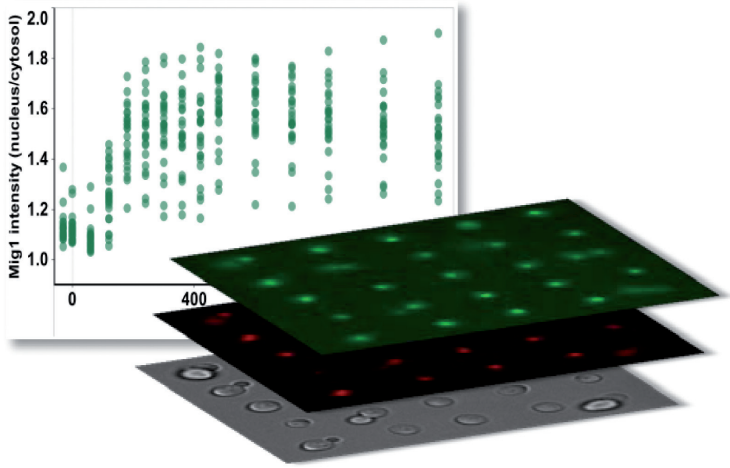
Dynamic Control of the Yeast AMPK/SNF1 Pathway in Response to Glucose Signals



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Ph.D. thesis

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THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Dynamic control of the yeast AMPK/SNF1 pathway in response to glucose signals

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Cover picture: Brightfield, GFP and mCherry images showing the localization of Mig1 and Nrd1 localization. The graph represents the ratio of the fluorescence intensity of Mig1 in the nucleus relative to the cytoplasm.

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Abstract

The SNF1/AMP-activated protein kinase (AMPK) belongs to a family of energy sensors that is conserved in all eukaryotes. Activated by ATP depletion, AMPK plays a vital role in restoring the energy balance by enhancing energy-generating and damping energy-requiring processes. Yeast SNF1 is activated by depletion of glucose in the growth medium but is also affected by other environmental stresses such as salt, oxidative and alkaline stresses. Currently the regulatory mechanism by which glucose controls the activity of SNF1 is incompletely understood. The aim of this thesis was to achieve a better understanding of the glucose regulation of the SNF1/AMPK pathway in the yeast *Saccharomyces cerevisiae*. By employing time-lapse imaging of the nucleo-cytoplasmic shuttling of the transcription factor Mig1, which is directly controlled by Snf1, we revealed the ability of the Snf1-Mig1 system to monitor not only the changes in glucose concentrations but also the absolute levels of glucose. It was also found that this system is highly flexible and rapidly adapts to glucose changes. Monitoring Mig1 migration in cells expressing different glucose uptake systems indicated that the profile of Snf1-Mig1 activity parallels the characteristics of the expressed hexose transporter, suggesting a firm link between glucose uptake and the regulation of the SNF1 pathway. Single cell studies of Mig1 nuclear/cytoplasmic shuttling revealed a significant cell-to-cell variability, which was studied using nonlinear mixed effects modelling. Our model was able to quantify characteristics of Mig1 translocation which cannot be directly measured experimentally such as the time, amplitude and duration of Mig1 transient response. SNF1 shares a number of structural and functional similarities with its mammalian ortholog AMPK. We show that different AMPK isoforms confer growth of the *snf∆* mutant on SNF1-dependend carbon sources indicating functional complementation. Moreover, mammalian AMPK expressed in yeast showed proper regulation by glucose suggesting a conserved mode of regulation. Our data also showed that compound 991, an AMPK activating drug candidate, was able to enhance the activity of yeast-expressed AMPK providing scope for employing yeast for the screening of drugs affecting AMPK activity.

Keywords: AMPK, SNF1, Mig1, glucose signalling, dynamic control, mechanism, *Saccharomyces cerevisiae*

Abbreviations

AMPK	AMP-activated protein kinase
O_2	Oxygen
MFS	Major facilitator superfamily
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
cAMP	Cyclic AMP
PP1	Protein phosphatase 1
AIS	Autoinhibitory sequence
LKB1	Liver kinase B1
	Calmodulin-dependant protein kinase kinase
STRAD	Sterile-20-related adaptor
MO25	Mouse-protein 25
	Transforming growth factor-beta- activated kinase 1
AMPKK	AMPK kinase
	Tumour necrosis factor (TNF)-related apoptosis-inducing ligand
PECK	Phosphoenolpyruvate carboxykinase
	Peroxisome proliferator-activated receptor-γ co-activator 1α
FOXO1	Forkhead box protein O1
	5-aminoimidazole-4-carboxamide riboside
SnRKs	SNF1-related protein kinases
FC	Flow cytometry
FACS	Fluorescence-activated flow sorting
CE	Capillary electrophoresis

LSC	Laser scanning cytometry
LOC	Lab-on-chip
µTAS	Micro-total analysis systems
LIC	Laboratory-in-a-cell
PDMS	Poly-dimethylsiloxane
Re	Reynolds number
GFP	Green fluorescent protein
FISH	Fluorescence in situ hybridization
BFP	Blue fluorescent protein
YFP	Yellow fluorescent protein
CFP	Cyan fluorescent protein
FRET	Fluorescence resonance energy transfer
FRAP	Fluorescence recovery after bleaching
FLIP	Fluorescence loss in photo bleaching
NLS	Nuclear localization signal
Species	
S. cerevisiae	*Saccharomyces cerevisiae*
D. melanogaster	*Drosophila melanogaster*
E. coli	*Escherichia coli*

Nomenclature

The term SNF1 refers to the heterotrimeric complex, whereas Snf1 denotes to the catalytic subunit of the complex

In *S. cerevisiae* it is common to refer to the gene as *SNF1* and to the protein as Snf1.

List of appended papers

This thesis is based on the work conducted in five different papers:

I. Bendrioua L, Smedh M, Almquist J, Cvijovic M, Jirstrand M, Goksör M, Adiels CB, Hohmann S- (2014) Yeast AMP-Activated Protein Kinase Monitors Glucose Concentration Changes as well as Absolute Glucose Levels. J Biol Chem 289: 12863-12875

II. Bendrioua L, Welkenhuysen N, Adiels CB, Goksör M, Hohmann S- The response profile of the Snf1-Mig1 glucose derepression system is tightly coupled to the cell's capacity for glucose uptake**.** *Manuscript*

III. Almquist J, Bendrioua L, Adiels CB, Goksör M, Hohmann S, Jirstrand M- A nonlinear mixed effects approach for modeling the cell-to-cell variability of Mig1 dynamics in yeast. *Manuscript for PLOS Computational Biology*

IV. Lubitz T, Welkenhuysen N, Bendrioua L, Shashkova S, Klipp E, Krantz M, Hohmann S- Network reconstruction of the yeast Snf1 pathway based on comprehensive literature mining and experimental evidence classification. *Manuscript*

V. Ye T, Bendrioua L, Carmena D, García-Salcedo R, Dahl P, Carling D and Hohmann S- The mammalian AMP-activated protein kinase complex mediates glucose regulation of gene expression in the yeast Saccharomyces cerevisiae. *FEBS Letters- In press*

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1. Introduction

All living cells require energy in order to ensure their biological functions such as viability, homeostasis, growth and proliferation. For an optimal cellular functioning, there needs to be a balance between energy supply (usually via means of glycolysis and respiration) and energy consumption (via e.g. biosynthesis, homeostasis and proliferation). It is now well established that any disorder in energy balance would lead to serious diseases such as obesity and obesity-related morbidities including type 2-diabetes, hypertension, cardiovascular and pulmonary diseases (Hamilton, et al. 2007, Kahn, et al. 2005, Schols, et al. 1991). The major energy sensor in mammalian cells is the AMP-activated protein kinase (AMPK). AMPK senses energy depletion and restores energy homeostasis by switching on energy-generating pathways and down-regulating energy-consuming pathways. Identified in 1981, the yeast *Saccharomyces cerevisiae* SNF1 was later found to be similar to AMPK and was therefore referred to as the yeast AMPK ortholog. It now appears that AMPK exists in all eukaryotes where this protein complex shares the same fundamental function: maintaining the energy balance. In this thesis, we employed *Saccharomyces cerevisiae* as a model eukaryote to investigate the dynamic regulation of the SNF1 and AMPK signalling pathways.

The SNF1 glucose repression pathway has been intensively studied for nearly 40 years. Although the core components of this pathway have apparently been identified, several questions regarding its regulation remain unanswered. For instance, it is still unknown how the glucose signal is transmitted to SNF1; it is unclear how glucose regulates SNF1 activity and how this pathway behaves in response to different glucose levels. In other words, how does SNF1 differentiate between high and low glucose levels? Is there a certain glucose concentration threshold for the response of the pathway? Is it the change in glucose concentration or the absolute glucose levels or both that trigger SNF1 activation? It is now believed that glucose generates an internal signal since altering SNF1 activity requires glucose uptake and phosphorylation. However we still do not know the nature of this stimulus. AMPK activity appears to be modulated by adenylate ligand binding but whether this mechanism holds for SNF1 as well is controversial. Recent studies suggest

that the main activator for AMPK is indeed AMP while yeast SNF1 appears to be activated rather by ADP (Chandrashekarappa, et al. 2013, Mayer, et al. 2011).

One way to deal with complex biological systems is to integrate different pieces of information generated from biological experiments with mathematical modelling. Hence the transition from molecular to systematic cell biology has enabled the contextualization and the structuration of biological networks (Alon 2007, Hartwell, et al. 1999). The non-linearity and interconnectivity of biological networks is not possible to study by basic molecular biology techniques. Rather, this interconnectivity requires large scale 'omics' approaches e.g. genomics, proteomics, as well as quantitative time-resolved data collection at cell population and single cell levels, combined with computational simulations. Maybe only then, it would be possible to achieve a comprehensive picture of biological systems.

In this work, we aimed to elucidate the dynamic properties and design principles of the Snf1 module in response to glucose from a systematic angle starting by addressing the questions raised above. By combining a range of cutting-edge biological techniques such as time-lapse fluorescence microscopy, confocal microscopy, microfluidics and optical tweezers with quantitative mathematical modelling and simulation, we present novel insights into the dynamics of yeast glucose signalling.

1.1. Aims of this study

The overall goal of this thesis was to achieve a better understanding of the control of the yeast AMP-activated protein kinase, the Snf1-Mig1 pathway. The AMPK Snf1-Mig1 pathway controls energy homeostasis and, in yeast, glucose repression/derepression and hence we surmised that this pathway responds rapidly to altered glucose levels. Therefore we were interested in the underexplored dynamics of the control of this crucial regulatory system.

The overall goal of this thesis can be broken down into five specific research objectives:

1. Employing Mig1 nucleo-cytoplasmic shuttling as a read-out in single cell analysis we wished to determine the threshold concentrations of glucose that caused changes in Snf1-Mig1 pathway activity. With this information at hand we wanted to determine the dynamic characteristics of the pathway around those thresholds as we expected those to reveal information of pathway control mechanisms.

2. Following up on aim (1) we further wished to determine the effects of altered glucose uptake activity on the dynamics of the Snf1-Mig1 system. By employing yeast strains expressing different uptake systems, we aimed at monitoring the effect of glucose uptake on Mig1 migration in real time.

3. Pursuing aim (1) we also wished to understand the characteristic features and the mechanisms associated with cell-to-cell variability.

4. Based on reliable information collected from the literature, we aimed to provide a reconstruction of the yeast Snf1-Mig1 network by using the publicly available *rxncon* software. In addition, we wanted to provide a detailed overview of our current understanding of the yeast Snf1-Mig1 pathway.

5. We aimed to test whether AMPK would be able to substitute SNF1 and mediate glucose derepression in yeast cells. To this end, we wished to functionally express the AMPK complex in a yeast strain that is devoid of its SNF1 complex. Furthermore, we wished to investigate the effect of anti-diabetic drug candidates on the activity of this yeast-expressed AMPK.

2. *Saccharomyces cerevisiae* as a eukaryotic model organism

The introduction in experimental research of the yeast *Saccharomyces cerevisiae* (*S. cerevisiae)*-baker's yeast- in the 1930's has attracted the attention of many biologists. This unicellular fungus (Fig. 1) combines a number of advantages. Its simplicity, reasonable growth time, ease of handling and disposal, and more importantly non-pathogenicity made this organism suitable for the study of various cellular processes. *S. cerevisiae* was the first eukaryote whose genome was fully sequenced (Botstein and Fink 2011). The wealth of genetic information owing to the completion of the yeast genome sequencing project in 1996 (Goffeau, et al. 1996) has opened the possibility for functional analysis. Moreover, the availability of sequence information has also allowed comparative studies between yeast and higher eukaryotes e.g. animals and plants. In addition to genome sequencing, genetic tractability and ease of manipulation has made yeast a versatile system for investigation of the function and the regulation of genes from other eukaryotes by expressing those in yeast. A number of the Nobel Prize winning-studies in physiology and medicine have employed yeast as a model organism. This involves the discovery, by Hartwell and Nurse, of the different yeast cell cycle, *CDC*, genes and the roles of checkpoints in monitoring the different stages of the cell division cycle. Another example is the striking findings of the role of telomeres and telomerase in the protection of eukaryotic chromosomes and hence the maintenance of genome stability by Blackburn, Greider and Szostak. Very recently, Schekman was also awarded for his seminal studies of the genes that are involved in vesicular trafficking in yeast cells.

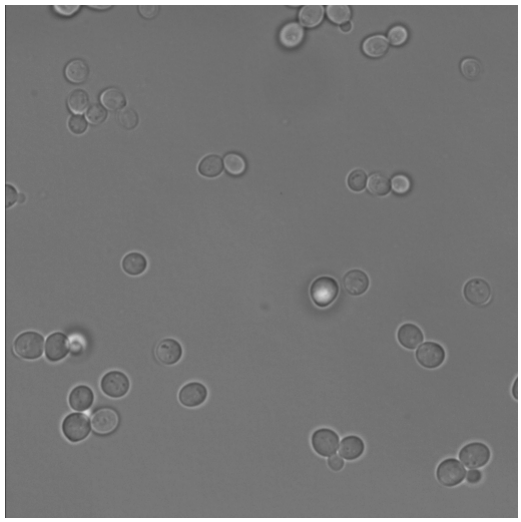

Figure 1. Bright field image showing the yeast *S. cerevisiae*

3. Glucose metabolism: glucose uptake and phosphorylation

3.1. Yeast metabolism

Metabolism refers to the overall biochemical reactions that take place within living cells and includes energy-driven anabolic pathways and energy-generating catabolic pathways. In *S. cerevisiae*, metabolism depends on two major factors: oxygen availability and the quality of carbon source in the surrounding environment (Lagunas 1976). Depending on oxygen levels, yeast can display a respiratory, a respiro-fermentative or a fermentative lifestyle. However, even though O_2 is present, alcoholic fermentation occurs in the presence of high glucose (Postma, et al. 1989, Pronk, et al. 1996). This effect is referred to as the Crabtree effect which is characteristic to *S. cerevisiae* and closely related yeasts. In addition to glucose, *S. cerevisiae* is able to ferment other mono-saccharides such as fructose, mannose and galactose. In its natural habitat, free glucose is rarely present and *S. cerevisiae* obtains glucose mainly from the disaccharides sucrose and maltose. While the hydrolysis of sucrose occurs outside the cell and requires the extracellular form of invertase, the cleavage of maltose takes place inside the cell. Maltose is taken up via maltose permease and is hydrolysed to glucose by maltase (α-glucosidase) (Carlson, et al. 1981b, Carlson, et al. 1983, Dubin, et al. 1985). The yeast *S. cerevisiae* is also able to consume non-fermentable (respirative) carbon sources such as glycerol, ethanol, acetate and lactate.

3.2. Glucose uptake

S. cerevisiae commonly lives on the surface of decaying fruits where sugar concentrations can be extremely high. Since environmental conditions may vary and the sugar concentration can drop drastically due to, for instance, sugar consumption, *S. cerevisiae* has developed a wide range of hexose transporters with distinct glucose affinities. Following its uptake, glucose is converted through glycolysis into pyruvate and through

the action of pyruvate decarboxylase and alcohol dehydrogenase into ethanol. Early studies (Becker and Betz 1972), have defined glucose uptake as the rate-limiting step of glycolysis. The yeast hexose transporters are integral plasma membrane proteins belonging to the major monosaccharide facilitator super family (MFS) (Marger and Saier 1993). The hexose transporter family includes 18 hexose transporters; Hxt1p-Hxt17p and Gal2p, and two glucose sensors Snf3p and Rgt2 (reviewed in (Horak 2013). These sensors seem to have lost their transport ability and serve as glucose sensors with distinct affinities toward glucose. While Snf3 represents the high affinity glucose sensor and is required for the induction of *HXT2* and *HXT4* genes in response to low glucose, Rgt2, its low affinity counterpart, is necessary for the induction by high glucose concentrations of the *HXT1* gene (Ozcan, et al. 1998, Ozcan, et al. 1996). As members of the major facilitator superfamily (MFS), the yeast hexose transporters contain 12 transmembrane α-helical domains and mediate transport of sugars by facilitated diffusion. Although these sugar facilitators have similar structures, they present different affinities toward glucose and distinct expression profiles (Marger and Saier 1993, Reifenberger, et al. 1997). Depending on the affinity of their products to glucose the hexose transporters genes can be subdivided into three different groups; those encoding low affinity transporters represented by *HXT1* and *HXT3,* expression of which is upregulated in the presence of high glucose concentrations, intermediate affinity transporters including *HXT2* and *HXT4* expressed at low glucose concentrations around 0.1% (Ozcan, et al. 1996) and finally high affinity transporters, *HXT6* and *HXT7,* that are highly expressed in the absence of glucose or at very low glucose conditions (Maier, et al. 2002, Reifenberger, et al. 1997). Several pieces of evidence have indicated that the high affinity transport system is repressed by moderate and high glucose, owing to the absent co-existence of the high and low affinity transport and to the observed repression of *HXT7* transcription at high glucose (Bisson 1988, Bisson, et al. 1993, Fuhrmann and Völker 1992, Ye, et al. 2001). *HXT5* on the other hand, is a moderate affinity transporter highly expressed at slow growth conditions such as sporulation, stationary phase and in the absence of non-fermentable carbon sources (Diderich, et al. 2001). The function and the regulation of Hxt8-Hxt17 are poorly documented. Although, when overexpressed, these transporters (except *HXT12*) were able to mediate glucose transport, they are thought to be involved in processes other than

hexose transport (Horak 2013, Wieczorke, et al. 1999). Indeed, Hxt9 and Hxt11 were found to be involved in pleiotropic drug resistance (Nourani, et al. 1997).

Construction of yeast strains expressing individual hexose transporters has enabled the characterization of different aspects of hexose transporters (Wieczorke, et al. 1999). Another approach based on the construction of chimeric hexose transporter proteins by fusing the amino-terminus of Hxt1 and the carboxy-terminus of Hxt7 has resulted in a strain with novel characteristics such that the mode of metabolism has been rewired from fermentation to respiration (Elbing, et al. 2004a, Otterstedt, et al. 2004). We took advantage of both approaches in order to investigate the effect of glucose uptake on the behaviour of the Snf1/Mig1 module (**Paper II**). For this purpose, we employed three strains expressing each, as a sole glucose uptake system, the low affinity glucose transporter Hxt1, the high affinity glucose transporter Hxt7 and their respective chimera *TM6**. Furthermore, we followed the migration of GFP-tagged Mig1 under different glucose up- and downshifts in single cells arrayed in microfluidic devices. We found that in cells expressing *HXT7* or *TM6** transporters and growing in high glucose, Mig1 displayed a cytosolic localization and did not respond to any glucose downshift. However, in *HXT1*-expressing cells, Mig1 exhibited a rapid and sustained response over a wide range of concentrations following either glucose up- or downshifts. However, in this strain Mig1 did not translocate to the nucleus when glucose starved cells were shifted to low glucose levels, consistent with the low affinity characteristic of Hxt1. These studies provide evidence for a firm link between the Snf1/Mig1 pathway and hexose transporters.

3.3. Glucose phosphorylation

In contrast to hexokinase PI and hexokinase PII which phosphorylate glucose and fructose, glucokinase appears to phosphorylate glucose and mannose. The fact that there is only 26 to 28% identity in the amino acid sequence between hexokinases and glucokinase might explain this divergence toward sugar substrate specificity and indicate different physiological roles (Albig and Entian 1988). Both hexokinases and glucokinase mediate

sugar phosphorylation by transferring a phosphoryl group from ATP to the sixth position of the sugar (Bisson, et al. 1993). *HXK2* plays a vital role in glucose phosphorylation and is highly expressed during growth on the fermentable carbon sources glucose, fructose and mannose, while expression of *HXK1* and *GLK1* occurs during growth on non-fermentable carbon sources or galactose (Herrero, et al. 1995). In addition to its catalytic activity, Hxk2 seems to also confer a regulatory role (Entian 1980, Entian and Frohlich 1984, Entian and Mecke 1982, Entian, et al. 1977, Pelaez, et al. 2010). In fact, mutants defective in hexokinase PII fail to properly repress expression of invertase and maltase in high glucose medium. Although overexpression of *GLK1* in a *hxk1Δhk2Δ* double mutant resulted in high phosphorylating activity, glucose repression remained impaired (Rose, et al. 1991). Characterization of random point mutants where catalytic and regulatory functions were differentially affected as well as two mutant Hxk2 alleles with either catalytic or regulatory activity suggested that catalytic and regulatory function might depend on different domains of Hxk2 (Hohmann, et al. 1999, Pelaez, et al. 2010). A 10-amino acid sequence, located between Lys_6 and Met_{15} was shown to be required for the nuclear localization of Hxk2. Deletion of this region impaired not only the nuclear enrichment of Hxk2 but also glucose repression of *SUC2* (a well characterized glucose-repressed gene encoding the invertase enzyme), *HXK1* and *GLK1* and the glucose induction of its own expression, suggesting a direct role of Hxk2 in transcription regulation (Herrero, et al. 1998, Rodriguez, et al. 2001). However it is worth mentioning that Hxk2 does not seem to be solely required for early glucose repression of *SUC2,* rather it seems to be only essential for the steady-state repression of *SUC2* (which occurs 3h after glucose addition) (Sanz, et al. 1996, Winde, et al. 1996). *In vitro* assays have indicated that Hxk2 interacts with Mig1 to form a repressor complex at the promoter of the *SUC2* gene. Mig1 Ser_{311}, which is phosphorylated by Snf1, is crucial for Hxk2-Mig1 interaction, suggesting that Hxk2 might play a role in protecting Mig1 against Snf1 phosphorylation upon growth on high glucose (Ahuatzi, et al. 2004, Ahuatzi, et al. 2007). Taken together, Hxk2 appears to be a protein with a dual function: it operates in the cytoplasm as a glycolytic enzyme and in the nucleus as a co-repressor for Mig1 mediating the repression of glucose repressed genes. Also Hxk1 seems to be involved in catabolite repression. Indeed, a *hxk1Δhxk2Δ* double mutant showed a more pronounced defect in catabolite repression of *SUC2* and the cytochrome c-encoding *CYC1*

genes as compared to the *hxk2Δ* single mutant (Ma and Botstein 1986). Moreover, when overexpressed, Hxk1 seemed to partially restore glucose repression of maltase and invertase (Rose, et al. 1991).

Hxk2 exists in a dimer-monomer equilibrium where only the monomeric form but not the dimer appears to be phosphorylated (Randez-Gil, et al. 1998). Hxk2 is phosphorylated in the absence of glucose at Ser_{14}, possibly by Snf1 (Fernandez-Garcia, et al. 2012, Kriegel, et al. 1994) and dephosphorylated in the presence of glucose by the Reg1-Glc7 phosphatase complex. It has been proposed that phosphorylation and dephosphorylation at Ser_{14} are responsible for Hxk2 nuclear export and import, respectively, since phosphorylated Hxk2 binds the exportin Xpo1 and dephosphorylated Hxk2 binds the α/β importin Kap60 (Fernandez-Garcia, et al. 2012). This nucleo-cytoplasmic shuttling pattern is analogous to other transcription factors such as the glucose repressor Mig1 (see section on Mig1 below) and the calcineurin-responsive zinc finger Crz1 (Cyert 2003, De Vit, et al. 1997). Hence, these results suggest a model where Snf1 phosphorylation of Hxk2 mediates its nuclear export under low levels of glucose, while Reg1-Glc7 dephosphorylates Hxk2 under high glucose levels promoting its nuclear localization, thereby participating in the repression of Mig1 repressible-genes. This is contradictory to the previously reported epistatic relationship of *SNF1* to *HXK2* suggesting that Hxk2 acts upstream of Snf1 in the glucose-repression pathway (Neigeborn and Carlson 1984). In the same line, Prieto's group has reported that Snf1 is not the kinase responsible for Hxk2 phosphorylation since the phosphorylation of the Hxk2 monomer upon shift from glucose to galactose or ethanol was unaffected by deletion of *SNF1* gene (Randez-Gil, et al. 1998). Altogether, the physiological relationship between Hxk2, Snf1 and Reg1-Glc7 remains to be elucidated.

3.4. Energy metabolism and ATP/ADP/AMP homeostasis

All living cells require energy as a fuel to drive diverse biosynthetic functions such as cell growth and proliferation. However, Lagunas has reported that as much as 60% of ATP produced by yeast cells appears to be spent on processes that are not directly associated to biomass production but rather to futile cycles and pH homeostasis in the cytoplasm and the

vacuole (Lagunas 1976, Verduyn, et al. 1991). *S. cerevisiae* utilizes sugars, most preferably glucose, as a source of energy. During glycolysis, two molecules of the energy-rich molecule ATP are consumed while four ATP molecules are generated from each molecule of glucose. In addition to ATP, some of the intermediary metabolites are used for biosynthesis. For an optimal functioning of the cell, there needs to be a balance between energy production and energy consumption. This energy balance is owed to the ubiquitous adenylate kinase which catalyses nucleotide interconversion reaction (2 ADP $\longleftrightarrow$ ATP + AMP) and hence plays an important role in the balance of cellular adenine nucleotide pool in all organisms (Dzeja, et al. 2007, Dzeja and Terzic 2009, Noma 2005). In yeast, the major form of the adenylate kinase, Aky2 is present in both mitochondria and cytoplasm and is required for growth on non-fermentable carbon sources (Bandlow, et al. 1988). According to the aforementioned reaction, any minor changes in the equilibrium between ATP and ADP would result in large alterations of AMP levels. Hence, a drop in cellular energy resulting in an increase in ADP, would cause adenylate kinase to shift the reaction toward the production of ATP and AMP (reviewed in (Hardie, et al. 2012). Thus, it is not surprising that the ratio of levels of AMP to ATP is an indicator of the energy status in the cell. The involvement of adenosine nucleotides in the regulation of energy homeostasis was proposed in 1964 when Atkinson et al., observed that ATP inhibits the activity of the yeast phosphofructokinase and that this inhibition is overcome by AMP (Ramaiah, et al. 1964). In addition to phosphofructokinase, the glycogen-catalysing enzyme, glycogen phosphorylase, and the gluconeogenic enzyme, fructose-1,6-biphosphatase are also regulated by the AMP:ATP ratio (Hardie, et al. 2012). Likewise, the mammalian AMP-activated protein kinase and its yeast counterpart SNF1 are regulated by AMP and ADP respectively (Chandrashekarappa, et al. 2013, Hardie, et al. 2003, Mayer, et al. 2011).

4. Glucose signalling pathways

All living cells are able to sense and respond to different environmental stimuli by the means of distinct signalling pathways leading thus to a meticulous regulation of gene expression. In the yeast *S. cerevisiae* as in other microorganisms, nutrient availability is one of the major factors affecting growth and differentiation. Being the most preferred carbon and energy sources, glucose (or fructose) represents a global regulator of cell growth and metabolism. Addition of glucose to yeast cells growing on non-fermentable carbon sources induces not only the genes required for its uptake and metabolism, but also the genes responsible for growth and proliferation. Moreover, glucose represses the expression of the genes that are required for respiration and the utilization of alternative carbon sources. This mechanism is referred to as glucose repression and is mediated by the Snf1/Mig1 pathway. By a mechanism called glucose inactivation, glucose triggers the inactivation and/or the degradation of a number of already existing enzymes such as fructose-1,6-bisphosphatase (Holzer 1976). This transcriptional and metabolic effect of glucose involves various signalling networks such as the Ras /PKA, Gpr1/PKA, Snf3/Rgt2, Snf1/Mig1, Hap and Sch9 (Conrad, et al. 2014). Although the core components of most of these pathways have presumably been identified, the mechanism by which glucose activates some of these pathways is poorly understood. It is now thought that the increase in intracellular pH generated by glucose is the stimulus of the Ras/PKA pathway. Moreover, it has been speculated that the G-protein coupled receptor Gpr1 functions as a glucose receptor and that the binding of glucose to Gpr1 promotes the activation of Gpa2 and subsequently the activation of the Gpr1/PKA pathway (Lemaire, et al. 2004). The fact that Snf1 activity is altered by glucose phosphorylation implies that the glucose signal occurs internally. Accordingly, a model that links metabolites such as adenosine nucleotides to the glucose regulation of Snf1 activity has been proposed. In this model, the low energy adenylate ligand seems to bind to the SNF1 heterotrimer protecting it from dephosphorylation by the phosphatase (Chandrashekarappa, et al. 2013, Mayer, et al. 2011, Townley and Shapiro 2007).

4.1. The Ras-cAMP/ PKA pathway

The Ras-cAMP pathway (Fig. 2) is a central glucose-regulated signalling pathway that is required for growth on glucose. Indeed, activation of this pathway by glucose addition affects 90% of glucose-induced transcriptional changes (Zaman, et al. 2009). In cells growing on non-fermentable carbon sources or in stationary phase, the cAMP-dependent protein kinase (PKA) is present as an inactive hetero-tetramer. This complex is composed of two catalytic subunits, encoded by three redundant genes, *TPK1*, *TPK2* and *TPK3,* and two identical regulatory subunits encoded by *BCY1* (Conrad, et al. 2014). Addition of rapidly fermentable carbon sources, such as glucose, results in the activation of the adenylate cyclase Cyr1 (Cdc35) which synthesizes cyclic AMP (cAMP) from ATP. This results in a rapid but transient increase in the intracellular cAMP concentration. Subsequently, cAMP binds to the Bcy1 subunits promoting its release from the tetramer and resulting in the activation of PKA. Similar to the situation in mammalian cells, yeast Cyr1 is activated by the small GTP-binding proteins Ras1 and Ras2 (Kataoka, et al. 1985). Ras1/2 are plasma membrane-attached proteins that continuously cycle between the GDP-bound inactive and the GTP-bound active states (Broek, et al. 1987, Toda, et al. 1985). Ras activation is promoted by the guanine nucleotide exchange factor Cdc25 and its homolog Sdc25 (Camonis, et al. 1986, Damak, et al. 1991), which exchange GDP for GTP. Ras is negatively regulated by Ras-GTPase activating proteins (Gap) Ira1 and Ira2 (Tanaka, et al. 1989, Tanaka, et al. 1990). In addition to Ras protein, the G-protein coupled receptor Gpr1 and the α-subunit of the G-protein Gpa2 also participate in the activation of PKA through the activation of adenylate cyclase (De Vries and Gist Farquhar 1999, Guan and Han 1999, Kraakman, et al. 1999, Kubler, et al. 1997). Hence two signalling branches seem to activate adenylate cyclase; the Ras system and the Gpa2/Gpr1 system. It has been argued that intracellular acidification caused by glucose addition, but not glucose itself, triggers the Ras system and that stimulation of the Gpa2/Gpr1 system specifically depends on glucose (Arguelles, et al. 1990, Colombo, et al. 1998, Dechant, et al. 2010, Kraakman, et al. 1999). A model where Gpr1 functions as a glucose receptor, which interacts with and activates Gpa2, which in turn stimulates adenylate cyclase, has been proposed (Xue, et al. 1998, Yun, et al. 1997). However, several pieces of evidence are not consistent with this

model; the lack of pharmacological assays for Gpr1-ligand binding, the weak affinity of Gpr1 for glucose and the absence of transcriptional effects after glucose addition in *gpa2Δ* and *gpr1Δ* mutants craves for the need of further studies (Broach 2012, Lemaire, et al. 2004, Zaman, et al. 2009). Active PKA targets a number of effectors such as Msn2, Msn4, Yak1 and Rim15 (Conrad, et al. 2014, Pedruzzi, et al. 2003, Thevelein and de Winde 1999) affecting the transcriptional regulation of a number of genes whose products are involved in the stimulation of mass accumulation, stress resistance and cell cycle progression. All these effects converge into one ultimate outcome, which is the regulation of cell growth in response to the quality and quantity of the available carbon source.

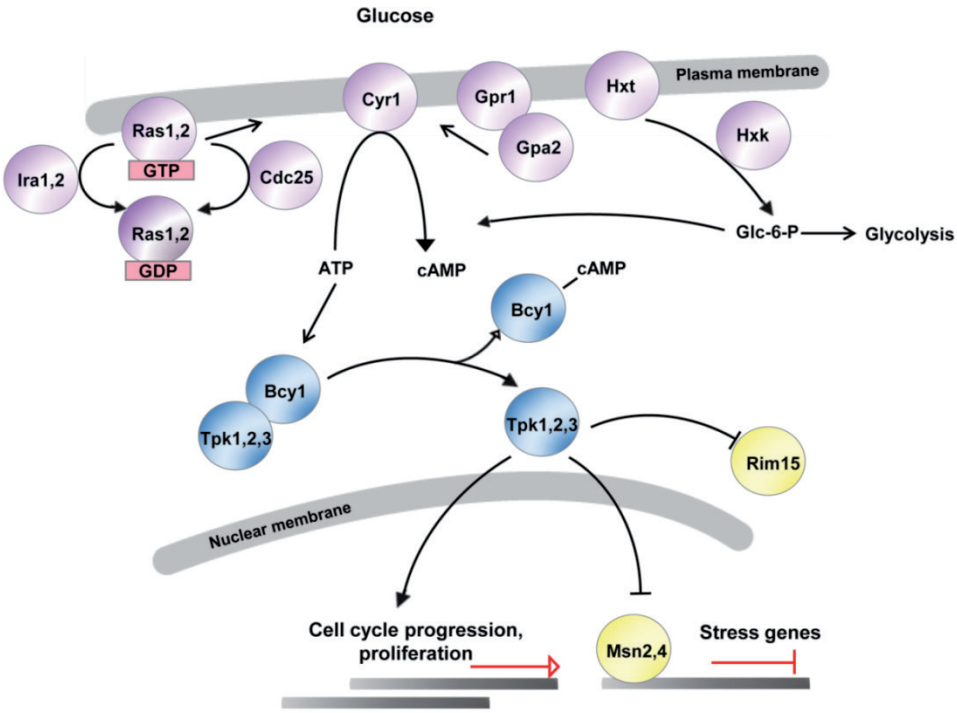

Figure 2. The Ras-cAMP/PKA pathway. The uptake of glucose by hexose transporters (Hxt) and its phosphorylation by hexokinases (Hxk) generates an intracellular signal that activates the Ras-cAMP pathway. Ras1,2 are two plasma membrane-attached proteins that constantly cycle between the GDP and GTP states due to the activity of the guanine nucleotide exchange Cdc25 and its homolog Sdc25 (only Cdc25 is depicted) and the Ras-GTPase activating proteins (Gap) Ira1 and Ira2. The Ras-GTP-bound form binds adenylate cyclase (Cyr1), which catalyses the production of cyclic AMP (cAMP) from ATP. Extracellular glucose seems to be detected by the Gpr1-Gpa2 system. The synthesized cAMP activates PKA by binding to its inhibitory subunit, Bcy1. Active PKA targets the effectors, Rim15, Msn2 and Msn4 in order to stimulate cell cycle progression and proliferation and dampen the expression of stress genes.

4.2. The Snf3/Rgt2 glucose induction pathway

To ensure an optimal production of energy, yeast cells have adapted a system (Fig. 3) for the upregulation of glycolytic genes and, most importantly, for the induction of glucose transporter encoding genes. Snf3 and Rgt2 are very similar sensor proteins (Ozcan, et al. 1996), which confer intracellular glucose signalling via their long cytoplasmic C-termini. The ultimate target of the Snf3/Rgt2 signalling cascade is the transcription factor Rgt1. Rgt1 is a Zn_2Cys_6 DNA-binding protein that negatively regulates the transcription of *HXT* genes during glucose depletion (Kim, et al. 2003). In the absence of glucose, Rgt1 forms a repressing complex at the promoter of *HXT* genes together with the co-repressors Mth1 and Std1 (Lakshmanan, et al. 2003) and the general repressors Tup1-Cyc8 (Ssn6). However, the presence of glucose promotes the phosphorylation of Std1 and Mth1 by the casein kinases I, Yck1 and Yck2, targeting them to degradation by ubiquitination (Flick, et al. 2003b, Moriya and Johnston 2004, Spielewoy, et al. 2004). Consequently, Rgt1 becomes hyper-phosphorylated causing an intramolecular interaction between its zinc-binding and central domains thus preventing DNA binding. Several pieces of evidence (Flick, et al. 2003a, Jeong, et al. 2004, Jouandot, et al. 2011, Mosley, et al. 2003) suggest that the hyper-phosphorylation of Rgt1 not only interferes with its DNA binding but it also converts Rgt1 into an activator required for the maximal induction of *HXT1*. Thus, Rgt1 has a dual function where it acts as a repressor in the absence of glucose and an activator in the presence of glucose, depending on its phosphorylation state (Jouandot, et al. 2011).

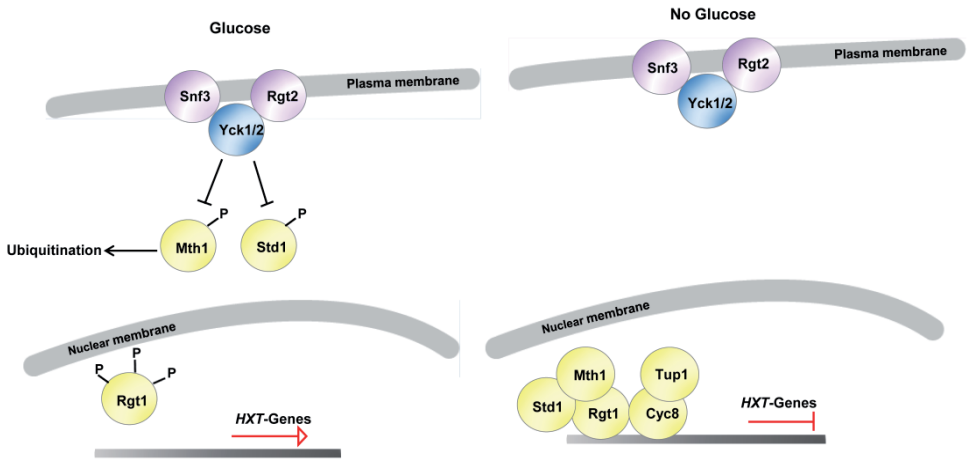

Figure 3. A simplified scheme of the Snf3/Rgt2 pathway. In the absence of glucose, the transcription factor Rgt1 forms a repressing complex at the promoter of *HXT* genes together with the co-repressor Mth1 and Std1 and the general repressor Tup1-Cyc8. When glucose becomes available, the casein kinases Yck1 and Yck2 promote the phosphorylation of Mth1 and Std1, targeting them to ubiquitination. This results in the hyper-phosphorylation and activation of Rgt1 and thereafter the derepression of *HXT* genes.

4.3. Glucose repression

Glucose represents the energy fuel for most cells. In yeast, both glucose and fructose are the most preferred carbon sources. The presence of one of these sugars exerts catabolite or simply glucose repression. Glucose repression is a widespread phenomenon in micro-organisms and affects transcription of a large set of genes. In *S. cerevisiae*, addition of glucose results in at least a twofold change in the expression of 40% of the 6,000 genes (Feldmann 2011). The genes targeted are those required for the uptake and metabolism of alternative carbon sources such as sucrose, maltose, galactose, glycerol, acetate, ethanol and others as well as the genes encoding enzymes for gluconeogenesis and respiration. Glucose mediates the repression of transcription by two mechanisms; on the one hand, glucose interferes with transcription activators by decreasing their transcription, preventing their interaction with the activating site at the promoter, hampering their activating

functions, or by inducing their nuclear exclusion. On the other hand glucose facilitates the action of transcription repressors (Gancedo 1998). The central pathway for glucose repression is the Snf1/Mig1 system.

4.3.1. The Snf1/Mig1 glucose repression pathway

The central regulator of glucose repression is the well-established Snf1/Mig1 pathway (Fig. 4). Snf1 was identified in 1981 by screening for mutants that are unable to utilize sucrose (Carlson, et al. 1981a) and was later on found to be allelic to *cat1* and *ccr1* mutants (Ciriacy 1977, Zimmermann, et al. 1977). The *SNF1* gene encodes a Ser/Thr kinase usually found in a complex (SNF1) together with one of the three β subunits (Sip1, Sip2 and Gal83) and the γ subunit Snf4 (Celenza and Carlson 1986, Celenza, et al. 1989). Activated by glucose depletion, Snf1 stimulates expression of genes that are required for the uptake and the utilization of other carbon sources, gluconeogenic genes and genes required for respiration (reviewed in (Carlson 1999, Gancedo 1998)). In addition to nutrient limitation, SNF1 is also involved in the regulation of other cellular processes such as meiosis and sporulation, aging, fatty acid metabolism, glycogen synthesis, fitness and stress resistance (Ashrafi, et al. 2000, Hardy, et al. 1994, Honigberg and Lee 1998, Navarro and Igual 1994, Zhang, et al. 2013). Activation of SNF1 requires phosphorylation on Thr_{210} in the activation loop of the catalytic subunit, Snf1 (Estruch, et al. 1992, Nath, et al. 2003). Any of the three partially redundant upstream kinases Sak1, Tos3 and Elm1 is able to mediate this phosphorylation, with Sak1 being the major activating kinase (Estruch, et al. 1992, Hong, et al. 2003a, Liu, et al. 2011, Nath, et al. 2003, Sutherland, et al. 2003). In addition to Snf1 phosphorylation, Snf4 is also required for Snf1 activity. In response to glucose limitation, Snf4 binds the C-terminal regulatory domain of Snf1 preventing the auto-inhibition of the kinase domain by the C-terminus (Jiang and Carlson 1996). This mechanism was confirmed by structural analyses of the yeast SNF1 heterotrimer indicating that the interaction between Snf1 and Snf4 occurs at the regulatory region of Snf1 illustrating the role of this interaction in the regulation of Snf1 activity (Amodeo, et al. 2007). The β-subunits interact with both Snf1 and Snf4 and serve not only

to stabilize the Snf1-Snf4 association in the complex but also to regulate the subcellular localization of the SNF1 complex and substrate definition (Jiang and Carlson 1996, Schmidt and McCartney 2000, Vincent, et al. 2001). Under glucose abundance, all the β-subunits are distributed in the cytosol. However, upon glucose limitation each subunit is differentially localized with Gal83 being the only subunits that localizes to the nucleus (Hedbacker, et al. 2004). The β-subunits seem to also confer upstream kinase specificity depending on growth conditions (Hedbacker, et al. 2004, McCartney, et al. 2005).

Recent structural studies (Chandrashekarappa, et al. 2013) suggested that Snf1 phosphorylation promotes the association of the kinase domain with the heterotrimer core resulting in the remodelling of the activation loop and its exposure to both kinases and phosphatases. SNF1 is negatively regulated mostly by the Reg1-Glc7 protein phosphatase1 (PP1). The catalytic subunit Glc7 is targeted to SNF1 by the Reg1 subunit resulting in Snf1 dephosphorylation and return to the auto-inhibitory status (Sanz, et al. 2000, Tu and Carlson 1995). It has been proposed that the recruitment of Glc7 by Reg1 affects the conformation of SNF1 complex (Sanz, et al. 2000). Under derepressing conditions, Snf1 phosphorylates Reg1, probably promoting its release from the kinase complex (Sanz, et al. 2000). Additional phosphatases including Sit4 and Ptc1 appear to also contribute to Snf1 dephosphorylation (Ruiz, et al. 2011, Ruiz, et al. 2013). Whether glucose regulates SNF1 activity through either phosphorylation or dephosphorylation or both is not clear. Rubenstein and co-workers (Rubenstein, et al. 2008) have shown that glucose most likely regulates the accessibility of the phosphatase to the Snf1 activation loop. This is consistent with the recent structural analyses of both AMPK and SNF1 suggesting that ADP might induce a reorganization of the complex into a phosphatase-resistant form (Chandrashekarappa, et al. 2013, Gowans, et al. 2013, Mayer, et al. 2011).

It is still unclear how the presence of glucose affects Snf1 activity. Given the structural and functional similarities between Snf1 and its mammalian ortholog AMPK it is thought that the activity of SNF1 is also regulated by adenosine nucleotides. Indeed, *in vivo* studies have indicated the existence of a positive correlation between SNF1 activity and nucleotide ratios (e.g. AMP: ATP) (Wilson, et al. 1996). However in these, and in other studies (Mitchelhill, et al. 1994, Woods, et al. 1994), SNF1 was not activated allosterically by

AMP. Comparative studies of the AMP-binding site between the AMPK γ-subunit and Snf4 revealed that the latter contains a $His_{151}Gly$ substitution. This residue is critical for AMP binding to the AMPK γ-subunit and may explain why AMP does not bind to the Snf1-Snf4 complex. Recent studies (Mayer, et al. 2011) suggested that ADP, but not AMP, might be the "long-sought" stimulator for Snf1 phosphorylation. Although, ADP did not allosterically regulate SNF1 activity, it protected the enzyme from dephosphorylation. Moreover, binding studies showed that Snf4 contains two nucleotide-binding sites and that ADP binding to the weaker site confers protection of Thr_{210} against dephosphorylation. ADP was also found to bind to the γ-subunit of the AMPK heterotrimer and participates in AMPK activation albeit not to the same extent as AMP (Oakhill, et al. 2011). In addition it appears that ADP-binding to the ATP-binding pocket of the Snf1 active site independently of the β- and γ- subunits may also protect Snf1 from dephosphorylation (Chandrashekarappa, et al. 2013). Taken together, although the regulation of AMPK by adenylate ligands appears to be well established, the effect of these nucleotides on Snf1 regulation remains opaque and hence requires further investigations, in particular *in vivo*.

Active Snf1 promotes the phosphorylation of several effectors including the transcriptional activators Sip4, Cat8, Adr1, Rds2, Hsf1 and the repressor Mig1 (Hahn and Thiele 2004, Lesage, et al. 1996, Randez-Gil, et al. 1997, Ratnakumar, et al. 2009, Smith, et al. 1999, Soontorngun, et al. 2007).

4.3.1.1. The Transcriptional repressor Mig1

The Multi-copy Inhibitor of *GAL* gene expression, Mig1, is a Cys_2His_2 zing finger DNA binding protein that binds the promoter of several glucose-repressed genes including *SUC*, *GAL* and *MAL* genes (Flick and Johnston 1992, Griggs and Johnston 1991, Hu, et al. 1995, Nehlin and Ronne 1990). Active Snf1 phosphorylates Mig1 at at least four different sites disrupting its interaction with the Tup1-Cyc8 complex and promoting its nuclear extrusion (Papamichos-Chronakis, et al. 2004, Smith, et al. 1999, Treitel, et al. 1998, Östling and Ronne 1998). The phosphorylation of Mig1 by Snf1 seems to be regulated by glucose

since phosphorylated active Snf1 does not mediate Mig1 phosphorylation in the presence of glucose (García-Salcedo, et al. 2014, Ye, et al. 2008). A model was proposed where Hxk2 interacts with both Mig1 and Snf1 in the presence of glucose thereby conferring protection of the Ser_{311} residue of Mig1 from phosphorylation by Snf1 (Ahuatzi, et al. 2004, Ahuatzi, et al. 2007). DNA-binding of Mig1 requires a GC-box core and an adjacent AT-rich region which facilitates Mig1 access to the DNA (Lundin, et al. 1994). Mig1 mediates repression by interaction with the Ssn6-Tup1 general co-repressor complex (Papamichos-Chronakis, et al. 2004, Treitel and Carlson 1995). This interaction appears to be disrupted by the conformational change of Mig1 following phosphorylation by Snf1, leading to derepression of glucose-repressed genes (Papamichos-Chronakis, et al. 2004). It is now well established that the nuclear-cytoplasmic localization of Mig1 is glucose regulated. Mig1 enters to the nucleus within a few seconds after glucose addition and quickly exits the nucleus after glucose removal (Bendrioua, et al. 2014, DeVit and Johnston 1999). In addition to Snf1, the nuclear export of Mig1 is dependent on the nuclear transporter, Msn5 (De Vit, et al. 1997, DeVit and Johnston 1999, Papamichos-Chronakis, et al. 2004). Msn5 is a β-importin homolog that mediates the export of Mig1 from the nucleus after its phosphorylation by Snf1. In the absence of Msn5, Mig1 is normally phosphorylated and properly represses *GAL1* transcription but its ability to exit the nucleus is impaired (DeVit and Johnston 1999). Although the export mechanism of Mig1 has been established, the mechanism by which Mig1 is imported to the nucleus remains elusive. Mig1 has a nuclear localization signal (NLS) in the C-terminal domain which is required for its nuclear import after glucose addition (DeVit and Johnston 1999).

Snf1-dependent phosphorylation of Mig1 is antagonized by the Reg1-Glc7 phosphatase complex although it is not entirely clear if Glc7-Reg1 directly dephosphorylates Mig1 or mediates its effect by controlling Snf1. In glucose-grown cells bearing *reg1Δ* mutation, Mig1 displayed a reduced mobility shift reflecting its partial phosphorylation (McCartney and Schmidt 2001). Moreover, in the absence of Reg1 Mig1 displayed an inefficient nuclear localization (De Vit, et al. 1997). Finally, Schmidt and co-workers have demonstrated that deletion of *REG1* increased Mig1 phosphorylation under both high and low glucose suggesting a constitutive activity of Reg1-Glc7 toward Mig1 (Rubenstein, et

al. 2008). However, although the Snf1-Mig1 system has been studied extremely well in terms of protein-protein interactions, a direct interaction between Mig1 and Glc7-Reg1 has not been reported so far. Hxk2 seems to also participate, most likely indirectly, in Mig1 dephosphorylation. Genetic analyses from our group have indicated that Hxk2 appears to facilitate the role of the phosphatase Reg1 in Mig1 dephosphorylation (Ye et al., unpublished results).

Mig2 and Mig3 are two other Cys_2His_2 zinc finger transcription factors that share similar DNA-binding specificity with Mig1 (Lutfiyya, et al. 1998, Lutfiyya and Johnston 1996) but their function and the mode of regulation are significantly different. Like Mig1, Mig2 binds to GC-rich sequences at the promoter DNA. However, in contrast to Mig1, Mig2 does not seem to be phosphorylated by Snf1 and its nuclear-cytoplasmic localization does not seem to be regulated by glucose (Lutfiyya, et al. 1998). Instead, expression of *MIG2* is upregulated by glucose in a Snf3/Rgt2 dependent manner (Kaniak, et al. 2004). Mig3 on the other hand is regulated by glucose at different levels; the expression of *MIG3* gene is upregulated by glucose and Mig3 is degraded after phosphorylation by Snf1 in the absence of glucose (Dubacq, et al. 2004). Mig1 and Mig2 cooperate to mediate repression of glucose-repressed genes where the individual contributions of Mig1 and Mig2 vary in a gene-specific manner (Westholm, et al. 2008). Mig3 seems to marginally participate in catabolite repression but appears to be involved in genotoxic stress and aging (Dubacq, et al. 2004, Lutfiyya and Johnston 1996, Westholm, et al. 2008).

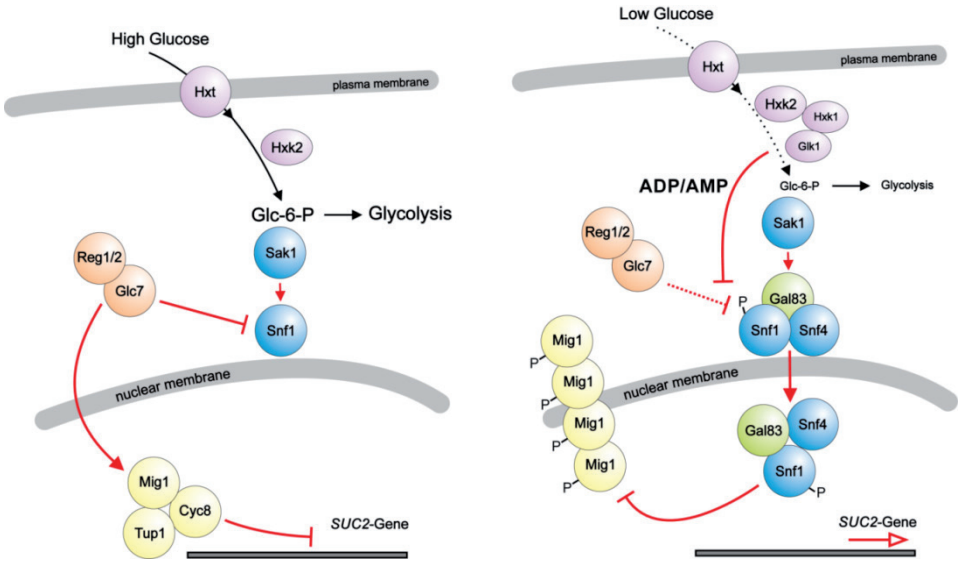

Figure 4. The Snf1/Mig1 pathway (adapted from (Bendrioua, et al. 2014)). Glucose is transported by hexose transporter (Hxt) and phosphorylated by mainly hexokinase PII (Hxk2) generating glucose-6-phosphate (Glc-6-P), which is further metabolized by glycolysis. Snf1 and Mig1 are dephosphorylated by the Reg1-Glc7 phosphatase complex leading to nuclear localization of Mig1 which together with Cyc8 (Ssn6)-Tup1 co-repressor represses the transcription of glucose-repressed genes. When glucose is depleted, hexokinase PI (Hxk1) and glucokinase (Glk1) work in concert with Hxk2 to phosphorylate glucose. This results in an increase in ADP which protects Snf1 from dephosphorylation. Snf1 becomes activated by phosphorylation by the upstream kinase Sak1 and subsequently phosphorylates Mig1. Phosphorylated Mig1 dissociates from the Cyc8-Tup1 complex and translocates to the cytoplasm leading to the relief of gene repression.

4.4. The AMPK pathway, the mammalian SNF1 ortholog

AMP-activated protein kinase (AMP-PK or simply AMPK) is the highly conserved mammalian ortholog of SNF1 and prototype of this kinase family. Activated by AMP, this multi-substrate protein kinase was originally reported to be a negative regulator of lipid synthesis (Fig. 5) (Hardie, et al. 1989). As an energy sensor, AMPK is activated by a drop in ATP levels, which results in an increase in the AMP concentration, and is therefore

activated by metabolic stresses including exercise, glucose depletion, hypoxia, hyperosmotic and heat shock, a number of hormones and pharmacological drugs (reviewed in (Hardie 2007, Hardie, et al. 2012)). Active AMPK operates by turning off ATP-consuming anabolic pathways such as lipid, protein and glycogen synthesis and by turning on ATP-generating catabolic pathways for instance glucose and fatty-acid metabolism. In the brain, activation of AMPK by low glucose stimulates eating desire. In analogy to SNF1, AMPK is an αβγ heterotrimeric complex. While yeast possesses only one isoform of the α- and γ-subunits and three isoforms of the β-subunits, the AMPK trimer contains two alterative α-catalytic subunits (α_1 and α_2), two scaffolding β-subunits (β_1 and β_2) and three regulatory γ-subunits (γ_1, γ_2 and γ_3). Sequencing of the mammalian AMPK α-subunit kinase domain has revealed a strong amino acid sequence homology with that of the *S. cerevisiae* Snf1 catalytic subunit indicating conserved structural and functional features (Gao, et al. 1995, Mitchelhill, et al. 1994). Like Snf1, the AMPKα contains the auto-inhibitory sequence (AIS) and binds to β- and γ-subunits through its C-terminus (Crute, et al. 1998, Dyck, et al. 1996).

AMPK is activated by phosphorylation at the Thr_{172} in the activation loop of the catalytic α-subunit (Hawley, et al. 1996). This phosphorylation is mediated by the upstream kinases, the tumour suppressor LKB1 and the two calmodulin-dependent protein kinase kinases, CaMKKα and CaMKKβ. LKB1 is the major activating kinase (Hawley, et al. 2003, Hawley, et al. 2005). LKB1 functions conjointly with the pseudo-kinase STRAD (sterile-20-related adaptor) and the scaffolding protein MO25 (mouse-protein 25) (Hawley, et al. 2003). The identification in a genetic screen of the transforming growth factor-beta-activated kinase 1 (TAK1) as a Snf1-activating kinase in a triple mutant lacking all the upstream kinases suggest that this mitogen-activated protein kinase kinase kinase might be an additional AMPKK (Momcilovic, et al. 2006). Indeed, further genetic analyses indicated that the cytokine tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induced activation of AMPK is dependent on TAK1 (Herrero-Martín, et al. 2009). However, how TAK1 regulates the activity of AMPK *in vivo* needs to be further investigated. While activation of AMPK by LKB1 appears to occur in response to a raise

in AMP, AMPK activation by CaMKKs is enhanced by an increase in the intracellular concentration of Ca^{2+} (Mihaylova and Shaw 2011, Sanders, et al. 2007).

The mechanism of AMPK regulation by AMP has long been debated. It is now proposed that AMP acts at three different levels: (1) AMP allosterically activates AMPK, (2) promotes phosphorylation of the α-Thr_{172} following the myristoylation of the β-subunit and (3) prevents its dephosphorylation by one or several protein phosphatases (Davies, et al. 1995, Gowans, et al. 2013, Oakhill, et al. 2011, Sanders, et al. 2007, Suter, et al. 2006).

Active AMPK targets metabolic enzymes and regulates gene expression by phosphorylating a number of effectors such as the transcription factors HNF-4α, required for the activation of glucose, cholesterol and fatty acid metabolism, and the nuclear receptor co-activator p300 (Hong, et al. 2003b, Leclerc, et al. 2001, Leff 2003, Yang, et al. 2001).

In addition to their high structural and functional conservation, the mammalian AMPK and the yeast SNF1 complexes also seem to be functionally interchangeable ((Woods, et al. 1994) and **Paper V**). Based on this concept, we expressed entire mammalian complexes in yeast and investigated the regulation of AMPK by the yeast regulatory machinery in cells lacking the endogenous SNF1 complex (**Paper V**). Different combinations of the AMPK isoforms were constructed. All combinations comprising the α1-, β1- or β2- and γ1- or γ3- subunits conferred growth on alternative carbon sources such as raffinose, ethanol and glycerol. Most importantly, the yeast-expressed AMPK was normally regulated by glucose availability. We investigated the phosphorylation status of AMPK in different mutants lacking, *REG1*, *REG2* and *SIT4* in order to elucidate if AMPK was controlled by the same phosphatases as SNF1. While the yeast SNF1 phosphorylation state is unresponsive to changes in glucose availability, AMPK was normally regulated in the *reg1*Δ and *reg1*Δ *reg2*Δ double mutant. It appears that the type 2A-related protein phosphatase Sit4 may contribute to some extent to the dephosphorylation of AMPK in yeast (our unpublished data). Altogether, these studies revealed that it is possible to express a functional AMPK that is properly regulated by glucose in yeast.

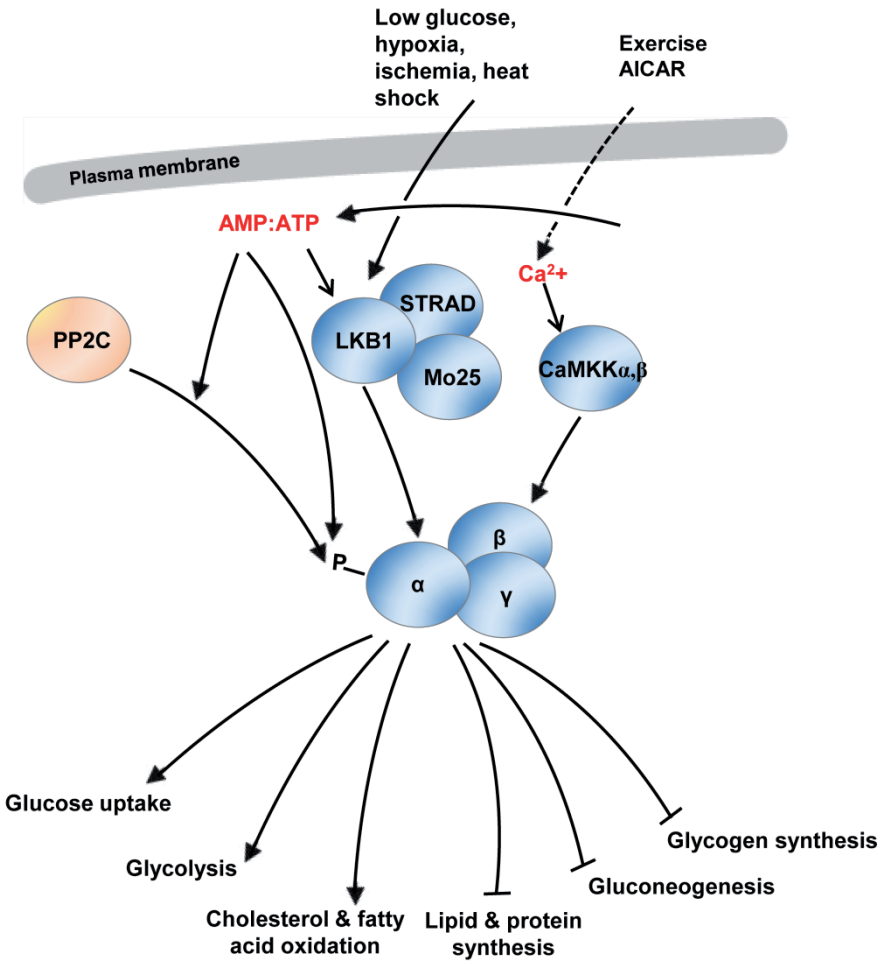

Figure 5. The AMPK pathway. The AMP-activated protein kinase is activated by various stimuli including low glucose, hypoxia, ischemia and heat shock which lead to a raise in the AMP: ATP ratio. AMP acts by stimulating AMPK phosphorylation by LKB1, directly binding to AMPK and by protecting AMPK against dephosphorylation by PP2C. An increase in the levels of Ca^{2+} is most likely due to exercise and drugs (e.g. AICAR). This leads to the activation of AMPK by either CaMKKα or CaMKKβ. Active AMPK stimulates glucose uptake, glycolysis and lipid oxidation while it hampers lipid and protein biosynthesis, gluconeogenesis and glycogen synthesis.

4.4.1. Significance of the AMPK/SNF1 pathway

Given its pleiotropic actions in the regulation of lipid and glucose metabolism, AMPK has become a potential target for therapeutic interventions in diabetes and metabolic diseases. AMPK is expressed in multiple tissues that are metabolically active such as pancreas, liver, lung, skeletal muscle, adipose tissue and hypothalamus, where it exerts different functions (reviewed in (Zhang, et al. 2009)). In liver, active AMPK functions to repress hepatic gluconeogenesis by down-regulating the transcription of the genes encoding the rate-controlling gluconeogenic steps phosphoenolpyruvate carboxykinase (PECK) and glucose-6-phosphatase (Horike, et al. 2008, Lochhead, et al. 2000, Shaw, et al. 2005). These results suggest a role of AMPK in counteracting hyperglycemia-related type 2-diabetes. In muscle, AMPK activation by muscle contraction stimulates mitochondrial fatty acid oxidation and upregulates mitochondrial and lipid utilization genes through the activation of the transcriptional regulators PGC-1α and FOXO1 respectively (Canto, et al. 2010, Suwa, et al. 2003). In parallel, activated AMPK promotes muscle glucose uptake by upregulating GLUT-4 transcription and stimulating its translocation to the cell surface in the muscle tissue (Hayashi, et al. 1998, Merrill, et al. 1997, Russell, et al. 1999). Altogether, activation of AMPK seems to be a potential approach for bypassing the defects of insulin-resistance and metabolic syndrome-related diseases.

A number of chemical compounds and anti-diabetic drugs such as phenformin, metformin and pioglitazone activate AMPK suggesting that at least some of their beneficial effects are mediated through AMPK (Cool, et al. 2006, Saha, et al. 2004, Xiao, et al. 2013, Zhou, et al. 2001). While metformin and phenformin activate AMPK indirectly and independently on adenosine nucleotides, the 5-aminoimidazole-4-carboxamide riboside (AICAR) mimics the allosteric effect of AMP and hence directly activate AMPK (Corton, et al. 1995, Sakamoto, et al. 2004, Zhou, et al. 2001). Our studies (**Paper V**) indicated that metformin, phenformin and AICAR did not affect the phosphorylation of yeast-expression AMPK suggesting that these drugs are probably not imported into or quickly exported out of the cells. Another possibility could be that the mechanism of AMPK activation by these drugs (especially metformin and phenformin) in mammalian cells is distinct from that in

yeast. Moreover, these studies highlight the use of humanized yeast where mammalian genes and proteins homologs can be functionally expressed and serve to complement yeast deletion mutants (Botstein and Fink 2011). Studies involving the expression of heterologous proteins in yeast have been successfully applied not only to the study of the structural and functional regulation of these proteins but also for the pharmacological purposes such as the screening of novel drugs.

4.5. Snf1 orthologs in other eukaryotes

In addition to yeast and mammals, the AMPK/SNF1 pathway is present in most, if not all, eukaryotes. In plants, there are numerous SNF1-related protein kinases (SnRKs) with SnRK1 being most similar to Snf1 (reviewed in (Ghillebert, et al. 2011, Halford and Hardie 1998, Halford, et al. 2003, Hardie 2007). SnRK1 was originally isolated from rye in 1991 and restored SNF1 function when expressed in the yeast *snf1Δ* mutant (Alderson, et al. 1991). Arabidopsis SnRK1 displays 48% sequence similarity with SNF1 and AMPK (Halford and Hardie 1998). Similar to SNF1 and AMPK1, SnRK1 is regulated by phosphorylation and has a direct effect on metabolism by regulating metabolic enzymes and an indirect role by controlling gene expression. SnRK1 plays an important role in starch biosynthesis by enhancing the expression of the sucrose synthase gene Sus4 (Purcell, et al. 1998). In the moss *Physcomitrella patens*, two SNF1-related protein kinase encoding genes, *PpSNF1a* and *PpSNF1b,* play an important role in coping with darkness since the double knockout mutant *snf1asnf1b* was unable to grow in a normal day-night light cycle (Thelander, et al. 2004). The nematode *Caenorhabditis elegans* has two homologs of the AMPKα subunit, Aak1 and Aak2, which are activated by AMP and involved in the extension of lifespan (Apfeld, et al. 2004). The fruitfly *Drosophila melanogaster* contains single isoforms of the α-, β- and γ- subunits that are essential for the activity of the AMPK complex. In analogy to mammalian AMPK, *D. melanogaster* AMPK is sensitive to the AMP: ATP ratio and the α-subunit is activated by phosphorylation at Thr_{184} within its activation loop (Pan and Hardie 2002). Deletion of AMPK causes severe abnormalities in cell polarity and mitosis (Lee, et al. 2007). SNF1

has also been studied in other yeasts than *S. cerevisiae*. While *Kluyveromyces lactis* Snf1 (KlSnf1) is responsible for sugar (e.g. lactose) uptake and metabolism (Lodi, et al. 2001, Wiedemuth and Breunig 2005), *Candida tropicalis* Snf1 (CtSnf1) was found to be indispensable for cell growth and viability (Kanai, et al. 1999). Taken together, AMPK belongs to an evolutionary conserved family of energy sensors which seem to exist in all eukaryotes. These energy sensors are able to detect energy depletion issued by nutrient limitation or environmental stresses and respond to these cues by readjusting energy metabolism. Hence these AMPK orthologs share the same fundamental function, i.e. control of energy homeostasis.

5. Single cell analysis

5.1. Why single cell analysis?

It is not until recently that the fields of molecular and cellular biology have started to apprehend the existence of cell-to-cell variability and estimate the importance of single cell analysis. Such variability can have a significant impact on the overall behaviour of the population. Since a population does not always manifest a normal distribution but it can display a bimodal or even a multimodal distribution, the average result obtained from this population represents therefore an intermediate response that is false and misleading. In addition to the widespread diversity of the population, sample concentration due to filtering and centrifugation of a cell culture might be a major problem in functional genomics. Hence the results obtained might reflect the response of the cells to the concentration rather than to the condition under study (Lidstrom and Meldrum 2003). Another major benefit using single cell analysis is the significantly reduced cell amount enabling the study of cells that are hard or impossible to culture (Lidstrom and Meldrum 2003). Hence, now that we are aware of the limits of bulk studies, the use of single cells becomes imperative in order to attain a complete picture of biological processes and cell design.

5.2. Cell variability

Traditional microbiology has focused on the study of large populations where the obtained information is mostly based on the inference from population-level data. Such studies are subject to the averaging effects obtained from bulk measurements and mask important parameters such as cellular discrepancies that exist within a clonal cell population (Irish, et al. 2006). This data only reflects the overall behaviour of the population and is unable to describe the behaviour of individual cells that constitute this population. A clear example of the disconnection between population and single cell measurements is the recent study undertaken by Mathies' group where they examined siRNA knockout of GAPDH gene in

single cells. Their single cell measurements suggest the presence of two populations of cells with partial (50%) and complete (0%) gene silencing. These results were clearly different from the average of all the 50 cells where gene knockout corresponded to ~ 80% (Toriello, et al. 2008). Early studies (Raser and O'Shea 2004) have demonstrated that cell variability is a result of cellular noise which is the sum of extrinsic noise, defined by the size, shape and the cycle stage of the cell and intrinsic noise which reflects the stochasticity at gene expression level. Such stochasticity is often explained by genetic noise resulting from transcriptional and translational fluctuations and was shown to largely contribute to the heterogeneity in the cellular behaviour within an isogenic eukaryotic population (Blake, et al. 2003). It has long been argued that in living cells, most regulatory genes, mainly involved in cell growth and survival, are expressed in low copy numbers (Guptasarma 1995). This low molecular copy number would give rise to large variances (noise) in molecules' concentrations and consequently in the rate of the reactions they regulate (Thattai and van Oudenaarden 2001). Cell-to-cell variability does not necessarily represent an obstacle in cellular biology but rather an evolutionary feature that enables microorganisms to survive hostile conditions and adapt to novel milieus. Population modelling of bacterial gene expression and growth has suggested that the emergence of a heterogeneous population with broader phenotypes as a result to changing environments might be a beneficial property that bacterial and yeast cells acquire in order to survive sudden external changes (McAdams and Arkin 1999, Thattai and van Oudenaarden 2004). Moreover, simulations of genetic regulation in single cells have shown that the production of signal proteins occurs at random time intervals and results in a variable number of proteins. This non-genetic diversity would account for diverse fates of cellular events reflected by diverse phenotypes between cells (McAdams and Arkin 1997). However, cells have also adapted other features such as gene redundancy and feedback loops in order to reduce the noise when a more deterministic and precise cellular response is desired (McAdams and Arkin 1999). Cell heterogeneity is inherent to all cells and especially cancer cells. Hence, dissection of cell-to-cell variability is highly appreciated in order to understand and survey tumour development steps and target therapeutic responses (Wang and Bodovitz 2010).

5.3. Single cell dynamics-Time-resolution

The cell is able to sense and respond to external signals through the interplay of a complex system. This includes a number of signal messengers and protein interactions, which convey the signal to the nucleus leading subsequently to the regulation of target genes expression. In order to completely understand the structural architecture and the function of these signalling cascades, it is vital to accurately define not only the spatial but also the temporal integration of their components. The importance of the spatiotemporal relationships of these complex systems has become evident since it has been demonstrated that it affects the biological response (Murphy, et al. 2002). Analysis of cell signalling at this level of resolution is not possible to achieve by traditional experimental tools which provide snapshots of averaged behaviours. Hence the use of real-time single cell methodology has become imperative to unravel inter and intracellular dynamics.

Owing to the advances in optics and engineering and to the wealth of fluorescent probes, light microscopy has revolutionized into fluorescence time-lapse microscopy. This sophisticated technique has made it possible to follow the chronological incidence of distinct biological events. For instance, fluorescence time-lapse studies were applied to investigate the coordination of cell apoptosis events and cell cycle progression in single cells (Chen, et al. 2006, Goldstein, et al. 2000, Munoz-Pinedo, et al. 2005). Combination of multi-photon auto-fluorescence imaging with second harmonic generation has allowed visualization of human skin with a significantly high temporal resolution in orders of picoseconds (Konig and Riemann 2003). Fluorescent microscopy is widely applied to examine protein localization. Because protein function is related to its subcellular localization, it is fundamentally plausible to unravel the regulatory function of the protein of interest by fluorescently tagging it and tracking it in real-time (Losick and Shapiro 1999). Such studies have become possible with the introduction of immunofluorescence microscopy in 1993 for *E. coli* (Maddock and Shapiro 1993). Improvements in confocal microscopy have enabled visualization of fluorescent proteins at high resolutions (Betzig, et al. 2006, Hell 2003). We have combined fluorescence time-lapse microscopy, optical tweezers and microfluidics in order to investigate the dynamic shuttling of Mig1 in

response to various glucose up- and downshifts in single yeast cells (**Paper I** and **Paper II**). Nevertheless, time-lapse techniques generate a large set of data that is not possible to be handled by the human eye and therefore necessitates the use of automated software (reviewed in (Meijering, et al. 2008)).

6. Methods for single cell analysis

Studies at the level of single cell would not have been possible without the development of new tools that allow isolation of single cells from a cell population. Techniques such as flow cytometry (FC) and its extensions (e.g. fluorescence-activated flow sorting (FACS) and capillary electrophoresis (CE)) have allowed the study of individual cells and their chemical contents respectively (Krylov and Dovichi 2000). The principle of flow cytometry relies on the measurements of flowing cells passing through the flow cytometer (Shapiro 2005). This technique is applied for various purposes such as cell counting, sorting, and determination of cell size, shape and other biochemical and physiological aspects of single cells. The introduction of fluorescence to flow cytometry in the late 1960's has allowed the application of FACS (Dean and Hoffman 2007). This technique uses not only light scattering but also fluorescence properties of the single cells passing through the flow cytometer in order to dissect cell populations into subpopulations. Although FC techniques are able to simultaneously measure several parameters of several thousands of cells and can generate high-throughput data within a considerably short time, they have some limitations. Since these techniques are based on fluid stream where the cells once measured are streamed away, they do not allow the examination of the same cells over a certain time. That is to say, FC does not allow either cell manipulating or the study of solid substrates-attached cells (Shapiro 2005). Techniques such as fluorescence microscopy and laser scanning cytometry (LSC) are able, to some extent, to measure similar features to flow cytometry but dispose of fewer limitations (Deptala, et al. 2001, Golchin, et al. 2012). Instead of flowing cells, LSC is performed on cells attached to a solid support (e.g. glass slides). In the LSC, cells are scanned by a moving laser beam and the scattered fluorescence is then detected at multiple wavelengths by a microscope. This technique provides information on cell morphology and allows sequential analyses on the same cells (Darzynkiewicz, et al. 1999). However as any other technique, LSC has also some restrictions such as its lack of cell sorting.

Advances in micro and nanotechnologies have contributed to the emergence of tools such as the lab-on-chip (Lochhead, et al.) or micro-total analysis systems (µTAS). Introduced in

the 90's, these portable micro-chambers are designed for various experimental purposes including electronic, mechanical and fluidic functions (Abgrall and Gue 2007). Due to their low cost, reduced scale and the significantly short experimental time these tools have attracted many investigators (Hatch, et al. 2001). The success of this technology has allowed the extension of this concept and hence the development of novel field-specific technologies such as the laboratory-in-a-cell (LIC) where a single cell is considered as a laboratory for detailed investigations of, for instance, intracellular processes (Andersson and van den Berg 2004). A number of additional LOC-based techniques have emerged within the past few years. Techniques such as counting low-copy number proteins, micro-PCR, patch-clamp and single cell-mRNA purification and analysis have enabled studies of single cells at different levels of details to be carried out (Bontoux, et al. 2008, Chen and Folch 2006, Huang, et al. 2007, Marcus, et al. 2006).

6.1. Microfluidics

Microfluidics is the technology that handles small amounts of fluids running through channels of micrometre scales. Microfluidics offers a number of advantages, namely, the use of small volumes of samples and reagents, short reactions time, liquids separation and the significantly low cost (Whitesides 2006). In addition, and most importantly, microfluidics offers laminar flow.

The development of microfluidics technology has largely benefited from the application of photo lithography. In fact, original works in the field of microfluidic systems have combined the use of silicon and glass. However, due to their high cost and significant opacity to visible and ultraviolet light, the use of silicon and glass became less desirable in optics. Besides, both silicon and glass turned out to be inappropriate for the study of biological systems and more specially for the study of mammalian cells (Whitesides 2006). The introduction of elastomers namely the polymer poly-dimethylsiloxane (abbreviated as PDMS) to microfluidics has been revolutionary. PDMS is a soft elastomer, relatively inert and optically transparent, which favoured its use in optical methods. Its softness has

enabled the fabrication of different shapes of microstructures and its high permeability to oxygen and carbon dioxide has allowed cell culturing inside microfluidic chambers. Working with microfluidics requires the knowledge of a set of parameters. The dimensionless Reynolds number (Re) defines the flow regime, which can be either turbulent or laminar, and is considered one of the most important parameters when dealing with fluids (Whitesides 2006). Re corresponds to the ratio between inertial and viscous forces on fluids. At low Reynolds number, the viscous forces dominate over the inertial forces resulting in a laminar flow. That is to say, when introduced into the microfluidics, two fluid streams flow in parallel without disruption. A Re that is below 2,300 dictates that the flow is laminar (Beebe, et al. 2002). In our experiments, employing microfluidics, the Re is normally below 1, indicating that the flow inside the micro-channels is laminar and that the only way for two fluid streams to mix is by diffusion.

6.2. Optical tweezers

The study of how single cells communicate with each other and with their environment and how cells adapt and operate in different environmental conditions requires the ability to manipulate cells. Techniques such as optical tweezers have been developed for such purpose. The ability to use light for trapping and manipulating single particles such as bacteria and viruses (Ashkin and Dziedzic 1987) has made a major breakthrough in many scientific and engineering fields. Particle manipulation reflects acceleration, deceleration, guidance and deflection (Ashkin 1997). Originally this technique was designed for atoms trapping but extended later on in order to trap bacteria, yeast, mammalian and even plant cells (Ashkin 1997). By using an infrared trapping, Ashkin et al., 1987 developed a damage-free procedure, allowing bacterial cells to reproduce inside the optical trap. The principle of this technique relies on the use of a single strong Gaussian-distributed laser beam, which is a collection of rays with different intensities and with the highest intensity being in the middle of the beam. The incidence of the laser beam on a spherical particle results in a scattering force, in the direction of the incident light, and a gradient force in the direction of the intensity gradient (Ashkin and Dziedzic 1987). In order to allow particle

trapping, the gradient force must be dominating over the scattering force. This can be achieved by strongly focusing the light beam through a high numerical aperture objective creating a three-dimensional stable trap.

6.3. Fluorescence

The fact that molecules are able to fluoresce has made a major breakthrough in the history of single cell (Brehm-Stecher and Johnson 2004). Fluorescence occurs when a photon from a light source is absorbed by a fluorophore, a molecule with fluorescence properties (Lichtman and Conchello 2005). This leads to the transition of an electron in the fluorophore to an excited energy state. Eventually, the excited molecule returns to a lower energy state. This return is accompanied by the emission of light in form of fluorescence as a result of energy loss. This separation in energy or rather wavelength is referred to as Stokes shift, *in other words*, fluorescence shift (Davey and Kell 1996). Stokes shift separates the emitted light by optical filters and hence quantify the amount of fluorescence. When dealing with fluorescence measurements, certain parameters should be considered. Hence two major fluorescence characteristics can be distinguished: the extinction coefficient corresponding to the amount of light absorbed at a certain wavelength (Davey and Kell 1996) and the quantum yield which describes the ratio between the absorbed and the emitted photons (Lichtman and Conchello 2005). In addition to these two parameters, the photo-stability of the fluorophore is another factor to consider. Photo-stability defines the number of times a dye can be excited before decaying (Davey and Kell 1996). Hence an excess of excitation results in a decrease of photo-stability and consequently in an increase of photo-bleaching. The excited-state lifetime of a fluorophore is also of some importance. It refers to the time a fluorophore remains excited before decaying to the ground state (Lichtman and Conchello 2005). The lifetime is affected by the proximity of an energy acceptor. Hence the nearer the acceptor is to the donor the shorter the lifetime.

The discovery of the green fluorescent protein (GFP) from the *Aequorea victoria* jellyfish has revolutionized biosciences and especially live imaging. Irradiated by the near

ultraviolet or blue light, GFP is largely used as an indicator of gene expression and protein localization (Chalfie, et al. 1994). This 238 amino-acid β-barrel-shaped protein contains the tripeptide Ser-Tyr-Gly which after oxidation becomes a fluorophore (Heim, et al. 1994). Given its many advantages such as its fairly small size, non-toxicity and most importantly its ability to fuse to almost any protein without altering its endogenous function, GFP has become a versatile tool for the dynamic investigation of many cellular events including protein abundance, motility and interactions (Chalfie, et al. 1994, Lippincott, et al. 2001, Zimmer 2002). In addition to its role as a reporter, GFP can be combined with fixation-based staining techniques namely fluorescence in situ hybridization (FISH) (Brehm-Stecher and Johnson 2004). Further improvement of GFP generated GFP proteins with distinct emission and absorbance spectra such as blue fluorescent (BFP), yellow fluorescent (YFP) and cyan fluorescent (CFP) proteins, designed to simultaneously look at different targets at the same time and within the same specimen (Lippincott-Schwartz and Patterson 2003). This prosperity in fluorescent proteins has allowed the development of techniques such as fluorescence resonance energy transfer (FRET). The concept of this technique is to detect the interaction between two proteins based on their intermolecular distance. Technically, a transfer of energy occurs from an excited fluorophore called the donor to another molecule called the acceptor (Sekar and Periasamy 2003). Moreover, the expansion of the large range of fluorophores and the rise of diverse GFP variants has enabled multicolour microscopy where different cellular compartments and/or components can be monitored simultaneously.

In parallel to the development of fluorescence molecules, fluorescence microscopy has evolved. Given the Stokes shift, a fundamental characteristic of fluorescence microscopy, it is possible to illuminate an object with a given wavelength and filter the resulting light in order to solely see longer wavelength (Lichtman and Conchello 2005). A wide range of fluorescence microscopes, extending from a conventional wide-field to a super-resolution microscope, with different levels of resolution has been established. In our experiments involving fluorescence microscopy, we employed an epi-fluorescence microscope where the incident light which is used to excite the sample can be separated from the relatively weaker light that is emitted from the specimen. This latter is then being detected (Webb and Brown 2013). In addition to an EM-CCD camera, this inverted epi-fluorescence

microscope is equipped with a fluorescence light source EL 6000, a GFP filter cube (472/30 nm exciter, 520/35 nm emitter and 495LP dichroic mirror) and an mCherry filter cube (560/40x exciter, 630/75m emitter and 585LP dichroic mirror) (for a detailed description of this step, see Materials and Methods section in **Paper I** and **Paper II**). This set-up has allowed us to perform time-lapse imaging to monitor localization dynamics of the glucose repressor Mig1.

6.3.1. Bleaching

The estimated number of excitation-emission cycles for a good fluorophore ranges between 10,000 and 40,000 (Lichtman and Conchello 2005). After that the fluorescence signal of the fluorophore irreversibly fades. This phenomenon is called bleaching (or photobleaching). In contrast, when the fluorescence signal fades reversibly then it is the quenching which takes place. Although the bleaching is a common issue in fluorescence microscopy, the photochemistry of this phenomenon still remains obscure. One explanation could be that photobleaching is related to the triplet states of the fluorophore as it could be a result of an interaction between the fluorophore's triplet state and the molecular oxygen.

6.3.1.1. Bleaching techniques

Despite the undesirable effect of bleaching, techniques such as fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) take advantage of bleaching. FRAP and FLIP techniques allow the study of the mobility of fluorescent tagged molecules in living cells. In the case of FRAP, fluorescent molecules of a small region of the cell are irreversibly photobleached using a laser beam. The recovery of non-bleached fluorescent molecules from the neighbouring areas into the irradiated region can be quantitatively monitored by time-lapse microscopy. Such recovery would reflect the movement of the fluorescent particles between these two compartments. The recovered fluorescence into the bleached region is plotted over time (Ishikawa-Ankerhold, et al.

2012). From this graph, many parameters can be distinguished namely the mobile and immobile fractions which correspond respectively to the fraction of the proteins able or unable to diffuse to the bleached region. Another important parameter is the half time, half maximum or the half-life corresponding to the time it takes to reach the half recovery plateau (Ishikawa-Ankerhold, et al. 2012). Thus, the shorter the half time the faster the recovery hence the diffusion.

Complementary to FRAP, FLIP is used to investigate protein redistribution and the inter-connectivity between two compartments (Ellenberg, et al. 1997). FLIP technique corresponds to the repetitive photobleaching of a small region of the cell. This results in the loss of fluorescence in the irradiated region which is, soon after, recovered by the inflow of fluorescent molecules from the surrounding regions. The loss of fluorescence in the unbleached area which represents the mobile fraction of the fluorescent particles is monitored over time. Both FRAP and FLIP can be used in order to study the transit of fluorescent tagged protein of interest between two cellular compartments. In **Paper I**, we employed both FRAP and FLIP in order to explore the kinetics of the rate of Mig1 nuclear cytoplasmic shuttling.

6.4. Technical limitations and drawbacks

As discussed above studies at the single cell level unveil the apparent phenotypic distinction between individual cells within a clonal population. However, given the large influence of biological and technical noises, single cell studies are technically challenging.

A number of factors might contribute to the complexity of single cell analysis. For instance, the small size of microfluidic channels increases the risk of clogging of cells or PDMS, which subsequently affect the laminar flow through the channel. Further, photobleaching, which occurs during imaging, might also affect the results. In our case, we have dealt with this issue by reducing the intensity of the fluorescence lamp. However, at low intensities the lamp is not always stable and can show small irregular intensity fluctuations over time. This could affect fluorophore excitation and therefore fluorescence

intensity. One approach to correct for the differences in the starting intensity between cells is to perform data normalization. In principle, normalization would be performed by dividing the intensity of all time points by that of the initial time point. Since we took only one image at the start of the experiment (prior to changing the microfluidic flow), normalizing to the intensity resulting from this image would be biased. A proper normalization would be to divide by the average intensity acquired from several images before the shift. However, data normalization is not always desirable since it might also mask important aspects of biological variation.

In addition to the technical noise, the biological noise emerging from intrinsic and extrinsic noises also adds to the complexity of single cell analysis. In fact, the low copy number of proteins, inherent to components of signaling pathways and transcriptional regulators (Huang, et al. 2007), enhances the challenges of single cell analysis considerably. It might be possible to reduce the extrinsic noise by synchronizing the cell-cycle of the cells. We presently do not know if the cell cycle stage affects Snf1-Mig1 signalling, a question that could be addressed in our setting.

Another limitation arising from the small sample size and the various stochastic effects described above, is the difficulty to interpret single cell data generated from one single run and the possibility to compare data between different runs. Therefore experimental and biological replicas are necessary to minimize the uncertainties and generate accurate results. Statistics and quantitative mathematical modelling are two frameworks that might allow discerning between technical and biological noises. Nevertheless, due to the sample size, standard statistical methods such as standard deviation cannot be applied. Hence, specific statistical approaches, such as e.g. mixed effects models, need to be developed for the analysis of single cell data. This is an area of active research but still in its infant stages.

7. Summary of appended papers

Since its emergence as an energy sensor and regulator in all eukaryotes, the AMPK/SNF1 has attained a considerable increase in attention. In this thesis, we examined different aspects of the regulation of the AMPK Snf1-Mig1 pathway. We surmised that the analysis of time-dependent signalling behaviour provide insights into the dynamic properties of this system. By employing the real-time kinetics of Mig1 nucleo-cytoplasmic shuttling as a read-out, we were able to elucidate characteristics of the Snf1/Mig1 pathway in response to different glucose levels (**Paper I**, **Paper II** and **Paper III**). In order to fully control glucose concentrations, we constructed arrays of individual yeast cells in a microfluidic device allowing a shift in glucose concentration within 2 s time. The cell arrays were then exposed to different glucose up- and downshift regimes (**Paper I** and **Paper II**).

In **Paper I**, we found that following glucose upshifts, Mig1 displayed a rapid and sustained nuclear entry over a wide range of the tested concentrations including actually derepressing concentrations. However, after a shift to glucose concentrations that are below 0.01%, Mig1 migration was slower and not all the cells responded. On the other hand, following glucose downshifts from 4% to concentrations ranging between 1.5% and 0.5%, we found that Mig1 migrated rapidly but transiently to the cytosol, before returning to the nucleus. Below these concentrations, Mig1 remained in the cytosol. Our FLIP and FRAP data indicated that Mig1 nucleo-cytoplasmic mobility is constant regardless of glucose concentration. In summary, this data suggests that the Snf1/Mig1 signalling pathway is highly sensitive to glucose changes. It is also able to monitor glucose change as well as the absolute glucose level. Our single cell analyses displayed a significant cell-to-cell variability throughout a wide range of the conditions tested. This variability may indicate an evolutionary adaptive feature to assure an optimal utilization of the available glucose in the surrounding environment of the population. We assumed that analysis of the cell-to-cell variability might bear information about the regulatory mechanism of the system. Hence this issue was addressed in **Paper III.**

Our data from **Paper I** showed that the Snf1/Mig1 system displayed different sensitivities depending on glucose up- and downshifts suggesting a fast adaptation of metabolism to

glucose availability. This behaviour may be connected to glucose uptake whose characteristics depend on glucose availability (Horak 2013, Ozcan and Johnston 1995). We hypothesized that the behaviour of Mig1 migration would be affected by the characteristics of the uptake system (**Paper II**). To this end, we monitored Mig1 translocation, using the same experimental set up described above, in three different strains, expressing each as a sole uptake system the low affinity hexose transporter Hxt1, the high affinity Hxt7 or their chimeric, TM6* under different glucose up- and downshifts. We found that in cells expressing either *HXT7* or *TM6** and growing in high glucose, Mig1 was constitutively cytosolic and did not respond to any glucose downshift. However in the Hxt1-only strain, the Mig1 migration profile was consistent with the affinity profile of this transporter. When we shifted these cells from 4% to 1% glucose, we did not observe the transient behaviour observed in the wild type strain. This data clearly indicates that Mig1 migration, and hence the Snf1/Mig1 system is affected by glucose uptake.

It is now accepted that cell-to-cell variability is ubiquitous to any population and might bear important information regarding the underlying regulatory mechanisms of biological systems (Li and You 2013). By applying nonlinear mixed effects modelling, we aimed to quantify the dynamic behaviour of Mig1 shuttling in response to glucose downshifts (**Paper III**). Based on this approach, we were able to construct a dynamical mathematical model successfully simulating the observed transient nuclear exit following glucose shift from 4% to intermediary concentrations ranging between 1.5% and 0.5%. Interestingly, the estimated parameter distributions showed a significant correlation between the two parameters determining the initial time scale of Mig1 exit and the time scale of the adaptation process. These findings suggest that Mig1 nuclear exit and re-entry might likely be controlled by the same mechanism. By applying Monte Carlo simulation of the established model we were able to determine various characteristics of the transient behaviour of Mig1. We found that Mig1 exits the nucleus more quickly and re-enters it more slowly as glucose concentrations decreases. Also at these low concentrations, there was an enhanced cell-to-cell variability. These results elucidate biological features of the Snf1/Mig1 module which could not be directly measured experimentally and represent a step toward the understanding of the mechanism underlying Mig1 shuttling.

The aim of **paper IV** was to provide a reconstruction of the Snf1 network and a detailed review about our current understanding of the Snf1/Mig1 pathway based on solid and structured pieces of evidence collected from over 400 published articles and on the publically available *rxncon* software. Moreover, we discuss various aspects of the pathway including the mechanistic architecture, the environmental perturbations, and the functional response. In addition, we review the cross-talk with other pathways and the conservation among other species.

Given the remarkable conservation between yeast and human proteins, including those involved in human diseases, it is now possible to study the biological function of such orthologous proteins in yeast (Dolinski and Botstein 2007). This approach has been adopted by Woods et al., who provided evidence for the functional interchangeability between the yeast SNF1 and its mammalian ortholog AMPK (Woods, et al. 1994). In **Paper V**, we tested whether mammalian AMPK could functionally substitute for the SNF1 complex. To this end, we expressed the mammalian AMPK heterotrimeric complex in yeast cells lacking the entire endogenous SNF1 complex. Using twelve different combinations of AMPK subunits, we found that combinations comprising α1-, β1- or β2- and γ1- or γ3 subunits appeared to be functional since they conferred growth on alternative carbon sources. Remarkably, heterologous AMPK is properly regulated by glucose. AMPK regulation was not affected by deletion of *REG1* and *REG2* however the phosphatase Sit4 seemed to contribute, to some extent, to the negative regulation of AMPK. In contrast to the anti-diabetic drugs phenformin and metformin (Sakamoto, et al. 2004, Zhou, et al. 2001), which indirectly activate endogenous AMPK, the drug candidate compound 991 seemed to moderately activate the yeast-expressed AMPK. Taken together, this data highlights the conservation of the structural and functional AMPK and SNF1 regulatory systems and opens up possibilities for the screening of drugs affecting AMPK activity in yeast.

8. Conclusions and future perspectives

The Snf1/Mig1 pathway plays a vital role in energy homeostasis. Although this pathway has been extensively studied over the past decades, several questions remain yet to be answered. Given that the overall structural and functional organization is conserved between SNF1 and its mammalian ortholog AMPK, it is likely that also the mechanism of their activation is related, while life style specific adaptation have probably evolved. Indeed, in analogy to AMPK, SNF1 activity seems to be affected by adenosine nucleotides, but still the mechanistic and physiological underpinnings of this regulation remain to be elucidated. Thus, this area clearly requires more investigations. In this thesis, we aimed to elucidate the mechanisms governing the dynamics of glucose signal transmission in single cells and at population level.

We provide for the first time a detailed and time-resolved study describing different characteristics of the Snf1/Mig1 pathway in response to various glucose levels such as a fast response and a rapid adaptation to changes in glucose levels. We also demonstrate that characterization of Mig1 translocation could be a proper approach for the study of the regulation of the Snf1/Mig1 pathway. A number of open questions concerning Mig1 translocation remain yet to be addressed. For instance, it is still unclear whether the regulation occurs at the level of Mig1 phosphorylation by Snf1 or at its nuclear transport, or both. Recent studies have indicated that active Snf1 does not automatically phosphorylate Mig1 but that there seems to be an additional glucose-regulated step that controls Mig1 phosphorylation by Snf1 (García-Salcedo, et al. 2014, Ye, et al. 2008). Perhaps access of active Snf1 to Mig1 is controlled by glucose. Evidence has been reported that Hxk2 interacts with both Snf1 and Mig1 in order to prevent Mig1 phosphorylation under repressing conditions (Ahuatzi, et al. 2007). While details about Mig1 nuclear export have been reported it is still unknown how Mig1 is imported to the nucleus. Nuclear import of the related Mig2 is mediated by the importin-β Kap95 and requires the presence of GTP-binding protein Gsp1 and the nuclear localization signal (NLS) motif of Mig2 (Fernandez-Cid, et al. 2012). Also Mig1 has an NLS region between the amino acid residues 359 to 373 and hence it is possible that the nuclear translocation of

Mig1 might also involve a similar importin (De Vit, et al. 1997, DeVit and Johnston 1999). Screening for importins and importin-like proteins that physically interact with Mig1 might provide some clues about the regulation of Mig1 nuclear cytoplasmic translocation. The finding that Mig1 constantly shuttles between the nucleus and the cytoplasm regardless of glucose concentration raises the question of the physiological relevance of the nuclear localization of Mig1 in absence of glucose. We hypothesize that phosphorylated and unphosphorylated Mig1 coexist under all conditions as consequence of a balance between the phosphorylation and dephosphorylation reactions, which could allow a fast response when glucose levels change. Our data is consistent with the previously observed constitutive presence of Mig1 on the promoter of glucose-repressible genes under both repressing and derepressing conditions (Papamichos-Chronakis, et al. 2004). These observations raise the question whether Mig1 could act like Sko1. This transcriptional repressor also mediates repression in complex with Cyc8 (Ssn6)-Tup1 co-repressor but under hyperosmotic stress is converted to an activator following phosphorylation by Hog1 (Proft and Serrano 1999, Proft and Struhl 2002). Mig1 was previously found to act as an activator in the absence of Ssn6 suggesting that the Mig1-Ssn6 interaction might prevent Mig1 to either act as an activator or bind to an activator (Treitel and Carlson 1995). However, given the significant derepression of *SUC2* in the absence of *MIG1* and the predominant cytoplasmic distribution of Mig1 under derepressing conditions, it is unlikely that Mig1 might be an activator, at least not in a wild-type background ((Bendrioua, et al. 2014, Bu and Schmidt 1998, De Vit, et al. 1997) and our unpublished data). Instead, it is possible that the absence of Mig1 allows other transcriptional activators to occupy Mig1-DNA binding site.

By monitoring Mig1 nucleo-cytoplasmic shuttling in strains with different glucose uptake rates, we confirm a tight link between glucose metabolism and the activity of the Snf1/Mig1 pathway. The Snf1/Mig1 pathway does not directly perceive the level of glucose but rather the glycolytic flux and/or the level/turnover of AXPs in the cell. Our data is in accordance with the previously observed correlation between glycolytic rates and Mig1 phosphorylation status (Elbing, et al. 2004b). Strains expressing a high affinity glucose transporter are in a derepressed state and Gustafsson and co-workers (Bosch, et al. 2008) have demonstrated that in the Tm6* strain the ratio of ATP: ADP and of ATP:

AMP is in fact higher than in wild type. This observation is in contradiction with the paradigm stating that AMPK is activated by a drop in the ATP: ADP. Hence, it remains still elusive how adenosine nucleotide levels are linked to SNF1 activity *in vivo*. Further studies are clearly necessary to investigate the mechanism of regulation of the Snf1/Mig1 pathway by glycolytic metabolism. The newly emerging time-resolved technology involving aptamer-based nanosensing could be an appropriate approach to elucidate the link between AXP and the Snf1/Mig1 pathway in real-time. Technically, it would be feasible to simultaneously monitor the levels of AXP-conjugated aptamers in the cell and the shuttling of fluorescently-tagged Mig1. The strains used in this study, Hxt7 and Tm6*, might provide valuable information about the governing mechanism but given their re-wired metabolism (e.g. respirative glucose metabolism) also a control strain expressing all hexose transporters needs to be studied.

It has proven difficult to functionally swap the Snf1 protein with the AMPK α-subunit. Previously, Carling and his co-worker (Daniel and Carling 2002) reported that the yeast-expressed chimeric AMPKα1-Snf1 was able to rescue growth of *snf1Δ* mutant only on raffinose indicating limited activity of the chimaera. By replacing the entire SNF1 complex by different combinations of the AMPK subunits, we were able, for the first time, to fully complement SNF1 function by heterologous AMPK. Interestingly, AMPK was properly regulated by glucose implying that it was phosphorylated by yeast kinases and dephosphorylated by yeast phosphatases. While we did not test which yeast protein kinase phosphorylates AMPK, it appears that Glc7-Reg1, which normally is the main Snf1 phosphatase, does not act on AMPK. The type 2A-like protein phosphatase Sit4 seems to marginally contribute to AMPK dephosphorylation ((Ye, et al. 2014) and our unpublished data). Sit4 was reported to participate in Snf1 dephosphorylation (Ruiz, et al. 2011) indicating some analogy in the regulation between SNF1 and AMPK. Hence, screening for the phosphatases affecting phosphorylation of yeast-expressed AMPK might provide information on the regulation of endogenous AMPK.

It appears that AMPK recognizes and accurately regulates Snf1 targets in yeast. Active Snf1 translocates to the nucleus in order to phosphorylate its effector Mig1 (Vincent, et al. 2001). Therefore, it would be intriguing to monitor the localization of AMPK under

derepressing conditions. This would probably help gain insight into the regulation of AMPK not only in yeast but also in higher eukaryotes.

Altogether, the finding that AMPK was not affected by Glc7-Reg1 but still properly regulated by glucose indicates that glucose control of the AMPK/SNF1 complex may not be mediated by the phosphatase alone, but rather by the kinase complex.

The fact that compound 991 affected the activity of yeast-expressed AMPK is of a major interest. This small molecule activator binds to the α-subunit of the AMPK complex (Xiao, et al. 2013), therefore it would be tempting to investigate the mechanism of AMPK activation by compound 991 in yeast. The modest activation of yeast-expressed AMPK activation by compound 991 compared to that observed for the endogenous AMPK and the lack of a dose-response may be due to a restricted uptake into the cell, an active efflux from the cell or a decreased accessibility of the compound to the AMPK complex. It would be plausible to distinguish between these scenarios by measuring the uptake and the efflux rate of radiolabeled compound 991, as described for the analysis of vinorelbine transport in lung cancer cells (Stuckler, et al. 2005). Moreover, it would be intriguing to determine whether 991-activated AMPK regulates its targets. This could be done by performing kinase activity and invertase assays. Unfortunately, due to the limited amount of the compound, we were unable to perform such assays. Furthermore, the binding ability of compound 991 to yeast expressed AMPK could be investigated by structural analyses such as electron microscopy and X-ray crystallography.

Taken together, our studies with AMPK activating compounds will open up new possibilities for the identification of drug candidates against metabolic disorders such as insulin-resistance, obesity and type 2-diabetes, employing the humanized yeast as a platform.

We have learned from dynamic quantitative modelling of cell-to-cell variability that the initial time scale of Mig1 exit and the time scale required for the adaptation process strongly correlate upon shift from high to intermediate glucose concentrations. Surprisingly, we found that despite the dispersed profile between cells, it appears that the

mechanism underlying the behaviour of different cells is similar. Our single cell results (Bendrioua, et al. 2014) indicated that upon a shift to a concentration ≤0.2% glucose, Mig1 rapidly localized to the cytoplasm and remained cytosolic under the entire experimental running time. Thus, it seems that the glucose concentration threshold for Mig1 nuclear exit is between 0.5% and 0.2% glucose. It would be appealing to further extend the current model in order to predict the exact glucose threshold. Such information could, for instance, serve to investigate experimentally the behaviour of Mig1 exit around the glucose threshold concentration. Moreover, it would be useful to develop this model, which is mainly descriptive so far, to a mechanistic model providing more insights into the mechanism controlling Mig1 shuttling.

Taken together, the work in this thesis has provided a better understanding of the glucose control of the Snf1/Mig1 pathway and more importantly a solid ground for further research in this field.

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