Weissman JS, Thorner J (2011) Protein kinase Ypk1 phosphorylates regulatory proteins Orm1 and Orm2 to control sphingolipid homeostasis in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 108:19222–19227

Rudge SA, Morris AJ, Engebrecht J (1998) Relocalization of phospholipase D activity mediates membrane formation during meiosis. J Cell Biol 140:81–90

Ruenwai R, Neiss A, Laoteng K, Vongsangnak W, Dalfard AB, Cheevadhanarak S, Petranovic D, Nielsen J (2011) Heterologous production of polyunsaturated fatty acids in *Saccharomyces cerevisiae* causes a global transcriptional response resulting in reduced proteasomal activity and increased oxidative stress. Biotechnol J 6:343–356

Saba JD, Nara F, Bielawska A, Garrett S, Hannun YA (1997) The *BST1* gene of *Saccharomyces cerevisiae* is the sphingosine-1-phosphate lyase. J Biol Chem 272:26087–26090

Saerens SMG, Verstrepen KJ, Van Laere SDM, Voet ARD, Van Dijck P, Delvaux FR, Thevelein JM (2006) The *Saccharomyces cerevisiae EHT1* and *EEB1* genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. J Biol Chem 281:4446–4456

Saerens SMG, Delvaux FR, Verstrepen KJ, Thevelein JM (2010) Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb Biotechnol 3:165–177

Sajbidor J, Ciesarová Z, Smogrovicová D (1995) Influence of ethanol on the lipid content and fatty acid composition of *Saccharomyces cerevisiae*. Folia Microbiol (Praha) 40:508–510

Santos AXS, Riezman H (2012) Yeast as a model system for studying lipid homeostasis and function. FEBS Lett 586:2858–2867

Sasser T, Qiu Q-S, Karunakaran S, Padolina M, Reyes A, Flood B, Smith S, Gonzales C, Fratti RA (2012) Yeast lipin 1 orthologue pah1p regulates vacuole homeostasis and membrane fusion. J Biol Chem 287:2221–2236

Sawai H, Okamoto Y, Luberto C, Mao C, Bielawska A, Domae N, Hannun YA (2000) Identification of *ISC1* (*YER019w*) as inositol phosphosphingolipid phospholipase C in *Saccharomyces cerevisiae*. J Biol Chem 275:39793–39798

Schneiter R, Brügger B, Sandhoff R, Zellnig G, Leber A, Lampl M, Athenstaedt K, Hrastnik C, Eder S, Daum G et al (1999) Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of the lipid molecular species composition of yeast subcellular membranes reveals acyl chain-based sorting/remodeling of distinct molecular species en route to the plasma membrane. J Cell Biol 146:741–754

Schorling S, Vallée B, Barz WP, Riezman H, Oesterhelt D (2001) Lag1p and Lac1p are essential for the Acyl-CoA-dependent ceramide synthase reaction in *Saccharomyces cerevisae*. Mol Biol Cell 12:3417–3427

Schrag JD, Cygler M (1997) Lipases and alpha/beta hydrolase fold. Meth Enzymol 284:85–107

Schulz TA, Prinz WA (2007) Sterol transport in yeast and the oxysterol binding protein homologue (OSH) family. Biochimica et Biophysica Acta (BBA)-Molecular Cell Biol Lipids 1771:769–780

Shang F, Wen S, Wang X, Tan T (2006) Effect of nitrogen limitation on the ergosterol production by fed-batch culture of *Saccharomyces cerevisiae*. J Biotechnol 122:285–292

Shen H, Heacock PN, Clancey CJ, Dowhan W (1996) The *CDS1* gene encoding CDP-diacylglycerol synthase in *Saccharomyces cerevisiae* is essential for cell growth. J Biol Chem 271:789–795

Simocková M, Holic R, Tahotná D, Patton-Vogt J, Griac P (2008) Yeast Pgc1p (Ypl206c) controls the amount of phosphatidylglycerol via a phospholipase C-type degradation mechanism. J Biol Chem 283:17107–17115

Simons K, Sampaio JL (2011) Membrane organization and lipid rafts. Cold Spring Harb Perspect Biol 3:a004697

Siniossoglou S, Santos-Rosa H, Rappsilber J, Mann M, Hurt E (1998) A novel complex of membrane proteins required for formation of a spherical nucleus. EMBO J 17:6449–6464

Skrzypek M, Lester RL, Dickson RC (1997) Suppressor gene analysis reveals an essential role for sphingolipids in transport of glycosylphosphatidylinositol-anchored proteins in *Saccharomyces cerevisiae*. J Bacteriol 179:1513–1520

Souza CM, Pichler H (2007) Lipid requirements for endocytosis in yeast. Biochim Biophys Acta 1771:442–454

Spanova M, Daum G (2011) Squalene—biochemistry, molecular biology, process biotechnology, and applications. Eur J Lipid Sci Technol 113:1299–1320

Spanova M, Czabany T, Zellnig G, Leitner E, Hapala I, Daum G (2010) Effect of lipid particle biogenesis on the subcellular distribution of squalene in the yeast *Saccharomyces cerevisiae*. J Biol Chem 285:6127–6133

Spanova M, Zweytick D, Lohner K, Klug L, Leitner E, Hermetter A, Daum G (2012) Influence of squalene on lipid particle/droplet and membrane organization in the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1821:647–653

Sreenivas A, Patton-Vogt JL, Bruno V, Griac P, Henry SA (1998) A role for phospholipase D (Pld1p) in growth, secretion, and regulation of membrane lipid synthesis in yeast. J Biol Chem 273:16635–16638

Strahl T, Thorner J (2007) Synthesis and function of membrane phosphoinositides in budding yeast, *Saccharomyces cerevisiae*. Biochim Biophys Acta 1771:353–404

Stroud DA, Oeljeklaus S, Wiese S, Bohnert M, Lewandrowski U, Sickmann A, Guiard B, van der Laan M, Warscheid B, Wiedemann N (2011) Composition and topology of the endoplasmic reticulum-mitochondria encounter structure. J Mol Biol 413:743–750

Stukey JE, McDonough VM, Martin CE (1990) The *OLE1* gene of *Saccharomyces cerevisiae* encodes the Δ9–fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. J Biol Chem 265:20144–20149

Sumby KM, Grbin PR, Jiranek V (2010) Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. Food Chem 121:1–16

Sun Y, Taniguchi R, Tanoue D, Yamaji T, Takematsu H, Mori K, Fujita T, Kawasaki T, Kozutsumi Y (2000) Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. Mol Cell Biol 20:4411–4419

Tamura Y, Iijima M, Sesaki H (2010) Mdm35p imports Ups proteins into the mitochondrial intermembrane space by functional complex formation. EMBO J 29:2875–2887

Tamura Y, Onguka O, Hobbs AEA, Jensen RE, Iijima M, Claypool SM, Sesaki H (2012) Role for two conserved intermembrane space proteins, Ups1p and Ups2p, in intra-mitochondrial phospholipid trafficking. J Biol Chem 287:15205–15218

Tanaka S, Maeda Y, Tashima Y, Kinoshita T (2004) Inositol deacylation of glycosylphosphatidylinositol-anchored proteins is mediated by mammalian PGAP1 and yeast Bst1p. J Biol Chem 279:14256–14263

Tehlivets O, Scheuringer K, Kohlwein SD (2007) Fatty acid synthesis and elongation in yeast. Biochim Biophys Acta 1771:255–270

Tevzadze GG, Pierce JV, Esposito RE (2007) Genetic evidence for a *SPO1*-dependent signaling pathway controlling meiotic progression in yeast. Genetics 175:1213–1227

Thoms S, Debelyy MO, Nau K, Meyer HE, Erdmann R (2008) Lpx1p is a peroxisomal lipase required for normal peroxisome morphology. FEBS J 275:504–514

Thoms S, Debelyy MO, Connerth M, Daum G, Erdmann R (2011) The putative *Saccharomyces cerevisiae* hydrolase Ldh1p is localized to lipid droplets. Eukaryot Cell 10:770–775

Tinkelenberg AH, Liu Y, Alcantara F, Khan S, Guo Z, Bard M, Sturley SL (2000) Mutations in yeast *ARV1* alter intracellular sterol distribution and are complemented by human *ARV1*. J Biol Chem 275:40667–40670

Tiwari R, Köffel R, Schneiter R (2007) An acetylation/deacetylation cycle controls the export of sterols and steroids from *S. cerevisiae*. EMBO J 26:5109–5119

Toth MJ, Huwyler L (1996) Molecular cloning and expression of the cDNAs encoding human and yeast mevalonate pyrophosphate decarboxylase. J Biol Chem 271:7895–7898

Trotter PJ, Voelker DR (1995) Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (*PSD2*) in the yeast *Saccharomyces cerevisiae*. J Biol Chem 270:6062–6070

Tuller G, Hrastnik C, Achleitner G, Schiefthaler U, Klein F, Daum G (1998) *YDL142c* encodes cardiolipin synthase (Cls1p) and is non-essential for aerobic growth of *Saccharomyces cerevisiae*. FEBS Lett 421:15–18

Uemura H (2012) Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: current state and perspectives. Appl Microbiol Biotechnol 95:1–12

Uemura S, Kihara A, Inokuchi J-I, Igarashi Y (2003) Csg1p and newly identified Csh1p function in mannosylinositol phosphorylceramide synthesis by interacting with Csg2p. J Biol Chem 278:45049–45055

Vallée B, Riezman H (2005) Lip1p: a novel subunit of acyl-CoA ceramide synthase. EMBO J 24:730–741

Van der Laan M, Bohnert M, Wiedemann N, Pfanner N (2012) Role of MINOS in mitochondrial membrane architecture and biogenesis. Trends Cell Biol 22:185–192
Van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9:112–124
Van Roermund CW, Elgersma Y, Singh N, Wanders RJ, Tabak HF (1995) The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. EMBO J 14:3480–3486
Van Roermund CW, Drissen R, van Den Berg M, Ijlst L, Hettema EH, Tabak HF, Waterham HR, Wanders RJ (2001) Identification of a peroxisomal ATP carrier required for medium-chain fatty acid beta-oxidation and normal peroxisome proliferation in *Saccharomyces cerevisiae*. Mol Cell Biol 21:4321–4329
Van Roermund CWT, Ijlst L, Majczak W, Waterham HR, Folkerts H, Wanders RJA, Hellingwerf KJ (2012) Peroxisomal fatty acid uptake mechanism in *Saccharomyces cerevisiae*. J Biol Chem 287:20144–20153
Veen M, Lang C (2004) Production of lipid compounds in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 63:635–646
Veen M, Stahl U, Lang C (2003) Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in *Saccharomyces cerevisiae*. FEMS Yeast Res 4:87–95
Vehring S and Pomorski T (2005) Dynamics and regulation of phospholipid distribution in the yeast. In: Daum G (ed) Cell biology and dynamics of yeast lipids, Research Signpost, Trivandrum (Kerala), pp. 85–104
Verger R (1997) "Interfacial activation" of lipases: facts and artifacts. Trends Biotechnol 15:32–38
Verstrepen KJ, Van Laere SDM, Vercammen J, Derdelinckx G, Dufour J-P, Pretorius IS, Winderickx J, Thevelein JM, Delvaux FR (2004) The *Saccharomyces cerevisiae* alcohol acetyl transferase Atf1p is localized in lipid particles. Yeast 21:367–377
Verwaal R, Wang J, Meijnen JP, Visser H, Sandmann G, van den Berg JA, van Ooyen AJJ (2007) High-level production of beta-carotene in*Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from Xanthophyllomyces dendrorhous. Appl Environ Microbiol 73:4342–4350
Voelker DR (2003) New perspectives on the regulation of intermembrane glycerophospholipid traffic. J Lipid Res 44:441–449
Wagner A, Grillitsch K, Leitner E, Daum G (2009) Mobilization of steryl esters from lipid particles of the yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 1791:118–124
Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J Am Chem Soc 93:2325–2327
Wilcox LJ, Balderes DA, Wharton B, Tinkelenberg AH, Rao G, Sturley SL (2002) Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. J Biol Chem 277:32466–32472
Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H et al (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 285:901–906
Wolinski H, Kolb D, Hermann S, Koning RI, Kohlwein SD (2011) A role for seipin in lipid droplet dynamics and inheritance in yeast. J Cell Sci 124:3894–3904
Wriessnegger T, Pichler H (2013) Yeast metabolic engineering—Targeting sterol metabolism and terpenoid formation. Prog Lipid Res 52:277–293
Wu WI, Carman GM (1994) Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by nucleotides. J Biol Chem 269:29495–29501
Wu WI, Carman GM (1996) Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by phospholipids. Biochemistry 35:3790–3796

Wu WI, Lin YP, Wang E, Merrill AH Jr, Carman GM (1993) Regulation of phosphatidate phosphatase activity from the yeast *Saccharomyces cerevisiae* by sphingoid bases. J Biol Chem 268:13830–13837

Xue Z, Sharpe PL, Hong SP, Yadav NS, Xie D, Short DR, Damude HG, Rupert RA, Seip JE, Wang J, Pollak DW, Bostick MW, Bosak MD, Macool DJ, Hollerbach DH, Zhang H, Arcilla DM, Bledsoe SA, Croker K, McCord EF, Tyreus BD, Jackson EN, Zhu Q (2013) Production of omega-3 eicosapentaenoic acid by metabolic engineering of *Yarrowia lipolytica*. Nat Biotechnol 31:734–740

Yamano S, Ishii T, Nakagawa M, Ikenaga H, Misawa N (1994) Metabolic engineering for production of beta-carotene and lycopene in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 58:1112–1114

Yang H, Bard M, Bruner DA, Gleeson A, Deckelbaum RJ, Aljinovic G, Pohl TM, Rothstein R, Sturley SL (1996) Sterol esterification in yeast: a two-gene process. Science 272:1353–1356

Yazawa H, Iwahashi H, Kamisaka Y, Kimura K, Aki T, Ono K, Uemura H (2007) Heterologous production of dihomo-gamma-linolenic acid in *Saccharomyces cerevisiae*. Appl Environ Microbiol 73:6965–6971

York JD (2006) Regulation of nuclear processes by inositol polyphosphates. Biochim Biophys Acta 1761:552–559

Yu C, Kennedy NJ, Chang CC, Rothblatt JA (1996) Molecular cloning and characterization of two isoforms of *Saccharomyces cerevisiae* acyl-CoA:sterol acyltransferase. J Biol Chem 271:24157–24163

Yu X, Zheng Y, Dorgan KM, Chen S (2011) Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresour Technol 102:6134–6140

Zanghellini J, Natter K, Jungreuthmayer C, Thalhammer A, Kurat CF, Gogg-Fassolter G, Kohlwein SD, von Grünberg H-H (2008) Quantitative modeling of triacylglycerol homeostasis in yeast–metabolic requirement for lipolysis to promote membrane lipid synthesis and cellular growth. FEBS J 275:5552–5563

Zerbes RM, Bohnert M, Stroud DA, von der Malsburg K, Kram A, Oeljeklaus S, Warscheid B, Becker T, Wiedemann N, Veenhuis M et al (2012) Role of MINOS in mitochondrial membrane architecture: cristae morphology and outer membrane interactions differentially depend on mitofilin domains. J Mol Biol 422:183–191

Zhao X, Hu C, Wu S, Shen H, Zhao ZK (2011) Lipid production by Rhodosporidium toruloides Y4 using different substrate feeding strategies. J Ind Microbiol Biotechnol 38:627–632

Zinser E, Daum G (1995) Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*. Yeast 11:493–536

Zinser E, Sperka-Gottlieb CD, Fasch EV, Kohlwein SD, Paltauf F, Daum G (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. J Bacteriol 173:2026–2034

Zinser E, Paltauf F, Daum G (1993) Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. J Bacteriol 175:2853–2858

Zweytick D, Leitner E, Kohlwein SD, Yu C, Rothblatt J, Daum G (2000) Contribution of Are1p and Are2p to steryl ester synthesis in the yeast *Saccharomyces cerevisiae*. Eur J Biochem 267:1075–1082

Chapter 9
Molecular Mechanisms in Yeast Carbon Metabolism: Bioethanol and Other Biofuels

Volkmar Passoth

Abstract Biofuels, such as ethanol, biodiesel and biogas, have the potential to replace a large proportion of transportation fuels that presently are mainly produced from fossil raw materials. Bioethanol, which is the product of the fermentative energy metabolism of yeasts, is currently the major biofuel on the global market. It is to a large extent generated from first-generation substrates, i.e. food grade raw materials. There are huge research efforts to develop ethanol processes based on non-food lignocellulosic materials. Using—omics technologies, metabolic and evolutionary engineering, strains of, predominantly, *Saccharomyces cerevisiae* have been isolated that display enhanced inhibitor and general stress tolerance, lowered glycerol production and a broadened substrate spectrum (including the fermentation of pentose sugars released from hemicellulose). Expression of these features in industrial isolates may within a relatively short time generate strains robust enough for commercial ethanol production from lignocellulose. *S. cerevisiae* has also been modified to produce the advanced biofuel butanol. Although yields and production rates are still below the threshold for industrial applications, tools for further developments are now available. Biodiesel production by either oleaginous yeast species that can naturally accumulate high amounts of lipids or by genetically engineered *S. cerevisiae* are further examples of how yeasts can be used for biofuel production. Sustainable production of biofuels requires the integration of all steps of handling biomass, including preservation, pretreatment, fermentation and conversion of side products into high value compounds. In all these steps, yeasts have great technological potential.

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_9, © Springer-Verlag Berlin Heidelberg 2014

9.1 Introduction

Biofuels are reduced organic compounds, whose oxidation energy is used for heating, producing electricity and running combustion engines, mainly for transportation. The metabolic activity of photosynthetic organisms is the basis for both the origin of fossil fuels and biofuel generation, but, in contrast to fossil fuels, biofuels are generated from renewable biomass. Currently, bioethanol, biogas and biodiesel are those biofuels that are commercially used in transportation. However, the present global society primarily runs on fossil fuels; biofuels represent less than 4 % of the total global transportation energy. To move away from this large-scale consumption of fossil resources requires both, measures to reduce the global energy consumption and a considerable development of sustainable energy technologies (Cheng and Timilsina 2011; Vanholme et al. 2013). Yeasts are able to produce a variety of reduced organic molecules and have good potential to play a central role in these developments (Nielsen et al. 2013).

Bioethanol is the major biofuel on the global market (Cheng and Timilsina 2011; Caspeta et al. 2013; Amorim et al. 2011). It is mainly produced by the yeast *Saccharomyces cerevisiae* as the final product of fermentative sugar metabolism. The production of ethanol is one of the mankind's oldest biotechnologies. Ethanol has had a major impact on the development of human civilisation as a beverage component, conservation agent and drug (Vallee 1998; McGovern et al. 2004). It also has a history as a transportation fuel. The combustion engine developed by Nikolaus Otto in 1860 ran on ethanol, and, similarly, cars developed by Henry Ford at the end of the nineteenth and the beginning of the twentieth centuries could be driven with ethanol. However, at the beginning of the twentieth century, ethanol was no longer competitive with the relatively cheap gasoline made from mineral oil. In the following years, the interest in ethanol as a fuel declined, except on certain occasions when the supply with mineral oil was perturbed. During the oil crisis of the 1970s, the interest in ethanol as a fuel increased again, but with the exception of Brazil, this interest decreased with decreasing oil prices. Only towards the end of the twentieth and the beginning of the twenty-first centuries did ethanol became increasingly regarded as an alternative motor fuel, at first because it can replace lead-containing compounds as an octane booster, but nowadays, more and more because of growing concerns about the environmental impact of using fossil fuels and issues of supply and access (Solomon et al. 2007; Gnansounou 2010).

Considering the long history of ethanol production, it might be surprising that there is still a huge demand for research in this field. However, biofuel production has been debated during recent years, mainly because of low efficiency of production, low or occasionally negative impact on greenhouse gas and fossil fuel balance, and potential conflicts between food and energy production (Caspeta et al. 2013). New substrates derived from second-generation, lignocellulosic materials must be introduced into the production process to address these shortcomings. Lignocellulose provides the most abundant biomass resource on earth, and the

amount of lignocellulose produced by land plants has been estimated to be about 10–200 $\times$ 10^9 t per year (Vanholme et al. 2013). However, due to its recalcitrance and its heterogenic composition, conversion of lignocellulose into biofuels requires new methods of pretreatment and modified yeast strains. Newly developed methods of metabolic analyses and metabolic engineering are providing a variety of opportunities for optimising ethanol production (Nielsen et al. 2013; Van Vleet and Jeffries 2009). Even ethanol production from first-generation substrates could still be greatly improved by identifying optimal strains, metabolic engineering and optimising fermentation conditions (Amorim et al. 2011; Nielsen et al. 2013).

Apart from ethanol production, research efforts have also focused on producing biofuels such as biodiesel or biobutanol with the help of yeasts. Microbial biodiesel could overcome the low energy yield per hectare obtained from oil plants, and if produced from lignocellulose a food versus fuel conflict might be avoided (Caspeta et al. 2013). Biodiesel production can be achieved either by oleaginous yeasts, which can accumulate lipids to more than 30 % of their biomass, or by genetically engineered *S. cerevisiae*. Investigation and manipulation of lipid metabolism in yeasts will also provide new insights into the carbon metabolism of yeasts, as pathways towards lipid accumulation require oxygen, in contrast to the well-investigated alcoholic fermentation (Buijs et al. 2013; Ratledge and Wynn 2002).

Butanol, compared to ethanol, has a higher energy density, can be better blended with gasoline and is less hygroscopic. In 2008, 2.8 million t were produced, corresponding to a market value of about 5 billion US dollars. Most butanol is currently produced by chemical synthesis from mineral oil compounds. A fermentative process based on solventogenic clostridia was commercialised already in 1912, but is currently not competitive with chemical synthesis (Green 2011). Several approaches for producing biobutanol with genetically engineered yeasts have been developed (Buijs et al. 2013).

Sustainable conversion of biomass into biofuels and chemicals requires the integration of production, storage, pretreatment, processing of the feedstock in a biorefinery, treatment and generating value from the remnant feedstock (Vanholme et al. 2013). Yeasts can play important roles in such processes. This chapter aims to provide a survey on the efforts to understand and manipulate the yeast carbon metabolism to develop ethanol, butanol and biodiesel production for a biofuel refinery.

9.2 Ethics of Biofuel Production: Food Versus Fuel?

Biofuels are seen as a step towards a more sustainable society that is less dependent on fossil raw materials and that produces less or no surplus greenhouse gases (Cheng and Timilsina 2011; Vanholme et al. 2013; Solomon et al. 2007). However, production of biofuels, as well as human food, is based on plant

biomass. Recent years have seen an increased demand for biofuels and, in parallel, a dramatic increase in worldwide food prices. Moreover, there are examples where the effect of biofuel production on saving fossil resources and reducing greenhouse gas emission was negligible (Hill et al. 2006). In some cases, biofuel production has had negative impacts on the environment, for instance, when vast areas of rainforest in Borneo were deforested to grow oil palms (Graham-Rowe 2011). Given that marginalised members of the population, particularly in developing countries, struggle to meet their basic nutritional requirements, this raises the question of whether it is ethically acceptable to use food grade raw materials for producing biofuels (Thompson 2012). There is, however, no simple answer. Prices of food are influenced by additional factors beyond the competitive use of agricultural products for biofuel generation. Current agriculture is to a large extent dependent on an input of fossil fuels and increasing oil prices have a major influence on food prices. Moreover, there is also a global increase in average meat intake per capita, and animal production requires 2.5–10-fold more energy input per generated nutritive calorie compared to plant-based food, which is also driving prices up (Pelletier et al. 2011). Increasing prices should not necessarily be viewed as a negative phenomenon *per se*, they may support food producers in developing countries. According to the United Nations, about 50 % of people in extreme poverty (i.e. with an income of less than 1 Euro per day) are food producers, 30 % are to some extent involved in agricultural production, and thus, a majority of extremely poor people might benefit from price increases. Moreover, farmers might be encouraged by higher prices to produce more food, which might in the long term even help marginalised non-producers. On the other hand, there are a number of examples where poor farmers have been evicted due to the increasing value of land. Such cases may not specifically be linked issues of biofuel production, but rather may stem from political circumstances (Thompson 2012). Arising from this background, principles have been formulated to evaluation situations in which production of biofuels could be considered ethically acceptable. These criteria include: keeping essential rights of people (including food, water, health rights or land entitlements); sustainability of biofuels; greenhouse gas savings; and fair trade. If all of these criteria are met even a duty to develop these biofuels is postulated (Buyx and Tait 2011).

There is a certain consensus that the development of lignocellulose as raw material for biofuel production will solve the issue of competing food and fuel production. Exploitation of underutilised feedstocks may indeed provide a broader basis for biofuel production (Tilman et al. 2009). However, if second-generation energy plants turn out to be profitable, it is likely that they will also be produced in areas that can be used for food production (Graham-Rowe 2011). In this case, second-generation biofuels would also compete with food production. Moreover, if technologies are developed to gain access to the nutrients stored in the lignocellulose structure, it might be possible that these nutrients can also be used for food production (Thompson 2012). This has, in principle, been a long-established alternative in the feed industry by producing fodder yeast from lignocellulose residues (Johnson 2013).

Current biofuel production cannot be seen as the major cause of hunger in developing countries. The refinement of second- and higher generation biofuels will indeed provide essential technologies for a sustainable global economy, but technologies cannot in themselves change political circumstances. However, the scientific community developing these technologies can and should emphasise well-formulated ethical criteria regarding biofuels when communicating with society at large. Moreover, by sharing knowledge with colleagues from developing countries and especially supporting projects that enable local farmers to introduce low-tech innovations for food preservation or processing into their food value chains, scientists can actively support a development that empowers people and preserves their fundamental rights. Yeast-based technology can also play a role in such low-tech applications (e.g. Hellström et al. 2010; Leong et al. 2012).

9.3 First- and Second-Generation Ethanol Production

9.3.1 Towards Optimisation of First-Generation Biofuel Production

As ethanol production from first-generation substrates is still of great economic importance, significant research efforts are directed towards improving these well-established processes, especially in Brazil. Most Brazilian ethanol factories rely on a fermentation method in which yeast cells are recycled and inoculated to the next round of fermentation. This decreases the amount of sugar spent on biomass formation, increases the ethanol yield and shortens the fermentation time. On the other hand, the yeast cells are exposed to considerable stresses, e.g. high ethanol concentrations, high temperatures, osmotic stress, low pH, sulphite and contamination by bacteria. Thus, there are specific demands placed on strains used in these environments (Amorim et al. 2011; Della-Bianca et al. 2013). During a time frame of 12 years highly competitive, non-foaming and non-flocculating strains have been isolated and further tested for fermentation performance and competitiveness in commercial distilleries. However, most of the isolates had a poor implantation capability, highlighting that many factors influencing population dynamics in industrial ethanol fermentation are still unknown (Basso et al. 2008). In fermentations running with cell recirculation contamination with *Dekkera bruxellensis* has frequently been observed (de Souza Liberal et al. 2007; Passoth et al. 2007). *D. bruxellensis* is a slow-growing yeast with a highly efficient energy metabolism and, most probably, a high affinity to the limiting sugar substrate (Blomqvist et al. 2010, 2012; Tiukova et al. 2013), indicating that these characteristics play a major role in the ecosystem of industrial ethanol fermentation. Several genome analyses of *S. cerevisiae* isolates obtained from industrial ethanol fermentations have been performed. Almost all isolates were diploid strains with a high degree of heterozygosity, i.e. with a high number of polymorphisms between different alleles.

The number of transposable elements was comparably low, which may contribute to genome stability (Della-Bianca et al. 2013; Zheng et al. 2012). On the other hand, chromosomal rearrangements have been observed, but these were mostly restricted to the chromosome ends. Suppression of flocculation-related genes may contribute to competitiveness of Brazilian fermentation strains (Babrzadeh et al. 2012; Della-Bianca et al. 2013). A variety of genes may be responsible for efficiency in industrial ethanol production, including those involved in vitamin and glycerol metabolism (Babrzadeh et al. 2012; Della-Bianca et al. 2013; Zheng et al. 2012).

9.3.2 Strains for Second-Generation Ethanol Production

The situation is more complicated for second-generation substrates. A variety of inhibitors are formed during lignocelluloses pretreatment (Fig. 9.1), including furfural, hydroxymethylfurfural (HMF), acetate and other organic acids, and aromatic compounds (Palmqvist and Hahn-Hägerdal 2000; Klinke et al. 2004). Apart from this, lignocellulose hydrolysate contains a number of sugars, mainly released from hemicellulose, which cannot be fermented by wild-type *S. cerevisiae*. The most prominent of these sugars is the pentose xylose, which is, after glucose, the second most abundant sugar in nature. Several studies have demonstrated that the ability to convert this sugar into a valuable product is a key factor for the economic feasibility of ethanol production from lignocellulose (Kuhad et al. 2011). Attempts have been taken to manipulate strains to convert xylose and other lignocellulose sugars into ethanol (see below).

Occasionally, strains have been isolated from spent sulphite liquor (SSL) plants (Margeot et al. 2009). One isolated strain exhibited enhanced ethanol productivity and yield in SSL. This strain had high furaldehyde reductase activity and flocculated heavily, which may contribute to resistance against inhibitors in the hydrolysate (Sanchez et al. 2012). In terms of flocculation, the optimal characteristics for a production strain in second-generation substrates may thus differ from those for first-generation substrates (see above). Metabolic manipulation of industrial isolates has been recently performed regarding xylose fermentation and may result in strains that can be used in commercial plants (Garcia Sanchez et al. 2010b).

9.4 Second-Generation Ethanol Production on Commercial and Pilot Scale

Commercially available bioethanol is currently mainly produced from first-generation substrates such as cereal grain or sugar cane. Although it is desirable to develop second-generation processes, for both environmental and global food security reasons, their costs are still too high to replace first-generation ethanol

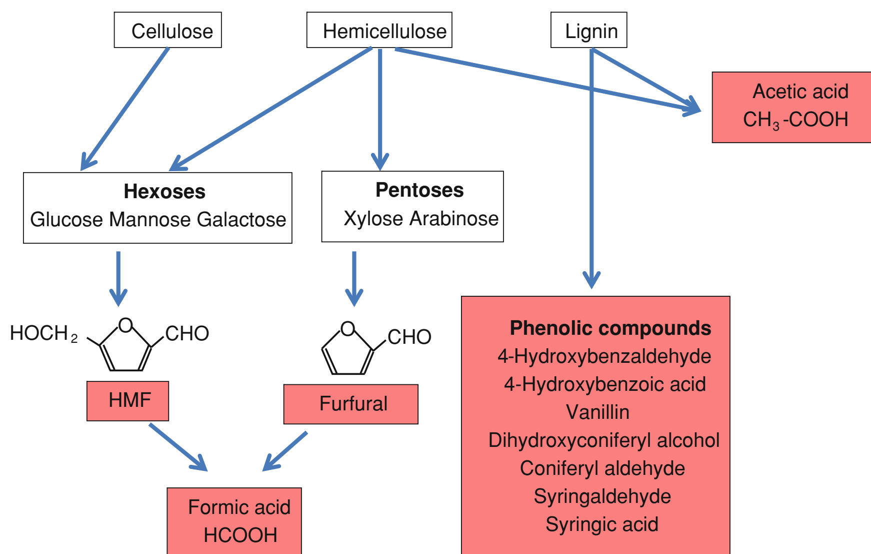


Fig. 9.1 Inhibitors (*red boxes*) released during pretreatment of lignocellulosic biomass. Acetic acid is released from acetylated hemicellulose and lignin. The major degradation products of lignin are phenolic compounds (the most prominent examples are given); sugars are primarily degraded to furans, especially furfural and hydroxymethylfurfural (HMF). Furans can be further degraded to weak acids, mainly formic acid

(Stephen et al. 2012). However, there are examples of commercial second-generation ethanol production and an increasing number of near-commercial ethanol production on a pilot scale. Borregaard (http://www.borregaard.com/Business-Areas/Borregaard-ChemCell) is a Norwegian company, mainly producing cellulose products from spruce. Sugars released from the cellulose process are used for producing second-generation ethanol, with an annual capacity of 20 million litres (Rødsrud et al. 2012). There is also a plant in Russia (Kirov Biochemical Plant) that converts wood biomass into pellets, furfural, fodder yeast, bioethanol and biogas. The ethanol process is based on thermophilic bacteria, and production capacity is not described on the company website (http://biochim.org/). In October 2013, the largest second-generation bioethanol plant to date was started in Crescentino (Italy), with a capacity of 75 million litres (60,000 metric tons) per year. The plant uses mixed feedstocks: wheat or rice straw, or the energy plant, giant cane (*Arundo donax*). Apart from this, energy is provided from burning of lignin. In the future, production of butanol and other chemicals are also planned. The plant's construction was driven by the BIOLYFE project, which was co-financed by the seventh framework program for Energy Research (FP7) of the European Union (http://www.biolyfe.eu/). The Danish company Inbicon AS has developed a process called Integrated Biomass Utilization System (IBUS). In this process, the cellulose of wheat straw is converted into ethanol, the lignin to a solid fuel, delivering process energy, and the hemicellulose fraction is planned to be converted into animal feed

(Larsen et al. 2008). With support of the Danish energy authority and FP7, a pilot plant has now been built and is able to fully operate 7 days per week. The production potential is 576 kg (730 l) per hour, which is still not at profitable levels. However, the produced ethanol is sold and distributed at more than 100 filling stations in Denmark (Larsen et al. 2012). In an ongoing European project (KACELLE), the fermentation of C5 sugars with genetically engineered strains (see below) will also be investigated (http://www.inbicon.com/projects/kacelle/pages/kacelle_project.aspx). A variety of international projects are currently supported by FP7, with the aim to develop new enzymes, new pretreatment strategies and new yeast strains for commercial second-generation ethanol production (http://www.biofuelstp.eu/cell_ethanol.html#ce2). Similar efforts are underway all around the world. In 2007, the US Department of Energy (DOE) announced an investment of up to $385 million for six biorefinery projects (http://www.biofuelstp.eu/cell_ethanol.html#ce8). In July 2013, INEOS Bio announced the start of a commercial ethanol plant in Vero Beach, Florida, using vegetative and yard waste, citrus, oak, pine and pallet wood waste. Its annual output is projected to be 8 million gallons (24,000 t) (http://www.ineos.com/en/businesses/INEOS-Bio/News/INEOS-Bio-Produces-Cellulosic-Ethanol/). There are also projects for demonstration and commercial plans in Canada and Brazil (http://www.biofuelstp.eu/cell_ethanol.html) and also China is encouraging efforts to obtain lignocellulosic ethanol production by offering specific subsidies (Qiu et al. 2012). The above-mentioned examples demonstrate that, although costs are still quite high compared to both first-generation ethanol and mineral oil, lignocellulosic ethanol has reached the threshold of commercial reality. Ongoing research efforts towards more efficient enzymes and pretreatment methods, novel strains fermenting all sugars present in hydrolysates, and learning effects when running large-scale lignocellulose-based ethanol production will most probably rapidly result in technologies with increased economical robustness and higher acceptance within society.

9.5 Manipulation of Yeast Carbon Metabolism to Obtain Higher Ethanol Production

9.5.1 Enabling Fermentation of Pentoses and Other Sugars

Lignocellulose mainly consists of the polysaccharides, cellulose and hemicellulose, and the polyaromatic compound, lignin. Hemicellulose, in contrast to cellulose that consists of polyglucose, contains a variety of sugars. Among them, xylose comprises the highest proportion. In softwood xylose comprises 5–10 % of the total lignocellulose biomass, however, in straw and hardwood its percentage can be higher than 20 and 25 %, respectively. Other sugars include the pentose arabinose (prominent in some softwoods and grasses, up to 10 % of the total

biomass) and the hexoses mannose (up to 15 % of the total biomass in softwoods) and galactose (about 4 % of the total biomass in softwoods and some agricultural materials) (Girio et al. 2010). Whereas the hexoses can be converted into ethanol by *S. cerevisiae*, this yeast is unable to assimilate pentoses (Kurtzman et al. 2011). The predominant pathway for xylose assimilation in fungi consists of two steps: NAD(P)H-dependent reduction by xylose reductase (XR) to xylitol and subsequent NAD^+-dependent re-oxidation by xylitol dehydrogenase (XDH) to xylulose. In most known xylose-assimilating fungi, the XR utilises only NADPH as co-factor, whereas for ethanol production from this sugar NADH utilisation by XR is essential (Bruinenberg et al. 1984). Interestingly, *S. cerevisiae* has enzymes that can reduce xylose to xylitol and oxidise xylitol to xylulose. The NADPH-dependent aldose reductase encoded by *GRE3* is the main XR in *S. cerevisiae*, and it is obviously involved in xylitol production by recombinant strains. Its disruption significantly decreases the xylitol production of those strains (Träff et al. 2001, 2002), and thus in many constructs that have been engineered during the recent years, this gene is knocked out (see below). There is also a gene encoding an XR in the *S. cerevisiae* genome (*YLR070c* or *XYL2*). This gene seems only to be expressed in the presence of a non-repressing carbon source and xylose. Thus, *S. cerevisiae* has the xylose assimilation pathway in its genome, but it is expressed at too low levels to enable the yeast to grow on this sugar (Richard et al. 1999). These results are supported by earlier findings that *S. cerevisiae*, although not able to grow on xylose as sole carbon source, can metabolise this sugar (van Zyl et al. 1989). A few years ago, *S. cerevisiae* strains were isolated that could grow slowly on xylose. When applying a series of mass matings and selection experiments on xylose medium, strains were obtained that could grow on xylose with doubling times down to 6 h, forming some ethanol (Attfield and Bell 2006). Several xylose-fermenting yeast species are known, but these have drawbacks such as low tolerance of ethanol and of inhibitors released during pretreatment of lignocellulose, or a requirement for a very controlled regime of oxygenation (Hahn-Hägerdal et al. 2007). Therefore, substantial efforts have been taken to engineer *S. cerevisiae* to ferment xylose. Bacteria directly convert xylose into xylulose using a co-factor-independent xylose isomerase (XI). However, early attempts to express bacterial XIs in *S. cerevisiae* did not result in active enzymes (Amore et al. 1989; Sarthy et al. 1987), and thus, a two-step, redox factor-dependent fungal xylose assimilation pathway was introduced. Xylose fermentation in *S. cerevisiae* could be achieved by expressing the genes of XR and XDH, encoded by *XYL1* (active both with NADPH and NADH) and *XYL2* (active with NAD^+) of the xylose-fermenting yeast *Scheffersomyces* (*Pichia*) *stipitis* (Kötter and Ciriacy 1993). The first recombinant strains produced substantial amounts of the side product xylitol, indicating a redox imbalance in the cell. Since then, several targets have been approached to improve *S. cerevisiae*'s ability to ferment xylose, including xylose transport, the xylose assimilation pathway, reduction of xylitol formation, redox factor regeneration or the general performance of the sugar metabolism (summarised in Hahn-Hägerdal et al. 2007). One example to approach the redox factor imbalance was the expression of *GDP1* of *Kluyveromyces lactis* in *S. cerevisiae*.

GDP1 encodes an $NADP^+$-dependent glycerol-aldehyde-phosphate dehydrogenase, which results in a lower production of NADH from glycolysis. Deletion of the gene encoding the naturally NADPH-generating glucose-6-phosphate dehydrogenase (*ZWF1*) resulted in an additional increase of ethanol production and lowered CO_2 production (Verho et al. 2003). The first functional XI expressed in *S. cerevisiae* was the xylA gene of the thermophilic bacterium *Thermus thermophilus;* however, as this enzyme operated far away from its temperature optimum, its activity was rather low (Walfridsson et al. 1996). Higher XI activity was obtained by expressing the XylA gene from the cellulolytic fungus *Pyromyces* sp. E2 (Kuyper et al. 2003). When this gene was expressed in a strain overexpressing all enzymes involved in converting xylulose into ethanol, i.e. xylulokinase, ribulose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transketolase and transaldolase, and with a deleted *GRE3* gene encoding aldose reductase, xylose was relatively rapidly converted into ethanol with comparatively low xylitol production and an ethanol yield of 0.43 g per g consumed xylose (Kuyper et al. 2005). Karhumaa et al. compared strains either expressing XR/XDH or XI. The genetic background was the same as in the strain of Kuyper et al. The ethanol yield was quite high in the XI strain, and accordingly, the xylitol yield rather low. On the other hand, xylose uptake was lower than in the XR/XDH-strain and also lower as described by Kuyper et al. 2005. Table 9.1 summarises important fermentation characteristics of selected manipulated strains. Obviously, some unknown factors are influencing the performance during xylose fermentation. Apart from the laboratory strains, an industrial isolate was also engineered to ferment xylose, using XR/XDH. This isolate essentially behaved like the XR/XDH strain on xylose as sole carbon source. However, in contrast to the two strains, which were derived from the laboratory strain CEN.PK, the industrial strain could ferment un-detoxified lignocellulose hydrolysate. Interestingly, in contrast to the cultivation on pure xylose, no xylitol was formed from the hydrolysate, and a substantially higher ethanol yield was obtained (Karhumaa et al. 2007). Expression of an XI from *Orpinomyces* in an *S. cerevisiae* strain overexpressing the homologous xylulokinase and the *S. stipitis* sugar transporter *SUT1* resulted in a xylose-fermenting strain that obtained an ethanol yield of 0.39 g per g consumed xylose (Table 9.1). However, not all xylose was consumed and substantial amounts of xylitol were produced (Madhavan et al. 2009). XI activities were still quite low, resulting in a carbon-starvation response of an engineered strain (Bergdahl et al. 2012). High XI activity was reached by expressing a codon-optimised *XylA* gene from *Clostridium phytofermentans*. An industrial strain transformed with this gene was able to grow on xylose after four serial transfers in xylose medium, without additional manipulations. In contrast to the eukaryotic XI, this enzyme was not inhibited by xylitol, which is always formed in *S. cerevisiae* due to the action of unspecific aldose reductases (Brat et al. 2009).

To identify genes critical for xylose fermentation, genomes and transcriptomes of yeasts naturally fermenting or assimilating xylose have been compared to other yeasts. Forty-three genes were specific for xylose-assimilating yeasts, including a putative xylose transporter and a variety of endoglucanases. Global gene

Table 9.1 Ethanol production parameters of selected genetically engineered *Saccharomyces cerevisiae* strains

Strain	Expressed genes[a]	Initial sugar $(g\ l^{-1})$[b]	Consumed sugar $(g\ l^{-1})$[b]	Specific sugar consumption $(g\ g^{-1}\ h^{-1})$[b]	Specific ethanol production $(g\ g^{-1}\ h^{-1})$	Yields[c]	Final concentration (g/l)[c]	Reference
H2684	*XYL1*, *XYL2*, *XKS1*, *GDP1*, Δ*zwf1*	X 50	X 14.7	NP[e]	0.018	E 0.41, X-OH 0.35	E 4.6, X-OH 5.2	Verho et al. 2003
RWB 217	*XylA*, *XKS1*, araA, araB, araD, *RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	X 20	X 20	X 1.06	NP[e]	E 0.43, X-OH 0.003	E 8.7, X-OH 0.058	Kuyper et al. 2005
		Mixed: G 20, X 20	G 20, X 20	NP[e]	NP[e]	E 0.43, X-OH 0.006[f]	E 17.1, X-OH 0.12	
TMB 3057	*XYL1*, *XYL2*, *XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	X 50	X 39.6	X 0.13	0.04	E 0.33, X-OH 0.22, Glyc 0.11	E 13.3, X-OH 8.7[d], Glyc 4.4[d]	Karhumaa et al. 2007
TMB 3066	*XylA, XKS1, RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	X 50	X 16.8	X 0.05	0.02	E 0.43, X-OH 0.04, Glyc 0.07	E 7.3, X-OH 0.7[d], Glyc 1.2[d]	Karhumaa et al. 2007
TMB 3400	*XYL1*, *XYL2*, *XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	X 50	X 36.5	X 0.06	0.02	E 0.34, X-OH 0.29, Glyc 0.04	E 12.1, X-OH 10.6[d], Glyc 1.5[d]	Karhumaa et al. 2007
		LCH: G 16, M 10, Gal 4, X 7, A 3	G 16, X 7, M 8, Gal 0, A NP[e]	G 0.021, M 0.013, X 0.005	0.02	E 0.41, Glyc 0.035	E 16, X-OH 0, Glyc 1.4	

(continued)

Table 9.1 (continued)

Strain	Expressed genes[a]	Initial sugar (g l^{-1})[b]	Consumed sugar (g l^{-1})[b]	Specific sugar consumption (g g^{-1} h^{-1})[b]	Specific ethanol production (g g^{-1} h^{-1})	Yields[c]	Final concentration (g/l)[c]	Reference
INVSc1 (engineered)	*XKS1*, *XylA*, *SUT1*	X 50	X 15.55	NP[e]	NP[e]	E 0.39, X-OH 0.08	E 6.05, X-OH 1.28, Glyc 0.66	Madhavan et al. 2009
IMS0010	*XylA*, *XKS1*, araA, araB, araD, *RPE1*, *RKI1*, *TKL1*, *TAL1*, Δ*gre3*	Mixed: G 30, X 15, A 15	G 30, X 15, A 15	X 0.35 A 0.53 [g]	NP[e]	E 0.43	E 25.8, X-OH 0, A-OH 0	Wisselink et al. 2009
BWY10Xyl	xylA	X 25	X 18	X 0.06	0.03	E 0.42	E 7.9, X-OH 3	Brat et al. 2009
TMB 3130	*XYL1*, *XYL2*, *XKS1*, araA, araB, araD	Mixed: G 20, X 20, A 20	G 20, X 17.89, A 18.39 [g]	X 0.09, A 0.1 [g]	NP[e]	E 0.29, X-OH 0.31[f], A-OH 0.95, Glyc 0.04		Garcia-Sanchez et al. 2010a, b
GS1.11-26	*XylA*, *XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, *TKL2*, *TAL2*, *HXT7* araA, araB, araD, *ARAT*	Mixed: G 36, X 37	G 36, X 37	G 2.71 X 1.1	1.38	E 0.46, X-OH 0.04[f], Glyc 0.06	E 33.6, X-OH 1.5, Glyc 4.3	Demeke et al. 2013b
		LCH (*Arundo donax*): G 59.3, X 22.18, M 6.19	G 59.3, X 22.18, M 6.19			E 0.47, X-OH 0.07[f], Glyc 0.04	E 41.2, X-OH 1.5, Glyc 3.5	

(continued)

Table 9.1 (continued)

Strain	Expressed genes[a]	Initial sugar (g l^{-1})[b]	Consumed sugar (g l^{-1})[b]	Specific sugar consumption (g g^{-1} h^{-1})[b]	Specific ethanol production (g g^{-1} h^{-1})	Yields[c]	Final concentration (g/l)[c]	Reference
GSF767	*XylA*, *XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, *TKL2*, *TAL2*, *HXT7* araA, araB, araD, *ARAT*	Mixed: G 36, X 37	G 36, X 37	G NP, X 0.65	NP[e]	E 0.46	E 33, X-OH 4, Glyc 4.5	Demeke et al. 2013a
		LCH (amended with sugars) G 62, X 18, M 15	G 62, X 16, M 15	NP[e]	NP[e]	E 0.42[d], X-OH 0, Glyc 0.05	E 39.8, X-OH 0, Glyc 4.65[d]	

[a] Overexpressed genes have been derived from *S. stipitis* (*XYL1*, *XYL2*, *SUT1*, *ARAT*), *S. cerevisiae* (*XKS1*, *RPE1*, *RKI1*, *TKL1*, *TAL1*, *HXT7*) and *K. lactis* (*GPD1*). Genes of the arabinose pathway (araA, araB, araD) were from *L. plantarum* (strains IMS0010 and GS1.11-26), or *B. subtilis* (araA) and *E. coli* (araB, araD) (strain TMB 3130). Xylose isomerase genes were from *Pyromyces* (*XylA*, strains RWB217, TMB 3066 and IMS0010), *Orpinomyces* (*XylA*, strain INVSc1), *C. phytofermans* (xylA, strains BWY10Xyl, GS1.11-26 and GSF767)

[b] G—glucose, X—xylose, M—mannose, Gal—galactose, LCH—lignocellulose hydrolysate

[c] E—ethanol, X-OH—xylitol, A-OH—arabitol, Glyc—glycerol

[d] Calculated from values provided in the original publications

[e] Information not provided by the original article

[f] Calculated on consumed xylose

[g] Xylose and arabinose were consumed after glucose was exhausted

expression analysis of cells grown in glucose or xylose revealed additional genes critical for xylose assimilation and fermentation. Besides the known genes of the xylose assimilation pathway, genes involved in plant biomass degradation like glucosidases and cellulases were strongly induced. Moreover, genes involved in redox metabolism and the pentose phosphate pathway were activated. Several of the identified genes were expressed in an *S. cerevisiae* strain engineered to ferment xylose. Two genes, a *Candida tenuis* aldo/keto reductase and a *Spathaspora passalidarum* gene, with homology to uncharacterised fungal-specific proteins had the greatest effect on xylose assimilation (Wohlbach et al. 2011).

L-Arabinose is the second most abundant pentose in plant biomass, and several attempts have been made to generate yeast strains able to ferment this sugar. In L-arabinose-assimilating fungi, the sugar is first reduced to L-arabinitol (NADPH dependent), which is then re-oxidised to L-xylulose (NAD^+ dependent). L-xylulose is reduced to xylitol (NADPH dependent), which is subsequently converted into D-xylulose by the NAD^+-dependent XDH. The redox factor imbalance generated by this pathway makes it almost impossible to ferment L-arabinose in fungi (Richard et al. 2002). In contrast, the bacterial pathway of L-arabinose assimilation is independent of redox factors and consists of L-arabinose isomerase, ribulokinase and L-ribulose-phosphate 4-epimerase. Genes encoding this pathway, derived from several species, have been expressed in *S. cerevisiae* and L-arabinose-fermenting strains have been obtained (Weber et al. 2010). Expressing AraA, AraB and AraD from *Lactobacillus plantarum* in an *S. cerevisiae* strain did not immediately result in arabinose assimilation. At first, cells were precultivated in galactose, as it has been shown that cells grown on galactose can transport arabinose into the cell. Subsequently, cells were cultivated in several passages in medium containing arabinose as sole carbon source. Finally, arabinose-assimilating cells were transferred to oxygen limited conditions, which resulted in arabinose-fermenting cells. The strain used for this selection had also been engineered to ferment xylose. However, this ability was lost during the selection procedure (Wisselink et al. 2007). Therefore, a strain containing genes for xylose and arabinose assimilation was selected in consecutive selection cycles on medium containing (i) glucose, xylose and arabinose, (ii) xylose and arabinose and (iii) only arabinose as carbon sources. The resulting strain (Table 9.1) was able to ferment all provided sugars (30 g/l glucose, 15 g/l xylose and 15 g/l arabinose) within 35 h, reaching an ethanol yield of 0.44 g per g total sugar (Wisselink et al. 2009). Another potential step towards efficient arabinose-fermenting *S. cerevisiae* strains may be the cloning and expression of L-arabinose transporters from naturally L-arabinose-fermenting yeasts (Verho et al. 2011).

The majority of the above-mentioned manipulations were performed in laboratory strains, which are very efficient tools in research but most probably not competitive under harsh industrial conditions. Recently, the xylose and arabinose fermentation pathways have been introduced in industrial strains. *S. stipitis XYL1* and *XYL2*, the *S. cerevisiae XKS1* and bacterial genes of the arabinose assimilation pathway were overexpressed in the diploid wine strain USM21 (Garcia Sanchez et al. 2010b; Westhuizen and Pretorius 1992). The resulting strain was then further

improved by evolutionary engineering using continuous cultivation for about 65 generations with xylose and arabinose as carbon sources, and with gradually increasing dilution rates as soon as a steady state was reached. In test fermentations with mixed sugars, the evolved strain completely consumed xylose and arabinose. A significant amount of ethanol was produced from xylose, however, also a substantial amount of xylitol. Arabinose was almost completely converted into arabitol in the mixed sugar fermentation (Garcia Sanchez et al. 2010b). In another attempt (Demeke et al. 2013b), the industrial Ethanol Red strain was transformed with a cassette containing a modified *HXT7* (transporting both glucose and xylose into the cell), the codon-optimised *C. phytofermentans XylA* gene, and genes coding enzymes of the pentose phosphate pathway. In addition, bacterial genes of the arabinose assimilation pathway and a codon-optimised arabinose transporter from *S. stipitis* (Subtil and Boles 2011) were introduced. However, these manipulations still did not result in efficient xylose fermentation. Resulting strains were chemically mutagenised and xylose-assimilating strains were further manipulated by genome shuffling (mass mating of isolated spores) with each other and the parental strain. The isolated strain fermented both glucose and xylose at the same time with comparable high rates and ethanol yields. Xylitol production was substantially diminished (0.04 g per g consumed xylose) compared to other studies. On the other hand, the evolved strain showed a decreased glucose uptake rate compared to the strain before random mutagenesis (2.71 and 3.83 g g^{-1} cell dry weight h^{-1}, respectively) and also a reduced ethanol productivity (1.38 and 1.79 g g^{-1} cell dry weight h^{-1}). Moreover, it was less ethanol tolerant and had a reduced respiratory capacity compared to the parental strain. Fermentation of arabinose has not been tested yet, although the arabinose assimilation pathway had also been expressed in the strain (Demeke et al. 2013b). To improve the fermentation capacity, several crossing/segregation experiments of this strain with isolates showing exceptional inhibitor tolerance have been performed. Three superior strains have been isolated. Although showing a lower xylose consumption rate compared to the original strain in complete medium, they exhibited significantly improved tolerance to spruce hydrolysate, higher glucose consumption rates, higher aerobic growth rates and higher maximum ethanol accumulation in high gravity ethanol production (Demeke et al. 2013a). These results illustrate that although there are still unknown factors when it comes to the manipulation of industrial isolates, the application of engineered pentose-fermenting strains in industrial fermentations may soon become a reality.

Apart from xylose and arabinose fermentation, some efforts have been directed towards a more efficient fermentation of other sugars. Overexpressing the *PGM2* gene encoding a phosphoglucomutase improved galactose fermentation, but at the same time also xylose fermentation. This indicates that interactive effects may occur when engineering multiple metabolic pathways in one strain (Garcia Sanchez et al. 2010a). Sucrose is the major substrate of sugar cane- and sugar beet-based ethanol production, and thus, its improvement would have a substantial effect on sustainability of global ethanol production. *S. cerevisiae* hydrolyses most of the sucrose extracellularly by secreting invertase. There is also some capacity to

directly transport sucrose into the cell via a proton symport system. This system consumes energy, hence, less biomass would be formed and thus more ethanol. Basso et al. constructed a strain where the signal sequence of the invertase was removed. This strain hydrolysed most of the sucrose intracellularly; however, growth rate was diminished and the residual sucrose concentration in the medium was high. Selection in anaerobic, sucrose-limited continuous fermentation finally resulted in a strain with higher affinity and higher growth and ethanol production rates and increased ethanol yield compared to the wild type (Basso et al. 2011). This indicates that even on a conventional substrate such as sucrose there is potential for process improvement.

Surface engineering of *S. cerevisiae* may provide an approach to obtain consolidated bioprocessing of biomass. Surface-engineered strains express extracellular, polymer-degrading enzymes such as cellulases, hemicellulases or amylases fused to a glycosylphosphatidylinositol anchoring system, resulting in the display of these enzymes on the surface of the engineered cells. Using those strains, direct conversion of lignocellulosic biomass into ethanol and other compounds has been demonstrated (Hasunuma and Kondo 2012).

9.6 Metabolic Engineering of Non-conventional Yeasts for Ethanol Production from Lignocellulose and Other Substrates

Apart from *S. cerevisiae*, other yeasts have been manipulated to ferment xylose and other sugars. Engineering of thermotolerant yeasts may be of special interest, as fermentation at higher temperatures reduces cooling costs, enables simultaneous saccharification and fermentation closer to the optimum temperature of polysaccharide-degrading enzymes and decreases the risk of contamination. The yeast, *Ogataea polymorpha* (widely known by its previous name *Hansenula polymorpha*), had originally not been described to ferment xylose to ethanol. However, carefully performed growth tests demonstrated that this yeast can convert xylose into ethanol at higher fermentation temperatures (Ryabova et al. 2003). Interestingly, expression of a bacterial XI gene (*xylA* of *Escherichia coli*) resulted in an active protein in this yeast. Expressing this gene in a strain with deleted natural XR/XDH-dependent xylose assimilation pathway resulted in higher ethanol production compared to the wild type (Dmytruk et al. 2008). Further improvements could be obtained for instance by overexpressing the *PDC1* gene, or by overexpressing heat shock proteins and at the same time deleting *ATH1*, encoding for an acid trehalase. The latter resulted in an even more thermotolerant strain with increased ethanol formation (Ishchuk et al. 2008, 2009). Expression of genes encoding for α-amylase, glucoamylase, xylanase and β-xylosidase resulted in a starch- and xylane-fermenting strain (Voronovsky et al. 2009). However, ethanol productivities and yields of all engineered *O. polymorpha* strains are still far below

levels obtained by other xylose fermenting yeasts or engineered *S. cerevisiae*; thus, further efforts are required to introduce this yeast into industrial bioethanol production.

A thermotolerant strain of *Kluyveromyces marxianus* has been manipulated by surface engineering to display endoglucanase and β-glucosidase at its cell surface. The resulting strain converted glucan into ethanol with a yield close to the theoretical maximum (Yanase et al. 2010).

9.7 Inhibitor Tolerance

Yeasts detoxify furfural and HMF (Fig. 9.1) by converting them into the less toxic alcohols, furfuryl alcohol (mainly NADH dependent) and 2, 5-bis-hydroxymethylfuran (mainly NADPH dependent), respectively (Liu 2006). Inhibitor-tolerant strains isolated from pilot-scale lignocellulose fermentations showed increased NADPH- and NADH-dependent furfural and HMF reduction abilities (Sanchez et al. 2012). In accordance with this, several successful attempts of manipulation included overexpression of enzymes with NADPH- or NADH-dependent aldehyde reduction activity. Other approaches increased the flux through the pentose phosphate cycle, which, apart from improving pentose fermentation, may also have provided higher amounts of NADPH, which can be utilised as co-factor in the reduction of furfural and HMF (Hasunuma and Kondo 2012; Liu et al. 2009b), and to detoxify reactive oxygen species (ROS). It has been shown that ROS are released in yeast cells upon exposure to furfural. Interestingly, in contrast to ROS generated by acetic acid (see below), this does not seem to induce programmed cell death (Allen et al. 2010). Overexpression of *ALD6*, encoding an $NADP^+$-dependent cytosolic aldehyde dehydrogenase also increased the tolerance against lignocellulose hydrolysate, probably by generating NADPH. However, a strain overexpressing this gene also generated more acetate (Zheng et al. 2012), which is itself a potent inhibitor present in lignocellulose hydrolysate (Fig. 9.1).

Acetic acid is a powerful antimicrobial agent, which is used for food preservation. At a pH below the pK_a value 4.76, acetic acid is protonated and can diffuse into the cell either directly through the hydrophobic cell membrane or via the facilitator Fps1. Inside the cell the molecule dissociates, which affects the intracellular pH, and by this a variety of metabolic reactions. Yeast cells exposed to acetic acid stress have been described to undergo programmed cell death (Ludovico et al. 2001). This is probably tightly connected to the development of ROS, which are formed as response to acetic acid stress. A variety of other disturbances are caused by acetic acid, including inhibition of amino acid uptake and inhibition of glycolysis (Sousa et al. 2012). As a response to acetic acid stress, the Hog1 MAP kinase and the transcription factor Haa1 are activated. These factors in turn deactivate Fps1 and activate a corresponding stress response, including energy demanding proton transport out of the cell (Piper 2011). Acetic acid-tolerant strains were isolated by long-time cultivation in increasing acetate

concentrations at pH below the pK_a of acetic acid: resulting strains could tolerate up to 6 g/l acetate. Remarkably, during the selection procedure, a very high specific xylose uptake rate was monitored, probably due to the increased energy demand of the cell to run the energy-dependent defence mechanisms (Wright et al. 2011). However, after storing the cells under non-selective conditions, the resistance phenotype disappeared and could only be re-established by precultivating in sub-lethal acetate concentration, indicating an inducible resistance mechanism. It has been shown that catalase is activated upon pre-incubation at sub-lethal concentrations of acetic acid, pointing towards a mechanism detoxifying ROS by the inducible resistance (Martani et al. 2013). A loss of resistance after cultivation under non-selective conditions has also been observed in other systems (e.g. Tiukova et al. 2014), indicating a demand for further investigation of the mechanism of adaptation. In another attempt, an acetate-tolerant strain was isolated by screening about 500 isolates from different origins. The strain showed higher expression of HAA1p- and HOG1p-regulated genes, although transcription of *HOG1* was less enhanced compared to a more sensitive strain. Remarkably, additional genes regulated by another transcription factor, Aft1p, were upregulated. These genes are mainly involved in iron transport. It is to date unclear which role they play in acetate resistance (Haitani et al. 2012). Genetic engineering of *S. cerevisiae* towards production of vitamin C (L-ascorbic acid) resulted in lowered production of ROS and a higher viability of cells exposed to acetic acid stress (Martani et al. 2013). Overexpression of *HAA1* resulted in constitutive increased acetate tolerance in *S. cerevisiae* (Tanaka et al. 2012). Guadalupe-Medina et al. expressed an NADH-dependent aldehyde dehydrogenase in *S. cerevisiae*. The resulting strain was able to use acetate as electron acceptor and converted it into ethanol, which is an example of combining detoxification with product generation (Guadalupe-Medina et al. 2013). Deletion of the *PHO13* gene, encoding *p*-nitrophenyl phosphatase, improved xylose fermentation in genetically engineered *S. cerevisiae* (Van Vleet et al. 2008). Moreover, increased ethanol production by the deletion strain was also observed in the presence of common inhibitors such as acetate, formic acid, furfural and HMF, and in lignocellulose (rice straw) hydrolysate. The physiological basis for this improvement is not clear, but increased expression of genes involved in the pentose phosphate cycle, glycolysis and alcoholic fermentation has been observed (Fujitomi et al. 2012).

A formic acid-tolerant strain has been constructed by overexpression of the *FDH1* gene, encoding formate dehydrogenase. The resulting strain produced ethanol in the presence of 10 mM formic acid, almost as efficient as the original strain in a control fermentation (Hasunuma et al. 2011). Several attempts have been made to construct strains resistant to inhibitors released from lignin, e.g. by expressing the *lcc2* gene from *Trametes versicolor*, encoding a laccase, in *S. cerevisiae* (Larsson et al. 2001).

9.8 Lowering Glycerol Formation

Glycerol is formed during alcoholic fermentation to re-oxidise NADH formed by processes other than glycolysis. It is also a compatible solute, which is produced in response to extracellular stress (Ansell et al. 1997). Glycerol production removes carbon from ethanol formation, decreasing the yield of ethanol production. Disrupting one or both genes encoding cytosolic glycerol-3-P-dehydrogenases in *S. cerevisiae*, *GPD1* and *GPD2*, resulted in decreased glycerol and increased ethanol yield. On the other hand, manipulating the glycerol production genes resulted in slow growth and low specific ethanol production rates. The double disruptant lacked the ability to grow anaerobically (Ansell et al. 1997; Valadi et al. 1998). This is similar to the situation in *D. bruxellensis*, which naturally produces low amounts of glycerol and has a high ethanol yield, but shows low growth and ethanol production rates and requires addition of amino acids for anaerobic growth (Blomqvist et al. 2010, 2012). Apart from directly manipulating the genes involved in glycerol production, attempts have been made to influence the redox balance in the cell. For instance, the NADPH-dependent pathway of ammonium assimilation has been replaced by an NADH- and ATP-dependent pathway in *S. cerevisiae* by disrupting the gene of the NADPH-dependent glutamate dehydrogenase, *GDH1*, and overexpressing *GLN1* and *GLT1*, encoding glutamine synthetase and glutamate synthase (Nissen et al. 2000). Replacement of the natural glycerin-aldehyde-3-P-dehydrogenase by a non-phosphorylating $NADP^+$-dependent bacterial equivalent also significantly decreased glycerol production. When an NADH-dependent aldehyde dehydrogenase was expressed in a Δ*gpd1*, Δ*gpd2* strain, anaerobic growth was restored in the presence of acetate, which served as alternative electron acceptor and was converted into ethanol (Guadalupe-Medina et al. 2010, 2013). This is a promising approach, as acetate is one of the inhibitors of fermentation released during pretreatment of lignocellulose (see above). The lowered tolerance towards osmotic stress of the Gpd^- strain could be compensated by overexpressing the genes of the trehalose pathway *TPS1* and *TPS2* (Guo et al. 2011). Apart from this, both approaches reduced the amount of ATP produced per mol sugar, and due to this, the flux through the fermentation pathway was increased, resulting in higher ethanol productivity. Deleting *FPS1* encoding an aquaglyceroporin involved in glycerol efflux and acetate uptake (see above) resulted in lower glycerol production but also slower ethanol formation (Wang et al. 2012; Zheng et al. 2012). However, strains with low capabilities of glycerol production are often sensitive to osmotic and other stresses, and thus, they are not suited to the stressful environment of industrial ethanol production. Apart from overexpressing stress-related genes like those of the trehalose synthesis pathway, strategies of genetic manipulation have been combined with genome shuffling by multi-parental protoplast fusion of strains with desired phenotypes, which resulted in the generation of stress-tolerant, low glycerol producing strains (Guo et al. 2011; Tao et al. 2012; Wang et al. 2012).

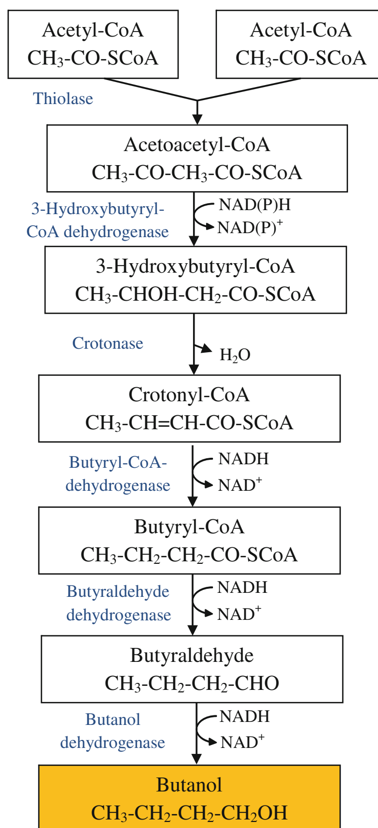


Fig. 9.2 Biosynthesis of n-butanol in engineered *S. cerevisiae*. Two acetyl-CoA are combined and subsequently reduced to n-butanol in a multi-enzyme cascade. Most of the enzymes required for n-butanol formation have been introduced by expressing the corresponding *Clostridium beijerinckii* genes in *S. cerevisiae*. Only thiolase (acetyl-coenzyme A acetyltransferase, encoded by *ERG10*) is present in *S. cerevisiae*. The most productive strain overexpressed thiolase from *S. cerevisiae* or *E. coli*. Expression of NADH-dependent *C. beijerinckii* 3-hydroxybutyryl-CoA dehydrogenase resulted in better n-butanol production than expression of NADPH-dependent enzymes from other bacteria (Steen et al. 2008)

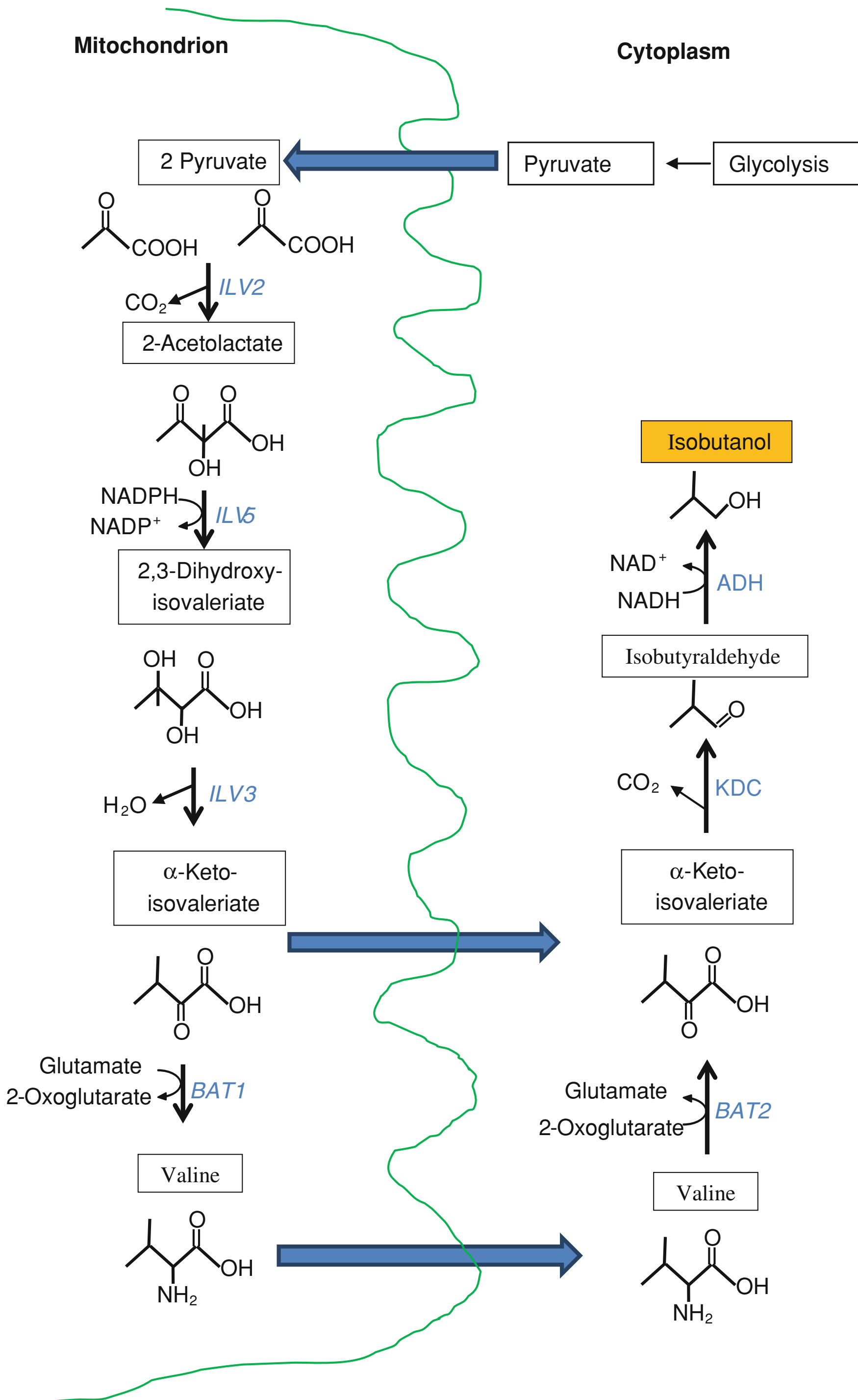
9.9 Manipulating Yeasts for Butanol Production

Yeasts may have several advantages for producing butanol compared to the established clostridia, as yeasts produce fewer side products, are less sensitive to oxygen and are probably more robust. An n-butanol production pathway has been introduced into *S. cerevisiae*. In this pathway two acetyl-CoA first form aceto-acetyl-CoA, which is then reduced by 3-hydroxybutyryl-CoA dehydrogenase to 3-hydroxybutyryl-CoA. This reduction step can be NADH or NADPH dependent in different organisms. From this, crotonyl-CoA is formed by the crotonase reaction. Crotonyl-CoA is then in several NADH-dependent steps finally reduced to butanol (Fig. 9.2). Since *S. cerevisiae* lacks most of the enzymes required for these steps, several genes from *Clostridium beijerinckii*, *E. coli*, *Ralstonia eutropha*, *Streptomyces collinus* and *S. cerevisiae* were overexpressed. Higher butanol production was obtained when an NADH-instead of an NADPH-dependent dehydrogenase was introduced for reduction of acetoacetyl-CoA. However, the highest concentration reached (2.5 mg/l) was below that of an engineered

E. coli strain and of the *Clostridium*-based system. Accumulation of butyryl-CoA indicated a bottleneck at the butyraldehyde dehydrogenase reaction (Steen et al. 2008).

More intense efforts have been made to produce isobutanol, which has similar characteristics as a biofuel compared to n-butanol. Yeasts naturally produce isobutanol during the catabolism of valine through the Ehrlich pathway (Hazelwood et al. 2008). Valine is first deaminated to α-ketoisovalerate, which is then decarboxylated and reduced to isobutanol. Valine biosynthesis starts from pyruvate, and α-ketoisovalerate is formed as an intermediate also in the synthesis pathway (Fig. 9.3). Thus, it is possible to establish an isobutanol production pathway by combining the valine synthesis and degradation pathways. However, the valine synthesis pathway is localised in the mitochondrial matrix, whereas valine degradation takes place in the cytoplasm. Thus, strategies of metabolic engineering aimed, in many cases, to express the corresponding enzymes in the cytoplasm (Buijs et al. 2013; Matsuda et al. 2012; Brat et al. 2012). Further optimisations included enhancement of the pyruvate levels by disrupting *PDC* genes, expressing an optimal α-ketoisovalerate decarboxylase (*Lactococcus lactis* KivD) and alcohol dehydrogenase (*ScADH6*) (Kondo et al. 2012; Matsuda et al. 2012). Brat et al. transferred the valine synthesis pathway into the cytosol by overexpressing codon-optimised *ILV2*, *ILV5* and *ILV3* with truncated mitochondrial import signal sequences. The *S. cerevisiae ARO10* (encoding an α-ketoacid decarboxylase, KDC) and *ADH2* were found to encode the optimal genes for the final two steps of isobutanol production. When these genes were expressed in a Pdc^{-} strain (Δ*pdc1*, Δ*pdc5*, Δ*pdc6*) with blocked mitochondrial valine synthesis pathway, up to 0.63 g/l, with a yield of 15 mg/g glucose could be obtained (Brat et al. 2012). When additionally XI, transaldolase and xylulokinase genes were overexpressed, the strain was able to ferment xylose to isobutanol, with a final concentration of 1.36 mg/g xylose and a yield of 0.16 mg isobutanol/g xylose (Brat and Boles 2013). A different strategy was employed by Avalos et al., who expressed the whole isobutanol production pathway inside the mitochondria. Overexpressing *S. cerevisiae ARO10* and the *Lactococcus lactis* AdhA (encoding *L. lactis* ADH7) with mitochondrial targeting sequences, together with overexpression of *ILV2*, *ILV3* and *ILV5*, resulted in a maximal isobutanol titer of 630 mg/l in complete medium. No further gene deletions were required to attain this level (Avalos et al. 2013). Although these values are still much lower than in bacterial production hosts, they can be regarded as starting points for further optimisation. In the patent literature, final concentrations up to 18.6 g/l and yields up to 0.33 g/g (i.e. about 80 % of the theoretical maximum) have been reported (Buijs et al. 2013).

Using amino acids as substrate may be another way of producing butanol and isobutanol with *S. cerevisiae*. Branduardi et al. (2013) obtained isobutanol and butanol production from glycine, and introducing a heterologous glycine oxidase gene from *Bacillus subtilis* (*goxB*) increased butanol/isobutanol formation. Maximum concentrations of 92 and 58 mg/l of butanol and isobutanol, respectively, were reached.


Mitochondrion
Cytoplasm
2 Pyruvate
Pyruvate
Glycolysis
COOH
COOH
ILV2
CO_2
2-Acetolactate
OH
OH
NADPH
$NADP^+$
ILV5
2,3-Dihydroxy-isovaleriate
OH
O
OH
OH
H_2O
ILV3
α-Keto-isovaleriate
Glutamate
2-Oxoglutarate
BAT1
Valine
NH_2
Isobutanol
OH
NAD^+
NADH
ADH
Isobutyraldehyde
CO_2
KDC
α-Keto-isovaleriate
Glutamate
2-Oxoglutarate
BAT2
Valine
NH_2

◀ **Fig. 9.3** Biosynthesis of isobutanol in *S. cerevisiae*. Isobutanol is produced as a result of the Ehrlich pathway of valine degradation. α-Ketoisovalerate is a common intermediate of both the mitochondrially localised valine synthesis and the cytoplasmatic degradation pathways, and by combining both pathways, isobutanol can be generated from pyruvate. α-Ketoacid decarboxylase (KDC) and alcohol dehydrogenase (ADH) activities are encoded by several genes, including all *PDC*-genes and *ARO10* (KDC), and a variety of *ADH* genes, respectively (Hazelwood et al. 2008). Increased isobutanol production was obtained by either expressing KDC and ADH proteins with a mitochondrial signal sequence, thus expressing the whole pathway in the mitochondria, or by expressing cytoplasmic *ILV2* (encoding acetolactate synthase), *ILV5* (acetohydroxyacid reductoisomerase), and *ILV3* (dihydroxyacid dehydratase) (Avalos et al. 2013; Brat et al. 2012). Transamination is performed by branched chain amino acid transaminase (*BAT1* and *BAT2*)

9.10 Yeasts for Biodiesel Production

Biodiesel is currently the only liquid biofuel that is produced on a commercial scale, apart from ethanol. It is generated from oil plants such as soy, oil palms or oilseed rape, which can accumulate triglycerides. The triglycerides are extracted from the plant material. Subsequently, the triglycerides are converted by the transesterification reaction, in which the glycerol is replaced by the short chain alcohols methanol or ethanol, forming fatty acid methyl or ethyl esters (FAME or FAEE), respectively (Fig. 9.4). However, the methanol that is preferably used is generated from mineral oil. Thus, biodiesel cannot completely be considered a renewable fuel. The oil plants are cultivated on arable land, and thus, biofuel production may compete with food production. The energy yield per hectare of oil plants is relatively low compared to sugar plants. Moreover, in contrast to sugar plants that are the basis of ethanol production, oil plants can be cultivated on rainforest areas; and examples of deforestation in such areas for oil plant production have been documented (Graham-Rowe 2011; Azócar et al. 2010).

To overcome the obvious disadvantages of plant-based biodiesel, microbial lipids may represent an alternative. Lipid-accumulating microalgae obtained considerable attention. Indeed, the concept of producing biofuels just from sunlight and assimilated CO_2 is appealing. However, algae have several disadvantages, including slow growth, low lipid accumulation rates, high costs and a high risk for contamination (Cheng and Timilsina 2011). Certain oleaginous yeasts can form triglycerides with high specific rates, and to a proportion of their biomass exceeding 50 %, which is higher than in all other known lipid-accumulating organisms. Thus, yeasts may have a great potential also for biodiesel production. There are about 30 known oleaginous yeast species. They belong to different phylogenetic groups, including ascomycetous species such as *Lipomyces starkeyi* and *Yarrowia lipolytica* or basidiomycetes such as *Rhodotorula glutinis* and *Rhodosporidium toruloides* (Ratledge and Wynn 2002).

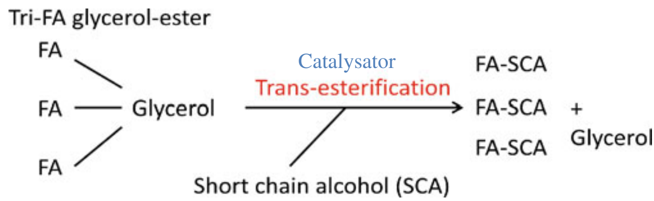


Fig. 9.4 Survey of the transesterification process. Glycerol is replaced by a short chain alcohol, either methanol or ethanol, generating fatty acid methyl or ethyl esters (FAME or FAEE). Alkaline (NaOH), acid (H_2SO_4) or lipases can be used as catalysator

9.11 Physiology of Lipid Accumulation in Yeasts

Lipid accumulation (Fig. 9.5) follows a common pattern in all known oleaginous yeasts (Ratledge and Wynn 2002), in spite of their phylogenetic distance: upon nitrogen limitation, AMP concentration decreases to less than 5 % of its value under C-limitation. The AMP is deaminated to inosine monophosphate by AMP deaminase. In oleaginous yeasts, isocitrate dehydrogenase (IDH) is strictly dependent on AMP. Thus, the activity of the tricarboxylic acid cycle (TCC) decreases, isocitrate accumulates and is equilibrated with citrate. Citrate is transported out of the mitochondria and in the cytoplasm it is converted into acetyl-CoA and oxaloacetate by the ATP citrate lyase (ACL). Oxaloacetate is channelled back to the mitochondria, while acetyl-CoA is the substrate of the acetyl-CoA carboxylase (ACC) which forms malonyl-CoA, the substrate of the fatty acid synthase (FAS) to elongate the acyl-CoA chain (Tehlivets et al. 2007). Nitrogen concentration should, however, not be below a certain threshold, as under those circumstances, citrate is secreted from the cells, decreasing the lipid yield (Morin et al. 2011; Ratledge and Wynn 2002). Lipid accumulation also occurs upon P and S and other non-carbon limitations, but in these cases, the cellular processes are less well documented. FAS requires NADPH as co-factor, and although there are several enzymes in the cell producing NADPH, malic enzyme was the sole enzyme supposedly involved in fatty acid synthesis. In the transhydrogenase cycle, pyruvate is carboxylated to oxaloacetate, which is converted into malate (NADH dependent). Malic enzyme converts malate into pyruvate, generating CO_2 and NADPH. A physical interaction between malic enzyme and other lipid synthesis enzymes has been suggested (Ratledge and Wynn 2002). However, in many yeasts, no cytoplasmic malic enzyme has been found. The oleaginous yeast *L. starkeyi* possesses a cytoplasmic malic enzyme, but this has a preference for NADH over NADPH (Tang et al. 2010). *Y. lipolytica* contains only a mitochondrial malic enzyme, and its overexpression did not affect lipid accumulation (Beopoulos et al. 2011). Malic enzyme has not been identified among the highly expressed enzymes in proteome studies of *L. starkeyi* and *R. toruloides*; instead, upregulation of the NADPH-generating 6-P-gluconat dehydrogenase has been reported (Liu et al. 2009a, 2011). However, an increased level of malic enzyme

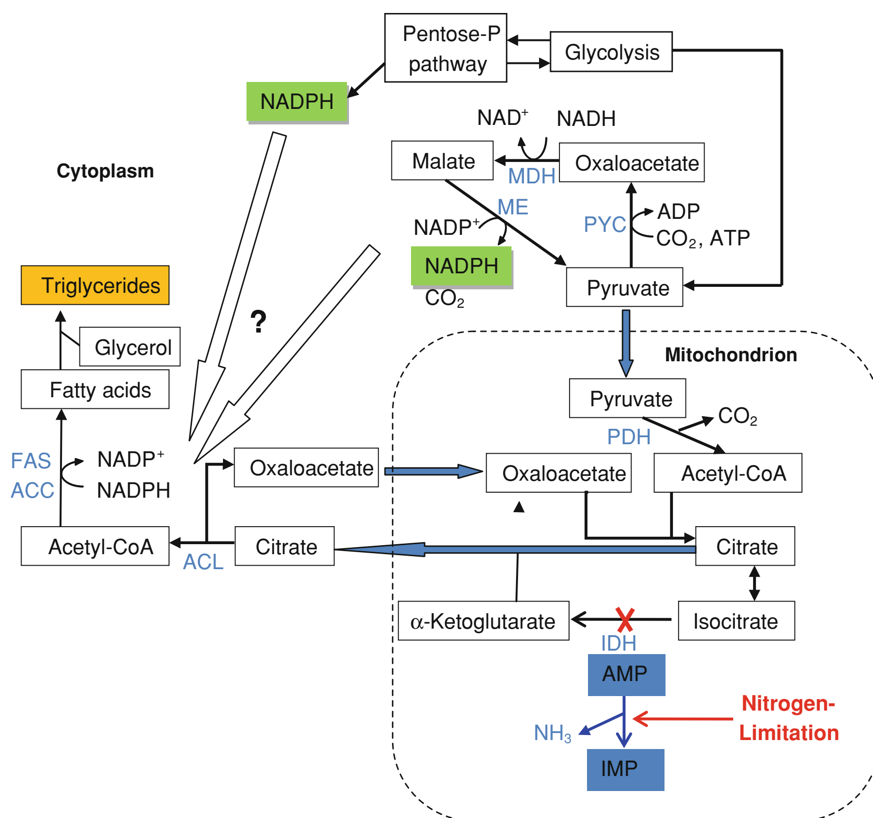


Fig. 9.5 Lipid production in oleaginous yeasts. Sugars are metabolised via glycolysis and the pentose phosphate pathway to pyruvate, which is transported into the mitochondria, converted into acetyl-CoA by PDH and further metabolised via the TCC. In oleaginous yeasts, isocitrate dehydrogenase (IDH) depends on AMP. At nitrogen limitation, AMP is deaminated to IMP, halting the IDH reaction. Isocitrate accumulates and is equilibrated with citrate. Citrate is transported out of the mitochondria and degraded to acetyl-CoA and oxaloacetate by the ACL. Oxaloacetate can be transported back to the mitochondria and feed the TCC. Acetyl-CoA is substrate of the fatty acid synthesising enzymes ACC and FAS. ACC forms malonyl CoA from two acetyl-CoA. Malonyl-CoA reacts in the FAS reaction with acyl-CoA, prolonging the chain by two carbons. Finally, the fatty acids react with glycerol and form, in several steps, triglycerides. The source of the NADPH that is required for the FAS reaction is unclear. It is supposedly generated in the transhydrogenase cycle of pyruvate carboxylase (PYC), malate dehydrogenase (MDH) and malate enzyme (ME), but some recent studies rather indicated that the pentose-P pathway might be the source of NADPH (see text)

upon lipid production conditions has been recently observed in *R. toruloides* (Zhu et al. 2012). Transcriptome analysis of *Y. lipolytica* in nitrogen-limited fed-batch culture showed significant transcriptional regulation of 569 genes. Interestingly, genes encoding assumed key enzymes for fatty acid synthesis like ACL, ACC, FAS or malic enzyme were not transcriptionally regulated, similar to genes

encoding the TCC enzyme IDH (Morin et al. 2011). In the *L. starkeyi* and *R. toruloides* proteomes, ACC was more abundant in lipid accumulation conditions (Liu et al. 2009a, 2011), indicating either a physiological difference to *Y. lipolytica* or regulation at the post-transcriptional level. However, fatty acid accumulation seems to be mainly dependent on regulation of enzyme activities, with inactivation of IDH due to AMP degradation as a key event. In general, nitrogen assimilation enzymes were upregulated at onset of lipid accumulation, which is most likely a physiological response to nitrogen limitation. On the other hand, enzymes involved in glycolysis and the pentose phosphate pathway were downregulated, probably as a response to prevent carbon overflow of the metabolism (Liu et al. 2009a, 2011; Morin et al. 2011). Similar results have been reported in a recently performed multi-omic study of *R. toruloides*, especially in terms of both transcription and protein concentrations of enzymes involved in nitrogen metabolism. However, enhanced transcription of genes encoding FAS has been noted; transcription of most of the other genes involved in fatty acid synthesis was not significantly altered. On a protein level, increased levels of several enzymes involved in fatty acid synthesis have been observed, including, apart from ACC, also ACL, FAS and ME. The behaviour of ME was, to some extent, unusual, as its transcription was decreased under these conditions. This once again shows that not all cellular processes influencing lipid production are yet well understood (Zhu et al. 2012). For lipid production from oleaginous yeasts, obviously cultivation conditions have to be carefully optimised. The optimal pH value differs from species to species, different C:N ratios from 25 to 100 have also been stated to be optimal (Ageitos et al. 2011; Shen et al. 2013; Ykema et al. 1986). Growth rate and lipid production have been found to be inversely correlated (although at very low growth rates, lipid content may decrease), and thus, a compromise between yield and volume-time productivity must be made (Shen et al. 2013; Ykema et al. 1986). Thus, fed-batch cultivation where the growth rate can be regulated probably represents the most efficient fermentation technique for lipid production (Beopoulos et al. 2011).

9.12 Metabolic Engineering to Improve Biodiesel Production by Yeasts

Analysing and manipulating the metabolism of oleaginous yeasts is severely hampered due to the paucity of molecular tools for genetic engineering of these yeasts. Recently, experiments to improve lipid formation in oleaginous yeasts have been performed by a kind of evolutionary engineering. After random mutagenesis, cells of the yeasts *R. glutinis* and *L. starkeyi* were plated onto medium containing cerulenin, an inhibitor of lipid synthesis. Isolates that were able to form bigger colonies accumulated higher amounts of intracellular lipids (Tapia et al. 2012; Wang et al. 2009). Identifying the altered genes in those mutants may identify

targets for improvement of lipid accumulation by oleaginous yeasts. *Y. lipolytica*, which can convert glucose, acids, glycerol and hydrophobic substances such as alkanes into fatty acids, is the only oleaginous yeast for which molecular genetic tools have been developed (Beopoulos et al. 2009). However, in some oleaginous yeasts, initial efforts have been taken to perform molecular manipulation (e.g. Tully and Gilbert 1985), and, with increasing interest in these yeasts, these tools may rapidly be developed. In *Y. lipolytica*, disruption of *GUT2*, encoding a glycerol-3-P dehydrogenase that converts glycerol-3-P into dihydroxyacetone, together with the disruption of genes encoding acyl-CoA oxidases (*POX1-6*) involved in beta oxidation of fatty acids, resulted in a substantial increase in lipid production (Beopoulos et al. 2008).

Several efforts have been undertaken to engineer *S. cerevisiae* to produce biodiesel. *S. cerevisiae* is not oleaginous; in fact, storage lipid synthesis is not essential for this yeast (Sandager et al. 2002). However, it is an established cell factory with well-developed tools for molecular manipulation and a well-investigated metabolism. In yeasts, acetyl-CoA, the precursor of fatty acid synthesis, is formed from pyruvate: in the cytosol, by the action of pyruvate decarboxylase, aldehyde dehydrogenase and acetyl-CoA synthase; and in the mitochondria by pyruvate dehydrogenase (PDH) (Holzer and Goedde 1957). In contrast to oleaginous yeasts, the pathway for synthesising cytosolic acetyl-CoA from surplus citrate from the mitochondria is absent in *S. cerevisiae* (Beopoulos et al. 2011). Thus, increasing the intracellular acetyl-CoA level and redirecting the flux from ethanol production towards producing precursors of lipids is one of the challenges when producing biodiesel or related products from *S. cerevisiae*. An increased acetyl-CoA level has been achieved by overexpressing the aldehyde dehydrogenase gene *ALD6* and a mutated acetyl-CoA synthase gene from *Salmonella enterica* in *S. cerevisiae* (Shiba et al. 2007). This system was further improved by (Chen et al. 2013), who additionally overexpressed *ADH2*, encoding the assimilatory alcohol dehydrogenase. Moreover, *ERG10*, encoding an acetyl-CoA acetyltransferase, was overexpressed. By this strategy, substantial amounts of acetyl-CoA were redirected from ethanol and biomass production towards desired compounds, in this case the production of α-santalene. This platform can be the basis for producing a variety of compounds, including biodiesel or n-butanol. Overexpression of the genes of the isoprenoid synthesis pathway and repressing ergosterol synthesis, or overexpressing a phosphatase dephosphorylating farnesol pyrophosphate resulted in strains overproducing farnesol, which can be used as biodiesel or jet fuel (Hong and Nielsen 2012; Zhang et al. 2011). Expression of heterologous wax synthases in *S. cerevisiae* for in vivo production of FAEE, which can be directly used as biodiesel, has also been reported (Kalscheuer et al. 2004; Shi et al. 2012). Overexpression of ACC additionally increased FAEE production by 30 %, resulting in a biodiesel production of 8.2 mg/l (Shi et al. 2012).

Glycerol is a side product from transesterification and its conversion into biofuels can contribute to sustainability of biodiesel production. By overexpression of genes of the glycerol assimilation pathway (glycerol kinase, *GUT1*) and the triacylglycerol formation pathway (diacylglycerol acyltransferase, *DGA1* and

LRO1), lipid concentrations of 23 mg/l could be obtained from glycerol as sole carbon source (Yu et al. 2013). Overexpressing the glycerol assimilation pathway (including a glycerol transport protein) and disruption of the genes of dihydroxyacetone phosphate degradation and glycerol export resulted in ethanol production from glycerol in *S. cerevisiae*. When a wax ester synthase was also overexpressed, the engineered strain was able to condense the formed ethanol with externally added oleic acid to ethyl oleate, thus representing FAEE production from glycerol (Yu et al. 2012).

9.13 Biodiesel Production from Lignocellulose

Lignocellulose hydrolysate seems to have a good potential for lipid production, as it usually has a high C/N ratio (Hyvönen et al. 2000; Reinertsen et al. 1984). Moreover, most oleaginous yeasts (unfortunately, except the well-investigated yeast *Y. lipolytica*) have the potential to assimilate xylose and other sugars present in lignocellulose hydrolysate (Kurtzman et al. 2011). On the other hand, the inhibitors released during pretreatment (Fig. 9.1) also influence oleaginous yeasts. A variety of oleaginous yeast species have been tested to convert residue materials such as wheat and rice straw, corn stover hydrolysate or sewage sludge into lipids (Angerbauer et al. 2008; Galafassi et al. 2012; Huang et al. 2009; Yu et al. 2011). The final lipid concentrations in these experiments rarely reached more than 10 g/l, which was relatively low compared to, say, a high cell density cultivation of an *R. toruloides* strain on glucose, where a lipid concentration of 151.5 g/l was obtained (Li et al. 2007). However, most of these tests were performed in batch cultivation, so there is still a great potential to optimise the fermentations. Remarkably, the diversion between growth and lipid accumulation as observed in artificial growth medium was not seen in these experiments. Lipid accumulation mainly followed biomass formation. In later stages of fermentation, the lipid content remained constant or slightly decreased, while the biomass was still increasing. Thus, the lipid proportion on the total biomass was relatively low towards the end of the fermentation (Huang et al. 2009; Yu et al. 2011). Inhibitors had differing effects on growth and lipid accumulation. Reports on the inhibitory action of acetate are conflicting to some extent. In a screening experiment, 5 g/l acetate completely prevented growth of strains of *L. starkeyi*, *R. glutinis* and *R. toruloides*. *Trichosporon cutaneum* could grow at this concentration, but was strongly inhibited (Chen et al. 2009). Similarly, *Rhodotorula graminis* was already inhibited at acetate concentrations above 2 g/l (Galafassi et al. 2012). However, only a slight inhibitory effect of acetate on the growth and even some stimulation of lipid accumulation was observed for *R. toruloides* (Hu et al. 2009). Obviously, species and strains differ substantially. In some studies, acetate seemed to be rather a substrate than an inhibitor (Lian et al. 2012; Yu et al. 2011). In mixed substrate, it was consumed faster than the sugars in the medium, and it contributed to lipid accumulation

(Yu et al. 2011). HMF, which is a strong inhibitor during ethanol production, does not seem to have a similar deleterious effect on lipid production, whereas furfural and vanillin were toxic for the yeasts (Chen et al. 2009; Hu et al. 2009).

9.14 Lipid Extraction from Yeasts

In contrast to ethanol, lipids are not secreted into the medium and thus have to be extracted from the cells. The relatively robust cell walls represent a serious barrier for extraction. Moreover, several other lipophilic compounds are present in the lipid bodies, which have to be removed before using the fatty acids to produce biodiesel. Thus, lipid extraction from the cells represents a major challenge in establishing yeast-based biodiesel production. Several extraction methods have been tested at pilot scale, mainly by using ethanol–hexane mixtures, but further optimisation is required to obtain an optimal process running under commercial conditions (Ageitos et al. 2011; Jacob 1992). The final step in biodiesel production, transesterification, is currently mainly done with the help of alkaline catalysts, which can result in undesirable saponification reactions with biolipids, due to the high availability of free fatty acids. Thus, the development of an economically viable enzyme-based transesterification is critical for developing a commercial biodiesel process based on microbes (Azócar et al. 2010; Robles-Medina et al. 2009).

Forcing secretion of the formed lipids out of the cell might be an alternative to extraction. Recently, experiments were performed in bacteria to express transporters exporting hydrophobic molecules out of the cell (Dunlop et al. 2011). In particular, the approach of expressing specific ABC transporters may be also relevant for yeasts, as ABC transporters are ubiquitous among all kingdoms of life. When the transporters are expressed under an inducible promoter, the cells can, after a lipid-accumulating cultivation, be transferred into a biphasic system with an aquatic and an organic phase. Secreted lipids will then accumulate in the organic phase, while the cells stay in the liquid phase. After secreting the lipids, the cells can be re-used in further fermentations (Doshi et al. 2013). Another approach makes use of the native capacity of yeasts to excrete esterified fatty acids. Overexpression of a gene encoding Acyl-coenzyme A: ethanol O-transferase can result in the production of fatty acid ethyl esters. Moreover, it has been shown that *Candida tropicalis* excretes free fatty acids to the medium when transferred to oxygen limited conditions (Phadnavis and Jensen 2013).

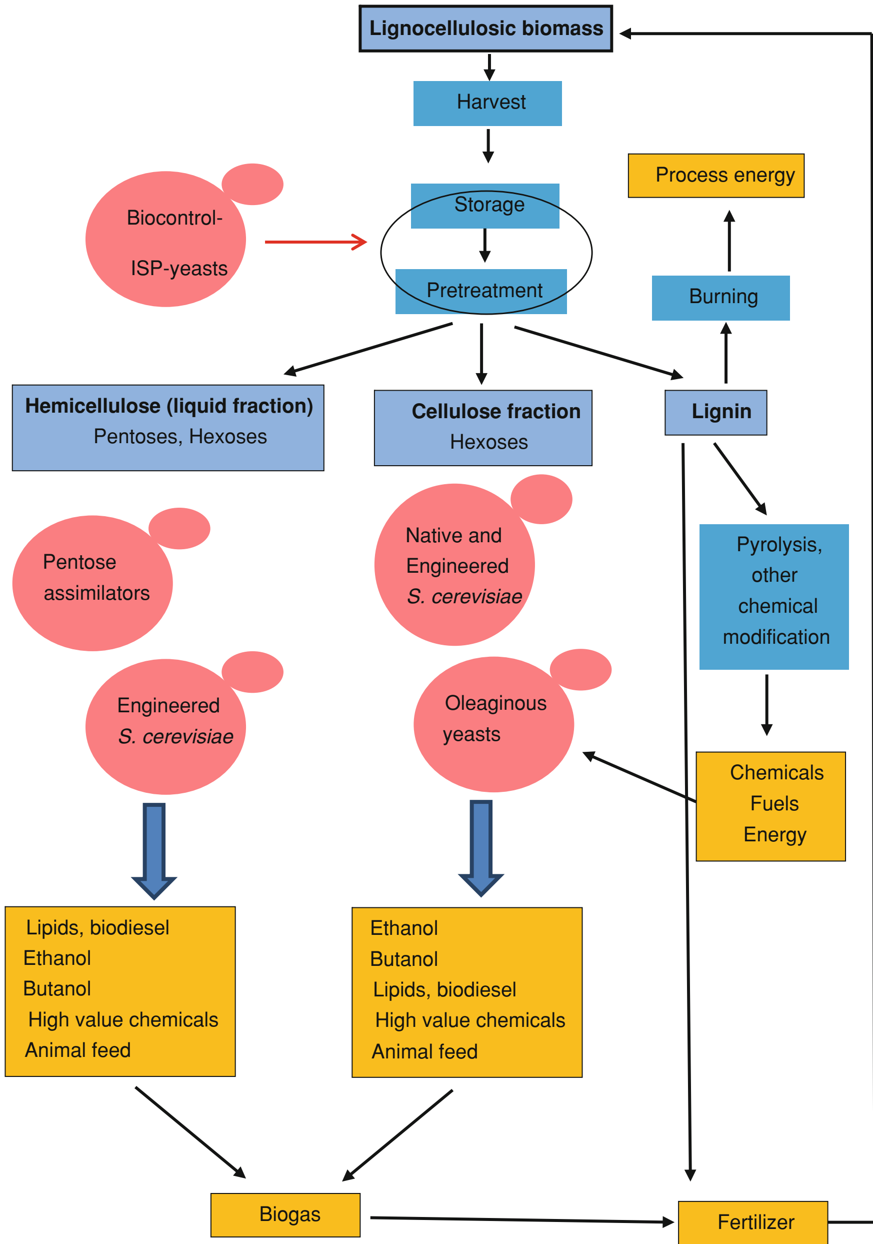
9.15 Outlook: Yeasts in a Biofuel/Biochemicals Refinery

The above-mentioned examples illustrate that, based on knowledge of physiology appropriate yeasts can be selected, processes can be optimised and metabolic pathways can be manipulated, which leads to substantial improvements in

producing the desired biofuels. However, it should be noted that currently there is almost no commercial scale biofuel production from second-generation raw materials, except biogas production, which is not yeast based. As mentioned above, high costs are among the major obstacles to commercialising second-generation biofuel production (Stephen et al. 2012; Cheng and Timilsina 2011). Integration of the different steps of handling lignocellulose biomass is a way of improving the process economy. Biomass handling for biofuel production includes growth, harvest, storage, pretreatment, fermentation, handling side and residual products, and if possible, generating value out of them (Vanholme et al. 2013; Liguori et al. 2013). Several of these partial processes can make use of the metabolic capacities of yeasts (Fig. 9.6).

Storage of biomass is an essential process, as biomass is seasonally produced, whereas it is highly desirable that biofuel production facilities run continuously. Therefore, it is necessary to preserve the harvested biomass until use. The most frequently used and safest means of biomass preservation is drying; however, especially in temperate climates, this can require a substantial input of energy (Olstorpe and Passoth 2011). In the case of lignocellulose material such as straw, which is usually passively dried in the field, excessively high moisture contents can result in losses of produced biomass (Nilsson 2000). For the handling of feed biomass, several methods of biopreserving moist biomass have been developed (Olstorpe and Passoth 2011; Zheng et al. 2011). When these methods were applied to preserve raw materials for biofuel production, yeast-based biopreservation of the moist biomass not only saved the energy that would have been consumed for drying, but also made the biomass more accessible for the subsequent pretreatment. Biopreservation of wheat straw with a yeast able to partially degrade hemicellulose (*S. stipitis*) had a positive effect on the pretreatment efficiency (Passoth et al. 2009, 2013). Integration of storage and pretreatment can thus streamline the pretreatment input in the process. This is one of the most critical issues in obtaining sustainable biofuel production, as pretreatment contributes to high costs, requires a major input of energy and releases inhibitors (Sassner et al. 2008). Consolidated bioprocessing, in which enzymatic degradation of the biomass and ethanol production are combined, represents another opportunity to reduce the impact of pretreatment (Hasunuma and Kondo 2012).

Handling fermentation residues from lignocellulose-based ethanol production is an important issue for the total process. Fermentation residues are nutrient rich and have a high COD value, and their cleaning represents a major cost factor (Wilkie et al. 2000). Biogas production is one opportunity for generating value out of the costly residues. Interestingly, some studies indicated that biogas production from lignocellulose was positively influenced when the material was fermented to ethanol and the residues were introduced into the biogas process. The total energy output from a combined ethanol/biogas production was higher than biogas production alone, and the biogas production rate was also enhanced. In this way, ethanol production acted like a pretreatment for the biogas process (Dererie et al. 2011; Kreuger et al. 2011). Residues from biogas production, in turn, have very



Lignocellulosic biomass
Harvest
Storage
Pretreatment
Biocontrol-
ISP-yeasts
Process energy
Burning
Hemicellulose (liquid fraction)
Pentoses, Hexoses
Cellulose fraction
Hexoses
Lignin
Pentose
assimilators
Native and
Engineered
S. cerevisiae
Pyrolysis,
other
chemical
modification
Engineered
S. cerevisiae
Oleaginous
yeasts
Chemicals
Fuels
Energy
Lipids, biodiesel
Ethanol
Butanol
High value chemicals
Animal feed
Ethanol
Butanol
Lipids, biodiesel
High value chemicals
Animal feed
Biogas
Fertilizer

◄ **Fig. 9.6** Integrative approach for generating biofuels and chemicals from non-food (lignocellulose) biomass and the role of yeasts in such a process. After harvest, biomass has to be preserved until pretreatment, which includes thermochemical and enzymatic processing. Biocontrol yeasts can be used for low-energy biopreservation of the biomass. Certain yeasts (ISP yeasts) can even de-stabilise the material during storage, and by this, integrate storage and pretreatment (ISP). After thermochemical treatment, most of the hemicellulose is present in the liquid fraction, whereas the cellulose together with the lignin is present in the solid fraction. If necessary, both can be separated by further treatment steps. Enzymatic treatment releases monosaccharides from the polysaccharides. The sugars can be converted into biofuels, chemicals and animal feed using appropriate yeasts. Residues from yeast fermentations can be converted into biogas. Lignin that cannot be degraded to ethanol or biogas can be burned to obtain process energy, or it can be converted into chemicals and biofuels by chemical processes (pyrolysis). Residues from pyrolysis can be transformed into lipids using oleaginous yeasts. Biogas residues and lignin are excellent fertilisers to produce new biomass

good potential as fertilisers and can thus close the loop to generate new biomass for biofuel and food production (Odlare et al. 2011).

Residues from first-generation raw material ethanol production are frequently used as animal feed. For instance, in the EU, feeding fermentation residues to animals may result in saving 0.7 Mio ha maize cultivation area for animal feed production (Özdemir et al. 2009). Utilisation of the fermentation residues as animal feed in a corn-based ethanol production process contributed to about one-sixth of the total energy output and was essential to obtain a positive energy balance for the whole process (Hill et al. 2006). However, residues from lignocellulose-based fermentation may not be suitable for direct incorporation into animal feed, due to inhibitors and lignin-derived compounds that influence palatability. Recently, the extraction of proteins from lignocellulose to use them as animal feed or even human food has been suggested (Chiesa and Gnansounou 2011). As discussed above, biofuel production from first-generation raw materials may raise ethical concerns (Buyx and Tait 2011) and even lignocellulosic feedstock may compete with food production. Thus, feed and food production from side streams of biofuel production may be a way to overcome potential food versus fuel debates. Moreover, as animal feed production in particular is one of the major consumers of fossil resources and arable land in agriculture, feed generation from biofuel production will substantially improve the overall environmental balance of the entire process (Graham-Rowe 2011).

In a future biofuel refinery, different compounds will be generated according to corresponding demands. The pentose fraction can primarily be used to produce: biodiesel using naturally pentose-assimilating oleaginous yeasts; bioethanol, using engineered *S. cerevisiae*; or yeast biomass for animal feed. The hexose fraction is the preferred substrate for ethanol and butanol production; lipid generation by oleaginous yeasts or engineered *S. cerevisiae* is also possible. Apart from this, high value compounds, e.g. platform chemicals for the pharmaceutical or cosmetic industries can be co-generated, which will significantly improve the total economic basis of the biofuel process (Nielsen et al. 2013; Zhang et al. 2011). Lignin

is difficult to degrade due to its complex structure. It is typically burned to generate process energy. However, it is also possible to convert it into valuable chemicals (Zhu and Pan 2010). Pyrolysis is one method to obtain low molecular weight chemicals from lignin and other compounds of lignocellulose, and during this process, several residues, including carboxylic acids, are generated. These acids can be converted into lipids by oleaginous yeasts (Lian et al. 2012). When used as fertiliser, lignin plays an important role for the carbon balance of soil (Jarecki and Lal 2003).

This chapter illustrates that biofuel-related yeast research has undergone an impressive development: based on the bulk of knowledge about yeast physiology, culture conditions and metabolic pathways, cells have been manipulated to optimise production of the desired biofuel. The ongoing efforts to obtain strains producing sufficient amounts of biofuels or platform chemicals in an industrial environment, and in integrating the processing steps of a biorefinery, are, in their turn, boosting scientific developments towards understanding yeast and thus eukaryotic physiology. In this way, biofuel research can be seen as an excellent example of mutual positive effects when combining fundamental science with an emerging technology development.

Acknowledgments I gratefully acknowledge the support for the biofuel-related research of my group from the *MicroDrivE* programme of the Swedish University of Agricultural Sciences, the Swedish Energy Authority (Energimyndigheten), the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas) and the Visby programme of the Swedish Institute. I wish to thank Mattias Carlsson for his advices during writing the manuscript and Dr Su-Lin Leong for linguistic advice.

References

Ageitos J, Vallejo J, Veiga-Crespo P, Villa T (2011) Oily yeasts as oleaginous cell factories. Appl Microbiol Biotechnol 90(4):1219–1227. doi:10.1007/s00253-011-3200-z

Allen SA, Clark W, McCaffery JM, Cai Z, Lanctot A, Slininger PJ, Liu ZL, Gorsich SW (2010) Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol Biofuels 3:2. doi:10.1186/1754-6834-3-2

Amore R, Wilhelm M, Hollenberg CP (1989) The fermentation of xylose—an analysis of the expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast. Appl Microbiol Biot 30:351–357

Amorim HV, Lopes ML, de Castro Oliveira JV, Buckeridge MS, Goldman GH (2011) Scientific challenges of bioethanol production in Brazil. Appl Microbiol Biotechnol 91(5):1267–1275. doi:10.1007/s00253-011-3437-6

Angerbauer C, Siebenhofer M, Mittelbach M, Guebitz GM (2008) Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. Bioresour Technol 99(8):3051–3056. doi:http://dx.doi.org/10.1016/j.biortech.2007.06.045

Ansell R, Granath K, Hohmann S, Thevelein JM, Adler L (1997) The two isoenzymes for yeast NAD^+ -dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J 16:2179–2187

Attfield PV, Bell PJ (2006) Use of population genetics to derive nonrecombinant *Saccharomyces cerevisiae* strains that grow using xylose as a sole carbon source. FEMS Yeast Res 6(6):862–868. doi:10.1111/j.1567-1364.2006.00098.x

Avalos JL, Fink GR, Stephanopoulos G (2013) Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. Nat Biotech 31(4):335–341. doi:10.1038/nbt.2509

Azócar L, Ciudad G, Heipieper HJ, Navia R (2010) Biotechnological processes for biodiesel production using alternative oils. Appl Microbiol Biotechnol 88(3):621–636. doi:10.1007/s00253-010-2804-z

Babrzadeh F, Jalili R, Wang C, Shokralla S, Pierce S, Robinson-Mosher A, Nyren P, Shafer R, Basso L, Amorim H, Oliveira A, Davis R, Ronaghi M, Gharizadeh B, Stambuk B (2012) Whole-genome sequencing of the efficient industrial fuel-ethanol fermentative *Saccharomyces cerevisiae* strain CAT-1. Mol Genet Genomics 287(6):485–494. doi:10.1007/s00438-012-0695-7

Basso LC, De Amorim HV, De Oliveira AJ, Lopes ML (2008) Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res 8(7):1155–1163. doi:10.1111/j.1567-1364.2008.00428.x

Basso TO, de Kok S, Dario M, do Espirito-Santo JC, Müller G, Schlölg PS, Silva CP, Tonso A, Daran JM, Gombert AK, van Maris AJ, Pronk JT, Stambuk BU (2011) Engineering topology and kinetics of sucrose metabolism in *Saccharomyces cerevisiae* for improved ethanol yield. Metab Eng 13(6):694–703. doi:10.1016/j.ymben.2011.09.005

Beopoulos A, Cescut J, Haddouche R, Uribelarrea JL, Molina-Jouve C, Nicaud JM (2009) *Yarrowia lipolytica* as a model for bio-oil production. Prog Lipid Res 48(6):375–387. doi:10.1016/j.plipres.2009.08.005

Beopoulos A, Mrozova Z, Thevenieau F, Le Dall MT, Hapala I, Papanikolaou S, Chardot T, Nicaud JM (2008) Control of lipid accumulation in the yeast *Yarrowia lipolytica*. Appl Environ Microbiol 74(24):7779–7789. doi:10.1128/AEM.01412-08

Beopoulos A, Nicaud JM, Gaillardin C (2011) An overview of lipid metabolism in yeasts and its impact on biotechnological processes. Appl Microbiol Biotechnol 90(4):1193–1206. doi: 10.1007/s00253-011-3212-8

Bergdahl B, Heer D, Sauer U, Hahn-Hägerdal B, van Niel EW (2012) Dynamic metabolomics differentiates between carbon and energy starvation in recombinant *Saccharomyces cerevisiae* fermenting xylose. Biotechnol Biofuels 5(1):34. doi:10.1186/1754-6834-5-34

Blomqvist J, Eberhard T, Schnürer J, Passoth V (2010) Fermentation characteristics of *Dekkera bruxellensis* strains. Appl Microbiol Biotechnol 87(4):1487–1497. doi:10.1007/s00253-010-2619-y

Blomqvist J, Nogue VS, Gorwa-Grauslund M, Passoth V (2012) Physiological requirements for growth and competitiveness of *Dekkera bruxellensis* under oxygen-limited or anaerobic conditions. Yeast 29(7):265–274. doi:10.1002/yea.2904

Branduardi P, Longo V, Berterame NM, Rossi G, Porro D (2013) A novel pathway to produce butanol and isobutanol in *Saccharomyces cerevisiae*. Biotechnol Biofuels 6(1):68. doi:10.1186/1754-6834-6-68

Brat D, Boles E (2013) Isobutanol production from D-xylose by recombinant *Saccharomyces cerevisiae*. FEMS Yeast Res 13(2):241–244. doi:10.1111/1567-1364.12028

Brat D, Boles E, Wiedemann B (2009) Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Appl Environ Microbiol 75(8):2304–2311. doi:10.1128/AEM.02522-08

Brat D, Weber C, Lorenzen W, Bode HB, Boles E (2012) Cytosolic re-localization and optimization of valine synthesis and catabolism enables inseased isobutanol production with the yeast *Saccharomyces cerevisiae*. Biotechnol Biofuels 5(1):65. doi:10.1186/1754-6834-5-65

Bruinenberg PM, de Bot PHM, van Dijken JP, Scheffers WA (1984) NADH-linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts. Appl Microbiol Biotechnol 19:256–260

Buijs NA, Siewers V, Nielsen J (2013) Advanced biofuel production by the yeast *Saccharomyces cerevisiae*. Curr Opin Chem Biol 17(3):480–488. doi:10.1016/j.cbpa.2013.03.036
Buyx A, Tait J (2011) Ethics. Ethical framework for biofuels. Science 332(6029):540–541. doi:10.1126/science.1206064
Caspeta L, Buijs NAA, Nielsen J (2013) The role of biofuels in the future energy supply. Energy Environ Sci 6(4):1077–1082. doi:10.1039/c3ee24403b
Chen X, Li Z, Zhang X, Hu F, Ryu DY, Bao J (2009) Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. Appl Biochem Biotechnol 159(3):591–604. doi:10.1007/s12010-008-8491-x
Chen Y, Daviet L, Schalk M, Siewers V, Nielsen J (2013) Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metab Eng 15:48–54. doi:10.1016/j.ymben.2012.11.002
Cheng JJ, Timilsina GR (2011) Status and barriers of advanced biofuel technologies: A review. Renewable Energy 36(12):3541–3549. doi:10.1016/j.renene.2011.04.031
Chiesa S, Gnansounou E (2011) Protein extraction from biomass in a bioethanol refinery–possible dietary applications: use as animal feed and potential extension to human consumption. Bioresour Technol 102(2):427–436. doi:10.1016/j.biortech.2010.07.125
de Souza Liberal AT, Basilio AC, do Monte Resende A, Brasileiro BT, da Silva-Filho EA, de Morais JO, Simoes DA, de Morais MA, Jr (2007) Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. J Appl Microbiol 102(2):538–547
Della-Bianca B, Basso T, Stambuk B, Basso L, Gombert A (2013) What do we know about the yeast strains from the Brazilian fuel ethanol industry? Appl Microbiol Biotechnol 97(3):979–991. doi:10.1007/s00253-012-4631-x
Demeke M, Dumortier F, Li Y, Broeckx T, Foulquie-Moreno M, Thevelein J (2013a) Combining inhibitor tolerance and D-xylose fermentation in industrial *Saccharomyces cerevisiae* for efficient lignocellulose-based bioethanol production. Biotechnol Biofuels 6(1):120. doi:10.1186/1754-6834-6-120
Demeke MM, Dietz H, Li Y, Foulquie-Moreno MR, Mutturi S, Deprez S, Den Abt T, Bonini BM, Lidén G, Dumortier F, Verplaetse A, Boles E, Thevelein JM (2013b) Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. Biotechnol Biofuels 6(1):89. doi:10.1186/1754-6834-6-89
Dererie DY, Trobro S, Momeni MH, Hansson H, Blomqvist J, Passoth V, Schnürer A, Sandgren M, Ståhlberg J (2011) Improved bio-energy yields via sequential ethanol fermentation and biogas digestion of steam exploded oat straw. Bioresour Technol 102(6):4449–4455. doi:10.1016/j.biortech.2010.12.096
Dmytruk OV, Voronovsky AY, Abbas CA, Dmytruk KV, Ishchuk OP, Sibirny AA (2008) Overexpression of bacterial xylose isomerase and yeast host xylulokinase improves xylose alcoholic fermentation in the thermotolerant yeast *Hansenula polymorpha*. FEMS Yeast Res 8(1):165–173. doi:10.1111/j.1567-1364.2007.00289.x
Doshi R, Nguyen T, Chang G (2013) Transporter-mediated biofuel secretion. Proc Natl Acad Sci USA 110(19):7642–7647. doi:10.1073/pnas.1301358110
Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A (2011) Engineering microbial biofuel tolerance and export using efflux pumps. Mol Syst Biol 7:487. doi:10.1038/msb.2011.21
Fujitomi K, Sanda T, Hasunuma T, Kondo A (2012) Deletion of the *PHO13* gene in *Saccharomyces cerevisiae* improves ethanol production from lignocellulosic hydrolysate in the presence of acetic and formic acids, and furfural. Bioresour Technol 111:161–166. doi:10.1016/j.biortech.2012.01.161
Galafassi S, Cucchetti D, Pizza F, Franzosi G, Bianchi D, Compagno C (2012) Lipid production for second generation biodiesel by the oleaginous yeast *Rhodotorula graminis*. Bioresour Technol 111:398–403. doi:http://dx.doi.org/10.1016/j.biortech.2012.02.004

Garcia Sanchez R, Hahn-Hägerdal B, Gorwa-Grauslund M (2010a) Cross-reactions between engineered xylose and galactose pathways in recombinant *Saccharomyces cerevisiae*. Biotechnol Biofuels 3(1):19

Garcia Sanchez R, Karhumaa K, Fonseca C, Sànchez Nogué V, Almeida JR, Larsson CU, Bengtsson O, Bettiga M, Hahn-Hägerdal B, Gorwa-Grauslund MF (2010b) Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. Biotechnol Biofuels 3:13. doi:10.1186/1754-6834-3-13

Girio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Lukasik R (2010) Hemicelluloses for fuel ethanol: a review. Bioresour Technol 101(13):4775–4800. doi:10.1016/j.biortech.2010.01.088

Gnansounou E (2010) Production and use of lignocellulosic bioethanol in Europe: current situation and perspectives. Bioresour Technol 101(13):4842–4850. doi:10.1016/j.biortech.2010.02.002

Graham-Rowe D (2011) Agriculture: beyond food versus fuel. Nature 474(7352):S6–S8

Green EM (2011) Fermentative production of butanol- the industrial perspective. Curr Opin Biotechnol 22(3):337–343. doi:10.1016/j.copbio.2011.02.004

Guadalupe-Medina V, Metz B, Oud B, van Der Graaf CM, Mans R, Pronk JT, van Maris AJ (2013) Evolutionary engineering of a glycerol-3-phosphate dehydrogenase-negative, acetate-reducing *Saccharomyces cerevisiae* strain enables anaerobic growth at high glucose concentrations. Microb Biotechnol. doi:10.1111/1751-7915.12080

Guadalupe Medina V, Almering MJ, van Maris AJ, Pronk JT (2010) Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. Appl Environ Microbiol 76(1):190–195. doi:10.1128/AEM.01772-09

Guo ZP, Zhang L, Ding ZY, Shi GY (2011) Minimization of glycerol synthesis in industrial ethanol yeast without influencing its fermentation performance. Metab Eng 13(1):49–59. doi:10.1016/j.ymben.2010.11.003

Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF (2007) Towards industrial pentose-fermenting yeast strains. Appl Microbiol Biotechnol 74(5):937–953

Haitani Y, Tanaka K, Yamamoto M, Nakamura T, Ando A, Ogawa J, Shima J (2012) Identification of an acetate-tolerant strain of *Saccharomyces cerevisiae* and characterization by gene expression analysis. J Biosci Bioeng 114(6):648–651. doi:10.1016/j.jbiosc.2012.07.002

Hasunuma T, Kondo A (2012) Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering. Biotechnol Adv 30(6):1207–1218. doi:10.1016/j.biotechadv.2011.10.011

Hasunuma T, Sung K-m, Sanda T, Yoshimura K, Matsuda F, Kondo A (2011) Efficient fermentation of xylose to ethanol at high formic acid concentrations by metabolically engineered *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 90(3):997–1004. doi: 10.1007/s00253-011-3085-x

Hazelwood LA, Daran JM, van Maris AJ, Pronk JT, Dickinson JR (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol 74(8):2259–2266. doi:10.1128/AEM.02625-07

Hellström AM, Vázques-Juárez R, Svanberg U, Andlid TA (2010) Biodiversity and phytase capacity of yeasts isolated from Tanzanian togwa. Int J Food Microbiol 136(3):352–358. doi:10.1016/j.ijfoodmicro.2009.10.011

Hill J, Nelson E, Tilman D, Polasky S, Tiffany D (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci USA 103(30):11206–11210

Holzer H, Goedde HW (1957) Zwei Wege von Pyruvat zu Acetyl-Coenzym A in Hefe. Biochem Z 329(3):175–191

Hong K-K, Nielsen J (2012) Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries. Cell Mol Life Sci 69(16):2671–2690. doi: 10.1007/s00018-012-0945-1

Hu C, Zhao X, Zhao J, Wu S, Zhao ZK (2009) Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. Bioresour Technol 100(20):4843–4847. doi:http://dx.doi.org/10.1016/j.biortech.2009.04.041

Huang C, Zong M-h, Wu H, Liu Q-p (2009) Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*. Bioresour Technol 100(19):4535–4538. doi:http://dx.doi.org/10.1016/j.biortech.2009.04.022

Hyvönen R, Olsson BA, Lundkvist H, Staaf H (2000) Decomposition and nutrient release from *Picea abies* (L.) Karst. and *Pinus sylvestris* L-logging residues. For Ecol Manage 126(2):97–112

Ishchuk OP, Voronovsky AY, Abbas CA, Sibirny AA (2009) Construction of *Hansenula polymorpha* strains with improved thermotolerance. Biotechnol Bioeng 104(5):911–919. doi:10.1002/bit.22457

Ishchuk OP, Voronovsky AY, Stasyk OV, Gayda GZ, Gonchar MV, Abbas CA, Sibirny AA (2008) Overexpression of pyruvate decarboxylase in the yeast *Hansenula polymorpha* results in increased ethanol yield in high-temperature fermentation of xylose. FEMS Yeast Res 8(7):1164–1174. doi:10.1111/j.1567-1364.2008.00429.x

Jacob Z (1992) Yeast lipids- extraction, quality analysis, and acceptability. Crit Rev Biotechnol 12(5–6):463–491. doi:10.3109/07388559209114236

Jarecki MK, Lal R (2003) Crop management for soil carbon sequestration. Crit Rev Plant Sciences 22(6):471–502. doi:10.1080/713608318

Johnson EA (2013) Biotechnology of non-*Saccharomyces* yeasts–the ascomycetes. Appl Microbiol Biotechnol 97(2):503–517. doi:10.1007/s00253-012-4497-y

Kalscheuer R, Luftmann H, Steinbüchel A (2004) Synthesis of novel lipids in *Saccharomyces cerevisiae* by heterologous expression of an unspecific bacterial acyltransferase. Appl Environ Microbiol 70(12):7119–7125. doi:10.1128/AEM.70.12.7119-7125.2004

Karhumaa K, Garcia Sanchez R, Hahn-Hägerdal B, Gorwa-Grauslund MF (2007) Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. Microb Cell Fact 6:5. doi:10.1186/1475-2859-6-5

Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66(1):10–26. doi:10.1007/s00253-004-1642-2

Kondo T, Tezuka H, Ishii J, Matsuda F, Ogino C, Kondo A (2012) Genetic engineering to enhance the Ehrlich pathway and alter carbon flux for increased isobutanol production from glucose by *Saccharomyces cerevisiae*. J Biotechnol 159(1–2):32–37. doi:10.1016/j.jbiotec.2012.01.022

Kötter P, Ciriacy M (1993) Xylose fermentation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 38(6):776–783. doi:10.1007/bf00167144

Kreuger E, Sipos B, Zacchi G, Svensson SE, Björnsson L (2011) Bioconversion of industrial hemp to ethanol and methane: the benefits of steam pretreatment and co-production. Bioresour Technol 102(3):3457–3465. doi:10.1016/j.biortech.2010.10.126

Kuhad RC, Gupta R, Khasa YP, Singh A, Zhang YHP (2011) Bioethanol production from pentose sugars: Current status and future prospects. Renew Sust Energ Rev 15(9):4950–4962. doi:10.1016/j.rser.2011.07.058

Kurtzman CP, Fell JW, Boekhout T (2011) The Yeasts, a taxonomic study, 5th edn. Elsevier, Amsterdam

Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MS, de Laat WT, den Ridder JJ, Op den Camp HJ, van Dijken JP, Pronk JT (2003) High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? FEMS Yeast Res 4(1):69–78

Kuyper M, Hartog MM, Toirkens MJ, Almering MJ, Winkler AA, van Dijken JP, Pronk JT (2005) Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res 5(4–5):399–409

Larsen J, Haven MØ, Thirup L (2012) Inbicon makes lignocellulosic ethanol a commercial reality. Biomass Bioenergy 46(0):36–45. doi:http://dx.doi.org/10.1016/j.biombioe.2012.03.033

Larsen J, Østergaard Petersen M, Thirup L, Wen Li H, Krogh Iversen F (2008) The IBUS process—lignocellulosic bioethanol close to a commercial reality. Chem Eng Technol 31(5):765–772. doi:10.1002/ceat.200800048

Larsson S, Cassland P, Jönsson LJ (2001) Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. Appl Environ Microbiol 67(3):1163–1170. doi:10.1128/AEM.67.3.1163-1170.2001

Leong SL, Niba AT, Ny S, Olstorpe M (2012) Microbial populations during maize storage in Cameroon. Afr J Biotechnol 11(35):8692–8697

Li Y, Zhao Z, Bai F (2007) High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. Enzyme Microb Technol 41(3):312–317. doi:http://dx.doi.org/10.1016/j.enzmictec.2007.02.008

Lian J, Garcia-Perez M, Coates R, Wu H, Chen S (2012) Yeast fermentation of carboxylic acids obtained from pyrolytic aqueous phases for lipid production. Bioresour Technol 118:177–186. doi:10.1016/j.biortech.2012.05.010

Liguori R, Amore A, Faraco V (2013) Waste valorization by biotechnological conversion into added value products. Appl Microbiol Biotechnol 97(14):6129–6147. doi: 10.1007/s00253-013-5014-7

Liu H, Zhao X, Wang F, Jiang X, Zhang S, Ye M, Zhao ZK, Zou H (2011) The proteome analysis of oleaginous yeast *Lipomyces starkeyi*. FEMS Yeast Res 11(1):42–51. doi:10.1111/j.1567-1364.2010.00687.x

Liu H, Zhao X, Wang F, Li Y, Jiang X, Ye M, Zhao ZK, Zou H (2009a) Comparative proteomic analysis of *Rhodosporidium toruloides* during lipid accumulation. Yeast 26(10):553–566. doi:10.1002/yea.1706

Liu ZL (2006) Genomic adaptation of ethanologenic yeast to biomass conversion inhibitors. Appl Microbiol Biotechnol 73(1):27–36. doi:10.1007/s00253-006-0567-3

Liu ZL, Ma M, Song M (2009b) Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways. Mol Genet Genomics 282(3):233–244. doi:10.1007/s00438-009-0461-7

Ludovico P, Sousa MJ, Silva MT, Leão C, Côrte-Real M (2001) *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology 147(Pt 9):2409–2415

Madhavan A, Tamalampudi S, Ushida K, Kanai D, Katahira S, Srivastava A, Fukuda H, Bisaria VS, Kondo A (2009) Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. Appl Microbiol Biotechnol 82(6):1067–1078. doi:10.1007/s00253-008-1794-6

Margeot A, Hahn-Hägerdal B, Edlund M, Slade R, Monot F (2009) New improvements for lignocellulosic ethanol. Curr Opin Biotechnol 20(3):372–380. doi:10.1016/j.copbio.2009.05.009

Martani F, Fossati T, Posteri R, Signori L, Porro D, Branduardi P (2013) Different response to acetic acid stress in *Saccharomyces cerevisiae* wild-type and l-ascorbic acid-producing strains. Yeast 30(9):365–378. doi:10.1002/yea.2969

Matsuda F, Kondo T, Ida K, Tezuka H, Ishii J, Kondo A (2012) Construction of an artificial pathway for isobutanol biosynthesis in the cytosol of *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 76(11):2139–2141

McGovern PE, Zhang J, Tang J, Zhang Z, Hall GR, Moreau RA, Nunez A, Butrym ED, Richards MP, Wang CS, Cheng G, Zhao Z, Wang C (2004) Fermented beverages of pre- and proto-historic China. Proc Natl Acad Sci USA 101(51):17593–17598

Morin N, Cescut J, Beopoulos A, Lelandais G, Le Berre V, Uribelarrea JL, Molina-Jouve C, Nicaud JM (2011) Transcriptomic analyses during the transition from biomass production to lipid accumulation in the oleaginous yeast *Yarrowia lipolytica*. PLoS ONE 6(11):e27966. doi:10.1371/journal.pone.0027966

Nielsen J, Larsson C, van Maris A, Pronk J (2013) Metabolic engineering of yeast for production of fuels and chemicals. Curr Opin Biotechnol 24(3):398–404. doi:10.1016/j.copbio.2013.03.023

Nilsson D (2000) Dynamic simulation of straw harvesting systems: Influence of climatic, geographical and biological factors on performance and costs. J Agric Eng Res 76(1):27–36
Nissen TL, Kielland-Brandt MC, Nielsen J, Villadsen J (2000) Optimization of ethanol production in *Saccharomyces cerevisiae* by metabolic engineering of the ammonium assimilation. Metab Eng 2(1):69–77. doi:10.1006/mben.1999.0140
Odlare M, Arthurson V, Pell M, Svensson K, Nehrenheim E, Abubaker J (2011) Land application of organic waste—Effects on the soil ecosystem. Appl Energy 88(6):2210–2218. doi:10.1016/j.apenergy.2010.12.043
Olstorpe M, Passoth V (2011) *Pichia anomala* in grain biopreservation. Antonie van Leeuwenhoek 99(1):57-62. doi:10.1007/s10482-010-9497-2
Özdemir ED, Härdtlein M, Eltrop L (2009) Land substitution effects of biofuel side products and implications on the land area requirement for EU 2020 biofuel targets. Energ Policy 37(8):2986–2996. doi:10.1016/j.enpol.2009.03.051
Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol 74(1):25–33. doi:http://dx.doi.org/10.1016/S0960-8524(99)00161-3
Passoth V, Blomqvist J, Schnürer J (2007) *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. Appl Environ Microbiol 73(13):4354–4356
Passoth V, Eriksson A, Sandgren M, Ståhlberg J, Piens K, Schnürer J (2009) Airtight storage of moist wheat grain improves bioethanol yields. Biotechnol Biofuels 2(1):16
Passoth V, Tabassum MR, Nair HA, Olstorpe M, Tiukova I, Ståhlberg J (2013) Enhanced ethanol production from wheat straw by integrated storage and pre-treatment (ISP). Enzyme Microb Technol 52(2):105–110. doi:10.1016/j.enzmictec.2012.11.003
Pelletier N, Audsley E, Brodt S, Garnett T, Henriksson P, Kendall A, Kramer KJ, Murphy D, Nemecek T, Troell M (2011) Energy intensity of agriculture and food systems. In: Gadgil A, Liverman DM (eds) Annual review of environment and resources, vol 36. Annual Reviews, Palo Alto, pp 223–246. doi:10.1146/annurev-environ-081710-161014
Phadnavis AG, Jensen PR (2013) Production of biodiesel by yeast from lignocellulose and glycerol. USA Patent US 20130137149A1
Piper PW (2011) Resistance of yeasts to weak organic acid food preservatives. Adv Appl Microbiol 77:97–113. doi:10.1016/B978-0-12-387044-5.00004-2
Qiu H, Sun L, Huang J, Rozelle S (2012) Liquid biofuels in China: current status, government policies, and future opportunities and challenges. Renew Sustain Energy Rev 16(5):3095–3104. doi:http://dx.doi.org/10.1016/j.rser.2012.02.036
Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 51:1–51
Reinertsen SA, Elliott LF, Cochran VL, Campbell GS (1984) Role of available carbon and nitrogen in determining the rate of wheat straw decomposition. Soil Biol Biochem 16(5):459–464. doi:http://dx.doi.org/10.1016/0038-0717(84)90052-X
Richard P, Putkonen M, Vaananen R, Londesborough J, Penttilä M (2002) The missing link in the fungal L-arabinose catabolic pathway, identification of the L-xylulose reductase gene. Biochemistry 41 (20):6432–6437. doi:10.1021/bi025529i
Richard P, Toivari MH, Penttilä M (1999) Evidence that the gene *YLR070c* of *Saccharomyces cerevisiae* encodes a xylitol dehydrogenase. FEBS Lett 457(1):135–138
Robles-Medina A, González-Moreno PA, Esteban-Cerdán L, Molina-Grima E (2009) Biocatalysis: towards ever greener biodiesel production. Biotechnol Adv 27(4):398–408. doi:http://dx.doi.org/10.1016/j.biotechadv.2008.10.008
Rødsrud G, Lersch M, Sjöde A (2012) History and future of world's most advanced biorefinery in operation. Biomass Bioenergy 46(0):46–59. doi:http://dx.doi.org/10.1016/j.biombioe.2012.03.028
Ryabova OB, Chmil OM, Sibirny AA (2003) Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res 4(2):157–164

Sanchez INV, Bettiga M, Gorwa-Grauslund MF (2012) Isolation and characterization of a resident tolerant *Saccharomyces cerevisiae* strain from a spent sulfite liquor fermentation plant. AMB Express 2(1):68. doi:10.1186/2191-0855-2-68

Sandager L, Gustavsson MH, Ståhl U, Dahlqvist A, Wiberg E, Banas A, Lenman M, Ronne H, Stymne S (2002) Storage lipid synthesis is non-essential in yeast. J Biol Chem 277(8):6478–6482. doi:10.1074/jbc.M109109200

Sarthy AV, McConaughy BL, Lobo Z, Sundstrom JA, Furlong CE, Hall BD (1987) Expression of the *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. Appl Environ Microbiol 53(9):1996–2000

Sassner P, Galbe M, Zacchi G (2008) Techno-economic evaluation of bioethanol production from three different lignocellulosic materials. Biomass Bioenerg 32(5):422–430. doi:10.1016/j.biombioe.2007.10.014

Shen H, Gong Z, Yang X, Jin G, Bai F, Zhao ZK (2013) Kinetics of continuous cultivation of the oleaginous yeast *Rhodosporidium toruloides*. J Biotechnol 168(1):85–89. doi:http://dx.doi.org/10.1016/j.jbiotec.2013.08.010

Shi S, Valle-Rodriguez JO, Khoomrung S, Siewers V, Nielsen J (2012) Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production. Biotechnol Biofuels 5:7. doi:10.1186/1754-6834-5-7

Shiba Y, Paradise EM, Kirby J, Ro D-K, Keasling JD (2007) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. Metab Eng 9(2):160–168. doi:http://dx.doi.org/10.1016/j.ymben.2006.10.005

Solomon BD, Barnes JR, Halvorsen KE (2007) Grain and cellulosic ethanol: history, economics, and energy policy. Biomass Bioenerg 31(6):416–425. doi:http://dx.doi.org/10.1016/j.biombioe.2007.01.023

Sousa MJ, Ludovico P, Rodrigues F, Leão C, Côrte-Real M (2012) Stress and cell death in yeast induced by acetic acid, cell metabolism. In: Bubulya P (ed) Cell homeostasis and stress response. InTech, Rijeka, Croatia, pp 73–100. doi:10.5772/27726

Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD (2008) Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. Microb Cell Fact 7:36. doi:10.1186/1475-2859-7-36

Stephen JD, Mabee WE, Saddler JN (2012) Will second-generation ethanol be able to compete with first-generation ethanol? Opportunities for cost reduction. Biofuels Bioprod Biorefin-Biofpr 6(2):159–176. doi:10.1002/Bbb.331

Subtil T, Boles E (2011) Improving L-arabinose utilization of pentose fermenting *Saccharomyces cerevisiae* cells by heterologous expression of L-arabinose transporting sugar transporters. Biotechnol Biofuels 4:38

Tanaka K, Ishii Y, Ogawa J, Shima J (2012) Enhancement of acetic acid tolerance in *Saccharomyces cerevisiae* by overexpression of the *HAA1* gene, encoding a transcriptional activator. Appl Environ Microbiol 78(22):8161–8163. doi:10.1128/AEM.02356-12

Tang W, Zhang S, Tan H, Zhao Z (2010) Molecular cloning and characterization of a malic enzyme gene from the oleaginous yeast *Lipomyces starkeyi*. Mol Biotechnol 45(2):121–128. doi: 10.1007/s12033-010-9255-8

Tao X, Zheng D, Liu T, Wang P, Zhao W, Zhu M, Jiang X, Zhao Y, Wu X (2012) A novel strategy to construct yeast *Saccharomyces cerevisiae* strains for very high gravity fermentation. PLoS ONE 7(2):e31235. doi:10.1371/journal.pone.0031235

Tapia VE, Anschau A, Coradini AL, T TF, Deckmann AC (2012) Optimization of lipid production by the oleaginous yeast *Lipomyces starkeyi* by random mutagenesis coupled to cerulenin screening. AMB Express 2(1):64. doi:10.1186/2191-0855-2-64

Tehlivets O, Scheuringer K, Kohlwein SD (2007) Fatty acid synthesis and elongation in yeast. Biochim Biophys Acta 1771(3):255–270. doi:10.1016/j.bbalip.2006.07.004

Thompson P (2012) The agricultural ethics of biofuels: the food versus fuel debate. Agriculture 2(4):339–358

Tilman D, Socolow R, Foley JA, Hill J, Larson E, Lynd L, Pacala S, Reilly J, Searchinger T, Somerville C, Williams R (2009) Energy. Beneficial biofuels–the food, energy, and environment trilemma. Science 325(5938):270–271. doi:10.1126/science.1177970

Tiukova I, de Barros Pita W, Sundell D, Momeni MH, Horn SJ, Ståhlberg J, de Morais MA, Jr., Passoth V (2014) Adaptation of *Dekkera bruxellensis* to lignocellulose-based substrate. Biotechnol Appl Biochem 61(1):51–57. doi:10.1002/bab.1145

Tiukova IA, Petterson ME, Tellgren-Roth C, Bunikis I, Eberhard T, Pettersson OV, Passoth V (2013) Transcriptome of the alternative ethanol production strain *Dekkera bruxellensis* CBS 11270 in sugar limited, low oxygen cultivation. PLoS ONE 8(3):e58455. doi:10.1371/journal.pone.0058455

Träff KL, Jönsson LJ, Hahn-Hägerdal B (2002) Putative xylose and arabinose reductases in *Saccharomyces cerevisiae*. Yeast 19(14):1233–1241. doi:10.1002/yea.913

Träff KL, Otero Cordero RR, van Zyl WH, Hahn-Hägerdal B (2001) Deletion of the GRE3 aldose reductase gene and its influence on xylose metabolism in recombinant strains of *Saccharomyces cerevisiae* expressing the xylA and *XKS1* genes. Appl Environ Microbiol 67(12):5668–5674. doi:10.1128/AEM.67.12.5668-5674.2001

Tully M, Gilbert HJ (1985) Transformation of *Rhodosporidium toruloides*. Gene 36(3):235–240

Valadi H, Larsson C, Gustafsson L (1998) Improved ethanol production by glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 50(4):434–439

Vallee BL (1998) Alcohol in the western world. Sci Am 278(6):80–85

Van Vleet JH, Jeffries TW (2009) Yeast metabolic engineering for hemicellulosic ethanol production. Curr Opin Biotechnol 20(3):300–306

Van Vleet JH, Jeffries TW, Olsson L (2008) Deleting the para-nitrophenyl phosphatase (pNPPase), *PHO13*, in recombinant *Saccharomyces cerevisiae* improves growth and ethanol production on D-xylose. Metab Eng 10(6):360–369. doi:10.1016/j.ymben.2007.12.002

van Zyl C, Prior BA, Kilian SG, Kock JL (1989) D-xylose utilization by *Saccharomyces cerevisiae*. J Gen Microbiol 135(11):2791–2798

Vanholme B, Desmet T, Ronsse F, Rabaey K, Breusegem FV, Mey MD, Soetaert W, Boerjan W (2013) Towards a carbon-negative sustainable bio-based economy. Front Plant Sci 4:174. doi:10.3389/fpls.2013.00174

Verho R, Londesborough J, Penttilä M, Richard P (2003) Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. Appl Environ Microbiol 69(10):5892–5897

Verho R, Penttilä M, Richard P (2011) Cloning of two genes (*LAT1,2*) encoding specific L: -arabinose transporters of the L: -arabinose fermenting yeast *Ambrosiozyma monospora*. Appl Biochem Biotechnol 164(5):604–611. doi: 10.1007/s12010-011-9161-y

Voronovsky AY, Rohulya OV, Abbas CA, Sibirny AA (2009) Development of strains of the thermotolerant yeast *Hansenula polymorpha* capable of alcoholic fermentation of starch and xylan. Metab Eng 11(4–5):234–242. doi:10.1016/j.ymben.2009.04.001

Walfridsson M, Bao X, Anderlund M, Lilius G, Bülow L, Hahn-Hägerdal B (1996) Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus* xylA gene, which expresses an active xylose (glucose) isomerase. Appl Environ Microbiol 62(12):4648–4651

Wang J, Li R, Lu D, Ma S, Yan Y, Li W (2009) A quick isolation method for mutants with high lipid yield in oleaginous yeast. World J Microbiol Biotechnol 25(5):921–925. doi:10.1007/s11274-009-9960-2

Wang PM, Zheng DQ, Liu TZ, Tao XL, Feng MG, Min H, Jiang XH, Wu XC (2012) The combination of glycerol metabolic engineering and drug resistance marker-aided genome shuffling to improve very-high-gravity fermentation performances of industrial *Saccharomyces cerevisiae*. Bioresour Technol 108:203–210. doi:10.1016/j.biortech.2011.12.147

Weber C, Farwick A, Benisch F, Brat D, Dietz H, Subtil T, Boles E (2010) Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. Appl Microbiol Biotechnol 87(4):1303–1315. doi:10.1007/s00253-010-2707-z

Westhuizen TJ, Pretorius IS (1992) The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. Antonie Van Leeuwenhoek 61(4):249–257. doi:10.1007/bf00713932

Wilkie AC, Riedesel KJ, Owens JM (2000) Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. Biomass Bioenerg 19(2):63–102

Wisselink HW, Toirkens MJ, del Rosario Franco Berriel M, Winkler AA, van Dijken JP, Pronk JT, van Maris AJ (2007) Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. Appl Environ Microbiol 73(15):4881–4891. doi:10.1128/AEM.00177-07

Wisselink HW, Toirkens MJ, Wu Q, Pronk JT, van Maris AJ (2009) Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. Appl Environ Microbiol 75(4):907–914. doi:10.1128/AEM.02268-08

Wohlbach DJ, Kuo A, Sato TK, Potts KM, Salamov AA, Labutti KM, Sun H, Clum A, Pangilinan JL, Lindquist EA, Lucas S, Lapidus A, Jin M, Gunawan C, Balan V, Dale BE, Jeffries TW, Zinkel R, Barry KW, Grigoriev IV, Gasch AP (2011) Comparative genomics of xylose-fermenting fungi for enhanced biofuel production. Proc Natl Acad Sci USA 108(32):13212–13217. doi:10.1073/pnas.1103039108

Wright J, Bellissimi E, de Hulster E, Wagner A, Pronk JT, van Maris AJ (2011) Batch and continuous culture-based selection strategies for acetic acid tolerance in xylose-fermenting *Saccharomyces cerevisiae*. FEMS Yeast Res 11(3):299–306. doi:10.1111/j.1567-1364.2011.00719.x

Yanase S, Hasunuma T, Yamada R, Tanaka T, Ogino C, Fukuda H, Kondo A (2010) Direct ethanol production from cellulosic materials at high temperature using the thermotolerant yeast *Kluyveromyces marxianus* displaying cellulolytic enzymes. Appl Microbiol Biotechnol 88(1):381–388. doi: 10.1007/s00253-010-2784-z

Ykema A, Verbree EC, van Verseveld HW, Smit H (1986) Mathematical modelling of lipid production by oleaginous yeasts in continuous cultures. Antonie Van Leeuwenhoek 52(6):491–506

Yu KO, Jung J, Kim SW, Park CH, Han SO (2012) Synthesis of FAEEs from glycerol in engineered *Saccharomyces cerevisiae* using endogenously produced ethanol by heterologous expression of an unspecific bacterial acyltransferase. Biotechnol Bioeng 109(1):110–115. doi:10.1002/bit.23311

Yu KO, Jung J, Ramzi AB, Choe SH, Kim SW, Park C, Han SO (2013) Development of a *Saccharomyces cerevisiae* strain for increasing the accumulation of triacylglycerol as a microbial oil feedstock for biodiesel production using glycerol as a substrate. Biotechnol Bioeng 110(1):343–347. doi:10.1002/bit.24623

Yu X, Zheng Y, Dorgan KM, Chen S (2011) Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresour Technol 102(10):6134–6140. doi:http://dx.doi.org/10.1016/j.biortech.2011.02.081

Zhang F, Rodriguez S, Keasling JD (2011) Metabolic engineering of microbial pathways for advanced biofuels production. Curr Opin Biotechnol 22(6):775–783. doi:10.1016/j.copbio.2011.04.024

Zheng D-Q, Wang P-M, Chen J, Zhang K, Liu T-Z, Wu X-C, Li Y-D, Zhao Y-H (2012) Genome sequencing and genetic breeding of a bioethanol *Saccharomyces cerevisiae* strain YJS329. BMC Genom 13(1):479

Zheng Y, Yu C, Cheng YS, Zhang R, Jenkins B, VanderGheynst JS (2011) Effects of ensilage on storage and enzymatic degradability of sugar beet pulp. Bioresour Technol 102(2):1489–1495. doi:10.1016/j.biortech.2010.09.105

Zhu JY, Pan XJ (2010) Woody biomass pretreatment for cellulosic ethanol production: technology and energy consumption evaluation. Bioresour Technol 101(13):4992–5002. doi:10.1016/j.biortech.2009.11.007

Zhu Z, Zhang S, Liu H, Shen H, Lin X, Yang F, Zhou YJ, Jin G, Ye M, Zou H, Zhao ZK (2012) A multi-omic map of the lipid-producing yeast *Rhodosporidium toruloides*. Nat Commun 3:1112. doi:10.1038/ncomms2112

Chapter 10
Wine, Beer and Cider: Unravelling the Aroma Profile

Amparo Gamero, Vicente Ferreira, Isak S. Pretorius and Amparo Querol

Abstract The aroma profile of alcoholic beverages is a major factor that distinguishes one product from another, and it is a key attribute that drives consumer preference at points of sale. A longstanding objective has, therefore, been to identify those aromatic compounds that are important to particular olfactory attributes of different styles of wine, beer and cider—whether perceived ortho- or retro-nasally—and to modulate them according to consumer preferences. That this has been achieved only to a relatively small extent to date is partly a reflection on the complexity of the perception of aroma mixtures and also the presence of very low concentrations of potent aroma compounds in these products. It is known, although perhaps not appreciated as widely as it should be, that aroma compounds will interact with each other, with masking or suppressing effects being probably universal for compounds at supra-threshold concentrations, together with additive interactions for compounds at sub-threshold concentrations. Thus it is likely that volatile compounds with marginal aroma impact when isolated, can together provide an influence on aroma. Some of these aroma-active compounds are produced during fermentation. Different yeasts produce differing ranges of aroma-active substances, which may greatly affect the complex flavour of a fermented product such as wine, beer and cider. While these secondary metabolites are often formed only in trace amounts, their concentrations may well determine the distinct aroma of these beverages. This chapter reviews the production of the most important aroma-active compounds produced by yeast at molecular level and seeks to understand how they might be perceived by consumers.

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_10, © Springer-Verlag Berlin Heidelberg 2014

10.1 The Fundamentals of Aroma and Flavour Perception of Fermented Alcoholic Beverages

The aroma of a given foodstuff is formed by the pool of volatile molecules contained in that product with the ability to impact the olfactory receptors located in the olfactory region in the human nose. Since volatile molecules can reach this olfactory region both via orthonasal and retronasal pathways, the information elicited by those receptors affects two different sensory properties of products, i.e. their smell and their flavour. Smell is primarily information originating from the excited olfactory receptors together with, eventually, information produced by trigeminal nerve terminals located in the nostrils, which are also present in the mouth, pharynx and eyes. The information generated by these terminals falls in the category of chemesthesis and is related to semi-tactile properties such as refreshing/cooling effects (menthol), irritation (acids and alkalis) or pungency (chilies' capsaicins) (Bandell et al. 2004; Bautista et al. 2007; Caterina et al. 1997; Macpherson et al. 2006). Many aroma chemicals are able to produce some chemesthesis, although in general the intensity of the response is smaller than the purely olfactory (Prescott 1999a). On the other hand, the flavour of a product is formed by the cerebral integration of the different sensory responses elicited during the consumption of a product. These sensory responses stem from three different chemical sensory systems (olfaction, taste and chemesthesis) and in the tactile and thermal sensory systems which give information about the temperature and rheological properties of the food. From a qualitative point of view, the olfactory system is the one carrying the biggest amount of information and that explains the limited amount of sensory information that can be perceived when the nose is blocked.

Flavour is an integrated cerebral response and it, therefore, not always possible to clearly assign the origin of the stimulus causing a particular flavour perception (Delwiche 2004; Prescott 1999b). For instance, whenever vanillin is present together with sweet tastants, the intensity of its smell increases (Green et al. 2012) and vice versa, the presence of vanillin can increase the perception of sweetness (Sakai et al. 2001). These complex phenomena are broadly included into the concept of perceptual interactions that are responsible for some unexpected and important observations in wine flavour chemistry, such as the prominent role played by fruity aromas on the perception of sweetness, bitterness and astringency (Saenz-Navajas et al. 2010). Although these phenomena are well documented in the general scientific literature, they are not yet well understood in the context of wine and other fermented alcoholic beverages. It is therefore important to note that aroma compounds play a sensory role in fermented beverages that it is not limited to the perception of an odour.

To fully understand the role played by individual chemicals, it is equally important to note that fermented beverages are considered by flavour chemists as 'complex' products. Although there is no definite border between what products are categorised as 'simple' and 'complex', the fact that the aroma of any fermented

beverage is formed by at least 25 different aroma chemicals—all of which are present at concentrations above their corresponding odour thresholds—classes wine, beer and cider as ‘complex’ products. So, given the fact that the sense of olfaction has to be by nature a ‘synthetic’ rather than ‘analytic’ sense (Wilson and Stevenson 2003), the overall odour of fermented beverages has to be a global perception in which the individual chemicals are just poorly identified. In addition, since the odour of fermented beverages has accompanied humankind since the beginning of time, we can postulate the existence of what psychophysicists define as an ‘odour object’ (Ferreira 2012; Stevenson and Wilson 2007; Yeshurun and Sobel 2010). In other words, the human brain transforms the complex signals produced by the interactions of the chemicals present in all alcoholic beverages (alcohol, fusel alcohols, fatty acids, branched acids, ethyl esters, acetates, etc.) into a single unified concept that would be defined as ‘alcoholic’ or ‘vinous’. This is a highly efficient and ‘economic’ of signal processing and this capability has great practical importance in the understanding the chemical basis of aroma perception.

Therefore, although in the following sections we refer to individual compounds or groups of compounds and mention the specific odour properties of a compound or groups of compounds, it should not be concluded that those odour properties are directly responsible for such odour perception in wine, beer or cider.

10.2 The Basic Hierarchy of Aroma Compounds in Fermented Alcoholic Beverages

The previous notes, together with many experimental observations obtained in different reconstitution studies performed mainly in wine, make it possible to provide a basic rationale for understanding the contribution of the different chemicals to the aroma of a naturally fermented beverage. At the core of this rationale lies the aforementioned complexity of aroma and flavour perception in relation to fermented beverages and the so-called ‘aroma buffering effect’. The ‘buffering aroma effect’ of a product’s base refers to the demonstrated resistance of a particular aroma mixture to change its aroma both upon the elimination of some of its components or upon the addition of some new aroma compounds (Ferreira et al. 2002; Escudero et al. 2004). This does not mean that such a base always bears the same aroma and has the same composition. The composition of the base depends on some basic factors such as, for example, the concentration of sugar in a grape must, the prevalent yeast strain(s) and the degree of anaerobiosis during fermentation. For instance, the latter factor makes white and rosé wines richer in fatty acids and their ethyl esters while containing less alcohols and isoacids than red wines (Ferreira et al. 1996). Another less well-known factor is that the concentrations of fusel alcohols, fusel alcohol acetates, isoacids and their ethyl esters, all of them related to the yeast amino acid metabolism, are related to the varietal origin of the must (Ferreira et al. 2000; Hernández-Orte et al. 2002). Different

compositions mean slightly different aromas and slightly different buffering abilities.

Notwithstanding of this, there are some compounds or combinations of compounds, that at the 'natural' concentrations at which they usually occur in fermented beverages, can break the buffer and transmit to the mixture their specific aroma or a particular feature of their aroma. There are five different possibilities for this to happen:

1. The aroma buffer will be broken whenever a beverage contains an 'aromatic vector' with enough odour intensity.
2. Such an aroma vector can be an individual compound (a so-called 'impact compound'), a family of aroma compounds belonging to the same chemical series, such as ethyl esters of fatty acids, or even an association of aroma compounds sharing a generic descriptor (such as 'fruity' or 'floral').
3. The vector will express in the beverage an aromatic nuance whose intensity and vicinity to the innate aroma character of the vector will be proportional to its concentration. For instance, isoamyl acetate bearing a characteristic smell of banana, if present at a low concentration does not transmit to the beverage its characteristic aroma. Rather it just transmits its generic 'fruity' character.
4. Hence, concentration modulates the role that the vector actually plays in the mixture. The following intensity categories can be identified:

    a. null
    b. minor contributor—the elimination of the vector does not bring about any clear aromatic change
    c. neat contributor—the elimination of the vector brings about a clear decrease in the intensity of the aroma nuance to which the vector contributes
    d. major contributor—in this case the elimination of such vector will cause a dramatic drop in the intensity of the aroma nuance with even possible qualitative changes in the overall aroma profile
    e. impact compound—in this case the elimination of the vector will cause a dramatic change on the aroma profile.

5. When several aromatic vectors coexist in the same beverage, they will interact at perceptual level following three different potential patterns of interaction:

    a. competitive—both aroma nuances are simultaneously and competitively perceived (Campo et al. 2005),
    b. destructive—only one of the vectors is perceived at a lower intensity,
    c. creative—a new aroma nuance emerges from the blend (San Juan et al. 2011).

Whenever it comes to fermentative compounds, they are the basic constituents of the base and some of them can also play the role of contributors to different aroma nuances. To the best of our knowledge, they only seldom play the role of

impact compounds. For instance, isoamyl alcohol or β-phenyl alcohols, even if they are present at relatively high concentrations never reach the level at which they would act as real impact compounds. So far, in wine, only isoamyl acetate, ethyl acetate, acetic acid, acetaldehyde and diacetyl can play such a role individually, and leaving aside some specific quirky wine styles, when they are clearly perceived the quality of the wine can be questioned. Fermentative compounds form the following aroma vectors:

1. Alcohols (ethanol, isobutanol, isoamyl alcohol).
2. Methionol.
3. Ethyl esters of fatty acids (ethyl butyrate, hexanoate, octanoate and decanoate).
4. Ethyl acetate.
5. Acetic acid.
6. Fatty acids (butyric, hexanoic, octanoic and decanoic acids).
7. Isoamyl acetate.
8. β-phenyl acetate and β-phenylethanol.
9. Branched fatty acids [isobutyric, isovaleric, 2-methylbutyric and the recently discovered 2-, 3 and 4-methylpentanoic and cyclohexanoic acids (Campo et al. 2007)].
10. Ethyl esters of the previously mentioned branched fatty acids.
11. Diacetyl.
12. Acetaldehyde.

As previously mentioned, these aroma vectors rarely reach the category of impact compounds, but they are important contributors to some key aroma nuances of fermented beverages. The role of two of the vectors, namely fatty acids and branched fatty acids, is quite complex, since apparently they form a kind of 'creative' interaction with the 'fruity' vectors (ethyl esters of fatty acids, branched acids and isoamyl acetate) to form the aroma of 'fresh fruit' (San Juan et al. 2011). Another aspect that must be kept in mind is that compounds of apparently 'bad' aroma, such as isovaleric and 2-methylbutyric acids, are in fact precursors for the strawberry-smelling ethyl isovalerate and ethyl 2-methylbutyrate which are formed by slow esterification of the acids with ethanol during ageing.

Bearing these fundamental aspects of aroma perception of fermented alcoholic beverages and the basic hierarchy of aroma compounds involved in mind, the following sections focus on the contribution of yeast to the aroma and overall quality of fermented beverages.

10.3 Wine Yeasts

Winemaking is a complex chemical and biological process in which different genera of yeast and bacteria are involved. During the early stages of spontaneous wine fermentation, different genera of non-*Saccharomyces* yeasts, such as *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora* and its asexual counterpart

Kloeckera, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces* and *Zygosaccharomyces* play a role (Pretorius et al. 1999). Yeasts of the genera *Kloeckera*, *Hanseniaspora* and *Candida* predominate in the early stages, followed by several species of *Metschnikowia* and *Pichia* in the middle stages when the ethanol concentration rises to 3–4 % (Fleet and Heard 1993). However, some species of *Schizosaccharomyces*, *Zygosaccharomyces*, *Brettanomyces* and its sexual ('perfect') equivalent, *Dekkera*, are more resistant to high concentrations of ethanol and SO_2 and, if present under certain conditions, can adversely affect the sensory quality of wine.

On the other hand, the principal species conducting the alcoholic fermentation in grape wine is *Saccharomyces cerevisiae*, but the closely-related *Saccharomyces uvarum* (*Saccharomyces bayanus var. uvarum*) can also participate (Demuyter et al. 2004; Massoutier et al. 1998; Naumov et al. 2000, 2001; Sipiczki 2002, 2008). Both *S. cerevisiae* and *S. uvarum* are able to grow on substrates characterised by high sugar and ethanol content, low pH, high sulphur dioxide concentrations and remains of fungicides, demonstrating that they are genetically well adapted to winemaking conditions (Sipiczki 2008). However, *S. cerevisiae* has higher resistance to high temperature stress (up to 37 °C) and ethanol levels (up to 15 %) than *S. uvarum* (Belloch et al. 2008). From an oenological point of view, these *Saccharomyces* species differ in several properties. Comparison between *S. uvarum* and *S. cerevisiae* reveals that the former is more cryotolerant, produces less acetic acid, lower levels of amyl alcohols, but higher concentrations of glycerol, succinic acid, malic acid, isobutyl alcohol, isoamyl alcohol and numerous secondary compounds (Sipiczki 2002). Wines produced by *S. uvarum* strains have a higher aromatic intensity than those produced by *S. cerevisiae* (Coloretti et al. 2006; Henschke et al. 2000). Specifically, *S. uvarum* produces more of 2-phenylethanol, 2-phenylethyl acetate and ethyl lactate than *S. cerevisiae* (Antonelli et al. 1999; Di Stefano et al. 1981; Gangl et al. 2009). On the other hand, *S. uvarum* is less common and appears mainly in fermentations at low temperatures (Antunovics et al. 2003; Demuyter et al. 2004; Masneuf-Pomarède et al. 2010; Sipiczki et al. 2001).

Other members of the genus *Saccharomyces* (*S. cariocanus, S. kudriavzevii, S. mikatae, S. paradoxus, S. arboricolus, S. pastorianus*) are not likely to play important roles in wine fermentation (Sipiczki 2008). Nevertheless, *S. paradoxus* has been found in grapes in the north-western region of Croatia and it is currently used to ferment wines (Redzepovic et al. 2002). Likewise, *S. kudriavzevii* has only been isolated in natural environments, like decayed leaves (Naumov et al. 2000) or oak barks (Sampaio and Gonçalves 2008; Lopes et al. 2010). However, there are reports that indicate that *S. kudriavzevii* may participate in hybrid formation with wine-related *S. cerevisiae* and *S. bayanus* species. For example, the genome sequence of a widely used wine yeast strain, VIN7, revealed an allotriploid hybrid genome with *S. cerevisiae* and *S. kudriavzevii* origins (Borneman et al. 2012). Physiological characterization of *S. kudriavzevii* strains has shown that they are able to grow at relatively low (10 °C) and high (up to 30 °C) temperatures;

however, they are not able to tolerate more than 5 % of ethanol (Belloch et al. 2008).

Natural hybrids of *S. cerevisiae, S. bayanus* and *S. kudriavzevii* conducting wine fermentations have been recently discovered and characterised by genetic approaches (Belloch et al. 2009; Borneman et al. 2012; Dunn and Sherlock 2008; González et al. 2006, 2008; Horinouchi et al. 2010; Masneuf et al. 1998; Nguyen et al. 2000; Sipiczki 2008). The hybridisation process between *Saccharomyces* species has been proposed as an adaptation mechanism of yeasts to ferment at low temperatures (de Barros Lopes et al. 2002; Barrio et al. 2006; Sipiczki 2008). Physiological data suggest that *Saccharomyces* hybrids might have inherited the ability to grow at high temperatures (30–37 °C) and their ethanol tolerance from their *S. cerevisiae* parent and the ability to grow at low temperatures (10–16 °C) from their *S. bayanus* and *S. kudriavzevii* parents. These physiological characteristics point to *Saccharomyces* hybrids as better adapted to meet the winemakers' trends, such as conducting wine fermentation at low temperatures, which may cause wine aroma improvement (Lambrechts and Pretorius 2000; Torija et al. 2003; Llauradó et al. 2002, 2005; Novo et al. 2003).

Oenological characterization of *S. cerevisiae* × *S. kudriavzevii* hybrid strains has demonstrated that the hybrids are well adapted to ferment at low and intermediate temperatures, producing moderate or higher levels of glycerol and less acetic acid with regard to reference strains of *S. cerevisiae* and *S. kudriavzevii* (Gangl et al. 2009; González et al. 2007). Similar comparative studies, which also included *S. uvarum* and a hybrid between *S. cerevisiae* and *S. uvarum*, in wine and cider (Masneuf et al. 1998; Nguyen et al. 2000), indicated that the highest production of glycerol was produced by *S. uvarum*, *S. kudriavzevii* and the *S. cerevisiae* × *S. uvarum* hybrid (Gamero et al. 2013). Regarding aroma formation, one study indicated that hybrids produced the same quantity of aromatic compounds as *S. cerevisiae* at high temperatures, and the same aromatic intensity as *S. kudriavzevii* at low temperatures (González et al. 2007), whereas in another study this trend was only observed in the case of fusel alcohol production (Gamero et al. 2013). In the latter study, *S. cerevisiae* strains yielded the highest aroma amounts at 28 °C were, whereas *S. uvarum* and some hybrids excelled at 12 °C. Altogether, these studies pointed to the fact that aroma formation is highly dependent on both yeast strain and fermentation temperature (Gamero et al. 2013).

10.4 Beer Yeasts

In brewing, a distinction is made between ale yeasts (*top fermentation*) and lager yeasts (*bottom fermentation*). Ale yeasts are classified as *S. cerevisiae* and are mostly used for the production of specialty beers where the fermentation temperatures are relatively high (15–25 °C). Lager yeasts are classified as *Saccharomyces pastorianus*, which include *S. carlsbergenis* and *S. monacensis* isolated by EC Hansen in 1908. Lager yeasts are used for the production of pilsner type beers,

fermented at lower temperatures than ale yeasts (6–14 °C). The genomes of lager yeasts are complex as they are aneuploid and consist of a hybrid of mixed genetic lines of the *Saccharomyces* genus (Kodama et al. 2006). DNA/DNA reassociation studies on the type strains of *S. bayanus* (CBS380), *S. carlsbergensis* (CBS1513) and *S. monacensis* (CBS1503) presented *S. bayanus* as one of the contributors to *S. pastorianus* genome (Vaughan-Martini and Kurtzman 1985). Thereafter many reports agreed with this fact (Tamai et al. 2000; Rainieri et al. 2006; Dunn and Sherlock 2008; Nakao et al. 2009). Later, some evidences pointed to *S. bayanus* to be a hybrid between *S. uvarum* and *S. cerevisiae* (Nguyen et al. 2000; Nguyen and Gaillardin 2005), which was recently confirmed (Nguyen et al. 2011) and being one of its parents *S. uvarum* and the other, a new species isolated from Patagonia and named *S. eubayanus* (Libkind et al. 2011). As *S. eubayanus* carries a 'pure' or monogenome it is very likely to be the common contributor of *S. bayanus* and *S. pastorianus*. Lager brewing yeast is now recognised by many authors as *S. eubayanus/S. cerevisiae* hybrid (Dunn et al. 2012; Piotrowski et al. 2012; Cousseau et al. 2013; Pengelly and Wheals 2012).

10.5 Cider Yeasts

Studies on population dynamics in cider have shown that the composition of yeast flora can vary according to climatic conditions, apple varieties, geographic location and the cider-making technology employed (Cabranes et al. 1990; del Campo et al. 2003; Suárez et al. 2007a). First, an oxidative phase carried out by autochthonous non-*Saccharomyces* yeasts with a low fermentation capacity and with the predominance of *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Hanseniaspora valbyensis* and *Candida* yeasts was observed (Michel et al. 1988; Morrissey et al. 2004; Coton et al. 2006; Suárez et al. 2007a). Furthermore, species of the genera *Pichia*, *Torulaspora*, *Rhodotorula*, *Cryptococcus, Zygosaccharomyces* and *Brettanomyces*/*Dekkera* yeasts, originating from apples or the environment have been also related to cider production (Beech 1993; Michel et al. 1988; Morrissey et al. 2004).

Second, strains with a greater tolerance to ethanol (*Saccharomyces* spp.) completed the cider fermentations. In the aforementioned studies on population dynamics in cider, the *Saccharomyces* species found to be present were *S. cerevisiae* and *S. bayanus*. The data indicated that *S. bayanus* was the predominant species at the beginning and the middle fermentation phases of the fermentation process, reaching a percentage of isolation between 33 and 41 %, whereas *S. cerevisiae* took over the process in the final stages of fermentation (Suárez et al. 2007a). A study that was carried out to examine the dynamics and variability of wild *Saccharomyces* spp. (Suárez et al. 2007b) determined that the number of strains observed was higher than those reported for *Saccharomyces* populations in some wine-growing regions in

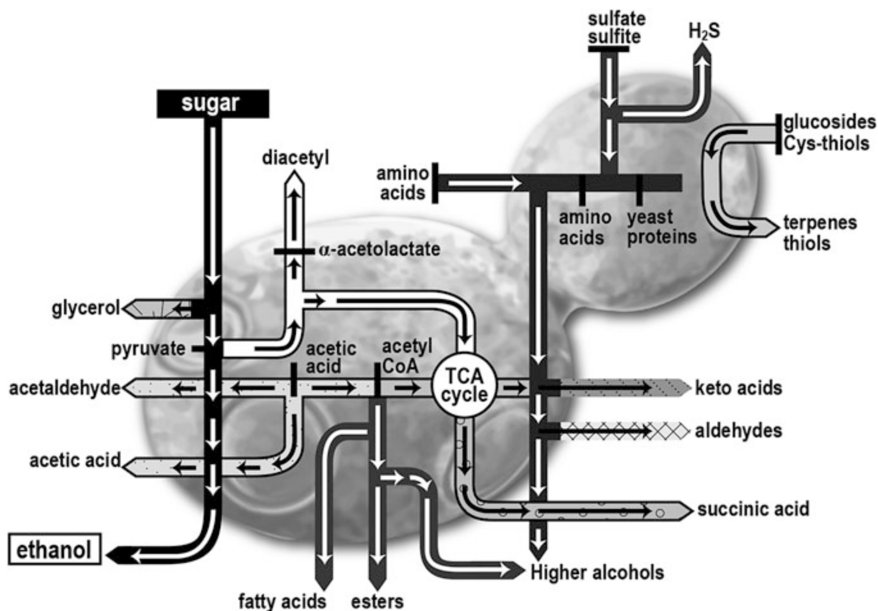

Fig. 10.1 Flavour-related metabolism in yeasts

other studies (Frezier and Dubourdieu 1992; Querol et al. 1994; Gutiérrez et al. 1999; Torija et al. 2001; Schuller et al. 2005).

Finally and as commented before, natural hybrids between *S. bayanus* and *S. cerevisiae* have been described by some authors in wine and cider some years ago (Masneuf et al. 1998; Nguyen et al. 2000).

10.6 Fermentative Aroma

The most important compounds within fermentative aroma are higher alcohols, acetate and ethyl esters, aldehydes (acetaldehyde), ketones (diacetyl), organic acids (acetic acid), volatile phenols (4-vinylphenol, 4-vinilguaiacol) and sulphurous compounds (hydrogen sulphide, mercaptans, volatile thiols). A scheme of the synthesis of the main fermentative aroma compounds is shown in Fig. 10.1, while Table 10.1 presents their aroma descriptors and odour thresholds. Finally, Table 10.2 depicts the most important genes involved in flavour-active compound synthesis identified in *S. cerevisiae*.

Table 10.1 Aroma and odour thresholds of the most representative aroma compounds appearing in alcoholic fermentations

Aromatic compounds	Aroma descriptor	Odour threshold
Higher alcohols (mg/l)		
Methanol	Chemical, medicinal	668[a]
1-Propanol	Ripe fruit, alcohol	0.830[a]
2-Methyl-1-propanol (Isobutanol)	Bitter, green, harsh	0.200[b]
3-Methyl-1-butanol (Isoamyl alcohol)	Alcohol, fusel	30[b]
3-Methyl-1-pentanol	Vinous, herbaceous, cacao	50[c]
4-Methyl-1-pentanol	Almond, toasted	50[c]
1-Butanol	Medicinal, phenolic	150[a]
2,3-Butanediol	Fruity	150[a]
1-Pentanol	Almond, synthetic, balsamic	64[a]
1-Hexanol	Green, grass	8[b]
(Z)-3-Hexenol	Green, cut grass	0.400[d]
1-Heptanol	Oily	2.500[a]
2-Phenylethyl alcohol	Roses, sweet	14[b]
Benzyl alcohol	Sweet, fruity	200[a]
Acetate esters (mg/l)		
Ethyl acetate	Fruity, solvent	7.500[d]
Isoamyl acetate	Banana	0.030[b]
Hexyl acetate	Green, floral	1.500[c]
2-Phenylethyl acetate	Rose, flowery	0.250[b]
Ethyl esters (mg/l)		
Ethyl hexanoate (ethyl caproate)	Green apple, anise	0.014[b]
Ethyl octanoate (ethyl caprylate)	Sweet, fruity, fresh	0.005[b]
Ethyl decanoate (ethyl caprate)	Pleasant, soap	0.200[b]
Ethyl butyrate	Fruity, apple	0.020[b]
3-Hydroxy ethyl butyrate	Caramel, toasted	20[a]
Ethyl succinate	Wine	6[b]
Diethyl succinate	Wine	200[a]
Ethyl pyruvate	Vegetable, caramel	100[a]
Ethyl lactate	Acid, medicine	155[c]
Aldehydes (mg/l)		
Acetaldehyde	Pungent, ripe apple	0.500[d]
Benzaldehyde	Bitter, cherry	2[b]
Phenylethanal	Flowery, rose, honey	0.005[b]
Ketones (mg/l)		
2,3-Butanedione (diacetyl)	Buttery	0.200–2.800[e]
Organic acids (mg/l)		
Acetic acid	Sour, pungent, vinegar	200[d]
Propanoic acid	Pungent, rancid, soy	8.100[c]
Benzoic acid	Chemical	1[a]
3-Methylbutanoic acid	Cheese, fatty, rancid	0.033[b]
Butyric acid	Rancid, cheese, sweat	0.173[a]
Isobutyric acid	Rancid, butter, cheese	2.300[a]

(continued)

Table 10.1 (continued)

Aromatic compounds	Aroma descriptor	Odour threshold
Isovaleric acid	Sweet, acid, rancid	0.033[c]
Hexanoic acid	Fatty acid, cheese	0.420[b]
Octanoic acid	Fatty acid, rancid	0.500[b]
Decanoic acid	Fatty, rancid, soap	1[b]
Phenylacetic acid	Honey, pollen, flowery	2.500[b]
Volatile phenols (mg/l)		
4-Vinylphenol	Stramonium, almond shell	0.180[f]
4-Vinilguaiacol	Clove, curry	0.010[f]
4-Ethyl guaiacol	Phenolic, sweet	0.110[e]
4-Ethyl phenol	Phenol, spicy	0.440[f]
2-Methoxyphenol	Medicine, sweet, smoke	0.010[c]
Sulphur compounds (µg/l)		
Hydrogen sulphide	Rotten egg	10000–80000[e]
3-(Methylthio)-1-propanol	Cooked vegetable	1000[d]
Methanethiol (methyl mercaptan)	Cooked cabbage, onion, putrefaction, rubber	0.300[e]
Ethanethiol (ethyl mercaptan)	Onion, rubber, natural gas	1.100[e]
Dimethyl sulphide	Asparagus, corn, molasses	25[e]
Diethyl sulphide	Cooked vegetables, onion, garlic	0.930[e]
Dimethyl disulfide	Cooked cabbage, intense onion	15, 29[e]
Diethyl disulfide	Garlic, burnt rubber	4.300[e]
3-(Methylthio)-1-propanol (methionol)	Cauliflower, cabbage, potato	500[e]
Benzothiazole	Rubber	50[e]
Thiazole	Popcorn, peanut	38[e]
4-Methylthiazole	Green hazelnut	55[e]
2-Furanmethanethiol	Roasted coffee, burnt rubber	0.001[e]
Thiophene-2-thiol	Burned, burned rubber, roasted coffee	0.800[e]
Monoterpenes (µg/l)		
Geraniol	Roses, geranium	30[d]
Linalool	Floral	15[d]
α-Terpineol	Pine, lily of the valley	250[f]
Citronellol	Green lemon	100[f]
Nerol	Rose, lime	400[f]
Volatile thiols (ng/l)		
4-Mercapto-4-methylpentan-2-one (4MMP)	Cat urine, box tree/blackcurrant, broom	3[e]
3-Mercaptohexan-1-ol (3MH)	Passionfruit, grapefruit	60[e]
3-Mercaptohexyl acetate (3MHA)	Riesling-type note, passionfruit, box tree	4[e]

[a] Etiévant (1991)
[b] Perestrelo et al. (2006)
[c] Ferreira et al. (2000)
[d] Guth (1997)
[e] Swiegers et al. (2005)
[f] Vilanova et al. (2010)

Table 10.2 Most important genes involved in flavour-active compound synthesis

Aromas	Enzymatic activity	Genes identified
Higher alcohols	Branched-chain amino acid transferases	*BAT1, BAT2*
	Aromatic amino acid transferases	*ARO8, ARO9*
	Decarboxylases	*ARO10, PDC1, PDC5, PDC6, THI3*
	Alcohol dehydrogenases	*ADH1-7, SFA1*
Esters	Alcohol acetyl transferases	*ATF1, ATF2*
	Acyl transferases	*EEB1, EHT1, YMR210 W*
	Esterases	*EEB1, EHT1, IAH2*
Aldehydes	Pyruvate decarboxylases	*PDC1-3*
Ketones	*ILV*-encoded enzyme forms and alcohol dehydrogenases	*ILV2, ILV3, ILV5, ADH*
Organic acids	Acetaldehyde dehydrogenases	*ALD2-6*
Volatile phenols	Phenolic acid decarboxylases	*PAD1* (=*POF1*)
Sulphur compounds	Sulphur metabolism related enzymes	*CYS4, MET5, MET10, MET14, MET17, MRX1*
Monoterpenes	*β*-glucosidases and glucanases	*BGL1, BGL2, BEG1, END1, EXG1*

10.6.1 Higher Alcohols

Higher or fusel alcohols are alcohols with two or more carbon atoms with molecular weights and boiling points higher than those of ethanol (Lambrechts and Pretorius 2000). From a quantitative point of view, higher alcohols are the most important group of volatile compounds produced by yeast during wine fermentation. Higher alcohols are classified in aliphatics like isobutanol, hexanol and isoamyl alcohol and aromatics like 2-phenylethanol and benzyl alcohol. Higher alcohols contribute with an intense aroma to the flavour of wine and other alcoholic beverages. According to Rapp and Versini (1991), concentrations of higher alcohols below 300 mg/l add desirable complexity to wine aroma, whereas higher concentrations (400 mg/l) can be detrimental to wine quality by disguising ester-based fruity aromas and imparting a strong, pungent smell and taste. On the other hand, the concentration of each higher alcohol acting positively or negatively on the aroma is variable. In beer, the flavour of the aliphatic alcohols is distinctly alcoholic (e.g. ethanol) and the aromatic alcohols have a rather sweet, alcoholic or bitter taste (Meilgaard 1975). Nevertheless, in spite of having aroma themselves, the main oenological importance of higher alcohols lies in the fact that they are precursors of acetate esters (Soles et al. 1982).

Many factors affect the levels of higher alcohols in alcoholic beverages. For example, in wine, viticultural conditions, yeast strain and species, initial sugar content of the grape must, pH and composition of the juice, fermentation temperature, assimilable nitrogen and aeration have a strong influence (Fleet and Heard 1993; Houtman et al. 1980a, b; Houtman and du Plessis 1981). In beer, the addition of fatty acids and sterols (Taylor et al. 1979), oxygenation (Quain and Duffield 1985)

or high temperatures (Landaud et al. 2001), cause an increase in higher alcohol content. Those factors also stimulate yeast growth in the fermenting wort. In addition, the production of 2-phenylethyl alcohol appears to be particularly sensitive to temperature, whereas the synthesis of other higher alcohols is relatively unaffected by this factor.

Several studies have demonstrated that *S. bayanus* produces higher amounts of several fusel alcohols (2-phenylethanol, isobutyl alcohol and isoamyl alcohol) than *S. cerevisiae* (Antonelli et al. 1999; Massoutier et al. 1998). Other authors observed that *Saccharomyces* species generally produce higher concentrations of fusels alcohols than non-*Saccharomyces* species (Gil et al. 1996; Herraiz et al. 1990).

Higher alcohols are synthesised by the Ehrlich pathway from branched-chain amino acids, leucine, valine and isoleucine; aromatic amino acids, phenylalanine, tyrosine and tryptophan; and the sulphur-containing amino acid methionine. In this metabolic pathway, the amino acids are transaminated to the corresponding α-ketoacid, followed by decarboxylation to aldehydes. Finally, these aldehydes are reduced to higher alcohols and NADH becomes NAD^+. These chemical reactions are carried out by amino acid permeases, transaminases, decarboxylases and dehydrogenases. Amino acid permeases are encoded by the genes *GAP1*, *BAP2*, BAP3, *MMP1* and *MUP3* (Didion et al. 1998; Grauslund et al. 1995; Isnard et al. 1996; Jauniaux and Grenson 1990; Mai and Lipp 1994; Rouillon et al. 1999); branched-chain amino acid transaminases by *BAT1* and *BAT2* and aromatic amino acids transaminases by *ARO8* and *ARO9* (Dickinson and Norte 1993; Eden et al. 2001; Hazelwood et al. 2008; Kispal et al. 1996; Lilly et al. 2006b; Ugliano and Henschke 2009). In the valine-degradation pathway, any one of the three isozymes of the pyruvate dehydrogenase complex (PDC), encoded by *PDC1*, *PDC5* and *PDC6*, will decarboxylate α-ketoisovaleric acid (Dickinson et al. 1998); in isoleucine catabolism, any one of the family of decarboxylases encoded by *PDC1*, *PDC5*, *PDC6*, *KID1* or *ARO10* is sufficient for the decarboxylation reaction (Dickinson et al. 2000); in the leucine-degradation pathway, the major decarboxylase is encoded by *KID1* (Dickinson et al. 1997); in the case of aromatic amino acids, *PDC1*, *PDC5*, *PDC6* or *ARO10* are involved (Dickinson et al. 2003). And finally, ethanol dehydrogenases are codified by *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5*, *ADH6*, *ADH7* and *SFA1* (encoding formaldehyde dehydrogenase) (Delneri et al. 1999; Hazelwood et al. 2008). On the other hand, aryl alcohol dehydrogenases, *AAD10* and *AAD14*, are believed to be responsible for the degradation of aromatic aldehydes into their corresponding higher alcohols (Delneri et al. 1999) and higher alcohols can also be produced de novo through carbohydrate metabolism (Äyräpää 1968, 1971).

Several studies have been carried out in order to understand the complexity of higher alcohol formation and to be able to modulate the aroma of alcoholic beverages by yeasts Recent screenings based on constructing double- and triple-deletion mutants presented *AAD6*, *BAT2*, *HOM2*, *PAD1*, *PRO2*, *SPE1* and *THI3* as the most important genes affecting higher alcohol production, being *BAT2* the dominant gene in this respect and suggesting that the initial transaminase step of

the Ehrlich pathway is rate-limiting (Styger et al. 2011, 2013). Other studies showed that overexpression of the branched-chain amino acids transaminases *BAT1* or *BAT2* under the control of the constitutive phosphoglycerate kinase I gene (*PGK1*) lead to an increase in the levels of isoamyl alcohol, isoamyl acetate and, to a lesser extent, isobutanol and isobutyric acid or an increase in isobutanol, isobutyric acid and propionic acid, respectively. In both cases, wines presenting higher ‘peach’ and ‘apricot’ notes were obtained (Lilly et al. 2006b). In the case of the wort fermentation, *BAP2* gene was overexpressed under the glyceraldehyde 3-phosphate dehydrogenase promotor (*TDH3*) in a brewer’s yeast (Kodama et al. 2001). As a result, accelerated assimilation rates of branched-chain amino acids resulted in an increased production of isoamyl alcohol derived from leucine, while no increases of isobutyl alcohol derived from valine or of active amyl alcohol derived from isoleucine were observed. These results suggest that the mechanisms for the production of each higher alcohol are, although interconnected, not the same. Finally, a recent study has shown that the synthesis of higher alcohols seems to be influenced by the NAD^+/NADH availability, having the redox balance an important impact (Jain et al. 2012).

10.6.2 Acetate Esters

Acetate esters such as ethyl acetate (‘solvent’-like aroma), isoamyl acetate (‘banana’ aroma), ethyl caproate and ethyl caprylate (‘sour apple’ aroma) and 2-phenylethyl acetate (‘flowery’, ‘roses’ and ‘honey’ aromas), give desirable ‘fruity’ and ‘floral’ aromas in the alcoholic beverages (Lambrechts and Pretorius 2000; Swiegers et al. 2005).

The concentration of acetate esters in wines is affected by different factors such as maturity and sugar content (Houtman et al. 1980a, b), yeast species, fermentation temperature (Piendl and Geiger 1980), alcoholic and malolactic fermentation, winemaking method (Herraiz and Ough 1993; Gómez et al. 1994) or the presence of non-soluble material in the must (Edwards et al. 1985). Besides, different factors after the fermentative process, such as time and temperature of ageing and storage, affects ester content in wine (Marais and Pool 1980; Ramey and Ough 1980). Regarding yeast species carrying out the fermentation, acetate ester production depends on each strain (Antonelli et al. 1999; Mateo et al. 1992). Some studies have demonstrated that *S. cerevisiae* produces high amounts of several acetate esters such as isopenthyl acetate, phenylethyl acetate, isoamyl acetate, hexyl acetate (Nykänen and Nykänen 1977; Soles et al. 1982; Suomalainen and Lehtonen 1979), whereas *S. bayanus* has demonstrated to be a good 2-phenylethyl acetate producer (Soles et al. 1982). Comparison between *Saccharomyces* and non-*Saccharomyces* regarding acetate esters production showed species dependence in the production of these aromatic compounds (Gil et al. 1996; Lema et al. 1996; Rojas et al. 2001).

Recently, it has been demonstrated that pure and/or mixed cultures of several non-*Saccharomyces* strains are able to increase ester levels in wine: *Hanseniaspora guillermondii* (2-phenyl ethyl acetate) and *H. uvarum* (isoamyl acetate) (Moreira et al. 2008); *Hanseniaspora osmophila, H.vinae* and *H. anomala* (2-phenyl ethyl acetate) (Viana et al. 2008, 2009, 2011; Izquierdo-Canas et al. 2011); *Pichia membranifaciens* and *Pichia klyuveri* (Viana et al. 2009; Swiegers et al. 2011); *Williopsis saturnus* and *T. delbrueckii* (ethyl and isoamyl acetate) (Erten and Tanguler 2010; Swiegers et al. 2011; Izquierdo-Canas et al. 2011; Tanguler 2012; Azzolini et al. 2012). Some of the strains of the genera *Hanseniaspora* spp., *Torulaspora* spp., *Kluyveromyces* spp., *Pichia* spp., and *Williopsis* spp. have been commercialised.

In lager beers, the only acetate ester that can be sensorially perceived is isoamyl acetate (Dufour and Malcorps 1995). However, the presence of multiple esters can have a synergistic effect, having an impact on the overall flavour (Meilgaard 1975). In addition, it has been demonstrated that small changes in ester concentration can have a significant impact on beer flavour (Hammond 1995). Several fermentation conditions have an important impact on ester formation during brewery fermentations (Verstrepen et al. 2003a): fatty acids (Saerens et al. 2008a), temperature (Saerens et al. 2008a), wort gravity (Saerens et al. 2008b; Piddocke et al. 2009; Lei et al. 2012), pitching rate (Verbelen et al. 2009a) and oxygen (Verbelen et al. 2009b).

Acetate esters are synthesised by a condensation reaction between higher alcohols and acetyl-CoA. This reaction is mediated by acetyltransferases codified by genes *ATF1*, *Lg-ATF1* and *ATF2* (Fujii et al. 1994, 1996; Fujiwara et al. 1999; Lilly et al. 2006a; Saerens et al. 2008b, 2010; Verstrepen et al. 2003c). *ATF1* and *ATF2* are present in both ale and lager strains, but Lg-*ATF1* is found only in lager strains (Yoshimoto et al. 1998). During fermentation, acetate ester production rates are dependent on alcohol acetyltransferases activity (Malcorps et al. 1991). Besides, the effect of esterases encoded by *IAH1* and *TIP1* is also important for the final concentration of acetate esters (Horsted et al. 1998; Lilly et al. 2006a; Saerens et al. 2008b, 2010).

Deletion/overexpression studies indicated that the *ATF2*-encoded enzyme of *S. cerevisiae* plays a minor role as compared with its *ATF1*-encoded enzyme (Lilly et al. 2000, 2006a; Verstrepen et al. 2003b). Additionally, the fact that the double-deletion strain produced considerable amounts of certain esters suggests the existence of additional, as yet unknown, ester synthases in the yeast proteome (Verstrepen et al. 2003b). Interestingly, overexpression of different alleles of *ATF1* and *ATF2* led to different ester-production rates, indicating that differences in the aroma profiles of yeast strains may be partially due to mutations in their *ATF* genes (Verstrepen et al. 2003b).

In addition, it has been recently postulated that the ratio acetyl-CoA/CoA could affect acetate ester synthesis (Cordente et al. 2007). The carnitine acetyltransferases catalyse the reversible reaction between carnitine and acetyl-CoA to form acetylcarnitine and CoA. Overexpression of *CAT2*-encoded mitochondrial and

cytosol carnitine acetyltransferases resulted in lower levels of acetate esters in the fermentation since less acetyl-CoA is available for acetate ester synthesis (Cordente et al. 2007).

10.6.3 Ethyl Esters

Ethyl esters such as ethyl propanoate, ethyl butanoate, ethyl hexanoate (ethyl caprylate), ethyl octanoate (ethyl caproate), ethyl decanoate (ethyl caprate) and ethyl lactate give desirable fruity and flowery aroma to the wine. They are produced by condensation between ethanol and acyl-CoA, reaction mediated by acyltransferases. These acyltransferases are encoded by the genes *EHT1* (ethanol hexanoyl transferase 1) and *EEB1* (ethanol hexanoyl transferase) (Rossouw et al. 2008; Saerens et al. 2006, 2008a, 2010), the latter being responsible for the majority of ethyl ester production in *S. cerevisiae* as shown in deletion studies (Saerens et al. 2006). The final concentration of ethyl esters in wine will therefore be influenced by the esterase activity of *EHT1* and *EEB1* encoded-transferases (Saerens et al. 2006), as well as the effect of esterases encoded by *IAH1* and *TIP1* (Horsted et al. 1998; Lilly et al. 2006a; Saerens et al. 2008b, 2010).

Ethyl ester concentrations in alcoholic beverages are affected by the same factors mentioned for acetate esters in the previous section. Regarding yeast species carrying out the fermentation, ester production depends on each strain (Mateo et al. 1992). Several studies have demonstrated that *S. cerevisiae* produced high amounts of several ethyl esters such as ethyl caproate, ethyl caprylate and ethyl caprate (Antonelli et al. 1999; Nykänen and Nykänen 1977; Soles et al. 1982; Suomalainen and Lehtonen 1979), whereas *S. bayanus* has demonstrated to be a good ethyl caprate and ethyl lactate producer (Antonelli et al. 1999; Soles et al. 1982). Comparison between *Saccharomyces* and non-*Saccharomyces* ethyl ester production showed *Saccharomyces* species produced equal or higher ethyl esters amounts (Gil et al. 1996; Lema et al. 1996).

10.6.4 Aldehydes

Acetaldehyde is the most important aldehyde present in alcoholic beverages from a quantitative point of view. In beer, acetaldehyde is normally present at close to its flavour threshold (Engan 1981), whereas different levels can be found in wines. The average values are about 80 mg/l for white wine, 30 mg/l for red wine and 300 mg/l for sherries (McCloskey and Mahaney 1981). At low levels, it gives a pleasant, fruity aroma, but at high concentrations it possesses a pungent irritating odour (Miyake and Shibamoto 1993). Excess acetaldehyde produces a 'green', 'grassy' or 'apple-like' off-flavour in beer (Margalith 1981; Adams and Moss 2000), cider (Williams 1974) and wine (Henschke and Jiranek 1993), with the

exception of sherry-type wines, where high acetaldehyde content is a characteristic feature (Sponholz 1993; Cortes et al. 1998).

Acetaldehyde, also called ethanal, is an intermediary of alcoholic fermentation obtained by the decarboxylation of pyruvate. Pyruvate decarboxylase enzymes encoded by *PDC1*, *PDC2* and *PDC3* participate in this process. Later on, acetaldehyde is reduced to ethanol by alcohol dehydrogenase enzymes, primarily the enzyme encoded by the *ADH1* gene (Pronk et al. 1996), although a little quantity always remains in the wine. The conversion of acetaldehyde to ethanol is required for the maintenance of the redox balance of the cell, since it re-oxidises NADH to NAD^+, which will be available for glycolysis. In this way, sugar is the primary substrate for acetaldehyde formation, but metabolism of amino acids such as alanine also contributes to the synthesis of this compound (Henschke and Jiranek 1993; Boulton et al. 1998).

In alcoholic beverages, acetaldehyde is mainly produced in the first stages of fermentation and its concentration drops at the end of the fermentation and during maturation due to yeast activity. The levels of acetaldehyde in vinification can be considerably affected, from 0.5 to 286 mg/l, depending on the yeast strain (Liu and Pilone 2000), but other factors can affect acetaldehyde level in wines, such as low quantity of zinc, presence of oxygen late in the fermentation, the nature of insoluble material used to clarify the must, increasing fermentation temperature or the excessive use of SO_2 in grape must (Delfini and Costa 1993; Romano et al. 1994; Liu and Pilone 2000). Excessive acetaldehyde levels contribute to a perception of oxidation, although in some Jerez wines such as Fino and Manzanilla high concentrations of this compound are desirable (Zamora 2009). In the case of the beer, high acetaldehyde concentrations reflect premature flocculation or a decrease in yeast viability.

10.6.5 Ketones

Vicinal diketones appear normally in beer fermentation and are undesirable compounds affecting lager beer flavour (Inoue 1992; Wainwright 1973), whereas it is a characteristic flavour of some ale beers. The two most important vicinal diketones are diacetyl (2,3-butanedione) and 2,3-pentanedione. Diacetyl confers a 'butterscotch'-like aroma and pentanedione, a 'honey'-like aroma. In the case of the wine, diacetyl can contribute to wine aroma complexity in low concentrations, giving 'nutty' or 'toasty' nuances, but it becomes undesirable at levels between 1 and 4 mg/l (Sponholz 1993).

Diacetyl is synthesised from α-acetolactate, an intermediate in the valine and leucine biosynthesis pathway, by spontaneous oxidative decarboxylation. Yeasts are also able to reduce diacetyl to acetoin, which may then be further reduced to 2,3-butanediol. Acetoin has a much higher flavour threshold (50 mg/l) than diacetyl, exhibits 'fruity', 'mouldy' and 'woody' flavours (Meilgaard 1975) and does not cause any off-flavours in the beer. The production of diacetyl in beer is

increased by low pH, high temperature, oxygen and the presence of metal ions (Haukeli and Lie 1978) and can be regulated in wort by nitrogen content, valine addition (Krogerus and Gibson 2013) and the enzyme α-acetolactate decarboxylase (Godtfredsen and Ottesen 1982).

The *ILV*-encoded enzyme forms α-acetolactate from pyruvate. This enzyme is subject to general amino acid control and very strong feedback inhibition by valine. In the case of lager yeasts, 90–95 % of the diacetyl reductase activity is accounted for alcohol dehydrogenases (Bamforth and Kanauchi 2004), whereas in the case of ale yeasts, enzymes other than alcohol dehydrogenases appear to be more important, these enzymes being only responsible for the 60 % of the reductase activity.

There have been several attempts to try to reduce diacetyl formation in brewing, such as the disruption of the gene *ILV2*. Mutants lacking this gene did not produce diacetyl but because of their inability to synthesise valine and leucine, such yeasts fermented poorly (Ryder and Masschelein 1983). Changing the upstream regulatory sequence of *ILV2* could reduce the level of this enzyme rather than to eliminate it completely (Petersen et al. 1983). An alternative approach was to increase the flux to amino acid synthesis, which has been achieved transforming yeasts with multiple copies of the *ILV5* gene (Villanueva et al. 1990; Goossens et al. 1991). Conversely, transformations with the *ILV3* gene had no effect on diacetyl concentration (Goossens et al. 1987).

10.6.6 Organic Acids

Acetic acid is the main responsible for volatile acidity of wines. Other contributors to volatile acidity are propionic acid and hexanoic acid. The optimal concentration in wine is 0.2–0.7 g/l (Corison et al. 1979; Dubois 1983). At high concentrations (0.7–1.1 g/l), acetic acid imparts a 'vinegar' flavour to the wine. *S. cerevisiae* wine strains can produce from 100 mg/l to 2 g/l of acetic acid depending on the conditions during fermentation and the type of strain (Radler 1993). *S. bayanus* and *S. uvarum* usually produce less acetic acid than *S. cerevisiae* (Giudici et al. 1995; Eglinton et al. 2000). Furthermore, certain strains of *T. delbrueckii* have been shown to reduce acetic acid production in wine (Bely et al. 2008; Van Breda et al. 2013).

Acetate is produced through acetaldehyde oxidation in a reaction catalysed by acetaldehyde dehydrogenases encoded by *ALD4* and *ALD5* (mitochondrial isoforms) and *ALD6*, *ALD2* and *ALD3* (cytosolic isoforms) (Navarro-Aviño et al. 1999). During winemaking, Ald6p, Ald5p and Ald4p are the main enzymes responsible for acetate formation (Saint-Prix et al. 2004). Deletion of both alleles of *ALD6* in a wine yeast caused a 2-fold reduction in the amount of acetate produced during fermentation, but as a consequence of the redox imbalance

generated, glycerol, succinate and 2,3-butanedediol production was slightly increased (Remize et al. 2000).

On the other hand, mutations in the stress response gene *YAP1* (Cordente et al. 2013) or use of non-*Saccharomyces* yeasts such as *T. delbrueckii* (Bely et al. 2008; Van Breda et al. 2013) constitute succesful examples of non-GMO approaches to decrease acetic acid formation during fermentation.

In high-gravity brewing, yeast cells are stressed because of high sugar and ethanol concentrations, which can lead to higher production of acetic acid, which can be a problem to beer quality (Mizuno et al. 2003). An alternative to solve this problem could be to employ a mutant overexpressing *ALD4* (Mizuno et al. 2006), which produced half the amount of acetic acid and 1.1 % more ethanol than beer brewed using the wild-type.

10.6.7 Volatile Phenols

Volatile phenols can appear in wine as a consequence of a non-oxidative decarboxylation of hydroxycinnamic acids *p*-coumaric and ferulic carried out by yeasts (Chatonnet et al. 1993; Grando et al. 1993) or through decarboxylation of phenolic acids, usually first into 4-vinyl derivatives that are then reduced to 4-ethyl derivatives through enzymes called phenolic acid decarboxylases (Cavin et al. 1993). The genes encoding phenolic acid decarboxylases include *PAD1* (also known as *POF1*). However, phenolic acid decarboxylase activity is very low in most *S. cerevisiae* strains (Barthelmebs et al. 2000a, b) and several attemps have been carried out to develop mutant strains to modulate volatile phenol production. Strains overexpressing the *Bacillus subtilis* phenolic acid decarboxylase gene (*padc*), the *Lactobacillus plantarum p*-coumaric acid decarboxylase gene (*pdc*) and strains in which *PAD1/POF1* gene was disrupted, are examples of succesful volatile phenol modulation (Smit et al. 2003). Contrarily, constructed strains overexpressing *S. cerevisiae* phenylacrylic acid decarboxylase gene (*PAD1/POF1*) has no significant effect in volatile phenol synthesis (Smit et al. 2003).

Volatile phenols possess low sensory thresholds and, in spite of the fact that they can be desirable in certain wines, normally they appear as off-flavours (‘stable’, ‘barnyard’, ‘pharmaceutical’) (Dubois 1983). Ethyl phenols (4-ethyl guaiacol and 4-ethyl phenol) present a special negative contribution and are derived from the reduction of vinyl phenols (4-vinyl guaiacol and 4-vinyl phenol). Vinyl reductase activity is typically associated with *Brettanomyces* and *Dekkera* spp.

On the other hand, volatile phenols can contribute positively or negatively depending on the beer product. The presence of excessive amounts of vinyl phenols is considered undesirable in bottom-fermented pilsners. Hence the term ‘phenolic off-flavour’ (POF) is attributed to beers with a strong aroma described as ‘pharmaceutical’, ‘medicinal’, ‘solvent’, ‘spicy’, ‘clove-like’, ‘smokey’ or ‘barbeque’. However, these compounds are crucial for the characteristic aroma of Belgian white beers (made with unmalted wheat), German rauch beers and Weizen

beers (made with malted wheat) and in many top-fermented blond and dark specialty beers.

10.6.8 Sulphur Compounds

Hydrogen sulphide imparts a 'rotten egg' aroma and has a very low odour threshold of 10–80 μg/l (Swiegers et al. 2005). The concentration of H_2S produced during wine fermentation depends on the presence of sulphur compounds, wine yeast strain, fermentation conditions, and the nutritional status of the grape juice (Henschke and Jiranek 1991; Rauhut 1993; Spiropoulos and Bisson 2000). However, some strains produce H_2S constitutively without being affected by environmental conditions (Jiranek et al. 1995; Spiropoulos and Bisson 2000; Mendes-Ferreira et al. 2002).

During wine fermentation, yeast can synthesise hydrogen sulphide from either inorganic sulphur compounds (sulphate and sulfite) or from organic sulphur compounds (cysteine and glutathionine) (Henschke and Jiranek 1993; Rauhut 1993; Hallinan et al. 1999; Spiropoulos and Bisson 2000).

The sulphate reduction sequence (SRS) is activated in response to the necessity to produce cysteine and methionine, usually insufficient in wine must (Henschke and Jiranek 1993). The firs step involves the transportation of suphate from the medium into the yeast cell by sulphate permease. Several steps follow to reduce sulphate to sulphide using the enzymes ATP-sulfurylase and sulfite reductase. Subsequently, *O*-acetylserine (from the amino acid serine) combines with sulphide to form cysteine, and *O*-acetylhomoserine (from the amino acid aspartate) combines with sulphide to form homocysteine, which can then be converted to methionine (Thornton and Bunker 1989; Yamagata 1989; Henschke and Jiranek 1993; Rauhut 1993; Jiranek et al. 1995; Spiropoulos and Bisson 2000). Nitrogen limitation leads to insufficient of these precursors and sulphide is accumulated and released to the medium as hydrogen sulphide (Henschke and Jiranek 1993; Rauhut 1993; Jiranek et al. 1995; Spiropoulos and Bisson 2000). Additionally, significant amounts of H_2S can be produced when the fermentation medium is rich in sulphite since it can diffuse into the cell.

Several attempts have been made to modulate H_2S production by using certain wine and brewing yeasts that are commercially available. The consequences of overexpression of the *MET17* gene, which encodes *O*-acetylserine and *O*-acetylhomoserine sulfhydrylase in *S. cerevisiae,* seemed to be strain dependent (Omura et al. 1995; Spiropoulos and Bisson 2000). Conversely, the deletion of the *MET14* gene (encoding an adenosylphosphosulfate kinase) or the *MRX1* gene (encoding a methionine sulfoxide reductase), might be the most effective way to prevent wine yeast from producing H_2S in fermentations (Pretorius 2000, 2003, 2004; Pretorius and Høj 2005). Another attempt to prevent H_2S formation was carried out through modifying the activity of the sulfite reductase enzyme by engineering one of the enzyme subunits codified by *MET10* (Sutherland et al. 2003).

This strategy has been succesfully applied in beer (Hansen and Kielland-Brandt 1996). On the other hand, classical mutagenesis which lead to mutants presenting mutations in *MET5* and *MET10* genes produced 50–99 % less H_2S than the parental strain (Cordente et al. 2009). Some of these wine strains are now in commercial use. Finally, increased expression of *CYS4* in brewing yeast, encoding the cystathionine β-synthase, has been shown to suppress the formation of H_2S (Tezuka et al. 1992).

Another sulphur compound that can be detrimental for the flavour of alcoholic beverages is ethanethiol ('onion' aroma), synthesised through the reaction of hydrogen sulphide and ethanol or acetaldehyde (Rauhut 1993). On the other hand, dimethyl sulphide (DMS), which present 'asparagus', 'corn' and 'molasses' notes, might be produced in wine via cleavage of *S*-methyl-L-methionine to homoserine and DMS. In beer production, heat decomposition during malting of *S*-methyl-methionine produces dimethyl sulfoxide (DMSO), which can be reduced to DMS, during storage (Rauhut 1993) or fermentation by yeasts. In *S. cerevisiae*, the *MXR1* gene has been shown to encode a methionine sulfoxide reductase and its disruption prevents DMS production (Hansen 1999). Finally, DMS formation during fermentation has also been linked to cysteine, cystine or glutathione metabolism in yeast (Rauhut 1993; Ribéreau-Gayon et al. 2000).

10.7 Yeasts and Its Role in the Development of Varietal Aroma in Wine

In addition to the aroma-active compounds synthesised by yeasts during alcoholic fermentations, some yeasts play a relevant role in the development of the primary or varietal aroma of wines (Gamero et al. 2011a, b). Wine's primary aroma consists of lactones, benzenes, volatile phenols, vanillins, norisoprenoids, terpenes and some polyfunctional mercaptans present at low concentrations in the ng/l–μg/l range (Loscos et al. 2007; Mateo-Vivaracho et al. 2010; Tominaga et al. 1998b). Most of these aromas appear in grapes as odourless precursors (glycosides, polyhydroxylated molecules or cysteinyl-derivatives). It has been demonstrated that some yeasts are able to release those aroma compounds by cleavage of the precursor molecules or are even able to synthesise new aroma molecules similar to the ones present in grapes (Darriet et al. 1988; Delcroix et al. 1994; Delfini et al. 2001; Fernández-González et al. 2003; Fernández-González and Di Stefano 2004; Gamero et al. 2011a, b; Hernández et al. 2003; Hernández-Orte et al. 2008; Loscos et al. 2007; Mateo and Di Stefano 1997; Spagna et al. 2002; Ugliano et al. 2006; Ugliano and Moio 2008). In this way, yeast can enhance wine varietal aroma. For instance, *Saccharomyces* species and hybrids are able to release and synthesise de novo vanillins, terpenes, lipid derivatives, volatile phenols and norisoprenoids (Gamero et al. 2011a, b).

In certain wines, varietal aroma compounds play a crucial role. This is the case of some polyfunctional mercaptans in certain white wines (Tominaga et al. 1998b; Mateo-Vivaracho et al. 2010), of linalool and other terpenols in Muscat wines

(Ribéreau-Gayon et al. 2000) or of *cis*-rose oxide in Gewürztraminer (Guth 1997). On the other hand, in most wines, varietal aroma is formed by combinations of many grape- and yeast-derived compounds, none of which play a predominant aroma role, and it is the overall aroma profile the responsible for varietal and origin related difference (Escudero et al. 2007; Loscos et al. 2007, 2010).

10.7.1 Monoterpenes

Among the most important key odorants in the so-called 'aromatic' grape varieties (e.g. Muscat) are monoterpenes such as linalool, geraniol, nerol, citronellol and α-terpineol. (Gunata et al. 1985; Loscos et al. 2007; Maicas and Mateo 2005; Strauss et al. 1986; Ugliano and Henschke 2009). The common precursor of all the monoterpenoids is isopentyl pyrophosphate.

During must fermentation the grape-derived glycosidic precursors are hydrolysed by the action of glycosidases and the aromatic volatile compounds released into the wine. Among the most important glycosidases are β-glucosidases, α-L-arabinofuranosidases, α-L-rhamnosidases and β-D-xylosidases (Maicas and Mateo 2005; Van Rensburg and Pretorius 2000; Sarry and Gunata 2004). Several research groups the world over have investigated various wine-related *Saccharomyces* and non-*Saccharomyces* (*Brettanomyces/Dekkera*, *Candida*, *Debaryomyces*, *Hanseniaspora* and *Pichia*) yeasts for their ability to produce suitable glycosidases and other enzymes that can release varietal aromas (Charoenchai et al. 1997; Esteve-Zarzoso et al. 1998; Fernández et al. 2000; Fleet 2008; McMahon et al. 1999; Strauss et al. 2001; Ugliano et al. 2006; Zoecklein et al. 1997).

In addition to the search for yeasts that naturally produce aroma-enhancing enzymes, several mutants have been constructed with the aim of enhancing monoterpene-based varietal flavours during wine fermentation. These mutants include a yeast expressing the β-1,4-glucanase gene from *Trichoderma longibratum* (Villanueva et al. 2000) and a wine yeast expressing the *BGL1* and *BGL2* β-glucosidase genes of *Saccharomycopsis fibuligera*, the *ABF2* α-L-arabinofuranosidase gene of *Aspergillus niger* and a glucanase-encoding gene cassette consisting of several glucanase genes (*BEG1*, *END1* and *EXG1*) (Pretorius 2000, 2003, 2004; Van Rensburg and Pretorius 2000; Pretorius and Bauer 2002; de Barros Lopes et al. 2006).

In addition to studies focused on the release of monoterpenes by yeasts, there were also several studies undertaken concerning the biotransformation of terpenes by *Saccharomyces* species and hybrids, such as the reduction of geraniol to citronellol, translocation of geraniol to linalool, isomerisation of nerol to geraniol and cyclicizations of linalool to α-terpineol (Gamero et al. 2011b; Gramatica et al. 1982; King and Dickinson 2000; Zea et al. 1995; Zoecklein et al. 1997).

In the case of brewing, it has also recently been shown that different hop varieties have different concentrations of monoterpenoids (Takoi et al. 2010).

10.7.2 Volatile Thiols

Volatile thiols are sulphur compounds that can appear in wines in very low concentrations, but they can have a profound impact on the aroma of certain wine varieties, such as Sauvignon Blanc, Colombard, Riesling, Semillon, Merlot and Cabernet Sauvignon, since they present very low sensory thresholds (ng/l level) (Tominaga et al. 1995, 1998a, b; Murat et al. 2001b). These compounds are responsible for the 'fruity' or 'tropical' organoleptic flavours. Some examples of volatile thiols are 4-mercapto-4-methylpentan-2-one (4MMP), reminiscent of 'box tree', 'passion-fruit', 'broom' and 'black current' bud; 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), responsible for 'passion-fruit', 'grapefruit' and 'citrus' aromas; 4-mercapto-4-methylpentan-2-ol (4MMPOH) that can also contribute to the characters of 'citrus', 'passion-fruit' and 'grapefruit', although its organoleptic role is more limited, due to its concentration in wines seldom exceeding its olfactory threshold of 55 ng/l and 2-furfurylthiol, which can contribute roast coffee aroma to the bouquet of wines aged in oak barrels (Darriet et al. 1995; Tominaga et al. 1996, 1998b, 2000; Tominaga and Dubourdieu 2006).

Most of the thiols that are present in grapes appear as non-volatile, cysteine-bound conjugates and can be released by the action of carbon–sulphur lyases of certain yeasts (Darriet et al. 1995; Tominaga et al. 1995). Deletion and overexpression of the genes encoding these enzymes resulted in a decrease and increase, respectively, in the levels of the corresponding thiols (Howell et al. 2005; Swiegers et al. 2007). The release of thiols occurs during fermentation in a low percentage since it has been detected that only a small fraction of cysteine-bound conjugates (1.6–3.2 %) is released as 3MH (Dubourdieu et al. 2006; Murat et al. 2001b). The efficiency of thiol release is strain dependent (Dubourdieu et al. 2006; Howell et al. 2004); however, some studies reported that *S. bayanus* and *S. bayanus/ S. cerevisiae* hybrid strains have stronger abilities than *S. cerevisiae* in this sense (Murat et al. 2001a; Swiegers et al. 2006a). In addition, certain non-*Saccharomyces* yeasts can have a significant impact on volatile thiol concentration. Co-inoculation of *Pichia kluyveri*, isolated from a spontaneous fermentation of Chardonnay must, with specific commercial wine strains of *S. cerevisiae* resulted in an increase of the concentration of 3MHA in Sauvignon Blanc wines (Anfang et al. 2009). Recent work also showed that some strains of *Metschnikowia pulcherrima*, *T. delbrueckii* and *K. thermotolerans* have relatively high capacities to release 3MH (Zott et al. 2011).

Thiols can also be synthesised by yeasts. For instance, it has been proposed that cysteine desulfhydrase enzyme catalyses the formation of furfurylthiol from furfural (Tominaga et al. 2000) and the formation of H_2S enhances this process. In this way, the production of furfurylthiol is linked to the production of the HS^- anion, which is not produced when sufficient ammonium sulphate is present in the medium (Tominaga et al. 2000). During fermentation, 3MH can be converted to 3MHA by the action of alcohol acetyltransferase, encoded by the *ATF1* gene (Swiegers et al. 2006b). There is significant variation in the conversion rates

present by the different yeast strains, which is not correlated with the ability to release 4MMP (Swiegers et al. 2006b).

In addition to the specific yeast strain conducting the fermentation, temperature is also a relevant factor in determining volatile thiol concentration. Concentrations of 4MMP, 3MH and 3MHA were higher when the alcoholic fermentation was conducted at 20 °C compared to 13 °C or a 18 °C compared to 23 °C and 28 °C (Masneuf-Pomarède et al. 2006; Swiegers et al. 2006a). So around 18–20 °C seems to be the optimum.

Regarding beer, there is not much knowledge about the occurrence of volatile thiols. However, Vermeulen et al. (2006) detected more than ten of these compounds in fresh lager beer. Thiols do not appear in wort. The most powerful thiol in beer is 3-methyl-2-buten-1-thiol, and this thiol together with 2-mercapto-3-methylbutanol and 3-mercapto-3-methylbutanol are thought to be derived from hop allylic alcohols (Vermeulen et al. 2006). On the other hand, it is hypothesised that the origin of 2-mercaptoethanol and 3-mercaptopropanol and their corresponding acetates could be Ehrlich degradation of sulphur amino acids, whereas 2-methyl-3-furanthiol could be produced through Maillard reactions (Vermeulen et al. 2006).

10.8 Concluding Remarks

The aroma profile of wine, beer and cider is a defining component of the value proposition to consumers. Producers are therefore keen to understand what the optimal ‘absolute’ and ‘relative’ concentrations of the most important aroma-active compounds are and how they can adapt their practices to gain control over the composition of their products. It is widely accepted that one way to adjust the aroma profile of certain styles of fermented beverages is choice of yeast strain(s) with which the fermentation is conducted. However, further research is required into the range of ‘aroma phenotypes’ that wine yeast exhibit, and how this knowledge can be applied to develop novel aroma-enhancing yeast strains or combinations of yeast strains or mixtures of different yeast species.

References

Adams MR, Moss MO (2000) Food microbiology. Royal Society of Chemistry, Cambridge
Anfang N, Brajkovich M, Goddard M (2009) Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon Blanc. Austr J Grape Wine Res 15:1–8
Antonelli A, Castellari L, Zambonelli C et al (1999) Yeast influence on volatile composition of wines. J Agric Food Chem 47:1139–1144
Antunovics Z, Csoma H, Sipiczki M (2003) Molecular and genetic analysis of the yeast flora of botrytized Tokaj wines. Bull de l’OIV 76:380–397
Äyräpää T (1968) Formation of higher alcohols by various yeasts. J Inst Brew 74:169–179

Äyräpää T (1971) Biosynthetic formation of higher alcohols by yeast. Dependence on the nitrogenous nutrient level of the medium. J Inst Brew 77:266–276

Azzolini M, Fedrizzi B, Tosi E et al (2012) Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone wine. Eur Food Res Tech 235:303–313

Bamforth CW, Kanauchi M (2004) Enzymology of vicinal diketone reduction in brewer's yeast. J Inst Brew 110:83–93

Bandell M, Story GM, Hwang SW et al (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron 41(6):849–857

Barrio E, González SS, Arias A et al (2006) Molecular mechanisms involved in the adaptive evolution of industrial yeasts. In: Querol A, Fleet GH (eds) Yeasts in food and beverages. The yeast handbook. Springer, Berlin, pp 153–174

Barthelmebs L, Divies C, Cavin JF (2000a) Knockout of the *p*-coumarate decarboxylase gene from *Lactobacillus plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. Appl Environ Microbiol 66:3368–3375

Barthelmebs L, Lecomte B, Divies C et al (2000b) Inducible metabolism of phenolic acids in *Pediococcus pentosaceus* is encoded by an autoregulated operon which involves a new class of negative transcriptional regulator. J Bacteriol 182:6724–6731

Bautista DM, Siemens J, Glazer JM et al (2007) The menthol receptor TRPM8 is the principal detector of environmental cold. Nature 448(7150):204–208

Beech FW (1993) Yeasts in cider-making. In: Rose AH, Harrison JS (eds) The yeasts. Yeast technology, vol. 5, 2nd edn. Academic Press Limited, London, pp 169–213

Bely M, Stoeckle P, Masneuf-Pomarède I et al (2008) Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. Int J Food Microbiol 122:312–320

Belloch C, Orlic S, Barrio E et al (2008) Fermentative stress adaptation of hybrids within the *Saccharomyces sensu stricto* complex. Int J Food Microbiol 122(1–2):188–195

Belloch C, Pérez-Torrado R, Gonzalez SS et al (2009) Chimeric genomes of natural hybrids of *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii*. Appl Environ Microbiol 75:2534–2544

Borneman AR, Desany BA, Riches D et al (2012) The genome sequence of the wine yeast *VIN7* reveals an allotriploid hybrid genome with *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii*. FEMS Yeast Res 12:88–96

Boulton RB, Singleton VL, Bisson LF et al (1998) Principles and practices of winemaking. Chapman & Hall, New York

Cabranes C, Moreno J, Mangas JJ (1990) Dynamics of yeast population during cider fermentation in the Asturian Region of Spain. Appl Environ Microbiol 56:381–384

Campo E, Cacho J, Ferreira V (2007) Solid phase extraction, multidimensional gas chromatography mass spectrometry determination of four novel aroma powerful ethyl esters: assessment of their occurrence and importance in wine and other alcoholic beverages. J Chromatogr A 1140(1–2):180–188

Campo E, Ferreira V, Escudero A et al (2005) Prediction of the wine sensory properties related to grape variety from dynamic-headspace gas chromatography-olfactometry data. J Agric Food Chem 53(14):5682–5690

Caterina MJ, Schumacher MA, Tominaga M et al (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389(6653):816–824

Cavin JF, Andioc V, Etievant PX et al (1993) Ability of wine lactic acid bacteria to metabolize phenol carboxylic acids. Am J Enol Vitic 44:76–80

Charoenchai C, Fleet GH, Henschke PA et al (1997) Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Austr J Grape Wine Res 3:2–8

Chatonnet P, Dubourdieu D, Boidron JN et al (1993) Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines. J Sci Food Agric 62:191–202

Coloretti F, Zambonelli C, Tini V (2006) Characterization of flocculent *Saccharomyces* interspecific hybrids for the production of sparkling wines. Food Microbiol 23:672–676

Cordente AG, Cordero-Bueso G, Pretorius IS et al (2013) Novel wine yeast with mutations in *YAP1* that produce less acetic acid during fermentation. FEMS Yeast Res 13(1):62–73

Cordente AG, Heinrich A, Pretorius IS et al (2009) Isolation of sulfite reductase variants of a commercial wine yeast with significantly reduced hydrogen sulfide production. FEMS Yeast Res 9(3):446–459

Cordente AG, Swiegers JH, Hegardt FG et al (2007) Modulating aroma compounds during wine fermentation by manipulating carnitine acetyltransferases in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 267:159–166

Corison CA, Ough CS, Berg HW et al (1979) Must acetic acid and ethyl acetate as mold and rot indicators in grapes. Am J Enol Vitic 30:130–134

Cortes MB, Moreno J, Zea L et al (1998) Changes in aroma compounds of sherry wines during their biological aging carried out by *Saccharomyces cerevisiae* races *bayanus* and *capensis*. J Agric Food Chem 46:2389–2394

Coton E, Coton M, Levert D et al (2006) Yeast ecology in French cider and black olive natural fermentations. Int J Food Microbiol 108:130–135

Cousseau FE, Alves SL Jr, Trichez D et al (2013) Characterization of maltotriose transporters from the *Saccharomyces eubayanus* sub-genome of the hybrid *Saccharomyces pastorianus* lager brewing yeast strain Weihenstephan 34/70. Lett Appl Microbiol 56(1):21–29

Darriet P, Boidron JN, Dubourdieu D (1988) Hydrolysis of the terpene heterosides of small-seed Muscat grapes by periplasmic enzymes of *Saccharomyces cerevisiae*. Connaissance de la Vigne et du Vin 22:189–195

Darriet P, Tominaga T, Lavigne V et al (1995) Identification of a powerful aromatic compound of *Vitis vinifera* L. var. Sauvignon wines: 4-mercapto-4-methylpentan-2-one. Flav Fragr J 10:385–392

De Barros Lopes MA, Bartowsky EJ, Pretorius IS (2006) The application of gene technology in the wine industry. In: Hui HY, Castell-Pérez E, Cunha LM et al (eds) Handbook of food science, technology, and engineering. CRC Taylor & Francis, New York, pp 1–21

de Barros Lopes M, Bellon JR, Shirly NJ et al (2002) Evidence for multiple interspecific hybridization in *Saccharomyces sensu stricto* species. FEMS Yeast Res 1:323–331

del Campo G, Santos JL, Berregi I et al (2003) Ciders produced by two types of presses and fermented in stainless steel and wooden vats. J Inst Brew 109:342–348

Delcroix A, Gunata Z, Sapis JC et al (1994) Glycoside activities of three enological yeast strains during winemaking: effects of the terpenol content of Muscat wine. Am J Enol Vitic 45:291–296

Delfini C, Cocito C, Bonino M et al (2001) Definitive evidence for the actual contribution of yeast in the transformation of neutral precursors of grape aromas. J Agric Food Chem 49(11):5397–5408

Delfini C, Costa A (1993) Effects of the grape must lees and insoluble materials on the alcoholic fermentation rate and the production of acetic acid, pyruvic acid, and acetaldehyde. Am J Enol Vitic 44:86–92

Delneri D, Gardner DCJ, Bruschi CV et al (1999) Disruption of seven hypothetical aryl alcohol dehydrogenase genes from *Saccharomyces cerevisiae* and construction of a multiple knock-out strain. Yeast 15:1681–1689

Delwiche J (2004) The impact of perceptual interactions on perceived flavor. Food Qual Pref 15(2):137–146

Demuyter C, Lollier M, Legras JL et al (2004) Predominance of *Saccharomyces uvarum* during spontaneous alcoholic fermentation, for three consecutive years, in an Alsatian winery. J Appl Microbiol 97:1140–1148

Di Stefano R, Ciolfi G, Delfini C (1981) Composti volatili prodotti dei lieviti. Rivista di Viticoltura e di Enologia 34:342–355

Dickinson JR, Harrison SJ, Dickinson JA et al (2000) An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae*. J Biol Chem 275:10937–10942

Dickinson JR, Harrison SJ, Hewlins MJE (1998) An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*. J Biol Chem 273:25751–25756
Dickinson JR, Lanterman MM, Danner DJ et al (1997) A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J Biol Chem 272:26871–26878
Dickinson JR, Norte V (1993) A study of branched-chain amino acid aminotransferase and isolation of mutations affecting the catabolism of branched-chain amino acids in *Saccharomyces cerevisiae*. FEBS Lett 326:29–32
Dickinson JR, Salgado LE, Hewlins MJE (2003) The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. J Biol Chem 278:8028–8034
Didion T, Regenberg B, Jorgensen MU et al (1998) The permease homologue *Ssy1p* controls the expression of amino acid and peptide transporter genes in *Saccharomyces cerevisiae*. Mol Microbiol 27:643–650
Dubois P (1983) Volatile phenols in wine. In: Piggott JR (ed) Flavours of distilled beverages. Ellis Horwood, Chichester, pp 110–119
Dubourdieu D, Tominaga T, Masneuf I et al (2006) The role of yeasts in grape flavor development during fermentation: the example of Sauvignon Blanc. Am J Enol Vitic 57:81–88
Dufour JP, Malcorps P (1995) Ester synthesis during fermentation: enzymes characterization and modulation mechanisms. In: Campbell I, Priest FG (eds) 4th Aviemore conference on malting, brewing and distilling. The Institute of Brewing, London, pp 137–151
Dunn B, Richter C, Kvitek DJ et al (2012) Analysis of the *Saccharomyces cerevisiae* pan-genome reveals a pool of copy number variants distributed in diverse yeast strains from differing industrial environments. Genome Res 22(5):908–924
Dunn B, Sherlock G (2008) Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. Genome Res 18(10):1610–1623
Eden A, Van Nedervelde L, Drukker M et al (2001) Involvement of branched-chain amino acid aminotransferases in the production of fusel alcohols during fermentation in yeast. Appl Microbiol Biotechnol 55:296–300
Edwards TL, Singleton VL, Boulton R (1985) Formation of ethyl esters of tartaric acid during wine aging: chemical and sensory effects. Am J Enol Vitic 36:118–124
Eglinton JM, McWilliam SJ, Fogarty MW et al (2000) The effect of *Saccharomyces bayanus*-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. Austr J Grape Wine Res 6:190–196
Engan S (1981) Beer composition: Volatile substances. In: Pollock JRA (ed) Brewing science, vol 2. Academic Press, London, pp 93–165
Erten H, Tanguler H (2010) Influence of *Williopsis saturnus* yeasts in combination with *Saccharomyces cerevisiae* on wine fermentation. Lett Appl Microbiol 50:447–449
Escudero A, Campo E, Farina L et al (2007) Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. J Agric Food Chem 55(11):4501–4510
Escudero AB, Gogorza MA, Melús N et al (2004) Characterization of the aroma of a wine from Maccabeo. Key role played by compounds with low odor activity value. J Agric Food Chem 52:3516–3524
Esteve-Zarzoso B, Manzanares P, Ramón D et al (1998) The role of non-*Saccharomyces* yeasts in industrial winemaking. Int Microbiol 1(2):143–148
Etiévant PX (1991) Wine. In: Maarse H (ed) Volatile compounds of food and beverages. Marcel Dekker, New York, pp 483–546
Fernández M, Úbeda JF, Briones AI (2000) Typing of non-*Saccharomyces* yeasts with enzymatic activities of interest in wine-making. Int J Food Microbiol 59:29–36
Fernández-González M, Di Stefano R (2004) Fractionation of glycoside aroma precursors in neutral grapes. Hydrolysis and conversion by *Saccharomyces cerevisiae*. Food Sci Technol 37(4):467–473

Fernández-González M, Di Stefano R, Briones A (2003) Hydrolysis and transformation of terpene glycosides from Muscat must by different yeast species. Food Microbiol 20:35–41

Ferreira V (2012) Revisiting psychophysical work on the quantitative and qualitative odour properties of simple odour mixtures: a flavour chemistry view. Part 2: qualitative aspects. A review. Flav Fragr J 27(3):201–215

Ferreira V, Fernandez P, Cacho JF (1996) A study of factors affecting wine volatile composition and its application in discriminant analysis. Food Sci Technol 29(3):251–259

Ferreira V, Lopez R, Cacho JF (2000) Quantitative determination of the odorants of young red wines from different grape varieties. J Sci Food Agric 80(11):1659–1667

Ferreira V, Ortín N, Escudero A et al (2002) Chemical characterization of the aroma of Grenache rosé wines. Aroma extract dilution analysis, quantitative determination and sensory reconstitution studies. J Agric Food Chem 50:4048–4054

Fleet GH (2008) Wine yeasts for the future. FEMS Yeast Res 8(7):979–995

Fleet GH, Heard GM (1993) Yeasts—Growth during fermentation. In: Fleet GH (ed) Wine microbiology and biotechnology. Harwood Academic Publishers, Chur, pp 27–54

Frezier V, Dubourdieu D (1992) Ecology of yeast strain *Saccharomyces cerevisiae* during spontaneous fermentation in a Bordeaux winery. Am J Enol Vitic 43:375–380

Fujii T, Nagasawa N, Iwamatsu A et al (1994) Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene. Appl Environ Microbiol 60(8):2786–2792

Fujii T, Yoshimoto H, Tamai Y (1996) Acetate ester production by *Saccharomyces cerevisiae* lacking the *ATF1* gene encoding the alcohol acetyltransferase. J Ferm Bioeng 81(6):538–542

Fujiwara D, Kobayashi O, Yoshimoto H et al (1999) Molecular mechanism of the multiple regulation of the *Saccharomyces cerevisiae ATF1* gene encoding alcohol acetyltransferase. Yeast 15:1183–1197

Gamero A, Hernández-Orte P, Ferreira V et al (2011a) Effect of aromatic precursor addition to wine fermentations carried out with different *Saccharomyces* species and their hybrids. Int J Food Microbiol 147(1):33–44

Gamero A, Manzanares P, Querol A et al (2011b) Monoterpene alcohols release and bioconversion by *Saccharomyces* species and hybrids. Int J Food Microbiol 145(1):92–97

Gamero A, Tronchoni J, Belloch C et al (2013) Production of aroma compounds by cryotolerant *Saccharomyces* species and hybrids at low and moderate fermentation temperatures. J Appl Microbiol 114:1405–1414

Gangl H, Batusic M, Tscheik G et al (2009) Exceptional fermentation characteristics of natural hybrids from *Saccharomyces cerevisiae* and *S. kudriavzevii*. N Biotechnol 25(4):244–251

Gil JV, Mateo JJ, Jiménez M (1996) Aroma compounds in wine as influenced by apiculate yeasts. J Food Sci 61:1247–1250

Giudici P, Zambonelli C, Passarelli P et al (1995) Improvement of wine composition with cryotolerant *Saccharomyces* strains. Am J Enol Vitic 46:143–147

Godtfredsen SE, Ottesen M (1982) Maturation of beer with α-acetolactate decarboxylase. Carlsberg Res Commun 47:93–102

Gómez E, Laencina J, Martínez A (1994) Vinification effects on changes in volatile compounds of wine. J Food Sci 59:406–409

González SS, Barrio E, Gafner J et al (2006) Natural hybrids from *Saccharomyces bayanus*, *S. cerevisiae* and *S. kudriavzevii* in wine fermentations. FEMS Yeast Res 6(8):1221–1234

González SS, Barrio E, Querol A (2008) Molecular characterization of new natural hybrids of *Saccharomyces cerevisiae* and *S. kudriavzevii* in Brewing. Appl Environ Microbiol 74(8):2314–2320

González SS, Gallo L, Climent MD et al (2007) Enological characterization of natural hybrids between *Saccharomyces cerevisiae* and *S. kudriavzevii*. Int J Food Microbiol 116(1):11–18

Goossens E, Debourg A, Villanueva K et al (1991) Decreased diacetyl production by site directed integration of the *ILV5* gene into chromosome XIII of *Saccharomyces cerevisiae*. In: Proceedings of the congress of the 23rd European Brewery Convention, Lisbon, 13–15 May 1991

Goossens E, Dillemans M, Debourg A et al (1987) Control of diacetyl formation by the intensification of the anabolic flux of acetohydroxy acid intermediates. In: Proceedings of the 21st congress of the European Brewery Convention, Madrid, 10–14 May 1987

Gramatica P, Manitto P, Ranzi BM et al (1982) Stereospecific reduction of geraniol to R-(+)-citronellol by *Saccharomyces cerevisiae*. Experimentia 38:775–776

Grando MS, Versini G, Nicolini G et al (1993) Selective use of wine yeast strains having different volatile phenols production. Vitis 32(43):50

Grauslund M, Didion T, Kielland-Brandt MC et al (1995) *BAP2*, a gene encoding a permease for branched-chain amino acids in *Saccharomyces cerevisiae*. Biochim Biophys Acta 1269(3):275–280

Green BG, Nachtigal D, Hammond S et al (2012) Enhancement of retronasal odors by taste. Chem Senses 37(1):77–86

Gunata YZ, Bayonove CL, Baumes RL et al (1985) The aroma of grapes. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. J Chromatogr 331:83–90

Guth H (1997) Identification of character impact odorants of different white wine varieties. J Agric Food Chem 45(8):3022–3026

Gutiérrez AR, Santamaría P, Epifanio S et al (1999) Ecology of spontaneous fermentation in one winery during 5 consecutive years. Lett Appl Microbiol 29:411–415

Hallinan CP, Saul DJ, Jiranek V (1999) Differential utilisation of sulfur compounds for H_2S liberation by nitrogen-starved wine yeasts. Austr J Grape Wine Res 5:82–90

Hammond JRM (1995) Genetically-modified brewing yeasts for the 21st century. Progress to date. Yeast 11:1613–1627

Hansen J (1999) Inactivation of *MXR1* abolishes formation of dimethyl sulfide from dimethyl sulfoxide in *Saccharomyces cerevisiae*. Appl Environ Microbiol 65:3915–3919

Hansen J, Kielland-Brandt MC (1996) Inactivation of *MET2* in brewer's yeast increases the level of sulfite in beer. J Biotechnol 50:75–87

Haukeli AD, Lie S (1978) Conversion of α-acetolactate and removal of diacetyl: a kinetic study. J Inst Brew 84:85–89

Hazelwood LA, Daran JM, van Maris AJA et al (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae*. Metabol Appl Environ Microbiol 74(8):2259–2266

Henschke PA, Jiranek V (1991) Hydrogen sulfide formation during fermentation: effect of nitrogen composition in model grape must. In: Proceedings of the international symposium on nitrogen in grapes and wine. American Society for Enology and Viticulture, Seattle, Washington, 18–19 June 1991

Henschke PA, Jiranek V (1993) Yeast—Metabolism of nitrogen compounds. In: Fleet GH (ed) Wine microbiology and biotechnology. Harwood Academic Publishers, Chur, pp 77–164

Henschke PA, Kwiatkowski MJ, Fogarty MW et al (2000) The effect of *Saccharomyces bayanus*-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. Austr J Grape Wine Res 6:190–196

Hernández LF, Espinosa JC, Fernández-González M et al (2003) β-glucosidase activity in a *Saccharomyces cerevisiae* wine strain. Int J Food Microbiol 80:171–176

Hernández-Orte P, Cacho J, Ferreira V (2002) Relationship between the varietal amino acid profile of grapes and the wine aromatic composition. Experiments with model solutions and chemometric study. J Agric Food Chem 50:2891–2899

Hernández-Orte P, Cersosimo M, Loscos N et al (2008) The development of varietal aroma from non-floral grapes by yeasts of different genera. Food Chem 107:1064–1077

Herraiz T, Ough CS (1993) Formation of ethyl esters of amino acids by yeasts during the alcoholic fermentation of grape Juice. Am J Enol Vitic 44:41–48

Herraiz T, Reglero G, Herraiz M et al (1990) The influence of the Yeast and type of culture on the volatile composition of wines fermented without sulfur dioxide. Am J Enol Vitic 41:313–318

Horinouchi T, Yoshikawa K, Kawaide R et al (2010) Genome-wide expression analysis of *Saccharomyces pastorianus* orthologous genes using oligonucleotide microarrays. J Biosci Bioeng 110(5):602–607

Horsted MW, Dey ES, Holmberg S et al (1998) A novel esterase from *Saccharomyces carlsbergensis*, a possible function for the yeast *TIP1* gene. Yeast 14(9):793–803

Houtman AC, Du Plessis CS (1981) The effect of juice clarity and several conditions promoting yeast growth on fermentation rate, the production of aroma components and wine quality. S Afr J Enol Vitic 2:71–81

Houtman AC, Marais J, Du Plessis CS (1980a) Factors affecting the reproducibility of fermentation of grape juice and of the aroma composition of wines. Grape maturity, sugar, inoculum concentration, aeration, juice turbidity and ergosterol. Vitis 19:37–54

Houtman AC, Marais J, Du Plessis CS (1980b) The possibilities of applying present-day knowledge of wine aroma components: Influence of several juice factors on fermentation rate and ester production during fermentation. S Afr J Enol Vitic 1:27–33

Howell KS, Swiegers JH, Elsey GM et al (2004) Variation in 4-mercapto-4-methyl-pentan-2-one release by *Saccharomyces cerevisiae* commercial wine strains. FEMS Microbiol Lett 240:125–129

Howell KS, Klein M, Swiegers JH et al (2005) Genetic determinants of volatile-thiol release by *Saccharomyces cerevisiae* during wine fermentation. Appl Environ Microbiol 71:5420–5426

Inoue T (1992) A review of diacetyl control technology. In: Proceedings of the 22nd Institute of Brewing Convention, Melbourne, 1–6 March 1992

Isnard AD, Thomas D, Surdin-Kerjan Y (1996) The study of methionine uptake in *Saccharomyces cerevisiae* reveals a new family of amino acid permeases. J Mol Biol 262(4):473–484

Izquierdo-Canas PM, Palacios-Garcia AT, Garcia-Romero E (2011) Enhancement of flavour properties in wines using sequential inoculations of non-*Saccharomyces* (*Hansenula* and *Torulaspora*) and *Saccharomyces* yeast starters. Vitis 50:177–182

Jain VK, Divol B, Prior BA et al (2012) Effect of NAD^{+}-regenerating pathways on the formation of primary and secondary aroma compounds in a *Saccharomyces cerevisiae* glycerol-defective mutant. Appl Microbiol Biotechnol 93:131–141

Jauniaux JC, Grenson M (1990) *GAP1*, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. Eur J Biochem 190(1):39–44

Jiranek V, Langridge P, Henschke PA (1995) Validation of bismuth-containing indicator media for predicting H_2S-producing potential of *Saccharomyces cerevisiae* wine yeasts under enological conditions. Am J Enol Vitic 46:269–273

King A, Dickinson JR (2000) Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*. Yeast 16:499–506

Kispal G, Steiner H, Court DA et al (1996) Mitochondrial and cytosolic branched-chain amino acid transaminases from yeast, homologs of the *myc* oncogene-regulated Eca39 protein. J Biol Chem 271:24458–24464

Kodama YK, Kielland-Brandt MC, Hansen J (2006) Lager brewing yeast. In: Sunnerhagen P, Piskur J (eds) Comparative genomics. Springer, Berlin, pp 145–164

Kodama Y, Omura F, Miyajima K et al (2001) Control of higher alcohol production by manipulation of the *BAP2* gene in brewing yeast. J Am Soc Brew Chem 59:157–162

Krogerus K, Gibson BR (2013) Influence of valine and other amino acids on total diacetyl and 2,3-pentanedione levels during fermentation of brewer's wort. Appl Microbiol Biotechnol. doi:10.1007/s00253-013-4955-1

Lambrechts MG, Pretorius IS (2000) Yeasts and its importance to wine aroma: a review. S Afr J Enol Vitic 21:97–129

Landaud S, Latrille E, Corrieu G (2001) Top pressure and temperature control the fusel alcohol/ ester ratio through yeast growth in beer fermentation. J Inst Brew 107:107–117

Lei H, Zhao H, Yu Z et al (2012) Effects of wort gravity and nitrogen level on fermentation performance of brewer's yeast and the formation of flavor volatiles. Appl Biochem Biotechnol 166:1562–1574

Lema C, García-Jares C, Orriols I et al (1996) Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some components of Albariño wine aroma. Am J Enol Vitic 47:206–216

Libkind D, Hittinger CT, Valério E et al (2011) Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. Proc Natl Acad Sci USA 108(35):14539–14544

Lilly M, Bauer FF, Lambrechts MG et al (2006a) The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. Yeast 23:641–659

Lilly M, Bauer FF, Styger G et al (2006b) The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. FEMS Yeast Res 6:726–743

Lilly M, Lambrechts MG, Pretorius IS (2000) Effect of increased yeast alcohol acetyltransferase activity on flavour profiles of wine and distillates. Appl Environ Microbiol 66:744–753

Liu SQ, Pilone GJ (2000) An overview of formation and roles of acetaldehyde in winemaking with emphasis on microbiological implications. Int J Food Sci Technol 35:49–61

Llauradó JM, Rozès N, Bobet R et al (2002) Low temperature alcoholic fermentation in high sugar concentration grape must. J Food Sci 67:268–273

Llauradó JM, Rozès N, Constantí M et al (2005) Study of some *Saccharomyces cerevisiae* strains for winemaking after preadaptation at low temperatures. J Agric Food Chem 53(4):1003–1011

Lopes C, Barrio E, Querol A (2010) Natural hybrids of *S. cerevisiae* × *S. kudriavzevii* share alleles with European wild populations of *Saccharomyces* kudriavzevii. FEMS Yeast Res 10(4):412–421

Loscos N, Hernández-Orte P, Cacho J et al (2007) Release and formation of varietal aroma compounds during alcoholic fermentation from nonfloral grape odorless flavor precursors fractions. J Agric Food Chem 55(16):6674–6684

Loscos N, Hernández-Orte P, Cacho J et al (2010) Evolution of the aroma composition of wines supplemented with grape flavour precursors from different varietals during accelerated wine ageing. Food Chem 120:205–216

Macpherson LJ, Hwang SW, Miyamoto T et al (2006) More than cool: promiscuous relationships of menthol and other sensory compounds. Mol Cell Neurosci 32(4):335–343

Mai B, Lipp M (1994) Cloning and chromosomal organization of a gene encoding a putative amino-acid permease from *Saccharomyces cerevisiae*. Gene 143:129–133

Maicas S, Mateo JJ (2005) Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. Appl Microbiol Biotechnol 67:322–335

Malcorps P, Cheval JM, Jamil S et al (1991) A new model for the regulation of ester synthesis by alcohol acetyl transferase in *Saccharomyces cerevisiae* during fermentation. J Am Soc Brew Chem 49:47–53

Marais J, Pool H (1980) Effect of storage time and temperature on the volatile composition and quality of dry white table wines. Vitis 19:151–164

Margalith PZ (1981) Flavour microbiology. Charles C. Thomas Publishers, Springfield

Masneuf I, Hansen J, Groth C et al (1998) New hybrids between *Saccharomyces sensu stricto* yeast species found among wine and cider production strains. Appl Environ Microbiol 64:3887–3892

Masneuf-Pomarède I, Bely M, Marullo P et al (2010) Reassessment of phenotypic traits for *Saccharomyces bayanus* var. *uvarum* wine yeast strains. Int J Food Microbiol 139:79–86

Masneuf-Pomarède I, Mansour C, Murat ML et al (2006) Influence of fermentation temperature on volatile thiols concentrations in Sauvignon blanc wines. Int J Food Microbiol 108:385–390

Massoutier C, Alexandre H, Feulliat M, Charpentier C (1998) Isolation and characterization of cryotolerant *Saccharomyces* strains. Vitis 37:55–59

Mateo JJ, Di Stefano R (1997) Description of the β-glucosidase activity of wine yeasts. Food Microbiol 14:583–591

Mateo JJ, Jiménez M, Huerta T et al (1992) Comparison of volatiles produced by four *Saccharomyces cerevisiae* strains isolated from Monastrell musts. Am J Enol Vitic 43:206–209

Mateo-Vivaracho L, Zapata J, Cacho J et al (2010) Analysis, occurrence and potential sensory significance of five polyfunctional mercaptans in white wines. J Agric Food Chem 58:10184–10194

McCloskey LP, Mahaney P (1981) An enzymatic assay for acetaldehyde in grape juice and wine. Am J Enol Vitic 32:159–162

McMahon H, Zoecklein BW, Fugelsang K et al (1999) Quantification of glycosidase activities in selected yeasts and lactic acid bacteria. J Ind Microbiol Biotechnol 23:198–203

Meilgaard MC (1975) Aroma volatiles in beer: Purification, flavour, threshold and interaction. In: Drawert F (ed) Geruch und Geschmackstoffe. Verlag Hans Carl, Nürnberg, pp 211–254

Mendes-Ferreira A, Mendes-Faia A, Leao C (2002) Survey of hydrogen sulphide production by wine yeasts. J Food Prot 65:1033–1037

Michel A, Bizeau C, Drilleau JF (1988) Flore levurienne présente dans les cidreries de l'ouest de la France. Sciences des Aliments 8(3):359–368

Miyake T, Shibamoto T (1993) Quantitative analysis of acetaldehyde in foods and beverages. J Agric Food Chem 41:1968–1970

Mizuno A, Amano H, Mukai N et al (2003) High gravity brewing utilizing an alpha-glucosidase. J Brew Soc Japan 98:376–385

Mizuno A, Tabei H, Iwahuti M (2006) Characterization of low-acetic-acid-producing yeast isolated from 2-deoxyglucose-resistant mutants and its application to high-gravity brewing. J Biosci Bioeng 101:31–37

Moreira N, Mendes F, Guedes de Pinho P et al (2008) Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. Int J Food Microbiol 124:231–238

Morrissey WF, Davenport B, Querol A et al (2004) The role of indigenous yeasts in traditional Irish cider fermentations. J Appl Microbiol 97(3):647–655

Murat ML, Masneuf I, Darriet P et al (2001a) Effect of *Saccharomyces cerevisiae* yeast strains on the liberation of volatile thiols in Sauvignon blanc wine. Am J Enol Vitic 52:136–139

Murat ML, Tominaga T, Dubourdieu D (2001b) Assessing the aromatic potential of Cabernet Sauvignon and Merlot musts used to produce rose wine by assaying the cysteinylated precurser of 3-mercaptohexan-1-ol. J Agric Food Chem 49:5412–5417

Nakao Y, Kanamori T, Itoh T et al (2009) Genome sequence of the lager brewing yeast, an interspecies hybrid. DNA Res 16(2):115–129

Naumov GI, Masneuf I, Naumova ES et al (2000) Association of *Saccharomyces bayanus var. uvarum* with some French wines: genetic analysis of yeast populations. Res Microbiol 151:683–691

Naumov GI, Naumova ES, Aigle M et al (2001) Genetic reidentification of the pectinolytic yeast strain SCPP as *Saccharomyces bayanus* var. *uvarum*. Appl Microbiol Biotechnol 55:108–111

Navarro-Aviño JP, Prasad R, Miralles VJ et al (1999) A proposal for nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterization of the stress-inducible *ALD2* and *ALD3* genes. Yeast 15:829–842

Nguyen HV, Gaillardin C (2005) Evolutionary relationships between the former species *Saccharomyces uvarum* and the hybrids *Saccharomyces bayanus* and *Saccharomyces pastorianus*; reinstatement of *Saccharomyces uvarum* (Beijerinck) as a distinct species. FEMS Yeast Res 5:471–483

Nguyen HV, Legras JL, Neuvéglise C et al (2011) Deciphering the hybridisation history leading to the Lager lineage based on the mosaic genomes of *Saccharomyces bayanus* strains NBRC1948 and CBS380. PLoS ONE 6(10):e25821

Nguyen HV, Lepingle A, Gaillardin C (2000) Molecular typing demonstrates homogeneity of *Saccharomyces uvarum* strains and reveals the existence of hybrids between *S. uvarum* and *S. cerevisiae* including the *S. bayanus* type strain CBS 380. Syst Appl Microbiol 23:71–85

Novo MT, Beltrán G, Torija MJ et al (2003) Fermentaciones a bajas temperaturas: análisis químico y sensorial. Tecnol Vino 9:51–55

Nykänen L, Nykänen I (1977) Production of esters by different yeast strains in sugar fermentations. J Inst Brew 83:30–31

Omura F, Shibanoy Y, Fukui N et al (1995) Reduction of hydrogen sulfide production in brewing yeast by constitutive expression of *MET25* gene. J Am Soc Brew Chem 53:58–62

Pengelly RJ, Wheals AE (2012) Rapid identification of *Saccharomyces eubayanus* and its hybrids. FEMS Yeast Res 13(2):156–161

Perestrelo R, Fernandes A, Albuquerque FF et al (2006) Analytical characterization of the aroma of Tinta Negra Mole red wine: Identification of the main odorants compounds. Anal Chim Acta 563:154–164

Petersen JGL, Kielland-Brandt MC, Holmberg S et al (1983) Mutational analysis of isoleucine-valine biosynthesis in *Saccharomyces cerevisiae.* Mapping of *ILV2* and *ILV5*. Carlsberg Res Comm 48:21–34

Piddocke MP, Kreisz S, Heldt-Hansen HP et al (2009) Physiological characterization of brewer's yeast in high-gravity beer fermentations with glucose or maltose syrups as adjuncts. Appl Microbiol Biotechnol 84:453–464

Piendl A, Geiger E (1980) Technological factors in the formation of esters during fermentation. The Brewer's Digest 55:26–35

Piotrowski JS, Nagarajan S, Kroll E et al (2012) Different selective pressures lead to different genomic outcomes as newly-formed hybrid yeasts evolve. BMC Evol Biol 12(1):46

Prescott J (1999a) Flavour as a psychological construct: implications for perceiving and measuring the sensory qualities of foods. Food Qual Pref 10(4/5):349–356

Prescott J (1999b) Introduction to the trigeminal sense: The role of pungency in food flavours. In: Bell GA, Watson AJ (eds) Tastes and aromas. The chemical senses in science and industry. UNSW Press, Sydney, pp 38–49

Pretorius IS (2000) Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. Yeast 16:675–729

Pretorius IS (2003) The genetic analysis and tailoring of wine yeasts. In: Hohmann S (ed) Topics in current genetics. Springer, Heidelberg, pp 99–142

Pretorius IS (2004) The genetic improvement of wine yeasts. In: Arora DK, Bridge PD, Bhatnagar D (eds) Handbook of fungal biotechnology. Marcel Dekker, New York, pp 209–232

Pretorius IS, Bauer FF (2002) Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol 20:426–432

Pretorius IS, Høj PB (2005) Grape and wine biotechnology: challenges, opportunities and potential benefits. Austr J Grape Wine Res 11:83–108

Pretorius IS, Van der Westhuizen TJ, Augustyn OPH (1999) Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. S Afr J Enol Vitic 20:61–74

Pronk JT, Steensma HY, van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12:1607–1633

Quain DE, Duffield ML (1985) A metabolic function for higher alcohol production by yeast. In: Proceedings of the 20th congress of the European Brewery Convention, Helsinki, 2–7 June 1985

Querol A, Barrio E, Ramón D (1994) Population dynamics of natural *Saccharomyces* strains during wine fermentation. Int J Food Microbiol 21:315–323

Radler F (1993) Yeast: Metabolism of organic acids. In: Fleet GH (ed) Wine microbiology and biotechnology. Harwood Academic Publishers, Chur, pp 165–182

Rainieri S, Kodama Y, Kaneko Y et al (2006) Pure and mixed genetic lines of *Saccharomyces bayanus* and *Saccharomyces pastorianus* and their contribution to the lager brewing strain genome. Appl Environ Microbiol 72:3968–3974

Ramey DD, Ough CS (1980) Volatile ester hydrolysis or formation during storage of model solutions and wines. J Agric Food Chem 28:928–934

Rapp A, Versini G (1991) Influence of nitrogen compounds in grapes on aroma compounds of wine. In: Rantz J (ed) Proceedings of the international symposium on nitrogen in grapes and wines. Davis American Society For Enology and Viticulture, California

Rauhut D (1993) Yeasts: Production of sulfur compounds. In: Fleet GH (ed) Wine microbiology and biotechnology. Harwood Academic Publishers, Chur, pp 183–223

Redzepovic S, Orlic S, Majdak A et al (2002) Identification and characterisation of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains isolated from Croatian vineyards. Lett Appl Microbiol 35:305–310

Remize F, Andrieu E, Dequin S (2000) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*: Role of the cytosolic Mg^{2+} and mitochondrial K^{+} acetaldehyde dehydrogenases *Ald6p* and *Ald4p* in acetate formation during alcoholic fermentation. Appl Environ Microbiol 66:3151–3159

Ribéreau-Gayon P, Dubourdieu D, Doneche B et al (2000) Handbook of enology volume 1: the microbiology of wine and vinification. John Wiley & Sons Ltd., Chichester

Rojas V, Gil JV, Piñaga F et al (2001) Studies on acetate ester production by non-*Saccharomyces* wine yeasts. Int J Food Microbiol 70:283–289

Romano P, Suzzi G, Turbanti L et al (1994) Acetaldehyde production in *Saccharomyces cerevisiae* wine yeasts. FEMS Microbiol Lett 118:213–218

Rossouw D, Næs T, Bauer FF (2008) Linking gene regulation and the exo-metabolome: a comparative transcriptomics approach to identify genes that impact on the production of volatile aroma compounds in yeast. BMC Genom 9(1):530–547

Rouillon A, Surdin-Kerjan Y, Thomas D (1999) Transport of sulfonium compounds. Characterization of the s-adenosylmethionine and s-methylmethionine permeases from the yeast *Saccharomyces cerevisiae*. J Biol Chem 274(40):28096–28105

Ryder DS, Masschelein CA (1983) Aspects of metabolic regulatory systems and physiological limitations with a view to the improvement of brewing yeast performance. In: Proceedings of the 19th European Brewery Convention symposium, London

Saenz-Navajas MP, Campo E, Fernandez-Zurbano P et al (2010) An assessment of the effects of wine volatiles on the perception of taste and astringency in wine. Food Chem 121(4):1139–1149

Saerens SMG, Delvaux FR, Verstrepen KJ et al (2010) Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microbial Biotechnol 3:165–177

Saerens SMG, Delvaux F, Verstrepen KJ et al (2008a) Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. Appl Environ Microbiol 74:454–461

Saerens SMG, Verbelen PJ, Vanbeneden N et al (2008b) Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast. Appl Microbiol Biotechnol 80:1039–1051

Saerens SMG, Verstrepen KJ, van Laere SDM et al (2006) The *Saccharomyces cerevisiae EHT1* and *EEB1* genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. J Biol Chem 281:4446–4456

Saint-Prix F, Bonquist L, Dequin S (2004) Functional analysis of the *ALD* gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the NADP(+)-dependent *Ald6p* and *Ald5p* isoforms play a major role in acetate formation. Microbiol 150:2209–2220

Sakai N, Kobayakawa T, Gotow N et al (2001) Enhancement of sweetness ratings of aspartame by a vanilla odor presented either by orthonasal or retronasal routes. Percept Mot Skills 92(3):1002–1008

Sampaio JP, Gonçalves P (2008) Natural populations of *Saccharomyces kudriavzevii* in Portugal are associated with oak bark and are sympatric with *S. cerevisiae* and *S. paradoxus*. Appl Environ Microbiol 74(7):2144–2152

San Juan F, Escudero A, Cacho J et al (2011) Quality and main aromatic sensory descriptors (mainly fresh and dry fruit character) of Spanish red wines can be predicted from their aroma-active chemical composition. J Agric Food Chem 59. doi:10.1021/jf1048657 (in press)

Sarry JE, Gunata Z (2004) Plant and microbial glycoside hydrolases: volatile release from glycosidic aroma precursors. Food Chem 87:509–521

Schuller D, Alves H, Dequin S (2005) Ecological survey of *Saccharomyces cerevisiae* strains from vineyards in the Vinho Verde region of Portugal. FEMS Microbiol Ecol 51:167–177
Sipiczki M (2002) Taxonomic and physiological diversity of *Saccharomyces bayanus*. In: Ciani M (ed) Biodiversity and biotechnology of wine yeasts. Research Signpost, Kerala, pp 53–69
Sipiczki M (2008) Interspecies hybridization and recombination in *Saccharomyces* wine yeasts. FEMS Yeast Res 8:996–1007
Sipiczki M, Romano P, Lipani G et al (2001) Analysis of yeasts derived from natural fermentation in a Tokaj winery. Antonie Van Leeuwenhoek 79:97–105
Smit A, Cordero Otero RR, Lambrechts MG et al (2003) Enhancing volatile phenol concentrations in wine by expressing various phenolic acid decarboxylase genes in *Saccharomyces cerevisiae*. J Agric Food Chem 51:4909–4915
Soles RM, Ough CS, Kunkee RE (1982) Ester concentration differences in wine fermented by various species and strains of yeasts. Am J Enol Vitic 33:94–98
Spagna G, Barbagallo RN, Palmeri R et al (2002) Properties of endogenous β-glucosidase of a *Pichia anomala* strain isolated from Sicilian musts and wines. Enzyme Microb Technol 31(7):1036–1041
Spiropoulos A, Bisson LF (2000) *MET17* and hydrogen sulfide formation in *Saccharomyces cerevisiae*. Appl Environ Microbiol 66:4421–4426
Sponholz WR (1993) Wine spoilage by microorganisms. In: Fleet GH (ed) Wine microbiology and biotechnology. Harwood Academic Publishing, Chur, pp 395–420
Stevenson RJ, Wilson DA (2007) Odour perception: an object-recognition approach. Perception 36(12):1821–1833
Strauss MLA, Jolly NP, Lambretchs MG et al (2001) Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J Appl Microbiol 91:182–190
Strauss MLA, Wilson B, Gooley PR et al (1986) Role of monoterpenes in grape wine flavor. ACS Symp Ser 317:222–242
Styger G, Jacobson D, Bernard P et al (2011) Identifying genes that impact on aroma profiles produced by *Saccharomyces cerevisiae* and the production of higher alcohols. Appl Microbiol Biotechnol 91:713–730
Styger G, Jacobson D, Bernard P et al (2013) Genetic analysis of the metabolic pathways responsible for aroma metabolite production by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 97:4429–4442
Suárez B, Pando R, Fernández N et al (2007a) Yeast species associated with the spontaneous fermentation of cider. Food Microbiol 24:25–31
Suárez B, Pando R, González A et al (2007b) A molecular genetic study of natural strains of *Saccharomyces* isolated from Asturian cider fermentations. J Appl Microbiol 103:778–786
Suomalainen H, Lehtonen M (1979) The production of aroma compounds by yeast. J Inst Brew 85:149–156
Sutherland CM, Henschke PA, Langridge P et al (2003) Subunit and cofactor binding of *Saccharomyces cerevisiae* sulfite reductase-towards developing wine yeast with lowered ability to produce hydrogen sulfide. Austr J Grape Wine Res 9:186–193
Swiegers JH, Bartowsky EJ, Henschke PA et al (2005) Yeast and bacterial modulation of wine aroma and flavour. Austr J Grape Wine Res 11:139–173
Swiegers JH, Bjerre K, Corfitzen LB et al (2011) Winemaking returning back to nature. Austr New Zealand Grapegrower Winemaker 564:46–48
Swiegers JH, Capone DL, Pardon KH et al (2007) Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. Yeast 24:561–574
Swiegers JH, Francis IL, Herderich MJ et al (2006a) Meeting consumer expectations through management in vineyard and winery: the choice of yeast for fermentation offers great potential to adjust the aroma of Sauvignon Blanc wine. Austr New Zealand Wine Ind J 21:34–42

Swiegers JH, Willmott RL, Hill-Ling A et al (2006b) Modulation of volatile thiol and ester aromas in wine by modified wine yeast. In: Bredie W, Petersen M (eds) Developments in food science vol 43: flavour science recent advances and trends. Elsevier, Amsterdam

Takoi K, Koie K, Itoga Y et al (2010) Biotransformation of hop-derived monoterpene alcohols by lager yeast and their contribution to the flavor of hopped beer. J Agric Food Chem 58:5050–5058

Tamai Y, Tanaka K, Umemoto N et al (2000) Diversity of the *HO* gene encoding an endonuclease for mating-type conversion in the bottom fermenting yeast *Saccharomyces pastorianus*. Yeast 16(14):1335–1343

Tanguler H (2012) Evaluation of *Williopsis saturnus* inoculum level on fermentation and flavor compounds of white wines made from Emir (*Vitis vinifera* L.) grown in Anatolia. Food Biotechnol 26:351–368

Taylor GT, Thurston PA, Kirsop BH (1979) Influence of lipids derived from malt spent grains on yeast metabolism and fermentation. J Inst Brew 85:219–227

Tezuka H, Mori T, Okumura Y et al (1992) Cloning of a gene suppressing hydrogen sulfide production by *Saccharomyces cerevisiae* and its expression in a brewing yeast. J Am Soc Brew Chem 50:130–133

Thornton RJ, Bunker A (1989) Characterisation of wine yeasts for genetically modifiable properties. J Inst Brew 95:181–184

Tominaga T, Baltenweck-Guyot R, Peyrot de Gachons C et al (2000) Contribution of volatile thiols to the aromas of white wines made from several *Vitis vinifera* grape varieties. Am J Enol Vitic 51:178–181

Tominaga T, Darriet P, Dubourdieu D (1996) Identification of 3-mercaptohexyl acetate in Sauvignon wine, a powerful aromatic compound exhibiting box-tree odor. Vitis 35:207–210

Tominaga T, Dubourdieu D (2006) A novel method for quantification of 2-methyl-3-furanthiol and 2-furanmethanethiol in winesmade from *Vitis vinifera* grape varieties. J Agric Food Chem 54:29–33

Tominaga T, Furrer A, Henry R et al (1998a) Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. Flav Fragr J 13:159–162

Tominaga T, Masneuf I, Dubourdieu D (1995) A *S*-cysteine conjugate, precursor of aroma of white sauvignon. J Int des Sci de la Vigne et du Vin 29:227–232

Tominaga T, Peyrot des Gachons C, Dubourdieu D (1998b) A new type of flavour precursors in *Vitis vinifera* L. cv. Sauvignon Blanc: *S*-cysteine conjugates. J Agric Food Chem 46:5215–5219

Torija MJ, Beltrán G, Novo M et al (2003) Effects of fermentation temperature and *Saccharomyces* species on the cell fatty acid composition and presence of volatile compounds in wine. Int J Food Microbiol 85:127–136

Torija MJ, Rozés N, Poblet M et al (2001) Yeast population dynamics in spontaneous fermentations, comparison between two different wine-producing areas over a period of three years. Antonie Van Leeuwenhoek 79:345–352

Ugliano M, Bartowsky EJ, McCarthy J et al (2006) Hydrolysis and transformation of grape glycosidically bound volatile compounds during fermentation with three *Saccharomyces* yeast strains. J Agric Food Chem 54(17):6322–6331

Ugliano M, Henschke PA (2009) Yeasts and wine flavour. In: Moreno-Arribas MV, Polo MC (eds) Wine chemistry and biochemistry. Springer, New York, pp 251–274

Ugliano M, Moio L (2008) Free and hydrolytically released volatile compounds of *Vitis vinifera* L. cv. Fiano grapes as odour-active constituents of Fiano wine. Anal Chim Acta 21(1):79–85

Van Breda V, Jolly N, Van Wyk J (2013) Characterisation of commercial and natural *Torulaspora delbrueckii* wine yeast strains. Int J Food Microbiol 163:80–88

Van Rensburg P, Pretorius IS (2000) Enzymes in winemaking: harnessing natural catalysts for efficient biotransformations: a review. S Afr J Enol Vitic 21:52–73

Vaughan-Martini A, Kurtzman CP (1985) Deoxyribonucleic acid relatedness among species of *Saccharomyces sensu stricto*. Int J Syst Bact 35:508–511

Verbelen PJ, Dekoninck TM, Saerens SM et al (2009a) Impact of pitching rate on yeast fermentation performance and beer flavour. Appl Microbiol Biotechnol 82:155–167
Verbelen PJ, Saerens SM, Van Mulders SE et al (2009b) The role of oxygen in yeast metabolism during high cell density brewery fermentations. Appl Microbiol Biotechnol 82:1143–1156
Vermeulen C, Lejeune I, Tran TTH et al (2006) Occurrence of polyfunctional thiols in fresh lager beers. J Agric Food Chem 54:5061–5068
Verstrepen KJ, Derdelinckx G, Dufour JP et al (2003a) The *Saccharomyces cerevisiae* alcohol acetyl transferase gene *ATF1* is a target of the cAMP/PKA and FGM nutrient-signalling pathways. FEMS Yeast Res 4:285–296
Verstrepen KJ, Derdelinckx G, Dufour JP et al (2003b) Flavor-active esters: adding fruitiness to beer. J Biosci Bioeng 96:110–118
Verstrepen KJ, van Laere SDM, Vanderhaegen BMP et al (2003c) Expression levels of the yeast alcohol acetyltransferase genes *ATF1, Lg-ATF1*, and *ATF2* control the formation of a broad range of volatile esters. Appl Environ Microbiol 69:5228–5237
Viana F, Belloch C, Valles S et al (2011) Monitoring a mixed starter of *Hanseniaspora vineae–Saccharomyces cerevisiae* in natural must: Impact on 2-phenylethyl acetate production. Int J Food Microbiol 151:235–240
Viana F, Gil JV, Genoves S et al (2008) Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiol 25:778–785
Viana F, Gil JV, Valles S et al (2009) Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. Int J Food Microbiol 135:68–74
Vilanova M, Genisheva Z, Masa A et al (2010) Correlation between volatile composition and sensory properties in Spanish Albariño wines. Microchem J 95:240–246
Villanueva KD, Goossens E, Masschelein CA (1990) Subthreshold vicinal diketone levels in lager brewing yeast fermentations by means of *ILV5* gene amplification. J Am Soc Brew Chem 48:111–114
Villanueva A, Ramón D, Valles S et al (2000) Heterologous expression in *Aspergillus nidulans* of a *Trichoderma longibrachiatum* endoglucanase of enological relevance. J Agric Food Chem 48:951–957
Wainwright T (1973) Diacetyl: a review. J Inst Brew 79:451–470
Williams AA (1974) Flavour research and the cider industry. J Inst Brew 80:455–470
Wilson DA, Stevenson RJ (2003) The fundamental role of memory in olfactory perception. Trends Neurosc 26(5):243–247
Yamagata S (1989) Roles of *O*-acetyl-L-homoserine sulfhydrylases in microorganisms. Biochimie 71:1125–1143
Yeshurun Y, Sobel N (2010) An odor is not worth a thousand words: from multidimensional odors to unidimensional odor objects. Annual Rev Psychol 61:219–241
Yoshimoto H, Fujiwara D, Momma T et al (1998) Characterization of the *ATF1* and *Lg-ATF1* genes encoding alcohol acetyltransferases in the bottom fermenting yeast *Saccharomyces pastorianus*. J Ferment Bioeng 86:15–20
Zamora F (2009) Biochemistry of alcoholic fermentation. In: Moreno-Arribas MV, Polo MC (eds) Wine chemistry and biochemistry. Springer, New York, pp 3–26
Zea L, Moreno J, Ortega JM et al (1995) Content of free terpenic compounds in cells and musts during vinification with three *Saccharomyces cerevisiae* races. J Agric Food Chem 43:1110–1114
Zoecklein BW, Marcy JE, Williams JM et al (1997) Effect of native yeasts and selected strains of *Saccharomyces cerevisiae* on glycosyl glucose, potential volatile terpenes and selected aglycones of white Riesling (*Vitis vinifera* L.) wines. J Food Compos Anal 10:55–65
Zott K, Thibon C, Bely M et al (2011) The grape must non-*Saccharomyces* microbial community: impact on volatile thiol release. Int J Food Microbiol 151:210–215

Chapter 11
Production of Metabolites and Heterologous Proteins

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11.1 Introduction

Yeasts and particularly *Saccharomyces cerevisiae* play a significant role in biotechnology. They have been employed since ancient times for the production of alcoholic beverages and bread and are nowadays not only of importance for chemical and enzyme production but also for the production of biopharmaceutical ingredients. Clearly, the central carbon metabolism plays a crucial role for every microbial process. This is quite obvious for the production of central metabolites such as ethanol, which is a direct end product of the central carbon metabolism. In this case, the impact is very clear-cut in a direct stoichiometric relation of the carbon source, which is converted by the central carbon metabolism into the product. For secondary metabolites and proteins, this relation is less clear. While it is evident that every fermentation process starts with the carbon source and is therefore inevitably connected to the central carbon metabolism, various regulatory layers control the synthesis of end products and no simple rule exists for identifying the metabolic bottlenecks for their accumulation. These bottlenecks might be regulatory events within the metabolic pathways—upstream or downstream. Moreover, also less defined phenomena such as energy supply or electron balances might directly influence the production and the yield of conversion of the

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J. Piškur and C. Compagno (eds.), *Molecular Mechanisms in Yeast Carbon Metabolism*,
DOI: 10.1007/978-3-642-55013-3_11, © Springer-Verlag Berlin Heidelberg 2014

Table 11.1 Products of the primary metabolism with industrial relevance, their central precursor and important organisms for their production

Chemical	Precursor metabolite	Organisms
Butanol	Acetyl-CoA	*S. cerevisiae*
Citric acid	Acetyl-CoA, pyruvate	*Yarrowia lipolytica*
Erythritol	Glucose-6-phosphate	*Y. lipolytica*
Lactic acid	Pyruvate	*S. cerevisiae, Candida* spp.
Mannitol	Glucose-6-phosphate	*Y. lipolytica*
Succinic acid	Acetyl-CoA, pyruvate	*S. cerevisiae*

carbon source. In this chapter we want to outline our current knowledge about these relations, by describing some major products, divided by their category as primary or secondary metabolites or protein.

11.2 Products of the Primary Metabolism

The metabolic activities of the primary metabolism are required for cellular growth, maintenance and survival: as a consequence, the concentration of end-product molecules of the primary metabolism is directly related to the fluxes through these reactions. They depend mainly on substrate availability and uptake and on environmental conditions, such as oxygen levels, pH and temperature. Remarkably, while the central carbon metabolism is apparently quite simple, it is very strictly regulated. Some primary metabolites are easily accumulated, while others are very hard to produce. Table 11.1 summarizes some industrially important primary metabolites. Special challenges relate to their production. First of all, most of them are low value added products, requiring the need for optimizing product yield and productivity to minimize costs of goods. This also implies that many of these products turn out to be toxic even if naturally produced by the cells because of the high levels of production required for the viability of the process. Secondarily, cells are usually reluctant to manipulations occurring to their central metabolism, meaning that eventual manipulations (genetic or environmental) have to be fine-tailored for being successful.

11.2.1 Main Pathways: Main Nodes

The core pathway of yeast's central metabolism is the glycolysis (Fig. 11.1). The major substrate is glucose, but thanks to other pathways converging into glycolysis, other sugars can also serve as a carbon and energy source. Mannose, fructose, sucrose, lactose, galactose, glycerol, xylose and arabinose, among others, are all relevant for biotechnology. Further principal pathways are the TCA cycle, the

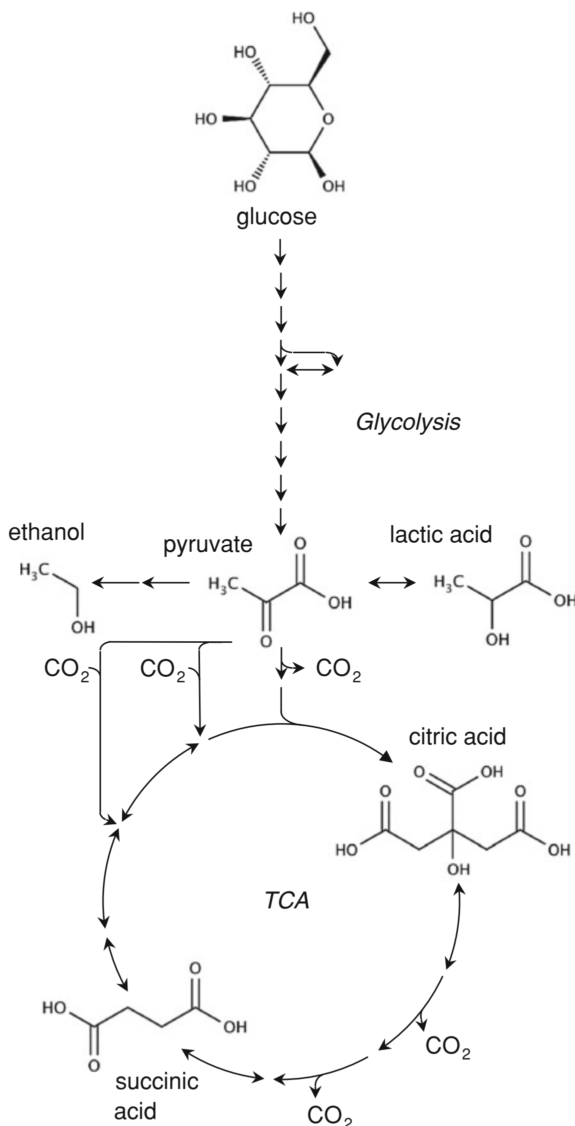

Fig. 11.1 Schematic overview about the primary metabolism. Ethanol, lactic acid, succinic acid and citric acid are depicted as major products of the primary metabolism, which have industrial relevance

pentose phosphate pathway and the fermentative pathways. Inside the cell, pathways can be differently compartmentalized, in the cytosol, mitochondria, peroxisomes, or vacuole.

From a biotechnological point of view, ideally all the carbon of the substrate should end up in a product. Besides high yield, also high production rates are required for an economically viable biotech process.

Conceptually, the flux rate of a 'substrate' along the different pathways towards the final product can be regulated at least at four main levels:

1. Transport mechanisms—substrate uptake, product secretion and trafficking of compounds between the various cellular compartments
2. Enzyme synthesis—induction, repression and derepression of gene expression and translation
3. Enzyme activity—activation, inhibition or interconversion of isoenzymes.
4. Redox balance.

In the model yeast *S. cerevisiae*, fluxes and kinetic activities of glycolytic enzymes are only minimally controlled by their transcript levels (Daran-Lapujade et al. 2007). Instead, the modulation of the transport processes for taking up of substrate by the cells and for the trafficking of substrate(s) between the various cellular compartments and product secretion have been proven to substantially influence the production rates. Different hexose transporters (HXTs) are responsible for glucose uptake by *S. cerevisiae*. At least 20 *HXT* genes encoding these transporters have been identified. Remarkably, a simple manipulation of the glucose uptake can strongly alter the mode of metabolic control (Otterstedt et al. 2004). Analyses of the effect of *HXT* gene inactivation have shown that the hexose carriers *HXT*1 to *HXT*7 are the main transporters. In this respect, it has been shown that the ethanol (and CO_2) productivity and yield (gram of ethanol produced per gram of glucose consumed) can be improved by overexpression of the *HXT*1 or *HXT*7 transporters in *S. cerevisiae* (Gutiérrez-Lomelí et al. 2008; Rossi et al. 2010). Lager yeast strain improvement has been shown in terms of substrate uptake after modulation of the *AGT*1 (or *MAL*11) gene encoding for a maltose and maltotriose transporter (Vidgren et al. 2009). A similar approach has been applied to improve the xylose consumption. Xylose—constituting a significant fraction of lignocellulosic biomass—is a key substrate for second-generation productions of metabolites. However, *S. cerevisiae* lacks xylose-specific transporters and it takes up xylose by facilitated diffusion mainly through the non-specific hexose transporters encoded by the *HXT* gene family. These transporters have lower affinity for xylose than for glucose, and their xylose transport properties have been mainly characterized with regard to sugar affinity. The heterologous expression of xylose-specific transporters in recombinant xylose-utilizing *S. cerevisiae* strains is crucial (Tanino et al. 2012; Young et al. 2012).

In addition to trafficking and compartmentalization phenomena, the maintenance of the cellular redox balance strongly modulates the accumulation of metabolites. As an example, products which differ in their degree of reduction from the substrate alter the cellular redox balance which in turn impacts their production and yield. The intracellular redox state is to a large extent dependent on the intracellular concentration ratios of the two pyridine nucleotide systems $NADH/NAD^+$ and $NADPH/NADP^+$. In *S. cerevisiae*, redox cofactors participate in more than 300 different biochemical reactions involving oxidation and/or reduction. During growth, NADH is preferentially used in catabolic pathways, whereas NADPH functions mainly as a reducing equivalent in anabolism (Bakker et al. 2001). If a product has a higher degree of reduction than the substrate, the cells need to provide reduction equivalents out of their metabolism. This clearly

consumes a fraction of the substrate and decreases the product yield. If the degree of reduction is lower in the product, the cells accumulate redox equivalents which have to be re-oxidized in the respiratory chain—where oxygen is the terminal electron acceptor—or under oxygen-limited or anaerobic conditions through alternative oxidases, or by reduction of a metabolite, what usually leads to by-product formation.

Last but not least, the operative conditions of an industrial production process determine that microorganisms meet multiple stresses such as non-optimal pH, temperature, oxygenation and osmotic stress. Besides these, the product itself is an important source of stress, due to the high product concentrations required for the economical viability of the process. All together these factors impair cellular metabolism and growth and, as a consequence, reduce the productivity of the process. For said reason, for some of the products reported here several studies focused on the development of strains more tolerant to the final product. Organic acids and alcohols are the chemical species where toxicity has been more extensively studied (e.g. reviewed in Abbott et al. 2009; Piper et al. 2001; Teixeira et al. 2011). This focus on organic acids and alcohols is also due to the fact that a relevant number of products already available on the market (or close to appear on the market) belong to these categories (see sections below for further details).

Remarkably, the highly desirable evolution of robust cell factories can rarely be ascribed to a single molecular element, since it requires a complex cellular reprogramming, implying the simultaneous modification of many regulatory and operative elements. In the last years, different cellular engineering approaches, spanning from the global transcription machinery engineering to the genome shuffling and evolutionary engineering, have been described and applied (i.e. as reviewed for ethanol in Ma and Liu 2010). These studies will be crucial for closing the gap between our deep knowledge about many different single key elements and their still unknown function in the operative networks of the biological systems. However, it has to be mentioned that most of the protocols developed and applied for selecting tolerant strains were not simultaneously coupled with production properties: this means that an improved tolerance might not be reflected in an improved yield or productivity.

11.2.2 Organic Acids

For a future biobased economy, organic acids constitute central chemical building blocks and are now extensively used in the chemical industry, food industry, agriculture and in medicine (Sauer et al. 2008). Weak acids are typically produced using bacterial hosts. During fermentation, the decreasing pH (due to the weak acid accumulation) has an inhibitory effect on metabolic activities of the producing microbial cells. The addition of KOH, $Ca(OH)_2$, $CaCO_3$, NaOH or NH_4OH, among others, to neutralize the organic acid is a conventional operation to minimize the negative effects. The neutralization of the organic acid during

fermentation has major disadvantages. Additional operations are required to regenerate undissociated weak acid from its salt and to dispose of or recycle the neutralizing cation.

All these extra operations and expenses can be reduced if the undissociated weak acid is accumulated by microorganisms able to grow and metabolize substrates at low pH levels. Yeasts can generally grow and survive at very low pH values. Indeed, metabolically engineered yeast strains have been proven to be successful cell factories for the production of lactic acid, succinic acid and citric acid (Raab and Lang 2011; Rymowicz et al. 2010; Sauer et al. 2010).

Lactic acid is a key example of the impact of the central metabolism on industrial biotechnology (Fig. 11.1). Lactic acid is commercially available and produced by microorganisms (Sauer et al. 2008, 2010). The monomer can be used as an acidulant. More importantly, it can also be used for the production of the biodegradable plastic PLA (polylactic acid). The global use of bioplastics was 0.85 million metric tons in 2011 and is projected to increase up to 3.7 million metric tons by 2016.

Nowadays, the production of lactic acid approximates 150,000 tons per year. The costs related to the purification of the monomer still represent an important fraction of the overall costs. The production from recombinant yeasts appears well suited for this task due to the yeast's tolerance to low pH and the possibility to grow them on mineral media. Nevertheless, volumetric productivities are significantly lower compared to bacterial strains under optimal conditions.

A process employing *Candida utilis* obtains an overall productivity of 3.1 g/l h with a final pH of 4 reaching a maximum of 4.9 g/l h during the process. For *S. cerevisiae*, production rates of about 1 g/l h reaching a pH of about 2.5 have been published. While data for industrially achieved productivities are not available, the order of magnitude can be estimated from the values above. They remain behind the values of bacterial production hosts. Nevertheless, the advantage of the easier downstream processing outweighs this fact, so industrial production of lactic acid is mainly taking place with recombinant yeasts as production host.

According to a study published by the US Department of Energy (DOE) in 2004, succinic acid is one of the twelve most promising 'platform chemicals' that can be sustainably produced from biomass with an estimated 15,000 t/year worldwide demand. The demand is predicted to expand to commodity chemical status with 270,000 t/year. Succinic acid is a building block used in the manufacture of polymers, resins, food and pharmaceuticals, among other products (i.e. synthesis of 1,4-butanediol, tetrahydrofuran, butyrolactone, maleic succinimide, itaconic acid, 2-pyrrolidinone and N-methylpyrrolidinone and as a monomer for the production of biodegradable polymers). DSM and Roquette have recently developed a new commercial production facility with a capacity of about 10 kilotons/year, the plant will be the largest of its kind in Europe. The process is based on recombinant *S. cerevisiae* hosts growing at low pH values. Also in this case, data for industrially achieved productivities are not available. However, the pathways leading to the accumulation of secondary metabolites like glycerol and ethanol have been deleted, while the production of the organic acid is based on a

combination of the reductive and oxidative TCA cycle. The overexpression of the transporter leading to the accumulation of succinic acid in the culture medium has also been considered.

Remarkably, the metabolic pathway is also based on sequestering some of the carbon dioxide developed by the process itself. The new production facility will, for the time being, use starch derivatives as feedstock. In the longer term, the aspiration is to switch to lignocellulosic biomasses.

Citric acid is an important microbial product used in a wide variety of applications. It is widely used as an acidulant in pharmaceutical and food industries. Global citric acid production has reached 2 million tons/year. The classical microbial process for the production of citric acid is based on the filamentous fungus *Aspergillus niger*. The critical parameters, which must be addressed to get an efficient production, include high substrate concentration, low and finite content of nitrogen and certain trace metals, thorough maintenance of high dissolved oxygen and low pH. Many of the biochemical and physiological mechanisms underlying the process remain unknown. These mechanisms are currently undergoing investigation, in order to allow the improvement in the citric acid production process, which is hardly improvable by traditional means like mutagenesis or cultivation optimization. Because of this and considering the ever-increasing demand for citric acid, alternative fermentation processes using high-yield yeast strains like *Yarrowia lipolytica* and different *Candida* species are investigated for its production. More in detail, *Y. lipolytica* yeast attracts the interest of the biotechnologists due to its ability to produce a wide spectrum of organic acids from the TCA cycle such as citric, isocitric, pyruvic and alpha-ketoglutaric acid from various substrates. The citric acid production by wild-type and mutant yeasts is primarily based on batch cultures. The carbon source is glucose, ethanol, plant oil, n-paraffins or sucrose. Citric acid concentrations of 140 g/L are easily reached nowadays using *Y. lipolytica*. High productivity and yield can also be obtained with acetate-negative mutants of *Y. lipolytica* in batch cultures by using a low-cost carbon substrate like glycerol, a by-product generated in large amounts during the production of biodiesel (da Silva et al. 2009).

11.2.3 Sugar Alcohols

Sugar alcohols are major compounds synthesized through the pentose phosphate pathway. These sugars are calorie-free and can be mainly used as sweetener or flavour additives. Erythritol is a four-carbon sugar alcohol. In comparison with other sugar alcohols currently used as sucrose replacers, erythritol has a much lower energy value ($\sim$0.2 kcal g^{-1}) when compared to sucrose (4 kcal g^{-1}). Moreover, this polyol is non-cariogenic, generally free of gastric side effects in regular use and its use in food is largely desirable. Mannitol—a six-carbon sugar alcohol—has several applications in the food, pharmaceutical and medical industries. It is nowadays produced industrially by chemical synthesis using

hydrogenation of fructose at high temperature and pressure. This process is not very efficient and requires a high purity of substrates.

During the production of citric acid from glycerol by *Y. lipolytica*, erythritol and mannitol can also be produced. High production of erythritol (80 g/L) and mannitol (28 g/L) were achieved from a glycerol feedstock (Tomaszewska et al. 2012). Extracellular as well as intracellular erythritol and mannitol ratios depended on the glycerol used and on the presence of NaCl in the medium.

11.2.4 Alcohols

Ethanol is clearly the major product of yeast's primary metabolism on the market. Chapter 9 is dedicated to its biochemistry and production. However, other alcohols are moving into the focus of biotechnology, above all butanol and isobutanol, which have superior liquid-fuel characteristics compared to ethanol.

While *Clostridia* are natural producers of butanol, *Escherichia coli* and *S. cerevisiae* have been engineered in recent years for butanol production (Atsumi et al. 2008; Steen et al. 2008). The engineering of *E. coli* has been quite successful, whereas productivities of yeasts remain limited up to now (Lan and Liao 2013). Figure 11.2 summarizes the major metabolic pathways to butanol, isobutanol and 2,3-butanediol, including a recently proposed biosynthetic pathway taking advantage of ketoacids (intermediates of amino acid biosynthesis and degradation) to produce fusel alcohols in the yeast *S. cerevisiae* (Branduardi et al. 2013).

11.2.5 Diols

Diols are compounds with two hydroxyl groups having a wide range of applications as chemicals and fuels, receiving much interest in the new wave of industrial biotechnology.

1,3-Propanediol (1,3-PDO) and its desirable properties have long been known. The demand for 1,3-PDO is constantly increasing, since new applications of this chemical compound are described regularly. It can be used in polymers, cosmetics, lubricants or drugs production, just to name a few. Of particular interest is its use as a monomer in a polycondensation reaction yielding polyesters, polyethers and polyurethanes. The aromatic polyester, polytrimethylene terephthalate (PTT), is commercially produced and available as Corterra™ or Sorona®.

Although 1,3-PDO cannot be naturally formed from sugars via fermentation, a wide range of microorganisms (e.g. *Klebsiella pneumoniae*) is able to ferment glycerol to 1,3-PDO. However, these organisms lack the enzymes required to form glycerol from glycolytic dihydroxyacetone phosphate (DHAP). In contrast, many organisms, including baker's yeast, are excellent glycerol producers but are unable to accomplish its subsequent conversion into 1,3-PDO. Therefore, the challenge

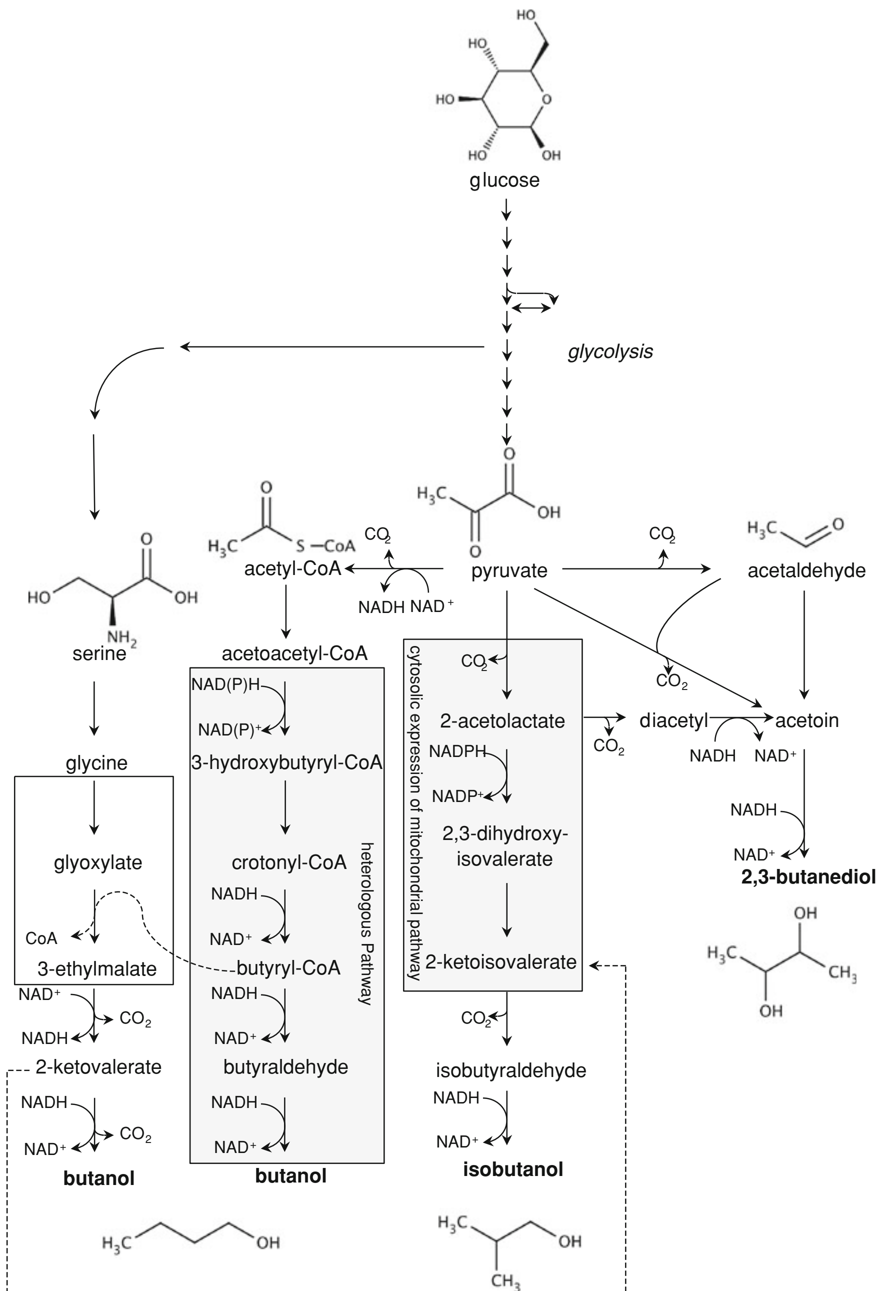

Fig. 11.2 Schematic overview about the primary metabolism with special respect to butanol, isobutanol and 2,3-butanediol production. *Grey boxes* highlight recombinant pathways. The dotted box relates to enzymatic activities present in *S. cerevisiae*, to which no gene has been annotated up to now (Branduardi et al. 2013)

for metabolic engineering is to integrate both parts of the pathway into a single organism. Till now, 1,3-PDO was produced only in small detectable amounts by yeasts from sugars (Celińska 2010).

2,3-Butanediol is an interesting metabolic product as its derivatives can be used in wide arrays of industries ranging from synthetic rubber, solvents and drugs. This important metabolite can be produced efficiently via mixed acid fermentation with bacteria cells such as *K. pneumoniae, Klebsiella oxytoca, Enterobacter aerogenes, Serratia* sp. and *Bacillus polymyxa.* Unfortunately, the use of most of these bacteria is undesirable in industrial-scale fermentation in terms of safety regulations. Despite having more endogenous pathways leading to 2,3-butanediol starting from pyruvate, the productivity is extremely poor in *S. cerevisiae* strains when compared to bacteria. Several reconstructions of yeast genome-scale metabolic models have been published. Using one of these constraint-based stoichiometric models of yeast, an in silico strain design was performed. High 2,3-butanediol titer (2.29 g l^{-1}) and yield (0.113 g g^{-1}) were achieved by a Δ*adh*1 Δ*adh*3 Δ*adh*5 yeast strain growing under anaerobic conditions (Ng et al. 2012).

11.3 Products of the Secondary Metabolism

Primary metabolites are of major importance as bulk or fine chemicals. Titers and productivities are generally high. This is in contrast to the products of the secondary metabolism, which are emerging as food and feed additives or pharmaceuticals. Table 11.2 summarizes some industrially important secondary metabolites. Productivities and titers for these products are generally lower. Here we outline three main classes of secondary products: vitamins, phenolics and isoprenoids.

11.3.1 Vitamins

Vitamins are a group of very different chemical compounds defined historically as being essential in small amounts in the diet of humans or in that of other organisms. Because of their chemical heterogeneity, also the metabolic pathways for their biosynthesis lack common features. Here we outline the developments for industrial production of two vitamins: riboflavin and ascorbic acid.

11.3.1.1 Riboflavin

Riboflavin (vitamin B2) is the precursor of coenzymes FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide), which serve as cofactors in various enzymatic redox reactions. Riboflavin is synthesized by microorganisms and plants, while higher eukaryotes have to take up riboflavin as part of their nutrition

Table 11.2 Products of the secondary metabolism with industrial relevance, their central metabolism precursor and important organisms for their production

Chemical	Precursor metabolite	Organisms
Artemisinic acid	Acetyl-CoA	*S. cerevisiae*
Ascorbic acid	Glucose	*S. cerevisiae, Zygosaccharomyces bailii*
Astaxanthin	Acetyl-CoA	*Xanthophyllomyces dendrorhous*
ß-Carotene	Acetyl-CoA	*S. cerevisiae, C. utilis*
Ergosterol	Glucose-6-phosphate	*S. cerevisiae*
Flavonoids	Phenylalanine, tyrosine, malonyl-CoA	*S. cerevisiae*
Paclitaxel	Acetyl-CoA	*S. cerevisiae*
Resveratrol	Phenylalanine, tyrosine, malonyl-CoA	*S. cerevisiae*
Riboflavin	GTP, ribulose-5-phosphate	*Ashbya gossypii, Candida famata*

(Dmytruk et al. 2011). The microbial production of riboflavin competes with chemical synthesis. However, the biotechnological production is advantageous in terms of cost-effectiveness, sustainability and the use of renewable feedstock (Stahmann et al. 2000). According to Abbas and Sibirny (2011), the annual riboflavin production capacity in 2008 was 10.000 tons. Most of this vitamin is produced by a microbial process with *A. gossypii* or *Bacillus subtilis*. A process applying the yeast *C. famata* (*Candida flareri*) has been abandoned due to the genetic instability of the production strain.

Riboflavin is synthesized by microbes from the precursors guanosine triphosphate (GTP) and ribulose-5-phosphate. The connection to the central carbon metabolism is evident, as ribulose-5-phosphate is derived from the oxidative branch in the pentose phosphate pathway. Although supply of ribulose-5-phosphate is not the limiting precursor in most cases, it has been shown that the overexpression of glucose dehydrogenase in *B. subtilis* increases the pool of ribulose-5-phosphate, which enhances riboflavin production (Zhu et al. 2006). In the natural riboflavin producers *A. gossypii* and *C. famata*, the supply of GTP and its precursors are of highest importance. By deregulating the purine biosynthesis pathway and supply of glycine as a GTP precursor, high-level producers of riboflavin have been engineered (Heefner et al. 1988; Jiménez et al. 2005). Overexpression of rate-limiting enzymes and the positive regulator *SEF1* enhanced riboflavin synthesis (Dmytruk et al. 2011). The coenzyme FMN itself was produced up to 200 mg/L by high-level overexpression of riboflavin kinase (FMN1) in *C. famata* (Yatsyshyn et al. 2009).

11.3.1.2 Ascorbic Acid

Ascorbic acid or vitamin C is a naturally occurring organic compound with antioxidant properties. Most higher eukaryotic organisms produce L-ascorbic acid. Humans are among the few organisms, which are dependent on exogenous provision

of this vitamin. It works as an antioxidant and scavenger of ROS (Padh 1991), and it has been ascribed various positive health effects. Microbial production of ascorbic acid is desirable. However, neither yeasts nor bacteria produce this compound naturally. Yeasts produce the structurally related compound erythroascorbic acid, which shows chemical properties similar to those of ascorbic acid (Bremus et al. 2006). This natural metabolic pathway has been employed for ascorbic acid production from L-galactose by *S. cerevisiae* and *Z. bailii* (Sauer et al. 2004). The biosynthesis of vitamin C from D-glucose by recombinant baker's yeast was obtained by employing the biosynthetic pathway from plants (Branduardi et al. 2007; Fossati et al. 2011), which was heterologously expressed in yeast. Glucose is the starting point for this metabolic pathway. That means that the central carbon metabolism is directly competing for the same substrate. However, the productivities obtained so far are apparently too low to exert any metabolic influence of ascorbic acid production on the primary metabolism.

11.3.2 Phenolics

Phenolics constitute a class of chemical compounds characterized by at least one hydroxylated aromatic ring. They constitute a large group of secondary metabolites, which has attracted considerable scientific but also commercial interest due to various promising health applications.

The most important phenolics in this respect are the flavonoids and the stilbenes with high value as nutritional and/or therapeutic agent (Ververidis et al. 2007). Other phenolics which are biologically important are the precursors for lignins and tannins. However, their commercial value as monomers is limited at the time being.

The biosynthesis of these compounds follows the phenylpropanoid metabolic pathway (Fig. 11.3). In the plant pathway, the amino acid phenylalanine is converted into coumaric acid. However, also tyrosine is a possible starting point, because it can be converted into coumaric acid by bacterial enzymes. The backbone of the flavonoids—the two phenyl ring containing chalcones—is formed from coumaric acid and malonyl-CoA. Conjugated ring closure of chalcones results in the three-ringed structure of the flavones, which are derivatized further down the metabolic pathway to yield flavanones, dihydroflavonols and so on.

11.3.2.1 Stilbenoids: Resveratrol as Commercial Example

Stilbenoids are hydroxylated derivatives of stilbene belonging to the family of phenylpropanoids. The commercially most important stilbenoid is resveratrol, a compound found in grapes and wine and connected to potential positive health effects. It can be produced from coumaric acid through expression of two plant-derived enzymes in yeast. The first attempts aimed at the conversion of exogenously added coumaric acid (Becker et al. 2003). De novo synthesis of resveratrol

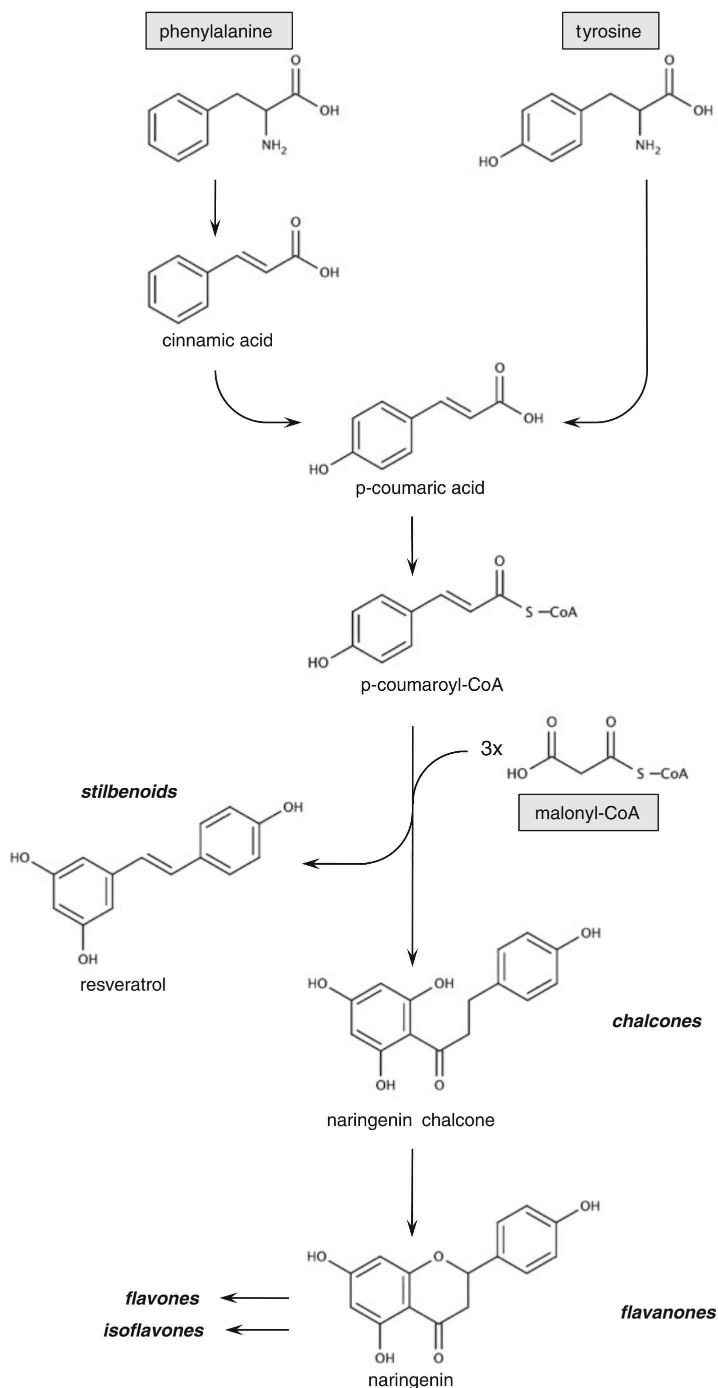

Fig. 11.3 Metabolic pathways for phenolics biosynthesis. Precursors from central metabolism are indicated by a *grey* background

starting from the primary metabolism requires the introduction of the pathway synthesizing coumaric acid from phenylalanine and/or tyrosine and has been accomplished only recently (Wang et al. 2011). Titers and productivities are apparently still too low to see any impact of the central carbon metabolism.

11.3.2.2 Flavonoids

By the introduction of plant genes, various flavonoids have been produced by yeast cells, such as naringenin, genistein and kaempferol (Naesby et al. 2009; Trantas et al. 2009). While biosynthesis of flavonoids from glucose was shown, most of the approaches aimed at the bioconversion of exogenously added precursors such as coumaric acid. *De novo* production of a flavonoid from glucose in baker's yeast has been analysed in detail only recently (Koopman et al. 2012). The key intermediate flavonoid, naringenin, was produced by heterologous expression of specific naringenin biosynthetic genes from *Arabidopsis thaliana*. However, this led to only marginal amounts of the desired flavonoid. It turned out that the connection point to the central carbon metabolism, namely the provision of aromatic amino acids was a major bottleneck. Deregulation by alleviating feedback inhibition of two key enzymes for aromatic amino acid biosynthesis appeared to be a prerequisite for efficient flavonoid biosynthesis. Additionally, a reduction in by-product formation, an increased copy number of the chalcone synthase gene and expression of a heterologous tyrosine ammonia lyase, allowing the direct conversion of tyrosine into coumaric acid, led to the accumulation of over 400 μM of naringenin under optimal bioprocess conditions (Koopman et al. 2012).

11.3.3 Isoprenoids

Isoprenoids (or terpenoids) are the largest group of plant secondary metabolites. Over 40,000 different compounds have been isolated up to now (Misawa 2011). They are assembled from the phosphorylated five-carbon building blocks isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Interestingly, it has been estimated that about 20 % of all carbon fixed by plant photosynthesis is channelled into this pathway (Ralston et al. 2005). Paclitaxel and artemisinin are plant-derived isoprenoids, which are used as powerful drugs for the treatment of the life-threatening diseases cancer or malaria, respectively. Other isoprenoids, such as the carotenoids are valued in the food industry for their nutritional, colour or flavour properties.

However, not only plants produce isoprenoids, but most other organisms including bacteria, fungi and mammals synthesize these secondary metabolites. Many key functions are controlled by isoprenoids, such as the membrane fluidity of yeasts by ergosterol.

Microorganisms, such as yeasts, offer themselves as cell factories for isoprenoid biosynthesis because the precursors and some intermediates are supplied by native metabolic processes.

Most prokaryotes and plants (within their plastids) synthesize the central precursors for isoprenoids—IPP and DMAPP—through the non-mevalonate pathway starting with the conversion of pyruvate and glyceraldehyde-3-phosphate in seven enzymatic steps. In contrast, IPP is synthesized via the mevalonate pathway (see Fig. 11.4) in the cytoplasm of other eukaryotes (and a few bacteria). IPP and DMAPP are further condensed to higher terpenes as outlined in Fig. 11.4.

11.3.3.1 Carotenoids

Carotenoids are widespread in nature, more than 700 natural variants are known so far. The major part is synthesized by plants. Animals are not able to synthesize carotenoids. They are forced to satisfy their demand for carotenoids by vitamin A ingestion. However, there are some coloured yeasts, bacteria and algae, which produce a variety of carotenoids. Of special interest is *Xanthophyllomyces dendrorhous* (also known as *Phaffia rhodozyma*), which naturally accumulates significant amounts of astaxanthin (up to 400 μg g^{-1} for wild-type strains). These amounts are too low for industrial production, so strain improvement is required (Schmidt et al. 2011). Most of the approaches have been dedicated to mutagenesis and screening, although the metabolic pathway and the involved enzymes have been described in detail. The rational engineering approaches have been mainly centred around the product-specific enzymes catalysing the conversion of geranylgeranyl-PP into astaxanthin. However, interestingly it has been shown that the astaxanthin titers could be increased by feeding precursors, such as glutamate, citrate or mevalonate (Schmidt et al. 2011), indicating that the connection to the central carbon metabolism is a crucial bottleneck. However, this has not been addressed by metabolic engineering yet.

The metabolic pathway for carotenoid biosynthesis has also been introduced into *C. utilis* (Shimada et al. 1998) and *S. cerevisiae* (Verwaal et al. 2007; Yamano et al. 1994). Only two genes are necessary to allow the production of ß-carotene in baker's yeast. However, in order to allow efficient production, the provision of the geranylgeranyl-PP has to be optimized. Verwaal et al. (2007) achieved this by overexpression of the endogenous geranylgeranyl-PP synthase and an increase in HMG-CoA reductase activity.

11.3.3.2 Sesquiterpenes: Artemisinic Acid

Artemisinin, a sesquiterpene endoperoxide lactone from *Artemisia annua*, is an important medicinal drug for the treatment of malaria. Artemisinic acid is a precursor for chemical synthesis of artemisinin. Recently, all genes encoding the enzymes required for efficient accumulation of artemisinic acid have been identified

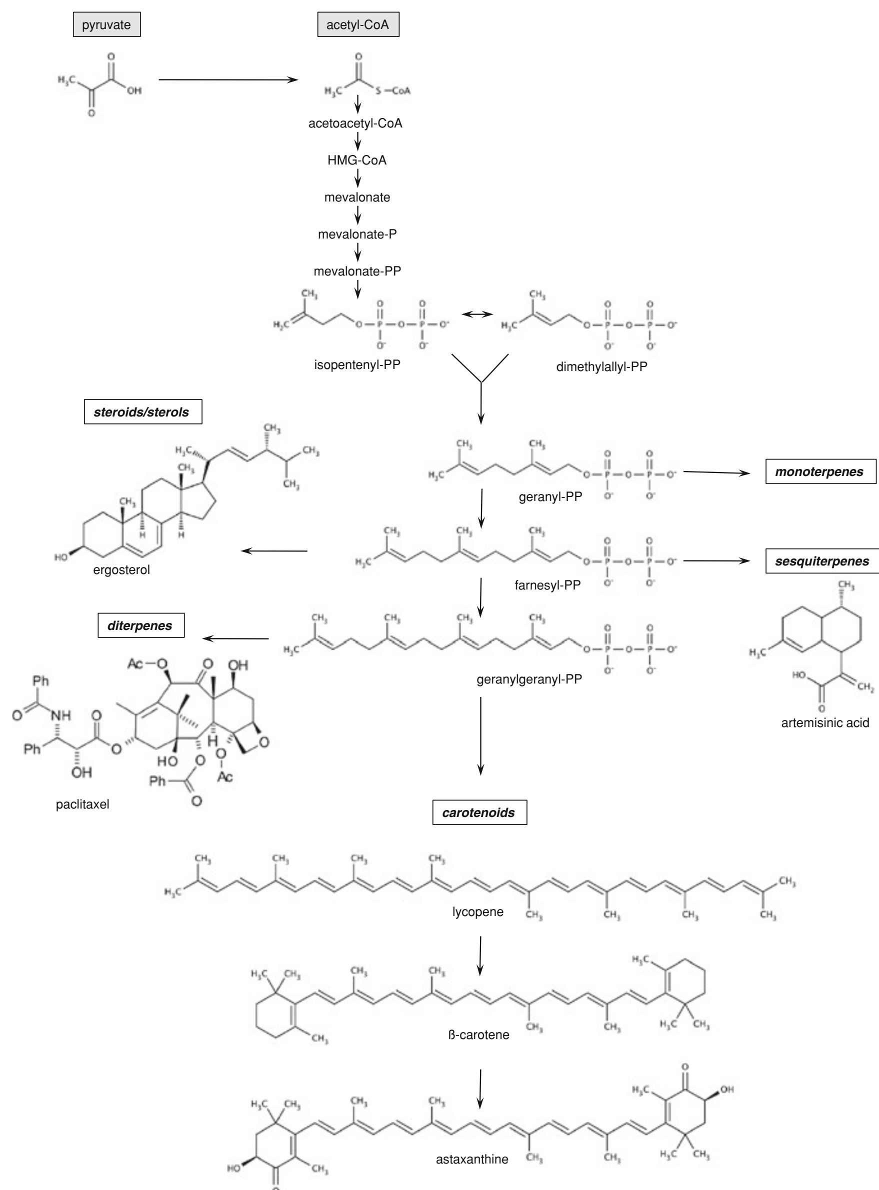

Fig. 11.4 Metabolic pathways for isoprenoid biosynthesis. Precursors from central metabolism are indicated by a *grey* background. *P* phosphate, *PP* pyrophosphate, *HMG-CoA* 3-hydroxy-3-methylglutaryl-coenzyme A

(Paddon et al. 2013). The starting point for the committed biosynthetic pathway of artemisinic acid is farnesyl-PP (Fig. 11.4). Various downstream genes have been identified and expressed in *E. coli* and *S. cerevisiae* (Ye and Bhatia 2012) and both

organisms readily accumulated artemisinin precursors. Interestingly, it turned out that the flux into the isoprenoid pathway is a major bottleneck for their production. Significant amounts of the desired compound were only produced upon upregulation of several genes in the mevalonate pathway (Ro et al. 2006); the key regulatory gene there is HMG-CoA reductase. Overexpression of this gene increased the artemisinin precursor production fivefold. However, it turned out that competing metabolic pathways, particularly sterol biosynthesis also limits productivity. Downregulation of the sterol biosynthesis is consequently a prerequisite for efficient artemisinic acid production. By a combination of the acquired knowledge with two newly identified specific genes, a yeast-based production process could be established, which leads to artemisinic acid titers of 26 g/L (Paddon et al. 2013).

11.3.3.3 Diterpenes: Paclitaxel (Taxol)

Paclitaxel (taxol) is a potent drug with excellent activity against a range of cancers. It is a diterpenoid derived from *Taxus* species. Particularly, it can be isolated from the bark of *Taxus brevifolia*. Currently, industrial production of taxol depends on semisynthesis from other taxoids such as baccatin III, isolated from the needles of *Taxus baccata*.

The entire taxol biosynthetic pathway is considered to involve 19 enzymatic steps starting from the diterpenoid precursor geranylgeranyl-PP (Jiang et al. 2012). While most of the enzymes catalysing these steps have been identified, some remain putative or even unknown up to date. Nevertheless, approaches for microbial production of taxol precursors have been reported: *E. coli* has been engineered to produce taxadiene—the first dedicated precursor of the taxol biosynthetic pathway (Ajikumar et al. 2010; Huang et al. 2001). Furthermore, genetic engineering of baker's yeast was employed to gain more insight into this complex biosynthetic pathway (DeJong et al. 2006). However, the desired compounds taxol or baccatin III have not been produced heterologously yet. Interestingly, also in case of taxol precursor biosynthesis, it was shown that the provision of the building blocks by the primary metabolism is a major limiting factor. Taxadiene accumulation in *S. cerevisiae* was only possible after heterologous expression of a geranylgeranyl-PP-synthase, catalysing the formation of geranylgeranyl-PP (Engels et al. 2008). Also in this case the productivity could be further increased by deregulating the pathway at the HMG-CoA reductase node and downregulation of sterol biosynthesis. This underlines once more that the central carbon metabolism is naturally tightly controlled and not easily prone to provide building blocks to heterologous biosynthetic pathways, a fact that one should keep in mind when designing synthetic microbial cell factories for secondary metabolites.

11.3.3.4 Sterols/Steroids

Sterols and steroids are essential for the physiology of eukaryotic organisms. Cholesterol or ergosterol affect the cell membranes' fluidity and form therefore essential parts of the cellular membrane. In vertebrates, corticosteroids, such as cortisol, act as hormones and play a crucial role for cellular communication.

In order to construct a yeast strain, which accumulates high amounts of ergosterol, various genes of the respective biosynthetic pathway have been overexpressed in *S. cerevisiae* (Veen et al. 2003). Starting point was in this case a strain which already produced high amounts of squalene due to a deregulation of HMG-CoA reductase (Polakowski et al. 1998). Again this step was a bottleneck for precursor production. Overexpression of downstream genes changed the pattern of sterols accumulated. The total sterol content in this strain increased threefold compared to a wild-type strain.

11.4 Protein Production

Based on the development of recombinant DNA technology in the 1970s, the production of recombinant proteins has become a multi-billion dollar market. The biopharmaceutical industry has been the driving force of this development, so that up to now more than 200 biopharmaceuticals have been approved by the US Food and Drug Association. According to Berlec and Strukelj (2013), 66 (31 %) of the approved biopharmaceuticals were produced in *E. coli*, 31 (15 %) in yeast (30 in *S. cerevisiae* and 1 in *Pichia pastoris*) and 91 (43 %) in mammalian cells. In 2009, the market value of recombinant proteins was 99 billion dollars, representing the fastest growing segment of the pharmaceutical industry (Walsh 2010).

With respect to the production of recombinant proteins, not only biopharmaceuticals are of importance. The production of industrial enzymes is a market of more than 5 billion dollars (Porro et al. 2011). Industrial enzymes are for example proteases, lipases and carbohydrases, a large portion of them being produced using yeast as a host organism. The growing demand for lignocellulolytic enzymes further drives the production of new recombinant enzymes.

Yeasts bear several advantages compared to other expression systems. Yeasts can grow to high cell densities, perform eukaryotic post-translational modifications related to folding and secretion, produce high product titers and do not contain pyrogens or viruses which are harmful for humans. These advantages make yeasts interesting for industry as an expression host for protein production. Up to now mainly *S. cerevisiae*, *P. pastoris* and *Hansenula polymorpha* are used in an industrial process to produce recombinant proteins, but there are several more yeast expression systems with a great potential in recombinant protein production (Mattanovich et al. 2012).

11.4.1 Impact of Recombinant Protein Production on the Yeast Metabolism

Production costs of recombinant proteins are an issue for high-volume therapeutic proteins where manufacturing costs significantly impact total treatment costs per patient. Even more pronounced, the large-scale use of technical enzymes is often not feasible unless production costs decrease below a critical threshold. Therefore, research is focused on a detailed understanding of the impact of recombinant protein production on the yeast cell physiology (Graf et al. 2009). This knowledge can be used to engineer cellular processes, e.g. protein synthesis, processing and secretion, to maximize the titer of the protein produced. Protein folding and secretion is a complex process involving several hundred cellular proteins (Delic et al. 2013). Engineering of the secretory pathway has successfully improved productivity of recombinant proteins in yeast (reviewed in Idiris et al. 2010 and Delic et al. 2014).

It is well known that the production of a foreign protein causes an additional stress for the host cell and leads to a decrease in the overall cell fitness. The phenotype of such cells is a decreased maximum specific growth rate, decreased by-product formation and increased cell lysis (Gonzalez et al. 2002; Heyland et al. 2011). In addition, cellular stress may also lead to reduced product formation (Mattanovich et al. 2004).

Limitations in the primary metabolism of a yeast cell can cause a stress situation, resulting in the aforementioned defects. The changes in metabolism due to recombinant protein production are summarized under the term metabolic burden or metabolic load, which can be defined as a portion of host cell's resources, in form of either energy or precursor metabolites such as amino acids and nucleotides (Fig. 11.5), which is required to maintain and express foreign DNA (Glick 1995). The metabolic processes which lead to a burden on yeast metabolism under protein production conditions are poorly understood. The extent of the metabolic burden on a yeast cell is influenced by bioprocess parameters (temperature, dissolved oxygen, substrate choice or growth rate) and gene dosage (Dragosits et al. 2009; Hohenblum et al. 2003; Liu et al. 2013).

As stated in the definition of the metabolic burden two possible explanations can be proposed. First, a limitation in synthesis capacity of certain amino acids can lead to a burden for the yeast cell. Also a limited supply of amino acid biosynthetic pathways with precursors from the central carbon metabolism may be a reason for the observed burden. Second, a higher energy demand (ATP) due to recombinant protein production is plausible, as additional protein has to be synthesized, folded and secreted, all processes needing high amounts of energy.

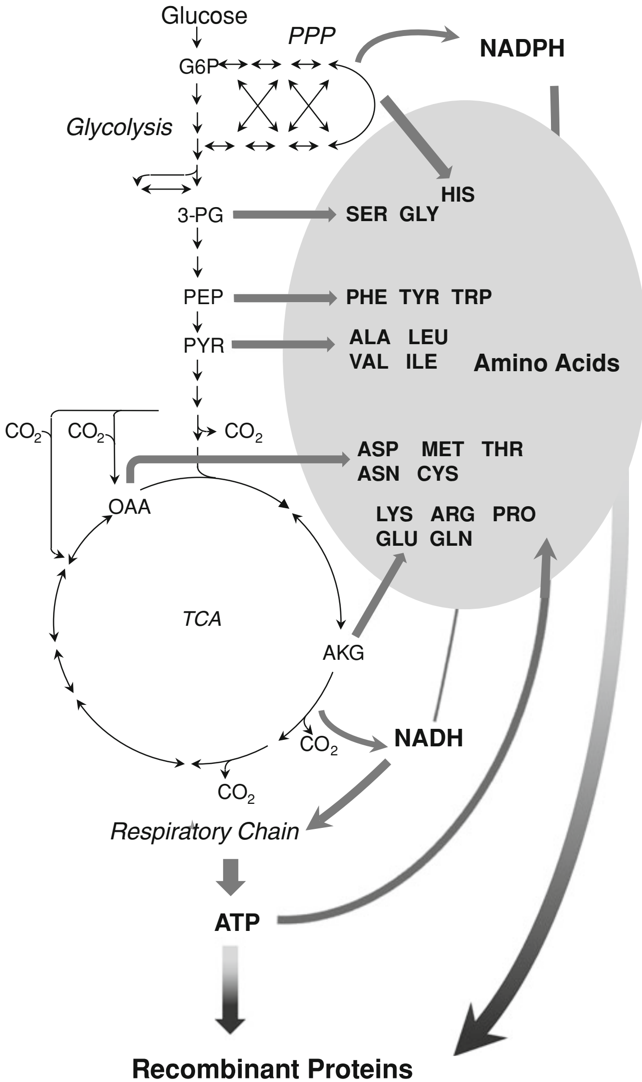

Fig. 11.5 Schematic representation of the primary metabolism with special respect to protein production. *PPP* pentose phosphate pathway, *TCA* tricarboxylic acid cycle, *G6P* glucose-6-phosphate, *3-PG* 3-phosphoglycerate, *PEP* phosphoenolpyruvate, *PYR* pyruvate, *OAA* oxaloacetate, *AKG* α-ketoglutarate. Amino acids are indicated by the three-letter code

11.4.2 Amino Acid Metabolism

The production of a foreign protein exhibits a certain metabolic burden even when the amount of recombinant protein is low compared to total host cell protein. The building blocks of proteins are amino acids. To produce a recombinant protein, the

cell metabolism has to ensure that enough of the needed amino acids are available. The amino acid composition of the recombinant protein may influence protein production if the frequency of certain amino acids is higher compared to the yeast cell proteome. In other words, heterologous proteins, especially secreted human proteins, have a significantly different amino acid composition than total cellular yeast protein. The effect depends on the metabolic costs of respective amino acids with higher frequency in the recombinant protein. Metabolic costs of amino acid synthesis have been calculated by Wagner (2005) based on the amount of activated phosphate bonds needed for synthesis, and the loss of energy by not using the precursor for energy production. Thus, aromatic or branched chain amino acids are most cost intensive. Additionally, amino acids with a very low abundance in the cellular protein may constitute a bottleneck. For *P. pastoris*, it has been reported that cysteine levels are very low (Carnicer et al. 2009), however, cysteine occurs at a rather high frequency in many recombinant proteins of interest (e.g. antibodies).

Several studies showed that amino acids supplementation to the cultivation media partly released the metabolic burden and improved heterologous protein production. Heyland et al. (2011) showed improved production of a bacterial β-aminopeptidase in *P. pastoris* by the addition of glutamine, a mixture of TCA-cycle-derived amino acids (Glu, Gln, Lys and Pro) and energetically expensive amino acids (His, Leu, Ile, Lys, Met, Phe and Tyr). They suggested that glutamine as a carbon and nitrogen source is a possible candidate for partly relieving the metabolic burden. A second study in *S. cerevisiae* by Görgens et al. (2005) showed an improvement in xylanase production. A higher production rate of xylanase was observed by the addition of a mixture of preferred amino acids (Ala, Arg, Asn, Glu, Gln and Gly) to the cultivation media. The selection of these amino acids was based on previous screening results. The two main reasons for using this mixture was the increased production rate of the recombinant protein and the decreased cellular proteolysis. In a second experiment, they added just the TCA cycle intermediate succinate to the cultivation media, which also improved recombinant xylanase production.

Both studies showed that the addition of amino acids derived from TCA cycle intermediates enhanced recombinant protein production. Additionally, the supplementation of media only with succinate showed the same effect. This suggests that exogenous amino acids are not only directly incorporated into the recombinant protein, but metabolized via the TCA cycle, and that both TCA-cycle-related amino acids and TCA cycle intermediates may have a beneficial effect on protein production. Heyland et al. (2011) also showed that glutamine is co-metabolized with glucose and was used for the production of other amino acids.

In summary, the above-mentioned studies showed that under recombinant protein production condition, a limitation of the supply with amino acids and TCA cycle intermediates may lead to the observed metabolic burden.

11.4.3 Energy Metabolism

For the production of a protein, not only the sufficient supply with amino acids is necessary, but also energy in form of ATP, GTP and reduction equivalents (NADH, NADPH) is indispensable. Translation costs 4 energy equivalents per amino acid residue, 2 for charging the tRNA and 2 for ribosomal synthesis. Protein folding and eventual secretion involve further energy consuming steps like ATP-dependent chaperone activity. Taken together, yeast cells producing a heterologous protein have an additional demand for energy, because of the fact that more protein has to be translated, folded and secreted.

The cellular response to the higher need for energy in the form of ATP can be a redirection of fluxes within the central carbon metabolism towards the TCA cycle. To verify this change in the fluxes, metabolic flux analysis is the method of choice and has been used in different studies to investigate the host cell physiology under recombinant protein production conditions.

Several studies in *P. pastoris* showed exactly this redirection of fluxes under recombinant protein production conditions (Dragosits et al. 2009; Heyland et al. 2010). Dragosits et al. applied glucose-limited chemostat cultures to study the effect of different growth temperatures on productivity and cell physiology. At 30 °C, the flux through the TCA cycle was higher in a recombinant protein production strain as compared to a control strain. At lower temperatures (20 and 25 °C), they observed up to threefold higher protein productivity but no changes in the TCA cycle flux between the reference strain and the production strains. They suggest that the higher TCA cycle flux at 30 °C is a cellular response to the extra energy needed for folding, refolding and secretion of the recombinant protein. It is proposed that at lower temperature, reduced stress due to less misfolding of the recombinant protein may also lead to a lower energy demand. In a second study, Heyland et al. (2010) showed that also in glucose-limited high cell density fed-batch cultivation of *P. pastoris*, a higher flux through the TCA cycle was seen. The calculated ATP generation rate showed a slight increase for the recombinant protein production strain. These results again indicate the need of extra energy during heterologous protein production.

The above-mentioned studies with *P. pastoris* were performed on glucose as a carbon source, employing the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter for recombinant gene expression. Alternatively, the strong alcohol oxidase (*AOX1*) promoter is used which requires methanol as an inducer, and usually also as the carbon source for cell growth. Growth on methanol requires efficient regeneration of reduced redox equivalents and reactive oxygen scavenging (Yano et al. 2009). Mixed substrate feeds (e.g. glycerol/methanol, glucose/methanol) have been employed to reduce the oxidative stress onto *P. pastoris* during methanol induction. Additionally, it was observed that by using multiple carbon sources, the productivity of the cell in making the desired protein was elevated (Ramón et al. 2007). Methanol can be metabolized either directly to CO_2, generating 2 mol NADH per mol methanol. NADH can be used for the generation of

ATP via the respiratory chain. On the other hand, methanol can be assimilated into biomass. Jordà et al. (2012) analysed the core metabolic fluxes of *P. pastoris* growing on glucose/methanol mixtures. Under recombinant protein production conditions (production of a lipase from *Rhizopus oryzae*), the contribution of methanol to biomass production was decreased, which suggests a higher rate through the dissimilatory pathway which oxidizes methanol to CO_2. The additional NADH generated by this pathway might lead to a lower metabolic burden and the observed higher productivities under the mixed feed condition. The calculated NADH and ATP generation rate increased for the production strain, which speaks again for the higher energy demand due to recombinant protein production.

11.5 Conclusions

Here, we outlined our current knowledge about the interrelation of the central carbon metabolism and various microbial production processes. While it is obvious that every production process is inevitably connected to and dependent from the central carbon metabolism, our knowledge how these relations translate into flux and titer of product are surprisingly scarce. For primary metabolites, these relations are known and described in detail. However, for secondary metabolites and proteins, many points remain unclear. Precursor feeding experiments clearly show for a lot of products that the provision of central metabolites for secondary product biosynthesis is often limited. However, very few results have been published about addressing this problem by metabolic engineering.

References

Abbas CA, Sibirny AA (2011) Genetic control of biosynthesis and transport of riboflavin and flavin nucleotides and construction of robust biotechnological producers. Microbiol Mol Biol Rev 75:321–360. doi:10.1128/MMBR.00030-10

Abbott D, Zelle RM, Pronk JT, Van Maris AJ (2009) Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges. FEMS Yeast Res 9:1123–1136. doi:10.1111/j.1567-1364.2009.00537.x

Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. Science 330:70–74. doi:10.1126/science.1191652

Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJY, Hanai T, Liao JC (2008) Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab Eng 10:305–311

Bakker BM, Overkamp KM, van Maris AJ, Kötter P, Luttik MA, van Dijken JP, Pronk JT (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25:15–37. doi:10.1111/j.1574-6976.2001.tb00570.x

Becker JVW, Armstrong GO, van der Merwe MJ, Lambrechts MG, Vivier MA, Pretorius IS (2003) Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. FEMS Yeast Res 4:79–85. doi:10.1016/S1567-1356(03)00157-0

Berlec A, Strukelj B (2013) Current state and recent advances in biopharmaceutical production in *Escherichia coli*, yeasts and mammalian cells. J Ind Microbiol Biotechnol 40:257–274. doi:10.1007/s10295-013-1235-0

Branduardi P, Fossati T, Sauer M, Pagani R, Mattanovich D, Porro D (2007) Biosynthesis of vitamin C by yeast leads to increased stress resistance. PLoS ONE 2:e1092. doi:10.1371/journal.pone.0001092

Branduardi P, Longo V, Berterame NM, Rossi G, Porro D (2013) A novel pathway to produce butanol and isobutanol in *Saccharomyces cerevisiae*. Biotechnol Biofuels 6:68. doi:10.1186/1754-6834-6-68

Bremus C, Herrmann U, Bringer-Meyer S, Sahm H (2006) The use of microorganisms in L-ascorbic acid production. J Biotechnol 124:196–205. doi:10.1016/j.jbiotec.2006.01.010

Carnicer M, Baumann K, Töplitz I, Sánchez-Ferrando F, Mattanovich D, Ferrer P, Albiol J (2009) Macromolecular and elemental composition analysis and extracellular metabolite balances of *Pichia pastoris* growing at different oxygen levels. Microb Cell Fact 8:65. doi:10.1186/1475-2859-8-65

Celińska E (2010) Debottlenecking the 1,3-propanediol pathway by metabolic engineering. Biotechnol Adv 28:519–530. doi: http://dx.doi.org/10.1016/j.biotechadv.2010.03.003

Da Silva GP, Mack M, Contiero J (2009) Glycerol: a promising and abundant carbon source for industrial microbiology. Biotechnol Adv 27:30–39. doi: http://dx.doi.org/10.1016/j.biotechadv.2008.07.006

Daran-Lapujade P, Rossell S, van Gulik WM, Luttik MAH, de Groot MJ, Slijper M, Heck AJ, Daran J-M, de Winde JH, Westerhoff HV, Pronk JT, Bakker BM (2007) The fluxes through glycolytic enzymes in *Saccharomyces cerevisiae* are predominantly regulated at posttranscriptional levels. Proc Natl Acad Sci U S A 104:15753–15758. doi:10.1073/pnas.0707476104

Dejong JM, Liu Y, Bollon AP, Long RM, Jennewein S, Williams D, Croteau RB (2006) Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*. Biotechnol Bioeng 93:212–224

Delic M, Göngrich R, Mattanovich D, Gasser B (2014) Engineering of protein folding and secretion—strategies to overcome bottlenecks for efficient production of recombinant proteins. Antioxid Redox Signal. doi:10.1089/ars.2014.5844

Delic M, Valli M, Graf AB, Pfeffer M, Mattanovich D, Gasser B (2013) The secretory pathway: exploring yeast diversity. FEMS Microbiol Rev 37:872–914. doi: 10.1111/1574-6976.12020

Dmytruk KV, Yatsyshyn VY, Sybirna NO, Fedorovych DV, Sibirny AA (2011) Metabolic engineering and classic selection of the yeast *Candida famata* (*Candida flareri*) for construction of strains with enhanced riboflavin production. Metab Eng 13:82–88. doi:10.1016/j.ymben.2010.10.005

Dragosits M, Stadlmann J, Albiol J, Baumann K, Maurer M, Gasser B, Sauer M, Altmann F, Ferrer P, Mattanovich D, Cerdanyola B (2009) The effect of temperature on the proteome of recombinant *Pichia pastoris*. J Proteome Res 8:1380–1392. doi:10.1021/pr8007623

Engels B, Dahm P, Jennewein S (2008) Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab Eng 10:201–206. doi: http://dx.doi.org/10.1016/j.ymben.2008.03.001

Fossati T, Solinas N, Porro D, Branduardi P (2011) L-ascorbic acid producing yeasts learn from plants how to recycle it. Metab Eng 13:177–185. doi:10.1016/j.jbiotec.2010.09.539

Glick BR (1995) Metabolic load and heterologous gene expression. Biotechnol Adv 13:247–261. doi:10.1016/0734-9750(95)00004-A

Gonzalez R, Andrews BA, Molitor J, Asenjo JA (2002) Metabolic analysis of the synthesis of high levels of intracellular human SOD in *Saccharomyces cerevisiae* rhSOD 2060 411 SGA122. Biotechnol Bioeng 82:152–169. doi:10.1002/bit.10556

Görgens JF, van Zyl WH, Knoetze JH, Hahn-Hägerdal B (2005) Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium. Appl Microbiol Biotechnol 67:684–691. doi:10.1007/s00253-004-1803-3

Graf A, Dragosits M, Gasser B, Mattanovich D (2009) Yeast systems biotechnology for the production of heterologous proteins. FEMS Yeast Res 9:335–348. doi:10.1111/j.1567-1364.2009.00507.x

Gutiérrez-Lomelí M, Torres-Guzmán JC, González-Hernández GA, Cira-Chávez LA, Pelayo-Ortiz C, Ramírez-Córdova J de J (2008) Overexpression of ADH1 and *HXT*1 genes in the yeast *Saccharomyces cerevisiae* improves the fermentative efficiency during tequila elaboration. Antonie Van Leeuwenhoek 93:363–371. doi: 10.1007/s10482-007-9213-z

Heefner D, Weaver CA, Yarus MJ, Burdzinski LA, Gyure DC, Foster EW (1988) Riboflavin producing strains of microorganisms, method for selecting, and method for fermentation. International Patent Application WO 88/09822

Heyland J, Fu J, Blank LM, Schmid A (2010) Quantitative physiology of *Pichia pastoris* during glucose-limited high cell-density fed-batch cultivations for recombinant protein production. Biotechnol Bioeng 107:357–368. doi:10.1002/bit.22836

Heyland J, Fu J, Blank LM, Schmid A (2011) Carbon metabolism limits recombinant protein production in *Pichia pastoris*. Biotechnol Bioeng 108:1942–1953. doi:10.1002/bit.23114

Hohenblum H, Gasser B, Maurer M, Borth N, Mattanovich D (2003) Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnol Bioeng 85:367–375. doi:10.1002/bit.10904

Huang Q, Roessner CA, Croteau R, Scott AI (2001) Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. Bioorg Med Chem 9:2237–2242. doi:10.1016/S0968-0896(01)00072-4

Idiris A, Tohda H, Kumagai H, Takegawa K (2010) Engineering of protein secretion in yeast: strategies and impact on protein production. Appl Microbiol Biotechnol 86:403–417. doi:10.1007/s00253-010-2447-0

Jiang M, Stephanopoulos G, Pfeifer BA (2012) Downstream reactions and engineering in the microbially reconstituted pathway for Taxol. Appl Microbiol Biotechnol 94:841–849. doi:10.1007/s00253-012-4016-1

Jiménez A, Santos MA, Pompejus M, Revuelta JL (2005) Metabolic engineering of the purine pathway for riboflavin production in *Ashbya gossypii*. Appl Environ Microbiol 71:5743–5751. doi:10.1128/AEM.71.10.5743-5751.2005

Jordà J, Jouhten P, Cámara E, Maaheimo H, Albiol J, Ferrer P (2012) Metabolic flux profiling of recombinant protein secreting *Pichia pastoris* growing on glucose:methanol mixtures. Microb Cell Fact 11:57. doi:10.1186/1475-2859-11-57

Koopman F, Beekwilder J, Crimi B, van Houwelingen A, Hall RD, Bosch D, van Maris AJ a, Pronk JT, Daran JM (2012) De novo production of the flavonoid naringenin in engineered *Saccharomyces cerevisiae*. Microb Cell Fact 11:155. doi: 10.1186/1475-2859-11-155

Lan EI, Liao JC (2013) Microbial synthesis of n-butanol, isobutanol, and other higher alcohols from diverse resources. Bioresour Technol 135:339–349. doi:10.1016/j.biortech.2012.09.104

Liu Z, Hou J, Martínez JL, Petranovic D, Nielsen J (2013) Correlation of cell growth and heterologous protein production by *Saccharomyces cerevisia*e. Appl Microbiol Biotechnol 1–8. doi: 10.1007/s00253-013-4715-2

Ma M, Liu ZL (2010) Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 87:829–845. doi:10.1007/s00253-010-2594-3

Mattanovich D, Gasser B, Hohenblum H, Sauer M (2004) Stress in recombinant protein producing yeasts. J Biotechnol 113:121–135. doi:10.1016/j.jbiotec.2004.04.035

Mattanovich D, Branduardi P, Dato L, Gasser B, Sauer M, Porro D (2012) Recombinant protein production in yeasts. Methods Mol Biol 824:329–358. doi:10.1007/978-1-61779-433-9_17

Misawa N (2011) Pathway engineering for functional isoprenoids. Curr Opin Biotechnol 22:627–633. doi:10.1016/j.copbio.2011.01.002

Naesby M, Nielsen SV, Nielsen CA, Green T, Tange TO, Simón E, Knechtle P, Hansson A, Schwab MS, Titiz O, Folly C, Archila RE, Maver M, van Sint Fiet S, Boussemghoune T, Janes M, Kumar AS, Sonkar SP, Mitra PP, Benjamin VA, Korrapati N, Suman I, Hansen EH, Thybo T, Goldsmith N, Sorensen AS (2009) Yeast artificial chromosomes employed for random assembly of biosynthetic pathways and production of diverse compounds in *Saccharomyces cerevisiae*. Microb Cell Fact 8:45. doi: 10.1186/1475-2859-8-45

Ng CY, Jung MY, Lee J, Oh MK (2012) Production of 2,3-butanediol in *Saccharomyces cerevisiae* by in silico aided metabolic engineering. Microb Cell Fact 11:68. doi:10.1186/1475-2859-11-68

Otterstedt K, Larsson C, Bill RM, Ståhlberg A, Boles E, Hohmann S, Gustafsson L (2004) Switching the mode of metabolism in the yeast *Saccharomyces cerevisiae*. EMBO Rep 5:532–537. doi:10.1038/sj.embor.7400132

Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Fleck M, Bajad S, Dang G, Dengrove D, Diola D, Dorin G, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T, Henry R, Hepp M, Horning T, Iqbal T, Jiang H, Kizer L, Lieu B, Melis D, Moss N, Regentin R, Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J, Covello PS, Keasling JD, Reiling KK, Renninger NS, Newman JD (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496:528–532. doi:10.1038/nature12051

Padh H (1991) Vitamin C: newer insights into its biochemical functions. Nutr Rev 49:65–70. doi:10.1111/j.1753-4887.1991.tb07407.x

Piper P, Calderon CO, Hatzixanthis K, Mollapour M (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 147:2635–2642

Polakowski T, Stahl U, Lang C (1998) Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Appl Microbiol Biotechnol 49:66–71. doi:10.1007/s002530051138

Porro D, Gasser B, Fossati T, Maurer M, Branduardi P, Sauer M, Mattanovich D (2011) Production of recombinant proteins and metabolites in yeasts: when are these systems better than bacterial production systems? Appl Microbiol Biotechnol 89:939–948. doi:10.1007/s00253-010-3019-z

Raab AM, Lang C (2011) Oxidative versus reductive succinic acid production in the yeast *Saccharomyces cerevisiae*. Bioeng Bugs 2:120–123. doi:10.4161/bbug.2.2.14549

Ralston L, Subramanian S, Matsuno M, Yu O (2005) Partial reconstruction of flavonoid and isoflavonoid biosynthesis in yeast using soybean type I and type II chalcone isomerases. Plant Physiol 137:1375–1388. doi:10.1104/pp.104.054502

Ramón R, Ferrer P, Valero F (2007) Sorbitol co-feeding reduces metabolic burden caused by the overexpression of a *Rhizopus oryzae* lipase in *Pichia pastoris*. J Biotechnol 130:39–46. doi:10.1016/j.jbiotec.2007.07.133

Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940–943. doi:10.1038/nature04640

Rossi G, Sauer M, Porro D, Branduardi P (2010) Effect of *HXT*1 and *HXT*7 hexose transporter overexpression on wild-type and lactic acid producing *Saccharomyces cerevisiae* cells. Microb Cell Fact 9:15. doi:10.1186/1475-2859-9-15

Rymowicz W, Fatykhova AR, Kamzolova SV, Rywińska A, Morgunov IG (2010) Citric acid production from glycerol-containing waste of biodiesel industry by *Yarrowia lipolytica* in batch, repeated batch, and cell recycle regimes. Appl Microbiol Biotechnol 87:971–979. doi:10.1007/s00253-010-2561-z

Sauer M, Branduardi P, Valli M, Porro D (2004) Production of L-ascorbic acid by metabolically engineered *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. Appl Environ Microbiol 70:6086–6091. doi:10.1128/AEM.70.10.6086-6091.2004

Sauer M, Porro D, Mattanovich D, Branduardi P (2008) Microbial production of organic acids: expanding the markets. Trends Biotechnol 26:100–108. doi:10.1016/j.tibtech.2007.11.006

Sauer M, Porro D, Mattanovich D, Branduardi P (2010) 16 years research on lactic acid production with yeast—ready for the market? Biotechnol Genet Eng Rev 27:229–256. doi:10.1080/02648725.2010.10648152

Schmidt I, Schewe H, Gassel S, Jin C, Buckingham J, Hümbelin M, Sandmann G, Schrader J (2011) Biotechnological production of astaxanthin with *Phaffia rhodozyma*/*Xanthophyllomyces dendrorhous*. Appl Microbiol Biotechnol 89:555–571. doi:10.1007/s00253-010-2976-6

Shimada H, Kondo K, Fraser PD, Miura Y, Saito T, Misawa N (1998) Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. Appl Environ Microbiol 64:2676–2680

Stahmann KP, Revuelta JL, Seulberger H (2000) Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. Appl Microbiol Biotechnol 53:509–516. doi:10.1007/s002530051649

Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD (2008) Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. Microb Cell Fact 7:36. doi:10.1186/1475-2859-7-36

Tanino T, Ito T, Ogino C, Ohmura N, Ohshima T, Kondo A (2012) Sugar consumption and ethanol fermentation by transporter-overexpressed xylose-metabolizing *Saccharomyces cerevisiae* harboring a xyloseisomerase pathway. J Biosci Bioeng 114:209–211. doi:10.1016/j.jbiosc.2012.03.004

Teixeira MC, Mira NP, Sá-Correia I (2011) A genome-wide perspective on the response and tolerance to food-relevant stresses in *Saccharomyces cerevisiae*. Curr Opin Biotechnol 22:150–156. doi:10.1016/j.copbio.2010.10.011

Tomaszewska L, Rywińska A, Gładkowski W (2012) Production of erythritol and mannitol by *Yarrowia lipolytica* yeast in media containing glycerol. J Ind Microbiol Biotechnol 39:1333–1343. doi:10.1007/s10295-012-1145-6

Trantas E, Panopoulos N, Ververidis F (2009) Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. Metab Eng 11:355–366. doi:10.1016/j.ymben.2009.07.004

Veen M, Stahl U, Lang C (2003) Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in *Saccharomyces cerevisiae*. FEMS Yeast Res 4:87–95. doi:10.1016/S1567-1356(03)00126-0

Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G, Panopoulos N (2007) Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: chemical diversity, impacts on plant biology and human health. Biotechnol J 2:1214–1234. doi:10.1002/biot.200700084

Verwaal R, Wang J, Meijnen J-P, Visser H, Sandmann G, van den Berg JA, van Ooyen AJ (2007) High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. Appl Environ Microbiol 73:4342–4350. doi:10.1128/AEM.02759-06

Vidgren V, Huuskonen A, Virtanen H, Ruohonen L, Londesborough J (2009) Improved fermentation performance of a lager yeast after repair of its *AGT*1 maltose and maltotriose transporter genes. Appl Environ Microbiol 75:2333–2345. doi:10.1128/AEM.01558-08

Wagner A (2005) Energy constraints on the evolution of gene expression. Mol Biol Evol 22:1365–1374. doi:10.1093/molbev/msi126

Walsh G (2010) Biopharmaceutical benchmark. Nat Biotechnol 17:917–924. doi:10.1038/nbt0910-917

Wang Y, Halls C, Zhang J, Matsuno M, Zhang Y, Yu O (2011) Stepwise increase of resveratrol biosynthesis in yeast *Saccharomyces cerevisiae* by metabolic engineering. Metab Eng 13:455–463. doi:10.1016/j.ymben.2011.04.005

Yamano S, Ishii T, Nakagawa M, Ikenaga H, Misawa N (1994) Metabolic engineering for production of beta-carotene and lycopene in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 58:1112–1114. doi:10.1271/bbb.58.1112

Yano T, Takigami E, Yurimoto H, Sakai Y (2009) Yap1-regulated glutathione redox system curtails accumulation of formaldehyde and reactive oxygen species in methanol metabolism of *Pichia pastoris*. Eukaryot Cell 8:540–549. doi:10.1128/EC.00007-09

Yatsyshyn VY, Ishchuk OP, Voronovsky AY, Fedorovych DV, Sibirny AA (2009) Production of flavin mononucleotide by metabolically engineered yeast *Candida famata*. Metab Eng 11:163–167. doi:10.1016/j.ymben.2009.01.004

Ye VM, Bhatia SK (2012) Metabolic engineering for the production of clinically important molecules: Omega-3 fatty acids, artemisinin, and taxol. Biotechnol J 7:20–33. doi:10.1002/biot.201100289

Young EM, Comer AD, Huang H, Alper HS (2012) A molecular transporter engineering approach to improving xylose catabolism in *Saccharomyces cerevisiae*. Metab Eng 14:401–411. doi:10.1016/j.ymben.2012.03.004

Zhu Y, Chen X, Chen T, Shi S, Zhao X (2006) Over-expression of glucose dehydrogenase improves cell growth and riboflavin production in *Bacillus subtilis*. Biotechnol Lett 28:1667–1672. doi:10.1007/s10529-006-9143-2