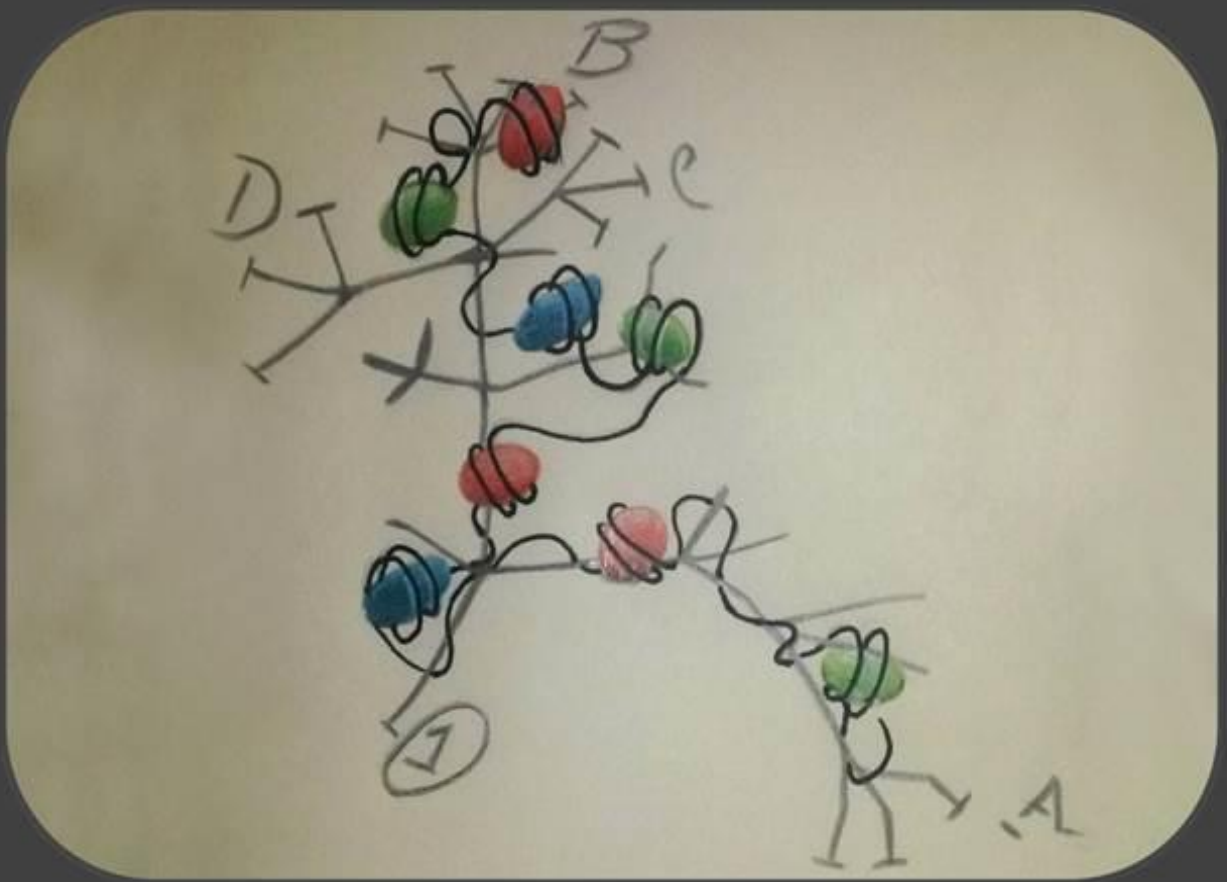
The role of epigenetic mechanisms in the adaptive evolution

Dragan Stajic



Dissertation presented to obtain the Ph.D degree in Evolutionary Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
February, 2018

UNIVERSIDADE NOVA DE LISBOA

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Research work coordinated by:

FUNDAÇÃO CALOUSTE GULBENKIAN
Instituto Gulbenkian de Ciência

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The research presented in this thesis was financially supported by Fundação para a Ciência e a Tecnologia (FCT) through a fellowship to D. Stajic [SFRH/BD/52170/2013]. This thesis was printed with support from Instituto Gulbenkian de Ciência.

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Acknowledgments

It is humbling and rewarding to see five years of my work taking shape in the form of this thesis. These years were a journey of personal growth. The change that happened during this process, of which I am very proud off, will be forever part of me. I have been very fortunate to share this journey with many wonderful people.

Firstly, I would like to mention my supervisor Lars Jansen. It has been a privilege to be part of your team. I learned from you what it means to be a scientist. Thank you for your support and the knowledge you shared. I will carry that with me on the next step I take.

I would also like to thank my co-supervisor Lillia Perfeito. I started my PhD as a molecular biologist. Thanks to you I can say I am finishing this project as evolutionary biologist as well.

I would also like to thank João Mata for his valuable help while conducting the experiments and to all current and past members of Epigenetic Mechanisms Group for the discussions and their contribution to this project.

The support and guidance by my Thesis Committee, Patricia Beldade and Miguel Godinho was very valuable, especially in the times when experiments would fail.

I would also like to thank Élio Sucena for his support, both professional and personal. Your work and contribution to the education of young scientists is highly respected and appreciated by current generations and many more that are coming.

I would also like to mention Isabel Gordo. Thank you for all the thought provoking discussions and valuable scientific input. Thank you for believing in science and in people.

These years would have been a bigger challenge if not for the support of Manuela Cordeiro. Thank you for your care and for bringing up the generations of students that passed through this Institute.

In these past five years I heard many times that IGC is a special place. It is not its walls and facilities that make it stand out. It is its people. I would like to thank all current and past members of this Institute for all the conversations and time we spent together.

I would also like to thank one special person. She is not a scientist, but her artistic contribution is woven into this thesis. I consider myself lucky that I can share my life with you. Thank you, Inês Amaro, for your support and love that gave me strength in tough moments. We learned many things from each other, and here I do not count the fact that you learned how to “run a gel”. I doubt that would be at any time useful to you.

Our friends are the family that we choose. I would like to thank my newly acquired family, Elvira Lafuente, Vania Silva, Ojas Deshpande, Sascha Werner, Zoe Ederlin and Marina Fridman for being a part of my life. Thank you for unforgettable moments we shared. I am very happy I had opportunity to grow up with you.

I would also like to thank the family I chose in Serbia, Mina Krnjajic, Danka Turtic, Zvezdana Jemuovic and Blagoje Murganic. Thank you for being part of my life. I am proud to be your friend. After so many years of friendship I am sure your thoughts and opinions are intrinsic part of my personality that I will proudly carry with me.

Last, but not least, I would like to thank the people who provided genetic material for this study, my family. I would like to give my thanks and appreciation to my mother Dragica, my father Radoslav, my aunt Stana, my sister Manuela, my niece Marija and my brother Aleksandar for your unconditional support and love you gave me over the years. I am proud of calling you my family and of all the things I learned from you during all these years.

Summary

Natural selection acts upon the variation in fitness-related traits within a population. Inheritance of this variation is of paramount importance for this process. Evolution of any inherited trait related to reproductive success will proceed, regardless of its molecular mechanism of inheritance. Mendel´s discoveries and definition of the particulate form of inheritance alongside the discovery that the instructions for phenotypic traits are encoded in the nucleotide sequence within DNA strand lead to the formalization of the Modern Synthesis. Within its framework Mendel´s principles of particulate inheritance were combined with Darwin´s principle of natural selection. In the view of Modern Synthesis, genes are carriers of the phenotypic information. Variation in the phenotypes within a population is caused by spontaneous mutations (changes in genes) that bring about the existence of different gene variants (alleles). These genes are inherited in a particulate manner, as separate entities that are assorted in the offspring. Natural Selection acts upon the phenotypic variation caused by the differences in genes, preserving more favorable variants. As a consequence a change in frequency of alleles within a population will happen during the course of evolution. In this view, inheritance has a genetic basis, independent of the environment, which has a role only as a selective force.

However, information for the development for a particular phenotype can be transmitted by mechanisms outside of the DNA sequence. These epigenetic forms of inheritance are comprised of many molecular mechanisms that are typically involved in the control of gene expression and can be maintained across cell divisions during the development of an individual or even across generations independently of the DNA sequence. Those mechanisms include processes such as DNA methylation and histone modification. They

can have profound effect upon the phenotype and were shown to persist for several generations. Therefore, in principle, natural selection could act upon the variation induced by these modes of inheritance. However, whether this is happening is still unknown. I set out to understand their potential contribution to the evolutionary processes. In this thesis I will describe the experiments conducted to address this question.

In Chapter 1, I will introduce the importance of inheritance in the process of evolution. Additionally, I will cover the examples of transgenerational epigenetic inheritance, as well as the model system that I will be using in my experiments.

In Chapter 2, I will present our results on how epigenetic silencing impacts the rate of adaptation. To understand this, we constructed several yeast strains with a reporter gene inserted at different positions within subtelomeric, chromatin silenced, region. This resulted with a range of yeast strains with different degrees of heritable epigenetic repression. We placed the populations of these strains in an environment where it is deleterious for them to express the reporter gene and monitored their adaptation. We observe that populations with higher levels of epigenetic silencing survive better in the novel environment. Surprisingly, the beneficial mutations appear faster in the populations with intermediate levels of silencing, indicating that there is an optimal level of silencing that is beneficial for the long-term evolution.

In Chapter 3, I will present our results on how epigenetic silencing impacts the mechanisms of adaptation. To better understand this, we performed the measurements of mutation rates in our yeast strains. The mutation rates did not show any significant difference, indicating that epigenetic silencing had the dominant role in determining the differences in adaptation pattern that we observed. Moreover, by whole genome sequencing of the evolved clones we

detected an expansion of mutational targets at intermediate level of silencing. We discovered mutations in the genes encoding proteins of the epigenetic silencing machinery, indicating that adaptation occured through a mutation that modulated the stability of epigenetic silencing.

In Chapter 4, I will cover the implications of our discoveries on the evolutionary theory and open questions that should be addressed. Also I will propose a mathematical model of adaptation of a biological system in the presence of epigenetic silencing.

Overall, in this thesis I provide one of first direct evidences how heritable control of gene expression can impact long-term evolution.

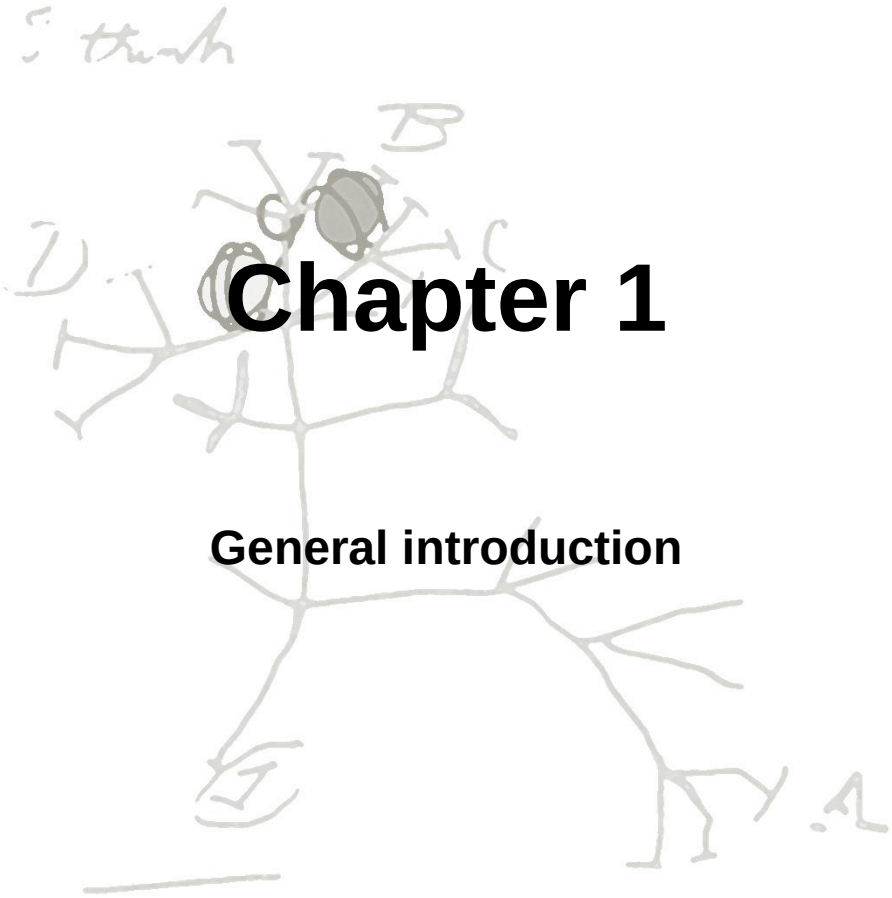
Sumário

A seleção natural atua na variação de características relacionadas com a aptidão (fitness) dentro de uma população. Herdar esta variação é de extrema importância para o processo de seleção natural. A evolução de qualquer característica herdada que esteja relacionada com o sucesso reprodutivo irá prosseguir, independentemente do mecanismo molecular por detrás dessa hereditariedade. As descobertas feitas por Mendel, bem como a sua definição da forma de hereditariedade por unidades elementares, em paralelo com a descoberta que as características fenotípicas estão codificadas em nucleótidos no ADN, conduziram à formalização da síntese moderna. Através da síntese moderna, os princípios de Mendel sobre hereditariedade por unidades elementares foram combinados com o princípio de seleção natural de Darwin. Segundo a visão da síntese moderna, são os genes que carregam a informação fenotípica.Dentro de uma população, a variação de fenótipos é causada por mutações espontâneas (alterações nos genes) que proporcionam a existência de diferentes variantes de genes (alelos). Estes genes são herdados através de unidades como entidades separadas que são distribuídas na descendência. A Seleção Natural atua na variação fenotípica causada pelas diferenças nos genes, preservando as variantes que são mais favoráveis. Consequentemente, uma alteração na frequência de alelos numa população acontecerá no curso da evolução. Segundo esta perspetiva, a hereditariedade tem uma base genética, independente do ambiente cujo papel é somente de força seletiva. Contudo, a informação para o desenvolvimento de um fenótipo particular pode ser transmitida através de mecanismos fora da sequência de ADN. Estas formas epigenéticas de hereditariedade são

compostas por vários mecanismos moleculares que estão tipicamente envolvidos no controlo de expressão de genes e que podem ser mantidos ao longo das divisões celulares durante o desenvolvimento de um individuo; podem até mesmo ser transversais a várias gerações independentemente da sequência de ADN. Estes mecanismos incluem processos como metilação de ADN e modificação de histonas, podem ter um efeito profundo no fenótipo e, tal como foi demonstrado, podem persistir por várias gerações. Assim, em princípio, a seleção natural poderia atuar na variação induzida por estes modos de hereditariedade. No entanto, se assim acontece ou não, ainda é desconhecido. Eu propus-me tentar compreender a sua possível contribuição para o processo evolutivo. Nesta tese descreverei as experiências conduzidas para abordar esta questão. No Capítulo 1 introduzirei a importância da hereditariedade no processo de evolução. Adicionalmente, abordarei os exemplos de herança epigenética transgeracional, bem como o sistema modelo que usarei nas experiências.

No Capítulo 2 apresentarei os nossos resultados em como silenciamento epigenético tem impacto na taxa de adaptação. Para compreender isto, construímos várias estirpes de levedura com genes repórteres inseridos em diferentes posições dentro da região subtelomérica e silenciada pela cromatina. Isto resultou numa gama de estirpes de levedura com graus diferentes de repressão epigenética hereditária. Populações destas estirpes foram introduzidas num ambiente onde é prejudicial expressar o gene repórter e monitorizámos a sua adaptação. Observámos que populações com maior nível de silenciamento epigenético sobrevivem melhor no novo ambiente. Surpreendentemente, as mutações benéficas aparecem mais depressa nas populações com níveis de silenciamento intermédio, indicando que há um nível ideal de silenciamento que é benéfico para a evolução a longo prazo.

No Capítulo 3 apresentarei os nossos resultados que mostram como o silenciamento epigenético tem impacto nos mecanismos de adaptação. Para melhor entender esta questão, procedemos às medições de taxas de mutação nas nossas estirpes de levedura. As taxas de mutação não demonstraram nenhuma diferença significativa, indicando que as diferenças no silenciamento epigenético foram determinantes para as diferenças no padrão de adaptação que observámos. Além disso, através do sequenciamento do genoma completo dos clones evoluídos, detetámos uma expansão dos alvos de mutação no nível intermédio de silenciamento. Descobrimos mutações nos genes que codificam proteínas implicadas no silenciamento epigenético, sugerindo que a adaptação ocorre pela mutação que modelou a estabilidade do silenciamento epigenético. No Capítulo 4 abordarei as implicações das nossas descobertas na teoria de evolução e questões que deverão ser discutidas no futuro. Também proporei um modelo matemático de adaptação de um sistema biológico na presença de silenciamento epigenético. De modo geral, providenciamos uma das primeiras provas de como a herança do controlo da expressão dos genes pode ter impacto na evolução a longo prazo.



Chapter 1

General introduction

Abstract

The study of variation in characters across biological systems conducted by early taxonomists showed clearly that different species share many similar characters and are closely related to each other. The formulation of the Theory of Evolution provided a mechanism underlying this similarity and the relations between species. In its terms, through the accumulation of beneficial variation in the observable characters (phenotypes) over time, groups of individuals diverge and form new species. Inheritance is an essential component of this process. If the variation in the phenotypes is not inherited evolution would not proceed. The developmental program of phenotypic traits (information) can be passed to the next generation by several interconnected mechanisms. The most important of those is the genetic, DNA based, system in which the information is contained within the sequence of nucleotides that makes up the DNA molecule. This information is independent from environmental regulation but subject to occasional random changes. These alterations in genes affect the variation in phenotypes and are subject to natural selection during the course of evolution. However, information can be passed also through the heritable changes in gene expression. These mechanisms include processes such as DNA methylation, histone modifications and gene silencing mediated by RNAs. Contrary to mutation, these processes can be influenced by the environmental cues but are inherited to a lesser extent than in the case of genetic information. Nevertheless, they can have a significant impact on phenotypes that are under selection. In this chapter, I will cover the importance of the principle of inheritance in evolutionary terms, the characteristics of epigenetic based control of gene expression and its possible implications in evolution.

Historical perspectives on evolution and the principle of inheritance

The diversity of the natural world and abundant variation of its biological systems has captivated and inspired philosophers and scientists for centuries. From the work of early taxonomists in the 17th century it became clear that many separate biological groups share similar morphological characters. John Ray (1627-1705), who was the first to give a biological definition of species, used to classify these groups according to their similarity/difference in certain morphological traits. The classification of the species by Carl Linaeus into higher order groups reflected this morphological relatedness. However, for him and many of his contemporaries species were fixed and unchangeable (Mayr, 1982). Nevertheless, it remained unclear how these species are related to one another and where this similarity comes from. Continued research on the variation within and between species, as well as the study of their geographical distribution, lead to the first formulations of evolutionary theory that provided the connection. The first scientist to introduce the concept of slow transformation of species was Georges-Louis Leclerc, Compte de Buffon (1707-1788). He proposed, based on the analysis of geographical distribution of animals, that species can improve or degenerate certain characters. Also he was the first scientist to point out the importance of inheritance of parental characters in this process. However, he is not considered an evolutionary biologist, in spite the claims that species can change (Mayr, 1982). Buffon in the end did not provide the mechanism by which species can modify. This was attempted by Jean-Baptiste Lamarck (1744-1829) in his work "Zoological Philosophy-an exposition with regard to the natural history of animals". He postulated two laws that govern the change of species. The first one is that individuals within a population use and disuse

certain organs, which is in turn dictated by the environmental conditions, leading to the improvement or degeneration of that organ within individual´s lifetime. Moreover, the second law stated that this characteristic that has now been acquired will be inherited by the next generation (Lamarck, 1809). In Lamarck´s terms, environment plays a crucial role in which the same environmental cues act as an inductive and selective force (Figure 1.1). More importantly, for Lamarck inheritance is an essential and inseparable part from the process of evolution.

Later formulations of evolutionary theories by Charles Darwin (1809-1882) and Alfred Wallace (1823-1913) also acknowledged the importance of inheritance. Darwin, in his work “On the origin of species”, defined several processes that are part of long-term evolution (Darwin, 1859). For him, being a naturalist, an abundant variation in observable characters between individuals within a population was evident. This variation forms a basis of evolutionary process. However, the sole existence of variation is not enough. It has to be transmitted to the next generation, otherwise evolution would not happen. In Darwin´s words, “Any variation that is not inherited is unimportant for us.” The variation that occurs within a single generation and is not transmitted to the next one has no effect on evolution. The next important component comes from the Malthusian principle, by which populations with unlimited resources will always geometrically increase in number. However, since resources are never limitless and conditions of life not always favorable, this will bring a struggle for existence between individuals. During this struggle, favorable variation is going to be preserved. Over time these slight beneficial variations will accumulate leading to the formation of new species. This forms the core of the process of Natural Selection. In Darwin´s terms, the environment has a sole role as a selective force for more favorable variants (Figure 1.1). Variation comes from the

reproductive system of parents, independently of the environment. This is in contrast to Lamarck´s view of the environment as an inducer of variation (Figure 1.1). Although he allows the possibility that processes such as disuse of organs or environmental influence on reproductive organs can happen, this influence he regarded as marginal. Darwin did not know the basis of the inheritance system nor

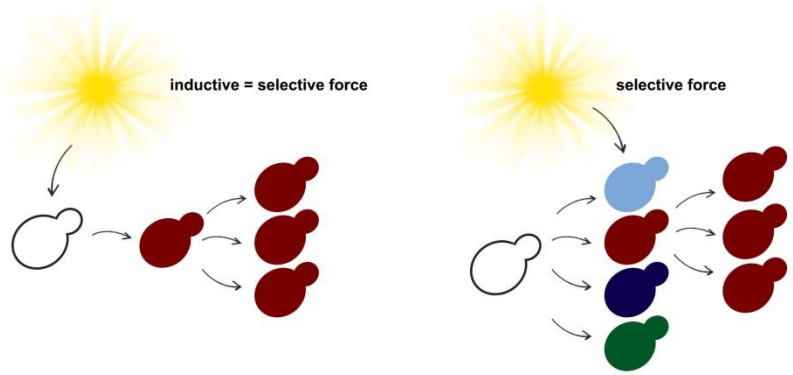


Figure 1.1 Principal differences between Lamarck´s and Darwin´s view on evolution. For Lamarck the environment has a primary role in inducing a phenotype in an individual that is subsequently inherited (left). For Darwin the variation comes from the reproductive system of the individual. The environment selects for beneficial characteristics that are then propagated (right). For the consistency with the remainder of the thesis individuals are here represented as budding yeast cells.

how the variation comes about in the reproductive organs, even though he did have his own views on it. He was a supporter of pangenesis. In his book, “The variation of animals and plants under domestication” (1868), he argued that each part of the body produces particles, called gemmules, that are transported to the reproductive organs (Darwin, 1868). In the offspring these gemmules coming from the parents would blend and determine the characteristic of the individual. The environment can influence different organs causing changes in gemmules. This allows the inheritance of acquired characteristics, which Darwin thought was a principle cause of variation observed in nature.

Nevertheless, he claimed that the process of Natural Selection will occur on any variation in a trait that is inherited, regardless of its basis of inheritance. In his words “owing to this struggle of life, any variation, however slight and from whatever cause proceeding, if it be in any degree profitable to an individual of any species…, if useful, is preserved” (Darwin, 1859).

In parallel to Darwin´s work, Gregor Mendel (1822-1884) was conducting his experiments on the basis of inheritance. His paper, which was published in 1865, showed clearly the particulate nature of inheritance, that observable characters are based on invisible factors that are segregated in parent´s reproductive organs and assorted independently in the offspring (Mendel, 1865). While remaining separate during their own time both Darwin and Mendel´s works would complement each other and lead to unified theory.

Another work that greatly influenced evolutionary biologists was that of August Weismann (1834-1914), who published his book “The germ plasm: a theory of inheritance”(reviewed in Churchill, 2003). By his concept, the information is passed to the next generation only by germ cells that separate early on from the cells of the rest of the body (somatic cells). Information in the germ cells is therefore independent from the environmental influences that might exist on organs. In that way Weismann discarded Lamarck´s notion of environment as an inductive force.

Rediscovery of Mendel´s work in 1900 by Hugo de Vries (1848-1935) made it clear that it was Mendel´s factors of inheritance in the germ cells that were responsible for the transmission of heritable traits (Lenay, 2000). It was Hugo de Vries who named these factors “genes” (de Vries, 1892), and who used the term “mutation” to describe the appearance of variation in characters differing significantly from other individuals of the same species (Lenay, 2000).

Thomas Morgan (1866-1945) continued the work of studying mutations and their inheritance in *Drosophila melanogaster*. He was the first to place genes on chromosomes, cellular structures discovered earlier and suggested to be carriers of information by Theodor Boveri and Walter Sutton (Gilbert, 1998). Morgan´s work finally paved way to the unification of Mendel´s principle of heredity and Darwin´s evolution by Natural Selection. The process of unification of the two theories lead to the formulation of Modern Synthesis. The first contribution to this came from Ronald Fisher. As a statistician he made considerable contributions to population genetics. He wrote "The Genetical Theory of Natural Selection" in which he used the particulate nature of Mendelian inheritance and incorporated it into the evolutionary framework (Fisher, 1930). He showed that the fitness increase depends on genetic variance at any given time. Fitness here represents the reproductive success of an organism, or in other words the average contribution to the gene pool of the next generation. He also defined the impact of spontaneous mutations on fitness during the process of evolution, arguing that only mutations with small effects on phenotypes would be beneficial. With the work of Sewall Wright and John Haldane, who devised methods of calculating gene frequency change during the process of evolution, the Modern Synthesis was born. Many population geneticists such as Theodosius Dobzhansky and Ernst Mayr, to name a few, contributed to further development of the theory. Covering all their work and contributions is out of the scope of this thesis. However, it is important to summarize the formulation of the Modern Synthesis. Genes are carriers of the phenotypic information. Variation in the phenotypes within a population is caused by spontaneous mutations (changes in genes) that bring about the existence of different gene variants (alleles). These genes are inherited in a particulate manner, as separate entities that are assorted in the offspring. Natural Selection acts upon the phenotypic variation

caused by the differences in genes, preserving more favorable variants. As a consequence a change in frequency of alleles within a population will happen during the course of evolution (Huxley, 1942). In this view, inheritance has a genetic basis, independent of the environment, which has a role only as a selective force. In Fisher´s view, phenotypic variation (Vp) that can be selected for, comes from the genetic variation (Vg) (Fisher, 1930).

However, a way that a gene produces a phenotype (genotype-phenotype map) is not always straight forward. It was known for some time that individuals with the same genotype can produce different phenotypes depending on the environment that they are experiencing, a phenomena known as phenotypic plasticity (Bradshaw, 1965). In this case the environment acts as an inductive force. For example, butterflies exhibit different wing color patterns depending on the temperature of the environment (Shapiro, 1984). Also water fleas (*Daphnia longicephala*) develop differently if a predator is present in the water. This phenotypic change is maintained for a few subsequent generations even when the predators (initial cue) are removed from the environment (Eads et al., 2008).

Conrad Waddington (1905-1975) also noticed that different mutant phenotypes in *Drosophilla* are extremely sensitive to environmental influences. Moreover, also in his work, it seemed that an induced phenotype can be transmitted to the next generation in the absence of the initial cue (Waddington, 1942). To reconcile these findings with the views of the Modern Synthesis he proposed a concept named “genetic assimilation”. In this process, the environment interacts with the underlying genetic developmental network to produce a phenotype. Through this interaction the so-called epigenetic landscape is created that determines the cell fate during development. The landscape itself can be changed either by mutation or by an environmental influence. In these terms the environment acts as inductive and selective force.

The environment first induces a plastic phenotype that then subsequently becomes gradually fixed by a mutation in the genetic network (Waddington, 1959). In this way, certain acquired characteristics can be selected for in a Darwinian manner.

Therefore, the phenotypic variation (V_p) depends not only on the genetic component (Vg), but also on the environmental contribution (V_e) and the variation that is a result of the interaction between the genes and the environment (V_{gxe}), or $V_p = V_g + V_e + V_{gxe}$ (Falconer et al., 1996). The ratio of genetic variance and total phenotypic variance represents the proportion of phenotypic variation that is due to genetic component. This parameter is known as heritability.

Although the environment cannot influence the types of changes that occur in the DNA, it can modulate the rate of that change. One such example is found in bacteria. In the stationary phase of growth, under starvation, bacteria produce a subunit of DNA polymerase, rpoS, who in turn regulates the transcription of particular set of genes as a response to stress. Consequently, there is rpoS-dependent decrease of mismatch repair proteins that recognize the wrongly incorporated nucleotide and removes it. As a result there is an increase of the mutation rate (Saint-Ruf and Matic, 2006). Within a certain range this mutation rate can be advantageous for the process of adaptation. Genetic variation created in such process contributes to the phenotypic variation and subsequent process of adaptation (Tenaillon et al., 2001; Bjedov et al., 2003).

Moreover, the effect of a mutation on a phenotype may also depend on the presence of other alleles in the genome, a phenomenon known as epistasis that was first described by Wiliam Bateson (1861-1926). This further complicates the way genotype-phenotype maps are constructed (Phillips, 2008).

In this context, the aim of the thesis is to understand the effect of additional components of phenotypic variation that are imposed by non-genetic inheritance.

We are taking an experimental evolution approach to define this phenomenon and to understand its underlying mechanisms. Modern day experimental evolution experiments provide detailed insight into mechanisms and paths of adaptation. Whole genome sequencing has revolutionized our understanding how beneficial mutations determine the fate of a population under selection. A prominent example is one that has been maintained for over 60 000 generations. The data shows rapid adaptation with several mutant clones arising and competing with each other, in a phenomenon known as clonal interference (Good et al., 2017). Attempts are being made to understand also the contribution of non-genetic elements to the phenotypic characteristics and to the process of adaptation. One such phenomenon is antibiotic tolerance, a physiological condition that enables bacteria to survive the antibiotic treatment without acquiring resistance through mutation. This process was shown to affect the subsequent molecular evolution and adaptation (Levin-Reisman et al., 2017).

As we have seen above, if there is a variation in phenotypes that are inherited, no matter the basis of that inheritance, the process of Natural Selection will occur. Genetic inheritance is one clear mechanism by which information is passed to the next generation. However, more recently, studies from molecular and cell biology clearly showed that in certain cases phenotypes can be induced by the environment and inherited independently of genetic change. These phenomena, their molecular mechanisms and their potential contribution to the phenotypic variation and to the process of evolution will be discussed in the next section.

Transgenerational epigenetic inheritance

The first biological definition of epigenetics was given, as mentioned above, by Conrad Waddington. He used it to describe the interaction of a genetic network with the environment that results in epigenetic (beyond genes) canalization or instruction for the developing organism. In this context, epigenetics is restricted to the development of an individual.

Here, I will talk about epigenetics in more modern and restricted definition. By epigenetic inheritance I consider any phenotype that is inherited yet not directly instructed by the genes but rather by independent self-perpetuating mechanisms. Epigenetic forms of inheritance are comprised of many molecular mechanisms that are typically involved in the control of gene expression and can be maintained across cell divisions during the development of an individual or even across generations independently of the DNA sequence. Those mechanisms include processes such as DNA methylation, histone modification, regulation of gene expression mediated by RNA and other self-sustaining feedback loops (Jablonka and Raz, 2009). Here, I will mostly focus on DNA methylation and histone modifications, since there is clear evidence that the gene expression pattern established by these epigenetic marks can be inherited transgenerationally.

However, it is also important to mention that there are forms of non-genetic inheritance that are not involved in control of gene expression, as is the case for inheritance of prions. Prions are proteins that adopt particular conformation and can act as templates in determining the conformation of other proteins. More importantly, it was shown in budding yeast that they can be inherited across cell division through

cytoplasm and also can have significant impact on the phenotype (Halfmann et al., 2012).

Before I present examples of epigenetic mechanisms of control of gene expression and of their inheritance it is important to point out that no epigenetic molecular machinery is indeed independent of the genome. Firstly, because the machinery itself is encoded by genes. Secondly, many if not most epigenetic marks are sequence determined, e.g. via transcription factor binding. However, what makes them epigenetic is that within a given DNA sequence context, different states can be propagated, e.g. active or silent gene expression (Moazed, 2011). Therefore, while the DNA sequence is important for encoding the epigenetic machinery and for targeting it, the epigenetic "state" is inherited independently as a self-maintaining system.

In eukaryotes, methylation of DNA comprises the addition of a methyl group to the cytosine residue commonly, but not exclusively, in the context of CpG sequence stretches. The methyl mark recruits other chromatin remodeler protein complexes that perform modification of neighboring histones leading to the compaction of local chromatin and consequently shutting down of expression of neighboring genes. While the addition of methyl groups is mostly sequence depended, these marks can be inherited independently. Methylation of DNA has been implicated in determining many phenotypic characteristics, such as leaf and seed color in plants (Heard and Martienssen, 2014). In bacteria, methylation occurs on adenine residues within GATC sequence stretches and is involved in mediating DNA-protein interactions, regulating gene expression and mismatch-repair mechanisms (Casadesús and Low, 2006).

Other important epigenetic molecular mechanisms include those based on histone modifications. Histones are proteins found in eukaryotic organisms that form octameric multi-protein complexes around which DNA is wrapped. However, their role is not solely

packaging DNA. Histones H3 and H4, that are part of this complex, have extensive N terminal tails that can be modified by addition of chemical functional groups such as methyl, acetyl, phosphate or ubiquitin. Unlike in the case of DNA methylation where modification leads to silencing of the genes, histone modifications have different outcomes on the gene activity depending on the type of modification. Acetylation of H3 leads to the activation of the underlying genes, in part due to opening or loosening of chromatin. Methylation on the other hand can have opposite effects depending on the residue modified. Addition of a methyl group on the lysine residue of histone H3 at positions 9 or 27 leads to repression. However, the same mark on lysine 4 activates genes (Berger, 2007). Another aspect that can impact gene expression is different histone variants. The histone H3 variant H3.3, for example, is selectively located at active gene loci and enhancers (Mito et al., 2005). Another prime example of epigenetic self-perpetuating systems is centromeres. Centromeres are chromatin structures that connect chromosomes to the mitotic spindle and insure correct separation of chromatids during cell division. In most organisms, centromeres are determined by the presence of a histone variant, CENP-A (Fukagawa and Earnshaw, 2014). The localization of this histone is largely independent of DNA sequence. For instance in animals, specific DNA sequences are neither sufficient nor necessary for the formation of the centromere. Indeed, artificial seeding of CENP-A chromatin to a previously naïve part of the genome is sufficient to lead to the formation of the centromere at that locus, independently of the underlying sequence (Hori et al., 2013; Mendiburo et al., 2011). Importantly, this newly formed centromere is stably propagated through subsequent generations (Hori et al., 2013). Moreover, germline depletion of CENP-A results in the loss of centromeres in the next generation, indicating the centromeres are inherited epigenetically through the gametes (Raychaudhuri et al., 2012).

All of the mentioned epigenetic marks can be inherited somatically, across cell division, during the development of an organism. One such example is the vernalization phenomenon in plants. Vernalization is a process by which exposure to cold is promoting flowering during spring. This phenomenon was already known in the 19th century. However, the first paper on the subject was published by Gustav Gassner in 1918. Today, most of molecular components of the process are known. It involves a repressor gene, Flowering Locus C (FLC), that represses flowering during winter (Michaels and Amasino, 1999). As a response to cold, Polycomb repressive complex 2 (PRC2) is recruited to FLC and performs methylation of lysine 27 of neighboring histone H3 at a nucleation center. Once the plants are exposed to warmer temperatures the methylation mark spreads over the histones that are located across the entire gene, causing a stable repression of the gene even at higher temperatures (Yang et al., 2017).

More importantly, some epigenetic marks can be inherited not only somatically but also transgenerationally. The extent of transgenerational effect of these marks differs among organisms. In animals, these effects are suppressed by reprogramming in the early embryo, including the removal of methyl marks (Monk et al., 1987). This process is quite limited in plants, which might explain the widespread epigenetic inheritance observed in these organisms (Heard and Martienssen, 2014). However, some particular epigenetic loci seem to resist germline erasure in mice (Messerschmidt, 2012). These loci correspond mostly to imprinted genes, that are differentially silenced depending on parental origin (Daxinger and Whitelaw, 2012). All histones show certain degree of retention and are randomly distributed between two cells during the cell division (Probst et al., 2009). However, similar to DNA methylation marks, histones are reprogrammed in animals such as mice. This is happening during spermatogenesis when most histones are being replaced by

protamines (Platz et al., 1975). Nevertheless, histones with particular modifications seem to be selectively retained at promoters of certain genes in the germline (Brykczynska et al., 2010; Hammoud et al., 2009). This indicates that the capacity to pass on information through histones to the next generation is higher in mothers than in fathers. Moreover, histone modifications are believed to be inherited not only because of histone stability but also by driving their own formation in cis. For instance silent chromatin is propagated through enzymes that bind to the modification they produce (Bannister et al., 2001; Margueron et al., 2009).

The first documented description of transgenerational epigenetic inheritance came from Barbara McClintock. She noticed that transposable elements, selfish genetic elements that can jump and insert at different places of the genome, cycle between active and silent phase, and that these periods can last for a number of generations, even though the plants analyzed were isogenic (McClintock, 1961). Later it was confirmed that this process is due to the methylation of DNA located near the insertion site of the transposon. The most striking epigenetic effect of DNA methylation is flower shape in toad flax (*Linaria vulgaris*). This plant can have two types of flowers, bilateral or radial. The difference between the two groups even made Linaeus classify them as two separate species (Diggle and Coen, 2000). The cause of this variance was shown to be the methylation pattern of the gene *Lcyc* which is involved in the determination of the flower shape (Cubas et al., 1999). Importantly, the epigenetic expression state of the gene is inherited transgenerational for at least two generations.

In plants, the addition of the methyl group is performed by an enzyme, DNA methyltransferase, *MET1*. The process includes other chromatin remodelers such as *DDM1*. The mutants in these genes were shown to have completely unmethylated DNA (Vongs et al., 1993). They have

an increased transposition rate and overall mutation rate (Mlura et al., 2001), suggesting that methylation has an important role in maintaining genomic stability. These mutant lines were used to create epigenetic recombinant inbred lines (epiRILs). Recombinant inbred lines are a genetic model system that are constructed by the inbreeding of genetically diverse populations for several generations and used for determining genetic loci responsible for a particular phenotypic trait (QTL mapping). In the same line of thought, epiRILs are created by crossing the homozygous mutants in *DDM1* and *MET1* that have unmethylated DNA with wild-type counterparts. In the subsequent steps of inbreeding homozygous wild-type plants are selected (Figure 1.2).

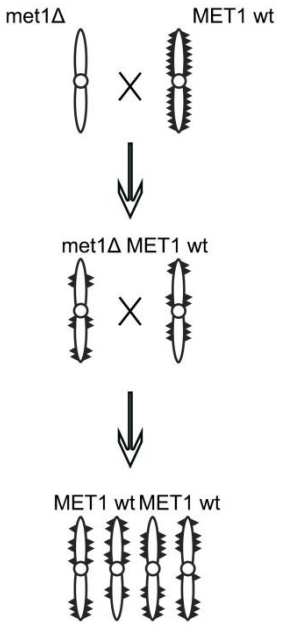


Figure 1.2 The construction scheme of epiRIL lines. Chromosomes represent genetic composition of individuals used during inbreeding. Black triangles indicate differentially methylated regions. The homozygous mutant in methylation machinery is crossed with homozygous wild-type. The heterozygous offspring is inbred and homozygous wild-type individuals are selected that have different methylation pattern across the genome.

As a consequence of this, the genome in the selected lines will be a mosaic of unmethylated parts that come from homozygous mutants and methylated parts coming from wild-type parents (Johannes et al., 2009; Reinders et al., 2009). Certain epigenetic, unmethylated loci were shown to be maintained for the length of the experiment, up to

seven generations (Johannes et al., 2009). These lines are used for mapping epigenetic determinants of particular traits (epiQTLs). In these studies it was determined that certain epigenetic markers can have heritability values close to those of genetic loci, up to 90% (Cortijo et al., 2014). This means that 90% of variance in a phenotype can be explained by the variance in epigenetic markers. In general, cases of epigenetic inheritance are more common in microbes and plants, probably due to the lack of segregation between somatic cells and germline (Jablonka and Raz, 2009). However, they do occur in animals as well.

One very good example of epigenetic inheritance in mice was described at the *agouti* locus. Upstream of this gene insertion of an IAP transposon was discovered that drives the expression of the *agouti* gene giving various characteristics to the individual, including yellow fur color. This transposon is differentially methylated and therefore, renders individuals with variegating levels of yellow fur (Morgan et al., 1999). Importantly, the particular epigenetic state, i.e. fur color, is maternally transmitted. In the case of a yellow mother, offspring tends to be yellow as well. It appears that this version of the gene in the father undergoes demethylation process in sperm cells. This transmission persists even when the fertilized eggs are implanted into another female that does not exhibit the phenotype (Morgan et al., 1999). Moreover, the degree of gene silencing and corresponding fur color can be induced by the diet of the animal which is subsequently inherited. If the mother is fed with food with high concentration of methyl donors, this results in a decrease of the frequency of yellow offspring (Daxinger and Whitelaw, 2012). This is an example of germline transmission of an acquired state and an apparent violation of the Weismann principle (see previous section). More importantly, this environmentally induced epigenetic change can be maintained for two generations (Morgan et al., 1999).

In mice it was shown that even behavioral traits can be epigenetically transmitted to the next generation. In one study, individuals were fear conditioned using a particular odor. The fear response persisted to the F2 generation even though the offspring never encountered that specific odor. The trait was inherited both maternally and paternally, even when the offspring was fostered by other parents. Although the complete molecular mechanism is unknown, differential DNA methylation of the odor receptor was implicated (Dias and Ressler, 2014). The mechanism must involve communication between nervous system and the germ line. The mediators of this communication might be RNA molecules implicated in the control of the transcription. These molecules were shown to be transported as part of exosomes, cell derived vesicles, across the body. In this case they would function as Darwin´s gemmules in transferring information from somatic to germ cells (Heard and Martienssen, 2014).

Another example of epigenetic inheritance comes from rats, where it was shown that different protein diet can induce differential epigenetic modification of glucocorticoid receptor gene that can persist for two generations (Burdge et al., 2007; Lillycrop et al., 2007).

In fission yeast, tethering of the histone methyltransferase, Clr4, through the fusion with tetracycline repressor protein (TetR), to the operator sequence inserted near a reporter gene, has been shown to cause the methylation of surrounding histones and consequently silencing of the reporter. Similar to the chromatin-based centromere, this epigenetic silencing is stably inherited through mitotic and meiotic divisions, even when the initiating sequence or the fusion protein is removed (Ragunathan et al., 2015). The histone methylation mark can be propagated for up to 20 generations in the presence of a histone demethylase, a protein that actively removes the histone mark, or even up to 50 generations in the case of absence of the demethylase (Ragunathan et al., 2015).

Transgenerational epigenetic inheritance was also shown in *Caenorabtitis elegans*. In one study it was shown that exposure to high temperatures causes a derepression of gene array, comprised of several copies of HSP90 gene, that persists for up to 14 generations once they are transferred to lower temperatures. Moreover, it seems that the extent of inheritance depends on the time during which individuals were exposed to elevated temperatures. If exposed for only one generation, inheritance persisted for 7 generations. If the exposure was extended for 5 generations, the inheritance increased to 14 generations. This phenomenon was linked to a demethylation of lysine 9 at histone H3 on the array, that was established at the elevated temperature and maintained upon transfer to the lower one (Klosin et al., 2017).

All of these examples show clearly that epigenetic inheritance of gene expression states (e.g. via DNA methylation and histone modifications) can produce strong phenotypes, observable characters, which can be inherited to a certain extent. However, to understand their true biological significance further quantification of the extent of transgenerational inheritance is needed. It is also important to obtain the data about the epigenetic variation within populations. Further ecological studies should include a genome-wide methylome and histone data alongside the whole-genome sequences (Richards et al., 2017). In many studies conducted in plants, that used bisulfite sequencing, that reveals the position of methylated cytosines, it was shown that many epigenetic marks correlate with environmental factors. However, it remains unclear how much of causal link there is (Richards et al., 2017; Verhoeven et al., 2016).

In summary, natural selection acts upon the phenotypic variation that exists within a population. The necessary component of this process is stable inheritance of these phenotypes. Inheritance is mediated by genes as stable carriers of information to the next generation.

However, as we have seen in examples presented in this section, epigenetic control of gene expression can have an impact on the phenotype. In many organisms there is evidence that these epigenetic states can be passed to the next generations, though not as stably as in the case of genetic inheritance. The fidelity of epigenetic transmission is context dependent, differing for different organisms as well as environmental conditions.

Under strong selection, adaptation can proceed rapidly. For example, the seasonal change in allele frequencies in *Drosophila* occurs within a ten generation time frame (Behrman et al., 2018). This time period is corresponding to the extent of inheritance of some epigenetic states (Figure 1.3). This leads to the question whether epigenetic mechanisms can be selected for? What is their contribution to the process of evolution? How do those mechanisms interact with the underlying genetic network during the process of evolution?

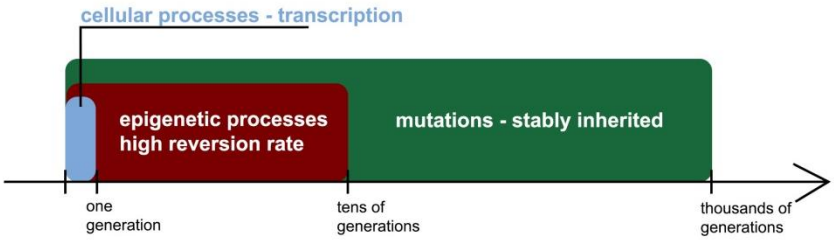


Figure 1.3 The timescale of inheritance. The arrow represents a time period for which particular form of inheritance (differently colored boxes) persists. Mutations are stably inherited and are a substrate for natural selection. On the other hand, certain cellular processes are maintained for only one generation and are of no importance for evolution. Epigenetic states of gene expression can be maintained for 10s of generation which is the time period we can observe adaptation under strong selection.

I set out to answer those questions. As my model system I chose budding yeast, *Saccharomyces cerevisiae,* an organism that allows me to control for both genetic and epigenetic variation. In the next section I will cover more in depth the epigenetic control of chromatin and gene expression in this unicellular organism.

Epigenetic inheritance in budding yeast

Budding yeast (*Saccharomyces cerevisiae*) is a unicellular eukaryote that belongs to the fungus phylium of *Ascomicota* (Figure 1.4). The name comes from the feature of its asymmetric cell division. It divides by the formation of a bud on the surface of the mother cell that enlarges and separates, forming a new daughter cell (Figure 1.4B). Yeast represents an easily controllable genetic system. The generation time is around 90 minutes. Growth conditions and construction of mutant strains is fairly similar to the manipulation of bacteria. Insertion of DNA cassettes into the genome is relatively straightforward, due to efficient recombination in yeast. Moreover, plasmids, circular episomes that are propagated outside of the genome, can be introduced stably into yeast cells. Selection of strains can be performed using auxotrophic markers like in bacteria. Auxotrophic strains carry a mutation in a gene that renders it unable to synthetize a particular essential compound (Duina et al., 2014).

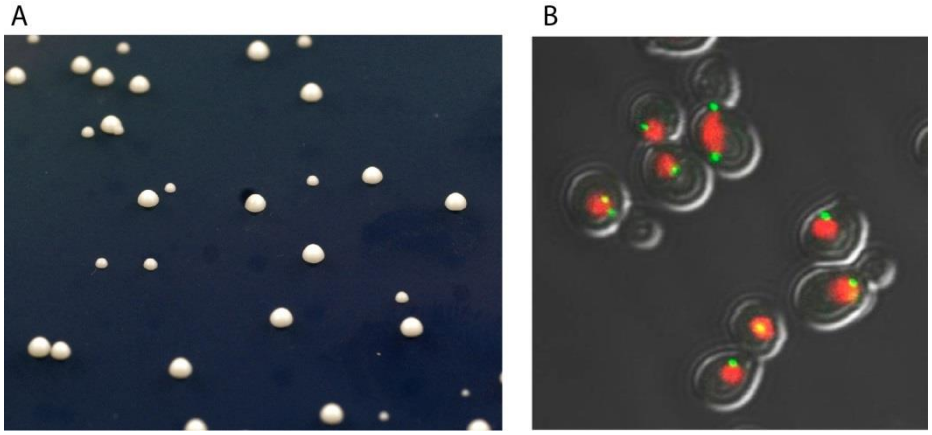


Figure 1.4 Yeast as a model system. (**A**) Photo showing yeast colonies forming on an agar plate. (**B**) Photo of yeast cells taken using confocal microscope. Red fluorescent protein is used for labeling the nucleus of the cell and green fluorescence protein is marking the spindle pole body, a cellular structure involved in the formation of the mitotic spindle during cell division. Photo adapted from Duina et al., 2014.

Some of the most common marker/reporter genes used in manipulation with yeast are *URA3* and *ADE2*. The protein product of the *ADE2* gene is involved in adenine biosynthesis. When the gene is active, the yeast colonies have normal white color (Figure 1.5A). However, when the gene is inactive and adenine is limiting, a precursor of adenine biosynthesis accumulates in cells giving colonies a red color (Grunstein et al., 2014; Roman, 1956).

Analogous to adenine biosynthesis, the Ura3 protein is essential for the biosynthesis of uracil. However, if a drug called 5-fluoroorotic acid (5-FOA) is added to the yeast medium, Ura3 protein transforms the drug into 5-fluorouracil (5-FU) that is toxic for the cell and causes its death (Figure 1.5B)(Boeke et al., 1987). As a result *URA3* can be selected for both positively and negatively where absence of uracil renders expression essential, while in the presence of 5-FOA, *URA3* gene expression is lethal. Such “marker” genes have been central to the development of yeast as a genetic powerhouse.

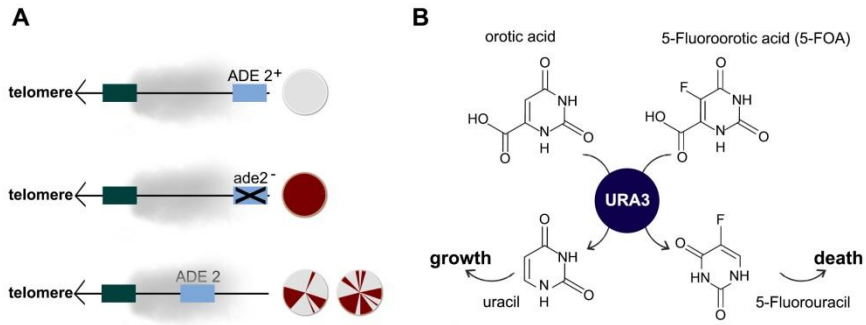


Figure 1.5 Most commonly used reporter genes in budding yeast. (**A**) The activity of *ADE2* gene confers color to the yeast colony. If it is active the colony is of white color. In the case of the *ADE2* knock-out the colony is of red color. However, if the gene is being epigenetically silenced colonies have variegating white and red color sectors. The pattern of these sectors depends on the strength of the silencing. (**B**) Scheme outlining the chemistry and principle of the counter-selection using *URA3* as reporter gene. Ura3 is essential for biosynthesis of uracil (positive selection in the absence of uracil), but converts the drug 5-FOA into a toxin that kills the cell (negative selection).

DNA methylation and RNA mediated gene silencing mechanisms are completely absent from yeast. Instead, the epigenetic control of gene expression is largely achieved through chromatin structure. Unlike in higher eukaryotes, budding yeast has only two copies of each of histone H3, H4, H2A and H2B genes. This makes the analysis of histone deletion mutants and mutants at the sites of histone modifications much easier (Grunstein et al., 2014).

The heterochromatin-like, histone mediated silencing in yeast is occurring at three distinct loci, mating type loci, telomeres and rDNA locus. There are many protein components involved in this process, but the most important of those are the Silent Information Regulator (SIR) proteins. The components of this complex are Sir1, Sir2, Sir3 and Sir4 (Rine and Herskowitz, 1987).

Silencing in mating type loci is crucial for the sexual reproduction in yeast. The yeast cells can be in either a haploid or diploid state. While typically diploid cells that are under stress undergo meiosis forming four haploid cells. Haploid cells can be of two mating types, **a** and α. When haploid, the main role of silencing the mating type loci is to ensure that the yeast cell is in one of two opposing mating types. For cells to mate two cells have to be of opposite mating types. The "**a**" cell produces a protein called **a** factor that binds to the **a** receptor on the α cell. Conversely, α cells produce α factor that binds to the α receptor on **a** cell. As a result protrusions on each cell are formed that end in fusion of haploid cells forming a diploid.

The mating type is determined by the genes expressed in the MAT locus. For example, an **a** cell will have **a** determining genes in the MAT locus (Rine et al., 1979). However, there are additional copies of **a** genes at HMR**a** locus, as well as α genes at HMLα locus. These genes are silenced to enable only the copies present at MAT locus to determine the mating type. However, cells can undergo a switch of the mating type, each haploid generation. This process is performed by

the HO endonuclease that causes a double strand cut in the MAT locus that is repaired by recombination from either HMLα or HMR**a** locus (Oshima and Takano, 1971). If the repair is performed by recombination with the gene from opposite type, the gene in the MAT locus will change and consequently the mating type. Chromatin plays crucial role in this process. Defects in the silencing machinery express both mating types and consequently behave as diploids, i.e. they cannot mate. Using this characteristic as a read-out, protein components responsible for silencing, such as Sir proteins, were discovered (Rine et al., 1979).

The rDNA locus is comprised of an array of 150-200 copies of rDNA genes. Since they all have similar sequences, they are prone to recombination. During this process circular rDNAs are formed and accumulated in mother cell. Their accumulation was linked to the process of senescence (Sinclair and Guarente, 1997). The Sir2 protein was shown to have important role in silencing the rDNA locus and lowering recombination rates. Mutations in *SIR2* shows an increase in recombination and amount of circular rDNA leading to faster senescence (Gottlieb and Esposito, 1989).

Telomeres are another genomic region that exhibit epigenetic silencing. If a reporter gene, such as *ADE2*, is positioned within the subtelomeric region, variegation of its expression is detected, observed as a colony on the plate with white and red sectors indicating clonal populations of cells in which the gene is either expressed or silent, either state being inherited within that clonal population. This is due to stochastic switching between silenced and active state of the gene at a timescale that exceeds that of the cell cycle (Gottschling et al., 1990). The phenomenon is reminiscent to that of position effect variegation of the *white* phenotype in the eye of *Drosophila melanogaster*, when the *white* gene is placed close to silent heterochromatin (Elgin and Reuter, 2013).

The molecular mechanism underlying silencing at all these yeast loci is similar. It is dependent on silencer sequences (core X element in case of the telomere) to which the protein Abf1 is recruited. Additionally, Rap1 is recruited at the telomere end, in a sequence dependent manner. These sequence binding proteins interact with the Sir complex through Sir3 or Sir4 (Figure 1.6) (Moretti et al., 1994). Sir1 is acting in the initial nucleation step by connecting the Origin Recognition Complex and Sir4. However it is not crucial for the maintenance of the epigenetic state (Triolo and Sternglanz, 1996). Although the sequence is required for the establishment of the mark, its maintenance is based on self-perpetuating mechanism and the extent of silencing itself is independent of the sequence (Moazed, 2011).

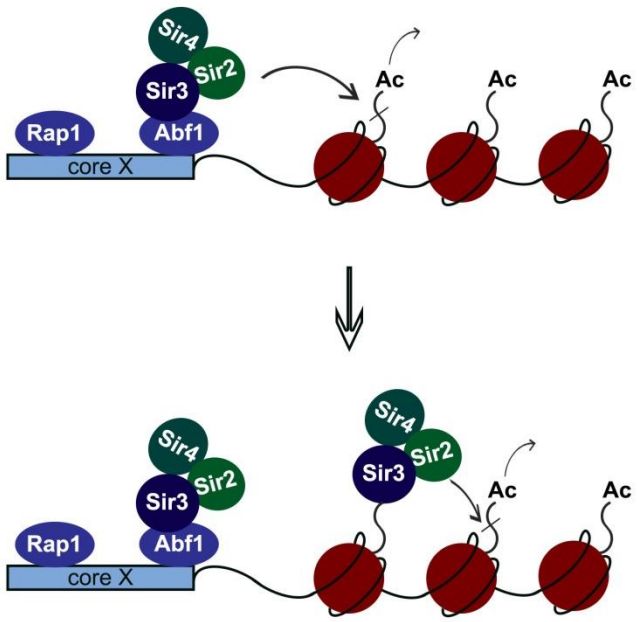


Figure 1.6 Dynamics of subtelomeric silencing. Sir complex is recruited to the nucleation center in a sequence dependent manner. Sir2 deacetylates neighboring histone that recruits new Sir complex through binding of hypoacetylated chromatin done by the interaction with Sir3. In this way epigenetic silencing is established and can spread to neighboring nucleosomes.

Importantly, while sequence is necessary for establishment of silencing it is not sufficient. Silencing only occurs once deacetylated histones are nearby. The crucial role in this step is that of Sir2. Sir2 is a NAD^+ dependent histone deacetylase that removes acetyl group from lysine at position 16 of histone H4 (Imai et al., 2000). Deacetylated histone acts as a recruiting center for Sir3 protein bringing the Sir2-Sir4 heterodimer, consequently removing the acetyl group on the neighboring nucleosome (Figure 1.6) (Carmen et al., 2002; Johnson et al., 1990). In this manner, the deacetylation wave spreads, leading to the compaction of the local chromatin and silencing of the genes (Grunstein et al., 2014; Moazed, 2011).

The spread of epigenetic silencing is counteracted by the action of histone acetyltransferases who add an acetylation mark to lysine residues of histones. The deletion of one such protein, Sas1, was shown to extend the telomere silencing (Kimura et al., 2002). Other histone modifiers, like the histone methyltransferase, Dot1, can also counteract the spreading of repressive marks by adding methyl group to lysine at position 79 of histone H3 (Van Leeuwen et al., 2002). Sir3 and Dot1 compete for the same binding site at H4. In this process Sir3 has a higher affinity for deacetylated histones. However, Dot 1 doesn´t discriminate between the two acetylation states. Consequently, an equilibrium is being established, where Sir3 binds to histones in silenced regions and Dot1 binds H4 in euchromatin regions and methylates H3. The methyl mark interferes with binding of Sir3 to the deacetylated histone and in such a manner stops the spreading of the deacetylation wave (Altaf et al., 2007).

Telomeres and their subtelomeric regions can be quite different in their sequence content, depending on the chromosome or even chromosomal arm. In general, there are two types of subtelomeric regions, the ones that contain only a core X element that contains the binding site for Abf1, and ones that have an additional coding Y´

element. The Y´ element usually contains a gene encoding a DNA helicase and is located between the telomere and core X element (Louis et al., 1994). Consequently, telomeres containing this element show discontinuous silencing with peaks of silencing right before the Y´ element and after the core X (Pryde and Louis, 1999). As a result silencing between these regions can vary significantly. However, it is in all cases Sir-dependent (Pryde and Louis, 1999).

The already mentioned reporter genes were used in many studies in which genes were inserted into subtelomeric region and used as a readout for silencing and its permanence. For *ADE2* the appearance of red color was used as a proxy for the strength of silencing. In the case of *URA3* resistance to the drug, 5-FOA was used as a measure (Grunstein et al., 2014). In one study, the red color resulting from *ADE2* gene silencing was used to estimate the epigenetic switching rate of silent chromatin at yeast telomeres. From a clonal population of red cells (silent) the rate of appearance of white sectors (active) was used as a measure to indicate a switch happened in a lineage that activated the gene. To measure the number of generations by which the silent epigenetic state was maintained the distance from the center of the colony to the white sector was measured. In this way it was estimated that a particular epigenetic state was maintained for around 20 generations (Gottschling et al., 1990).

In a more recent study, the epigenetic switching rate was measured more directly. Yeast cells were labeled with GFP flanked with LoxP sites with a downstream RFP gene. Upon recombination of GFP, cells lose green fluorescence and gain red fluorescence. Cre recombinase, the enzyme that catalyzes recombination between LoxP sites and performs the recombination and excision of, in this case GFP, was integrated in the silenced mating type loci, HMLα and HMR**a**. Effectively, Cre is used as a reporter for silencing where loss of silencing results in a switch in the color of the cell. In this way, it was

determined that the epigenetic switching rate is $1.6*10^{-3}$ in HMLα and $7*10^{-4}$ in the case of HMR**a** (Dodson and Rine, 2015).

Studies using *URA3* inserted in the subtelomeric region at different position showed that silencing is dependent on the distance from the telomere (Pryde and Louis, 1999). Moreover, the frequency of resistance to the 5-FOA in this case is a result of an equilibrium being established between cells that have the gene inactive and those ones that can still express *URA3*. This equilibrium will depend on the switching rates from on to off and vice versa. The switching rates can be measured by preselecting the cells to be in an active or repressed state, by growing them on media lacking uracil or on media with high concentrations of 5-FOA, respectively and then transferring them to non-selective media. In this case, changes of frequency of resistant and 5-FOA sensitive cells over time will be a result of switching rate of the gene to an off and on state, respectively (Jeffery et al., 2013).

All of these examples show that the epigenetic control of gene expression in yeast can be easily characterized and the strength of silencing quantified. Moreover, budding yeast enables me to disentangle the genetic variation from epigenetic one by controlling for clonality using isogenic strains. To answer questions mentioned in previous section, on how epigenetic mechanisms gene regulation contribute to the adaptation, I applied the current understanding of epigenetic gene silencing in yeast and developed an experimental setup whose outline and results I will describe in the next Chapter.

Acknowledgements

I would like to thank above all my supervisors, Lars Jansen and Lilia Perfeito, for their valuable help in conceiving the study and designing the experiments and most of all for their patience and support. Also I would like to thank Patricia Beldade, Miguel Godinho, Isabel Gordo, Èlio Sucena and to the whole IGC community for thought provoking discussions that expanded my intellectual horizons. I would also like to acknowledge Inês Amaro who helped with her valuable artistic skills in designing figures in this chapter

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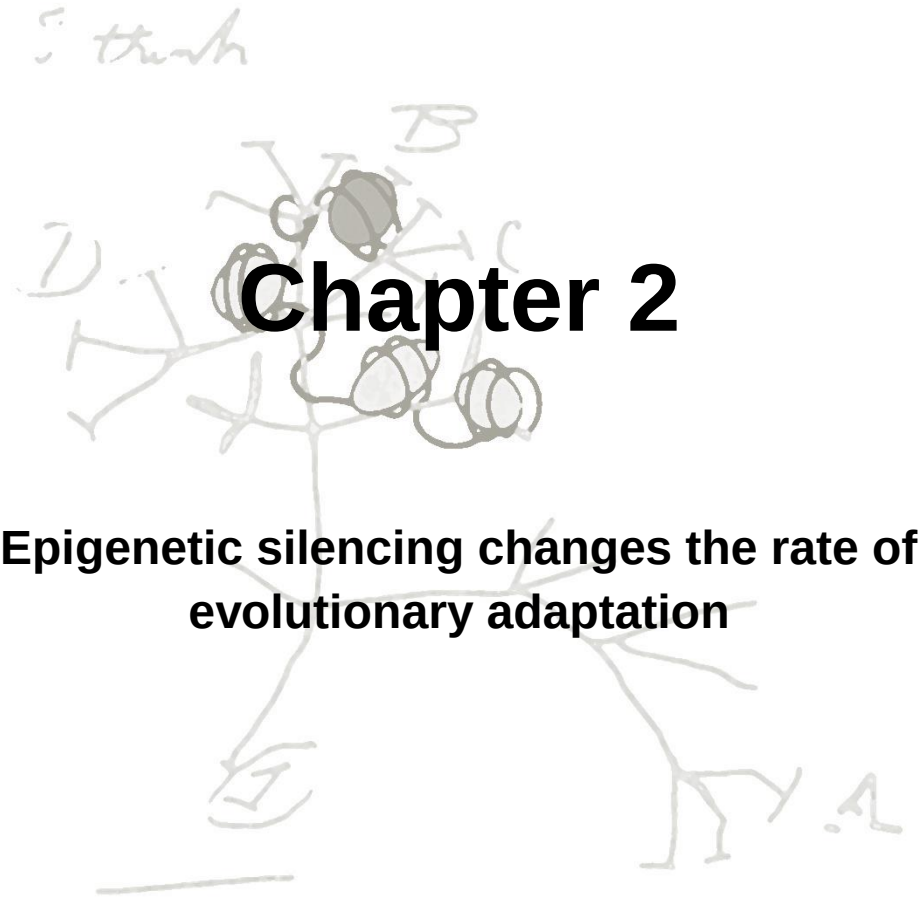

Chapter 2

Epigenetic silencing changes the rate of evolutionary adaptation

Abstract

Natural selection acts upon heritable variation in fitness-related traits. Genetic information is stably inherited and has clear phenotypic effects, which makes it a perfect substrate for Natural Selection. However, information can be transmitted to the next generation by mechanisms that are independent of genetic information. These epigenetic mechanisms of inheritance can also modulate the phenotype and can be stably inherited, although to a lesser extent than in the case of genetic information. This raises the question if these non-genetic mechanisms can have an effect on long-term evolution. Here, we test this notion by dissecting the contribution of epigenetic variation to adaptation in a novel experimental evolution setup in yeast. We placed *URA3* reporter gene at different position within subtelomeric, chromatin-silenced regions of the *Saccharomyces cerevisiae* genome. This resulted with a range of yeast strains with different degrees of heritable epigenetic *URA3* repression. Short-term heritable gene silencing results in higher rates of survival when we select against *URA3* expression. Moreover, as populations evolve the original epigenetically silenced clones are replaced with genetic mutants that reduce or abolish *URA3* expression in a process known as genetic assimilation. Interestingly, the rate of accumulation of adaptive mutations is faster in populations with an intermediate level of heritable silencing, suggesting that there is an optimal frequency of epigenetic switching that enables genetic assimilation.

Introduction

As I have introduced in the previous chapter, the existence of variation in phenotypic traits that affect survival or reproduction (fitness-related traits) within a population is an essential component of an evolutionary process. However, for Natural Selection to act upon such a population this variation should be stably inherited by the next generation. The variation that exists within a single generation and is not transmitted to the next one is of no importance for evolution (Darwin, 1859).

The understanding of the inheritance mechanisms and the discovery of the DNA as the carrier of that information led to the formulation of The Modern Synthesis. The information for phenotypic traits is encoded in the sequence of the genome. The variation is a result of the differences in this DNA sequence among individuals that arise through the accumulation of random mutations. Natural Selection leads to the survival of more favorable phenotypes, resulting in the change of the frequency of beneficial alleles within a population (Huxley, 1942).

However, information can be transmitted to the next generation also by the mechanisms outside of the DNA sequence. These epigenetic mechanisms of inheritance consist of many different phenomena that converge on regulation of gene expression, out of which DNA methylation and histone modifications are prime examples (Jablonka and Raz, 2009). The changes in epigenetic pattern (e.g. methylation status of DNA) can have large effects on the phenotype. Some of these epi-alleles can have heritability values comparable to those of genetic alleles, and can behave as a bona fide epigenetic Quantitative Trait Loci (Cortijo et al., 2014). Epigenetic inheritance is relatively short-lived, but can persist for a number of generations and contribute to selectable phenotypic variation, as highlighted e.g. by work in plants and worms (Cortijo et al., 2014; Heard and Martienssen, 2014; Johannes et al., 2009). Unlike genetic mutations, epi-mutations occur

frequently, are generally transient and reversible, a property fundamentally different from mutations in DNA where reversion of a change is typically rare (Rando and Verstrepen, 2007). The fact that epigenetic changes can affect the phenotype and persist for a number of generations raises a question to what extent and with what consequences can epigenetic mechanisms contribute to adaptation?

The first conceptual work related to this topic was formulated long before the molecular basis of the epigenetic mechanisms was known. Conrad Waddington was the first to propose that through the interaction of the underlying genetic developmental network with the environment a phenotype is produced. This allows the population to survive better in that environment and gives time for the mutation, that produces the same phenotype, to arise. This mutation would be advantageous in such environment and would quickly fix in the population. This process by which phenotype comes first, as a response to the environmental cue, followed by the genetic change is known as genetic assimilation (Lande, 2009; Waddington, 1959).

There is a large body of more contemporary work regarding the impact of epigenetic mechanisms on the process of evolution. This work predicts that an epigenetic form of inheritance can be an effective means of reaching a fitness optimum quickly by acting as a buffering step until mutations fix in the population. This can be achieved by facilitating the survival of the population in a new environment, giving it time to accumulate adaptive mutations (Bonduriansky and Day, 2009; Klironomos et al., 2013). However the benefit of a particular epigenetic state will depend on its rate of change. Models suggest there might be an optimal degree of the stability of epigenetic inheritance that can speed up evolution (Kronholm and Collins, 2015). Also, epigenetic systems of control of gene expression could help populations to explore fitness landscapes more efficiently. This would be the case, for example, when the transition between the peaks would involve

acquisition of a mutation that is deleterious and would bring population to extinction, i.e. passing through a deep fitness valley. Epigenetic silencing could buffer the deleterious effect of the mutation i.e. make the fitness valley shallower, and facilitate the survival during the transition between the peaks (Klironomos et al., 2013).

Despite the growing body of theoretical efforts, direct experimental evidence is still lacking as pointed out recently (Charlesworth et al., 2017). However, there are experimental efforts to address the problem. In a recent study done in *Chlamydomonas*, it was shown that strains that have knock-out mutations in epigenetic machinery that controls gene expression, observed reduced adaptation to stressful environments compared to the wild-type clones (Kronholm et al., 2017). Nevertheless, since the loss of epigenetic machinery can have profound pleiotropic effects on genome integrity and regulation (see Chapter 1 and 3), the reduced capacity to adapt to the new environment can be the result of many confounding effects and not directly due to the absence of epigenetic gene regulation itself.

Here we test the theoretical predictions in the presence of epigenetic mechanisms, with a novel and powerful experimental evolution setup that allows us to distinguish the contribution to adaptation of genetic mutations versus transiently heritable changes in gene expression. The strength of this system relies on the ability to control the clonality (i.e. no standing genetic variation) of the population and the rate of epigenetic modification. Key questions that I address are: i) how does short-lived but heritable gene silencing impact the rate of fitness increase, ii) the spread of new beneficial mutations and ultimately iii) the long-term adaptation of a population?

To answer these questions we are using budding yeast (*Saccharomyces cerevisiae*) as a model system. Epigenetic gene silencing in this unicellular organism occurs in three distinct genomic loci. Genes proximal to mating type loci, rDNA arrays and telomeres

undergo heritable gene silencing that is mediated by SIR protein complex (Aparicio et al., 1991; Ivy et al., 1986; Rine and Herskowitz, 1987). A protein that is part of this complex, Sir2, removes acetyl moieties from neighboring nucleosomes, which in turn act as anchors for recruitment of additional SIR complexes. This self-perpetuating feedback mechanism leads to an establishment of heterochromatin-like local spreading of silencing (Moazed, 2011). The inheritance of this silencing state was shown to persist for up to 20 generations (Gottschling et al., 1990).

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Results

We constructed an epi-allelic series of *S. cerevisiae* strains in which we inserted a functional *URA3* cassette at seven different positions ordered along the subtelomeric region of the left arm of chromosome XI (Figure 2.1A), thereby tuning the degree of epigenetic silencing in otherwise isogenic clones. *URA3* is a widely used reporter gene that can be subjected to both positive and negative selection. Functional Ura3 protein is essential for uracil biosynthesis and required for growth in the absence of uracil, but induces cell death in the presence of the drug 5-Fluoroorotic acid (5-FOA) (Figure 2.1B) (Boeke et al., 1987). Consequently, *URA3* gene silencing leads to resistance to 5-FOA (Figure 2.1C), a phenotypic trait previously used to study the nature of heritable epigenetic silencing in yeast.

Phenotypic characterization of the model system

To assess the levels of epigenetic silencing at these seven subtelomeric loci we measured the growth of the strains on 5-FOA in spot assays (Figure 2.1C) and in real-time growth rate measurements under different concentrations of 5-FOA, titrating in that way the

selective pressure within our experimental evolution setup (Figure 2.S1). As expected from previous reports (Pryde and Louis, 1999), we find that the degree of silencing in our allelic series roughly scales with the distance from the telomere, but is discontinuous, with strongest silencing observed adjacent to the core X element from which the peak

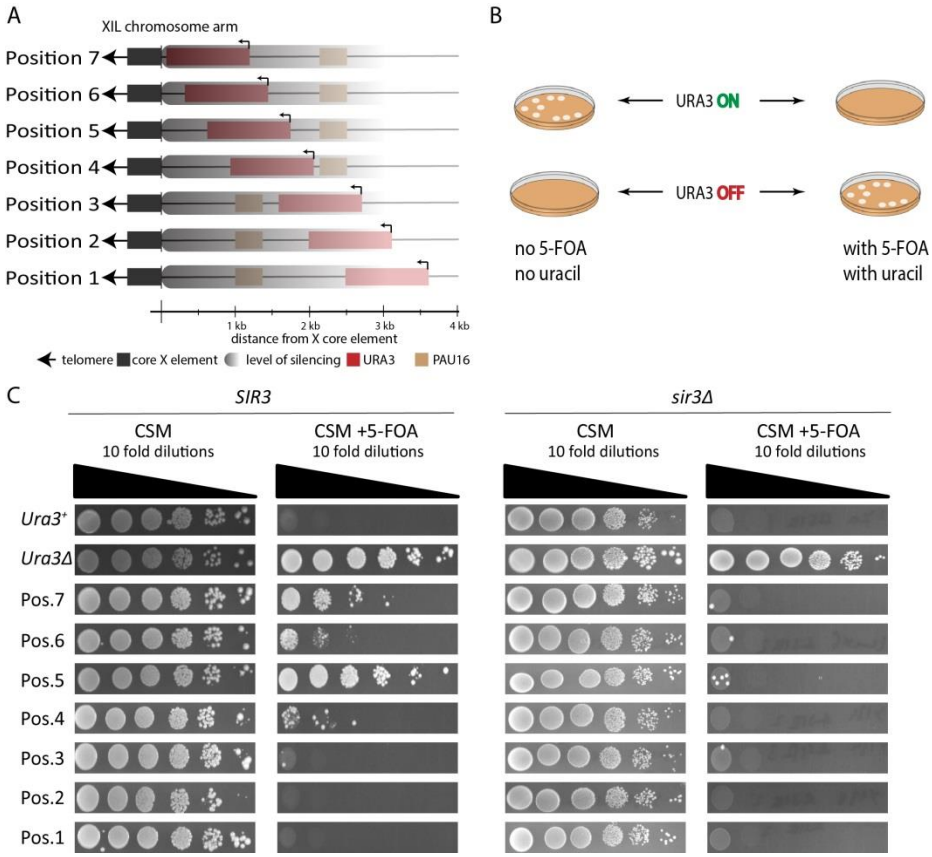

Figure 2.1. Outline and characterization of experimental system. (A**)** Scheme showing positions of *URA3* gene cassette in different yeast strains relative to the core X element near the telomere on the left arm of chromosome XI. (**B**) Scheme outlining principle of the counter-selection using *URA3* as reporter gene. *Ura3* is essential for biosynthesis of uracil (positive selection in the absence of uracil), but converts the drug 5-FOA into a toxin that kills the cell (negative selection). (**C**) Measurement of the relative strength of silencing. Ten-fold dilutions were spotted on plates containing 5-FOA or minimal non-selective media plates (CSM) as control for growth. *URA3Δ* and *URA3*$^{+}$ strains were used as positive and negative controls respectively. Deletion of *sir3* abrogates silencing at all *URA3* insertion positions (Rine and Herskowitz, 1987).

of SIR complex nucleation spreads (Ellahi et al., 2015). Silencing peaks at about 2 Kb from the telomere (Position 5) and declines in either direction where Positions 6 and 7 (closer to the telomere end) display intermediate levels of silencing and Positions 4, 3, 2, and 1 (distal to the SIR seeding point) represent low silencing levels (Figure 2.1C and Figure 2.S1). As expected (Aparicio et al., 1991), silencing at all positions is SIR complex dependent, since the deletion of *SIR3* gene completely abrogates the effect (Figure 2.1C, right panel and Figure 2.S2). From this epi-allelic series, we selected five *URA3* insertion loci for the experimental evolution representing Low (Position 4, 3, and 1), intermediate (Position 7) and high (Position 5) silencing.
Position 6 and Position 2 strains were not analyzed, since they showed very similar characteristics as Position 7 and Position 1 strain, respectively. These strains, that are otherwise isogenic, form the basis of our experimental evolution strategy where we can vary the level of epigenetic gene silencing (*URA3* position) as well as the selective pressure (5-FOA concentration). This allows us to determine the dynamics and mechanisms of adaptation in a highly defined system where a single pathway is under selection in the presence or absence of short-term heritable gene silencing.

Epigenetic silencing increases survival in a new environment

Cells were first preselected, in complete synthetic media (CSM) lacking uracil, to ensure all strains are in the URA^+ state at the onset of the experiment, irrespective of their ability to silence. Each population was then evolved by serial daily passage into liquid culture containing 0,05% 5-FOA (Figure 2.2A), a dose conferring an intermediate selection pressure in which the strains showed a range in growth rates in a manner dependent on the degree of *URA3* silencing (Figure 2.S1).

Half a million cells were transferred daily under continued selection and the number of living cells in each of the 24 replicates per strain was determined by plating on non-selective media.

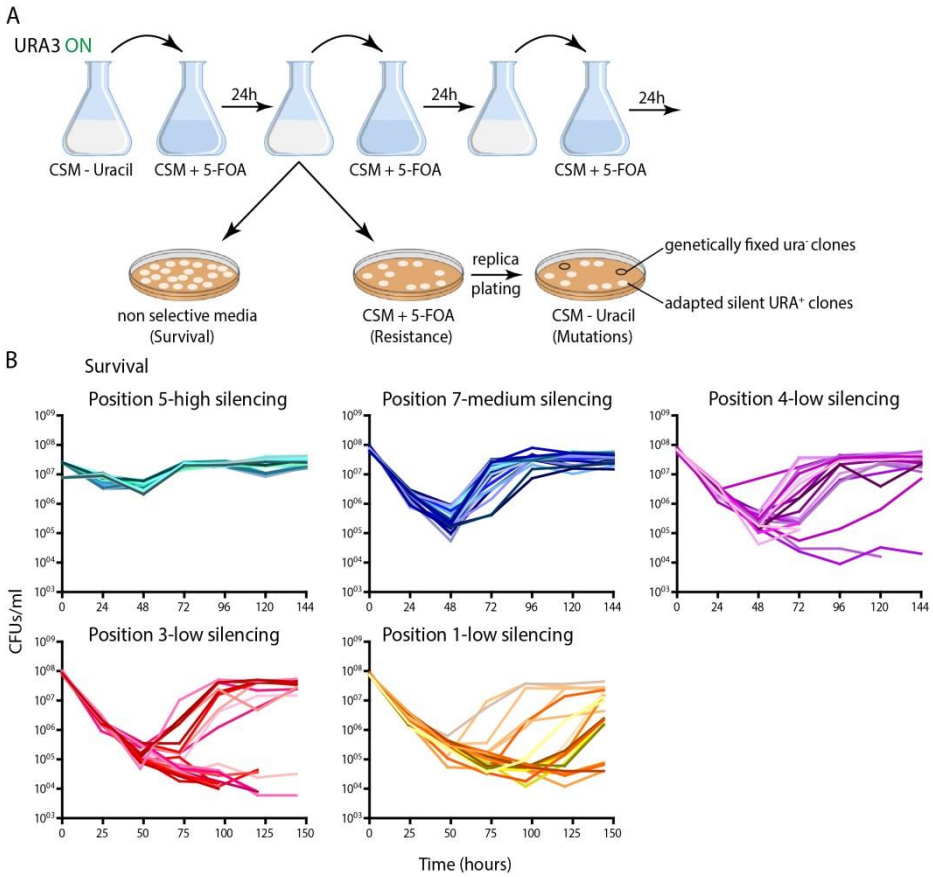

Figure 2.2 Epigenetic gene silencing enables better survival in the new environment. (**A**) Experimental setup. Populations were preselected in media lacking uracil (all cells are expressing *URA3*) and evolved in liquid CSM media with 0.05% 5-FOA. Every 24 hours cells were passed for further selection, scored for viability (based on the number of colonies on non-selective plates), and for acquisition of 5-FOA resistance (number of colonies growing on 5-FOA selection). 5-FOA resistant clones were replica plated onto CSM plates without uracil (growth indicates cells are *ura*$^{+}$, despite being 5-FOA resistant (epigenetically silenced), lack of growth indicates cells are *ura*$^{-}$. (**B**) Survival through the course of selection (as determined by the number of colony forming units (CFUs/ml). Each line represents the number of living cells in each replicate population - 24 parallel populations for positions 5 (high level of silencing) and position 7 (medium level of silencing), 21 for position 4 (low level of silencing), 21 for position 3 (low level of silencing), and 24 for position 1 (low level of silencing).

Populations were considered extinct if there were no more than half a million cells to transfer. In parallel, cells were also plated on high concentrations of 5-FOA to determine the fraction of cells adapted to growth under selection. As expected, the strain with higher levels of epigenetic silencing survived better upon encountering 5-FOA (Figure 2.2B, Pos.5-Pos.7: $P<0.0001$ at 48h, Pos.5-Pos.4;Pos.3;Pos.1: $P<0.0001$ at 48h, Tukey HSD). Contrary to the strong capacity of the high silencing strain (Pos.5) to withstand a novel stressful environment, in the strains with low levels of silencing (Pos.4,Pos.3,Pos.1), population sizes drop by more than 2 orders of magnitude, due to slow adaptation. In addition, extinction was detected in 4/21 replicates in Position 4, 9/21 in Position 3, and 5/24 in Position 1 (Figure 2.2B), demonstrating the power of epigenetic silencing in rescuing populations.

Population´s recovery is highly dependent on the level of silencing. All the populations with high and medium level of silencing recovered after certain time. In the case of the strains with lower level of silencing the recovery happened to a lesser extent, in 16/21 replicates for Position 4, 10/21 for Position 3, and 15/24 for Position 1.

Epigenetic gene silencing changes the dynamics of evolutionary adaptation

As we have seen, epigenetic silencing helps population survive better once they are exposed to a new selective environment, allowing for the subsequent adaptation step. However, to understand how this adaptation happens we need to disentangle the dynamics between epigenetically silenced cells and the cells that acquired a mutation in the URA biosynthesis pathway during the course of the experiment. Our experimental setup allows us to distinguish between these two subgroups within each population (Figure 2.2A). To this end, 5-FOA

resistant clones were replica plated on medium lacking uracil to determine whether adaptation occurred while maintaining the ability to switch on *URA3* expression (where growth indicates ability to reactivate *URA3*) or by mutation, affecting the uracil biosynthetic pathway, in which case cells can no longer grow in the absence of uracil.

Populations with higher levels of epigenetic silencing adapted very rapidly. The resistant clones in these cases appeared already after 24-48h (Figure 2.3A and Figure 2.S3). Importantly, the frequency of resistance in the Position 5 (high silencing) populations, after the initial rapid increase, reached an equilibrium at approximately 50% that was maintained throughout the length of the experiment (~90 generations) presumably reflecting the frequent switching of the silent URA^{off} state to an $URA3^{on}$ state. Genetically ura^- clones appear late (median time of initial detection is 72h) and take more time to spread in the population. Such dynamics likely results from competition of clones carrying *de novo* mutations with heritably silenced clones, delaying their increase in frequency, a phenomenon analogous to clonal interference (Gerrish and Lenski, 1998). Low levels of epigenetic silencing seem to delay the acquisition of beneficial mutations, since the median time of initial detection of ura^- mutations for Positions 4, 3 and 1 is 72h. Interestingly, at intermediate levels of silencing (Position 7), cells adapt rapidly by epigenetic means followed by the earlier appearance of ura^- mutants (median time of initial detection is 48h) and a fast rise in frequency. In order to quantify these differences, we calculated i) the frequency of appearance of resistance and ii) the frequency of appearance of adaptive ura^- mutations across all evolved populations (Figure 2.3B). The first measure gives us the rate of adaptation, while the second is proportional to the rate of acquisition of genetic mutations. This analysis shows that the rate of initial adaptation scales with the degree of silencing while the appearance and spread of adaptive ura^-

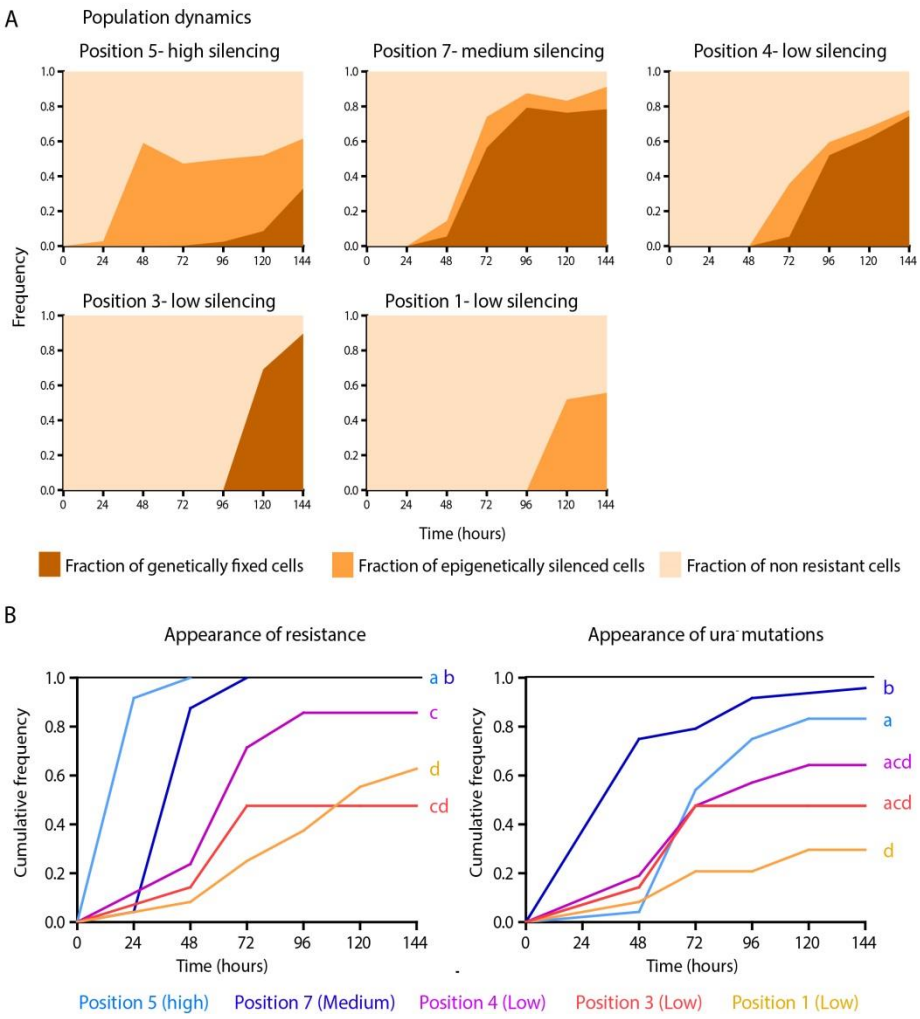

Figure 2.3 Intermediate levels of epigenetic gene silencing result in faster adaptation. (**A**) Dynamics of the acquisition of 5-FOA resistance. Colored areas represent the different phenotypic subgroups within each population (as indicated), plotted as the size fraction of each subgroup within the total population across time. The graphs represent the median value of all replicate populations (see Figure 2.S2 for complete data set). (**B**) Cumulative frequency of populations that acquired at least one detectable resistant clone (left) or resistant and *ura*- mutant phenotype (right) during the course of the experiment. Letters indicate statistical significance. The lines marked with the same letter are not statistically different from each other. Strains were compared using the Log-rank test with Bonferroni correction (Resistance: $P_{pos7\text{-}pos5}<0.0001$, $P_{pos7\text{-}pos4}<0.0001$, $P_{pos7\text{-}pos3}<0.0001$, $P_{pos7\text{-}pos1}<0.0001$, $P_{pos5\text{-}pos4}<0.0001$, $P_{pos5\text{-}pos3}<0.0001$, $P_{pos5\text{-}pos1}<0.0001$, $P_{pos4\text{-}pos3}=0.019$, $P_{pos4\text{-}pos1}=0.0018$, $P_{pos3\text{-}pos1}=0.75$; Mutations: $P_{pos7\text{-}pos5}=0.0016$, $P_{pos7\text{-}pos4}=0.0011$, $P_{pos7\text{-}pos3}<0.0001$, $P_{pos7\text{-}pos1}<0.0001$, $P_{pos5\text{-}pos4}=0.396$, $P_{pos5\text{-}pos3}=0.072$, $P_{pos5\text{-}pos1}=0.0003$, $P_{pos4\text{-}pos3}=0.37$, $P_{pos4\text{-}pos1}=0.017$, $P_{pos3\text{-}pos1}=0.19$).

mutations is accelerated at intermediate level of silencing (Figure 2.3B). In 18 out of 24 (strain with *URA3* at Position 7) populations, over 50% of clones were genetically mutated to be *ura*$^-$, while only 8 lines reached this mutant frequency at high silencing (Position 5) (P=0.008, Fisher exact Test). This indicates there is an optimal degree of short-term heritable gene silencing that is beneficial for the long-term adaptation via the acquisition of mutations. More importantly, in populations with higher level of silencing (Position 5 and Position 7) the first step in adaptation seems to happen via epigenetic means followed by the acquisition of a beneficial mutation.

This suggests a form of genetic assimilation where short-term epigenetic silencing precedes adaptation by mutation. Moreover, certain replicate populations within these strains seem to have adapted through epigenetic means, since we could not detect any causative mutation in these clones (see Chapter 3, Figure 3.3), probably by modulating the rate of epigenetic silencing itself (Figure 2.3A and Figure 2.S3). This suggests that epigenetic, heritable, gene silencing can confer a similar fitness advantage to that of a mutation, at least temporarily.

Discussion

Genetic information is relatively stable and largely independent from the environment. The change of that information can happen through spontaneous mutations which are the result of errors during the replication of the DNA. However, the rate of this change can be influenced by the environment, but not in a directional manner.

Epigenetic inheritance, on the other hand, involves the transmission of e.g. gene expression states that is based on many molecular mechanisms that are under the influence of the environment. Some of

this epigenetic “information” can be induced and inherited completely independent from any changes in the underlying DNA sequence and have a profound effect on the phenotype. Although these mechanisms are different at the molecular level, they all have the potential to act as mediators of the interaction between genotype and the environment. They can be modified by the environmental cues and are less stable than the genetic, DNA-based system. Since epigenetic information can impact the phenotype and can persist for a number of generations, it can also potentially impact the process of evolutionary adaptation, the premise of this thesis. To better understand this evolutionary problem we used budding yeast as a model system. As yeast can be grown rapidly and asexually from a clone we can control for any genetic variability. Further, previously developed tools in this unicellular organism allow us to control for the strength of epigenetic silencing. By positioning our reporter gene, *URA3*, at different position within the subtelomeric region we succeeded in obtaining different levels of epigenetic silencing. The degree of silencing decreases with the distance from the telomere. However, this decrease is not linear. We observe the highest level of silencing for Position 5, although Position 7 is closer to the core X element, the seeding center of SIR complex (Figure 2.1C). The particularity of the silencing at the Position 5 could be the result of the higher maintenance of the nucleosomes in the region or the characteristics of the subtelomeric chromatin organization. Such patterns, however, were not observed in previous studies (Pryde and Louis, 1999). This might be due to the different length scales in our study. While previous studies correlated silencing with relatively large distances from the telomere, we placed the genes closer to each other (250bp). This might allow us to see local differences in the strength of silencing that bigger spacing between genes would not uncover. Importantly, the silenced region that we detect in our strains corresponds to the SIR complex occupancy that

was described in previous studies using ChIP-seq methodology (Ellahi et al., 2015).

We used these strains in our experimental evolution approach, in which we selected against the activity of our reporter gene, using a counter-selection technique by administering a drug, 5-FOA.

We preselected the populations to ensure all of them are *ura*$^{+}$, and that they have the same starting fitness value at the onset of the experiment (Figure 2.2A). Epigenetic silencing helps populations survive better in a novel environment by acting as a buffering system to the stressful conditions that the populations are exposed to (Figure 2.2B). The switchable *ura*$^{+}$ clones appeared very quickly in populations of Position 5 strain (Figure 2.3A). We cannot, with complete certainty, claim that these clones are epigenetically silenced. The dual, *ura*$^{+}$ and 5-FOA resitant, phenotype could arise from a particular mutation. This can only be confirmed by whole genome sequencing data (see Chapter 3). However, their dynamics during the experiment indicates their capacity to switching between the *URA3*^{on} and *URA3*^{off} state. The switching frequency stabilizes around 50% and is maintained until the appearance of *ura*$^{-}$ clones (Figure 2.3A). This frequency is established because the epigenetically silent clones are switching the *URA3* gene on again and therefore supplying the subpopulation of cells in which *URA3* is active and are susceptible to the drug. The frequency at which the equilibrium between these two subpopulations will be established depends on switching rate of the chromatin states (Jeffery et al., 2013).

The mutational supply in populations with high silencing is large. However, we see that the appearance of mutant clones is delayed in these populations, probably because these *ura*$^{-}$ clones need to compete with already well epigenetically silenced cells for resources.

The first step in adaptation in populations with higher levels of silencing is always through the epigenetic system followed by the

appearance of *ura*$^-$ clones. This is very reminiscent to the genetic assimilation process we described earlier. Although, we cannot claim this with certainty since we cannot deduce if the *ura*$^-$ clones appeared from the epigenetically silenced subpopulation or from cells in which the gene was active.

We observe the fastest rate of appearance of *ura*$^-$ clones at an intermediate level of epigenetic silencing (Figure 2.3A and Figure 2.S3). This indicates that there is an optimal level of silencing that is beneficial for the acquisition of mutations in long-term evolution, confirming the claims of previous theoretical studies (Kronholm and Collins, 2016).

On the other hand, in populations with low level of silencing mutational supply is smaller, since we have fewer cells that are alive, which prolongs the acquisition of the mutation (Figure 2.2B and Figure 2.3A). At medium levels of silencing the mutational supply is high enough and the effect of clonal interference not as severe as in Position 5 populations, resulting in mutant clones arising more quickly.

There seems to be a small, residual, level of silencing in Position 4 populations, since we observe switchable *ura*$^+$ clones in these replicates (Figures 2.S3). However, this level doesn´t seem to be sufficient to significantly alter the adaptation pattern, since these populations behave rather similar to Positions 3 and 1 (Figure 2.3B). All adapted clones in Position 3 replicates are of the *ura*$^-$ phenotype (Figure 2.S3). We do observe *ura*$^+$ clones in Position 1, but they do not show the characteristic switching pattern we observe in Position 5 (Figure 2.S3). This suggests that these clones have mutations in some other genes beside *URA3* that are related to the uracil biosynthesis pathway.

Epigenetically silenced clones are less fit than *ura*$^-$ individuals, due to their frequent reactivation of the gene. However, even in Position 7 populations these clones persist in the population throughout the

length of the experiment, and are not being replaced by the more fit mutant clones (Figure 2.S3). The reason for this might be the cost of having the capacity for epigenetic silencing. This cost will be proportional to the rate of the reactivation of the gene and number of silenced cells in the population. As this number diminishes during the experiment, the probability of switching the gene on, and becoming sensitive to the drug, decreases as well. This enables these cells to still linger on in the population. This might be especially beneficial if the environment suddenly changes resulting in the need to reactivate the gene, such as in environment that lacks uracil. In such environments, populations with only genetically *ura*$^{-}$ adapted clones (e.g. Position 3) would probably go extinct. Mixed populations, on the other hand, would have better chances of surviving in changing environments. In these alternating conditions we would expect only epigenetically silenced clones to survive. Moreover, we would also anticipate that their rate of switching would evolve to correspond to the rate of change in the environment, assuming of course that the rate itself is constant, as in cases of regularly fluctuating environments.

Certain environmental conditions, like glucose concentration, were shown to influence the level of silencing in budding yeast (McCleary and Rine, 2017). This observation, along with the results from our experiments, might point us in direction to better understand how certain environmental cues might affect the adaptation process to a novel environment.

There are around 350 naturally occurring genes within the range of subtelomeric gene silencing in yeast. Six percent of these have been shown to be silenced in a SIR-dependent manner, and this percentage might be higher under stressful conditions (Ellahi et al., 2015). Some of these genes are involved in stress response. Of particular interest is the *FLO1* gene that is involved in flocculation, a process of forming structures similar to microbial biofilms. Flocculation is a cooperative

action that increases drug resistance and stress response of a population. However, the expression of *FLO1* confers significant fitness cost for an individual (Smukalla et al., 2008). The gene is located in the subtelomeric region and is subjected to chromatin silencing (Halme et al., 2004). Our results indicate that there may be an evolutionary advantage of maintaining *FLO1* and other stress response related genes within this region, since the epigenetic control of their expression may allow fast adaptation to new environmental conditions.

Materials and Methods

Strains and growth conditions

All Saccharomyces cerevisiae strains used in this study were derived from the S288c (*MATα ura3-52*, *trpΔ63*, *hisΔ200).* Y170, a *Ura*$^{+}$ strain was created by restoring the endogenous *URA3* allele by integration of a *URA3* construct amplified from the FEP193 strain, originally derived from pFEP24 (10). Y181, a *ura*$^{-}$ strain was created in which all *URA3* sequences were deleted by integration of a KanMX6 cassette amplified from pLJ32 (pLoxB-KMX; kind gift form Jaap Brouwer, Leiden University) into Y170, deleting nucleotides between positions 115,929 and 117,048 on chromosome V. This strain was used for the integration of *URA3* cassettes at 7 different positions in the subtelomeric region of the left arm of chromosome XI. Insertion were made telomere proximal of the following nucleotide coordinates: position 1 (strain Y182)- 3,305; position 2 (strain Y183)- 2,805; position 3 (strain Y184)- 2,400; position 4 (strain Y185)- 1,624; position 5 (strain Y186)- 1,374; position 6 (strain Y187)- 1,124; position 7 (strain Y188)- 874. In all strains *URA3* is transcribed in the telomere direction.
SIR3 knock-out strains were constructed by replacing the whole ORF (between nucleotide positions 1,019,287 and 1,022,281 on chromosome XII) with a *hphMX4* cassette (hygromycin resistance) amplified from the plasmid pAG32 (Goldstein and McCusker, 1999). *SIR3* was deleted in all 7 strains bearing a telomeric *URA3* insertion (Y182 through Y188), generating strains Y189 through Y195, respectively.
All strains were maintained either in rich medium [YPD; 1% Bacto yeast extract- BD (Fisher; #212720), 2% Peptone (Fisher; #BP1420-500), 2% Glucose (Merck; #1.08342.1000), either as liquid medium or supplemented with 2% Agar (Roth; #2266,4) for solid medium] or in complete synthetic dropout medium [CSM; 0.7% Yeast nitrogen base

(Sigma; #Y0626), 0.1% Complete synthetic medium (MP Biomedicals; #4560-222), 0.005% Tryptophan (Sigma; #T0254), 0.002% Histidine (Sigma; #H8000), 0.001% Uracil (Sigma; #U0750), 2% Glucose (Merck; #1.08342.1000), either as liquid medium or supplemented with 2% Agar (Roth; #2266,4) for solid medium].

Spot assay

After 2 days of growth on rich YPD media plates, fresh single colonies were picked and suspended into 100µl of distilled, demineralized water. Six 10-fold dilutions were prepared and 10 µl was plated onto plates of CSM or CSM supplemented with 0.1% 5-FOA (Apollo Scientific; #PC4054). Plates were imaged and scored after three days of incubation at 28°C.

Growth curves

Cultures were grown in liquid YPD at 28°C for 16 hours. Then, approximately 10^5 cells were diluted into 200µl cultures of CSM supplemented with different concentrations of 5-FOA (0%, 0.025%, 0.05%, 0.1%). Growth was monitored in Bioscreen C by measuring optical density (OD) every half an hour.

Experimental evolution

All strains were preselected to be in $URA3^+$ state by growing cells in liquid CSM lacking uracil for 16 hours. Next, 10^6 cells were diluted into 1ml liquid CSM supplemented with 0.05% 5-FOA. Every 24h OD_{600} was determined and $5*10^5$ cells were transferred into fresh medium. If populations had less than $5*10^5$ they were considered extinct. At each passage cells were analyzed for viability, 5-FOA resistance and growth on medium lacking uracil (scoring URA state). For this, approximately 100 cells are plated onto non-selective plates (YPD or CSM) or at higher densities on plates containing 0.1% 5-FOA. Plates

were incubated at 28°C for five days, after which colonies were counted and cell numbers in each population estimated. The number of cells plated on nonselective media imposes a 1% threshold of detection of resistance. In some cases, a low number of epigenetically silenced cells were detected on plates with 5-FOA at time 0h. Since these cells could not survive in medium lacking uracil during preselection, we assume these to have arisen after plating, due to their high frequency of switching. Plates with 5-FOA were replica plated onto CSM plates lacking uracil and incubated for three days at 28°C, after which the fraction of ura^+ and ura^- clones in each population were estimated. Experiments were performed three times independently with eight population replicates for each strain (a total of 24 replicates per strain across all experiments).

Acknowledgments

I would like to thank my supervisors, Lars Jansen and Lilia Perfeito, for helping and guiding me in conceiving this study, also for their support and advices in conducting the experiments. I would also like to acknowledge João Mata for the help he has provided me while performing the experiments. The scientific input from the members of my thesis committee, Patricia Beldade and Miguel Godinho, was of great importance in determining the direction of the project.

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Supplementary figures

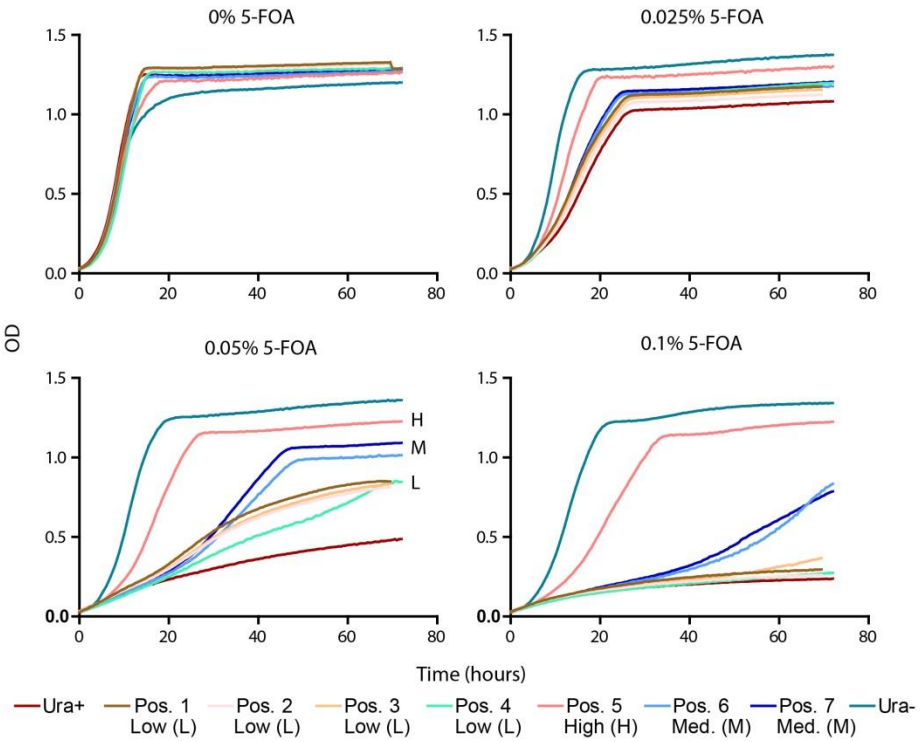


Figure 2.S1 Titration of 5-FOA selection for evolution experiments. Strains with uniquely positioned *URA3* insertions were grown in indicated concentration of 5-FOA. Growth was monitored using a Bioscreen C setup to obtain real-time growth curves at 30 minute intervals. Data was obtained for 9 replicate cultures per strain (of which one representative population is shown for clarity). The positions representing High (H), Medium (M) and Low (L) silencing are indicated in the 0.05% 5-FOA condition which is used for subsequent experimental evolution experiments.

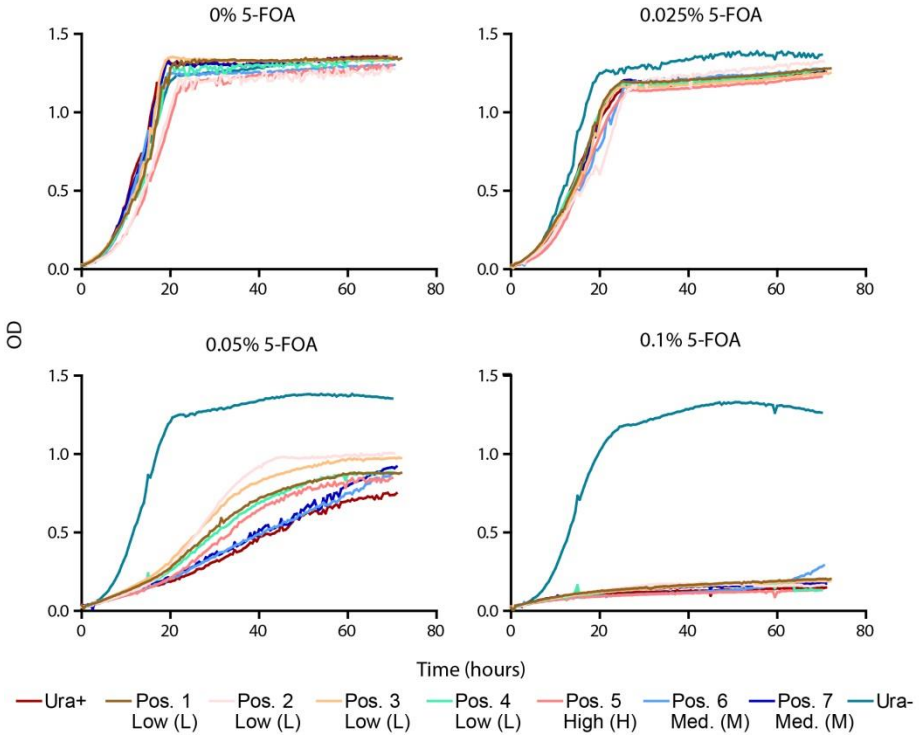


Figure 2.S2 Growth curves measurement for sir3 knock-out strains. Strains with uniquely positioned *URA3* insertions and deletions of SIR3 gene were grown in indicated concentration of 5-FOA. Growth was monitored using a Bioscreen C setup to obtain real-time growth curves at 30 minute intervals. Data was obtained for 9 replicate cultures per strain (of which one representative population is shown for clarity).

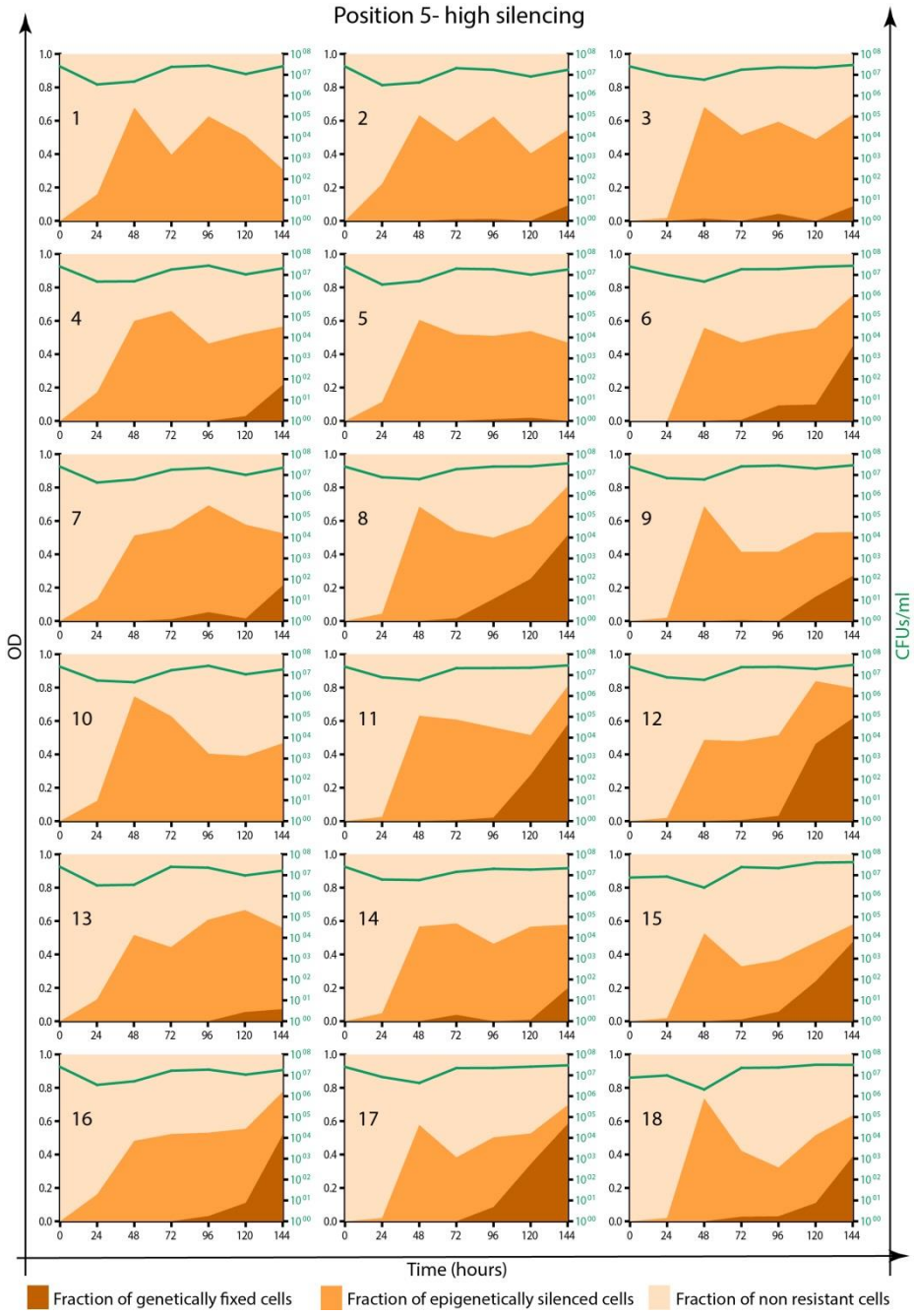
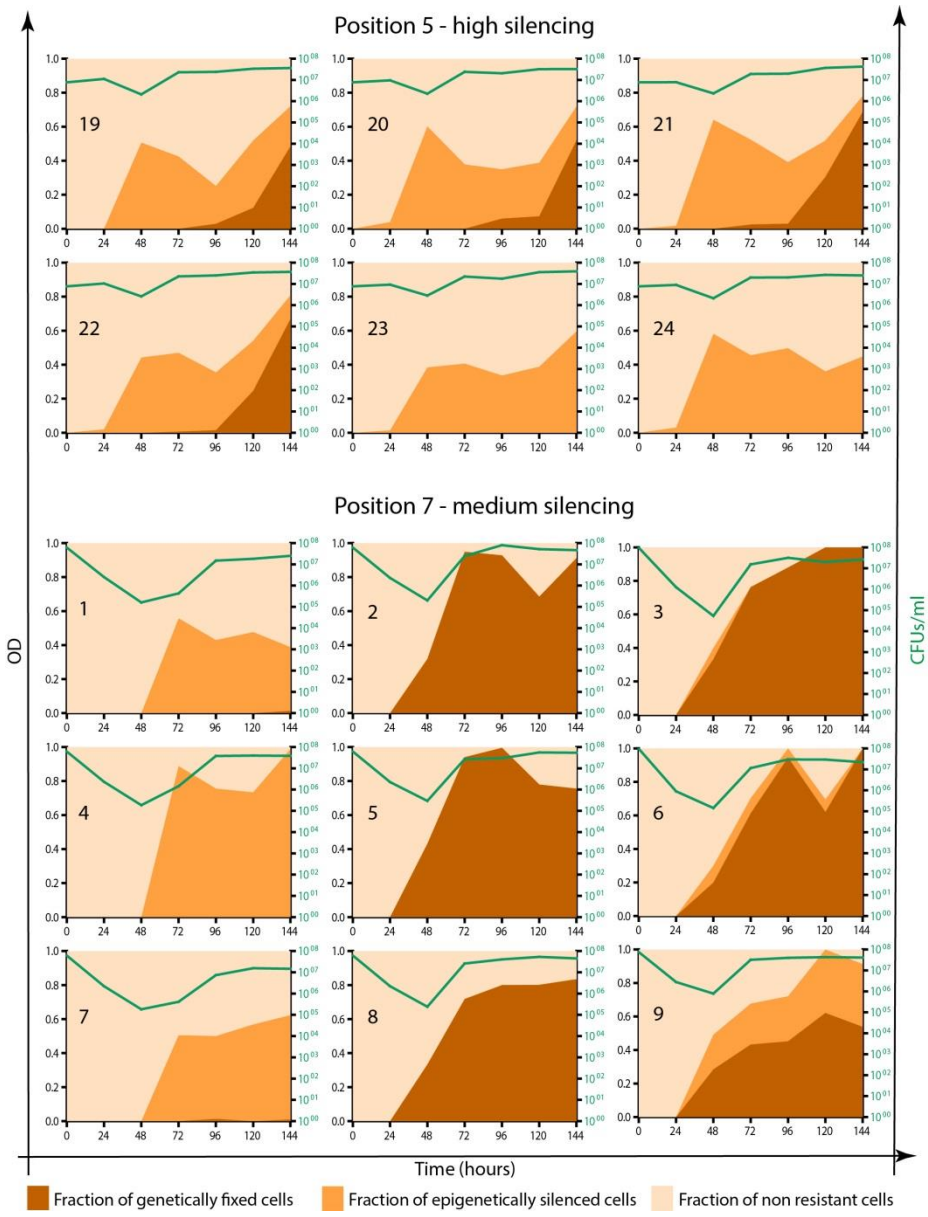


Figure 2.S3 Population dynamics of acquisition of 5-FOA resistance during experimental evolution. Plots as represented in Figure 2.2B and Figure 2.3A. All replicate populations are indicated. Green line indicates the total number of living cells within a population during the course of the experiment [colony forming units (CFUs)/ml]. Colors represent the different phenotypic subgroups within each population (as indicated), plotted as the size fraction of each subgroup within the total population across time.


Position 5 - high silencing
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Position 7 - medium silencing
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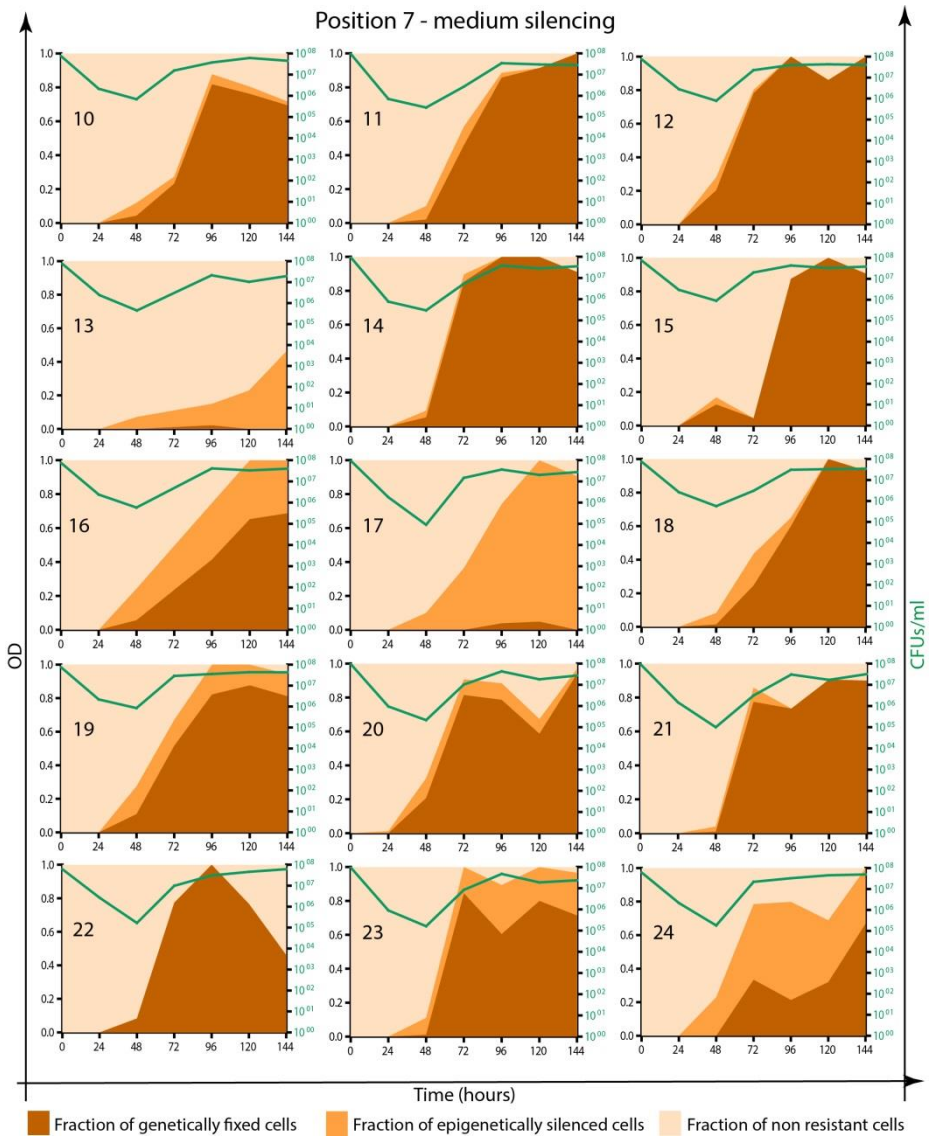
CFUs/ml
Time (hours)
Fraction of genetically fixed cells
Fraction of epigenetically silenced cells
Fraction of non resistant cells


Position 7 - medium silencing
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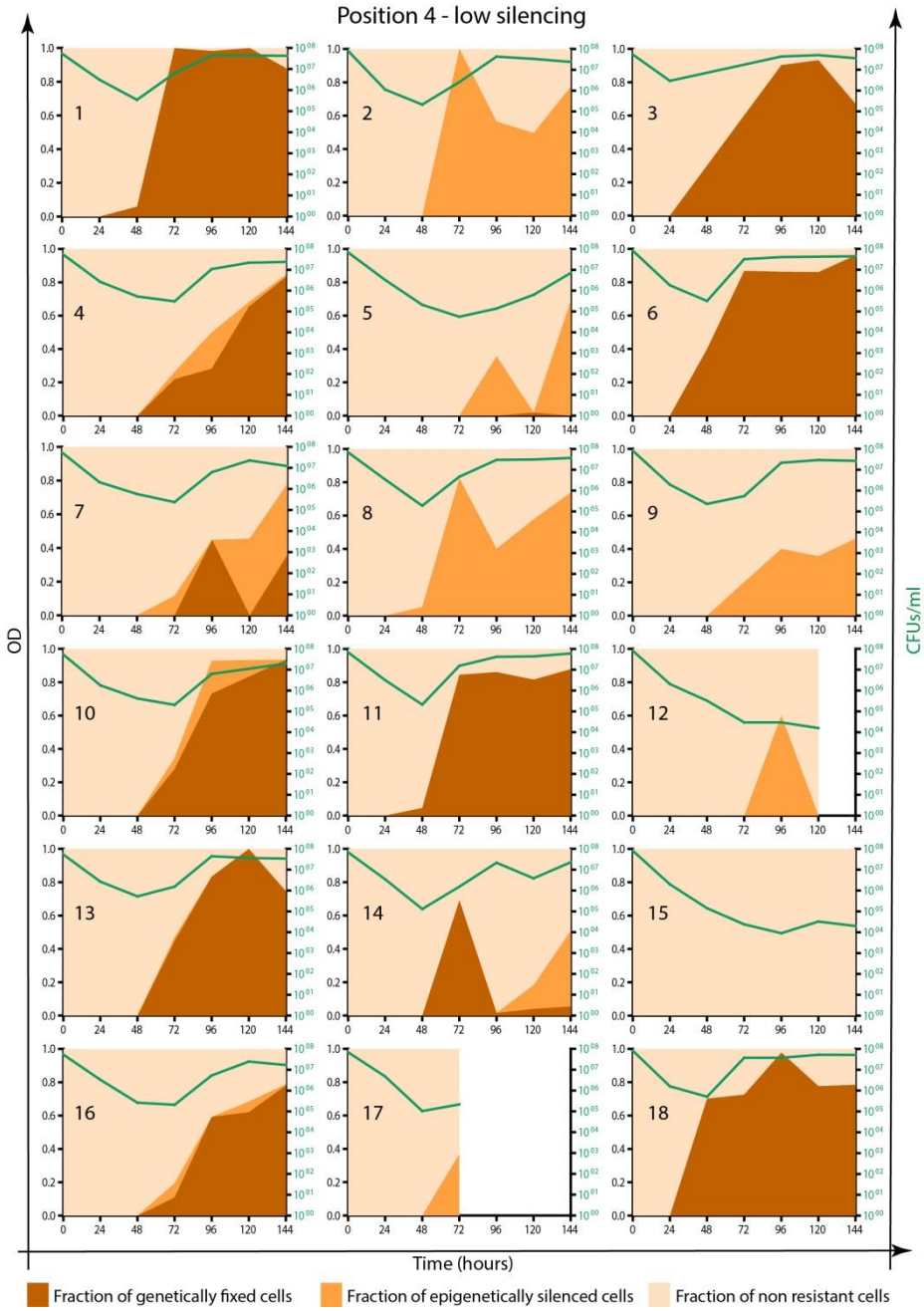
CFUs/ml
Time (hours)
Fraction of genetically fixed cells
Fraction of epigenetically silenced cells
Fraction of non resistant cells


Position 4 - low silencing
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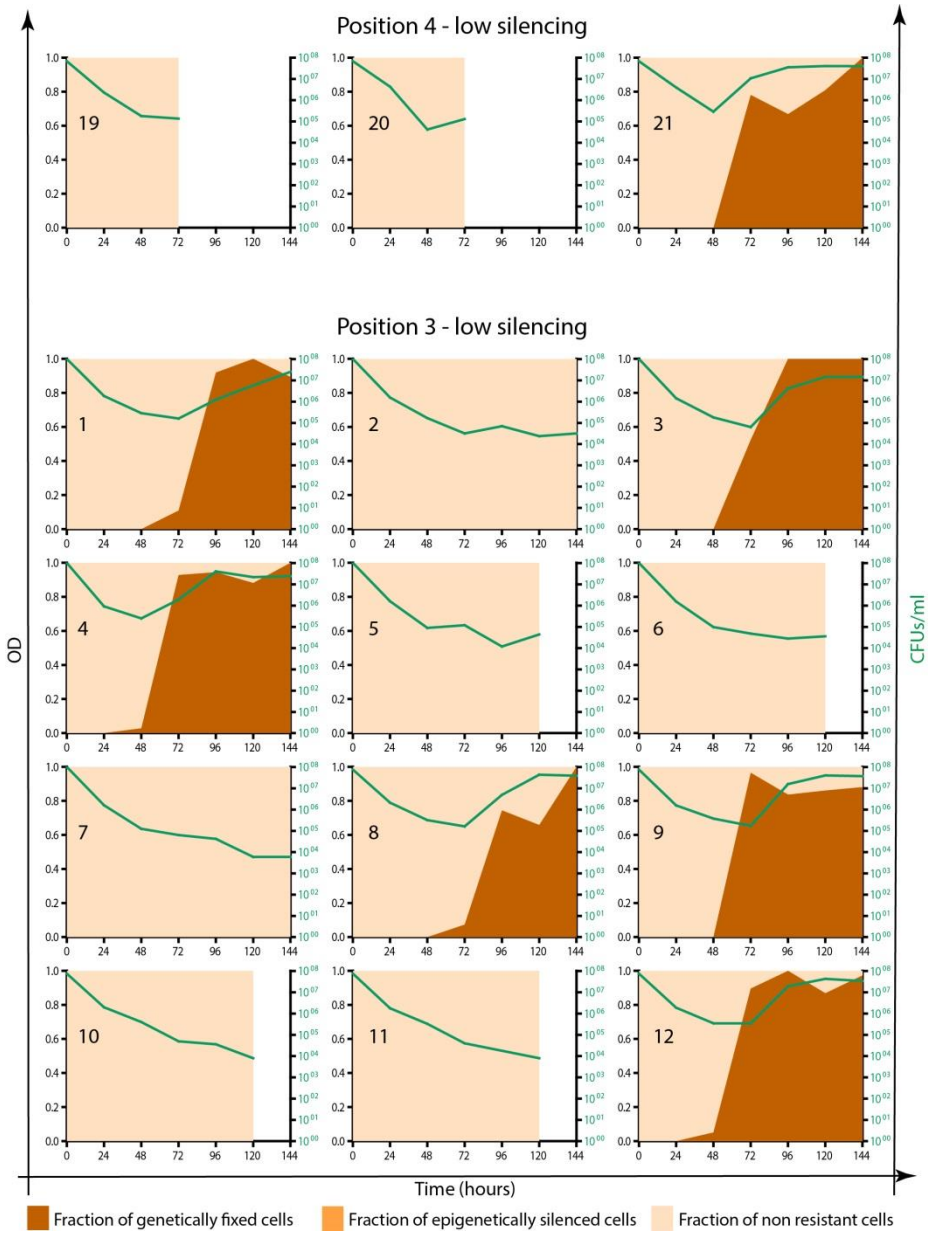
Fraction of genetically fixed cells
Fraction of epigenetically silenced cells
Fraction of non resistant cells


Position 4 - low silencing
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Position 3 - low silencing
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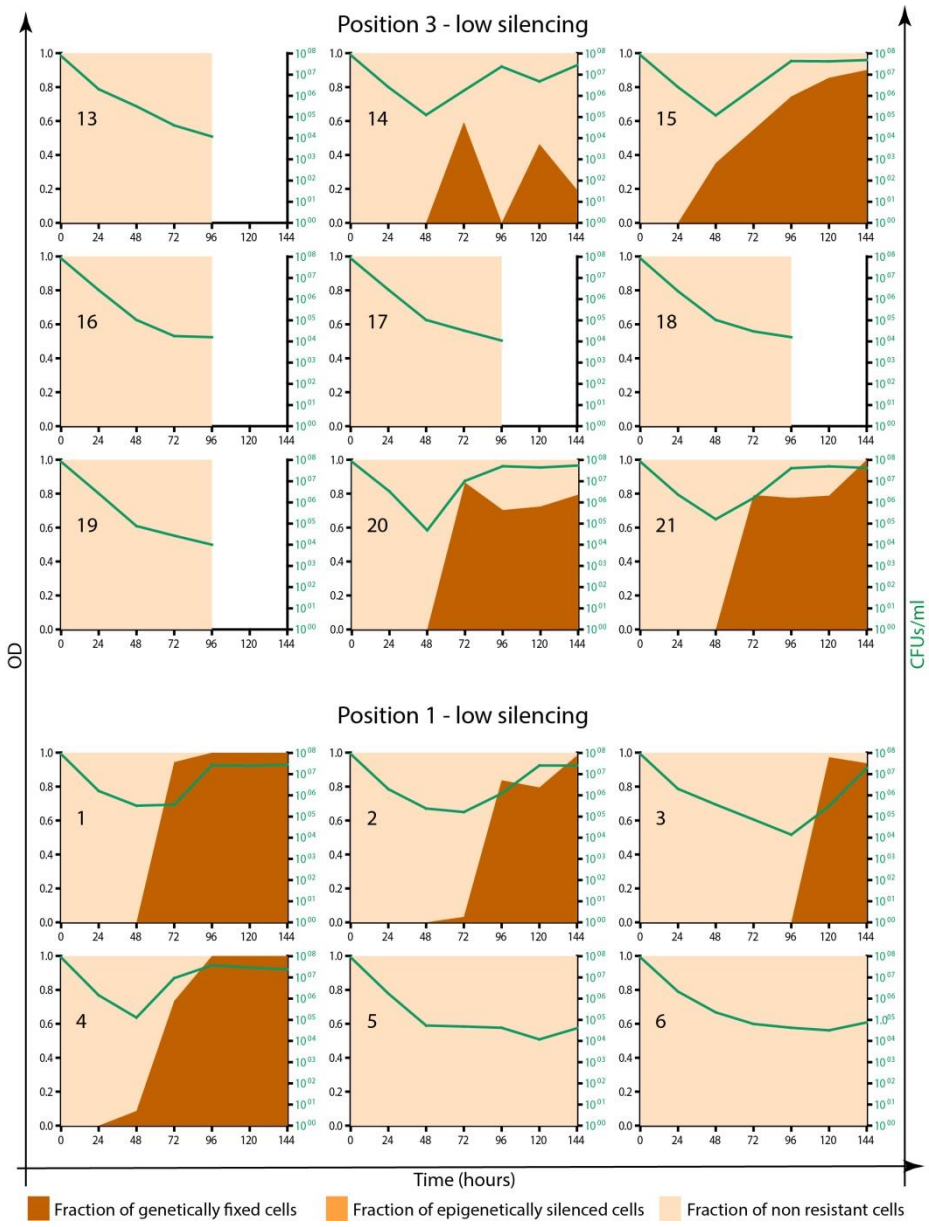
CFUs/ml
Time (hours)
Fraction of genetically fixed cells
Fraction of epigenetically silenced cells
Fraction of non resistant cells


Position 3 - low silencing
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Position 1 - low silencing
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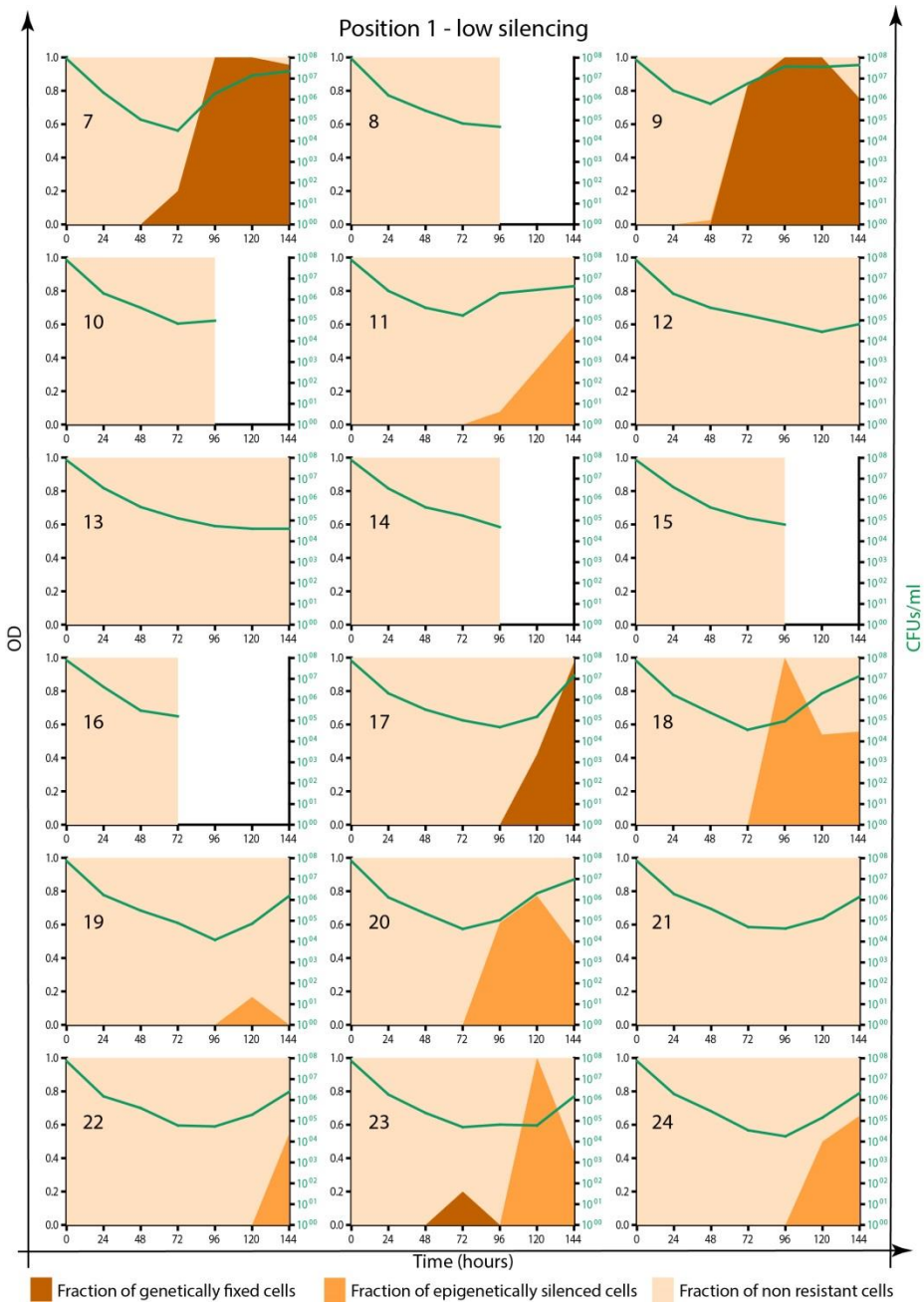
CFUs/ml
Time (hours)
Fraction of genetically fixed cells
Fraction of epigenetically silenced cells
Fraction of non resistant cells


Position 1 - low silencing
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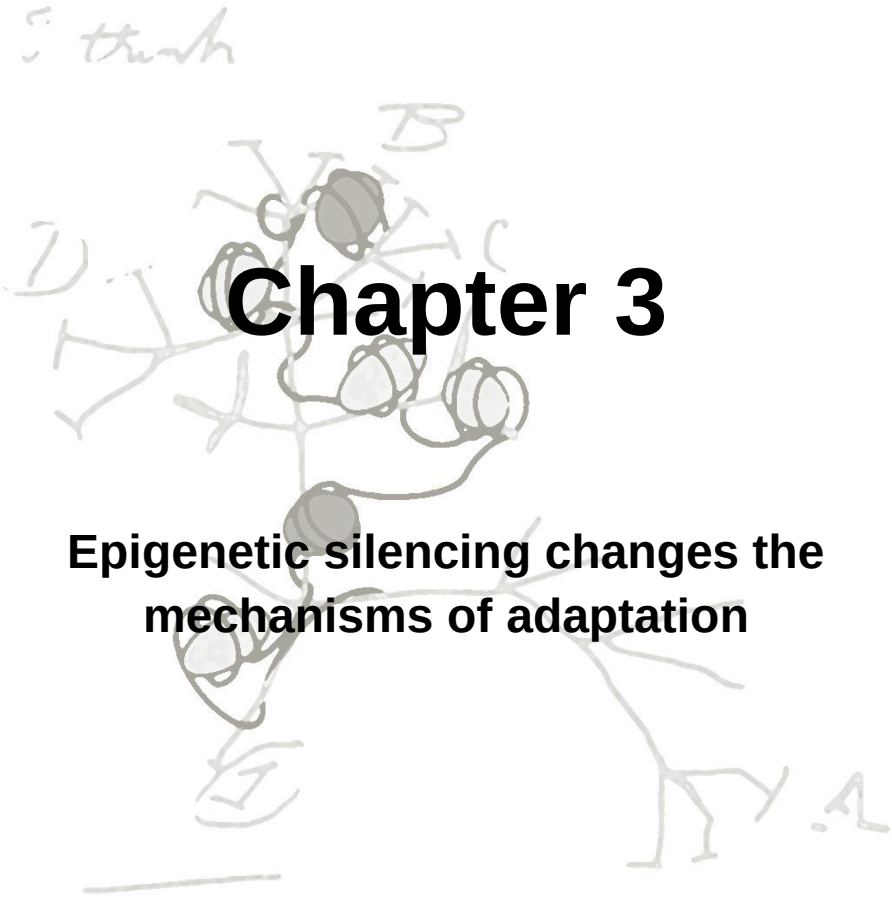
CFUs/ml
Time (hours)
Fraction of genetically fixed cells
Fraction of epigenetically silenced cells
Fraction of non resistant cells



Chapter 3

Epigenetic silencing changes the mechanisms of adaptation

Abstract

Epigenetic, non-DNA sequence-based inheritance, can potentially contribute to adaptive evolution, but it is often thought to only affect transient dynamics, with little effect on long-term evolution. However, short-term epigenetic inheritance may interact with the underlying genetic system during the course of evolution. The presence of epigenetic silencing might change the selective pressure on a particular gene and alter the mutational spectrum during the process of adaptation. This non-genetic inheritance system might also increase the mutational target size and help adaptation through the acquisition of mutations that would impact epigenetic silencing itself, which would otherwise in the absence of silencing have no effect. And importantly, epigenetic modifications could modulate the mutation rate and in that way change the speed of the appearance of beneficial mutations. In the previous chapter I described our finding that intermediate levels of silencing can accelerate the rate of genetic adaptation to a new environment. In this chapter, I will describe the mechanisms that may explain this increased rate. We firstly measured the mutation rates in our strains, with different positions of URA3 gene in subtelomeric region, in the presence and in absence of epigenetic silencing. We see that chromatin structure can impact the rate of mutations, however not in a manner that could explain the pattern of adaptation we described previously. We also set out to understand the mutational spectrum that a population under epigenetic silencing acquired by performing whole genome sequencing of the evolved clones from experimental evolution experiment described in Chapter 2. The result shows that heritable gene silencing changes the types of mutations that are beneficial, and drives new alleles that enhance gene silencing, enlarging the mutational target size and aiding adaptation.

Introduction

Evolutionary adaptation rate is directly dependent on mutation rate (Allen Orr, 2000; Neher et al., 2010). The rate of genetic changes is controlled by many mechanisms such as epigenetic regulation of gene expression that has a role not only in instructing the activity of genes, but also in maintaining genome stability and integrity. For instance, DNA methylation plays an important part in cellular immunity in prokaryotes. Bacteria use the methyl marks to distinguish between host and foreign DNA (bacteriophage) and degrades the pathogen´s unmethylated, genome (Wilson and Murray, 1991). In eukaryotes many transposable elements are highly methylated which renders them transcriptionally inactive. Indeed, in plants it was shown that mutants that are deficient in the methylation machinery have increased activity of transposable elements (Kakutani et al., 1995). However, CpG regions that are methylated have a higher propensity of acquiring SNPs as shown in sequencing data done on human samples (Xia et al., 2012). It seems that even though the methylation of the DNA has a role in protecting the genome from deleterious effect of transposable elements, it also can increase the local mutation rate in methylated regions. Moreover, methylation of the DNA is tightly linked with the process of DNA repair. In bacteria the methylation status of the DNA strand is used to distinguish between the old and newly synthetized strand during the replication of the DNA. This ensures that the repair machinery, once it encounters an incorrectly incorporated nucleotide, would correct the mistake in the newly synthesized strand (Saint-Ruf and Matic, 2006; Schofield and Hsieh, 2003). The overproduction of the DNA methylase was shown to cause the rise in mutation rate as a consequence of the alteration in the recruitment of the mismatch-repair machinery (Herman and Modrich, 1981).

DNA methylation and histone modification are tightly connected processes. In mouse, methylated DNA can cause the deacetylation of neighboring nucleosomes and probably recruit histone methyltransferases, leading to the compaction of local chromatin (Cedar and Bergman, 2009). Histone modifications are also highly correlated with local mutation rates. In human cancer cells it was shown that a variation in the heterochromatin mark, methylation of lyisine 9 of H3, can account for nearly 40% in variation of mutation rate (Schuster-Böckler and Lehner, 2012).

In budding yeast, this link between histone modifications and mutation rate is better characterized. Sequence comparison between closely related species of *S. cerevisiae* revealed high divergence in single nucleotide polymorphisms within the subtelomeric, chromatin silenced region (Teytelman et al., 2008). This indicates a higher mutation rate in this region compared to other parts of the genome. Indeed, it was shown that subtelomeric regions are more prone to recombination events and repressive chromatin plays an important role in this process (Batté et al., 2017). Many genes that have high sequence homology and are members of extensive protein families, such as the PAU genes, can be found close to the telomeres. The amplification of their number and localization is probably a consequence of the high rate of recombination at chromosome ends (Luo and van Vuuren, 2009).

Telomere-associated proteins are located at the periphery of the nucleus driving perinuclear localization of telomeres. The yeast knock-out strains for SIR proteins show delocalization of the telomeres away from the nuclear membrane (Palladino et al., 1993). Silent chromatin in the subtelomeric region has also been shown to play important role in homologous recombination by promoting gene conversion events in the case of double strand break repair process (Batté et al., 2017). All of these examples demonstrate clearly that chromatin structure

(including DNA methylation and histone modifications) can influence significantly the rate of genetic change, on which the adaptation rate to a novel environment is directly depended.

In addition, epigenetic changes can also impact the type of mutations that arise in the population during the course of adaptation. The change of the genetic material can occur indirectly if natural selection acts on epigenetic component, taking into account of course there is an interaction between the two systems. This interaction can be complex. The epigenetic change can be dependent on the genetic alleles, and as we saw epigenetic changes of DNA and chromatin can influence directly the genetic change (Bonduriansky and Day, 2009).

Additionally, if an epigenetic mark acts by silencing a gene, a phenotype that is under selection and that can be achieved equally by a mutation, then a particular form of epistasis between the two inheritance systems emerges. In other words, epigenetic silencing of a gene can potentially change the adaptive landscape, and therefore the selective pressure on the gene, and render all the subsequent mutations neutral. The acquisition of the corresponding genetic change would in turn change the selective pressure on the epigenetic system. In such a way population can explore fitness landscape more efficiently (Klironomos et al., 2013).

Results

Although theoretical work has made predictions on how the two modes of inheritance can interact and influence each other during the process of adaptation, whether this can actually occur in the course of evolution is still unclear.

To answer this question we performed measurement of mutation rates in our silenced clones with subtelomeric positions of *URA3* gene. Moreover, we sequenced the evolved clones obtained from the

evolution experiment described in Chapter 2 to determine whether epigenetic silencing of the gene under selection changes the spectrum of mutations that accumulate during adaptation.

Epigenetic silencing has a dominant role in explaining the differences in adaptation

As we have seen, heritable changes in chromatin structure can change the local mutation rate in the genome. To understand if this is also the case in our adaptation experiment we have to disentangle silent chromatin from mutation rate. In our analyses, we used previously constructed strains with a *URA3* cassette inserted in seven different positions within the subtelomeric region of *S. cerevisiae* genome (Figure 2.1).

To measure mutation rate we performed a slightly modified Luria-Delbruck fluctuation assays (Lang and Murray, 2008). We grow 96 parallel populations that were inoculated with a small initial number of cells (around 10 per population) for a number of generations. After the growth period we estimate the average number of divisions (N_d) which is effectively equal to the final number of cells. We then plate all of these populations as separate spots onto selective plates and score the number of populations that did not form a resistant colony (zero class event, $P0$) after an incubation time. The mutation rate is determined using equation: $\mu = -\ln(\frac{P0/Pt}{Nd})$, where $P0$ is number of zero class events, Pt total number of populations and N_d number of divisions (for more details see Materials and Methods section). We performed this test in strains with a knock-out of *SIR3*, an essential part of the protein complex that is responsible for silencing of the genes in the subtelomeric region (see Chapter 1). In a *sir3* mutant background, any difference in mutation frequency would be due to local sequence differences imposed by the different *URA3* positions

along the chromosome end. In this way we can uncover a possible direct effect of local sequence differences on adaptation rates rather then those imposed by epigenetic gene silencing of *URA3*. Positions 5, 7 and 4, that in a SIR^+ wildtype background display, high, medium and low silencing, respectively, do not show significant intrinsic differences in mutation rate when silencing is absent (*sir3Δ*) (Figure 3.1A). This indicates that intrinsic differences in mutation rate cannot explain the

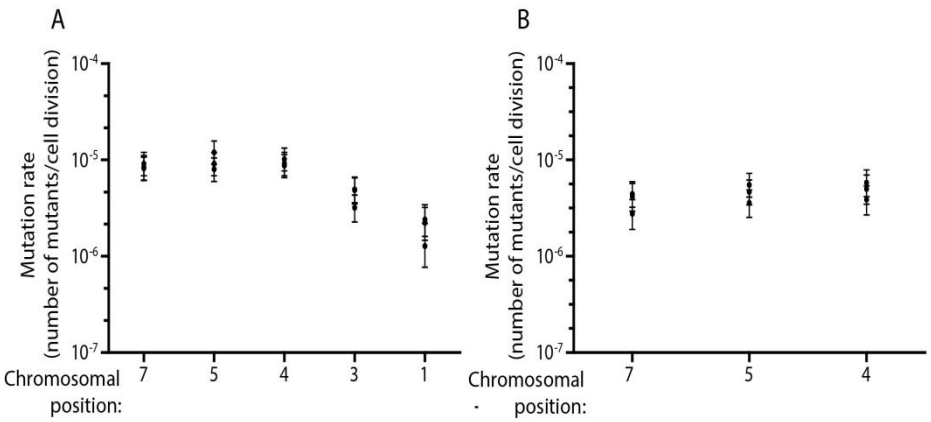


Figure 3.1 Mutation rate patterns cannot explain the differences in the adaptation rate. (**A**) Fluctuation tests were performed for indicated *URA3* positions in a *sir3Δ* background. Three independent clones were analyzed for each position. The mutation rate was estimated using the p0 method (Rosche W.A., 1947), and confidence interval for each value was determined using binomial exact test. Whiskers indicate 95% confidence interval. (**B**) We performed fluctuation tests in the presence of nicotinamide for indicated positions in a *SIR3* wild-type background. Three independent clones were analyzed.

differences in adaptation (described in Chapter 2), and that instead, epigenetic silencing has the dominant role in driving the differences in adaptation. However, Positions 3 and 1 (low silencing in a SIR^+ background), that are further away from the telomere have two or three folds lower mutation rates in the absence of silencing (Figure 3.1A). For these strains it is difficult to disentangle the contribution of mutation rate from epigenetic silencing in the process of adaptation. Although, it is important to point out that even though these strains

have lower mutation rate than a strain with *URA3* at Position 4, they have similar levels of epigenetic silencing, and show a similar adaptation pattern (Figure 2.3). This analysis shows us the effect of local sequence differences on mutation rate in the absence of silencing. However, SIR-induced changes in local chromatin structure could alter the frequency of genetic change significantly. To account for this, we performed a slightly modified fluctuation test. In this case, we performed the experiment in wild-type (*SIR3*$^{+}$) yeast clones, which are the ancestral strains used in experimental evolution. We scored zero class events (populations with no mutations) on plates that contained the inhibitor of Sir2, nicotinamide (NAM) (Bitterman et al., 2002), to unmask any mutational events scored on 5-FOA from any epigenetic silencing that would confer 5-FOA resistance. We find that under epigenetic silencing the mutation rate is 2-3 fold lower compared to the *sir3Δ* background, probably because chromatin decreases the probability of a recombination event from happening (Figure 3.1B). However, we find the mutation rate to be independent from the level of silencing, since all three strains (Position7, 5 and 4) have similar mutation frequencies. These results clearly demonstrate that mutation rates cannot explain the differences that we see in adaptation during the experimental evolution (Figure 2.3). However, to further confirm this, we performed experimental evolution experiments with the same setup as before (described in Chapter 2, Figure 2.2) using strains with a *sir3Δ* background, that do not exhibit any epigenetic silencing (Figure 2.1 and Figure 2.S2). All populations in each experimental group acquired resistance during the course of the experiment (Figure 3.S1). Furthermore, the adaptation pattern is virtually the same across all of the strains (Figure 3.2). The median time of appearance of 5-FOA resistant clones is 72h and is the same for all clones with different positions of *URA3*.

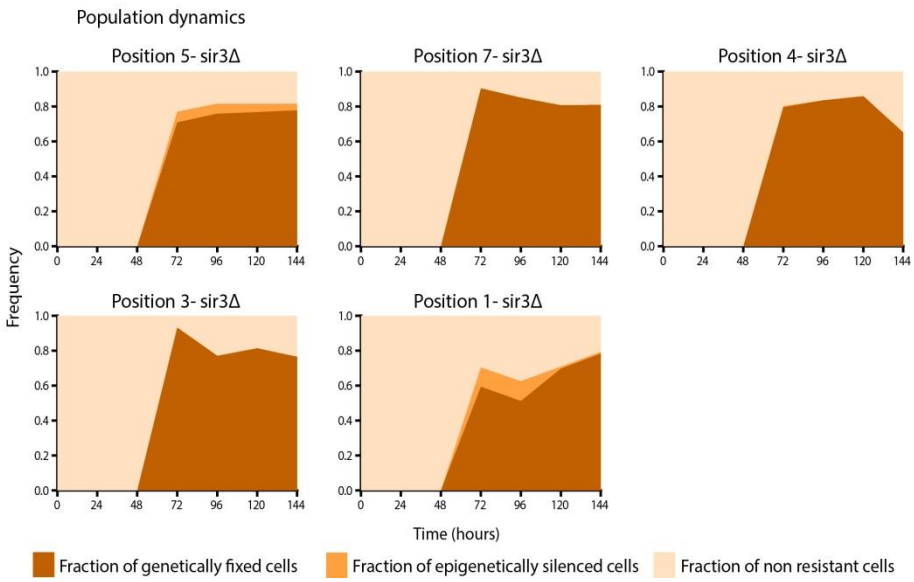


Figure 3.2 Different URA3 positions have the same adaptation pattern in the absence of epigenetic silencing. Graphs represent dynamics of the acquisition of 5-FOA resistance. Colored areas show the different phenotypic subgroups within each population (as indicated), plotted as the size fraction of each subgroup within the total population across time. The graphs represent the median value of eight replicate populations per strain (see Figure 3.S1 for complete data set).

Moreover, all the resistant clones are of *ura*$^-$ phenotype, probably due to the higher mutation rate we observed previously in this background (Figure 3.1A).As expected, we do not find any epigenetically silent clones in the populations of bearing a *URA3* cassette at Position 7-*sir3Δ* and Position 4- *sir3Δ* (Figure 3.S1), unlike in the case of populations that have epigenetic machinery intact (Figure 2.S3). Taken together, these results, alongside the fluctuation test analysis, clearly show that the observed differences in adaptation patterns in the presence of Sir silencing, are solely due to the differences in the levels of epigenetic silencing. As we have observed, the presence or absence of SIR protein complex can impact the overall mutation rate across the subtelomeric region. However, this doesn´t seem to be the mechanism by which epigenetic system is aiding adaptation.

Epigenetic silencing changes the mutational spectrum during adaptation

As discussed in the introduction, epigenetic silencing can potentially change the type of mutations and aid populations to survive a change in environment by enlarging the mutational supply. To uncover this possible interaction between epigenetic gene silencing and genetic mutations, we performed whole genome sequencing (for details see Materials and Methods section) of independently evolved clones, both from the *ura*$^-$ class and from resistant but switchable *URA*$^+$ clones (See Chapter 2, Figure 2.S3). Most *ura*$^-$ clones were caused by a subtelomeric deletion that contains the *URA3* cassette, probably due to a high rate of gene conversion at subtelomeric regions (Figure 3.3A) (Batté et al., 2017), consistent with the high mutation rate estimate in the fluctuation test (Figure 3.1). Interestingly, in the case of adapted *URA*$^+$ clones bearing *URA3* at Position 5 (high silencing), we cannot detect any causative mutation. While we cannot exclude that these clones bear an undetected mutation, it is possible that cells can adapt purely epigenetically by transiently enhancing their capacity to silence, although this is not easily tractable and may be reversible.

Importantly, we observe an expansion of the mutational target beyond the *URA3* gene in clones from populations with low or intermediate levels of silencing that show rapid adaptation (Figure 3.3A). In these strains, we find, in addition to *URA3* mutations in *ura*$^-$ clones, distal mutations in adapted *URA*$^+$ clones. These mutations map e.g. to genes involved in metabolism and signaling pathways (NAB3, GPB1) (Figure 3.3A and Table 3.S1) (Darby et al., 2012; Peeters et al., 2006). Of specific interest is *PPR1*, a known transactivator of *URA3* that has previously been shown to collaborate with Sir-mediated silencing. *PPR1* mutants enhance telomere repression of *URA3* at more distal positions from the telomere in a Sir-dependent manner (Aparicio and

Gottschling, 1994; Renauld et al., 1993). This exemplifies how heritable gene silencing can render specific mutational targets such as *PPR1* relevant for adaptation. We find this form of adaptation in three independent clones in populations with low and intermediate silencing. Furthermore we recovered a nonsynonymous mutation in the *RPD3* gene (Figure 3.3 and Table 3.S1), a histone deacetylase with known roles in gene silencing, where it has been shown to directly oppose SIR mediated silencing (Zhou et al., 2009).

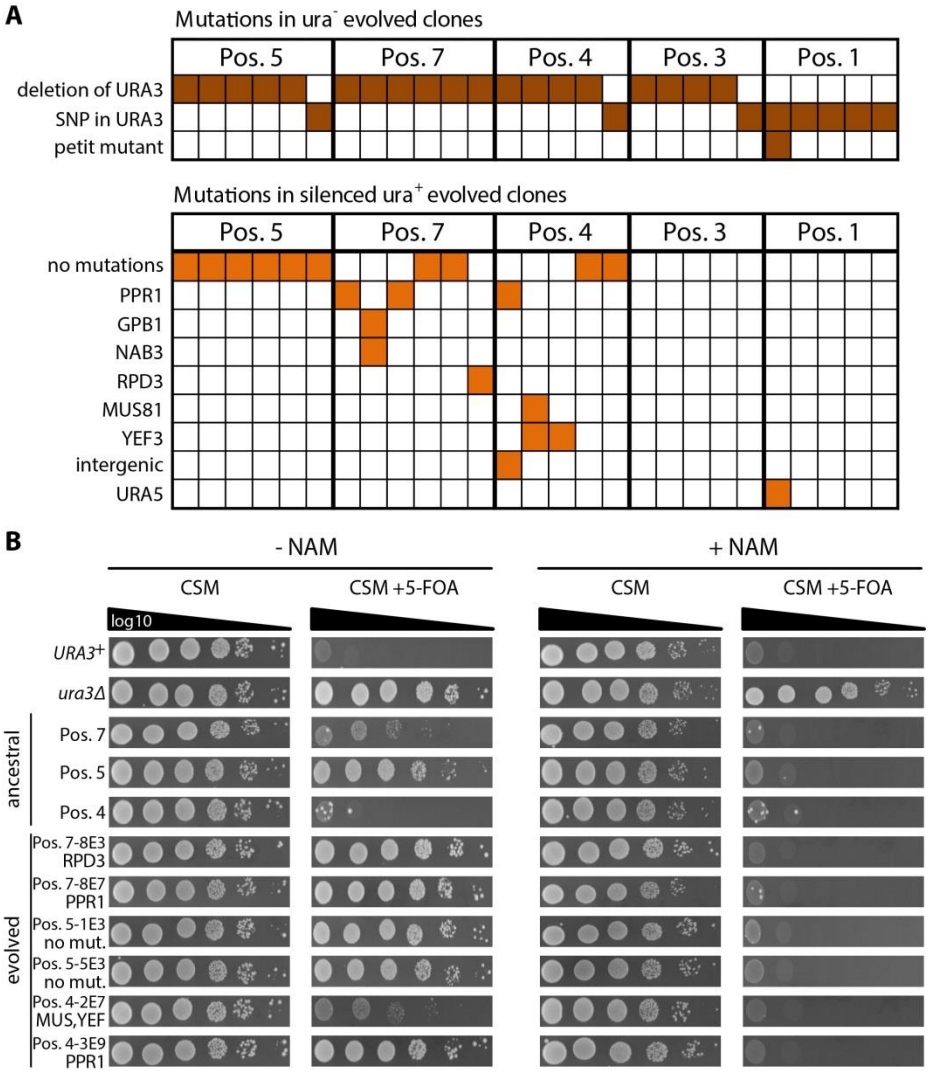


Figure 3.3 Strains carrying epigenetically silenced *URA3* evolve by expanding the mutational target in collaboration with the epigenetic machinery. (**A**) Type of mutations in the evolved clones. For each *URA3* position, evolved clones were isolated at the end of the 144 hour experiment. Both ura$^-$ and silenced (5-FOA resistant but switchable) ura$^+$ clones were analyzed by whole genome sequencing. Columns represent individual clones. Colored cells indicate the clone has a mutation in the indicated gene. (**B**) Nicotinamide abrogates the resistance in the adapted URA3$^+$ silenced clones. Ten-fold dilutions of indicated ancestral and evolved clones were spotted on plates containing nicotinamide (NAM; with and without 5-FOA) or minimal media plates (CSM-with and without 5-FOA) to control for the level of resistance to the 5-FOA. URA3$^+$ and *ura3Δ* strains were used as negative and positive controls respectively. Corresponding mutations for each evolved clone are indicated. See Figure 3.S2 for NAM analysis of all sequenced evolved URA3$^+$ clones.

To determine whether URA3$^+$ evolved clones adapted by modulating epigenetic silencing or through independent means, we exposed these clones to nicotinamide (NAM), a known inhibitor of Sir2 activity that abrogates silencing (Bitterman et al., 2002). Strikingly, Sir2 inhibition abolished 5-FOA resistance in all evolved clones that have maintained epigenetic switching (Figure 3.3B and Figure 3.S2), indicating that the acquired resistance to 5-FOA is increased in SIR-dependent manner. This demonstrates a mechanism of adaptation where a genetic change is only beneficial in the presence of an active epigenetic system. In other words, these mutations collaborate with epigenetic silencing as a means of increasing fitness.

Discussion

As we have seen in the introduction of this Chapter, heritable epigenetic control of gene expression (e.g. through methylation of DNA and histone modifications) have a profound impact on the rate of genetic changes. Our results clearly show that the SIR protein complex, and consequently epigenetic silencing caused by it, are essential for the maintenance of stability of subtelomeric region, in agreement with previous reports (Batté et al., 2017; Palladino et al.,

1993). The absence of this protein complex causes a rise in mutation rate by several folds (Figure 3.1). As the distance from the telomere increases the mutation rate seems to drop, with a clear transition between Positions 4 and 3. Interestingly, at this genomic locus a member of the extensive PAU gene family, PAU16, is located. These genes show a high level of sequence homology between themselves. They are mostly found in subtelomeric regions, and their increase in number is a likely the result of frequent recombination events (Luo and van Vuuren, 2009). Probably their high sequence similarity is also a direct cause of these recombination events, consistent with our sequence data of evolved clones. Most of our *URA3* deletion events in *ura*$^-$ clones are deletions of the part of the chromosomal arm between the *PAU16* gene and the telomere (Figure 3.3 and Table 3.S1). This is possibly because of the homologous recombination event that originated between *PAU16* gene and another gene from the same family from different subtelomeric region of another chromosome. The recombination will result in the loss of genomic sequences of chromosome XI and an exchange with other subtelomeric sequence. The fact that further supports this claim is that we do not observe deletion events in *ura*$^-$ clones of Position 1 that is more centromere proximal and further away from *PAU16* gene. Recombination events at *PAU16* would not cause a deletion of the *URA3* gene in this strain and hence are not selected for. This also explains the lower mutation rate in strain with *URA3* at Position 1.

Even though strains in which *URA3* is more telomere distal, such as Position 3 and 1, have a different mutation rate from other clones (Figure 3.1A), this cannot explain the adaptation difference observed previously (Figure 2.3). Strain with *URA3* at Position 4 has a higher mutation rate, yet the appearance of resistant and mutant clones in this population has similar dynamics to strains with *URA3* at Position 3 and 1. Moreover, *URA3* at Positions 7, 5 and 4 have virtually the same

mutation frequencies both in *sir3Δ* and sir3 wild-type background. This clearly shows that the cause of different evolutionary dynamics between these strains is not due to a modulation of mutation frequency.

Our results from experimental evolution performed with *sir3Δ* clones seem to corroborate this. Populations lacking epigenetic silencing display the same patterns of adaptation (Figure 3.2 and Figure 3.S1). However, all populations, regardless of the *URA3* position, acquire resistance during the course of the experiment, probably due to the higher recombination rate. The median time of acquiring the resistance, 72h, is the same for all *sir3Δ* strains, even for the Position 7-*sir3Δ* strain. It is important to note that the median time for acquiring *ura*$^-$ resistant clones for the strain with *URA3* at Position 7 in the *SIR3* wild-type counterpart is only 48h, even though it has several folds lower mutation rate than its corresponding *sir3Δ* clone that acquires mutations later. This clearly demonstrates the power that epigenetic silencing has in speeding up evolutionary processes. Most of the resistant clones in *sir3Δ* strains are of the *ura*$^-$ phenotype (Figure 3.S1), which is expected since there is no epigenetic silencing in these strains. Nevertheless, we do observe some switchable *ura*$^+$ clones in Position 5 strain. This could be due to some residual level of silencing performed by other histone deacetylases independently of SIR complex.

Whole genome sequencing of the SIR3 wild-type evolved clones recovered in the evolution experiment described in Chapter 2 (Figure 2.S3) uncovers the molecular mechanisms of adaptation. Most of *ura*$^-$ clones are deletions of a part of the chromosomal arm that contains *URA3*, except in clones with *URA3* at Position 1 where we do not detect such events (Figure 3.3). All of the sequenced epigenetically silenced (switchable *ura*$^+$) evolved clones from Position 5 have no underlying genetic mutation. This suggests that the adaptation might

have occurred solely based on epigenetic silencing. We observe these cases also in strains with *URA3* at Position 7 and 4. Populations with *URA3* at Position 3 did not have any switchable *URA*$^+$ clones. In this case the adaptation occurred only through the mutations in the *URA3* gene. At Position 1, however, we do observe switchable *ura*$^+$ clones in 7/24 populations. However, these cells showed very slow growth and proved resistant to recovery for sequencing. Nevertheless, we succeeded to sequence one of them that still showed a mutation in uracil biosynthesis pathway, but production of uracil does not seem to be affected (Figure 3.3 and Table 3.S1). These clones are still switchable, since their *URA3* gene is still intact.

Importantly, we do observe an expansion of the mutational target in strains with *URA3* at Position 7 and 4. The detected mutations occur in genes that are outside of the canonical uracil pathway. We found mutations in YEF3 (Qin et al., 1987), a factor that is involved in the elongation process during translation, and MUS81, endonuclease involved in DNA repair (Interthal and Heyer, 2000), in populations carrying *URA3* at Position 4. These evolved clones might have an increased mutation rate. In populations with an intermediate level of epigenetic silencing we found mutations in NAB3, a RNA-binding protein involved in responses to nutrient deprivation (Darby et al., 2012; Wilson et al., 1994) and GPB1, a regulator of protein kinase A signaling pathway (Peeters et al., 2006). This clone possibly adapted by the modulation of the metabolic response or by the regulation of the effectors of epigenetic silencing. More importantly, we found mutations in PPR1, a known regulator that interacts with Sir silencing machinery (Aparicio and Gottschling, 1994; Renauld et al., 1993), and RPD3, a histone deacetylase that was shown to interact with Sir2 protein that is part of the silencing complex (Zhou et al., 2009). These clones adapted probably by changing the epigenetic switching rate. This finding shows a possible mechanism by which a genetic change

affects the rate of epigenetic switching leading to the adaptation to a new environment.

As we have seen, epigenetic silencing increases the variety of options available for adapting to a novel environment. With this additional mechanism of adaptation, populations can adapt by the changing the epigenetic switching rate without the need of acquiring a genetic change in the gene under selection. Instead populations can adapt by acquiring mutations in the genes that control the epigenetic machinery itself. This provides a clear mechanism by which gene silencing can speed up adaptation and shows a tight epistatic interaction between the underlying genetic sequence and heritable gene expression states.

Materials and Methods

Strains and growth conditions

All Saccharomyces cerevisiae strains used in this study were derived from the S288c (*MATα ura3-52*, *trpΔ63*, *hisΔ200).* Y170, a *Ura*$^{+}$ strain was created by restoring the endogenous *URA3* allele by integration of a *URA3* construct amplified from the FEP193 strain, originally derived from pFEP24 (Pryde and Louis, 1999). Y181, a *ura*$^{-}$ strain was created in which all *URA3* sequences were deleted by integration of a KanMX6 cassette amplified from pLJ32 (pLoxB-KMX; kind gift form Jaap Brouwer, Leiden University) into Y170, deleting nucleotides between positions 115,929 and 117,048 on chromosome V. This strain was used for the integration of *URA3* cassettes at 7 different positions in the subtelomeric region of the left arm of chromosome XI. Insertion were made telomere proximal of the following nucleotide coordinates: position 1 (strain Y182)- 3,305; position 2 (strain Y183)- 2,805; position 3 (strain Y184)- 2,400; position 4 (strain Y185)- 1,624; position 5 (strain Y186)- 1,374; position 6 (strain Y187)- 1,124; position 7 (strain Y188)- 874. In all strains *URA3* is transcribed in the telomere direction.
SIR3 knock-out strains were constructed by replacing the whole ORF (between nucleotide positions 1,019,287 and 1,022,281 on chromosome XII) with a *hphMX4* cassette (hygromycin resistance) amplified from the plasmid pAG32 (Goldstein and McCusker, 1999). *SIR3* was deleted in all 7 strains bearing a telomeric *URA3* insertion (Y182 through Y188), generating strains Y189 through Y195, respectively.
All strains were maintained either in rich medium [YPD; 1% Bacto yeast extract- BD (Fisher; #212720), 2% Peptone (Fisher; #BP1420-500), 2% Glucose (Merck; #1.08342.1000), either as liquid medium or supplemented with 2% Agar (Roth; #2266,4) for solid medium] or in complete synthetic dropout medium [CSM; 0.7% Yeast nitrogen base

(Sigma; #Y0626), 0.1% Complete synthetic medium (MP Biomedicals; #4560-222), 0.005% Tryptophan (Sigma; #T0254), 0.002% Histidine (Sigma; #H8000), 0.001% Uracil (Sigma; #U0750), 2% Glucose (Merck; #1.08342.1000), either as liquid medium or supplemented with 2% Agar (Roth; #2266,4) for solid medium]. Sequenced evolved strains are used from populations that were isolated previously (Figure 2.S3).

Spot assay

After 2 days of growth on rich YPD media plates, fresh single colonies were picked and suspended into 100µl of distilled, demineralized water. Six 10-fold dilutions were prepared and 10 µl was plated onto plates of CSM or CSM supplemented with 0.1% 5-FOA (Apollo Scientific; #PC4054) and also onto plates that were supplemented with 5 mM nicotinamide (Fisher; # 1663C) Plates were imaged and scored after three days of incubation at 28°C.

Experimental evolution

All *sir3Δ* strains were preselected to be in $URA3^+$ state by growing cells in liquid CSM lacking uracil for 16 hours. Next, 10^6 cells were diluted into 1ml liquid CSM supplemented with 0.05% 5-FOA. Every 24h OD_{600} was determined and $5*10^5$ cells were transferred into fresh medium. If populations had less than $5*10^5$ they were considered extinct. At each passage cells were analyzed for viability, 5-FOA resistance and growth on medium lacking uracil (scoring URA state). For this, approximately 100 cells are plated onto non-selective plates (YPD or CSM) and on plates containing 0.1% 5-FOA. Plates were incubated at 28°C for five days, after which colonies were counted and cell numbers in each population estimated. The number of cells plated on nonselective media imposes a 1% threshold of detection of

resistance. Plates with 5-FOA were replica plated onto CSM plates lacking uracil and incubated for three days at 28°C, after which the fraction of ura^+ and ura^- clones in each population were estimated. Experiment was performed once with eight population replicates for each strain.

Sequencing

Representative evolved clones from both the ura^+ and ura^- class were isolated at the final time point (144 hours) (Figure 2.S3 and Table 3.S1) and genomic DNA was extracted using standard procedures based on beads lysis followed by phenol/chloroform extraction and ethanol precipitation of genomic DNA. Libraries were prepared with Nextera kit (according to manufacturer's instruction) and were sequenced at approximately 30x coverage using an Illumina MiSeq Sequencer. The reads were processed using SeqTK (github.com/lh3/seqtk) to trim low-quality reads. Alignment was performed by BreSeq (Deatherage and Barrick, 2014) based on the yeast reference genome taken from Yeast Genome Database (SGD; www.yeastgenome.org). The alignments of all called mutations were manually checked. Mutations in homopolymers were very common and discarded because they are difficult to distinguish from sequencing error and were all in intergenic regions. The list of mutations in each evolved clone was compared with the list of mutations in its ancestor in order to determine which were acquired during the experiment. The full list of all mutations considered can be found in Table 3.S1. Whole genome sequence data for all strains listed are available at the NCBI Sequence Read Archive (SRA), Accession number: SRP129019.

Mutation rates

Mutation rates were determined by fluctuation tests and were performed on sir3Δ clones and SIR3 wild-type used previously in experimental evolution (Figure 2.1), as previously described (Lang and Murray, 2008). Three clones per each position were analyzed to account for possible inter clonal differences. In brief, cultures were grown in CSM without uracil to saturation to select cells in the $URA3^{+}$ state. Cells were then transferred to 96 YPD cultures at a concentration of 10^{3}cells/ml. After 3 days of nonselective grown, 7 cultures were pooled together and appropriate dilutions were plated on YPD plates to estimate the average number of cells per well. The remaining 89 wells were plated as lawns onto CSM plates with 0.1% 5-FOA, in the case of sir3Δ strains, to estimate number of populations in which ura^{-} mutations occurred. Additionally SIR3 wild-type clones were pleated on plates containing 5mM nicotinamide, so that only cells with ura^{-} mutation would be able to grow. Plates were incubated at 28°C for 5 days, after which the number of mutants per lawn was scored. The mutation rates were calculated using the p0 method (Rosche W.A., 1947) and 95% confidence interval (CI) was determined with binomial exact test. The values for mutation rates are as follows:

Position 4 (sir3Δ) - $8.74*10^{-6}$ (CI: $1.14*10^{-5}$-$6.55*10^{-6}$), $9.18*10^{-6}$ (CI: $1.2*10^{-5}$-$6.86*10^{-6}$), $1.02*10^{-5}$ (CI: $1.33*10^{-5}$-$7.69*10^{-6}$);

Position 5 (sir3Δ) - $9.15*10^{-6}$ (CI: $1.19*10^{-5}$-$6.86*10^{-6}$), $1.2*10^{-5}$ (CI: $1.57*10^{-5}$-$9.03*10^{-6}$), $8.03*10^{-6}$ (CI: $1.05*10^{-5}$-$5.98*10^{-6}$);

Position 7 (sir3Δ) - $8.19*10^{-6}$ (CI: $1.07*10^{-5}$-$6.13*10^{-6}$), $9.16*10^{-6}$ (CI: $1.2*10^{-5}$-$6.86*10^{-6}$), $8.41*10^{-6}$ (CI: $1.11*10^{-5}$- $6.19*10^{-6}$);

Position 3 (sir3Δ) – $4.86*10^{-6}$ (CI: $3.5*10^{-6}$-$6.53*10^{-6}$), $4.94*10^{-6}$ (CI: $3.59*10^{-6}$-$6.61*10^{-6}$), $3.19*10^{-6}$ (CI: $2.27*10^{-6}$-4.34*10-^{6});

Position 1 (sir3Δ) - $2.22*10^{-6}$ (CI: $1.47*10^{-6}$-$3.2*10^{-6}$), $2.4*10^{-6}$ (CI: $1.61*10^{-6}$-$3.41*10^{-6}$), $1.28*10^{-6}$ (CI: $7.69*10^{-7}$-$2.2*10^{-6}$);

Position 4 - $5.01*10^{-6}$ (CI: $3.47*10^{-6}$-$6.97*10^{-6}$), $3.88*10^{-6}$ (CI: $2.69*10^{-6}$-$5.4*10^{-6}$), $5.81*10^{-6}$ (CI: $4.16*10^{-6}$-$7.86*10^{-6}$);
Position 5 - $3.58*10^{-6}$ (CI: $2.54*10^{-6}$-$4.89*10^{-6}$), $5.52*10^{-6}$ (CI: $4.1*10^{-6}$-$7.25*10^{-6}$), $4.66*10^{-6}$ (CI: $3.41*10^{-6}$-$6.2*10^{-6}$);
Position 7 - $4.44*10^{-6}$ (CI: $3.23*10^{-6}$-$5.95*10^{-6}$), $2.77*10^{-6}$ (CI: $1.9*10^{-6}$-$3.88*10^{-6}$), $4.17*10^{-6}$ (CI: $2.95*10^{-6}$-$5.69*10^{-6}$);

Acknowledgments

I would like to thank my supervisors, Lars Jansen and Lilia Perfeito, for helping and guiding me in conceiving this study, also for their support and advices in conducting the experiments. I would also like to acknowledge João Mata for the help he has provided me while performing the experiments. The scientific input from the members of my thesis committee, Patricia Beldade and Miguel Godinho, was of great importance in determining the direction of the project.

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Supplementary figures

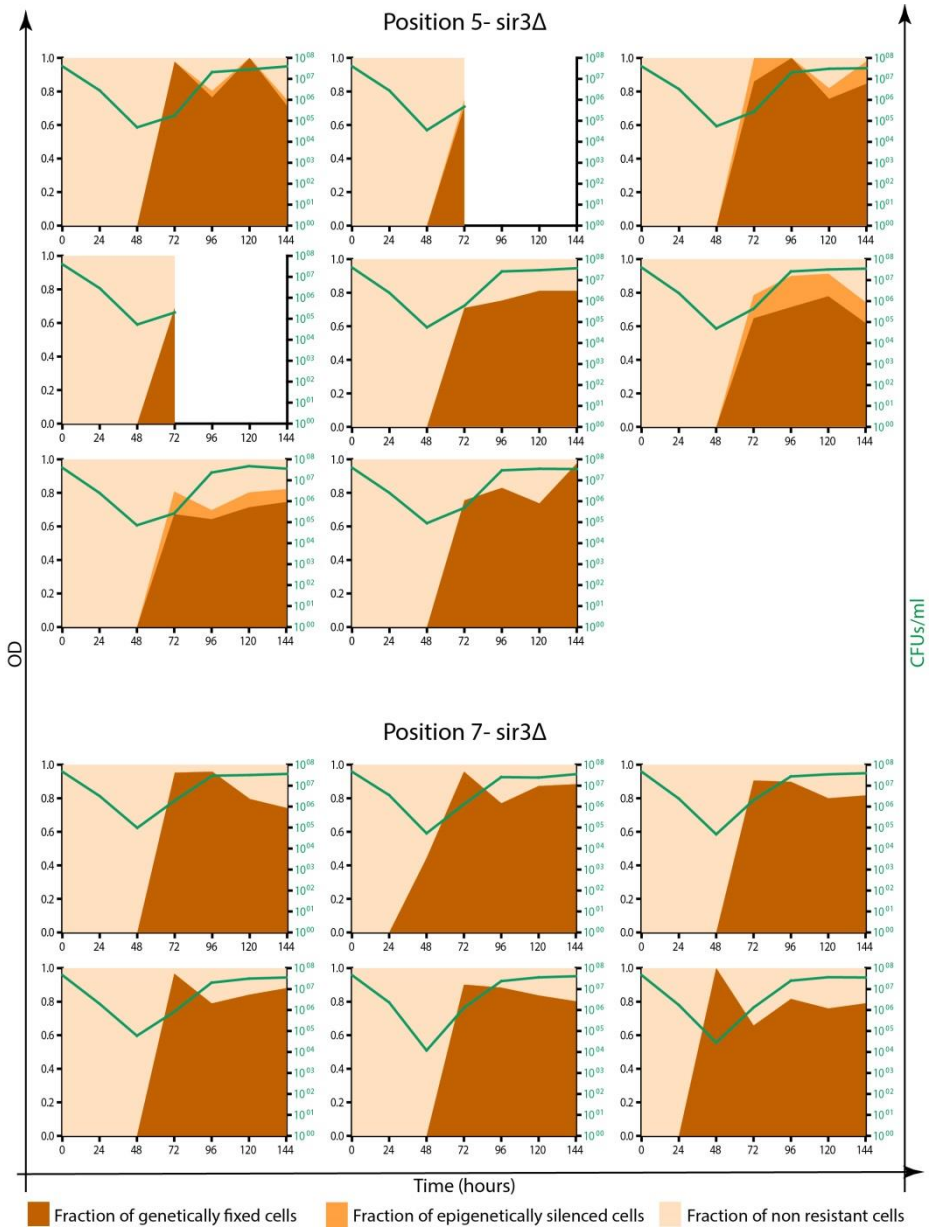
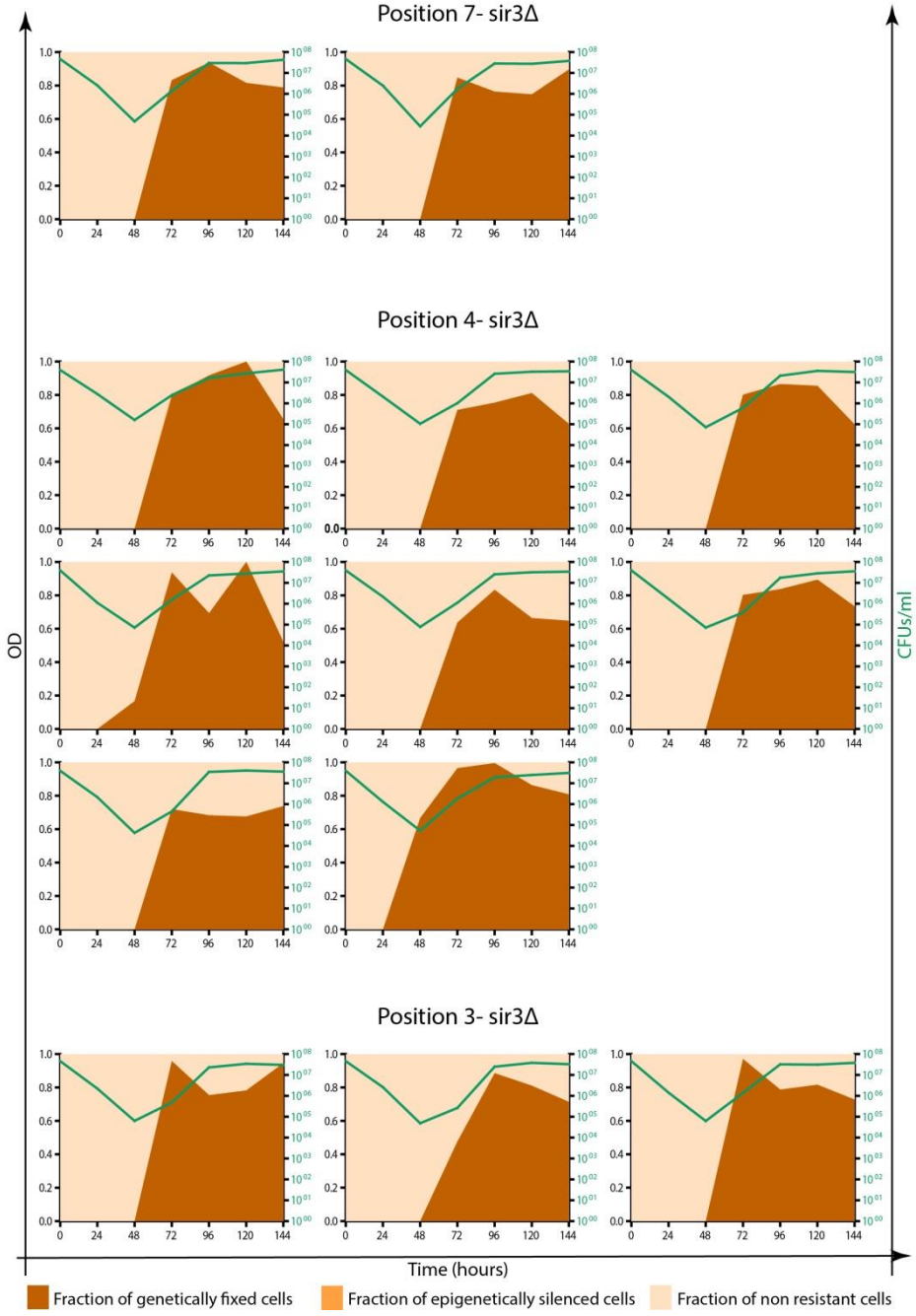
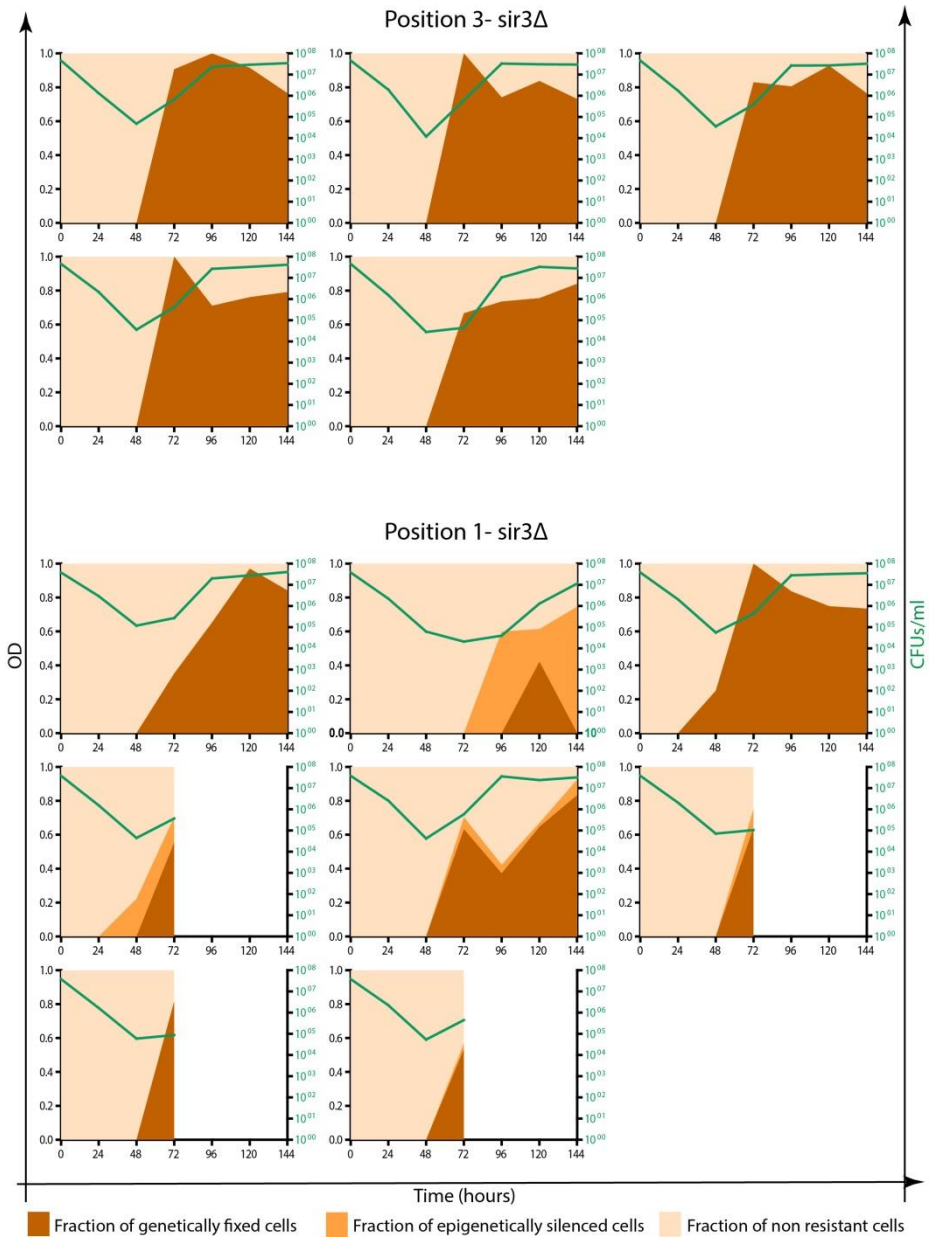


Figure 3.S1 Population dynamics of acquisition of 5-FOA resistance during experimental evolution in *sir3Δ* background. Plots as represented in Figure 3.2. All replicate populations are shown. Green line indicates the total number of living cells within a population during the course of the experiment [colony forming units (CFUs)/ml]. Colors represent the different phenotypic subgroups within each population (as indicated), plotted as the size fraction of each subgroup within the total population across time.


Position 7- sir3Δ
Position 4- sir3Δ
Position 3- sir3Δ
OD
CFUs/ml
Time (hours)
Fraction of genetically fixed cells
Fraction of epigenetically silenced cells
Fraction of non resistant cells


Position 3- sir3Δ
Position 1- sir3Δ
OD
CFUs/ml
Time (hours)
Fraction of genetically fixed cells
Fraction of epigenetically silenced cells
Fraction of non resistant cells

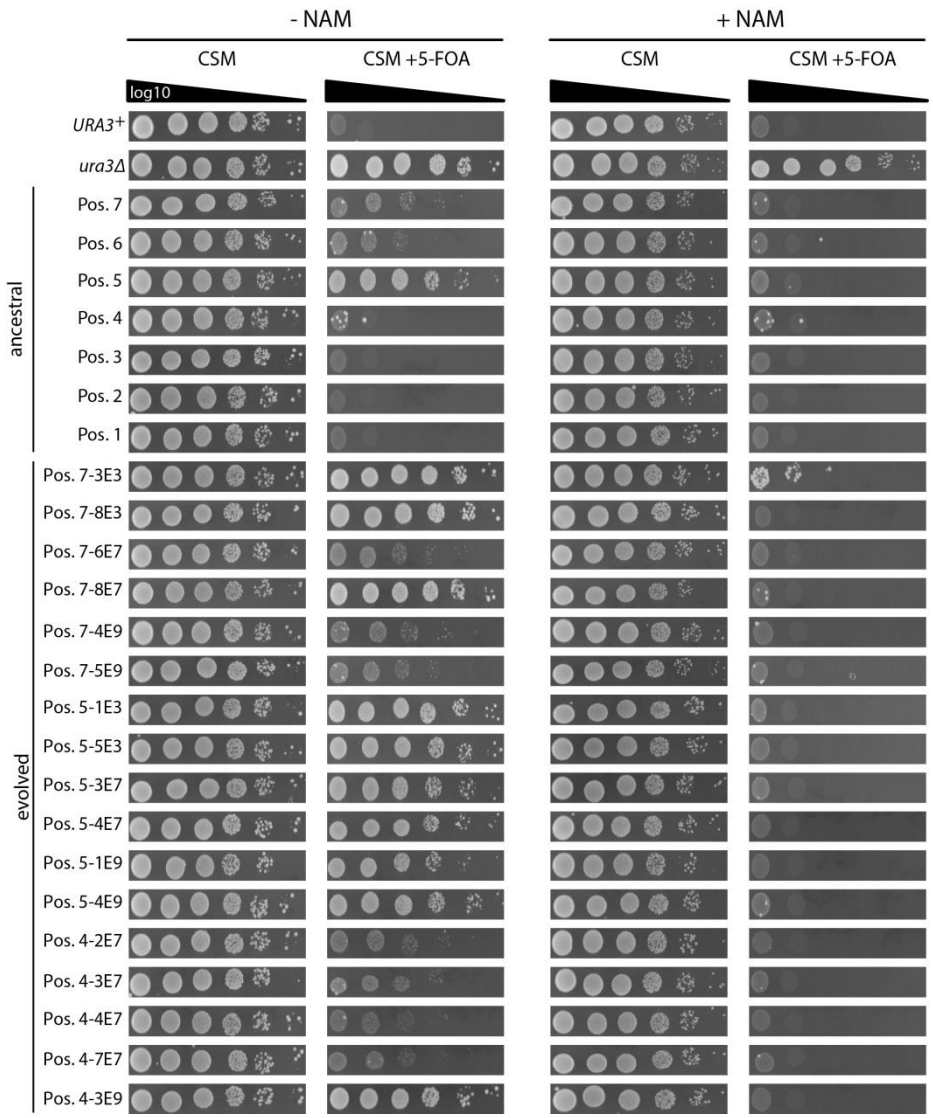
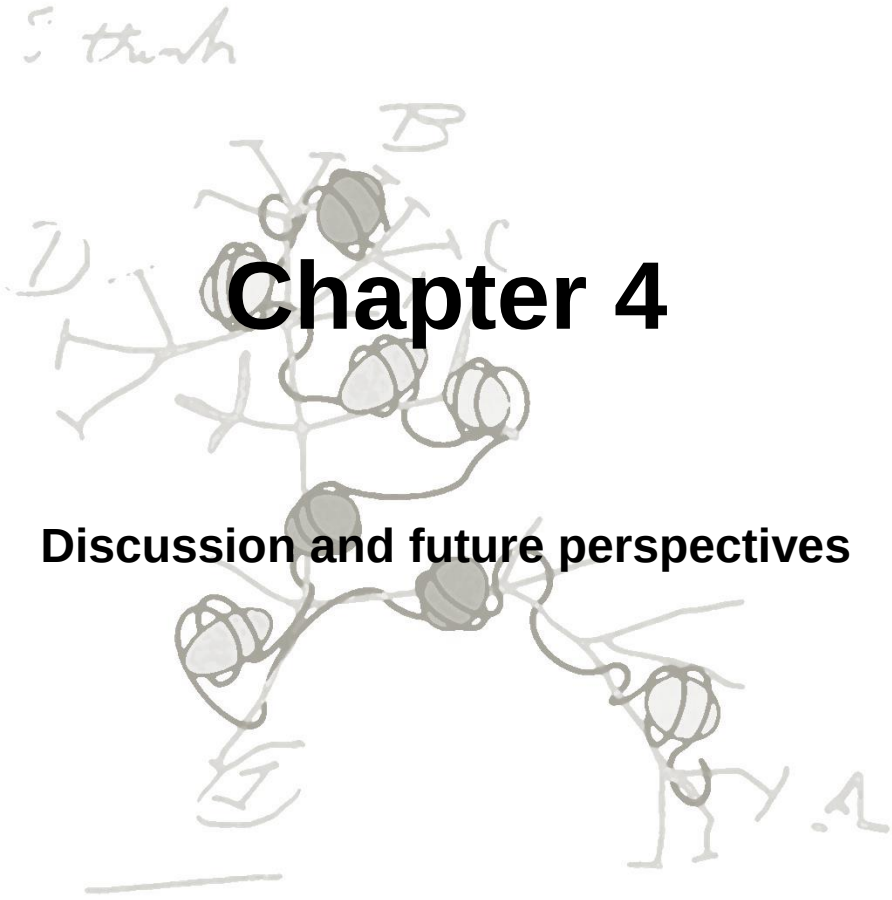


Figure 3.S2 Evolved *URA3*$^{+}$ clones adapt by enhancing epigenetic gene silencing. Ten-fold dilutions of indicated ancestral and evolved clones (for detailed list see Table 3.S1) were spotted on plates containing nicotinamide (with and without 5-FOA) or minimal media plates (CSM-with and without 5-FOA) as control for the level of resistance to the 5-FOA. *URA3*$^{+}$ and *ura3Δ* strains were used as negative and positive controls respectively.

Evolved silent ura+ clones									
Strain	Clone	Repl. Pop.	Chromosome	Position	Ancestral allele	Mutant allele	Type	Gene	AA change
Pos. 7	3E-3	17	XII	173,691	A	C	Nonsynonymous	PPR1	L→stop
Pos. 7	8E-3	6	XIV	18,043	A	C	Nonsynonymous	RPD3	Y→stop
Pos. 7	6E-7	13	XVI	185,419	T	G	Nonsynonymous	NAB3	N→T
Pos. 7	6E-7	13	XVI	1,032,708	C	G	Nonsynonymous	GPB1	G→A
Pos. 7	8E-7	19	XII	173,179	C	A	Nonsynonymous	PPR1	E→stop
Pos. 7	4E-9	7					no mutation		
Pos. 7	5E-9	1					no mutation		
Pos. 5	1E-3	1					no mutation		
Pos. 5	5E-3	13					no mutation		
Pos. 5	3E-7	14					no mutation		
Pos. 5	4E-7	17					no mutation		
Pos. 5	1E-9	15					no mutation		
Pos. 5	4E-9	22					no mutation		
Pos. 4	2E-7	4	IV	1,247,059	G	A	Nonsynonymous	MUS81	A→T
Pos. 4	2E-7	4	XII	638,857	T	C	Nonsynonymous	YEF3	L→S
Pos. 4	4E-7	10					no mutation		
Pos. 4	7E-7	16					no mutation		
Pos. 4	3E-7	7	XII	638,857	T	C	Nonsynonymous	YEF3	L→S
Pos. 4	3E-9	9	XII	173,515	T	A	Nonsynonymous	PPR1	R→stop
Pos. 4	3E-9	9	XI	575,988	T	C		intergenic	
Pos. 1	3E-7	11	XIII	57,359	T	A	Nonsynonymous	URA5	L→stop

Evolved ura- clones									
Strain	Clone	Repl. Pop.	Chromosome	Position	Ancestral allele	Mutant allele	Type	Gene	AA change
Pos. 7	6M-3	21	XI	1-1,012			deletion		
Pos. 7	8M-3	6	XI	1-1,335			deletion		
Pos. 7	3M-7	15	XI	1-2,645			deletion		
Pos. 7	8M-7	19	XI	1-2,343			deletion		
Pos. 7	5M-9	1	XI	1-1,550			deletion		
Pos. 7	1M-9	22	XI	1-1,592			deletion		
Pos. 5	2M-3	4	XI	1-2,405			deletion		
Pos. 5	5M-3	13	XI	1-2,348			deletion		
Pos. 5	3M-7	14	XI	1-1,356			deletion		
Pos. 5	4M-7	17	XI	1-2,552			deletion		
Pos. 5	4M-9	22	XI	1-1,356			deletion		
Pos. 5	1M-9	15	V	116,293	G	T	Nonsynonymous	URA3	E→stop
Pos. 5	1M-9	15	XV	124,046	C	A	Nonsynonymous	ITR2	L→I
Pos. 4	1M-7	1	V	116,795			1bp deletion	URA3	
Pos. 4	5M-7	13	XI	1-2,407			deletion		
Pos. 4	8M-7	3	XI	1-1,696			deletion		
Pos. 4	1M-9	6	XI	1-1,597			deletion		
Pos. 4	7M-9	18	XI	1-2,180			deletion		
Pos. 3	1M-3	1	V	116,569	C	T	Nonsynonymous	URA3	Q→stop
Pos. 3	3M-3	3	XI	1-2,446			deletion		
Pos. 3	4M-3	4	XI	1-2,365			deletion		
Pos. 3	1M-9	8	XI	1-2,304			deletion		
Pos. 3	6M-9	12	XI	2,314-2,399			deletion		
Pos. 1	1M-3	1	V	116,251	C	T	Nonsynonymous	URA3	Q→stop
Pos. 1	2M-3	2	V	116,341	A	T	Nonsynonymous	URA3	K→stop
Pos. 1	2M-3	2	MitDNA				deletion		
Pos. 1	3M-3	3	V	116,526	T	G	Nonsynonymous	URA3	N→K
Pos. 1	7M-3	7	V	116,341	A	T	Nonsynonymous	URA3	K→stop
Pos. 1	1M-7	9	V	116,627	C	T	Nonsynonymous	URA3	S→L

Table 3-S1. List of position and identity of mutations identified in evolved clones. Repl. Pop. is replicate population (corresponding to plots in Figure 2.S3) from which clone was recovered.



Chapter 4

Discussion and future perspectives

Abstract

Our results presented in previous chapters show that transgenerational inheritance of gene expression states can have significant impact on patterns of evolutionary adaptation. In this chapter I will discuss how epigenetic effects fit into the more general evolutionary framework. Recently, there have been calls for a new, extended evolutionary theory based mostly on new discoveries and research on the nature of epigenetic control of gene expression. However, such claims should reflect direct experimental evidence. It is has been proposed that epigenetic mechanisms of gene expression control will have the biggest significance to adaptation in fluctuating environments, considering the high switching capability between epigenetic states. The mechanisms of adaptation will depend on the fluctuation pattern of the environment. In a regularly fluctuating environment I would expect that the switching rate of epigenetic silencing would change to accommodate to the rate of change of the environment. However, in an irregularly fluctuating environment a bet hedging-like phenomenon or a more plastic response would evolve. Silencing in the budding yeast subtelomeric region can be subject to various environmental influences. This creates a possibility that the environment can induce or influence indirectly the adaptation to that environment. This is reminiscent to a neo-Lamarckian type of mechanism that allows for some aspects of Lamarck´s view on evolution to happen. Our study clearly demonstrates an approach by which this hypothesis can be tested.

Summary of the results

Before I discuss the future perspectives and the general implications of our results on evolutionary theory, I will first briefly summarize our observations.

We constructed seven yeast strains carrying a *URA3* reporter gene inserted in different positions within subtelomeric chromatin, causing a variable degree of epigenetic silencing of the gene (Chapter 2, Figure 2.1). We evolved these strains in an environment in which we selected against the expression of the URA3 reporter. We observed that the survival in the new, selective environment scales with the level of epigenetic silencing (Chapter 2, Figure 2.2). Cells with high epigenetic silencing adapted faster, possibly through more stable silencing of the reporter (Figure 4.1). However, these strains acquired adaptive mutations relatively late, probably due to clonal interference at high silencing. Similarly, strains with low silencing acquired mutations late due to the small population size resulting in a low mutational supply. Interestingly acquisition of mutations was the fastest at intermediate levels of silencing, balancing mutation supply and selection pressure. In populations with high and intermediate levels of gene silencing, the first step in adaptation most likely occurs through epigenetic gene silencing followed by the acquisition of adaptive mutations. Moreover, at the intermediate level of silencing we observed an expansion of mutational targets. In addition to mutations in uracil biosynthesis pathway, we identified mutations in several other genes, including some that are known to be involved in epigenetic states. This provides an important possible mechanism underlying accelerated adaptation as it suggests cells adapted by changing the strength of silencing through the acquisition of a mutation. In other words, we found evidence of transient heritable silencing directing long-term adaptation

by genetic mutations that in turn modifiy the heritability of silencing. We confirmed this notion by exposing the evolved cells to an inhibitor of the histone deacetylase, Sir2 known to be involved in silencing. Loss of silencing completely abolished the resistance to the drug used for selection (Chapter 3, Figure 3.S2).

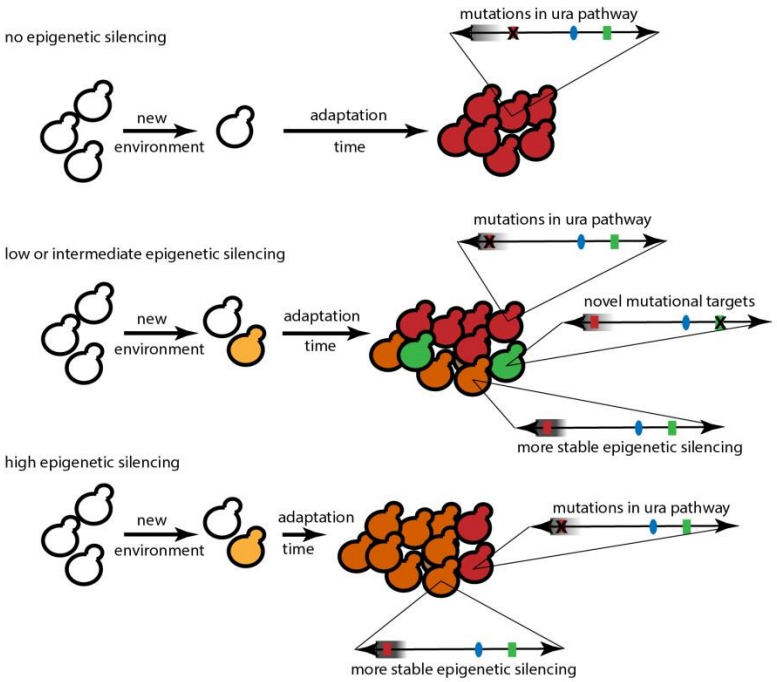


Figure 4.1 Observed and possible paths to adaptation and the contribution of epigenetic silencing. Populations with no epigenetic silencing adapt through mutation mostly through the acquisition of mutations in the *URA3* gene locus(Lang and Murray, 2008) at a low frequency (red cells). Intermediate levels of heritable gene silencing allows population size to expand (yellow cells) and results in the appearance of novel mutational targets (green cells) that may accelerate adaptation. Strong heritable gene silencing leads to preadapted populations and interferes with mutation accumulation. Possibly these non-mutant cells are epigenetically adapted (orange cells). Chromosomes are represented by lines with telomeres (arrows), centromeres (blue circles), *URA3* gene (red boxes) and additional non-*URA3* genes (green). Shaded area represents the region of epigenetic gene silencing.

Thus, these evolved clones adapted by mutation in a way that is dependent on the silencing machinery. Importantly, the differences in mutation rates we observe are not a consequence of intrinsic differences in mutation frequencies at the *URA3* insertion positions. Strains with telomere proximal position of *URA3* gene have similar mutation rates, although they show different adaptation patterns. This indicates that the main contributor to accelerated adaptation is epigenetic silencing. This notion is further supported by our observation that in strains that are genetically defective in silencing, the position of URA3 has no influence on the rate and type of adaptation (Chapter 3, Figure 3.2).

Towards a model of adaptation under epigenetic inheritance

As we have seen, the pattern of adaptation in a biological system in which expression of a gene under selection is controlled both by genetic and epigenetic means, can be quite different compared to a scenario where adaptation depends solely on genetic inheritance.

In principle, epigenetic inheritance can confer transmission of information for a phenotype in a similar manner as genetic forms of inheritance. The main difference between the two systems is their rate of change. Additionally, as epigenetic states are typically bistable, i.e. ON and OFF, this form of inheritance has a high reversion rate, rare in genetic mutations. Furthermore, epigenetic switching can depend on environmental cues.

To further elucidate the interaction between these two systems I set out to define a mathematical model that can correspond to the experimental setup we used. The results from the simulations of the model can be compared with experimental data and parameters determined more accurately. Moreover, our data suggests that there is an optimal degree of epigenetic silencing that is beneficial for the subsequent acquisition of mutations. The model can help us determine the range of epigenetic switching rates that are conferring advantage in that process. Furthermore, the model can make predictions how the system will behave in other types of environments.

In our experimental setup, populations have a structure composed of three subgroups, cells that are sensitive to the drug and have the *URA3* gene active (N_s), those that have a mutation in the gene and are of the *ura3*$^-$ phenotype (N_m) and those ones that are *URA3*$^+$, but have the gene silenced (N_e) (Figure 4.2).

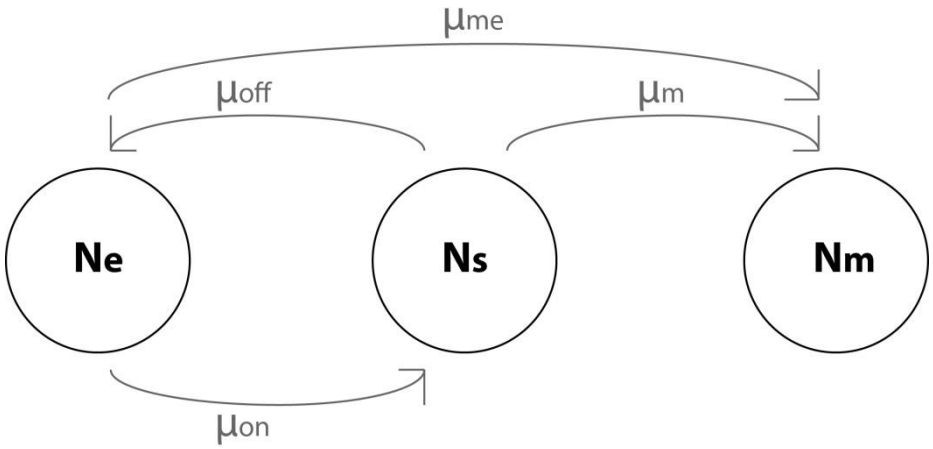


Figure 4.2 Schematic representation of population with epigenetic silencing. Circles represent each subgroup within a population, N_s- *URA3*$^+$ drug-sensitive cells, N_e-epigenetically silenced ura3$^+$ cells and N_m-cells with ura3$^-$ mutation. Arrows represent corresponding rates of change from one subgroup to another, μ_{off} -rate of silencing the gene, μ_{on}-rate of activating the gene, μ_m and μ_{me}, mutation rates without and with epigenetic silencing respectively.

The frequency of each subgroup will depend closely on the corresponding rates of change and the number of cells. From this setup we can easily derive equations for the frequencies of each subpopulation for a given time point as a function of a previous generation.

$$p_s(t) = (1-\mu_{off} - \mu_m) * p_s(t-1) + \mu_{on} * p_e(t-1)$$

$$p_e(t) = (1 - \mu_{on} - \mu_{me}) * p_e(t-1) + \mu_{off} * p_s(t-1)$$

$$p_m(t) = p_m + \mu_m * p_s + \mu_{me} * p_e$$

In the equations, rates are represented as μ_{off} -rate of silencing the gene, μ_{on}-rate of activating the gene, μ_m and μ_{me} represent mutation rates without and with epigenetic silencing respectively. The frequencies of epigenetically silenced, genetically mutated and drug sensitive subpopulations are denoted as p_e, p_m and p_s respectively. Moreover, the frequencies of each group will change due to the action

of selection. To account for that each generation I am adding a particular fitness advantage to epigenetically silenced and genetically mutated group. The mentioned frequencies are recalculated each generation using equations

$$p_e = \frac{(1 + s_e) * p_e}{\bar{W}}$$

$$p_m = \frac{(1 + s_m) * p_m}{\bar{W}}$$

$$\bar{W} = (1 + s_e) * p_e + (1 + s_m) * p_m + p_s$$

Where s_e and s_m are selection coefficients associated with epigenetic silencing and ura$^-$ mutation respectively, and $\bar{W}$ represents the mean fitness of a population. The selection coefficients, the advantage of silencing or mutating the gene, here are assumed to be the different. However, this is very challenging to confirm experimentally. Because of the switchable nature of epigenetically adapted clones it is difficult to perform head to head competition assays between these subgroups. We also assumed there is no fitness advantage of drug sensitive subpopulation.

This is very simplistic, deterministic model, since it is not taking into account processes such as clonal interference. However, it can give us a preliminary insight into the dynamics within a population under selection that has epigenetic silencing mechanisms. With particular set of values of the parameters we succeeded in recapitulating the dynamics of adaptation we observed in strain with strong epigenetic silencing (*URA3* at Position 5), where we see first rise of frequency of epigenetically silenced clones that establish an equilibrium around 50%, followed by the appearance of mutation (Figure 4.3)(Figure 2.S3).

The simulation was performed for 200 generations. The values for frequencies for $t = 0$ were $p_s = 1, p_e = 0$ and $p_m = 0$.

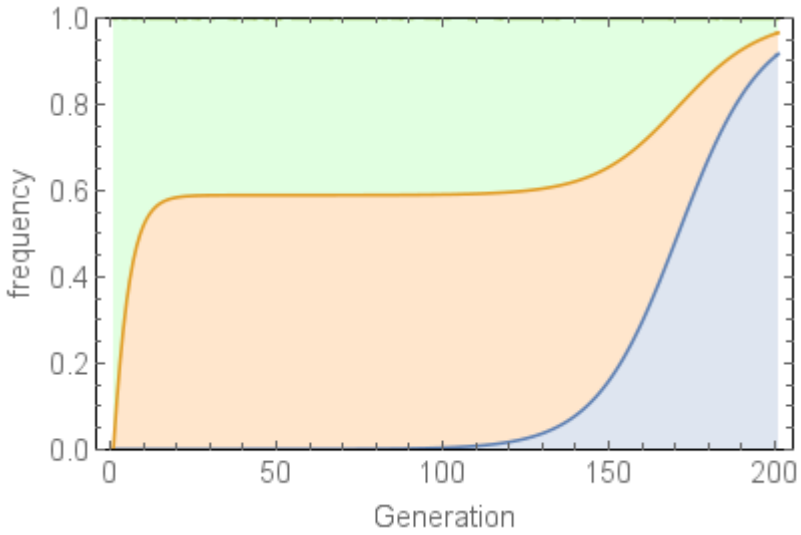


Figure 4.3 Simulation of the model. Light green color represents the fraction of drug sensitive cells, orange the fraction of epigenetically silenced and light blue the fraction of genetically mutated cells. The parameter values were set as follows $\mu_{off} = 0.018$, $\mu_{on} = 0.001$, $\mu_m = \mu_{me} = 10^{-7}$, $s_e = 0.046$, $s_m = 0.086$. Simulation was performed using Mathematica.

Although the mutation rates associated with epigenetically silenced and drug sensitive subgroups are different (Figure 3.1), here we assumed they have the same value. The epigenetic switching rates (μ_{off} and μ_{on}) are typically much higher than the mutation rate. More importantly, they are related to one another, although probably do not have the same value. In the case of strong silencing, such as we observed in strain with *URA3* at Position 5, the rate of silencing of the gene is higher than the rate of activating it. On the other hand, in the case of low levels of epigenetic silencing μ_{on} would be higher than μ_{off}.

Since the epigenetic switching rates are higher than DNA mutations, the appearance of epigenetically silenced cells will precede the appearance of mutations. Nevertheless, it is important to point out that μ_{on} and μ_{off} can themselves change and be subject to selection, through, for example, the acquisition of a mutation that might regulate their stability, as we have seen in Chapter 3.

All of these parameters can be estimated experimentally. Mutation rates can be measured using fluctuation assays (Chapter 3, Figure 3.1) in the presence of the inhibitor of epigenetic silencing or without it, defining $\mu_m$and μ_{me}, respectively. In contrast, the measurement of epigenetic switching rates is more challenging. One way to measure the rate of silencing of the gene would be to preselect the cells to activate the gene, by growing them in a medium where the activity of the gene is essential for survival, and subsequently transferring them into a non-selective media. The initial rise in frequency of resistant clones that inactivated the gene would reflect the switching rate, as described previously (Jeffery et al., 2013).

The estimated parameters can be used to inform the model and to theoretically test the predictions and further confirm the experimental observations.

Implications on the evolutionary theory and future perspectives

Our results indicate that current models of evolution need also to account for non-genetic forms of inheritance in order to better understand adaptation of biological systems that have heritable epigenetic control of gene expression. In other words, when determining phenotypic variance for particular traits it is crucial to understand the epigenetic component of that variance. Environmentally-induced epigenetic variation contributes to the variation imposed by the interaction of genes and the environment (V_{gxe}) (see Chapter 1). However, some epigenetic alleles are independent of the environment. Therefore, it is crucial to take into account also this component (V_{epi}) when determining phenotypic variance. Moreover, phenotypic variation might come also from the interaction between genes and those environmentally-independent epigenetic alleles (V_{gxepi}). Thus, the total phenotypic variation in that case would be

$$V_p = V_g + V_e + V_{gxe} + V_{epi} + V_{gxepi}$$

It is important to point out that the contribution of epigenetic mechanisms of gene control to the selectable phenotype will differ between organisms and environments. Additionally, future efforts should be aimed at determining the distribution of fitness effects of epigenetic changes. I would expect this distribution to be quite different from mutations, since epigenetic control of genes largely impacts on expression levels of a gene, while mutations can completely alter the function of a gene.

Our experimental setup is designed to determine the fate of a population when a single gene is under selection in order to have maximal control over both genetic and epigenetic variation. While serving well as a proof of concept, it is by necessity highly artificial. In

nature very rarely selection acts against the activity of a particular gene. However, one example that occurs naturally and is reminiscent of our experimental setup is the role of tumor-suppressor genes during clonal expansion in tumors. These genes are typically involved either in the control of the cell cycle or in the induction of a cell death (apoptosis) and suppress the progression of neoplastic transformation. Therefore, for cancer cells survival and proliferation it is crucial to inactivate these genes. Similar to our system, selection pressure is high under such conditions and the number of genes involved limited. In many instances it was shown that epigenetic silencing is one of the mechanisms of tumor suppressor inactivation (Buchholtz et al., 2014; Fatemi et al., 2014; Lomberk, 2011). Our results indicate that the epigenetic silencing of tumor suppressors can increase the rate of cancer evolution and also expand the mutational targets that would promote the clonal expansion of tumor cells. Such targets might be genes that would further stabilize the silencing of tumor suppressors. These mutations would have no effect on the cancer progression in the absence of silencing. However, if the silencing is present the mutations in these target genes might have severe consequences for the cancer patients. There are already attempts to develop cancer treatment drugs that would target the epigenetic machinery. Indeed, it was shown in cell cultures that the addition of inhibitors of DNA methylation and of histone deacetylases reduces cell proliferation, migration and invasion (Fatemi et al., 2014).

Another example in which our experimental setup could be relevant is that of genes that confer a large fitness advantage e.g. when in a stressful environment, but have enormous fitness cost associated with their expression under normal environmental conditions. Such is the case of the *FLO1* gene (Chapter 1) that is involved in flocculation process in budding yeast. The biofilm-like structures formed in this process were shown to be involved in stress response. Since yeast is

not always under stress conditions, having it under epigenetic control might enable populations to adapt faster to a novel environment. Indeed, this gene is located in subtelomeric region and was shown to be under epigenetic silencing.

Switchable gene expression states are expected to have significant fitness advantage in fluctuating environments. Under highly variable conditions a system that has a higher rate of change would be beneficial (Bonduriansky et al., 2012; Klironomos et al., 2013; Rando and Verstrepen, 2007). In regularly fluctuating environments the rate of epigenetic switching might evolve to correspond to the rate of change of selective cues. On the other hand, in irregularly fluctuating environments mechanisms reminiscent to phenotypic plasticity might evolve, in which an environmental sensor would develop that would control the epigenetic expression state and change it accordingly to the environmental cue. Alternatively, under such conditions a bet hedging type of mechanism might arise. Bet hedging is a phenomenon in which a phenotype of reduced fitness is produced in a given environment that will be beneficial and better adapted to a future environment that an organism might experience (Grimbergen et al., 2015). Our model system allows the possibility to test these predictions. In such experiments, we would grow yeast strains with different levels of epigenetic silencing in alternating media containing 5-FOA and media lacking uracil (negative and positive selection) and measure the impact of such alternating environments on the rate and type of adaptation. I would expect under those conditions that a clone with an intermediate level of silencing would become fixed in the population. Having very strong epigenetic silencing or too low would prove deleterious once environment changes. Moreover, I would not expect to see the appearance of the ura3^{-} mutations. They might be beneficial in the environment containing 5-FOA. However, these

clones would not be able to survive in the environment that lacks uracil.

More importantly, some epigenetic changes are under environmental control. Indeed such is the case with silencing in subtelomeric region of yeast. Under osmotic stress a genome-wide remodeling of histone modifications occurs (Magraner-Pardo et al., 2014). Moreover, the Sir2 protein, histone deacetylase, is dependent on NAD^+, whose concentration relies on the metabolic state of the cell. Indeed, it was shown that glucose concentration can alter the epigenetic silencing at mating type loci (McCleary and Rine, 2017). This puts us in a unique position to test how adaptation would proceed if the environment acts as an inductive force (see Chapter 1). Using our experimental setup, we could change the concentration of glucose causing altered level of silencing which would in turn result in different adaptation patterns to 5-FOA as we observed. Here the inductive and selective forces are different. However, it is not difficult to conceive a setup in which a gene related to glucose metabolism would be brought under epigenetic control and the adaptation to different glucose concentrations monitored. This would put us in position to directly test Lamarckian mechanisms of adaptation.

In recent years, there have been calls for a reformulation of the evolutionary theory, or Extended Evolutionary Synthesis (Laland et al., 2014, 2015). The proponents claim that current evolutionary theory is gene-centered and should incorporate phenomena such as developmental bias, phenotypic plasticity, non-genetic inheritance and niche construction. Developmental bias represents a developmental constraint on the effect of a mutation. Consequently, the probability of acquiring a particular developmental phenotype is higher than others. Phenotypic plasticity, as described in Chapter 1, is a phenomenon where one genotype can produce several phenotypes depending on the environment. Niche construction is a phenomenon in which

organism alters the environment and as a result changes the selective pressure upon itself. However, some of those phenomena are already incorporated in the evolutionary theory and are being investigated, such is the case with phenotypic plasticity. Other claims still need to be substantiated with experimental evidence (Charlesworth et al., 2017; Futuyma, 2017). The mechanisms and stability of non-genetic inheritance have been investigated extensively. However, their contribution to evolutionary processes was unknown. This is what we tried to change. Our observations follow the current evolutionary principles and can be easily incorporated into the evolutionary models by taking into account the rate and effect of epigenetic switching. Such attempts are already being made, as in the work of Bonduriansky and Day, 2009, who introduced non-genetic inheritance into the fitness calculations during the process of selection. However, our observations open door for testing other potential mechanisms of adaptation.

Evolutionary theory since its conception in the beginning of the 19th century has been continuously modified and is itself evolving to a form that would better describe the natural phenomena. This project reflects our attempt to understand how one such phenomenon, epigenetic inheritance, influences evolutionary outcomes. However, as outlined in this section it also opens more questions that are waiting to be answered.

Acknowledgements

I would like to thank above all my supervisors ,Lars Jansen and Lilia Perfeito for their invaluable help in conceiving the study and designing the experiments and most of all for their patience and support. Also I would like to thank Patricia Beldade, Miguel Godinho, Isabel Gordo, Èlio Sucena and to the whole IGC community for thought provoking discussions that expanded my intellectual horizons. I would also like to acknowledge Inês Amaro who helped with her valuable artistic skills in designing figures in this chapter.

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