RICE UNIVERSITY

Understanding dynamic activities of the yeast galactose utilization network under environmental changes

by

Truong Huu Nguyen

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

Matthew R. Bennett, Chair
Assistant Professor of BioSciences

Kathleen M. Beckingham,
Professor of BioSciences

John S. Olson,
Ralph and Dorothy Looney Professor of BioSciences

Laura Segatori,
Associate Professor of Chemical and Biomolecular Engineering

Yizhi Jane Tao,
Associate Professor of BioSciences

HOUSTON, TEXAS
JANUARY 2016
ABSTRACT

Understanding dynamic activities of the yeast galactose utilization network under environmental changes

by

Truong Huu Nguyen

Cellular adaptability to environmental changes depends on the collective actions of genes, mRNA, proteins and ligands, all of which are components of a “genetic network”. To understand the dynamics of a gene network in response to temporally and spatially environmental changes, we focus on the galactose utilization network in the yeast *Saccharomyces cerevisiae*. This network allows yeast cells to metabolize galactose in the absence of glucose and is tightly repressed when glucose is available in the environment. The main question is how the Gal network is activated when glucose is depleted since both sugars cannot be metabolized simultaneously. Using a microfluidic device, we supplied yeast cells with both glucose and galactose before linearly depleting glucose at different rates. We tracked the onset and accumulation of a yellow fluorescent reporter-tagged Gal1p, the first enzyme of the Gal network. Our data shows that the glucose-depletion rate plays an important role in the activation of the Gal network. The onset of the network’s activation depends on the time it takes to pass a specific threshold of the glucose concentration. On the other hand, the full induction of the Gal network, represented by the Gal1-accumulation time, is strongly influenced by the depletion rates. In particular, the mean of the Gal1-accumulation time increases significantly when glucose is depleted instantaneously. Furthermore, the variability of the Gal1-accumulation time also increases in short depletion rates and achieves a minimum at intermediate depletion rates. Using a mathematical simulation, we demonstrate that the increase in the accumulation time is due to the loss of energy when glucose is instantaneously depleted. This loss of energy also correlates with the length of diauxie, a
period of catabolic transition from glucose to a secondary carbon source. Thus, changes in
the glucose-depletion rate not only affect the dynamics of the Gal network’s activation, but
can also affect the phenotypic outcomes of the single cells within the population. Our results
contribute to growing sets of evidence that a gene network can exhibit complex, dynamic
behaviors under environmental changes to shape the fitness and survival of individual and
collective members of a microbial population.
I want to acknowledge Dr. Matthew Bennett for being a wonderful mentor and teacher. Thank you for being kind, patient and generous. You have given me a lot of advice not only on my research but also in life. You understand that I have a life and other interests outside of the lab and generously allowed me to pursue them. For that, I am truly grateful.

I would like to thank my committee members, Dr. Beckingham, Dr. Tao, Dr. Olson and Dr. Segatori for their advice and comments on my research and thesis.

My research would not be successful without many lab members who have helped me throughout the years. I would like to thank Dr. Faiza Hussain, Dr. Bo Ma, Erin O’Brien, Dr. David Shis, Andrew Herning, Yu-Yu Cheng and Chen Ye for their help and for the many fun memories we shared with each other in the lab and in Valhalla. I also like to thank Dr. Chinmaya Gupta and members of the Josić lab (University of Houston) for their collaboration in my project.

I would like to thank my wife, Dan, and my daughter, An, for your love and support throughout my research at Rice. You always warm my heart and encourage me for all the up and down in my life. My success is not fulfilled without you.

Xin cảm ơn ba mẹ, các em, và cô chú bác trong gia đình đã quan tâm và giúp đỡ Trưởng trong thời gian học tập. Thành quả ngày hôm nay đã không có được nếu thiếu lòng yêu thương của mọi người.

Nguyễn Hữu Trưởng
Contents

1 Glucose signaling in *Saccharomyces cerevisiae* ................................. 1
  1.1 Glucose sensing and import .................................................. 3
  1.2 Glucose-induced repression of the metabolism of secondary sugars .... 8
  1.3 Effects of glucose depletion on cellular activity in yeast .............. 10

2 The galactose metabolic network in *Saccharomyces cerevisiae* ............. 15
  2.1 Enzymatic reactions of the Gal network .................................. 15
  2.2 Regulatory components of the Gal network ................................ 16
    2.2.1 Gal4p, a continuously, weakly expressed transcriptional activator . 16
    2.2.2 Gal3p, a galactose sensor evolved from the galactokinase Gal1p ... 18
    2.2.3 Gal80p, a transcriptional repressor of the Gal network .......... 20
  2.3 Activity of the Gal network under glucose-dependent regulation ....... 21
    2.3.1 The Gal network is repressed in the presence of glucose .......... 21
    2.3.2 The Gal network is activated in the presence of galactose and the absence of glucose .................................................. 23
  2.4 Dynamic behaviors of the Gal network .................................... 25
    2.4.1 The Gal network is hyper-sensitive to galactose concentrations ... 27
    2.4.2 The Gal network filters environmental signals based on a specific threshold ............................................................ 28
    2.4.3 The Gal network retains its expression level from a previous exposure to galactose ......................................................... 30
    2.4.4 The activity of the Gal network can be stochastic .................. 31
    2.4.5 The Gal network can be activated in the presence of glucose ..... 32

3 Materials and methods ............................................................... 38
  3.1 Construction and maintenance of yeast strains ............................ 38
    3.1.1 Culture media ..................................................................... 38
    3.1.2 Construction of integration plasmids .................................. 38
    3.1.3 Construction of a fluorescent-tagged *GAL1* yeast strain ......... 39
### 3.2 Design and construction of microfluidic devices

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.1 Layout of the microfluidic device</td>
<td>41</td>
</tr>
<tr>
<td>3.2.2 Poly-dimethylsiloxanse (PDMS)</td>
<td>42</td>
</tr>
<tr>
<td>3.2.3 Steps for making microfluidic devices</td>
<td>43</td>
</tr>
<tr>
<td>3.2.4 Troubleshooting</td>
<td>44</td>
</tr>
</tbody>
</table>

### 3.3 Glucose-depletion assay

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.1 Priming yeast cells in galactose and glucose media</td>
<td>48</td>
</tr>
<tr>
<td>3.3.2 Calibrating a microfluidic device</td>
<td>49</td>
</tr>
<tr>
<td>3.3.3 Programming a scope run</td>
<td>53</td>
</tr>
<tr>
<td>3.3.4 Analyzing digital images</td>
<td>55</td>
</tr>
<tr>
<td>3.3.5 Calculating glucose-depletion times</td>
<td>60</td>
</tr>
<tr>
<td>3.3.6 Calculating activation times of the Gal network</td>
<td>60</td>
</tr>
<tr>
<td>3.3.7 Quantifying lengths of yeast cell cycles and diauxic phases</td>
<td>62</td>
</tr>
</tbody>
</table>

### 3.4 Constructing repression curves of the Gal network | 64 |

### 3.5 Mathematical simulation for the activation of the Gal network | 66 |

### 4 Activities of the Gal network in static environments | 67

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Repression of the Gal network follows a sigmoidal function</td>
<td>67</td>
</tr>
<tr>
<td>4.2 Activity of the Gal network in single-sugar environments</td>
<td>69</td>
</tr>
</tbody>
</table>

### 5 Activation of the Gal network is dynamically affected by the rate of glucose depletion | 73

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Cells exhibit wide ranges of $GAL1$ expression under different schemes of glucose depletion</td>
<td>73</td>
</tr>
<tr>
<td>5.2 The initiation of $GAL1$ expression depends linearly on the glucose depletion time</td>
<td>75</td>
</tr>
<tr>
<td>5.3 Gal1p’s accumulation changes non-monotonically with glucose depletion rates</td>
<td>78</td>
</tr>
<tr>
<td>5.4 Yeast cells showed three distinct growth phases during glucose depletion scheme</td>
<td>79</td>
</tr>
<tr>
<td>5.5 An energy model for GAL network induction</td>
<td>84</td>
</tr>
</tbody>
</table>

### 6 Conclusions | 88

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 The activation of the Gal network is dependent on the depletion of glucose</td>
<td>88</td>
</tr>
<tr>
<td>6.2 Future directions</td>
<td>90</td>
</tr>
</tbody>
</table>
## List of Figures

1.1 Roles of nutrients in metabolic pathways and cellular fitness .......................... 2  
1.2 Changes in glucose concentration affect widespread cellular activities .............. 4  
1.3 The Snf1-Mig1 pathway regulates the metabolism of alternative sugars ............. 9  
1.4 Growth phases of *S. cerevisiae* in a glucose environment ............................... 12  
1.5 Effects of the glucose depletion on the diauxie and the cellular physiology ....... 13  
2.1 Structures of galactose and glucose ................................................................. 16  
2.2 The galactose metabolic pathway ...................................................................... 17  
2.3 A Gal4p dimer binds to the UAS\(_{GAL}\) site ......................................................... 18  
2.4 Glucose dependent repression of the Gal network ............................................. 24  
2.5 The Gal network is activated when glucose is exhausted ............................... 26  
2.6 The Gal network acts as a low pass filter ......................................................... 29  
2.7 The expression of the Gal network is influenced by a previous exposure of galactose ................................................................. 31  
2.8 The stochastic expression of the Gal network ................................................... 33  
2.9 The activation of the Gal network depends on the glucose:galactose ratio .......... 34  
2.10 The Gal network can be activated in the anticipation of glucose depletion ...... 35  
2.11 Wild-type yeast strains have different adaptation levels to diauxie ................. 37  
3.1 The K699-1y strain .......................................................................................... 39  
3.2 Design of the microfluidic chip used in this project ......................................... 42  
3.3 A mold for making microfluidic chips ............................................................... 45  
3.4 Examples of bad and good chip features. ......................................................... 46  
3.5 Steps of the glucose depletion assay ................................................................. 49  
3.6 Materials for running a microfluidic assay ....................................................... 50  
3.7 Connected materials for a microfluidic assay .................................................. 51  
3.8 Equipment for a microfluidic assay .................................................................. 52  
3.9 The iDAW software for controlling glucose-depletion steps ......................... 54  
3.10 Images captured by the microscope in two different experimental runs .......... 56
3.11 Using ImageJ plugins to analyze digital images ............... 59
3.12 Calculating expression times of the Gal network ............... 62
3.13 Characterizing cell cycle based on scope images ............... 65
3.14 Constructing repression curves of the Gal network ............... 66

4.1 Repression of glucose on the expression of the Gal network follows a sigmoidal function ......................................................... 68
4.2 Characteristics of cells grown in exclusive galactose media .......... 70
4.3 Characteristics of Gal1p degradation in glucose media ............... 72

5.1 Experimentally tracking single cells during glucose depletion assays .... 74
5.2 Experimentally measured single-cell responses to different glucose-depletion times ................................................................. 76
5.3 The initiation of GAL1 expression depends linearly on the glucose depletion time ................................................................. 77
5.4 Increase in glucose priming period does not affect Gal1-initiation time .... 78
5.5 Gal1p accumulation changes non-monotonically with the glucose depletion rate 79
5.6 Changes in cell cycles’ length at different phases of glucose depletion .... 81
5.7 Changes in cell cycles’ length in different glucose depletion schemes .... 82
5.8 An energy model for the glucose/galactose switch ............................ 86
5.9 Results of the mathematical model ............................................. 87

6.1 The loss of energy contributes to the delay of the Gal network’s accumulation 91
6.2 Yeast cells in a family share a similar behavior in the face of environmental changes .............................................................. 92
List of Tables

3.1 Yeast strains used in this project ............................................. 40
3.2 PCR primers for constructing plasmids and yeast strains ................. 40
3.3 Equipment for setting up a scope run ....................................... 53
3.4 Roles and working positions of the five reservoirs .......................... 55
3.5 Programming the glucose-depletion time for the microfluidic assay ...... 55
3.6 Plugin 1 for measuring fluorescence values .................................. 58
3.7 Plugin 2 for correction of bad image segmentation ........................ 60
3.8 Linear depletion of glucose concentration .................................... 61

5.1 Values of Gal1p initiation and accumulation times ........................ 79
5.2 Distribution of cell cycle lengths in each phase of glucose depletion .... 83
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinases</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary unit</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CSM</td>
<td>Complete synthetic media</td>
</tr>
<tr>
<td>DAW</td>
<td>Dial-a-wave</td>
</tr>
<tr>
<td>FI</td>
<td>Fluorescent intensity</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HF</td>
<td>High Fidelity</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>P-bodies</td>
<td>Processing bodies</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly-dimethylsiloxane</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescence protein</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>scfm</td>
<td>Standard cubic feet per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>UCSD</td>
<td>University of California, San Diego</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UEP</td>
<td>Ultimate eroded point</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescence protein</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast media containing 1% yeast extract, 2% peptone, 2% dextrose (D-glucose)</td>
</tr>
</tbody>
</table>
Chapter 1

Glucose signaling in *Saccharomyces cerevisiae*

Systems biology is the study of systems of biological components, including molecules, cells, organisms or entire species (https://sysbio.med.harvard.edu). The collection of intracellular components, including nucleic acids, proteins, inducers, metabolites, and their interactions, is called a gene network. A gene network is a dynamic and complex system, making it hard to understand the network’s activity from static measurements of a single protein. In contrast, by dynamically changing the environment of a cell, we can better understand the gene network within it (Bennett *et al.*, 2007; Bennett and Hasty, 2008; Bennett *et al.*, 2008; Bennett and Hasty, 2009b).

Metabolic pathways are an important focus of systems biology. There are several reasons for this. A metabolic gene network is a direct link from environmental conditions to cellular physiology (Bennett *et al.*, 2008; Bennett and Hasty, 2008). Changes in extracellular concentrations of nutrients, such as carbon, nitrogen and amino acids, can directly affect the growth and reproduction of a cell (Gray *et al.*, 2004). For each cell cycle, the transition from the G1 to S phase depends on the availability of nutrients (Figure 1.1A). The lack of nutrients, especially a carbon source, can force a cell into a quiescent and stationary state (Gray *et al.*, 2004). As a result, a metabolic network is very sensitive to changes in environmental parameters (Pannala *et al.*, 2009). Therefore, most nutrients act as not only metabolites that are consumed to generate energy and building parts, but also inducers that
A, the cell cycle is dependent on the availability of nutrients. In a cell cycle, the transition from the G1 to S phase and the exit of the quiescent state are dependent on the availability of nutrients, especially the carbon source. This figure was adapted from (Gray et al., 2004).

B, the dual roles of nutrients. After being imported into a cell, nutrients are metabolized (blue arrow) to generate intermediates and energy for cell growth. Nutrients and intermediates can act as inducers to trigger the activation (red arrow) of their metabolic pathway (red star). The activation of the metabolic pathway contributes to the general signaling process and the regulation of the metabolic pathway itself. This figure was adapted from (Saad et al., 2013).

trigger the activation or repression of genes (Figure 1.1B). Thus, in a natural environment in which multiple sources of nutrients are available, the interplay between different metabolic pathways is an important topic of systems biology (Saad et al., 2013).

In nature, carbon sources are primarily sugars, such as hexoses, pentoses, and polysaccharides, though other sources, such as alcohol and glycerol, can also be used. Most microorganisms cannot consume multiple carbon sources simultaneously (Monod, 1942). Instead, cells metabolize carbon sources sequentially starting with the most preferred (usually glu-
cose), and then the less preferred, such as galactose. As a result, when glucose is present in the environment a cell needs to quickly metabolize glucose while simultaneously repressing alternative metabolic pathways. When glucose becomes scarce in the environment, the cell needs to make a quick catabolic transition to the metabolic pathway of an alternative sugar, or risk losing energy. The transition from glucose metabolism to the metabolism of an alternative sugar is the main topic of my thesis. Specifically, I investigated the relationship between glucose and galactose, an alternative sugar, in the yeast *Saccharomyces cerevisiae*. The pathway for metabolizing galactose, called the “Gal network”, is tightly repressed when glucose is available in the environment (Johnston *et al.*, 1994). Only when glucose is depleted will the Gal network be activated. However, the transition from glucose to galactose metabolism was shown to be very dynamic especially when the environmental level of glucose fluctuates (Bennett *et al.*, 2008), but the details of these dynamics are still poorly understood.

In the introduction of my thesis, I will discuss two aspects of galactose utilization that are relevant to my research. First, I will describe the role of glucose in cellular physiology, especially the glucose-induced repression of the Gal network and intracellular effects caused by glucose depletion. Second, I will discuss the galactose metabolic pathway, how it is repressed and activated in the presence and absence of glucose and what one can learn about the activity of genetic networks through our investigation of the Gal network.

1.1 Glucose sensing and import

Glucose is the most preferred carbon source for *S. cerevisiae* and thus, changes in the glucose concentration can affect widespread activities of a yeast cell. These activities include oxidative phosphorylation, biosynthesis of amino acids and ribosomes, and the metabolism

---

*Following a standard yeast nomenclature, in this thesis, a yeast gene is written in italic uppercase letters. A yeast protein is written in normal letters with the first letter uppercase and the last letter is “p” for protein. Thus, *GAL1* is a gene, Gal1p is the protein encoded by the *GAL1* gene. For a gene lacking a number at the end, such as *HXT*, the protein is depicted as Hxt. In other situations, explanations will be given for clarification. For example, the “Snf1-Mig1 pathway” represents the *SNF1* and *MIG1* genes and Snf1p and Mig1p proteins that involve in the process.*
of alternative carbon sources. It has been estimated that the expression levels of more than 1000 genes are affected by changes in the glucose concentration (Figure 1.2). It should be noted that the concentration of extracellular glucose is rarely stable and can fluctuate from micromolar to molar concentrations (Busti et al., 2010). Due to the fluctuation in glucose levels, yeast cells do not oxidize this sugar into water and carbon dioxide through the TCA cycle, but metabolize it by alcoholic fermentation (Busti et al., 2010). While alcoholic fermentation yields less energy than respiration, this process allows yeast cells to consume glucose at a much faster rate. Consequently, yeast cells are able to utilize the limited amount of available glucose and outgrow competing microorganisms (Busti et al., 2010). I will not go into details about the enzymatic reactions of the glucose metabolism, since the main difference between the glucose and galactose metabolism is the number of steps taken to convert the sugars to glucose-6-phosphate. Rather, I will focus on three aspects of glucose signaling and metabolism: 1) how glucose is sensed and imported into the cell; 2) how glucose represses the metabolism of alternative sugars via the Snfl-Mig1 pathway; and 3) changes in cellular physiology when glucose is depleted in the environment.
The importance of glucose to yeast cells can be highlighted by the fact that yeast has evolved a diverse range of transporters and sensors in order to efficiently capture glucose at different concentrations (Busti et al., 2010). There are 17 transporters (encoded by \textit{HXT1 - HXT17}), two sensors (\textit{SNF3, RGT2}) and one receptor (\textit{GPR1}) for glucose. These proteins are transmembrane proteins with diverse, cytoplasmic-facing amino- and carboxyl-terminal tails (Reifenberger et al., 1997; Ozcan and Johnston, 1999; Zaman et al., 2008; Santangelo, 2006; Rolland et al., 2002).

The transporters encoded by \textit{HXT1 - HXT17} are responsible for transporting glucose into the cell for metabolism (Ozcan and Johnston, 1999). Each of these transporters has a different affinity to glucose ranging from low (Hxt1p and Hxt3p), medium (Hxt2p and Hxt4p) to high affinities (Hxt6p and Hxt7p) (Reifenberger et al., 1997; Maier et al., 2002). The wide range of glucose affinities allows a cell to adapt to different growth conditions. In high glucose concentrations, a low-affinity but high-capacity Hxt1p transporter is suitable, whereas the Hxt6p and Hxt7p transporters work best in low glucose concentrations (Reifenberger et al., 1997; Maier et al., 2002). The differences in the glucose affinities among these transporters correlate with the glucose levels that regulate their expression. Some transporters are induced only in high (\textit{HXT1}) or low (\textit{HXT2 and HXT4}) glucose concentrations. \textit{HXT3} and \textit{HXT5} are regulated independently of glucose concentrations whereas \textit{HXT6} and \textit{HXT7} are repressed under high glucose levels (Diderich et al., 1999; Zaman et al., 2009; Diderich et al., 2001; Verwaal et al., 2002; Verwaal et al., 2004). The expressions of these transporters are regulated directly by Snf1 and Snf3/Rgt2 pathways and indirectly through Protein kinase A (PKA), the hyperosmotic response and the Target of Rapamycin (TOR) pathways (Busti et al., 2010).

G protein-coupled receptors Gpr1/Gpa2 are responsible for cAMP synthesis and activating the PKA pathway. The G protein-coupled receptors (GPCRs) consist of the receptor Gpr1p, the G\alpha protein Gpa2p and its regulator of heterotrimeric G protein-signaling (RGS) protein Rgs2p (Colombo et al., 1998; Versele et al., 1999). Gpr1p is only activated when
it binds directly with glucose or sucrose. It is not activated by fructose or galactose, and is inhibited by mannose (Lemaire et al., 2004). Interestingly, Gpr1p has a higher affinity for sucrose than glucose, which may reflect the fact that sucrose is more abundant in nature than glucose (Rubio-Texeira et al., 2010; Busti et al., 2010). The binding of glucose to Gpr1p activates Gpa2p, leads to a direct activation of adenylate cyclase, increases cAMP concentration and activates the PKA pathway. Furthermore, the activation of the Gpr1-Gpr2 system also inhibits activities of Krh proteins, PKA inhibitors, which further increases the concentration of cAMP and the activity level of the PKA (Rubio-Texeira et al., 2010). However, sensing extracellular glucose is not enough to activate the PKA pathway. An increase in intracellular glucose is also needed. Imported glucose is phosphorylated into glucose-6-phosphate by Glk1p, Hxk1p, and/or Hxk2p (Rolland et al., 2000). An increase in the level of glucose-6-phosphate promotes the concentration of small GTP-bound proteins, Ras1p and Ras2p, and maintains the level of the adenylate cyclase Cyr1p which catalyzes ATP to cAMP (Colombo et al., 2004).

The glucose sensors Snf3p and Rgt2p are responsible for regulating the expression of Hxt transporters. These sensors are homologs of nutrient transporters, however their transporting capabilities have been lost. For glucose, Snf3p and Rgt2p are two non-transporting transceptrors that generate intracellular signals for the induction of hexose transporter (HXT) genes (Rubio-Texeira et al., 2010). Both Snf3p and Rgt2p contain long C-terminal tails that act as signaling domains and interact with the regulatory proteins Mth1p and Std1p (Ozcan et al., 1998). In the absence of glucose, Mth1p and Std1p mainly stay in the nucleus and bind to Rgt1p (Polish et al., 2005; Papamichos-Chronakis et al., 2004; Treitel and Carlson, 1995). Rgt1p, in turn, binds to the promoters of the HXT genes and inhibits their transcription through the activity of the corepressor complex Cyc8p/Ssn6p and Tup1p (Polish et al., 2005). Snf3p is a high-affinity sensor which induces high-affinity HXT2, HXT6, and HXT7 transporters in low glucose conditions. On the other hand, Rgt2p is a low-affinity sensor which induces HXT1 transporter in high glucose conditions (Ozcan et al., 1998; Moriya
and Johnston, 2004). In the presence of glucose, both proteins induce the phosphorylation of Mth1p and Std1p via kinase Yck1/2 and result in the ubiquitination and degradation of Mth1p and Std1p by ligase Grr1p and proteasomes (Moriya and Johnston, 2004; Santangelo, 2006; Kim et al., 2006). This proteolysis releases Rgt1p from \textit{HXT} genes promoters allowing \textit{HXT} and downstream genes to be transcribed (Santangelo, 2006). Kim and Johnston elucidated that PKA, which is activated by the Gpr1p-Gpr2p complex, directly phosphorylate Rgt1p. Both PKA and five conserved Serine residues near the N-terminal of Rgt1p are required for the removal of Rgt1p from the \textit{HXT} promoters and the transcription of these \textit{HXT} genes (Kim and Johnston, 2006).

The presence of the non-transporting transceptors Snf3p and Rgt2p implies that sensing and importing glucose are two activities that can run parallel to one another. In one research article, Youk and van Oudenaarden tested different yeast mutants, each of them containing either Snf3p, Rgt2p, or one of the six hexose transporters (Hxt 1-4, 6-7) (Youk and van Oudenaarden, 2009). By controlling their transcription separately, Youk et al. demonstrated that cells expressing a fixed level of hexose transporters can grow faster, slower or enter a stationary phase, even with an increase in glucose uptake rates and extracellular concentrations of glucose (Youk and van Oudenaarden, 2009). When both the sensors Snf3p and Rgt2p are absent, cell growth depends entirely on the glucose concentration, albeit with a significant reduction in sensitivity. These results suggest that the interaction between the sensing and importing modules actually controls cell growth. The reason is that yeast cells cannot measure the glucose uptake rate in real time, and thus cells must rely on the extracellular glucose concentration to set their internal activities. By using Snf3p and Rgt2p, cells can measure extracellular glucose concentrations, anticipate the glucose import rate, regulate the intracellular activities based on the import rate and then put the expression of the hexose transporters under the control of Snf3p and Rgt2p (Youk and van Oudenaarden, 2009). This approach reduces the energy cost of a direct measurement of the glucose import. However, it cannot prepare cells for a situation in which glucose depletes instantaneously,
as demonstrated by our research.

**1.2 Glucose-induced repression of the metabolism of secondary sugars**

Glucose metabolism generates enough energy to support fast exponential growth of yeast cells. As a result, pathways for metabolizing alternative sugars are tightly repressed in the presence of glucose. This repression prevents interference from different carbon influxes and production of toxic intermediates (de Jongh et al., 2008), and is regulated by the Snf1-Mig1 pathway.

Snf1p is a Ser/Thr-specific protein kinase and a yeast homolog of the mammalian AMP-activated protein kinases (AMPK) and plant kinases (Celenza and Carlson, 1986). Plant kinases have been shown to substitute for Snf1p’s function in yeast (Celenza and Carlson, 1986). The Snf1p complex is a heterotrimer that is composed of α, β and γ subunits (Vincent et al., 2001; Momcilovic et al., 2008). The α subunit, Snf1p, has a kinase domain in its N-terminus and an auto-inhibitory domain in its C-terminus. The β subunit, interchangeable among Sip1p, Sip2p and Gal83p proteins, regulates the localization of the complex. The γ subunit, Snf4p, is responsible for counteracting the auto-inhibitory domain and for Snf1p phosphorylation at its T210 residue (Vincent et al., 2001; Momcilovic et al., 2008; Jiang and Carlson, 1996). In the presence of glucose, Glc7p/Reg1p phosphatases interact with Snf1p and dephosphorylate the T210 residue (Vincent et al., 2001; Momcilovic et al., 2008; Jiang and Carlson, 1996). As a result, the C-terminal inhibitory domain of Snf1p binds to the N-terminal kinase domain and inhibits its activity (Figure 1.3). The Snf1 complex is inactivated and stays in the cytoplasm (García-Salcedo et al., 2014). When glucose is exhausted, changes in the ATP/AMP ratio trigger the activation of Snf1p (Wilson et al., 1996). Within 5 minutes, Sak1p kinase phosphorylates the T210 residue and triggers the conformation change of Snf4p (Wilson et al., 1996; Hedbacker and Carlson, 2008). The Snf4p protein binds to the auto-inhibitory domain and prevents its interaction with the kinase domain of Snf1p.
Figure 1.3: The Snf1-Mig1 pathway regulates the metabolism of alternative sugars. When glucose is abundant in the environment, glucose is imported into the cell by hexose transporters (Hxt). Glucose is converted to glucose-6-phosphate mainly by hexosekinase Hxk2 before entering glycolysis. The phosphatases Glc7 and Reg1/2 dephosphorylate the Snf1 kinase and keep it in the cytoplasm. As a result, the repressor Mig1p inhibits the expression of metabolic pathways of alternative carbon sources. When glucose level is low, glucose is still imported into the cell and is phosphorylated by the hexosekinases Hxk1/2, however, glycolytic activity is reduced. As a result, the Sak1 kinase phosphorylates Snf1 and activates it through the Snf4 protein. The phosphorylated Snf1 moves to the nucleus and phosphorylates Mig1p. Phosphorylated Mig1p is exported to the cytoplasm and its repression on alternative pathways is alleviated. As a result, the metabolism of secondary sugars is initiated (Bendrioua et al., 2014; García-Salcedo et al., 2014).

The Snf1 complex is delivered to the nucleus via the activity of the β subunit. In the nucleus, Snf1p phosphorylates its target proteins (García-Salcedo et al., 2014; Vincent et al., 2001; Sanz et al., 2000).

Among 500 genes that are directly and indirectly regulated by Snf1p, there are several important downstream targets (Busti et al., 2010; Hardie et al., 1998). One of Snf1’s main
targets is Mig1p, a glucose-dependent repressor. Mig1p is a C$_2$H$_2$ zinc finger protein (Nehlin and Ronne, 1990) and was first identified as a multicopy inhibitor of GAL genes (Nehlin and Ronne, 1990). It contains two N-terminal zinc-fingers of the C$_2$H$_2$ type and binds to a 6-bp GC-rich core sequence with a motif (G/C)(C/T)GGGG (Griggs and Johnston, 1991). The GC box is essential, all but three mutations within the box abolish binding whereas no mutation outside the box affects its interaction (Griggs and Johnston, 1991). The specificity of Mig1p is determined by the base-triplet recognition within the box and also by the presence of an adjacent AT box. Lundin demonstrated that Mig1p bends its target site within the AT box, suggesting the requirement of AT nucleotides at this site (Lundin et al., 1994). In the presence of glucose, the Snf1 complex is inactivated, and thus Mig1p is dephosphorylated and stays in the nucleus. Consequently, it binds to the specific GC-rich sequence on promoters of target genes, including GAL4 and GAL1 of the Gal network, and represses their transcription through the Ssn6p-Tup1p complex (Nehlin et al., 1991; Treitel and Carlson, 1995). The Tup1-Ssn6 complex interacts with the mediator of RNA polymerase II and halts its transcriptional activity. This complex also recruits histone deacetylases to the target’s promoter. As a result, histone proteins are deacetylated and the chromatin is compacted to ensure a complete repression of the target gene (Malavé and Dent, 2006). When glucose is exhausted, the Snf1 kinase phosphorylates Mig1p and facilitates Mig1p’s exit to the cytoplasm and alleviates its repression on the GAL4 and GAL1 genes (De Vit et al., 1997). As a result, these genes are transcribed and the Gal network is activated (Figure 1.3).

### 1.3 Effects of glucose depletion on cellular activity in yeast

Typically, a cell may exist in three distinct states; proliferation, slow proliferation and quiescence (Gray et al., 2004). These three states match three growth phases in cell culture, namely exponential, post-diauxic and stationary phase (Figure 1.4). When glucose is abundant in the environment, cells are able to maintain proliferation. However, when glucose
is exhausted, cells undergo a period called the “diauxic shift” (Figure 1.5A). If there is no alternative carbon source, the diauxic shift marks the transition from exponential growth to post-diauxic growth (Gray et al., 2004). If there is an alternative carbon source, like galactose, the diauxic shift marks the transition from glucose-induced proliferation to galactose-induced proliferation. In both cases, the diauxic shift is the period when a cell rearranges its gene expression to adapt to a new environment (Li et al., 2013).

A rich nutrient environment that contains fermentable sugars, essential nutrients, and amino acids allows cells to proliferate in the exponential growth phase. This period is characterized by continuous cell division with a doubling time on an average of 90 minutes (Gray et al., 2004). Also, the rates of protein and mRNA syntheses do not fluctuate during the cell cycle (Elliott and McLaughlin, 1978; Di Talia et al., 2007). DNA synthesis is initiated just before the budding event, peaks when a new bud emerges and ceases when the daughter and mother cells separate (Elliott and McLaughlin, 1978). Cell size is controlled throughout the cell cycle by the activity of G1 cyclins, such as Cln3p, to ensure that all cells divide at the same size (Di Talia et al., 2007).† Importantly, during exponential growth, a large portion of cellular activity is devoted to protein synthesis, and the creation of a sufficient number of ribosomes. Indeed, *S. cerevisiae* has 138 genes encoding 79 ribosomal proteins, 150 rDNA repeats encoding 4 rRNAs (5S, 5.8S, 18S, and 25S), as well as 236 genes encoding components of the transcriptional and translational machineries (Zaman et al., 2008). As such, more than 50% of cellular and polymerase activities and up to 90% of total energy is devoted to ribosome synthesis (Warner, 1999). Consequently, any decrease in the metabolic rate will directly affect the synthesis of ribosomes and the protein synthesis of a cell.

When glucose is exhausted from the environment, one direct consequence is the loss of energy generated by glucose metabolism. Van de Brink demonstrated that the transition from glucose to galactose metabolism, in aerobic conditions, results in a loss of up to 30%

†However, some researchers have argued that cell growth, and especially cell volume, varies at different stages in the cell cycle. Specifically, the growth rate is fixed before budding, then slows down just before the budding event, and then increases (Goranov and Amon, 2010). The difference in these findings may depend on strains and culture conditions.
Figure 1.4: Growth phases of *S. cerevisiae* in a glucose environment. Different phases of cell growth and the inoculated culture are depicted through a log scale. Yeast cells go from slow proliferating to proliferating, slow proliferating and quiescent states. The transition from the proliferating to the slow proliferating is called a diauxic shift. The switch from the slow proliferating to the quiescence is called saturation and it has been shown that cells prepare for this switch before nutrients are depleted. If the environment has only glucose as the sole carbon source, then cells metabolize ethanol, a by-product of fermentation, as their carbon source in the post-diauxic growth. The dash lines depict changes in the concentrations of glucose and ethanol in different phases of the yeast growth (Gray *et al.*, 2004; Busti *et al.*, 2010).

of the total ATP level in the cell (van den Brink *et al.*, 2009), while in anaerobic conditions the cell cannot make this transition and dies (van den Brink *et al.*, 2009). The significant loss of ATP results in more than a 10-fold reduction in the expression of ribosomal genes (Radonjic *et al.*, 2005). Furthermore, Ashe *et al.* demonstrated that, within 5 minutes of glucose depletion, the poly-ribosome chains in a yeast cell are disintegrated into single ribosomes (Ashe *et al.*, 2000). The combined effect of reducing ribosomal genes’ transcription and disintegrating poly-ribosome chains significantly decreases a cell’s capability for protein synthesis, especially when the environmental glucose concentration is lower than 30 mM (Castelli *et al.*, 2011). As a result, the cell cannot accumulate new proteins for a budding event. Consequently, the population undergoes a lag phase, the diauxic shift, in which cell growth is halted (Gray *et al.*, 2004).

If there is no alternative carbon source available, a cell undergoes a slow proliferation process and then enters quiescence. Sizes of processing bodies (P-bodies) increase under
Figure 1.5: Effects of the glucose depletion on the diauxie and the cellular physiology.

A, the first experiment that demonstrated diauxie. E. coli cells were grown in media containing glucose and a secondary sugar. Their growth curves demonstrated that the bacterial cells consumed glucose first, before switching to the secondary sugar as glucose was exhausted. Diauxie was defined as a lag phase between the two exponential growth phases during which cells made the catabolic transition to the secondary pathway (Monod, 1942). B, the transition from glucose to galactose metabolism results in a 30% loss of the ATP in a yeast cell (van den Brink et al., 2009). C, during diauxie, the transcription of ribosomal genes is reduced more than 10-fold in a yeast cell (Radonjic et al., 2005). D, within 5 min after glucose is depleted from the environment, the poly-ribosomes are disintegrated into single ribosomes which significantly reduces the rate of protein translation (Ashe et al., 2000). Figures A, B and C are reproduced with permissions from (Monod, 1942; van den Brink et al., 2009; Radonjic et al., 2005).

glucose deprivation, indicating an increase in mRNA degradation during diauxie (Teixeira et al., 2005). However, while the translational initiation factors eIF4E and eIF4G accumulate at a low level in the P-bodies during glucose deprivation, the levels of the initiation factors increase significantly during quiescence, suggesting that mRNA accumulated in the P-bodies can be returned to translation (Brengues and Parker, 2007; Kurischko et al., 2011; Kaeberlein and Guarente, 2002). During quiescence, a cell does not proliferate or increase its mass...
and volume (Choder, 1991). Its transcriptional rate is 3 to 5 times lower than that of a cell in exponential phase and it has a special translation mechanism that is dependent on internal ribosomal entry sites (Paz et al., 1999). As a result, the rate of protein synthesis during quiescence is reduced to around 0.3% compared to that during the exponential growth (Fuge et al., 1994). This reduction is based on lines of evidence that yeast chromosomes are condensed during quiescence and mRNAs are delivered to the P-bodies for degradation and sequestration (Pi non, 1978; Li et al., 2013). However, it has been shown that RNA polymerase II binds to upstream regions of hundreds of genes allowing these genes to be transcribed when the cell exits quiescence (Radonjic et al., 2005).

If there is another sugar, such as galactose, in the environment after glucose has been depleted, the activity of the Snf1p kinase allows the alternative metabolic pathway to be activated. In this work, we will determine whether the rate of glucose depletion can affect the activation of an alternative pathway.
Chapter 2

The galactose metabolic network in *Saccharomyces cerevisiae*

2.1 Enzymatic reactions of the Gal network

The galactose metabolic pathway is responsible for metabolizing galactose when galactose is available and glucose is depleted in the environment. Galactose is a C-4 epimer of glucose (Figure 2.1), and thus, requires additional enzymatic steps to be converted into glucose-6-phosphate before entering alcoholic fermentation. The steps of this pathway were elucidated by Luis Federico Leloir (Holden *et al.*, 2003). As shown in Figure 2.2, the conversion from galactose to glucose-6-phosphate is achieved through several enzymatic steps in the Gal network, compared to just one step for glucose (Scott and Timson, 2007). As a result, yeast cells only metabolize galactose when glucose is exhausted in the environment. The Gal network is regulated by a small, well-defined regulatory core that includes Gal3p, Gal4p, and Gal80p. These three regulatory components, together with the permease Gal2p, comprise three feedback loops that control the dynamics of the Gal network. These loops include Gal3p and Gal2p-dependent positive feedback loops and a Gal80p-dependent negative feedback loop (Bennett *et al.*, 2008). The galactose metabolic pathway is conserved among species and has been studied in great detail (Horak, 2013). As a result, it is considered a model for the activity of gene networks.
2.2 Regulatory components of the Gal network

2.2.1 Gal4p, a continuously, weakly expressed transcriptional activator

The Gal4p protein is the main transcription factor for the Gal network. GAL4 is a continuously, weakly transcribed gene that is not induced by galactose or repressed by the Gal80p repressor (Laughon and Gesteland, 1982). Gal4p is a classic Zn-finger protein of 881 amino acids (aa). Its structure includes a Zn-Cys DNA binding domain, a nine amino-acid linker, a dimerization domain and two acidic activation domains (Traven et al., 2006).

The N-terminal DNA binding domain, residues 1-147, is highly basic since lysine and arginine residues compose more than 25% of the first 80 amino acids (Laughon and Gesteland, 1984). This region is characterized by six cysteine residues clustered within a 30-aa stretch (Laughon and Gesteland, 1984). These six cysteine residues interact with two closely-spaced Zn ions to form a Zn$_2$Cys$_6$ binuclear cluster (Liang et al., 1996). The zinc cluster binds to a DNA motif of 5’-CGGN$_{11}$CCG-3’ (called UAS$_{GAL}$, Figure 2.3) and recruits the transcriptional machinery to the target gene (Campbell et al., 2008; MacPherson et al., 2006; Marmorstein et al., 1992) (Ma and Ptashne, 1987b). The specific binding of the Gal4p protein to its 17-bp UAS$_{GAL}$ happens in the following manner. First, the two Zn domains of a Gal4p dimer make base pair-specific contacts to the CGG and CCG triplets at both ends.
Figure 2.2: The galactose metabolic pathway. In glucose metabolism, glucose is imported into the cell via Hxt transporters. Glucose is then converted into glucose-6-phosphate mainly through the activity of hexokinase Hxk2. In galactose metabolism, the conversion of galactose into glucose-6-phosphate is achieved through several enzymatic steps. In brief, galactose is imported into the cell via Gal2p permease. Galactose is then converted into galactose-1-phosphate through the activity of ATP-dependent galactokinase Gal1p. Galactose-1-phosphate is converted to glucose-1-phosphate by galactose-1-phosphate uridylyltransferase Gal7p and galactose mutarotase/UDP-galactose 4-epimerase Gal10p. Finally, glucose-1-phosphate is converted to glucose-6-phosphate by phosphoglucomutase 1 and 2. Enzymatic reactions of the Gal network are regulated by a regulatory core includes a galactose sensor Gal3p, an activator Gal4p and a repressor Gal80p. In general, Gal4p activates the transcription of all GAL genes, Gal80p inhibits the Gal4p’s transcriptional activity and Gal3p is an antagonistic factor of Gal80p. The regulatory core of the Gal network is repressed by the master repressor Mig1p in the presence of glucose (Scott and Timson, 2007).

of the UAS\textsubscript{GAL}, and the linker and dimerization elements contact the phosphate backbone within the inner 11 bp (Liang \textit{et al.}, 1996). At the same time, the two acidic activation domains, residues 148-196 and 768-881 of the C-terminus, interact with RNA polymerase and
stimulate transcription (Ma and Ptashne, 1987a). In uninduced conditions, Gal4p dimers remain bound to the UAS\textsubscript{GAL} sites of its target genes, however, its transcriptional activation is blocked by the activity of Gal80p proteins (Laughon and Gesteland, 1984).

![A Gal4p dimer binds to the UAS\textsubscript{GAL} site.](image)

Since Gal4p is the main activator of the Gal network, the number of the UAS\textsubscript{GAL} sites in the promoters of \textit{GAL} genes is important for their activity. Genes with one UAS\textsubscript{GAL} site are \textit{MEI1}, \textit{GAL3} and \textit{GAL80}. Genes with two UAS sites are \textit{GAL1}, \textit{GAL2}, \textit{GAL7}, \textit{GAL10}, \textit{MTH1}, \textit{PCL10} and \textit{FUR4} (Kew and Douglas, 1976). In genes with two UAS\textsubscript{GAL} sites, the close position of these two sites facilitates a cooperative binding and formation of a Gal4p dimer-dimer structure (Kang et al., 1993). As a result, these \textit{GAL} genes have a much higher transcriptional level in induced conditions. For example, \textit{GAL1} and \textit{GAL2} genes can be induced up to 1000-fold in the presence of galactose (Griggs and Johnston, 1991; Bajwa et al., 1988). However, in uninduced conditions, these genes are also more repressed due to the tetramerization of Gal80p dimers which bind to the Gal4p dimer-dimer structure (Melcher and Xu, 2001). On the other hand, \textit{GAL} genes with one UAS\textsubscript{GAL} site, like \textit{GAL3} and \textit{GAL80}, have a leaky transcription as they are not strictly repressed by the Gal80p tetramers. However, they also express at a lower level, 2-100 fold, in the presence of galactose (Kang et al., 1993).

### 2.2.2 Gal3p, a galactose sensor evolved from the galactokinase Gal1p

Gal3p was shown to be evolved by the duplication of the galactokinase Gal1p (Thoden et al., 2005; Bajwa et al., 1988). The proteins share 70% of their amino acid sequences and Gal3p can be converted to Gal1p through the addition of two amino acids (Serine and Alanine...
after the Serine 164 residue) (Platt et al., 2000). In a yeast cell that has lost GAL3, the Gal network can be induced at a very slow rate and this rate can be restored by the overexpression of Gal1p (Thoden et al., 2005). The loss of both Gal1p and Gal3p renders the yeast cell unable to catabolize galactose as the sole carbon source (Thoden et al., 2005). However, the constitutive expression of Gal3p allows the expression of the Gal network in the absence of galactose (Thoden et al., 2005).

The Gal3p protein comprises two domains, which is responsible for the interaction with the Gal80p protein. The phosphate-binding loop connects the two domains and binds the Mg^{2+}-ATP phosphoryl tail (Thoden et al., 2005; Lavy et al., 2012). The binding of ATP and galactose to this loop changes the Gal3p structure from an open to a closed conformation. In Gal1p galactokinase, the closed conformation facilitates the phosphorylation of galactose to galactose-1-phosphate in an ATP-dependent process (Thoden et al., 2005). In Gal3p, the loss of the two residues Ser and Ala near the ligand-binding site prevents the galactose C1 hydroxyl group from contacting the ATP γ-phosphate for the phosphorylation reaction (Lavy et al., 2012). However, the closed conformation allows Gal3p dimers to interact with Gal80p dimers through hydrogen bonds between their β sheets. Consequently, these two proteins form a Gal80p-Gal3p-ATP-galactose heterotetramer complex (Lavy et al., 2012). Also, the binding of Gal3p happens at a different surface on Gal80p than the surface at which Gal80p interacts with the activation domain of Gal4p (Kumar et al., 2008; Thoden et al., 2008; Lavy et al., 2012). This raises a question about the formation of Gal3p-Gal80p-Gal4p complex and contributes to a school of thought that Gal3p and Ga80p remain bound to Gal4p even when Gal4p is activated and carries out its transcriptional activities (Platt and Reece, 1998; Egriboz et al., 2013).

It is known that Gal3-Gal80 complex formation is required for relieving Gal80 inhibition of the activation domain of Gal4p and Gal4-mediated transcription activation of the GAL genes. However, the precise nature of the interaction between Gal3p and Gal80p remains unknown. In one school of thought, the association of Gal3p with Gal80p does not cause
Gal80p to dissociate from Gal4p, but rather results in the formation of a tripartite Gal4p-Gal80p-Gal3p complex. However, there is no direct interaction between Gal3p and Gal4p. These data suggest that Gal80p interacts with Gal4p in two regions, one of them overlapping the activation domain. Activation by Gal3p may release the activation domain while not affecting the other interaction site. Thus, Gal3p binding to Gal80p reduces Gal80 self-association and/or the stability of preexisting Gal80 oligomers allowing the Gal4p to carry out its activity (Egriboz et al., 2013; Platt and Reece, 1998; Wightman et al., 2008). In another school of thought, Gal3p proteins remain exclusively in the cytoplasm and its interaction with the Gal80p facilitates Gal80p shuttling to the cytoplasm (Diep et al., 2008; Peng and Hopper, 2000). The nucleo-cytoplasmic shuttling of the Gal80p reduces its concentration in the nucleus and thus, alleviates its inhibitions on Gal4p proteins (Peng and Hopper, 2000; Peng and Hopper, 2002). Furthermore, the sequestration of Gal80p proteins into the nucleus is believed to contribute to the hypersensitivity of the Gal network to the galactose concentration (Verma et al., 2003; Ruhela et al., 2004).

2.2.3 Gal80p, a transcriptional repressor of the Gal network.

Gal80 is a purely negative regulator in the sense that it does not mediate the glucose repression signal. Thus, a deletion of the Gal80p protein does not bypass the need for a functional Gal4p activator (Torchia et al., 1984).

Compared to Gal4p, Gal80p is a small protein of 450 amino acids (Thoden et al., 2008). Its structure has not been solved, but a structure of Kluyveromyces lactis Gal80p, which has 82% similarity with S. cerevisiae Gal80p, has been solved (Thoden et al., 2007). Based on that structure, Gal80p was shown to have two domains, an N-terminus comprised of 6 β sheets flanked on one side by two α helices and by three α helices on the other side, and a C-terminus composed of nine mixed β sheets (Thoden et al., 2007). These two domains are connected by a cleft structure where Gal80p binds to the transcriptional activation domain of Gal4p, a 30 aa sequence at the carboxyl terminus of Gal4p (Ma and Ptashne, 1987a; Thoden et al., 2007; Thoden et al., 2008). In solution, Gal80p was shown to exist in a dimer formed
by extensive interaction by the $\beta$ sheets in the C-terminal domain (Thoden et al., 2008).

Melcher and Xu showed that Gal80p dimerizes with a high affinity and this dimerization stabilizes the Gal4p-Gal80p interaction in a Gal4p dimer-Gal80p dimer-DNA complex (Melcher and Xu, 2001; Egriboz et al., 2013). Also, the Gal80p dimers transiently interact with each other to form a higher order tetramer. This self-associated tetramerization is dependent on the spacing of the UAS$_{GAL}$ sites. Two UAS$_{GAL}$ sites, when correctly spaced, greatly stabilize Gal80p dimer-dimer interaction, resulting in a complete shielding of the Gal4p’s transcriptional activation domain. In contrast, the genes with a single Gal4p binding site do not stabilize Gal80p multimers, resulting in biologically-important, transcriptional leakage (Melcher and Xu, 2001).

### 2.3 Activity of the Gal network under glucose-dependent regulation

Since galactose is not the preferred carbon source for yeast, the activity of the Gal network is dictated by the presence of glucose in the environment. In general, the Gal network’s activity is regulated mainly by the interplay between Mig1p, Gal80p and Gal3p proteins.

#### 2.3.1 The Gal network is repressed in the presence of glucose

The Gal network is tightly repressed in the presence of glucose. In fact, this repression can happen within minutes from when glucose is added into a galactose environment (Figure 2.4A). Recent research has shown that the repression of glucose on the Gal network happens not only at the transcriptional level but also at the translational and post-translational levels (Bennett et al., 2008).

First, Mig1p binds to the promoters of GAL genes and facilitates transcriptional repression through the repressor complex Tup1-Ssn6 (De Vit et al., 1997; Papamichos-Chronakis et al., 2004; Treitel and Carlson, 1995). In cells grown in galactose, the mRNA concentration of Gal4 was shown to be around to be $1.6 \times 10^{-5}$ of the total mRNA concentration, or around 0.1 mRNA molecule per cell (Laughon and Gesteland, 1982). In glucose media,
however, its concentration is reduced two- to five-fold (Griggs and Johnston, 1991; Laughon and Gesteland, 1984). While the reduction is modest, this leads to 40- to 100-fold reduction in Gal1 expression (Griggs and Johnston, 1991; Kang et al., 1993). Since Gal4p is the main activator of the Gal network, the inhibition by Mig1p of Gal4p significantly contributes to the network’s repression (Figure 2.4B).

Second, transcriptional activity of Gal4p is inhibited through the binding of Gal80p. It has been shown that Gal80p dimers bind to free Gal4p and to Gal4p in a complex with the UAS\textsubscript{GAL} sites (Figure 2.4C). This interaction is mediated by nicotinamide adenine dinucleotide phosphate (NADP) and stabilizes both the interaction between Gal4p and Gal80p as well as that between Gal4p and its DNA element (Kumar et al., 2008). In \textit{GAL} genes with two UAS\textsubscript{GAL} sites, the spacing of these two sites allows two Gal80p dimers to form a tetramer complex (Leuther and Johnston, 1992; Lue et al., 1987; Melcher and Xu, 2001; Griggs and Johnston, 1991). However, there is no interaction between Gal80p and the DNA sequence (Thoden et al., 2008). The concentration of Gal80p required for half saturation of a Gal4p-DNA complex corresponds to a dissociation constant of about 5 x 10^{-9} M for Gal80p-Galp4 interaction (assuming a 1:1 stoichiometry) \textit{in vivo} (Lue et al., 1987). Overexpression of Gal4 results in partial expression of the \textit{GAL} structural genes in the absence of galactose. This partial expression in the absence of galactose is presumably due to “titration” of Gal80p by Gal4p (\textit{i.e.}, the presence of an excess of Gal4p molecules), because overexpression of Gal80p in the same cell abolishes this partial expression. The binding of Gal80p dimers to Gal4p dimers physically block the interaction of the Spt-Ada-Gcn5 acetyltransferase (SAGA) and NuA4 histone acetyltransferase complexes with Gal4p-activation domain (Carrozza et al., 2002). Consequently, this blocking prevents the recruitment of TATA-binding protein (TBP) and transcription factor IIB (TFIIB) to the promoter and inhibits transcription (Wu et al., 1996).

Third, \textit{GAL} mRNAs and Gal proteins are degraded at a faster rate in a glucose environment. These findings have added another layer of glucose-related repression on the Gal
network. By measuring mRNA decay, Bennett et al. have shown that the half-lives of \textit{GAL1} and \textit{GAL3} mRNA are reduced four to five-fold in glucose compared to their half-lives in a galactose environment (Bennett \textit{et al.}, 2008). As a result, less protein is produced (Figure 2.4E). Also, the half-life of Gal2p protein was shown to reduce from two to five-fold in glucose compared to that in galactose (Figure 2.4D). Horak \textit{et al.} showed that Gal2p was ubiquitinated and delivered to intracellular vacuoles via the endocytic pathway and degraded by vacuolar proteolysis (Horak and Wolf, 1997).

2.3.2 The Gal network is activated in the presence of galactose and the absence of glucose

In the presence of galactose and the absence of glucose, several things happen that affect the Gal network’s activity. First, Snf1p kinase alleviates Mig1p’s binding to the promoters of \textit{GAL} genes. It has been shown that when the glucose concentration is dropped from 200 mM to 5.6 mM, cells respond rapidly to the glucose shift (Bendrioua \textit{et al.}, 2014). Within 1 min, the Snf1p complex is phosphorylated. The activated Snf1p phosphorylated Mig1p, and the phosphorylated Mig1p is shuttled to the cytoplasm (Figure 2.5B). Within 150s, Mig1p reaches a steady state concentration in the cytoplasm and Snf1p remains phosphorylated for at least 420s, through the activity of Sak1p kinase, ensuring Mig1p stays in the hypophosphorylated form (Bendrioua \textit{et al.}, 2014). When glucose is at an intermediate level, Mig1p moves transiently to the cytoplasm. From 200 mM to 100 mM glucose concentration, Mig1p remains in the nucleus. However, when the glucose concentration drops to 80 mM, Mig1p moves transiently to the cytoplasm but then moves back into the nucleus. At less than 10 mM of glucose concentration, Mig1p remains in the cytoplasm (Bendrioua \textit{et al.}, 2014). In general, the activity of Mig1p is reduced before glucose is consumed completely, suggesting that a cell is ready to activate the Gal network before glucose is completely exhausted (Bendrioua \textit{et al.}, 2014).

Second, Gal3p alleviates the binding of Gal80p to Gal4p. Gal3p-Gal80p complex formation is required for relieving the Gal80p inhibition of the activation domain of Gal4p
and Gal4p-mediated transcription activation of the \( GAL \) genes (Figure 2.5A). These events occur rapidly, resulting in readily detectable \( GAL \) mRNA within 3-4 min of exposure to galactose (Egriboz et al., 2013). Data from SDS-PAGE showed a reduction in Gal80p self-association in response to the formation of Gal3p-Gal80p complexes. They also showed that the addition of galactose to the environment resulted in a complete dissipation of the
Gal80p tetramers within 30 min. When the galactose was removed after the dissipation of clusters, the Gal80p tetramers reappeared within 30 min (Egriboz et al., 2013). Mutation in Gal3p results in a slow induction of the Gal network since the mutant only reaches the full induction in 3-5 days after the addition of galactose (Egriboz et al., 2013). In an in vitro transcription system, Gal4p activates transcription 10-fold, and this activity is specifically inhibited by approximately equimolar amounts of Gal80p. Gal3p or Gal1p have no effect on the basal level of transcription or on the transcriptional activity of Gal4p in the absence of Gal80p, but increasing the amount of these two proteins relieves the inhibitory effect of Gal80p on Gal4p. For Gal3p, a 20- to 30-fold molar excess of Gal3p over Gal80p is required to alleviate Gal80p repression. This large amount is needed probably due to the weak interaction between Gal3p and Gal80p. This weak interaction may be used by yeast as a way to modulate its response to galactose. Thus, the intracellular levels of galactose may have to exceed a threshold to saturate the available Gal3p, before activation of GAL gene expression occurs (Platt and Reece, 1998). For Gal1p, a 40-fold molar is needed for the alleviation to happen. The absence of either galactose or ATP reduces Gal4p-Gal80p-Gal3p complex formation by 30-35%. In the absence of both galactose and ATP, formation of the tripartite complex is reduced by 50% (Platt and Reece, 1998).

Third, Gal4p binds to the UAS\textsubscript{GAL} sites and drives the transcription of downstream GAL genes (Figure 2.5A & C). The activation domains of Gal4p proteins interact with the Spt-Ada-Gcn5 acetyltransferase (SAGA) and NuA4 histone acetyltransferase complexes. This interaction drives the recruitment of the TATA-binding protein (TBP) and transcription factor IIB (TFIIB) to the promoter and the transcription is started through the activity of RNA polymerase II (Bhaumik et al., 2004).

### 2.4 Dynamic behaviors of the Gal network

The Gal network is considered a model for understanding gene network dynamics because it displays complex behaviors that cannot be elucidated just from knowledge of its components.
Figure 2.5: The Gal network is activated when glucose is exhausted. A, a diagram depicts the activation of the Gal network in the absence of glucose. After Mig1p’s repression is alleviated, Gal3p proteins are expressed and activated through the binding of galactose and ATP. Gal3p proteins bind to Gal80p repressors and alleviate their inhibition on Gal4p activators. Gal4p proteins drive the expression of other GAL genes. Gal2p permeases import galactose into the cell. Galactose is metabolized to generate energy and also induces the full induction of the Gal network.

B, fluorescent images show the exportation of Mig1p repressors (green colors) to the cytoplasm when the concentration of glucose decreases (García-Salcedo et al., 2014). C, fold-inductions of several GAL genes compare in the galactose environment in comparison to that of in the glucose environment. The GAL1 and GAL2 genes have up to 1000-fold inductions, whereas GAL3 and GAL80 genes only have 3 to 9-fold inductions. D, the cooperative binding of the Gal4p activators on genes with the two UASGAL sites allows the Gal network to be hyper-sensitive to galactose concentrations. Compares to a Michaelis-Menten system (dashed line, Hill function number $\eta_H =1.0$), the Gal network (solid line, Hill function number $\eta_H =3.0$) has a switch-like response to changes in the galactose concentrations (Pannala et al., 2009). Figures B and D are reproduced with permissions from (García-Salcedo et al., 2014; Pannala et al., 2009).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL1</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>GAL2</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>GAL3</td>
<td>3 - 5</td>
</tr>
<tr>
<td>GAL80</td>
<td>~ 9</td>
</tr>
</tbody>
</table>

### Table: Fold Inductions of GAL Genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL1</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>GAL2</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>GAL3</td>
<td>3 - 5</td>
</tr>
<tr>
<td>GAL80</td>
<td>~ 9</td>
</tr>
</tbody>
</table>
From a topological perspective, the Gal network consists of three feedback loops, a negative feedback loop dictated by the Gal80p and two positive feedback loops dictated by the Gal3p and the galactose permease Gal2p. Ruhela et al. showed that the Gal80p-dependent negative feedback loop allows the Gal network to function in three environmental scenarios: repressed in glucose, activated in galactose, and partially repressed in non-inducing-non-repressing carbon sources (Ruhela et al., 2004). Disabling either the Gal80p or Gal3p feedback loop renders the Gal switch non-functional (Verma et al., 2003). However, deletion of both loops allows cells to achieve an induction pattern similar to that of wild-type cells, though these double-mutant cells grow 20% slower than wild-type cells in an environment where the cells are exposed to alternating glucose and galactose (Bhat and Iyer, 2009). Thus, it is suggested that these feedback loops help reduce heterogeneity in network expression among individual cells, which allows a cell population to grow at a similar pace (Bhat and Iyer, 2009). Aspects of the Gal network’s dynamics are further discussed in subsequent sections.

2.4.1 The Gal network is hyper-sensitive to galactose concentrations

A biochemical system is said to be “hypersensitive” if it is more sensitive to changes in the ligand concentration than a normal response dictated by the Michaelis-Menten equation (Koshland, 1998). In contrast to the hyperbolic, graded response of a Michaelis-Menten-like system, a hypersensitive gene network has a switch-like, all-or-none response to changes in the ligand concentration (Koshland, 1998). This behavior comes from mechanisms such as multistep binding, cooperative binding, cascades, and feedback loops (Pannala et al., 2009). One possible consequence of hypersensitivity is that the system becomes bistable, meaning it can transition between two alternative stable steady states. Under a transient stimulus, the switch of a bistable system to another state is an irreversible process (Ferrell, 2002).

A well-known example of an ultrasensitive system is the Mos-MEK-p42 mitogen-activated protein kinases (MAPK) cascade in Xenopus oocytes (Ferrell and Machleder, 1998). An exposure of the oocytes to progesterone triggers an irreversible maturation process (Ferrell and Machleder, 1998; Ferrell, 2002). The maturation is dependent on a positive feedback loop
in the MAP kinase pathway and a cooperative phosphorylation at two amino acid residues on the MAPK protein (Gotoh et al., 1995; Ferrell, 1996; Burack and Sturgill, 1997; Ferrell and Bhatt, 1997).

The Gal network is also hypersensitive, as a result of the cooperative binding of Gal4p protein and the nucleocytoplasmic shuttling of Gal80p protein (Figure 2.5D). Similar to the MAPK phosphorylation, several GAL genes require the cooperative binding of Gal4p proteins at the two UAS\textsubscript{GAL} sites in order for transcription to occur (Pannala et al., 2009). It has been demonstrated that the binding of a Gal4p dimer on the first site accelerates the subsequent binding at the second site (Pannala et al., 2009). Consequently, genes with two UAS\textsubscript{GAL} sites like \textit{GAL1} and \textit{GAL2} can be upregulated 1000-fold in the presence of galactose (Johnston and Davis, 1984). Also, the nucleo-cytoplasmic shuttling of Gal80p likely contributes to this hypersensitivity property (Verma et al., 2003). In the presence of galactose, the Gal3p-induced nucleo-cytoplasmic shuttling of Gal80p reduces the number of Gal80p dimer-dimers that bind and inhibit Gal4p activity. Since there is a significant difference in the inhibition effect of the dimer-dimer and single dimer forms of Gal80p, a small loss of Gal80p proteins allows the Gal network to respond at a faster rate to galactose (Verma et al., 2003). Another reason for the hypersensitivity is that galactose is not only an inducer but also a catabolite, thus, the induction of the Gal network needs to be adjusted to the level of galactose. Also, during full induction, GAL enzymes constitute 5% of the total cellular proteins, thus the Gal network has evolved to be highly sensitive to system parameters (Verma et al., 2003).

\section*{2.4.2 The Gal network filters environmental signals based on a specific threshold}

Fluctuations of an extracellular stimulus, at a specific frequency, can be used to understand how a gene network reacts to its input signal. From a signal transduction perspective, a bandwidth is proportional to the amount of information that can be transmitted through a pathway. A larger bandwidth means the pathway has a shorter response time and a more accurate reaction. This bandwidth can be measured by fluctuating input signals at different frequencies and measuring frequencies of the network responses. Based on these
The Gal network acts as a low pass filter. Top row, input glucose signal that was used to stimulate the Gal network. Middle row, normalized fluorescence trajectories of a yeast cell population as they respond to glucose simulations in the top row. In the absence of glucose, Gal1p-YFP expressed and caused an increase in fluorescence. As glucose was introduced, transcription of \textit{GAL1-YFP} was inhibited, causing a decrease in fluorescence level due to protein degradation. Periods of glucose waves are 4.5, 3.0, 2.25, 1.5, 1.125 and 0.75 h. When oscillation periods were lower than 1.125 h, cells no longer responded faithfully to the glucose signal as they filtered out high-frequency environmental fluctuations. Bottom row, simulation data from the same frequencies. This figure is reproduced with permission from (Bennett et al., 2008).

responses, we can classify gene networks based on their filtering characteristics (Hersen\textit{ et al.}, 2008; Behar \textit{et al.}, 2007). For instance, a low-pass filter will respond to inputs that have lower frequencies than its cutoff frequency, and integrates inputs that have higher frequencies (Hersen\textit{ et al.}, 2008; Mettetal \textit{et al.}, 2008).

The Gal network was demonstrated to be a low-pass filter system (Bennett \textit{et al.}, 2008). Bennett \textit{et al.} used a microfluidic device and subjected yeast cells to a series of sinusoidal waves of glucose concentrations, from 0% to a full induction level of 0.25% (w/v) in a galactose background environment (Bennett \textit{et al.}, 2008). They varied the period of the glucose signals from 0.75 to 4.5 hr and showed that the Gal network has a cut-off frequency at
$2\pi/1.125\ \text{hr}^{-1}$. At frequencies lower than this cutoff threshold, the levels of Gal1p protein oscillated in-sync with the glucose fluctuations. These oscillations disappeared when the frequencies were higher than $2\pi/1.125\ \text{hr}^{-1}$ (Figure 2.6). They demonstrated that the degradation of Gal1p contributed to the low-pass filter property (Bennett et al., 2008). This is similar to other low-pass filter systems, such as the high-osmolarity glycerol mitogen-activated protein kinase pathway in *S. cerevisiae* (Mettetal et al., 2008; Hersen et al., 2008) and the epidermal growth factor (EGF)-dependent Akt pathway in mammalian cells (Fujita et al., 2010).

### 2.4.3 The Gal network retains its expression level from a previous exposure to galactose

An interesting aspect of some gene networks is their ability to maintain their active state even after a transient stimulus diminishes. As a result, such gene networks are said to have “memory”. Factors that contribute to this memory come from positive feedback loops that allow the network to have two or more discrete, stable states of network activity (Acar et al., 2005). Interestingly, in the Gal network, Acar et al. showed that each feedback loop contributes differently to the memory capacity. Only the Gal3p positive feedback loop is responsible for creating two stable activity states of the Gal network. The Gal2p positive feedback loop only increases the difference in the expression of the two states, whereas the Gal80p negative feedback loop reduces the memory capacity (Figure 2.7). One reason that the Gal network does not have a strong memory is because galactose acts as both an inducer and a metabolite for yeast cells. Thus, this network is very sensitive to its parameters such as glucose and galactose concentration, and the expression level of its components (Bhat and Iyer, 2009). Besides the Gal network, memory capability have been demonstrated in the maturation of olfactory sensory neuron into odorant receptor (Shykind et al., 2004), MAPK cascade (Markevich et al., 2004), osmotic stress adaptation (You et al., 2012) and PKA pathway in yeast (Pérez-Landro et al., 2015).
Figure 2.7: The expression of the Gal network is influenced by a previous exposure of galactose. Yeast cells were grown in media with and without galactose and then subjected to different levels of galactose. While the expression levels of Gal1p at low and high galactose concentrations are the same in these cells, at intermediate galactose concentration, there are significant differences in the expression level of Gal1p. This suggests that these cells retained a memory of previous expression state. And this memory is independent with the positive feedback loop of Gal2p, but is abolished in Gal3p mutant and is expanded in Gal80p mutant. This figure is reproduced with permission from (Acar et al., 2005).

2.4.4 The activity of the Gal network can be stochastic

Stochasticity refers to random fluctuations in the expression level of genes in a clonal population (Raser and O’Shea, 2005). Since components of a gene network, especially mRNA, exist in small numbers (often less than 20 molecules per cell), these components are subjected to Brownian motion (Maheshri and O’Shea, 2007). The effect of random Brownian movements cause transcription and translation to be noisy and stochastic. Consequently, individual cells of a homogeneous population can exhibit different expression levels of the same gene (Raser and O’Shea, 2004). Stochasticity in gene expression has been demonstrated, for instance, in PHO5 and GAL1 genes in yeast (Figure 2.8). Feedback loops, especially nega-
tive feedback, have been shown to dampen stochasticity and make gene networks function more reliably (Thattai and van Oudenaarden, 2001; Becskei and Serrano, 2000).

2.4.5 The Gal network can be activated in the presence of glucose

Recent developments have demonstrated a more dynamic relationship between glucose and the Gal network. In a static environment in which high levels of glucose are available, the Gal network is tightly repressed. However, the composition of carbon sources in nature is much more complex. First, there are many types of sugars coexisting in the environment, such as glucose, sucrose, galactose and fructose. Second, the concentration of glucose is rarely optimal. Third, glucose can be consumed at different rates depending on the growth rate of yeast strains and the competitive landscape. Several research projects were based on these observations and have yielded surprising data about the relationship between glucose and the Gal network.

In one recent publication, Escalante-Chong et al. showed that the Gal network can be activated in the presence of glucose (Escalante-Chong et al., 2015). They subjected yeast cells to more than 500 combinations of glucose and galactose concentrations that span a range of 1000 fold (Figure 2.9A). Based on expression levels of a Gal1 promoter-driven reporter, they demonstrated that the expression of the Gal network is independent of glucose concentration by itself, but instead, dependent on the ratio of galactose-to-glucose (Figure 2.9B). This ratio sensing was maintained even when the MIG1 gene was deleted, suggesting that the Gal network was repressed by glucose by a combination of mechanisms, such as mRNA and protein degradation (Figure 2.4). A deletion of the GAL80 gene allowed cells to express the Gal network at a threshold concentration of glucose, whereas a double deletion of MIG1 and GAL80 genes allowed cells to constitutively express the Gal network (Escalante-Chong et al., 2015). Escalante-Chong et al. believe the ratio sensing is caused by competitive bindings of glucose and galactose to hexose transporters (Hxt1 - Hxt17 and Gal2p) and is independent of the activity of Gal2p. Consequently, cells can tune their transporters to adapt to changes in environmental conditions, such as the depletion of glucose (Escalante-Chong et al., 2015).
A, two reporters were driven by GAL1 promoters and were fused to same alleles in a diploid yeast cell. Two questions were asked. First, whether the expression levels of these two reporters different within a cell due to the stochasticity in gene expression. Second, whether the expression levels of these two reporters different among cells in a homogeneous population. B, Stochasticity in gene expression is caused by intrinsic factors (i.e. stochastic events within the gene network) and extrinsic factors (i.e. stochastic events outside the gene network). Using time-lapse flow cytometry, the effects of intrinsic and extrinsic factors can be measured by the vertical and diagonal spread of the expression levels of the two reporters, respectively. C, Raser et al. showed that GAL1 promoters have low intrinsic noise. D, promoters have high extrinsic noise result in variabilities in the expression levels of the two fluorescent reporters among cells in the homogeneous population. This figure is reproduced with permission from (Raser and O'Shea, 2004).
Figure 2.9: The activation of the Gal network depends on the glucose:galactose ratio.

A. Escalante-Chong et al. subjected yeast cells to more than 500 combinations of glucose and galactose concentrations which span a range of 1000 fold and measured the expression level of a YFP reporter driven by the GAL1 promoter. An induced fraction was calculated as the difference between cells growing in glucose and cells growing in glucose plus galactose. B. Data showed that the Gal network was activated at a constant ratio of glucose:galactose which is independent of the absolute glucose concentration. This figure is reproduced with permission from (Escalante-Chong et al., 2015).

Using a similar approach, Venturelli et al. subjected yeast cells to a wide range of glucose and galactose concentrations, and quantified expression levels of a Gal10 promoter-driven reporter in single cells using flow cytometry (Venturelli et al., 2015). They showed that between 0.03 to 0.5% glucose and 0.06 to 2% galactose, there were two subsets in a homogeneous yeast population, each exhibiting a different patterns of the Gal network expression (Figure 2.10A). In one subset, yeast cells expressed the Gal network very early but had a slower growth rate in the glucose environment. The other subset, however, delayed its expression 7.3 hr after the fast-expression subset (Figure 2.10B) and had a much faster growth rate in the glucose environment. The situation was reversed in galactose-rich environment,
Figure 2.10: The Gal network can be activated in the anticipation of glucose depletion. A, yeast cells containing a GAL10 promoter-driven fluorescent reporter were subjected to a wide range of glucose and galactose concentrations. Between 0.03 to 0.5% of glucose concentrations and 0.06 to 2% of galactose concentrations, yeast cells exhibited different patterns of the Gal network’s expression. B, there are two subpopulations with two different patterns of the Gal network’s expression. One had an early activation of the Gal network, which is 7.3 hr earlier than the second subpopulation, the one with a delay activation of the Gal network. This figure is reproduced with permission from (Venturelli et al., 2015).

as cells in the fast group grew much faster and caught up with cells in the delayed group (Venturelli et al., 2015). Interestingly, both groups could not consume galactose even when the Gal network was activated, and galactose metabolism was only initiated after glucose was exhausted. Venturelli et al. proposed that the existence of these two subpopulations is a form of bet hedging. Here, cells rely on stochastic fluctuations to create variability in the Gal network’s expression, allowing cells to adapt to changing environments without a dominant environmental signal (Venturelli et al., 2015).

A cell’s ability to adapt to the diauxic transition also indicates how it regulates the Gal network in response to changes in glucose concentrations. Wang et al. subjected 43 natural isolates of S. cerevisiae to media containing 0.25% of glucose and 0.25% of galactose concentrations and measured their growth rates for 44 hr (Wang et al., 2015). They observed that these strains exhibited diverse diauxic lengths, ranging from 0 to 9 hr with a mean of 3.2 ± 1.6 hr (Figure 2.11). They further showed that in strains with short diauxic peri-
ods, the Gal network activated 3 hr before glucose was exhausted (Wang et al., 2015). In contrast, long-diauxic strains expressed the Gal network 2 hr after glucose was exhausted. Interestingly, all these strains completely consumed glucose and galactose, and reached the same growth density at the end of the experiment (Wang et al., 2015). Based on the growth rates of these strains, it appeared that these strains also employed bet hedging to anticipate glucose depletion. Wang et al. proposed that the trade-off between growth rate and network activation is decided within individual cells while contributing to the population’s overall response to environmental changes.

In general, the relationship between glucose and the Gal network is very dynamic and is not constrained to a simple ON-OFF switch. One important question remains unanswered: does the rate of glucose depletion affect the dynamic activation of the Gal network? The motivation to answer this question is based on the observation that yeast cells consume glucose at different rates based on their growth rate and the competitive landscape. Also, in an industrial setting and especially during fermentation, the activation of a gene network, through adding inducers at different rates, can directly affect the yield and production cost of a metabolic product. Understanding the dynamics of the Gal network in response to continuously changing concentrations of glucose can provide invaluable information about the capability of the Gal network and how it functions. With technical advancements of single-cell observation methods, understanding the Gal network’s dynamics at a single-cell level, we believe, can provide us new insights into this important gene network’s activities.
Figure 2.11: Wild-type yeast strains have different adaptation levels to diauxie. 43 natural isolates of *S. cerevisiae* were grown for 44 hr in media containing 0.25% glucose and 0.25% galactose. Changes in culture density and growth rate over time showed significant variabilities between these isolates. Strains in the top rows, represented by a blue line, had a short, undistinguished diauxic phase, whereas strains in the bottom rows, represented by a red line, had a long diauxic phase and a long lag time in the growth curve. This figure is reproduced with permission from (Wang *et al.*, 2015).
Chapter 3

Materials and methods

3.1 Construction and maintenance of yeast strains

3.1.1 Culture media

*S. cerevisiae* strains used in this project are listed in Table 3.1. The unit used for calculating media ingredients is weight per volume (w/v). Wild-type (WT) strains, K699 and K700, were routinely grown in YPD (1% yeast extract, 2% peptone, 2% dextrose). Fluorescent-tagged *GAL1* strains, K699-1y, K700-1m and K701-1ym, were grown in selective dropout media. The dropout media include 6.7% yeast nitrogen base, 1.92% yeast synthetic dropout medium, 20% agar and 2% glucose. The selective dropout markers are histidine for K699-1y, uracil for K700-1m and histidine and uracil for K701-1ym.

For glucose-depletion assays, yeast cells were grown in complete synthetic media (CSM), which are the selective dropout media plus any missing auxotrophic marker. The inducing medium (galactose) contained 2% galactose, whereas the repressing medium (glucose) contained 2% glucose and 2% galactose. Hereafter, they will be called as the galactose and the glucose media, respectively.

3.1.2 Construction of integration plasmids

Since Gal1p, the galactokinase enzyme, is considered the output of the Gal network, a common approach for observing the Gal network’s activity is to tag a fluorescent reporter to
Gal1p. We obtained from the Hasty lab (UCSD) strain K699-1y, in which a yeast-enhanced VENUS gene, encoding a variant of the yellow fluorescent protein (excitation/emission, 515/528 nm), was fused to the C-terminal of GAL1 (Shaner et al., 2005). This strain was constructed using the integration plasmid pKT90 from a collection of Sheff and Thorn (Sheff and Thorn, 2004). To construct a complementary haploid strain, I built another integration plasmid based on the collection.

A new integration plasmid was created from plasmid pKT175 (Sheff and Thorn, 2004). yeMCHERRY, encoding a derivative of red fluorescent protein (excitation/emission, 587/610 nm), was amplified from plasmid pAW8-mCherry (Watson et al., 2008) by PCR using Phusion polymerase (New England Biolabs) in HF buffer and primers mCherry-F and mCherry-R (Table 3.2). The reaction parameters were 98°C for 30 s; then 30 cycles of 98°C for 10 s, 61°C for 30 s, and 72°C for 15 s; and finally 72°C for 5 min. The PCR product and the pKT175 plasmid were each digested with PacI and AscI, gel purified and ligated together to create the plasmid pKT175-mCherry.

In this plasmid, the fluorescent tag/selectable marker construct is yeMCHERRY-T_{ADH1}, P_{TEF1}-URA3-T_{TEF1}. This construct is similar to that of the plasmid pKT90, which includes yeVENUS-T_{ADH1}, P_{TEF1}-HIS5-T_{TEF1}, and was used to create the strain K699-1y (Bennett and Hasty, 2008).

### 3.1.3 Construction of a fluorescent-tagged GAL1 yeast strain

To compare the Gal network’s activity in haploid and diploid strains, I constructed a complementary haploid strain for the K699-1y (mating type MATa). Strain K700 (mating type...
MATα) was obtained from ATCC. This strain has an identical genotype with the K699 strain, except for the mating locus, and these two strains can be mated to form a diploid strain.

Table 3.1: Yeast strains used in this project. The K699 and K700 strains were obtained from ATCC. The K699-1y strain is from the Hasty lab (Bennett et al., 2008). The K700-1m and K701-1ym strains were constructed in the lab.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>K699</td>
<td>MATa ade2-1 trp1-1 leu2-3 leu2-112 his3-11 his3-15 ura3 can1-100</td>
</tr>
<tr>
<td>K700</td>
<td>MATα ade2-1 trp1-1 leu2-3 leu2-112 his3-11 his3-15 ura3 can1-100</td>
</tr>
<tr>
<td>K699-1y</td>
<td>GAL1::yeVENUS, PTEF1-HIS5-TEF1</td>
</tr>
<tr>
<td>K700-1m</td>
<td>GAL1::yeMCHERRY, PTEF1-URA3-TTEF1</td>
</tr>
<tr>
<td>K701-1ym</td>
<td>Diploid of K699-1y &amp; K700-1m</td>
</tr>
</tbody>
</table>

Table 3.2: PCR primers for constructing plasmids and yeast strains.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCherry-F</td>
<td>ctgatgtttaaatacatttgagcaagggcga</td>
</tr>
<tr>
<td>mCherry-R</td>
<td>catgtcgaagtgccgcgcctacttg</td>
</tr>
<tr>
<td>Gal1-tagF</td>
<td>cgtctctaaccacagcttggcagctgtctatatgaatagttgagcgtgcttgtta</td>
</tr>
<tr>
<td>Gal1-tagR</td>
<td>tgagaagtgggtctgcaacaaagttaaagaaaaagaattatactcgagatgtcgctcg</td>
</tr>
<tr>
<td>Gal1-checkF</td>
<td>cagcgaagcgtatgttttg</td>
</tr>
<tr>
<td>Gal1-checkR</td>
<td>ggttgtttatatgtcggagatgtag</td>
</tr>
</tbody>
</table>

The DNA sequence, linker-\textit{MCHERRY}-\textit{URA3}, in the pKT175-mCherry plasmid was amplified by PCR using Phusion polymerase (New England Biolabs) in HF buffer and the primers Gal1-tagF and Gal1-tagR (Table 3.2). These primers contains 50 bp of homologous sequences of the C-terminus of the \textit{GAL1} gene flanking the stop codon. Reaction parameters were 98°C for 30 s; then 30 cycles of 98°C for 10 s, 58°C for 30 s, and 72°C for 75 s; and finally 72°C for 5 min. PCR products were gel purified and transformed into the K700 cells using a lithium acetate method (Gietz and Woods, 2002). Transformants were selected for uracil auxotrophy and screened for fluorescence (Amberg et al., 2005). The integration was verified by colony PCR using Taq polymerase (New England Biolabs) in a standard buffer plus the Lyse and Go PCR reagent (Thermo Scientific) and the primers Gal1-checkF and Gal1-checkR (Table 3.2). The \textit{GAL1}-plasmid junction was amplified with reaction parameters: 95°C for
2 min; 80°C for 10 min; 94°C for 60 s; then 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min; and finally 72°C for 5 min. K701-1ym strain was created by mating the K699-1y and K700-1m strains and selected on synthetic dropout media with two selectable markers, histidine and uracil.

3.2 Design and construction of microfluidic devices

Previous experiments have demonstrated the repression effect of glucose on the activity of the Gal network (Johnston et al., 1994; Ashe et al., 2000; Griggs and Johnston, 1991). However, in most cases, their data represented population-average values and were obtained at a steady-state level. Recent advances have demonstrated that there are many important biological processes that can only be discovered when one looks at cellular behavior at the single cell level (Bennett and Hasty, 2008). Among many techniques for single-cell level research, microfluidic devices allow one to dynamically manipulate the environmental conditions and quantify the effects of these changes simultaneously (Bennett and Hasty, 2009a).

3.2.1 Layout of the microfluidic device

Microfluidics is a truly versatile technique in which one can design a layout that fits a specific experiment. The microfluidic device (microfluidic chip or chip) used in this study was designed in the Hasty lab (Bennett and Hasty, 2008; Ferry et al., 2011). The chip resembles a miniature chemostat in which cells are continuously grown in a single layer. Its layout consists of a cell trapping chamber, five ports, a dial-a-wave (DAW) junction and staggered herringbone mixers (Figure 3.2). The cell trap is where cells are grown and their behaviors are captured by a microscope’s camera (Figure 3.2D). The five ports are connected to media inputs and waste outlets to provide a continuous, one-way flow of nutrients. The DAW junction is used to adjust the amount of the two media inputs supplied to the cell trap (Figure 3.2B). The mixers mix the two media and generate a homogeneous environment for the cell trap (Figure 3.2C). The height of the trapping chamber and the mixers are 3.5 μm
Figure 3.2: Design of the microfluidic chip used in this project. A, layout of the chip. Port 1 and 2 are for media, port 3 and 4 are waste outlets, port 5 is for cell loading. Red feature is the dial-a-wave (DAW) junction. Green features are the staggered herringbone mixers. Yellow feature is the cell chamber. Black arrows depict the fluid flow direction during experimental runs. Red arrows depict the flow direction when cells are loaded into the chip. The master mold consists 16 identical chips arranged in a 4x4 square. B, details of the DAW junction with flow direction. C, details of the staggered herringbone mixer with flow direction. D, fluid flow direction when cells (red circles) are loaded into the chip and during the experimental run. Figures are adapted from (Ferry, 2010; Ferry et al., 2011).

and 12 μm, respectively (Ferry, 2010; Ferry et al., 2011). The height of the rest of the chip is 10 μm (Bennett and Hasty, 2008; Ferry et al., 2011). In general, microfluidic devices are made followed two processes, photolithography and soft lithography. In the first process, the features of the chip are etched on a silicon wafer. Photoresist (i.e. liquid chemicals that react with light) is applied on top of a wafer and the chip’s features are etched on the photoresist using UV light (Ferry, 2010; Ferry et al., 2011). In the end, a master mold is created allowing microfluidic chips to be made multiple times with high reproducibility. For our design, the mold contains 16 chips arranged in an array of 4x4 (Figure 3.3). Next, microfluidic chips are made by the soft lithography process using an elastomer called poly-dimethylsiloxanese (PDMS).

3.2.2 Poly-dimethylsiloxanese (PDMS)

Among many materials for making microfluidic chips, polydimethylsiloxanese (PDMS) has many advantages. First, it is optically transparent down to 280 nm, thus detection methods
at different light wavelengths can be conducted. Second, it is nontoxic and gas-permeable so that organisms can be grown on it. Third, it exists in liquid form at room temperature and can be cured at low temperatures (McDonald et al., 2000). Its liquid form allows it to reproduce features on the mold with high fidelity and later, in a solid form, it can binds to another solid PDMS layer or glass reversibly through van der Waals bonds or irreversibly through covalent bonds (McDonald et al., 2000; Xia and Whitesides, 1998). PDMS is the preferred material for rapid prototyping of microfluidic devices (Duffy et al., 1998).

3.2.3 Steps for making microfluidic devices

The soft lithography process for making microfluidic devices was described in detail previously (Ferry, 2010; Ferry et al., 2011). In brief, steps for making a microfluidic chip include:

1. **Preparing a PDMS solution:** Ten parts of PDMS base and one part of curing agent were mixed vigorously for 5 min (PDMS, Sylgard 184, Dow Corning). For this chip design, 30 grams of the PDMS base and 10 grams of the curing agent were used. The final solution was placed inside a vacuum desiccator and the internal pressure was maintained at -30 inHg for 15 - 30 min to eliminate air bubbles generated from the mixing.

2. **Pouring a PDMS layer:** The mold (Figure 3.3) was wrapped around by a piece of aluminum foil to hold the liquid PDMS solution. The solution was taken out of the desiccator and poured onto the center of the mold. The mold was then placed into the vacuum desiccator again to eliminate any air bubbles generated by the pouring. In this step, the balance of the desiccator surface was checked to ensure the liquid PDMS spread evenly. The pressure was dropped to -30 inHg and maintained for 30 min. After that, the mold was placed in a dry oven and was baked at 80°C for 1 hr. This step allows the PDMS solution to be cured into a solid layer. Next, the PDMS layer was peeled of the mold. This layer has two sides, the feature side is the one that faces the mold and the non-feature side is the one that does not touch the mold.
3. **Cutting features on the PDMS layer:** The PDMS layer, feature-side up, was placed on a thick glass plate under a dissecting scope. The five ports of the chip were cut using a 0.5 mm-biopsy punch (Harris Uni-Core, Sigma-Aldrich, St. Louis, MO). In this step, it was critical to ensure the port is punched in a straight line to prevent a ragged, tilted port. After all the ports were cut, the PDMS layer was cut into individual chips. These chips were placed in a beaker and covered with a 100% methanol solution. The beaker was placed in a water sonicator and was sonicated for 5 min, three times, to clean the insides of the ports. After that, chips were dried in an oven at 80°C for 1 hr.

4. **Bonding a chip to a glass coverslip:** Each chip was cleaned on both sides with a piece of scotch tape (matte finish, Office Depot). A glass coverslip was sprayed both sides with a 100% isopropanol solution and was blown dry with clean nitrogen gas. A chip, feature-side up, and one coverslip were placed inside an UVO cleaner (Jelight Company Inc.). Oxygen gas was flown to the UVO cleaner at a 0.45 scfm. The cleaner was turned on, and the chip and the coverslip were exposed to UV light for 3 min. The treatment of air plasma creates a layer of oxygen atoms on the surfaces of the chip and the coverslip. Next, the chip, feature-side down, was placed on top of the coverslip within 1 min. This step was done quickly so that the covalent bonds can be formed between the oxygen atoms. The finished chip was placed in a dry oven overnight to enhance bonding strength between the chip and the coverslip. After that, chips were placed in a clean Petri dish before use in an experiment.

### 3.2.4 Troubleshooting

Microfluidic devices need to be made at a high level of precision in order to function properly. Below are several factors that can affect the quality of a chip.

One critical factor is the quality of the port. A port connects cells with media sources and waste outlets. Some problems include rough openings, improper placement (Figure 3.4A),
Figure 3.3: A mold for making microfluidic chips. The mold contains 16 chips arranged in an array of 4x4. Each chip is a one-centimeter square.

or a tilted port (not shown). The first one is caused by a blunt biopsy punch and thus can be solved by sharpening the punch. The second and third problems are caused by placing the punch outside the chip area and not punching them in a straight-down direction. Thus, these two problems require more practice in making chip.

Features of a chip could be collapsed because the chip was pushed too hard onto the coverslip. While the problem is not critical in other areas, a reduced height at the cell trap can render the chip unusable, because this height of 3.5 μm is critical for maintaining a single layer of cells. The solution is to not apply force on top of the chip, especially near the center where the cell trap is. Instead, the corner of the chip can be gently tapped to ensure that the entire chip is in contact with the coverslip.

Dust or hair particles can block the flow and disrupt the function of a chip. One solution is to eye-check the chip right after the bonding step. If these objects are found, the chip can be lifted up from the coverslip and the bonding procedure can be repeated with a cleaner chip and coverslip.
3.3 Glucose-depletion assay

While effects of a glucose-induced repression on the activity of the Gal network have been explored using different approaches (Johnston et al., 1994; Wang et al., 2015; Escalante-Chong et al., 2015; Venturelli et al., 2015), one important aspect of this relationship was often overlooked. In both natural conditions and industrial fermentation, the concentration of glucose never stays constant. Instead, this concentration often decreases as cells consume glucose during growth. The question is whether the rate of glucose depletion affects the activity of the Gal network at a population and single cell level. To answer this question, we used a platform developed in the Hasty lab (UCSD) to investigate the activation of the Gal network at different rates of the glucose depletion (Ferry, 2010; Ferry et al., 2011; Bennett et al., 2008). For this glucose-depletion assay, I employed the microfluidic device mentioned in the previous section together with time-lapse fluorescence microscopy. A procedure to setup and conduct the assay is mentioned below. All steps of the experiment were done at 30°C (Figure 3.5).

1. Priming yeast cells in galactose and glucose media: Yeast cells were first grown in galactose media (CSM + 2% galactose) overnight at 30°C, 250 rpm. This galactose inoculation step prevents a long delay and high variability in the activation of the Gal network in later stage of the experiment (Acar et al., 2005). This variability, which is presented in cells without the galactose background, can mask the effect of the glucose depletion on the Gal network. Cells were then transferred to glucose media and the Gal network was repressed. Since Gal1p is a very stable protein (Johnston et al., 1994) and is degraded mainly through cell divisions over six generations (around 12 hr), this step was done mainly outside of the chip.

2. Setting up the microfluidic device: This step is for setting up the microfluidic chip on the fluorescence microscope. A chip was inspected and primed with dH2O. Waste and media reservoirs were prepared and connected to the chip. Since variations
in glucose-depletion time can affect the expression of Gal1p, it is important to ensure that glucose is supplied into the cell trap at a precise concentration. The DAW junction was calibrated and glucose-depletion times were programmed using a custom iDAW software.

3. **Programming the experimental run:** Changes in cell physiology and Gal1p expression level were tracked through phase contrast and fluorescent images of yeast cells inside the chip. Changes of glucose concentration were also tracked through a fluorescent tracking dye. The scope was programmed to run for 15 hr in which images from all channels were collected every five minutes.

4. **Analyzing digital images:** After the experiment was done, digital images were collected and analyzed using ImageJ software with custom plugins. Numerical data that were extracted from the digital images include cell lineages, cell cycle times, changes in glucose concentration, changes in cell size, mean and standard deviation of the fluorescence intensity. The relationship between glucose-depletion rates and the activation of Gal1p-YFP was analyzed using Matlab, Excel and Inkscape.

3.3.1 **Priming yeast cells in galactose and glucose media**

A glycerol stock of the K699-1y strain was streaked on an Histidine-dropout plate. The plate was incubated for 3 to 4 days, which allowed yeast cells to grow into 3-4 mm colonies. This plate is good for one week. One day prior to the depletion assay, a single yeast colony was inoculated into 5 mL of galactose media at 250 rpm shaking for 16-18 h. On the day of the experiment, an aliquot of the overnight culture was transferred into fresh glucose media, at a 1:50 volume ratio of galactose:glucose media. The new culture was grown to an OD$_{600}$ 0.3-0.6 (8 hr at 250 rpm). As mentioned in Section 3.3, six cell divisions (around 12 hr) are needed for the complete degradation of Gal1p. Eight hours of the repression time was done in flask, and an additional four hours were set after cells were loaded into the chip.
Figure 3.5: Steps of the glucose depletion assay. A single yeast colony was grown in galactose media overnight, in the next day, an aliquot was transferred to fresh glucose media, at a 1:50 ratio of overnight culture:fresh media, and the culture was grown for another 8 hrs. After the microfluidic chip was setup, cells were loaded into the chip and grown in glucose media for another 4 hrs. Glucose media were then linearly depleted follow setup program. Images were taken every 5 min in phase contrast, yellow and red fluorescent channels. All steps of the experiment were done at 30°C (Ferry, 2010).

3.3.2 Calibrating a microfluidic device

The setup of the microfluidic device and the time-lapse fluorescence microscope was described previously (Bennett and Hasty, 2008; Ferry et al., 2011). First, the temperature of the microscope’s hood was set at 30°C (Figure 3.8A). A chip was inspected to ensure it was free of defects and dust within its features. If the chip was good, it was secured to the sample holder.

Next, the chip was primed with dH₂O. A short tubing with a luer stub adapter (blunt needle) was connected to a connection pin (Figure 3.6). The adapter was connected to a 10-mL syringe containing dH₂O. In this step, it is essential that there is no air bubble in the tubing and the needle nozzle. The pin was inserted into port 4 and water was pushed slowly into the chip. In a good chip, the water should completely fill the chip and flow out through other ports. The pin was pulled out and connected to port 5, then port 3, 2 and 1 and they were each filled with water. When the chip was completely filled with water, the
Figure 3.6: Materials for running a microfluidic glucose-depletion assay.

syringe was kept connected to port 5.

20 mL of fresh glucose and galactose media were prepared. A red fluorescent dye, Sulforhodamine 101 (excitation/emission, 586/605 nm, Invitrogen) was mixed into the glucose media for a final concentration of 1 μg/ml for tracking the environmental concentration of glucose (Bennett and Hasty, 2008). The two media were warmed to 30°C. They were then poured into two 50-mL syringes that were connected to two 90-inch long pieces of tubing. At the same time, two 10-mL syringes that connected to two 60-inch long pieces of tubing were filled with dH₂O. Again, it was essential that no air bubbles were inside the syringes and tubes. If they were, the nozzle of the syringe could be gently turned clockwise for the air bubbles to be released. The liquid, water and media, was gently pushed out of the pin. Once the water flowed out of the tubing in a steady drop-wise rate, an indication that there
Figure 3.7: Connected materials for a microfluidic assay. Left, a piece of parafilm was used to cover the 50-mL syringe containing media. The plunger of the syringe was taken out. A 23-gauge luer stub adapter was connected to the syringe. For the media reservoir, a 90-inch long piece of Tygon Micro-bore tubing was used. At the end is a connection pin which will be connected to a port on the microfluidic chip. Right, the same setup with a 10-mL syringe for a water reservoir. A 60-inch long piece of Tygon Micro-bore tubing was used.

were no bubbles in the system, a piece of parafilm was used to cover the top of the syringe (Figure 3.7).

The two media-containing syringes and the two water-containing syringes were hung on linear actuators and hanging towers, respectively (Figure 3.8A and B). Their heights were set according to the Load column in Table 3.4. In general, all four syringes should be kept at a height higher than the microscope’s stage to prevent backward flow of water into the media reservoirs. Also, the two media reservoirs were kept higher than all other reservoirs.

The NIS-Element Advanced Research software program, which controls the microscope, was turned on and the four reservoirs were connected to the chip (Figure 3.8C). The two water-containing syringes were connected to port 4 and 3. Then, the two media-containing syringes were connected to ports 1 and 2. This connection was done while observing the
Figure 3.8: Equipment for a microfluidic assay. A, hanging towers for waste outlet and cell loading, temperature-controlled incubator for maintaining temperature during scope run and inverted fluorescence microscope. B, linear actuators for controlling the ratio of the two media supplied to the chip. C, a microfluidic chip under microscope objective with connecting tubing.

chip with the 10X objective lens. Next, the scope was switched to the 40X objective lens for calibrating the DAW junction.

For calibrating the DAW junction, the two water reservoirs were moved to their respective running positions, as indicated in Table 3.4. The fluorescent signal of the Sulforhodamine 101 tracking dye at the junction was monitored at 50 ms-exposure time. The heights of the media reservoirs were calibrated and controlled using a custom software called “iDAW” (written by the Hasty lab). Steps for calibrating and setting glucose depletion steps are explained in Figure 3.9. In general, the two actuators first were moved in opposite directions so that 100% of glucose media was presented in the junction. The heights of these two actuators were recorded in the iDAW software. Next, the two actuators were moved so that 0% of glucose media was presented in the junction and the height values of the two actuators were also recorded. More steps can be done, for example at 25%, 50% and 75% of glucose media, to increase the accuracy of the calibration. Then, the glucose-depletion steps were programmed into the iDAW software. Some examples of the program file are listed in Table
### Table 3.3: Equipment for setting up a scope run.

| Equipment                        | Qty    | Role                        | Vendor                      |
|----------------------------------|--------|-----------------------------|                            |
| 0.02in x 0.06in Tygon            | 10in   | Chip wetting                | Saint-Gobain Plastics      |
| Micro-Bore tubing                | 3 x 60in | Cell & waste input          |                            |
|                                  | 2 x 90in | Media input                 |                            |
| 23-gauge Luer stub adapter       | 6      | Syringe & tubing connection |                            |
| Connection pin                   | 6      | Tubing & chip connection    |                            |
| 50-mL syringe                    | 2      | Media reservoirs            |                            |
| 10-mL syringe                    | 4      | Cell & waste reservoirs     |                            |
| parafilm                         | 5 pieces | Cover syringe barrels     |                            |
| Kimwipe                          | 1 box  | Wiping                      |                            |
| **Chemical**                     | Qty    | Role                        | Vendor                      |
| Galactose media                  | 5 mL   | Overnight induction         |                            |
|                                  | 20 mL  | Assay media                 |                            |
| Glucose media                    | 10 mL  | Repressing media            |                            |
|                                  | 20 mL  | Assay media                 |                            |
| Sulforhodamine 101               | 20 μL  | Glucose tracker             |                            |
| Cascade Blue                     | 29 μL  | Glucose tracker             |                            |
| dH$_2$O                          | 50 mL  | Chip wetting & waste outlet |                            |

#### 3.3.3 Programming a scope run

Once the media have been set up and the DAW junction calibrated, the next step is to load cells into the chip. Cell culture was loaded into a 10-mL syringe. The syringe was hung on the hanging tower (Figure 3.8A) and the tubing was connected to the chip. Cells were loaded to the chip and they flowed to the gate of the cell trap. Cells were forced into the cell trap by hand flicking the tubing connected to port 4. This flicking action generated a force that temporarily expanded the cell trap and allowed cells to be loaded into the trap. One disadvantage of this approach is that we cannot control the number of cells loaded into the trap. In general, more than five cells should be loaded. The viewing field of the objective lens was moved so that these cells stayed in the center of it.

The distance of the lens to the chip was adjusted to get the best phase contrast image, *i.e.* a black cell with a clear white ring outside the cell (Figure 3.11A). Parameters for capturing cell images were set. Capturing interval was set to 5 min for a duration of 15 hrs. Phase
Figure 3.9: The iDAW software for controlling glucose-depletion steps. 1, axis 1 and axis 2 represents two media reservoirs, glucose and galactose respectively. The “LINK?” box was checked so that both reservoirs were moved together. 2, “GOTO position” boxes allowed manual adjustments of the two reservoirs’ heights. The two reservoirs were moved to 300 mm-positions which were equal to the Load positions in table 3.4. 3, for calibrating the two media reservoirs, the moving distance of the linear actuator was set between 5 or 10 mm. 4, two buttons “Jog +” and “Jog -” were used to move one media reservoir up or down, respectively. When one reservoir moved up, the other reservoir will moved down and the changes in the fluorescent signal of Sulforhodamine at the DAW junction was tracked on the scope. 5, when 100% glucose was flown to the cell trap, positions of the two reservoirs in the “Current position” boxes were added into calibration table (No. 6). Steps 4, 5 and 6 were repeated but in the reserved direction so that 0% of glucose media was flown to the cell trap. 7, additional calibration steps, such as 25, 50 and 75% of glucose media could be added by using “Add” and “Delete” buttons. 8, the values in the calibration table were fitted into the movement curve by clicking the “Fit to Curve” button. 9, the curve was shown in the graph. 10, the calibration can be tested by moving the ruller from 0 to 1 which represent 0 to 100% of glucose reservoirs. 11, we used a custom data for the linear depletion of glucose. 12, the “Generate Signal” button was clicked to select the file containing the programing steps (table 3.5). 13, once the program file was selected, its trajectories were showed in this graph. 14, postitions of each moving steps of the two reservoirs can be recorded by clicking the “Save Data?” box. 15, steps for glucose depletion were initiated. The iDAW software was run at the same time with the scope so that the capture of fluorescent-image and the depletion of glucose were synchronized (Ferry, 2010; Ferry et al., 2011).
contrast, yellow and red fluorescent channels (YFP and RFP) were selected to capture the cell morphology, Gal1p-YFP and tracking dye signal, respectively. The exposure time for YFP was 100 ms and for RFP 300 ms. The iDAW and the NIS-Element Advanced Research were set to run at the same time so that the glucose-depletion steps and image-capturing steps were synced.

Table 3.4: Roles and working positions of the five reservoirs. The numbers in the run and load columns are the height in inches of the five reservoirs. In the run column, the heights of media 1 and 2 are at the beginning of the experiment. They were changed follow the setting of the glucose-depletion steps.

<table>
<thead>
<tr>
<th>Ports</th>
<th>Reservoirs</th>
<th>Content</th>
<th>Run (inH₂O)</th>
<th>Load (inH₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Media 1</td>
<td>glucose + dye</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Media 2</td>
<td>galactose</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Shunt waste</td>
<td>dH₂O</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>4</td>
<td>Waste output</td>
<td>dH₂O</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>Yeast cells</td>
<td>glucose</td>
<td>6</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 3.5: Programming the glucose-depletion time for the microfluidic assay. Two examples for programing the glucose-depletion time. Numbers on the left column are the code. Italic texts on the right column are the functional explanations. The codes include step 1, starts the experiment with a 100% glucose media; 2, maintains the glucose media for 4 hr; 3, linearly switches to the galactose media in a specific time period; 4, maintains a 100% galactose media until the end of the experiment. Top, glucose depletion happens in 1 min. Bottom, glucose depletion in 6 hr.

Depletion time = 1 min

| 0   | 100 | %Start with 100% glucose media |
| 240 | 100 | %Keep glucose media for 4 hr |
| 240.01 | 0 | %Change to galactose media in 1 min |
| 4000 | 0 | %End with 100% galactose media |

Depletion time = 6 hr

| 0   | 100 | %Start with 100% glucose media |
| 240 | 100 | %Keep glucose media for 4 hr |
| 600 | 0   | %Change to galactose media in 6 hr |
| 4000 | 0 | %End with 100% galactose media |

3.3.4 Analyzing digital images

The microscope only captures images from the microfluidic chip. At the end, I obtained several series of digital images, or image sequences, from the three channels which include
phase contrast, red and yellow fluorescence (Figure 3.10). For each channel, a sequence of 181 images were obtained. Analysis of these digital images was an important and integral part of the experiment. The goal was to extract all information from these images. For the phase contrast images, changes in cell morphology and lineage, such as cell sizes, lengths and numbers of budding events or cell divisions, and time points of these events could be collected. The phase contrast images were also modified and used as a positioning tool from which changes in the fluorescence intensity, represented by the mean gray value, in a cell can be calculated. For my experiment, ImageJ software was the tool of choice.

**Algorithms for tracking changes in fluorescent intensity in a single cell.** To track changes in the fluorescence intensity (FI) of a reporter in a single cell, two tasks have to be done simultaneously. First, the single cell needs to be tracked and followed throughout the experiment, in this case, a sequence of 181 digital images. Second, the FI value of the whole cell has to be quantified. For this quantification, a region of interest (ROI) needs to
be defined from which a mean gray value, representing the fluorescence intensity, can be quantified. This ROI must cover the whole cell and not overlap with other neighboring cells. Defining a ROI is a non-trivial task since yeast cells always touch each other as the cell trap becomes crowded during the later stages of the experiment. A simple approach is to trace a cell’s outline in the fluorescence image, then measure the gray value in that region. However, this is an extremely time-consuming, irreproducible approach since one needs to draw 181 ROIs to track a single cell. An alternative approach is to use an imaging tool for automatically defining the ROI. One of these tools is the Wand tool in ImageJ. This tool traces the outline of an object that has uniform color. Thus, this tool works efficiently with a binary image, i.e. an image that has only two polarized colors like black and white. Phase contrast images are suitable for this conversion. Here, I describe two main algorithms and two custom plugins for converting phase contrast images into binary images and for tracking a single cell throughout an experiment.

In a phase contrast image, there are distributions of gray values of both an object’s pixels and the background’s pixels. If there is a significant difference between these two gray values, a threshold can be defined. Based on this threshold, a pixel with a gray value larger than the threshold value will have its pixel converted to the value 1. On the other hand, a pixel with a gray value smaller than that of the threshold will have its pixel converted to the value 0. As a result, every pixel of the phase contrast image will be classified into two groups, 0 and 1, and hence that image becomes a binary image. By optimizing a criterion function to minimize the average pixel classification error rate, Kittler and Illingworth develop the Minimum Error algorithm, from which an optimal, minimum error threshold value can be selected (Kittler, 1986). An example of a binary image obtained using Minimum Error thresholding is shown in Figure 3.11A & B.

The next step after converting the phase contrast image into binary is to separate, or segment, single cells in the viewing field. One approach to object segmentation is to use the Watershed algorithm in ImageJ. This algorithm, based on the Euclidian distance map, finds
the ultimate eroded point (UEPs) for each object in the image. This point is then dilated until it reaches its particle edge or a neighbor UEP region. This approach is based on the work of Vincent and Soille and is best applied to particles with smooth convex shapes, like yeast cells (Vincent, 1991).

One side effect caused by object segmentation is the problem of over-segmentation. Since most yeast cells have vacuoles, the presence of this structure can result in two or more local regions in a single cell. Consequently, each of these local regions have their own UEP. These UEPs, in turn, cause a cell to be segmented into two or more objects instead of one object (Figure 3.11B). The problem of over-segmentation can be corrected using a Brush tool which works to connect these segmented parts together. I wrote two plugins to solve the over-segmentation problem and to speed up the analysis process. The first one allows measuring the grayscale value of an object in a fluorescent image based on its selection on the binary, phase contrast image (Table 3.6). This plugin also allows correcting some mistakes caused by the over-segmentation. The second plugin allows manual segmentation of some objects that have not separated properly using the Watershed algorithm (Table 3.7).

Table 3.6: Plugin 1 for measuring fluorescence values

```java
//macro“Truong_aa [7]”
{ setTool(17);
  run(“Colors...”,
   “foreground=black
   background=white
   selection=green”);
  var brushWidth = 20;
  getCursorLoc(x, y, z, flags );
  doWand(x, y);
  run("Measure");
  run("Next Slice [>]”); } %Plugin name and shortcut key %Choose tool set %Set color %Foreground color is black %Background color is white %Selection color is green %Width of the brush is 20 pixel %Determine cursor position %Run selection using wand tool %Measuring follow measurement setup %Run to next image
```

For analyzing the digital images captured from the glucose-depletion assay, the phase contrast image sequence was loaded into the ImageJ software (Figure 3.11A). These images were converted to binary, black and white images by adjusting their color threshold using the MinError algorithm (Figure 3.11B & E). Segmentation was done using the Watershed
Figure 3.11: Using ImageJ plugins to analyze digital images. Two single cells were tracked in a phase contrast image and a fluorescent image. A-D, example 1. A, a single cell was tracked in an original phase contrast image. B, the phase contrast image was converted to a binary image and was segmented using the Watershed algorithm. Due to the presence of a vacuole, the segmentation splitted the cell into two objects. C, the Brush tool, incorporated in plugin 1, was used to link the two objects into one single cell. D, the fluorescence intensity of that cell was quantified using the fluorescent image. E-H, example 2. E, another cell was tracked in the same converted binary image. F, correction by plugin 1 caused the target cell to link with a neighbor cell. G, the Erase tool, incorporated in plugin 2, was used to split the one object into two cells. H, the fluorescent intensity of that cell was quantified correctly using the fluorescent image.

algorithm. After that, the image sequence of Gal1p-YFP was loaded into the software and linked to the binary image sequence using the “Redirect” function in the “Set Measurements” setting. If a cell was over-segmented as in Figure 3.11B, a Brush tool was used to link the two fragments into one object as in Figure 3.11C. That cell was then selected by pointing
Table 3.7: Plugin 2 for correction of bad image segmentation

```javascript
//macro“Paintbrush Toggle [8]”
{ setTool(17);
  while (true) {
    getCursorLoc(x, y, z, flags);
    run(“Colors...”,
      “foreground=white
      background=white
      selection=green”);
    var brushWidth = 2;
    draw(brushWidth); } }
```

%Plugin name and shortcut key
%Choose tool set
%Run while loop
%Determine cursor position
%Set color
%Foreground color is white
%Background color is white
%Selection color is green
%Width of the brush is 2 pixel
%Draw using brush tool

the mouse cursor to it and running plugin 1. The cell size, mean and standard deviation of the fluorescence intensity of the cell were recorded (Figure 3.11D). If the segmentation was not done properly by the Watershed algorithm (Figure 3.11F), plugin 2 was used to separate them before plugin 1 was run (Figure 3.11G & H). The process was done in 181 images and the raw data of every cell were collected. For each experiment, all the cells present at the beginning of the experiment (the first generation or mother cells) were measured unless they died or were pushed out of the viewing field before the conclusion of the experiment.

3.3.5 Calculating glucose-depletion times

Experimentally, the image sequence of the red fluorescence channel was loaded into ImageJ software. A rectangular ROI, covering most of the viewing area, was selected. The mean gray value was measured for the whole image sequence. Obtained values were plotted against time to track changes in the glucose concentration. Also, since glucose was linearly depleted from the environment, the exact concentration at any time point can be calculate using simple linear equations. For each glucose-depletion times, Table 3.8 lists the changes in glucose concentration at different time points.

3.3.6 Calculating activation times of the Gal network

The activity of the Gal network has multiple stages, including early expression of Gal components, their accumulation, and finally the saturation of these proteins’ concentrations.
Table 3.8: Linear depletion of glucose concentration. Top row, glucose-depletion times (hr). Left column, change in glucose concentration from 2% to 0%. Remaining columns, time point of each glucose concentration (hr).

<table>
<thead>
<tr>
<th>Conc.</th>
<th>0.01</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>8 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>2%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 hr</td>
</tr>
<tr>
<td>1.9</td>
<td>0.0005</td>
<td>0.0125</td>
<td>0.025</td>
<td>0.05</td>
<td>0.075</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>1.8</td>
<td>0.001</td>
<td>0.025</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>1.7</td>
<td>0.0015</td>
<td>0.0375</td>
<td>0.075</td>
<td>0.15</td>
<td>0.225</td>
<td>0.3</td>
<td>0.45</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>1.6</td>
<td>0.002</td>
<td>0.05</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>1.5</td>
<td>0.0025</td>
<td>0.0625</td>
<td>0.125</td>
<td>0.25</td>
<td>0.375</td>
<td>0.5</td>
<td>0.75</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>1.4</td>
<td>0.003</td>
<td>0.075</td>
<td>0.15</td>
<td>0.3</td>
<td>0.45</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
<td>1.8</td>
<td>2.4</td>
</tr>
<tr>
<td>1.3</td>
<td>0.0035</td>
<td>0.0875</td>
<td>0.175</td>
<td>0.35</td>
<td>0.525</td>
<td>0.7</td>
<td>1.05</td>
<td>1.4</td>
<td>2.1</td>
<td>2.8</td>
</tr>
<tr>
<td>1.2</td>
<td>0.004</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.2</td>
<td>1.6</td>
<td>2.4</td>
<td>3.2</td>
</tr>
<tr>
<td>1.1</td>
<td>0.0045</td>
<td>0.1125</td>
<td>0.225</td>
<td>0.45</td>
<td>0.675</td>
<td>0.9</td>
<td>1.35</td>
<td>1.8</td>
<td>2.7</td>
<td>3.6</td>
</tr>
<tr>
<td>1</td>
<td>0.005</td>
<td>0.125</td>
<td>0.25</td>
<td>0.5</td>
<td>0.75</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>0.9</td>
<td>0.0055</td>
<td>0.1375</td>
<td>0.275</td>
<td>0.55</td>
<td>0.825</td>
<td>1.1</td>
<td>1.65</td>
<td>2.2</td>
<td>3.3</td>
<td>4.4</td>
</tr>
<tr>
<td>0.8</td>
<td>0.006</td>
<td>0.15</td>
<td>0.3</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
<td>1.8</td>
<td>2.4</td>
<td>3.6</td>
<td>4.8</td>
</tr>
<tr>
<td>0.7</td>
<td>0.0065</td>
<td>0.1625</td>
<td>0.325</td>
<td>0.65</td>
<td>0.975</td>
<td>1.3</td>
<td>1.95</td>
<td>2.6</td>
<td>3.9</td>
<td>5.2</td>
</tr>
<tr>
<td>0.6</td>
<td>0.007</td>
<td>0.175</td>
<td>0.35</td>
<td>0.7</td>
<td>1.05</td>
<td>1.4</td>
<td>2.1</td>
<td>2.8</td>
<td>4.2</td>
<td>5.6</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0075</td>
<td>0.1875</td>
<td>0.375</td>
<td>0.75</td>
<td>1.125</td>
<td>1.5</td>
<td>2.25</td>
<td>3</td>
<td>4.5</td>
<td>6</td>
</tr>
<tr>
<td>0.4</td>
<td>0.008</td>
<td>0.2</td>
<td>0.4</td>
<td>0.8</td>
<td>1.2</td>
<td>1.6</td>
<td>2.4</td>
<td>3.2</td>
<td>4.8</td>
<td>6.4</td>
</tr>
<tr>
<td>0.3</td>
<td>0.0085</td>
<td>0.2125</td>
<td>0.425</td>
<td>0.85</td>
<td>1.275</td>
<td>1.7</td>
<td>2.55</td>
<td>3.4</td>
<td>5.1</td>
<td>6.8</td>
</tr>
<tr>
<td>0.2</td>
<td>0.009</td>
<td>0.225</td>
<td>0.45</td>
<td>0.9</td>
<td>1.35</td>
<td>1.8</td>
<td>2.7</td>
<td>3.6</td>
<td>5.4</td>
<td>7.2</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0095</td>
<td>0.2375</td>
<td>0.475</td>
<td>0.95</td>
<td>1.425</td>
<td>1.9</td>
<td>2.85</td>
<td>3.8</td>
<td>5.7</td>
<td>7.6</td>
</tr>
<tr>
<td>0</td>
<td>0.01</td>
<td>0.25</td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
</tbody>
</table>

Regarding the effect of the glucose depletion, we focused on the onset and the accumulation of Gal1p-YFP. The question is whether changes in the glucose-depletion rate can affect the mean and variability of the Gal network’s activation in a cell population. The mean and variability also reflect how each cell in the population reacts to the rate of change. An increase in the variability is a signal that each cell in the population achieves a different level of Gal1p expression (i.e. a different phenotype) in a homogeneous environment. Since the yeast population has a galactose background, we considered the onset of the Gal network as the time point when the FI of Gal1p-YFP increases irreversibly. We called this time point the “initiation point” at which the FI value equals to 0 (arbitrary unit, AU). The period from the beginning of the glucose depletion to this initiation point is called the “Gal1-initiation time” (Figure 3.12). Also, we measured an “accumulation point” at which the FI value
Figure 3.12: Calculating expression times of the Gal network. The glucose-depletion time represents the time it takes for glucose concentrations to decrease from 2% to 0%. The Gal1-initiation time and Gal1-accumulation time represent the times it take for the fluorescent intensity of Gal1p-YFP proteins to reach 0 and 200 (AU).

equaled 200 AU. Again, the period from the beginning of the glucose depletion to this accumulation point is called the “Gal1-accumulation time” (Figure 3.12). Our research goals are to elucidate the relationship between Gal1-initiation and Gal1-accumulation times and the depletion times of glucose.

3.3.7 Quantifying lengths of yeast cell cycles and diauxic phases

As mentioned in Section 3.3.4, information about the cell lineage, budding and division events can be obtained by tracking changes in cellular morphology in the sequence of phase contrast images. Tracking cell lineages allows us to compare the expression pattern of Gal1p in cells of different generations, including the mother, daughter, and granddaughter cells. We can
then elucidate the correlation in Gal1p expression among these cells. This correlation can signal a background effect in gene expression that works antagonistically with environmental effects (Acar et al., 2008). In my experiments, cells that are present in the chip at the beginning of the experiment are considered “mother” cells (the first generation). In the later stages of the experiments, these cells divide and generate multiple offspring of the second generation. These “daughter” cells (the second generation) then generate their offspring which are “granddaughter” cells. The birth of an offspring is called a “budding event”, since a new cell asymmetrically buds from the mother cell. Due to the asymmetry, the mother cell and the daughter cell can be clearly distinguished, as shown in Figure 3.13. In these experimental runs, I tracked cells in the first, second and third generations that were born before the initiation of the glucose depletion. In most cases, the mother, two daughters, and one granddaughter were tracked.

The length of a cell cycle is an important indication of the environmental effect on an individual cell. Yeast cells have a short cell cycle time, around 90 min, when they are grown in glucose. The cell cycle length is around 120 min in galactose. However, Monod demonstrated that a transition from glucose to another sugar can cause a cellular arrest for a period of time (Monod, 1942). This period of time is called “diauxie” and its length was suggested to be correlated with the secondary carbon source. The presence of diauxie is typically quantified by the measurement of OD_{600}, and is depicted as a lag phase between two exponential growth periods. In my experiments, the two exponential growth periods were characterized by continuous budding events. Thus, by tracking the length of a cell cycle, as demonstrated in Figure 3.13, one can determine the longest cell cycle which strongly indicates diauxie.

Besides the cell lineage and cycle length, cellular fitness is also an important aspect. The transition between the two metabolic pathways results in diauxie, i.e. a growth-arrested period in the cell. We hypothesized that one reason for growth arrest is the lack of energy, due to a reduction in ATP generated from glucose metabolism (van den Brink et al., 2009).
The severity of the energy loss can force cell into a quiescent stationary phase (Venturelli et al., 2015). In the phase contrast images, these cells may stop their budding for an extended period of time. However, these cells are not dead. Thus, the ability to distinguish dead cells and quiescent cells is important. In most cases, a dead cells emits a red fluorescence signal that can be detected in the red fluorescence channel. Another way to distinguish it is to look at the change in the cellular morphology in the phase contrast image. Dead cells tend to collapse and their shape becomes wrinkled due to the loss of osmotic pressure. In contrast, quiescent cells maintain a round and smooth body. In most cases, dead cells were discarded from the dataset.

3.4 Constructing repression curves of the Gal network

Beside elucidating the effect of glucose-depletion rate on the Gal network’s activation, it is also essential to understand the range of glucose concentrations that affect the Gal network’s activity. We constructed repression curves of the Gal network based on three techniques: fluorimetry, flow cytometry, and microfluidic-aided microscopy.

For the fluorimetry and flow cytometry assays, a colony of the K699-1y strain was grown in 5 mL of galactose media, overnight at 30°C and 250 rpm shaking. On the next day, 1% of the overnight culture was transferred into 5 mL of fresh galactose media. The new culture was aliquoted and different amounts of glucose were added. Glucose concentrations ranged from 0% to 5% (w/v). These subcultures were distributed into a clear, U-bottom, 96-well plate (Corning) and grown for 8 hr at 30°C, 250 rpm shaking. At the end of the growth, the plate was read in a Infinite M1000 Pro plate reader (Tecan), in which the absorbance (OD$_{600}$) of the cell culture and the FI of the Gal1p-YFP (excitation/emission: 515 nm/528 nm) were collected. For the flow cytometry experiment, 4 μL of formaldehyde was added to each well of the same plate (2% of total volume) for cell fixation. The plate was gently shaken for 10 min and centrifuged for 10 min at 3000 rpm. Cells were then washed twice using phosphate buffered saline (PBS) solution. Finally, the cell size, granularity and FI of
Figure 3.13: Characterizing cell cycle based on scope images. Cell cycle is determined by change in cell morphology observed in digital images. The numbers are time in minutes. M, mother cell; D1, first daughter cell. Red arrow at 5 min, start of a budding event. Red arrow at 80 min, end of a budding event and birth time of the first daughter. Red arrow at 90 min, start of the subsequent budding event. From 5 to 80 min, length of the budding event. From 5 to 90 min, length of a cell cycle.

The Gal1p-YFP were collected by a Guava easyCyte HT flow cytometer (Millipore).

For the microfluidic-based assay, a single colony of the K699-1y strain was grown in 5 mL of galactose media with different amount of glucose overnight at 30°C and 250 rpm shaking. Glucose concentrations ranged from 0% to 2% (w/v). On the next day, the cell culture was transferred into a microfluidic chip and grown in the same media for 8 hr at 30°C. Phase contrast and fluorescent images of the Gal1p-YFP were taken every 5 min. At the end of the experiment, images were analyzed using the imageJ software.
Figure 3.14: Constructing repression curves of the Gal network. A single yeast colony was picked and grown in galactose overnight at 30°C, 250 rpm shaking. In the next day, for the plate reader and flow cytometry, the culture was transferred to fresh media and grown in a 96-well plate with 2% galactose in addition to a range of glucose concentrations. For the plate reader, the plate was grown at 30°C and the fluorescent intensity of Gal1p-YFP was collected every 5 min for 8 hr. For the flow cytometry, the plate was grown for 8 hr, then cells were fixed followed standard protocol and the FI of Gal1p-YFP from single cells were collected using the Guava easyCyte HT flow cytometer (Millipore). For microfluidic assay, the overnight culture was transferred to fresh media containing 2% galactose and a specific concentration of glucose. Cells were loaded into the chip and observed for 8 hr.

3.5 Mathematical simulation for the activation of the Gal network

To uncover the effect of the glucose depletion on the activation of the Gal network, a mathematical simulation was conducted by Chinmaya Gupta, a post-doc in Josic lab, University of Houston. The results will be described and discussed in the Results section.
Chapter 4

Activities of the Gal network in static environments

To understand the dynamics of the Gal network, I first investigated activities of the Gal network in static environments. Three different approaches, fluorimetry, flow cytometry, and microfluidic-aided microscopy were used. In each method, cells were exposed to different environments even though the initial experimental conditions were the same. Doing so allowed us to understand different behaviors of the Gal network in similar settings.

4.1 Repression of the Gal network follows a sigmoidal function

The first goal was to understand the effect of glucose on the activation of the Gal network. Previous studies have shown that the relationship between the expression levels of Gal1p and the glucose concentration follows a decreasing sigmoidal curve (Bhat and Iyer, 2009; Bennett et al., 2008). In this thesis, using three different approaches, microfluidic assay, fluorimetry, and flow cytometry, I examined this relationship in different contexts. In fluorimetry, the amount of media is limited and Gal1p signals come from the whole population. In flow cytometry, the amount of media is limited, and the Gal1p signals come from individual cells. In the microfluidic device, the amount of media is unlimited and the Gal1p signals come from individual cells. Details of these methods are explained in Section 3.3.5. The fitting of these plots were done with help from Dr. Chinmaya Gupta.
Figure 4.1: Repression of glucose on the expression of the Gal network follows a sigmoidal function. Data were obtained using A) fluorimetry, B) flow cytometry and C) microfluidic-aided microscopy. D) Distributions of cell densities in some glucose concentrations from the microfluidic assays demonstrated bimodal patterns of the Gal1p expression.
Although all three methods showed sigmoidal relationships between glucose concentration and the expression level of Gal1p-YFP (Figure 4.1A, B and C), the slopes of these curves were different, suggesting variability in the cellular sensitivity to glucose concentrations. In both the fluorimetry and flow cytometry curves, the slopes were steeper with a range from 0.05% to 1% glucose (Figure 4.1A, B). These results suggested an effect of a limited carbon source that forces cells to degrade Gal1p faster. Also, the variability of the Gal1p signals in the fluorimetry was smaller than that in flow cytometry, suggesting that the signals were averaged out in the cell population. In contrast, cells in the microfluidic device have a more gradual change in the Gal1p-YFP level as a function of glucose with a higher variability than that of the two other methods. It was shown that in an abundance of glucose and galactose, cells maintain a higher-than-background level of Gal1p, even in the presence of glucose (Braun and Brenner, 2004). Also, data from Figure 4.1D shows that cells exhibited bimodal patterns in the Gal1p-YFP’s expression level within an intermediate range of glucose concentrations, from 0.4% to 0.8%. These data explained the high variability in the Gal1p signals from the microfluidic assay that were not observed in the fluorimetry and flow cytometry experiments.

4.2 Activity of the Gal network in single-sugar environments

To understand the dynamics of the Gal network, we first looked at characteristics of Gal1p-YFP’s expression in cells grown exclusively in galactose. Cells were grown overnight in galactose, transferred and grown in fresh galactose media for 8 hr, then observed on chip for 15 hr in galactose media. We quantified three parameters from the collected cell images using the ImageJ software: area, mean gray value, and standard deviation. The area value is the cell area in square pixels. The mean gray value is the fluorescence intensity (FI) of the Gal1p-YFP in arbitrary unit (AU). The standard deviation has been used to demonstrate a spatial variance of Gal1p-YFP signals within a cell body (Braun and Brenner, 2004). The spatial variance is an indication of Gal1p-YFP’s distribution within a cell. A small value indicates that the Gal1p-YFP proteins are distributed evenly in the cell, whereas a high value indicates
Figure 4.2: Characteristics of cells grown in exclusive galactose media. A) Cellular area increase linearly over time. B) While values of the fluorescence intensities fluctuate, in general, cells maintain a stable level of Gal1p-YFP expression. C) Values of the spatial variance of Gal1p-YFP decrease over time, indicating the protein is expressed or distributed evenly within cells’ bodies. Green lines depict the average. Red lines depict the standard deviation within the cell population. D) Cell cycle length in exclusive galactose media.

that Gal1p proteins are colocalized or concentrated at a specific cellular region. In Figure 4.2A, our data showed that the cellular area generally increased over time. In contrast, the spatial variance of Gal1p signals decreased over time (Figure 4.2C). The fluorescence intensities of Gal1p-YFP were shown to be around 8000 (AU) on average (Figure 4.2B). It should be noted that standard deviation values of the area, fluorescence intensity and spatial variance were consistent over time, suggesting that the whole cell population reacted uniformly to this steady state environment.

One important parameter is the cell cycle length. This value is defined as the time period
between two successive budding events. In Figure 4.2D, our data showed that more than 90% of cells have cycle lengths between 1 to 3 h (>60% of these cell cycles are between 1.5 to 2.5 h).

Another aspect we need to understand is the degradation rate of Gal1p-YFP protein. It has been shown that Gal1p is very stable requiring at least 6 generations for complete degradation (Johnston et al., 1994). Furthermore, these proteins are degraded mainly through dilution as cells grow and divide. We tested the degradation rate of Gal1p-YFP proteins by growing cells in galactose overnight before transferring them to fresh glucose media and growing for another 8 hr. These cells were transferred to microfluidic devices and grown for another 15 hr.

One problem we encountered in this experiment was that cells grow very fast in glucose media, thus they moved out of the chip very quickly. We were therefore only able to obtain data for 9 hr of the microfluidic assay, instead of 15 hr. In Figure 4.3, we examined similar characteristics of Gal1p-YFP’s expression in yeast cells as mentioned in previous section. We saw that cells grown in glucose increased their area at a similar rate to those grown in galactose media (Figure 4.3A). On average, however, the cellular area was bigger than that of cells grown in galactose media. On the other hand, we saw that after a fast decrease in the fluorescence intensity of Gal1p-YFP, these values fluctuated above a background level, even after 9 hr in the chip (Figure 4.3B). The spatial variance of Gal1p-YFP was close to zero indicating that Gal1p-YFP was sparsely distributed in the cell (Figure 4.3C). For the cell cycle length, 90% fell between 1 and 1.5 hrs, consistent with previous results for cells grown in glucose (Figure 4.3D).

In general, by comparing profiles of Gal1p-YFP in galactose and glucose media we saw that Gal1p-YFP was maintained at a steady state level during activation and repression states. Since galactose is considered a secondary carbon source, cells grown in galactose are smaller than those grown in glucose.
Figure 4.3: Characteristics of Gal1p-YFP degradation in glucose media. A) Cellular area stays roughly constant over time. B) Values of the fluorescence intensities significantly decrease over time. C) Values of the spatial variance of Gal1p-YFP decrease over time, indicating the proteins are degraded within cells’ bodies. Green lines depict the average. Red lines depict the standard deviation within the cell population. D) Cell cycle length in glucose media.
Chapter 5

Activation of the Gal network is dynamically affected by the rate of glucose depletion

The results of Gal1p-YFP in steady state indicate low and high levels of Gal1p expression in the presence and absence of glucose, respectively. Next, I want to understand how the transition between the presence and absence of glucose affects the expression of Gal1p-YFP. Specifically, the question is how glucose depletion rates influence the dynamics of Gal1p-YFP expression.

5.1 Cells exhibit wide ranges of \( GAL1 \) expression under different schemes of glucose depletion

The setup of the depletion trial experiments is described in Section 3.3. During loading and set-up of the microfluidic device, cells were exposed to media containing 2% galactose and 2% glucose. Figure 5.1 depicts the general procedure of the experiments. At the beginning of each trial, we began to decrease the concentration of glucose at a constant rate until it reached zero, while maintaining a constant level of galactose. Due to this change, cells eventually switched from glucose metabolism to galactose metabolism. To determine the times at which cells turned on the galactose network, we monitored the YFP fluorescence of individual cells which served as a proxy for \( GAL1 \) activity (Figure 5.1A).

In each experiment, there are three times that are of particular interest (Figure 5.1B).
Figure 5.1: Experimentally tracking single cells during glucose depletion assays. A) Cells were trapped within a microfluidic flow chamber and the environmental concentration of glucose was depleted as a function of time, while holding the galactose concentration constant. As glucose levels dropped, individual cells heterogeneously activated Gal1p production, and the resulting fluorescence trajectories were recorded. B) Glucose concentration as a function of time (red line). Here, the depletion time is 4 hrs. Also shown is the experimentally measured fluorescence trajectory of an individual cell to the 4 hr depletion time (green line). This cell first initiates Gal1p production and then accumulates protein (below). C) Images of yeast cells in the microfluidic device at successive events from a 4 hr glucose-depletion assay. [glu] = 2%, t = 0 (hr) indicates the beginning of glucose depletion; [glu] = 0% indicates total depletion of glucose; labels FI = 0 and 200 (AU) correspond to the times at which FI reaches these values; end of run, the end of glucose depletion assay (Nguyen-Huu et al., 2015).

First is the “depletion time” – the time between the beginning of glucose depletion until its concentration reaches zero. Second, the “initiation time” is the time at which fluorescence in a cell first begins to increase. At the beginning of the experiment, each cell has higher fluorescence intensity (FI) than the background as depicted by the fluorescent intensity value before the time point t= 0 hr in Figure 5.1B. This is expected since these cells were grown in galactose overnight before transferring to glucose media. Thus, these cells still contained remained Gal1p-YFP proteins from previous galactose exposure. We observed that, during glucose depletion, FI values slowly decrease before increasing again as cells acclimated to
the galactose environment. We call the time at which a FI trajectory changes from having a negative to a positive slope the “initiation point.” We rescaled the FI so that it equals zero at the initiation point. We called the interval between the beginning of glucose depletion and the initiation point the “initiation time” (Figure 5.1B). Finally, the “accumulation time” is the interval between the initiation of glucose depletion and the time at which cell fluorescence reaches an arbitrary threshold above that at the initiation point (Figure 5.1B).

We tested ten different depletion rates, varying the depletion time between 0 and 8 hrs. We chose this range of depletion times to cover both unnatural induction events, in which glucose is quickly removed from a culture to induce protein expression, and more naturally occurring scenarios, in which glucose is more gradually depleted by a growing colony. Even when glucose is being naturally depleted from the environment, it has been shown that cells exhibit heterogeneity in their growth rates under diauxic shift (Wang et al., 2015; New et al., 2014). Figure 5.2 shows representative single-cell YFP fluorescence trajectories of YFP-tagged Gal1p and time-dependent concentrations of environmental glucose for each of the depletion times tested.

5.2 The initiation of GAL1 expression depends linearly on the glucose depletion time

Mathematical simulation and calculation in this section was done by Dr. Chinmaya Gupta. When we plotted the mean and standard deviation of the initiation times against the depletion times, we observed that the initiation time was linearly related to the depletion time (Figure 5.3A, \( R^2 = 0.974 \)). This result fits an intuitive hypothesis about GAL network activity: The GAL network activates some time, \( \tau \), after glucose has dropped below a threshold concentration, \( g^* \). This simple model would indicate that the initiation time obeys

\[
T_i = \left[ \frac{g_s - g^*}{g_s} \right] T_d + \tau, \tag{5.1}
\]
Figure 5.2: Experimentally measured single-cell responses to different glucose-depletion times. Ten glucose-depletion times were tested. Red lines depict changes in glucose concentration from 2% to 0% (w/v). Gray curves depict individual fluorescence trajectories of Gal1-YFP. Green curves show mean FI (Nguyen-Huu et al., 2015).

where $T_i$ is the initiation time, $T_d$ is the depletion time, and $g_s$ is the initial glucose concentration (here, $g_s = 2\%$). Fitting Eq. 5.1 to data gives us approximate values for the threshold and the delay time, namely $g^* \approx 1.4\%$ ($P < 0.001$, 95% CI= (1.17%, 1.63%) and $\tau \approx 17$ min. ($P < 10^{-7}$, 95% CI = (15.4 min, 18.6 min)). Furthermore, the standard deviation of the initiation time as a function of depletion time (Figure 5.3B) suggests that the threshold concentration of glucose and the delay time vary from cell to cell. As depicted in Figure 5.3C, the time it takes to sweep past the distribution of thresholds increases as the depletion time increases. Therefore, assuming that variations in $\tau$ and $g^*$ are independent, this simple model predicts that the standard deviation of the initiation times is given by

$$\sigma^2(T_i) = \frac{\sigma^2(g^*)}{g_s^2} T_d^2 + \sigma^2(\tau),$$

(5.2)

where $\sigma(g^*)$ is the standard deviation of the repression threshold, and $\sigma^2(\tau)$ is the variability in the activation delay. Indeed, our data support this relationship between $T_d^2$ and $\sigma^2(T_i)$, and fitting Eq. 5.2 to the data gives us $\sigma(g^*) \approx 0.2\%$ and $\sigma(\tau) \approx 18$ min.
Figure 5.3: The initiation of *GAL1* expression depends linearly on the glucose depletion time. A) The mean initiation time increases linearly with increasing depletion time. Shown are the mean (dots), standard deviations (error bars), and best fit line. B) The standard deviation of the initiation time is related to the variability of the threshold $g^*$ and the delay time $\tau$ according to Eq. (5.2). Error bars represent the standard deviation of the measurement as calculated with a bootstrapping method. C) The dependence of both the mean and the standard deviation of the initiation time can be explained by a simple threshold model. As glucose depletes (red curves, left) a variable threshold (green distribution, right) is crossed that relieves repression of the galactose genes. The slower the transition through the repression thresholds (horizontal shaded region) the greater the variability in the initiation time (vertical shaded region) (Nguyen-Huu *et al.*, 2015).

Note that the initiation time for the 0 hr depletion was not significantly different when we held cells at 2% galactose for 6 hr (instead of 4 hr) before the depletion of glucose. With a 6 hr “pre-growth,” the initiation time was $0.35 \pm 0.21$ hr, compared to an initiation time of $0.30 \pm 0.20$ hr with a 4 hr pre-growth (Figure 5.4).
5.3 Gal1p’s accumulation changes non-monotonically with glucose depletion rates

We next examined how the accumulation time depends on the depletion time. While the initiation of genes within the Gal network is an important step in the process of changing from glycolysis to galactose metabolism, the proteins encoded by the GAL genes must accumulate, or reach a specific level, before the Gal network achieves full induction and galactose can be metabolized (Venturelli et al., 2015). To measure this accumulation time, we first chose an arbitrary threshold FI value that was above the background level, but still well below the mean steady-state levels. The interval between the initiation point and the time at which a cell’s FI trajectory first reached this threshold value was recorded as the accumulation time.

As a function of the depletion time, the accumulation time looks similar to the initiation time (Figure 5.5). However, there are two important differences. First, the accumulation time is larger for near instantaneous than for intermediate depletion times (Figure 5.5A). This indicates that cells accumulate proteins more quickly if glucose is depleted slowly rather than instantaneously. Second, the standard deviation of the accumulation time is not an increasing function of the depletion time (Figure 5.5B).
Figure 5.5: Gal1p accumulation changes non-monotonically with the glucose depletion rate. A) Average accumulation time as a function of the depletion time (error bars represent SD). B) The standard deviation of the accumulation time as a function of the depletion time. In fast and slow depletion schemes, the accumulation of Gal1p is highly variable, and achieves a minimum at intermediate depletion rates. Error bars represent the standard deviation of the measurement as calculated with a bootstrapping method (Nguyen-Huu et al., 2015).

Table 5.1: Values of Gal1p initiation and accumulation times

<table>
<thead>
<tr>
<th>Depletion schemes (hrs)</th>
<th>Initiation time (hrs)</th>
<th>Accumulation time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std. Dev.</td>
</tr>
<tr>
<td>0</td>
<td>0.3025</td>
<td>0.2020</td>
</tr>
<tr>
<td>0.25</td>
<td>0.2872</td>
<td>0.1972</td>
</tr>
<tr>
<td>0.5</td>
<td>0.3801</td>
<td>0.2269</td>
</tr>
<tr>
<td>1</td>
<td>0.4563</td>
<td>0.3386</td>
</tr>
<tr>
<td>1.5</td>
<td>0.6637</td>
<td>0.3728</td>
</tr>
<tr>
<td>2</td>
<td>0.8917</td>
<td>0.4028</td>
</tr>
<tr>
<td>3</td>
<td>1.3371</td>
<td>0.4980</td>
</tr>
<tr>
<td>4</td>
<td>1.5928</td>
<td>0.5901</td>
</tr>
<tr>
<td>6</td>
<td>1.8253</td>
<td>1.0003</td>
</tr>
<tr>
<td>8</td>
<td>2.5167</td>
<td>0.9907</td>
</tr>
</tbody>
</table>

5.4 Yeast cells showed three distinct growth phases during glucose depletion scheme

We next wanted to understand why the accumulation time behaved differently than the initiation time when glucose was depleted rapidly. One piece of evidence was cell cycle length. We noticed that cells exposed to rapid glucose depletion temporarily stopped growing after glucose was totally depleted. By contrast, cells experiencing more gradual depletion
did not exhibit this behavior. We hypothesized that the cessation of growth during fast depletion times represented a diauxic shift as the cells transition from glucose metabolization to galactose metabolization. For yeast cells growing on a single carbon source, diauxie is a hallmark of the stationary and quiescence phase, when cells exhaust their preferred carbon source and start to metabolize another. As a result, cells delay or stop their division to conserve energy while their translational machinery is poised to exit diauxie when new carbon sources become available. We further hypothesized that during these slow cell cycles, protein production within the Gal network became very slow. Therefore, even though the network might be initiated, the cells did not have sufficient energy to produce the Gal network’s proteins rapidly, leading to accumulation times that were much longer than normal.

To better characterize how the glucose depletion rate influences the growth dynamics of cells during the diauxic shift, we tracked the budding events of individual cells throughout each experiment, and calculated the cell cycle lengths (the time from one budding event to the next) of each cell (Figure 5.6). We observed that, before glucose depletion, the average cell cycle length was 1.38 ± 0.25 hr (Figure 5.6A). Well after glucose was completely depleted and the cells were growing only in galactose, the average cell cycle length was 1.65 ± 0.38 hr (Figure 5.6C). These two times represent normal growth in glucose and galactose media, respectively. However, between these two extremes, cell cycle lengths tended to be longer and highly variable, especially for fast depletion times (Figure 5.6B).

The longest cell cycles occurred just as glucose was nearly depleted and we used these cell cycle lengths as estimates of the diauxic cell cycle lengths. We found that the length of the cell cycle during the diauxic phase was, on average, 3.19 ± 1.95 hr (Figure 5.6B). An increase in the SD values is an indication of increasing variability as cells make the transition to galactose metabolism. However, the diauxic cell cycle length depended greatly on the depletion time. For instance, the diauxic cell cycle length was longest for instantaneous depletion times, at 4.74 ± 3.04 hr, and shortest for a depletion time of 8 hrs, at 2.32 ± 1.03 hr (Figure 5.6B). Additionally, the SD of the diauxic cell cycle length was largest for instantaneous depletion
Figure 5.6: Changes in cell cycles’ length at different phases of glucose depletion. A) Before the depletion schemes, the average length of cell cycles is $1.38 \pm 0.25$ hr. B) During diauxie, the length of cell cycles increases significantly, especially in fast depletion schemes, and reaches $3.19 \pm 1.95$ hr. C) After the diauxie, in an exclusive galactose environment, the length of the cell cycle decreases to $1.65 \pm 0.38$ hr (Nguyen-Huu et al., 2015).

times, at 3.04 hr (Figure 5.6B). Thus, while galactose is always present in the environment, the transition from glucose to galactose metabolism can be dictated by the depletion rate of glucose. Specifically, instantaneous depletion of glucose appeared to have two correlated effects: 1) an increase in the accumulation time, and 2) an increase in the diauxic cell cycle length.

It should be noted that those cells that exhibited the largest cell cycle times ($> 9$ hr in Figure 5.7B1) during diauxie also had large accumulation times. In fact, when these cells were removed from the 0 hr depletion time data, the average accumulation time of the remaining cells is significantly shorter ($1.78 \pm 0.92$ hr), and better fits into the overall linear relationship between accumulation time and depletion time ($R^2=0.97$).

The simple model given in Eq. (5.1) cannot explain why the cell cycle lengths vary with the rate of glucose depletion, nor can it explain the non-monotonicity in the accumulation times or their SD with increasing depletion times (Figure 5.5B). We therefore next wanted to use a simple mathematical model that could explain: 1) the depletion time dependence of the diauxic cell cycle length, 2) the increase in accumulation times for short depletion times, and 3) the non-monotonicity of the SD of the accumulation times as a function of depletion time.
Figure 5.7: Changes in cell cycles’ length in different glucose depletion schemes. Cell cycle lengths in glucose (green bars) and exclusive galactose (red bars) are comparable in different glucose depletion schemes (A1 to A10 and C1 to C10). Distributions of diauxie lengths change as depletion time was increased. Cells exposed to short depletion times (0 to 1 hr) has higher mean of diauxie length and a subpopulation of cells in quiescence state. Cells exposed to long depletion time (6 to 8 hrs) has diauxie length comparable to that of in exclusive galactose.
Table 5.2: Distribution of cell cycle lengths in each phase of glucose depletion

### In glucose

<table>
<thead>
<tr>
<th>Length (hr)</th>
<th>0</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75 - 1</td>
<td>0.02</td>
<td>0.01</td>
<td></td>
<td>0.03</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>1 - 1.25</td>
<td>0.46</td>
<td>0.45</td>
<td>0.36</td>
<td>0.26</td>
<td>0.50</td>
<td>0.62</td>
<td>0.42</td>
<td>0.14</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>1.25 - 1.5</td>
<td>0.42</td>
<td>0.36</td>
<td>0.44</td>
<td>0.40</td>
<td>0.32</td>
<td>0.27</td>
<td>0.56</td>
<td>0.46</td>
<td>0.48</td>
<td>0.32</td>
</tr>
<tr>
<td>1.5 - 1.75</td>
<td>0.08</td>
<td>0.11</td>
<td>0.14</td>
<td>0.20</td>
<td>0.10</td>
<td>0.03</td>
<td>0.00</td>
<td>0.19</td>
<td>0.19</td>
<td>0.26</td>
</tr>
<tr>
<td>1.75 - 2</td>
<td>0.04</td>
<td>0.04</td>
<td>0.01</td>
<td>0.06</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 - 2.25</td>
<td></td>
<td>0.02</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>2.25 - 2.5</td>
<td></td>
<td>0.02</td>
<td>0.03</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>2.5 - 2.75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### During diauxie

<table>
<thead>
<tr>
<th>Length (hr)</th>
<th>0</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 1</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - 2</td>
<td>0.03</td>
<td>0.29</td>
<td>0.24</td>
<td>0.23</td>
<td>0.10</td>
<td>0.23</td>
<td>0.27</td>
<td>0.64</td>
<td>0.42</td>
<td>0.55</td>
</tr>
<tr>
<td>2 - 3</td>
<td>0.29</td>
<td>0.27</td>
<td>0.25</td>
<td>0.45</td>
<td>0.54</td>
<td>0.39</td>
<td>0.64</td>
<td>0.11</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td>3 - 4</td>
<td>0.38</td>
<td>0.29</td>
<td>0.25</td>
<td>0.23</td>
<td>0.19</td>
<td>0.13</td>
<td>0.05</td>
<td>0.14</td>
<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td>4 - 5</td>
<td>0.05</td>
<td>0.07</td>
<td>0.04</td>
<td>0.05</td>
<td>0.05</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>5 - 6</td>
<td>0.01</td>
<td>0.04</td>
<td>0.06</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.13</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>6 - 7</td>
<td>0.04</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>7 - 8</td>
<td>0.03</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>8 - 9</td>
<td>0.01</td>
<td>0.04</td>
<td>0.02</td>
<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>&gt; 9</td>
<td>0.15</td>
<td>0.05</td>
<td>0.10</td>
<td>0.04</td>
<td>0.04</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### In galactose

<table>
<thead>
<tr>
<th>Length (hr)</th>
<th>0</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 1.25</td>
<td>0.03</td>
<td>0.04</td>
<td>0.10</td>
<td>0.06</td>
<td>0.06</td>
<td>0.01</td>
<td>0.10</td>
<td>0.08</td>
<td>0.10</td>
<td>0.27</td>
</tr>
<tr>
<td>1.25 - 1.5</td>
<td>0.37</td>
<td>0.25</td>
<td>0.15</td>
<td>0.43</td>
<td>0.35</td>
<td>0.46</td>
<td>0.32</td>
<td>0.47</td>
<td>0.48</td>
<td>0.45</td>
</tr>
<tr>
<td>1.5 - 1.75</td>
<td>0.27</td>
<td>0.35</td>
<td>0.38</td>
<td>0.29</td>
<td>0.33</td>
<td>0.26</td>
<td>0.39</td>
<td>0.17</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>1.75 - 2</td>
<td>0.15</td>
<td>0.15</td>
<td>0.25</td>
<td>0.11</td>
<td>0.18</td>
<td>0.14</td>
<td>0.15</td>
<td>0.19</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>2 - 2.25</td>
<td>0.08</td>
<td>0.15</td>
<td>0.05</td>
<td>0.03</td>
<td>0.03</td>
<td>0.07</td>
<td>0.02</td>
<td>0.06</td>
<td>0.10</td>
<td>0.18</td>
</tr>
<tr>
<td>2.25 - 2.5</td>
<td>0.05</td>
<td>0.04</td>
<td>0.01</td>
<td>0.06</td>
<td>0.04</td>
<td>0.04</td>
<td>0.02</td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>2.5 - 2.75</td>
<td>0.04</td>
<td>0.03</td>
<td>0.01</td>
<td></td>
<td>0.01</td>
<td>0.03</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.5 An energy model for GAL network induction

This section was added for a complete understanding of the dynamics of the Gal network. Simulation and data were computed by Dr. Chinmaya Gupta.

When glucose depletion occurs, the repression on the GAL network is released; however, achieving full induction levels requires translation of multiple regulatory components of the network. Since translation is known to require up to 50% of the cell’s free energy (van den Brink et al., 2009), incorporating energy availability into our model may help us understand the behavior of the network. Further, a lack of available energy is what causes the diauxic lag (Albers et al., 2007). We therefore hypothesized that the noisy intermediate cell cycle lengths, as well as the initial decrease in the variability of the accumulation times of the GAL network with increasing glucose depletion times, are due to the more gradual changes in energy availability to the cell which occur at longer depletion times.

To test our hypothesis, we derived a minimal model of the GAL network including only two proteins, Gal2p and Gal4p, as well as a unitless time scaling parameter, $E$, that depends on the energy available to the cell. This energy term reflects the basal metabolic rate of the cell under different carbon sources (Figure 5.8). We used the time-delayed stochastic simulation to simulate realizations of the model given by the reactions

$$\emptyset \xrightarrow{E^{34}} g_4$$  \hspace{1cm} (5.3)

and

$$\emptyset \xrightarrow{E^{32}} g_2,$$  \hspace{1cm} (5.4)
where

\[
\beta_4 = \frac{\alpha_4}{1 + (\text{glu}/c_4)^{n_4}} \quad (5.5a)
\]

\[
\beta_2 = \frac{\alpha_2(g_4/c_2)^{n_2}}{1 + (g_4/c_2)^{n_2}} \quad (5.5b)
\]

\[
E = \max \{ -E_{\text{glu}}([\text{glu}(t)]), E_{\text{gal}}(g_2), \epsilon \} \quad (5.5c)
\]

Here, \(\emptyset\) is the empty set, \(g_4\) and \(g_2\) are the concentrations of Gal4p and Gal2p, respectively, and \(\alpha_4\) and \(\alpha_2\) are their respective maximal production rates; Gal4p production is repressed by glucose, with a Hill coefficient \(n_4\) and a half-maximal concentration \(c_4\); Gal2p production is activated by Gal4p, with a Hill coefficient \(n_2\) and half-maximal concentration \(c_2\); \(E_{\text{glu}}([\text{glu}(t)])\) is the energy time scaling due to glucose metabolism, and depends on the time-dependent concentration of glucose, \([\text{glu}(t)]\); \(E_{\text{gal}}(g_2)\) is the energy time scaling due to galactose metabolism, which is a function of the concentration of Gal2. Both \(E_{\text{glu}}([\text{glu}(t)])\) and \(E_{\text{gal}}(g_2)\) are piecewise-linear functions of their arguments, increasing from zero until reaching a maximum (representing a maximum metabolic flux rate which depends on the carbon source, see Figure 5.8). Note that Gal2p is the transporter for galactose, and hence intracellular galactose concentrations will depend on \(g_2\). In this manner, Gal2p provides positive feedback for the galactose network. In addition, when energy from both glucose and galactose is low, we assume that the time scaling parameter is at a minimum, \(E = \epsilon\). This represents a basal metabolic activity of the cells after glucose has been depleted but before there is enough Gal2p to effectively transport galactose into the cells.

The time varying rate, \(\gamma_t\), at which cells grow was computed as \(E\gamma\) where \(\gamma\) is a basal growth rate. Since the energy term \(E\) scales the cell growth rate, the maximum levels \(E_{\text{glu}}^{\text{max}}\) and \(E_{\text{gal}}^{\text{max}}\) were fit to the experimentally measured cell cycle lengths in glucose and galactose, respectively. In addition, since \(\epsilon\) represents the growth rate in the diauxic phase (which was highly variable), we sampled \(\epsilon\) from a Beta distribution on the interval \((0, \epsilon_{\text{max}})\).
Figure 5.8: An energy model for the glucose/galactose switch. A) A schematic of the regulation in the heuristic stochastic model. Glucose inhibits expression of the GAL genes, but increases the cellular energy needed for protein production. Gal4p up-regulates transcription of gal2, whose gene product, Gal2p, imports galactose. The ability of the cell to metabolize galactose is therefore dependent on the availability of Gal2p. In addition, intercellular galactose increases cellular energy and up-regulates gal2. B) We used piecewise linear functions for $E_{\text{glu}}([\text{glu}])$ and $E_{\text{gal}}([\text{Gal2p}])$. The $E_{\text{glu}}$ term is assumed to only depend on the environmental glucose concentration. The $E_{\text{gal}}$ term does not depend on environmental galactose because it is held constant; what changes is the amount of galactose that is utilized by the cell, a good proxy for which is the concentration of Gal2p. The maximum (steady-state) energy levels $E_{\text{glu}}^{\max}$ and $E_{\text{gal}}^{\max}$ are inferred from the cell-cycle lengths in the two conditions. The threshold $th_{\text{glu}}$ is the glucose threshold at which the cell is assumed to obtain maximal energy. The equivalent threshold for galactose is $th_{\text{gal}}$; this is the threshold at which the cell has sufficient Gal2p to maximally utilize environmental galactose (Nguyen-Huu et al., 2015).

with two shape parameters and the range parameter $\epsilon_{\text{max}}$. We obtained the range and shape parameters for this distribution by fitting the model to the cell cycle lengths observed experimentally.

The constant $c_4$ in Eq. (5.5a) is the glucose concentration at which the GAL network is half-maximally repressed. While functionally different from the $g^*$ term in Eq. (5.1), any variability in the threshold at which glucose repression on the GAL network is released will affect both $c_4$ and $g^*$ in the same way. In order to make the stochastic model consistent with the simple model from Eq. (5.1), we sample $c_4$ from a normal distribution. The contribution of the variability in $c_4$ is analogous to that of $g^*$ in Eq. (5.1); variability in the accumulation times increases with increasing depletion times.

Our mathematical modeling suggests the non-monotonicity in the variance of accumulation times is a consequence of the variability in the energy availability to cells when they transition between carbon sources. To test whether this was indeed the driving force behind
Figure 5.9: Results of the mathematical model. A) Simulated standard deviation of the accumulation time as a function of the depletion time for the full model (red dots) and a model lacking the energy scaling (blue dashed line). Note that the energy scaling recreates the non-monotonicity observed in the experiments. B) Box plots of the distributions of the different types of cell cycle lengths for the 3 hr depletion scheme. The red box plots are the experimental data, whereas the blue box plots are obtained from a simulation of Eq. (5.5). The distribution of the energy availability in the diauxic phase ($\epsilon$) is obtained by fitting the model to the diauxic cell cycle data (Nguyen-Huu et al., 2015).

The non-monotonicity in the model, we also fit a model in which the scaling term $E$ is set to a constant value independent of the carbon source being utilized. Our model shows that in the absence of a time-varying energy term $E$, the standard deviation of the accumulation time monotonically increases with the depletion time (blue dashed line, Figure 5.9A). When the scaling term $E$ is allowed to vary depending on the glucose concentration and Gal2p accumulation, the non-monotonic behavior observed experimentally is recreated (red dots, Figure 5.9A).
Chapter 6

Conclusions

6.1 The activation of the Gal network is dependent on the depletion of glucose

Our data in Section 5.2 demonstrated that Gal1p-YFP signals appear at 17 min after glucose concentration is reduced to 1.4%. The threshold concentration of glucose, 1.4%, is in agreement with a galactose:glucose ratio of 2%:1% which was demonstrated to trigger the activation of GAL1pr-YFP (Escalante-Chong et al., 2015). Our work also demonstrates that this onset of the activation of the Gal network is independent of the glucose-depletion rate (Escalante-Chong et al., 2015). Thus, our data contribute to the growing body of evidence that the Gal network can be activated in the presence of glucose (Escalante-Chong et al., 2015; Venturelli et al., 2015; Wang et al., 2015). Further, this activation can happen very early, up to 4-5 hr, before glucose is totally depleted, as in the case of 6 and 8 hr-depletion time (Figure 5.3A). While the mean of the Gal1p initiation time is independent of the glucose-depletion time, the variability of the initiation time is linearly dependent on the depletion time. This dependence is demonstrated by the increase of the SD of the initiation time as the depletion time increases (Figure 5.3B). We demonstrated that the increase in the variability is due to the time it takes for the glucose concentration to pass through the distribution of threshold concentrations. Since there is variability in the threshold concentration,
the longer the glucose concentration spends near this threshold, the higher the variability among cells to initiate the Gal network.

The accumulation of the Gal network is significantly dependent on the depletion rate of glucose (Figure 5.5). We demonstrated that when glucose was depleted instantaneously, the Gal1p accumulation time was significantly increased, larger than the Gal1p accumulation time of several intermediate glucose depletion times (0.5 hr to 1.5 hr). Significantly, the SD of these accumulation times does not follow a linear relationship with the glucose depletion times. Rather, they are significantly higher at near instantaneous depletion times and reach a minimum at intermediate glucose depletion times (3 - 4 hr). A direct consequence of the increase in the variability of the accumulation time is that individual cells of the homogeneous population achieve different phenotypes ranging from early expression to delayed expression of the Gal network. While other researches (Venturelli et al., 2015; Wang et al., 2015) have demonstrated an effect of stochasticity in gene expression to explain the variability in a population expressing the Gal network, our work points to another reason for this variability. We showed that, by tracking the cell cycle times of these individual cells, loss of energy generated by glucose metabolism significantly contributes to the delay. Two cellular mechanisms contribute to the loss of energy (Figure 6.1A&B). First, a yeast cell does not have a mechanism to track glucose influx in real-time. As mentioned in the Introduction, the two receptors Snf3 and Rgt2 trigger the expression of Hxt genes for importing glucose into the cell (Ozcan et al., 1998). By separating the expression of Snf3 and Rgt2 with the expression of Hxt transporters, Youk et al. showed that a cell can have a decreased growth rate even when the extracellular concentration of glucose and its uptake rate are increased (Figure 6.1A). Since a cell does not have a mechanism to track the glucose influx rate, it must measure the extracellular concentration of glucose, anticipate the glucose import rate and then adjust its expression level accordingly (Youk and van Oudenaarden, 2009). As a result, a cell is already locked into its expression state based on exponential growth when glucose is depleted instantaneously. This depletion triggers a massive loss of energy and a
cell cannot turn over its protein fast enough for the galactose metabolism. In addition, a cell cannot consume galactose in the presence of glucose even when the Gal network is already activated (Figure 6.1B). As a result, it cannot trigger the full induction of the Gal network to exit the catabolic transition of the diauxic phase (Venturelli et al., 2015). These two effects create significant delay and high variability in the cell population. Only at intermediate glucose depletion rates can cells within the population make the catabolic transition in a uniform manner.

Variability in the catabolic transition during diauxie has been demonstrated not only in the Gal network, but also in other networks and species like E. coli (Boulineau et al., 2013). Our work contributes to a growing body of evidence that the activity of a gene network is much more dynamic than once thought and can dictate the phenotypic outcomes of single cells within a homogeneous population. Significantly, our work is one of the first to highlight detailed steps of the transition between different gene networks and how each network, while maintaining an antagonistic effect on each other, ultimately contributes to the transition and fitness of a cell. Furthermore, we elucidated another aspect of adaptation, where the rate of change in environmental factors can contribute to the variability in the phenotypic outcomes of single cells and trigger early steps of evolution and natural selection.

6.2 Future directions

As mentioned in the Introduction, our work was motivated by questions of cellular adaptation in natural conditions where multiple types of nutrients coexist, and by a desire for improvements in fermentation. The next step is to understand the effect of glucose depletion on diploid yeast. A diploid yeast strain, K701-1ym, was created (Section 3.1.3) and ready for experiments. In our preliminary data, the effect of instantaneous depletion of glucose on the Gal1p-accumulation time was significantly reduced in the diploid strain. One reason is that the presence of two sets of genes reduces the probability of disrupted gene expression and allows a quicker turnover of proteins to accelerate the catabolic transition (Cook et al.,
Figure 6.1: The loss of energy contributes to the delay of the Gal network’s accumulation. A, Youk *et al.* demonstrated that yeast cells cannot measure glucose influx rates in real-time. By deleting all genes encoding hexose transporters (Hxt1 - Hxt17 and Gal2p) and glucose sensors (Snf3p and Rgt2p) and expressing only one of these genes, the growth rates of these mutants can be manipulated and decreased even when glucose concentrations were increased (solid lines in comparison to dashed lines of the wild type). This figure is reproduced with permission from (Youk and van Oudenaarden, 2009). B, yeast cells cannot consume galactose in the presence of glucose, even when the Gal network has been expressed. Venturelli *et al.* showed that the galactose concentration (blue line) decreased only when the glucose concentration (green line) reached zero, even though the Gal network turned on 4 hr before (red line). This figure is reproduced with permission from (Venturelli *et al.*, 2015).

Another question is the effects of environmental changes on subsequent generations of yeast cells. Yeast cells have been shown to stochastically switch between two expression states of the Gal network, and these cells can maintain one specific state for many generations (Kaufmann *et al.*, 2007). Our preliminary data showed that cells from multiple generations in the same family respond similarly to glucose depletion (Figure 6.2). These preliminary data raise interesting questions about antagonistic effects between a family background and an environmental change. An environmental change is a force that creates variabilities within a cell population and can act as a natural selection process to enhance cellular adaptability and evolution. In contrast, a family background, created by previous exposure of environmental cues, can create a behavioral framework from which a cell can rely to respond to uncertainty.
Figure 6.2: Yeast cells in a family share a similar behavior in the face of environmental changes. Trajectories of fluorescence intensities of five families are shown. Blue lines depict mother cells which presented in the beginning of the glucose-depletion assays. Gray lines depict daughter and granddaughter cells. These cells were collected in multiple glucose-depletion assays in which glucose was instantaneously depleted (glucose-depletion time = 0 hr).

It would be very interesting to investigate the dynamics between these two aspects of cellular adaptation. In this regard, evolution is not only a process created by random mutations but also created by stochastic fluctuations of gene expression in response to the dynamics of environmental changes.
Bibliography


