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Abstract

Snf1p is a protein in Baker's yeast, *Saccharomyces cerevisiae,* which is homologous to the mammalian AMP-activated protein kinase (AMPK). The AMPK protein is important for the energy metabolism of mammals, since it is central in a control circuit for the carbohydrate and fat metabolism of liver, fat, and muscle cells. It therefore has medical implications in terms of the metabolic syndrome and type 2 diabetes. In yeast, the enzyme is required for the process of glucose de-repression, i.e., the process of switching from (respiro)fermentative to fully respiratory metabolism in response to low glucose concentrations. The goal of this thesis was to create a mathematical model of the glucose de-repression process in yeast, including signalling, transcription and translation, and effects on the metabolic pathways.

Based on a comprehensive review of the field and of the different partial models available in the open literature, a conceptual, integrated model of the complete glucose de-repression process is presented, on the levels of signaling, transcriptional, post-transcriptional, and post-translational regulation, and the dependent metabolic processes. A web application was designed in order to summarize the collated information.

A kinetic model was implemented, summarizing those aspects where knowledge was deemed good enough, and making the best possible assumptions in other aspects. The model describes the time-courses of the unphosphorylated and phosphorylated Snf1 protein complex, the unphosphorylated and phosphorylated Mig1 protein complex, free Mig1, active and inactive Pak1, active and inactive Elm1, bound and released Reg1/Glc7 protein phosphatase, bound and released Msn2 protein, Mig1 mRNA and Suc2 mRNA.

By adjusting the kinetic parameters, it was possible to modulate the modelled time responses of the Snf1 and Msn2 protein species, as well as the responses of the Mig1 and Suc2 mRNA transcripts, so that a reasonable agreement with known experimental data could be obtained. For the proteins, the steady-state was reached in less than five minutes, and in the case of the transcripts, in less than fifty minutes.

There is, presently, not enough experimental data available to allow a validation of the estimated time responses of many of the entities, such as the free Mig1, the Pak1 species, the Elm1 species and phosphatase complexes. In this model, the upstream kinases and the phosphatase just played an intermediate role between Snf1 and the low glucose signal, whereas the effect of free Mig1 could be neglected.

The model can be used as a starting point for creating a more rigorous model, and as an aid in the design of future experiments, e.g. for the purpose of parameter estimation and clarifying biological research. Simulations carried out here, in order to reproduce the expected behavior, show that the present model can be used also for didactical purposes.

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1. Kinetic Modelling Concepts

"An idea which can be used once is a trick. If it can be used more than once, it becomes a method". G.Polya and S.Szego (quoted by Wolkenhauer, homepage, 2004).

1.1. Generalities

The metabolism of the cell is well known from a qualitative point of view (components and interactions). We also have good quantitative descriptions of the kinetics of many individual reactions. However, the quantitative in vivo description of metabolic pathways is not clear at all. As Hynne et. al. say in their study about glycolysis, "Glycolysis is at the heart of classical biochemistry and, as such, it has been thoroughly studied. When viewed as a collection of individual steps, it is very well described. When viewed as a whole, our understanding leaves much to be desired. A model assembled from mechanistic data alone will not reproduce any significant part of the experimental findings straight away; describing a complete pathway (including its dynamics) quantitatively requires a higher level of understanding in which delicate balances of different processes may become important"[1].

The construction of signalling pathways is currently a very active area of research in molecular biology, mainly due to their relation to diseases such as cancer or diabetes. The construction of transcriptional networks is also an increasingly important area. However, different to metabolism, in these two cases the researchers are still working on the qualitative description of the pathways, discovering their components and interactions.

This way, kinetic metabolic models are the most common of the three cellular pathways; however, the rigorous models are very recent. In the case of the carbon metabolism of yeast, we could highlight the models of glycolysis of Rizzi et al.[2], Teusink et al.[3] and Hynne et al.[4], among others (models for the TCA cycle or respiratory chain are not that popular). In the case of signalling pathways, the first kinetic models of signalling and transcription appeared in the late nineties; several kinetic models have been presented in the last years, but more work wait to be done:

[1] HYNNE, F., DANØ, S., SØRENSEN, P., Full-scale model of glycolysis in S. cerevisiae, *Biophysical Chemistry*, **94**, 2001
[2] RIZZI, M., BALTES, M., THEOBALD, U., REUSS, M., In vivo analysis of metabolic dynamics in S. cerevisiae: II. Mathematical Model, *Biotechnology and Bioengineering*, **55**, 4, 1997
[3] TEUSINK, B., PASSARGE, J., REIJENKA, C., et al., Can yeast glycolysis be understood in terms of in vitro kinetics of the constituent enzymes? Testing Biochemistry, *Eur. J. Biochem.*, **267**, 2000
[4] HYNNE, F., et al., Op.Cit.

Kholodenko et al. on epidermal growth factor[5], Sedaghat, et al. on insulin[6], are examples of this kind of modelling. In the case of transcriptional pathways, other approaches have been more popular (e.g., Boolean behaviour). Rigorous signalling or transcription models on glucose de-repression have not been reported. The research about joint models of signalling, transcription and metabolism has also started and the method known as "Hierarchical Control Analysis" seems to be the most promissory tool; however, rigorous kinetic models using this approach to integrate signalling, transcription and metabolism wait to be designed.

In this chapter, we will present some basic concepts on kinetic modelling. Such concepts have been extensively used in metabolic modelling and not that much in signalling or transcription. However, following some of the authors who we will introduce, these concepts will be adopted as a theoretical framework in order to model our process.

1.2. Basic concepts

1.2.1. Enzymatic reactions

An enzyme is a protein which stimulates the rate of a biochemical reaction. The reactants whose reaction is catalyzed by the enzyme are known as substrates. In this thesis, we will work with protein-metabolites, protein-protein and protein-DNA reactions, so it is important to point out that, in our kinetic model, our substrates are also proteins.

1.2.2. Kinetic models

A kinetic model is a set of equations with the form:

$$\frac{d[M]}{dt} = \sum_i \upsilon_{formation} - \sum_j \upsilon_{consumption}$$

where d[M]/dt represents the change in the concentration of M on time, and each ν_i or ν_j (reaction rate of the reaction "i" or "j") is a function of constant values named "parameters" and variable values (metabolite concentrations). In fact, the parameters are also subject to fluctuations, but need to be assumed as constants.

1.2.3. Types of kinetics models

According to Hofmeyr et al., there are three types of kinetic models: "The lowest rung on this part of the hierarchy is where reactions are described in terms of elementary reactions where two molecules associate to form a complex (e.g. protein-ligand association), a complex dissociates into two molecules, or a molecule or a complex isomerises. The rates of these reactions are described in terms of first or second-order rate constants in combination with reactant concentrations. This can be called the mass-action level where molecular events happen in the micro to millisecond time-scale. Biochemists usually aggregate the elementary steps associated with an enzyme catalytic process into a single reaction, the rate of which

[5] KHOLODENKO, B., DEMIN, O., MOEHREN, G., HOEK, J., Quantification of short term signalling by the epidermal growth factor receptor, *The Journal of Biological Chemistry*, **274**, 42, 1999

[6] SEDAGHAT, A., SHERMAN, A., QUON, M., A mathematical model of metabolic insulin signaling pathways, *Am.J.Physiol.Endocrinol.Metab.*, **283**, 2002

is described by a rate-law, the most familiar being the Michaelis-Menten rate equation. At this level of the time scale events happen within seconds to minutes – this we regard as the intermediary metabolic time scale. The processes of transcription, translation, protein synthesis and degradation are slower (minutes to hours) and can be thought of as the genetic time-scale"[7].

The first approach (mass-action form) demands the knowledge of the detailed mechanism with all kinetic constants, which is commonly difficult to obtain. The second approach (rate-law form) uses rate-laws that aggregate elementary rate equations, coming from steady-state studies. Finally, in the last form, all production and consumption reactions for a given metabolite are aggregated in a power-law equation. The second approach will be followed from now on.

1.2.4. Moieties

The chemical groups that remain constant between different forms of some chemical species are called moieties. For example, adenylate is conserved between ATP, ADP and AMP, whereas pyridine is conserved between NAD^+ and NADH. In both cases, the formation and degradation of the moiety is slow compared to the interconversion between the different forms.

1.2.5. Steady-state

In steady-state, the concentration of metabolites does not change with time (d[M]/dt = 0); however, the individual reaction rates υ_i are different to zero: they are called the "fluxes" J_i.

Two common assumptions in kinetic studies are: First, the steady-state is "asymptotically stable", and second, the distribution of metabolites in the cell is uniform (no concentration gradients in space).

1.3. Kinetic equations

Many rate laws have been derived, each of them according to specific assumptions. The mass-action, Michaelis-Menten and Hill kinetics are the most frequently used. Here, we will review these equations together with others that were considered in this work.

1.3.1. Constant flux

"The rate of reaction is not sensitive to any chemical species and therefore is constant. This is also known as a zero-order reaction or a pump"[8].

$\upsilon = V$

where the constant V is given in μmol/s.

1.3.2. Mass-action kinetics

In this case, the rate of reaction depends only on the substrate(s) S_i.

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[7] HOFMEYR, J., SNOEP, J., WESTERHOFF, H., Kinetics, Control and Regulation of Metabolic Systems, *Tutorial*, 2002

[8] MENDES, P., Manual of Gepasi 3.3, http://www.gepasi.org, 2002

$$\upsilon = k \cdot \prod_i S_i^{a_i}$$

where the constant k is given in amount/time, depending on the number of substrates (e.g., in a first-order reaction, l/s; in a second-order reaction, l^2/mol.s; etc…) and a_i is the kinetic order.

1.3.3. Henri-Michaelis-Menten kinetics

In this case, one substrate (S) is converted to one product (P) ("isomerisation" reaction), catalysed by one enzyme (E). The enzyme and the substrate associates in a reversible way, and then the complex irreversibly releases the enzyme and the product (S + E <-> SE -> E + P).

$$\upsilon = \frac{V_{max} \cdot S}{K_m + S}$$

where the parameters V_{max} and K_m are given in mol/s, and $V_{max} = k_{cat} . E$

1.3.4. Hill kinetics

Here, one only substrate binds cooperatively to the enzyme, producing a sigmoidal response. "If the Hill coefficient is greater than 1, the rate shows positive cooperativity (one molecule of S bound facilitates another molecule of S to bind to the enzyme) – the shape of the curve is a sigmoid; if the Hill coefficient is smaller than 1 then the rate shows negative cooperativity (one molecule of S bound makes the binding of another molecule of S to the enzyme harder) – the shape of the curve is a false (but similar to) hyperbola"[9].

$$\upsilon = \frac{V_{max} \cdot S^h}{(K_{0.5})^h + S^h}$$

where the parameter V_{max} is given in mol/s and h is the Hill coefficient.

1.3.5. Uncompetitive inhibition kinetics

This reaction presents one substrate, one product and one inhibitor (I), and the affinity of the ES complex for the inhibitor is higher than the affinity of the free E for the inhibitor (formation of ESI instead of EI). This equation approaches to Michaelis-Menten when [I] approaches zero.

$$\upsilon = \frac{V_{max} \cdot \frac{S}{K_m}}{1 + \frac{S}{K_m} \cdot \left(1 + \frac{I}{K_i}\right)}$$

where the parameter V_{max} is given in mol/s.

1.3.6. Catalytic activation kinetics

This reaction presents one substrate, one product and one activator (A), and the ES complex needs to bind the activator in order the reaction to take place (essential activation). This equation approaches to Michaelis-Menten when K_a approaches zero or A approaches infinity.

[9] MENDES, P., Op.Cit.

$$\upsilon = \frac{V_{max} \cdot \frac{S}{K_m}}{1 + \frac{K_a}{A} + \frac{S}{K_m} \cdot \left(1 + \frac{K_a}{A}\right)}$$

where the parameter V_{max} is given in mol/s.

1.3.7. Iso Uni Uni kinetics

This reaction presents one substrate and one product, and the free enzyme released from P is different to the free enzyme that binds S. This equation has been used for permeases, where the substrate is the same compound as the product (K_{eq} = 1) and the change of the enzyme corresponds to the reorientation of the protein in the membrane.

$$\upsilon = \frac{V^f \cdot \left(S - \frac{P}{K_{eq}}\right)}{S \cdot \left(1 + \frac{P}{K_{ii}}\right) + K_{mS} \cdot \left(1 + \frac{P}{K_{mP}}\right)}$$

where the parameter V^f is given in mol/s, K_{eq} is the equilibrium constant, K_{mS} is the Michaelis constant at zero P, K_{mP} is the Michaelis constant at zero S and K_{ii} is the isoinhibition constant.

1.4. Metabolic Control Analysis (MCA)

Control of flux is often distributed among all enzymes in a pathway. MCA is a kind of sensitivity analysis where a perturbation is applied to a parameter in the metabolic step under study in order to see the local effect on the activity of this step and/or the global effect on a steady-state variable (fluxes or concentrations) of the system. The two main concepts of MCA are the elasticity coefficients and the control coefficients.

1.4.1. Elasticity coefficients

Elasticity coefficients are "local" properties, which consider the effect of the perturbation of one parameter of an isolated reaction (frequently, concentration of substrate, product or modifiers) on the correspondent reaction rate, assuming that all other parameters of the pathway remain constant. "The elasticity coefficients are defined as the ratio of relative change in local rate to the relative change in one parameter (normally the concentration of an effector). Infinitesimally, this is written as

$$\varepsilon_p^{\upsilon_i} = \frac{\partial \upsilon_i}{\partial p} \cdot \frac{p}{\upsilon_i}$$

where ν is the rate of the enzyme in question and *p* is the parameter of the perturbation"[10].

[10] MENDES, P., Op. Cit.

1.4.2. Control coefficients

Different to elasticity coefficients, the control coefficients are "global" properties, relating changes in local rates to changes in any variable of the pathway. A control coefficient can be understood as the relative change of a steady-state variable of the system (e.g. fluxes or concentrations) in response to the relative change of the activity of the perturbed step. It is defined as

$$C^{y}_{\upsilon_i} = \frac{\partial y / y}{\partial \upsilon_i / \upsilon_i}$$

where y is the global variable and ν_ι the steady-state rate of the perturbed step. Frequently, "y" means flux or concentration.

According to the "summation theorem", for a given flux, the sum of the flux-control coefficients of all steps is equal to one; and, for a given metabolite concentration, the sum of the concentration-control coefficients of all steps is equal to zero

$$\sum_i C^{J}_{\upsilon_i} = 1$$
$$\sum_i C^{[M]}_{\upsilon_i} = 0$$

The consequence of these equations is that changes in some control coefficients originate changes on the rest, so that the relations are sustained. This way, Control is a global or systemic property.

1.4.3. Response coefficients

A response coefficient is a measurement of "the sensitivity of a steady-state variable to a perturbation of the local rate (activity) through a specific step caused by a change in a parameter that affects this step directly"[11]. This way, the response coefficient combines the effects measured by the elasticity and control coefficients. It is defined as

$$R^{y}_{x} = \frac{\partial y / y}{\partial x / x} = C^{y}_{\upsilon_i} \cdot \varepsilon^{\upsilon_i}_{x}$$

where "x" is a local parameter and "y" is a global variable.

1.4.4. Connectivity relations

Finally, in order to describe how the local changes of an enzyme propagate to the whole system, we also need to know the relations that are held between the local elasticity coefficients and the global control coefficients. These are called connectivity relations or connectivity properties

$$\sum_i C^{J_m}_{\upsilon_i} \cdot \varepsilon^{\upsilon_i}_{s_j} = 0$$

[11] HOFMEYR, J., et al., Op. Cit.

where s_j is the concentration of a variable metabolite pool and J_m is a specific system flux.

$$\sum_i C^{s_k}_{\upsilon_i} \cdot \varepsilon^{\upsilon_i}_{s_j} = 0, \quad k \neq j$$

$$\sum_i C^{s_j}_{\upsilon_i} \cdot \varepsilon^{\upsilon_i}_{s_j} = -1$$

2. System to Model

> "*And so, bringing her gift of assiduity to bear, she began to train herself to love him. To do this she divided him, mentally, into every single one of his component parts, physical as well as behavioural, compartmentalizing him into lips and verbal tics and prejudices and the likes... she resolved to fall in love with her husband bit by bit*". Salman Rushdie, Midnight's Children (quoted by Huetteman in "Micro-Explanation", 2003).

2.1. Glucose Repression / De-Repression

The baker's yeast *Saccharomyces cerevisiae* is an unicellular eukaryotic organism, frequently used as a model organism for eukaryotic cells. For this reason, it is also one of the organisms with more available information. Its chemical complexity is summarized in Table 2.1, compared to three simpler organisms.

It can be observed that the percentage of ORFs involved in metabolism decreases when the number of metabolites and reactions increases (which suggests that simplest organisms have comparatively more genes directly involved in metabolism, meanwhile complex organisms have comparatively more genes involved in regulation of gene expression and enzyme activities).

Glucose is the main carbon source (or the preferred food) for yeast. It is known that a glucose input generates a big change in the levels of metabolites, transcripts and proteins, which "activates glycolysis, decreases respiratory activity, increases ribosome biogenesis and regulates growth and development"[12]. We may appreciate the yeast's global regulatory response to glucose in Table 2.2. Evidently, such a massive response needs to be simplified in biological pathways or processes able to be modelled (in our case, we will focus on a process known as "glucose de-repression").

Table 2.1. Overview of reactions, metabolites, and ORFs in reconstructed metabolic networks[13].

Organism	No. of reactions	No. of metabolites	No. of metabolic ORFs	Total No. of ORFs	% of ORFs involved in metabolism
H. Pylori	444	340	268	1638	16
H. Influenzae	477	343	362	1880	19
E. Coli	720	436	695	4485	15
S. cerevisiae	1175	584	708	5773	12

[12] YIN, Z., et al., Glucose triggers different global responses in yeast, depending on the strength of the signal, and transiently stabilizes ribosomal protein mRNAs, *Molecular Microbiology*, **48**, 3, 2003
[13] NIELSEN, J., It is all about metabolic fluxes, *Journal of Bacteriology*, **185**, 24, 2003

Table 2.2. Number of genes that showed two-fold regulation or more, in response to different glucose signals, compared to control cells (no glucose), after 30 min of exposure[14].

	All ORFs	Very Low Level (0.01% Glucose)		Low Level (0.1% Glucose)		High Level (1% Glucose)	
		Up	Down	Up	Down	Up	Down
Number of ORFs	6450	32	238	191	294	352	368

From a biological (macro) point of view, it is known that *S. cerevisiae* can live in either aerobic or anaerobic conditions, and it is known that it can have a fermentative, respirofermentative or respiratory metabolism. The preferred state for anaerobic conditions and glucose as a carbon source is fermentation. Aerobic conditions and low levels of glucose stimulate respirofermentation, but aerobic conditions and high levels of glucose also stimulate fermentation. The switch from a high to a low level of glucose under aerobic conditions, generates a switch from fermentative to respirofermentative metabolism, and viceversa, in a process known as "Crabtree effect".

The "Crabtree effect" also depends on the type of culture: Batch cultures with low glucose have a respiro-fermentative metabolism. Continuous cultures with low glucose to a high dilution rate, also have a respiro-fermentative metabolism. But continuous cultures with low glucose to a low dilution rate, show a fully respiratory metabolism. We will not work with the latter case. It is also possible to create a temporal fermentation in a non-fermenting culture adding an excess of glucose; this "short-term Crabtree effect" is explained as a saturation of respiration leading to overflow of Pyruvate into Ethanol[15]. We will not review this effect, but the "long-term Crabtree effect", which is a steady-state condition as a consequence of regulation. For the rest of this text, we will assume aerobic conditions.

From a biochemical (micro) point of view, nutrients have two roles: They are processed in metabolic pathways and, at the same time, they regulate signalling pathways that control transcription. In the first case, glucose follows the well-characterized "central carbon metabolism" (glycolysis and connected pathways); however, due to the high degree of connectivity of the central carbon metabolism (e.g., H (229 reactions), ATP (188 reactions), ADP (146 reactions), CO_2 (90), NAD (78), and NADH (65)), glucose provokes a global cell response in the "long term". The second case is a current area of research: In the switch from a high to a low level of glucose, a signal is transduced up to release a number of genes that were repressed in high glucose. For this reason, this process is known as "Glucose de-repression". Even if we could think in the repression and de-repression processes as the same reversible process, some lines of evidence suggest that the signalling mechanisms could be qualitatively different[16]. Most of the studies have been performed in the high-to-low glucose conditions (also referred as starvation or glucose depletion), this is, on the glucose de-repression process and, for this reason, we will follow the same approach.

[14] Adapted from: YIN, Z., et al., Op.Cit.
[15] WALKER, G., Yeast Physiology and Biotechnology, p. 216, John Wiley & Sons, 1998
[16] J. Norbeck and L. Gustafsson, personal communication, 2004

Table 2.3. Summary of characteristics of aerobic metabolism in yeast in response to low and high levels of glucose.

Glucose Repression / De-Repression	
HIGH GLUCOSE	**LOW GLUCOSE**
Snf1 protein kinase is inactive	Snf1 protein kinase is active
Mig1 protein is repressing genes (TCA cycle, glyoxylate cycle, gluconeogenesis, respiratory chain and use of alternative carbon sources)	Mig1 protein is phosphorylated; Genes are De-repressed (expressed)
High ATP by Glycolysis	High AMP
Mig1 is repressing the High Affinity Hexose Transporters Hxt2 and Hxt4	Mig1 is phosphorylated; Hexose Transporter Genes are De-repressed
Glycolytic flux coming to Pyruvate mainly goes to Ethanol production (TCA cycle, glyoxylate cycle, gluconeogenesis, respiratory chain and use of other carbon sources are not favoured)	TCA cycle, glyoxylate cycle, gluconeogenesis, respiratory chain and use of non-fermentative carbon sources (such as ethanol or glycerol) are favoured
Metabolism mainly fermentative	Metabolism mainly respiratory
Glucose Induction	
HIGH GLUCOSE	**LOW GLUCOSE**
Rgt2 glucose sensor detects high signal	Snf3 glucose sensor detects low signal
Rgt1 is a transcriptional activator. High Capacity Transporters Hxt1 and Hxt3 are induced (Low capacity transporters Hxt2 and Hxt4 are not repressed by this pathway, but they are repressed by Mig1)	Rgt1 is neutral. High Capacity Hexose Transporter Hxt1 is Std1-repressed[17]. Other Hexose Transporters (Hxt2, Hxt4, Hxt6, Hxt7) are expressed
Glycolysis is enhanced	Glycolysis is enhanced

Table 2.3. shows two different glucose-signalling pathways and its transcriptional and metabolic consequences (there is also a third pathway known as the Ras-cAMP pathway). Apparently, the Snf1 signalling pathway is enough to explain the glucose de-repression process, reason for which we will focus on it. However, in a recent work[18], Kaniak et al. suggest that all these pathways are part of a network.

In the following pages, we will discuss all these pathways as detailed as possible, trying to extract the main elements of the network that are involved in glucose de-repression. For a graphical representation, see Appendix 1-4.

2.2. Snf1 signalling pathway

The net effect of the Snf1 kinase pathway is to downregulate certain enzymes in order to conserve ATP energy and to relieve glucose repression and gene expression. This chapter is meant to give the description of the Snf1 signal transduction pathway and to point out the different uncertainties and questions arisen during its study.

2.2.1. Snf1-kinase complex

Snf1 kinase is a heterotrimer with a catalytic alpha subunit and two regulatory subunits denoted by beta and gamma. A common regulatory switch observed in protein kinases is the phosphorylation of one or more residues in the activation loop

[17] LAFUENTE, M., et al., Mth1 receives the signal given by the glucose sensors Snf3 and Rgt2 in Saccharomyces Cerevisiae, *Molecular Microbiology*, **35**, 1, 2000

[18] KANIAK, A., et al., Regulatory Network connecting two Glucose Signal Transduction Pathways in Saccharomyces Cerevisiae, *Eukaryotic Cell*, **3**, 1, 2004

of the catalytic subunit. According to Schmidt, et al., genetic and biochemical evidence suggest that the Snf1 kinase is regulated by phosphorylation of threonine 210.

Snf1 belongs to a highly conserved family of serine-threonine protein kinases found in fungi, plants, Drosophila, C. Elegans and mammals. The mammalian AMP-activated protein kinase (AMPK) has similar subunits as the Snf1 kinase. AMPK is involved in the cellular response to a variety of stresses like heat shock and nutrient starvation. Likewise Snf1 is responsible for triggering de-repression and is directly involved in the generation of ATP by enabling the cell to metabolize alternative carbon sources in the absence of fermentable amounts of glucose.

2.2.1.1 Gamma subunit (Snf4) and its role in regulating Snf1 activity

It is known that the alpha subunit of the Snf1 complex has the catalytic function, the beta subunit acts as an anchor, controls the localization and plays a role in substrate definition, and the gamma subunit, encoded by Snf4, plays an important regulatory role: Upon high glucose, Snf1 remains in an autoinhibited state but, under low glucose, Snf4 binds to the regulatory domain of Snf1, releasing the catalytic domain and, this way, relieving the autoinhibition (Figure 2.1.). McCartney and Schmidt[19] have shown that the role of Snf4 in regulating Snf1 is distinct from the modification of threonine: "Cells that are lacking the Snf4 protein are still able to phosphorylate Snf1 on threonine 210 in response to glucose limitation. Therefore the Snf4 protein is not required to recruit or to stimulate the activity of the Snf1-activating kinase"[20]. The same authors also conclude that Snf4 is not sufficient to activate the Snf1 kinase in the absence of phosphorylation on Thr-210.

As a final comment, Jiang and Carlson (1997) have shown that Snf4 binds to Snf1 both in high and low levels of glucose.

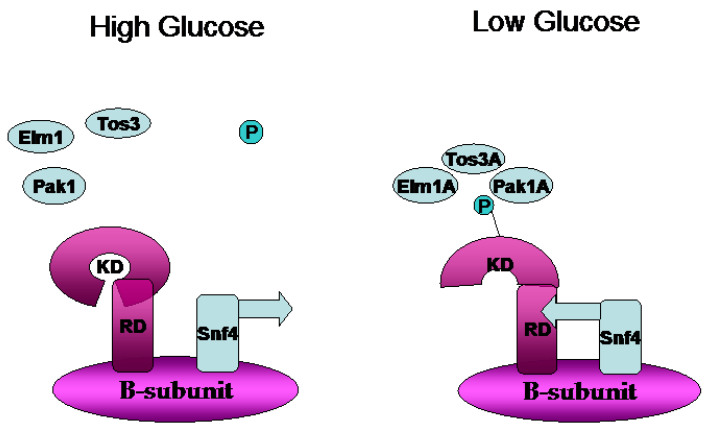

Figure 2.1. Role of Snf1 subunits and upstream kinases in high and low glucose.

[19] McCARTNEY, R., SCHMIDT, M., Regulation of Snf1 kinase: activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit, *JBC Papers in Press*, 2001
[20] McCARTNEY, R., et.al, Op.Cit.

2.2.1.2 Beta subunit (Sip1/Sip2/Gal83)

According to Hedbacker et al., under high glucose Gal83 is the most abundant of the three beta subunits. "Snf1-Gal83 kinase is, correspondingly, responsible for the majority of Snf1 kinase activity… In the *sip1*Δ*sip2*Δ mutant, which expresses only Snf1-Gal83, the level of activity was 75% of wild-type levels. Conversely, in *sip2*Δ*gal83*Δ and *sip1*Δ*gal83*Δ strains, which express only Snf1-Sip1 and Snf1-Sip2, respectively, activity was reduced to 10% of the wild type"[21].

The process of activation of Snf1 involves conformational changes of the kinase. These changes have been shown to be due to the beta subunit, which binds to the complex and functions as a scaffold by anchoring the Snf1 kinase and its regulating subunit Snf4. Jiang and Carlson showed that the beta subunit has this function but, at the same time, it is not essential for Snf1 function. In a *sip1*Δ*sip2*Δ*gal83*Δ triple mutant, half of the cellular Snf4 proteins still bind to Snf1 which may lead to the hypothesis that "other proteins may be functionally redundant with Sip1, Sip2, and Gal83"[22] or that "under conditions of glucose limitation, Snf1 and Snf4 can interact directly, without assistance from other proteins"[23]. The second hypothesis seems to be more accepted.

It has also been observed that under high glucose all three beta subunits are localized in the cytoplasm, while under low glucose they have different locations: Snf1-Gal83 goes to the nucleus, Snf1-Sip1 is found around the vacuole, and Snf1-Sip2 stays in the cytoplasm. The proteins Sip1/Sip2/Gal83 may have another function besides scaffolding as is suggested in the same article: "Previous genetic evidence suggested that these proteins have different functions in the Snf1 pathway and may serve as adaptors that mediate the interaction of the kinase with different substrate proteins or as targeting proteins that direct the kinase to different intracellular locations"[24]. Vincent et al.[25] reported that Gal83 directs Snf1 to the nucleus under glucose limitation.

2.2.2. Kinases upstream Snf1

In the last years, three main kinases have been reported as responsibles for Snf1 phosphorylation. These kinases are Pak1, Elm1 and Tos3 (see Figure 2.1.). According to Sutherland et al., they present partially redundant function: "…a *pak1*Δ*tos3*Δ*elm1*Δ triple mutant had an *snf1*- phenotype, i.e., it would not grow on raffinose and did not display hyperphosphorylation of the Snf1 target, Mig1p, in response to glucose starvation"[26].

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[21] HEDBACKER, K., HONG, S., CARLSON, M., Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase, *Molecular and Cellular Biology*, **24**, 18, 2004

[22] JIANG, R., CARLSON, M., The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex, *Molecular and Cellular Biology*, **17**, 1997

[23] JIANG, R., et al., Op.Cit.

[24] JIANG, R., et al., Op.Cit.

[25] VINCENT, O., TOWNLEY, R., KUCHIN, S., CARLSON, M., Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism, *Genes and Development*, **15**, 2001

[26] SUTHERLAND, C., HAWLEY, S., MCCARTNEY R., LEECH, A., STARK, M., SCHMIDT, M., HARDIE, G., Elm1p is one of three upstream kinases for the S. cerevisiae SNF1 complex, *Current Biology*, **13**, 2003

It has been suggested that each upstream kinase (Pak1, Elm1, Tos3) is specific for each Snf1 complex (Snf1-Gal83, Snf1-Sip1, Snf1-Sip2). However, Hedbacker et al. show that there is no such a correlation. In the case of Snf1-Gal83, "Pak1 is the most important kinase for activating Snf1-Gal83 in response to glucose limitation, but Elm1 also has a significant role… we assayed the *pak1*Δ*sip1*Δ*sip2*Δ mutant for in vitro kinase activity. In the triple mutant, activity was reduced 12-fold relative to that of the *sip1*Δ*sip2*Δ mutant, indicating that Pak1 has a major role in the activation of Snf1-Gal83… the *elm1*Δ*sip1*Δ*sip2*Δ mutant showed a twofold reduction in activity. The activity level of the *tos3*Δ*sip1*Δ*sip2*Δ mutant was not significantly different from that of *sip1*Δ*sip2*Δ mutant, although it remains possible that Tos3 has a minor effect"[27]. In the case of Snf1-Sip2, "Snf1-Sip2 activity was reduced more than threefold by the *pak1*Δ mutation and twofold by the *elm1*Δ". The *pak1*Δ mutation also seems to cause some reduction of Snf1-Sip1 activity.

Hedbacker et al. also report that Pak1 is not localized in the nucleus as a response to low glucose, but both Pak1 and Gal83 are required for the nuclear enrichment of Snf1. The role of Pak1 seems to be dual: First, phosphorylation of Snf1 by Pak1 is required for nuclear localization of Snf1-Gal83. And second, Pak1 seems to regulate the localization of Gal83 in the presence of Snf1; however, in the absence of Snf1, Gal83 can still be found in the nucleus, due to an unknown Pak1-independent regulatory mechanism.

According to Nath et al.[28], Pak1 associates to Snf1 upon glucose depletion and activates it.

2.2.3. The role of Reg1/Glc7

Protein phosphatase 1, which is formed by the regulatory subunit Reg1 and the catalytic subunit Glc7, is believed to be involved in the dephosphorylation of Snf1. It is known that Reg1 interacts with Snf1, but the mechanism is not clearly understood.

Sanz and co-workers[29] propose the following model (see Figure 2.2.):

- In low glucose, Reg1 binds to Snf1 phosphorylated. Reg1 is phosphorylated by Snf1, which seems to be necessary for posterior release of the phosphatase from the kinase complex.
- In high glucose, Reg1/Glc7 dephosphorylates Snf1 and it is released from the complex. Glc7 then dephosphorylates Reg1.

[27] HEDBACKER, K., et al., Op.Cit.
[28] NATH, N., McCARTNEY, R., SCHMIDT, M., Yeast Pak1 kinase associates with and activates Snf1, *Molecular and Cellular Biology*, **23**, 11, 2003
[29] SANZ, P., ALMS, G., HAYSTEAD, T., CARLSON, M., Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase, *Mol. Cel. Biol*. **20**, 2000

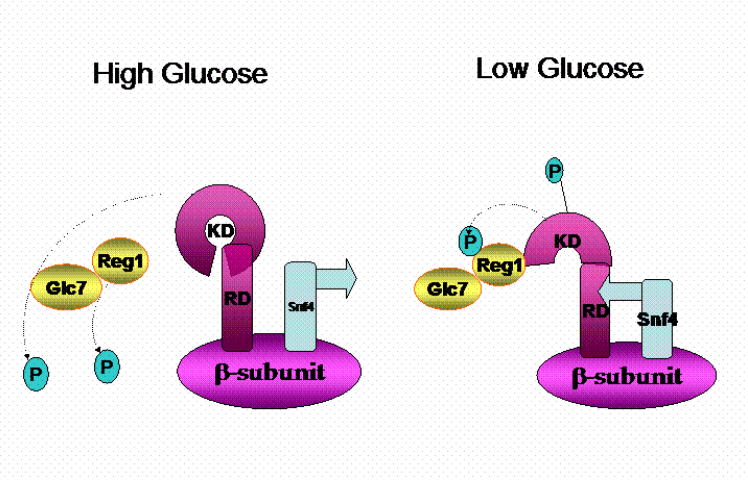

Figure 2.2. Role of Reg1/Glc7. First hypothesis.

This model suggests that Snf1 phosphorylated binds either Mig1 in nucleus or Reg1 in cytoplasm, because Reg1 is not a nuclear protein and Mig1 phosphorylation is a nuclear event. It is not clear when Reg1 recruits Glc7.

Nath et al. present a model which differ from the previous one because Snf1 is in a complex with Reg1 during high glucose (Figure 2.3); however, they summarize the low-glucose behaviour of Snf1 in a coincident way: "Pak1 associates with Snf1 kinase complex and phosphorylates Snf1 at threonine 210. Snf4 also participates in activation of the Snf1 kinase through direct interaction with Snf1 regulatory domain. Once activated, Snf1 phosphorylates its beta subunit (Sip1, Sip2, Gal83), the Reg1 protein, and additional substrates, such as Mig1"[30].

Additionally, Sanz et. al. performed a screening in a two-hybrid system for the protein Sip5 and found that this protein interacts both with Snf1 kinase and the Reg1/Glc7 phosphatase complex in response to glucose limitation. The genetic findings of the experiments "suggest that Sip5 negatively regulates the Snf1 kinase

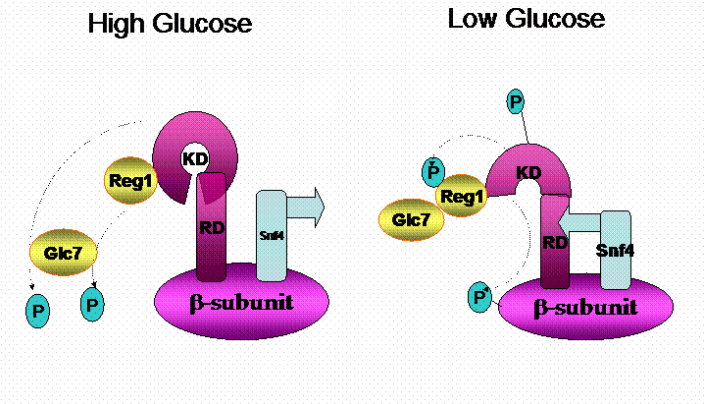

Figure 2.3. Role of Reg1/Glc7. Second hypothesis.

[30] NATH, N., et. al., Op. Cit.

by promoting the interaction of Reg1/Glc7 with the kinase"[31]. The data also shows that Sip5 binds both to Snf1 and to Reg1 in the phosphatase complex and this way directly facilitates the interaction between Snf1 and Reg1/Glc7 when glucose is limiting. Data indicates that Sip5 does not interact with the Glc7 subunit.

According to Johnston, the protein phosphatase that acts on Mig1 has not been identified, but Reg1-Glc7 is an attractive candidate, because *reg1* and *glc7* mutations cause Mig1 to be hyperphosphorylated and always in the cytoplasm[32].

Finally, Sanz et al. report the presence of three possible Snf1 phosphorylation sites on Reg1. However, there is no evidence that these sites are used by Snf1 in vivo.

2.2.4. Mig1-complex formation

Mig1 is a $Cys_2 – His_2$ zinc finger protein which binds to glucose-repressed promoters of SUC, GAL, MAL and other glucose-repressible proteins.

Treitel and Carlson[33] show that LexA-MIG1 fusion represses target genes transcription and that the repression requires Ssn6 and Tup1. In addition, interaction between Mig1 and Ssn6 was detected in a two-hybrid system. The two-hybrid experiment showed that Ssn6 and Mig1 interact with each other and this supports the idea that Ssn6 is a mediator between Mig1 and Tup1 but do not exclude the possibility of a direct interaction between the two proteins.

Conclusively, "the findings support the model that Mig1 recruits SSN6-TUP1 to promoters containing MIG1-binding sites… (but)… this model does not exclude the possibility that other proteins assist Mig1 in tethering SSN6-TUP1"[34]. Besides this, the experiments revealed "unexpected transcriptional activation capability when Ssn6 and Tup1 are absent"[35]. According to the writers, "this result suggests that SSN6 not only functions with MIG1 in repression but also masks an activation domain of the MIG1 or blocks interaction with an activator protein"[36]. Mutation of TUP1 also converted Mig1 to a weak activator, which also suggests that TUP1 might mask an activator domain of Mig1.

Ssn6/Tup1 complex, different to Mig1, resides permanently in the nucleus independently of the glucose level. Treitel et al. show that the repressor function of Ssn6/Tup1 complex is not directly regulated by the glucose signal. According to Schuller, "It has been proposed that the Cyc8-Tup1 complex (molecular stoichiometry is one subunit of Cyc8 with four subunits of Tup1; Varanasi et al. 1996) does not directly bind DNA but is brought to target genes through interactions

[31] SANZ, P., LUDIN, K., CARLSON, M., Sip5 interacts with both the Reg1/Glc7 protein phosphatase and the Snf1 protein kinase of S. Cerevisiae, *Genetics*, **154**, 2000
[32] JOHNSTON, M., Feasting, fasting and fermenting: glucose sensing in yeast and other cells, *TIG*, **15**, 1999
[33] TREITEL, M., CARLSON, M., Repression of SSN6-TUP1 is directed by Mig1 a repressor/activator protein ,*Genetics*, **92**, 1995
[34] TREITEL, M., et al., Op.Cit.
[35] TREITEL, M., et al., Op.Cit.
[36] TREITEL, M., et al., Op.Cit.

with sequence-specific DNA-binding proteins"[37]. Ssn6(Cyc8) / Tup1 complex is also found in association with Mig2 and Rgt1, among other proteins.

2.2.5. Snf1 phosphorylates Mig1

According to Treitel and co-workers, "Several lines of genetic and biochemical evidence support the view that Snf1 phosphorylates Mig1 in vivo"[38]:

- Snf1 and Mig1 interact in a two-hybrid system, showing that "…Mig1 is a substrate of Snf1 in vivo"[39].
- By using immunoblot analysis of Mig1p, it was found that the Mig1p band of a *snf1Δ* mutant "…migrated at the position predicted for the unmodified protein in both glucose-repressed and derepressed cells… Thus, the Snf1 protein kinase is required for the phosphorylation of Mig1"[40].
- In vitro, Snf1 is able to phosphorylate Mig1 at four sites. "Mutations of all four putative Snf1 recognition sites eliminate most of the differential phosphorylation of Mig1 in response to glucose"[41].

Three of the putative sites exactly match with the proposed motif. But, according to Smith and co-workers, mutation of two of them (Ser-278 and Ser-310/Ser-311) was responsible for "…almost complete abolition of the effect of SNF1 (from 6.1-fold to 1.6-fold)"[42], meanwhile mutation of each of them generated a decrease to 2.0-fold (for Ser-278) and to 2.6-fold (for Ser-310/Ser-311).

Finally, we highlight that the authors reported experimental problems to prevent the degradation of Mig1p, reason for which it is difficult to calculate the stoichiometry of the phosphorylation. "However, assuming an average molecular mass of 40 kDa, the maximum labeling was around 0.63 mol phosphate per mole protein"[43].

2.2.6. Snf1 interacts with Sip3, Sip4, Msn2, Cat8 and Adr1 proteins

Lesage et al. show that Snf1 and Sip4, a Snf1-regulated transcription activator of gluconeogenic genes, interact in a two-hybrid system. They report that "the transcriptional activation of Sip4 is regulated by glucose and depends on the Snf1 protein kinase"[44]. Moreover, "Sip4 is differentially phosphorylated in response to glucose availability, and phosphorylation requires Snf1… as no phosphorylation was detected in a snf1-K84R mutant"[45]. The authors don't exclude the hypothesis that

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[37] SCHULLER, H., Transcriptional control of nonfermentative metabolism in the yeast S. Cerevisiae, *Curr. Genet.*, **43**, 2003
[38] TREITEL, M., KUCHIN, S., CARLSON, M., Snf1 protein kinase regulates phosphorylation of Mig1 repressor in S. Cerevisiae, *Molecular and Cellular Biology*, **18**, 11, 1998
[39] TREITEL, M., et al., Op.Cit.
[40] TREITEL, M., et al., Op.Cit.
[41] TREITEL, M., et al., Op.Cit.
[42] SMITH, F., DAVIES, S., WILSON, W., CARLING, D., HARDIE, G., The Snf1 kinase complex from S. cerevisiae phosphorylates the transcriptional repressor protein Mig1p in vitro at four sites within or near regulatory domain 1, *FEBS letters*, **453**, 1-2, 1999
[43] SMITH, F., et al., Op.Cit.
[44] LESAGE, P., YANG, X., CARLSON, M. Yeast Snf1 protein kinase interacts with Sip4, a C_6 zinc cluster transcriptional activator: a new role for Snf1 in the glucose response, *Mol. And Cellular Biolog.*, **16**, 1996
[45] LESAGE, P., et al., Op.Cit.

Sip4 might be phosphorylated by another kinase which in its turn is phosphorylated by Snf1.

The interaction of Snf1 with Sip4 shows a new regulatory role of Snf1 protein kinase in the glucose response. Besides its role in the derepression of the different respiratory and gluconeogenic genes by phosphorylation of Mig1-complex, Snf1 is revealed to have a role in activating transcription in response to glucose limitation, by regulating the function of the Sip4 protein. Vincent and Carlson showed that Gal83 mediates the association of Snf1 kinase with Sip4. Gal83 also facilitates the rapid Snf1-dependent phosphorylation and activation of Sip4 in response to glucose limitation.

Lesage et al. also show that "Sip3 is functionally related to the Snf1 protein kinase pathway"[46]. First, the Sip3 protein was identified to interact with Snf1 protein kinase in a two-hybrid system. Second, Sip3 was shown to interact with DNA-bound Snf1 and to activate the transcription of the target gene GAL1. Third, the negative effect of Sip3 on the regulation of SUC2 can be suppressed by overexpressing Snf1[47].

According to Lesage et. al.[48], genetic evidence also suggests interaction between Snf1 and other transcriptional activators as Msn2 and its homolog Msn4, zinc finger transcriptional activators of the SUC2 gene.

"Similarly, CAT8 encodes a zinc cluster transcriptional activator necessary for derepression of gluconeogenic enzymes and is a multicopy suppressor of the growth defect of a *snf1* mutant on ethanol"[49]. Klein et al. reported that it has been proposed that Snf1 also phosphorylates (and this way activates) the transcriptional activator Cat8: "Cat8… requires phosphorylation for activation (hypothetically by Snf1)"[50]. According to Schuller, "The biosynthetic derepression of Cat8 by deactivation of Mig1 and transcriptional activation mediated by Cat8, require a functional SNF1 gene. Indeed, Cat8 becomes phosphorylated in the course of the derepression of target genes. Since Snf1 recognition sites are apparently absent from the sequence, it remains unclear whether Cat8 is a direct substrate of the Snf1 kinase complex"[51].

According to Zaragoza et al.[52], during growth on a non-fermentable carbon source (such as ethanol or glycerol), Snf1p turns off the repressor Mig1p and turns on the activators Cat8p and Sip4p (Sip4p, in its turn, also needs Cat8p for activation); during growth on galactose or low levels of glucose, Snf1 also inactivates Mig1, but it cannot activate Cat8p or Sip4p; and, during growth on high levels of glucose, Snf1

[46] LESAGE, P., YANG,X.,CARLSON, M., Analysis of Sip3 protein identified in a two-hybrid screen for interaction with Snf1 protein kinase, *Nucleic Acid Research,* **22,** 1994
[47] LESAGE, P., et al., Op.Cit.
[48] LESAGE, P., YANG, X., CARLSON, M. Yeast Snf1 protein kinase interacts with Sip4, a C_6 zinc cluster transcriptional activator: a new role for Snf1 in the glucose response, *Mol. And Cellular Biolog*., **16**, 1996
[49] LESAGE, P., et al., Op.Cit.
[50] KLEIN, C., OLSSON, L., NIELSEN, J., Glucose control in S. Cerevisiae: the role of MIG1 in metabolic functions, *Microbiology*, **144**, 1998
[51] SCHULLER, H., Op.Cit.
[52] ZARAGOZA, O., VINCENT, O., GANCEDO, J., Regulatory elements in the FBP1 promoter respond differently to glucose-dependent signals in S. Cerevisiae, *Biochem. J.*, **359**, 2001

is inactive and Mig1 is fully active, repressing the transcription of the activator Cat8p.

Adr1p is a transcriptional activator required for Acetyl-CoA production and glycerol utilization, among other uses. According to Schuller, "Similar to Cat8, Adr1 also requires a functional Snf1 protein kinase… It is unclear whether Adr1 is a substrate of the Snf1 protein kinase. Nevertheless, recent results provide evidence for a stimulation of the Adr1 binding to chromatin by Snf1 under derepressing conditions, while Glc7+Reg1 inhibit binding in the presence of glucose… No influence of glucose on nuclear localization of Adr1 could be detected"[53].

2.2.7. Mig1 transport

Mig1 translocation depends on both Msn5 protein and Snf1 kinase. Phosphorylation of Mig1 affects its subcellular localization causing it to be in the nucleus when glucose is present and in the cytoplasm when glucose is absent. According to DeVit and Johnston, the exportation of Mig1 from the nucleus requires Msn5, a member of the importin beta family of nuclear transport receptors. Msn5 is found to recognize two sequences in Mig1 which overlapped with the serine residues predicted to be Snf1 phosphorylation sites. DeVit and Johnston conclude that "Mig1 contains a new nuclear export signal that is phosphorylated by Snf1 upon glucose removal, causing it to be recognized by the nuclear exportin Msn5 and carried out of the nucleus in the cytoplasm where it contributes to derepression of glucose-repressed genes"[54].

However, according to Schuller, "An increased level of Msn5 allows export of the Mig1 repressor, even in the absence of phosphorylation"[55]. Finally, the importin Cse1p has been proposed to be responsible for importing Mig1p to the nucleus[56].

2.2.8. Switch between transcriptional repression and de-repression (the dissociation of the Ssn6-Mig1-Tup1 complex)

There have been reported at least two hypotheses regarding the key regulatory step for releasing glucose repression: A first hypothesis was that cytoplasmic translocation of Mig1 was the key regulatory step for releasing glucose repression. In another study, Papamichos-Chronakis, et al. re-evaluate the previous model and propose an alternative hypothesis: Snf1 phosphorylation of Mig1 is "the molecular switch that controls transcriptional repression/de-repression"[57]. This study involved the analysis of Mig1 behaviour in relation to Cyc8/Tup1 complex in their repressive action on GAL1 transcription, upon Snf1 phosphorylation.

It has been shown that Mig1 regulates the transcription of GAL1 gene by recruiting the general co-repressor complex Cyc8 (Ssn6)/Tup1[58]. In a first hypothesis it was suggested that Mig1 dissociation from the repressor complex Ssn6-Tup1 depended

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[53] SCHULLER, H., Op.Cit.
[54] DEVIT M., JOHNSTON M., The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of S. cerevisiae, *Curr. Biol.*, **9**, 21, 1999
[55] SCHULLER, H., et al., Op.Cit.
[56] SCHULLER, H., et al., Op.Cit.
[57] PAPAMICHOS-CHRONAKIS, M., GLIGORIS, T., TZAMARIAS, D., The Snf1 kinase controls glucose repression in yeast by modulating interactions between the Mig1 repressor and the Cyc8-Tup1 co-repressor, *EMBO*, **5**, 2004
[58] NEHLIN et al.,1991; TREITEL & CARLSON, 1995; TZAMARIAS & STRUHL, 1995

on Mig1 translocation to cytoplasm. After a series of experiments, Papamichos-Chronakis et al. showed that the phosphorylation of Mig1 by Snf1 kinase modulates Mig1 interaction with Cyc8 (Ssn6). To exclude the possibility that Mig1 localization in the cytoplasm during glucose depletion was responsible for the lack of interaction between Mig1 and Cyc8, *msn5*Δ mutants were used, and this way Mig1 was continuously localized in the nucleus. "The above experiments demonstrate that Cyc8 interacts specifically with the non-phosphorylated form of Mig1 and, upon glucose depletion, Snf1-dependent phosphorylation of Mig1 releases its interaction with Cyc8-Tup1"[59]. Papamichos-Chronakis, et al. conclude that "Mig1 phosphorylation and lack of Cyc8 interaction, rather than Mig1 nuclear export, is the important regulatory step that releases transcription from glucose repression"[60].

2.2.9. Input to the pathway

It is still unclear how glucose is sensed and how the information is transduced to Snf1. Two main hypotheses have been proposed:

Hypothesis 1: AMP/ATP ratio is triggering the phosphorylation of the Snf1 kinase: The previous was one of the first popular hypotheses due to: "Although it has been shown that Snf1 is not directly activated by AMP, a good correlation between Snf1 activity and AMP/ATP ratio was reported"[61]. In glucose-growing cells, ATP generation by glycolysis depletes AMP and, when glucose is exhausted, the AMP level is repleted. This means that the AMP/ATP ratio is high and could activate Snf1 and de-repress the genes involved in glucose transport.

Before knowing the existence of kinases upstream Snf1, it was proposed that the AMP/ATP ratio regulates Snf1 activity indirectly. "AMP may activate Snf1 by activating a Snf1 kinase kinase which appears to exist"[62], by analogy to AMPK, which is phosphorylated by another protein kinase that is also regulated by AMP. Surprisingly, nowadays, there are no reports on the connection between the AMP/ATP ratio and Pak1/Elm1/Tos3.

Hypothesis 2: Hxk2 has a regulatory function in the glucose de-repression pathway: Rolland, et al., support the idea that Hxk2 have a specific function in the activation mechanism of glucose de-repression. It has been shown that "no further metabolization beyond the sugar phosphorylation step appears to be necessary for triggering glucose repression"[63]. This is supported by the finding that the phosphorylation of 2-deoxyglucose, which is not further metabolized, trigger repression. Overexpression of Glk1 did not restore glucose repression in a Hxk mutant indicating that glucose phosphorylation by itself is not sufficient to trigger glucose repression. This way, Hxk2 might have the regulatory function which is needed for the activation of glucose de-repression.

Hxk2 is found in two isoforms, both as a monomer and as a dimer. The low level of glucose induces a shift of the equilibrium of the two isoforms, from a monomer-

[59] PAPAMICHOS-CHRONAKIS, M., et al., Op.Cit.
[60] PAPAMICHOS-CHRONAKIS, M., et al., Op.Cit.
[61] ROLLAND, F., WINDERICKX, J., THEVELEIN, J., Glucose-sensing and –signalling mechanisms in yeast, *FEMS Yeast Research*, **2**, 2002
[62] JOHNSTON, M., Op.Cit.
[63] ROLLAND, F., et.al, Op.Cit.

dimer equilibrium to a mainly-monomer[64]. Hxk2 is also a phospho-protein and the level of its phosphorylation correlates inversely with the level of glucose. Randez-Gil et al. prove this correlation: "After 120 min of incubation, the presence of glucose reduced the abundance of the phosphorylated monomer and increased that of the unphosphorylated forms"[65]. The same authors showed that the Hxk2p-S15A mutant enzyme, a mutant which presents a defect in the phosphorylation site, was not able to form monomers and had lower glucose affinity. Alms et. al. give an interpretation to these findings: "These findings suggest that Ser15 phosphorylation may enable Hxk2p to scavenge glucose more efficiently under conditions when the sugar is limiting"[66].

It has been found that Hxk2 interacts with Reg1-Glc7 complex which, in its turn, regulates Snf1 activity. According to Sanz et al., "…Hxk2 either stimulates the binding and/or phosphorylation of Reg1 or inhibits the dephosphorylation of Reg1 by Glc7"[67]. However, these authors suggest that the low-glucose signal could be due to the upstream kinases, and the high-glucose signal could act either inhibiting the phosphorylation of Snf1 or activating Reg1/Glc7 function. "The complexity of these regulatory interactions suggests that the Snf1 kinase activity is finely tuned in response to glucose"[68].

However, according to Randez-Gil et. al., phosphorylation of Hxk2 seems to be essential for signaling and the interaction with the Reg1-Glc7 (Cid1-Hex2) complex can be the next step for mediating the signal of low glucose: "…Hxk2p is a direct or indirect target of the protein phosphatase Cid1p-Hex2p complex, suggesting a putative interaction between the Hxk2p phosphorylated monomer and this protein complex. As a result of this interaction downstream elements would be activated in the transmission of the glucose signal. In this model, the phosphate group would likely be critical for controlling the affinity of the interaction site of Hxk2p with the Cid1p-Hex2p complex. By removing the phosphate, the strength of the interaction would be decreased, making the signal transduction weaker"[69].

A last scenario for Hxk2 is the possibility of a Hxk2-pathway independent of Snf1, as suggested by Ahuatzi et al.: "The mechanism of Hxk2-dependent glucose repression pathway is not well understood, but the Mig1-dependent part of the pathway has been elucidated in great detail. Here we report that Hxk2 has a glucose-regulated nuclear localization and that Mig1… is required to sequester Hxk2 into the nucleus. Mig1 and Hxk2 interacted *in vivo* in a yeast two-hybrid assay and *in vitro* in immunoprecipitation... We conclude that Hxk2 operates by interacting with Mig1 to

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[64] BISSON, L., KUNATHIGAN, V., On the trail of an elusive flux sensor, *Research in Microbiology*, **154**, 2003

[65] RANDEZ-GIL, F., SANZ, P., ENTIAN,,K., PRIETO, J., Carbon Source-Dependent Phosphorylation of Hexokinase II and Its Role in the Glucose-Signaling Response in Yeast, *Molecular and Cellular Biology*, 18, **5**, 1998

[66] ALMS, G., SANZ, P., CARLSSON, M., HAYSTEAD, T., Reg1p targets protein phosphatase 1 to dephosphorylate hexokinase II in S. cerevisiae: characterizing the effects of a phosphatase subunit on the yeast proteome, *The EMBO Journal*, 18, **15**, 1999

[67] SANZ, P., ALMS, G., HAYSTEAD, T., CARLSON, M., Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase, *Mol. Cel. Biol*. **20**, 2000

[68] SANZ, P., et al., Op.Cit.

[69] RANDEZ-GIL, F., et al. Op. Cit.

generate a repressor complex located in the nucleus of *S. cerevisiae* during growth in glucose medium"[70].

2.2.10. Discussion

The state-of-the-art of the Snf1-signalling pathway shows that enough knowledge has been accumulated regarding the structural modifications of Snf1, the influence of three upstream kinases and one phosphatase on it, the role of Gal83 in its localization and its role in Mig1 phosphorylation. In the same way, the switch between the Mig1 repressor complex and the Mig1 phosphorylated protein, as well as the role of Msn5 in Mig1 export from the nucleus, are well known.

However, the exact mechanisms behind the action of the kinases and the phosphatase are unknown, as well as the transduction mechanisms of the glucose signal to these proteins. For this reason, in order to design a biochemical model, it is necessary to postulate the existence of, at least, an active and an inactive state for each kinase/phosphatase which respond to a simulated glucose signal producing the phosphorylating/de-phosphorylating effects on Snf1. In the same way, transport mechanisms of both Snf1 and Mig1 are not well understood, which makes difficult the construction of a model including transport reactions; however, cytoplasmic de-phosphorylation of Mig1 and Cse1-mediated transport to the nucleus have been proposed and seem to be the best available assumption for Mig1 behaviour.

The Snf1-Gal83 pathway can be observed in Appendix 4.

2.3. Transcriptional Regulation

2.3.1. Mig1-repressed genes

The main Mig1-repressed genes have been found to be those that code for the enzymes of the Central Carbon Metabolism (Glycolysis, TCA Cycle, Alcoholic fermentation, Gluconeogenesis, Glyoxylate Cycle and Respiratory chain) and peripheral functions (such as sugar transport and the catabolism of sugars to the level of Glucose-6-Phosphate).

2.3.1.1. Introduction

Two main strategies have been used to detect Mig1 repression:

- The "Biochemical strategy" consists in searching for putative Mig1-binding sites on the promoters of candidate genes. This purpose can be accomplished in two main ways:
    - Looking for a consensus motif: It has been suggested that such a motif is "(G/C)(C/T)GG(G/A)G"[71].
    - In vitro assays: Klein et al. review two main assays: "In a footprint assay, the endonuclease DNAse I hydrolyses DNA that is not covered by bound Mig1, the undigested sequence representing the putative binding sequence. In an oligonucleotide gel shift experiment… if the

[70] AHUATZI, D., HERRERO, P., DE LA CERA, T., MORENO, F., The glucose-regulated nuclear localization of Hexokinase 2 in S. Cerevisiae is Mig1-dependent, *J. Biol. Chem.*, **279**, 14, 2004
[71] KLEIN, C., et al., Op.Cit.

mobility of the DNA fragment in an electrophoresis gel is decreased, Mig1 must have bound to it"[72]. Agarose gel shift assays have also been used.

- The "Physiological strategy" consists in looking for the physiological consequences of the MIG1 gene disruption or overexpression.

2.3.1.2. List of genes

Glucose de-repression involves a transcriptional network formed by several protein-DNA reactions exhibiting different degrees of repression. In this network, some genes are directly repressed by Mig1p, including the genes Gal4, Hap4, Cat8 and MalR, which encode regulatory proteins. However, some other genes are regulated in an indirect way (by one of the regulatory proteins but not by Mig1p).

Finally, some other genes display both a direct and an indirect control, phenomenon that is called "dual-level control". According to Klein, "such a dual-level control is a potential amplifier of glucose repression"[73]. Gal4p and Hap4p are transcriptional activators; Cat8p acts as a de-repressor of some gluconeogenic and glyoxylate cycle genes, and MalRp is involved in maltose induction and glucose repression of MALT and MALS genes. Table 2.4. and Appendix 2 summarize the previous information.

However, the panorama is even more complex: Some transcriptional reactions have been reported to need transcriptional activators and some are known to form complete networks with different activator and inhibitor elements involved. The following are the best studied cases:

GAL genes: According to Klein, et al., GAL4 regulation of GAL-genes is, at the same time, regulated by at least four effects: "Concomitant binding of Gal80 to the activation region prevents the transcriptional activation and is neutralized by the galactose-dependent binding of the regulatory protein Gal3, or, less efficiently, by that of the related Gal1 protein, to Gal80… Phosphorylation of Gal4 seems to be necessary for its competency to activate GAL transcription…"[74].

FBP1: According to Zaragoza et al., "FBP1 is controlled by glucose through the upstream activating sequences UAS1 and UAS2 and the upstream repressing sequence URS1 in its promoter"[75]. The protein binding UAS1 is not known, whereas Cat8 and Sip4 are known to bind UAS2, and Mig1 is known to bind URS1 and to block transcription by recruiting Cyc8 and Tup1.

As it was said before, during growth on a gluconeogenic carbon source (such as glycerol), Snf1p turns off the repressor Mig1p and turns on the activators Cat8p and Sip4p. Therefore, both Cat8p and Sip4p activate the transcription of the FBP1 gene. During growth on galactose or low glucose, Snf1 also inactivates Mig1, but it can't activate Cat8 or Sip4. "…in low glucose Cat8 expression is low and on galactose Cat8 is only partially derepressed. Under neither set of conditions is FBP1 expressed,

[72] KLEIN, C., et al., Op.Cit.
[73] KLEIN, C., et al., Op.Cit.
[74] KLEIN, C., et al., Op.Cit.
[75] ZARAGOZA, O., et al., Op.Cit.

Table 2.4. Genes believed to be repressed by Mig1.

Gene name	MIG1 direct control	Indirect control	COMMENTS
Self-repression			
MIG1	Yes	---	(Lundin, et al., 1994) reported that Mig1 represses Mig1.
Peripheral			
SUC2	Yes	---	"Nehlin & Ronne (1990) reported that MIG1 overexpression results in an aggravated repression of SUC2 expression and proved by DNAse I footprinting that Mig1 binds to the SUC2 upstream region".
MEL1	Yes	GAL4	---
HXT2, HXT4	Yes	---	Using ΔMig1-mutants, (Özcan and Johnston, 1995) found strong glucose repression of HXT2, HXT4 and SNF3.
SNF3	Yes	---	
HXT3	Yes	---	This gene is glucose-induced but, at the same time, shows a weak Mig1-repression. This apparent paradox can be explained as a "fine-tuned Mig1-mediated repression, i.e. a balance between glucose induction and repression".
FPS1	Yes	---	Lundin et al. proved Mig1 binding to a consensus sequence on the FPS1 promoter, using an oligonucleotide gel shift experiment. "ΔMig1 strains were shown to secrete significantly higher amounts of glycerol".
Galactose / Maltose Metabolism			
GAL2	No	GAL4	"The transcription of GAL2, GAL1, GAL7, GAL10 and GAL5 is activated by the binding of… GAL4 on to their upstream sequences… Both GAL4 and GAL1 are repressed by Mig1, as proven biochemically by DNase I footprinting, and physiologically by MIG1 deletion and MIG1 overexpression (Nehlin & Ronne, 1990, Nehlin et al., 1991)".
GAL1	Yes	GAL4	
GAL3	Yes	---	
GAL7	No	GAL4	
GAL10	No	GAL4	
GAL5 / PGM2	Yes	GAL4	
MALT	Yes	MALR	Using In Vitro assays, (Lundin, et al., 1994) reported Dual-Level control (Mig1p and MalRp) for both MALT and MALS.
MALS	Yes	MALR	
Glycolysis			
GLK1	Yes	---	---
HXK1	Yes	---	
TCA Cycle			
LPD1	No	HAP4	According to Klein et al., (de Winde & Grivell, 1993) proved that all three genes are activated by HAP4.
KGD1, KGD2	No	HAP4	
CIT1	No	HAP4	According to Klein et al., (Rosenkrantz et al., 1994) proved that CIT1 expression requires HAP activation complex.
Gluconeogenesis			
FBP1	Yes	CAT8 and HAP4	Lundin et al. found a Mig1-binding site in FBP1 using In Vitro assays. FBP1 and PCK1 have three consensus sequences: for Mig1, for the HAP activating complex and for Cat8. However, according to deletion and overexpression experiments, Mig1 influence is very low. "The exceptionally high sensitivity of the mRNAs of these genes towards glucose can at least be partly explained by stimulated mRNA degradation".
PCK1	Yes	CAT8 and HAP4	
Glyoxylate cycle			
ICL1	Yes	CAT8	According to Klein et al., (Schöler and Schuller, 1993) reported the presence of putative Mig1 binding sites in the promoters of both genes.
MLS1	Yes	---	

Table 2.4. Continued.

Gene name	MIG1 direct control	Indirect control	COMMENTS
Ethanol Fermentation			
PDC1	Yes	---	Lundin et al. found a Mig1-binding site by DNase I footprinting, but PDC1 expression increases in the presence of glucose. This paradox could be explained by an activating function of Mig1 or a similar protein, or an interaction between glucose induction and glucose repression pathways.
ACS1	No	CAT8	---
Respiratory chain			
CYT1	Yes	HAP4	According to Klein et al., (de Winde & Grivell, 1993; Crawford et al., 1995) proved the effects of HAP4 on oxidative phosphorylation.
COX5B	Yes	HAP4	
QCR8,9	Yes	HAP4	
QCR2,7	No	HAP4	
COX4,5A,6	No	HAP4	
CYC1	No	HAP4	
CYB2	No	HAP4	
HEM1	No	HAP4	
YHG	No	HAP4	
Trehalose metabolism			
GGS1 / TPS1	Yes	MALR	Trehalose-6-Phosphate Synthase / Phosphatase Complex has been reported to be subject to glucose repression (Francois et al., 1991; Thevelein and Hohmann, 1995). "There is a putative Mig1-binding sequence on the GGS1 / TPS1 promoter… but in vitro binding with Mig1 could not be confirmed in oligonucleotide gel shift experiments… MAL genes accelerates trehalose synthesis during growth on glucose… a dual-level control of GGS1 / TPS1, both directly by Mig1 and indirectly by gene products of MAL genes, can be assumed".
NTH1	Yes	---	---
Regulatory proteins			
GAL4	Yes	---	(See GAL genes)
MALR	Yes	---	---
CAT8	Yes	---	According to Klein, Cat8 is believed to interact with both Mig1 and Snf1.
HAP4	Yes	---	"HAP4 has a Mig1-binding site on its promoter as verified by oligonucleotide gel shift experiments (Lundin et al., 1994)… HAP4 overexpression has been reported to result in a more oxidative metabolism with a higher specific growth rate… a MIG1 deletion did not result in a derepression of HAP4, suggesting a redundant repression mechanism (Lundin et al., 1994)".

*Source: Adapted from: KLEIN, et al., Op.Cit. (all quotations come from this article); additional information taken from: LUNDIN,M., NEHLIN, J., RONNE, H., Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein MIG1, Molecular and Cellular Biology, 14, **3**, 1994*
Klein et al. report the existence of Mig1-repression with different degrees of confidence: All genes with a "Yes" on the list were found to have putative Mig1-binding sites, but the physiological effects were only confirmed for the genes in a white cell. The genes in a yellow cell are those without information about physiological effects. Besides, the genes in a blue cell are those for which there is no physiological effect after Mig1 disruption or over-expression (an apparent contradiction that can be explained by the presence of additional mechanisms of control). Another special case is TPS1, for which Mig1-binding information seems to be contradictory, as explained before.

and there is only a slight derepression of Sip4 during growth on galactose"[76]. During growth on high glucose, Mig1 is fully active and represses the transcription of both FBP1 and the activator Cat8, so FBP1 is "completely turned off"[77].

SUC2: SUC2 is known to be repressed by high-glucose and no-glucose conditions, and to reach the higher transcription rate under low-glucose levels. Geng and Laurent have presented a detailed study of SUC2 transcription, where transcription is report to proceed in two phases. "We first monitored SUC2 RNA levels in a time course following a rapid shift of yeast cells from glucose to raffinose (containing low glucose) medium… SUC2 RNA accumulated rapidly and in two distinct phases: a short first phase in which transcript levels peaked at 45 min after induction and a prolonged second phase in which RNA reached steady-state levels at 2 h. Shifting to a medium containing only low glucose (no raffinose) resulted in a similar biphasic pattern, except that SUC2 RNA levels dropped to noninducing levels by 4 h. These results suggest that the biphasic transcription of SUC2 is primarily a response to low glucose and that raffinose is required for the maintenance of SUC2 transcription"[78]. The authors report that RNA polymerase II levels at the SUC2 promoter "also showed a biphasic pattern that slightly preceded that of the SUC2 RNA" and that Msn2p/Msn4p are involved in early induction (e.g., "…Msn2p crosslinking at the SUC2 promoter increased more than three-fold 5 min following gene induction but quickly returned to repression levels within 30 min and did not increase further throughout the induction"[79]).

The chromatin-remodeling enzymes were also studied: According to Geng et al., "…the activity of a gene is largely dictated by the chromatin structure in which it resides, which can be modulated by enzymes that reversibly remodel chromatin. Chromatin-remodeling enzymes… have been grouped into two major categories – ATP-dependent chromatin remodelers and covalent histone modifiers. ATPase remodelers, such as the yeast SWI/SNF complex, induce conformational changes in nucleosomes by altering DNA-histone interactions; histone modifiers catalyze post-translational modifications of histones. Histone acetylation, the first modification shown to correlate strongly with transcriptional competence, is controlled by the antagonistic activities of histone acetyltransferases (HATs) and deacetylases (HDACs)"[80]. In the case of SUC2, "SWI/SNF is essential for both phases of gene induction and associates with the SUC2 promoter in a biphasic manner… both the Gcn5p and Esa1p HATs facilitate the association of SWI/SNF with the promoter for optimal SUC2 induction. Gcn5p is recruited to the promoter concurrently with SWI/SNF, whereas Esa1p associates constitutively with SUC2"[81].

As a final remark, "…the early induction of SUC2 resembles a stress response in that SWI/SNF, Gcn5p, and pol II are recruited to the promoter within 2 min"[82].

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[76] ZARAGOZA, O., et al., Op.Cit.
[77] ZARAGOZA, O., et al., Op.Cit.
[78] GENG, F., LAURENT, B., Roles of SWI/SNF and HATs throughout the dynamic transcription of a yeast glucose-repressible gene, *EMBO Journal*, **23**, 2004
[79] GENG, F., et al., Op.Cit.
[80] GENG, F., et al., Op.Cit.
[81] GENG, F., et al., Op.Cit.
[82] GENG, F., et al., Op.Cit.

HXT2 and HXT4: HXT2 and HXT4 are known to be Mig1-repressed at high-glucose levels and Rgt1-repressed at no-glucose; the maximum transcription occurs at low-glucose. These mechanisms will be explained later on.

2.3.1.3. Mig1-independent mechanisms

None of the commented strategies give us an absolute proof: The finding of a Mig1-binding motif is just an indication that MIG1 "could" bind. And the changes in protein levels after a MIG1 disruption or overexpression are not an enough guarantee because MIG1 does not seem to explain the whole phenomenon of glucose repression.

Related to the last consideration, Klein et al. report that "…a deletion/disruption of MIG1 is not able to eliminate glucose repression entirely of galactose, maltose and sucrose metabolism, as substantial Mig1-independent glucose control mechanisms do exist for the GAL, MAL and SUC systems…"[83]. First of all, Klein et al. discuss the existence of a Mig1-redundant protein ("i.e. a protein that is similar to Mig1 and that can partly substitute for it"[84]). The main candidate seems to be the repressor MIG2, which is 71% identical to MIG1. "Mig2 has been shown to bind to some of the Mig1-binding sites. A double deletion mutant ΔMig1ΔMig2 yielded a considerably higher derepression than the single ΔMig1 mutant for SUC2 expression, but not for PCK1, FBP1, HXT2 and GAL1"[85]. Post-translational modifications also need to be taken into account: "The finding that CAT8 is derepressed in a Δmig1 strain, whereas Cat8-controlled FBP1, PCK1 and ICL1 not, gives a clear hint of the existence of other repressors such as Mig2 or a post-translational modification of an effector such as the phosphorylation of the derepressor Cat8"[86].

In general terms, besides Mig1 transcriptional repression, we found Snf1-independent mechanisms such as Mig1-redundant transcriptional repressors, stimulated mRNA degradation, translational efficiency decrease, catabolite inactivation and enzyme inhibition, which need to be taken into account in order to explain the glucose de-repression phenomenon. Therefore, to consider all the available experiments would be desirable.

2.3.2. Adr1p and Imp2p transcriptional regulators

According to Alberti et al., "Imp2p is a transcriptional activator involved in glucose derepression of the maltose, galactose and raffinose utilization pathways… IMP2 is required for rapid glucose derepression of the utilization pathways of the above-mentioned carbon sources. In fact, the mutants display a long growth delay in galactose after metabolic shifts from glucose but not after metabolic shifts from derepressing or inducing carbon sources… during metabolic shifts, Imp2p regulates the expression of galactose permease, maltose permease and maltase at the transcriptional level… Imp2 was shown to have a positive effect on glucose derepression of Leloir pathway genes and their activator gene GAL4. The effect of

[83] KLEIN, C., et al., Op.Cit.
[84] KLEIN, C., et al., Op.Cit.
[85] KLEIN, C., et al., Op.Cit.
[86] KLEIN, C., et al., Op.Cit.

Imp2 on galactose metabolism was shown to be partially dependent on Mig1p. The Mig1-independent role depends on Nrg1p"[87].

Young et al. highlight that "…expression of almost one half of the 40 most highly glucose-repressed genes is ADR1-dependent… ADR1 was discovered as a regulatory gene that is required for expression of the glucose-repressed ADH2 gene. ADH2 encodes an alcohol dehydrogenase isozyme that is required for the first step in ethanol oxidation"[88]. According to Young and co-workers, the following carbon metabolism genes are ADR1-dependent: CIT3 (Citrate synthase), ACS1 (Acetate CoA ligase), ADH5 (Alcohol dehydrogenase), ICL2 (2-Methylisocitrate lyase), ADH2 (Alcohol dehydrogenase), DIC1 (Dicarboxylate transport), ALD4 (Aldehyde dehydrogenase), CYB2 (L-Lactate dehydrogenase), ALD5 (Aldehyde dehydrogenase), OAC1 (Oxaloacetate transport), GUT1 (Glycerol kinase), GUT2 (Glycerol-3-P dehydrogenase) and CTP1 (Citrate transport).

Young et al. also reported a new group of gene targets of Cat8: ADH2, IDP2, REG2, ICL2, GUT1 and the peroxisomal POX1 and CTA1, among others.

2.3.3. Glucose-transcriptional network

Looking after Mig1-redundant proteins, Luftiyya et al. studied Mig2p and Yer028p, which are similar to Mig1p. Mig2p have been reported to perform glucose repression by recruiting Ssn6p and Tup1p, just like Mig1p does. SUC2 gene has been reported to be repressed by both Mig1 and Mig2. After deletion experiments, they also report that Mig1 and Mig2, but not Yer028, are almost completely responsible of Mig1-repression. GAL genes have been reported to be repressed just by Mig1p.

As differences, Mig2p is located on the nucleus both in presence and absence of glucose, and Snf1 does not seem to have any effect on it. According to Luftiyya et al., "Suc2 expression is abolished in a snf1Δ mutant. Mig1 is clearly responsible for this, because deletion of Mig1, but not Mig2, restores expression. Snf1 appears not to inhibit Mig2 function. This is clearly seen in a snf1mig1 mutant, in which Mig2 is primarily responsible for the 10-fold glucose repression of Suc2 expression observed in this mutant: Mig2-mediated repression and derepression of Suc2 expression are not affected by loss of Snf1. Thus, it appears that Snf1 does not inactivate Mig2. Nevertheless, Mig2 function is regulated in response to glucose"[89]. Besides, "Yer028 appears to have evolved a role in regulating expression of a set of genes separate from those regulated by Mig1 and Mig2"[90].

The authors report repression mediated by both Mig1 and Mig2, in: Reg2, Hxt1, Hxk1 and many other genes. Based on this observation, they suggest that "…as far as we can tell, Mig2 always work in conjunction with Mig1. However, Mig1 appears to

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[87] ALBERTI, A., LODI, T., FERRERO, I., DONNINI, C., MIG1-dependent and MIG1-independent regulation of GAL gene expression in S. cerevisiae: role of Imp2p, *Yeast*, **20**, 2003

[88] YOUNG, E., DOMBEK, K., TACHIBANA, C., IDEKER, T., Multiple pathways are co-regulated by the protein kinase Snf1 and transcription factors Adr1 and Cat8, *The Journal of Biological Chemistry*, **278**, 28, 2003

[89] LUTFIYYA, L., IYER, V., DERISI, J., DEVIT, M., BROWN, P., JOHNSTON, M., Characterization of three related glucose repressors and genes they regulate in S.Cerevisiae, *Genetics*, **150**, 1998

[90] LUTFIYYA, L., et al., Op.Cit.

Table 2.5. Genes repressed by Rgt1, Mig1 and Mig2.

Transcription Repressor	Repressed Genes
Rgt1p (Glucose Induction pathway)	• Hxt2, Hxt4 • Hxt1, Hxt3 • Std1 • Mig2
Mig1p (Glucose Repression pathway)	• Hxt2, Hxt4 • Snf3 • Mth1 • Mig1 • Suc2, etc...
Mig2p (Mig1-redundant protein)	• Hxt2, Hxt4 • Snf3 • Mth1 • Mig1 • Suc2

Source: Adapted from: KANIAK, A., et al., Op.Cit.

be more important than Mig2, since it is sufficient to cause nearly full repression of most of the genes it regulates. An effect of deleting Mig2 alone is observed only for a few genes"[91]. In order to explain this fact, they suggest: "Perhaps Mig2 binds to DNA with lower affinity than Mig1, causing it to repress gene expression only in the absence of Mig1. Alternatively, Mig2 could be regulated post-transcriptionally, so that it is present only at low levels in the presence of Mig1"[92].

In a more recent work, Kaniak et al. show a different picture. They reported the repression effects summarized in Table 2.5. The authors highlight that glucose induction (Snf3/Rgt2 to Mth1/Std1 to Rgt1) and glucose repression (Hxk2? to Snf1 to Mig1) pathways are interconnected in two main ways: Glucose induction contributes to glucose repression (Rgt1p represses Mig2, which represses Mig1); and viceversa (Mig1 represses both Snf3 and Mth1, which are involved in glucose induction, this way regulating Rgt1p). The authors also highlight the self-regulation of glucose induction (Rgt1p controls Std1) and the self-regulation of glucose repression (Mig1p controls Mig1). Therefore, we want to highlight three main consequences:

1. ***Mig2 behavior:*** Mig2p is controlled by Rgt1p (glucose induction pathway) and affects the Mig1-dependent genes listed in Table 2.5.. Kaniak et al. also suggest a similar role for the protein Mig3.
2. ***Hexose transporters:*** According to Kaniak et al.[93], Hxt2 and Hxt4 seems to be regulated by Rgt1, Mig2 and Mig1, whereas Hxt1 and Hxt3 are just regulated by Rgt1. This way, we can explain the observed expression patterns of Hexose Transporters (see section 2.5.2.) in the following way:
   - Glucose induction pathway represses Hxt1 and Hxt3 (for no-glucose or low-glucose). Hxt1 and Hxt3 are de-repressed in high glucose.
   - Both Mig1p (for high-glucose) and Rgt1p (for no-glucose) repress Hxt2 and Hxt4. These genes are de-repressed in low glucose.

[91] LUTFIYYA, L., et al., Op.Cit.
[92] LUTFIYYA, L., et al., Op.Cit.
[93] KANIAK, A., et al., Op.Cit.

3. ***SUC2:*** According to Özcan et al., "SUC2 expression, which has long been known to be repressed by high glucose concentrations, is modestly induced by low amounts (0,1%) of Glucose or Fructose, the products of Invertase cleavage of Sucrose. The cell must strike a balance between the amount of Sucrose that is cleaved and the amount utilized: cells growing on Sucrose must cleave enough of it to generate sufficient Glucose and Fructose to induce SUC2 expression and serve as a carbon source, but not so much that Glucose Repression ensues"[94]. The work of Kaniak et al. suggests that "SUC2 Induction" can be explained from the presence of Mig2 and Mig1 repression sites, and the interactions of Mig1 and Mig2 in the induction/repression network.

Finally, the authors also highlight the regulation of MTH1: "MTH1 expression is not induced by glucose but is nevertheless modestly repressed by Rgt1. In addition, MTH1 expression is modestly induced by galactose due to the regulation by the Gal4 transcriptional activator. Thus, Gal4 sustains and Rgt1 attenuates MTH1 expression in galactose-grown cells"[95].

2.3.4. RTG pathway

Liu and Butow reported a transcriptional switch between HAP and RTG (not Rgt) control in cells with compromised mitochondrial function (when mutations or conditions lead to, e.g., "inhibition of respiration, loss of TCA cycle activity, or loss of mitochondrial DNA"[96]). According to them, "…as the cell's respiratory function is reduced or eliminated, the expression of four TCA cycle genes, CIT1, ACO1, IDH1, and IDH2, switches from HAP control to control by three genes, RTG1, RTG2, and RTG3"[97]. However, the expression of four TCA cycle genes downstream α-ketoglutarate (KGD1, SDH1, FUM1 and MDH1) is independent of the RTG genes.

Besides, the authors report two regulatory loops: First, glutamate acts as a feedback regulator: α-ketoglutarate produces glutamate, which inhibits expression of RTG genes (which, in turn, control enzymes upstream α-ketoglutarate). Second, RTG genes control CIT2 gene from glyoxylate cycle when a mitochondrial dysfunction is present; in their words, "…RTG genes control the retrograde pathway, defined as a change in the expression of a subset of nuclear genes, e.g., the glyoxylate cycle CIT2 gene, in response to changes in the functional state of mitochondria"[98]. As a consequence, they propose that "…in cells with compromised mitochondrial function, the RTG genes take control of the expression of genes leading to the synthesis of α-ketoglutarate to ensure that sufficient glutamate is available for biosynthetic processes and that increased flux of the glyoxylate cycle, via elevated

[94] ÖZCAN, S., VALLIER, L., FLICK, J., CARLSON, M., JOHNSTON, M., Expression of the SUC2 gene of S. Cerevisiae is induced by low levels of glucose, *Yeast*, **13**, 1997
[95] KANIAK, A., et al., Op.Cit.
[96] LIU, Z., BUTOW, R., A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function, *Molecular and Cellular Biology*, **19**, 10, 1999
[97] LIU, Z., et al., Op.Cit.
[98] LIU, Z., et al., Op.Cit.

CIT2 expression, provides a supply of metabolites entering the TCA cycle sufficient to support anabolic pathways"[99].

2.3.5. Peroxisomal enzymes

Dmochowska et al.[100] reported the regulation of POX1, which encodes the peroxisomal acyl-coenzyme A oxidase; this enzyme catalyzes the degradation of fatty acids (myristic acid, palmitate, stearate) to acetyl CoA. Igual et al.[101] reported the regulation of POT1, which encodes the peroxisomal thiolase; this enzyme catalyzes the conversion of oxoacyl CoA to acetyl CoA. Young et al.[102] reported that Pox1, Cta1 (catalase), Fox2 (enoyl-CoA hydratase), Pot1 and Idp3 (isocitrate dehydrogenase NADP) are in the list of the 40 most highly glucose repressed genes and all of them are ADR1-dependent.

2.3.6. More on the gluconeogenic genes

Some authors (Yin et al., 2000) have also proposed that the Ras-cAMP pathway leads to the transcriptional repression of FBP1 and PCK1.

2.3.7. Discussion

The Mig1 transcriptional network, including the regulators Cat8, Hap4, Gal4 and MalR, can be easily reconstructed, based on reviews such as those written by Klein (1998) and Young (2003). Klein's review was made in 1998, but it is still the reference reported in yeast's databases such as CYGD.

The work of Kaniak et al. (2004) shows the high complexity derived from the cross-talk between glucose induction and repression pathways. This information leads us to propose the following model for Mig1 repression:

There will be a balance between three forces: The Mig1 phosphorylation (Snf1 reaction, transport from/to nucleus, recruiting of SSn6/Tup1), which is the main force; the feedback from the glucose induction pathway; and the feedback from self-regulation. This way, under low levels of glucose, we will have:

- More Mig1 phosphorylated: Less Mig1 repression.
- Mig1 repression is weak, so we will have more Snf3p and Mth1p. More Rgt1p means more repression of Mig2, i.e., more levels of Mig1 and more Mig1-repression.
- Mig1 repression is weak, so we will have more Mig1p and more Mig1-repression.

Under high levels of glucose, we will find the opposite situation.

The regulators Adr1, Imp2, Ngr1, among others, have been proved to be a part of this complex network but most of their connections still remain unclear. On the other

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[99] LIU, Z., et al., Op.Cit.
[100] DMOCHOWSKA, A., et al., Structure and transcriptional control of the Saccharomyces Cerevisiae POX1 gene encoding acyl-coenzyme A oxidase, *Gene*, **88**, 1990
[101] IGUAL, J., et al., The POT1 gene for yeast peroxisomal thiolase is subject to three different mechanisms of regulation, *Molecular Microbiology*, **6**, 1992
[102] YOUNG, E., Op.Cit.

Table 2.6. Post-transcriptional regulation of SDH1 and SDH2.

Evidence	*Details*
Glucose phosphorylation, and no further glycolytic metabolism, is required.	"Phosphorylation of glucose was required to trigger the rapid degradation of the Ip mRNA after a shift from YPG to YPG+D, but either Glk1p or Hxk1p can initiate the signalling"[103]. The authors report that addition of Fructose, instead of Dextrose, also triggers the Ip mRNA turnover. Other glycolytic enzymes, such as Pgi1, don´t seem to have any role in mRNA degradation; however, the metabolite Fructose-2,6-Bisphosphate has been proposed to have a sensor role.
REG1 affects Ip mRNA degradation.	After an arrest of transcription, Ip mRNA levels were measured by Northern analysis. "In the presence of REG1 gene product, the transcript was degraded rapidly in YPD. On the other hand, in the *reg1* mutant there was a rapid drop in the transcript level in YPD during the first 5 min, but then the level stabilized at ~50% of the original level. The Ip mRNA was equally stable in glycerol in the presence or absence of REG1 function. Thus, Reg1p appears to play a role in the glucose-sensitive turnover of the Ip transcript, but the biphasic decay of the mRNA observed in the above experiment suggests additional complexity in the process"[104].
Snf1 and Ume5 protein kinases are not required.	The Ip mRNA turnover doesn't seem to be affected by the absence of the Snf1 and Ume5 genes.
Acceleration depends on Cyc8 (Ssn6).	The *cyc8* and *tup1* mutants were not expected to be involved in mRNA turnover due to this process occurs in the cytoplasm; however, Ip mRNA turnover in the *cyc8* mutant was affected.
mRNA turnover and translation are linked.	"In our model, a signalling mechanism exists by which glucose regulates the competition between translation and turnover"[105].

hand, detailed information about transcription reactions of SUC2 and other genes is available in the literature, which, at least, supports the construction of a model for SUC2.

2.4. Post-Transcriptional Regulation

2.4.1. Evidence

According to Yin et al., "…a relatively small number of yeast mRNAs are known to be regulated post-transcriptionally by glucose"[106]. The main regulatory mechanism is the increase of the mRNA degradation in the presence of glucose, which is reflected in shorter half-lives of the mRNAs.

Cereghino and Scheffler studied the glucose expression regulation of the SDH1 and SDH2 genes (encoding the iron protein (Ip) and flavoprotein (Fp) subunits of the succinate dehydrogenase complex). According to them, the steady-state levels of their mRNAs are determined by their rate of turnover: "…the half-lives of the mRNAs appear to be < 5 min in glucose and >30 min in glycerol… Our findings indicate that most mutations in the transcriptional signalling mechanism do not alter

[103] CEREGHINO, G., et al., Op.Cit.
[104] CEREGHINO, G., et al., Op.Cit.
[105] CEREGHINO, G., et al., Op.Cit.
[106] YIN, Z., et al., Op.Cit.

the Ip transcript's turnover"[107]. The authors also highlight some facts (see Table 2.6.), which could indicate the presence of a different signalling mechanism.

SUC2 has also been proved to be post-transcriptionally controlled; Invertase mRNA stability decreases in the presence of glucose: "…the mechanism controlling mRNA stability not only modulates the expression of Ip and Fp mRNAs but also plays a role in the SUC2 gene expression… After yeast cells were shifted… the SUC2 transcript was degraded slowly after the cessation of transcription. However, if glucose was added to the same culture after transcription was halted, the same transcript was degraded much more rapidly"[108].

The Ume5 protein was initially proposed (Surosky et al., 1994) to be involved in glucose- post-transcriptional- regulation of some meiotic transcripts: "The half-lives of select meiotic transcripts are increased 2-fold in this mutant strain"[109]; e.g., "…SPO13 mRNA degradation accelerates about twofold in response to a high glucose signal (2%), but this response is blocked in *ume5* mutants"[110].

Additional complexity has been found in the genes FBP1 and PCK1 after glucose addition. They are known to be subject to transcriptional repression, mRNA degradation, allosteric inhibition, covalent modification and protein degradation. According to Yin et al., accelerated gluconeogenic mRNA degradation can be triggered by low concentrations of glucose (<0.02%). "Different amounts of glucose were added to mid-exponential yeast cultures growing on lactate, and the effects upon the mRNA degradation were tested by measuring mRNA levels after 15 min. The FBP1 and PCK1 mRNAs responded reproducibly to glucose concentration as low as 0.001%, whereas the Ip mRNA was only slightly affected by 1% glucose under these conditions… the FBP1, PCK1 and Ip mRNA were cleared rapidly from the cytoplasm after the addition of 2% glucose to mid-exponential gluconeogenic cultures"[111]. The authors reported a number of additional facts, summarized in Table 2.7.

Finally, "The Ras-cAMP pathway has been shown to be involved in the stimulated degradation of MALT mRNA and maltose permease inactivation (Wanke et al., 1997)"[112]. Klein et al. reports that "Ggs1 / Tps1 has been shown to be a positive regulator of fructose-1,6-bisphosphatase and maltose permease inactivation and of the stimulated mRNA degradation of FBP1, PCK1 and MALT transcripts (Bell et al., 1992; Wanke et al., 1997)"[113].

[107] CEREGHINO, G., SCHEFFLER, I., Genetic analysis of glucose regulation in S. cerevisiae: control of transcription versus mRNA turnover, *EMBO Journal*, **15**, 2, 1996
[108] CEREGHINO, G., et al., Op.Cit.
[109] CEREGHINO, G., et al., Op.Cit.
[110] YIN, Z., HATTON, L., BROWN, A., Differential post-transcriptional regulation of yeast mRNAs in response to high and low glucose concentrations, *Molecular Microbiology*, **35**, 3, 2000
[111] YIN, Z., et al., Op.Cit.
[112] KLEIN, C., et al., Op.Cit.
[113] KLEIN, C., et al., Op.Cit.

Table 2.7. Post-transcriptional regulation of FBP1 and PCK1.

Evidence	*Details*
Glucose phosphorylation, and no further glycolytic metabolism, is required.	Glucose and fructose triggered both transcriptional repression and mRNA degradation. Galactose, glycerol and lactate did not trigger either response. Besides, "…glycolytic blocks caused by mutations in the phosphoglucoisomerase, phosphofructokinase or pyruvate kinase genes did not inhibit the response"[114].
The acceleration of mRNA degradation depends on Snf3p.	"…rapid gluconeogenic mRNA degradation by *rgt2* mutation, but was blocked in the *snf3* strain. This suggests that the low glucose sensor Snf3p, but not the high glucose sensor Rgt2p, is required for post-transcriptional regulation of the gluconeogenic mRNAs in response to 0.02% glucose"[115]. The authors suggested that "Snf3p could mediate this effect either directly, by activating a signalling cascade, or indirectly, by promoting expression of the glucose transporters required for the generation of the intracellular signal"[116]. After knowing the above mentioned transcriptional network, we give more credit to the second hypothesis.
The acceleration of mRNA degradation depends on Reg1p.	"A low glucose signal (0.02%) did not promote accelerated PCK1 mRNA degradation in the *reg1* mutant, and this effect was reproducible"[117].
Ras-cAMP pathway does not seem to be necessary.	"…we examined FBP1 and PCK1 mRNA metabolism in a yeast strain without a functional Ras-cAMP pathway (*ras1, ras2, Δtpk1, tpk2^{wt}, Δtpk3, Δbcy1*)… Gluconeogenic mRNA degradation rates increased in this mutant after the addition of 0.02% glucose... Hence, an additional pathway can trigger this response"[118].
The acceleration of mRNA degradation depends on Ume5.	"Gluconeogenic mRNA degradation did accelerate in response to this signal, even in the *ume5* mutant. However, PCK1 degradation rates were consistently slower in the *ume5* mutant compared with its isogenic parent. Although this effect was not large, it was reproducible. Therefore, although Ume5p is not essential for accelerated gluconeogenic mRNA degradation in response to a low glucose signal, it does seem to play a positive role in this process. This further supports the existence of distinct glucose signalling pathways for the differential activation of gluconeogenic and Ip mRNA degradation in yeast"[119].
Sugar phosphates and low AMP levels have been proposed as potential signals that trigger mRNA degradation.	Some observations make the authors to support the first hypothesis: "First, the response was triggered by low levels of glucose (<0.01%) which would not be expected to cause significant changes in AMP levels. Second, the response did not require the glycolytic metabolism, but this would presumably be required to raise ATP levels after glucose addition"[120].

2.4.2. Discussion

Given that the information on the post-transcriptional control is limited to the influence of specific genes, but there is not a clear picture of a pathway to model, the only way to include this effect in our work is to generate a glucose post-transcriptional signal that represents all the processes involved.

[114] YIN, Z., et al., Op.Cit.
[115] YIN, Z., et al., Op.Cit.
[116] YIN, Z., et al., Op.Cit.
[117] YIN, Z., et al., Op.Cit.
[118] YIN, Z., et al., Op.Cit.
[119] YIN, Z., et al., Op.Cit.
[120] YIN, Z., et al., Op.Cit.

2.5. Microarray experiments related to glucose de-repression

A high-throughput technique, such as micro-arrays, can be combined with the specific mechanisms presented in the previously discussed studies in order to get a broader perspective of glucose de-repression. The concentration of each mRNA is correlated with the transcription efficiency and the mRNA degradation, so micro-arrays may report the results after both transcriptional and post-transcriptional changes, depending on the time of sampling.

We will present the work of Yin et al. (2003). Although they generated the results presented in Table 2.2. (global response to glucose signal), their article is focused on the significant primary responses found in carbon metabolism, hexose transporters and ribosome biogenesis.

2.5.1. Central Carbon Metabolism

As Nielsen states: "…this part of the metabolism is tightly connected to most other parts of the cellular metabolism and it is therefore also the part of the complete metabolic network that is most interesting to study"[121]. Besides, it is directly connected to the glucose de-repression switch (fermentation / respiration). For this reason, we start showing the fold-change response of genes involved in carbon metabolism in Table 2.8.

Yin et al. conclude that: "Most glycolytic mRNAs displayed significant increases within 30 min of exposure to a high glucose signal… The only exceptions were the mRNAs for Glk1, Gpm2, Gpm3 and Pyk2, all of which play minor roles in fermentation compared with their functionally redundant homologues. As expected, all gluconeogenic and glyoxylate cycle mRNAs and most TCA cycle mRNAs were repressed by the high glucose signal"[122].

2.5.2. Hexose transporters

According to Reifenberger, "…it has been thought that glucose transport in *S. cerevisiae* is mediated by two kinetically distinct systems. One is a glucose-repressible high-affinity system… and the other is a constitutive low-affinity system…"[123]. Yin adds that there are "…genetic and biochemical data that indicate that HXT1-7 are the major players in glucose uptake… HXT8-17 are not thought to contribute significantly to glucose uptake"[124].

According to Johnston, there are four main high affinity hexose transporters (Hxt2, Hxt4, Hxt6, Hxt7) and two main low affinity hexose transporters (Hxt1 and Hxt3). High affinity implies low capacity (Km ~ 1-10 mM); the promoters of these genes have Mig1 binding sites, so they are repressed when high glucose, and repressed by

[121] NIELSEN, J., Op.Cit.
[122] YIN, Z., et al., Glucose triggers different global responses in yeast, depending on the strength of the signal, and transiently stabilizes ribosomal protein mRNAs, *Molecular Microbiology*, **48**, 3, 2003
[123] REIFENBERGER, E., Kinetic characterization of individual hexose transporters of S.Cerevisiae and their relation to the triggering mechanisms of glucose repression, *Eur. J. Biochem.*, **245**, 1997
[124] YIN, Z., et al., Op.Cit.

Table 2.8. Fold-change of genes involved in carbon metabolism, in response to low and high glucose signals, compared to control cells (no glucose), after 30 min of exposure. (Conventions: Yellow = Induced, Cyan = Repressed, Red = Highly Induced, Blue = Highly Repressed).

Glycolysis			***TCA Cycle***		
Gene	**0.10 %**	**1.0%**	**Gene**	**0.10 %**	**1.0%**
Glk1	1.04	-1.81	Aco1	-3.76	-8.85
Hxk1	5.00	1.21	Cit1	-2.43	-5.77
Hxk2	1.42	1.39	Cit2	-1.02	1.59
Pgi1	1.12	1.68	Cit3	-1.42	-1.49
Pfk1	-1.05	1.53	Fum1	-1.64	-2.25
Pfk2	2.10	2.65	Idh1	-1.73	-2.90
Fba1	3.72	4.61	Idh2	-1.58	-2.31
Tpi1	2.27	3.69	Idp1	-1.11	1.32
Tdh1	2.20	2.22	Idp2	-7.46	-7.84
Tdh2	1.61	1.76	Kgd1	-2.06	-3.55
Tdh3	1.40	1.90	Kgd2	-1.63	-2.69
Pgk1	2.87	3.46	Lpd1	-2.10	-2.13
Gpm1	1.80	2.05	Lsc1	-1.40	-1.63
Gpm2	1.22	-1.18	Lsc2	-1.28	-2.66
Gpm3	-1.32	-1.17	Mdh1	-1.49	-3.05
Eno1	2.73	4.15	Osm1	-1.65	-1.40
Eno2	3.88	6.29	Sdh1	-1.52	-2.15
Pyk1	3.64	5.36	Sdh2	-1.31	-2.71
Pyk2	1.28	-1.30	Sdh3	-1.59	-2.47
Pda1	1.12	-1.21	Sdh4	-1.24	-2.21
Pdb1	-2.63	-2.03			
Gluconeogenesis			***Glyoxylate Cycle***		
Gene	**0.10 %**	**1.0%**	**Gene**	**0.10 %**	**1.0%**
Fbp1	-3.81	-3.28	Icl1	-5.31	-4.82
Pck1	-4.80	-5.98	Mls1	-3.79	-3.29
Pyc1	-1.73	-2.42			
Pyc2	-2.39	-2.11			

Source: Adapted from: Yin, Z., et al., 2003, Op.Cit.

Rgt1 when no glucose; only expressed in low levels (which is appropriate, because they have low capacity). Low affinity means high capacity (Km ~ 50-100 mM); they are induced in high glucose, which is also appropriate.

Reifenberger, Johnston, Lafuente and Yin present different reports about hexose transporters (Table 2.9.a,b). However, they agreed that Hxt1 and Hxt3 work for high glucose and Hxt2, Hxt6 and Hxt7 for low glucose.

Table 2.9.a. Differences in reports about Hexose Transporters.

REFERENCE	LOW AFFINITY	MEDIUM AFFINITY	HIGH AFFINITY
Reifenberger, 1997	Hxt1 (~100 mM) and Hxt3 (~60 mM)	Hxt4 (~10 mM)	Hxt2 (1,5 or 60 mM in low glucose), Hxt6 and Hxt7 (~1 mM)
Johnston, 1999	Hxt1 and Hxt3 (50-100 mM)	-	Hxt2, Hxt4, Hxt6 and Hxt7 (1-10 mM)

Table 2.9.b. Differences in reports about Hexose Transporters.

REFERENCE	HIGH GLUCOSE	LOW GLUCOSE
Lafuente, 2000	Hxt1 and Hxt3	Hxt2, Hxt3 and Hxt4
Yin, 2003	Hxt1, Hxt3 and Hxt4 (1%)	Hxt2, Hxt6 and Hxt7 (0,1%)

Lafuente and Yin differ in their reports for Hxt3 and Hxt4. According to the transcriptional control information (section 2.3.3.), Hxt3 is Rgt1-repressed in low glucose and de-repressed in high (which doesn't fit with Lafuente's observation); the same way, Hxt4 is Mig1-repressed in high glucose and de-repressed in low glucose, which doesn't fit with the data of Yin et al (cf. Table 2.10.).

Finally, Yin et al. report the significant increasing of ribosome biogenesis, due to cell needs to translate more proteins. We will ignore Yin's considerations about ribosomes, because they are not directly related to fermentation or respiration processes.

Table 2.10. Fold-change of Hexose Transporter mRNAs, in response to low and high glucose signals, compared to control cells (no glucose), after 30 min of exposure. (Conventions: Yellow = Induced, Cyan = Repressed, Red = Highly Induced, Blue = Highly Repressed).

Hexose Transporter Family					
Gene	**0.10%**	**1.0%**	**Gene**	**0.10%**	**1.0%**
Hxt1	5.18	8.97	Hxt12	-1.10	-1.26
Hxt2	14.99	5.47	Hxt12	-1.24	-1.08
Hxt3	11.38	9.05	Hxt13	1.01	-1.10
Hxt4	1.41	1.66	Hxt14	-1.34	-1.45
Hxt5	1.46	-1.24	Hxt15	-1.00	-1.06
Hxt6	10.25	3.28	Hxt16	-2.08	-2.12
Hxt7	12.06	4.06	Hxt17	-1.16	-1.17
Hxt8	-1.37	-1.65	Gal2	1.13	1.06
Hxt9	1.05	-1.01	Rgt2	-1.54	-1.25
Hxt10	1.17	1.12	Snf3	-1.08	-1.18
Hxt11	1.17	-1.00			

Source: Adapted from: Yin, Z., et al., Op.Cit.

2.5.3. Limitations of this approach

Essentially, taking samples in just two points of time (pre-post assays) can obscure the complex dynamics of transcription.

There are different scales of time for each gene to act (ranging from 1 min to more than 30 min); some responses are short-term responses, some present a peak of activity and return to previous levels, some present biphasic patterns (as SUC2 does). Besides, glucose triggers metabolic effects in the whole cell, so we could detect primary (transcriptionally controlled) or secondary effects (changes due to signals coming from changes in primary responses; no direct control). According to Yin et al., a long-term measure would show all type of effect (primary or indirect), so they choose a period of time of 30 min (short-term) because, in this period, "…primary glucose responses would be activated, but activation of secondary and tertiary responses to glucose addition was less likely"[125]. However, they found that "…most glucose-responsive mRNAs had not reached their new steady-state levels within 30 min and, as a result, most fold changes in expression were relatively low"[126]. For this reason, they used statistical methods to "assess the significance of each fold change for every gene" and to determine what a significant effect is.

2.5.4. Summary

Mixing all reviewed reports (in a non-rigorous way), we summarize the candidate gene products (mRNA and proteins) to be included in a rigorous glucose de-repression model in Table 2.11.

Table 2.11. Gene products reported as involved in glucose de-repression studies.

Category	Gene products
Proteins involved in Snf1-signalling pathway	Hxk2, Snf1, Sip1, Sip2, Snf4, Gal83, Reg1, Glc7, Sip5, Mig1, Ssn6, Tup1
mRNA (and proteins) involved in Mig1-transcriptional network	Mig1, Mig2, Rgt1, Snf3, Mth1, Std1
mRNA (and proteins) of transcriptional regulators	Gal4, MalR, Hap4, Cat8, Msn2, Adr1, Sip4, Imp2, Nrg1, Rtg1-3
mRNA in peripheral pathways of carbon metabolism	Suc2, Hxt1-4, Hxt6-7, Adh2, Adh5, Gut1, Glk1, Hxk1-2, Gal2, Gal1, Fps1, MalT, MalS
Proteins involved in post-transcriptional modification	Ras, PkA
mRNA in central carbon metabolism	Gluconeogenesis (Fbp1, Pck1), Pyruvate (Pdc1, Ald2, Ald4-5, Acs1), Glx cycle (Icl1-2, Mls1, Mdh2) and TCA cycle (Aco1, Cit1, Cit3, Idh1, Lpd1, Kgd1-2, Lsc2, Sdh2, Mdh1)
	Glycolysis (Pfk2, Fba1, Tpi1, Pgk1, Gpm1, Eno1, Eno2, Pyk1), Galactose (Gal3, Gal7, Gal10, Pgm2), Trehalose (Tps1, Nth1), Respiratory (Cyt1, Cyb2, …) and Transport (Oac1, Ctp1)
mRNA in peroxisome biogenesis and β-oxidation	Pox1, Cta1, Fox2, Pot1, Idp2-3

[125] YIN, Z., et al., Op.Cit.
[126] Idem.

2.6. Post-Translational modifications

Translational and post-translational modifications of glucose-related transcripts also need to be taken into account, specially the efficiency of translation and the degradation of protein (because the concentration of a protein depends on the balance between them).

On the translational level, decrease of the translational efficiency due to glucose has been reported; however, this effect seems to be linked to mRNA turnover (Caponigro and Parker, 1996). More important is the post-translational effect known as "carbon catabolite inactivation" (accelerated protein degradation). According to Klein, "Carbon catabolite inactivation affects enzymes of peripheral functions, for example maltose permease and galactose permease… as well as enzymes of central functions such as the gluconeogenic enzymes fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase… Vacuolar and proteasomal proteolysis have recently been identified as possible mechanisms of degradation, the former particularly for carrier proteins"[127]. According to Busturia and Lagunas (1986), glucose transporters are also subject to catabolite inactivation.

The Ras-cAMP pathway seems to be involved in this process: "Sugar phosphorylation by any hexokinase (Hxk1, Hxk2, Glk1) leads to an accumulation of cAMP via the Ras-cAMP pathway, inducing a massive increase of cAMP-dependent protein kinase, which modifies a number of proteins post-translationally"[128].

2.7. Related Metabolic pathways

2.7.1. Introduction

In principle, a "related pathway" could be defined as each well-known metabolic pathway (a preferred pathway for flux through the network) whose enzymes (most of them, or some of them in a strong way) are controlled by glucose. With this definition we are skipping pathways whose flux could be affected by the "directly-controlled" pathways via some metabolites (in our case, we will not consider anabolic reactions such as the production of aminoacids, structural polysaccharides, phospholipids, sterols or fatty acids from glycolytic metabolites).

However, even using the previous definition, it would be impossible to study all "related pathways" because not all glucose-regulated proteins have been well-studied. Summarizing (see Table 2.11.), it has been reported that glucose represses enzymes of TCA cycle, gluconeogenesis, glyoxylate cycle, respiratory chain, trehalose pathway, and sucrose/raffinose, galactose, melibiose and maltose uptake, whereas activates enzymes for glycolysis and production of hexose (glucose, fructose) transporters. Activation of ribosome biogenesis and repression of peroxisomal enzymes has also been reported. Microarray data shows that enzymes belonging to additional pathways need to be characterized but, for our purposes, the previous list is good enough.

[127] KLEIN, C., et al., Op.Cit.
[128] KLEIN, C., et al., Op.Cit.

Those processes not directly connected to the "Crabtree effect" will be skipped, as well as the presence of certain sugars in the medium. Therefore, in this chapter we will consider glycolysis, TCA cycle, glyoxylate cycle, gluconeogenesis, raffinose metabolism (and, therefore, fructose and galactose metabolism), ethanol / acetate branch from pyruvate, glycerol metabolism and hexose transporters (notice that we skipped melibiose and maltose uptake, storage carbohydrates metabolism (glycogen and trehalose), respiratory chain, peroxisomal enzymes and ribosome biogenesis).

Metabolic pathways are better characterized than signalling pathways. In fact, the complete metabolic network of *S. cerevisiae* has been reported by Förster et al.[129]. In this chapter, we will describe the announced pathways and we will discuss their localization, full set of reactions and control issues. A complete list of metabolites and the symbols we will use for them is in Appendix 1. The complete metabolic maps can be seen in the appendices 3 and 4.

2.7.2. Glycolysis in *S. cerevisiae*

2.7.2.1. Description

According to Berg et al., "Glycolysis is the sequence of reactions that metabolizes one molecule of glucose to two molecules of pyruvate with the concomitant net production of two molecules of ATP"[130]. In general, glycolysis can be summarized in three stages:

- In early glycolysis, glucose binds to a hexokinase, which phosphorylates it to glucose-6-phosphate (which cannot diffuse through the membrane) (reaction 4 in Table 2.12.); then, phosphoglucose isomerase changes the aldehyde group into a keto group, producing fructose-6-phosphate (reaction 5); and, finally, phosphofructokinase adds a second phosphoryl group to the molecule, generating fructose-1,6-bisphosphate (reaction 8).
- In middle glycolysis, aldolase transform fructose-1,6-bisphosphate to two trioses: DHAP (a ketose) and GAP (an aldose) (reaction 9). Triose phosphate isomerase interconvert DHAP and GAP (reaction 10).
- In late glycolysis, GAP dehydrogenase replaces the H from the aldehyde group of GAP with a phosphoryl group, to form 1,3-bisphosphoglycerate (reaction 11). Such a compound has a high phosphoryl-transfer potential, which is useful to generate ATP. Phosphoglycerate kinase produces 3-phosphoglycerate (reaction 12). Phosphoglycerate mutase changes the position of the phosphoryl group to produce 2-phosphoglycerate (reaction 14). Enolase catalyzes the dehydration of 2-phosphoglycerate to form phosphoenolpyruvate, that also has a high phosphoryl-transfer potential (reaction 15). And, finally, pyruvate kinase produces a stable ketone (pyruvate) and two molecules of ATP (profit of glycolysis).

2.7.2.2. Compartmentalization

Glycolysis occurs in the cytoplasm.

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[129] FÖRSTER, J., et al., Genome-scale reconstruction of the Saccharomyces Cerevisiae metabolic network, *Genome Res.*, **13**, 2003

[130] BERG, J., TYMOCZKO, J., STRYER, L., Biochemistry, 5th ed., W.H. Freeman & Co., 2002

Table 2.12. Glycolytic reactions in *Saccharomyces cerevisiae*.

	Reaction	Enzyme	ORF(s)	Gene(s)	Reaction Type
Early Glycolysis (Glucose is trapped and converted to less-stable compounds)					
1	GLC + ATP -> G6P + ADP	GLUCOKINASE	*YCL040W*	*GLK1*	Phosphoryl Transfer
2	bDGLC + ATP -> bDG6P + ADP	GLUCOKINASE	*YCL040W*	*GLK1*	Phosphoryl Transfer
3	bDGLC + ATP -> G6P + ADP	HEXOKINASE (HEXOKINASE I, HEXOKINASE II)	*YFR053C, YGL253W*	*HXK1, HXK2*	Phosphoryl Transfer
4	GLC + ATP -> G6P + ADP	HEXOKINASE (HEXOKINASE I, HEXOKINASE II)	*YFR053C, YGL253W*	*HXK1, HXK2*	Phosphoryl Transfer
5	G6P <-> F6P	GLUCOSE-6-PHOSPHATE ISOMERASE	*YBR196C*	*PGI1*	Isomerization
6	G6P <-> bDG6P	GLUCOSE-6-PHOSPHATE ISOMERASE	*YBR196C*	*PGI1*	Isomerization
7	bDG6P <-> F6P	GLUCOSE-6-PHOSPHATE ISOMERASE	*YBR196C*	*PGI1*	Isomerization
8	F6P + ATP -> FDP + ADP	PHOSPHOFRUCTOKINASE (BETA SUBUNIT + ALPHA SUBUNIT)	*YMR205C* + *YGR240C*	*PFK2* + *PFK1*	Phosphoryl Transfer
Mid Glycolysis (Six-carbon molecules are converted to three-carbon molecules)					
9	FDP <-> DHAP + GAP	FRUCTOSE-BISPHOSPHATE ALDOLASE	*YKL060C*	*FBA1*	Aldol cleavage
10	DHAP <-> GAP	TRIOSEPHOSPHATE ISOMERASE	*YDR050C*	*TPI1*	Isomerization
Late Glycolysis (ATP generation)					
11	GAP + PI + NAD <-> NADH + 13PDG	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (1 2 3)	*YJL052W YJR009C YGR192C*	*TDH1 TDH2 TDH3*	Phosphorylation coupled to Oxidation
12	13PDG + ADP <-> 3PG + ATP	PHOSPHOGLYCERATE KINASE	*YCR012W*	*PGK1*	Phosphoryl Transfer
13	13PDG <-> 23PDG	PHOSPHOGLYCERATE MUTASE	*YKL152C*	*GPM1*	Phosphoryl Shift
14	3PG <-> 2PG	PHOSPHOGLYCERATE MUTASE, PROTEIN SIMILAR TO GPM1, PHOSPHOGLYCERATE MUTASE	*YKL152C, YDL021W, YOL056W*	*GPM1, GPM2, GPM3*	Phosphoryl Shift
15	2PG <-> PEP	ENOLASE (ENOLASE I, ENOLASE, PROTEIN WITH SIMILARITY TO ENOLASES, ENOLASE RELATED PROTEIN, ENOLASE RELATED PROTEIN)	*YGR254W, YHR174W, YMR323W, YPL281C, YOR393W*	*ENO1, ENO2, ERR1, ERR2, ERR1*	Dehydration
16	PEP + ADP -> PYR + ATP	PYRUVATE KINASE	*YAL038W*	*CDC19*	Phosphoryl Transfer
17	PEP + ADP -> PYR + ATP	PYRUVATE KINASE, GLUCOSE-REPRESSED ISOFORM	*YOR347C*	*PYK2*	Phosphoryl Transfer

Source: Adapted from: Genetic Circuits Research Group (Bernhard Palsson), Website Data, 2004, and Berg, Op.Cit., 2002

2.7.2.3. Full list of reactions

The full list of reactions and enzymes (with the ORFs and genes coding for each enzyme) are presented in Table 2.12.

2.7.3. Pyruvate metabolism in *S. cerevisiae*

2.7.3.1. Description

Pyruvate is a key point for the respiration/fermentation switch. If most of the pyruvate is processed following reactions 1 and 2 (in Table 2.13.), the yeast will be in a fermentative state. If most of the pyruvate goes to reactions 6 and 7, the yeast will be in a respiratory state (pyruvate also has a role in gluconeogenesis, which we will review later, and metabolism of aminoacids, which is not the subject of this work).

Fermentation was defined by Louis Pasteur as "la vie sans l'air" (the life without air)[131]. In fermentation, pyruvate is decarboxylated to acetaldehyde (pyruvate wins a H and loses a CO_2) (reaction 1). Acetaldehyde is converted to ethanol in a reversible reaction (ethanol loses two hydrogens to form an aldehyde, or vice versa) (reaction 2).

The other option is the transport of pyruvate to the mitochondria (reaction 6), where pyruvate dehydrogenase complex transforms it to acetyl CoA by means of an oxidative decarboxylation (reaction 7).

2.7.3.2. Compartmentalization

Table 2.13. shows both cytosolic and mitochondrial reactions, as well as the transport between the two phases.

2.7.3.3. Full list of reaction

The full list of reactions and enzymes involved in pyruvate metabolism in *S. cerevisiae* are presented in Table 2.13.

2.7.4. TCA Cycle in *S. cerevisiae*

2.7.4.1. Description

In the respiration alternative, acetyl CoA can produce CO_2 and water, after the TCA cycle and the oxidative phosphorylation. In general, the TCA cycle (citric acid cycle, Krebs cycle or tricarboxylic acid cycle) is the oxidation of C_2 compounds, in order to generate two molecules of CO_2 and five energy carrier molecules: one of ATP, three of NADH and one of $FADH_2$. The NADH and $FADH_2$ molecules possess eight high-energy electrons that are used to convert 9 more ADPs to ATPs in the respiratory chain.

In summary, there are eight important enzymes / reactions:

- Citrate synthase (reaction 16 in Table 2.14.): Oxaloacetate condenses with acetyl CoA to form citryl CoA, which is then hydrolyzed to citrate and CoA.
- Aconitase (reaction 17): Isomerization of citrate is accomplished by: A dehydration, where citrate becomes cis-aconitate; and a hydration, where cis-aconitate becomes isocitrate.
- Isocitrate dehydrogenase (reaction 18, 19 and 20): Isocitrate is oxidized and decarboxylated to alpha-ketoglutarate.

[131] BERG, J., et al., Op.Cit.

Table 2.13. Pyruvate metabolism in *Saccharomyces cerevisiae*.

	Reaction	Enzyme	ORF(s)	Gene(s)
Cytosolic Reactions				
1	PYR -> CO2 + ACAL	PYRUVATE DECARBOXYLASE	*YGR087C, YLR134W, YLR044C*	*PDC6, PDC5, PDC1*
2	ETH + NAD <-> ACAL + NADH	ALCOHOL DEHYDROGENASE (ISOENZYME IV, II, ISOENZYME V, I and I)	*YGL256W, YMR303C, YBR145W, YOL086C, YDL168W*	*ADH4, ADH2, ADH5, ADH1, SFA1*
3	ACAL + NADP -> NADPH + AC	CYTOSOLIC ALDEHYDE DEHYDROGENASE	*YPL061W*	*ALD6*
4	ATP + AC + COA -> AMP + PPI + ACCOA	ACETYL-COENZYME A SYNTHETASE	*YAL054C, YLR153C*	*ACS1, ACS2*
5	ACCOA -> COA + AC	ACETYL COA HYDROLASE	*YBL015W*	*ACH1*
Transport Reactions				
6	PYR <-> PYRm + Hm	PYRUVATE CARRIER	*Unknown*	*Unknown*
	CO2 <-> CO2m, ETH <-> ETHm and ACm <-> AC, DIFFUSE THROUGH THE INNER MITOCHONDRIAL MEMBRANE IN A NON-CARRIER-MEDIATED MANNER			
Mitochondrial Reactions				
7	PYRm + COAm + NADm -> NADHm + CO2m + ACCOAm	PYRUVATE DEHYDROGENASE (E1 COMPONENT, ALPHA UNIT + E1 COMPONENT, BETA UNIT + E2 COMPONENT + E3 COMPONENT)	*YER178W + YBR221C + YNL071W*	*PDA1 + PDB1 + LAT1 + LPD1*
8	ETHm + NADm <-> ACALm + NADHm	ALCOHOL DEHYDROGENASE ISOENZYME III	*YMR083W*	*ADH3*
9	ACALm + NADm -> NADHm + ACm	MITOCHONDRIAL ALDEHYDE DEHYDROGENASE	*YOR374W*	*ALD4*
10	ACALm + NADPm -> NADPHm + ACm	MITOCHONDRIAL ALDEHYDE DEHYDROGENASE	*YOR374W, YER073W*	*ALD4, ALD5*

Source: Adapted from: Genetic Circuits Research Group (Bernhard Palsson), Website Data, 2004. LPD1 information from www.yeastgenome.org.

- Alpha-ketoglutarate dehydrogenase complex (reaction 21): Catalyzes the oxidative decarboxylation of alpha-ketoglutarate.
- Succinyl CoA synthetase (reaction 22): CoA is displaced by orthophosphate, which generates succinyl phosphate; the enzyme takes the phosphoryl and releases succinate.
- Succinate dehydrogenase (reaction 23): Succinate is oxidized (to fumarate).
- Fumarase (reaction 26): Fumarate is hydrated to L-malate.
- Malate dehydrogenase (reaction 27): Malate is oxidized to form oxaloacetate.

2.7.4.2. Compartmentalization

TCA cycle takes place in mitochondria.

2.7.4.3. Full list of reactions

A full list of TCA cycle reactions and enzymes in *S. cerevisiae* are presented in Table 2.14.

Table 2.14. TCA cycle in *Saccharomyces cerevisiae*.

	Reaction	Enzyme	ORF(s)	Gene(s)
Cytosolic Reactions				
1	ACCOA + OA -> COA + CIT	CITRATE SYNTHASE, NON-MITOCHONDRIAL CITRATE SYNTHASE	*YCR005C*	*CIT2*
2	ICIT + NADP -> NADPH + OSUC	ISOCITRATE DEHYDROGENASE (NADP+)	*YLR174W, YNL009W*	*IDP2, IDP3*
3	OSUC -> CO2 + AKG	ISOCITRATE DEHYDROGENASE (NADP+)	*YLR174W, YNL009W*	*IDP2, IDP3*
4	FUM <-> MAL	FUMARATASE	*YPL262W*	*FUM1*
5	MAL + NAD <-> NADH + OA	MALATE DEHYDROGENASE, PEROXISOMAL	*YDL078C*	*MDH3*
6	MAL + NAD <-> NADH + OA	MALATE DEHYDROGENASE, CYTOPLASMIC	*YOL126C*	*MDH2*
Transport Reactions				
7	OA <-> OAm + Hm	MITOCHONDRIAL OXALOACETATE CARRIER	*YKL120W*	*OAC1*
8	CIT + MALm <-> CITm + MAL	CITRATE TRANSPORT PROTEIN	*YBR291C*	*CTP1*
9	CIT + PEPm <-> CITm + PEP	CITRATE TRANSPORT PROTEIN	*YBR291C*	*CTP1*
10	CIT + ICITm <-> CITm + ICIT	CITRATE TRANSPORT PROTEIN	*YBR291C*	*CTP1*
11	AKGm + OXA <-> AKG + OXAm	2-OXODICARBOYLATE TRANSPORTER	*YPL134C, YOR222W*	*ODC1, ODC2*
12	MAL + SUCCm <-> MALm + SUCC	DICARBOXYLATE CARRIER	*YLR348C*	*DIC1*
13	SUCC + PIm -> SUCCm + PI	DICARBOXYLATE CARRIER	*YLR348C*	*DIC1*
14	SUCC + FUMm -> SUCCm + FUM	MITOCHONDRIAL MEMBRANE SUCCINATE-FUMARATE TRANSPORTER, MEMBER OF THE MITOCHONDRIAL CARRIER FAMILY (MCF) OF MEMBRANE TRANSPORTERS	*YJR095W*	*SFC1*
15	MAL + PIm <-> MALm + PI	DICARBOXYLATE CARRIER	*YLR348C*	*DIC1*
Mitochondrial Reactions				
16	ACCOAm + OAm -> COAm + CITm	CITRATE SYNTHASE (NUCLEAR ENCODED MITOCHONDRIAL PROTEIN, MITOCHONDRIAL ISOFORM OF CITRATE SYNTHASE)	*YNR001C, YPR001W*	*CIT1, CIT3*
17	CITm <-> ICITm	MITOCHONDRIAL ACONITASE, ACONITATE HYDRATASE HOMOLOG	*YLR304C, YJL200C*	*ACO1, YJL200C*
18	ICITm + NADm -> CO2m + NADHm + AKGm	ISOCITRATE DEHYDROGENASE (NAD+) MITOCHONDRIAL (SUBUNIT1 + SUBUNIT2)	*YNL037C + YOR136W*	*IDH1 + IDH2*
19	ICITm + NADPm -> NADPHm + OSUCm	ISOCITRATE DEHYDROGENASE (NADP+)	*YDL066W*	*IDP1*
20	OSUCm -> CO2m + AKGm	ISOCITRATE DEHYDROGENASE (NADP+)	*YDL066W*	*IDP1*
21	AKGm + NADm + COAm -> CO2m + NADHm + SUCCOAm	ALPHA-KETOGLUTARATE DEHYDROGENASE COMPLEX (E1 COMPONENT + E2 COMPONENT)	*YIL125W + YDR148C*	*KGD1 + KGD2 LPD1*

Table 2.14. Continued.

	Reaction	Enzyme	ORF(s)	Gene(s)
Mitochondrial Reactions, continued				
22	ATPm + SUCCm + COAm <-> ADPm + PIm + SUCCOAm	SUCCINATE--COA LIGASE (GDP-FORMING)	*YGR244C*	*LSC2*
23	SUCCm + FADm <-> FUMm + FADH2m	SUCCINATE DEHYDROGENASE (CYTOCHROME B + CYTOCHROME B + IRON-SULFUR PROTEIN SUBUNIT + MEMBRANE ANCHOR SUBUNIT)	*YKL141W* + *YKL148C* + *YLL041C* + *YDR178W*	*SDH3* + *SDH1* + *SDH2* + *SDH4*
16	ACCOAm + OAm -> COAm + CITm	CITRATE SYNTHASE (NUCLEAR ENCODED MITOCHONDRIAL PROTEIN, MITOCHONDRIAL ISOFORM OF CITRATE SYNTHASE)	*YNR001C, YPR001W*	*CIT1, CIT3*
17	CITm <-> ICITm	MITOCHONDRIAL ACONITASE, ACONITATE HYDRATASE HOMOLOG	*YLR304C, YJL200C*	*ACO1, YJL200C*
18	ICITm + NADm -> CO2m + NADHm + AKGm	ISOCITRATE DEHYDROGENASE (NAD+) MITOCHONDRIAL (SUBUNIT1 + SUBUNIT2)	*YNL037C* + *YOR136W*	*IDH1* + *IDH2*
19	ICITm + NADPm -> NADPHm + OSUCm	ISOCITRATE DEHYDROGENASE (NADP+)	*YDL066W*	*IDP1*
20	OSUCm -> CO2m + AKGm	ISOCITRATE DEHYDROGENASE (NADP+)	*YDL066W*	*IDP1*
21	AKGm + NADm + COAm -> CO2m + NADHm + SUCCOAm	ALPHA-KETOGLUTARATE DEHYDROGENASE COMPLEX (E1 COMPONENT + E2 COMPONENT)	*YIL125W* + *YDR148C*	*KGD1* + *KGD2* *LPD1*
22	ATPm + SUCCm + COAm <-> ADPm + PIm + SUCCOAm	SUCCINATE--COA LIGASE (GDP-FORMING)	*YGR244C*	*LSC2*
23	SUCCm + FADm <-> FUMm + FADH2m	SUCCINATE DEHYDROGENASE (CYTOCHROME B + CYTOCHROME B + IRON-SULFUR PROTEIN SUBUNIT + MEMBRANE ANCHOR SUBUNIT)	*YKL141W* + *YKL148C* + *YLL041C* + *YDR178W*	*SDH3* + *SDH1* + *SDH2* + *SDH4*
	ORFS YLR164W, YMR118C AND YJL045W, HAVE STRONG SIMILARITY TO SOME OF THE PREVIOUS GENES			
24	FADH2m + FUMm -> SUCCm + FADm	MITOCHONDRIAL SOLUBLE FUMARATE REDUCTASE INVOLVED IN OSMOTIC REGULATION	*YJR051W*	*OSM1*
25	FADH2m + FUM -> SUCC + FADm	SOLUBLE FUMARATE REDUCTASE, CYTOPLASMIC	*YEL047C*	*YEL047 C*
26	FUMm <-> MALm	FUMARATASE	*YPL262W*	*FUM1*
27	MALm + NADm <-> NADHm + OAm	MITOCHONDRIAL MALATE DEHYDROGENASE	*YKL085W*	*MDH1*

Source: Adapted from: Genetic Circuits Research Group (Bernhard Palsson), Website Data, 2004. LPD1 information from www.yeastgenome.org.

2.7.5. Glyoxylate cycle and gluconeogenesis in *S. cerevisiae*

2.7.5.1. Description

Gluconeogenesis is the generation of glucose from noncarbohydrate precursors (ethanol, glycerol, etc); however, here we will start the description from cytosolic pyruvate. In summary, gluconeogenesis uses most of the reversible reactions of glycolysis (following the opposite direction), and by-passes the two highly irreversible reactions (F6P to FDP, and PEP to PYR) with three new reactions (3 new enzymes). This way, the pathway starts with reactions 5 (PYR to OA) and 3 (OA to PEP) from Table 2.15., which are driven by the enzymes pyruvate carboxylase and PEP carboxylkinase, respectively; then, it uses the same enzymes of glycolysis, from enolase to phosphoglucose isomerase (PEP to 2PG, 2PG to 3PG, 3PG to 13PG, 13PG to GAP, GAP and DHAP to FDP), because equilibrium leads reactions from bottom to top. At the end, FDP is converted to F6P by fructose 1,6-bisphosphatase (reaction 4), and F6P converts to G6P, as in glycolysis. Although there is a high degree of overlapping, "Both pathways are stringently controlled by intercellular and intracellular signals, and they are reciprocally regulated so that glycolysis and gluconeogenesis do not take place simultaneously in the same cell to a significant extent… Both glycolysis and gluconeogenesis are highly exergonic under cellular conditions, and so there is no thermodynamic barrier to such simultaneous activity. However, the amounts and activities of the distinctive enzymes of each pathway are controlled so that both pathways are not highly active at the same time"[132].

2.7.5.2. Compartmentalization

Glyoxylate cycle and gluconeogenic reactions take place in the cytoplasm.

2.7.5.3. Full list of reactions

A list of glyoxylate and gluconeogenic reactions is shown in Table 2.15.

Table 2.15. Anaplerotic reactions in *Saccharomyces cerevisiae*.

	Reaction	Enzyme	ORF(s)	Gene(s)
Glyoxylate Pathway				
1	ICIT -> GLX + SUCC	ISOCITRATE LYASE	*YER065C*	*ICL1*
2	ACCOA + GLX -> COA + MAL	MALATE SYNTHASE	*YIR031C, YNL117W*	*DAL7, MLS1*
Gluconeogenesis Reactions				
3	OA + ATP -> PEP + CO2 + ADP	PHOSPHOENOLPYRUVATE CARBOXYLKINASE	*YKR097W*	*PCK1*
4	FDP -> F6P + PI	FRUCTOSE-1,6-BISPHOSPHATASE	*YLR377C*	*FBP1*
5	PYR + ATP + CO2 -> ADP + OA + PI	PYRUVATE CARBOXYLASE	*YGL062W, YBR218C*	*PYC1, PYC2*
Mitochondrial Reactions				
6	MALm + NADPm -> CO2m + NADPHm + PYRm	MITOCHONDRIAL MALIC ENZYME	*YKL029C*	*MAE1*

Source: Adapted from: Genetic Circuits Research Group (Bernhard Palsson), Website Data, 2004.

[132] BERG, J., et al., Op.Cit.

2.7.6. Assimilation of other sugars

Yeast can metabolize other sugars than glucose, but, in an interesting regulatory mechanism, lack of glucose allows the expression of genes needed for assimilation of galactose or maltose, and presence of glucose represses those genes, one of the essential features of glucose repression. Fructose and mannose has been reported to exert a similar phenomenon to glucose repression, but to a lesser extent[133]. Here, we will review the monosaccharides fructose, galactose and maltose, the disaccharides sucrose and melibiose, and the trisaccharide raffinose (see Table 2.16.).

Fructose metabolism is the conversion of fructose to one of the intermediate metabolites of glycolysis. Two pathways are known to occur in fructose metabolism: In the most common one, fructose is phosphorylated to F6P by hexokinase (reaction 1 in Table 2.16.); in the second one, fructose is phosphorylated to F1P (missing reaction in Palsson´s data) and, then, F1P is phosphorylated again to FDP (reaction 4), entering this way to glycolysis.

Galactose is taken up by the galactose permease. Then is phosphorylated to galactose-1-phosphate by the galactokinase (reaction 7). And, finally, it enters to the Leloir pathway, where it is converted first to glucose-1-phosphate (reaction 9) and, finally, to glucose-6-phosphate (reactions 13 and 14), entering this way to glycolysis.

Sucrose, melibiose and raffinose are externally hydrolyzed into monosaccharides (reactions 5 and 6), due to secreted enzymes such as invertase and melibiase. Raffinose can be a good option in glucose repression studies. According to Reifenberger, "Degradation of raffinose leads to very low concentrations of glucose causing induction of SUC2 expression. In contrast, sucrose is a better substrate for invertase than raffinose, suggesting that higher concentrations of glucose are liberated leading to a partial repression of SUC2"[134]. According to Özcan, et al., "While SUC2 transcription in cells grown on 2% Glucose is completely repressed, cells grown on 2% Raffinose exhibit the highest level of SUC2 transcript"[135].

[133] KLEIN, C., et al., Op.Cit.
[134] REIFENBERGER, E., Op.Cit.
[135] ÖZCAN, S., et al., Op.Cit.

Table 2.16. Fructose, galactose, sucrose and raffinose metabolism in *S. cerevisiae*.

	Reaction	Enzyme	ORF(s)	Gene(s)
Fructose Metabolism				
1	ATP + FRU -> ADP + F6P	HEXOKINASE I, HEXOKINASE II	*YFR053C, YGL253W*	*HXK1, HXK2*
2	ATP + F6P -> ADP + F26P	6-PHOSPHOFRUCTOSE-2-KINASE	*YIL107C, YOL136C*	*PFK26, PFK27*
3	F26P -> F6P + PI	FRUCTOSE-2,6-BIPHOSPHATASE	*YJL155C*	*FBP26*
4	F1P + ATP -> FDP + ADP	1-PHOSPHOFRUCTOKINASE (FRUCTOSE 1-PHOSPHATE KINASE)	*Unknown*	*Unknown*
Sucrose Metabolism				
5	SUCxt -> GLCxt + FRUxt	INVERTASE (SUCROSE HYDROLYZING ENZYME)	*YIL162W*	*SUC2*
Raffinose Metabolism				
6	RAF -> GLAC + SUC	INVERTASE	*YIL162W*	*SUC2*
Galactose Metabolism				
7	GLAC + ATP -> GAL1P + ADP	GALACTOKINASE	*YBR020W*	*GAL1*
8	UTP + GAL1P <-> PPI + UDPGAL	GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE	*YBR018C*	*GAL7*
9	UDPG + GAL1P <-> G1P + UDPGAL	UDPGLUCOSE--HEXOSE-1-PHOSPHATE URIDYLYLTRANSFERASE	*YBR018C*	*GAL7*
10	UDPGAL <-> UDPG	UDP-GLUCOSE 4-EPIMERASE	*YBR019C*	*GAL10*
11	G1P + UTP <-> UDPG + PPI	UTP--GLUCOSE 1-PHOSPHATE URIDYLYLTRANSFERASE	*YHL012W*	*YHL012 W*
12	G1P + UTP <-> UDPG + PPI	URIDINEPHOSPHOGLUCOSE PYROPHOSPHORYLASE	*YKL035W*	*UGP1*
13	G1P <-> G6P	PHOSPHOGLUCOMUTASE 1	*YKL127W*	*PGM1*
14	G1P <-> G6P	PHOSPHOGLUCOMUTASE	*YMR105C*	*PGM2*

Source: Adapted from: Genetic Circuits Research Group (Bernhard Palsson), Website Data, 2004, except for Raffinose metabolism (Palsson reported Melibiase as the enzyme catalyzing this reaction).

2.7.7. Glycerol metabolism

Glycerol metabolism is summarized in Table 2.17.

Table 2.17. Glycerol metabolism in *S. cerevisiae*.

	Reaction	Enzyme	ORF(s)	Gene(s)
Glycerol Metabolism				
1	GLYN + ATP -> DHAP + ADP	DIHYDROXYACETONE KINASE, PUTATIVE DIHYDROXYACETONE KINASE	*YFL053W, YML070W*	*DAK2, DAK1*
2	DHAP + NADH -> GL3P + NAD	GLYCEROL-3-PHOSPHATE DEHYDROGENASE (NAD)	*YDL022W, YOL059W*	*GPD1, GPD2*
3	GL3P -> GL + PI	DL-GLYCEROL-3-PHOSPHATASE	*YER062C, YIL053W*	*HOR2, RHR2*
4	GL + ATP -> GL3P + ADP	GLYCEROL KINASE	*YHL032C*	*GUT1*
5	GL3P + FADm -> DHAP + FADH2m	GLYCEROL-3-PHOSPHATE DEHYDROGENASE	*YIL155C*	*GUT2*

Source: Adapted from: Genetic Circuits Research Group (Bernhard Palsson), Website Data, 2004.

2.7.8. Metabolite transporters in *S. cerevisiae*

Hexose transporters are fundamental in our process. According to Walker, "…it is known that the rate of alcohol production by yeast is primarily limited by the rate of sugar uptake. Thus in winemaking, the loss of hexose transport toward the end of fermentation may be responsible for reduced alcohol yields"[136].

According to Walker, "Some strains of *Saccharomyces cerevisiae* have been reported possessing a sucrose proton symporter, but predominantly in this species sucrose is firstly converted to glucose and fructose. This is accomplished at the yeast cell envelope by a periplasmic invertase"[137]. Klein et al. adds that "…direct sucrose uptake has been reported, playing an important role under repressing conditions"[138]. There are no transport systems reported for Melibiose or Raffinose.

Other metabolites that are able to diffuse through the membrane are: Glycerol, ethanol, CO_2, pyruvate, acetaldehyde, acetate, citrate, alpha-ketoglutarate, succinate, fumarate and malate. In the glycerol case, it is known that the Mig1-repressed channel protein Fps1p facilitates glycerol uptake and efflux.

[136] WALKER, G.M., Op.Cit.
[137] WALKER, G.M., Op.Cit.
[138] KLEIN, C., et al., Op.Cit.

3. Graphical Model of Glucose De-repression

Hofmeyr et al. emphasize that "The field of systems biology falls within the context of Bioinformatics where its role lies in the integration of the data sets obtained within the Genomics, Transcriptomics, Proteomics and Metabolomics fields"[139]. In this sense, all the biological information has been integrated and summarized in a graphical application, whose main goal is to allow the researchers to retrieve general information, as well as references and quotations from the original articles, for each protein/gene involved in the glucose de-repression process, on the signalling, transcriptional, and metabolic levels.

3.1. Functional requirements

The application was thought to fulfil the following requirements:

- To present each pathway in a graphical format, so that the user could identify the protein/gene of interest in the context of the glucose de-repression process.
- To retrieve the information directly from a graphical interface without the necessity of searching machines.
- To facilitate the sharing of this information with researchers all over the world.
- To permit the integration of the graphical environment with other Systems Biology software.

3.2. Design

3.2.1. Type of graphical interface

According to requirements 2 and 3, a web-based graphical interface was chosen. All the files were created using HTML without any other sophisticated tool.

3.2.2. Website structure

The application would consist of the following components:

- Start page: This page would contain a short presentation and links to the "Pathway pages".

[139] HOFMEYR, J., SNOEP, J., WESTERHOFF, H., Kinetics, Control and Regulation of Metabolic Systems, *Tutorial*, 2002

- Pathway pages: Four pages containing inserted pictures of the Snf1-Gal83 signalling pathway, Mig1 transcriptional network and Central Carbon Metabolism inside and outside the mitochondria. The regions in each picture corresponding to each protein would have links to the "Description pages".
- Description pages: One page per protein/gene, containing all information related to glucose de-repression in the signalling, transcriptional, posttranscriptional, posttranslational and metabolic levels, if available, including references to some of the important articles and relevant quotations from the original articles.

3.2.3. Designing procedure

The pathways were drawn using Microsoft Visio 2002, which facilitate both the drawing and the creation of links to graphical structures. The other web pages were created directly in HTML, using the information collected in the previous chapter.

Drawing of the pathways using Visio would also permit us to fulfil the requirement number 4, due to Systems Biology simulators such as Pathway Lab[140] run under a Visio environment. However, no such software was used in this thesis.

3.3. Sample

The graphs of the pathways are shown in the appendices. The complete application is presented on the accompanying CD.

[140] INNETICS, Pathway Lab, http://www.innetics.com/, 2004

4. Kinetic Model of Glucose De-Repression

> *"Give me 15 free parameters, and I can make an elephant. Give me 16, and I can make it dance". Stanislaw Ulam.*

According to the biological evidence presented in the previous chapters, we decided to design a model of glucose de-repression including the Snf1-Gal83 signaling pathway, Mig1 transcription and one metabolic response (Suc2 transcription). This model can be used as a basis for the creation of a rigorous model of glucose de-repression, when experimental data becomes available. The pathway is presented in Figure 4.1.

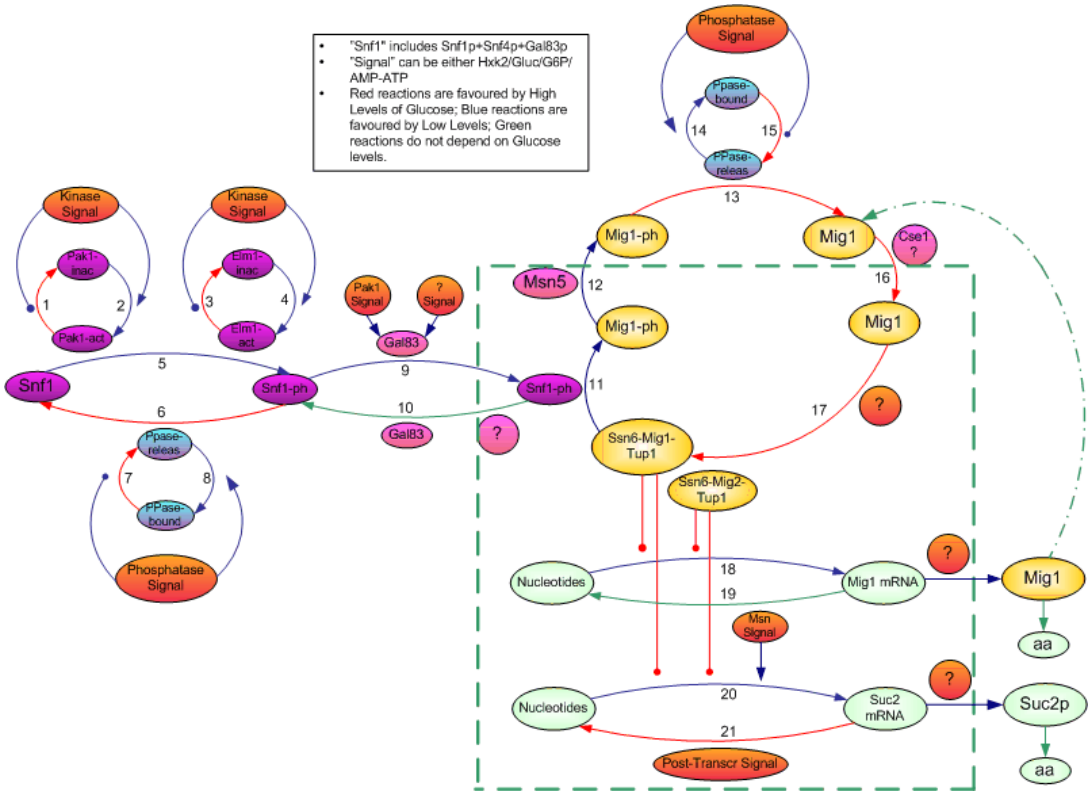

Figure 4.1. Basic model of glucose de-repression including Snf1-Gal83 signalling pathway, Mig1 transcription and Suc2 transcription.

Table 4.1. Model without transport (i.e., skipping reactions 9, 10, 12 and 16).

$\frac{d[Pak1]}{dt} = \upsilon_1 - \upsilon_2$	$\frac{d[Mig1]}{dt} = \upsilon_{13} - \upsilon_{17}$
$\frac{d[Pak1A]}{dt} = -\upsilon_1 + \upsilon_2$	$\frac{d[Mig1ph]}{dt} = \upsilon_{11} - \upsilon_{13}$
$\frac{d[Snf1]}{dt} = -\upsilon_5 + \upsilon_6$	$\frac{d[Ssn6Mig1Tup1]}{dt} = \upsilon_{17} - \upsilon_{11}$
$\frac{d[Snf1ph]}{dt} = \upsilon_5 - \upsilon_6$	$\frac{d[Mig1RNA]}{dt} = \upsilon_{18} - \upsilon_{19}$
$\frac{d[Reg1]}{dt} = -\upsilon_7 + \upsilon_8 + \upsilon_{14} - \upsilon_{15}$	$\frac{d[Suc2RNA]}{dt} = \upsilon_{20} - \upsilon_{21}$
$\frac{d[Reg1A]}{dt} = \upsilon_7 - \upsilon_8 - \upsilon_{14} + \upsilon_{15}$	

4.1. Snf1-Gal83 signaling pathway model

4.1.1. Rate equations

Given that the reaction mechanisms of all these reactions are unknown, we don't present a detailed model of elementary reactions but an aggregated model (Michaelis-Menten type rate-laws), where inactive kinases act as substrates and active kinases act as products and enzymes, and all unknown signals are input parameters of the model. The stoichiometries of these reactions are also unknown; for this reason, all reactions are assumed to be dependent on substrate and enzyme concentrations, ignoring the role of ATP, and the 1:1 stoichiometry is assumed unless the results suggest something different. In the case of Protein Phosphatase I, the kinetic mechanism was summarized in two steps named active and inactive; the conversion of active to inactive (binding to Snf1-complex) is favored by low glucose, whereas the opposite reaction (dephosphorylation of Snf1 and release of Reg1-Glc7) is favored by high glucose. This scheme leads to six assumed moieties, for Pak1, Elm1, Snf1, Reg1, Mig1 and Msn2. According to Figure 4.1, Table 4.1. and Table 4.2. list the rate equations for two different models, respectively.

4.1.2. Kinetic types

Mendes (1997)[141] created a didactic model of a kinase cascade controlled by a signal, using Gepasi 3. In this model, the phosphorylation reactions activated by a signal follow catalytic activation kinetics, the dephosphorylation reactions inhibited by a signal follow uncompetitive inhibition kinetics, and the phosphorylation reactions catalyzed by another known kinase follow irreversible Michaelis-Menten kinetics.

We follow a similar scheme in our model. Reactions 5, 6, 11 and 13 will follow, in principle, irreversible Michaelis-Menten kinetics. Reactions 1, 2, 3, 4, 7, 8, 17, 19 and 21, are not well documented, so we will assume irreversible mass-action kinetics (one only parameter), introducing the effect of the signals by means of changes in the kinetic parameter. Notice that, from now on, we are just considering the model without transport.

[141] MENDES, P., Biochemisty by numbers: simulation of biochemical pathways with Gepasi 3, *TIBS*, **22**, 1997

Table 4.2. Model including transport.

Cytoplasm	Nucleus
$\frac{d[Pak1]}{dt} = \upsilon_1 - \upsilon_2$	$\frac{d[Snf1ph]}{dt} = \upsilon_9 - \upsilon_{10}$
$\frac{d[Pak1A]}{dt} = -\upsilon_1 + \upsilon_2$	$\frac{d[Mig1]}{dt} = \upsilon_{16} - \upsilon_{17}$
$\frac{d[Snf1]}{dt} = -\upsilon_5 + \upsilon_6$	$\frac{d[Mig1ph]}{dt} = \upsilon_{11} - \upsilon_{12}$
$\frac{d[Snf1ph]}{dt} = \upsilon_5 - \upsilon_6 - \upsilon_9 + \upsilon_{10}$	$\frac{d[Ssn6Mig1Tup1]}{dt} = \upsilon_{17} - \upsilon_{11}$
$\frac{d[Reg1]}{dt} = -\upsilon_7 + \upsilon_8 + \upsilon_{14} - \upsilon_{15}$	$\frac{d[Mig1RNA]}{dt} = \upsilon_{18} - \upsilon_{19}$
$\frac{d[Reg1A]}{dt} = \upsilon_7 - \upsilon_8 - \upsilon_{14} + \upsilon_{15}$	$\frac{d[Suc2RNA]}{dt} = \upsilon_{20} - \upsilon_{21}$
$\frac{d[Mig1]}{dt} = \upsilon_{13} - \upsilon_{16}$	
$\frac{d[Mig1ph]}{dt} = \upsilon_{12} - \upsilon_{13}$	

Reaction 5 also needs to take into account the possibility of an inhibitory signal (so, the Michaelis-Menten model would become an uncompetitive inhibition model); however, we will assume that this is not the case and we will use normal Michaelis-Menten. Besides, given that two different enzymes (Pak1 and Elm1, in our model) are catalyzing the reaction at the same time, we will use an additive model, as proposed by Schulz[142]

$$\upsilon = \frac{V_1[S]}{K_1 + [S]} + \frac{V_2[S]}{K_2 + [S]}$$

For reactions 18 and 20, Mendes has proposed a model in which the basal rate of transcription changes with the multiplication of all the activation or inhibition contributions, each of them following a Hill-like kinetics (in our case, Mig1 and Mig2 repression)[143].

Other type of models can be taken into account: Ferrell (1996), e.g., proposes a Hill kinetics for the MAP kinase cascade due to the exhibited switch-like response[144].

4.1.3. Kinetic parameters

As there is no report of kinetic parameters for the Snf1-pathway in the literature, we looked for data coming from protein kinases of the Snf1-AMPK family in other

[142] SCHULZ, A., Enzyme kinetics: From diastase to multi-enzyme systems, *Cambridge University Press*, 1994

[143] MENDES, P., SHA, W., YE, K., Artificial gene networks for objective comparison of analysis algorithms, *Bioinformatics*, **19**, 2003

[144] FERRELL, J., Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs, *TIBS*, **21**, 1996

organisms such as *D. melanogaster*, *C. elegans*, mammals and plants, as well as other phosphorylation schemes.

Among other models, Tyson et al. (2003)[145] presented a phosphorylation-dephosphorylation model with sigmoidal response of the product to a signal. In this model, they used Michaelis-Menten kinetics with hypothetical values of parameters $k_{cat} = 1$ and $K_m = 0.05$.

Gong et al. reported the kinetic parameters for the protein kinase Sos2 (Snf1-homolog in *Arabidopsis*) in the phosphorylation of three synthetic peptides called p1 (LRRASLG), p2 (VRKRTLRRL) and p3 (ALARAASAAALARRR). According to them, the values of K_m for the three peptides (ranging from 99 to 211 μM), "are similar to those for SNF1/AMPK from yeast, mammalian, and higher plants with Ser-containing peptides as substrates. The preferred peptide substrate p3… contains within it the following sequence: hydrophobic-X-basic-X(2)-Ser-X(3)-hydrophobic residue. The same motif has been previously established as a minimal recognition motif for the cauliflower AMPK/SNF1 homolog"[146]. The reported k_{cat} values ranges from 1.89 to 2.76 s^{-1}.

It is also known that Snf1 and AMPK (Snf1 homolog) share acetyl-CoA carboxylase and the peptide AMARA as substrates for phosphorylation. Salt et al.[147] tested the SAMS and AMARA peptides with an α1 kinase domain of AMPK and native rat liver AMPK, obtaining k_{cat} values around 4.2 s^{-1} and K_m values ranging from 10 to 29 μM. They also tested different point mutations of rat acetyl-CoA carboxylase (as a substrate) against AMPK α1 kinase domain, obtaining values ranging from 0.56 to 6.33 s^{-1} for k_{cat} and 1.85 to 36.3 μM for K_m. Finally, it has been reported that human PAS kinase with AMARA peptide gives values around $k_{cat} = 1$ s^{-1} and $K_m = 210$ μM.

We will try to use values in this range for our model, assuming that mutations between Snf1 and its homologs have a similar (inoffensive) effect on the kinetic parameters.

4.1.4. Initial concentrations

Information about estimated numbers of molecules of each protein per cell was adapted from SGD[148]. Following Ferrell[149], the values were converted to μM assuming a constant cell volume of 0.1 pl. As these concentrations were measured in growing cells, they are a good estimate for our initial (high glucose) conditions (see Table 4.3.).

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[145] TYSON, J., CHEN, K., NOVAK, B., Sniffers, buzzers, toggles and blinkers: dynamics of regulatory and signaling pathways in the cell, *Current Opinion in Cell Biology*, **15**, 2003

[146] GONG, D., GUO, Y., JAGENDORF, A., ZHU, J., Biochemical characterization of the Arabidopsis protein kinase Sos2 that functions in salt tolerance, *Plant Physiology*, **130**, 2002

[147] SALT, I., CELLER, J., HAWLEY, S., PRESCOTT, A., WOODS, A., CARLING, D., HARDIE, G., AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the α2 isoform, *Biochemical Journal*, **334**, 1998

[148] SGD, http://yeastgfp.ucsf.edu, 2004

[149] FERRELL, J., Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs, *TIBS*, **21**, 1996

Table 4.3. Total initial concentrations.

Protein	Molecules/cell	Concentration (μM)
Pak1	752	0.01249
Elm1	149	0.00247
Snf1	589	0.00978
Mig1	830	0.01379
Reg1	2560	0.04252
Glc7	14600	0.24252
Mig2	504	0.00837
Msn2	125	0.00208

4.2. Model implementation

4.2.1. Generalities

All simulations were made using the software Gepasi 3.3. Reactions held the following assumptions:

- Similar values of kinetic parameters are used for similar reactions (based on Snf1-homologs data).
- Mass-action constants and basal transcription rates were arbitrarily fixed to values that generate the expected behavior.

4.2.2. Model construction

Due to the lack of parameters and values of concentrations/activities for finding these parameters, we need a strategy to use the available information in constructing an approximate model. This way, our modelling approach consists in creating a model for the two Snf1 reactions and progressively add the other reactions, upstream and downstream Snf1, checking the consequences on the system. The reason for starting with Snf1 is that we not only know its high glucose concentration but also the kinetic parameters of several homologous proteins and the expected behaviour of its concentration-time plots, which is a good help for defining an estimated value of the parameters.

Our first model included reactions 5 and 6, following Michaelis-Menten kinetics, controlled by constant values of Pak1A and Reg1A; the kinetic constants were fixed at k_{cat}=8 and K_m=10, which are feasible values for Snf1-homologs. In the ***first scenario***, we assumed all the Snf1 being phosphorylated (low glucose state) and then we added a high glucose signal (100%-Reg1A and a very small amount of Pak1A); the result is shown in Figure 4.2. Snf1-phosphorylated quickly disappears whereas Snf1 is generated, everything in around 120 s, as expected.

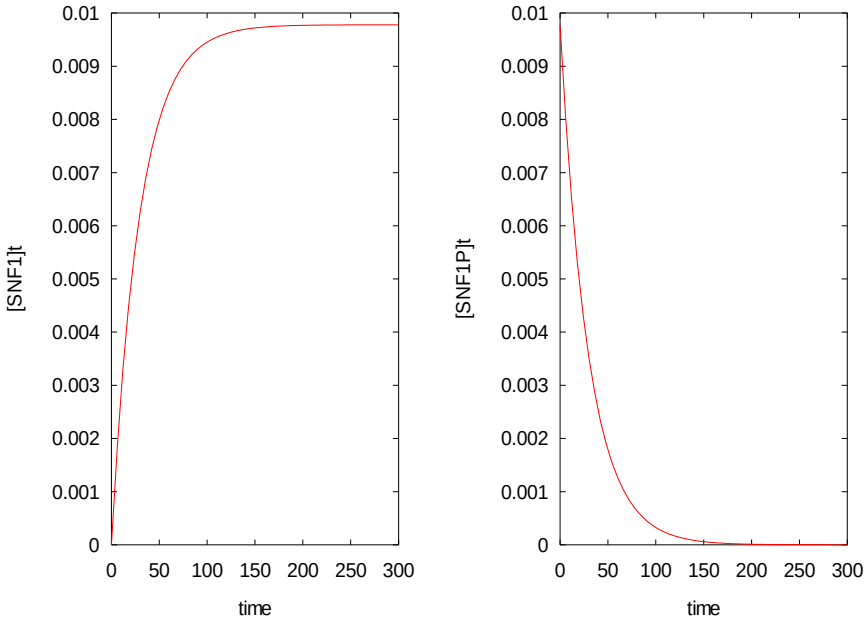

Figure 4.2. Changes in Snf1 and Snf1P concentrations, in response to a high glucose signal (100%-Reg1A and approx. 0%-Pak1A). First scenario.

In the ***second scenario***, we assumed all the Snf1 being inactive (high glucose state) and then we added a low glucose signal (100%-Pak1A and a very small amount of Reg1A); the result is showed in Figure 4.3. Now Snf1 quickly disappears whereas Snf1P is generated; however, this process is slower than the previous one, due to the kinases having smaller concentrations than the phosphatases. Due to the fact that we are interested in glucose de-repression, we will continue working in the second scenario.

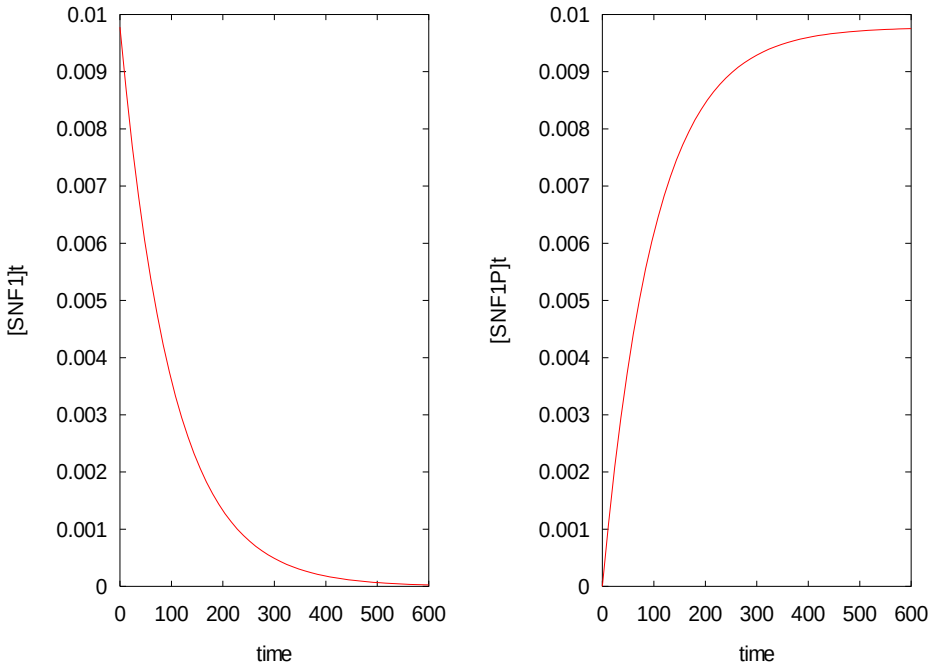

Figure 4.3. Changes in Snf1 and Snf1P concentrations, in response to a low glucose signal (100%-Pak1A and approx. 0%-Reg1A). Second scenario.

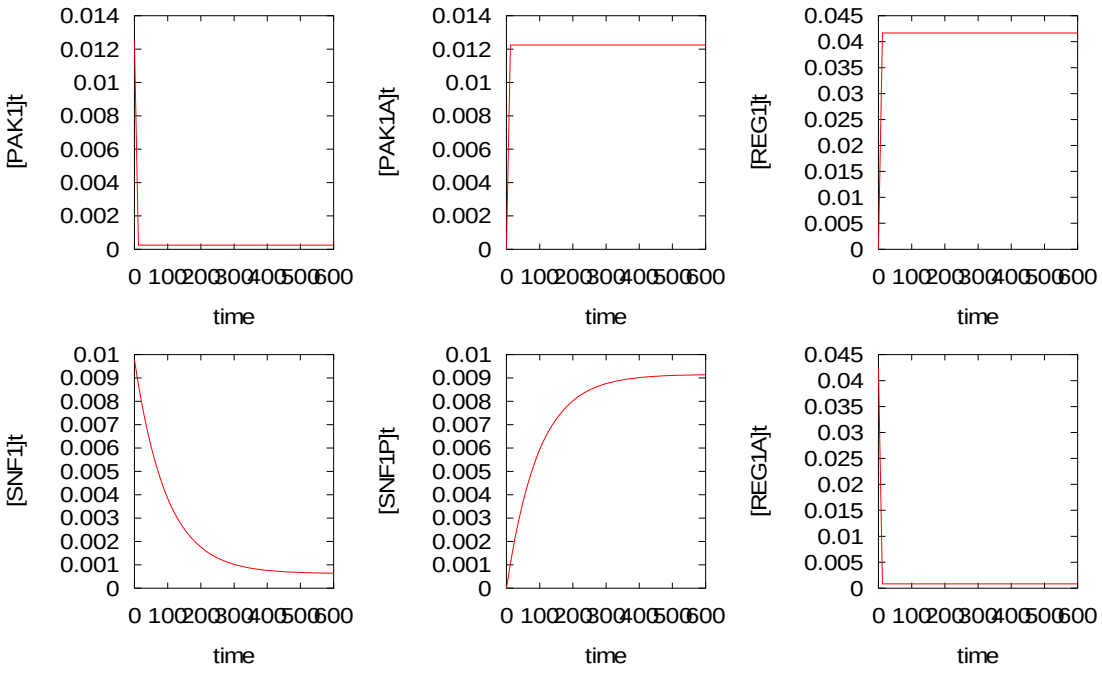

Figure 4.4. Changes in Snf1, Snf1P, Pak1, Pak1A, Reg1 and Reg1A concentrations, in response to a low glucose signal (generation of Pak1A and degradation of Reg1A are 50 times faster than their reverse reactions, i.e., k = 5 and k = 0.1). Third scenario.

In our ***third scenario***, initial high values of Pak1A and Reg1 are not the low glucose signal anymore. Instead, an unknown signal is now changing the concentration values from Pak1 to Pak1A and from Reg1A to Reg1 in a gradual way, so that they are influencing Snf1 phosphorylation in this gradual way (see Figure 4.4). Mass-action kinetics were assumed for interconversion of Pak1/Pak1A and Reg1/Reg1A, and the reaction rates of generation of Pak1A and degradation of Reg1A were forced to have values 50 times higher than their reverse reactions, in order to reflect how the low glucose signal is stimulating them or inhibiting the others. The values of both the kinetic parameters and the ratio of rates (for reactions 1, 2, 7 and 8) were also defined according to the expected behaviour of Snf1.

In our ***fourth scenario***, we added to the model the effect of Elm1p, assuming the same kinetic parameters as those of Pak1p (it is assumed that the smaller influence of Elm1 compared to Pak1 is due to its smaller concentration value). As expected, Snf1 phosphorylation is slightly favoured (Figure 4.5.).

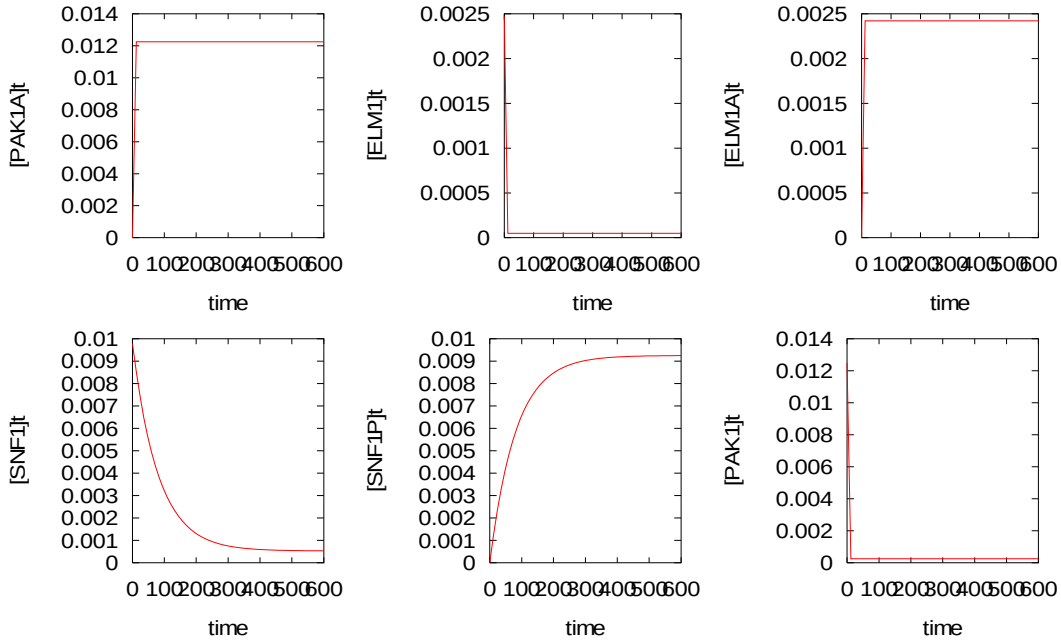

Figure 4.5. Changes in Snf1, Snf1P, Pak1, Pak1A, Elm1 and Elm1A concentrations, in response to a low glucose signal (generation of Pak1A and Elm1A, as well as degradation of Reg1A are 50 times faster than their reverse reactions, i.e., k = 5 and k = 0.1). Fourth scenario.

In low glucose, reaction 6 (de-phosphorylation of Snf1) is not favoured; for this reason, it is not interesting to study its sensitivity to changes in its parameters: these changes do not affect the reaction rate (not shown). However, reaction 5 (phosphorylation of Snf1) seems to respond to changes in the kinetic parameters; for instance, a change in the V_{max} for Pak1 leads to a faster response (Figure 4.6.).

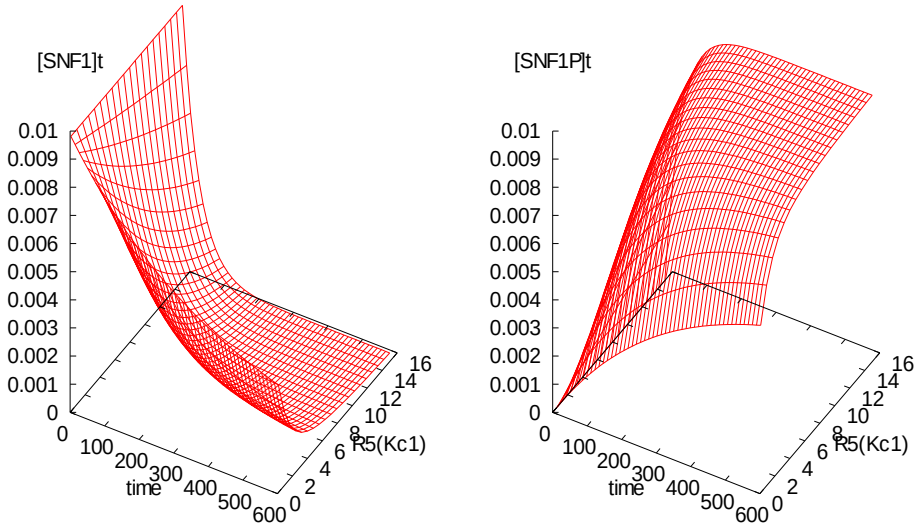

Figure 4.6. Changes in Snf1 phosphorylation in response to a low glucose signal, according to changes in k_{cat} for Pak1A (all other parameters remained fixed).

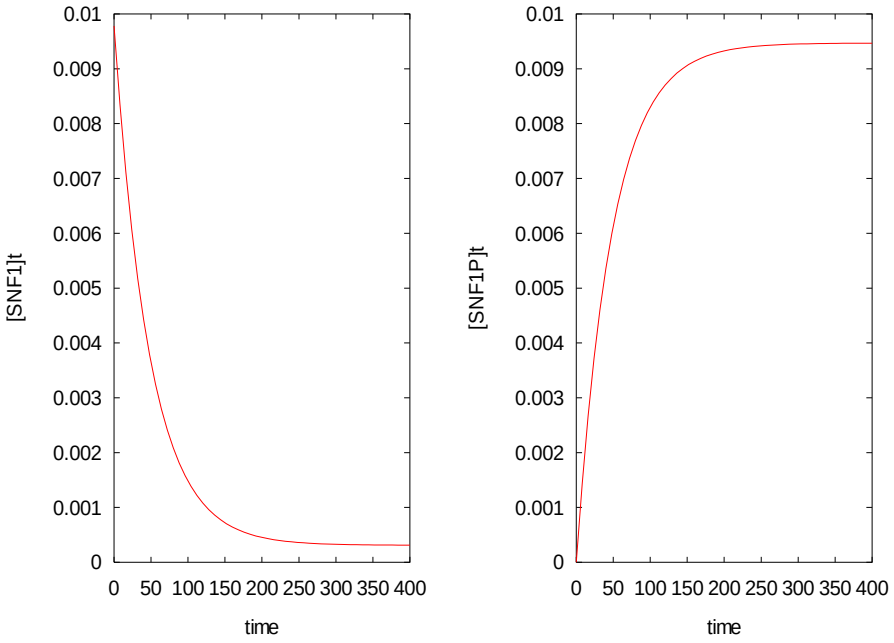

Figure 4.7. Output of the final model of Snf1 phosphorylation in response to a low glucose signal, depending on the combined action of Pak1p, Elm1p and Reg1p/Glc7p, and with k_{cat} = 15 for Pak1A.

Based on this new information, we decided to use in our model the value k_{cat} = 15, which gives a better response (Figure 4.7.).

Our ***fifth scenario*** takes into account the effect of Snf1P on the nuclear phosphorylation of Mig1 (reaction 11). We assume Michaelis-Menten kinetics for Mig1 phosphorylation, using the same parameters as in the Pak1 contribution to Snf1 phosphorylation. As expected, Mig1C decreases and Mig1P increases due to the low signal.

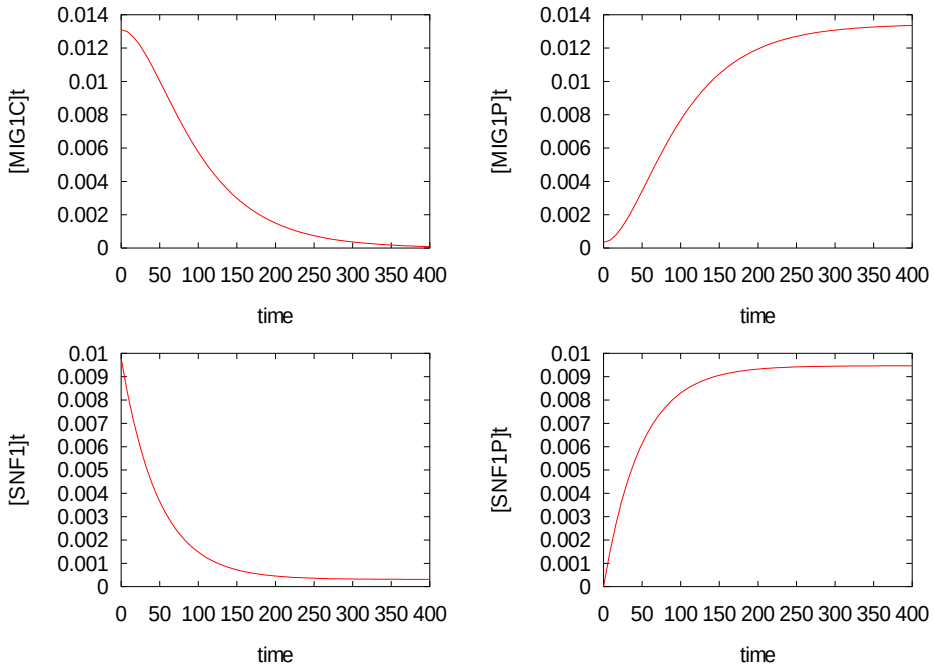

Figure 4.8. Output of the Snf1 and Mig1 phosphorylation in response to a low glucose signal, without taking into account degradation of Mig1P and formation of Mig1C (assumed initial ratios: 95%Mig1C, 2.5%Mig1P, 2.5%Mig1). Fifth scenario, no phosphatase activity on Mig1.

It is possible to test two different models for Mig1 behavior. In the first one, the Mig1 repressor is degraded and regenerated *de novo*, and action of phosphatases or re-entrance to the nucleus are not important. Assuming that times of degradation and formation are very high compared to our time-scale, we can assume that this scenario correspond to the one shown in Figure 4.8. In the second model, Mig1P is dephosphorylated and then reform the Mig1C, as depicted in the general diagram of the model (Figure 4.1.). In order to construct this second scenario, we added reactions 13 and 17 with mass-action kinetics, assuming small mass-action constants but still ignoring the catalytic effect of the phosphatase (which will be added later on). It can be observed that the levels of Mig1P and Mig1C are conserved, as well as the slight increment of free Mig1 concentration (Figure 4.9.).

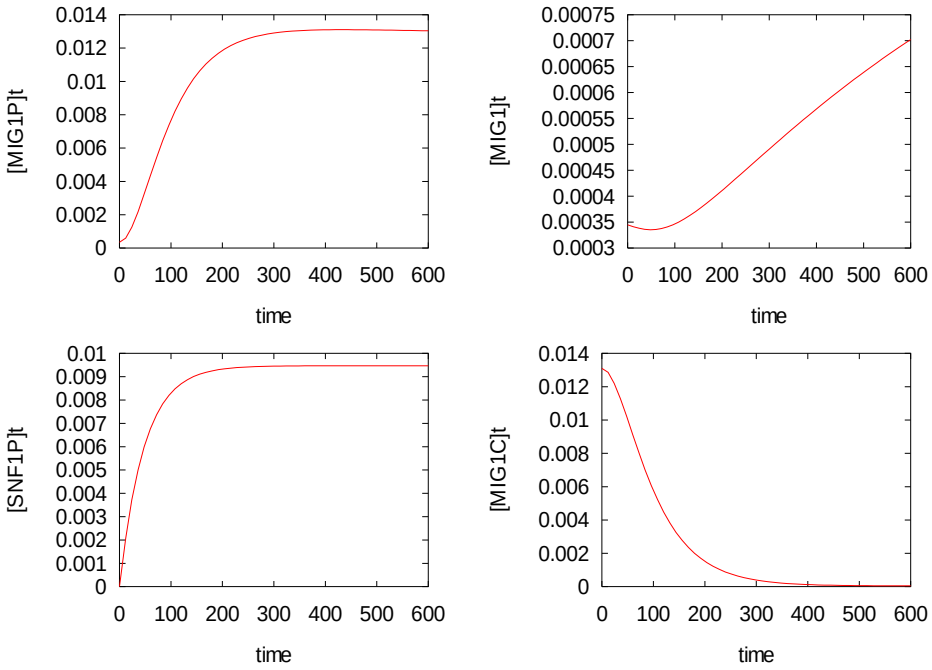

Figure 4.9. Output of the Snf1 and Mig1 phosphorylation in response to a low glucose signal, including dephosphorylation of Mig1P and formation of Mig1C (assumed initial ratios: 95%Mig1C, 2.5%Mig1P, 2.5%Mig1, and mass-action kinetics). Fifth scenario.

In our ***sixth scenario***, the dephosphorylation of Mig1P is assumed to follow a Michaelis-Menten kinetics, whereas the Reg1/Glc7 complex plays the same role as in Snf1P dephosphorylation (Figure 4.10.).

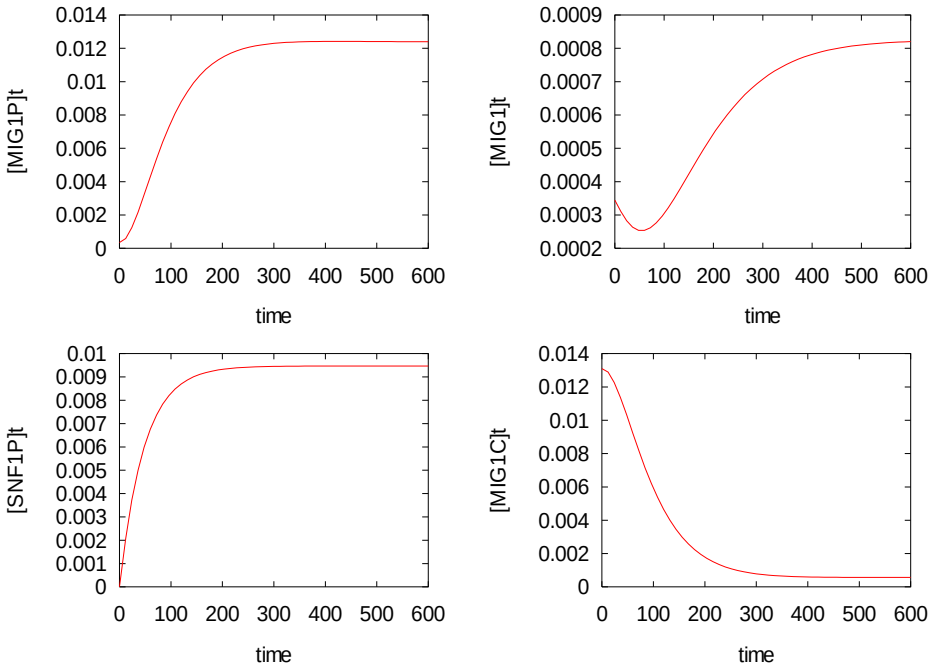

Figure 4.10. Output of the Snf1 and Mig1 phosphorylation in response to a low glucose signal, including dephosphorylation of Mig1P (using Michaelis-Menten kinetics) and formation of Mig1C (using mass-action kinetics). Sixth scenario.

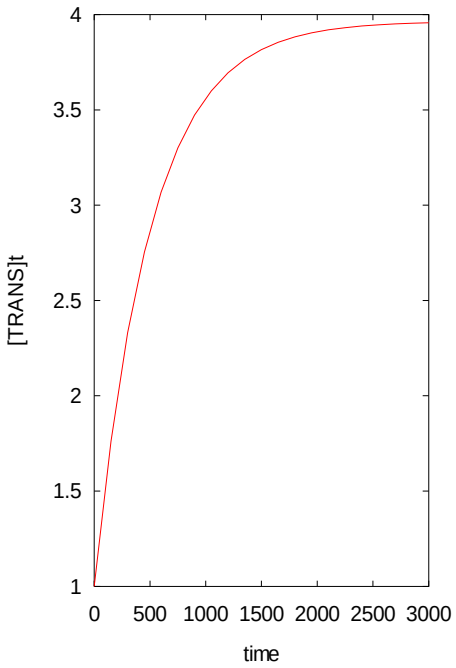

Figure 4.11. Output of the Mig1 transcripts in response to a low glucose signal (concentration of transcripts is given in fold change units relative to t=0).

Finally, our ***last scenario*** includes the formation of Mig1 mRNA in response to glucose de-repression. The kinetic equation proposed by Mendes was used, assuming a constant and high enough supply of nucleotides and two inhibitors: Mig1C and Mig2C, and using parameter values which give the expected behaviour (Figure 4.11.). The initial concentration of mRNA was said to be equal to 1 arbitrary unit, so the change in mRNA concentration could be understood as a fold-change. It can be seen that the curve shows an increase in mRNA concentration due to the low glucose signal; however, it was not possible to verify whether the time-scale of the changes is correct or not.

Transcription of SUC2 was also modelled. The same kinetic model as before was used, adding the activating effect of Msn2. This protein was supposed to change from an inactive (unbound) to an active state (binding to SUC2 promoter) following mass-action kinetics. As expected, levels of active Msn2 increased within the first five minutes and levels of SUC2 mRNA increased to reach a steady-state around 45 minutes after the low glucose signal was initiated (Figure 4.12.). However, it has been reported that the levels of Msn2 bound to the SUC2 promoter will return to the repression levels within 30 minutes and that SUC2 mRNA levels enter to a second phase where: a) they will reach a steady-state after 2 hours if raffinose is present, or b) they will return to repression levels after 4 hours if raffinose is absent. Since we used a linear mass-action kinetics for the conversion of Msn2 from the inactive to the active state, the second behaviour could not be simulated.

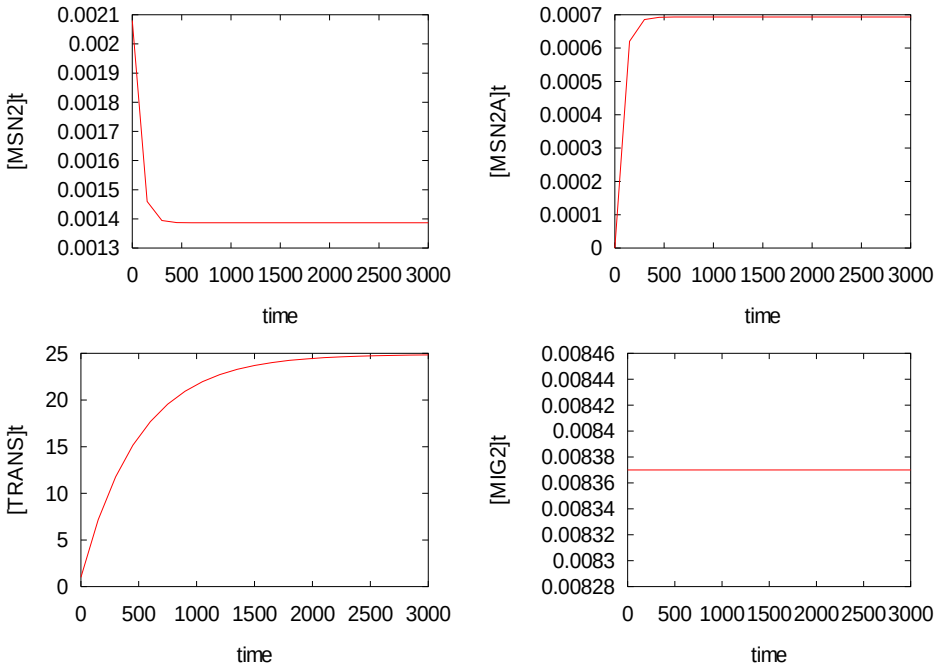

Figure 4.12. Output of the Suc2 transcripts, Msn2 activator and Mig2 repressor complex in response to a low glucose signal (concentration of transcripts is given in folding units).

4.3. Summary of the model (last scenario)

4.3.1. Phenomenological description

The model includes the time-courses for the following species: Snf1-complex, Snf1-phosphorylated, Mig1-complex, Mig1-phosphorylated, free Mig1, Pak1-active, Pak1-inactive, Elm1-active, Elm1-inactive, phosphatase bound, phosphatase released, Msn2-bound, Msn2-released, Mig1-mRNA and Suc2-mRNA.

The response of the Snf1 and Msn2 species (in terms of time of response), as well as the response of the Mig1 and Suc2 transcripts, seems to agree with experimental data. In the case of the proteins, the steady-state was reached in less than five minutes, and, in the case of the transcripts, in less than fifty minutes.

In the case of entities such as free Mig1, Pak1 species, Elm1 species and phosphatase species, there is no way to associate the information to experimental data. In this model, the upstream kinases and the phosphatase just played an intermediate role between Snf1 and the low glucose signal, meanwhile the effect of free Mig1 could be neglected.

A summary of the time-courses is shown in Appendix 5.

Table 4.4. Initial and final concentrations (after 2.80e+003 s) for all the species.

Protein	Initial concentration (µM)	Final concentration (µM)
Pak1	1.249000e-002	2.449020e-004
Pak1A	0	1.224510e-002
Elm1	2.470000e-003	4.843137e-005
Elm1A	0	2.421569e-003
Snf1	9.780000e-003	3.107632e-004
Snf1P	0	9.469237e-003
Reg1A	4.252000e-002	8.337255e-004
Reg1	0	4.168627e-002
Mig1C	1.310050e-002	5.808468e-004
Mig1P	3.447500e-004	1.238417e-002
Mig1	3.447500e-004	8.249785e-004
Mig2	8.370000e-003	8.370000e-003
Msn2	2.080000e-003	1.386667e-003
Msn2A	0	6.933333e-004

4.3.2. Relative concentrations

The concentrations were assumed to start from 100% of the high-glucose species (Pak1, Elm1, Snf1, Reg1A, Msn2) and 0% of the low-glucose species (Pak1A, Elm1A, Snf1A, Reg1, Msn2A), with exception of the Mig1 species (95% of Mig1C, 2.5% of Mig1P and 2.5% of Mig1). Table 4.4. shows the initial and final concentrations of the included proteins and mRNAs.

4.3.3. Kinetic parameters

The kinetic parameters used in the final model are listed in Table 4.5.

4.3.4. Steady-state solution

The steady state solution of the final model can be found in Table 4.6.

Table 4.5. Kinetic parameters used in the simulation.

Reaction	Kinetic parameters
R1: $\upsilon_1 = k \cdot [Pak1A]$	k = 1.0000e-001
R2: $\upsilon_2 = k \cdot [Pak1]$	k = 5.0000e+000
R3: $\upsilon_3 = k \cdot [Elm1A]$	k = 1.0000e-001
R4: $\upsilon_4 = k \cdot [Elm1]$	k = 5.0000e+000
R5: $\upsilon_5 = \frac{Kc1 \cdot [Pak1A] \cdot [Snf1]}{Km1 + [Snf1]} + \frac{Kc2 \cdot [Elm1A] \cdot [Snf1]}{Km2 + [Snf1]}$	Kc1 = 1.5000e+001 Km1 = 1.0000e+001 Kc2 = 8.0000e+000 Km2 = 1.0000e+001
R6: $\upsilon_6 = \frac{Kc \cdot [Reg1A] \cdot [Snf1P]}{Km1 + [Snf1P]}$	Kc = 8.0000e+000 Km1 = 1.0000e+001
R7: $\upsilon_7 = k \cdot [Reg1]$	k = 1.0000e-001
R8: $\upsilon_8 = k \cdot [Reg1A]$	k = 5.0000e+000
R11: $\upsilon_{11} = \frac{Kc \cdot [Snf1P] \cdot [Mig1C]}{Km1 + [Mig1C]}$	Kc = 1.5000e+001 Km1 = 1.0000e+001
R13: $\upsilon_{13} = \frac{Kc \cdot [Reg1A] \cdot [Mig1P]}{Km1 + [Mig1P]}$	Kc = 8.0000e+000 Km1 = 1.0000e+001
R17: $\upsilon_{17} = k \cdot [Mig1]$	k = 1.0000e-002
R18 : $\upsilon_{18} = V \cdot \left(\frac{K1^{n1}}{[Mig1C]^{n1} + K1^{n1}} + \frac{K2^{n2}}{[Mig2C]^{n2} + K2^{n2}} \right)$	V = 8.0000e-003 K1 = 1.0000e+000 n1 = 1.0000e+000 K2 = 1.0000e+000 n2 = 1.0000e+000
R19: $\upsilon_{19} = k \cdot [Mig1mRNA]$	k = 2.0000e-003
R20: $\upsilon_{20} = V \cdot \left(\frac{K1^{n1}}{[Mig1C]^{n1} + K1^{n1}} + \frac{K2^{n2}}{[Mig2C]^{n2} + K2^{n2}} + \frac{[Msn2A]^{n3}}{[Msn2A]^{n3} + K3^{n3}} \right)$	V = 2.5000e-002 K1 = 1.0000e+000 n1 = 1.0000e+000 K2 = 1.0000e+000 n2 = 1.0000e+000 n3 = 1.0000e+000 K3 = 1.0000e+000
R21: $\upsilon_{21} = k \cdot [Suc2mRNA]$	k = 2.0000e-003
R22: $\upsilon_{22} = k \cdot [Msn2]$	k = 5.0000e-003
R23: $\upsilon_{23} = k \cdot [Msn2A]$	k = 1.0000e-002

Table 4.6. Steady-state solution.

Protein	Steady-state concentration (μM)	Rate (μM/s)
Pak1	2.449020e-004	2.168e-019
Pak1A	1.224510e-002	-2.168e-019
Elm1	4.843137e-005	2.711e-020
Elm1A	2.421569e-003	-2.711e-020
Snf1	3.107632e-004	-8.171e-015
Snf1P	9.469237e-003	8.171e-015
Reg1A	8.337255e-004	0.000e+000
Reg1	4.168627e-002	0.000e+000
Mig1C	5.808468e-004	-2.412e-014
Mig1P	1.238417e-002	-1.069e-014
Mig1	8.249785e-004	3.481e-014
Mig2	8.370000e-003	0.000e+000
Msn2	1.386667e-003	-1.625e-014
Msn2A	6.933333e-004	1.625e-014
MIG1RNA	3.964495e+000	0.000e+000
SUC2RNA	2.489765e+001	-4.056e-014

4.3.5. Metabolic Control Analysis

The numerical values of the elasticity coefficients have the following interpretations:

Reactant elasticities (elasticity to substrate):

- If e = 0, the rate of the reaction does not vary with the substrate.
- If e > 0, the rate of the reaction increases as the substrate concentration increases.
- If e < 0, the rate of the reaction decreases as the substrate concentration decreases.
-

Enzyme elasticities (elasticity to enzyme):

- If the enzyme is an activator, e > 0 (always).
- If the enzyme is an inhibitor, e < 0 (always).
- If the enzyme catalyses its own reaction, e = 1. If not, e = 0.

According to this, the elasticity coefficients of the final model (Table 4.7.) agree with the known behaviour.

Table 4.7. Elasticity coefficients (protein reactions).

Reaction	Elasticity to substrate	Elasticity to enzyme(s)
Protein reactions		
R1	e(R1,[PAK1A]) = 1.0000e+000	No
R2	e(R2,[PAK1]) = 1.0000e+000	No
R3	e(R3,[ELM1A]) = 1.0000e+000	No
R4	e(R4,[ELM1]) = 1.0000e+000	No
R5	e(R5,[SNF1]) = 9.9997e-001	e(R5,[PAK1A]) = 9.0459e-001 e(R5,[ELM1A]) = 9.5408e-002
R6	e(R6,[SNF1P]) = 9.9905e-001	e(R6,[REG1A]) = 1.0000e+000
R7	e(R7,[REG1]) = 1.0000e+000	No
R8	e(R8,[REG1A]) = 1.0000e+000	No
R11	e(R11,[MIG1C]) = 9.9994e-001	e(R11,[SNF1P]) = 1.0000e+000
R13	e(R13,[MIG1P]) = 9.9876e-001	e(R13,[REG1A]) = 1.0000e+000
R17	e(R17,[MIG1]) = 1.0000e+000	No
R22	e(R22,[MSN2]) = 1.0000e+000	No
R23	e(R23,[MSN2A]) = 1.0000e+000	No
Transcriptional reactions		
R18	e(R18,[MIG1C]) =-5.8051e-004 e(R18,[MIG2]) =-8.3005e-003	No
R20	e(R18,[MIG1C]) =-2.9128e-004 e(R18,[MIG2]) =-4.1327e-003	e(R18,[MSN2A]) = 3.4761e-004

4.4. Computational experiments

4.4.1. Generalities

In general terms, the effect of decreasing the value of both kinetic constants in a pair of reactions (reactions 1-2, 3-4, 7-8, etc), keeping the same ratio between these constants, is that the reactions become slower but the same steady-state is reached. On the contrary, changing the ratio between kinetic constants will change the final steady-state value. E.g., figure 4.13 shows the effect of decreasing the value of all the parameters for reactions 1, 2, 3, 4, 7 and 8 by a factor 50. It can be observed that Pak1, Elm1 and Reg1 species are converted in the first 50 seconds while Snf1 conversion becomes slightly slower than in the original scenario (see also appendix 5).

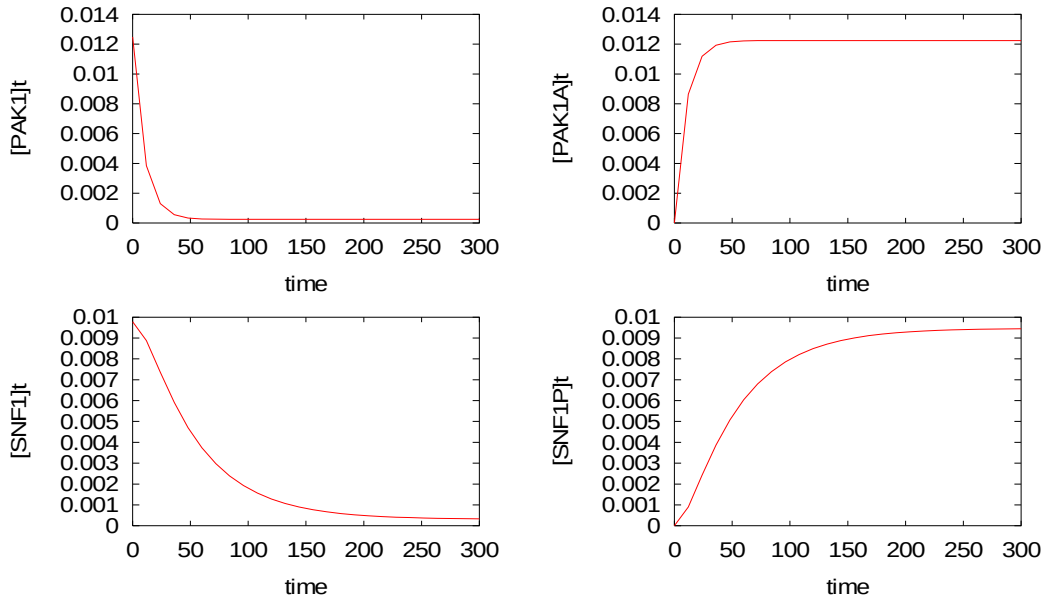

Figure 4.13. Response of Pak1 and Snf1 species to extremely low values of the parameters.

4.4.2. "Kinase" signal

The role of the signal upstream Snf1 was simulated by modifying the kinetic parameter of Pak1 activation (which was set to k = 5 in the previous model), and observing the response in the phosphorylation of both Snf1 and Mig1 (Figure 4.14.). It could be observed that small values (equivalent to a low "low-glucose signal") made both reactions slower (phosphorylation of Snf1 was more dramatically affected); it could also be observed that, for values larger than 2, the signal does not play any role.

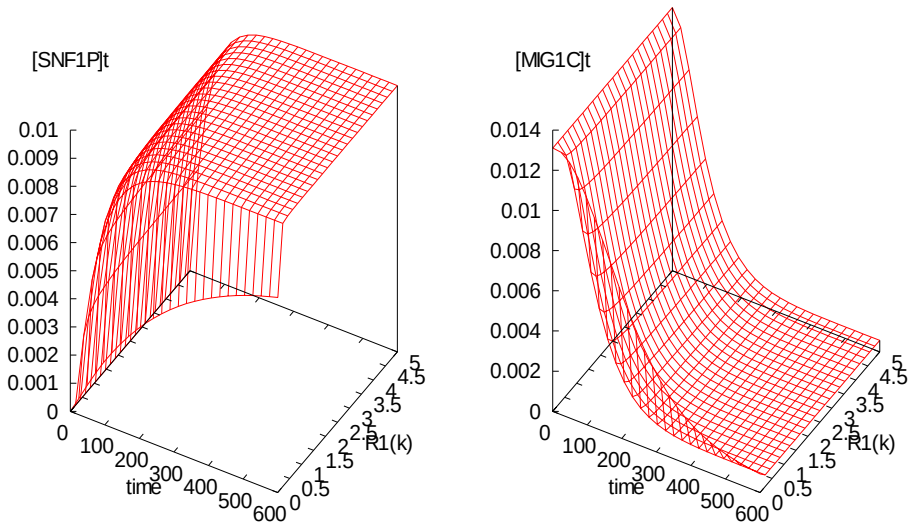

Figure 4.14. Response of Snf1P and Mig1C to different degrees of the "kinase" low-glucose signal (simulated as a value multiplying the kinetic parameter of Pak1 activation).

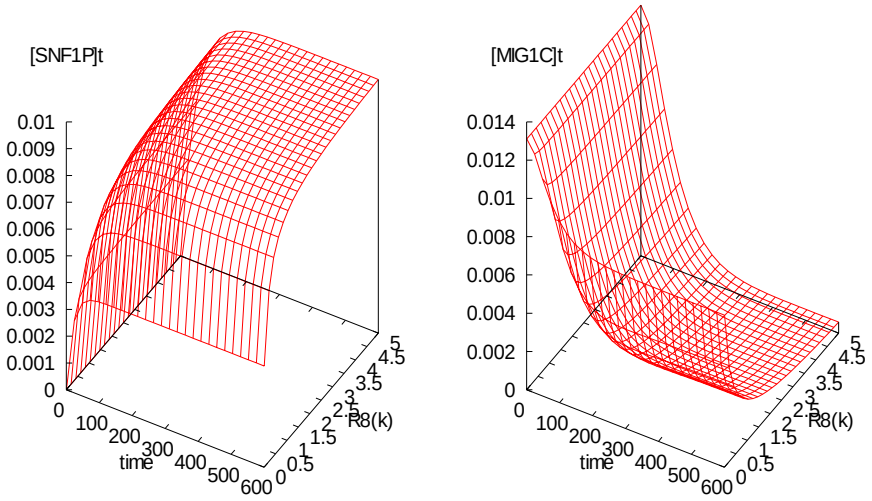

Figure 4.15. Response of Snf1P and Mig1C to different degrees of the "phosphatase" low-glucose signal (simulated as a value multiplying the kinetic parameter of Reg1 inactivation).

4.4.3. "Phosphatase" signal

The signal responsible for the de-phosphorylation was simulated by modifying the kinetic parameter of Reg1 inactivation (which was set to k = 5 on the previous model) and observing the response in the phosphorylation of both Snf1 and Mig1 (Figure 4.15.). A similar response to the first signal was observed but, in this case, it was more drastic. It affected both Snf1P and Mig1C equally, and it continued affecting both rates also at high values of the low-glucose signal.

4.4.4. Suc2 post-transcriptional signal

Finally, the accelerated degradation of Suc2 transcripts was simulated in the same way as in the two previous experiments (k = 0.002), now observing the response in Suc2-folding (Figure 4.16.). It could be observed that Suc2-folding increased dramatically at small values of the signal.

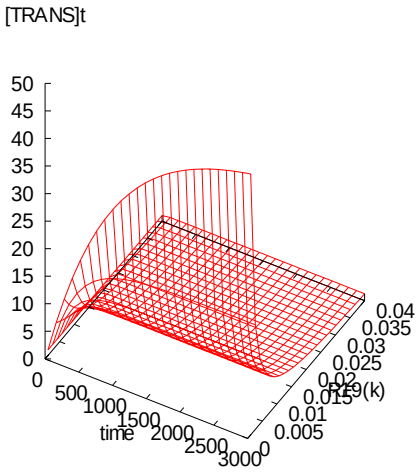

Figure 4.16. Response of Suc2-transcripts to different degrees of the "post-transcriptional" low-glucose signal (simulated as a value multiplying the kinetic parameter of Suc2-transcript degradation).

5. Conclusions and Future Work

"Prediction is difficult, especially if it concerns the future". Mark Twain.

5.1. Introduction

Two main results were presented:

- A conceptual model of the glucose de-repression process, on the signaling, transcriptional, post-transcriptional, post-translational and metabolic levels, from a comprehensive review of the state-of-the-art of the field and the discussion of the different partial models available in literature. A web application was designed in order to summarize that updated knowledge.

- A kinetic model was implemented, summarizing those aspects where knowledge is good enough, and making the best possible assumptions in all other aspects. The model can be used as a basis for a more rigorous model, waiting for experimental parameters and more biological research to be done. Simulations were carried out in order to reproduce the expected behavior, showing in this way how to use the model for didactical purposes.

5.2. Recommendations concerning the kinetic model of glucose de-repression

Besides that the model can be used as a first approximation to the construction of a rigorous model, and that nowadays it is possible to use it for didactical purposes, the process of creation of the model gave us important information regarding the parts of the de-repression process where emphasis in experimental characterization needs to be done. The weak points of our model are, at the same time, the guidelines for research to be developed:

- The kinetic constants are unknown. We used some estimates of k_{cat} and K_m for kinases homolog to Snf1p; the other proteins and the other parameters are even less correct. However, the model needs to be fitted with experimental measures; Gepasi also provides a good set of tools with this purpose.

- Measurements of concentrations/activities for proteins in each state (bound/unbound, phosphorylated/non-phosphorylated) are required. In this work, these ratios were also assumed. The main limitation is that some of these proteins seem to be very difficult to separate.

- Stoichiometries are also unknown. Research needs to be done regarding number of phosphorylation sites in the proteins, ATP stoichiometry, and so on.

- Knowledge about the glucose repression and glucose induction mechanisms might also be included.

- Finally, the mechanisms of the original signals, as well as the transport mechanisms of both Snf1 and Mig1, are unknown. However, this is the area in which a kinetic model would be more useful. A more realistic kinetic model would permit simulations of the different hypotheses regarding the nature of the signals, as well as the degree to which each of the signals is responsible for the phenomenon, besides all the studies regarding dynamics and control.

5.3. Recommendations concerning the web application

The idea of a web-based application with graphical and up-to-date evidence, interpretation and references of biological processes in all their signaling, transcriptional, post-transcriptional, post-translational and metabolic levels, seems to be an important tool for the academic/research community world-wide. This way, we would like to recommend the continuation of our efforts in the following directions:

- To keep the information updated, as soon as new evidence, discussions, partial models or important references will appear in the literature.

- To improve the application, by adding progressively related pathways (e.g. trehalose pathway in metabolism, or glucose induction in signalling).

- To design mechanisms in order to facilitate the curation of the information from the community.

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Appendices

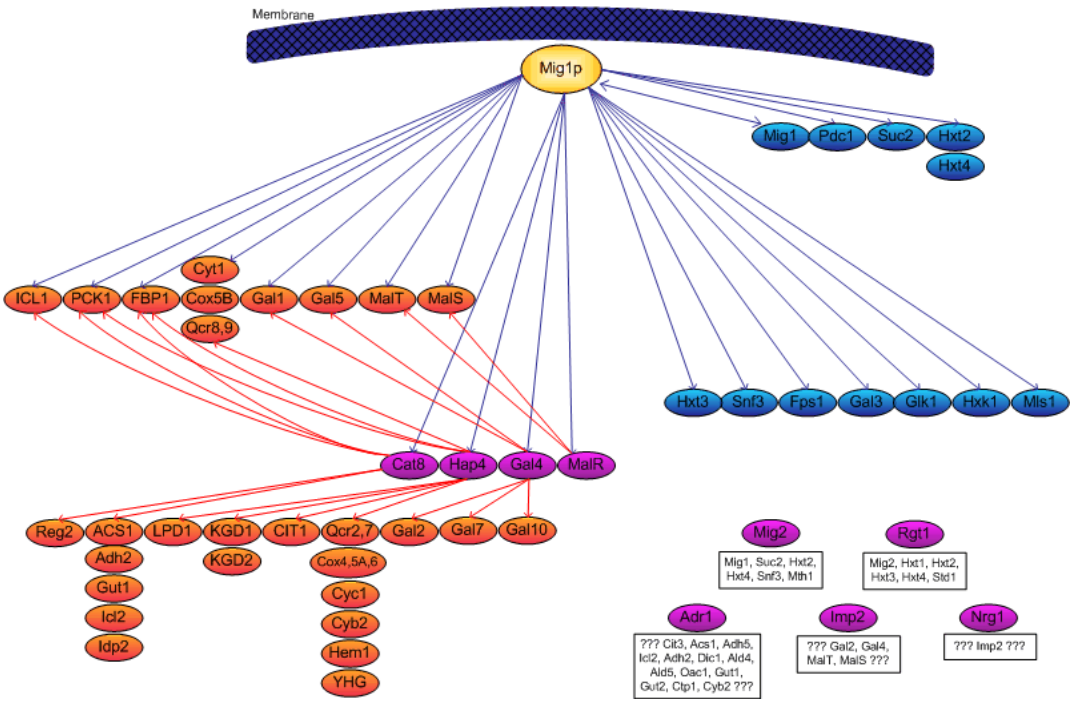
Appendix 1. Metabolites involved, symbols and connectivity degrees (the subindex "m" stands for "mitochondrial").

Abbreviation	Name	Real Connectivity Degree	Abbreviation	Name	Real Connectivity Degree
Extracellular reactions			Pyruvate Metabolism		
GLCxt	External alpha-D-Glucose	2	COAm	CoAM	5
FRUxt	External D-Fructose	2	CO2m	CO2M	24
SUCxt	External Sucrose	2	ACCOAm	Acetyl-CoAM	7
GLxt	External Glycerol	1	CO2	CO2	54
PYRxt	External Pyruvate	1	ACAL	Acetaldehyde	5
ACALxt	External Acetaldehyde		AC	Acetate	11
ACxt	External Acetate		COA	CoA	29
ETHxt	External Ethanol	1	ACCOA	Acetyl-CoA	20
CO2xt	External CO2	1	ETH	Ethanol	3
CITxt	External Citrate	1	ETHm	EthanolM	2
AKGxt	External 2-Oxoglutarate	2	ACALm	AcetaldehydeM	3
SUCCxt	External Succinate	1			
FUMxt	External Fumarate	1	TCA / Glyoxylate Cycle		
MALxt	External Malate	2	OAm	OxaloacetateM	4
			CITm	CitrateM	5
Glycolysis / Gluconeogenesis			OA	Oxaloacetate	5
GLC	alpha-D-Glucose	8	CIT	Citrate	5
bDGLC	beta-D-Glucose	2	ICITm	IsocitrateM	4
G6P	alpha-D-Glucose 6-phosphate	8	AKGm	2-OxoglutarateM	10
bDG6P	beta-D-Glucose 6-phosphate	3	ICIT	Isocitrate	3
F6P	beta-D-Fructose 6-phosphate	13	AKG	2-Oxoglutarate	18
FDP	beta-D-Fructose 1,6-bisphosphate	4	OSUCm	OxalosuccinateM	2
GAP	D-Glyceraldehyde 3-phosphate	8	OSUC	Oxalosuccinate	2
DHAP	Glycerone phosphate	8	SUCCOAm	Succinyl-CoAM	3
13PDG	3-Phospho-D-glyceroyl phosphate	3	SUCCm	SuccinateM	6
3PG	3-Phospho-D-glycerate	3	FUMm	FumarateM	4
23PDG	2,3-Bisphospho-D-glycerate	1	FUM	Fumarate	8
2PG	2-Phospho-D-glycerate	2	SUCC	Succinate	7
PEP	Phosphoenolpyruvate	6	MALm	MalateM	6
PYR	Pyruvate	13	MAL	Malate	8
PYRm	PyruvateM	5	GLX	Glyoxylate	4
Fructose Metabolism			Energy Currencies		
FRU	D-Fructose	3	ATP	ATP	121
F26P	D-Fructose 2,6-bisphosphate	2	ADP	ADP	97
F1P	D-Fructose 1-phosphate	1	PI	Orthophosphate	93
			NAD	NAD+	39
Glycerol Metabolism			NADH	NADH	34
GL	Glycerol	4	NADm	NAD+M	18
GL3P	sn-Glycerol 3-phosphate	6	NADHm	NADHM	13
GLYN	Glycerone	1	NADPm	NADP+M	23
			NADPHm	NADPHM	21
			NADP	NADP+	53
			NADPH	NADPH	51
			ATPm	ATPM	20
			ADPm	ADPM	13
			PIm	Orthophosphate M	17
			FADm	FADM	11
			FADH2m	FADH2M	9
			AMP	AMP	32
			PPI	Pyrophosphate	52

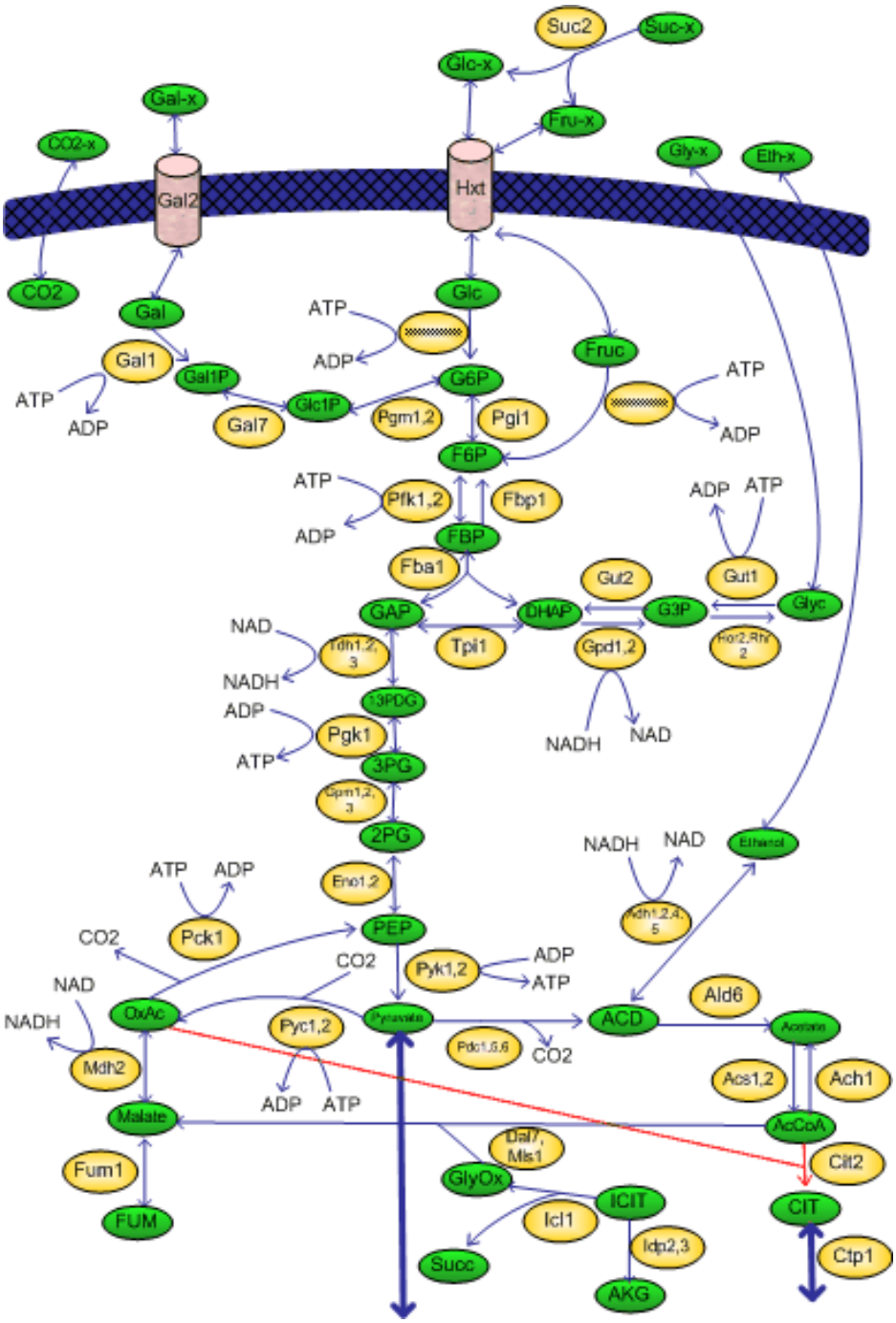
Note 1: In Reversible reactions, reactants can be considered an input AND an output, so that it would be possible to count two connections for each reactant in a reversible reaction. However, in this table we count just one connection.

Note 2: All reactions involving hexose transporters were summarized as two reactions: one for glucose transporters and one for fructose transporters

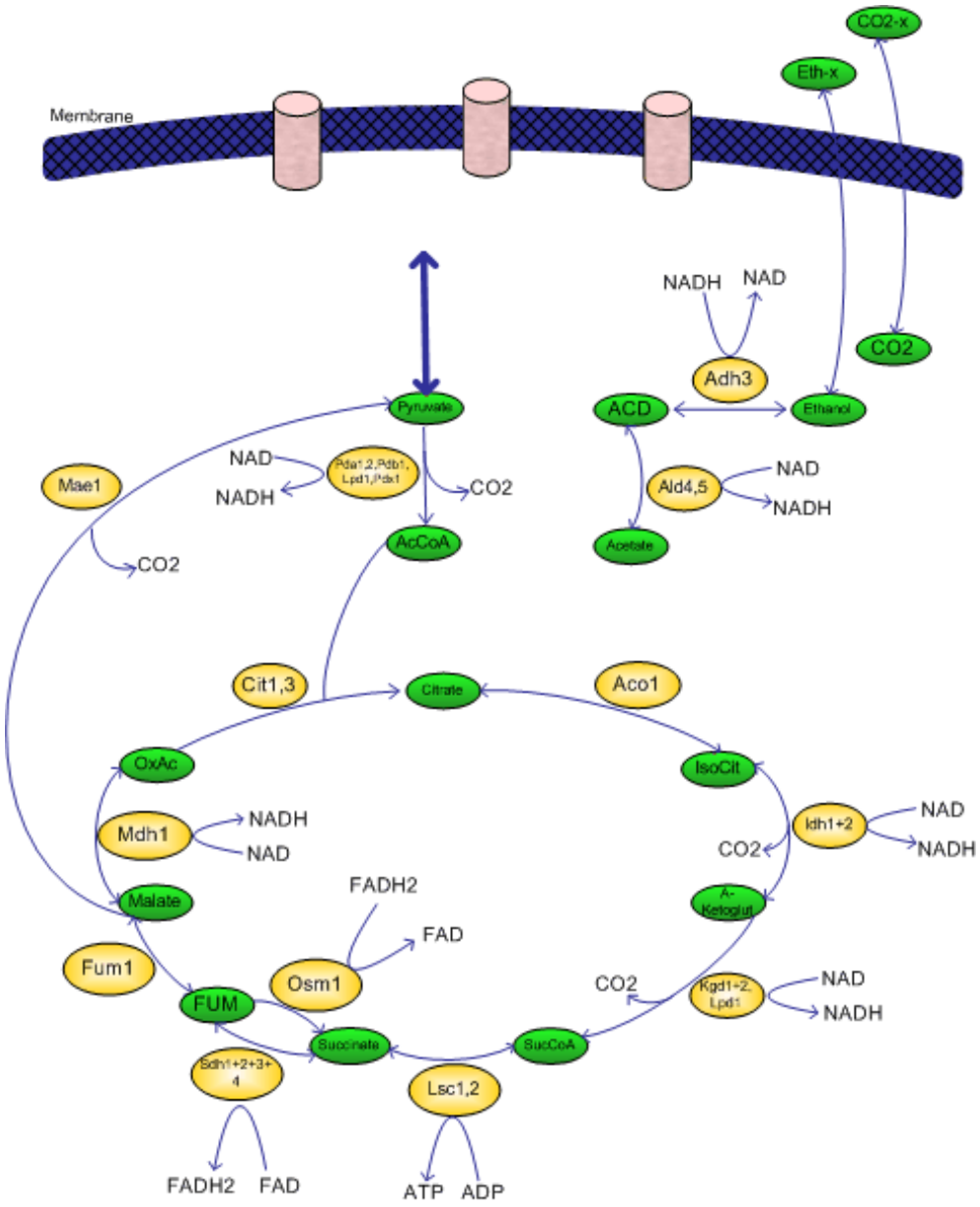
Appendix 2. Mig1 Transcription Network.



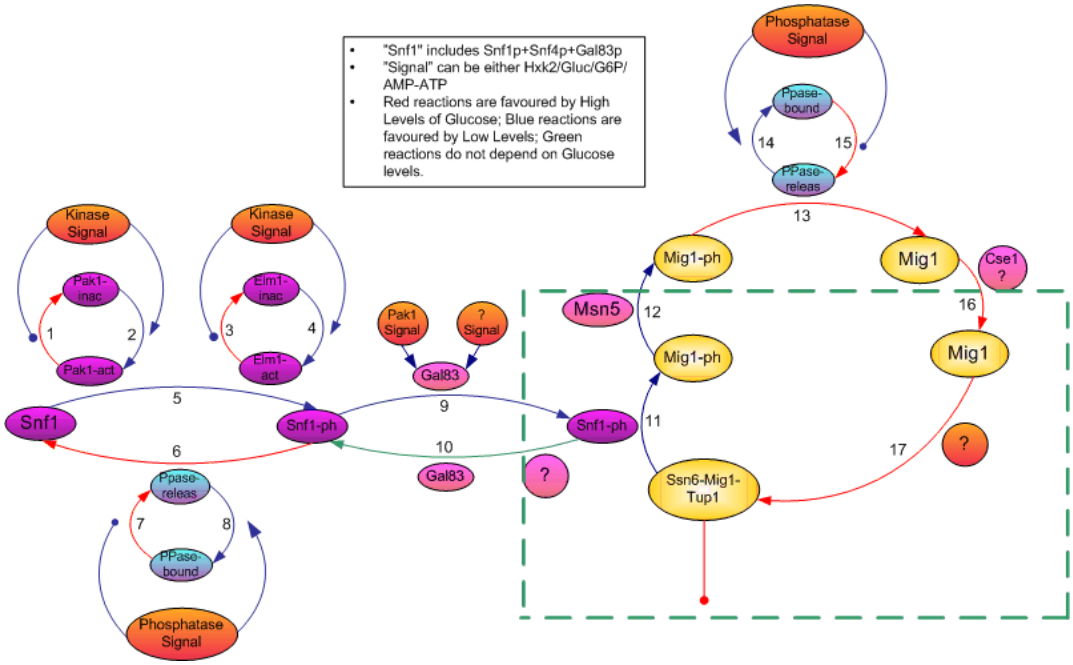
Appendix 3.A. Metabolic Map (Cytosolic reactions).



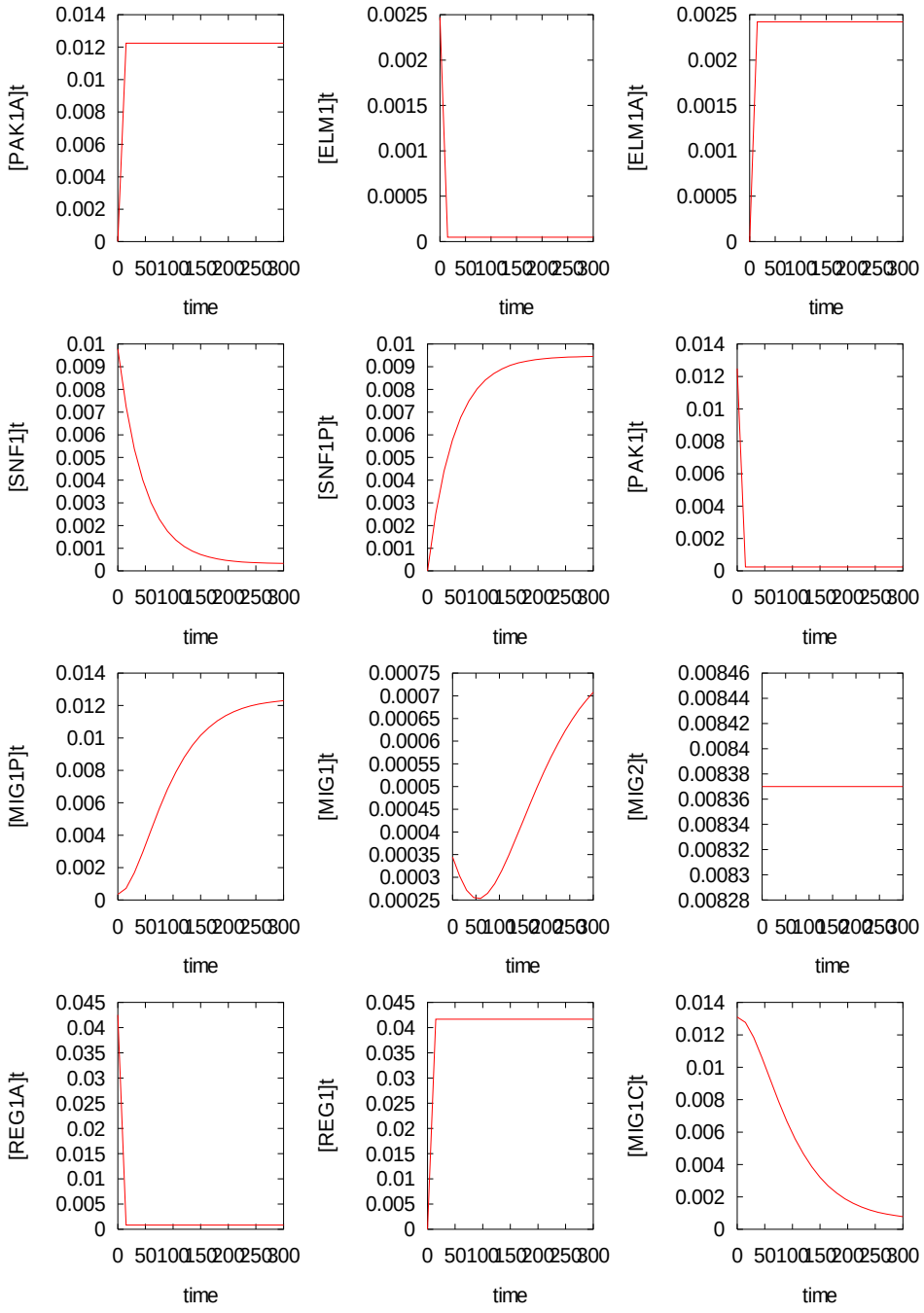
Appendix 3.B. Metabolic Map (Mitochondrial reactions).

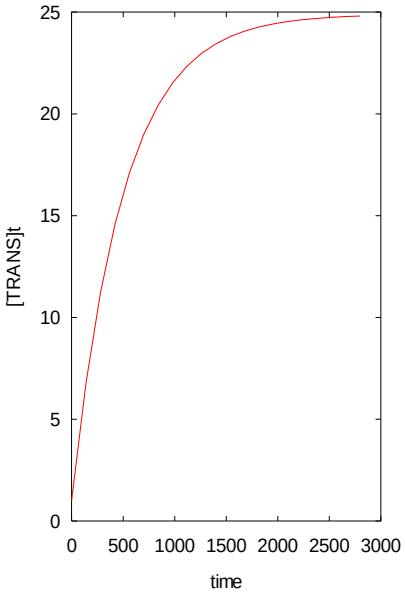


Appendix 4. Snf1-Gal83 signalling pathway.



Appendix 5. Summary (Time-courses):

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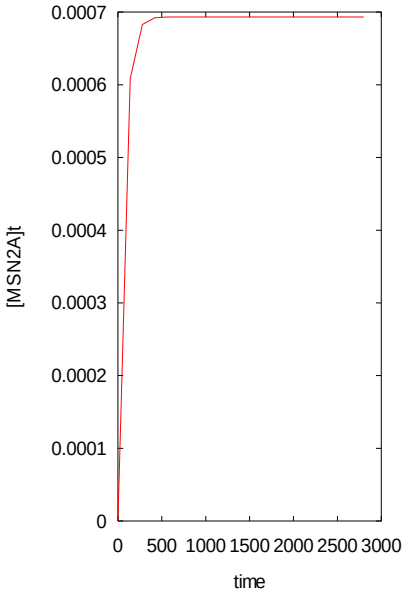
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