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„Development of mutations in DNA repair deficient stationary phase cells of Saccharomyces cerevisiae“

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Abstract

Genome integrity is essential for the health of the individual. Mutations, which can occur in resting cells as well as in proliferating cells, may probably contribute to cancer and aging. Since a large fraction of human somatic cells are in a non-replicative state it is necessary to understand mechanisms in non-replicating cells.

In this diploma thesis I observed the different aspects of the onset of mutations in replicating and non-replicating cells of *Saccharomyces cerevisiae* (*S. cerevisiae*). The recently discovered different populations of resting cells, in particular quiescent (Q) and non-quiescent (NQ) cells, were analyzed separately regarding their development of mutations. Quiescence is a poorly understood state of the cell cycle. Q cells are dense, unbudded daughter cells in contrast to NQ cells, which war less dense, heterogeneous and become rather apoptotic. *S. cerevisiae* cells in glucose-limited culture become Q and NQ cells and due to their different physical properties they can be isolated using density gradient centrifugation. The isolation is the basis for further experiments regarding oxidative stress levels and their mutation frequencies. The experiments were carried out with different yeast strains. I analyzed the wildtype (concerning DNA repair mechanisms) strain YLBM/EH150 and strains with imbalanced or knocked out DNA repair pathways YLB4, YLB14, YLBA1, and YLB14A1.

For the detection of oxidative stress via microscope and FACS I used MS for the detection of superoxide anion.

Furthermore, the mutations frequencies were determined using the canavanine assay, which is a forward mutation assay. In the course of this assay I determined the number of mutations in a gene that is independent of the selective pressure.

In addition the differences of DNA Double strand breaks (DSBs) occurring during glucose starvation in Q and NQ cells were analyzed via Western Blot.

All these experiments were needed to reveal undiscovered properties and behaviour of stationary phase cells. The understanding of the accumulation of mutations in resting cells is important, because they are a potential risk for cancer development.

Zusammenfassung

Die Stabilität des Genoms ist essentiell für die Gesundheit des Individuums. Mutationen entstehen nicht nur in replizierenden Zellen, sondern auch in ruhenden Zellen und können Krebs und Alterungsprozesse verursachen. Potentieller Auslöser für Mutationen sind DNA Läsionen. Ein großer Teil der menschlichen somatischen Zellen befindet sich im nicht replizierenden Zustand. Daher ist es von entscheidender Bedeutung, die Mechanismen in ruhenden Zellen kennenzulernen, die solchen Läsionen, bzw. den entstehenden Mutationen, zugrundeliegen.

Im Rahmen der Diplomarbeit wurden die verschiedenen Aspekte der Mutationsentstehung in replizierenden und nicht replizierenden Zellen von *Saccharomyces cerevisiae* (*S. cerevisiae*) untersucht wurden. Die erst kürzlich entdeckten unterschiedlichen Populationen ruhender Zellen - „eigentlich ruhende“, sogenannte „Quieszente Zellen“ (Q), und eine heterogenere Gruppe, die eher apoptotisch werdenden „Nicht Quieszenten Zellen“ (NQ) - wurden getrennt voneinander untersucht. Q Zellen sind im Gegensatz zu NQ Zellen dichtere Tochterzellen, während NQ Zellen weniger dicht, heterogen und eher apoptotisch sind. *S. cerevisiae* Zellen in Kultur mit limitierten Nährstoffen entwickeln sich zu Q und NQ Zellen und können durch Dichtegradientenzentrifugation isoliert werden. Die Isolierung ist die Basis für weitere Experimente hinsichtlich des oxidativen Stresses sowie der Mutationsfrequenzen. Die Experimente wurden über einen Zeitverlauf mit unterschiedlichen Hefestämmen durchgeführt. Es wurden folgende Stämme untersucht: Wildtyp (hinsichtlich DNA Reparatur Mechanismen) YLBM/EH150, sowie die Stämme YLB4, YLB14, YLBA1 und YLB14A1, bei denen DNA Reparaturwege beinträchtigt oder völlig ausgeschalten sind. Oxidativer Stress wurde unter Verwendung des Fluoreszenzfarbstoffes MitoSox Red mittels FACS detektiert. Die Mutationsfrequenzen wurden durch einen „forward mutation assay“ (canavanine assay) bestimmt. Zusätzlich wurden die

Unterschiede an DNA Schäden, die im Verlauf der Glukose-Hungerung auftreten, durch Western Blot untersucht. Diese Experimente sollen zur Aufklärung der Mechanismen der Mutationsentstehung in ruhenden Zellen beigetragen. Diese sind insofern von Bedeutung, als sie ein Risiko für die Entstehung von Krebs darstellen.

Abbreviations

AA	amino acid
APS	ammoniumpersulphate
BER	base excision repair
BSA	bovine serum albumine
ONC	over night culture
can	canavanine
cfu	colony forming units
c/ml	cells per milliliter
c/pl	cells per plate
d0	day 0
ddH2O	double distilled water / aqua bidest
DSB	double strand break
DTT	dithiothreitol
exp	exponential cells
EXT	external
f.c.	final concentration
HR	homologous recombination
INT	internal
incl	inclusive
kDa	kilodalton
MeOH	methanol
MS	MitoSox Red
MMR	mismatch Repair
NER	nucleotide excision repair
NHEJ	non homologous end joining
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
rpm	revolutions per minute
ROS	reactive oxygen species
SB	sterile bidest water
SC	synthetic complete

SDS	sodium dodecyl sulfate
SM	synthetic minimal
SSB	single strand break
TEMED	N, N, N, N-tetramethylethylenediamine
YNB w/o AA	yeast nitrogen base without amino acids
βME	β-mercaptoethanol
YAC	yeast artificial chromosome

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1. Introduction

1.1. General Information

The term "yeast" is a morphological term that refers to eukaryotic micro-organisms classified in the kingdom of fungi, with about 1.500 species. Yeasts are unicellular and exhibit a great diversity with respect to cell size, shape and colour. Their size can vary depending on the species, typically measuring 3 - 4 µm diameter, although some yeasts can reach more than 40 µm. This is mainly due to alterations of physical and chemical conditions in the environment **[1]**.

Yeast cells share most of the structural and functional features of higher eukaryotes, which has rendered yeast as an ideal model for eukaryotic cell biology. In contrast to mammalian cells, yeast cells are surrounded by a rigid cell wall and develop birth scars during cell division. These scars are protrusions at the cell surface which remain on the mother cell of budding yeast **[2]**.

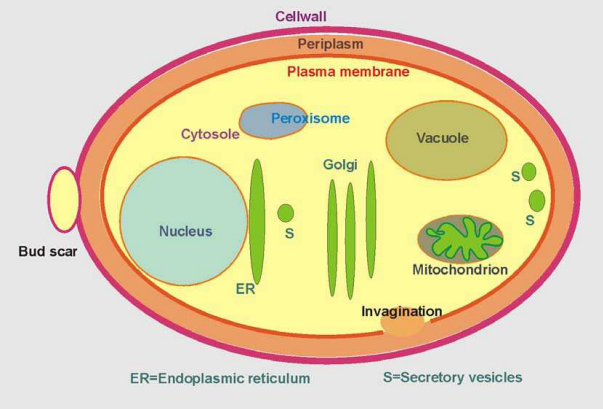


Fig. 1: Scheme of organelles and compartments in a yeast cell

Yeasts hold a wide dispersion of natural habitats. They are common on plant leaves and flowers, soil and salt water. Yeasts are also found on the skin surfaces and in the intestinal tracts of warm-blooded animals. Macromolecular constituents of yeast comprise proteins, glycoproteins, polysaccharides, polyphosphates, lipids and nucleic acids. Because yeast is also a rich source of B vitamins, it is often taken as a vitamin supplement **[1,3]**.

The world's most important yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*), has been a very useful fungus for humans for many millennia. It lives on sugars and has been used for beer brewing already 6000 B.C. in Babylonia. Because of the fermentation of the sugars of rice, wheat, barley and corn it is used for the production of alcoholic beverages and in the baking industry to expand or raise dough in the oven. That is where the names brewer's and baker's yeast come from **[1].** Today, they are also used in a variety of commercial fermentation and biomass conversion processes.

1.2. Reproduction and Life Cycle

Many species of yeast are unicellular and reproduce asexually. Most yeasts, including *S. cerevisiae,* reproduce by budding, but a few yeasts, like *Schizosaccharomyces pombe* (*S. pombe*), reproduce by simple binary fission.

In *S. cerevisiae,* the process of budding takes place at the poles of the cell. In these areas the mother cell builds out a lateral outgrowth, called the bud, which will become the new cell. While the bud is enlarging the chromosomes of the parent cell replicate. Then mitosis occurs, with one of the nuclei moving into the newly formed cell. The bud grows in size while still being attached to the parent body. When the new cell bud reaches the approximate size of the parent cell, it separates, leading to a daughter cell.

The cycle begins again for both cells. The result is an exponential increase in the number of cells with a doubling time equal to the mean cell-division cycle time (90 - 120 min).

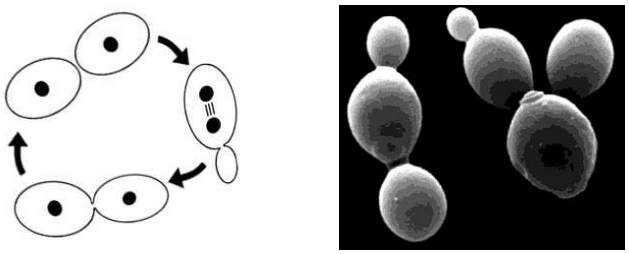


Fig. 2: Budding Yeast

S. pombe practices fission, which is a simpler process. During fission mitosis occurs, followed by an elongation of the cell and the formation of a cell wall that divides the cell in halves, separating the two nuclei **[4].**

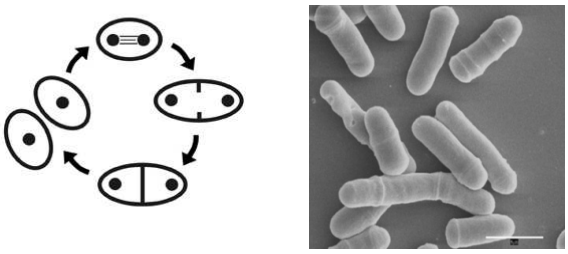


Fig. 3: Fission Yeast

The life cycle of *S. cerevisiae* alternates between diplophase and haplophase. Both ploidies can exist as stable cultures. In heterothallic strains, haploid cells exist as two mating types: **a** and α. Mating only occurs between haploid cells resulting in **a** / α diploids which are unable to mate but can undergo meiosis to produce four haploid spores: two **a** and two α spores.

1.3. Metabolism

Yeast cells have simple nutritional needs. They are unable to carry out photosynthesis; they require a reduced carbon source as well as a nitrogen source. Furthermore biotin and a variety of salts and trace elements are essential as well.

S. cerevisiae is facultative anaerobe. They can grow either aerobically in the presence of oxygen where they grow by oxidizing carbon sources, such as sugars, ethanol, acetate or glycerol. If they have adequate oxygen, they will oxidize their carbon sources to carbon dioxide and water. In the presence of high Glucose concentrations they also produce Ethanol aerobically, called the Crabtree effect. Under anaerobic conditions, yeasts convert sugars only to carbon dioxide and ethanol. The growth will be limited in both cases by essential nutrients.

The major source for energy production in yeast is glucose and glycolysis is the general pathway for the conversion of glucose to pyruvate. The use of pyruvate in further energy production can be distinguished either in respiration or fermentation. In the presence of oxygen glycolysis is linked to the citric acid cycle which produces CO_2 and ATP. Under anaerobic conditions yeast starts fermentation. The fermentation of sugar, which is one of the oldest and largest applications of biotechnology with *S. cerevisiae* proceeds according to the chemical reaction:

- $C_6H_{12}O_6 + 2\ ADP + 2\ P_i \dashrightarrow 2\ CO_2 + 2\ C_2H_5OH + 2\ ATP + H_2O$

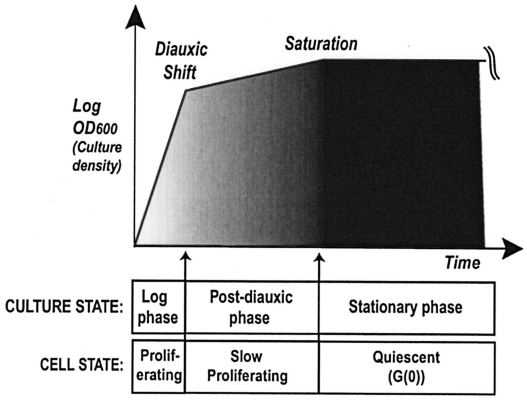


Fig. 4: Growth Phases of S. cerevisiae [13]

The stationary phase, also called the G0 Phase, enables yeasts to survive prolonged periods without nutrients **[5, 6]**.

1.4. Cell Cycle and Growth of Yeast in Culture

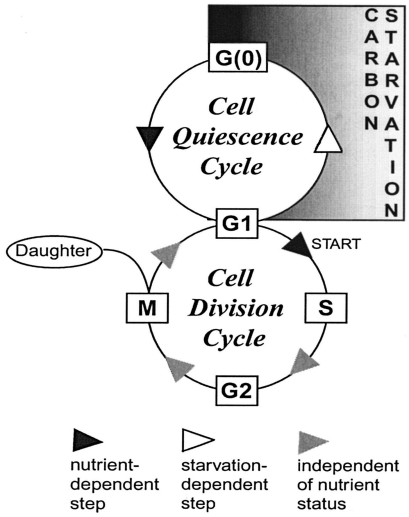


Fig. 5: Cell quiescence and division cycle [13]

In general, the cell cycle can be defined as the period between division of a mother cell and subsequent division of its daughter cell. In yeasts the cell cycle

follows a predictable schedule of events: G0/G1, S, G2, which are subphases within the interphase, followed by mitosis M and cytokinesis.

G1 - Phase is the first growth phase of the interphase, where the cell increases in mass in preparation for the cell division. Biosynthetic activities of the cell work at a high rate.

During S - Phase the DNA of the cell, which is packed into chromosomes, is replicated. This is necessary to produce two similar daughter cells. Once DNA replication is complete the cell contains twice its normal number of chromosomes and is ready to enter G2 phase.
In G2 - Phase again, significant biosynthesis occurs, mainly involving the production of microtubules, which are required for mitosis. G2 is a safety gap in which a cell can check if its DNA and other intracellular components have been duplicated properly. Therefore, it represents the cell's final chance to grow before the split into two independent cells.
Mitosis is the process by which a cell separates its previously duplicated genome. As Mitosis is completed the cell starts cytokinesis, which allocates the nuclei, Cytoplasm, Organelles and cell membrane into the daughter cells, which are mostly genetically identical. *S. cerevisiae* undergoes a "closed" mitosis, where chromosomes divide within an intact cell nucleus **[7]**.

1.4.1. Growth of Yeast in Culture

- Lag Phase
- Log Phase
- Stationary Phase (led in by diauxic shift)
- Death Phase

The lag phase is the time during which the cells become acclimatized to the environment and prepare to reproduce and consume large amounts of sugar.

During lag phase oxygen is used for synthesis of sterols and fatty acids which are essential growth factors.

The log phase is the time of exponential growth of the yeast culture. While the cells are reproducing they consume sugar and other nutrients like nitrogen or amino acids.
External conditions like temperature and nutrient availability have an important influence on this phase.

Yeast cells may run through the cell division cycle or arrest in G0. The decision is made in early G1 phase. At this point (START) the cell has to check if all nutrients and growth factors are available to start the cell division cycle. If the cell passes this START point, it has to complete a whole turn of the cell division cycle with a daughter cell as final product.
If sugar is exhausted, the cells use the ethanol - which they have produced themselves throughout the log phase - as a carbon source. This period (of readjustment) is called diauxic shift. After the depletion of ethanol and other nonfermetable carbon sources the culture enters the stationary phase. In this phase all cells should be arrested in G0 phase - the exit from active proliferation into a stable non-proliferating state is performed.
Cells in G0 phase cycle display various characteristics, which distinguish them from proliferating cells: transcription rate and protein synthesis are strongly reduced and the chromosomes are condensed. They fail to accumulate mass and volume and are arrested as unbudded cells. The cell wall thickens and thus the cells are more resistant to digestion with zymolyase. Furthermore, these cells are more thermotolerant and osmotolerant than their proliferating counterparts **[6]**.

In the study of Aragon A.D. et al **[8]** it was shown that *S. cerevisiae* cells in stationary phases are not homogenous, in contrast to the expectations. Glucose exhaustion leads to the formation of so called Q and NQ cells. Although they both are stationary-phase cells, they exhibit significant different morphological and physiological characteristics **[9]**, as mentioned below.

Quiescent cells:

Quiescent cells can be separated from non quiescent cells one or two days after glucose exhaustion. They are unbudded daughter cells arrested upon glucose exhaustion during the diauxic shift. They are much smaller and more dense than non quiescent cells. It is suggested that they are in G0 state and have the ability to reenter synchronously the mitotic cell cycle when nutrients and growth factors become available. Their cell wall is thickened and their metabolic rate is decreased. Furthermore, quiescent cells are more thermotolerant and retain viability without added nutrients.

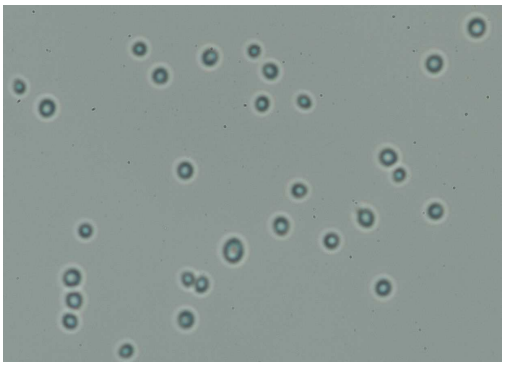


Fig. 6: Quiescent (Q) yeast cells

Non Quiescent cells:

In contrast, non quiescent cells are mother cells. They are less dense and comprise of budded and unbudded cells. They rapidly lose the ability to reproduce and become apoptotic faster than quiescent cells do. In addition non quiescent cells exhibit more signs of oxidative stress and apoptosis.

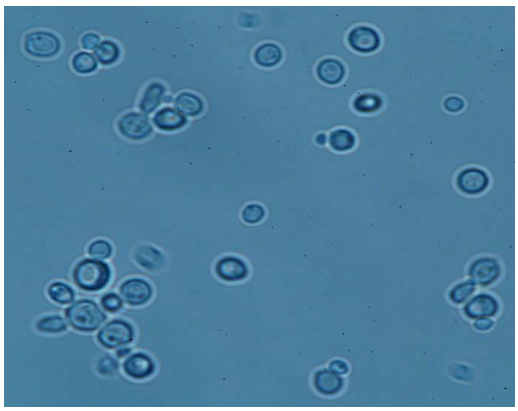


Fig. 7: Non-quiescent (NQ) yeast cells

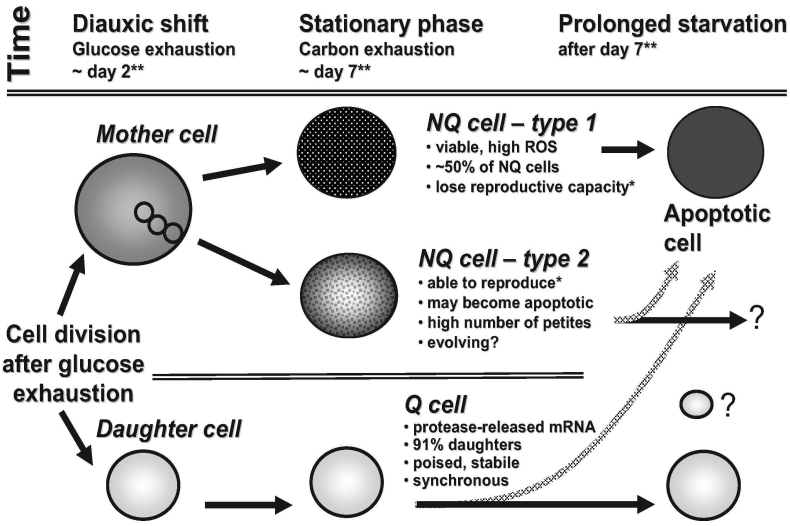


Fig. 8: Quiescent (Q) and Non quiescent (NQ) cells [8]

1.5. Yeast as a Model System

The unique properties of the yeast *S. cerevisiae* and its enormous potential which has been exploited for thousands of years made it a preferred organism also for research.
S. cerevisiae has been used in genetic studies for many decades. It has been introduced as an experimental organism in the mid-thirties of the 20th century. Furthermore, it was the first eukaryote whose genome was sequenced completely. Subsequently, it became one of the key organisms for genomic research. The size of the genome is approximately 12 Mb, comprising about 6000 genes, distributed to 16 chromosomes.

S. cerevisiae combines several advantages: it is small and unicellular and provides a cheap alternative for biochemical research. Large numbers of cells can be grown in culture in a very small amount of space, and the cells are dispersed. Unlike many other organisms *S. cerevisiae* is viable with many markers and can be handled with little precautions. However, yeast has the advantage of being an eukaryotic organism, therefore the results of genetic studies with yeast are applicable to human, animal or plant genetics.

High efficiency transformation of yeast cells is achieved, e.g., by the lithium acetate procedure or by electroporation. Integrating recombination of transformed DNA proceeds via homologous recombination (HR). Exogenous DNA can therefore be directed to specific locations in the genome.

A large variety of vectors was designed to introduce and to maintain or express recombinant DNA in yeast cells. In addition, yeast has proved to be valuable for studies of other organisms, including the use of the two-hybrid-screening system for the general detection of protein-protein interactions, or the use of yeast artificial chromosomes (YAC) for cloning large DNA fragments.

YAC is a vector which is considered to be a self replicating element including several specific DNA elements/sequences, like telomeres, a centromere, and

an origin of replication. Due to these elements a yeast cell will replicate the artificial chromosome together with the other, natural chromosomes. By using a circular Plasmid, which is cut into a linear molecule using a restriction enzyme, YAC allows the insertion of - a sequence or a gene of interest - even large DNA fragments **[10].**

Furthermore single step gene replacement is unique in *S. cerevisiae* and offers an outstanding advantage for experiments. Marker proteins like GFP can be fused to yeast genes, thus allowing to localize functional expressed gene products in living cells by fluorescence microscopy.

Strains of *S. cerevisiae* have a stable haploid and diploid state which means that mutations are manifested in haploids strains, whereas complementation tests can be carried out with diploid strains.

Altogether, *S. cerevisiae* provides an exceedingly appropriate system for molecular biological research, especially if rare events - as in our case mutation frequencies in eukaryotic cells - are investigated.

1.6. DNA Damage and DNA Repair

1.6.1. DNA Damage

DNA in cells is under constant bombardment. E.g. it is known that the DNA in a single human cell is damaged over 10.000 times a day. The integrity of the genome is essential for the health of the individual, it is necessary that any damage is detected quickly and repaired.
DNA can be damaged by many influences – from ultraviolet (UV) radiation in sunlight to chemicals in our environment such as those present tobacco smoke. Another major cause of damage are the chemical reactions that continuously take place by default in our cells.

In my study I focused on reactive oxygen species (ROS) which are byproducts of the normal metabolism and play an important role in cell signaling. Due to specific conditions - e.g. UV exposure or, as in my study glucose starvation - ROS levels can increase and can damage macromolecules, like DNA, RNA and proteins.

Although our cells are able to repair these damages, errors can accumulate over the years. This explains why most types of cancer usually increasingly affect older people.

Types of DNA Damage:

Modification of bases

All four DNA bases (Adenine, Thymine, Cytosine, and Guanine) can be modified at various positions. One of the most frequent modifications is the loss of an amino group ("deamination"). This may e.g. result in a Cytosine being converted to an Uracil. A normal C-G DNA base pair is changed to a U-G base pair. Uracil is not a regular part of the DNA, but this modification can easily be detected and repaired by BER. Unrepaired Uracil would cause a mutation, because, if replicated, an Adenine would be created in the complementary strand

In addition oxidation and alkylation of the nucleobases can happen.

Loss of bases

This means that the purine or pyrimidine base is removed from a nucleotide. Such locations are called AP sites (apurinic/apyrimidinic sites) and are mostly a consequence of spontaneous depurination.

Mismatches

Mismatch of bases commonly occurs due to a failure during DNA replication, more precisely a failure in the proofreading function of a DNA polymerase

during DNA replication. A wrong DNA base is inserted in a newly forming DNA strand. Mismatches can also happen during recombination, due to branch migration in the heteroduplex not all of the sequences are homologous.

Breaks in the DNA-backbone

If only one strand is broken it is called a single strand break or a single strand gap. If both strands are affected, this is referred to as a double strand break. The most frequent reason for DSBs is ionizing radiation. But also radicals and chemical agents may cause this severe type of damage.

Crosslinks

Covalent crosslinks can occur within the same DNA double strand (intrastrand crosslink) or between opposite strands (interstrand crosslink). Several chemotherapeutic drugs (e.g. cisplatin) used against various types of cancer crosslink DNA.

Pyrimidine Dimers

Cyclobutane pyrimidine dimers consist of two adjacent pyrimidine bases and are induced by UV light. As a consequence of this lesion the transcription as well as the replication is blocked until the dimers are excised and repaired **[11, 12].**

1.6.2. DNA Repair

As mentioned above the integrity of the genome is essential for the health of the individual. Therefore it is necessary to maintain genomic fidelity by the coordinated action of surveillance and repair pathways **[13]**.

Damaged or inappropriate bases can be repaired by several mechanisms:

Direct chemical reversal of the damage

The formation of pyrimidine dimers due to UV light damage can directly be reversed by the activation of the enzyme photolyase. The enzyme which becomes activated by light has a high affinity for pyrimidine dimers, binds them reversibly and converts them back to the original state. Photolyase has been found in many living organisms but it has not been identified in placental mammals, including humans.

Methyl groups can be removed by a protein encoded by the MGMT (O^6 Methylguanine Methyltransferase) gene. Regrettably the protein can only act once (it is therefore despite its name no enzyme), hence the removal of each methyl group requires a new protein molecule.

Excision Repair

The damaged base or bases are removed and then replaced with the correct ones in a localized burst of DNA synthesis. There are three modes of excision repair, each of which employs specialized sets of enzymes.

- Base Excision Repair (BER)
- Nucleotide Excision Repair (NER)
- Mismatch Repair (MMR)

BER

DNA bases may be modified by deamination or alkylation as mentioned above. The BER pathway starts with the excision of a damaged base by an enzyme called DNA glycosylase, creating an AP site. There are several types of DNA glycosylases, each one specifically excising a defined type of damaged base.

The generated AP site is a signal for the AP endonuclease (Apn1 is the major AP endonuclease in yeast), which nicks the damaged DNA strand upstream (5') of the AP site creating a 3' OH terminus and a 5' phosphate end without a base. The nucleotide will be replaced by the extension of the 3'-OH terminus by a short-patch or long-patch base excision repair.

Short patch means that only a single nucleotide is replaced by polymerase activity, whereas in the case of long patch base excision repair 2 - 10 nucleotides are replaced.

For the experiments within my diploma thesis I used the *S. cerevisiae* strains YLBA1 and YLB14A1. In both strains the *APN1* gene is replaced by *apn1::URA3*. This means that the strain is highly restricted in its ability to repair AP sites.

NER

The NER pathway is responsible for the elimination of bulky DNA lesions such as UV caused pyrimidine dimers. The importance of this repair mechanism is documented by several diseases (e.g. Xeroderma pigmentosum) concerning defects in the NER system. People who suffer from Xeroderma pigmentosum are extreme photosensitive and predisposed to skin cancer due to their lacking ability to repair UV light caused DNA damages.

The NER enzymes recognize bulky lesions of the DNA double helix. Recognition of these distortions leads to the removal of a short single-stranded DNA segment that includes the lesion, creating a single-strand gap in the DNA, which is subsequently filled by a DNA polymerase which uses the undamaged strand as a template.

Two of the *S. cerevisiae* strains used in this thesis (YLB14, YLB14A1) are carrying a mutation in the *RAD14* gene and therefore lack the ability to perform nucleotide excision repair.

MMR

This pathway corrects errors made by DNA polymerases during DNA replication as well as errors during recombination. If the cell does not correct mismatches, the lesions will be incorporated in the genome and future generations of cells

will possibly have modified proteins and cell functions. MMR requires recognition and removal of wrongly inserted nucleotides.

In order to remove the correct part of the mismatch the MMR machinery must distinguish the daughter strand from the parental strand. In *E. coli*, this is achieved by methylation. E.g. by a special methylase called Dam methylase, which methylates all adenines that occur within (5')GATC sequences. The newly synthesized strand is not methylated. The repair itself is accomplished in *E.coli* by Mut Proteins, which detect, nick and excise the wrongly incorporated bases from the unmethylated daughter strand. DNA polymerase III and DNA ligase fill and seal the arisen gaps, respectively.

The MMR in eukaryotes is similar to that in *E. coli*. Homologues of Mut proteins have been identified in yeast as well as in mammals. However, the mechanism to differentiate the template strand from the newly synthesized strand is still unclear. **[13].**

Repair of double strand breaks

The most severe kind of DNA damage are DSBs, in which both strands of the double helix are disrupted. If left unrepaired, they lead to broken chromosomes, genome rearrangements and cell death. Mostly exogenous sources like ionizing radiation are the reason for this kind of DNA damage. Cells possess two independent pathways for the repair of DSB. HR takes advantage of proteins that are able to obtain information from the sister chromatide to perform an error free repair. The other mechanism is non-homologous DNA endjoining which allows joining of ends even if there is no sequence similarity.

During NHEJ some specific proteins identify and detect the broken DNA ends and bring them in close vicinity. A specialized DNA ligase (DNA ligase IV) that forms a complex with some cofactors, directly joins the two ends.

In the *S. cerevisiae* strain YLB4, which is one of the strains I used in the course of my diploma thesis, the gene coding for DNA ligase IV is knocked out.

Therefore this strain lacks the ability to carry out NHEJ to repair double strand breaks.

If DNA damages will not be recognized and repaired by cell systems, the lesions can be inherited during cell division, becoming mutations. In contrast to DNA damages, a mutation is a change in the base sequence of the DNA that cannot be recognized by enzymes, once the base change is present in both DNA strands, thus a mutation cannot be repaired **[14, 15]**.

1.7. Replication-Dependent and Adaptive Mutations

The appearance of mutations kept scientists busy since Darwin claimed that mutations have a key role in natural selection and that they occur randomly and spontaneously. Selection takes place as a consequence of environmental conditions **[16].**

The latter mutations are called adaptive mutations and occur in non-dividing or slowly dividing cells and relieve the selective pressure. They appear to be specific to the challenge of the selection in the sense that they provide a growth advantage to the cell. They typically can be observed by plating a population of bacteria or yeast onto a medium on which growth cannot occur unless a known, cell-cycle arrest triggering mutation reverts.

Many experiments and studies investigating mutations and natural selection were carried out throughout the last decades.

Already in 1943 Luria and Delbrück **[17]** proved the existence of random mutations which arise without the selection pressure. For this purpose they exposed bacteria to the bacteriophage T1 and observed that nearly all of the bacteria were killed, representing a lethal selection pressure. But few bacteria

survived and gave rise to colonies that were permanently and specifically resistant to the bacteriophage. This fact implied that bacteria with a previous mutation, which caused resistance, survived. These bacteria acquired the resistance already before the time of the attack, hence without selection pressure.

Furthermore, they noticed that the number of resistant colonies on each plate varied drastically. Luria and Delbrück proposed, that the earlier the arise of the mutation happens, the more mutant cells exist. Based on these assumptions Delbrück derived the Luria-Delbrück distribution that gives information about the relationship between moments consistent with the experimentally obtained values. The distribution that follows shows that mutations in bacteria, as in other organisms, are rather randomly than directed.

However, the work of Luria and Delbrück did not exclude the possibility that adaptive mutations can arise.

In 1954 Ryan **[18]** provided evidence of mutations occurring without replication. To prove this he exposed bacteria to a non-lethal pressure by lowering the metabolizable ingredients of the medium. The bacteria were not able to replicate in the minimalized medium. He observed that mutants developed that were able to metabolize the ingredients, indicating that these mutations emerged replication-independent.

Although Ryan proved replication-independent mutations, he could not show that the mutations referred to a specific selection pressure.

A similar experiment was carried out by Shapiro **[19]**, who used a bacterial strain containing an araB-promoter and lacZ, flanking a Mu insertion. On plates with arabinose-lactose selection medium he observed mutants which means that araB-lacZ-fusion occurred, or, in other words, that the integrated Mu fragment was excised. Excision allows transcription of *lacZ* under the control of the neighbouring *araB* promoter.

The mutation event did not occur in growing cells immediately after exposure to the medium, but after starving for a couple of days. With his work he proved that these mutations depend on selection.

1988 Cairns **[20]** also demonstrated that mutations can arise in non-proliferating cells when they are exposed to a non-lethal selection. Therefore he used *E. coli* with a point mutation in the *LacZ* gene. The bacteria were grown in full medium before plated onto lactose minimal medium. Cairns was not interested in mutations that arised early, but in mutations, reactivating the LacZ gene, appearing several days after plating. The conclusion was that these late mutations, occurring as a result of the selective agents, were adaptive mutations.

Adaptive mutation is not restricted to prokaryotes. Steele and Jinks-Robertson **[21]** made prosperous experiments with *S. cerevisiae*. Their examination of the reversion of a *Lys2* frameshift (+4 frameshift) mutation in yeast provided evidence for adaptive mutation in a simple eukaryote as well. After incubation of the strain in full medium cells were plated on synthetic complete medium lacking lysine. The numbers of Lys(+) revertant colonies accumulated over a period of 8 days, caused by random and adaptive reversion events. Up to day 4 revertants resembled a Luria-Delbrück distribution, which means a high variance. An examination of the distribution of late appearing Lys(+) colonies (day 5 to day 8) exhibited a Poisson distribution, indicating that the underlying reversion events occurred after selective plating **[22].**

To eliminate the suspicion that late arising colonies are the same as early arising colonies just growing slower, early and late revertants were plated on a non - reverting background. Both kinds of revertants arised within the same time. Also the argument of a phenotypic lag, which would mean the delay of the expression of a newly acquired character, was excluded with yeast plasmid transformation experiments.

Finally, it seems that the phylogenetic distribution of the adaptive mutation phenomenon indicates that it is a constitutive and conserved mechanism of the cell.

1.8. Approach

Mutations that might cause cancer do not only occur in proliferating cells but also in non-proliferating cells. Therefore it is necessary to understand mechanisms in non-replicating cells because a large fraction of human somatic cells are in a non-replicating state.

In this diploma thesis I isolated quiescent and non-quiescent stationary phase cells of *S. cerevisiae* using density gradient centrifugation according to Allen et al. **[9]** as a basis for experiments concerning oxidative stress. Allen et al. reported the occurrence of quiescent and non quiescent cells in stationary phase yeast cultures differing in physiological and morphological properties and behaviour. I now analyzed the mutation frequencies of quiescent and non quiescent yeast cells.

For the experiments different strains of *S. cerevisiae* were used. I analyzed the wildtype (concerning DNA repair mechanism) strain EH150 and strains with imbalanced or knocked out DNA repair pathways YLBA1, YLB4, YLB14A1, and YLB14.

In order to determine the number of mutations in a gene that is independent of the selective pressure a forward mutation assay was performed - the canavanine assay.

The differences in the amount of DNA damages - more precisely double strand breaks in quiescent and non quiescent cells - were examined via the occurrence of γH2A protein, a member of the histone H2A family, which is involved in nucleosomal organization of chromatin. H2A can be phosphorylated at serine residue 139, then being termed γH2A, which excessively binds to DSBs. Due to this feature, specific antibodies against γH2A are widely used to detect DSBs.

Furthermore I focused on the amount of ROS in the cells. In order to track the amount of oxidative stress throughout the stationary phase the fluorescent dye MitoSOX™ Red (MS) was used and the cells were analyzed by fluorescence activated cell sorting (FACS) **[23]**.

2. Material and Methods

All solutions and media were made with ddH2O, except stated otherwise.

2.1. Cultivation

2.1.1. Yeast Strains

The following strains of *S. cerevisiae* were used for this study:

YLBM / EH150 **[24]**

MAT **a**, *trp1–Δ, his3–Δ200, ura3-52, ade2 –1$_o$, lys2-ΔBgl*II

YLB4 **[25]**

MAT **a**, *trp1−Δ, his3−Δ200, ura3–52, ade2−1$_o$, lys2−ΔBgl*II, *dnl4::HIS3*

YLB14 **[26]**

MAT **a**, *trp1−Δ, his3−Δ200, ura3–52*, *ade2−1$_o$, lys2−ΔBgl*II, *rad14::TRP1*

YLBA1 **[14]**

MAT **a**, *trp1–Δ, his3−Δ200, ura3−52, ade2−1$_o$, lys2−ΔBgl*II, *apn1::URA3*

YLB14A1 **[unpublished data]**

MAT **a**, *trp1-Δ, his3−Δ200, ura3–52, ade2−1$_o$, lys2−ΔBgl*II, *rad14::TRP1, apn1::URA3*

In the wild type strain YLBM/EH150 formation of tryptophan, histidine, uracil, adenine and lysine is disabled, consequently these substances are essential in growth media of this strain.

In the strain YLB4 the DNA ligase IV gene (*DNL4*) was disrupted with *HIS3*. DNA ligase IV is the unique ligase in the NHEJ DSB repair pathway. These *DNL4* mutants are not able to ligate DSBs via NHEJ.

In the strain YLB14 the Rad14 gene was disrupted with *TRP1*. Rad14 is a UV-damaged DNA binding protein with a zinc-finger motif, and it is similar to the protein encoded by the human xeroderma pigmentosum gen. Its knockout leads to a NER pathway deficiency because Rad14 is required for the incision step.

In the strain YLBA1 *APN1*, the gene coding for the major AP endonuclease in yeast, was disrupted with *URA3*. This leads to a substantially impaired BER pathway.

The strain YLB14A1 was produced by crossing the strains YLB14 and YLBA1. Therefore this strain lacks both, Rad14 and Apn1, hence being imbalanced in its ability to perform BER, and deficient for NER.

2.1.2. Media

For the experiments, yeast was usually grown at 30°C on complete medium (YPD) or on synthetic complete medium (SC).

Yeast peptone dextrose (YPD); (Tab. 1) was used as liquid and solid medium. 2 % Agar (Oxoid) was added for the use of solid medium. Liquid YPD was used for the cultivation of overnight cultures (onc) as well as for survival determination. Furthermore, stocks were also stored on YPD plates.

To avoid depletion of adenine, upon which growing yeast colonies turn red, adenine was added to the YPD medium, now termed YPDA (Tab2)

Synthetic complete (SC) medium, which consists of synthetic minimal (SM) medium (Tab. 3), was prepared for the Canavanine assay. L-canavanine sulfate (Can) was added instead of the amino acid arginine. Furthermore, amino acids and nucleobases were added to the SM. (Tab. 4, Tab. 5).

To produce SC medium the stock solutions listed in table 4 and 5 were added to SM-medium. All media were autoclaved for 12 minutes.

- YPD Medium

YPD	per 1 l	per 400 ml
Glucose	20.0 g	8.0 g
Peptone	20.0 g	8.0 g
Yeast Extract	10.0 g	4.0 g
Agar	20.0 g	8.0 g

Tab. 1: YPD Medium

- YPDA Medium

YPDA	per 1 l	per 400 ml
Glucose	20.0 g	8.0 g
Peptone	20.0 g	8.0 g
Yeast Extract	10.0 g	4.0 g
Adenine	80.0 ml	32.0 ml
Agar	20.0 g	8.0 g

Tab. 2: YPD Medium

- SM Medium

SM	per 1 l	per 400 ml
Glucose	20.0 g	8.0 g
YNB (yeast nitrogen base)	6.7 g	2.68 g
Agar	20.0 g	8.0 g

Tab. 3: SM Medium

Amino acids	stock-conc.	final conc.	per 400 ml	per 1 l
	[mg/ml]	[mg/l]		
Arginine	2.0	30.0	6.0 g	15.0 g
Histidine	1.0	20.0	8,0 g	20.0 g
Isoleucine	6.0	60.0	4,0 g	10.0 g
Leucine	2.0	60.0	12.0 g	30.0 g
Lysine	2.0	40.0	8.0 g	20.0 g
Methionine	2.0	20.0	4.0 g	10.0 g
Tryptophane	2.0	30.0	6.0 g	15.0 g
Valine	15.0	30.0	0.8 g	2.0 g
Threonine	10.0	30.0	1.2 g	3.0 g
Phenylalanine	1.0	50.0	20.0 g	50.0 g
Tyrosine	0.25	30.0	48.0 g	120.0 g

Tab. 4: Stock solutions for SC-Medium

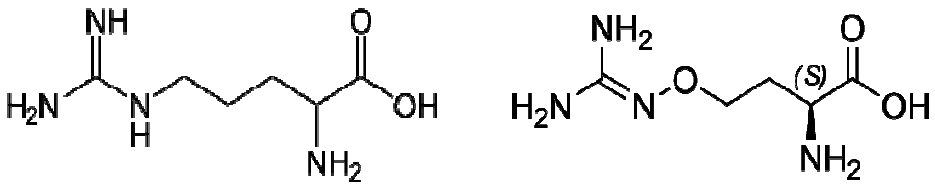
In addition to the amino acids the nucleobases adenine and uracil were added to the medium because the strains are adenine and uracil auxotroph.

Nucleotide bases	stock-conc.	final conc.		per 400 ml	per 1 l
	[mg/ml]	[mg/l]			
Adenine	0.50	30.00		24.0 g	60.0 g
Uracil	1.00	20.00		8.0 g	20.0 g

Tab. 5 Stock solutions for SC-Medium

To minimize the risk of bacterial infections, the antibiotic ampicillin was added at a final concentration (f.c.) of 0.05 mg/ml.

Can is used as a Monohydrat salt (98%). The molecular formula is $C_5H_{12}N_4O_3 \cdot H_2SO_4 \cdot H_2O$ and the molecular weight is 274.3.



L-Arginine **L- Canavanine Sulfate**

Fig. 9: L-Arginine and L-Canavanine sulfate

The non-protein aminoacid L-canavanine sulfate, chemically named L-2-Amino-4-(guanidiooxy)butyric acid, is the guanidinooxy analogue of the aminoacid arginine.

Can was prepared as a stock solution at a concentration of 20 mg/ml. The solution was sterile filtered (0.22 µm), when dissolved completely. The Can was added to the warm medium before pouring the plates. The f.c. of can in the medium was 60 mg/l.

2.1.3. Cultivation

The used strains were permanently stored in liquid YPD medium at 4℃ and periodically streaked on fresh YPD plates and incubated (Hera cell 150, Thermo) for three days at 30℃ to obtain single colonies for a new experiment.

Overnight culture

For the experiments liquid YPDA was used. To avoid upcoming bacterial infections ampicillin was also added to the medium to a f.c. of 0.05 mg/ml.

For inoculation either a single colony from the stored plate or a certain cell number was used. In case of a single colony, it was removed from a plate with an inoculation loop and suspended in 1 ml SB by vortexting for a while. To ascertain the starting concentration the suspension was diluted and counted in a hemicytometer. After cell counting the YPDA was inoculated with an aliquot of the cell suspension with about 5×10^4 to 1×10^5 cells and incubated in an airbath shaker (Model G25 Incubator shaker; New Brunswick Scientifix) at 250 rpm and 30℃ until the cell density reached 5×10^7 cells/ml.

2.1.4. Glucose starvation

In this study I focused on starvation of cells, more precisely glucose starvation. Diauxic shift and finally starvation of the cells starts approximately 24 hours after inoculation. As long as enough nutrients are in the medium, the yeast cells grow exponentially. By and by the glucose is exhausted and the cells shift into the diauxic phase and, finally, into starvation.

To be sure that the cells for the first density gradient centrifugation, the so-called exponential cells, were indeed exponential and not already in the diauxic shift, an aliquot was taken at a cell density of 5×10^7 cells per ml.

To determine the cell density an aliquot of the cells was counted with a hemicytometer in an appropriate dilution at the morning following inoculation.

2.2. Density gradient centrifugation

Due to the existence of quiescent and non-quiescent cells in the yeast culture, differing in their density and size, a density gradient centrifugation was performed. This centrifugation enabled me to separate the two cell fractions from yeast cultures. To achieve this experiment, I used Percoll, a silica-based colloidal medium for cell separation by density gradient centrifugation. The silica particles of the medium are coated with polyvinylpyrrolidone (PVP), which gives low osmolarity and low viscosity. Percoll can be used for cell separation. It facilitates the isolation of a variety of cells, subcellular particles and viruses. The density range of 1.0 to 1.3 g/ml for Percoll gradients can be optimized for separation of cells, subcelluar particles and larger viruses, with a boyant density of 1.0 to 1.2 g/ml.

The taken aliquots used for centrifugation:

- exponential cells (200 ml of 450 ml)
- cells after 48 hours of starvation (50 ml of 250 ml)
- cells after 120 hours of starvation (50 ml of 200 ml)
- cells after 168 hours of starvation (50 ml of 150 ml)
- cells after 288 hours of starvation (50 ml of 100 ml)

The protocol for density gradient centrifugation was established in our lab by a former lab member according to the publication of Allen et al [15]. However, the protocol has been modified in the way that Percoll was not diluted 8+1 but 8.5+1.5 (vol/vol) with 1.5 M NaCl.

- 85% Percoll solution with 15% 1.5 M NaCl (25.5 ml Percoll, 4.5 ml 1.5 M NaCl) in Beckmann Ultra-Clear Centrifuge Tubes (25x89 mm).
- For gradient formation Percoll-NaCl solution was put into centrifugation tubes and was centrifuged for 17 min at 12 000 rpm (19 238xg) in a swing-out rotor (L7-55 Ultracentrifuge Beckman; Rotor SW28; 95E1982) at 20℃
- A defined aliquot of the cell culture was harvested and washed in SB
- The cell pellet was resuspended in approximately 2 ml Tris buffer (50 mM, pH 8.5)
- The resuspended cells were then overlaid onto the preformed gradient and centrifuged for 50 min at 2000 rpm (530xg) in a swing - out rotor (L7-55 Ultracentrifuge Beckman; Rotor SW28; 95E1982) at 20℃

After centrifugation either one band in case of exponential cells or two separated bands in case of starved cells were obtained in the tube. The upper band represents the non-quiescent cell fraction, the lower band the quiescent cell fraction. The fractions were separately collected with a pipette, washed twice with SB and finally resuspended in an appropriate amount of SB. The number of total cells and of cfus for each fraction was determined for subsequent assays.

With proceeding density gradient centrifugations I could confirm the results of Aragon et al **[8]** according the inhomogeneity of non-quiescent cells. Beyond that I found out that even within the non-quiescent fraction the cells exhibited different properties, depending on the position of removal from the band. Their behaviour varied in relation to the survival as well as in the forward mutation assay.
Therefore I decided to investigate two different aliquots of non-quiescent cells, terming them „lower non-quiescent cells“ and „upper non-quiescent cells“, according to their sampling.

2.3. Canavanine assay

Can naturally occurs in many legumes. Due to its structural similarity with the naturally occurring L-aminoacid it interferes with L-arginine-utilizing enzymes.

S. cerevisiae contains an arginine permease, which is responsible for the uptake of arginine with high affinity. This arginine permease is encoded by the *CAN1* gene in budding yeast.
In the absence of arginine L-canavanine is mistaken for it by the transmembrane protein and enters the cell. Due to the structural similarity the yeast cells incorporate it into their proteins instead of arginine, which leads to structurally aberrant proteins that may not function properly, causing cell death.

If there is a mutation in the *CAN1* gene that leads to a non-functional arginine permease, the cell is not able to import can, has to produce arginine on its own and will thus not be poisoned by canavanine. In other words, an inactivating mutation in the *CAN1* gene makes cells resistant to L-canavanine.
Therefore, the yeast *CAN1* gene is widely used in genetic experiments as a selectable locus. It allows the selection of canavanine resistant mutants. In my study I used the canavanine assay to determine the mutation frequency in exponential and quiescent cells.

Therefore, the fractions obtained from Percoll density gradient centrifugation were washed twice with SB (5 min, 3500 rpm; Heraeus Multifuge 1 L-R). After the last washing step the pellet was resuspended in an appropriate amount of SB depending on the size of the pellet.
Furthermore, dilutions of the suspended fractions were made to be counted in a hemicytometer. The counting was necessary because only a defined amount of cells was plated to Sc–arg+can plates. This defined amount of cells ranged between $2.5x10^6$ and $5x10^7$, depending on the strain used.
Usually 10 plates were used for the exponential cells and 7 plates for the starved cells, at each point of time. The plates were incubated at 30℃ for 4

days (Hera cell 150 Thermo). Subsequently, all colonies grown on the Sc–arg+can plates were counted.

2.4. Survival Determination

As mentioned before the survival determination was carried out with the use of YPD plates. I used three YPD plates for each day and each fraction. Therefore, dilutions were made of the obtained cell suspensions from the density gradient centrifugation. The cell numbers and the amount of cfus were counted and 100 cfus were plated onto each YPD plate. After incubating the cells for three days at 30℃ (Hera cell 150 Thermo), the colonies were counted and the percentage of survival was calculated.

2.5. Flow cytometry and FACS

Flow cytometry is a method for counting and/or separating cells by suspending them in an ionizable buffer (sheath fluid) and passing them through an electrostatic deflection system.

This technique is based on the labelling of cellular components using a fluorescent marker, usually a type of dye. These dyes fluoresce only when light of the appropriate wavelength hits them, causing an emission at a different wavelength. In a flow cytometer, single cells pass an excitation source and the light hitting the cells is either scattered or absorbed and re-emitted (fluorescence).

The collected data are usually shown either as single parameter histograms or as two parameter correlated plots, often called cytograms.

In my study I focused on the presence of ROS in the cell. ROS are chemically reactive molecules containing oxygen, like hydrogen peroxide or radicals like the hydroxyl radical. Radicals are clusters of atoms which contain an unpaired electron in their valence shell. They are byproducts of the normal metabolism and have important roles in cell signalling.

I supposed that the ROS levels increase during glucose starvation of the cells. In times of environmental stress, ROS levels increase dramatically, which may result in damages to cell structures. This situation is called oxidative stress, which describes the inability of a biological system to detoxify the reactive molecules before they start to damage cell components. My presumption was that quiescent cells are possibly less exposed to oxidative stress than non-quiescent cells.

To compare the burden of oxidative stress in quiescent and non-quiescent cells FACS analysis was performed. In my experiments MS was used for the selective detection of superoxide anion, which is generated in the mitochondria of living cells **[27].** MS is oxidized by superoxide anion and exhibits red fluorescence.

For this analysis exponential cells and cells glucose starved for 12 days (quiescent and non-quiescent cells), were prepared. At first, the cells were incubated with MS (f.c. 5 µM) shaking at 30℃ for 10 minutes protected from light. Subsequently, the cells were washed twice with 1 x PBS, diluted to approximately $1x10^7$ cells/ml and used for the FACS analysis in a FACS Calibur flow cytometer, equipped with an air cooled 15 mW argon ion laser system (excitation wave length 488 nm, detection at 570 nm with FL3-H).

2.6. Protein Preparation from Yeast

After density gradient centrifugation $1x10^8$ cells were stored in liquid nitrogen until protein preparation or for prolonged periods of time.

The protein preparation to perform western blots was done according to the established laboratory protocol described below:

- Resuspend $1x10^8$ cells in 1 ml 10 mM Tris-HCl pH 7.5 + 10% glycerine (4℃) and centrifuge in an Eppifuge at 13.200 rpm f or 30 seconds.
- Resuspend the pellet in 200 µl 10 mM Tris-HCl pH 7.5 + 10% glycerine (4℃), add one volume of glass beads (0.5 mm).
- Vortex 20x30 seconds, with breaks of 30 seconds on ice; every 10 times of vortexing leave the reaction tubes on ice for 5 minutes.
- Check in the microscope if at least 90% of the cells are destroyed.
- Remove the glass beads: puncture the reaction tubes, put each into a new reaction tube and centrifuge at 3.000 rpm for 30 seconds at 4℃.
- The lysates were collected in the new reaction tubes.

2.6.1. Bradford protein assay

The concentrations of the obtained protein lysates were measured with a Bradford protein assay.

1 ml of Bradford Reagent was pipetted each into 1.5 ml cuvettes. Additionally one blank sample (only Bradford Reagent) was prepared. BSA concentrations of 5, 10, 15, 20 and 25 µg/µl were used as reference. 5 µl of each protein lysate were added to the cuvettes. After mixing by inversion and incubation for 5 minutes the protein concentration was measured at a wavelength of 595 nm.

The absorbance of all samples was read. The values of the unknown samples were calculated according to the BSA calibration line.

To denature the protein lysates 2x sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol (β-ME) was added in a ratio 1:1 and heated for 4 minutes at 99℃. Subsequently the protein lysates were stored at 4℃.
SDS has a hydrophobic end which interacts with amino acids in proteins. This leads to the denaturation of the tertiary structures of proteins; the detergent converts the proteins into linear molecules coated with negatively charged SDS groups, masking the native charge of proteins. The negative charge generated by SDS is analogously to the size of the molecules - proteins with the same size have the same charge.

Therefore, after boiling in SDS, proteins will move through a polyacrylamide gel towards the positive electrode with a velocity according to their size.

The reason why β-ME is usually included in the sample buffer is to reduce disulfide bonds within or between molecules, allowing the molecules to assume an extended monomeric form.

2.7. SDS-PAGE

SDS Polyacrylamide Gel Electrophoresis is a useful system to separate proteins according to their size.
Proteins were separated by electrophoresis on 12.5% SDS-polyacrylamide gels using the Hoefer Mighty Small system (SE 215, Hoefer Scientific Instruments).

For non-continuous SDS PAGE a separation gel and a stacking gel had to be prepared. At the border between the stacking gel and the separation gel the proteins are focused before they enter the separation gel in which the molecules migrate according to their size.

4 x 12.5% separation gels:

- 7.3 ml acrylamide/bisacrylamide (40%, 29:1)
- 5.8 ml Tris-HCl pH 8.8
- 10.2 ml ddH2O
- 200 µl 10% APS
- 15 µl TEMED

4 x 4.5% stacking gels:

- 1.4 ml acrylamide/bisacrylamide (40%, 29:1)
- 3.1 ml Tris-HCl pH 6.8
- 8.0 ml ddH2O
- 25 µl 10% APS
- 80 µl TEMED

10 x SDS-PAGE running buffer:

- 192 mM glycine
- 250 mM Tris base
- 1% SDS

2 x sample buffer:

- 100 mM Tris-HCl pH 6.8
- 200 mM DTT
- 4% SDS
- 20% glycerol
- 0.2% bromphenole blue

As a molecular weight standard PageRuler^{TM} Plus Prestained Protein Ladder (Fermentas) was used.

All ingredients for the 12.5 % separation gel were mixed shortly and then poured between two fixed glass plates located in the chamber of a mighty small multiple gel caster (SE 215, Hoefer Scientific Instruments) to approximately 2-3 cm underneath the upper edge. The separation gel was topped with butanol to remove air bubbles and to get an even surface. Following polymerization (15 - 20 min) the butanol was rinsed of with ddH_2O, and the stacking gel was poured onto the separation gel. Immediately a suitable comb was put in the stacking gel and it was left at least 20 min to polymerize. Once the stacking gel has polymerized, the comb was gently removed. The gels were stored at 4℃ if not needed subsequently.

Loading the gel: the gels were placed in the electrophoresis unit (SE250, Hoefer Scientific Instruments) and the upper and lower pools of the unit were filled with 1x PAGE-buffer. Prior to loading, the slots of the gel were washed with 1 x PAGE buffer. The protein samples as well as the protein ladder were loaded onto the gels. Unused slots were filled with sample buffer to avoid a "smiling" front. The gels were run at 15 mA/gel for approximately 1 h and stopped when the dye front passed the lower edge of the gel.

During PAGE, electrophoretic mobility of SDS-treated proteins has a linear correlation to the log of the molecular weight.

2.7.1. Coomassie Staining

I used a Coomassie solution to visualize the protein bands after electrophoretic separation. Therefore, the polyacrylamide gels were incubated in a Coomassie solution for 15 min at room temperature under constant shaking. To destain, excess staining solution was removed and the gels were covered with destaining solution for at least 1 hour. Finally, the gels were dried with a slab gel dryer (483, Biorad).

Coomassie staining solution:

- 45% ethanol
- 10% acetic acid
- 0.25% Coomassie Brilliant Blue R-250

Destaining solution:

- 10% acetic acid

2.8. Western Blotting

Western Blotting allows to determine the relative amount of a protein present in different samples. This is carried out with specific primary antibodies. Separated samples on the gel were transferred to an Immobilon-P PVDF membrane for detection. Before blotting the membranes were activated by incubation in MeOH for some seconds.

The transfer sandwich was composed of one sheet of filter paper, the PVDF membrane, the polyacrylamide geld and two more filter papers. The filter papers were soaked with transfer buffer before. A roller was used to remove existing air bubbles from the sandwich. Finally, the gels were blotted for 1 h at about 1mA/cm^2 with the Semi Dry Transfer Cell (Biorad).

10 x Transfer buffer:

- 192 mM glycine
- 250 mM Tris base
- 1% SDS

After blotting the membrane was incubated with PBS + 0.1% Tween + 5% BSA buffer to block any remaining unsaturated places on the membrane to avoid unspecific antibody adherence to the membrane itself. Blocking was carried out at 4℃ over night on a rocking platform.

2.8.1. Immunological Detection

- Washing: After blocking the membranes were washed with PBS + 0.1% Tween20 for 3 x 5 minutes and 3 x 30 seconds, rocking at room temperature.
- First antibody:
  Anti-phospho-Histone H2A (Ser129); yeast specific; rabbit polyclonal IgG
  The blots were incubated with the first antibody in 10 ml PBS + 0.1% Tween + 5% BSA, at room temperature for about 1.5 h on a rocking platform. The primary antibody specifically binds to its antigen.
- Washing
- Second antibody:
  Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L)
  The membranes were incubated with the second antibody in 10 ml PBS + 0.1% Tween20 + 5 % BSA, rocking for 45 minutes at room temperature. I used a peroxidase-linked secondary antibody in conjunction with the chemoluminescent detection solution ECL-Plus (Amersham)
- The blots were incubated with the detection solution for 5 minutes at room temperature. The reaction produces luminescence proportionally to the amount of protein.
- A chemoluminescent film was exposed to the membrane and developed.

10 x PBS:

- 80 g/l NaCl
- 2.0 g/l KCl
- 2.4 g/l KH_2PO_4
- 18.1 g/l Na_2HPO_4 x 2 H_2O

3. Results

3.1. Density Gradient Centrifugation

As mentioned in Material and Methods, Percoll density gradient centrifugation was the first step to obtain exponential cells as well as quiescent and non-quiescent cells. In case of exponential cells I obtained only one band in the upper region of the gradient, whereas cells starved for 2, 5, 7, and 12 days were separated into an upper and a lower band, representing non-quiescent cells and quiescent cells, respectively. It should be mentioned that the lower band should not be located at the bottom of the tube like a pellet; this would indicate an improper density gradient centrifugation.

After density gradient centrifugation pictures of the bands which can be seen below (Figure 10) were taken to verify the result of the Percoll density gradient centrifugation. The obtained bands are indicated with arrows.

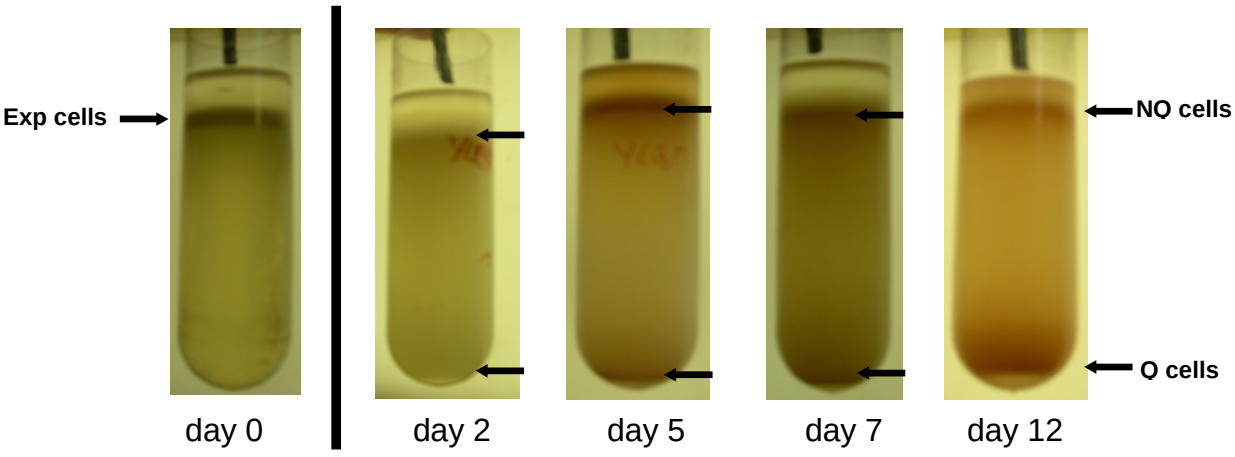


Fig. 10: Density gradient centrifugation and obtained bands

Checking the different cell types (Exponential, Q and NQ) obtained upon density gradient centrifugation via microscope or FACS, I could confirm some of the results of Allen et al **[9]**.

3.2. FACS

With the performance of a FACS analysis, which is a quantitative method, I wanted to analyze the burden of oxidative stress in quiescent and non-quiescent cells, especially the potential differences between these two types of stationary phase cells.

Oxidative stress can be detected by means of different fluorescent dyes - in my study I focused on the fluorescent dye MS as described in material and methods. Exponential cells, as well as quiescent and non-quiescent cells of day 12 of all 5 yeast strains were quantified regarding the ratio of oxidative stress.

In my study exponential cells served as a reference, because I already knew that the level of oxidative stress is low in these cells **[14]**. This observation was confirmed by my experiments. In Figure 11 the detailed FACS results of all used strains are shown. The plots display the amount of oxidative stress in the different cell types in the course of time. Peaks at the left side (FL3 ~10^1) of the plot represent more or less unaffected cells. Exponential cells of all strains exhibited one homogenous cell population, represented by one significant peak in the plot. Furthermore, the shift of the peak to the right was marginal, implying low levels of oxidative stress. Exponential cells of the strains YLBM, YLB4 and YLB14 tended to be marginally more affected than YLBA1 and YLB14A1. D12 quiescent cells appeared to be similar as exponential cells. I observed only low levels of oxidative stress, the lowest for Q cells of the strain YLBA1. Furthermore, YLBA1 as well as YLB4 d12 quiescent cells seemed to consist of one homogenous cell population, whereas the peaks of YLBM/EH150 as well as YLB14 d12 quiescent cells tended to form shoulders which may indicate the beginning of the development of different cell types. The peak of YLB14A1 was not clear but rather broad containing several shoulders, also indicating different subpopulations. This was also the strain which exhibited the highest amounts of oxidative stress, although still on a relative low level. In NQ upper and lower cells I observed that levels of ROS were significantly increased compared to

quiescent cells. The peaks of NQ upper as well as lower cells of all strains shifted to the right (FL3 ~10^2 - 10^3), correlating with an increased amount of oxidative stress in these cells. Furthermore, in none of the used strains I observed a homogenous cell population. All strains exhibited more than one subpopulation, indicated by different peaks in the plot. YLBM/EH150 NQ upper and NQ lower cells comprised at least three different populations, YLBA1 (both cell fractions) only two, but clearly separated from each other. Furthermore, all peaks of the strains were broadened compared to the quiescent cell fractions, especially YLB14A1. YLB14 showed one clear major cell population, but surrounded by broad shoulders, which as well indicates subpopulations.

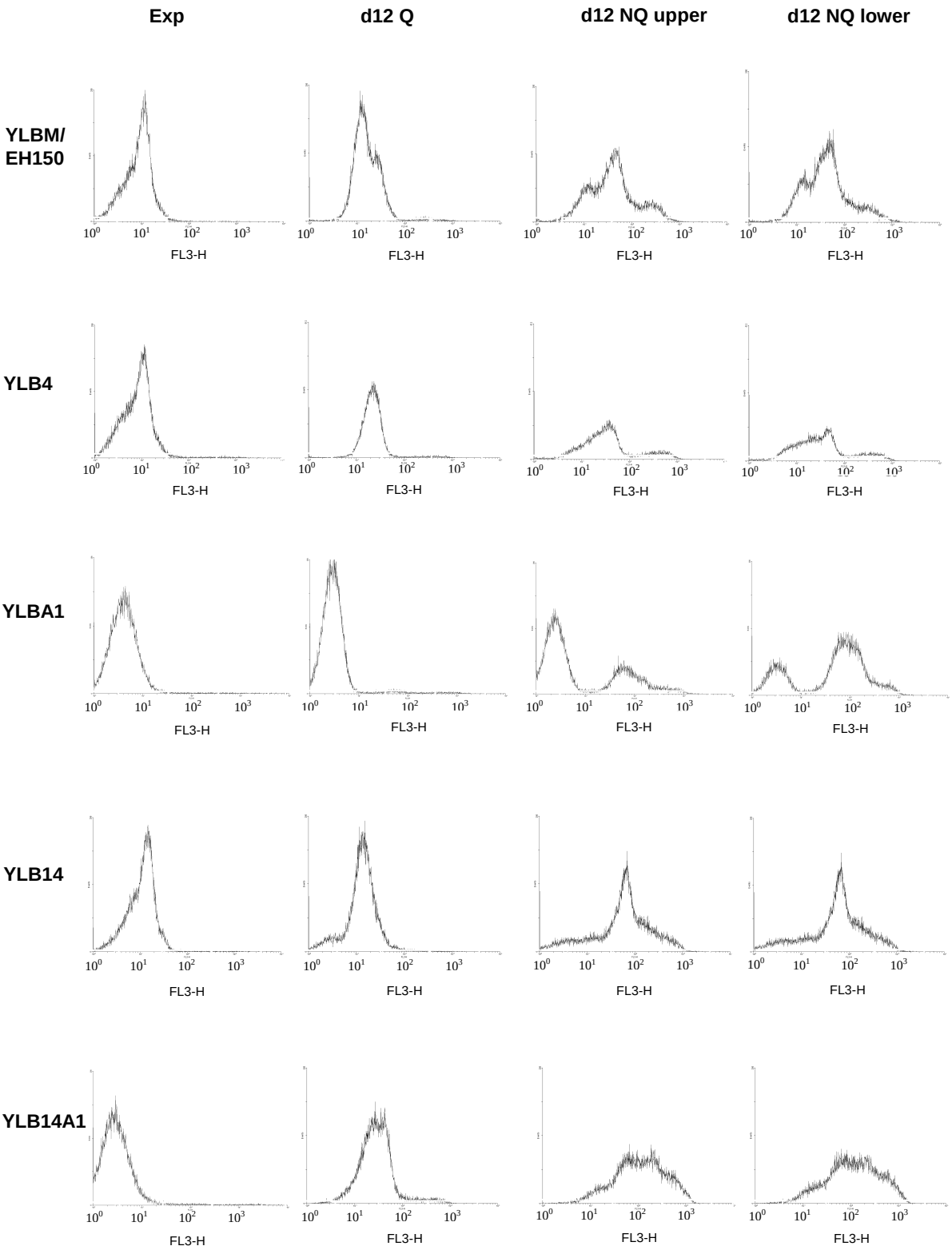


Fig. 11: FACS analyzes of yeast strains YLBM, YLB4, YLB14, YLBA1 and YLB14A1

Summing up, I observed that the amount of oxidative stress in exponential cells was very low, in agreement with the results of previous experiments. The ROS levels of Q cells on d12 were only slightly increased, largely exhibiting a homogenous cell population. In contrast, NQ upper and lower cells were heterogeneous after 12 days of glucose starvation and contained high levels of ROS. The significant difference between d12 NQ cells and Q cells was the most striking result of this assay.

3.3. Canavanine Assay

Performing this forward mutation assay, I wanted to analyze the potential influence of deficient repair pathways on the mutation frequency in stationary-phase cells.

After centrifugation the separated cells were washed twice with SB, counted and grown on SC-arg+can plates in a range between $2.5x10^6$ and $5x10^7$ cells/plate for four days at 30℃. In addition, the survival of the cells was determined (YPD plates) to know the rate of cells which stay replicable during glucose starvation.

After incubation for four days the colonies on the SC-arg+can plates (10 for each cell fraction) as well as the colonies on the YPD plates (3 for each fraction) were counted and the mean values were determined.

$$\frac{\text{Mean value of counted canavanine colonies per } 5x10^7 \text{ plated cells x } 100}{\text{Mean value of counted survival colonies per 100 cfus}}$$

The result displays the mutation frequency, which is the number of inactivating mutations within the *CAN1* gene, occurring per $5x10^7$ living cells.

Figure 12 shows the results - mutations per $5x10^7$ living cells versus time elapsed - plotted in a line chart, in which the exponential cells define the start point d0.

In general, all strains responded to glucose starvation with an increase of mutations. The amount of mutations was different depending on the cell fractions as well as the yeast strain.

3.3.1. Replication-dependent mutations from day 0 (exponential) to day 2

Mutations in exponential cells (d0) are potentially replication-dependent. Due to residual growth replication-dependent mutations may occur even after d0. Therefore, the cells of d2 were also categorized as potentially replication-dependent.

Within these cells (d0, d2) I observed significant differences between the different strains: more detailed, I observed approximately 80 replication-dependent mutations per 5 x 10^7 living cells on d0 in wildtype strain cells, but about 2000 in YLB14A1 cells, which was about 25 times more. The number of mutations of the strains YLB4, YLBA1 and YLB14 ranked between the wildtype strain and YLB14A1.

However, no significant difference could be found between the different cell fractions (Q, NQ upper and NQ lower): all exhibited nearly the same number of mutations as well as a slight increase in the number of mutations from d0 to d2.

3.3.2. Replication-independent mutations from day 2 to day 12

Later arising mutations (day 5, 7, and 12) are replication-independent. These mutations rose constantly over time for the strains YLBM/EH150 and YLBA1 for all cell fractions. The other strains like YLB4, YLB14 and YLB14A1 underwent up- and downturns concerning the number of mutations. Between d2 and d5 the

number of mutations increased only marginally in yeast strains YLBM/EH150, YLB14 and YLBA1, whereas I observed even a slight decrease in the strains YLB4 for all three cell fractions and YLB14A1 in NQ lower and Q cells.

The most distinctive increase of mutations of all cell fractions happened within the period of d5 to d7.

Upon prolonged starvation, NQ upper cells exhibited a steady increase until d12, which was more pronounced than that of NQ lower and Q cells in which the number of mutations often rose only marginally between d7 and d12. In the strains YLB4 and YLB14 I even observed a decline of mutations. Furthermore, I observed nearly the same, or a rather slightly increased number of mutations on d12 in NQ lower compared to Q cells in case of the wildtype, YLB4 as well as YLB14A1. In contrast, mutations in NQ lower cells of YLB14 and YLBA1 are similarly high as in NQ upper cells. Finally, it should be mentioned that the error bars tended to become larger with progression of time, because some strains responded very sensitive to glucose starvation. As shown in Fig 13, the survival on d12 is already significantly decreased.

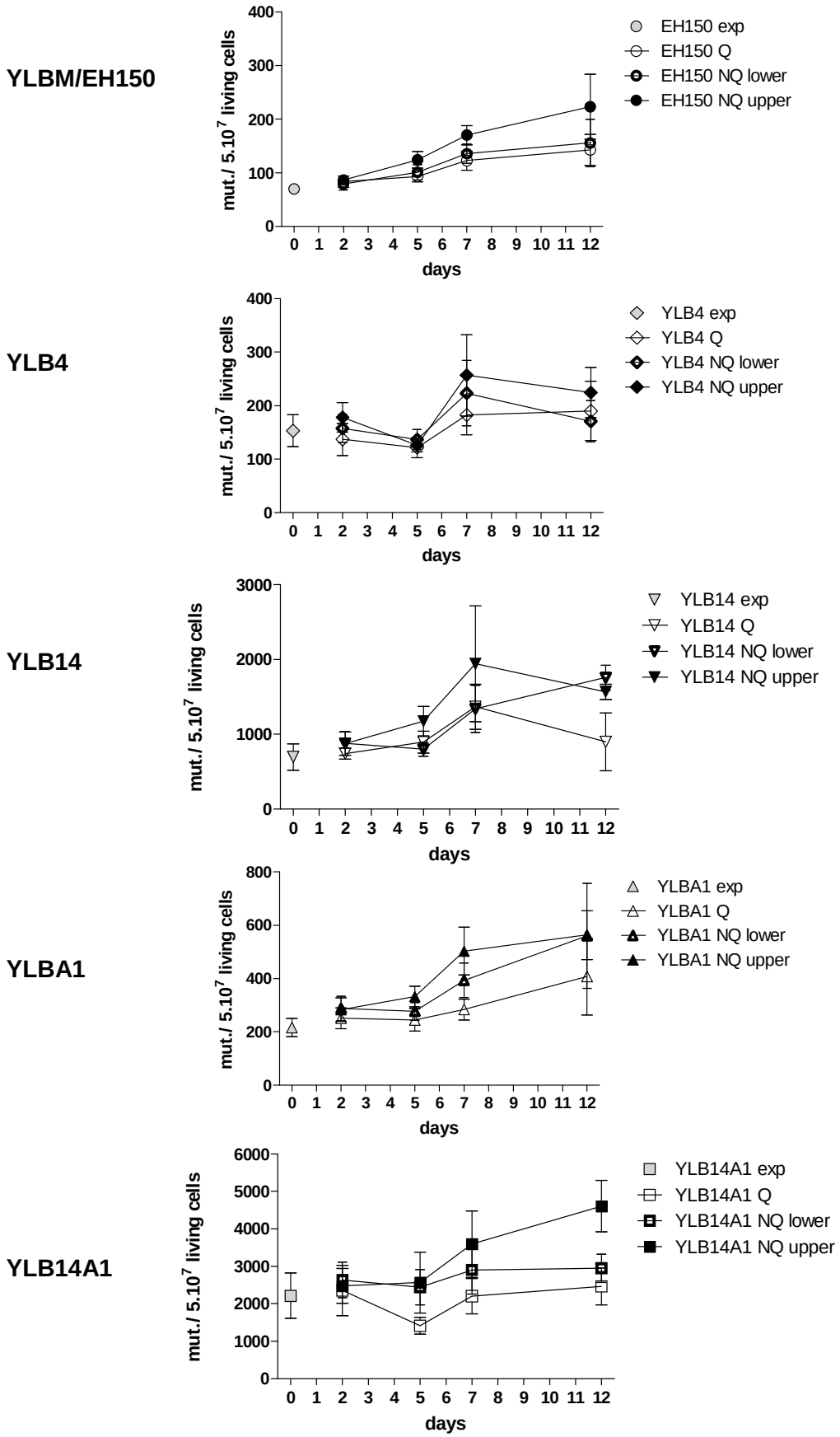


Fig. 12: Replication-independent mutations per $5x10^7$ living cells after a period of time as indicated; yeast strains YLBM, YLB4, YLB14, YLBA1 and YLB14A1

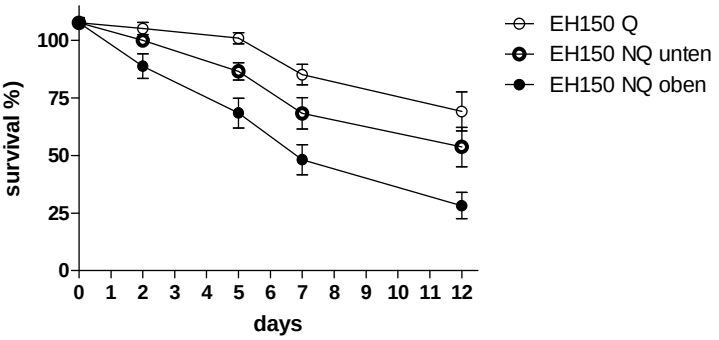
Summing up, the lowest mutation frequency I observed for the wildtype YLBM/EH150. YLB4 showed a slightly increased mutation frequency compared to the wildtype. Approximately two times more mutations I observed for YLBA1. In contrast YLB14 and YLB14A1 exhibited dramatically more mutations, already on d0. I observed different numbers of mutations within the three cell fractions of all used strains. In general the absolute number of mutations was increased for NQ upper cells, which exhibited a bigger increase of mutations until d12 followed by NQ lower cells. In contrast, in Q cells the fewest mutations occurred.

3.3.3. Survival

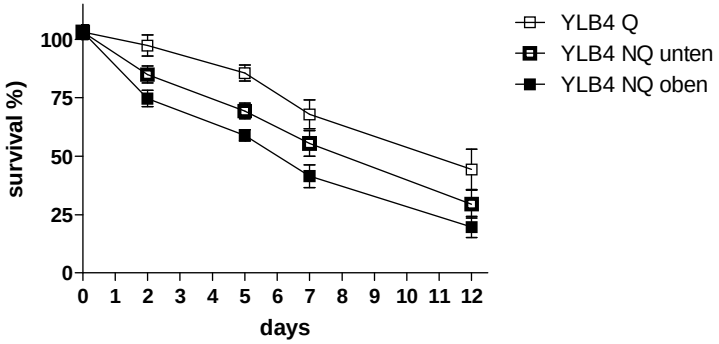
To obtain the information how many cells of a respective culture are replicable, I determined the survivals (CFUs) of all strains at all time points. The survival of d0 (usually already around that value) was set to 100%.

Our results (Fig. 13) showed a decrease in the survival of yeast cells with proceeding glucose starvation. Comparing the strains among each other I found the best viability for YLBM/EH150, followed by YLB4. The lowest survival was observed for the strains YLBA1 and YLB14A1. Concerning of the different cell fractions, I observed that quiescent cells showed the highest survival in all strains, as supposed. The survival rate of NQ lower cells was between that of Q cells and NQ upper, the latter exhibiting the lowest survival. YLBM/EH150 Q cells revealed at d12 still a survival rate of approximately 75%, whereas Q cells of the other strains only reached about 50%. Interestingly, in the majority of cases the most distinctive decline took place between d5 and d7 in all strains, which is the same period of time where I observed the most distinctive increase of mutations.

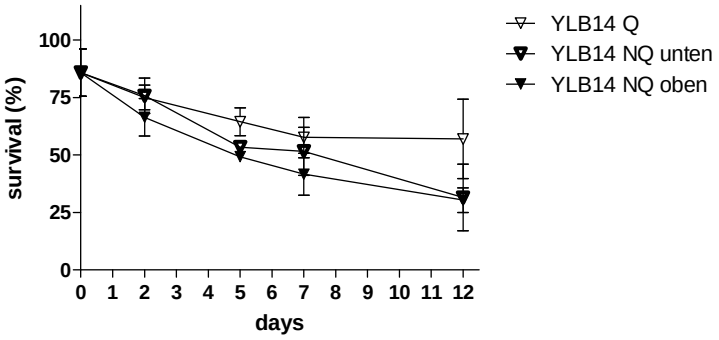
YLBM/EH150



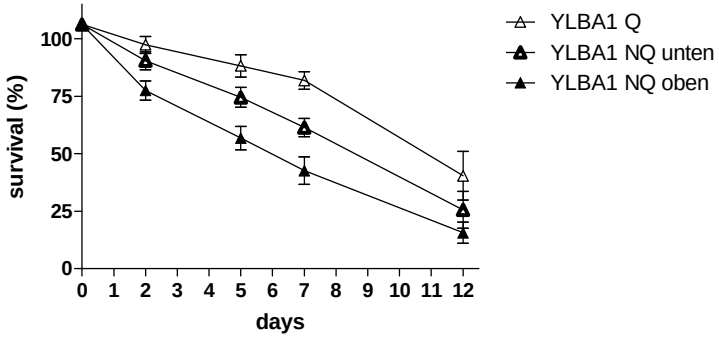
YLB4



YLB14



YLBA1



YLB14A1

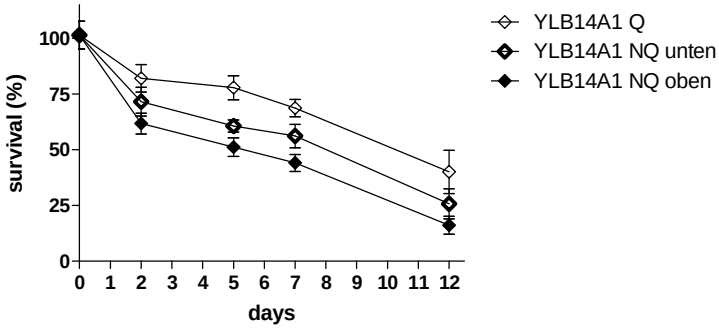


Fig. 13: Survivals of CUFs at defined time points

3.4. Western Blot

In my study I performed Western Blots to analyze the different cell fractions obtained after density gradient centrifugation. I was interested in the amount of DSBs.

If a DSB happens, phosphorylation of the C-terminal end of the histone H2A occurs and the phosphorylated protein (γH2A) exceedingly binds around the broken ends of the DNA molecule. Therefore, antibodies against γH2A are widely used to label DNA DSBs. The molecular masses of the phosphorylated and the non-phosphorylated histone H2A protein are 17 kDa and 14 kDa, respectively. Because *H2A* is a so-called housekeeping gene, I used an antibody against the unphosphorylated histone H2A as a loading control.

The following antibodies were used:

Primary antibody:

Anti-phospho-Histone H2A (Ser129); rabbit polyclonal IgG; Active Motif; (1:10.000)

Anti-Histone H2A; rabbit polyclonal IgG; Millipore; (1:20.000).

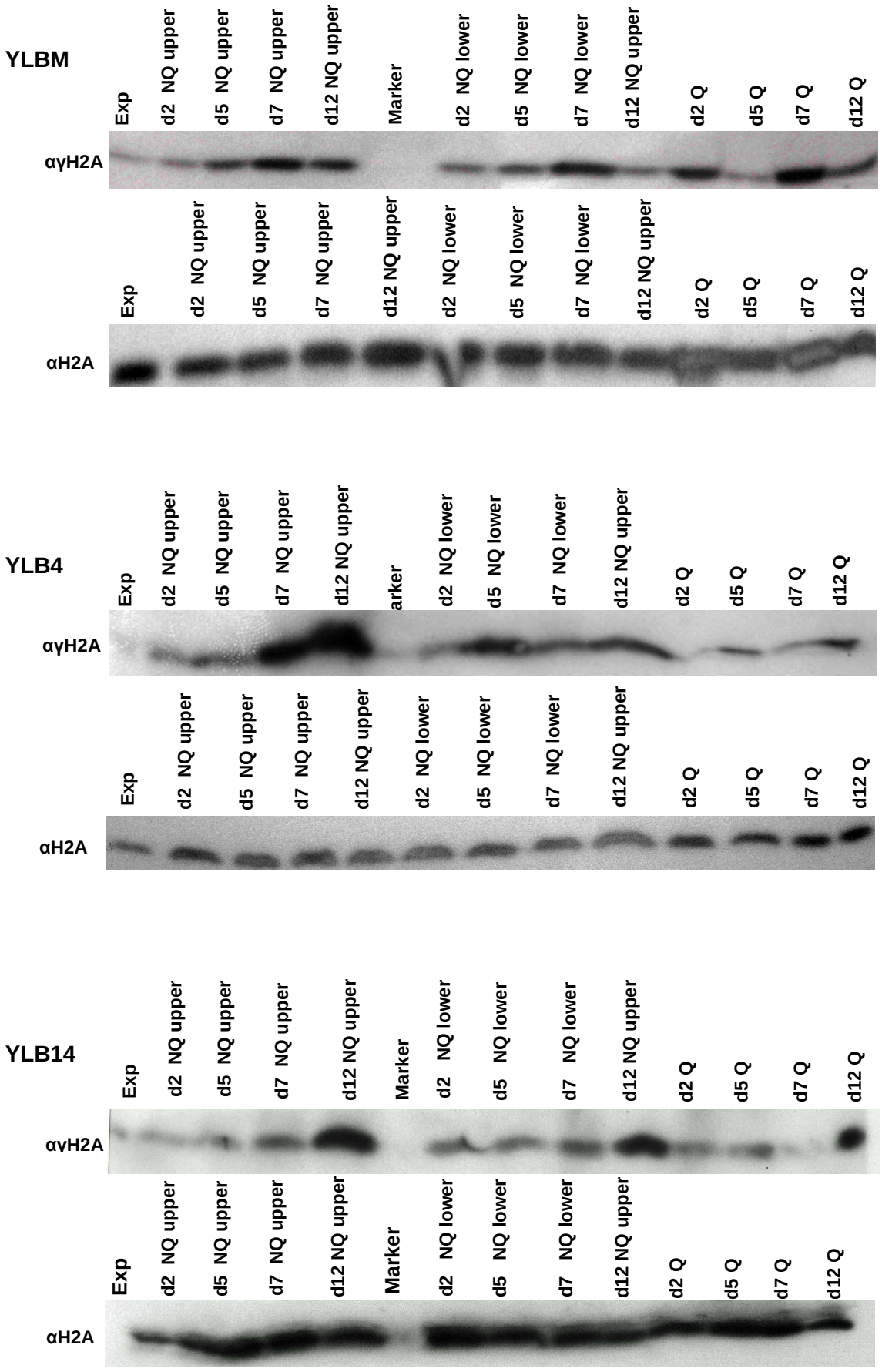
Secondary antibody:

Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L); Promega; (1:10.000)

Protein extracts of the different cell fractions after defined periods of time (d0, d2, d5, d7, d12) were used and western blots were performed as described in chapter 2.8 in material and methods.

With progression of glucose starvation the amount of DSBs and hence the detectable amount of γH2A increased significantly. In contrast, the control blots, with the unphosphorylated H2A, exhibited constant signals for all cell types at all time points, indicating that the amount of H2A, and therefore the amount of cells used, was more or less constant. Generally, in all strains exponential cells revealed the lowest amounts of γH2A, as assumed. In contrast, NQ upper cells exhibited the most DSBs, followed by NQ lower and finally Q cells. DSBs also

occurred in Q cells, but more rarely than in NQ upper and lower cells. D2 cell fractions looked similar to exponential cells; the differences in the expression of γH2A were only marginal. Cells which were in stationary phase for a prolonged period of time revealed higher amounts of DSBs. The wildtype YLBM/EH150 showed the weakest signals in the blot, regardless of the cell fraction. The highest levels of γH2A were reached on d7 in NQ upper, lower as well as Q cells. The appearance of YLBA1 was similar to the wildtype, apart from d12 NQ upper cells, where the blot did not exhibit a signal. A constant and strong enrichment of phosphorylated H2A from exponential to d12 was observed for the strains YLB4, YLB14 as well as YLB14A1 comprising all cell fractions. In the case of YLB4 I observed the biggest differences within the cell types. NQ upper cells showed much stronger signals on d12 than Q cells. YLB14 and YLB14A1 were related concerning their amounts of γH2A, and again, the NQ upper cells revealed much stronger signals than NQ lower and Q cells, respectively.


YLBM
Exp
d2 NQ upper
d5 NQ upper
d7 NQ upper
d12 NQ upper
Marker
d2 NQ lower
d5 NQ lower
d7 NQ lower
d12 NQ upper
d2 Q
d5 Q
d7 Q
d12 Q
αγH2A
αH2A
YLB4
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αH2A
YLB14
αγH2A
αH2A

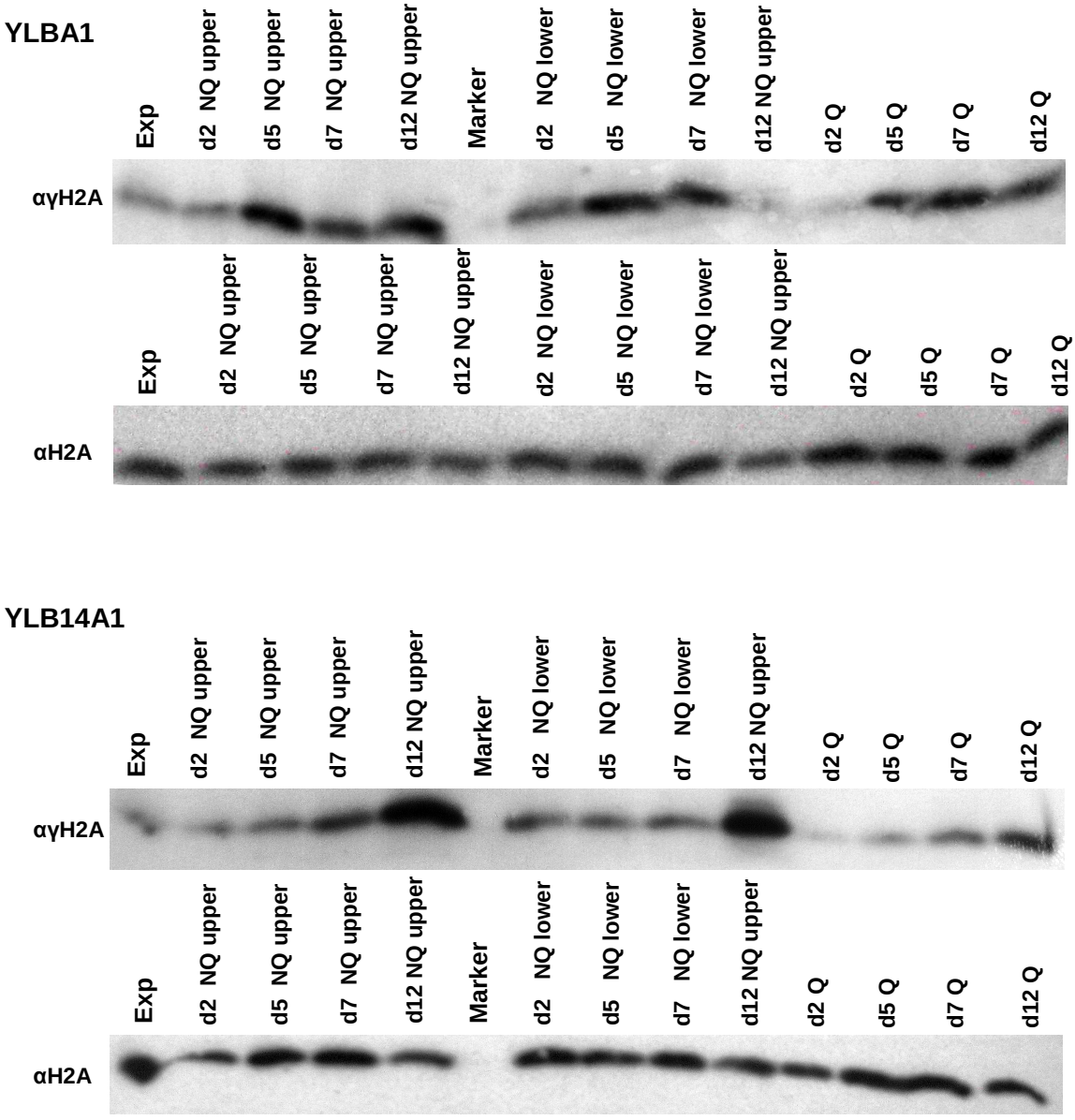

Fig. 14: Western Blot of yeast strains YLBM/EH150, YLB4, YLB14, YLBA1 and YLB14A1

4. Discussion

It is well known that spontaneous mutations not only arise in replicating cells, but also in cell cycle-arrested cells. The incidence of mutations is essential for the potential risk of cancer development. Therefore, I analyzed the occurrence of mutations in stationary phase cells of different yeast strains with impaired or knocked out repair pathways. Stationary phase was triggered by glucose starvation.

Using density gradient centrifugation, according to Allen et al [**9**], I obtained quiescent (Q), non-quiescent (NQ) upper and non-quiescent lower cells. After separation I performed several methods to study the different situations in Q and NQ cells.

4.1. Oxidative Stress

Oxidative damage represents a significant insult to organisms. ROS are permanently produced during normal cell metabolism. With the FACS method I wanted to compare the burden of oxidative stress within the different modified yeast strains by MS, which is taken up by the cell and oxidized by ROS forming a fluorescent molecule.

In the exponential cells of all strains I observed only low levels of oxidative stress. This is in agreement with the (common) expectations. The stress levels in Q cells are similar to the ones of exponential cells. I observed that d12 starved Q cells are significantly less burdened with ROS than NQ upper and lower cells are. Their metabolic rate is lower than in NQ cells, thus the production of ROS, which are byproducts of the metabolism, is decreased. The metabolic rate is associated with aging and ROS can accumulate more quickly

at higher metabolic rates. Furthermore, thickening of the cell wall is a characteristic feature of Q cells. Since it is proven that also extracellular stimuli can induce the production of ROS inside the cell [**27**], Q cells are more protected against extracellular stimuli and hence the production of ROS is reduced. Thereby, due to the specific properties of Q cells, their level of oxidative stress is lower than in non-quiescent cells.

The main repair mechanism of oxidative damage is BER. In my studies I used two BER impaired strains, YLBA1 and YLB14A1. For these strains I expected high levels of oxidative stress, indicated by a bigger shift to the right in the graph compared to other strains. Surprisingly, this was not the case. Both strains did not show higher levels of ROS than other strains did.
A possible explanation for this is, that BER is only impaired, which means in more detail, that only *APN1,* coding for (Apn1), the major AP endonuclease in yeast [**28**] which provides the main apurinic endonuclease activity*,* is knocked out but *APN2* is still intact. Probably the lack of Apn1 can be compensated by Apn2 and the contribution of other proteins. In addition to Apn2, Ogg1, which possesses AP lyase activity for the repair of oxidative damaged DNA, could be another backup protein. For further investigations it might be useful to design a new yeast strain with inactivated *APN1*, *APN2*, and/or *OGG1*.

4.2. Mutation Frequencies

The mutation frequencies in stationary phase cells I studied using the canavanine assay, which is a forward mutation assay. I distinguished between replication-dependent mutations, occurring up to d2, and replication-independent mutations, arising later.
I expected the fewest mutations for the wildtype strain YLBM/EH150, because this strain does not suffer from a deficient repair pathway. The results confirmed my expectation. The amount of replication-dependent (80 per 5 x 10^7 cells) as

well as replication-independent (90 per 5 x 10^7 cells on d5), was lower compared to other strains.

In the yeast strains with deficient or impaired repair pathways the mutation frequencies were increased.
The most significant increase of replication-independent mutations I detected between d5 and d7 in nearly all strains. Astonishingly the number of replication-independent mutations between d2 and d5 was slightly decreased in some of the used strains. I observed this decrease for all three cell fractions (Q, NQ upper and NQ lower) of the strain YLB4, as well as for the Q cells of YLB14A1, the NQ lower cells of YLB14 and the NQ upper cells of YLBA1. However, in consideration of the error bars, except for Q cells of YLB14A1 the decline was not significant. I assume that cells with mutations have a higher death rate than cells without mutations. This assumption would explain the decrease in stationary phase cells. Based on our data further studies are currently in progress.

YLB4 lacks the ability to carry out NHEJ to repair double strand breaks. I observed an increase up to about 150 per 5 x 10^7 cells replication-dependent as well as replication-independent mutations (d2). Heidenreich et al [25] showed that the knockout of NHEJ caused a reduced number of frameshift mutations. However, in this study only frameshift mutations were observed and not - as in my study - all kinds mutations, including DSBs. This could explain the higher mutation frequency compared to the wildtype.

The strain YLBA1 is impaired to accomplish BER properly and therefore restricted in its ability to repair AP sites. Our data show about 200 per 5 x 10^7 cells replication-dependent and approximately 250 per 5 x 10^7 cells replication-independent mutations. This is about the double amount of mutations compared to the wildtype, although the characteristics of both graphs look similar - in both strains the amount of mutations stays nearly constant up to d2 and then constantly rises until d12.

The knockout of Rad14 in YLB14 and YLB14A1 is the reason why both strains lack the ability to perform NER. For YLB14 I detected around 800 per 5 x 10^7 cells replication-dependent and about 900 per 5 x 10^7 cells replication-independent (d2) mutations. In fact NER deficient strains have a highly increased number of mutations compared to the wildtype. In contrast to the BER deficient strain YLBA1 it seems that the defect of NER has a more severe effect on the occurrence mutations than an impaired BER pathway.
But: BER is only impaired. *APN1* deficiency can be at least partially bypassed by other proteins like Apn2 and Ogg1. Therefore, it would be interesting which impact on the mutation frequency a BER deficient yeast strain would have.

Furthermore data revealed that the mutation frequency in case of YLB14A1 has the most distinct increase of all strains. The total amount of replication-dependent mutations mounted up to 2000 per 5 x 10^7 cells and 2500 replication-independent mutations (d12). Compared to the strain YLB14, which is only affected by a deficient NER, the additional burden of an impaired BER as an impact on the appearance of mutations, which is more than additive.

I can conclude that the different repair pathway deficiencies have varying impacts on the mutation frequencies of the cells. Interestingly, NER deficiency has the most severe effect on the cells and contributes more to the development of mutations than NHEJ deficiency or an impaired BER. This suggests the assumption that the majority of mutations are not a consequence of error-prone repair of DNA DSBs, but of helix distorting lesions (e.g. pyrimidine dimers or large adducts).

4.3. Double Strand Breaks

I detected the amount of DSBs using an antibody against the phosphorylated histone H2A (γH2A). Histone H2A is phosphorylated in response to DSBs, around which it is accumulated. Therefore antibodies against γH2A are widely used as a marker for DNA DSBs. DSBs occurred in all stationary phase cells of all strains, but the amount (of the DSBs) differed significantly in Q and NQ cells. In exponential cells there were only marginal amounts of DSBs in all strains used.

YLBM/EH150 reveals an increase of DSBs in the course of starvation for all three kinds of cell fractions. The amount of DSBs is nearly the same for NQ upper, lower and Q cells. On d7 the cells exhibited a bigger amount of DSBs compared to d12. It seems that a lot of cells with DSBs die before d12, although they haves the possibility to repair DSBs.

Due to the fact that YLB4 is unable to repair DSBs via NHEJ, I supposed that YLB4 would exhibit a bigger amount of DSBs compared with the other strains. Indeed, our data showed a significant increase in the amount of DSBs until d12, but only in NQ upper cells.

Budding of the yeast cells leads predominantly to Q (daughter) cells but also to NQ upper and lower (mother) cells. As mentioned in the introduction they are different in their morphology as well as in their physiology. Therefore, Q cells have a better chance of survival than NQ cells, especially under deteriorating living conditions. This could be the explanation why all NQ upper cells of all yeast strains showed higher amounts of DSBs compared to the other fractions. The increased accumulation of DSBs in NQ upper cells is probably one of the reasons why these cells become rather apoptotic than quiescent cells do.

As mentioned above YLB14 is NER deficient, which leads to an increased occurrence of mutations. Similarly, the amount of DSBs is significantly increased. An explanation for this results could be that an increased amount of unrepaired lesions may lead to a higher number of DSBs (and finally to apoptosis).

YLBA1 shows nearly the same signal intensity throughout all cell fractions, as well as not much more DSBs than the wildtype. I conclude that the impaired BER pathway has not that severe effect on the occurrence of DSBs. On the other hand, I had observed high amounts of oxidative stress in this strain. This leads to the conjecture that oxidative stress is most likely not the major reason for DNA DSBs.

Our experiments demonstrate the accumulation of DSBs in stationary phase cells. NQ cells are more affected by DSBs than cell-cycle arrested Q cells, most likely because they are not arrested in G0. Repair pathway deficiencies differ in their influence on the generation of DSBs. Impaired BER does not play an important role in the rise of DSBs, whereas I detected a correlation between NER as well as NHEJ deficiency and the increased occurrence of DSBs.

Because plenty of human cells spend long periods of their life in stationary phase, mutations occurring in resting cells should be considered with more importance. Further investigations need to be done to reveal undiscovered properties and behaviour of stationary phase cells, because the occurrence of mutations in resting cells is a potential risk of cancer development.

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Children:	1 daughter (9 years)

Education

2009 - 2011	Diploma Thesis; Institute of Cancer Research at the Medical University; Group: Dr. Ferdinand Steinböck
2001 - 2009	Undergraduate Studies of Molecular Biology at the University of Vienna (with parental leave)
1995 - 1999	"Höhere Lehranstalt für wirtschaftliche Berufe", Wiener Neustadt, Austria
1990 - 1995	"Bundesgymnasium", Neunkirchen, Austria

Employments and Internships

2005 - 2008	Secretary at Eccona GmbH, Vienna, Austria (part-time)
2000 - 2001	Secretary at KOPAS Patent Attorney, Vienna, Austria
1999 - 2000	Doctors office receptionist, Neunkirchen, Austria
07/1997 - 09/1997	Summer job in hotel and restaurant industry, Mörbisch
07/1996 - 08/1996	Summer job at Henkel Central Eastern Europe, Vienna

Research Experiences

09/2009 - 11/2010 **Medical University of Vienna, Institute for Cancer Research**, **Department of Cellular and Molecular Tumorbiology** (Group Dr. Ferdinand Steinböck)

Diploma Thesis: Development of mutations in DNA repair deficient stationary phase cells of Saccharomyces cerevisiae

Methods: Isolation of quiescent and non-quiescent cells by density gradient centrifugation, forward-mutation-assays, FACS, Western Blot

05/2008 - 07/2008 **Medical University of Vienna, Institute of Immunology, Department of Immuneregulation** (Group: Dr. Johannes Stöckl)

Project: A physiological approach to differentiate human Th-17 cells

Methods: FACS, isolation of PBMNC and T-cells with MACS, cell culture (dendritic cells, monocytes, T-cells)

04/2008 - 05/2008 **University of Vienna, Max F. Perutz Laboratories, Department of Nucleus and Chromosome Biology** (Group: Dr. Roland Foisner)

Project: Characterization of a novel antibody against mLEM3
The functional role of LEM3 in DNA Damage Response

Methods: Cell culture (HeLa, U2OS, oREC, mFib, MCF7), transfection with Lipofectamine^{Tm}, pulsed field gel electrophoresis, co-immunoprecipitation, PCR, immunofluorescence, Gateway Cloning, Western Blot

11/2007 - 12/2007 **University of Vienna, Max F. Perutz Laboratories, Department of Developmental Biology and Diseases** Mechanism (Group: Dr. Marcela Hermann) - no Project

Methods: Western Blot, RNA isolation, cDNA synthesis, PCR, transformation with *E.coli*