Chapter 4
Systems Biology: Developments and Applications

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

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

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Box 4.1 Key Technological Developments

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

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

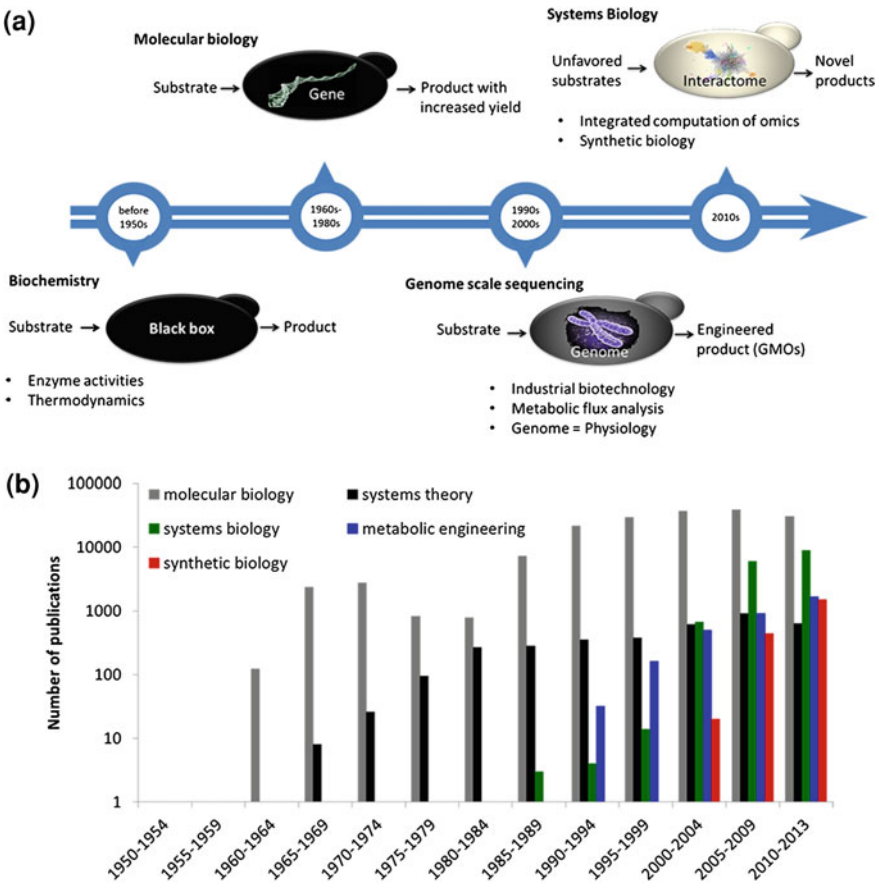

Fig. 4.1 Toward holistic understanding of biological systems: **a** An overview of the scientific progress from the "*black box*" model to the mechanistic details at molecular level that may help to explain phenotypes. Beginning from the determination of the DNA structure, key innovations (see Box 4.1)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

4.1 Yeast Carbon Metabolism: Progress

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

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

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

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

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

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

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

4.2 Molecular Mechanisms in Glucose Metabolism

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

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

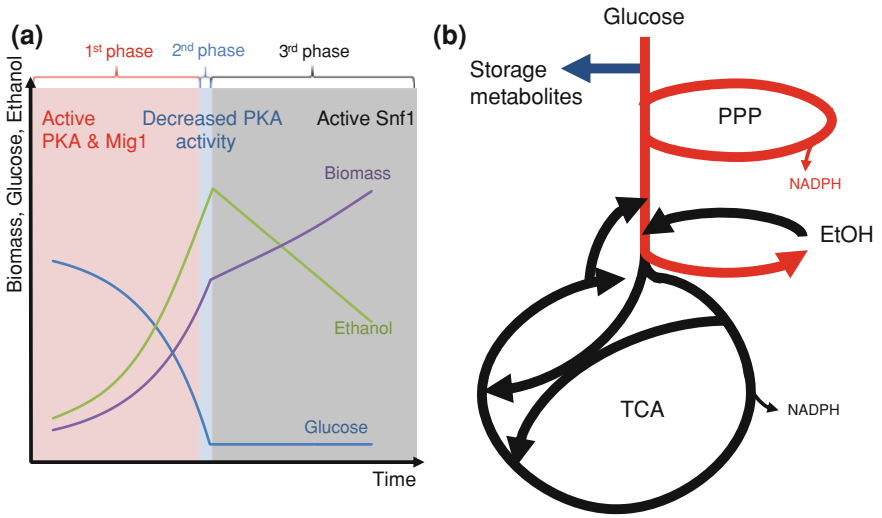

Fig. 4.2 **a** Typical time profile of the diauxic shift. In the 1st phase, there is consumption of glucose with co-current formation of ethanol and biomass. In the 2nd phase, there is transition, and in the 3rd phase, there is consumption of ethanol and further growth of the biomass. Activity of key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

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

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

4.3 Molecular Mechanisms in Galactose Metabolism

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

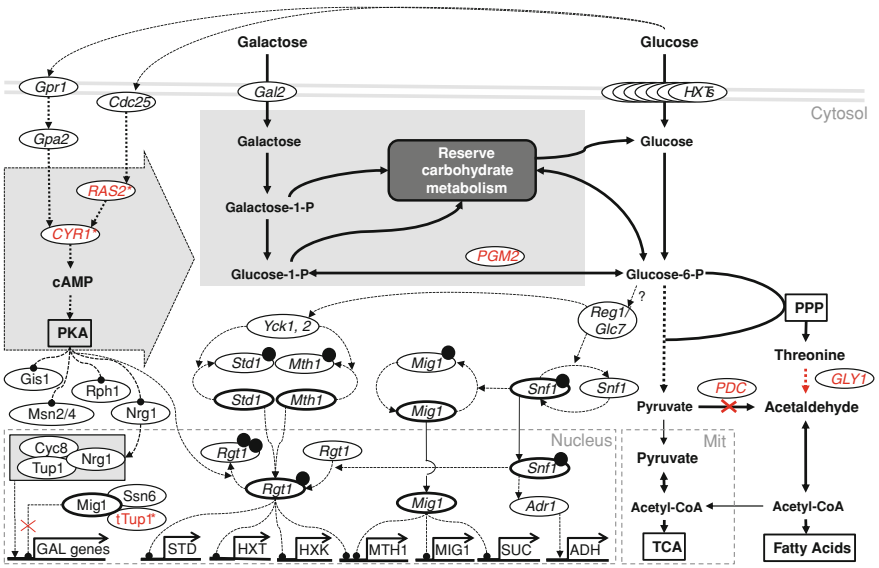

Fig. 4.3 Glucose and galactose metabolism in *S. cerevisiae*: Glucose causes carbon catabolite repression, where other carbon sources cannot be consumed in the presence of glucose. Systems-level analysis has suggested mechanisms for some of the key metabolic regulation related problems in *S. cerevisiae*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

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

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

4.4 Perspective

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

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

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

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