Characterization of carbon metabolism and glucose repression in yeasts

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Characterization of carbon metabolism and glucose repression in yeasts

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Contents

Abstract 3
Zusammenfassung 5
Chapter 1 7
General introduction
Chapter 2 23
Intracellular characterization of aerobic glucose metabolism in seven yeast species by $^{13}$C flux analysis
Chapter 3 46
Impact of glucose signaling on Saccharomyces cerevisiae physiology under differential repressive conditions
Chapter 4 69
Metabolic regulation through the four glucose signaling pathways in yeast under differential repressive conditions
Chapter 5 94
Impact of Ras/PKA signaling on metabolism after glucose relief of starvation
Chapter 6 119
Other projects where metabolomics and flux analysis could be applied
Chapter 7 127
Summary and Outlook
Acknowledgements 131
Curriculum vitae 132
Abstract

Due to its relevance in biotechnology and as a model organism, yeast and its metabolism received a lot of attention. The core of metabolism, hence central carbon metabolism, serves yeast to catabolize various nutrients and is conserved among different species. However, metabolic operation at the level of metabolic flux is highly variable. This variability is primarily caused by the species and the environment, hence condition-dependent regulation. In this thesis we quantitatively investigate growth and metabolism in dependency on both of these factors. Specifically, we characterize Crabtree metabolism in the context of 7 yeast species and on the other hand we determine the condition dependency of growth and metabolism in the context of glucose repression in *Saccharomyces cerevisae*.

In Chapter 2 we found via $^{13}$C flux analysis, that the yeast species can be clearly grouped into Crabtree-positive and Crabtree-negative types of metabolism at the level of intracellular fluxes, although the extracellular rates suggested a gradually evolved metabolism. This further implied that redox metabolism was either dependent on either ethanol fermentation or respiration. Additionally, we measured the intracellular metabolome of the species and found that it seems to be species-specific, since only fructose-1,6-bisphosphate and dihydroxyacetone-phosphate showed a strong correlation with glycolysis. Thus we concluded that the kinetic properties around these two metabolites might be conserved among yeasts.

In Chapter 3 we explored the conditional impact of the glucose signaling network components on growth and physiology during shifts between the differential repressive carbon sources glucose, galactose, and ethanol. This allowed us to verify key signaling components, which might constitutively activate or interrupt a signaling pathway. The different growth parameters of each component revealed that a well-balanced activity of the Ras/PKA pathway and the Snf1 pathway were important for growth and physiology under all conditions. An active Hap complex was needed under respiratory carbon sources and the Snf3/Rgt2 pathway was required only during growth on glucose. Besides the pathway-specific phenotypes, we could show, that the Ras/PKA signaling pathway and Hxk2 from the Snf1 pathway seem to redundantly control glucose repression.

The overall difference in the growth profiles led us to the hypothesis, that metabolism is differentially affected by the four signaling pathways in a condition-dependent manner. Therefore we utilized a metabolomics approach in Chapter 4 to quantify the impact of the glucose signaling components on central carbon metabolism during steady-state growth under the three differential repressive conditions glucose, galactose, and ethanol. This readout enabled us to set condition-dependent links between signaling pathway activity and metabolic phenotypes. For example on glucose, upper glycolysis seemed to be dependent on a balanced Snf1 activity and on the Snf3/Rgt2 pathway. On galactose, glycolysis was still
dependent on Snf1 pathway, but interruptions of the Snf3/Rgt2 pathway did not exhibit a metabolic phenotype.

In Chapter 5 we investigated the interdependency of the Ras/PKA pathway and the metabolic response upon glucose relief of starvation. For this purpose we determined the metabolic response upon glucose relief of starvation in wild type yeast and compared it to single deletion mutants of Ras/PKA signaling components. Wild type yeast showed a fast response in glycolytic intermediates and nucleotide phosphates, which occurred before the peak in cAMP, a main mediator of Ras/PKA signaling. Other metabolites had a general slow dynamic or peaked after cAMP. We further found that several signaling components of the Ras/PKA signaling pathway, i.e. Ras2, Ira1, Ira2 and Pde2 seem to be highly relevant for a fast metabolic response upon glucose relief of starvation. Specifically, these components caused changes in the amplitude and timing of the metabolic response, but not in the sequence of metabolic events. Presumably this happened due to their importance in the maintenance of an adequate repression prior to glucose addition. In a second step we attempted to determine, where the pH-signal enters the Ras/PKA signaling pathway besides the well-known metabolic inputs of ATP and GTP. In collaboration with the research group of Prof. Stelling, we set up a mathematical model, which was parameterized with the novel approach of modular decomposition and in consideration of Ras knockout mutants. The model allowed us to narrow down the possible entry points of the pH-signal to the Ira and Pde proteins. However, the model could not predict the cAMP response of the Ira and Pde deletion mutants, probably due to the assumption of a wild-type like pH trajectory.
Zusammenfassung


In Kapitel 2 erkannten wir durch $^{13}$C Flussanalyse, dass die Spezies aufgrund ihrer intrazellulären Flussraten klar in die Gruppen von Crabtree-positivem und Crabtree-negativem Stoffwechsel unterteilt werden können, obwohl die extrazellulären Raten eine schrittweise Entwicklung des Stoffwechsels nahelegen. Das bedeutet ferner, dass der Redox-Stoffwechsel entweder von der Ethanol-Fermentation oder der Respiration abhängig ist. Zusätzlich haben wir das intrazelluläre Metabolom gemessen und fanden, dass intrazelluläre Metabolit-Konzentrationen artspezifisch zu sein scheinen, da nur Fructose-1,6-bisphosphat und Dihydroxyacton-phosphat stark mit der Glykolyse korrelierten. Daraus schlossen wir, dass die kinetischen Eigenschaften um diese zwei Metaboliten zwischen Hefen konserviert sind.


Der generelle Unterschied zwischen den Wachstumsprofilen bringt uns zu der Annahme, dass die vier Signalwege den Stoffwechsel auf eine bedingungsabhängige Art differenziert affektieren. Folglich benutzten wir Metabolomics in Kapitel 4, um den Einfluss der Signalwegekomponenten auf den zentralen Kohlenstoff-Stoffwechsel während Steady-

Chapter 1

General Introduction
1.1 Yeast metabolism

Due to their relevance in biotechnology [1, 2] and as a eukaryotic model organisms [3-5], yeasts have been the scope of investigation for decades. Nowadays, they are used for classical production of food and beverages, as well as for the production of proteins, fine chemicals, and small molecular weight compounds of industrial and medical relevance (Fig 1). Most of these applications take advantage of yeast’s metabolism, a network of more than 1000 reactions [6-8]. This network enables yeast to consume extracellular nutrients, to catabolize them, and to build precursors for biomass accumulation and proliferation. The core network, hence central metabolism and amino acid metabolism, consists of about 170 reactions [9] and is highly conserved [10-12]. The use of different reactions within this network depends on i) the yeast species and on ii) the environmental condition.

![Fig 1: An overview of applications of yeast in biotechnology](from Johnson et. al, 2013)

Species specific metabolism was mostly studied on glucose as the sole carbon source, since it is important for biotechnology and environment [13-16]. Specifically, aerobic glucose catabolism was classified into the groups of fermentative, respiro-fermentative, respiratory and obligate aerobic metabolism [14-16]. Respiro-fermentative yeasts, such as *Saccharomyces cerevisiae*, are characterized by the occurrence of the long-term Crabtree effect; *i.e.* they exhibit high glucose uptake rates that are mainly fermented to ethanol under...
aerobic glucose excess conditions. In contrast, respiratory and obligate respiratory yeasts, summarized as aerobic yeasts, exhibit low glucose uptake rates that are fully channeled into respiration without secretion of fermentation by-products. These differences of metabolic operation between species are mostly caused by evolutionary reasons. Specifically, Crabtree-positive metabolism evolved to a large extent by horizontal gene transfer and whole genome duplication [10, 14, 17-19], through which the possibility for anaerobic growth, and high glycolytic and fermentation rates were acquired. Whether or not evolution led to a gradual change from Crabtree-negative to –positive metabolism, however, is unclear.

The environment provides consumables like carbon sources, nitrogen sources and oxygen that enter metabolism at specific localities, but it might also expose yeast to stresses such as heat, oxidants, pH and osmotic concentrations. All these influences trigger condition-specific regulation [20-33] and alterations in metabolic fluxes, that were most detailed described in *S. cerevisiae* [34-39]. In particular, the quality of the carbon source was found to affect metabolism at the level of transcripts, proteins, metabolites and fluxes [9, 35, 40-42]. These effects are usually caused by carbon catabolite repression, hence glucose repression [31]. The quantitative effect of glucose repression on metabolism was recently exemplified during a classical diauxic shift [39]. On the preferred carbon source glucose, metabolism was characterized by a repression of the utilization of alternative substrates, the tricarboxylic acid (TCA) cycle, respiration and gluconeogenesis. Consequently, glucose was mainly fermented to ethanol and other by-products, eventually leading to a low biomass yield. However, the overall fast fermentative metabolism allowed a high specific growth rate. After glucose depletion yeast entered a lag phase, when it rearranged metabolism to grow on the before excreted ethanol. Specifically, during the lag phase ethanol utilization, respiration, TCA cycle and gluconeogenesis were ‘derepressed’, which enabled fully respiratory growth without by-product formation. The more efficient metabolism caused an increased yield compared to glucose, albeit at a lower growth rate. Most of these glucose repression effects are mediated by a signaling network that is introduced below.

1.2 Signaling pathways that are involved in glucose repression

In the context of different carbon sources and glucose repression, regulation is mainly achieved by an interconnected glucose signaling network that receives carbon source dependent signals and affects various cellular processes. This signaling network consists of about 60 components and is classically divided into four signaling pathways; i.e. the Ras/PKA pathway, the Snf1 pathway, the Snf3/Rgt2 pathway and the Hap complex. These signaling pathways jointly regulate metabolism at different levels, such as i) regulation of transcription and expression [43-48] ii) regulation of post-translational modifications [42, 49-51] and rather indirectly iii) regulation via intracellular metabolites such as fructose-2,6-bisphosphate inhibition of fructose-1,6-bisphosphatase [52], trehalose-6-phosphate inhibition
of Hxk2 [53, 54], or the dependency of many reactions on a proper NAD(P)$^+$/NAD(P)H ratio [55, 56].

**Basic structure of the four glucose signaling pathways**

The Ras/PKA signaling pathway is the main mediator of glucose repression, since 90% of the transcript changes upon glucose addition (about 2200 transcripts) are dependent on it [47, 48]. These targets include metabolism, ribosome biogenesis, cell cycle and stress response [43, 44, 47, 48]. Topologically the Ras/PKA signaling pathway has a glucose-responsive upstream network that modulates the activity of adenylate cyclase (Cyr1) for generation of the internal signal cAMP. This upstream network has two branches [30, 57-59]: i) the G-protein coupled glucose receptor Gpr1 together with Gpa2 and ii) the guanine nucleotide exchange factor Cdc25 and the GTPases Ras1 and Ras2. Both branches are connected via the inhibitory GTPase-activating components Ira1 and Ira2, but also directly to Cyr1 (Fig 3).

Once Cyr1 is activated by the upstream network, cAMP is generated and provokes the release of the binding between the downstream inhibitor Bcy1 and the main kinases of this signaling pathway (Tpk1, Tpk2 and Tpk3). In turn, these kinases regulate the various cellular functions. Additionally, the Tpk kinases negatively feed back to cAMP via the cAMP phospho-diesterases Pde1 and Pde2, which degrade cAMP to AMP, and to the upstream network by hyper-phosphorylating Cdc25 [60] (Fig 3). Thus, the normal response of cAMP to glucose addition is a transient peak before going back to a basal level.

Besides sensing external glucose through the G-protein coupled glucose receptor Gpr1, intracellular glucose metabolism is needed to fully activate Ras/PKA signaling. Specifically it was proposed, that besides the known substrates ATP and GTP, pH serves as a metabolism-dependent intracellular signal for the Ras/PKA pathway [61-63]. However, mechanistically it remains unclear, where such a signal would enter the signaling pathway.

With 260-400 genes, the Snf1 pathway has an intermediary spectrum of transcriptional targets [25, 30, 46, 48, 64] that include utilization of alternative carbon substrates, gluconeogenesis and fatty acid metabolism. At the core of the Snf1 pathway is the hetero-trimeric Snf1 kinase and its inhibitory Glc7-Reg1 protein phosphatase1, which regulate overall pathway activity in a carbon source dependent manner [65-67] (Fig 4). On alternative and respiratory substrates, Snf1 is phosphorylated by one of its three upstream kinases (Sak1, Tos3 or Elm1 [68]) and assembles to its active heterotrimeric form [64]. This trimer consists of the Snf1 kinase, the $\gamma$-subunit Snf4, and one of the redundant $\beta$-subunits Sip1, Sip2 or Gal83 [70], that regulate Snf1 kinase localization [71] (Fig 4). Specifically, Gal83 enhances nuclear localization and thus the regulation of several transcription factors, which includes the inactivation of Mig1 or the activation of Adr1, Cat8 and Sip4 [64, 72-74]. Furthermore, the Snf1 kinase seems to negatively regulate Glc7-Reg1 via association with Sip5, which interacts with both Snf1 kinase and Glc7-Reg1 [67], and subsequent
phosphorylation [66]. On glucose, Glc7-Reg1 dephosphorylates Snf1, which leads to an auto inhibitory state of Snf1 and its γ-subunit Snf4. Besides Glc7-Reg1, hexokinase 2 (Hxk2) is suggested to contribute to Snf1 inactivation either directly or via interaction with the transcription factor Mig1 [32, 75] (Fig 4). Similar to the Ras/PKA pathway, it is so far unclear, how the activating upstream kinases, Sak1, Tos3 and Elm1 receive signals or how the upstream inhibitor Glc7-Reg1 is controlled in a glucose-dependent manner.

The Snf3/Rgt2 pathway is generally thought to be the main regulator of hexose transport [30, 32, 33, 58], albeit it might also affect genes in TCA cycle, respiration and gluconeogenesis [76]. On glucose, the membrane spanning low- and high-affinity glucose sensors Snf3 and Rgt2 [77], respectively, activate the intracellular associated casein kinases Yck1 and Yck2 (Fig 5). These kinases further phosphorylate the transcriptional co-repressors of the main transcription factor Rgt1, Mth1 and Std1, which makes them susceptible to ubiquitinylation by SCF-Grr1 [78, 79] (Fig 5). Upon ubiquitinylation Mth1 and Std1 get degraded in the proteasome and Rgt1 relieves its repression of low affinity hexose transport.
Fig 4: Core structure of the Snf1 pathway. Black arrows indicate an activating interaction, flat arrows indicate an inhibiting interaction, open arrows indicate an enzymatic reaction, and the dotted arrow depicts Snf1 trimerization. The green plus indicates a positive feedback and question marks indicate that an interaction or a component is unknown. (For details, see text)

On alternative carbon substrates Rgt1 is associated with its co-repressors Mth1 and Std1 and represses expression of low affinity hexose transport. Lastly, the Hap complex is a transcriptional complex that mainly induces the expression of proteins involved in mitochondrial functions and respiration [25, 80, 81]. Specifically, Hap2, Hap3 and Hap5 build the DNA binding part of the complex and Hap4 seems to regulate its activity [82, 83]. Although it was shown that Hap4 expression is glucose-regulated, the input signal is still elusive [25].

Cross-talk between signaling pathways and regulatory overlaps

With the exploration of the genetic and physical interactions of the glucose signaling network components [69], and its transcriptional regulation [84], it became clear that the four glucose signaling pathways are not stand-alone modules, but rather constitute a highly interconnected network with extensive cross-talk (Fig 6). For example at the level transcriptional regulation it was shown that the transcription factors Mth1, Std1 and Rgt1 of the Snf3/Rgt2 pathway regulate the Snf1 pathway transcription factors and vice versa [45]. At the level of transcription factor phosphorylation it was shown that the Snf1 pathway and the Ras/PKA pathway could regulate the transcription factors Adr1 [72, 85-88], Msn2 [89], and
Rgt1 [90]. Within the layer of the signaling network components it was examined, that Glc7-Reg1 might be important for the activity of the casein kinases Yck1 and Yck2 from the Snf3/Rgt2 pathway [91], and that Sip1, a beta-subunit of Snf1, is PKA regulated [92]. Additionally it was suggested that Ras/PKA pathway regulates the Snf1 pathway via phosphorylation of upstream kinases and other targets [93].

**Fig 5: Core structure of the Snf3/Rgt2 pathway.** Black arrows indicate an activating interaction, dotted arrows indicate a change in localization or the state of a component, P depicts a phosphorylation and small ovals indicate protein degradation. (For details, see text)

Besides the crosstalk between the signaling pathways, it was also shown that many target genes might be redundantly regulated at the transcriptional level. Besides these transcriptomic screens, the redundant regulation of only a few enzymes was characterized in more detail [76, 87, 94-100]. For example it was shown that Reg1 and Grr1 are needed for cytosolic proteolysis of the gluconeogenic enzyme fructose-1,6-bisphosphatase [98, 100], and that the Ras/PKA pathway might play a role in its vacuolar degradation [99]. Besides
these degradation processes, all three signaling pathways might transcriptionally regulate fructose-1,6-bisphosphatase [48, 76].

Cross-talk, multilayered regulation, and unknown input signals, result in a complex network structure, where it is impossible to predict the contribution of a particular signaling component or pathway to a metabolic response. In order to elucidate when and how strong a signaling component or pathway contributes to quantitative metabolic regulation it is therefore inevitable to genetically perturb the signaling system, and to verify yeast’ physiology and metabolism at the level of growth, flux, and metabolites.

Fig 6: The components of the glucose signaling pathways build a highly intertwined network. The circles represent different glucose signaling network components of the four glucose signaling pathways (grey scale and sections) and the color around the circles (red, green, black) shows their assumed activity on glucose. The edges represent physical interactions (solid dark), genetic interactions (solid light) or transcriptional regulation (dotted) that were reported in SGD [69] or YeastRACT [84].

1.3 Quantitative readouts of growth and metabolism

The quantitative response of yeast physiology and metabolism can be assessed by several methods that deliver functional readouts at different resolution. The most broad readout of yeast’s fitness and physiology under particular conditions is given by its growth
profile [101]. From a growth profile during a nutrient shift one can determine different parameters. Specifically, the growth rate is an integrated readout of the function of all cellular processes, and thus generally reflects the fitness of a cell. The growth phase specific biomass yield is more related to metabolism, since it is a readout of metabolic efficiency; *i.e.* how much biomass is generated per unit of consumed substrate. Finally, the lag phase between two substrates serves as a general readout for the ability of a cell to adapt to new conditions. With the development of automated growth devices it is nowadays possible to online-monitor growth of cell-cultures under different conditions in a medium throughput. Although this makes a growth screen a suitable tool to investigate the quantitative impact upon genetic or environmental perturbation, a growth profile does not directly resolve metabolic effects.

More resolution about metabolic operation could be achieved by flux analysis [102, 103]. Specifically, fluxes are a readout of how much substrate is converted in a reaction per unit of time. Classically, $^{13}$C-substrates are added to cell cultures and resulting proteinogenic labeling patterns are used to estimate so-called flux ratios [104], hence the fate of carbon at key branching points within metabolism. These flux ratios could be further combined with physiological data and a stoichiometric reaction network to estimate an absolute flux distribution within a cell [105]. Whereas, the classic $^{13}$C-flux analysis is mostly restricted to central metabolism and steady state conditions, new approaches were developed where dynamic labeling data and metabolite concentrations from a small set of reactions are integrated to investigate fluxes in peripheral metabolism or in a dynamic environment [106-108]. However, since all these approaches for absolute flux determination need labeled substrates and tedious experiments to determine physiology or labeling trajectories, they are only suited for a small number of organisms or mutants at a time.

Another approach to explore functionality of metabolism is the measurement of intracellular metabolites, hence metabolomics, which delivers a readout of metabolism at the reaction-level [55, 109, 110]. Intracellular metabolites can be assessed in cell cultures, but also in single cells by a multitude of methods, that range from specific assays, such as resonance energy transfer (FRET) sensors [111], to mass spectrometry and NMR based methods that allow the simultaneous measurement of multiple metabolites [112-117]. These metabolomic readouts could potentially be used to find genetic interactions between different mutants by statistical analysis (reviewed in [118]), or to state hypothesis about their metabolic targets [110, 119]. Additionally, metabolite concentrations could be analyzed on the basis of metabolic network models, such as network-embedded thermodynamic analysis [120] or kinetic modeling [121-123], which might allow to elucidate the effect on metabolic operation.
1.4 Outline of the Thesis

In this thesis we quantitatively explore yeast physiology and metabolism dependent on species evolution and the glucose signaling network. Specifically, in Chapter 2 we investigate whether evolution led to gradual change in intracellular metabolic operation of Crabtree-positive and –negative yeast species, and whether metabolites correlate with flux in a species-overarching manner. Therefore we determined physiology, absolute flux distribution and intracellular metabolites of seven yeast species, that exhibit either respiro-fermentative or aerobic glucose catabolism.

In Chapter 3 and Chapter 4 we investigated the condition-dependent impact of the glucose signaling network in *S. cerevisiae* on growth and metabolism. Specifically, in Chapter 3 we asked which components of the glucose signaling network are required for growth under differential repressive conditions. Therefore we generated a knockout library including single and selected double knockouts, and recorded their growth profiles during nutrient shifts between differential repressive carbon substrates. From the obtained growth profiles and the derived growth parameters we could determine the key signaling mutants that quantitatively regulate growth and physiology under differential repressive conditions. Since, the impact on these macroscopic parameters indicated a vast re-routing of flux we asked in Chapter 4, which metabolic reactions might be affected by the different signaling pathways and its components. Therefore we determined the metabotypes of the knockout mutants of the glucose signaling components on the differential repressive substrates glucose, galactose and ethanol and could verify signaling-pathway specific targets within central carbon metabolism.

In Chapter 5 we more specifically investigated the function of the Ras/PKA pathway after glucose relief of repression. The activation of the Ras/PKA signaling pathway is dependent on metabolism. However, since it also regulates metabolism, a double-reciprocal feedback system exists. Therefore we here characterized, how the response to glucose relief of starvation is influenced by the Ras/PKA signaling pathway, and in turn, where a putative pH signal might enter the signaling pathway. We chose an interdisciplinary approach with the group of Prof. Jörg Stelling and combined dynamic metabolomics, pH measurements and mathematical modeling to characterize this feedback in several single knockout mutants of the Ras/PKA pathway.

In Chapter 6 we give a summary of additional projects that were done during this PhD, and with Chapter 7 we conclude this thesis by summarizing the main results and giving an outlook on future questions in this research area.
1.5 References


Chapter 2

Intracellular characterization of aerobic glucose metabolism in seven yeast species by $^{13}$C flux analysis and metabolomics

Stefan Christen$^{1,2}$, Uwe Sauer$^1$
2.1 Abstract

Key distinguishing characteristics of yeast glucose metabolism are the relative proportions of fermentation and respiration. Crabtree-positive yeast species exhibit a respirofermentative metabolism, whereas aerobic species respire fully without secretion of fermentation byproducts. Physiological data suggest a gradual transition in different species between these two states. Here, we investigate whether this gradual transition also occurs at the intracellular level by quantifying the intracellular metabolism of *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces exigus*, *Kluyveromyces thermostolerans*, *Yarrowia lipolytica*, *Pichia angusta* and *Candida rugosa* by $^{13}$C-flux analysis and metabolomics. Different from the extracellular physiology, the intracellular fluxes through the tricarboxylic acid cycle fall into two classes where the aerobic species exhibit much higher respiratory fluxes at otherwise similar glycolytic fluxes. More generally, we found the intracellular metabolite concentrations to be primarily species-specific. The sole exception of a metabolite-flux correlation in a species-overarching manner was found for fructose-1,6-bisphosphate and dihydroxyacetone-phosphate, indicating a conservation of the functional properties around these two metabolites.
2.2 Introduction

Although the reaction network topology of central carbon metabolism is conserved among different yeasts, magnitude and distribution of flux through these pathways varies among them (Blank et al. 2005; Cannizzaro et al. 2004; Fiaux et al. 2003; Flores et al. 2000). This is particularly true for aerobic catabolism of glucose, the preferred carbon source for yeast and many other organisms. Aerobic glucose catabolism is classified into the groups of fermentative, respiro-fermentative, respiratory and obligate aerobic metabolism (Pronk et al. 1996; van Dijken et al. 1993). Respiro-fermentative yeasts, such as *Saccharomyces cerevisiae*, are characterized by the occurrence of the long-term Crabtree effect (Zimmermann and Entian 1997); i.e. they exhibit high glucose uptake rates that are routed to ethanol under aerobic, glucose excess conditions. In contrast, respiratory and obligate respiratory yeasts, summarized as aerobic yeasts, exhibit low glucose uptake rates that are fully channeled into respiration without secretion of fermentation by-products.

These metabolic states evolved to a large extent by horizontal gene transfer and whole genome duplication, through which metabolism acquired the ability for anaerobic growth, including high glycolytic and fermentation rates in Crabtree-positive yeasts (Conant and Wolfe 2007; Merico et al. 2007; Piskur and Langkjaer 2004; Seoighe and Wolfe 1999; van Hoek and Hogeweg 2009). In present day yeasts, metabolism is subject to additional levels of regulation. These include glucose repression of the tricarboxylic acid cycle and respiration (De Deken 1966; Gancedo 2008) and overflow metabolism at the pyruvate decarboxylase branch point due to its high capacity and limited flux through pyruvate dehydrogenase (Pronk et al. 1996; van Urk et al. 1989). Additionally different metabolites with regulatory roles affect respiration and other cellular functions. This regulation might either occur directly, via the redox or energy state of the cell, or via glucose signaling (Diaz-Ruiz et al. 2008; Gancedo 2008; Muller et al. 1995; Tisi et al. 2002; Vemuri et al. 2007).

Here we ask whether evolution of metabolism and its regulation led to a gradual transition between Crabtree-positive and aerobic steady-state metabolism in modern yeast. Physiological studies of *S. cerevisiae* and other yeasts revealed a gradual transition from respiratory to fermentative metabolism with increasing glucose uptake rates (Blank et al. 2005; van Urk et al. 1989), which would suggest the existence of intermediate metabolic states. At present it remains unclear whether the intracellular distribution of fluxes reflects such a gradual transition between respiro-fermentative and fully respiratory, aerobic states. Additionally, we ask whether absolute metabolite concentrations are species-specific or rather correlate with fluxes in a species-overarching manner, as one might conclude from the good flux correlations of glycolytic intermediate concentrations in *S. cerevisiae* under different conditions (Bosch et al. 2008; Elbing et al. 2004; Kresnowati et al. 2008a; Kresnowati et al. 2008b; Tai et al. 2007). Furthermore, it was shown qualitatively that the metabolite levels in different organisms respond similarly to environmental perturbations.
(Brauer et al. 2006). Whether or not such circumstantial observations are more generally true is investigated here.

To address these questions we chose seven yeast species, including *S. cerevisiae*, the biotechnological relevant *Yarrowia lipolytica* (Abbott et al. 2009; Beopoulos et al. 2009; Gellissen et al. 2005; Vakhlu and Kour 2006), and the less characterized biotechnologically emerging *Candida rugosa* (Dominguez de Maria et al. 2006; Lee and Park 2009). The other four species were chosen for a gradual coverage of extracellular rates between the Crabtree-positive *S. cerevisiae* and the obligate aerobic *Y. lipolytica* (Blank et al. 2005; Merico et al. 2007). Specifically, we quantified their glucose excess metabolism in batch culture by $^{13}$C flux analysis (Sauer 2006) and metabolomics (Roessner and Bowne 2009).
2.3 Results

**Physiology and growth**

To verify presence and to quantify the degree of the respiro-fermentative metabolism in the seven selected yeast species, we characterized their physiology by determining specific growth, uptake and secretion rates in aerobic batch cultures with 10 g/L glucose (Table 2). The seven species covered a wide range of glucose uptake and ethanol secretion rates. As expected, the Crabtree-positive species *S. cerevisiae, S. bayanus, S. exiguus* and *K. thermotolerans* exhibit high glucose uptake rates between 6.3 and 15.4 mmol g\(^{-1}\) h\(^{-1}\), coupled with ethanol secretion and to a minor extent with acetate and glycerol secretion. The aerobic species *Pichia angusta* and *Y. lipolytica* exhibited much lower glucose uptake rates, ranging from 2.8 to 4.4 mmoles g\(^{-1}\) h\(^{-1}\), without detectable secretion of any metabolic by-products. Consequently, the aerobic species grew more efficiently with a yield of at least 0.5 g\(^{\text{biomass}}\)/g\(^{\text{glucose}}\), whereas the Crabtree-positive species achieved yields between 0.13 and 0.27 g\(^{\text{biomass}}\)/g\(^{\text{glucose}}\) during exponential growth on glucose. Despite their slower overall metabolism, the aerobic species *P. angusta, Y. lipolytica* and *C. rugosa* grew faster than the Crabtree-positive species.

**Intracellular flux distribution**

Given the differences in macroscopic physiological rates, we ask whether the intracellular distribution of fluxes relied on the same pathways within each group of aerobic glucose metabolism. For this purpose, separate isotopic tracer experiments with [U\(^{\text{13}}\)C] and [1\(^{\text{13}}\)C]-labelled glucose were performed in at least duplicate batch cultures for each case. Specifically, we determined the mass isotopomer distributions of proteinogenic amino acids to calculate ratios of converging fluxes at key branch points in central metabolism (Blank et al. 2005; Zamboni et al. 2009). The largest variability between species was in the pentose phosphate pathway and the tricarboxylic acid (TCA) cycle, indicated by large differences in the split ratios *serine derived through glycolysis* and *mitochondrial oxaloacetate originating from anaplerosis*, respectively (Fig 1A).

For a more detailed insight, we estimated network-wide absolute fluxes by using these intracellular flux ratios (Fig 1A) and secretion rates (Table 2) as input values for net flux analysis using the FiatFlux software (Zamboni et al. 2009; Zamboni et al. 2005). Specifically, this method estimates the best flux distribution by iteratively fitting intracellular fluxes to the experimental data within a model of yeast metabolism (Appendix). These absolute flux values clearly differed between Crabtree-positive and negative metabolism (Fig 1B). In the
<table>
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<th>Species</th>
<th>Growth rate (h⁻¹)</th>
<th>Glucose uptake (mmol g⁻¹ h⁻¹)</th>
<th>Glycerol secretion (mmol g⁻¹ h⁻¹)</th>
<th>Acetate secretion (mmol g⁻¹ h⁻¹)</th>
<th>Ethanol secretion (mmol g⁻¹ h⁻¹)</th>
<th>Yield (g g⁻¹)</th>
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<td>P. angusta</td>
<td>0.42 ± 0.05</td>
<td>4.4 ± 0.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td>C. rugosa</td>
<td>0.45 ± 0.09</td>
<td>2.8 ± 0.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.79 ± 0.02</td>
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Table 2. Growth parameters of the different yeast species in glucose minimal medium batch.
Figure 1: Flux distribution in 7 yeast species on glucose minimal medium. (A) Ratios of converging fluxes at branch points of central carbon metabolism. The ratios are given by the division of the flux indicated by the solid line over the flux indicated by the dotted line (B) The quantitative flux distribution of Crabtree positive (left) and aerobic (right) yeasts in mmol g\(^{-1}\) h\(^{-1}\). The thickness of the arrows indicates the glucose uptake normalized flux distribution with a variability that is depicted in gray.
Crabtree-positive species, glucose is almost exclusively catabolized through glycolysis and finally to the fermentative product ethanol, while the respiratory TCA cycle flux was negligible. This single pathway metabolism was essentially independent of the overall flux rate that increased about 3-fold from *K. thermotolerans* to *S. cerevisiae* (Fig 1B). In aerobic species, in contrast, the pentose phosphate pathway and TCA cycle became major pathways that equal the glycolytic flux. However, since the overall flux was much lower in the aerobic species, the pentose and NADPH-producing pentose phosphate pathway remained largely constant in all species at 1 to 1.7 mmol g$^{-1}$ h$^{-1}$ and contributed between 50% and 80% to the overall NADPH required for biomass production. Since we considered only the NAD-dependent isoform of the isocitrate dehydrogenase to be active, we could not further distinguish the isocitrate dehydrogenase or acetaldehyde dehydrogenase contribution to NADPH regeneration.

**Figure 2: Interrelationship between fluxes of central carbon metabolism in Crabtree positive (●), aerobic (○) species, and *S. cerevisiae* under Galactose and Pyruvate conditions (+).** The fitted values from the net flux analysis were used. (A) Correlations to Glucose uptake and Glycolysis. (B) Correlations to yield. (C) Correlations to PP pathway.
Figure 3: Intracellular metabolite concentrations of 7 yeast species. CR = C. rugosa, PA = P. angusta, YL = Y. lipolytica, KT = K. thermotolerans, SE = S. exiguus, SB = S. bayanus, SC = S. cerevisiae. The species are sorted according to their glucose uptake rate, hence the Crabtree effect. The concentrations are given in nmoles/mg or arbitrary units (AU).
To elucidate whether there was a gradual shift between respiratory and fermentative metabolism, we correlated key fluxes from all species. We also included previously published *S. cerevisiae* fluxes during growth on the respiratory substrates galactose and pyruvate (Fendt and Sauer 2010). On these substrates, the glycolytic flux was low and the TCA cycle flux high, representing an in between state of metabolism. Consistent with their role as the key pathways, substrate uptake rate and yield correlated both with glycolytic flux and ethanol secretion, independently of whether respiratory metabolism was triggered genetically or environmentally (Fig 2 A). Including the data of *S. cerevisiae* on galactose and pyruvate further underlines the key role of the pentose phosphate pathway in supplying the anabolic cofactor NADPH for biomass formation in yeasts, which differs from its primarily catabolic role in some bacteria (Fischer and Sauer 2005). These monotonous changes in various fluxes across genetic and environmentally changes suggest similar underlying regulatory mechanisms. A similarly good correlation was found between the substrate uptake rate or glycolytic flux with the respiratory TCA cycle flux among all Crabtree-positive yeast on glucose and *S. cerevisiae* on respiratory substrates. The aerobic yeasts, however, fall completely out of these correlations; *i.e.* exhibited several-fold higher TCA cycle fluxes than would be expected for the same glycolytic flux in galactose-grown *S. cerevisiae* (Fig 2 C). This suggests a different mode of regulation in the aerobic species relative to the environmentally triggered regulation of respiratory metabolism in *S. cerevisiae*. Generally, the increased TCA cycle flux of the Crabtree-positive yeasts is a function of higher biomass yield as their metabolism becomes more efficient with reduced ethanol secretion. In aerobic species, in contrast, the biomass yield is not increased any further.

![Figure 4: Fractionation of the measured metabolome into the different metabolites.](image)

The amino acids make up 90% of the total metabolite concentrations measured. Nevertheless the portioning of this pool between the different amino acids is quite diverse.
Relations between fluxes and metabolites

Finally, we asked whether intracellular metabolite concentrations are species-specific or generally related to the flux through their pathway. For this purpose, we quantified 26 intermediates of central metabolism plus 10 amino acids by LC-MS/MS (Buscher et al. 2009) from mid-exponential growing aerobic batch cultures of the seven species (Fig 3). Generally our metabolite concentrations were in good agreement with published data (Bolten and Wittmann 2008; Canelas et al. 2009; Fendt et al. 2010). The sole exception were systematically higher AMP concentrations in the Crabtree-positive strains that resulted in slightly lower energy charges of about 0.7 compared to 0.9 in the other yeasts (Wiebe and Bancroft 1975). Our data do not allow differentiating whether this difference was biological or a putative artefact from imperfect quenching by cold methanol. None of the species featured systematically lower or higher concentrations than the others, indicating a comparable quality of extraction by the employed hot ethanol procedure. Generally, the intracellular pool of amino acids accounted for about 90% of all determined metabolite concentrations with glutamate having concentrations up to 150 nmoles/mg (Fig 4). In Y. lipolytica and C. rugosa, glutamate was the dominating metabolite, accounting for about 50% of the total determined metabolite pool. In the two Saccharomyces species S. bayanus and S. cerevisiae, three amino acids contributed each about one quarter to the total pool; i.e. glutamate, glutamine and alanine or arginine (Fig 4). All other concentrations varied significantly between species at much lower concentrations between 0.02 and about 10 nmoles/mg (Fig 4).

To assess the relationship of intracellular metabolite concentrations to fluxes or species, we correlated the determined pathway fluxes with all metabolite concentrations (Fig 5). There was clearly no general correlation between metabolite levels and fluxes in a species-overarching manner, hence metabolite levels are rather species-specific. The exception were the good correlations of fructose-1,6-bisphosphate and dihydroxyacetone phosphate with glycolysis and ethanol secretion ($R^2>0.9; p<10^{-9}$). Since both metabolites span a relatively large range of concentrations of about one order of magnitude in either case, they could potentially function as general glycolytic flux indicators, a mechanism that then would be conserved across species.
Figure 5: Correlation between fluxes and metabolites in 7 yeast species. In the heat map the correlation coefficients ($R^2$) between all metabolites (left) and the representative fluxes (top) are illustrated. Fructose-1,6-bisphosphate and dihydroxyacetone-phosphate showed a high correlation with glycolysis and ethanol secretion in a species-overarching manner (right).
2.4 Discussion

We quantified metabolic fluxes in seven yeast species with different strengths of the Crabtree effect by $^{13}$C flux analysis and metabolomics. For six species the physiological metabolic states of aerobic glucose metabolism were in agreement with published data (Blank et al. 2005; Fendt and Sauer 2010; Merico et al. 2007). As expected, decreasing glucose uptake rates concur with gradually decreasing ethanol secretion until complete absence of fermentation. Despite this gradual physiological transition, the corresponding intracellular flux distributions fall into two clearly different modes of Crabtree-positive and aerobic metabolism. Specifically, the aerobic species featured about 3-fold higher respiratory TCA cycle fluxes than respiring Crabtree-positive *S. cerevisiae* at otherwise equal rates of overall metabolism on galactose and pyruvate. This difference in respiratory rates at equal glycolytic flux are potentially explained by the stronger repression of the TCA cycle and respiration, with concomitant activation of overflow metabolism at the pyruvate decarboxylase in the Crabtree-positive species (Gancedo 2008; Pronk et al. 1996; Zaman et al. 2008). In this case the fermentation route is the only possibility to reoxidize NADH, hence to maintain redox homeostasis. For the aerobic species *K. lactis* and *P. stipitis*, in contrast, it was shown that glucose repression of respiratory genes is absent and overflow metabolism at the pyruvate decarboxylase branch point is negatively regulated in an oxygen-dependent manner (Kiers et al. 1998; Passoth et al. 1996). Thus, aerobic species channel their glycolytic flux into the TCA cycle for complete oxidation and NAD$^+$ regeneration via respiration (Snoek and Steensma 2007). Interestingly, aerobic species rely primarily on energy-dependent glucose transport (van Dijken et al. 1993; van Urk et al. 1989), which might further require an energy-efficient mode of metabolism.

Previous studies with *S. cerevisiae* revealed glycolytic intermediate concentrations to vary with differentially repressive substrates and thus with glycolytic fluxes (Bosch et al. 2008; Elbing et al. 2004). If this was indeed a general feature of glycolysis, one would expect a correlation between flux and the according pathway intermediate concentrations across all species. Here, we show that intracellular metabolites do not generally correlate with flux and are therefore species-specific. Most striking, up to 90% of the total metabolite pool was made up of amino acids. Different to *Escherichia coli* (Bennett et al. 2009), the total amino acid pool in yeast species was not only dominated by glutamate but rather by a combination of glutamate, glutamine, arginine, alanine and aspartate. The large variability of other metabolite concentrations was not correlated with fluxes, which indicates diverse kinetic properties and expression levels of enzymes in the different yeast species. The exception were the good correlations between fructose-1,6-bisphosphate and dihydroxyacetone-phosphate concentrations with glycolytic flux, which indicates that the functional properties of enzymes around these two metabolites are conserved among the different species. Since fructose-1,6-bisphosphate inhibition of the respiratory complex III in Crabtree-positive
species was proposed earlier (Diaz-Ruiz et al. 2008), it is tempting to speculate that this inhibition causes the negative correlation between the TCA cycle and glycolytic flux. The almost equally good correlation of dihydroxyacetone-phosphate might then be explained by the near equilibrium with fructose-1,6-bisphosphate via the aldolase.
2.5 Material and Methods

**Strains medium and cultivation conditions**

The investigated yeasts are listed in Table 1. All liquid cultivations were carried out in minimal medium with 10g/L glucose (Blank and Sauer 2004). After pre-cultivation overnight in glucose minimal medium, 25-50 ml cultures were inoculated to a starting optical density at 600 nm (OD$_{600}$) of about 0.05 and grown in 500 ml shake flasks at 30°C and 250 r.p.m. Aliquots were withdrawn during the exponential growth phase on glucose. For flux analysis experiments, natural abundance glucose was replaced by either 100% of the $^{13}$C glucose or a mixture of 20% of the U-$^{13}$C isotopologue and 80% natural abundance glucose ($^{13}$C-enrichment $\geq$ 99%, Cambridge Isotope Laboratories, Andover, USA).

<table>
<thead>
<tr>
<th>Species</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tr>
<td><em>S. cerevisiae</em></td>
<td>FY4 Mata – wild type</td>
<td>Winston et al. 1994</td>
</tr>
<tr>
<td><em>S. bayanus var. uvarum</em></td>
<td>wild type</td>
<td>CLIB 251$^a$</td>
</tr>
<tr>
<td><em>S. exiguus</em></td>
<td>wild type</td>
<td>CLIB 179$^a$</td>
</tr>
<tr>
<td><em>K. thermotolerans</em></td>
<td>wild type</td>
<td>CLIB 292$^a$</td>
</tr>
<tr>
<td><em>Y. lipolytica</em></td>
<td>wild type</td>
<td>H222</td>
</tr>
<tr>
<td><em>P. angusta</em></td>
<td>wild type</td>
<td>CLIB 421$^a$</td>
</tr>
<tr>
<td><em>C. rugosa</em></td>
<td>wild type</td>
<td>IFO 0750</td>
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</table>

Table 1. Yeast species used in this study $^a$ CLIB: Collection de levures d'intérêt biotechnologique (http://www.inra.fr/Internet/Produits/clib/) – (2)

**Biomass and extracellular metabolite concentrations**

Biomass concentrations were determined by recording OD$_{600}$ with a spectrophotometer (Novaspec II, Pharmacia Biotech, Uppsala, Sweden). For each species, we determined mass to OD$_{600}$ conversion factors by determining cellular dry weight from 5-10 ml filtrate with pre-dried and pre-weighted membranes (0.45 µM, Sartorius, Goettingen, DE), followed by three wash steps with 4°C ddH$_2$O. These membranes were dried overnight at 85°C and the weight difference was measured.

Extracellular metabolite concentrations were determined with an HPX-87C Aminex, ion-exclusion column (Biorad, Munich, Germany) as described in (Heer and Sauer 2008) on an HPLC HP1100 system (Agilent Technologies, Santa Clara, USA). The column temperature was 60°C and a flow rate of 0.6 ml/min of 5 mM H$_2$SO$_4$ as the eluant was used.

Biomass yields were obtained from a linear fit of substrate or by-product concentrations during exponential growth as a function of corresponding biomass concentrations. Multiplication with the growth rate then yielded specific glucose uptake and by-product secretion rates. The physiological parameters were determined from at least two independent biological replicates.
**13C-flux analysis**

For labelling experiments at least two replicate cultures were inoculated to an OD$_{600}$ of 0.05 or less. During sampling, 1 ml of culture was harvested during exponential growth followed by two wash steps with ddH$_2$O and stored at -20°C for further analysis. The processing for GC-MS analysis was done as described previously (Zamboni et al. 2009). The pellets were hydrolyzed with 6M HCl over night at 105°C and then dried at 95°C under a constant air stream. We dissolved the hydrolysates in 30 µl of the solvent DMF (Sigma-Aldrich, Buchs, Switzerland) and added 30 µl of the derivatization agent N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide with 1% tert-butyldimethyl-chlorosilane (Sigma-Aldrich, Buchs, Switzerland). Upon incubation at 85°C for 1 h, mass isotopomer distributions of the protein-bound amino acids were determined on a 6890N GC system (Agilent Technologies, Santa Clara, USA) combined with a 5875 Inert XL MS system (Agilent Technologies, Santa Clara, USA).

The mass isotopomer distribution of the amino acid fragments was corrected for the amount of naturally occurring stable isotopes and unlabeled biomass. From the corrected mass isotopomer distribution of the amino acids, ratios of converging fluxes were calculated with the analytical equations described in (Blank and Sauer 2004; Zamboni et al. 2005). These ratios together with the extracellular fluxes and a general stoichiometric network were then used as constraints for the netto subprogram of the FiatFlux software to iteratively identify an absolute flux solution that best described the data (Zamboni et al. 2009; Zamboni et al. 2005).

**Intracellular metabolite concentrations**

For rapid quenching of metabolism (Buscher et al. 2009; Ewald et al. 2009) a 1 ml culture aliquot was transferred to 4 ml -40°C 60% methanol, 10 mM ammonium acetate (pH 7.5) within 10 seconds. The quenching was followed by 3 min centrifugation with a swing-out rotor at 4500 g and -9°C (Centrifuge 5804R, Eppendorf, Germany). Pellets were stored at -80°C until extraction. The extraction was done in 80°C 75% boiling ethanol, 10mM ammonium acetate (pH 7.5). At this step 100 µl fully labelled $^{13}$C-biomass was added as an internal standard (Wu et al. 2005). The extracts were dried using a vacuum centrifuge (Christ-RVC 2-33 CD plus, Kuehner AG, Birsfeld, Switzerland). The dried extracts were dissolved in 50-100 µl ddH$_2$O before being separated by ion pairing-reverse phase liquid chromatography coupled to a ultra-high performance system.

We used a Waters Acquity UPLC (Waters Corporation, Milford, MA, USA) with a Waters Acquity T3 end-capped reverse phase column with dimensions 150mm x 2.1 mm x 1.8 µm (Waters Corporation) for metabolite separation as described in detail in (Buscher et al. 2009). The chromatography was coupled to a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a heated electrospray ionization source (Thermo Fisher Scientific) in negative mode with multiple
reaction monitoring. Acquisition and peak integration was done with a in-house software (Begemann and Zamboni, unpublished) and the peak areas were further normalized to fully $^{13}$C-labeled internal standards and the amount of biomass.

The metabolome of all species was measured with at least four separately quenched replicates. To exclude artifacts, we performed an outlier-detection on the raw data. With four data-points the outlier detection discards the data point furthest away from the mean. With more data points we calculated the 0.2 and 0.8 quantiles. From these data points we determined the mean and the standard deviation. The points being higher/ lower than the average $\pm 2$ times the standard deviation were discarded.
2.6 References


### 2.7 Acknowledgements

We thank the Degussa AG and the Swiss initiative for systems biology (SystemsX.ch) project YeastX for financial support.
2.8 Appendix

Metabolic network for flux analysis:

Reactions:
glc uptake:glucose + ATP > G6P
v1:G6P = F6P
v2:G6P > P5P + 2 * NADPH + CO2
v3:F6P + ATP > 2 * T3P
v4:2*P5P = S7P + T3P
v5:P5P + E4P = F6P + T3P
v6:S7P + T3P = E4P + F6P
v7:T3P > SER + NADH
v8:SER + NADH > GLY + C1
v9:GLY + C1 > SER + NADH
v10:C1 + CO2 + NADH = GLY
v11:T3P = PEP + ATP + NADH
v12:PEP > cPYR + ATP
v13:mPYR > mAcCoA + CO2+ NADH
v14:mOAA + mAcCoA > CIT
v15:CIT > OGA + CO2+ NADH
v16:OGA > SUCC + CO2 + 0.5*ATP + NADH
v17:SUCC = FUM + NADH
v18:MAL = mOAA + NADH
v19:FUM = MAL
v20:MAL > mPYR + CO2 + NADPH
v21:cOAA + ATP > PEP + CO2
v22:cPYR + CO2 + ATP > cOAA
v23:acetate + 2 * ATP > cAcCoA
v24:acetaldehyde = acetate + NADPH
v25:acetaldehyde + NADH = ethanol
v26:T3P + NADH = glycerol
v27:cOAA > mOAA
v28:mOAA > cOAA
v29:cAcCoA = mAcCoA
v30:cPYR > mPYR
v31:O2 + 2*NADH > 2*PO*ATP
v32:cPYR > acetaldehyde + CO2
Biomass requirements are expressed in µmol(Precursor)/gCDW.
Chapter 3

Impact of glucose signaling on *Saccharomyces cerevisae* physiology under differential repressive conditions

Stefan Christen, Uwe Sauer
3.1 Abstract

In *Saccharomyces cerevisiae*, the cellular response to available carbon sources is relayed by a signaling network arranged into the Ras/PKA pathway, the Snf1 pathway, the Snf3/Rgt2 pathway and the Hap complex. Although many putative targets of these signaling pathways are known, the condition-dependent relevance of a particular signaling pathway for growth and physiology is unclear. Here we address this question by conducting growth profiles of 63 deletion mutants within the signaling pathways during nutrient shifts between the differential repressive carbon substrates glucose, galactose and ethanol. Specifically, we determined the parameters of growth rate, phase specific yield and lag phase between the carbon sources. From the affectedness of the growth parameters we could identify key signaling components, which interrupt or constitutively activate a signaling pathway, i.e. deletions of SNF1 and SNF4 interrupt and HEX2 constitutively activates the Snf1 pathway, deletion of GRR1 interrupts the Snf3/Rgt2 pathway, the RAS1 RAS2 double knockout interrupts and deletion of BCY1 constitutively activates the Ras/PKA pathway, and deletions of each component of the Hap complex interrupts its activity. Their specific influence on growth and physiology implied that well-balanced Ras/PKA pathway and Snf1 pathway activities are required under all conditions, whereas the Hap complex and the Snf3/Rgt2 pathway were needed under respiratory conditions and on glucose, respectively. Moreover, we could verify the redundant roles of Hxk2 and the Ras/PKA pathway in glucose repression.
3.2 Introduction

All cells are able to recognize environmental signals and translate them into cellular responses through a multitude of signaling pathways that most prominently consist of kinases, phosphatases, g proteins, ubiquitin ligases and transcription factors [1-4]. These signaling pathways are not isolated modules but constitute an intertwined network with extensive crosstalk. It is therefore generally not possible to explain a given cellular response such as in cell cycle, stress response or in metabolism by a single pathway [1].

Due to its relevance in environment, nutrition and medicine, the impact of glucose on cellular metabolism has received a long standing attention [5]. In the model organism *Saccharomyces cerevisiae* [6], signaling pathways are known to either directly or indirectly respond to extracellular glucose and to modulate a number of cellular processes that include among others metabolism, ribosome biogenesis and stress response [1, 2]. This regulation is generally known as glucose repression and the involved signaling pathways are made up by about 60 highly interconnected components [7-11] (Fig. 1A) that are classically arranged into the Ras/PKA, Snf1, Rgt2/Snf3 pathways and the Hap complex (Fig 1B) [1-3]. With the exception of the Hap complex, each pathway has both activating and inhibiting components, which can modulate total pathway activity in a condition-dependent manner. The Ras/PKA signaling pathway is the main repressing pathway with the largest number of targets, since about 90% of all transcript changes (~2200 genes) during a shift between differentially repressive carbon sources can be assigned to this pathway [12-15]. Its main targets are in metabolism and ribosome biogenesis. The role of the Snf1 pathway lies in derepression and it has an intermediate number of targets (260 - 400 genes) that include gluconeogenesis and metabolism of carbon substrates other than glucose [14, 16-18]. The transcriptional Hap complex and the Snf3/Rgt2 pathway are more specifically acting on mitochondrial respiration and hexose transport, respectively [14, 17, 19, 20].

Although the putative network structure and potential targets are known, it remains unclear in how far the four pathways work together in controlling a given function. Here we ask how the condition-dependent information flows through the four glucose signaling pathways and controls growth and physiology. For this purpose we made 51 single deletion mutants within the signaling pathways and quantified the physiological consequences under dynamic transitions between different states of glucose repression. Specifically, we recorded growth profiles from which we analyzed the parameters of growth rate, phase-specific yield and duration of the lag phase. These parameters serve as readout of general fitness, metabolic efficiency and ability to adapt, respectively.
Chapter 3

Figure 1: Glucose signaling network is classically divided into four signaling pathways. A) The glucose signaling pathways (sections) and their components (nodes) build a intertwined network of physical interactions [8], genetic interactions [8] and transcriptional regulation [11]. The circle color indicates, whether a particular component is thought to be active (red) or inactive (green) on glucose [1-4], and black circles indicate, that its substrate-specific activity is unknown. B) According to [1-4] the components of signaling network (circles) are topologically divided into The Snf1 pathway, Ras/PKA pathway, Snf3/Rgt2 pathway and the Hap complex (top), which may have a transmembrane receptors (circle on dotted line), a cytosolic signaling network (light grey) and a transcriptional layer in the nucleus (dark grey). The main signaling components are tagged with their standard name [8] and an asterisk (*) indicates whether their deletion is thought to be lethal. The numbers below the transcriptional layer indicate how many genes are transcriptionally regulated by the signaling pathways [12-16, 19] and the boxes describe the putative targets of the signaling pathways according to [1-4].
3.3 Results

*Characterization of growth profiles during differential repressive nutrient shifts*

For later testing of signaling mutants, we selected three so-called diauxic growth transitions, where a preferred carbon source that is consumed in the first phase represses the utilization of one or more alternative substrates [18, 21-24]. Specifically we analyzed shifts between glucose, galactose and ethanol. In *S. cerevisiae*, extracellular glucose leads to a fast-growing repressed state, where glycolysis is highly active and gluconeogenesis, tricarboxylic acid (TCA) cycle and respiration are repressed. Consequently glucose is mainly converted to ethanol and other by-products and thus the biomass yield is low [25, 26]. On ethanol in contrast, *S. cerevisiae* is in a derepressed respiratory state that is manifested by activation of TCA cycle and gluconeogenesis without by-product formation. Although this respiratory metabolism is more efficient in terms of energy-production and biomass yield, overall metabolism and growth rate are slow compared to glucose. Galactose represents an intermediary repressing condition with simultaneous fermentative and respiratory metabolism.

As expected, all nutrient shifts lead to a diauxic growth profile, where glucose is always consumed first and ethanol last (Fig 2 A). Independent of the culture history, *S. cerevisiae* displayed highly reproducible maximal specific growth rates on a given substrate (Fig 2B). Consistent with glucose repression, growth rate is highest on glucose (0.35 h⁻¹), followed by galactose (0.26 h⁻¹) and ethanol (0.07 h⁻¹) (Fig 2 B). Expectedly, the longest lag phase of 7.27 ± 1.03 h was detected between the metabolically least related conditions of glucose and ethanol growth, which represent glucose repressed and derepressed states, respectively. The lag phase between the intermediary repressing galactose and ethanol was 4.53 ± 0.18 h and shortest from glucose to galactose at 2.53 ± 0.58 h. The energetically more efficient respiratory state on galactose compared to glucose was further confirmed by the higher yield on galactose.

*The dependency of the growth phenotype on the glucose signaling network*

To quantify the relevance of the glucose signaling pathways and their components under dynamically changing conditions, we constructed 51 prototrophic single deletion mutants within the four signaling pathways. Under the three diauxic shift conditions we characterized these mutants by the number and strength of their physiological effects on growth rate, biomass yield and length of lag phases (Fig 3).

As expected [1, 2, 16], deletion of the central activating and inhibiting components Snf1, Snf4 and Hex2 in the Snf1 pathway resulted in the most severe phenotypes, affecting
Figure 2: Growth profiles and derived parameters in wild type *S. cerevisiae*. **A)** Each nutrient shift condition, i.e. from glucose to ethanol or galactose and from galactose to ethanol (top), led to a diauxic growth profile that is characterized by two growth phases (blue) and lag phase (red) between them. The growth profile further allowed us to determine the depicted biomass yields in particular growth phases (black arrow, dotted line). The relative biomass accumulation was measured with a plate reader delivering a relative biomass accumulation (scattering signal arbitrary units, [AU]) over time. **B)** The parameters of absolute growth rate (blue), absolute lag duration (red) and relative biomass yield (arbitrary units [AU], black) were determined for the single growth phases during different nutrient transitions (axis). Glc: Glucose; Gal: Galactose; EtOH: Ethanol. '-' specifies the particular nutrient shift experiment.
Figure 3: Effect of single deletions within the glucose signaling network on growth parameters during different nutrient transitions. The heat map illustrates the relative effect of each glucose-activated and — inactivated component (left, black/ white/ grey) of the four glucose signaling pathways (left) on determined growth parameters during the three nutrient shift experiments (top). The bar chart on the right indicates the impact of each mutant and the grey area depicts a cutoff of the number of effects (< 6 effects). Components, whose single deletion had a high impact (green bar: > 6 effects and > 60% these cause a fold change of >25%) were further classified as key signaling nodes. Glc/glc: Glucose, Gal/gal: Galactose, EtOH/etoh: Ethanol, μ: growth rate.
more than 10 parameters (60% led to a change of > 25%) compared to wild type. Thus we can confirm their essential role for information flow through the Snf1 pathway. Only few additional deletions within this pathway were effective, such as the deletion mutant of CAT8, a Snf1 activated transcription factor that did not grow on ethanol and deletion of ELM1, a Snf1 activating kinase that mildly affected most parameters determined. The much lower impact of deleting these genes indicates that their gene products have a more modulatory role in this pathway. Within the Ras/PKA pathway, the deletions of the inhibitors Ira2 and Bcy1 had a high impact; i.e. more than 10 parameters changed and in more than 60% of the cases more than 25%. This was expected because both deletions are main inhibitors of the up- and down-stream network of this pathway, respectively [1, 2, 27, 28]. Unexpectedly, deletions of the activators (Ras1, Ras2 and Gpr1) and the PKA kinases themselves only resulted in mild effects, although 90% of the glucose signal reaching the transcriptional level was proposed to be dependent on Ras2 [13, 14]. This suggests that they are functionally redundant in the regulation of Ras/PKA pathway activity under the conditions measured. In the glucose-activated Snf3/Rgt2 pathway, the main signal receiver Grr1 expectedly affected 10 growth parameters (60% led to a change of > 25%) [29-31]. On the other side, deletion of the Grr1-inactivated components of this pathway, such as of the central transcription factor Rgt1, only caused mild effects. Also here, the absence of an effect suggests explained by redundancy within the signaling network or with other pathways[32]. As expected [19, 25, 33], each deletion mutant of the Hap complex resulted in the same growth profile, which is characterized by strong effects on at least 10 parameters.

**Key signaling components render yeast more repressed or derepressed**

Having verified the key components for each signaling pathway, we next attempted to unravel their conditional relevance. Specifically, we assumed that the strongest effects exerted by any of the signaling components of a pathway is the closest to rendering a pathway constitutively active or blocked (Fig 4). Furthermore, this constitutive activation or interruption of pathway would force yeast into a mal-adapted metabolic state that is either more repressed or more derepressed, than it would naturally be in wild-type.

Constitutive activation of the Ras/PKA pathway, as well as interruption of the Snf1 pathway and the Hap complex were expected to lead to a more repressed state. Constitutive activation of the Ras/PKA pathway generally led to a severe growth defect under all conditions investigated; i.e. growth rate and yield were reduced on glucose and growth was not possible on alternative substrates. This is consistent with the reported essentiality of a down-regulated Ras/PKA pathway on respiratory substrates and underlines its broad impact [13-15, 27, 28, 34].

Compared to constitutive activation of the Ras/PKA pathway, interruption of the Hap complex and interruption of the Snf1 pathway were less severe. As expected, both led to
prolonged lag phases from glucose to galactose, suggesting that they cannot adequately adapt to a more derepressed condition, and consistently growth was substantially reduced on galactose and absent on ethanol. However, the biomass yield was more differentially affected, which indicates that the Snf1 pathway and the Hap complex act on different targets. Specifically, the Hap complex led to a decreased yield, hence a reduced metabolic efficiency, presumably due impaired TCA cycle and respiration [19, 25]. The Snf1 mutant only mildly affected yield on glucose, but on galactose it was increased. Since it is known, that Snf1 pathway induces galactose metabolism [16-18], it might be that a reduced glycolysis and a relatively increased respiration are responsible for this effect [24, 30, 35].

On the other side, constitutive activation of the Snf1 pathway and interruption of the Snf3/Rgt2 pathway were expected to enforce a derepressed physiological state. Here, constitutive Snf1 activation caused a reduced growth rate under all conditions and a shortened lag phase from glucose and galactose to ethanol. Besides, it exhibited an increased yield on glucose, whereas on galactose yield was not affected. This is consistent with Herwig et al [36], who showed that constitutive Snf1 activation leads to reduced glucose uptake and ethanol secretion, and with Heyland et al, and Blank et al [30, 35], who showed that reduced glucose uptake coincides with increased respiration. On galactose, in contrast, metabolism was expected to depend on Snf1 induction of substrate utilization [18, 21], which

![Figure 4: The impact interruption or constitutive activation of a signaling pathway on growth.](image)

From single deletion mutants, which had the strongest effect in consideration of their role in signaling pathway activation or interruption (left), the effects on growth parameters during transitions between three differential repressive carbon substrates (top, arrows) were summarized and used as readouts for the impact of a their particular signaling pathway.
would further explain why constitutive activation did not strongly affect biomass yield. During growth on a mixture of glucose and galactose, the first phase yield was increased and the second growth phase was short and less defined (Sup Fig 1A). Since the yield of the first growth phase roughly reached the level of both growth phases (glucose and galactose) in the wild type, we speculate that a constant induction of galactose metabolism led to substrate co-consumption.

Similar to constitutive Snf1 activation, Snf3/Rgt2 interruption caused a decreased growth rate and an increased yield on glucose. Thus also here, metabolism seems to be forced into a more efficient mode carbon utilization, hence derepression on glucose. This change in repression probably emerged from a lowered glucose uptake rate [30], since this is a main target of the signaling pathway [20, 37]. Consistently, it did not affect growth on the more respiratory substrates galactose and ethanol, where glucose transport might be less important. Similar to constitutive Snf1 activation, we recorded a substantially increased yield in the first growth phase during growth on glucose and galactose, whereas the following phase was characterized by slow growth (Sup Fig 1A). Since it is known that Grr1 regulates the degradation of the galactose transporter Gal2, we hypothesize that the reduced Gal2 degradation [38] might also cause a co-consumption phenotype. Moreover, as this mutant did not reach a similar biomass as the wild-type after sequential growth on glucose and galactose, it might be that co-consumption led to a substantially increased glycolysis and hence increased overflow metabolism.

**Redundancies within the signaling pathways**

So far, we determined the condition-dependent relevance of most of the four signaling pathways by their constitutive activation or interruption, but unexpectedly deletion of most components were physiologically silent, in particular the PKA activators and the main Snf3/Rgt2 pathway transcription factors. This suggests that these components are functionally redundant. To test this hypothesis, we generated double deletions within each of these pathways. Specifically, within the upper PKA pathway we deleted combinations of GPR1, RAS1 and RAS2 and in the lower PKA pathway combinations of TPK1, TPK2 and TPK3, the PKA kinases. Additionally, we combined the PKA upstream signaling deletions with an HXK2 mutant, which represses Snf1, but acts also as transcription factor [39-42], and therefore might be redundant to PKA for glucose repression [36, 40]. Within the Snf3/Rgt2 pathway we deleted all three pairs of the main transcription factors RGT1, MTH1 and STD1.

Out of PKA upstream signaling combinations, the simultaneous deletion of RAS1 and RAS2 had the most severe phenotype (Fig. 5), hence we show that these components jointly constitute the main node of the upstream network of the Ras/PKA signaling pathway. Similarly to constitutive PKA activation, this mutant did not grow under any condition besides glucose. Since it is known, that full absence of PKA signaling leads to a G1 arrest in cell
cycle [1, 2, 43], the low growth rate on glucose might be achieved from minimal activation via the glucose receptor Gpr1 [44], which is not given on any other substrate tested. Unexpectedly, although interruption of the Ras/PKA pathway was expected to relieve repression of the TCA cycle and respiration [13, 14, 34] the mutant had a low yield, suggesting that overall metabolic efficiency is reduced. This low yield phenotype might further emerge from induction of gluconeogenic genes, which provoke futile cycles that might lead to extensive waste of energy [45].

The simultaneous deletion of either Ras2 or Gpr1 with Hxk2 led to a similar growth phenotype as interruption of Snf3/Rgt2 or constitutive activation of Snf1. Specifically, these mutants had a high yield on glucose and shortened lag phases when shifting to more respiratory substrates, whereas growth on galactose was only mildly affected. Thus we suggest that repression is at least partially shared between the Snf1 and the PKA pathways. Interestingly, they also had a high-yield phenotype during the mixed substrate growth with glucose and galactose (Sup Fig 1B). Specifically, they exhibited one major growth phase, where yield almost reached wild-type level after sequential growth on glucose and galactose. Thus, also here we hypothesize that galactose metabolism was derepressed and caused

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**Figure 5: Redundancy within PKA pathway and Snf3/Rgt2 pathway.** The effect of chosen double deletion mutants within the PKA and Snf3/Rgt2 pathway (left) during three nutrient shift experiments (top) is depicted in the heat map. Glc: Glucose, Gal: Galactose, EtOH: Ethanol, μ: growth rate. Total effect is given on the right, where the grey area indicates a cutoff of at least 6 effects.
substrate co-consumption. A resulting increased glycolytic rate might explain why the overall yield rather tends to be below wild-type level instead of being increased. Other double deletions of three PKA kinases and the transcription factors of the Snf3/Rgt2 pathway did not exhibit a strong phenotype. Whereas it was known that the PKA kinases have broad functional overlap [1, 2], we show here that also the transcription factors of the Snf3/Rgt2 pathway are functionally redundant, eventually even with other signaling pathways [32]. Moreover, it might be that regulation of the Snf3/Rgt2 pathway is mostly mediated by ubiquitinylation instead of transcriptional regulation.

**Co-consumption restores glycolysis and overall ‘repression’**

From the growth profiles on glucose we detected an increased yield for interruption of the Snf1 and the Snf3/Rgt2 pathway, as well as for the simultaneous deletions of GPR1 or RAS2 with HXK2. Therefore these mutants were suggested to lead to a more efficient physiological state, hence derepression on glucose. Consistently, on the more derepressed galactose condition the mutant yield was similar to wild type. During growth on glucose and galactose, they exhibited only one major growth phase that did not result in an increased yield compared to the total yield of wild-type yeast that sequentially grew on glucose and galactose (Sup Fig 1). Thus, we hypothesized that derepression on mixed substrate glucose and galactose leads to co-consumption, which in turn increases absolute glycolysis and maybe fermentation. In order to verify whether co-consumption occurred, we enzymatically measured glucose and galactose concentrations of the deletions of GRR1 and HEX2, as well as from the double deletions of GPR1 or RAS2 with HXK2 during growth on glucose and galactose.

Confirming our hypothesis during growth on the mixed substrate, wild-type yeast sequentially consumed glucose followed by galactose (Fig 6). In contrast, all mutants, *i.e.* deletions of GRR1, HEX2 and double deletions of GPR1 or RAS2 with HXK2, co-consumed glucose and galactose and therefore did not grow diauxic on these substrates (Fig 6). This co-consumption would inevitably lead to increased glycolysis and thus might explain the overall reduced or similar metabolic efficiency of these mutants compared to the wild-type.
Figure 6: Co-consumption phenotypes within the glucose signaling network. Wild type yeast and the single deletion mutants of GRR1 and HEX2, as well as the double deletion mutants of HXK2 and either RAS2 or GPR1 were grown on minimal medium containing glucose and galactose. Whereas the wild-type sequentially consumes glucose and galactose (black triangles, grey dotted arrows), the mutants co-consumed both substrates (colored symbols).
3.4 Discussion

Here we characterized the impact of the four glucose signaling pathways on growth and physiology during three nutrient shifts between the differential repressive substrates glucose, galactose and ethanol [25]. Besides the impact of enforced repression and derepression by single pathways, we additionally found that Hxk2 and the Ras/PKA signaling pathway partially act redundant in the regulation of repression.

As expected, wild type yeast showed a diauxic growth profile where one substrate represses the utilization of another, less preferred substrate [22, 24]; i.e. glucose is consumed before galactose, and ethanol is consumed last. From these growth profiles we determined growth rate, duration of the lag between the two growth phases and biomass yield. Consistent with the repressive state that is caused by these nutrients, growth was fast on glucose and slowest on ethanol [22], and the lag phase was longest between glucose and ethanol, where metabolism has to be entirely rearranged from glycolytic fully fermentative growth to gluconeogenic growth, where TCA cycle, glyoxylate shunt and respiration have to be induced [22]. Consistent with the energetically higher efficiency with increasing respiration, we confirmed that the biomass yield on galactose was increased compared to glucose [25].

We further asked when and how strong these macroscopic characteristics of growth are controlled by the four glucose signaling pathways. Therefore we generated a knockout library of 51 single knockouts covering most glucose signaling components, and selected double knockouts to eliminate potential redundancies. From these mutants we recorded growth profiles during the above mentioned diauxic shift conditions, and the mutants causing strongest effects in the growth parameters were further classified as key signaling components that either constitutively activate or interrupt signal flow through a particular signaling pathway (Fig 7)( i.e. deletions of SNF1 and SNF4 interrupt the Snf1 pathway, whereas the deletion of HEX2 constitutively activates the Snf1 pathway [16, 46]. the RAS1 RAS2 double deletion interrupts and deletion of BCY1 constitutively activates the Ras/PKA pathway [1, 2, 4, 27, 28, 47, 48]. The deletion of all components of the Hap complex interrupt its activity [25, 33] and the deletion of GRR1 interrupts the Snf3/Rgt2 pathway [4, 20, 29, 31].)

The most severe effect on growth was found from constitutive activation or interruption of the Ras/PKA signaling pathway (Fig 7). Specifically, both had a strong growth defect and a low yield on glucose and did not grow on any other substrate, which underlines the broad impact of this signaling pathway at the transcriptional level and the essentiality of a low basal activity. The decreased yield, hence lowered metabolic efficiency, in both cases is probably caused by differential regulation of metabolism. Specifically, constitutive activation of the pathway is expected to fully repress respiration and gluconeogenesis and thus a decrease in metabolic efficiency and a lethal phenotype on alternative substrates were
expected [15]. In the case of Ras/PKA interruption, however, TCA cycle and respiration were expected to be increased. Thus we speculate, the low yield might be explained by a malinduction of gluconeogenesis [1, 3, 13, 14, 34], which would lead to futile cycles and thus impair the overall energy yield [45]. The additional lethality of PKA interruption on alternative carbon substrates might be explained by the absence of a minimal activation via the glucose receptor Gpr1 [44] and a resulting complete interruption of signal flow, which led to a G1 arrest in cell cycle[43].

The Snf1 pathway also always affected the growth phenotype, but less severely than the Ras/PKA pathway (Fig 7). Consistent with its important role in inducing alternative carbon substrate utilization and gluconeogenesis [1, 14, 16-18, 21], interruption of the Snf1 pathway caused a decreased growth rate on more respiratory substrates, finally being lethal on ethanol. On galactose it further provoked an increased yield, which presumably is caused by a lower glycolysis and a coinciding increased respiration [24]. On the other hand, constitutive activation of the Snf1 pathway caused a general growth defect, and thus even on the derepressed ethanol condition a controlled pathway activity, hence active Hex2-Glc7 phosphatase seems to be crucial. Consistent with its role in prohibiting derepression, constitutive Snf1 pathway activation led to a shortened lag phase from galactose or glucose to ethanol. Furthermore on glucose and while growing on glucose and galactose, it exhibited an increased yield, whereas on galactose as a single substrate it did not. On glucose, the increased yield probably emerged from a deregulation of glycolysis and concomitant increase in respiration [26, 35, 36]. However, during mixed substrate growth we found, that constitutive Snf1 activation leads to a co-consumption phenotype, which is consistent with the finding that Snf1 activates galactose utilization. Moreover, the yield only reached wild-type level after sequential growth on glucose and galactose, instead of being increased. This reduction in metabolic efficiency might emerge from an increased glycolysis and consequently more overflow metabolism and suggests that metabolic efficiency might be a rather indirect target of the Snf1 pathway.

Interruption of the Snf3/Rgt2 pathway was primarily effective on glucose where it caused a low growth rate and an increased yield, whereas it did not cause a phenotype on galactose and ethanol (Fig 7). This is further consistent with reported physiology data [30], which show that on glucose substrate consumption is decreased, whereas TCA cycle gets increased. Overall this effect on glucose presumably emerges from deregulation of glucose transporters, since they are main targets of the Snf3/Rgt2 pathway [20, 31, 49, 50]. Similarly to constitutive Snf1 activation it also co-consumed glucose and galactose during growth on both substrates, and furthermore the yield was substantially decreased compared to the wild type, which sequentially grew on glucose and then on galactose. Thus also here, the reduction in metabolic efficiency is probably caused by increased overflow metabolism, which further underlines the important role of the Snf3/Rgt2 pathway in the co-repression of galactose metabolism [38]. The absence of an effect in the main transcription factor deletion
mutants of the pathway might emerge from a high redundancy with other pathways under the conditions tested [32], or because the repressing upstream network does not regulate metabolism mainly via transcription.

As expected [25, 33], the importance of the Hap complex increases with more important with respiratory conditions as it had a decreased growth rate on galactose and was lethal on ethanol. Consistent with its importance for the regulation of mitochondrial functions, it had decreased yield under all conditions tested [19].

Besides the impact of signaling pathway interruption or constitutive activation, we tested, whether Hxk2 is redundant with the Ras/PKA pathway. Specifically, all single deletions of Ras/PKA activators were silent, as well as Hxk2, the major regulator of glucose repression in the CEN.PK genetic background, where adenylate cyclase (Cyr1) carries a point mutation [40]. Furthermore, it was shown that the effect of HXK2 deletion could be partially rescued by insertion of an intact CYR1 gene, and thus it was postulated, that these pathways are partially redundant [40]. However, the role of Hxk2 in FY4 background was unclear. Here we showed that Hxk2 indeed partially co-regulates repression with the Ras/PKA signaling pathway. Similarly to constitutive Snf1 activation, yield was increased on glucose and during growth on glucose and galactose. Whereas on glucose metabolic efficiency was increased, presumably due to lower glycolysis, overall metabolic efficiency during mixed substrate growth was decreased, where the mutant co-consumed glucose and galactose. The overall similar phenotype compared to constitutive Snf1 activation indicates, that the Ras/PKA pathway co-inhibits Snf1 or that it acts together with Hxk2 at the level of the downstream targets, hence transcriptional regulation [40, 42, 46, 51, 52]. However, more combinatorial knockouts of the signaling pathway components and a subsequent analysis of substrate-specific growth are needed to test this hypothesis and enlighten redundant regulation in general.

Overall, we could determine the key signaling mutants that quantitatively regulate growth and physiology under differential repressive conditions. Moreover, we could verify the exclusive quantitative impact of high and low signaling pathway activity on growth and metabolic efficiency under differential repressive conditions. The high variation in these macroscopic parameters implies a vast rearrangement in metabolic fluxes. In order to find causal reactions, hence major metabolic targets of the signaling pathways that contribute to the macroscopic phenotype and to distinguish, whether these metabolic targets might be the same or not, it is inevitable to measure intracellular metabolites, and further to relate signaling pathways and metabolic perturbation with quantitative flux analysis.
Figure 7: The impact of constitutive signaling pathway activation or interruption on growth on differentially repressive substrates. The table specifies, which single and double deletion mutants of the glucose signaling pathways lead to constitutive activation or interruption. ‘#’ indicates a double deletion mutant that hinted on redundant regulation and ‘*’ double deletion mutants that were expected to cause a constitutive activation, but did not exhibit a strong effect on growth. The chart below indicates whether constitutive activation or interruption is expected to lead to enforced repression (dark grey) or derepression (light grey). The black half circles illustrate whether constitutive activation or interruption of a pathway had a strong impact on growth rate relative to the wild type under differential repressive conditions.
3.5 Material and Methods

**Strains used in this study**

The supplementary Table 1 lists the mutants that were generated in this study and mutants that were received from Dr. Sarah-Maria Fendt.

The here generated single knockout library was derived from the haploid Yeast Orf library (BY4741 Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). Specifically, primers were designed on the basis of gene sequences in SGD and the provided software [8]. After cell lysis with 0.2% SDS at 2 min at 90°C, fragments of single gene deletions (KanMX cassette) with at least 200 base pairs flanking regions were amplified and transformed into the prototrophic yeast background FY4 *Mata* [53] by using the LiAc/SS carrier DNA/PEG method [54]. After selective growth on YPD agar plates with geneticine (G-418) the transformants were verified with an internal primer and an additional primer from the flanking region outside the original primers.

Prototrophic double deletion mutants carrying a KanMX and a NatMX resistance cassette [55] were obtained by exchanging the KanMX cassette in the above mentioned strains with a NatMX cassette and subsequent re-transformation into a strain carrying the deletion of the gene of interest. Specifically, all MX resistance cassette carry the TEF promotor and thus have enough overlap for homologous recombination [55].

**Cultivation medium**

All cultivations were performed in batch on minimal medium as described in [35]. For diauxic shifts carbon sources were added as follows: 10 g/L glucose, 10 g/L galactose, and a mixture at equal amounts (7.5-10 g/L) glucose and galactose.

**Online measurements**

We first tested growth on 10 g/L glucose, galactose or ethanol by cultivating the mutants in 96 deep-well plates and measuring OD$_{600}$ in a microplate reader (TECAN sunrise, Tecan group). For later shift experiments between the differential repressive substrates glucose, galactose and ethanol we performed online measurements of growth with a BioLector plate reader (m2p-labs GmbH, Germany). Specifically, we used 48-well flower plates with 1.5 mL medium volume. The back scatter signal was recorded in 5-10 min intervals at culture conditions of 30°C, >90% air humidity and a shaking frequency of 900 rpm. The obtained growth profiles were background corrected smoothed with an moving average over 6-10 points. Growth rate, yield and lag phases were calculated in a half-automatic way in Matlab. Specifically, growth phases and yield were manually tagged and the growth rates and the yield were calculated within these phases. Lag phases were
calculated by intersecting two growth curves with the yield height. All measurements were done in at least biological duplicates.

**Determination of Co-Consumption**

For co-consumption experiments, the strains were cultivated in 96-deep-well plates (Kuehner AG, Switzerland) with 1.2 mL medium and a 4mm diameter glass bead, and incubated in a shaker at 30°C and 300 rpm. $\text{OD}_{600}$ measurements were done with a microplate reader (TECAN sunrise, Tecan Group). The galactose and glucose content in supernatants were analyzed with in glucose and galactose contents were determined by enzyme assays (Megazyme International Ireland, Ireland) in a microplate reader (TECAN sunrise, Tecan Group).
3.6 References


3.7 Appendix

Sup Fig 1: High-yield growth profiles during a glucose-galactose shift. A) Depicts the growth profile of single deletions of HEX2 (left, blue) and GRR1 (right, blue) compared to the wild type (red) during a shift from galactose to glucose. B) illustrates the growth profile of the double deletions of HXK2 with either RAS2 (left, blue) or GPR1 (right, blue) compared to the wild type (red) during a nutrient shift from galactose to glucose.

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Table 1.1 all strains used in this study were in the FY4 background. * from Dr. Sarah-Maria Fendt
Chapter 4

Metabolic regulation through four glucose signaling pathways in yeast under differential repressive conditions

Stefan Christen, Uwe Sauer
4.1 Abstract

Central metabolism connects catabolism of various carbon sources to biosynthesis pathways by providing energy, redox, and biomass precursors. Each carbon source leads to an alternative metabolic operation that may range from repressed, fermentative on glucose to derepressed, respiratory on ethanol. The acquisition and maintenance of these different metabolic states depends on a glucose signaling network consisting of four signaling pathways, i.e. the Ras/PKA pathway, the Snf1 pathway, the Snf3/Rgt2 pathway and the Hap complex that mediate glucose repression. Due to the regulatory overlap between these signaling pathways, it is unclear which central metabolic pathways are controlled by a particular signaling pathway under a given condition. To quantitatively assess metabolism in the context of glucose signaling, we performed targeted metabolomics of deletion mutants of most glucose signaling components under steady-state conditions with the differentially repressive carbon substrates glucose, galactose and ethanol. Generally, all signaling pathways affected central metabolism in a condition-specific manner. Thus we could functionally confirm the exclusive control of the signaling pathways over known metabolic targets (e.g. on glucose the Ras/PKA pathway was the main regulator of the TCA cycle, whereas the Snf1 and the Snf3/Rgt2 pathway mainly affected upper glycolysis) and we newly found that the Hap complex regulates the branch point between upper and lower glycolysis on galactose.
4.2 Introduction

Yeast can synthesize biosynthetic precursors and generate energy from a variety of carbon substrates. Growth on these carbon substrates leads to distinct physiological states that are particularly prominent at the level of central metabolic fluxes, protein and metabolite concentrations [1-3]. Two of the most distinct physiological states of respiration or fermentation are found during growth on glucose and ethanol and lead to ‘repressed’ and ‘derepressed’ metabolism, respectively. The repressed state on glucose is also referred to as the Crabtree effect [4], specified by a repression of the tricarboxylic acid (TCA) cycle and respiration and a high specific growth rate that is largely fueled by glycolysis. Consequently, glucose is mainly fermented to ethanol and other incompletely oxidized by-products and thus the biomass yield is low. On the non-fermentable substrate ethanol, respiration and the TCA cycle are ‘derepressed’, leading to fully respiratory growth without by-product formation, albeit at a lower growth rate than on glucose. Other substrates such as galactose or mannose cause an intermediary repressed state with simultaneously active glycolysis and TCA cycle [2].

Metabolic adaptation to these different substrates and establishment of exponential growth are primarily mediated by a glucose signaling network that consists of four pathways: Ras/PKA pathway, Snf1 pathway, Snf3/Rgt2 pathway and the Hap complex [4-14]. Since these four glucose signaling pathways are still being explored by different screening approaches at the level of transcriptomics [15-17], proteomics and phosphoproteomics [15, 18-20], as well as at the level of multiple gene deletions [21], new genetic and physical interactions with metabolism are continuously found and extend far beyond central metabolism [22]. Understanding the specific regulatory function of each pathway in metabolism is complicated by extensive transcriptional co-regulation, where a given metabolic gene is regulated by more than one signaling pathway [15, 16, 19, 23-27], and by the fact that many metabolic reactions in yeast are catalyzed by two or more so-called isoenzymes [28] (Fig 1). Given this regulatory overlap, it remains unclear which reactions are controlled by which signaling pathway, how strong, and under which condition.

In Chapter 3 we characterized the growth phenotype of single deletion mutants in components within the glucose signaling network during shifts between differential repressive carbon sources, thus determining the condition-specific relevance of each signaling pathway. Consistent with its impact on the transcriptional level, both, Ras/PKA pathway interruption and constitutive activation severely impaired growth on glucose and caused a lethal phenotype on other substrates, suggesting that Ras/PKA pathway activity has to be generally controlled within a narrow range. The Snf1 pathway affected the growth phenotype more differentially where interruption of signal flow was more severe on non-glucose substrates and even lethal on the obligatory respiratory ethanol, and constitutive activation
Fig 1: Central metabolic targets of the four glucose signaling pathways. The figure gives an overview of generally assumed [6, 9-11] metabolic targets of the Snf1 pathway (green area), the Ras/PKA pathway (red area), the Snf3/Rgt2 pathway (blue area) and the Hap complex (yellow area). Circles represent particular metabolites within the metabolic pathways. If a genetic or a physical interaction with a particular metabolic enzyme is reported [22], the participating metabolites (circles) are colored.
caused a general growth defect under all conditions [Chapter 3]. Specifically, constitutive activation of Snf1 led to slow growth but increased yield on glucose that we suggest to be caused by derepression of metabolism. The reverse situation was found on galactose where interruption of Snf1 signal flow caused an increased yield, indicating that metabolic efficiency seems to be indirectly regulated by this signaling pathway. The Snf3/Rgt2 pathway caused decreased growth and increased yield on glucose, whereas it was phenotypically silent on any other substrate, which was consistent with its role in the regulation of hexose transport. As expected, Hap complex mutants were increasingly impaired in growth and yield with increasing respiration, eventually being lethal on ethanol.

Given the Influence of the signaling pathways on these macroscopic cellular parameters, we attempt here to determine which metabolic pathways are when and how strongly regulated by signaling under differential repressive conditions. To assess a metabolic phenotype, we measured intracellular metabolite concentrations in deletion mutants of most signaling components in the four glucose signaling pathways on glucose, galactose and ethanol by targeted liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) [29].
4.3 Results

**Strongest metabolic effects are discernible upon complete interruption or constitutive activation of glucose signaling pathways**

To determine, which metabolic reactions are affected by glucose signaling, we analyzed intracellular metabolite pools of 50 single deletion mutants within the glucose signaling network under the three differential repressive conditions of steady-state exponential growth on glucose, galactose and ethanol. Additionally, we included the RAS1/RAS2 double mutant to investigate the impact of Ras/PKA pathway interruption (Chapter 3). Using a targeted mass spectrometric approach [29] we were able to detect 32 metabolites in all mutants that cover most of central carbon metabolism and also include amino acids and nucleotide phosphates (Fig 2).

Fig 2: Measureable metabolites within this screen. The scheme on the left represents central metabolism. Black tagged circles represent central carbon metabolites that could be detected within this screen. Single letters indicate whether a metabolite represents a precursor for other metabolic pathways, which are listed on the right. Bold names indicate, that some amino acids and nucleotide phosphates could be additionally measured. T6P: Trehalose-6-phosphate; G1P: Glucose-1-phosphate; G6P: Glucose-6-phosphate; F6P: Fructose-6-phosphate; FBP: Fructose-1-6-bisphosphate; DHAP: Dihydroxyacetone-phosphate; GlyP: Glycerol-phosphate; xpG: 2-3-phosphoglycerate; PEP: Phosphoenol-pyruvate; AcCoA: Acetyl-CoA; Cit/Ici: Citrate & Isocitrate; Aco: Aconitate; Succ: Succinate; Fum: Fumarate; Mal: Malate; 6PG: 6-phosphogluconate; RSP: Ribose-5-phosphate; S7P: Seduheptulose-7-phosphate.
As reported in Chapter 3, the most severe impact was found on ethanol, where 9 out of 51 mutants had a lethal phenotype (Fig 3). Nonetheless, since most mutants grew on galactose and glucose, we were able to elucidate the metabolic pathways that are regulated by the glucose signaling network in a condition-dependent manner. Specifically, we classified metabolite concentration changes by a log2 fold-change of at least 0.75 and a multiple testing corrected p-value (h-value) below 0.01. Using this cutoff, trehalose-6-phosphate and citrulline were most frequently affected (in 20 and 29 cases, respectively) and also exhibited the strongest log2 fold-changes in the range between -3.7 – 7.6 and -5.6 – 1.5, respectively (Fig 3). All other responding metabolites were more differentially influenced (6 ± 4 times) and their average maximal log2 fold-change was about 2. The deletion mutants causing these differential effects were the same as found in the growth screen for interruption or constitutive activation of the signaling pathway; i.e. SNF1, SNF4 or HEX2 deletions in the Snf1 pathway, BCY1 deletion or the RAS1 and RAS2 double deletion in the Ras/PKA pathway, GRR1 deletion in the Snf3/Rgt2 pathway, and deletion of any component of the Hap complex (Fig 3). To identify the signaling-controlled metabolic pathways, we thus focused on these mutants that either fully interrupt or constitutively activate each signaling pathway.

*The Snf1 pathway mainly affects storage carbohydrate metabolism and glycolysis*

The growth parameter screen demonstrated that Snf1 pathway interruption or constitutive activation affected growth rate and physiology under all conditions. At the level of intracellular metabolites the Snf1 pathway mainly caused changes in intermediates of storage carbohydrate metabolism and upper glycolysis (Fig 4). Specifically on glucose, constitutive activation of Snf1 increased the concentration of trehalose-6-phosphate, a storage carbohydrate metabolism intermediate (88 - fold). Similarly about 2-fold increases were found for glucose-1-phosphate and glucose-6-phosphosphate concentrations, whereas other metabolites of glycolysis such as fructose-1,6-bisphosphate, glycerol-3-phosphate and pyruvate were 0.5-fold decreased, suggesting that phosphofructokinase was blocked. Since it is known, that the Snf1 pathway can affect the flux at phosphofructokinase either directly [30, 31], or via regulation of fructose-1,6-bisphosphatase [15, 16, 32], we suggest that this blockage might be the main cause for a reduced glucose uptake. Consistently, Snf1 pathway interruption resulted in the opposite effect in storage carbohydrate intermediates and glucose-6-phosphate. Since other glycolytic intermediates were not affected by Snf1 pathway interruption, our results indicate that glycolysis was only mildly regulated (Fig 3).

On galactose, constitutive activation of Snf1 provoked a similar increase of trehalose-6-phosphate (164 ± 71 – fold), but the effect on fructose-1,6-bisphosphate was reversed compared to the glucose condition. Consistently, Snf1 pathway interruption caused a decrease in fructose-1,6-bisphosphate. Since it is known that galactose uptake and utilization
**Fig 3: Impact of signaling components on metabolism.** The heat-map represents impact of all glucose signaling components (left) on the determined metabolites, metabolic pathways, and growth (bottom) under three differential repressive steady-state conditions glucose, galactose and ethanol (top). The mutants were sorted according to their signaling pathways (left) and to the expectation of them taking part in derepression (black) or repression (white). The arrows indicate, whether the mutants were assumed to be essential for (black) or main modulators (grey) of signal flow through a particular signaling pathway. The metabolic log2-fold changes were derived from intracellular metabolite measurements, and the growth defect was adopted from Chapter 3.
Fig 3 continued. Abbreviations: T6P: Trehalose-6-phosphate; G1P: Glucose-1-phosphate; G6P: Glucose-6-phosphate; F6P: Fructose-6-phosphate; FBP: Fructose-1-6-bisphosphate; DHAP: Dihydroxyacetone-phosphate; GlyP: Glycerol-phosphate; xPG: 2-3-phosphoglycerate; PEP: phosphoenol-pyruvate; AcCoA: Acetyl-CoA; Cit/lci: Citrate & Isocitrate; Aco: Aconitate; Succ: Succinate; Fum: Fumarate; Mal: Malate; 6PG: 6-phosphogluconate; R5P: Ribose-5-phosphate; S7P: Seduheptulose-7-phosphate.
Fig 3 continued.
are activated by the Snf1 pathway, our results are in agreement with the hypothesis that Snf1 pathway interruption prevents activation of galactose metabolism, which in turn impairs glycolytic flux and causes a decrease in the concentration of fructose-1,6-bisphosphate. On the other side, a constitutively active mutant over-induces Snf1 signaling [33] and galactose metabolism such that upper glycolytic intermediates accumulate. On ethanol, consistent with the other conditions, constitutive Snf1 pathway activation still provoked a substantial increase of trehalose-6-phosphate (~13-fold) and glucose-6-phosphate (~3-fold), but pathway interruption was lethal, probably because it is an important regulator for the activation of gluconeogenesis and carbon utilization [7, 16, 34-37]. Thus, our results confirm that the Snf1 pathway is directly needed for the regulation of storage carbohydrate metabolism and utilization of alternative carbon sources, and suggest that glycolysis is differentially regulated in a condition-dependent manner.

The TCA cycle was only mildly affected by the Snf1 pathway on glucose with a 2-fold increase in citrate as the sole effect in the constitutively active mutant. This indicated an increased TCA cycle activity, which was consistent with the increased yield under this condition. On galactose conversely, Snf1 interruption caused 2-fold increased citrate concentrations, which was consistent with higher TCA cycle flux and increased biomass yield (Chapter 3). Here, constitutive activation additionally resulted in a 2-fold increase of the other TCA cycle intermediates oxoglutarate, fumarate and malate. Since the constitutively active Snf1 pathway mutant did not exhibit a higher biomass yield and thus TCA cycle flux, and it is known that Snf1 regulates the glyoxylate cycle that might bypass respiration [15, 34, 38], we hypothesize that a mal-induction of this metabolic pathway partially explains this response.

**The Ras/PKA pathway is the main regulator of TCA cycle and respiration on glucose**

Chapter 3 revealed that the Ras/PKA signaling pathway was crucial for growth under all conditions. Specifically both, the constitutively active BCY1 deletion mutant and the interrupted RAS1/RAS2 double deletion mutant could not grow on any substrate besides glucose. Within metabolism, Ras/PKA interruption caused a decrease in the upper glycolytic intermediates fructose-1,6-bisphosphate and dihydroxy-acetone phosphate, indicating reduced glycolytic flux. The additional depletion of pentose phosphate pathway intermediates suggests a misbalanced redox metabolism, although it is unclear whether this regulation is a direct or an indirect effect. The strongest effect was detected in the TCA cycle, where both, interruption and constitutive activation caused large increases in pyruvate and TCA cycle intermediate concentrations (Fig 3). In the case of the constitutive active Ras/PKA pathway, succinate was most increased (8-fold), whereas fumarate was reduced. This strongly supports the notion that respiration was blocked at succinate dehydrogenase [1]. Interruption of Ras/PKA signaling caused a pronounced increase in citrate (17 ± 1.2-fold). The overall
high impact on TCA cycle intermediates suggests that the Ras/PKA pathway constitutes the major signaling regulator of the branch point between respiration and fermentation.

**The Snf3/Rgt2 pathway and the Hap complex regulate glycolysis and TCA cycle in a condition-dependent manner**

Phenotypically, the exclusive effect obtained in Snf3/Rgt2 signaling was by pathway interruption in the GRR1 mutant with slow growth and increased yield on glucose (Chapter 3). Consistently, interrupting the Snf3/Rgt2 pathway led to depletion of metabolites in upper glycolysis, the non-oxidative pentose phosphate pathway, and storage carbohydrate metabolism on glucose, but in none of the other conditions (Fig 3, Fig 4). Since it is known, that a deletion mutant of GRR1 has a reduced glucose uptake rate [39], we suggest reduced glucose uptake as the cause for the depletion of the metabolites in upper central metabolism. Additionally, Snf3/Rgt2 interruption resulted in increased succinate and citrate concentrations, indicating derepression of the TCA cycle, which would be consistent with the higher yield that was found in Chapter 3. Consistent with this view, deletion of all glucose transporters leads to a comparable effect in glycolysis and pentose phosphate pathway (A.-M. Schmidt, personal communication, June, 2013). The absence of metabolic response on galactose or ethanol is fully consistent with the hypothesis that the Snf3/Rgt2 pathway regulates primarily the glucose transporter (HXT) genes that are not required on these substrates.

The Hap complex deletion mutants exhibited more effects with increasing respiration, and the functional complex was essential on the fully respiratory substrate ethanol. This is in agreement with its known role in the activation of mitochondrial functions that are required for respiratory growth [40, 41]. Consistently, Hap complex mutants had little metabolic effects on glucose with succinate as the only increased metabolite (Fig 3A). As expected, on galactose the effect of the Hap complex on the TCA cycle became more accentuated. Specifically, we detected significantly changed concentrations of oxoglutarate (0.7-fold), succinate (1.7-fold) and malate (2-fold). Surprisingly, the major impact was detected at the connection between upper and lower glycolysis in fructose-1,6-bisphosphate, dihydroxyacetone-phosphate, 2/3-glycerol-phosphate and phosphoenol-pyruvate (Fig 3, Fig 4). These results indicate a potential new regulatory interaction of the Hap complex at glyceraldehyde-phosphate-dehydrogenase since no targets of the Hap transcription factor complex are known at this site.
**Fig 4: Impact of signaling pathways on central carbon metabolism.** The effect of each glucose signaling pathway (Snf1 pathway: green; Ras/PKA pathway: red; Snf3/Rgt2 pathway: blue; Hap complex: orange) on central metabolism under the differential repressive conditions glucose, galactose and ethanol (left) is summarized on a simplified central metabolic network. The small circles within the metabolic network depict metabolites (black: detected; grey: not detected) and the big circles indicate whether a signaling pathway affected a particular metabolite. ‘*’ indicates that only constitutive Snf1 activation could be measured, since its interruption was lethal.
Only few modulating components in the signaling pathways are essential for metabolic regulation

As expected, interrupting the signal transmission entirely by deleting the main pathway components revealed specific metabolic consequences of changing signal flow under conditions where specific signaling pathways were active. In each of the signaling pathways, however, are also many additional protein components whose role in signal transduction is less clear and their deletion does not lead to clear signal phenotypes such as pathway interruption or constitutive activation. These additional proteins include many potentially redundant components such as the Snf1 upstream kinases Sak1, Tos3 and Elm1, where only the triple knockout leads to a Snf1 phenotype [10, 11], or the Ras/PKA signaling kinases Tpk1, Tpk2 and Tpk3 that have a high regulatory overlap [10, 11]. Here we elucidate, whether some of these components are more important for the modulation of signal flow under particular condition and whether such an effect is consistent with pathway interruption or constitutive activation. Overall, only few deletions of these additional components had a significant and strong effect. These few components with strong effects, that are therefore apparently major modulators of signal flow, are associated with the Snf1 and the Ras/PKA pathways.

Within the Snf1 pathway, the deletion of ELM1, an upstream-kinase of Snf1 provoked a decrease in amino acid concentrations on glucose. Since there was little metabolic overlap with Snf1 pathway interruption (Fig 3), we propose that Elm1 acts partially Snf1-independent. On ethanol, deletion of the upstream Snf1 kinase SAK1 and deletion of the Snf1-activated transcription factor CAT8 were effective. Similar to Snf1 pathway interruption, CAT8 deletion was lethal on ethanol. Since it is known that Cat8 particularly up-regulates gluconeogenic genes, we hypothesize that this is the main cause of Snf1 lethality under this condition [16]. The impact of SAK1 deletion was less detrimental. Specifically, it provoked decreased seduheptulose-7-phosphate concentrations (~0.2 -fold) on ethanol. Since this effect is opposite to constitutive activation of Snf1, our results support the previously formulated hypothesis that Sak1 is the main upstream kinase of Snf1 under this condition [10, 11, 42]. Additionally Sak1 deletion caused an increase of phosphoenol-pyruvate, which together with lethality of Cat8 deletion confirms the major role of the Snf1 pathway in the regulation of gluconeogenesis.

From the PKA pathway components we found the strongest effects for the deletions of the upstream inhibitors IRA1 and IRA2. On glucose they caused an increase in succinate and a decrease in amino acids, resembling an attenuated effect of constitutive Ras/PKA activation (Fig 3). Consistent with the role of this signaling pathway in glucose repression [16, 24, 43], the effect of these components was most pronounced on the respiratory substrates galactose and ethanol (Fig 3). Since in contrast to full constitutive activation of the Ras/PKA pathway, these mutants were viable on respiratory substrates, we could investigate the
metabolic effect of partial constitutive activation of the Ras/PKA pathway. On galactose the IRA mutants generally had decreased levels of glycolytic compounds, whereas citrate was increased (Fig 3). These results support the hypotheses that besides Snf1 pathway activation, a lowered PKA activity is required for derepression of galactose uptake and utilization [44]. On ethanol, the effect of these inhibitors affected the lower glycolytic intermediates phosphoenol-pyruvate and 2/3-phosphoglycerate, which were both about 3.5-fold increased. This increase in lower glycolytic intermediates indicated a block within gluconeogenesis and is in line with the role of Ras/PKA in repression of gluconeogenesis [16, 24, 43]. Besides the general impact of the upstream inhibitors, the deletion of the gene encoding the PKA inactivated transcription factor Msn2 had a pronounced effect on galactose (Fig 3). Specifically, its phenotype resembled the effect of IRA2 deletion, and thus we propose that Msn2 is a main transcriptional mediator of derepression on this particular substrate.
4.4 Discussion

Here we characterized when and how strong different metabolic pathways are regulated by the four glucose signaling pathways on the differential repressive carbon substrates glucose, galactose and ethanol. Specifically, we measured the central carbon metabolome response of single deletion mutants of most components in these signaling pathways. As expected, most of the components causing a strong metabolic effect were the same as those previously found in Chapter 3 to affect growth and physiology. On the basis of these results, we classified these mutants as interrupting or constitutively activating signal flow through each of the four pathways; i.e. deletions of SNF1, SNF4 interrupt and deletion of HEX2 constitutively activates the Snf1 pathway, the double knockout of RAS1 and RAS2 interrupt and the deletion of BCY1 constitutively activates the Ras/PKA pathway, GRR1 interrupts the Snf3/Rgt2 pathway and all components of the Hap complex interrupt its activity. Deletion of the many other components of these signaling pathways had much less metabolic effects. The exception were the two Ras/PKA upstream inhibitors Ira1 and Ira2, the Snf1 activating kinase Sak1, and the transcription factor Msn2 whose deletion mutants exhibited an alleviated metabolic response of pathway interruption of constitutive activation. Additionally the deletion of the transcription factor CAT8 of the Snf1 pathway was lethal on ethanol. Thus these additional components seem to be main modulators of the signal flow and they could be further used to decipher the potential impact of signal flow interruption under conditions where a signaling pathway was essential. As expected, metabolic pathway regulation varied with the activity of the signaling pathways under different conditions (Fig 5A). I will first discuss the impact of the glucose signaling pathways on the fermentable carbon substrates glucose and galactose with the focus on upper and lower central metabolism, hence glycolysis and TCA cycle, respectively. At the end I will examine the influence of the glucose signaling pathways on ethanol, which was the only gluconeogenic substrate tested.

Impact of the glucose signaling pathways on upper central metabolism

On glucose, upper glycolysis was differentially affected by blocking the Snf3/Rgt2 and Ras/PKA pathways, as well as by constitutive activation of the Snf1 pathway. Specifically, interruption of the Snf3/Rgt2 pathway provoked a strong depletion of all glycolytic intermediates, as well as storage carbohydrates and pentose phosphate pathway intermediates. Since the Snf3/Rgt2 pathway is a main regulator of glucose uptake [16, 27], this depletion in glycolytic intermediates emerges probably from a highly reduced glucose consumption [39] (A.-M. Schmidt personal communication, June, 2013). Interruption of Ras/PKA signaling provoked a decrease in fructose-1,6-bisphosphate and dihydroxyacetone-
phosphate, indicating a reduction in glycolytic flux [45] that might be explained by the joint absence of the following functions: i) Ras/PKA was shown to co-regulate hexose transport at the transcriptional level [16, 24, 43] ii) Ras/PKA transcriptionally represses the gluconeogenic enzyme fructose-1,6-bisphosphatase and is needed for its degradation [16, 24, 43, 46] iii) Ras/PKA activates fructose-2,6-bisphosphate biosynthesis [11, 24, 31], which might inhibit fructose-1,6-bisphosphatase [47]. The additional depletion in pentose phosphate pathway intermediates could potentially result from direct transcriptional deregulation of 6-phosphogluconolactonase (SOL4) and 6-phosphogluconate dehydrogenase (GND2), or indirectly via transcriptional deregulation of NADP⁺-dependent isocitrate dehydrogenase (IDP2, IDP3) or NADP⁺-dependent acetaldehyde-dehydrogenase (ALD4) [17, 23, 24, 43].
Second would alter NADPH homeostasis and thus affect pentose phosphate pathway activity [48]. To distinguish these possibilities one could further determine the in vitro activity of these enzymes in cell lysates and evaluate their in vivo contribution to NADP+ reduction by quantitative flux analysis [49]. Finally, constitutive activation of the Snf1 pathway caused increased concentrations of glucose-6-phosphate and storage carbohydrates, whereas fructose-1,6-bisphosphate was depleted. This suggests that it blocked metabolism at the phosphofructokinase reaction. This block might explain why glucose uptake is reduced in a REG1 knockout [50]. There are two sources of regulation that could account for this particular effect. i) constitutive Snf1 activation by HEX2 deletion transcriptionally induces fructose-1,6-bisphosphatase at the transcriptional level and inhibits its degradation [15, 16, 32], which would counteract phosphofructokinase. ii) Snf1 might directly inhibit Pfk2 [30], albeit it is unclear whether this happens via phosphorylation [31], since the functionality of the targeted phosphosites could not be shown [51]. However, increased fructose-1,6-bisphosphatase activity would lead to futile cycling and thus a lower yield [51, 52], which is opposite to the finding that constitutive Snf1 activation provoked an increased yield. Therefore inhibition of Pfk2 by constitutive Snf1 activation is the more plausible reason for this effect.

Regulation of glycolysis is different on the still glycolytic substrate galactose, where derepression for the utilization of alternative carbon substrates has to be present [53] (Fig 5B). This derepression is mediated by the activation of Snf1 pathway [5, 8, 10, 11, 53] and reduced activity of the Ras/PKA pathway [44, 54]. Consistently, Snf1 pathway interruption reduced intermediate concentrations of glucose-1-phosphate and upper glycolytic intermediates, which suggests, that galactose uptake and consequently glycolysis are impaired. Conversely, increased intermediate concentrations in upper glycolysis and storage carbohydrates upon constitutive Snf1 activation indicate hyperactivation of galactose uptake, which is consistent with Shanks et. al [33], who showed that a REG1 deletion mutant has an accelerated galactose uptake. However, overall fermentation flux through glycolysis to ethanol seems not to be increased, since the growth screen in Chapter 3 did not reveal an altered biomass yield. The deletion of the main modulators of the Ras/PKA pathway caused a lethal phenotype on galactose. However, a mild constitutive activation by deleting IRA1 and IRA2, as well as deletion of MSN2, led to a decrease in most glycolytic intermediates while glucose-1-phosphate was mildly increased. Thus, our results support the finding that a relief of phosphoglucomutase repression is needed for galactose utilization, besides a general activation via the Snf1 pathway [44]. Unexpectedly, deletions of the Hap complex strongly affected the glycolytic intermediates at the branch point between upper and lower glycolysis where no direct Hap target is known. Specifically, upper glycolytic intermediates were increased, whereas lower glycolytic intermediates were reduced. Thus, the Hap complex seems to regulate the branch point at glyceraldehyde-3-phosphate dehydrogenase. It might be that this regulation is indirectly mediated via an imbalance NADH metabolism.
Specifically, with the absence of respiration an important sink of NADH would be missing [55], and this imbalance could block glyceraldehyde-3-phosphate dehydrogenase. In order to test this hypothesis, one might inhibit respiration on galactose with chemicals such as antimycin A and verify, whether a similar effect occurs.

**Impact of the glucose signaling components on lower central metabolism**

The TCA cycle is known to be at least partly co-regulated by all signaling pathways at the transcriptional level [2, 15, 16, 24, 27, 34, 38, 40]. Consistently on glucose, all signaling pathways affected TCA cycle intermediates, and the resulting altered TCA cycle fluxes might explain the differences in yield described in Chapter 3 (Fig 5A). In particular, modulation of the Ras/PKA pathway had an exceptionally high impact on TCA cycle intermediates. Specifically, constitutive activation of the Ras/PKA caused a pronounced accumulation of succinate, whereas fumarate was decreased. This indicates a block at succinate dehydrogenase that prevents respiration, potentially causing decreased biomass yields (Chapter 3). Interruption of the Ras/PKA pathway caused a strong increase in citrate, circumstantially indicating increased flux into the TCA cycle, as also indicated by transcriptional derepression of the TCA cycle in mutants, where all PKA kinases were deleted [16, 17, 24]. Inconsistently, we found in Chapter 3 that Ras/PKA pathway interruption led to a decreased biomass yield, instead of an expected increase. This discrepancy might happen due to derepression of gluconeogenesis that might lead to several futile cycles within metabolism that cause a substantial decrease in yield [52]. Overall, our results show that on glucose the Ras/PKA pathway is a key regulator of proper TCA cycle function.

On galactose, strong modulation of the Ras/PKA pathway was lethal. However, a mild constitutive activation of the Ras/PKA pathway did not affect TCA cycle intermediates and thus we hypothesize that repression of the TCA cycle by Ras/PKA might be less strong. From the other pathways main effects were found for deletions in the Hap complex and constitutive Snf1 activation (Fig 5B). Specifically, the Hap complex led to an accumulation of succinate, fumarate and malate, which might emerge from an expected mal-induction TCA cycle and respiratory chain genes [40]. This is further supported by the fact, that the biomass yield was decreased in the deletion mutants of the Hap complex components, whereas relative ethanol secretion was increased [56]. In the case of constitutive Snf1 activation, several TCA cycle intermediates were increased. However, here these effects did not coincide with an altered yield. Since Snf1 regulates the glyoxylate shunt at the transcriptional level [15, 34, 38], we hypothesize that an induction of the glyoxylate shunt causes these effects, whereas overall respiration would stay similar to the wild type. To test this hypothesis one could further use a labeling experiment and estimate the ratio between TCA cycle and glyoxylate shunt.
**Impact of the glucose signaling pathways during growth on a gluconeogenic substrate**

On ethanol, yeast metabolism relies on gluconeogenesis, TCA cycle and respiration. Constitutive activation and interruption of the Ras/PKA pathway led to a lethal phenotype and thus we could only specify a phenotype for a partial activation in the deletions of IRA1 and IRA2. This partial activation led to an increase of phosphoenol-pyruvate and 2/3-phosphoglycerate, which might be caused by repression of fructose-1,6-bisphosphatase (Fig 5C). Consistent with their essentiality for the induction of gluconeogenic genes [41], the blockage of the Snf1 pathway and deletion of its regulated transcription factor Cat8 caused a lethal phenotype. Furthermore, we found that the deletion of the gene encoding the Snf1 upstream kinase Sak1 caused an increase in lower glycolytic intermediates (Fig 5C), and constitutive Snf1 activation led to increased glucose-6-phosphate and trehalose-6-phospshate, which indicated impaired or fully induced gluconeogenesis, respectively. As expected [2], also deletions within the Hap complex caused a lethal phenotype, since it is needed for the transcriptional induction of TCA cycle and respiratory genes [40]. Thus, on ethanol, an active Snf1 pathway and a low PKA activity are essential for derepression of gluconeogenic genes, whereas the Hap complex might be of major importance for the induction of TCA cycle and respiration.

**Conclusion**

Overall, we characterized the impact of the four glucose signaling pathway on central carbon metabolites in dependency of the condition-induced state of repression (Fig 5). Although we could hypothesize on the signaling pathway targets that lead to these effects, it is still unclear how this regulation is achieved. There are three partially interconnected ways of regulation, which might contribute to the functional impact [57]: i) transcriptional regulation and expression [15, 16, 23, 24, 43, 58] ii) post-translational modifications [18, 31, 51, 59] and iii) regulation via intracellular metabolites such as fructose-2,6-bisphosphate inhibition of fructose-1,6-bisphosphatase [47], trehalose-6-phosphate inhibition of Hxk2 [60, 61], or the dependency of many reactions on a proper NAD(P)⁺/NAD(P)H ratio [62]. Thus, in order to systematically generate hypothesis about the way of metabolic regulation it is inevitable to integrate signaling-dependent changes in the transcriptome, proteome, and metabolome and to relate them with flux. After verification of regulatory hypothesis via more specific experiments one might close the mechanistic gap between signaling metabolism.
4.5 Material and Methods

**Strains and cultivation**

The strains used here, were obtained in Chapter 3. Batch cultures in 600 uL minimal medium [63] with either 10g/L glucose, galactose or ethanol were done in 96-deep-well plates with fritted bottoms (Cat. No. 278011, Nunc, USA), and a 4mm glass bead was added to each well for efficient mixing. The cultures were incubated in a plate shaker at 30°C and 300 rpm. To confirm the mid-exponential growth phase during sampling and to later normalize intracellular metabolite concentrations, culture growth was assessed by measuring OD$_{600}$ in a microplate reader (TECAN sunrise, Tecan group).

**Quenching and Metabolomics**

Metabolite samples were obtained by the application of the method described in [64]. Specifically, a 48-well plate containing 3.6 mL quenching solution (60% methanol, 10 mM ammonium acetate, pH 7.5) was precooled at -40°C. For sampling this plate was put into a vacuum manifold, and cultures in fritted plates were put on the top, before being harvested by applying a vacuum. After harvesting the 48-well plate was immediately cooled in an dry ice containing ethanol bath at ~-50°C. After centrifugation (4000 rpm, 5 min, -9°C) and discarding the quench solution, the 48-well plate with the cell pellets was transferred back to the cold ethanol bath (~-50°C). Cell pellets were resuspended in precooled extraction solution (75% ethanol, 10 mM, pH 7.5) and 30-50 µL $^{13}$C-labelled biomass was added as an internal standard for later measurements. For extraction the plate was transferred to a 80°C water bath for 3 minutes with a vortexing step at about every 30-45 seconds. After extraction, the plate was transferred back into the cold ethanol bath and cell debris was removed by centrifugation for 5 min at 4000 rpm and -9°C. The extracts were transferred into a 96-deep-well plate and stored at -80°C before being dried vacuum centrifuge (Christ-RVC 2–33 CD plus, Kuehner AG, Switzerland). Prior to determination of intracellular metabolites, extracts were dissolved in 50-100 µL ddH$_2$O.

Intracellular metabolite concentrations were obtained with the method described in [65]. Specifically we separated the metabolites with a Waters Acquity UPLC (Waters Corporation, Milford, MA) with a Waters Acquity T3 end-capped reverse-phase column (dimensions 150 mm × 2.1 mm × 1.8 µm; Waters Corporation) and assessed their intensity with a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray ionization source (Thermo Fisher Scientific) in negative mode with multiple reaction monitoring. The peak integration was done with an in-house software (B. Begemann & N. Zamboni, unpublished data) and peak areas were normalized to the $^{13}$C internal standard and to biomass. Fold-changes were obtained by the metabolite-wise normalization of all samples to the mean of wild-type intensities. From
these fold-changes we calculated the mean and the standard-deviation of at least biological duplicates and further determined the p-values by a two-sided t-test, assuming equal variance. The p-values were further metabolite-wise corrected for multiple testing with the mafdr function in Matlab, which is depicted with an q-value.
4.6 References


Chapter 5

Impact of Ras/PKA signaling on metabolism after a fast nutritional perturbation

Stefan Christen, Mikael Sunnaker, Sean Summers, Jörg Stelling, Uwe Sauer

Own contributions:

- Experimental setup for dynamic metabolomics
- Sampling with the assistance of Mikael Sunnaker and Sean Summers
- Measurement of intracellular metabolites by quantitative LC-MS/MS
- Metabolome analysis in the scope of this chapter
5.1 Abstract

*Saccharomyces cerevisiae* responds to extra- and intracellular signals via signal receptor systems. A prominent example is the Ras/PKA signaling pathway as the main mediator of glucose repression. Since this signaling pathway not only regulates various metabolic functions, but also depends on metabolic input signals, a double-reciprocal feedback system emerges. Here, we explore this feedback between the Ras/PKA signaling pathway and metabolism after glucose relief of starvation by dynamic metabolomics in deletion mutants of the Ras/PKA pathway. We found that the sequence of metabolic events was largely unchanged, but the amplitude and the timing varied a lot among the mutants. This implied that the RAS2 deletion mutant leads to less repression, whereas the deletion mutants of IRA1, IRA2 and PDE2 caused more repression prior to glucose addition. To contextualize the altered metabolic response with Ras/PKA pathway activity and to evaluate where a putative pH signal could enter, we used a mathematical model, which was parameterized after modular decomposition. We could identify the Ira and Pde proteins as the most likely receivers of a pH signal. Overall our study provides a first step to mechanistically explain how balanced Ras/PKA activity can be achieved in complex environments that might lead to conflicting signals, or to characterize its regulatory role in cell cycle progression under constant conditions.
5.2 Introduction

In order to achieve and maintain homeostasis, single-celled microorganisms have to cope with fast-changing nutritional environments. Thus they respond to extra- and intracellular signals via dedicated signal receptor systems or via internal feedback mechanisms in metabolism. As the main mediator of glucose repression the Ras/PKA signaling pathway is a sensing system of particular relevance in the yeast *Saccharomyces cerevisiae* that is activated by glucose [1-8] (Fig 1). Topologically, the Ras/PKA signaling pathway has a glucose-responsive upstream network that modulates the activity of adenylate cyclase (Cyr1) for generation of the internal signal cAMP (Fig 1). This upstream network has two branches: i) the G-protein coupled glucose receptor Gpr1 together with Gpa2 and ii) the guanine nucleotide exchange factor Cdc25 and the GTPases Ras1 and Ras2. Both branches are connected via the inhibitory GTPase-activating components Ira1 and Ira2, but also by directly targeting Cyr1. Once Cyr1 is activated by the upstream network, cAMP is generated and provokes the release of the binding between the downstream inhibitor Bcy1 and the main kinases of this signaling pathway (Tpk1, Tpk2 and Tpk3). In turn, these kinases regulate various cellular functions such as metabolism, ribosome biogenesis and cell cycle [2, 4, 5, 9]. Additionally, the Tpk kinases negatively feed back to cAMP via the cAMP phosphodiesterases Pde1 and Pde2 and to the upstream network by hyper-phosphorylating Cdc25 [1, 3, 6, 10, 11]. Therefore, the normal response of cAMP to glucose addition is a transient peak before going back to a basal level [1, 2, 6, 12]. Besides sensing external glucose through the G-protein coupled glucose receptor Gpr1, intracellular glucose metabolism is needed to fully activate Ras/PKA signaling [13, 14]. Specifically it was proposed, that besides the known substrates ATP and GTP, pH serves as a metabolism-dependent intracellular signal for the Ras/PKA pathway [6, 15-22]. However, mechanistically it remains unclear, where such a signal would enter the signaling pathway (Fig 1).

Given a reciprocal feedback between metabolism and the Ras/PKA pathway (Fig 1), we asked, to what extent are the signaling components of the pathway relevant for glucose relief of a derepressed physiological state such as starvation, and in turn, where a putative pH signal would enter the Ras/PKA pathway and modulate its activity. Thus, it is important to know how metabolism and the Ras/PKA jointly react upon glucose relief of derepression.

Upon glucose addition to derepressed wild-type yeast cultures first, glycolysis, pH and nucleotide phosphates respond and are followed by a cAMP peak [13, 14, 16, 24-27]. Specifically, the pH drops within the first seconds, and slowly recovers after about 30 seconds to a new steady state [15, 16, 24]. Upper glycolytic intermediates immediately increase and stabilize at about 30 seconds after glucose addition, whereas lower glycolytic intermediates decrease to low concentrations within the same time scale [25, 26, 28]. This opposite response is most probably caused by an imbalance of NAD+/NADH, which in turn
inhibits glyceraldehyde-dehydrogenase [25, 26, 28]. Nucleotide phosphates exhibit transient response within the same time scale. Specifically, ATP and the total AXP pool decrease within the first seconds due to hexose phosphorylation and interconversion to inosine. These metabolite pools subsequently recover to basal level due to mitochondrial activity, hence respiration, and remobilization of inosine [24, 29]. Other metabolites, such as TCA cycle intermediates or trehalose-6-phosphate either respond with a slow dynamic or a transient response after the cAMP peak, respectively [25, 30]. Particularly, the transient peak of trehalose-6-phosphate seems to be important as a feedback inhibitor for glycolysis to prevent accelerated cell death, hence a severe loss of energy due to fast hexose phosphorylation [31-33]. It is likely, that at least some of these events are regulated by PKA after glucose addition [2, 5, 30, 34-37]. However, although for most Ras/PKA signaling components the influence on the cAMP response was shown [15, 19, 21, 22, 30, 34, 38-41], their quantitative effect on entire central metabolic response is unclear.

Thus, in order to investigate, how metabolism and the Ras/PKA pathway are connected, we recorded the response of central carbon metabolites, nucleotide phosphates and cAMP after glucose relief of starvation in selected single deletion mutants of components of the Ras/PKA pathway. Specifically, we chose mutants covering both Cyr1 activating
branches of the upstream Ras/PKA network (Gpa2, Ras1 and Ras2), the upstream inhibitors Ira1 and Ira2, and the cAMP phosphodiesterases Pde1 and Pde2 from the downstream network. The trajectories of ATP, GTP, cAMP and wild-type pH were then used as in- and output for a mathematical model, which distinguishes the likelihood of the Ras/PKA components to be the receivers of a putative pH signal.
5.3 Results

**Metabolic response to a sudden relief of glucose starvation**

To verify the fast metabolic response upon glucose relief of starvation in dependency of the applied glucose concentration, we recorded dynamic profiles of central metabolic intermediates, nucleotide phosphates and cAMP for 5 minutes after adding 2% w/v, 0.1% w/v or 0.01% w/v glucose to 20 min starved cells. Generally, the course of metabolic intermediates was in agreement with reported data in similar experiments [15, 24-29]. The timing of the immediate metabolite response upon a glucose step was not affected by the applied glucose concentration, but the amplitude of change in metabolite levels was generally highest upon addition of 2% glucose (w/v), decreased at 0.1% (w/v) and entirely absent at 0.01 % glucose (Fig 2). This change in the amplitude is similar to Beullens et. al [27], who measured glycolytic intermediates after addition of different glucose concentrations, and reflects the response to an altered hexose transport [13]. Therefore we suggest, that the low response in glycolysis is caused by altered glucose uptake kinetics [42, 43]. The cAMP level peaked at about 30-45 seconds after glucose was added and decreased to the basal level after about 100 seconds, which is further consistent with [6, 14, 15, 27]. As expected, other metabolic changes occurred before this event (Fig 2). Specifically, upper glycolytic compounds (glucose-6-phosphate, fructose-1,6-bisphosphate and dihydroxyacetone-phosphate) increased immediately after glucose addition and stabilized within 30 seconds, whereas the lower glycolytic compounds 2-/3-phosphoglycerate and phosphoenol-pyruvate decreased. Nucleotide phosphates also changed within this time scale at higher glucose concentration and exhibited a sharp peak at 15 seconds (Fig 2). Specifically, ATP was decreasing, whereas ADP and AMP increased, which caused a transient drop of the energy charge. Thus, consistent with other observations [25, 29], yeast is transiently misbalanced in its energy homeostasis, that gets immediately restored, probably via still derepressed TCA cycle and respiration [24, 29]. Other metabolites exhibited either a slow continuous dynamic or they peaked after the cAMP signal. Specifically, 6-phosphogluconate in the PP pathway and the TCA cycle intermediates citrate, isocitrate, aconitate, succinate, and malate had a slow dynamic and did not reach a new steady state within 300 seconds. This continuous change might happen due to remodeling at the transcript level that starts after about 120 seconds [25]. Finally, the storage carbohydrate trehalose-6-phosphate transiently peaked about 30 seconds after cAMP. From the cAMP-like, but delayed trajectory we support the finding that the Ras/PKA pathway might activate trehalase, which degrades trehalose-6-phosphate [30, 37, 39].
Fig 2: Response of the wild-type to glucose relief of starvation with different glucose concentrations. A) intracellular metabolite trajectories upon glucose relief of starvation (t=0, dotted line) with different glucose concentrations (red: 2% (w/v); blue: 0.1% (w/v); green: 0.01% (w/v)). The light areas indicate the standard deviation and concentrations are given in nmoles/OD600 if not otherwise indicated ([AU]: arbitrary units). The time is given on the x-axis in seconds. B) intracellular pH trajectory of wild type yeast that grew on 2% glucose (white) and then was starved (grey) upon relief by 2% glucose (white). The time is given on the x-axis in minutes. (Adopted from [44])
Overall, we could confirm the assumed timing of metabolic events upon glucose relief of starvation in dependency on glucose concentration and set a basis for comparison in later experiments. Since the addition of 2% w/v glucose caused the strongest effect, we further used this concentration for later experiments.

**Ras2 is the main upstream activator and substantially reduces the cAMP response**

The upstream activators of the Ras/PKA signaling pathway are known to influence the transient cAMP peak after glucose addition to starved or carbon-limited yeast [14, 19, 34, 40, 45]. Furthermore, besides their potential transient relevance, these components might modulate basal Ras/PKA activity and thus alter metabolism prior to glucose addition. Therefore we here characterized the metabolic response of the single deletions of the Ras/PKA upstream activators RAS1, RAS2 and GPA2 after a glucose relief (2% w/v) of starvation.

All single deletions of the activators caused a reduction of the transient cAMP peak with the RAS2 deletion mutant being the most severe (Fig 3, Fig 4). This is consistent with [34, 40], which showed that a RAS2 deletion mutant abolishes the response in cAMP, and with the suggestion that Ras2 is a main regulator of the pathway [2, 4]. Besides reducing the cAMP peak, the RAS2 and GPA2 deletions affected the response in central carbon metabolism (Fig 3, Fig 4). Specifically, the glycolytic compounds responded with virtually identical dynamics as the wild-type, but, in the case of the GPA2 deletion mutant fructose-1,6-bisphosphate was reduced, and in the RAS2 deletion mutant additionally glucose-6-phosphate reached a lower steady state concentration (Fig 3). These effects might be explained by the deregulation of two Ras/PKA targets: i) hexose transport is reduced and thus less glucose can enter metabolism [2] and ii) less biosynthesis of fructose-2,6-bisphosphate causes less forward-activation of Pfk2 [35, 36]. Besides glycolysis, the TCA cycle compounds citrate/isocitrate and aconitate accumulated more rapidly in the RAS2 deletion mutant than in the wild type. Since it was shown that the Ras/PKA pathway represses TCA cycle [2, 4, 5], this indicates that the RAS2 deletion mutant led to increased TCA cycle activity. For the GPA2 deletion mutant, the measurements of TCA cycle showed an inconsistent behavior between the replicates and also compared to the wild type (Fig 4). Thus we could not derive a conclusion about its impact on respiratory activity. In the RAS2 deletion mutant trehalose-6-phosphate peaked at a much lower concentration than wild type yeast and further was slower degraded than in the wild type. The lower amplitude might be explained by a reduced glucose uptake, whereas the slow degradation might happen due to less trehalase activity, since this is a well-known target of the Ras/PKA pathway [30, 37, 39].
Fig 3: Response to glucose relief of starvation in mutants of the Ras/PKA upstream activators Ras1 and Ras2. Intracellular metabolite trajectories upon glucose relief (2% (w/v)) of starvation (t=0, dotted line) in deletion mutants of RAS1 (green), RAS2 (blue) compared to wild type yeast (red). The light areas indicate the standard deviation and concentrations are given in nmoles/OD$_{600}$ if not other indicated ([AU]: arbitrary units). The time is given on the x-axis in seconds.
Fig 4: Response to glucose relief of starvation in mutants of the Ras/PKA upstream activator Gpa2. Intracellular metabolite trajectories upon glucose relief (2% (w/v)) of starvation (t=0, dotted line) in GPA2 deletion mutant (blue) compared to wild type yeast (red). The light areas indicate the standard deviation and concentrations are given in nmoles/OD$_{600}$ if not other indicated ([AU]: arbitrary units). The time is given on the x-axis in seconds.
Overall this suggests, that Ras2 and to a smaller extent also Gpa2 seem to control basal activity of the Ras/PKA pathway and thus their deletions lead to decreased repression, hence an altered metabolic state, prior to glucose addition.

**Inhibitors of upstream PKA signaling affect metabolite dynamics and delay cAMP evolution**

In a second step we elucidated the response to glucose relief of repression in deletion mutants of the Ras/PKA inhibitors IRA1 or IRA2. In contrast to the RAS2 deletion, cAMP peaked in both mutants at an increased level, presumably due to pathway hyper-activation (Fig 5), but the cAMP peak was delayed by 120 seconds. However, generally the sequence of other metabolic events before and after the cAMP peak was similar to the wild type with some alterations. Fructose-1,6-bisphosphate overshoots at first, whereas trehalose-6-phosphate peaked with a delay and at a lower level. This is consistent with the finding that increased PKA activity enhances glycolysis via i) the activation of fructose-2,6-bisphosphate biosynthesis, which allosterically activates Pfk2 [35, 36] and ii) the activation of trehalase, which degrades the hexokinase inhibitor trehalose-6-phosphate and thus prevents accelerated cell death [30-32, 37, 39]. A proposed block at glyceraldehyde-3-phosphate dehydrogenase via redox metabolism [25, 26, 28] might further explain, why the pronounced accumulation occurs at fructose-1,6-bisphosphate. Additionally, the TCA cycle intermediates succinate, malate and aconitate did not respond at all and ATP dropped to a lower level and recovered with a strong delay compared to the wild-type. This indicates that increased PKA activity repressed TCA cycle and respiration already during starvation and thus ATP utilization upon glucose addition is less counteracted. Consistently, this would further explain, why the recovery of the energy charge is delayed, since it is exclusively dependent on glycolysis [29]. The overall consistent opposite effect compared to RAS2 deletion suggests, that Ira1 and Ira2 lead to stronger repression, which causes an altered response to glucose relief of repression.

**Downstream feedback to cAMP can be divided into transiently and constantly active components**

Finally, we characterized the metabolic response to glucose relief of starvation in the knockout mutants of PDE1 and PDE2, the downstream cAMP phosphodiesterases of the Ras/PKA pathway. Whereas Pde1 is a low affinity phosphodiesterase, which is thought to be responsible for the transient response of cAMP, Pde2 is a high-affinity phosphodiesterase that is suggested to control basal cAMP concentrations [11, 22]
Fig 5: Response to glucose relief of starvation in mutants of the Ras/PKA upstream inhibitors Ira1 and Ira2. Intracellular metabolite trajectories upon glucose relief (2% (w/v)) of starvation (t=0, dotted line) in deletion mutants of IRA1 (blue), IRA2 (green) compared to wild type yeast (red). The light areas indicate the standard deviation and concentrations are given in nmoles/OD$_{600}$ if not other indicated ([AU]: arbitrary units). The time is given on the x-axis in seconds.
Fig 6: Response to glucose relief of starvation in mutants of the Ras/PKA downstream feedback inhibitors Pde1 and Pde2. Intracellular metabolite trajectories upon glucose relief (2% (w/v)) of starvation (t=0, dotted line) in deletion mutants of PDE1 (green), PDE2 (blue) compared to wild type yeast (red). The light areas indicate the standard deviation and concentrations are given in nmoles/OD$_{600}$ if not other indicated ([AU]; arbitrary units). The time is given on the x-axis in seconds.
Consistent with their role in long- and short-term regulation of PKA activity, the responses in mutants carrying a PDE2 or PDE1 deletion were very different (Fig 6). The PDE2 deletion mutant responded similar to IRA1 and IRA2 deletions with a delayed cAMP peak, but no change in the sequence of metabolic events. Specifically, glycolytic intermediates responded first, with a strong overshoot of the fructose-1,6-bisphosphate and dihydroxyacetone-phosphate, whereas trehalose-6-phosphate peaked with a strong delay. The TCA cycle did not respond at all, and consistent with [29] the recovery of ATP concentration and the energy charge were delayed. Thus we can support the finding, that Pde2 is a long-term inhibitor of Ras/PKA and that upon its removal PKA is hyper-active. The PDE1 knockout, in contrast, responded similar to the wild-type at the level of central metabolic intermediates, but however, with a highly increased cAMP peak. The absence of a metabolic response supports the finding that Pde1 constitutes an exclusively transient regulator [22].

A model to identify the entry of a putative pH signal into the Ras/PKA pathway

Since the Ras/PKA signaling pathway is thought to be dependent on a putative pH signal, we asked if and where it would enter the pathway. To distinguish the contribution of various metabolic inputs (ATP, GTP and pH) and signaling components, as well as to handle the complex feedback-structure of the Ras/PKA pathway, it is indispensable to use a mathematical model. Specifically, we defined a mechanistic core model of the Ras/PKA pathway which is described in [44]. The model consisted of 26 state variables (abundance of PKA components), 3 measurable input signals (pH, ATP and GTP), 2 measurable output signals (cAMP, active Ras) and 38 unknown parameters. Additionally the glucose input was either set to 1 (2% w/v) or 0 (absent). Potential entry points, i.e. Ira1, Ira2, Pde1, Pde2, and Cyr1 [16, 19-22], were defined as individual hypothesis, of which each represents an extension of the model that adds 0-10 unknown parameters. Together with unknown initial conditions, the complete model has a total number of 64 unknown parameters that had to be fitted.

In order to reduce the number of unknown parameters, we made the assumption that the signaling system was in homeostasis, hence the signaling components were abundant, prior to the glucose step. An additional reduction of complexity was achieved by using data driven modular decomposition and genetic knockouts [46]. Specifically, data driven decomposition was newly developed to obtain sub-modules of the complete model, where each in- and output represented a measurable parameter. Since each module consisted of less unknown parameters than the complete model, complexity, hence the parameter space, could be reduced. An additional reduction of unknown parameters was achieved by the consideration of gene deletions of components within the Ras/PKA signaling pathway, since
all chemical species and reactions associated with a component would be eliminated. Specifically, deletions of RAS1 and RAS2 caused the highest reduction of unknown parameters, and thus these mutants were of major interest for parameter fitting and to rank different hypothesis about pH signal entry. However, since RAS2 deletion experimentally abolished cAMP evolution, it was considered to be the least informative and only used for model verification. Iterative Bayesian analysis was further used to fit the parameters of modules. Specifically, in each round of parameter fitting the size of the modules increased and thus step-wise additional parameters could be estimated until the full model was analyzed. This analysis suggested that either the Ira or the Pde proteins serve as receivers of a putative pH signal, whereas Cyr1 could be excluded.

Given the fitted parameters and the most likely hypothesis of signal entry, we aimed to verify the complete wild type model. Specifically, we analyzed whether the model could successfully predict the output signals (cAMP and active Ras) for wild-type and single deletion mutants of RAS1, RAS2, and the deletion mutants GPA2 and GPR1, which were not used for parameter fitting. In all cases, the model could predict the cAMP and active Ras trajectories (Fig 7 A), and supported that the Ira components serve as receivers of a pH signal, although they might not be exclusive. Finally, the model was used to predict the effect of single deletion mutants of IRA1, IRA2, PDE1, PDE2. However, the predictions did not capture the delay of the cAMP peak that was seen in the experiments (Fig 7 B). There are three possibilities why the model could not capture the dynamics: i) It might be that the not all parameters are sufficiently fitted, or ii) that basic assumptions, such as a similar initial state that all mutants exhibit a wild-type like pH trajectory, might be wrong, or iii) that the model structure is incorrect due to an unknown link. The first problem might be solved by a sensitivity analysis, to elucidate, which parameters were not constraint. However, since a global sensitivity analysis is computationally not feasible [47], a further fitting process with an unpredicted response, such as the deletions of the Ira proteins, might be more suitable to improve the model. The second problem needs a better definition of the initial state variables in dependency of the signaling knockouts, hence regulation of basal activity. This might be achieved by incorporation of transcript data done by [4, 5, 9] that indicate that Bcy1 is negatively and Ras1 positively feed-backed by the Ras/PKA pathway. Besides, we elucidated above, that some deletions led to a different metabolic state. Therefore one might speculate that also intracellular acidification and a following response of the pH are affected. In order to exclude that, it is important to determine the response of pH to glucose relief of starvation in the different single deletion mutants. The third problem might be most difficult, since it includes the definition of new hypothesis about either an additional signal to the Ras/PKA pathway or a new feed-back link within the pathway. From the above results we suggested that a link might emerge dependent from an ATP-dependent process, as ATP recovery had a similar delay as the cAMP signal. Since it was shown in other organisms, that ATP-linked compounds like CTP, UTP or pyrophosphate inhibit adenylate cyclase [48]
Fig 7: Model verification and predictions. (Adopted from [44]) A) Verification of the mathematical model of the Ras/PKA signaling pathway in wild type yeast and single deletions of RAS1, RAS2, GPA2 and GPR1. Measured output data of cAMP (nM) and active RAS (arbitrary units [AU]) are illustrated with red crosses and standard deviations. The best fit is depicted as black line, the mean is given as a blue line and 95% confidence intervals are given as grey areas. B) Predictions of cAMP (nM) and active RAS (arbitrary units [AU]) trajectories in deletion mutants of IRA1, IRA2, PDE1 and PDE2. The mean of the of the predictions is depicted in blue and the 95% confidence intervals are given by grey areas.
and these metabolites responded fast after glucose addition [25, 26, 29] one might speculate that they serve as additional signals for the timing of cAMP. To test such a hypothesis, one could firstly include an additional ‘ATP’-trajectory and evaluate, whether such a link would improve the model’s prediction.
5.4 Discussion

Here, we explored the reciprocal feedback between the Ras/PKA signaling pathway and metabolism after glucose relief of starvation. Specifically, we characterized for the first time the metabolic response in context of deletions of the Ras/PKA signaling pathway components, and attempted to elucidate where a putative pH signal would enter the signaling pathway.

First, we characterized the wild-type response to the addition of different glucose concentrations (2% w/v, 0.1% w/v and 0.01% w/v) after 20 min of starvation, which depicts a derepressed condition. Generally, the response to these different glucose concentrations was at similar speed, but decreasing with lower concentrations. This gradual effect was probably caused by kinetic properties of the hexose transport, since it was shown that hexose transporter mutants, which alter glucose affinity and capacity, provoke a similar response in glycolytic intermediates [13, 42, 43]. As expected, cAMP peaked about 30-45 seconds after glucose addition [13, 14, 27]. Consistent with [25, 26, 28], who measured central metabolic intermediates after glucose relief of starvation or carbon-limitation we found that several intermediates responded before cAMP peaked. Specifically, upper glycolytic intermediates increased and lower glycolytic intermediates decreased within the first seconds before reaching a new plateau after about 30 seconds. This opposite behavior of glycolysis was suggested to be caused by an altered NAD^+/NADH ratio that affects glyceraldehyde-dehydrogenase [25]. The response in nucleotide phosphates was within the same time scale, but transient. Specifically, ATP dropped and recovered within 30 seconds. The drop of ATP, hence an imbalance of energy homeostasis, is probably caused by fast hexose phosphorylation, whereas a subsequent recovery of ATP might at least be partly dependent on respiration, since it was shown that inhibition of respiration provoked a strong delay in ATP regeneration [29]. Consistent with [25], other metabolites such as the TCA cycle intermediates had a slow dynamic, whereas trehalose-6-phosphate transiently peaked about 30 seconds after cAMP.

Having characterized wild-type response to glucose relief of starvation, we asked whether this metabolic response is altered in mutants of the Ras/PKA signaling components. Since it is known, that the Ras/PKA pathway is not only involved during nutritional transitions, but its basal activity is also essential in steady state, the deletion of the signaling components might alter the metabolic system prior to glucose addition and alter a following metabolic response. Specifically, we characterized the metabolic response in deletions of the Ras/PKA upstream activators RAS1, RAS2 and GPA2, the upstream inhibitors IRA1 and IRA2 and in deletions of the downstream inhibitors PDE1 and PDE2. From these deletion mutants, we found that the upstream activator RAS2 and the inhibitors IRA1, IRA2 and PDE2 had a high
impact on the metabolic response upon glucose relief of repression and proposed that they provoked an enforced derepression or repression.

The RAS2 deletion was suggested to lead to more derepression. It did not affect the timing of metabolic events, but the amplitude of the response. Specifically, it highly reduced the peak of the cAMP level, which is consistent with Rolland et. al [14]. From the events before the cAMP peak, RAS2 deletion also affected the amplitude of glucose-6-phosphate and fructose-1,6-bisphosphate, which stabilized at a lower level than the wild-type. Moreover, the glycolytic response reflected the response to low glucose addition and since the Ras/PKA pathway is known to regulate hexose transport transcriptionally [2, 4, 49], we suggested, that RAS2 causes an impaired glucose uptake. Besides the response in glycolysis, the TCA cycle intermediates increased faster in the RAS2 deletion mutant. Since the TCA cycle is a known repression target of the Ras/PKA signaling pathway we suggested, that TCA cycle activity might be enhanced.

From the inhibitors of the PKA pathway, most metabolic effects were found for the deletion of the upstream GTPase-activating components IRA1 and IRA2, and the downstream phosphodiesterase PDE2. Therefore we suggested that they lead to more repression. Specifically, they provoked a delayed evolution of the cAMP signal. However, the sequence of the metabolic events before and after the cAMP peak did not change. In contrast to RAS2 deletion, these mutants exhibited overshooting levels of fructose-1,6-bisphosphate and a delayed build-up of trehalose-6-phosphate. This indicated that glycolysis was enhanced and not properly feed-backed. Specifically, such an effect could be explained by the view that i) Ras/PKA induces the production of fructose-2,6-bisphosphate, which activates Pfk2 [35] and ii) it induces trehalose-6-phosphate degradation [39, 50], which would otherwise inhibit hexokinase and accelerated cell death [32]. Additionally, the response in TCA cycle intermediates malate and succinate was absent. Thus we suggested that in contrast to the RAS2 deletion, that TCA cycle was probably already repressed prior to glucose addition. Consistent with this suggestion we found that ATP recovery is delayed as it got exclusively dependent on glycolysis. This is further in agreement with Walther et. al [29], who tested the effect of an inhibited respiration by addition of antimycin on ATP recovery. Since ATP was the only metabolite with a delayed recovery that occurred concomitant to the start of cAMP build-up, we speculated that ATP or an ATP-dependent metabolite might be relevant for the timing of the cAMP response.

Additionally we asked where a putative metabolic signal, specifically pH, would enter the Ras/PKA signaling pathway. To connect the metabolic response and the Ras/PKA signaling pathway, we defined a mathematical core model of the Ras/PKA signaling pathway that included ATP and GTP as input, and cAMP and active RAS as output. Furthermore hypotheses about pH signal entry were used as extensions. After complexity reduction via the novel approach of modular decomposition and the consideration of most informative knockout mutants (i.e. RAS1 and RAS2), parameters were iteratively fitted and the model
verified. The model could successfully predict the response upon deletion of the Ras/PKA activators and the Ira proteins were considered to be the most plausible receivers of a pH signal. However, later predictions could not capture the delayed cAMP response of the deletions of IRA1, IRA2 and PDE2. Since we suggested above, that several mutants lead to an altered metabolic response in central carbon metabolism, one might speculate, that they also affected intracellular pH. Thus the determination of the pH-trajectories in these mutants upon glucose relief of starvation might be the key step to shed more light on the double-feedback between metabolism and the Ras/PKA signaling pathway. Moreover the mechanistic exploration of this double-feedback might further explain how balanced Ras/PKA activity can be achieved in complex environments that might lead to conflicting signals, or how it regulates cell cycle progression under constant conditions.
5.5 Material and Methods

Strains

The strains were obtained from the haploid yeast or f library (Open Biosystems). Specifically, wild type and single deletion mutants of RAS1, RAS2, IRA1, IRA2, PDE1 and PDE2 carrying a KanMX resistance cassette were in the BY4741 background (ura3Δ0; leu2Δ0; his3Δ1; met15Δ0).

Cultivation, Quenching and Extraction

Precultures were done overnight in 500 mL shake flasks containing 30 mL SD medium (synthetic medium with 2% w/v glucose, 0.17% yeast nitrogen base, 0.5% (NH₄)₂SO₄). During mid-exponential growth the cells were centrifuged and washed two times with SC medium (SD without glucose). For carbon starvation they were again resuspended in 30 mL SC medium, transferred to a shake flask and incubated in a 30°C water bath under constant stirring (magnetic stirrer). After 20 min starvation glucose was added to a final concentration of either 2% w/v, 0.1% w/v or 0.01% w/v. In all experiments samples were taken at -5min and -1min prior to glucose addition, and afterwards in 10-15 second interval during the first minute, 20-30 second intervals during the second minute, and 30-60 seconds until the end of the experiment (usually 5 minutes). Specifically, at each sampling point 1mL of the culture was manually pipetted into precooled falcon tubes (ethanol bath with dry ice at ~-40°C) containing 4 mL quenching solution (60% methanol, 10 mM NH₄-acetate, pH 7.5) [51]. After centrifugation (5 min, 4000 rpm, -7 °C), the quenching solution was discarded and the cell pellets were stored at -80°C until extraction. At the beginning of the extraction 50-100 µL ¹³C-labelled biomass were added as an internal standard [52], and extraction was performed for 3 minutes at 80 °C with 1mL 75% boiling ethanol and 10 mM ammonium acetate (pH 7.5)[51]. After centrifugation (5 min, 4000 rpm, -9°C) supernatants, hence extracts were stored at -80°C until they were dried in a vacuum centrifuge (Christ-RVC 2–33 CD plus, Kuehner AG, Switzerland).

Intracellular metabolomics

Extracts were dissolved in 50-150 µL ddH₂O and metabolite concentrations were determined with liquid chromatography tandem mass spectrometry as described in [53, 54]. Specifically, we separated the metabolites with a Waters Acquity T3 end-capped reverse-phase column on a Waters Acquity UPLC (Waters Corporation, Milford, MA) and detected the metabolites with an Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with an ESI in negative mode. The acquired data was processed with an in-house software (Zamboni, Begemann, unpublished) and absolute concentrations of most central metabolites could be determined after normalization to
biomass and $^{13}$C-internal standard. Due to the consistent timing protocol, the mutant trajectories were batch-wise compared to the wild-type in at least biological duplicates.

Detailed model construction, additional data processing, and model evaluation will be described in the thesis of Mikael Sunnaker from the group of Prof. Jörg Stelling.
References

44. Sunnaker, M., Supplementary Information, 2013.
47. Sunnaker, M., 2013.

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Applications of metabolomics and flux analysis

Mass spectrometry-based metabolomics has found a wide range of biological applications in determining the presence and concentration of small molecules. Furthermore, mass spectrometry is often used to determine specific labeling patterns or dynamic label incorporation in metabolic intermediates. In combination with extracellular rates or intermediate concentrations these data could be used to estimate intracellular fluxes.

During my PhD I had the opportunity to take part in several collaboration projects, where we could successfully establish and apply metabolomics and flux analysis to answer biological questions in different organisms ranging from yeast to mammalian cells. Besides applying established methods we could develop new assays such as the sample preparation and a mass spectrometric method for the determination of the growth hormone 20-Ecdysone in *Drosophila melanogaster* larvae. Here we give an overview of these projects by providing the abstract and a short description of our contribution.
Local requirement of the *Drosophila* insulin binding protein imp-L2 in coordinating developmental progression with nutritional conditions

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In *Drosophila*, growth takes place during the larval stages until the formation of the pupa. Starvation delays pupariation to allow prolonged feeding, ensuring that the animal reaches an appropriate size to form a fertile adult. Pupariation is induced by a peak of the steroid hormone ecdysone produced by the prothoracic gland (PG) after larvae have reached a certain body mass. Local downregulation of the insulin/insulin-like growth factor signaling (IIS) activity in the PG interferes with ecdysone production, indicating that IIS activity in the PG couples the nutritional state to development. However, the underlying mechanism is not well understood. In this study we show that the secreted Imaginal morphogenesis protein-Late 2 (Imp-L2), a growth inhibitor in *Drosophila*, is involved in this process. Imp-L2 inhibits the activity of the *Drosophila* insulin-like peptides by direct binding and is expressed by specific cells in the brain, the ring gland, the gut and the fat body. We demonstrate that Imp-L2 is required to regulate and adapt developmental timing to nutritional conditions by regulating IIS activity in the PG. Increasing Imp-L2 expression at its endogenous sites using an Imp-L2-Gal4 driver delays pupariation, while Imp-L2 mutants exhibit a slight acceleration of development. These effects are strongly enhanced by starvation and are accompanied by massive alterations of ecdysone production resulting most likely from increased Imp-L2 production by neurons directly contacting the PG and not from elevated Imp-L2 levels in the hemolymph. Taken together our results suggest that Imp-L2-expressing neurons sense the nutritional state of *Drosophila* larvae and coordinate dietary information and ecdysone production to adjust developmental timing under starvation conditions.

**Contribution:**

In *Drosophila melanogaster* the progression through larval stages and pupariation are tightly controlled by dietary and 20-Ecdysone. In this study we investigated the role of Imp-L2 in this process with the specific question, whether it rules 20-Ecdysone. To determine 20-Ecdysone concentrations in Drosophila larvae we developed a mass spectrometric assay on an Agilent 1290 Infinity liquid chromatography-system coupled with an AB Sciex Qtrap 5500 in positive mode. Specifically, we generated an MRM method based on the online fragmentation of 20-Ecdysone in the enhanced product ion mode. Besides, we established a
protocol to extract 20-Ecdysone from Drosophila larvae, which included the homogenization in methanol with glass beads and delipidation with hexane. From the obtained data we found that 20-Ecdysone levels were delayed in Imp-L2 overexpressing larvae, whereas Imp-L2 mutant larvae showed a strong increase 20-Ecdysone. Thus, together with 20-Ecdysone feeding experiments, we concluded that Imp-L2 controls 20-Ecdysone production during larval development and consequently the transition to pupariation.
Quantitative Phosphoproteomics Reveal mTORC1 Activates de Novo Pyrimidine Synthesis

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The Ser-Thr kinase mammalian target of rapamycin (mTOR) controls cell growth and metabolism by stimulating glycolysis and synthesis of proteins and lipids. To further understand the central role of mTOR in cell physiology, we used quantitative phosphoproteomics to identify substrates or downstream effectors of the two mTOR complexes. mTOR controlled the phosphorylation of 335 proteins, including CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase). CAD catalyzes the first three steps in de novo pyrimidine synthesis. mTORC1 indirectly phosphorylated CAD-S1859 through S6 kinase (S6K). CAD-S1859 phosphorylation promoted CAD oligomerization and thereby stimulated de novo synthesis of pyrimidines and progression through S phase of the cell cycle in mammalian cells. Thus, mTORC1 also stimulates the synthesis of nucleotides to control cell proliferation.

Contribution:

In a phosphoproteomic screen we found that CAD, a key enzyme complex of de novo pyrimidine biosynthesis, is a phospho-target of mTORC1 signaling. In order to functionally verify the control of mTORC1 over de novo pyrimidine biosynthesis, we measured $^{15}$N-incorporation into the intermediates of this pathway, as well as their concentration. In particular, we contributed to the experimental design for labeling experiments and established a mass spectrometry method on the basis of [1]. Our main results suggested that both, starvation and rapamycin addition strongly reduced de novo pyrimidine biosynthesis. In conjunction with cell cycle analysis and rescue experiments by uridine addition to CAD mutant cells we further concluded that mTORC1 stimulates pyrimidine biosynthesis via CAD phosphorylation and therefore controls cell cycle progression through S phase.
Aneuploid yeast strains exhibit defects in cell growth and passage through START

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Aneuploidy, a chromosome content that is not a multiple of the haploid karyotype, is associated with reduced fitness in all organisms analyzed to date. In budding yeast aneuploidy causes cell proliferation defects, with many different aneuploid strains exhibiting a delay in G1, a cell cycle stage governed by extracellular cues, growth rate, and cell cycle events. Here we characterize this G1 delay. We show that 10 of 14 aneuploid yeast strains exhibit a growth defect during G1. Furthermore, 10 of 14 aneuploid strains display a cell cycle entry delay that correlates with the size of the additional chromosome. This cell cycle entry delay is due to a delayed accumulation of G1 cyclins that can be suppressed by supplying cells with high levels of a G1 cyclin. Our results indicate that aneuploidy frequently interferes with the ability of cells to grow and, as with many other cellular stresses, entry into the cell cycle.

Contribution:

Aneuploid yeast cells have an abnormal number of chromosomes that often goes together with reduced fitness. To determine whether reduced fitness is caused by a lack of amino acids, we measured intracellular amino acid pools in 5 different aneuploid yeast strains with a GC-TOF [2]. We found that the aneuploid yeast strains did not have depleted amino acid concentrations compared to the wild type and therefore suggested that amino acids are not limiting for growth.
A prototrophic deletion mutant collection for yeast metabolomics and systems biology

Michael Mülleder, Floriana Capuano, Pinar Pir, Stefan Christen, Uwe Sauer, Stephen G Oliver & Markus Ralser


Description:

Auxotrophic markers are widely used in yeast genetics but alter cellular physiology. Here, the physiological effect of all combinations of auxotrophic markers was characterized. Auxotrophic markers interact epistatically and affect fundamental processes such as growth rate and chronological lifespan. To alleviate such unwanted interferences, a prototrophic deletion library was constructed.

Contribution:

To evaluate the effect of auxotrophic markers on intracellular metabolites we performed quantitative metabolomics [3] on yeast strains carrying all combinations of them. Particularly, we were interested in the determination of the adenylate energy charge, which serves as a readout of energy homeostasis. We found that although auxotrophic yeast strains have an altered physiology, the adenylate energy charge was not affected. Thus auxotrophic markers do not cause a deregulation of energy homeostasis.
Unraveling condition-dependent networks of transcription factors that control metabolic pathway activity in yeast

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Which transcription factors control the distribution of metabolic fluxes under a given condition? We address this question by systematically quantifying metabolic fluxes in 119 transcription factor deletion mutants of *Saccharomyces cerevisiae* under five growth conditions. While most knockouts did not affect fluxes, we identified 42 condition-dependent interactions that were mediated by a total of 23 transcription factors that control almost exclusively the cellular decision between respiration and fermentation. This relatively sparse, condition-specific network of active metabolic control contrasts with the much larger gene regulation network inferred from expression and DNA binding data. Based on protein and transcript analyses in key mutants, we identified three enzymes in the tricarboxylic acid cycle as the key targets of this transcriptional control. For the transcription factor Gcn4, we demonstrate that this control is mediated through the PKA and Snf1 signaling cascade. The discrepancy between flux response predictions, based on the known regulatory network architecture and our functional $^{13}$C-data, demonstrates the importance of identifying and quantifying the extent to which regulatory effectors alter cellular functions.

Contribution:

Many transcription factors are suggested to be involved in the regulation of central metabolism. However, their single deletion often did not lead to a detectable phenotype at the level of intracellular flux ratios. An explanation for this is given by the potential redundancy within the transcription regulatory network. To test this hypothesis we performed $^{13}$C-flux analysis [4] with double and triple knockouts of selected transcription factors (*i.e.* Nrg1 and Nrg2, Msn2 and Msn4, and Mig1, Mig2 and Mig3). We found that these mutants did not have alterations in flux ratios, which further indicated that the observed metabolic robustness was not caused by transcription factor redundancy.
6.1 References


Chapter 7

Summary and Outlook

In this thesis we quantitatively explored the dependency of metabolism on the evolution of Crabtree metabolism, and on conditional regulation in the context of glucose repression in *S. cerevisiae*.

In Chapter 2 we characterized aerobic glucose metabolism in seven yeast species by $^{13}$C flux analysis and metabolomics to answer the questions, whether the Crabtree-metabolism hence respiro-fermentative metabolism, evolved gradually, and whether metabolites could serve as general flux readout. We found, that although extracellular rates indicate a gradual change between aerobic and respiro-fermentative metabolism, on the basis of intracellular fluxes the species could be clearly classified into the distinct groups of Crabtree-negative and positive lifestyle. From the determined metabolites we found, that they mostly do not correlate with intracellular flux in a species–overarching manner, and thus were rather species-specific. The exception was found in fructose-1,6-bisphosphate and dihydroxyacetone-phosphate, which both correlated with glycolytic flux. Since it was shown, that fructose-1,6-bisphosphate could regulate respiration [1] and in *E. coli* it was suggested, that fructose-1,6-bisphosphate is a glycolytic flux sensor that could mediate regulation via the transcription factor Cra, one might speculate that this compound has a similar, even conserved, function in yeast. In order to elucidate the role of fructose-1,6-bisphosphate as a flux sensor, one might firstly uncover, with which proteins this compound interacts. A brute force approach to identify these proteins might be to pack a column with fructose-1,6-bisphosphate, to apply whole cell broth extract and to perform proteomics on the eluted fractions. From the found candidate proteins, one could derive hypothesis about a regulatory system, which are then evaluated by computational models [2]. Finally, one could verify the proposed system by interrupting the system, for example via genetic manipulation of the binding sites for the metabolite.

In Chapter 3 we investigated the impact of glucose signaling on condition-dependent growth and physiology in *S. cerevisiae* by the analysis of growth profiles of prototrophic single deletion mutants of most glucose signaling components and selected double deletion mutants during nutrient shifts between differential repressive conditions. From the derived growth parameters we could verify key signaling components, which interrupt or constitutively activate a signaling pathway. Specifically, deletions of SNF1 and SNF4 interrupt and the deletion of HEX2 constitutively activates the Snf1 pathway, deletion of GRR1 interrupts the Snf3/Rgt2 pathway, the double deletion of RAS1 and RAS2 interrupts and deletion of BCY 1 constitutively activates the Ras/PKA pathway, and the deletion of each component of the Hap complex interrupts its activity. Their signaling pathway specific effect on growth and
physiology implied that a well-balanced Ras/PKA pathway activity Snf1 pathway activity are important for optimal, hence wild type-like, growth and physiology under all conditions, whereas the activity of Hap complex and the Snf3/Rgt2 pathway were needed under respiratory conditions and glucose, respectively. Besides, we could verify the redundant role between Hxk2 and the Ras/PKA pathway in glucose repression. However, to topologically explain this redundant regulation and redundancies in general one might need more selected multiple deletions and evaluate the growth phenotypes emerging from genetic interactions [3] in a condition-dependent manner.

The overall variable growth profiles, especially differences in yield that were found in Chapter 3 indicated differential metabolic regulation. In order to specify the quantitative impact of the glucose signaling components on metabolism we determined their metabotypes by LC-MS/MS under the differential repressive conditions glucose, galactose and ethanol in Chapter 4. We found that central metabolic pathways were differentially regulated dependent on the particular signaling pathway and the applied condition. For example on glucose, upper glycolysis was primarily affected by constitutive activation of the Snf1 pathway and interruption of the Snf3/Rgt2 pathway, whereas on galactose glycolysis was dependent on a balanced Snf1 activity and interruption of the Snf3/Rgt2 pathway did not exhibit an effect. Surprisingly, we found that the Hap complex seems to regulate the branch point between upper and lower glycolysis, although no direct transcriptional target was known. Thus, overall we could set quantitative links between signaling and metabolism. However, since the quantitative phenotype resembles the integrated outcome of a response that emerges at various and so far only partially explored regulatory layers, the question about the mechanism behind these links remains to be answered. First steps to analyze the involved regulatory layers were done in the wild-type yeast, where by now datasets about the condition-dependent proteome, transcriptome and phospho-proteome and fluxome exist [4-9]. These datasets might be used to make condition dependent interaction networks between regulatory layers. For the Snf1-pathway it was further demonstrated, that the integration of existing networks and the according data in knockout mutants within the signaling pathway, could lead to the identification of regulatory sub-networks. Moreover, this allowed the reconstruction of the functional Snf1 kinase regulatory network and to mechanistically resolve its function as a low-energy check-point [10]. Thus, additional complementary condition- and signaling-pathway-dependent readouts at different regulatory layers, such as the proteome and its post-translational modifications, or the fluxome, might help to further investigate functional signaling regulatory networks of one or more signaling pathways. Another, yet unresolved issue is the nature of the signals that activate the signaling pathways [11-14]. Moreover, it was suggested that various signaling pathways might be regulated by metabolism. A key step to find these signals might lie in the simultaneous assessment of the metabolome and signaling activity (e.g. by measuring phosphorylation of the targets) under dynamically changing conditions. From the obtained trajectories one could extract candidate
metabolites that respond prior to the signaling regulation. These candidates might further be used as hypothesis in a mathematical model that evaluates their plausibility as signaling inputs by setting them into relation with the output, which might be non-linear. The last step would then be to experimentally prove predictions about the output of the model upon perturbation of the signal, or the signaling pathway.

Another important aspect is that the Ras/PKA pathway might not exclusively rely on a receptor that receives an extracellular signal, but rather is dependent on an intracellular signal, such as pH, that is derived from metabolism. This leads to a complex double-reciprocal feedback system between signaling and metabolism. In Chapter 5, we attempted to shed more light on this feedback system in the Ras/PKA signaling pathway by an interdisciplinary approach together with the Prof Stelling group that included metabolomics, intracellular pH-measurements and mathematical modeling. In wild type yeast we could confirm the assumed sequence of metabolic events, that suggested that glycolytic intermediates and nucleotides respond before cAMP, a main mediator of PKA activity, whereas trehalose-6-phosphate peaked after cAMP. We further found in deletion mutants of the Ras/PKA signaling pathway that the response upon glucose relief of starvation is highly dependent on Ras2, Ira1, Ira2 and Pde2. Specifically, RAS2 deletion led to a more derepressed and the deletion mutants of IRA1, IRA2 and PDE2 led to a more repressed state prior to perturbation. The enforced derepression and repression majorly affected the amplitude and the timing of the metabolic response, but not the sequence. In a second step we used specific metabolite trajectories, i.e. ATP, GTP and wild-type measurements of pH as an input, and cAMP and active Ras as an output for a mathematical model of the Ras/PKA pathway. Specifically, we aimed to verify where besides the defined entry points of ATP and GTP, pH would affect signaling pathway activity. The model was parameterized by the application of the newly developed computational approach of modular decomposition and by the inclusion of the wild-type and Ras knockout data. After model verification, we concluded that the Pde or Ira proteins are the most plausible receivers of a pH signal. However, the model could not capture the response of the deletion mutants of IRA1, IRA2 and PDE2, presumably due to the assumption, that pH was not affected in these individual mutants. In order to close the double-reciprocal feedback between the Ras/PKA pathway and metabolism it is therefore inevitable to determine pH trajectories in these particular signaling mutants. Future questions lie in the elucidation, how pH is mechanistically linked to metabolism. Overall, given the dependency of the Ras/PKA signaling pathway on specified intracellular signals and in line with the idea of flux sensing [15, 16], one might further use metabolomics, intracellular pH and the here created model to elucidate how a balanced PKA signaling activity can be achieved in complex environments that might lead to conflicting signals, or to characterize its regulatory role in cell cycle progression under constant conditions.
7.1 References


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