INSTITUTIONEN FÖR KEMI OCH MOLEKYLÄRBIOLOGI

Systems-level investigation of the interaction between glucose metabolism and the Snf1/Mig1 signalling pathway

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Systems-level investigation of the interaction between glucose metabolism and the Snf1/Mig1 signalling pathway

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”Excelsior!”

- Stan Lee

Abstract

Saccharomyces cerevisiae Snf1 and its mammalian homolog, AMPK, are members of a protein kinase family present throughout the Eukaryotic kingdom. AMPK plays an essential role in different cellular processes and is involved in diseases such as diabetes, obesity and cancer. Snf1 in yeast is a central component of metabolic switching and influences a broad spectrum of cellular processes such as lipid synthesis, glucose uptake and glucose metabolism. This kinase also plays a distinct role in other stress responses. When glucose becomes limiting, the Snf1 kinase phosphorylates, among others, the Mig1 transcriptional repressor causing it to exit the nucleus, resulting in derepression of gene expression. Many components of glucose signalling are already known, however there are still some caveats in our knowledge. Here, additional details are presented on how glucose metabolism influences the functioning of the Snf1/Mig1 pathway and how the glucose signalling interaction network is integrated with other cellular processes. Another aspect of this work centred on the individual yeast cells responses to glucose. Both empirical observations and mathematical modelling was used to predict the outcome of glucose signalling and to identify the source(s) of the significant cell-to-cell variability in the response to carbon source availability. We report a novel modelling approach to explain cell-to-cell variability in the response of individual yeast cells to glucose and reconstruct large signalling networks. Taken together, the importance of individuality of single yeast cells is highlighted by glucose signalling displaying considerable variability at the level of individuals. Furthermore, this work shows that glucose metabolism mediates a dynamic and stringent regulation of Snf1/Mig1 pathway dynamic.

Keywords: glucose signalling**,** microfluidics, *Saccharomyces cerevisiae*.

List of papers

I. **Niek Welkenhuysen**, Johannes Borgqvist, Mattias Backman, Loubna Bendrioua, Mattias Goksör, Caroline B. Adiels, Marija Cvijovic, Stefan Hohmann. Single-cell study links metabolism with nutrient signalling and reveals sources of variability. **FEBS Journal.** *under review*

II. **Niek Welkenhuysen**, Gregor Schmidt, Stefan Hohmann. Mig1 requires glucose phosphorylation for transient nuclear localization but Hxk2 to repress *SUC2*. *manuscript in preparation*

III. Timo Lubitz , **Niek Welkenhuysen**, Sviatlana Shashkova, Loubna Bendrioua, Stefan Hohmann , Edda Klipp and Markus Krantz. Network reconstruction and validation of the Snf1/AMPK pathway in baker's yeast based on a comprehensive literature review. **npj Systems Biology and Applications** 2015 1, 15007.

IV. Adam J. M. Wollman*, **Niek Welkenhuysen***, Stefan Hohmann, Mark C. Leake. Dynamic time-resolved sub-cellular proteomics. **Nature Methods brief communication**, *manuscript in preparation*

V. Kristofer Bodvard*, Ken Peeters*, Friederike Roger*, Natalie Romanov, Aeid Igbaria, **Niek Welkenhuysen,** Wolfgang Reiter, Michel B. Toledano, Mikael Käll and Mikael Molin, Light-sensing via hydrogen peroxide and a peroxiredoxin. **Nature Communications**, *manuscript resubmitted*

*: authors contributed equally

Paper contributions

Paper I-II, IV

I performed all the experimental work, contributed to the modelling and contributed to a major part of the writing for the manuscript.

Paper II

I performed the major part of the experimental work, and contributed to a major part of the writing for the manuscript.

Paper III

I performed a part of the literature review and wrote a minor part of the manuscript.

Paper IV

I performed the major part of the experimental work, and contributed to a major part of the writing for the manuscript.

Paper V

I assisted in data analysis and contributed to the script for data analysis.

Papers not included

Soheil Rastgou Talemi*, Carl-Fredrik Tiger*, Mikael Andersson, Roja Babazadeh, **Niek Welkenhuysen**, Edda Klipp, Stefan Hohmann, Jörg Schaber, Systems Level Analysis of the Yeast Osmo-Stat, **Scientific Reports** 2016 6,30950.

Sviatlana Shashkova *, **Niek Welkenhuysen** *, Stefan Hohmann. Molecular communication: crosstalk between the Snf1 and other signaling pathways. **FEMS Yeast Research**. 2015 Jun; 15(4):fov026. **[review]**

Niek Welkenhuysen, Caroline B. Adiels, Mattias Goksör and Stefan Hohmann. Applying microfluidic device to study effects of glucose at single cell level. Glucose **Transport volume, Methods in Molecular Biology**, *under review* **[bookchapter]**

*: authors contributed equally

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1 Preface: what is life?

Life is defined by systems which can reproduce, respond to stimuli, process information and maintain balances. These systems can consist of millions of cells organized in complex structures, such as humans, or be composed of a single individual cell such as the yeast *Saccharomyces cerevisiae.* Living cells are able to operate due to molecules from metabolites such as adenosine triphosphate (ATP) to macromolecules such as proteins, deoxyribonucleic acid (DNA) and complexes thereof (e.g. ribosomes). Accordingly, cells can carry out a wide variety of chemical reactions to produce and consume a broad spectrum of molecules. We still do not completely understand the workings of living cells, particularly about how information is transduced and how cells generate an appropriate response. With this work and the scientific articles produced during my PhD education I hope to have contributed to the knowledge about how living cells work and define further what life is.

2 Nutrients: the building blocks of life

Nutrients, such as carbon sources, provide the cell with the energy and the building blocks that are essential for its survival and proliferation. In the cell environment, a broad spectrum of usable nutrients is present, comprising the basic building blocks such as C, N, P, H etc. The cell needs to import these nutrients and afterwards metabolize them into the various cellular components. Many organisms are able to compensate for the decreasing availability of one substrate through the utilization of another. Typically, the cell prefers to use richer substrates before the substrates with a lower nutritional value. To alter the substrate which is used, the cell requires a switch in its gene expression profile. This extensive cell reprogramming requires a rigorous regulation of nutrient uptake and usage. To achieve this switch, several nutrient-controlled signalling pathways are activated or inactivated. As will be discussed later, the yeast *Saccharomyces cerevisiae* has been a favoured model organism to study metabolism and metabolic regulation (Rodkaer and Faergeman 2014).

Some key nutrients are sensed extracellularly, but for many other nutrients, a sensing system remains to be discovered. It is hard to imagine that one organism would have extracellular sensors for all conceivable nutrients in the environment. Therefore it could be that the cell identifies these metabolites in another way. It seems that for the majority of nutrients there is a need of at least partial metabolism before a stimulus is generated (Huberts *et al.* 2012). For example, sensing of glucose occurs through membrane receptors, such as in the Snf3-Rgt2 pathway, or by intracellular sensing mechanisms, such as in the Snf1-Mig1 pathway (Conrad *et al.* 2014). Glucose sensing pathways that employ membrane-localized receptors are relatively well understood. However, the sensing mechanism of intracellular glucose or metabolites from glycolysis is not completely understood (Broach 2012,

Conrad *et al*. 2014). Among those pathways sensing metabolites intracellularly is the AMPK/Snf1 system. This system controls energy homeostasis and is mainly known for its role in glucose de/repression. *Saccharomyces cerevisiae* preferentially uses rapidly fermentable sugars, like glucose, fructose or mannose as a carbon source. In the presence of preferable carbons sources the Snf1 pathway is inactivated. However, in absence of preferable carbon sources the Snf1 pathway is activated, and this allows for the upregulation of components required for the utilization of alternative carbon sources.

3. Yeast as a model organism

The unicellular budding yeast *Saccharomyces cerevisiae* is commonly used in beer brewing, winemaking, food production and synthesis of many useful compounds (Figure 1). *S. cerevisiae* has served as research subject in many fundamental and ground-breaking studies, some of which have earnt their authors a Nobel prize (Hohmann 2016). Yeast distinguish themselves as model organisms to study mammalian cells because, in contrast to other unicellular organisms such as bacteria and archaea, they have organelles such as mitochondria and nuclei. Despite the evolutionary distance between yeast and mammals, they still share elementary cellular processes on a fundamental level (Figure 1). Shared processes between mammalians and yeasts include, but are not restricted to, metabolism, transcriptional regulation, cytoskeleton dynamics, organelle synthesis, protein folding and secretion (Botstein and Fink 2011). Since basic cellular processes in yeast and mammalian cells are similar, the cellular implications of human diseases such as Alzheimer and Parkinson can be studied in yeast cells (Khurana and Lindquist 2010) (Figure 1).

A large toolbox of experimental methods has been established for *S. cerevisiae*. Databases and collections, such as the genomic deletion, epitope and fluorescence tagged protein collections, and yeast two-hybrid screening, are available to study *S. cerevisiae* (Ghaemmaghami *et al.* 2003, Khurana and Lindquist 2010). Yeast was the first eukaryotic organism whose complete genome sequence was deciphered (Goffeau *et al.* 1996). In the $1.3x10^4$ kbp long genome there are over 6000 ORFs of which 80% have a known function and 60% have a homologue in the human genome (Ghaemmaghami *et al.* 2003, Khurana and Lindquist 2010). Further advantages are that yeast has a rather short generation time, is inexpensive, easy to handle, can be stored for longer periods, is harmless to humans, and is genetically

relatively stable. Another important advantage is that the yeast genome is straightforward to manipulate as a result of an efficient system for homologous recombination (Miller-Fleming *et al.* 2008). Information about the genome and associated discoveries about genes and proteins can be easily found in the Saccharomyces Genome Database (http:/www.yeastgenome.org). Finally, its capacity to grow under a wide variety of conditions has made yeast a fruitful model to study metabolic phenomena and metabolic signalling (Rodkaer and Faergeman 2014).

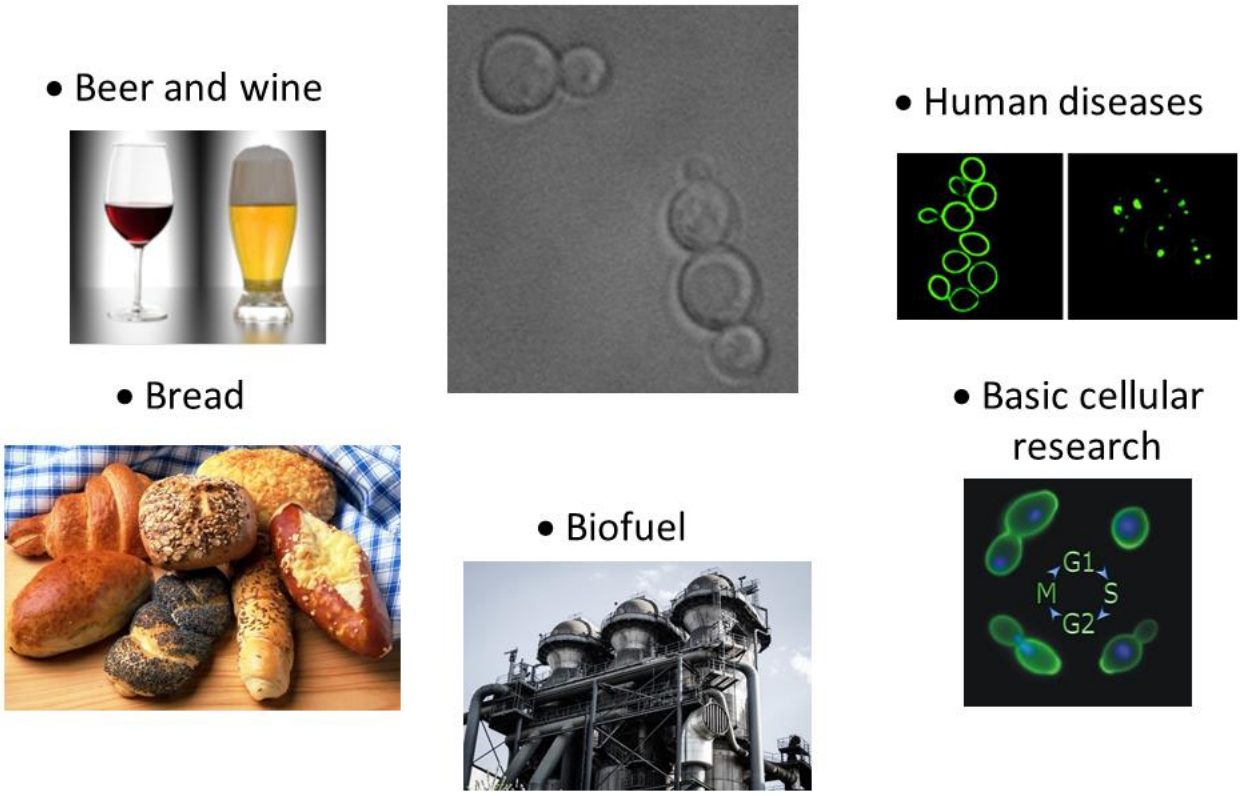

Figure 1: The yeast *Saccharomyces cerevisiae* and its applications. In the middle a microscopic transmission image of *Saccharomyces cerevisiae* cells obtained during a typical microfluidic experiment. Around the middle picture are several images of applications in which *Saccharomyces cerevisiae* can be utilized. From left hand side counter clockwise is beer and winemaking, bread production, synthesis of chemical components, basic research (indicated as cell cycle control) and human diseases (indicated as protein aggregation of α-synuclein).

4 Yeast metabolism: processing the building blocks

As discussed in the previous chapter; the environment of the cell contains many valuable nutrients. An efficient and rapid metabolism gives an organism a big evolutionary advantage when competing with other organisms for the same nutrients. Metabolism comprises a series of chemical reactions, and can be divided into two separate parts, catabolism and anabolism. Anabolism is the process whereby the cell produces complex molecules for the build-up of cell mass from simple chemical building blocks. Catabolism provides the cell with energy to balance the energy homeostasis. This process is similar to combustion, whereby fuel is converted into water and energy. In the cell this happens stepwise, which increases the efficiency of the process, prevents too much energy from being released at once, and enables the capture of energy in the form of ATP (Lodish *et al.* 2008). *S. cerevisiae* is a facultative anaerobic organism and has two modes of catabolism; respiration and fermentation. During growth, yeast has several phases, in the first phase rapid growth is achieved by fermentation of high yield carbon sources such as glucose. During fermentation, genes required for respiration and other carbon sources are repressed. Fermentation is an anaerobic process in which sugars are converted into ethanol and CO_2. When these high yield fermentable carbon sources become limited, the yeast switches to respiration. The switch requires a change in metabolic activity and is called a diauxic shift (Galdieri *et al.* 2010). In respiration, the substrates are fully oxidized to H_2O and CO_2 in an aerobic process. Fermentation is preferred over respiration in *S. cerevisiae*, despite fermentation having a lower energy yield (Pfeiffer and Morley 2014). This phenomenon is called the Crabtree effect (Coleman *et al.* 2015).

The Crabtree effect has arisen through several evolutionary events, such as a whole-genome duplication, regulatory rewiring of yeast energy metabolism and hexose transporter duplications (Pfeiffer and Morley 2014). The Crabtree effect is very similar to the Warburg effect, a phenomenon where cancer cells prefer fermentation over respiration (Gatenby and Gillies 2004). Interestingly, fermentation produces ethanol in yeast and lactic acid in cancer cells. It has been suggested that the toxicity of these end-products could be a plausible explanation for the evolutionary choice for respiration. It has been shown that the production of lactic acid acidifies the cancer microenvironment and thereby gives an advantage to the rapidly adapting cancer cells (Gatenby and Gillies 2004, Alfarouk *et al.* 2011). Alcohol production can also be advantageous for yeast in the competition for nutrient source with competing organisms, since alcohol has toxic effects on most organisms. Further, in later growth stages alcohol can be consumed by yeast when sugars are depleted, and therefore serve as alternative carbon source (Pfeiffer and Morley 2014). Other arguments suggest that respiration requires more enzymes than fermentation, and that the cost of producing these would make fermentation more efficient (Pfeiffer and Morley 2014). It has been, and remains, a subject of discussion why fermentation is advantageous over respiration in certain situations, and its evolutionary origin remains ambiguous.

4.1 Glucose uptake: the Hexose transporters

The permeability of molecules through membranes is confined to small molecules such as CO_2, O_2 and small uncharged polar molecules such as ethanol and H_2O. These molecules can pass membranes by passive diffusion. Other molecules require active or passive transporters to pass through membranes (Lodish *et al.* 2008).

For the transit of the hexose sugars glucose, fructose and mannose through the cell membrane, facilitated diffusion is necessary. In *S. cerevisiae* this is achieved by a sub-group in a class of membrane transport proteins named the major facilitator superfamily (MFS). This superfamily is expressed ubiquitously in all biological kingdoms and is accountable for the import and export of a large range of metabolites (Marger and Saier 1993). The hexose transporter group consist of 20 different hexose transport-like proteins and includes Hxt1-17, Snf3, Rgt2 and Gal2 (Kruckeberg 1996, Özcan and Johnston 1999, Horak 2013).

The hexose transporters can be subdivided into three sub-groups based on their kinetic properties for glucose. These groups are the low affinity transporters (Km = 50-110 mM), intermediate affinity transporters (Km =10-20 mM) and finally the high affinity transporters (Km = 1-10 mM) (Table 1) (Reifenberger *et al.* 1997, Maier *et al.* 2002, Horak 2013). Besides having different kinetic properties, another distinction between the subgroups are the conditions in which they are expressed. The low affinity group is mainly responsible for glucose uptake in high extracellular glucose concentrations, andin low extracellular glucose concentrations, the high affinity transporters are expressed. Hxt1 and Hxt3 are considered low affinity transporters; Hxt2, Hxt4, Hxt5 are considered intermediate affinity transporters; Hxt6 and Hxt7 are considered high affinity transporters. The genes for Hxt8 to Hxt17 are not included in this classification since they are insufficiently characterized. Knock-out strains of these genes do not cause any specific phenotypes. Experimental evidence has suggested that the function of Hxt8

to Hxt17 could be in drug resistance rather than hexose transport (Nourani *et al.* 1997). However when overexpressed in a complete knock out strain for hexose transporters they are able to complement the transport of hexoses with the exception of Hxt12 (Wieczorke *et al.* 1999). Snf3 and Rgt2 are outliers in this hexose transporter family because they have evolved from hexose transporters to hexose sensors. Their main function as a glucose sensor is in regulation of hexose transporter gene expression; they will be discussed more extensively below (chapter 5.2 Snf3/Rgt2 pathway). Gal2 is a galactose transporter which also is able to transport glucose. Expression of the *GAL2* gene is induced by galactose and repressed by glucose (Boles and Hollenberg 1997).

Table 1: Overview of the yeast transporter. The transporters can be divided into three classes. For each transporter the experimentally determined Michaelis–Menten import kinetics (Km) for glucose and the known expression conditions are given. Hxt8 to Hxt17 are poorly defined and not included in the table.

transporter	Km (mM)	expression	Citations
Low affinity transporters			
Hxt1	+/-100	300 fold by high extracell. glc (>1%)	(Reifenberger *et al.* 1997, Maier *et al.* 2002)
Hxt3	+/-30-60	weakly dependent on glucose	(Reifenberger *et al.* 1997, Maier *et al.* 2002)
Intermediate affinity transporters			
Hxt2	+/-10	expressed around 0.1% glc, repressed in high glc 10-20 fold	(Wendell and Bisson 1994, Özcan and Johnston 1996, Maier *et al.* 2002)
Hxt4	+/-10	expressed around 0.1% glc, repressed in high glc 10-20 fold	(Özcan and Johnston 1996, Maier *et al.* 2002)
Hxt5	+/-10	regulated by growth (max at slow growth rate) STREs and HAP elements in promoter	(Diderich *et al.* 2001, Verwaal *et al.* 2002)
High affinity transporters			
Hxt6	+/-1	highly expressed at very low glc conc	(Reifenberger *et al.* 1997, Maier *et al.* 2002)
Hxt7	+/-1	highly expressed at very low glc conc	(Reifenberger *et al.* 1997, Maier *et al.* 2002)

The regulation of hexose transporter expression and degradation is highly complex. Expression of *HXT1-4* and *HXT6* and *HXT7* is mainly controlled by the Snf3/Rgt2 pathway. However, additional pathways are involved in expression of these transporters, such as the Snf1-Mig1 and the cAMP-PKA pathway (Boles and Hollenberg 1997, Özcan and Johnston 1999, Horak 2013). Micro-array studies show that the expression of the HXT genes is regulated by both Mig1 and Mig2. The activity of these transcription factors in the expression and repression of the HXT genes depends on the extracellular glucose levels (Westholm *et al.* 2008). Expression of *HXT5* is not directly controlled by glucose concentration (Verwaal *et al.* 2002). The *HXT5* promoter has several different types of regulatory elements: stress-responsive elements (STREs), one putative post-diauxic shift (PDS) element and two putative Hap2/3/4/5p (HAP) complex binding elements. This suggests that the expression of *HXT5* to be regulated by multiple pathways (Verwaal *et al.* 2004).

It is not only the expression of the hexose transporters which are regulated, but also their turn-over rate. It is known that the high affinity transporters Hxt7 and Hxt6 are internalized and degraded after the cell is exposed to high glucose concentrations (Krampe *et al.* 1998). Degradation of Hxt7 requires inactivation of TORC1, through rapamycin treatment, or Ras2 through growth on gluconeogenic carbon sources (Snowdon *et al.* 2008, Snowdon and van der Merwe 2012). Moreover, it has been found that also Hxt1 is actively internalized and degraded when glucose is depleted, possibly regulated by PKA (Roy *et al.* 2014). This clearly demonstrates that there is a complex regulation network in place to control the hexose transporter levels in the yeast cell.

The rate of glycolysis is determined by the glucose uptake rate into the cell. Glucose uptake and other steps in glucose metabolism are often hard to study since genetic manipulation of metabolism often causes severe secondary effects or lethality. Fortunately, a set of isogenic strains is available with each strain expressing only single hexose transporters and displaying different glucose uptake

rates. This set allows for the investigation of the connection between glucose signalling and different glycolytic rates (Elbing *et al.* 2004, Elbing *et al.* 2004). Some strains of this set have been used in **paper I**.

4.2 Glycolysis: the first steps of glucose metabolism

Glycolysis is the pathway where glucose is degraded into pyruvate through a series of enzymatically catalysed reactions. During the reactions, ATP and NADH are produced. The overall net yield of glycolysis is two ATPs per glucose molecule. Glycolysis does not require any specific organelles and happens in the cytosol (Lodish *et al.* 2008). The cells have to adjust the glycolysis rate according to the energy need. This requires high control of the glycolytic rate. To achieve this control of glycolysis, the cell regulates several enzymes of the glycolysis pathway and the hexose transporters. Several pathways are involved in this process which we discuss further in other chapters.

In the first phase of glycolysis D-glyceraldehyde 3-phosphate (GDAP) is produced. This phase does not generate energy, but instead two ATP molecules per glucose molecule are consumed (Figure 2) (Lodish *et al.* 2008). During this preparatory phase, fructose enters straight into the glycolytic pathway after import and is phosphorylated by the hexokinases Hxk1 and Hxk2 to fructose-6-phosphate (F6P) (Lobo and Maitra 1977). Glucose and mannose are converted into glucose-6-phosphate (G6P) and mannose-6-phosphate (M6P), respectively, by all the sugar kinases (Hxk1, Hxk2 and Glk1) (Lobo and Maitra 1977, Maitra and Lobo 1983). To enter glycolysis, G6P is converted to F6P by the homo-tetrameric phosphoglucose isomerase Pgi1 (Lowe and Reithel 1975, Aguilera and Zimmermann 1986). Pmi40, a M6P isomerase, catalyses the isomerization between M6P and F6P (Gracy and Noltmann 1968). F6P is converted into fructose-1,6-bisphosphate (F1,6BP), catalysed by yeast phosphofructokinase.

Phosphofructokinase is a hetero-octamer and this enzyme complex consists of four α-subunits Pfk1 and 4 β-subunits Pfk2 (Heinisch 1986). The β-subunits are catalytically active, whereas the α-subunits serve a regulatory function. However, upon loss of function from one type of subunit, the other type of subunit seems to be able to compensate for the loss (Arvanitidis and Heinisch 1994). F1,6BP is the first point where these metabolic pathways merge. It has been shown that F1,6BP allosterically controls pyruvate kinase activity (Kochanowski *et al.* 2013, Ros and Schulze 2013). In the next step of glycolysis, the C6 molecule F1,6BP is split by Fba1 into two 3C molecules, D-glyceraldehyde 3-phosphate (GDAP) and dihydroxyacetone phosphate (DHAP) by Fba1 (Schwelberger *et al.* 1989). For further glycolysis, DHAP is converted to GDAP by triosephosphate isomerase which is a Tpi1 dimer (Alber and Kawasaki 1982, Lolis *et al.* 1990). From DHAP, glycerol can be produced as a by-product of glucose metabolism through two distinct steps by glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase. Glycerol production is important in the response to osmotic stress and for redox-balancing (Hohmann 2002).

Energy is generated during the second phase of glycolysis. In the conversion of GDAP to pyruvate two ATP and one NADH is generated. GDAP is converted into 1,3-bis-phosphoglycerate (BPG) by Tdh1,2 and 3. Each isoenzyme contributes differently to the GDAP dehydrogenase activity, and none of them seems to be essential (McAlister and Holland 1985). In the next step, Pgk1 catalyses transfer of a high-energy phosphoryl group from the acyl phosphate of BPG to ADP to produce ATP and 3-phosphoglycerate (3PGA) (Hitzeman *et al.* 1980, Blake and Rice 1981). Phosphoglycerate mutase mediates the conversion of 3PGA to 2-phosphoglycerate (2PGA). In yeast, phosphoglycerate mutase contains four subunits which consist of Gpm1(Blake and Rice 1981). Gpm1 has two paralogs Gpm2 and Gpm3, which might be catalytically non-functional because they seem unable to compensate for loss of Gpm1 function (Heinisch *et al.* 1998). Conversion of 2PGA to phosphoenolpyruvate (PEP) is catalysed by the phosphopyruvate

hydratase complex which consists of the enolases Eno1 and Eno2 (McAlister and Holland 1982). The pyruvate kinases, Cdc19 and Pyk2, catalyse the conversion of PEP into pyruvate. Cdc19 seems to be the main pyruvate kinase for growth on glucose (Sprague 1977), while Pyk2 deletion only has noticeable defects in combination with the Cdc19 deletion (Boles *et al.* 1997). Pyruvate is the final metabolite in glycolysis and can then be used in anaerobic (fermentation) or aerobic (respiration) metabolism.

The hexokinases, and especially Hxk2, have been attributed an important role in the glucose signalling network and therefore have been a source of much debate. Time-lapse analyses show that glucose repression consists of a short- and a long-term response. The short-term repression can be mediated by any one of the three glucose kinases, but if Hxk2 is absent, this response is only transient. For full long-term repression Hxk2 is required (De Winde *et al.* 1996, Sanz *et al.* 1996). Hxk2 does not seem to have a unique role in glucose repression, since Hxk1 also contributes to repression, especially when fructose is the available carbon source (De Winde *et al.* 1996). The work in **paper II** suggests that the initial response requires only the presence of a carbon source , while for a sustained repression, a high rate of glycolytic flux is needed**.** A detailed mechanism about how Hxk2 regulates glucose signalling has been suggested, where Hxk2 would play a stabilizing role in the interaction between Snf1 and downstream transcription factor Mig1 under high glucose conditions. In the proposed mechanism, Hxk2 would act as an intracellular glucose sensor (Vega *et al.* 2016), however, some of these claims have been controversial and disputed (Kriegel *et al.* 2016). The precise regulatory role of Hxk2 in the glucose signalling network remains obscure.

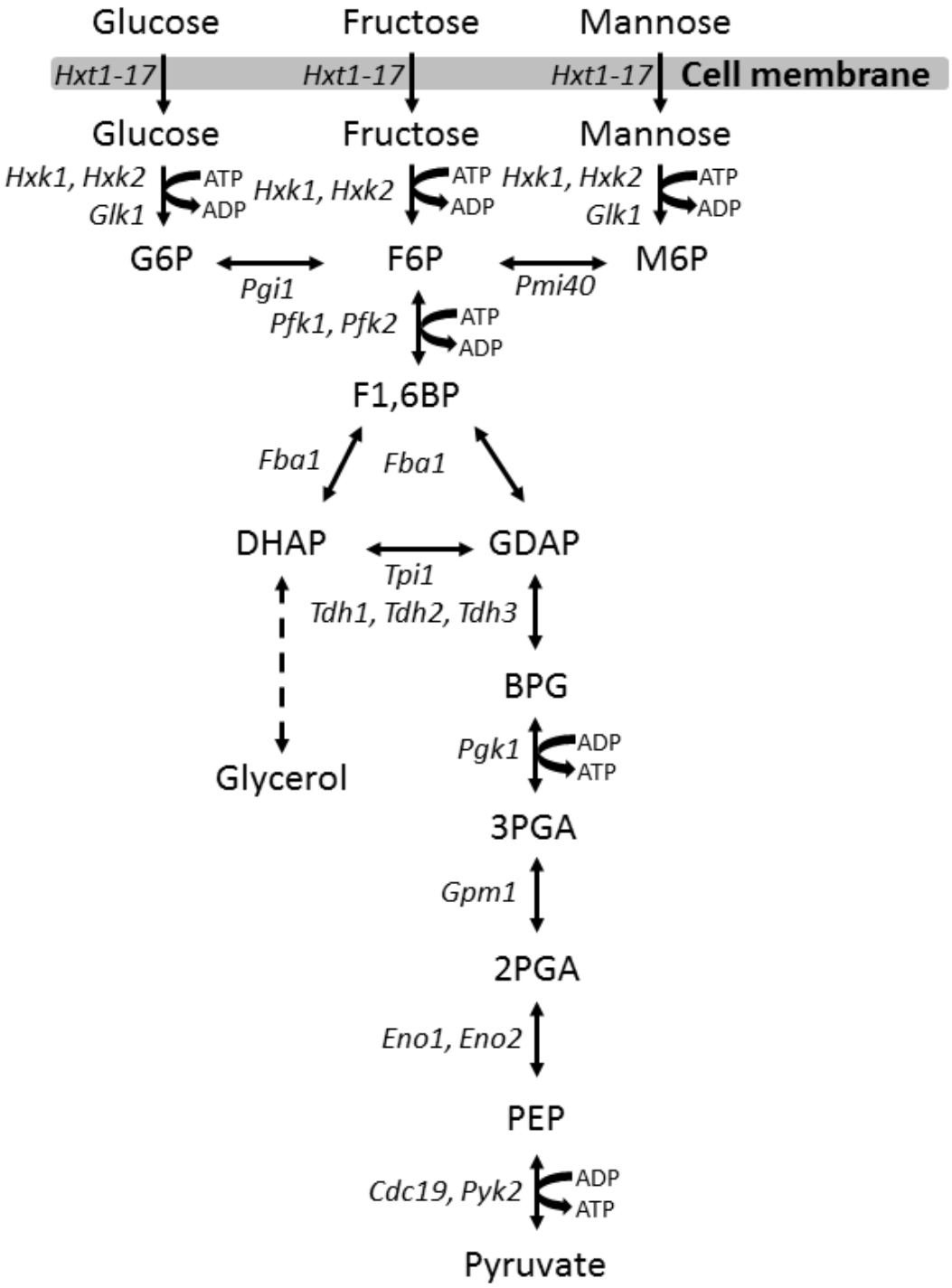

Figure 2: First steps of the glycolysis. Abbreviations; F6P: fructose-6-phosphate, G6P: glucose-6-phosphate, M6P: mannose-6-phosphate, F1,6BP: fructose-1,6-bisphosphate, DHAP: dihydroxyacetone phosphate, DGAP: D-glyceraldehyde 3-phosphate, BPG: 1,3-bis-phosphoglycerate, 3PGA: 3-phosphoglycerate; 2PGA: 2-phosphoglycerate, PEP: phosphoenolpyruvate. Only the enzymes for the forward reactions are given, not for the reverse reactions, if any specified.

4.3 Further metabolism

When glucose is metabolized to pyruvate in glycolysis, only a fraction of the energy available in glucose has been extracted and converted to ATP and NADH. Yeast preferably converts the pyruvate to ethanol and CO_2 via acetaldehyde. This anaerobic degradation of glucose, called fermentation, the basis of alcohol production, only serves as redox regulation (NADH re-oxidation) and produces no additional energy. Acetaldehyde is generated from pyruvate by one of three pyruvate decarboxylase isozymes (Schmitt and Zimmermann 1982). Pdc1 is the major pyruvate decarboxylase and is highly expressed during growth on glucose (Seeboth *et al.* 1990). Pdc5 seems to be expressed when Pdc1 loses its function (Hohmann and Cederberg 1990). The exact role of Pdc6 is unknown, but as it expressed during sulphur limitation, Pdc6 might have a role in sulphur-limited growth (Hohmann 1991, Boer *et al.* 2003). Finally, acetaldehyde is converted to ethanol by alcohol dehydrogenases. In yeast there are five genes that encode alcohol dehydrogenases, *ADH1* to *ADH5* (Smith *et al.* 2004). Only Adh1, Adh3 and Adh5 are involved in ethanol production, Adh4 functions as a formaldehyde dehydrogenase (Drewke *et al.* 1990) and Adh2 is an alcohol dehydrogenase involved in alcohol consumption (Ganzhorn *et al.* 1987).

When oxygen is available, yeast will first ferment glucose to ethanol and some glycerol, followed by a diauxic shift and thereafter a purely respiratory phase will take place. Respiration requires the presence of oxygen. Aerobic degradation happens via oxidative phosphorylation of pyruvate with O_2 to CO_2 in the mitochondria. In respiration, pyruvate is imported into the mitochondria. The uptake into the mitochondria is mediated by the mitochondrial pyruvate carrier (Bricker *et al.* 2012). Pyruvate is converted into acetyl-CoA through oxidative decarboxylation by the pyruvate dehydrogenase complex (PDC). The complex consist of three major catalytic components called El, E2 and E3 (Pronk *et al.*

1996). These last steps connect glycolysis and the tricarboxylic acid (TCA) or citric acid cycle by generating acetyl-CoA which is the start product of the TCA cycle.

During the TCA cycle more ATP and NADH is produced through eight biochemical reactions which oxidize acetyl-CoA to CO_2 and H_2O. The produced NADH is consumed to generate an electrochemical proton gradient across the inner mitochondrial membrane by mitochondrial cytochrome c oxidase complex. This complex consists of a large number of polypeptide subunits (Cooper *et al.* 1991). Mitochondrial cytochrome c oxidase catalyses the electron transfer and proton translocation reactions across membranes (Lodish *et al.* 2008). ATP synthase is another large complex in the mitochondria which utilizes the electrochemical gradient to produce ATP (Velours and Arselin 2000).

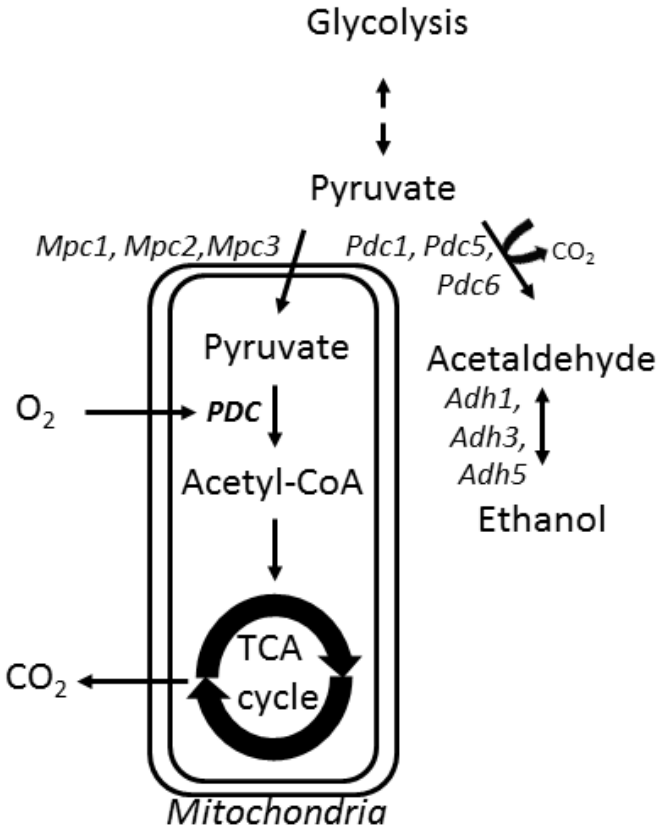

Figure 3: Fermentative and oxidative glucose metabolism. Pyruvate is generated in glycolysis. During respiration (left hand side) pyruvate is converted to Acetyl-CoA where it enters the TCA cycle. During fermentation pyruvate is converted into ethanol via acetaldehyde (right hand side). Only enzymes for forward reactions or given not for the reverse reactions, if any specified.

5 Glucose signalling

Signalling encompasses the entire process of sensing stimuli, generating intracellular signals, signal transduction and the generation of an appropriate response. Signalling processes are employed by cells to monitor their environment and respond to changes in environmental or internal conditions. Efficient use of nutrients requires extensive readjustment of metabolism following exposure to new, or depletion of, substrates. Glucose signalling therefore represses a large set of yeast genes to achieve reprogramming of the cell in response to changes in glucose concentrations. In S. c*erevisiae,* an upshift in glucose concentration results in at least a threefold change in the levels of 20%, and a twofold expression change of 40%, of all genes (Wang *et al.* 2004). Three pathways play a major role in the response to glucose signalling in *S. cerevisiae*. These are the PKA-cAMP pathway, the Snf3/Rgt2 pathway, and the Snf1 pathway, which are involved in cellular programming following depletion of fermentable carbon sources, and together they form a large gene regulatory network.

5.1 cAMP-PKA pathway

The protein Kinase A (PKA) is not solely part of a glucose-sensing pathway, but appears to monitor the cumulative presence of all essential nutrients as well as stress factors. When an essential nutrient is missing or depleted, the PKA pathway is downregulated (Conrad *et al.* 2014). PKA, the central component in this pathway, is a heterotetramer consisting of two catalytic subunits Tpk1, Tpk2 or Tpk3, and two regulatory subunits (Bcy1) (Toda *et al.* 1987, Toda *et al.* 1987) (Figure 4). Glucose is sensed by the PKA pathway through two independent systems, one extracellullar and one intracellullar. Extracellular glucose is sensed by

the G-protein coupled receptor Gpr1 and its associated Gα protein, Gpa2 (Nakafuku *et al.* 1988, Xue *et al.* 1998). The PKA-pathway can also be activated through Ras1/2 by stimuli originating from glycolysis (Rolland *et al.* 2000, Colombo *et al.* 2004). It has been suggested that intracellular acidification would activate the Ras proteins (Colombo *et al.* 1998). However, glucose phosphorylation through Hxk1, Hxk2, or Glk1 is still required for Ras activation (Colombo *et al.* 2004). The Ras GTPase activity is regulated through binding with GDP/GTP (Broach and Deschenes 1990). The switch from the GDP-bound inactive form to the GTP-bound active form is catalyzed by Cdc25 and Sdc25, two Guanine nucleotide exchange factor (GEF). The reverse action, the hydrolysis of GTP to GDP, is driven by the GTPase-activating proteins (GAP) Ira1 and Ira2 (Broek *et al.* 1987, Tanaka *et al.* 1990, Boy-Marcotte *et al.* 1996). It has been implied that the Ras1/2 activity would be regulated through inhibition of Ira1 and Ira2 (Colombo *et al.* 2004). The signaling from Ras 1/2 and Gpr1 converge on adenylate cyclase Cyr1 by increasing its activity (Kataoka *et al.* 1985). Adenylate cyclase catalyzes the synthesis of the secondary messenger cAMP from ATP (Lodish *et al.* 2008). Antagonistically, cAMP is degraded to AMP by phodiesterases Pde1 and Pde2 (Sass *et al.* 1986, Nikawa *et al.* 1987). cAMP, in turn, increases PKA activity by binding to Bcy1.

Active PKA has a wide variety of targets in the cell. PKA directly phosphorylates several cytosolic enzymes and regulates gene expression by interacting with transcription factors such as Msn2 and Msn4 (Conrad *et al.* 2014). The nuclear localization of Msn2 is inhibited by PKA when glucose is available (Huh *et al.* 2003). Under low glucose Msn2 binds to stress response promoter element (STRE) and increases transcription (Schmitt and McEntee 1996). In addition, components of other signalling pathway are target of the PKA kinase (Conrad *et al.* 2014).

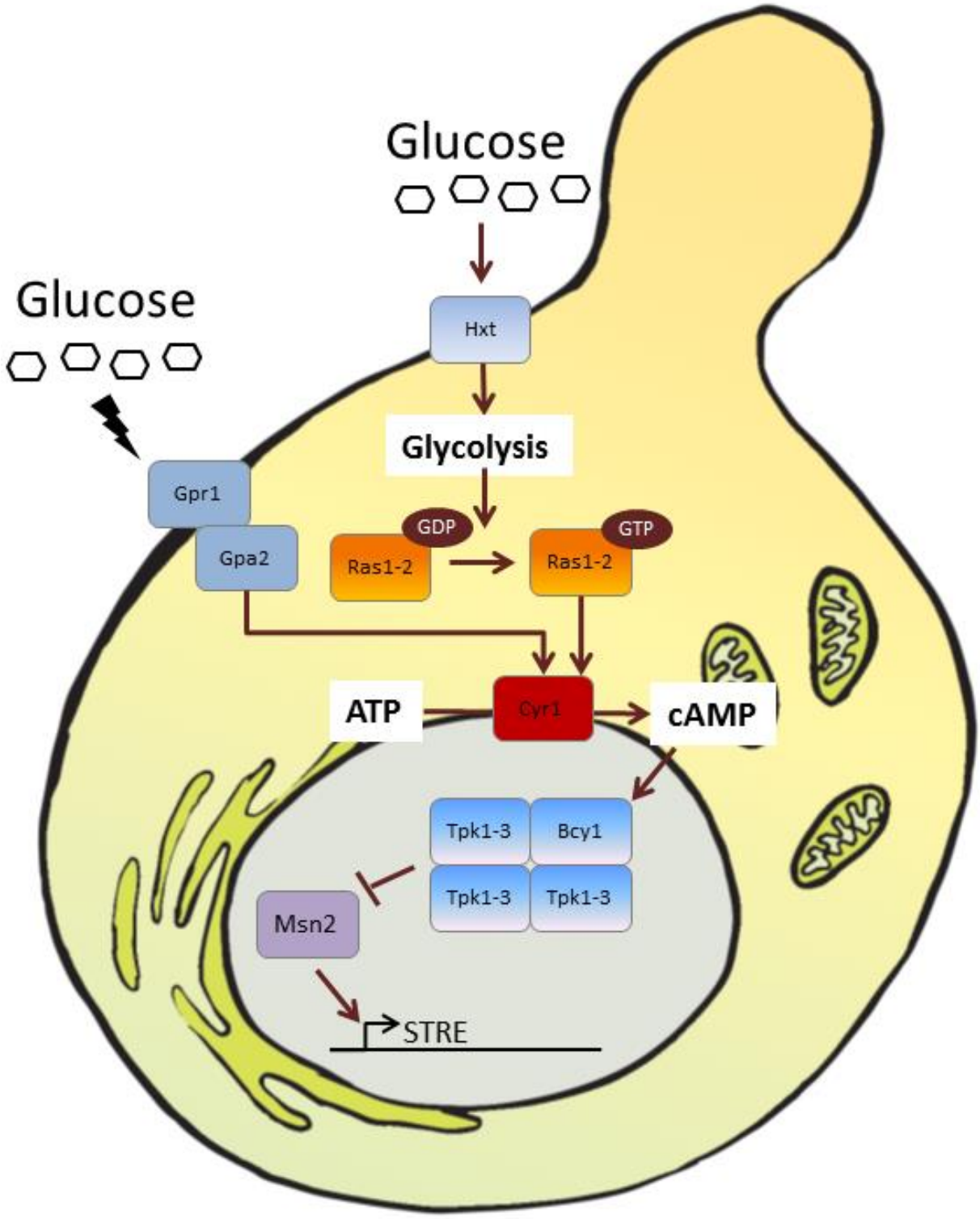

Figure 4: The cAMP-PKA pathway. A simplified schematic representation of the cAMP-PKA pathway. Gpr1 and Gpa2 sense glucose extracellularly. Via an unknown mechanism, Ras1-2 is activated by guanine nucleotide exchange. These two pathways converge on Cyr1 which catalyses the synthesis of cAMP. cAMP activates the PKA complex which inactivates transcriptional activators such as Msn2 who bind STRE genes in active form.

The cAMP-PKA pathway is not restricted to roles in nutrient sensing. It is also activated under a range of different stress factors, including light. Sensing of blue light involves Pox1, a Fatty-acyl coenzyme A oxidase, which is located in the peroxisomal membrane. Upon excitation by light, Pox1 catalyzes the removal of hydrogen atoms from specific organic substrates and transfers these to molecular oxygen and this reaction creates H_2O_2 (Dmochowska *et al.* 1990, Hockberger *et al.* 1999). The 2-Cys peroxiredoxin Tsa1 reduces H_2O_2 in H_2O, and this creates an inactive oxized form of Tsa1 (Peskin *et al.* 2013). The reactivation of Tsa1 requires the donation of electrons from cytosolic thioredoxins Trx1 and Trx2 to restore its catalytic activity and the Trx1 become themselve oxidized. The oxidized thioredoxins modulate the PKA-pathway through a currently unknown mechanism and this results in Msn2 dephosphorylation. This implies that H_2O_2 acts as a secondary messenger that inhibits PKA function. The inhibition of phosphorylation of Msn2 allows Msn2 to localise to the nucleus and consequently a general stress response is established, even in the presence of glucose. Work included in this thesis proposed a mechanism for light detection in organisms which lack dedicated light sensors **(Paper V)**.

5.2 Snf3/Rgt2 pathway

The Snf3/Rgt2 regulatory network controls hexose transporter expression and glycolytic genes in cooperation with the other glucose sensing pathways (Özcan and Johnston 1996, Özcan and Johnston 1999, Palomino *et al.* 2006). The number of genes regulated by the Snf3-Rgt2 regulatory network is limited in comparison with the Snf1/Mig1 pathway and the cAMP-PKA (Kaniak *et al.* 2004, Zaman *et al.* 2009, Horak 2013). The function of the Rgt2/Snf3 pathway is thereby more specific and restricted mainly to fine-tuning glucose uptake (Westholm *et al.* 2008). The

key components of this pathway are the glucose sensors Snf3 and Rgt2 and the repressor Rgt1 (Figure 5). As discussed earlier, Snf3 and Rgt2 both belong to the hexose transporter family (Özcan *et al.* 1998). Through evolution these have lost their transporter function and now function solely as a glucose sensor (Özcan *et al.* 1996). Snf3 has a high binding affinity for glucose and senses low glucose concentrations, while Rgt2 binds glucose with a low affinity and therefore senses high glucose concentrations (Moriya and Johnston 2004). Rgt1 functions both as a transcriptional repressor and activator (Özcan and Johnston 1995, Özcan *et al.* 1996, Kim *et al.* 2006).

The sensors Snf3 and Rgt2 inhibit the functions of Std1 and Mth1 when bound to glucose (Kim *et al.* 2006). This inhibition is mediated by recruiting Std1 and Mth1 to the plasma membrane, where they become phosphorylated by the casein kinases I; Yck1 and Yck2 (Moriya and Johnston 2004). Both Yck1 and Yck2 are activated by stabilization through binding with Sod1 in response to glucose (Reddi and Culotta 2013). Phosphorylation targets Mth1 and Std1 for ubiquitination by SCF (Grr1) ubiquitin-protein ligase. Consequently, ubiquitination targets Mth1 and Std1 to the proteasome for degradation (Flick *et al.* 2003, Kim *et al.* 2006). Mth1 and Std1 degradation makes Rgt1 available for phosphorylation on multiple sites by PKA subunit Tpk3 or by a Tpk3-dependent protein kinase. Consequently, Rgt1 becomes hyper-phosphorylated and is converted into a transcription activator (Mosley *et al.* 2003).

Under glucose limitation, Rgt1 acts as an inhibitor together with Mth1 and Std1 as it forms a repressor complex (Tomas-Cobos and Sanz 2002, Lakshmanan *et al.* 2003, Polish *et al.* 2005). Inhibition is achieved by binding to the promoters and recruiting the general repressor complex Ssn6-Tup1 to these promoters (Özcan and Johnston 1995, Tomas-Cobos and Sanz 2002).

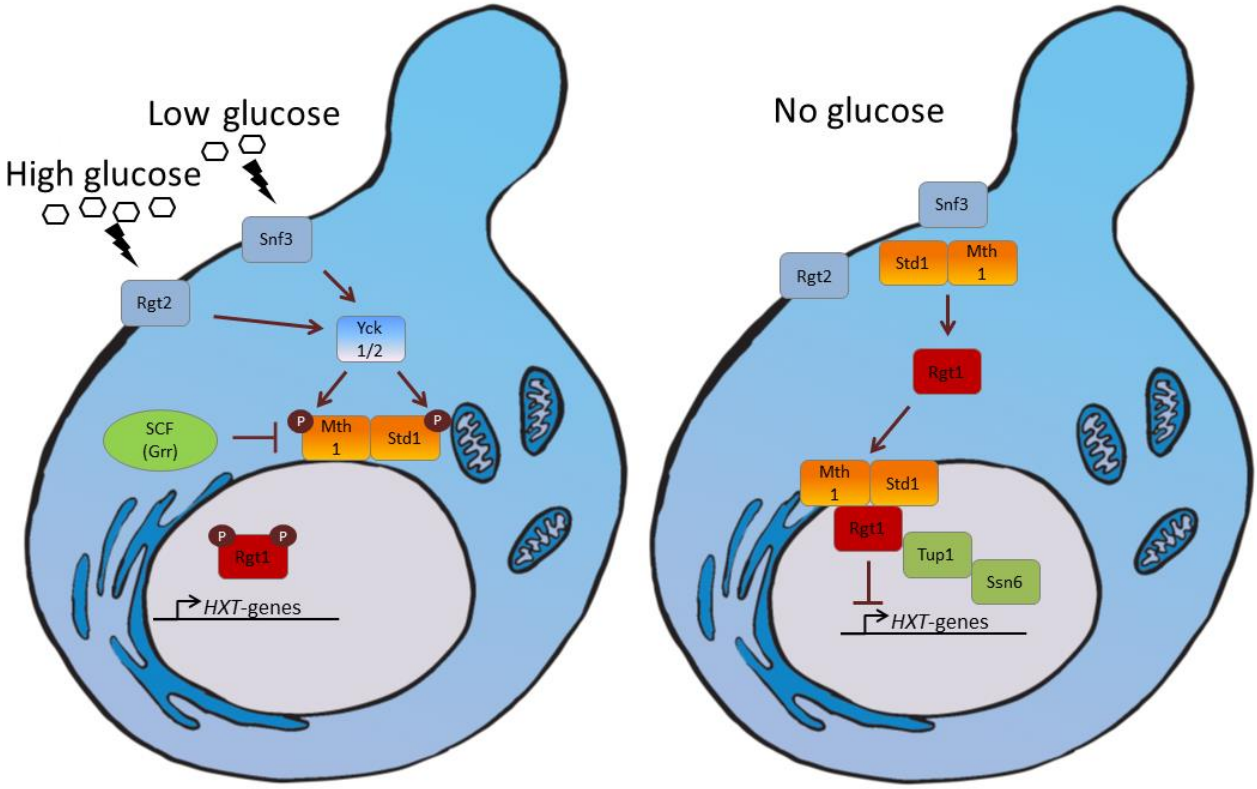

Figure 5: The Snf3/Rgt2 pathway. A simplified schematic representation of the Snf3/Rgt2 pathway. On the left hand side a representation of the pathway in low glucose and high glucose conditions. Snf3 and Rgt2 recruit Mth1 and Std1 to the plasma membrane where they get phosphorylated by Tck1/2 and this leads, via ubiquitination by the SCF (Grr1) ubiquitin-protein ligase, to degradation in the proteasome. On the right hand side a scheme for in zero glucose conditions where Rgt1 forms a repressor complex with Std1 and Mth1 and recruits Tup1 and Ssn6 which represses transcription of genes such as the *HXT* genes.

5.3 Snf1 pathway

The Snf1 pathway or glucose repression pathway plays a major role in metabolic regulation, where it transduces information about energy and nutrient availability. Snf1 is activated when energy and nutrients become limited, and alters the global energy regulation in yeast cells when glucose or fructose become limiting. The function of the Snf1 pathway is to balance energy homeostasis. In its function, it regulates a broad spectrum of processes such as lipid biogenesis and gluconeogenesis (Usaite *et al.* 2009).

5.3.1 AMPK in higher Eukaryotes

Snf1 belongs to a larger group of AMP-activated kinases (AMPK) which is represented throughout the entire Eukaryotic kingdom. The only eukaryotes lacking clear AMPK orthologues are some intracellular pathogens such as *Plasmodium falciparum*, which are typically not exposed to altered nutrient availability (Ward *et al.* 2004). Like Snf1, mammalian AMPK is activated in situations where ATP production is impaired (Hardie 2015). AMPK activation restores the energy balance in a twofold process: (I) by activation of ATP production through increased activity or expression of proteins involved in catabolism. (II) Through conserving ATP by turning off non-essential pathways that consume energy. Due to its central roles in metabolism, AMPK is a plausible target for treating metabolic conditions associated with type 2 diabetes, obesity, cancer and inflammation (Hardie *et al.* 2012, Hardie 2015).

Mammalian AMPK studies have shown that the AMP/ADP/ATP ratio plays an important role in protecting AMPK against dephosphorylation on Thr172 (Xiao *et al.* 2011). While mammalian AMPK seems to be regulated by AMP, ADP may be the regulating adenylate nucleotide for Snf1 (Mayer *et al.* 2011, Xiao *et al.* 2011, Chandrashekarappa *et al.* 2013). Despite this difference it is still possible to

complement an *S. cerevisiae* strain with deletions of all five genes encoding SNF1 complex subunits with certain mammalian AMPK complexes and restore glucose repression. These mammalian complexes expressed in yeast were glucose regulated although not by the Glc7-Reg1 phosphatase complex, suggesting that the role of Glc7 as phosphatase is not conserved (Ye *et al.* 2014). It has been shown that other phosphatases can also dephosphorylate Snf1 (Ruiz *et al.* 2011, Ruiz *et al.* 2013). This suggests that dephosphorylation of SNF1/AMPK is mainly regulated by itself and in a lesser extent by glucose control of the phosphatase.

It appears that mammalian AMPK can correctly interpret the metabolic stimuli produced by yeast. Hence, AMPK is strongly conserved through evolution, consistent with its fundamental role in nutrient signalling.

5.3.2 Components of the Snf1 pathway

The central component of this pathway is the SNF1 kinase complex. The SNF1 complex is activated by glucose depletion. It is composed of three different subunits, the catalytic α-subunit Snf1, the regulatory γ-subunit Snf4 and the three alternative stabilizing β-subunits Gal83, Sip1 or Sip2 (Jiang and Carlson 1997, Schmidt and McCartney 2000). The β-subunits have several functions such as targeting the complex to different subcellular locations and stabilization of the complex. The catalytic subunit alone is not sufficient to mediate glucose derepression: both Snf4 and at least one β-subunit are required for stable Snf1 activity (Celenza *et al.* 1989, Schmidt and McCartney 2000). Fluorescence microscopy studies reveal that in high glucose conditions the subunits seem to be located in the cytosol, while upon the shift to ethanol as sole energy source, Sip1 localizes to the vacuole, Gal83 to the nucleus and Sip2 stays localized in the cytosol. Upon the shift from high glucose to ethanol as carbon and energy source, a

major proportion of Snf1 and Snf4 seems to localize together with Gal83 to the nucleus (Vincent *et al.* 2001).

Phosphorylation of Snf1 is required for its activity (McCartney and Schmidt 2001). Snf1 is constitutively phosphorylated on Thr210 by the upstream kinases (UKs) Elm1, Sak1 and Tos3 (Hong *et al.* 2003, Nath *et al.* 2003, Garcia-Salcedo *et al.* 2014). Sak1 seems to be the most important of the three UKs (Clement *et al.* 2013). Recently it has also been shown that Ser214 in Snf1 is phosphorylated by Sak1 under glucose depletion, and point mutation of this site turns Snf1 inactive. This suggests that the activity of Snf1 can be modulated in two ways (McCartney *et al.* 2016). It has been shown that the UKs also fulfil functions in other pathways. Elm1 is involved in a salt stress response both dependent and independent of Snf1 (Ye *et al.* 2008). Snf1 is dephosphorylated by the yeast PP1 phosphatase Reg1/2-Glc7 when a rapidly-fermentable sugar like glucose is available (Zhang *et al.* 2011). Additional phosphatases, Sit4 and Ptc2, have also been shown to dephosphorylate Snf1 and have been implicated in glucose regulation of the Snf1 pathway (Ruiz *et al.* 2011, Ruiz *et al.* 2013). A model of how these components interact with each other is provided and mathematically encoded in **Paper III.**

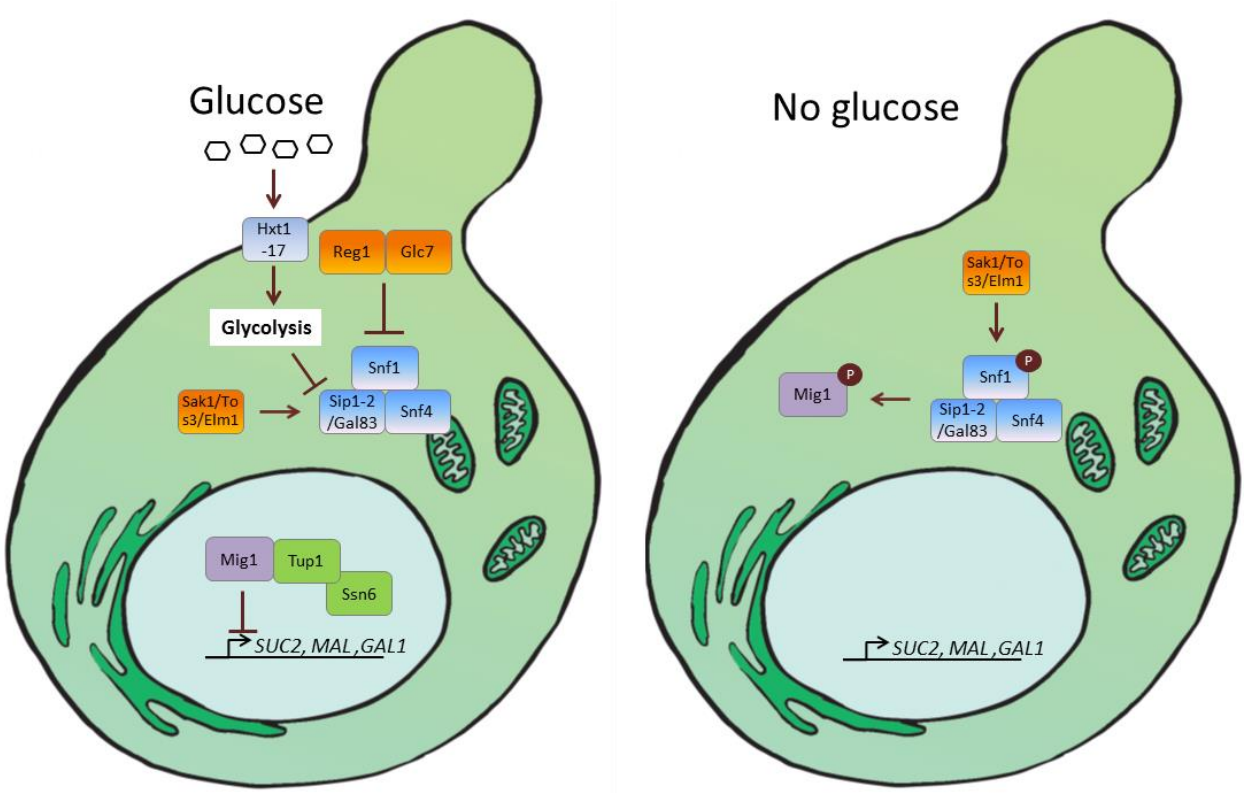

Figure 6: The Snf1/Mig1 pathway. A simplified schematic representation of the Snf1/Mig1 pathway. On the left hand side a representation of the pathway in glucose conditions. Glucose enters the cell through hexose transporters and in glycolysis a signal is generated which inhibits the SNF1 complex and results in dephosphorylation by the phosphatase complex Reg1-Glc7. This allows transcription factors such as Mig1 to bind to promoters and recruit the transcription repressor complex Tup1-Ssn6. On the right hand side in no or low glucose conditions. Snf1 is activated through phosphorylation by Sak1, Tos3 or Elm1. The active SNF1 complex phosphorylates transcription factor such as Mig1.

5.3.3 Snf1 pathway targets

Active Snf1 has a broad spectrum of downstream effects (Zhang *et al.* 2010). The transcriptional repressor Mig1 is one of the principally studied downstream targets of Snf1 (Gancedo 1998, Treitel *et al.* 1998). Mig1 is the best-studied, but not the only, downstream target phosphorylated by Snf1. An overview of Snf1 targets with known Snf1 phosphorylation site is given in Table 2. The consensus site for Snf1-mediated phosphorylation is Hyd-X-Arg-XX-Ser-XXX-Hyd (Dale *et al.* 1995).

When the SNF1 complex is active under glucose limitation, it phosphorylates Mig1 on at least four phosphorylation sites resulting in Mig1 nuclear exit (Treitel *et al.* 1998, DeVit and Johnston 1999). When glucose is available, Mig1 resides in the nucleus where it binds numerous promoters controlling expression of genes that encode metabolic functions. A well-studied example is the gene coding for invertase; *SUC2* (Carlson *et al.* 1981, Lutfiyya and Johnston 1996, Klein *et al.* 1998). When bound on a promoter, Mig1 recruits the repressor complex Ssn6-Tup1 (Keleher *et al.* 1992, Treitel and Carlson 1995). It has been shown that Mig1 displays pulsatile movements in and out of the nucleus when observed at single cell level (Dalal *et al.* 2014, Lin *et al.* 2015). For Mig1, the catabolic or regulatory function of Hxk2 seems to be involved in these pulses since in an *hxk2Δ* mutant only an initial transient entry of Mig1 into the nucleus is observed during growth on glucose **(Paper II)**. This suggests that the Mig1 import and export is regulated dynamically, possibly through signals derived from glucose metabolism. Since Mig1 export out of the nucleus is regulated by phosphorylation through Snf1 (DeVit and Johnston 1999). Snf1 activity may also have pulsatile character.

Cat8, Adr1 and Sip4 are transcription factors controlled by Snf1 in two ways (Vincent and Carlson 1998, Vincent and Carlson 1999, Roth *et al.* 2004). Snf1/Mig1 control the expression of the genes encoding these transcription factors and Snf1 directly controls the activity of Cat8, Adr1 and Sip4 (De Vit *et al.* 1997). Cat8 is a major contributor to cellular reprogramming during the diauxic shift

together with Adr1 and Sip4 (Vincent and Carlson 1998, Haurie *et al.* 2001, Young *et al.* 2003). Both Cat8 and Sip4 regulate genes with a carbon source-responsive element (CSRE) (Lesage *et al.* 1996, Vincent and Carlson 1998). Hcm1 is a forkhead transcription factor that activates genes involved in chromosome segregation during S-phase in the cell cycle. Hcm1 has been shown to be regulated through Snf1 during glucose limitation, suggesting that Snf1 affects cell cycle progression under those conditions (Pramila *et al.* 2006).

Apart from modulating transcription activators and repressors, Snf1 also directly targets enzymes: glycerol-3-phosphate dehydrogenases Gdp1 and Gdp2, which play a key role in glycerol production, are phosphorylated by Snf1 when glucose is limiting to decrease glycerol production (Lee *et al.* 2012). Snf1 also phosphorylates subunits of the SNF1 complex. Gal83 is phosphorylated by Snf1, although it is unclear how this influences the Snf1 pathway (Mangat *et al.* 2010). Snf1 further phosphorylates and thereby regulates the general stress-response transcriptional activator Msn2 (De Wever *et al.* 2005). The osmotic stress response pathway HOG has been reported to be activated by glucose limitation in a Snf1 dependent way (Piao *et al.* 2012).

Snf1 interacts directly with the RNA polymerase II holoenzyme through the Srb/mediator proteins, suggesting that Snf1 regulates RNA polymerase activity under glucose limitation (Kuchin *et al.* 2000, Young *et al.* 2012). It has been shown that histone H3 is phosphorylated and modulated by Snf1 to enhance *INO1* expression and this increases inositol synthesis (Lo *et al.* 2001, Lo *et al.* 2005). Hence Snf1 functions as a histone kinase controlling the Gcn5-dependant acetylation of histone H3 (Abate *et al.* 2012). It has also been suggested that Snf1 stimulates the decay of mRNAs (Braun *et al.* 2016). This shows that Snf1 regulates the expression of glucose-repressed genes not only through transcription factors but also through chromatin remodelling, modulation of polymerase activity and mRNA

stability. An overview of how the regulatory network of Snf1 regulates all these targets is presented in **Paper III**.

Table 2: Snf1 phosphorylation targets. An overview of known sites that are directly phosphorylated by Snf1 in response to glucose limitation.

Protein	Site	Effect of Snf1 phosphorylation	Citations
Acc1	Ser659	Deactivation	(Shi *et al.* 2014)
	Ser1157	Deactivation	(Shi *et al.* 2014)
Cat8	S553	Activation	(Charbon *et al.* 2004)
	S803	Activation	(Charbon *et al.* 2004)
Gdp1	S24	Deactivation	(Lee *et al.* 2012)
Gdp2	S72	Deactivation	(Lee *et al.* 2012)
Mig1	S222	Deactivation	(Treitel *et al.* 1998)
	S278	Deactivation	(Treitel *et al.* 1998)
	S311	Deactivation	(Treitel *et al.* 1998)
	S3811	Deactivation	(Treitel *et al.* 1998)
Msn2	S582	Deactivation	(De Wever *et al.* 2005)
Pfk27	S68	Degradation	(Benanti *et al.* 2007)
	S144	Degradation	(Benanti *et al.* 2007)
Sip4	S217	Activation	(Lesage *et al.* 1996)
Rod1	S447	/	(Shinoda and Kikuchi 2007)

5.3.4 Glucose derepression through the Snf1/Mig1 pathway is a two-step process

The mechanisms regulating the activity of the SNF1 complex activity are not entirely elucidated. It is well known that the establishment of glucose repression requires glucose uptake and phosphorylation, but no further glucose metabolism is required (Rose *et al.* 1991). The UKs do not seem to be upregulated upon glucose depletion and Snf1 seems to be constitutively phosphorylated by the UKs (Hong *et*

al. 2005, Rubenstein *et al.* 2008). The catalytic subunit Snf1 is sufficient for glucose derepression, since strains with a Snf1 form that is unable to interact with the β and γ subunits, maintained Snf1 function. However, it could not establish glucose derepression to the same extent as the wild-type form of Snf1. In addition, in a *snf4Δ* mutant, Snf1 is still glucose regulated, although to a lesser extent than the wild-type (Ruiz *et al.* 2012). Protection of the Snf1 phosphorylation site Thr210 by the subunits is required to maintain an active form of Snf1 under glucose depletion.

Glucose repression is not established by Mig1 nuclear localization alone. Deletion of the exportin Msn5 results in constitutive nuclear Mig1 localization. However, such a strain is still capable of glucose derepression (DeVit and Johnston 1999), suggesting that Mig1 nuclear localization does not automatically result in glucose repression. It has further been observed that under low glucose conditions, a portion of Mig1 remains bound on both the *GAL1* and *SUC2* promoters (Papamichos-Chronakis *et al.* 2004). In addition, irrespective of the glucose condition, Mig1 moves in and out of the nucleus (Bendrioua *et al.* 2014). It has also been shown that Snf1 can be phosphorylated in the presence of glucose without causing glucose derepression (Ye *et al.* 2008, Garcia-Salcedo *et al.* 2014). Taken together, is seems that an additional step is required that enables active Snf1 to phosphorylate Mig1. This additional step is complex and dynamic since upon glucose addition Mig1 is initially dephosphorylated, but remained dephosphorylated only at high glycolytic rates (Elbing *et al.* 2004). Furthermore, the cellular localization of Mig1 appears to be highly correlated with the rate of glucose metabolism (**Paper I**).

It has been proposed that changes in glucose repression are linked to altered catalytic Hxk2 activity and hence metabolic flux, since relevant metabolite levels inside these cells were not changed (Ma *et al.* 1989, Rose *et al.* 1991). Interaction between Hxk2 and Mig1 has been demonstrated and it has been shown that the nuclear localization of Mig1 is dependent on Hxk2 (Moreno and Herrero 2002,

Ahuatzi *et al.* 2007, Vega *et al.* 2016). Overexpression of Glk1 in an *hxk1Δ hxk2Δ* strain resulted in restoration of glucokinase activity but not glucose repression (Rose *et al.* 1991). Finally, mutants displaying a low catalytic Hxk2 activity could still trigger full repression (Mayordomo and Sanz 2001). To further test the direct link between Hxk2 and glucose signalling the hexokinases from *Schizosaccharomyce pombe* and *Yarrowia lipolytica* were stably expressed in an *hxk2Δ* strain. Both homologues could restore repression of invertase in *S. cervevisiae* (Rose *et al.* 1991, Petit *et al.* 2000). Additionally, overexpression of Hxk1 in an *hxk1Δ hxk2Δ* strain, cannot restore glucose repression to its full extent **(Paper II)**. This suggests that Hxk2 might play a regulatory role besides its catalytic activity. Nonetheless, this role might not be specific to Hxk2 since Hxk1 could replace Hxk2 to some extent (De Winde *et al.* 1996, Vega *et al.* 2016), and heterologous hexokinases share a regulatory capability of Hxk2 in *S. cerevisiae*. Taken together, Hxk2 and Hxk1 both have a regulatory and catalytic function and Hxk2 is the primary hexokinase in both functions.

The ADP/ATP ratio has been suggested to control dephosphorylation of Snf1 and thereby regulate Snf1 activity on downstream targets (Mayer *et al.* 2011, Xiao *et al.* 2011, Chandrashekarappa *et al.* 2013). Phosphorylation of threonine 210 could require a distinct step mediated by the Snf4 subunit (McCartney and Schmidt 2001). It seems that the SNF1 complex activity is regulated by the steric availability of Thr210 to the Snf1 phosphatases (Rubenstein *et al.* 2008). It has been reported that ADP binds to Snf4 and this binding leads to allosteric protection from dephosphorylation of the SNF1 complex (Mayer *et al.* 2011). The SNF1 β-subunit has multiple binding sites for adenylate nucleotides. Binding of ADP to these sites results in a conformational change that protects Snf1 from dephosphorylation by phosphatases (Chandrashekarappa *et al.* 2011, Mayer *et al.* 2011, Chandrashekarappa *et al.* 2013).

Several additional mechanisms have been suggested to regulate Snf1 function. The cAMP-PKA pathway contributes to regulation of the Snf1 pathway by controlling Reg1 activity (Castermans *et al.* 2012). The function of the SNF1 complex may also be regulated by SUMOylation of Lys549 in Snf1. Regulation by SUMOylation seems to include two steps: (I) The SUMO protein attached to Snf1 interacts with a SUMO interacting region near the catalytic site of Snf1, thereby reducing Snf1 activity. (II) The SUMO protein targets Snf1 to ubiquitin-dependent degradation (Simpson-Lavy and Johnston 2013). Taken together, Snf1 degradation is regulated in a highly dynamic fashion by multiple regulatory mechanisms that are not mutually exclusive.

5.3.5 Functions of the Snf1 regulatory network in other stress responses

It is known that Snf1 plays a role in stress responses other than carbons source depletion. Under NaCl stress, alkaline pH and oxidative stress Snf1 is phosphorylated and its activity is increased (Hong and Carlson 2007). Under NaCl stress in the presence of glucose, Mig1 is not phosphorylated (Ye *et al.* 2008). Instead, Snf1 phosphorylates the Mig1 homologue Mig2 (Serra-Cardona *et al.* 2014). In addition, it has been shown that for a successful response to NaCl stress Hxk2 is not required, but instead the transcriptional repressor Nrg1 appears to be involved (Ye *et al.* 2008). This suggests that Snf1-mediated responses are regulated differently by NaCl stress than under glucose depletion. In order to accommodate these differences there should be mechanism which can distinguish between these two stresses. The observation that active Snf1 cannot phosphorylate Mig1 under NaCl stress in the presence of glucose supports the notion that there are two regulatory steps (Snf1 activation and Mig1 phosphorylation by Snf1) in glucose derepression. Snf1 has also been suggested to play a role in the response to the toxic agents like hydroxyurea, methyl-methane sulfonate, and cadmium (Dubacq *et*

al. 2004, Thorsen *et al.* 2009). Snf1 appears to play a role in tolerance against toxic cations through the Sip4 transcriptional activator. Phosphorylation on Thr210 of Snf1 does not seem to be required for this mechanism (Portillo *et al.* 2005). Recently it has been reported that the β-subunits play a role in appropriate responses to alkaline stress (Chandrashekarappa *et al.* 2016). Overall it seems that Snf1 is able to accommodate a variety of stress responses and the mechanisms by which this is achieved are not well understood.

5.3.6 Crosstalk between Snf1 pathway and other nutrient signalling pathways

The three main glucose signalling pathways; the cAMP-PKA pathway (see 5.1), the Snf3/Rgt2 pathway (see 5.2) and the Snf1 pathway (see 5.3) do not simply work in parallel. There is extensive cross-talk between the glucose signalling pathways as well as with other signalling pathways (Shashkova *et al.* 2015). Here we provide a short overview of signalling pathway cross-talk involving Snf1.

As mentioned above (see 5.1), the cAMP-PKA system controls the activity of the phosphatase subunit Reg1, which acts on Snf1 (Castermans *et al.* 2012). It has been reported that components of the cAMP-PKA pathway, Ira1, Ira2 and Bcy1, are required for Snf1 pathway activation in response to glucose limitation (Barrett *et al.* 2012). Snf1 also affects the cAMP-PKA pathway as Bcy1 is phosphorylated in a Snf1 dependent way (Braun *et al.* 2014).

Snf1 interacts with the Rgt1/Snf3 pathway at different levels. It has been shown that Std1, Mth1 and Snf3 directly interact with Snf1 (Kaniak *et al.* 2004). The expression of *MTH1* and *SNF3*, both encoding components of the glucose induction pathway, is repressed by Mig1 in cooperation with Mig2. This implies that the expression of proteins in the glucose induction pathway is partly under control of the glucose repression pathway (Kaniak *et al.* 2004). In the collaborative operation

of the Snf1/Mig1 and the Rgt1/Snf3 pathway, Mig1 is the major transcriptional repressor, while Mig2 only targets a subset of Mig1 genes and thereby appears to only adjust the repression in response to glucose signalling (Westholm *et al.* 2008). Snf1 also plays a role in preventing ubiquitination of Std1 and Mth1 (Pasula *et al.* 2007). These observations illustrate that Snf1 influences expression of the hexose transporters by interacting with the glucose induction pathway.

Snf1 also interacts with the TOR pathway. TOR is a key player in nutrient sensing and global metabolic regulation (Nandy *et al.* 2010). Gat1 and Gln3, two transcriptional activators of genes involved in nitrogen catabolite repression, are both phosphorylated in a Snf1-dependent manner following carbon starvation (Bertram *et al.* 2002, Kulkarni *et al.* 2006). Rapamycin, a compound that blocks the TOR complex, and nitrogen limitation stimulate Snf1 phosphorylation. Snf1 phosphorylation in response to rapamycin treatment and nitrogen limitation is inhibited in *sak1Δ* strains, suggesting that the signal of nitrogen limitation requires Sak1, the Snf1 activating kinase (Orlova *et al.* 2006). Taken together, it seems that the Snf1 pathway is also controlled by nitrogen limitation and that Snf1 also controls transcription factors involved in nitrogen limitation repression.

In conclusion, the different glucose signalling pathways seem to form a larger glucose signalling network.

6. Cell-to-cell variability

Biological processes are often assumed to be deterministic. In other words, the resulting behaviour of a biological process is entirely determined by its initial state and inputs (Heldt *et al.* 2015). Dynamic imaging and single-cell studies have revealed the stochastic nature of biological processes. These processes are subjected to stochastic fluctuations that can give rise to cell-to-cell variability. The phenomenon of cell-to-cell variability refers to the fact that no two genetically identical cells have identical behaviour and appearance. Often this variability is referred to as cellular noise and consists of intrinsic and extrinsic noise. The intrinsic noise is attributed to the inherent probabilistic nature of intracellular biochemical reactions (Raj and van Oudenaarden 2009). Intrinsic noise increases the probabilities in all dimensions (i.e., time points) autonomous from one another. Extrinsic noise is caused by variability in cellular states and is constrained by the signalling network that generates the dynamics. Therefore, the variability in components due to extrinsic noise at different time points is deterministically dependent on each other (Selimkhanov *et al.* 2014). Fluctuations in cellular states generating extrinsic noise can be caused by factors such as cell size, shape and cell cycle stage (Raser and O'Shea 2004). Further, other sources of extrinsic noise in biological processes that have been described are mutations (Levy and Siegal 2008), chemicals in the cell environment (Dar *et al.* 2014), thermal fluctuations (Jo *et al.* 2005) and age (Bahar *et al.* 2006). Overall, many factors contribute to extrinsic noise in cellular processes.

Extrinsic and intrinsic noise leads to variability which can be experimentally observed inside single cells and leads to considerable variability between individuals. These two types of noise are hard to separate from external noise. The two former are part of the biological system, while the latter is created due to

experimental setup. It is challenging to distinguish between biological noise and noise caused by the experimental setup. Therefore, we should be vigilant that not all observed noise has biological significance, but can be caused by the experimental setup.

The abundance of proteins involved in responses to environmental conditions has been shown to be more variable than proteins involved in protein synthesis (Newman *et al.* 2006). Large cell-to-cell variability in nutrient signalling pathways has been reported, as for example in a single cell study of yeast cells exposed to a shift in sulphur sources the transcriptional adaptation displayed a large cell-to-cell variability (Schwabe and Bruggeman 2014). We also observed a large cell-to-cell variability in response to glucose uptake **(Paper I)**. Evolution might have preferred intrinsic noise in certain cellular regulatory systems to modulate for the uncertainty of future events (Ben-Jacob and Schultz 2010, Eldar and Elowitz 2010). For instance, random expression patterns of signalling proteins can result in probabilistic outcomes when the cells are faced with a decision between two different cell fates (McAdams and Arkin 1997). Systems that reduce noise in biological cells exist, and are used when a precise cellular response is desired. However these systems are not used in regulatory mechanisms where stochastic outcomes might be advantageous (McAdams and Arkin 1999). This suggests that the cell could have evolved systems to keep all noise in check, but for some systems evolution has preferred not to suppress the noise, implying that noise in biological systems has been integrated and plays a role in some biological processes. Unfortunately, much of our knowledge about cellular processes is based on population level experiments (Altschuler and Wu 2010). In population level experiments, noise, which is caused by cell-to-cell variability, is often seen as a nuisance. However, the resulting cell-to-cell variability should not be disregarded as it is a relevant biological phenomenon.

Not all variability in cells could be contributed to the random noise on the level of biochemical reactions. Processes resulting in cell decisions that before have been thought to be stochastic, have been shown to be more deterministic than initially assumed (St-Pierre and Endy 2008, Robert *et al.* 2010). Therefore, it seems that not all noise can be attributed to the stochasticity of nature, but that some of the variability might be deterministic and should be treated as such (Paulsson 2004).

In conclusion, it seems that biological processes, although displaying stochasticity, are still deterministic, and that the stochasticity is used to determine probabilistic events. To further unravel the characteristics of variability and its impact on, or handling by, biological systems, requires studying processes on the single cell level.

7. Microfluidic systems

Conventional experimental methods measure the average of all individuals of a population. As discussed in the previous chapter, cells within a population show considerable heterogeneity which cannot be disregarded. Single-cell characteristics can be captured by many different experimental methods, such as flow cytometry, electrophysiology, microscopy, and single cell PCR or sequencing (Altschuler and Wu 2010). However, these techniques measure data only at one time point, therefore they do not allow a dynamic study of cellular processes. Without single cell studies, dynamic localization behaviours such as the pulsating characteristics of transcription factors Msn2 and Mig1 would not have been observed (Dalal *et al.* 2014).

Microfluidic systems have emerged as key tools to study the dynamics of processes, since it allows time lapse (fluorescence) microscopic imaging. The development of microfluidic systems has been driving the emergence of single cell analysis techniques. These microfluidic systems enable the culture of cells in controlled and constant environments (e.g. Growth media) and further offer the possibility to reliably shift between media with different composition rapidly (Eriksson et al. 2010). This makes microfluidics an excellent tool to study the effect of changing environmental conditions on biological cells **(Paper I, Paper II, Paper IV).** Even more challenging environmental changes such as light intensity can be studied using microfluidic tools (**Paper V**). Most microfluidics systems work with capillary systems in the micrometre scales (10-100 µm) in which small volumes (nl - pl) are processed. At these scales, the physical properties of flow differ significantly from flow in larger channels. At these small dimensions, the mixing between the flows is restricted to diffusion and therefore there is a sharp concentration gradient between the flows. Other advantages are the small number

of reagents and samples, and the lower experimental costs (Whitesides 2006). A commonly used bio-compatible material for the fabrication of microfluidic systems is polydimethylsiloxane (PDMS). This polymer is very suitable for the use of studying biological systems optically, since it is chemically inert, optically transparent, permeable to gasses such as oxygen and has widely controllable mechanical characteristics (Cademartiri and Ozin 2009). Single cell analysis has a big potential, and has already moved in the other "omics" fields, such as single cell genomics, transcriptomics, proteomics and metabolomics (Wang and Bodovitz 2010).

8. Systems biology

Methods such as microfluidics, but also high throughput “omics” measurements produce data in large quantities allowing the identification and quantification of components in biological processes. However, it is not sufficient just to know the components of a biological system. To gain a complete oversight of the biological system we need to understand where and what the functions of the components are. A system can be described as “components that interact in such a way that they form a functional unit” (Alberghina and Westerhoff 2005). According to this definition many biological processes can be seen as a system. E.g. glycolysis is a system of enzymes working towards metabolizing carbon sources and glucose signalling is a system that works towards signalling the glucose status. Biological systems can include many processes and encloses many hierarchical levels in biology.

The components of a typical signalling pathway consist of sensors, signal transduction molecules and proteins mediating responses. For the Snf1/Mig1 pathway however, the stimulus remains unclear. This will be discussed later in chapter 9.1.

The common denominator of biological systems is that all these components interact as a functional unit. If we understand the function of the components and how they interact on a systems level in a biological system, we can reproduce the system. This reproducibility can be computationally or mathematically modelled. With the help of computational and mathematical modelling we can predict the outcome of a biological system. Systems biology thrives to understand systems in their whole, i.e. the properties that emerge from the interaction between the components of the system.

Systems biology approaches have already shown their value (Wolkenhauer 2014). An early success of mathematical modelling was a model that provided a

mechanistic explanation of the propagation of action potentials in axons, based on the combined behaviours of a system of ionic channels (Hodgkin and Huxley 1952). Another example of the success of systems biology is the modelling of organs such as the heart and liver. These models can simulate diseases and predict the effect of candidate therapies on diseases (Noble 2004, Kuepfer *et al.* 2014). The temporal modelling of expected cell-to-cell variability in different tumour cell types helps in constructing drug-dosing schedules for chemotherapy (Foo and Michor 2009, Liao *et al.* 2012).

For the future, systems biology will gain more importance as a scientific field. Integrating a systems biology approach to the stochastic nature of biological processes could revolutionize medicine. As systems in which each individual patient is extensively examined by new technologies such as genomic, proteomic, single-cell analyses and high throughput phenotypic assays would generate a considerable amount of data points on each individual, powerful computational methods would reduce this vast amount of data to simple hypotheses about the health and disease of the person in question. This would allow healthcare to be personal, predictive and preventive (Hood et al. 2004, Hood and Friend 2011, Tian et al. 2012).

8.1 Mathematical modelling

Modelling is an essential tool of systems biology. Due to the complexity and size of systems we cannot predict the behaviour or dynamics of such system through human intuition alone. By modelling a biological system, we can predict or simulate the outcome of complex systems. Accordingly, simulation results should be compared with experimental measurements characterizing the dynamic behaviour of biological processes. There are several types of models available, and to model a dynamic biological process the correct mathematical model needs to be defined. Most dynamic mathematical models are continuous since they have to deal with high concentrations of molecules in a time dependent way. Ordinary differential equations (ODEs) are fundamental tools for studying the dynamics of a continuous system. ODE models recapitulate a series of biochemical reactions that take place over time, and therefore simulate response dynamics in a signalling network. Larger models are often static since they are time invariant and work at a steady state (Murray 2002). Important to note is that there is no fully correct model, a model is a simplification of reality and will therefore only be able to approximate reality. However, in combination with high quality experimental data they can be predictive and used to test hypotheses (Xia *et al.* 2014). Microfluidics systems can provide the high quality experimental data mathematical modelling requires (Breslauer *et al.* 2006).

Several modelling approaches have been published which characterize the interplay between the components of the Snf1 pathway. An overview is given in Table 3. These models show that the Snf1 pathway displays complex dynamics and influences many cellular processes in its function as global energy regulator.

The heterogeneity poses practical challenges for building accurate models. However, models could be suited for situations where biological variability, as well

as uncertainty in measurements, may affect data interpretation. This adds a new level of complexity to modelling. Several attempts have been made to produce models that include cell-to-cell variability. Bayesian and Monte Carlo methods have been used to recover the full probability distributions of free parameters (Eydgahi *et al.* 2013, Almquist *et al.* 2015). In our work we have used Nonlinear mixed effects (NLME) to try to understand the variability within a cell population **(paper I)**. NLME modelling is a theoretical approach that provides a framework to account for cell-to-cell variability. The NMLE approach is already common practise in pharmacokinetic studies and pharmacodynamics, since this approach allows for the analysis of sparse and unbalanced datasets (Niepel *et al.* 2009, Ribba *et al.* 2014). Further, NMLE has been proposed and used to model dynamic single cell data (Zechner *et al.* 2014, Karlsson *et al.* 2015). Recently, a phenomenological model with four components describing the Snf1-Mig1 pathway using the NLME approach has been constructed (Almquist *et al.* 2015). This model could simulate the cell-to-cell variability observed in the experimental data.

Overall, the Snf1-Mig1 pathway has been subject to several modelling attempts, which have allowed a better overview and understanding of the pathway. In some cases modelling approaches have led to new insight such as the identification of new Snf1 targets (Usaite *et al.* 2009), or suggested novel regulatory mechanisms (Garcia-Salcedo *et al.* 2014), thereby validating the usage of systems approaches to biological questions.

Table 3: Overview of the modelling approaches in studies on the Snf1 pathway.

Model purpose	Type of model	source
Describes the crosstalk between the Snf1 pathways and the Snf3/Rgt2 pathway	Boolean model	(Christensen *et al.* 2009)
Identification of the Snf1's global regulation on gene and protein expression levels based on global mRNA, protein and metabolite levels.	DOGMA analysis based regulatory network	(Usaite *et al.* 2009)
Larger network reconstructions based on exhaustive and manually curated literature review	Boolean model	**(Paper III)**
Elucidate Snf1 pathway design and control by generating 24 different networks structures and analyse their performance.	ODE model	(Garcia-Salcedo *et al.* 2014)
Describes Mig1 shuttling in and out of the nucleus upon changes in glucose concentration. This model can takes bleaching into account.	ODE model	(Frey *et al.* 2011)
Describes Mig1 shuttling in and out of the nucleus upon changes in glucose concentration. This model takes cell-to-cell variability into account.	NMLE and hidden Markov models	(Almquist *et al.* 2015)

9. Summary of the appended papers

9.1 Main findings Paper I

We present a multidisciplinary approach combining single cell time-scale fluorescence microscopy in microfluidics system and mathematical modelling. We find that the Mig1 nuclear import profile is different for every single transporter strain and correlates with the known kinetic properties of the glucose transporters. This suggests that glucose metabolism controls the Snf1-Mig1 system rather than the absolute glucose concentrations. We further applied the mixed-effect modelling approach to quantify the dynamic behaviour of glucose repression at single cell level. This model allowed us to: simulate cell-to-cell variability, suggest regulatory steps and predict sources of stochasticity in the Snf1-Mig1 pathway.

9.2 Main findings Paper 2

We revisited the catabolic and regulatory function of the sugar kinase in glucose metabolism and the Snf1-Mig1 pathway. We find that Mig1 displays a short transient nuclear import upon addition of fermentable carbon sources and that this import requires phosphorylation by any of the sugar kinases. However, for prolonged Mig1 pulsatile behaviour, Hxk2 is required during growth on glucose and mannose, and Hxk1 or Hxk2 are required during growth on fructose. This suggests that Mig1 import is controlled via two different mechanisms, (i) the initial import is regulated by metabolism and (ii) continuous shuttling is regulated by Hxk2 and Hxk1. Mig1 nucleocytoplasmic shuttling is required but not essential for the establishment of glucose repression in which Hxk2 seems to play an essential role.

9.3 Main findings Paper 3

We present a workflow that enables large scale network reconstruction, validation and gap filling of signal transduction pathways. With this workflow we created a machine-readable Snf1 regulatory network which is based on a comprehensive literature review.

9.4 Main findings Paper 4

We present a method for time-resolved subcellular optical proteomics using single-cell microfluidics, fluorescence microscopy and image analysis to quantify dynamic protein copy numbers in subcellular compartments *in vivo*. Therefore, this method does not monitor relative protein movement as in traditional fluorescence microscopy, but generates a complete overview of protein abundance and subcellular localization in response to environmental perturbations. We apply this method on the Snf1-Mig1 pathway and observe only a part of the Mig1 molecules displaying nuclear import upon addition of glucose.

9.5 Main findings Paper 5

We show that the nutrient sensing pathways also transduce signals other than nutrient signals. We find the Msn2 oscillations are induced by blue light in yeast through counteraction of PKA-dependent Msn2 phosphorylation. In this mechanism H_2O_2 functions as secondary messenger of light and this process involves the oxidase Pox1, peroxiredoxin Tsa1, and thioredoxin Trx1 and Trx2. Our data identify a mechanism by which light could be sensed in all cells lacking dedicated photoreceptors.

10. Conclusion and perspectives

10.1 Glucose repression

The Snf1 regulatory network is extensive and complex. In **paper III** we have constructed a large Boolean model with many components in the Snf1 pathway. This model can serve as the lay-out for a large dynamic model of the Snf1 regulatory network and can even be extended to cover further glucose signalling pathways.

It remains up for discussion how Snf1 regulates Mig1 and other downstream targets in response to glucose depletion. A complete hypothesis on how Hxk2 would regulate the Snf1-Mig1 interaction has been suggested (Vega *et al.* 2016). However, this concept has been contested (Kriegel *et al.* 2016). According to the suggestion of Vega *et al.* and others, Hxk2 would have two roles in glucose repression; a catabolic role and regulatory role. Due to the importance of Hxk2 in glucose phosphorylation it is hard to discriminate between the catabolic role and the regulatory function. Furthermore, this proposed mechanism only explains how the step between active Snf1 and Mig1 could be regulated, but does not explain how other downstream targets of Snf1 would be regulated. In **paper II** it is confirmed that Hxk2 does play an important role in glucose repression. However, it is not completely clear how Hxk2 would regulate its target, as no mechanism have been suggested as to how Hxk2 would connect Mig1 to Snf1. The exact regulatory role of Hxk2 in glucose signalling remains to be elucidated and might not be as specific to Hxk2 as has been suggested.

Previous modelling approaches have suggested the importance of the glycolytic flux rate in glucose signalling (Bosch *et al.* 2008). **Paper I** shows experimentally the close correlation between glucose flux and glucose signalling. The ADP/ATP ratio could be an ideal stimulus. A system where ATP/ADP/AMP turnover provides input to the regulatory machinery would allow the cell to measure intracellular flux and the energy status. Elbing *et al.* 2004, suggest that the control

of glucose metabolism is distributed over several metabolic reactions (Elbing *et al.* 2004). Many metabolic reactions consume or produce adenylate nucleotide derivatives. It is therefore imaginable that the control could be conveyed by adenylate nucleotides binding to components of signalling pathways. Allosteric regulation of proteins provides a rapid and highly dynamic method to control activity and is therefore suitable for systems that need stringent regulation. The consumption of NAD^+ has been shown to mediate signalling events,thereby linking the cellular energy state to gene expression in mammalian cells (Koch-Nolte *et al.* 2011). Molecular mechanisms that dynamically measure the concentration of metabolites have been suggested to function as "flux sensors" and might be used by the cell to provide feedback for the regulation of metabolism (Kotte *et al.* 2010, Kochanowski *et al.* 2013). Moreover, the regulation by adenylate nucleotides of AMPK seems to be conserved in the Eukaryotic kingdom (Xiao *et al.* 2011, Chandrashekarappa *et al.* 2013). This suggests that the indicated regulation mechanism is strongly conserved, thus implying that it would be an important control mechanism in the Snf1 regulatory network. It has also been shown that the PKA pathway controls AMPK in mammalian cells, although not in a similar way as in yeast (Djouder *et al.* 2010).

Taken together, sensing of the status of cofactors and coenzymes could function as an ideal flux sensor. If ADP stabilized active Snf1, this would also allow the cell to measure the energy status in certain cell compartments. In this case, adenylate nucleotides binding to signalling proteins would serve as a "flux sensor" in glycolysis. Binding of metabolites to signalling proteins would be a suitable strategy for cells, since metabolites such as ATP/ADP/AMP are rapidly turned over and therefore a good marker for the energy status. It is important to keep in mind that flux is not directly connected to metabolite concentrations but more defined as a flow of, in our case, metabolites. High flux does not directly indicate high concentrations of metabolites or *vice versa*.

10.2 Cell-to-cell variability

We have observed considerable cell-to-cell variability in response to glucose uptake **(paper I)**. Light stress also induced considerable cell-to-cell variability within a yeast population **(Paper V)**. The variability in expression and translation of hexose transporters may cause a variability for glucose uptake within a population and consequently variability in further glucose metabolism. Such variability in glycolysis might lead to cells responding differently to nutritional changes or even lead to failure in the activation of a nutrient signalling pathway in a subpopulation. Systems to restore unbalanced dynamics in glycolysis have already been reported (van Heerden *et al.* 2014).

We have also observed pulsatile behaviour of Mig1 **(Paper II)**, as others before (Dalal *et al.* 2014, Lin *et al.* 2015)**.** It has been shown that this pulsatile behaviour of transcription factors is regulated by the cell. Regulation of pulsatile behaviour would avoid two transcription factors with the same target promoter being active at the same time, thereby increasing their efficiency (Lin *et al.* 2015). The reason why transcription factors show this pulsatile behaviour and how it is modulated is not yet understood (Dalal *et al.* 2014).

Seemingly, the cell allows certain variability in some cellular processes. In some cases, cells have modulated this variability into a part of its cellular processes and this insinuates that stochastic events might be more deterministic then initially assumed. Therefore, if we understand the stochasticity of biological processes, we would contribute to the understanding of the biological system in its whole.

10.3 What is life now really?

In 1944 Erwin Schrödinger wrote his seminal work “What is life?”. This work attracted many scientists such as James Watson and Francis Crick, who later

discovered the molecular structure of DNA, to the biological sciences. The huge attraction to biology lead to a massive expansion of the biological field and a golden age for discovery in biology; such as the aforementioned molecular DNA structure (Watson and Crick 1953). Protein, DNA and RNA sequencing have also been major breakthroughs of recent times (Berg 2014), together with the discovery and characterization of restriction enzymes (Loenen *et al.* 2014) and transposable element or so called “jumping genes”(Mc 1950), which both lead to the development of recombinant DNA technology. Among other major achievements is the complete sequencing of the first eukaryotic genome, that of *Saccharomyces cerevisiae* (Goffeau *et al.* 1996).

In his influential book Schrödinger states that most physical laws can be explained by chaos on a small scale, by the principle "order-from-disorder". He claims that, in contrast to physical laws, since molecules are stable structures, biology can be explained by an “order-from-order” principle. However, as our knowledge of the biological cell, and biological processes have increased, it has been shown that biology is not as ordered as Schrödinger suggested. This lack of order is exemplified by the Snf1 pathway throughout this thesis. The Snf1 pathway is not linear and in parallel to other signalling pathways. These glucose pathways reside within a large regulatory network and display a considerable amount of crosstalk between nutrient sensing pathways. Further, the whole regulatory process displays extensive stochasticity and turns glucose sensing into a highly chaotic network.

Fully integrating systems biology in biological research could help to understand the stochastic nature of biological processes and drive us towards a new age of discovery within biology. Perhaps we should change our perception of the biological cell from a simple order-from-order approach, towards a highly unordered and chaotic system which displays a high degree of variability between individuals. Would this view lead to a new area of biological discovery and advancement?

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