THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Towards a comprehensive modelling framework for studying glucose repression in yeast

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Abstract

The yeast *Saccharomyces cerevisiae* is an important model organism for human health [1, 2] and for industrial applications as a cell factory [2, 3]. For both purposes, it has been an important organism for studying glucose repression. Glucose sensing and signaling is a complex biological system, where the SNF1 pathway is the main pathway responsible for glucose repression. However, it is highly interconnected with the cAMP-PKA, Snf3-Rgt2 and TOR pathways. To handle the complexity, mathematical modeling has successfully aided in elucidating the structure, mechanism, and dynamics of the pathway [4]. In this thesis, I aim to elucidate what the effect of the interconnection of glucose repression with sensory and metabolic pathways in yeast is, and specifically, how crosstalk influences the signaling cascade; what the main effects of nutrient signaling on the metabolism are and how those are affected by intrinsic stress, such as damage accumulation. Here, I have addressed these questions by developing new frameworks for mathematical modeling.

A vector based method for Boolean representation of complex signaling events is presented. The method reduces the amount of necessary nodes and eases the interpretation of the Boolean states by separating different events that could alter the activity of a protein. This method was used to study how crosstalk influences the signaling cascade.

To be able to represent a diverse biological networks using methods suitable for respective pathways, we also developed two hybrid models. The first is demonstrating a framework to connect signaling pathways with metabolic networks, enabling the study of long-term signaling effects on the metabolism. The second hybrid model is demonstrating a framework to connect models of signaling and metabolism to growth and damage accumulation, enabling the study of how the long-term signaling effects on the metabolism influence the lifespan. This thesis represents a step towards comprehensive models of glucose repression. In addition, the methods and frameworks in this thesis can be applied and extended to other signaling pathways.

Keywords: budding yeast, glucose repression, metabolism, signaling, mathematical modelling.

Abbreviations

AMPK AMP-activated kinase

ROS Reactive oxygen spices

GAM Growth associated maintenance

NGAM Non-growth associated maintenance

PTMs Post-translational modifications

HXT Hexose transporters

STRE Stress response element

PDS Post diauxic shift element

CSRE Carbon source-responsive elements

SNF1 Sucrose non-fermenting 1

ODE Ordinary Differential Equation

SAEM Stochastic approximation expectation maximization

MCMC Markov chain Monte Carlo

FBA Flux balance analysis

FRAP Fluorescence Recovery after Photobleaching

NLME Non-linear mixed effect

bBM Bipartite Boolean model

GEMs Genome-scale models

FVA Flux variability analysis

GECKO GEM with Enzymatic Constraints using Kinetic and Omics data

ec Enzyme constrained

List of Publications

1. Welkenhuysen N, Schnitzer B, Österberg L, Cvijovic M. Robustness of nutrient signaling is maintained by interconnectivity between signal transduction pathways. Front Physiol. 2019;9:1964. doi:10.3389/fphys.2018.01964

2. Österberg L, Domenzain I, Münch J, Nielsen J, Hohmann S, Cvijovic M. A novel yeast hybrid modeling framework integrating Boolean and enzyme-constrained networks enables exploration of the interplay between signaling and metabolism. PLoS Comput Biol. 2021;17(4):e1008891. doi:10.1371/journal.pcbi.1008891

3. Österberg L, Welkenhuysen N, Persson S, Hohmann S, Cvijovic M. Localization and phosphorylation in the Snf1 network is controlled by two independent pathways. bioRxiv; 2021. doi:10.1101/2021.06.14.448401

4. Schnitzer, B.*, Österberg, L.*, Skopa, I., & Cvijovic, M. Multi-scale model suggests the trade-off between protein and ATP demand as a driver of metabolic changes during yeast replicative ageing. bioRxiv; 2022. doi:10.1101/2022.03.07.483339 (submitted)

5. Schnitzer, B., Österberg, L., & Cvijovic, M. The choice of the objective function in flux balance analysis models is crucial for predicting replicative lifespans in yeast. bioRxiv; 2022. doi:10.1101/2022.03.08.483444 (submitted)

Review Persson, S., Sviatlana S., Österberg L., and Cvijovic, M. (2022). Modelling of glucose repression signalling in Yeast Saccharomyces Cerevisiae. FEMS Yeast Research. 2022;22(1):foac012. doi:10.1093/femsyr/foac012

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Contribution summary

Paper I NW and MC conceptualized the study. NW and BS designed the model and the implementation. BS performed the model simulations. NW and LÖ carried out the experiments. MC supervised the execution of the work. All authors discussed the results and contributed to the final manuscript.

Paper II LO and MC conceptualized the study. Data curation, model design and implementation was done by LO, ID and JM. LO and ID did the formal analysis, validation and drafted the manuscript. All authors discussed the results and contributed to the final manuscript.

Paper III NW, MC and LO conceptualize the study. LO conducted the FRAP experiments and analyzed the results together with SP using NLME. LO conducted the steady-state experiments and NW performed the time-laps microfluidic experiments. LO and NW drafted the manuscript. All authors discussed the results and contributed to the final manuscript.

Paper IV LO, BS and MC conceptualized the study. LO and BS proposed the methodology. BS implemented the model and performed the model simulations. LO and BS interpreted the results and drafted the manuscript. All authors discussed the results and contributed to the final manuscript.

Paper V LO, BS and MC conceptualized the study. BS performed the simulations and drafted the manuscript. All authors discussed the results and contributed to the final manuscript.

Review SP, SS and MC conceptualized the review. LO drafted the Modelling nutrient sensing + metabolism = profit? section. All authors discussed and contributed to the final manuscript.

Preface

This doctoral thesis serves as a partial requirement for a PhD degree at the Department of Biology and Biological Engineering at Chalmers University of Technology. The doctoral studies were carried out between October 2017 and March 2022 at the Division of Systems and Synthetic Biology and supported by the Swedish Research Council (VR2016-03744). The work was supervised by Professor Marija Cvijovic, Division of Applied Mathematics and Statistics, Gothenburg University, and co-supervised by the late Professor Stefan Hohmann, Division of Systems and Synthetic Biology, Chalmers University of Technology and Research Professor Verena Siewers, Division of Systems and Synthetic Biology, Chalmers University of Technology. The thesis was examined by Professor Ivan Mijakovic.

In memoriam of Stefan Hohmann:
They existed. They existed.We can be.
Be and be better. For they existed.

When Great Trees Fall'
Maya Angelou

Linnea Österberg
March, 2022

Contents

'accurate descriptions of our pathetic thinking'

James Black

Chapter 1

Introduction

In presence of glucose, yeast uses fermentative metabolism, and represses genes for respiration along with genes for utilization of alternative carbon sources and gluconeogenesis, otherwise referred to as glucose repression. The SNF1 pathway is central in the control of glucose repression, where Snf1 is the yeast homolog of the highly conserved human AMP-activated kinase (AMPK). SNF1/AMPK acts as a global energy regulator to balance the anabolic and catabolic processes in the cell with glucose (de-)repression as its main tool [5]. Understanding glucose repression is of large interest for the development of biotechnological processes, where it is desirable to direct flux towards the product in a variety of culturing conditions, as well as for the biological understanding of associated diseases and conditions [5]. Dysregulation of SNF1/AMPK has been associated with human diseases and conditions such as aging, obesity, diabetes, cardiovascular disease, cancer and dementia [6]. Understanding the mechanism of the regulation and how the pathway interacts with other sensory and metabolic pathways in yeast, can guide efforts to help elevate or cure associated diseases and conditions.

In this thesis, I aim to elucidate the effect of the interconnection of the SNF1 pathway with sensory and metabolic pathways in yeast, specifically, how crosstalk influences the signaling cascade; what the main effects of nutrient signaling on the metabolism are and how those are affected by intrinsic stress, such as damage accumulation. In this thesis I have addressed these questions by developing and establishing new frameworks for mathematical modelling. The ideas and work put forward in this thesis demonstrate a step towards comprehensive models of glucose repression, enables improved predictions for models commonly used in the design of cell factories and presents a framework connecting the effect signaling imposes on metabolism to longevity traits and aging.

Chapter 2 and **3**, introduce mathematical frameworks and biology, focusing on the general structure and function of central carbon metabolism and glucose signaling pathways. The choice of mathematical framework is dependent on the question or context, as the frameworks come with both advantages and disadvantages. Other considerations include the frameworks' ability to capture the biology, the scalability of the framework and the availability of data. As data is a central component of mathematical modelling, a short introduction of the type of data commonly used in these types of models is presented, together with the results from **Paper III**, as an example of how mathematical regression models can be used to estimate kinetic parameters from time-lapse data.

Chapter 4 focuses on the existing models of metabolism and glucose repression including framework implementation. An exhaustive review of the mathematical models on SNF1 signalling is presented, based on the **review** paper, along with the method developed in **Paper I**. There are many metabolic models, therefore this chapter focuses on the development of metabolic models, lifting up a few examples. The result of **Paper V** and how it should be considered when simulating metabolic FBA models is also discussed.

Multi-scale models combine processes acting on different scales, such as processes at different time scales. **Chapter 5** discusses two different goals of multi-scale models and lifts a few examples of the historical development towards a multi-scale model of glucose repression. Here, the model construction from **Paper II** and **IV** is presented.

It is my sincerest hope that the ideas put forward here in this thesis will be further developed. **Chapter 7** focuses on the lessons learnt from the included papers, summarized in **Chapter 6**, and on ideas for possible developments, including my view of the future of this field and the central question: How can we continue the road towards a comprehensive model of glucose repression?

Chapter 2

Yeast metabolism and glucose signaling pathways

Life has been defined many times. In a recently published reflection in Molecular Biology Reports, Gómez-Márquez defined life as "a process that takes place in highly organized organic structures and is characterized by being pre-programmed, interactive, adaptative and evolutionary" [7]. The organized organic structure, i.e. the carbon chemistry that makes up the metabolism, transforms the nutrients and energy from its environment to enable the low entropy, necessary to maintain life. The cells' ability to sense and signal, builds up the ability to interact and adapt to, and with, the environment and systems in the cell itself. It is the foundation for life to evolve into higher organisms. These two features, metabolism and signaling, are at the core of our conceptual understanding of life. Understanding how these features work by themselves, but also how they connect and interact are central in our ability to understand life.

2.1 Central carbon metabolism

The metabolism transforms nutrients into energy (catabolism) and synthesizes building blocks for new cells or other products (anabolism). As the substrate for one reaction is a product generated by a previous reaction in a pathway, the reaction rates are dependent as much on the substrate and product metabolite ratios as they are on the kinetic properties of the enzymes in the network. The metabolism needs to be tightly regulated to avoid energy loss in futile cycles as well as to avoid depletion of important metabolites, such as the energy storage molecule ATP, and the redox cofactors NADH and NADPH, and therefore balance the rate of catabolism and biosynthesis.

The central carbon metabolism, in general, includes the pathways that enable aerobic respiration and fermentation. These include glycolysis, pentose phosphate pathway, TCA cycle, anaplerotic reactions, oxidative phophorylation and anaerobic excretion pathways (**Table 2.1**). In our model (**Paper II, IV and V**) we also include galactose metabolism **(Figure 2.1)**, representing an alternative carbon source. In the upper glycolysis, glucose is cleaved into three-carbon units, consuming two ATPs. In the lower glycolysis these units get oxidized into pyruvate generating two ATP and one NADH each, resulting in net two ATP and two NADH molecules per glucose. [9, p.469-483] In fermentation, decarboxylation of pyruvate produces CO_2, the NAD^+ is regenerated trough a reduction

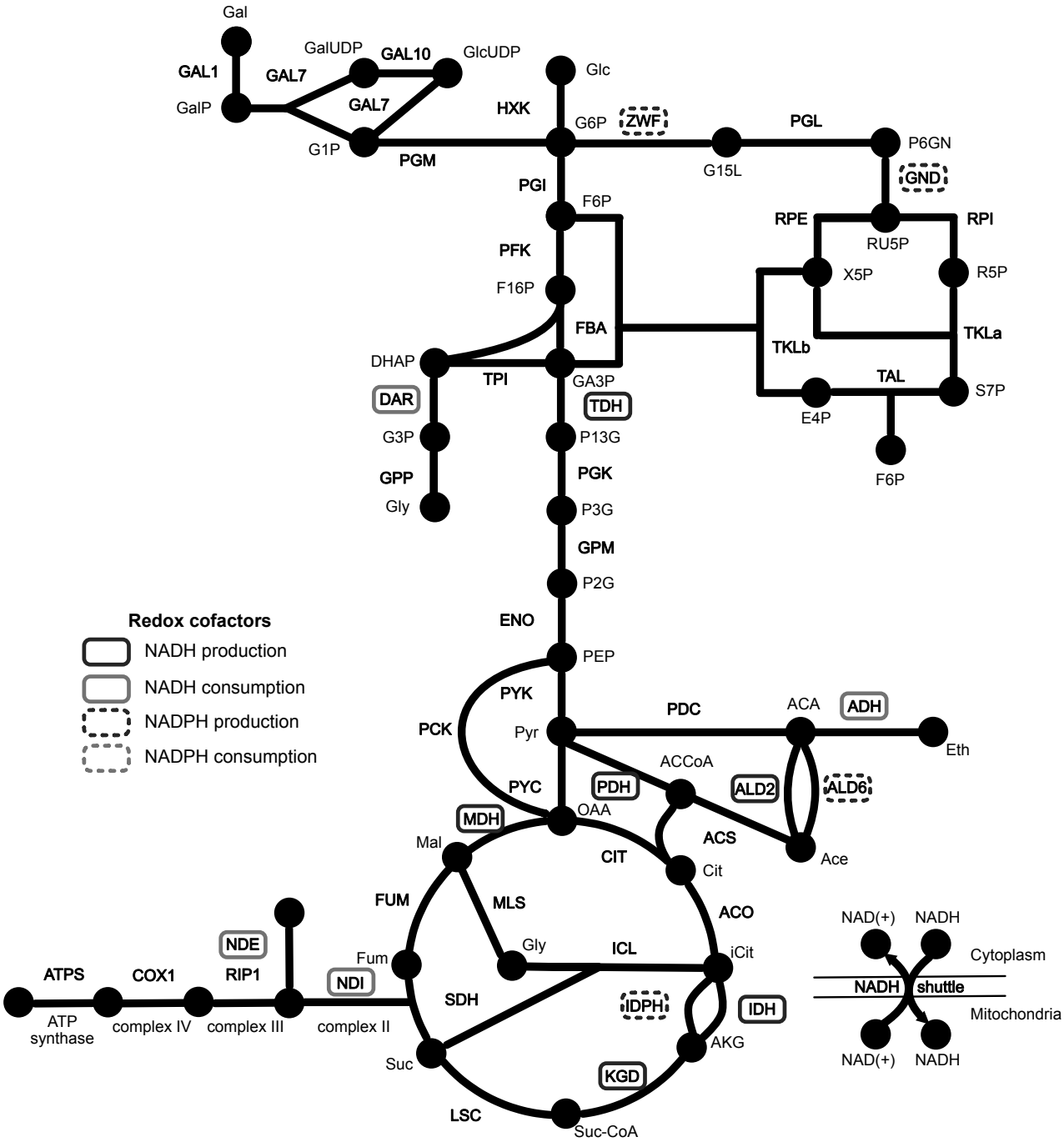

Figure 2.1: The central carbon metabolism in *Saccharomyces cerevisiae*, include pathways allowing for aerobic respiration and fermentation. Figure adapted from paper II [8].

Table 2.1: Net reactions of the pathways in the central carbon metabolism participating in the conversion of glucose into ATP.

Glycolysis	$\text{Glucose} + 2\,P_i + 2\,ADP + 2\,NAD^+ \rightarrow$ $2\,\text{pyruvate} + 2\,ATP + 2\,NADH + 2\,H^+ + 2\,H_2O$
TCA cycle	$\text{pyruvate} + CoA + NAD^+ \rightarrow$ $\text{acetyl}\,CoA + CO_2 + NADH + H^+$ $\text{acetyl}\,CoA + 3\,NAD^+ + FAD + ADP + P_i + 2\,H_2O \rightarrow$ $2\,CO_2 + 3\,NADH + FADH_2 + ATP + 2\,H^+ + CoA$
Anaplerotic reactions	$2\,\text{acetyl}\,CoA + NAD^+ + 2\,H_2O \rightarrow$ $\text{succinate} + 2\,CoASH + NADH + 2\,H^+$
Oxidative phophorylation	$NADH + 1/2\,O_2 + H^+ \rightarrow H_2O + NAD^+$ $ADP + P_i + H^+ \rightarrow ATP + H_2O$
Anaerobic excretion	$\text{pyruvate} + 2\,H^+ + NADH \rightarrow$ $\text{ethanol} + CO_2 + NAD^+$

of acetaldehyde to ethanol, maintaining the redox balance [9, p.483]. As no oxygen is required, fermentation can be carried out both aerobically and anaerobically.

In aerobic respiration, pyruvate is decarboxylated to acetyl CoA and enters the TCA cycle in the mitochondria. The citric acid cycle harvests electrons by oxidizing acetyl CoA and stores them in the electron carrier molecules NADH and $FADH_2$, in the process ATP and CO_2 are produced [9, p.515-529]. The TCA cycle is also a source of intermediates for biosynthesis, thus intermediates need to be replenished by anaplerotic reactions. These anaplerotic reactions are also important for growth on acetate in yeast. In oxidative phosphorylation, electrons from $FADH_2$ and NADH, move trough the electron transport chain and facilitate a proton gradient across the mitochondrial inner membrane and reduce oxygen to water in the mitochondrial matrix. The pH gradient powers a proton-motive force that allows ATP synthesis. From the NADH generated in the mitochondria, the electrons yield 2.5 ATP each while the ones generated in the cytoplasm only yields 1.5, depending on which path they take. The $FADH_2$ yields 1.5 ATP molecules. This sums up to 26 molecules of ATP per glucose [9, p. 573] together with the ATP generated in the glycolysis and citric acid cycle, the total reaction yields about 30 ATP molecules per glucose and can be compared to the 2 ATP per glucose generated by fermentation.

$$\text{Glucose} + 30\,ADP + 30\,P_i + 28\,H^+ + 5\,O_2 \rightarrow 30\,ATP + 6\,CO_2 + 34\,H2O$$

Although aerobic respiration is one of the most important pathways to produce ATP in higher organisms, the electron transport chain is the main source of reactive oxygen species (ROS) and oxidative stress in the cell, contributing to aging, damage and disease [10, 11]. The glycolysis branches out into the pentose phosphate pathway which mitigates oxidative stress and supplies the reducing carrier molecule NADPH as well as precursors for nucleotides and aromatic amino acids. In addition to biosynthesis, ATP and redox cofactors are also used to maintain the cell functions. The ATP demand for maintenance,

the maintenance energy, is usually divided into, growth associated maintenance (GAM) and non-growth associated maintenance (NGAM) [12].

Yeast maintenence energy
A Very Short Introduction

Probably the first expression of maintenance energy was formulated by Duclaux: $ds = Adx + mxdt$, where s = substrate, A = energy substrate consumed , x = biomass and m = energy for maintenance [12]. Thus, the maintenance energy was defined as the processes in a cell that are not directly coupled to growth, for example, repair of cell damage, protein turnover, maintenance of homeostasis in pH, osmotic pressure, energy etc. Using the Duclaux expression $ds/dt = (ds/dt)_M + (ds/dt)_G$, substituted with growth rate, where Y is observed growth yield and Y_G is the true growth yield,

$$ds/dt = -\mu x/Y$$
$$(ds/dt)_M = -mx$$
$$(ds/dt)_G = -\mu x/Y_G$$

results in:

$$1/Y = m/\mu + 1/Y_G.$$

By plotting the observed growth yield at varying growth rates, $1/Y$ against $1/\mu$, the linear fit will have the slope m and the intercept of $1/Y_G$. It is noteworthy that a linear regression model would work only when m is a constant and that the extrapolation of $1/Y_G$ is only valid while the metabolic conditions of the measurements holds true. As the maintenance constant varies greatly between the carbon source in limited continuous cultures it was postulated that the maintenance energy has two terms, growth associated maintenance (GAM) and non-growth associated maintenance (NGAM) [12]. From the postulation in the 1960's until today, there is no consensus of what constitutes the GAM and NGAM [12–15] and the terms continues to be defined by what it is not and quantified by the discrepancy between the theoretical yield and the data.

2.2 Regulation of central carbon metabolism

How the flux is distributed and thus how the resources are utilized in the cell is determined by many factors. The cell needs to not only be primed for the current situation but it also needs to be prepared for sudden changes in the environment. To tackle this, the cell exploits metabolites as reporters of the intracellular state and receptors are used to sense the extracellular environment [16]. The cell responds using post-translational modifications (PTMs) that either directly or trough a network modulate a plethora of

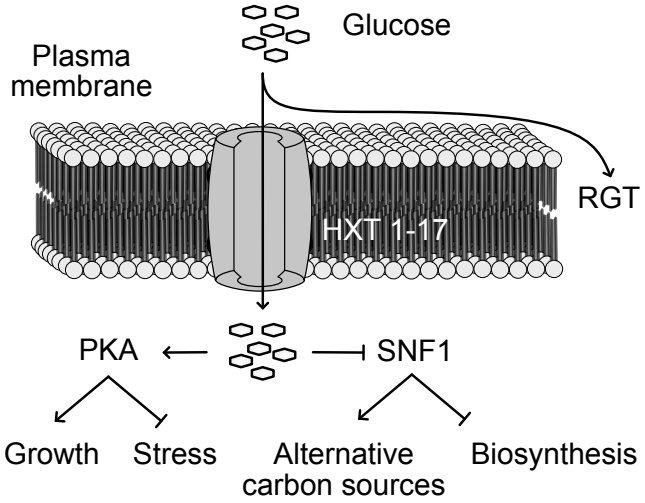

Figure 2.2: Glucose is sensed by several pathways. The cAMP-PKA pathway (PKA) is activated by glucose and regulates growth as well as inhibits stress signaling while the SNF1 pathway is inhibited by glucose. When there is no glucose available the SNF1 pathways become active and inhibits biosynthesis and induces genes necessary for growth on alternative carbon sources. The Snf3/Rgt2 pathway (RGT) is sensing extracellular glucose and regulating what type of hexose transporters (HXT) are expressed.

cellular functions. For example, the activity of proteins, the localization or activity of transcription-factors, chromatin structure or even targeting proteins for proteolysis. In addition metabolites can also by themselves act as modulators and change activity of proteins. The triunal role of these molecules as metabolites, reporters and modulators builds up a fine tuned system, able to respond with different magnitude on different time-scales and even carry memory of previous experiences.

2.2.1 Glucose sensing pathways

When specifically looking at the regulation of the cell in response to glucose availability, we already have an immensely complicated system of several pathways working together to sense and regulate the glucose response.

Snf3/Rgt2 pathway

Monosaccarids such as glucose enter the cell through transporters in the plasma membrane where most operate trough facilitated diffusion [17]. When the amount of sugar in the medium is scarce, the cell uses an active proton-sugar symporter system, coupling glucose uptake to proton uptake [18]. Yeast has about 20 genes in the hexose transporter (HXT) gene family. Snf3 senses low levels of extra-cellular sugar while Rgt2 senses high levels [5]. The other genes in the family consist of Hxt1-17 and Gal2, where all but Hxt12 can transport glucose[18]. The transporters are generally divided into three categories based on affinity for glucose (high, medium and low affinity transporters) and are dy-

namically regulated by the Snf3/Rgt2 pathway to facilitate efficient glucose uptake. Snf3 and Rgt2 are transmembrane proteins; when sensing glucose, the membrane-attached kinases Yck1 and 2 are activated, which are necessary for Mth1 and Std1 degradation. Phosphorylation of Mth1 and Std1 leads to ubiquitination and proteosomal degradation. In high glucose Mth1 is repressed by the SNF1 pathway to ensure glucose repression of HXT genes while Std1 expression is being increased, priming the cell for expression of high affinity HXT if glucose is being exhausted. When Mth1 and Std1 are being degraded, PKA hyperphospphorylates Rgt1, a transcriptional repressor, inactivating it. When glucose is depleated, Mth1 and Std1 interact with Rgt1, concealing the target site for PKA. Rgt1 gets phosphorylated by Snf1 and triggers repression activity. [5]

cAMP-PKA pathway

In *S. cerevisiae*, PKA is a tetramer with two catalytic subunits (Tpk1, Tpk2 or Tpk3) and two regulatory subunits (Bcy1) [19]. The binding of cAMP to the regulatory Bcy1, triggers the release of the Bcy1 dimer and thus activates PKA. The kelch repeat proteins Krh1 and Krh2 stimulate the association of catalytic and regulatory subunits resulting in an increased amount of cAMP required for PKA activation [20]. The different Tpk isoforms are in large functionally redundant but display different roles in PKA signalling [19]. The cAMP-PKA pathway represses genes associated with stress and induces genes associated with growth. By phosphorylating Rim15 protein kinase, it becomes inactive and can not activate the transcription factors Msn2, Msn4 and Gis1 [21] that otherwise induce genes with stress response element (STRE) and post diauxic shift element (PDS) respectively. Phosphorylation of Rim15 can also be achieved through crosstalk with the TOR pathway [22], and it has been shown that PKA crosstalks with the SNF1 pathway by inactivating the transcription factor Adr1 [23]. PKA targets include the phosphodiesterases Pde1 and Pde2 which mediate a negative feedback mechanism on PKA by degrading cAMP [24]. PKA also directly phosphorylates metabolic enzymes such as trehalase [25], phosphofructokinase 2 [26], pyruvate kinase [27] and fructose-1,6-bisphosphatase [28].

The cAMP-PKA pathway senses intracellular glucose through Ras proteins and extracellular glucose through the G-protein coupled receptor Gpr1 that interacts with Gpa2 [29]. Ras1 and 2 are GTP-binding proteins that are activated by Cdc25, triggering the exchange from GDP to GTP [30–32] while Ira1 and Ira2 trigger GTP hydrolysis [33, 34]. Extracellular glucose causes a Gpr1-mediated nucleotide exchange in Gpa2 from GDP to GTP yielding its activation [35]. Gpa2 and Ras activity stimulate cAMP production via adenylate cyclase [29, 36] however activation of adenylate cyclase also requires activity in the upper metabolism [29] and is deactivated by crosstalk with the SNF1 pathway [37].

2.2.2 Glucose repression

The transcription factor Mig1 mediates glucose repression by interacting with Cyc8/Ssn6 and Tup1 to repress transcription of genes involved in metabolism for alternative carbon sources, gluconeogenesis, respiration and peroxisome biogenesis, in high glucose conditions [38–40]. In low/no glucose conditions SNF1 is active and phosphorylates Mig1 on

at least four sites [41, 42], relieving the repression. SNF1 also activate the transcription factors Cat8, Sip4 and Adr1. Cat8 and Sip4 induce expression of genes regulated by carbon source-responsive elements (CSRE) [19] and Adr1 induces genes involved in the use of alternative carbon sources as well as peroxisome biogenesis and fatty acid utilization [19]. Active SNF1 can also directly phosphorylate and inhibit Acc1 [43].

The *SNF1* gene was identified in 1977 as *CAT1* [44], *CCR1* [45] and in 1981 as sucrose non-fermenting 1 (*SNF1*) [46] by the mutant version's inability to grow on alternative carbon sources. SNF1 is a heterotrimeric complex consisting of the catalytic α subunit (Snf1), the regulatory β subunit (Gal83, Sip1 or Sip2) and the γ subunit (Snf4) [47–49]. Snf1 contains an autoinhibitory domain that inhibits phosphorylation of the Thr210 residue needed for kinase activity. Interaction with Snf4, relieves the autoinhibition and allows for activation of the complex. Binding of ADP to Snf4 protects Snf1 from being dephosphorylated, however ATP competes with ADP for binding [50, 51]. The β subunits are partially redundant, as only the triple mutant *sip1*Δ*sip2*Δ*gal83*Δ strain show a *snf1*Δ phenotype [52]. The β subunit interacts with both the α and γ subunits and confers the localization pattern upon glucose starvation [53], where the Sip1 isoform is associated with the vacuolar membrane, Sip2 is located in the cytoplasm, and Gal83 accumulates in the nucleus [53, 54]. The subcellular localization is important for SNF1 response to alkaline stress, however, in glucose depletion all isoforms can phosphorylate Mig1, though they cannot completely complement each other [52, 54].

The SNF1 complex is activated trough phosphorylation of Thr210 on Snf1 by the constitutively active kinases Sak1, Tos3 or Elm1 [55, 56]. Dephosphorylation is mediated through Glc7 that targets Snf1 through Reg1 [57, 58].

Chapter 3

Frameworks for mathematical modelling of the cell

To handle the complexity of a biological system, mathematical modelling has proven to be an excellent tool [59–63]. Mathematical modelling itself consists of various techniques and when constructing a model, there are many considerations to make before choosing or developing a specific framework. The right approach is always depending on the context and the question i.e reason the model was created. A few of the most important considerations are:

- What abstractions and assumptions are reasonable to make?
- What level of explanation is needed?
- What type of data is available?
- What is the timescale or time dependence of the system?
- What type of predictions are expected?

The answer to these questions determines what model properties are necessary but also limitations of what is possible. In this thesis, important model properties that will be discussed are quantitative or qualitative, linear or non-linear, static or dynamic mechanistic or non-mechanistic. It is also good to have in mind that depending on which

Framework	Properties
ODE	Quantitative Linear OR Nonlinear Dynamic
FBA	Quantitative Linear Static
Boolean	Qualitative Linear Dynamic

Table 3.1: Different frameworks and their key properties

framework that are used, the implication of the model properties are different. For example, what is a disadvantage in one framework could be an advantage in a different framework.

3.1 Ordinary differential equations

Ordinary Differential Equation (ODE) based models are dynamic, implicit models, describing the rate of change. In systems biology this framework is most often used in kinetic modelling [64], where a set of non-linear ODEs are used to describe the state derivatives of the model states, x, usually corresponding to an amount or concentration of interest, such as a metabolite, protein or cell mass. The states, x, are described by the model parameters, θ, and the model input, u.

$$\frac{dx}{dt} = f(x, \theta, u) \tag{3.1}$$

The function, f, is describing the kinetic rate expressions of the chemical reactions affecting the state quantities. These are usually formulated based on the law of mass action, Michaelis-Menten kinetics, and Hill kinetics, and naturally, require knowledge on the kinetic constants, as well as time-series data on states.

Experimental kinetic values are available for some reactions, however, they are often measured *in vitro*, and even if kinetic properties of some proteins have been re-measured in an *in vivo*-like environment [65], these are usually limited to the central carbon metabolism and the availability of measurements still emprise a problem.

Time-series data of states, like phosphorylation dynamics and protein concentrations, are often done by immunoassays, which are limited by the availability and specificity of antibodies, or omics approaches. In both approaches isoforms of complexes and single proteins can be impossible to distinguish between and experiments are usually done in triplicates which is not sufficient for precise parameter estimations [66]. Hence, many parameters are formulated as fractions or measured indirectly, with low specificity, and with a low time frequency.

All types of data are associated with measurement noise in addition to the method specific considerations which propagate to the estimated values, typically computed using Monte Carlo sampling approaches [64]. The uncertainty in both, the measured and estimated parameters, impedes the development of large kinetic models [64, 66, 67] as the uncertainty scales with model size.

3.1.1 Mixed effect regression models

ODEs are also often encountered in regression models, especially when generating parameter values for kinetic models. Mixed-effect models are a class of statistical regression models that incorporate both fixed effects and random effects [68] in data based on repeated measurements of multilevel and/or longitudinal data. To decide what are fixed effects and what are random effects for a biological system is both a technical issue and a bit of a philosophical question, where fixed effects are properties of the population

and random effects are parameters that are associated to the individual belonging to the population[68].

The purpose of mixed effect regression models is to describe a response variable as a function of covariates while taking into account correlation among observations in the same group [69], meaning that there are two types of variability, the inter-subject variability and the intra-subject variability. The population parameters are assumed to follow a distribution where the fixed effect captures the overall behavior of a population. Individual subjects can be described using the fixed effect with a deviation coming from random effects, which are described by the inter-subject variability, η. Measurement errors affect all measurements and are described by ϵ. In biology, normal or log normal distributions are commonly assumed [70, 71]. For example, consider a growth model, assuming that the parameter for doubling time, T, are independent and normal distributed with a variance of α^2, the individual, i, can be described as a linear combination of fixed effects, population parameters and random effects.

$$T_i = T_{pop} + \eta_i, \tag{3.2}$$

where $T_i \sim \mathcal{N}(T_{pop}, \alpha^2)$ and $\eta_i \sim \mathcal{N}(0, \alpha^2)$. The error between the predicted values and the measured values are assumed to be normally distributed, with a variance of σ^2, centered around 0, giving:

$$y_{ij} = f(X_{0i}, t_j, T_i) + \epsilon_{ij} = X_{0i} * 2^{tj/T_i} + \epsilon_{ij}, \tag{3.3}$$

where, $\epsilon_i \sim \mathcal{N}(0, \sigma^2)$. For a general expression using the probability distributions, Ψ_{pop}, is a vector of the n population parameters and Ω is the $n \times n$ variance-covariance matrix:

$$y_{ij} \sim \mathcal{N}(f(t_{ij}, \Psi_i), \sigma^2) \tag{3.4}$$

$$\Psi_i \sim \mathcal{N}(\Psi_{pop}, \Omega) \tag{3.5}$$

y is the complete set of observations and Ψ is the complete set of individual parameters. The parameter can be estimated by using either maximum likelihood estimation or Bayesian inference.

Using the maximum likelihood approach with a stochastic approximation expectation maximization (SAEM) algorithm [72], the unknown parameters are denoted θ, and $\theta = (\Psi_{pop}, vec(\Omega), \sigma)$. The algorithm is designed to find estimates of θ which maximizes the likelihood of the data points y_{ij} giving the likelihood function as follows: $\mathcal{L}(\theta; \mathbf{y})$. If measurements are independent, the likelihood is calculated as the product of the individual likelihoods and written in terms of the joint probability distributions.

$$\mathcal{L}(\theta; \mathbf{y}) = \prod_{i=1}^{N} p(y|\theta) \tag{3.6}$$

Non-linear regression functions can not be analytically solved, the SAEM algorithm iterativly converges to the maximum likelihood [72]. The algorithm starts by generating

individual parameters based on the probability distributions of Ψ given the data y, and the population parameters from either the previous iteration or a starting guess. SAEM has implemented a Markov chain Monte Carlo (MCMC) procedure to generate individual parameters, since there are no analytical expression for this. The population parameters i.e the fixed effects are updated to the average of the individual parameters and are used in the next step as population parameters. When the values have converged, the algorithm uses the average over all the individual parameters generated in this phase of the approximation, to update the new population parameters.[72]

3.2 Flux balance analysis and constraint-based modelling

Flux balance analysis (FBA), was developed in the mid 1990s in the context of modelling metabolic networks and utilizes linear optimization to get around the need of kinetic parameters that an ODE system is limited by. The emergence of the genomics, proteomics and metabolomics fields generated an overflow of information with few tools to generate knowledge. FBA is often referred to as a top-down approach where, instead of starting with detailed information about the biological functions and mechanisms of a system and simulate a model to understand the exact characteristics of it, the idea was to start with the possible solution space of the system and successively narrow the range of possible solution based on physio-chemical constraints [73] generated by data from the new fields.

The dynamic mass balance of integrated metabolic networks is formulated based on that the change of a metabolite over time equals the difference in the production and consumption rates.

$$\frac{d[M_n]}{dt} = \sum v_{prod} - \sum v_{cons} \tag{3.7}$$

If S is a stoichiometric matrix describing the relationship between the metabolites, represented by rows, the vector of states, can be described as the product of the stoichiometric and the reaction rates, including exchange reactions [73].

$$S = \begin{array}{c} \begin{matrix} r_1 & r_2 & \cdots & r_i \end{matrix} \\ \begin{pmatrix} -1 & 0 & \cdots & 0 \\ 1 & 1 & \cdots & 0 \\ \vdots & \vdots & \ddots & \vdots \\ 0 & -2 & \cdots & 1 \end{pmatrix} \end{array} \begin{matrix} m_1 \\ m_2 \\ \vdots \\ m_n \end{matrix}$$

$$\frac{d[\mathbf{M}]}{dt} = S\mathbf{v} \tag{3.8}$$

In steady state, $S\mathbf{v} = 0$, and the null space can be reduced using both physio-chemical constraints and condition specific knowledge on the system. However, the system is usually underdetermined, $n < i$ and can not be algebraically solved. By defining an objective, linear programming can be used to calculate the optimal solution based on the objective, subject to the defined constraints, which have proven to be surprisingly accurate during a number of conditions.

3.3 Logic and Boolean models

Boolean models are qualitative, discrete, scaleable and dynamic models where kinetic parameters are not needed to infer dynamics. Generally, Boolean models were introduced in systems biology in 1969 by Kauffman [74], and have since then been a standard for modelling gene regulatory networks. They have also been used to model signaling networks[75–77] and specifically for glucose repression [4]. The model consists of n states or nodes.

$$p = \{p_1, p_2, \ldots, p_n\} \tag{3.9}$$

The nodes, or Boolean variables are constricted to a binary state, $\{0, 1\}$, but variations exists where a range are allowed $\{0, 1, ..., max_k\}$. As follows, the state space is defined as:

$$\mathcal{S} = \prod_{i=1,\ldots,n} \{0, 1, \ldots, max_k\} \tag{3.10}$$

The state of a component can be altered by a Boolean function that define the interactions that switches the state of a variable, $p = \{p_1, p_2, ..., p_n\}$. Each p_i, has a rule $\mathcal{B}_i$ The states are either updated synchronous, where all nodes are updated every time unit, or asynchronous, where in most cases, a node is chosen at random(or with a given probability) to be updated for each time unit [78]. In the synchronous case, the model is deterministic, but the time dimension of the model does not take into account that reaction dynamics through different pathways may be different and that the level of detail in each pathway is directly translated to time. If a pathway is well studied and has more interactions, the steady state takes longer to reach than for the pathway where either there is a lack of knowledge or simplifications have to be made to represent the interactions in a Boolean fashion. As stochastic behavior is characteristic for chemical reactions and biochemistry is no exception, the asynchronous approach is better at capturing that behaviour. However, the asynchronous approach leads to complicated state-transition graphs and gives the model a non-deterministic property, which can complicate the analysis. The complexity of the result and the resulting properties have to be weighted against the relevance of catching stochastic behaviours in the model.

Many biological interactions cannot be represented in a Boolean fashion and abstractions have to be made. Different approaches have been used, for example activity is achieved if at least one activator and no inhibitor is present. Another approach is to use the (weighted) sum of positive and negative regulatory contributions.

3.4 Data generation for mathematical modelling

One of the determinants of which modelling framework to work with is data availability. There are three types of data dominating the integration in mathematical modelling: 1) omics, 2) immunoblotting, and 3) fluorescence microscopy data.

The use of omics datasets such as transcriptomics, proteomics, metabolomics, fluxomics, etc. is a common way to validate models (as done in **Paper II**), reduce the solution space in constraint-based modelling frameworks and to determine parameters in

the construction of dynamic models. There are no doubts of how powerful these methods are and how large the impact has been on the diverse fields in biology and particularly in mathematical modelling. However, there are some pitfalls for omics studies that might impact our confidence in the data and affect how it should be handled. In omics studies, when looking at sample size and statistical power, the false discovery rate scales with the entities measured [79]. A microarray study looking at 461 miRNAs needed about 19 samples per condition to achieve a statistical power of 0.8 at a 1.5 fold change [80], while an RNA-seq study of 10,000 transcripts with 10 counts minimum would need 35 samples per condition for the same statistical power [81]. The effect size relates to statistical power and sample size, so that when the sample size is low we can only expect to capture large effects. It should also be noted that all type of omics data do not have straight forward ways to calculate appropriate sample size and a small sample size also gives rise to false negatives [79, 82].

Yeast proteome studies are typically conducted in two to five replicates [83–85]. A meta study of 21 proteome studies in yeast [86] concluded that the variance in data sets are highest in low abundant ($\leq$ 866 molecules per cell) and high abundant ($\geq$14,923 molecules per cell) proteins, where the median coefficient of variance was 94.8% respectively 80.9% for the respective category. The lowest median coefficient of variance was 60.2% (2786-3586 molecules per cell) [86]. Many commonly used methods for absolute quantification of protein abundance yield high experimental errors, spanning over orders of magnitude, when measuring proteins of known concentration [87–89]. Therefore, when validating a model or when using omics data in the construction, it is important to be aware of these numbers especially when using the data for quantitative modelling.

Another commonly used type of data in mathematical modelling is immunoblotting data (also called Western blot). This is a low throughput technique that measure population averages, that can be quantitative or qualitative [90] and is highly dependent on the availability of antibodies. By developing antibodies for phosphorylated epitopes one can follow dynamics of phosphorylation (as done in **Paper I**). Some proteins also display a migration shift when phosphorylated and can therefore be followed even if the antibody with affinity to the phophorylated epitope is not available [91].

Fluorescence microscopy data is mostly used to validate models or to determine parameters in the construction of dynamic models. Fluorescence Recovery after Photobleaching (FRAP) is a common technique to study protein movement and diffusion, compartmentalization and connections between intracellular compartments, speed of protein exchange between compartments, binding characteristics between proteins, or immobilization of proteins binding to larger structures [92]. In FRAP, the fluorochrome is irreversibly bleached in a small area by a high intensity illumination. The diffusion of surrounding non-bleached fluorescent molecules into the bleached area leads to recovery of fluorescence. This can be used to evaluate kinetic properties like the half life $\tau_{1/2}$ which is per definition the speed to recovery of half the plateau intensity I_∞. The fraction of the molecules that are able to diffuse is called a mobile fraction. M_f and the fraction unable to participate is called the immobile fraction I_f [92]. This method was used in **Paper III** to evaluate the speed of protein exchange between the cytosol and the nucleus for SNF1 in 2% glycerol and 0.05% glucose, when SNF1 is active, as well as in 2% glucose, when SNF1 is not active. There as distinct kinetic behaviour for all conditions where

SNF1 followed the dynamic of a single exponential recovery, indicating that all SNF1 proteins that exchange between the cytosol and the nucleus display the same kinetic behavior, consistent with previous studies where only the Snf1-Gal83 isoform locates to the nucleus [53]. The data from the FRAP experiment were fitted with a non-linear mixed effect (NLME) regression model and the correlation matrix show a negative correlation between the mobile fraction and the half life, indicating a negative feedback.

Chapter 4

Modelling of metabolism and signaling

Mathematical models of both signal transduction and metabolism have been widely used to study the biology of the respective pathways. In mathematical modelling, one has to explicitly formulate relationships between species and states which both highlights lack of knowledge and simultaneously help to form knowledge from information.

4.1 Modelling signaling

Signal transduction systems are usually modeled using ODEs [93] or logical models such as Boolean and specifically for glucose repression (Table 4.1) both Boolean and dynamic models have aided our understanding of the dynamics and topology of the SNF1 signalling pathway. Reliable quantitative models of signal transduction are hard to construct, and require extensive knowledge, not only due to the scale and uncertainty in parameters of quantitative models, but also due to the complexity added by crosstalk with other signal transduction pathways. Often experimental data is condensed to a smaller model or a simpler formulation, generating knowledge that can be used to construct a more comprehensive consensus model and iteratively our knowledge of the pathway and the models of the pathway improves.

The presence of feedback loops in regulatory systems are common. Negative feedback loops can act as a mitigating factor after initial response even when the external signal is present, a transient response or it can give rise to oscillatory behaviour. Positive feedback loops can amplify a signal but also result in a bi-stable system where a weak signal gives a transient response and a strong signal gives a sustained activation [94].

4.1.1 Boolean models of signal transduction

Boolean models of glucose signal transduction have been used to evaluate our knowledge on the topology of the SNF1 pathway and the role of crosstalk with other signaling pathways [95–97]. It has been evident that there is a lack of understanding of the topology, in particular when it comes to the activation of the SNF1 pathway [96], and that the crosstalk between different signaling pathways play an important role [95, 97]. Christensens et al. highlighted the shortcomings of Boolean models, as some complex interactions are hard to represent in a Boolean fashion [95].

Table 4.1: Frameworks of mechanistic models including the SNF1 pathway in chronological order and their main contribution.

Framework	Description
Boolean, Christensen et al. 2009 [95]	Literature based, containing 77 components, of which 22 are proteins, covering the SNF1 pathway, the Snf3/Rgt2 pathway along with glucose, galactose and maltose metabolism. Evaluated by steady-state analysis of deletion mutants compared to expression data. Contributed with knowledge on the complexity of, as well as the crosstalk between, pathways. Highlighted the lack of understanding of glucose regulatory network and the limitations of Boolean formalism in signaling transduction networks.
ODE, Kuttykrishnan et al. 2010 [98]	Mechanistic ODE model, containing 24 components, of which 12 are proteins, where 81 parameters were calculated from measurements and 10 parameters estimated. Covering the SNF1 pathway and the Snf3/Rgt2 pathway. Evaluated by steady-state and dynamic analysis of the Hxt1-4 mRNA levels. Contributed with highlighting the importance of Mth1 and Mig2 in *HXT* regulation and suggested novel interactions.
ODE, García-Salcedo et al. 2014 [99]	24 mechanistic ODE models, containing 7 components in different network structures, of which 6 are proteins, covering the SNF1 pathway. Evaluated by χ^2 and Akaike information criterion (AIC) after comparing model outcome with data on localization and phosphorylation. Contributed with better understanding of Snf1 and Mig1 regulation.
NLME, Almquist et al. 2015 [100]	Single-cell model containing 2 components, covering Mig1 nuclear accumulation upon glucose addition. Contributed with estimations of the parameters as well as insight in the cell-to-cell variability and possible mechanisms explaining the dynamics of Mig1 re-entering the nucleus after an up shift and the co-regulation with the process regulating Mig1 nuclear exit.
Boolean, Lubitz et al. 2015 [96]	Literature based, containing 52 components, of which 44 are proteins, covering the SNF1 pathway. Evaluated by steady-state analysis of different input compared to known outcomes for specific components. Contributed with identifying knowledge gaps and forming hypothetical reactions to fill the gaps.
NLME, Welkenhuysen et al. 2017 [101]	Single-cell model containing 8 components and 18 parameters, covering Mig1 localisation. Contributed with suggesting a link between glycolytic flux and Mig1 dephophorylation and the Mig1 localization as a source of cell-to-cell variability.

Boolean, Welkenhuysen et al. 2019 [97] **(Paper I)**	Literature based, containing 80 components, of which 63 are proteins, covering the SNF1 pathway, the Snf3/Rgt2 pathway and the cAMP-PKA pathway. Evaluated by steady-state localization under different conditions and phosphorylation status of key components. Contributed with a new type of Boolean formalism to easier describe complex interactions, identifying knowledge gaps and forming hypothetical reactions to fill the gaps and knowledge on the importance of crosstalk for pathway function and robustness.
NLME, Person et al. 2020 [102]	Single-cell model containing 5 components, covering Mig1 glucose repression. Contributed with the possible mechanism for *SUC2* expression dynamics where the recovery upon long term starvation is likely due to energy recovery.
ODE, Jalihal et al. 2021 [103]	Mechanistic ODE model, containing 30 components, where 128 parameters estimated. Covering the SNF1 pathway, the cAMP-PKA pathway and the TOR pathway. Evaluated by time-series data as well as perturbation analysis. Contributed with a consensus dynamic model of nutrient signaling highlighting the challenges in parameter estimation for large systems.
NLME, Persson et al. 2021 [104]	Single-cell model containing 4 components, covering Mig1 localisation. Contributed with developing a framework to improve parameter estimations in state-space mixed-effects stochastic dynamic single-cell models and suggested that the heterogeneity in Mig1 localisation is due to hexokinase activity.
NLME, Österberg et al. 2022 (**Paper III**)	Single-cell regression model containing 1 component, covering SNF1 localization dynamics. Contributed with estimates of kinetic parameters and indication of additional regulation mechanism of SNF1.
ODE, Montano-Gutierrez et al. 2021 [105]	Small mechanistic model covering the Snf3/Rgt2 and SNF1 pathways, showing that a push-pull system is a plausible mechanism for regulation of *HXT*.

To tackle the challenge of representing complex interactions, a rxncon-based approach of bipartite Boolean modelling (bBM) has been developed and implemented for the HOG pathway[106]. In addition, the model in **Paper I** tackled this challenge by a new vector-based Boolean formalism. In a vector-based Boolean models each of its N components is represented by a p-dimensional vector of binary states $\mathbf{p}_i \in \{0,1\}^k$, $i = 1..N$, that represents chosen properties. The state of each component i is altered based on all other states by Boolean rules or functions $\mathcal{B} : (\mathbf{p}_1, \mathbf{p}_2, .., \mathbf{p}_N) \rightarrow \mathbf{p}_i$. This approach has also been implemented in hybrid (**Paper II**) and multi-scale model(**Paper IV**), representing the signal transduction layer [8, 107].

4.1.2 ODE models of signal transduction

When constructing a kinetic model of signal transduction the typical procedure is to 1) decide on the topology of the network, 2) derive kinetics rate expressions, 3) construct ODE equations, 4) define initial conditions 5) perform parameter estimation based on quantitative data and 6) simulate the model [93, 108]. As highlighted by the Boolean models, the topology is missing key components and crosstalk reactions are important for the proper functioning of signal transduction. García-Salcedo et al. approached the uncertainty of the topology by constructing 24 models with different topology and evaluated them by the χ^2 and (AIC) [99]. However, considering the importance of crosstalk to understand glucose repression, and hence the importance of inclusion of other signal transduction pathways, it is evident that the parameter estimation empires the key problem for comprehensive models of glucose repression using the ODE framework. In Kuttykrishnan's model the parameters were mined from literature and only a few needed estimation [98]. The result was a model with high predictive power that could predict novel behaviour in dynamics. When Jalihal et al. constructed a consensus model of nutrient singling all 128 parameters were estimated and after fixing 49 parameters, additional 18.000 parameter sets were found, also fitting the data used for estimation [103]. Out of 15 prediction experiments only four of these were predicted by all parameter sets, while three were predicted by fewer than half of the sets.

NLME models have been used to model single-cell dynamics both producing mechanistic knowledge elucidating the topology and regulatory relationships and to estimate specific parameters aiding in the quest for curated parameters, enabling kinetic models with high predictive power (**Paper III**) [100–102, 104]. In **Paper III**, NLME was used to determine the kinetic characteristics of SNF1 nuclear-cytoplasmic shuttling at different glucose concentrations. A strong negative correlation between the kinetic constant for the recovery and the mobile fraction which indicates a negative feedback loop regulating SNF1 activity.

4.2 Modelling metabolism

The metabolic network was originally modeled using kinetic ODE-based models and some of our key findings on the dynamics of glycolysis originate from these types of models [64]. Whilst it is still an active field, FBA and constraint-based modelling approaches are

Table 4.2: A few examples of the available metabolic models and applications in yeast.

Model	Reference	Type	Description
Yeast1	Herrgård et al.[110]	GEM	First genome-scale metabolic consensus model of *S.cerevisiae*.
Yeast8	Lu et al. [111]	GEM	Latest consensus model with a biomass equation containing cofactors and metal ions.
iAN50	Nilsson and Nielsen [112]	CORE	First yeast implementation of an ecModel. The method enabled prediction of the Crabtree effect.
ecYeast7	Sánchez et al. [113]	GEM	First enzyme-constrained (ec) GEM in yeast. The method GECKO was developed that integrates kinetic information and proteomic data to GEMs.
—	Covert et al. [114]	TOY	First transcriptional regulatory constraind model.
pcYeast	Elsemman et al. [115]	GEM	Added reactions for protein production metabolism, maintenance and dilution. Extension of enzyme-constrained models.

dominating metabolic modelling. Genome-scale models (GEMs) are popular where the most recent applications are in metabolic flux predictions, culture condition optimization, cell factory design and multi-yeast comparative analysis [109] but for some applications core models are more appropriate.

4.2.1 Modelling metabolism with FBA

FBA and constraint-based modelling was driven by the rise of the omics era where the systematic collection of large-scale datasets not only enabled the construction of large biochemical networks but instigated the need for them [73, 116, 117]. Besides to enable the large size of the biochemical networks, constraint-based modelling also popularized a new way of thinking in regards to mathematical models. Instead of fighting the identifiability problem of ODEs the approach minimizes the need for parameters and instead focuses on exploring the solution space. In yeast, key efforts to constrain the solution space consist of transcriptional regulatory constraints, thermodynamical constraints, and protein constraints.

The first transcriptional regulatory constraint FBA was done 2001 by Covert et al. [114] and was implemented on a toy model of metabolism and a regulatory network. Covert used thermodynamical constraints, implemented by setting the rate of known thermodynamically unfavorable reactions to zero, and capacity constraint by setting the upper bound to a maximum uptake rate for transporters. The transcriptional regulatory constraints were implemented by building a Boolean model of the gene regulatory network. Metabolites are sensed by the flux that creates or imports the metabolite,

meaning if the flux is larger than zero the metabolite is present. If a gene is affected by the Boolean regulation and turned off, the upper bound for the fluxes associated with that gene is set to zero. This was implemented in *E. coli* [118] and in *S. cerevisiae* [119]. The yeast model consists of the 750 metabolic genes where 82 intra- and extracellular metabolites act on 55 nutrient-regulated transcription factors [119].

The basis for protein constraints is that the cell has a finite amount of proteins due to a limitation in cell size and space [120]. Several approaches have been applied to constrain metabolic models based on protein limitations where one example is total mass of proteins. This approach was first used in yeast on a core model [112] and later implemented in the tool *GEM with Enzymatic Constraints using Kinetic and Omics data* (GECKO) and applied to the yeast7 GEM. Here, the enzyme usage, E, and k_{cat} value are introduced and the stoichiometric reaction is written:

$$A + \frac{1}{k_{cat}} E \rightarrow B \tag{4.1}$$

This extends the stoichiometry matrix to also contain the k_{cat} for the reactions and the rate vector to also contain enzyme usages [113]. The model is then constrained either by experimental data on specific enzyme abundances or by the total enzyme abundance.

Recently, protein constraints have been implemented by also adding 1) a protein dilution reaction, where the dilution is equal to the growth rate, 2) degradation of proteins that generate free amino acids, 3) ribosome capacity constraints, 4) proteome maintenance constraints and 5) cytoplasmic, mitochondrion and membrane volume/area constraints [115]. This implementation contain features that would be expected from a hybrid model, only using one framework. While this allows for the simulation of quasi-steady states, the dynamic component of the interactions between the metabolism and protein production machinery is lost.

In essence, applying these constraints introduces parameters to the model and thus also introduces uncertainty to the model; however, one only needs to introduce parameters that we have prior knowledge on, and there is still no need for parameter estimation of unknown parameters.

Linear optimization and the objective function

Linear optimization is used to identify optimal solutions after applying constraints. The objective function used for optimization has been formulated based on different biological assumptions, but there is not much literature evaluating the performance of the commonly used objectives in different contexts [55, 121, 122]. The effect of the objective on the prediction power of intracellular fluxes is context-dependent [121, 122] and depending on the structure of the solution space; multiple states can fulfill the objective, leading to multiple alternate optima [123]. One method to explore the range of optimal solutions is flux variability analysis, (FVA). By fixing the values for the optimal solution, the minimal and maximal fluxes can be calculated for each reaction in the network [124]. A common strategy is to use the parsimonious FBA rationale and minimize gene-associated reaction fluxes [125] supported by a similar approach where the maximization of ATP

yield per flux unit showed a high prediction power of intracellular fluxes during nutrient-rich conditions [121]. In **Paper V** the effect of the objective function is systematically evaluated for the prediction of replicative ageing using a multi-scale model developed in **Paper IV**. The objective function is often motivated by evolutionary arguments but has not previously been evaluated against properties such as reproduction or ageing. In the study, single and sequential optimizations were tested for nutrient-rich conditions where maximal growth was found to be the only objective that by itself could generate a combination of wildtype growth rates and replicative lifespans. When allowing a deviation from optimal solutions when optimising for the sequential objective or the regulation step in the multi-scale model, more objectives could reach a wildtype phenotype. In line with previous research [121] the parsimonius solution improved prediction of lifespans for all sequential objectives where maximal growth was used as the first objective.

Chapter 5

Multi-scale modelling

Glucose repression has hitherto mostly been studied from two distinctive perspectives, the signalling perspective and the metabolic perspective. From the signalling perspective important questions have been: How does the cell sense glucose starvation? How is the signal transduced? How is the activated SNF1 specifically recognised as a glucose signal when SNF1 also is activated by other stress signals? The aim has been to gain mechanistic insight, not only for purely biological insight, but also in order to elucidate the connection between glucose regulation and human diseases and conditions such as cancer and ageing. The metabolic perspective has focused on the main question: How do components of the glucose repression pathway affect the metabolism? [126] Besides gaining knowledge on biological stress response, the focus of the metabolic perspective has been on how stress adaptation affects yeast in the industrial setting where yeast's role as a cell factory has had a huge impact on human health, world economy and culture. [127]

Recently, both the signalling and the metabolic perspectives have highlighted the importance of integrating knowledge from the opposite perspective to improve the model predictions and increase the mechanistic insight [127]. However, as demonstrated in the earlier chapters, there are good reasons for using different modelling approaches and integrating knowledge from the respective perspective, presents a technical challenge. Models of signalling focus mainly on changes and dynamics while metabolic models require at least a quasi-steady state. Multi-scale and hybrid models aim to integrate both perspectives in the mathematical models of glucose repression. When joining the two perspectives of glucose repression there are three modules that need to be accounted for: 1) signal transduction network, 2) the transcriptional regulatory network and 3) the metabolism. The first steps towards a multi-scale model of yeast and glucose repression was the development of a transcriptional regulatory constrained FBA (**Table 4.2**) which accounted for the second and third module. This represents a steady state model that allows the use of such information from the signalling models in the context of the metabolic models. Additional developments on the integration of transcriptional regulatory constraints in FBA include valve methods instead of the Boolean on/off and have recently been reviewed [128]. In large, the methods include penalties, non-linear inferred expression scores or normalized expression levels based on experimental data. This approach is limited to studying steady states, however, by solving the modules iteratively, the metabolic state can also feed back to the transcription layer. This allows for some feedback and dynamics, following the methodology of Covert et al. [114]. However, if the formulations are not Boolean, a threshold must be decided for when a metabolite is

considered present. As metabolite concentrations are not predicted using FBA and signalling cascades are typically metabolite-oriented, strong assumptions need to be made in order to connect the two. In contrast to the steady state metabolic focus, there is a model focusing on dynamics that manage to integrate all three modules, however not implemented for signalling related to glucose repression. This model implemented a detailed module of the osmotic shock phosphorelay and of the signalling cascade, together with a gene expression module and a reduced module of the glycolysis [129]. However, the formulation using only ODEs still limits the scaleability of the network and limits the usefulness from the metabolic perspective. In addition, the connections are in one direction. This assumes that the module is in steady state before it affects the following module and only allows for the effect of signaling on the metabolism and does not allow feedback signals.

The next step towards multi-scale models of yeast glucose repression was built on the development of enzyme-constrained models (**Table 4.2**) that could be coupled with heterogeneous networks [130]. To create a model of glucose repression connecting all three modules, a Boolean model of nutrient signalling pathways related to glucose repression was integrated with a core model of metabolism trough a gene regulatory network in **Paper II** [8]. The signal transduction module was modeled using a vector-based Boolean methodology presented in **Chapter 3** and **Paper I** and included the cAMP-PKA pathway, the TOR pathway and the SNF1 pathway. The output of the Boolean module consists of transcription factor activity and PTMs. The advantage of using a vector-based Boolean methodology is to be able to construct a large dynamic network, fairly easy, and still capture complex regulatory relationships and crosstalks. Another key advantage of using the Boolean formulation is the short computational time, which is beneficial for multi-scale models which are typically large and where the dominating strategy is to iteratively solve modules.

Choosing to represent the metabolism trough an enzyme-constrained model, formulated on the form: $A + \frac{1}{k_{cat}} E \rightarrow B$, gives the possibilities to implement regulation based on PTMs. The PTMs typically change the k_{cat} of an enzyme while the transcriptional regulatory constraints can be used to alter the enzyme concentrations, E. In **Paper II** a flexible pipeline has been implemented for integrating condition specific k_{cat}-values based on PTMs as outputed from the Boolean module. However, as literature-curated values on PTM specific k_{cat}s rendered the ecFBA unsolvable, that approach was dropped in the context of **Paper II** and further discussed in **Chapter 7**. Nonetheless, the k_{cat} values could be directly modified based on the output of the Boolean model, but to get from transcription factor activity to enzyme constraints, a transcriptional regulatory network/gene regulatory module is needed.

Transcriptional regulatory networks can be represented as bipartite graph, but inferring the connections is an under determined problem and inferring the right relationship still imposes a challenge. In the IDREAM approach, a probabilistic method [131] to infer these connections is implemented based on the YEASTRACT database [132] while the CoRegFlux algorithm uses statistical inference and experimental data to establish the network structure, relationships and effect size [133]. In **Paper II**, a more naive Boolean approach is used for simplicity. The active transcription factors have either a negative or a positive regulatory contribution to a set of genes that show binding evidence in the

YEASTRACT database [132]. If the sum of regulatory contributions is positive, the gene is up-regulated whilst if it is negative the gene is down regulated. The magnitude of the regulation is implemented as a fraction of the enzyme variability, set either on the upper or lower bounds of the enzyme concentration, E, that is set globally.

The resulting multi-scale model of glucose repression was based on the predictions of enzyme abundance compared to a purely enzyme-constrained model. In addition to significantly improving the protein abundance predictions, the effect of the regulatory constraints was evaluated both for respiratory metabolism and for mixed metabolism in wildtype cells and when components of the regulatory pathways were perturbed [8]. This adds to the aims of the metabolic perspective, particularly in the context of flux control theory. Flux control theory is commonly used for rational design in the development of cell factories [134]. In the implemented framework, the addition of regulatory constraints generates futile flux cycles and changes the flux control distribution. The flux distribution after the additional constraints represents a more realistic metabolic landscape compared to the optimal solution using only enzymatic constraints. In this multi-scale model of glucose repression, the glucose uptake rate was the only metabolite that fed back information to the Boolean model and with the course on/off threshold, the model contributes very little to the aims of the signalling perspective.

The model developed in **Paper II** [8] has been extended to also include a Boolean network of ROS signaling, and an ODE model of damage accumulation and growth (**Paper IV**), enabling the study of glucose repression on long term phenotypes such as aging. While still using a the limited feedback signal from the metabolism to the signal transduction module, the additions to the model in **Paper IV** can connect the aims of the metabolic perspective with the aims of the signalling perspective and elucidate how regulation of metabolism affect the replicative aging phenotype.

Yeast aging
A Very Short Introduction

There are two definitions used for ageing in yeast [135, 136]:

- Chronological ageing: the absolute time a non-dividing cell can survive
- Replicative ageing: the number of daughter cells a mother can produce

In replicative aging, loss of proteostasis is recognised as one of the hallmarks [137], and is linked to the accumulation of damaged proteins over time and cell divisions [138]. The main time-dependent source of damage is oxidative stress [139] and some have argued that it is the lone cause of ageing and the plethora of age-correlated phenotypes [140, 141]. The age-dependent accumulation of damage is mainly driven by the retention mechanism of the mother that causes an asymmetric distribution of damage after division [138].

The ODE module describe growth and the accumulation of damage. The growth rate is directly retrieved from the metabolic module and the damage is represented as a fraction of intact and damaged proteins, dependent on the oxidative stress in the metabolic

module, a non-metabolic damage formation and the damage repair. The ODE module feeds back to the metabolic module by constraining the enzyme pool based on the intact protein fraction, and through the NGAM, so that the ATP cost for maintenance scales with protein damage.

The model could only reproduce replicative lifespans and growth rates of the wildtype cells when regulatory constraints were applied. The distinct metabolic phases yeast undergoes during replicative lifespan, characterised by a switch from fermentation to mixed metabolism, followed by a switch to respiration [142, 143], could be mechanistically explained by the model and showed dynamics tightly associate with the NGAM.

Evolutionary properties are often used to motivate objectives for the FBAs but the objective functions have not been evaluated on evolutionary traits such as the replicative lifespan. The multi-scale model developed in **Paper VI** enables studies of phenotypes emerging at long time scales and serves as an excellent platform to evaluate how different objectives perform in this context, as done in **Paper V**.

Chapter 6

Summary of papers

Paper I In paper I, we investigated the role of crosstalk in glucose-sensing pathways. We developed a vector format rule-based Boolean logic model to capture the complexity of the cAMP-PKA, Snf1, and the Snf3-Rgt2 signaling pathways. I contributed to the model validation by confirming phosphorylation status by western blot. Using gap filling, we highlighted the lack of knowledge on phosphatases. By systematically perturbating the system and analyzing the component states, we found that some crosstalk contribute to robustness while others play an important role for the functioning of the system.

Paper II In paper II, we look at the effect of signaling on the metabolism. We created a hybrid model, combining an enzyme-constrained flux balance model of the central carbon metabolism and a Boolean model of the SNF1, TOR and PKA pathways. We used the strategy as in paper I, to capture the complexity of the pathways, and constrained the metabolic model using the output. To investigate the effects, we compared the metabolic model with the hybrid model in the ability to predict protein concentrations and we were able to replicate key regulatory effects of the flux distributions when transitioning from fermentation to respiration.

Paper III In paper III we wanted to further characterize the SNF1 pathway in response to different carbon sources. We looked at a common read out for the activity of SNF1, the Mig1 localization and the dynamic behavior of the SNF1 complex during steady state. I contributed to the analysis of the SNF1 complex using FRAP and used non-linear mixed effect modelling to capture the cell-to-cell variability and the population behavior.

Paper IV In paper IV we studied the dynamics of the metabolism and signaling network in relation to aging phenotypes, such as replicative lifespan and growth rate. We further built on the models we developed in paper I and II by expanding the hybrid model with ROS signaling and ROS production and connecting it to a growth model using an ordinary differential equation approach. In the growth model we include damage formation, damage repair and growth. We found that we could reproduce an age-dependent shift in metabolism to respiration in nutrient rich conditions as well as identify non-growth-related maintenance (NGAM) as a possible factor in this transition.

Paper V In paper V, we used the model developed in paper IV and systematically evaluated the effect on aging phenotypes of different objective functions in the FBA module. The core successful objective is maximum growth. Having a second objective, either to maximize the NGAM or to use the parsimonious solution, increases the variability in parameters that could reach wildtype behavior. This enabled either upregulated ROS removal or an increased respiratory metabolism in conjunction with a slower growth rate.

Review paper In the review paper, we address the progress of the bottom-up mathematical models of SNF1 signaling and cover the advantages and disadvantages of the available mathematical modelling frameworks for SNF1 models. This includes Boolean, dynamic kinetic, single-cell models. Based on the existing models, we provide a perspective on how to move forward with a consensus dynamic mechanistic model of the entire SNF1 pathway that can provide novel insights into the dynamics of nutrient signalling.

Chapter 7

Conclusions and future perspectives

The aims of mathematical models portraying the signaling system of glucose repression focus on unanswered questions on the mechanism. This knowledge is important for us to be able to connect the dysfunction of the signalling system with human diseases and conditions such as cancer and aging. The aims of the mathematical models portraying the metabolism are focused on how glucose repression and glucose starvation affect the metabolism. In this thesis, the aim was to elucidate the factors that interconnect glucose repression with sensory and metabolic pathways in yeast. This is an important piece of the puzzle to enable studies of glucose repression from both the signalling and the metabolic perspective.

In **Paper I** we developed a framework for Boolean modelling and addressed questions regarding crosstalk between nutrient sensory pathways. The method proved successful and we gained insight to the robustness of the signaling cascade. This was built on in **Paper II** where a vector-based Boolean model of signalling was created and further extended with a module of gene regulation and a module of the central carbon metabolism. The multi-scale model predicts how signalling affects metabolism and provides further insight on how the interconnectivity affects the robustness of the metabolism. The model from **Paper II** was further extended in **Paper IV** to include damage accumulation and growth. By investigating the adaptation to intrinsic stress, we elucidated traits of the age-dependent metabolic shift and the long-term effect of regulation on the metabolism.

In addition, we demonstrated that using our framework, we could improve predictions of metabolic FBA models (**Paper II**) and evaluate the impact of objective functions on longevity traits (**Paper V**), contributing to constraint-based modelling outside the scope of glucose repression.

The model from **Paper IV** works fairly well in elucidating the effects of regulation on metabolism and has potential to serve as a basis for exploring effects of a dysregulated glucose repression pathway on aging. To work as an efficient tool to study the effect of glucose repression the future development of the model would go in two directions depending on the focus of the studies, the metabolic direction or the signalling direction. However, there are improvements that would benefit both perspectives. Firstly, for simplicity, the gene regulatory network was implemented in a naive fashion. Instead, there are more sophisticated methods that predict the relationship between transcription factor and gene, and the magnitude of the regulation [133]. Secondly, the implementation of soft constraints using a penalty function could enable the implementation of PTMs. Applying constraints based on experimentally determined values and logical assumptions

leads to an unfeasible model. We could consider the confidence in the values and assumptions to create a penalty function that allows the model to break constraints, but to a cost, depending on how certain we are in the knowledge behind the constraint.

7.1 Mechanism of glucose repression and the link to human diseases and aging

To gain more mechanistic insight to the dynamics and topology of glucose repression, and the effect on longevity and health, the signaling module would benefit from being represented in a more fine-grained way. There are frameworks for extending Boolean models to be more quantitative [144, 145], and to have a more time-interpretive dynamics using Boolean delay equations [146]; however, Jalihals large scale kinetic model [103] would be a more ideal base for testing hypotheses on the signalling network. Modelling the signal transduction network in a kinetic model would allow the feedback to be more fine-grained both in terms of magnitude and time. However, it does not address the assumption that a sub-module is in steady state before the output is relayed to the next module. If the changes in the environment are sufficiently small and slow, the kinetic effects on the metabolism are negligible. However, if they are not, kinetic effects in the metabolism and the signal transduction are happening at the same timescale and the assumption that the signalling system is in steady state before affecting the metabolism is not holding. When the extracellular environment changes, we violate the steady state assumption of the FBA and metabolites can accumulate or be consumed, which is often what is detected by the signalling system. In the case of SNF1, this is believed to be the accumulation of ADP [50]. The change in the metabolites would be interesting to relay to the signalling module and could possibly be identified in the ecFBA framework by fixing the upper and lower bounds for the enzyme usage based on the first condition and then solve the model using the new extracellular environment. The subtracted flux distributions or the subtracted minimal and maximal flux for each reaction, could give an indication of which metabolites accumulate and which are depleted and could be used as candidates for unknown metabolic regulations that has been hypothesised in models of the signalling pathways [4, 101, 104]. To simulate how the system relaxes at steady state, a progressive constraint model could be implemented in the growth model, that is dependent on the level of activation in the model of glucose repression.

For development in this direction there are two major issues: the computational demand and the parameter estimation. As demonstrated by Jalihal et al. the parameter space is large [103] and not only is parameter estimation difficult but also computationally demanding [147]. However, improvements in solvers and algorithms for parameter estimation move into a promising direction [148–150].

7.2 Glucose repression and the effect on metabolism

To get a better picture of how glucose repression affect metabolism, the metabolic model should be expanded to genome scale. Two recently published models, the pcYeast [115]

and the ecYeast8 [111] serve as good bases for the metabolic modules. The ecYeast model is the enzyme-constrained version of the latest yeast consensus model and could be directly implemented. The pcYeast model is based on Yeast 7.6. using the following constraints: 1) mass balance constraints, 2) biomass composition (excluding protein), 3) enzyme capacity constraints, 4) ribosome capacity constraints, 5) compartment -specific proteome constraints, and 6) cytosolic volume constraints. In this implementation the enzyme level is determined by its synthesis rate, where regulatory constraints could be applied. The pcYeast model has more constraints implemented than the ecYeast model. This means both that the pcYeast incorporates more of our knowledge on yeast metabolism and physiology but as mentioned in **Chapter 4** and **3** the uncertainty and noise in both estimated and measured values scales with size. By implementing further constraints, one also introduces more uncertainty. If the constraints do not contribute to the explanatory power in the model, the uncertainty introduced could also push the solution further away from the biologically relevant solution and decrease the prediction power. However, if implementing soft constraints the concern can be lessened.

As demonstrated in **Paper V**, the objective function plays a large role. It would be interesting to further explore the solution space of genome-scale versions of both the model from **Paper II** and **Paper IV**.

Within my thesis, I demonstrated that even with a coarse representation of systems we benefit from comprehensive hybrid models. The notion of explicitly formulate relationships in a formal language such as mathematics, highlights our lack of knowledge and enables conversion from information to knowledge. This thesis represents a step on the road towards a comprehensive model of glucose repression. Ahead on the road, there are still challenges. However, computational and statistical developments to ease parameter estimation and reduce the computational demand, as well as community-driven data generation, will take us down the road. It will allow us to harness the ability of mathematical models to handle the complexity of biological systems and elucidate how the features of metabolism and sensory pathways work by themselves, but also how they connect and interact. It is the foundation for life to evolve into higher organisms and it is central to our ability to understand life.

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