Universidade de Aveiro Departamento de Química
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Resistência a antifúngicos gerada por erros na tradução em levedura

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em biotecnologia molecular, realizada sob a orientação científica do Doutor Manuel António da Silva Santos, Professor associado do Departamento de Biologia da Universidade de Aveiro e co-orientação do Dr. Tobias Franz Anton Ludwig Weil, investigador pós-doutorado do Departamento de Biologia da Universidade de Aveiro.

Dedico o final desta etapa ao meu pai, que vai finalmente poder ir de férias sem ter de contar os “trocos”.

o júri

presidente

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agradecimentos

Gostaria de agradecer, primeiramente, ao professor Manuel Santos por me ter dado a oportunidade de trabalhar no seu laboratório. Foi uma experiência completamente nova para mim. Aprendi muito.
Agradeço imenso ao Dr. Tobias Weil por ter supervisionado o meu trabalho ao longo do ano.
Estou extremamente agradecida a todos os membros do laboratório de biologia RNA, quer os actualmente presentes, quer os que já seguiram com as suas vidas. O bom humor que existe todos os dias no laboratório é graças a eles. Em especial, agradeço à Rita Bezerra e ao João Simões, que tinham sempre um tempinho no meio das suas experiências. Aos meus colegas de mestrado estou grata pelo companheirismo.
Agradeço aos meus amigos, que ouviram demasiadas vezes a palavra tese nos nossos últimos encontros.
Tenho de agradecer ao meu pai, que quando me via em baixo ou com vontade de barafustar, tentava sempre dar-me alento à sua maneira.
E finalmente agradeço ao Telmo, que esteve sempre comigo, sempre me apoiou, ouviu e, claro, aturou. Infelizmente, o tempo passa demasiado depressa e nem sempre tive tempo para aqueles de quem gosto.

palavras-chave erros de tradução, resistência a antifúngicos, RNA de transferência.

resumo

A resistência a antifúngicos é, hoje em dia, um problema sério a nível clínico, pelo que há necessidade de descobrir novos alvos que possibilitem o desenvolvimento de novos antifúngicos. Investigação a decorrer no nosso laboratório indica que a ambiguidade no reconhecimento de codões em *Candida albicans*, um patogénio humano, acelera a resistência a antifúngicos. O presente trabalho teve como objectivo elucidar se a ambiguidade em leveduras não patogénicas aumenta a resistência a antifúngicos. Para tal foi induzida artificialmente ambiguidade no reconhecimento de diferentes codões em *Saccharomyces cerevisiae*. As estirpes resultantes possuem um plasmídeo de replicação reduzida contendo um $tRNA_{UGA}^{Ser}$ de *C. albicans* sujeito a mutagénese dirigida, de modo a mutar o anticodão. Os novos anticodões reconhecem codões de diferentes aminoácidos mas o tRNA mantém os elementos de reconhecimento, sendo acilado com serina. Os tRNAs mutantes vão competir com os nativos, formando-se um proteoma estatístico. Para verificar se estas estirpes apresentam um fenótipo mais vantajoso em resposta a variados antifúngicos, foram expostas a diferentes classes dos mesmos. Adicionalmente, foram analisados microarrays de estirpes não expostas a qualquer stress adicional, de modo a perceber se as mesmas apresentam já tendência para responderem de modo diferente perante os diferentes antifúngicos.

keywords mistranslation, antifungal drug resistance, transfer RNA

abstract

Antifungal drug resistance has become a severe clinical problem and new targets for the development of new antifungal drugs need to be discovered. Ongoing work in our laboratory indicates that codon mistranslation due to codon ambiguity accelerates antifungal drug resistance in the human pathogen *Candida albicans*. The present work aimed to elucidate if non pathogenic yeasts behave similarly. Therefore, mistranslation was artificially induced in *Saccharomyces cerevisiae* strains by the expression of chimeric tRNAs. Each of the constructed strains carried a low-copy number plasmid, containing a *C. albicans* $tRNA_{UGA}^{Ser}$ gene, whose anticodon was changed by site-directed mutagenesis, in order to replace it by several other anticodons. As the identity elements of the tRNA remained unchanged it was still acylated with serine. These mutant tRNAs are expected to compete with the native ones and have an impact on the proteome. To verify if mistranslation leads to an advantageous phenotype regarding antifungal drug resistance, cells were exposed to different antifungals. Additionally, microarray analyses were performed on non-exposed mutant strains in order to detect a possible pre-disposition to resist antifungal exposure.

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List of Abbreviations

aaRS – tRNA-aminoacyl synthetase
aa-tRNA – aminoacyl-tRNA
AMP – adenosine monophosphate
AP – amphotericin B
ATPase – adenosine triphosphatase
CTZ – clotrimazole
CS – caspofungin
DHA – drug H^+ antiporter
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
eEF – eukaryotic elongation factor
eIF – eukaryotic initiation factor
eRF – eukaryotic release factor
FDR – false discovery rate
FLZ – fluconazole
GTP – guanosine 5'-triphosphate
IRES – Internal ribosome-entry site
MCZ – miconazole
MDR – multidrug resistance
mRNA – messenger RNA
NBD – nucleotide binding domain
PDR – pleiotropic drug resistance
PP_i – pyrophosphate
ROS – reactive oxygen species
RNA – ribonucleic acid
rRNA – ribosomal RNA
TMD – transmembrane domain
tRNA – transfer RNA
TSM – translation stress-induced mutagenesis

Other abbreviations will be explained along the text.

List of Figures

Thesis outline

This work was aimed to elucidate if mistranslation due to codon ambiguity accelerates antifungal drug resistance in *Saccharomyces cerevisiae*, as it was previously tested for *Candida albicans* (data not published).

S. cerevisiae is a good model organism to study the antifungal action upon mistranslation. It is well characterized, its genome was fully sequenced and it is closely related to *C. albicans*, a major opportunistic pathogen.

Experiments were performed using mutant *S. cerevisiae* strains, in which artificial mistranslation was induced by the expression of a mutant *Candida albicans* serine tRNA. This mutant tRNA was previously subjected to site-directed mutagenesis in order to change its anticodon to several other anticodons. The anticodon of serine-tRNAs is not an identity element and therefore, aminoacylation is not compromised. These mutant tRNAs will compete with the native ones by recognizing different amino acid codons but inserting always serine into the nascent polypeptide. A fraction of mutant proteins will not fold or will be unstable. These unstable proteins are expected to either unfold, have impaired function or eventually gain new functions. As each of the different strains carries a unique amino acid substitution (e.g. replacement of threonine by serine), severe consequences are expected to happen especially when very different chemically and structurally amino acids are exchanged.

First, we accessed the growth rate of the mistranslating strains. Then, an evolution experiment was carried out in order to investigate if mistranslation accelerates antifungal drug resistance and if translational stress-induced mutagenesis (TSM) occurs also in eukaryotes. To check for differences in antifungal drug resistance susceptibility tests were performed using different classes of antifungals (e.g. azoles, echinocandins, pyrimidine analogs and polyenes). Additionally, a long term experiment was performed using these strains, aimed to answer the question whether mistranslation over time increases mutation rate on a genome wide view. Therefore, whole genome sequencing will be applied in order to check for single nucleotide polymorphisms, indels and chromosomal rearrangements, among others. Finally, microarray analyses of the mistranslating strains were used to support the data obtained in a phenomics study in which several antifungals and translation inhibitors were used.

Introduction

1. The genetic code

The genetic code represents a well thought but degenerate system to translate nucleic acid sequences into proteins. Initially, 20 amino acids were described (Crick, 1966b), but a few years later, selenocysteine and pyrrolysine were discovered (Bock et al., 1991; Hao et al., 2002).

DNA sequences consist of four deoxyribonucleotides which can be purines - adenine (A) and guanosine (G) – or pyrimidines – cytosine (C) and thymine (T). In eukaryotes, these sequences are transcribed into non processed messenger RNAs (mRNA) by RNA polymerase II and after maturation are translated into proteins by ribosomes. mRNA sequences consist of four ribonucleotides - adenine (A), uridine (U), guanosine (G) and cytosine (C) - which are organized in triplets, enabling a set of $4^3 = 64$ possible codons. Those codons are either specifically assigned to amino acids or lead to translation termination (figure 1). Furthermore, the genetic code is considered to be degenerate, as with the exception of methionine and tryptophan, every amino acid can be decoded by two or more synonymous codons. Those codons similar to each other are assigned to chemically similar amino acids. This biased display of triplets is very important in order to reduce the incorporation of dissimilar amino acids into a protein. Hence a near cognate amino acid can be misincorporated but rarely will a non-cognate one. Due to their chemical differences the introduction of dissimilar amino acids could cause major consequences (e.g. improper protein folding). Another important feature is that most synonymous codons have the same ribonucleotide at the same position, usually the second one. Such feature also contributes to reduce the consequences of misincorporation as errors happen more easily by misreading of the third ribonucleotide position known as the wobble position.

Actually, the wobble hypothesis predicted that ribonucleotides could be displaced and form a non-Watson-Crick base pair (Crick, 1966a). Recent research has confirmed this statement, verifying that the wobble position often contains a modified base, which can have more than one correspondent ribonucleotide (Agris et al., 2007).

Another aspect to be considered is the correlation between sets of codons and the two groups of tRNA-aminoacyl synthetases. Group I synthetases deal with codons containing uridine in the second position (with the exception of the phenylalanine codon), while group II synthetases handle all codons containing cytosine in this position (Wetzel, 1995).

Thus, the organization of the genetic code contributes to the improvement of translation accuracy.

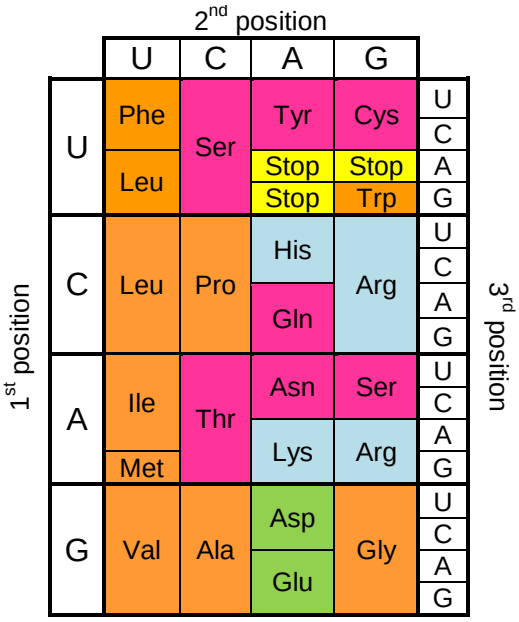

Figure 1 - Standard genetic code: Pink-polar uncharged; blue- positively charged; green – negatively charged; orange – non polar.

1.1 Transfer RNA

Besides the wobble hypothesis, Crick postulated his adaptor hypothesis, which states that adaptor molecules possibly link nucleotides to amino acids(Crick, 1958). These adaptor molecules, which recognize codons in the mRNA were discovered to be transfer RNAs (tRNA). Due to the wobble position mentioned above, tRNAs exist in isoacceptor families, and each family is recognized by a cognate aminoacyl-tRNA synthetase (aaRS).

tRNAs' secondary structure resembles a cloverleaf and their tertiary structure is L-shaped. The cloverleaf shape forms due to base pairing and consists of a stem with a loose 3'-CCA end, called acceptor stem as it binds the corresponding amino acid, and three stem loops: the D loop, the anticodon loop and the T loop. Some tRNAs have an extra variable loop located between the anticodon and the T loops. Regarding the tertiary structure, the anticodon is located on the first major domain of the "L" shape, while on the second domain the correspondent amino acid is attached (figure 2).

In eukaryotes, post-transcriptional processing of tRNAs is required to allow fine tuning of structure and identity in order to obtain diverse tRNA types (Hopper et al., 2010). Part of this process is the customization of nucleotides in specific positions. Some modifications are quite complex, requiring more than one modifying enzyme. A well known example is the ubiquitous pseudouridine (Ψ), the first discovered tRNA modification. So far, 81 modifications concerning tRNA are known and from those, 47 belong to the Eukarya domain (the RNA modification

database). These contribute to the stability and recognition of the carrying molecule, and tend to be conserved within phylogenetic domains. In particular, they contribute to tRNA folding, Mg^{2+} binding, intron removal, protein recognition, codon recognition and fidelity of the translational reading frame (Agris, 2008). Variation in modification level appears along with disease, under exposure to stresses such as starvation and drug exposure, and seem to be age and localization dependent (Dirheimer et al., 1995).

Besides such nucleoside modifications, transfer RNAs are subjected to further post transcriptional processing, such as splicing and trimming of 5'- and 3'-ends by specific RNAses (reviewed by Hopper et al., 2010; Nakanishi and Nureki, 2005). Unlike in many bacteria, eukaryote tRNA genes do not encode the 3'-CCA tail, thus, it has to be added enzymatically. An important role of this loose end is to protect the tRNA from degradation due to its function as an anti determinant against the 3'end ribonucleases (Phizicky and Hopper, 2010).

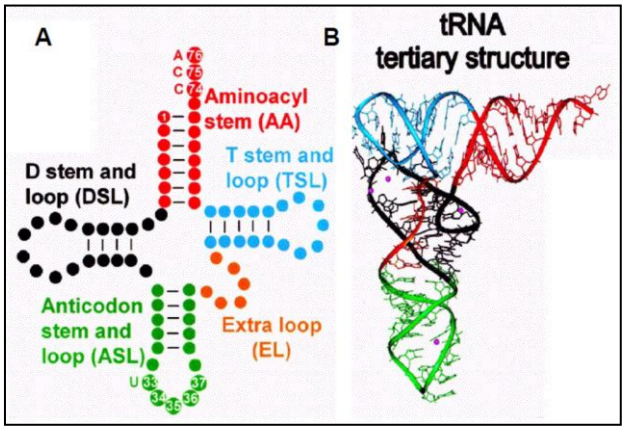

Figure 2 - The structure and domains of tRNA. The cloverleaf secondary structure (A) is color coded to identify the structural domains of the crystal structure: amino acid accepting stem, or aminoacyl-stem (AA) in red; dihydrouridine stem and loop domain (DSL) in black; anticodon stem and loop domain (ASL) in green; variable or extra loop (EL) in orange; and the ribothymidine, or TYC, stem and loop (TSL) in light blue. The positions of the (almost) invariant U33 and the amino acid accepting 3'-terminus (C74, C75 and A76) are shown. The three-dimensional structure of tRNA is represented by the crystallographic structure of yeast $tRNA^{Phe}$(B). Adapted from Agris (2004).

tRNA identification by aaRS owes less to modified nucleotides than to structural elements along the tRNA. However, if located at the wobble position, the modified residue can contribute both for precise codon-anticodon pairing and the recognition of the tRNA. One example is the hypermodified nucleoside mnm^5s^2U at the wobble position of $tRNA^{Lys}$ and $tRNA^{Glu}$ (Nakanishi and Nureki, 2005). tRNAs may interact with aaRSs at several regions (e.g. Lenhard et al., 1999; McClain et al., 1998; Sekine et al., 1996; Senger et al., 1995), but mainly with the discriminator base (position 73), the acceptor stem pairs 1-72, 2-71 and 3-70, and the anticodon (McClain, 1993). These identity elements serve not only specific recognition purposes but also can inhibit aminoacylation by non-cognate aaRSs (antideterminants or negative elements) (McClain, 1993).

1.2 Aminoacyl tRNA synthetases

Aminoacyl tRNA synthetases catalyze the 3'- esterification of tRNAs with their cognate amino acid. Esterification mostly happens in a direct way, according to the following reactions:

$$\text{Amino acid} + \text{ATP} + \text{aaRS} \leftrightarrows \text{aaRS}\cdot(\text{amino acid})\text{-AMP} + PP_i$$
$$\text{aaRS}\cdot(\text{amino acid})\text{-AMP} + \text{tRNA} \leftrightarrows \text{aaRS} + (\text{amino acid})\text{-tRNA} + \text{AMP}$$

Although similar in action aaRSs can be divided into two evolutionary distinct classes. Each class gathers those containing a certain structural motif in their active site. Even though not all synthetases have been extensively studied, these two motifs seem to result in two different ways of binding ATP and approaching the cognate tRNA: Class I binds ATP in an extended conformation and approaches the tRNA's acceptor stem from its minor groove side, and class II binds ATP in a bent conformation and approaches the tRNA's acceptor stem from the major groove side (Ibba and Soll, 2000). Only LysRS can be found in both classes, depending on the organism.

Although evolutionary distinct, it has been suggested by Rodin and Ohno (1995) that they could be complementary to each other, giving rise to the idea that in early genomes both strands of RNA were used for protein synthesis. Further, each class can be divided in three subclasses (*a*, *b*, and *c*) representing closer related enzymes (Schimmel, 2008).

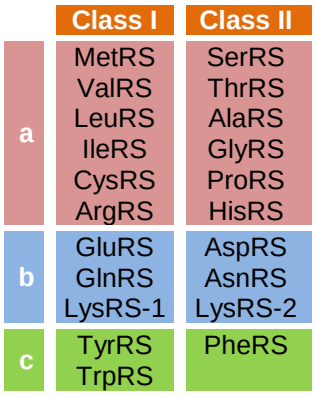

Figure 3 - Classes and subclasses of aminoacyl tRNA synthetases. Subclasses include enzymes that are most closely related to each other in their sequences. Significantly, the subclasses also group aminoacyl tRNA synthetases according to their amino acid chemical types. Adapted from Schimmel (2008).

2. Protein translation in Eukaryotes

Messenger RNA (mRNA) is subjected to maturation resulting in a ready-to-go template that can be frequently used by the translation machinery, as long as it remains stable and is needed. The translation machinery consists of cytoplasmic ribosomes, tRNAs and translational factors, and is assisted by aaRS. Eukaryotic ribosomes are large complexes of proteins and ribosomal RNA (rRNA). They can be divided into a large 60S and a small 40S subunit. These two subunits bind to each other during translation. The complete ribosome 80S permits the binding of three tRNAs, whose binding sites are located on the 40S subunit. These are called aminoacyl (A site), peptidyl (P site) and exit (E site) sites.

Protein translation can be divided in three steps: initiation, elongation and termination. These steps are followed by the recycling of the ribosome.

2. 1 Initiation

Initiation is assisted by the initiation factors, which interact with each other, resulting in a solid and stable initiation complex, able to bind strongly to the mRNA. Many of these factors are also macrocomplexes.

Initiation requires the formation of a ternary complex, which is formed by the initiator transfer RNA bound to methionine (Met-$tRNA_i^{Met}$), the eukaryotic initiation factor 2 (eIF2) and GTP. eIF2 is assisted by eIF2B which recycles GDP back to GTP. This complex binds to the small ribosomal subunit (40S) and to eIF1, 1A and 3, resulting in a larger complex, the 43S. These factors appear to help scanning the mRNA (eIF1 and 1A) and to avoid premature binding of the large ribosomal subunit (eIF3) (reviewed by Kapp and Lorsch, 2004).

Before the 43S complex attaches to the 5'end of the mRNA, forming the 48S complex, unwinding of the mRNA's secondary and tertiary structure in 5'-untranslated region (UTR) takes place and an anchor is assembled. The unwinding seems to be performed by the helicase eIF4A, stimulated by eIF4B (Grifo et al., 1982; Lawson et al., 1989; Ray et al., 1985; Rozen et al., 1990). The anchor assembly seems to be accomplished by the binding of eIF4E to the 5' 7-methylguanosine cap. The anchor eIF4E, together with eIF4A bind to eIF4G. These three factors together form the eIF4F complex. eIF4G functions like a hub and is thought to be very important in unusual cases of initiation, such as the absence of the 5' cap, the poly A tail, or both simultaneously, and in the presence of an internal ribosome-entry site (IRES) mediated translation which is cap-independent (Holcik and Sonenberg, 2005; Johannes et al., 1999). Finally, the 48S complex can start scanning the 5'UTR for the initiation codon (AUG).

With the localization of the initiation codon, the 60S ribosomal subunit, binds to the small subunit, assisted by eIF5B. As ligation happens, eIF 1, 1A, 3 and 5 are released. Premature association of ribosomal subunits is thought to be blocked in part by the binding of eIF6 to the 60S.

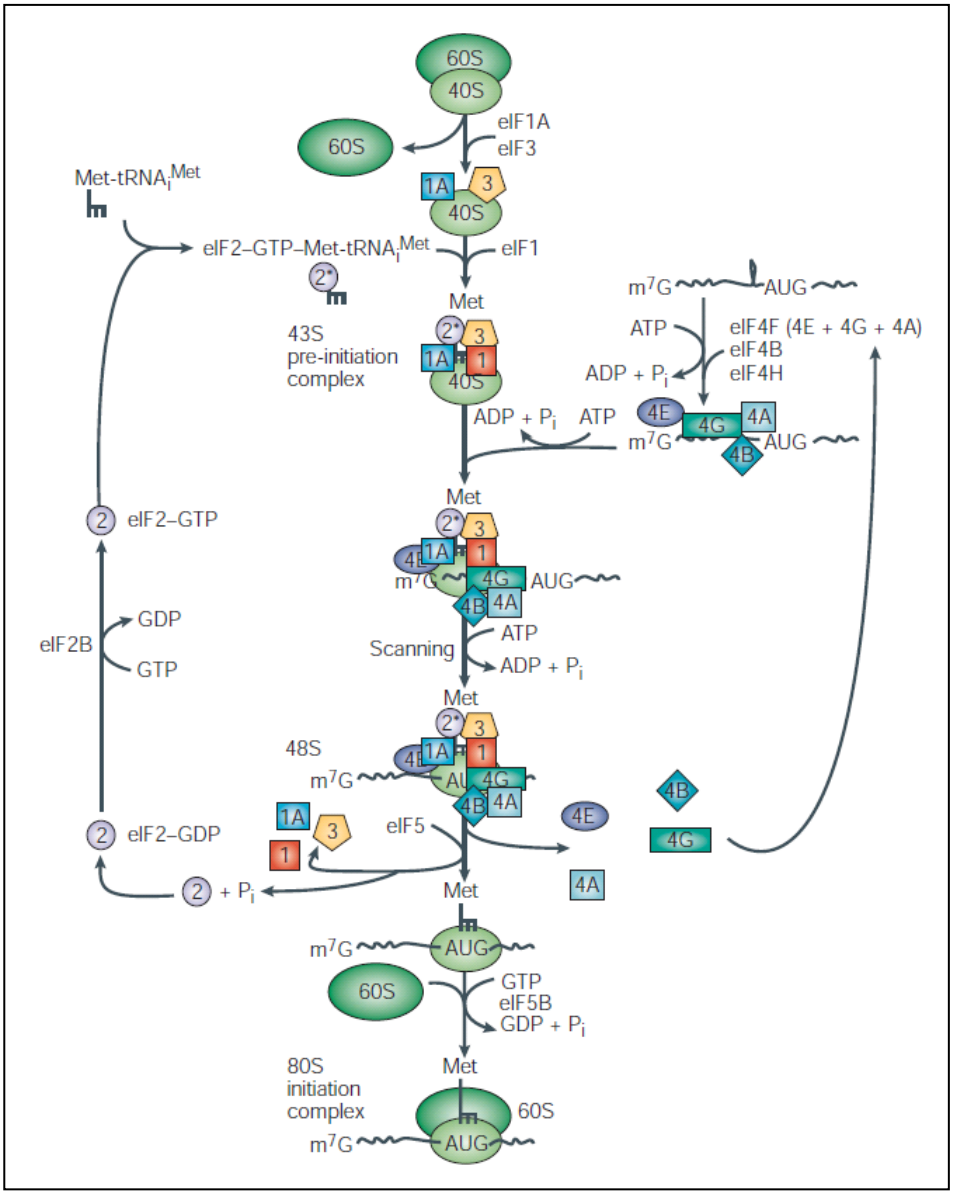

Figure 4 - Translation initiation in eukaryotes (Holcik and Sonenberg, 2005).

2.2 Elongation

After the binding of the met-tRNA$_i^{Met}$ anticodon to the initiation codon (AUG) elongation starts. Met-tRNA$_i^{Met}$ binds at the P site while the following tRNAs bind to the A site of the ribosome. Elongation ternary complexes bind to the elongation factor eEF1A instead of eIF2. eEF1A triggers GTP hydrolysis, and eEF1A•GDP releases the aminoacyl tRNA. Codon-anticodon base pairing and the GTP hydrolysis by eEF1A are part of the quality control steps (see below). The formation of a peptide bond between the incoming amino acid and the peptidyl aminoacyl-tRNA is carried out by the ribosomal peptidyl transferase center. Then, translocation takes place with the deacylated tRNA moving to the E site. During

translocation the tRNA passes through a hybrid state in which it is present in both sites. The same happens to the tRNA at the A site by first translocating its acceptor stem to the P site and only then the anticodon end. Translocation is possible due to GTP hydrolysis stimulated by the factor of the eEF2·GDP complex. After translocation the A site is free for the next aa-tRNA to bind. However, eEF1A·GDP has to be recycled and this is accomplished by the eEF1B complex, consisting of eEF1Bα and eEF1Bβ (Kapp and Lorsch, 2004). During translation the three ribosome sites will always be occupied. Once a deacylated tRNA is released from the E site, a new ternary complex binds to the A site. This process is repeated until a stop signal appears.

Particularly, in fungi a third factor, eEF3, exists which was shown to be essential in yeast survival (Qin et al., 1990). It interacts with eEF1A (Kovalchuke et al., 1998), and is required for every round of peptide bond formation. Two possible roles are described for eEF3: a) to aid in the release of the deacylated tRNA from the E site and b) to increase efficiency in the binding of eEF1A·GTP·aa-tRNA to the A site (Trianaalonso et al., 1995).

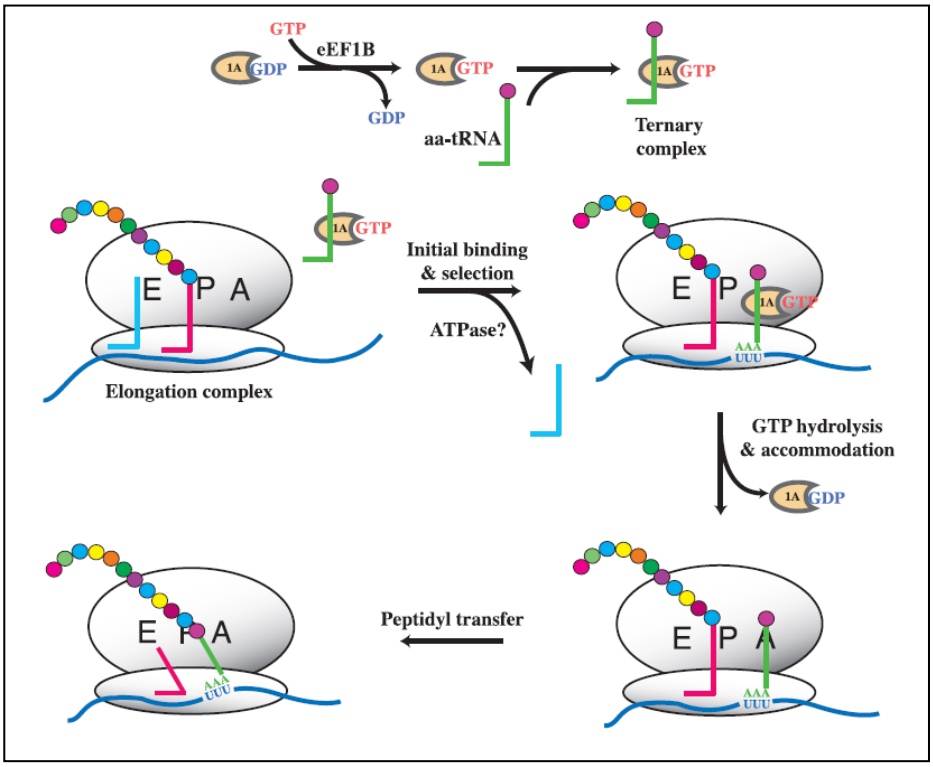

Figure 5 - Translation elongation in eukaryotes. Adapted from Kapp and Lorsch (2004)

It was previously demonstrated that the rate-limiting step in elongation is tRNA selection and that different tRNAs exist in different concentrations leading to variation in the translation rate along the mRNA (Varenne et al., 1984). Thus, to maintain translation at a specific relevant rate (Reynolds et al., 2010) it is important that a set of correctly acylated tRNAs is available at any time. Otherwise the elongation complex will get stalled and/or a near-cognate tRNA, more

abundant at that moment, may eventually take the correct one's place, resulting in a mistranslated protein.

2.3 Termination

Once a stop signal enters the A site the release of the peptide is initiated. There are no cognate tRNAs to decode stop signals. Instead, a release factor with a similar 3-D structure binds to the ribosome. Only two release factors exist in eukaryotes. eRF1 decodes all three stop signals (UAA, UAG or UGA) while eRF3 is discussed to be a GTPase that stimulates the release of eRF1 from the ribosome, leading to consequent peptidyl tRNA hydrolysis. eRF3 is essential in eukaryotes and its interaction with eRF1 leads to an optimal termination efficiency in *S. cerevisiae* (Kapp and Lorsch, 2004).

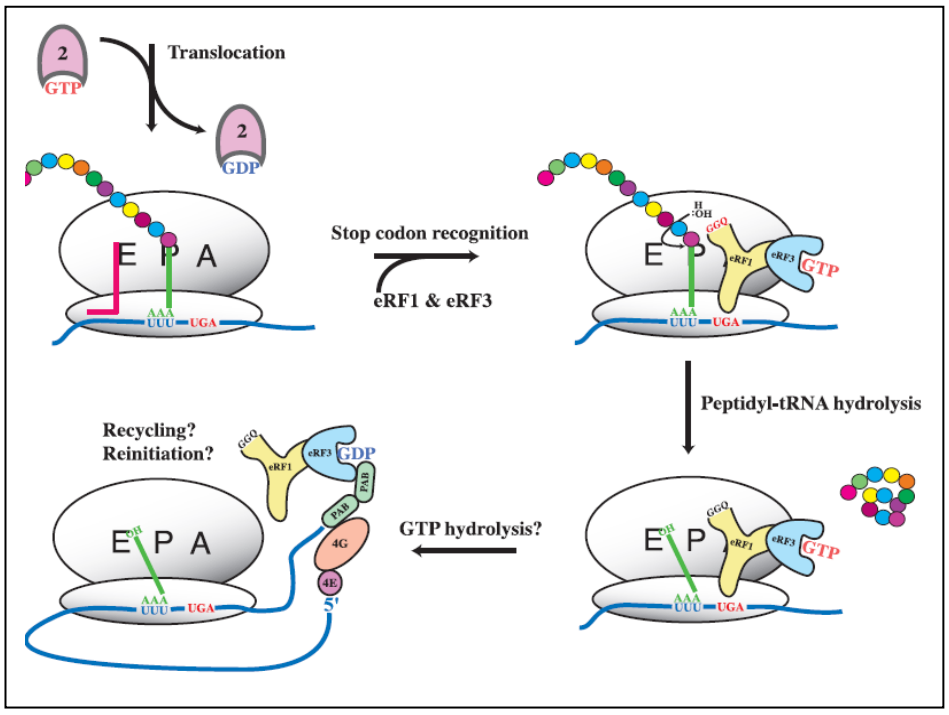

Figure 6 - Translation termination and recycling. adapted from Kapp and Lorsch (2004).

2.4 Recycling and re-initiation

After termination the ribosomes can be used again for another translation round on the same mRNA (re-initiation) or reassembled on a new mRNA (recycling). Regarding recycling it is known that a post-termination complex (PoTC) remains, consisting at least of mRNA, tRNA and ribosome. Recently, eIF3, eIF1 and eIF1A have been suggested to cooperatively disassemble PoTCs into

free 60S subunits and mRNA and tRNA bound 40S subunits (Pisarev et al., 2007). In yeast eEF3 and ATP have been implicated in the disassembly of this complex, suggesting that they catalyze the simultaneous split of the ribosomal subunits and the detachment of mRNA and deacylated tRNA (Kurata et al., 2010). There is still much to unravel about recycling especially concerning which factors are involved and how.

The discovery of the interaction of poly-A binding protein (PABP) with eIF4G led to the assumption that mRNA circularizes and re-initiation of translation takes place. Hence, after termination, the 40S subunit might not be released, but instead transferred back to the 5' end via 3' and 5' translation factors (figure 7) (Tarun and Sachs, 1996).

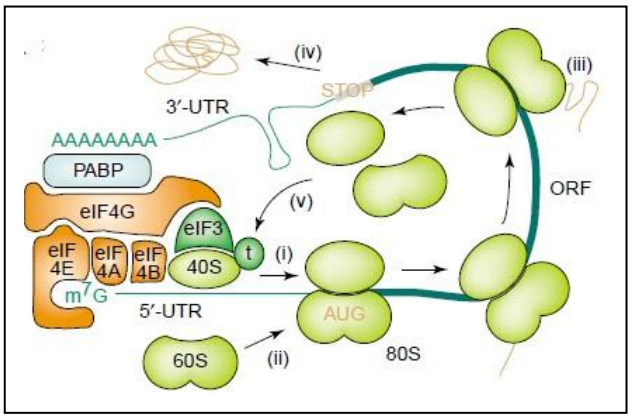

Figure 7 - 'Circular' or 'closed-loop' mRNA model showing circularization mediated by poly(A)-binding protein (PABP) binding to both the poly(A) tail and eIF4G: (i) scanning of the 40S containing 43S initiation complex to the initiation codon, (ii) joining of the 60S ribosomal subunit to form a translation-competent 80S complex, (iii) protein elongation, (iv) termination of translation, and (v) re-initiation. Adapted from Mazumder et al. (2003).

3. Mistranslation

Translation is the most error-prone step of protein synthesis. The overall translational error rate is about 1 in 10^4 polymerized amino acids which is approximately the sum of transcription ($\sim10^{-4}$), aa-tRNA synthesis ($\sim10^{-4}$), and ribosomal decoding ($\sim10^{-4}$) errors. Mainly for eukaryotes, the known mechanisms controlling protein translation are important to avoid wasting energy by producing unnecessary amounts of non-functional proteins. Actually, it has been estimated that translation can consume up to 50% of the cell's energy (Holcik and Sonenberg, 2005), and therefore, it is important for cells to have different possibilities to control translation (Gebauer and Hentze, 2004; Hinnebusch, 2005). In cases of stress global translation can be reduced, while selective translation can be activated in order to synthesize specific proteins that aid cell survival. Protein synthesis errors can arise from DNA mutations, during transcription and/or splicing, during the translation process or even during folding (figure 8). Only errors occurring during translation are considered in the following.

Mistranslation has a plethora of sources and contributes greatly to the production of non functional proteins. In general, mammalian cells have proven to be more sensitive to mistranslation than bacteria and yeast (Nangle et al., 2006).

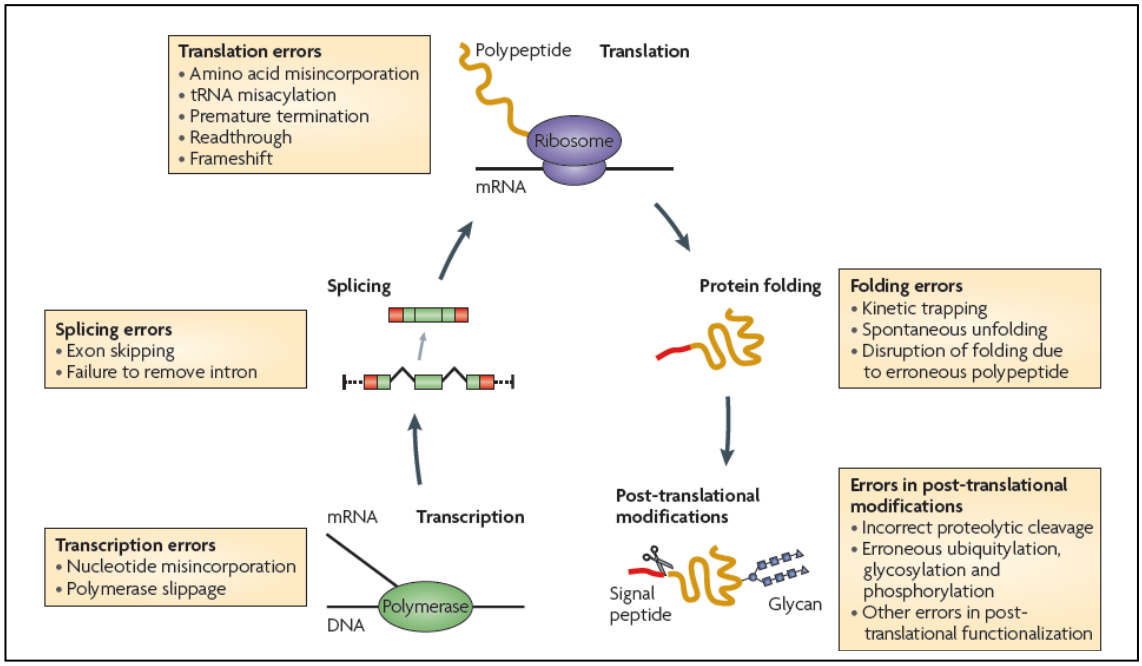

Figure 8 - Sources of errors in eukaryotic protein synthesis. Adapted from Drummond and Wilke (2009)

3.1 tRNA aminoacylation errors

As mentioned, direct tRNA aminoacylation is a two-step process meticulously controlled by aaRS (e.g. “double-sieve” model proposed by Fersht (1977)), but still errors can occur at a significant error rate (Reynolds et al., 2010). The errors at this stage include:

1. Editing failures
2. tRNA misrecognition by aaRS
3. Amino acid misactivation

1) Editing is needed to amend amino acid misactivation. These types of failures are probably due to mutations. To date, the only known case which seriously affects mammals is caused by a heritable missense mutation in the editing domain of AlaRS in mice (Lee et al., 2006). This enzyme mischarges $tRNA^{Ala}$ with serine, giving rise to a statistical protein population. As a result, the unfolded protein response (UPR) is triggered, leading to Purkinje cells (in cerebellum) degeneration and consequently, ataxia[1]. *In vitro* cultures of human cells, using an editing-defective ValRS have also shown increased degradation and apoptosis (Nangle et al., 2006). Regarding prokaryotic cells, heritable mutations in the editing site of an IleRS were verified to result in the induction of error-prone DNA polymerases (Bacher and Schimmel, 2007).

2) The structural diversity of different base combinations, and a complex network of sequence-specific aaRS-tRNA interactions, sometimes aided by binding enhancers, ensures the accurate selection of the cognate tRNA. Further, *in vivo* aaRSs compete for their cognate tRNAs. For these reasons, misrecognition of tRNAs seems to be rare compared to other errors and thus, there is no need for proofreading ability. In eukaryotes, despite some exceptions, tRNA transcription and maturation occur in the nucleus. This “boundary” guarantees to aaRSs that only functional tRNAs are exported to the cytoplasm (Ibba and Soll, 1999).

3) Amino acids are very small molecules and therefore pose a challenge to aaRSs. For instance, class I synthetases can deal with wrong attached amino acids in two ways (Cochella and Green, 2005; Reynolds et al., 2010):

   - Pre-transfer (before the linkage to the tRNA): First, the correct amino acid has to be selected by the aaRS. This is a grand challenge as some of them have similar chemical or physical properties and hence, must be distinguished mainly by their side chains. During selection amino acids with larger side chains are easily discarded but those with smaller ones can pass through the “sieve”. If the aaRS detects the erroneous selection, the misactivated amino acid is transferred to a second catalytic site, called editing-site (second sieve) in which, in contrast to

[1] Ataxia is a non-specific clinical manifestation implying dysfunction of the parts of the nervous system that coordinate movement, such as the cerebellum.

the smaller misactivated amino acid, the cognate amino acid does not fit. There, the misactivated amino acid is hydrolyzed.

- Post transfer (after linkage): If the aaRS does not detect the error, the amino acid will be linked to the tRNA, which still might be correctly selected due to its numerous identification elements. Misacylation can also be detected by the aaRS followed by the hydrolysis of the ester bound between the 3'-CCA end of the tRNA and the amino acid in the editing site. If the misacylated tRNA is released it might be re-sampled by the aaRS or recognized by trans-acting factors (*trans* editing), such as D-aminoacyl-tRNA deacylases.

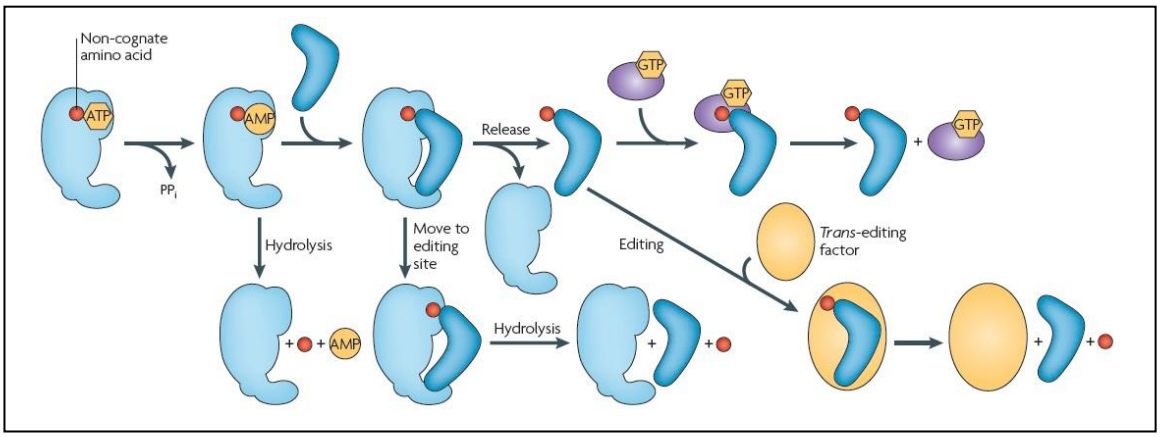

Figure 9 - Quality control steps during the formation of a non-cognate aminoacyl-tRNA. Adapted from Reynolds *et al*. (2010)

If all of these check points fail, there is still one last possibility of preventing the use of a misacylated tRNA during the formation of the ternary complex with GTP and eIF2 (or eEF1A). Both amino acid and tRNA contribute to the binding affinity to the ternary complex. If a non-cognate amino acid is attached to the tRNA, this binding affinity decreases immensely (up to 700-fold) (Asahara and Uhlenbeck, 2002).

Although not common for most organisms, the stereospecificity (D or L) of each amino acid can contribute to erroneous discrimination. In response to this problem one D-Tyr-tRNA^{Tyr} deacylase has been found to act upon D-aminoacyl-tRNAs in bacteria, yeast and more recently human cells (Soutourina et al., 2000; Soutourina et al., 1999; Zheng et al., 2009).

Two commonly referred examples of misacylation are: 1) the selection of L-valine instead of L-isoleucine by *E. coli* IleRS, which differ only by one methyl group and 2) the selection of L-threonine by ValRS, which has a hydroxyl group instead of a methyl group like L-valine (Fersht, 1977; Fersht and Kaethner, 1976). So far, a second catalytic site, necessary for the double sieve model, has not been described for all aaRSs.

Also, regulated misacylation events are known, for instance, the transamidation pathway, which was described in bacteria and archaea. Here, GluRS is used instead of GlnRS to produce Gln-tRNA^{Gln} (Becker and Kern, 1998; Wilcox, 1969; Wilcox and Nirenber.M, 1968) and AspRS is used to produce Asn-tRNA^{Asn}

(Curnow et al., 1996). Some organisms can only use this pathway (Curnow et al., 1997). Beside bacteria and archaea this pathway was also described for yeast mitochondria (Frechin et al., 2009) and other eukaryotes (Nabholz et al., 1997; Schon et al., 1988).

Other examples using different pathways are the production of cysteine which requires the O-phosphoseryl-tRNA synthase (SepRS) to acylate $tRNA^{Cys}$ and the formation of selenocysteine, which starts with the attachment of serine to $tRNA^{Sec}$ by a SerRS. It is worth noting that these examples, together with codon reassignments, show the evolutionary relevance of genetic code ambiguity (Moura et al., 2009).

3.2 Decoding errors

Decoding errors can happen mainly due to missense and nonsense errors and less frequently due to processivity errors. aa-tRNA selection has to be fast to achieve the optimum translation rate, leaving the translation machinery only a short time interval to explore the full discrimination potential between cognate and near cognate aa-tRNAs. Acceptance of near cognate tRNAs is avoided because of kinetic discrimination mechanisms. In a healthy organism the acceptance of non cognate tRNAs is rare. Besides codon organization the reciprocal relationship between E- and A-sites also contributes to tRNA discrimination. An occupied E site induces a low affinity A site and vice versa. So, the accommodation of the ternary complex will trigger the release of the tRNA in the E site (Nierhaus, 2006). The referred kinetic mechanisms are (Cochella and Green, 2005; Hopfield, 1974):

1. Kinetic proofreading: this strategy resembles the double-sieve editing mechanism but instead of two different selective steps, the same basic principle is repeated. The first selection step explores the ability of the ternary complex to hydrolyze GTP. This is an easy task for the cognate complex but problematic for near cognate complexes, as they will most likely dissociate. The second selection step scrutinizes binding differences between codon and anticodon, as the cognate tRNA will more easily accommodate. Non cognate aa-tRNAs are essentially excluded in the first step, while near cognate ones can pass this step with a frequency of approximately 1 in 30.

2. Induced fit: during initial selection, the rate of GTPase activation is significantly higher for the cognate aa-tRNA. During proofreading the accommodation rate is also higher. Summarizing, this strategy counts on the ability of cognate molecules to induce conformational changes on the enzyme and/or substrate, resulting in downstream effects on catalysis.

Missense substitutions are quite common errors, occurring approximately at a frequency of $4x10^{-4}$ per codon translated in prokaryotes (Parker, 1989). However, only 1 in 400 misincorporations completely inactivate the protein by affecting its folding, structure or function (Nierhaus, 2006). Such substitutions result from mischarging of tRNAs or codon-anticodon mismatches on the

ribosome. Their detection is difficult because the resulting protein has essentially the same size and composition as the native one. Also, missense substitutions, can lead to frameshifting, as described below.

Suppression of stop codons, known as translational readthrough, is another decoding error. The stop codon is misread either by a mutated tRNA or a near-cognate tRNA, which pairs with at least two bases of the codon. Here, the quality control mechanisms fail, and competition between the elongation and the termination machineries occurs. Nucleotide sequences surrounding stop codons can influence the readthrough efficiency. These context sequences can be quite complex (e.g. pseudoknots) (reviewed by Beier and Grimm, 2001). It is worth noting that premature stop codons lack this context sequences. In eukaryotes the nonsense-mediated mRNA decay mechanism can detect these premature codons and degrade the mRNA to avoid translation. Mutations like those in the yeast genes encoding eRF1 (SUP45) and eRF3 (SUP35), lead to suppression of the three stop codons (UAA, UAG, UGA) (Serio and Lindquist, 1999).

Processivity errors can be widely defined as those causing the release of the nascent polypeptide chain prior to its completion. The resulting truncated peptide can be toxic to the cell and will probably have an energetic cost, especially if highly expressed.

Frameshifts, which can be considered as processivity errors, are rare events (estimated error frequency of $3x10^{-5}$) that imply the alteration of the reading frame forward (+) or backwards (-) by one or more nucleotides. The most probable consequence is the production of truncated proteins due to early emergence of nonsense codons. For instance, tRNA slippage can happen if in the coding sequence the nucleotides before or after the target triplet are the same as the first and third of the latter, respectively. More rarely (10^{-15}) the ribosome carrying a peptidyl-tRNA can slide along the sequence (Nierhaus, 2006). Further, the occupation of the E-site of the ribosome is also of great importance, because an empty one augments the probability of +1 frameshifts by 25% (Marquez et al., 2004). Although not common, there are specific cases where frameshifts can be programmed like in viral genes, and in a single case in *E. coli* (Craigen et al., 1985). A programmed frameshifting error has an efficiency of nearly 100%. Under these circumstances the mechanism controlling frameshifting seems to be switched off. In eukaryotes, programmed frameshifting has been observed as well (Manktelow et al., 2005; Weiss-Brummer et al., 1987; Wills et al., 2006).

Nonsense errors that arise during replication or transcription can originate frameshifts and ribosome drop-off during translation. Other types of premature termination, which are not due to the presence of stop codons are thought to happen in two different ways: 1) the growing peptide could be released from the ribosome, followed by hydrolysis of the bond attaching it to the peptidyl-tRNA, or 2) a release factor could misread a sense codon. It is hard to distinguish them experimentally and both are difficult to distinguish from frameshifts and ribosome pauses. *In vivo*, drop-off of peptidyl-tRNA from the ribosome seems to happen at frequencies between $3x10^{-3}$ and 10^{-4} per elongation event (Parker, 1989).

4. Translation Stress-induced Mutagenesis

The great amount of possible mistranslation errors arising in cells activates intrinsic mechanisms, as described above. However, to cope with different types of stress and/or accumulation of errors, cells may be forced to evolve faster, in order to survive. Usually, a certain accumulation level of aberrant proteins results in a decrease in fitness, culminating in cell death. However, below this limit, cells can present a statistical population of proteins, consisting of a minor part of mutant proteins together with a major part of wild-type proteins. The mutant proteome fraction is expected to include unfolded proteins but also unstable proteins which can misfold and form toxic aggregates. The subsequent overloading of protein control systems might result in the activation of stress responses and alteration of gene expression. Nonetheless, this minor proteome grants genetic and phenotypic diversity and if phenotypic advantage arises, fixation of mutation may happen (Moura et al., 2009).

If error-prone polymerases and defective DNA repair enzymes are produced they might originate hypermutagenic clones possibly exhibiting high adaptation potential (Moura et al., 2009). Recently, a hypermutagenesis phenotype associated with codon ambiguity was discovered in *E. coli* (Michaels et al., 1990) and named translation stress-induced mutagenesis (TSM). TSM has similarities with the SOS response (reviewed in Humayun, 1998), the best studied inducible mutagenic pathway in bacteria, as it also requires *recABC* and *ruvAC* (Ren et al., 2000). It is associated to two mutator loci named *mutA* and *mutC* which exhibit similar phenotypes. Compared to the wild-type strain they presented a transversion increase, namely the A•T→ T•A and G•C→ T•A changes, followed by the less frequent A•T→ C•G. These two mutators were found to encode glycine tRNAs, which mutations affected the anticodon (Slupska et al., 1996). The *mutA* allele consists of a tandem set of three identical tRNA genes (*glyV*), while *mutC* contains four copies of also identical tRNAs (*glyW*). Both alleles code tRNAs that misread the 5'-GAU/C aspartic acid codon as glycine (5'-GGU/C). In each case, only one copy is affected and so the existence of a statistical tRNA population, resulting in a low level of mistranslation was proposed. After comparison with *mutD* mutations, it was suggested that the low mistranslation level might be due to the action of an error-prone polymerase. The *mutD/dnaQ* gene mutations are implicated in the impairment of polymerase III (pol III) editing function, particularly the ε subunit, and implicate also the loss of an aspartic acid. DNA pol III was later established to be error-prone (Al Mamun *et al.*, 2002; Dorazi, 2002). However, the exact changes of pol III are unknown.

A mutator phenotype was also observed when cells were exposed to streptomycin, a translation inhibitor (Ren et al., 1999). Later on, using this same stressor, it was proven that mistranslation in general could induce TSM (Balashov and Humayun, 2002), possibly through enhanced protein misfolding and turnover, and culminating in the expression of an error-prone DNA polymerase (Dorazi *et al*., 2002).

Finally, there is evidence in the literature supporting the appearance of other mutator phenotypes due to translational stress (Connolly and Winkler, 1989; Connolly and Winkler, 1991).

5. Antifungal drug resistance in fungi

Microorganisms' resistance to drugs is a growing problem nowadays mainly as a consequence of treatment failures. In the present thesis we focused on antifungal drug resistance, in particular resistance to azoles, using *S. cerevisiae* as a model system. To date, most of the available information on this topic concerns the genus *Candida* and the antifungal fluconazole. Besides these two, other genera will only be mentioned briefly.

In the mid-80's a significant amount of reports on antifungal drug resistance emerged. These concerned mainly resistant clinical isolates of *Candida albicans*, that were attributed to prolonged treatment with miconazole and ketoconazole (Sanglard et al., 1998). Nowadays about 200 yeast species are associated with humans, which are either commensals or pathogens. Actually, fatal invasive fungal infections are mostly caused by the commensals *Candida*, *Aspergillus* and *Cryptococcus*, which are opportunistic fungi. The costs associated with treatment of fungal infections are considerably high, even for the most common infections such as candidiasis. Patients' survival (specially for immunocompromised patients) is often time dependent and usually a correct diagnosis needs more than the available time. Therefore, patients are not always treated with the right antifungal from the beginning, thus favoring resistance increment.

Currently, four antifungal classes are mainly used for treatment: echinocandins, fluorinated pyrimidine analogs, polyenes and azoles. Besides these, there are two other less used antifungal classes, the allylamines and the morpholines. The effectiveness of each antifungal depends on the fungus, the dose, the exposure time and the mechanism of action. Two types of drug-induced stresses, the short- and the long-term stress, can contribute to the survival of fungi.

Briefly, echinocandins are lipopeptide molecules that inhibit beta-glucan synthesis by impairing the enzyme complex beta-(1,3)-glucan synthase, located in the cell wall. The first echinocandin, anidulafungin, was discovered in 1974 and more than ten years later, caspofungin and its precursor micafungin were developed (Denning, 2003).

Fluorinated pyrimidine analogs such as 5-fluorocytosine, inhibit DNA and RNA synthesis by mimicking the structure of pyrimidines. This compound enters the cell via a permease and is converted into 5-fluorouracil by a cytosine deaminase. 5-fluoroacil can then be converted into 5-fluorouridylic acid, which is incorporated in RNA after phosphorylation or into 5-fluorodeoxyuridine monophosphate which interferes in DNA synthesis by inhibiting the enzyme thymidylate synthase (Ghannoum and Rice, 1999). It is used frequently in combination with polyenes.

Widely used, polyenes such as amphotericin B, bind to ergosterol, a component of the fungal plasma membrane and thereby cause the formation of membrane-spanning channels. These channels provoke leakage of ions with consequent loss of cell integrity. Resistance to polyenes seems to be related to low ergosterol content, probably due to defective ERG3 function like described for azoles (Cowen and Steinbach, 2008).

There are two strong reasons why azole drugs are the most commonly used antifungals nowadays: firstly because unlike other drugs such as amphotericin B (polyene), mammalian cells tolerate them quite well and secondly because azoles act against a wide spectrum of fungi. This class comprises two generations named imidazoles (first generation, e.g.: ketoconazole and miconazole) and triazoles (second generation, e.g.: fluconazole and itraconazole). The first generation comprises, among others, miconazole, used in the current study, and ketoconazole. The primary target of azoles, is the ergosterol biosynthesis, in particular the cytochrome P450 Erg11p or lanosterol-14α demethylase (CYP51) encoded by the Erg11 gene. Azoles mimic lanosterol and compete for enzyme binding. This step is rate-limiting in ergosterol biosynthesis (figure 10). Ergosterol is a key component of fungi plasma membranes, analogous to mammalian cholesterol.

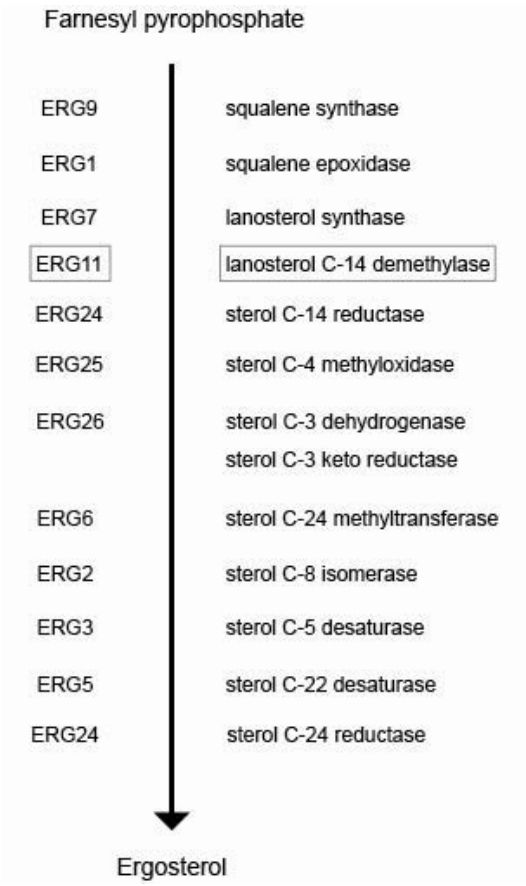

Figure 10 - Part of the ergosterol pathway. Squares indicate azoles' target enzyme and the gene encoding it. Adapted from Bammert and Fostel (2000).

5.1 Resistance Mechanisms

Luckily, fungi do not generally spread their resistance determinants like bacteria, nor mate often. Also, resistant isolates in human patients have never been reported to pass to other hosts. Still, several resistance mechanisms are known and the combination of two or more can result in synergy, antagonism or addition (Anderson, 2005).

Membrane or osmotic stresses are short-term consequences of azole exposure, as cells are more and more depleted in their fundamental membrane molecule ergosterol. Briefly, such stresses can be fought by activation of conserved signaling pathways like the mitogen-activated protein kinase (MAPK) and the cyclic AMP-protein kinase A pathway (reviewed in Roman et al., 2007).

Further, the serine/threonine protein phosphatase calcineurin, has an opposite mechanism of action, and is known to play a role in membrane stress. Its inhibition in *C. albicans* turns azoles from fungistatic into fungicidal drugs. Acting in the same signaling pathway as calcineurin, heat shock protein 90 (Hsp90) was also shown to contribute to fluconazole resistance in *S. cerevisiae* and *C. albicans* (Cowen et al., 2006; Cowen and Lindquist, 2005). However, the precise mechanism behind azole tolerance remains to be discovered.

In most fungi, azoles cause growth arrest by reducing the ergosterol content of membranes and by accumulating toxic ergosterol precursors (e.g. 14α-methylergosta-8,24(28)-dien-3β,6α-diol). However, there are species for which some azoles are fungicidal (e.g. *Arpergillus fumigatus*) (Espinel-Ingroff, 2001; Manavathu et al., 1998; Manavathu et al., 2000). Resistance to azoles seems to be acquired due to multiple mechanisms. These are, among others, overexpression or point mutations (confirmed to reduce fluconazole binding) of *ERG11*, efflux via ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters, tolerance to methylated sterols via mutation in *ERG3*, stress tolerance induction and aneuploidy (Cannon et al., 2009).

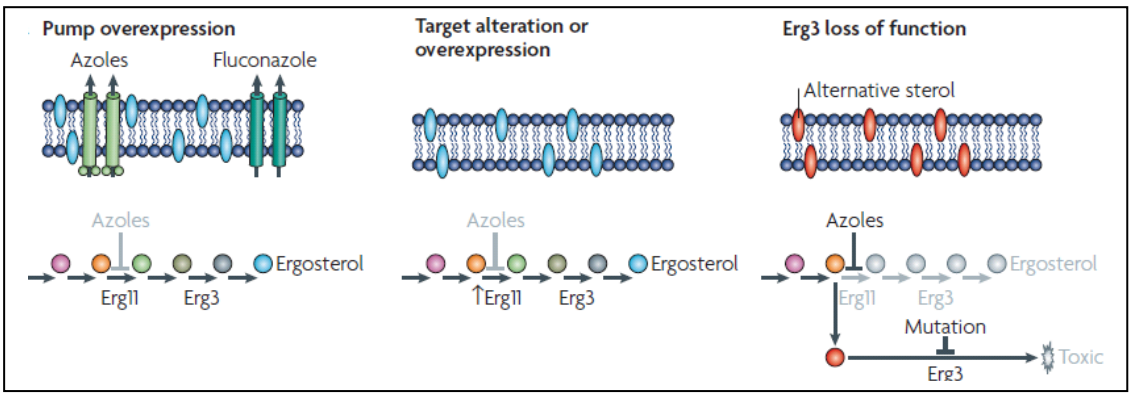

Figure 11 – Azole resistance: Three main forms of resistance to azoles can result from the upregulation of ABC and MFS transporters that remove the drug from the cell, through the mutation or overexpression of Erg11, which minimizes the impact of the drug on the target, or alterations in ergosterol biosynthesis, such as the loss-of-function mutation of Erg3, which blocks the accumulation of a toxic sterol intermediate that is produced when Erg11 is inhibited by azoles (Cowen, 2008).

A gain of fitness is often associated with the development of azole resistance and therefore resistant isolates may persist in the cell population after the cessation of azole therapy. Evolution of resistance strongly depends on the population size, as each propagule (any fungal structure capable of dissemination) represents an independent possibility of resistance acquisition (Anderson, 2005).

Yet to be precisely determined, certain levels of overexpression of drug efflux pumps, present in the plasma membrane, could reduce azoles to non toxic levels (Cannon et al., 2009). Lately, great interest has turned to these efflux pumps, especially in order to understand how these pumps bind and transport substrates, and to determine their "regular" substrates. Therefore, they will be described in more detail below.

5.2 ABC and MFS transporters

ABC proteins are primary transporters that require ATP hydrolysis and can be found in every organism's plasma membrane. Their basic structure consists of two nucleotide binding domains (NBD) and two transmembrane domains (TMD), which are arranged accordingly to the type of ABC protein. In *S. cerevisiae* ABC proteins can be divided in three subfamilies: pleiotropic drug resistance (PDR), multidrug resistance[2] (MDR) and multidrug resistance-associated protein (MRP). The PDR subfamily is most frequently associated to antifungal resistance, with *S. cerevisiae*'s Pdr5p considered as an archetype.

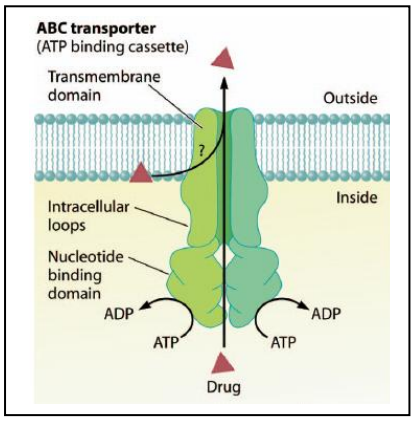

Figure 12 - Possible arrangement of an ABC transporter (Cannon *et al*., 2009)

As *S. cerevisiae* cells contain numerous ABC genes, a new system (Decottignies et al., 1998) was developed in a mutant species, which allowed background reduction. Using this system it was demonstrated that the genes *YOR1* and *PDR5* share miconazole as common a substrate. A derived system overexpressing CaCdr1p[3] showed increased itraconazole resistance (1,000-fold) (Lamping et al., 2007).

[2] The term Multidrug Resistance is also used as a broader concept, covering other subfamilies, such as PDR and MDRp.
[3] CaCdr1p – "Ca" stands for *Candida albicans*, the "p" usually stands for protein.

Clinical isolates of *C. albicans* were found to have increased levels of *CDR* genes (Perea et al., 2001), known to confer resistance to multiple azoles. Disruption of *CaCDR1* led to hyper susceptibility to azoles and deletion of both *CaCDR1* and *CaCDR2* provoked even higher susceptibility (Sanglard et al., 1996; Sanglard et al., 1997). *CaCDR1* overexpression in *S. cerevisiae* was found to confer cross-resistance to different azoles, including fluconazole, itraconazole and ketoconazole (Sanglard et al., 1995). Regarding *CDR* proteins, there is evidence that CaCdr1p, 2p and 3p are involved in phospholipid transport, having some influence in membrane leaflets composition. Thus, it was suggested that overexpression can indirectly contribute to antifungal resistance (e.g. effects on membrane function or membrane protein activity). Further, *C. glabrata* azole resistance is also associated with PDR ABC pumps (CgCdr1p and CgCdr2p).

C. dubliniensis is another organism for which the genes Cd*CDR1* and *CdMDR1* (MFS transporter) have been implicated in azole resistance. Contrary to *C. albicans* that preferentially uses *CaCDR1* for drug efflux, *CdMDR1* is directly linked to fluconazole resistance. *CdCDR1*, is not as commonly used, although it has been found to efflux ketoconazole and itraconazole and its deletion showed increased susceptibility to both azoles (for more detailed information see Sullivan et al., 2004).

MFS pumps belong to protein superfamilies, that are widespread across phylogenetic domains. Their expression reveals a much weaker association with azole resistance than ABC transporters. They are secondary transporters, containing only TMDs that require a proton gradient and can be divided in two subfamilies regarding the number of transmembrane spans (TMS): DAH1 (12 TMS) and DHA2 (14 TMS).

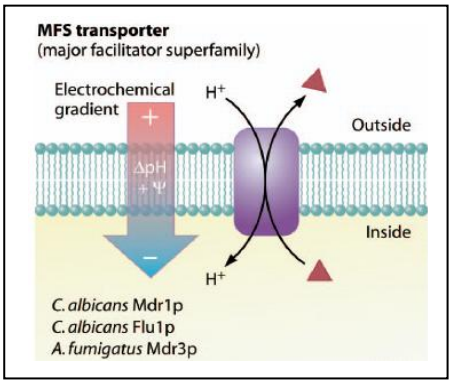

Figure 13 - Arrangement of a MFS transporter in *C. albicans* ans *A. fumigatus*. (Cannon *et al*., 2009)

The first MFS gene characterized was *C. albicans'* Ca*MDR1*. Later, the same gene was identified in fluconazole resistant mutants (Albertson et al., 1996) and clinical isolates of *C. albicans* (Perea et al., 2001; White, 1997). The same clinical isolates also showed increased levels of the *ERG11* gene (White, 1997) as

well as increased resistance to itraconazole, voriconazole, and posaconazole (Perea *et al*., 2001) Overexpression of *CaMDR1* in *S. cerevisiae* conferred, I addition to fluconazole resistance, ressitance to ketoconazole (Lamping et al., 2007), while only very high levels of overexpression led to fluconazole resistance in clinical *C. albicans* isolates (Hiller et al., 2006). Additionally, *CaMDR1* confers resistance to other drugs (e.g. cerulenin and brefeldin A).

Both types of efflux pumps are related to transcriptional factors that act as regulators. While exposed to antifungals, these can be subjected to gain-of-function (point) mutations. Elements acting upon the PDR ABC subfamily of *S. cerevisiae* are among the best studied. Gain-of-function mutations have been identified in the genes of two transcription factors, ScPdr1p and ScPdr3p (Carvajal et al., 1997; Nourani et al., 1997). ScPdr1p is required to induce compensatory upregulation of efflux pumps if individual efflux pump genes are deleted (e.g. *ScPDR5*) (Kolaczkowska et al., 2008).

Also, aneuploidy might play a role in azole resistance. A duplicated arm of the chromosome 5 of *C. albicans* clinical isolates was discovered to contain duplicates of the transcription factor gene *CaTAC1*, which regulates *CaCDR1* and *CaCDR2* (Selmecki et al., 2006). Transcriptional control mechanisms of the genus *Candida*, seem to be similar to those of *S. cerevisiae*. Finally, one other gene, *CAP1*, similar to *S. cerevisiae*'s *YAP1*, was expressed in the latter and it was shown that it could activate the transcription of *FLR1*, which has functional similarity to the *C. albicans MDR1* gene (Alarco et al., 1997).

Solutions to efflux-mediated resistance are currently under investigation. Proposed solutions so far comprise 1) the use of antifungals that are not substrates of efflux pumps, 2) the development of systems that prevent efflux, 3) removal of the energy required by these pumps to function, and 4) attempt to shift the balance between antifungal uptake and efflux. Studies in *S. cerevisiae* have been carried out to identify pump inhibitors, as these possibly chemosensitize the cells to the antifungals (Cannon *et al*., 2009). Besides solving efflux-mediated resistance, other approaches under discussion, such as the development of stronger azole molecules and the discovery of new antifungal classes. Still, resistance will not disappear and more strains will evolve resistance more rapidly than expected. Research and drug use have to be directed in a way that channel resistance to a less harmful direction. The first attempt to channeling, currently in use, is combination therapy (two different antifungal drugs used simultaneously or in succession). Also, the development of new research tools (e.g. microarrays) can increase the knowledge available so far, providing more rapidly the information needed to find innovative solutions.

Materials and Methods

1. Strains and growth conditions

Mistranslating strains used in this work were constructed previously in our laboratory and are based on the *S. cerevisiae* BY4743 (MATa/αhis3Δ1/his3Δ1, leu2Δ0/leu2Δ0, LYS2/lys2Δ0, met15Δ0/MET15, ura3Δ0/ura3Δ0) strain. The control strain used has a single-copy plasmid, pRS315, while the mistranslating strains carry the same plasmid with a mutated $tRNA_{UGA}^{Ser}$ inserted between the BamHI and SalI restriction sites. The $tRNA_{UGA}^{Ser}$ anticodon was mutated by site-directed mutagenesis, resulting in several other anticodons (Table 1) (Mateus, 2011; Paulo, J. unpublished). Unlike the case of most aaRS, the SerRS recognition mechanism does not depend on the anticodons sequence, so acylation is not blocked.

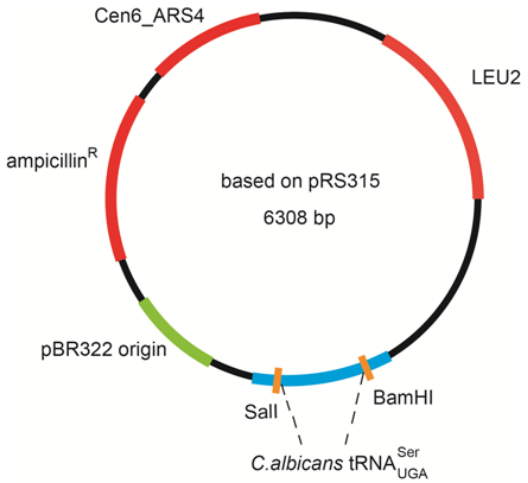

Figure 14 - Single copy plasmid used for the expression of *C. albicans* $tRNA_{UGA}^{Ser}$ in *S. cerevisiae* BY4743. The UGA serine anticodon was mutated by site-directed mutagenesis to several other anticodons (see table 1)

Table 1 – List of plasmids carried by strains used in this work.

plasmid ID	Description
pUA262	pRS315 with Ser-$tRNA_{CAG}^{Leu}$
pUA263	pRS315 with Ser-$tRNA_{GUA}^{Tyr}$
pUA265	pRS315 with Ser-$tRNA_{CGU}^{Thr}$
pUA266	pRS315 with Ser-$tRNA_{CAC}^{Val}$
pUA268	pRS315 with Ser-$tRNA_{UGC}^{Ala}$
pUA269	pRS315 with Ser-$tRNA_{UCC}^{Gly}$
pUA809	pRS315 with Ser-$tRNA_{CAU}^{Met}$
pUA810	pRS315 with Ser-$tRNA_{AGG}^{Pro}$

All strains were grown at 30^{o}C in minimal medium without leucine (MM-Leu) (0.67% yeast nitrogen base w/o amino acids, 0.2% amino acid drop out mix w/o leucine, 2% agar, 2% glucose (all from Formedium)).

Stressor agents were added to the medium when required. Miconazole, itraconazole, amphotericin and cycloheximide (all from Sigma) were dissolved in DMSO (Sigma). Geneticin (Formedium) and caspofungin (Merck) were dissolved in Milli-Q water.

2. Growth Curves

Cell cultures of mistranslating strains and the control were grown at 30^{o}C until they reached a late stationary phase and re-inoculated into 10mL of selective media with a starting OD_{595} of 0.100. At various time points aliquots of the cultures were harvested and OD_{595} was measured. The growth rates of each mistranslating strain were calculated relative to the control by using exponential phase values. Growth rates were compared with a one-way ANOVA coupled with Dunnett's multiple comparison test, with a 95% CI relative to pRS315, using GraphPad Prism software (GraphPad, Inc.).

3. Antifungal susceptibility

In vitro susceptibility was determined according to the guidelines of the EUCAST[4] method for the determination of broth dilution minimum inhibitory concentrations (MICs) of antifungal agents (Rodriguez-Tudela et al., 2008). Briefly, for the inoculum, strains were grown on MM plates. 3 to 5 colonies were resuspended in PBS 1x and diluted to an OD_{530} of 0.15. Sterile 96-well plates containing a two-fold dilution series with twice the final concentrations of miconazole or fluconazole were inoculated with 100µL of cell suspension (3-4replicas) or 100µL of PBS 1x (negative control). The final concentration of the antifungal ranged from 0.125 to 64mg/L. After 24h and 48h of incubation at 30ºC, the most suitable MIC values were determined (OD_{595}).

For all other antifungal susceptibility tests, the Etest® assay for MIC determination was utilized according to manufacturer's instructions (Biomerieux). All strains were tested for amphotericin B, caspofungin, itraconazole and flucytosine (except for proline- and methionine-to-serine strains). Therefore cell suspensions were grown in liquid MM-Leu medium until exponential phase and diluted to an optical density of 0.05. 150µL of cell suspension was inoculated onto selective agar plates and spread evenly with glass beads. Plates were incubated at 30^{o}C. MIC endpoints were determined after 24h, 48h and 72h of incubation. A minimum of three independent tests was performed.

[4] European Committee on Antimicrobial Susceptibility Testing

4. Evolution of antifungal drug resistance

Cells grown until exponential phase were inoculated into 15mL Falcon tubes containing liquid selective media and a miconazole concentration of 0.5µg/mL. Once a cell suspension reached the exponential phase it was changed to two new Falcon tubes, one containing twice the last used concentration of miconazole and the other containing the last used concentration as a backup. The experiment was carried on until the cells stopped growing.

5. Development of translational-caused stress along with generation increment

Cells were inoculated into 100mL Erlenmeyer flasks containing 20mL of MM-Leu and ampicillin (100µg/mL, Sigma). As cultures reached the stationary phase they were changed to new media. MIC endpoints for the antifungals caspofungin and amphotericin were determined at the end of the experiment using the Etest® assay.

6. Phenomics of mistranslating strains

This experiment was based on the methods described by Homann (2009) and Kvitek (2008). BY4743 mistranslating strains were grown until exponential phase and $1x10^7$ cells were collected and ressuspended in 1mL of PBS 1x. From this initial dilution, a series of 5 dilutions were made and transferred to 96-well plates. Six ten-fold serial dilutions were plated in MM-Leu agar plates supplemented with the following stressors:

stress compound	concentration (µg/mL)
amphotericin B	0,5
caspofungin	0,05
itraconazole	0,25
miconazole	0,05
cycloheximide	0,06
geneticin	75

Cell suspensions were inoculated using a Sciclone liquid handling workstation and plates were incubated at 30°C. After 3 and 4 days photos were taken (Quantity One® software, Biorad) and areas of colonies were measured using Image J software (Abramoff, 2004). Due to the half time of antifungals, only data obtained at day 3 were considered for further analysis. Day 4 measurements were used in the case of the xenobiotic cycloheximide, as colonies sizes were too small to score after 3 days. The growth rates of each strain were calculated by dividing the average area of the triplicates of each strain exposed to the stressor by the average area of the triplicates of each strain grown in MM-Leu only. This ratio was calculated for all measurable dilutions. Statistics were made using GraphPad Prism software (GraphPad, Inc.).

7. Microarray analysis

Images of the microarray hybridizations were previously acquired using the Agilent G2565AA microarray scanner (Agilent). Fluorescence intensities were quantified with Quantarray v3.0 software (PerkinElmer). Using R2.2.1 limma software (Smyth and Speed, 2003) and BRB-ArrayTools v3.4.0 (developed by Dr. Richard Simon and BRB-ArrayTools Development Team), $\log_2$ intensity ratios were median normalized, to correct for differences in genomic labeling efficiency between samples. One-class (to compare each mistranslating strain with the control) and multiclass (for comparison between mistranslating strains) Significance Analysis for Microarrays (SAM), with a false discovery rate (FDR) <0.001, were performed using MultiExperiment Viewer (MeV) 4.7.4 software (Saeed et al., 2003). Individual hybridizations of two clones with two dye-swaps were used as the input data for each strain.

Results

1. Growth curves

Growth curves were performed in order to understand how strains are affected by the toxicity caused by the introduction of the mutated tRNAs. Apart from the strain misincorporating serine at alanine codons, the results revealed that the growth rate of the mistranslating strains is slightly affected even though there were no significant differences in growth rate. Misincorporation of serine at glycine and leucine codons led to lowest growth rates.

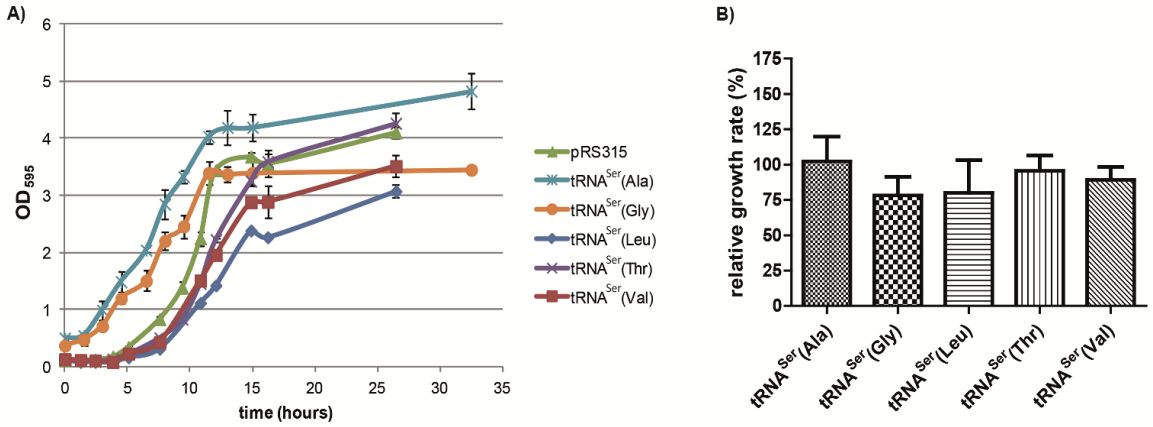

Figure 15 – Growth curves (A) and specific growth rates (B) of yeast cells transformed with pRS315 only (control) and with pRS315 carrying the mistranslating serine tRNAs. Data represent the mean ± SD of duplicates of three independent experiments.

2. Evolution of the mistranslating strains

2.1. Antifungal susceptibility – broth dilution test

A preliminary antifungal susceptibility test using the broth dilution method was performed with fluconazole (FLZ) to verify if this antifungal was suitable for an evolution experiment. As expected, the control strain BY4743 transformed with pRS315 tolerates the same concentration of FLZ as the wild-type (WT) BY4743. The mistranslating strains all showed the same inhibitory concentration (16mg/L). According to NCCLS guidelines, a MIC<16mg/L is considered as susceptible and a MIC>64mg/L is regarded as resistant to FLZ (NCCLS, 2002). Thus, this azole in particular is well tolerated by many fungal strains. For instance, according to EUCAST data, a small part of *Candida* strains tested grows up to 256 mg/L of FLZ[5]. Although our mistranslating strains fit in the NCCLS dose dependent

[5] http://www.eucast.org/mic_distributions/

category, they tolerate relatively high concentrations of FLZ. Thus, this antifungal was discarded as a potential drug for an evolution experiment.

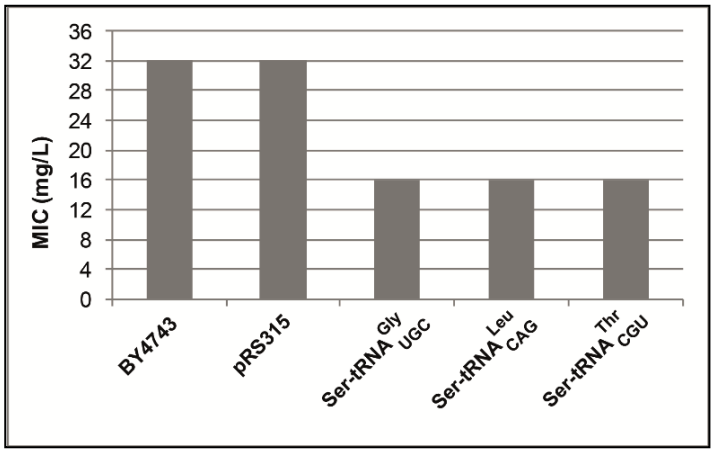

Figure 16 - 24h MIC_{90} for fluconazole determined by broth microdilution (EUCAST methodology).

Hence, a further commonly used antifungal, the imidazole miconazole, was tested and two more mutant strains were included which misincorporate serine at tyrosine and alanine codons. It was very effective against our strains. All of them were inhibited at a concentration of 0.5mg/L at 24h. For its high inhibition ability for most of the tested strains miconazole was chosen as a good candidate for an evolution experiment.

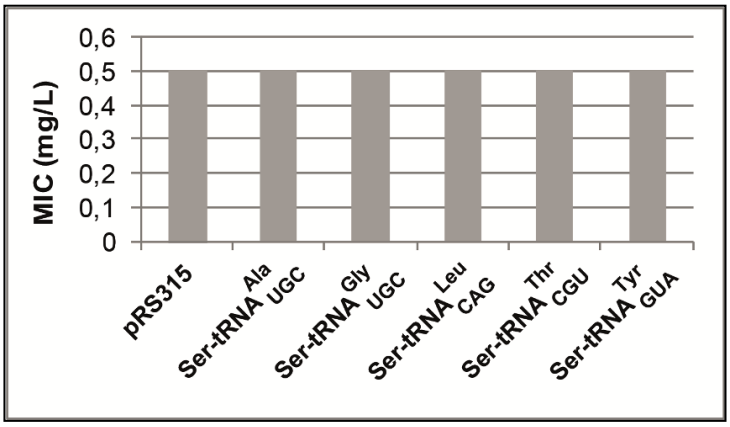

Figure 17 - 24h MIC_{90} for miconazole determined by broth microdilution (EUCAST methodology).

2.2. Evolution of antifungal drug resistance

As mentioned before, the *S. cerevisiae* strains used in this work have an increased frequency of misincorporation. Ongoing work in our laboratory led to the assumption that codon mistranslation, due to codon ambiguity, accelerates antifungal drug resistance in *C. albicans*. Here, we tried to force *S. cerevisiae* to evolve azole resistance, by exposing the strains to increasing concentrations of miconazole. All strains stopped growing at concentrations higher than 8mg/mL but they reached this concentration limit at different time points.

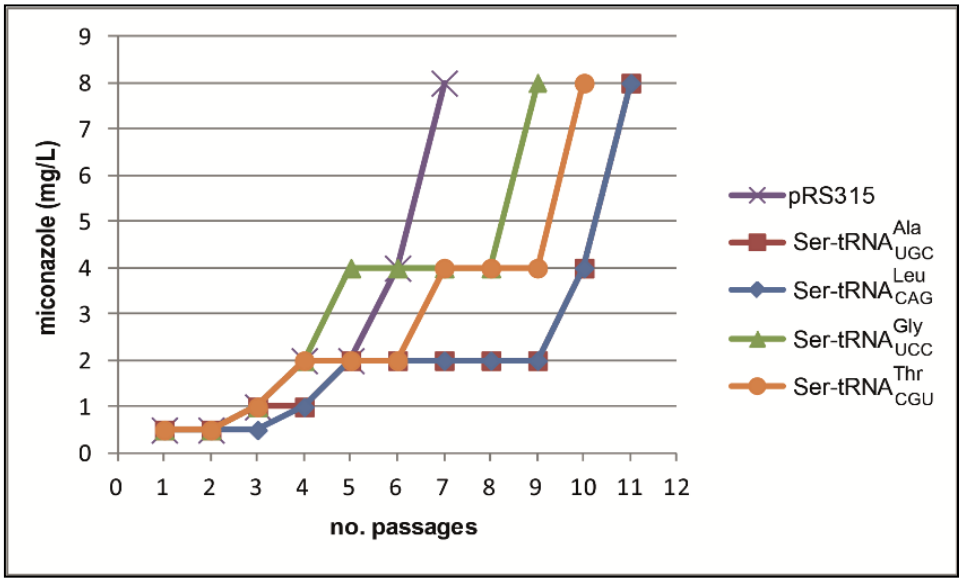

Figure 18 – Evolution of the mistranslating *S. cerevisiae* BY4743 strains exposed to increasing concentrations of miconazole.

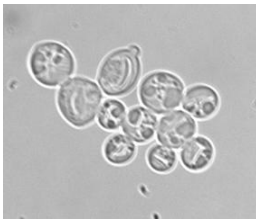

Figure 19 - Strain misincorporating serine at alanine codons showing irregular intracellular structures, in 8µg/mL of MCZ (63x optical magnification). This picture is illustrative for all strains.

3. Antifungal susceptibility – Etest® assay

We tested eight mistranslating strains plus the control for four different classes of antifungals (caspofungin, amphotericin B, itraconazole and flucytosine). The manufacturer's *interpretation guide* for *C. albicans* MIC endpoints was used as there are no indications available for *S. cerevisiae*.

Caspofungin (CS) seemed to be a quite effective antifungal as none of the tested strains grew in concentrations higher than 0.25µg/mL. Amphotericin B (AP) inhibited the strains to a maximum concentration of 1.5µg/mL. In both tests the strains misincorporating serine at leucine and valine codons showed the weakest performance. The strain misincorporating serine at tyrosine codons had a similar performance to the control, when exposed to AP. All the other mistranslating strains were in general more resistant to AP and more susceptible to CS than the control.

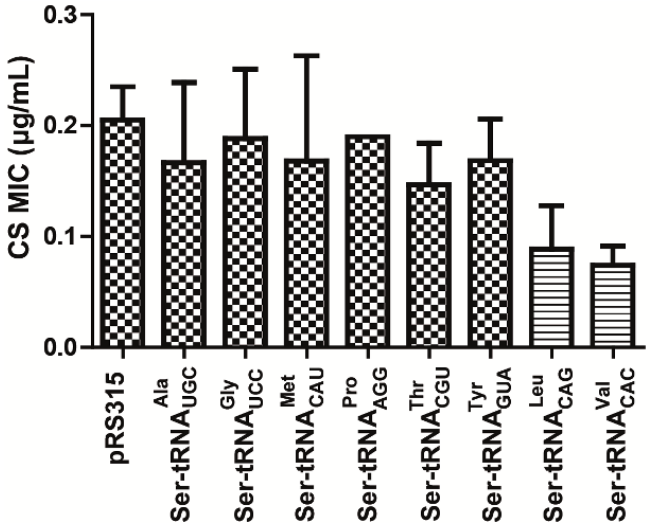

Figure 20 - Caspofungin 48h MIC_{80} endpoints of the mistranslating strains and control, determined with the Etest assay. Strains misincorporating serine at leucine and valine codons did not grow sufficiently to determine the MICs at 48h and therefore 72h MICs are presented.

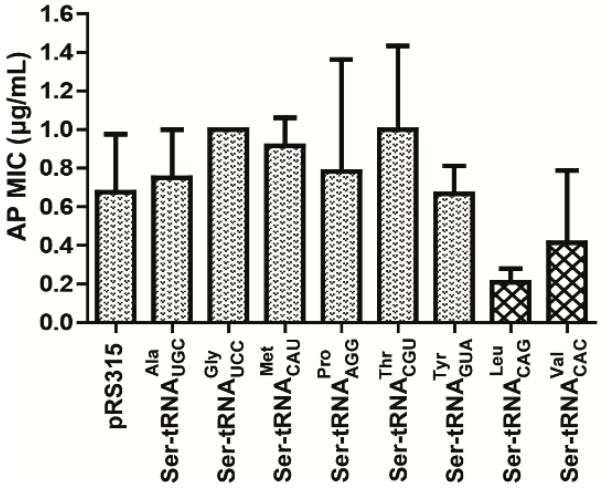

Figure 21 – Amphotericin B 48h MIC_{100} endpoints of the mistranslating strains and control, determined with the Etest assay. Strains misincorporating serine at leucine and valine codons did not grow sufficiently to determine the MICs at 48h and therefore 72h MICs are presented.

Itraconazole Etests were performed only once and not repeated as strains have shown high resistance (figure 22). It is important to refer that ITZ Etest strips range from 0.002 to 32µg/mL. Performance differences between the strains were still determined: the control strain and the strains misincorporating serine at threonine and glycine codons were inhibited at 16µg/mL, while the strains misincorporating serine at methionine, proline and valine codons were inhibited at 24µg/mL; the strains misincorporating serine at leucine and alanine codons had the best performance (32µg/mL) and the one misincorporating at tyrosine codons has the weakest (12µg/mL) (figures 22 and 23).

The strains showed no inhibition towards flucytosine (FC) exposure except for the one misincorporating serine at leucine codons (fig 24). For this reason FC Etests were not repeated.

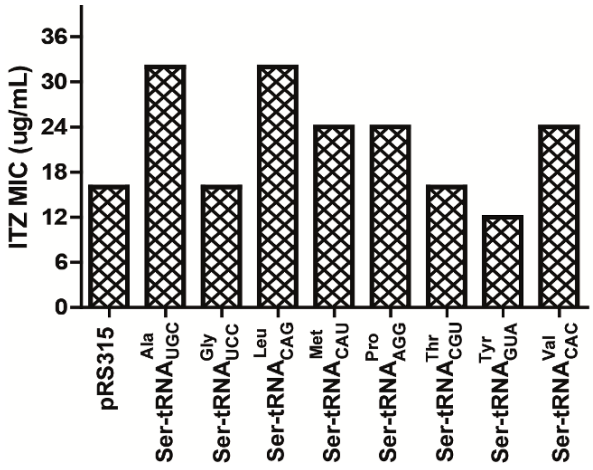

Figure 22 - Itraconazole 48h MIC_{80} endpoints of the mistranslating strains and control, determined with the Etest assay

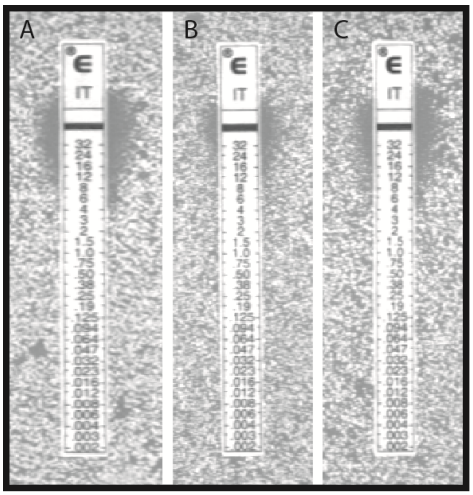

Figure 23 –Examples of the itraconazole Etest assay presented in figure 18: control (A) and the strains misincorporating serine at alanine and glycine codons (B and C respectively).

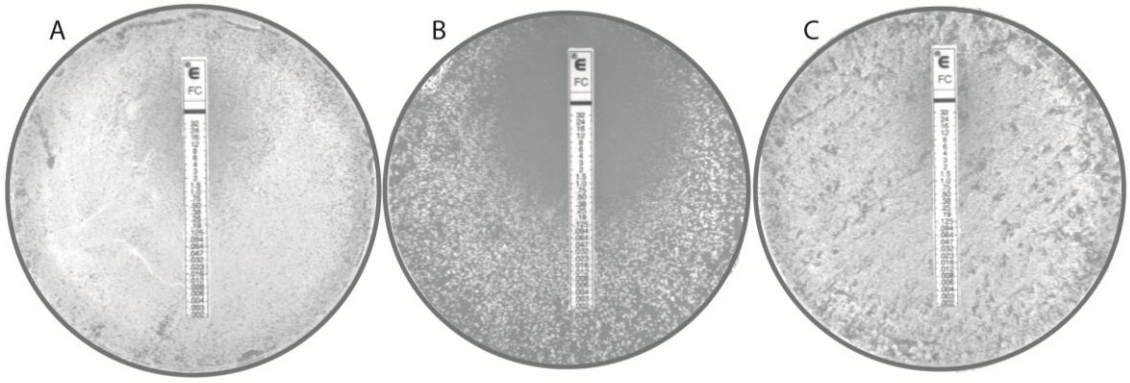

Figure 24 – Examples of 5-flucytosine Etest assay: Control strain (A) and strains misincorporating serine at leucine and alanine codons after 48h incubation period (B and C, respectively).

4. Development of translational-caused stress along with generation increment

Here we intended to understand if the accumulation of errors due to induced codon ambiguity could be *per se* a major stressor to change the antifungal resistance of the tested strains. There were small differences regarding antifungal resistance between strains in generation “zero” and after 230 generations for the two antifungals tested (figure 25). Cell morphology did not seem to be altered (figure 26).

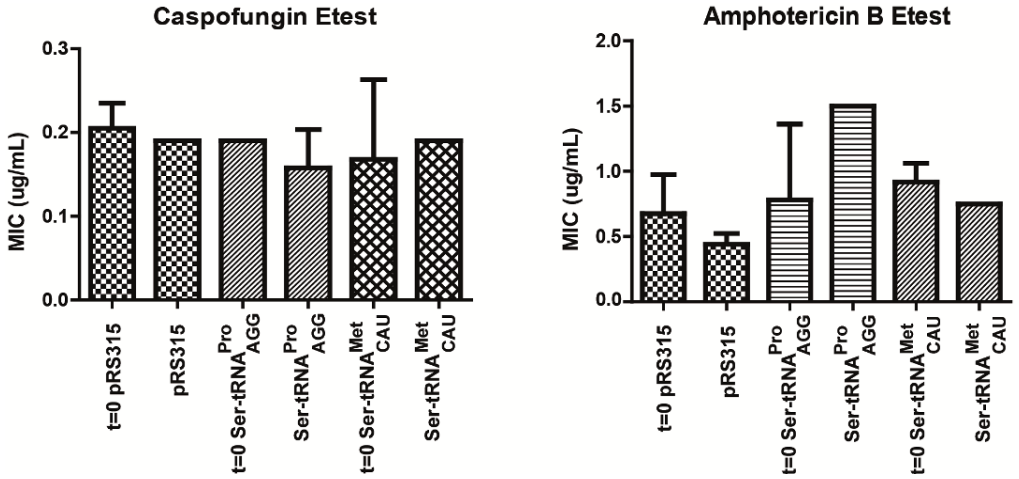

Figure 25 – Comparison of 48h MICs of strains at the beginning of the experiment (t=0) and strains grown until the 230 generation approximately, determined by the Etest for caspofungin (MIC_{80}) and amphotericin B (MIC_{100}).

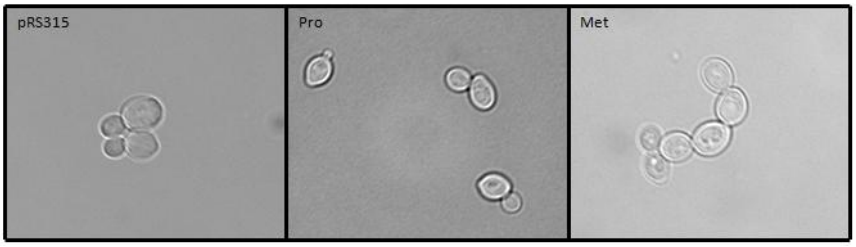

Figure 26 – *S. cerevisiae* strains – control, strain misincorporating serine at proline codons and at methionine codons respectively - morphology after approximately 230 generations (63x optical magnification)**.** These pictures are illustrative of what was visualized for all of the strains.

5. Phenomics of mistranslating yeasts

Induced mistranslation is expected to trigger a stress response that may have more severe effects in the cells carrying the most toxic mutated tRNAs. Confirmation of its degenerative character in these strains was previously done in the laboratory. However, it was also shown that some of these strains have a selective advantage under certain environmental conditions (e.g. copper sulphate) (Mateus, 2011). To verify whether mistranslation induces a selective advantage towards exposure to different antifungals and translation inhibitors we performed a phenotypic screening. From the available set of antifungals, we could test two from the azole family (miconazole and itraconazole), one echinocandin (caspofungin) and one polyene (amphotericin). Additionally, geneticin and cycloheximide, two translation inhibitors, were tested (figure 27).

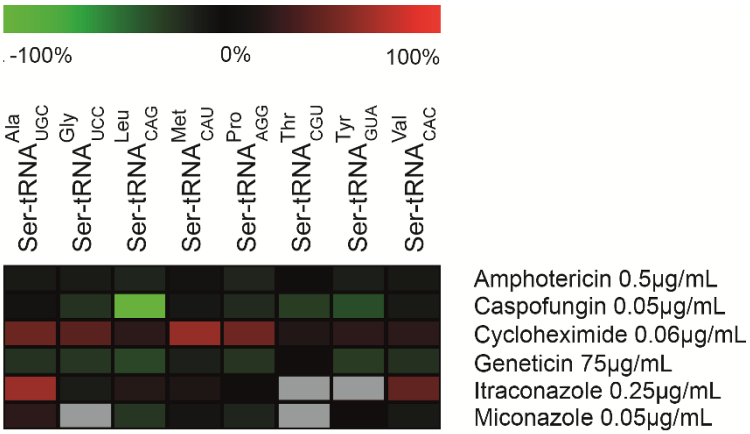

Figure 27 – Growth performance of yeast cells transformed with pRS315 containing the mutant $tRNA^{Ser}$ under 6 stress conditions. Growth is represented as advantageous (red), disadvantageous (green) or neutral (black) compared to growth of the control strain pRS315. Grey squares represent eliminated values.

The results obtained show that mistranslating strains cope better the exposure to cycloheximide than the control strain. Cycloheximide is a powerful translation inhibitor and the growth rate of all strains was affected when compared to the growth in media without the stressor (figure 28). The strain misincorporating serine at methionine codons had the best relative growth rate (+64%), followed by strains misincorporating at proline and alanine codons (about +50%).

Geneticin, another translation inhibitor, was less effective. It did not affect mistranslating strains' growth as much as cycloheximide (figure 28) but when compared to the control strain (figure 27), their performance decreased. Misincorporation of serine at leucine codons was the most disadvantageous (-30%); at threonine codons growth was similar to the control (+1%); and the only advantageous strain was the one misincorporating serine at proline codons (+22%).

Regarding caspofungin resistance the results revealed that the strain misincorporating serine at leucine codons did not grow at all, while the strain misincorporating serine at valine codons grew similarly to the control (only -8% of growth). The strains misincorporating serine at tyrosine (-33%) and threonine (-27%) codons are the most affected, followed by the one misincorporating serine at glycine codons (-21%).

The tested amphotericin concentration of 0.5µg/mL did not seem to highly affect cells' growth (figure 28). Therefore, 1 µg/mL of this compound was also tested during this experiment and none of the strains grew during the first three days. After 5 days colonies were still not suitable for ImageJ analysis. These results did only partly match the Etest assay results.

The response to both azoles was quite different. Mistranslating cells tolerated itraconazole better than the control while miconazole seemed to affect them more. The strain misincorporating serine at alanine codons showed better growth than the control when exposed to both azoles, but it was quite obvious in the case of

ITZ (+66%). The strain misincorporating serine at valine codons showed the second best performance (+44%). However, the leucine-to-serine strain presented half the growth rate (+20%) of the valine-to-serine one. There was also a difference between them in MCZ supplemented media but it was more obvious in the case of ITZ.

Some strains have not grown measurable colonies beside for the initial dilution, which left us with a poor set of values and hence, increase erroneous interpretation. This concerned the strains misincorporating serine at glycine and threonine codons for the antifungal MCZ and serine at tyrosine and threonine codons for itraconazole. For the reasons mentioned these results were not taken into account.

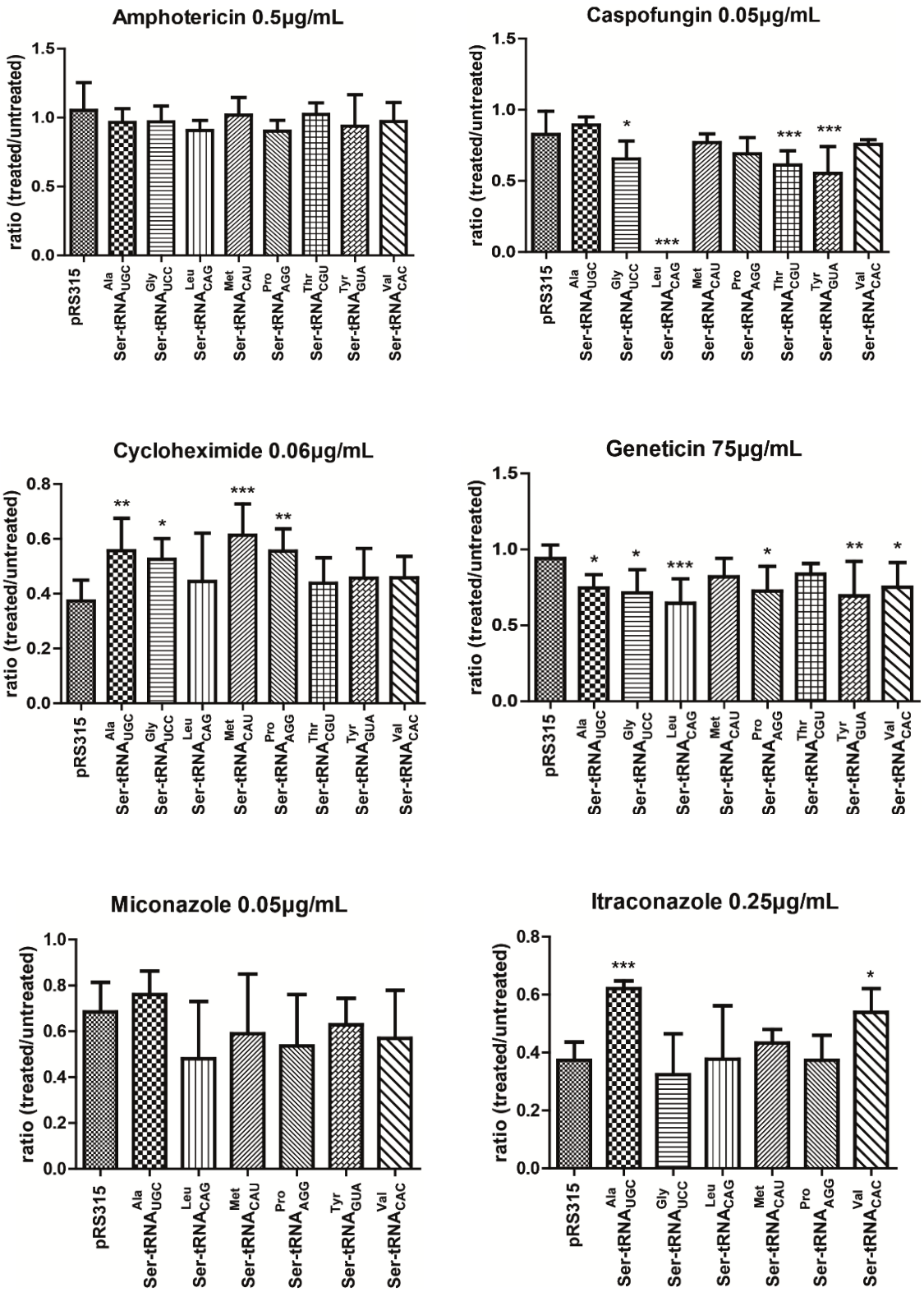

Figure 28 – Relative growth of yeast cells transformed with mutant $tRNA^{Ser}$. Data represent the mean ± SD of triplicates of three independently grown replicates (***p<0,0001. One-way ANOVA coupled with Dunnett's multiple comparison post test, with a 95% CI relative to pRS315).

6. Microarrays

As an attempt to explain the results of the phenomics study in molecular terms, microarray data obtained previously in our laboratory were analyzed. This analysis was meant to find a possible predisposition of the mistranslating strains regarding resistance to antifungals and translation inhibitors. Unfortunately, no microarray data was available for the strains misincorporating serine at proline and methionine codons.

Table 2 – Total number of significant differentially expressed genes by the mistranslating strains compared to the control strain, determined by one-class SAM analysis (FDR≤0.0001).

strain	down-regulated	up-regulated	total
Ser-tRNA$_{CAG}^{Leu}$	18	92	110
Ser-tRNA$_{GUA}^{Tyr}$	29	187	216
Ser-tRNA$_{CGU}^{Thr}$	3	25	28
Ser-tRNA$_{CAC}^{Val}$	32	136	168
Ser-tRNA$_{UGC}^{Ala}$	106	34	140
Ser-tRNA$_{UCC}^{Gly}$	4	110	114

Table 3 - Number of genes related to amino acid biosynthesis (previously described by Singh et al., 1979) that were significantly differentially expressed by the mistranslating strains compared to the control strain, determined by one-class SAM analysis (FDR≤0.0001).

strain	down-regulated	up-regulated	amino acids
Ser-tRNA$_{CAG}^{Leu}$	0	10	Glu, Lys, Met
Ser-tRNA$_{GUA}^{Tyr}$	3	17	Glu, Lys, Met, Ser, Gly
Ser-tRNA$_{CGU}^{Thr}$	0	2	Lys
Ser-tRNA$_{CAC}^{Val}$	0	11	Glu, Lys, Met
Ser-tRNA$_{UGC}^{Ala}$	3	2	Glu, Lys, Ser, Gly
Ser-tRNA$_{UCC}^{Gly}$	0	4	Glu, Lys, Ser

Our SAM (Significance Analysis for Microarrays) analysis revealed that, when compared to the control, the mistranslating strains up-/down-regulate more genes that have been previously connected to amphotericin B response (55 genes) than to any other antifungal used in our tests. Further 25 genes related to azole response and 36 genes related to CS response were differentially expressed by the mistranslating strains (figure 29).

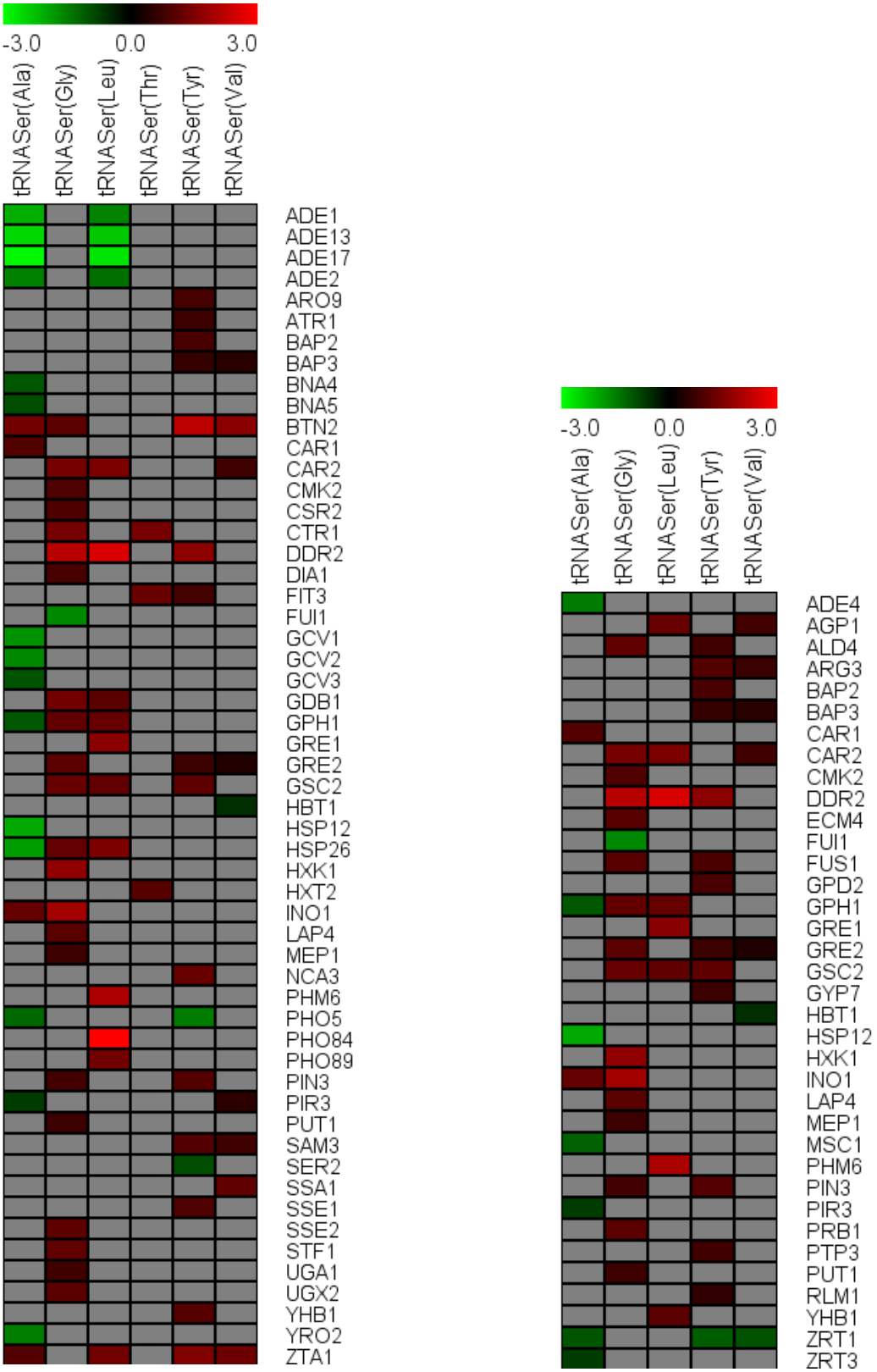

Figure 29 – One-class SAM analysis – selected genes previously related to *S. cerevisiae*'s response towards amphotericin B (left) and caspofungin (right).

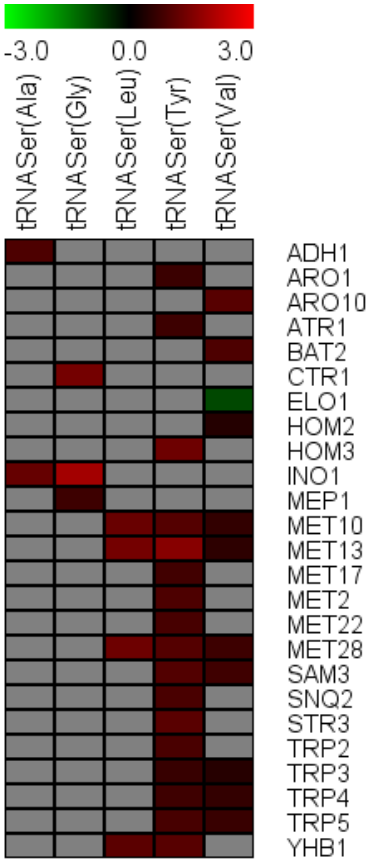

Figure 30 – One-class SAM analysis – genes previously related to *S. cerevisiae*'s response towards azoles

The multiclass Significance Analysis for Microarrays (SAM) revealed a total of 182 differentially expressed genes. A list of these genes is given in the *annex*.

Discussion

1. Antifungal susceptibility – Etest® assay

The mistranslating strains showed MIC endpoints (80% inhibition) that did not surpass 0.3µg/mL of caspofungin (CS), revealing that this antifungal is very effective. Mistranslation did not seem to alter the strains susceptibility as the mutant strains showed MICs similar to the control. Echinocandins were previously shown to be very effective, even fungicidal, against most common *Candida* spp, presenting lower MICs than those described for amphotericin B (AP). On the other hand, CS was shown to be mainly fungistatic against *Aspergillus* spp. (Denning, 2003). A previous study on clinical isolates of *S. cerevisiae* using the Etest assay revealed a 50% inhibition in the range of 0.25 to 0.5µg/mL of CS (48h) (Chryssanthou and Cuenca-Estrella, 2002). Our laboratory strains do not greatly differ from these values.

The MIC endpoints (100% inhibition) obtained for the AP Etest assay showed more interesting results. A slight advantage towards AP exposure was observed for the strains misincorporating serine at glycine, methionine and threonine codons. Threonine is chemically and structurally similar to serine while glycine is a special amino acid, as it can fit in hydrophobic and hydrophilic parts of a protein. The introduction of serine into a hydrophobic part of a protein could have severe consequences, such as, e.g., loss of function due to incorrect folding. In principle, a decrease in antifungal resistance would be expected, as the cell fitness also decreases (Mateus, 2011). Also, the result obtained for the strain mistranslating methionine was surprising. *A priori* a methionine substitution by serine should have severe consequences due to their chemical and structural differences. Methionine is hydrophobic and contains a sulphur atom, which confers stability to the protein and helps it to fold due its ability to form disulfide bonds. Other studies using the NCCLS or the EUCAST methodologies showed MIC endpoints (90% inhibition) in the range of 0.5 to 1µg/mL (Lass-Florl et al., 2008; Munoz et al., 2005; Pfaller et al., 1997; Swine et al., 2004; Thompson et al., 2005).

Moreover, it was also interesting to see that three of the mistranslating strains had AP MIC endpoints similar to the control. These were the strains mistranslating serine at alanine, proline and tyrosine codons. Again, if the chemical and structural differences of serine and these three amino acids were solely taken into account, a decrease in their performance would be expected. Further investigation should be done in order to clarify such results.

The MIC endpoints of the strains misincorporating serine at leucine and valine codons were assessed 24h later (72h), for both CS and AP, as the strains were not sufficiently grown for an accurate determination after 48h. Both strains showed lower MIC endpoints than the other mistranslating strains and the control. Such results might be due to the fact that the strains seem to be dealing with a growth delay, and are less fit to cope with the antifungals. Furthermore, it was previously shown in our laboratory that mistranslation, in different ways, affects these two strains more than the other strains studied here (Mateus, 2011).

The MIC endpoints (80% inhibition) determined for itraconazole (ITZ) were very high (12-32µg/mL) for the mistranslating strains and the control strain (figure 18). As mentioned before, these strips range from 0.002 to 32µg/mL. Because of

this range, the resulting ellipses presented similar small areas (figure 19). The Etest guidelines include interpretive criteria for the various antifungals tested. However, it concerns uniquely Candida strains. For instance, for ITZ a strain is considered susceptible dose dependent (SDD) if the MIC is in the range of 0.25 to 0.5 µg/mL. According to NCCLS official breakpoints, SDD strains present MIC_{50} in the range of 0.25 to 0.5µg/mL. Above and below these values, strains are considered as being resistant and susceptible, respectively. MIC endpoints such as the ones obtained were not in accordance with most published MICs (50 to 90% inhibition, determined with NCCLS and EUCAST methodologies) obtained for clinical isolates of *S. cerevisiae* (Espinel-Ingroff, 1998; Lass-Florl et al., 2008; Munoz et al, 2005; Permán J., 2006; Pfaller et al., 1997; Swine et al., 2004; Zerva et al., 1996). It is important to stress that an agar diffusion test was used in this work while the NCCLS and EUCAST methodologies use liquid media. Cuenca-Estrella et al. (2005) compared the EUCAST methodology with several commercial systems using *Candida* isolates and an overall 90% agreement was observed for Etest (amphotericin B, flucytosine and several azoles).

Regarding 5-flucytosine (FC) Etest results we observed that all of the strains exhibited resistance but for leucine. MIC endpoints obtained previously for *S. cerevisiae* strains using different methods seem to be much lower (Berenguer et al., 1995; Espinel-Ingroff et al., 1991; Pfaller et al., 1995; Pfaller et al., 1997; Thompson et al., 2009). To our knowledge, there is at least one case in which a laboratory strain, the haploid BY4741, was shown to be resistant to FC, presenting a MIC_{95} of 100µg/mL (microdilution broth, 48h) (Markovich et al., 2004). BY4741 is a haploid strain that is very similar to the diploid strain, BY4743, used in this work. Both strains share the same deletions except for the gene LYS2 that is only deleted in the BY4743 strain. Resistance to 5-FC can be mediated by two basic mechanisms (Vermes et al., 2000): (i) certain mutations can result in a deficiency in the enzymes necessary for cellular transport and uptake of 5-FC or for its metabolism (i.e. cytosine permease (FCY2), uridine monophosphate pyrophosphorylase (FUR1) or cytosine deaminase (FCY1)); (ii) resistance may result from increased synthesis of pyrimidines, which compete with the fluorinated antimetabolites of 5-FC and thus diminish its antimycotic activity. The multiclass Significance Analysis for Microarrays (SAM) showed that FUI1 and URA1 genes are differentially expressed in mistranslating strains. The FUI1 gene codes for a high affinity uridine permease, localized to the plasma membrane and null mutants have showed increased resistance to FU (Jund and Lacroute, 1970). Up-regulation of FUI1 in the strain misincoporating serine at alanine codons might contribute to the uptake of uridine and balance the ratio U/FU. The URA1 gene codes for a dihydroorotate dehydrogenase which catalyzes a key reaction in the superpathway of histidine, purine, and pyrimidine biosynthesis. This reaction takes place immediately before the branching of the pathway, which can prevent or increase the formation of uridine-triphosphate (UTP). The strain misincorporating serine at alanine, tyrosine and valine codons showed up-regulation of this gene. Curiously, a point mutation that replaced serine by leucine in *C. dubliniensis* was suggested to cause FC resistance, by disrupting the quaternary structure of the enzyme, distorting the active site (McManus et al., 2009). We verified that *S. cerevisiae*'s FCY1 gene contains only one CUG codon (leucine), the FUR1 gene contains two CUG codons and FCY2 contains nine CUG codons. Thus there is the

possibility that one of these genes' products is affected. A previous study that tested a set of 39 yeast proteins of the SWISS-PROT database, has shown that leucine is the most abundant amino acid, appearing in these proteins with a frequency of almost 10%. Valine, for instance, appears with a frequency around 6% only (Pratt et al., 2002). Microarray analysis of the mistranslating strains exposed to FC could shed light on this matter. Previous microarray studies using *S. cerevisiae* have already shown that FC triggers differential regulation of genes involved in several different functions (e.g. amino acid metabolism, cell cycle, protein degradation) (Agarwal et al., 2003).

2. Evolution of antifungal drug resistance

The forced evolution experiment revealed that mistranslating strains were as resistant to the antifungal miconazole (MCZ) as the control was and no visible differences in cell aggregation were qualitatively observed using the microscope. Although the initially determined MICs were quite low and the experiment ended with a final concentration of 8μg/mL, it is known that laboratory strains such as BY can grow in 10μg/mL MCZ supplemented media and others reach similar concentrations (Francois et al., 2009; Parker et al., 2008; Portillo and Gancedo, 1984; Thevissen et al., 2007). Azoles are mainly fungistatic, high MIC variations are expected when grown in different media and volume, presence or absence of oxygen and even when temporal independent experiences are performed (Agarwal et al., 2003; Sud and Feingold, 1981). As the final limiting MCZ concentration of our evolution experiment (8μg/mL) was similar to other published inhibitory concentrations for this specific antifungal in baker's yeasts we assumed that our mutant strains are as resistant to MCZ as wild type strains and mistranslation did not increase MCZ resistance in the course of the experiment. It was interesting to observe that the strains misincorporating serine at leucine and alanine codons needed more time to reach 8μg/mL, while the strains misincorporating at glycine and threonine codons reached the same concentration quicker. Such observation might be due to the fact that both alanine and leucine amino acids are non polar, while threonine is polar like serine and glycine is a special case, as it has no side chain.

The observations on the microscope at the final steps of this experiment showed structures that might be vacuoles. Vacuoles are common in every growth stage of a yeast cell but their morphology does not remain unchanged along the growth process. Azoles cause a decrease in cell wall integrity, by impairing ergosterol biosynthesis. As a consequence, ion leakage increases leading to osmotic stress, which in turn causes the formation of small, fragmented vacuoles. It has been suggested that such fragmentation may increase the efficiency of ion uptake by vacuolar transporters (higher contact area) (Li and Kane, 2009). Also, vacuoles play an important role in detoxification, thus the cells might try to sequester MCZ inside the vacuoles.

3. Development of translational-caused stress along with generation increment

This long term experiment was carried out for approximately 230 generations in order to study the effects of mistranslation on the genome over time. It did not lead to neither growth nor cell morphology differences. Only slight differences in resistance to AP of the strain misincorporating serine at proline codons were observed. High resistance was not expected as cells are not under additional antifungal-caused stress. The main goal of this experiment is to do whole genome sequencing (WGS) to verify if induced mistranslation indeed augments DNA mutation rate over time. As mentioned before, mistranslation can affect DNA synthesis, repair and recombination proteins. At a certain point there is the possibility that cells produce a significant amount of mal-functioning (or unfolded) proteins. If a DNA polymerase subunit is affected (e.g. production of error-prone holoenzyme), like shown before in bacteria (Al Mamun et al., 2002), it could introduce more mutations in the DNA. The more DNA mutations, the higher the possibility of producing erroneous proteins, and the closer the cell gets to the death threshold. We had no evidence for an advantageous phenotype in the forced evolution experiment, although experiments using streptomycin in bacteria showed that mistranslation in general could induce TSM (Balashov and Humayun, 2002). The WGS will allow us to screen for the expected higher level of point mutations and Indels spread across the genome. Even though theoretically possible, it is worth noting that the introduction of a mutant tRNA such as *glyV*^{Glu} and *alaV*^{Glu}, immediately caused the emergence of a mutator phenotype in bacteria (Dorazi et al., 2002). Nevertheless we can predict some changes in DNA polymerases. For instance, the quantity of proline codons CCU and methionine codons AUG in some polymerases is:

- The sliding clamp for DNA polymerase delta (POL30) has 7 proline codons of which 5 (CCU) can be recognized by the suppressor $tRNA_{AGG}^{Ser}$ and 5 methionine codons (initiator excluded).
- The subunit of DNA polymerase delta (gene POL31), which is involved in DNA replication and repair and is essential for cell viability, contains 28 proline codons of which 7 (CCU) can be recognized by the suppressor $tRNA_{AGG}^{Ser}$ and 9 methionine codons.
- A subunit of DNA polymerase delta (gene POL32) contains 21 proline codons of which 7 (CCU) can be recognized by the suppressor $tRNA_{AGG}^{Ser}$ and 7 methionine codons.

Thus, there is the possibility that amino acid exchange can produce a fair amount of unstable DNA polymerases. If WGS confirms this theory, a logical step would be to study the other DNA polymerases involved only in DNA repair (e.g. zeta subunits). As it is known that mistranslation involves several different events, later on, it could be interesting to check RNA polymerases sequences too.

4. Phenomics study and microarray analysis

In general, there was no visible pattern regarding the antifungal drug response of the mistranslating strains, nor seemed to be similarities for both translation inhibitors tested. Examples are leucine- and valine-to-serine strains, two of the most toxic codon changes, which were expected to have more similar responses to, for instance, itraconazole and caspofungin. Our laboratory showed recently by northern blot analysis and beta-galactosidase assays that the cell tries to shut down the expression of the mistranslating tRNAs and the most affected strains have been these two. Also, these showed higher percentages of cell death, and together with isoleucine-to-serine strain (not used in this work), were the only ones affected by aneuploidy. So, these two strains are always on the spotlight of most of the experiments already made in our lab (Mateus, 2011). It seems that the antifungal response is greatly affected by each specific type of mistranslation.

It was previously shown that CUG ambiguity in *S. cerevisiae* strains expressing the *C. albicans* ser-tRNA$_{CAG}$ induced tolerance to oxidants, high osmolarity (Santos et al., 1999) and temperature shifts (Santos et al., 1996). Some of our mistranslating strains showed a growth advantage in antifungals.

In the phenomics study all of the strains grew in agar plates supplemented with 0.5µg/mL of amphotericin B (AP) and reacted similarly to the control which was not greatly affected by this polyene. Curiously, the growth rate of the strains mistranslating serine at leucine and valine codons was similar to all others.

It was surprising to observe that the strains misincorporating serine at proline and methionine codons were not highly affected by any of the drugs, regarding that both amino acids are hydrophobic and with complex side chains. On the contrary, in some situations they showed a growth advantage (e.g. ITZ Etest). Such observation is in agreement with the Etest results (see above). The study by Pratt and colleagues (2002) further confirmed that methionine and proline are amongst the least abundant amino acids. The fraction of mutant proteins produced may be lower in these two strains, and thus, may have less influence on the cells' fitness. Further investigation might add an explanation to our current knowledge.

The microarray data, revealed that there were no differentially expressed genes related to antifungal targets such as those decoding the ergosterol pathway or ABC and MFS pumps (with two exceptions for the tyrosine-to-serine strain). However, we had the curiosity to use false discovery rates (FDRs) lower than 1% (instead of the considered FDR<0.0001) and noticed that when compared to the control strain (one-class SAM), valine-to-serine misincorporation showed down-regulation of ERG1 and ERG3. Next to ERG11, ERG3 is probably the most important gene for azole resistance, as cells tend to introduce point mutations in order to stop the accumulation of toxic ergosterol intermediates (Cowen, 2008; Cowen and Steinbach, 2008). Thus, this could in part account for its better response towards ITZ and MCZ, when compared to leucine-to-serine strain's response and to the other strains in general.

It was previously shown in our laboratory that mistranslating strains increase the level of ROS production mainly during the exponential phase and the most affected are those misincorporating serine at leucine, isoleucine (not used in this work) and valine codons (Mateus, 2011). An increased level of ROS was

shown to be characteristic of yeast cells exposed to MCZ and its forced reduction decreased MCZ action (Kobayashi et al., 2002). Such ROS levels might have reduced the growth rate of leucine- and valine-to-serine strains under MCZ treatment, even though (to our knowledge) a direct comparison of the effect of both azoles on the intracellular ROS level was not made yet. Still, a comparison between MCZ, CTZ and FLZ, showed that only the first one induces high levels of ROS in *C. albicans*, which can possibly be due to the combined inhibition of peroxidase and catalase observed (Francois et al., 2006).

A curious observation was that the generally highly affected strain misincorporating serine at leucine codons did not seem to be highly affected by ITZ exposure. It grew similarly to the control strain pRS315 in both the phenomics test and the Etest assay. Further investigation could shed light on such startling observation.

The strain misincorporating serine at tyrosine codons was one of the weakest strains under ITZ exposure and grew only in the initial dilution. This result is in accordance with the observed before for the ITZ Etest assay. However, it grew almost similarly to the control under MCZ exposure. Such structurally different amino acids are expected to provoke stronger consequences when exchanged. They are both polar and uncharged but tyrosine's side chain is longer and contains an aromatic ring. Besides structure, some tyrosine residues can be (de-) phosphorylated. Several cell components transmit signals via pathways involving tyrosyl phosphorylation of specific proteins. Such pathways influence survival or death and regulation of phosphorylation is critical for homeostasis (Neel and Tonks, 1997). Impediment of such important events by the insertion of a serine instead of tyrosine could, in principle, reduce the cells' fitness and their ability to deal with antifungals. Interestingly, the microarray analysis showed that this strain had the highest up-regulation of genes related to azole response. Singh and colleagues (Singh et al., 1979) showed that yeast cells with low ergosterol content accumulate a selective set of amino acids (lysine, glycine, glutamic acid, proline, methionine and serine) at a higher rate and level. Also, Thevissen and colleagues (2007) observed that the deletion of several tryptophan genes decreased MCZ resistance in yeast. Interestingly, our strain up regulates several genes related to amino acid biosynthesis, which could possibly indicate a predisposition to cope with azole exposure. In addition, it up regulates ATR1, which codes for a MFS pump, whose overexpression is known to confer aminotriazole resistance (Kanazawa et al., 1988) and SNQ2, which codes for an ABC pump of the PDR family, known to confer multidrug resistance (Ernst et al., 2005). Curiously, the threonine-to-serine strain, which did not tolerate both azoles well, had a low number of up-regulated genes related to amino acid biosynthesis. Additionally, we verified that there was down-regulation of FRE1 gene, a ferric reductase, only in the tyrosine-to-serine strain (see annex). This gene was previously connected to ITZ resistance in *S. cerevisiae* (Barker et al., 2003).

The strain misincorporating serine at glycine codons had, except for cycloheximide, a weaker performance than the control towards the compounds tested. Such observation was expected as these two amino acids differ structurally and chemically: glycine is the tiniest amino acid, without side-chain, while serine is polar and with a small side chain. This means that glycine has the unique ability to fit in small spaces of a folded protein and it can also be found in hydrophilic and

hydrophobic environments. Thus, the substitution of glycine with serine can lead to incorrect folding of proteins.

Interestingly, the strain misincorporating serine at alanine codons copes better than the control with azoles. Alanine is the second tiniest amino acid but carries a non polar side chain, which makes this substitution harder for the cells to deal with, as serine is preferentially located at the outer side of the proteins. Another curious observation was that this strain presented the most down-regulated genes, when compared to the control (table 2). Actually, its genes linked to AP resistance are mostly down-regulated. This down-regulation differs from published results for exposed yeast cells (Agarwal and colleagues (2003), which observed up-regulation of genes such as GPH1, HSP12, HSP26, PHO5, PIR3 and YOR2 after exposing *S. cerevisiae* strains to AP). However, the strain might change gene regulation accordingly if exposed to the antifungal.

Regarding CS the leucine-to-serine strain had the weakest performance as it did not grow at all. Such result is startling and leads one to question the reason why beta-1,3-glucans are so important for this strain. Glycine-, leucine- and tyrosine-to-serine strains present the most up regulated genes previously related to CS response. From the microarray data, the up-regulated gene that immediately draws one's attention to it is GSC2, a catalytic subunit of the 1,3-beta-glucan synthase. The other catalytic subunit is GSC1, is not significantly differentially expressed. This is not surprising as they are thought to act as alternate subunits (Denning, 2003). These two proteins are controlled by Rho1p, which in turn is controlled by Tor2p, a TOR signaling pathway protein (Schmidt et al., 1997). In addition, Rho1p regulates Pkc1p, a MAPK pathway protein, which in turn seems to play a role in GSC1 expression (Gustin et al., 1998; Igual et al., 1996). GSC1 was previously shown to confer specific resistance to CS (Markovich et al., 2004). For their proximity, GCS2 may possibly play a direct role in CS resistance too. It would be interesting to see if our strains exposed to the antifungal would up-regulate both GSC2, FSK1 and RHO1 genes.

DDR2 is also up-regulated in the strains misincorporating serine at glycine, leucine and tyrosine codons. Although not many information is available about this gene it has been suggested a possible role in the response of cells to diverse environmental stresses. So far it is known that it is rapidly activated after heat shock and DNA damage (Kobayashi et al., 1996).

It was surprising to verify that the control strain was more affected by cycloheximide (CHX) than most of the mistranslating strains. The opposite was verified for geneticin (even if a higher concentration was used), leading us to conclude that it is a much weaker translation inhibitor in yeast. Both compounds act during the elongation step, but their chemical nature is distinct. Geneticin is an aminoglycoside antibiotic, that acts against prokaryotic and in much lower extent against eukaryotic cells. In prokaryotes it binds to the 16S ribosomal subunit and blocks translocation of the tRNA from the A-site to the P-site. In general, aminoglycoside antibiotics bind to regions of the prokaryotic ribosome that are not conserved in eukaryotes. Also, the fact that such drugs are positively charged, reduces their uptake by eukaryotic cells. Besides blocking translocation it was also suggested that aminoglycoside antibiotics decrease the proofreading ability of the ribosome, decreasing translation fidelity (reviewed by Hermann (2007)). CHX also binds to the ribosome and blocks eEF2-mediated translocation but the mechanistic

details of its action remain unclear. It was suggested that mutations in genes coding for ribosomal proteins, such as CYH2 and L41, might increase CHX resistance (Kaufer et al., 1983; Kawai et al., 1992). Our mistranslating strains might be more prepared to handle ribosome inhibition, as they are already coping with an increased level of translational errors. Curiously, Santos et al. (1996) observed an increase in cycloheximide resistance of a constructed *S. cerevisiae* mistranslating strain. Additionally, the deletion of the ABC pump gene PDR5 showed decreased resistance not only to common antifungals such as FLZ but also to very low CHX concentrations (reviewed by Bauer et al., 1999).

Conclusion and Outlook

Our analysis shed the first light on the understanding of the role of mistranslation in yeasts' antifungal drug response but it requires further investigation to draw meaningful conclusions.

According to the Etest results, the echinocandin caspofungin (CS) was the most effective antifungal tested. The strains misincorporating serine at glycine, threonine and alanine codons seemed to have a slight advantage when exposed to the polyene amphotericin B (AP). The strains misincorporating serine at leucine and valine codons had lower MIC endpoints for both CS and AP.

The phenomics study showed that certain types of mistranslation can be advantageous in terms of antifungal drug resistance. Such observation was in agreement with previous studies which revealed a selective advantage with other environmental stressors (Mateus, 2011; Santos et al., 1999). Specifically, the strain misincorporating serine at alanine codons showed a significant growth advantage in itraconazole (0.25µg/mL) supplemented media and surprisingly also did the strain misincorporating serine at valine codons which deals with a very toxic amino acid exchange.

We verified that the mistranslating strains are in general less affected by the translation inhibitor cycloheximide than the control, which is in agreement with previous observations (Santos et al., 1996). The strain that showed the best growth rate is the one misicorporating serine at methionine codons. Curiously, none of the strains had a significantly increased relative growth rate when exposed to geneticin, another translation inhibitor.

Our results together with the ones previously obtained in our laboratory, seem to point to the fact that *S. cerevisiae* does not tolerate the substitution of leucine by serine as well as for the other amino amino acids. Such findings are surprising regarding that *C. albicans* naturally copes with a serine-to-leucine ambiguity (3% leucine insertion) and even could sustain a reversion of its genetic code (100% leucine insertion).

The forced evolution experiment revealed that by using miconazole, mistranslation did not lead to an acceleration of antifungal drug resistance in *S. cerevisiae*.

Other surprising result verified was that, in general, the substitution of proline and methionine by serine did not highly affect the cell fitness. Regarding their chemical and structural differences a sharp decrease in fitness would be expected. It would be interesting to further investigate such strains in order to understand the "why" and "how" of such results.

In the future it could be interesting to do microarrays of cells exposed to several antifungal drugs in different time intervals. It was already seen that short time intervals can make a difference in gene expression (Kuo et al., 2010) and doing so, could allow us to understand which genes are really important for the cell in different growth stages and separate them from those that are always differentially expressed.

We will do WGS of the strains grown until the 230^{th} generation in order to verify changes in the genome (e.g. SNP, Indels). Another experiment could be done in order to check if TSM is also present in eukaryotes. Exposing the cells to

the same concentration of antifungal for several generations instead of increasing it, could allow the cells to survive for longer periods of time at reduced growth rates and eventually overcome that stress. WGS results might include amongst others, a higher mutation rate in the ergosterol pathway genes (mainly ERG3 and ERG11), ABC and MFS pump genes, which are directly related to antifungal drug response.

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Annex I

List of genes differentially expressed by the mistranslating strains. A comparison among mistranslating strains was performed by multiclass SAM analysis (FDR≤0.0001). Values correspond to the means of fold variation presented by each clone. Positive values correspond to up-regulated genes and negative values correspond to down-regulated genes.

Systematic name	*Gene name*	*$tRNA^{Ser}$(Ala)*	*$tRNA^{Ser}$(Gly)*	*$tRNA^{Ser}$(Leu)*	*$tRNA^{Ser}$(Thr)*	*$tRNA^{Ser}$(Tyr)*	*$tRNA^{Ser}$(Val)*
YJR155W	AAD10	-1,45	0,39	-0,53	0,82	-0,70	-0,15
YPL267W	ACM1	0,18	-0,15	-0,49	0,43	-0,16	0,02
YAR015W	ADE1	-2,05	0,55	-1,56	0,04	-1,53	0,08
YLR359W	ADE13	-2,54	0,01	-2,34	-0,01	-2,53	-0,66
YMR120C	ADE17	-2,94	0,49	-2,70	-0,03	-2,61	-0,97
YOR128C	ADE2	-1,52	0,55	-1,31	0,32	-1,45	0,15
YMR300C	ADE4	-1,44	0,31	-1,00	0,09	-1,06	0,09
YGL234W	ADE5,7	-1,91	0,03	-1,61	-0,20	-1,67	-0,50
YGR061C	ADE6	-1,54	0,01	-1,03	-0,49	-1,04	-0,58
YDR408C	ADE8	-0,88	-0,06	-0,47	-0,02	-0,58	0,06
YMR303C	ADH2	0,87	0,41	0,77	-0,32	0,55	0,53
YGL256W	ADH4	-2,73	-0,50	-3,36	-0,62	-3,82	-1,05
YJL122W	ALB1	0,50	-1,20	-0,04	0,09	-0,03	-0,25
YER073W	ALD5	0,34	-0,41	0,83	0,12	1,00	0,14
YPL061W	ALD6	0,11	-0,06	-0,12	-0,72	0,64	0,03
YOL058W	ARG1	1,10	0,17	1,59	0,62	1,96	1,37
YHR018C	ARG4	0,74	0,18	1,23	0,47	1,14	0,81
YER069W	ARG5,6	0,74	-0,03	1,14	0,43	1,42	0,71
YOL140W	ARG8	0,40	-0,48	0,58	0,24	0,79	0,23
YDR127W	ARO1	-0,08	0,04	0,84	-0,12	0,70	0,42
YDR380W	ARO10	-0,16	0,46	0,78	0,53	1,21	1,04
YBR249C	ARO4	0,52	-0,02	1,11	0,25	0,76	0,52
YBR068C	BAP2	0,22	0,08	1,01	0,23	0,87	0,47
YDR046C	BAP3	0,46	-0,17	0,47	0,12	0,64	0,49
YKR099W	BAS1	0,94	-0,30	0,42	0,61	1,00	0,09
YHR208W	BAT1	0,16	-0,01	1,06	0,20	0,70	0,48
YLR412W	BER1	0,37	-0,14	0,68	0,04	0,34	0,15
YNR058W	BIO3	-2,07	-0,35	-1,20	0,85	-0,79	-0,94
YNR057C	BIO4	-1,41	-0,07	-0,61	1,44	-0,47	-0,45
YNR056C	BIO5	-2,13	-0,10	-1,35	1,45	-0,89	-0,85
YBL098W	BNA4	-1,03	0,41	-0,25	0,01	-0,73	-0,39
YLR231C	BNA5	-0,92	-0,13	-0,12	0,08	-0,77	-0,62
YFR047C	BNA6	-0,57	0,38	-0,05	0,22	-0,44	0,00

YLR267W	BOP2	0,70	0,68	1,32	0,30	-0,19	0,14
YGR142W	BTN2	1,37	1,08	0,86	0,33	2,22	1,64
YPL111W	CAR1	0,99	0,43	0,41	-0,46	-0,22	0,66
YLR438W	CAR2	0,89	1,39	1,45	-0,10	0,02	0,78
YCL064C	CHA1	1,26	-0,48	0,10	-0,48	1,33	0,22
YHR122W	CIA2	0,66	-0,23	0,53	0,30	0,77	0,24
YCR005C	CIT2	0,78	0,90	1,33	0,81	1,39	2,09
YOR303W	CPA1	0,92	-0,05	1,50	0,64	1,45	0,87
YJR109C	CPA2	0,58	-0,15	1,35	0,21	1,17	0,68
YBR233W-A	DAD3	-0,94	1,63	1,38	0,09	0,00	0,47
YOR173W	DCS2	-0,54	1,77	1,31	0,48	0,74	0,36
YPL265W	DIP5	0,77	0,76	1,27	0,49	1,34	1,55
YEL071W	DLD3	0,73	0,31	1,00	0,43	1,03	1,22
YIL103W	DPH1	0,13	-1,10	-0,54	-0,12	-0,24	-0,25
YKL191W	DPH2	0,58	-0,68	0,00	0,22	0,43	0,00
YBR208C	DUR1,2	0,34	0,89	-0,35	-0,45	-0,51	0,25
YGR146C	ECL1	0,68	0,59	1,30	0,31	1,38	1,01
YMR062C	ECM40	0,83	0,39	1,37	0,49	1,34	1,01
YPL095C	EEB1	-0,17	0,01	-1,26	-0,53	-0,42	-0,04
YAL003W	EFB1	0,59	-0,07	-0,02	0,19	0,29	0,35
YMR323W	ERR3	0,04	0,92	0,01	0,11	0,02	0,15
YOR388C	FDH1	-0,31	0,36	0,55	5,28	0,30	0,12
YPL276W	FDH2	-0,57	-0,06	0,09	3,75	-0,47	0,02
YPL275W	p-FDH2	-0,59	0,25	0,53	4,87	-0,50	0,05
YMR319C	FET4	-0,19	0,24	-0,18	0,84	-1,33	0,16
YDR070C	FMP16	-0,21	2,40	2,27	0,90	0,86	0,24
YBR047W	FMP23	1,21	0,88	1,88	1,15	2,01	1,46
YDL222C	FMP45	-0,18	1,68	0,08	-0,13	0,18	0,03
YLR214W	FRE1	0,71	1,25	0,38	2,15	-0,92	0,35
YOL152W	FRE7	1,62	4,05	1,74	4,62	-0,50	0,52
YBL042C	FUI1	0,22	-1,60	-0,73	-0,36	-0,17	-0,51
YDR019C	GCV1	-1,73	-0,04	-1,18	-0,14	-0,81	-0,17
YMR189W	GCV2	-1,61	-0,11	-0,99	-0,15	-0,95	-0,10
YPR184W	GDB1	-0,25	1,36	1,07	0,32	0,35	0,29
YEL011W	GLC3	-0,54	1,32	0,91	-0,19	0,20	-0,04
YKR058W	GLG1	-0,82	0,89	0,40	-0,21	-0,08	0,14
YGR256W	GND2	-0,34	1,42	0,97	0,97	-0,14	-0,06
YPR160W	GPH1	-1,03	1,17	1,21	0,00	0,05	-0,12
YDL021W	GPM2	-0,97	0,70	-0,08	0,08	-0,54	-0,05
YPL223C	GRE1	-0,43	0,94	1,63	0,20	-0,12	-0,07
YFR015C	GSY1	-0,88	1,11	0,23	-0,64	-0,76	-0,36
YJR055W	HIT1	-0,28	0,68	-0,23	0,45	-0,41	-0,06
YLR205C	HMX1	0,00	0,15	1,01	-0,28	0,37	0,31
YER052C	HOM3	0,72	0,23	1,17	0,39	1,29	0,49

YOL155C	HPF1	-1,09	0,31	-0,57	-0,14	-0,55	-0,50
YDR399W	HPT1	Nd[1]	Nd	Nd	Nd	Nd	-1,57
YBR072W	HSP26	-1,85	1,20	1,48	0,03	0,25	-0,52
YCR021C	HSP30	0,04	0,77	1,76	-0,14	1,13	0,82
YHR096C	HXT5	0,27	1,11	1,12	0,12	-0,59	0,05
YDR342C	HXT7	-0,23	0,33	0,68	0,45	0,14	-0,33
YLR099C	ICT1	0,75	0,74	1,23	0,22	0,74	0,29
YPL250C	ICY2	0,56	0,19	1,33	0,50	1,42	0,73
YJR016C	ILV3	0,40	0,03	1,06	0,21	1,05	0,25
YCL009C	ILV6	0,62	0,48	1,27	0,40	1,44	0,82
YAR073W	IMD1	0,59	-1,09	0,03	0,21	-1,00	-0,91
YJL153C	INO1	1,21	1,94	0,21	0,33	0,07	0,08
YHR085W	IPI1	0,59	-0,95	-0,06	0,40	0,15	0,17
YMR081C	ISF1	-0,93	0,56	0,83	-0,71	-0,54	0,08
YLL019C	KNS1	-0,31	0,35	0,43	-0,11	0,39	-0,03
YKL103C	LAP4	-0,22	1,06	-0,23	0,04	0,13	-0,32
YCL018W	LEU2	-1,23	-0,45	-2,07	-1,09	-1,00	-1,41
YNL104C	LEU4	0,40	0,40	1,43	0,38	1,37	0,56
YIR034C	LYS1	0,95	0,71	1,24	0,72	1,70	0,68
YBR115C	LYS2	0,54	0,30	1,24	0,34	1,31	0,67
YDL182W	LYS20	1,83	1,30	2,64	1,45	2,43	1,70
YKL021C	MAK11	0,41	-0,63	-0,17	0,08	-0,10	-0,11
YGL125W	MET13	0,58	0,84	1,38	0,47	1,61	0,54
YML128C	MSC1	-1,15	1,10	0,34	0,22	-0,15	-0,14
YKR080W	MTD1	-1,64	0,57	-1,25	0,16	-1,50	0,37
YNL240C	NAR1	0,65	-0,18	0,53	0,45	0,88	0,08
YNL036W	NCE103	0,74	0,84	1,73	0,58	1,41	1,33
YOL144W	NOP8	0,41	-0,91	-0,12	0,30	0,36	-0,18
YKL120W	OAC1	0,32	-0,12	0,99	0,24	0,81	0,65
YPR194C	OPT2	-1,49	-0,67	-0,16	-0,77	-1,16	0,40
YOR269W	PAC1	0,78	0,04	0,37	0,22	0,96	0,17
YHR071W	PCL5	1,07	0,62	1,75	0,93	1,80	1,09
YGR239C	PEX21	0,42	-0,15	0,84	0,43	0,98	0,52
YDR281C	PHM6	0,12	0,23	2,02	0,33	-0,15	-0,13
YBR093C	PHO5	-1,23	-1,06	-0,03	-0,46	-1,47	-0,92
YML123C	PHO84	-0,47	0,16	6,68	-0,62	-0,64	-0,06
YBR296C	PHO89	0,22	0,37	1,35	0,14	-0,14	0,10
YKL163W	PIR3	-0,72	-0,41	0,67	-0,85	-0,52	-0,33
YBL018C	POP8	0,36	-0,82	-0,17	-0,10	0,02	0,05
YDR075W	PPH3	0,29	-0,53	0,19	0,26	0,07	-0,04
YJL079C	PRY1	-1,00	-0,23	0,44	0,08	0,10	-0,75
YML017W	PSP2	0,05	0,02	-1,20	0,20	0,52	-0,08

[1] Nd – No hibridization was detected.

YER075C	PTP3	0,57	-0,06	0,47	0,36	0,79	-0,27
YLR142W	PUT1	0,22	0,73	0,57	0,04	-0,17	0,02
YGL062W	PYC1	0,48	0,98	1,18	0,29	1,16	1,26
YJL217W	REE1	1,65	2,54	1,66	3,22	0,53	1,41
YLR073C	RFU1	0,95	-0,97	0,58	0,38	0,36	-0,22
YER067W	RGI1	-0,33	1,98	0,93	0,02	-0,07	0,64
YBR256C	RIB5	0,65	0,14	1,00	0,48	1,09	0,74
YOR287C	RRP36	0,47	-0,99	-0,26	0,24	0,11	0,01
YIL127C	RRT14	0,30	-0,85	-0,31	0,12	-0,23	-0,10
YHR056C	RSC30	-0,30	0,41	0,16	0,68	-0,07	-0,37
YER125W	RSP5	-0,52	-0,01	-0,78	-0,44	-0,03	-0,66
YDL204W	RTN2	-0,48	1,97	1,08	0,09	1,04	0,02
YPL274W	SAM3	0,42	-0,10	0,73	0,41	0,98	0,76
YBR214W	SDS24	-0,51	0,94	0,63	-0,18	0,23	0,62
YGR208W	SER2	-0,51	0,15	-0,96	-0,17	-0,90	0,00
YER081W	SER3	-0,84	1,10	-0,60	0,39	-0,88	0,20
YLR058C	SHM2	-1,52	0,10	-1,53	0,15	-1,14	-0,54
YMR175W	SIP18	-0,38	0,73	1,99	0,16	-0,08	0,00
YMR095C	SNO1	1,44	0,94	2,24	1,43	2,20	1,81
YMR096W	SNZ1	1,15	0,78	2,07	1,16	2,02	1,76
YMR107W	SPG4	0,52	1,76	1,78	0,46	-0,73	0,86
YHR136C	SPL2	-0,19	-0,19	5,15	-0,36	-0,65	-0,01
YHR184W	SSP1	-0,22	-0,19	-0,52	0,42	-0,69	-0,13
YDL048C	STP4	-0,34	-0,14	0,74	-0,29	0,78	-0,31
YDL244W	THI13	-0,05	0,38	0,56	0,57	-0,36	-0,40
YOL055C	THI20	-1,40	-0,24	-0,71	-1,08	-1,13	-0,24
YLR327C	TMA10	-0,33	1,96	1,47	0,02	0,88	0,79
YGL026C	TRP5	0,58	0,25	0,98	0,35	0,85	0,68
YLR193C	UPS1	0,07	0,14	0,90	0,09	0,44	0,07
YKL216W	URA1	0,75	-0,86	-0,36	-0,08	0,20	0,21
YOR004W	UTP23	0,23	-1,69	-0,29	0,03	-0,20	-0,30
YGL258W	VEL1	-3,79	-0,94	-4,00	0,23	-4,35	-2,09
YIL056W	VHR1	0,80	0,55	1,46	0,56	1,42	0,71
YGR065C	VHT1	-1,15	-0,01	-0,75	0,81	-0,38	-0,78
YER072W	VTC1	0,71	0,44	2,96	0,42	0,37	0,24
YFL004W	VTC2	-0,26	-0,16	0,78	-0,23	-0,17	-0,18
YPL019C	VTC3	-0,46	0,00	1,64	-0,23	-0,46	-0,12
YJL012C	VTC4	-0,20	-0,04	1,47	-0,07	-0,05	-0,23
YER024W	YAT2	0,38	0,49	1,36	0,38	1,18	0,87
YGR234W	YHB1	0,12	0,10	1,08	-0,19	1,04	0,50
YHR029C	YHI9	0,90	0,49	1,76	0,51	1,46	1,07
YDL198C	YHM1	0,55	0,08	0,95	0,29	1,18	0,59
YPR058W	YMC1	0,65	0,07	0,96	0,17	0,78	0,28
YBR104W	YMC2	0,63	-0,64	0,68	0,15	0,59	0,36

YGR211W	ZPR1	0,63	-0,49	0,11	0,10	0,73	0,20
YOL154W	ZPS1	-2,19	-0,49	-2,79	0,31	-3,06	-0,93
YEL073C		-0,82	0,46	-0,41	-0,45	-0,83	-0,76
YBL028C		0,28	-1,21	-0,44	-0,14	-0,22	-0,70
YLR162W		-1,37	-0,28	-2,27	-1,33	-0,98	-0,05
YDR161W		0,47	-0,94	-0,08	0,11	0,00	-0,10
YMR321C		0,38	-0,37	0,53	-0,12	0,44	0,18
YHR054C		-0,43	0,41	0,25	0,58	-0,17	-0,15
YIL165C		0,65	0,17	0,92	0,43	1,26	0,56
YGR153W		-0,20	-0,33	-0,62	0,19	-0,96	0,03
YPR074W-A		0,68	-1,15	-0,18	0,61	0,04	-0,08
YJR079W		-0,75	0,77	0,01	0,50	-0,49	0,06
YBR285W		-0,98	1,22	1,16	0,78	-0,37	-0,29
YLR179C		0,50	0,42	1,20	0,31	0,88	0,90
YNL058C		-0,36	-0,45	0,54	0,02	-0,27	0,21
YGL117W		1,12	0,28	1,53	0,62	1,63	1,09
YJL012C-A		-0,24	-0,10	1,58	-0,18	-0,26	-0,29
YOR387C		-3,79	-0,94	-4,16	0,17	-4,72	-1,94