Functional profiling of the quiescence-to-proliferation dynamics in fission yeast

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I, Bruno Lages, confirm that the work presented in this thesis is my own and has not been submitted for a degree or any other qualification at any other University. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Bruno Lages

3rd January 2017
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Abstract

Cellular quiescence can be defined as a proliferation stand-by, whereby cells direct their focus towards survival and endurance until growth is favored.\textsuperscript{10,25} In fact, most cells spend most of their time in a quiescent state.\textsuperscript{25} Therefore, for microbial cells, a fast and steady transition from this non-dividing state into proliferation confers a fitness advantage and an increased likelihood of self-perpetuation.\textsuperscript{9,89,90} Unicellular organisms in the wild must thus exhibit a highly efficient regulation of this crucial step or otherwise risk gradual elimination. Moreover, the cellular machineries responsible for this transition appear to be in place, ready for quick activation upon requirement, although it is not yet clear how this is accomplished.\textsuperscript{102,105}

Barcode-sequencing (bar-seq) is a state-of-the-art strategy that allows massive parallel profiling of genome-wide deletion libraries in a pooled environment.\textsuperscript{136,137} We implemented, established and optimized this high-throughput platform in our Lab, with the purpose of routinely employing this technique in future research.

We successfully validated bar-seq to allow kinetic studies during a time-course experiment. Furthermore, we employed this technique to carry out a functional profiling study focusing on the dynamics of the quiescence-proliferation (q-p) transition. Together with the q-p transition profiles for over 2,000 mutants, we identified several genes whose deletion leads to altered q-p dynamics, together with others involved in biological processes important for this transition, namely autophagy and chromatin regulation. Furthermore, we uncovered several promising players exhibiting unusual q-p transition profiles, where the kinetics of this transition
was found to be clearly affected. This study thus constitutes a valuable framework for follow-up studies aiming to further elucidate the basis of the dynamic behavior associated with the quiescence-proliferation transition. In addition, we carried out a detailed characterization of the dynamics of this transition in fission yeast using mathematical models specifically developed for that purpose.

Furthermore, we carried out growth fitness profiling of a genome-wide deletion library of non-essential genes in a pool environment, using bar-seq. We identified the mutants that were most affected with respect to growth kinetics during the q-p transition, and estimated their doubling times and respective growth rates. This project should prove to be a valuable resource to guide future work using this deletion library in general, and research on the q-p transition in particular.
List of abbreviations

ANOVA - Analysis of Variance
Bar-seq - Barcode-sequencing
bp - base-pair
CDK - Cyclin-Dependent Kinase
CFU - Colony Forming Unit
cont. - continued
cv - coefficient of variation
DNA - Dexoribonucleic Acid
DN/DNTG - downtag
DT - doubling time
e.g. - exempli gratia, for example
EMM/EMM2 - Edinburgh Minimal Medium
EMM+N - Edinburgh Minimal Medium + (plus) Nitrogen
EMM-N - Edinburgh Minimal Medium - (minus) Nitrogen
ER - Endoplasmic Reticulum
FACS - Fluorescence Activated Cell Sorting
FDR - False Discovery Rate
i. e. - id est, that is
KRIIBB - Korea Research Institute of Bioscience & Biotechnology
Max - Maximum
mTOR/mTORC1/2 - mammalian TOR/TORC1/2
m/s - metres per second
nm - nanometer
NGS - Next-Generation Sequencing
OD - Optical Density
OD$_{600}$ - OD at wavelength 600 nm
ov - overnight
PCG - Protein Coding Genes
PCR - Polymerase Chain Reaction
qPCR - quantitative PCR/real-time PCR
q-p - Quiescence-proliferation
RNA - Ribonucleic Acid
ROS - Reactive Oxygen Species
<table>
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<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>Set1C</td>
<td>Set1 Complex</td>
</tr>
<tr>
<td>Spec</td>
<td>Spectrophotometer</td>
</tr>
<tr>
<td>SVM</td>
<td>Support Vector Machine</td>
</tr>
<tr>
<td>ts</td>
<td>temperature sensitive</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous Sclerosis Complex</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YE</td>
<td>Yeast Extract</td>
</tr>
<tr>
<td>YES</td>
<td>Yeast Extract + Supplements</td>
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Key Words

. Fission Yeast

. *Schizosaccharomyces pombe*

. Cellular Quiescence

. Proliferation

. Quiescence-proliferation transition

. Quiescence’proliferation dynamics

. Quiescence-proliferation transition profile

. Barcode-Sequencing

. Genome-Wide Deletion Library

. Mutant Screen

. Autophagy

. Chromatin Regulation

. Cell Cycle

. BioLector
1 Introduction
1.1 Fission yeast as a model system

*Schizosaccharomyces pombe* (*S. pombe*) is a unicellular eukaryote that has been successfully established as a model organism for the study of a multitude of cell biology phenomena and events. It is commonly named as fission yeast for its characteristic mode of division by binary fission. It has become an increasingly popular model system in recent decades. The relative simplicity, ease of manipulation and the associated multitude of state-of-the-art technologic strategies available for its study make fission yeast a key reference for cell biology research.¹,²

Phylogenetically, fission yeast is part of the Fungi kingdom, Ascomycota phylum, Taphrinomycotina subphylum, Schizosaccharomycetes class, Schizosaccharomycetales order, Schizosaccharomycetaceae family and *Schizosaccharomyces* genus. The phylum Ascomycota represents the largest and most diverse group of fungi. Free-living ascomycetes are commonly found in tree exudates, on plant roots and in surrounding soil, on ripe and rotting fruits, and in association with insect vectors that transport them between substrates. Many of these associations are symbiotic or saprophytic, although numerous ascomycetes represent important plant pathogens that target a myriad of plant species. However, the natural ecology of *Schizosaccharomyces* yeasts has not been adequately covered and studied thus is far from understood.¹,²

The genus *Schizosaccharomyces* is deeply rooted among ascomycetous fungi, comprises three species. From these, *S. pombe*, has been the subject of most extensive experimental research spanning now some 50 years. The two remaining species, *S. octosporus* and *S. japonicus* also have had their genomes sequenced. The potential information associated with these new resources will considerably broaden
the informative potential of fission yeast research and open the door for novel analytical options, namely evolutionary and comparative studies.\textsuperscript{1,2}

\textit{S. pombe} has indeed become one of the best known model organisms in the entire fungal kingdom, together with its distantly related cousin budding yeast (\textit{Saccharomyces cerevisiae}). The fact that a considerable long evolutionary distance separates the two yeasts (the same evolutionary distance as that between \textit{S. pombe} and humans), of about 1 billion years old, makes them highly complementary systems for cell biology research.\textsuperscript{3} As fungi share a common ancestor with the animal kingdom and the fungal-specific specializations of fission yeast are relatively few, much of basic cell biology observed in \textit{S. pombe} is also relevant for basic mechanisms operating in animal cells.\textsuperscript{1}

### 1.1.1 Fission yeast biology and physiology

\textit{S. pombe} are rod-shaped cells that typically measure 3-4 micrometres in diameter and 7-14 micrometres in length (figure 1.1).\textsuperscript{1} These cells maintain their shape by growing by elongation exclusively through the cell tips (apical growth) and divide by medial fission to produce two daughter cells of equal sizes.\textsuperscript{1} Division occurs by the formation of a septum in the centre of the rod. It appears that only a restricted range of cell shapes is possible for the fission yeast cell.\textsuperscript{4} They are usually haploid as their diploid state is usually followed by meiosis.\textsuperscript{1}
Figure 1.1. Fission yeast cells are rod-shaped cells that grow by elongation. Adapted from Morgan, D. (2007)\textsuperscript{5}

1.1.2 Fission yeast genome

The genome of fission yeast is composed of approximately 12.6 million base pairs or mega bases (Mb), organized as three linear chromosomes (table 1.1). Chromosome I is 5.6 Mb long, chromosome II is 4.5 Mb and chromosome III is 2.5 Mb long.\textsuperscript{139} Currently, it is estimated to comprise ~5,118 protein-coding genes and at least 1522 non-coding RNAs, with gaps in the sequence of centromeric (~40-100 kilo bases (kb)) and telomeric (20 kb) regions.\textsuperscript{6,7} It is the smallest number of genes recorded for a eukaryote, fewer even than that of some prokaryotes.\textsuperscript{7}

Around two-thirds (~67%) of the predicted fission yeast coding genome (3,397 protein coding genes) have metazoa orthologs, evidence that the genome of \textit{S. pombe} has maintained a reasonably high degree of conservation.\textsuperscript{6,7,139}

4,515 genes have no paralogs within the \textit{S. pombe} genome itself, reflecting a low degree of redundancy in \textit{S. pombe}’s genome. The fission yeast genome only contains ~41% of duplicated genes.\textsuperscript{139}
Most fission yeast genes contain introns, often rather short, providing some level of resemblance with more complex genomes like that of human.

643 genes from a pool of 4843 analyzed genes revealed no Gene Ontology (GO) process annotation, from which 574 (89.2%) exhibited a deletion phenotype. As an example, there are 513 genes associated with the cell cycle. This shows the fact that some kind of information is already available for most genes of *S. pombe*, reflecting the vast catalogue of knowledge already acquired regarding the genome of this model organism, reinforcing fission yeast as a very valuable tool for biology, and more concretely genome regulation, research.

The relatively large fission yeast chromosomes have features reminiscent of multicellular eukaryotes, including large centromeres mostly comprising sequences of highly repetitive elements, conserved heterochromatin proteins and epigenetic silencing mechanisms, large replication origins, and conserved telomere proteins. 

For more detailed information on *S. pombe* genome features and statistics please see section 1 in the Appendices.

**1.2 Quiescence and proliferation**

**1.2.1 Overview**

The cell is the basic unit of life. Two fundamental cellular states exist: quiescence and proliferation.
Proliferation, or growth, encompasses all the metabolic and cellular events necessary for ensuring the synthesis of a new cell. Proliferation refers to the multiplication of the number of cells, hence usually denoting a population-wise perspective. Growth on the other hand refers to the increase in the size and dimensions of a cell, thus more focused on a single-cell level. It constitutes the state whereby cells prosper by availing environment’s permissive and amenable growth conditions. It is also the way to ensure the perpetuation of the species by continuously originating progeny. In multicellular organisms the situation is different. As progeny is almost exclusively obtained through sexual reproduction (via gametes originating from meiosis) cellular proliferation is key for other reasons. It is the way the single-cell zygote originates the adult individual throughout the developmental process. It also allows the regeneration of damaged cells, tissues and organs, together with the renewal of the body’s own old cells, amongst other less fundamental tasks.\textsuperscript{8,9}

Quiescence on the other hand could be considered a proliferation stand-by mode, a physiological condition whereby proliferation is absent due to cell cycle arrest, and the cell is engaged in endurance and survival until growth is permitted. Therefore, quiescence is a reversible state and quiescent cells remain viable, readily resuming proliferation when growth conditions become present.\textsuperscript{9,10}

Quiescence is the most common cellular state as most cells spend the majority of their time in quiescence.\textsuperscript{10,11} In fact, through a simple arithmetic exercise it is possible to conclude that it would take a single yeast cell a mere 8 days in order to cover the entire surface of the Earth with its progeny, if it was continuously proliferating at its intrinsic standard growth rate.\textsuperscript{10} It is thus evident that proliferation could not constitute the default mode of cells in the wild. Additionally, in multicellular organisms growth is only dominant throughout the initial developmental stages that lead to the adult
individual, after which cellular proliferation usually only occurs for specific biological and physiological tasks other than a mere increase in cell numbers, such as cell renewal, immune responses, amongst others.\textsuperscript{10,11}

Proliferating and quiescent cells are expected to have very different metabolic requirements. While proliferating cells must devote much of their metabolic capacity to biosynthetic processes in order to create the materials necessary to form a new cell, quiescent cells are relieved of this steep metabolic requirement, focusing instead on endurance-promoting biological processes, such as stress resistance responses and energy storage.\textsuperscript{9}

1.2.2 Cell cycle and proliferation

1.2.2.1 Overview

All major processes taking place in the cell are tightly regulated to ensure its proper functioning. In that line it has long been known that cells undergo a particular cycle upon engaging in growth and proliferation. The cell cycle comprehends a unidirectional series of events whereby a cell replicates its DNA, together with other essential cellular macro-components, followed by nuclear and cell division through (the processes of) mitosis and cytokinesis respectively, giving rise to two genetically identical daughter cells.\textsuperscript{5,12}

The cell cycle is divided into two main phases: Interphase, which is then subdivided in G1 (G for Gap), S (S for DNA Synthesis) and G2 phases, and M phase (M for Mitosis), which includes mitosis and cytokinesis. (figure 1.2). Several strict check-points assess and confirm the general conditions, viability and health status of the cell at various key points throughout the cell cycle, ultimately being responsible for making the decision of the cell’s outcome. If the cell possesses any significant damage and/or fails to
successfully meet the criteria required for passing the check-points, it could incur in programmed cell death, or apoptosis\textsuperscript{156}, ensuring that it does not risk originating aberrant progeny\textsuperscript{5,12}.

\textbf{A}

\begin{center}
\includegraphics[width=\textwidth]{cell_cycle_diagram.png}
\end{center}

\textbf{B}

\begin{center}
\includegraphics[width=\textwidth]{cycle_concentration_graph.png}
\end{center}
Figure 1.2 Schematic representation of the cell cycle. A) An overview of the fission yeast general cell cycle. Adapted from Morgan, D. (2007). B) Oscillatory behavior of master cell cycle regulators Cyclins throughout the cell cycle. Adapted from Wikimedia. In S. pombe: Puc1, Cdk1 (Cyclin D); Cig1, Cdk1 (Cyclin E); Cig1, Cig2, Cdk1 (Cyclin A); Cdc13, Cdk1 (Cyclin B).

Interphase is responsible for generating an additional replica of the DNA material, accomplished through the process of DNA replication during S phase. Although the various stages of Interphase are not in general morphologically distinguishable, each phase of the cell cycle has a distinct set of specialized biochemical processes that prepare the cell for initiation of cell division. Most non-genetic cell components are synthesized continuously throughout the cycle, especially during G1 and G2 phases.

Interphase starts with the G1 phase. This could arguably be considered the most pivotal point in the cell cycle (notably in S. pombe), as it is upon it where the cell decides and commits to its fate. Three outcomes could happen from G1: cells can commit to the cell cycle, proceed to mating and consequent meiosis or enter quiescence. Therefore this is a highly regulated step, whereby a precise interpretation of the current conditions needed in order for the cell to undertake the appropriate decision. G1 is then followed by S phase, which as already mentioned refers to the specific stage within Interphase whereby the genetic material is duplicated to give rise to two identical DNA copies. G2 phase, or pre-mitotic phase, is the third and final subphase of Interphase, directly preceding Mitosis. It follows the successful completion of S phase. G2 phase is a period of rapid cell growth and protein synthesis during which the cell readies itself for mitosis.
After Interphase, more specifically G2, cells enter M phase, where chromosome segregation and cell division occur. M phase is much shorter when compared to Interphase, requiring significantly less time as typically Interphase lasts for at least 90% of the total time required for the cell cycle to be completed. Nevertheless, M phase is complex and highly regulated as it is mechanically and morphologically a highly delicate and intricate process. M phase involves a series of dramatic events that start with nuclear division, or mitosis. The mitotic phase begins with chromosome condensation: the duplicated DNA strands of the elongated chromosomes become condensed into the much more compact chromosomes required for their segregation. The nuclear envelope then breaks down, and the replicated chromosomes, each consisting of a pair of sister chromatids, become attached to the microtubules of the mitotic spindle. As mitosis proceeds, the cell pauses briefly in a state called metaphase, when the chromosomes are aligned at the equator of the mitotic spindle, poised for segregation. The sudden separation of sister chromatids marks the beginning of anaphase, during which the chromosomes move to opposite poles of the spindle, where they decondense and reform intact nuclei. The cell then divides through the process of cytokinesis, which separates the nuclei, cytoplasm, organelles and cell membrane into two cells containing roughly equal amounts of each cellular macro-component. With the end of cytokinesis the cell cycle is complete.

1.2.2.2 Key regulators and metabolic pathways

Some features of the cell cycle, including the time required to complete certain events, vary greatly across different species. The basic organization of the cycle and its control system, however, are essentially the same in all eukaryotic cells.
Regulation of the cell cycle involves processes crucial to the survival of a cell, including the detection and repair of genetic damage as well as the prevention of uncontrolled cell division. The molecular events that control the cell cycle are ordered and directional with each process occurring in a sequential fashion that is not reversible.\textsuperscript{5,12-18}

The master players of cell cycle control are proteins called cyclins. These proteins are responsible for regulating all major cell cycle events, exhibiting an fluctuating behaviour that helps directing the cell cycle in a tightly orchestrated sequential manner.\textsuperscript{5,12} Cyclins have their own effector players, or catalytic subunits, with whom they complex and which they ultimately activate, termed cyclin-dependent kinases (CDKs), to undertake and unroll the cascade of regulatory events that ultimately drive the cell cycle forward. Moreover, CDKs remain inactive without their corresponding cyclin partners.

In summary, there are two main groups of cyclins. G1/S cyclins - essential for the control of the cell cycle at the G1/S transition - and G2/M cyclins - essential for the control of the cell cycle at the G2/M transition.

1.2.2.3 Cell cycle in fission yeast

In fission yeast, within Interphase G2 phase is particularly long, with G1, S phase (DNA replication) and M phase (Mitosis) each taking about 10\% of the division time. Generally fission yeast is a haploid, with diploid growth only a transient stage in sexual differentiation.\textsuperscript{14-21,96}

Fission yeast cell cycle progression is controlled by a single CDK, namely Cdc2 (the protein encoded by the cdc2+ gene), in combination with three B-type cyclins (Cdc13
(paralog of cyclin B), Cig1, and Cig2 (paralogs of cyclin A)).\textsuperscript{14-17} Several cyclins associate with Cdc2 in G1-phase and the proteins that regulate inhibitory phosphorylation of Cdc2 during S-phase and G2-phase.\textsuperscript{97} The most important cyclin partner of Cdc2 protein is Cdc13. The complex of Cdc2 and Cdc13 (known as M-phase promoting factor, MPF) is absolutely essential to initiate mitosis, and in the absence of other cyclins, this complex can trigger S phase as well. Cdc13 level fluctuates dramatically during the cell cycle, reaching a maximum as cells enter mitosis, dropping precipitously as cells exit mitosis, and reappearing after S phase is initiated. The activity of Cig2-dependent kinase peaks at the G1/S transition, and it, together with Cdc13-dependent kinase, is responsible for S phase initiation during normal cell cycles. Cig1-dependent kinase peaks at M phase; its physiological role is not known. In addition to the three B-type cyclins, fission yeast has a CLN-type cyclin with unknown function, Puc1 (paralog of cyclin D), whose concentration is roughly constant (small compared to Cdc13 level at mitosis) over the cell cycle.\textsuperscript{14-24}

1.2.3 Cellular quiescence

1.2.3.1 Overview

Ensuring the continuity of life requires that individual cells and entire organisms can survive not only in ideal environments, but also in conditions of scarcity. When conditions are unfavorable for proliferation, many cells have the capacity to become quiescent for extensive periods of time, sometimes years, while retaining the ability to re-enter the proliferative cell cycle.\textsuperscript{10,11,25}

Quiescence can be observed in stem cells, eggs and spores. Some quiescent cells are extremely resistant: they can survive long periods of nutrient starvation, cold temperatures and even desiccation. Entry into quiescence is often associated with
dramatic changes in metabolism, namely the use of intra-cellular substrates and nutrients for the synthesis of macromolecules and energy.\textsuperscript{10,11,25}

There are many contexts whereby a cell might be induced to enter a quiescent state in the wild.\textsuperscript{10,11,25} In unicellular organisms such as bacteria and yeast the critical factors mainly concern nutritional and energy availability, and environmental conditions such as temperature, pH, oxygen and water supply, to name a few.\textsuperscript{25,26} This means that unicellular organisms are highly reliant upon their milieu for growth. Throughout harsh and stressful times, or simply during periods where growth would be ill advised, quiescence becomes a useful adaptive strategy.\textsuperscript{25,26} Quiescent cells have been shown to be more resistant to a myriad of different stress conditions,\textsuperscript{27-31} in great part because they exhibit a characteristic thickened and more resistant cell wall. This extra-protective cell wall defends the cells from harmful conditions such as thermal fluctuations and dehydration, as well as functioning as a stronger and more robust physical barrier, protecting the cell from mechanical disruption of its outer layers.\textsuperscript{27-30}

Many of the conditions that induce cells to become quiescent are also promoters of sexual reproduction and differentiation. For example, exhaustion of a nitrogen source induces fission yeast cells to become quiescent; however, if there are suitable mating partners within the vicinity then yeast cells preferably undergo sexual reproduction and consequent meiosis and sporulation.\textsuperscript{25}

The signals for quiescence vary for different types of cells. Bacteria and yeast can enter quiescence in response to deprivation for a specific nutrient fundamental for growth. Indeed, nitrogen depletion is a particularly efficient way to induce quiescence to fission yeast cells (fig. 1.3, fig. 2.1B), as they have shown to remain viable for extensive periods lasting up to several months.\textsuperscript{25,32} Therefore, this has recently
become an extremely useful strategy for investigating this cellular state in fission yeast, with successful results.\textsuperscript{25,26}

**Figure 1.3** *S. pombe* quiescent cells in nitrogen-depleted media. (a) When exponentially growing *S. pombe* cells are shifted to minimal medium lacking a nitrogen source (quiescence media), cells undergo two rounds of cell division before arresting into quiescent (also called G\textsubscript{0}) cells (~6 h after nitrogen-depletion) to represent this particular phase within a cell cycle perspective. Cells are able to mate and go into meiosis if they find sexual partners of opposite mating type. Otherwise, cells become quiescent G\textsubscript{0} cells that are not able to mate (>12 h). Quiescent cells are small and round, differing from proliferating rod-shaped cells of variable length. *S. pombe* G\textsubscript{0} phase is defined as a cell state in which cells never divide but remain
viable. Quiescent $G_0$ cells are thus in a reversible state, and can return to proliferative cell growth and division upon induction with a growth stimulus (e.g. replenishment of the nitrogen source). Three distinct control windows exist: entry into, maintenance of, and exit from the $G_0$ phase. $G_0$ quiescent cells are fully arrested, non-growing cells, thus differing from pre-replicative $G_1$ or post-replicative $G_2$ phase cells within these cell-cycle stages. (b) A thin-section electron micrograph of $S. \text{pombe}$ $G_0$ quiescent cell. CW, cell wall; L, lipid droplet; M - mitochondrion; N - nucleus; V - vacuole. (c) If the carbon source, such as glucose, is completely depleted from the growth media, cells arrest without further division. The resulting arrested cells are rod-shaped, more similar to proliferative growing cells than nitrogen-depletion induced quiescent cells. $S. \text{pombe}$ cells growing under nutrient-limitation, such as in 0.08% glucose show a decreased cell size. (d) Stationary (ST)-phase $S. \text{pombe}$ cells are obtained by exhausting nutrients in the growth medium after overgrowth. These stationary cells are pear-shaped, and ~20% of them contain 1C DNA. Cells and DNA content of vegetatively growing (VE) and nitrogen-depleted $G_0$ phase quiescent cells are shown for comparison. Synchronous culture analysis indicated that the frequencies of post-replicative with 2-copy DNA (2C) quiescent cells varied (0-20%) depending on the cell cycle stage upon transfer to quiescent media. (e) The stationary phase of $S. \text{cerevisiae}$ is characterized by a diauxic shift, a metabolic shift from glucose to ethanol as an energy source). With this adaptation strategy, $S. \text{cerevisiae}$ cells slowly and continuously grow until reaching stationary phase after 6–7 days. In sharp contrast, $S. \text{pombe}$ cells are unable to utilize ethanol (because it lacks a glyoxylate cycle), thus entering stationary phase within 1 day of exponential growth. Adapted from Yanagida, M. (2009)^83

Conversely, in multicellular organisms like animals and plants, one of the outcomes of their higher systemic complexity translates into a panoply of evolutionary adaptations and mechanisms that contribute to increase survivability and reduce direct exposure to a variety of potentially prejudicial environmental factors. Together with the development of energy reserves, this results into a considerable detachment from a
clear-cut dependence upon these otherwise critical factors. For these reasons, quiescence in multicellular life forms is a much less straightforward feedback response to the corresponding environmental context. With the exception of some well-defined groups or types of cells, for the vast majority of cells their main purpose is other than to proliferate.\textsuperscript{10,11,25} Therefore, cell cycle exit is critical for normal cell physiology and is generally permanent as in the case of fully differentiated, functional cells, which make up for the majority of adult tissues. Here, epigenetic mechanisms that stably silence tracts of the genome by the generation of heterochromatin operate, along with feedback and feed-forward transcriptional loops that induce and stably maintain tissue-specific genes.\textsuperscript{10,11} However, adult tissues also contain reversibly arrested cells, like stem or progenitor cells, that are maintained in a temporarily quiescent state\textsuperscript{10,11}. For these cells, quiescence is not achieved or even maintained by nutritional or environmental signals, but rather actively induced by the organism. This is typically accomplished by signaling molecules synthesized and secreted by the organism, such as hormones that work by inhibiting growth and promoting other important biological processes contributing to the homeostasis and correct functioning of the organism.\textsuperscript{10} Moreover, in mammals the cellular state of individual cells is regulated by a host of factors that include cues from proliferative signaling molecules such as mitogens.\textsuperscript{10} These growth-related factors are crucial for the body to maintain its cellular balance and levels. Moreover, disruptions occurring in the regulation of cellular growth, metabolism and physiology could lead to serious complications within multicellular organisms like humans, namely tumorigenesis and cancer\textsuperscript{10,25,33,34}, amongst other severe disorders\textsuperscript{10,25}.

Despite the major differences driving cells into quiescence in unicellular versus multicellular organisms, the strategies and mechanisms employed remain strikingly similar on a molecular level.\textsuperscript{25} The cellular pathways involved for establishing whether
a cell is quiescent or proliferating are well-conserved from yeast to human.\textsuperscript{25} Furthermore, quiescent yeast and mammalian cells share a similar set of responses to conditions that are not suitable for proliferation: cell cycle arrest in $G_1$, condensed chromosomes, reduced rRNA synthesis, decreased translation levels, decreased cell size, activation of autophagy and increased resistance to a variety of stresses.\textsuperscript{10,11,25-31}

Most cells in our body lose the capacity for dividing once differentiation is complete, ultimately becoming quiescent.\textsuperscript{10} Therefore, an adequate understanding of this cellular state should prove a decisive requirement for carrying out biomedical research. Therefore, instead of regarding quiescence almost as a cell cycle outcast when compared to the spotlight given to the cell cycle in Cell Biology research, one could argue that a more prominent and pivotal role should be awarded to this cellular state, as more than merely a state opposing proliferation, quiescence likely constitutes the default state of cells in most if not all living organisms during most of their life cycle.\textsuperscript{25}

On a physiological level, the link between quiescence and proliferation must be tightly regulated during the development of a single embryonic cell into the complex pluricellular structure that makes up the adult organism, as well as in the further homeostatic maintenance of the adult organism.\textsuperscript{9,10,25,35} As already mentioned, disruption of this tightly-regulated balance can lead to numerous complications, from cancer and other degenerative diseases to various sorts of metabolic disorders.\textsuperscript{25,33,34} Therefore, the relevance of understanding how quiescence is regulated, and importantly the mechanisms that govern the transition between these two basic cellular states should not be underestimated. However, all these processes associated with such cellular state transition remain relatively poorly understood, again especially when compared to the level of detail dedicated to the study of the cell cycle.
1.2.3.2 Key regulators and metabolic pathways

- Pathways that control the metabolic state: TOR pathway

One pathway that is critical for determining the metabolic state of cells is the highly evolutionarily conserved target of rapamycin (TOR) pathway. Extracellular and intracellular information about the availability of nutrients and the cell’s energetic state are integrated into this pathway, greatly contributing to the rate of cell growth. When nutrients are plentiful, the TOR regulatory protein kinase, the upstream main player of this pathway, phosphorylates and activates downstream targets that promote cell growth and increase cell size. When nutrients are scarce, namely nitrogen, and in response to other triggers (like stress agents), the TOR pathway becomes inhibited or even inactive. Cells with inactive TOR reduce energy-intensive processes and induce catabolic programs that allow the cell to recover metabolites and energy from existing macromolecules through autophagy to ensure their survival.

In fission yeast as in multicellular eukaryotes, two TOR protein complexes exist, namely TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 is required for cell growth, while TORC2 is important during suboptimal conditions, such as nutrient depletion, promoting growth inhibition.

TORC2 has a cell cycle role through determining the proper timing of Cdc2 Tyr15 dephosphorylation and the cell size under limited glucose, whereas TORC1 restrains mitosis and opposes securin-separase, which are essential for chromosome segregation.
Loss of the TOR Kinase Tor2 mimics nitrogen starvation. TOR, in association with cooperating proteins, phosphorylates targets that promote protein synthesis and cell growth. TORC1, which is sensitive to rapamycin (TORC2 is not), plays an important role in promoting translation and two important targets of its kinase activity are 4E-BP1 and S6 kinase. Phosphorylation of 4E-BP1 leads to eIF-4E activation, which can in turn recruit the translation initiation complex and increase cap-dependent translation rates. TOR also phosphorylates and activates S6 kinase. S6K phosphorylates S6 protein of the 40S ribosomal subunit and other targets and activates translation. TORC2, in contrast, phosphorylates and activates Akt (also called protein kinase B), which inhibits apoptosis and promotes survival. TORC2 also targets proteins important for cytoskeletal organization. Excessive TOR activity results in increased cell growth while TOR inhibition results in reduced cell growth and smaller cell size.

TOR protein kinases play a vital role in the regulation of cell growth and metabolism in response to environmental cues. Tor2 containing-TORC1 complex (TORC1) directly promotes protein translation and ribosome biogenesis in response to nutrient levels through the TOR-mediated signal transduction pathway. ∆tor2 temperature sensitive (ts) mutant exhibits a phenotype in growth-promoting medium that includes a pear-shaped cell morphology and unreplicated DNA, typical of quiescent cells, and fails to initiate growth and replication from a state of G0. The TORC2 complex containing the Tor1 catalytic subunit is not essential for growth and division, but ∆tor1 is sensitive under various stresses including nutrient starvation and DNA damage.

The S. pombe TOR signaling pathway includes the tuberous sclerosis complex (TSC) Tsc1 and Tsc2 proteins and Rheb GTPase Rhb1, which are involved in amino acid import. Additionally, Tel2, Tti1 and Tti2 proteins form a stable complex that
interacts with all Pik3 related kinases (PIKKs), TOR kinases, Tra1/Tra2 (TRRAP homologues) and Rad3/Tel1 (ATR/ATM mammalian homologues).\textsuperscript{50,51} It is believed that Tel2 might act as a chaperone that stabilizes PIKKs or possibly upon signal transduction of the PIKK cascades. TRRAP proteins are common components of various histone acetyltransferase complexes and mediate a diversified array of cellular responses by recruiting these complexes to chromatin.\textsuperscript{50} ATR and ATM homologues play a major role in the response to DNA damage and in the regulation of replication delay, with Rad3/ATR proving essential for DNA damage repair in \textit{S. pombe} quiescent cells.\textsuperscript{28}

- Pathways that Regulate Metabolism: Pik3 pathway

The phosphatidylinositol-3-kinase (Pik3 in fission yeast) pathway is the pathway through which extracellular insulin and growth factors affect TOR activity and thereby modulate the cell’s metabolic state.\textsuperscript{36,37} In mammals Pik3 phosphorylates the lipid phosphatidylinositol-4,5-bisphosphate to generate phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P\textsubscript{3}], which creates lipid docking sites on the cytoplasmic face of the plasma membrane (figure 1.4).\textsuperscript{35-37} This activity is opposed by the dephosphorylating action of tumor suppressor PTEN phosphatase, Tep1 in budding yeast\textsuperscript{42} and not identified in \textit{S. pombe}.

Serine/threonine protein kinases Sck1 and Sck2 (Sck1-2) can bind to PtdIns(3,4,5)P\textsubscript{3} on the cytosolic face of the lipid membrane, become activated and phosphorylate downstream targets.\textsuperscript{36,37,95} Among the targets of Sck1-2 is the tuberous sclerosis complex (TSC), which it inhibits.\textsuperscript{48,49} TSC acts as a GTPase-activating protein and inhibits the activity of the Rhb1 GTPase, which in turn activates TOR. When nutrients are plentiful, Sck1-2 is active; TSC is inactive, and Rhb1 activates TOR. When nutrients
are scarce, Sck1-2 is inactive; the TSC complex is active; Rhb1 is inhibited, and TORC1 is inactivated. Mutations in the TSC complex result in constitutive activation of TOR and tuberous sclerosis disease, an autosomal dominant disorder characterized by benign tumors affecting virtually all organ systems of the body.\textsuperscript{48,49}

TOR activity is also responsive to intracellular energy levels through Ssp1 (fission yeast ortholog of mammalian AMP activated protein kinase AMPK). A high ratio of AMP to ATP, indicative of an energy-depleted state, leads to restoring of cellular ATP levels through phosphorylation of key regulatory proteins involved in protein synthesis, fatty acid and glucose metabolism and glucose transport, resulting in a reduction in energy-intensive cellular activity.\textsuperscript{36-45} Ssp2 also phosphorylates TSC, which results in inactivation of TOR and a decrease in biosynthesis when the cell is depleted of energy equivalents. Thus, when extracellular conditions indicate that nutrients are rich and ATP is plentiful, TOR leads the cell into a growth program. If the cell is in an energy-poor state or nutrients are unavailable, TOR inactivation results in decreased translational activity, and, instead, there is a shift to an alternative metabolic program in which the cell reclams energy and metabolites through autophagy.\textsuperscript{36-45}

Therefore, through the control of energy and nutrient availability, the Pik3-TOR pathway becomes largely implicated in control of quiescence exit and entry.\textsuperscript{43} Furthermore, there is a close connection between TOR activation and the cell cycle. Treatment with TOR inhibitor rapamycin causes fission yeast to arrest in a quiescence-like state.\textsuperscript{47}
Figure 1.4 External factors induce cell growth and suppress autophagy via Akt/Sck1-2Spo and TOR. Upon stimulation by external factors, such as growth factors, the receptor tyrosine kinase is phosphorylated and recruits Pik3 (fission yeast ortholog of mammalian PI3K). PI3K leads to recruitment of Pkp1 (fission yeast ortholog of mammalian PDK) and Sck1 and Sck2 (fission yeast ortholog of mammalian Akt) to the cell membrane. Akt, when phosphorylated by TORC2 (mTORC2 (mammalian-TORC2) in figure) and PDK, inhibits TSC, which in turn inhibits Rhb1 (fission yeast Rheb1 GTPase mammalian ortholog). When activated, Rheb promotes TORC1 (mTORC1 (mammalian-TORC1) in figure) action, which leads to an increase in biosynthesis and suppression of autophagy via Atg1 (fission yeast ortholog of mammalian ULK1). Thus, nutrient-rich conditions lead to high activity of Pik3, Sck1-Sck2 and Tor2-containing TORC1, while nutrient depletion causes reduced Pik3 activity and a resulting decrease in TORC1 activity. Adapted from Valcourt, J. (2012)\(^\text{10}\)

- Autophagy

Cells that are in nutrient-poor environments or are not actively growing tend to regulate two distinct processes in a coordinate manner: on the one hand, they reduce the rate at which macromolecules including proteins are synthesized, and on the
other hand they activate catabolic processes that allow them to recapture energy by breaking down macromolecules. One approach to achieving an increase in catabolism is the activation of autophagy, as mentioned before when describing the Pik3-TOR pathways.

Autophagy, more specifically macroautophagy (autophagy of specifically cellular macro-components)\textsuperscript{53}, is an evolutionarily conserved mechanism through which cytoplasmic proteins and organelles are engulfed into autophagosomes and degraded in lysosomes.\textsuperscript{54,55} The biological functions of autophagy include the reclamation of metabolites and ATP, the elimination of damaged proteins and organelles\textsuperscript{56-61}, the elimination of pathogens, and in multicellular organisms tumor suppression and antigen presentation. In multicellular organisms defects in autophagy have been implicated in liver disease, neurodegeneration, Crohn’s disease and metabolic syndrome.\textsuperscript{58,59,61-64}

Autophagy is required for long-term survival in quiescence in yeast.\textsuperscript{65-71} Furthermore, Reactive Oxygen Species (ROS) accumulate in the cell, both in the nucleus but especially in mitochondria during quiescence.\textsuperscript{72-74} For that reason, mitochondrial degradation by autophagy (mitophagy) is also one of many vital anti-ROS protection events that occur during quiescence.\textsuperscript{72-74}

The TOR complex can act as an intermediary between the presence of glucose and amino acids, and the catabolic process of autophagy (fig 1.5). Amino acid depletion results in reduced TOR activity and an induction of autophagy, which releases free amino acids from lysosomes for the rebuilding of proteins.\textsuperscript{60,61,64,66} TOR actively represses autophagy, and thus, when cells are in a nutrient-rich, high-energy state, only basal autophagy is activated. TOR controls autophagy levels in part by
phosphorylating and inhibiting the autophagy specific kinase ULK1, Atg1 in fission yeast.\textsuperscript{75} When TOR is inactivated, Atg1 is released from inhibitory phosphorylation events. Atg1 in conjunction with other coordinating proteins can then form complexes that initiate the formation of autophagosomes.\textsuperscript{54,55,76} Thus, under conditions of starvation signaled by low levels of glucose or amino acids, autophagy can provide nutrients to an energetically depleted or starved cell.\textsuperscript{60,62,66,72,74}

\textbf{Figure 1.5 Autophagy is activated by low TOR signaling and activation of ULK1/Atg1\textsubscript{Spo}.} In starvation conditions, high TSC activity represses TOR, which allows ULK1/Atg1\textsubscript{Spo} to become active. After recruiting additional proteins to form a complex, ULK1/Atg1\textsubscript{Spo} promotes autophagosome formation and autophagy. In contrast, high mTOR activity in high-nutrient conditions phosphorylates ULK1/Atg1\textsubscript{Spo} and suppresses the induction of autophagy. Adapted from Valcourt, J. (2012)\textsuperscript{10}
During starvation, autophagy can provide a nutrient source and thereby promote survival. Autophagy is required immediately after mating in fission yeast, to provide energy for differentiation and spore formation.\textsuperscript{66,69}

The Atg proteins required for all autophagy-related pathways are referred to as the core Atg proteins, and most of them are involved in the generation of a double membrane-enclosed transport vehicle called autophagosome.\textsuperscript{54,55,76}

Two protein complexes are important for initiating the autophagosome formation process (figure 1.6). One complex consists of Atg1 kinase and its associated proteins.\textsuperscript{54,55} The other is the phosphatidylinositol 3-kinase (Pik3) complex composed of Vps34, Vps15, Atg6, and Atg14, which generates phosphatidylinositol 3-phosphate (PI3P) at the sites where autophagosomes are assembled.\textsuperscript{77} PI3P is recognized by Atg18 (homolog of mammalian WIPI proteins), which together with Atg2, regulates the retrograde trafficking of Atg9, the only core Atg protein with transmembrane domains.\textsuperscript{78,79,82}
The expansion of the autophagosome precursor, called the isolation membrane or phagophore, requires the conjugation of an ubiquitin-like protein Atg8 to phosphatidylethanolamine. Factors involved in this conjugation include the Atg8 processing enzyme Atg4, the E1 enzyme Atg7, the E2 enzyme Atg3, and the E3-like complex Atg12–Atg5–Atg16. Atg12 is another ubiquitin-like protein whose conjugation to Atg5 requires the E2 enzyme Atg10.\textsuperscript{54,55}

In addition, Atg6, Atg11, Atg17, and Atg101, Atg10, Atg14, Atg16, Ctl1, and Fsc1 were also found to be required for substrate-Atg8 processing.\textsuperscript{81} More concretely, Atg10 is required for the conjugation of Atg12 to Atg5 and Atg16 protein interacts with Atg5 both in the presence and in the absence of Atg12. Atg6 is the expected
binding partner of Atg14 in a PI3K complex.\textsuperscript{77} Fsc1 localizes to the vacuole membrane and is specifically required for the fusion of autophagosomes with vacuoles.\textsuperscript{81} In S. cerevisiae, a constitutive biosynthetic route termed cytoplasm-to-vacuole targeting (Cvt) pathway utilizes the Atg proteins to transport cytosolic hydrolases into the vacuole, and thus the assembly of Atg proteins also occurs under nutrient-rich conditions. In contrast, the Cvt pathway has not been identified in S. pombe. A striking difference in autophagy between the 2 yeast model systems is that a budding yeast cell has one or a few large vacuoles, whereas an S. pombe cell has about 80 small vacuoles.\textsuperscript{67,68,70,71,81}

1.2.3.3 Genetic control of cellular quiescence in fission yeast

In fission yeast, when compared to cellular proliferation, quiescence is characterized by lower rates of transcription, translation and metabolic activity, as well as reduced cell size and a heterochromatic nucleus.\textsuperscript{25,83-87,133-135} The cellular protein content is approximately one-fifth of that of vegetative growing cells.\textsuperscript{25,86} They also show a significant increase in the number of vacuole structures, and are more resistant to a variety of stresses.\textsuperscript{25,28,135} Moreover, for all the reasons described and more, it is increasingly becoming clear that quiescence is not a just a passive state of cell cycle arrest characterized merely by a depressed macromolecular metabolism, but an actively regulated one maintained by a typical transcriptional profile that includes induction of a large number of genes\textsuperscript{10,11,25,26,83,86,87}. Furthermore, S. pombe quiescent cells require proteasome function during the maintenance of G\textsubscript{0} quiescence.\textsuperscript{72,74,88}

Transcription factors that promote quiescence are distinct from factors that determine irreversible cell differentiation events and cell fate decisions.\textsuperscript{10,11} However, the
mechanisms regulating these genes are not yet well understood. Cell cycle regulation in response to nutrient deprivation or quiescence cues is often mediated through cyclin-dependent kinase (CDK) inhibitors. Quiescence signals, such as hormone withdrawal, nutrient starvation and contact inhibition result in the upregulation of CDK inhibitors. CDK inhibitors also maintain cells in a quiescent state, and their elimination can result in inappropriate cell cycle entry.\textsuperscript{10,11}

Around 300 genes were found to be required for both quiescence and proliferation, covering a broad range of distinct cellular functions.\textsuperscript{83} These were called super housekeeping genes (fig 1.7). Also, \textasciitilde 750, genes uniquely essential during quiescence, were reported.\textsuperscript{25,83}

However, a central player inducing the transition into a quiescence state in a similar fashion to that of cyclin-dependent kinases (CDKs) towards cell cycle progression has not been identified.

Quiescent cells contain abundant Rum1 protein, a CDK inhibitor.\textsuperscript{18,25} No S-phase cyclin has been detected (Cig2) in quiescent cells, but were found to exhibit low levels of Cdc13, a mitotic cyclin B.\textsuperscript{18,25}

The transcriptional program of quiescent cells has been shown to be very distinct from that of proliferating ones.\textsuperscript{89} In fact, more than half of the fission yeast transcriptome was shown to be differentially regulated upon transition from quiescence to proliferation.\textsuperscript{25,89} Most prevailing quiescence-specific transcripts encode glycolytic enzymes and other catabolic factors.\textsuperscript{89} In addition, transcripts for stress-related proteins, such as heat shock proteins, transporters and ubiquitin ligases are also abundant, contrasting with reduced levels of protein biosynthesis.\textsuperscript{89} Several quiescence (G\textsubscript{0})-lethal mutants relate to transcriptional control, such as Fcp1, Klf1 or
Rsv2. Rsv2 is involved in the transcriptional control of proteasome genes. Fcp1 regulates transcription through phosphorylation of the C-terminal repeat domain (CTD) of RNA polymerase II. It also seems to play a central role in differentiating quiescence from proliferation, as several transcripts essential for both physiological states, were severely affected in Δfcp1. Other genes that were found to be essential in both quiescent and proliferative states cover a broad range of cellular functions (figure 1.8). These include Sty1 (p38 MAPK in human) and Wis1 dependent stress responses, vacuole formation, autophagy, endosome formation (through interactions with cortical actin) and protein trafficking. The Sty1 pathway, whose control is exerted through TOR signaling, regulates the main changes that occur upon entry into quiescence, including nuclear organization and Rum1 levels. Cdk1 serine/threonine kinase, a key player in cell cycle regulation, is also required during quiescence, contrary to what was observed for other essential cell cycle regulators. Another essential protein, the histone H4 chaperone RbAP, is required for the stability and maintenance of centromere regions. Although most of the functions of these proteins are partially understood in proliferating S. pombe cells, their roles in quiescence remain to be properly elucidated. Nonetheless, the fact that most of them are conserved in mammals and perform vital cellular functions for the metabolism of DNA, RNA, protein and metabolites is consistent with the notion of a conserved mechanism regulating quiescence. In summary, during quiescence in S. pombe control of the cell cycle and growth is largely inhibited and diminished, whereas stress-responsive signaling is fully functional. Protein translation is decreased, contrasting with highly active sugar catabolism, protein and nucleic acid recycling mechanisms, trafficking, and vesicle function.
1.2.4 Quiescence-proliferation transition

1.2.4.1 Cellular and transcriptome dynamics

Transition from a quiescent, non-dividing state into a proliferative state constitutes a critical step for the life of an organism in the wild. Failure to do so in an efficient and successful manner could greatly reduce the chances of survival. Therefore, a tightly regulated highly responsive and sensitive system is required in order to ensure...
that organisms are well-equipped to perform such transition with high capability and success.$^{9,10}$

In *S. pombe* nitrogen-deprived quiescent cells it was observed that upon quiescence-proliferation transition, cells that had been quiescent for a longer period of time (24 days) exhibited a longer delay re-entering the cell cycle than cells that had been in quiescence for a shorter period (1 day).$^{91}$ The reasons behind these delayed kinetics could only be partly accounted for, and involved the duration of nuclear decondensation and cell elongation processes, suggesting the involvement of both chromatin and growth factors.$^{91,93,94}$ However, it has also been argued that metabolic rather than cell cycle signals control quiescence entry and exit$^{92}$, even though this is still a matter of intense debate.$^{93}$ A study aiming to understand the changes upon this transition occurring at the level of the transcriptome was performed in *S. pombe*.$^{89}$ In this study, cells were kept quiescent for 2 days and then induced to undergo the q-p transition and return to proliferation, whereby the transcriptome was analyzed. Marked differences were observed in the transcriptional patterns between quiescent and proliferative cells (figure 1.8). Two-fold changes (increase and decrease) were detected in the levels of about 2,700 transcripts upon q-p transition. Of the 30 most abundant transcripts in quiescent cells, 23 were replaced by ribosome- and translation-related transcripts upon q-p transition. During this transition, a two-step alteration of the transcriptional profile occurred before the reduction of Rum1 and the onset of DNA replication. Significant increase in the proteome content was detected one hour after q-p transition, but only after two hours did the cell length began to increase. The timing of DNA replication was ~6 hours. The greatest change to the transcriptome was observed 1-2 hours after the q-p transition in cells that morphologically still resembled a quiescent state. Genes that showed a faster decrease in transcript level while staying down-regulated during proliferation were
strongly enriched in catabolic and oxidoreductive processes, whereas those exhibiting a sharp increase in transcript levels were implicated in protein biosynthesis and ribosome biogenesis. A group of approximately 980 transcripts, whose intensity was not altered, belonged to families of genes related to chromatin remodelling, cell cycle control and RNA splicing. These transcripts did not show any significant changes between proliferation and quiescence, which suggests that they could be ubiquitously required by the cells in a steady-state level.

![Graphical representation of transcript ratios and gene ontology analysis.]

<table>
<thead>
<tr>
<th>Cluster name</th>
<th>Genes</th>
<th>Gene ontology</th>
</tr>
</thead>
<tbody>
<tr>
<td>DN1</td>
<td>127</td>
<td>amine catabolism, oxido reductase, hydrolase</td>
</tr>
<tr>
<td>DN2</td>
<td>358</td>
<td>vacuole, carbohydrate metabolism, autophagy, carbohydrate transporter, reverse transcriptase</td>
</tr>
<tr>
<td>DN3</td>
<td>483</td>
<td>mitochondria, generation of precursor metabolites, ATP and energy, ubiquitin-dependent proteolysis, protein modification, mitochondrial ATPase</td>
</tr>
<tr>
<td>DN4</td>
<td>389</td>
<td>membrane, cell communication, signal transduction, response to nutrient levels</td>
</tr>
<tr>
<td>UP1</td>
<td>287</td>
<td>cytosolic ribosome, nucleolus, protein biosynthesis, ribosome biogenesis and assembly, rRNA processing</td>
</tr>
<tr>
<td>UP2</td>
<td>400</td>
<td>nucleolus, nuclear lumen, RNA binding, helicase, translation factors, ribosome biogenesis and assembly, rRNA processing</td>
</tr>
<tr>
<td>UP3</td>
<td>362</td>
<td>tRNA modification, nuclease</td>
</tr>
<tr>
<td>UP4</td>
<td>347</td>
<td>mitochondria, ER to Golgi, translation, protein transporter, chaperones, secretion, glycoprotein metabolism</td>
</tr>
<tr>
<td>SA</td>
<td>979</td>
<td>Chromosome, chromatin, organelle ribosome, DNA metabolism, transcription, splicing, mRNA processing, cell cycle</td>
</tr>
</tbody>
</table>

*TRENDS in Cell Biology*
Figure 1.8 Transcriptome profile changes upon the transition from quiescence to proliferation. The transcriptional program of fission yeast was analyzed during the q-p transition. Time-points depicting 1 (R10), 2 (R2), 3.5 (R3.5) and 6 (R6) hours upon q-p transition initiation, and upon full proliferation (VE), were analyzed by comparison with transcriptomes during quiescence (G0). Over 2700 genes were classified into eight cluster groups. Green indicates down-regulation (< 0.5-fold), while red indicates up-regulation (> 2-fold). ~1200 genes did not show reliable scores and were thus eliminated from the initial pool of 4932 analyzed genes. Of the remaining 3732 genes, 980 classified as SA (small alteration) with ratios of 1 at any time-point. A brief summary of the gene ontology for biological processes, cellular components, and molecular functions of each gene cluster is also shown. Adapted from Shimanuki et al. (2007)\(^9\)

1.2.4.2 Chromatin dynamics

The massive and highly coordinated transcriptional response that occurs when transiting between states of quiescence and proliferation imply a tight regulation of the associated broad gene expression programs.\(^89\) Chromatin dynamics, namely nucleosome positioning and histone post-translational modifications, play a critical role in this challenging task at a genome-wide level.\(^98-101,103\) Accordingly, shortly upon quiescence entry, pronounced nucleosome eviction from both promoter and coding regions was observed at the loci of up-regulated genes, many of which are found to be clustered in the genome in a non-random manner.\(^99,100\) This lower nucleosome density persisted for at least 60 minutes. Also it was found for two gene clusters that their subnuclear localization suffered drastic changes upon quiescence entry in a way that was transcription dependent.\(^99,100\) On proliferating cells these gene clusters localized to the nuclear periphery in a histone deacetylase Clr3 dependent manner, but 20 minutes after entry into quiescence they were found deep inside the nucleus. These observations support the importance of chromatin dynamics in regulating gene
expression upon the transition between proliferation and quiescence, and point to an important role of chromatin architecture remodelers for achieving such control. Moreover, a study in budding yeast showed that during stationary phase high levels of RNA polymerase II were detected at intergenic regions directly upstream of hundreds of genes immediately induced upon stationary phase exit.\textsuperscript{102}

Another striking and highly dynamic phenomenon is that of transcriptional memory.\textsuperscript{106-125} Initially observed on the yeast Gal gene cluster, which is repressed by glucose, it describes the peculiar reactivation kinetics exhibited by these genes upon transcription induction. In summary, it was shown that in Gal cluster genes that have been recently transcribed, further reactivation of these genes leads to a faster transcription kinetics, suggesting that somehow the gene remained poised for activation, even after transcriptional repression.\textsuperscript{105} Moreover, the promoter and coding regions of these genes were found to remain highly tri-methylated at histone 3 lysine 4 (H3K4me3) for up to five hours, or the equivalent to 2 rounds of division in yeast.\textsuperscript{105} An epigenetic regulation of this phenomenon has since been suggested to be possibly behind such effect.\textsuperscript{104-106} However, this matter remains to be properly elucidated and is the subject of strong debate.

1.3 Heterogeneity and bet-hedging in yeast clonal cell populations

Bet-hedging can be defined as investing in opposite outcomes.\textsuperscript{127} This is a phenomenon that happens at the population level, with the advantage of spreading the risks amongst individuals of a population.\textsuperscript{130} It has been widely studied in bacteria, where it is used to explain antibiotic resistance.\textsuperscript{126,129} Clonal populations of bacteria
were shown to exhibit two distinct types of cells: some cells that grew at the maximum growth rate and others, named persister cells, that were slow growing, almost quiescent. The rapid growing cells were shown to be very sensitive to an array of different types of stress and drugs, whilst the slow growing persister ones exhibited high resistance to both kinds of perturbations. Bet-hedging also describes one of the strategies undergone by cancer cells in tumors to escape death and acquire resistance to therapeutic agents.\textsuperscript{127} A study has recently shown that a similar type of strategy is present in budding yeast.\textsuperscript{128} In yeast, no clear-cut distinction between two sub-populations was observed, rather cells exhibited a continuum of growth rates, from very fast to very slow. Importantly, progeny of both fast- and slow-growing cells could ultimately reproduce the population’s ancestral growth-rate range of values, demonstrating that, like bacteria, yeast can switch between growth states. Quiescent cell populations might exhibit a similar heterogenous feature, providing some cells with a metabolic or physiological state more approximated to that of proliferative cells, allowing these cells to quickly resume proliferation as soon as environmental conditions become favorable. Understanding whether this interpretation is in any way observed in yeast quiescent populations would be of great biological interest and might bring value to improving the approaches available for tackling quiescence-proliferation related disorders like cancer.\textsuperscript{127}

1.4 Aim

The main scope of this project concerned the functional profiling of the transition occurring when cells engage into proliferation from a quiescent state, and the identification of a genetic basis regulating the dynamics of this transition.
2 Methodology
2.1 Cell biology techniques

2.1.1 *S. pombe* wild-type and mutant strains

*S. pombe* wild-type strains were obtained from Leupold 1948 original *S. pombe* isolate. *S. pombe* wild-type strains used in this study included heterothallic strains 972 h- and 975 h+, and homothallic strain 968 h90 (figure 2.1).

![Image of fission yeast cells](image.png)

**Figure 2.1 Wild-type *S. pombe* 972 h- cells visualized under light microscope. A) During Proliferation. B) During quiescence induced by nitrogen depletion.** Nitrogen-starvation response induces cells to enter mitosis at a reduced cell size. Imaging of fission yeast cells was performed with a Zeiss microscope (magnification 40x) coupled with the image analysis software Volocity version (v) 5.5.1 as indicated by Perkin Elmer. Images were captured using the Auto-Exposure setting.

All gene deletion mutant strains used in this work were obtained by transformation of the aforementioned wild-type strains through PCR recombination following the methodology developed by Bahler et al. (1998). Primer design for gene deletion was obtained with the pppp bioinformatic resource available at
Unless otherwise specified all gene deletion mutant strains used in this work were obtained from the comprehensive fission yeast strain collection available at the lab. These strains are preserved in a 25% glycerol stock solution stored at -80°C.

To make a glycerol stock of a fission yeast strain, a pre-culture of that strain was grown to near saturation. 700 µl were then collected from the pre-culture to a vial tube. 700 µl 50% glycerol sterile solution were then added and mixed by pipetting. Tubes were then stored at -80°C.

### 2.1.2 Cell culture and cell media

To start and set up a cell culture of a fission yeast strain, a bit of biomass was scraped from the strain’s glycerol stock stored at -80°C and spread onto a 2% agar culture plate. The cells were then incubated at 32°C for 2-3 days after which small *S. pombe* colonies started to appear. For preserving these colonies for prolonged duration, we kept them in culture plates wrapped in Parafilm tape at 4°C for up to 2-3 weeks usage.

To grow fission yeast cells we used culture media from Formedium. For rich media we used Yeast Extract (YE) media plus supplements (YES), for minimal media we used Edinburgh Minimal Media (EMM) media with (+N) or without (-N) a nitrogen source (solution of NH₄⁺ (Sigma). All culture media content’s concentrations are as recommended by Formedium. Standard temperature for growing fission yeast cells was 32°C, unless otherwise stated. Standard shaking speed was 180 rpm (rotations per minute), unless otherwise stated. Solid media used was YES in 2% agar or EMM+N.
in 2% agar in culture plates (identical to Petri dishes). Liquid media was used for liquid cultures grown in Erlenmeyer culture flasks, from all volumes ranging 10-2,000 ml. All culture media was prepared as indicated by Formedium.

To start a fission yeast cell culture we scraped a bit of a single colony from the desired strain on a culture plate using a inoculation loop and added the collected biomass to a culture flask containing YES liquid media, with a short vigorous shaking of the inoculation loop immersed in the liquid media. Flasks containing this pre-culture were placed in shaking incubators and allowed to grow overnight (ov). Then a small sample was collected to inoculate new culture flasks, containing the fresh media of choice. The volume of liquid culture media used depended on the experiment, ranging from 10-250 ml, and was usually pre-heated to the designated experimental temperature. Once the *S. pombe* cell culture was obtained the experimental study would be initiated. As a rule, fission yeast liquid cultures would be grown up until their culture reached the optical density (OD$_{600}$) value of 0.6 as a starting point for beginning the experimental assay. This cellular density ensured all the cells were in the mid-logarithmic phase of exponential growth and thus retaining a high degree of physiological homogeneity and uniform growth across the whole cell population. By default, 3 or more biologically independent cultures were used to replicate each tested condition or fission yeast strain.

To induce fission yeast cells to become quiescent, nitrogen was removed from the culture media. For this, *S. pombe* cells in EMM+N liquid cultures were transferred to EMM-N liquid cultures. For that, cells were moved to 50 ml Falcon tubes and centrifuged for 3 minutes (min) at 2,300 rpm at room temperature (RT) to remove the EMM+N media, washed 2x in EMM-N media to remove remaining traces of EMM+N media and resuspended in culture flasks with EMM-N media.
To induce cells to exit quiescence and resume proliferation, *S. pombe* cells were transferred from EMM-N to EMM+N liquid media. Cells were collected in 50 ml Falcon tubes and centrifuged for 3 minutes at 2,300 rpm at room temperature (RT) to remove the EMM-N media and resuspended in culture flasks with EMM+N media.

The centrifuge machine used when handling fission yeast liquid cell cultures was model 5804R by manufacturer Eppendorf. This applies for all steps that include centrifugation of fission yeast liquid cell cultures.

### 2.1.3 Cell mating

For mating *S. pombe* cells we used a common standard mating protocol followed by a Glucurosidase test to select for the mating products, spores.\(^{132}\) We used either homothallic or heterothallic strains from different mating types, designated accordingly. Protocol is described next.

The first step consisted on cell mating, where we:

- picked a colony of the first strain and mixed it in 50 µl sterile H\(_2\)O previously placed in a sterile Eppendorf tube;
- picked a colony of the second strain and mixed it in the same Eppendorf tube;
- mixed colonies well with pipetting up and down;
- placed 10 µl of the mix in an malt extract agar (MEA) plate as a small spot (repeated process for 5 replicate spots);
- spread solution spots with a hockey stick;
- wrapped culture plate in Parafilm tape;
- placed culture plate at 25°C in an incubator non-shaking for 2-3 days to allow mating;
After that, we performed a selection of spores with Glucurosidase (Sigma), an enzyme that kills cells but not spores:

- took a scoop of the biological material and put it in 200-300 µl glucuronidase 1:100* at RT ov;
- washed spores 2-4 times in 1 ml distilled-(d)H₂O at 2,400 rpm for 2 min;
- resuspended in 100 µl dH₂O;
- took 20 µl spores and plated them in 10-fold serial dilutions in a culture plate with EMM+N solid media;
- waited for 5-6 days for spores to germinate and form colonies;
- re-streak the newly-formed colonies in a new culture plate with fresh EMM+N or YES solid media**;
- allowed growth for 4-5 days to obtain positive colonies;

* from the commercial solution

** used a colony of strain JB574 as the auxotroph negative control in the experimental work presented in chapter VII

2.1.4 Cell density and cell viability assays

In order to assess *S. pombe* cell concentration or cell density levels in liquid cell cultures or suspensions, we used two independent measuring systems, a spectrophotometer (spec) and a particle counter. We used a spectrophotometer model WPA CO 8000 Cell Density Meter (Biochrom), which measures the relative amount of unscattered light passing through a sample at 600 nm, providing a measure of optical density referred to as OD₆₀₀. This functions as an indirect measure
of a cell culture’s density. A reference control sample was used which contained only
the media used to grow the cells.

We also assessed cell density values of our cell cultures by counting the number of
cells in a solution using a particle counter. This was a slightly more complex and time-
consuming approach than measuring optical density, but offered a considerably
higher sensitivity that allowed for a significantly higher resolution output. We used a
Z Series Coulter Counter Cell and Particle Counter (Beckman) instrument. A 0.05-1 ml
sample was collected, mixed in 10 ml Coulter Counter salt solution. We performed
measurements using a blank containing only Coulter Counter special salt solution, as
indicated by Beckman. Cells were measured in the recommended range of 10,000-
45,000 particles per ml. Size threshold for particle detection was set at 3 µm for both
vegetative and quiescent S. pombe cells.

Cell viability assesses the number of cells in a liquid cell culture that can give rise to
progeny and are thus viable. First, a small 1 ml sample was collected from the cellular
suspension. Cell sample was diluted in a 10-fold serial manner in dH2O, and then 100
µl of these samples were plated in YES culture plates, covering a concentration range
of 100,000 to 10 million cells per ml. Cells were spread evenly throughout the solid
media using sterile micro glass beads. Cells in culture plates were then incubated at
32° C for 3-4 days to allow cell colonies to appear. By assessing the number of
colonies formed it is possible to determine how many viable cells are present in the
cell culture, as we know that each multicellular colony initially originates from a single
cell. We calculated cell viability using the values from plates with 100-200 colonies per
plate, which corresponds to the confidence zone of this technique. Through the
number of colonies in a plate, we estimated the number of viable cells in a cellular
suspension.
2.1.5 Cell population growth curves/profiles

We assessed the growth dynamic profiles of fission yeast liquid cultures by monitoring alterations in their relative cell biomass over time using a BioLector high-throughput platform (m2p). BioLector detects alterations in light scattering as an indirect measure of relative biomass continuously with high temporal resolution.

For BioLector analysis fission yeast cell cultures were previously grown or kept in standard Erlenmeyer culture flasks until the designated time-point of profiling. Then, cell cultures were diluted to OD 0.2* and plated onto 48-well BioLector flowerplates in numbers of 3 or more technical replicates, 3 ml per well. Conditions during BioLector profiling were:

- temperature: 25° C or 32° C,
- shaking speed: 180 rpm;
- \([\text{CO}_2]\) levels: 5%;
- Humidity: >80%.

We designated 5-10 min as interval times between BioLector measurements as we found it suited our requirements for high sampling frequency. We were thus able to collect fission yeast relative biomass information with very high temporal resolution. Data acquired by the BioLector platform was then analyzed as explained in section 3 in this chapter.

* OD 0.2 constitutes the threshold upon which the BioLector platform starts to produce reliable measurements.
2.1.6 Cell microscopy

Fission yeast cells, experimentally growing in flasks as cellular suspensions or on agar plates as multicellular colonies, were routinely inspected under the microscope to monitor their physiological state.

To visualize cells from fission yeast cell colonies growing in solid media, we added a fraction of a colony collected using a small sterile wood pick to a 10 µl dH₂O droplet placed onto a microscope slide. After evenly spreading the biomass-containing droplet throughout the microscope slide, a coverslip was placed on top of the spread biological material. Cells were then visualized under an optical microscope (Leica) on 40x magnification.

To visualize fission yeast cells in suspension, we added a 10 µl sample collected from the cellular suspension onto a microscope slide. A small square coverslip slide was placed on top of the droplet. Cells were then visualized under an optical microscope (Leica) on 40x magnification.

2.1.7 Calcofluor cell staining, fluorescence microscopy and cell size analysis

*S. pombe* cells grow by elongation, which reduces the number of dimensions varying with growth to that of cell length or cell size. To determine the cellular size of fission yeast single cells we employed the protocol described next.

First, small samples of fission yeast cellular suspensions were collected at selected time-points and fixed. For that ~0.5 ODs (1 ml OD 0.5) from a cellular suspension sample were:
• immersed in 3.7% formaldehyde;
• mixed several times by pipetting and inversion, without vortexing;
• left for 30 minutes at RT;
• spun for 30” (seconds) at 8,000 rpm at RT;
• washed 3 times in 1x phosphate buffered saline (PBS) solution.

Cells were now fixed and samples could then be stored at 4° C for up to a month if needed.

Second, we performed a common technique of cell staining with calcofluor, a fluorescent dye that stains the cell wall and division septum of fission yeast cells, highlighting the outer limits of the cell and thus facilitating its detection and further visualization. For that, we took an aliquot of the fixed cell sample’s pellet (by pipetting) and resuspended it on 100 µl 1x PBS solution. Then, 1 µl of resuspended cell pellet is placed onto a microscope slide. Finally, we added 1 µl calcofluor (1:500 from available stock solution) to the cell sample on the microscope slide.

Third, we visualized the calcuofluor stained samples with a fluorescence microscope (Zeiss) using the designated GFP filter under 63x magnification. We then performed imaging for fission yeast cells stained with calcuofluor with the image analysis software Volocity version (v) 5.5.1 as indicated by Perkin Elmer. Images were captured using the Auto-Exposure setting. We could then import the captured images to dedicated software ImageJ (NIH). ImageJ allowed us to measure the dimensions of fission yeast single cells as given by the fluorescent signals of their cell walls. We quantified these dimensions using pixel units as a reference baseline. These data allowed us to then estimate the absolute cell size of fission yeast cells by converting pixel units to µm length units.
2.1.8. Cell sampling and storage

Fission yeast cell cultures were routinely sampled for downstream processing and analysis. Sample volume harvested depended on the experimental requirements and the culture’s cell density at the time of sampling, typically ranging from 1-50 ml. At least 10 ODs* of cells were collected per cell culture being tested, as it corresponded to the minimum amount usually required for downstream sample processing. If further sample processing was not possible immediately after, samples could be stored at -80°C for a prolonged period of time lasting up to several months. Samples harvested were centrifuged in Falcon tubes for 5 min at max speed at RT, their supernatants discarded and the remaining cell pellets stored at -80°C.

* 1 OD in cell density conventional terms corresponds to the equivalent of the number of cells present in 1 ml of a cell culture at OD 1.

2.2 Molecular biology techniques

2.2.1 Genomic DNA extraction

For DNA extraction we used two commercial kits, depending on the experiment. We used a Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) as indicated by Zymo Research. Unless otherwise stated, we used MasterPure Yeast DNA Purification Kit (Epicentre) as indicated by Epicentre as our standard DNA extraction method. Any subsequent alterations to the protocol are stated appropriately.

If further sample processing was not possible immediately, samples could be stored at cold temperatures for a prolonged period of time lasting up to several months or
years. Extracted DNA in elution buffer could be stored at 4°C for short-time storage (a few weeks) or at -20°C for a prolonged storage (several months).

### 2.2.2 Genomic DNA quantification

Three approaches were utilised for the quantification of extracted DNA: Qubit Fluorometer, NanoDrop spectrophotometer and BioAnalyzer platform.

- **Using Qubit**

  Qubit Fluorometer instrument (Life Technologies) was used to quantify DNA concentration with elevated accuracy using only 1-10 µl sample volume, as indicated by Life Technologies.

- **Using Nanodrop**

  NanoDrop 1000 spectrophotometer instrument (Thermo Scientific) was used to quantify DNA concentration using only 1 µl sample volume, as indicated by Thermo Scientific. Nanodrop was less precise than Qubit but noticeably less time-consuming.

- **Using BioAnalyzer**

  2100 BioAnalyzer instrument (Agilent) quantifies DNA concentrations with extremely high precision, using up to 1 µl as sample volume. It also accurately determines the size of DNA fragments. This is the most time-consuming of the three approaches but also the most precise and reliable one. DNA was quantified analysed as indicated by Agilent.

  Information on the method used for DNA quantification is provided accordingly in the respective sections.
2.2.3 Genomic DNA purification

For DNA purification we performed a standard 1:1 phenol-chloroform extraction and ethanol purification protocol as described in detail in section 2.3.5.3 in this chapter.

2.2.4 PCR, cPCR and PCR product purification

Extracted and purified genomic DNA was used for different applications including sequence amplification through Polymerase Chain Reaction (PCR). Fission yeast colonies were also used for sequence amplification through colony PCR (cPCR). PCR and cPCR settings were defined according to the required purpose, for which specific PCR programs were created. High fidelity TaKaRa Ex Taq Hot Start DNA polymerase (TaKaRa) was used for PCR amplification reactions. BIOTAQ DNA polymerase (Bioline) was used for cPCR amplification reactions. Standard PCR reaction mix and conditions used are described next.

PCR/cPCR reaction mix was:

- 10× BIOTAQ Reaction Buffer 5.0 µl
- MgCl$_2$ Buffer 1.5 µl
- dNTPs (10 mM) 1.5 µl
- Primer F (5 µM) 1 µl
- Primer R (5 µM) 1 µl
- BIOTAQ Taq (5 µM) 0.2 µl
- DNA template 2 µl
- Add ddH$_2$O to 50 µl
PCR/cPCR reaction settings were:

- 94°C 3 min
- 94°C 30 sec
- Tm* - 5 30 sec
- 72°C 1 min/kb**
- 72°C 5 min

×30 cycles

* Tm stands for primer melting temperature and is specific for each primer. This value was provided by the company that synthesized the primer, usually Invitrogen.

** DNA fragment size determined the extension time for each specific case.

Primer sequences are available at section 2 at the Appendices. PCR products were then purified using QIAquick PCR purification Kit (Qiagen), as indicated by Qiagen.

2.2.5 Gel electrophoresis

For investigating the molecular size and amount of DNA samples we used agarose gel electrophoresis. The agarose concentration varied according to the expected size of the product being analyzed and ranged from 0.5-2%. Typically the volume for an agarose gel ranged from 100-250 ml. Ethidium Bromide (EtBr) (Life Technologies), used to stain DNA, was added to the agarose gels at 0.4-0.5 µg/ml. DNA samples to be analyzed were mixed 1:5 with 5x Loading Buffer (BioLine). Each sample contained ~50 ng DNA. DNA ladders or molecular reference scales used included HyperLadders I-V (Bioline). Upon loading the DNA samples onto the gel wells, we switched on the electric current at ~80-120 volts (V), and gels were usually run for the time necessary to allow visualization of DNA samples at maximum resolution, typically 30-90 min. The agarose gel would then be observed under a UV-light using a gel documentation GelDoc-Imager station (UVP). Pictures of the agarose gels, under
visible spectrum and UV light were be acquired using the dedicated camera and software included in the gel doc system.

2.3 Barcode-sequencing assay using a fission yeast deletion library

The functional profiling studies presented in chapters 4, 5 and 6 constituted the main focus of this PhD project. To conduct such work we mainly employed a barcode-sequencing (bar-seq) strategy for screening a fission yeast deletion library in a high-throughput fashion.\textsuperscript{136-138}

Bar-seq constituted a novel technique introduced in the Lab. Therefore, this technology required its establishment, optimization and validation to ensure it could be successfully utilized to perform high quality experimental research. We reserved this Methodology section to describe all the technical information related to the bar-seq assays performed in this project. We trust that the information presented in this section could represent a valuable resource for understanding and performing this technique in the Lab hereafter.

2.3.1 Overview

The arrival of next-generation sequencing (NGS) platforms roughly with the end of the last decade brought with it the possibility for feasibly undertaking massive high-throughput sequencing endeavours within a short period of time and with relatively low associated costs.\textsuperscript{136} Furthermore, the technology developed for deep sequencing could finally be afforded by the typical average-sized research group, greatly facilitating their access.
The implications of this new reality could not be underestimated as it practically meant that sequencing was now readily available with all sorts of applications. It was then possible to analyze entire genomes, chromatin landscapes (via chromatin immunoprecipitation sequencing (ChIP-seq) technology), transcriptomes (via RNA-seq technology), and more. Importantly, it was now possible to cover the same target regions over multiple times which dramatically amplified the resolution power and depth of our analyses leading to a marked increase on the quality of the results obtained. This was of particular significance for research groups from fields that involved the generation and manipulation of large quantities of genomic data. Our group focuses on global cellular responses to environmental perturbations, namely the tightly regulated gene expression programs that drive such adaptive behaviour, with the aim of acquiring a holistic and systematic view of the cell’s mechanistic nature. Consequently, we had a lot to gain with this novel sequencing technology.

**Barcode-sequencing** (bar-seq) is another technology that originated with the advent of NGS platforms. Basically, bar-seq consists of sequencing libraries of small molecular tags in the form of short DNA sequences of ~20 bp. These individual tags or barcodes can then be quantified and this information used as a measure of relative abundance of each molecular tag within a given library. We employ bar-seq to determine changes in relative abundance occurring within a barcoded fission yeast gene deletion library over the course of an experimental assay. Each deletion strain contained in the deletion library is tagged with a 20 bp barcode inserted in its genome and therefore mutant pools of the deletion library can be screened and analysed via relative quantification of each individual strain present in the library. Mutant pools are sampled and their barcodes prepared into a library, via DNA extraction and PCR amplification that is then analysed through NGS.
2.3.2 Fission yeast deletion library

One of the many valuable resources available at our Lab is a fission yeast gene deletion strain collection, *S. pombe* Genome-Wide Deletion Mutant Library version 2, co-developed by the KRB-Bioneer-CRUK consortium in collaboration with Dr. Paul Nurse of CR-UK-LRI in the S. pombe Genome Deletion Project.\textsuperscript{139}

This *S. pombe* Gene Deletion Mutant Library targets every open reading frame (ORF) (4,914 types) in the *S. pombe* genome using a targeted mutagenesis method for a total of 4,836 genes in heterozygous diploid deletion mutants representing 98.4\% of the fission yeast genome and 3,400 haploid deletion mutants with 95.3\% genome coverage.\textsuperscript{139} The deletion cassette module construct contains a KanMX4 selection marker gene, 2 unique tag sequences (molecular barcodes), and the sequences for homologous recombination trough PCR (figure. 2.2). The deletion cassette modules were constructed by PCR and transformed into *S. pombe* SP286 diploid host strain (h\(^{+}\)/h\(^{+}\), ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32). Deletion of the target ORF was screened for by G418 antibiotic selection.

One of the ingenuous features of this gene deletion library is the incorporation of the small molecular barcodes in the genome of each single mutant strain. These tags are unique for each strain functioning as specific markers. By sequencing these molecular signatures via bar-seq, it is possible to acquire highly sensitive information on the relative abundance of any given mutant strain present in an analysed sample.\textsuperscript{137} As these tag sequences are specific for each strain it is possible to pool an entire genome-wide mutant library for massive parallel profiling. This greatly reduces the costs and time associated while strongly increasing the extracted throughput and depth. The deletion cassette present in each deletion mutant contains two distinct
and unique barcodes (figure 2.2), constituting two identical but independent ways by which to acquire information on a mutant's relative abundance. The barcode upstream the KanMX4 gene is accordingly labelled uptag, while the one downstream the Kan marker is named downtag. They are surrounded on one side by the KanMX4 gene and in the other by universal sequences, which function as regions of primer complementarity for barcode amplification through PCR.

![Figure 2.2 Deletion cassette used for making the fission yeast deletion library developed by Bioneer.](image)

Adapted from Han et al. (2009)\(^{137}\)

### 2.3.3 Construction of a prototroph haploid deletion mutant pool

This mutant library is originally auxotroph for several amino acids that functioned as selective markers during its construction. As we performed our functional profiling studies in well-defined minimal culture media we required a prototroph collection without any selective markers or special nutritional requirements. Using the haploid version of this fission yeast haploid collection, which comprehends all *S. pombe* non-essential gene deletion mutants, we generated a fission yeast prototroph haploid deletion mutant library, as described in section 2 of the Appendices section.\(^{140}\) Due to the characteristics of the approach employed, deletion mutants with both mating
types were created, meaning that each mutant generated could have either one or
even both mating types, in a random fashion manner. Information on the mating type
of each individual mutant is absent due to technical limitations. Conveniently this
information was found to be neither critical nor required.

Due to unknown reasons, some mutant strains could not be made prototroph. Although it would have been valuable to understand the basis underlying the inability for several mutant strains to be made prototroph, we did not possess enough information that could allow us to determine that. More specifically we could not identify for each mutant strain the basis of this impracticality. There could have been several possibilities behind this basis: some mutants could not have been made prototroph for intrinsic biological reasons; some mutants could have been lost due to the prototroph creation technical process; and finally some mutants could not have been viable from the start or possible to be awoken from the auxotroph collection. With a special regard to this last possibility, as we did not possess the information about the mutant strains unable to be revived from the initial auxotroph deletion library it was not possible to produce a list containing only the mutants lost during prototroph creation. All information about this process has been meanwhile made available through a recent publication by our Lab.170

The total number of prototroph haploid mutants obtained in this approach was
\(~3,000. A complete list of all the mutants present in the prototroph collection is available in section 2 of the Appendices section.

We then produced mutant pools of the prototroph deletion library where all the prototroph mutants were pooled together to a single sample, as described by Han et al.137 We made glycerol aliquots of these mutant pools for ease of manipulation,
which were stored at -80\(^\circ\) C for future use. We could then easily awake a whole prototroph mutant collection by simply awaking one of these aliquots.

To start and set up a culture from a mutant pool aliquot, see section 2.1.2 in the Methodology.

**2.3.4 Establishment and optimization**

Whenever the written information is not adequately clear or becomes too complex, please refer to figures 2.11 and 2.12 for a visual and schematic representation of the bar-seq protocol and its various steps.

**2.3.4.1 Mating test through a Glucuronidase assay**

Some of our bar-seq assays were conducted in EMM-N media. Nitrogen depletion was used as a way to induce fission yeast cells to become quiescent. Absence of nitrogen from the culture media is also one of the conditions required for inducing fission yeast to engage in mating or sexual reproduction, together with lower temperatures, typically 25\(^\circ\) C. Although all bar-seq assays were performed at 32\(^\circ\) C we evaluated whether any mating occurred within a mutant pool in media without nitrogen. Any mating detected could compromise the validity of the assay, as it would dramatically alter the nature and composition of the mutant pool, the subject of study, in a fashion we could not control or even monitor. For this reason it was imperative to ensure that mating would not occur and affect the course of a bar-seq experimental assay.

To test this we employed a **Glucuronidase Test** assay. We set up a prototroph mutant pool culture in 250 ml rich YES media, as described in section 2.2.1. We then moved the mutant pool to EMM-N as follows:
• 100 ml cell culture were collected;
• cells were centrifuged at 2,300 rpm for 3 min and YES media was discarded;
• cells were resuspended in EMM-N and washed x2 in EMM-N by centrifuging at 2,300 rpm for 3 min;
• cells were resuspended in 100 ml EMM-N;

Cells were then incubated at 32°C for 2 days to allow for mating to occur. The cell culture was then adjusted to OD 0.8 (this constituted the expected maximum OD a cell culture would reach during quiescence in a bar-seq assay). After that, we performed a glucuronidase assay, as described in section 1.1.3 in this chapter. Our aim was to assess whether mating was occurring when our prototroph mutant pools were in liquid cultures of EMM-N minimal media, as it constituted one of the conditions used in the bar-seq assays. To test that we directly sampled the mutant pool culture in EMM-N. We added glucuronidase 1:100 to the culture media and proceeded with the glucuronidase assay scaled for this volume of sample. As a negative control we used a culture that did not undergo the treatment with glucuronidase. We then performed a viability assay to precisely determine if any mating had occurred. Results obtained for biological triplicates are shown in fig. 2.3.
Figure 2.3 Cell viability assay after glucurosidase treatment. Student t-test showed statistical significance, *** p < 0.001.

No mating was detected upon inspecting the mutant pool cultures in EMM-N under a light microscope, even after 3 days in EMM-N. A viability assay performed without previous glucurosidase treatment depicts the overall cell viability of the mutant pool, which was found to be ~2M CFUs/ml at this particular OD (OD 0.8) (figure 2.3). This shows the total number of cells that can give rise to progeny in a mutant pool, either from asexual or sexual reproduction. Treatment with glucurosidase allowed us to discern with high sensitivity if mating had occurred within a mutant pool in EMM-N. We found that some mating had occurred, originating ~250 CFUs/ml. However, this represents only 0.013% of the overall viable population, meaning that only one mating event was detected per ~10,000 viable cells. We therefore concluded that this residual level of mating constituted no significant interference to the validity of our bar-seq assays and could thus be neglected.
2.3.4.2 Multiplex indexes, barcode amplification primers and sequencing adaptor primers: design and verification

In our profiling assays, the mutant collection is analysed through the quantification of the individual barcodes, integrated in each deletion mutant, through bar-seq. To obtain the bar-seq library, DNA had to be initially extracted followed by the amplification of the barcode regions so to attain the levels required for sequencing, as explained in detail in section 2.3 in this chapter. Finally, adaptor sequences necessary for the sequencing step had to be added to the bar-seq library. For amplifying the barcodes, special primers had to be designed that displayed complementarity to their flanking regions, universally identical throughout the entire mutant collection so that any pool of mutants could be simultaneously processed in parallel. For this we followed the rationale employed by Han et al. Another useful strategy incorporated in this technique was the inclusion of multiplex indexes, or multiplexing. These are short ~3-4 bp sequences included in the amplification primer’s design, which can be used to specifically identify each sample. This allows the pooling of multiple samples and consequent bar-seq analysis in a single sequencing run, drastically reducing associated time and cost.
Figure 2.4 Representation of the multiplex strategy used for bar-seq. A) One of 20 available unique amplification primers, incorporating a specific multiplex index (depicted in orange), is used for barcode (uptag or downtag) amplification. Adapted from Han et al. 2009. B) (this results in) PCR products tagged for the sample they originated from thus allowing for multiple sample parallel analysis (multiplexing). UR - universal regions.

We designed 40 unique amplification primers for bar-seq, 20 for uptag (labelled Up1-20) and 20 for downtag (labelled Dn1-20) amplification, following the rationale employed by Han et al. All the primers’ full sequences, including the multiplex indexes, are available in section 2 in the Appendices. This provided the possibility for analysing both uptag and downtag barcodes in parallel for up to 20 independent bar-seq samples.

Next, an additional round of PCR was required for the incorporation of the sequencing adaptors. Again, primers for this step were designed following Han et al.’s rationale. Their full sequence is available in section 2 at the Appendices. Due to the strategy employed, the obtained bar-seq products containing uptags consisted of 146 bp fragments, while those enclosing downtags sized 153 bp.

All primers had to be verified as they had never been tested. For that we grew a mutant pool as described in the protocol in section 2.4.1 in this chapter. When the mutant pool reached mid-log phase (OD600 0.6) several samples were collected, each
comprising ~20ml (>10 ODs). Then, DNA was extracted from these samples as described in section 2.4.3 in this chapter. Next, barcodes were amplified using the newly designed primers described here, as indicated by Han et al.\textsuperscript{54} followed by the incorporation of sequencing adaptors, also using the primers described here. The resulting PCR products, i.e. the multiplex-indexed bar-seq library integrating the sequencing adaptors, were then analysed on a 2% agarose gel (uptags 1-10 and downtags 1-10 are shown in figure 2.5).

\textbf{Figure 2.5 Verification of the primers designed for bar-seq library preparation.} A barcoded mutant pool was processed with designed primers. After barcode amplification through PCR, bar-seq products were analysed on an agarose gel. \textbf{A)} uptags 1-10. \textbf{B)} downtags 1-10.

As it is possible to observe in fig. 2.5 all primers worked successfully and were thus considered valid for bar-seq library preparation.

To infer that all barcodes (therefore all different deletion mutants) were successfully being covered through this method we randomly picked 3 strains (\textit{\Delta}rgf1, \textit{\Delta}sgf73 and \textit{\Delta}pck1) from the deletion library to test if their barcodes were being correctly
amplified. We grew the cells in YES media to mid-log phase and then collected ~20 ml (>10 ODs) samples. The remaining protocol was identical to that done for the mutant pool. For this test we used amplification primers Up1 and Dn1. Results are shown in figure 2.6.

Barcode amplification was successful in all strains randomly tested for both uptags and downtags, allowing us to theoretically infer that in general all barcodes could be successfully targeted and monitored, which demonstrated the validity of the designed primers (figure 2.6).

![Figure 2.6 Verification of the validity of the primers designed for bar-seq library preparation. Δrgf1, Δsgf73 and Δpck1 deletion mutants were processed with designed primers Up1 and Dn1 (Up5 in Δpck1 was used as an additional test). After barcode amplification through PCR, bar-seq products were analysed on an agarose gel.](image)

2.3.4.3 Bar-seq on quiescent cells

We tested the primers designed for the generation of the bar-seq library using samples of proliferating cells, in a similar fashion to what was described by Han et al.\textsuperscript{54}. Given that our profiling studies were conducted in both quiescent and proliferating cells we needed to ensure that we could perform the bar-seq assay in this condition as well. This is especially pertinent given that, as explained in more detail in the Introduction in chapter I, quiescent cells are known to exhibit a large number of differences when compared to proliferating cells. Any or some of these
differences could, for unexpected or uncontrollable reasons, be found incompatible with our bar-seq experimental pipeline. For that, we decided to perform the same experiments as described before using quiescent cells collected from a mutant pool that in EMM-N media for 48 hours. Apart from the change of culture media, the remaining processing steps were as described in section 2.3 in this chapter. Results are shown in figure 2.7.

All primers tested successfully amplified the barcode regions of quiescent cells (fig. 2.7), meeting another essential requisite for performing our functional studies using bar-seq. This confirms the possibility of performing bar-seq with quiescent cells.

![Figure 2.7 Barcodes extracted from quiescent cells were amplified through PCR and analysed on an agarose gel. Amplification primers Up1-3 and Dn1-3 were used.](image)

2.3.4.4 Assay optimization: cell disruption, library preparation and sample purification settings

We adopted this bar-seq protocol from a published study by Han et al.\textsuperscript{137}. Therefore, many aspects of this assay benefited from adjustments and further optimization to adequately suit our particular requirements and experimental studies. All the alterations and adjustments we introduced to our bar-seq assay are described in this section.
- Cell disruption settings

One of the first steps of bar-seq library preparation concerns the lysis of the cells to be analysed, followed by DNA extraction and purification (see section 2.4.3 for a description of the protocol). In the bar-seq protocol by Han et al.\textsuperscript{137} cell disruption is achieved first through a mechanical approach followed by a chemical non-enzymatic treatment. The bar-seq protocol was developed using samples from cells harvested during a proliferative state. We also aimed to perform bar-seq on quiescent cells, which as mentioned before exhibit many different characteristics compared to proliferating cells, namely a thickened and more rigid cell wall. We noticed that the amount of genomic material extracted from quiescent cells was significantly lower than that obtained from proliferative cells. We suspected that this could be due to a greater resistance from the former to the lysis treatments provided by their reinforced cell walls. For that we decided to try different settings during the cell lysis treatments in the attempt of improving the DNA yield obtained from quiescent cells.

Mechanical cell disruption was achieved through multidirectional beating of small glass beads (~3 mm diameter in size) on the sample material using a high-speed homogenizer instrument, FastPrep-24 (MP). We decided to try different homogenization settings, namely speed, duration and number of times the treatment was applied, to samples of both quiescent and proliferation cells, collected as described in section 2.3.2 in this chapter. DNA was then extracted and purified as described in section 2.2.1 and quantified with Qubit as described in section 2.2.2 in this chapter. Results are shown in figure 2.8.
Figure 2.8 DNA yields obtained after mechanical cell lysis using different settings.

Proliferating and quiescent cells were lysed using a FastPrep-24 homogenizer and their extracted DNA was quantified with Qubit. Columns were compared to the corresponding reference (the two columns on the left) using One-way ANOVA statistical tests. ** p < 0.01. *** p < 0.001. +N depicts samples in EMM+N media (proliferating cells). -N depicts samples in EMM-N media (quiescent cells). Green columns represent the samples from proliferating cells. The dark green column depicts the original protocol recommended settings, whilst the light green columns depict the different conditions tested. Blue columns represent the samples from proliferating cells. The dark blue column depicts the original protocol recommended settings, whilst the light blue columns depict the different conditions tested.

As indicated by the adopted bar-seq protocol the original cell disruption settings were of 5.5 meters/second (m/s) for 16 sec ('). These values constituted the reference starting point. Unexpectedly, as we expected the assay to be optimized for
proliferating cells, we found that increasing the duration of treatment to 1 min and the speed to 6.5 m/s gave rise to higher DNA yields even in proliferating cells. Regarding the quiescent cells’ treatment, we found that increasing the speed of treatment but especially increasing its duration from the default 16 sec to 1 min lead to much higher DNA yields. Repeating the treatment twice did not seem to increase the yield any further. However, by visualizing cell lysates from quiescent cells disrupted at 6.5 m/s for 1 min under the microscope it was noticeable that some cells representing ~5 % were not disrupted by the treatment and were still intact when treatment was only performed once. On the contrary, after undergoing the treatment twice we observed no unbroken or intact cells under the microscope, which suggested that performing the treatment 2 times would ensure that all cells were disrupted, thus maximizing the efficiency of this step and the corresponding yield obtained. For that reason we decided to change the FastPrep-24 cell disruption settings to 6.5 m/s for 1 min, repeated once.

Regarding the chemical non-enzymatic treatment, we used that of the DNA extraction protocol by MasterPure (see section 2.2.1). We also increased the duration of the chemical cell lysis step from 15 min to 45 min as we found that this alteration also lead to higher DNA yields (data not shown).

Finally, we increased the duration of RNAse treatment (to remove contaminant RNA) as we found that the indicated time of 15 min was insufficient (data not shown). We defined a period >2 hours as an adequate duration for the RNAse treatment.

- PCR processing steps settings

After quantifying (for information on the quantification assay see section 2.2.2 in this chapter) the amount of amplified barcodes product obtained after the PCR
processing steps using Qubit and BioAnalyzer (data not shown) we concluded that the attained levels were lower than those reported by Han et al. using the same conditions.\textsuperscript{137} For that reason, we decided to investigate with closer detail how the amplified material was behaving throughout the PCR steps. Also, we wanted to test higher amounts of starting material in the second PCR, as we feared that the amount indicated in the adopted bar-seq protocol (0.5 µl of a 200-fold dilution of the first round PCR products) could be too low to adequately cover the entire mutant pool in a robust and deep fashion. For that we decided to analyse both PCR processing steps (barcode amplification and integration of sequencing adaptors) through qPCR.

We used a KAPA Library Quantification Kit code KK4824 for performing this qPCR assay. The protocol was performed as indicated by Kapa Biosystems.

We were interested in examining the amplification curves from the amplified genetic material throughout the PCR processing steps, as it would provide us with the information on the behaviour of the PCR events. Results are shown in figure 2.9.
Figure 2.9 Amplification curves of PCR processing steps obtained through qPCR analysis. A) Amplification curve from 50 ng DNA as obtained with uptag primers. B) Amplification curve from 50 ng DNA as obtained with downtag primers. C) Amplification curve from serial volumetric dilutions of the first round PCR products (after 26 cycles of PCR).
as obtained with up-tag primers. Curve dilutions used were respectively (starting with the curve on the left): 1:6.25, 1:12.5, 1:25, 1:50 and 1:100 from the barcode amplification PCR products’ total volume. **D** Amplification curve from serial volumetric dilutions of the first round PCR products (after 26 cycles of PCR), as obtained with down-tag primers. Curve dilutions used were respectively (starting with the curve on the left): 1:6.25, 1:12.5, 1:25, 1:50 and 1:100 from the barcode amplification PCR products’ total volume.

The adapted bar-seq protocol indicates 30 cycles for the first round of PCR (barcode amplification) and 20 cycles for the second round (integration of sequencing adaptors). By inspecting the results obtained depicted in figure 16.A and 16.B, it was possible to understand that, for the first PCR, 30 cycles is dangerously approaching the PCR saturation point after which DNA would start to be amplified unevenly and therefore leading to the disruption of the relative abundance’s information across barcodes. For that reason we decided to alter the protocol to 26 cycles, which we regarded as adequate to meet our requirements of thorough barcode amplification without risking any saturation of the PCR products. We also decided to use 50 ng DNA as starting material as quantified by Qubit as we found that amounts proposed in the adopted protocol were being inaccurately determined*.

In figure 2.9C and 2.9D it was possible to observe at 20 cycles PCR had reached saturation point for all dilutions analysed. For that reason we decided to change the number cycles of the second PCR to 13. We regarded 1:25 dilution from the first PCR, corresponding to a 2 µl sample given the total reaction of 50 µl, as an adequate amount as it ensured a significant amount of the originating sample was carried down the pipeline, which increased the depth of the library being produced and consequently the coverage of each barcode. Moreover, the amplification curve
exhibited by this dilution indicated no risks of saturation after the newly designated number of PCR cycles (13).

To improve the quality and efficiency of the second PCR step (the step where sequencing adaptors were added to the bar-seq samples), we decided to introduce a purification step between both PCR steps. Therefore, after the first PCR where barcodes were amplified, the obtained products were purified using QIAquick PCR Purification Kit from Qiagen, as indicated by Qiagen.

* Han et al.\textsuperscript{137} report DNA quantification using Nanodrop, which is a platform that also detects RNA, which might represent up to 10-fold of the amount of genetic material present, especially using the original RNase treatment conditions. This non-sensitive method lead to erroneous results and therefore was discarded as an invalid quantification method for this step.

- Bar-seq library purification

The final alteration to the adopted bar-seq protocol regarded the purification step after the PCR processing steps (see section 2.3.3.3 in this chapter). It consisted in the substitution of the method used for purification. According to Han et al.’s protocol bar-seq library final purification is performed using a gel extraction PCR purification kit. However, because of the low amounts of product obtained with this method and the associated DNA mutagenic risks, even if low, posed by using a UV light throughout a significant part of the process, we decided to change the purification method for a MiniElute PCR purification Kit by Qiagen, as indicated by Qiagen. This lead to higher amounts of PCR product recovered with a significant decrease in the time consumed performing this purification step.
2.3.4.5 Assessing the dynamic range of bar-seq’s resolution window with external spikes

All primers were successfully working but for their final validation it was crucial to verify whether the relative differences amongst different barcodes within a sample were being maintained throughout the PCR steps of barcode amplification and integration of sequencing adaptors. The whole assay relied on this information so it was vital to ensure that none of the bar-seq assay processing steps, including library preparation, significantly interfered with or altered the nature of the biological material to be analysed.

We introduced barcoded strains as external spikes, as explained next, to assess if relative differences amongst amplified barcodes were accurately transported throughout the PCR processing steps of bar-seq library preparation. We prepared aliquots of 7 barcoded strains (for information concerning this procedure refer to section 2 in the Appendices), from a previous version of the deletion library and therefore not present in the mutant pool, in gradual amounts. These were added to 5 independent mutant pool samples (previously prepared as described in section 2.3.1 and 2.3.2 in this chapter) ready for library preparation. The spikes ranged from 500 to 40,000 cells, an interval that enclosed the estimated number of cells per deletion strain in each sample (~5,000 cells per deletion strain per 10 ODs sample) (table 1). We then aimed to assess whether these differences were still present after the PCR processing steps.
Table 2.1 External barcoded strains were used to spike the dynamic range of bar-seq PCR products through qPCR analysis.

<table>
<thead>
<tr>
<th>Cells/sample</th>
<th>Spike ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>40,000</td>
<td>Spk1</td>
</tr>
<tr>
<td>20,000</td>
<td>Spk2</td>
</tr>
<tr>
<td>10,000</td>
<td>Spk3</td>
</tr>
<tr>
<td>5000</td>
<td>Spk4</td>
</tr>
<tr>
<td>2000</td>
<td>Spk5</td>
</tr>
<tr>
<td>1000</td>
<td>Spk6</td>
</tr>
<tr>
<td>500</td>
<td>Spk7</td>
</tr>
</tbody>
</table>

Samples containing external spikes were processed as described in sections 2.3.3.1 and 2.3.3.2 in this chapter and then analysed through quantitative PCR (qPCR), a real time PCR assay. qPCR primers were designed with complementary regions within the barcodes of the external spikes strains so to specifically quantify the levels of those barcodes only. Information on the primers and external spikes strains is available in section 2 at the Appendices section.

We used a KAPA Library Quantification Kit code KK4824 for performing this qPCR assay. The protocol was as described by Kapa Biosystems.

In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). The Ct values obtained with qPCR can work as an indirect quantification
measure, as they are proportional (direct logarithmic relation) to the amount of analysed material (in this case the barcode regions). By comparing the Ct values obtained for each spike within a given sample it was possible to indirectly infer about spikes’ intrinsic relative levels and associated differences within that sample. Then we could test if the relationship between the spikes barcode levels’ after the PCR processing steps mirrored that initially present between the numbers of cells of each spiked strain. Consequently we could conclude whether relative differences amongst mutants were kept intact throughout the preparation of the bar-seq library, namely the PCR processing steps. Results are shown in figure 2.10.

Figure 2.10A shows that the relative abundances of all spikes were highly consistent across independently processed samples, which demonstrated the robustness of the bar-seq designed primers and associated PCR processing steps.

Figure 2.10B depicts the relation between the initial number of cells from each spiked mutant and the relative abundance from the corresponding amplified barcodes after bar-seq PCR processing steps as obtained after qPCR quantification. A non-linear regression with associated $R^2 = 0.92$ demonstrates the strong reliability of this assay in conserving the relative differences between mutants within a wide analytical window comprising from at least 500-40,000 cells, representing an 80-fold coverage. We thus successfully verified and consequently validated the (designed) bar-seq primers and associated PCR processing steps.
Figure 2.10 Relative abundances are maintained throughout bar-seq PCR processing steps as shown by external spikes analysis through qPCR. A) All spikes behaved consistently across 5 independent mutant pool samples. B) Relative abundances across spikes are unchanged during PCR processing steps throughout a high dynamic range. For unknown reasons data from Spk6 could not be retrieved. x-axis is in log scale.
2.3.4.6 Deletion library de novo decoding

In the published study describing the bar-seq protocol we adopted, Han et al.\textsuperscript{137} infer that the information provided by Bioneer regarding the mutant strains from the deletion library contains some degree of error. Han et al. report that from their haploid mutant pools of \(~2,800\) deletion mutants, only \(~90\%\) were correctly detected after performing de novo sequence decoding. Several reasons underlie the basis for the percentage obtained. They refer several inaccuracies regarding the information provided by Bioneer such as: duplicated barcodes within a given strain or strains with more than one barcode sequence, misplaced barcode insertions, different strains with identical barcode sequences, strains with barcode sequences differing from those provided by Bioneer, amongst other minor sources of error\textsuperscript{137}. All these sources of error could potentially lead to significant poorer outputs when analysing the results obtained with bar-seq against the information provided by Bioneer.\textsuperscript{139}

For the reasons mentioned above and to improve the quality of the information on the deletion library (and all the subsequent information deriving from its use) we decided to undergo the decoding of our own haploid deletion library.

For that we analysed a pooled sample aliquot from the deletion library. We processed it following the bar-seq protocol described in section 2.3 in this chapter. Bar-seq libraries were then sequenced externally. Data was processed and analysed according to the pipeline described in section 2.4.1.

We managed to identify barcodes for 2473 deletion mutants. Amongst them, 1871 mutant strains had both uptag and downtag decoded. 254 mutants had only uptag decoded and for 348 mutants only downtag was decoded. Detailed information regarding the decoding process is available at section 2 in the Appendices section.
The number of detected barcodes was relatively lower than that reported by Han et al. (~2,800 mutants identified), which might be explained by the fact that we analysed a prototroph deletion library as opposed to the auxotroph library (as provided by Bioneer by default) analysed by Han et al. As mentioned before in section 2.1.2 in this chapter, making the prototroph library lead to the loss of some mutants whose number might have been underestimated.

In conclusion, the results from decoding our prototroph deletion library indicated that its featured analytical screening capacity, when undergoing fitness profiling studies through bar-seq, comprises a magnitude of ~2,500 mutant strains, roughly covering half the genome of *S. pombe*.

### 2.3.5 Barcode-sequencing protocol

This section describes the bar-seq protocol as finally implemented in our Lab and used for the functional profiling studies presented in chapters 4 and 5. A visual representation of the overall bar-seq assay is depicted in figure 2.11.

![Barcode-sequencing experimental pipeline](image)

**Figure 2.11 Barcode-sequencing experimental pipeline.** Fission yeast deletion library is pooled and made into aliquots, which are used to set up mutant pool cultures of the deletion library. Mutant pool cultures are then sampled according to the experimental assay, processed and finally analysed through deep sequencing.
Figure 2.12 Barcode-sequencing library preparation. After DNA extraction the molecular tags or barcodes (here depicted as cDNA1 and cDNA2) are tagged with the sample index (multiplexing), the sequence adaptors are integrated and the resulting DNA fragments are amplified through PCR. After that the library of amplified barcodes is purified and finally sequenced.

2.3.5.1 Setting up a mutant pool culture

Bioneer prototroph pooled library aliquots are stored at -80°C. Each aliquot contains a volume of ~500 µl. To wake-up a pooled deletion library, take an aliquot, thaw it and spin down for 3 min at 2,300 rpm RT. Remove supernatant and resuspend pellet in equal volume of YES media. If doing the experiment in YES rich media, cells can be resuspended in desired volume (100-200 ml) and proceed with the experimental plan, taking into consideration that cells require some time (some hours) to wake up and adjust before they start growing. If the experiment is to be performed in EMM
minimal media, add the resuspended aliquot to 200 ml fresh YES media in a 500 ml culture flask and incubate for five hours at 25°C non-shaking to allow for the cells to adjust and resume normal metabolic activity. After that, measure $\text{OD}_{600}$ and centrifuge cells. Discard supernatant and resuspend cells in 200 ml EMM+N minimal media. Once cells are resuspended in minimal media initiate the desired experimental assay.

2.3.5.2 Experimental assay and sampling

The experiment is performed in liquid culture, as planned. Cell samples are collected as designated by the experimental assay to be performed. At least ~10 ODs (~17 ml at OD 0.6) should be harvested per sample. Spin cells for 5 min at 4,000 or max speed, discard supernatants and store pellets at -80°C.

2.3.5.3 Bar-seq library preparation

- DNA extraction, purification and quantification

Take desired cell pellets from -80°C and centrifuge at maximum speed for 5 min RT. Discard supernatant and resuspend pellet in 1 ml fresh media. Wash cells once at max speed for 3 min RT. Discard supernatant and resuspend cells in 100 µl TE buffer solution. Some cells show a great deal of resistance to enzymatic cell lysis, especially nitrogen-depleted quiescent cells (in EMM-N media). For this reason, a mechanical lysis of the cells may be required. For this, transfer the TE-cells mixture to fastprep plastic veil tubes previously loaded with ~400 µl glass beads, and mix thoroughly.

Disrupt cells using the FastPrep-24 homogenizer (MP). Settings may vary depending on the type of cells and may require optimization. For best yields in minimal media use [6.5 m/s for 1 min] x2, allowing a 5-min resting period between runs. After that,
swiftly pierce fastprep tubes with a pre-heated needle and quickly insert them inside an Eppendorf tube; then insert this object (fastprep tube in the Eppendorf tube) inside a 50 ml falcon tube and centrifuge them at 3,000 rpm for 1 min. Collect cell lysates in the clean Eppendorf tubes and discard the rest.

Proceed to DNA extraction using a MasterPure Complete DNA and RNA Purification Kit (Epicentre), as indicated by Epicentre. Perform final RNase A treatment for >2 hr at 37°C.

For DNA purification, re-extract DNA with equal volumes of phenol:chloroform. Add 0.1 volumes of NaOAc pH 5.5 and 1 volume of 2-propanol. Mix well and keep at -20°C for 30 min. Centrifuge the DNA using a Speed-Vac machine until all supernatant has evaporated. Resuspend DNA with 35 µl of TE buffer solution.

Perform DNA quantification using Qubit Fluorometer (Life Technologies), as indicated by Life Technologies.

- Library generation

We amplify the barcodes through PCR. We designed 40 different barcode amplification primers, 20 uptags and 20 downtags, each containing small unique 3-4 bp sequences which can be used to trace the bar-seq data back to their original corresponding samples. This allows sequencing multiple samples simultaneously, or multiplexing. Barcode amplification primers are labeled Up1-20, for uptags, and Dn1-20, for downtags.

On a subsequent round of PCR we integrate the sequencing adaptors, small ~50 bp sequences complementary to the primers used for deep sequencing, into our bar-seq
library. Designed primers SeqF and SeqR integrate sequencing adaptors compatible with an Illumina assay, as indicated by Illumina.

All primers’ complete sequences and respective specific multiplex indexes are available at section 2 in the Appendices section.

We use a high-fidelity DNA polymerase in the PCR processing steps, TaKaRa Ex Taq Hot Start DNA Polymerase (TaKaRa).

- Barcode amplification step

This step is performed through PCR. Use one Up1-20 and Dn1-20 barcode amplification forward primer per sample. Perform PCR as described next.

Reaction system (total volume 50 μl):

- 10× EX Reaction Buffer 5.0 μl
- dNTPs (2.5 mM) 4.0 μl
- Up1-20F / Dn1-20F (5 μM) 2.0 μl
- UpR / DnR (5 μM) 2.0 μl
- EX HS Taq (5 U/μl) 0.4 μl
- Mutant pool DNA (50 ng total amount) 0.5 μl**
- Add ddH₂O*** to 50 μl

* dd - double-distilled

** Volume of DNA depends on the concentration of genomic purified DNA. 50-100 ng can be used.

*** Adjust ddH₂O volume accordingly so that total volume is 50 μl.

Both of uptag and downtag barcodes are amplified under identical conditions. PCR conditions are as follows:

94°C 4 min
- Purification of PCR products

After the first round of PCR to amplify the barcodes, purify the PCR products using the QIAquick PCR Purification Kit (Qiagen), as indicated by Qiagen.

- Integration of sequencing adaptors

Next, sequencing adaptors are added to the bar-seq library through PCR. Perform PCR as described next.

Reaction system (total volume 50 μl):

- 10× EX Reaction Buffer 5.0 μl
- dNTPs (2.5 mM) 4.0 μl
- SeqF (5 μM) 2.0 μl
- SeqR (5 μM) 2.0 μl
- EX HS Taq (5 U/μl) 0.4 μl
- Amplified barcodes 2 μl*
- Add ddH2O to 50 μl

* 1:25 dilution from previous barcode amplification PCR’s products.

PCR conditions are as follows:

- 94° C 4 min
- 94° C 20 sec
- 56° C 20 sec
- 72° C 20 sec
- 72° C 20 sec ×13 cycles
- Library purification

Next, purify the PCR products obtained after library preparation using a MiniElute PCR Purification Kit (Qiagen), as indicated by Qiagen, using a Speed Vac (Savant) to help accelerate the evaporation of ethanol leftovers.

- Library quantification and pooling

Purified bar-seq library is then quantified through a high sensitivity assay. We utilize a 2100 BioAnalyzer Instrument (Agilent) to accurately quantify DNA, as indicated by Agilent. We then use this information to pool all the samples into a single tube at the desired concentration, which can vary depending on the NGS platform and method used.

Due to unknown reasons and as reported by Han et al.\textsuperscript{137}, bar-seq data obtained is skewed towards a bias for downtags. To circumvent that we pool the libraries in the opposite direction, in a uptags:downtags ratio of 3:2.

- Library deep sequencing

We can then proceed to deep sequencing the newly prepared bar-seq library.

Note: We used an Illumina MiSeq for deep sequencing all the samples analysed and presented in chapters 4 and 5, as indicated by Illumina.
2.3.6 Bar-seq data analysis

2.3.6.1 NGS data conversion, mapping and quality control

Bar-seq libraries were sequenced using a MiSeq platform which has seen its associated depth increased with recent updates to ~20 million (M) reads. Aiming for 1M counts per deletion mutant per sample (~400-fold coverage) it is possible deep sequencing up to 20 bar-seq samples in a single run.

Fastq files containing the raw bar-seq data composed of ~10 (M) 50 bp sequencing reads obtained with bar-seq were first investigated on the FastQC bioinformatics tool, as indicated by developers. Practically all sequencing runs worked successfully as confirmed by the various parameters analysed in FastQC. If sequencing data failed the FastQC tests it would be discarded and samples were re-sequenced.

We then converted the Fastq files to count data using an in-house script based on short read aligner tool Bowtie, using R software. This script allowed mapping the obtained read sequences to a custom made reference database containing all the existing combinations of any given multiplex index and deletion mutant barcode sequences. We allowed 1 mismatch across the whole 50 bp sequencing read.

We could then acquire information on the number of reads per deletion mutant per sample. Furthermore, this analytical step allowed us to better understand the quality of the data obtained through the interpretation of valuable information such as: the number of reads that successfully mapped to the reference database, the number of reads that failed to map, reads with multiple mapping ‘sites’, duplicated barcode sequences on both uptag and downtag and more. Overall, data quality was good (~80 % successfully mapped reads in average) although it is relevant to note that for
some runs a significant part of data failed to produce results (68.4 % successfully mapped reads reported in the poorest sequencing run). Information summaries on the bar-seq count data analysis performed are shown in section 2 in the Appendices section.

2.3.6.2 Overall analysis

We analysed over 50 samples and identified ~2,500-2,700 deletion mutants in our analyses. As previously mentioned when discussing the de novo decoding, the total number of deletion mutants detected was somehow lower than that reported by Han et al.\textsuperscript{54}, but still although within the same range (see section 2.3.2.6). All indexes worked successfully. Bar-seq samples analysed in independent sequencing runs exhibited high similarity that correlated strongly, demonstrating the robustness and reproducibility of this assay (figure 2.12.A and 2.12.B).
Figure 2.13 Bar-seq assay was robust providing highly reproducible data in independent sequencing runs. Identical samples were analysed in independent sequencing runs. Correlation test Sp. R with $p < 0.001$. Dashed lines depict the start of analytical confidence zones as obtained with DESeq analysis. A) Data from uptags. B) Data from downtags. r reads.

Moreover, we analyzed the relative abundances exhibited by external spikes after bar-seq (figure 2.13). Most spikes behaved consistently across 5 independent mutant pool samples. Relative abundances across spikes were practically unchanged during PCR processing steps throughout a high dynamic range of at least ~40-fold (1,000 cells - 40,000 cells), confirming the robustness and reproducibility of this assay. Spike 7, corresponding to an initial pool of 500 cells, was not detected. This could likely mean that an amount of 500 cells is too low to be detected by this assay, and therefore outside its resolution window.
Figure 2.14 Relative abundances are maintained throughout deep sequencing. Most spikes behaved consistently across 5 independent mutant pool samples. Relative abundances across spikes are practically unchanged during PCR processing steps throughout a high dynamic range. x-axis is in log scale.

2.3.6.3 DESeq data analysis and sample cross-examination

Processed bar-seq data was then analysed using NGS data analysis bioinformatics tool DESeq, as indicated by the developers.\textsuperscript{143} DESeq is a freely available R package. All results obtained with DESeq analysis are available at the Lab’s digital archives. All significant results obtained with DESeq analysis are available at section 2 in the Appendices. The results from each sample were cross-examined using a custom made R script. p-value adjusted for multi-test correction was 0.1.
2.3.6.4 Gene Ontology data analysis

Gene Ontology Term Enrichment analysis was performed using AmiGO bioinformatics resource, as indicated by the developers.\textsuperscript{144} Multi-test adjusted p-value used was 0.1, as calculated by a hypergeometric distribution test.

2.3.6.5 Clustering analysis

Clustering analysis was performed using expression analysis software GeneSpring GX 10 (Agilent). Clustering applied using a default Gene Tree analysis, as indicated by the developers.

2.3.6.6 Protein-interactions prediction analysis

Gene lists of significant results obtained from bar-seq data analysis with DESeq were analysed using pINT\textsuperscript{145}, a computational tool for predicting protein-interactions, as indicated by the developers.

2.3.6.7 Estimating growth rates and quiescence-proliferation profiles for deletion mutants in a pooled environment

Results obtained in the work presented in chapter 4 were used to extract the quiescence-proliferation transition profiles presented in section 6.2 from chapter 6 and the results obtained in chapter 5 were used to estimate the growth rates of the deletion mutants present in our prototroph deletion library presented in section 5.6 from chapter 5, acquired from a mutant pool growing in a pooled fashion (thus representing a competitive environment). To do so, we multiplied the mutant pool overall cell density numbers (fig. 4.1) for the relative abundance fold change values obtained for each analyzed time-point with bar-seq (the unlogged values of Log\textsubscript{2} Fold
Change values ("log2FoldChange.T(time-point ID).UP/DN" depending if it comes from uptag or downtag information) at excel files “barseq_QtoP_DESeq_UP.csv” for uptag information and “barseq_QtoP_DESeq_DN.csv” for downtag information (present in section 4 of the Appendices) for obtaining q-p transition profiles. For the growth rates we used the mutant pool overall cell density numbers (fig. 5.1) for the relative abundance fold change values obtained for each analyzed time-point with bar-seq ((the unlogged values of Log₂ Fold Change values ("log2FoldChange.T(time-point ID).UP/DN" depending if it comes from uptag or downtag information) at excel files “barseq_EMM_DESeq_UP.csv” for uptag information and “barseq_EMM_DESeq_DN.csv” for downtag information (present in section 5 of the Appendices). With the obtained values it was possible to plot the q-p transition profiles and estimate the growth rate of each deletion mutant present in the mutant pools assayed. Information on the deletion mutants’ estimated growth rates and doubling times (DTs) are available in section 5 in the Appendices.

2.3.6.8 Data repository

All raw data obtained in the bar-seq experimental studies is deposited in our Lab’s sequencing repository as fastq files. For more information on this please address the Lab (www.bahlerlab.info/).

2.4 Analysis and modelling of fission yeast quiescence-proliferation dynamic profiles

This section describes the analytical methods used for analysing the growth curves presented in chapters 3, 4, 5 and 6.
2.4.1 Analysis of quiescence-proliferation profiles using GroFit R package

Growth curves obtained with BioLector when studying quiescence-proliferation transition dynamics, presented in chapters 3 and 6, were performed using GroFit, an R package developed for analysing biological growth curves, as indicated by the developers.146

2.4.2 Analysis of quiescence-proliferation profiles using a custom mathematical model.

The methods used in the analysis of quiescence-proliferation profiles presented in chapter 3 and chapter 6 section 3 were developed in collaboration with Pablo Crotti and Dr. Vahid Shahrezaei (Faculty of Natural Sciences, Department of Mathematics, Imperial College London).147

2.4.3 Using high-density relative biomass information

A robust mathematical method was used to analyse the growth curves obtained using the BioLector platform when studying quiescence-proliferation transition dynamics presented in chapter 3 and chapter 6. A detailed description of this analytical method is presented in the Appendices.

2.4.4 Using bar-seq NGS information

An adapted version of the method referenced in the previous section (section 2.4.3 in this chapter) was used to analyse the data obtained with the bar-seq profiling studies presented in chapter 4 and chapter 5. A detailed description of this analytical method is presented in section 2 of the Appendices.
3 Quiescence-proliferation transition dynamics in fission yeast
3.1 Overview

The transition undergone by cells from a quiescent to a proliferative state constituted the main focus of this work. We were particularly interested in the dynamics associated with this transition as the kinetics of this process were found to be associated with or depend upon the amount of time cells had been quiescent for. Anecdotally it is thought that the longer cells are kept “dormant”, for example when kept in culture plates in the fridge as the source for setting up new cell cultures, the longer it takes them to engage in growth. Moreover, we know that many biological processes, such as morphogenetic development and immuno-responses, as well as several disorders and pathogenesis that affect humans with high incidence arise from errors occurring when cells are undergoing this transition. However, the existing knowledge regarding the scope of this dynamic behaviour and its underlying basis is still relatively sparse. This is especially startling if considered under the context of the pivotal role of this event upon cell fate decision.

In an attempt to expand our understanding of this key cellular event, particularly on the extent of the dynamic relation between time in quiescence and its effect on the kinetics of the subsequent return to proliferation, we decided to use fission yeast to study this phenomenon. This chapter describes the results obtained in this study.

3.2 Fission yeast quiescence-proliferation transition profiles

Fission yeast wild-type cell cultures were grown in liquid minimal media until mid-log phase. Then, nitrogen was depleted from the media inducing cells to become quiescent. Cells were starved for nitrogen for a variable amount of time ranging from...
24 hours up to roughly 1 month. After that, nitrogen was replenished in the media. This led cells to exit quiescence and start growing, eventually resuming proliferation. The period from when cells exit quiescence until they start to proliferate represents a lag phase where only cellular growth, but not division, is observed. This lag phase where cellular growth is occurring depicts the period when cells engage in huge levels of de novo biosynthesis at very high metabolic rates. This is because during quiescence much of the cellular components and macromolecules required for growth are degraded and need to be re-synthesized. As discussed in the introduction, the metabolic down-regulation that occurs during quiescence has several consequences all seemingly aiming towards increasing of the cell’s survival chances. Using a high-throughput BioLector platform (see section 2.1.5 in Methodology chapter for more information on this assay), we recorded the relative biomass - an indirect indicator of cell culture density levels and thus cell numbers - of these cultures over time, from the moment the media was replenished with nitrogen, to trigger cells to exit quiescence and return to proliferation. Results are shown in figure 3.1.
**Figure 3.1 Fission yeast wt cultures: quiescence-proliferation transition profiles after increasing periods in quiescence.** Cells were kept in quiescence for the designated amount of time and then triggered to return to proliferation. Relative biomass was assessed using a BioLector high-throughput platform. Quiescence was induced by removal of a nitrogen source from the culture media.

Figure 3.1 shows the q-p profiles of fission yeast cell cultures after triggering nitrogen-starved cells to exit quiescence and return to proliferation through nitrogen replenishment. Each profile curve represents the growth of a cell culture previously in quiescence for the depicted amount of time. Cells initially experienced a lag phase period, where they undergo massive changes in nuclear morphology in order to unwrap all the compacted chromatin in order to make available all the genetic material that needs to be transcribed (particularly rRNA), in order to allow all the de
novo synthesis of cellular material required for the cell cycle and the cell to resume
growth and proliferation.\textsuperscript{[90-92,98]} Cells thus grow in size before starting to divide. We
can thus define lag phase in this context to correspond to the period between the
trigger or cellular cue for quiescence exit and the first cellular division. More
concretely it represents the time required for cells to undergo the transition from
quiescence to proliferation, ie, the amount of time that starts upon triggering cells to
exit quiescence until the first cell division occurs.

As expected from our current knowledge, the length of the lag phase varied with the
amount of time cells were in quiescence in a positive relation. As can be easily
confirmed by observing figure 3.2, delimiting the exact moment in time where the lag
phase gives way to the log phase stage (the exponential phase of microbial growth,
wherein cells in the population are undergoing the cell cycle, and therefore increasing
in number in an exponential manner, at their optimal rate) is not a clean task. This is
likely because cells within the same culture probably do not resume proliferation all at
the same time. This results in a very smooth transition between the two phases. This
heterogeneity could be due to internal variability present within a (clonal) population
of cells. Nevertheless, it was clearly shown that cells that had been quiescent for
longer periods required more time until engaging in full proliferation. We were thus
able to reproduce the kinetics observed by Su et al.\textsuperscript{[91]} with higher sensitivity, by using
a highly accurate platform, and covering a temporal period with higher resolution.

It is worth mentioning that the plateau reached by fission yeast cells (around unit 3 for
relative biomass in fig. 3.1) corresponds to the saturation point of these cell
populations when in BioLector plate wells. That is the reason why no further growth is
observed after those cell density levels.
We then estimated the duration of the lag phases exhibited by the cultures shown in figure 3.1 by employing a mathematical model created for analyzing microorganism profile curves available using the groFit R package (figure 3.2), developed for analyzing growth curves and providing reliable estimates on several relevant parameters extracted from these curves, like maximum growth levels, time to reach several important growth steps, like the aforementioned maximum growth level, lag phase duration, log phase start, growth rate, and more.

![Graph showing lag phase duration vs. time in quiescence](image)
<table>
<thead>
<tr>
<th>Time in quiescence (days)</th>
<th>Lag phase duration during q-p transition (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.1 ± 0.23</td>
</tr>
<tr>
<td>2</td>
<td>16.4 ± 1.34</td>
</tr>
<tr>
<td>3</td>
<td>19.1 ± 0.24</td>
</tr>
<tr>
<td>6</td>
<td>19.4 ± 1.47</td>
</tr>
<tr>
<td>7</td>
<td>20.2 ± 1.71</td>
</tr>
<tr>
<td>8</td>
<td>21.4 ± 3.72</td>
</tr>
<tr>
<td>9</td>
<td>21.9 ± 1.54</td>
</tr>
<tr>
<td>10</td>
<td>22.0 ± 2.69</td>
</tr>
<tr>
<td>14</td>
<td>22.5 ± 1.53</td>
</tr>
</tbody>
</table>

Figure 3.2 Q-p transition profiles depicted in figure 3.1 were used to estimate lag phase durations using the groFit R package. Quiescence was induced by removal of a nitrogen source from the culture media. A) Relationship between time in quiescence and duration of lag phase upon q-p transition for up to 14 days in quiescence. B) Lag phase duration values for q-p transition after different periods in quiescence.

groFit analysis further confirmed the association between time in quiescence and lag phase duration q-p transition. This relationship was very pronounced at shorter quiescent periods for up to 8-9 days. After that time, lag phase differences appeared to gradually fade away. This could mean that for some reason, after that amount of time in quiescence the duration of the lag phase upon q-p transition would no longer be affected. This observation did not contradict that reported by Su et al.\textsuperscript{91}, as the
longer duration of quiescence for which q-p transition was analysed (24 days) was only compared to another quiescence period (1 day). This analytical window thus did not cover the time-points in question, after circa 8 days in quiescence. Nevertheless, we aimed to confirm whether this relation was in fact only prevalent on a short-term basis and not after more prolonged periods in quiescence. For this reason we decided to test a broader window of quiescent periods ranging over more than a month (32 days).

Given the gradual nature and relative complexity of the q-p transition profile curves our concern was that the computational resource groFit did not possess the necessary accuracy and sensitivity to allow an adequate analysis of this biological event. Moreover, from observing cells during this transition under the microscope, we could see that not all cells were undergoing the transition with the same kinetics, instead exhibiting some degree of variation (data not shown). This reinforced the idea of a non-linear and relatively complex phenomenon for which groFit was found unsuitable, as it tackled profiles from cell populations without considering any variables that could be providing heterogeneity to the system.

For these reasons we decided to apply a mathematical model specifically developed by Pablo Crotti and Dr. Vahid Shahrezaei\textsuperscript{147} to analyse the q-p transition*. One of the main advantages of this approach was the fact that it incorporated the variable of cell heterogeneity and elongation rate (or cellular growth rate) in their models. This meant that it was possible to accurately analyse the q-p profiles and predict the basis of their dynamics with high precision based on robust mathematical models. In this model lag phase we test a broad hypothesis that instead of having all cells emerging after a random amount of time, some of the cells might have died or simply stopped their cycle during the quiescence period. This model thus studies the growth of the
population of cells when a fraction of the cells does not grow. The model consists of computing the proliferation curve (or also called growth curve) by fitting an exponential function, subject to a variational parameter representing the fraction of living cells by taking into account the growth or elongation rate of the cells and thus being able to accurately estimate when the cells start dividing after the lag phase of the q-p transition. This model does not consider the cell itself but the population of cells. The model then extracts several important parameters from the growth curves analyzed, notably the lag phase duration, with high sensitivity and accuracy (shown in section 3 of the Appendices). In this model, lag phase is defined and estimated as explained in section 2 and section 3 of the Appendices.

We performed several independent experiments where we analysed the q-p transition profiles of cell cultures in EMM-N media for a large set of quiescence periods lasting over a month in quiescence (32 days). Q-p transition profiles obtained were then analysed using the aforementioned model especially developed for this effect. A representation of this analysis is depicted in figure 3.3.

* This model is explained in detail in section 3 of the Appendices where a description of its successful validation process, performed by Pablo Crotti as part of his PhD studies, is also provided.
Days in quiescence:

Red: 30
Green: 27
Purple: 19
Black: 15
Blue: 13
Orange: 9
Yellow: 7
Blue: 5
Orange: 2
Green: 1

A

Relative biomass (through light scattering)

Time after quiescence exit (hr)

B

WT

Lag (mean)

Days

$r = 0.78$
Figure 3.3 Relationship between time in quiescence and duration of lag phase upon q-p transition for up to 32 days in quiescence. Q-p profiles depicted were analysed using a specifically developed mathematical model. Quiescence was induced by removal of a nitrogen source from the culture media. A) Quiescence-proliferation profiles of S. pombe cell cultures after increasing periods in quiescence. Legend indicates the number of days in quiescence prior the q-p transition depicted in the q-p profiles show. To facilitate visualization, only one q-p profile is shown per each time (number of days) in quiescence analyzed. B) Lag phase duration versus amount of time in quiescence of the S. pombe cell cultures represented in A). Symbol size varies with days in quiescence. Wild-type S. pombe cell cultures were induced to remain in quiescence for increasing periods of time (x-axis), after which they were triggered to exit quiescence and resume proliferation. The x-axis indicates the number of days in quiescence. The y-axis indicates the number of hours required for the cell population to resume proliferation. C) Coefficient of variation (cv) of lag phase duration versus amount of time in quiescence for the same cell populations. D) Elongation (growth) rate versus amount of time in quiescence for the same cell populations. The elongation rate represents the average growth rate of the cell population.

The method employed revealed a good correlation between days in quiescence (x-axis) and the duration of the lag phase (y-axis) (figure 3.3). However, this relation appeared to be stronger up to ~10 days in quiescence after which it only seems to increase again after ~27 days in quiescence.

Cell survival during quiescence was monitored through a viability assay by plating and counting colony forming units (CFUs) (figure 3.4). Cell viability gradually decreased slowly until ~20 days after which it decreased moderately to 68%. The decline may represent a fraction of fission yeast cells that are unable to cope with long quiescent periods. This could explain the outlying values obtained for the cultures that had been quiescent for more than 20 days. Moreover, these data seem to reproduce that of
figure 3.1, where the duration of lag phase seemed unaffected after ~9 days. Notably, this decrease in viability was not observed by Su et al. They reported unchanged viability for up to 24 days in quiescence. However, our experience with cells throughout long quiescence in quiescence followed the results obtained in this assay. Su et al. perform their experimental work at 25°C. Different temperatures could have been the basis for the differences reported for cell viability, although with the information available it was not possible to definitively address what gave rise to these distinct observations.

We also analysed the coefficient of variation (cv) and estimated elongation rate of the q-p transition profiles obtained (figure 3.3.C). cv values are a measure of the intrinsic variability exhibited by behaviour of the cell culture upon this transition. No meaningful correlation was observed. However, it is worth noting that the q-p profiles after >20 days appeared to exhibit a lower cv. This could be a reflection of their lower viability. Elongation rate, a measure comparable to growth rate, depicted the estimated time at which cells grew. These values were extracted through the analysis of the q-p profile curves and thus without any information on individual cell size. Elongation rates determined (figure 3.3.D) did not appear to be affected by the amount of time in quiescence. This was somehow expected and probably merely reflected that elongation rate constitutes an intrinsic property of the cells and is unaffected by a previous period in quiescence.
Figure 3.4 Cell survival during quiescence up to 32 days. Cell survival was analysed during quiescence until 32 days through a CFU viability assay. Quiescence was induced by removal of a nitrogen source from the culture media.

3.3 Quiescence-proliferation transition dynamics at the single-cell level

In order to thoroughly study and understand the q-p transition it was imperative to zoom in to the single-cell level to grasp how individual cells were behaving upon this transition, as opposed to population-focused studies. This was important to acquire valuable information on what was happening within a population. Whatever dynamics could be occurring within a cell population, even clonal, would not be detected through a population-level analysis, whereby any specific differences between cells would be diluted across the population. As mentioned in the previous section, upon
examining clonal cell populations under the microscope undergoing the q-p transition, we noticed that not all of them exhibited the same morphology or cell size. All the cells appeared to exhibit similar dimensions during the quiescence periods that preceded this transition (fig. 3.5A). Therefore, no differences on cell size were present at the start of the transition that could justify those observed throughout the q-p transition. Therefore, we hypothesised that the differences observed across cells from the same population (which are in theory clonal cells) could represent an existent intrinsic variation on the kinetic properties of clonal cells. This variation could then be providing the basis for a certain degree of heterogeneity in the kinetics manifested during the q-p transition.

In order to understand how cells were behaving throughout the q-p transition we measured the cell length of individual cells upon exit from quiescence at the start of q-p transition, using a calcofluor staining assay combined with fluorescence microscopy (see section 1.1.7 in the Methodology chapter). Data relative to cell length at the time where the q-p transition was induced (T₀), representing the cell lengths of quiescent cells after the designated amounts of time in quiescence, and 390 minutes upon the q-p transition (T₃₉₀), roughly when the first dividing cells started to be detected*, is depicted in figure 3.5.

* We routinely sampled the fission yeast cell cultures during the q-p transition to visualise cells under the light microscope (data not shown). This allowed us to determine the amount of time after quiescence exit induction where the first cell divisions started to be observed.
Upon q-p transition induction

Time in quiescence (days)

Cell length (pixels)

390 min upon q-p transition induction

Time in quiescence (days)

Cell length (pixels)
Time in quiescence:
- Green: 14
- Black: 8
- Orange: 6
- Red: 3
- Blue: 2
- Pink: 1

(c) Histogram of cell length (pixels) frequency for different time in quiescence.

(d) Histogram of cell length (pixels) frequency for different time in quiescence.
**Figure 3.5. Single-cell analysis of the q-p transition after increasing amounts of time in quiescence.** Quiescence was induced by removal of a nitrogen source from the culture media. **A)** Cell length values measured upon quiescence exit induction, after the designated amount of time in quiescence. **B)** Cell length values measured 390 minutes upon quiescence exit induction, after the designated amount of time in quiescence. Statistical significance was calculated through one-way analysis of variance (ANOVA) and Bonferroni multi-comparison tests. * p < 0.05; ** p < 0.01; *** p < 0.001. **C)** Cell length distributions upon quiescence exit induction, after the designated amount of time in quiescence. **D)** Cell length distributions 390 minutes upon quiescence exit induction, after the designated amount of time in quiescence. All samples with n > 150.

Analysis of the individual cell lengths of quiescent cells exposed to increasing periods in quiescence revealed significant differences in the mean of cell lengths. This observation agrees with previously published studies. There it was reported that as cells become exposed to longer quiescence periods, their cell volume slightly decreases gradually across time and DNA becomes more compacted and heterochromatin-like. Therefore, our observations are in accordance with those reported in the specialized literature. An important factor that could be the basis of the cellular shrinkage observed, is cellular autophagy. Autophagy has been shown to be a crucial biological process during cellular quiescence, as it allows the internal recycling of nutrients, damaged and unneeded organelles and macromolecular contents by the cell, greatly increasing its pool of energy reserves and biosynthetic substrates. It also allows degradation of mitochondria, which in the high numbers observed in proliferative cells can become highly toxic to the cell during quiescence, mainly due to the elevated production of ROS.

Interestingly, cell lengths measured 390 minutes after the start of the q-p transition showed a similar trend to that initially present at T₀, across the different quiescence
periods analysed. Therefore, it would seem likely that differences at T_{390} might merely reflect the differences already present upon quiescence exit. In addition, the q-p transition lag phase differences reported and discussed in the previous section in this chapter might thus be a consequence of smaller cells requiring more time to reach the size necessary for division to occur.

To acquire a more global view on the information on single-cell length values, we then analysed the dispersion of all individual cell length values across a frequency histogram. Here, quiescent cells appeared to be distributed more or less around the same range (figure 3.5C), exhibiting an approximation to a Gaussian distribution. Interestingly, at T_{390} the shape of the cell length frequency histograms did not reproduce the pattern observed at T_{0} (figure 3.5D). Cells after 1 day in quiescence clearly showed two distinct sub-groups in respect to cell length. It was possible to detect a very narrow group of cells (frequency peak on the right-hand within the pink histogram at figure 3.5D) exhibiting an average cell length above that which we interpreted as representing the bulk of the population cell lengths (frequency peak on the left-hand side within the same pink histogram). These peaks appeared to originate from different sub-groups of cells within the same clonal population exhibiting distinct kinetics upon the q-p transition. This interpretation agrees with observations acquired from routinely inspecting cells upon the q-p transition under the microscope (data not shown).

On a related note, after 1 day in quiescence it was possible to observe that some cells, slightly bigger than the population average, had not yet become fully quiescent (data not shown). Therefore it could be that the sub-group of cells exhibiting faster kinetics might have been constituted by these not yet fully quiescent, bigger cells. Interestingly, however, traces of this sub-population were still detectable in the
histograms of 2-day and 3-day (although not clearly) (figure 3.5D) quiescent cells, which are known to be fully quiescent exhibiting a high degree of homogeneity (through microscopy analysis, data not shown). This suggested that the kinetic heterogeneity exhibited by fission yeast wt clonal populations were not due to differences initially present in the quiescence cell population, as it was not possible to identify any sub-groups across quiescent cell populations, at least in respect to cell size.

Interestingly, the frequency distribution at T_{390} of the cell lengths from cells that had been quiescent for longer periods (6, 8 and 14 days in quiescence) did not show any evidence of population heterogeneity (figure 3.5D). This could mean that whatever intrinsic heterogeneity existed within a (clonal) population regarding the kinetics of q-p transition, it could become less pronounced as the duration of quiescence extended over time. This observation appears to be supported by the analysis of the results obtained with the mathematical model employed to examine the q-p profile curves presented in section 1 in this chapter. This model analyses the q-p profile curves obtained without any prior information on single-cell values or even population growth rates. All is simulated with the model, which made predictions based on the power associated with the q-p profile models developed (see section 3 at the Appendices for more information about this model). One of the variables analysed was the variation exhibited by the q-p profile curves (figure 3.5B). As suggested in the interpretation of this result, the intra-population variation estimated across cells appeared to diminish as the amount of time in quiescence became larger, although this was not entirely clear. Consequently, this information agreed with that extracted from single-cell analysis (figure 3.5D) where it appeared that deep quiescent cells exhibited a higher cell length homogeneity upon the q-p transition. In addition, a higher degree of heterogeneity was observed in cells that had been quiescent for
shorter periods, both in the analysis of the q-p profile curves coefficient of variation depicted in figure 3.5B as in the analysis of the distribution of cell length values shown in figure 3.5D. The fact that these constituted completely independent approaches leading to similar conclusions strongly reinforces these observations, calling for their definitive validation through subsequent experimental work. Moreover, the existence of different subgroups might very likely be related to the fact that upon nitrogen starvation some cells (those in G1) might be able to arrest without additional division, whereas most cells will be in G2 and require a mitotic division before they can arrest in G1. This means that the latter cells will have relatively few resources compared to the cells that can enter G1 directly making the latter group of cells better equipped to undergo an efficient return to proliferation, resulting in a faster and shorter transition than the former group. This could be the basis for the observations shown in fig. 3.5C-D.

No reports have been made regarding the existence of sub-groups of cells within quiescent clonal populations in fission yeast. Interestingly however, sub-groups of cells have been reported within budding yeast quiescent populations. In that study, cells were separated based on a density gradient, demonstrating the existence of at least two cell sub-populations within quiescent cell cultures. Whether these sub-populations are in any way related to the apparent sub-populations hereby identified and described (figure 3.5B) constitutes an interesting research question that would be pertinent to address.

Also for many cancer tumours it has been shown that a high degree of heterogeneity is observed across the cells that constitute them. Here, whilst the majority of cells are engaged in high rates of proliferation, other cells are maintained quiescent in a non-dividing state. This has been suggested to be a defensive mechanism that
develops in many tumours, in which these quiescent cells exhibit higher levels of stress resistance and survival rates that could be in the basis for tumour resistance-acquiring mechanisms, especially to drugs and therapeutics developed for eliminating tumour cells.\textsuperscript{33,34} Furthermore, it has been suggested that this could constitute the underlying reason for why cancer-treatments become so drastically ineffective after relatively short periods of time.\textsuperscript{33}

Alternatively, the existence of 2 sub-groups might represent those cells that have already divided (peak of lower size values) and those yet to divide (peak of higher size values), which would not be present in the populations of cells that been under longer quiescence periods as the time-point sampling time (390’ after quiescence exit induction) had not allowed for any cells to have divided yet. Still, the fact that some cells have divided whilst others have not highly suggests the presence of a dynamic heterogeneity upon q-p transition. Moreover this heterogeneity could still be retained after longer quiescence periods, but were undetected in this analysis using these time-points. Further experiments with higher temporal resolution could easily address this question.

3.4 Interpretation of the results obtained in the analysis of the quiescence-proliferation dynamics in fission yeast

Interpreting this chapter at an evolutionary and biological level, cells have developed countless different strategies and mechanisms that ultimately increase their chances for survival. Moreover, this can be observed inclusively at the group level, where a few cells sacrifice themselves for the sake of the entire population. Expanding this
rationale could eventually lead to (at least in part) some of the theories explaining the advent of multicellular organisms, where cells become highly differentiated and specialized in particular and highly-defined tasks within a multicellular organism. Remaining on the unicellular level though, it is interesting to consider the observations here discussed under the concept of bet-hedging, where cells (even within clonal populations) invest in different outcomes on many levels by exhibiting variation across a whole plethora of characteristics. In this view, one could interpret the heterogeneity in the q-p transition kinetics exhibited by the cells as a possible strategy of bet-hedging. Here, some cells could be dedicated to quickly respond to growth or proliferation signals by immediately triggering the q-p transition investing in high growth rates aiming towards a quick resumption of proliferation and consequently ensuring that more progeny is originated. On the other hand, other cells could be exhibiting a much slower response to engage on the q-p transition with the purpose or advantage of maintaining higher stress resistance levels and an augmented capacity for producing a meaningful and robust response to any eventual environmental perturbations arising from the possibility that the conditions suitable for growth might steadily and unexpectedly be significantly altered. Whilst quickly growing, cells could be too sensitive to any drastic or even relatively moderate perturbations, quiescent of slow growing cells exhibiting slower q-p transition kinetics would be much better suited to face any eventual challenges by relying on the whole horde of characteristics that make them more resistant to stress and possessing higher survival chances towards adversities.
4 Functional profiling of the quiescence-proliferation transition
4.1 Overview

As explained in detail in the methodology section, the bar-seq assay we developed made it possible to acquire highly sensitive and dynamic information on the relative abundance of a fission yeast prototroph deletion library over time. In this particular case we were interested on the transition from quiescence-to-proliferation (q-p). By analyzing a time-course of the deletion mutants’ kinetics upon this transition we were able to indirectly detect those that were undergoing this transition faster or slower than (the mutant pool’s) average. The genes deleted in the mutants exhibiting differential kinetics would then represent very interesting candidates for further studies to identify regulatory players of this process’ dynamics.

4.2 Experimental design

The bar-seq screen was designed in the attempt to capture the most crucial stages of the quiescence-to-proliferation transition. In the same method as the experiments conducted with the fission yeast wild-type in the quiescence-to-proliferation dynamics analysis presented in chapter 3, a haploid deletion library was grown to mid-log phase, and then induced to enter quiescence through nitrogen source depletion - representing the first sampled time-point of this assay (Q_{entry} in figure 4.1). After the designated periods in quiescence, the mutant pool was again sampled (time zero or T_{0}) and allowed to exit quiescence towards a return to proliferation. The mutant pool was then regularly sampled over time throughout the whole lag phase between these two biological states, until cells had already fully entered the growth exponential
phase. A visual representation of this assay's time-course can be observed in figure 4.1.

**Figure 4.1 Experimental design of the quiescence-proliferation functional profiling assay.** Red arrows depict sampled time-points selected for downstream analysis using bar-seq. The time-points were taken and are labelled as follows: $Q_{\text{entry}}$ - upon quiescence entry; $T_0$ - time zero, upon quiescence exit; $T_{390}$, $T_{530}$, $T_{750}$, $T_{890}$, $T_{980}$ - time-points taken at $T_n$ minutes after quiescence exit. 2 days in quiescence represent a short quiescence period. 10 days in quiescence represent a deep quiescence state. Each of the 2 green profiles depicts an independent biological mutant pool replicate, after a 2-day short quiescence state. Each of the 2 blue profiles depicts an independent biological mutant pool replicate, after a 10-day deep quiescence state.

Next, bar-seq libraries were prepared for each of the samples selected for downstream bar-seq analysis, and then sequenced using a MiSeq Illumina sequencing platform. A comprehensive explanation of all the processing steps
performed for this assay can be consulted in the corresponding Methodology section. A full and thorough description of all the results obtained from the bar-seq screen analysis will be presented next.

Note 1: The sample from time-point $T_{390}$ - taken 390 minutes upon triggering the exit from quiescence - from q-p time-course after deep quiescence was not sequenced and thus did not take part in the analysis of this condition. This was because no growth had been detected until a much later stage, leading us to the conclusion that no great advantage would result from analysing this time-point. Moreover, after analyzing the sample from this time-point after short quiescence we realized that it was also not qualitatively informative for this condition, as low consistency was observed between that and the remaining time-points analyzed. We believe this to be likely due to the little action or change yet undergone in overall cell density 390 min upon triggering the exit from quiescence. This could have led to not enough resolution in which to retrieve robust data, together with the interference of high levels of noise. Ultimately this would have led to inconsistent and/or low-quality data and eventually overestimation of the marginal differences observed in relative abundance, that could have appeared to be larger and more marked than what was actually the case, leading to deceiving results. For all this, the sample taken from time-point 390 min upon triggering the exit from quiescence was not considered for the analysis of q-p transition from both short and deep quiescence.

Note 2: Even though time-point $Q_{\text{entry}}$ was sampled, sequenced and analyzed, due to complexity constraints it was not directly included in this analysis of the q-p transition. With the exception of some particular cases where it is clearly stated, data from this time-point was not considered in the analysis presented here. Still, it would constitute an interesting addition to this analysis to understand in some detail the “behaviour” of
the analysed mutants throughout periods of short and deep quiescence. This could still be pursued further as data should be available for future use.

4.3 Short and deep quiescence states

The analysis of this genetic screen will initially tackle each condition - short and deep quiescence - separately. We believe this will allow us to better understand the particularities and specificities of the different quiescent states and the respective transitions to proliferation, namely which genes could be required or important for the dynamics of the transitions from each of the quiescence states analyzed, presented in this chapter. This will be followed by a more global interpretation aiming to integrate the main results obtained for both quiescent states where we hope it will be possible to capture the differences and similarities of the genetic programs regulating each transition (chapter 6).

The rationale for selecting upon the quiescence period duration to represent these two distinct quiescence states - short and deep quiescence - was based on both published\(^{91}\) and our own observations. Su et al.\(^{91}\) had described that as the duration of quiescence increased, fission yeast cells became more round and small, and their heterochromatin became further compacted. This was in accordance with our own observations from visualizing fission yeast quiescent cells on the light microscope. Cells would appear roundish and oval like at the initial stages of quiescence (upon the first days in quiescence), become more and more round and small as time in quiescence progressed. We wanted to capture the short quiescence period at the shortest duration where we were confident enough that practically all cells would
have fully become quiescent. For that reason one day in quiescence was considered to be insufficient as we noted that some of the cells still resembled rod-shape proliferative cells. After 2 days this was not the case, as all cells appeared to be smaller roundish and fully quiescent. The duration for the deep quiescent period was 10 days for it became clear by visualizing cells under the microscope that after this period in quiescence cells were already fully in a deeper quiescence state. They were considerably smaller than after 2 days in quiescence, rounder. Also, this was roughly the plateau where we observed that the relationship between the duration of quiescence and the lag phase during the q-p transition started to dilute and become unclear or not present. Moreover, by visualizing cells under the microscope, it became clear that some cell death, even if negligible, had begun to occur and become more prominent after this period (data not shown). For this reasons we considered 10 days to be the adequate duration for representing a deep quiescence state.

4.4 Identification of genes affecting the dynamics of the quiescence-proliferation transition

In order to try to identify genes affecting the q-p transition dynamics we looked for gene-deletion mutants exhibiting differential kinetics upon this transition using a mutant pool consisting of a near genome-wide non-essential gene deletion library (containing >2,700 mutants). Such mutants with differential q-p kinetics could be characterized by the display of both faster/early or slower/delayed q-p transitions. Therefore we focused our analytical enquiry on the recognition of the mutants exhibiting such behaviour.
It is analytically challenging and complex to straightforwardly assess these altered kinetics for such a high number of mutant strains. However, using a bar-seq approach it is possible to acquire accurate and highly sensitive information on the relative abundance of all the mutants present in the pool across a sampled q-p transition time-course. Consequently it is possible to indirectly infer which mutants are undergoing this transition faster or slower than average. Mutants significantly changing their relative abundance over the q-p time-course would therefore constitute strong candidates for displaying altered dynamics upon this transition. This could then be suggestive of an important role for the corresponding deleted genes upon the q-p transition.

Importantly, a mutant exhibiting an early q-p transition might be more valuable and informative than a delayed one where a plethora of reasons, direct and indirect, might be affecting such transition by keeping it from occurring in a normal and unimpaired fashion.

A functional and more comprehensive analysis of this study is presented in chapter 6. Here, an overview of the genes highlighted in this profiling study was conducted with the valuable help of the fission yeast online resource PomBase. PomBase (www.pombase.org) is a new model organism database established to provide access to comprehensive, accurate, and up-to-date molecular data and biological information for the fission yeast Schizosaccharomyces pombe to effectively support both exploratory and hypothesis-driven research. PomBase encompasses annotation of genomic sequence and features, comprehensive manual literature curation and genome-wide data sets, and supports sophisticated user-defined queries.
Note: There were more samples collected throughout the q-p time-course (mostly before time-point T₃₉₀) that were not further selected for downstream analysis. We did not see great additional value on doing so, especially when put into perspective the sequencing costs associated against the limited extra output they would allow us to acquire. Also conducted was a 3rd biological replicate for each quiescence condition, which was likewise not analysed mainly due to sequencing costs. The full list of the time-points selected for sampling can be assessed in the Appendices section and all the remaining samples could still be further processed and analysed, if required.

4.4.1 Analysis of the quiescence-to-proliferation transition in a time-course fashion

Upon sequencing the selected bar-seq samples, we obtained several large data sets, which were then analyzed using the BioConductor package DESeq, developed for analyzing next-generation sequencing (NGS) data. More details on the analytical steps employed can be found in the corresponding Methodology section 2.3.6. Although this package had been available for some time, this represented a novel bar-seq analytical pipeline as all the bar-seq studies published by the time of this analysis had been examined using statistical less robust approaches. Namely, these approaches were based upon standard statistical analysis for outlier identification, not taking into account the intrinsic variance between biological replicates and the depth of the raw data. This kind of analysis can lead to high number of false positives. Our approach, by taking into account such variance, and by using a statistical analysis optimized for this type of data that incorporates information on the depth and robustness of the raw data, successfully tackles these issues, leading to a much more reliable output.
Given the complexity and size of the data sets obtained we designed our analytical approach with the aim of obtaining an output with fewer false negatives. For that we decided to include only those mutants found to be significantly changing their relative abundance at the time-points considered most critical (as described later in this chapter) for the detection of an early or delayed q-p transition for both uptags and downtags, following the rationale described next.

4.4.2 Fast or early q-p transition mutants

In order to try to identify mutants that undergo a faster or early q-p transition we employed an interpretation of the corresponding time-course that we believed to optimise the power of our analysis. For this, we considered that the time-points most important for the identification of fast q-p mutants were those time-points taken earliest in the q-p time-course, more specifically those situated at the boundaries/borders between the lag phase and log-phase of growth (T_{390}, T_{530} and T_{750}) (figure 4.2). However, as T_{390} provided inconsistent results, with little or no overlap with those for the other time-points considered for this analysis, we decided to discard and not use the information resulting from its analysis. We believe that 390 min was not enough time to allow for significant changes (in this particular case cell divisions) to occur to allow for the detection of fast or early q-p transition mutants, and thus its results, even if found statistically significant, were likely due to noise, or fluctuations and associated error arising from either technical aspects or limitations intrinsic to the model.
Figure 4.2 Time-points selected for identification of early quiescence-proliferation transition mutants after short quiescence. Red arrows depict time-points selected for downstream analysis using bar-seq. The time-points were taken and are labelled as follows: $Q_{\text{entry}}$ - upon quiescence entry; $T_0$ - time zero, upon quiescence exit; $T_{390}$, $T_{530}$, $T_{750}$, $T_{890}$, $T_{980}$ - time-points taken at $T_n$ minutes after quiescence exit. The orange box surrounds the time-points selected for detection of early q-p transition mutants in the semi-conservative analysis. 2 days in quiescence represent a short quiescence period. 10 days in quiescence represent a deep quiescence state. Each of the 2 green profiles depicts an independent biological mutant pool replicate, after a 2-day short quiescence state.
Figure 4.3 Time-points selected for identification of early quiescence-proliferation transition mutants after deep quiescence. Red arrows depict time-points selected for downstream analysis using bar-seq. The time-points were taken and are labelled as follows: $Q_{\text{entry}}$ - upon quiescence entry; $T_0$ - time zero, upon quiescence exit; $T_{390}$, $T_{530}$, $T_{750}$, $T_{890}$, $T_{980}$ - time-points taken at $T_n$ minutes after quiescence exit. The orange box surrounds the time-points selected for detection of early $q$-$p$ transition mutants in the semi-conservative analysis. 2 days in quiescence represent a short quiescence period. 10 days in quiescence represent a deep quiescence state. Each of the 2 blue profiles depicts an independent biological mutant pool replicate, after a 10-day deep quiescence state.

These are the time-points that better allow us to detect over-represented mutants that resume the cell cycle earlier as they are the first ones within the $q$-$p$ transition time-course. Later time-points could also be considered for this analysis, although there is a substantial risk that they could confound the results obtained. This is because mutants that undergo this transition more rapidly are not necessarily over-represented at later time-points, where practically the full mutant pool has already entered the log-phase of growth and their initial over-representation quickly becomes diluted as a result of the increase in relative abundance of fast-growing mutants. For
this reason, they were not taken into account in the identification of fast q-p transition mutants in the semi-conservative analysis.

4.4.3 Slow or delayed q-p transition mutants

Regarding slow q-p transition mutants, it should be valid to include all the time-points of the time-course in order to extract the mutants that are slow or defective in undergoing this transition. However, as there is hardly any action going on at the earliest time-points, where practically no mutants exhibit changes in relative abundance so to allow the uncovering of the slow q-p transition under-represented ones, the information arising from these time-points is inevitably very limited, decreasing the power and sensitiveness of the overall analysis. Moreover, by visually inspecting figure 4.4, it is easily perceptible that the near absence of any change in the overall mutant pool cell numbers at the earliest time-points could not give rise to the identification of slow q-p transition time-points. These numbers need to considerably increase to allow the extraction of the mutants that are significantly becoming under-represented and thus exhibiting a slower than average transition, or no transition at all. For this reason, the focus of the analysis aiming to identify the slower transition mutants comprehended those mutants found to be under-represented in the latest time-points of the q-p transition, where larger changes in the overall pool density facilitate the detection of those slow transition mutants (figure 4.4).
Figure 4.4. Time-points selected for identification of delayed quiescence-proliferation transition mutants. Red arrows depict time-points selected for downstream analysis using bar-seq. The time-points were taken and are labelled as follows: $Q_{\text{entry}}$ - upon quiescence entry; $T_0$ - time zero, upon quiescence exit; $T_{390}$, $T_{530}$, $T_{750}$, $T_{890}$, $T_{980}$ - time-points taken at $T_n$ minutes after quiescence exit. Orange square surrounds the time-points selected for detection of early q-p transition mutants in the semi-conservative analysis. 2 days in quiescence represent a short quiescence period. 10 days in quiescence represent a deep quiescence state.

4.4.4 Quiescence-proliferation transition after short quiescence

4.4.4.1 Fast or early q-p transition mutants

2 mutants were found over-represented at the critical time-points for this analysis (fig. 4.2) and thus suggestive of an early or fast q-p transition (table 4.1 and fig. 4.5).
Table 4.1. Gene list of fast or early q-p transition mutants after short quiescence. GI - Genetic Interactions; PhI - Physical Interactions; TF - Transcription Factors. Description and Function were retrieved using the PomBase online resource.6

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Common Name</th>
<th>Description</th>
<th>Function and other information</th>
<th>Doubling Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPAC22F3.13</td>
<td>tsc1</td>
<td>hamartin</td>
<td>TOR signalling, regulation of intracellular protein transport, cellular response to nitrogen starvation</td>
<td>146.2</td>
</tr>
<tr>
<td>SPAC458.06</td>
<td>atg1803</td>
<td>WD repeat protein involved in autophagy Atg18c</td>
<td>Autophagy, cellular response to nitrogen starvation; GI ash2, cph1 (clr6 HDAC)</td>
<td>142.9</td>
</tr>
</tbody>
</table>

A

![Graph showing relative abundances over time](image-url)
Figure 4.5 Quiescence-proliferation transition profiles of fast or early q-p transition mutants after short quiescence. A) Quiescence-proliferation transition profiles, uptags. B) Quiescence-proliferation transition profiles, downtags. * $p$-value < 0.05, ** $p$-value < 0.01, *** $p$-value < 0.001.

According to PomBase$^6$ tsc1, whose common name product is hamartin, is a highly conserved gene. It is part of the TOR signalling pathway and present in the TSC complex, involved in the response to nutrient availability, including nitrogen starvation cues. It is responsible for inhibiting the Rheb GTPase upon nutrient scarcity and thereby inhibiting TORC1. tsc1Δ is therefore unable to adequately inhibit TORC1, which could mean that tsc1Δ cells are unable to fully enter a quiescent state. This could explain why it exhibited an early q-p transition. tsc1Δ is a highly haploproficient deletion mutant.$^{139}$ Given the fact that it is important for autophagy activation (see fig. 1.4), it is interesting it can clearly survive through a period of short quiescence (alternatively it might not have adequately entered this state and been able to survive for a short period of time, thus remaining poised for a quick return to proliferation).
This mutant exhibits a variety of phenotypes, namely decreased levels of various amino acids but not all, as according to PomBase. Hamartin, the corresponding protein, is more abundant during vegetative growth than quiescence, but still it retains relatively high levels during quiescence, which is suggestive of its relevance also during this cellular state. Its name, meaning tuberous sclerosis 1, derives from the respective human orthologue. The physical interactions identified so far connect tsc1 with tsc2, with whom it forms the TSC1-TSC2 complex, initially found in humans when studying the disease with the same name. Also found physically interacting with Tsc1 were stress-response protein Bis1 and serine/threonine protein kinase Hhp1. Bis1, despite not being detected during quiescence, shows some interesting genetic interactions with chromatin silencing factors, namely Rik1, Cph2, involved in histone 3 lysine 9 methylation. It also interacts with two factors complexed with Cdc5, namely Cwf19 and Cwf15. Overexpression of Bis1 results in a cell elongation phenotype, as reported in Pombase. Hhp1 is amongst other functions involved in bipolar cell growth and chromosome segregation, two fundamental processes in order to engage in proliferation. This is a highly abundant protein both during vegetative growth and quiescence.

In humans the TSC1-TSC2 complex is strongly associated with the SWI/SNF chromatin complex and also the small nuclear RNA activating complex. It was found in cancer cells where TSC1 opposes mTORC1 activation of glycolysis. Recently it was also found that the tuberous sclerosis complex (TSC), through the suppression of mTORC1, induces autophagy in response to ROS at the peroxisome.

atg1803Δ also exhibited an early q-p transition (fig. 4.5). atg1803 is also part of the deep quiescence early q-p transition mutants’ gene list, and there a more detailed analysis is made about the possible involvement of this gene in the q-p transition.
4.4.4.2 Slow or delayed q-p transition mutants

We obtained 20 mutants suggestive of a slow q-p transition with this approach. The corresponding gene list together with some information relative to those genes is presented in table 4.2 and fig. 4.6.

Table 4.2 Gene list of slow or delayed q-p transition mutants after short quiescence. GI - Genetic Interactions; PhI - Physical Interactions; TF - Transcription Factors. Description and Function were retrieved using the PomBase online resource.6

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Common Name</th>
<th>Description</th>
<th>Function and other information</th>
<th>Doubling Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPAC1635.01</td>
<td>-</td>
<td>mitochondrial outer membrane voltage-dependent anion-selective channel (predicted)</td>
<td>Ion transport; GI png2; PhI hhp1; porin</td>
<td>203.8</td>
</tr>
<tr>
<td>SPAC1F5.10</td>
<td>-</td>
<td>ATP-dependent RNA helicase (predicted)</td>
<td>rRNA processing</td>
<td>142.9</td>
</tr>
<tr>
<td>SPAC3F10.09</td>
<td>-</td>
<td>1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase (predicted)</td>
<td>Histidine biosynthetic process</td>
<td>133.5</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Function</td>
<td>Value</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>SPBC106.07c</td>
<td>N alpha-acetylation related protein Nat2 (predicted)</td>
<td>N-terminal peptidyl-methionine acetylation</td>
<td>146.3</td>
<td></td>
</tr>
<tr>
<td>SPBC1539.03c</td>
<td>argininosuccinate lyase (predicted)</td>
<td>Arginine biosynthetic process</td>
<td>240.0</td>
<td></td>
</tr>
<tr>
<td>SPCC126.01c</td>
<td>conserved fungal protein</td>
<td>GI clr4, TF mca1, sft1 (hit std-cons)</td>
<td>163.7</td>
<td></td>
</tr>
<tr>
<td>SPCC1739.06c</td>
<td>uroporphyrin methyltransferase (predicted)</td>
<td>Heme and methionine biosynthetic process</td>
<td>161.0</td>
<td></td>
</tr>
<tr>
<td>SPAC1D4.03c</td>
<td>aut12 associated protein Aut12 (predicted)</td>
<td>Autophagy</td>
<td>155.7</td>
<td></td>
</tr>
<tr>
<td>SPAC25G10.05c</td>
<td>ATP phosphoribosyltransferase</td>
<td>Histidine biosynthetic process</td>
<td>138.1</td>
<td></td>
</tr>
<tr>
<td>SPAC227.07c</td>
<td>alpha-1,2-mannosyltransferase Omh6 (predicted)</td>
<td>Cell wall mannoprotein biosynthetic process; protein mannosylation</td>
<td>196.9</td>
<td></td>
</tr>
<tr>
<td>SPAC3H8.07c</td>
<td>prefoldin subunit 3 Pac10 (predicted)</td>
<td>Microtubule cytoskeleton, tubulin complex assembly</td>
<td>202.6</td>
<td></td>
</tr>
<tr>
<td>Gene ID</td>
<td>Gene ID</td>
<td>Description</td>
<td>Functions</td>
<td>Function Score</td>
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<td>----------------</td>
</tr>
<tr>
<td>SPCC790.02</td>
<td>pep3</td>
<td>HOPS/CORVET complex subunit, ubiquitin-protein ligase E3 (predicted)</td>
<td>Intracellular protein transport, vacuole organization</td>
<td>154.2</td>
</tr>
<tr>
<td>SPAC323.01c</td>
<td>pos5</td>
<td>mitochondrial NADH kinase Pos5 (predicted)</td>
<td>NAD metabolic process, NADP biosynthetic process</td>
<td>147.5</td>
</tr>
<tr>
<td>SPBC11C11.09c</td>
<td>rpl502</td>
<td>60S ribosomal protein L5</td>
<td>Ribosome biogenesis, translation, PI epe1</td>
<td>327.9</td>
</tr>
<tr>
<td>SPCC126.15c</td>
<td>sec65</td>
<td>signal recognition particle subunit Sec65 (predicted)</td>
<td>SRP-dependent cotranslational protein targeting to membrane, signal sequence recognition</td>
<td>154.3</td>
</tr>
<tr>
<td>SPAC31A2.13c</td>
<td>sft1</td>
<td>SNARE Sft1 (predicted)</td>
<td>Intra-Golgi vesicle-mediated transport</td>
<td>165.9</td>
</tr>
<tr>
<td>SPAC23H3.05c</td>
<td>swd1</td>
<td>Set1C complex subunit Swd1</td>
<td>Histone methylation, chromatin remodelling</td>
<td>175.6</td>
</tr>
<tr>
<td>SPBC32F12.11</td>
<td>tdh1</td>
<td>glyceraldehyde-3-phosphate dehydrogenase Tdh1</td>
<td>Gluconeogenesis, glycolysis, stress-activated protein kinase signaling</td>
<td>155.7</td>
</tr>
<tr>
<td>SPBC365.14c</td>
<td>uge1</td>
<td>UDP-glucose 4-epimerase Uge1</td>
<td>Galactose catabolism</td>
<td>207.2</td>
</tr>
</tbody>
</table>
Figure 4.6 Quiescence-proliferation transition profiles of slow or delayed q-p transition mutants after short quiescence. A1) Quiescence-proliferation transition profiles (1), *uptags. A2) Quiescence-proliferation transition profiles (2), *uptags. A3) Quiescence-proliferation transition profiles (3), *uptags. B1) Quiescence-proliferation transition profiles (1), *downtags. B2) Quiescence-proliferation transition profiles (2), *downtags. B3) Quiescence-proliferation transition profiles (3), *downtags. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.
Although no GO term was found significantly enriched within this gene list, there are some common features that are worth noting. One feature that immediately stands out is the number of genes involved in biosynthetic and biogenesis processes (8 out of 20), as amino acid synthesis and processing (5 out of 20). According to PomBase, within this last group, two genes, SPAC3F10.09 and his1, are involved in histidine biosynthesis, two genes, SPBC106.07c and SPCC1739.06c, are implicated in the biosynthesis of the start codon amino acid methionine. Finally, SPBC1539.03c is concerned with arginine biosynthesis.

Interestingly, SPCC126.01cΔ and sec65Δ showed statistical significance upon all time-points analysed (fig 4.6A1, fig. 4.6B1).

According to PomBase, SPCC126.01c is an unassigned conserved gene although with no apparent curated orthologues. Its deletion mutant is viable though exhibits an abnormal cell shape. Its protein product is quite abundant in quiescence (730 molecules per cell) when compared to its abundance in vegetative growth (1146 molecules per cell), considering that upon quiescence there is a great diminishment of the proteomic content of a cell, which might indicate a relevant role of this gene during quiescence. Some genetic interactions have been reported, namely with clr4, a histone methylase involved amongst other processes in chromatin silencing.

According to Pombase, sec65, a highly conserved gene, codes for a 7S RNA binding protein involved in SRP-dependent co-translational protein targeting to the cell membrane and Endoplasmic Reticulum (ER) and signal sequence recognition. GO terms associated with protein trafficking were enriched in gene lists extracted from the analysis of q-p transition from deep quiescence (see section 4.4.5.3 in this chapter), which could explain why Sec65 appears to be important either for
quiescence of for the q-p transition. Sec65 is relatively abundant during both vegetative growth and quiescence.\textsuperscript{86} Two physical interactions have been reported: with Hhp1, a serine/threonine protein kinase and Cid14, a poly(A) polymerase involved in RNA processing. Interestingly, Hhp1 was also found to physically interact with Tsc1, whose gene deletion mutant was found to undergo a fast q-p transition (table 4.1). However, given the extremely high number of physical interactions reported for Hhp1 in PomBase, this common feature might very well just be coincidental. \textit{sec65}\textsuperscript{Δ} exhibits normal cell morphology, although recently it was found that the deletion of sec65 leads to improper cytokinesis and DNA re-replication, contributing to diploidization or abnormal segregation in the mutants\textsuperscript{4}. Given that these phenotypes represent defects impairing the cell cycle or some of its stages it is possible they could be the basis of the slow q-p transition observed.

According to PomBase\textsuperscript{6}, 3 genes, \textit{pep3, sec65} and \textit{sft1}, function in protein trafficking and vesicle transport, also important processes upon the q-p transition (see GO Term Enrichment analysis of q-p transition from deep quiescence gene lists presented in section 4.4.5.3 of this chapter), which is not surprising given the great amount of de novo protein biosynthesis associated with this transition.\textsuperscript{25} \textit{pep3} and \textit{sft1} are involved in vacuole organization and vesicle-mediated transport respectively\textsuperscript{168}, which might reflect on one hand the importance of vacuoles and transport vesicles for crucial processes like autophagy and protein biosynthesis, during quiescence. On the other hand, a consequence from the intense metabolism and dynamics occurring at the q-p transition might render it very important to have a properly functioning vesicle-mediated transport system for the newly synthesized products to be correctly integrated at their respective destinations. \textit{SPCC126.01c}, a gene with no apparent orthologs for which very little is known has been reported to have a genetic interaction with \textit{sft1}.\textsuperscript{6}
According to PomBase one gene, *aut12*, is involved in autophagy, a process also found to be essential during quiescence. The problems caused by the deletion of this gene during quiescence could be the basis for its slow and/or defective q-p transition. Mon1, the budding yeast ortholog, is a guanine exchange factor and transmembrane protein in vacuoles involved in nucleotide recycling.\(^{153,162,163}\) Given that vacuolar metabolism is crucial during quiescence, it could likely be that *aut12* is essential during quiescence and therefore *aut12\(\Delta\)* cells were already dead after 2 days, upon q-p transition induction.

According to PomBase, *uge1* is involved in galactose catabolism.\(^{168}\) This might suggest that galactose metabolism and/or the corresponding pathway could be important upon this transition and/or during the quiescent state.

*SPAC1635.01* is involved in mitochondrial ion transport.\(^6\) *pos5*, another mitochondrial-target gene, relates with NADP biosynthesis.\(^6\) *SPBC106.07c*, involved in methionine acetylation, also codes for a mitochondrial product, making a total of 3 out of 20 listed genes associated with mitochondrial processes. In this view, it was recently found that quiescence-related autophagy is specially dependent on the autophagy of mitochondria (mitophagy) for proper cell functioning under quiescence.\(^{27}\) It is therefore necessary for the cell to re-synthesize de novo mitochondria to fuel the growth required for return to a proliferative state following a period in quiescence. One might thus speculate the possibility that mitochondrial-related events could be severely affected by the absence of this genes, which could lead to an impaired q-p transition or affected the cell’s ability to be quiescent.\(^6\)

Not surprising is also the inclusion of a RNA processing gene, *SPAC1F5.10*, given the high amount of RNA biosynthesis associated with the q-p transition.\(^{90,92}\)
The q-p transition is also associated with high levels of ribosome biogenesis\textsuperscript{83,90,92} so it is not surprising to find a ribosomal gene, *rpl502*, amongst this list. On the contrary, it could be considered rather surprising not to have found more ribosome metabolism related genes in this gene list.

According to PomBase\textsuperscript{6}, another gene, *pab1*, is involved in the regulation of septation initiation, also not unexpected given this is a process tightly associated with all the events occurring upon the q-p transition. Failure to properly undergo septation would likely give rise to a defective q-p transition.\textsuperscript{6}

*tadh1* possesses several roles involved in glycolysis, gluconeogenesis and stress response.\textsuperscript{6} Given the intense metabolism occurring during the q-p transition, it is not surprising to find that a gene involved in glycolysis can significantly impair this transition, specially considering the cell deficit of mitochondria due to the high levels of mitochondrial autophagy that occurs during quiescence. It could also be the case that being glycolysis important during quiescence that this mutant became impaired during quiescence and thus could not undergo q-p transition.\textsuperscript{6}

The q-p transition is characterized by a lag phase where the cells grow in size before engaging in proliferation.\textsuperscript{91} This growth in size is accompanied by the necessary de novo synthesis of new cell wall.\textsuperscript{25} We interpret the presence of *omh6* - a gene involved in cell wall biosynthesis - in this gene list to likely reflect the cell wall biosynthesis as an essential requirement upon the q-p transition.\textsuperscript{10,25}

In a similar view, *pac10*, a gene associated with cytoskeleton organization, might reflect the high and intense amount of cytoskeleton dynamics associated with a transition where the cell architecture is shifted rapidly and dramatically, such as it happens when the cell switches from a quiescent to a proliferative state.\textsuperscript{10,25}
Finally there is *swd1*. This is a gene that unlike most other genes present in this list, is not concerned with metabolism, biosynthesis or biogenesis processes. This gene encodes for a component of the Set1 complex (Set1C), involved in chromatin organization and regulation of gene expression.\textsuperscript{150} Set1C has been extensively investigated principally under the scope of epigenetics and chromatin architecture. Its best known role is to mono-, di- and tri-methylate the fourth lysine amino acid residue of the histone 3 tail (H3K4me1-2-3).\textsuperscript{104,151,152} These particular histone post-translational modifications are reversible, highly dynamic and strongly associated with active transcription. More concretely, its tri-methylation form is primarily found more abundantly in the promoter, 5’- and 3’-ends of coding regions, whilst the di-methylated form is more common across the open reading frames (ORFs) of gene bodies.\textsuperscript{104} These marks have been associated with developmental hereditary programs in multicellular organisms, cancer amongst others.\textsuperscript{104,151} The presence of the *swd1Δ* in the group of mutants exhibiting a slow q-p transition is particularly interesting, especially as it constitutes the only mutant depicting a gene expression regulatory gene (table 4.2). Under the scope of identifying factors regulating the dynamics of the q-p transition, this might represent a strong and exciting candidate to follow-up with further, more detailed studies.

### 4.4.5 Quiescence-proliferation transition after deep quiescence

We used a similar approach for the analysis of the q-p transition following a deep quiescence period of 10 days. We did not analyse the time-point $T_{390}$ for reasons explained before (see section 4.2 in this chapter). Also, the analysis of the time-point $T_{530}$ produced a set of results with little to no overlap with those from subsequent time-points. We attribute this to the fact that upon this time-point during the q-p transition there had not been yet time for enough cells to start dividing and allow the
detection of meaningful significant differences in their relative abundance. This justification is supported by the information on the overall pool cell density values where it is possible to conclude that little to no growth had occurred upon 530 minutes upon exit from 10 days in quiescence (figure 4.1).

Therefore, in the data analysis of the q-p transition after deep quiescence we selected the subsequent two time-points for the identification of fast or early q-p transition mutants (figure 4.3). Regarding the identification of slow or delayed q-p transition mutants, we focused on the two latest time-points, taken 890 and 980 minutes after the exit from quiescence (figure 4.4).

4.4.5.1 Fast or early q-p transition mutants

The data analysis of the bar-seq screen upon 10 days in quiescence identified 5 mutants as strong candidates for undergoing a fast q-p transition. The respective deleted genes are listed in table 4.4 and fig. 4.7.

Table 4.3 Gene list of fast or early q-p transition mutants after deep quiescence. GI - Genetic Interactions; PhI - Physical Interactions; TF - Transcription Factors. Description and Function were retrieved using the PomBase online resource. * info from downtag; ** info from uptag

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Common Name</th>
<th>Description</th>
<th>Function and other information</th>
<th>Doubling Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPBC1685.04</td>
<td>-</td>
<td>sequence orphan; no apparent orthologs</td>
<td>GI swd1, set1, prw1 (clr6 HDAC)</td>
<td>165.2</td>
</tr>
<tr>
<td>Strain</td>
<td>Gene</td>
<td>Protein Function</td>
<td>Cellular Response</td>
<td>Relative Abundance</td>
</tr>
<tr>
<td>-------------</td>
<td>--------</td>
<td>----------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>SPBC1711.08</td>
<td>-</td>
<td>Chaperone activator Aha1 (predicted)</td>
<td>Cellular response to stress, protein folding, PhI hsp90</td>
<td>92.3*</td>
</tr>
<tr>
<td>SPAC458.06</td>
<td>atg1803</td>
<td>WD repeat protein involved in autophagy Atg18c</td>
<td>Autophagy, cellular response to nitrogen starvation; GI ash2, cph1 (clr6 HDAC)</td>
<td>142.3</td>
</tr>
<tr>
<td>SPAC29B12.08</td>
<td>clr5</td>
<td>Clr5 protein</td>
<td>GI swi6, clr3, dcr1, ago1, clr4, Clr6C (pst2, cph1)</td>
<td>139.1</td>
</tr>
<tr>
<td>SPAC15A10.10</td>
<td>mde6</td>
<td>Muskelin homolog (predicted)</td>
<td>Meiotic chromosome segregation, regulated by mei4</td>
<td>112.5**</td>
</tr>
</tbody>
</table>

**Figure A**

A graph showing the relative abundances of different strains over time, with significant markers indicated at various time points.

**Notes:**
- * indicates significance at p < 0.05.
- ** indicates significance at p < 0.01.
- *** indicates significance at p < 0.001.
Figure 4.7 Quiescence-proliferation transition profiles of early or fast q-p transition mutants after deep quiescence. A) Quiescence-proliferation transition profiles, uptags. B) Quiescence-proliferation transition profiles, downtags. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

According to PomBase\textsuperscript{6}, \textit{SPBC1685.04} is a gene with no known function and no apparent orthologs. The information available regarding this gene is scarce. Interestingly, it shows genetic interactions with a gene whose deletion mutant was a delayed q-p transition hit from the screen upon short quiescence, \textit{swd1}, and another gene from the same complex, \textit{set1}, which shares a very high degree of homology with \textit{swd1}. It also interacts with a member of the Clr6 histone deacetylase (HDAC) complex.\textsuperscript{6}

According to PomBase\textsuperscript{6}, \textit{SPBC1711.08} codes for a chaperone activator involved in stress-response including heat shock. Moreover it physically associates with hsp90, a key protein in heat shock stress-response. This result might suggest an involvement of
*SPBC1711.08* in the response to quiescence following nitrogen starvation. It is also possible it could be involved in the response to nutrients, namely nitrogen. Both these hypotheses could be the basis for the altered (fastened) dynamics upon the q-p transition.6

According to PomBase6, the product of *atg1803* is interestingly more abundant during quiescence than during vegetative growth, which might suggest that this gene is more important during this cellular state. Actually, it was found that it plays a role in the cellular response to nitrogen starvation and autophagy.57,82 Given the fundamental role of autophagy during quiescence, especially after longer periods, one might speculate that the absence of this gene prevented the cells entering a deeper quiescent state, which could then have resulted in a faster q-p transition. Also, this gene displays genetic interactions with *ash2*, a member of the Set1C and Lid2 complex (Lid2C), and *cph1*, a component ofClr6C, all regulators of chromatin organization.6

Another gene, *clr5*, is also more abundant during quiescence, which might point to a more critical role during quiescence.6 Not much is known regarding *clr5*, especially as it is not a highly conserved gene. It has been found to play an important role in histone 3 lysine 9 methylation (H3K9me) independent gene silencing heterochromatin, together with several deacetylases.6 *clr5* genetically interacts with several regulators of chromatin organization, namely *clr3*, *clr4*, two members of the Clr6 complex, *swi6*, and two members from the RNAi-mediated heterochromatin formation pathway (*ago1* and *dcr1*).6 The absence of this gene might have led to the failing in silencing gene promoters of a full quiescent state, ultimately leading to the early or fast q-p transition suggested by the bar-seq screen.
Finally, the remaining gene mde6 has a role in meiotic chromosome segregation. It is regulated by the meiotic gene mei4 and interestingly it was not detected by brute force transcriptomic or proteomic approaches, suggesting that it might occur at very low levels. It shows genetic interactions with several transcription factors, such as toe4, msc2, sfp1 and prz1, suggestive of a regulatory role. Given that the meiotic program in fission yeast can, like quiescence, be induced by nitrogen depletion - thus exhibiting a relatively high degree of commonality with quiescence - it might be that this gene could be preventing the cells adopting or engaging into a fully quiescent state, eventually leading to its deletion mutant’s fast q-p transition.

4.4.5.2 Slow or delayed q-p transition mutants

45 mutants were found to be significantly under-represented and thus suggestive of a delayed q-p transition in the semi-conservative analysis, following a state of deep quiescence. The corresponding gene list is presented at table 4.4 and fig. 4.8.

Table 4.4 Gene list of slow or delayed q-p transition after deep quiescence. Description according to Pombase. GI - Genetic Interactions; PhI - Physical Interactions; TF - Transcription Factors. Description and Function were retrieved using the PomBase online resource.

<table>
<thead>
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<th>Common Name</th>
<th>Description</th>
<th>Function and other information</th>
<th>Doubling Time (min)</th>
</tr>
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<tr>
<td>SPAC1635.01</td>
<td>-</td>
<td>mitochondrial outer membrane voltage-dependent anion-selective channel (predicted)</td>
<td>Ion transport, mitochondrion organization, mitochondria location</td>
<td>203.8</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Description</td>
<td>Function</td>
<td>Meyer's Score</td>
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</tr>
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<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>---------------</td>
<td></td>
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<tr>
<td>SPAC1805.16c</td>
<td>purine nucleoside phosphorylase (predicted)</td>
<td>NAD biosynthesis via nicotinamide riboside salvage pathway, purine nucleoside catabolic process</td>
<td>174.1</td>
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<tr>
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<td>DUF1748 family protein</td>
<td></td>
<td>179.1</td>
<td></td>
</tr>
<tr>
<td>SPAC2F7.09c</td>
<td>mitochondrial GTPase related protein (predicted)</td>
<td></td>
<td>180.1</td>
<td></td>
</tr>
<tr>
<td>SPAC343.04c</td>
<td>WD repeat protein, human WDR26 family, ubiquitin ligase complex subunit (predicted)</td>
<td>Negative regulation of gluconeogenesis, proteasome-mediated ubiquitin-dependent protein catabolic process</td>
<td>158.8</td>
<td></td>
</tr>
<tr>
<td>SPAC3F10.09</td>
<td>1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase (predicted)</td>
<td>Histidine biosynthetic process</td>
<td>133.5</td>
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<td>SPBC106.13</td>
<td>ubiquitin ligase complex subunit, involved in proteasome-dependent catabolite inactivation of FBPase (predicted)</td>
<td>Proteasome-mediated ubiquitin-dependent protein catabolic process, negative regulation of gluconeogenesis</td>
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<td>SPBC25B2.03</td>
<td>zf-C3HC4 type zinc finger</td>
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<td>SPBC29A3.03c</td>
<td>ubiquitin-protein ligase E3 (predicted)</td>
<td>Inferred negative regulation of gluconeogenesis, protein ubiquitination</td>
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<td>156.7</td>
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<tr>
<td>SPBC2D10.04</td>
<td>arrestin Aly1 related, implicated in endocytosis</td>
<td>Signal transduction, ubiquitin-dependent endocytosis</td>
<td></td>
<td>142.6</td>
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<td>SPCC126.01c</td>
<td>conserved fungal protein</td>
<td>GI clr4, TF mca1, sft1 (hit std-cons)</td>
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<td>sequence orphan</td>
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<tr>
<td>SPCC594.06c</td>
<td>vacuolar SNARE Vam7 (predicted)</td>
<td>Golgi to vacuole transport, meiosis</td>
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<td>160.9</td>
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<td>Macroautophagy</td>
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<td>SPAC7D4.04</td>
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<td>Macroautophagy, Cvt pathway</td>
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<td>SPAC25A8.02</td>
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<td>Macroautophagy, Cvt pathway</td>
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<td>CVT pathway, macroautophagy, meiosis</td>
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<td>162.3</td>
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<td>WD repeat protein involved in autophagy Atg18a</td>
<td>Autophagy, cellular response to nitrogen starvation, macroautophagy</td>
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<td>195</td>
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<td>Function and Pathways</td>
<td>Score</td>
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<tr>
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<td>atg2</td>
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<td>SPAC1D4.03c</td>
<td>aut12 (predicted)</td>
<td>Autophagy, protein targeting to vacuole</td>
<td>155.6</td>
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</tr>
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<td>SPBC215.03c</td>
<td>csn1</td>
<td>COP9/signalosome complex subunit Csn1, Cellular response to DNA damage stimulus, regulation of DNA-dependent DNA replication, regulation of meiotic cell cycle, protein catabolic process</td>
<td>199.1</td>
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<td>SPAC688.11</td>
<td>end4</td>
<td>Huntingtin-interacting protein homolog, Required for G1 to G0 transition during nitrogen starvation, actin cytoskeleton organization, cellular response to nitrogen starvation, endocytosis</td>
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<td>SPAC1952.05</td>
<td>gcn5</td>
<td>SAGA complex histone acetyltransferase catalytic subunit Gcn5, Chromatin remodelling through H3 acetylation</td>
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<td>gsa1</td>
<td>Glutathione synthetase large subunit Gsa1</td>
<td>-1007.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutathione biosynthetic process, cellular response to cadmium ion, glutathione transport</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPAC6G10.08</td>
<td>idp1</td>
<td>Isocitrate dehydrogenase Idp1 (predicted)</td>
<td>170.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutamate biosynthetic process, NADPH regeneration, isocitrate metabolic process</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPBC19C7.01</td>
<td>mni1</td>
<td>Mago Nashi interacting protein Mni1 (predicted)</td>
<td>172.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mRNA cis splicing, via spliceosome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPAC23C11.10</td>
<td>mpn1</td>
<td>RNA processing protein (predicted)</td>
<td>175.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNA splicing, U6 snRNA 3'-end processing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPBC146.11c</td>
<td>mug97</td>
<td>Meiotically upregulated gene Mug97</td>
<td>149.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ascospore formation, meiotic chromosome segregation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPAC1805.04</td>
<td>nup132</td>
<td>Nucleoporin Nup132</td>
<td>182.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell division, nuclear pore distribution, poly(A)+ mRNA export</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPAC227.07c</td>
<td>pab1</td>
<td>Protein phosphatase regulatory subunit Pab1</td>
<td>151.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative regulation of GTPase activity, negative regulation of septation initiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPAC1A6.04c</td>
<td>plb1</td>
<td>Phospholipase B homolog Plb1</td>
<td>159.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cellular response to nutrient, cellular response to osmotic stress, glycerophospholipid catabolic process</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Protein Name</td>
<td>Description</td>
<td>Gene Length</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>SPAC19A8.10</td>
<td>rfp1</td>
<td>SUMO-targeted ubiquitin-protein ligase subunit Rfp1</td>
<td>187.1</td>
<td></td>
</tr>
<tr>
<td>SPBC1685.10</td>
<td>rps27</td>
<td>40S ribosomal protein S27 (predicted)</td>
<td>165.4</td>
<td></td>
</tr>
<tr>
<td>SPBP4H10.09</td>
<td>rsv1</td>
<td>transcription factor Rsv1</td>
<td>166.6</td>
<td></td>
</tr>
<tr>
<td>SPCC126.15c</td>
<td>sec65</td>
<td>signal recognition particle subunit Sec65 (predicted)</td>
<td>154.3</td>
<td></td>
</tr>
<tr>
<td>SPAC16E8.01</td>
<td>shd1</td>
<td>cytoskeletal protein binding protein Sla1 family, Shd1 (predicted)</td>
<td>156.8</td>
<td></td>
</tr>
<tr>
<td>SPAC1071.04c</td>
<td>spc2</td>
<td>signal peptidase subunit Spc2 (predicted)</td>
<td>169.3</td>
<td></td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Description</td>
<td>Function</td>
<td>Value</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>SPAC20G4.07c</td>
<td>sts1 C-24(28) sterol reductase Sts1</td>
<td>Cellular response to cation and osmotic stress, endocytosis, establishment of protein localization to plasma membrane, ergosterol biosynthetic process</td>
<td>168.2</td>
<td></td>
</tr>
<tr>
<td>SPAC23H3.05c</td>
<td>swd1 Set1C complex subunit Swd1</td>
<td>Histone methylation, chromatin remodelling</td>
<td>175.5</td>
<td></td>
</tr>
<tr>
<td>SPAC23H4.10c</td>
<td>thi4 bifunctional thiamine-phosphate diphosphorylase/hydroxyethylthiazole kinase</td>
<td>Thiamine biosynthetic process</td>
<td>615.7</td>
<td></td>
</tr>
</tbody>
</table>

**A1**

![Graph showing relative abundances over time](image-url)
**Figure 4.8 Quiescence-proliferation transition profiles of slow or delayed q-p transition mutants after deep quiescence.**


*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001.

Interestingly, *SPBC2D10.04Δ* and *idpΔ* showed statistical significance upon all time-points analysed (fig 4.8A1, fig. 4.8B1).

According to PomBase, *SPBC2D10.04* is believed to be a calcium ion binding gene and involved in signal transduction and ubiquitin-dependent endocytosis (Pombase). *SPBC2D10.04Δ* exhibits normal cell morphology. It is eight-fold more abundant during quiescence than vegetative growth, which likely indicates a more essential role in quiescence. Its budding yeast ortholog controls nutrient-mediated intracellular
sorting of permease Gap1p and may regulate endocytosis of plasma membrane proteins. It physically interacts with epe1, which is essential for stabilising silent chromatin, in a way not dependent on the RNAi pathway. It also shows genetic interactions with the stress response MAP kinase gene sty1, two Clr6C genes (pst2 and prw1), the gene coding for histone promoter control protein Hip4 and mug80, which codes for cyclin Clg1. There is not much more information available on this gene in order to be able to produce any likely explanation for why it could be exhibiting a delayed or impaired q-p transition.⁶

The other highlighted gene of this analysis is idp1, a predicted isocitrate dehydrogenase.⁶ According to PomBase it codes for a highly abundant mitochondrial product, both during quiescence and proliferation. idpΔ shows a viable and normal cell morphology. The corresponding budding yeast orthologue catalyzes the oxidation of isocitrate to alpha-ketoglutarate. It is not required for mitochondrial respiration and may function to divert alpha-ketoglutarate to biosynthetic processes. Idp1 has been recently found to physical interact with Epe1, mentioned before regarding the other gene on this list, and Bdf2, involved in transcription initiation at TATA-containing promoters and key for Epe1 function of establishing heterochromatin boundaries. Regarding genetic interactions, from the ~70 identified, we would highlight the sty1, SWI/SNF complex subunit snf5, SAGA complex gcn5 and ngg1, Clr6C cph2, nap1 involved in nucleosome assembly, transcription factor (TF) gene prz1 and protein kinase gene pom1, histone deacetylase complex subunit gene rxt2.⁶ The apparent delayed q-p transition might suggest the importance of this gene during quiescence or throughout the transition to proliferation.
4.4.5.3 Gene Ontology Term Enrichment Analysis of the gene list of slow or delayed q-p transition mutants after deep quiescence.

GO Term Enrichment analysis is a potent tool for the interpretation of systematic and global studies.\textsuperscript{144} Therefore, it should prove of great value towards yielding highly pertinent information regarding the big picture of what is occurring during the q-p transition and what cellular processes and components could be of critical importance upon said transition. As mentioned previously in the corresponding sections, no GO term was found enriched for the other gene lists obtained, in respect to the q-p transition after a period of short quiescence, and after deep quiescence regarding early q-p transition mutants. This was likely due to the small size of the gene lists obtained, a possibility that becomes reinforced when compared to the positive GO Term Enrichment results obtained for this larger gene list.

After performing a Gene Ontology (GO) Term Enrichment analysis of the gene list of the deep quiescence statistically significantly delayed q-p transition mutants, several biological processes (table 4.7), as well as cellular components, (table 4.8) were found to be significantly enriched. The employed GO analysis thus strongly points to an important and relevant role of these biological processes upon the q-p transition (at least) from a state of deep quiescence.
Table 4.5 List of the biological processes uncovered by GO Term Enrichment analysis performed with the gene list from the slow or delayed q-p transition mutants after deep quiescence. This data analysis referred to the gene list of slow or delayed q-p transition mutants after deep quiescence. GO Term represents the Gene Ontology Term associated with the biological process identified by the GO Term Enrichment analysis. Sample Frequency depicts the number of genes associated with the GO Term over the total amount of genes in the slow or delayed q-p transition mutants gene list. Background Frequency depicts the total number of genes associated with the GO Term over the total number of genes covered in the analysis.

<table>
<thead>
<tr>
<th>GO Term</th>
<th>p-value</th>
<th>Sample Frequency</th>
<th>Background Frequency</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006914 autophagy</td>
<td>1.40E-07</td>
<td>9/45 (20.0%)</td>
<td>26/2610 (1.0%)</td>
<td>atg11 atg16 atg2 aut12 atg1801 atg9 fsc1 atg14 atg10</td>
</tr>
<tr>
<td>GO:0016236 macroautophagy</td>
<td>2.57E-07</td>
<td>8/45 (17.8%)</td>
<td>19/2610 (0.7%)</td>
<td>atg11 atg16 atg2 atg1801 atg9 fsc1 atg14 atg10</td>
</tr>
<tr>
<td>GO:0071496 cellular response to external stimulus</td>
<td>2.83E-06</td>
<td>11/45 (24.4%)</td>
<td>62/2610 (2.4%)</td>
<td>atg11 atg16 plb1 atg2 atg1801 end4 rsv1 atg9 fsc1 atg14 atg10</td>
</tr>
<tr>
<td>GO:0031668 cellular response to extracellular stimulus</td>
<td>2.83E-06</td>
<td>11/45 (24.4%)</td>
<td>62/2610 (2.4%)</td>
<td>atg11 atg16 plb1 atg2 atg1801 end4 rsv1 atg9 fsc1 atg14 atg10</td>
</tr>
<tr>
<td>GO:0031667</td>
<td>response to nutrient levels</td>
<td>2.83E-06</td>
<td>11/45 (24.4%)</td>
<td>62/2610 (2.4%)</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------------------</td>
<td>----------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>GO:0031669</td>
<td>cellular response to nutrient levels</td>
<td>2.83E-06</td>
<td>11/45 (24.4%)</td>
<td>62/2610 (2.4%)</td>
</tr>
<tr>
<td>GO:0009605</td>
<td>response to external stimulus</td>
<td>2.83E-06</td>
<td>11/45 (24.4%)</td>
<td>62/2610 (2.4%)</td>
</tr>
<tr>
<td>GO:0009991</td>
<td>response to extracellular stimulus</td>
<td>2.83E-06</td>
<td>11/45 (24.4%)</td>
<td>62/2610 (2.4%)</td>
</tr>
<tr>
<td>GO:0042594</td>
<td>response to starvation</td>
<td>4.82E-06</td>
<td>10/45 (22.2%)</td>
<td>50/2610 (1.9%)</td>
</tr>
<tr>
<td>GO:0009267</td>
<td>cellular response to starvation</td>
<td>4.82E-06</td>
<td>10/45 (22.2%)</td>
<td>50/2610 (1.9%)</td>
</tr>
<tr>
<td>GO:0072594</td>
<td>establishment of protein localization to organelle</td>
<td>3.72E-04</td>
<td>9/45 (20.0%)</td>
<td>59/2610 (2.3%)</td>
</tr>
<tr>
<td>GO:0072665</td>
<td>4.05E-04</td>
<td>7/45 (15.6%)</td>
<td>30/2610 (1.1%)</td>
<td>atg11 atg16 atg2 aut12 atg1801 atg9 atg14</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------</td>
<td>--------------</td>
<td>----------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>protein localization to vacuole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0006623</td>
<td>4.05E-04</td>
<td>7/45 (15.6%)</td>
<td>30/2610 (1.1%)</td>
<td>atg11 atg16 atg2 aut12 atg1801 atg9 atg14</td>
</tr>
<tr>
<td>protein targeting to vacuole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0072666</td>
<td>4.05E-04</td>
<td>7/45 (15.6%)</td>
<td>30/2610 (1.1%)</td>
<td>atg11 atg16 atg2 aut12 atg1801 atg9 atg14</td>
</tr>
<tr>
<td>establishment of protein localization to vacuole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0032258</td>
<td>8.43E-04</td>
<td>5/45 (11.1%)</td>
<td>12/2610 (0.5%)</td>
<td>atg11 atg16 atg2 atg9 atg14</td>
</tr>
<tr>
<td>CVT pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0006605</td>
<td>1.00E-03</td>
<td>9/45 (20.0%)</td>
<td>66/2610 (2.5%)</td>
<td>atg11 atg16 spc2 sec65 atg2 aut12 atg1801 atg9 atg14</td>
</tr>
<tr>
<td>protein targeting</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0045912</td>
<td>4.92E-03</td>
<td>4/45 (8.9%)</td>
<td>8/2610 (0.3%)</td>
<td>SPBC106.13 SPAC343.04c rsv1 SPBC29A3.03c</td>
</tr>
<tr>
<td>negative regulation of carbohydrate metabolic process</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6 List of the cellular components uncovered by GO Term Enrichment analysis performed with the gene list from the slow or delayed q-p transition mutants after deep quiescence. This data analysis referred to the gene list of slow or delayed q-p transition mutants after deep quiescence. GO Term represents the Gene Ontology Term associated with the cellular component identified by the GO Term Enrichment analysis. Sample Frequency depicts the number of genes associated with the GO Term over the total amount of genes in the slow or delayed q-p transition mutants gene list. Background Frequency depicts the total number of genes associated with the GO Term over the total number of genes covered in the analysis.

<table>
<thead>
<tr>
<th>GO Term</th>
<th>p-value</th>
<th>Sample Frequency</th>
<th>Background Frequency</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0010677 negative regulation of cellular carbohydrate metabolic process</td>
<td>4.92E-03</td>
<td>4/45 (8.9%)</td>
<td>8/2610 (0.3%)</td>
<td>SPBC106.13 SPAC343.04c rsv1 SPBC29A3.03c</td>
</tr>
<tr>
<td>GO:0007034 vacuolar transport</td>
<td>5.15E-03</td>
<td>8/45 (17.8%)</td>
<td>60/2610 (2.3%)</td>
<td>atg11 atg16 atg2 aut12 atg1801 atg9 SPCC594.06c atg14</td>
</tr>
<tr>
<td>GO:0034045 pre-autophagosomal structure membrane</td>
<td>4.58E-06</td>
<td>7/45 (15.6%)</td>
<td>17/2610 (0.7%)</td>
<td>atg11 atg16 atg2 atg1801 atg9 atg14 atg10</td>
</tr>
<tr>
<td>GO:0000407</td>
<td>pre-autophagosomal structure</td>
<td>7.40E-06</td>
<td>7/45 (15.6%)</td>
<td>18/2610 (0.7%)</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------</td>
<td>----------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>GO:0000329</td>
<td>fungal-type vacuole membrane</td>
<td>8.31E-05</td>
<td>9/45 (20.0%)</td>
<td>50/2610 (1.9%)</td>
</tr>
<tr>
<td>GO:0005774</td>
<td>vacuolar membrane</td>
<td>1.68E-04</td>
<td>9/45 (20.0%)</td>
<td>54/2610 (2.1%)</td>
</tr>
<tr>
<td>GO:0044437</td>
<td>vacuolar part</td>
<td>2.33E-04</td>
<td>9/45 (20.0%)</td>
<td>56/2610 (2.1%)</td>
</tr>
<tr>
<td>GO:0000323</td>
<td>lytic vacuole</td>
<td>4.24E-03</td>
<td>9/45 (20.0%)</td>
<td>78/2610 (3.0%)</td>
</tr>
<tr>
<td>GO:0000322</td>
<td>storage vacuole</td>
<td>4.24E-03</td>
<td>9/45 (20.0%)</td>
<td>78/2610 (3.0%)</td>
</tr>
<tr>
<td>GO:0000324</td>
<td>fungal-type vacuole</td>
<td>4.24E-03</td>
<td>9/45 (20.0%)</td>
<td>78/2610 (3.0%)</td>
</tr>
</tbody>
</table>

This GO analysis allowed the identification of a clear trend amongst all of the highlighted biological processes, for two main reasons. The first is that most, if not all of the GO analysis results rely on the same or similar sets of genes, which underline a
functional connection existent amongst them, namely autophagic and vacuolar processes (Table 4.7). Secondly, the nature of the GO Terms themselves reveals a high degree of convergence towards nutrient-response and external stimulus-response processes. Interestingly, autophagic, vacuolar and transport processes have already been found to be intrinsically associated with nutrient-induced quiescence.\textsuperscript{10,65-72} The enriched nutrient-response associated GO Terms could probably be interpreted given the fact that q-p transition is induced by nitrogen source replenishment, as nitrogen constitutes an essential nutrient.

The fact that all of the enriched GO Terms are consistent in the light of current knowledge regarding cellular quiescence reinforces the validity and sensitivity of the bar-seq approach for performing this analysis of the q-p transition.

- **Autophagy**

The biological processes more strongly emphasized in the GO analysis of this particular q-p transition from a state of deep quiescence are those of autophagy (p-value 1.4E\textsuperscript{-7}) and macroautophagy (p-value 2.57E\textsuperscript{-7}). Autophagy is the core catabolic process whereby the cells degrade and recycle their own constituents and macromolecules, namely proteins, organelles, cytoplasmic portions and even DNA, amongst others, in the vacuole or lysosome.\textsuperscript{10,65-72} Macroautophagy refers to the autophagy main degradation pathway, which is responsible for the degradation processes of damaged cell parts, such as organelles, or unused proteins.\textsuperscript{53,153}

Autophagy has long been shown to be a general response of the cells to nutrient deprivation having then been found to actually be indispensable for protein degradation in the vacuoles under starvation conditions and thus vital for cell survival during nutrient limitation induced quiescence.\textsuperscript{10,65-72}
Also, it was relatively recently found that autophagy, especially mitophagy, plays a crucial role during quiescence. Autophagy becomes a necessary requirement for the cells to be able to sustain large periods in a quiescent state induced by nutrient limitation or starvation. This is thought to be mainly because it allows the recycling of internal sources of biological substrates like amino acids and also importantly the diminishment of ROS (through the elimination of mitochondria, a major source of ROS) that would otherwise reach toxic levels over time throughout the quiescence period.

Therefore, it is not surprising to find the process of autophagy strongly represented in the gene list of mutants exhibiting a delayed q-p transition.

Interestingly, no proteasome genes or associated GO enrichment was detected in this study, given that the proteosome degradation pathway has been reported to be required during quiescence in autophagy deficient mutants. This could be due to the fact with the exception of the autophagy gene deletion mutants, autophagy was not impaired in mutants of the proteosome system.

These results strongly suggest that these processes are of crucial importance for undergoing a normal q-p transition. However, one should always consider that any of these defective q-p transitions might arise because of the difficulties previously suffered by these autophagic mutants during the preceding period in quiescence. It is thus extremely important during this stage of the research to always have that consideration in mind. Consequently, one cannot exclude from this analysis though, the fact that any given result might also have some basis on eventual problems that the corresponding mutant might have had during or entering the quiescence state. For this it would be highly useful to analyze the mutant pool constitution before and
after the quiescence period, which will constitute part of the follow-up studies deriving from these studies.

4.4.6 Comparison of the distribution of overall quiescence-proliferation transition profiles after short and deep quiescence

This is probably as a result of a longer period in quiescence, which causes the differences between mutants to become more pronounced as they enter states of deeper quiescence. This is likely the result of sensitive mutants becoming sicker and sensitive with time, i.e., decreasing in viability or “health” as time goes by, or as a result of aging. Some mutants might exhibit a faster q-p transition due to their genotype being more advantageous during quiescence, conferring a greater fitness during quiescence that would then put them in a better status for when eventually the transition occurs, possibly even enhancing the kinetics of such transition. It might be that a state of deeper quiescence naturally enhances the intrinsic kinetic differences existent within the collection of mutants upon the q-p transition.

4.4.7 Compilation of all the results obtained in this functional profiling analysis

We compiled all the significant and non-significant results with the aim of making the information gathered in this profiling study available for future reference and work. We believe that it should constitute a great resource of valuable information on the quiescence-proliferation transition of more than 2,100 gene deletion mutants. The information obtained in this study could significantly complement and add to the existing information regarding the mutants, and corresponding deleted genes, analyzed in this work. This information is available in section 5 in the Appendices.
We also tried the mathematical model used in chapters 3 and 6, but there was not enough temporal resolution in the data to allow for a good performance (we only had 4-5 time-points). Nevertheless, there were some results with interesting potential for further investigation (see Appendices).

Note: The analysis of reproducibility for the bar-seq assay employed and whose results are presented in this chapter is described in section 2.3.6.2 in the Methodology chapter.
5 Parallel growth fitness profiling of haploid deletion strains
5.1 Overview

The main scope of this project concerns the functional profiling of the transition occurring when cells enter proliferation from a quiescent state, and the identification of factors regulating the kinetics of this transition. We decided to employ our fission yeast deletion library for that effect. Since this was a resource only relatively recently available to our laboratory, not much information had yet been gathered. A general profiling study of the deletion mutant collection developed by Bioneer had been published recently that showed that a certain degree of variability and unpredictability should be expected in the library behaviour. Moreover, in the profiling of their own Bioneer fission yeast deletion library, Han et al. also reported some discrepancy between the data they obtained and the information provided by Bioneer. This was likely the result of some degree of error associated with the evaluation of the deletion library made by Bioneer or with the down-stream processing events taking place after the library had been acquired. This means that when using the profiling data published by another group, this data will need to be considered with great care, as one cannot anticipate the extent and specifics of the variation between our collection and the one used for the published study, even though theoretically they should be identical. For all these reasons, we decided to profile our own deletion library together with an independent decoding of each mutant’s individual tags and respective flanking regions (see section 2.2.6 in Methodology chapter). Also, there had not been any previous profiling of our own deletion library whilst growing in a pooled competitive environment, i.e. where all mutants were sharing the same liquid culture media, so this was also a valuable opportunity for doing so. This would then allow us to more accurately interpret the results obtained with our own experimental work when using the deletion library or
parts of it. Moreover, it could then become a valuable resource for anyone using the library (or parts of it) in the future, as the information on each mutant’s growth rate would be available for immediate access. Finally, The findings and work presented in this chapter have ultimately led to a recent publication.¹⁷⁰

For the success of our aim to identifying factors regulating and affecting the dynamics of the q-p transition, it was important to identify and distinguish any mutants displaying differential kinetics solely as an indirect effect of any growth-related differences from those with a specific effect upon this transition. For this reason, we decided to analyse the mutant pool upon several cycles of growth in minimal media (the same used in the quiescence-to-proliferation screen), which allowed us the acquisition of valuable information regarding the growth rate of the mutants present in our bar-seq pools used for the quiescence-to-proliferation screen. This information allowed us to correct for such differences when analyzing our quiescence-to-proliferation experiments presented in chapter 4.

**5.2 Experimental design**

The general rationale for this mutant growth profiling in EMM media was that, whilst maintaining the exactly same experimental conditions over time, and given that the media contained everything for fission yeast cells to grow, any changes in the relative abundances of deletion mutants in the mutant pool would relate to intrinsic differences in their growth rates. A mutant whose relative abundance increases over time would reflect a fast grower, whilst a mutant gradually fading from the pool would indicate a slow grower. We designed this experimental profiling placing the sampling
step within the exponential phase of fission yeast growth, to analyze mutants at their optimal or maximal growth rates, which minimized associated variation that would be more difficult to control at sub-optimal conditions.

We set up a deletion library pool culture in EMM+N liquid media as described in section 2.3.1 in the Methodology chapter. Once the mutant pool had been growing for several cycles and fully adjusted to the experimental conditions, we started our sampling process.

To estimate the growth rate of a cell culture the analysis of 2 spaced time-points suffices. These would also suffice for profiling our deletion library, provided we had the information on the relative abundance of the mutant pool at the sampled time-points.

Nevertheless, in the growth profiling assay presented in this chapter, we sampled the mutant pool regularly over time for several growth cycles to maximize the quality and sensitivity of the analysis and to decrease the effect of intrinsic sources of error and variation.

Information on the relative abundance of the deletion mutants in the mutant pool was used to determine their respective growth rates, given that we monitored the overall pool density through \( \text{OD}_{600} \) and cellular concentration. Also, in order to account for intrinsic technical variance, we performed the experiment with two independent biological replicates, which has been found sufficient for a robust analysis of deep sequencing data with the cutting-edge bioinformatics tools developed for that effect.\(^{143} \)
Information on the relative abundance of each deletion mutant present in the pool was obtained through bar-seq, as described hereafter and in the dedicated Methodology section (see section 2.3 in the Methodology chapter).

An overall visual representation of the experimental design is illustrated in figure 2.12 and 2.13 in the Methodology chapter section dedicated to the detailed description of the bar-seq assay. The time-course of the experimental assay performed for this profiling study and the time-points the mutant pool was sampled for downstream analysis are depicted in figure 5.1.
**Table C**

<table>
<thead>
<tr>
<th></th>
<th>Pool A</th>
<th>Pool B</th>
<th>Average $P_A + P_B$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R²</strong></td>
<td>0.999</td>
<td>0.999</td>
<td>-</td>
</tr>
<tr>
<td><strong>Doubling Time (min)</strong></td>
<td>153.8 (144.0 to 165.0)</td>
<td>154.2 (150.4 to 158.2)</td>
<td>154</td>
</tr>
<tr>
<td><strong>OD_{600}</strong></td>
<td>0.996</td>
<td>0.996</td>
<td>-</td>
</tr>
<tr>
<td><strong>Doubling Time (min)</strong></td>
<td>154 (136.6 to 176.5)</td>
<td>151.3 (133.6 to 174.5)</td>
<td>152.7</td>
</tr>
<tr>
<td><strong>Average DT (min)</strong></td>
<td>153.9</td>
<td>152.8</td>
<td>153.4</td>
</tr>
</tbody>
</table>
**Figure 5.1 Representation of the mutant pools’ overall culture density over time.**

Samples were taken regularly over time within the mutant pools’ exponential phase of growth. **A)** Linear representation. **B)** Logarithmic representation. Left axis depicts total number of cells per ml. Right axis depicts optical density \( \text{OD}_{600} \). Red arrows depict the time-points selected for sampling the mutant pools. Blue lines depict data for Pool A. Red lines depict data for Pool B. \( \text{OD - OD}_{600} \) values. cc - coulter-counter particle counts (representing cells/ml). **C)** Pool cultures’ kinetic information as extracted with regression analysis depicted in B). DT - doubling time. \( P_A \) - Pool A. \( P_B \) - Pool B. cc - Coulter-counter. Pool A and Pool B represent mutant pool (from pooling the deletion library in a single cell culture) biological replicates A and B, respectively.

To visually confirm the pool was in the exponential phase of growth at the sampled time-points, we plotted the data with the cell density values displayed on a logarithmic scale. We then statistically tested this by performing a non-linear regression and exponential fit to the data (figure 5.1B). We obtained solid regression stats clearly demonstrating the exponential nature of the data, particularly the strong and robust \( R^2 \) scores. The marked linearity present in the data thus confirmed that all samples had been collected within the exponential phase of growth.

In addition, we used the regression analyses employed to extract the growth rate and respective doubling times of the mutant pools analyzed (figure 5.1B and 5.1C). As no previous profiling had been performed on the haploid prototroph mutant pools, these data provided novel and valuable information for using and interpreting results obtained with this valuable resource. This was accomplished following the interpretation and analysis described in section 2.4.7 in the Methodology chapter. By averaging all the values together, as obtained from \( \text{OD}_{600} \) and Coulter-counter, we concluded that the average doubling time of the mutant pool in liquid minimal media to be \( \sim 153.4 \) minutes (figure 5.1C). Interestingly, and perhaps contrary to what would
be expected given the intrinsic sub-optimal condition of a deletion mutant (usually translated to a slower growth rate), this value was remarkably close to that known for the standard fission yeast wild-type Lab strain, which doubles every ~150 min (2.5 hours) under the same experimental conditions (32°C, 180 rpm, see section 2.1.2 in the Methodology chapter). Given that we measured the overall, average kinetics of all deletion mutants in the pool, one could speculate that the similarity observed could be a reflection of the natural intrinsic balance of a genome’s genetic constitution, in which some genes work towards, whilst other contrary to, a certain functional aim or purpose, which in this case would be or could be reflected upon promoting or inhibiting cellular growth. Another possible explanation could be that the competitive environment present in the mutant pool eventually leads to a certain rearrangement or optimization of growth where some mutants would benefit from the slower growth of other mutants to boost their own growth kinetics. In the same angle, it is known that cheaters can arise even in clonal populations of microbial organisms including yeast.\textsuperscript{126,128-130} Cheaters - individuals in a population that do not engage in tasks required for achieving a common goal, but still retrieve the associated benefits - have been reported to be exceptionally impactful regarding the setting of the overall mutant pool’s growth kinetics, especially when originating from deletions in key pathways such as energy metabolism. Given that we are profiling a mutant pool constituted exclusively by deletion mutant cells, it is plausible that the insurgence of cheaters within the mutant pool could be the basis of such competitive overall growth rates. This question could possibly be addressed by inspecting the growth rates of such putative cheater mutants, like mutants lacking important genes regarding biosynthetic processes, in the mutant pool. A comparative analysis with the growth rates exhibited in respective single-mutant cultures might provide valuable information regarding the possible extent of cheating dynamically occurring in a
competitive environment such as a mutant pool. This would be of special importance when interpreting results arising from mutants for genes that were found to lead to a high degree of cheating within a cell population.

Finally, the statistical analysis employed allowed us to conclude that no significant differences were observed between OD\textsubscript{600} and coulter-counter data, reinforcing the robustness of these methods for assessing cellular density levels in liquid cultures of fission yeast cells (figure 5.1C).

### 5.3 De novo decoding analysis

As explained before, we performed a de novo decoding analysis of our prototroph deletion library (refer to section 2.2.6 in the Methodology chapter).

We managed to identify barcodes for 2473 deletion mutants. Amongst them, 1871 mutant strains had both uptag and downtag decoded. 254 mutants had only uptag decoded and 348 mutants had only downtag decoded. Detailed information regarding the decoding process is available at section 5 in the Appendices section. The number of detected barcodes was relatively lower than that reported by Han et al. (~2,800 mutants identified), which might be explained by the fact that we analysed a prototroph deletion library as opposed to the auxotroph library (provided by Bioneer by default) analysed by Han et al.\textsuperscript{37} As mentioned before in section 2.1.2, making the prototroph library lead to the loss of some mutants whose number might have been underestimated.
In conclusion, the results from decoding our prototroph deletion library indicated that its featured analytical screening capacity, when undergoing fitness profiling studies through bar-seq, comprises a magnitude of ~2,500 mutant strains, covering roughly half the genome of *S. pombe*. (>60% of total of ~4,000 non-essential genes).\(^6,7\)

The information acquired through the decoding process of the prototroph deletion library can be assessed in the Lab’s archives (please address the Bähler Lab at www.bahlerlab.info/).

### 5.4 Overall analysis of the prototroph haploid deletion library

With the bar-seq data obtained for the profiling studies presented in this chapter we also employed an overall interpretation of the mutant pool.

In total, 2566 deletion mutants were identified in this study. This value was highly comparable to the number of strains detected in the de novo decoding presented in the previous section (2473 deletion mutants identified). The fact that it was even higher than the ‘reference’ data source, although counter-intuitive at first interpretation, could simply represent the fact that some mutants might be, likely due to technical limitations or error, greatly under-represented in the original mutant pool starting sample to an extent that could render them undetectable through deep sequencing. In our profiling studies, however, the mutant pool is grown, which affords to any lowly abundant mutants the possibility to increase their representation in the mutant pool, provided that their growth rates are higher than that of the pool mean. This could be the basis for the greater number of mutants detected after the profiling studies than that using an unprocessed original mutant pool sample.
5.5 Mutant profiling data analysis

After collecting the samples at the designated time-points (figure 5.1), we proceeded to bar-seq library preparation, as described in section 2.3 in the Methodology chapter. Samples were then deep sequenced using an Illumina MiSeq platform. Upon the retrieval of the sequencing raw data, we converted it into biologically meaningful count data, following a custom pipeline, as described in section 2.4 in the Methodology chapter. Bar-seq count data constituted a highly sensitive measure of the relative abundance of each mutant detected in the analyzed mutant pools.

As explained in chapter 4, we used Bioconductor package DESeq\textsuperscript{143} to analyse the bar-seq count data obtained in our profiling studies. Most significant results obtained in the growth profiling of our deletion library are presented next in this chapter.

We regarded the first sampled time-point ($T_{120}$; see figure 5.1) as our time zero or reference sample. The other 4 time-points sampled ($T_{230}$, $T_{340}$, $T_{460}$ and $T_{550}$; figure 5.1) were used for profiling the mutant pool through multi-pairwise comparative analysis, as described in section 2.4 in the Methodology chapter.

In order to integrate the results obtained for all time-points, we decided to employ two approaches with different levels of stringency - a more stringent conservative one and another one less stringent or semi-conservative -, as done for the q-p transition profiling presented in chapter 4. In the conservative approach (presented in sections 5.5.1.1 and 5.5.2.1 regarding the analysis of fast and slow growing mutants, respectively) we identified only those mutants that were significantly called by DESeq at all time-points, whilst in the semi-conservative approach (presented in sections 5.5.1.2 and 5.5.2.2 regarding the analysis of fast and slow growing mutants, respectively) we included all the mutants significantly altered in at least one time-
point. Results had to be statistically significant at both up-tag and down-tag analysis. For both methods we employed a false discovery rate (FDR) set at 0.1, the common maximum threshold value for this type of NGS data analysis considering the number of biological replicates.\textsuperscript{153}

The first analysed time-point, T\textsubscript{230}, was taken 110 min after the reference, T\textsubscript{120}, a period shorter than the pool’s doubling time (~153 min). This could have limited the scope of the analysis employed for the respective data set, which in turn could have been the basis for the smaller set of mutants identified (as exhibiting significant changes in their relative abundance), when compared with the corresponding numbers obtained for the other time-points (except T\textsubscript{460}). The size of the gene list obtained for T\textsubscript{230} sized about a third of those from other time-points. The limited size of the results obtained for T\textsubscript{230} could in turn have lead to the relatively short gene list obtained in this conservative analysis.

In this view, it was possible to observe that the gene lists obtained for later time-points were larger than those obtained for the earlier ones. Moreover and as expected, changes in relative abundance exhibited by the mutants became gradually more pronounced over time. This also reflected the need for some time in order for the differences to become noticeable. We believe that 430 min (the difference between T\textsubscript{550} and T\textsubscript{120}) constituted an adequate amount of time for realizing a global fitness profiling study by a large margin, covering almost 3 complete fission yeast division cycles (2.9 rounds).

As explained in the respective section, the conservative analysis only addressed those mutants commonly found significantly over- or under-represented at all time-points. In the semi-conservative analysis however, we included all mutants found to exhibit
significant changes in relative abundance in at least one of the time-points analysed for at least one the tags. Thus, as expected by its lower stringency, the lists of genes obtained were markedly larger than those obtained in the conservative analysis. Given the extent of the gene lists obtained in this semi-conservative analysis, all the results and information regarding the identification of fast and slow growing mutants are available at section 5 in the Appendices. A total of 479 deletion mutants were identified in this analytical approach. No GO Term was found enriched in the gene list obtained.

Note: One of the time-points, T\textsubscript{460}, was not included in this analysis. This was because the number of significant results obtained for this time-point was extremely sparse (only ~6 mutants were found significantly altered for both tags) and disrupted the trend exhibited by the other time-points, where the number of significant results increased gradually with time. As this would drastically alter the nature of our analysis, possibly even rendering it unusable, the decision was thus to discard this time-point and the corresponding data. We believe that some technical reason might have been the basis of the abnormal results obtained for this time-point. However, to the extent of our knowledge, we could not find anything unusual associated with the sample or respective data. The nature and distribution of the count data was normal and similar to those obtained for the other time-points. Also, all quality control tests employed led us to conclude that data from this time-point was valid (data not shown).
5.5.1 Fast growing mutants

5.5.1.1 Fast growing mutants at all time-points

The genes whose mutants were found to significantly exhibit an increased relative abundance over time at all time-points, and thus to be growing faster than the overall pool mean, are listed in table 5.1 (conservative analysis).

Table 5.1 List of genes whose mutants were significantly over-represented at all time-points. * info from uptag only.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Common Name</th>
<th>Description</th>
<th>Doubling Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPAC11D3.02c</td>
<td></td>
<td>ELLA family acetyltransferase (predicted)</td>
<td>135.9</td>
</tr>
<tr>
<td>SPAC13F5.05</td>
<td></td>
<td>thioredoxin family protein (predicted)</td>
<td>138.6</td>
</tr>
<tr>
<td>SPAC1F12.02c</td>
<td></td>
<td>translationally controlled tumor protein homolog (predicted)</td>
<td>134.1</td>
</tr>
<tr>
<td>SPAC26H5.04</td>
<td></td>
<td>vacuolar import and degradation protein Vid28 (predicted)</td>
<td>136.3</td>
</tr>
<tr>
<td>SPBC1539.02</td>
<td></td>
<td>conserved eukaryotic nuclear protein implicated in meiotic chromosome segregation</td>
<td>123.1</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>SPBC16H5.14c</td>
<td>short chain dehydrogenase DHRS family (predicted)</td>
<td>115.2</td>
<td></td>
</tr>
<tr>
<td>SPBP4H10.17c</td>
<td>carboxyl methyl esterase (predicted)</td>
<td>128.7</td>
<td></td>
</tr>
<tr>
<td>SPBPB10D8.04c</td>
<td>membrane transporter (predicted)</td>
<td>132.2</td>
<td></td>
</tr>
<tr>
<td>SPAC15A10.16</td>
<td>actin interacting protein 3 homolog Bud6</td>
<td>137.6</td>
<td></td>
</tr>
<tr>
<td>SPAC4G9.11c</td>
<td>cytosine-mismatch binding protein 1</td>
<td>142.9</td>
<td></td>
</tr>
<tr>
<td>SPAC824.08</td>
<td>guanosine-diphosphatase Gda1</td>
<td>116.0</td>
<td></td>
</tr>
<tr>
<td>SPAC15A10.10</td>
<td>Muskelin homolog (predicted)</td>
<td>112.5*</td>
<td></td>
</tr>
<tr>
<td>SPCC1620.04c</td>
<td>Cdc20/Fizzy subfamily WD repeat protein</td>
<td>116.3</td>
<td></td>
</tr>
<tr>
<td>SPBC6B1.02</td>
<td>Ark1/Prk1 family protein kinase Ppk30</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>SPCC790.03</td>
<td>rhomboid family protease</td>
<td>137.9</td>
<td></td>
</tr>
<tr>
<td>SPAC1B9.02c</td>
<td>serine/threonine protein kinase Sck1</td>
<td>147</td>
<td></td>
</tr>
</tbody>
</table>
18 mutants, whose genes are depicted in table 5.1, were found to be significantly over-represented at all times analysed. No GO term was enriched in this gene list. However, it was interesting to note that 3 genes related to actin filament or cytoskeleton processes, namely mde6, bud6 and ppk30. bud6 is an actin nucleation promoting-factor involved in cytoskeleton organization and cell polarity. mde6 refers to the ortholog of human protein Muskelin, an intracellular mediator of cell adhesive and cytoskeletal transport events. ppk30 is a serine/threonine Ark1/Prk1 family protein kinase involved in actin filament organization. In addition, it is also involved in regulation of the cortical actin cytoskeleton and control of endocytosis.

Regarding the other genes portrayed in table 5.1, SPAC11D3.02c is a predicted acetyltransferase whose product exhibits physical interactions with CK1 isoforms Hhp1 and Hhp2. CK1 is required for a mitotic checkpoint that delays cytokinesis. Therefore, it could be plausible that the physical interactions displayed with Hhp1 and Hhp2 could be essential for their mitotic checkpoint-associated roles. This could impair the mitotic checkpoint in question, leading to an accelerated cytokinesis, which could in turn explain the fast growth observed in this experiment. The other physical interaction identified concerns Bdf2, which is recruited by Epe1 to establish heterochromatin boundaries. Also, protein disulfide isomer activity is important in ER and ER folding events, which might relate to autophagy.

SPAC13F5.05, with predicted protein disulfide isomerase and oxireductase activity, has been shown to be involved in the stress response to heat (Pombase). It is also predicted to be associated with protein-folding activities in the endoplasmic reticulum.
(ER) (Pombase). Not much information is known regarding this gene. Nevertheless, being a stress-response related gene its absence could provide a growth advantage to the cell, as is often the case when disrupting genes involved in stress responses.

SPAC1F12.02c codes for an ortholog of human tumor protein TPT1, a well-known cancer player, which has been identified as a prognostic factor in breast cancer and a critical regulator of the tumor suppressor p53. Its budding yeast ortholog has been found associated with ribosomes. Given that it is highly associated with cancer and involved in the regulation of p53 it is not unexpected that its deletion could lead to an elevated growth rate.

SPAC26H5.04, another unassigned gene, codes for an ortholog of budding yeast Vid28, a protein involved in proteasome-dependent catabolic degradation processes, namely fructose-1,6-bisphosphatase (FBPase).

SPBC1539.02 concerns a conserved eukaryotic nuclear gene implicated in meiotic chromosome segregation. The predicted human ortholog codes for cytokine IK. Not much information appeared to be known regarding this gene.

SPBC16H5.14c codes for a putative protein of unknown function. It is predicted to belong to the short chain dehydrogenase DHRS family and thus to exhibit oxidoreductase activity.

SPBP4H10.17c is thought to be involved in protein demethylation. Its product’s budding yeast ortholog, PPE1, was also identified as a small subunit mitochondrial ribosomal protein.
SPBPB10D8.04c is predicted to be involved in transmembrane transport. The budding yeast ortholog SSU1 is a Plasma membrane sulfite pump involved in sulfite metabolism and required for efficient sulfite efflux. 

cmb1 is a cytosine-mismatch binding protein involved in DNA repair and part of the genome integrity checkpoint. Its impairment could thus possibly lead to an associated increase in growth rate, as DNA repair mechanisms tend to be time-consuming tasks when regarded under the scale/magnitude of the molecular clock. However, this is unclear as many DNA damage gene deletion mutants grow exhibit slow growth.

gda1 is a gene involved in the guanosine diphosphate (GDP) and uracil diphosphate (UDP) catabolic process. More specifically it has been shown in budding yeast that GDA1 is involved in the transport of GDP-mannose into the lumen of Golgi vesicles.

mug55 codes for a Cdc20/Fizzy subfamily WD repeat protein ortholog of human Cdc20, which is an essential regulator of cell division. Cdc20’s main function is to activate the Anaphase Promoting Complex (APC), which is responsible for initiating chromatid separation and entrance into the anaphase stage of mitosis. Cdc20 appears to act as a regulatory protein interacting with many other proteins at multiple points in the cell cycle. It is required for two microtubule-dependent processes: nuclear movement prior to anaphase, and chromosome separation. Moreover it has been recently found that Cdc20 may function as an oncoprotein. Therefore, data from this study indicating Mug55 as a fast grower falls in agreement with the current knowledge regarding its human ortholog Cdc20.

rbd2 is rhomboid-related serine protease with a serine-type endopeptidase activity. In budding yeast, cells lacking Pcp1p (ortholog of fission yeast’s Rbp2) contain partially
fragmented mitochondria, instead of the long tubular branched mitochondria of wild-type cells. In addition, pcp1Δ cells lack mtDNA and therefore are unable to grow on non-fermentable medium. Therefore, it is not clear how this mutant could be achieving such high grow rates (doubling time ~114 min).

sck1 is a serine/threonine kinase involved in many biological processes. Mainly, it is an upstream factor of the TOR pathway, which is involved in nutrient sensing responses. Interestingly, it has been recently shown to negatively regulate Gpa2 mediated glucose signalling. In addition, its corresponding human ortholog is a well-known oncogene AKT1. Therefore, it is not at all unexpected that it was found in this study that its deletion lead to an increased growth rate.

Finally, sts5 is a RNA binding protein involved in RNA catabolic process. Sts5 protein is a crucial determinant of polarised growth and functionally interacts with the serine/threonine phosphatase protein kinase C, and an osmosensing MAP-kinase to maintain cell morphology. Its deletion leads to actin cortical abnormalities. Its 2 budding yeast orthologs have been reported to be involved in cell cycle control. Thus, its disruption in growth would most likely affect the stability and kinetic properties of the cell cycle and possibly lead to faster growth.

5.5.1.2 Fast growing mutants in at least one time-point

A total of 223 mutants were found to exhibit a significant increase in relative abundance in at least one of the time-points analyzed, an average of ~28 mutants per time-point. On average, each detected mutant in this analysis was roughly found significant over 2 different time-points (~1.6 time-points with significant relative abundance changes per deletion mutant, ~2.1 when T460 was excluded). In theory we would have hoped for all time-points to share the same or highly similar set of results.
However, and as mentioned before, differences in relative abundance tend to become more pronounced over time, resulting in mutants only found significantly altered upon the latter time-points analyzed.

All the mutants found suggestive of fast growth are listed in the Appendices section in the file named “barseq_EMM_Sig.csv”, together with the Log2 Fold Change and the p-value adjusted for multi-test correction as obtained with DESeq.

### 5.5.2 Slow growing mutants

#### 5.5.2.1 Slow growing mutants at all time-points

The genes whose mutants were found to be significantly decreasing in relative abundance over time at all time-points, and thus to be growing slower than the overall pool mean, are listed in table 5.2.

**Table 5.2. List of genes whose mutants were significantly under-represented at all time-points.**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Common Name</th>
<th>Description</th>
<th>Doubling Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPCC191.09c</td>
<td>gst1</td>
<td>glutathione S-transferase Gst1</td>
<td>172.4</td>
</tr>
<tr>
<td>SPBC1198.11c</td>
<td>reb1</td>
<td>RNA polymerase I transcription termination factor Reb1</td>
<td>216.7</td>
</tr>
</tbody>
</table>

Only 2 mutants, whose genes are depicted in table 5.2, were found suggestive of slow growth rates in this analytical approach. The reason for this unexpected low output of
only 2 results might be the number of biological replicates used in this assay. It could be that with only 2 biological replicates and for a period of time as that comprising the time-course of this assay, it was not possible to statistically conclude about other mutants, i.e., there was not enough power and resolution in the data, using these settings, to statistically infer about other mutants. Therefore, one possible and obvious way that could lead to a more informative set of results would be firstly to increase the number of replicates and secondly to increase the time-span for the experiment to allow more variation as the number of division cycles increases.

According to PomBase⁶ gsa1 codes for glutathione synthetase large subunit Gsa1, mainly involved in glutathione biosynthetic process and transport, but also in the response to heavy metal, oxidative and heat stress. It catalyzes the ATP-dependent synthesis of glutathione, an important antioxidant preventing damage to important cellular components caused by ROS.¹⁵⁵

Finally, reb1 codes for the RNA polymerase I transcription termination factor Reb1. It has recently been shown to be involved in RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription.⁶ It was also reported that the deletion of reb1 lead to a delayed cell cycle phase transition, which could be the basis for the slow growth phenotype observed, despite the same study reporting normal vegetative growth.⁶ It would be interesting to profile this mutant individually to test whether this delayed growth phenotype could be specific to a competitive environment as is the case in mutant pools where all mutants present compete within the same culture media.
5.5.2.2 Slow growing mutants in at least one time-point

A total of 264 mutants were found to exhibit a significant decrease in relative abundance in at least one of the time-points analyzed, an average of 33 mutants per time-point. On average, each detected mutant in this analysis was roughly found significant over 2 different time-points (~2.1 time-points with significant relative abundance changes per deletion mutant). Notably, the consistency of this detection increased to an average of ~3 significant time-points per deletion mutant when T_{460} was excluded from the analysis, which corresponded to an almost complete consensus between the remaining 3 time-points analysed (~2.7 time-points with significant relative abundance changes per deletion mutant). In general, this observation highly reinforced the robustness and sensitivity of the bar-seq assay for the execution of profiling studies in a high-throughput fashion such as those presented here. More particularly, it ensured the quality and validity of the results presented in this chapter.

All the mutants found suggestive of slow growth are listed in the Appendices section in the file named “barseq_EMM_Sig.csv”, together with the Log_{2} Fold Change and the p-value adjusted for multi-test correction as obtained with DESeq.

5.5.3 Overall interpretation of the results obtained

No GO term was found enriched when taking both fast and slow growing mutants’ gene lists altogether.

Regarding the overall numbers obtained, 18 fast growers versus 2 slow growers results in a fast-to-slow growers ratio of 9:1. Perhaps an explanation for this result might lie in the fact that as the amount of time between compared time-points
augments, an increasing fraction of the overall pool becomes occupied by fast growing mutants due to their higher growth rate, leading to the dilution and loss of slow growing ones, resulting in a better detection of fast growers through the cost of losing sensitivity on opposite side where the slow growing mutants reside.

5.6 Analysis of growth profiles of deletion mutants growing in pooled environment

Results obtained with DESeq provided fold change values for each deletion mutant at all time-points analyzed. Together with the overall pool cell density numbers it is possible to produce for each mutant the respective growth profile in the same fashion as obtained for the overall mutant pools, as portrayed in figure 5.1B. For more information about this approach see section 2.4.7 in the Methodology chapter.

We thus used the fold change values obtained for both uptag and downtag barcodes and the overall mutant pool cell density values used displayed by both mutant pools analyzed. This resulted into 4 growth rate values per deletion mutant exhibiting a high degree of consistency (fig. 5.1C). For this reason, we decided to average the growth rate values obtained for each deletion mutant into a single final figure that could be further used as the corresponding deletion mutant’s intrinsic growth rate in a pooled competitive environment. This information could be of value for any experimental studies using this deletion library (or parts of it) as they can work as a reference baseline to help in the interpretation of the results obtained. Given the dimension of the data set in question, comprehending information regarding ~2100 deletion mutants (for the remaining detected mutants the corresponding bar-seq data did not provide reliable results mainly due to lack of sufficient coverage), it was placed at
A representation of the doubling times obtained for all deletion mutants analyzed is depicted in figure 5.2.

**Figure 5.2 Frequency distribution of the doubling times obtained for barcoded deletion mutants growing in a pooled environment.** Growth rates for each deletion mutant, obtained from profiling a deletion library mutant pool, were converted to doubling time. Values depicted represent the estimated mean doubling time from all the deletion mutants examined in min. UP - data from *uptags*; DN - data from *downtags*. Pool A and Pool B represent pool biological replicates A and B, respectively. Values topping the histograms represent the median doubling time for the respective histogram. Pool A and Pool B represent mutant pool (from pooling deletion library) biological replicates A and B, respectively.

An obvious consideration retrieved from observing figure 5.2 regarded the consistency between the different sets of doubling times obtained. It was possible to observe that the results obtained for each of the barcodes, uptag and downtag,
exhibited greater resemblance within the same tag than those referring to the same biological mutant pool (each of the two mutant pools represents an independent biological replicate of a pooled deletion library). This was rather unexpected, as we expected that data originating from the same biological subject would display a greater similarity. This could have been due to technical aspects related with particularities of each tag, although we tried to minimize any possible sources of variation between the bar-seq processing of each tag. Complementarily, technical reasons related to the deep sequencing analytical step could have led to distinct analytical yields between each tag. Nevertheless, results were still greatly consistent and comparable.

It was interesting to observe that the deletion mutants’ doubling time mean obtained for downtags (~164 min for both analyzed mutant pools) was higher than that of uptags (~159 min was the mean of both pools) (figure 31). The overall average of both tags and pools analysed was ~161.6 min). Interestingly, this value is slightly higher than that obtained for the overall mutant pool mean (~153.4 min). This fact could be due to the uneven frequency of the mutants present in the pool, where, inferring by the raw NGS data, differences in absolute numbers might be of up to ~100-fold (data not shown). Fast growing mutants rapidly increase in frequency within a mutant pool, therefore it could be that the average obtained for the overall mutant pool endured a greater contribution from fast growing mutants (probably more abundant in the mutant pool) than slower growing strains, leading to the decrease of the overall doubling time exhibited by the mutant pool.

In addition, it was possible to confirm the Gaussian distribution of the doubling times across the mutant pool. This observation stayed in line with current understanding that most deletion mutants would not exhibit drastic alterations in growth fitness,
which constituted one of the assumptions required for the validity of the bar-seq assay (if the majority of mutants exhibited aberrant fitness kinetics, the overall mutant pool would be unreliable for working as the reference upon data analysis, as is the case with DESeq and other similar analytical bioinformatics tools). Finally, regarding the distribution of the data, results obtained with uptags exhibited lower associated dispersion and standard deviation values (uptag:downtag standard deviation ratio of 0.86) than those originating from downtags. This observation is clearly exemplified by the greater peak frequencies exhibited upon the bars most central to the mean in the doubling time histograms depicted in figure 5.2.
6 Functional analysis of the quiescence-proliferation transition
6.1 Overview

The functional profiling studies conducted and presented (mainly) in chapter 4, but also in chapter 5, yielded a comprehensive set of results and novel valuable information. For that reason there were many possible analytical routes that could be subsequently tackled towards improving the understanding of the q-p transition.

Enlightened by the information available after the q-p transition profiling presented in chapter 4, we decided to follow-up and focus on 2 biological processes highlighted by such analysis - autophagy and chromatin-mediated genome regulation.

Many more other exciting and promising research paths and questions were uncovered by the q-p transition profiling analysis, with equal potential for leading to promising and successful outcomes. For that reason, we believe that the information gathered in these studies could provide a valuable resource for future research, a point that we address in more detail in the Discussion presented in chapter 7.

A functional analysis regarding the understanding of these two key biological processes upon the regulation of the q-p transition dynamics is presented next.

6.2 Autophagy genes in the quiescence-proliferation transition

6.2.1 Overview

A GO Term Enrichment analysis of gene lists obtained in the q-p transition profiling presented in chapter 4 revealed the biological process of autophagy to be strongly enriched in the list of mutants exhibiting an altered (either faster or slower than the
mean) q-p transition from a state of deep quiescence (p-value $1.4 \times 10^{-7}$ for autophagy and p-value $2.6 \times 10^{-7}$ for macroautophagy; table 8). Therefore, we decided to investigate the q-p transition profiles exhibited by the mutants of this particular pathway in a closer inspection. According to the digital fission yeast online genetic database PomBase, there are 35 genes annotated with the biological process autophagy (GO:0006914 - autophagy; section 5 in the Appendices). Together with 2 additional autophagy genes, atg22 and atg24, that for unknown reasons are not present in the group of genes associated with this GO term, they make the list of genes selected hereafter for undergoing the analysis of autophagy during the q-p transition, for a total of 35 inspected autophagy genes (section 5 in the Appendices). In fact, practically all atg (autophagy) gene deletion mutants are known to be required for autophagy.$^{81,161}$ We then analyzed the q-p transition profiles displayed by the corresponding deletion mutants. We used the mutant pool overall cell density values acquired during the q-p transition combined with the deletion mutants’ associated fold change figures from the time-points analyzed in the q-p transition profiling presented in chapter 4 to obtain the q-p transition profiles of all the autophagy mutants present in the deletion library, as described in section 2.4.7 in the Methodology chapter. Results from time-point $T_{390}$ were not included in the q-p profiles as they were found to deviate considerably from the overall trend. This was likely due to the fact that most cells had not yet undergone the first division upon q-p transition, resulting in technical associated variation surpassing actual biological detected changes in relative abundance, rendering the corresponding data unreliable for biological valid interpretations.

From the 37 genes inspected, 25 had the corresponding deletion mutants detected in our bar-seq experiments. More specifically, there were 24 autophagy mutants with a detected uptag, and 24 with a detected downtag, 22 of those mutants with both their
tags detected. This means that 12 of these genes did not have the corresponding mutant in the mutant pool. Since none of the undetected genes are reported as being essential for viability, we suspect that either some technical aspect in the construction of the mutant pools (section 2.1.2 in the Methodology) or any eventual problems of the mutants growing in a pooled fashion could comprise the basis for their absence from the mutant pool. Given that the pool contained ~2700 from a total of ~3500 reported viable deletion mutants, it is inevitable that some genes will be absent from our analysis. Additionally there were some mutants whose tags (2 uptags and 4 downtags), even though being detected, did not give rise to a satisfactory number of reads and therefore could not be taken into account for this analysis. For one uptag and 4 downtags however, the consistency and/or statistic significance of the profile trends obtained, as a result of the low variation exhibited by these tags, led us to add their information to our analysis, even though the relatively low number of counts. This information is shown in tables 6.1 and 6.2.

Table 6.1 Autophagy genes and respective uptag representation in the q-p profiling. * represents $p < 0.2$ after correcting for FDR. Valid results comprehend those originating from >50 reads at $T_0$. NA - information not available.

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Table 6.2. Autophagy genes and respective *downtag* representation in the q-p profiling.

Valid results comprehend those originating from >50 reads at T₀. NA - information not available.

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In total, there were 24 deletion mutants with valid uptag information (table 6.1) and 20 with valid downtag information (table 6.2) regarding the mutants’ relative abundance when undergoing the q-p transition. 18 of these mutants had valid data arising from both their barcodes, meaning that more than half of the genes identified with

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autophagy in fission yeast were adequately covered by this analysis, providing a fair representation of this biological process. Amongst these 18 well-covered mutants, 14 showed similar or identical trends upon the q-p transition from a period of short quiescence, and 16 exhibited equally consistent q-p profiles upon transition from deep quiescence, representing a consensus of 78% (14/18) and 89%, respectively, which was highly reassuring. Moreover, there were statistically significant q-p transition profiles obtained for 7 uptags and 10 downtags after a short quiescence period and for 16 uptags and 15 downtags upon deep quiescence, underlining the importance of autophagy for a normal q-p transition. By using the data obtained from the bar-seq screen, namely the fold change from the mutant pool mean and the absolute mutant pool cell density values, we were able to obtain the relative abundances of each mutant in the pool at any given analysed time-point. With this information we were able to reproduce the q-p transition profiles for any mutant analysed in the bar-seq screen (for more details on this please address section 2.3.6.7 in the Methodology chapter). The resulting autophagy mutants’ q-p transition profiles, as obtained with the bar-seq screen presented in chapter 4, are depicted in figure 6.1 and 6.2. The analysis of the results obtained is presented next. These hits were not picked up in the original screen, because they did not make the significance cutoff.

6.2.2 Autophagy genes in the quiescence-proliferation transition after short quiescence

The q-p transition profiles obtained for the autophagy mutants after a period of short quiescence are shown in figure 6.1.
Figure 6.1 Quiescence-proliferation transition profiles of autophagy mutants after short quiescence. 

A) Early q-p transition mutants, *uptags*. 

B) Early q-p transition mutants, *downtags*. 

C) Delayed q-p transition mutants, *uptags*. 

D) Delayed q-p transition mutants, *downtags*. *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001.
In a global view, autophagy genes clearly showed a common trend towards an early q-p transition after a period of short quiescence (2 days) (figure 6.1A, 6.1B). 2 mutants, fasclΔ and aut12Δ (figure 6.1C, 6.1D), exhibited an impaired q-p transition. The remaining analyzed mutants however, did not completely fit into this tendency, as they showed no clear difference or only a very slight early q-p transition (data not shown).

The clear trend observed for most of the autophagy mutants analyzed indicated an early q-p transition. This could be considered an unexpected observation as autophagy is known to be essential for enduring quiescence.65-74 Indeed, autophagy mutants have been known to drastically lose almost all viability after 10 days in quiescence.69 In quiescence, especially through nutrient limitation, cells reset their metabolic programs from growth to endurance. Autophagy has been shown to be required during quiescence as the main process that provides for alternative sources of energy and biosynthetic units when they are scarce.56 Autophagy allows recycling of macromolecules that result from the degradation of unneeded organelles, proteins and other macromolecules. In addition mitophagy, the degradation of mitochondria, was found crucial for controlling the level of ROS during quiescence.72,74 It is thus interesting to consider that upon a short period of quiescence autophagy did not appeared to have affected the cells to the point of complicating the further q-p transition. On the other hand, loss of autophagy perturbed the q-p transition by decreasing the time required for this transition to occur. Autophagy in proliferating cells has been shown to play a role in balancing the levels of growth of the cells through the degradation of cellular material, such as extra levels of biosynthetic products.56 In tumour growing cells, disruption of autophagy leads to increased growth rates due to lower degradation of biosynthetic products, thus increasing their availability.56
Clearly autophagy seems to be dispensable or at least not at all essential for enduring a period of short quiescence. Alternatively, these mutants might not even enter nitrogen-depletion-induced quiescence properly\textsuperscript{68,70} and be able to survive with a slow or arrested growth state for some time. If they would be able to endure 2 days, then this might also explain the fastened q-p transition observed for these autophagy mutants.

It is possible that the disruption of autophagy leads to an increased growth rate and consequently a shortened q-p transition. However, most autophagy mutants revealed growth rates within or close to the norm, as obtained with the fitness profiling of the deletion library presented in chapter 5 (section 5 in the Appendices). Therefore, it appeared that the early q-p transition observed could be due to an advantage conferred during short periods of quiescence or specifically during the q-p transition. Moreover, it could be that for shorter periods in quiescence the impairment of autophagy is not critical whilst on the other hand means less protein and organelle degradation conferring the cells with higher levels of cellular components and consequently less required de novo biosynthesis for returning to a proliferative state. In any case, this constituted an unexpected and interesting result. These cells might not have entered G\textsubscript{0} at all.

\textit{fsc1}\textsuperscript{Δ} and \textit{aut12}\textsuperscript{Δ} revealed an impaired q-p transition. As it is possible to observe in figure 6.1C and 6.1D, these 2 mutants did not appeared to have been successful in returning to proliferation, as their cell numbers are kept unchanged even after 980 min after nitrogen replenishment inducing the move from quiescence to proliferation. It appears that thus these genes are essential either for (entering and/or maintenance in) short quiescence or a successful q-p transition. \textit{fsc1} and \textit{aut12} are also required for autophagy.\textsuperscript{81} They are involved in vacuolar membrane fusion, including
autophagosome fusion with the lysosome. The autophagosome includes the double membrane structure enclosing the materials targeted for degradation. As there were strong indications that autophagy did not impair the q-p transition, on the contrary, we believe that the disruption observed in the q-p transition of these 2 mutants is probably due to other roles related to their general function in vacuolar membrane fusion. Accordingly, *fsc1* and *aut12* were present in the gene lists of GO cellular components vacuole, vacuolar membrane, and other vacuole-related components (table 4.6). Furthermore, Fsc1 data suggests it could have other roles than just being specific for autophagosome fusion with vacuoles. In a recent study they show it is dispensable for what other AtPh-vacuole fusion proteins are used for (like Aut12) (cpy, homotypic vacuole fusion). However, our data suggests it has other roles besides only autophagy.

### 6.2.3 Autophagy genes in the quiescence-proliferation transition after deep quiescence

The q-p transition profiles obtained for the autophagy mutants after a period of deep quiescence are shown in figure 6.2.
Figure 6.2 Quiescence-proliferation transition profiles of autophagy mutants after deep quiescence. A) Early q-p transition mutants, uptags. B) Early q-p transition mutants, downtags. C1) Delayed q-p transition mutants (1), uptags. C2) Delayed q-p transition mutants (2), uptags. C3) Delayed q-p transition mutants (3), uptags. D1) Delayed q-p transition mutants (1), downtags. D2) Delayed q-p transition mutants (2), downtags. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Note: Fig. 6.1A and fig. 6.1B depict only one mutant due to scale differences, rendering the q-p profiles of these mutants incompatible with others, in the y-axis.

After a deep quiescence period, the q-p profiles of autophagy mutants were utterly different from those exhibited after short quiescence. Practically all autophagy mutants appeared to be unable to undergo the q-p transition and return to proliferation figure 6.2C-D). This was not at all surprising, given that autophagy mutants have been shown to lose almost all viability after 10 days in quiescence. Therefore, the results here obtained agree with current studies and appeared to
confirm the significance of autophagy during quiescence, especially after prolonged periods of time.

Interestingly, 2 mutants appeared to exhibit different behaviours upon the q-p transition. The information from up tags revealed a delayed but seemingly successful q-p transition undergone by \textit{atg20}Δ, as it is possible to observed in figure 6.2A (the q-p profile for \textit{atg20}Δ relative to the down tag information could not be obtained because it was not possible to retrieve any down tag data from this mutant). Moreover, \textit{atg20}Δ appeared to have maintained at least a significant part of its viability allowing the possibility for a successful return to proliferation. \textit{atg20} has been curated as coding sorting nexin Atg20, involved in the CVT pathway, which has been identified in budding yeast, but not in \textit{S. pombe}. Actually no dedicated information is available regarding \textit{atg20} in fission yeast, except for a handful of global studies employing a genome-wide brute force approach. Therefore, the fact that \textit{atg20} does not follow the trend of most autophagy genes could mean that the gene curated as \textit{atg20} is not the ortholog of budding yeast \textit{atg20}. Alternatively, it may have been the budding yeast \textit{atg20} ortholog, although it is not associated with the autophagy GO term, but with the CVT pathway. Given the enrichment of this biological process upon the GO analysis conducted in the q-p transition functional analysis (table 8), this observation could hint towards the existence of a CVT pathway in \textit{S. pombe}, or at least some evolutionary related equivalent.

The other autophagy mutant exhibiting a distinct behaviour from the common trend observed of an impaired q-p transition was \textit{SPAC1734.07c}. This is an unassigned gene, ortholog of budding yeast TRS85, a subunit of protein transport particle complex TRAPPIII, a multimeric guanine nucleotide-exchange factor, required for membrane expansion during autophagy and the CVT pathway. The fact that this
mutant was also unimpaired in the resume of proliferation suggests that it is not essential for autophagy. Given the findings in budding yeast’s corresponding ortholog, this result also adds to the idea of the possibility for the existence of a CVT pathway in S. pombe.

There were two mutants, \textit{atg18cΔ} (localized in different places in the cell) and \textit{SPAC14C4.11Δ}, that behaved differently to the pattern observed. Their q-p profiles strongly indicated a markedly early q-p transition (figure 6.2A for \textit{atg18cΔ} q-p transition profile and 6.2B for \textit{atg18cΔ} and \textit{SPAC14C4.11Δ} q-p transition profiles). The q-p transition of \textit{SPAC14C4.11Δ} was especially pronounced, revealing an exceedingly early q-p transition. However, these results do not originate from an adequately reliable data source due to the relatively low number of sequencing reads obtained for this mutant’s barcodes (table 6.1 and 6.2). For that reason, we decided that the respective information leading to the atypical q-p profile observed could not be legitimately considered. The other mutant found to be exhibiting an early q-p transition, \textit{atg18cΔ}, is known to be involved in autophagy in fission yeast\textsuperscript{42}. However, its q-p profile was strikingly distinct from that exhibited by most autophagy mutants. Instead of a disrupted q-p transition, \textit{atg18cΔ} appeared to undergo a very early q-p transition. \textit{atg18c} is part of a trio of homologous genes together with \textit{atg18a} and \textit{atg18b}. \textit{atg18a} and \textit{atg18b} showed a completely impaired q-p transition, most likely, as with the other impaired autophagy mutants, due to a complete loss of viability upon deep quiescence (10 days) (figure 6.2C2 and 6.2D2). This suggests that \textit{atg18c} might have a significantly different, non-essential role in autophagy, contrary to what has been reported\textsuperscript{81}. Alternatively, it could be non-essential for autophagy during quiescence induced by nitrogen depletion. In the same study\textsuperscript{81} \textit{atg18c} was reported to be required for correct autophagy. Atg8 puncta (small autophagosome precursor units, indicative of normal pre-autophagy activity) were not abolished in \textit{atg18cΔ}, as
happened in \textit{atg18b}\textsubscript{Δ}, as observed for most autophagy mutants, including \textit{atg18a}\textsubscript{Δ}. On the contrary, Atg8 puncta were detected in high numbers throughout the cell, indicating that pre-autophagy was occurring normally throughout the cell. This observation suggested a functional similarity between \textit{atg18b} and \textit{atg18c}. Nevertheless, our results, more exactly the q-p profile exhibited by \textit{atg18c}\textsubscript{Δ}, highly suggested that \textit{atg18c}\textsubscript{Δ} might (also) have a markedly different role. According to the q-p profiles obtained, this gene was the earliest of autophagy mutants to undergo the q-p transition after short quiescence, where most exhibited an early q-p transition (figure 6.2A, 6.2B). Notably, the fact that \textit{atg18c}\textsubscript{Δ} was clearly viable after a deep quiescence period of 10 days, where all other autophagy mutants were found to have lost almost all viability, strongly points towards a different role of \textit{atg18c}, not necessarily related to autophagy. Atg18C quantification indicated that this protein is more abundant during quiescence than vegetative growth or proliferation\textsuperscript{86}, suggesting that its role might be more important or required during quiescence than proliferation. In addition, \textit{atg18c} growth rates suggested a relatively faster than normal growth rate (~135 min doubling time as obtained with \textit{uptags}, 150 with \textit{downtags}), also suggesting that this gene is not involved in growth-promoting associated activities. This gene thus constitutes a particularly interesting subject for subsequent investigation work and ideally should be the target of a complete functional characterization, as we believe that interesting insights could arise from such analysis, in light of the surprising results exhibited by the respective deletion mutant.

Overall, the consistency of the q-p transition profiles obtained for the deletion mutants of this biological process, which should be somehow expected given that they share the same functional pathways, demonstrated the high sensitivity and reliability of bar-seq data in extracting such profile information.
6.3 Chromatin-mediated genes in the quiescence-proliferation transition

An overall interpretation of the q-p transition profiling was presented in chapter 4. In this analysis, we noted the presence of swd1 in the delayed q-p transition mutants’ gene lists obtained for both short and deep quiescence states analyzed (section 4.4, chapter 4). swd1 is part of the Set1 complex involved in chromatin-mediated genome regulation, more specifically in mono-, di- and tri-methylating the fourth amino acid residue of histone 3 tail (H3K4me, H3K4me2, H3K4me3).105,150-152 This epigenetic mark has been extensively shown to represent a mark of recent transcriptional activity.105 swd1Δ was found to exhibit a delayed q-p transition in the q-p profiling study presented in chapter 4 (table 4.4; table 4.8). We particularly focused on this gene as an interesting candidate for follow-up studies, especially under the light of the dynamic behavior characterizing the q-p transition addressed in chapter 3. In addition, we were interested in addressing the question whether the q-p transition delay identified could be somehow associated with any possible genome regulatory abnormalities arising from the absence of swd1 in the corresponding deletion mutant. However, before any specific molecular analysis could be employed a proper validation of the results obtained with bar-seq in the profiling study of chapter 4 was required. Moreover, given the important role of chromatin-mediated genome regulation and genome architecture in the establishment of specific transcriptional programs, we were interested in understanding whether other chromatin-mediated regulatory factors could be affecting the dynamics of the q-p transition. This was especially pertinent under the light of the drastic alterations in gene expression occurring upon the transition between biological states exhibiting such opposing and distinctive transcriptional programs, as is the case with those of quiescence and
proliferation. For that reason, we decided to examine the q-p transition profiling employing a special emphasis on the chromatin-mediated regulatory factors found to exhibit any suggestions of altered q-p transition kinetics. To this end, we investigated the combined set of significant results obtained for all time-points analyzed in the q-p transition profiling after short and deep quiescence presented in chapter 4 (a total of 479 genes (see section 6 in the Appendices for this gene list)), excluding the mutants found to exhibit significant differences in growth rate as obtained in the fitness profiling presented in chapter 5, i.e., those mutants with a growth rate found significantly different from the mutant pool mean were excluded from this analysis as it would be unclear whether the significant differences in q-p transition were due to their affected growth rate (see section 5 in the Appendices for the specific growth rates obtained for all mutants analysed), using two methods of global exploratory investigation: clustering analysis and protein-protein interactions prediction.

6.3.1 Clustering analysis of the quiescence-proliferation bar-seq screen

Clustering analysis provides a means by which to organize the results obtained based on the similarities exhibited by different mutants across the timecourse. It is a potent way to retrieve meaningful patterns across large data sets and to find valid associations between seemingly unrelated objects. As mentioned previously, to perform the clustering analysis, we used the entire data set of significant results obtained in the q-p transition profiling presented in chapter 4. For more information regarding the clustering method, see section 2.4.5 in the Methodology chapter). A visual representation of the gene tree obtained with clustering is depicted in figure 6.3.
Note: We experimented with several clustering methods/algorithms using different parameters, trying to obtain biologically coherent clusters with functional enrichments but did not obtain any meaningful insights from these analyses.
Figure 6.3 Clustering analysis of the results obtained in the q-p transition profiling. A) Overall view of the clustering analysis gene tree. B) Focus on the swd1 genes cluster. Clustering analysis was performed with GeneSpring GX 10 through a Gene Tree method. Expression bar applies to all figures. Red color indicates over-representation in relation to T₀. Green color indicates under-representation in relation to T₀. Red boxes indicate Set1C genes.

The clustering analysis yielded an abundant source of information (figure 6.3). From the various clusters obtained, and guided by the results obtained from the q-t transition profiling analysis, one particularly caught our attention. This referred to the subset of genes that contained swd1 (figure 6.3B), one of the hits from the bar-seq screen presented in chapter 4 (table 4.6, section 4.4.5.2). As previously mentioned, swd1 codes for a subunit of the Set1C complex and is required for Set1, the catalytic
domain that methylates H3K4.\textsuperscript{105,150-152} Interestingly, in this cluster there were two more Set1C subunits, set1, the catalytic domain itself, and spf1, also essential for Set1C activity.\textsuperscript{150-152} All the corresponding deletion mutants were significantly delayed upon undergoing q-p transition, as is depicted by the green colors indicating a decrease in relative abundance over time. All remaining mutants exhibited similar q-p transition profiles as in figure 6.3B. We therefore became interested in the possibility that the Set1C complex could be affecting the q-p transition dynamics. For this reason these genes were selected for follow-up investigation with the aim of asserting their scope of their effect upon the q-p transition dynamics.

We believe the clustering analysis performed could be a valuable resource for posterior re-interpretations of the q-p transition, especially under a different analysis, as for example a more detailed analysis of gene clusters highlighting biological processes other than chromatin-mediated genome regulation. For that reason, we believe that this resource should be revisited in subsequent studies addressing cellular quiescence or more specifically the q-p transition.

6.3.2 Protein interactions of the Set1 Complex subunits highlighted in the clustering analysis of the bar-seq screen of the quiescence-proliferation transition

The results obtained with the clustering analysis described in the previous section uncovered the Set1 Complex as a promising target for follow-up investigation.

A novel bioinformatics resource had been recently developed within the Lab: plnt (pombe Interactome), a protein interactions prediction tool.\textsuperscript{145} This resource retrieves predictions of fission yeast protein interactions, based on more than 100 features of the proteins. Predictions are generated using two machine learning algorithms and
the confidence score returned is used to rank the predictions according to their probability of being true. Furthermore, plnt allows retrieval of scores for each protein interaction prediction with a protein of choice. It also generates a ranking of protein-protein interactions with associated likelihood probabilities.

We decided to use plnt to analyse the list of proteins coded by all the genes whose deletion mutants were found to display altered q-p transition kinetics from short and deep quiescence as obtained in the q-p transition profiling presented in chapter 4 in a total of 479 genes (see section 6 in the Appendices for this gene list). Our aim was to determine if any of the proteins revealed any strong probabilities of interacting with the Set1 complex subunits identified in the clustering analysis. A visual representation of the predictions obtained for the Set1 complex genes is depicted in figure 6.4. The list of protein predictions for Set1 and the stronger protein-protein interactions amongst all proteins selected for this analysis are available at section 6 in the Appendices.
Figure 6.4 Set1C subunits Set1, Swd1 and Spf1 protein interactions with the list of targets obtained in the q-p profiling analysis, as predicted by pInt\textsuperscript{145}, a machine learning based bioinformatics tool. Green lines depict known protein interactions in fission yeast. Blue lines depict known genetic interactions in fission yeast. Yellow lines depict known genetic interactions in budding yeast. Red lines depict known genetic interactions experimentally confirmed in fission yeast. Grey lines depict putative protein interactions predicted by pInt. Thickness of connecting lines indicates the probability of interactions: the thicker the line the greater the likelihood of the interaction.

Figure 6.4 shows all the protein interactions predicted for the 3 Set1C subunits highlighted by the clustering analysis - Set1, Swd1 and Spf1. This examination with pINT clearly shows a strong functional relationship underlying this set of proteins, furthermore suggesting a range of new links amongst these proteins (depicted by the grey lines in fig. 6.4). Strikingly, mostly all of them are regulators of gene expression, namely many chromatin-mediated genome regulation factors.
As all these proteins were obtained from the significant results obtained in the q-p profiling study presented in chapter 4, it was interesting to note the presence of several regulators of gene expression. Given the fact that there are massive changes in the gene expression program upon this transition, with over half of the *S. pombe* genome undergoing differential regulation, it was not at all unexpected to find genome regulatory factors in the list of mutants with altered q-p transition kinetics. Amongst the proteins plnt clustered with the Set1 are complex subunits Spt8 and Gcn5 (part of the SAGA complex), Swc5 (Swr1 complex), Pst2 and Cph2 (Clr6 histone deacetylase complex), Set7 (histone lysine methyltransferase), Png1 and Png2 (genome regulation), Mcl1 (chromosome replication), these are all important players in genome regulation. This fact reinforced plnt's protein predictions in suggesting that they could be in fact collectively involved in the regulation and establishment of the q-p transition dynamics.

For this reason, we decided to proceed with a follow-up investigation incorporating this additional genome regulation factors.

### 6.3.3 Quiescence-proliferation dynamics

The results obtained in the previous sections 6.5.1 and 6.5.2, referring to the clustering analysis and protein interactions prediction, respectively, uncovered a set of genes indicating that various gene expression regulators are playing a role in the q-p transition. For this reason, we decided to focus our study on these factors. The results obtained so far constituted important progress towards uncovering these genome regulation players as strong candidates for being somehow involved in the regulation of the q-p transition dynamics. However this required further validation through an alternative independent approach that could confirm the interesting q-p
transition kinetics observed in the bar-seq profiling studies presented in chapter 4 (even though only swd1 was a brute force hit of the screen). We thus decided to individually test the corresponding deletion mutants upon the q-p transition using the BioLector high-throughput platform. This would provide us with adequate information to allow the verification and validation of the results so far obtained.

For that purpose, we developed an experimental assay identical to that employed for the q-p transition dynamics analysis presented in chapter 3. We set up liquid cultures in minimal media of these deletion mutants, allowing them to grow to OD$_{600}$ 0.6 as described in section 1.1.2 in the Methodology section. We then induced them to enter quiescence, through nitrogen depletion, for the equivalent periods as those employed in the functional profiling study presented in chapter 4, 2 and 10 days, corresponding to a short and deep quiescence states, respectively. After that, nitrogen was replenished triggering the cells to return to a proliferative state.

We then assayed the q-p transition profiles using a BioLector high-throughput platform that measures relative biomass of a liquid cell culture over time with high resolution, as described in section 1.1.5 in the Methodology chapter (figure 6.5).

In addition, to broaden the spectrum of our analysis and given that this constituted a high-throughput platform allowing parallel profiling of up to 48 individual cell cultures, we decided to include in this profiling analysis several other mutants deleted for genes involved in chromatin-mediated genome regulation, that were found to exhibit significantly altered q-p transition kinetics in the q-p transition profiling described in chapter 4, but that had not exhibited any predictions with the Set1 complex. The full list of additional mutants’ selection for this profiling analysis is depicted in table 6.3.
Table 6.3 List of deletion mutants selected for downstream BioLector high-throughput analysis. Gene list of chromatin-associated gene deletion mutants selected for follow-up analysis.

<table>
<thead>
<tr>
<th>Deleted Gene</th>
<th>Systematic name</th>
<th>Bioneer ID and plate location</th>
</tr>
</thead>
<tbody>
<tr>
<td>set1</td>
<td>SPCC306.04c</td>
<td>V2-11-G09</td>
</tr>
<tr>
<td>swd1</td>
<td>SPAC23H3.05c</td>
<td>V2-19-G02</td>
</tr>
<tr>
<td>spf1</td>
<td>SPCC594.05c</td>
<td>V2-20-D09</td>
</tr>
<tr>
<td>gcn5</td>
<td>SPAC1952.05</td>
<td>V2-23-A10</td>
</tr>
<tr>
<td>png1</td>
<td>SPAC3G9.08</td>
<td>V2-06-H04</td>
</tr>
<tr>
<td>png2</td>
<td>SPBC1709.11c</td>
<td>V2-21-H04</td>
</tr>
<tr>
<td>mcl1</td>
<td>SPAPB1E7.02c</td>
<td>V2-22-E09</td>
</tr>
<tr>
<td>mms19</td>
<td>SPAC1071.02</td>
<td>V2-04-A08</td>
</tr>
<tr>
<td>pst2</td>
<td>SPAC23C11.15</td>
<td>V2-28-B05</td>
</tr>
<tr>
<td>swc5</td>
<td>SPCC576.13</td>
<td>V2-20-H01</td>
</tr>
<tr>
<td>spt8</td>
<td>SPBC14C8.17c</td>
<td>V2-19-E07</td>
</tr>
<tr>
<td>set7</td>
<td>SPCC297.04c</td>
<td>V2-21-C10</td>
</tr>
</tbody>
</table>
Results were analysed using the same pipeline as that employed for the analysis of fission yeast wt \( q-p \) transition dynamics presented in chapter 3, described in section 3.2.1 in the Methodology chapter. Although we used Bioneer fission yeast wt as a control and reference in all BioLector profiling assays, we also included the data obtained for the wt \( q-p \) transition dynamics study presented in chapter 3 in order to increase the power and robustness of our analysis. Results obtained are shown in figure 6.5 (\( q-p \) transition BioLector profiles) and 6.6 (analysis of \( q-p \) transition BioLector profiles).

It should be noted that before proceeding with the high-resolution \( q-p \) transition profiling assay, we tested the deletion mutants for the correct integration of the respective deletion cassettes through PCR, as indicated by the company Bioneer. This was because, as reported by Han et al.\(^{137}\), a significant number of mutants were found to contain incorrect or misplaced deletion cassettes, rendering any results arising from such mutants misleading. We verified the deletions of the following mutants: \( set1\Delta, set2\Delta, swd1\Delta, png1\Delta, mms19\Delta, swc5\Delta, spt8\Delta, rhn1\Delta, pst2\Delta, clr5\Delta, stc1\Delta, png1\Delta \) and \( mms19\Delta \) (data not shown).
Some deletion mutants, however, could not be verified (data not shown). It remained unclear whether verification through PCR was not successful for some reason or whether the deletion mutants possessed any abnormality regarding their constructs. The following deletion mutations could not be verified: \textit{gcn5Δ, png2Δ, mcl1Δ, set7Δ, SPCC1393.08Δ, cph2Δ}. Consequently, they were not included in the q-p high-resolution profiling presented next.

We also constructed a Bioneer wt prototroph strain to use as a control in the high-resolution profiling experiments, as the prototroph library did not contain a wt strain. For that we successfully employed a protocol as described in section 1.1.3 in the Methodology chapter (data not shown).
B

Time after N refeed (hr)

Relative biomass (through light scattering)

C1

Time after N refeed (hr)

Relative biomass (through light scattering)
**Graphs:**

**C4**

- **Graph Title:**
  - Relative biomass (through light scattering)

- **X-axis:** Time after N refeed (hr)

- **Y-axis:**
  - 0 to 3

- **Legend:**
  - wt_2d_A1
  - msc1Δ_2d_A1
  - shg1Δ_2d_A1
  - spf1Δ_2d_A1

**D1**

- **Graph Title:**
  - Relative biomass (through light scattering)

- **X-axis:** Time after N refeed (hr)

- **Y-axis:**
  - 0 to 3

- **Legend:**
  - wt_10d_A1
  - sww1Δ_10d_A1
  - epe1Δ_10d_A1
  - jmj1Δ_10d_A1
  - set1Δ_10d_A1
  - swd3Δ_10d_A1

Figure 6.5 Quiescence-proliferation transition BioLector profiles of the chromatin-mediated genes selected for downstream analysis. Q-p transition profiles were obtained as described in section 2.1.5 in the Methodology chapter. **A)** Q-p transition profiles of the deletion mutants of several Set1C subunits after short quiescence (2 days); BioLector run #1. **B)** Q-p transition profiles of the deletion mutants of several chromatin-mediation factors after short quiescence (2 days); BioLector run #2. **C1-4)** Q-p transition profiles of the deletion mutants of several Set1C subunits and chromatin-mediation factors after short quiescence (2 days); BioLector run #3. **D1-2)** Q-p transition profiles of the deletion mutants of several Set1C subunits and chromatin-mediation factors after deep quiescence (10 days); BioLector run #4. **E1-2)** Q-p transition profiles of the deletion mutants of Set1C subunits after short quiescence (2 days); BioLector run #5. **F)** Q-p transition profiles of the deletion mutants of Set1C subunits after deep quiescence (10 days); BioLector run #6. 2d - 2 days; 10d - 10 days; A-C - Biological replicates A-C. A1-5 - Technical replicates 1-5.
Figure 6.6 Quiescence-proliferation transition dynamics of various deletion mutants of factors involved in genome regulation. Symbol sizes are proportional to the number of days in quiescence. The red ellipse represents the zone of normality as obtained with fission yeast wild-type analysis. The plots represent A) the average lag time against the coefficient of variation, B) the average lag time against the elongation rate, C) the coefficient of variation against the elongation rate, and D) the number of days in quiescence against the average lag-time. r(m) - r score for the deletion mutants. r(wt) - r score for the wt.

We examined the average lag time of the q-p transition against the elongation rate (Figure 6.6B) obtained by the analytical model employed. This analysis clearly revealed a diagonal trend for the wild-type, representing the relationship between
time in quiescence and the kinetics of the q-p transition. This relationship exhibited by the wt allowed us to calculate a normality zone whereby any differences would be included within the standard variation range and thus unlikely to be biologically meaningful (see section 3.2.1 in the Methodology chapter). Any deletion mutants laying outside this normality zone would thus be exhibiting statistically significant altered q-p transition kinetics. We thus proceeded to interpret the results obtained, which allowed us to identify 3 deletion mutants behaving outside the trend: set1Δ, swd1Δ and spf1Δ constituted obvious outliers. The same mutants were also found to behave outside the norm regarding the remaining variables analyzed, namely coefficient of variation and days in quiescence (figure 36). Interestingly, the mutants appeared to group together, suggesting a similar common behaviour upon the q-p transition. Although the lag times obtained for set1Δ, swd1Δ and spf1Δ are clearly much higher than would be considered within the norm, the very low elongation rates estimated for these 3 deletion mutants suggest that the delayed q-p transitions observed could be due, at least in part, to an intrinsic lower growth rate. This was not the case when analyzing the growth rates obtained in the fitness profiling presented in chapter 5 (section 5 in the Appendices). Still, further validation was required to test this hypothesis. All the remaining mutants did not appear to exhibit any strong detectable significantly altered kinetics when undergoing q-p transition.

All the information regarding the results obtained for the variables analyzed with this mathematical approach is available in section 6 in the Appendices.

Although the elongation rates calculated by this model are a reliable measure of growth rate, we noted throughout the course of the experimental work, in the manipulation of cell cultures, that the growth rate they exhibited upon resuming proliferation from a quiescent state was often different from that displayed when
routinely growing cell cultures under standard conditions (in a culture flask placed in a standard Lab shaking incubator). After exiting quiescence cell cultures (wt included) appeared not to reach as high growth rates as when growing in a culture typically set up from a pre-culture inoculation (thus not coming from a quiescent state). We thus further performed a fitness profiling in minimal media of the deletion mutants analyzed here, using the BioLector platform. This was to control for any eventual inherent growth rate differences exhibited by the deletion mutants that could thus be significantly affecting the q-p transition kinetics, therefore merely constituting an indirect effect. This would then allow us to discard the corresponding genes from our subsequent studies, as they would not constitute such interesting research targets as genes whose mutants were found to display altered q-p transition kinetics that could not be accounted for intrinsic differences in growth rate. Results regarding the fitness profiling analysis described here are shown in figure 6.7.

![Figure 8: Plots of the mutants growing without quiescence. The four plots show (A) the average lag-time against the coefficient of variation of the lag-time, (B) the average lag-time against the elongation rate, (C) the coefficient of variation of the lag-time against the elongation rate and (D) the elongation rate without quiescence against the elongation rate with quiescence.](image-url)
Figure 6.7 Fitness profiling in minimal media of various deletion mutants of factors involved in genome regulation. Symbol size is proportional to the number of days in quiescence as applied in the q-p profiling shown in figure 6.6. The plots show A) the average lag time against the coefficient of variation of the lag-time, B) the average lag-time against the elongation rate, C) the coefficient of variation of the lag time against the elongation rate, and D) the elongation rate obtained with the growth profiling shown here against the elongation rate obtained in the q-p transition profiling depicted in figure 36. \(r(m)\) - \(r\) score for the deletion mutants. \(r(wt)\) - \(r\) score for the wt.

A further fitness profiling analysis of the mutants growing in minimal media showed that when the cell comes from a quiescent state, the whole cellular machinery becomes perturbed. As it is possible to observe in figure 6.7, mutants grown without any prior quiescence experience did not reveal the same behavior. The scatterplot in Figure 6.7D shows the relation between the elongation rates of the mutants growing with and without previously undergoing a quiescent state. The correlation (Pearson’s \(r = 0.34\)) indicated that the mutants tend to keep a similar rate as they grow. Unexpectedly however, if examined with more scrutiny, the elongation rates reached after cells had been in quiescence were slightly higher than those obtained in the fitness profiling (figure 6.7D). This observation was contrary to what we had sensed from our empiric observations throughout the routine manipulation of cell cultures in the Lab. On the other hand, this lower associated variation could be reflecting a lower degree of population heterogeneity at the single-cell level exhibited by the deletion mutants regarding the q-p transition dynamics. This would constitute an interesting question to address.

In figure 6.7 it was possible to note that the coefficient of variation obtained for the deletion mutants appeared to be consistently lower than that of the wt. We could not find any reasonable explanation for this observation. We wondered though whether
this could have been due to a technical bias displayed by the mathematical model used for this analysis.

Figure 6.7B and 6.7C show that the elongation rates obtained in the fitness profiling analysis are very much comparable to those displayed by the wt. It became evident that the delayed q-p transition kinetics exhibited by set1Δ, swd1Δ and spf1Δ could not be attributed to inherently lower growth rates (total differences in the doubling time were lower than 10%). The exact elongation or growth rate values calculated with this model are available in section 6 in the Appendices.

All the information regarding the variables analyzed with this mathematical approach is available in section 6 in the Appendices.

In conclusion, we confirmed that Set1 complex subunits genes set1, swd1 and spf1 exhibit a significantly delayed q-p transition that is not accounted for intrinsic differences in growth rate. Therefore, and given that they constitute important chromatin-mediated genome regulation factors, they provide promising targets for subsequent studies regarding the elucidation of the basis for the q-p transition dynamics phenomenon observed.

Note: Data for quiescence-proliferation transition profiles was obtained for these mutants also at 25°C and included in the analysis. Despite that, and due to the lack of additional information they provided and to facilitate flow of information, we decided not to add their q-p transition plots.
7 Discussion
7.1 Quiescence-proliferation dynamics in fission yeast

It is known almost as a piece of conventional wisdom, that the deeper the sleep, the harder it is to wake up. Furthermore, we have known almost anecdotally, through acquired experience and sensibility when manipulating and working with fission yeast, that the longer cells have been kept dormant, as it happens for example when we store cells in the fridge for prolonged use, the longer it takes them to start growing.

7.1.1 Quiescence-proliferation population profiles

As had been previously noted by Su et al.\textsuperscript{44}, cells subjected to long periods in quiescence (induced by deficiency of a nitrogen source) exhibit a longer transition when resuming the cell cycle upon return to proliferation when compared to cells that had been quiescent for shorter periods of time. This transition is characterized by a lag phase, whereby cells do not divide but grow only in size. We have shown that this association seems to be consistent at least up to ~8-9 days in quiescence, where the longer the duration of quiescence, the longer the lag phase that characterizes the dynamics of the quiescence-proliferation transition (figure 3.1 and 3.2). However, for longer than this time (8/9 days), it was unclear whether the duration of the lag phase remained to be as affected by the duration of the quiescence period (figure 3.3). Actually, the results obtained suggested that this relation between time in quiescence and duration of succeeding lag phase upon return to proliferation were detected even after over a month in quiescence (figure 3.3). However, a slight but still considerable gradual decrease in viability started to be detected reaching ~80% viability after around 2 weeks in quiescence (figure 3.4). Thus, although the analytical model developed for the analysis of the q-p profiles theoretically controls for
variability in viability, it was not possible to simulate the full weight of the effect that a decrease in viability could have had on the dynamics upon q-p transition and to understand whether differences in cellular viability affected the dynamics of the q-p transition.

7.1.2 Quiescence-proliferation at the single-cell level

As we were interested on the dynamic behavior across the q-p transition, we further analyzed cell size upon this transition. We noted that (at least) two distinct sub-populations were detectable for cell populations that had been quiescent for a relatively short period (1-3 days). Our interpretation was that a given subgroup of cells within the entire population was somehow faster to undergo the q-p transition and resume growth. We believe that this observation likely reflected a certain degree of heterogeneity featured within a fission yeast population regarding the kinetics of the cellular response to environmental changes providing the required growth promoting signals. It is possible that some cells within a population might be readily poised for a quick transition to a proliferating state, whilst other cells of the population could instead be focused on maximizing survival and endurance during a period of adverse or sub-optimal external conditions unsuitable for growth. Such heterogeneity could have been a strategy developed by organism populations to help tackling the inevitable unpredictability and dynamics of their surrounding environment, notably fluctuations in fundamentally vital conditions such as temperature, pH, energy and food availability, water and oxygen levels, amongst others. For future work we suggest to do a time-course experiment with septation index to better assess the kinetics of this transitions.
Interestingly, many related observations have been made extensively at the single-cell level, mainly upon examination of bacterial growing populations\textsuperscript{126,129}, but also more recently in a study performed on budding yeast.\textsuperscript{128} In budding yeast it was reported that clonal cell populations growing in optimal conditions exhibit a highly diverse and gradual range of growth rates.\textsuperscript{128} Conversely, in bacteria this heterogenous behavior is reduced to 2 sub-populations, resulting in two distinct growth rates overall detected in bacterial populations.\textsuperscript{126} Our data suggested that a type of growth kinetics heterogeneity similar to that observed in bacteria could exist in fission yeast quiescent cell populations upon return to proliferation.

A highly pertinent question to address regarding this highly dynamic phenomenon (that constitutes the transition from quiescence to proliferation) concerned with whether the dynamics exhibited in the duration of the corresponding lag phase could be specifically attributed to a defined subset of cells from the quiescent population exhibiting a differential behavior. This could prove to be of great value in fields of oncobiology and biomedicine, given the fact tumor formation and growth is highly associated with the q-p transition, as massive disruptions in the metabolic and cellular program of quiescent differentiated cells within the body lay in the basis of neoplasry and tumorigenesis.\textsuperscript{25,33}

It would be very interesting to further analyze and compare the q-p transition kinetics from different sub-populations of quiescent cells. As these quiescent sub-populations would still require further validation and characterization, such heterogeneity could further be associated with other characteristic features. More specifically, one example comprises budding yeast quiescent populations, which exhibit marked differences in cellular density across cells within a population, amongst other characteristics.\textsuperscript{128}
Interestingly, the single-cell analysis of deep quiescent populations that had been quiescent for longer periods of time (over 8 days) revealed a different scenario (figure 3.5). Here, cells contrastingly exhibited a highly homogeneous behavior, resulting in a very uniform cell size across cells that had undergone the q-p transition. Strikingly, this low variation in cell size across cells of deep quiescent populations was consistent with the degree of variation exhibited by the overall populations when profiled in a high-resolution manner (figure 3.5). This fact strongly reinforced the observations from (single-cell) cell size analysis, whereby the level of dynamic heterogeneity associated with the q-p transition was found to gradually fade away upon longer exposures to quiescence.

Moreover, the fact that deep-quiescent cells did not appear to exhibit the same cell size heterogeneity might suggest that upon short quiescence cell could still retain a certain approximation or similarity with proliferation mechanisms and characteristics. This could mean that a certain metabolic memory\(^92\) could somehow be in place that resembled the previous state of vegetative growth, fading away as cells delve deeper into a full quiescence state. This "metabolic memory" could thus be advantageously utilized to equip the cells with the capacity for a prompt response as soon as conditions are met that allow them to return to proliferation. It is interesting to conceive that the cellular mechanisms used for attaining growth and cell cycle progression could still be in place for some time even during a state of cellular quiescence. Together with the finding that RNA polymerase II was greatly enriched at intergenic regions directly upstream of hundreds of genes immediately induced upon stationary phase exit\(^102\), these observations are consistent with the idea that cells are somehow poised for return to a proliferative state once the necessary external conditions are met. Furthermore, the fact that transcriptional memory has been observed in several gene clusters upon their re-induction suggests that a "memory" of
recent metabolic state could in fact be in place, carrying great biological significance as it would strongly impact by allowing cells to respond more rapidly to perturbations to their surroundings.\textsuperscript{106-111}

### 7.2 Establishment of barcode-sequencing strategy for screening a fission yeast barcoded mutant deletion library in a pooled environment

In this project we have successfully introduced, established and optimized a high-throughput strategy for massive parallel profiling of fission yeast gene deletion strains in a pooled environment, accomplished by using a barcode-sequencing technology for screening a barcoded deletion library.

We adopted a published protocol by Han et al.\textsuperscript{137} to which we introduced several improvements (explained in detail in section 2.2.2) that lead to an increase in the assay’s overall quality.

This strategy was fruitfully applied to carry out a functional profiling analysis of the quiescence-proliferation transition dynamics, presented in chapter 4. The high yield obtained generated valid biological information for over 2,500 deletion mutant strains. In addition, this approach proved very efficient with relatively reduced/low costs and time especially when compared to classical genetic approaches.

We also employed this high-throughput strategy to perform a fitness profiling study of the gene deletion strains present in the deletion library, presented in chapter 5.
7.3 Functional profiling of quiescence-proliferation transition dynamics

The main objective of this project concerned a functional profiling of the dynamics of the transition from quiescence to proliferation, and the identification of factors affecting the kinetics of this transition. For that we successfully employed a high-throughput genome-wide profiling analysis for screening a barcoded deletion library in a pooled environment using barcode-sequencing (bar-seq). We carried out an extensive functional profiling of the quiescence-proliferation transition after two distinct quiescence states - short and deep quiescence. We defined 2 days as the duration of short quiescence and 10 days as a period reflecting deep quiescence. We statistically identified 21 gene deletion strains leading to an altered q-p transition after short quiescence and 50 gene deletion strains significantly affecting the kinetics of this transition after deep quiescence (section 4.4). We successfully used the results obtained with this profiling study as a valuable framework for follow-up studies aiming to elucidate the nature of the dynamics exhibited upon quiescence-proliferation transition, presented in chapter 6. We selected the most promising targets to carry out an extensive and detailed investigation of this dynamic phenomenon. We also used the whole data set from all mutants that lead to statistically significant results to perform a gene clustering analysis, presented in chapter 6. We further employed a mathematical model to estimate the kinetics associated with the q-p transition for all mutants successfully analyzed in this profiling study (section 4 in the Appendices). However, this model was specifically developed for analyzing high-resolution q-p transition profiles, as used for the analysis presented in chapter 3 and section 6.2.3. We were expecting more translation/transcription/ribosomal targets with delayed q-p transition judging by the q-p transcriptional program. The fact that they did not
emerge in the bar-seq screen could be likely due to the fact that these genes are highly redundant.\textsuperscript{89}

7.4 Validation of the experimental design and assay

The utilization of bar-seq to carry out a profiling analysis, monitoring the dynamic kinetic changes occurring over time in a gene deletion mutant pool of more than 2500 mutant strains, was a novel approach. Thus, validation of the assay constituted one prior requirement. Validation experimental analysis using both positive and negative controls confirmed the high-sensitivity and robustness of this assay, although the assay likely missed several relevant genes. The identification of several genes known to play a role upon the transition between quiescence and proliferation and the identification of key players of this transition further asserted the validity of this state-of-the-art approach and its successful incorporation in the Lab’s vast resource portfolio.

7.5 Fitness profiling of haploid deletion strains

We profiled the growth fitness of our fission yeast prototroph deletion library growing in a pooled environment. This was done using a barcode-sequencing high-throughput platform. Our aim was to acquire information on the growth rates of the mutants present in the deletion library in order to analyze the results obtained in the quiescence-proliferation transition dynamics study presented in chapter 4. In addition, this revealed to be a great opportunity for profiling our deletion library as it had been
recently acquired by the Lab therefore little experience and knowledge had been gathered on this mutant collection. Moreover, a certain degree of imprecision was reported regarding the information provided by the manufacturer of the deletion library, Bioneer. Namely, mismatches in the barcode sequences, incorrect deletions, duplications and false positives were shown to make up for up to 5% of the entire collection, corresponding to ~250 deletion strains. In addition, barcodes with sequences differing from those provided by Bioneer was also reported to occur above neglectable level. For that reason we also conducted a de novo decoding of the deletion library, which allowed us to correct the errors contained in the information provided by Bioneer regarding the deletion library (section 5.3).

We determined the mutant pool overall doubling time to be ~158 min, roughly 2 hours and 30 minutes. This was strikingly similar to the doubling time exhibited by fission yeast standard wild-type strain when grown in the same conditions, namely at 32°C. Although one could have expected that the deletion of a gene could have an effect on growth rate, given the sub-optimal conditions carried by the absence of a gene, most mutants grew within the pool mean, confirming the fact that disruption of most genes does not necessarily imply an observable effect on growth rate.

In this profiling study we identified the deletion mutants significantly growing above the mutant pool mean, or fast growers, and below (slow growers), in section 5.5 The same applied for all significant slow growing mutants. The growth rates obtained for each deletion mutant analyzed in the pool, comprehending over ~2,500 mutants is available at section 5 in the Appendices.

With the information obtained with this fitness profiling study we also acquired valuable information regarding the specific growth rates of all detected mutants
present in our deletion library. This should prove to be a valuable resource for using as a comparative reference when interpreting and analyzing posterior studies using the same deletion library or parts of it. It has already been of great value in helping with the interpretation of the quiescence-proliferation transition profiling presented in chapter 5.

An important examination that could be performed would be an extensive comparative analysis between the growth profiling of our fission yeast haploid deletion library presented in chapter 5 and other important growth screen studies performed with the same deletion library source Kim et al.\textsuperscript{139} and Han et al.\textsuperscript{137} Also, with similar studies (in a pooled fashion) in budding yeast.\textsuperscript{138}

7.6 Functional analysis of the q-p transition - focus on autophagy and chromatin mediation

The main aim of this PhD study concerned the study of the quiescence-proliferation transition, shedding some light on our understanding of the basis of the dynamics exhibited upon the q-p transition and what could be affecting or controlling its kinetics. For this we employed the massive profiling study presented in chapter 5, together with a global analysis and interpretation of the results obtained. We then decided to undertake a more systematic and functional analysis of the large amount of information gathered in this study, in order to uncover which specific biological processes, and how, were affecting the dynamics of the q-p transition. Guided by the results obtained in the overall q-p profiling presented in chapter 5 we decided to follow-up two of the highlighted biological processes in a thorough examination,
autophagy and chromatin-regulation. Autophagy was the most enriched biological process in the gene lists obtained in the q-p profiling analysis (table 4.5). Regarding chromatin-regulation, we identified swd1 in the gene lists of mutants exhibiting altered q-p transition kinetics from both short and deep quiescence. swd1 is part of chromatin-regulation complex Set1C, that methylates H3K4, involved in regulation of gene expression and other DNA-related processes. Given our interest in the regulation and control of the q-p transition dynamics, together with the fact that in fission yeast there is a massive change in the transcriptional program with over half the genome undergoing differential regulation upon this transition, we decided to investigate chromatin-regulation and swd1 in particular in a more comprehensive manner.

7.6.1 Autophagy

Autophagy has been shown to be an essential biological process during cellular quiescence. This is mainly because it allows the recycling of biosynthetic substrates through the degradation of the cellular macro-components, such as organelles, proteins and other macromolecules. During a quiescent state, cells rely on the degradation of their own proteome to obtain an amino acid reservoir mainly required for de novo protein biosynthesis, which ensures that cells are able to maintain an active metabolism and prolong viability. Furthermore, cells mutated for autophagy factors have been shown to rapidly lose viability during quiescence, underlining the importance of this process during this cellular state. Moreover, studies have shown that mitophagy is an essential process during quiescence in proteosome-disrupted mutants. This was shown to be caused by accumulating levels of ROS that resulted from the activity of mitochondria. In this study the proteosome system was found essential for maintenance of viability in quiescence. Proteasome
dysfunction in quiescence elicited defensive responses, mainly the production of antioxidant components and the degradation of mitochondria by autophagy. It was further concluded that mitochondrial degradation by autophagy constituted one of many anti-ROS protection events that occurred after proteasome dysfunction in quiescence.\textsuperscript{72-74} We thus decided to investigate the q-p profiles of all the autophagy mutants profiled during the q-p transition (figure 6.1; figure 6.2) to obtain an idea on how were these mutants behaving upon this transition.

Unexpectedly, we found that most autophagy mutants analyzed exhibited an early q-p transition after a short quiescence period of 2 days, when autophagy has been found to be required during quiescence (where most mutants have been shown to have lost almost all viability after \textasciitilde 10 days in quiescence).\textsuperscript{68-70} Moreover, auxotrophic mutants (mutants with a nutritional requirement not present in the wild-type organism) were found to drastically lose all viability after only a few hours upon entering quiescent. It was thus shown that autophagy plays an important role as soon as cells start entering a quiescence state. For that reason, it would be expected that autophagic mutants would undergo some difficulties during quiescence. However, the early q-p transition obtained for all mutants suggest that autophagy mutants likely retain significant levels of cellular viability. In addition, autophagy has been shown to balance growth-related processes by controlling the levels and availability of biosynthetic products. More specifically, autophagy has been shown to contribute to growth inhibition in tumor cells.\textsuperscript{56,57} As autophagy mutants did not exhibit significant differences in growth rate in the fitness profiling presented in chapter 5 (section 5 in the Appendices), we hypothesize that the early q-p transitions observed could be due to an intrinsic advantage exhibited by autophagy mutants during the switch from quiescence to proliferation. One possible explanation is that the disruption of autophagy could lead to lower levels of degradation of cellular macro-components, which could allow a
steady return to proliferation. However, the q-p profile information is not sufficient to adequately address the question for the basis of the interesting q-p profiles reported here.

Two mutants were found to deviate from the general early q-p transition trend (fig. 6.1C and 6.1D). These mutants were deleted for two genes involved in vacuolar-membrane processes, *fsc1* and *aut12*. *aut12* budding yeast ortholog Mon1 is part of a guanine exchange factor dimmer that regulates Rab GTPases delivery to target membranes. Ultimately, it is involved in organelle maturation by incorporating new cargo through vesicle fusion events regulated by organelle-specific Rab GTPases.\textsuperscript{162-164} Vacuole membrane protein Fsc1 has been reported to be uniquely required for autophagosome-vacuole fusion and to constitute a specific control of autophagic traffic at the vacuolar fusion step.\textsuperscript{81} However, the impaired q-p profiles exhibited by *fsc1*Δ after short quiescence indicated that this mutant is unable to return to proliferation after short quiescence. This strongly suggests an alternative or broader roles for Fsc1, at least during quiescence or q-p transition. Indeed, in budding yeast all known mutants blocking autophagosome-vacuole fusion are also defective for vacuolar fusion in the CPY and ALP pathways, as well as vacuole–vacuole homotypic fusion.\textsuperscript{54,55,76,81} Thus, although it was found that *fsc1* was not involved in autophagosome-vacuole fusion of these pathways, it is possible that it possesses other unidentified roles, especially given the little to no attention dedicated to this gene in these two yeast model systems. *fsc1* Human ortholog periostin gene POSTN is a matricellular protein mainly known as a tumor promoting factor.\textsuperscript{165,166} Thus it could be that deletion of *fsc1* could lead to growth defects. Growth rate estimations obtained for *fsc1*Δ in our fitness profiling analysis were inconsistent between tags, with uptag revealing normal growth (~158 min) and downtag indicating a growth rate much slower than the average (~248 min). Therefore, it was not possible to conclude
whether growth rate was significantly affected in \( fsc1\Delta \). Interestingly, Periostin is transiently upregulated during cell fate changes, either physiologically or pathologically.\(^{165,166}\) Therefore, it could also be considered that the impaired q-p transition observed in \( fsc1\Delta \) could be reflecting an intrinsic defect upon q-p transition. Autophagy did not appear to be crucial during short quiescence, as suggested by the early q-p transition profiles exhibited by autophagy mutants (figure 6.1). Therefore, the impaired q-p transitions revealed by these vacuole-membrane associated mutants suggest a more general and wide vital role for membrane fusion events and organelle maturation during quiescence not necessarily involving autophagy.

The q-p transition profiles exhibited by the autophagy mutants from a state of deep quiescence (10 days) strongly suggested that these mutants were unable to undergo this transition and resume proliferation. In fact, autophagy mutants are known to have lost all viability after 10 days in quiescence. This is an expected observation, as autophagy mutants would not have survived the deep quiescence period induced to the cells.

\( atg20\Delta \) revealed an early q-p transition profile after deep quiescence (figure 6.2). The fact that this mutant was able to resume proliferation demonstrated that contrary to most autophagy mutants, \( atg20\Delta \) retained viability after deep quiescence. \( atg20 \) owes its name to the fact that it is predicted to be the ortholog of the budding yeast gene with the same name. There, \( atg20 \) codes for a sorting nexin involved in the CVT pathway.\(^6\) Interestingly, although the enrichment of genes associated with CVT pathway GO term, in the GO analysis of the q-p transition profiling, this pathway has not been identified in \( S. pombe \).\(^{153,158,160}\) Nonetheless, the fact that \( atg20 \) was able to resume proliferation alone demonstrates that it remained alive and viable and therefore is not essential for autophagy (again, autophagy mutants markedly loose
viability after 5 days in quiescence). This suggests that the main role of atg20 could be unrelated to autophagy and concerned with a different biological process. Another possible explanation is that a CVT pathway could exist in S. pombe, but that for some reason, for example due to great divergence from that characterized in budding yeast, it is yet to be identified.

Interestingly, one autophagy mutant, atg1803Δ or atg18cΔ, revealed a q-p profile strongly suggesting that atg18cΔ probably retained high viability after deep quiescence thus being able to exhibit an unimpaired q-p transition (figure 6.2). Furthermore, the time required for this transition to occur was strongly reduced. Therefore, it appears that atg18c might not be as crucial for autophagy as other autophagic factors. However, it was recently found that atg18c is required for processing of Atg8, a central event in autophagy. There are 3 atg18 homologues in fission yeast: atg18a, atg18b and atg18c. Thus, it is possible that there is some degree of redundancy regarding the role of atg18c. Another possibility is that atg18c could function as an inhibitor of autophagy of some sort, thus explaining why it did not fit the common trend observed for autophagy mutants. However, even if this was the case for both hypotheses mentioned, it would still not fully explain the atypical early q-p transition profile obtained. Furthermore, the fitness profiling study revealed that atg18cΔ exhibited a comparable growth rate to that of fission yeast wt (section 5 in the Appendices). All together, this information seems to suggest a role for atg18c that somehow contributes to regulate or restrain the kinetics of q-p transition.

7.6.2 Chromatin-regulation factors

In the analysis of the q-p transition profiling we identified a chromatin regulation-related mutant - swd1Δ - as significantly delayed upon this transition. Swd1 is a
subunit of the Set1 complex involved in chromatin modification through the mono, di and tri-methylation of H3K4. This histone post-translational modification has been shown to constitute a mark of recent transcriptional activity, leading to its association with epigenetic phenomena. Also, it has been shown to be required for the transmission of transcriptional programs during development. Given our interest in the identification of genetic factors involved in the control and regulation of the q-p transition dynamics we became interested in this gene when we observed that its deletion mutant exhibited a delayed q-p transition. We thus selected swd1 for follow-up investigation. We carried out a clustering analysis of the data set of q-p profiles from the mutants found to exhibit significantly altered q-p transition kinetics (figure 6.3). This analysis allowed us to identify 2 other Set1C subunits deletion mutants with similar altered q-p transition profiles, set1Δ and spf1Δ, reinforcing the idea that Set1C could be affecting this transition. We then searched for other possible partners of Set1C by analyzing the data set from the mutants exhibiting altered q-p transition profiles with a fission yeast protein interactions prediction tool developed by our group. This bioinformatics resource, named plnt, identified several targets as interactors with the Set1C, with several of these proteins interactions already identified (figure 6.4). Interestingly but not surprisingly, most of these genes were involved in genome regulation. We thus added the deletion mutants of these regulatory factors to our analysis. We profiled these deletion mutants with a high-throughput high-resolution microfermenter platform, which provided high quality informating regarding their q-p transition upon short and deep quiescence. With this approach, and employing a mathematical model specifically developed to analyze the high-resolution q-p profiles obtained, we were able to confirm the delayed q-p transition exhibited by Set1C mutants set1, swd1 and spf1 (fig. 6.5 and fig. 6.6). Unfortunately, no other regulatory factor was found to have significantly altered q-p
transition behaviour. Therefore, we believe that Set1C, namely its set1, swd1 and spf1 subunits, might have a significant role upon the regulation of the dynamic behavior upon q-p transition and thus constitute promising targets for the continuation of follow-up studies aiming to elucidate this intriguing cellular phenomenon. Set1, the catalytic subunit of Set1C, is the protein responsible for methylating the fourth lysine of the tail of histone 3. Its role has been the object of extensive study, particularly its role in the regulation of gene expression.\textsuperscript{150-152} H3K4me3 has been shown to act as a memory of recent transcriptional activity.\textsuperscript{105} Upon transcription initiation RNA Polymerase (Pol) II recruits the Set1C complex that in turn methylates the histones associated with the transcribed genes. This histone modification can remain even long after transcription had ceased. In budding yeast H3K4me3 was detected in Gal gene cluster for five hours, or the equivalent to 2 complete yeast cell cycles, after Gal transcription had been suppressed.\textsuperscript{105}

We believe that it would be important to address the basis of the delayed q-p transition observed in these Set1C subunits. It would be interesting to know whether this delay is associated with a delay in the onset of S phase and respective DNA replication occurring during the q-p transition. This could be relatively easily addressed by performing fluorescence activated cell sorting (FACS) analysis on these Set1C mutants upon q-p transition. Additionally, it would be interesting to analyze the expression profiles of these mutants upon q-p transition, as this would allow us to understand whether Set1C plays a role in the regulation of the gene expression program associated with this transition. Finally, we should gain valuable insights by observing the chromatin-associated profiles upon q-p transition, specifically the distribution over time of H3K4 post-translational methylation events conducted by Set1C during the transition from q-p. This could prove extremely insightful towards
the understanding of the role played by Set1C upon q-p transition and characteristic dynamics.

7.7 Final remarks

We believe that the profiling studies performed in this project generated valuable sources of information regarding a central key cellular event - the transition from a quiescent to a proliferative state. Furthermore, the work conducted helped delineating novel exciting research routes for understanding this intriguing phenomenon by uncovering several promising targets for the regulation of the highly dynamic transition between quiescence and proliferation.

In addition, the fitness profiling of the deletion library presented in chapter 5 provided extremely important and useful information that should helpful for the interpretation of any future work conducted with referred mutant collection (when performing barcode sequencing), greatly improving (and facilitating) the potential of this valuable resource.

Furthermore, we successfully implemented a state-of-the-art strategy in the Lab - barcode-sequencing - for high-throughput genome-wide profiling and screening studies in fission yeast, that should prove highly valuable and yield important contributions for the use of this technique in future work developed in the Lab.
References


38. Loewith, R., et al. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* 10, 457-468.


43. Weisman, R., Roitburg, I., Schonbrun, M., Harari, R., & Kupiec, M. (2007). Opposite effects of Tor1 and Tor2 on nitrogen starvation responses in fission yeast. *Genetics* 175, 1153-1162.

44. Ikai, N., Nakazawa, N., Hayashi, T., & Yanagida, M. (2011). The reverse, but coordinated, roles of Tor2 (TORC1) and Tor1 (TORC2) kinases for growth, cell cycle and separase-mediated mitosis in Schizosaccharomyces pombe. *Open Biol* 1, 110007.


74. Takeda, K., & Yanagida, M. (2010). In quiescence of fission yeast, autophagy and the proteasome collaborate for mitochondrial maintenance and longevity. Autophagy 6, 564-565. [Epub ahead of print].


147. http://www.imperial.ac.uk/people/v.shahrezaei


170. Sideri et al. (2015) Parallel Profiling of Fission Yeast Deletion Mutants for Proliferation and for Lifespan During Long-Term Quiescence. *G3* vol. 5 no. 1 145-155
Appendices

In this section we have included all the supplementary information addressed throughout the Thesis. The numbering of each Section refers to the respective Thesis chapter.

Whenever any given information or topic was not feasible for being included in this text document, due to its size or nature, a digital file was added instead and included in the corresponding Section folder.

Section 1

*S. pombe* Genome Statistics as currently present in PomBase are in excel file “Fission yeast Genome Statistics.xlsx”.

Section 2

This section displays the supplementary information regarding the Methodology chapter (chapter 2).

Construction of a fission yeast prototroph deletion library

For the construction of a fission yeast prototroph deletion mutant collection a procedure similar to *S. pombe* Synthetic Genetic Array (SGA) was followed. A Bioneer fission yeast deletion library v2.0 was crossed with a *S. pombe* 972- strain on smart-pool-array (SPA) plates and left to sporulate at 25°C for 2 days. The plates were transferred at 42°C and left for 3 days in order to eliminate vegetative cells and enrich
for spores. Spores were then transferred to YES media and left to germinate for 2 days. Following that, the library was successively spotted on EMM minimal media to select for prototrophy and YES media containing antibiotic G418 to select for the presence of the kanMX4 cassette used for the deletion mutant generation. We performed 3 rounds of EMM and YES+G418 selection, as indicated by Bioneer.

**Construction of a pooled fission yeast prototroph deletion library**

Using the Singer RoToR HDA we compacted the prototroph mutant library in nine 384-well density plates. Mutants were grown on YES containing Ampicillin, Kanamycin and G418 for 2 days. 5 ml of freezing YES medium containing 20% Glycerol were added on each plate. Colonies were washed off the plates, pooled together, aliquoted and stored at -80 °C. Pooled library cultures of OD\textsubscript{600} 0.6 at 32 °C in EMM minimal media without nitrogen tested negative for the existence of spores.

**Fission yeast prototroph deletion library strains list**

All the fission yeast prototroph deletion mutants obtained with our prototroph library construction that could be detected through our decoding analysis are listed, together with the respective barcode (uptag and downtag) information, on the excel file labelled “FY_Prototroph_Library.xlsx”. This file is included in the folder corresponding to Section 2.

**Primer sequences used for barcode amplification through barcode-sequencing**

Primers designed for processing uptag barcodes are labelled “Up”. Those designed for processing downtag barcodes are labelled “Dn”.
40 different primers were designed to allow multiplexing of up to 20 uptag and 20 downtag samples. They are labelled as “Up1-20” and “Dn1-20”.

An additional pair of primers was designed for the integration of the Illumina sequencing adaptors, as indicated by Illumina. These were labelled “SeqF” and “SeqR”.

“F” depicts forward primers whilst “R” refers to reverse primers, in relation to the barcodes orientation, 5’->3’.

**Primers for bar-seq PCR 1 - barcode amplification and integration of multiplex-indexes**

The letters in bold correspond to the multiplex-index sequence, specific for each primer and used to identify the sample of origin.

**Uptags**

UpF1

CACGACGCTCTTCCGATCTACGTGAGGCAAGCTAAGATATC

UpF2

CACGACGCTCTTCCGATCTTCTTGAGGCAAGCTAAGATATC

UpF3

CACGACGCTCTTCCGATCTTGCAGAGGCAAGCTAAGATATC
UpF4
CACGACGCTCTTCCGATCTTGTTAGGCAAGCTAAGATATC
UpF5
CACGACGCTCTTCCGATCTCTGAGGCAAGCTAAGATATC
UpF6
CACGACGCTCTTCCGATCTCGATGAGGCAAGCTAAGATATC
UpF7
CACGACGCTCTTCCGATCTAGCTGAGGCAAGCTAAGATATC
UpF8
CACGACGCTCTTCCGATCTCAGTGAGGCAAGCTAAGATATC
UpF9
CACGACGCTCTTCCGATCTATATGAGGCAAGCTAAGATATC
UpF10
CACGACGCTCTTCCGATCTTATATGAGGCAAGCTAAGATATC
UpF11
CACGACGCTCTTCCGATCTACTGAGGCAAGCTAAGATATC
UpF12
CACGACGCTCTTCGATCTAGAGGGCAAGCTAAGATATC
UpF13
CACGACGCTCTTCGATCTTGTTAGGGCAAGCTAAGATATC
UpF14
CACGACGCTCTTCGATCTCATAGGGCAAGCTAAGATATC
UpF15
CACGACGCTCTTCGATCTGAAGGGCAAGCTAAGATATC
UpF16
CACGACGCTCTTCGATCTATCGGGCAAGCTAAGATATC
UpF17
CACGACGCTCTTCGATCTCTAGGGCAAGCTAAGATATC
UpF18
CACGACGCTCTTCGATCTTCAAGGGCAAGCTAAGATATC
UpF19
CACGACGCTCTTCGATCTGCCAGGGCAAGCTAAGATATC
UpF20

CACGACGCTCTTCCGATCTGTGTGGGCAAGCTAAGATATC

UpR

AGCAGAAGACGGCATACGAGATATTGGCGTACTGGAGTTTCAGACGTGTGCTCTTC
CGAT CT GCCTTACTTTCGATTTTA

Downtags

DnF1

CACGACGCTCTTCCGATCTGACTCCAGTGTCGAAAATC

DnF2

CACGACGCTCTTCCGATCTGAGCCAGTGTCGAAAATC

DnF3

CACGACGCTCTTCCGATCTGTCAACAGTGTCGAAAATC

DnF4

CACGACGCTCTTCCGATCTGTGTCAGTGTCGAAAATC

DnF5

CACGACGCTCTTCCGATCTGCTACAGTGTCGAAAATC
DnF6
CACGACGCTTTCCGATCTTATAACCAGTGTCGAAAAGTATC

DnF7
CACGACGCTTTCCGATCTTCGAACCAGTGTCGAAAAGTATC

DnF8
CACGACGCTTTCCGATCTATTACCAGTGTCGAAAAGTATC

DnF9
CACGACGCTTTCCGATCTCGTACCAGTGTCGAAAAGTATC

DnF10
CACGACGCTTTCCGATCTAGGACCAGTGTCGAAAAGTATC

DnF11
CACGACGCTTTCCGATCTAACCCAGTGTCGAAAAGTATC

DnF12
CACGACGCTTTCCGATCTAGTCCAGTGTCGAAAAGTATC

DnF13
CACGACGCTTTCCGATCTTCCAGTGTCGAAAAGTATC
DnF14
CACGACGCTCTTCGATCTTCTCCAGTGTCGAAAAGTATC

DnF15
CACGACGCTCTTCGATCTCTTCCAGTGTCGAAAAGTATC

DnF16
CACGACGCTCTTCGATCTATGCCAGTGTCGAAAAGTATC

DnF17
CACGACGCTCTTCGATCTGCGCCAGTGTCGAAAAGTATC

DnF18
CACGACGCTCTTCGATCTGATCCAGTGTCGAAAAGTATC

DnF19
CACGACGCTCTTCGATCTGGCCAGTGTCGAAAAGTATC

DnF20
CACGACGCTCTTCGATCTTAGCCAGTGTCGAAAAGTATC

DnR
AGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCGATCTTTTAGCGTGGTAGG
**Primers for bar-seq PCR 2 - integration of Illumina sequencing adaptors**

SeqF

AATGATACGGCGACCACCGAGATCTACACTCTTTCTACACGACGCTCTTCCGATCT

SeqR

CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTTTCCGATCT

**External strains used to spike the fission yeast pooled prototroph library**

7 barcoded strains, not present in the prototroph pooled library, were introduced to spike the prototroph library and allow performing the control tests presented in chapter 2. For this, a defined number of cells from each mutant strain, as shown in table S2.1 were mixed, aliquoted and added to the pooled library samples. The exact number of cells for each spike strain was determined with a Coulter Counter instrument so that a 40 µl external spikes sample, containing the numbers shown in table S2.1, was added to each pooled library sample before bar-seq processing.
Table S2.1. External deletion strains used as external spikes for bar-seq.

<table>
<thead>
<tr>
<th>Gene barcoded</th>
<th>Cells/sample</th>
<th>Barcode sequence</th>
<th>Spike ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPBC11C11.09c</td>
<td>40 000</td>
<td>AAGGATTGTGTGTGGCCCCGC</td>
<td>Spk1</td>
</tr>
<tr>
<td>SPAC806.04c</td>
<td>20 000</td>
<td>AATTCACCACTCAACG</td>
<td>Spk2</td>
</tr>
<tr>
<td>SPBC29A10.05</td>
<td>10 000</td>
<td>CACCATTTTCTCGCGTCCC</td>
<td>Spk3</td>
</tr>
<tr>
<td>SPAC1A6.08c</td>
<td>5 000</td>
<td>TAGCTCCATCCGCGAC</td>
<td>Spk4</td>
</tr>
<tr>
<td>SPBC1718.07c</td>
<td>2 000</td>
<td>GCGGGAAAGAGGGGCAGGAC</td>
<td>Spk5</td>
</tr>
<tr>
<td>SPAC3G9.11c</td>
<td>1 000</td>
<td>GTGGCTCTCCGGGCTCTTTG</td>
<td>Spk6</td>
</tr>
<tr>
<td>SPAC23A1.16c</td>
<td>500</td>
<td>TAGCACATGCCGGGGGGGTTG</td>
<td>Spk7</td>
</tr>
</tbody>
</table>

Bar-seq sequencing data quality information

Table S2.2 depicts the information regarding the yield and quality of all bar-seq sequencing runs performed in this project. All bar-seq data obtained derived from these sequencing runs.
Table S2.2. Information on the output obtained for the sequencing runs performed in the bar-seq experiments presented in chapters 4 and 5.

<table>
<thead>
<tr>
<th>Sequencing Run</th>
<th>Reads processed*1</th>
<th>Aligned reads*2</th>
<th>Unaligned reads*3</th>
<th>Mismatch reads*4</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>4,474,061</td>
<td>3,605,651 (80.59%)</td>
<td>675,377 (15.10%)</td>
<td>193,033 (4.31%)</td>
</tr>
<tr>
<td>#2</td>
<td>6,154,742</td>
<td>4,284,609 (69.61%)</td>
<td>1,326,481 (21.55%)</td>
<td>543,652 (8.83%)</td>
</tr>
<tr>
<td>#3</td>
<td>8,546,680</td>
<td>7,077,830 (82.81%)</td>
<td>1,159,162 (13.56%)</td>
<td>309,688 (3.62%)</td>
</tr>
<tr>
<td>#4</td>
<td>21,303,444</td>
<td>17,576,350 (82.50%)</td>
<td>2,887,636 (13.55%)</td>
<td>839,458 (3.94%)</td>
</tr>
<tr>
<td>#5</td>
<td>7,579,697</td>
<td>5,600,212 (73.88%)</td>
<td>1,633,917 (21.56%)</td>
<td>345,568 (4.56%)</td>
</tr>
<tr>
<td>#6</td>
<td>9,969,798</td>
<td>6,821,993 (68.43%)</td>
<td>2,564,584 (25.72%)</td>
<td>583,221 (5.85%)</td>
</tr>
</tbody>
</table>

*1 total number of reads processed;

*2 number of successful reads processed with at least one reported alignment;

*3 number of reads that failed to align;
number of reads with alignments suppressed due to more than 1 mismatch (defined cut off).

**Archive of the data obtained with the bar-seq experiments presented in this project**

All the data obtained with the bar-seq experiments presented in this project were compiled and made available as both comma separated values (“.csv”) and text (“.txt”) digital files. These files are available in folder for Section 4 or 5, depending to which experimental study they refer.

These data refer to: the raw unprocessed sequencing bar-seq data, characterized by the number of counts a given read/sequence has been detected or processed; the data obtained with the further computational and statistical analysis performed with DESeq, as presented in the Methodology chapter. The data obtained after DESeq data analysis displays the fold change difference between the given reference sample and the given analysed “treatment” sample, according to the experimental designs described in chapters 4 and 5, and depicted as Log 2 values. Also, each column is labelled with the time-point it refers to. For example, “log2foldchange.T530” refers to the time-point T 530, following the same rationale of the experimental design of the studies presented in chapter 4 and 5.

**Bar-seq raw data**

The bar-seq raw count sequencing data obtained is included in Section 4 and Section 5 folders.

Files corresponding to the studies presented in chapter 4 are included in section 4 and are labelled as follows: “barseq_QtoP_RAW_counts.csv”,

"barseq_QtoP_RAW_counts.csv"
“barseq_QtoP2d_DN.txt”, “barseq_QtoP2d_UP.txt” and “barseq_QtoP10d_DN.txt”, “barseq_QtoP10d_UP.txt”. “QtoP2d” or “QtoP10d” refer to the quiescence-to-proliferation profiling experiments after 2 and 10 days, respectively.

Files corresponding to the studies presented in chapter 5 are included in the folder of section 5 and are labelled as follows: “barseq_EMM_DN.txt” and “barseq_EMM_UP.txt”.

“UP” and “DN” refer to data originating from barcodes uptag or downtag, respectively.

**Bar-seq results upon DESeq data analysis**

Results and information obtained with bar-seq data analysis, as described in the corresponding Methodology section, is included in Section 4 and 5 folders, depending to which chapter each experimental study refers.

Files corresponding to the studies presented in chapter 4 are included in section 4 and are labelled as follows: “barseq_QtoP_DESeq_DN.txt” and “barseq_QtoP_DESeq_UP.txt”.

Files corresponding to the studies presented in chapter 5 are included in section 5 and are labelled as follows: “barseq_EMM_DESeq_DN.txt” and “barseq_EMM_DESeq_UP.txt”.

“log2FoldChange” refers to the log2 of the fold change values as obtained by comparing a given time-point with the reference time-point, time zero or T₀, using DESeq. “padj” refers to the p-value of the corresponding log2 value, adjusted for
multi-test correction. “UP” and “DN” refer to data originating from barcodes uptag or downtag, respectively.

**Estimating the growth rate and doubling time of the deletion mutant library in a pooled environment**

The growth profiling studies presented in chapter 5 were used to estimate the growth rate and corresponding doubling or generation time of the mutants present in the deletion library, when growing in a pooled competitive fashion.

As explained in the Methodology chapter (chapter 2), we combined the mutant pool overall cell density values with the fold change values obtained with DESeq analysis for each of the surveyed time-points, for each mutant with available valid bar-seq data. This resulted in typical discrete exponential growth profiles. By semi-logarithmically transforming the values obtained we could then fit a simple linear model, using R, that allowed us to retrieve the growth rate and doubling time values for each analysed mutant present in the pool. Given that we obtained fold change values for 2 biologically independent mutant pools, for which both uptag and downtag information was available, this resulted in 4 different growth rate and doubling time values for each deletion mutant. The fact that most values exhibited a high degree of consistency within mutants provided a good indicator of the robustness of the results obtained. The growth rate and doubling time values are displayed in the following files, included in section 5: “Growth_Rates_EMM_PoolA_DN.csv”, “Growth_Rates_EMM_PoolA_UP.csv”, “Growth_Rates_EMM_PoolB_DN.csv”, “Growth_Rates_EMM_PoolB_UP.csv”. Predictably, “PoolA” refers to one of the tested mutant pools, labelled as Pool A, and “PoolB” refers to the other mutant pool tested, named Pool B, as described in chapter
5. Also, “UP” and “DN” refer to data originating from barcodes *uptag* or *downtag*, respectively.

*Note:* as it is possible to conclude by observing these growth rate tables, probably due to the minor stringency employed in this approach, some of the mutants were found to exhibit opposite behaviour upon different time-points and/or tags. However, by norm only one of the outcomes was statistically significant which suggested that the values obtained for the converse trend were not a reflection of any real difference but in fact most likely the result of associated technical variance. This apparent ambiguous behaviour could probably be resolved by examining the growth kinetics of the corresponding mutants in a single culture.

**Estimating the quiescence-proliferation transition profiles using a robust mathematical model**

*Using high-density relative biomass information*

In the experimental study presented in chapters 3 and 6, non-growing quiescent cells are induced to resume growth after a designated amount of time in quiescence. Upon induction, after a lag phase whereby cells do not divide and only grow in size, cells eventually start dividing and ultimately resume proliferation. A mathematical model was developed, in collaboration with Dr. Shahrezaei and Pablo Crotti. The description of the model is as follows.

**Description of the analytical model:**

The stochastic process associated with the model is mainly based on the time the cell waits before starting to grow. We define the length of a particular cell $i$ at time $t$ with
where \( n_0 \) is the total number of cells initially present in the population. As a first assumption, we define the lag-time as a random vector \( \tau \) following the probability distribution \( f_{\mu, \sigma^2} \), i.e.,

\[
\tau = (\tau_1, \ldots, \tau_{n_0}) \quad \tau_i \sim f_{\mu, \sigma^2} \quad i = 1, \ldots, n_0,
\]

where the parameters \( \mu \) and \( \sigma^2 \) are respectively the average lag-time and the variance of the lag-time. In addition, under the hypothesis that all cells possess the same initial length \( l_0 \) (variability in the division lengths is not considered here), we formally describe the length of a particular cell \( i \) at time \( t \) as

\[
L_i(t) = \begin{cases} 
  l_0 & t \in [0, \tau_i] \\
  l_0 e^{\lambda(t-\tau_i)} & t > \tau_i 
\end{cases} \quad i = 1, \ldots, n_0
\]

where the elongation of \( L_i(t) \) is exponential with an elongation rate \( \lambda \). The total mass of the system at time \( t \) is then defined as the sum of all lengths, i.e.,

\[
s(t, \mu, \sigma^2, \lambda) := \sum_{i=1}^{n_0} L_i(t).
\]

To approximate the process, based on real data, we employed the average total mass of the system \( s(t, \mu, \sigma^2, \lambda) \), i.e.,
The total number of cells \( n \) describe the length of a particular cell length \( l \). Moreover, and for simplicity, we normalise the data (see below) to obtain

\[
\tilde{s}(t, \mu, \sigma^2, \lambda) := \mathbb{E}[s(t, \mu, \sigma^2, \lambda)] = \mathbb{E} \left[ \sum_{i=1}^{n_0} L_i(t) \right] = \sum_{i=1}^{n_0} \mathbb{E}[L_i(t)] = n_0 \mathbb{E}[L_1(t)] = n_0 \mathbb{E} \left[ l_0 \mathbbm{1}_{\{t \leq \tau_1\}} + l_0 e^{\lambda(t-\tau_1)} \mathbbm{1}_{\{t > \tau_1\}} \right]
\]

(5)

The variable parameters involved in the process that need to be accounted for are the initial number \( n_0 \) of cells in the population, the initial length \( l_0 \) of the cell, the average lag-time \( \mu \) of the cells, the variance \( \sigma^2 \) of the lag-time and the elongation rate \( \lambda \) of the cells. The total number of cells \( n_0 \) and the initial length \( l_0 \) can be deducted from the experimental data. Hence, the initial length of the cells is simply the ratio of the real total mass of the system (denoted \( s_{\exp}(t) \) and found experimentally) at time \( t = 0 \) by a fixed \( n_0 \). In other words,

\[
l_0 = \frac{s_{\exp}(0)}{n_0}.
\]

(6)

Moreover, and for simplicity, we normalise the data (see below) to obtain

\[
n_0 l_0 = 1.
\]

(7)
This ansatz reduces the problem to three unknown parameters: $\mu$, $\sigma^2$ and $\lambda$. We numerically compute the starting and finishing points of each real process by finding

\[
\begin{align*}
\text{start} & := \arg \min_{t} \left\{ \frac{d\bar{s}(t)}{dt} = 0 \right\}, \\
\text{stop} & := \arg \min_{t} \left\{ \frac{d\bar{s}(t)}{dt} < 0 \mid t > \text{start} \right\}.
\end{align*}
\]

The averaged trajectory amongst the technical replicates is then normalised with the minimum mass, to start the process with a unit mass and thus agree with Eq.(7). For each triplet of parameters in

\[
\{(\mu, \sigma^2, \lambda) \mid \mu > 0, \sigma^2 > 0, \lambda > 0\},
\]

we run an optimisation algorithm on the error function

\[
e(\mu, \sigma^2, \lambda) := \sum_{t \in T} \left( \frac{s_{\text{exp}}(t) - \bar{s}(t, \mu, \sigma^2, \lambda)}{\sigma_{\text{exp}}(t)} \right)^2,
\]

where $T$ is the time points interval of the real data, $s_{\text{exp}}(t)$ is the average trajectory over multiple $s_{\text{exp}}(t)$ and $\sigma_{\text{exp}}(t)$ is the standard deviation between all trajectories from the real data.
**Using bar-seq NGS information**

An adapted version of the method presented above was also used to estimate the quiescence-proliferation transition profiles of all the mutants analyzed in the bar-seq quiescence-proliferation profiling studies. The quiescence-proliferation profiles obtained are displayed in the file “bar_seq_lagtime.xlsx”, included in section 4.

In the data frame present in this excel file, “mu” refers to the character μ used to designate the average lag time in this model, as explained in the previous sub-section. Also, “sigma” refers to the character σ² used to designate the variance of the lag time, and “lambda” refers to the character λ, designated to represent the cellular elongation or growth rate, more thoroughly explained in the previous sub-section. Finally, “up” and “dn” refer to data originating from barcodes uptag or downtag, respectively. “avg” refers to the average between the uptag and downtag values. All values are in hours, except for those regarding the elongation or growth rate.

Due to a relatively poor consistency across the full data sets obtained (as a result of the low density of the data set, as compared to the data originating from BioLector analysis), the list of mutants containing valid information was filtered by considering the mutants with an absolute difference for the uptag and downtag values equal or less than:

a) 1 hour for the lag-time

b) 0.25 hour for the std.deviation

c) 0.005 length/hour for the elongation rate.
By discarding the mutants whose data did not comply with the conditions delineated above, we obtained the following correlations (cor) between uptag and downtag barcodes.

**Quiescence-proliferation from short quiescence (2 days) - 756 mutants**

\[
cor(\mu_{up}, \mu_{down}) = 0.64932
\]

\[
cor(\sigma_{up}, \sigma_{down}) = 0.5208695
\]

\[
cor(\lambda_{up}, \lambda_{down}) = 0.3350419
\]

**Quiescence-proliferation from deep quiescence (10 days) - 476 mutants**

\[
cor(\mu_{up}, \mu_{down}) = 0.8465322
\]

\[
cor(\sigma_{up}, \sigma_{down}) = 0.3207684
\]

\[
cor(\lambda_{up}, \lambda_{down}) = 0.6643687
\]

We considered these values to be rather reasonable. The filtering steps applied led to a decrease of the number of mutants with valid information, from an initial dataset with ~2500 mutants to 756 and 476, from short (2 days) and deep (10 days) quiescence states, respectively.
**Section 3**

This section displays the supplementary information regarding chapter 3.

Estimating the quiescence-proliferation transition profiles using a robust mathematical model

Please see sub-section with the same name in section 2 for a detailed description of this method, more specifically the part titled “Using high-density relative biomass information”.

Results and data discussed in chapter 3

All the data concerning the figures and results analyzed and discussed in chapter 3 section 3.2 is displayed in table S3.

**Table S3. Results obtained from the analysis of the quiescence-proliferation profiles after applying the mathematical model described in section 2 of the Appendices.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Days in Quiescence</th>
<th>Average Lag-time</th>
<th>St.Dev Lag-time</th>
<th>Elongation Rate</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1</td>
<td>6.53</td>
<td>2.73</td>
<td>0.109</td>
<td>0.0002</td>
</tr>
<tr>
<td>wt</td>
<td>1</td>
<td>14.78</td>
<td>2.88</td>
<td>0.115</td>
<td>0.003</td>
</tr>
<tr>
<td>wt</td>
<td>1</td>
<td>12.52</td>
<td>2.73</td>
<td>0.147</td>
<td>0.099</td>
</tr>
<tr>
<td>wt</td>
<td>2</td>
<td>3.31</td>
<td>1.41</td>
<td>0.095</td>
<td>0.059</td>
</tr>
<tr>
<td>wt</td>
<td>2</td>
<td>15.21</td>
<td>5.79</td>
<td>0.128</td>
<td>0.003</td>
</tr>
<tr>
<td>wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>wt</td>
<td>2</td>
<td>17,72</td>
<td>6,19</td>
<td>0,133</td>
<td>0,003</td>
</tr>
<tr>
<td>wt</td>
<td>2</td>
<td>14,62</td>
<td>5,41</td>
<td>0,117</td>
<td>0,001</td>
</tr>
<tr>
<td>wt</td>
<td>2</td>
<td>11,28</td>
<td>4,17</td>
<td>0,103</td>
<td>0,012</td>
</tr>
<tr>
<td>wt</td>
<td>2</td>
<td>12,18</td>
<td>5,47</td>
<td>0,106</td>
<td>0,036</td>
</tr>
<tr>
<td>wt</td>
<td>2</td>
<td>12,92</td>
<td>5,46</td>
<td>0,115</td>
<td>0,005</td>
</tr>
<tr>
<td>wt</td>
<td>2</td>
<td>14,50</td>
<td>2,73</td>
<td>0,118</td>
<td>0,0317</td>
</tr>
<tr>
<td>wt</td>
<td>2</td>
<td>16,21</td>
<td>4,94</td>
<td>0,153</td>
<td>0,037</td>
</tr>
<tr>
<td>wt</td>
<td>3</td>
<td>14,68</td>
<td>2,89</td>
<td>0,122</td>
<td>0,083</td>
</tr>
<tr>
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<td>24,86</td>
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<td>0,136</td>
<td>0,062</td>
</tr>
</tbody>
</table>

**Section 4**

This section displays the supplementary information regarding chapter 4.

List of time-points sampled in the quiescence-proliferation profiling studies
The full list of time-points sampled in the quiescence-proliferation profiling studies presented in chapter 4, and respective labelling, were:

1. before inducing cells to stop proliferating and enter quiescence (upon depleting the nitrogen source from the media) - $Q_{\text{entry}}$
2. before inducing cells to exit quiescence and resume proliferation (upon replenishing the nitrogen source from the media) - time zero or $T_0$
3. 150 min upon quiescence exit - $T_{150}$
4. 220 min upon quiescence exit - $T_{220}$
5. 330 min upon quiescence exit - $T_{330}$
6. 390 min upon quiescence exit - $T_{390}$
7. 530 min upon quiescence exit - $T_{530}$
8. 625 min upon quiescence exit - $T_{625}$
9. 690 min upon quiescence exit - $T_{690}$
10. 750 min upon quiescence exit - $T_{750}$
11. 890 min upon quiescence exit - $T_{890}$
12. 980 min upon quiescence exit - $T_{980}$

For a clearer visualisation of this time-course see the experimental design of the quiescence-proliferation profiling studies described in chapter 4, figure 26.

**Compilation of all the results obtained in the quiescence-proliferation profiling studies**

*All results obtained, including non-significant results*
Please see the sub-section “Archive of the data obtained with the bar-seq experiments presented in this project” in section 2. As explained there, all data regarding these studies is located in the folder of section 4.

*All the significant results obtained*

We also compiled only the significant results obtained in the quiescence-proliferation profiling studies, for an easier inspection of this information. There we selected only those mutants found to exhibit a statistically significant result in at least one of the time-points analyzed. These results are located in the files labelled “barseq_QtoP2d_Sig.csv”, referring to the data from the quiescence-proliferation studies from a period of short quiescence (2days), and “barseq_QtoP10d_Sig.csv”, referring to the data from the quiescence-proliferation studies from a period of deep quiescence (10days), included in the folder respective of section 4.

“log2FolChange” refers to the log2 of the fold change values as obtained by comparing a given time-point with the reference time-point, time zero or T₀, using DESeq. “padj” refers to the p-value adjusted for multi-test correction. “UP” and “DN” refer to data originating from barcodes uptag or downtag, respectively.

**Section 5**

This section displays the supplementary information regarding chapter 5.

Results from the semi-conservative analysis of the growth profiling study

In the semi-conservative analysis we included all the mutants found to exhibit statistically significant results in at least one of the analyzed time-points.
The full data set of the results obtained in the semi-conservative analysis of the growth profiling study presented in chapter 5 is available in the file named “barseq_EMM_Sig.csv”, included in the folder respective of section 5.

“log2FoldChange” refers to the log2 of the fold change values obtained by comparing a given time-point with the reference time-point, time zero or T₀, using DESeq. “padj” refers to the p-value adjusted for multi-test correction. “UP” and “DN” refer to data originating from barcodes uptag or downtag, respectively.

**Estimating the growth rate and doubling time of the mutants profiled using bar-seq**

Please see section 2 regarding this subject.

We also used the mean of all 4 values obtained in order to obtain a single growth rate and doubling time value associated with each deletion mutant strain. This information is contained in the file “GrRate_DT_EMM.xlsx”, included in the folder of section 5. “pA” and “pB” refer to pool A and pool B, respectively. Also, “UP” and “DN” refer to the information obtained from uptag and downtag barcodes, respectively. The columns depicting “avg” refer to the averaged final value.

**Section 6**

This section displays the supplementary information regarding chapter 6.

**Genes associated with the Gene Ontology Term - Autophagy**

All the genes associated with the GO Term Autophagy (GO:0006914 - autophagy), as described in the reference fission yeast database PomBase, were compiled in the
excel document “GO_Autophagy_genes.xlsx”, included in the folder respective of section 6.

**Clustering analysis of the altered q-p transition profiles as obtained with bar-seq screen**

For this clustering analysis we used all the q-p transition profiles found statistically significant with DESeq as suggestive of an altered q-p transition. The list of deletion mutants analyzed is depicted at the excel documents “barseq_QtoP2d_Sig.csv” and “barseq_QtoP10d_Sig.csv”, present in the folder of section 4.

**pINT analysis - protein predictions for Set1 Complex subunits highlighted in the clustering analysis presented in chapter 6**

The clustering analysis presented in chapter 6 highlighted Set1 and two other subunits from the Set1 Complex, with the corresponding deletion mutants exhibiting an altered quiescence-proliferation transition. We thus investigated, amongst the complete set of proteins whose corresponding gene deletion mutants were also found to exhibit altered dynamics upon the quiescence-proliferation transition, which of these proteins could be associated with Set1 and Set1 Complex, using the bioinformatics resource pINT. The list of Set1 protein interactions as predicted by pINT were compiled in the file “Set1_pIntScores.txt”, included in the folder of section 6.

SVM (support vector machine) score and RF-score, used in this approach for the quantification of the interactions predicted, constitute standard machine learning measures that quantify the likelihood of an interaction prediction.
**Gene list of chromatin-associated deletion mutants considered for follow-up analysis**

The proteins found in pINT to exhibit a greater likelihood of interacting with the Set1 Complex subunits highlighted in chapter 6, and whose deletion mutants were also found to exhibit an altered quiescence-proliferation transition, were considered for downstream analysis. The full list of the corresponding genes and respective deletion mutants are available in table S6.1.

**Results from the sensitive analysis of the BioLector relative biomass high-density data**

The robust mathematical model applied to analyze the quiescence-proliferation transition data obtained with the BioLector high-sensitivity platform returned a rich data set regarding the kinetics of this transition from many analyzed mutants. This information is available at table S6.2.

**Table S6.2 Quiescence-proliferation transition kinetics.** Estimated lag time during the quiescence-proliferation transition (and associated growth/elongation rates), after variable periods in a quiescent state.

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<thead>
<tr>
<th>Strain</th>
<th>Days in Quiescence</th>
<th>Lag-time</th>
<th>St.Dev Lag-time</th>
<th>Elongation Rate</th>
<th>Error</th>
</tr>
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<tr>
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</tr>
<tr>
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<td>0,105</td>
<td>0,002</td>
</tr>
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<td>0,101</td>
<td>0,006</td>
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</table>

Also, in order to acquire more sensitive information regarding the intrinsic growth rate of the deletion mutants, we analyzed some deletion mutants without having been subjected to a preceding period in quiescence. The information regarding the intrinsic growth rate and kinetics displayed by these mutants is available in the table S6.3.
Table S6.3 Estimated growth/elongation rates for some deletion mutants grown without a prior quiescent state.

<table>
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<th>Growth Rate</th>
<th>Error</th>
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<tr>
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<td>0.011</td>
</tr>
<tr>
<td>swd3Δ</td>
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<td>0.006</td>
</tr>
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</tr>
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<td>0.0004</td>
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<td>snt2Δ</td>
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<td>0,041</td>
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</table>