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Dynamic regulation of the Mig1 transcriptional repressor under glucose de/repression

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Dynamic regulation of the Mig1 transcriptional repressor under glucose de/repression

Doctoral thesis

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If we knew what it was we were doing,

it would not be called research, would it?

-attributed to A. Einstein

Abstract

The budding yeast *Saccharomyces cerevisiae* AMP-activated protein kinase/SNF1 is a member of a highly conserved protein family present in all eukaryotes. Snf1 regulates energy homeostasis; in yeast, it is best-known for its role in cellular adaptation to glucose limitation. Utilisation of carbon and energy sources other than glucose is controlled, among others, via regulation of gene expression. Expression of genes essential for metabolism of alternative sources, such as maltose, galactose and sucrose is regulated by the transcriptional repressor Mig1, which in turn is controlled by Snf1. Mig1 shuttles in and out of the nucleus in response to glucose availability, which makes it a convenient read-out for Snf1 pathway activity in single cell analysis. The overall goal of this thesis is to achieve a better understanding of the dynamic control of an AMPK/SNF1 signal transduction pathway using the budding yeast *S. cerevisiae* as a model system. We combined classic biochemical, molecular and microbiology approaches with cutting-edge biophysical and imaging methods to fill gaps in our understanding of signal transduction mechanisms. Being the first ones to use millisecond imaging to monitor signal transduction, thus, the first ones to observe single Mig1 molecules in live cells, we found that regardless of glucose availability Mig1 is present in the cytoplasm and the nucleus as monomer and oligomers. We observed similar clusters of the transcription activator Msn2. Thus, we suggest that eukaryotic gene regulation is mediated through transcription factors which act as multimeric clusters. The structure of those clusters is stabilised by depletion forces that mediate interactions between intrinsically disordered regions of transcription factors. Classic biochemical approaches revealed a dual mechanism of Mig1 dephosphorylation which includes glucose-dependent and glucose-independent events. We also found evidence for a novel step of Mig1 regulation which includes tyrosine phosphorylation. We show that the expression of Mig1 is itself glucose-regulated in a Snf1-dependent manner. Taken together, this work provides novel concepts in understanding of the AMPK/Snf1 signal transduction pathway with specific emphasis on Mig1 regulation.

Keywords: cell signalling, glucose repression, Mig1, phosphorylation, *Saccharomyces cerevisiae*

Abbreviations

AAK	AMP-activated kinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
cAMP	Cyclic AMP
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HXT	Hexose transporter
IDP	Intrinsically disordered protein
IDR	Intrinsically disordered region
LCM	Laser capture microdissection
LMPTP	Low molecular weight tyrosine phosphatases
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
SnRK1	Snf1-related kinase 1
PPM	Protein phosphatase dependent on manganese/magnesium
PPP	Protein Ser/Thr phosphatase

PP1	Protein phosphatase 1
PP2	Protein phosphatase 2
PTP	Protein tyrosine phosphatases
NSOM/SNOM	Near-field scanning optical microscopy
PKA	Protein kinase A
PALM	Photoactivated localisation microscopy
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
TIRF	Total internal reflection fluorescence
TIVA	Transcriptome *in vivo* analysis
TF	Transcription factor
UAS	Upstream activation sequence
URS	Upstream repression sequence

Nomenclature

SNF1 denotes to the yeast AMPK heterotrimeric complex.

Snf1 refers to the protein, the catalytic subunit of the protein.

SNF1 defines the gene.

List of papers

I. Wollman AJM*, **SHASHKOVA S***, Hedlund EG, Friemann R, Hohmann S, Leake MC. Transcription factor clusters regulate genes in eukaryotic cells. **PNAS** *Manuscript in preparation*

II. **SHASHKOVA S***, Wollman AJM*, Leake MC, Hohmann S. The yeast Mig1 transcriptional repressor is dephosphorylated by glucose-dependent and independent mechanisms. **FEMS Microbiology Letters** *Submitted*

III. Lubitz T, Welkenhuysen N, **SHASHKOVA S**, Bendrioua L, Hohmann S, Klipp E and Krantz M. Network reconstruction and validation of the Snf1/AMPK pathway in baker's yeast based on a comprehensive literature review. **npj Systems Biology and Applications** 2015 1, 15007.

IV. **SHASHKOVA S**, Cvijovic M, Hohmann S. Transcriptional regulation of Mig1 in yeast *Saccharomyces cerevisiae*. *Manuscript in preparation*

*Authors contributed equally

Paper contributions

Paper I: I constructed some of the strains (YSH2856, YSH2862, YSH2863, YSH2896) and plasmids (pMig1-mGFP and pmGFPS), purified the protein, performed all non-super resolution imaging experiments, parts of bioinformatics analysis (PONDR: sequence disorder prediction, PyMOL: secondary structure prediction, RNABOB and following Excel analysis: potential target sequences identification) and made related figures and tables, participated in super-resolution imaging experiments, contributed to a major part to the writing and commenting on the manuscript.

Paper II: I performed all non-super resolution imaging experiments, participated in super-resolution imaging experiments and analysis, wrote the whole manuscript and made all the figures.

Paper III: I participated in literature review and data collection, contributed to a minor part to the writing of the paper.

Paper IV: I performed all experiments, contributed to the writing of the manuscript.

Papers not included:

SHASHKOVA S, Welkenhuysen N, Hohmann S. Molecular communication: crosstalk between the Snf1 and other signaling pathways. **FEMS Yeast Res**. 2015 Jun; 15(4):fov026.

Table of Contents

1 Introduction

In order to survive in constantly altering environments living organisms must adapt to new conditions by rapidly responding to the multitude of external stimuli. Once a stimulus is received by the membrane receptors, the signal is processed within the cell through the cascades of biochemical reactions. Signal transduction results in a specific response, which is mediated by spatial and temporal dynamics of signalling networks. The output of the signalling pathways is alterations in gene expression, metabolism, cell division, growth and hence an adjustment of cellular processes to the surrounding conditions (Krauss 2008).

The mammalian AMP-activated protein kinase (AMPK) is the main energy sensor. Its main role is to integrate information regarding energy sources availability and environmental stress factors in order to mediate an adaptive response. AMPK is a highly conserved protein present in all eukaryotes: yeast (Snf1), plants (SnRK1), roundworms (AAK), insects and mammals (AMPK) (Ghillebert et al. 2011). The budding yeast *Saccharomyces cerevisiae* AMPK/SNF1 signalling pathway controls energy homeostasis and is best known for its role in glucose derepression. One of the main direct targets of Snf1 in glucose derepression is the transcriptional repressor Mig1 which controls expression of genes essential for metabolism of carbon sources such as sucrose, maltose, galactose (Nehlin et al. 1991; Hu et al. 1995; Wu and Trumbly 1998).

The glucose repression pathway is well characterised biochemically and genetically and crosstalk between SNF1 and other nutrient pathways has been studied (Shashkova et al. 2015). However, some aspects of the mechanisms controlling glucose de/repression via Snf1-Mig1pathway remain unclear. For instance, it is still unknown if Mig1 dephosphorylation is glucose-regulated or mediated by constitutive phosphatases, and thus only depends on Snf1 activity. Also, mechanisms controlling rapid dynamics of Snf1-Mig1 signalling as well as how Mig1 finds its target sequences and regulates genes expression are not explained.

It is not sufficient to study individual components of the system to understand how complex biological systems integrate and coordinate the activity of all their elements. It is also necessary to take into account interactions of the components and reaction kinetics. Systems biology is an interdisciplinary field that merges experimental data collection with mathematical and computational methods. Based on a holistic approach, mathematical modelling describes how multiple complex

regulatory modules and cellular processes are connected. Mathematical modelling provides an invaluable tool to develop hypotheses and test them by computational simulations as well as targeted experiments (Fischer 2008).

Our ability to address many unresolved questions in fundamental biological mechanisms is limited by traditional technologies. With the development of super-resolution imaging techniques in living cells, we can study biological processes on a single-molecule level following cell signalling dynamics without disrupting the cell or an organism. In this work we used cutting-edge biophysical and imaging methods to fill gaps in our understanding of signal transduction mechanisms. We combined live cell measurements with genetic, pharmacological and physiological perturbations in order to shed light on mechanisms of gene expression regulation. Here we propose novel concepts in understanding of the downstream events of signal transduction.

2 Specific aims

We wish to better understand mechanisms that control the dynamics of cell signalling. To achieve this goal, we employ the yeast *S. cerevisiae* as a model organism and the AMPK/SNF1 pathway as a model for signal transduction to monitor signalling activity. The focus of this PhD work is on the dynamics of the Snf1-Mig1 regulatory module in yeast metabolic regulation. Specific emphasis is on the molecular mechanisms that regulate the dynamics of Mig1 de/activation on systems level control mechanisms as monitored in cell populations and at the single-cell and single-molecule levels.

Thus, we defined four specific parallel research objectives for this work:

1. The mechanism of DNA target sequence recognition by Mig1

We aimed to understand the dynamic mechanism of how Mig1 transcriptional repressor recognises its target sequences in a way to achieve rapid and efficient repression of genes. We employed cutting-edge Slimfield microscopy on live cells in order to follow the behaviour of single Mig1 molecules.

2. The role of Glc7-Reg1 phosphatase in Mig1 regulation

Despite the fact that the Snf1/Mig1 glucose repression pathway has been extensively studied, the mechanism of Mig1 dephosphorylation remains unclear. We combined classic western blotting with novel single-molecule live-cell imaging to study the effect of the Glc7-Reg1 phosphatase on Mig1 phosphorylation status. Moreover, we wanted to provide a link between the Mig1 phosphorylation state and its cellular localisation under different glucose conditions.

3. Snf1/Mig1 network reconstruction

By using the publicly available software *rxncon* we performed a Snf1-Mig1 network reconstruction based on experimental data collected through comprehensive literature review. Moreover, employing simulations of the signal transfer we tried to identify and fill the gaps in the network.

4. *MIG1* expression regulation

We wished to understand the importance of the glucose repression pathway components for *MIG1* expression. We employed mathematical modelling in an

attempt to understand the dependencies between Mig1 and Snf1 presence upon glucose repression/derepression.

3 *Saccharomyces cerevisiae* as a model organism

The bakers' yeast *Saccharomyces cerevisiae* (**Figure 1**) has been established as a model organism in 1935 (Mortimer 2000) and has been used since in many research laboratories around the world (Botstein and Fink 2011). It is also the first eukaryotic organism whose genome was fully sequenced in 1996 (Goffeau et al. 1996). *S. cerevisiae* is easy to grow, store and manipulate genetically. Many fundamental cellular mechanisms are conserved across eukaryotic species. In fact, several human disease genes have functional homologues in yeast, e.g. genes involved in mitochondrial diseases (Perocchi et al. 2008). Yeast is also extensively used for studies of Huntington's, Alzheimer's and Parkinson's diseases (Outeiro and Giorgini 2006; Pimentel et al. 2012; Verduyckt et al. 2016). On average, the similarity of amino acid sequences between the human and yeast proteomes is about 32%. More than 31% of yeast genes have clear homologues in the mammalian genome (Botstein et al. 1997). Studies on 414 essential yeast genes indicate that 47% of those can be successfully replaced with human orthologues complementing lethality caused by gene deletion (Kachroo et al. 2015). Besides that, a budding yeast cell represents the whole organism just in one cell, which makes yeast an ideal model for various studies on eukaryotic organisms. Several discoveries that won the Nobel Prize in physiology and medicine or chemistry were carried out on yeast. Just this year (2016) the prize was awarded to Yoshinori Ohsumi for the discovery of autophagy mechanisms. Several years earlier Randy Schekman received the prize for studies on vesicular trafficking machinery. In 2001 the prize was awarded to Hartwell and Nurse who worked with *S. cerevisiae* and *Schizosaccharomyces pombe*, respectively, for the discoveries of *CDC* genes, key regulators of the cell cycle (Hohmann 2016).

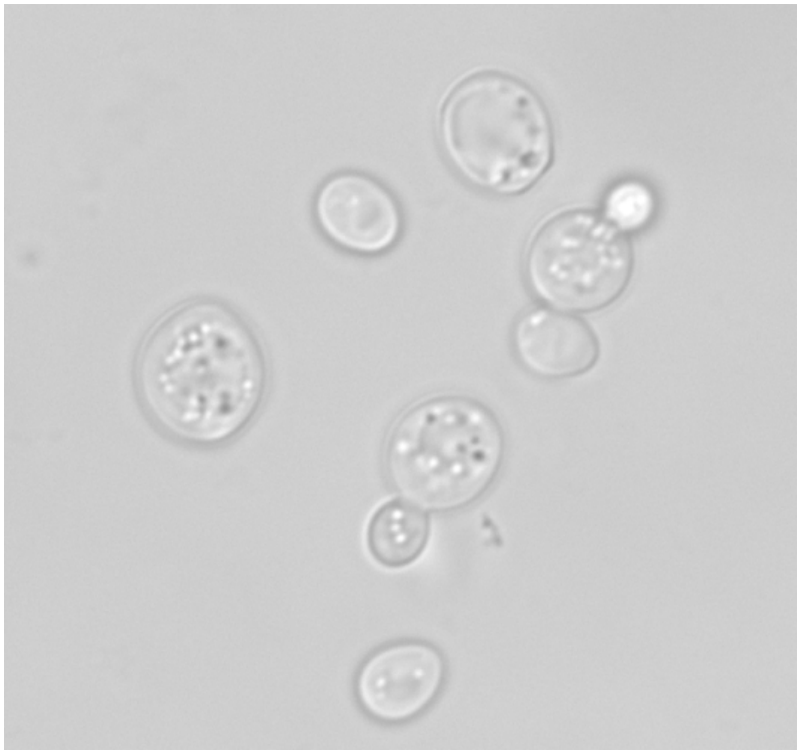

Figure 1. Bright field image of *Saccharomyces cerevisiae* (100x)

4 Signal transduction in yeast

4.1 cAMP-PKA pathway

The cyclic AMP-dependent protein kinase A (cAMP-PKA) is one of the most well-characterised glucose-induced signalling pathways in yeast. PKA regulates cell growth and division, metabolism and stress resistance in response to glucose availability (Thevelein and de Winde 1999; Conrad et al. 2014). The pathway is activated via two systems working in parallel (**Figure 2**) that sense intracellular (Ras proteins) and extracellular (G-protein coupled receptor (GPCR) system) glucose (Rolland et al. 2000). It has been suggested that acidification of the cytoplasm caused by glucose addition acts as a stimulus for glucose metabolism and thus activation of PKA (Dechant et al. 2010).

Ras proteins are small GTPases that in yeast are encoded by the *RAS1* and *RAS2* genes. Ras was the first signalling molecule shown to be conserved throughout the eukaryotic kingdom (Hunter 2000). Activation of Ras1 and Ras2 is promoted by guanine nucleotide exchange factors, Cdc25 and Sdc25 (Camonis et al. 1986; Damak et al. 1991), that mediate the GDP/GTP exchange. It has been suggested that the increase in the GTP/GDP ratio, and hence activation of Ras, is caused by intracellular acidification which occurs upon glucose addition (Colombo et al. 1998). Under glucose-rich conditions Ras binds GTP, which results in its activation and subsequent increase of intracellular levels of cAMP (Zaman et al. 2009). However, Ras activity has been shown to be insensitive to extracellular glucose in mutants unable to phosphorylate glucose (Colombo et al. 2004). Therefore, not only the presence of glucose but also subsequent glycolytic reactions are required for Ras signalling (Rolland et al. 2000; Colombo et al. 2004; Santangelo 2006). Inactivation of Ras is triggered by the GTPase activating proteins Ira1 and Ira2 (Tanaka et al. 1989; Tanaka et al. 1990).

The GPCR system includes Gpr1 and an associated α-subunit Gpa2 of the heterotrimeric G protein (Colombo et al. 1998; Kraakman et al. 1999). Extracellular glucose is sensed by Gpr1, which in turn activates Gpa2 (Xue et al. 1998). Signals from both GPCR and Ras activate adenylate cyclase Cyr1 (Cdc35), which catalyses the conversion of ATP to cAMP, mediating a rapid increase of the intracellular cAMP level (Rolland et al. 2000; Gelade et al. 2003). It has been shown that the Cyr1 adenylate cyclase can be activated by the GPCR system in a Ras-dependent

manner (Beullens et al. 1988). Hence it appears that extracellular glucose sensing via GPCR is dependent on intracellular glucose metabolism (Conrad et al. 2014).

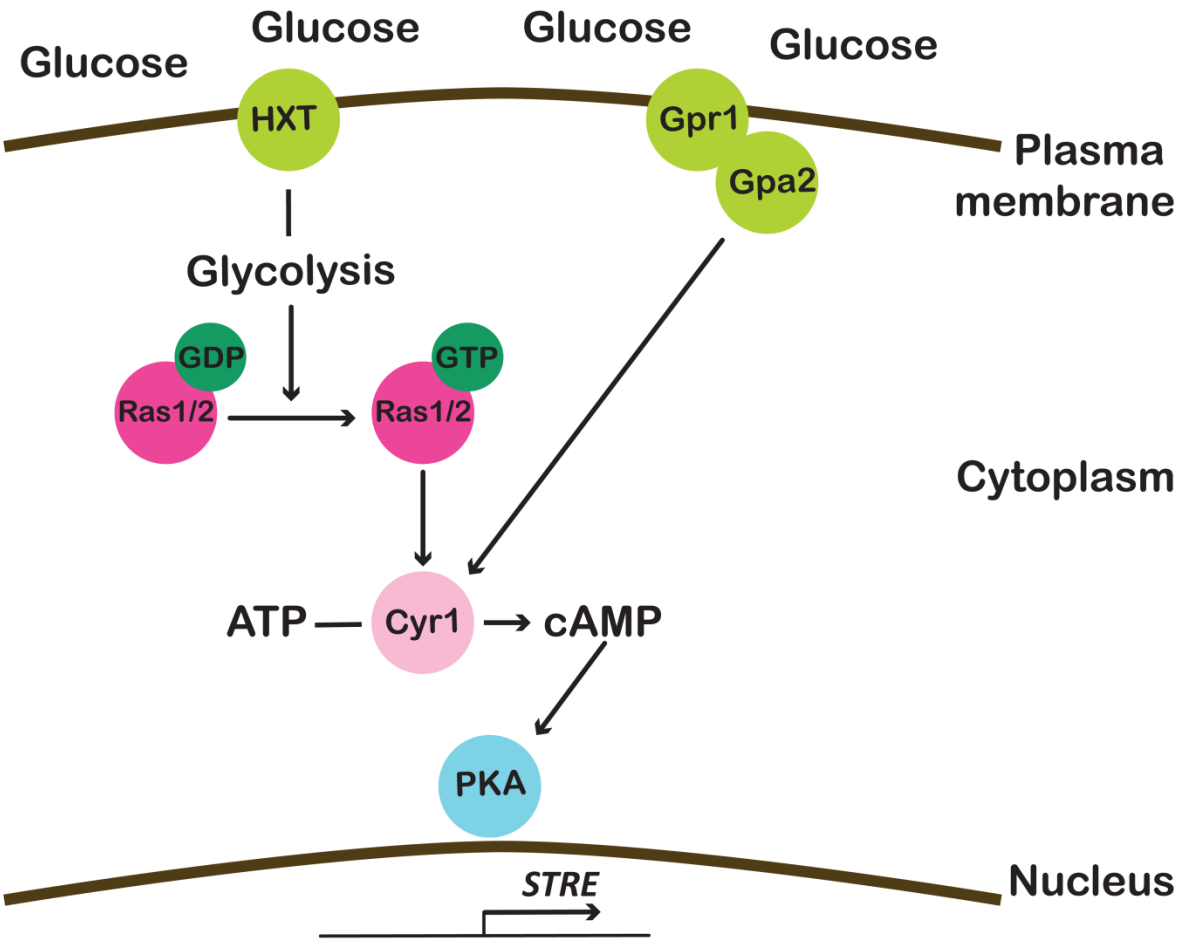

Figure 2. The cAMP-PKA pathway. The pathway senses glucose via two systems. One of them is triggered by Ras1 and Ras2 proteins in response to signals from glycolysis and results in GDP/GTP exchange. The other mechanism is activated via GPCR system (Gpr1 and Gpa2 proteins). The signals from both systems stimulate production of cAMP through the Cyr1 adenylate cyclase. Activation of PKA leads to repression or stimulation of expression of target genes and activity or stability changes of other target proteins.

PKA is a hetero-tetramer that consists of a dimeric regulatory subunit encoded by *BCY1* and two catalytic subunits encoded by the *TPK1-3* genes (Toda et al. 1987) . Intracellular cAMP binds to Bcy1 which results in PKA complex dissociation and activation of the enzymatic activity (Broach 2012). Generally, PKA stimulates rapid fermentative growth and acts negatively on cellular processes that occur

during stationary phase or respiratory growth (Thevelein and de Winde 1999). Active PKA controls cell cycle progression, diauxic shift and glycogen accumulation via phosphorylation of the Rim15 protein kinase (Swinnen et al. 2006). Mutations that inactivate the Ras/PKA pathway lead to permanent stationary phase-like arrest (Matsumoto et al. 1983). Hence, mutants lacking all three types of catalytic subunits of PKA (Tpk1, Tpk2, Tpk3) are inviable (Toda et al. 1987). The PKA-regulated transcription factors, Msn2 and Msn4, are involved in stationary phase induction of stress resistance genes that contain STRE elements (Gorner et al. 1998). High PKA activity prevents stress gene expression and hence leads to stress sensitivity (Shin et al. 1987; Gorner et al. 1998). It has also been shown that elevated activity of PKA by overexpression of *RAS2*, cAMP addition or *BCY1* deletion inhibits autophagy via preventing the autophagosome formation (Budovskaya et al. 2004; Yorimitsu et al. 2007).

4.2 Rgt2/Snf3 pathway

The Rgt2/Snf3 pathway (**Figure 3**) is also glucose regulated, although, compared to the PKA pathway, displays higher sensitivity to lower glucose concentrations (Zaman et al. 2009). It has been proposed that the membrane glucose sensors Rgt2 and Snf3 detect the relative external/internal glucose ratios (Karhumaa et al. 2010). Under high glucose conditions Rgt2 induces transcription of low affinity transporters encoded by *HXT1*. Low glucose concentrations stimulate expression of *HXT1-4* through Snf3 (Ozcan et al. 1996; Ozcan et al. 1998; Ozcan and Johnston 1999).

Rgt2 and Snf3 belong to the hexose transporters (HXT) family but they have lost their ability to transport glucose (Ozcan et al. 1998). Rgt2 and Snf3 show 60% overall identity and are also similar to mammalian and yeast glucose transporters but have a long cytoplasmic C-terminus to transmit the glucose signal to downstream regulatory proteins (Ozcan et al. 1996; Moriya and Johnston 2004). Rgt2 and Snf3 sense high and low concentrations of external glucose, respectively, and transmit the signal to Rgt1, a Zn-finger transcription factor (Ozcan and Johnston 1999; Ozcan 2002; Kim et al. 2003). In the absence of glucose in the medium, Rgt1 is recruited together with Mth1, Std1 and the Tup1-Ssn6 corepressor complex to the promoters and represses *HXT* genes (Ozcan and Johnston 1995; Kim et al. 2003). Addition of glucose mediates Rgt1 phosphorylation and

intramolecular interaction between its Zn-binding and central domains. In glucose starved cells Mth1 prevents this interaction by changing the conformation of Rgt1 (Polish et al. 2005) and preventing its phosphorylation (Lakshmanan et al. 2003).

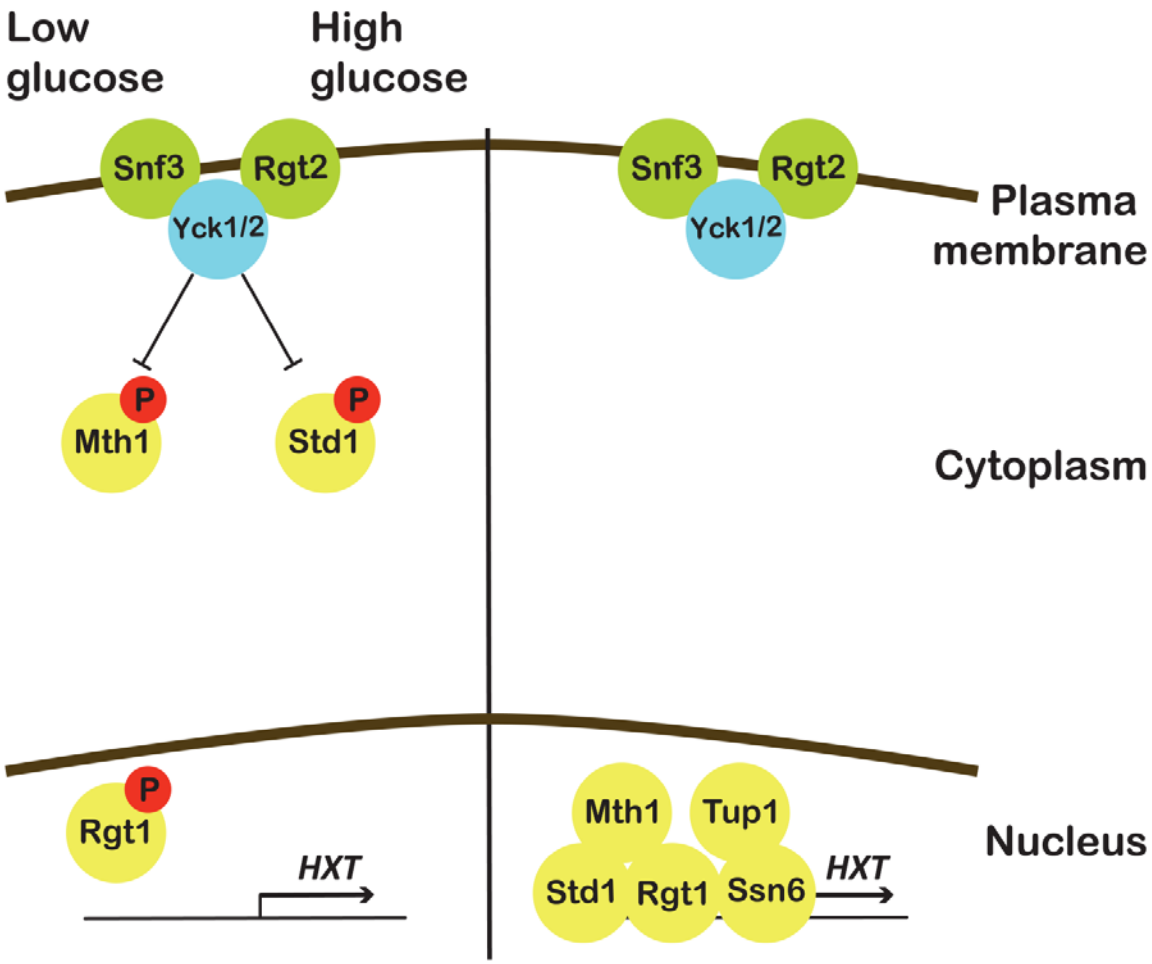

Figure 3. The Rgt2/Snf3 pathway. When glucose is available, Snf3 and Rgt2 sense low and high glucose concentrations, respectively. The casein kinases Yck1 and Yck2 phosphorylate Std1 and Mth1 and direct them to degradation. Rgt1 becomes hyper-phosphorylated and activated which results in its dissociation from the DNA, releasing *HXT* genes expression. In the absence of glucose, Rgt1 together with Std1 and Mth1 and a co-repressor Ssn6-Tup1 forms a repressing complex on *HXT* promoters.

Glucose binding to either Rgt2 or Snf3 mediates nuclear export of Mth1 and Std1 and their phosphorylation by the casein kinases I, Yck1 and Yck2 (Moriya and Johnston 2004). This phosphorylation subjects Mth1 and Std1 to degradation via the SCF Grr1 ubiquitin-protein ligase in the proteasome (Ozcan and Johnston 1995; Flick et al. 2003). Rgt1 becomes hyper-phosphorylated by PKA on multiple sites (Jouandot et al. 2011) resulting in its dissociation from the DNA. Thus, expression

of the *HXT* genes is released (Kim et al. 2003). It has been suggested that Rgt1 phosphorylation on all PKA sites converts it into a transcriptional activator required for maximal activation of target genes (Mosley et al. 2003).

4.3 Snf1/Mig1 pathway

The *S. cerevisiae* Snf1 (for *sucrose non-fermenting*) protein kinase is a yeast analogue of the mammalian AMP-activated protein kinase (AMPK). Both, Snf1 and AMPK are members of a highly conserved serine/threonine protein kinase family present throughout all eukaryotes (Carling et al. 1994). AMPK is activated by a decrease of the ATP concentration which results in an increased level of AMP. In turn, Thr172 in the activation loop becomes activated by phosphorylation via the tumour-supressor kinase LKB1 and Ca^{2+}/calmodulin-dependent protein kinase kinases CaMKKα and CaMKKβ (Hedbacker and Carlson 2008). Active AMPK phosphorylates its targets, such as transcription factors to regulate gene expression, but also metabolic enzymes to adjust metabolic activity. Moreover, pharmacological activation of AMPK has been suggested as treatment for metabolic diseases like obesity, heart diseases, type II diabetes mellitus and cancer (Bairwa et al. 2016; Jeon 2016; Martinez de Morentin et al. 2016).

The yeast Snf1/AMPK controls energy homeostasis and the adaptation to glucose limitation (Celenza and Carlson 1986). Snf1 regulates expression of genes required for respiration, metabolism of non-glucose carbon sources and gluconeogenic genes (Carlson 1999). It is also involved in autophagy, meiosis and sporulation, cell aging and response to various environmental stresses (Ghillebert et al. 2011). It has been shown, that Snf1 is also required for filamentous growth (Cullen and Sprague 2000). In response to glucose limitation Snf1 regulates *FLO11*, a gene required for invasive growth, through transcriptional repressors Nrg1 and Nrg2 (Cullen and Sprague 2000; Kuchin et al. 2002; Vyas et al. 2003).

The kinase domain of Snf1 shares a high level of similarity with mammalian AMPK (Rudolph et al. 2005). Like AMPK, SNF1 is a heterotrimer that consists of one catalytic, α (Snf1), two regulatory, β (Sip1, Sip2 or Gal83) and γ (Snf4) subunits (Nayak et al. 2006). Under glucose depletion, Snf4 binds to the C-terminal regulatory domain of Snf1 thereby eliminating autoinhibition of Snf1 (Jiang and Carlson 1996). The β-subunits interact with both Snf1 and Snf4 stabilising the whole complex as well as defining its subcellular localisation and substrate

definition (Jiang and Carlson 1996; Schmidt and McCartney 2000; Vincent et al. 2001). Subcellular localization of the Snf1 complex is regulated via Gal83, Sip1 and Sip2 N-termini in a glucose-dependent manner. Under glucose-rich conditions, all three β-subunits are cytoplasmic. When glucose becomes limited, Sip2 remains in the cytoplasm, whereas Sip1 and Gal83 are redirected to the vacuole and the nucleus respectively. Nuclear translocation of Gal83 can be inhibited by the addition of any fermentable carbon source (Vincent et al. 2001). Subcellular localization of the complex containing Sip1 or Sip2 is mediated by myristoylation of their N-termini (Lin et al. 2003; Hedbacker et al. 2004b).

The Ser/Thr kinase domain is about 330 amino acids long and located on the N-terminus of Snf1 (Vincent et al. 2001), whereas the C-terminal region contains sequences for binding to the other two subunits. Structurally, N- and C-termini represent small and large lobes, respectively. Disordered Gly-rich domains between β-sheets of the N-terminal lobe are believed to interact with phosphates of ATP (Rudolph et al. 2005). Snf1 is phosphorylated and thereby activated in the activation T-loop on Thr210 by upstream kinases (McCartney and Schmidt 2001). The T-loop has been shown to be disordered (Rudolph et al. 2005). Full activation of Snf1 is a two-step process that requires phosphorylation of the kinase domain in the disordered activation loop, which promotes binding of the kinase domain to the heterotrimer core which assumes an active conformation. Active Snf1 can bind an ATP molecule in the active site and transfer its terminal phosphate to a substrate (Chandrashekarappa et al. 2013).

Under glucose limiting conditions three upstream kinases, Sak1, Tos3 and Elm1, phosphorylate Snf1 at Thr210 in the activation loop (**Figure 4**) (Hong et al. 2003; Nath et al. 2003; Elbing et al. 2006; Rubenstein et al. 2008). Glucose does not seem to regulate their activity (Rubenstein et al. 2008), but decreases interaction with Snf1 (Nath et al. 2003). All three isoforms of the Snf1 complex can be activated by any of the three upstream kinases (McCartney et al. 2005), while Sak1 seems to have highest activity towards Snf1 (Hong et al. 2003; Kim et al. 2005). Despite the fact that Sak1 is the major activating kinase, Elm1 and Tos3 do not require Sak1 presence to be able to phosphorylate Snf1 (Hong et al. 2003; Nath et al. 2003). In *sak1* deficient cells, Snf1 shows decreased kinase activity and Gal83 remains cytoplasmic (Hedbacker et al. 2004a; Hong et al. 2005). Recent studies showed that the C- and N-termini of Sak1 are essential, while the carboxy-terminal domain of Tos3 is important but not crucial and the C-terminus of Elm1 does not play any role

in Snf1 signalling (Rubenstein et al. 2006; Liu et al. 2011). Sak1 also plays a major role in Snf1 activation in response to salt stress, alkaline pH and hydrogen peroxide (Hong and Carlson 2007). However, only the lack of all three upstream kinases results in constant inactivation of Snf1, regardless of the salt concentration (Ye et al. 2008).

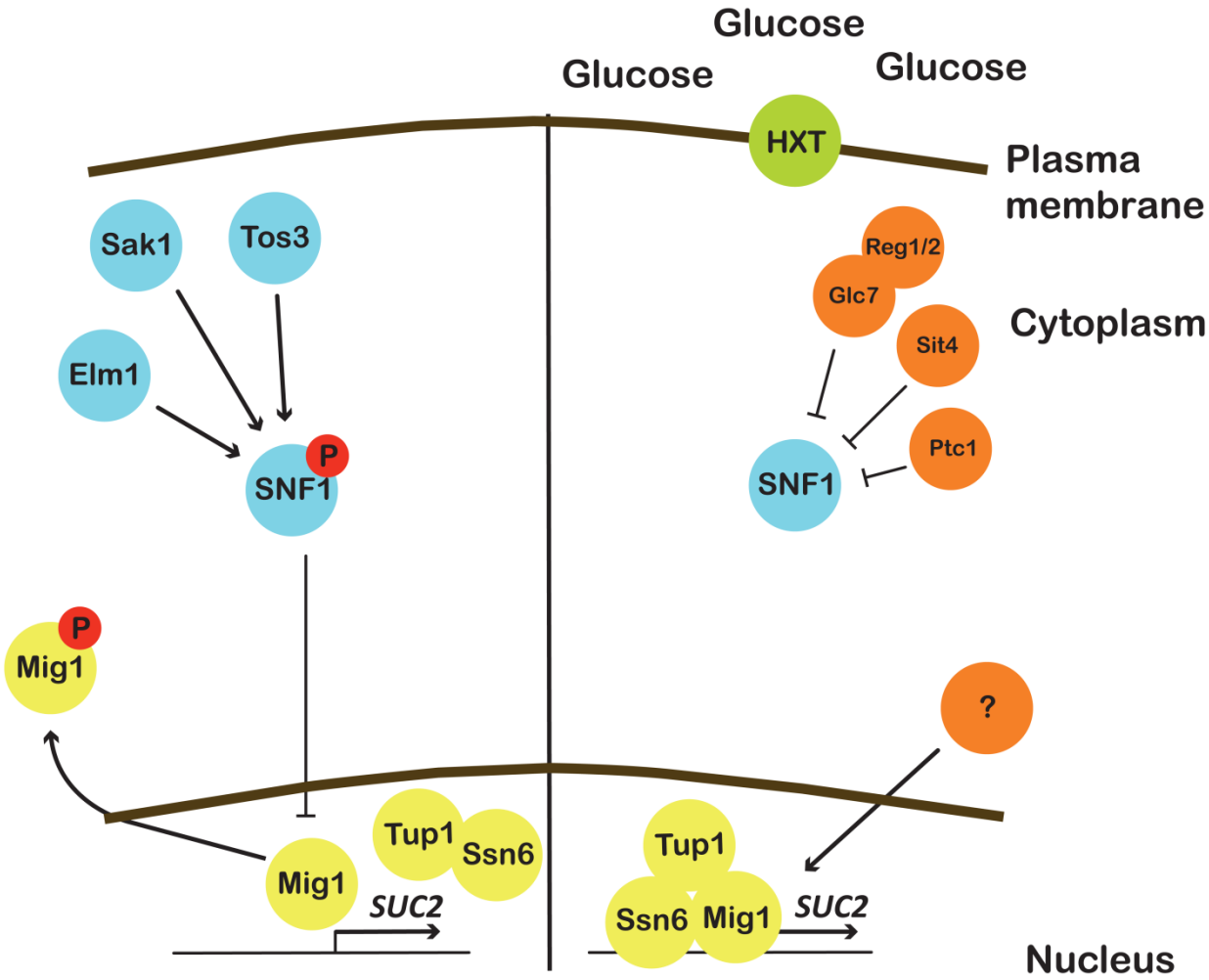

Figure 4. The Snf1/Mig1 glucose repression pathway. Under glucose-rich conditions Snf1 is cytosolic and Mig1 together with Ssn6 and Tup1 forms a repressor complex that binds to the promoters of target genes to repress them. If glucose becomes limited Snf1 is activated by phosphorylation via three upstream kinases. Snf1 then translocates into the nucleus where Mig1 is phosphorylated and the repression complex dissociates to release gene expression. Mig1 is exported from the nucleus.

Protein phosphatase Glc7-Reg1 is the main negative regulator of Snf1 (Tu and Carlson 1994). Sit4 and Ptc1 phosphatases have also been shown to participate in Snf1 dephosphorylation (Ruiz et al. 2011; Ruiz et al. 2013). The presence of

glucose, probably via its effect on the ATP/ADP/AMP ratio, makes the Snf1 activation loop accessible for protein phosphatases (Rubenstein et al. 2008). The activation of the complex correlates with a high AMP/ATP ratio (Wilson et al. 1996). ADP binds to the regulatory subunit of the SNF1 complex, which results in protection of Thr210 from dephosphorylation. Consequently, it has been suggested that ADP is a metabolic signal resulting in SNF1 activation (Mayer et al. 2011). SNF1 regulates cellular processes through the control of different transcription factors, such as Hsf1, Sip4, Cat8, Adr1, Rds2 and others (Lesage et al. 1996; Randez-Gil et al. 1997; Hahn and Thiele 2004; Soontorngun et al. 2007; Ratnakumar et al. 2009). The transcriptional repressor Mig1 regulates metabolism of alternative carbon sources, such as sucrose and galactose (Nehlin and Ronne 1990). Under glucose derepression, Snf1 inactivates and redirects Mig1 to the cytoplasm (Lutfiyya et al. 1998; Smith et al. 1999; Ahuatzi et al. 2007) through phosphorylation on Ser278 and Ser311 (Ostling and Ronne 1998). All three subunits of the SNF1 complex are required for Mig1 phosphorylation (Schmidt and McCartney 2000; Leech et al. 2003). Together with co-repressors Ssn6 and Tup1, Mig1 forms a repression complex that under high glucose conditions binds to the promoters of the genes essential for utilization of carbon sources other than glucose (Treitel and Carlson 1995). A recent study suggests that in cells grown in the presence of glucose, Mig1 also interacts with Hxk2 specifically through Ser311 of Mig1 resulting in repression of *SUC2* expression. Moreover, Hxk2 was reported as an essential factor for nuclear localization and dephosphorylation of Mig1 under high glucose conditions (Ahuatzi et al. 2007). It has also been suggested that Hxk2 functions as a cytoplasmic glucose sensor and changes its conformation in response to the presence of glucose in the cytoplasm. Those conformational alterations regulate Hxk2 interaction with Mig1, and thereby its nuclear import (Vega et al. 2016). At the same time Hxk2 exists in multiple molecular forms with different phosphorylation states and conformations (Kuettner et al. 2010). The exact role of Hxk2 in Mig1 regulation remains unclear.

4.3.1 The Mig1 transcriptional repressor

The Multicopy Inhibitor of Galactose gene expression, Mig1, is a Cys2His2 zinc finger transcription factor (Nehlin and Ronne 1990) that binds to the promoters of genes required for metabolism of non-glucose carbon sources, such as sucrose (Wu and Trumbly 1998), maltose (Hu et al. 1995) and galactose (Nehlin et al. 1991;

Johnston et al. 1994). Mig1 binding sites upstream of the target genes include a GC-rich region and an AT-box 5' to facilitate protein access to the DNA (Lundin et al. 1994). In glucose-grown cells, Mig1 recruits a co-repressor complex consisting of Ssn6 (Cyc8) and Tup1 transcription factors (Treitel and Carlson 1995), where Tup1 has been suggested to perform a repression function and Ssn6 to be a link between Tup1 and DNA-binding proteins (Tzamarias and Struhl 1994). Snf1-dependent phosphorylation prevents Mig1 association with the complex (Papamichos-Chronakis et al. 2004). *In vitro* assays showed that Mig1 binding to its target sites does not depend on glucose or Ssn6 and Tup1 presence (Wu and Trumbly 1998). On the other hand, absence of Ssn6 results in Mig1 being capable to strongly activate gene expression whereas *tup1Δ* mediates a weaker Mig1 activator function (Treitel and Carlson 1995). Thus, glucose repression of maltose metabolism can be relieved by *MIG1* and/or *SSN6* deletion but not by that of *TUP1* (Lin et al. 2014).

Despite the fact that Mig1 has been convincingly shown to locate mainly in the cytoplasm and the nucleus upon glucose depletion/repletion, respectively (De Vit et al. 1997), Mig1 constantly shuttles between the nucleus and the cytoplasm regardless of glucose availability (Bendrioua et al. 2014). Mig1 nuclear export is mediated by a β-importin homologue, Msn5, in response to Snf1-dependend phosphorylation of the Nuclear Localisation Signal (NLS) sequence on the C-terminus (DeVit and Johnston 1999). However, how exactly Mig1 is imported/exported into/out of the nucleus and the dependence on the environmental conditions as well as the relevant stoichiometry of transported molecules remain to be resolved.

Protein database analysis suggests more than 90 homologues for yeast Mig1. Some of them where identified quite some time ago. Thus, it has been shown that the zinc fingers of Mig1 are similar to those of mammalian Egr1 and Egr2 proteins expressed upon the early growth response, as well as a protein encoded by a gene deleted in Wilms' tumour cells. Egr proteins and Mig1 share the same motif, FSRSD, which is believed to be important for DNA binding (Nehlin and Ronne 1990). A nerve growth factor, NGF, has also been shown to contain similar zinc fingers to those of Wilms' tumour gene product (Crosby et al. 1991). Another Cys2His2 zinc finger protein, a carbon catabolite repressor of *Aspergillus nidulans,* CreA, has 84% similarity with Mig1 within the zinc fingers (Dowzer and Kelly 1991). All of these proteins, Mig1, CreA, Egr1 and Egr2, Wilms' tumour gene

product, recognise similar GC-rich target sequences (Brambl et al. 1996). Thus, Egr proteins and Mig1 were suggested to have a similar role in growth and glucose metabolism in mammals and yeast, respectively (Nehlin and Ronne 1990). Both Mig1 and CreA are involved in catabolite repression (Dowzer and Kelly 1991).

Another transcriptional repressor, Mig2, a functional homolog of Mig1, binds to target sites similar to those of Mig1, although with different affinity (Lutfiyya et al. 1998). However, Mig2 is not phosphorylated by Snf1 and its expression is not repressed by glucose (Lutfiyya et al. 1998). Mig3 is similar to Mig1 and Mig2, although it is not able to repress genes involved in non-glucose carbon sources metabolism (Lutfiyya et al. 1998). Snf1 directly phosphorylates Mig3 in the absence of glucose, resulting in Mig3 degradation (Dubacq et al. 2004). It has also been shown that Mig1, Mig2 and Mig3 together repress expression of *MTH1*, encoding a negative regulator of the glucose-sensing signal transduction (Kaniak et al. 2004).

4.4 MAP-kinase signalling pathways

In yeast, mitogen-activated protein kinases (MAPKs) are involved in the control of mating, sporulation, adaptation to hyperosmotic stress, filamentous growth and cell wall stress. Yeast MAPK pathways are among the best understood signal transduction pathways is biology.

MAPK cascades are evolutionary conserved and found in fungi, plants and animals (Gustin et al. 1998) and consist of three tiers of kinases: MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). The latter must be activated to initiate the cascade (Chen and Thorner 2007). The MAPKKK is normally activated either through phosphorylation or by interaction of a small GST-protein Ras in response to external stimuli (Cargnello and Roux 2011). Then MAPKKK activates MAPKK through phosphorylation on two Ser or Thr residues in the activation loop. Subsequently, MAPKK, a dual specificity (tyrosine and serine/threonine) kinase, transmits the signal onto MAPK via tyrosine and threonine phosphorylation on a conserved Thr-X-Tyr motif of the activation loop. Though having cytosolic functions too, active MAPKs often translocate from the cytoplasm to the nucleus in order to regulate the activity of various targets including transcription factors, phosphatases and MAPK activated protein kinases (Marshall 1994; Hunter 2000;

Chen and Thorner 2007). It has also been suggested that MAPKK can play a role of a MAPK carrier in and out of the nucleus (Qi and Elion 2005).

The yeast *S. cerevisiae* has five MAPK pathways controlling pseudohyphal growth (MAPK: Kss1), the response to mating pheromone (Fus3), cell wall integrity (Slt2), high osmolarity glycerol (Hog1) and sporulation (Smk1) (Gustin et al. 1998; Qi and Elion 2005) , respectively. However, the meiosis-specific Smk1 is not activated through MAPK cascades but via auto-phosphorylation (Whinston et al. 2013). Some kinases act in several pathways. Thus, Hog1 not only regulates osmoadaptation but also prevents the crosstalk between the HOG and mating pathways (O'Rourke and Herskowitz 1998). Other kinases that control hyperosmotic stress and the pheromone response also function in filamentous growth (Liu et al. 1993; Cullen and Sprague 2012). Kss1 not only regulates pseudohyphal growth but also plays a role in cell wall integrity control and mating in response to pheromones (Qi and Elion 2005).

4.5 Crosstalk

Crosstalk between the pathways enables integration of external and internal stimuli, which serves quick and the most appropriate response to environmental alterations. Thus, the Snf1 pathway is massively involved in crosstalk with other glucose signalling and MAPK pathways as well as pathways not discussed here, such as Ras and TOR pathways (Shashkova et al. 2015).

For instance, Snf1 and Glc7-Reg1 have been suggested as upstream activators of Yck1 and Yck2 in the Rgt2/Snf3 pathway. Mig1, in cooperation with its paralogue Mig2, represses expression of the *HXT2* and *HXT4* genes. Glucose-regulated mechanisms that activate Glc7-Reg1 seem to be involved in posttranslational modifications mediated by the cAMP-PKA pathway. At the same time, the Glc7-Reg1 phosphatase participates in regulation of Msn2, one of the PKA targets. Msn2, initially identified as multi-copy suppressor of *SNF1* deletion, has also been shown to be phosphorylated by Snf1. Overall it appears that the PKA pathway affects activation and localisation of the SNF1 complex (Shashkova et al. 2015).

One of the Snf1 upstream kinases, Elm1, appears to phosphorylate a Gpa1 protein, which is involved in the MAPK pheromone response pathway. Reg1 has been shown to regulate Gpa1, which influences the mating response. Snf1 has been

shown to participate in glucose-dependent activation of the HOG pathway. Glucose depletion seems to stimulate the pseudohyphal growth system via the transcriptional repressors Mig1 and Mig2 possibly through their association with cytosolic regulators of the filamentous pathway (Karunanithi and Cullen 2012). In general, regulation of invasive growth appears to be an orchestrated action of several pathways: Snf1, PKA, TOR and MAPK (Cullen and Sprague 2012).

Various interactions between the pathways were described in a number of reviews (McClean et al. 2007; Saito 2010; Schmidt 2013).

5 Protein structure

Proteins are one of the main components of living cells preforming a large variety of functions. They represent the most abundant molecules in live organisms after water. The way the protein is folded corresponds to the most stable or the lowest energy state. It also defines native structure of proteins. In order to be functional most of the proteins fold into a defined three-dimensional structure.

The three-dimensional organisation of the protein, the tertiary structure defines biological activity and function of the protein. This structure is mainly characterised by non-specific hydrophobic interactions arising between secondary structured regions stabilised by several types of forces. There are also some small assembles of secondary structure elements which are called super secondary structure and serve as a structural and/or functional motif, for instance leucine zipper, zinc finger, helix-turn-helix domains of transcription factors (Aung and Li 2007).

5.1 Intrinsic disorders

Despite precise structural hierarchy, many functional proteins possess regions without defined structure – intrinsically disordered regions (IDRs). Under physiological conditions IDRs are characterised by low sequence complexity, specific amino acid composition and high predicted flexibility. IDRs can be detected by various techniques: NMR and circular dichroism spectroscopy approaches, X-ray crystallography. There is also a number of online software tools that were created to predict unstructured regions from an amino acid sequence, such as DisEMBL, DISOPRED3, GLOBPLOT2.3, PONDR (Dyson and Wright 2005; Jones and Cozzetto 2015). The number of intrinsically disordered proteins (IDPs) in cells increases with a complexity level of an organism (Dyson and Wright 2005). In fact, disorder prediction indicates 6-33% of the whole proteome to be disordered in bacteria and 35-51% in eukaryotes (Dunker et al. 2002). Intrinsically disordered proteins undergo faster protease digestion, which provides for a quick response to rapid alterations in protein concentration; however, there are several mechanisms that protect these proteins from proteolysis *in vivo*, e.g. the lack of protease sensitive residues, unavailability to proteases due to steric factors and also protection by chaperons (Dunker et al. 2002).

The amino acid sequence determines the properties of the protein. Hydrophobic amino acids (Val, Leu, Ile, Met, Phe, Trp and Tyr) form the core of folded protein. Low hydrophobicity and high content of charged amino acids (Ala, Arg, Gly, Gln, Ser, Pro, Glu, Lys) are main characteristics of intrinsically disordered proteins (Uversky 2011). IDPs have no specific three-dimensional conformation although many of them obtain a more ordered structure or stable secondary structure upon binding to their targets (Dyson and Wright 2005). For example, Shoemaker et al proposed a "fly-casting" mechanism which suggests that the folding of an IDP associated with binding provides a greater capture radius than a compactly folded protein, therefore allows faster target finding (Shoemaker et al. 2000). Some IDRs, like flexible linkers and/or spacers, are functional without undergoing disorder-order transition which enables domains to move relative to each other and/or regulates the distance between them (Dunker et al. 2002).

The conformation that IDRs obtain upon association with their interactors is determined by those proteins as well as by the amino acid content of an IDR. It allows IDPs to have a wide variety of partners with high specificity and low affinity which results in quick dissociation and termination of signal transduction (Wright and Dyson 1999). Protein complex formation has been associated with disorder-to-order transition which are mainly localised on binding motifs (Fong et al. 2009). It has been shown that the content of IDRs is higher in homodimers than heterodimers. Many proteins in the cell form oligomers. Symmetrical arrangement of the same protein can regulate accessibility to the binding partners, generate new binding sites or increase complex specificity and diversity (Fong et al. 2009). Moreover, homo-oligomerisation is more energy beneficial (Goodsell and Olson 2000). At the same time, homo-dimeric complexes could inhibit protein evolution and function optimisation (Andreeva and Murzin 2006).

Disordered sequences have been shown to play an important role in molecular recognition (protein-protein interactions, binding to DNA and various types of RNA, ligands and metal ions), thus, in various cellular processes, such as signal transduction, regulation of transcription and translation (Dyson and Wright 2005). Indeed, signalling proteins were predicted to have a higher content of IDRs than other proteins (Iakoucheva et al. 2002). IDPs have been shown to have kinetic advantages as their fast association rates enable rapid signal transduction (Pontius 1993). The involvement of disordered regions in those interactions can serve more efficient association or dissociation due to reduced dependence on structural

orientation, large interaction surfaces and one molecule can bind to differently shaped partners (Dunker et al. 2002). The number of unstructured regions within a protein correlates with the number of their interacting partners (Iakoucheva et al. 2002). Thus, intrinsically disordered proteins were shown to function as hub proteins in protein-protein interaction networks having tens and even hundreds of interacting partners (Tsai et al. 2009; Wright and Dyson 2015).

Various studies indicate the presence of posttranslational modification sites, such as phosphorylation, ubiquitination, etc, within IDRs, which tune the protein according to its interactor at a given time providing faster and more efficient binding to a wide variety of targets (Uversky 2011). Modifications within the structured region are slow or significantly inhibited due to steric factors which prevent close association of the modifying enzyme with the target protein. Instead, a disordered region would facilitate this binding as it would fold directly onto the modifying enzyme (Dunker et al. 2002). Studies show that disorder-order transitions are tightly linked with phosphorylation and protein-protein interactions. This correlation is especially pronounced on serine phosphorylation sites (Nishi et al. 2013). Indeed, serine and threonine are often found in IDRs whereas tyrosine is more characteristic for structured regions. In **Paper I** we propose a novel concept that transcription factors operate in spherical clusters with the purpose of faster target sequence recognition and binding. We suggest that those clusters are stabilised by weak depletion forces that arise between intrinsically disordered regions of zinc fingers transcription factors. This model is supported by the fact that at least 50% of all phosphorylation sites lie within IDRs, and the correlation between binding affinity and phosphorylation state of the protein has already been reported (Nishi et al. 2013).

6 Posttranslational modifications

Once a protein is folded and stabilised it can undergo various modifications on a side chain or backbones catalysed by enzymes (Walsh et al. 2005). Covalent posttranslational modifications occur in prokaryotes but their frequency is much higher and the variety is broader in more complex organisms. Genes that encode enzymes for posttranslational modifications occupy about 5% of the whole genome in higher eukaryotes. Therefore, posttranslational modifications that occur on one or more sites of proteins greatly expand the proteome.

Covalent posttranslational modifications are defined based on the catalysing enzyme, target amino acid chain and reversibility. The most common types of such modifications are phosphorylation, glycosylation (O- and N-glycosylation), alkylation (methylation and prenylation), acylation (ubiquitination, acetylation, etc), oxidation (Walsh et al. 2005). Proteins can undergo different types of posttranslational modifications simultaneously or sequentially which mediates a flexibility of metabolism in response to internal and environmental alterations. For instance, transcription factors can be modified by several reactions, such as phosphorylation, methylation and ubiquitination (Leach and Brown 2012). The order and combination of phosphorylation, acetylation, methylation, sumolyation and ubiquitination on histones form a “histone code” which is then read by other proteins, thus, the downstream events are defined (Strahl and Allis 2000). About 54% of enzymes involved in carbon metabolism of *S. cerevisiae* are targets for more than one type of modifications including phosphorylation, ubiquitination and acetylation. Moreover, it has been shown that evolutionary conserved signalling pathways have evolutionary conserved posttranslational modifications as a mechanism controlling enzymatic activity (Tripodi et al. 2015).

6.1 Protein phosphorylation

In order to transmit the signal from the outside to the target gene, living cells use signal transduction. This transduction occurs via consequent conformational changes of the protein on the posttranslational level. Phosphorylation is one of the best characterised posttranslational modifications. Phosphorylation is a reversible modification which is mediated by protein kinases and inverted by protein phosphatases through dephosphorylation (**Figure 5**). An orchestrate action of

kinases and phosphatases determine the general dynamics of cells responding to extracellular or intracellular stimuli, thus balance of protein phosphorylation (Bononi et al. 2011). More than 30% of the yeast proteome is altered by phosphorylation at any given moment (Ficarro et al. 2002). Proteins in eukaryotic cells can be phosphorylated on several phosphorylation sites with different kinetics and regulation simultaneously which provides a platform for integration of various signals (Olsen et al. 2006). Multi-omics analysis on the *S. cerevisiae* genome showed that proteins without phosphorylation sites are involved in much fewer protein-protein interactions than phosphoproteins (Yachie et al. 2011). Hence, phosphorylation of a protein mediates a wide variety of molecular interactions, thus, modulation of signal transduction pathways.

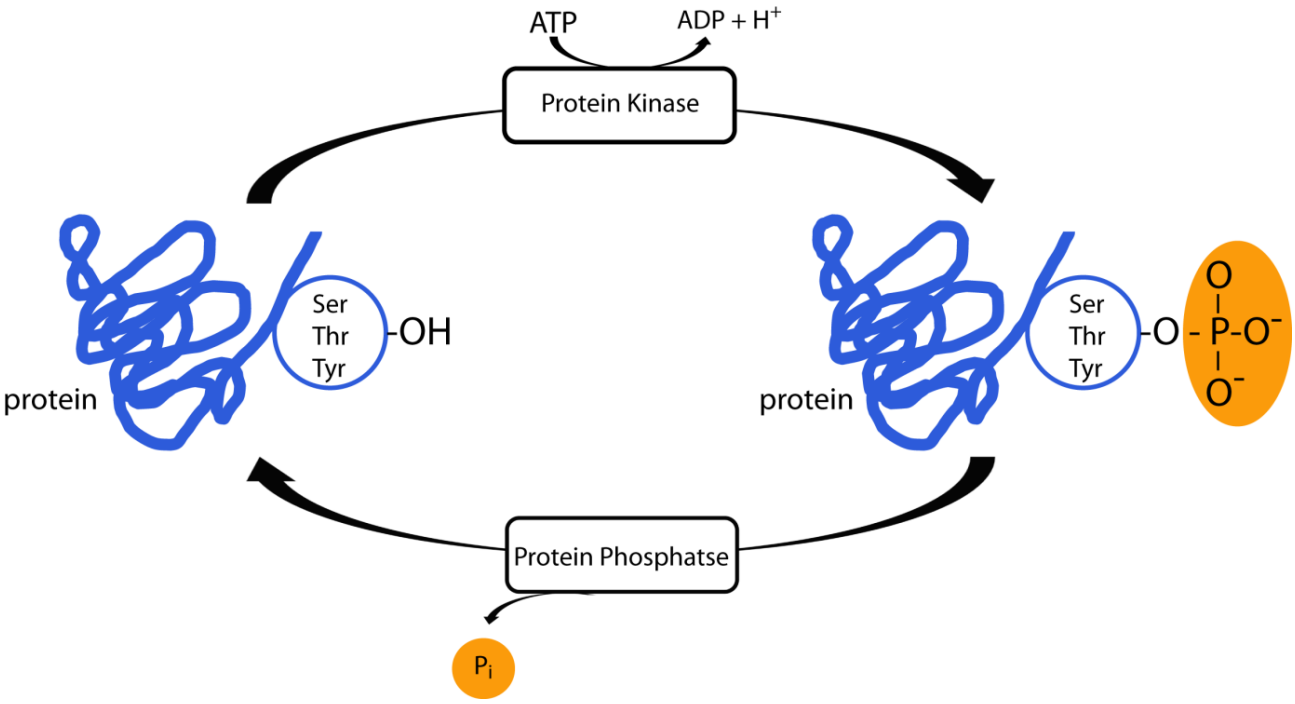

Figure 5. Protein phosphorylation. A phosphate group released during ATP conversion into ADP, covalently binds to the side chain hydroxyl group on Ser, Thr or Tyr residues. This reaction is catalysed by protein kinases. A protein phosphatase removes inorganic phosphate by dephosphorylation.

Phosphorylation plays a crucial role in a broad spectrum of cellular processes such as growth, stress response and cell cycle. The phosphate PO_4^{3-} group donated from ATP covalently binds to proteins, mainly to serine, threonine and tyrosine residues, resulting in changing protein functions through altering its confirmation or

interaction ability (Humphrey et al. 2015). Most commonly phosphorylation occurs on serine and threonine residues, more rarely on tyrosine. Other amino acids, such as arginine, lysine, histidine (Ciesla et al. 2011), aspartate (Li et al. 1998), cysteine (Guan and Dixon 1991) can also be phosphorylated; and this phosphorylation has also been shown to play an important role in cellular processes (Ciesla et al. 2011). However, there are no examples of Cys phosphorylation as well as no arginine and lysine kinases have been yet identified in eukaryotes (Gomperts et al. 2009; Kramer 2015).

6.1.1 Protein Kinases

1.5-2.5% of eukaryotic genes encode protein kinases (Manning et al. 2002). The budding yeast contains at least 121 protein kinases (Fiedler et al. 2009), and most of them are Ser/Thr kinases (Hunter and Plowman 1997). The catalytic domain responsible for the phosphorylation reaction is highly conserved and can be used for classification of kinases based on structural similarities (Hunter and Plowman 1997). The active catalytic site is located in a deep cleft between the lobes (Bossemeyer 1995). The binding site of a substrate protein is directed towards the cleft that shelters a whole ATP molecule with the γ-phosphate group out, facilitating the covalent attachment to the protein (Bossemeyer 1995).

Most commonly, more than 82% of protein phosphorylation in yeast occurs on serine, much less on threonine (17.5%) and very little on tyrosine (less than 0.03%) residues. Although yeast does not have tyrosine-specific kinases, there are about 10 dual specificity kinases which participate either in cell cycle control or MAPK signalling (Chi et al. 2007). A large scale screening identified 27 protein kinases that were able to phosphorylate poly(Tyr-Glu) motif, a typical artificial substrate for Tyr-specific kinases, suggesting that there are more kinases that are potentially able to phosphorylate proteins on tyrosine residues (Zhu et al. 2000). A preferred substrate sequence for Tyr-specific kinases in eukaryotic cells has been reported to contain hydrophobic and acidic residues downstream and upstream of a tyrosine, respectively (Blom et al. 1999; Miller 2003).

An example of a Ser/Thr protein kinase family is an AMP-activated protein kinase (AMPK), represented in yeast by SNF1 (Carlson et al. 1981).

6.1.2 Protein Phosphatases

Protein phosphatases are enzymes responsible for removing phosphate groups from the substrate. At least 38 protein phosphatases were identified in the budding yeast (Fiedler et al. 2009).

There are four major classes of protein phosphatases defined based on the catalytic domain and substrate preference (Moorhead et al. 2009). The tyrosine phosphatases (PTP) are characterised by CX_5R catalytic domain. Yeast PTPs include tyrosine-specific phosphatases, dual specificity phosphatases that can remove phosphate from Ser/Thr and Tyr residues (e.g. Cdc14), low molecular weight tyrosine phosphatases (LMPTP) and Cdc25 (Moorhead et al. 2009). Ser/Thr dephosphorylation is carried out by serine/threonine-specific (PPP), Mn^{2+}/Mg^{2+}-specific (PPM) and the aspartate-specific phosphatases (Moorhead et al. 2009; Shi 2009). The first two groups are highly similar in their catalytic domain, even though their sequences are unrelated (Moorhead et al. 2009). PPPs are the most conserved phosphatases throughout all eukaryotes showing 80% identity across the species (Brautigan 2013). A great number of PPPs consists of a catalytic subunit that associates with various regulatory subunits (Shi 2009). Based on substrate specificity, metal requirement and sensitivity to inhibitors, eukaryotic PPPs are divided into eight groups (Cohen 1997). PP1 is the major one and is expressed in all eukaryotic cells (Shi 2009). The catalytic domain of mammalian PP1 shares 76-88% similarity with plant and 90% with fungi PP1 (Moorhead et al. 2009). At least three PPPs participate in the glucose repression pathway in yeast. A PP2A phosphatase, Sit4, and a PP2C phosphatase, Ptc1, have been shown to play a role in Thr210 dephosphorylation of Snf1 (Ruiz et al. 2011; Ruiz et al. 2013). Glc7 is a PP1 catalytic subunit that participates in regulation of a broad variety of cellular processes depending on a regulatory subunit it is associated with. The *GLC7* gene is essential for yeast viability (Wu and Tatchell 2001). In a complex with the Reg1 regulatory subunit, the Glc7-Reg1 phosphatase controls glucose repression, cell growth and glycogen accumulation (Cui et al. 2004).

To preserve the phosphorylation state of proteins, sodium fluoride (NaF) and orthovanadate (Na_3VO_4) phosphatase inhibitors are routinely included in extraction buffers to prevent dephosphorylation by endogenous phosphatases. Vanadate anions are known to inhibit tyrosine phosphatases (Gordon 1991) due to structural similarity to orthophosphate ions (Crans et al. 2004; Korbecki et al. 2012). Vanadate compounds were also reported to inhibit protein phosphatases other than

tyrosine specific ones, such as alkaline phosphatases and ATPase (Parra-Diaz et al. 1995; Reiter et al. 2002). NaF is another protein phosphatases inhibitor that prevents dephosphorylation on serine and threonine residues (Shenolikar and Nairn 1991). It has been shown that NaF does not change the total activity of these phosphatases but selectively inhibits some of them, e.g. inhibition of myosin-specific phosphatase results in endothelial cell barrier function via its effect on actin (Wang et al. 2001). In **Paper II** we use phosphatase inhibitors to study their effect on Mig1 transcriptional repressor phosphorylation. Although Mig1 has only serine and threonine phosphorylation sites, we found that incubation with Na_3VO_4 resulted in constant Mig1 phosphorylation whereas NaF did not affect the phosphorylation pattern of Mig1.

6.2 Other types of protein posttranslational modifications

Acetylation is catalysed by acetyl-transferase and depends on the concentration of acetyl-CoA (Tripodi et al. 2015). Acetylation normally takes place on Lys residues of histones and non-histone proteins and participates in various cellular processes, such as aging, cell cycle progression, stress adaptation via affecting protein functions (Leach and Brown 2012). Acetylation of Sip2 subunit of the SNF1 complex increases its affinity to the catalytic subunit, Snf1, which in turn decreases kinase activity of SNF1 resulting in cell growth retardation and replicative life span extension (Lu et al. 2011).

Protein methylation is a form of alkylation that typically occurs on the side chains of Arg or Lys residues, where a hydrogen atom is replaced by a methyl group, in particular on already acetylated histone tails (Walsh et al. 2005). Methylation is catalysed by methyltransferases. This type of protein posttranslational modification can affect protein functions, stability and protein-protein interactions. Recent studies show that about 2.6% of identified *S. cerevisiae* proteins are methylated (Wang et al. 2015). For a long time, methylation has been thought to be irreversible. However, in the early 2000s the first histone demethylase has been discovered (Shi et al. 2004). In general the turnover of a methyl group seems to be slower than for other posttranslational modifications. Nowadays a wide variety of demethylases have been identified and the importance of their action has been shown (Greer and Shi 2012). For instance, reversible carboxymethylation of Leu of

protein phosphatase 2 plays an essential role in growth regulation under stress conditions (Tolstykh et al. 2000).

Ubiquitination is mediated by a sequential action of a hierarchical cascade of E1-E2-E3 enzymes (Hochstrasser 1996). Most commonly ubiquitin covalently binds to Lys residues (Pickart 2001) but also on serines, threonines and cysteines (Finley et al. 2012). The number of ubiquitin molecules attached to the target defines the fate of the protein. Thus, polyubiquitination preferentially results in directing the modified protein to the proteasome for degradation whereas a small number of ubiquitin molecules targets the protein to the endosome (Leach and Brown 2012). By altering protein functions ubiquitin serves as a signalling agent for various cellular processes such as membrane protein trafficking and extracting proteins from multi-subunit complexes (Finley et al. 2012). In yeast ubiquitination has been shown to play a role in cell growth, metabolism and stress response (Leach and Brown 2012).

Proteins can undergo glycosylation in endoplasmic reticulum (ER) or the Golgi. The addition of carbohydrates to asparagine side-chains (N-glycosylation) of newly synthesised proteins in ER serves regulation of protein folding and quality control (Xu and Ng 2015). O-linked glycosylation plays roles in regulation of cell cycle, growth, morphology (Goto 2007).

7 Transcription regulation

Transcription is the first step of gene expression which starts with the recognition of specific DNA regions within gene promoter sequences by transcription factors (TF) represented by transcriptional activators or repressors which bind to upstream activation (UAS) or repression (URS) sequences, respectively. Several UAS present within the same promoter allow combinatorial control of gene expression.

There are at least 169 genes of *S. cerevisiae* genome encoding TFs. TFs are divided into three groups based on their DNA-binding domain: zipper type (22 members), helix-turn-helix (8 members) and Zn^{2+}-stabilised TFs (at least 113). The latter class is further subdivided into Cys2His2, C6 and C4 or GATA fingers depending on residues liganded to Zn^{2+} (Hahn and Young 2011). Zinc stabilised TFs are the most abundant, however the C6 type is unique for fungi. Cys2his2 TFs were generally believed to bind to DNA as monomers, although in **Paper I** we present a novel concept that transcription factors of this type operate in spherical multimeric clusters. Gene localisation in the nucleus can be determined by regulatory signals. For instance, it has been suggested that genes together with associated transcriptional activators move close to the nuclear pore. Repressors normally recruit co-repressor complexes, which, by altering chromatin configuration, inhibit binding of an activator and prevent chromatin remodelling and transcription activation. Some transcription factors may act as repressors and activators depending on a context DNA sequence (Hahn and Young 2011). Generally, the binding of TFs to the target sequence does not depend on whether it is 5' or 3'-oriented, however, most of the yeast activators do not function 3' of the promoter.

RNA polymerase II assembles with general TFs including TATA-binding protein (TBP), TFIIB, TFIID, TFIIE, TFIIF and TFIIH into a preinitiation complex at the site called core promoter in front of the transcription start site. This complex together with coactivators, such as TFIIA, histone acetyltransferase and ATP-dependent chromatin remodelling system, then binds to and opens the promoter DNA, thus, RNA synthesis and RNA polymerase escape are initiated (Maldonado et al. 1999; Sainsbury et al. 2015). In yeast *S. cerevisiae* RNA Pol II initiates transcription at preferred sites but always downstream the TATA sequence, usually 50-120bp in yeast (Hampsey 1998). The open complex formation involves severe conformational changes occurring upon promoter insertion into the jaw and downstream cleft of polymerase II. DNA strands are then separated and a single-

strand DNA template is incorporated into the polymerase II active site. However, the exact mechanism of open complex formation in yeast is still unclear.

There are several proposed mechanisms of transcription activation. These include: recruitment of general TFs and coactivators to the promoters; conformational changes induced by an activator; chromatin modification and remodelling by ATP-dependent remodellers such as SWI/SNF; and enhancing steps following preinitiation complex formation, although it does not seem to be common in yeast (Hahn and Young 2011).

8 Population vs single-cell and molecule studies

Through decades scientific research was mainly carried out on cell cultures accepting a population as a uniform organism with similar behaviour of all its cells. Frequently the behaviour of the population cannot be described by normal distribution but consists of several subpopulations. With the population approach, the result represents the average behaviour of all units in a sample, which might cause masking outliers (Sott et al. 2008). In fact, bulk analysis will cover drug-resistant bacteria by the general population. In case subpopulations are identified, the only way to determine which cell contributes to which group, hence, to separate competing signals is to analyse the whole population cell by cell (Perkel 2015).

Single-cell approaches are implemented in different fields of studies. Transcriptome *in vivo* analysis (TIVA) is based on capturing mRNA of interest by biotin-tagged photoactivatable oligonucleotides. The technique allows quantification of gene expression within the whole cell or separated cellular compartments (Lovatt et al. 2014). However, an isolated cell loses its natural environment with active connections and communication between the cells. This issue is of particular importance in studies on neurons as their gene expression is controlled by signals received from their neighbours (Lovatt et al. 2014). Combination of fluorescent microscopy with mass spectral imaging enables the analysis of about 10,000 separated cells simultaneously (Lanni et al. 2012). Another approach, Mass-cytometry, is a flow- based method that uses heavy metal-tagged antibodies to avoid the channels overlap of standard flow cytometry and mass spectrometry (Leipold and Maecker 2012).

It has been shown that budding yeast *S. cerevisiae* can form biofilms (Reynolds and Fink 2001) as well as grow as filaments (Gimeno et al. 1992). Therefore, various techniques can be applied for yeast studies depending on the research interest. For example, single-cell invasive growth assay allows investigation of yeast filamentous growth (Cullen and Sprague 2000; Cullen 2015).

Population heterogeneity arises due to environmental alterations and /or genetic mutations that affect gene expression and can provoke fluctuations in various cellular components (Elowitz et al. 2002). Differences in transcriptional regulation affect signal transduction pathways and hence responses to various stress factors,

such as pH and oxidative stress. Therefore, an ideal single-cell experiment should be performed under precise environmental control. Moreover, the age of a cell and its phase in the cell cycle may influence the cell response (Sott et al. 2008).

Even a unicellular organism represents a heterogeneous system on a molecular level. Depending on a biological function, single molecules can have multiple states. Analysis of mean conformation will broaden molecular parameters, which would lead to misinterpretation of the physiological role (Leake 2013a). By performing single-molecule experiments it is possible to investigate molecular subpopulations. Moreover, it allows researchers to study not only cellular response but also precise underlying mechanisms (Leake 2013a). Single-molecule biophysics enables to observe and study many biological processes that were previously not considered due to technological limitations, e.g. bacterial flagella rotation, protein folding, movement (Deniz et al. 2008; Leake 2013a).

One of the main fundamental characteristics of all single-cell and single-molecule approaches is the ability to detect signal over noise. Isolation and harvesting of cells is critically important in single-cell and single-molecule studies. There are various strategies for capturing individual cells. One of the most popular techniques is flow cytometry-based fluorescence-activated cell sorting (FACS) which was developed in the 1970s. This method allows separation of cells from a population and collects them into different containers (Julius et al. 1972). Laser capture microdissection (LCM) allows single cell isolation directly from heterogeneous tissue upon microscopic visualisation (Emmert-Buck et al. 1996). However, there is a high probability of cell damage or contamination with neighbouring cells (Hodne and Weltzien 2015). With the development of microfluidics systems the long-term dynamic studies in controlled environment on the same isolated cells became possible (Eriksson et al. 2010). In **Paper I** we used microfluidic chambers that were described previously (Gustavsson et al. 2012) to follow the dynamic behaviour of a protein of interest (Mig1) under different glucose concentrations. For Slimfield microscopy experiments in **Papers I** and **II** we immobilised pre-grown cell cultures on 1% agarose pads well perfused with medium enclosed between a slide and a plasma-cleaned cover slip. This allowed cells to continue growing and dividing during the experiment.

9 Single-molecule biophysics

Single-molecule biophysics is a new scientific approach that uses physics to understand life. Focusing on molecules as the minimal units representing a biological system, single-molecule biophysics makes a big impact on various fields of research, such as medical immunology, synthetic and systems biology and others, by enhancing both spatial and temporal resolution of data (Leake 2013a). Modern techniques allow for probing the dynamics of cellular signal transduction directly which enables deeper and more precise understanding of important biological processes, e.g. immune response, gene expression and cellular differentiation. One of the main techniques used in single-molecule biophysics is fluorescence microscopy (Leake 2013a).

9.1 Fluorescent proteins

Fluorescence is a process occurring in atoms and/or molecules due to absorption of light by atoms or molecules and subsequent emission of light of a longer wavelength. The absorption of an incoming photon leads to a transition of a fluorophore, a compound that can undergo the process of electron shift and is attached to a larger molecule, to a higher excitation state. After a brief interval the molecule relaxes, i.e. returns to the ground energy state, emitting a photon of a longer wavelength than was eventually absorbed (Lichtman and Conchello 2005). The British scientist Sir George G. Stokes was the first to describe the event of fluorescence as well as the shift to longer wavelengths, also called Stokes shift (Stokes 1852). Various energy states and electrons transitions between them are represented on the Jablonski diagram (**Figure 6**) (Jabłoński 1933).

In 1960 the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was identified (Shimomura et al. 1962). Although GFP has become extensively used only 30 years later (Chalfie et al. 1994), this discovery, so called “Green revolution” (Stearns 1995), pushed experimental tools in biosciences to a completely new level. GFP is a 238 amino acid β-barrel protein consisting of 11 β-sheets and an α-helix with the chromophore. The wild type GFP chromophore is encoded by the Ser65-Tyr66-Gly67 sequence and forms spontaneously by intramolecular rearrangement and subsequent oxidation (Heim et al. 1994). Numerous mutations of wild type GFP have been generated in order to improve its biophysical characteristics. Ones of the major improvements were achieved by the

S65T mutation resulting in increased photo-stability and fluorescence as well as excitation peak shift to 488 nm (Heim et al. 1995). A206K was reported to inhibit self-oligomerisation (Zacharias et al. 2002). Colour mutations, e.g. Y66H, Y66W, T203Y for blue, cyan, yellow, respectively, exist (Lippincott-Schwartz and Patterson 2003). Fluorescent proteins are often fused with a protein of interest to monitor protein expression, movement, protein-protein interactions and degradation (Chalfie et al. 1994; Lippincott-Schwartz and Patterson 2003). Several newly engineered fluorescent proteins (e.g. mEos2) can be photo-activated and photo-converted from green to red-emitting state (McKinney et al. 2009). Long-term experiments on protein dynamics with continuous illumination are limited by the life time of standard fluorescent proteins as they can be tracked no longer than the photobleaching point while the photo-conversion approach increases experimental time (Baker et al. 2010). Thus, various combinations of different mutations of fluorescent proteins serve a wide range of application in both *in vitro* and *in vivo* types of experiments as well as simultaneous use of several proteins within the same specimen.

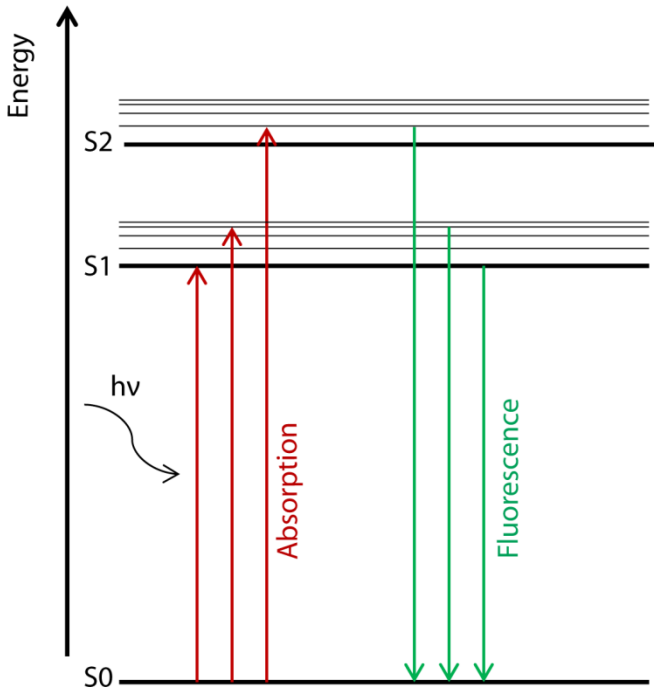

Figure 6. Simplified Jablonski diagram. An electron of a fluorophore at the ground state (S0) receives energy (hv) which results in electron transition to an excited energy state (Absorption). When the excited electron relaxes to the ground state the loss of energy is emitted as photon which causes fluorescence (Fluorescence).

Fluorescence microscopy is an indispensable tool in single-molecule investigations. It provides a high signal to noise ratio with rather small perturbation of the native system. Genomically integrated fusions of fluorescent dyes with the proteins under native promoters of the target proteins serves the most precise labelling. Organic dyes are also commonly used in single-molecule imaging. They are brighter and more photo-stable compared to fluorescent dyes, but they are not genetically encodable which makes their use nonspecific to the targets (Chiu and Leake 2011).

9.2 Super-resolution and single-molecule imaging

Resolution is the minimal distance at which two points can be visible under the microscope as separated units. In 1873 Ernst Abbe described the diffraction limit (Abbe limit) that defines margins of the conventional microscopy resolution (Abbe 1873). Development of super-resolution techniques was invaluable for single-molecule imaging as it allowed the scientists to break the resolution limit down to 1-50nm thus to study localisation and behaviour of the proteins at the level of a single molecule (Chiu and Leake 2011). In 2014 Eric Betzig, Stefan W. Hell and William E. Moerner were awarded the Nobel prize in chemistry “for the development of super-resolved fluorescence microscopy” (Choquet 2014). The super-resolution methods can be generally divided into near- and far-field microscopy approaches. Some of these techniques are described below.

Total internal reflection fluorescence (TIRF) is the most frequently used near-field approach. The method is based on total internal reflection of the excitation light from the glass-water interface to 100-200 nm beyond the cover slip (Chiu and Leake 2011). As there is no signal detected from regions out of focus, TIRF serves high signal to noise ratio. Although the lateral diffraction limit is still 200-300nm, TIRF enhances the axial resolution (Schermelleh et al. 2010). This approach is widely used in surface-related area of cell biology (adhesion, endocytosis in the plasma membrane, cytoskeleton studies) (Schermelleh et al. 2010).

Near-field scanning optical microscopy (NSOM, SNOM) was developed by Eric Betzig. It is another near-field technique where the objective is placed very close to the specimen (a distance less than the optical resolution limit). Typically the method involves scanning of a fluorescently labelled sample with a thin optical fibre. However the scanning is slow and the resolution is limited by the size of the detector aperture (Schermelleh et al. 2010; Chiu and Leake 2011; Leake 2013c).

Confocal microscopy systems enable imaging further into the sample. This is achieved by reducing the illuminated part of the sample and collecting only in-focus light. However, in single-molecule studies this technique is often used to excite fluorescently tagged molecules which are then detected by other imaging approaches (Leake 2013b; Wollman et al. 2015).

Photoactivated localisation microscopy (PALM) and *stochastic optical reconstruction microscopy* (STORM) are far-field imaging approaches that detect fluorescence of photo-activatable or photo-switchable fluorophores, respectively (Chiu and Leake 2011; Leake 2013c). One or a very small number of fluorophores is excited at a given time, thus, their diffraction-limited areas do not overlap. Excitation cycles can be repeated until all locations of target molecules are detected, which then can be assembled into the final image (Schermelleh et al. 2010; Chiu and Leake 2011).

In 1994 Stefan Hell proposed a technique to break down the diffraction limit. *Stimulated emission depletion* (STED) microscopy is a far-field method based on minimising the area of illumination at the focal point by controlled selective de-excitation of the fluorophores. The focal plane is scanned by two laser beams. The first one excites the fluorophores. The second beam of a longer wavelength is specifically altered that at the focal plane it has a donut shape. Therefore, only a small area from the centre of the "donut" is left to be able to emit the light. Thus the STED technique allows taking images below the diffraction limit (Schermelleh et al. 2010; Wollman et al. 2015).

Further development of super resolution microscopy made it possible to obtain three-dimensional information. Some of these 3D methods, including double-helix microscopy developed by the group of William Moerner (Pavani et al. 2009), and astigmatism can be implemented as an addition to many currently developed fluorescence microscopes as the required equipment is simply placed between the objective lens and the camera (Wollman et al. 2015).

In **Papers I** and **II** in order to observe single fluorescently tagged molecules we employed Slimfield fluorescence microscopy which uses a non-Gaussian collimated laser beam combined with a highly sensitive CCD camera enabling millisecond temporal resolution.

10 Summary of the papers

The aim of **Paper I** was to uncover the mechanisms by which Zn finger transcription factors (Mig1 in particular) bind to DNA. Our data show that irrespective of glucose availability Mig1 is present in both the nucleus and cytoplasm as a monomer and various multimers. However, the nuclear fraction of Mig1 is characterised by a higher stoichiometry and a lower diffusion coefficient compared to the cytoplasmic fraction. To test whether clusters were unique for Mig1 or a more general feature of eukaryotic zinc finger transcription factors we analysed the behaviour of the transcriptional activator Msn2. Direct observation of the localisation pattern revealed that Msn2 also operates as a multimer. We propose that those multimers are stabilised by weak depletion forces representing a micelle organised by interactions of intrinsically disordered domains as an inner part and zinc fingers as an outer. We suggest that Mig1 clusters serve faster and more efficient target sequence recognition as well as repression of several promoters at a time.

The aim of **Paper II** was to understand the mechanism of Mig1 de/phosphorylation and the role of Glc7-Reg1 in this process. Our data suggest two unrelated events of Mig1 dephosphorylation. One of them, glucose-independent, seems to be triggered by inhibition of the kinase activity of Snf1. Mig1 dephosphorylation by the Glc7-Reg1 phosphatase appear to be regulated by glucose. We also studied the influence of commonly used phosphatase inhibitors on Mig1 behaviour under different glucose conditions. We found that despite the fact that Mig1 phosphorylation sites were identified only on serine and threonine residues, tyrosine-specific phosphorylation is crucial for Mig1 regulation. The agent that contains this Tyr phosphorylation site remains to be determined. Overall, Mig1 de/phosphorylation appears to be a complex process controlled in glucose-dependent and independent manner, in line with the importance for rapid and sensitive regulation upon altered glucose concentrations in the medium.

In **Paper III** we wanted to reconstruct the Snf1 network based on experimental evidence collected from available research articles. A comprehensive literature review allowed us to collect and assemble pieces of experimental evidence of biochemical reactions taking place in Snf1 signal transduction pathways regulation. We used of the *rxncon* software to create and validate a model as well as to identify missing links.

In **Paper IV** we study regulation of *MIG1* transcription under different glucose conditions. We show that the expression of Mig1 is itself glucose-regulated in a Snf1-dependent manner. Mathematical modelling showed strong Mig1 levels dependency on Snf1presence. We also show that the amount of Snf1 is strongly dependent on glucose, whereas Mig1 production is stable within the cell regardless of glucose availability when Snf1 is not present.

11 Future perspectives

Although this work provides novel insight into Mig1 regulation and action, there are still several questions that remain unresolved.

Employing super-resolution millisecond microscopy we found that Mig1 is present as monomer and oligomers in both compartments regardless of glucose availability. We propose that the purpose of those multimers in the nucleus is to mediate faster and more efficient gene repression. We show that Mig1 oligomers also exist in the cytoplasm. However, the functional aspects of these clusters are less clear. The role of Mig1 phosphorylation in oligomer formation is still unknown. Therefore, our next steps include creation of mutant strains of single and multiple Mig1 phosphorylation sites and the subsequent investigation of complex formation. Although we propose a model where the clusters are stabilised by depletion forces arising between intrinsically disordered Mig1 regions, we need to further investigate the structure of those multimers. For instance, live cell imaging of GFP-tagged truncated Mig1 variants containing only zinc finger domains as well as *in vitro* assays on purified mutant proteins will provide solid evidence for the architecture of those oligomers. The crystal structure of Mig1 has not been resolved yet either. This knowledge would certainly help uncover mechanistic aspects of Mig1 interaction with its partners. Although we suggest a model of Mig1 cluster architecture, knowing the exact structure of the Mig1 protein aid understanding the mechanism of cluster association and dissociation supported by experimental evidence. The exact machinery and dynamics of Mig1 clusters operating in the nucleus are also not completely understood. To solve this issue one can study Mig1 binding to the target sequences on horizontally extended DNA by using novel biophysical imaging systems (Cross et al. 2016). These data would provide an enormous impact in the field for understanding mechanisms of transcription factors-DNA interactions.

The dynamic properties of the Mig1 transcriptional repressor and other key components of the Snf1/Mig1 pathway under various environmental conditions have not been studied yet. The main interest would be to answer several fundamental questions, such as when and where Mig1 associates with other proteins under various glucose concentrations and how fast those interactions occur once environmental stress was applied. We will follow protein-protein interactions of key components in real time to elucidate the role and interdependencies of

different steps/proteins in the pathway. By combining super resolution microscopy techniques with microfluidic devices, we will observe and characterise dynamic properties of the molecular mechanisms alterations in response to different environmental stresses. Thus, we could also understand how altered environmental conditions affect DNA recognition by Mig1 as well as Mig1 nuclear import/export.

In this thesis, I provide evidence that Mig1 dephosphorylation is a complex process which includes at least two mechanisms: a glucose-dependent mechanism mediated through the Glc7-Reg1 phosphatase and a glucose- and Glc7-Reg1 independent mechanism. We also state that there is a tyrosine phosphorylation-dependent event which plays an essential role in Mig1 regulation. Mig1 has several serine/threonine phosphorylation sites but no tyrosine phosphorylation site has been identified to date. Therefore, some additional work is needed to determine the protein that istyrosine-phosphorylated in the glucose repression pathway. Reg1 dephosphorylation was proposed to be promoted by the serine/threonine phosphatase Glc7 (Sanz et al. 2000). The presence of tyrosine 480 as phosphorylation site on Reg1 makes this protein a possible candidate carrying the regulatory tyrosine phosphorylation. Analysis of the Mig1 phosphorylation status in *REG1* mutants in combination with phosphatase inhibitors will confirm whether Tyr480 of Reg1 is essential for Mig1 control. It is still unclear if other phosphatases play roles in Mig1 activation. For instance, it has been suggested that the transcriptional repressor activity and stability of Mig1 is also controlled by Sit4 (Jin et al. 2007). We are currently studying roles of serine/threonine phosphatases other than Glc7-Reg1 in Mig1 activation and localisation. We have already tested roles of various serine/threonine phosphatases in expression regulation of Mig1 target genes, such as *SUC2* (unpublished data). Although in all cases the pattern was similar to the wild type strains, we will further study the Mig1 phosphorylation status in single and double/multiple phosphatase deletion mutants. Another approach is to analyse the behaviour of Mig1 upon overexpression of various phosphatases. We are also planning to test whether the ability of Mig1 to bind to its target DNA sequence is dependent on various phosphatases by employing the gel shift assay. The results will provide more detailed information about mechanisms controlling dynamics and action of Mig1.

Our results suggest that following a rapid decrease of glucose levels in the medium different phosphorylated forms of Mig1 appear over time (unpublished data). Further work is needed to characterise the dynamics of Mig1 phosphorylation.

Collection and purification of Mig1 at different intervals after the switch to glucose-limited conditions will allow to study different forms of phosphorylated Mig1 by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS). Several Mig1 phosphorylation sites were shown to be important for Mig1 localisation and function. Ser222 was proposed to be involved in cytoplasmic import of Mig1 upon glucose depletion (DeVit and Johnston 1999), Ser311 was shown to be required for *SUC2* derepression under glucose depletion and also for the interaction between Mig1 and Hxk2, which seems to be important for glucose repression (Ahuatzi et al. 2007). We suggest that some the apparent Mig1 phosphorylation sites responsible for the complex band shift pattern of Mig1 in protein gelsare not critical for its localisation. However, further work is needed to identify those sites.

Together, those data would provide a more complete picture of the architecture of the yeast Snf1-Mig1 module as well as deeper understanding of signal transduction mechanisms and gene expression regulation.

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