# Cell Cycle Commitment in *Saccharomyces cerevisiae* is a Multi-Step Process and *Start* is Reversible

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# Abstract

Cells need to coordinate their metabolism with the cell division cycle to survive under changing nutrient conditions. In eukaryotic cells, nutrient signalling is integrated into the cell cycle during the cell cycle commitment at the end of G1. This point of irreversible commitment is determined by a positive feedback loop of CDK activation and is called Restriction Point in mammals and *Start* in yeast. Under unfavourable nutrient conditions, yeast cells arrest at *Start* until the conditions improve. However, once the yeast cells past *Start* the effect of nutrient signalling on the cell cycle machinery, is poorly understood. A comprehensive picture of how the metabolism interacts with the cell cycle and through which regulators the nutrient signals are received is still lacking. Thus, in this project, we aimed to understand how the yeast cell cycle responds to nutrient signalling, by imposing acute nutrient deprivation.

Using live cell imaging and single cell analysis, I tracked the nuclear localization of the *Start* inhibitor Whi5, whose phosphorylation by CDK leads to its export and determines *Start*. I detected that post-*Start* cells respond to nutrient deprivation by delaying their cell cycle, or by going into a stable arrest until glucose is replenished. Cells that were more progressed mostly only delayed their cell cycle, whereas most of the cells that were exposed to starvation within the first 20 minutes permanently arrested. When I further characterized these early permanent arrests, we found that many of the cells can re-import Whi5 when faced with acute starvation. I demonstrate that, for the cells that were within the first 25 minutes after passing *Start*, re-importing Whi5 corresponds to an interruption of the CDK positive feedback loop. I show that, this group of cells become sensitive to the mating pheromones just like regular pre-*Start* cells. Thus, upon nutrient starvation, cells can functionally reverse *Start*. We next sought to identify the mechanism responsible for reversing *Start*. We tested several regulatory mechanisms including Msn2,4, Msa1,2, Xbp1, Sic1, Cip1, Snf1, Rim15 and the non-CDK phosphorylation of Whi5. While we could not unveil the complete mechanism, we found that neither cyclin repression, nor Whi5 phosphorylation is responsible for interrupting the feedback loop. We therefore suggest that the CDK-cyclin complex itself is target of nutrient signalling.

With these findings, we show that the textbook model of the one-step irreversible cell

cycle commitment point in budding yeast does not hold true under nutrient deprivation. In mammals, the idea of a single restriction point has been previously challenged. Since cell cycle regulation is well conserved among eukaryotes, our findings can help understand cell cycle commitment as a multi-step process beyond yeast. Even though the proteins that the networks comprise of may be structurally different, the mechanism of the cell cycle commitment is very similar. As a result, our findings will lead to a better understanding of cell cycle control related disease states such as cancer.

# Zusammenfassung

Zellen müssen ihren Stoffwechsel mit dem Zellteilungszyklus koordinieren, um unter sich ändernden Nährstoffbedingungen zu überleben. Im Zellzyklus eukaryotischer Zellen werden Nährstoff-Signale nur bis zum Ende der G1-Phase verrechnet. Der Punkt der irreversiblen Entscheidung wird durch eine Positive-Feedback-Schleife bestimmt, die CDK aktiviert. Bei Säugetieren heißt dieser Punkt im Zellzyklus Restriktionspunkt, in Hefe, *Start*. Unter ungünstigen Nährstoffbedingungen arretieren Hefezellen bei *Start* bis sich die Bedingungen verbessern. Kaum bekannt ist jedoch, wie Nährstoff-Signale auf den Zellzyklus von Hefezellen wirken, wenn diese *Start* überschritten haben. Bisher fehlt ein umfassendes Bild darüber wie Stoffwechsel und Zellzyklus interagieren und über welche Regulatoren die Nährstoff-Signale empfangen werden. Mit diesem Projekt beabsichtigten ich aufzuklären, wie der Zellzyklus der Hefezellen reagiert, wenn ihnen ein akuter Nährstoffmangel auferlegt wird.

Mit Live-Cell-Imaging und Einzelzellanalyse habe ich die Nucleus-Lokalisation des *Start* Inhibitors Whi5 verfolgt. Whi5 kann von CDK phosphoryliert werden, was zum Export aus dem Nucleus führt und *Start* bestimmt. Ich habe festgestellt, dass Zellen auch nach *Start* auf Nährstoffmangel reagieren, indem sie ihren Zellzyklus verzögern oder in einen stabilen Arrest eintreten, bis wieder Glukose hinzugegeben wird. Zellen, deren Zellzyklus weiter fortgeschritten war, haben ihren Zellzyklus meist nur verzögert. Wenn Zellen aber innerhalb von 20 min nach *Start* Nährstoffmangel ausgesetzt wurden, arretierten sie meist permanent. Als ich diese frühen, permanenten Arretierungen weiter charakterisierte, stellte ich fest, dass bei Nährstoff-Mangel Whi5 oft wieder in den Nucleus importiert wird. Ich zeige, dass bei Zellen, die *Start* um bis zu 25 min überschritten haben, der erneute Import von Whi5 mit einer Unterbrechung der Positive-Feedback-Schleife auf CDK einhergeht. Diese Gruppe von Zellen wird wieder für Pheromone empfänglich - genau wie gewöhnliche Prä-*Start* -Zellen. Somit können Zellen bei Nährstoffmangel *Start* funktionell umkehren. Als nächstes betrachtete ich die Regulatoren Msn2,4, Msa1,2. Xbp1, Sic1, Cip1, Snf1, Rim15 und die nicht CDK-abhängige Whi5-Phosphorylierung. Obwohl der vollständige Mechanismus nicht enthüllt werden konnte, fand ich heraus, dass weder Cyclin-Repression noch Whi5-Phosphorylierung für die Unterbrechung der Positive-Feedback-Schleife verant-

wortlich sind. Daher schlage ich vor, dass der CDK-Cyclin-Komplex selbst Ziel der Nährstoff-Signale ist.

Diesen Ergebnissen zeigen, dass die Vorstellung des einstufigen, irreversiblen Zellzyklus-Entscheidungspunkts in knospenden Hefen unter Nährstoffmangel nicht zutrifft. Auch wenn in Säugetieren die Proteine, aus denen die Netzwerke bestehen, strukturell unterschiedlich sein können, ist der Entscheidungsmechanismus über den Zellzykluseintritt sehr ähnlich. Somit liefert unsere Forschung wesentliche Hinweise auch auf höhere eukaryotische Organismen und kann so zu einem besseren Verständnis von Krankheiten führen, die wie z.B. Krebs mit der Zellzykluskontrolle zusammenhängen.

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"The data and figures described in this thesis will be part of a manuscript which is currently in preparation titled:
When Yeast Cells Change their Mind: Cell Cycle "*Start*" is Reversible under Starvation
Deniz Irvali, Fabian P. Schlottmann, Prathibha Muralidhara, Ilya Nadelson, N. Ezgi Wood, Andreas Doncic, and Jennifer C. Ewald

All of the work described in the following thesis was completed by myself unless specifically indicated otherwise."

# List of Abbreviations

| | |
|---|---|
| **Amp** | Ampicilin |
| **bZip** | Basic Leucine Zipper Domain |
| **CDK** | Cyclin Dependent Kinase |
| **CKI** | Cyclin-dependent Kinase Inhibitor |
| **Clb** | B type cyclin |
| **Cln** | G1 cyclin |
| **DNA** | Deoxyribonucleic Acid |
| **dPSTR** | Dynamic Protein Synthesis Translocation Reporter |
| **E. Coli** | Escherichia coli |
| **GFP** | Green Fluorescent Protein |
| **HPH** | Hygromycin |
| **KAN** | Kanamycin |
| **KHP** | Potassium Hydrogen Phthalate |
| **KOH** | Potassium Hydroxide |
| **LiAc** | Lithium Acetate |
| **LB** | Luria Bertani |
| **MAPK** | Mitogen-Activated Protein Kinase |
| **MAT** | Mating-type |
| **MBF** | Mlu1 Cell Cycle Box [MCB] Binding Factor |
| **MgCl2** | Magnesium Chloride |
| **NaCl** | Sodium chloride |
| **NLS** | Nuclear Localization Signal |
| **NPC** | Nuclear Pore Complex |
| **OD** | Optical Density |
| **PCR** | Polymerase Chain Reaction |
| **PEG** | Polyethylene Glycol |
| **PKA** | Protein Kinase A |
| **RB** | Retinoblastoma Protein |
| **RP** | Restriction Point |
| **RNA** | Ribonucleic Acid |

| | |
|---|---|
| **SC** | Synthetic Complete |
| **S.cerevisiae** | Saccharomyces cerevisiae |
| **TBE** | Tris/Borate/EDTA |
| **TOR** | Target of Rapamycin |
| **TFP** | Teal Fluorescent Protein |
| **YPD** | Yeast Extract Peptone Dextrose |

# 1 Introduction

The cell cycle is the series of tightly regulated, consecutive events that leads to cell division. In one round of the cell cycle, the cell grows in size, duplicates its genetic material and passes it on to its daughter cells. For complex multicellular eukaryotic organisms, cell division provides multiple purposes such as growth, regeneration, and differentiation, whereas for the single cell organisms, the main purpose is multiplication [Morgan, 2007].

To be able to maintain cellular homoeostasis, cells need to coordinate their cell cycle with metabolism. In the eukaryotic cell, a proper cell cycle regulation is ensured by a complex network where cellular and environmental signals are monitored, evaluated, and integrated into the cell cycle machinery. Many aspects of this control network is conserved among eukaryotes. Through signalling networks, the cells can sense the cues to give proper responses to the changing conditions via a fine-tuned control mechanism [Broach, 2012].

For single cell organisms like yeast, nutrient signals do not only act as energy source, but also function as metabolic signals. Thus, for a well-regulated cell cycle progression, the yeast cells must integrate the nutrient signals into its cell cycle machinery. This integration should be through a robust mechanism where nutrient signals are sensed to give a fine-tuned response to avoid cell cycle irregularities [Broach, 2012], [Ewald, 2018].

Yeast cells monitor and integrate nutrient signals at the cell cycle commitment point called *Start*. At this point, the cells decide to stop the cell cycle progression under poor nutrient conditions [Morgan, 2007]. However, how this coordination is achieved after the cell passes this checkpoint is poorly understood. Also, through which mechanism the cell cycle responds to nutrient signals is still not broadly understood. In the following chapter, the reasons why we decided to use yeast as the model organism to study nutrient signalling will be briefly explained, and the current knowledge of the cell cycle regulation and nutrient integration will be summarized.

## 1.1 Yeast as a Model Organism for the Eukaryotic Cell Cycle

Yeast is one of the simplest eukaryotic organisms. Due to its simplicity, among all eukaryotic cell cycles, that of the budding yeast " *Saccharomyces cerevisiae* (S. cerevisiae)" has been one of the most extensively studied. The yeast genome is the first eukaryotic genome that was completely sequenced. As a result the gene functions are easily accessible in databases such as Comprehensive Genome Database and the Saccharomyces Genome Database. From its genome, 954 yeast genes belongs to orthologous gene families which are found to be related to a human disease [Heinicke et al., 2007]. Thus, yeast provides a good opportunity to study disease states in more complex eukaryotic organisms as it has a simpler cell cycle organisational system. Additionally, yeast grows and duplicates faster compared to the cells of higher eukaryotic organisms. Multiple cell cycles can be observed in a very short amount of time (Figure 1) [Botstein and Fink, 2011], [Nielsen, 2019].

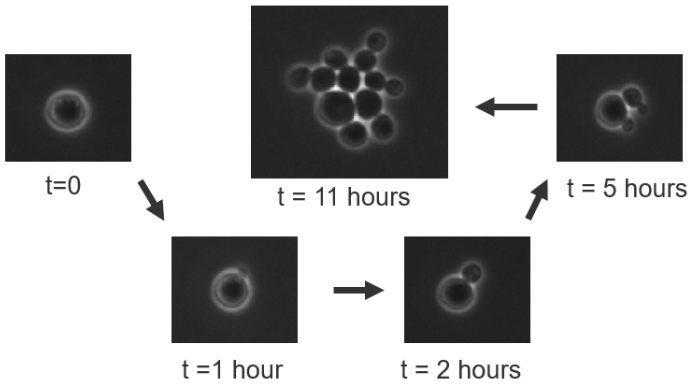


**Figure 1:** Example scheme showing yeast growth

Yeast cells duplicate fast compared to the higher eukaryotic cells. In short amount of time, a colony can be formed from a single yeast cell.

The mechanisms that regulate the cell cycle are conserved in all eukaryotic organisms. Even though the regulatory proteins involved in the cell cycle network are not all homologous and differ widely, the network structure itself is conserved from yeast to more complex eukaryotes. The organisational structure of cell cycle progression is conserved among eukaryotes. Also, the step by step control of the cell cycle is maintained by resembling mechanisms [Cross et al., 2011]. Additionally, the mechanism and struc-

ture of the signal transduction mechanisms are conserved among eukaryotic organisms. In both mammalian and yeast cells, the control mechanism of G1/S is conserved in some aspects and shares a lot of properties such as functionally similar feedback loops thought to be leading to the cell cycle commitment. Also, in both organisms, the proper progression into the cycle is assured by control points called checkpoints which function similarly. For example, the cell cycle commitment point in yeast called *Start* is analogous to the Restriction Point (RP) in mammals [Johnson and Skotheim, 2013]. Additionally, the pioneer cyclin dependent kinase of the budding yeast, Cdk1, is homologous to the human Cdc2. To sum up, due to numerous advantages, S. cerevisiae has been used extensively as a model organism for a lot of different fields of cell cycle and cell cycle control. Due to these similarities, the information gained from yeast has been very applicable for human cells and more complex organisms [Cross et al., 2011], [Bertoli et al., 2013]. In 2001, Leland H. Hartwell, R. Timothy Hunt, and Paul M. Nurse were awarded a Nobel Price in Medicine for their work on "key regulators of the cell cycle" in which they provided important contributions to the field of cell cycle control. Their work led to the characterization of the major Cdk of budding yeast, Cdk1, and the discovery of cyclins [Angadi et al., 2004].

## 1.2 Cell Cycle Control in Yeast

The eukaryotic cell cycle consists of four consecutive phases: G1, S, G2 and M phases where G1 and G2 are considered as gap phases in which the cell prepares itself for the next stage (Figure 2). The eukaryotic cell cycle stages are defined on the basis of consecutive chromosomal and molecular events with the final goal of duplication. Most eukaryotic cells go through those cell cycle phases and crucial cell cycle events. However, the details of the organisational structure might vary among different eukaryotic organisms [Morgan, 2007].

The budding yeast cell cycle lacks a distinct G2 phase but has a long G1 phase compared to most other eukaryotes [Hartwell and Weinert, 1989]. A new round of S. cerevisiae´s cell cycle starts with the gap phase G1. While in G1, the cell grows in size and prepares itself for the further cell cycle events. During late G1, the cell goes through the cell cycle commitment point called *Start* where cellular and environmen-

tal conditions are evaluated. During G1, the cell additionally decides whether it wants to undergo another cell division cycle or enter a non-dividing, quiescent (G0) state, if the environmental conditions are not favourable, from which it goes back into its regular progression when the conditions are improved [Lillie and Pringle, 1980]. Also, unfavourable growth conditions can cause the cell to extend its G1 phase. If the conditions are favourable, budding initiation starts and the cell transitions towards S phase [Morgan, 2007].

During S phase, the cell replicates its DNA. Histones are synthesized to condensate DNA into chromatins. Replication starts at the replication origins, which are present in large numbers across the genome. Certain proteins specifically bind the DNA sites and open the DNA double helix, thereby exposing it to enzymes which copy both DNA strands [Morgan, 2007].

S phase is followed by the second gap phase, G2. The cell goes through the G2/M DNA damage checkpoint. If the DNA replication is successfully completed, the cell then proceeds with the M phase. The preparations for mitosis, however can start before the end of DNA replication process. During M phase, nuclear division (mitosis) and the cell division (cytokinesis) take place. In early mitosis (prophase), the chromatids are attached to the mitotic spindle by microtubules until the sister chromatids in each chromosome pair are connected to the opposite pole of the spindle (metaphase). Then, the sister chromatids are separated (anaphase) and the microtubules pull the two sets of chromosomes to opposite ends of the cell, where the daughter nuclei are formed. Yeast cells go through asymmetric division. The point where the cell will divide is determined at the end of G1 when the budding is initiated. During cytokinesis, the cell divides from that bud neck and the daughter cell is formed [Morgan, 2007].

Yeast cells govern a robust cell cycle control system which ensures the events of the cell cycle are properly regulated with precise timing. The transitions through cell cycle phases are controlled by periodic transcription and degradation of proteins. The master regulatory switch for the cell cycle progression is the sequential activation and inactivation of the cyclin-dependent kinases [Hartwell et al., 1974]. For the cell to progress through a new event, the previous steps must be completed successfully. Otherwise, the cell becomes more sensitive to external disruptions which may eventually

result in defective DNA replication, mutations and even in cell death. The proper cell cycle progression is achieved by this system which makes sure that the events are coordinated and not prematurely performed before the timing is correct [Morgan, 2007]. The yeast genome includes more than 800 cell cycle related genes which is more than 10 % of the yeast genome [Spellman et al., 1998], showing how important the cell cycle regulation is on transcriptional level.

The cell cycle control system consists of positive regulatory proteins called cyclin dependent kinases, cyclins and also negative regulators, which function as inhibitory proteins, that are expressed or transferred into where needed only during certain times of the cell cycle. Budding yeast is regulated trough six cyclin dependent kinases: Kin28, Ssn3, Pho85, Ctk1, Bur1, and Cdk1. However, only one major CDK is sufficient to drive the cell cycle, Cdk1, which is involved in all the cell cycle events and pushes the cell cycle progression further as a positive regulator, where the mammalian cell cycle requires multiple CDKs [Satyanarayana and Kaldis, 2009], [Enserink and Kolodner, 2010].

During different stages of the cell cycle various transcriptional programs are activated. During G1, genes need to push the cell forward to DNA replication. When the cell reaches S phase, histone genes are expressed. At G1, transcriptional factors and genes needed for replication are transcribed with the final aim of duplication [Koch and Nasmyth, 1994].

A crucial part of the cell cycle regulatory mechanism is the control points called checkpoints. Checkpoints are the control points which are specific to the cell cycle stage the cell is in and all of them contributes to the cell cycle machinery in a different way, but with the same end purpose. This purpose is to make the cell evaluate specific inner and outer cues to decide if it will continue through the cell cycle progression or stop until the conditions are favourable again. Thus, checkpoints act as switch-like brake systems for the cell. Additionally, by halting the cell cycle when necessary, they give the cell time to repair the damage. When the conditions are favourable, and a specific checkpoint is passed successfully, the cell cycle event becomes irreversible and the cell proceeds to the following stage of its cell cycle progression [Hartwell and Weinert, 1989], [Murray, 1992], [Elledge, 1996].

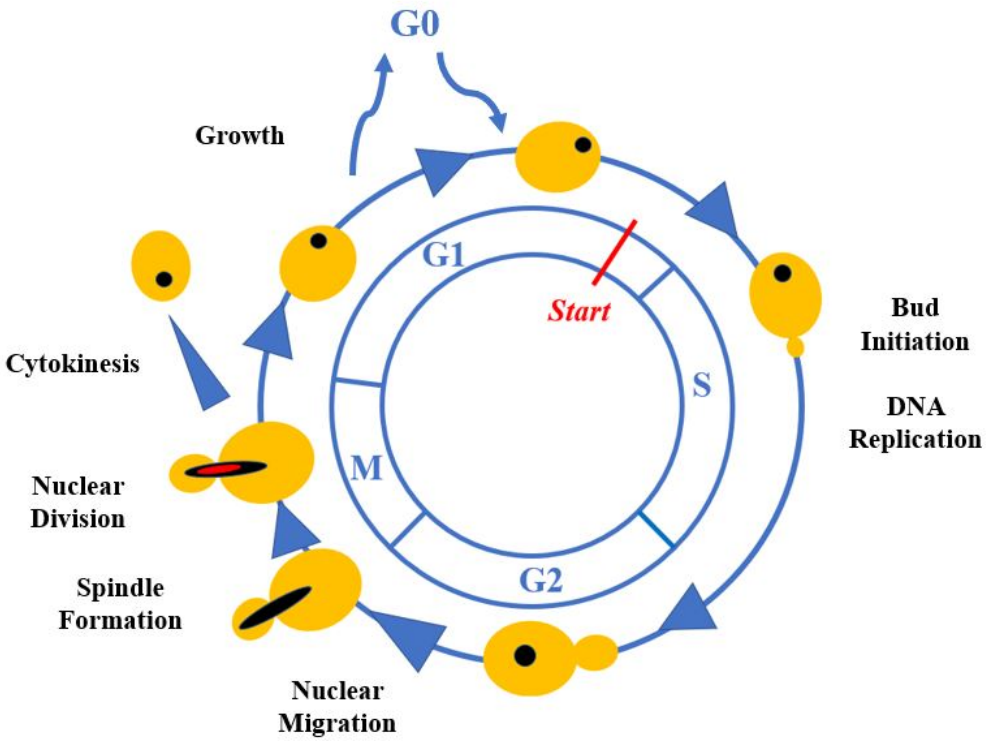


**Figure 2:** The budding yeast cell cycle

The schematic representation of budding yeast cell cycle phases. During G1, the cell grows in size and passes the cell cycle commitment point called *Start*. Bud initiation and DNA replication occur during S phase. Following S phase, the cell divides asymmetrically and a daughter cell is formed.

A lot of common checkpoints in eukaryotes were firstly identified in yeast. All the checkpoints serve the regulation of another cell cycle event such as the regulation of cell size, DNA replication, mating, G1/S transition, inhibition of entering premature mitosis, and cell cycle dependent DNA synthesis. Among the discovered checkpoints, the most characterized ones are the DNA damage checkpoint [Lydall and Weinert, 1995], spindle assembly checkpoint [Straight and Murray, 1997], and the cell cycle commitment point called *Start*, which are the three major checkpoints in yeast. The first one is the cell cycle commitment point called *Start* which regulates the transition into S phase. The second one is the G2/M DNA damage checkpoint which controls the entry into the mitotic phase and the last one is the metaphase to anaphase spindle assembly checkpoint where the last stages of the cell cycle are initiated [Elledge, 1996], [Morgan, 2007].

The dysregulation of checkpoints results in inevitable cell cycle defects. For example,

a defective spindle checkpoint leads to aneuploidy and chromosome missegregation which results in genetic defects [Sheltzer et al., 2011]. A malfunctioning DNA replication checkpoint leads to genomic instability [Longhese et al., 1998].

## 1.3 Regulation of the G1/S Transition

G1, also described as the gap phase, functions as a transition period before cell cycle events, such as DNA replication, are initiated. When in G1, cells prepare themselves and wait until the conditions and requirements of entering S phase are met. During G1 phase, the cells grow in volume, size and mass. Until a certain size threshold is reached, the cell remains in G1. Once the cell reaches S phase, the cell initiates budding and the DNA replication process is triggered [Morgan, 2007].

While the cell is progressing into S phase, a specific group of genes responsible for cell growth, DNA replication, and budding is expressed. In addition to this set of genes, many others responsible for cell cycle control are transcribed to ensure a proper transition [Bertoli et al., 2013]. The transcription of more than 200 genes is activated and these changes in transcription lead to the expression of many regulators and proteins responsible for the initiation of S phase and DNA replication [Spellman et al., 1998], [Iyer et al., 2001].

As S phase is accompanied with one of the most crucial cell cycle events, DNA replication, the transition into S phase is also tightly regulated. For the cell to go into S phase and initiate DNA replication, it needs to make sure that the inner and surrounding conditions are favourable. This control mechanism is achieved by the checkpoint called *Start* [Morgan, 2007]. *Start* is the irreversible cell cycle commitment point where the cell monitors various cues to decide if the cell will progress through the rest of the cell cycle. If the conditions are not favourable to start the replication process, at *Start*, the cell cycle is halted. The cell waits in arrest until the conditions are improved [Hartwell et al., 1974].

## 1.4 The Molecular Basis of *Start* and its Regulation

The initiation of *Start* depends on the G1 cyclins, namely Cln1, Cln2, and Cln3, which activated the main cyclin dependent kinase of yeast: Cdk1 [Morgan, 2007]. In terms of

structure and functionality, *Start* resembles to the mammalian Restriction point, despite some differences in how the signals are processed [Johnson and Skotheim, 2013]. *Start* as a checkpoint was primarily described as the point where the cells are no longer sensitive to mating pheromones and commit to going through another round of division [Morgan, 2007], [Doncic et al., 2011]. The mating is mainly regulated by the mitogen activated-protein kinase (MAPK) pathway. Fus3, which is downstream of MAPK, leads to the activation of Ste12 which then leads to the expression of Far1 during pre-*Start* stages. The G1 CDK-cyclin complexes are inhibited by the CDK inhibitor Far1 which faces a four to five fold increase in its expression when facing mating pheromones. The inhibition of the cyclin-CDK complexes leads to pre-*Start* G1 arrest, opening a path towards mating. The mating of yeast cells has been studied in detail for the last 40 years [Merlini et al., 2013]. Forming diploid cells is achieved through the existence of two different mating types: MAT a and MAT $\alpha$. The mating is triggered by the interactions between these types by the mating pheromones called $\alpha$ factor and a factor. To attract the opposite type of yeast cells, MAT $\alpha$ cells produce the pheromone called $\alpha$ factor and MAT a cells produce a factor. The peptide pheromones are then recognized by G-protein coupled receptors present on the the surface of the cell membrane called Ste2 and Ste3. After recepting the pheromones, the opposite mating type responds by creating nodule-like projections called shmoos [Strazdis and MacKay, 1983]. Even though how these pheromones are sensed differs between the mating types, the pheromone response pathways are the same [Chang and Herskowitz, 1990], [Cross and Mckinney, 1992], [Peter et al., 1993], [Holt et al., 2009]. To find a mate and transition into diploid state, the cells need to make sure to be at the same cell cycle progression stage. Thus, when exposed to the mating pheromones, the haploid cells synchronize their cell cycle at *Start*. As a result, the cells that already passed *Start* chose to go through another round of division and arrest at the *Start* point of the next cell cycle round [Hartwell et al., 1974], [Reid and Hartwell, 1977].

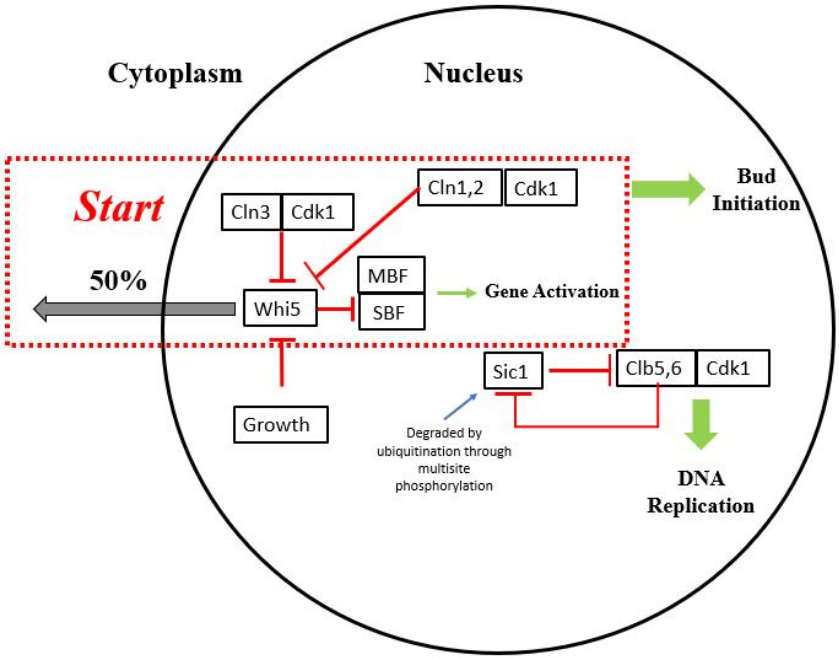


**Figure 3:** G1/S transition in budding yeast

The cell cycle inhibitor Whi5 gets deactivated by growth and the activated Cln3-Cdk1 complex towards the end of G1. This deactivation results in the relief of SBF which in return triggers the expression of Cln1,2. The newly expressed Cln1,2 forms activated complexes with Cdk1 which hyperphosphorylates Whi5 which leads to its nuclear export. At the point where 50 % of Whi5 is exported, the positive feedback loop becomes self-sustaining and defined as *Start*. After passing *Start*, the cell then progresses towards S phase.

The regulation of *Start* is achieved through a complex CDK positive feedback loop. *Start* is triggered in a switch-like manner rather than the gradual accumulation of cell cycle events. This switch-like mechanism leads to the activation of *Start* in an all-or-none way [Skotheim et al., 2008]. *Start* has then been accepted as the point of irreversibility through its non-linearity which mostly depends on the inhibitory regulator Whi5 [Charvin et al., 2010].

*Start* is inhibited by Whi5 which shuttles between the nucleus and the cytoplasm. Whi5 is the functional homolog of the retinoblastoma protein (RB) which leads to the Restriction Point in mammalians. During cell cycle progression, Whi5 is transported into the nucleus from the cytoplasm at the end of mitosis. When it is imported into the nucleus, it binds to the transcriptional factor SBF to inhibit the transcription of the early G1 cyclins Cln1 and Cln2, stopping the cell from going into premature S phase transition [De Bruin et al., 2004]. The inhibition of Whi5 is relieved by growth and

the phosphorylation through Cdk1. The size growth of the cell during G1 results in the dilution of Whi5. However, the concentration of the G1 cyclin Cln3 stays the same throughout the whole G1 period. The gradually diluted Whi5 gets less active and becomes more prone to the deactivation by the Cln3-Cdk1 complex [Wagner et al., 2009], [Schmoller et al., 2015]. When SBF inhibition is partially relieved, Cln1 and Cln2 transcription starts and they form activated complexes with Cdk1. Whi5, in turn, gets hyperphosphorylated by these active complexes which results in its export from the nucleus. With partial inactivation of Whi5, its inhibition of SBF is relieved, leading to the self-initiation of the transcriptional expression of cyclins Cln1 and Cln2 which completes the CDK positive feedback loop [Skotheim et al., 2008]. 50 % export of Whi5 from the nucleus leads to the self-sufficiency of the positive feedback loop, which is defined as *Start* checkpoint. After passing *Start*, the cells fully commit to the cell cycle progression and progress through the next stages of its cell cycle. Due to its switch-like structure, *Start* has been accepted the point which marks the point of irreversibility [Skotheim et al., 2008], [Charvin et al., 2010], [Doncic et al., 2011].

## 1.5 The *Start* Inhibitor Whi5

Whi5 is the functional analog of the mammalian cell cycle inhibitor retinoblastoma protein (RB). They posses the same functional properties, however they do not share any homology or similarities in their sequence [Cooper, 2006], [Hasan et al., 2014]. The transcription of Whi5 is triggered by the forkhead protein Hcm1 which is transcribed during S phase [Pramila et al., 2006], [Schmoller et al., 2015].

Whi5 plays an important role in the G1/S transition as the key transcriptional inhibitor of the G1 cyclins Cln1 and Cln2. It shuttles between the cytoplasm and the nucleus in a cell cycle dependent manner. At the end of mitosis, Whi5 goes into the nucleus due to the decreased activity of cyclin-Cdk1 complexes and increased Cdc14 phosphatase activity. During early G1, when it is in the nucleus, Whi5 inhibits the transcription of the genes required for the transition to S phase. Thus, its export from the nucleus during late G1 stops the inhibition of the genes responsible for G1/S transition and marks the cell cycle commitment point [Morgan, 2007].

Whi5 is imported into the nucleus at the end of mitosis and exported at *Start*. The

nuclear transport of Whi5 is achieved through the classical nuclear import pathway that includes classical nuclear localization signals and Msn5 [Lange et al., 2007]. In the classical nuclear import pathway, the proteins and molecules are shuttled between cytoplasm and nucleus through nucleo-cytoplasmic transport which is enabled by nuclear pore complexes (NPC). NPCs are proteinaceous complexes embedded in the nuclear envelope to facilitate the transportation via creating a semi-permeable barrier. The structures smaller than 40 kDA can easily diffuse through the NPCs via passive transportation. The proteins with bigger sizes need active transportation that requires energy [Görlich and Mattaj, 1996], [Adam, 2001]. However, the proteins, that play active roles inside the nucleus such as Whi5 (32.9 kDA) are also imported through active transportation even though they have smaller sizes than 40 kDA [Marfori et al., 2011]. The targeted transport of proteins is enabled by the amino acid sequences called nuclear localization sequences (NLS) that are recognized by the transport receptors of the beta karyopherin family and allow access into the target nuclear location [Lu et al., 2021].

The nuclear import of Whi5 is achieved through the beta karyopherins Cse1 and Kap95. Whi5 has two NLSs responsible for its import into the nucleus. The first one, which is called NLS1 is between the amino acids six to ten and the second one , NLS2 is between the amino acids 72 to 95 [Taberner et al., 2009].

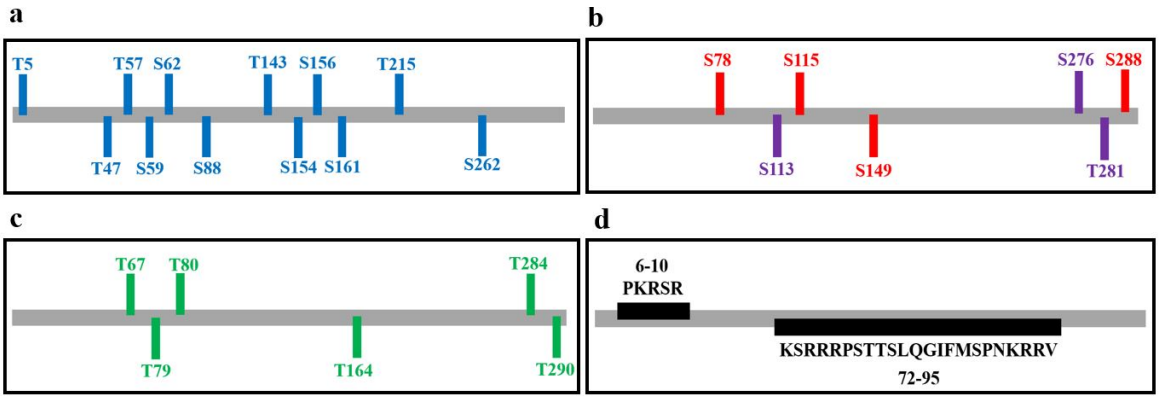


**Figure 4:** Phosphorylation sites and NLSs of Whi5

a) CDK phosphorylation sites responsible for nuclear export of Whi5 (blue) b) G1 non-CDK phosphorylation sites of Whi5 (red). Non-G1 non-CDK phosphorylation sites of Whi5 (purple). The function of these phospho-sites are unknown. c) Uncharacterised phosphorylation sites of Whi5 (green) d) NLSs responsible for nuclear import of Whi5.

The export of Whi5 from the nucleus is accepted as irreversible and marks the point of irreversibility of the cell cycle commitment point. As a result, the irreversibility of Whi5 comes in hand with the irreversibility of the CDK positive feedback loop [Skotheim et al., 2008], [Charvin et al., 2010], [Doncic et al., 2011]. The nuclear export of Whi5 is dependent on the exportin Msn5. The export of Whi5 is driven by Msn5 through the amino acid region between 51–167 through phosphorylation. The CDK phosphorylation sites Ser 154, 156, and/or 161 have been found to play a role in this phosphorylation. The import of Whi5 through this mechanism has been found to be independent of the cell cycle progression. However its export is completely cell cycle dependent. Thus, the cell cycle dependent transport of Whi5 depends on the export mechanism [Taberner et al., 2009].

Whi5 has 19 well-characterized phosphorylation sites (Figure 4). 12 of those phosphorylation sites are phosphorylated by CDK and responsible for its export from the nucleus (Figure 4a) [De Bruin et al., 2004],[Smolka et al., 2007], [Kosugi et al., 2009], [Wagner et al., 2009], [Holt et al., 2009], [Gnad et al., 2009], [Bodenmiller et al., 2010]. The release of Whi5 from SBF is initiated by the 4 G1 specific CDK sites (Figure 4b). The remaining 8 CDK sites do not have any effect on the binding activity. Unlike the 12 CDK phospho-sites, the role of the 7 non-CK sites and which regulator phosphorylates these sites are still not known (Figure 4c) [Chi et al., 2007], [Smolka et al., 2007], [Albuquerque et al., 2008], [Holt et al., 2009], [Huber et al., 2009], [Wagner et al., 2009], [Bodenmiller et al., 2010]. In addition to the 19 well-characterized phospho-sites, there are also six additional sites that have not been characterized yet [Albuquerque et al., 2008], [Holt et al., 2009], [Huber et al., 2009], [Bodenmiller et al., 2010].

## 1.6 Integration of Nutrient Signals

### 1.6.1 Nutrient Signalling in Yeast

For yeast cells, nutrients do not only function as an energy source, but also act as signalling molecules affecting the metabolism. Nutrients provide direct signals for cell growth and the regulation of the metabolism. Thus, nutrients can directly affect the cell cycle progression, cell growth, and division [Bharatula and Broach, 2018]. As a single cell organism, throughout its lifetime, yeast cells face environmental changes in the form of nutrients very often. Thus, for a healthy cell cycle regulation, a finely tuned control mechanism is especially important for single cell organisms like yeast. To survive under limiting nutrient conditions, yeast cells developed a complex nutrient response system which helps them adapt to the fluctuating nutrient sources and increase survival chance [Smets et al., 2010]. To minimize the negative effects of nutrient limitation on the cell cycle progression, yeast cells respond to nutrient stress through complex and multi level adaptation mechanisms.

Even though they primarily prefer fructose and glucose, yeast cells can metabolize various carbon sources, but through different metabolic pathways [Käppeli, 1987], [Kresnowati et al., 2006], [Zaman et al., 2009]. The cells should have a fast and precise response to adapt itself to the changing nutrient conditions to protect itself from the potential damage. Additionally, this response should be specific to the cells needs depending on the metabolic or cell cycle state the cell is in, at the time of the nutrient depletion. However, through which mechanism the cell cycle is affected by metabolic signalling is still not completely known and lacks a lot of aspects [Ewald, 2018].

Through nutrient sensing pathways, yeast cells can sense nutrient changes in the surrounding environment. Compared to multicellular eukaryotic organisms, yeast cells have a relatively simpler metabolic organisational structure which lacks hormones and other signalling mechanisms. Therefore, nutrients do not solely act as an energy source, but also functions as metabolic signals triggering multiple aspects in metabolism growth and proliferation. Budding yeast has several types of direct nutrient-sensing receptor proteins present in the plasma membrane to sense and transmit the nutrient signals. The growth and metabolism can be directly affected by the availability and the type of nutrient sources. With having nutrients as direct metabolic

signals, the cells can adjust how fast they grow and when they divide [Broach, 2012], [Conrad et al., 2014].

Yeast cells can metabolize different types of carbon sources in the lack of glucose by quickly adapting their metabolism via nutrient sensing kinases such as Snf1, Tor1, and PKA. Yeast cells have network systems for signal transduction to make sure to adapt themselves to a variety of nutrient conditions. As a response to nutrient switches, transcription levels of more than 40 % of the genes go through a change showing how the nutrient adaptation is also controlled through a transcriptional level [Smets et al., 2010].

In the absence of nutrients, cells may enter a state termed "quiescence". Quiescent cells show very low levels of metabolic activity, that also includes protein synthesis and transcription rates, which makes them easier to survive in the lack of energy sources [Fuge et al., 1994], [Jona et al., 2000]. However, not all cells go into quiescence under nutrient limitation. The reason of the heterogeneity among the yeast population is still not completely understood, but thought to be dependent on the factors such as cell age, size, or cell density [Sagot and Laporte, 2019]. However, the cells that are in quiescence share many common features that make them different from the regular proliferating cells such as not being able to grow in terms of mass and volume, and also not being able to proliferate [Gray et al., 2004], [de Virgilio, 2012]. When the nutrient conditions improve, the cells leave this state and continue with their regular cell cycle progression.

Cells reach quiescence as a result of a wide crosstalk among multiple pathways. TOR and PKA pathways play roles in quiescence as negative regulators where Snf1 and PKC pathways positively regulate the entry into G0 [Gray et al., 2004], [Miles et al., 2020]. The target of rapamycin (TOR) was first discovered in yeast and then found to be involved in many other eukaryotic organisms [Heitman et al., 1991]. PKA and TOR pathways are activated by rich nutrient conditions. Conversely, they get downregulated and thus inactivated under glucose depletion. TOR proteins prevent the cells from going through quiescence in nutrient rich conditions. However, through which mechanism yeast TOR is regulated is still not completely known in detail. The PKA pathway is conserved among the eukaryotic family and its downregulation leads to

the quiescence transition. Additionally, its activation is essential for the exit from quiescence. On the contrary, as a positive regulator of quiescence, Snf1 is inactive when the cells are grown under rich carbon sources. Within five minutes after glucose depletion is imposed, Snf1 becomes active to activate the transcription of genes responsible for utilizing alternative carbon sources [Wilson et al., 1996]. In the absence of other nutrient sources, this process helps the cell to go into G0 [Gray et al., 2004], [Barbet et al., 2017]. However, there are still many things that remain unknown about how the cells transition into quiescence, remain quiescent and how to make the way out of it due to the diversity of the quiescence states [Sagot and Laporte, 2019].
Following poor nutrient conditions, when the cell is again exposed to an environment that is rich in glucose, it goes through changes both in terms of how their transcriptional structure is organized, how the growth is re-organized and how fast the cell cycle progression is maintained [Kresnowati et al., 2006]. Even though many aspects of how nutrients affect yeast cells are discovered, there are still many things to uncover especially on its effect on the cell cycle progression.

### 1.6.2 Nutrient Signalling During Cell Cycle Progression

Slow depletion of an essential nutrient such as carbon will allow the completion of an ongoing cell division cycle but will not allow passage through the cell cycle commitment called *Start* [Hartwell et al., 1974]. At the cell cycle commitment point, before fully committing into division, the cells do the final check on inner and outer cues to make sure the nutrient conditions are favourable [Morgan, 2007]. During pre-*Start* stages, if the nutrient conditions are not favourable, the cells might exit proliferating state and choose to go into quiescence where it slows down its metabolic activities [Gray et al., 2004].
Nutrients can directly and indirectly alter the timing or the initiation of the G1 checkpoint *Start* by the regulation of growth and size. To progress in the cell cycle and committing itself to division by *Start*, cells must reach a certain size threshold through which the cells attain by the nutrients in their surrounding environment [Jorgensen and Tyers, 2004]. A cell that is grown under poor nutrient conditions can pass *Start* when they reach a smaller size threshold in G1, compared to a cell which

is grown under rich nutrient conditions [Ferrezuelo et al., 2012].

Yeast quiescence has been initially assumed to be achieved only during pre-*Start* G1 stages [Hartwell et al., 1974]. Studies suggest that a high percentage of the cells that go into quiescence are considered to be G1 arrested cells. However, not all the cells that arrest at G1 are quiescent cells [de Virgilio, 2012], [Sagot and Laporte, 2019]. 10% of the cells that go into quiescence were found to be at later stages than G1. However, their cell cycle progression stage remained unknown. It was also proposed that the entry into quiescence might be independent of the cell cycle progression, but was a result of metabolic status [Laporte et al., 2011]. A recent publication shows that the cells can transition into quiescence during all stages of the cell cycle. Using a single cell microscopy approach it was shown that a higher amount of cells than previously assumed could go into a quiescence-like state under glucose limiting conditions [Wood et al., 2020]. However, through which mechanism the cells go into quiescence after G1 and what kind of metabolic organisations are needed is still not known.

# 2 Aim of the Project

As a single cell organism, yeast is exposed to continuously changing nutrient conditions throughout its life span. Thus, to survive under changing nutrient conditions, it should have a response system, that adapts metabolism, growth and division to the nutrient supply. Thus, cells need a robust regulatory system through which they can sense and evaluate nutrient signals and integrate them into the cell cycle machinery.

It is known that the nutrient signalling affects cell cycle progression before the cell passes the cell cycle commitment point called *Start*. The current model states that, the integration of nutrients is achieved during G1 at *Start*. Under poor nutrient conditions, the cells do not transition through *Start* and arrest at a pre-*Start* stage until the conditions become favourable [Morgan, 2007]. However, how nutrient signalling affects cell cycle progression after the cell passes *Start* is still poorly understood. It has been shown in two recently published papers that the cells can respond to nutrient deprivation also during post-*Start* stages [Wood et al., 2020], [Argüello-Miranda et al., 2021]. However, the mechanisms behind cell cycle specific nutrient responses are yet to be discovered.

In this project, we aimed to understand how nutrients are integrated into the cell cycle *after* the cells pass the commitment point *Start*. We established a live-cell imaging work flow to study how glucose deprivation affects cell cycle progression in single cells. Using this approach, we could avoid population averaging and managed to get the heterogeneous responses from the cells. We hope that our findings will lead to a broader understanding of the cross-talk between nutrient signalling and cell cycle control in yeast and beyond.

# 3 Results

## 3.1 Yeast Cells Respond to Acute Starvation During All Stages of the Cell Cycle

According to the commonly accepted model of cell cycle regulation, nutrients should be monitored and integrated into the cell cycle machinery at the cell cycle commitment point called *Start*. When the cell reaches the commitment point, it evaluates the nutritional conditions to decide if it will progress with its cell cycle or stop it until the conditions become favourable again [Morgan, 2007]. However, how the cells coordinate their cell cycle along with nutrients after passing the *Start* point is still not thoroughly understood. To understand if and how the cells integrate nutrient signals during post-*Start* stages of the cell cycle, we used a single cell analysis approach and monitored unsynchronized cells individually using time-lapse microscopy imaging. In order to detect the cell cycle progression dependent response from each cell better, we avoided population-based approaches which would lead to generalizations instead of providing the heterogeneous responses we aimed to get from single cell analysis.

To track the cell cycle, we used endogenously tagged cell cycle marker proteins and quantified the fluorescence intensities or localization changes which oscillate according to cell cycle progression. To distinguish between post-*Start* and pre-*Start* cells, we used Whi5, the negative *Start* regulator as the cell cycle marker protein (Figure 5a). It had been shown that 50 % of Whi5 nuclear export defines the *Start* checkpoint by initiating the self sufficiency of the positive G1 CDK feedback loop [Skotheim et al., 2008], [Doncic et al., 2011]. We tracked the nuclear signal intensity of Whi5 for each cell in the data set and determined the point where 50 % of Whi5 was exported from the nucleus.

To investigate how post-*Start* budding yeast cell cycle progression is affected by nutrient signals, we performed acute glucose starvation experiments. The cells were grown using a microfluidic cell cultivation platform, which enables fast and precise nutrient switches, while being imaged in time-lapse using inverted fluorescence microscopy. During the experimental setup, the cells were grown on 1 % glucose minimum media for eight hours, then the media was switched to 1 % sorbitol minimum media for ten

hours which did not contain any amino acids or other additional carbon sources the cells could metabolize in order to achieve complete starvation conditions. Sorbitol was used as the starvation media to maintain the osmotic pressure of the medium to avoid imposing any additional osmotic stress on the cells. After supplying the cells with the starvation media for ten hours, glucose was then replenished again for at least four hours to detect if the cells behaviour under acute starvation is reversible.

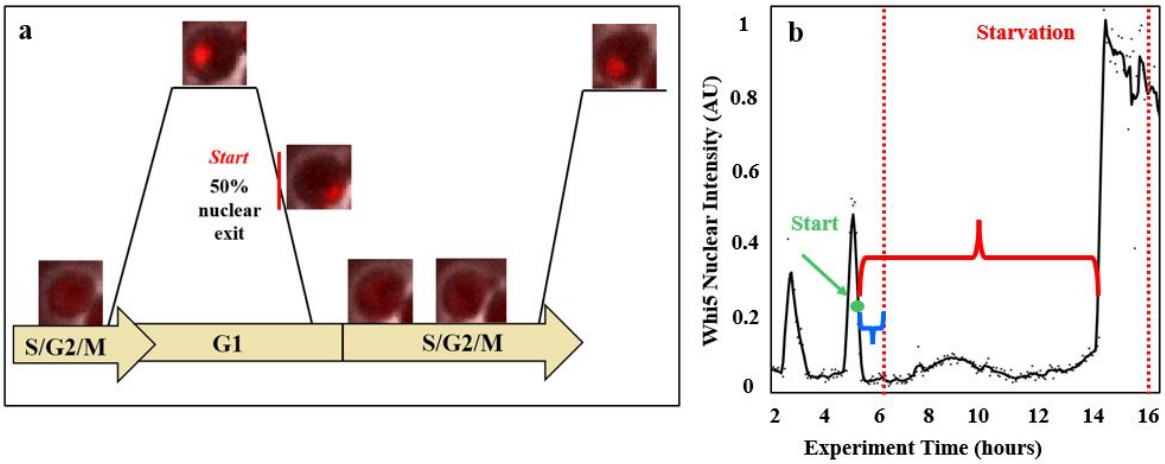


**Figure 5:** Monitoring nuclear Whi5 during cell cycle progression

Nuclear Whi5 was monitored during time-lapse microscopy experiments. a) Whi5 is imported into the nucleus during late M phase. 50 % nuclear export marks the cell cycle commitment point. Using Whi5, the time between *Start* and mitotic exit is calculated. b) The cell cycle progression under starvation was tracked using nuclear Whi5. For each single cell, the time between 50 % nuclear exit and starvation was calculated to detect the cell cycle progression (blue line). The time between 50 % Whi5 exit and mitotic exit was calculated (red line) to detect how the cell cycle progression was affected by starvation.

Following the microscopy experiments, the yeast cell colonies were further analysed individually using a customized semi-automated algorithm [Doncic et al., 2013]. Using Whi5 as our cell cycle marker, we firstly calculated the unperturbed cell cycle duration from *Start* to mitotic exit under glucose media and found it as 90 minutes +-16. Then, to understand how the cell cycle progression was affected by acute glucose starvation we used a total of 425 post-*Start* cells in five independent experiments. Using the same approach, we calculated the time passed from *Start* until starvation to determine the cell cycle progression stage the cells were in (blue line, Figure 5b). Then, we calculated the the cell cycle duration from *Start* to mitotic exit under starvation by again using

Whi5. We calculated the time between 50 % Whi5 export and the initiation of Whi5 import at the end of the cell cycle to determine the time from *Start* to mitotic exit under starvation (red line, Figure 5b). These values were then compared to the average unperturbed cell cycle duration. We detected that post-*Start* cells could respond to acute carbon depletion in three different ways. All the cells in our data set that had a cell cycle duration lower than or same as 90 +-14 minutes were classified as cells that did not respond to glucose deprivation (green cells in Figure 6). The cells with cell cycle duration, longer than the ten hour starvation period were considered as being permanently arrested (red cells in Figure 6). The cells that had their cell cycle duration between 90 minutes +-16 and 10 hours were classified as the ones that delayed their cell cycle progression (blue cells in Figure 6). After determining the cells that fit into these three groups, we then calculated the percentage of the cells in each group accordingly to the cell cycle progression level.

We showed that of all post-*Start* cells, only 2 % continued their cell cycle within the same duration as the unperturbed cells. 59 % of the cells arrested their cell cycle until glucose was replenished and 39 % delayed their cell cycle progression for various extents of time (Figure 6). Additionally, we detected that almost all the cells, independently of how they progressed under acute starvation, progressed with their cell cycle at a normal rate after glucose was supplied back. Also, no increase in the death rate during and after starvation was detected. Almost all cells, independently of the arrest state they had been in, continued with their regular cell cycle progression after glucose was supplied back, showing long term starvation did not affect their viability or post-starvation behaviour. With this, we concluded that the behaviour of the cells under acute starvation was reversible, recoverable, and only limited to the starvation period. To understand the effect of the cell cycle progression the cells were divided into 20 minute bins and the percentages of the cells within these three groups were calculated. With this, we saw that a majority of the cells within the first 20 minutes after passing *Start* permanently arrested their cell cycle until the glucose was replenished. The rest of the cells delayed their cell cycle and none of the cells continued their cell cycle progression on their normal course. As the cells were progressed more into their cell cycle, the percentage of cells that delayed their cell cycle started to show

an increase. After the 40th minute, more cells completed their cell cycle with a delay instead of staying arrested during the starvation period (Figure 6). The earlier in its cell cycle progression the cell was exposed to starvation, the more likely it is to arrest than finishing the cell cycle with or without a delay. With this, we concluded that the cell cycle progression had a clear effect on the cells behaviour under acute glucose starvation and the cells give cell-cycle dependent responses to nutrient stress.

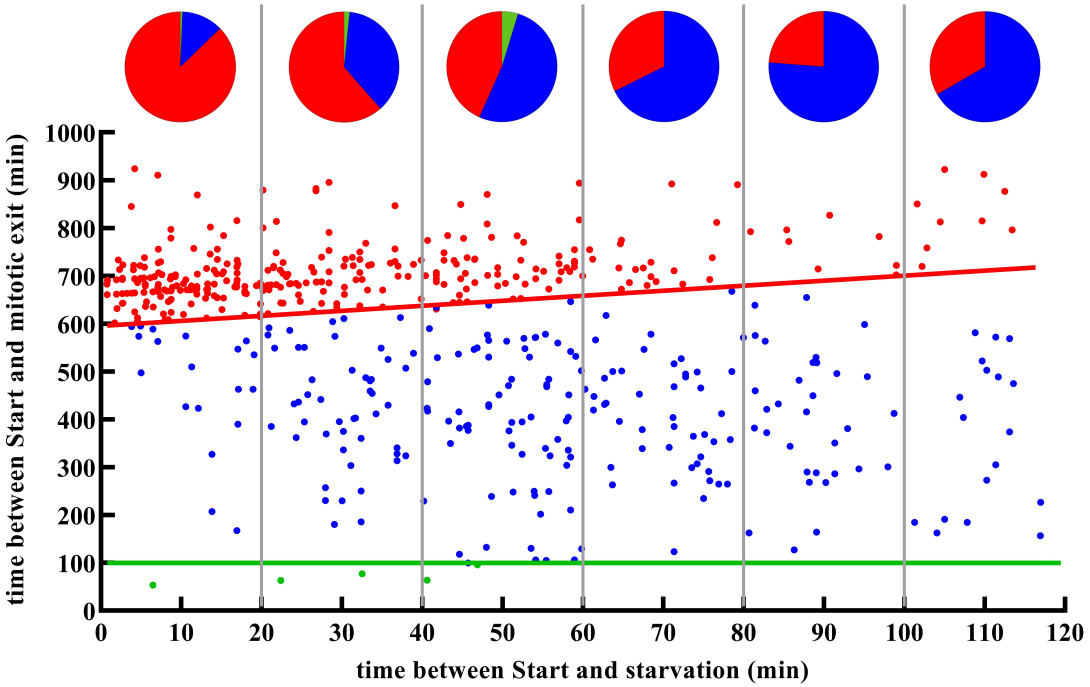


**Figure 6:** Response of post-*Start* cells to acute glucose starvation

Budding yeast cells harbouring Whi5-mCherry were grown in a microfluidic cell growth platform for at least six hours using glucose minimal media. The cells were then exposed to starvation for ten hours using osmotically balanced glucose-free minimal media. After the starvation period, glucose was replenished for at least four hours. In the graph, each dot represents a single cell (n=425, from four independent experiments). The x-axis shows the time that had passed since *Start* until the cell was first exposed to glucose starvation. The y-axis shows the time between *Start* and the end of mitosis. These values were calculated according to the method explained in Figure 5b. The red line indicates the end of the ten hour glucose starvation period and the yellow line shows the average cell cycle duration of unperturbed cells under glucose minimal media. Cells that are above the red line, which continue their cell cycle after glucose replenishment are classified as "permanently arrested". Cells between the green and the red line are categorized as the cells that "delayed" their cell cycle. Pie charts show the fractions of "permanently arrested" (red), "delayed" (blue), and "normal duration" (green) in 20 minute bins.

The cells could integrate nutrient signals into their cell cycle and give a cell cycle pro-

gression dependent response during all stages of the cell cycle. We showed that how the cell responds to acute glucose starvation depended on the cell cycle phase the cell was going through when nutrient limitation was imposed. We detected, that the more the cell progressed, the more likely it would finish its cell cycle even under nutrient scarcity where it stayed arrested until the nutrient conditions were improved.
Our findings indicate that cells can respond to nutrient starvation during all stages of the cell cycle are also in a good agreement with two studies that have been recently published. They showed that cell cycle progression was affected by nutrients throughout its course [Wood et al., 2020], [Argüello-Miranda et al., 2021] . Even though these studies showed that the cell cycle could be affected, they did not investigate the molecular mechanisms behind the decision-making process. Thus in the next part of the project, we decided to focus more on the mechanistic reasons of why the cells chose to behave in certain ways when exposed to acute starvation. As the next step of the project, we decided to focus on those cells that enter a stable arrest to explore the mechanisms leading to stabilizing this arrest.

## 3.2 Post-*Start* Cells Can Re-Import Whi5 Back into Their Nuclei

The further analysis of post-*Start* cells which arrest their cell cycle under acute glucose starvation, lead us to discovering a surprising phenomenon which could not be explained by the current model of cell cycle commitment. By monitoring Whi5 as the cell cycle marker to track cell cycle progression, we detected that the cells could arrest in two different ways. The first group of arrests took place in an anticipated way as the cells arrested at a post-*Start* stage with Whi5 remaining outside of the nucleus throughout the starvation period. However, interestingly the second group of cells, after being exposed to starvation, re-imported Whi5 into their nucleus. We saw, that this type of an arrest was prominent especially among cells that were relatively early in their cell cycle progression stage. Those cells kept Whi5 in their nucleus throughout the whole starvation period and remained arrested at that stage (Figure 7). After detecting the existence of two groups of cell cycle arrest stages, we then analysed how frequently these arrest types could occur.

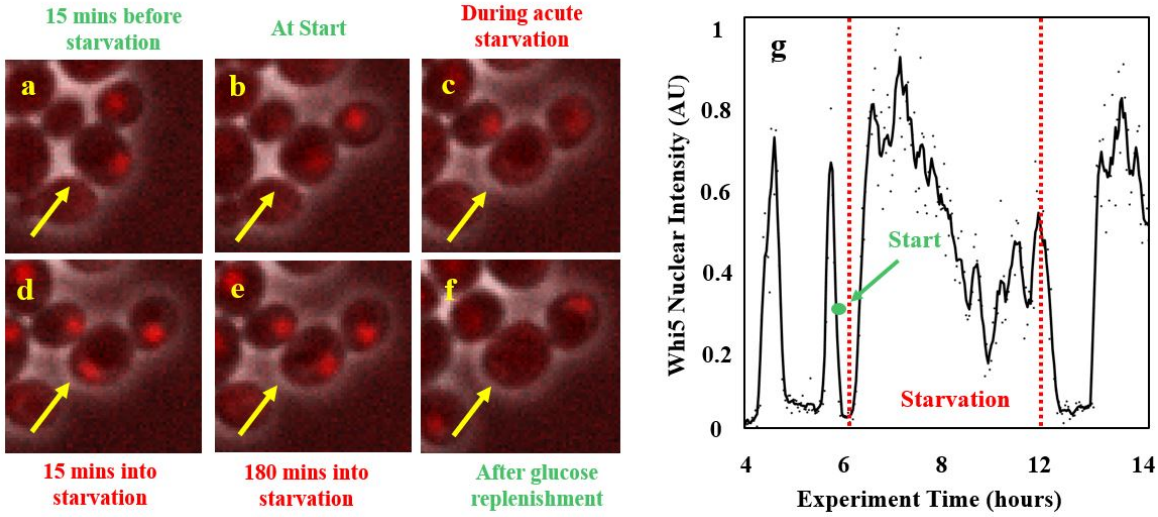


**Figure 7:** Example cell showing nuclear Whi5 re-import as a response to acute glucose starvation

Cells were grown and starved as explained in figure 8. a) The cell is in pre-*Start* where the Whi5 is completely in the nucleus under glucose minimal media. b) 50 % of Whi5 is exported from the nucleus as the cell passes *Start*. c) Acute starvation is imposed by switching glucose minimal media to sorbitol minimum media. d) After several minutes Whi5 re-enters the nucleus. e) During the whole starvation Whi5 remains in the nucleus. f) After the starvation period is over Whi5 leaves the nucleus and the cell continues its cell cycle. g) Nuclear Whi5 trace over the course of the experiment. The time period between dotted red bars mark starvation. The time cell passed *Start* is marked with a green arrow and a dot.

Single cell analysis was performed on a total of 350 post-*Start* cells from five independent starvation experiments. To determine the effect of cell cycle progression, nuclear Whi5 intensities were monitored on the onset of glucose removal, during the starvation period, and after glucose replenishment. The time between starvation and *Start* was calculated for each cell to detect the cell cycle progression stage on the onset of starvation. Our data showed that 95 % of the cells that were within the first five minutes of the cell cycle arrested by re-importing Whi5 into their nuclei. The probability of re-entering Whi5 decreased gradually according to the cell cycle progression and was 82 % within the first 20 minutes and after this time the decrease got faster. From the results, we also detected that being arrested after re-entering Whi5 could even happen among the cells that were very far in the cell cycle. However, it was not as frequent as in cells that were within the first 25 minutes after passing *Start* and decreased to less than 1 % after the 100th minute. This confirmed that cells respond differently to

starvation depending on their cell cycle phase (Figure 8).

As we demonstrated the cell cycle progression dependency of the Whi5 re-entry probability, the next step, was to determine the biological implications of this phenomenon on the cell cycle. By its definition as the cell cycle commitment point, the CDK positive feedback loop and thus *Start* point is accepted as the point of irreversibility of the cell cycle [Charvin et al., 2010]. From previous research, it was shown that the initiation of *Start* is triggered by the CDK positive feedback loop, which revolves around the cell cycle inhibitor protein Whi5 [Skotheim et al., 2008]. As a result, the irreversibility of the positive feedback loop is directly connected with the irreversibility of Whi5 export from the nucleus. However, our data showed that Whi5 export was not necessarily irreversible when the cells faced acute glucose starvation condition. This strongly suggested, that *Start* point could be reversible with nutrient signalling. This challenges the definition of *Start* as the point of irreversibility under all stress conditions. The phenomenon of nuclear Whi5 re-entries under acute starvation was briefly mentioned in two recently published research papers [Liu et al., 2015] [Wood et al., 2020]. However, its biological indications or the molecular mechanisms behind the re-entries have not been investigated yet. As a result, we decided to analyse this phenomenon in more detail to see if the cells could really reverse the cell cycle commitment point.

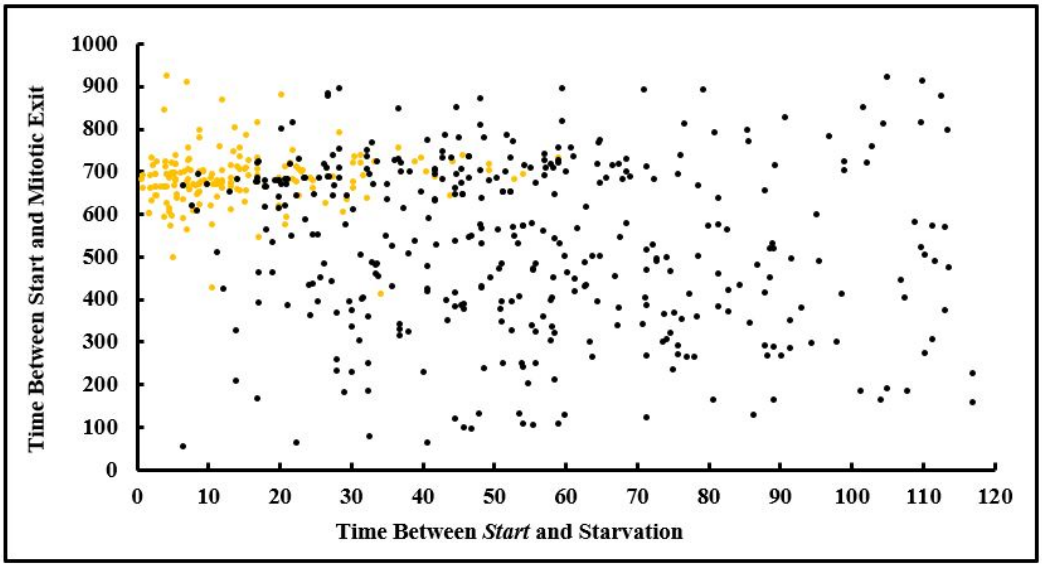


**Figure 8:** Whi5 re-entries among post-*Start* cells exposed to glucose starvation

Cells were grown and starved as explained in Figure 6. Every dot represents one single post-*Start* cell. The y-axis is the time between *Start* and mitotic exit. The x-axis is the time period between *Start* and starvation. Yellow dots depict the cells that re-entered Whi5 into the nucleus after the exposure to acute starvation. The black dots are cells that do not re-import Whi5 into their nuclei.

We detected that the earlier the cell was exposed to starvation, the more likely it was to decide to re-import Whi5 into its nucleus. We then decided to understand the mechanism and reason behind this tendency. For that purpose, we aimed to detect if there was a point in the cell cycle that could affect how the cell behaved. Our first guess was the transition into S phase. We decided to detect when the G1/S transition took place by using core histone proteins which were used as determinants of the initiation of DNA replication. The amount of histones are proportional to the amount of DNA present in the nucleus. Thus, by measuring the fluorescent intensity, detecting the timing of DNA replication under glucose is possible. Using histones, we calculated the required time from *Start* until the initiation of DNA replication as 24 +-4 minutes. We then compared this time frame with the time period in which the re-entries occurred frequently. We detected that the Whi5 re-entries were observed at a very high frequency within the first 25 minutes of the cell cycle (Figure 8). The time frames were found to be matching. Thus, this suggested that transitioning into S phase could be a milestone in the decision of re-entering Whi5. The export and import of Whi5 is known to be taking place during late M and G1 phases and there is no research linking

the involvement of Whi5 in later cell cycle events. Consequently, even a re-entry after 30 minutes was surprising, given that the replication had already started. Thus, we investigated if the Whi5 re-entries at early and later stages were caused by the same mechanism. We hypothesized that, the Whi5 translocations occurring further in the cell cycle should be initiated by a different mechanism. Therefore, if the feedback loop was indeed interrupted, then the interruption should be happening in a time frame close to *Start* before the cell progressed in its cycle more and passed additional checkpoints by completing further cell cycle events.

Our further analysis of nuclear Whi5 re-entries led to the detection of two qualitatively different Whi5 re-entry patterns which mostly depended on the cell cycle progression stage. We saw, that if the cells were approximately within the first half an hour into their cell cycle after passing *Start*, they re-entered Whi5 steep and fast. If the cells progressed further from the first half an hour and had visibly big bud formations, they re-imported Whi5 into their nuclei slowly and gradually. To understand the mechanistic differences behind the two different types of Whi5 translocations, we decided to analyse this phenomenon in more detail.

### 3.2.1 There are Two Types of Whi5 Re-entries

Our qualitative analysis of the cells suggested that, there might be at least two different types of Whi5 re-entries. We detected that the cells that were approximately within the first 25 minutes after passing *Start* and with no or very small buds showed immediate and steep Whi5 re-entries. In contrast, the cells that were at later stages of cell cycle progression re-imported Whi5 back into their nuclei more gradually (Figure 9). These differences implied that there might be different underlying mechanisms behind the re-entries which could take place during different cell cycle progression phases. Thus, to understand the reasons behind mechanistically different Whi5 re-entry patterns and their biological implications, we decided to test several cell cycle parameters such as bud size and the initiation of DNA replication.

Our first attempt in distinguishing between Whi5 re-entry mechanisms was to analyse the size of the buds at the time of Whi5 translocation. For the cells grown under glucose, we calculated the time between passing *Start* and bud initiation as 13.5 +-5.7

minutes. Even though this timing did not completely match with the 25 minute time frame where most steep Whi5 re-entries took place, we tested if there could still be a correlation between the re-entry mechanisms and how progressed the cells were in budding. However, measuring bud sizes did not result in a reliable classification due to the varieties in the mother cell size and age. Additionally, we saw that the cells could still re-enter Whi5 slowly with small buds, and vice versa fast with big buds. Thus, we did not detect any bud size thresholds. As a result, we concluded that there was not any mechanistic link between the process of budding and the Whi5 re-entry types.

Next, we tested if the initiation of S phase could indeed be the point in the cell cycle progression where the re-entry mechanism changed. Unfortunately, unlike mammalian cells, we do not have a good fluorescent read-out for the initiation of S-phase for yeast [Easwaran et al., 2005]. However, as the initiation of DNA replication and the transition to S phase is directly coupled, many studies used histones as marker of S phase initiation for the growth under glucose. The amount of Htb2 present in the cell is proportional to the amount of DNA content [Morgan, 2007]. Thus, to track the G1/S transition, we used a core histone protein called Htb2. Using the total signal of endogenously tagged Htb2 under glucose, we previously calculated the time when the DNA replication was initiated after *Start* as an average of 24.5 minutes with a standard deviation of 4 minutes. This time gap matched our initial qualitative analysis and strongly suggested that the critical point leading to different Whi5 re-entry mechanisms could be when DNA replication process was initiated. Then, we decided to image Htb2 in starvation experiments. Theoretically, using microscopy imaging techniques, by measuring the total Htb2 intensity for each cell, it should be possible to detect the exact timing when Htb2 amount started doubling and thus the initiation of DNA replication. However, in an unexpected way, we saw that total and nuclear Htb2 intensity increased continuously under glucose starvation independently of the cell cycle progression stage the cells were in. As a result, the Htb2 increase could only be detected for the cells that were at late stages if DNA replication started before the starvation started. However, unfortunately, this artefact prevented us from detecting the real Htb2 increase for the cells that were within 30 minutes after *Start*. Therefore,

we were not able to use Htb2 in starvation experiments as a DNA marker to distinguish between Whi5 re-entries during this critical time frame in which the cells showed visibly different qualitative re-entries. To overcome this problem, we tried to divide the total intensity by the cell area to check if it was resulted from a shrinkage of the nucleus. However, we could still see the same increase in the signal intensities.

In addition to Htb2, another histone protein Htb1 was tested by our lab in starvation experiments and the same intensity increase problem was encountered. Thus, even though the timing of DNA replication was very close to our initial qualitative analysis results, we could not find a feasible way to detect it under starvation conditions using microscopy techniques. We, therefore could not precisely determine the onset of histone production in our starved cells. In the future, the effect of DNA replication on the Whi5 re-entry types should be analysed further by using other markers or techniques.

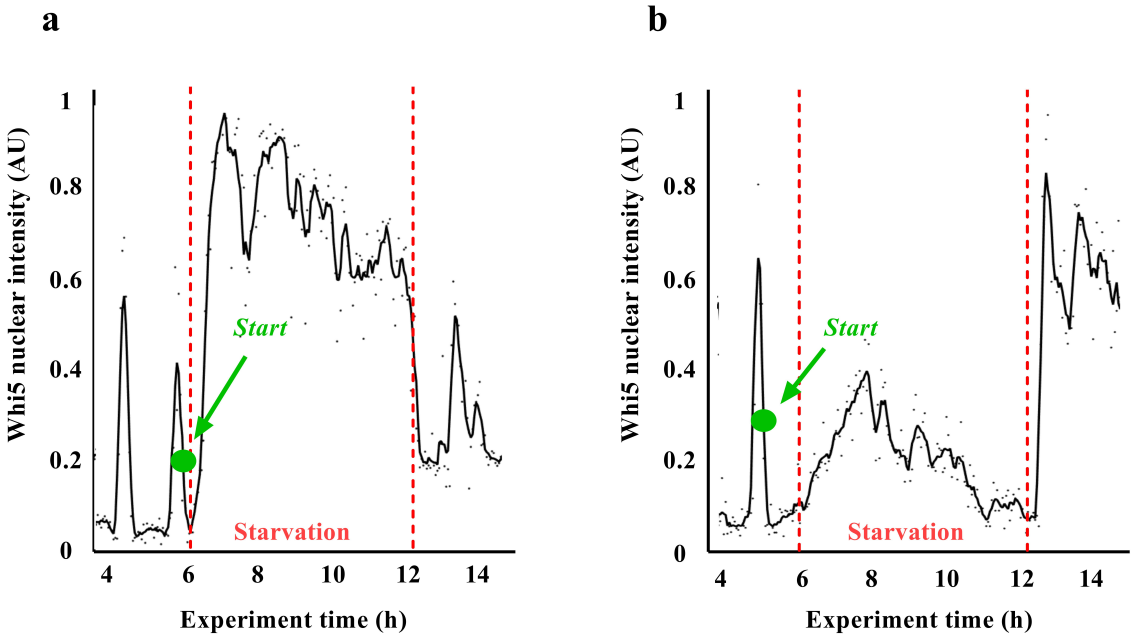


**Figure 9:** Example traces showing the Whi5 re-entry types

The cells harbouring Whi5-mCherry are grown and starved as explained in in figure 6. a) Cells that are approximately within the first half an hour after passing *Start* and have no bud or very small buds re-enter Whi5 fast and steep. In this group of cells, the intensity of nuclear Whi5 after the re-entry is higher than in the pre-*Start* phase. b) The cells that are at later stages of the cell cycle re-import Whi5 slower and more gradually. The nuclear intensity after re-entering Whi5 is less than or equal to the previous G1.

As we could not get feasible results from the cell cycle markers and bud sizes, we next, focused on the nuclear fluorescent intensity of Whi5 after it re-entered into the nucleus and compared these intensities to the previous pre-*Start* intensity of the same cell cycle round. We noticed was that, with the gradual Whi5 re-entries, the Whi5 intensities after Whi5 was translocated into the nucleus, were mostly lower and not as bright as the sharp re-entries. To analyse it further, and see if this tendency was significant enough to be used to classify cells, we measured the nuclear Whi5 intensities before and after the cell re-imported Whi5. Then we compared these two values with each other for each cell. We saw that, even though there was a tendency for the cells that re-enter Whi5 gradually to have lower nuclear Whi5 intensities, it was not consistent enough to be used for making re-entry classifications. Additionally, the results were found to be inconsistent both among different fluorescent proteins that Whi5 was endogenously tagged with, and even sometimes among the same type of fluorescent proteins. A reason for this inconsistency might be lying behind the detected instability of the fluorescent proteins under changing pHi conditions. Some fluorescent proteins, especially on the green spectrum were shown to be going through physiological and structural modifications in response to changing pH levels [Marc-André Elsliger et al., 1999]. It was reported that the intercellular pH of yeast cells changes as a result of environmental perturbations including nutrient stress. It was shown that the intercellular pH drops [Petrovska et al., 2014]. Therefore, the fluorescent proteins that we used to tag Whi5 were also most likely going through physiological changes which caused the signal intensity to fluctuate. This could explain the differences within the same and among different fluorescent proteins. Therefore, we concluded that the nuclear signal intensity itself alone was not a reliable factor and did not use it to make any strong claims.

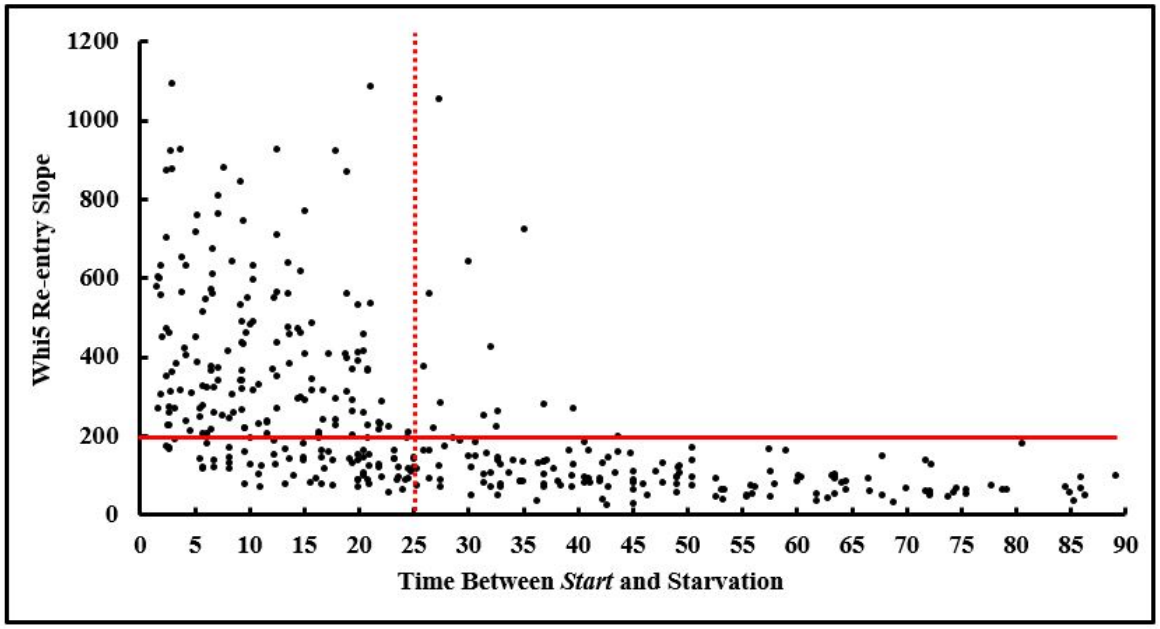


**Figure 10:** Whi5 re-entries among post-*Start* cells that were exposed to acute starvation

The cells harbouring Whi5-mCherry were grown and starved as explained in in figure 6. Every dot represents one single post-*Start* cell that was exposed to starvation. The x-axis is the time between *Start* and starvation. The y-axis is the slope of nuclear Whi5 re-entries. The red bar is the selected threshold (200) for nuclear Whi5 re-entries. The dotted red bar marks the 25th minute after *Start*.

As the nuclear Whi5 intensity differences before and after starvation did not result in consistent results, as a next step we investigated how the slopes of the re-entries change depending on the cell cycle progression level. As mentioned before, the cells within the first 25 minutes tended to re-import Whi5 faster and steeper. We calculated the re-entry slopes for all individual cells in our data set. The average Whi5 re-entry slope within the first minutes was calculated as 348, where as for the cells that re-entered Whi5 after 25 minutes this value was 147, less than half of the first group of cells. As we expected, there was indeed a high slope difference between the two consecutive time frames. To determine if the majority of the cells also fit in this criterion, we then plotted the individual re-entry slopes against time. The data shows that, the re-entry slopes indeed decreased after around the 25th minute. Additionally, we saw that only a very small percentage of the cells that passed the first 25 minutes re-entered Whi5 with a slope higher than 200 (Figure 10). Thus, we chose 200 as the threshold to distinguish between the re-entries. To understand how the budding affects the re-entry dynamics, we then calculated the average re-entry slopes for the

cells that initiated budding and the ones that did not. We calculated the re-entry slope for the unbudded cells as 480 and for the budded cells as 230. Just like the 25 minute time frame, there was approximately two-fold difference between the budded and unbudded cells. However, the average slope for the budded cells exceeded 200 which could be explained by the cells that had just initiated budding and were within the first 25 minutes. As the re-entry sloped of these cells were plotted against time, it was seen that the budded cells tended to re-enter Whi5 with slopes lower than 200, if the time between starvation and *Start* exceeded 30 minutes. As a result, we chose 200 to be the slope threshold, as it could helped distinguishing the cells for the time frame we were interested in.

Now that we found a reliable way to distinguish between the two Whi5 re-entry mechanisms, in the next step we aimed to understand what those re-entries under starvation meant for the cell cycle progression. Whi5 is a known cell cycle inhibitor that plays an active role in the positive CDK feedback loop that leads to the cell cycle commitment point *Start* during G1. As a result, the irreversibility of *Start* comes in hand with the irreversibility of Whi5. Whi5 being exported, and then in the face of acute starvation being re-imported into the nucleus without the completion of the cell cycle strongly suggested that the export of Whi5 was not necessarily irreversible under acute glucose limitation. Thus, this also indicated the potential reversibility of the cell cycle commitment point. As the role of Whi5 as a cell cycle inhibitor during G1, we focused on the cells that were within the 25 minutes after passing *Start* and had a slope above 200 which we consider to be pre-replication. In the next chapter, we further analysed these cells.

### 3.2.2 The Feedback Loop that Defines *Start* is Activated

The steep and fast Whi5 re-entries within the first half an hour suggested that the CDK positive feedback loop defining *Start* could be reversible. To see if this is the case, we first aimed to establish if the positive feedback loop had been indeed activated when cells first exported Whi5 into their nuclei under starvation [Doncic et al., 2011], [Charvin et al., 2010]. To check if the cells that re-enter Whi5 back into the nucleus under starvation were truly post-*Start* cells, we tracked the promoter activity of the

early G1 cyclin Cln2. Following 50 % Whi5 exit, Cln1 and Cln2 triggers the self-sufficiency of the feedback loop by leading to their own expression. Therefore, we aimed to test if we would see an increase in Cln2 promoter activity by measuring its fluorescent intensity increase after our target cells exported Whi5 before the starvation period started. To achieve this, we needed to be able to image Cln2 activity together with Whi5 in time-lapse imaging experiments. The G1 cyclins oscillate fast and thus, have very high turnover rates. As a result, tagging the cyclins themselves does not work well for fluorescent microscopy since maturation of the fluorophore is longer than the half-life of the cyclin. Thus, imaging Cln2 using conventional fluorescent tagging is not possible.

To surpass this problem and increase the time resolution, we integrated a published expression reporter system called dPSTR (dynamic protein synthesis translocation reporter) into our imaging system [Aymoz et al., 2016]. It is a specific method designed to bypass the maturation time of the fluorophore in fluorescent proteins, thereby allowing measurements of gene expression at a minute time-scale in live-cell imaging experiments. The dPSTR constructs are constituted of two transcriptional units: a constitutive promoter controlling the expression of a fluorescent protein fused to a synthetic bZip domain and a promoter of interest controlling the expression of two nuclear localization signals (NLS) fused to another synthetic bZip domain. The constitutively expressed fluorescent protein diffuses freely in the cell, but once the promoter of interest is activated, the bZip domains of both constructs dimerize, leading to the translocation and enrichment of the fluorescent protein in the nucleus. The changes in nuclear signal intensity therefore correspond to changes in gene expression, while essentially bypassing the maturation time of the fluorophore and only depending on the import rate of the dimer into the nucleus [Aymoz et al., 2016]. To monitor the Cln2 promoter activity, Cln2-dPSTR constructs were used in conjunction with endogenously tagged Whi5 which was previously optimized for the imaging strains in our lab [Nadelson, 2020].

To test the precision and time sensitivity of the Cln2- dPSTR reporter system before using it in starvation experiments, we monitored this construct and Whi5 together under high glucose first. Then we calculated the time passed between 50 % nuclear

Whi5 exit and the appearance of nuclear Cln2 reporter peak and found it as, on average, 4.2 minutes. Considering the switch-like structure of the positive feedback loop, 4.2 minutes showed, that there was no time delay in acquiring accurate signals from the Cln2 reporters except the cells that were exposed to starvation within 4.2 minutes after passing *Start*. In addition to the risk of time delay, we also tested if these constructs could lead to any toxic effects on the cell that could negatively affect the cell cycle progression. Our data showed that, the cell cycle duration of the cells which harboured dPSTR constructs were the same as the ones that did not. Additionally, the cell death rate during or after starvation did not show any difference. As a result, we concluded that adding dPSTR constructs did not have any toxic effect on the cells.

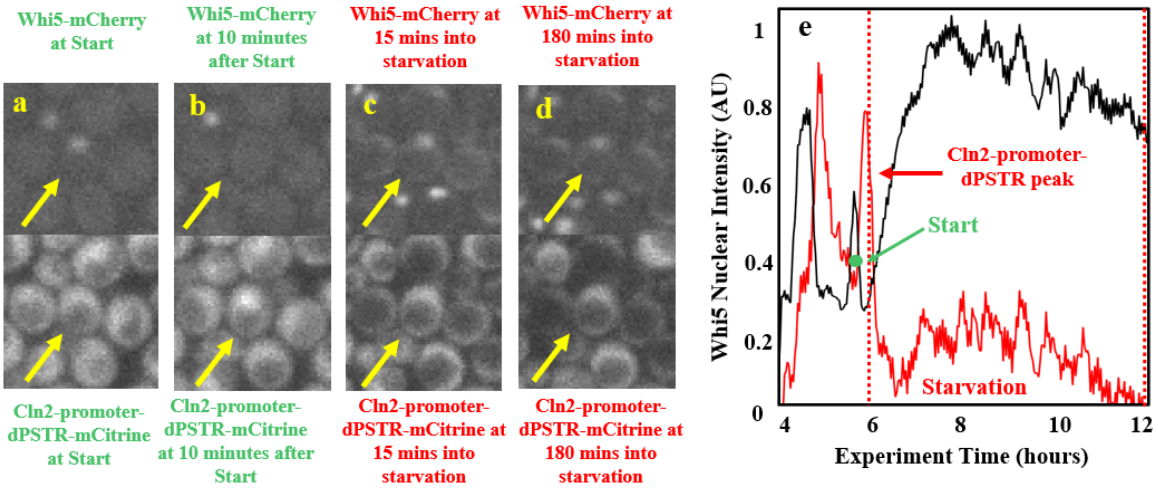


**Figure 11:** Example Cln2-promoter-dPSTR and Whi5 traces under glucose starvation

The cells harbouring Whi5-mCherry and Cln2-promoter-dPSTR-mCitrine are grown and starved as explained in in figure 6. a) At *Start*, there is no Cln2-promoter-dPSTR signal b)10 minutes after passing *Start*, Cln2-promoter-dPSTR signal becomes nuclear. c) Whi5 re-enters the nucleus. Cln2-promoter-dPSTR signal is lost d) Whi5 stays in the nucleus during the starvation period. e) The nuclear Whi5-mCherry (black) and nuclear Cln2-promoter-dPSTR-mCitrine (red) traces throughout the time lapse experiment. The red bars show the starvation period.

Acute starvation experiments were repeated using the same experimental setup. 150 post-*Start* cells, which re-imported Whi5 into the nucleus in response to glucose deprivation, were analysed. For all the cells that re-entered Whi5 into the nucleus, we detected nuclear Cln2-reporter peaks. Figure 11 shows an example cell that re-entered Whi5 within 15 minutes after being exposed to starvation. From there it was seen

that the cells had activated the Cln2 promoters before re-entering Whi5 under starvation suggesting that the CDK positive feedback loop was already initiated when Whi5 was re-imported into the nucleus. The Cln2- reporter peak was observed in both budded and unbudded cells except the ones that were within four minutes after passing *Start*. As the initiation of the positive CDK feedback loop leads to *Start* [Skotheim et al., 2008] [Doncic et al., 2011], we came to the conclusion that the cells that could re-enter Whi5 were at a post-*Start* phase of their cell cycle progression stage. As a result, we confirmed that the cells could re-enter Whi5 when faced to acute starvation conditions after passing *Start* as we predicted. For the next step we checked what the physiological consequences of Whi5 re-entries could possibly be. As the irreversibility of Whi5 export is tightly correlated with the irreversibility of *Start*, the reversibility of Whi5 export from the nucleus strongly suggests the reversibility of the *Start* point. For that reason, we firstly investigated if re-importing Whi5 could mean reversing the cell cycle progression and going back to a pre-*Start* phase.

### 3.2.3 Post-*Start* Cells Can Reverse Their Cell Cycle

The cells were able to reverse the export of the *Start* inhibitor Whi5 after the CDK positive feedback loop activation was initiated. In response to acute nutrient depletion, post-*Start* cells could interfere with the CDK positive feedback loop by reversing the export of Whi5 from the nucleus. The reversibility of Whi5 export which had been accepted as irreversible, also strongly indicates the possibility of the positive CDK loop of being reversible.

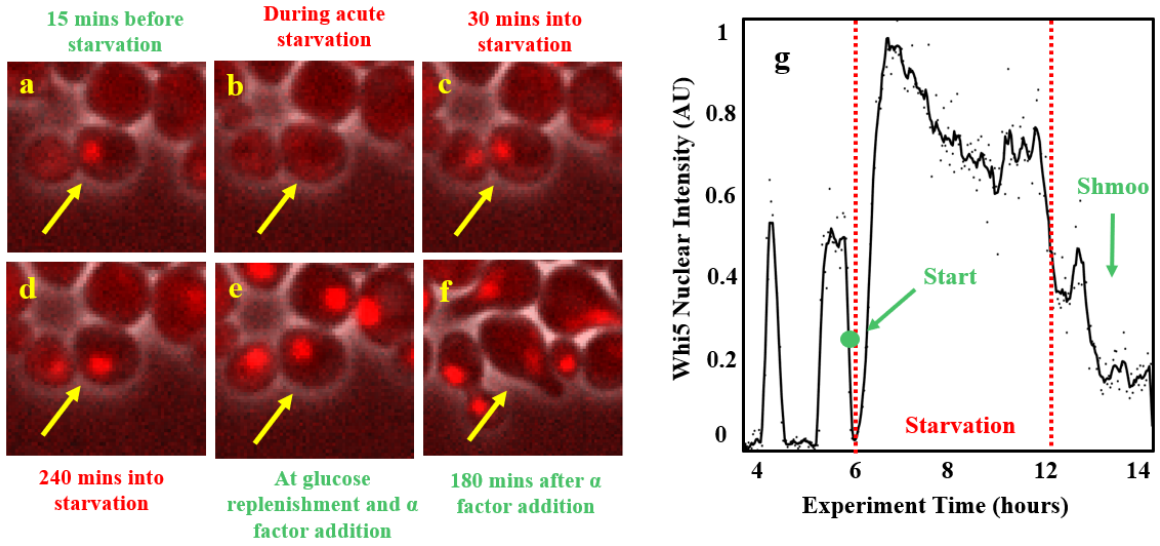


**Figure 12:** Example cell that re-enters Whi5 into the nucleus and shmoos

The cells harbouring Whi5-mCherry are grown and starved as explained in figure 6. Following the starvation period, 100 nM $\alpha$ factor is added to the glucose media and the cells are grown for 4 hours. a) The cell is in pre-*Start* and the Whi5 is in the nucleus completely. b) The post-*Start* cell is exposed to acute glucose starvation. Whi5 is completely out of the nucleus. c) Whi5 re-enters nucleus after several minutes after being exposed to glucose starvation. d) Whi5 remains in the nucleus throughout the 6 hour starvation period. e) After glucose replenishment, Whi5 does not leave the nucleus. f) The cell starts to shmoo just like a regular pre-*Start* G1 cell. g) Nuclear Whi5-mCherry trace. The period between the dotted red bars is the starvation period. *Start* and the time the cell starts to shmoo are marked with green.

To investigate this further, we tested the sensitivity of the cells to $\alpha$ factor mating pheromone to see if the cells could reverse their cell cycle. To test whether cells were indeed functionally reversing *Start*, we exposed them to the mating pheromone, $\alpha$ factor, to see if they would show sensitivity to mating signalling. *Start* was primarily defined as the point where the cells lose sensitivity to mating pheromones and go through another round of division instead. While post- *Start* cells first finish their cell cycle, pre-*Start* cells respond to mating pheromones by producing nodule shaped structures called shmoo to find a suitable mate [Hartwell et al., 1974]. Therefore, these two group of cells can be distinguished between each other and characterized easily using microscopy techniques. We tested if the post-*Start* cells that re-enter Whi5 would be sensitive to the mating pheromones just like regular pre-*Start* cells. If they did, it would strongly indicate that these cells could reverse their cell cycle and go back to a pre-*Start* stage.

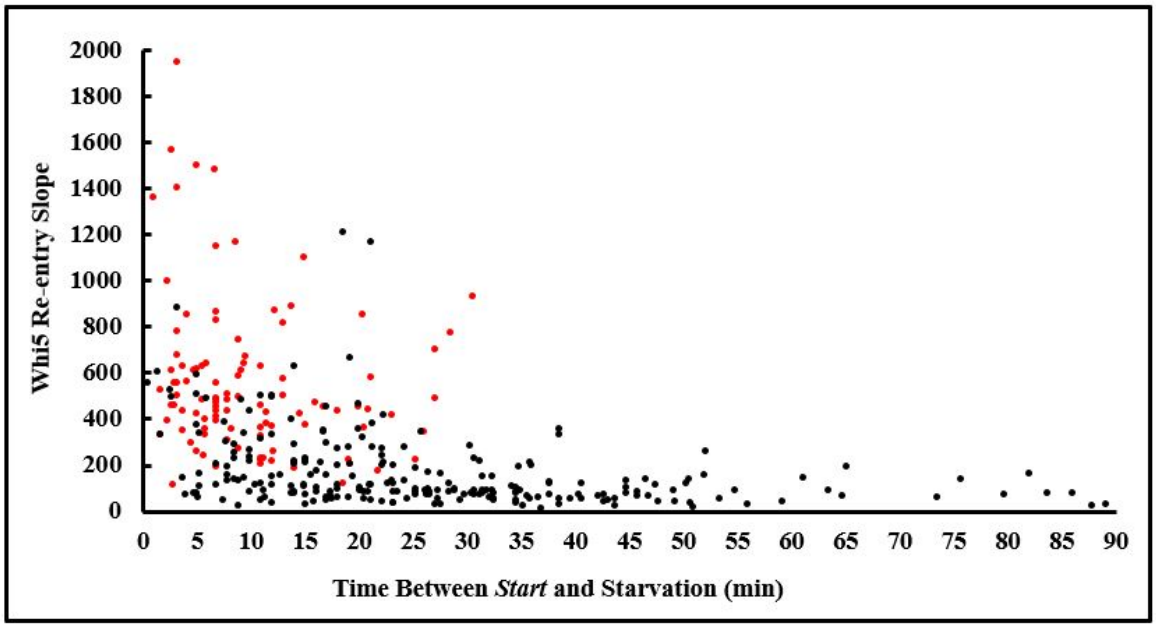


**Figure 13:** Cells that shmoo and do not shmoo classified by the Whi5 re-entry slopes

The cells harbouring Whi5-mCherry are grown and starved as explained in figure 6. Following the starvation period, 500 nM $\alpha$ factor is added to the glucose media and the cells are grown for four hours. Each dot depicts a single cell. The red dots represent the cells that shmoo in response to the mating pheromones. Black dots show the cells that finish another round of cell cycle.

Acute glucose starvation experiments were repeated, and after the starvation period $\alpha$ factor mating pheromone with a concentration of 500 nM was added to the glucose media. From three independent experiments, 350 post-*Start* cells were categorized by their sensitivity to the mating pheromone. We saw that the cells that re-entered Whi5 fast and during earlier stages of the cell cycle progression could respond to $\alpha$ factor by shmooing (Figure 12). We detected that none of the cells that initiated budding showed sensitivity to the mating pheromone and finished the round of cell cycle. From the cells that did not initiate budding, 65 % of the cells that re-entered Whi5 within the first 10 minutes after passing *Start*, acted like regular pre- *Start* G1 cells and showed sensitivity to the mating pheromone and chose shmooing over budding (Figure 14a). Within 20 minutes after *Start*, the probability of shmoo decreased to 53 % for the unbudded cells. None of the cells responded to mating pheromones if the time between *Start* and starvation was more than 35 minutes (Figure 13).

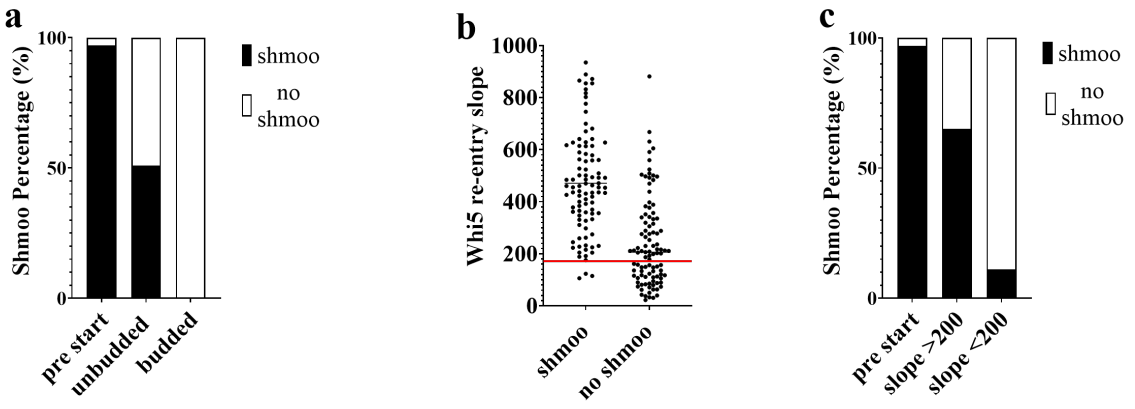


**Figure 14:** The probability dynamics of shmooing

The group of cells that show sensitivity to mating pheromones and shmoo are determined. a) 100 pre-*Start* G1 cells from three independent experiments are analysed. 97 % of the cells shmood in response to the mating pheromones. 200 cells that re-enter Whi5 from the same experiments are analysed and categorized for their budding status. 53 % of the unbudded cells show sensitivity and none of the cells that initiated budding shmoo. b) The Whi5 re-entry slopes of 200 cells used in (a) are calculated. The slopes of the cells that shmoo and shmoo are plotted. The red line shows the 200 slope threshold. c) The cells used (a) are classified according to their budding.

The sensitivity to the mating pheromone was detected to be dependent on the slope of Whi5 re-entry. For each cell, the slope of Whi5 re-entry was calculated and the cells were divided into two groups accordingly. Consistent with our previous classification, the first group of cells was chosen as the ones with a slope higher than 200 and the second group as the ones with a slope lower than 200. We calculated that 95 % of the cells that shmood in response to the mating pheromone had re-entered Whi5 fast and with a slope higher than 200 (Figure 14b). Among those cells, the average re-entry slope was calculated as 583. For the cells that did not respond to the mating pheromone and went through another round of cell division, the average Whi5 re-entry slope was calculated as 170. The clear distinction between the slopes strongly suggested that the slope of the Whi5 re-entry was correlated with the decision to shmoo.

This clear distinction supported our previous proposal of the existence of two mechanistically different Whi5 re-entries. We showed that, cells which re-entered Whi5 more gradually and during later phases of their cell cycle did not reverse their cell cycle, and acted like post-*Start* cells. However the cells which were in earlier phases in the cell cycle and re-entered Whi5 fast and with a slope higher than 200, reversed their cell

cycle and went back to pre-*Start* G1 and stayed at that stage throughout the whole starvation period. The sensitivity to mating pheromone confirmed that, the positive feedback loop defining *Start* could be reversed within the first 25 minutes. Interestingly, not all cells that had Whi5 re-entry sloped higher than 200 showed sensitivity to the mating pheromone. This also suggested that, the group of cells with slopes above 200 could also be diverged among themselves.

### 3.2.4 The Cells Go Through The CDK Feedback Loop Twice in The Same Cell Cycle Round

For the post-*Start* cells to go back to a pre-*Start* stage and continue the cell cycle progression from that phase after glucose replenishment, they should go through the positive feedback loop once more again. In other words, the cells should go through the same CDK feedback loop twice in one round of cell division. To confirm this, we used the Cln2 promoter which should be activated when the positive CDK loop is triggered. In the previous chapters we already showed that the cells that re-entered Whi5 had a Cln2 promoter peak after Whi5 exit on the onset of the starvation period. Now, to show that they go through *Start* the second time, we monitored Cln2 activity after the glucose replenishment where the cells continued their regular cell cycle progression.

To confirm that the cells go through a second round of CDK positive feedback loop after glucose replenishment when the cells proceeded with the cell cycle progression, we monitored Cln2 promoter activity, right after the starvation period. The cells were exposed to acute starvation for six hours, after been grown on glucose minimum media. Then, glucose was replenished again for four hours. Cln2 promoter activity was monitored together with the nuclear Whi5 signal. Single cell analysis was performed and 145 post-*Start* cells that had Whi5 re-entry slopes higher and lower than 200 from two experiments were further analysed. In the previous chapter, we had determined that the cells which re-entered Whi5 with low slopes did not show sensitivity towards mating pheromones, thus it was concluded that they did not go back to pre-*Start*. Therefore, by checking the post-starvation Cln2 promoter activity also for these group of cells, we wanted to confirm our findings.

In the previous chapters, to detect the Cln2 promoter activity on the onset of starvation, we used a Cln2-dPSTR construct to avoid the delay in the signal due to the maturation time. However, since nutrient perturbations like carbon starvation influence the cellular dynamics and mobility of the proteins [Joyner et al., 2016], they also affect dPSTRs, as the translocation rate of the dimer is the biggest time-limiting factor for the detection of a change in the nuclear signal intensity. We observed that the time dPSTRs needed to recover strongly interfered with Cln2 promoter activity signal, we could not get good signal from those constructs when glucose is replenished. In the dPSTRs constructs, the fluorophore to be imaged was under a constitutive promoter. However, as it was a ribosomal promoter, it was not active under starvation conditions. As a result, no signal could be received under starvation. Additionally, the recovery time after starvation prevented us from getting data for the post-starvation Cln2 promoter activity. Consequently, to monitor Cln2 promoter activity after the starvation, we used a Cln2 promoter on a plasmid directly driving the mNeongreen fluorophore expression. With this promoter construct, we detected the Cln2 promoter activity in the form of Cln2 promoter signal intensity peaks.

We demonstrated that 97 % of the cells that re-enter Whi5 with a slope higher than 200, had detectable Cln2 promoter activity after glucose replenishment, including the cells that initiated budding. Figure 15 depicts an example signal we obtained from the Cln2-promoter-mNeongreen construct. The cell re-entered Whi5 and went into a stable arrest during the starvation period. After glucose replenishment, Whi5 left the nucleus and we detected a Cln2 promoter peak indicating increasing Cln2 promoter activity. In contrast, only 6 % of the cells with a slope lower than 200 showed Cln2 promoter peaks, confirming that these cells did not reverse their cell cycle and the later-stage Whi5 re-entries were mechanistically different than the early ones. Our data agreed with our findings from the $\alpha$ factor mating pheromone experiments. By using the Cln2 promoter activity, we confirmed that the cells that showed sensitivity towards the mating pheromones continued with their cell cycle progression from the pre-*Start* G1 phase they previously went to after starvation.

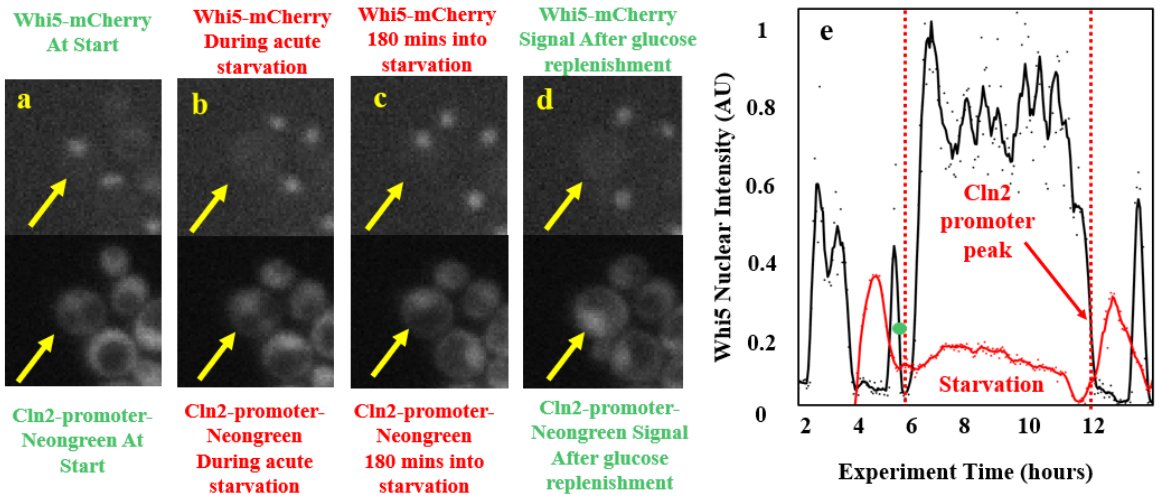


**Figure 15:** Example Cln2-promoter-dPSTR and Whi5 traces under glucose starvation

The cells harbouring Whi5-mCherry and Cln2-promoter-mNeongreen are grown and starved as explained in figure 6. a) At Start, there is no Cln2-promoter activity b) During acute starvation, Whi5 is already out of nucleus and Cln2-promoter signal is undetectable c) Whi5 stays in the nucleus throughout the starvation period d) After glucose replenishment, Cln2-promoter signal starts increasing when Whi5 left the nucleus. e) The nuclear Whi5-mCherry (black) and total Cln2-promoter-mNeongreen (red) traces throughout the time lapse experiment. The red lines show the starvation period.

So far, we established, that the cells which passed the cell cycle commitment point could go back to a pre-*Start* G1 phase under acute glucose starvation conditions. By using Cln2 promoter activity, we showed, that these cells which act like regular pre-*Start*, had previously activated the CDK positive feedback loop before re-entering Whi5. As defined in the literature, *Start* was primarily defined as the point where the cell loses its sensitivity to the mating pheromones [Hartwell et al., 1974]. Our data showed that the cells which re-enter Whi5 with a slope higher than 200 and did not start bud initiation, could respond to the mating pheromones which showed that they acted like regular pre-*Start* cells. However, none of the cells that initiated budding were responsive to the mating pheromones. This might indicate that these cells could still have previous memory from CDK activation. Additionally, by again using Cln2 promoter activity, we demonstrated that both budded and unbudded cells that re-enter Whi5 with a slope higher than 200 reactivated the CDK loop for the second time, which showed that the cells passed *Start* twice in one cell cycle round which suggested the reversibility of the cell cycle commitment point of the yeast cell cycle.

### 3.2.5 Through Which Mechanism do the Whi5 Re-entries Happen?

We have demonstrated, that under acute glucose deprivation, post-*Start* cells could reverse the G1 CDK positive feedback loop suggesting that *Start* could be reversed under nutrient perturbations. This indicated that the feedback loop was interrupted by a yet unknown mechanism. In the second part of the project, we aimed to find the underlying mechanism behind this phenomenon and how it is triggered. For that purpose, we first tested if the transcriptional regulators that were known to be involved in nutrient regulation and stress could be playing a role.

### 3.2.6 Established Nutrient-Sensitive Transcriptional Factors are not Required

Through testing nutrient-sensitive regulators, we aimed to see if the feedback loop was interrupted through repression of G1 cyclin transcription. To understand how the CDK positive feedback loop was interrupted, we firstly investigated the role of five important transcriptional factors that are known to be responsive to nutrient changes. To test that, we created deletion mutants of the transcriptional regulators Msn2, Msn4, Xbp1, Msa1, and Msa2 and tracked nuclear Whi5 in time-lapse experiments over time under starvation conditions.

Msn2 and Msn4 are two of the core elements in the coordination of all kinds of stress responses including acute nutrient stress. They function as transcriptional activators and repressors that activate hundreds of stress related genes under exerted stress conditions which in return help the cell to regulate its response towards unfavourable situations. They bind to the Cln1 and Cln2 promoters when the cell faces stress and inhibit the Cln1 and Cln2 expression. Msn2 and Msn4 was shown previously to be expressed and localized in the nucleus under various sources of stress including osmotic shock, heat shock, and different types of nutrient deprivation [Martínez-Pastor et al., 1996]. Together, these two regulators also play a key role in the activation of several genes which are responsible for the regulation of the carbohydrate metabolism. Through this, Msn2 and Msn4 support the adaptation process to glucose limiting conditions by activating several genes responsible for the transition into quiescence [Kuang et al., 2017]. In addition to the general stress conditions and overall nutrient deprivation, the tran-

scription of Msn2 and Msn4 was shown to be specifically activated as a response to glucose starvation [Görner et al., 2002]. Through our microscopy data, we also confirmed their localization under acute glucose starvation. We detected that Msn2 is enriched in the nucleus approximately six minutes and Msn4 after nine minutes after the cells were exposed to glucose deprivation (Figure 16). The Msn2 and Msn4 pulses were observed throughout the starvation period and the nuclear signal disappeared after glucose replenishment. Therefore, we concluded that Msn2 and Msn4 were activated during the acute starvation experiments.

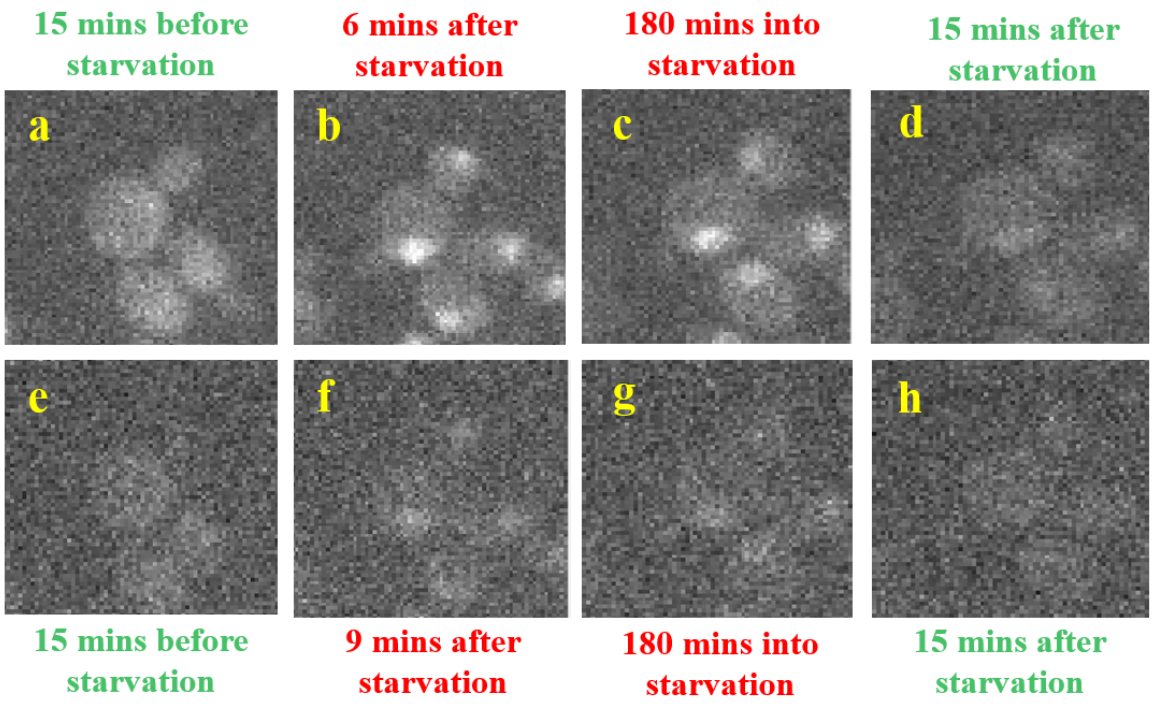


**Figure 16:** Example cells showing nuclear localization of Msn2 and Msn4 under glucose starvation

The cells harbouring Msn2-GFP and Msn4-mNeongreen were grown and starved as explained in figure 6. a) Msn2-GFP signal 15 mins. before starvation b) Msn2-GFP signal after 6 minutes of starvation c) Msn2-GFP signal after 180 minutes of starvation d) Msn2-GFP signal after 15 mins. of glucose replenishment. e) Msn4-mNeongreen signal 15 mins. before starvation f) Msn4-mNeongreen signal after 9 mins. of starvation g) Msn4-mNeongreen signal after 180 minutes of starvation h) Msn4-mNeongreen signal after 15 mins. of glucose replenishment.

Xbp1 is a transcriptional repressor that is known to be induced under glucose limitation, heat shock, DNA damage, and oxidative stress conditions and plays a role in cell cycle regulation. It has high homology with two important cell cycle related transcrip-

tional factors called Swi4 and Mbp1. Cln1 and Cln3 promoters have been shown to have Xbp1 binding sites, suggesting that Xbp1 may be a part of the complex network leading to the repression of these G1 cyclins [Mai and Breeden, 1997]. It has been shown that, Xbp1 contributes to the repression of several metabolic pathways. Together with Cln3 repression, Xbp1 plays an important role in a stable G1 arrest under nutrient limitation. Additionally, in the same study it has been shown that Xbp1 is responsible for going through quiescence during G1 as it prevents the cells from choosing other types of fates when nutrients are limiting [Miles et al., 2013]. Interestingly, it has also been shown recently that quiescence during cell cycle stages later than G1 is also regulated through the nuclear accumulation of Xbp1. The nuclear accumulation and the regulation of Xbp1 is caused by stress response and takes place independently from the cell cycle progression stage [Argüello-Miranda et al., 2021]. Xbp1 was thought to be good candidate due to its role in G1 arrest, but also its regulation by stress not only during G1 but throughout all cell cycle progression.

Msa1 is an SBF and MBF associated protein where Msa2 is only associated with MBF. Unless any type of stress is exerted on the cell, these two proteins are not specifically needed for any cell function. Msa1 is found to be playing a role in the the cells transition to G1 quiescence under poor nutrient conditions [Ashe et al., 2008]. Additionally, under stress conditions, G1 cyclin transcription is down-regulated through Msa1, together with Whi5 which are phosphorylated by Hog1 [González-Novo et al., 2015]. However, later it was found that both Msa1 and Msa2 are involved in this transition. Also, the $\Delta$msa1,2 mutation contributes to the initiation of G1 arrest. Both proteins are directly involved in the repression of SBF related gene expression which also includes the early G1 cyclin Cln2 under glucose deprivation. Therefore, Msa1,2 is involved in the Cln2 repression. The repression of all these genes then, leads to quiescence during G1. However, it was shown that, even though Msa1,2 have an important function in the transition to quiescence and G1 arrest, they are definitely not involved in the maintenance of quiescence or stable G1 arrest in the long run [Miles et al., 2016]. Thus, Hog1 regulates G1 cyclin transcription upon osmostress to ensure coherent passage through *Start*. To sum up, Msa1 and Msa2 were chosen due to their role in the G1 arrest in response to nutrient deprivation that had been shown

only in bulk experiments.

To test if these transcriptional regulators played a role in the Whi5 re-entries, a deletion mutant of Xbp1, a double deletion mutant of Msn2,4 and a double deletion mutant of Msa1,2 were used. Firstly, in order to understand if these deletions had any visible effect on the cell cycle progression or on the viability of the cell, the mutants were grown on glucose rich media. It was seen that the cell cycle durations were very similar to the wild type. As a result, we decided to use these mutants in time lapse starvation experiments.

To understand if any of these transcription factors contributed to the process of reversing *Start*, acute starvation experiments were repeated. We monitored nuclear Whi5 signal in the mutant strains that were exposed to six hours of starvation. Post-*Start* cells from two independent experiments were analysed for each mutant. For all mutants, the re-entry probabilities were calculated for the cells that re-entered Whi5 with a slope higher than 200 and compared with the wild type strain (Figure 17a).

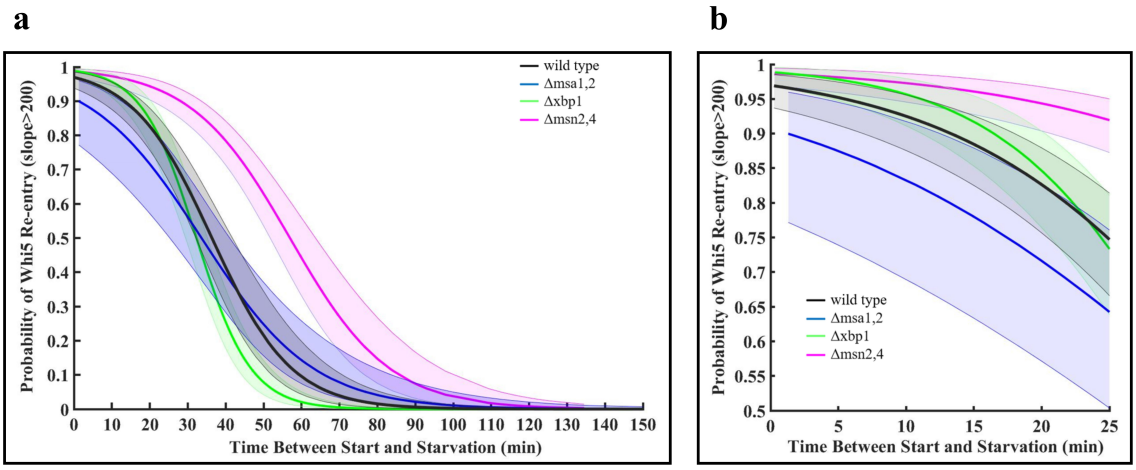


**Figure 17:** Whi5 re-entries above the slope of 200 in the wild type strain, Δmsa1,2, Δxbp1 and Δmsn2,4 mutants

Cells are grown and starved as explained in in figure 6. The binary data (re-entry yes/no) if the re-entry slope>200 from n>100 cells per strain was fit to a logistic regression to estimate the re-entry probabilities as a function of time passed between *Start* and being exposed to starvation. The re-entries within the first 25 minutes were considered as the reversal of *Start*. The re-entries after 25 minutes were classified as regular re-entries. The wild type probability is estimated (black)and compared to the mutant strains. a) Δxbp1 strain (green) show a similar re-entry profile as the wild type strain. Δmsa1,2 (blue) has slightly lower Whi5 re-entry frequencies in the first 40 minutes than the wild type. Δmsn2,4 (magenta) increases Whi5 re-entry probability at every stage of the cell cycle progression. Shaded areas represent 95 % confidence intervals of the regression. b) Δxbp1 cells (green) reverse *Start* just like wild type cells. Δmsa1,2 (blue) results in 10 % decrease in the probability of reversing *Start*. Δmsn2,4 (magenta) causes an increase in the reversal of *Start*.

All the mutant cells could still translocate Whi5 back into their nuclei under glucose deprivation. Δxbp1 mutant showed almost the same probability pattern as the wild type strain with a slight decrease after the 30th minute and a slight increase in the first 20 minutes. The Δmsa1,2 strain however, had a lower re-entry frequency within the first 40 minutes, then showed slightly higher probability frequency between 40th and 100th minute. The most interesting effect was observed with the Δmsn2,4 strain. During all stages of the cell cycle progression, the Whi5 re-entries were visibly higher than the wild-type strain and all the other deletion mutants. Within the first 25 minutes the re-entry probability was 92 % where it was 75 % in the wild-type strain. This showed that the deletion of msn2,4 increased the likelihood of cells reversing their cell cycle and going back to a pre-*Start* stage. After the 50th minute, the probabilities started to decrease more steeply, but always remained higher than the wild-type strain.

One of the reasons of the higher re-entry probabilities during late stages was that the percentage of the Whi5 re-entries with slopes higher than 200 were more commonly encountered even at further stages of the cell cycle. Taken these together, the deletion of msn2,4 strongly indicated that these two regulators might have an effect on the dynamics and the types of the Whi5 re-entries, but were not essential for the re-entry decision. To test how much deleting Msn2 and Msn4 affected the underlying biology behind it, in the future the cells should be tested against mating pheromones to see if the cells that re-enter Whi5 after the 30th minute with a slope higher than 200 could be sensitive to mating pheromone and thus able to reverse their cell cycle.

After testing five transcriptional regulators that were known to be a part of nutrient stress response regulation throughout the cell cycle, we concluded that none of them completely stopped the Whi5 re-entries or prevented the cells to reverse their cell cycle (Figure 17a, Figure 17b). The only strain that showed a slight decrease in the cell cycle reversals within the first 20 minutes was $\Delta$msa1,2 which overall showed a 10 % decrease. With these results, we concluded that even though the mutants changed the probability frequencies to some extent, none of them caused a decrease in the occurrence of re-entering Whi5 back under acute starvation. Despite their previously known involvements in the regulation of starvation response, these transcriptional regulators did not have any direct negative effect on the cell cycle reversal under acute glucose starvation conditions. Therefore, we concluded that, even though they could be contributing it, these transcriptional regulators were not essential for the interruption of the CDK positive feedback loop through the G1 cyclins directly or indirectly. Thus, in the next step we decided to directly test the effect of the changes in the transcriptional regulation of the early G1 cyclins that play a key role in the CDK positive feedback loop. Instead of trying more transcriptional regulators, we directly tested if the repression of cyclin expression was needed for the Whi5 re-entries.

### 3.2.7 Repression of Cyclin Transcription is not Essential for Interrupting the CDK Feedback Loop

The cell cycle commitment point, *Start* is initiated when the G1 cyclins Cln1 and Cln2 trigger their own expression and thereby enable the self-sufficiency of the CDK positive

feedback loop [Skotheim et al., 2008]. As they contribute to the irreversibility of the feedback loop, we decided to check if the reason for the interruption of the CDK positive feedback loop could be the through cyclin repression. Consequently, we aimed to see if the Whi5 re-entries could still happen when over-expressing the G1 cyclin Cln1. We used a strain in which cyclins were not under its endogenous control and independent of the cell cycle progression. The remaining G1 cyclins were deleted and the cell cycle progression was driven completely by a synthetic system. Cln1 is overexpressed continuously from a synthetic promoter induced by beta-estradiol throughout the starvation experiments [Ewald et al., 2016]. The promoter is also shown to retain activity under starvation (Fabian Schlottmann). As a result, the cell is always has Cln1 present including the time frame in which the re-entries occur.

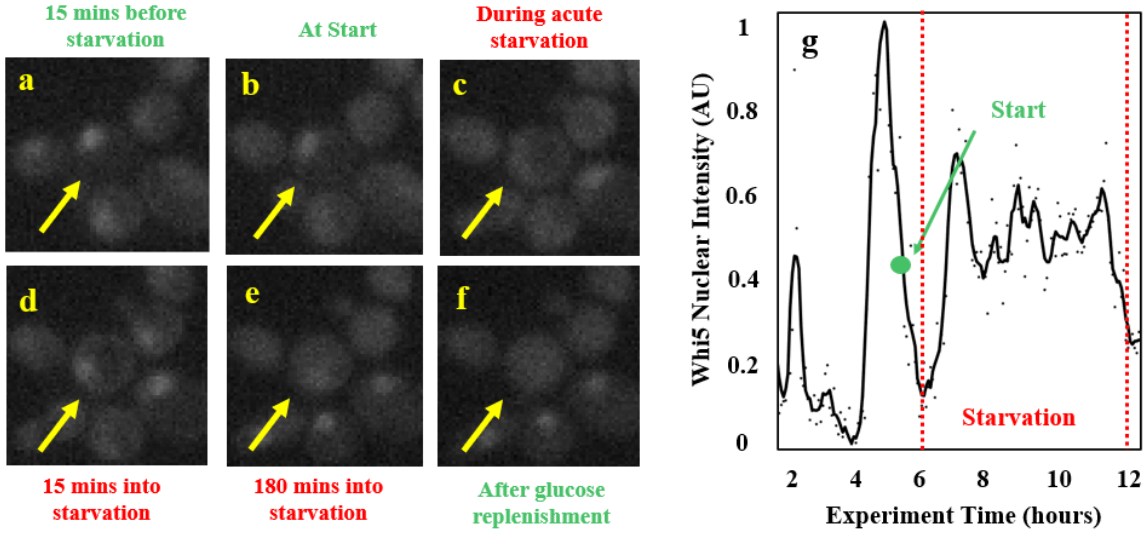


**Figure 18:** Example Whi5 re-entry traces in a Cell in which Cln1 is overexpressed during glucose starvation

The cells harbouring Whi5-mCherry are grown and starved as explained in figure 6, with the addition of 1000 nM beta-estradiol during all stages of the experiment. a) The cell is in pre-*Start* where the Whi5 is completely in the nucleus under glucose minimal media. b) 50 % of Whi5 is exported from the nucleus as the cell passes *Start*. c) Acute starvation is imposed by switching from glucose minimal media to sorbitol minimum media. d) After several minutes Whi5 re-enters the nucleus. e) During the whole starvation Whi5 remains in the nucleus. f) After the starvation period is over Whi5 leaves the nucleus and the cell continues its cell cycle.

We tested the induced strain against acute glucose starvation using the similar setup we had used with the wild-type strain. The cells were grown for five hours on glucose

media and then exposed to starvation for six hours. Beta-estradiol concentrations were adjusted to achieve wild-type like doubling times under unperturbed conditions (1000 nM). These concentrations were maintained throughout the experiment. We detected that beta-estradiol concentrations below and above 1000 nM during microfluidic cell cultivation caused cell cycle and division defects. When we tracked nuclear Whi5 throughout the course of the experiment, we saw, that the cells did not fit into the 200 slope threshold we previously set to distinguish Whi5 re-entries. We noticed that even under constant glucose conditions, Whi5 dynamics were slightly different than WT with more gradual re-entry at the end of mitosis. This is likely due to the overexpression of Cln1 outside of the normal time window. Consistently, during starvation, the Whi5 re-entry slopes were more gradual. As a result, instead of just using the cells that re-entered Whi5 with slopes higher than 200, we decided to use all 50 cells from 2 independent experiments for further statistical analysis. Nevertheless, Whi5 re-entries occurred at a similar fraction as in wild-type cells. Additionally, we detected fluctuations in the nuclear Whi5 signal during the starvation period which results from the low nuclear intensities (Figure 18).

Our data demonstrated that even under exogenous control of Cln1, Whi5 re-entries could still take place indicating that repression of early G1 cyclins was not required. With this, we came to the conclusion that the feedback loop was not interrupted under acute starvation through the repression of early cyclins. Thus, Cln1 repression is not a requirement for re-importing Whi5 back into the nucleus.

Secondly, to test the upstream regulator factors of *Start*, we tested a strain in which the cyclin Cln3 gene was inserted in multiple copies (5X) (kind gift of Linda Breenden). We aimed to see if Cln3 overexpression could suppress the re-import of Whi5 from the nucleus. The strain was first tested under glucose minimum media. It was seen that the cell cycle duration was significantly longer than the wild-type strain. Using Whi5, the length of one cell cycle round was calculated as 7.5 hours, where the cell cycle duration of the wild-type strain was 1.5 hours. However, it was seen that the cell was able to complete its cell cycle and Whi5 was cycling regularly. Due to the length of the cell cycle duration during starvation experiments, the cells were grown under glucose for 12 hours prior to being exposed to starvation to be able to detect at

least one round of cell cycle. After glucose growth, the cells were exposed to starvation for 6 hours just as our previous experiments. The cells could still re-enter Whi5 back into their nuclei even in the presence of overly expressed Cln3. Due to the length of the cell cycle duration, the re-entry slopes were not calculated and the re-entries were not classified accordingly to the cell cycle progression. Instead, an example cell was used to demonstrate that the re-entries could still take place.

After showing that nutrient-sensitive transcriptional factors were not essential for the cell cycle reversal and overall Whi5 re-entries, using Cln1 we also showed that cyclin repression was not needed. Since we could largely exclude transcriptional control over the cyclins, we next decided to change our approach and investigate post-transcriptional mechanisms. Thus, we started with the kinases which are involved in the nutrient metabolism.

### 3.2.8 Nutrient Responsive Kinases Snf1 and Rim15

As we showed that transcriptional regulation was not the primary target on Whi5 re-entries, we decided to investigate post-translational mechanisms. Phosphorylations are the most prominent for the cell cycle progression and thus kinases are important for signalling. The most prominent kinases for starvation signalling are Snf1 and Rim15, which are known to be involved in the nutrient mechanism and known to be responding to carbon limitation [Broach, 2012]. We decided to check their potential role in the Whi5 re-entries and cell cycle reversal.

Rim15, also known as the Greatwall Kinase, is the main regulator in the transition to quiescence. It mainly integrates signals coming from several nutrient-sensing kinases and pathways such as PKA and TOR which are known to be causing early G1 arrest when inactivated [Cameroni et al., 2004], [Swinnen et al., 2006]. Rim15 contributes to the regulation of cell cycle progression both directly and indirectly by leading to the transcription of some genes under nutrient stress and starvation. Also, $\Delta$rim15 strain are shown to pass *Start* even under poor nutrient conditions, suggesting Rim15 plays a crucial role in the coordination of nutrient signals before the cell cycle commitment decision takes place [Bisschops et al., 2014]. Additionally, Rim15 contributes to the transition into G0 from the end of the previous round of cell cycle

[Bisschops et al., 2014]. It has been shown recently that, the amount of Rim15 present in the cell when exposed to starvation, can be predictive on the cells fate after the starvation period is over and glucose is replenished [Wood et al., 2020]. As its role in nutrient signalling and cell decision making process was considered, we thought Rim15 would be a good candidate to investigate the reversal of *Start* and nuclear Whi5 re-imports.

Snf1 (Sucrose Non-Fermenting 1), is a protein kinase homologous to AMPK in mammalian cells which is conserved among the eukaryotic family. Snf1 is involved in the cellular responses for both nutritional and environmental stress conditions. Snf1 activity is directly controlled by glucose signalling and it is known that Snf1 phosphorylates its targets during carbon limitation. Snf1 coordinates the starvation response by suppressing many genes under glucose limitation, and also leading to a more than 100-fold increase in the expression of several other genes [Jiang and Carlson, 1996]. Snf1 pathway controls glucose signalling in budding yeast together with other signalling pathways. Although Snf1 regulates a limited number of genes, it mediates a significant branch of the glucose repression mechanism not subject to PKA regulation [Zaman et al., 2009]. In addition to its role in stress response, Snf1 is also found to be involved in the cell cycle regulation. Its absence is shown to be down-regulating Clb5 expression and activity which points out the crucial role of Snf1 in the positive regulation the G1/S transition [Pessina et al., 2010]. This G1/S regulation was then suggested to be through the initiation of the binding of Swi4, Mbp1, and Swi6 to the early G1 promoter elements SCB and MCB [Busnelli et al., 2013]. Due to its high involvement in carbon metabolism and starvation responses, we decided to test Snf1 as a reasonable candidate for the cells to go back to a pre-*Start* stage as a response to acute glucose starvation.

To test the possible roles of these two kinases on Whi5 re-entries and the reversibility of *Start*, we created $\Delta$rim15 and $\Delta$snf1 mutant strains and tracked nuclear Whi5 signal during acute glucose starvation using time-lapse microscopy. Before, exposing them to starvation conditions, we first tested for their viability and potential cell cycle defects by growing them on rich glucose media. It was seen that both strains were viable. Additionally, no increase in death rate was observed. We detected that the cell cycle

progression of Δrim15 mutant was slower compared to the wild-type, however we saw that the cells could still cycle regularly without showing any sign of cell cycle defect. Additionally, nuclear Whi5 signal was in line with the cell cycle progression and did not show any unexpected fluctuating behaviour. Therefore, we decided to use this strain in the acute starvation experiments to test their effects on the Whi5 re-entry dynamics. The cell cycle duration of the Δsnf1 mutant strain, however, was very similar to the wild-type strain. Just like the Δrim15 mutant, no additional death rate was observed, and the cells were viable. Therefore, we concluded, that under glucose, the Δsnf1 mutant proceeds with its cell cycle progression in its normal course. This observation was also compatible with the previous findings stating that the negative effects on the cell cycle progression resulting from Snf1 deletion may be kept at minimum by using glucose rich medium [Pessina et al., 2010].

Using the mutant strains, acute starvation experiments were performed. The strains were grown in glucose minimum media for eight hours before being exposed to acute glucose deprivation for six hours. Nuclear Whi5 re-entry frequencies were calculated for the cells that had Whi5 re-entry slopes higher than 200.

The Whi5 re-entry frequencies were calculated and compared to the wild-type strain (Figure 22a). We saw, that the deletion of the two kinases did not completely stop the cells from re-entering Whi5. However, when the Whi5 re-entry profiles were analysed for the two mutants and compared to the wild-type strain, we detected, that the re-entry probabilities in the Rim15 deletion strain was higher. However, the average cell cycle duration of the Δ mutant was found to be approximately 30 minutes longer than the wild-type strain. Due to this difference in the cell cycle durations, the re-entry frequencies were also delayed by 30 minutes within the cell cycle progression. As a result, the Whi5 re-entries were observed for a longer period of time after *Start* and were higher within the first 25 minutes (Figure 19a). However, the probability graph had the same pattern as the wild-type strain which suggested that Rim15 did not have a change in the Whi5 re-entries when the cell cycle durations were considered. Our data showed, that even though Rim15 might be playing a role in the long-term starvation response of the cells, it did not affect the acute reaction of cell cycle reversal.

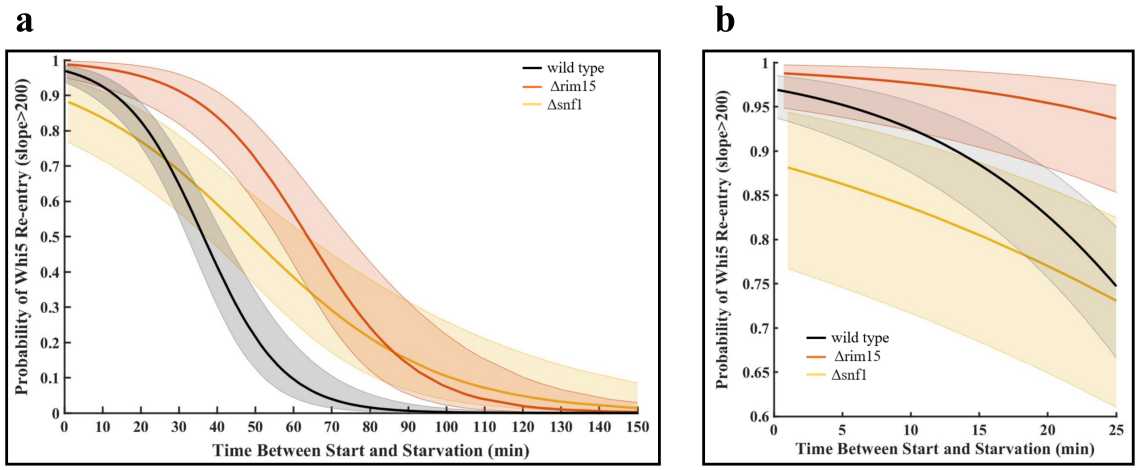


**Figure 19:** Whi5 re-entries above the slope of 200 in the wild-type strain, Δrim15 and Δsnf1 mutants

Cells are grown and starved as explained in figure 6. The binary data (re-entry yes/no) if the re-entry slope>200 from n>100 cells per strain was fit to a logistic regression to estimate the re-entry probabilities as a function of time passed between *Start* and being exposed to starvation. The re-entries within the first 25 minutes are considered as the reversal of *Start*. The re-entries after 25 minutes were classified as regular re-entries. The wild-type probability is estimated (black)and compared to the mutant strains. a) Δrim15 strain (orange) shows a shift in the graph only as a result of its longer cell cycle duration. Δsnf1(yellow) results in a more gradual decrease than the wild-type strain. Shaded areas represent 95 % confidence intervals of the regression. b) Δrim15 cells (orange) reverse *Start* with a similar pattern, but shows a shift because of the extended cell cycle duration. Δsnf1 (yellow)result in more gradual decrease in reversing *Start*, than the wild-type strain.

However, the Δsnf1 mutant strain showed a different Whi5 re-entry probability pattern than the wild-type and Δrim15 mutant strains. Even though the probability decreased gradually, Snf1 mutants could re-import Whi5 back into their nucleus until the end of their cell cycle progression with slopes higher than 200 (Figure 19a). We saw that the Whi5 re-entry probability within the first 25 minutes was lower than the wild-type strain. Unlike the wild-type strain, even within the first five minutes the re-entry frequency was lower than 0.9 (Figure 19b). The re-entry frequency then became equal to the wild-type strain after the 26th minute. After that point, the Whi5 re-entry probabilities always stayed higher for the Δsnf1 mutant strain and in contrast to the wild-type strain, the decrease in the probabilities were more gradual compared to the relatively steeper decrease within the wild-type strain (Figure 20).

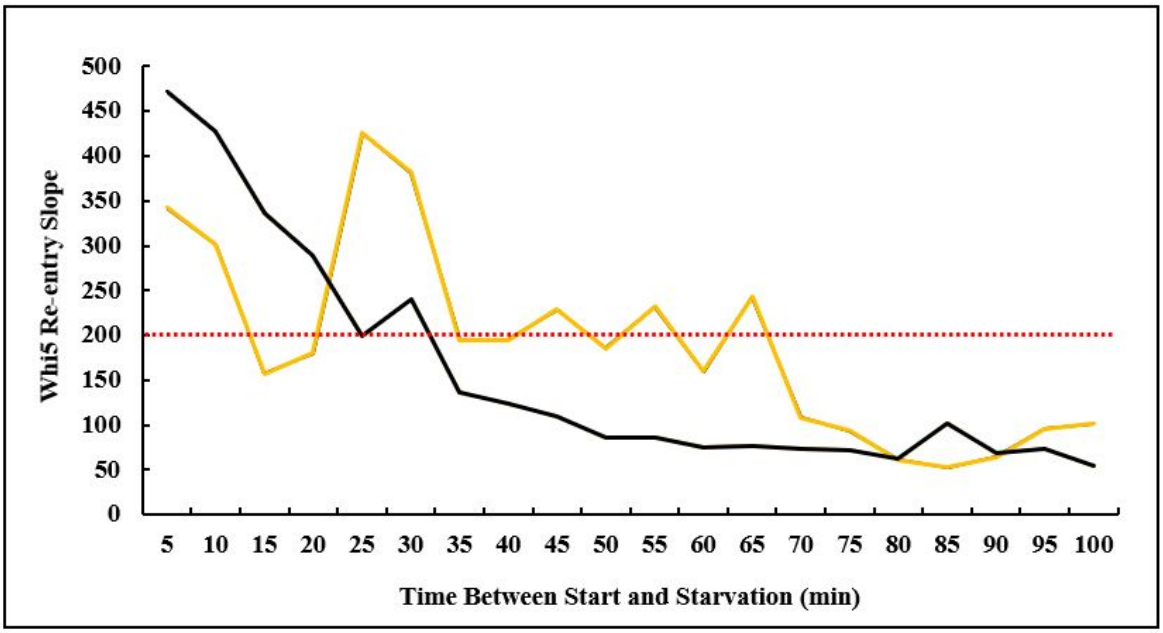


**Figure 20:** Whi5 re-entry slopes of the wild-type strain and Δsnf1 changing with time

The changes in Whi5 re-entry slopes of Δsnf1 mutant and wild-type strains analysed in Figure 19, are shown against time. The black line shows the wild-type strain. The yellow line shows Δsnf1 mutant.

Another interesting thing was that not all Snf1 re-entries fit to the 200 slope classification. We detected that some cells could re-enter Whi5 very fast and steep even when they were visibly more progressed in their cell cycle with a big bud. Additionally, more cells within the first 25 minutes re-imported Whi5 more gradually and with lower slopes than 200 compared to the wild-type strain. This indicated that, in addition to its effect on the frequency, Snf1 might also be affecting the re-entries mechanistically, causing the two types of mechanistically different re-entries to be more indistinguishable. As a result, we concluded that even though Snf1 did not completely stop the cells to reverse their cell cycle or entering Whi5 back into the nuclei, it decreased the frequency within the first 25 minutes and caused some cells to re-enter Whi5 in a mechanistically different way. Thus, in the future the strain should be tested using mating pheromones, to see if the mechanistically different re-entries could also result in changing the sensitivity towards mating pheromones.

### 3.2.9 Whi5 is not Directly Targeted

We found that the CDK positive feedback loop was not interrupted through cyclin repression. Thus, we decided to focus on the other members of the positive feedback loop. In the previous chapter, we detected the possible effect of Snf1 on the probability and dynamics of Whi5 re-entries. To test if Snf1 or any other kinases indirectly act on Whi5, or if Whi5 was being directly targeted by another regulator, we first decided to focus on Whi5 itself and analyse it further.

Whi5 has a total of 19 previously well- characterized phosphorylation sites. 12 out of the 19 identified sites are found to be phosphorylated by Cdk1 and their roles were discovered and investigated thoroughly [Costanzo et al., 2004], [Smolka et al., 2007], [Huber et al., 2009], [Wagner et al., 2009], [Bodenmiller et al., 2010]. It is known that phosphorylation of these sites leads to the nuclear export of Whi5 at *Start*. The phosphorylation of four of these phosphosites leads to the dissociation of Whi5 from the transcriptional factor complex SBF where the other eight are thought to be leading to the inactivation of Whi5. However, evidence suggests that Whi5 translocation and activity may be more complicated and multi-layered than solely being directly controlled by Cdk1. Unlike the CDK phosphorylation sites, the function and the role of the remaining seven non-CDK phosphosites (S78, S113, S115, S149, S276, S281, S288) on Whi5 and on the cell cycle progression is not completely understood [Smolka et al., 2007], [Chi et al., 2007], [Albuquerque et al., 2008], [Wagner et al., 2009], [Holt et al., 2009], [Bodenmiller et al., 2010]. In addition to the identified sites, Whi5 also has six additional uncharacterised phospho-sites that have not been characterized yet. It has been suggested that several kinases may be physically interacting with Whi5 directly through the non-CDK sites. Research indicates that a more complex kinase system may also be contributing to the changes in the Whi5 activity in response to metabolic and nutrient signal integration [Hasan et al., 2014] Thus, there are strong indications that Whi5 activity and localisation may not be only controlled through Cdk1 phosphorylation.

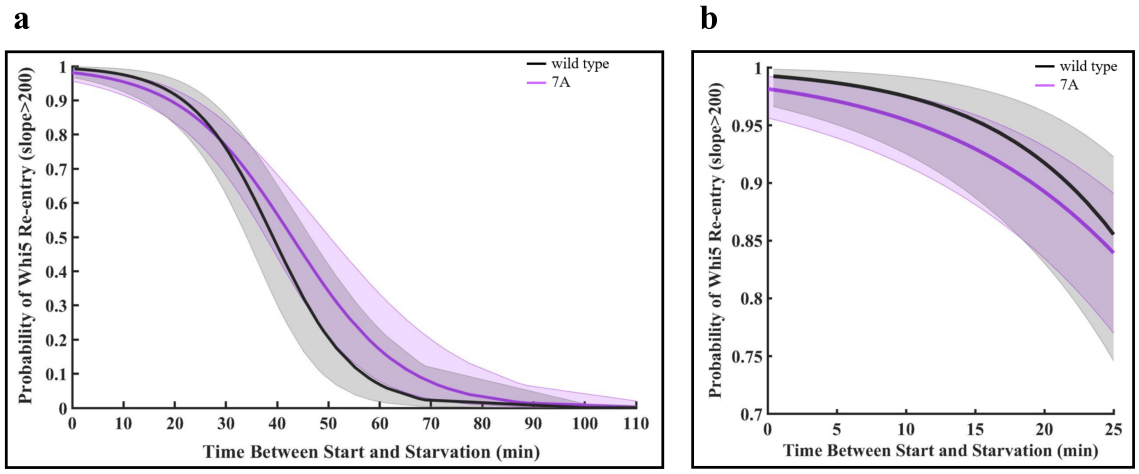


**Figure 21:** Whi5 re-entries above the slope of 200 in the wild-type strain and non-CDK phosphorylation mutant

Cells are grown and starved as explained in figure 6. a) The binary data (re-entry yes/no) if the re-entry slope>200 from n>100 cells per strain is fit to a logistic regression to estimate the re-entry probabilities as a function of time passed between *Start* and being exposed to starvation. The re-entries within the first 25 minutes are considered as the reversal of *Start*. The re-entries after 25 minutes are classified as regular re-entries. The wild-type probability is estimated (black) and compared to the mutant strains. a) 7A non-CDK phospho-site mutant strain (purple) has a similar Whi5 re-entry probability profile. Shaded areas represent 95 % confidence intervals of the regression. b) Mutating the seven non-CDK sites does not affect the probability of reversing *Start*.

To see if direct phosphorylation of Whi5 from the non-CDK sites leads to its nuclear import during acute glucose starvation, we decided to deactivate these seven phospho-sites by replacing the serine residues with alanine. The cells were first grown under glucose to see if the cells were viable. It was seen that the cells were healthy and cycle regularly without showing any cell cycle defects. The cell cycle duration was calculated as for the wild-type strain. Additionally, we did not detect any different phenotypes compared to the wild-type strain.

As we detected that the seven non-CDK mutant was viable and healthy, acute glucose starvation experiments were preformed. The cell cycle dependent probabilities of Whi5 re-entries and the reversal of *Start* were calculated and compared to the wild-type strain. Deleting the non-CDK sites had no effect on re -entering Whi5 (Figure 21a). We also saw, that within the first 25 minute time frame after passing *Start*, the Whi5 re-entry probabilities were the same (Figure 21b). Therefore we concluded, mutating non-CDK phosphorylation sites on Whi5 did not have any inhibitory effect.

In addition to not observing any effect of deleting the seven non-CDK sites on reversing *Start* and re-entering Whi5, we found it notable that these phospho-sites also did not have any influence on how Whi5 cycled on glucose. We detected no delay in the nuclear import and export patterns or timings. Additionally, the cell cycle duration and the time required for bud initiation was the same as the wild-type strain. As we combine the results for the Whi5 re-entries and the cell cycle progression under glucose feed, we concluded that the non-CDK sites did not have any visible effect on the cell cycle. Thus, the role of these sites remains unknown.

### 3.2.10 CDK-Cyclin Inhibitors Sic1 and Cip1 are not Required for Whi5 Re-entries

As explained in the previous chapters, the positive CDK loop defining the *Start* checkpoint is initiated by the activation of CDK-cyclin complexes. Additionally, the nuclear localization of Whi5 over the course of the cell cycle progression is determined by the phosphorylation states of the Cdk1 phospho-sites. As we demonstrated that neither repression of cyclins nor phosphorylation of Whi5 was the reason why the positive feedback loop was interrupted, we then hypothesized that inhibition of Cdk1-Cyclin complex activity could lead to the cell cycle reversals. To test our hypothesis, we decided to check the role of two well known CDK inhibitors, Sic1 and Cip1, that are involved in the G1/S transition.

Sic1 is a well known stoichiometric inhibitor of Cdk1-B-type cyclin complexes which regulate the transition into S phase from G1 [Schwob et al., 1994]. The expression of Sic1 is initiated after the cell passes the *Start* checkpoint, then Cdk1 forms complexes with the B-type cyclins Clb5 and Clb6. Soon after, Sic1 is phosphorylated by the transcription complex SBF which results in Sic1 degradation and in return initiates the transition towards S phase [Tyers, 1996] The degradation of Sic1 takes place through a multi-step process involving many regulators including the early G1 cyclins [Köivomägi et al., 2011]. In addition to its role as a cell cycle inhibitor, multiple research indicate that Sic1 may be a key player leading to the integration of metabolic signals into the cell cycle [Rossi et al., 2005], [Kono et al., 2016]. It was

shown, that Sic1 is directly targeted and in return, phosphorylated by the kinase Hog1 as a response to the osmotic stress conditions. This phosphorylation then leads to the stabilization of Sic1, thus, leading to G1 arrest during the period of the osmotic stress [Escoté et al., 2004].

Cip1 is an inhibitor of Cdk1-cyclin complexes which only interacts with the complexes Cdk1 forms with early G1 cyclins Cln1, Cln2, and Cln3. It has been shown that Cip1 does not interact with B-type cyclin complexes or M-phase cyclins, which makes it a down-regulator of early cell cycle [Ren et al., 2016]. Cip1 was also shown to function in a similar way as the mammalian tumour suppressor p21 as it responds to environmental stress and its overexpression initiates cell cycle arrest during G1 through the inhibition of all G1-specific cyclins. Members of the Cip/Kip family, including Dacapo and p27, control multiple cyclin–Cdk1 complexes by interacting with both the cyclin and Cdk [Morgan, 2007]. Additionally, Cip1 stops inactivation of Whi5 by leading to the downregulation of Cln3-Cdk1 activity and as a result prevents the cells to go through G1/S transition. The Whi5 inactivation and the stress dependent G1 arrest was found to be independent of Sic1, which makes Cip1 the main inhibitory factor. The regulation of Cip1 is achieved through the stress mediated transcriptional activators Msn2 and Msn4 together with the cell cycle mediated factor Mcm1. This strongly indicates that Cip1 might be regulated through both metabolic and cell cycle signals. Under osmotic stress conditions, Cip1 is also directly phosphorylated by Hog1 to additionally stop Cdk1 activity. Msn2,4 complexes are also found to be responsible for the expression of the inhibitory protein Cip1. Cip1 expression results in the inhibition of all G1 Cdk1-Cln complexes under environmental stress, resulting in a delay in G1 progression, and in extreme stress conditions, also resulting in G1 arrest [Chang et al., 2017]. However, it is shown that in $\Delta$snf1 mutants, the nutrient stress response regulated through Msn2 does not show any difference which indicates that Cip1 transcription by Msn2 is most likely independent of Snf1 [Jiang et al., 2017]. It was found recently that Cip1 also acts as an additional negative regulator of *Start*. In addition to the cyclin-CDK complexes, Cip1 also directly acts on a second pathway, namely Ccr4-Caf120 for transcriptional Whi5 downregulation and thus indirectly on the positive CDK feedback loop that initiates *Start*. These research strongly indicated

that Cip1 may have other unknown functions than only being just a cyclin-CDK regulator protein [Li et al., 2020]. To sum up, Cip1 helps to regulate *Start* by combining cell cycle elements with metabolic cues by targeting the CDK complexes and other secondary pathways.

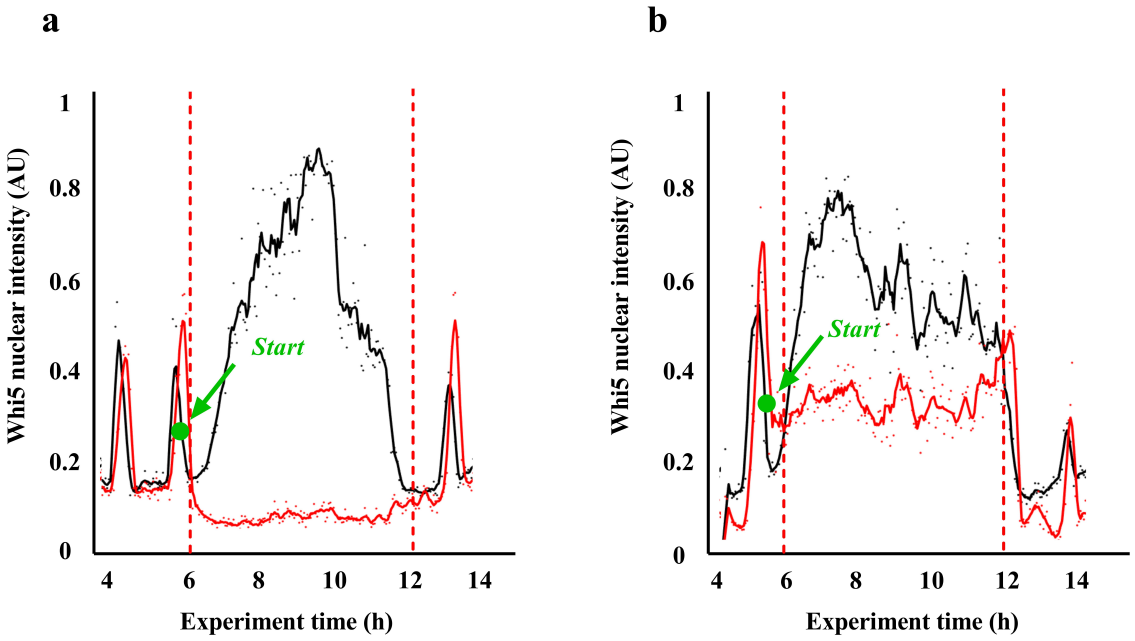


**Figure 22:** Example Sic1 traces during Whi5 re-entries under glucose starvation

Cells are grown and starved as explained in figure 6. The traces show cells that re-entered Whi5 with a slope higher than 200. The red trace shows total Sic1-mNeongreen signal. The black trace shows nuclear Whi5-mCherry signal. a) Sic1 is completely degraded when the cell re-entered Whi5. Sic1 intensity stayed zero during the whole starvation period. b) Sic1 intensity stayed at the level it reached before starvation and stayed at that level during the starvation period.

As a first step, we decided to test if Sic1 stabilization was essential for re-entering Whi5. We endogenously tagged Sic1 with mNeongreen and monitored it together with Whi5 in acute starvation experiments. The total signal intensity during the onset of starvation and when Whi5 re-entries took place was tracked. The time between *Start* and complete Sic1 degradation under glucose media was calculated. This value was found on average as 22 minutes and the time required for Sic1 intensity to reach its maximum was found to be 7 minutes. Then, acute starvation experiments were performed and 220 cells from two independent experiments were analysed. We found that the re-entries did not require Sic1 stabilization. Our data shows that Whi5 re-entries could

take place with a slope higher than 200 in the absence of Sic1 stabilization. In all the cells in which the time between starvation and *Start* was more than 10 minutes, Sic1 is degraded completely (Figure 22a). If the cells were exposed to starvation within the first 10 minutes, Sic1 level stays high throughout the whole starvation period (Figure 22b). However, in none of the cells, any Sic1 increase during starvation was observed. As a result, we concluded, that Sic1 concentration was not predictive of whether or not a cell reverses *Start*.

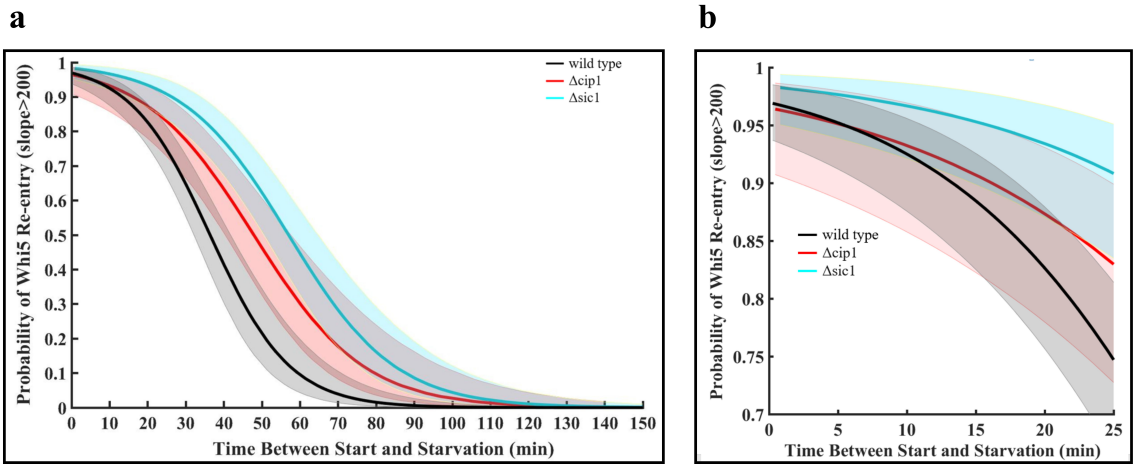


**Figure 23:** Whi5 re-entries above the slope of 200 in the wild-type strain, Δcip1 and Δsic1 mutants

Cells are grown and starved as explained in figure 6. a) The binary data (re-entry yes/no) if the re-entry slope>200 from n>100 cells per strain is fit to a logistic regression to estimate the re-entry probabilities as a function of time passed between *Start* and being exposed to starvation. The re-entries within the first 25 minutes are considered as the reversal of *Start*. The re-entries after 25 minutes are classified as regular re-entries. The wild-type probability is estimated (black) and compared to the mutant strains. a) Δcip1 strain (red) shows a similar re-entry profile as the wild type strain. After the 15th minute, a slight increase in the Whi5 re-entries is observed. Due to its longer cell cycle duration Δsic1 (turquoise) re-enters Whi5 for a longer period of time which causes a shift in its probability graph. Shaded areas represent 95 % confidence intervals of the regression. b) Δcip1 cells (turquoise) reverse *Start* with a slightly increased probability. Its extended cell cycle duration causes Δsic1 (turquoise) cells to re-enter Whi5 with a higher frequency within the first 25 minutes.

After demonstrating that Sic1 stabilization was not required for the reversal of *Start*, we then tested if deleting Sic1 would change the re-entry probabilities. Growth under glucose showed that the cells could cycle regularly, but 22 minutes slower than the

wild-type cells. Also, the time required for bud initiation for the Sic1 deficient cells were found to be slightly longer than the wild-type strain. However, the cells could cycle without showing any defect. Then, the strain was tested against acute starvation. 185 post-*Start* cells from two independent experiments were analysed and the Whi5 re-entry probabilities were calculated over time. The data showed, that as the pre-*Start* stage took longer, the $\Delta$sic1 mutant strain could re-enter Whi5 longer with an approximately 20 minute shift. However, the re-entry pattern was similar to the wild-type strain. Thus, we concluded that Sic1 did not cause any visible changes in the re-entry probability frequencies.

After showing that Sic1 was not involved in the cell cycle reversals, we then tested the cyclin-Cdk1 inhibitor (CKI), Cip1. The average cell cycle duration of the $\Delta$cip1 mutant was the same as the wild-type strain and it did not show any cell cycle defects. As the cells were healthy and viable, we then tested them against acute starvation conditions. 200 *Start* cells from three independent experiments were chosen for statistical analysis. Within the first 7 minutes, the re-entry frequencies were the same as the wild-type strain. Within the first 25 minutes, the re-entry probability was calculated as 83 % where it was 75 % for the wild-type strain. After 25 minutes, it always remained higher than the wild-type strain and reached zero after the 110th minute. The data demonstrated a slight positive effect of deleting Cip1 on the re-entry and cell cycle reversal possibilities. During none of the stages of the cell cycle progression, an inhibitory effect of Cip1 on re-importing Whi5 was observed.

When our results were combined, we concluded that CDK activity was not inhibited through the inhibitory proteins Cip1 and Sic1. If and how the CDK activity is affected when the cells reverse their cell cycle, should be further investigated in the future.

# 4 Discussion

## 4.1 Post-*Start* Cells Exhibit a Cell Cycle Dependent Response to Starvation

Yeast cells integrate internal and external signals into the cell cycle commitment point called *Start* checkpoint. Cells can delay or accelerate *Start* depending on the nutrient availability, and thereby integrate nutrient signalling into the *Start* decision. However, how the cell integrates nutrient signals after passing *Start* is still poorly understood. Thus, in this project we aimed to investigate how post-*Start* cells respond to nutrient signals by imposing acute starvation.

In order to understand the cell cycle dependent responses to nutrient stress, we avoided population-based approaches and adapted a fluorescence imaging based single-cell analysis method to track the cell cycle progression precisely without synchronizing the cells. To distinguish between the post-*Start* and pre-*Start* cells, the nuclear localization of the cell cycle inhibitor Whi5 was used, as 50 % of nuclear export marks *Start* [Doncic et al., 2011].

We showed that the cells could respond to acute glucose deprivation even after passing-*Start*, in agreement with recent publications suggesting that cells can respond to nutrient starvation during all stages of their cell cycle progression [Wood et al., 2020], [Argüello-Miranda et al., 2021]. We detected that the cells respond to glucose starvation by either delaying their cell cycle or permanently arresting until glucose replenishment. We determined that the responses depend on the cell cycle progression. We showed that since the earlier the cells were exposed to starvation, the more likely they were to enter a stable permanent arrest. Independent of the arrest or delay stage, almost all the cells continued with their cell cycle normally after glucose was supplied back. This shows that the reaction towards acute glucose deprivation, the interruption of the cell cycle was a specific response to starvation and completely reversible. Additionally, the death rate during and after starvation was seen to be approximately the same as during glucose conditions. However, it was seen that if the starvation period exceeded 10 hours, the cell death during and after the starvation started increasing. This could be either due to a gradual decrease in viability during long-term starvation,

or technical difficulties with the microfluidic platform or phototoxicity.

We showed that the yeast cells could also receive nutrient signals even after passing *Start* and integrate these signals into their cell cycle to give a cell cycle progression dependent response. The cell cycle dependency showed that the less the cell was progressed in the cell cycle, the more likely that it will chose to arrest. This might be due to its attempt to protect itself from DNA damage or cell cycle defects. If the cell has not started DNA replication or another crucial event, it might decide to arrest at an early cell cycle stage before an important cell cycle event starts, avoid finishing in the lack of nutrients. Even though we saw the effect of cell cycle progression, we also detected that the cells did not give homogeneous responses. If the cells at further stages are on the edge of starting a cell cycle event, they might decide to arrest at a gap phase to make sure the event will proceed during high nutrient conditions. Our data strongly suggests a crosstalk between metabolism and the cell cycle during all stages of the cell cycle progression and we thus investigated the molecular basis of how it is achieved. To understand the mechanism behind the starvation reaction types, we first decided to start by understanding how the cells go into a stable arrest.

## 4.2 The Feedback Loop that Defines *Start* is Interrupted and Cells can Reverse *Start* Under Acute Starvation

We found, that, when exposed to acute glucose starvation, some cells could arrest by re-entering the key cell cycle inhibitor of the CDK positive feedback loop, Whi5. This phenomenon was also observed in three recent research papers, however the underlying molecular mechanism and the biological significance has not been unveiled [Qu et al., 2019],[Yang et al., 2020], [Wood et al., 2020].

For eukaryotic cells, the safest cell cycle stage is considered G1 phase which is also known as the first gap phase of the cell in which DNA replication or any other crucial cell cycle event has not been initiated yet [Morgan, 2007]. Re-entering Whi5 strongly suggested the reversibility of the feedback loop and thus the *Start* checkpoint. We also found that the probability of reversing *Start* depended on the cell cycle progression. Within the first five minutes, 95 % of the cells responded by re-entering Whi5. As the cells progressed more, the probability of re-entering Whi5 started decreasing, but

re-entries occurred until the end of the cell cycle. Our data also indicated that even the cells that passed the cell cycle commitment point *Start*, could go back to a pre-*Start* stage when exposed to extreme glucose limitation conditions.

We showed, that the more the cell is progressed the less likely it would re-enter Whi5. The main goal of the cells that re-enter Whi5 and arrest at G1 when exposed to acute starvation is to protect its DNA and ensure a properly regulated cell cycle. Thus, it is logical to assume, if the DNA replication is already initiated when the cell faces glucose limitation, the cell chooses not to reverse its cell cycle and re-initiate DNA replication for the second time. This could make the cell more vulnerable to the effects of nutrient limitation and could potentially result in defects in DNA replication. As a result, we hypothesized, DNA replication might be the determining point in the cells decision to reverse *Start* or not.

The closer the cells were to *Start*, the more likely they would re-enter Whi5. This showed the impact of the cell cycle progression state on the cells decision to re-enter Whi5 or not. This also supports our hypothesis that the cells can choose to arrest at G1 if they did not initiate a crucial cell cycle event such as DNA replication. However, in addition to the cells that were early at their cell cycle progression, there were cells that could re-import Whi5 into the nucleus even during the very final stages of the cell cycle. Reversing *Start* from a very late stage of the cell cycle meant reversing multiple crucial cell cycle events. Thus, we suspected that there might be more than one type of Whi5 re-entries with different biological indications.

After investigating the Whi5 re-entries further, we found that cells at later stages of the cell cycle and with visibly big buds re-entered Whi5 slower and more gradually than the cells that were earlier in their cell cycle progression. Also, we detected that nuclear Whi5 intensity after the re-entry was lower than during the pre-*Start* phase. For the first group of cells, re-entering Whi5 could be as fast as three minutes whereas for the gradual re-entries this time could be extended to even more than 45 minutes. This firstly indicated, that the cells at earlier stages, gave a faster and more immediate response to nutrient depletion, meaning that these cells respond to starvation more quickly. This may imply the existence of a fast emergency response. The reason why the cells that are at later stages of the cell cycle re-enter Whi5 slower

might be that the S-phase CDK complexes are less sensitive to nutrient signalling. Thus, the cells might be progressing through S phase until the cyclins Clb2 and Clb3 are expressed, which are more sensitive to the nutrient signalling [Broach, 2012], [Conrad et al., 2014], [Ewald, 2018], [Bharatula and Broach, 2018]. As a result, the time difference might be due to the time the cell spends in the S phase. Thus, if there are more than one type of Whi5 re-entries through different response mechanisms, the biological consequences would also not be the same. To understand the indications of the Whi5 re-entries, we analysed the cells further. We first tested if the onset of G1/S transition might be the determining point. In yeast, unlike mammals, unfortunately there is not a reliable S phase marker [Easwaran et al., 2005]. However, as the transition of S phase and the initiation of DNA replication is coupled, core histone proteins are commonly used to detect the start of DNA replication, and thus the beginning of the S phase under glucose. For that purpose, we used the core histone Htb2 to detect the initiation of DNA replication. However, under starvation, we detected that the intensity of the histone signal increased independently from the cell cycle stage the cell was in. This can be due to the post-translational modifications on the histone protein resulting from the poor nutrient conditions [Lee et al., 2021]. To test if this was a fluorescence artefact, we tested additional fluorescent proteins. However we still encountered with the same problem even when using Htb1 instead of Htb2. Therefore, we could not track the initiation of DNA replication under starvation. To understand if DNA replication is the key factor distinguishing the Whi5 re-entries, in the future a more reliable S phase marker should be adapted.

As the first difference among the Whi5 re-import mechanisms we noticed was the slope of the re-entry, and so we finally decided to focus on the slopes of Whi5 translocations. We detected that almost all the cells which were further than 25 minutes from passing *Start* and had visibly big buds, re-entered Whi5 with slopes lower than 200. With this we showed that the qualitatively different Whi5 re-entries indeed depend on the cell cycle progression.

We used the slope of the Whi5 re-entry to classify cells into two groups, which we consider to be pre- and post-replication initiation cells. We hypothesized, that those cells with steep Whi5 re-entries were post-*Start*, but before replication initiation and

were re-importing Whi5 to reverse *Start*. We provide two different lines of evidence that these cells were indeed reversing back the positive feedback loop of *Start*.

Firstly, we performed mating pheromone experiments and found that some cells could reverse *Start*. As explained in the previous chapters in more detail, *Start* was first defined as the point where the cells no longer respond to pheromone signalling and instead prioritize division and the completion of the cell cycle [Hartwell et al., 1974]. We showed, that the cells at earlier stages of their cell cycle progression and, re-entered Whi5 fast and steep were more responsive to the mating pheromones. We detected that the average re-entry slopes of the cells that shmood was 3.5 fold higher than the cells that did not shmoo. We also detected, that almost all the cells that responded to the mating pheromones fit the 200 slope threshold we previously set. This showed that the slope threshold indeed held true in reversing *Start*.

However, our results also showed additional factors affecting the cells´ decisions to shmoo. The percentage of the shmooing cells for the regular pre-*Start* was 97 % indicating that almost all the cells were responsive. However, for cells with Whi5 re-entries, we saw that the percentage of shmooing cells never exceeded 65 % even within as early as five minutes. Additionally, we found that none of the cells that initiated budding were sensitive to the mating pheromones. This suggests, that cells that choose division may have a remaining memory from the previous pre-starvation CDK activation leading to choosing cell cycle progression over shmooing. Additionally, we detected that none of the cells that initiated budding showed sensitivity to mating pheromones. These strongly suggest that the cells might have previous memories from the previously activated CDK loop.

It has been shown that yeast cells can keep memories of the past, that in return affect cell cycle related decisions. This is especially apparent in the case of exposure to mating pheromones. The memory keeping mechanism is so robust that it can even be transferred to the daughter cell [Caudron and Barral, 2013]. The daughter cells formed after transient pheromone exposure tend to be unresponsive to the mating pheromones, whereas long term exposure of mating pheromones leads to shmooing daughters [Doncic et al., 2015], [Reichert and Caudron, 2021]. The cells choose mating over cell cycle progression through the inhibitor protein Far1 which inhibits the

activity of G1 cyclins Cln1 and Cln2. However, if the cell still has memories from the previous CDK activation, it might surpass the inactivation caused by Far1. To test this idea, the cell cycle progression during pre-starvation should be monitored more precisely and carefully to understand during which stage the cell cycle and the positive feedback loop is interrupted. Additionally, the cells should also be exposed to mating pheromones with higher and lower concentrations than we used in the experiments to see if there is a threshold variability among the cells that re-enter Whi5 and the regular G1 pre-*Start* cells. This could give us an idea on how the competition between the cell cycle progression and the mating pathway differs among the two groups of cells.

Our second piece of evidence for *Start* reversal comes from Cln2 promoter activity: using Cln2 promoter activity reporters, we showed that the cells that reversed *Start*, went through the CDK feedback loop once again, when the nutrient conditions were improved. With this, we proved that, the cells which reversed *Start*, stayed at the pre-*Start* stage they went to during the starvation period. They, then, after glucose was replenished, initiated *Start* for the second time. In contrast, all cells that re-entered Whi5 with slopes lower than 200 and at late stages did not show any Cln2 promoter activity, which confirms the distinction between these groups of cells. Cells with steep Whi5 re-entries and small buds seem to represent a special case. While we did not see shmooing in the cells that initiated budding, we saw Cln2 promoter activity in the cells that already had buds. This also confirms our hypothesis of previous cell memory resulting in the insensitivity towards the mating pheromones.

Taken together, the results of the two experimental approaches strongly imply that post- *Start* cells can reverse their cell cycle when exposed to acute nutrient depletion. Thus, the currently accepted notion of having only one irreversible single commitment point is in fact not true vis a vis nutrient perturbations. We showed that the current model of CDK activation at *Start* is not sufficient to explain the irreversibility of the G1/S transition. However, by the definition of *Start*, it has been considered as the point of no return even under stress conditions [Morgan, 2007]. Therefore, our findings strongly challenged the concept of a one-step irreversible cell cycle commitment. Thus, there might exist a multi-level commitment system.

## 4.3 The Underlying Mechanism Behind the Reversibility of *Start*

In the previous part of the project, we showed that cell commitment in yeast could be reversed under acute carbon starvation. Having established that, we then focused on uncovering the mechanism behind the Whi5 re-entries and cell cycle reversals.

We first hypothesized that the CDK positive feedback loop could be interrupted through the transcriptional repression of G1 cyclins. For that purpose, we tested five transcriptional regulator proteins that are activated during glucose limitation and known to be involved in the regulation of nutrient responses. Out of these five, we found that Xbp1, Msa1, and Msa2 were not responsible for primary signals for Whi5 re-entries. Even though they are important in the general starvation response [Miles et al., 2013], [Miles et al., 2016], [Sagot and Laporte, 2019], deletion of these three transcription factors did not stop the Whi5 re-entries. However, we detected an interesting Whi5 re-entry profile in the Δmsn2,4 strain. We saw that deleting these two transcriptional factors, which can act as activators and repressors of many genes including Cln1 and Cln2, resulted in a slight increase in the re-entry probability throughout the whole cell cycle progression period. We saw, unlike the wild-type strain, the Δmsn2,4 strain could re-enter Whi5 with high slopes at a higher frequency even when having visibly big buds.

In conclusion, we tested five transcription factors linked to starvation and quiescence, but none of them prevented the Whi5 re-entries or the reversal of *Start*. To test if another transcriptional regulator or regulators could be involved in the repression of cyclin expression, we used a strain in which Cln1 was constitutively expressed from a strong synthetic promoter. We detected, that these cells could still re-enter Whi5. Thus, we concluded, that cyclin repression was not necessary for the cells to reverse *Start* and the CDK positive feedback loop was most likely not interrupted through the repression of cyclin expression.

As the transcriptional regulation did not seem to be essential, we decided to investigate the role of kinases known to be involved in starvation signalling. We constructed deletion mutants for the kinases Snf1 and Rim15 which are involved in nutrient regulation and the entry to quiescence [Broach, 2012]. The Rim15 mutant showed that

the probability of re-entering had a similar pattern to the wild-type strain under acute starvation, with a half an hour shift due to the elongated cell cycle durations. Thus, we concluded that Rim15 does not play a role in reversing the cell cycle. However, the Δsnf1 mutant strain showed a re-entry probability profile that was different than the wild-type strain. While the re-entry probability in the early cell cycle was slightly lower, throughout the remaining stages of the cell cycle progression the probability did not decrease as sharply as in the wild-type strain, remained the same between the 35th to 70th minutes, and never completely reached zero.

Additionally, we also noticed that the Δsnf1 mutant strain exhibited a different Whi5 transport profile in many ways compared to the wild-type strain. Firstly, we detected that a small amount of cells could not import Whi5 into their nuclei accordingly with cell cycle progression. Whi5 always remained in the nucleus and the signal intensity was higher than the other group of cells. After these cells were exposed to starvation, the nuclear signal intensity of Whi5 was observed to be brighter than the other group of cells. The difference between the glucose rich and starvation conditions indicate that the nuclear import and export dynamics might be changing depending on the nutrient conditions. However, we did not investigate this phenomenon further. Once possibility is that this observation is linked to general nuclear transport mechanisms. Protein nuclear export in yeast is achieved through four nuclear carriers from importin beta family, Xpo1/Crm1, Cse1, Los1, and Msn5. Under nutrient stress, Snf1 controls the localization of these importins and catalyses the export of many proteins through them. The deletion of Snf1, as a result, affects the export and import of proteins when the cells are faced to acute starvation [Quan et al., 2007]. Msn5 was shown to be phosphorylated by Snf1 leading to the export of the glucose repressor Mig1 showing Snf1 and Msn5 act together under glucose limitation [DeVit and Johnston, 1999].

As none of the factors we have tested so far stopped the cells from re-entering Whi5 under acute starvation, we then investigated if Whi5 itself was being targeted. Whi5 has a total of 19 phosphorylation sites, 12 of these get phosphorylated by Cdk1. Cdk1 phosphorylation leads to the nuclear export of Whi5 resulting in the initiation of *Start* in regular cell cycle progression [De Bruin et al., 2004], [Costanzo et al., 2004], [Wagner et al., 2009]. However, the role of the remaining seven non-CDK phospho-

rylation sites has not been uncovered yet. Thus, we decided to test if these seven non-CDK sites could be playing an active role in Whi5 re-entries. For that purpose, we mutated these phospho-sites and tested the resulting 7A mutant against acute starvation conditions. We detected that the re-entry probability was not affected by the mutation of these phosphorylation sites. This shows that, re-importing Whi5 was not caused by direct phosphorylation of Whi5 through these seven sites. However, in addition to these phospho-sites, Whi5 has an additional six sites that have been identified through mass spectrometry techniques, but have not been characterized yet [Chi et al., 2007], [Albuquerque et al., 2008], [Huber et al., 2009], [Holt et al., 2009], [Wagner et al., 2009], [Bodenmiller et al., 2010]. Without testing those, it is not possible to make a conclusion that Whi5 is not directly targeted. Thus, when more is known about the function of those sites, their potential roles in the cell cycle reversals can also be tested.

A notable thing we detected was that, for the 7A phosphorylation mutant, during growth under glucose, the cellular localization of Whi5 was changing regularly accordingly with cell cycle progression. The Whi5 export from the nucleus was not affected at all and the phenotype of the cell was the same as the wild-type strain. This showed, that also under glucose feed, these seven sites had no visible affect on the cell cycle progression. Whi5 is known to be targeted by one phosphatase called PP2A-Cdc55 during G1 [Talarek et al., 2017]. To test if the direct interaction between Whi5 and PP2A-Cdc55 was the resulting in reversing *Start*, we also tested the $\Delta$cdc55 mutant (data not shown). Despite the cell cycle defects, we still were able to get Whi5 signal from some of the cells. In those cells we saw, that the cells could still re-enter Whi5 into the nucleus. As there are not any known phosphatases acting on Whi5, we excluded the effect of phosphatases and the possibility of Whi5 being directly targeted. Lastly, we hypothesized that the CDK-cyclin complex might be targeted, resulting in the interruption of the positive feedback loop. To check it, we focused on two inhibitory proteins which target the early CDK-Cyclin complexes: Cip1 and Sic1. Apart from being the main inhibitor of the second feedback loop following *Start*, Sic1 is also known to be responding to environmental stress conditions, and building a bridge between metabolism and cell cycle [Escoté et al., 2004]. We showed that Sic1 stabi-

lization was not necessary for the cells to reverse their cell cycle. We detected that the cells could arrest with different levels of undegraded Sic1 in the nucleus showing that Sic1 degradation was not essential for the reversal of *Start*. In agreement with this, a $\Delta$sic1 mutation did affect Whi5 re-entries. The other known inhibitor Cip1 also did not have an effect on the re-entry frequencies or dynamics. Consequently, we reached the conclusion, that under acute starvation, CDK activity was not being inhibited by these two inhibitors. To detect what might be acting on the CDK-cyclin complexes, more inhibitors should be tested additionally.

Even though, we could not unveil the complete mechanism behind the cells decision to reverse *Start*, we managed to exclude several factors. We found that repression of cyclin expression levels were not causal for the reversal of *Start*. By deleting the non-CDK phosphosites of Whi5, we made sure that Whi5 itself was not the reason why the feedback loop was interrupted. Thus, this strongly suggests that the CDK-Cyclin complexes were the main target of glucose starvation signalling. On our search of finding the mechanism by which the cyclin-Cdk1 complexes are targeted, we managed to exclude the two main G1 CKI inhibitors Cip1 and Sic1. However, other than these two inhibitors, there are not any other known inhibitors. Thus, we concluded that CDK activity was being targeted by an unknown inhibitor yet to be discovered.

# 5 Open Questions and Outlook

Our findings raise a lot of new interesting questions. We have found that *Start* is not the point of no return. However, we still do not know the actual point of irreversibility in the cell cycle progression. Even though we could not detect this point, we managed to exclude some steps and narrow the time frame. Firstly, we detected that stabilization of Sic1, the stoichiometric inhibitor of B-type cyclins is not essential for reversing *Start*. Also, we showed that cells can reverse *Start* with high and low amounts of Sic1 present in the cell. We detected that Sic1 concentration stays the same when starvation is imposed and does not show any increase. By showing that *Start* can be reversed even when Sic1 is degraded, we proved that the second feedback loop following the initiation of *Start* has already been initiated when the cells reverse their cell cycle. Together with the Δsic1 results we concluded that Sic1 does not play a role in the nuclear Whi5 re-entries, and also is not the point of irreversibility.

As we pointed out that the second feedback loop is already activated, as a next step, the potential involvement of the further elements of this feedback loop should be investigated. Firstly, as the degradation of Sic1 leads to the activation of Clb5,6-Cdk1 complexes [Morgan, 2007], the role of the B-type cyclins should be tested. Some of our preliminary results (data not shown) indicate that the levels of Clb5 in the cell can be the determining factor in the decision of reversing *Start*. We saw different Clb5 profiles in the cells that re-enter Whi5. In some cells we detected that Clb5 reaches its maximum and stays at that level throughout the starvation period and then drops accordingly with the cell cycle progression when glucose is replenished. However, in some cells, we saw that Clb5 does not reach its maximum and then increases after glucose replenishment. In this group of cells, Clb5 either stays at the same level as it is when exposed to starvation or shows a slight decrease. We also determined that these different Clb5 profiles are observed for the cells within similar time periods after passing *Start*. Thus, the cells may be stuck at the point where Clb5,6 expression has started and that point might be the step where the cell cannot go back from a pre-*Start* stage. However, for a better understanding, Clb5 should be further investigated. In addition to directly monitoring the endogenously tagged Clb5 intensity, a strain with inducible Clb5 expression should also be used and tested against acute starva-

tion. Testing if the Whi5 re-entries can take place under stable, non-fluctuating Clb5 activity can tell us if the second feedback loop poses as the point of irreversibility.

To find the point of no return, in addition to Clb5, the initiation of S phase should also be tested. Unfortunately, there is no good fluorescent read-out for yeast cells to track the initiation of S phase [Easwaran et al., 2005]. Thus, we decided to use the initiation of DNA replication as an indicator of S phase transition. In our experiments we calculated the time between *Start* and DNA replication as 24 +-4 minutes using the histone Htb2. This time frame is close to the time threshold we determined to distinguish between the cells that reverse their cell cycle and the ones which do not. Thus, it suggests that DNA replication can be the point of no return. Unfortunately, as the Htb2 intensity during starvation increases continuously, we could not determine the time of DNA initiation. Additionally, another histone Htb1 showed the same increase under starvation. Thus, in the future, a reliable S phase or DNA replication initiation marker should be found.

Another important step in understanding how the cell cycle commitment takes place is to understand through which mechanism the irreversibility take place. So far, we managed to detect that the CDK positive feedback loop is not interrupted by the repression of G1 cyclins or by additional phosphorylation of Whi5. The V5-tagged Whi5 by Phostag-SDS PAGE and Western blot experiments in our lab (Katja Kleemann), showed Whi5 actually loses its hyperphosphorylated forms within 10 minutes when exposed to starvation. Considering that Whi5 gets hyperphosphorylated with CDK activity [Wagner et al., 2009], this suggests that Whi5 is actually dephosphorylated by its CDK phosphorylation sites while reversing *Start*. However how this dephosphorylation takes place remains unknown.

One of our theories is, that the starvation signalling might be received by a phosphatase which leads to the dephosphorylation of Whi5. Thus, it might be causing the reversal of *Start*. We tested the PP2A-Cdc55, which is shown to be targeting Whi5 during G1 [Talarek et al., 2017] by creating a $\Delta$cdc55 mutant and testing under starvation. Even though the strain had a cell cycle defect, we still managed to detect Whi5 re-entries. Thus, we excluded the role of PP2A-Cdc55. Other than this phosphatase, there are no other known phosphatases that are targeting Whi5 or re-

sponding to nutrient deprivation. Thus, our conclusion is, it is most likely that Whi5 is not targeted by a phosphatase. By deleting the non-CDK phosphorylation sites of Whi5, we also showed that the cells could still reverse *Start*, showing that Whi5 is not directly targeted. As Whi5 itself is not likely to play a role in the reversal of *Start*, our assumption is that the cyclin-Cdk1 complexes should be the target of the nutrient signalling. We have already tested the role of Sic1 and Cip1 the known inhibitors of Cyclin-Cdk1 complexes and showed that these two do not change the frequency or the dynamics of Whi5 re-entries. Thus, they are not responsible for the decreased Cyclin-Cdk1 activity which leads to Whi5 re-import. Therefore, the activity of these complexes must be inhibited by another mechanism. There are not any known Cyclin dependent kinase inhibitors (CKI) other than these two in budding yeast. Potential discoveries of new CKIs might resolve this question in the future.

The beta-karyopherin Msn5 is involved in the cell cycle dependent export of Whi5 [Taberner et al., 2009], and so Whi5 in cells lacking Msn5 remains nuclear. However the failure to export Whi5 is not due to Cdk1 phosphorylation [Wagner et al., 2009]. However, how the export of Whi5 through Msn5 works under starvation is yet unknown. To test if the inability of exporting Whi5 in the absence of msn5 will be affected under starvation, an $\Delta$msn5 strain might be created and tested under starvation. This way, how Msn5 is affected can be understood and further experiments can be designed to see if it can also be involved in the Whi5 re-entries.

Recently, another repressor protein of *Start* called Whi7 has been found which is a paralog of Whi5. It is also regulated by the cell cycle through Cdk1. Even though it is not the main player in the transition into *Start*, it helps this transition in a transcription way [Gomar-Alba et al., 2017]. In the level of functionality and localization Whi7 is being regulated in different ways than Whi5 and suggested as being an emergency break for the cells facing cell wall stress [Méndez et al., 2020]. However, in any research Whi7 has not been tested under nutrient stress conditions, so how the behaviour of Whi7 changes remains interesting work for the future. Additionally, to detect its contribution to the reversal of *Start*, the deletion mutant should be tested under starvation. This way, the bigger picture can be more thoroughly comprehended.

Going back to a pre-*Start* phase of the cell cycle is a surprising phenomenon that

changes the course of the current cell cycle progression. A decision this important should have physiological consequences on the cell. One of the consequences might be the memory from the previous CDK activation. The function of memory might be determinant in the responsiveness of the cell to the alpha factor mating pheromones. In addition to its role in the sensitivity to the mating pheromones, the previous memory might also have even more extensive consequences. Firstly, if re-importing Whi5 into the nucleus means re-binding to the transcription factor, SBF should be detected. This way, if the transcription of G1/S genes from SBF is affected by the re-import of Whi5. For that purpose, Co-IP Western blot approach can be optimized to see if Whi5 re-binds to SBF after the starvation.

Secondly, how re-entering Whi5 affects the transcription of the genes from SBF. It is known that, when in the nucleus, Whi5 binds to the transcription factor SBF and inhibits its activity. When Whi5 leaves the nucleus, the inhibition is relieved and many genes responsible for G1/S transition are transcribed in a temporal order [De Bruin et al., 2004], [Harris et al., 2013]. To understand how expressions of these genes are affected, a reliable method to monitor the targets of SBF should be implemented. Multiple SBF targets can be endogenously tagged and imaged. For the proteins that have high turnover numbers, the dPSTR reporter system, that we have already successfully used in this project [Aymoz et al., 2016], can be implemented. This way, it can be determined which proteins are already transcribed when the cell reverses *Start*. As a result, which proteins might be contributing to the memory can be understood. Even though Whi5 does not bind to MBF, the targets of MBF should also be tested to see if Whi5 re-entry also affects the activity of MBF using the same approach.

Reversing *Start* might also have consequences on the cell cycle progression after glucose replenishment whch makes it an interesting target to track. The differences between the regular G1 cells and the ones that re-entered Whi5 in terms of cell cycle durations, death rates, and budding initiation should be analysed.

During this project, we only investigated the effect of acute glucose starvation on the cell cycle progression on post-*Start* cells. However, different nutrient sources are sensed through different pathways and affect the cell metabolism differently [Broach, 2012],

[Rødkær and Færgeman, 2014]. Additionally, deprivation of specific nutrients affect the transcription of various group of proteins [Conway et al., 2012]. Thus, in addition to acute glucose limitation, cells should also be tested under other nutrient conditions, such as nitrogen, to understand the extent of the nutrient signalling. For example, nitrogen starvation can be imposed on the cells. This way, we can see if reversing *Start* is a glucose specific response or a more universal reaction that can occur when faced to other nutrient resources.

# 6 Conclusions

The eukaryotic cell cycle consists of multiple steps in the form of feedback loops and checkpoints. Among them all, one of the most crucial is the cell cycle commitment point, which is known as *Start* for the yeast cell cycle and as Restriction Point in mammals. Even though the elements of the commitment points may differ, the overall structure and functionality is conserved among eukaryotic cells [Cross et al., 2011], [Johnson and Skotheim, 2013]. The currently accepted model for cell cycle commitment states that both yeast and mammalian cells irreversibly commit to the cell cycle through these one-step commitment points. However, for mammalian cells, the concept of a one-step commitment has been strongly challenged [Fisher, 2016]. Several studies showed that the mammalian cells could sense and evaluate inner and outer signals after passing the restriction point and could give a cell cycle dependent response to perturbations. It has been shown, that there might be more than one commitment stages, specific for nutrients [Foster et al., 2010], stress and cell growth [Cappell et al., 2016]. However, the whole picture still has not been understood completely and the current model lacks a lot of information, such as how the cells integrate the nutrient signals into the cell cycle and existence of a real point of no return [Patel et al., 2017], [Lo et al., 2020], [Yang et al., 2020], [Pennycook and Barr, 2020].

Here, we discovered a new and interesting phenomenon. We detected, that post-*Start* cells can re-import the *Start* inhibitor Whi5 back into their nucleus when facing acute glucose starvation conditions. As the irreversibility of the nuclear export defines the irreversibility of *Start*, we suspected that the initiation of *Start* could also be reversible. By applying mating pheromones, we demonstrated that these cells indeed reverse their cell cycle and act like regular G1 pre-*Start*. This showed that, yeast cells can go into a stable starvation-induced arrest by reversing *Start*.

Through our results, we provided strong evidence, that the current model stating that *Start* is the final point of irreversible cell cycle commitment point is indeed incomplete. We demonstrated, that in yeast cells, the cell cycle commitment point called *Start* is not necessarily irreversible under acute glucose starvation. Unlike the commonly accepted textbook model [Morgan, 2007], we showed that, *Start* is not the final point of cell cycle commitment. Our findings, thus strongly suggests that cell cycle

commitment is a multi-step process. We demonstrated that for nutrient signalling, the point of no return is at a further stage than *Start*. We detected that the cells can reverse their cell cycle after the initiation of the second feedback loop that follows *Start*. Also, even though we could not get a good read-out of DNA replication under starvation, our data from the glucose rich period indicates that the point of no return should be before DNA replication. Additionally, once the DNA replication is initiated, reversing it would be damaging to the integrity of the division cycle. Thus, we suggest that the point of irreversibility for nutrient signals is within a time frame between Sic1 degradation and DNA replication.

Our findings rise many important questions on the cell cycle regulation. Firstly, even though we showed that *Start* can be reversible under nutrient signalling, we have not found the underlying mechanism behind this decision. We managed to exclude certain elements that could potentially cause reversing *Start*, such as cyclin repression and Whi5 itself, and hypothesized that cyclin-Cdk1 activity is targeted. However, the mechanism still remains unknown. The second question that waits to be answered is: As *Start* is not the point of no return, then what is? Even though we managed to to narrow the possible time frame, we could not provide a certain answer. Thus, to get a broader understanding of how the cell cycle commits itself to the division, these two questions should be answered first.

Our research on yeast will provide a good opportunity to study the cell cycle commitment in eukaryotic organisms. As the cell cycle commitment is conserved among eukaryotes, our discoveries might also be applicable to more complex organisms. Once the cell cycle commitment with nutrient signalling is more thoroughly discovered, how the early cell cycle regulation is achieved will be better understood. Understanding this in yeast, will also help to understand how early cell cycle regulation is achieved in mammals. As many disease states are related to misregulation of early cell cycle regulation, our findings will hopefully reflect more light on the future research on many cell cycle control related diseases.

# 7 Materials

## 7.1 Growth Media used for Yeast Growth

All the solid growth media were autoclaved before the addition of glucose. After the media were cooled, the glucose and, if needed, antibiotics were added. For each Petri dish, approximately 25 ml of media was poured and stored at 4 $°C$.
Yeast liquid growth media were not autoclaved before being used, but prepared using autoclaved MilliQ and under the sterile hood unless indicated otherwise. The ingredients were listed for a final media of 1 liter.

**Table 2:** Liquid media used for yeast growth

| **Name** | **Ingredients** |
|---|---|
| 1 % Glucose Minimal Medium +0.5M KPH | 100 ml 10X Yeast Salts, 100 ml 0,5M KHP, 50 ml 20 % Glucose, 750 ml autoclaved MilliQ water |
| 1 % Sorbitol Minimal Medium +0.5M KHP | 100 ml 10X Yeast Salts, 100 ml 0,5M KHP, 50 ml 20 % Glucose, 750 ml autoclaved MilliQ water |
| 2 % YPD Medium | 10 g Yeast Extract, 20 g Casein Peptone, 1-2 KOH Pellet, 900 ml MilliQ water,100 ml 20 % Glucose (added after autoclave) |

**Table 3:** Solid media used for yeast growth

| Name | Ingredients |
|---|---|
| 2 % SC Complete Agar Plates | 5g Ammonium Sulfate, 1.7 g Yeast Nitrogen Base without Amino Acids, 1.4 g amino acid stock powder complete, 20 g Agar, 900 ml autoclaved MilliQ water, 100 ml 20 % glucose (added after autoclave) |
| 2 % SC-HTULA Agar Plates | 5g Ammonium Sulfate, 1.7 g Yeast Nitrogen Base without Amino Acids, 1.2 g amino acid stock powder-HTULA complete, 20 g Agar, 900 ml autoclaved MilliQ water, 100 ml 20 % glucose (added after autoclave) |
| 2 % YPD Agar Plates | 10 g Yeast Extract, 20 g Casein Peptone, 20 g Agar, 900 ml MilliQ water, 100 ml 20 % Glucose (added after autoclave) |

## 7.2 Media Used for Bacterial Growth

All the media were autoclaved before being used. The listed amounts are for 1 liter of media.

**Table 4:** Liquid media used for bacterial growth

| Name | Ingredients |
|---|---|
| LB (Luria-Bertani) Media | 5 g Yeast Extract, 10 g Bacterial Peptone (Tryptone), 5 g NaCl, 950 ml MilliQ water |

**Table 5:** Solid media used for bacterial growth

| **Name** | **Ingredients** |
|---|---|
| LB Agar Plates | 10 g Yeast Extract, 20 g Casein Peptone, 20 g Agar, 900 ml MilliQ water |

### 7.2.1 Antibiotic Stocks

**Table 6:** Antibiotic solutions used in growth media

| **Name** | **Added Amount** | **Final Concentration** |
|---|---|---|
| Hygromycin B (Hph) | 4 ml 100 mg/ml in $ddH_2O$ 1000x Stock | 50 mg/l (200 µg/ml) |
| G-418 Sulfate (G-418) | 4 ml 200 mg/ml in $ddH_2O$ 1000x Stock | 800 mg/l (200 µg/ml) |
| Nourseothricin Sulfate (Nat) | 4 ml 100 mg/ml in $ddH_2O$ 1000x Stock | 200 mg/l (200 µg/ml) |
| Ampicilin | 1 ml 100 mg/ml in $H_2O$ | 1000x (100 mg/ml) |

### 7.2.2 Stock Solutions for Growth Media

**Table 7:** Stock solutions used for growth media

| **Name** | **Ingredients** |
|---|---|
| 10X Yeast Salts | 10 g Ammonium Sulfate, 17 g Yeast Nitrogen Base without Amino Acids and without Ammonium Sulfate, up to 1000 ml autoclaved MilliQ water |
| 20 % Glucose | 200 g Glucose, 900 ml autoclaved MilliQ water |
| 20 % Sorbitol | 200 g Glucose, 900 ml autoclaved MilliQ water |
| 0,5 M KHP (Potassium hydrogen phtalate), pH 5,0 | 102,11 g KHP, up to 1000 ml autoclaved MilliQ water |
| Amino Acid Stock Powder | 2.5 g Adenine (hemisulfate salt), 1.2 g L-arginine (HCl),6 g L-aspartic acid, 6 g L-glutamic acid (mono sodium salt), 1.2 g L-histidine, 7.2 g L-leucine, 1.8 g L-lysine(mono-HCl), 1.2 g L-methionine, 3 g L-phenylalanine, 22,5 g L-serine, 12 g L-threonine, 2.4g L-tryptophan, 1.8 g L-tyrosine, 9 g L-valine,1.2 g Uracil |
| Amino Acid Stock Powder-HTULA | 1.2 g L-arginine (HCl),6 g L-aspartic acid, 6 g L-glutamic acid (mono sodium salt), 1.8 g L-lysine(mono-HCl), 1.2 g L-methionine, 3 g L-phenylalanine, 12 g L-threonine, 1.8 g L-tyrosine, 9 g L-valine |

### 7.2.3 Stock Solutions used in the Experiments

**Table 8:** Stock solutions used for growth media

| **Name** | **Ingredients** |
|---|---|
| 50 % PEG | 500 g Polyethylene glycol Powder (50 % w/v) , up to 1000 ml autoclaved MilliQ water |
| Yeast DNA Extraction Buffer (100 ml) | 2 g 2 % Briton X-100, 1 % ADS, 0.58 g 100 mM NaCl, 0.157 g 10 mM Tris-HCl, 0.037g 1 mM Na2EDTA, up to 100 ml autoclaved MilliQ water |
| 1M LiAc | 102.1g LiAc, up to 1000 ml autoclaved MilliQ water |
| 0.1M LiAc (50 ml) | 5 ml 1M LiAc in MilliQ water, 45 ml autoclaved MilliQ water |
| 5x KCM (150 ml) | 5.59 g 0.5M KCl, 3.31 0.15 M $CaCl_2$, 7.62 g 0.25M MgCl2, 150 ml autoclaved MilliQ water |
| 2 mg/ml Salmon Sperm DNA (100 ml) | 200 mg DNA, 100 ml autoclaved MilliQ water, filtrate with a sterile 0.2 µm Filter |
| TE Buffer | 10 ml 1 M Tris-Cl (pH 8.0), 2 ml 0.5M EDTA (pH 8.0), 998 ml autoclaved MilliQ water |
| 20 % Glycerine | 20 ml Glycerine, 80 ml autoclaved MilliQ water |
| Yeast Freeze Culture (-80 $°C$) | 500 µl 20 % Glycerine Solution in Cyro Tube, 1 scoop of yeast |

## 7.3 Yeast Strains and Plasmids

The yeast and E.Coli strains that were used during the project are listed below.

### 7.3.1 Yeast Strains

**Table 9:** Yeast strains used in this study

| Name | Genotype | Source |
|---|---|---|
| FS042<br>Figure 8,13<br>17, 19, 20<br>21,23,25 | MAT a, ADE, TRP, LEU, URA, HIS | Schlottmann et al<br>in prep |
| JE401<br>Figure 8 | MAT a, Whi5-GFP-natMX, Htb2-mCherry-his,<br>URA3, LEU2, TRP1, ADE2 | Ewald Lab<br>[Ewald et al., 2016] |
| DI002<br>Figure 18 | MAT a, Htb2-mTFP-Nat, Whi5-mCherry-KanMX,<br>Sic1-mNeongreen-hph,ADE, LEU, HIS, TRP, URA | This Study |
| DI028<br>Figure 17 | MAT a,Whi5-mCherry-KanMX, Cln2-promote-<br>mNeongreen, ADE, TRP, LEU, URA | This Study |
| DI011<br>Figure 23 | MAT $\alpha$, ADE2, Whi5-mNeongreen-Ura | This Study |
| FS032<br>Figure 13 | MAT a, Whi5-mCherry-KanMX,<br>leu2::clb2-promoter-dPSTR-mCitrine-leu2,<br>ADE, TRP, URA | Constructed by<br>Iliya Nadelson |
| FS002<br>Figure 24 | MAT a,Whi5-mCherry-KanMX,<br>Sic1-mNeongreen-hph<br>ADE, TRP, LEU, URA, HIS | Schlottmann et al.<br>in prep |
| DI030<br>Figure 8 | ,MAT a, Whi5-mCherry-KanMX, Clb5-mNeongreen<br>- NAT, ADE, TRP, LEU, URA | This Study |
| VS001<br>Figure 18 | MAT a,Whi5-mCherry-KanMX, Msn2-GFP-His3,<br>Msn4-TFP-Nat, ADE, TRP, LEU, URA | Constructed by<br>Vidiyaah<br>Santhanakumaran |

**Table 10:** Yeast strains used in this study-continued

| Name | Genotype | Source |
|---|---|---|
| DI041<br>Figure 23 | MAT $\alpha$, Whi5-7Ala-mNeongreen-URA,ADE2<br>TRP, LEU | This Study |
| DI031<br>Figure 25 | MAT a, Whi5-mCherry-KanMX, Cip1::Hph<br>ADE, TRP, LEU, URA | This Study |
| DI040<br>Figure 21 | MAT a, Whi5-mNeongreen-hph, Rim15::KanMX,<br>ADE, TRP, LEU, URA, HIS | This Study |
| DI045<br>Figure 19 | MAT $\alpha$, Whi5-mCherry-KanMX, Msn2::HphMX<br>Msn4::His, TRP, LEU, URA | This Study |
| BY6883 | MAT a, Msa1::HIS3, Msa2::KanMX<br>ADE, TRP, LEU, URA | Breeden Lab<br>[Miles et al., 2016] |
| DI047<br>Figure 19 | MAT a, Msa1::HIS3, Msa2::KanMX<br>Whi5-mNeongreen-Hph, TRP, LEU, URA | This Study |
| KK086<br>Figure 21, 22 | MAT a,Whi5-mCherry-KanMX, Htb2-TFP-Nat,<br>Snf1::HphMX, ADE, TRP, LEU, URA | This Study,<br>Katja Kleemann |
| BY5654 | MAT a, 5X CLN3 W303<br>ADE2 HIS3 LEU2 TRP1 URA3 | Breeden Lab<br>[Miles et al., 2016] |
| KK081 | MAT a,Whi5-mCherry-KanMX, 5X CLN3 W303<br>ADE2 HIS3 LEU2 TRP1 URA3 | This Study,<br>Katja Kleemann |
| BY6602 | MAT a, Xbp1::KanMX | Breeden Lab<br>[Miles et al., 2016] |
| DI043<br>Figure 19 | MAT a, Whi5-mNeongreen-hph, Xbp1::KanMX<br>Msn4::His, TRP, LEU, URA | This Study |
| BY6828 | MAT a, Sic1::KanMX, ADE,LEU,TRP, HIS,URA | Breeden Lab<br>[Miles et al., 2016] |
| DI038<br>Figure 25 | MAT a, Whi5-mNeongreen-hph, Sic1::KanMX<br>ADE, LEU, TRP, HIS,URA | This Study |
| JE616<br>Figure 20 | MAT $\alpha$, cln1$\Delta$, cln2$\Delta$, cln3::leu2, lexOPr-Cln1-Leu2,<br>ADE2, his3::cyc1-Pr-lexO TF-his3, TRP, URA,<br>Whi5-GFP-NatMX | Ewald Lab<br>[Ewald et al., 2016] |

### 7.3.2 Plasmids

**Table 11:** Plasmids for endogenous tagging, all based on the pFA series

[Longtine et al., 1998]

| Name | Description | Resistance | Source |
|---|---|---|---|
| pYLB9 | mNeongreen - NAT | Amp | Doncic lab, unpublished |
| pYLB10 | mNeongreen - Hph | Amp | Doncic lab, unpublished |
| pYLB11 | TFP - Hph | Amp | Doncic lab, unpublished |
| pYLB12 | TFP - NAT | Amp | Doncic lab, unpublished |
| pYLB17 | TFP-KanMX | Amp | Doncic lab, unpublished |
| pYLB18 | TFP-His | Amp | Doncic lab, unpublished |
| pJH007 | mNeongreen-KanMX | Amp | This study, Julia Heinzelbecker |
| pFS016 | Cln2-promoter-dpstr-mCitrine | Amp | Pelet Lab, [Aymoz et al., 2016] |
| pMS50 | pFA6a-3PK-HphMX6(V5tag) | Amp | Skotheim Lab |
| pMS52 | pFA6a-3PK-KanMX6(V5tag) | Amp | Skotheim Lab |

**Table 12:** Plasmids for integration into the Ura locus based on pRS406

| Name | Description | Resistance | Source |
|---|---|---|---|
| pMK14 | pRS406 - Whi5pr-WHI5-mCitrine | Amp | Skotheim Lab, Mardo Koivomagi |
| pMK19A | pRS406 -WHI5-3Flag-Whi5-19A | Amp | Skotheim Lab, Mardo Koivomagi |
| pDI001 | pMK14 - Whi5- 2 Ala[1] | Amp | This Study |
| pDI002 | pMK14 - Whi5-2 Ala[2] | Amp | This Study |
| pDI003 | pRS406 - Whi5pr-WHI5-mNeongreen | Amp | This Study |
| pDI004 | pMK14 - Whi5-2 Ala[2]-mCitrine:mNeongreen | Amp | This Study |
| pDI005 | pMK14 - Whi5-2 Ala[1]-mCitrine:mNeongreen | Amp | This Study |
| pDI006 | pDi004 - Whi5-19 Ala[3] | Amp | This Study |
| pDI008 | pDI004 - Whi5- 4 Ala[4] | Amp | This Study |
| pDI009 | pDI004 - Whi5- 7 Ala[5] | Amp | This Study |
| pDI010 | pDI004 - Whi5- 6 Ala[6] | Amp | This Study |
| pJE206 | Estradiol promoter mkok | Amp | Ewald Lab, Katja Kleemann |

[1]: Whi5 phosphorylationsites on 78 and 149 are changed to alanine

[2]: Whi5phosphorylation sites on 115 and 288 are changed to alanine

[3]: Whi5 replaced by pMK19A

[4]: Whi5 phosphorylation sites on 78 and 149 are changed to alanine

[5]: Whi5 phosphorylation sites on 78, 149, 161, 276 and 281 are changed to alanine

[6]: Whi5 phosphorylation sites on 78, 149, 276, and 281 are changed to alanine

### 7.3.3 Control Primers

**Table 13:** Primers used in control PCRs

| Name | Sequence |
|---|---|
| TFP_C_fw | GCGAGGAGACCACAATGG |
| TFP_C_rev | GGTCACCTTGTTGTAGTCCTT |
| mCherry_C_fw | GAGGAGGATAACATGGCCAT |
| mCherry_C_rev | ACTGTTCCACGATGGTGTAG |
| mNeongreen_C_fw | AGGAGGACAATATGGCTTCG |
| mNeongreen_C_rev | GCCCATAACATCCGTAAAGG |
| Msn4_C_fw | GGCTCAGGATCAACAGTTTATC |
| Msn4_C_rev | CCAATCCTTGAATGCTTCCC |
| Htb2_C_fw | GCTACTGAAGCTTCTAAATTGGC |
| Htb2_C_rev | AATGAATGCTCGTGTAGTGAACC |
| Msn2_C_fw | GAAGTCTGCTGTGCCTTTG |
| Msn2_C_rev | TACAAAAGCAAGAGGGGTGCC |
| Sic1_C_fw | CCAGCGACAAGGTGATAACATT |
| Sic1_C_rev | CGGTAAGGAAAAGTTAAAAATCCTG |
| Whi5_C_fw | AAGAGAAGCAGGACTAGCGA |
| Whi5_C_fw | CTCCACTTCGGTATCCGACT |
| Cip1_KO_C_fw | CTTAGTAAAGCGAACTAGAACCAGTTTAAT AGGATATAGACGGATCCCCGGGTTAATTAA |
| Cip1_KO_C_rev | GGAGGTATACTCCTTTACGC |
| Rim15_KO_C_fw | GGCAGCTTGGTGTTGTTTTTC |
| Snf1_KO_C_fw | AAGCTGGAAATGGCAGTGTG |
| Msn2_KO_C_fw | TTTTTCAACTTTTATTGCTCATAGAAGAACT AGATCTAAAGACATGGAGGCCCAGAATAC |
| hph_Cnew_rev | CATCAGGTCGGAGACGCTGTC |
| Msn4_KO_C_fw | CCTTAGATGCATGAGCATACC |
| kan_C_rev | GTTGGAATTTAATCGCGGCC |

# 8 Methods

## 8.1 Cell Growth Conditions

All liquid yeast cultures were grown using shake flasks in an incubator shaker operated at 30°C and rotating with a speed of 200 rpm.

For live cell imaging experiments, 15 ml of liquid yeast culture was prepared using 1 % Glucose Minimum Media and grown overnight in 50 ml shake flasks. For growing the strain JE616, beta-estradiol was added to the growth media with a final concentration of 15 nM. The next morning, the cells were transferred to a fresh culture by applying 1:15 dilution and grown for at least another 5 hours. When the cell culture reached log phase($OD_{600}$ between 0.4 and 0.6), they were taken out and prepared for the live-cell imaging experiment.

The yeast cells were grown on solid media using 2 % YPD agar plates. Cells were streaked from -80°C, 20 % glycerol stock onto a YPD agar plate. The cells were grown between 2 to 4 days at 30°C. The plate was kept at the refrigerator operated at 4°C up to 4 weeks. During live cell imaging experiments, starvation was imposed on the yeast cells by using 1 % Sorbitol Minimum Media. Sorbitol was used as a carbon source yeast cannot metabolize and was only used for osmotic balance to prevent the cells from being exposed to osmotic shock. The starvation media did not contain any amino acids that the cells could metabolize unless indicated otherwise.

Bacteria cells were grown using LB-agar plates at 37°C. Liquid bacteria cultures were prepared using 500 ml LB media and grown overnight at 37°C using an incubator shaker operated at 300 rpm.

## 8.2 Cell Preparation for Live Cell Imaging

From the yeast cultures that reached log phase, 500 µl was transferred to a 1.5 ml sterile micro-centrifuge tube and mixed with 1 ml 1 % glucose minimal media. The diluted culture was sonicated for 3 seconds at 15 % efficiency to prevent the cells from sticking together during the microscopy experiments.

## 8.3 Microfluidic Cell Growth Conditions

For cell growth and imaging during the time lapse experiments CellAsic ONIX microfluidics cell cultivation system was used. This system provides continuous media flow and prevents the accumulation of unwanted metabolic by products. The temperature was kept constant at 30°C using an incubator chamber surrounding the imaging system to avoid temperature fluctuations. Before transferring the cells into the microfluidic plates, the antimicrobial preservative liquid inside the wells was emptied. Using MilliQ water, any remaining fluid was removed to make sure that no trace of the antimicrobial fluid was left inside. For time lapse experiments shorter than 20 hours, only three wells were filled with 300 µl of target growth media. The prepared cells were transferred to another well. At the beginning of the experiments, the liquid levels of all the used and unused wells were kept the same to avoid uneven media flow due to pressure differences .

Before loading the cells into the imaging chamber, the wells that supplied media were opened for 5 minutes with 5 psi pressure to ensure the remaining antimicrobial liquid inside the microfluidic channels is flown out. Then the cells were loaded into the chamber with 8 psi or higher pressure to avoid accumulation of cells the at the entrance of the cell growth chamber. After the cells were loaded, one of the wells containing glucose media was opened for 15 seconds at 5 psi to avoid clogging of the media outlet and to immobilize the loaded cells. While loading the cells, the well which contains the starvation media was kept open at 1.5 psi to avoid any back flow from the glucose containing wells into the glucose-free media.

For the cells to adjust to their new environment and, before starting the imaging process, the cells were grown inside the microfluidic chamber for at least the duration of 2 rounds of cell cycle. The glucose media was supplied with a pressure of 3 psi. During glucose feed, the well that contained the starvation media was also pressurized with 0.5 psi to avoid any backflow into the starvation media from the glucose media. For media feed, one well was not used longer than 8 hours.

The mating pheromone experiments were performed using the same setup from the beginning until the end of the starvation. 500 nM $\alpha$ factor was added to the glucose media supplied after the starvation period. To prevent $\alpha$ factor from adhering to the

cell wall, 20 μg casein was added only to the media that contains $\alpha$ factor. The cells were grown for 5 hours.
For the growth of JE616, a final concentration of 1000 nM beta-estradiol was added to both glucose and sorbitol minimal media.

## 8.4 Live Cell Imaging

The images during time lapse experiments were taken with a frequency of 3 minutes except for the experiments that the strain FS032 was used. The images of FS032 were taken in every 2 minutes. To detect if the imaging conditions caused phototoxicity, the cells were both grown under glucose and sorbitol without using any fluorescent lights and tracked under the microscope. Then the growth rates were compared to the cells imaged with fluorescent lighting. No difference in the cell growth rate was detected. Photo-bleaching was detected in some experiments if the cells were exposed to starvation approximately more than 12 hours. For that reason, the starvation period did not exceed 10 hours. No photobleaching was detected when the cells were grown under rich glucose conditions.

### 8.4.1 Microscopy Details and Settings

Images were taken with a Nikon Ti2 inverted fluorescence microscope using a Lumencor SPECTRA X light engine (Lumencor, Beaverton, OR) and Photometrics Prime 95 (Teledyne Photometrics, USA). The time lapse images were taken using Nikon PlanApo oil-immersion 60X objective (NA=1.4). During imaging, cells were cultivated in a controlled environmental chamber surrounding the microscopy setup (Okolab Cage Incubator, Okolab USA INC, San Bruno, CA) that operated at 30°C. Settings for fluorophore illumination are described in tables 2 and 3.

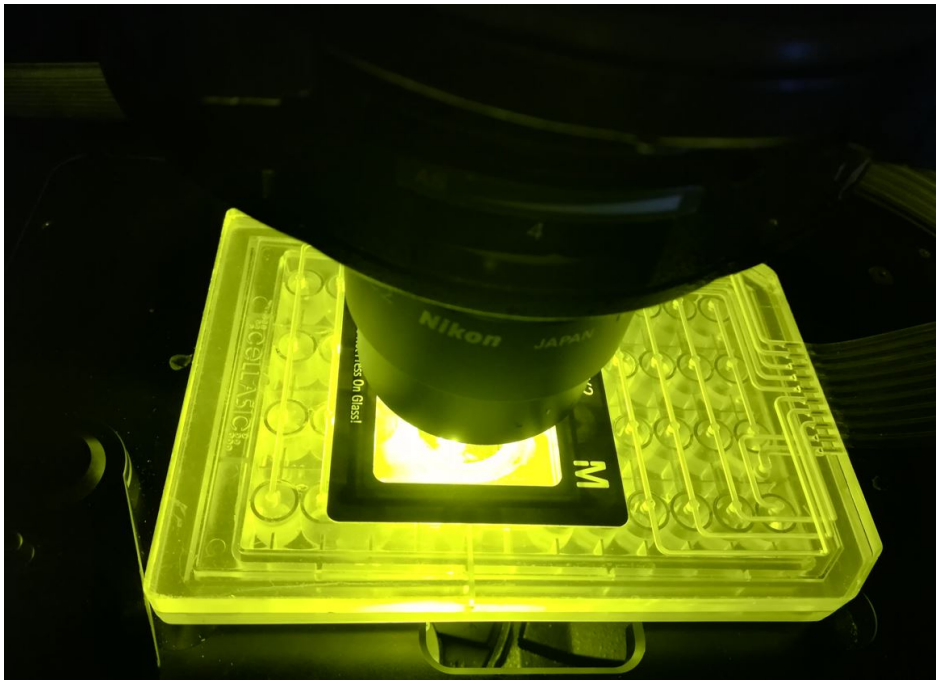


**Figure 24:** Time Lapse Imaging System

The cells are grown and imaged using CellAsic ONIX microfluidic platform. The growth media was loaded in the wells on the right side. The cells were loaded in the wells on the left side. The cells were transferred to the imaging chamber in the middle and imaged while being supplied by continuous media flow.

**Table 14:** Time lapse microscopy imaging settings

| **Fluorophore** | **Imaged Protein** | **Intensity** | **Exposure Time** |
|---|---|---|---|
| mTFP | Htb2 | 10 % | 100 ms |
| GFP | Whi5, Msn2 | 20 % - 40 % | 200 ms - 300 ms |
| mNeongreen | Whi5, Sic1, Cln2-Promoter, Msn4 | 15 % - 30 % | 200 ms - 300 ms |
| mCitrine | Cln2-promoter-dPSR | 30 % | 300 ms |
| mCherry | Whi5 | 40 % - 60 % | 300 ms - 600 ms |

**Table 15:** Microscopy filter settings

Filters used for imaging. All filters are manufactured at Chroma and purchased from AHF.

| **Fluorophore** | **LED Wavelength** | **Filter Set** | **Excitation Filter** | **Dichroic** | **Emission Filter** |
|---|---|---|---|---|---|
| mTFP | 475 nm | (Teal FP)ET Filter Set | ET445/30x | T470lpxr, Di 25 mm x 36 mm | ZET488/10x |
| GFP | 475 nm | DAPI/FITC /Cy3/Cy5 Quad LED ET | Quadband Exciter ET391 /479 /554/638 | Beamsplitter (89402bs), Di 25 mm x 36 mm | Emitter ET435/520 /595/695 |
| mNeongreen | 513 nm | YFP ET Filter Set | ET500/20x | T515lp, Di 25 mm x 36 mm | ET535/30m |
| mCitrine | 513 nm | YFP ET Filter Set | ET500/20x | T515lp, Di 25 mm x 36 mm | ET535/30m |
| mCherry | 575 nm | CFP/YFP /mCherry/ Cy7 QUAD LED ET | Quadband Exciter ET/422-449 /496-517/ 566-588/ 709-752 | Quadband Beamsplitter (89402bs) | Quadband Emitter ET/463-484/ 529-550/ 605-678/ 773-845 |

## 8.5 Data Analysis

The time lapse microscopy experiments were analysed using a custom built Matlab Algorithm [Doncic et al., 2013], [Wood and Doncic, 2019] which automatically segments cells and extracts the fluorescent intensity information from the individual cells by

tracking them backwards throughout the experiments. The segmentation of the nucleus was achieved by applying a two-dimensional Gaussian fit to the average brightest pixel [Doncic et al., 2011]. The algorithm is suitable for the cells with different sizes and shapes and sizes, which enabled us to track the cells since they were born. For the proteins that get degraded during cell cycle progression, total (nuclear+cytoplasmic) fluorescence intensity per area was used when analysing the signals. For the proteins that change nuclear localization, nuclear intensity per area was used which is based on the total nuclear fluorescence intensity divided by the area of the nucleus. The autofluorescence was subtracted using FS034 which did not contain any fluorophore.
To determine *Start*, a 5 point moving average spline was fit to the Whi5 nuclear signal as described above and the point of 50 % exit was determined [Doncic et al., 2011]. Whi5 re-entries during starvation were defined as re-importing Whi5 back into the nucleus after exporting at least 50 % of nuclear Whi5.
When analysing the intensity values obtained from Matlab, the plots were smoothed using Savitzky-Golay filter to decrease the noise and make the figures visually more convenient to analyse. However, when determining the 50 % Whi5 exit, the non-smoothed data points were used. Using this method, through polynomial regression, a smoothed value from the two neighbouring data points were smoothed over one point. When calculating Whi5 re-entry slopes, normalized nuclear Whi5 intensity per area was used. To normalize the intensity, the nuclear intensity per area at each time point was divided to the maximum signal intensity.

## 8.6 Yeast Transformation

To introduce DNA, integrative plasmids were integrated into the locus of the respective auxotrophic marker (Ura3 for pRS406 and pMK14). A restriction digestion was performed which cut within the auxotrophic marker to make the plasmid linear.
For endogenous tagging with fluorophores, PCR-based homologous recombination method based on the pFA plasmid series [Longtine et al., 1998] was used. Forward and reverse primers containing 40 overlapping base pairs with the protein to be tagged and the (CGGATCCCCGGGTTAATTAA) [Longtine et al., 1998] were designed for each tagging. Using these primers, a PCR using the plasmid DNA containing the

desired fluorophore was performed to amplify the assembled DNA.
To make gene deletions, PCR based homologous recombination using the KanMX or HphMX6 cassettes of the pFA series was used.(plasmids pMS50, pMS52).
To introduce the DNA into the yeast, lithium acetate (LiAc)-mediated transformation method was used. To make the yeast cells competent, 50 ml YPD cultures with 1:10, 1:50 and 1:100 dilutions were prepared 1 day before the transformation process. The next morning the culture that had an OD between 0.7 and 1.5 was chosen and poured into a 50 ml screw cap, centrifuged at 4000 rpm for three minutes. The pellet was washed with 20 ml water and centrifuged using the same settings. After the supernatant was discarded, 0.8 ml 0.1 mol/l LiAc was added. The mixture was centrifuged for 30 seconds at 6000 rpm. The supernatant was discarded and the competent cells were suspended in 400 µl 0.1 mol/l LiAc.
A receiving tube that contained 240 µl 50 % PEG, 32 µl 1 mol/l LiAc, 25 µl 2 mg/ml carrier DNA (salmon sperm) ,boiled 5 min, quick chilled on ice and up to 4 ng DNA was prepared and mixed by vortexing. 50 ml from the competent cell solution was added and the final mixture was gently vortexed again. The mixture was incubated for 30 minutes at 30 $^{\circ}C$ and then heat shocked at 42 $^{\circ}C$ for 30 minutes. The mixture was centrifuged for 30 seconds at 6000 rpm and the supernatant was discarded. For drug resistance markers, the cells were grown in YPD for three hours, then plated on selection plates. In case of metabolic markers, the cells were plated directly.
After 3 days, at least 5 newly formed yeast colonies were chosen and re-plated. When the cells grew enough, DNA was extracted. To check if the transformations worked, a control PCR was performed. The control primers used for each cloning are listed in Table 16.

## 8.7 Gibson Cloning

Gibson Cloning Method [Gibson et al., 2009] was used specially when integrating small DNA molecules into the vectors with significantly bigger sizes (plasmids pDI001, pDI002, pDI003, pDI004, pDI005, pDI008, pMK14). Gibson assembly [Gibson et al., 2009] was performed using the New England Biolabs (NEB) Gibson Assembly R Master Mix protocol and cells were transformed according to the NEB Chemical Transformation

Protocol .

## 8.8 E.Coli Transformation

To transform E. Coli, the cells were made competent using KCM Method. A transformation mixture that contains 20 µl 5X KCM solution (0.5 mol/l KCl, 0.15 mol/l $CaCl_2$, 0.25 mol/l $MgCl_2$), 60-79 µl $H_2O$ and 100 µl KCM-competent E. coli cells was prepared and mixed with 20 µl ligation mixture or 1 µl plasmid. The mixture was incubated on ice for 20 minutes, then heatshocked at 42 $°C$ using a water bath for 90 seconds. After that, the cells were recovered at 37 $°C$ for 60 minutes by adding 1 ml of LB. The cell culture was spun down by centrifuging for 1 minutes at 6000 rpm and re-suspended in 100 µl LB medium. As the last step, the cells were plated on LB plates that contain the antibiotic resistance fitting the plasmid´s marker.

## 8.9 DNA Extraction from Yeast

To extract DNA from yeast, a colloquial scoop of yeast is mixed with 200 ml extraction buffer (2 % Triton X 100 ; 1 % SDS; 100 mmol/l NaCl; 10 mmol/l Tris-Cl; 1 mmol/l Na 2 EDTA) in 1.5 ml a safe-lock tube. 200 µl phenol:chloroform:isoamyl alcohol were added and the mixture was vortexed. After letting the samples sit for 5 minutes, 200 µl of 1X TE buffer (pH 8.0) was added and the tube was inverted to mix. The mixture was then centrifuged at 13,300 rpm (or at full speed) for 5 minutes. The aqueous phase was transferred to a new reaction tube that already contains 1 ml of of 100 % ethanol. The reaction tube was inverted a couple times to mix at centrifuged again for 5 minutes at 13,300 rpm. The supernatant was disposed and 1 ml of 70 % ethanol was added to the reaction tube. The samples were centrifuged this time for 1 minute at $13,300$ rpm.

The supernatant was disposed off and the pellet was air dried by opening the cap until the ethanol is completely evaporated. When the ethanol is completely evaporated, 50 ml of sterile deionized water was added and the pellet was dissolved. Afterwards DNA concentration was measured via the Nucleic Acid Method on the NanoPhotometer NP 80.

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# 9 Supplementary

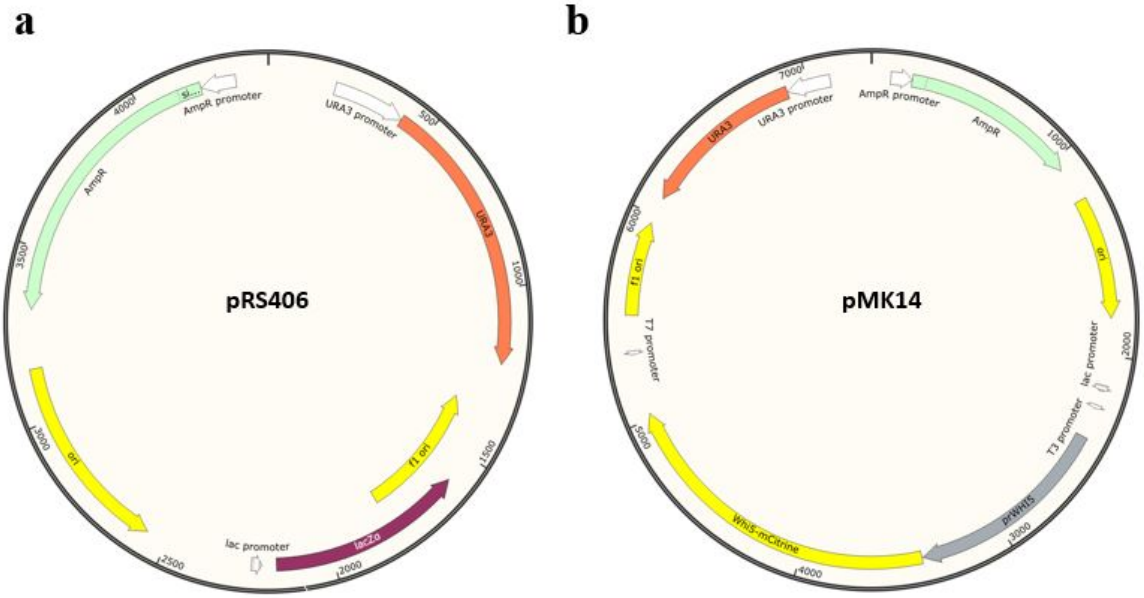


**Figure 25:** maps of plasmids used in the construction of Whi5 deletion mutants.

a) pRS406(Stratagene) b) pMK14

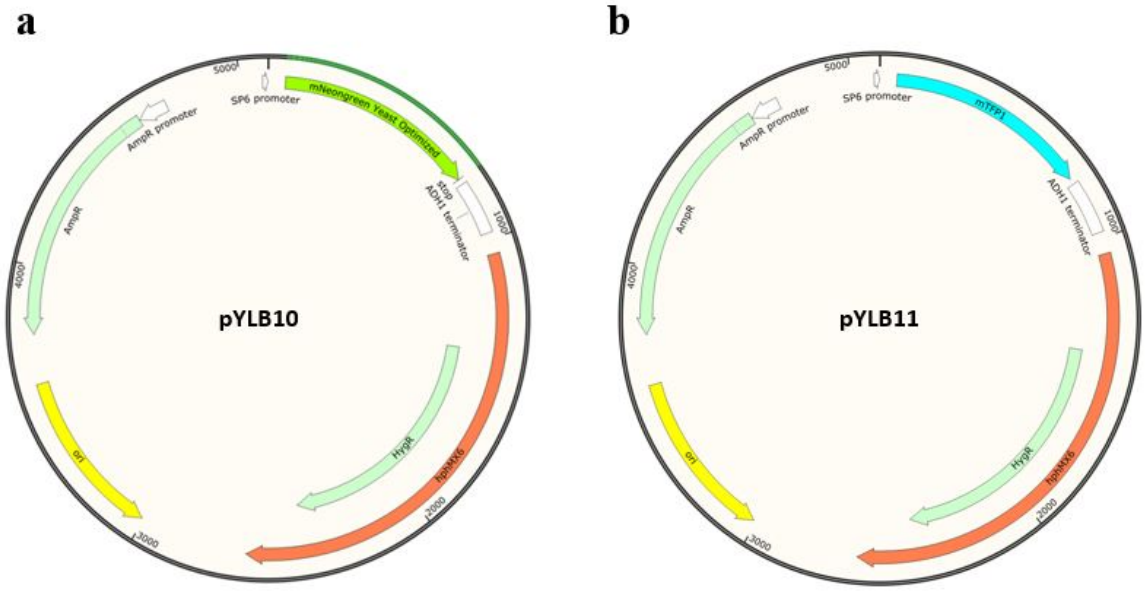


**Figure 26:** Maps of example plasmids used for endogenous tagging

[Longtine et al., 1998] a) pYLB10 b) pYLB11