Universidade de Aveiro Departamento de Biologia
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Mistranslation in *Candida albicans*

Erros na tradução do mRNA em *Candida albicans*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Manuel António da Silva Santos, Professor Associado do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Código genético, erros da tradução do mRNA, ambiguidade do codão, mRNA, tRNA, resposta ao stress, caracterização fenotípica, evolução, *Candida albicans*

resumo

O código genético estabelece regras que determinam a transferência de informação genética a partir dos ácidos nucleicos para proteínas. A importância do código genético na descodificação do genoma e sua alta conservação sugere que a sua evolução é altamente restrita. Apesar disso, várias alterações no código genético dos procariotas e eucariotas têm sido encontradas, mostrando que o código é surpreendentemente flexível. Por exemplo, o patogénico humano *Candida albicans* contém um $tRNA_{CAG}$ ambíguo que descodifica o codão CUG como Ser (97%) e como Leu (3%).

Para continuar o estudo da ambiguidade noutros codões, induzimos 8 $tRNA^{Ser}$ mutantes, que incorporam incorretamente o aminoácido serina a 8 codões diferentes, pertencentes a distintas famílias de aminoácidos (Glu, Arg, Asn, Cys, Phe, Gln, His e Pro), em *Candida albicans*. O tRNA não mutado foi submetido a mutagénese dirigida, a fim de modificar o seu anticodão UGA para CUC, CCU, GUU, GCA, GAA, CUG, GUG e GGG. A estabilidade do tRNA, as alterações celulares e resposta ao stress das estirpes mutantes resultantes foram avaliadas através da análise de *Northern blot*, da eficiência de transformação das células, da taxa de crescimento e da expressão do sistema repórter HSP104-GFP. Além disso, a caracterização fenotípica em determinadas condições de stress foi realizada com o intuito de caracterizar melhor essas estirpes.

Os dados experimentais sugerem que essas ambiguidades ao código genético afetam negativamente a aptidão das células em condições de crescimento normais e introduzem vantagens no crescimento na presença de condições de stress. Assim, a resposta ao stress provocada pela ambiguidade dos codões pode aumentar o potencial de adaptação.

keywords

Genetic code, mistranslation, codon ambiguity, mRNA, tRNA, anticodon, stress response, phenotypic screening, evolution, *Candida albicans*

abstract

The genetic code establishes the rules that determine the transfer of genetic information from nucleic acids to proteins. The importance of the genetic code in genome decoding and its high conservation suggests that its evolution is highly restricted or even frozen. Despite this, various prokaryotic and eukaryotic genetic code alterations have been found, showing that the code is surprisingly flexible. For instance, the human pathogen *Candida albicans* contains an ambiguous $tRNA_{CAG}$ that decodes a CUG codon as Ser (97%) and as Leu (3%).

To further study ambiguity in other amino acid codons, we have engineered 8 mutant $tRNA^{Ser}$ that misincorporate Ser at 8 different codons belonging to distinct amino acids families (Glu, Arg, Asn, Cys, Phe, Gln, His and Pro) in *Candida albicans*. The wild-type tRNA was subjected to site-directed mutagenesis in order to change its anticodon to CUC, CCU, GUU, GCA, GAA, CUG, GUG and GGG. The tRNA stability, the cellular changes and the stress response of the resulting mistranslating strains were evaluated through northern blot analysis, cell transformation efficiency, growth rate and expression of a HSP104-GFP reporter system. A phenotypic screening probing various environmental stress conditions was performed in order to further characterize these strains.

Experimental data suggest that these genetic code ambiguities affect fitness negatively in standard growth conditions and introduce growth advantages in presence of stress conditions. Thus, stress response triggered by codon ambiguity increase adaptation potential.

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aaRS: Aminoacyl tRNA synthetase

aa-tRNA: Aminoacyl-tRNA

ACT1- Actin 1

Amp: Ampicillin

ANOVA: Analysis of variance

APS: Ammonium persulphate

A-site: Aminoacyl site

ATP: Adenosine 5’-triphosphate

bp: Base pairs

CI: Confidence interval

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

dsDNA: double-stranded deoxyribonucleic acid

EDTA: Ethylenediamine tetraacetic acid

eEF: Eukaryotic elongation factors

eIF: Eukaryotic initiation factors

ER: Endoplasmic reticulum

eRF: Eukaryotic release factors

E-site: Exit site

g (mg, µg, ng) gram (milligram, microgram, nanogram)

GDP: Guanosine 5'-diphosphate

GTP: Guanosine 5'-triphosphate

HSP: Heat shock protein

kDa: KiloDalton

L (mL, µL) liter (mililiter, microliter)

LB: Lysogeny broth

M (mM, µM) molar (milimolar, micromolar)

mA: Miliampère

Mb: Megabit

$MgCl_2$: Magnesium chloride

$MgSO_4$: Magnesium sulphate

MM: Minimal médium

$MnCl_2$: Manganese chloride

mol (mmol, μmol, pmol) mole (nanomole, micromole, picomole)

MQ: MilliQ

mRNA: Messenger ribonucleic acid

OD: Optical density

OD_{595}: Optical density at 595nm

ORF: Open reading frame

PCR : Polymerase chain reaction

PEG: Polyethylene glycol

P-site: Peptidyl site

RNA: Ribonucleic acid

ROS: Reactive oxygen species

rpm: Revolutions per minute

RPS10: Ribosomal protein S10

rRNA : Ribosomal ribonucleic acid

S: Svedberg

SAPK: Stress-activated protein kinase

SD: Standard deviation

SDS: Sodium dodecyl sulphate

Sp. and *spp.* : *species* and "several *species*"

TEMED: Tetramethylethylenediamine

TFB: Standard transformation buffer

Tris: Tris(hydroxymethyl)aminomethane

tRNA: Transfer ribonucleic acid

U: Units

UPS: Ubiquitin proteasome system

V: Volt

yEGFP: Yeast-enhanced green fluorescent protein

YEPD: Yeast extract peptone dextrose, rich medium

YFP: Yellow fluorescent protein

Other abbreviations will be explained when used in the text.

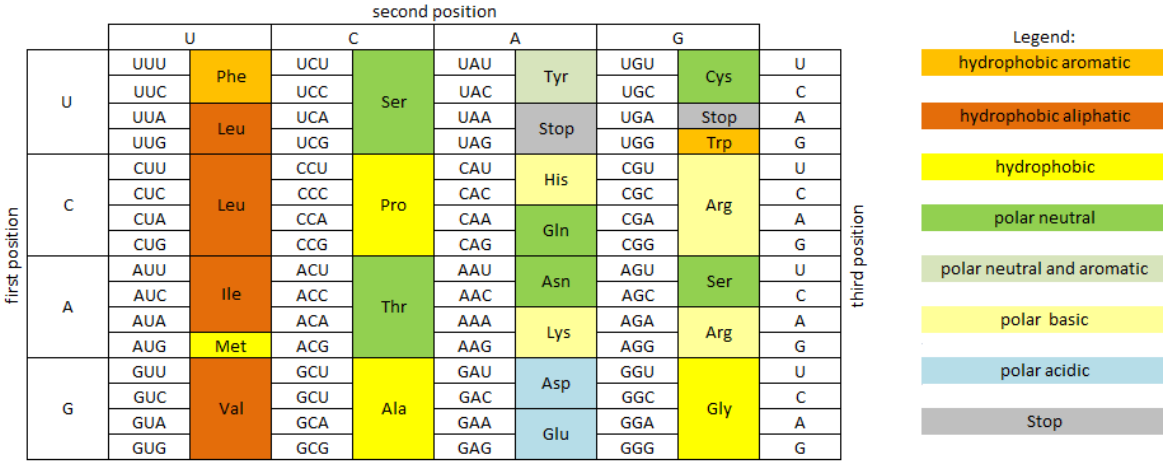
1.Introduction

1.1. Genetic code

The standard genetic code was established in 1966 and the rules that govern the transfer of genetic information from nucleic acids (DNA and mRNA) to protein were defined (Crick, 1966a). By this time, the hypothesis of a frozen and universal genetic code was also established by Crick. This hypothesis postulated that the genetic code is common to all organisms (Crick, 1968). In the mRNA, the genetic information comes from 4 ribonucleotide base combinations [**C**(cytosine), **G**(guanine), **A**(adenine) and **U**(uracil)], organized into nucleotide-triplets (codons). The combinations of these bases in three different positions define 64 possible codons, where 61 different codons are translated into 20 amino acids and 3 are stop codons. This means that the same amino acid is encoded by more than one codon, named "synonymous codons", except methionine (Met) and tryptophan (Trp), which are specified by only one codon, AUG codon and UGG codon, respectively. The "synonymous codons" tend to share the first two nucleotides with variation in the third position, except in case of leucine (Leu), serine (Ser) and arginine (Arg), which are encoded by six "synonymous codons", consisting of a "family box" or a "four-codon box" and a "two-codon set". Nine amino acids: asparagine (Asn), aspartate

(Asp), cysteine (Cys), glutamine (Gln), glutamate (Glu), histidine (His), lysine (Lys), phenylalanine (Phe) and tyrosine (Tyr) are encoded by two "synonymous codons" (a two-codon set); five amino acids: alanine (Ala), glycine (Gly), proline (Pro), threonine (Thr) and valine (Val) are encoded by four "synonymous codons" (a family box). One amino acid, isoleucine (Ile), has three "synonymous codons". So, we can say that the standard genetic code is degenerate or redundant (Table 1.1). During the last 25 years, two new amino acids were discovered, namely selenocysteine (Sec) (Chambers et al., 1986; Zinoni et al., 1987) and pyrrolysine (Pyl) (Hao et al., 2002; Srinivasan et al., 2002), designated as the 21st and 22nd amino acids, respectively. In this case, some organisms translate the UGA stop codon as Sec and UAG stop codon as Pyl. This confirms the expansion of the code genetic and its flexibility.

Table 1.1: The standard genetic code. The colors on the table indicate the fundamental chemical properties of the amino acids. The orange ones are hydrophobic aromatic, the red ones are hydrophobic aliphatic and the yellow ones hydrophobic. The other colors are hydrophilic. From these, the green ones are polar neutral, the green water ones are polar neutral aromatic, the yellow water ones are polar basic and the blue ones are polar acidic. The three stop codons are indicated by the grey color.



This table shows a correlation between codons encoding amino acids with similar chemical properties. Every codon with an **U** in second position encodes for hydrophobic amino acids (Phe, Leu, Ile, Met and Val), where the Leu, Ile and Val are connected by a single base mutation at the first codon base. The codons with an **A** in second position encode for hydrophilic amino acids: acidic amino acids (Glu, Asp), their amides (Gln, Asn), basic

amino acids (His, Lys) and neutral amino acid (Tyr). Also, Asp and Asn, and Glu and Gln only differ in the first codon position. Thus, the second base of the codon is the major base to determine the amino acid assignment of a codon. It also controls the charge (positive and negative) of the amino acids when they are purines (**A** or **G**), and specifies hydrophobic amino acids when they are pyrimidines (especially **U**) (Biro et al., 2003). However, the third position in many cases leaves the amino acid assignment unchanged (Woese, 1965). This biased codon organization and redundancy may minimize decoding error (Castro-Chavez, 2010; Woese, 1965; Woese et al., 1966).

When the first two nucleotides in the codon (doublet) are **G** and/or **C**, which are the "strong codons", because they have a three hydrogen bonds, they always represent a "family box"– GG**N** (Gly); CG**N** (Arg); GC**N** (Ala); CC**N** (Pro), **N** is any nucleotide - while "weak codons" formed by the AU doublet (**A** and **U** have a two hydrogen bond) never represent a "family box". All GC doublets have a strong binding to their cognate tRNA anticodons and do not require the third base pair, while all AU doublets have a weak binding and need a third base pair (Lagerkvist, 1978). Therefore, the degeneracy of the code appears to be controlled by the GC content of codons, where the maximum GC content in codon may be reducing ''the rate of mutations and mistranslation'' (Goldberg and Wittes, 1966).

1.2. Natural genetic code alterations - Evolution of the genetic code

The hypothesis of an universal and frozen genetic code, mentioned in previous section, was questioned in 1979, when it was discovered that the UGA stop codon codes for Trp and the AUA codon codes for Met and not Ile, in human mitochondria (Barrell et al., 1979). Since then, alterations to the standard genetic code have been found in mitochondrial and cytoplasmic translation systems of various organisms (Hanyu et al., 1986; Kuchino et al., 1985; Ohama et al., 1990; Yamao et al., 1985).

Most of the alterations occur in nonplant mitochondrial genomes. For example, the AAA codon is translated as Asn and not as Lys, the AG**R** (**R** is purine - A or G) is translated as Gly or Ser, the CU**N** (**N** is any nucleotide) is translated as Thr and not as Leu, the UAA codon stop is translated as Tyr, and the UAG codon stop is translated as Ala or Leu, in various mitochondrial systems [reviewed in (Osawa et al., 1992)].

Also, there are several examples of cytoplasmic genetic code alterations. For example, in the eubacteria *Mycoplasma* and *Spiroplasma*, the UGA stop codon codes for Trp, while in some holotrichous ciliates *Tetrahymena spp*., *Paramecium spp.*, in the hypotrichous ciliates *Oxytricha spp.*, *Stylonichiathe spp.*, and in the unicellular green algae *Acetabularia spp*. the UAA and UAG stop codons code for Gln. In other hypotrichous ciliate *Euplotes sp.*, the UGA stop codon codes for Cys and UAA is used as a stop codon [reviewed in (Osawa et al., 1992)]. Another example is the genus *Candida* where several species decode the leucine CUG codon as serine (Santos and Tuite, 1995; Santos et al., 1997). So, most organisms use a standard genetic code, but some prokaryotic and eukaryotic organisms use slightly different genetic codes.

1.3. Control of protein synthesis fidelity

1.3.1. mRNA translation

Gene expression needs two basic steps, one consists on transcription, where information is transferred from DNA into mRNA molecules, and the other is translation, where the information is transferred from mRNA molecules into proteins (Crick, 1970). The process of mRNA translation takes place in the ribosome, which is a supramolecular complex composed by more than 50 different ribosomal proteins (RPN) and several ribosomal ribonucleic acid (rRNA) molecules, which form a small and a large subunit. In eukaryotes, the 80S (S is Svedberg unit sedimentation velocity) ribosome contains a 40S small subunit (one molecule of 18S rRNA plus 30-32 proteins) and a 60S large subunit (three molecules of rRNA, named 5S, 5.8S and 25-28S plus 41-49 proteins) [reviewed in (Kozak, 1983; Marintchev and Wagner, 2004)].

The ribosome contains three sites for binding the transfer ribonucleic acids (tRNAs): the aminoacyl site (A-site), where aminoacyl-tRNA (aa-tRNA) has high affinity; the peptidyl site (P-site), where peptidyl-tRNA has high affinity; and the exit site (E-site), where deacylated tRNA has high affinity before exit from ribosome (Figure 1.1) [reviewed in (Marintchev and Wagner, 2004)].

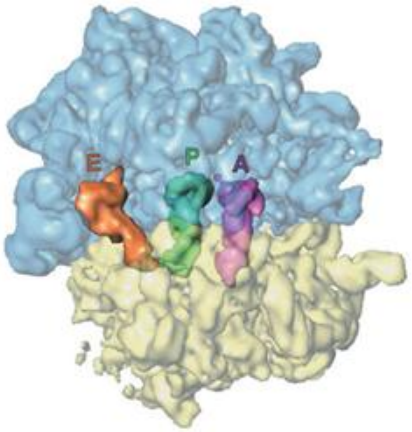


Figure 1.1: Cryo-EM maps showing ribosome structure with decoding sites, the ribosomal A-site (magenta), P-site (green) and E-site (orange) where the tRNAs anticodons interact with mRNA codons [adapted from (Valle et al., 2003)].

Protein synthesis is only possible with the participation of ribosomes, tRNAs, amino acids, translational factors and aminoacyl tRNA-synthetases (aaRS), that read the mRNA messages. Translation can be divided in three stages: initiation, elongation and termination.

1.3.1.1 Translation Initiation

In eukaryotes, mRNA binds to the 40S subunit of the ribosome, which is dissociated from the 60S subunit. The anticodon of the initiator methionyl-tRNA (Met-tRNAi^{Met}) interacts with start codon (AUG) on mRNA through base pairing, in the ribosomal P-site. Then, the 60S subunit of the ribosome joins this initiation complex. These steps are only possible with the help of at least twelve eukaryotic initiation factors (eIF), which dissociate at the end of the initiation step [reviewed in (Acker and Lorsch, 2008; Marintchev and Wagner, 2004)].

During initiation the translational factors eIF1A, eIF3 and eIF1 associate with the 40S ribosomal subunit. In this complex, the eIF1A binds near the A-site to prevent Met-tRNAi^{Met} from binding to it and directs it to the ribosomal P-site, while the elF3 and elF6 inhibits the reassociation of the 40S with the 60S subunit. Meanwhile, the eIF2B activates eIF2 by replacing its guanosine diphosphate (GDP) with guanosine triphosphate (GTP), regulating the formation of the eIF2•GTP•Met-tRNAi^{Met} ternary complex, where the eIF2 and GTP bind to the initiator methionyl-tRNA. Then, with the help of eIF1A, eIF3 and eIF1, the ternary complex binds to the 40S subunit, resulting in the formation of a 43S pre-initiation complex. At the same time, the eIF4F complex, which includes the factors eIF4E, eIF4G and eIF4A, is assembled on the 5'-end cap structure (**m7GpppN** - where **m7G** represents 7-methylguanylate, **p** represents a phosphate group and **N** represents any base) of the mRNA. In this case, the factor eIF4E is the cap-binding protein, while the eIF4A has RNA helicase activity and is an ATPase, which is used to unwind secondary structures that may exist in the 5´ untranslated region (5´UTR) of mRNA. RNA helicase activity of the elF4A is enhanced by eIF4B and elF4H, which stimulates its binding to mRNA. The eIF4G is a scaffolding protein capable of binding to a variety of other proteins, including eIF4E, elF4A, eIF3 (the 40S ribosomal subunit-binding protein) and PABP (a polyA-binding protein), which binds to the 3´ poly(A) tail, thus joining the mRNA and the 43S complex. This complex starts scanning down the message in the 5′→3′ direction until the AUG start codon is recognized. This localization is improved by the presence of the Kozak consensus sequence [(GCC)GCC**R**CC**AUG**G where **R** is a purine (A or G)] that surrounds the start codon (**AUG**). This assembly results in the formation of the 48S initiation complex. Here, the eIF1 and eIF1A may play a role in the scanning process (Kozak, 1981), where elF1 recognizes the correct initiation codon. When the start codon is found and codon-anticodon is base paired, the elF2•GTP is hydrolyzed to elF2•GDP and inorganic phosphate (Pi) by eIF5. The elF2•GDP and the other initiation factors are released from the 40S subunit. Then, the complex eIF5B•GTP stimulates the joining of 60S ribosomal subunit with Met-tRNAi•mRNA•40S ternary complex, through an increase of the GTP hydrolysis by eIF5B. As eIF5B•GDP complex has a low affinity for the ribosome, it dissociates from the complex and 80S ribosome is assembled, which marks the end of the translation initiation phase (Figure 1.2) [review in (Acker and Lorsch, 2008; Gebauer and Hentze, 2004; Holcik

et al., 2000; Kapp and Lorsch, 2004)].

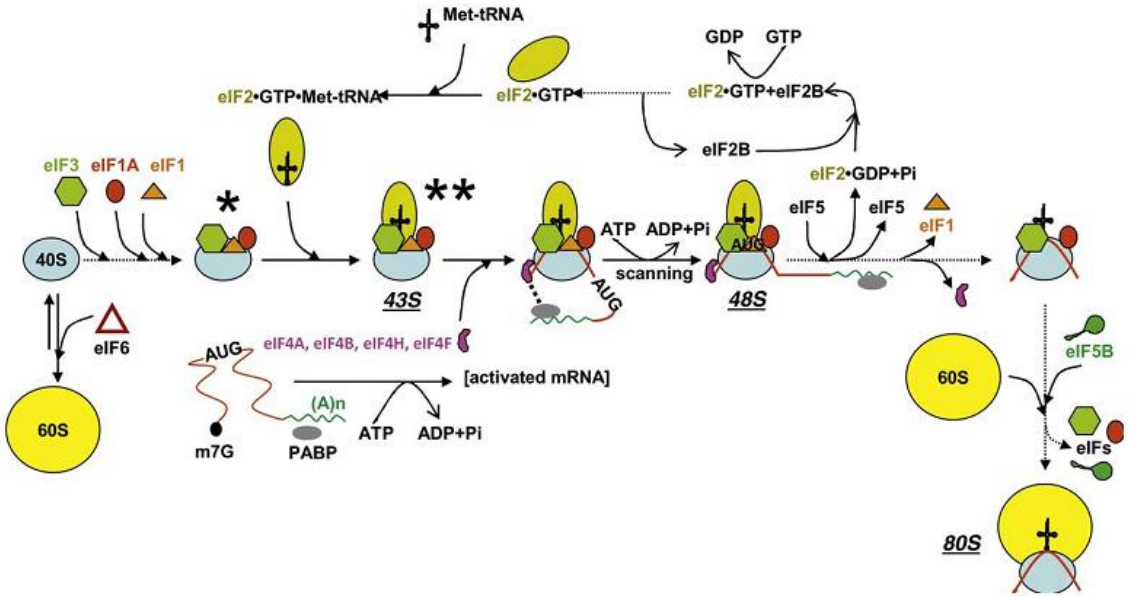


Figure 1.2: A model of eukaryotic translation initiation. The ribosomal 40S and 60S subunits are dissociated with help of elF6, elF3, elF1A and elF1. The ternary complex is formed by elF2, GTP and Met-tRNAi^{Met}, in presence of elF2B. This complex associates with the 40S subunit to form the 43S pre-initiation complex. Then, the 43S complex assembles and scans the activated mRNA until the AUG start codon is recognized, in presence of elF4 and PABP (polyA-binding protein). The anticodon of the Met-tRNAi^{Met} interacts with AUG start codon and the 48S initiation complex is formed. Finally, two GTPs are hydrolyzed in presence of the elF5 and elF5B, and the 60S subunit binds to the 48S complex to form the 80S ribosome [adapted from (Myasnikov et al., 2009)].

1.3.1.2 Translation Elongation

Translation elongation is the stage where the ribosome assembled at the start codon, moves in the direction 5′→ 3′ along the mRNA until a stop codon is found. The codons encode amino acids that are connected through peptide bonds for the synthesis of a polypeptide chain.

Eukaryotic cells require three elongation factors, namely eEF1A, eEF1B and eEF2. In fungi there is an additional factor called eEF3 that interacts with the E-site of the ribosome.

The aa-tRNA (tRNA charged with its cognate amino acid) is selected at the A-site of the ribosome as part of an eEF1A•GTP•aa-tRNA ternary complex, through cognate mRNA codon and tRNA anticodon base pairing, which activates the eEF1A•GTPase activity, inducing GTP hydrolysis and releasing eEF1A•GDP from the aa-tRNA placed in the A-site. Subsequently, cognate aa-tRNA is accommodated in the decoding site and non-cognate tRNA is rejected from the A-site. The eEF1A•GDP is recycled to eEF1A•GTP by eEF1B, to participate in successive rounds of polypeptide chain elongation. After aa-tRNA accommodation, the ribosomal peptidyl transferase centre catalyzes the transference of the polypeptide chain from the peptidyl-tRNA in the ribosomal P-site to the newly arrived amino acid of the aa-tRNA located into the A-site. Following this peptide bond formation, the ribosome translocates the peptidyl-tRNA in the A-site and the deacylated tRNA in the P-site into the P- and E-sites, respectively. The sequential movement of tRNAs through the A-, P-, and E-site moves the ribosome in the mRNA to leave the A-site free to accept a new aa-tRNA, decoding the next codon. This translocation is catalyzed by a second ribosomal GTPase, eEF2, which hydrolyzes GTP [review in (Aitken et al., 2010; Kapp and Lorsch, 2004; Marintchev and Wagner, 2004)]. This cycle is repeated until a stop codon enters the A-site (Figure 1.3, A)).

1.3.1.3 Translation Termination

Termination of translation is initiated when one of the three stop codons, UAG, UGA or UAA is recognized on the mRNA at the ribosomal A-site. These codons are recognized by a protein called class-I release factor (RF), eRF1, which is associated with eRF3 (class-II RF) and GTP to form the ternary eRF1•eRF3•GTP complex. Then, the eRF1 catalyzes the hydrolysis of the ester bond between polypeptide chain and tRNA at the P-site, releasing the nascent polypeptide from the ribosome. In this complex, eRF3, which is a GTP-binding protein with GTPase activity, stimulates the binding of eRF1 to the ribosome and peptidyl-tRNA hydrolysis, with GTP consumption (Mitkevich et al., 2006). Finally, all eRFs are released from ribosome, ribosomal subunits are separated and recycled for another cycle of the translation process (Figure 1.3, B)) (Frolova et al., 1996; Nakamura and Ito, 1998; Petry et al., 2008; Zhouravleva et al., 1995).

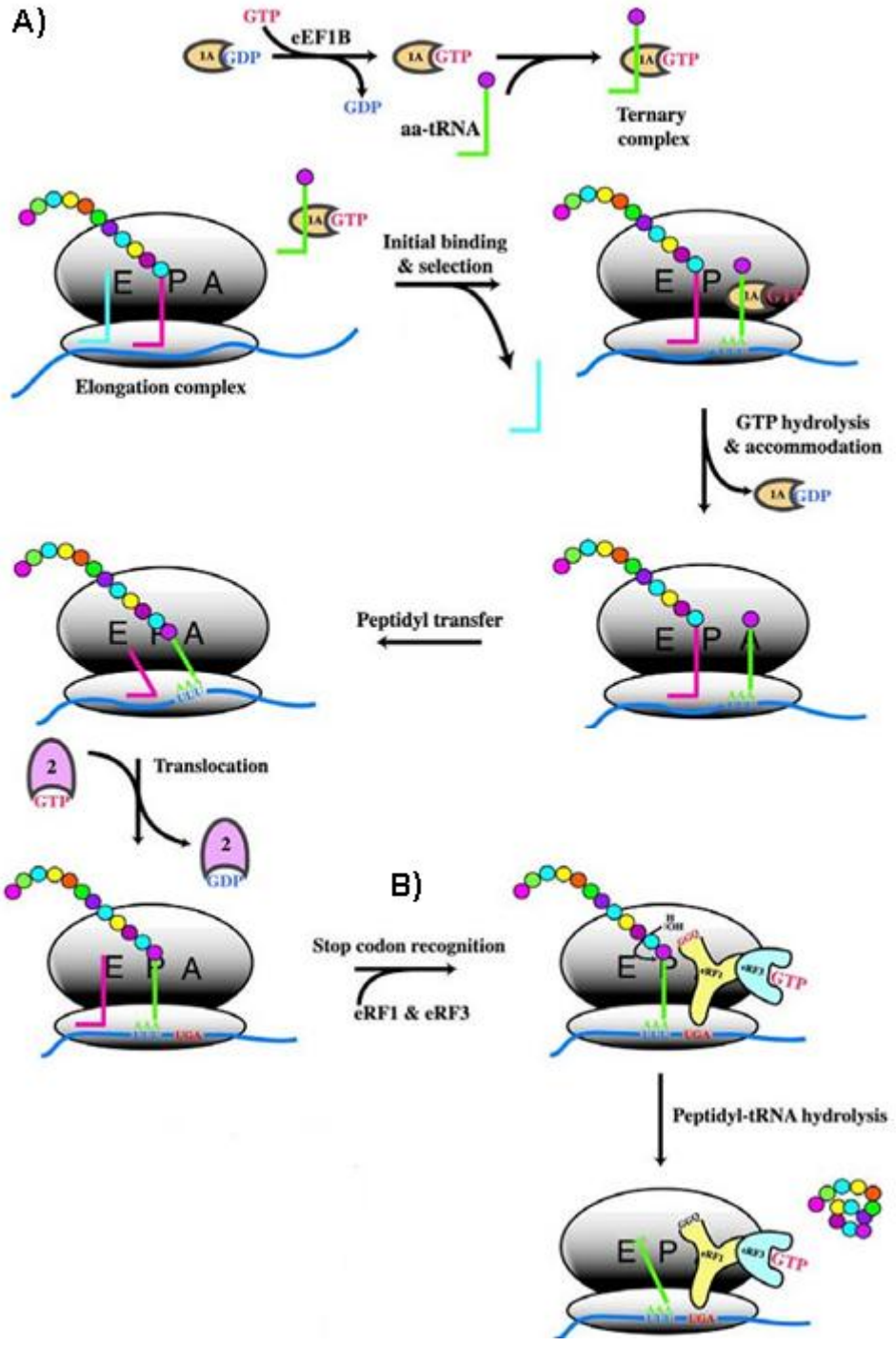


Figure 1.3: A model of translation elongation (A), termination (B) in eukaryotes. A) The ternary complex is formed by eEF1A, GTP and aminoacyl-tRNA (aa-tRNA), in presence eEF1B. This complex is selected by the ribosome and binds to the ribosomal A-site, through correct mRNA codon and tRNA anticodon interaction. This activates the GTPase activity of the ribosome and eEF1•GDP is released by GTP hydrolysis. Then, aa-tRNA is accommodated and polypeptide chain is transferred from the peptidyl-tRNA in the

ribosomal P-site to the aa-tRNA into the A-site. Next, the peptidyl-tRNA and the deacylated tRNA are translocated respectively from the A- and P-site into the P- and E-sites, with GTP hydrolysis by eEF2. **B)** The eRF1•eRF3•GTP ternary complex binds to ribosomal A-site and recognizes one of the three stop codons of the mRNA. The ternary complex promotes hydrolysis of the peptidyl-tRNA bond, releasing the polypeptide chain [adapted and modified from (Kapp and Lorsch, 2004)].

1.3.2. Transfer RNA (tRNAs)

tRNAs are small RNA molecules of 75 to 95 nucleotides in length. During mRNA translation, tRNAs are aminoacylated with cognate amino acids, which are then transferred to a growing polypeptide chain, in response to base pairing between codons of the mRNA and complementary tRNA anticodons.

tRNAs can be grouped in 20 families of isoacceptors which correspond to 20 cognate amino acids. Each family of isoacceptors is recognized by a single aaRS that charges them with cognate amino acids. Isoacceptor tRNAs have different anticodons that recognize different mRNA codons of the same amino acid family. Ideally, each organism should have 61 tRNAs, one for each sense codon, but some organisms have less than 61 tRNAs. This is explained by the wobble hypothesis, which postulates that the first two bases of codons pair correctly with the last two base of tRNA anticodons, according to Watson-Crick base pairing rules, while the first base of tRNA anticodons can pair with more than one base in last base position of codons through non-Watson-Crick base pairing (Crick, 1966b). Consequently, the same aa-tRNA anticodon can recognize more than one codon, reducing the number of tRNAs required to translate the genetic code. For example, 46 tRNAs in *Encephalitozoon cuniculi* decode the 20 amino acids (Genomic tRNA database).

1.3.2.1 tRNA structure

All tRNAs discovered to date, about 74,000 in 740 species (Chan and Lowe, 2009), have a cloverleaf secondary structure (Figure 1.4), which was first predicted by Holley (Holley, 1965). This structure consists of three arms composed of a D-arm (**D** is dihydrouridine), an anticodon-arm and a TΨC-arm (**T** is ribothymidine and **Ψ** is pseudouridine). This structure

also contains a variable region and an acceptor stem with the 3´single-stranded **N**CCA$_{OH}$ end (**N** is any nucleotide at position 73), where the amino acids are attached. The D-arm has a stem of 3 or 4 base pairs (in class I and class II of tRNAs, respectively) and a loop of 8 to 11 unpaired bases. The anticodon-arm and TΨC-arm have a stem of five base pairs and a loop of 7 unpaired bases. The anticodon is located in the center of the anticodon loop between positions 34-36. The acceptor stem is longer than the other stems with seven base pairs. The variable region has a variable number of nucleotides, and its length differentiates tRNAs in two families. Class I tRNAs comprise the majority of tRNAs, which have a short variable loop of 4 or 5 bases, while class II tRNAs have a long variable arm of 10-24 bases. This last class is formed by Leu and Ser tRNAs in eukaryotes, and in bacteria by Leu, Ser and Tyr tRNAs (Dirheimer et al., 1995a).

The cloverleaf structure can assume an L-shaped three-dimensional structure, where tRNA has a high stability. The acceptor stem is stacked onto the TΨC-arm, while D-arm is stacked onto anticodon-arm, thus defining two functional domains. One of the domains has an amino acid attachment site and the other has the anticodon, which are at the opposite ends of the tRNA, separated by 75 Å. This L-shaped structure is only maintained with base paring of the conserved and semiconserved nucleotides between D-loop and TΨC-loop, and D-loop and variable region. For example, in *E. coli*, the tRNA^{Cys} has G15•G48 base, and all other tRNAs contain a G15•C48 or an A15•U48 that establish the tertiary interaction known as the *Levitt base pair* (Dirheimer et al., 1995b; Hou et al., 1993).

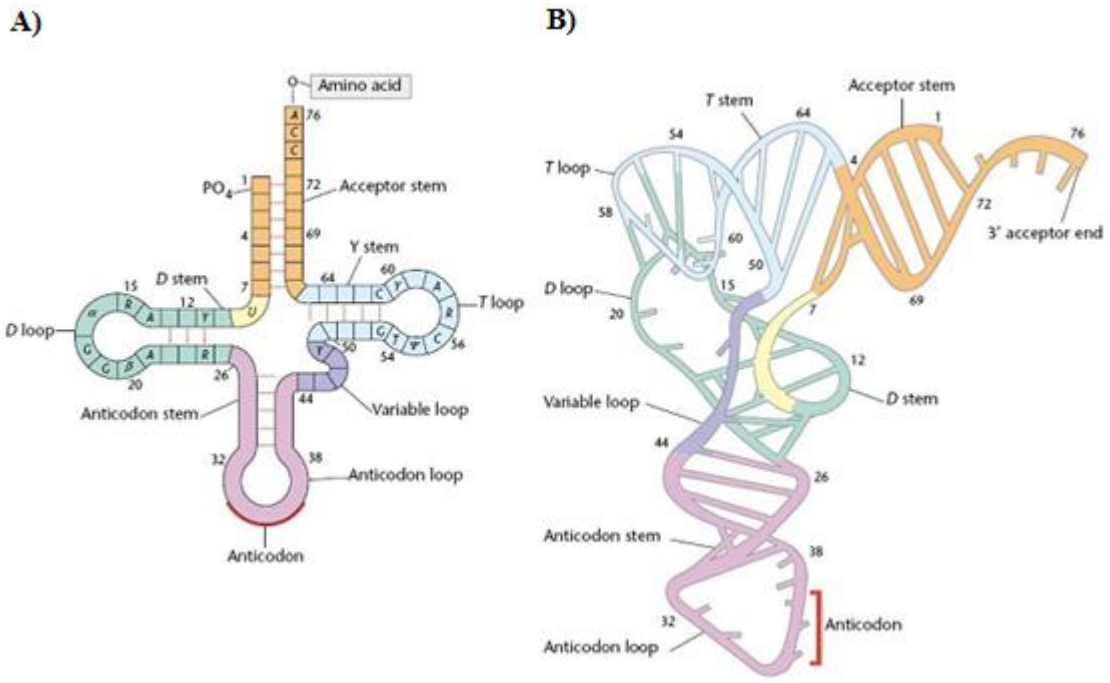


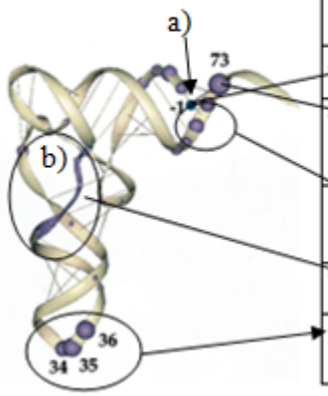
Figure 1.4: The structures and domains of tRNA. A) The cloverleaf secondary structure of tRNA. The conserved nucleotides are indicated. **B)** The L-shaped tertiary structure of tRNA. The colors correspond to the domains indicated in A. The orange color is acceptor stem, the green water color is D-arm, the rose color is anticodon-arm, the purple color is variable region and blue water color is T-arm [adapted from (Goldman, 2002)].

1.3.2.2 Identity elements

Each family of tRNAs is recognized by one of the 20 aaRS. This recognition is possible due to identity elements present in tRNA, which are divided in two types: identity determinants (positive elements) and anti-determinants (negative elements). The identity determinants contribute to the specific recognition and aminoacylation of tRNA by cognate aaRS, while the anti-determinants prevent the recognition and aminoacylation of tRNA by non-cognate aaRSs (Crothers et al., 1972; McClain, 1993).

The identity determinants are located mainly in the anticodon, in the unpaired base at position 73 (discriminator base) and in three first base pairs (1-72, 2-71 and 3-70) of the acceptor stem, where they vary from one tRNA to another. In other cases, the positive elements can be located in the variable region (ex: $tRNA^{Ser}$) and in nucleotide -1 (ex:

tRNA^{His}) - Figure 1.5.



Identity Elements for aaRS		
	Class I	Class II
Nucleotide -1		His
Discriminator base (position 73)	Cys, Glu, Ile, Leu, Met, Gln, Arg, Val, Trp, Tyr	Ala, Asp, Phe, Gly, His, Lys, Asn, Pro, Ser, Thr
Acceptor stem	Met, Gln, Val, Trp	Ala, Asp, Phe, Gly, His, Lys, Ser, Thr
Variable region		Ser
Anticodon	Cys, Glu, Ile, Met, Gln, Arg, Val, Trp, Tyr	Asp, Phe, Gly, His, Lys, Asn, Pro, Thr

Figure 1.5: Distribution of the identity elements in the structure of tRNAs. The -1 nucleotide (a) is important for His identity, while the variable domain (b) indicates its participation in Ser identity [adapted and modified from (Giege et al., 1998)].

The tRNA identity elements change between tRNA families within the same organism, and between the same tRNA family of different organisms. For example, in *E. coli*, almost all families of tRNAs have identity elements in the anticodon, except in the tRNA^{Leu}, tRNA^{Ser} and tRNA^{Ala} families (Figure 1.5). This is because the tRNA^{Leu} and tRNA^{Ser} families decode six codons, and tRNA^{Ala} family decodes four codons.

Each tRNA family has its own discriminator base, and most tRNAs accepting chemically similar amino acids are characterized by an identical residue at this position (Crothers et al., 1972).

Table 1.2: A few examples of anti-determinants. Adapted from (Giege et al., 1998)

Anti-determinants	In tRNA	Against aaRS
Lysidine$_{34}$ (modified C)	tRNA^{Ile} (*E. coli*)	MetRS
A$_{36}$	tRNA^{Arg} (*E. coli*)	TrpRS
U$_{34}$	tRNA^{Ile} (*yeast*)	MetRS
G$_{37}$	tRNA^{Ser} (*yeast*)	LeuRS
A$_{73}$	tRNA^{Leu} (*human*)	SerRS
m^{1}G$_{37}$ (methylated G)	tRNA^{Asp} (*yeast*)	ArgRS
G$_3$•U$_{70}$	tRNA^{Ala} (*yeast*)	ThrRS
U$_{30}$•G$_{40}$	tRNA^{Ile} (*yeast*)	GlnRS/LysRS

The identity elements can be modified or unmodified nucleotides. Some of the tRNA determinants for cognate aaRSs may act as anti-determinants for other aaRSs. Several examples of anti-determinants are listed in Table 1.2.

1.3.2.3 Modified bases

Eighty percent of the modifications known in RNA molecules, occur in tRNAs (Engelke and Hopper, 2006). Up to now, the tRNA contains more than 100 different base modifications (Globisch et al., 2011) which are introduced post-transcriptionally, with the exception of queuosine (**Q**). The major diversity of modifications is found in the anticodon loop of the tRNA, especially in the first anticodon position (**N**34) and in 3´anticodon position (**N**37). The anticodon region is also the only structural domain that contains hypermodified bases, where **Q**, in substitution of **G**, is found at position 34 in Tyr, His, Asn and Asp tRNAs and the **G** derivative wybutosine is found at position 37 in tRNAs^{Phe} (Agris, 2004). These modifications in anticodon are important for codon recognition and efficiency of cognate aminoacylation (Yokoyama et al., 1985). Methylation is evenly distributed over the entire tRNA structure (Brahmachari and Ramakrishnan, 1984).

Surprisingly, in some stress conditions, as in presence of hydrogen peroxide, the tRNAs can lose their modified nucleotides, due to decrease expression of modifying enzymes (Chan et al., 2010).

The modified base at the first base pair of the anticodon (position 34) that interacts with the third base pair of the codon alters the decoding properties of the tRNA. For example, inosine (**I**) is a purine formed by the deamination of **A**, common at position 34 of eukaryotic tRNAs and can base pair with **A**, **U** and **C**; and the **Q** pairs with all four nucleotide (**A**, **U**, **C**, **G**). In this case, the wobble interaction (describe in section 1.3.2) occurs and the first base of the anticodon can pair with more than one nucleotide located in the last base of the codon through Watson-Crick base pairing or non-Watson-Crick base pairing (Figure 1.6).

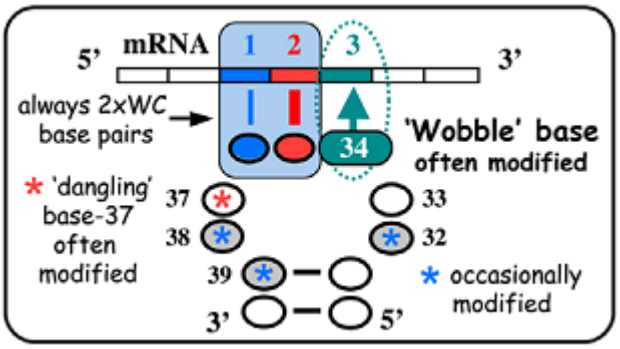


Figure 1.6: The anticodon-codon interaction. Base 1 (blue) and 2 (red) of the codon are paired to base 36 and 35 of the anticodon through Watson-Crick (WC) base pairing, respectively. Base 3 of the codon is paired to base 34 (cyan, the ‘‘wobble” base) of the anticodon. The wobble base 34 and the base 37 are often modified, while bases 32, 38 and 39 are occasionally modified. Adapted from (Grosjean et al., 2010).

Modified bases at position 37 strengthens the base pairing between the last base of the anticodon (position 36) and the first base of the codon, being the case of isopentenyladenosine (**i^{6}A**) and threonylcarbamoyladenosine (**t^{6}A**) in tRNAs that read codons starting with **U** and **A**, respectively. However, the most conserved modification found in position 37 is the 1-methylguanosine (**m^{1}G**) in tRNAs that decode codons starting with **C**. This modification acts as an identity anti-determinant for ArgRS, in *yeast* (Table

1.2).

1.3.3. Aminoacyl-tRNA synthetases (aaRSs)

Aminoacyl-tRNA synthetases (ARSs) are enzymes that catalyze the charging of cognate amino acids to their cognate tRNA. Each amino acid and isoacceptor tRNA (tRNAs of the same family) is recognized by their specific aaRS.

1.3.3.1 Aminoacylation reaction and aaRS editing mechanisms

The aminoacylation reaction by aaRS is a two step reaction. First, the ligation of the cognate amino acid (aa) to the active site of the aaRS is activated with ATP•Mg^{2+} to form an aminoacyl adenylate, releasing inorganic pyrophosphate (PPi) – Equation 1. Subsequently, the active amino acid is transferred, with the formation of an ester bond, at the 3´ end of the cognate tRNA, thus generating aa-tRNA and adenosine monophosphate (AMP) – Equation 2 (Figure 1.7: A) (Ibba and Soll, 2000).

Equation 1- Activation: aa + ATP•Mg^{2+} + aaRS→aaRS•aa•AMP + PPi•Mg^{2+}

Equation 2- Transfer: aaRS•aa•AMP + tRNA→ aa•tRNA + AMP +aaRS

The correct selection of an amino acid and its attachment to the appropriate tRNA by aaRSs guarantees the fidelity of the translation process, but the errors in translation of the genetic code can occur at a significant rate. Error rate in tRNA selection by aaRSs is 10^{-6} or lower and in amino acid selection by aaRSs is 10^{-4} to 10^{-5} [reviewed in (Jakubowski and Goldman, 1992)]. The error rate in tRNA selection is lower than the error rate in amino acid selection. It is because tRNA has a large contact surface area that allows easy establishment of molecular interactions between identity elements of the tRNA and domains of the aaRS. Thus, the correct tRNA is easily recognized by specific aaRS. The discrimination of the cognate amino acid by aaRS is more difficult, because amino acids have a small size and share very similar structure and chemical proprieties. [(Bacher et al., 2005); reviewed in (Reynolds et al., 2010); (Ling et al., 2009)]. To prevent mischarged

tRNA with near- and non-cognate amino acids, the aaRSs use editing mechanisms. These mechanisms can occur before or after the transfer of the amino acids to the tRNA, namely pre-transfer editing or post-transfer, respectively [reviewed in (Reynolds et al., 2010)].

Pre-transfer editing involves mechanisms that promote hydrolysis of the misactived aminoacyl adenylate, releasing the non-cognate amino acid, AMP and PPi. In the post-transfer editing, the misacylated tRNA may be translocated from active site into the editing site of the aaRS, where the tRNA–amino acid ester linkage is hydrolyzed, releasing tRNA and amino acid. If the misacylated tRNA is released from the aaRS without being edited, it is subjected to hydrolysis by specific proteins named trans-editing factors. Finally, misacylated tRNAs can be discriminated by elongation factors (eEF-1A), which bind them too weakly for efficient delivery and release in the ribosome (Figure 1.7 B) [reviewed in (Reynolds et al., 2010)].

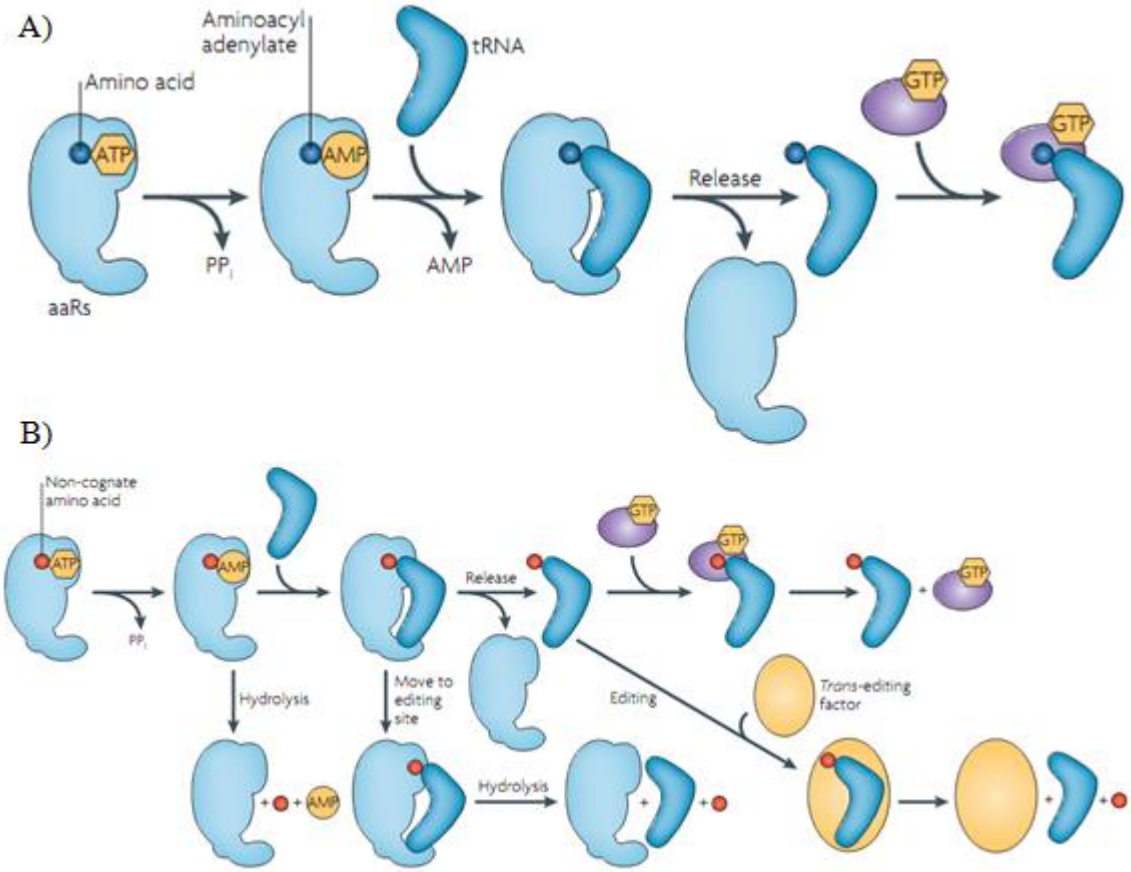


Figure 1.7: Some steps in translation quality control. A) Aminoacylation reaction. The cognate amino acid (blue) is activated at the aminoacyl-tRNA synthetase (aaRS) active site, in the presence of ATP, to form the aminoacyl adenylate with release of inorganic pyrophosphate (PPi). Then, the cognate tRNA binds to the aaRS, and the amino acid is attached to the 3′ end of the tRNA with release of AMP. The aminoacyl-tRNA (aa-tRNA) is released and binds to the elongation factor (purple) associated with GTP which is used in elongation of translation. **B) Editing mechanisms of aaRS: pre-transfer editing and post-transfer editing**. After activation of the non-cognate amino acid (red), the aminoacyl adenylate may be hydrolyzed and released (pre-transfer editing). The non-cognate aa-tRNA may be moved to the editing site of the aaRS for hydrolysis. If the mischarged aa-tRNA is formed and released from the aaRS, it may be subjected to editing by trans-editing factors. Finally, the non-cognate aa-tRNA can also be recognizing by elongation factor, but the ligation may be too weak that the non-cognate aa-tRNA may be released from the ribosome. Adapted and modified from (Reynolds et al., 2010).

1.3.3.2 Two classes of aaRS

The 20 aaRSs are divided into two classes (class I and II) of 10 enzymes each, where each

class is originated from an independent ancestor. This class division is based on the active site (catalytic domain) architecture of the aaRSs and on their catalytic domains, which have a large structural diversity in secondary, tertiary and quaternary structures. For example, class I synthetases are generally monomers while class II are dimmers (Figure 1.8).

The class division is very rigid and it is almost impossible to change the enzymes between classes. However, the LysRS in some organisms belongs to class I, while in most cases it is a typical class II enzyme – Figure 1.8 (Schimmel, 2008).

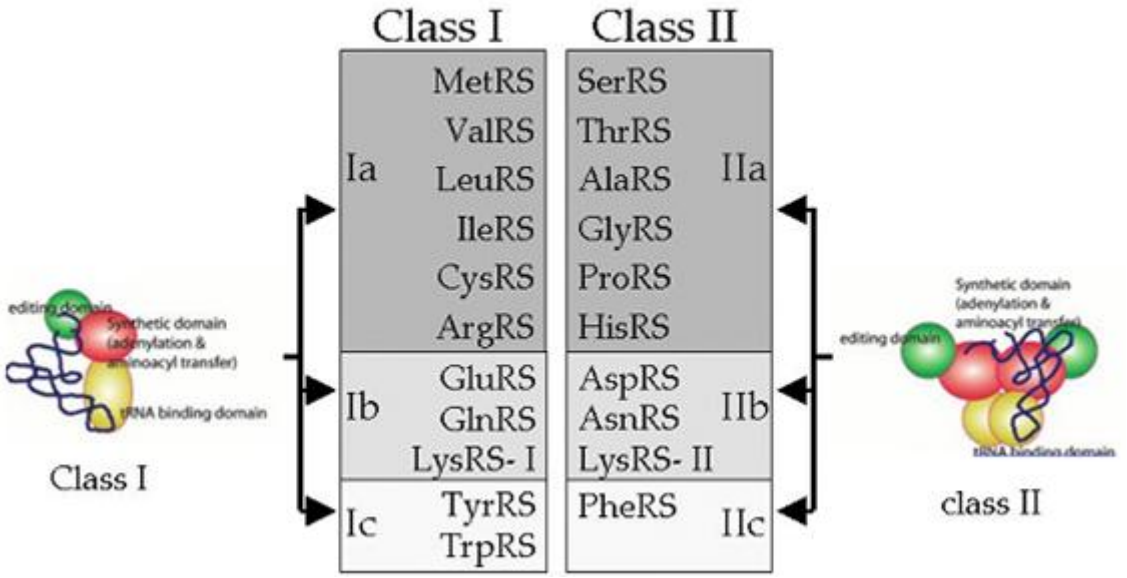


Figure 1.8: Two subclasses of aminoacyl-tRNA synthetases that evolved from two independent single-domain proteins. The class I of the aaRS is represented by IleRS and class II of the aaRS is represented by ThrRS. Adapted and modified from (Bori-Sanz, 2007; Francklyn, 2008).

Class I enzymes are characterized by an active site domain containing a Rossmann nucleotide-binding fold composed of alternating β-strands and α-helices with the conserved peptidic motifs HIGH and KMSKS, which are responsible for the interaction with subtracts. These synthetases can be divided into three subclasses, Ia, Ib and Ic, and the enzymes in each subclass generally recognize chemically and sterically similar amino acids. The class Ia enzymes recognize hydrophobic amino acids, such as the branched aliphatics (Ile, Leu, and Val), amino acids with sulfur-containing residues (Met and Cys)

and also Arg. The class Ib enzymes recognize the charged amino acid Glu and its derivate Gln. The class Ic enzymes recognize the aromatic amino acids (Tyr and Trp) – Figure 1.8.

Class II enzymes contain in their active sites, seven-stranded β-sheets with flanking α-helices with three degenerate sequence motifs, a N-terminal motif 1 which is responsible for subunits interaction with the dimmer, a central motif 2 and a C-terminal motif 3 which are responsible for the interaction with subtracts. Class II enzymes show similarities with their class I counterparts, being also divided into three subclasses. Subclass IIa enzymes recognize hydrophobic amino acids (Ala, Pro, Gly) and polar (Ser, Thr) amino acids as well as His. Subclass IIb enzymes recognize charged amino acids (Asp, Lys) and Asp derivative (Asn), whereas subclass IIc synthetase recognizes the aromatic Phe – Figure 1.8.

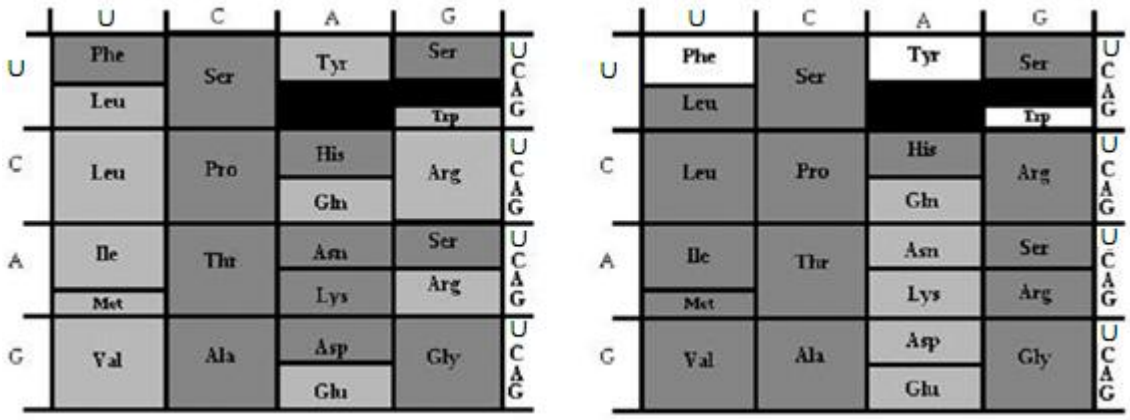


Figure 1.9: The genetic code. On the left, codons are colored according to the class of the synthetase (class I in light gray, class II in dark gray). For simplicity, the lysine codon is colored as class II. On the right, codons are colored according to the subclasses of the aaRs (Ia–IIa in dark gray, Ib–IIb in light gray, Ic–IIc in white). Stop codons are colored in black. Adapted and modified from (Bori-Sanz, 2007).

The two classes and the respective three subclasses of synthetases can be organized in the genetic code. Thus, the codons standard distribution, with the amino acid assignment, can be ordered according to the proposed pairings of synthetase classes and subclasses (Figure 1.9). Specific patterns can be observed on the left of the Figure 1.9, such as a **U** at the second base of the codon corresponding almost exclusively to class I enzymes, and a **C** for class II enzymes. By observing the right panel of Figure 1.9 we can consider that

subclasses Ic and IIc recognize aromatic residues and their codons share the same first base (**U**), and subclasses Ib and IIb recognize charged amino acids and the derivatives with same second base (**A**) for their codons. In the largest subclasses – Ia and IIa – a distinct pattern of second-base relationships is noticed. Four subclass Ia enzymes have a **U** at the second position of the codon, while four subclass IIa enzymes have a **C** at the middle position (Ribas de Pouplana and Schimmel, 2001).

The hypothesis of a common ancestor for the synthetases of the same class is manifested mechanistically in the aminoacylation reaction. During the first step of the aminoacylation, the conformation of ATP bound is different. In the class I enzymes the ATP is in a straight conformation, while in class II enzymes the ATP exhibits a bent conformation. Also, during the second step of the reaction, the class I synthetases systematically aminoacylate the 2'-hydroxyl (OH) group of the last ribose of the tRNA and then move the amino acid to the 3´-OH group, while class II enzymes aminoacylate the 3'-OH group (Cavarelli and Moras, 1993; Woese et al., 2000). These differences in the reaction mechanism are a direct consequence of the manner that aaRSs bind to tRNA. Class I enzymes approach the acceptor arm of tRNA through the minor groove side and bind on D-loop side, whereas class II aaRSs interact with the major groove side of the acceptor arm of the tRNA and bind on the variable-loop side.

1.4. Mistranslation – errors in protein synthesis

The high efficiency and accuracy in DNA replication, DNA transcription and mRNA translation are fundamental to maintain cellular fitness, homeostasis and survival in all organisms. This assures the production of stable and functional proteomes. However, errors in DNA replication, DNA transcription and mRNA translation occur, albeit a low level. The DNA replication error rate is estimated at 10^{-8} to 10^{-11} [reviewed in (Parker, 1989)], whereas the DNA transcription error rate is in order of 10^{-4} to 10^{-6} (Edelmann and Gallant, 1977). The mRNA translation error rate in the range of 10^{-3} to 10^{-4} (one error in 1000 to 10000 codons translated) (Ogle and Ramakrishnan, 2005). So, DNA replication and DNA transcription have lower error rates than mRNA translation. The errors in mRNA

translation (mistranslation) can result from incorrect aminoacylation of tRNAs by aaRSs, from incorrect selection of the aa-tRNA by the ribosome and from incorrect mRNA decoding by the ribosome, such as frameshifting errors, processivity errors, nonsense errors and missense errors. Mistranslation can be the source for genetic code ambiguity and, subsequently of aberrant proteins (Drummond et al., 2005; Geslain et al., 2010; Nangle et al., 2006).

The incorrect aminoacylation may be caused by the failure of aaRSs to recognize their cognate tRNA or by the inability to differentiate between amino acids with similar chemical properties. In cells, the quality control mechanisms (as describe in section 1.3.3.1, in aaRSs editing) reduce mischarging, but tRNA aminoacylation errors occur at 10^{-4} to 10^{-5} (Francklyn, 2008).

Translational frameshifting errors (shift in the mRNA reading frame) alter the reading frame to the -1 or to the +1 frames by tRNA splippage during elongation. The erroneous product might be longer or shorter, depending on the position of the nearest stop codon in the new frame. But, normally, frameshifting errors lead to premature termination of translation. This alteration in mRNA reading frame has a frequency of 10^{-5} per codon (Huang et al., 2009). The majority of processivity errors, lead to premature drop-off of the peptidyl-tRNAs from the ribosome. In rare cases, these errors can be caused by failure in stop codon recognition (termination readthrough), leading to the production of mutant proteins with extended C-termini. The frequency of this error is around 4×10^{-4} (four drop-offs in 10.000 amino acid incorporations). The nonsense error is a false stop that causes premature termination of translation. A false stop is a rare event with a probability of about 10^{-6} per codon. In these three errors, the premature termination of translation, normally results in production of truncated and frequently non-functional proteins. Missense errors are mistakes in the decoding of a sense codon leading to the incorporation of an incorrect amino acid, which result in production of mutant proteins. Misreading occurs more often in the third nucleotide of a codon (wobble position), followed by the first nucleotide. However, misreading in the second codon base is almost undetectable, due to its importance for the decoding process. Errors in third codon base normally lead to

incorporation of the same amino acid or one with similar chemical proprieties that do not disrupt protein structure and function. Nevertheless, 1 in about 400 missense errors will affect folding, structure and/or function of proteins and do have an impact on cell fitness. These errors are the result of a mischarged tRNA or of an anticodon-codon mismatch in the ribosome (Parker, 1989). The frequency of missense errors was estimated at 3×10^{-4} per codon decoded (Drummond and Wilke, 2008; Moura et al., 2009; Nierhaus, 2006).

The protein synthesis error frequency also increases due to the high speed of the process, where 4 to 8 amino acids are incorporated into peptides per second (Ruusala et al., 1982; Thompson and Stone, 1977). Also, translation errors may increase under stress conditions, namely carbon and amino acid starvation due to reduction in the pool of charged tRNAs (Fredriksson et al., 2007; Parker and Precup, 1986).

1.5. Mistranslation - Negative and positive effects

Mistranslation can produce aberrant proteins (mistranslated protein), although their impact on the cell fitness may be variable. Mistranslated proteins can maintain the wild type structure and function, or can retain wild type structure and loose its function (altered protein), or can fold abnormally (misfolded/unfolded protein) and loose function. Misfolded/Unfolded proteins can lead to direct toxicity, by destabilizing membranes of cells or due to inappropriate interactions with other cellular components. Also, these can increase sensitivity to stress (Drummond and Wilke, 2008). The hypothesis that cells are sensitive to cytotoxic misfolded proteins has been studied as a contributor to neurodegenerative diseases. For example, mistranslation generated by a single missense mutation in the editing domain of the alanyl-tRNA synthetase (AlaRS), which misacylates $tRNA^{Ala}$ with Ser induces Purkinje cell loss and ataxia in the brain of a mouse model. In particular, these cells die due to intracellular accumulation of unfolded/misfolded proteins that is accompanied by failure of the mechanism of protein quality control (Lee et al., 2006). In all cases, mistranslation results in activation of quality control mechanisms, i.e. protective mechanisms of cells to minimize mistranslation, such as the upregulation of cytoplasmic protein chaperones, the heat-shock response, the unfolded protein response

(UPR) in the endoplasmic reticulum (ER), the activation ubiquitin-proteasome system (UPS), the chaperone-mediated autophagy and eventually cell death via apoptosis. Firstly, misfolded/unfolded proteins promote the heat-shock response by enhancing expression of heat-shock proteins (Hsp-one type of the chaperones), which refold various misfolded proteins. The expression of Hsps in response to elevated temperatures or other cellular stresses reduces protein aggregation, but when the proteins cannot refold, they may be targeted with ubiquitin for degradation by the proteasome (Geslain et al., 2010; Lee et al., 2006).

In mammalian cells, a mutation in the editing domain of ValRS causes dramatic changes in cell phenotype. In this case, apoptotic response occurs and is characterized by cell contraction, membrane blebbing and caspase-3 activation (Nangle et al., 2006). In bacteria, the oxidative stress-induced mistranslation causes editing site defects of threonyl-tRNA synthetase (ThrRS), that misactivates serine (Ser), leading to Ser-tRNA^{Thr} formation. This results in accumulation of misfolded proteins, and in growth and viability defects. The lack of major heat-shock proteases severely exacerbates the growth defect caused by mistranslation (Ling et al., 2010). In general, mistranslation brings negative consequences to cells and decreases cell fitness, but quality control mechanisms protect cells against aberrant proteins. However, these mechanisms are not sufficient in order to prevent several neurodegenerative diseases, such as Alzheimer, Parkinson and Huntington that are characterized by presence of protein aggregates.

Surprisingly, mistranslation can also have positive effects in several organisms. For example, *C. albicans* contains a $tRNA_{CAG}$ that decodes CUG codon as Ser (major: 97%) and as Leu (minor: 3%) and can tolerate up to 28.1% of Leu misincorporation, which represents a 28,000-fold increase in decoding error. Wild-type *C. albicans* cells misincorporate Leu at 3.9% to 4.95% under stress conditions. This CUG codon ambiguity expands exponentially the proteome size and generates phenotypic diversity in *C. albicans* (Gomes et al., 2007). Miranda and colleagues support the hypothesis that genetic code alterations speed up evolution of new phenotypes due to increased phenotypic diversity (Miranda et al., 2007). Also, codon ambiguity is noticeably detrimental to growth, but there

is no indication of cell death and under some stressful environmental conditions such ambiguity can be advantageous (Gomes et al., 2007; Santos et al., 1999; Santos et al., 1996; Santos et al., 1997; Silva et al., 2007). In *S. cerevisiae,* genetic code ambiguity reprogrammed genes belonging to the stress response, general metabolism, protein synthesis, folding and degradation pathways, increases proteasome activity and pre-adapts cells to tolerate adverse growth conditions. Such ambiguity induces the accumulation of carbohydrate reserves like glycogen and trehalose. Moreover, alterations in gene expression and physiological remodeling creates stress tolerance and stress cross-protection that allows cells overcome the negative impact of proteome disruption (Silva et al., 2007). So, codon ambiguity has the capacity to generate new adaptive traits, under certain physiological conditions (Gomes et al., 2007). Interestingly, in *C. albicans* each stress response is unique, there is no stress cross-protection and glycogen and trehalose do not accumulate in stressed *C. albicans* cells (Enjalbert et al., 2003).

Finally, mutations in the editing site of IleRS confer a growth rate advantage in *Acinetobacter baylyi* under limiting Ile conditions (Bacher et al., 2007) and misincorporation of Met into the proteome of mammalian cells is an apparent positive response to the exposure of the cells to various environmental stressors that generate ROS. This is apparently an adaptive response, where Met residues may be a protector of cells from the damage caused by oxidative stress (Netzer et al., 2009).

1.6. Stress response and Heat-shock protein

Every cell develops mechanisms, termed the adaptive response, to respond appropriately to unfavorable conditions. These unfavorable conditions that threaten survival promote the stress response. Cells respond to stress with transient changes in the expression of genes encoding “stress proteins”, namely molecular chaperones. So, these “stress proteins” are used for rapid molecular responses, in order to repair the damage and protect against additional exposure to stress. These mechanisms increase tolerance and maintain cell capacity to proliferate (Estruch, 2000).

A number of genomic studies show that stress responses are more robust in *C. albicans* than in the model yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [reviewed in (Quinn, 2007)]. For example, *C. albicans* is more resistant to oxidative and osmotic stress than *S. cerevisiae* and *S. pombe* (Nakamoto, 2009), but these fungi induce expression of similar functional genes in response to different types of stress, namely oxidative, osmotic or heavy metals (Enjalbert et al., 2003; Enjalbert et al., 2006). Also, the stress-responsive regulatory proteins that regulate transcriptional responses to stress are conserved between *C. albicans*, *S. cerevisiae* and *S. pombe*. Nevertheless, *C. albicans* has diverged significantly when compared with *S. cerevisiae* and *S. pombe* in nature and regulation of its general stress response, in particular in the stress cross-protection mechanism [reviewed in (Quinn, 2007)]. These general stress responses are characterized by the presence of genes that respond in a stereotypical manner to diverse stress conditions. As noted in the previous section, *C. albicans* does not have a common transcriptional response to several stress conditions which is characteristic of *S. cerevisiae* and *S. pombe* (Enjalbert et al., 2003). Nevertheless, stress cross protection was observed in *C. albicans* in response to a diverse range of stress conditions, such as osmotic, oxidative and heavy metal stress, where the Hog1 SAPK pathway is activated (Enjalbert et al., 2006).

The first step of response to stress is the synthesis of heat-shock proteins that control protein folding and refolding and keep protein complexes in a functionally competent state. The expression of these proteins occurs in response to heat shock, ethanol, nutritional stress and thermo tolerance followed by heat shock. In *Candida albicans*, heat shock from 23 to 37 ºC induces proteins at 18, 22, 40, 68 and 70 kDa (Dabrowa and Howard, 1984), while at 42 ºC induces the Hsps 28, 38, 47 and 60 kDa and predominantly Hsps with 70–110 kDa range (Franklyn and Warmington, 1994). In this organism, homologues of *S. cerevisiae*, Hsp90 (82 kDa), Ssa1/Hsp70 (74 kDa), Ssa4/Hsp70 (69 and 71 kDa), Ssb1/Kar2/Hsp70 (66 and 74-78 kDa), Ssz1/Pdr13/Hsp70 (66 kDa) and Hsp104 (99 kDa) have been identified (Pitarch et al., 2002). In *Candida psychrophila*, various Hsps were induced upon a temperature shift from 25 ºC to 37 ºC, namely Hsps with molecular mass of 80 and 110 kDa, while Hsps with a molecular mass of 60, 70 and 90 kDa were

expressed constitutively. These Hsps have a great diversity of functions, but their interactions contribute to the stress response. For example, mutants of *C. albicans* that accumulate misfolded/unfolded proteins induce heat-shock proteins, but also form insoluble protein aggregates to prevent exaggerated toxicity (Burnie et al., 2006). The intracellular aggregates can be disaggregated and dissolved by action of chaperones. Hsp104 is a stress tolerance factor that in cooperation with Hsp70 and Hsp40 releases polypeptide from protein aggregates and facilitates their refolding by Hsp90. Presumably, Hsp70 and Hsp40 alter the structure of a protein aggregate such that it represents a better substrate for Hsp104. However, the molecular details of this process are still poorly understood (Boesl et al., 2006; Glover and Lindquist, 1998).

1.7. The *Candida albicans* model system

Candida albicans is a fungus that normally resides asymptomatically in the gastrointestinal tracts of healthy humans and other warm-blooded healthy animals. However, it is also an opportunistic pathogen for hosts with compromised immune systems due to age, disease, or medical treatment. *C. albicans* can cause a range of medical problems, such as infections of the skin, nails, mucous membranes (oral and vaginal mucosa) and in the bloodstream. It is the most common human fungal pathogen, where the bloodstream infections lead to a mortality rate of 50% (Noble and Johnson, 2007).

C. albicans has the ability to grow in three different morphological forms, including budding yeast cells and filamentous forms that are either pseudohyphae or true hyphae (Figure 1.10). These distinct morphological forms have both common and specific features. The yeast cells are small, oval cells that can originate either pseudohyphae or hyphae. In pseudohyphal cells, the buds elongate and do not separate from the mother cell, retaining constrictions at the septal junctions. True hyphae develop from an unbudded yeast cell (also termed a blastospore) and have no constrictions at the septal junctions from the mother cell. Yeast forms grow at 30°C and in acidic pH (pH 4.0). Pseudohyphal forms grow at 35°C in pH 6.0 in the presence of high concentration of phosphate and nitrogen limitation. The hyphal form is believed to be more important for virulence, grows in

different environmental conditions, namely at 37°C, neutral pH (pH 7.0), in the presence of serum, in Lee´s medium, high CO_2, and N-acetylglucosamine. So, the hyphal cells support more severe conditions (higher temperature and pH) and pseudohyphae is considered an intermediate state between yeast and true hyphae (Sudbery et al., 2004).

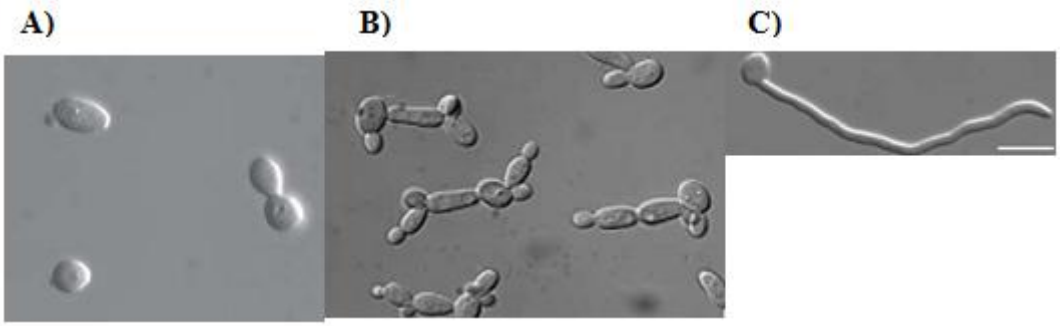


Figure 1.10: Different growth morphologies of *C. albicans*. A) Yeast morphology. **B)** Hyphal morphology. **C)** Pseudohyphal morphology. Adapted from (Sudbery et al., 2004).

C. albicans forms white, smooth and dome-shaped colonies in solid media. The cells within these "white" colonies are oval-shaped, and are able to form grey colored colonies at low frequency (10^{-4}), which have a flatten appearance on agar plates. The cells within these colonies have an elongated shape. This form is called "opaque" (Kim and Sudbery, 2011).

C. albicans is a diploid organism with eight chromosomes. The size of the *C. albicans* genome is 13.3-13.4 Mb encoding 6,100-6,200 genes (Kim and Sudbery, 2011). This organism lacks a complete sexual cycle, called parasexual cycle, where mating of diploid cells is followed by mitosis and chromosome loss instead of meiosis. This feature differentiates this species from the great majority of other fungal species. Diploid cells (2n) of *C. albicans* are typically heterozygous (**a**/**α**, white) at the **m**ating **t**ype **l**ocus (**MTL**). Strains normally lose their heterozygosity by loss of one copy of Chromosome (Chr) 5, eliminating the **a** or **α** allele, to create **α**/**α** or **a**/**a** diploid strains (2n, "white"), respectively. These homozygous strains at the MTL locus can undergo an epigenetic alteration from "white" to "opaque" cells, which involves transcriptional and morphology (referred above) alterations. The transition between "white-opaque" cells alters expression of more than 400

genes. When diploid **a/a** and **α/α** "opaque" cells mate form tetraploid **aa/αα** cells (4n, "white") which undergo mitosis, and concerted chromosome loss to return to diploid **a/α** cells (2n, "white"). This parasexual cycle is completed with no recognized meiosis (Figure 1.11). Thus, *C. albicans* genetic diversity and genome instability is due to mating and random chromosome loss (Lee et al., 2010; Lohse and Johnson, 2009; Noble and Johnson, 2007).

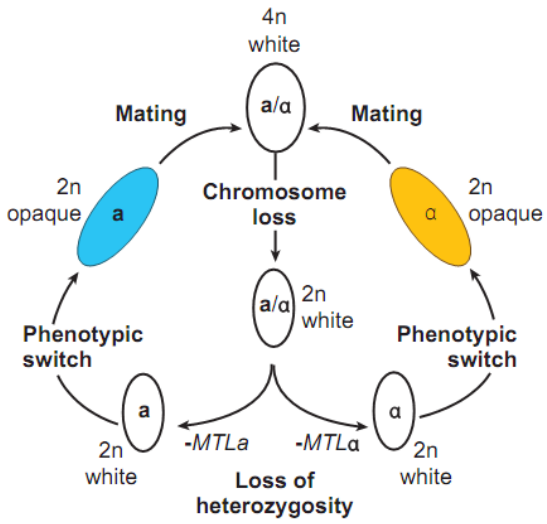


Figure 1.11: Parasexual cycle of *C. albicans*. The diploid (2n) **a/a** or **α/α** "opaque" cells mate to produce tetraploid (4n) **aa/αα** cells. These cells can lose chromosomes to return to the diploid (2n) state; conventional meiosis has not been observed in *C. albicans*. MTL is Mating Type Like locus. Adapted from (Noble and Johnson, 2007).

Therefore, the study of *C. albicans* pathogenesis requires the development of alternative genetic strategies to overcome the difficulties of the diploid genome, incomplete sexual cycle, lower frequency of the homologous recombination and its non-standard genetic code (Kim and Sudbery, 2011; Noble and Johnson, 2007). The new tools include: genome sequence, gene disruption technologies, microarray technology for expression profiling and mammalian animal model systems for both localized and disseminated infection (Noble and Johnson, 2005). The majority of homozygous knockout mutants of this organism have been generated with the selectable auxotrophic marker *URA3*. Deletion of a particular gene requires two successive transformations to delete both alleles of the target

gene with repeated use of the *URA3* selectable marker. In addition, the *URA3* marker can be recycled for disrupting the second allele of target genes by passage on medium containing 5-fluro-orotic acid (5-FOA), which selects intragenic recombinations that delete the *URA3* gene. Exposure of *C. albicans* to 5-FOA also generates chromosomal abnormalities, making this step a potential source of unwanted genetic changes. However, it is necessary to be careful when using the *URA3* marker because poor expression levels of *URA3*, which depends on chromosome position, can influence the virulence phenotypes, as well as morphogenesis and adhesion (Kim and Sudbery, 2011; Noble and Johnson, 2007). These complications lead to the development of a new strategy, using alternative auxotrophic markers. For example, a strain with deletions of the *ARG4*, *LEU2* and *HIS1* genes (Strain SN152) exhibits wild-type or nearly wild-type virulence in a mouse model. Thus, new disruption marker cassettes and a fusion PCR protocol permit rapid and highly efficient generation of homozygous knockout mutations in the new *C. albicans* strains (Noble and Johnson, 2005). Finally, protein visualization in situ can also be achieved using fusions with green fusion protein (GFP) or its derivatives.

A further complication arises from translation of the leucine CUG codon as serine (Santos et al., 1999; Santos and Tuite, 1995) which prevents the use of heterologous reporter genes in *C. albicans* and impairs gene replacement studies.

1.8. Objectives of the study

The main objective of this work was to study the effects of mRNA mistranslation in *C. albicans*. For this, we have created an artificial system of mistranslation based on induction of Ser misincorporation at eight codons belonging to eight distinct amino acids (Glu, Arg, Asn, Cys, Phe, Gln, His and Pro), through the insertion of alternative anticodons (CUC, CCU, GUU, GCA, GAA, CUG, GUG and GGG) in a $tRNA_{UGA}^{Ser}$ gene. In other words, we have engineered eight different mutagenic $tRNAs^{Ser}$ that could misincorporate Ser at those codons.

In order to study the impact of mistranslation on *C. albicans* we have:

- Checked expression and stability of the mutant tRNAs by Northern blot analysis;
- Studied effects of mistranslation in cell fitness;
- Characterized the mistranslation strains using a phenotypic screening;
- Studied the formation of protein aggregates using a Hsp104-GFP reporter system.

2.Materials and Methods

2.1. Strains and Growth Conditions

Escherichia coli strain JM109 (endA1, recA1, *gyr*A96, *thi, hsd*R17(r_k^-, m_k^+), *rel*A1, *sup*E44, Δ[*lac-pro*A], F´[*tra*D36, *pro*AB, *laq*I^qZΔM15]) and *E. coli* strain DH5α (F^-, φ80d*lacZ*ΔM15, Δ[*lacZYA-argF*]U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1) were used as hosts for manipulation of recombinant DNAs. These strains were grown at 37ºC on nutritionally rich medium LB [1% (w/v) peptone from casein, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride (NaCl); (Merck)] or in LB/2% (w/v) agar. Transformed *E. coli* strains were grown in the LB supplemented with 75 μg/ml of ampicillin sodium (Sigma-Aldrich). Strains were preserved at -80ºC in LB-Amp/20% (v/v) glycerol.

Candida albicans strain SN148 (arg4Δ/arg4Δ, leu2Δ/leu2Δ, his1Δ/his1Δ, ura3Δ::imm^{434}/ura3Δ::imm^{434}, iro1Δ::imm^{434}/iro1Δ::imm^{434}) (Noble and Johnson, 2005) was grown at 30°C on liquid complete medium YEPD [2% (w/v) glucose; 1% (w/v) yeast extract, 1% (w/v) peptone], or in YEPD/2% (w/v) agar. Transformed *C. albicans* strains were grown in minimal medium without uridine (MM-URI) [0.67% (w/v) yeast nitrogen

base without amino acids, 2% (w/v) glucose, with 100 µg/ml of each essential amino acids, without uridine], and without uridine and leucine (MM-URI-LEU) [0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, with 100 µg/ml of each essential amino acids, without uridine and leucine], or in MM-URI or MM-URI-LEU/2% (w/v) agar. Strains were preserved at -80ºC in MM-URI or MM-URI-LEU/40% (v/v) glycerol.

For phenotypic screens, mistranslating strains were incubated and grown in MM-URI agar plates supplemented with the indicated different environmental stress conditions – Table 2.1.

Table 2.1: Growth conditions used for characterization of *C. albicans* mistranslating strains.

Conditions tested	Medium Supplementation
Control	MM-URI without any supplementation, at 30 ºC
Temperature	25 ºC, 37 ºC, 42 ºC
Antifungal Drugs	Fluconazole (0.5 µg/ml), Itraconazole (0.5 µg/ml)
Cell wall stress	Calcofluor white (20 µM)
Metal/Toxicity	Lithium chloride (300 mM)
Protein denaturation	Guanidine hydrochloride (5 mM), Urea (25 mM)
Alkalinity	pH 8.6
Ionic/osmotic/toxicity	Calcium chloride (300 mM), Sodium chloride (1.3 M)
Ionic/osmotic/carbon source/toxicity	Sorbitol (1.5 M)
Carbon source/toxicity	Substitution of glucose by ethanol 2% (w/v)
Carbon source	Without glucose (C-absent), substitution of glucose by [Glicerol (3% (w/v)), Galactose (2% (w/v))]

2.2. DNA Manipulations

2.2.1. Oligonucleotides

Oligonucleotides were purchased from IDT (Integrate DNA Technologies, Belgium) and

STABvida (Portugal). These were resuspended in ultra pure MilliQ (MQ) water to a final concentration of the 100 pmol/µl.

Table 2.2: Oligonucleotides sequences used to construct pUA850 containing the tRNA^{Ser} gene and other plasmids with tRNA^{Ser} mutant genes, with respective temperature melting (Tm). The green shading indicates sequence of *XhoI* restriction enzyme. The gray shading indicates site-directed-mutagenesis to change the anticodon UGA of tRNA^{Ser} to new anticodons of tRNAs^{Ser}.

Oligo	Sequence 5´-3´	Tm (ºC)
Site-directed-mutagenesis of pUA526 plasmid cut site to *XhoI*		
Oli_Di 6	GTACAATTCATCCATACTCGAGGTAATACCAGCAGCAG	62.8
Oli_Di 7	CTGCTGCTGGTATTACCTCGAGTATGGATGAATTGTAC	62.8
Confirmation of Site-directed-mutagenesis of the pUA526 plasmid		
oUA1511	GCCAGAAGGTTATGTTCAAG	55.3
Confirmation of the construction of pUA850 plasmid by colony PCR and sequencing		
oUA1617	CCAGGCTTTACACTTTATGC	51.5
oUA1556	GGTATAGAAATGCTGGTTGG	50.8
Site-directed-mutagenesis of tRNA$_{UGA(Ser)}^{Ser}$ for tRNA$_{CUC(Glu)}^{Ser}$		
oUA1771	TAAGGCGACAGACGCTCAATCTGTTGGGCTC	65.9
oUA1772	GAGCCCAACAGATTGAGCGTCTGTCGCCTTA	65.9
Site-directed-mutagenesis of tRNA$_{UGA(Ser)}^{Ser}$ for tRNA$_{CCU(Arg)}^{Ser}$		
oUA1775	TAAGGCGACAGACGCCTAATCTGTTGGGCTC	65.7
oUA1776	GAGCCCAACAGATTAGGCGTCTGTCGCCTTA	65.7
Site-directed-mutagenesis of tRNA$_{UGA(Ser)}^{Ser}$ for tRNA$_{GUU(Asn)}^{Ser}$		
oUA1777	TAAGGCGACAGACGGTTAATCTGTTGGGCTC	64.2
oUA1778	GAGCCCAACAGATTAACCGTCTGTCGCCTTA	64.2
Site-directed-mutagenesis of tRNA$_{UGA(Ser)}^{Ser}$ for tRNA$_{GCA(Cys)}^{Ser}$		
oUA1779	TAAGGCGACAGACGGCAAATCTGTTGGGCTC	66.3
oUA1780	GAGCCCAACAGATTTGCCGTCTGTCGCCTTA	66.3
Site-directed-mutagenesis of tRNA$_{UGA(Ser)}^{Ser}$ for tRNA$_{GAA(Phe)}^{Ser}$		
oUA1781	TAAGGCGACAGACGGAAAATCTGTTGGGCTC	64.6
oUA1782	GAGCCCAACAGATTTTCCGTCTGTCGCCTTA	64.6
Site-directed-mutagenesis of tRNA$_{UGA(Ser)}^{Ser}$ for tRNA$_{CUG(Gln)}^{Ser}$		

oUA1783	TAAGGCGACAGACGCTGAATCTGTTGGGCTC	65.9
oUA1784	GAGCCCAACAGATTCAGCGTCTGTCGCCTTA	65.9
Site-directed-mutagenesis of $tRNA_{UGA(Ser)}^{Ser}$ for $tRNA_{GUG(His)}^{Ser}$		
oUA1785	TAAGGCGACAGACGGTGAATCTGTTGGGCTC	65.8
oUA1786	GAGCCCAACAGATTCACCGTCTGTCGCCTTA	65.8
Site-directed-mutagenesis of $tRNA_{UGA(Ser)}^{Ser}$ for $tRNA_{GGG(Pro)}^{Ser}$		
oUA1787	TAAGGCGACAGACGGGGAATCTGTTGGGCTC	67.2
oUA1788	GAGCCCAACAGATTCCCCGTCTGTCGCCTTA	67.2
Confirmation of the site-directed-mutagenesis of the mutant tRNA´s		
oUA1617	CCAGGCTTTACACTTTATGC	51.5
Probe to detect $tRNA_{UGA}^{Ser}$ by northern blot analysis		
oUA1757	TTAACCACTCGGCCATAGT	53.3
Probe to detect $tRNA_{UGU}^{Thr}$ by northern blot analysis		
oUA1699	ACGCTCTACCACTAAGCTAA	50.6

Table 2.3: Oligonucleotides sequences used to construct the fusion protein Hsp104-GFP to elucidate the stress response. The green, gray, purple and blue shading indicates sequence of the *XhoI*, *SmaI*, *SpeI* and *NotI* restriction enzymes, respectively.

Oligo	Sequence 5´-3´	Tm (ºC)
Isolation of Hsp104 gene from strain SN148		
Oli_Di 1	TTTCTCGAGATTATGGTTTTGTGCGGGTT	60.4
Oli_Di 8	AAACCCGGGGTCAAGTCCAGGTGAAGTGA	66.2
Oli_Di 3	GTGGGATGATTCTCTTTTAT	47.1
Oli_Di 4	TATACACAAAACAAGTTCCT	46.6
Site-directed-mutagenesis of pACT1GFP plasmid cut site to *SmaI*		
Oli_Di 9	CCTTTAGACATTTTAATACCCGGGTTTGAATGATTATATTTT	59.0
Oli_Di 10	AAAATATAATCATTCAAACCCGGGTATTAAAATGTCTAAAGG	59.0
Confirmation of Site-directed-mutagenesis of the pACT1GFP plasmid		
oUA1512	CTGGTCTCTCTTTTCGTTTG	55.3
Confirmation of cloning Hsp104 gene in pGFP plasmid by Colony PCR		
Oli_Di 5	AGACCATTGAACCGCTTGAT	54.5

oUA1512	CTGGTCTCTCTTTTCGTTTG	55.3
Sequencing of pHsp104-GFP plasmid		
oUA1617	CCAGGCTTTACACTTTATGC	51.5
oli_Di 8	AAACCCGGGGTCAAGTCCAGGTGAAGTGA	66.2
Construction, Colony PCR and sequencing of pHsp104-GFP-LEU plasmid		
oUA1820	TTTACTAGTGGATCCAATCATCACTGGTG	58.0
oUA1821	TTTGCGGCCGCACCTACCCATGTCTAGAAAG	66.2

2.2.2. Plasmids

In this work we used three original plasmids, namely pUA526, pACT1GFP and pSN40. Following the description of the original plasmids and other plasmids constructed. The plasmids maps and sequences of genes cloned in plasmids are in Annex A.

Table 2.4: Original plasmid and plasmids constructed in this work.

Name	Description
pUA526	Original plasmid (8448 bp) containing the URA3 auxotrophic marker, the RPS10 gene of chromosomal integration, the YFP and mCherry fluorescence reporter genes, ACT1 promoter and *S. cerevisiae* $tRNA_{UGA}^{Ser}$ gene inserted between *KpnI* (6188 bp) and *ApaI* (5483 bp) restriction enzyme sites (Simões, J., unpublished).
pUA850	This plasmid was constructed by extracting YFP and mCherry fluorescence reporter genes and ACT1 promoter from pUA526, using the *XhoI* restriction sites. This plasmid containing the URA3 auxotrophic marker, the RPS10 gene of chromosomal integration and *S. cerevisiae* $tRNA_{UGA}^{Ser}$ gene.
pUA851	Plasmid based on pUA850. This plasmid was constructed by Site-direct-mutagenesis of $tRNA_{UGA}^{Ser}$ gene for $tRNA_{CUC}^{Glu}$ gene.
pUA852	Plasmid based on pUA850. This plasmid was constructed by Site-direct-mutagenesis of $tRNA_{UGA}^{Ser}$ gene for $tRNA_{CCU}^{Arg}$ gene.
pUA854	Plasmid based on pUA850. This plasmid was constructed by Site-direct-mutagenesis of $tRNA_{UGA}^{Ser}$ gene for $tRNA_{GUU}^{Asn}$ gene.
pUA855	Plasmid based on pUA850. This plasmid was constructed by Site-direct-mutagenesis of $tRNA_{UGA}^{Ser}$ gene for $tRNA_{GCA}^{Cys}$ gene.

pUA856	Plasmid based on pUA850. This plasmid was constructed by Site-direct-mutagenesis of $tRNA_{UGA}^{Ser}$ gene for $tRNA_{GAA}^{Phe}$ gene.
pUA857	Plasmid based on pUA850. This plasmid was constructed by Site-direct-mutagenesis of $tRNA_{UGA}^{Ser}$ gene for $tRNA_{GGG}^{Pro}$ gene.
pUA858	Plasmid based on pUA850. This plasmid was constructed by Site-direct-mutagenesis of $tRNA_{UGA}^{Ser}$ gene for $tRNA_{CUG}^{Gln}$ gene.
pUA859	Plasmid based on pUA850. This plasmid was constructed by Site-direct-mutagenesis of $tRNA_{UGA}^{Ser}$ gene for $tRNA_{GUG}^{His}$ gene.
pACT1GFP	Original plasmid (7031 bp) containing the URA3 auxotrophic marker, yEGFP fluorescence reporter gene and ACT1 promoter.
pHsp104-GFP	This plasmid was constructed by extracting the ACT1 promoter from pACT1GFP, using the *XhoI* and *SmaI* restriction sites. Following, the cloning of the Hsp104 gene and promoter (3006 bp – sequence in annex A) into *XhoI* and *SmaI* restriction sites. This plasmid contains the URA3 auxotrophic marker, yEGFP fluorescence reporter gene, Hsp104 gene and Hsp104 promoter.
pSN40	Original plasmid (6000 bp) with *C. maltosa* LEU2 auxotrophic marker (Noble and Johnson, 2005).
pHsp104-GFP-LEU	This plasmid was constructed by extracting the URA3 auxotrophic marker from pHsp104-GFP, using the *NotI* and *BcuI (SpeI)* restriction enzyme sites. Following, the cloning of the LEU2 auxotrophic marker (2119 bp – sequence in annex A) into *NotI* and *BcuI (SpeI)* restriction sites. This plasmid contains the *C. maltosa* LEU2 auxotrophic marker, yEGFP fluorescence reporter gene, Hsp104 gene and Hsp104 promoter.

2.2.3. DNA amplification

Amplification of DNA fragments was done by polymerase chain reaction (PCR). Sample reaction mix (50µl) were made with 100 µg of template DNA, 1X DreamTaq ™ buffer (5 µl) with 2 mM of $MgCl_2$, 0.2 mM dNTPs mix (Bioron), 10 pmol of each primer (forward and reverse), 1.25 U of DreamTaq™ DNA polymerase (Fermentas). PCR was performed using a thermocycler (Eppendorf, Bio-Rad). The cycling programme consisted of an initial cycle at 95 ºC for 3 min followed by 30 cycles of 95 ºC for 30 s, primers annealing temperature was Tm-5 (3rd column of Table 2.2 and 2.3) for 30 s and amplifications were at

72ºC for 1.5 min. In general a final cycle at 72ºC for 5 min was carried out and the temperature was then held at 20ºC.

2.2.4. DNA purification

PCR products, digestion products with restriction enzyme and DNA dephosphorylation reaction products were purified using QIAquick PCR Purification Kit (Qiagen), according to the manufacturer´s instructions.

2.2.5. DNA quantification

The DNA concentration and quality were determined using the spectrophotometer ND-1000 (Nanodrop). Quality of DNA solutions was determined using the ratio A_{260}/A_{280}. DNA solutions with ratios between 1.7 and 2.2 were used for additional manipulations.

2.2.6. Agarose Gel electrophoresis

DNA fragments from PCR products and restriction digestion were electrophoresed on 1% (w/v) agarose gels. Agarose was dissolved in **TAE** buffer [40 mM **T**ris-acetic **a**cid, 10 mM **E**thyleneDiamineTetrAcetic acid (EDTA), pH 8.0] by melting. 0.2 µg/mL of ethidium bromide (EtBr) (Invitrogen) was added to the melted agarose and gels were mounted in an electrophoresis apparatus (Bio-Rad). DNA samples were mixed with 1x loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol] and loaded into the wells, and GeneRuler™ 1 kb DNA Ladder (Fermentas) was loaded into one well. The DNA ladder was used to estimate the size of the DNA fragments. The gels were electrophoresed at 80V for 10 min followed by 40 min at 120V with power supply units (Bio-Rad) using submerged horizontal electrophoresis systems (Mini-Sub Cell GT, Bio-Rad). For DNA band visualization, the gels were exposed to ultraviolet (UV) light using a Gel Doc System (Bio-Rad) coupled to a computer. The images were obtained and analyzed with the Quantity One software (Bio-Rad).

2.2.7. Site directed mutagenesis

Site-directed-mutagenesis was carried out with the QuikChange® Site-Directed-Mutagenesis Kit (Stratagene), according to the manufacturer´s instructions. Briefly, two synthetic oligonucleotide primers, each complementary to opposite strands of the plasmid, containing the desired mutation in the middle and flanked by 10-20 nucleotides were incorporated and amplified during temperature cycling by Pfu Turbo DNA polymerase, resulting in nicked circular strands in the mutated plasmid. Sample PCR was performed with 0-10 ng of dsDNA template, 10-12 pmol of each primer, 2.5 U of PfuTurbo DNA polymerase (Fermentas), 1x reaction buffer, 2 mM $MgSO_4$ and 200 µM of dNTP mix in a final volume of 25 µl.

A thermocycler (Eppendorf, Bio-Rad) programme consisting in a first cycle at 95ºC for 30 s, followed by 14 cycles of 95ºC for 30 s, 55°C for 1 min and 68°C for 13 min. The amplification was checked using 1% agarose gel electrophoresis (as describe in section 2.2.6). After band visualization, the non mutated parental DNA templates were digested with 1 of µl *DpnI* restriction enzyme (Fermentas) at 37 ºC, for 2h. The nicked plasmid containing the desired mutation was stored at -20 ºC, and then transformed into *E. coli* competent cells.

2.2.8. Transformation of *E. coli* cells

For preparation of *E. coli* competent cells the TFB method was used. For this, 200 µl of an overnight stationary phase culture were inoculated in 5 ml of LB medium at 37°C with 180 rpm agitation, to an OD_{550nm} of 0.3 (2h). 100 ml of LB medium were inoculated with 4 ml of anterior culture and were left to grow at 37°C with 180 rpm agitation, until an OD_{550nm} of 0.3 (3h). The cells were collected in 50 ml falcon tubes and were incubated on ice for 5 min, and centrifuged at 2500 rpm, for 5 min at 4 ºC. Each pellet was resuspended in 20 ml of cold TFB I [100 mM $RbCl_2$, 50 mM $MnCl_2.4H_2O$, 30 mM potassium acetate (KOAc), 10 mM $CaCl_2.2H_2O$, 15% glycerol, pH 5.8] and cells were centrifuged at 2500 rpm, for 5 min at 4ºC. Finally, each pellet was resuspended in 5 ml of cold TFB II [10 mM 4-

Morpholinepropanesulfonic acid (MOPS Na), 10 mM $RbCl_2$, 75 mM calcium chloride ($CaCl_2$), 15% glycerol, pH 6.5] and incubated on ice for 5 min, and 200 µL of cell suspension were distributed for each cold 1.5mL eppendorf tube. Cell suspensions were then directly used for transformation or immediately frozen in liquid nitrogen and stored at -80°C.

For each transformation reaction, we added 20 µl of site directed mutagenesis final product (as describe in section 2.2.7) or ligation reaction (as describe in section 2.2.12) to 200 µL competent cells and incubated the mixtures on ice for 30 min. Cells were submitted to heat shock at 42°C, for 90 s and incubated, for 2 min on ice. Then, 800 µL of SOC medium [20 mM glucose, 2% tryptone, 0.5 % yeast extract, 0.05% NaCl, 2.5 mM potassium chloride (KCl), pH 7.0] were added and cells were incubated for 1 h at 37 ºC with 180 rpm, in order to allow cell regeneration. Cells were centrifuged for 1 min at 2500 rpm, and 950 µL of the supernatant was removed. Pellets were homogenized with pipette tips and the suspensions were plated onto selective LB medium plates (LB-agar-Amp plates). Plates were inverted and incubated overnight at 37°C.

2.2.9. Plasmid isolation and purification

For plasmid isolation, a single colony was picked from a recently streaked LB-agar-Amp plate and used to inoculate into 2 ml LB-Amp in 15 ml falcon tubes. These cultures were incubated overnight, 180 rpm, at 37 ºC. Plasmid DNA was extracted using GeneJET™ Plasmid Miniprep Kit (Fermentas) or QIAprep Spin Miniprep Kit (Qiagen), as described by the manufacturer.

2.2.10. DNA digestion with restriction enzymes

5 µg of DNA were digested with restriction enzymes (Fermentas) with appropriated buffer, in a final volume of 50 µl, normally at 37 ºC overnight. Usually, enzymatic digestions were inactivated at 80 ºC for 20 min.

2.2.11. DNA Dephosphorylation with SAP

To prevent self-ligation DNA vectors were treated with SAP (shrimp alkaline phosphatase, Promega). DNA dephosphorylation reactions consisted on 5 µg of digested vector DNA, 1 U of SAP, 1 X dephosphorylation buffer (0.5 M Tris-HCl, 50 mM $MgCl_2$, pH 8.5) in a final volume of the 50 µl, at 37 ºC, for 2 h. Then, SAP was inactivated at 65°C for 15 min.

2.2.12. Ligation reaction

50 ng of vector DNA were mixed with insert DNA fragments, in four independent ligation reactions, with different vector: insert molar ratios, namely 1:0 (negative control), 1:1, 1:2 and 1:5 or 20-60 ng of the digested DNA fragments were ligated with 1 U T4 DNA ligase (Fermentas) in appropriate buffer (final volume of the 20 µl), at 16 ºC during overnight. After the ligations, enzymes were inactivated at 65°C for 10 min.

2.2.13. Transformation of *C. albicans* cells

C. albicans SN148 cells were transformed using the lithium acetate (LiAc) method (Walther and Wendland, 2003). Briefly, overnight stationary phase cultures were diluted in 10 ml of fresh YEPD medium at an OD_{600nm} of 0.3 and incubated at 30 ºC, 180 rpm shaking, for an additional 4 h to an OD_{600nm} of 1-1.2. Cells were centrifuged at 3220 rpm for 5 min, the pellet was washed once with 20 ml of water and resuspended in 1.5 ml LiAc solution (100 mM LiAc, 10 mM Tris-Cl, 1 mM EDTA, pH 7.5). 200 µL of cell suspension were transferred into 1.5 mL eppendorf tubes and 50 µl of single-stranded carrier DNA (sheared salmon sperm DNA at 2 µg/µl) previously denatured were added, 50 µl of plasmid DNA (3-5 µg of DNA) and 600 µl of **PEG**/LiAc solution (50% (w/w) **p**oly**e**thylene**g**lycol 4000 in LiAc-sol). These mixtures were briefly vortexed and used for one transformation. Subsequently, the transformation mixture was incubated at 30 ºC for at least 3 h up to overnight, followed by heat-shock at 44 ºC for 15 min. Cells were then centrifuged, at 5000 rpm, 1 min, and the pellet was resuspended in 100 µL of minimal medium. Each suspension was plated onto a selective plate (MM-URI or MM-URI-LEU

agar plates) and incubated at 30ºC for 4 days to allow colonies formation.

2.2.14. Colony PCR

For colony PCR, single colonies were picked from a freshly streaked selective plate and cells were denaturated at 95 ºC for 5 min in 5 µl MQ sterile water. Following 1X DreamTaq TM buffer, 0.3 µl dNTPs mix (10 mM, Bioron), 0.3 µl of each primer diluted 1:10 (forward and reverse), 0.075 µl of DreamTaqTM DNA polymerase (Fermentas) and 7,525 µl MQ water were added to denaturated cells. PCR was executed using a thermocycler (Eppendorf, Bio-Rad) programme, which consisted of an initial cycle at 95 ºC for 3 min followed by 30 cycles of 95 ºC for 30 s, primers annealing temperature was Tm-5 (3rd column of Table 2.2 and 2.3) for 30 s and amplification was at 72ºC for 1.5 min. A final extension cycle was carried out at 72ºC for 5 min.

2.2.15. *C. albicans* genomic DNA extraction

Genomic DNA of all strains was extracted using the Wizard® Genomic DNA Purification Kit (Promega), as described by the manufacturer. Then, the samples were quantified using the Nanodrop.

2.2.16. Construction of plasmids carrying the $tRNA_{UGA}^{Ser}$ gene

The pUA526 plasmid with a $tRNA_{UGA}^{Ser}$ gene was used to construct the pUA850 plasmid (Table 2.4). For this, the pUA526 plasmid was submitted to site-directed-mutagenesis (as describe in section 2.2.7) using the oli_Di 6 and oli_Di 7 oligonucleotides (Table 2.2.) to insert the *XhoI* restriction site. After transformation into *E. coli* JM109 competent cells, the plasmid DNA was isolated and purified using a plasmid mini prep kit (as mentioned in section 2.2.9), quantified (as mentioned in section 2.2.5) and sequenced using the primer oUA1511 by STABvida (Table 2.2). Mutated plasmid was digested with 0.1 U of the *XhoI* restriction enzyme (as describe in section 2.2.10) which removed the YFP, mCherry fluorescence reporter genes and ACT1 promoter from the mutated plasmid. The product of

restriction digestion was purified (as mentioned in section 2.2.4), quantified (as mentioned in section 2.2.5) and then digestion efficiency was verified by gel electrophoresis (as describe in section 2.2.6). Next, 20-60 ng plasmid was ligated using T4 DNA ligase (Fermentas) in appropriate buffer (as describe in section 2.2.12). Ligations reactions were transformed into *E. coli* DH5α competent cells (as describe in section 2.2.8). Ligation and transformation efficiency was verified using agarose gel electrophoresis of the PCR products amplified with oUA1617 and oUA1556 oligonucleotides (Table 2.2) by colony PCR (as describe in section 2.2.14). The pUA850 plasmid was isolated and purified by plasmid mini prep kit (as mentioned in section 2.2.9), quantified (as describe in section 2.2.5) and sequenced with primer oUA1617 by STABvida (Table 2.2).

2.2.17. Construction of mutant $tRNA_{UGA}^{Ser}$ genes

The $tRNA_{UGA}^{Ser}$ gene cloned into plasmid pUA850 was submitted to site-directed-mutagenesis for modification of the anticodon of that $tRNA^{Ser}$. The oligonucleotides used to introduce the anticodons CUC (Glu), CCU (Arg), GUU (Asn), GCA (Cys), GAA (Phe), CUG (Gln), GUG (His), GGG (Pro) in $tRNA^{Ser}$ are mentioned in Table 2.2, and plasmid constructions are described in Table 2.4. After transformation in *E. coli* JM109 competent cells the plasmids DNA were isolated and purified using mini prep kit (as mentioned in section 2.2.9), quantified (as describe in section 2.2.5) and sequenced by STABvida using the primer oUA1617 (Table 2.2). 3-5 µg of each DNA (plasmid) were linearized with 2 µl of *StuI* restriction enzyme (as describe in section 2.2.10), for transformation of *C. albicans* cells (as describe in section 2.2.13). Positive clones were selected and inoculated into minimal medium lacking uridine, and genomic DNA was extracted from 3 clones of each mutant $tRNAs^{Ser}$ strain (as mentioned in section 2.2.15). Genomic DNA was amplified with oUA1617 and oUA1554 oligonucleotides (Table 2.2) to confirm the authenticity of the integrations (as describe in section 2.2.3). PCR products were purified (as describe in section 2.2.4), quantified (as describe in section 2.2.5) and sequenced using primer oUA1617, STABvida.

2.3. Artificial system of mistranslation

In order to evaluate the impact of Ser misincorporation at 8 codons belonging to different amino acids (Glu, Arg, Asn, Cys, Phe, Gln, His and Pro) in *Candida albicans*, we have engineered a $tRNA^{Ser}$ by substituting the wild-type anticodon (UGA) with specific anticodons (CUC, CCU, GUU, GCA, GAA, CUG, GUG and GGG). The modifications in anticodon of $tRNA^{Ser}$ do not interfere with its ability to be aminoacylated with serine, because the specific recognition of $tRNA^{Ser}$ by seryl-tRNA synthetase does not depend on the sequence of the anticodon of the $tRNA^{Ser}$. Consequently, the mutants of the *S. cerevisiae* $tRNA_{UGA}^{Ser}$ expressed in *C. albicans* cells introduce errors into proteins producing misfolded and/or aggregated proteins (Figure 2.1) (Geslain et al., 2010). These mistranslated proteins can lead to toxicity that will be evaluated by this strategy.

The codons chosen for each amino acid had similar usage at the *C. albicans* genome level (Table 2.5).

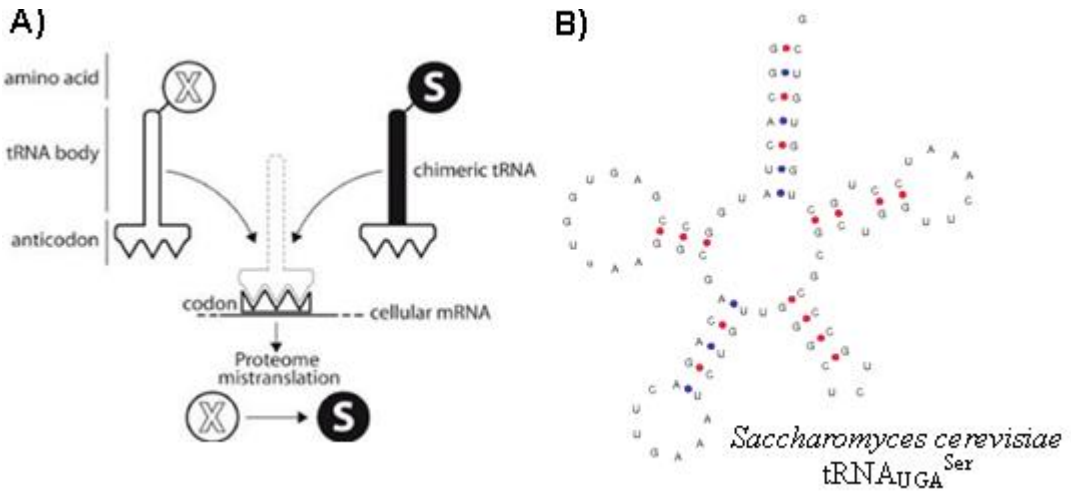


Figure 2.1: Representation of chimeric tRNA construction (A) using *S. cerevisiae* $tRNA_{UGA}^{Ser}$ (B). Chimeras of $tRNA^{Ser}$ were engineered by site-directed mutagenesis of the wild type anticodon (UGA) of the $tRNA_{UGA}^{Ser}$. Chimeric $tRNA^{Ser}$ is charged with serine, regardless of the sequence of the anticodon. The expression of chimeric $tRNA^{Ser}$ leads to proteome mistranslation. Adapted and modified from (Geslain et al., 2010) and (Genomic tRNA database).

Table 2.5: **Codons usage used for artificial system of mistranslation (*Candida* Genome Database).**

$tRNA^{Ser}$ engineered	Amino acid	Anticodon	Codon	Codon usage
$tRNA^{Ser}_{CUC(Glu)}$	Glutamic acid	CUC	GAG	0.21
$tRNA^{Ser}_{CCU(Arg)}$	Arginine	CCU	AGG	0.08
$tRNA^{Ser}_{GUU(Asn)}$	Asparagine	GUU	AAC	0.28
$tRNA^{Ser}_{GCA(Cys)}$	Cysteine	GCA	UGC	0.18
$tRNA^{Ser}_{GAA(Phe)}$	Phenylalanine	GAA	UUC	0.32
$tRNA^{Ser}_{CUG(Gln)}$	Glutamine	CUG	CAG	0.17
$tRNA^{Ser}_{GUG(His)}$	Histidine	GUG	CAC	0.26
$tRNA^{Ser}_{GGG(Pro)}$	Proline	GGG	CCC	0.17

2.4. tRNA detection by northern blot analysis

2.4.1. Total RNA isolation from *C. albicans*

For total RNA extraction, 50 ml of cell culture grown to middle exponential phase (OD_{600nm} of 0.6) in MM-URI at 30 ºC were centrifuged and the resulting cell pellets were immediately frozen in liquid nitrogen and stored at -80 ºC until further use.

Cell pellets were resuspended in 600 µl of Acidic Phenol-Chloroform 5:1 pH 4.7 (Sigma) preheated at 65 ºC and in 600 µl of **TES**-buffer (10 mM **T**ris pH 7.5, 10 mM **E**DTA, 0.5% **S**DS). Cell suspensions were vigorously shaken for 20 s and were incubated in water bath for 1 h at 65ºC with agitation every 10 min. Next, cells were shaken for 20 s, transferred to 1.5 ml eppendorf-tubes and centrifuged at 13000 rpm for 20 min at 4 ºC. The 600 µl of water-phase containing RNAs were added in new eppendorfs-tubes containing 600 µl of Acidic Phenol-Chloroform, and were shaken for 20 s and centrifuged for 10 min at 13000 rpm at 4ºC. The 500 µl of water-phase was re-extracted with 500 µl of Acidic Phenol-

Chloroform in new eppendorf-tubes by centrifugation at 13000 rpm at 4 ºC for 10 min and subsequently the 400 µl of water-phase was re-extracted with 400 µl of Chloroform Isoamyl Alcohol 25:1 (Fluka) using new eppendorf-tubes, by centrifugation at 13000 rpm, 4 ºC for 10 min. The 350 µl water-phase was incubated overnight at -20 ºC with 35 µl of 3M Sodium Acetate pH 5.2 and 800 µl of ethanol 100% at -20 ºC in new eppendorf-tubes. The next day, RNA precipitates were harvested by centrifugation at 13000 rpm for 5 min, washed with 500 µl of ethanol 80 % at -20 ºC, air-dried for 1 min and finally dissolved in sterile water MQ. The RNA samples were quantified using a Nanodrop (describe in section 2.2.5) and stored a -80 ºC.

2.4.2. Northern Blot Analysis

The integrity of RNA was checked by polyacrylamide gel electrophoresis. 50 µg of total RNA was fractionated on 15 % polyacrylamide gels, prepared with 40% of 19:1 acrylamide/bisacrylamide (Bio-Rad) containing 7 M urea buffered with 1x TBE pH 8.0. Polymerization of the gel was done by addiction of 100 µl of TEMED and 10% of APS. Each RNA sample was mixed with 1x loading buffer and loaded. Gels were electrophoresed at 500 V for about 16 h. Then, fractionated RNA were stained with EtBr buffered with 1x TBE pH 8.0 for 3 min, exposed to UV light using a Gel Doc System (Bio-rad) coupled to a computer, where the image was visualized and analyzed with the Quantity One software (Bio-Rad). The portion of the gels containing the tRNAs were cut and transferred onto nitrocellulose membranes (*Hybond-N*, Amersham) using a blotting system (Semy-Dry Trans Blot, Bio-rad) for 37 min at [($Area_{membrane}/cm^2$) x 0.8] milliamp. Membranes were pre-hybridized for at least 30 min in 10 ml of hybridization buffer containing 7xSSPE (20x SSPE = 3 M NaCl, 0.2 M sodium phosphate monobasic, 0.02 M EDTA, pH 7.4 with 10 M NaOH), 5xDenhardt´s solution (50x Denhardt´s solution = 0.02% Ficoll 400, 0.02 % polyvinylpyrrolidone, 0.02 % bovine serum albumin) and 1 % sodium dodecyl sulfate, with agitation, at probes annealing temperature (Tm-5) (3^{rd} column of Table 2.2). Probes were prepared using 10 pmol of dephosphorylated oligonucleotide, 3 µl of γ -^{32}P-ATP (5000Ci/mmol, Perkin Elmer), 0.1 mM spermidine and 15 U of T4 kinase (Takara) and 1x T4 kinase buffer. Phosphorylation reactions of 5´-OH

termini of oligonucleotides were incubated for 1 h at 37 ºC and extracted with 100 µl of Phenol:Choloroform:Isoamyl alcohol (25:24:1) by centrifugation for 5 min at 13000 rpm. Membranes were hybridized in 10 ml of hybridization buffer, at 53 ºC (oUA1757 annealing temperature) and 51 ºC (oUA1699 annealing temperature) overnight, with ɣ-^{32}P-ATP labeled probe. The probe used to detect mutant and wild type (wt) $tRNA_{UGA}^{Ser}$ was oUA1757 (Table 2.2), while the positive control $tRNA_{UGU}^{Thr}$ was detected using the probe oUA1699 (Table 2.2). Membranes were washed 2 times for 3 min each time at hybridization temperature in washing buffer at room temperature (2x SSPE, 0.5 % SDS), washed 2 times for 3 min each time at hybridization temperature in pre-warmed washing buffer, and finally washed at hybridization temperature overnight in pre-warmed washing buffer, with agitation. The membranes were sealed in a plastic bag and exposed 1 to 3 days to a K-Screen and scanned using a Bio-rad scanner with appropriate settings.

2.5. *C. albicans* cells fitness assays

2.5.1. Transformation efficiency

Transformation efficiency of *C. albicans* transformed with plasmid pUA850 carrying the wt and mutant $tRNA^{Ser}$ genes were calculated by determination the number of transformants per plate (MM-URI agar plates). The number of transformants was normalized relative to the control.

2.5.2. Growth rate

C. albicans cell cultures transformed with plasmid pUA850 carrying the $tRNA_{UGA}^{Ser}$ wt gene (control) and with plasmids carrying mutant $tRNA^{Ser}$ gene were grown until stationary phase, at 30 ºC, at 180 rpm, in liquid MM-URI. For main cultures, cells from the previous cultures were removed and inoculated in 10 ml fresh liquid MM-URI to an initial OD_{595} of 0.05 (100 ml Erlenmeyer´s). These main cultures were grown at 30 ºC, 180 rpm until late stationary phase. At several time points the OD_{595} were measured every hour using a microplate reader. Growth rate was calculated for cells expressing the control and

mutant tRNAs, taking into account the growth of *C. albicans* cells in exponential phase.

2.6. Phenotypic screening of strains mistranslation

First, agar screening plates (50 ml of MM-URI) were prepared to test different stress conditions (as described in section 2.1 - Table 2.1). For each condition 3 plates were prepared, with exception of the control condition (MM-URI without supplement, at 30 ºC), where 9 plates were used.

C. albicans cultures transformed with control plasmid (pUA850) and with different plasmids carrying the mutant $tRNA^{Ser}$ gene were grown at 30 ºC, 180 rpm, in 15 ml falcon tubes until late stationary phase. Aliquots of these cultures were then removed and inoculated in 50 ml falcon tubes, and grown at 30 ºC, 180 rpm, until an OD_{600}=0.5 to 0.8 (until middle exponential phase). Cell density was determined by counting the number of cells using a Neubauer chamber/hemocytometer. Then, cells from each strain were diluted in water to obtain $1x10^7$ cells in each sample. 200 µl of each first dilution were transferred to a first and fifth line of 96-well plates (mother plates) where three dilutions additional of 1:10 were prepared as show in Figure 2.2. In total, three "mother plates" were prepared, each one with two clones of each mistranslation strain and three dilutions (each clone was tested twice). Cells contained in the "mother plate" were plated in three plates previously prepared using a liquid handling station (Caliper LifeSciences).

Finally, the plates were incubated in different temperature conditions (16°C, 30°C, 37°C, 42°C) and the area of the colonies was measured using ImageJ, after 3 days of growth for temperature and antifungal drugs conditions and after 5 days of growth (30 ºC) for other conditions tested. To score the growth of each strain, the area of each colony grown in presence of each stressor was divided by the area of the colonies in the control medium lacking the stressor. The growth score of each strain in each stress condition was obtained by the average growth score of three clones in all dilutions of the same strain.

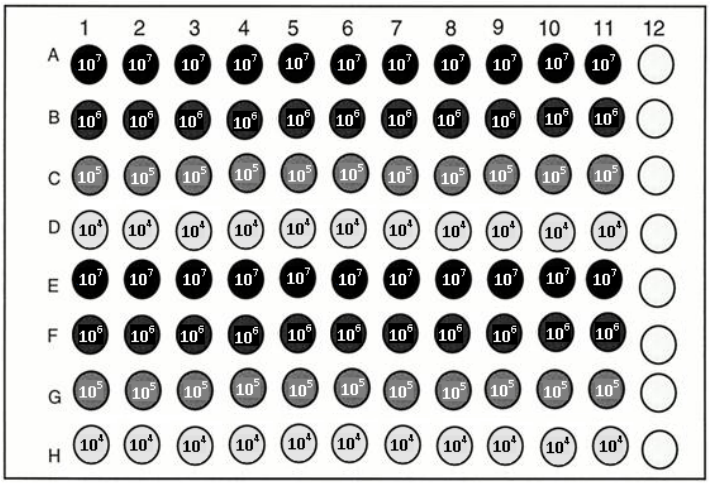


Figure 2.2: 96-well plate (Mother plate) used in phenotypic screening of mistranslating strains. The 1, 2, 3, 4, 5, 6, 7, 8 correspond to the mistranslating strains and 9,10,11 correspond to three different clones of the control strain. The A,B,C,D and E,F,G,H correspond to the different clones of each strain. The A and E correspond to the first dilution with $1x10^7$ cells, and B and F to the second dilution with $1x10^6$ cells, C and G correspond to the third dilution with $1x10^5$ cells, and D and H correspond to the fourth dilution with $1x10^4$ cells.

2.7. Construction the Hsp104-GFP reporter system

DNA genomic was extracted from the strain SN148 (as describe in section 2.2.15). The Hsp104 promoter and coding region (3006 bp, sequence in annex A) were amplified from the genomic DNA by standard DNA amplification (as describe in section 2.2.3) using oli_Di 1 and oli_Di 8 oligonucleotides (Table 2.3). To determine amplification efficiency, 5 µl of PCR product were analyzed by gel electrophoresis (as describe in section 2.2.6). 45 µl of PCR product were purified (as describe in section 2.2.4), quantified (as describe in section 2.2.5) and digested using 0.2 U of *SmaI* restriction enzyme, at 30 ºC overnight. This digestion reaction was inactivated at 65 ºC, for 15 min. Then, this sample was digested with 0.1 U *XhoI* restriction enzyme (as describe in section 2.2.10), purified (as describe in section 2.2.4) and quantified (as describe in section 2.2.5). The Hsp104 gene then ligated to the pGFP plasmid.

Before submitting the plasmid pACT1GFP to the same restriction digestions it was subjected to a single round of site-directed-mutagenesis (as describe in section 2.2.7) using the oligonucleotides oli_Di 9 and oli_Di 10 (Table 2.3) to insert a *SmaI* restriction site. Site-direct-mutagenesis of the plasmid was confirmed by sequencing (STABvida) with the oUA1512 primer (Table 2.3). Mutated plasmid was digested with the same restriction enzymes used for the Hsp104 gene digestion, separating ACT1 promoter from mutated plasmid. After digestion, the plasmid was dephosphorylated with SAP (as describe in section 2.2.11) and subsequently ligated with Hsp104 gene (as describe in section 2.2.12). Cloning of Hsp104 gene into the pGFP plasmid was confirmed by colony PCR (as describe in section 2.2.14) using oli_Di 5 and oUA1512 primers (Table 2.3) and by sequencing using oUA1617 and oli_Di 8 primers (Table 2.3). The URA3 gene was then removed from the pHsp104-GFP plasmid, using the *NotI* and *BcuI (SpeI)* restriction enzymes. Then, the LEU2 gene was amplified with oUA1820 and oUA1821 oligonucleotides from pSN40 plasmid and cloned into *NotI* and *BcuI (SpeI)* restriction sites of the pHsp104-GFP lacking the URA3 gene (Table 2.4). Cloning efficiency was carried out using colony PCR, where the samples were amplified with oUA1820 and oUA1821 oligonucleotides (Table 2.3) (as describe in section 2.2.14). Further sequencing was also performed with oligonucleotide oUA1821. The pHsp104-GFP-LEU was transformed into mistranslating *C. albicans* cells. Positive transformants were selected in minimal medium lacking leucine and uridine.

2.7.1. Visualization of *C. albicans* cells by light microscopy

Mistranslating *C. albicans* cells transformed with the Hsp104-GFP plasmid were observed using epifluorescence microscopy. Cells were grown overnight, at 30°C and 180rpm. After that, aliquots of pre-cultures were removed and inoculated into 50 ml falcon tubes, and grown at 30 ºC, 180 rpm, until an OD_{600}=0.5 (middle exponential phase). The cell suspensions were spread on microscope slides covered with a thin layer of agarose for visualization using a fluorescence microscope (Zeiss) equipped with Brightfield and GFP filters and an oil-immersion objective, 63x (Zeiss). The maximum fluorescence, area and circularity of the cells were measured using the software ImageJ.

3. Results

3.1. Effect of mistranslation in the expression of mutant tRNAs^{Ser} in *C. albicans*

In order to determine whether the mutations introduced in the $tRNA_{UGA(Ser)}^{Ser}$ gene affect the stability and expression level of the tRNAs a northern blot analysis was performed. All mutant tRNAs were detected. The northern blot results showed that the mutant $tRNA_{CUC(Glu)}^{Ser}$, $tRNA_{CCU(Arg)}^{Ser}$ and $tRNA_{GGG(Pro)}^{Ser}$ were expressed at very low level, while $tRNA_{GAA(Phe)}^{Ser}$, $tRNA_{GUG(His)}^{Ser}$, $tRNA_{GUU(Asn)}^{Ser}$, $tRNA_{GCA(Cys)}^{Ser}$ and $tRNA_{CUG(Gln)}^{Ser}$ were expressed at higher level, when compared with the $tRNA_{UGA(Ser)}^{Ser}$ (control). The control $tRNA_{UGU}^{Thr}$ was expressed at identical level in all transformed strains, as expected (Figure 3.1).

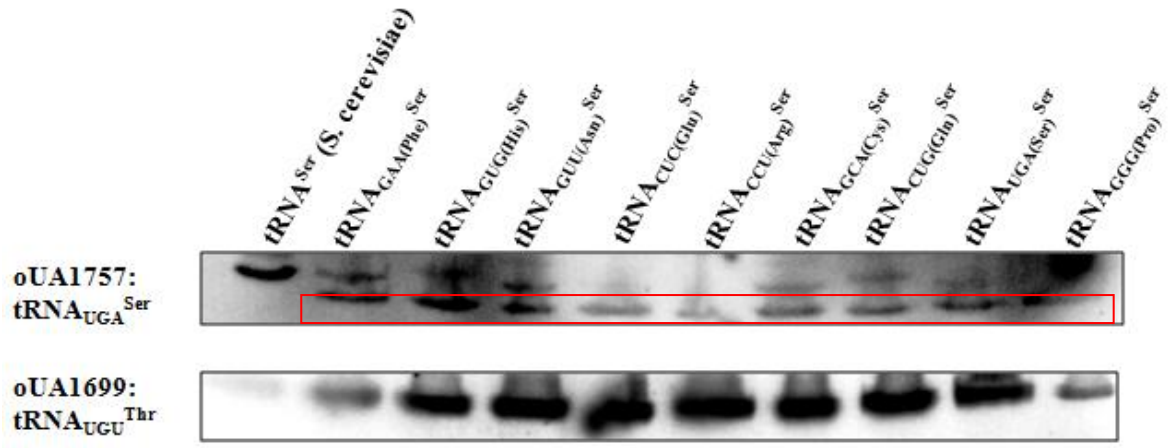


Figure 3.1: Expression of mistranslating tRNAs detected by northern blot analysis. Total RNA was extracted from transformed *C. albicans* clones and fractionated on 15 % polyacrylamide gel containing 7M urea. $tRNA_{UGA}^{Ser}$ and $tRNA_{UGU}^{Thr}$ were detected using γ-^{32}P-ATP-$tRNA_{UGA}^{Ser}$ (oUA1757) and γ-^{32}P-ATP-$tRNA_{UGU}^{Thr}$ (oUA1699) probes (as describe in section 2.4). The $tRNA_{UGU}^{Thr}$ was used as control. The bands that are included in the red rectangle are non-specific bands.

3.2. Transformation efficiency of *C. albicans*

Transformation efficiencies of *C. albicans* were determined for strains expressing the mutant $tRNA^{Ser}$ (Figure 3.2). The results revealed that the mutant $tRNA_{CUC(Glu)}^{Ser}$, $tRNA_{GAA(Phe)}^{Ser}$, $tRNA_{GCA(Cys)}^{Ser}$, $tRNA_{CCU(Arg)}^{Ser}$, $tRNA_{GUG(His)}^{Ser}$, $tRNA_{CUG(Gln)}^{Ser}$, $tRNA_{GGG(Pro)}^{Ser}$ and $tRNA_{GUU(Asn)}^{Ser}$ decreased transformation efficiencies to 23,3 %, 30,6 %, 46,7 %, 50,8 %, 59,1 %, 66,1 % 69,8 % and 86,5 %, respectively, relative to the control [$tRNA_{UGA(Ser)}^{Ser}$]. The transformation efficiency of the control was considered as 100 %. Only the strains expressing the $tRNA_{CUC(Glu)}^{Ser}$ and $tRNA_{GAA(Phe)}^{Ser}$ had significantly lower transformation efficiencies than the control strain.

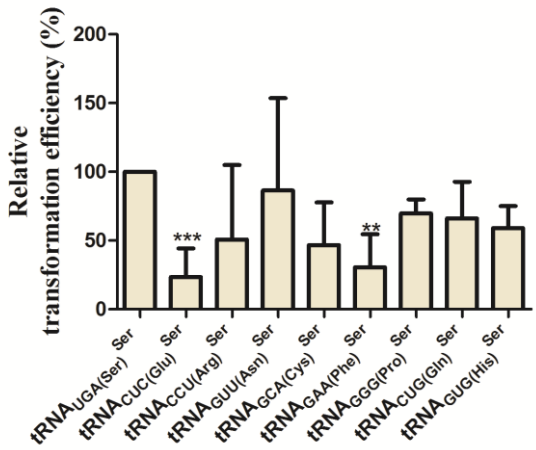


Figure 3.2: Effect of mistranslation on transformation efficiency. Transformation efficiency of *C. albicans* cells transformed with plasmid pUA850 carrying the control $tRNA_{UGA(Ser)}^{Ser}$ gene or carrying $tRNA^{Ser}$ mutant genes. The results represent the mean ± SD of four experiments (***p□ 0.001; **p□ 0.01; *p□ 0.05 one-way ANOVA post Dunnett´s multiple comparison test with CI 95% relative to control).

3.3. Effect of mistranslation on growth rate in *C. albicans* cells expressing mutant $tRNA^{Ser}$

Previous studies showed that mistranslating *S. cerevisiae* strains grow slower of the cells due to toxicity caused by genetic code ambiguity (Santos et al., 1996). We have also tested the effect of expression of our mutant tRNAs on *C. albicans* growth rate. The data showed that growth rate of all mistranslating strain is slightly affected, although these data are not statistically significant, relative to the control (Figure 3.3), indicating that *C. albicans* tolerates codon ambiguity better than *S. cerevisiae*.

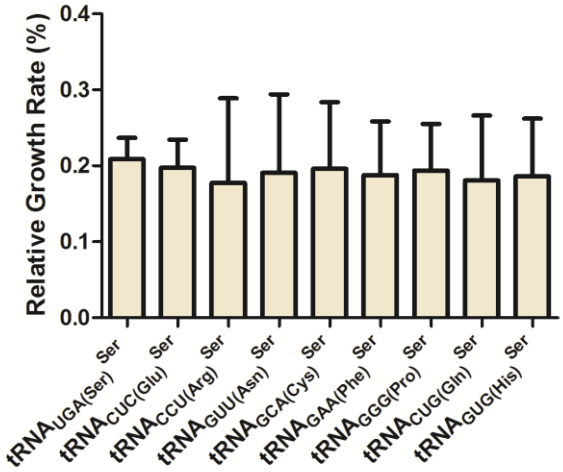


Figure 3.3: Growth rate of *C. albicans* cells expressing mutant tRNA^{Ser}. Cultures were grown at 30ºC in selective medium without uridine. Growth rates were calculated using exponential growth phase values of cultures. The results are expresses as mean ± SD of four experiments of 3 independent clones (***p□ 0.001; **p□ 0.01; *p□ 0.05 oneway ANOVA post Dunnett´s multiple comparison test with CI 95% relative to control).

3.4. Phenotypic screening

Previous studies showed that CUG ambiguity in yeast creates a competitive advantage under stress conditions, due to expression of a novel set of stress proteins that induce a constitutive stress response (Santos et al., 1999). This stress response is an important mechanism of pre-adaptation to severe stress. *S. cerevisiae* expressing CUG ambiguity becomes tolerant to high temperature, oxidation, salts, heavy metals and drugs (Santos et al., 1999). In order to determine whether the mutant $tRNA_{UGA(Ser)}^{Ser}$ introduced in *C. albicans* also create selective advantages in variable environmental conditions, a phenotypic screening was performed. The mistranslating strains expressing mutant tRNAs were grown in solid medium supplemented with several stressors, namely high temperature (25 ºC, 37ºC, 42ºC), antifungal drugs (fluconazole, itraconazole), cell wall stress (calcofluor white), heavy metal [lithium chloride (LiCl)], protein denaturation [guanidine

hydrochloride (GdnHCl), urea], alkalinity (pH 8.6), osmotic/ionic stress [calcium chloride ($CaCl_2$) and sodium chloride (NaCl), sorbitol], carbon sources (without glucose, 2% galactose, 3% glycerol, 2% ethanol) (Table 2.1, in section 2.1). The results of the phenotypic screening were compared with control strain (cells expressing $tRNA_{UGA(Ser)}^{Ser}$) and showed that mistranslating display high phenotypic diversity (Figure 3.4). Misincorporation of Ser at Glu and Arg codons lead to increased sensitivity or tolerance depending on the stressors tested, although there was no significant difference in growth. The strain misincorporating Ser at Asn codon showed a trend to tolerate almost all stressors tested, although these growth advantages were not significant. On other hand, the strain misincorporating Ser at Cys, Phe, Pro, Gln and His codons showed tolerance and sensitive to some of the stress conditions with significant growth advantage and disadvantage, respectively.

The data also showed significant growth disadvantages of one or more of the mistranslating strains, in media containing different carbon sources (C-absent, 2% galactose, 3% glicerol), osmotic/ionic stress (NaCl) and antifungal drug (fluconazole). Conversely, media containing GdnHCl, $CaCl_2$, LiCl, temperature at 37 ºC and calcofluor white provided good growth substrates for one or more of the mistranslating strains. The high pH medium (pH=8.6) also differentiated the mistranslating strains as some grew better than controls while others grew more poorly.

Strain misincorporating Ser at the Cys codon showed tolerance to GdnHCl and $CaCl_2$, but this strain had a strong growth disadvantage in media lacking glucose. The strain misincorporating Ser at the Phe codon grew better than controls at pH 8.6, but grew poorly in presence of fluconazole, galactose 2% and very poorly in absence of carbon source. The strain misincorporating Ser at the Pro codon grew very poorly at pH 8.6, in presence of NaCl and in media lacking glucose, and poorly in presence of fluconazole. This mistranslation strain showed selective advantages when grown at 37ºC and in presence of GdnHCl. The strain misincorporating Ser at the Gln codon grew better than the control strain at pH 8.6, in media containing $CaCl_2$, GdnHCl and calcofluor white. This particular strain only grew more poorly than the control strain in absence of glucose. Conversely, the strain misincorporating Ser at the His codon had significant disadvantages when grown at

pH 8.6, in absence of carbon source, in presence of galactose and in media containing glicerol. This strain only grew with strong growth advantage in media with heavy metal (LiCl).

In order to analyze each mistranslating strain a scoring system was developed, which involves the application of positive and negative values depending on the difference in growth. Values were scored as: extremely significant (***), highly significant (**) and significant (*) advantage, corresponding to values of 3, 2 and 1, respectively. Growth disadvantages were also scored as: extremely significant (***), highly significant (**) and significant (*) disadvantage corresponding to values of -3, -2 and -1, respectively. This showed that most of the mistranslating strains had a negative score, namely the strains misincorporating Ser at the His (-6), Ser at the Pro (-5) and Ser at the Phe (-4). The strain misincorporating Ser at the Cys had a score of zero and the only strain with a positive score misincorporated Ser at the Gln (+4).

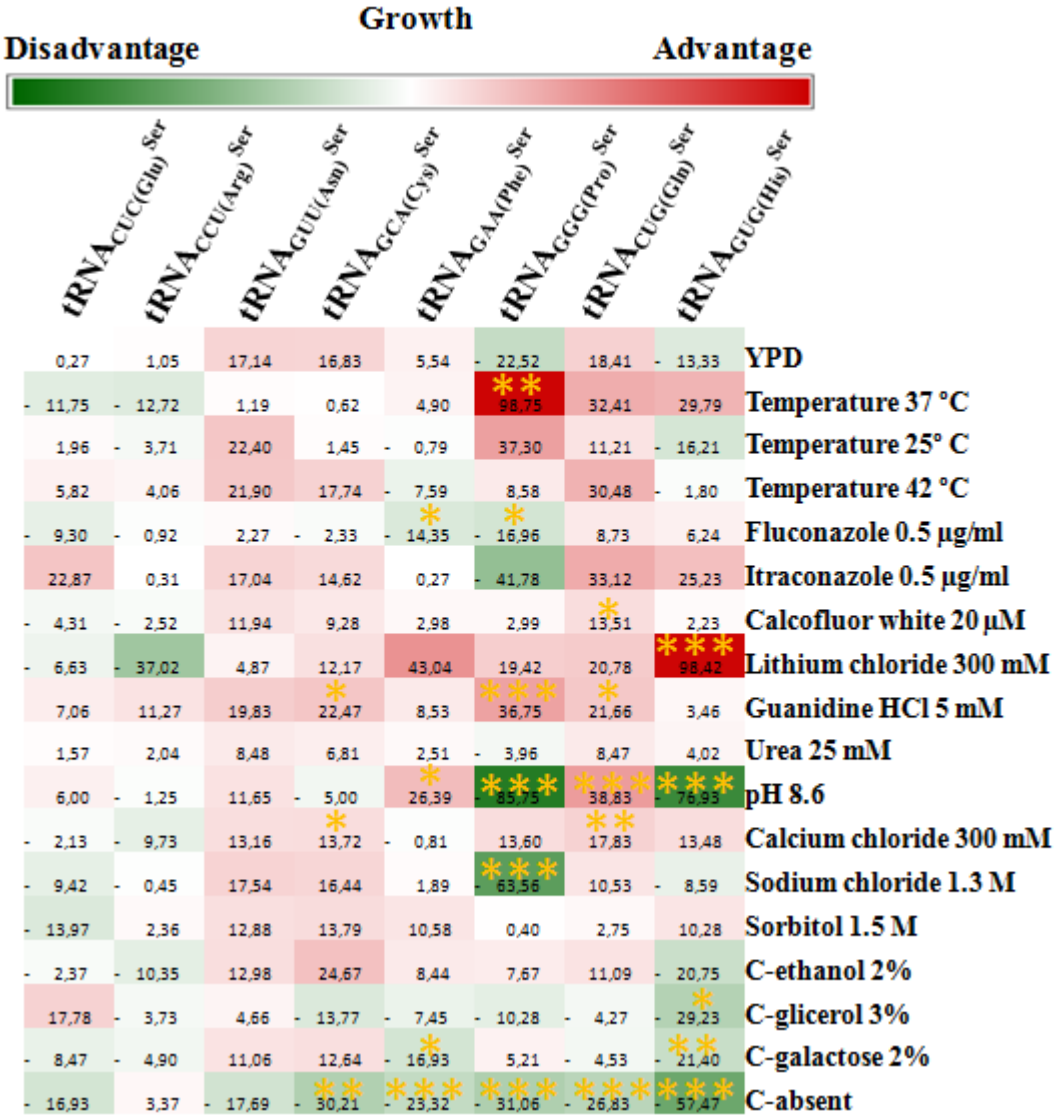


Figure 3.4: Phenotypic profile of the mistranslating strains. Growth performance of *C. albicans* strains misincorporating Ser at eight non-cognate codons, in presence of 18 growth conditions, was determined after 5 days of growth, and in presence of Fluconazole and Itraconazole and variable temperature (25ºC,37ºC,42ºC) was determined after 3 days of growth. The red, white and green colors represent growth advantages, neutral and disadvantages, relative to the control strain (pUA850), respectively. Results were scored as extremely significant (***), highly significant (**) and significant (*) compared to control. The data represents duplicate of three independent clones of each strain. To see results in more detail, see annex B.

3.5. Hsp104-GFP reporter system

In this work, the molecular chaperone Hsp104 was chosen to test whether mistranslation induces protein aggregation in *C. albicans* because of its involvement in disaggregation of protein aggregates (Doyle and Wickner, 2009; Lee et al., 2004). The objective was to determine the presence or absent of cytoplasmic protein aggregates in mistranslating strains by epifluorescence microscopy. However, the Hsp104-GFP reporter system was not functional because diffuse fluorescence was observed in all strains (Figure 3.5). This suggests that mistranslated proteins do not aggregate in *C. albicans* or that Hsp104 is not involves in protein disaggregation in this pathogen. Therefore, Hsp104 expression was monitored by measuring the maximum fluorescence using epifluorescence microscopy (as describe in section 2.7.1) (Figure 3.5). The results revealed that the strain transformed with the mutant $tRNA_{CCU(Arg)}^{Ser}$ and $tRNA_{GGG(Pro)}^{Ser}$ expressed higher level of Hsp104 while strain expressing the mutant $tRNA_{GUU(Asn)}^{Ser}$ expressed lower level.

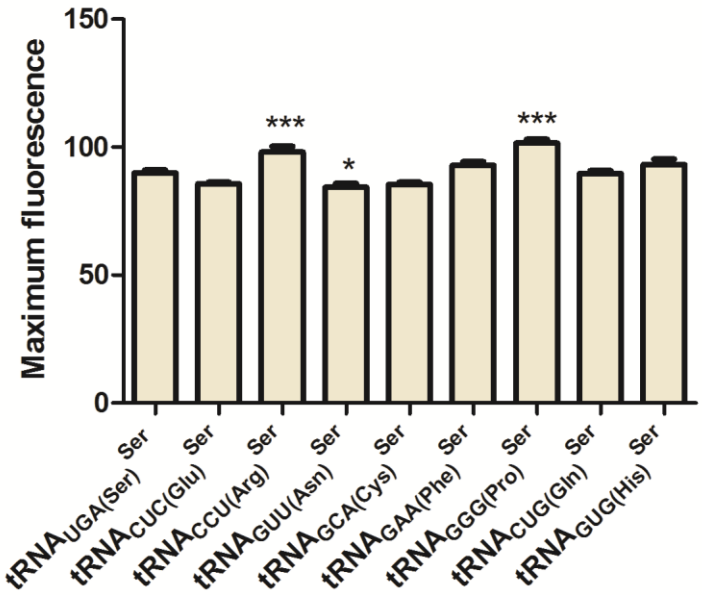


Figure 3.5: Hsp104 expression in mistranslating *C. albicans* cells. Cells were grown at 30ºC in selective medium without leucine and uridine, collected at middle exponential phase, observed by fluorescence microscopy (63x objective) and analyzed by Image J. The results are expressed as mean ± s.e.m (standard error the mean) of three images of three independent clones (***p□ 0.001; **p□ 0.01; *p□ 0.05 one-way ANOVA post Dunnett´s multiple comparison test with CI 95% relative to control).

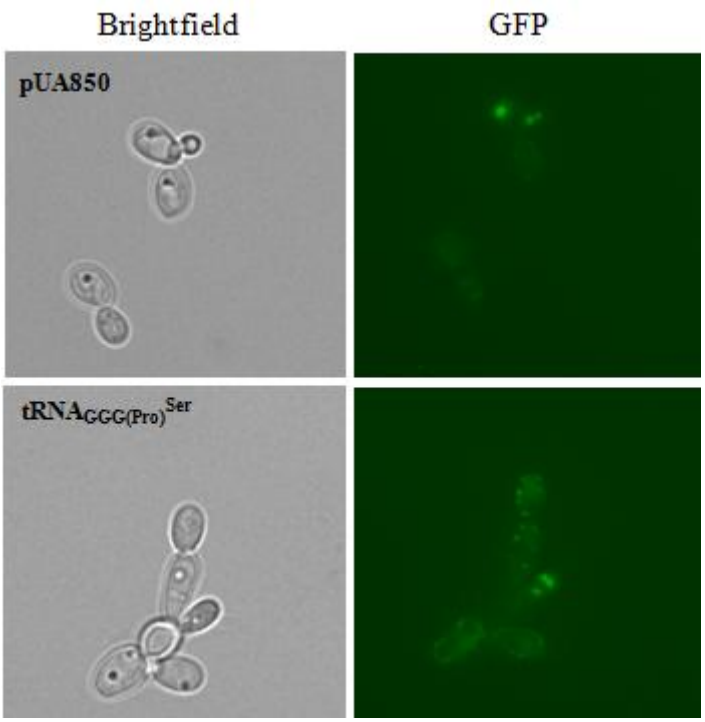


Figure 3.6: Representative pictures of mistranslating *C. albicans* cells transformed with Hsp104-GFP reporter protein observed by epifluorescence microscope (63X objective). Cells were grown in liquid medium without uridine and leucine until middle exponential phase, at 30 ºC. Images of the control cells with pUA850 plasmid and mistranslating cells expressing $tRNA_{GGG(Pro)}^{Ser}$ were observed with Brightfield and GFP filters. Similar images were obtained for other mistranslating cells (data not shown).

Also, mistranslating cells transformed with Hsp104-GFP reporter were analyzed for circularity and area. These parameters show that the cells were mostly in the yeast form, having an elongated-ovoid form (Figure 3.7 A)). In almost all cases pseudohyphal form (Figure 3.7 B)) appeared in very low quantity. The $tRNA_{GCA(Cys)}^{Ser}$, $tRNA_{GAA(Phe)}^{Ser}$ and $tRNA_{GUG(His)}^{Ser}$ were exception since pseudohyphal form were common (graphs of the circularity and area of the cells are in annex C). These three specific strains had only elongated-ovoid shaped. The other strains had two types of morphology, namely elongated-ovoid form and pseudohyphal.

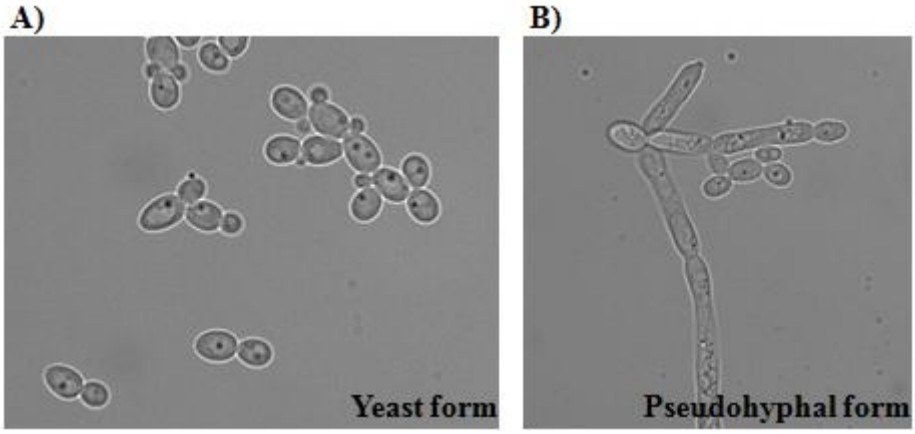


Figure 3.7: *C. albicans* cells morphology observed by light microscope (63X objective). A) Yeast form, showing elongated-ovoid cells. **B)** Pseudohyphae were common in almost all strains expressing mutant $tRNA^{Ser}$.

4.Discussion

Misincorporating Ser at codons belonging to different amino acids can cause a series of changes in structure, stability and function of normal proteins. These potential mistranslated proteins can lead to cellular damage and death. Possible negative effects are due to substitution of Ser for amino acids with different physical, chemical and structural proprieties.

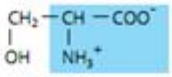
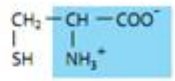
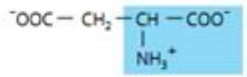
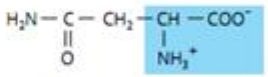
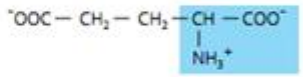
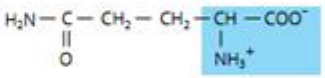
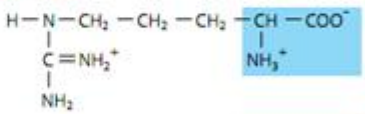
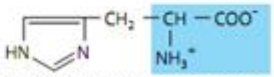
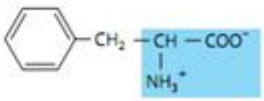
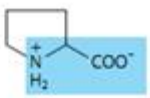
All amino acids contain both amino ($-NH_2$) and carboxyl (-COOH) terminal functional groups. Table 4.1 shows the structure of each amino acid substituted by Ser. Other characteristics essential to interpret our results are amino acids polarity, charge and weight, which are describing below.

Serine is a neutral polar and a small amino acid that contains a moderately reactive aliphatic hydroxyl group (-OH) which is able to form hydrogen bounds with several polar molecules. Cysteine (Cys) is a polar neutral and small amino acid that contains a sulfhydryl, or thiol (-SH) group in place of the hydroxyl group of Ser. Both groups allow Ser and Cys to act as nucleophiles during enzymatic catalysis. Glutamine (Gln) and Asparagine (Asn) are similar to Ser, both are polar neutral and Asn has a carboxamide group smaller than Gln, being considered a small amino acid. Arginine (Arg) and Histidine

(His) are polar and basic amino acids. Arg has a relatively long side chain that terminates with a guanidinium group, while His has an imidazole ring with a pK_a value near 6 that can be uncharged or positively charged near neutral pH, depending on its local environment. Indeed, His is frequently found in the active site of enzymes, where the imidazole ring can bind and release protons in the course of enzymatic reactions. Glutamate (Glu) is polar acidic with a carboxylic acid side chain. Finally, Proline (Pro) and Phenylalanine (Phe) are nonpolar (hydrophobic). Pro is a small amino acid which has a distinctive cyclic structure, where the alpha amino group is incorporated into the ring, while Phe contains a phenyl ring (aromatic side chain). So, Arg, His, Glu, Pro and Phe are more dissimilar to Ser than Asn, Gln and Cys (Lehninger et al., 2008; Murray et al., 2003). In other words, amino acid substitutions involving amino acids with similar polarity, charge and weight, are expected to induce low toxicity. If substitutions involve amino acids with different polarity, charge and weight, they should stimulate medium or high toxicity, depending on the chemical differences of these amino acids.

The distribution of amino acids in proteins is essential for their function and is related with the amino acids chemical proprieties. The amino acids that are predominantly hydrophobic (Trp, Met, Phe, Ile, Val, Leu and Ala) are more frequently located in the interior than in the surface of proteins, while polar amino acids (Thr, Ser, Asn, Gln, Arg, Asp, Glu and Lys) reside frequently on the surface. Gly and His are located equally in the interior and surface of proteins, while Pro is located predominantly on the surface, presumably due to its frequent location structural turns. Cys is the most highly conserved amino acid and the rarest on the surface, probably because it has the most reactive side chain (Fiser et al., 1996). The physical and chemical properties and the frequency of the amino acids in proteins are correlated with protein structure. Phe, Leu, Ile, Met and Val (hydrophobic) are more frequent in β-sheets, while Tyr, His, Gln, Asn, Lys, Asp, Glu and Ala (hydrophilic) are more frequent in α-helices. Some amino acids, namely Ser, Pro, Thr, Cys, Trp and Arg do not exhibit preference for specific protein structure. The aperiodic structures contain more hydrophilic amino acids than hydrophobic amino acids (Chiusano et al., 2000).

Table 4.1: The structure of the amino acids. The blue rectangles represents amino (-NH_2) and carboxyl (-COOH) groups (Murray et al., 2003).

Name	Symbol	Structural Formula
With Side Chains Containing Hydroxylic (OH) Groups		
Serine	Ser [S]	
With Side Chains Containing Sulfur Atoms		
Cysteine	Cys [C]	
With Side Chains Containing Acidic Groups or Their Amides		
Aspartic acid	Asp [D]	
Asparagine	Asn [N]	
Glutamic acid	Glu [E]	
Glutamine	Gln [Q]	
With Side Chains Containing Basic Groups		
Arginine	Arg [R]	
Histidine	His [H]	
Containing Aromatic Rings		
Phenylalanine	Phe [F]	
Imino Acid		
Proline	Pro [P]	

The above mentioned chemical properties and the polar requirement of the amino acids, result in differential usage in proteins. For example, yeast uses Ser, Glu, Asn, Phe, Pro, Arg, Gln, His and Cys at 9 %, 6.5 %, 6 %, 4.5 %, 4.5 %, 4.5%, 4%, 2% and 1% in the proteome, respectively (Pratt et al., 2002). The polar requirement of the amino acid

replaced by Ser in these thesis are indicated below (Woese et al., 1966).

Table 4.2: Polar requirement of the amino acids substituted by Ser.

Amino acids	Polar requirement
Phe	5.0
Pro	6.6
Ser	7.5
His	8.4
Gln	8.6
Arg	9.1
Asn	10.0
Cys	11.5
Glu	12.5

4.1. Impact of mistranslation

A previous study showed the impact of expression of mutant tRNAs on human cells and on mouse. In this study, Ser-tRNA^{Gln} and Ser-tRNA^{Asn} had low toxicity, while Ser-tRNA^{Arg}, Ser-tRNA^{Pro}, Ser-tRNA^{Glu} and Ser-tRNA^{His} had medium toxicity. These mutagenic tRNAs were ordered according to their increasing negative impact on mammalian cells (Geslain et al., 2010). These results are consistent with the low impact of substitutions of Ser for Gln and Asn in *C. albicans* cells shown in our study. Both Ser-tRNA$_{CUG}^{Gln}$ and Ser-tRNA$_{GUU}^{Asn}$ were detected by northern blot analysis with similar expression levels to Ser-tRNA$_{UGA}^{Ser}$, but the transformation efficiency and growth rate were only marginally lower than those of the control (Ser-tRNA$_{UGA}^{Ser}$). Therefore, the Ser-tRNA$_{CUG}^{Gln}$ and Ser-tRNA$_{GUU}^{Asn}$ do not induce high toxicity in *C. albicans*. This is likely related to the chemical characteristics of these amino acids, since Asn is a small, polar neutral amino acid and Gln is a polar neutral, similarity to Ser. Gln has a similar polar requirement to Ser, while Asn has a higher polar requirement (Table 4.2). In addition, these amino acids are normally present at proteins surface, similarity to Ser.

Northern blot analysis showed that the mutagenic tRNA$_{CUC(Glu)}^{Ser}$ was strongly

downregulated. This and its very low transformation efficiency suggest that this $tRNA_{CUC(Glu)}^{Ser}$ is highly toxic. This may be related to the chemical, structural and of weight dissimilarities between Glu and Ser, but also to its abundance since it is highly abundant in the yeast proteome. In other words, it has higher potential to disrupt the proteome, especially because its polar requirement is very different than Ser (Table 4.2.). Our results are also in agreement with those of Geslain and colleagues who showed that Ser-$tRNA^{Glu}$ have a negative impact on cells, confirming the results of our work (Geslain et al., 2010).

The northern blot analysis also showed that $tRNA_{CCU(Arg)}^{Ser}$ and $tRNA_{GGG(Pro)}^{Ser}$ genes were expressed at low level. However, the high levels of expression of Hsp104 in the respective mistranslating strains show that these tRNAs induce high level of proteome disruption. Again, the results are similar to those expression of Ser-$tRNA^{Arg}$ and Ser-$tRNA^{Pro}$ in mammalian cells (Geslain et al., 2010) and may be explained by the differences in polar requirement of Arg, Pro and Ser (Table 4.2). Both amino acids also have an abundance of the 4.5 % in the yeast proteome, which is an intermediate amino acid usage value in yeast. The toxicity of Pro may also be related to the fact that it influences protein architecture due to its ring structure which increases conformational rigidity and reduces the structural flexibility of polypeptide regions (Lehninger et al., 2008).

Supposedly, maximal toxicity should be observed for the substitution of Ser for Phe, because this amino acid is chemically distinct from Ser. Indeed, this substitution is considered an extremely rare event (Henikoff and Henikoff, 1992). It is because Phe has a phenyl group and very different polar requirement to Ser. It is more hydrophobic than Pro and localizes mainly in β-sheets of proteins hydrophobic cores. In other words, the substitution of Phe by Ser normally disrupts protein structure, which is consistent with the low transformation efficiency observed in our study.

The $tRNA_{GUG(His)}^{Ser}$ showed similar expression to the wild type (wt) tRNA and did not induce significant loss of fitness. This fact, is consistent with the low abundance of this amino acid in yeast, it can uncharged or positively charged near neutral pH and with its

similar polar requirement to Ser. Interestingly, this amino acid replacement is extremely toxic in mammalian (Geslain et al., 2010).

The Ser-tRNA$_{GCA}^{Cys}$ showed similar expression to the Ser-tRNA$_{UGA}^{Ser}$ and did not induce visible negative effects relative to the control, which may be related to the similar weight and structure with to Ser and with its low abundance in yeast. However, this amino acid rarely localizes on the surface of protein due to its reactivity (Fiser et al., 1996).

4.2. Phenotyping of the mistranslating strains

Genetic code alterations can induce phenotypic advantages under specific environmental conditions (Santos et al., 1999). In this work, advantages were observed in presence of guanidine hydrochloride (GdnHCl) in all mistranslating strains. GdnHCl is an inhibitor of the ATPase activity of the Hsp104 protein, although the function of this protein is maintained in presence of this stressor in *C. albicans* (Zenthon et al., 2006). Therefore, this unexpected resistance can favor the selective advantage of all mistranslating strains that were tested. Interestingly, the Ser-tRNA$_{GGG}^{Pro}$ showed a strong selective advantage in presence of GdnHCl and at high temperatures, which are protein unfolding agents. Therefore, it is likely that Hsp104 plays an important role in refolding proteins whose unfolding was induced by the Pro for Ser replacement.

On other hand, our results show that mistranslation has a negative impact on respiratory capacity. It is likely therefore that codon ambiguities leads to an alteration of the proteome and subsequently to an excessive consumption of energy by the cell and affects respiratory activity, increasing the energetic deficit. The substitution of Ser for Cys, Phe, Pro, Gln and His lead to a significant negative impact on growth in medium lacking a carbon source (Figure 3.4). The misincorporation of Ser at the His codon showed the highest impact on respiratory activity, as cells grew poorly in media where glucose was replaced by other carbon source, indicating that this ambiguity affects mitochondrial respiration.

Another important aspect is that misincorporation of Ser at Asn, Cys and Gln codons

produced a trend of selective advantages in almost all environmental stress conditions, although these results are only statistically significant for Gln. This is in agreement with the similar chemical properties between these amino acids and Ser. The misincorporation of Ser at Phe and Pro did not produce positive outcomes in the phenotypic screen, which is coherent with their polarity since both of them are hydrophobic amino acids and Ser is not.

5.Conclusions and Future Work

In this study, the artificial mistranslation system that we have engineered successful was at introducing mutations in the proteome of *C. albicans*. The mutant tRNAs created affect negatively cell fitness and the efficiency of transformation. The characterization of the mistranslating strains showed that almost all mutant tRNAs affected negatively energy production by the mitochondria. Overall, the negative effects were detected in strains misincorporating Ser at Glu, Arg, Phe, Pro and His codons. Polarity justified the distinct negative effects between the amino acids referenced above and Ser. Interestingly, misincorporation of Ser at Gln, Asn and Cys codons did not have significant effects on fitness likely due to the polarity similarities. Growth advantages under stress conditions were observed mainly when Ser was misincorporated at the Gln codon.

Future work should clarify the negative effects of misfolded proteins through additional experiments, namely as cell death and cell cycle analysis. This is essential for studying the cellular impacts of codons ambiguities, in *C. albicans*.

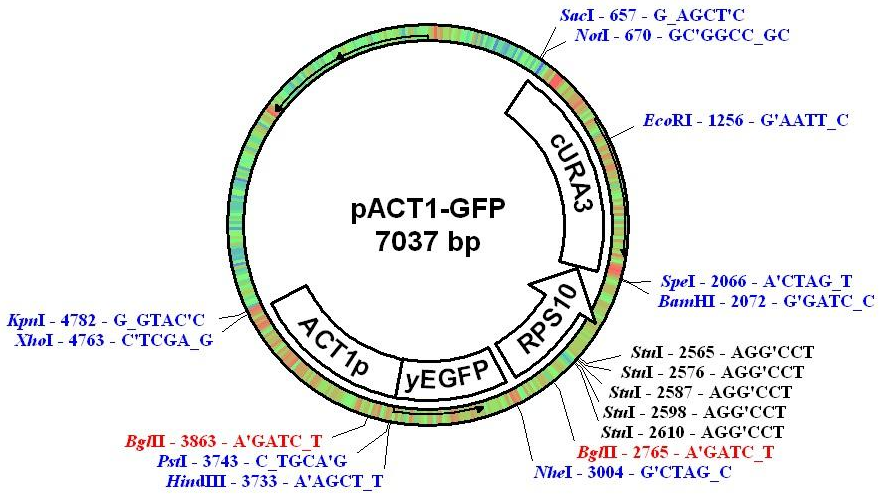
The experimental data on the effect of mistranslation on the expression of the Hsp104-GFP reporter shows that *C. albicans* responds to mistranslation by up-regulation this chaperone, however the expression of the Hsp104-GFP reporter should be confirmed by Western blot

analysis. These strains should also be characterized using a DNA microarray, in order to identity genes whose expression is deregulated by mistranslation. It will also be interesting to quantify the proteasome activity in these strains in order to determine the impact of mistranslation on protein turnover.

6. Annexes

Annex A: Map of plasmids and sequence cloned in plasmids

Map of pACT1- GFP (7037 bp)



Gene	Position (bp)
URA3	671-2059
RPS10	2072-2937
GFP	3003-3733
ACT1p	3734- 4762

Restriction enzyme	Restriction enzyme site	Position (bp)
XhoI	CTCGAG	4763
NotI	GCGGCCGC	670
SpeI	ACTAGT	2066

Sequence of *C. albicans* Hsp104 gene and Hsp104 promoter cloned in pGFP plasmid

Sequence Hsp104 gene with 1000 bp upstream and downstream.

At grey is the region cloned: Hsp104 gene and Hsp104 promoter

>orf19.6387 COORDS:Ca21chrR_C_albicans_SC5314:1788044-1792743C (4700 nucleotides)
TAGAGCGTAAGGAATATACTGTGGGAGTGGCGGATATCTGTTTTTTTAAAAGTTGCAGTT
CCTCATACACCACGGAAGTGTTGTTCAAACAGACAATCGAATTGCCCGGTACTTTCGAAT
GTTGTGCAACTACATTTTCTGTGCACTTTGATGCATAAAAGACAACTACAAATTGAAATA
AACACGTAATCGGTCTATAAATCATCTTATATTTGCATTGATTTTCTTGTTTGTATTTGT
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Sequence of *C. maltosa* LEU2 gene cloned in pHSP104-GFP plasmid

```
LOCUS       AY534142         2119 bp    DNA     linear   PLN 11-FEB-2005
DEFINITION  Candida maltosa strain ATCC 38041 3-isopropylmalate
            dehydrogenase(LEU2) gene, complete cds.
ACCESSION   AY534142
VERSION     AY534142.1  GI:46811100
KEYWORDS    .
SOURCE      Candida maltose
ORGANISM    Candida maltosa
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            Saccharomycetes; Saccharomycetales; mitosporic
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REFERENCE   1  (bases 1 to 2119)
  AUTHORS   Noble,S.M. and Johnson,A.D.
  TITLE     Strains and strategies for large-scale gene deletion studies
            of the diploid human fungal pathogen Candida albicans
  JOURNAL   Eukaryotic Cell 4 (2), 298-309 (2005)
  PUBMED    15701792
REFERENCE   2  (bases 1 to 2119)
  AUTHORS   Noble,S.M. and Johnson,A.D.
  TITLE     Direct Submission
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                     /db_xref="ATCC:38041"
                     /db_xref="taxon:5479"
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                     /gene="LEU2"
     mRNA            <816..>1937
                     /gene="LEU2"
                     /product="3-isopropylmalate dehydrogenase"
     CDS             816..1937
                     /gene="LEU2"
                     /note="Leu2; third step in the leucine biosynthesis
                     pathway"
                     /codon_start=1
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                     /protein_id="AAT01852.1"
                     /db_xref="GI:46811101"

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KHHLIGGAAIDATGVPLPDDALESAKKSDAVLLGAVGGPKWGTGAVRPEQGLLKIRKE
LNLYANIRPCNFASDSLLELSPLRPEVVKGTNLIIVRELVGGIYFGDREEQEESEDKQ
TAWDTEKYTVDEVTRITRMAAFMALQHNPPLPIWSLDKANVLASSRLWRRTVDKVISE
EFPTLSVQHQLIDSAAMILIQNPTKLNGIIITSNMFGDIISDEASVIPGSLGLLPSAS
LASLPDTNTAFGLYEPCHGSAPDLPANKVNPIATILSAASMLRLSLDCVKEAEALEEA
VKQVLDKGIRTADLRGTSSATEVGDAIVEAVTKILKEKA"

ORIGIN
        1 aggtttgaga tcaggtactt tggcaccacc attagtagct gggtttggtg aagctgcaag
       61 attaatgaaa caagaatctg catttgacaa gaaacatatt gagaggttat ctactaaatt
      121 gaagaacggt ttgttatcca ttccatctac tcagtttaac ggttgtaata acccaacata
      181 tcaataccca ggttgtgtga atgtttcctt tgcatacatt gaaggggaat ctttgttaat
      241 ggcattgaaa gatattgcat tgagttctgg ttctgcatgt acctctgcat ctttggaacc
      301 atcgtatgtt ttacatgctt tgggtgccga tgatgccttg gctcattctt ccattagatt
      361 cggtattggt agattcacca ctgaagcaga agttgactat gttattcaag caattaatga
      421 aagagttgat ttcttgagaa agatgtctcc attatgggaa atggttcaag aaggtattga
      481 cttgaactct atcgaatgga gtggacatta aggttttttg ttttttgta atttgtgttg
      541 ttatgtgtaa ttatcgtgta tagagtaatt tcggaaattg tactttcgtt catgtatctg
      601 aaccagatga ataatacgtt cgtattgttg aagaatgact aactcctgta acggcgtgcg
      661 tgggttccgc agccgggttc aatttttttt ttcttgttca ctataaaaat atataataaa
      721 cgaaaaaaat ccgagggact caggctatga aatttttcac tcaagaattt tttttccttc
      781 atccttaatt cctttattca ctacccaaac ccacaatgtc agttaaaact aaaactatta
      841 caatttttacc aggtgatcac gtcggtaccg aaattgtcaa tgaagctatc aaagttttag
      901 aagcaattga agctgccact ccttaccaaa aaatccattt cgatttcaaa catcatttaa
      961 tcggtggtgc tgccattgat gccactggtg tcccattgcc agatgacgca cttgaaagtg
     1021 ccaaaaaatc tgatgccgtc ttattgggtg ctgttggtgg accaaaatgg gggactggcg
     1081 ccgttcgtcc agaacaaggt ttattaaaga ttcgtaaaga attgaaccct tatgctaaca
     1141 tcagaccatg taactttgcc agtgactcct tattggaatt atccccattg agaccagaag
```
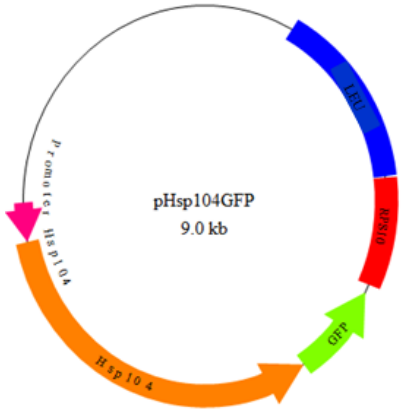

```
      1201 tcgttaaagg tacaaacttg atcattgttc gtgaattagt tggtggtatt tatttcggtg
      1261 atcgtgaaga acaagaagaa agtgaagata aacaaaccgc atgggatact gaaaaataca
      1321 ccgtcgatga agtcaccaga atcacccgta tggctgcctt catggcttta caacataacc
      1381 caccattacc aatctggtca ttggataagg ctaatgtttt agcttcttct agattatgga
      1441 gaagaactgt cgataaagtg atttctgaag agttcccaac tttgtctgtt caacatcaat
      1501 tgattgactc cgccgccatg attttaattc aaaacccaac caaattgaat ggtataatca
      1561 tcacttctaa catgtttggt gatatcattt ccgatgaagc ttcagttatt ccaggttcct
      1621 tgggtttatt accatctgca tctttggctt cattgccaga taccaacacc gcttttggtc
      1681 tttatgaacc atgtcacggt tctgctcctg atttaccagc caataaagtt aacccaattg
      1741 ctactatttt atctgctgct tctatgttga gattatcttt ggattgtgtg aaagaagctg
      1801 aagctttaga agaagccgtg aaacaagtct tggataaagg tatcagaact gcagatttaa
      1861 gaggtaccag ttcagctact gaagttggtg acgcgattgt cgaagctgtt actaaaatct
      1921 taaaagaaaa agcttaagta aagtatacac aaaatttaac gaatattatt aatacaaaat
      1981 gaacaccacc taaatacttt tttttaaaaa tgtaccgact cttctaaaaa ttgcgataat
      2041 tcaatatcgg aaatttctct caccggttta cttggatctt cggaaataaa tctatttgaa
      2101 gtaatggtag ccctcactt
//
```

Map of HSP104-GFP-LEU (9019 bp)

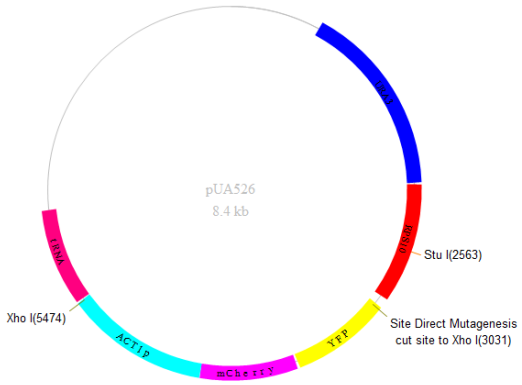


Gene	Position (bp)
LEU3	671-2059
RPS10	2072-2937
GFP	3004-3733
HSP104	3733- 6435
Promoter HSP104	6436-6750

Restriction enzyme	Restriction enzyme site	Position (bp)
XhoI	CTCGAG	6745

NotI	GCGGCCGC	670
SpeI	ACTAGT	2066
SmaI	CCCGGG	3733

Map of pUA526 (8448 bp)



Gene	Position (bp)
URA3	671-2059
RPS10	2071-2937
YFP	3005-3726
mCherry	3742-4449
ACT1p	4450-5480
tRNA with anticodon	5770-5851

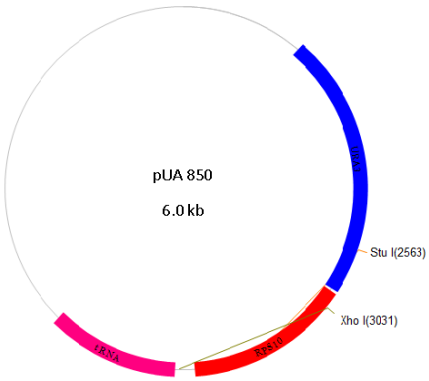
Sequence of tRNA^{Ser} tS(UGA)I Chr 9 from 247847 to 249928 from *S. cerevisiae*:

CTCGAGGGGGGGCCCTCCGTGCATAACGAATGACTCTTGTGTCGTGTTATCCGGGCTTTCTCCTTTTCCTTTC
CCCGAGAAAGCAACCGACGCATACGGATATAAGCGGATCCCACCGAAAAGGAGACTCCACGGTCTTGGTGAAT
AACATCGCGCAGATTATTCTGCGAACCGTGGCTAATAGGAGCATGCCCGAAGAAATGATTATGGGTGCGTGAT
CACCAATCTGTGTGAGGCTGTCGAAGATATCTGATAATCAGTAAGCGATAAAAAAAAAATAAAAATAAAAAAA
TAACGACACCAGCAGGATTTGAACCAGCGCGGGCAGAGCCCAACAGATTTCAAGTCTGTCGCCTTAACCACTC
GGCCATAGTGCCTAAAACAATGTAGGTTATTTAAGCAAGTATTGTAGATACTTTTCGTAATAAACTACAATGC
ACCCACGACTCGCGGTGTAATGATGGCATGAAATCATTGAACGAAGTTTTGCGGCTATACGGCTGAAGGACGA
GACTAAAGGGACAGGAATTATTAATGCGGGGTATAATTTGAATAGTATTAACGGGCACTGCCGTTTAGCCATC
AAATGCTATTGTTGGGGTATTCTCTCTACTTTTTGTTCTTGGCTTGAACCTTTTCGGCGGTTGGCAATCGTCC

GTATATAAGCATCGGCTGTCCCAATCCTCTATTGCCCTTTTCCCTTGCACCTCCTTCGGTACC

Restriction enzyme	Restriction enzyme site	Position (pb)
XhoI	CTCGAG	3032 and 5475
ApaI	GGGCCC	5484
KpnI	GGTACC	6189
StuI	AGGCCT	2563

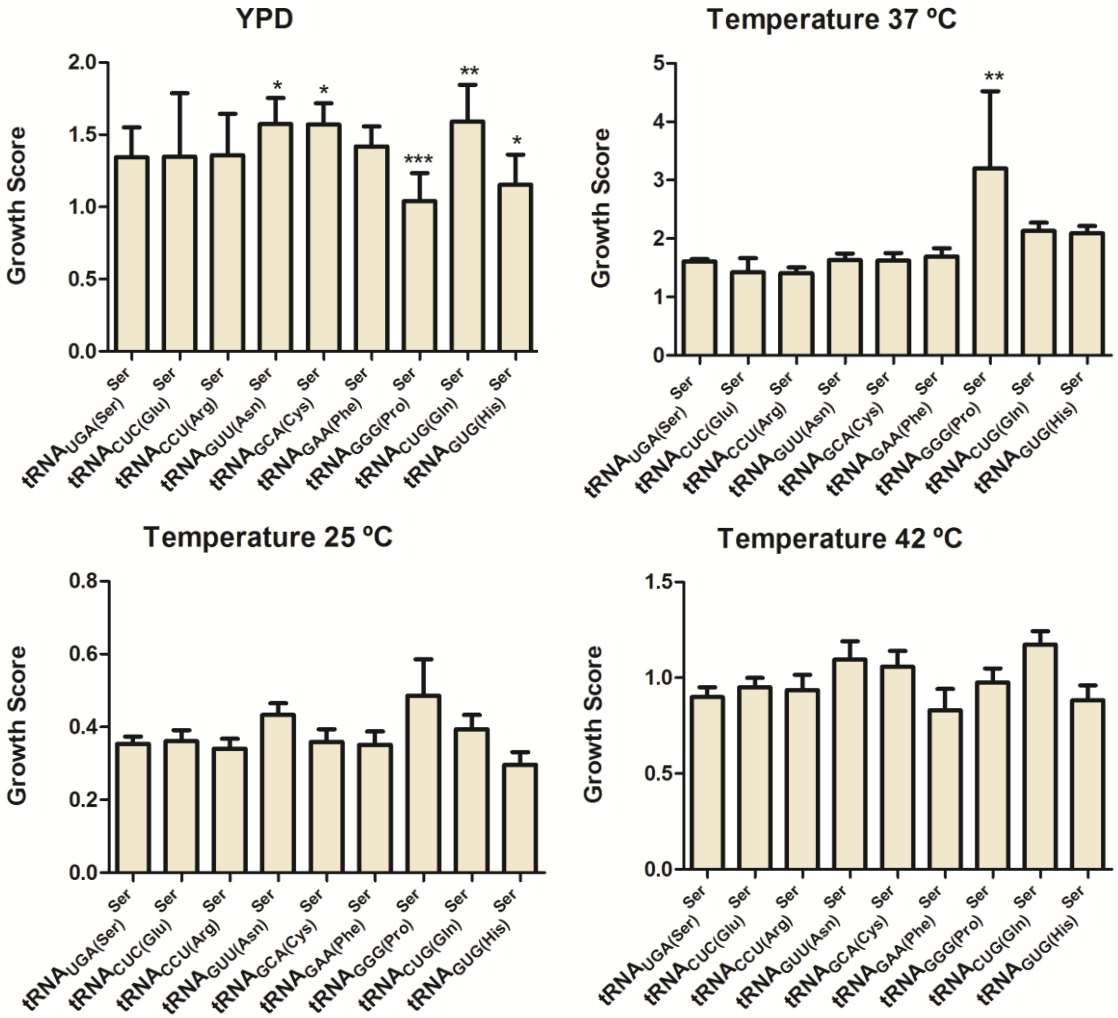
Map of pUA850 (6005 bp)

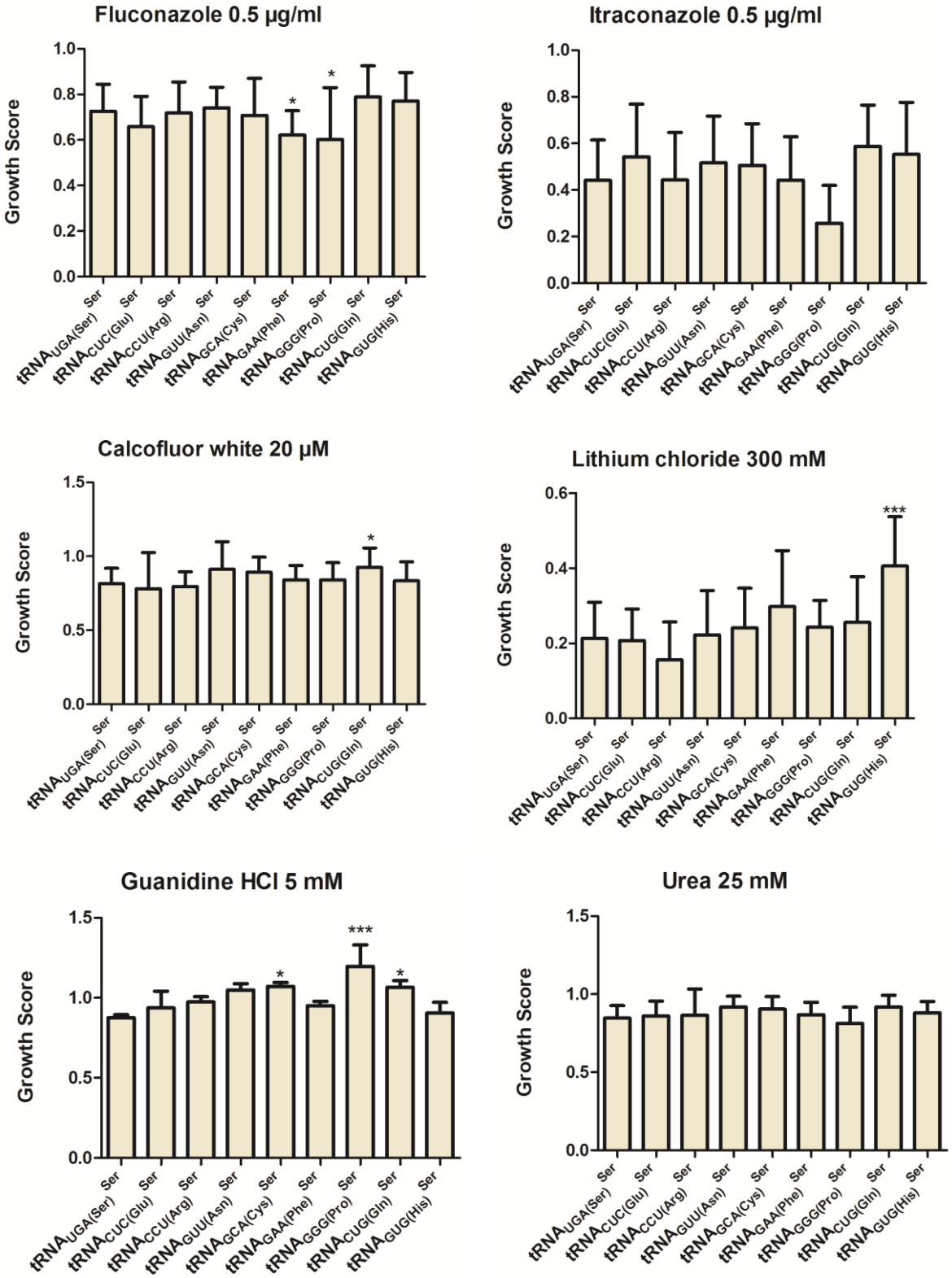


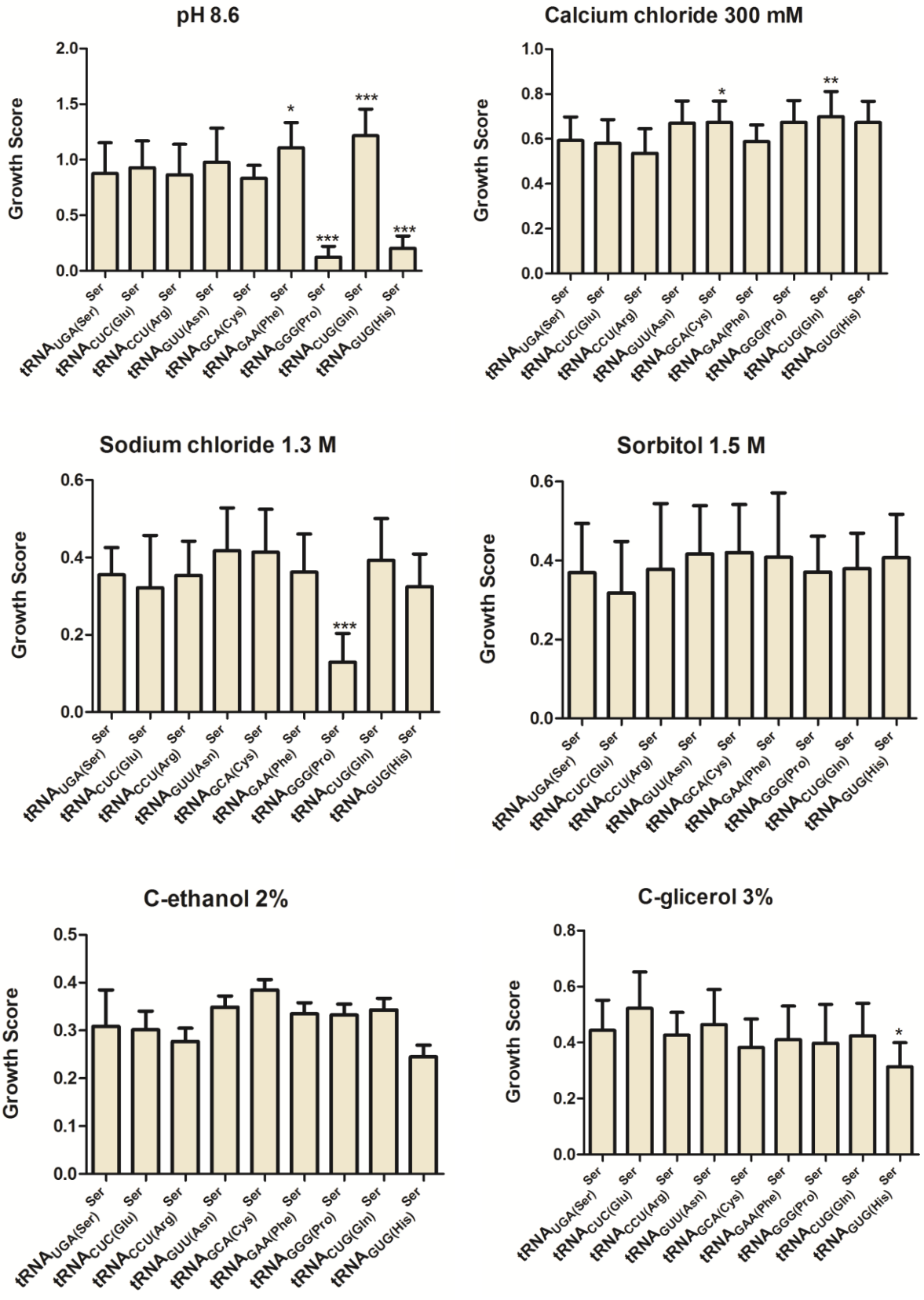
Gene	Position (bp)
URA3	671-2059
RPS10	2071-2937
tRNA with anticodon	3327-3408

Restriction enzyme	Restriction enzyme site	Position (bp)
XhoI	CTCGAG	3032
ApaI	GGGCCC	3041
KpnI	GGTACC	3746
StuI	AGGCCT	2563

Annex B: Phenotypic screening results




Fluconazole 0.5 µg/ml
Growth Score
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Ser
tRNAUGA(Ser)
tRNACUC(Glu)
tRNACCU(Arg)
tRNAGUU(Asn)
tRNAGCA(Cys)
tRNAGAA(Phe)
tRNAGGG(Pro)
tRNACUG(Gln)
tRNAGUG(His)
Itraconazole 0.5 µg/ml
Growth Score
1.0
0.8
0.6
0.4
0.2
0.0
Ser
tRNAUGA(Ser)
tRNACUC(Glu)
tRNACCU(Arg)
tRNAGUU(Asn)
tRNAGCA(Cys)
tRNAGAA(Phe)
tRNAGGG(Pro)
tRNACUG(Gln)
tRNAGUG(His)
Calcofluor white 20 µM
Growth Score
1.5
1.0
0.5
0.0
*
Ser
tRNAUGA(Ser)
tRNACUC(Glu)
tRNACCU(Arg)
tRNAGUU(Asn)
tRNAGCA(Cys)
tRNAGAA(Phe)
tRNAGGG(Pro)
tRNACUG(Gln)
tRNAGUG(His)
Lithium chloride 300 mM
Growth Score
0.6
0.4
0.2
0.0
***
Ser
tRNAUGA(Ser)
tRNACUC(Glu)
tRNACCU(Arg)
tRNAGUU(Asn)
tRNAGCA(Cys)
tRNAGAA(Phe)
tRNAGGG(Pro)
tRNACUG(Gln)
tRNAGUG(His)
Guanidine HCl 5 mM
Growth Score
1.5
1.0
0.5
0.0
***
*
*
Ser
tRNAUGA(Ser)
tRNACUC(Glu)
tRNACCU(Arg)
tRNAGUU(Asn)
tRNAGCA(Cys)
tRNAGAA(Phe)
tRNAGGG(Pro)
tRNACUG(Gln)
tRNAGUG(His)
Urea 25 mM
Growth Score
1.5
1.0
0.5
0.0
Ser
tRNAUGA(Ser)
tRNACUC(Glu)
tRNACCU(Arg)
tRNAGUU(Asn)
tRNAGCA(Cys)
tRNAGAA(Phe)
tRNAGGG(Pro)
tRNACUG(Gln)
tRNAGUG(His)


pH 8.6
Growth Score
2.0
1.5
1.0
0.5
0.0
*
***
***
***
Ser
tRNAUGA(Ser)
tRNACUC(Glu)
tRNACCU(Arg)
tRNAGUU(Asn)
tRNAGCA(Cys)
tRNAGAA(Phe)
tRNAGGG(Pro)
tRNACUG(Gln)
tRNAGUG(His)
Calcium chloride 300 mM
Growth Score
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Sodium chloride 1.3 M
Growth Score
0.6
0.4
0.2
0.0
***
Sorbitol 1.5 M
Growth Score
0.6
0.4
0.2
0.0
C-ethanol 2%
Growth Score
0.5
0.4
0.3
0.2
0.1
0.0
C-glicerol 3%
Growth Score
0.8
0.6
0.4
0.2
0.0
*

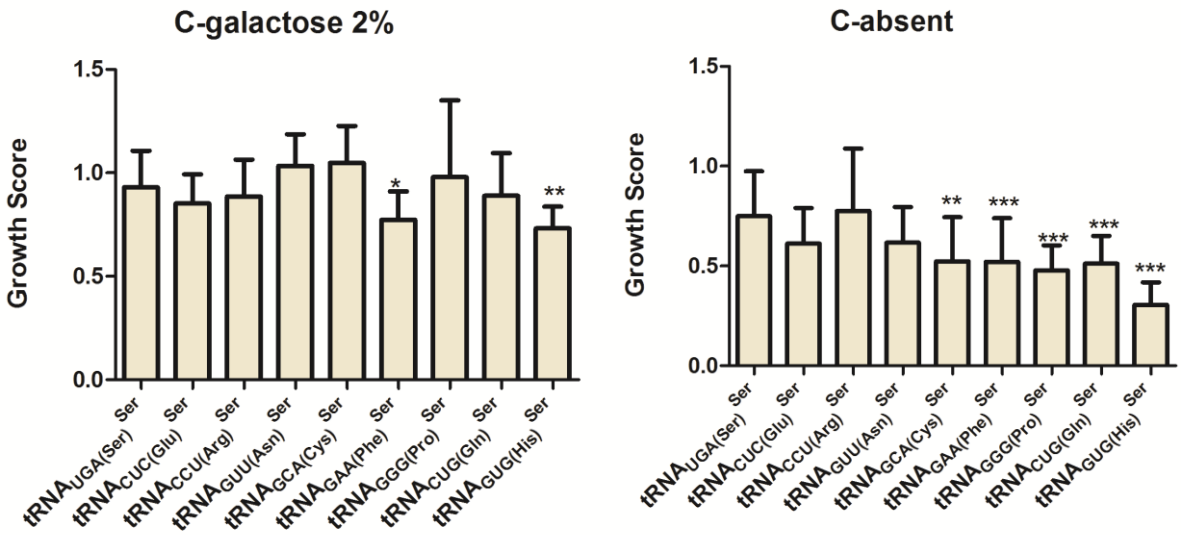
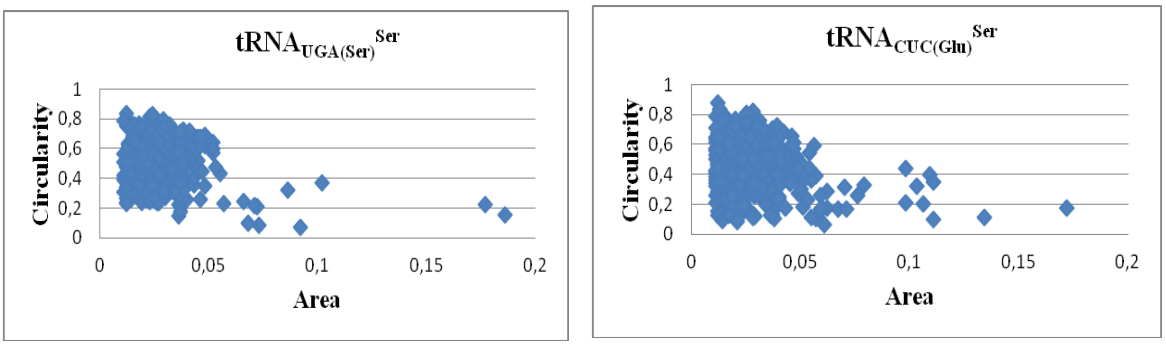


Figure 6.1: Phenomics of the mistranslating strains. Growth scores of *C. albicans* strains misincorporating Ser at eight non-cognate codons were determined after 5 days, and in presence of drugs (Fluconazole and Itraconazole) and temperature (25ºC, 37ºC, 42ºC) were determined after 3 days. Growth scores were calculated by dividing the area of each colony in the presence of each stressor by the area in control medium lacking stressor. The results are expresses as mean ± SD of duplicates of three independent clones of each strain (***p□ 0.001; **p□ 0.01; *p□ 0.05 one-way ANOVA post Dunnett´s multiple comparison test with CI 95% relative to control).

Annex C: Analysis of the circularity and area of the cells by Image J



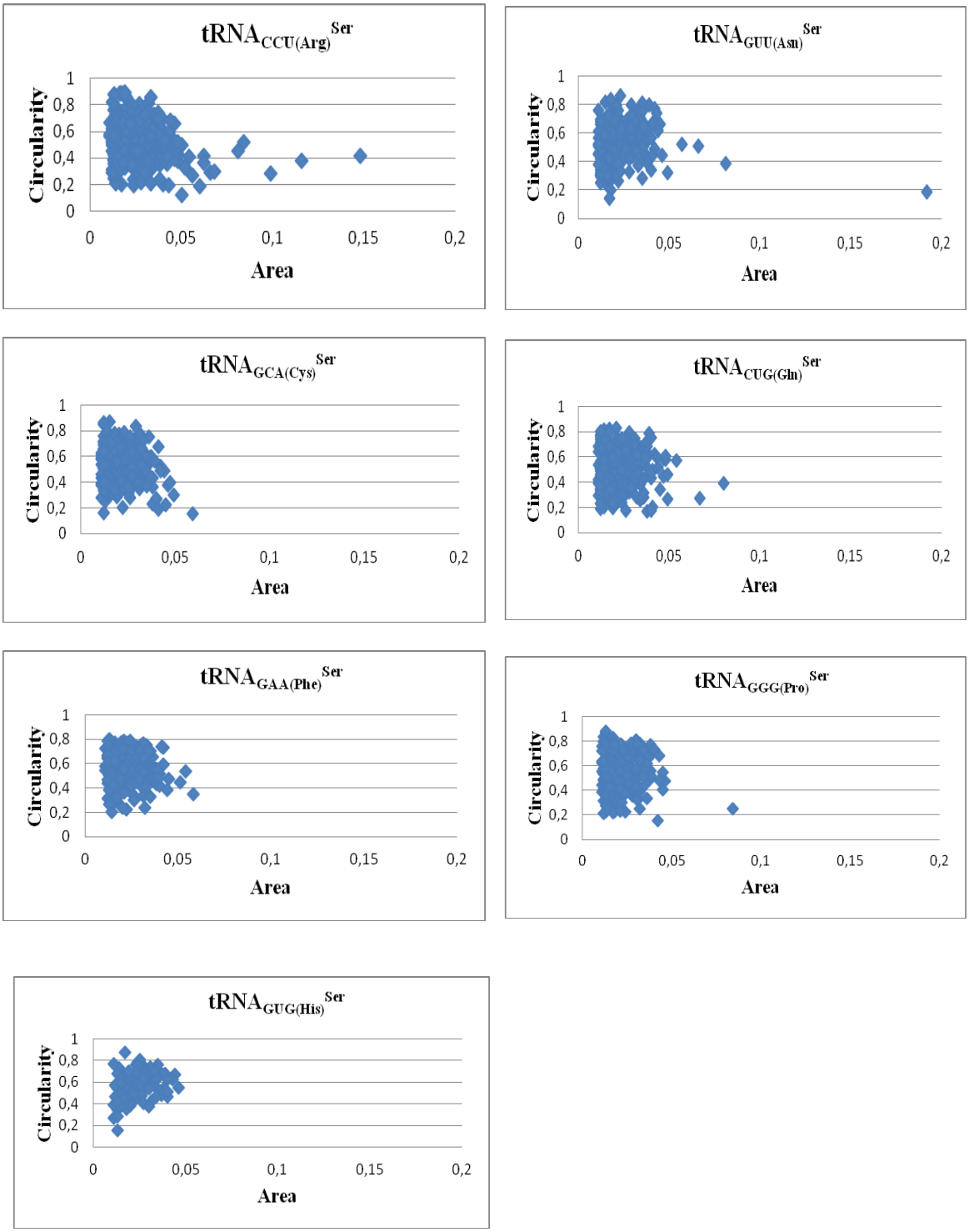


Figure 6.2: Circularity and area of the cells were analyzed for all strains by Image J.

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