UNIVERSITE SORBONNE PARIS CITE

UNIVERSITE PARIS DIDEROT (PARIS 7)

THÈSE

Pour obtenir le grade de DOCTEUR DE L'UNIVERSITÉ PARIS DIDEROT

Spécialité: Biophysique

École Doctorale Frontières du Vivant (ED 474)

Laboratoire de Pathologie et de Virologie Moléculaire

Laboratoire Matière et Systèmes Complexes

Memory of stress response in the budding yeast *Saccharomyces cerevisiae*

Présentée par

Zacchari Ben Meriem

Thèse dirigée par Emmanuelle FABRE et Pascal HERSEN

Soutenue le 26 Novembre 2018 à l'Université Paris Diderot

Teresa TEIXEIRA	Directrice de recherche, CNRS	Rapporteuse
Philippe NGHE	Maitre de conférence, ESPCI	Rapporteur
Isabelle SAGOT	Directrice de recherche, CNRS	Examinatrice
Gaël YVERT	Directeur de recherche, CNRS	Examinateur
Pascal HERSEN	Directeur de recherche, CNRS	Directeur de thèse
Emmanuelle FABRE	Directrice de recherche, CNRS	Directrice de thèse

Acknowledgements

Many people have contributed to the work presented here, through scientific exchange, personal support or both.

I would like to thank the members of the Lab513 past and present, especially Artémis Llamiosi for introducing me to microfluidics, molecular biology and your constant support, Jean-Baptiste Lugagne for all the help he provided me with at any time, Zoran Marinkovic for all the laughs, scientific talks and work that we did, Céline Cordier for your friendship and all the chats aound a coffee (I will buy the milk, I promise), Fabien Duveau for your friendship and all the great talks about science, especially evolution, Adrien Hallou and Adrien Marck for all the fun we had whether in poetry, politics or science. I would also like to thank, Jean-Louis Plouhinec (the fight always continues!), Sara Bonavia (cucumbers are awesome), Raphael Ponthieu (I will miss the skateboard), Marguerite Benony (thank you for giving me the opportunity to talk to you about science for your work), Tom Wyatt (I already miss our French/English talks), for their friendship, great scientific and personal exchanges and the great atmosphere they provided in the lab. A thank to Chiara Fracassi who has always been there for me, whether for personal issues or my experimental work. Although not technically a part of the Lab513, but part our family team nonetheless, I wish to thank Srajan Jain, my little brother from another mother, and Etthel Windels, a great friend with whom I have the greatest talks about musicals and biotechnology.

I would like to thank all the members of the MSC lab for all their help and support, in particular Jean-Marc Di Meglio, Gaelle Charron, Benoit Sorre, David Pereira and Alain Richert. I would also like to thank Florence Haudin for her friendship and her help, Victoria Reichel for all the laughs we had in the office and Camille Gaulon for her friendship during difficult times, your constant support and all the fun in the office.

I would also like to thank the members of the CBD team past and present, especially Pierre Thérizols for all his jokes and his great scientific insights, Antoine Canat who has always been greatly passionate about my work and suggested important experiments, Adeline Veillet for her friendship and support, availability for a lunch or a movie, Etienne Almayrac, my teammate on many experiments during the PhD and outside of work, Fabiola Garcia Fernandez who always kind words for me when things did not go as planned and who is a rock I can lean on whenever I needed to, Yasmine Khalil and Julie Renard who greatly helped me with my professional work (especially my PhD presentation, I will never forget your help) as well as with my personal issues, and boy did I bother these two fairies. I would also like to thank Alice Brion who throughout my PhD was a great friend I could always rely on.

I would also like to thank the members of the Laboratory of Pathology and Molecular Virology, in particular Pascale Lessage, Amandine Bonnet and all the GeBi team. I wish to particularly thank Anastasia Barkova for her friendship, great talks about science and philosophy and the fun during conferences.

Obviously I would like to thank my entire family for their support and help during this period of my life where I was not always available and confident. Thank you, sincerely. Also, I wish to thank Louis Olivier, a friend like no other. Thanks for all the adventures we had and I can’t wait for all the adventures we will have again.

I would like to thank Fabien Montel and Tereisa Teixera for agreeing to be members of my PhD committee and for being excellent supports and mentors.

And finally, a huge thank to both my advisers Pascal HERSEN and Emmanuelle FABRE for supporting me during the PhD, giving me the opportunity to do great work and collaborations. Thank you for believing in me.

Table of contents

Abstract

Cellular memory is a critical ability displayed by micro-organisms in order to adapt to potentially detrimental environmental fluctuations. In the unicellular eukaryote *S. cerevisiae*, it has been shown at the population level that cellular memory can take the form of a faster or a decreased response following repeated stresses.

We here present a study on how yeasts respond to short, pulsed hyperosmotic stresses at the single-cell level. We analyzed the dynamical behavior of the stress responsive *STL1* promoter fused to a fluorescent reporter using microfluidics and fluorescence time-lapse microscopy.

We established that pSTL1 displays a dynamical variability in its successive activations following two short and repeated stresses. Despite this variability, most cells displayed a memory of past stresses through a decreased activity of pSTL1 upon repeated stresses. We showed that this memory does not require *do novo* protein synthesis. Rather, the genomic location is important for the memory since promoter displacement to a pericentromeric chromatin domain leads to its decreased transcriptional strength and to the loss of the memory. Interestingly, our results points towards an unreported involvement of the SIR complex on the activity of pSTL1 only when displaced to the pericentromeric domain in our experimental conditions.

This study provides a quantitative description of a cellular memory that includes single-cell variability and points towards the contribution of the chromatin structure in stress memory. Our work could serve as a basis to broader studies on the positioning of stress response genes at subtelomeric positions in the budding yeast, from a genetic point of view as well as an evolutionary one.

Keywords: chromosome organization, cellular memory, single cell, stress response, yeast, microfluidics

Résumé

La mémoire cellulaire est une capacité critique dont font preuve les micro-organismes pour s'adapter aux fluctuations environnementales potentiellement néfastes. Chez l'eucaryote unicellulaire *S. cerevisiae*, il a été montré à l'échelle d'une population que la mémoire cellulaire peut prendre la forme d'une réponse plus rapide ou moins prononcée suite à des stress répétés.

Nous présentons ici une étude sur la façon dont les levures réagissent à des stress hyperosmotiques de courte durée à l'échelle de la cellule unique. Nous avons analysé le comportement dynamique du promoteur *STL1*, exprimé en condition de stress osmotique, et fusionné à un rapporteur fluorescent en faisant usage de microfluidique et de microscopie à fluorescence.

Nous avons établi que pSTL1 présente une variabilité dynamique dans ses activations successives après deux stress courts. Malgré cette variabilité, la plupart des cellules présentent une mémoire des stress passés caractérisée par une diminution de l'activité de pSTL1. Nous avons montré que cette mémoire ne nécessite pas de nouvelle synthèse de protéines. L'emplacement génomique est important pour cette mémoire puisque le déplacement du promoteur vers un domaine chromatinien péricentromérique entraîne une diminution de sa force transcriptionnelle ainsi que la perte de la mémoire. Nos résultats indiquent aussi une implication non rapportée du complexe SIR sur l'activité de pSTL1 lorsqu'il est déplacé dans le domaine péricentromérique, dans nos conditions expérimentales.

Cette étude fournit une description quantitative d'une mémoire cellulaire qui inclut la variabilité cellulaire et prend en compte la contribution de la structure de la chromatine sur la mémoire du stress. Nos travaux pourraient servir de base à des études plus larges sur le positionnement des gènes de réponse au stress en positions subtélomériques dans la levure, tant d'un point de vue génétique qu'évolutif.

Mots clés : organisation chromosomique, mémoire cellulaire, cellule unique, réponse au stress, microfluidique

Motivation

All living organisms evolve in constantly fluctuating environments that can take various forms. These environmental changes, defined as stresses, can be harmful and exert a selective pressure on cell survival. Thus, to ensure their survival, living organisms have developed various strategies throughout their evolution, such as adaptation in order to cope with a single environmental change. In some cases, adaptation to a first stress can serve as a learning process when the organisms face subsequent stresses. This type of survival strategy is defined as cellular memory. Therefore, the knowledge of the biological mechanisms of cellular memory is both an important feature to comprehend how micro-organisms as we know them now have evolved, as well as what could be their fate in fluctuating environments.

During my PhD, I aimed to understand the mechanisms behind the cellular memory in the budding yeast, model organism in biology. To that effect, I tested an endogenous yeast genetic system in a well-controlled environment which parameters I could change. This allowed me to test a natural genetic response to environmental change and ask: **do budding yeast develop a memory of short repeated hyperosmotic stresses and if so, what are the underlying biological mechanisms?**

Before explaining the methodology I used to reach my goals (Chapter II) and the results I obtained (Chapter III), I will introduce several key biological concepts in order to understand the importance of cellular memory in the budding yeast in response to environmental stresses as well as the genetic strategies it implies and the importance of single-cell analysis. This understanding requires the knowledge of certain key biological concepts.

To that effect, I will here present *S. cerevisiae*, the model organism on which I have performed my experiments, along with some of its main characteristics, especially nuclear organization. I will present the basis of gene expression, which is intimately linked to the nuclear architecture, and introduce the key notion of epigenetics with an emphasis on the SIR complex which role I have investigated in the context of my experimental work. I will next introduce what is known about cell adaptation to hyperosmotic stresses. The notion of adaptation to repeated changes in the environment, defined as cellular memory, will subsequently be presented through two examples.

My work is an attempt to go further than studies on memory usually performed at the population level, therefore without considering the differences that exist between clonal cells. To that effect, I will present in the last part of the introduction the origin of the cell-cell variability and the advantages of performing studies at the single-cell level, which I have extensively done during my PhD.

Chapter I: Introduction

Table of contents

I. Overview of the S. cerevisiae's genetics

A. Introducing the budding yeast

Fungi constitute a group among the eukaryotes, which includes yeast. *Saccharomyces cerevisiae*, also known as the budding yeast, the baker yeast or even the brewing yeast, is a type of yeast that is 3-6µm large and naturally found on ripped grapes. The budding yeast was first reported to have appeared in Mesopotamia (Liti, 2015), but a recent study tends to put its geographic origin in East Asia (Peter et al., 2018). The budding yeast is supposedly the first micro-organism to have ever been domesticated by mankind. Over the course of thousands of years of human civilization, yeast has been notoriously used for brewing[1] thanks to its ability to perform alcoholic fermentation, and baking.

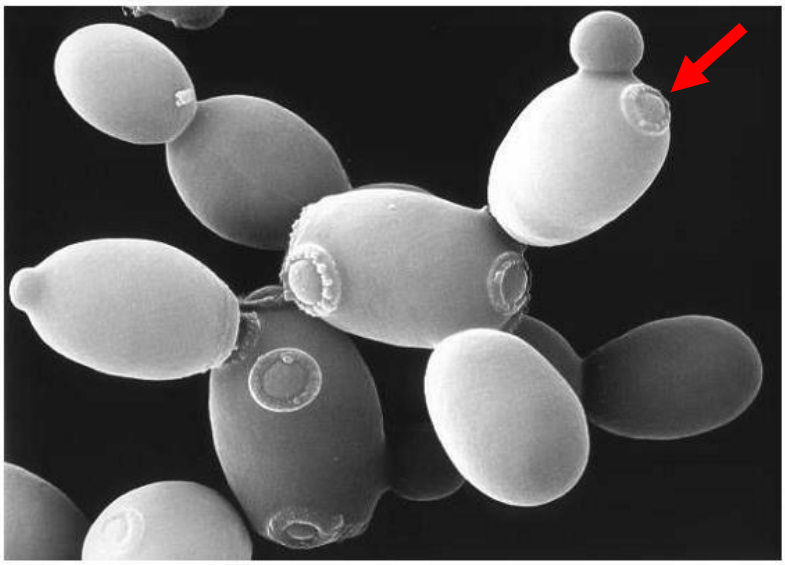


Figure 1-1. Electron microscopy image of haploid budding yeast. The budding yeast, in its haploid form, has an ovoid shape of 3-6µm. The red arrow shows an example of a scar that follows the budding process leading to the creation of a daughter cell. Picture from mpg.de

Although Fungi, plants and animals have evolved separately for several millions of years, they all are eukaryotes thus share many common genetic and metabolic features. However, the budding yeast is easy to select, cultivate, conserve and engineer compared to other eukaryotes. As a result, S. *cerevisiae* enabled the

[1] Amusingly, a study showed that the strains of yeast used by mankind in order to make alcoholic beverages are actually the evolutionary outcome of flies eating specific yeast because of their taste, therefore causing a natural selection that constrained mankind's choice (Christiaens et al., 2014).

establishment of most of the basis in molecular biology and stands out as a model organism in biology (Botstein et al., 2011)

The budding yeast can either be found in haploid or diploid form, respectively with one or two copies of each of its chromosomes where its genetic information is contained. I will detail in a later part the notion of chromosome. While human cells take on average 20h to divide, S. *cerevisiae* divides on average every 90-100min in standard laboratory conditions. The budding yeast's division is asymmetrical (Lord and Wheals, 1980). This allows the distinction between the original cell (mother) and its progeny (daughter).

The division of a haploid cell consists in the bud and the duplication of the genetic material. This constitutes the S phase. The cell then undergoes a checkpoint defined as G2 phase during which it prepares for the segregation of the duplicated DNA, then enters the M phase during which mitosis is performed. The genetic material will be equally distributed between the mother and the daughter cell which is still in the form of a bud almost the same size of the mother. The cell then undergoes cytokinesis, meaning that mother and newly formed daughter will separate from each other. Eventually, they will both enter the G1 phase, a checkpoint that will allow the entry in the S phase if no errors such as DNA damages are detected (Chen et al., 2000; Li et al., 2004).

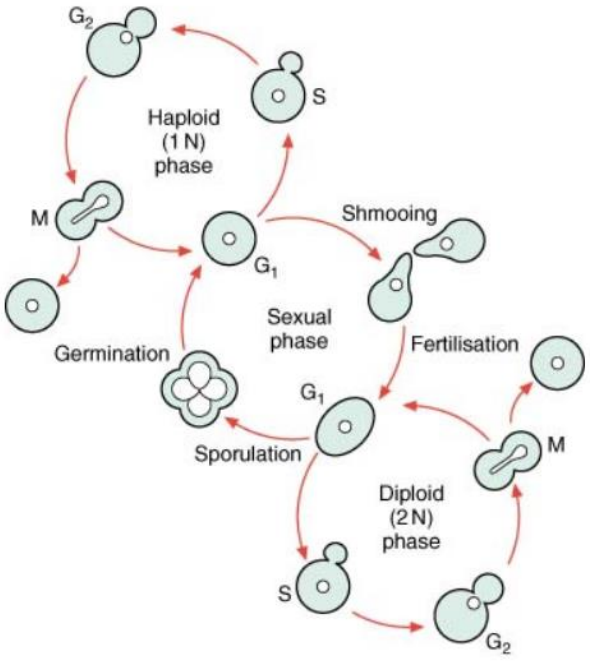


Figure 1-2. Cell-cycle of the budding yeast. Haploid yeast that successfully pass the G1 checkpoint will enter the S phase, characterized by duplication of the genetic material and start of the budding. After successfully passing the G2 checkpoint, the yeast will enter the M phase, during which it will perform mitosis, then cytokinesis. Haploid of different mating type will shmoo and form a diploid that will follow the same cell cycle as a haploid. Starvation of the diploid causes the formation of ascopores and a subsequent restoration of optimal growth conditions causes the sporulation. Image from Encyclopedia of Life Sciences.

However, because the division is asymmetric, the passive repartition of proteins between mother and daughter is not necessary identical. Specific complexes of proteins such as the polarisome (Casamayor, 2002) can allow the active passing of proteins to the daughter cell. Interestingly proteins can be retained in ageing mothers, such as extrachromosomal rDNA circles (ERCs) (Kennedy and McCormick, 2011; Sinclair and Guarente, 1997).

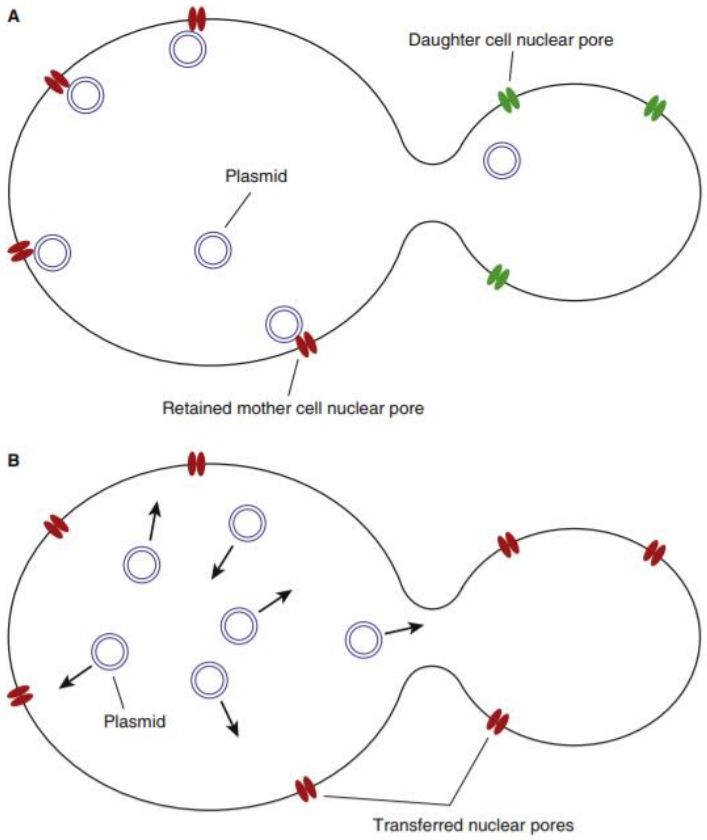


Figure 1-3. ERCs are not transmitted to the daughter cell. (A) Admitted model of retention of ERCs. Maternal nuclear pore complexes (NPCs) are retained in the mother nucleus during mitosis. The ERCs are tethered to the NPCs, thus retained. (B) New proposed model. The maternal NPCs are not retained during mitosis, but both the small size of the bud neck and the short duration of mitosis makes it impossible the diffusion of the ERCs, thus their heritability. Image from Kennedy and McCormick, 2011.

In its haploid form, the budding yeast displays two mating types defined as **a** and $\boldsymbol{\alpha}$. Haploid yeast of opposite mating type, when given the opportunity, will systematically mate and form a diploid (Schrick and Hartwell, 1997). As a result, in its natural environment, the budding yeast is usually found in diploid form in non-stressing condition (Liti, 2015). The diploid will perform its cell-cycle in a similar fashion than the haploid cell. However, in challenging environments, especially when

nutrients are lacking, the diploid will enter meiosis and form spores that will eventually lead to four haploids cells, two **a** and two $\boldsymbol{\alpha}$ (Neiman, 2005).

From a genomic point of view, a haploid budding yeast has over 6000 genes distributed among 16 chromosomes and a genome of 12Mb (Goffeau et al, 1996). Of comparison, a haploid human cells has an estimated 20000 genes distributed among 23 chromosomes with a total of 3400Mb (Venter et al., 2001). The yeast coding sequences represent almost 75% of the genome. Such compactness is much more pronounced than in most eukaryotes. For instance, only 10% of the human's genome codes for functioning proteins (Venter et al., 2001).

S. *cerevisiae* performs homologous recombination, *inter alia* as a mechanism to counter DNA damages such as double strand breaks. Although it is not clear why, its ability to perform homologous recombination is so efficient compared to other eukaryotes it allows the engineering of its genome through DNA fragments integration in the yeast genome through cell transformation (Struhl et al., 1979).

The ease to genetically engineer the budding yeast has led to major yeast collections widely used in the yeast community:

- The deletion collection (Giaever et al., 2002). The original idea was to identify the role of the budding yeast's genes through the study of yeast that are missing specific genes. To that effect, a collection of yeast strains deleted for 90% of the genes has been created. This eventually led to the creation of the Saccharomyces Genome Database (SGD)[2], a comprehensive database where the sequences and details on the functions of the yeast genes can be found.

- The GFP collection (Huh et al., 2003): the improvements of biological tools reached a peak with the use of the Green Fluorescence Protein (GFP) that allows to tag proteins of interest and analyze their dynamics and localization in a living cells. The GFP collection consists in a collection of strains where most genes are tagged with a fluorescence reporter.

B. Chromosome organization in the budding yeast

1. Necessity of the packaging of the genetic material

[2] https://www.yeastgenome.org/

As I have previously mentioned, the size of the yeast genome is 12Mbp. It has been established[3] that 1bp=0.33 nm, therefore the size of *S. cerevisiae*'s linear DNA in a cell is ~4mm. This length of molecule has to be contained in a nucleus that has an average size of 1µm. This means that for such genetic material to be contained in the nucleus, it has to be consequently compacted in it. To that effect, the DNA is coiled around structures called nucleosomes.

A nucleosome is an assembly of two copies of the four proteins H2A, H2B, H3, and H4 named core histones. 147 bp of DNA (~49 nm) is coiled around a nucleosome. A quick calculation shows that in order to have a completely packaged DNA in a yeast's nucleus, around 80 000 nucleosomes are required.

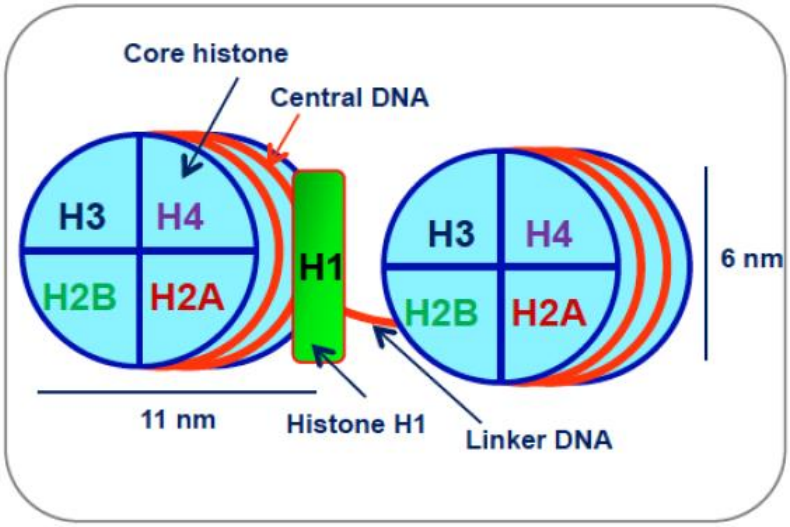


Figure 1-4. Schematic diagram of a nucleosome. Each nucleosome consists of two copies of the four types of core histones H2A, H2B, H3, and H4, around which 147 base pairs of DNA are wrapped forming 1.67 turns. Linker DNA is located between nucleosomes with a variable length ranging between 8 and 114 bp depending on the species, cell type, and chromatin region. From Koprinarova et al, 2016.

The DNA around nucleosomes forms fibers: this is defined as the chromatin (Kornberg, 1974). The chromatin itself is condensed into microscopic structures: the chromosomes[4]. Chromosomes are of different size, depending on the eukaryote considered. Importantly, the number of chromosomes does not necessarily reflect on the complexity of the organism. For instance, humans have 46 chromosomes and are

[3] Molecular Biology of the Cell 6th edition ISBN: 9780815344643

[4] First published by Schwan, Smith and Schleiden in 1847 in *Microscopical researches into the accordance in the structure and growth of animals and plants.*

viewed as more complex than goldfishes that have over a hundred chromosomes (Venter et al., 2001).

The yeast nucleus itself is characterized by two domains: the nucleoplasm and the nucleolus that can occupy 1/3 of the nucleus. The latter serves as a factory for biogenesis of ribosomes (Roeder and Rutter, 1970).

As chromosomes are densely packed in the nucleus, in a molecular crowded environment, it was long thought that this packing would drastically constrain the mobility of chromosomes, making impossible to have an emergence of a spatially functional organization. The nuclear functions would then rely on the biochemistry of the nucleus only. Research of the past 15 years have however established that the nucleus is a highly spatially non-random organized structure.

2. The Rabl conformation

In the budding yeast, chromosomes adopt a configuration within the nucleus that is called Rabl (Marshall et al., 1997). Chromosomes' centromeres, composed of a single nucleosome in the budding yeast, are tethered to a structure called the Spindle Pole Body (SPB) through nuclear microtubules. The SPB consists in a single structure embedded in the nuclear envelope, found opposite the nucleolus in the G1 stage of the cell-cycle (Rout, 1990).

In addition to the SPB and anchoring proteins, another key feature of the nucleus is a structure defined as the Nuclear Pore Complex (NPC). The NPC is an ensemble of 456 nucleoporins with a size of 50MDa that is embedded in the nuclear envelope (Alber et al., 2007; Rout et al., 2000). The budding yeast's nucleus is covered with about 200 NPCs. To make a physicist approximation, it resembles a cylinder of 96nm diameter and 35nm width. The NPC enables the exchange of small molecules (through diffusion) and active exchange of macro-molecules between the nucleus and the cytoplasm (Ishii et al.). In particular, the NPCs controls trafficking of proteins from nucleus to the cytoplasm, and vice versa.

The extremities of the chromosomes are important features named telomeres, which spread away from the centromeres and are tethered to the Nuclear envelop (Palladino et al., 1993). Telomeres have notoriously been linked to the process of biological ageing as they shorten over time (Austriaco and Guarente, 1997). They are also associated with a protective role against chromosome fusion and degradation (Sandell, 1993). Unlike the rest of chromosomes, yeast telomeres do not contain

nucleosomes (Wright et al., 1992). The haploid budding yeast has 32 telomeres that are clustered to the nuclear envelop into an average of 8 foci (Gotta, 1996).

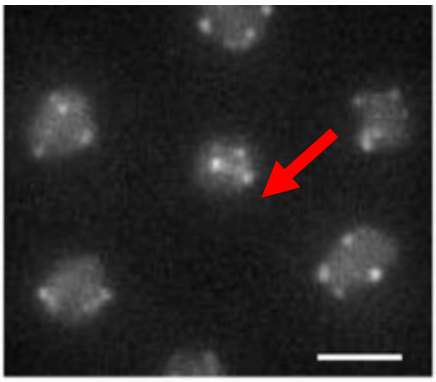


Figure 1-5. Fluorescence observation of telomeres clusters. A RAP1-GFP strain is observed under the fluorescence light. As the Rap1 protein is bound to the telomeres, observing this proteins allows the observation of telomeres clusters. The red arrow points to a Rap1 focci. From Hozé et al, 2013.

Telomeres consist in 200-300bp irregular $TG_{1\text{-}3}$ repeats in S. *cerevisiae*. As they do not contain any genes, they do not produce any functional proteins. Surprisingly, telomeres are transcribed into long non coding mRNA called Telomeric containing Repeated RNA (TERRA). One of the roles of the TERRA is the regulation of telomerase activity, linked to cellular ageing through the control of the shortening of telomeres (Iglesias et al., 2011; Luke and Lingner, 2009).

Upstream telomeres, up to 30-40kbp, are regions that contain highly repeated sequences and few genes, usually in several copies. Such regions are called subtelomeres (Louis, 2014). The current experimental characterizations of subtelomeres point towards an evolutionary and adaptive role. In the budding yeast, subtelomeres are also proposed to be the playground of the creation of novel genetic functions, since this is where new genes are created (Snoek et al., 2014). Thus subtelomeres are considered to be gene reservoirs.

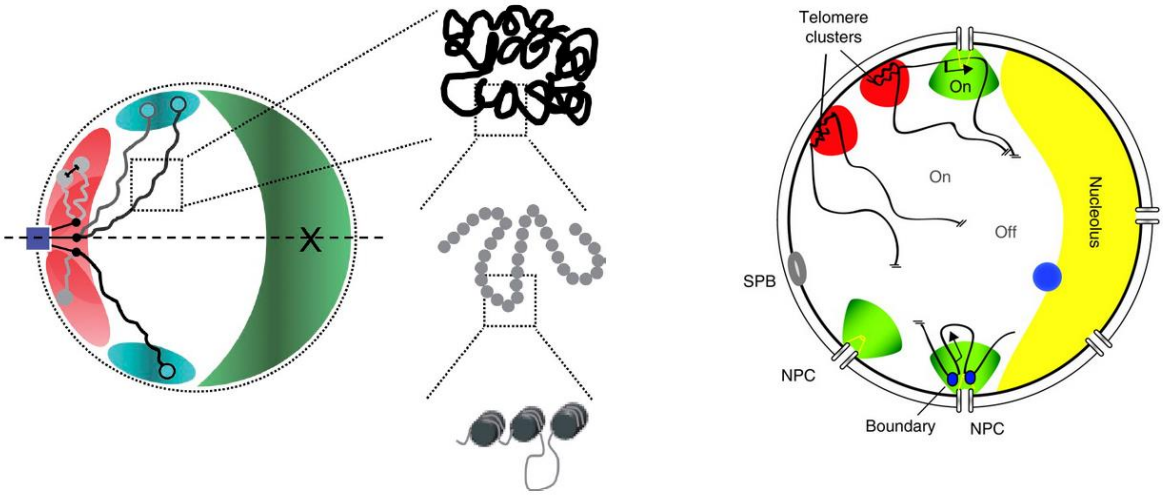


Figure 1-6. Nuclear organization in the budding yeast. (Left) Rabl conformation. Chromosomes (in black) have their centromeres tethered to the spindle pole body (purple). The extremities of the chromosomes are tethered to the nuclear periphery. (Right) Telomeres are clustered at the nuclear periphery (red) and chromosome in an "active state" relocate to the nuclear pore complex (green). From Zimmer & Fabre, 2010.

Chromosomes can be viewed as polymeric fibers but for a long time, no quantitative description was made to characterize their behavior. Physicists have shown that chromosomes can be described by polymer physics. As a result, various parameters such as persistence length, rigidity and compaction have been associated for the yeast chromosomes (Wong et al., 2012).

The modeling of chromosomes, along with experimental evidences, showed that chromosomes do not interact much between one another, but rather intra-chromosomal interactions occur. This lack of interaction between chromosomes led the way to thinking that one chromosome could be constrained to stay in specific areas of the nucleus (Burgess and Kleckner, 1999). This is the basis of the notion of chromosome territories.

The genetic information has to be treated in specific manners depending for instance on the external environment. It can therefore be asked if this implies that local changes on the chromosomes have to be made. This would mean that biophysical parameters associated to the chromosomes are not fixed in time, but subjected to changes that are triggered by proteins in the nucleus. As a result, part of the nuclear function and organization would be an interplay between local structural changes and biochemical changes.

C. Basics of gene expression

The genetic information has to be read in order to produce proteins of interest, necessary for any living organism to perform metabolic function throughout their lives. In the case of genes transcribed by RNA Polymerase II, mRNAs are produced upon binding of Pol II along with other factors to the promoter of a target gene. Once produced, mRNAs are exported from the nucleus through NPCs and serve as a template for translation.

Most studies on gene expression focused on investigating the sequences of genes, the characterization of the associated promoters and the specific transcription factors require to the transcription. More recently, thanks to the improvement and development of next generation sequencing and whole genome approaches, the role of the structure of the chromatin was acknowledged to be as important as the biochemical processes occurring in the nucleus.

The role of the structure of the chromatin is fairly apparent if we adopt a simple model that assumes that the packing of the chromatin is homogenous in an initial state. The budding yeast has genes with an average size of 2kb. As DNA, and consequently genes, are coiled every 147bp around histones, when the state of a gene changes (from not transcribed to transcribed for instance), a seemingly contradiction appears between the accessibility and realization of the transcription of DNA due to the fact that DNA is tightly compacted. An obvious solution would be a change in the chromatin structure in order to loosen the DNA around nucleosomes, or even locally evict the nucleosomes altogether, to make the DNA accessible to transcription factors.

The yeast has two genes encoding for each of the core histones, which incidentally makes easier the studies of histones during transcription. For instance, changes in the histones level have been shown to impact gene expression (Norris and Osley, 1987). Experimental studies have also shown that the depletion of the H4 histone led to a global change of the chromatin and to the activity of the PHO5 promoter under experimental conditions where it shouldn't be expressed (Han and Grunstein, 1988). Overall, this implies that the presence of histones and their level is essential for gene expression, and must be tightly regulated throughout all events that can occur in the life of the yeast such as environmental stress.

Analysis of certain genes that are in a repressed state, shows that the associated promoters contain a large amount of nucleosomes that block the access of transcription factors (Han and Grunstein, 1988). Conversely, when the promoter is activated, the nucleosomes are evicted with the help of chromatin remodeler complexes (see below), allowing factors to bind to the chromatin and the transcription of the gene (Gutiérrez et al., 2007). Gene expression is therefore an interplay between repression state of a promoter and transcription factors overcoming it.

Gene expression is a costly phenomenon in terms of energy. As a result, all genes are not expressed at all times, from one part to optimize the energy used in the cell, and because of the uselessness to transcribe proteins that won't be required in unnecessary physiological conditions. In particular, the yeast possess genes called housekeeping genes, involved in the day-to-day activity of the cell. The promoters of these genes are mostly nucleosome free regions which makes them highly expressed. (Basehoar et al., 2004; Huisinga and Pugh, 2004). The yeast also possesses stress genes that are usually in regions enriched with nucleosomes and are repressed in normal conditions (Bernstein et al., 2004). As a result, transcription factors binding sites at the promoter of stress genes are inaccessible, which creates a competition between the nucleosome repression of stress genes and transcription factors trying to bind to them. It has been hypothesized that this competition contributes to the cell-cell variability, a concept that will be detailed in a later part (SvarenS et al., 1994).

D. Basis of chromatin regulation and epigenetics

As I have presented above, gene expression is a dynamical process with local modifications on the chromatin enabling changes of chromatin configuration and protein production in a given period. This directly affects gene expression, and is done so in a well-regulated manner. An important point is that a regulated change in gene expression is never made directly through a change in the gene's sequence[5].

As gene expression is a dynamical process, the state of compaction of DNA around nucleosomes has to be temporarily and locally changed during transcriptional events, or even after[6] (Bernstein et al., 2004). The process of temporary alteration of histones can be done via two distinct processes.

On one hand, through the involvement of specific proteins called ATP-chromatin remodelers (Muchardt and Yaniv, 1999). In the budding yeast, there exists several families of such remodelers, including the SWItch/Sucrose Non-Fermentable (Swi/Snf) superfamily (Tsukiyama et al., 1999). The latter is historically the first identified chromatin remodeler able to modify the chromatin structure, favoring transcription.

Chromatin remodelers function through hydrolysis of ATP, which can induce local changes in the conformation of nucleosomes, sometimes through the recruitment

[5] Changes in a gene's sequence can occur through mutations, DNA damages or evolutionary mechanisms such as transposition (Hershberg, 2015; Langley'; Nei). As these events are random, they can either induce no changes in gene expression (silent mutation) or lead to an uncontrolled change of in gene expression that thereafter cause a malfunction in the gene that might be detrimental to the cell and lead to its death.

[6] This is referred to as post-transcriptional events.

of specific proteins, for instance a variant[7] of the histone H2A is H2A.Z. It is involved in several genetic processes including the regulation of transcription and DNA repair (Jackson, 2000; Santisteban et al., 2000). Although not essential to yeast viability, it is closely linked to the interaction between gene expression and chromatin structure.

I will here present two important points of the activity of the Swi/Snf system for the results that will be presented in this manuscript. It has been shown that the Swi/Snf complex is involved in stress response. It regulates the genes expressed under the control of heat-shock factor (Erkina et al., 2008). The response to heat-shock involves genes that are also expressed in different stress conditions, such as the osmotic stress. Another key feature of the Swi/Snf system is its implication in the regulation of genes that are repressed in glucose conditions (Geng and Laurent, 2004). The *STL1* promoter, which dynamics I have investigated during this study, is also repressed in glucose conditions (Ferreira et al., 2005).

DNA is a negatively charged molecule because of its phosphate composition. Depending on the charge on nucleosomes, DNA will either be attracted by nucleosomes or repelled by them. In the most basal state, nucleosomes are positively charged, therefore the interaction between DNA and nucleosome is strong: the chromatin is inaccessible to transcription factors (SvarenS et al., 1994). Changes of the nucleosomes are made through changes on the histones. In addition to ATP-chromatin remodelers, a possible way to modify histones is through covalent histone-modifying complexes.

The modification of the charge of a nucleosome is done through addition or removal of specific residues at N tails of histones (Strahl and Allis, 2000). It exists several processes allowing to perform nucleosomes modifications. To better understand the reasoning behind the experiments dealing with nucleosome modifications performed during my PhD, I will here present two processes[8] that are:

Acetylation: the addition of an acetyl group on histones causes the nucleosomes to be negatively charged, thus reducing the affinity between DNA and nucleosome and loosening the chromatin: transcription factors can bind the DNA (Grunstein, 1997). This process is made through proteins named Histones Acetyl Transferases (HATs).

[7] The budding yeast also possesses another histone H3 variant that is CenH3, found at centromeres' nucleosome.

[8] In addition to acetylation and deacetylation, it exists processes such as summoylation, phosphorylation, ubiquityation. A comprehensive summary can be found in Bannister and Kouzarides, 2011.

Deacetylation: the removal of an acetyl group on histones increases the affinity between nucleosomes and chromatin. This process is performed by Histone DeACetylases (HDACs). An example is the protein Rpd3. Its influence on transcription during hyperosmotic stress conditions will be described in a later section (de Nadal et al., 2004).

In general, acetylation and HATs are associated to gene expression; deacetylation and HDACs are associated to repression. However there are exceptions. It has been observed that they can behave either as activators or repressors. For instance, the HAT Gcn5 can repress certain genes in the budding yeast (Vogelauer et al., 2000).

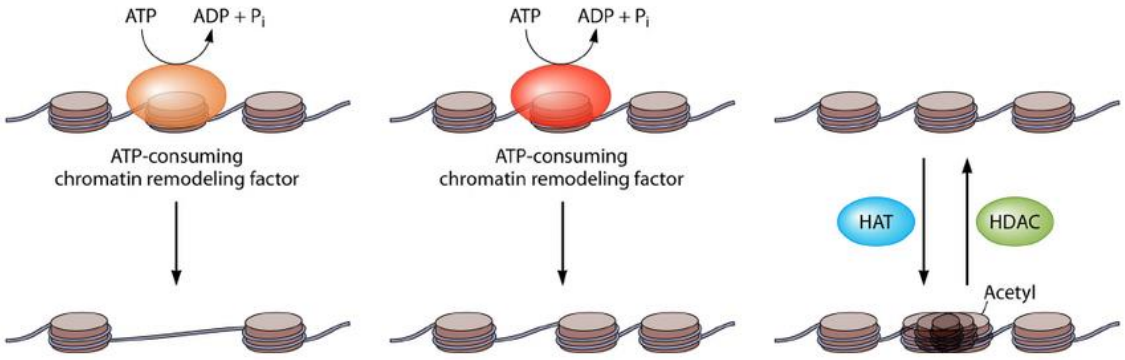


Figure 1-8. Schematic representation of the action of chromatin remodeler factors. Chromatin remodeling factors can remove (left) or slide (middle) nucleosomes. Histone-modifying enzymes can add or remove covalent modifications to certain histone residues. For example, histone acetyltransferases (HATs) can add an acetyl residue, which can be removed by histone deacetylases (HDACs). Acetyl groups add an electronegative charge to the histones, which repulses the negatively charged DNA polymer, resulting in a modified, "looser" DNA-histone interaction and an increased accessibility of the DNA. From Jansen and Verstrepen, 2011

The histone code corresponds to all modifications applied on histones and could give the keys to a complete description of gene regulation (Jenuwein, 2001). However, many exceptions to the mechanisms of chromatin remodeling[9], blurs the understanding of histone role on transcriptional regulation.

An important remark is that gene position along a chromosome can be important for its level of expression. As the chromatin can locally be different because of local changes of structure or the presence of a high amount of specific protein at specific locations, changing the position of a gene may alter its gene expression. Such

[9] An example is the fact that deacetylation is usually associated to gene repression but there are many counter examples to this assessment (Gcn5, Rpd3 for instance).

phenomenon is referred to as position effect[10]. A very particular example in the budding yeast is the Telomere Position Effect (TPE): the closer a gene is moved to telomeres, the more decreased its transcriptional activity is (Aparicio et al., 1991; Rine' and Herskowitz, 1987).

Any change in the current chromatin state that affects its gene expression without a change in the gene's sequence, and produces a stable subsequent phenotype that can be passed on through cellular division is referred to as **epigenetics**.

E. Example of epigenetic process: the SIR complex

In this paragraph, I will detail an epigenetic process occurring through specific proteins that I have investigated during my PhD and proved to be a key feature to my results.

As I have presented before, subtelomeres are chromosomal domains in the budding yeast that contain genes that are not expressed in normal conditions and are submitted to transcriptional silencing. This phenomenon occurs through the Silent Information Regulator complex (Guarente, 1999; Moazed et al., 1997).

The silencing of genes[11] was originally described in the case of the mating type in the budding yeast. In its haploid form, the **a** or $\boldsymbol{\alpha}$ mating type are expressed, never both at the same time. The genes responsible for the mating types are HML and HMR, both located at the subtelomeres of the chromosome III. Essentially, the HML or HMR information is copied at the MAT specific locus. Once inserted, the budding yeast has a fixed mating type (Kostriken et al., 1983).

A yeast of mating type **a** (resp. $\boldsymbol{\alpha}$) will express a-specific transcription factors (resp. α -transcription factors) at the mating-type locus. Haploids of different mating type can mate and form a diploid. Such diploid will both express **a** and $\boldsymbol{\alpha}$ factors which cancels out the ability of the diploid to mate. It was thus proposed that a yeast that has the **a** mating type strictly possess the HML gene, and a yeast of mating type $\boldsymbol{\alpha}$ only has the HMR gene. However, it was discovered that haploid mating-deficient **a**- yeast could switch to an $\boldsymbol{\alpha}$ mating type, implying that a haploid contains both mating type

[10] More information can be found in Wilson C, Bellen HJ, Gehring WJ. Position effects on eukaryotic gene expression. Annu Rev Cell Biol. 1990;6:679-714

[11] In the budding yeast, silencing is chromatin context dependent. Chromosomal regions of the budding yeast other than the subtelomeres are subjected to transcriptional silencing, notably part of the ribosomal DNA repeats (rDNA). Although the silencing of the subtelomere is performed through the SIR complex, the silencing of the rDNA is made via the REgulator of Nucleolar silencing and Telophase (RENT) (Huang, 2003). A common point between the SIR complex and the RENT complex is that both involve the protein Sir2 (Tanny et al., 2004).

genes in its genome, one of which has to be silenced[12]. This was one of the first introduction of the notion of gene silencing (Nakayama et al., 1988).

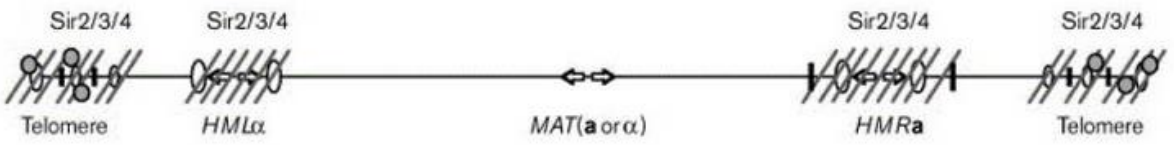


Figure 1-9. Sketch of the mating type loci on the chromosome III. The SIR complex is recruited at telomeres and to the HML and HMR locus (either to one of these locus in a haploid cell, to both on these locus in a diploid cell). From Gartenberg et al, 2000.

Subsequent screening experiments showed the existence of a complex of proteins that is responsible for the silencing at a mating type locus and form a family named the Silent Information Regulator (SIR) complex. The SIR complex is a family composed of 4 proteins Sir1/2/3/4 encoded by four genes required for transcriptional silencing (Moazed et al., 1997). Using truncated subtelomeres, it has been shown that the SIR complex binds to telomeres and spreads to the subtelomeres.

The SIR proteins are first recruited through specific factors and eventually form a complex that prevents the access of the transcription factors to their targets. For instance, RapI, a factor that binds to the $TG_{1\text{-}3}$ repeats of the telomeres, can recruit either Sir3 or Sir4 (Marcand et al. 1996; Moretti et al. 1994) . Other factors are more specific, such as AbfI that recruits exclusively Sir3 and the YKU complex that recruits exclusively Sir4[13] (Guarente, 1999; Luo, 2002). The proteins Sir3 and Sir2 can bind to Sir4 in order to form a complex. The Sir4 part of the complex will then bind to the chromatin. The protein Sir2 will deacetylate the H3K16ac, allowing Sir3 to bind to H3K16dac. In turn, Sir3 will recruit Sir2-Sir4, causing the spreading of the SIR complex along the chromatin. SIR spreading will prevent the access of the RNA Polymerase II and thus the transcription of the genes in the newly silenced domain (Liou et al., 2005). Spreading is stopped when a regulatory element is encountered. Also, the amount of available SIR proteins could limit the formation of SIR complexes and implicitly cause the arrest of the silencing spreading (Buchberger et al., 2008). Finally, deletion of a SIR protein compromises the formation of the SIR complex altogether.

[12] The budding yeast can naturally switch between mating types, through the HO endonuclease. This enzyme causes a double strand break at the MAT locus, permitting homothallic switching. Haploid strains used in labs are deleted for this endonuclease, which constrains a population of clonal yeast to always be in a specific mating type, therefore preventing the emergence of diploids.

[13] The clustering of the telomeres is caused by the dual role of Sir4, involved both in the silencing and the anchoring at the NPC .

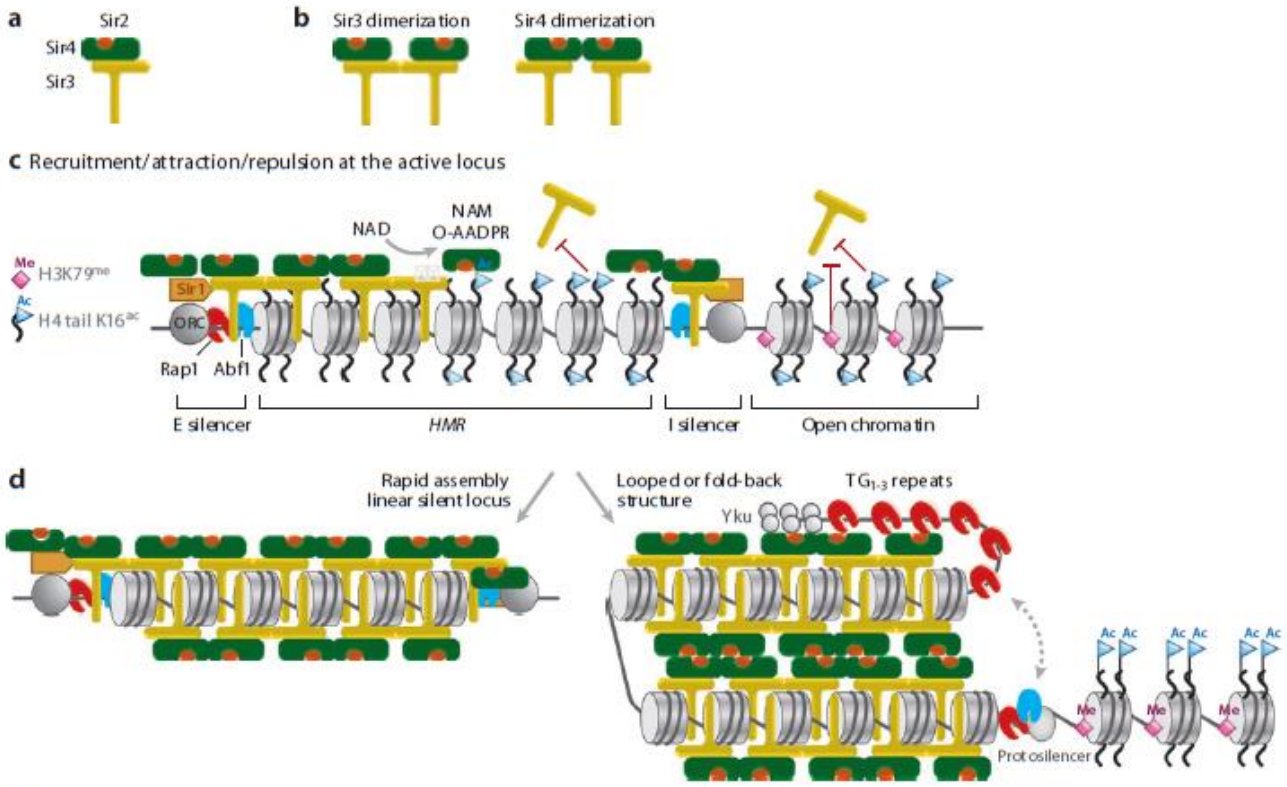


Figure 1-10. Comprehensive summary of the SIR complex assembly. (a) Schematic of the Sir2-3-4 complex as a heterotrimer. (b) Formation of subcomplexes of the SIR complex through dimerization of either Sir3 or Sir4 C-terminal domains. (c) Model of the assembly of the silent HMR locus: Sir2-Sir4 binds to the chromatin and Sir2 deacetylate the H3K16ac, allowing Sir3 to bind to H3K16dac.Sir3 will then recruit the Sir2-SIr4 complex that will bind the chromatin and start the process anew. (d) The conversion of SIR-bound nucleosomes into a repressed domain may require compaction or folding into a higher-order structure, depicted as a highly compact HMR locus, or as a folded telomeric domain. From Kueng et al, 2013.

Interestingly, a study has shown that a HMR loci which ability to recruit the SIR complex has been cancelled out can still be silenced if anchored to the nuclear periphery (Andrulis et al., 1998). This shows that nuclear organization plays an important in transcriptional regulation. However, two very recent studies are leading the way to a novel interpretation of the notion of nuclear organization in the budding yeast. In particular, both studies used synthetic biology tools in order to fuse all 16 chromosomes of the yeast into a single (or two) chromosome (Luo et al., 2018; Shao et al., 2018). All the notions presented above do not seem to apply anymore in the case of this new synthetic chromosome. Very surprisingly however, haploid cells with such a chromosome do not show growth defects and their transcriptome is almost similar to the wild type. Differences in growth were observed in challenging environments. These studies prove a great plasticity and robustness of haploid budding yeasts to

genome changes, but a necessity of a nuclear organization to better adaption to challenging environments.

II. Environmental stress and adaptation

All living organisms, whether unicellular or multicellular, evolve in fluctuating environments. This implies that some of the physiological properties of the cells (such as protein synthesis) need to be adjusted or fine-tuned dynamically. This is done through the use of specific sensors and regulatory networks working in a dynamic fashion (Gasch and Werner-Washburne, 2002).

When environmental fluctuations have mild intensities, they can be tolerated by those organisms, *i.e.* do not require a specific response. However, these fluctuations can be very intense and cause harm to the organisms. In this case, such fluctuations are defined as environmental stress. They necessitate an adequate strategy from the organisms in order to ensure their survival, and implicitly cause a selective pressure on them (Coleman and Chisholm, 2010).

Throughout their evolution, living organisms have developed strategies in order to actively try to survive such environmental stresses. The case where these organisms have successfully survived the stress through an active, functional strategy is defined as adaptation.

The adaptation to an environmental stress requires a very complex genetic response that is not universal. Indeed, micro-organisms have evolved different strategies in order to survive stresses are different, although similarities may occur. In addition, as there are numerous environmental stresses, numerous strategies exist. Such diversity adds a high level of complexity to studies on stresses.

It exists several definitions of how micro-organism can respond to environmental stresses:

- **Tolerance:** the fact that a micro-organism does not have an active strategy to fight a stress (either because it cannot or the stress is not strong enough to necessitate such a response) and manages to survive it.

- **Adaptation:** the fact that a cell has an active response to fight a stress and successfully survives it

- **Memory:** following an adaptation to a stress, a micro-organism improves its response to a subsequent stress in order to have a better adaptation. In a certain measure, this can be viewed as an extension of the adaptation to repeated stresses.

In the following parts, I will focus on the response to environmental changes in the budding yeast and emphasize on the response to hyperosmotic stresses. I will then present two key examples of cellular memory in response to environmental stresses.

A. Environmental stress response program

In their natural environment, budding yeast are subjugated to various stresses such as changes in the osmolarity, increase of the amount of UV lights, changes in the acidity, sudden presence of toxic products, food starvation.

The effects of specific stresses are different, so the response to a particular stress can differ from another stress. Interestingly, it has been observed in the budding yeast that when cells are exposed to a mild dose of a stress, it adapts better to lethal doses of other stresses (Berry and Gasch, 2008; Święciło, 2016). The adaptation to a specific stress can cause the yeast to better adapt to a different stress, suggesting the existence of a crosstalk between the strategies of responses to different stresses.

More surprisingly, with the advances in technology that permitted genome wide studies, it appeared that the genes involved in the response to a specific stress (heat shock) are also activated when the cells is subjugated to a different stress (Schmitt and McEntee, 1996). Sequences known as the STRE[14]-sequences were identified in most stress-response promoters and linked to two transcription factors Msn2 and Msn4, known as the general stress transcription factor (Martínez-Pastor et al., 1996). The general stress response to external environments was later better characterized and named **Environmental Stress Response** program (Gasch et al., 2000).

[14] Stress Response Elements (STRE) are DNA sequences found in promoters of stress response genes.

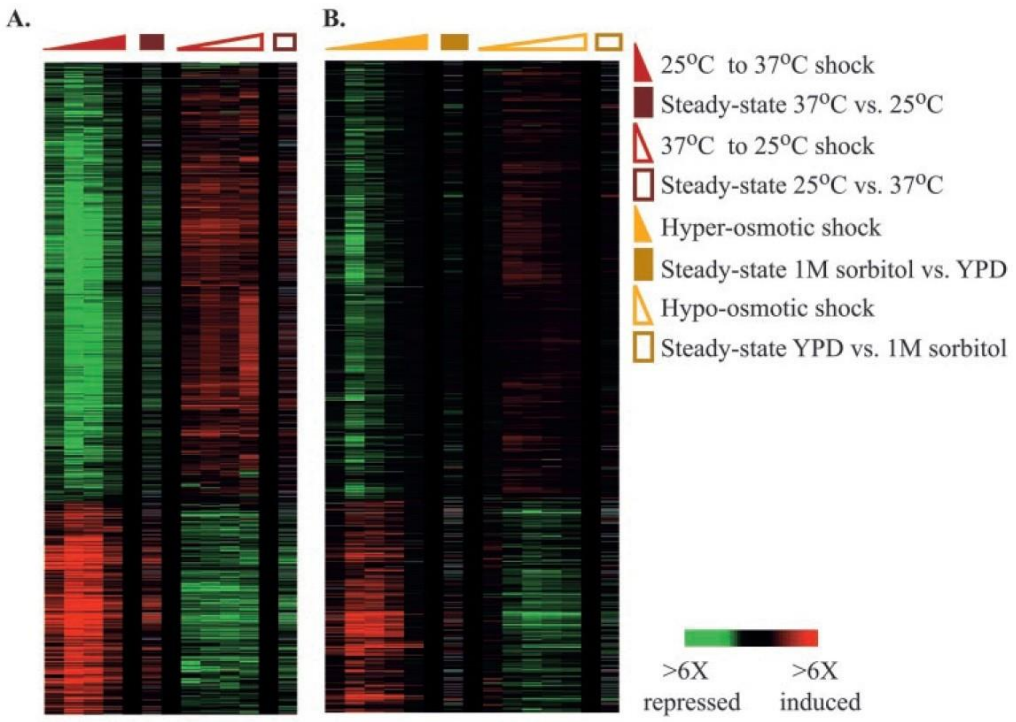


Figure 1-11. Transcriptomes of the genes from the ESR, in various stress conditions. Gene expression change over 1h following stress for genes composing the Environmental Stress Response (ESR) which are transcriptionally affected by most stress types. From Gasch et al, 2000.

B. Example of an adaptation: the budding yeast's response to hyperosmotic stresses

Budding yeasts are naturally found on grapes, which can rot and thus release molecules in their surroundings such as fructose. A change in the fructose concentration can lead to a local variation of the osmolarity in the environment. The response to osmotic changes has been extensively studied in *S. cerevisiae* and is well-characterized.

Osmotic stresses can happen in two fashions that lead to different physiological effects: either the external osmolarity increases or it decreases. An osmotic stress is by essence a physical constrain applied on the cell. In the case of a decrease in the osmolarity of the external environment, water will flow in the yeast. This causes an increase of the yeast volume which can sometimes causes the cell to burst. Conversely, in the case of an increase in the osmolarity of the environment, water will flow out of the yeast, leading to a shrinkage of the cell's volume. In both cases, these constraints applied on the cell compromise the integrity of the cell wall, and consequently biological events related to it, such as mating or budding (Morris et al., 1986). Moreover, as any osmotic stress causes a change in the water's activity since the

number of available water molecules either increases or decreases, the day-to-day biochemical reactions occurring in the cell are compromised (Morris et al., 1986). Therefore it is vital that following an osmotic stress, the yeast returns in homeostasis with its environment. The strategy to survive to hyperosmotic stresses consists in using glycerol (either by biosynthesis or by taking it from the environment if it is available) in order to increase the cell's volume, forcing the water to flow in and recovering the homeostasis (Albertyn et al., 1994). A hyperosmotic stress will cause an imbalance of pressure in the yeast. This will be detected by osmosensors that will trigger the activation of the High Osmolarity Glycerol (HOG) pathway (Dihazi et al., 2004; Miermont et al., 2011; O'Rourke et al., 2002).

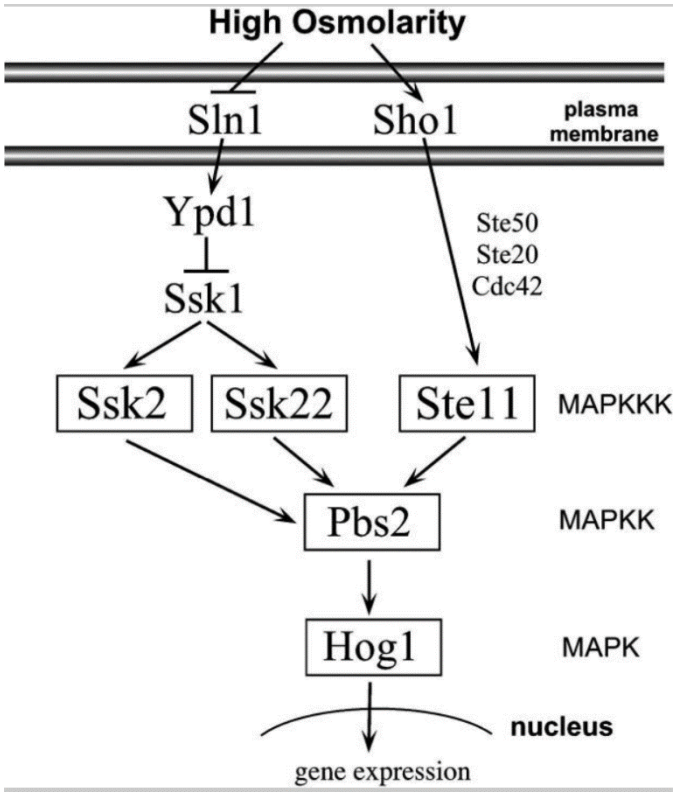


Figure 1-12. Comprehensive sketch of the Hog Pathway. The pathway is composed of a Sln1 and Sho1 modules that sense the osmotic change in the environment and transmit the information to the mitogen activated protein (MAP) kinase module, ending on the MAPKK Pbs2. The later phosphorylates the cytoplasmic protein Hog1, causing its translocation in the nucleus and the eventual upregulation of stress response genes. From Bettinger et al, 2007

The two branches of the pathway, related to specific osmosensors, will lead to the phosphorylation of the cytoplasmic protein Hog1, causing its translocation into the nucleus where it will participate in the activation and regulation of an estimated 10% of the genome, including the genes *GPD1* (responsible for the biosynthesis of the glycerol) and the glycerol transporter *STL1* that I will describe in more detail below (Ferreira et al., 2005).

Following the stress and activation of the HOG pathway, the cell will eventually adapt by accumulating glycerol and getting back in homeostasis with its external environment. This adaptation takes an estimated 15-30min (Miermont et al., 2011).

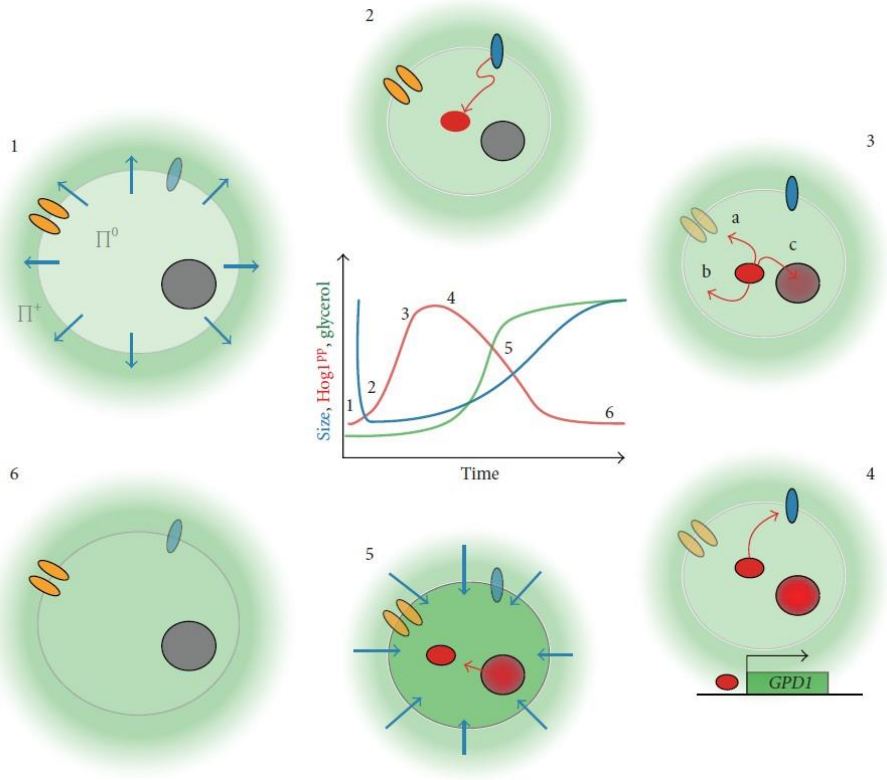


Figure 1-13. Sequential steps of the HOG pathway in response to a hyperosmotic stress. (1) Increase of the osmolarity of the environment causes the cells to lose their water and shrink. (2) Within less than 1min: osmosensors at the membrane detect the osmotic imbalance and start the activation of the Hog Pathway. (3) Within a minute, the Hog Pathway is activated. (a) Closure of the main glycerol membrane export channel Fps1 (b) Activation of several enzymes involved in glycerol synthesis. (c) Phosphorylated Hog1 accumulates into the nucleus and within 3min, upregulates 600 genes. (4) Proteins of osmo-responsive genes (such as GPD1) are produced, starting the physiological adaptation of the cell and the progressive deactivation of the Hog Pathway. (5) Glycerol production cause the water to flow back in the cell and the cell to regain its original size. (6) The cell is physiologically adapted: the Hog Pathway is fully deactivated. From Miermont et al, 2011

1. A few consequences of the compression due to hyperosmotic stress

From a transcriptional perspective, a hyperosmotic stress causes a dramatic change in the transcription profile in the budding yeast, as stress response genes will be activated and housekeeping genes downregulated (Schuller et al.,1994). Interestingly, the stability of the mRNA of housekeeping genes present at the moment of the stress is also affected: these mRNA will be destabilized. Conversely, mRNA

produced by the stress responses genes will be stabilized (Romero-Santacreu et al., 2009).

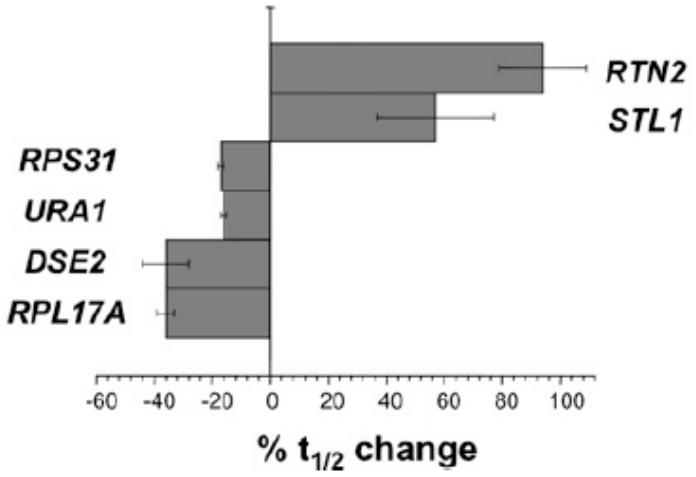


Figure 1-14. mRNAs half-life change after mild osmotic stress. Non osmo-responsive genes have their associated mRNA destabilized, whereas the mRNA following the transcription of osmo-responsive genes are stabilized through an increase of their half-life. From Romero-Santacreu et al, 2009.

As I have previously mentioned, a hyperosmotic stress is a physical phenomenon that compresses the cell. Experimental work along with computer simulations show that this physical compression not only impacts the cell wall, but also the nucleus (Morris et al., 1986). A notable consequence is that proteins that are bound to chromatin prior to hyperosmotic stress are removed from it upon stress. In particular, it has been shown that it is the case for the protein Sir3 (Mazor and Kupiec, 2009)

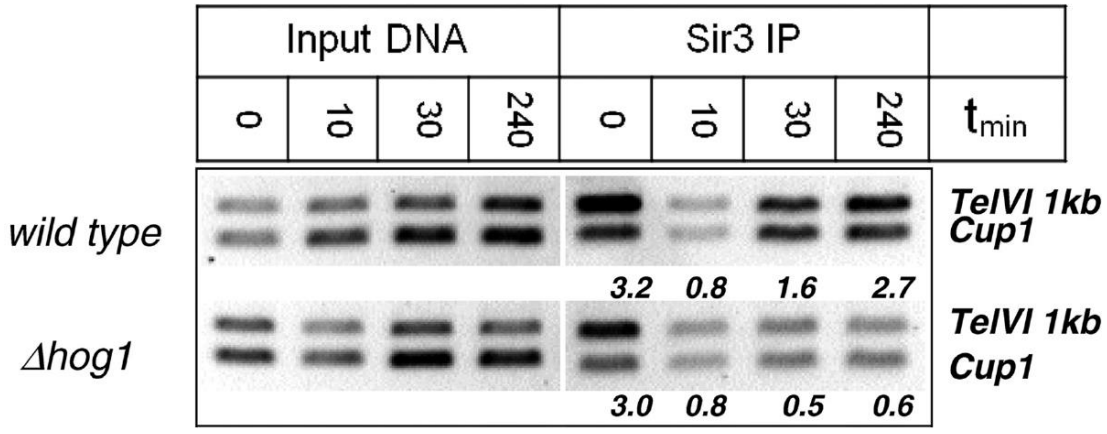


Figure 1-15. ChiP analysis of the presence of Sir3 on the telomere of chromosome VI in response to a hyperosmotic stress. The Sir3 is removed from the telomere of the chromosome VI after hyperosmotic stress. 30min upon stress, Sir3 is on the chromatin again. From Mazor et al, 2009.

2. A promoter of interest: pSTL1

As the protein Hog1 translocates in the nucleus after phosphorylation upon stress, the level of transcription of hundreds of genes is altered. Most of them are part of the ESR program and encode for chaperones and protective enzymes, but some of them are osmotic stress specific.

Among all osmo-responsive genes, 80% are Hog1 dependent. Although the mechanisms behind which Hog1 impacts these genes is still unknown, studies show that Hog1 works in association with several co-factors such as the transcription activators Hot1, Smp1, Msn1, Msn2, Msn4 and the transcription repressor Sko1 (Miermont et al., 2011). In particular, Hog1 and the transcription factor Hot1 co-activate the *STL1* gene (Bai et al., 2015).

As explained before, a possible strategy for a cell to accumulate glycerol is to take it from the surrounding environment. This is done via *STL1* which codes for a transporter that imports glycerol from the environment into the cell (Ferreira et al., 2005). In addition, a yeast cell constitutively synthesizes glycerol through the *GPD1* gene and its paralog, which activity increases upon stress in order to permit the yeast to adapt. Consequently, the deletion of *STL1* has limited effects on the cell viability during stress. Highly and specifically expressed upon hyperosmotic stress, *STL1* is a standard reporter of the HOG pathway activity (Llamosi et al., 2016; Uhlendorf et al., 2012).

Like most stress response genes, *STL1* is located at the subtelomere of the right arm of the chromosome IV. Upon activation, evidences suggest that this gene relocates to the nuclear pore complex, although this relocation does not appear to have an important effect on the adaptation or activity of this gene (Guet et al., 2015).

Interestingly, a study showed that Hog1 also works in tandem with the Histone Diacetyl (HDAC) Rpd3 to activate osmo-responsive genes (de Nadal et al., 2004). This is surprising considering that, as I have previously introduced, histone deacetylation is associated with repression. In particular, the study shows that removing Rpd3 actually reduces the activity of Hog1 osmo-responsive genes such as *STL1*.

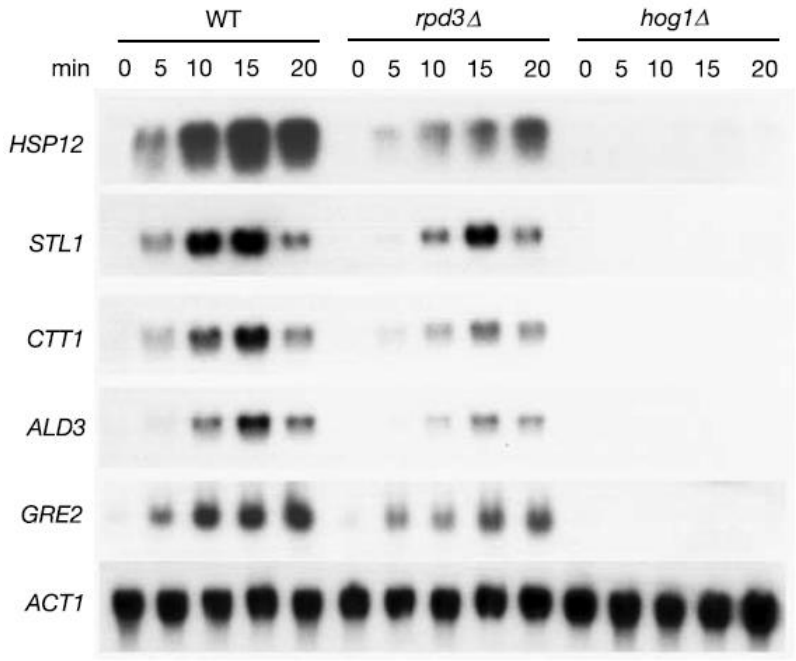


Figure 1-16. Northern blot study of the Influence of the deletion of Rpd3 on the activity of various osmo-responsive genes during a hyperosmotic stress. Deletion of the HDAC Rpd3 causes a decrease in the produced amount of Stl1p compared to the wild type, at similar times after hyperosmotic stress induction. From de Nadal et al, 2004.

The transcriptional activity of osmo-responsive genes such as *STL1* is in the vast majority mediated by the phosphorylated Hog1, i.e. Hog1 inside the nucleus. This transcriptional event occurs within minutes. Once the cell is adapted, Hog1 relocates inside the cytoplasm, ending the transcriptional response to the stress: Hog1 causes a negative feedback loop (Zi et al., 2010). This adaptation takes 15-30min to appear. A quantitative study on the transcriptional dynamics of *STL1* for instance is thus made more difficult as adaptation effects have to be taken into account. However, the study of the response to short hyperosmotic stresses would allow to dismiss the adaptation effect.

C. Cellular memory

As I have presented above, the budding yeast can adapt to environmental stresses through the activation of specific pathways. In the case of the hyperosmotic stress, the Hog Pathway. However, such adaptations can serve as a learning process in order to improve the adaptation when encountering a similar stress later or even

preparing the cell to a high adaptation when encountering a different stress, thus increasing the chances of survival. This is defined as cellular memory[15].

The budding yeast has proven to be an excellent model to study the emergence of cellular effects and the underlying mechanisms. I will describe two examples bellow.

1. The galactose memory

a) The GAL pathway

The budding yeast has the natural ability to process various sources of carbon such as raffinose, glucose, galactose and maltose. This is due to the presence of specific genetic pathways (Carlson and Botstein, 1983). The GAL cluster of genes has been extensively studied and is among the most well characterized gene network in the budding yeast. Therefore, many experiments on the galactose memory have been performed.

The model of activation of the GAL pathway is as follow. When yeast are grown in glucose, the GAL genes are repressed by the protein Gal80, which binds the protein Gal4 that is found at the *GAL1*, *GAL2*, *GAL80* promoters. When galactose is added to the external environment, the protein Gal2 imports the galactose in the cell. The galactose permits the activation of *GAL3* and the subsequent Gal3 protein binds to Gal80 and brings it in the cytoplasm. Without the Gal80 blockage, Gal4 along with the transcriptional machinery can activate the GAL cluster[16].

The repression implies that it will take time for the repression to be lifted.

[15] The notion of cellular memory exists for all unicellular. For instance in mammalian cells, cellular memory can be defined for cell differentiation and even cancer cells.

[16] Interestingly, the activation of the GAL cluster is made with a bimodality that has recently been linked to a long non-coding RNA and a bifunctional galactokinase (Zacharioudakis and Tzamarias, 2017).

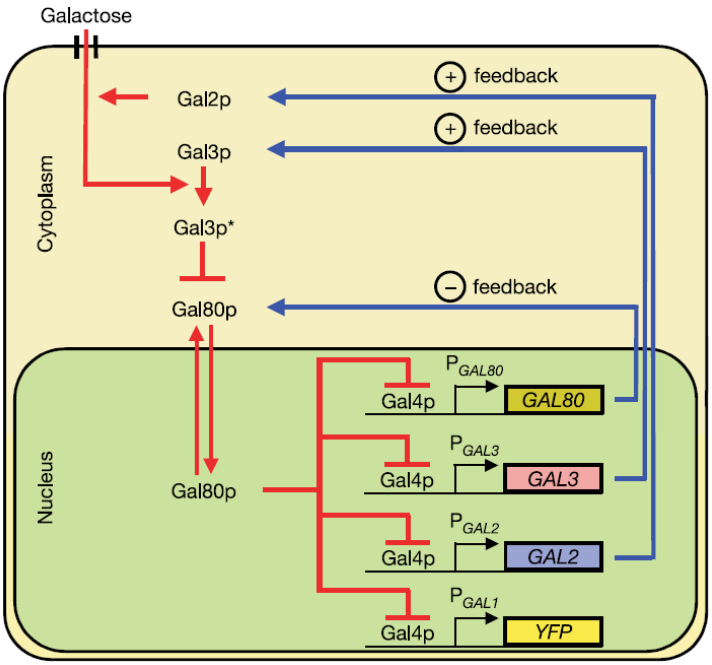


Figure 1-17. Comprehensive sketch of the galactose pathway in the budding yeast. From Stockwell et al, 2015.

b) Memory experiments

As I have presented above, the galactose pathway has been extensively studied and is very well understood. Since the repression in non-galactose conditions implies that it will take time for the repression to be lifted, the groups of J. Brickner and D. Tzamarias wondered back in 2007 if sequential activations of the GAL pathways could lead to a its faster reactivation. They published two different studies exposing similar results (Brickner et al., 2007; Zacharioudakis et al., 2007). I will here present Tzamarias' study.

The experiments on the galactose memory performed by this group followed a similar pattern which consists in:

- Yeast are grown in a medium containing glucose

- Yeast are then transferred to a medium containing galactose. The GAL cluster will then be activated and its dynamics of activation analyzed, usually using the fluorescence reporter GAL1-GFP.

- The population of yeast is then be transferred back to a medium with the original source of carbon, causing the GAL cluster to be deactivated. The

growth of the population will last several hours in order to allow them to divide and making sure that the GAL genes are not activated any more.

- A second induction in the galactose containing medium is performed and the dynamics of the GAL genes is then observed.

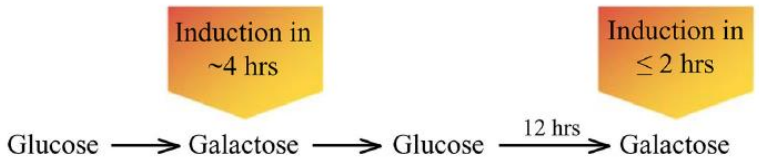


Figure 1-18. Principle of the galactose memory experiment. Yeast are grown in glucose, then switched to galactose until the GAL cluster is activated (4h). The cells are then switched to a glucose medium for 12h causing the deactivation of the GAL pathway, then switched to galactose and the dynamics of the GAL cluster is analyzed and compared to the dynamics during the first activation. From Kundu and Peterson, 2009.

The dynamics of activation of the genes during both activations is compared. A difference between the activations is likely to reflect a cellular memory.

Tzamarias' group observed that the GAL genes were expressed after 4h during the first induction and, interestingly, the time to induce the GAL genes was reduced by half after the second induction, triggered 12h after the first one. **It was inferred that previous activations of the GAL genes are remembered and lead to a faster activation: this is defined as the galactose memory.**

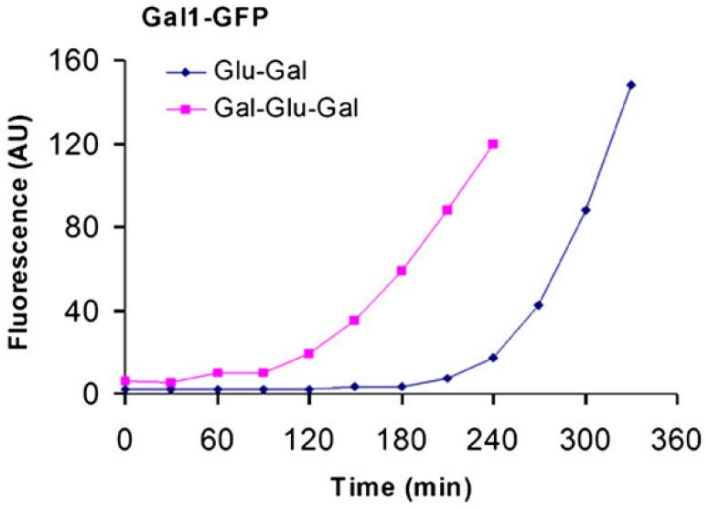


Figure 1-19. Fluorescence analysis of *GAL1* activation and reactivation. The GAL1 gene is fused to a fluorescence protein and its dynamics is analyzed upon a first and second galactose activation. Mean GFP values are analyzed every 30min. The activation of GAL1 occurs after 4h and its reactivation occurs after 2h.

However, a question was quickly raised by Perterson's group: could this reactivation be an artefact caused by variable time of glucose repression? In other words, does lifting the repression on the GAL cluster causes, after the first induction, the GAL cluster to be only slightly repressed compared to the previous time? To that effect, similar experiments were performed using raffinose[17] prior to the first induction then glucose prior to the second induction (Kundu et al., 2007).

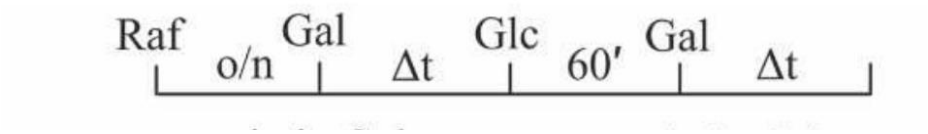


Figure 1-20. Principle of the galactose memory experiment. Yeast are grown in raffinose, then switched to galactose until the GAL cluster is activated (4h). The cells are then switched to a glucose medium for 1h causing the deactivation of the GAL pathway, then switched to galactose and the dynamics of the GAL cluster is analyzed and compared to the dynamics during the first activation. From Kundu et al, 2007.

In this case, the first galactose induction caused the GAL genes to be activated in less than 20min, and the second induction took less than 5min[18]. This means that the galactose memory is not an artefact of the glucose repression but a different biological phenomenon. However, such memory was lost after 6h (*i.e.* 3 divisions), leading the way to the notion of long-term and short-term memory.

[17] Raffinose is a sugar that causes the GAL cluster to not be expressed, but without an active repression.

[18] In this study, the analysis was performed using northern blots.

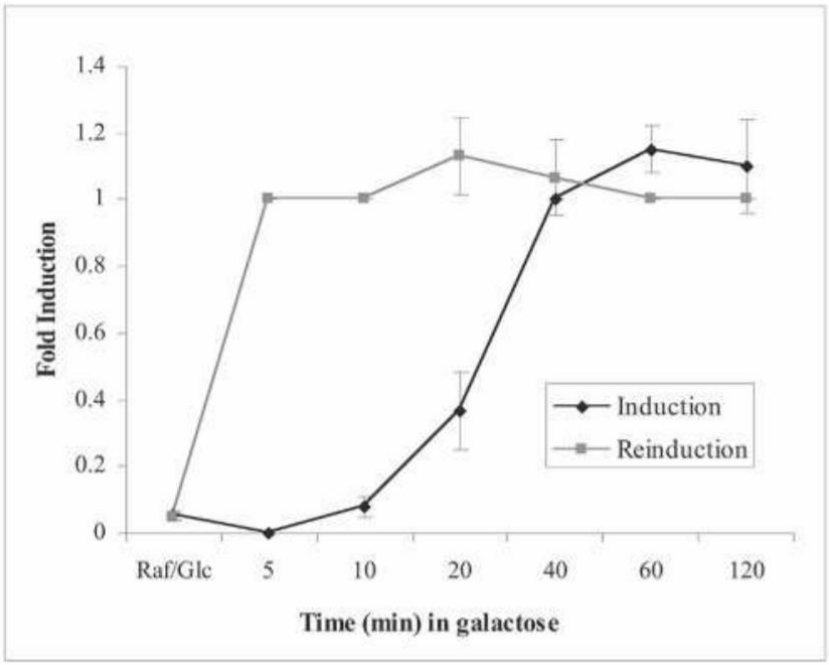


Figure 1-21. Transcriptional memory at the GAL1 gene. Comparison of the kinetics of GAL1 induction and reinduction following a sequential northern blot quantification. A faster GAL1 activity was observed after reinduction. From Kundu et al, 2007.

Because those two memories have different persistence and time dynamics, distinct biological mechanisms could explain them although their effects both consists in a faster reinduction of the GAL genes compared to a previous induction.

Long-term memory

This memory is characterized by a persistence between 4h-12h and has been associated to the presence of Gal1p which is synthetized during the first induction. Because of its long half-life, this protein is kept in the population of cells that received the first induction and is diluted throughout their progenies. Gal1p serves as a transactivator and its presence in the cell allows them to activate the GAL genes faster (Zacharioudakis et al., 2007).

Short-term memory

This memory is characterized by a persistence of 4h. It has been described to be a consequence of the activity of the SWI/SNF chromatin remodeler that poises the GAL genes for faster reactivation. Contradictory results however state that the relocation of GAL1 to the NPC forms a loop that is necessary for the memory. But such loop would only appear in the original population of cells (parents) as they keep their NPC, and

the memory is looked at after several hours, so in the daughters which have newly synthesized NPC (Brickner et al., 2007; Kundu et al., 2007).

The example of the galactose memory shows that depending on the time of repression between two inductions, different mechanisms can appear although the inductions have the same nature: there is no universality in the mechanisms of a memory to a specific stimuli as it appears to be time-dependent.

2. An example of hyperosmotic memory

As I have previously explained, the response and adaptation to hyperosmotic stresses has been extensively studied. There are however not many studies on the memory of the response to hyperosmotic stresses. I will here present the study performed by M. Proft's group (Rienzo et al., 2015).

Two identical populations of cells are grown in a synthetic glucose medium to exponential growth phase. The cells from one of the population are then submitted to a 1h (long) hyperosmotic stress using 0.7M of NaCl. Cells from the other population remain in the growth medium and serve as a negative control.

In a second time, both cultures were then transferred to a synthetic glucose medium and aliquots of those cultures were submitted to various concentration of NaCl. The dynamics of *GRE2* (an osmo-responsive gene) was analyzed using luciferin. The study shows that:

- Bellow 0.1M of NaCl there is no activity of *GRE2*, meaning that the stress is too mild to trigger an active transcriptional response: the cells tolerate such stress.

- From 0.25M to 0.8M of NaCl, *GRE2* displays a transcriptional activity that is always lower when the cells have already experienced the 1h hyperosmotic stress: this is defined as a cellular memory.

The deletion of *ENA1*, which codes for an ATPase sodium pump involved in the efflux of sodium, causes the loss of the memory. Therefore cells that have already experienced a stress have synthesized Ena1p that is kept in those cells and diluted to their progeny, therefore conferring them the memory of a previous stress. This memory does not rely on chromatin remodelers.

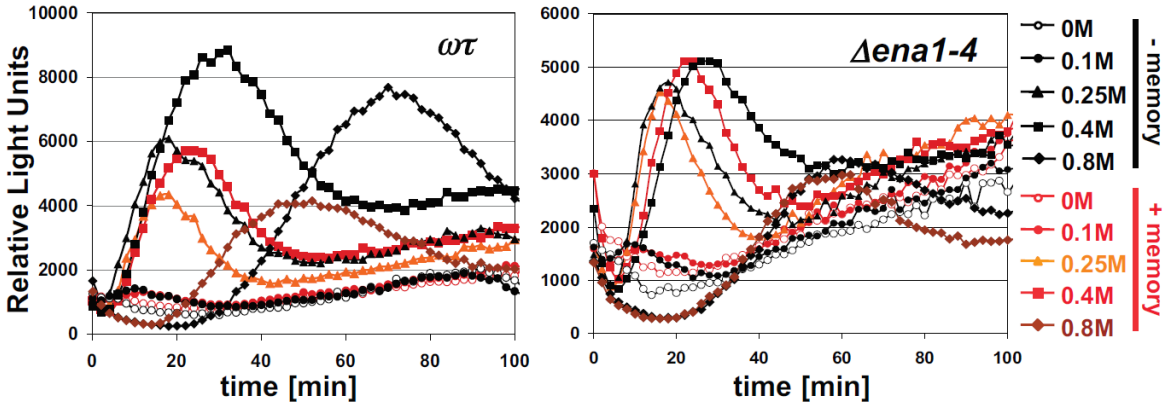


Figure 1-22. Memory of the GRE2 gene upon hyperosmotic streses depends on the Ena proteins. (Left) Fluorescence analysis of GRE2 fused to a luciferase in different hyperosmotic conditions. Cells pre-exposed to the hyperosmotic stress develop a memory characterized by a decrease of GRE2 activity (red) compared to cells that have never experienced a stress before (black). (Right) Deletion of the ENA complex causes the pre-exposed cells (red) to lose the memory and behave like cells that have never experienced a stress before (red). From Rienzo et al, 2015

Both these examples of cellular memory show that a cellular memory varies in its description depending on the stress. Indeed, the galactose memory is characterized by a faster gene activation whereas the previously described hyperosmotic memory is characterized by a reduced transcriptional activity.

All studies on cellular memory in the yeast converge on two mechanisms that are at their origin:

- **Cytoplasmic memory:** the fact that during the stress/induction, proteins are created and are still present, albeit in smaller concentration because of degradation or dilution, during the next stress/induction. Thus, they can help optimizing the response.

- **Epigenetic memory:** the memory is developed through changes on the chromatin state made by remodelers present prior to the stress. Although a cytoplasmic memory can technically be an epigenetic one, the distinction is always made between the two.

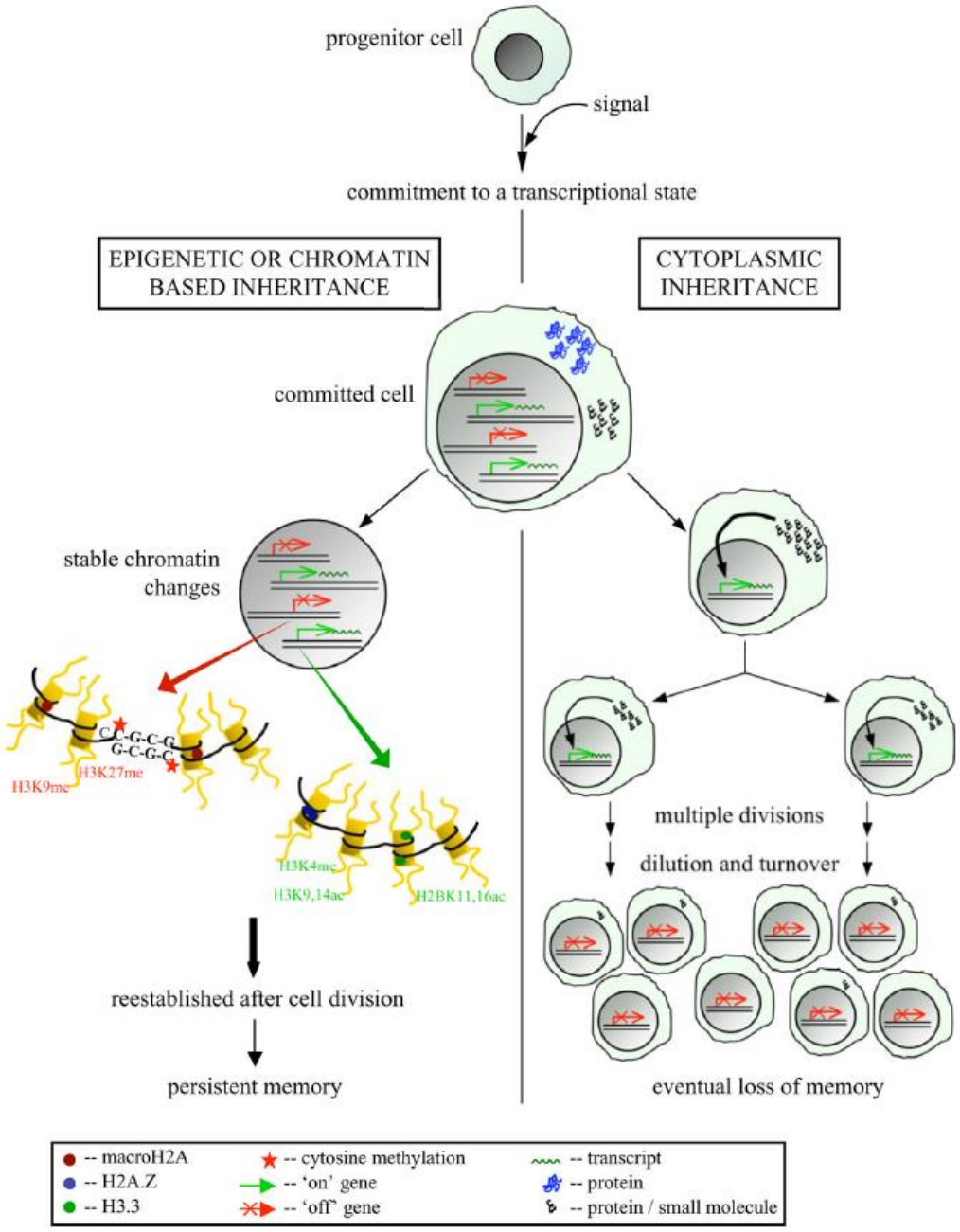


Figure 1-23. Summary of the different mechanisms of cellular memory. (Left) Epigenetic modifications occur following the event that caused a transcriptional response and causing the emergence of a cellular memory. Such epigenetic modifications are stable and can be inherited. (Right) Following a stress, proteins will be synthesized causing the emergence of a memory. These proteins are diluted to the progeny and the memory persistence is function of the half-life of the proteins. From Kundu and Peterson, 2009.

Here, I want to stress the fact that the studies on cellular memory are performed in a population of cells. Because of the division time of the budding yeast and the long inductions of the stresses, the memory is always shown to be transmitted to the progeny of the original population of cells. However, it is not known if the initial population that receive the first induction develops the memory as well and, more broadly, if the memory is systematic or if it takes some time to be set throughout the

generations of descendent. These questions can be answered by not looking at the entire population, but at specific individuals. It requires the ability to track individual cells and their progenies.

III. On the importance of single-cell measurements

Despite having similar genotypes, a clonal population of cells can display different phenotypes. This is due in part to the epigenome, environmental factors and the noise in gene expression. In this part, I will explain how genetic noise causes an isogenic population to be different and the use of performing studies at the single-cell level.

A. Mechanisms of stochastic gene expression

Gene expression is a phenomenon involving biochemical reactions, which are dynamical and subjected to some inherent randomness: stochasticity is an important feature. For instance, there is such a small amount of transcription factors available for a specific promoter to create a productive reaction that transcription of a gene becomes an unlikely event (Kepler and Elston, 2001). Thus, the description of biochemical processes occurring in the cells can be explained by stochastic processes.

The first evidences associated with the stochasticity of gene expression were provided experimental with the microorganisms *E. coli* and the budding yeast (Elowitz, 2002; Raser, 2004). The principle of the latter experiment is as follow: two identical *PHO5* promoters control the expression of two fluorescence proteins genes that are relatively similar in their sequence (*YFP* and *GFP*). The proteins are therefore relatively similar and expressed in a similar fashion, thus erasing the possible bias of the overexpression of one protein compared to the other. Moreover, the promoters are located at identical loci so that their activity would be identical and not linked to chromatin environment. Thus, for each cell, it is possible to perform fluorescence measurement of each fluorescence reporter in order to quantify it.

The analysis revealed that the spectrum of the two different fluorescence proteins under the control of the same promoter were different. The cells have a mixture of red (symbolizing the YFP) and green (symbolizing the CFP). Each cell is represented by a dot. The color of the dot represents cells from different time points from the start of the induction of the *PHO5* promoter. In the case of a system where the promoter could be described by a deterministic process, the two reporters would have the same expression level and all dots should fall along the bisector (YFP=CFP

line). However, a non neglectable number of cells are away from the bisector. This number is higher than what could be expected in deterministic conditions

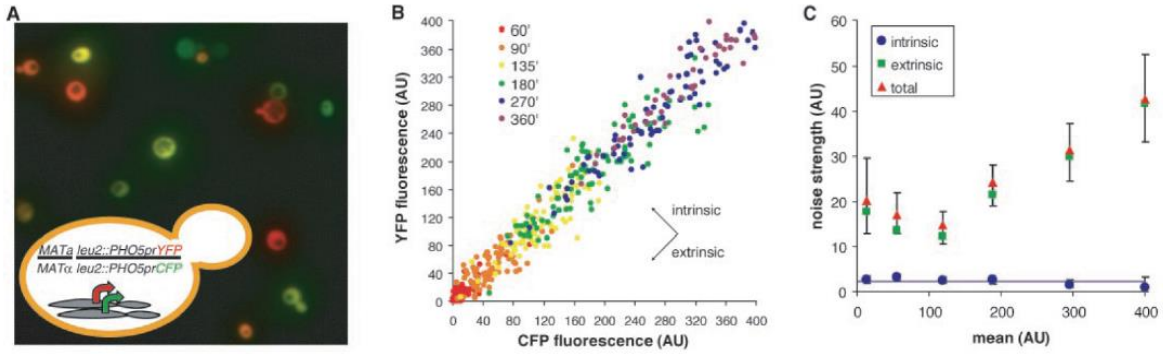


Figure 1-24. Two components of genetic noise. (A) Microscopic picture of the dual fluorescence strains. (B) CFP vs YFP fluorescence intensities measurements upon induction. (C) Decomposition into intrinsic and extrinsic components. From Raser et al, 2004.

The conclusion of the article is the existence of two components that justifies the differences of expression of YFP and CFP:

-the intrinsic component: represents the deviation from the situation YFP=CFP, i.e. the fact that in this experiment, clonal cells did not transcribed the two promoters equally.

-the extrinsic component: represents the fact that some cells are globally brighter than others, regardless of the proportion of YFP and CFP. Thus, this component is independent of transcription and is likely linked to cell-cell physiological differences.

B. Necessity of single-cell studies on adaptation

Most studies questioning adaptation effects are carried out on isogenic populations of cells giving information on the mean population behavior. Nevertheless such a population is heterogeneous and sometimes results at the single-cell level are crucial. In the case of the hyperosmotic stress for instance, a bimodality of the stress response is shown to exist, linked to a slow transition from a repressed state to an active one[19] (Pelet et al., 2011).

An overview of some of the techniques enabling single-cell studies is found bellow (Llamosi, 2016). Another tool enabling performing single-cell experiments that is not represented on the figure bellow is microfluidics. As it is the tool used during

[19] This will be presented in more details in the Chapter III.

the PhD, I will briefly present it in the next chapter, along with the specific devices I have used and how they were made.

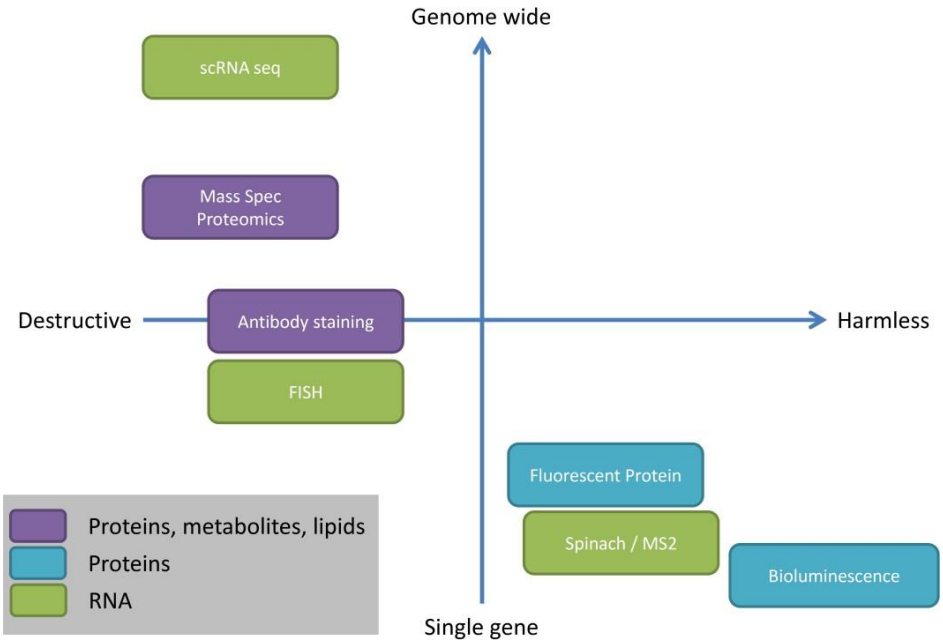


Figure 1-25. Example of various technological tools for genome wide and single-cell analysis, classified according to their dangerousness. Image from Artemis Llamosi.

C. Conclusion

The experimental evidence of the difference in gene transcription in a clonal population of cells can be linked to two parameters. As I have presented before, those two parameters can be described in a simplistic way as: 1/ the influence of the environment on a cell's physiology in a manner independent of the biochemical reaction occurring in the cell, so independent of gene transcription and 2/ the stochasticity of the biochemical reactions within the cell and the unlikeliness of gene expression occurring because of the low number of reactants. Those parameters are both referred to as extrinsic and intrinsic noise respectively. The figure bellow provides a good summary.

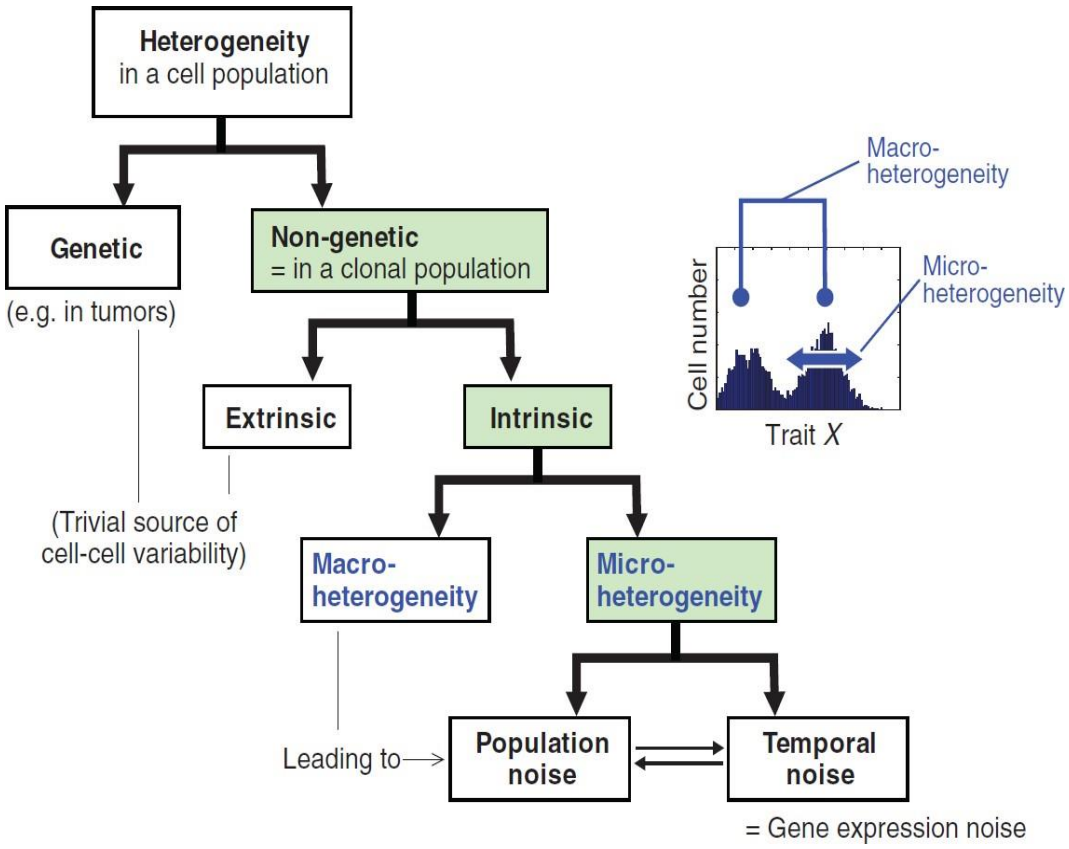


Figure 1-26. Tentative summary of the description of extrinsic and intrinsic noise. From Huang 2009.

As a brief conclusion on the stochasticity is its origins have been quickly described, but what could its biological purpose be? As I have presented before, stochasticity is an inevitable phenomenon determined by the laws of physics and chemistry. It has a natural repercussion in biology, which is causing a genetically identical population of cells to actually behave differently in response to external stimuli. In other words stochasticity permits, along with genetic variability, the emergence of diversity. Thus stochasticity is a necessary phenomenon in biology as diversity is the driving motor of evolution.

General conclusion

The response to hyperosmotic stresses was presented, and I here want to emphasize that adaptation to a long hyperosmotic stresses in the yeast involves a feedback loop that prevents the understanding of transcriptional events exclusively. However, short hyperosmotic stresses allows the investigation of transcriptional events and the eventual study of the possibility that the budding yeast could develop a memory in response to short stresses.

This is in such a framework that the studies I have performed took place. As I have presented in the motivations, I have investigated the dynamical response of budding yeast to pulsed hyperosmotic stresses in order to interrogate: **do budding yeast develop a memory of short repeated hyperosmotic stress at the genetic level solely and if so, what are the underlying biological mechanisms?**

I will present in the second chapter the various tools I have designed and used that made the experimental work possible. The third chapter will be dedicated to the results that I have obtained. The fourth and final chapter will serve as a discussion, conclusion and opening that, I hope, will be of counsel for whoever will perform akin or subsequent work dealing with cellular memory and dynamical variability effects in the budding yeast.

Chapter II: Materials and Methods

Table of contents

Performing long term experiments on single-cells is a challenge. Not only do the cells have to be constrained in a specific area so that they can be observed without getting out of the view field of observation, they also need to be as close as possible to their normal state. The latter can be perturbed by the constraint applied to keep the cells at specific locations. The cells also need to be fed, which requires flowing a constant fresh media on the cells. In addition, if one wants to investigate the behavior of cells in response to specific stimuli, there must be a way to switch between different media dynamically. Finally, since the experiments can last for hours, most of the processes such as image acquisition and input delivery, have to be automated. As a result, the creation and use of specific tools are required to tackle those issues.

I. Microfluidics

A. Introduction

Microfluidics is a field of science and technology aiming at studying the dynamics of fluids at micrometric dimensions, and to develop techniques to manipulate fluids at small scale. It is a very active field of research that has led to numerous technological applications, particularly in biology. Microfluidics developments are also known as "Lab-on-a-chip" and consists in miniaturizing and automatizing experiments usually performed in an experimental biology or chemistry lab, such as PCR (Khandurina et al., 2000), RNA sequencing (Wu et al., 2014), cell growth (Barbulovic-Nad et al., 2010), blood analysis (Dimov et al., 2011) and even identification of viral receptors (Patolsky et al., 2004), using small microfluidic devices. Microfluidics stands out as it allows not only to make single-cell observations for hours (even days), but also to control the external environment with fast dynamics (seconds to minutes).

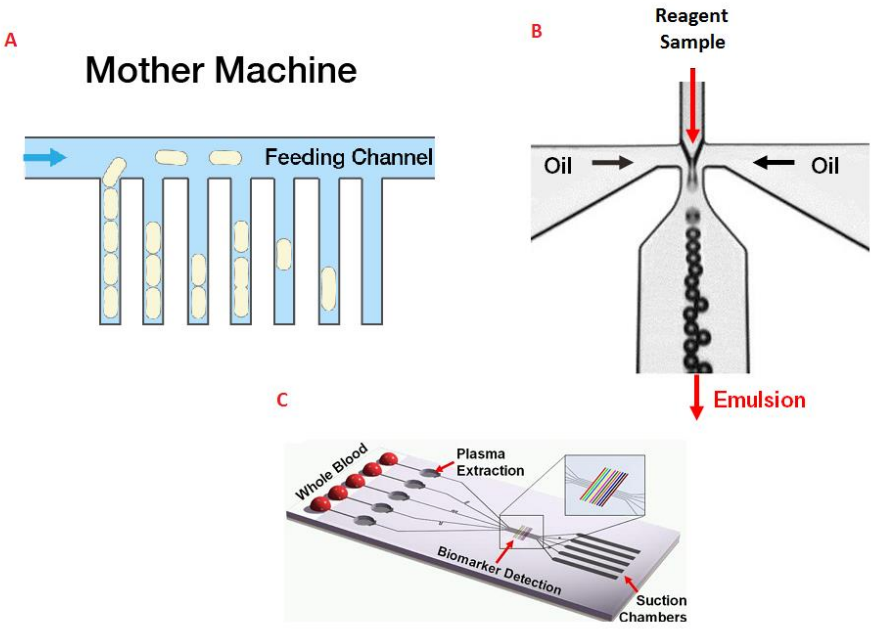


Figure 2-1. Sketches of several microfluidic devices. (A) Mother machine. Bacteria are loaded in chambers and grow there in line. A medium flows in the feeding channel and diffuses in the chambers, feeding the bacteria. (B) Microfluidic device enabling the formation of droplets. A hydrophilic reagent is flown in a chamber, and oil is flown from to the reagent. The ensemble exists through an opening of specific height, which leads to the creation of droplets. (C) Microfluidic device for blood analysis. Blood is flown in channels to a chamber containing biomarkers.

The majority of microfluidics devices are made out of synthetic polymers (mostly polydimethylsiloxane, PDMS) or glass welled in a mold called a wafer. Taking advantage of the experience in making microfluidic devices in one of my teams, I learnt how to design, micro fabricate and operate microfluidic systems for yeast.

B. Microfluidic device used during the PhD

The microfluidic device I used during my PhD is a H-shaped device composed of two elements:

- Two flow channels of 40µm height that possess inputs at each ends where tubes of 0.02mm diameter are connected perfuse culture medium at a velocity of 120µL/min.

- Squared observational chambers of 3.6µm heights and 400µm side. The yeast are confined in the chambers[20] and imaged. The medium flowing in the channels diffuses to the chambers, feeding the cells that are confined there.

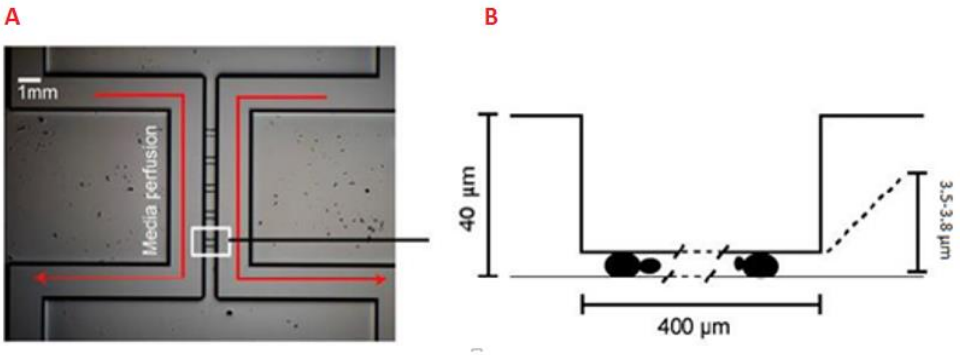


Figure 2-2 H-shaped microfluidic device used during the PhD. (A) picture of the microfluidic device. (B) The cells are constrained in a chamber where they receive the medium. From Uhlendorf et al, 2012.

[20] The cells are loaded using a 0.02mm needle syringe from one of the two input channels and access the chambers where they stay confined. The confinement is very important as it constrains cells to grow as a monolayer thus making them easier to segment and track.

C. Microfabrication: how to make the device?

1. Principle of photolithography

The dominant approach to make microfluidic devices is based on photolithography, a technique which purpose is to transfer the motifs of a support that can be a photomask or a film, to a light-sensitive substrate. The latter is, in my conditions, a silicon wafer on which is poured a photoresist. It is a chemical that becomes soluble (positive photoresist) or insoluble (negative photoresist) in a developer, after exposure to UV light. I had the opportunity to perform photolithography in the clean room facility of Paris Diderot University.

The principle is as follow:

-the wafer + photoresist[21] and the photomask are placed in contact, then exposed to intense UV light.

-the photoresist is then removed using a developer, thus only the motifs will remain on the wafer.

-PDMS can then be poured on the newly created mold and baked so that it gets solid. The removal of solid PDMS from the wafer provides a usable microfluidic device.

[21] The photoresist is poured on the wafer and spin-coated at a specific velocity. The faster the ensemble is spin-coated, the lower would the height of the photoresist be on the wafer.

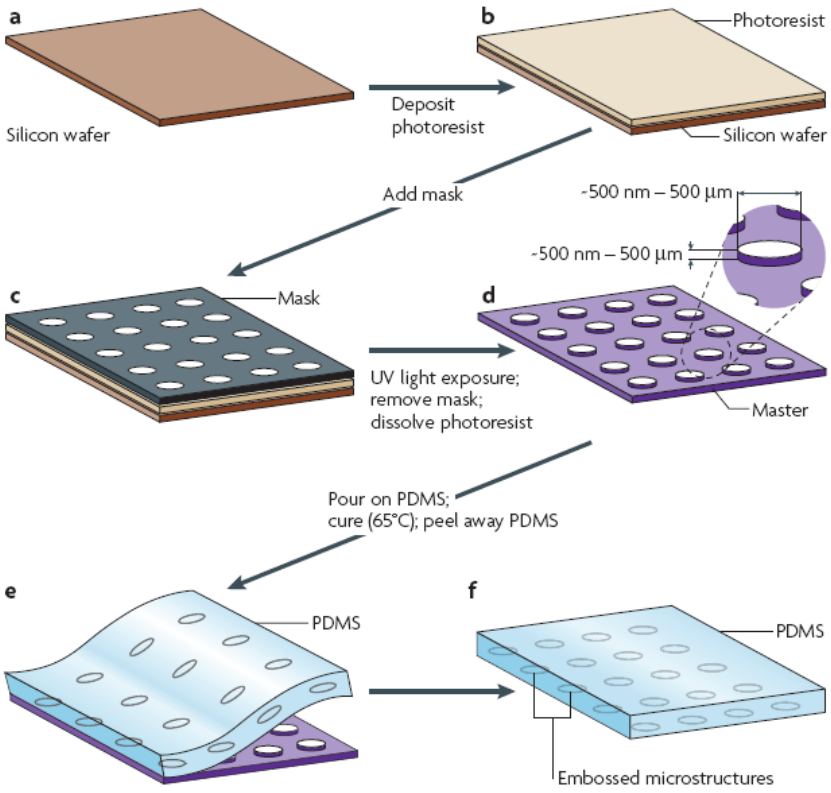


Figure 2-3. Principle of soft photolithography. (a) Silicon wafer that serves as a support to make a mold. (b) Photoresist is poured and spin-coated on the silicon wafer. The velocity of spin-coating determines the final height of the mold. (c) The photomask is put in contact with the wafer. The ensemble is then (d) exposed to UV light, which enables the engraving of the patterns of the mask on the wafer. The photoresist is then dissolved in a developer. (e) PDMS is poured on the new wafer and serves to make a (f) microfluidic device containing the patterns.

2. Design of the photomask

In order to make a design of the microfluidic device that will serve to make a photomask, I use the layout software L-Edit (Mentor). As I have presented above, the flow channels and the observation chambers have different heights. Therefore, I separate the design of the microfluidic in two parts. Once the photomask is received, the idea is to first engrave the patterns of smallest heights (observations chambers) on the wafer. Then, the flow channels will be engraved on the same wafer. In order to align the observations chambers and the flow channels properly, I added references crosses on the design. The alignment process is made in the cleaning room using a mask aligner MJB4 (Süss Microtec).

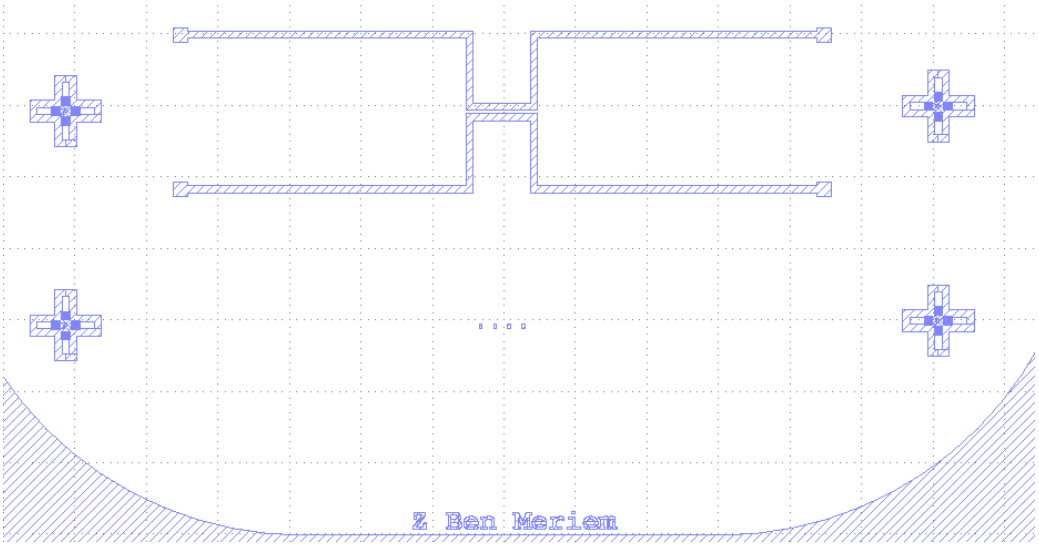


Figure 2-3. Design of the photomask used to make the microfluidic device use in the PhD. (top design) Flow channels (bottom) observation chambers. Reference crosses allows to align the chambers and the channels.

3. Protocol

The photoresist I use comes from a family of resist called SU-8 (Microchem, Newton, USA). In order to grow yeast in a monolayer, I aim to reach a height of 3.6µm of my microfluidic device's observation chambers.

I use the SU8-2010 that I dilute by 1/3 in order to reach a range of heights between 3µm and 5µm, something that cannot be obtained with a non-diluted SU-8 photoresist[22].

My experimental protocol is as follow:

1- Pour the photoresist on a silicon wafer[23] and spin-coat at 500 rpm for 10s with acceleration of 100 rpm/s then at 3000 rpm for 30s with acceleration of 300 rpm/s.
2- Soft bake at 95°C for 2min
3- Exposure to UV lights (100mJ/cm²)

[22] In theory it should be possible to reach heights above 3µm with the SU8-2 photoresist. However, this requires such low spin-coating velocities that the photoresist does not spread across the wafer in a homogeneous fashion: the subsequent microfluidic device will not necessarily have the same height everywhere.
[23] I use wafers from Silicon Prime Wafers, 76.2 mm diameter, N/Phosporus dopant, a resistivity of 1-10 Ohm.cm and 380µm thickness.

4- Post-exposure bake at 95°C for 3min
5- 1min treatment in the SU-8 developer
6- Hard bake at 150°C for 15min

After hard bake, I use a mechanical profilometer Dektak 150 in order to control the heights of the patterns.

Once the wafer is obtained[24], usually referred to as a master wafer[25], PDMS is poured on it then baked overnight at 65°C. It is then possible to cut microfluidic devices using a scalpel.

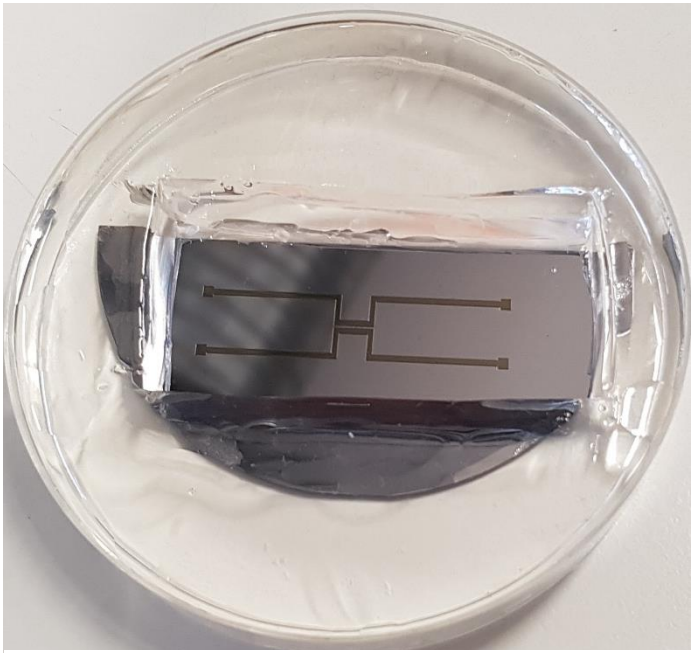


Figure 2-4. One of the wafer used during the PhD that shows the shape of the microfluidic device.

II. Yeast strains and methods

[24] Removing the PDMS from the mold can cause the patterns to be removed as well. After obtaining the wafer with the patterns, it is useful to treat its surface with silane (Sigma, ref 63800). This step makes it possible to remove the PDMS device from the mold without destroying the patterns.

[25] An alternative that I adopted is to cut a thick PDMS microfluidic device and make another master wafer with a resin called epoxy. This newly made wafer is extremely solid, won't break following strong stresses caused with a scalpel as it may be the case with the master wafer. Moreover, it provides microfluidic devices of the same quality than the original master wafer. A protocol detailing the making of an epoxy wafer can be found in the appendix.

During my PhD, I mostly made fluorescence measurements of a strain which has the *STL1* gene replaced by a fluorescence protein[26] (strain yPH53). This strain served as a parent strain, as the strains I later made are derived from this one.

A. Yeast transformation protocol

I use the standard Lithium Acetate transformation protocol to genetically engineer yeast strains. Unlike the common protocol however, I treat the cells for 20min with 0.5M $CaCl_2$, after the heat shock step[27]. The detailed protocol along with the list of primers used to make the strains of interest can be found in the appendix.

B. List of strains presented in the manuscript

STRAIN	GENOTYPE
yPH003	ura3Δ0, leu2Δ0, lys2Δ0, GPD1-GFP-HIS3
yPH015	leu2Δ0, lys2Δ0, HOG1::GFP-HIS3, HTB2::mCherry-URA3
yPH053	ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, pSTL1::yECITRINE-HIS5
yPH200	ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, Δ(pSTL1-STL1)::CaURA3
yPH212	ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, Δ(pSTL1-STL1)::CaURA3, Δtrp1::pSTL1-yECITRINE-HIS5
yPH213	ura3Δ0, his3Δ1, lys2Δ0, pSTL1::yECITRINE-HIS5, Δsir3::pLEU2-LEU2
yPH215	ura3Δ0, his3Δ1, lys2Δ0, Δ(pSTL1-STL1)::CaURA3, Δtrp1::pSTL1-yECITRINE-HIS5 , Δsir3::pLEU2-LEU2
yPH353	leu2Δ0, his3Δ1, lys2Δ0, pSTL1::yECITRINE-HIS5, Δtrp1::pURA3-URA3
yPH358	ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, pSTL1::yECITRINE-HIS5, SNF2::pLEU2-LEU2
yPH359	ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, pSTL1::yECITRINE-HIS5, HTZ1::pLEU2-LEU2
yPH360	ura3Δ0, lys2Δ0 , GPD1-GFP-HIS3, Δsir3::pLEU2-LEU2
yEF506	ura3Δ0, trpΔ1, lys2Δ0, his3Δ1, SIR3::GFP-TRP1

C. Culture media

[26] The fluorescence reporter is the yECITRINE, which excited at 516nm and emits at 529nm and has a half-life of 2h (Bionumbers).

[27] Such treatment is thought to increase the transformation efficiency.

I used a SC media (Synthetic Complete), which is one of the most used culture media for *S. cerevisiae*. It is especially useful if one wants to select for auxotrophic markers (*i.e.* remove one or more amino acid).

I typically make SC 1X solution in 180mL of H_2O. To that effect, I mix and autoclave[28]:

- 1.34g of Yeast Nitrogen Base w/o Amino Acid (Difco ref 291940)
- 0. 16g CSM dropout mix (MP Biomedicals ref 114500022)

In addition to the SC 1X, I add 20 mL of a 10X glucose autoclaved glucose solution (200g of glucose in 1L H_2O). This allows me to have a solution of SC 1X+ 2% glucose which is the media I use to feed the cells. It is important to autoclave the glucose separately from the SC to avoid auto fluorescence of the medium.

In order to trigger a hyperosmotic stress, I supplement the medium with a 2M sorbitol solution (Sigma, ref S1876) in order to reach a final concentration of 1M.

D. Protocol for Crispr experiments

I used the plasmid pAG414GPD-dCas9-VPR from Addgene (ref # 63801) to express the inactivated form of CAS9 fused to transcriptional activator VPR. This plasmid has a TRP1 selection. The guides RNA were cloned under SNR52 promoter in plasmid pEB002 (TRP1 selection) using the enzyme BsmBI. Digesting the resulting plasmid by NotI / XbaI and cloning the Guide containing fragment into pRS425 similarly digested, performed marker exchange (from TRP1 to LEU2), leading to the plasmid pZB004. I designed two guides targeting pSTL1 using the online software E-CRISP[29] targeting pSTL1. I then checked on SGD[30] that the guides were in a nucleosome free region. Strains yPH212 and yPH353 were used for Crispr transformation. The detailed protocol along with the maps of the plasmids can be found in the appendix.

[28] After autoclaving, SC sometimes presents some precipitate put dirt in the microfluidic device during experiments. To avoid such debris, I filter the medium used for the microscopy experiments.
[29] http://www.e-crisp.org/E-CRISP/
[30] https://www.yeastgenome.org/

E. Flow cytometry experiments

I performed all flow cytometry experiments using a flux cytometer Gallios (Beckman Coulter) equipped with 10 colors, 4 lasers (488nm Blue, 561nm Yellow, 638nm Red, 405 nm Violet). As all cells that I observed possess the yECITRINE fluorescence reporter, I used the excitation laser 488nm and the emission filter at 530nm +/- 30 nm. Cells were grown in SC+2% glucose. Experiments were performed at OD=0.5. Removal of the inducer is performed through a centrifugation at 4000rpm for 5min.

III. Long-term experiments

A. Experimental platform

To follow the fluorescence in the cells, I used an inverted microscope Olympus IX71. The yeast were observed with an objective x100 UplanFLN 100x/ 1.3 Oil Ph3 Ul2. The microscope was linked to a camera Cool Snap HQ2 Princeton Instruments. All experiments were made at 30°C thanks to a thermostat Cube-Life Imaging. The microscope also contains a piezo, used *inter alia* for autofocusing.

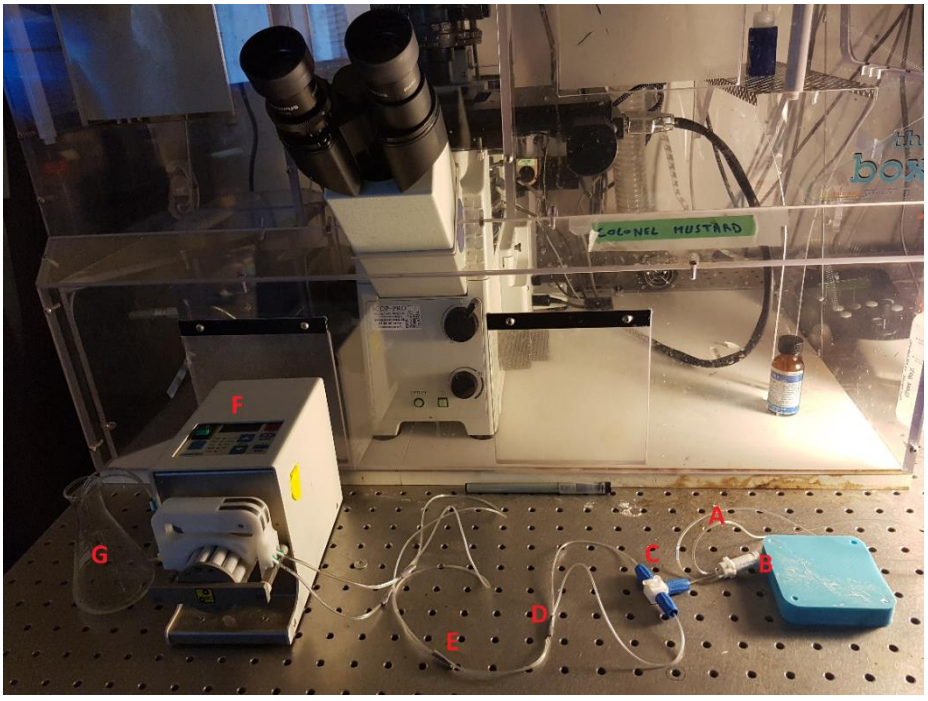


Figure 2-5. Image of the experimental platform. (A) Two tubes in the relevant medium are connected to the (B) arduino controlled valve. Exiting from the valve a tube going in the (C) T-deviation. The two following tubes will be connected to the (D) two inputs of the microfluidic (E) and its two outputs. A peristaltic pump (F) pulls the medium in the circuit and flows it in the trash (G).

Yeast are imaged every 5min with 20ms exposure in bright light and 200ms in fluorescence light, using a mercury lamp Olympus U-RFL-T. The microscope was controlled by the open source software MicroManager which was interfaced with Matlab[31].

Cells were grown in SC 1X+2% glucose. In order to trigger a hyperosmotic stress, the media was supplemented with 1M sorbitol. Both media are flown in the microfluidic chip using a peristaltic pump ISMATEC with a velocity[32] set at 120µL/min with tubes of 0.02mm diameter. A Matlab-controlled Arduino circuit[33] controls a valve[34] that allows the switching between growth media and inducer media.

B. Autofocus

I use an autofocus that was developed in the lab which allows to:

- impose a specific window as to where the autofocus should be performed. I impose the autofocus to work only on the sharp-edged patterns of my microfluidic device, which are fixed structure that never move over the course of the experiment.

- take an entire z-stack of one of the sharp-edged pattern that I set as a reference prior to the experiment. The sharpest image is determined using the entire stack and all the out-of-focus information. This new method proved itself to be very stable and was actually at the inception of a new segmentation method developed in the lab (see appendix).

[31] Associated Matlab script can be found on https://github.com/Lab513
[32] Such velocity allows diffusion in the chambers without flushing the cells out of the chambers.
[33] Associated Matlab script can be found on https://github.com/Lab513
[34] The Lee Co, ref LFAA1201418H

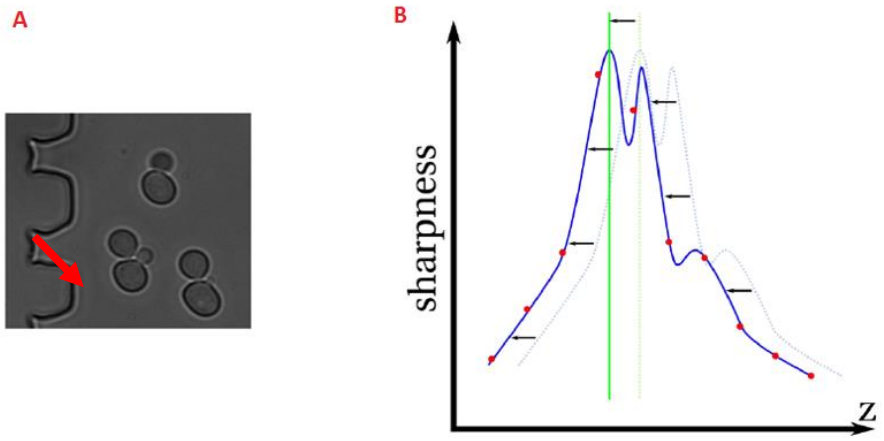


Figure 2-6. Microfluidic device and autofocusing. (A) Sharp edges of the observations chambers (red arrow) are used as reference structures to perform the autofocusing. (B) Typical autofocusing curve. The image sharpness measured through a stack during an acquisition (in red) is compared to the curve acquired at the beginning of the experiment (in blue) and the new in-focus frame is inferred from it (green line). Using the entire stack for comparison is more precise than using a single image as reference.

IV. Single-cell analysis

I made a simple program to have a semi-automatic method to perform the single-cell analysis. First, I manually segment a cell on a bright field image. Then, the program measures various parameters such as mean value of grey level inside the cell, on the corresponding fluorescence frame before moving to the next frames both in bright field and fluorescence

This method is time consuming, but factually gives an extremely robust way to segment and track cells[35]. The fluorescence for every cells can thus be analyzed.

[35] There are several available software to perform automatic segmentation and tracking. However, the implementation time for my image analysis was so long that using the semi-automatic method was more efficient.

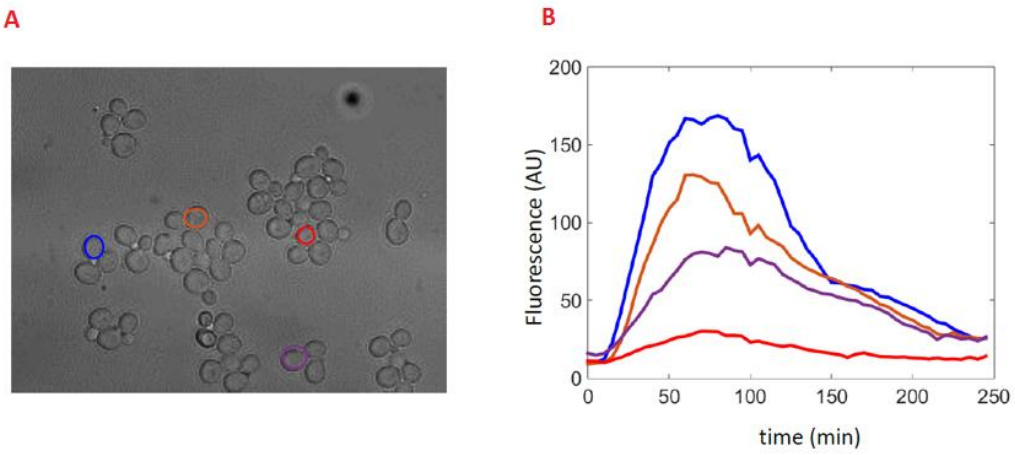


Figure 2-8. Manual segmentation and fluorescence trajectories. (A) Segmentation on four different cells from a bright field image. (B) Fluorescence trajectories of the four cells after segmentation and tracking over time.

Chapter III: Results

Table of contents

Most studies on cellular memories do not take into account the heterogeneity between cells in a population, yet cell variability is critical to understand how cells adapt and remember past stresses. Indeed, single-cell variability may cause genetically-identical cells to exhibit different behaviors when encountering the same stimuli. My first objective is to study, at the single-cell level the response of yeast cells to repeated, pulsed hyper osmotic stress. I will show that under such conditions, yeast cells do exhibit a genetic memory of past stresses at the population level, even though they display a large cell-cell variability that I have quantified. After establishing and characterizing this memory, I will expose how my experimental results show that the memory does not require de novo protein synthesis and is gene-positioning dependent. I will close this chapter by presenting preliminary results on the influence of chromatin modifying proteins and the inheritance of the cellular memory.

I. Introduction

A. Performing short stresses prevents the physiological adaptation

With the long-term experimental platform presented in the previous chapter **(figure 2-5)**, I am able to submit a population of cells confined in my microfluidic device to a long hyperosmotic stress, and follow the stress response of single cells over time. Bellow, an example of yeast submitted to a continuous hyperosmotic stress imaged in bright and fluorescence light, along with the corresponding single-cell quantification **(figure 3-1)**.

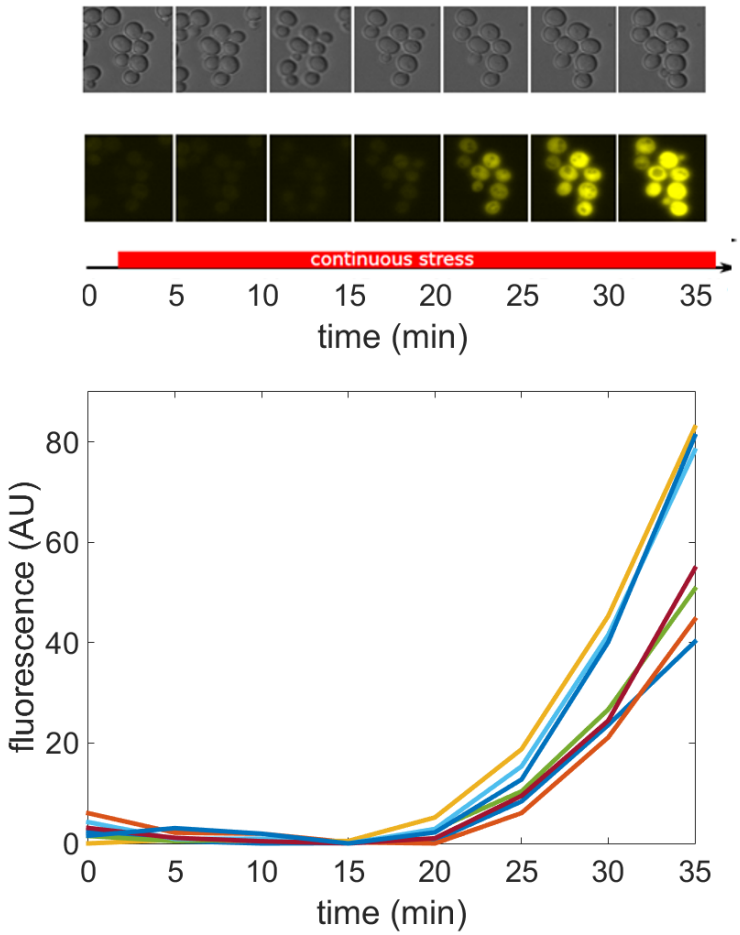


Figure 3-1. Observation and analysis of cells (yPH53 strain) submitted to a continuous hyperosmotic stress. The stress is triggered using sorbitol 1M. (Top) All cells respond to the long stress. The cells are exposed both to bright light (10ms exposure) and fluorescence light (200ms exposure). (Bottom) Fluorescence trajectories of cells in the view field that received the hyperosmotic stress.

I have presented in the introduction that a long hyperosmotic stress causes the cells to physiologically adapt to the stress within 15-30min (Miermont et al., 2011). This is due to a feedback loop orchestrated by the Hog1 protein (Zi et al., 2010). In such conditions, it is impossible to discriminate between the genetic response and the physiological adaptation. However, my aim is to focus on transcriptional events exclusively. This implies that the duration of the stress should not be longer than the duration of the main steps of physiological adaptation, *i.e.* shorter than 15min. However, the transcriptional events during hyperosmotic stresses start after several minutes, so the duration of the stress has to be long enough to trigger the activity of the transcriptional response, but also to have enough protein production in order to make relevant analysis. As Hog1 is responsible for the upregulation of stress response gene upon hyperosmotic stress, I decided to perform 8min stresses as such duration is long enough to cause a significant nuclear Hog1 enrichment **(figure 3-2)**, thus a consequent transcriptional activity, but short enough to not cause a physiological adaptation.

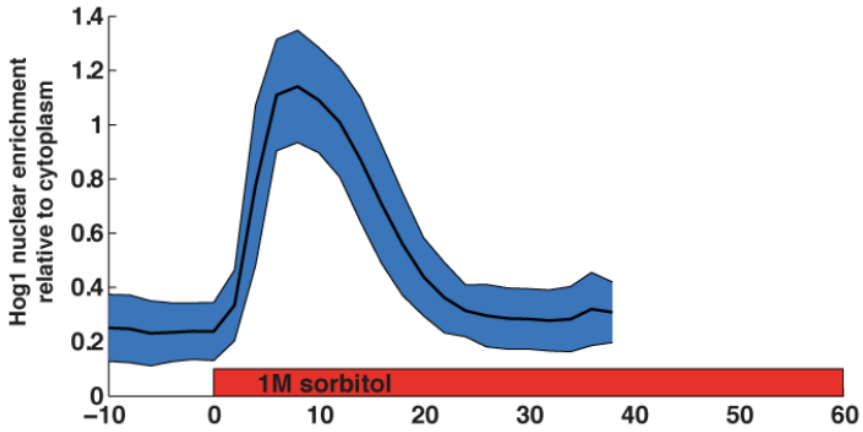


Figure 3-2. Analysis of the nuclear enrichment of the Hog1p. The strain used is yPH15 and imaged every 2min in GFP. Peak of enrichment is reached after 8min of hyperosmotic stress triggered with 1M sorbitol. This time is chosen as the time of the stress induction in my PhD. Data from Llamosi, 2016.

With this experimental platform, I am able to submit a population of cells confined in my microfluidic device to an 8min stress and perform the single-cell quantification of the fluorescence level (**figure 3-3**).

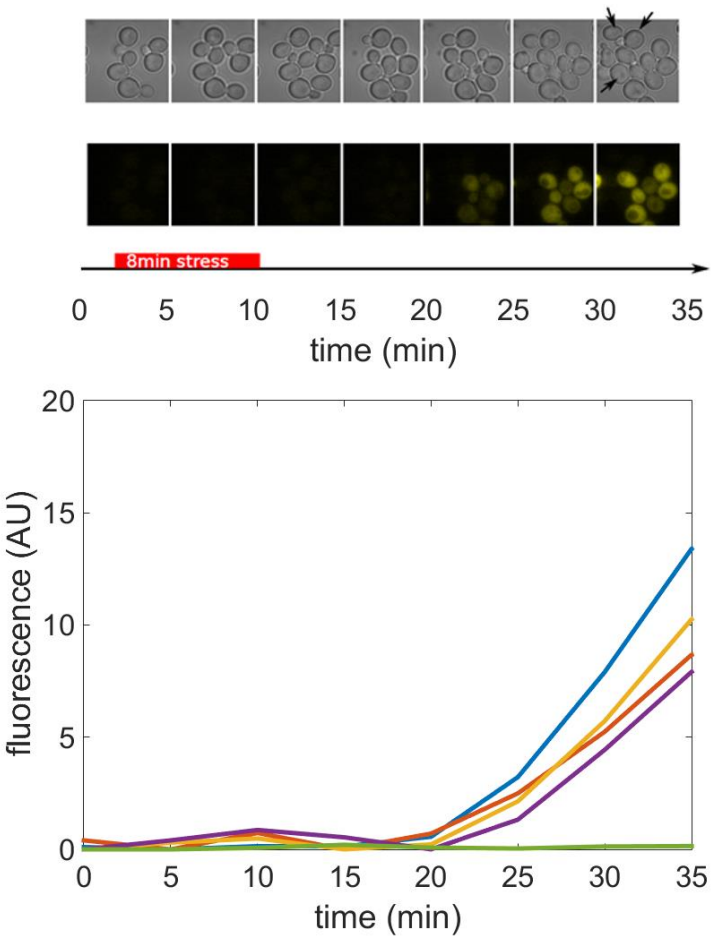


Figure 3-3. Observation and analysis of cells submitted to an 8min stress. The stress is triggered using sorbitol 1M. (Top) The cells are exposed both to bright light (10ms exposure) and fluorescence light (200ms exposure). Variability of the response is visible; the arrows point to cell that do not show a response to the stress. (Bottom) Fluorescence trajectories of cells in the view field that received the hyperosmotic stress.

A consequence of performing an 8min stress is that the cell-cell variability is pronounced as all cells in the view field do not express the fluorescence protein to identical levels **(figure 3-4)**. In particular, some cells do not express the fluorescence protein at all, a phenomenon referred to as bimodality (Pelet et al., 2011).

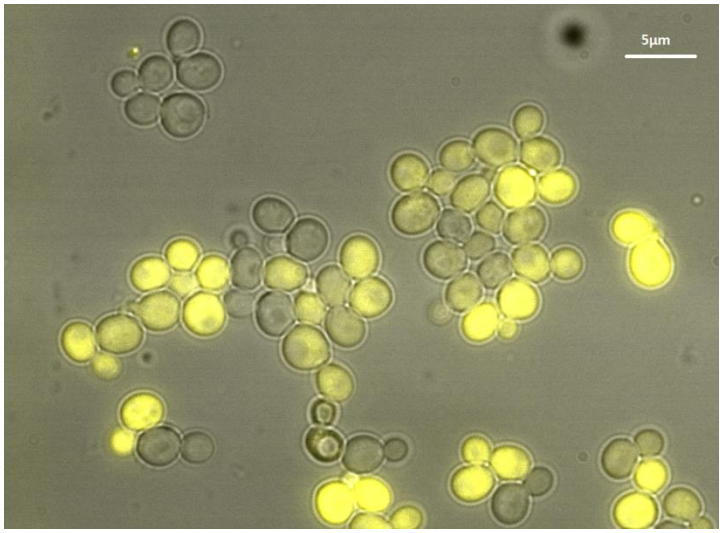


Figure 3-4. Single-cell variability in the expression of fluorescence. Merge of bright field (10ms exposure) and fluorescence (200ms exposure) images of a population of cells (strain yPH53) 70min after receiving an 8min hyperosmotic stress. Most cells express the fluorescence protein, with a large variability in its amount. Some cells, particularly (top left) do not express the fluorescence protein at all.

B. Amplitude and time parameters to study the response to repeated stresses

As my goal is to study the genetic response to short repeated hyperosmotic stresses, I studied a population of growing yeast in the microfluidic device that I submitted to 8min stresses separated by 4h. This is done by switching the culture medium to a medium supplemented with 1M Sorbitol. Image analysis enables me to obtain the fluorescence trajectories of all cells in response to two pulsed stresses **(figure 3-5)**. The rationale for this experimental plan is to activate the response to hyperosmotic stress, wait for the cell to recover fully from the stress, progress through the cell cycle and let the yECITRINE reporter protein to be degraded[36], then apply a second stress.

A remark that I wish to make here is that a common issue in fluorescence microscopy experiments is the impact of photobleaching on experiments. A fluorescence protein, when exposed too often to fluorescence light, becomes chemically altered and loses its ability to fluoresce. This phenomena is referred to as

[36] The half-life of the yECITRINE is 2h (Bionumbers). Waiting 4h allows to the degradation of the fluorescence protein and to discriminate between fluorescence proteins already present and newly created fluorescence protein.

photobleaching. During the first stress, fluorescence proteins are synthesized. Thus photobleaching should not blur the stress response.

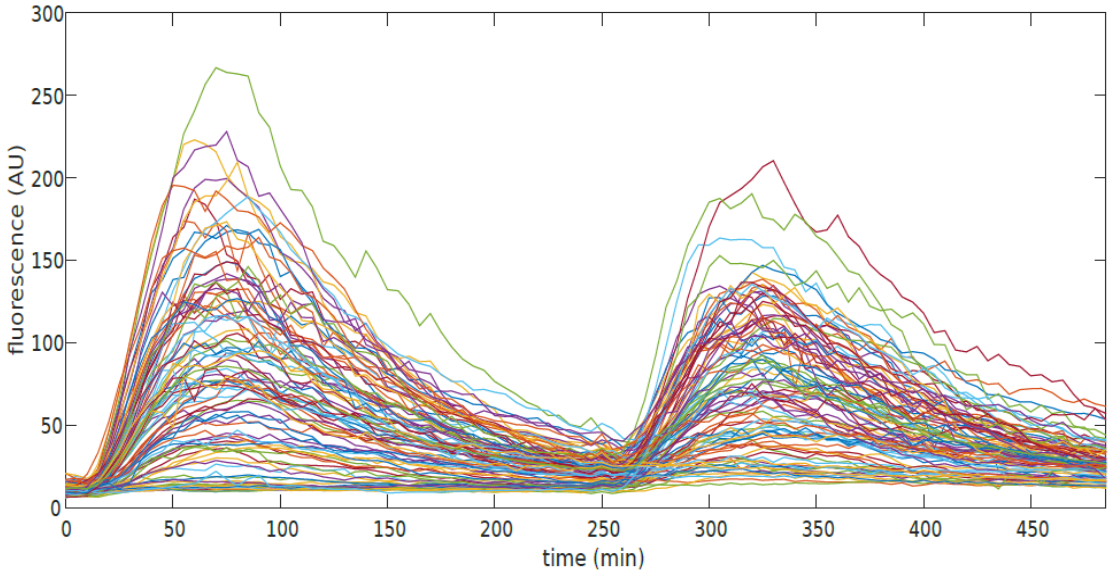


Figure 3-5 Variability of the fluorescence expression in response to pulsed stresses. Single-cell fluorescence trajectories of cells from the yPH53 strain submitted to two 8min hyperosmotic stresses triggered by 1M sorbitol and separated by 4h. The fluorescence trajectories show a difference in the level of fluorescence reached by the cells (N=103).

I focus on cells that receive both a first and a second stress. This means that in a growing population, not all cells are of interest for my study. On the contrary only the cells that were already present before the first stress appeared are considered for subsequent analysis.

The fluorescence trajectories of each cells is then used to compare the dynamics and amplitude of the transcriptional response to subsequent hyperosmotic stresses. Following the parameters of studies in the works on memory presented in the introduction, I chose to study two parameters[37] of the successive response to hyperosmotic stresses:

- The **amplitude of the response**: the highest level of fluorescence reached by a cell in response to a short stress compared to the basal fluorescence level.

- The **time of response** defined as the time it takes for the fluorescence of a cell to reach the maximum of amplitude from the moment the cell is stressed.

[37] The two parameters are calculated using a polynomial fit on the top of the fluorescence trajectory in order to determine these parameters with a higher precision.

In the following part, I report my investigations of the study of the response to pulsed hyperosmotic stresses in the budding yeast at the population level. All subsequent trajectories are plotted with the standard error since I estimate average values. However, because of cell-cell variability I will also present single-cell quantifications, which implicitly account for the standard deviations. Interestingly, this last point allowed the observation and quantification of interesting and unreported phenotypes.

II. Yeast cells display a cellular memory of past stress

In this part, I will describe the results showing the existence of a memory in response to repeated hyperosmotic stresses in my experimental conditions. Using single-cell analysis and comparison with a stochastic model, I will show that the memory effect is not a reflection of the stochasticity of gene expression, which is linked to cell-cell variability, but rather is likely caused by another biological mechanism.

A. The response of a population of cells to successive hyperosmotic stresses suggests the existence of a cellular memory

To start with, I quantified the fluorescence expression of pSTL1-yECITRINE of a population of cells receiving two pulsed hyperosmotic stresses separated by 4h. Because of the segmentation and tracking method I used, this process was time consuming. However, it allowed me to perform a robust population and single-cell study. The single-cell trajectories, along with the standard deviation, shows a large variability in the values of fluorescence reached by the cells **(figure 3-6).** For this reason, I focus my population study on how does the mean fluorescence value evolves with the stress, which requires an estimate of the mean with the mean on the error. To take into account the variability, I next perform a single-cell analysis.

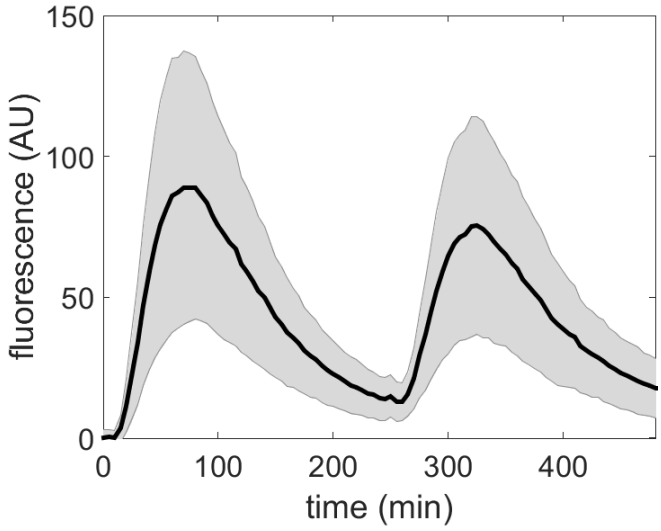


Figure 3-6. Large variability in the response to two short hyperosmotic stresses. Cells from the yph53 strain were submitted to two 8min hyperosmotic stresses separated by 4h using sorbitol 1M. The standard deviation shows the variability in the response of the cells. (N=150, 3 experiments).

The fluorescence analysis of such a population shows that the cells have a decreased response to the second stress by 30% in average compared to their response to the first stress **(figure 3-7)**. This effect is akin in its behavior to the one observed in the context of the hyperosmotic memory presented in the introduction (Rienzo et al., 2015). If the response of the two stresses was not correlated, it would be expected that in such a population there won't be significant differences in the average amplitudes of response to both stresses. This is not the case in my analysis, likely suggesting that the response to the first and second stress is indeed correlated. This points to the existence of a cellular memory in my experimental conditions. However, at this point, since there a decrease in the amplitudes of fluorescence it could be argued that either the rate of transcription decreases with the stress, or cells spend less time producing proteins during the second stress.

To answer this question, I analyzed the times of response to both stress. The subsequent analysis shows that there are no statistical differences between the times of response ($p>0.05$), implying that the decrease of fluorescence if caused by a reduction of the protein production rate upon stress rather than a shortened duration of transcription events **(figure 3-7)**.

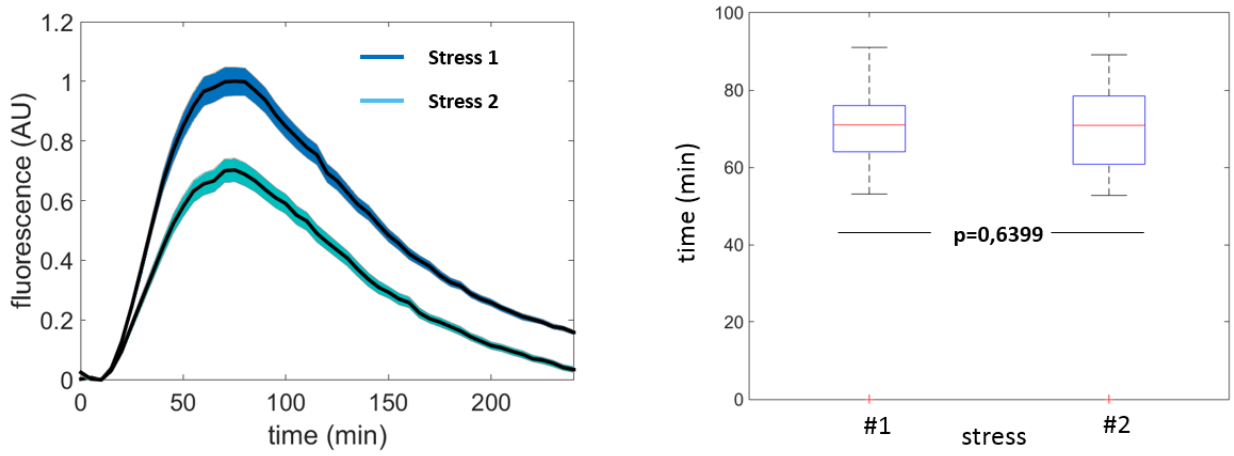


Figure 3-7. Decreased rate of fluorescence production with the stresses. *(Left)* Normalized fluorescence trajectories of a population (N=97, two independent experiments) of yeast (yPH53 strain) submitted to two repeated hyperosmotic stresses. The envelop represent the mean error calculated with the independent experiments. The amplitude of response decreases with the stress. *(Right)* There are no differences in the time of response between the two repeated stresses for the same population of cell.

I will now refer to the phenomena of decreased response with the stress as **memory effect**. As I established this result at the population level, I then proceed to quantify the single-cell variability in the context of my experiments to better characterize the memory effect.

B. At the level of single cells, most cells, but not all, show a cellular memory

1. There are several dynamical behaviors in response to pulsed stresses

As I have presented in **figure 3-5**, there is a variability in the fluorescence values of the cells. There is also a variability in the behaviors of the cells. Indeed, although at the population level the cells decrease their amplitude of response with the stress, some cells have, for instance, a behavior that is the opposite of the memory effect, namely they have a response to the second stress that is actually higher than during the first stress **(figure 3-8)**.

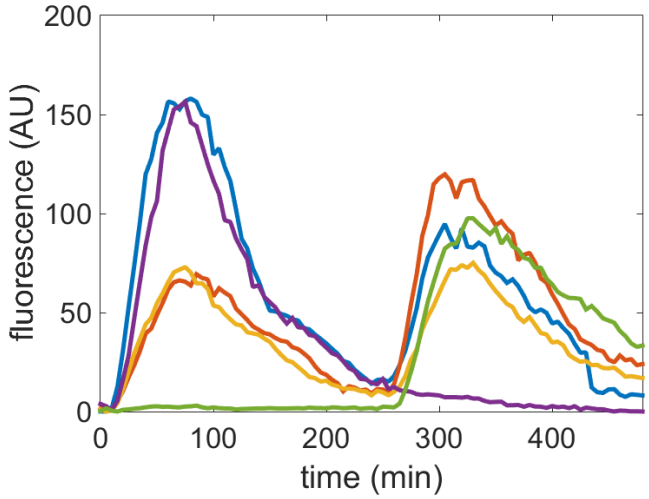


Figure 3-8. Dynamical behavior in response to pulsed stresses. Five examples of single-cell trajectories of cells (yPH53 strain) behaving differently in response to two stresses. These single-cell trajectories will serve as a reference for the subsequent single-cell analysis.

I named the ensemble of possible behaviors in response to pulsed stresses **dynamical variability**. Said differently, while the population exhibits a memory effect in my conditions, this is not the case for every cells, which can display different dynamical behavior. To quantify this competition between the leading effect (memory) and the dynamical variability, a way to make a relevant single-cell characterization of the cells would be to cluster them according to their dynamical behavior in response to two stresses.

2. The dynamical variability can be clustered according to typical profiles

Based on the analysis of all fluorescence trajectories in my experiments, I classified the behavior of the cells according to five typical trajectories (**figure 3-9A**). The subsequent single-cell quantification of the cells according to these profiles shows that the most predominant profile is the profile 1 -profile that corresponds to the **memory effect**. This stands to reason as it is also the effect that is apparent at the population level. Indeed, while 55%±11% of the cells display the memory effect, 18%±7% of the cells have an opposite response, *i.e.* a stronger amplitude during the second stress.

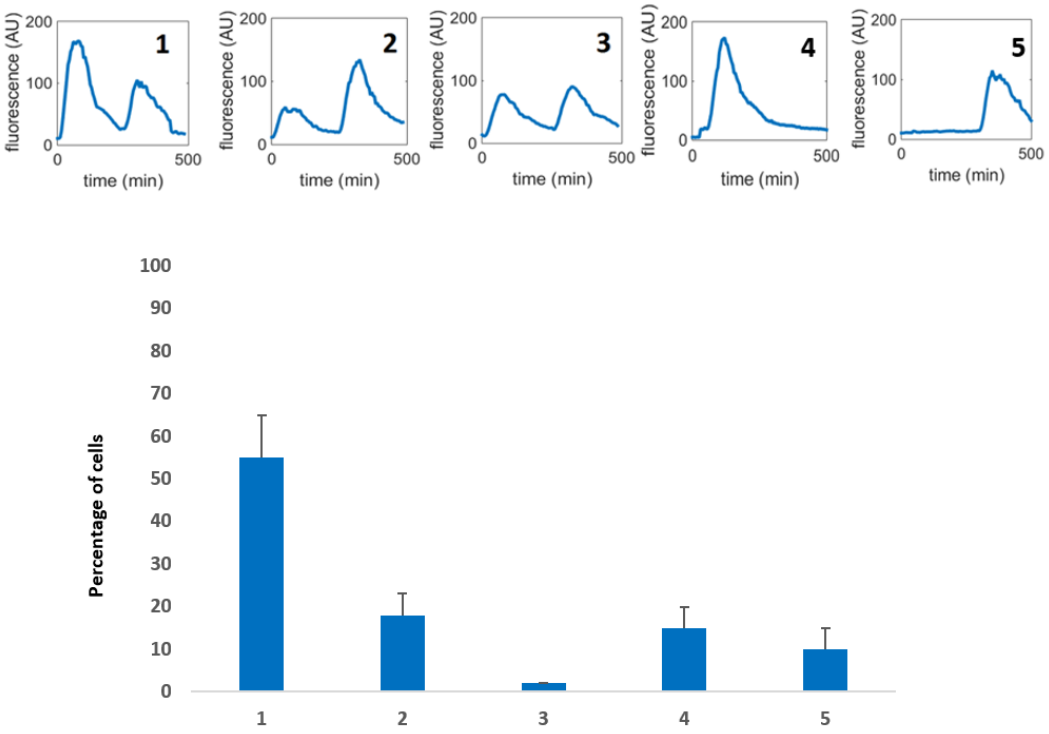


Figure 3-9. Most mothers behave according to the memory effect at the single-cell level
(Top) Single-cell fluorescence trajectories of that correspond to the five typical profiles of response that serves as reference for categorization. *(Bottom)*. Quantification of the response of cells (yPH53) to two pulsed stresses according to the five typical profiles of response. Most cells behave according to the profile 1, *i.e* memory effect. (N=700 performed on three independent experiments)

As expected, there are cells that show no response to a single stress, which is likely the result of the bimodality of the HOG response at the transcriptional level as

presented previously, and which is thought to be seen for short stress (Pelet et al., 2011). The single-cell analysis reveals a dynamical variability in the response to pulsed stresses among the cells. As an explanation to cell-cell variability is the stochasticity of gene expression, a legitimate question is: could the single-cell quantification be consistent with a purely stochastic behavior of the cells or does it imply that additional biological effects have to be taken into account?

3. Computing a population of "stochastic" cells

To determine if the variability of the response to the repeated stresses and if the eventual single-cell quantification could be explained by the stochasticity of gene expression solely, I computed a population of cells constrained to perform only transcription and translation upon receiving pulsed hyperosmotic stresses using the Gillespie algorithm[38] **(figure 3-10)**.

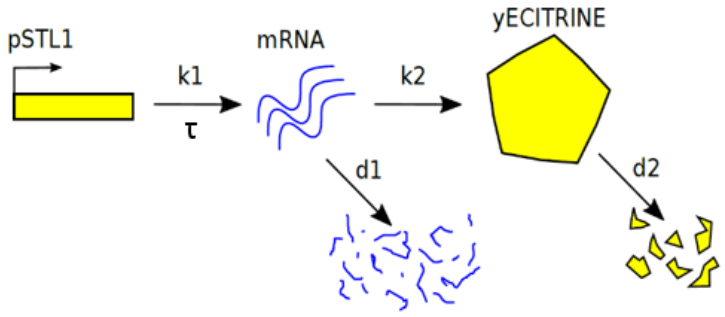


Figure 3-10. Principle of the simulation. A population of cells is modelled to perform the transcription of pSTL1 at the moment of the stress with a rate k1 and the translation of the yECITRINE with a rate k2. Degradation of the mRNA and the fluorescence occur a rates d1 and d2 respectively. The transcription can occur with a time delay τ.

Such a model would render possible to compute a cell-cell variability in the expression of the fluorescence protein. The rates of production and degradation of protein and mRNA that I used can be found in the table below and are from (Llamosi et al., 2016).

[38] The code can be found on https://github.com/Lab513

Parameter	Definition	Unit	Reference Value	Source
k1	transcription rate	min^{-1}	1.10^{1}	(Llamosi, 2016)
d1	mRNA decay	min^{-1}	$2.94.10^{-1}$	(Llamosi, 2016)
τ	time delay	min	Between 0 and 10 min	This study
k2	translation rate	min^{-1}	$9.47.10^{-1}$	(Llamosi, 2016)
d2	protein decay	min^{-1}	4.10^{-3}	(Llamosi, 2016)

Table 1. Parameters used in the stochastic simulations.

4. The cellular memory cannot be explained by a stochastic behavior only

From the computed single-cell fluorescence trajectories, I used the same principle of clustering in order to categorize the cells according to the same rules than the ones used experimentally.

The stochastic simulation allowed to obtain the profiles 1, 2, 3. Addition of a time delay randomly chosen between 0 and 10min caused the emergence of profiles 4 and 5. The model thus shows that a possible explanation to the temporal bimodality is a delay in the transcription[39]. Overall, the simulation shows that the stochasticity of gene expression solely can explain the existence of the five typical profiles of response. As expected in such simulations, profiles 1 and 2 have the same occurrences as they are ruled by the stochasticity of gene expression, therefore no biological mechanism should force the cells to behave according to one of the two profiles in particular. The same reasoning can be applied to the profiles 4 and 5. The profile 3 is a rare event that depends on the threshold imposed during the clustering process[40]-threshold that is identical in the simulations and experiments **(figure 3-11)**.

[39] As the bimodality exists for short stresses but not long stresses, the experiments suggest the existence of a critical time from which all cells will eventually respond to the stress.
[40] I use a 5% tolerance to evaluate the profiles for the experiments as well as the model.

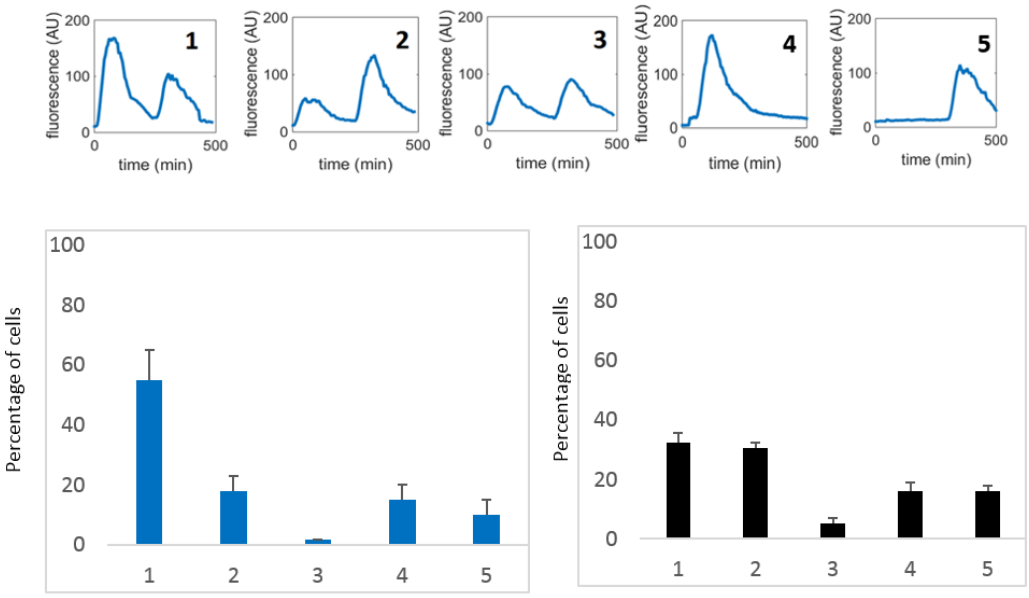


Figure 3-11. The stochasticity of gene expression alone cannot explain the memory effect. *(Top)* Typical fluorescence trajectories. (Bottom left) Single-cell quantification of the yPH53 strain as previously shown. *(Right)* Single-cell quantification of mothers computed with the Gillespie algorithm and in the case where a variable transcriptional delay is added (N=1000, 4 rounds of simulations).

Altogether, the five profiles of response can be explained by the stochasticity of gene expression. However, such stochasticity cannot explain why the profile 1 is privileged by the cells in my experimental conditions. Therefore, the results suggests that at least one other biological phenomenon is active to ensure that most of the cells dynamically behave by displaying the memory effect.

C. Conclusion

My experimental results show that a population of cells adapts to short, repeated hyperosmotic stresses by decreasing their response with the stresses, process that I call **memory effect**. Furthermore, the single-cell analysis shows that this mean behavior is actually a mixture between cells behaving according to several individual typical profiles, synonym of a dynamic response upon stress. The differences in clusters quantification observed in vivo and through the simulation, suggest that a biological mechanism is able to overcome the stochasticity of gene expression and force most cells (but not all) to display the memory effect. With these information, my next step is to find what this (these) biological mechanism(s) could be. As I have presented in the introduction, memory experiments performed in the budding yeast describe two mechanisms responsible for a cellular memory: a cytoplasmic memory or an epigenetic one.

I will describe in the following parts transcriptional inhibition experiments that eventually suggest that the memory effect I described in my experiments is not a cytoplasmic one.

III. The memory effect does not require de novo protein synthesis during stress.

To investigate the possibility of a cytoplasmic memory, the strategy I chose consisted in canceling out the cells' ability to perform transcription during the first stress and allow them to perform transcription during the second stress only. The idea behind this strategy is: if the cells still possess the memory of past stresses, a cytoplasmic factor would not be involved as none would have been created during the first induction. From an experimental point of view, a way to prevent the transcription in a fast and reversible manner is required.

I will here present my experimental results consisting in analyzing the dynamics of the fluorescence reporter upon repeated stresses when cells are treated with a transcriptional inhibitor, and pointing towards an origin of the memory effect that is not consistent with a cytoplasmic memory.

A. The transcriptional inhibitor thiolutin can successfully prevent the transcriptional response upon stress

There are many works on the stability and degradation of mRNA that requires the use of transcriptional inhibitors. As a result, many have been well characterized and are available on the market. A good summary can be found in this study (Bensaude, 2011).

To prevent the cells from producing any protein during a hyperosmotic stress, I used a well-characterized transcriptional inhibitor in *S. cerevisiae* which is the thiolutin[41]. It is a fast and reversible chemical that inhibits the activity of all three RNA polymerases in the budding yeast. I first searched for parameters of thiolutin treatment that would lead to the loss of the stress response in the cells (Jimenez et al., 1973). After a treatment of 1h with thiolutin at a concentration[42] of 50µg/µL, no cells showed a fluorescent signal upon 8 minutes of hyperosmotic stress[43] **(figures 3-12, 3-13)**.

[41] Abcam ref ab143556

[42] Typically, concentrations of 3µg/µL are used for transcriptional inhibition using thiolutin, although it has been shown that higher concentrations do not impact cell viability and can potentially also inhibit translation. Following a hyperosmotic stress, cells treated with a concentration of thiolutin lower than 50µg/µL still expressed the fluorescence reporter.

[43] Experiment made in batch for the treatment and on agar pad for the observation and quantification.

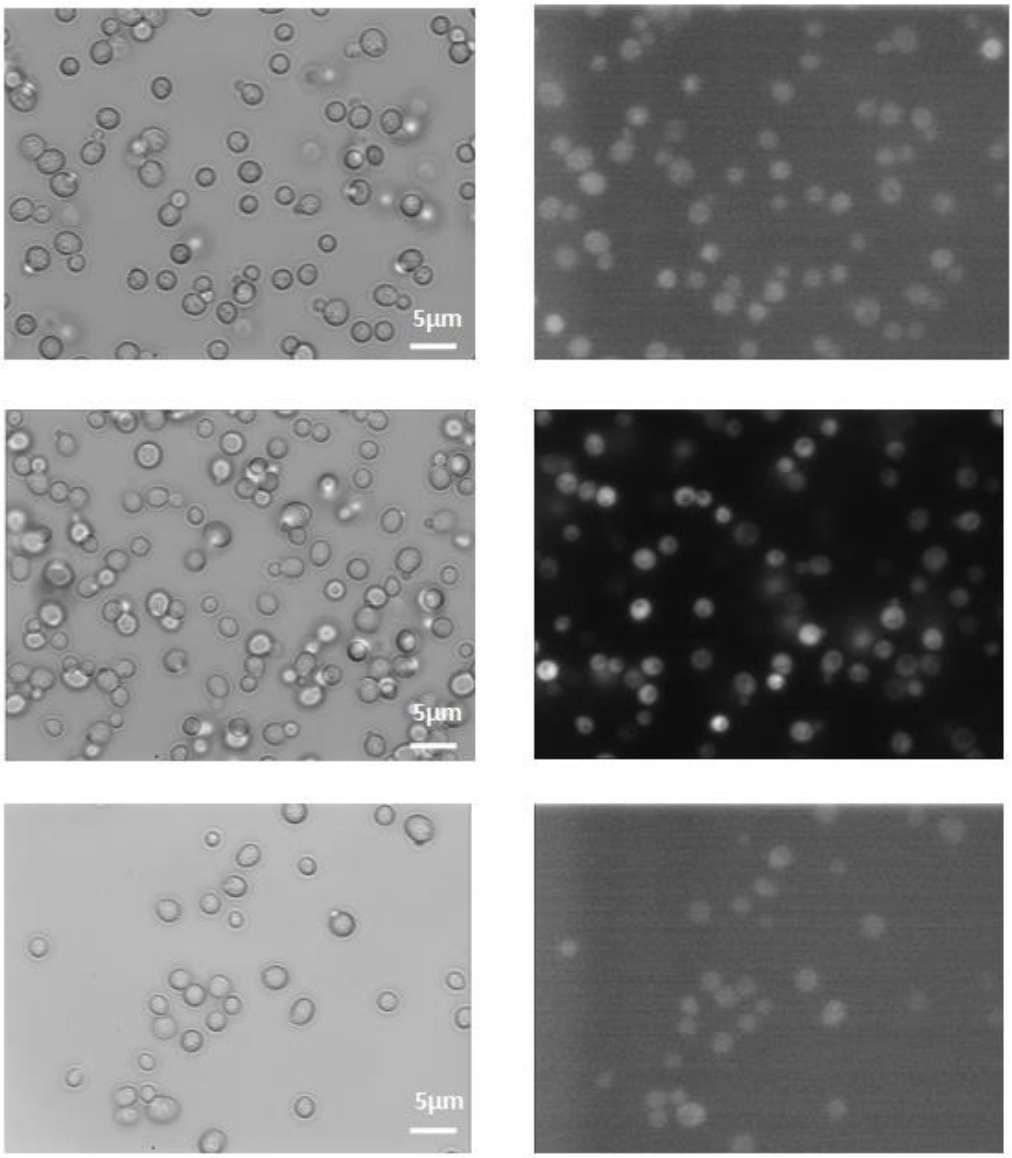


Figure 3-12. Thiolutin treatment prevents the activity of pSTL1 upon stress. *(Top)* yPH53 cells observed in bright and fluorescence light in SC. *(Middle)* Cells submitted to a 8min hyperosmotic stress using sorbitol 1M observed in fluorescence and bright light after 70min. *(Bottom)* Cells submitted to a 8min hyperosmotic stress using sorbitol1M complemented with thiolutin and observed in fluorescence and bright light after 70min. Prior to the stress, these cells were pretreated with thiolutin for 1h. The cells do not express the fluorescence protein.

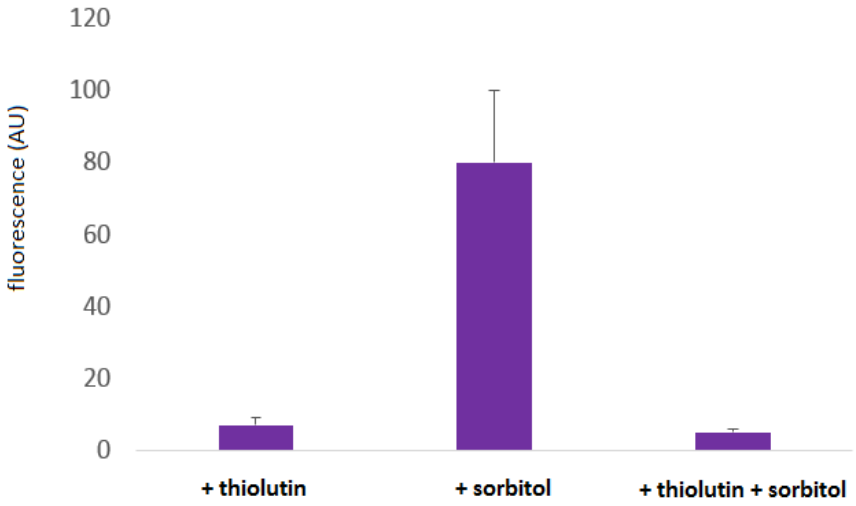


Figure 3-13. Fluorescence quantification of the different cases exposed in the previous figure. The amount of cells are respectively N=100.

B. Inhibition of the transcriptional response during the first stress does not prevent the emergence of the memory effect at the second stress

Having established that the thiolutin can successfully prevent transcription during a hyperosmotic stress, the next step was to test if a treatment with the thiolutin could alter the cells' ability to respond back to a hyper-osmotic stress **(figure 3-14)**. The idea was if the thiolutin persisted in the cell after treatment and altered the activity of pSTL1 upon stress, for instance by decreasing it, an effect resembling the memory effect could be observed, but would be a consequence of the treatment rather than the actual memory effect.

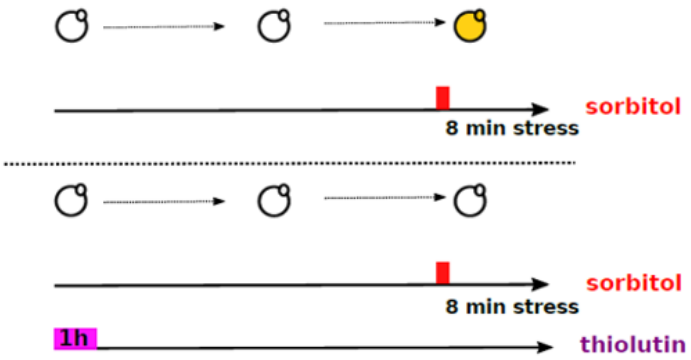


Figure 3-14. Principle of the control experiments. (Top) Cells are exposed to an 8min hyperosmotic stress and their fluorescence level analyzed. (Bottom) Cells are pretreated with thiolutin for 1h, then the inhibitor is washed away and the cells grow for 4h before being exposed to a hyperosmotic stress. Their fluorescence level will subsequently be analyzed.

To that effect, I treated the cells during 1h with the transcriptional inhibitor. After treatment, the inhibitor was washed out and cells were submitted to a hyperosmotic stress, 4h later in order to let them recover the treatment and because such duration is the resting time that I allow the cells in my experimental conditions. I successfully observed that such treatment did not affect the activity of pSTL1, as the response of treated cells is similar[44] to the response of non-treated cells **(figure 3-15)**. Therefore, any effect observed after transcriptional inhibitor treatment in the context of my memory experiments would likely not be a consequence of the treatment.

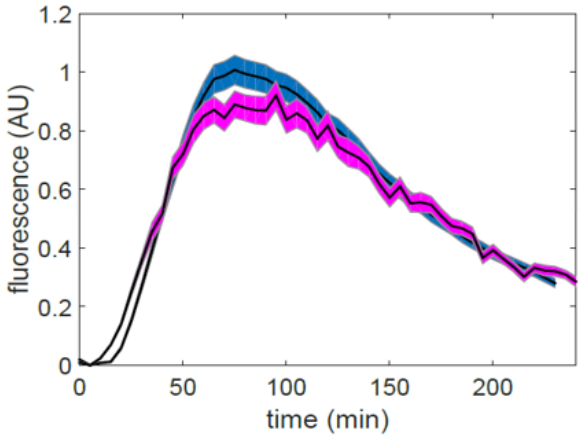


Figure 3-15. Thiolutin treatment does not impair the transcriptional response. Cells (yPH53 strain) treated with the transcriptional inhibitor do not present a pSTL1 transcriptional defect after the inhibitor is washed. Population quantification of the response to a stress in the case when the cells were untreated (blue) or treated with the inhibitor (purple)

Having verified that treatment by thiolutin does not impair stress response, I next performed thiolutin treatment 1h prior and during the first stress, washed thiolutin and submitted cells to a second stress 4h later. Interestingly, in these conditions, the cells responded to the second stress similarly with an amplitude decreased by 65%±5%. This is comparable[45] to the non-treated cells displaying the memory effect at the second stress with an amplitude reduced by nearly 50%±5%.

[44] Albeit a slight decrease in the case where the mothers are treated with the inhibitor. There *might* be a slight effect on the response of the thiolutin.

[45] A possible explanation would be that if the thiolutin indeed had a slight decrease effect on the response, it is possible that the memory effect emerged but because of the thiolutin, will be slightly more pronounced compared to non-treated mothers.

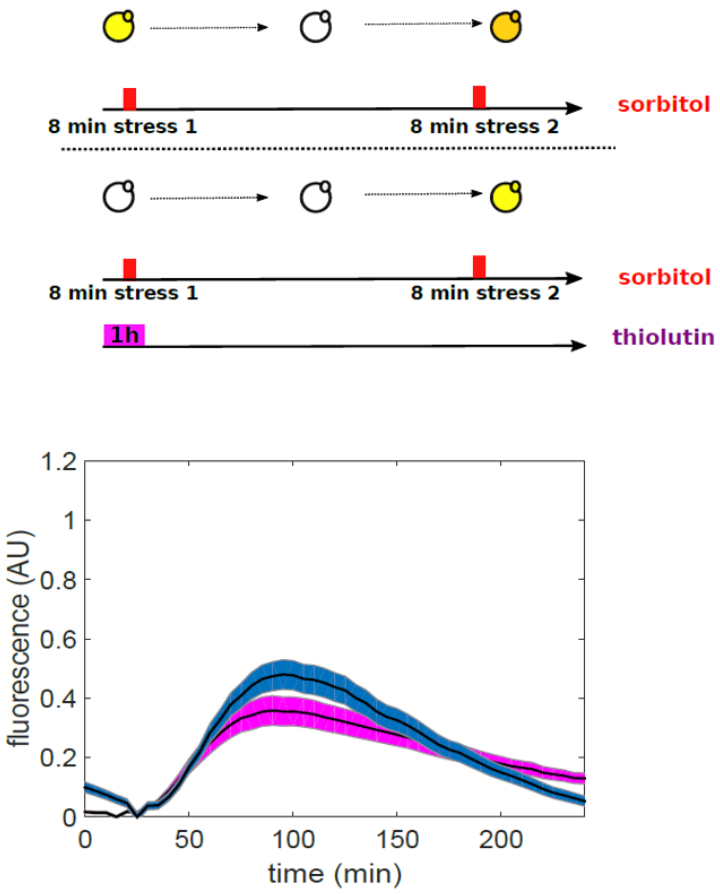


Figure 3-16. The memory effect does not require a transcriptional activity during the first stress. *(Top)* Non treated cells are exposed to two 8min hyperosmotic stresses separated by four hours. Treated cells are exposed to thiolutin for 1h and, still treated with the inhibitor, exposed to an 8min hyperosmotic stress. The inhibitor is then washed and the cells grow for 4h before being exposed to another 8min stress. *(Bottom)* Inhibition of the response to the first stress does not prevent the memory of the stress. Population quantification of the response to a second stress in the case when the cells (yPH53 strain) were submitted to a first 8min stress (blue) or submitted to a first stress + transcriptional inhibitior (purple).

C. Conclusion

The transcriptional inhibition experiment suggests that the memory effect is not driven by *de novo* protein synthesis during the stress. Therefore, **the memory effect that I observed is likely an epigenetic memory**. In addition, these experiments suggest that the first stress induces changes independent of the activity of any RNA polymerases, and are responsible for adaptation. One interesting possibility is that the chromatin is modified by marks independent of transcription. The modifications would appear in most cells during the first stress and remain to the second stress. Since

thiolutin inhibits RNA Polymerases but does not prevent other factors from binding to the chromatin, such marks are possibly dependent on transcription initiation.

As I ruled out the cytoplasmic memory hypothesis, I then focused on investigating the influence of several epigenetic factors on the memory effect, in order to characterize it and find its biological mechanism.

IV. The chromatin structure impacts both the transcriptional activity of pSTL1 and the memory effect

I will here present my experimental results showing that a change in the position of the genetic system that I analyzed not only alters the activity of pSTL1, but also its dynamical behavior and the memory effect. The results suggest that the position of pSTL1 on the genome may determine if the memory effect or the noise in gene expression is predominant.

A. Chromosome positioning alters both the activity and the dynamical behavior of pSTL1

1. Rationale to displace pSTL1

The *STL1* locus is located on the right arm of the chromosome IV, in its subtelomeric region. As I have presented in the introduction, silencing and epigenetics are known to partly depend on the locus positioning. Particularly, subtelomeres are regions prone to transcriptional silencing.

As the memory effect is characterized by a decreased amplitude of response upon stress, a possible explanation to it would be that it is a reflection of the silencing caused by the subtelomeric position of pSTL1. I thus investigated the influence of the chromatin context on the dynamics of activation of pSTL1. As subtelomeres are particular regions containing stress response genes[46], I have moved a region containing the promoter of *STL1* and the yECITRINE fluorescent reporter to a distinct, centromeric chromatin domain. Such a region is not prone to silencing, nor contains stress response gene. Therefore, pSTL1 is in a distinct transcriptional environment. More specifically the fluorescence reporter, along with 1kb of upstream sequence, enough to have a fully functional *STL1* promoter at its endogenous locus, was moved at the TRP1 locus **(figure 3-17)**.

[46] However, they can contain housekeeping genes which activity increase upon stress. For instance, *GPD1* is a housekeeping gene that is upregulated upon hyperosmotic stress, and is located at a pericentromeric location of the chromosome IV.

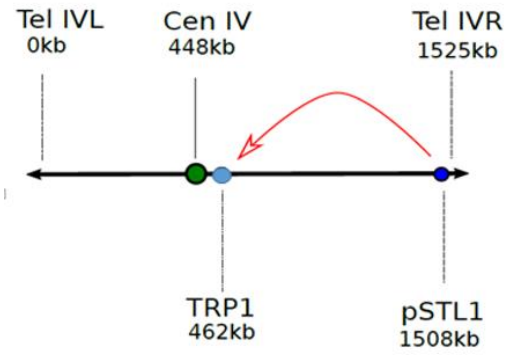


Figure 3-17. Genomic coordinates on the chromosome IV. For the following experiments, pSTL1 was moved to the Trp1 locus.

2. Quantification of the variation of the displaced pSTL1's behavior compared to the wild type

a) The activity of pSTL1 is decreased at the pericentromeric location

Before performing the dynamical analysis of the displaced pSTL1 in the context of the memory experiments, and as transcription level can depend on genome location, I first wanted to verify if the displacement caused any differences in its transcriptional activity. To that effect, I submitted a population of cells to a 2h hyperosmotic stress. Such long stress forces all cells to express the fluorescence protein under the control of promoter of interest. I performed flow cytometry experiment in order to have a robust and statistically relevant analysis of the potential differences between wild type strain and strain with the displaced pSTL1.

Interestingly, the experiments (triplicates on 15 000 cells) show that at the population level, the transcriptional strength of the displaced pSTL1 is 20% lower in average than the endogenous one, in the stress conditions **(figure 3-18)**. This result is quiet counter-intuitive since subtelomeres are notoriously known to be regions with lower transcriptional activity compared to others. However, this paradigm has been established in normal conditions. During stress conditions, subtelomeres are no longer silenced since stress response genes, mostly located at subtelomeres, have to be expressed. Cells, in these conditions, will stop most of their day-to-day processes, controlled by non-subtelomeric genes, in order to fight the stress. Therefore, it appears

that the paradigm stating that subtelomeres are silenced is true only in non-stress conditions. It can thus be hypothesized that upon stress, the activity of pSTL1 is likely to be stronger than at another location that might be downregulated upon stress.

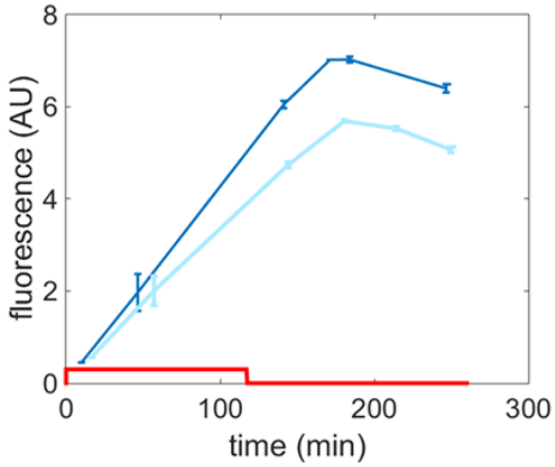


Figure 3-18. Displacement of pSTL1 causes a decrease in its transcriptional activity. Cells were submitted to a 2h hyperosmotic stress and the fluorescence activity of pSTL1 at its endogenous location (yPH53 strain, dark blue) and at the pericentromeric location (yPH212, light blue) was analyzed. The displaced pSTL1 expression decreases by 20% upon stress compared to the wild type. Represented, the error on the mean value between the triplicates for the various time points.

b) The memory effect is lost at the pericentromeric location

As there are differences at the population level between subtelomeric and pericentromeric positions, my next step was to perform microfluidic experiments on the centromeric strain in order to investigate the cells' behavior at the single-cell level. In this context, the cells still behave according to the five typical profiles of response that I have presented earlier for the wild type strain. I then compared the patterns of consecutive responses to two 8min hyperosmotic stresses separated by 4h between subtelomeric and centromeric pSTL1. Cells expressing the *STL1* promoter at the pericentromeric position showed a more uniform distribution into the five defined typical profiles of response[47] and there was a decrease in the amount of cells displaying the memory effect (from 55%±11% to 28%±4%, **figure 3-19**). Such a loss of memory effect is actually comparable to a solely stochastic process, as simulations have established. This result suggests that the chromatin environment determines the dynamical transcriptional activity of pSTL1 (see discussion).

[47] An interesting point is that the profiles 4 and 5 have similar occurrences in both the subtelomeric and centromeric strain. Consequently, these profiles do not depend on the genomic location which is another argument in favor of a transcriptional delay.

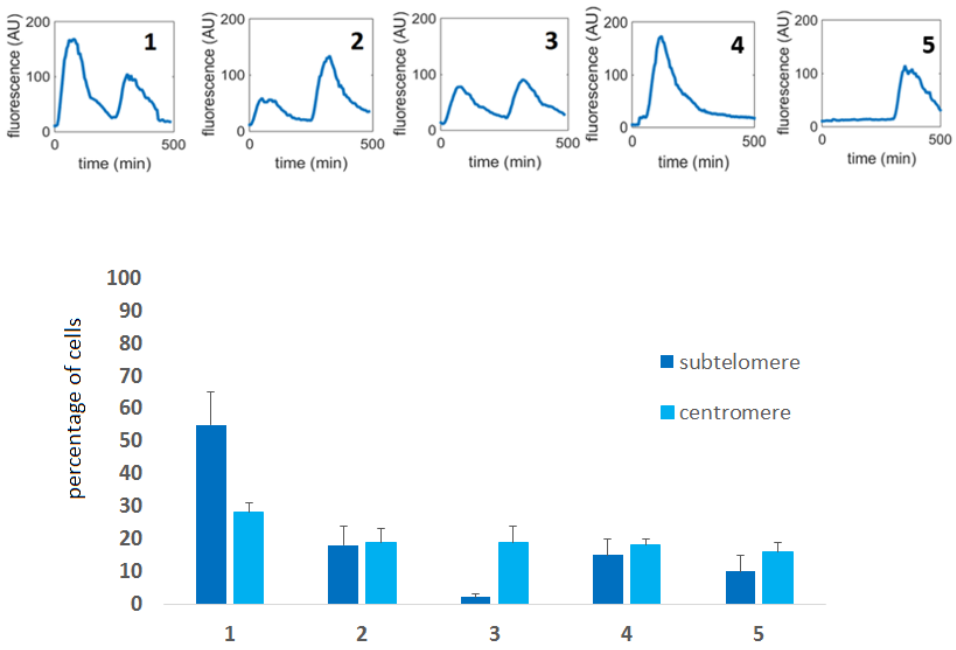


Figure 3-19. Displacement of pSTL1 causes a loss of the memory effect in favor of a uniform behavior. (Top) Five typical fluorescence trajectories. (Bottom) Single-cell quantification of cells with pSTL1 at its endogenous location (yPH53, dark blue, N=700), or pSTL1 at the pericentromeric location (yPH212, light blue, N=250). The memory effect is lost when pSTL1 is displaced, in favor of a homogenous distribution with regards to the five classes.

3. Influence of the chromatin environment in non-stress condition: a Crispr control

A key observation is that moving the promoter of interest from its endogenous location to a pericentromeric location on the same chromosome causes a decrease in its activity. It has been established that a decrease in the activity of pSTL1 can be obtained when the promoter is truncated, *i.e.* when the sequence upstream *STL1* is inferior to 658bp (Bai et al., 2015). This is not the case in this study, as 1kb upstream of the promoter sequence was moved[48]. A fully functional pSTL1 may, in addition to its sequence, require the presence of unreported genetic regulators located at its endogenous position.

In my study, only the *STL1* promoter was displaced in a pericentromeric domain and no potential regulators. Therefore, the hypothesis that the decrease of activity of pSTL1 at the pericentromeric at the population level is caused by the

[48] Also, there are no mutation in the displaced pSTL1 that could explain the reduced activity. Sequencing data available in https://github.com/Lab513

absence of genetic regulators has to be considered. To test this hypothesis, I used a Crispr/dCas9Vpr system in order to force the activation of pSTL1 in non-stress conditions (Chavez et al., 2015). The Cas9 is a DNA endonuclease associated with the CRISPR immune system of bacteria that is guided by a small RNA (gRNA). Recognition of a specific DNA sequence by gRNA induces DNA specific double stranded cleavage. Mutated, catalytically inactive "dead" Cas9 (dCas9) can still recognize a specific sequence in the presence of dedicated gRNA, but will leave DNA uncleaved. When dCas9 is fused to the Viral Protein R and given a specific RNA sequence, it is virtually possible to force the activation of any specific gene. By targeting a genomic sequence within the 1kb of upstream sequence of the fluorescence reporter in both the subtelomeric and centromeric strains and forcing the activity of pSTL1, it is possible to bypass stress induction of pSTL1 and therefore the potential effect of proximal regulators at the endogenous position. Consequently, any differences in the transcriptional activity of pSTL1 observed using the Crispr/dCas9Vpr would likely be linked to the chromatin environment.

Using such system on the yeast strains where pSTL1 is at its endogenous location and displaced, I have successfully bypassed the need to stress the cells with 1M sorbitol in order to activate pSTL1. However, the fluorescence quantification shows that the activity of the promoter is twice as strong as at its endogenous location than when displaced **(figure 3-20)**. As the Crispr/dCas9Vpr system bypasses the stress response and enables the distinction between endogenous regulatory mechanisms of the promoter and chromatin context, I have concluded that the differences of expression and dynamical behavior of pSTL1 when close to the centromere, are neither linked to the sequence of the promoter itself, nor the presence of regulatory elements. Rather, these differences in activity are related to the chromatin environment. Altogether, these experiments suggest that the chromatin environment is critical to respond in a manner distinct to a solely stochastic one in response to pulsed hyperosmotic stresses.

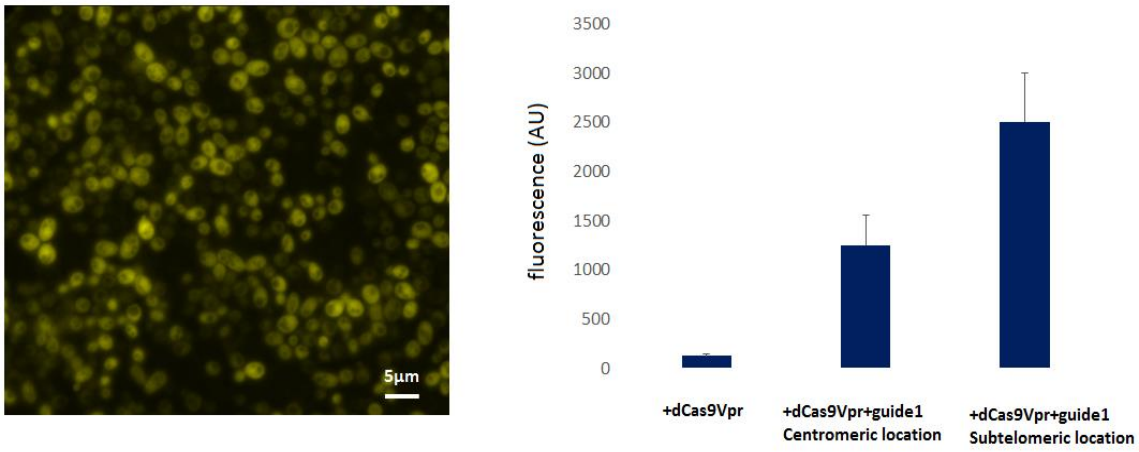


Figure 3-20. Successful activation of pSTL1 in non-stress conditions using Crispr/dCas9Vpr shows that the chromatin context determines the transcriptional strength of pSTL1. *(Left)* Fluorescence image of the yPH53 strain transformed with Crispr/dCas9Vpr and guide 1. The system successfully forces the transcription of the yECITRINE. *(Right)* Fluorescence quantification of the yPH53 and yPH212 strains with Crispr/dCas9Vpr and the guide 1.

B. Conclusion

The experimental results that I have presented points towards an influence of the chromatin on the memory effect, as a change in the genomic location of pSTL1 led to a loss of the memory effect among a population of cells and to a behavior that is comparable to the one described by cells performing only transcription and translation, *i.e.* without memory effect.

My next step was to try finding the epigenetic factor(s) that could be responsible for such differences between the two strains. Because of the previously presented observations, the factor(s) would likely be specific to the subtelomeres, or absent at the subtelomeres. The natural choice, as I have presented in the introduction, is the epigenetic SIR complex. I will thus present in the following part my investigation of the SIR complex on the transcriptional activity of pSTL1 and on the memory effect.

V. The SIR complex has an unreported influence at a pericentromeric location

In this part, I will here present the influence of the SIR complex on the memory effect at the subtelomeric and centromeric location. In particular, I will show that the SIR complex forms unreported hyperclusters upon stress, and the absence of these hyperclusters has no impact on the memory effect at the subtelomeric location, but enables the recovery of both an activity of pSTL1 comparable to the wild type and the memory effect at the pericentromeric location. However, the effect of the SIR complex on a location outside of subtelomeres appears not to be a global phenomenon as suggest experiments performed on pericentromeric osmo-responsive gene *GPD1*.

A. The SIR complex has no influence on pSTL1 at its endogenous position

1. The SIR complex forms hyperclusters upon hyperosmotic stress

a) Observation of the SIR3 hyperclusters

The SIR complex is bound to the telomeres and forms 3-8 clusters at the nuclear periphery (Guarente, 1999; Maillet et al., 1996; Taddei et al., 2009). Using a SIR3-GFP strain from the GFP collection, I could observe the various Sir3 clusters in fluorescence. In non-stress conditions, small clusters are observable. However, when I perform an 8min stress on the SIR3-GFP strain, I observed a change in the phenotype with the SIR3-GFP clusters larger and much brighter **(figure 3-21)**.

Several reports have stated that during a hyperosmotic stress, factors are depleted from the chromatin. In particular, Kupiec's group showed that Sir3 is removed from subtelomeres during a hyperosmotic stress. However, no studies have investigated in more depth the fate of Sir3 during a hyperosmotic stress. My experiments show that, assuming that Sir3 is removed from the chromatin, this removal ctually causes Sir3-GFP to form hyperclusters in the nucleus upon stress **(figure 3-21)**. Such a phenotype has never been reported in any stress conditions and would require a more consequent investigation.

b) Quantification of the Sir3 hyperclusters

To make a quantitative measurement of the fluorescence of the Sir3 hypercluster upon stress and to try to determine the location of this hypercluster in the nucleus, I examined the SIR3-GFP formation in a strain with NUP49-RFP[49].

Using a simple thresholding on the fluorescence images, I was able to quantify the fluorescence intensities of the Sir3 clusters in non-stress and stress conditions. On average, the hyperclusters formed upon stress are twice as fluorescent as the Sir3 clusters that are present in normal conditions (values on **figure 3-21**). However, no conclusions could be drawn about the position of the Sir3 hyperclusters in the nucleus compared to the position of the Sir3 clusters. This would require a deeper investigation that will probably necessitate the use of gene maps (Berger et al., 2008).

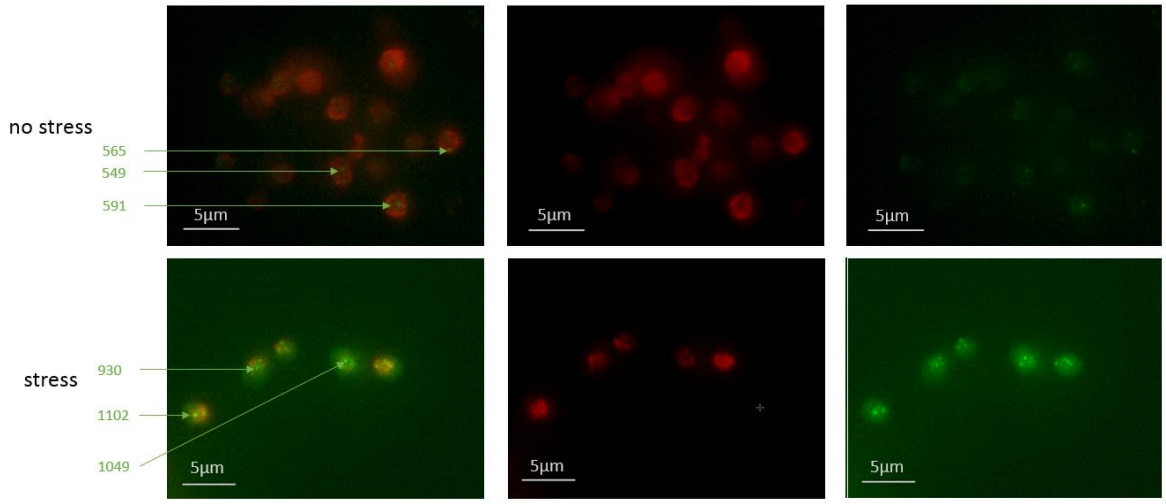


Figure 3-21. Formation of Sir3 hyperclusters upon hyperosmotic stress that are twice as fluorescent as the Sir3 focci in non-stress conditions. Cells (yEF506) were submitted to an 8min hyperosmotic stress using sorbitol 1M. Images were taken five minutes upon stress. *(Middle)* Fluorescence image of the budding yeast. The nuclear envelope is marked with a fluorescence marker. *(Top right)* Fluorescence image in GFP with Sir3 focci in normal conditions. *(Bottom right)* Fluorescence image in GFP with Sir3 hyperclusters upon hyperosmotic stress. *(Left)* Merged fluorescence images.

One could ask the question whether the Sir3 hyperclusters are not due to an increase of *SIR3* activity upon stress that could obviously make the original clusters

[49] Nup49 is a protein of the Nuclear Pore Complex. Therefore, tagging such a protein would allow the nuclear membrane to be visible under fluorescence light.

brighter. Since I am observing the production of the complex SIR3-GFP following a hyperosmotic stress, the folding time of the GFP in the yeast must be taken into account. This folding time is around 20-30min, therefore any newly produced proteins won't be visible under fluorescence light before such folding time. As I observe the formation of the Sir3 hyperclusters during the stress -namely after 5min of induction- the phenotype I observe cannot match an overproduction[50] of Sir3. Therefore, the experimental observation is consistent with a relocation of Sir3 that forms hyperclusters.

Interestingly, the hyperclusters of Sir3 that I observe upon stress are very similar to the Sir3 hyperclusters observed when cells reach a quiscence state (Guidi et al., 2015; Laporte et al., 2016). It is worth mentioning that it exists several common points between a quiescent cell and a cell under hyperosmotic stress **(figure 3-22)**. Although the phenomenon I observe might probably be different than the one described in the study, similarities could be still be drawn between the two phenotypes.

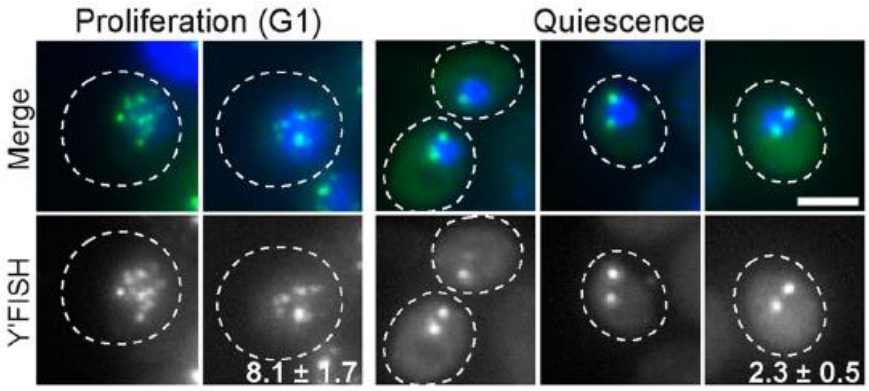


Figure 3-22. From Laporte et al, 2016. Hyperclusters of Sir3 in quiescence state.

2. Sir3 has no influence on the endogenous pSTL1

a) Sir3 does not impact the activity of pSTL1

In order to study the influence of the SIR complex on the activity of pSTL1 upon stress, and to potentially obtain an indirect information on the impact of the Sir3 hyperclusters on the transcriptional activity of pSTL1, I have deleted *SIR3* in the subtelomeric strain **(figure 3-23)**.

[50] It is however factually possible that there could be an overproduction of Sir3 during the stress. An RT-qPCR would be required to further investigate this possibility.

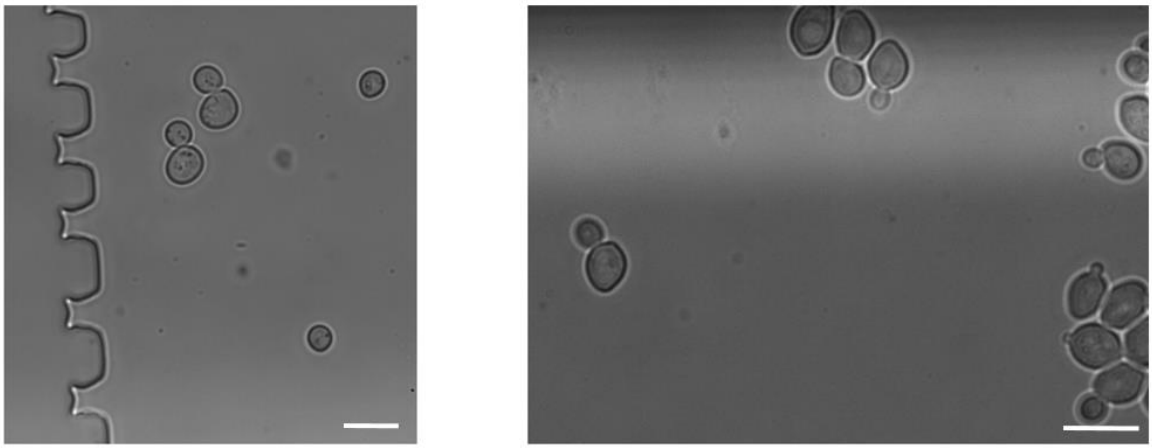


Figure 3-23. Deletion of SIR3 changes the phenotype of cells. (Left) Wild type cells growing in the microfluidic device and imaged in bright light. (Right). Cells deleted for SIR3 growing in the microfluidic device and imaged in bright light have a lemon-like shape. Scale bar at 5µm.

In a similar fashion than I have presented before, I submitted the cells to a long stress in order to force every cell to response and performed flow cytometry experiments in order to quantify potential differences of pSTL1's activity between wild type strain and the *SIR3* mutant. After exposing both strains to a 2h hyperosmotic stress, the fluorescence quantification of the flow cytometry experiment reveals that deletion of *SIR3* has no influence on the subtelomeric strain, albeit a slight increase of activity[51] of pSTL1 **(figure 3-24)**.

[51] Absence of the SIR complex means that Sir3 does not replace the H3 histone. Therefore, the spacing between nucleosomes might be different, so the chromatin would be less compacted in the case where SIR is absent, hence the slight increase in the activity of pSTL1.

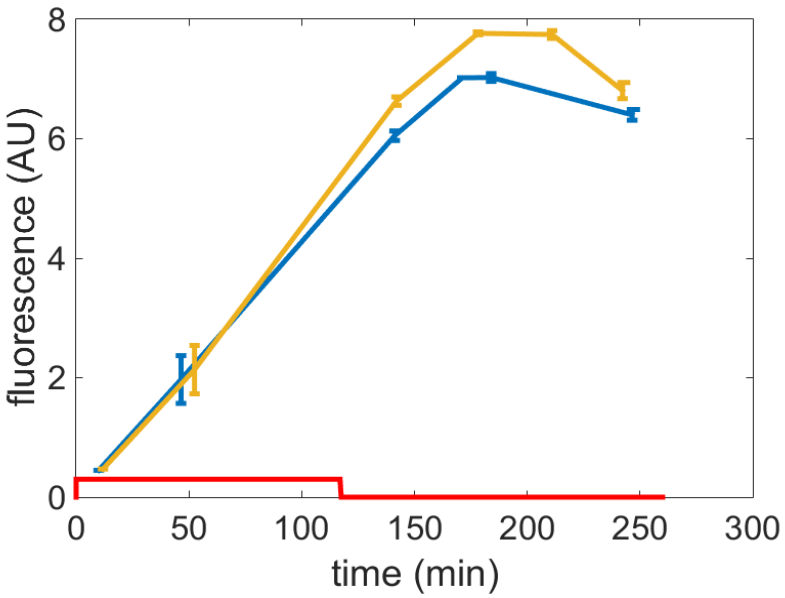


Figure 3-24. There is no influence of *SIR3* on the activity of pSTL1 in the wild type strain. The cells were submitted to a 2h hyperosmotic stress (red) and the fluorescence level was quantified in the yPH53 strain (blue) and the *SIR3* mutant (yPH213, gold) using flow cytometry. The analysis, made in triplicates on 15000 cells, shows that there is no influence of SIR3 on the transcriptional activity of pSTL1. Represented, the error on the mean between the triplicates for the various time points.

b) Sir3 does not impact the memory effect

The analysis of the experiments performed in the microfluidic device consisting in submitting a population of cells deleted for *SIR3* to pulsed hyperosmotic stresses separated by 4h shows that there are no differences in the single-cell quantification compared to the wild type strain **(figure 3-25)**, a result consistent with the population experiment.

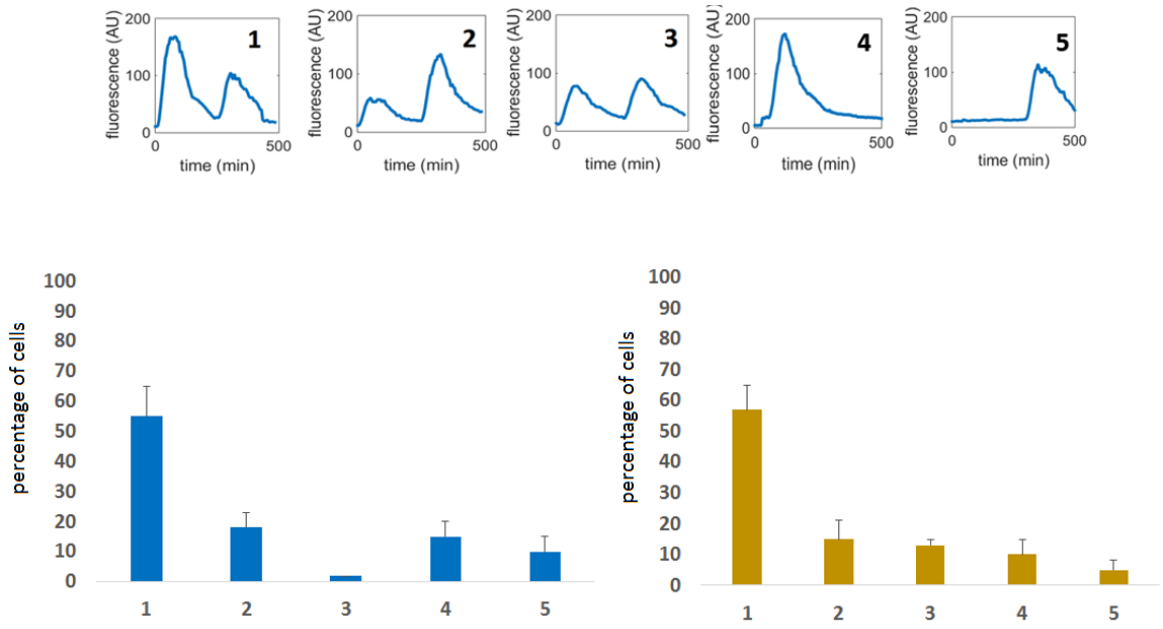


Figure 3-25. There is no influence of SIR3 on the memory effect at the single-cell level. Single-cell quantification according to the five profiles of response *(top)* of the wild type strain yPH53 (blue, N=700) and the *SIR3* mutant yPH213 (gold, N=100) in response to two pulsed hyperosmotic stresses. In both cases, most of the cells behave according to the profile 1 (memory effect), suggesting that there is no influence of SIR3 on the memory effect.

3. Conclusion

One possible explanation for the lack of effect of the *SIR3* deletion on stress response is that in the endogenous strain and upon hyperosmotic stress, pSTL1 has to be expressed, along with various stress response genes that are mostly located at subtelomeres. This implies that the subtelomeric repression, ensured by the SIR complex, has to be lifted during the stress. As a result, the SIR complex should not have any impact on pSTL1's activity, and the analysis of my experiments suggests that it actually does not. Another argument in favor of the absence of role of the SIR complex upon stress is the fact that a study has reported that the SIR complex is depleted from the subtelomeric chromatin upon stress (Mazor and Kupiec, 2009).

B. The SIR complex has an unexpected effect at the pericentromeric location

Although Sir3p does not impact the activity of pSTL1 at its endogenous location upon stress, the formation of Sir3 hyperclusters in the nucleus might impact the transcriptional activity of other regions of the chromatin, especially if the formation of

such hyperclusters is an active phenomenon. To that effect, I have investigated the influence of the Sir3 in the strain where pSTL1 is displaced at a pericentromeric location.

1. Deletion of SIR3 restores a wild-type like transcriptional activity of pSTL1

Similarly to the experiment performed on the subtelomeric strain, I have deleted *SIR3* in the centromeric strain and submitted a population of yeast to a 2h hyperosmotic stress and analyze the fluorescence activity of pSTL1. Unlike the wild type strain, I observe a very surprising effect: removal of *SIR3* actually causes an increase by 20% in the activity of pSTL1, to a level that is similar to the wild type strain **(figure 3-26)**. This result implies that **the SIR complex has an impact on the activity of pSTL1 displaced at the pericentromeric location**, which is unexpected as the SIR complex has never been reported to have any activity outside the subtelomeres and telomeres.

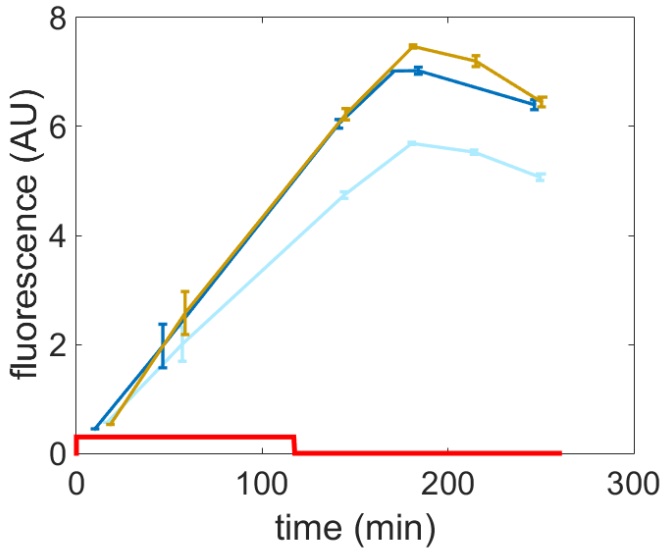


Figure 3-26. Deletion of *SIR3* causes an increased activity of pSTL1 in the centromeric context to a level comparable to the wild type. The cells were exposed to a 2h hyperosmotic stress (red) and their fluorescence level was quantified in the strain where (light blue) pSTL1 displaced at the centromere yPH212, (yPH215, gold) the same strain deleted for *SIR3*. Also, the wild type strain yPH53 is plotted (dark blue). Experiment made in triplicates on 15000 cells. The experiment shows that the deletion of SIR3 causes pSTL1 in the centromeric context to regain a transcriptional activity comparable to the wild type. . Represented, the error on the mean between the triplicates for the various time points.

2. Deletion of SIR3 restores the memory effect at the pericentromeric location

Following the patterns of microfluidic experiments that I have presented earlier, I then performed single-cell experiments in the microfluidic device on the pericentromeric strain deleted for *SIR3*. The single-cell quantification shows that the cells still display the five typical profile of response. However, as the pericentromeric strain was characterized by a pronounced decrease of the memory effect, deletion of *SIR3* causes most cells to regain the memory effect. The quantification **(figure 3-27)** shows an increase in the amount of cells displaying the memory effect in the centromeric compared to the same strain deleted for *SIR3* (from 28%±4% to 50%±5%). Such behavior is comparable to the dynamical behavior of the wild type strain as 55%±11% of the cells displayed the memory effect in the wild type strain.

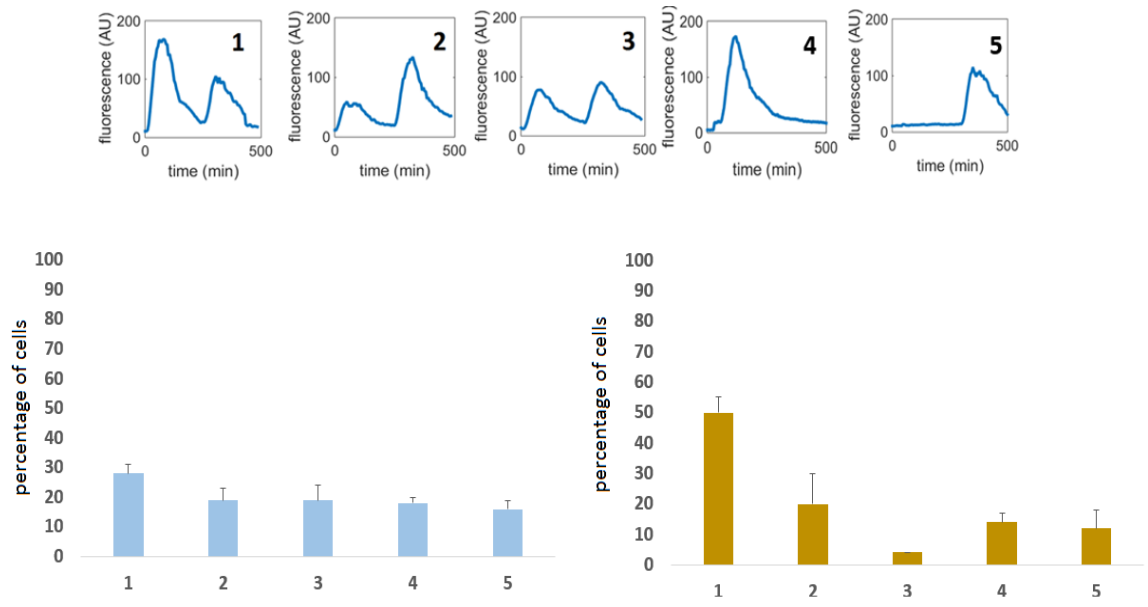


Figure 3-27. Deletion of SIR3 in the pericentromeric context allows the recovery of the memory effect. Single-cell quantification according to the five profiles of response *(top)* of the centromeric strain yPH212 (blue, N=100) and the *SIR3* mutant yPH215 (gold, N=100) in response to two pulsed hyperosmotic stresses. The memory effect is recovered in the mutant.

3. Conclusion

It has been established in a definitive manner that the SIR complex only acts at subtelomeres and telomeres in the budding yeast, through interactions with specific factors such as Rap1 and Abf1, in non-stress conditions (Maillet et al., 1996).

My experimental data show that the SIR complex does not have any influence on the activity and dynamical behavior of pSTL1 at its endogenous location, which can logically be linked to the fact that stress response genes must be expressed upon stress. However, my experimental data also point towards an influence of the SIR complex on pSTL1 when this promoter is moved to the pericentromere. As the SIR complex is likely removed from subtelomeres upon stress and appears to form hyperclusters according to my experiments, it is entirely possible that such hyperclusters have an influence on chromatin domains distinct from subtelomeres.

If such hypothesis were to be true, other genes expressed upon hyperosmotic stresses might be influenced by the SIR complex in stress conditions. I have therefore looked for a gene at the pericentromeric location of the chromosome IV and expressed upon hyperosmotic stress conditions in order to test the influence of the SIR complex on such gene.

C. The SIR complex does not have a global influence on osmo-responsive genes

1. *GPD1* is an osmo-responsive, pericentromeric gene

The budding yeast has the natural ability to produce glycerol for its day-to-day biochemical reactions, and does so in normal conditions through the constitutive expression of the *GPD1* gene. The latter is overexpressed upon hyperosmotic stress in order to ensure the physiological adaptation of the cell to the stress. *GPD1* is a pericentromeric gene, more precisely located at 34kb from Cen IV **(figure 3-28)**. Those characteristics of *GPD1* makes it an ideal candidate to verify if Sir3 has a broad influence on the chromatin, especially at pericentromeric regions.

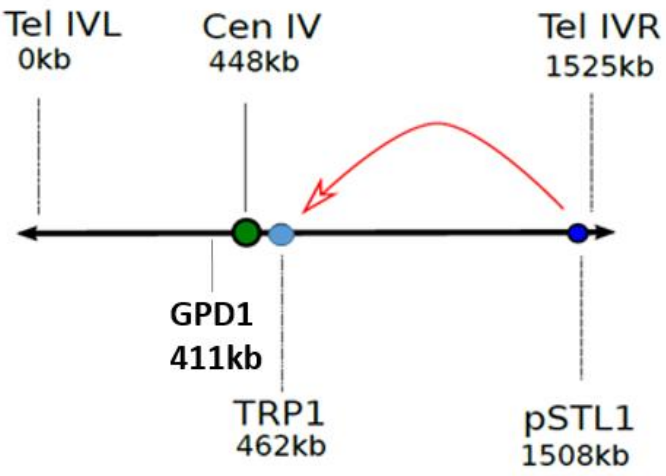


Figure 3-28. Coordinates of GPD1 on the chromosome IV.

2. The transcriptional activity of *GPD1* does not change upon short stresses, but increases upon long stresses

I used the GPD1-GFP strain from the GFP collection and first tried to make a fluorescence quantification of the overexpression of *GPD1* upon stress. As it turned out, an 8 min hyperosmotic stress did not cause any increase of the fluorescence. This is due to the adaptation time to a hyperosmotic stress that has been determined to start at least 15min after an induction and end at a maximum of 30min. As I have explained before, I only focus on the transcriptional response and perform short stresses in order to decorrelate the physiological adaptation from the transcriptional response. It is thus not surprising that I do not observe an overexpression of *GPD1* and actually confirms that an 8 min stress is a good choice to avoid the physiological adaptation. However, in response to a 1h hyperosmotic stress, the increase of *GPD1* fluorescence is clearly apparent[52] **(figure 3-29)**.

[52] Interestingly, I also observe a bimodality in the response to the stress: some cells do not show any increase in *GPD1* activity upon stress. As a result, these cells do not adapt to the stress and stop their cellular division. A justification based on a transcriptional delay could not explain such subpopulation as *GPD1* is constitutively transcribed. However, these cells are unable to overexpress the gene. As I have not investigated further this phenomenon, I am unable to propose a biological explanation.

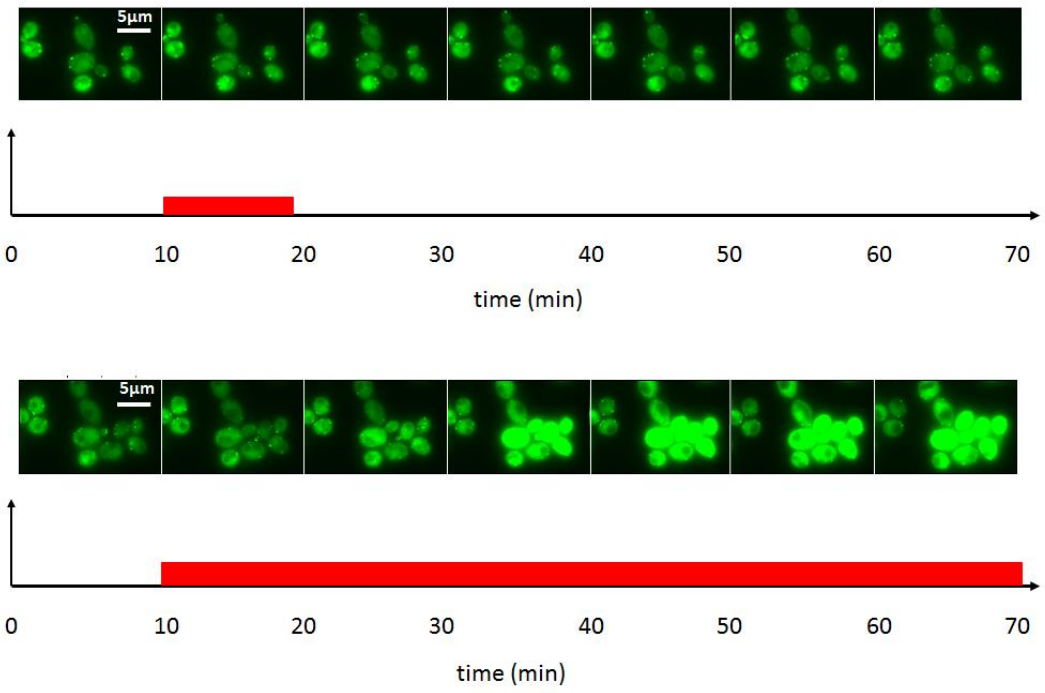


Figure 3-29. *GPD1* is overexpressed in long hyperosmotic stress conditions only. GPD1-GFP strain exposed to an 8min hyperosmotic stress *(top)* or a 1h hyperosmotic stress *(bottom)*. Cells were imaged in fluorescence light (200ms exposure). A short stress does not cause an increase of *GPD1* fluorescence level, unlike a long stress. This is because an 8min stress does not trigger a physiological adaptation.

Overall, performing the same patterns of microfluidics experiments on the GPD1-GFP strain that I did before is not possible. Thus, I decided to investigate the influence of the SIR complex on *GPD1* upon long stress only.

3. The SIR complex does not impact the activity of *GPD1*

I have therefore proceeded to the deletion of Sir3 from in the GPD1-GFP strain. The subsequent cells were then submitted to a 2h hyperosmotic stress and their fluorescence level quantified using flow cytometry. The quantification shows that there are no differences between the wild type strain and the strains deleted for *SIR3* **(figure 3-30)**. This suggests that the effect of Sir3p is specific to the inducible pSTL1 gene when pericentromeric.

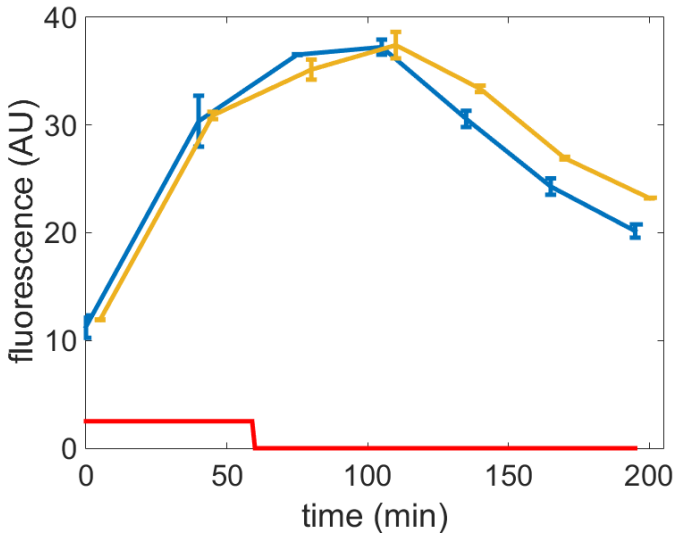


Figure 3-30. The SIR complex does not have an influence on GPD1 upon stress. The cells were exposed to a 2h hyperosmotic stress (red) and their fluorescence level was quantified in the GPD1-GFP strain (yPH003, blue) and the same strain deleted for *SIR3* (yPH360, gold). Experiment made in triplicates on 15000 cells. Represented, the error on the mean between the triplicates for the various time points.

One possibility is that the influence of the SIR complex at the *GPD1* locus is non-existent, however *GDP1* is constitutively expressed so the chromatin environment is already in a state prone to transcriptional activity. This is not the case with the displaced pSTL1 which is repressed in normal conditions. Thus it would be interesting to study an inducible hyper-osmotic pericentromeric gene in order to verify if the presence or absence of the SIR complex can influence its expression, or displacing the pSTL1 promoter and its fluorescence reporter to various locations of the chromatin.

The absence of effect of the SIR complex on the overexpression of *GPD1* suggests that the role of the Sir3 hyperclusters is not global. As of now, the role and effect of these hyperclusters is still elusive. Gene maps and ChIP experiments in order to look at the location of Sir3 upon stress would be a great starting point to study the likely locations where the hyperclusters would have an effect. Having such locations, investigations of the biological effects would be the natural following step (see discussion).

D. Conclusion

Upon hyperosmotic stress, I observed a previously unreported phenotype which consists in the formation of Sir3 hyperclusters. These hyperclusters, which resembles those observed during quiescence, a state that share many similarities with a cell upon stress, do not impact the activity of the endogenous pSTL1, nor the single-cell quantification of the cells that have the promoter of interest at the endogenous location.

At the pericentromeric position, the most obvious effect that the deletion of *SIR3* caused is the recovery of an activity of the displaced pSTL1 to a similar level to the wild type. In such conditions, the recovery of the memory effect was also observed. The SIR complex explains how to recover the memory effect in a particular case, but does not explain why it existed in the first place. However, the previously presented results on the influence of Sir3 on the displaced pSTL1 suggests that **a particular level of transcriptional activity is required for the emergence of memory effect.** This level of transcriptional activity would be similar to the one of wild type, explaining why this strain already has the memory effect. If such hypothesis were to be true, this would mean that particular loci (such as the TRP1 locus) caused the stochasticity of gene expression to trump a high transcriptional activity, thus overcoming any biological effect hence the absence of memory at the pericentromeric location. However, the subtelomeric position would allow to overcome the stochasticity of gene expression for some cells. Thus, a subpopulation of cells will switch to one specific profile, *i.e.* the profile 1 corresponding to the memory effect.

Based on the hypothesis of the necessity to have a high transcriptional activity, my next step was to look for epigenetic factors independent to any RNA Pol (as shown by the transcriptional inhibition experiment) that could influence the compaction of the chromatin which, as I have presented in the introduction, is linked to the likelihood of having a high or low transcriptional activity.

VI. Preliminary study of the impact of chromatin remodelers on the memory effect

In the previous part, I have presented results on the influence of the SIR complex on the memory effect. The analysis points to the necessity of an optimal level of transcription in order for most of the cells to obtain the memory effect. However, I do not have an answer as to why at the endogenous location the memory effect is predominant, nor what are the biological mechanisms behind it. As the transcriptional inhibition experiment has ruled out that *de novo* protein synthesis is at the origin of the memory effect, I have studied several factors that are known to influence the chromatin state. In particular, as we have presented during the introduction, I have investigated the roles of histone acetylation, the histone variant H2A.Z and the chromatin remodeler Snf2p. This work is however still preliminary.

A. Acetylation may not have an effect on the memory effect

As developed in the introduction, histones acetylation increases the accessibility of the chromatin to transcriptional factors. Since I observed a decrease of transcriptional activity upon successive stresses, a possible explanation would be that the state of the chromatin is modified between stresses, therefore causing the transcriptional response to vary. However, if changes in the acetylation levels upon stress is the factor responsible for the memory effect, forcing the same level of acetylation in order to constantly prepare the chromatin at the *STL1* locus for transcriptional activity should in theory lead to a decorelation of the transcriptional responses to the stresses and lead to a dynamical behavior of the cells comparable to the stochastic simulations[53].

To test this hypothesis, I have attempted to force an open state of the chromatin using Trichostatin A (TSA)[54]. This chemical inhibits histones deacetylases, thus the chromatin is primed to be in a constant hyperacetylated state, including at the *STL1* locus.

Cells were treated for 8h with 100nM TSA dissolved in ethanol and submitted to pulsed stresses in the microfluidic device. Following the pattern of my memory

[53] Alternatively, forcing a high level of acetylation on the strain with the displaced pSTL1 during the first stress only, might cause a recovery of the memory effect.
[54] Abcam ref ab120850

experiments, I quantified the fluorescence trajectories of the cells in order to analyze their dynamical behaviors. The subsequent single-cell quantification shows that despite hyperacetylation of the chromatin, this latter is likely not in the same state during the first and second stress as most of the cells still decrease their transcriptional activity with the stress (62%±21% in the hyperacetylated case compared to 55%±11% in the wild type strain, **figure 3-31**): the memory effect is predominant. Overall, this result suggests that there are no differences in the dynamical behavior compared to the non-treated cells, therefore TSA acetylation does not seem to be a critical factor to the memory effect. This work is however preliminary and will need to be repeated and TSA efficiency controlled[55].

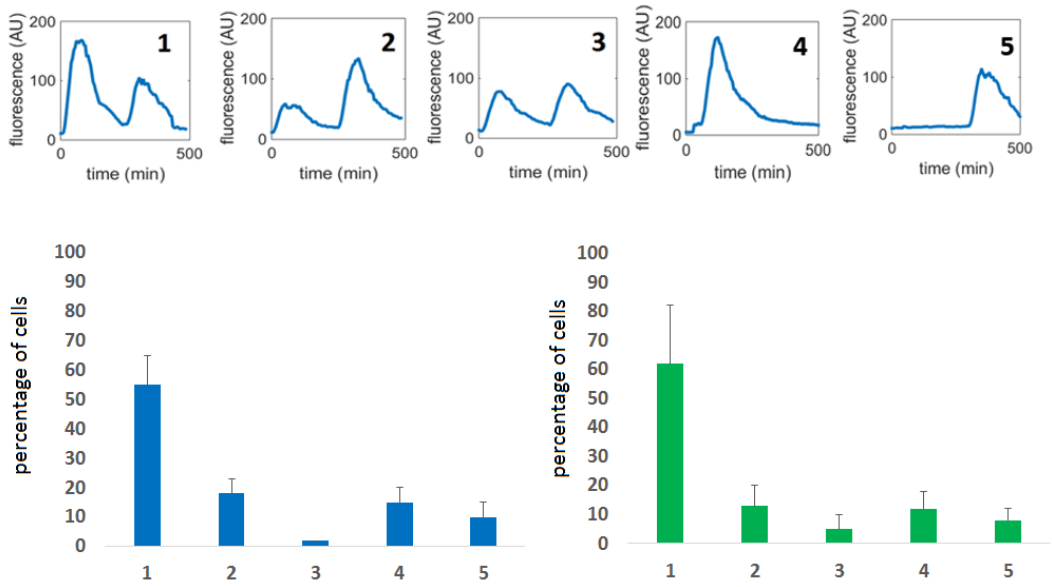


Figure 3-31. Acetylation does not permit to decorrelate the response to two hyperosmotic pulses. Single-cell quantification according to the five profiles of response (top) of the wild type strain yPH53 (blue, N=700) and the wild type strain treated with TSA (gold, N=100) in response to two pulsed hyperosmotic stresses. The single-cell quantification is similar in both cases.

B. Investigation of chromatin remodelers

Chromatin remodelers, through hydrolysis of ATP, induce local changes in the conformation of nucleosomes that can influence gene expression. As I have presented before, a possible hypothesis to the memory effect is the necessity to have a high transcriptional activity at the first stress. To that effect, I investigated the influence on

[55] I have used the TSA according to protocol available in Gael Yvert's lab website (http://www.ens-lyon.fr/LBMC/gisv/index.php/en/protocols/yeast-methods/92-tsa-treatment), but I did not perform a control consisting of doing a western blot targeting the acetylated histones exclusively. Therefore, the result could also be explained by a failed TSA treatment.

the memory effect of two well studied factors that affect the compaction of the chromatin. My reasoning here was to keep the chromatin in a constantly open state so that the activity of pSTL1 would constantly be at a high level and potential post-transcriptional regulation would not be effective: the responses to the pulsed stresses would be decorrelated.

1. The Swi/Snf complex does not impact the memory effect

The Swi/Snf complex is an assembly of proteins involved in the regulation of gene expression, including stress response genes. Moreover, it has implication in the regulation of genes that are repressed in glucose conditions. As the *STL1* gene is a stress response gene that is submitted to glucose repression in non-stress conditions, the Swi/Snf complex is a perfect candidate to the investigation of its potential role in the transcriptional regulation of *STL1* and maybe the memory effect.

I have deleted *SNF2*, the catalytic subunit of the Swi/Snf complex. The subsequent strain was submitted to pulsed stresses in the microfluidic devices and the dynamical behavior of the cells analyzed by fluorescence. The single-cell quantification shows that the cells still behave according to the five typical profiles of response. A high variability in the amount of cells behaving according to the profiles 1 and 2 impeaches to conclude on the effect of *SNF2* (50%±21% and 30%±22% respectively, **figure 3-32**). This experiment needs to be reproduced.

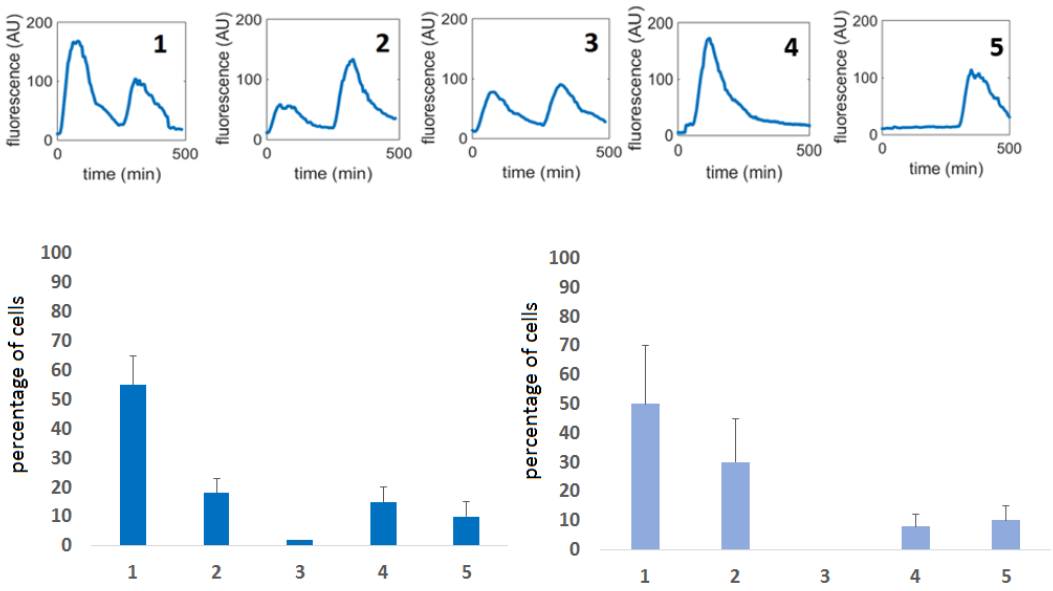


Figure 3-32. The memory effect is not impaired by *SNF2*. Single-cell quantification according to the five profiles of response (top) of the wild type strain yPH513 (blue, N=700) and the *SNF2* mutant yPH385 (light blue, N=120) in response to two pulsed hyperosmotic stresses. Although there is an increase in the cell-cell variability in the latter case, there is no real change in the single-cell quantification.

However, the high variability might suggest that the presence of the complex influences the cell-cell variability. Further work is required in order to have a definitive answer as to the role of the complex on the dynamical response.

2. The histone variant H2A.Z may not impact the memory effect

A stated in the introduction, H2A.Z is involved in the regulation of transcription and has been implied in the galactose memory.

This histone variant is encoded by the *HTZ1* gene. I have deleted this gene of interest in the yPH53 strain. I have then performed single-cell experiments during which the dynamical behavior a cells was studied in microfluidics.

The preliminary result does not point towards an influence of this factor on the memory effect as the profiles of responses are relatively similar to the wild type (62% of cells with the memory effect in the strain deleted for HTZ1 compared to 55%±11% in the wild type strain, **figure 3-33**). However, this preliminary work needs confirmation.

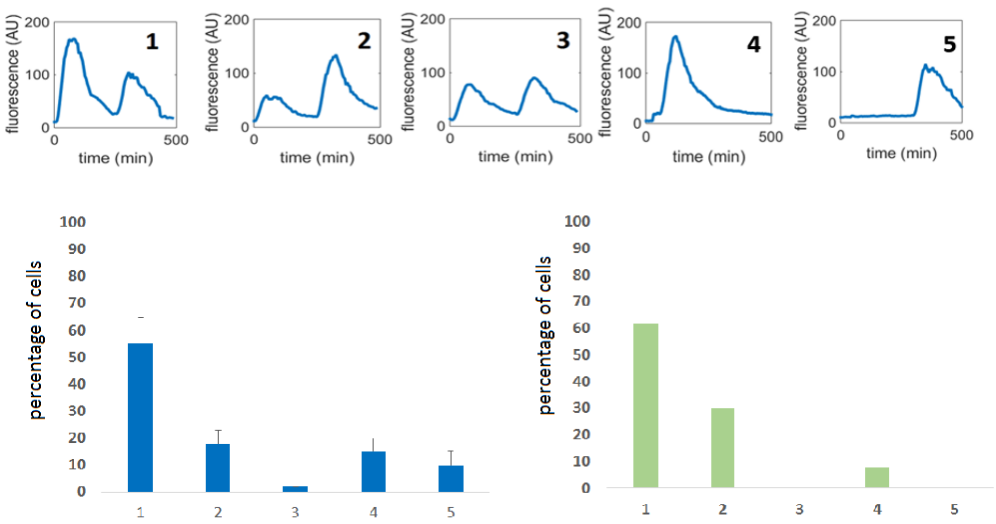


Figure 3-33. The memory effect is not impaired by *HTZ1*. Single-cell quantification according to the five profiles of response (top) of the wild type strain yPH53 (blue, N=700) and the *HTZ1* mutant yPH359 (gold, N=56) in response to two pulsed hyperosmotic stresses. Although there is an increase in the cell-cell variability in the latter case, there is no real change in the single-cell quantification.

C. Conclusion

My previous work pointed towards the fact that a high level of activity of pSTL1 during the first stress is required to have the emergence of the memory effect at the second stress, characterized by a decreased activity, probably linked to post-transcriptional activity that are independent of Pol II transcriptional marks. In this case, both stresses are correlated. By altering the activity factors involved in the structure of the chromatin, I intended to keep the chromatin in the same state during the pulsed stress in an effort to avoid the emergence of the memory effect.

Although preliminary work, my study points towards the absence of influence of at least two factors that modify the chromatin by opening it. Moreover, artificially inducing an open state of the chromatin with TSA does not impact the single-cell quantification either. Overall, the open state of the chromatin, usually associated with acetylation, does not seem to be involved in the memory effect.

However, it is known that a high expression of *STL1* is linked to the presence of the HDAC Rpd3 (de Nadal et al., 2004). In such a case, deacetylation might actually be linked to an open state of the *STL1* promoter. Since TSA inhibits HDACs, an effect should have been visible in the TSA experiment, which is not the case. Interestingly, the TSA is known to inhibit all HDACs except a specific class of HDACs called sirtuins. In the budding yeast, the archetypal sirtuin is Sir2.

Sir2 interacts with Rpd3 in an antagonist fashion. Therefore, a promising lead of study is the investigation of both deacetylation through the study of Rpd3 and Sir2 which can make a bridge between the memory effect at the subtelomeric location (the high activity of pSTL1 is at this position which is Rpd3 dependent) and the absence of it at the centromere (the low activity of pSTL1 at this location is linked to the presence of the SIR complex).

VII. Preliminary study of the heredity of the memory effect

I have so far presented an investigation on the existence of a cellular memory in a population of cells in response to short repeated hyperosmotic stresses. A memory, by essence, not only can be defined for the same individual, it can also be defined for different individuals since a memory can potentially be passed on. I here asked if the memory effect that I have observed among the cells that received pulsed stresses could be passed on to their progeny. To that effect, I have investigated the inheritance of the memory to the first generation of daughter cells that was born from the original population of cells from the yPH53 strain, which I will here referred to as mothers, during the first and second stress **(figure 3-34)**. As daughters are naïve cells in regard to the response to a hyperosmotic stress, the purpose is to investigate if the memory effect could be transmitted and, as I can do single-cell experiments, if such heredity happens with a cell-cell variability that we can quantify or is it absolute, i.e. if all daughters would systematically inherit the memory.

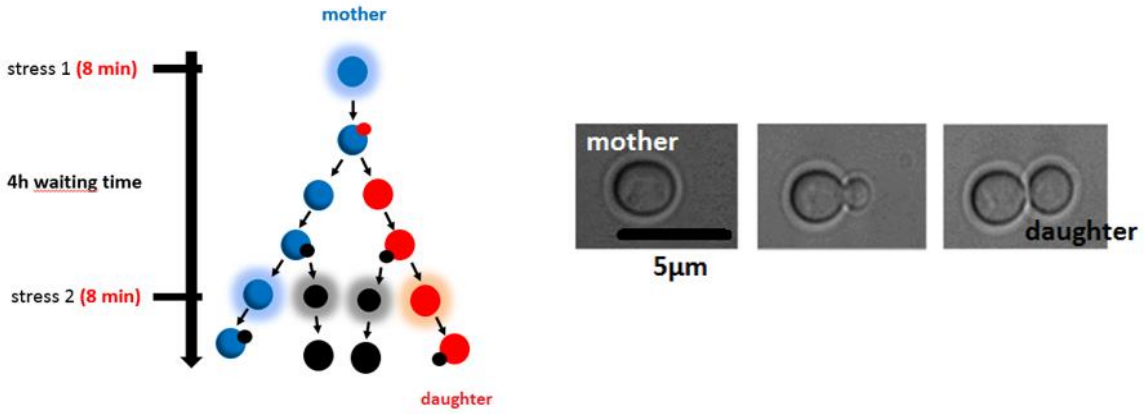


Figure 3-34. Denomination: notion of first generation daughters. *(Left)*. Sketch of a cell dividing over time and submitted to two pulsed stresses separated by 4h. The cell that receive the first and second stress is called mother. The daughter is the cell created by the mother between the two stresses. *(Right)* Bright field sequence images of a mother cell giving birth to a daughter cell.

A. The memory effect is inherited by the progeny

1. Analysis at the population level shows that the memory effect is transmitted

I have analyzed the response of the first daughters in some of my previous microfluidics experiments where a population of yeast was submitted to pulsed hyperosmotic stresses.

The response of the daughters to their first stress is on average 34% lower than the response of the mothers to their first stress (*i.e.* when they are still naïve cells in regard of the memory effect, **figure 3-35**). In fact, the response of the daughters is actually more comparable to the response of the mothers during their second stress, *i.e.* when the mothers display the memory effect. In this case, among the mothers, the difference of amplitudes of response between the two stresses is in average 30%.

Unlike the mothers, the daughter cells have never experienced a stress prior to the second stress. In the absence of heredity of memory, they should behave like the mothers during their first stress which is not the case here. Instead, the daughters behave like mothers with the memory effect: as a result, the memory effect is transmitted to at least the first generation of daughters.

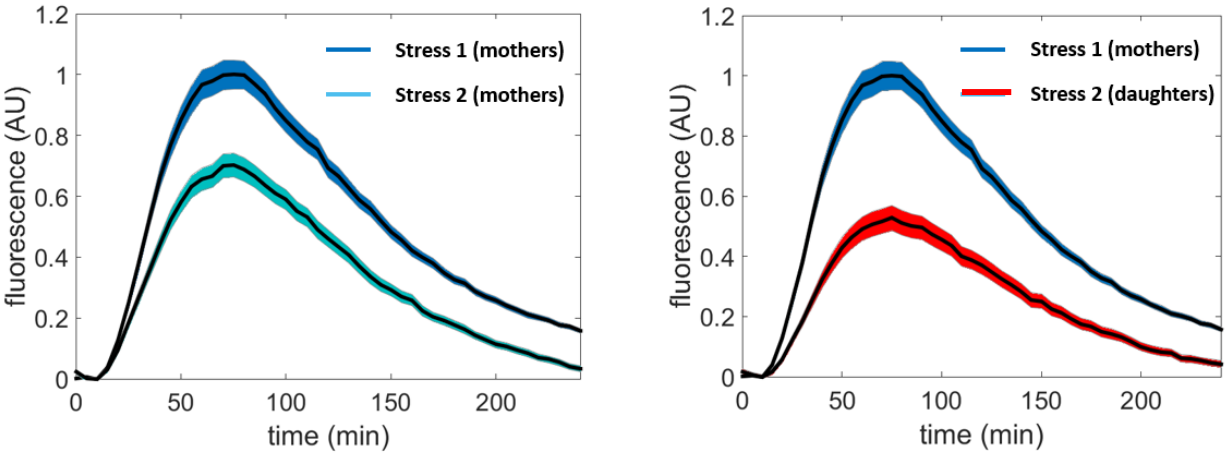


Figure 3-35. The memory effect is transmitted at the population level. *(Left)* Response of the mothers to the repeated hyperosmotic stresses. The mother have a decreased response to the second stress, which corresponds to the memory effect. *(Right)* Response of the daughters to their first stress. The response is different from the response of mothers during the first stress but closer to the response of the mothers to the second stress, meaning that the memory effect is inherited.

2. Single-cell quantification shows that the memory effect is partially transmitted to the progeny

I have analyzed various pairs mother-daughter in order to compare the response of the daughters to their second stress with the overall response of the mothers. In a similar fashion than the single-cell analysis performed on the response of the mothers, I have clustered the various pairs mother-daughter according to typical profiles.

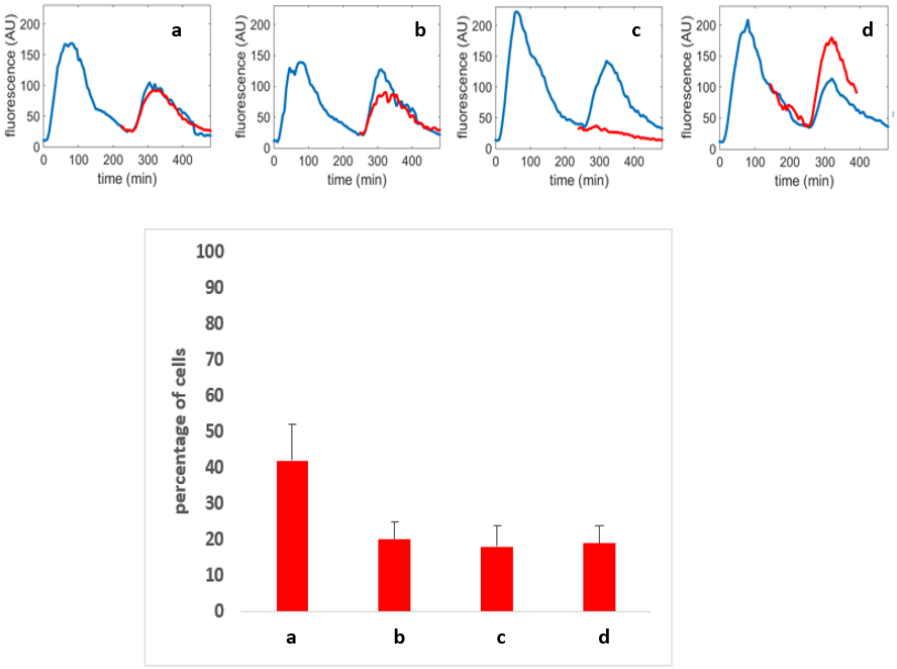


Figure 3-36. The heredity of the memory effect occurs with a single-cell variability. *(Top)* Typical profiles of response of the daughters (red) compared to the response of their corresponding mothers (blue). *(Bottom)* Single-cell quantification of the daughters according to the five typical profiles of response. Most cells respond exactly like their mothers (40%) but there is an equally distribution according to the three remaining patterns. As a result, the memory effect is a mixture between daughters not responding and daughters actually displaying the memory effect.

The single-cell quantification **(figure 3-36)** shows that 40% ± 10% of the daughters actually display a response to the second stress that is identical to the response of their mothers during the same stress (profile a). As most of the mothers have the memory effect, so do the daughters behaving according to profile a. In addition to this behavior, 20% ± 7% of the daughters have a response to the second stress that is lower than their mothers' (profile b). Moreover, 19%± 8% of the daughters do not show any response, which can be associated to the bimodality of the response to a hyperosmotic pulse (profile d).

B. Conclusion

As daughters have never experienced a stress before, in the absence of memory they should behave like their mothers during the first stress. It is however not the case, as daughters behave like their mothers once they have already experienced a stress and respond to the next with a decreased amplitude of fluorescence: at the population level, the memory effect appears to be transmitted.

However, the single-cell quantification shows that this heredity is a mixture between daughters that actually have the memory effect, daughters not responding to the stress and daughters even better responding to the stress compared to their mothers. Of note, a subpopulation of daughters behave according to the profile d, which means that they do not develop a memory. Therefore, unlike what the population analysis reveals, the memory effect is only partially transmitted.

The cell-cell variability among the transmission of the memory effect will obviously require more work, such as investigating the position in the cell cycle and the persistence of such memory by analyzing next generations of progenies and especially quantifying what happens to other generations of daughters, which could potentially point towards a slow establishment of the memory effect.

Chapter IV: Discussion

Table of contents

I. A possible interpretation of the memory effect

I have presented a study aiming at understanding how do individual yeast dynamically behave in response to short pulsed stresses and if a genetic adaptation does exist. In response to two pulsed hyperosmotic stresses, I have observed that a population of yeast reduce pSTL1 amplitude of response with the stress, without any difference in the time of response. This suggests that the rate of protein production decreases with the stress. I named this phenomenon **memory effect**. The use of the term memory comes from the fact that such behavior does not match a stochastic behavior: in my experimental conditions, the response to the first and the second stress is correlated.

As I have presented in the introduction, several examples of cellular memories in the budding yeast in response to repeated stresses exist. I described two possible ways to respond from a genetic point of view:

- with a faster dynamics of gene expression, *i.e* galactose memory (Brickner et al., 2007; Kundu et al., 2007; Zacharioudakis et al., 2007).

- with a lower amplitude of response, *i.e* of hyperosmotic memory (Rienzo et al., 2015).

Although I studied short stresses, the memory effect I described is similar in its behavior that the one observed in the context of the memory to long hyperosmotic stresses (Rienzo et al., 2015). However, two interesting questions could be asked:

- Why the memory effect I described does not consist in a faster dynamics of response, like the galactose memory?

- What does it mean for a cell to decrease its amplitude of response upon stress?

A. Comparison between the memory effect and the galactose memory

A hyperosmotic or galactose stress[56] both cause cells to stop their growth in order to counter the stress and eventually adapt to the stress. The subsequent growth rate is lower than in the original environment. The adaptation process consists in the activation of specific metabolic pathways in order to create enzymes allowing the cell to synthesize glycerol or process galactose. As a result, galactose memory and osmotic memory might be different because of the differences in the metabolic pathways they involve.

The genetic response to a switch from glucose to galactose starts with a 4h dynamics (Zacharioudakis et al., 2007). However, the genetic response to a hyperosmotic stress starts within 3 minutes. Signal transduction between the sensing of the stress and the transcriptional activity of osmo-responsive genes might make impossible to modify this time of response (Miermont et al., 2011). However, in the case of the galactose memory, this time could be optimized as it has been shown in the galactose memory experiments. As a result, faster dynamics of response in the context of the memory effect would not be a possible strategy.

B. Comparison with the memory of long hyperosmotic stresses

The hyperosmotic memory in response to long stresses described by Proft's group shows that a reactivation of the osmo-responsive gene *GRE2* leads to a decreased activity of the gene, without any difference in its time of reactivation. The stresses were triggered using NaCl, which is uptaken by yeast. In order to tolerate the stress, the yeast Ena complex of proteins that is involved in the efflux of Na+ ions. Accumulation of Ena proteins has been shown to be at the origin of the reduced transcriptional activity of *GRE2* upon reactivation.

In my experimental conditions, I observe a similar decrease in amplitude of pSTL1 response after pulsed stresses, without any difference in the time of reactivation (figure 3-7). A diminished transcriptional activity could be a way for the cells to lessen their burden compared to the case where transcriptional response to two stresses would be similar. Therefore, **the memory effect could be a way for the yeast to optimize the amount of energy dedicated to fighting the stress**

One could ask what are the potential factors explaining a decreased amplitude of response. It is probable that Ena proteins described to be at the origin of the

[56] When a cell experiences any environmental stress, the Environmental Stress Response will necessarily be triggered and upregulate stress response genes, controlled by PKA or Msn2/Msn4 (Gasch et al., 2000). This is indeed the case when the cell encounters a hyperosmotic change, but not a galactose switch. It could be argued that a galactose stress does not fit the definition of environmental stress.

hyperosmotic memory are not responsible for the memory effect I described since I use a non-salty inducer. However, it is still possible that the accumulation of others proteins could impact the response to the second stress. Transcriptional inhibition experiments with thiolutin that I have performed showed that proteins present prior to the first stress could be involved during the second stress in order to cause the emergence of the memory effect (figure 3-16). A potential candidate could be the protein Hog1 whose half-life is ca 10-11 hours. Since upon hyperosmotic stress, the cytoplasmic protein Hog1 is phosphorylated and translocates inside the nucleus where it participates in the activation of numerous osmo-responsive genes (Miermont et al., 2011), it could be hypothesized that some of the Hog1p remains in the nucleus after stress and ready osmo-responsive genes for activation with a different dynamics during a following stress. Although it has been shown that the entirety of Hog1p exits the nucleus after adaptation of cells to the stress (Muzzey et al., 2009), performing a dynamical analysis of Hog1p during the first and second stress could give an indication on its potential influence on the genetic response's dynamics to the stresses.

II. On the biological mechanisms of the memory effect

A. On the role of the SIR hyperclusters

Transcriptional inhibition experiments show that the memory effect does not seem to require *de novo* protein synthesis. This suggests that one or several factors, potentially epigenetic factors, already present during first the stress, would influence the transcriptional activity of pSTL1 during the second stress. It has been described that the SIR complex is removed from the chromatin upon hyperosmotic stress (Mazor and Kupiec, 2009). However, no additional information is available on SIR complex fate in such stress conditions. Interestingly, I observed that the SIR complex forms hyperclusters upon stress (figure 3-21). Such phenotype requires to be explored in more detail. These hyperclusters resemble the ones observed in quiescence (Guidi et al., 2015). Quiescence can be defined as a state where cells maintain viability under growth-arrested conditions and to resume mitotic growth once growth-promoting conditions are restored (Klosinska et al., 2011). Interestingly, a long hyperosmotic stress causes the arrest of the cell-cycle and the subsequent activity of the HOG pathway that allows viability under the growth-arrested condition. The mitotic growth is restored after adaptation to the hyperosmotic stress or removal of said stress. By this analogy, the physiological condition of a cell submitted to a long hyperosmotic stress

could be compared to the quiescence state. In my experimental conditions I performed short stresses which indeed cause the activity of the HOG pathway but does not lead to the physiological adaptation of the cell to the stress. However after stress, the cell will be back the original environment, which is suitable for mitotic growth. I have not quantified if such short stresses actually cause the arrest of the cell cycle, which will require a more in-depth analysis. A positive answer would encourage try to build a bridge between quiescence and stress response.

Nevertheless, in quiescent cells, the SIR complex is still on the subtelomeric and telomeric chromatin, and the hyperclusters cause a grouping of telomeres (Guidi et al., 2015; Laporte et al., 2016). It could be hypothesized that this clustering changes the yeast genome architecture in order to maintain longevity (Guidi et al., 2015). Based on the study showing that the SIR complex is stalled from the chromatin upon hyperosmotic stress (Mazor and Kupiec, 2009), the hyperclusters I observed may not, be on the subtelomeric chromatin anymore. This may explain why the Δsir3 mutant has no effect on the endogenous pSTL1 in my experimental conditions (figures 3-24, 3-25). Surprisingly, the SIR complex has an impact on the activity of pSTL1 moved to a pericentromeric area (figures 3-26, 3-27). This suggests that in stress conditions the hyperclusters might have a regulatory activity in this pericentromeric gene[57].

Potential ways to investigate the influence of the SIR hyperclusters on gene expression would be to:

- Perform Chip experiments in order to first ascertain that the SIR complex interacts with the pericentromeric pSTL1 upon hyperosmotic stress. A genome wide analysis of the distribution of the SIR complex on the genome upon stress could be performed eventually.

- Investigate where the SIR hyperclusters actually form in the nucleus in order to verify if their formation appears at the same locations upon stress or are randomly distributed in the nucleus. The use of gene maps (Berger et al., 2008) would be a good way to perform such investigation.

B. On the transcriptional activity in stress memory establishment

I observed that a low transcriptional activity during the first stress does not cause the memory effect, at least at the pericentromeric location (figure 3-19). Therefore, a high transcriptional strength of pSTL1 might be a prerequisite condition to have the emergence of the memory effect. To work towards confirming this hypothesis, I used TSA to force an open state of the chromatin during the first and

[57] This activity would not be global as no effect has been observed on the osmo-responsive pericentromeric gene *GPD1*

second stress in order to uncouple the response to these stresses and cause the loss of memory effect at the endogenous location. However, the result was inconclusive as the memory effect was still present (figure 3-31) and no experiments were performed to verify that the TSA actually worked. Nevertheless it is a relevant strategy that can be improved, along with additional experiments such as:

- Using specific chemicals in order to open the chromatin during the first stress only. This treatment could cause a high transcriptional activity of pSTL1 moved to the pericentromeric location during the first stress. Observing if the memory effect is restored at the pericentromeric location the second stress could inform on the role of the chromatin state in the establishment of the memory effect.

- Using a Crispr/dCas9 system with inducible guides (Farzadfard et al., 2013) in order to artificially force the activity of pSTL1 at the first stress to a high level, then observe at the second stress if the memory effect is restored. Inducing the guides could either be done during the first stress in order to enhance the natural activity of the displaced pSTL1, or without a stress in order to decorrelate the exclusive genetic activity of pSTL1 from the entire activity of the HOG pathway.

These experiments require an artificial way to induce or remove the memory effect, but won't show what the biological mechanisms behind the memory are. However, a positive result of these experiments could justify the search for factors known to be involved in the opening of the chromatin which is usually linked to a high transcriptional activity. I investigated the role of two factors known to open the chromatin, Snf2 and Htz1 (figures 3-32, 3-33). My work on the matter is however preliminary and would require more experiments and probably the investigation of others factors.

A way to ascertain the influence of factors on the transcriptional strength of pSTL1 would be to perform a screen by mating the strain with pSTL1 moved at the pericentromere, with strains from the yeast library deleted for non-essential ORFs. The subsequent screen would consist in the search of a strain with a higher activity of pSTL1 as compared to the centromeric strain by flow cytometry. This would allow to discriminate the factors that change the activity of pSTL1. The right strains would be further analyzed using microfluidics in order to test if the memory effect can be developed.

A factor that I would be keen on studying is the HDAC Rpd3. As I have presented during the introduction, this protein influences the transcriptional activity of pSTL1 (figure 1-16). Thus, Rpd3 might determine the transcriptional activity of

pSTL1 upon repeated stresses. Moreover, Rpd3 has been proposed to drive transcriptional quiescence in the budding yeast (McKnight and Tsukiyama, 2015), so investigating its role might also prove to be interesting as it could create a bridge between quiescence and memory effect.

Interestingly, the notion of developing a memory because of transcriptional events occurring above a certain threshold has been described in *B. subtilis* (Wolf et al., 2008). This organism develops a memory of competence with the expression of the ComK transcription factor above a certain threshold. Similarities in the regard to the mechanisms of the memory effect could imply the existence of a conserved mechanism through evolution.

III. Perspectives on the dynamical variability

Stochastic modelling shows that the dynamical variability in the response to pulsed stresses can be explained by the noise in gene expression (figure 3-11). Noise causes a diversity of behaviors that allows one or several choices when a selective pressure is exerted. Along with mutations, noise is a driving motor of evolution. The single-cell quantification of the microfluidics experiments shows a dynamical variability of the behaviors of mothers that respond to two pulsed stresses.

Noise in gene expression can be explained by the external stimuli influencing the physiology of the cell (extrinsic noise), cell-cell physiological differences and the stochasticity of gene expression (intrinsic noise). This latter is hardly controllable, so it would be interesting to try to experimentally quantify the influence of several factors involve in the extrinsic noise:

- The age of the cells could influence the response upon pulsed stresses since old cells will not necessarily be in the same physiological state as younger ones. Using calcofluor in order to stain the cells will allow counting the buds scars of the mothers and estimating their age. It will allow determining if a correlation between the amplitude of response with the age exists. This will also allow to evaluate if there is a likelihood that young mothers are more likely to display the memory effect than old ones.

- The cells in my microfluidic device are all in different stages of the cell cycle, which would partly account for the variability of the response. Determining

the position in the cell-cycle will require the use of strains with a tagged nucleus and/or bud neck.

These are parameters that can be estimated and, to a certain extent, controlled. As a result, the description of the mechanisms behind the dynamical variability would greatly be improved and open the way towards an evolutionary description of this mechanism.

However, the stochasticity of the biochemical reactions occurring during the genetic response to the stress must also be taken into account. This is something that I have tried to address by moving pSTL1 in a different transcriptional area (figure 3-17).

It has been established that changing the position of a gene on the locus can alters its expression (Aparicio et al., 1991). The change in the expression can be associated to a reduction of noise in the transcription, or a reduction in the noisy steps of transitioning from a repressed state to an expressed one. The latter case is important as it has been shown that such changes can actually enhance a cellular memory (Acar et al., 2005). A possible hypothesis would thus be that **the pericentromeric position might increase the transcriptional noise which can be translated by a reduced transcriptional activity**. As a result, the previously presented hypothesis stating that a high transcriptional activity is required to the memory effect would essentially mean that a high transcriptional activity allows to overcome the noise in gene expression, therefore making possible the emergence of various mechanisms such as the memory effect.

It would consequently be important to investigate the effect of displacements of pSTL1 on more locations, especially:

- If pSTL1 is displaced to different subtelomeres, do the mothers still display the memory effect with the same proportions?

- Does moving pSTL1 outside of a subtelomere necessarily cause a decrease of transcriptional activity along with the loss of the memory effect and a behavior close to a stochastic one?

- In the various positions chosen, does the SIR complex has in influence of the dynamical behavior of pSTL1?

Such investigation would broaden the understanding of the influence of the chromatin context on the memory effect and actually help find the factors involved in the emergence of the memory effect.

IV. On the dynamical bimodality

I have observed that cells submitted to two short stresses may or may not respond to either of the stresses (figure 3-3, 3-4). It could be argued that cells not responding are physiologically unable to respond, or have gone in a dormant state upon stress in order to try to tolerate it. However, such explanations are dismissed by the fact that extending the duration of the stress causes all cells to eventually respond (figure 3-1). The stochastic simulations showed that a possible explanation as to why some cells respond or not to a stress can be linked to a delay in the transcription. This is consistent with the original study by Pellet's group on the bimodality phenomenon (Pelet et al., 2011). However, such delay could have several explanations:

- pSTL1 is repressed in glucose conditions, and such repression has to be lifted upon expression. It is possible that some cells are not able to lift the repression with a fast dynamics compared to the rest of the population. Experiments consisting in modifying the amount of glucose could help determining if these new conditions allow obtaining a more uniform distribution of the time of response compared to my experimental conditions.

- The HOG pathway is activated through different sensors. This leads to two distinct branches of activation that eventually converge to a catalytic activity of Pbs2 (figure 1-12). However, these two branches have different dynamics. Deletion of either of the branches would be a good indication.

- The transcriptional activity of pSTL1 depends on the duration of the stress and its intensity. Although I perform stresses using 1M sorbitol, can increasing the dose of sorbitol change the amount of cells not responding to a stress?

Investigating such parameters would allow to better characterize this novel phenomenon. Also, it would allow to determine if such mothers can transmit the memory effect. Indeed, the absence of transcriptional activity is not a requirement for the absence of memory effect as I have established in the transcriptional inhibition experiment presented in the previous chapter (figure 3-16). An understanding of this bimodality could therefore help in, for instance, the context of heredity experiments.

V. Improvement of the experimental setup and parameters

I have presented a preliminary study of the heredity of the memory effect. At the population level, the memory effect appear to be transmitted to the first progenies of the mothers. However, the single-cell level analysis reveals that this transmission if only partial and actually consists in a mixture of various single-cell profiles, including daughters with the memory and not responding to the stress.

Because of the cellular crowding, I was unable to analyze more generations of daughter cells. Such analysis would enable to better characterize the memory effect, for instance by understanding if short pulsed only cause the first generation of daughters to mostly develop a memory or if the more generations, the more likely the daughters of a specific generation would be able to develop a memory. Such a study would require the use of a microfluidic device that would prevent cellular crowding. Such a tool has been developed by (Kim et al, 2010) to answer such questions. In particular, a microfluidic device where cells can only grow in one dimension, therefore the lineage can easily be tracked and studied (figure 4-1).

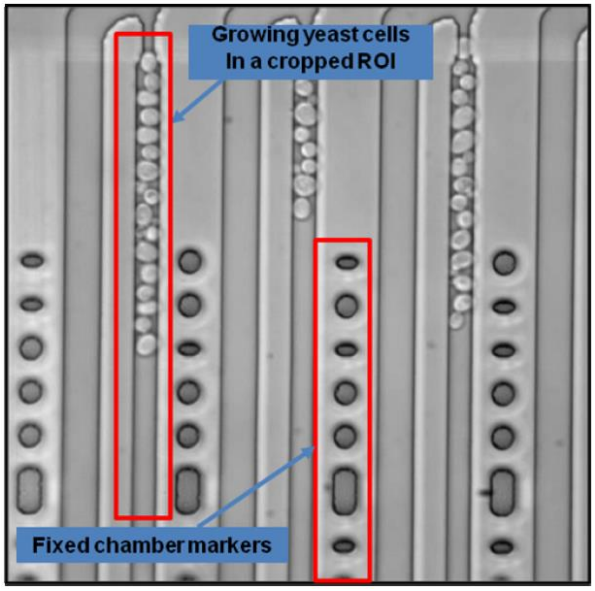


Figure 4-1. Microfluidic device for lineage experiments. Related cells grow in one dimension in a chamber, enabling the analysis of the genealogy of a particular cell. From Kim et al, 2010.

Cellular crowding is also a parameter that could limit the amount of cells receiving pulsed stresses and being subsequently analyzed. As a result, the persistence of the memory effect in such cells might be difficult to fully study. The Alcatras microfluidic system (figure 4-2) could be an interesting alternative (Crane et al., 2014). Mother cells are trapped between two pillars and the progeny of the cells is flushed away. More single-cell investigations of the memory effect can thus be made on the mothers, but any study of its heredity is impossible with such a device.

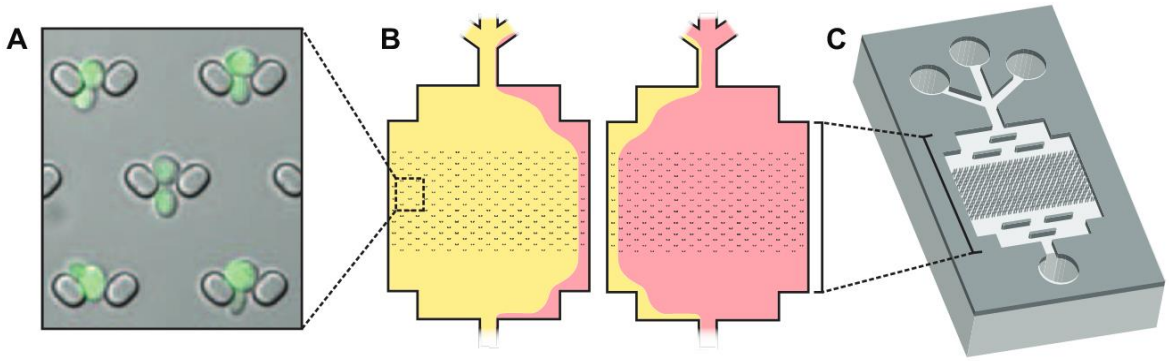


Figure 4-2. Alcatras microfluidic system. Mother cells are trapped in pillars and the progeny is flushed away. This enables the analysis of mothers exclusively. From Crane et al, 2014.

Over my PhD, I triggered the hyperosmotic stresses using sorbitol 1M for 8min every 4h. Constrained by the time it took to set up the experimental platform, the long time it takes to manually perform the single-cell analysis and the subsequent biological investigations I privileged, I did not change much the parameters of study. However, changing the concentration of sorbitol, even the chemical used to trigger the stress, the duration of the stresses and even their frequency would be interesting to better quantify the memory effect. The comparison of those various experiments would show if the phenotype of dynamical adaptation and/or the genetic mechanisms for them are always the same. In other words, is there a universality of the memory or, like the galactose memory, could different biological mechanisms exists for a short-term memory, a long-term memory and similar type of stresses caused by different sources.

VI. Final word

The stochasticity of gene expression gives a diversity of behaviors. From an evolutionary point of view, this diversity of responses to repeated stresses allows one or several choices to be selected. As it appears that the memory effect was the one selected, this suggests that the specific subtelomeric position of pSTL1 has been chosen in order to gain a regulation level in order to perform better adaptation instead of behaving in the same manner observed at the pericentromeric position. After time, additional biology effects would be added to enhance the memory effect. This research not only proves how critical single-cell studies are for such analysis, but it also indicates that the establishment and the transmission of the memory does not require a long stress and can start very early on, at least on the original population. Overall, my study suggests that the specific location of pSTL1 at the subtelomere is required

for an optimal level of transcription that can go beyond a simple stochastic behavior and lead to the emergence of a memory in response to osmotic stresses. Despite several improvements required, this work could serve as corner stone to broader studies of stress response genes at subtelomeric positions in the budding yeast. Moreover, the methodology developed during this work can help perform similar work with a different stress, and even cross-stress experiment. And finally, this work could lead the way of more investigation of the role of the SIR complex in stress conditions as not only unreported phenotypes appear in osmotic conditions, but genetic influence outside subtelomeres have also been observed which would possibly cause a reinterpretation of the way we think about transcriptional silencing at subtelomeres.

References

Acar, M., Becskei, A., and van Oudenaarden, A. (2005). Enhancement of cellular memory by reducing stochastic transitions. Nature *435*, 228–232.

Alber, F., Dokudovskaya, S., Veenhoff, L.M., Zhang, W., Kipper, J., Devos, D., Suprapto, A., Karni-Schmidt, O., Williams, R., Chait, B.T., et al. (2007). The molecular architecture of the nuclear pore complex. Nature *450*, 695–701.

Albertyn, J., Hohmann, S., Thevelein, J.M., and Prior, B.A. (1994). GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. Molecular and Cellular Biology *14*, 4135–4144.

Andrulis, E.D., Neiman, A.M., Zappulla, D.C., and Sternglanz, R. (1998). Perinuclear localization of chromatin facilitates transcriptional silencing. *394*, 4.

Aparicio, O.M., Billington, B.L., and Gottschling, D.E. (1991). Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell *66*, 1279–1287.

Austriaco, N.R., and Guarente, L.P. (1997). Changes of telomere length cause reciprocal changes in the lifespan of mother cells in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences *94*, 9768–9772.

Bai, C., Tesker, M., and Engelberg, D. (2015). The yeast Hot1 transcription factor is critical for activating a single target gene, *STL1*. Molecular Biology of the Cell *26*, 2357–2374.

Barbulovic-Nad, I., Au, S.H., and Wheeler, A.R. (2010). A microfluidic platform for complete mammalian cell culture. Lab on a Chip *10*, 1536.

Basehoar, A.D., Zanton, S.J., and Pugh, B.F. (2004). Identification and Distinct Regulation of Yeast TATA Box-Containing Genes. Cell *116*, 699–709.

Bensaude, O. (2011). Inhibiting eukaryotic transcription. Which compound to choose? How to evaluate its activity?: Which compound to choose? How to evaluate its activity? Transcription *2*, 103–108.

Berger, A.B., Cabal, G.G., Fabre, E., Duong, T., Buc, H., Nehrbass, U., Olivo-Marin, J.-C., Gadal, O., and Zimmer, C. (2008). High-resolution statistical mapping reveals gene territories in live yeast. Nature Methods *5*, 1031–1037.

Bernstein, B.E., Liu, C.L., Humphrey, E.L., Perlstein, E.O., and Schreiber, S.L. (2004). Global nucleosome occupancy in yeast. Genome Biology 11.

Berry, D.B., and Gasch, A.P. (2008). Stress-activated Genomic Expression Changes Serve a Preparative Role for Impending Stress in Yeast. Molecular Biology of the Cell *19*, 4580–4587.

Botstein, D., Chervitz, S.A., and Cherry, J.M. (2011). Yeast as a Model Organism. 4.

Brickner, D.G., Cajigas, I., Fondufe-Mittendorf, Y., Ahmed, S., Lee, P.-C., Widom, J., and Brickner, J.H. (2007). H2A.Z-Mediated Localization of Genes at the Nuclear Periphery Confers Epigenetic Memory of Previous Transcriptional State. PLoS Biology *5*, e81.

Buchberger, J.R., Onishi, M., Li, G., Seebacher, J., Rudner, A.D., Gygi, S.P., and Moazed, D. (2008). Sir3-Nucleosome Interactions in Spreading of Silent Chromatin in Saccharomyces cerevisiae. Molecular and Cellular Biology *28*, 6903–6918.

Burgess, S.M., and Kleckner, N. (1999). Collisions between yeast chromosomal loci in vivo are governed by three layers of organization. Genes & Development *13*, 1871–1883.

Carlson, M., and Botstein, D. (1983). Organization of the SUC gene family in Saccharomyces. Molecular and Cellular Biology *3*, 351–359.

Casamayor, A. (2002). Bud-site selection and cell polarity in budding yeast. Current Opinion in Microbiology *5*, 179–186.

Chavez, A., Scheiman, J., Vora, S., Pruitt, B.W., Tuttle, M., P R Iyer, E., Lin, S., Kiani, S., Guzman, C.D., Wiegand, D.J., et al. (2015). Highly efficient Cas9-mediated transcriptional programming. Nature Methods *12*, 326–328.

Chen, K.C., Csikasz-Nagy, A., Gyorffy, B., Val, J., Novak, B., and Tyson, J.J. (2000). Kinetic Analysis of a Molecular Model of the Budding Yeast Cell Cycle. Molecular Biology of the Cell *11*, 369–391.

Christiaens, J.F., Franco, L.M., Cools, T.L., De Meester, L., Michiels, J., Wenseleers, T., Hassan, B.A., Yaksi, E., and Verstrepen, K.J. (2014). The Fungal Aroma Gene ATF1 Promotes Dispersal of Yeast Cells through Insect Vectors. Cell Reports *9*, 425–432.

Coleman, M.L., and Chisholm, S.W. (2010). Ecosystem-specific selection pressures revealed through comparative population genomics. Proceedings of the National Academy of Sciences *107*, 18634–18639.

Crane, M.M., Clark, I.B.N., Bakker, E., Smith, S., and Swain, P.S. (2014). A Microfluidic System for Studying Ageing and Dynamic Single-Cell Responses in Budding Yeast. PLoS ONE *9*, e100042.

Dihazi, H., Kessler, R., and Eschrich, K. (2004). High Osmolarity Glycerol (HOG) Pathway-induced Phosphorylation and Activation of 6-Phosphofructo-2-kinase Are Essential for Glycerol Accumulation and Yeast Cell Proliferation under Hyperosmotic Stress. Journal of Biological Chemistry *279*, 23961–23968.

Dimov, I.K., Basabe-Desmonts, L., Garcia-Cordero, J.L., Ross, B.M., Ricco, A.J., and Lee, L.P. (2011). Stand-alone self-powered integrated microfluidic blood analysis system (SIMBAS). Lab Chip *11*, 845–850.

Elowitz, M.B. (2002). Stochastic Gene Expression in a Single Cell. Science *297*, 1183–1186.

Erkina, T.Y., Tschetter, P.A., and Erkine, A.M. (2008). Different Requirements of the SWI/SNF Complex for Robust Nucleosome Displacement at Promoters of Heat Shock Factor and Msn2- and Msn4-Regulated Heat Shock Genes. Molecular and Cellular Biology *28*, 1207–1217.

Farzadfard, F., Perli, S.D., and Lu, T.K. (2013). Tunable and Multifunctional Eukaryotic Transcription Factors Based on CRISPR/Cas. ACS Synthetic Biology *2*, 604–613.

Ferreira, C., van Voorst, F., Martins, A., Neves, L., Oliveira, R., Kielland-Brandt, M.C., Lucas, C., and Brandt, A. (2005). A Member of the Sugar Transporter Family, Stl1p Is the Glycerol/H^+ Symporter in *Saccharomyces cerevisiae*. Molecular Biology of the Cell *16*, 2068–2076.

Gasch, A.P., and Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. Functional & Integrative Genomics *2*, 181–192.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., and Brown, P.O. (2000). Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes□D. Molecular Biology of the Cell *11*, 17.

Geng, F., and Laurent, B.C. (2004). Roles of SWI/SNF and HATs throughout the dynamic transcription of a yeast glucose-repressible gene. The EMBO Journal *23*, 127–137.

Giaever, G., Chu, A.M., Ni, L., Connelly, C., Riles, L., Véronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., André, B., et al. (2002). Functional profiling of the Saccharomyces cerevisiae genome. Nature *418*, 387–391.

Gotta, M. (1996). The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. The Journal of Cell Biology *134*, 1349–1363.

Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. Nature *389*, 349–352.

Guarente, L. (1999). Diverse and dynamic functions of the Sir silencing complex. Nature Genetics *23*, 281–285.

Guet, D., Burns, L.T., Maji, S., Boulanger, J., Hersen, P., Wente, S.R., Salamero, J., and Dargemont, C. (2015). Combining Spinach-tagged RNA and gene localization to image gene expression in live yeast. Nature Communications *6*.

Guidi, M., Ruault, M., Marbouty, M., Loïodice, I., Cournac, A., Billaudeau, C., Hocher, A., Mozziconacci, J., Koszul, R., and Taddei, A. (2015). Spatial reorganization of telomeres in long-lived quiescent cells. Genome Biology *16*.

Gutiérrez, J.L., Chandy, M., Carrozza, M.J., and Workman, J.L. (2007). Activation domains drive nucleosome eviction by SWI/SNF. The EMBO Journal *26*, 730–740.

Han, M., and Grunstein, M. (1988). Nucleosome loss activates yeast downstream promoters in vivo. Cell *55*, 1137–1145.

Hershberg, R. (2015). Mutation—The Engine of Evolution: Studying Mutation and Its Role in the Evolution of Bacteria: Figure 1. Cold Spring Harbor Perspectives in Biology *7*, a018077.

Huang, J. (2003). Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. Genes & Development *17*, 2162–2176.

Huh, W.-K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O'Shea, E.K. (2003). Global analysis of protein localization in budding yeast. Nature *425*, 686–691.

Huisinga, K.L., and Pugh, B.F. (2004). A Genome-Wide Housekeeping Role for TFIID and a Highly Regulated Stress-Related Role for SAGA in Saccharomyces cerevisiae. Molecular Cell *13*, 573–585.

Iglesias, N., Redon, S., Pfeiffer, V., Dees, M., Lingner, J., and Luke, B. (2011). Subtelomeric repetitive elements determine TERRA regulation by Rap1/Rif and Rap1/Sir complexes in yeast. EMBO Reports *12*, 587–593.

Ishii, K., Arib, G., Lin, C., Houwe, G.V., and Laemmli, U.K. Chromatin Boundaries in Budding Yeast: The Nuclear Pore Connection. 12.

Jackson, J.D. (2000). Histone H2A.Z has a conserved function that is distinct from that of the major H2A sequence variants. Nucleic Acids Research *28*, 3811–3816.

Jenuwein, T. (2001). Translating the Histone Code. Science *293*, 1074–1080.

Jimenez, A., Tipper, D.J., and Davies, J. (1973). Mode of Action of Thiolutin, an Inhibitor of Macromolecular Synthesis in Saccharomyces cerevisiae. Antimicrobial Agents and Chemotherapy *3*, 729–738.

Kennedy, B.K., and McCormick, M.A. (2011). Asymmetric Segregation: The Shape of Things to Come? Current Biology *21*, R149–R151.

Kepler, T.B., and Elston, T.C. (2001). Stochasticity in Transcriptional Regulation: Origins, Consequences, and Mathematical Representations. Biophysical Journal *81*, 3116–3136.

Khandurina, J., McKnight, T.E., Jacobson, S.C., Waters, L.C., Foote, R.S., and Ramsey, J.M. (2000). Integrated System for Rapid PCR-Based DNA Analysis in Microfluidic Devices. Analytical Chemistry *72*, 2995–3000.

Klosinska, M.M., Crutchfield, C.A., Bradley, P.H., Rabinowitz, J.D., and Broach, J.R. (2011). Yeast cells can access distinct quiescent states. Genes Dev. *25*, 336–349.

Kornberg, R.D. (1974). Chromatin Structure: A Repeating Unit of Histones and DNA. Science *184*, 868–871.

Kostriken, R., Strathern, J.N., Klar, A.J.S., Hicks, J.B., and Heffron, F. (1983). A site-specific endonuclease essential for mating-type switching in Saccharomyces cerevisiae. Cell *35*, 167–174.

Kundu, S., Horn, P.J., and Peterson, C.L. (2007). SWI/SNF is required for transcriptional memory at the yeast GAL gene cluster. Genes & Development *21*, 997–1004.

Langley', C.H. THE EVOLUTION OF SELF-REGULATED TRANSPOSITION OF TRANSPOSABLE ELEMENTS. 25.

Laporte, D., Courtout, F., Tollis, S., and Sagot, I. (2016). Quiescent *Saccharomyces cerevisiae* forms telomere hyperclusters at the nuclear membrane vicinity through a multifaceted mechanism involving Esc1, the Sir complex, and chromatin condensation. Molecular Biology of the Cell *27*, 1875–1884.

Li, F., Long, T., Lu, Y., Ouyang, Q., and Tang, C. (2004). The yeast cell-cycle network is robustly designed. Proceedings of the National Academy of Sciences *101*, 4781–4786.

Liou, G.-G., Tanny, J.C., Kruger, R.G., Walz, T., and Moazed, D. (2005). Assembly of the SIR Complex and Its Regulation by O -Acetyl-ADP-Ribose, a Product of NAD-Dependent Histone Deacetylation. Cell *121*, 515–527.

Liti, G. (2015). The fascinating and secret wild life of the budding yeast S. cerevisiae. ELife *4*.

Llamosi, A. Effects of repeated osmotic stress on gene expression and growth: from cell-to-cell variability to cellular individuality in the budding yeast Saccharomyces cerevisiae. 236.

Llamosi, A., Gonzalez-Vargas, A.M., Versari, C., Cinquemani, E., Ferrari-Trecate, G., Hersen, P., and Batt, G. (2016). What Population Reveals about Individual Cell Identity: Single-Cell Parameter Estimation of Models of Gene Expression in Yeast. PLOS Computational Biology *12*, e1004706.

Lord, P.G., and Wheals, A.E. (1980). Asymmetrical Division of Saccharomyces cerevisiae. J. BACTERIOL. *142*, 11.

Louis, E.J. (2014). Introduction. In Subtelomeres, E.J. Louis, and M.M. Becker, eds. (Berlin, Heidelberg: Springer Berlin Heidelberg), pp. 1–12.

Luke, B., and Lingner, J. (2009). TERRA: telomeric repeat-containing RNA. The EMBO Journal *28*, 2503–2510.

Luo, K. (2002). Rap1-Sir4 binding independent of other Sir, yKu, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. Genes & Development *16*, 1528–1539.

Luo, J., Sun, X., Cormack, B.P., and Boeke, J.D. (2018). Karyotype engineering by chromosome fusion leads to reproductive isolation in yeast. Nature *560*, 392–396.

Maillet, L., Boscheron, C., Gotta, M., Marcand, S., Gilson, E., and Gasser, S.M. (1996). Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression. Genes & Development *10*, 1796–1811.

Marcand, S., Buck, S.W., Moretti, P., Gilson, E., and Shore', D. Silencing of genes at nontelomeric sites in yeast is controlled by sequestration of silencing factors at telomeres by Rapl protein. 14.

Marshall, W.F., Fung, J.C., and Sedat, J.W. (1997). Deconstructing the nucleus: global architecture from local interactions. Current Opinion in Genetics & Development *7*, 259–263.

Martínez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996). The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). The EMBO Journal *15*, 2227–2235.

Mazor, Y., and Kupiec, M. (2009). Developmentally regulated MAPK pathways modulate heterochromatin in Saccharomyces cerevisiae. Nucleic Acids Research *37*, 4839–4849.

McKnight, J.N., and Tsukiyama, T. (2015). The conserved HDAC Rpd3 drives transcriptional quiescence in S. cerevisiae. Genomics Data *6*, 245–248.

Miermont, A., Uhlendorf, J., McClean, M., and Hersen, P. (2011). The Dynamical Systems Properties of the HOG Signaling Cascade. Journal of Signal Transduction *2011*, 1–12.

Moazed, D., Kistler, A., Axelrod, A., Rine, J., and Johnson, A.D. (1997). Silent information regulator protein complexes in Saccharomyces cerevisiae: A SIR2/SIR4 complex and evidence for a regulatory domain in SIR4 that inhibits its interaction with SIR3. Proceedings of the National Academy of Sciences *94*, 2186–2191.

Moretti, P., Freeman, K., Coodly, L., and Shore, D. Evidence that a complex of SIR proteins interacts with the siIencer and telomere- binding protein RAP. 14.

Morris, G.J., Winters, L., Coulson, G.E., and Clarke, K.J. (1986). Effect of Osmotic Stress on the Ultrastructure and Viability of the Yeast Saccharomyces cerevisiae. Microbiology *132*, 2023–2034.

Muchardt, C., and Yaniv, M. (1999). ATP-dependent chromatin remodelling: SWI/SNF and Co. are on the job. Journal of Molecular Biology *293*, 187–198.

Muzzey, D., Gómez-Uribe, C.A., Mettetal, J.T., and van Oudenaarden, A. (2009). A Systems-Level Analysis of Perfect Adaptation in Yeast Osmoregulation. Cell *138*, 160–171.

de Nadal, E., Zapater, M., Alepuz, P.M., Sumoy, L., Mas, G., and Posas, F. (2004). The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes. Nature *427*, 370–374.

Nakayama, N., Kaziro, Y., Arai, K., and Matsumoto, K. (1988). Role of STE genes in the mating factor signaling pathway mediated by GPA1 in Saccharomyces cerevisiae. Molecular and Cellular Biology *8*, 3777–3783.

Nei, M. The new mutation theory of phenotypic evolution. 8.

Neiman, A.M. (2005). Ascospore Formation in the Yeast Saccharomyces cerevisiae. Microbiology and Molecular Biology Reviews *69*, 565–584.

Norris, D., and Osley, M.A. (1987). The two gene pairs encoding H2A and H2B play different roles in the Saccharomyces cerevisiae life cycle. Molecular and Cellular Biology *7*, 3473–3481.

O'Rourke, S.M., Herskowitz, I., and O'Shea, E.K. (2002). Yeast go the whole HOG for the hyperosmotic response. Trends in Genetics *18*, 405–412.

Palladino, F., Laroche, T., Gilson, E., Axelrod, A., Pillus, L., and Gasser, S.M. (1993). SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. Cell *75*, 543–555.

Patolsky, F., Zheng, G., Hayden, O., Lakadamyali, M., Zhuang, X., and Lieber, C.M. Electrical detection of single viruses. 6.

Pelet, S., Rudolf, F., Nadal-Ribelles, M., de Nadal, E., Posas, F., and Peter, M. (2011). Transient Activation of the HOG MAPK Pathway Regulates Bimodal Gene Expression. Science *332*, 732–735.

Peter, J., De Chiara, M., Friedrich, A., Yue, J.-X., Pflieger, D., Bergström, A., Sigwalt, A., Barre, B., Freel, K., Llored, A., et al. (2018). Genome evolution across 1,011 Saccharomyces cerevisiae isolates. Nature *556*, 339–344.

Raser, J.M. (2004). Control of Stochasticity in Eukaryotic Gene Expression. Science *304*, 1811–1814.

Rienzo, A., Poveda-Huertes, D., Aydin, S., Buchler, N.E., Pascual-Ahuir, A., and Proft, M. (2015). Different Mechanisms Confer Gradual Control and Memory at Nutrient- and Stress-Regulated Genes in Yeast. Molecular and Cellular Biology *35*, 3669–3683.

Rine', J., and Herskowitz, I. Four Genes Responsible for a Position Effect on Expression From HML and HMR in Saccharomyces cerevisiae. 14.

Roeder, R.G., and Rutter, W.J. (1970). Specific Nucleolar and Nucleoplasmic RNA Polymerases. Proceedings of the National Academy of Sciences *65*, 675–682.

Romero-Santacreu, L., Moreno, J., Perez-Ortin, J.E., and Alepuz, P. (2009). Specific and global regulation of mRNA stability during osmotic stress in Saccharomyces cerevisiae. RNA *15*, 1110–1120.

Rout, M.P. (1990). Components of the yeast spindle and spindle pole body. The Journal of Cell Biology *111*, 1913–1927.

Rout, M.P., Aitchison, J.D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B.T. (2000). The Yeast Nuclear Pore Complex: Composition, Architecture, and Transport Mechanism. The Journal of Cell Biology *148*, 635–652.

Sandell, L. (1993). Loss of a yeast telomere: Arrest, recovery, and chromosome loss. Cell *75*, 729–739.

Santisteban, M.S., Kalashnikova, T., and Smith, M.M. (2000). Histone H2A.Z Regulates Transcription and Is Partially Redundant with Nucleosome Remodeling Complexes. Cell *103*, 411–422.

Schmitt, A.P., and McEntee, K. (1996). Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences *93*, 5777–5782.

Schrick, K., and Hartwell, L.H. Mating in Saccharomyces cerevisiae: The Role of the Pheromone Signal Transduction Pathway in the Chemotropic Response to Pheromone. 14.

Schuller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C., and Ruis, H. The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the Saccharomyces cerevisiae CTT1 gene. 8.

Shao, Y., Lu, N., Wu, Z., Cai, C., Wang, S., Zhang, L.-L., Zhou, F., Xiao, S., Liu, L., Zeng, X., et al. (2018). Creating a functional single-chromosome yeast. Nature *560*, 331–335.

Sinclair, D.A., and Guarente, L. (1997). Extrachromosomal rDNA Circles— A Cause of Aging in Yeast. Cell *91*, 1033–1042.

Snoek, T., Voordeckers, K., and Verstrepen, K.J. (2014). Subtelomeric Regions Promote Evolutionary Innovation of Gene Families in Yeast. In Subtelomeres, E.J. Louis, and M.M. Becker, eds. (Berlin, Heidelberg: Springer Berlin Heidelberg), pp. 39–70.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modi®cations. *403*, 5.

Struhl, K., Stinchcomb, D.T., Scherer, S., and Davis, R.W. (1979). High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proceedings of the National Academy of Sciences *76*, 1035–1039.

SvarenS, J., Klebanow, E., and Sealya, L. Analysis of the Competition between Nucleosome Formation and Transcription Factor Binding. 10.

Święciło, A. (2016). Cross-stress resistance in Saccharomyces cerevisiae yeast—new insight into an old phenomenon. Cell Stress and Chaperones *21*, 187–200.

Taddei, A., Van Houwe, G., Nagai, S., Erb, I., van Nimwegen, E., and Gasser, S.M. (2009). The functional importance of telomere clustering: Global changes in gene expression result from SIR factor dispersion. Genome Research *19*, 611–625.

Tanny, J.C., Kirkpatrick, D.S., Gerber, S.A., Gygi, S.P., and Moazed, D. (2004). Budding Yeast Silencing Complexes and Regulation of Sir2 Activity by Protein-Protein Interactions. Molecular and Cellular Biology *24*, 6931–6946.

Tsukiyama, T., Palmer, J., Landel, C.C., Shiloach, J., and Wu, C. (1999). Characterization of the Imitation Switch subfamily of ATP-dependent chromatin-remodeling factors in Saccharomyces cerevisiae. Genes & Development *13*, 686–697.

Uhlendorf, J., Miermont, A., Delaveau, T., Charvin, G., Fages, F., Bottani, S., Batt, G., and Hersen, P. (2012). Long-term model predictive control of gene expression at the population and single-cell levels. Proceedings of the National Academy of Sciences *109*, 14271–14276.

Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A., et al. (2001). The Sequence of the Human Genome. THE HUMAN GENOME *291*, 51.

Vogelauer, M., Wu, J., Suka, N., and Grunstein, M. (2000). Global histone acetylation and deacetylation in yeast. Nature *408*, 495–498.

Wolf, D.M., Fontaine-Bodin, L., Bischofs, I., Price, G., Keasling, J., and Arkin, A.P. (2008). Memory in Microbes: Quantifying History-Dependent Behavior in a Bacterium. PLoS ONE *3*, e1700.

Wong, H., Marie-Nelly, H., Herbert, S., Carrivain, P., Blanc, H., Koszul, R., Fabre, E., and Zimmer, C. (2012). A Predictive Computational Model of the Dynamic 3D Interphase Yeast Nucleus. Current Biology *22*, 1881–1890.

Wright, J.H., Gottschling, D.E., and Zakian, V.A. (1992). Saccharomyces telomeres assume a non-nucleosomal chromatin structure. Genes & Development *6*, 197–210.

Wu, A.R., Neff, N.F., Kalisky, T., Dalerba, P., Treutlein, B., Rothenberg, M.E., Mburu, F.M., Mantalas, G.L., Sim, S., Clarke, M.F., et al. (2014). Quantitative assessment of single-cell RNA-sequencing methods. Nature Methods *11*, 41–46.

Zacharioudakis, I., and Tzamarias, D. (2017). Bimodal expression of yeast GAL genes is controlled by a long non-coding RNA and a bifunctional galactokinase. Biochemical and Biophysical Research Communications *486*, 63–69.

Zacharioudakis, I., Gligoris, T., and Tzamarias, D. (2007). A Yeast Catabolic Enzyme Controls Transcriptional Memory. Current Biology *17*, 2041–2046.

Zi, Z., Liebermeister, W., and Klipp, E. (2010). A Quantitative Study of the Hog1 MAPK Response to Fluctuating Osmotic Stress in Saccharomyces cerevisiae. PLoS ONE *5*, e9522.

Appendix

A-Protocol to make epoxy wafer from a microfluidic device

What you need

-PDMS and curing agent
-microfluidic device
-Cutter, scalpel and ideally a Dremel
-epoxy resin and associated hardener

In this protocol, the resin used is the epoxy resin R123 and hardener R614. (reference in here http://www.soloplast-vosschemie.fr/resine-r123.htm)

The protocol is fairly easy, but needs an overnight step, so consider two days to make an epoxy wafer.

Protocol

Step 1:

Pour some PDMS mixed with curing agent on a petri dish. Put the microfluidic chip you want to replicate on the petri dish. **Make sure the patterns of your device are on top!!** (figure 1). Bake the whole at 65°C so that the PDMS will get solid. Be very careful though, the device can move in the petri dish so you might want to hold it the center of the petri dish by putting some tape on it and the edges of the tape are stuck to the edges of the petri dish.

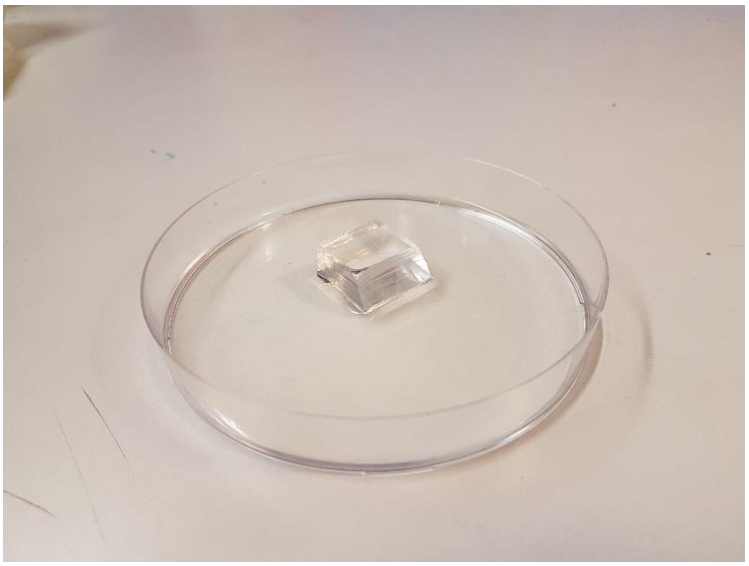


Figure 7. Microfluidic device on solid PDMS in a petri dish. The patterns are not in contact with the bottom of the petri dish.

Step 2:

Pour some epoxy and hardener in a 50mL falcon tube.
With the epoxy R123, you will need 100g of epoxy and 45g of hardener.
Although it is written on the protocol found on the epoxy bottle that you can work in terms of volumes (100 parts of epoxy and 49 parts of hardener). We have tried this method several times and it does not work.
For a standard petri dish, you need about 70-80g of epoxy.

Close the falcon tube and shake in order to mix everything. The whole should be very bubbly (figure 2 left), so you need to get rid of the bubbles, otherwise you will find them on your new wafer where your patterns are. It is impossible to get rid of the bubbles by putting the opened tube in a vacuum bell, as the epoxy is very viscous.

To get rid of the bubbles, centrifuge your falcon tube at 2000rpm for 10min (figure 2 right).

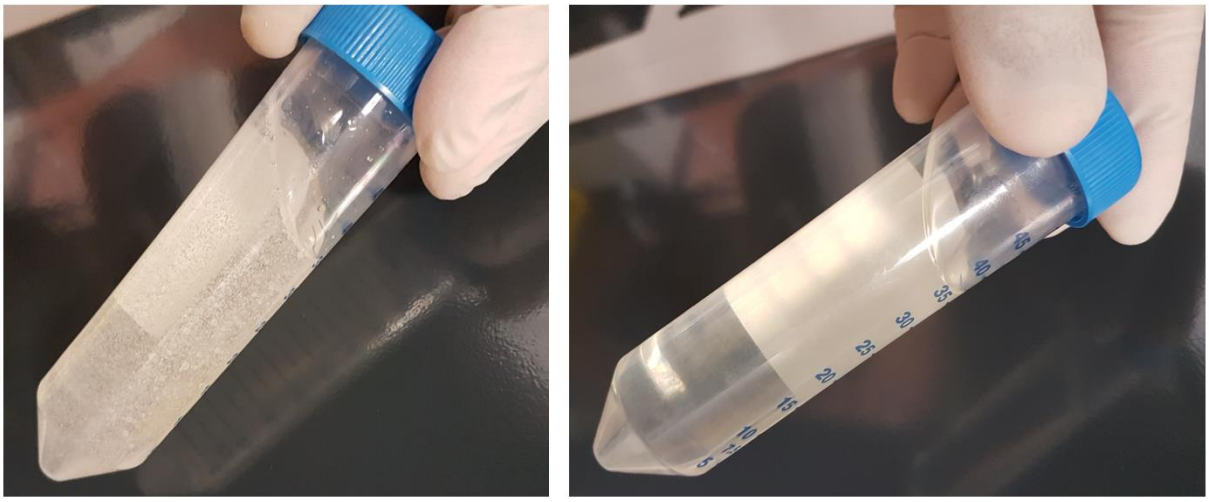


Figure 2: (left) before centrifugation (right) after centrifugation

Step 3:

Pour the epoxy on your wafer. Do it **slowly** and be careful as bubbles might form so try to get rid of them if they do, or you can leave them be if you are absolutely sure that the bubbles are not on the patterns of your microfluidic device. If you see bubbles, remove them using a pipette.
Leave the whole (ideally under a hood) overnight in order for the resist to solidify.

Another way to do that: after you pour the epoxy, put the petri dish at 30°C for 6h and check that there are no bubbles. If there are some, remove them with a pipette. Check every 30min that no more bubbles form. After 6h at 30°C, leave the petri dish at room temperature overnight.

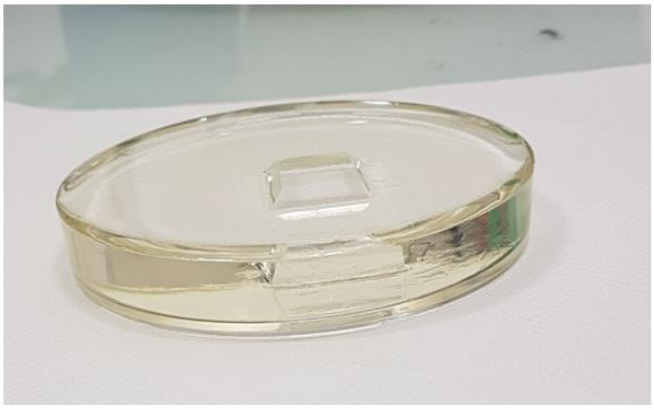
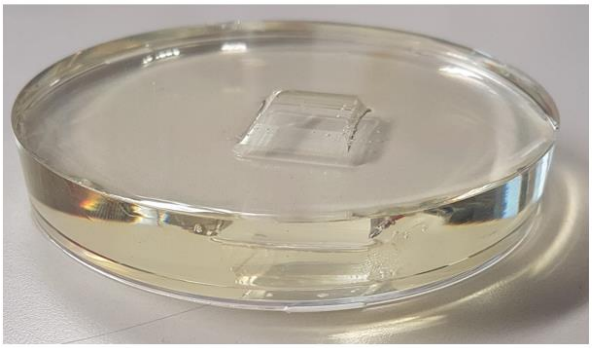


Figure 3. (left) pouring the epoxy slowly in the petri dish. (right) Make sure that you put enough resin to entirely cover the microfluidic device.

Step 4

Once the wafer is solid (figure 4 left), **flip the petri dish over** (so now the patterns of your device are facing **down**) and draw an area (figure 4 right).



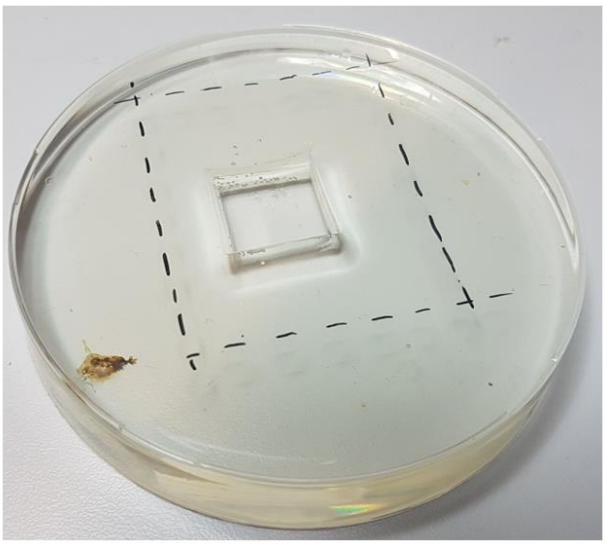


Figure 4. (left) solid mold. The chip is entirely covered in epoxy. (right) area drawn on the bottom of the petri dish that will serve to be cut.

You need to remove the petri dish part on the drawn area. Use a scalpel, a cutter or a dremel to do so (figure 5 left). This latter is, by experience, the safest and more efficient method. Once this is done, remove the cut petri dish (figure 5 center) and use a scalpel to cut the microfluidic device from the new epoxy wafer (figure 5 right). You can then pour PDMS in the wafer and cut chips.

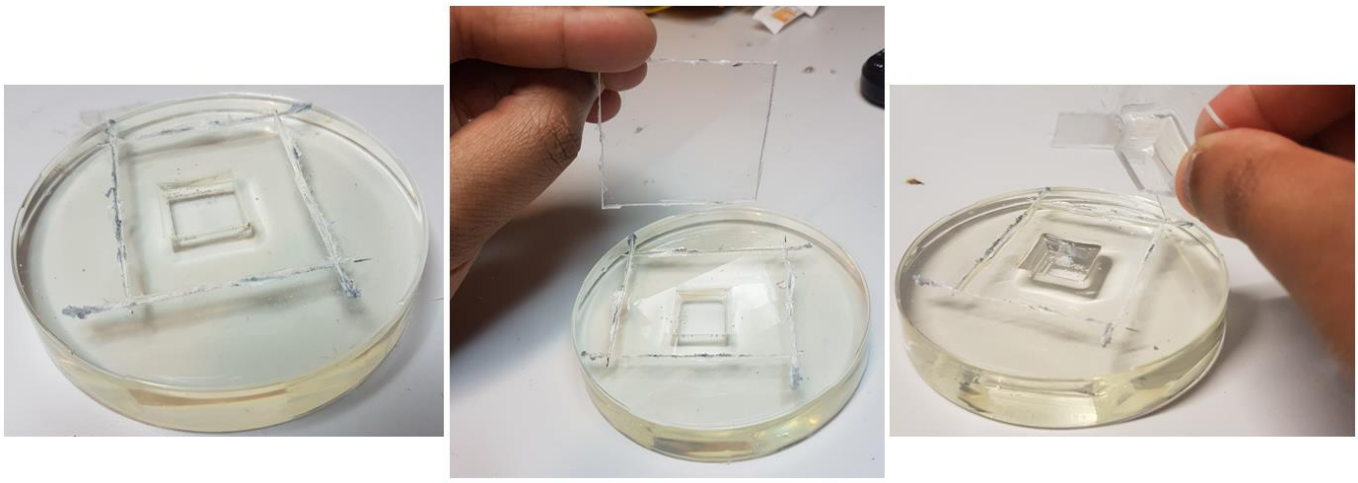


Figure 5. Final epoxy wafer.

B-Yeast transformation protocol

To do on the eve : launch a culture with the strain of interest in 5mL of YPD.

What you need :

Mother solutions:

- Solution TE 10x :		for 500ml
Tris HCl (pH 7.5)	100mM	50ml 1M
EDTA	10mM	10ml 0.5M

- Solution LiAc 10x : sterile

Acétate de lithium pH7.5	1M

- Solution PEG 3350

PEG 3350 (p/v)	50%	5g for 10ml
PEG 4000 (p/v)	50%	4.8g for 10ml

Final solutions:

- solution TE/LiAc : pour 100ml	- solution TE/LiAc/PEG :
10ml TE 10x	0,1ml TE 10x
10ml LiAc 10x	0,1ml LiAc 10x
Qsp 100ml eau	0,8ml PEG 3350

- DNA carrier 5mg/ml (3min à 95°C and sonication)

1. Diltion of the culture made on the eve at 1/50 in 50ml of YPD than put at 30°C in shaker untill the cell density reaches 1 to 3.10^7 cellules/ml (it usually takes 3h-4h).

2. Transfer the culture in a 50mL falcon and centrifuge for 5min at 5000rpm.

3. Remove the surpernatent and resuspend in 20ml of TE/LiAc solution. Centrifuge for 5min at 5000rpm.

4. Remove the supernatent and resuspend with TE/LiAc untill you reach 2.10^9 cellules/ml.

5. Incubate 15min at 30°C without shaking.

6. In an 1.5mL eppendorf, put :

	Negative control	Positive control	Transformation of interest
TE/LiAc/PEG	300µl	300µl	300µl
DNA carrier	50 µg	50 µg	50 µg
DNA solution to be transformed	/	500ng – 1µg	1 – 5 µg
	Vortex		
Competent cells	50µl	50µl	50µl
	Mix with the pipet		

7. Incubate 30min at 30°C without shaking.

8. Perform a heat shock at 42°C for 20min.

9. Centrifuge the tubes 5min at 5000rpm. Remove the supernatent.

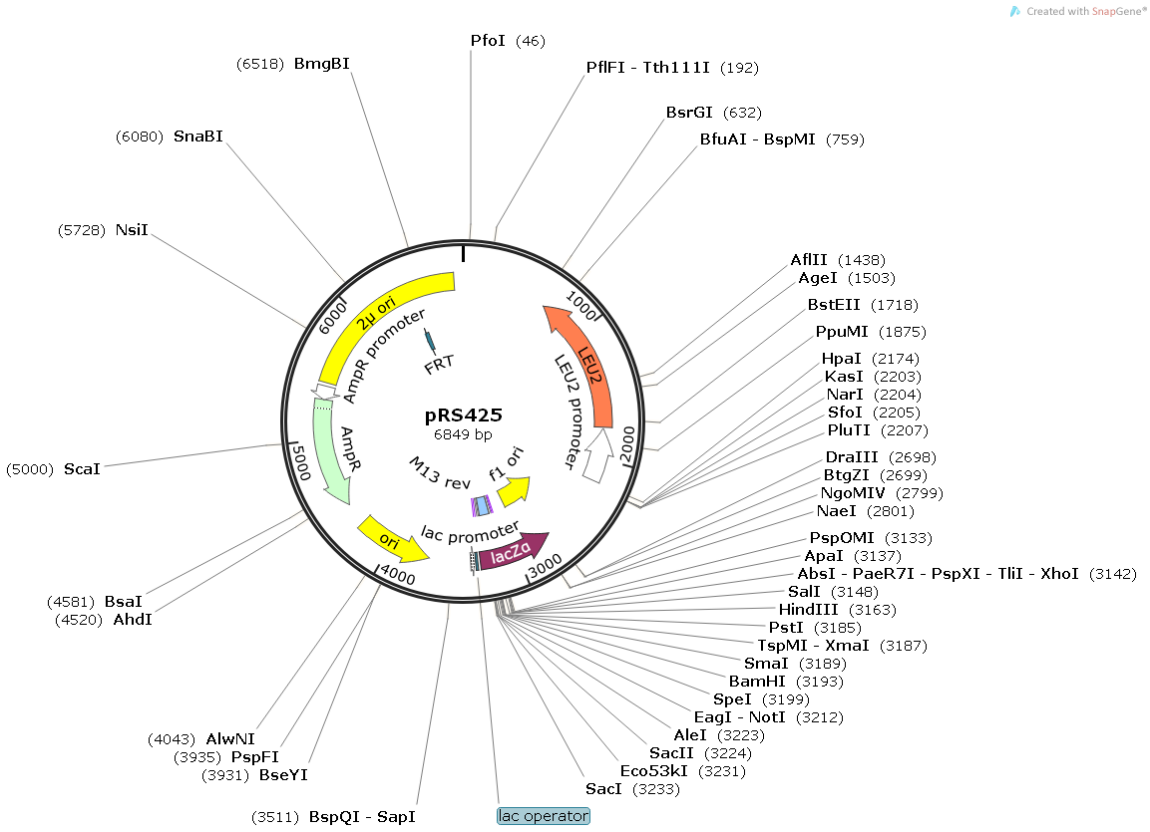
10. Resuspend in CaCl2 0.5M for 15min. Centrifuge the tubes 5min at 5000rpm. Remove the supernatent and resuspend in YPD.

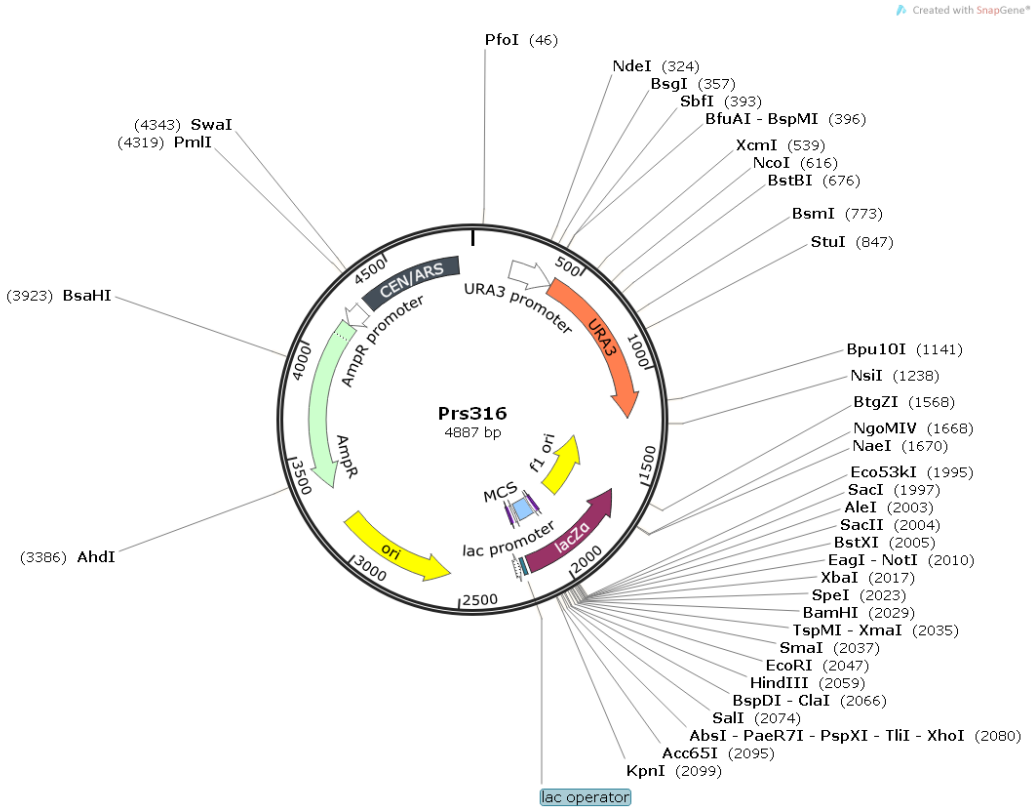
11. Plate the yeast on plates with the right auxotrophy markers for 48-72h.

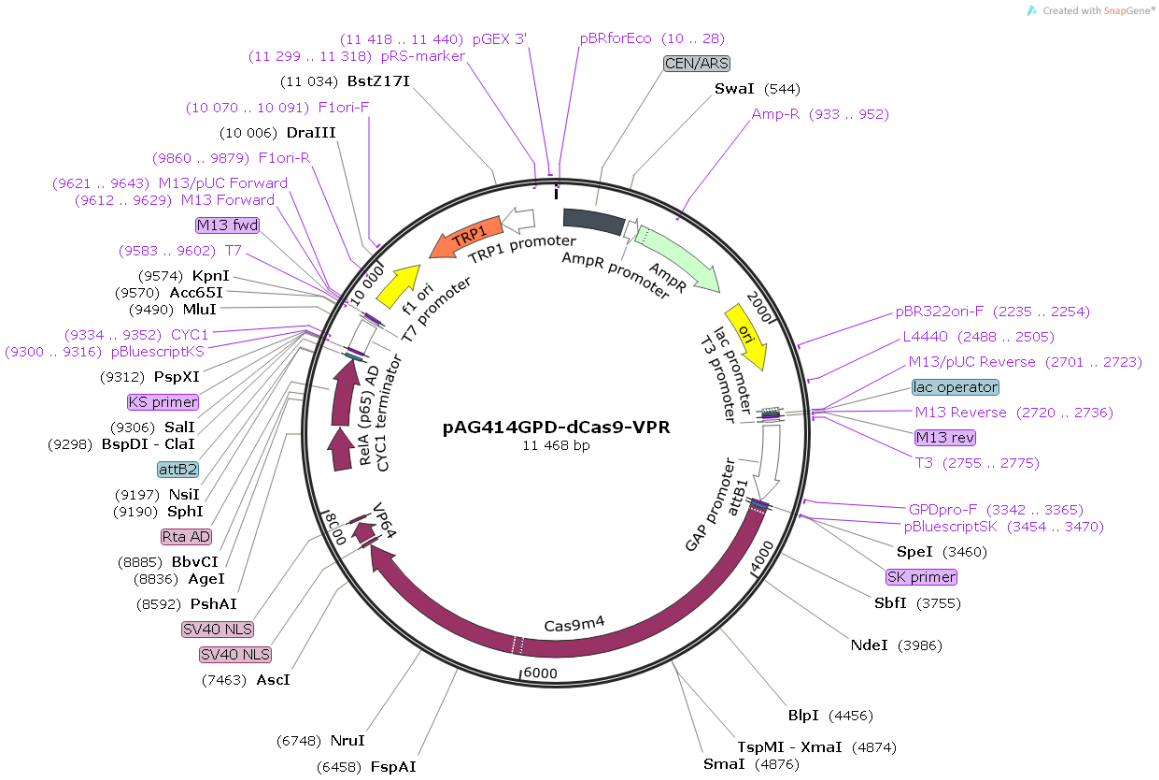
C- List of primers

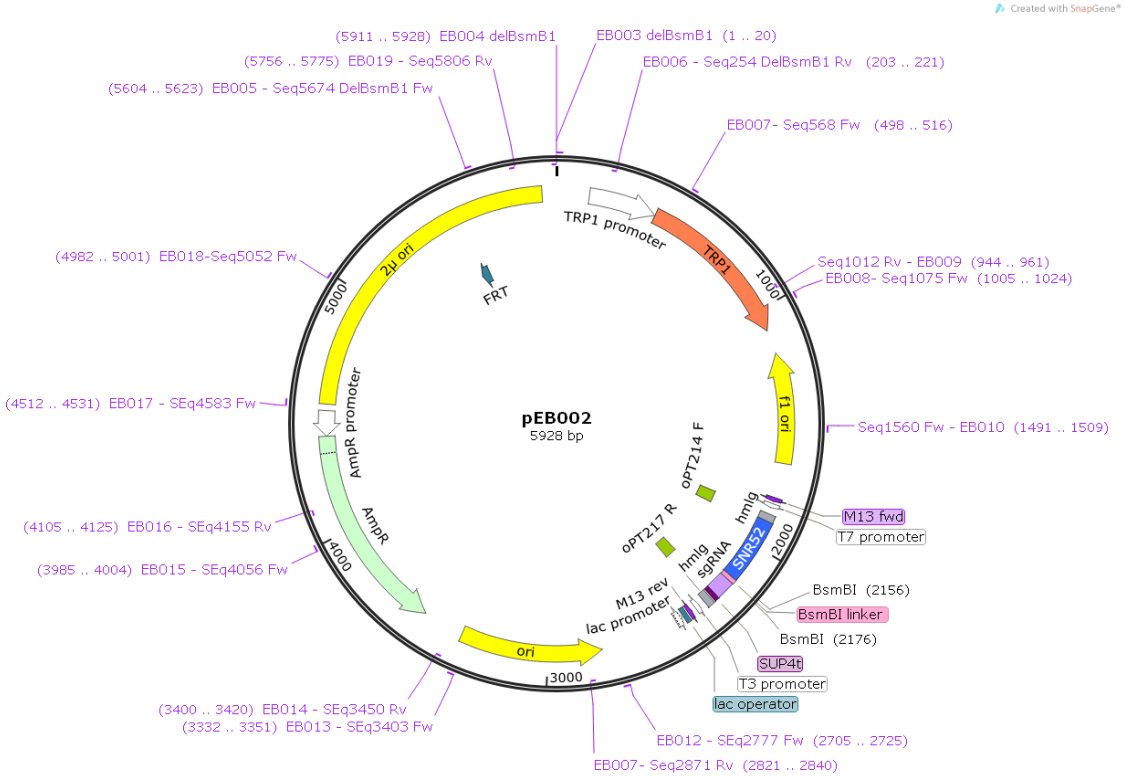
Sequence	Function
TGCAGGCAAGTGCACAAACAATACTTAAATAAATACTAC TCAGTAATAACATTATTGGTGCGGCAAGG	Reverse primer to move pSTL1-yECITRINE-HIS6 at the pericentromere domain of chromosome IV
TATTGAGCACGTGAGTATACGTGATTAAGCACACAAAGG CAGCTTGGAGTCAATGATTCTGAAATACTCCTTTTACA	Forward primer to move pSTL1-YECITRINE-HIS5 at the pericentromere domain of chromosome IV
CGCCAGATGGCAGTAGTGGAAG	Forward primer to amplify pSTL1-yECITRINE at the pericentromere
GCCTGCAGGCAAGTGCAC	Forward primer to amplify pSTL1-yECITRINE at the pericentromere
CCGATTAAGAATTCGGTCG	Forward sequencing primer 1 (for pSTL1-yECITRINE) at the pericentromere.
CATTGCCAAGGCTAGGAG	Forward sequencing primer 2 (for pSTL1-yECITRINE) at the pericentromere.
GGATCTGCACTTTCTCAG	Reverse sequencing primer 1 (for pSTL1-yECITRINE) at the pericentromere.
catcaccttcaccttcacc	Reverse sequencing primer 2 (for pSTL1-yECITRINE) at the pericentromere.
ATACAGGAGCAGGGAGAATTACGGGAAATGGGAAAGA AAAACTATTCTTCttaagcaaggattttcttaacttcttc	Htz1 deletion forward (via pRS405)
CGTTAAATTCAATTTCGCACTATAGCCGCACGTAAAAATA ACTTAACATAaactgtgggaatactcaggtat	Htz1 deletion reverse (via pRS405)
TGTTTGTCTACGTATAAACGAATAAGTACTTATATTGCTTT AGGAAGGTAttaagcaaggattttcttaacttcttc	Snf2 deletion forward (via 405)
TCTAATCGCGACTTTCTGCTATTTTCACGACTTTCGATTAA TTATCTGCCaactgtgggaatactcaggtat	Snf2 deletion reverse (via pRS405)
CATGTGTACATAGGCATATCTATGGCGGAAGTGAAAATG AATGTTGGTGGttaagcaaggattttcttaacttcttc	Sir3 deletion forward (via pRS405)
TTACAGGGGTTTAAGAAAGTTGTTTTGTTCTAACAATTGG ATTAGCTAAAaactgtgggaatactcaggtat	Sir3 deletion reverse (via pRS405)
TATTGAGCACGTGAGTATACGTGATTAAGCACACAAAGG CAGCTTGGAGTactatgcggcatcagagcag	Trp1 deletion forward (via pRS316)
gatcGAAAGTGCAGATCCCGGTAA	Crispr guide forward
aaacTTACCGGGATCTGCACTTTC	Crispr guide reverse

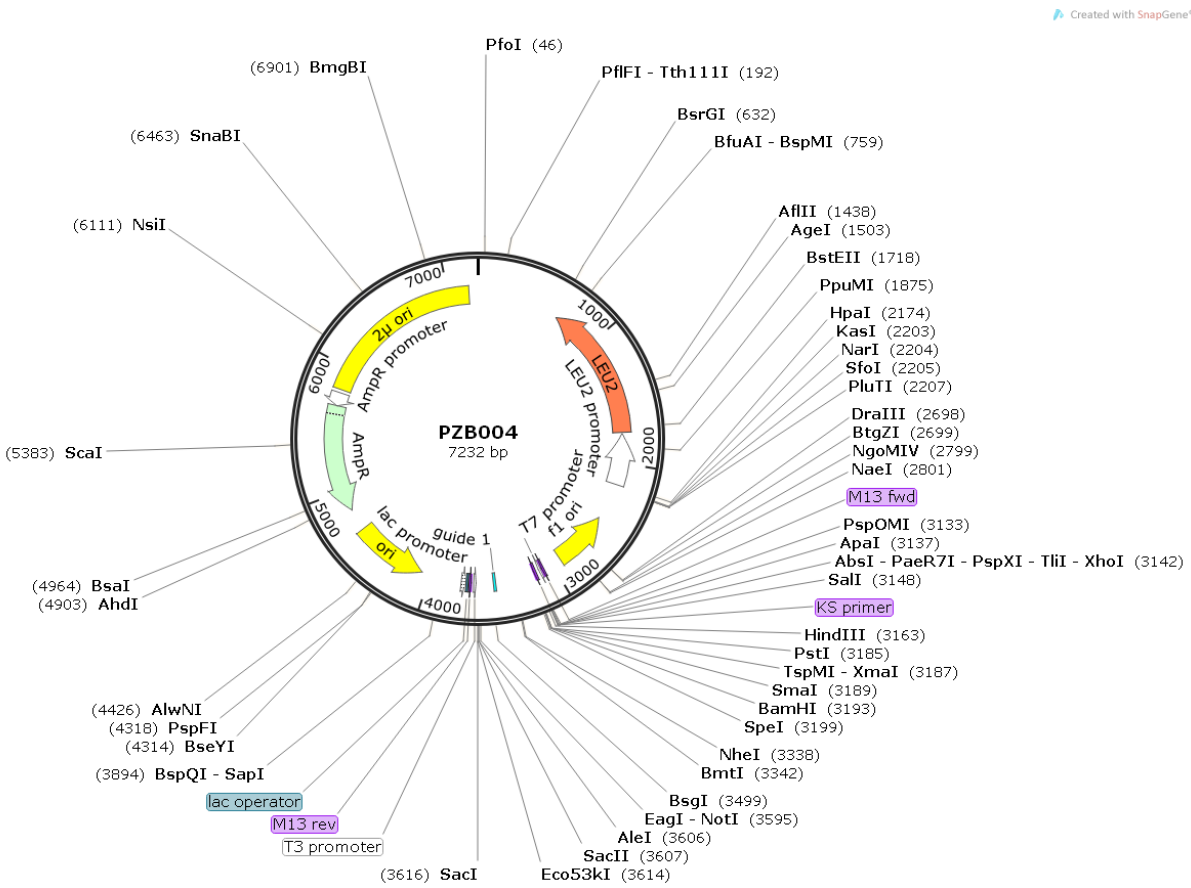
D- plasmid maps






Created with SnapGene®
(11 418 .. 11 440) pGEX 3'
(11 299 .. 11 318) pRS-marker
(11 034) BstZ17I
(10 070 .. 10 091) F1ori-F
(10 006) DraIII
(9860 .. 9879) F1ori-R
(9621 .. 9643) M13/pUC Forward
(9612 .. 9629) M13 Forward
M13 fwd
(9583 .. 9602) T7
(9574) KpnI
(9570) Acc65I
(9490) MluI
(9334 .. 9352) CYC1
(9300 .. 9316) pBluescriptKS
(9312) PspXI
KS primer
(9306) SalI
(9298) BspDI - ClaI
attB2
(9197) NsiI
(9190) SphI
Rta AD
(8885) BbvCI
(8836) AgeI
(8592) PshAI
SV40 NLS
SV40 NLS
(7463) AscI
(6748) NruI
(6458) FspAI
pBRforEco (10 .. 28)
CEN/ARS
SwaI (544)
Amp-R (933 .. 952)
pBR322ori-F (2235 .. 2254)
L4440 (2488 .. 2505)
M13/pUC Reverse (2701 .. 2723)
lac operator
M13 Reverse (2720 .. 2736)
M13 rev
T3 (2755 .. 2775)
GPDpro-F (3342 .. 3365)
pBluescriptSK (3454 .. 3470)
SpeI (3460)
SK primer
SbfI (3755)
NdeI (3986)
BlpI (4456)
TspMI - XmaI (4874)
SmaI (4876)
TRP1
TRP1 promoter
AmpR promoter
AmpR
ori
lac promoter
T3 promoter
GAP promoter
attB1
Cas9m4
Vp64
RelA (p65) AD
CYC1 terminator
f1 ori
T7 promoter
10 000
2000
4000
6000
8000
pAG414GPD-dCas9-VPR
11 468 bp


Created with SnapGene®
(5911 .. 5928) EB004 delBsmB1
EB003 delBsmB1 (1 .. 20)
(5756 .. 5775) EB019 - Seq5806 Rv
EB006 - Seq254 DelBsmB1 Rv (203 .. 221)
(5604 .. 5623) EB005 - Seq5674 DelBsmB1 Fw
EB007- Seq568 Fw (498 .. 516)
(4982 .. 5001) EB018-Seq5052 Fw
Seq1012 Rv - EB009 (944 .. 961)
EB008- Seq1075 Fw (1005 .. 1024)
(4512 .. 4531) EB017 - SEq4583 Fw
Seq1560 Fw - EB010 (1491 .. 1509)
(4105 .. 4125) EB016 - SEq4155 Rv
(3985 .. 4004) EB015 - SEq4056 Fw
M13 fwd
T7 promoter
BsmBI (2156)
BsmBI linker
BsmBI (2176)
SUP4t
T3 promoter
lac operator
(3400 .. 3420) EB014 - SEq3450 Rv
(3332 .. 3351) EB013 - SEq3403 Fw
EB012 - SEq2777 Fw (2705 .. 2725)
EB007- Seq2871 Rv (2821 .. 2840)
TRP1 promoter
TRP1
2µ ori
FRT
AmpR promoter
AmpR
ori
M13 rev
lac promoter
oPT214 F
oPT217 R
hmlg
sgRNA
SNR52
f1 ori
5000
1000
4000
3000
2000
pEB002
5928 bp


Created with SnapGene®
PZB004
7232 bp
7000
1000
2000
3000
4000
5000
6000
2μ ori
AmpR promoter
AmpR
LEU2
LEU2 promoter
T7 promoter
f1 ori
guide 1
lac promoter
ori
PfoI (46)
PflFI - Tth111I (192)
BsrGI (632)
BfuAI - BspMI (759)
AflII (1438)
AgeI (1503)
BstEII (1718)
PpuMI (1875)
HpaI (2174)
KasI (2203)
NarI (2204)
SfoI (2205)
PluTI (2207)
DraIII (2698)
BtgZI (2699)
NgoMIV (2799)
NaeI (2801)
M13 fwd
PspOMI (3133)
ApaI (3137)
AbsI - PaeR7I - PspXI - TliI - XhoI (3142)
SalI (3148)
KS primer
HindIII (3163)
PstI (3185)
TspMI - XmaI (3187)
SmaI (3189)
BamHI (3193)
SpeI (3199)
NheI (3338)
BmtI (3342)
BsgI (3499)
EagI - NotI (3595)
AleI (3606)
SacII (3607)
Eco53kI (3614)
(3616) SacI
T3 promoter
M13 rev
lac operator
(3894) BspQI - SapI
(4314) BseYI
(4318) PspFI
(4426) AlwNI
(4903) AhdI
(4964) BsaI
(5383) ScaI
(6111) NsiI
(6463) SnaBI
(6901) BmgBI

E- Article

Identification of individual cells from *z*-stacks of bright-field microscopy images.

Jean-Baptiste Lugagne[1,2,*], Srajan Jain[1], Pierre Ivanovitch[1], Zacchary Ben Meriem[1,3], Clément Vulin[4], Chiara Fracassi[1,2], Gregory Batt[2,5] and Pascal Hersen[1,*]

1 Laboratoire Matière et Systèmes Complexes, UMR 7057 CNRS & Université Paris Diderot, 10 rue Alice Domon et Léonie Duquet, 75013 Paris, France.
2 Inria Saclay – Ile-de-France and Université Paris Saclay, 1 rue Honoré d'Estienne d'Orves, Bâtiment Alan Turing, Campus de l'Ecole Polytechnique, 91120, Palaiseau, France
3 Laboratoire Biologie et Dynamique des Chromosomes, CNRS UMR 7212, Hôpital Saint-Louis, Paris, France.
4 ETH, Swiss Federal Institute of Technology, Zurich, Switzerland.
5 Institut Pasteur, 25-28 Rue du Docteur Roux, 75015, Paris, France.

*Correspondence and requests for materials should be addressed to Jean Baptiste Lugagne (*jean-baptiste.lugagne@inria.fr*) and Pascal Hersen (*pascal.hersen@univ-paris-diderot.fr*).

Abstract

Obtaining single cell data from time-lapse microscopy images is critical for quantitative biology, but bottlenecks in cell identification and segmentation must be overcome. We propose a novel, versatile method that uses machine learning (SVM, Random Forest, Neural Network) to identify cell morphologies from *z*-stack bright-field microscopy images. We show that axial information is enough to successfully classify the pixels of an image, without the need to add image transformation to outline morphological features. This fast, robust method can be used to identify different cell morphologies, including the features of *E. coli*, *S. cerevisiae* and epithelial cells, even in mixed cultures. Our method demonstrates the potential of acquiring and processing Z-stacks for single-layer, single-cell imaging and segmentation

Main Text

Thanks to the development of microfluidics and microscopy, it is now possible to measure the dynamics of single cells over time[1,2]. In recent years, longitudinal time-lapse studies have emerged as key methods in quantitative biology and are essential to understand the dynamics of cellular processes[2–4]. However, a robust and efficient cell segmentation method is required to obtain high quality single cell traces[1,2]. Despite years of development, a universal method to segment cells from microscopy images has not yet been established. The numerous existing methods were designed for specific cell types and usually rely on specific morphological features (*e.g.*, size, shape, fluorescent labeling). Although efficient for specific problems, these methods are not versatile, and usually fail when applied to different cell types or other experimental conditions. As a result, research groups design and tweak image analysis software[5–11] to match their specific segmentation problem. This is a considerable waste of time and energy, and highlights the need for a simple, versatile strategy to segment cells, irrespective of experimental design or cellular characteristics.
Notably, segmentation critically depends on obtaining high-quality images with a constant focus that outlines the borders and main morphological features of the cell. This is an important constraint, which – in practice – requires periodic auto-focusing or a control system to automatically maintain perfect focus. Here, we propose a different segmentation strategy inspired by hyperspectral imaging. Instead of relying on the in-focus image, we systematically acquired multiple stacks of images around the focal plane (*i.e.* a *z*-stack) of various cells, and use the information contained within the full *z*-stack to identify the focal region of the cells in the images. The central idea is that cell contours, the cellular interior or any objects within the field of view do not have the exact same intensity profile throughout the *z*-dimension. Here, we show it is possible to train an algorithm using machine learning to classify *z*-pixels (the vector of light intensity along the *z*-axis for a specific pixel in the image) based on their focal signature. The method is simple, robust, can be run in real-time and – importantly – gives excellent results for *E. coli* (rod-shaped), *S. cerevisiae* (round), mammalian epithelial (HeLa) cells, and even a mixture of bacteria and yeast cells.
We used a Piezzo drive (PIFOC, PI) to acquire *z*-stacks containing 100 images, 100 nm apart, of *E. coli* using a 100x oil objective (UPlanFL 1.3NA) and CoolSNAP HQ2 camera with a resolution of 1040 × 1392 pixels (Figure 1A-B). *E. coli* cells were loaded into a microfluidic device where they were cultured in narrow chambers (Figure 1C). A graphical user interface (GUI, Supplementary

Text) was developed to manually label a training dataset by defining regions of interest (classes), such as the interior of the cell and its contours, then a machine learning classifier was trained on this dataset (see Supplementary Text). Importantly, the algorithm was not trained using the morphological features in the (x-y) plane, but only a representative set of z-pixels (Figure 1B) for each class of objects that the user wants to identify and segment in the image. Indeed, many such z-pixels can be found in a z-stack of a monolayer since it usually contains tens to a few hundred *E. coli* and any 10 × 10 pixels area (representing the typical surface of a cell) contains as many as 100 different profiles. Principal component analysis was used to reduce the dimensionality of the problem from 100 dimensions (if all z-positions in the stack are considered) to a lower dimension space (typically between 5 and 20 dimensions, see Supplementary Text), that enabled separation of the different user-defined classes (Figure 1D-E). In practical terms, we first used Matlab and its Support Vector Machine library (*fitcsvm* package), to perform training and class identification (see Supplementary Figure S1). The classification of a z-pixel does not depend on the classification of other z-pixels, so the parallelization of the prediction process is straightforward.

After training, we acquired another z-stack and used the SVM to classify the pixels. As shown in Figure 2, the different parts of an image of *E. coli* cells were correctly identified with neither post-processing nor user intervention (see also Supplementary Figure S2). The cells were detected, and it was even possible to locate and classify the cell contour, cell interior and microfluidic chamber with excellent fidelity. Therefore, this method is markedly more powerful than classic segmentation, since it enables identification of more than one type of structure, without any *a priori* knowledge of their morphological features in the focal plane. Moreover, Figure 2C shows how automatic labeling of a pixel is associated with a confidence score that can be used to assess the quality of the classification and further refine cell segmentation (see Supplementary Text, Supplementary Figures S2-S6). Although it's often interesting to define several classes to identify important objects on the image, classification of cells only requires two classes: "cell" and "not cell" (see supplementary text). The number of images can also be decreased while achieving good performance; the method gave excellent identification scores (classification error less than 1%) if the z-stack contained at least seven images (Figure 2D, Supplementary Figure S3).

On our computer system (20-cores Xeon, DELL), training the SVM on a typical dataset took from 15 min to 1 hour at most, and a little longer than 1 minute to

attribute the pixels to their classes in a 1392 × 1040 × 100 *z*-stack. This is relatively slow, as expected for SVM on such data. Therefore, we also implemented two other classifiers, namely, Random Forest and Neural Network (See Supplementary Text, supplementary Figure S7) and used them within the same framework to classify each pixels. We obtained comparable classification accuracy than SVM, but at much faster speed. Specifically, Random Forest classification was typically 20 times faster than SVM classification. With a single core processor, a full image classification was obtained in typically a minute with a Random Forest classifier. It is thus possible to perform identification in real-time, at least with respect to the typical timescale of single cell microscopy imaging (*e.g.* several minutes between two frames when observing gene expression by fluorescence microscopy). Of course, the processing time could be also drastically decreased by relying on graphic processor unit (GPU) accelerated libraries.

Importantly, this method could be applied to different experimental designs. First, we confirmed our method could efficiently identify single bacteria in a dense monolayer of *E. coli* (instead of a few lines of cells) grown between a glass slide and an agar pad (Figure 3A). We then showed that the method also worked well to identify yeast cells, even though budding yeast cells are larger than *E. coli* cells (~5 μm vs. ~1 μm) and are round (Figure 3B, Supplementary Figure S4). We also successfully segmented a mixture of yeast cells and bacteria (Figure 3D, Supplementary Figure S5); the system could distinguish between the two types of cells based on their focal signature. This is a particularly hard task for any standard segmentation algorithm based on morphological features alone, indicating this method could represent an important tool for research on infectious diseases or microbial ecology. Additionally, we succeeded in identifying individual epithelial HeLa cells in a confluent monolayer (Figure 3C, Supplementary Figure S6). Mammalian cell segmentation is a particularly hard problem, and although deep learning methods have already demonstrated their potential to segment mammalian cells with complex shapes, it came at the expense of large dataset and long computing time[12]. In our case, a simple machine learning algorithm and limited training datasets were sufficient to enable rapid identification of cells with various shapes in a timescale that allow cell classification to be done on the fly. Importantly, our method showed better classification results (see Supplementary Figure S8) than the Ilastik classifier, a reference image analysis software, which does not use axial information, but encode information from neighboring pixels. This suggests that axial information contained in Z-stack contains enough information to be used

directly for pixel classification (see Supplementary Text). After classification, the cellular regions are already identified and the cellular segmentation becomes easier. The use of even simple segmentation methods downstream of the classification step provided good results (see Supplementary Text). We anticipate that advanced segmentation algorithms, such as active contour methods, could further improve the fidelity of cell segmentation and tracking.

The method presented in this manuscript does not require a complex imaging setup, since only a few images (~10) in a *z*-stack are required for robust identification of cells. We also developed a GUI to facilitate drawing of the regions of interest in the training dataset. Importantly, our method can identify bacteria, yeast, mixture of yeast and bacteria, and mammalian cells (Figure 3). Our method is versatile, can be integrated into any image analysis pipeline[13] and can thus tremendously facilitate segmentation for several cell types and morphologies.

Methods

Cell culture and imaging. Cells were cultured and imaged following standard protocols (E. coli were grown in LB at 37°C, yeast in SC at 30°C and HeLa cells in MDEM at 37°C and 5% CO_2). Unless noted otherwise, *z*-stacks were acquired using an IX71 Olympus equipped with a piezo (PIFOC, PI). This allows precision positioning of the objective at a resolution in the tens of nanometers.

Z-pixel classification.
A graphical user interface (GUI, see supplementary text) was used to simplify training set construction. Images were normalized by performing a standard histogram equalization procedure with 1% loss on the histograms. We used principal component analysis (PCA) to reduce dimensionality of the problem. Only a subset of the main principal component ($N < 20$) dimensions were used to represent the data used to train the classifiers and later generate predictions. For SVMs, we used a method known as winner-takes-all SVM (WTA-SVM), which has the double advantage of providing a classification score for each class and does not require the experimenter to establish a classification tree. The fit implementation of the SVMs was based on the Matlab *fitcsvm* package, which features automatic hyper-parameter optimization, with a Gaussian radial basis function as a kernel and a hinge loss function. We built a manually labeled set and divided it into two parts by random data subsampling: the first part, consisting of 90% of all data was used to train the SVMs. The remaining 10%

was used as an evaluation set. Once a satisfactory SVM set was obtained for a particular classification problem, it was used to process new stacks (captured under similar conditions) for cell identification. Stacks to be analyzed were first scaled to the same dynamic range as the training stacks (the histogram of the entire stack is equalized over the maximum range of the training data type). Then transformed into the principal components base. The best SVM set was then applied to the transformed data, and – for each *z*-pixel – a set of classification scores that correspond to each of the classes the SVMs were trained for was computed. Good to very good segmentations were obtained from the classification maps, has shown in supplementary materials. Classification and segmentation methods are described in details in supplementary materials. We also implemented classification with a Random Forest classifier and a neural network. Both methods show similar accuracy than SVMs classifier but, as expected, are much faster for the classification step and thus should be considered as the methods of choice.

Data Availability. The Matlab code and datasets generated for this study are available on our GitHub repository (https://github.com/Lab513/Zcells) to facilitate its diffusion to the scientific community.

Acknowledgments

We thank Peter Swain, Julian Pietsch, our team members and an anonymous referee for their suggestions and critical reading of this manuscript. This work was supported by the *Agence Nationale de la Recherche* (ANR-10-BINF-0006; ANR-16-CE12-0025; ANR-16-CE33-0018) and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No 724813).

Author Contributions

JBL and PH designed the research plan; JBL, ZBM, CF, CV performed experiments; JBL, SJ and PI wrote the software; JBL, GB and PH wrote the article. The authors do not have any competing financial interest.

Supplementary Information

Supplementary information contains an extended description of the Materials and Methods, several examples on how to use classification to get a proper segmentation and six supplementary figures.

References

1. Skylaki, S., Hilsenbeck, O. & Schroeder, T. Challenges in long-term imaging and quantification of single-cell dynamics. *Nat. Biotechnol.* **34,** 1137–1144 (2016).

2. Young, J. W. *et al.* Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. *Nat. Protoc.* **7,** 80–88 (2011).
3. Meijering, E., Carpenter, A. E., Peng, H., Hamprecht, F. A. & Olivo-Marin, J.-C. Imagining the future of bioimage analysis. *Nat. Biotechnol.* **34,** 1250–1255 (2016).
4. Mattiazzi Usaj, M. *et al.* High-Content Screening for Quantitative Cell Biology. *Trends Cell Biol.* **26,** 598–611 (2016).
5. Marr, C., Theis, F. J. & Schroeder, T. Software tools for single-cell tracking and quantification of cellular and molecular properties. *Nat. Biotechnol.* **34,** (2016).
6. de Chaumont, F. *et al.* Icy: an open bioimage informatics platform for extended reproducible research. *Nat. Methods* **9,** 690–696 (2012).
7. Carpenter, A. E. *et al.* CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* **7,** R100 (2006).
8. Wang, Q., Niemi, J., Tan, C.-M., You, L. & West, M. Image segmentation and dynamic lineage analysis in single-cell fluorescence microscopy. *Cytometry A* **77A,** 101–110 (2010).
9. Versari, C. *et al.* Long-term tracking of budding yeast cells in brightfield microscopy: CellStar and the Evaluation Platform. *J. R. Soc. Interface* **14,** 20160705 (2017).
10.Sommer, C., Straehle, C., Koethe, U. & Hamprecht, F. A. Ilastik: Interactive learning and segmentation toolkit. in *Biomedical Imaging: From Nano to Macro, 2011 IEEE International Symposium on* 230–233 (IEEE, 2011).
11.Kan, A. Machine learning applications in cell image analysis. *Immunol. Cell Biol.* **95,** 525–530 (2017).
12.Ronneberger, O., Fischer, P. & Brox, T. U-net: Convolutional networks for biomedical image segmentation. in *International Conference on Medical Image Computing and Computer-Assisted Intervention* 234–241 (Springer, 2015).
13.Carpenter, A. E., Kamentsky, L. & Eliceiri, K. W. A call for bioimaging software usability. *Nat. Methods* **9,** 666–670 (2012).

Figure 1

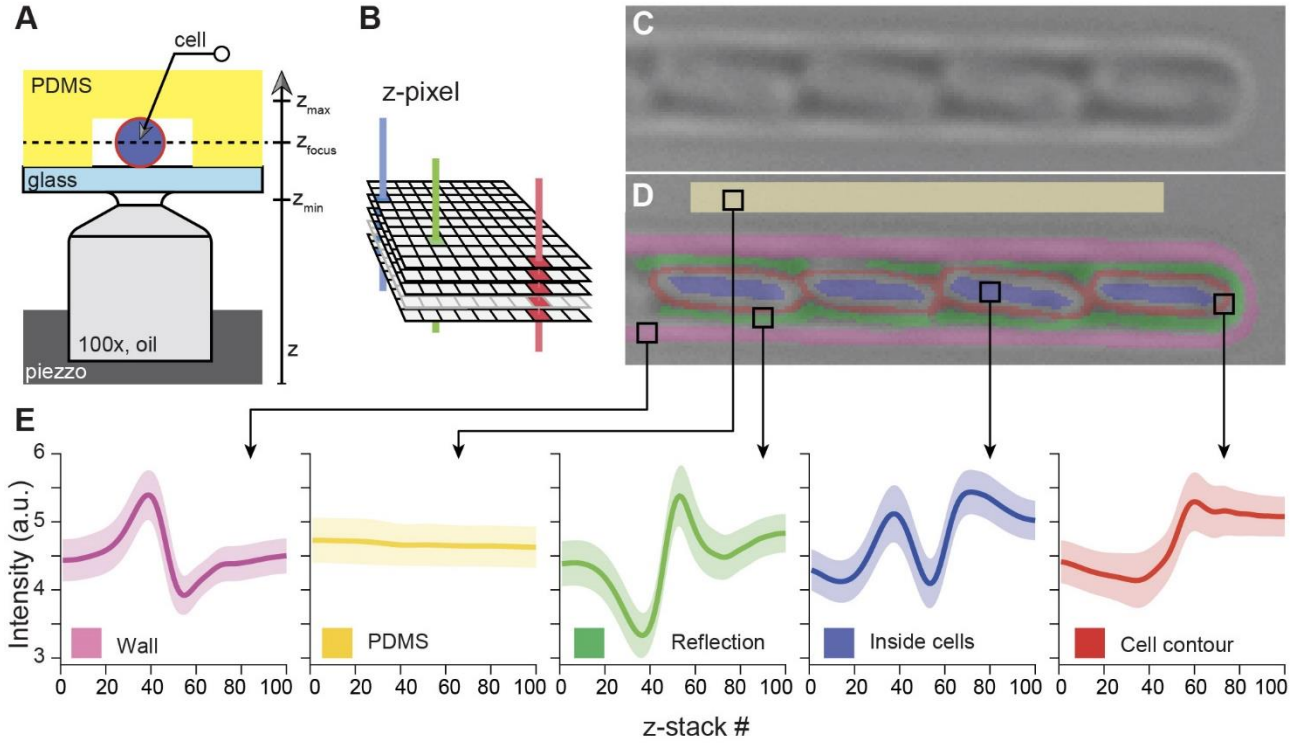


Figure 1. Principle of *z*-stack segmentation. A. A piezo-driven system is used to quickly and precisely move the objective and acquire a stack of images below, at and above the focal plane (*z*-stack). **B.** Acquisition of bright field *z*-stacks provides the focal signature of every *z*-pixel. **C.** *E. coli* cells (~1µm long, observed with a 100x objective) are cultured in a microfluidic device designed to keep them in lines. **D.** The graphical user interface is used to define different classes of object by directly drawing them. Each *z*-pixel in the image contains a profile of intensity as a function of the *z*-stack position. **E**. Average *z*-pixel profiles obtained from the example shown in **C-D,** demonstrating that different classes have different focal signatures. The shaded areas are +/- one standard deviation. From this training dataset and the definition of classes, it is possible to classify each pixel in a *z*-stack into one of the classes.

Figure 2

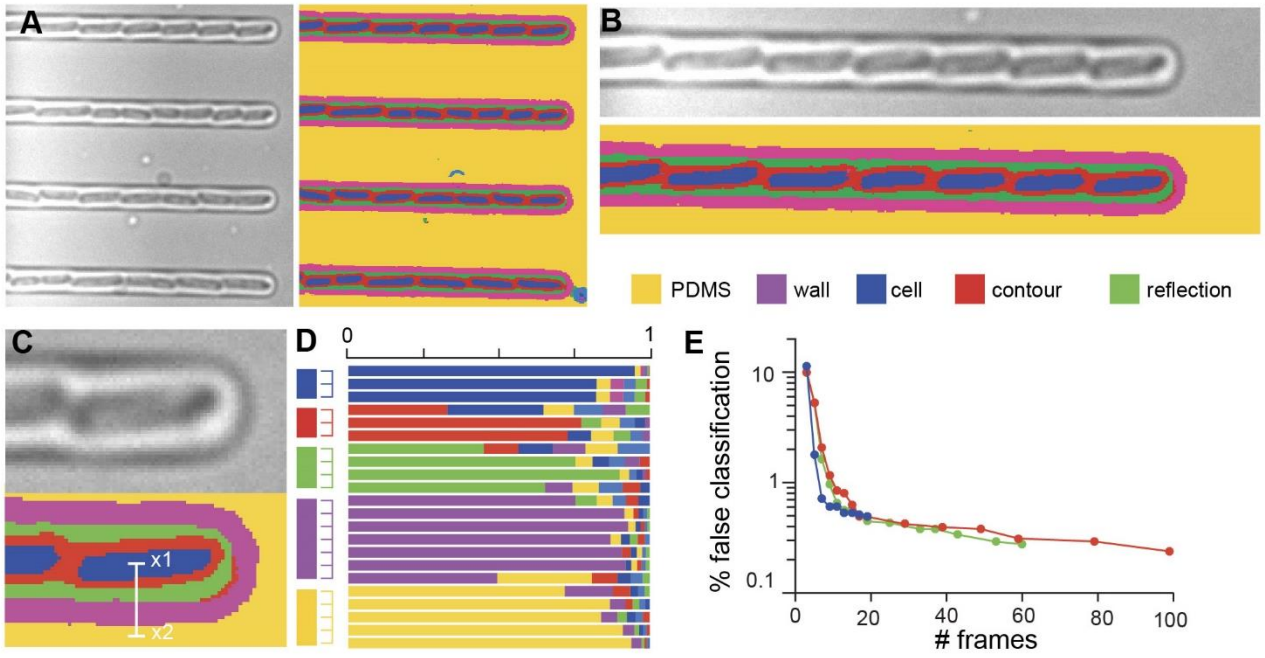


Figure 2. SVM classification enables robust, precise detection of cell features. **A**. Applying the procedure described in Figure 1, we can attribute *z*-pixels to different classes that are freely defined by the user (*e.g.* PDMS, microfluidic wall, cell interior, cell contour, halo between cells, microfluidic wall). **B-C**. High-magnification image showing perfect classification of the interior and contours of *E. coli* cells. **D.** Normalized confidence scores for each pixel of the *x1-x2* line shown in **C**. The score of each class is computed as a *softmax* function (see Supplementary Information). **E.** Validation of the method as a function of the number of frames used to identify the different object classes (red: evenly spaced images; blue: manually selected images; green: logarithmically spaced images). Irrespective of how the frames are chosen, misclassification was lower than 1% for a *z*-stack containing as few as 10 frames.

Figure 3

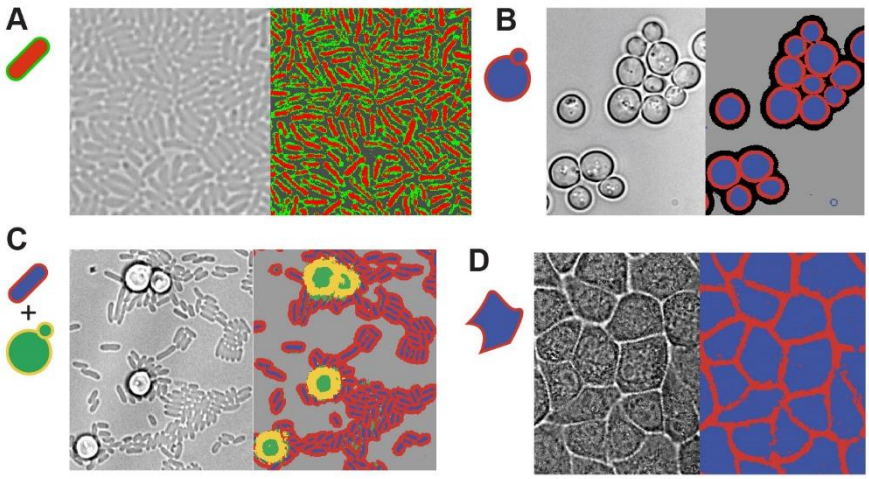


Figure 3. Segmentation of different cell types using the same method. **A**. *E. Coli* cells (~1 µm long, observed with a 100x objective) growing as a monolayer; cell interior and cell contour classes are indicated in red and green, respectively. From left to right: original image; identification results. **B**. Budding yeast cells (~4 µm wide, observed with a 100x objective) growing in a microfluidic device. Cells are growing as a monolayer and cell interior, cell contour and halo classes are shown in blue, red and black. **C**. A mixed culture of *E. coli* and budding yeast (observed with a 60x objective). Both cell types can be identified within the same image based only on their focal signatures. **D**. Monolayer of epithelial HeLa cells (observed with a 60x objective). Cell interior and cell contour classes are shown in blue and red. Adding elementary topological rules (*e.g.* cell contour cannot be inside a cell) and using basic segmentation method (*e.g.* watershed) we obtained good to very good segmentation on yeast, bacteria and mammalian cells (see supplementary text).

F- Article (prepared version)

The memory of hyperosmotic stress response in *S. cerevisiae* is modulated by Gene-positioning

Z.Ben Meriem, P. Hersen and E. Fabre

[1] Laboratoire Matière et Systèmes Complexes, UMR 7057 CNRS & Université Paris Diderot, 10 rue Alice Domon et Léonie Duquet, 75013 Paris, France.
[2] CNRS UMR 7212, INSERM U944, IUH, Université Paris Diderot Sorbonne Paris Cité, Paris, France.
[3] Equipe Biologie et Dynamique des Chromosomes, Institut Universitaire d'Hématologie, Hôpital St. Louis, Paris, France.

*Correspondence and requests for materials should be addressed to Pascal Hersen (pascal.hersen@univ-paris-diderot.fr) and Emmanuelle Fabre (emmanuelle.g-fabre@inserm.fr).

Keywords: cellular memory, yeast, single-cell, stress response, chromosome organization

Abstract

Cellular memory is a critical ability displayed by micro-organisms in order to adapt to potentially detrimental environmental fluctuations. In the unicellular eukaryote *S. cerevisiae*, it has been shown at the population level that cellular memory can take the form of a faster or a decreased response following repeated stresses. We here present a study on how yeasts respond to short-pulsed hyperosmotic stresses at the single-cell level. We analyzed the dynamical behavior of the stress responsive *STL1* promoter fused to a fluorescent reporter using microfluidics and fluorescence time-lapse microscopy. We established that pSTL1 shows variability in its successive activations following two repeated short stresses. Despite this variability, most cells displayed a memory of past stresses through a decreased activity of pSTL1 upon repeated stress. Notably, we showed that genomic location is important for the memory effect since

promoter displacement to a pericentromeric chromatin domain leads to a decreased transcriptional strength of pSTL1 and to the loss of the memory. This study provides a quantitative description of a cellular memory that includes single-cell variability and points towards the contribution of the chromatin structure in stress memory.

Introduction

Cellular memory can be defined as a cellular response to transient and repeated stimuli. These latter can emanate from constantly fluctuating and potentially stressful environments, thus possibly exerting a selective pressure on cell viability [1]. To ensure their survival, living organisms have developed various strategies to cope with environmental changes [2], [3]. One possible way for cells to maintain their biological functions in a challenged environment is to regulate gene transcription [4]. The active genetic response allowing cells to survive a single stimulus is referred to as cellular adaptation. Factors such as chromatin remodelers [3], specific proteins produced during the stress [5], [6] or even changes in chromatin conformation [7], [8] have been determined to be causal factors of adaptation to environmental changes.

What happens when cells encounter consecutive stresses is less well understood, however it has been observed in some cases that the adaptation to a first stress can serve as a learning process to a better adaptation to a consecutive stress. This process is defined as memory.

Two main pathways are proposed to explain underlying biological mechanisms for cellular memory. The first one involves the remodeling of the chromatin and is usually referred to as epigenetic memory, which may also be genomic-position dependent ref!. The second explanation is the creation of one or several proteins during the event that caused the emergence of the cellular memory. Such proteins are kept throughout the divisions, thus giving a trace of the event that happened in the first place: this is defined as cytoplasmic memory. Such memory should not be dependent on the genomic position.

In the budding yeast, studies on cellular memory showed that cells confronted to successive environmental stresses can respond differently. For instance, the so called galactose memory is characterized by a faster transcriptional reactivation of the GAL cluster, while an example of the memory of long hyperosmotic stresses is characterized by a reduced activity of the osmo-responsive gene *GRE2* without any difference in the time of reactivation of this gene [5], [6]].

All eukaryotes have a highly organized nucleus. In the case of the budding yeast, chromosomes follow a Rabl organization : centromeres are tethered to the spindle pole body, while telomeres are anchored to the nuclear periphery [26]–[28]. Interestingly, the galactose memory appears to rely on gene positioning inside the nucleus since the GAL cluster has been found to be repositioned towards the nuclear periphery in an H2AZ and Nup60 dependent manner Ref . The nuclear organization may also play a critical role in the genetic stress response as most stress response genes are located in the subtelomeres Ref . Subtelomeres are regions up to 40kb upstream the telomeres that are subjected to silencing by proteins of the Silent Information Regulator (SIR) complex [29]–[31]. However, stress conditions lift this repression, making possible the transcription of stress response genes. Most of those genes are globally expressed in response to any external stresses: this is defined as the environmental stress response (ESR) mechanism [32]. In addition to this mechanism, stress response genes that are specific to the nature of a stress will also be transcribed. This latter is in part the reason why there is no universality in the mechanisms of adaptation to different types of stresses.

Most studies questioning memory effects are carried out on isogenic populations of cells, giving information on the mean behavior. Nevertheless, a population of micro-organisms is heterogeneous. This is partly due to physiological differences between cells such as their age, size or position in the cell cycle, defined as extrinsic noise. Moreover gene expression is an inherently stochastic phenomenon, inter alia because of the low number and availability of specific molecules such as transcription factors, accessibility of the promoter or functional regulatory networks ref. Overall, stochasticity causes genetically identical cells to exhibit different behavior when encountering the same stimuli [33]–[36].

The response to osmotic changes in the budding yeast has proven to be a good tool to study the emergence of adaptation and cellular memories in this organism [9] [10]–[12]. When a yeast experiences an increase in the osmolarity of its surrounding environment (hyperosmotic stress), intracellular water will flow out of the yeast, leading to its shrinkage [13]. This causes a perturbation of the chemical reactions occurring in the cell. [15]– [17]. The imbalance of osmotic pressure is detected by osmosensors that activate the High Osmolarity Glycerol (HOG) pathway [18]–[20] [21]. This conducts to the phosphorylation of the cytoplasmic protein Hog1 and its eventual transient translocation into the nucleus where it will participate in the activation and regulation of an estimated 10% of the genome, including the osmo-responsive gene *STL1* [22], [23], [24]. Thanks to the HOG pathway, a yeast can physiologically adapt to a

hyperosmotic stress in 15-30 min [25], notably by producing glycerol which leads to homeostasis. The dephosphorylation of Hog1 and its exit from the nucleus signals the end of the adaptation to the hyperosmotic stress: this constitutes a negative feedback loop. However, such negative regulation makes impossible the distinction between physiological adaptation and genetic response. As the time required for this negative feedback loop matches the time of the physiological adaptation to the stress, the investigation of the genetic component of the hyperosmotic stresses response exclusively can only be made through the study of short stresses.

We here present a single-cell study of S. *cerevisiae* cells submitted to short pulsed hyperosmotic stresses in a well-controlled system based on time lapse fluorescence microscopy and microfluidics [39], [40]. Using computer science tools, we were able to track and analyze hundreds of single cells receiving repeated osmotic stresses. We found that in response to two consecutive hyperosmotic stresses separated by 4h, individual cells display a variability in the dynamical activity of pSTL1 in response to the two stresses- variability that we have clustered according to five typical profiles of response. Despite the existence of this pronounced dynamical variability, most of the cells adopt the same behavior, consisting in a decreased amplitude of response upon stress. We called this specific behavior memory effect. Importantly, we found that the chromatin environment dictates the manner by which cells respond to pulsed stresses as a relocation of the promoter of interest on the chromatin eventually causes a reduced activity of pSTL1 and the loss of the memory effect. Overall, our study suggests that the specific location of pSTL1 at the subtelomere is required for an optimal level of transcription that can go beyond a simple stochastic behavior and lead to the emergence of a memory in response to short osmotic stresses.

Results

The response of a population to successive hyperosmotic stresses suggests the existence of a cellular memory.

We studied a population of growing yeast cells in a microfluidic device and submitted to short and repeated hyperosmotic stresses, using 1M Sorbitol. We measured the fluorescence at the single cell level of the promoter pSTL1 tagged with the yECITRINE fluorescent reporter as a function of time [41] (figure 1a). This protein serves as a reporter of the activity of the HOG Pathway. During the time course of an experiment, each cell is tracked allowing observing, in the same cell, the first and second activation of the pSTL1 promoter due to the short

and transient activation of the HOG pathway. The limited duration of the stress (8 min) and the long delay between the two stresses (4 hours) guaranteed that cells fully recovered from the first stress, thus allowing us to compare the dynamics of response to the first and second osmotic stresses. Moreover cells born between the two stresses were not considered since they dis not receive the stress and may blur the stress response. Rather, we focus exclusively on the population that received both the first and the second stress (figure 1b).
We observed on such a population that the response to the second stress was lowered by 20% in average as compared to response to the first stress (figure 1c), but detected no difference in the time required to reach the peak of fluorescence (figure 1d). The decrease of fluorescence amplitude correlated with decreased protein amount, as detected by Western Blotting (supplementary figure 1), suggesting it is independent of photobleaching .
Altogether, these observations show that, somehow, there is a memory of the stress event at the level of the population of stressed cells. Moreover, the decrease of fluorescence intensity at the second stress is seemingly due to a reduction of protein production rate rather than a shortened duration of transcription events.

At the level of single cells, most cells, but not all, show a cellular memory.
Yet, this memory effect was not shared equally among cells. Indeed, single-cell analysis reveals a dynamic variability in the individual response to the repeated stresses. We classified single-cell fluorescent trajectories according to typical behaviors based on the first and second responses to stress (figure 2a). The most frequent behavior (55%±11%) is in line with the population memory effect in which the cells submitted to the second stress showed a lower fluorescence intensity. However, up to 18%±7% of cells display an opposite behavior, with a stronger response at the second stress. Very few cells showed similar responses to both stresses. Interestingly, we observed two subpopulations of cells that did not respond to one of the stress (figure 2b), although we confirmed that these cells indeed perceived the stress by visualization of transient cell shrinkage upon stress (Supplementary video & supplementary figure 2). Altogether our results show that the population behavior hides a richer set of dynamic behaviors of single-cell responses, which are likely the traces of the variability of the activation of pSTL1 by a hyper osmotic stress.

The cellular memory cannot be explained by a stochastic behavior only.
To determine the importance of the intrinsic variability on setting the different dynamical behaviors shown in figure 2a, that is the stochastic nature of the

pSTL1 activation, we performed stochastic simulations based on the Gillespie algorithm [42]. We simulated the transcription of pSTL1 and the translation of the fluorescent reporter of 1000 cells submitted to two 8min stresses separated by 4h (figure 2c). The rates of production and degradation of mRNA and proteins were set as established previously [39] (supplementary Table 1). Such a model implies that the computed cells will necessarily respond to both stresses. However, our experiments show that cells exposed to an 8min stress do not necessarily respond to a stress (figure 2d), conversely to cells submitted to a longer stress (figure 2e). More specifically, the absence of response disappears for longer stress duration since for a 1h stress 100% cells showed an activation of pSTL1, while for 8 min stress 80% of cells showed a response (figure 2f). This experimental observation suggests the existence of a critical time from which all cells will eventually respond to a stress. Therefore a stochastic time of activation of the *STL1* promoter was added to the model.
As expected in such a memory-free system, cells were clustered equally in the two main categories obtained experimentally, and the population did not display any memory effect. Of note, transcriptional delay made possible clusters 4 and 5 appearance (figure 2g).
The differences in clusters observed *in vivo* and through simulation, suggest that a biological mechanism other than noise in transcription and translation is at play in the memory effect.

Adaptation to pulsed hyperosmotic stress does not require de novo protein synthesis during the stress.

In order to investigate the biological origin of the memory effect, we first wanted to determine if the memory effect was linked to one or several long-lived proteins synthesized during the first episode of stress. To test this hypothesis, we inhibited transcription upon stress using thiolutin, a well-studied molecule that inhibits all three RNA polymerases in the yeast in a reversible manner [43], [44] (figure 3a). As expected, treatment of cells with thiolutin led to the loss of pSTL1 activity: when cells were treated for 1h with thiolutin (50µg/µL), no cells showed a fluorescent signal when stimulated by an 8 min hyperosmotic stress, while 80%±10% cells showed a signal in response to a hyperosmotic stress in the absence of thiolutin (figure 3b,c). We next tested the cells ability to respond back to a hyper-osmotic stress after thiolutin treatment. After 1h in the presence of thiolutin, the inhibitor was washed out and cells were submitted to an 8 min hyperosmotic stress, 4h later (figure 3d). At the population level, a response similar to the response to the first stress without thiolutin treatment was observed (figure 3e). We then performed the thiolutin treatment during 1h,

including during the first stress, then washed the inhibitor and submitted cells to a second stress 4h later (figure 3f). In these conditions, cells showed a marked decrease of the YFP signal by 50% +/-? (figure 3g), comparable to the response to a second stress without thiolutin treatment. Consequently, this experiment suggests that the memory effect is not primarily driven by *de novo* synthesis of proteins during the first stress, which could help the cells to respond to the second stress. To explain the observed memory effect, we can hypothesize that the first stress induces chromatin modifications independently of the activity of any RNA polymerases, but with an effect on subsequent transcription events at the pSTL1 locus. A possibility could be that chromatin marks would appear in most cells during the first stress and alter the response dynamics of cells during the second stress.

Chromosome positioning influences the dynamical activity of pSTL1.

The *STL1* locus is located on the right arm of the chromosome IV, in its subtelomeric region, prone to silencing in non-stress conditions. To investigate the influence of the chromatin context on the dynamics of activation of pSTL1, we moved a region containing the promoter of *STL1* and the yECITRINE fluorescent reporter to a distinct, centromeric chromatin domain (figure 4a). The displaced DNA region included 1kb upstream of the *STL1* locus, enough to have a fully functional *STL1* promoter [45]. To compare pSTL1 activity between its endogenous position and the centromeric one, we first submitted both strains to a 2h hyperosmotic stress and used flow cytometry to quantify the fluorescence at several time points. The activity of centromeric pSTL1 was significantly lower than in wild type cells (figure 4b), although the integrity of the promoter was preserved (supplementary figure 3).

Patterns of consecutive responses to two 8min hyperosmotic stresses separated by 4h were then compared between endogenous and displaced pSTL1. Cells expressing the *STL1* promoter at this centromeric position showed a more uniform distribution into the five defined clusters and there was a decrease in the amount of cells displaying the memory effect (from 55%±11% to 28%±4%, figure 4c) compatible with a purely stochastic process. Such a result indicated that the chromatin environment might be involved in the dynamical transcriptional activity of pSTL1. Although a functional pSTL1 promoter has been displaced [45] , potential subtelomeric regulatory elements could have been lost during gene displacement. We have ruled out this hypothesis using a Crispr/dCas9VPR system [46] to bypass regulatory elements and force the activation of pSTL1 in non-stress conditions (supplementary figure 4). We designed guide RNAs to target Crispr/dCas9VPR within the 1kb sequence of

displaced pSTL1 and successfully induced the fluorescent reporter expression independently of any stress at both the peri-centromeric and subtelomeric positions. We observed a decrease in the activity of pSTL1 at the peri-centromeric position compared to the endogenous one, meaning that the observed differences of expression of pSTL1 are not linked to the sequence of the promoter itself or the presence of regulatory elements, but is rather related to the chromatin environment.
Taken together, our results show that the chromatin environment sets the variability of single-cell dynamical response for short stresses and can consequently act on cellular memory in a time varying environment.

Discussion

In the current study, we investigated how individual yeast cells dynamically behave in response to pulsed hyperosmotic stresses. Focusing our study on short stresses allowed us to analyze the genetic response to hyperosmotic stresses exclusively and probe the cell-cell variability that finds its origin in the onset of transcriptional events.

The in-depth single-cell analysis reveals that the yeast display various behaviors in response to two repeated stresses Most cells decrease their responses with the stress, meaning that the memory effect profile is privileged compared to the others profiles we have defined. Using stochastic simulations, we have established that the five profiles of response could be explained by the stochasticity of gene expression. However the single-cell quantification obtained with such a model could not match the quantification obtained following the experiments where the memory effect is the dominant behavior, despite the existence of various profiles of response that are synonymous of variability. Thus, the memory effect is not a reflection of the stochasticity of gene expression.
Although cells mostly display the memory effect, characterized by a reduced activity of pSTL1, we have not observed any difference in the time of response to the pulsed stresses. This suggests that the cellular memory consists in a decrease in the rate of protein production, which could also mean a decrease in the rate of transcription.
It can be argued that the *STL1* stress response promoter is naturally fine-tuned to respond with the fastest dynamics to ensure cell survival as its transcription starts at the moment of the stress, apart for the bimodality phenomenon, explaining why no temporal differences could be observed. However, a possible

way for the yeast to improve their response to subsequent stresses is the optimization of the amount of energy dedicated to fighting the stress, hence the emergence of the memory effect.
We have then investigated the biological mechanisms behind such a memory. In order to examine the possibility of factors synthesized during the stress that could influence the response to the next stress, we used a transcriptional inhibitor in order to block *de novo* transcription during stress. Since blocking the transcription during the first stress does not prevent a decreased response to the second stress, it might imply that no new factors are required for the emergence of the memory effect. Such result also incidentally show that there is no universality in the memory effect to the same type of stress, as [5] describes an increase in the Ena1 proteins production to be at the origin of the memory effect they observed, which is not the case in our experiments. Furthermore, the use of a transcriptional inhibitor such as the thiolutin that inhibits all three RNA Pol and *de novo* transcription in the yeast does not prevent various factors from binding to the promoter's sequence, potentially causing specific marks of transcription on it. Those marks would likely serve as a trace of previous activities of the promoter. Alternatively, proteins present prior to the stress with a long half-life could also be implicated in the memory effect by displaying different dynamics with the stresses, or in the case of cytoplasmic proteins, being translocated in the nucleus upon the first stress and remaining there, poising the promoter of interest for a different activation.

We further showed that the dynamic variability distribution between single cells was dependent on the positioning of the pSTL1 locus on the chromosome. When displaced in a domain where pSTL1 shows a decreased activity, the stochasticity of gene expression prevails. Consequently, it appears that a biological mechanism, active in the subtelomeric regions upon transcription during stress, allows the memory effect to become predominant among the population of cells. One of the key differences between the two genomic positions analyzed is the variation of the transcriptional activity of pSTL1. We thus propose that transcriptional marks or transactivators will induce a high level of transcriptional activity during the first stress, required to overcome the stochasticity of gene expression and have the emergence of a cellular memory at the second stress. A parallel can be drawn with previous studies performed in *B. subtilis*, where transcriptional events occurring above a certain threshold have been described to lead to the emergence of a cellular memory Ref! Et en dire un peu plus . Although this organism is a prokaryote, some similarities

might exist between those two microorganisms in the regard to the biology of the memory effect.
It could be hypothesized that a high activity of the promoter of interest would mean and opened chromatin. The latter is more likely to exist in an area of high transcriptional activity upon stress such as the subtelomere. Although marks of acetylation are usually associated with a high level of gene expression, the activity of pSTL1 is known to be reduced in absence of the histone deacetylase Rpd3. This means that in our experimental context, marks of deacetylation might actually be responsible for a high level of transcriptional activity. It would be interesting to investigate the potential role of (de)acetylation by, for instance, forcing a high level of (de)acetylation during the first stress only.

The stochasticity of gene expression gives a diversity of behavior. From an evolutionary point of view, this diversity could favor adaptation since it gives several choices for selection. As it appears that the memory effect was the one selected, the specific position of pSTL1 would have been kept throughout evolution to gain a regulation level in order to perform better response to stresses as compared to other down-regulated regions.
Working at the single-cell level allowed us to observe and quantify additional phenotypes that would have been blurred by a population study. We also showed that the cellular memory does not require a long stress and can start very early on. Overall, our study suggests that the specific subtelomeric location of pSTL1 is required for an optimal level of transcription that can go beyond a simple stochastic behavior and lead to the emergence of a memory in response to repeated hyperosmotic stresses. Our work could serve as a basis to broader studies of the positioning of stress response genes at subtelomeric positions in the budding yeast, from a genetic point of view as well as an evolutionary once.

Materials and Methods

Flow Cytometry: all flow cytometry experiments were performed with a flux cytometer Gallios (Beckman Coulter) equipped with 10 colors, 4 lasers (488nm Blue, 561nm Yellow, 638nm Red, 405 nm Violet). We used the excitation laser 488nm and the emission filter at 530nm +/- 30 nm.

Yeast strains and cell culture: our experiments were made using a STL1::yECITRINE-His5 (yPH53) strain derived from S288C and gifted to us by Megan McClean. The yeast were grown overnight in SC+2% glucose at 30°C. The cells were diluted next morning to reach OD600=0.5 at the moment of the experiments. Genotype of all strains used in this study are indicated in table 2. To move pSTL1 reporter construct in the peri-centromeric region of chromosome IV, pSTL1-yECITRINE-HIS5 construct was PCR amplified using primers TATTGAGCACGTGAGTATACGTGATTAAGCACACAAAGGCAGCTTGGA GTCAATGATTCTGAAATACTCCTTTTACA and TGCAGGCAAGTGCACAAACAATACTTAAATAAATACTACTCAGTAATAA CATTATTGGTGCGGCAAGG with 50 bases homologies to the *TRP1* locus. [HIS^+ TRP^-] yeast transformants were verified by PCR and PCR fragment subsequently sequenced, ensuring the absence of any mutations in the construct.

Crispr experiments: we used the plasmid pAG414GPD-dCas9-VPR from Addgene plasmid (# 63801) to express the inactivated form of CAS9 fused to transcriptional activator VPR. The guides were cloned under SNR52 promoter in plasmid pEF534 using the enzyme BsmBI. Digesting the resulting plasmid by NotI / XbaI and cloning the Guide containing fragment into pRS425 similarly digested, performed marker exchange. We designed two guides targeting pSTL1, respectively gRNA1, GAAAGTGCAGATCCCGGTAA and gRNA2, GCGCCGAATACCCCGCGAAA.

Single-cell clustering: To categorize cells in different classes, we compared the maximum level of fluorescence reached during the first and second stress, while taking the basal fluorescence level prior to the corresponding stress as a reference. The ratio between the maximum amplitude of the first and the second stress was then evaluated. Cells categorized according to profile 1 had a ratio superior to 1, cells categorized according to profile 2 had a ratio inferior to 1 and cells displaying profile 3 had a ratio equal to 1. The cells with a maximum of amplitude equal to 0 (no expression) during the second or the first stress were

categorized separately (figure 2b). All ratio were established with a 5% threshold.

Microfluidics: we used an H-shaped microfluidic device to confine the yeast in channels of 3.7µm high. This microfluidic device was made using soft lithography techniques. The hyperosmotic stresses were triggered using SC+2% glucose supplemented with 1M sorbitol. The media were flown in the microfluidic chip using a peristaltic pump ISMATEC set at 120µL/min flow rate.

Transcriptional inhibition: cells were exposed to SC with addition of thiolutin (Abcam ref ab143556) at 50µg/mL (diluted in DMSO) for 1h prior and during the stress. To wash out Thiolutin in the microfluidics experiments, SC medium was delivered to the cells during 4h using a peristaltic pump set at a flow rate of 120µL/min.

Microscopy: we used an inverted microscope Olympus IX71. The yeast were observed with an objective x100 UplanFLN 100x/ 1.3 Oil Ph3 Ul2. Images were recorded with a camera Cool Snap HQ2 Princeton Instruments. All experiments were made at 30°C. Yeast were imaged every 5min with 20ms exposure in bright light and 200ms in fluorescence light. The microscope was controlled by the open source software MicroManager which was interfaced with Matlab.

FIGURE LEGENDS

FIGURE 1

(A) Experimental setup. We use a multi-layer H-shaped microfluidics device composed of two large flow channels of 50µm height and 40µm thin, observation chambers of 400µm x 400µm x 3.7µm. Cells are trapped in the chambers and grow as a monolayer, facilitating cell segmentation and tracking. The medium flowing in the channels diffuse in the chambers. The hyperosmotic stresses activating expression of pSTL1-yECRITRINE are triggered using sorbitol 1M for 8min. (B) Principle of the experiments. Cells are exposed to two 8min stresses separated by 4h, which allows the cells to recover upon stress. Cells are imaged every 5min in bright light (20ms exposure) and fluorescence light (200ms exposure) (C) Decrease of the amplitude of response upon stress at the population level. Fluorescence response of a population of a hundred cells. The mean response of cells submitted to a first stress (dark blue), followed by another stress 4h later (light blue) is represented with the standard error. The peak of fluorescence decreases upon stress. (B) Analyses of time response to two consecutive stresses in the same population. Time between stress induction and fluorescence peak reveals that the time of response is similar upon stress.

FIGURE 2

(A) Examples of five typical single-cell response profiles. Although the single-cell analysis reveals a dynamical variability of the response, we have defined five typical profiles of response adopted by the cells. (B) Single-cell clustering. Using criteria on the values of the fluorescence peaks, we have clustered the dynamical variability of response according to the five typical fluorescence response. (C) Modeling gene expression upon stress in a memory-free system. We use stochastic simulations to model the transcription of the fluorescent reporter and the protein translation upon stress. (D) Sequence images of cells submitted to an 8min stress. The arrows on the last bright field image shows the cells that do not respond to the stress. (E) Sequence images of cells submitted to a continuous stress. All cells show a response to such a stress. (F) Quantification of responsive cells. Upon receiving an 8min stress, 80% of cells show a response, whereas 100% of cells respond to a 1h stress. (G) Single-cell quantification of computed cells according to the five typical profiles. The model in this case includes a transcription delay randomly chosen between 0 and 10min for each computed cells. This delay is also different during the two stresses. The simulation is run twice and the fluorescence peaks values are used to cluster the computed cells responses according to the five typical profiles of response.

FIGURE 3
(A) Principle of the transcription inhibition experiment. In our experimental context, we perform a 1h thiolutin treatment prior and during the stress in order to prevent the activity of pSTL1 upon stress. (B) Bright field associated fluorescence pictures of cells in (top) non stress conditions (middle) hyperosmotic conditions (bottom) hyperosmotic conditions in the presence of thiolutin (C) Single-cell quantification of the fluorescence of the cell in these different conditions. (D) Principle of the control experiment (Up) The cells are in the microfluidic device for 4h before being exposed to an 8min hyperosmotic stress. (Down) The cells are treated with the transcriptional inhibitor for 1h in the microfluidic device. The cells then are allowed to grow for 4h before being exposed to an 8min hyperosmotic stress. (E) Cells treated with the transcriptional inhibitor do not present a pSTL1 transcriptional defect after the inhibitor is washed. Population quantification of the response to a stress in the case where the cells were untreated (blue) or treated with the inhibitor (purple). (F) Principle of the experiment. (Up) The cells are exposed to two 8min hyperosmotic stresses separated by four hours. (Down) The cells are treated with thiolutin for 1h and, still treated with the inhibitor, exposed to a 8min hyperosmotic stress. The inhibitor is then washed and the cells grow for 4h before being exposed to another 8min stress. (G) Inhibition of the response to the first stress does not prevent the memory of the stress. Population quantification of the response to a second stress in the case when the cells were submitted to a first stress (blue) or submitted to a first stress + transcriptional inhibitior (purple).

FIGURE 4
(A) Displacement of pSTL1 towards the peri-centromere of the chromosome IV. Sketch of the genomic position of pSTL1 on the chromosome IV. The promoter was moved at the TRP1 locus, on the same chromosome. (B) Decrease of activity of the displaced pSTL1 upon stress. Fluorescence quantification of the activity of the promoter in response to a 2h hyperosmotic stress in the endogenous case (blue) and when the promoter was moved (red). (C) Displacement of pSTL1 leads to the loss of the memory effect. Single-cell quantification of cells containing displaced promoter pSTL1 in response to two hyperosmotic stresses. Classification made according to the five typical profiles of response as in figure 2.

Data availability

All data are available on an open repository hosted on the ZENODO platform and with the following DOI : XXX

Acknowledgments
Yasmine Khalil is thanked for help in cells tracking, Zoran Marinkovic for FACS experiments, Antoine Canat and Pierre Thérizols for helpful advices in thiolutin experiment. The authors would like to thank their respective team members for very fruitful discussions. This work was supported by Labex "Who am I?" (ANR-11-LABX-0071, Idex ANR-11-IDEX-0005-02). EF further acknowledges support from Agence Nationale de la Recherche (ANR-13-BSV8-0013-01), IDEX SLI (DXCAIUHSLI-EF14) and Cancéropôle Ile de France (ORFOCRISE PME-2015). PH acknowledges support by the Agence Nationale de la Recherche (ICEBERG-ANR-10-BINF-06-01) and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No 724813).

Author Contributions
ZBM, PH and EF designed the experiments and wrote the article. ZBM performed the experiments.

Supplementary Information

FIGURE S1

Supplementary Table 1: Gillespie parameters

Parameter	Definition	Unit	Reference Value	Source
k1	transcription rate	min^{-1}	1.10^{1}	[39]
d1	mRNA decay	min^{-1}	$2.94.10^{-1}$	[39]
τ	time delay	min	Between 0 and 10 min	This study
k2	translation rate	min^{-1}	$9.47.10^{-1}$	[39]
d2	protein decay	min^{-1}	4.10^{-3}	[39]

Supplementary Table 2: strains genotype

yPH53	ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 pSTL1::yECITRINE-HIS5
yPH200	ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 Δ(pSTL1-STL1)::CaUra3
yPH212	ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 Δ(pSTL1-STL1)::CaUra3 Δtrp1::pSTL1-yECITRINE-HIS5
5	leu2Δ0 his3Δ1 lys2Δ0 pSTL1::yECITRINE-HIS5 Δtrp1::pURA3-URA3

Supplementary Table 3: list of primers

Deletion of pSTL1-STL1 forward	Gagtagaaaatttactaatgtggtctcgcgtgtgaatcaggtttagctt gcctcgtcccc
Deletion of pSTL1-STL1 reverse	taagtaaattacaaaatatgatttgtgagttgtgtgtgaaGTTTTC GACACTGGATGGCG
PCR of pSTL1-yECITRINE His selection with 50bp of TRP1 homology forward	TATTGAGCACGTGAGTATACGTGATTAAGCA CACAAAGGCAGCTTGGAGTCAATGATTCTG AAATACTCCTTTTACA
PCR of pSTL1-yECITRINE His selection with 50 bp of TRP1 homology reverse	TGCAGGCAAGTGCACAAACAATACTTAAAT AAATACTACTCAGTAATAACATTATTGGTGC GGCAAGG
Amplification of pSTL1-yECITRINE at TRP1 locus forward	CGCCAGATGGCAGTAGTGGAAG
Amplification of pSTL1-yECITRINE at TRP1 locus reverse	GCCTGCAGGCAAGTGCAC
Sequencing of pSTL1 at TRP1 locus forward 1	CCGATTAAGAATTCGGTCG
Sequencing of pSTL1 at TRP1 locus reverse 1	GGATCTGCACTTTCTCAG
Sequencing of pSTL1 at TRP1 locus forward 2	CATTGCCAAGGCTAGGAG
Sequencing of pSTL1 at TRP1 locus reverse 2	catcaccttcaccttcacc
Primer gRNA 1 forward	gatcGAAAGTGCAGATCCCGGTAA
Primer gRNA 1 reverse	aaacTTACCGGGATCTGCACTTTC
Primer gRNA 2 forward	gatcGCGCCGAATACCCCGCGAAA

Primer gRNA 2 reverse	aaacTTTCGCGGGGTATTCGGCGC

References

[1] A. A. Hoffmann et M. J. Hercus, « Environmental Stress as an Evolutionary Force », *BioScience*, vol. 50, nº 3, p. 217, 2000.

[2] S.-J. Li *et al.*, « Microbial communities evolve faster in extreme environments », *Scientific Reports*, vol. 4, nº 1, mai 2015.

[3] R. M. Nesse, S. Bhatnagar, et E. A. Young, « Evolutionary Origins and Functions of the Stress Response », *Encyclopedia of Stress*, vol. 1, p. 6, 2007.

[4] Q. Guan, S. Haroon, D. G. Bravo, J. L. Will, et A. P. Gasch, « Cellular Memory of Acquired Stress Resistance in Saccharomyces cerevisiae », *Genetics*, vol. 192, nº 2, p. 495-505, oct. 2012.

[5] A. Rienzo, D. Poveda-Huertes, S. Aydin, N. E. Buchler, A. Pascual-Ahuir, et M. Proft, « Different Mechanisms Confer Gradual Control and Memory at Nutrient- and Stress-Regulated Genes in Yeast », *Molecular and Cellular Biology*, vol. 35, nº 21, p. 3669-3683, nov. 2015.

[6] I. Zacharioudakis, T. Gligoris, et D. Tzamarias, « A Yeast Catabolic Enzyme Controls Transcriptional Memory », *Current Biology*, vol. 17, nº 23, p. 2041-2046, déc. 2007.

[7] S. C. Kerr et A. H. Corbett, « Should INO Stay or Should INO Go: A DNA "Zip Code" Mediates Gene Retention at the Nuclear Pore », *Molecular Cell*, vol. 40, nº 1, p. 3-5, oct. 2010.

[8] W. H. Light, D. G. Brickner, V. R. Brand, et J. H. Brickner, « Interaction of a DNA Zip Code with the Nuclear Pore Complex Promotes H2A.Z Incorporation and INO1 Transcriptional Memory », *Molecular Cell*, vol. 40, nº 1, p. 112-125, oct. 2010.

[9] D. Muzzey, C. A. Gómez-Uribe, J. T. Mettetal, et A. van Oudenaarden, « A Systems-Level Analysis of Perfect Adaptation in Yeast Osmoregulation », *Cell*, vol. 138, nº 1, p. 160-171, juill. 2009.

[10] M. Acar, A. Becskei, et A. van Oudenaarden, « Enhancement of cellular memory by reducing stochastic transitions », *Nature*, vol. 435, nº 7039, p. 228-232, mai 2005.

[11] D. G. Brickner *et al.*, « H2A.Z-Mediated Localization of Genes at the Nuclear Periphery Confers Epigenetic Memory of Previous Transcriptional State », *PLoS Biology*, vol. 5, nº 4, p. e81, mars 2007.

[12] D. R. Burrill et P. A. Silver, « Synthetic circuit identifies subpopulations with sustained memory of DNA damage », *Genes & Development*, vol. 25, n° 5, p. 434-439, mars 2011.
[13] E. Petelenz-Kurdziel, E. Eriksson, M. Smedh, C. Beck, S. Hohmann, et M. Goksör, « Quantification of cell volume changes upon hyperosmotic stress in Saccharomyces cerevisiae », *Integrative Biology*, vol. 3, n° 11, p. 1120, 2011.
[14] A. Miermont, J. Uhlendorf, M. McClean, et P. Hersen, « The Dynamical Systems Properties of the HOG Signaling Cascade », *Journal of Signal Transduction*, vol. 2011, p. 1-12, 2011.
[15] R. H. REED, J. A. CHUDEK, R. FOSTER, et G. M. GADD', « Osmotic Significance of Glycerol Accumulation in Exponentially Growing Yeasts », *APPL. ENVIRON. MICROBIOL.*, vol. 53, p. 5, 1987.
[16] H. Dihazi, R. Kessler, et K. Eschrich, « High Osmolarity Glycerol (HOG) Pathway-induced Phosphorylation and Activation of 6-Phosphofructo-2-kinase Are Essential for Glycerol Accumulation and Yeast Cell Proliferation under Hyperosmotic Stress », *Journal of Biological Chemistry*, vol. 279, n° 23, p. 23961-23968, juin 2004.
[17] J. Albertyn, S. Hohmann, J. M. Thevelein, et B. A. Prior, « GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. », *Molecular and Cellular Biology*, vol. 14, n° 6, p. 4135-4144, juin 1994.
[18] V. Reiser, D. C. Raitt, et H. Saito, « Yeast osmosensor Sln1 and plant cytokinin receptor Cre1 respond to changes in turgor pressure », *The Journal of Cell Biology*, vol. 161, n° 6, p. 1035-1040, juin 2003.
[19] F. Posas, « Osmotic Activation of the HOG MAPK Pathway via Ste11p MAPKKK: Scaffold Role of Pbs2p MAPKK », *Science*, vol. 276, n° 5319, p. 1702-1705, juin 1997.
[20] K. Tatebayashi *et al.*, « Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway », *The EMBO Journal*, vol. 26, n° 15, p. 3521-3533, août 2007.
[21] C. Schuller, J. L. Brewster, M. R. Alexander, M. C. Gustin, et H. Ruis, « The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the Saccharomyces cerevisiae CTT1 gene. », p. 8.
[22] S. M. O'Rourke, I. Herskowitz, et E. K. O'Shea, « Yeast go the whole HOG for the hyperosmotic response », *Trends in Genetics*, vol. 18, n° 8, p. 405-412, août 2002.
[23] P. J. Westfall, « When the Stress of Your Environment Makes You Go HOG Wild », *Science*, vol. 306, n° 5701, p. 1511-1512, nov. 2004.

[24] S. Zhao, N. W. Douglas, M. J. S. Heine, G. M. Williams, H. C. Winther-Larsen, et P. G. Meaden, « The STLl gene of Saccharomyces cerevisiae is predicted to encode a sugar transporter-like protein », p. 5.
[25] S. Hohmann, « Osmotic Stress Signaling and Osmoadaptation in Yeasts », *Microbiology and Molecular Biology Reviews*, vol. 66, nº 2, p. 300-372, juin 2002.
[26] E. Fabre et M. Spichal, « Subnuclear Architecture of Telomeres and Subtelomeres in Yeast », in *Subtelomeres*, E. J. Louis et M. M. Becker, Éd. Berlin, Heidelberg: Springer Berlin Heidelberg, 2014, p. 13-37.
[27] C. Zimmer et E. Fabre, « Principles of chromosomal organization: lessons from yeast », *The Journal of Cell Biology*, vol. 192, nº 5, p. 723-733, mars 2011.
[28] A. Taddei, H. Schober, et S. M. Gasser, « The Budding Yeast Nucleus », *Cold Spring Harbor Perspectives in Biology*, vol. 2, nº 8, p. a000612-a000612, août 2010.
[29] L. Guarente, « Diverse and dynamic functions of the Sir silencing complex », *Nature Genetics*, vol. 23, nº 3, p. 281-285, nov. 1999.
[30] P. Moretti, K. Freeman, L. Coodly, et D. Shore, « Evidence that a complex of SIR proteins interacts with the silencer and telomere- binding protein RAP », p. 14.
[31] M. Grunstein, « Molecular model for telomeric heterochromatin in yeast », p. 5.
[32] A. P. Gasch *et al.*, « Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes□D », *Molecular Biology of the Cell*, vol. 11, p. 17, 2000.
[33] A. Raj et A. van Oudenaarden, « Nature, Nurture, or Chance: Stochastic Gene Expression and Its Consequences », *Cell*, vol. 135, nº 2, p. 216-226, oct. 2008.
[34] J. M. Raser, « Control of Stochasticity in Eukaryotic Gene Expression », *Science*, vol. 304, nº 5678, p. 1811-1814, juin 2004.
[35] B. Snijder et L. Pelkmans, « Origins of regulated cell-to-cell variability », *Nature Reviews Molecular Cell Biology*, vol. 12, nº 2, p. 119-125, févr. 2011.
[36] M. B. Elowitz, « Stochastic Gene Expression in a Single Cell », *Science*, vol. 297, nº 5584, p. 1183-1186, août 2002.
[37] S. Pelet, F. Rudolf, M. Nadal-Ribelles, E. de Nadal, F. Posas, et M. Peter, « Transient Activation of the HOG MAPK Pathway Regulates Bimodal Gene Expression », *Science*, vol. 332, nº 6030, p. 732-735, mai 2011.
[38] L. T. Burns et S. R. Wente, « Casein Kinase II Regulation of the Hot1 Transcription Factor Promotes Stochastic Gene Expression », *Journal of Biological Chemistry*, vol. 289, nº 25, p. 17668-17679, juin 2014.

[39] A. Llamosi *et al.*, « What Population Reveals about Individual Cell Identity: Single-Cell Parameter Estimation of Models of Gene Expression in Yeast », *PLOS Computational Biology*, vol. 12, nº 2, p. e1004706, févr. 2016.
[40] J. Uhlendorf *et al.*, « Long-term model predictive control of gene expression at the population and single-cell levels », *Proceedings of the National Academy of Sciences*, vol. 109, nº 35, p. 14271-14276, août 2012.
[41] M. A. Sheff et K. S. Thorn, « Optimized cassettes for fluorescent protein tagging inSaccharomyces cerevisiae », *Yeast*, vol. 21, nº 8, p. 661-670, juin 2004.
[42] D. T. Gillespie, « Exact stochastic simulation of coupled chemical reactions », *The Journal of Physical Chemistry*, vol. 81, nº 25, p. 2340-2361, déc. 1977.
[43] D. J. TIPPER, « Inhibition of Yeast Ribonucleic Acid Polymerases by Thiolutin », *J. BACTERIOL.*, p. 12.
[44] A. Jimenez, D. J. Tipper, et J. Davies, « Mode of Action of Thiolutin, an Inhibitor of Macromolecular Synthesis in Saccharomyces cerevisiae », *Antimicrobial Agents and Chemotherapy*, vol. 3, nº 6, p. 729-738, juin 1973.
[45] C. Bai, M. Tesker, et D. Engelberg, « The yeast Hot1 transcription factor is critical for activating a single target gene, *STL1* », *Molecular Biology of the Cell*, vol. 26, nº 12, p. 2357-2374, juin 2015.
[46] A. Chavez *et al.*, « Highly efficient Cas9-mediated transcriptional programming », *Nature Methods*, vol. 12, nº 4, p. 326-328, avr. 2015.