Universidade de Aveiro
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Departamento de Ciências Médicas

João Pedro Ferreira Oliveira

Identification of transcription factors involved in *Candida albicans* mistranslation

Identificação de factores de transcrição envolvidos no processo de *mistranslation* 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 Biomedicina Molecular, realizada sob a orientação científica do Professor Manuel António da Silva Santos

Dedico esta tese à minha família e namorada

o júri

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.

Palavras-chave

Candida albicans, fatores de transcrição, *mistranslation,* ambiguidade de codões, aaRS

Resumo

Candida albicans é o fungo patogénico mais comum em humanos. Este fungo normalmente é um comensal, no entanto quando indivíduos imunodeprimidos são expostos a ele desenvolvem normalmente infeções desde irritações de pele a doença sistémica generalizada. *Candida albicans* é caracterizada pela reatribuição do codão CUG de Leucina para Serina por um tRNA híbrido de serina ($tRNA_{CAG}^{Ser}$) que em condições normais descodifica o CUG-leucina como leucina (3 a 5%) e como serina (93 a 95%). O $tRNA_{CAG}^{Ser}$ é aminoacilado por duas aminoacil tRNA sintetases, a leucil-tRNA sintetase (LeuRS) e a seril-tRNA sintetase (SerRS). Estudos anteriores mostraram que quando *Candida albicans* é exposta ao stress, nomeadamente temperatura, pH, osmolaridade e antifúngicos, o nível de *mistranslation* de leucina aumenta, sugerindo que *C. albicans* regula os níveis de *mistranslation* in resposta ao stress. Nesta tese começamos por caracterizar mecanismos que controlam a *misincorporation* de leucina em *C. albicans*. Para isto, transformamos estirpes de *C. albicans* que contêm deleções de genes de cinases selecionadas e de fatores de transcrição com sistemas repórter fluorescentes para monitorizar os níveis de incorporação de leucina e serina no codão CUG. A atividade dos promotores LeuRS (CDC60) e da SerRS (SES1) foi quantificada em várias condições fisiológicas diferentes utilizando um segundo sistema repórter florescente. Os resultados sugerem que a *misincorporation* de leucina nos codões CUG pode ser devido ao aumento da expressão de LeuRS ou a um decréscimo da expressão de SerRS. Na segunda parte do estudo, proteínas da coleção de estirpes KO de *C. albicans* foi extraída e os níveis de LeuRS e SerRS foram quantificadas por western blot utilizando anticorpos para ambas as enzimas. O rácio da expressão de LeuRS/SerRS nos mutantes em relação a estirpe selvagem permitiu a identificação de 3 fatores de transcrição putativos que regulam a expressão de LeuRS e SerRS, nomeadamente EFG1, MRR1 e ACE2.

Keywords *Candida albicans*, transcription factors, mistranslation, codon ambiguity, aaRSs

Abstract

Candida albicans is the main human fungal pathogen. It is usually commensal yet when immunocompromised individuals are exposed to it infections normally develop from mild rashes to systemic disease. *Candida albicans* is characterized by the reassignment of the CUG codon from Leucine to Serine by a hybrid serine tRNA ($tRNA_{CAG}^{Ser}$) which decodes the leucine-CUG as leucine (3 to 5 %) and as serine (95 to 97 %) under normal growth conditions. The $tRNA_{CAG}^{Ser}$ is aminoacylated by two aminoacyl tRNA synthetases, the leucyl-tRNA synthetase (LeuRS) and the seryl-tRNA synthetase (SerRS). Previous studies showed that when *Candida albicans* is exposed to stress, namely temperature, pH, osmolarity and antifungals, the level of leucine misincorporation rises, suggesting that *C. albicans* regulates mistranslation levels in response to stress. In this thesis we started characterizing the mechanisms that controls Leu misincorporation in *C. albicans.* For this, *C. albicans* strains harboring deletions in genes of selected kinases and transcription factors were transformed with fluorescent reporter systems to monitor the levels of leucine and serine incorporation at CUG codons. The activity of the LeuRS (CDC60) and SerRS (SES1) promoter was quantified in several different physiological conditions using a second fluorescent reporter system. The results suggested that Leu misincorporation at CUG codons could be due to increased LeuRS expression or decreased SerRS expression. In the second part of this study, protein from *C. albicans* KO strain collection was extracted and the levels of LeuRS and SerRS were quantified by western blot using antibodies against both enzymes. LeuRS/SerRS expression ratio in the mutant relative to WT strains allowed the identification of 3 putative transcription factors that regulate the expression of LeuRS and SerRS, namely EFG1, MRR1 and ACE2.

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List of Abbreviations

aa-AMP aminoacyl-adenylate

aaRS aminoacyl-tRNA synthetase

AAS amino acid accepting stem

AIDS Acquired immunodeficiency syndrome

APS Ammonium peroxodisulfate

ASL anticodon stem loop

aa-tRNA aminoacylated tRNA

BCA Bicinchoninic acid assay

Bp Base pairs

CaCl2 calcium chloride

CO_2 Carbon dioxide

CH_3CO_2K Potassium acetate

DNA deoxyribonucleic acid

DNTP´s deoxynucleotides

DSL D-Stem loop

EDTA Ethylenediamine tetraacetic acid

eRF eukaryotic release factors

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

GTP Guanosine triphosphate

GTF General transcription factor

GUT Gastrointestinally induced transition

KCl potassium chloride

KO knock-out

L (ml, µl) liter (mililiter, microliter)

Leu leucine

LeuRS leucyl tRNA synthetase

LiAc lithium acetate

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

MAPK mitogen activated protein kinase

MM minimal medium

MnCl2 manganese chloride

MOPS Na 3- (N-Morpholino) propanesulfonic acid sodium salt

mRNA messenger ribonucleic acid

NaCl sodium chloride

OD optical density

PBS Phosphate-buffered saline

PCR polymerase chain reaction

PEG polyethylene glycol

PMSF phenylmethane sulfonyl fluoride

RbCl2 rubidium chloride

RNA ribonucleic acid

Rpm revolutions per minute

SDS Sodium dodecyl sulfate

Ser serine

SerRS seryl tRNA synthetase

TEMED Tetramethylethylenediamine

TF transcription factor

Tris tris(hydroxymethyl)aminomethane

tRNA transfer ribonucleic acid

TSL T-stem loop

UV Ultra violet

yEGFP yeast enhanced green fluorescent protein

YPD yeast extract peptone dextrose

WT wild type

Other abbreviations will be explained when used in the text.

Chapter I – Introduction

tRNA biology

Overview

Transfer RNAs (tRNAs) are non-coding short ubiquitous RNAs with a low number of base pairs, usually 70 to 100 nucleotides, whose tertiary structure forms an inverted L(1). tRNAs have two main functions; chemically link a specific amino acid and transport it to the ribosome. The tRNA 2D canonical structure is a cloverleaf with 6 distinct subdomains; the first subdomain is the AAS (Amino acid accepting Stem) that contains 7 bp and is terminated at position 73 in the 3' and the second subdomain is the 2 nucleotide connector between the AAS and the DSL (D- Stem Loop), the third subdomain is the DSL with a 4 bp stem which is closed by a D-Loop with 7 to 11 nucleotides, the fourth subdomain is the ASL(Anticodon Stem Loop) with a 5 bp stem and a loop of 7 nucleotides, a variable region of 4 to 24 nucleotides arranged in a stem loop being the 5 subdomain and the 6 subdomain a TSL (T-Stem Loop) with a 5 bp stem and a loop of 7 nucleotides (2)

tRNA genes are transcribed by RNA polymerase III (Pol III) (3). The pre-tRNAs contain a 5' leader sequence and a 3' trailer sequence (4), which are processed post or co-transcriptionally. The 5' leader sequence is removed by ribonuclease P (RNase P) (5), producing a tRNA molecule with a mature 5′ (5–7). The processing of the 3' trailer sequence requires several enzymes(6); the first one is RNase E which plays a critical role in the maturation of the tRNA by cleaving in the middle of the 3' trailer (4).This enzyme is also necessary to initiate the 5' leader sequence cleavage since in its absence RNase P cannot cleave the 5' leader sequence (8). After the initial cleaving by RNase E, several RNases process the 3'- trailer, namely RNase PH and RNase T, RNase II, PNPase, and RNase BN (4,6,9). As a last step of the cleavage, the RNase Z cleaves the tRNA immediaty after the discriminator base, allowing for the addition of the CCA end by tRNA nucleotidyltransferase (Figure 1.3) (4,6).

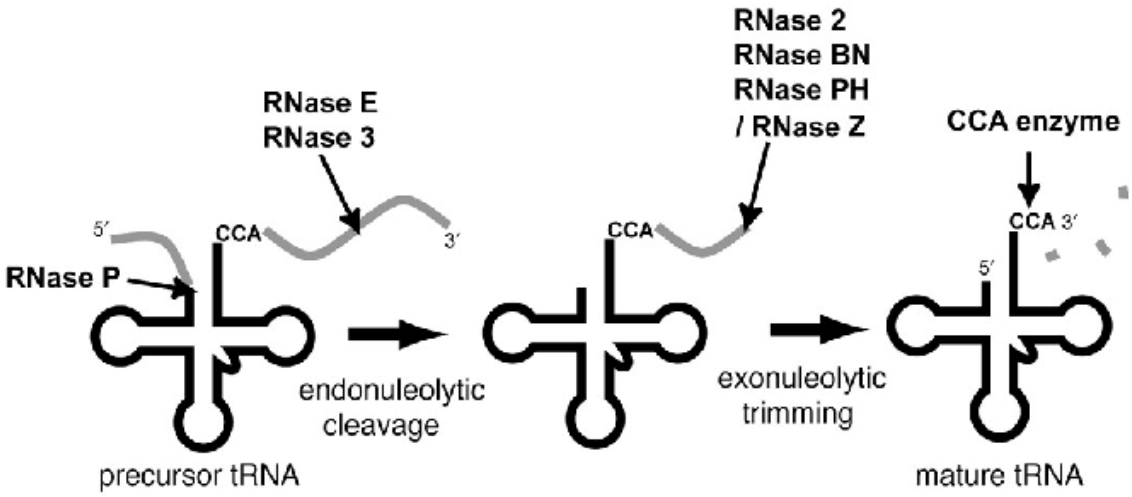

Figure 1.1: **The pre-tRNA processing of the 5′ leader and 3′ trailer sequences.** The 5'leader sequence is removed by RNase P, while 3'trailer is cleaved by RNase E and RNase 3, and trimmed by RNase 2, RNase BN, RNase PH and RNase Z. Finally, the CCA is added to the 3' trailer by a tRNA nucleotidyltransferase. Adapted from *(4)*

In some tRNAs maturation involves intron splicing, which happens in the cytoplasm (10). The splicing mechanism involves 3 distinct steps: the first step is the cleavage at the two splice sites by an endonuclease, which leads to two half tRNA molecules and a linear intron. The next step joins the two half tRNA molecules with the help of a tRNA ligase (Trl1) by adding a phosphate to the 3´-tRNA and forming a phosphodiester bond between the 5' and 3' halves. The last step removes the extra 2' phosphate from the junction of the two tRNA halves; this reaction is catalyzed by the 2' phosphotransferase (Tpt1) (Figure 1.4) (10,11).

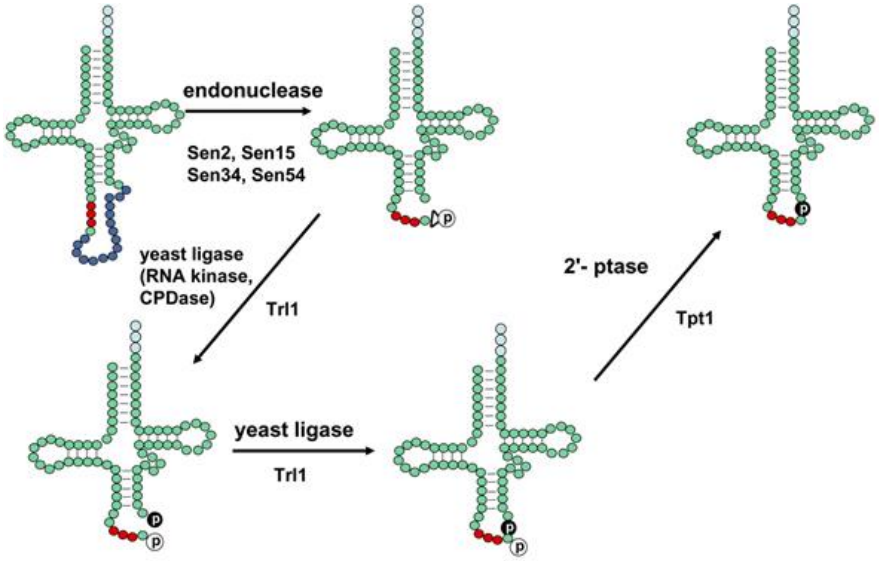

Figure 1.2: **tRNA splicing and ligation pathways in yeast.** tRNA is displayed in secondary structure with the anticodon indicated by red circles and the intron indicated by blue circles. The pre-tRNA is cleaved by an endonuclease, resulting in 5' half molecules with a cyclic phosphate (triangle with a white circle containing P) and a 5'-OH. Next, the Trl1promotes the ligation of the half molecules by adding a phosphate to the 3´-tRNA (black circle containing the P) half and binding the 5' and 3' halves by a phosphodiester bond. Finally, a tRNA Tpt1 removes the extra phosphate. Adapted from(6)

AARS

There are 20 aminoacyl-tRNA synthetases (aaRSs) that covalently link and catalyze the ligation of amino acids to their cognate tRNA (12–14). Structural and sequence analysis showed that the aaRS are divided into two distinct groups: class I and class II (14), each one with 10 different enzymes (12). The class I enzymes share the HIGH and KMSKS sequence motives in the active site of the Rossmann (nucleotide binding) fold and five-stranded parallel β-sheet connected by α-helices (12,15). These class synthetases are usually monomeric with a few exceptions. The class II enzymes share three degenerate motifs in their active site which are made up of seven stranded antiparallel β-sheets flanked by α-helices (16), the active site of this enzymes is composed by approximately 250 amino acid residues. This class is mostly composed of homodimers and few are monomers (12,16,17).

Aminacylation is a 2 step reaction: the first step activates the amino acids with ATP to produce aminoacyl-adenylates (aa-AMPs) a stable enzyme-bound intermediate: the second step consists of the transfer of the aa-AMP to the tRNA(12,17,18).

aaRs are very selective for their cognate tRNA, but some amino acids only differ by a single methyl group, ex: Ile and Val or Val and Ile and this small difference can lead to misacylation and protein structure disruption (19). In order to achieve accuracy in amino acylation the aaRS have an editing domain. Editing can be done through two different mechanisms: pre-transfer editing and post-transfer editing (15,16,18). In pre-transfer editing, the enzyme can hydrolyze noncognate aminoacyl adenylates releasing the amino acids and AMP, this reaction can be tRNA-independent or tRNA-dependent (15,16).

The post-transfer editing requires the 3' end of mischarged aa-tRNAs to migrate from the active site to the site of editing, where the tRNA incorrectly aminoacylated is hydrolyzed to free amino acid and tRNA (Figure 1.5) (15,16).

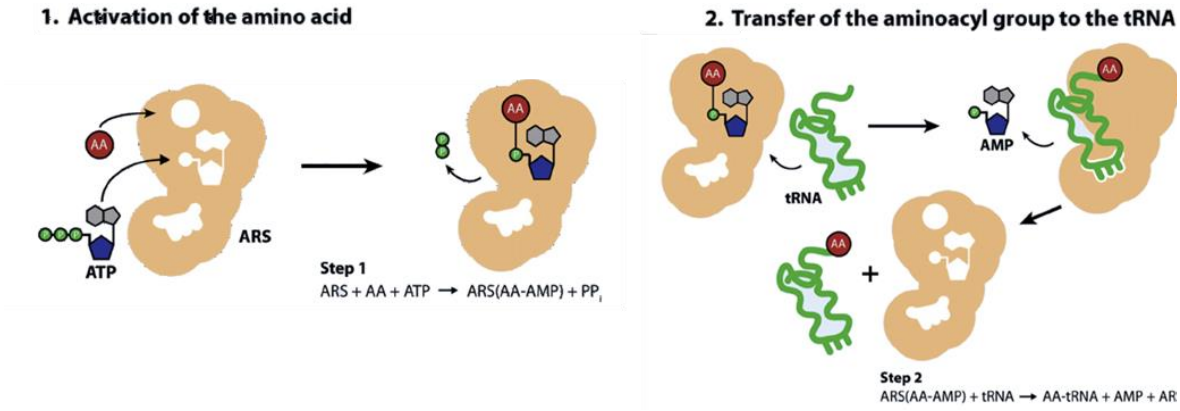

Figure 1.3: **Aminoacylation reaction.** In step 1, **t**he amino acid is activated with ATP at the aaRS active site to form aminoacyl adenylate (aa-AMP) coupled with the release of pyrophosphate molecule (PP_i). In step 2, **t**he aaAMP reacts with the tRNA Amino acid transfer and the aa is transferred to the tRNA (aa-tRNA), resulting in the tRNA esterification and release of AMP. Adapted from(15)

Translation

The translation process is divided into 3 phases, initiation, elongation and termination. The initiation starts with the formation of the 43S preinitiation complex, which results from the dissociation of the ribosome 80S, followed by the assembly of the ternary complex, composed by eIF2, a heterotrimer of α, β and γ subunits, methionyl-initiator tRNA and GTP. The binding of the ternary complex with the ribosomal 40S sub-unit forms the 43S pre-initiation complex. This pre-initiation complex is then able to recognize the m^7G cap

structure at the 5’ end of the mRNA to start the next step in the initiation process. The next step is the scanning of the mRNA from the 5’ to 3’ in order to find the AUG start codon, where the scanning machinery stops to form a 48S initiation complex. With the 48S initiation complex formed the 60S subunit joins by a process of GTP hydrolysis forming the 80S ribosome (20), ready for elongation.

Elongation depend on the formation of a ternary complex composed of the elongation factor (EF), aminoacyl-tRNA and GTP. The 80S ribosome has three tRNA binding sites the aminoacyl (A) site, the peptidyl (P) site, and the exit (E) site. During the process of elongation, an aa-tRNA is deposited in the aminoacyl (A) site along with the ternary complex and reacts with the peptidyl-tRNA which in turn leads to the elongation by one amino acid. After this deposition, the mRNA in the ribosome is removed with the help of the elongation factor EF-G, that uses the energy of the GTP hydrolysis to move the mRNA-tRNA complex through the aminoacyl(A) site and peptidyl (P) site to the exit (E) site (21,22). This process is then repeated leading to the formation of a chain of amino acids that is elongated until the ribosome encounters one of the three stop codons UAG, UAA, or UGA.

The last step of protein synthesis is termination. As mentioned above this step begins with a stop codon, at the A-site of the ribosome. The 3 stop codons this codons are recognized by class I release factor eRF1 (eukaryotic release factor 1) and class II release factor eRF3 (eukaryotic release factor 3) that activates a hydrolytic reaction leading to the release of the polypeptide chain from the tRNA (21,23). Once the complete chain has been removed, the ribosomes dissociate in the two subunits (40S and 60S) and the mRNA. Deacylated tRNA and the class I release factor eRF1 (23,24) are released .The translational machinery is then recycled for another translation cycle (23).

Mistranslation

The faithful translation of the genetic code is essential to life (25). Despite this, translation is not an error free process as 1 amino acid is misincorporated for each 10^3 to 10^4 codons decoded (26). Mutations and environmental conditions may increase error rate (27) . Errors occur during tRNA charging by aminoacyl-tRNA synthetase (aaRS) and during the mRNA decoding by the ribosomes. The aminoacylation errors are caused by failure of the aminoacyl-tRNA synthetases in distinguishing similar amino acids or by the incorrect

recognition of tRNAs (28) . The mRNA decoding errors are of 4 distinct types: missense errors, nonsense errors, frameshift errors, and processivity errors (Figure 1.6). Missense errors are normally the consequence of an incorrectly charged tRNA (misacylation or mischarging errors), or due to anticodon-codon mismatches on the ribosome known as misreading errors (29,30).

Nonsense errors occur when the ribosome stops prematurely the translation of a mRNA transcript, these errors are the consequence of improper translation by release factors, ribosome drop-off, missense mutations and frameshifts (31).

Frameshift errors normally require two-steps: a translational pause caused by an empty ribosomal A-site, that results from an insufficient amount of a cognate tRNA, and a slip where a weakly bound near-cognate tRNA at the ribosomal P-site can slip to the left or the right causing an erroneous decoding event. In the second (slip) step, while at the P-site, the weakly bound near-cognate tRNA might slip to the left or right in case its anticodon is also capable of binding the codon in the shifted frame (30,32). These types of errors usually generate a misfolded protein that aggregates or is degraded. Processivity errors are likely to affect protein function since they typically lead to early termination of translation, resulting in the synthesis of a truncated protein. These errors can be drop-off events, in which the tRNA is lost from the ribosome, frameshifting event, which leads to termination by a nonsense codon, and false termination events, where a codon is misread as a termination codon (32–35).

Mistranslation errors are costly and organisms developed adaptations for cost minimization. For example, the reduction of error frequencies through the preventive use of codons that are decoded with low error rates (25). Increased tolerance or robustness to errors is another adaptative mechanism that involves compensatory mutations (25). Despite these mechanisms, several diseases are caused by translational errors, in particular neurodegenerative disease, such as Alzheimer's disease, Parkinson's disease, Lewy body dementia, Huntington disease (36), but also by the loss of Purkinje cells (19).

Although mistranslation is usually associated with loss of fitness there are several cases describing mistranslation as being adaptative (37–39). For example, in in *Candida* sp., most particularly in *C. albicans*, the CUG codon is translated as Serine (95% to 97%) and Leucine (3% to 5%) by a mutant serine tRNA ($tRNA_{CAG}^{Ser}$) that is recognized by both the seryl and the leucyl tRNA synthetase (40)(41).

Surprisingly, RNA mistranslation is beneficial to *C. albicans* since it leads to better stress response and adaptability (42), allows evading macrophages while also leading to better infectivity (41). These observations prove that mistranslation is a powerful tool designed to help the invasion of new ecological niches and to adapt to them.

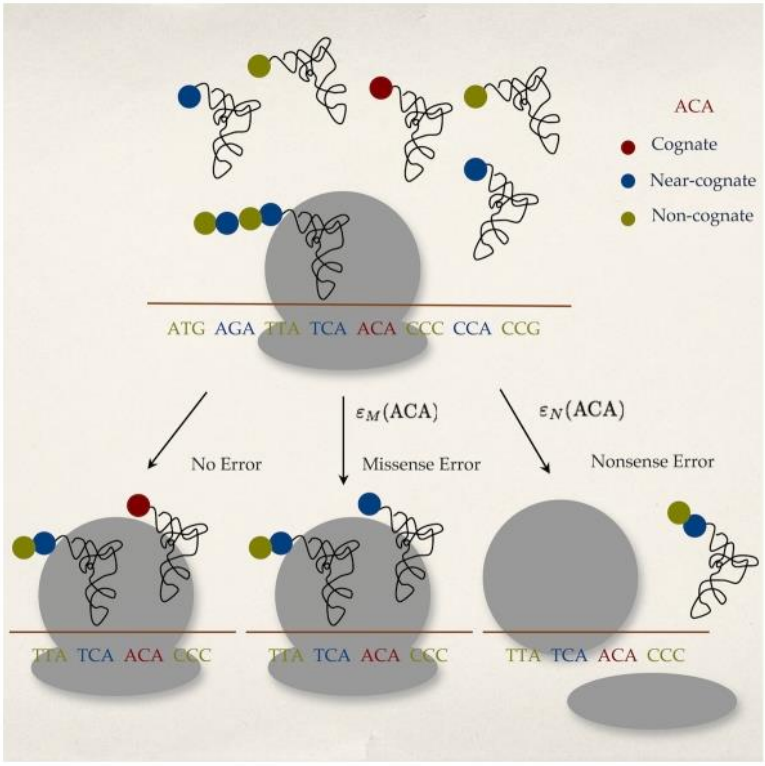

Figure 1.4: **Model of translation all errors.** During translation, a ribosome encounters a codon (in this example ACA). One of the three possible events can occur: elongation with a cognate tRNA leading to correct translation; elongation by a near-cognate tRNA leading to a missense error or premature termination of translation due to recognition and a sense codon by release factors and spontaneous ribosome drop-off or a frameshifting leading to a nonsense error. Adapted from(31)

The mechanism of transcriptional regulation

There are four RNA polymerases in eukaryotes I, II, III and IV. Polymerases I,II and III where identified in all eukaryotes(43), and polymerase IV was identified in plants (44), these polymerases share five common subunits .

The core promoter of protein coding genes is used as a platform for the formation of the transcription pre-initiation complexes, involving the general transcription factors: TFIIA,

TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and RNA polymerase II. The formation of the pre-initiation complex starts with the binding of TFIID to the TATA box, followed by the entry of another GTFs (general transcription factors) and the RNA polymerase II. The formation of this complex is necessary for basal transcription (45). Transcription factors can be divided in two different classes: transcription factors that control initiation, and transcription factors that control elongation, although some transcription factors could contribute to both elongation and initiation(46).Transcription factors bind to the promoter or enhancer regions, leading to a change in DNA conformation that helps recruiting the RNA polymerase II(47).

Transcription factor binding to promoters is influenced by :1) DNA packing proteins by histones. Histones have positively charged tails, that are subjected to covalent modifications acetylation, phosphorylation, methylation, and ubiquitination(48).These histones modifications constitute a histone code. The acetylation of the histones tails allow the transcriptions factors to connect to the DNA to promote DNA transcription initiation(49), this mechanism is also present in yeast(50,51). The histone methylation can be: the arginine methylation lysine methylation. The arginine methylation has three consequences, the blocking of the docking of effector molecules, the recruitment of repressor effector molecules and the recruitment of effector molecules with activator functions (49,52).

2) DNA methylation by methyltransferases. This process occurs by covalent addition of a methyl group at the 5' carbon of the cytosine ring, resulting in 5-methylcytosine(53), this process occurs mainly at the cytosine–guanosine dinucleotides. The DNA methylation in the promoter region of the genes is responsible for the inactivation or silencing (53,54), reversibly altering their expression. DNA methylation can interfere with transcription by two different processes: direct interference of the methyl group in binding of a protein to its cognate DNA sequence and protein attraction to methylated sites. In the first, TFs fail to bind to the DNA when it is methylated, these TFs normally would connect to the DNA in these now methylated sites. In the second process some proteins are attracted by the DNA methylation and bind to the methylated DNA repressing transcription.(53,55)

3) Phosphorylation of transcription factors by proteins kinases. Phosphorylation plays an important role in protein expression regulation and in many cellular processes. The process of phosphorylation is controlled by protein kinases and protein phosphatases. The proteins are phosphorylated at specific sites by one or more protein kinases and phosphates

are removed by the protein phosphatases (56). The phosphorylation can alter the transcription factor protein levels, their binding to the DNA and their interaction with regulation proteins(57).

Transcriptional regulation of gene expression by protein kinases.

The most common method of controlling gene expression is the phosphorylation and dephosphorylation of transcription regulators by protein kinases and protein phosphatases(58). For example, the MAP kinases (MAPKs) group of kinases composed by three kinases: the MAP kinase (MAPK), the MAP kinase kinase (MAPKK) and the MAP kinase kinase kinase (MAPKKK)(59), regulates transcriptional responses to extracellular signals. In *Saccharomyces cerevisiae* there are 5 known MAPK pathways that control diverse cellular processes: mating, sporulation, cell wall integrity, invasive growth and pseudohyphal growth, and the response to high osmolarity(59,60), while in mammals only 4 are known(60). In order to be able to activate or repress transcription the transcription factors need to be in the nucleus, bind DNA, and interact with the basal transcriptional apparatus(58), the phosphorylation of transcription factors by the MAPK pathway can regulate their intracellular location, their protein levels, their binding to DNA, and their interactions with regulatory proteins(57), activating or repressing the transcription factors. The intracellular location can be changed by stimulating the translocation of transcription factors to the nucleus to activate them or stimulate the export of the transcription factors from the nucleus, inactivating them. The regulation of transcription factor levels is also important. This can be achieved by modulating the transcription factor expression levels or the stability of the proteins. This is achievable because kinases regulate the expression levels of the transcription factor genes and the phosphorylation of transcription factors can also affect their stability, and or degradation. The regulation of DNA binding can be regulated positively or negatively by phosphorylation. This could occur by direct phosphorylation of the DNA binding domain or by indirectly regulating the DNA binding domain through phosphorylation. Finally, the interaction with regulatory proteins is also utilized. The phosphorylated residues can block the interacting surface, either by inducing conformational

changes in the binding surfaces or by forming part of the interacting surface(57,58,61,62) (Figure1.5).

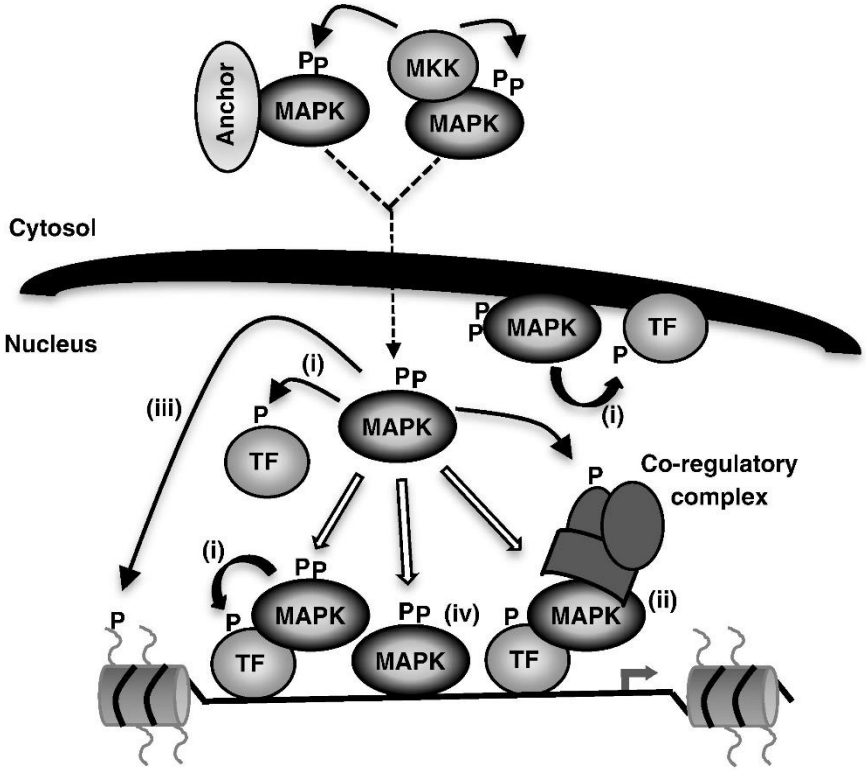

Figure 1.5 **Multiple mechanisms of subcellular localization and transcriptional regulation by MAPKs**. MAPKs can regulate transcription via many mechanisms. (i) phosphorylating transcription factors which may be either bound or unbound to DNA, (ii) recruiting co-regulators to DNA, (iii) phosphorylating histones, and (iv) binding directly to specific DNA sites.(57) Taken from (57)

Candida albicans

C. albicans is the most prevalent opportunistic pathogenic fungus being present in about 70% of healthy adults (63,64), in particular in the skin, gastrointestinal and vaginal tract ,where it is mostly asymptomatic. Since no other biological reservoir was discovered to date is it thought that *C.albicans* is exclusively adapted to mammalian hosts (64). In immunodepressed individuals, normally in chemotherapy and AIDS patients, *C.albicans* can manifest itself as a pathogen colonizing locations leading to infections that range from superficial thrush to systemic candidiasis, affecting brain, heart and kidney (65). It is also a major cause of nosocomial infections with mortality rate that can be as high as 40%, among the highest of nosocomial infections (66) .

The yeast form of *C.albicans* can go through a morphological switch that produces pseudo-hyphae and true hyphae (67,68), which are important for virulence (69,70). The yeast like cells (blastospores) are single rounded or ovoid shaped cells that grow best at 37ºC and pH 4.0 (67). Pseudo-hyphae cells are elongated buds of single cells that remain attached to each other and resemble true hyphae only to be distinguished by uneven parallel sides and constricted septal junctions (67), although similar to true hyphae they share more properties with single yeast cells than true hyphae cells (68) .Pseudo- hyphal cell grow best at 35ºC and pH 5,5 (71). True hyphae cells; cells not constricted septal junctions grow best at 37ºC in the presence of serum, neutral pH or high CO_2 (71).

Other phenotypes have been described recently like Chlamydospores and GUT (gastrointestinally induced transition) (72,73). Chlamydospores are large cells 3 or 4 times larger than yeast like cells, have a high amount of RNA, are rounded, have thick-wall and are produced at the end of the filaments of filamentous cells. This type of cells can be induced using a nutrient-poor media often supplemented with Tween-80 or other detergents when incubated in the dark with low oxygen content at room temperature (72).

GUT cells are formed in mouse gut due to over-expression of WOR1(White-opaque regulator 1) and are similar to the opaque cells, but darker, flattened elongated with large vacuoles. The GUT cells have enhanced fitness in the mammalian gastrointestinal tract because of a reorientation of its cellular metabolism to the nutrients that are available in the distal mammalian gastrointestinal tract (73).

Another important aspect of *C.albicans* morphology is the ability to undergo a reversible epigenetic change in its colonies, from white to opaque cells (74). Opaque cells are twice as large, asymmetric, elongated and flat relative to white cells (75).

The role of yeast like cells in the infection is to enhance dissemination through the bloodstream, while the other two phenotypes are responsible for the adherence, colonization and penetration of the host epithelium (68,69,76).

Candida albicans infection

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Without the morphological switching *C.albicans* is mainly avirulent (77) being stuck in one of its morphologic phases, although a strain modified to reproduce in the hyphal form only proved to be virulent. In order to promote the morphological switching, *C. albicans*

uses a variety of ways , including the cAMP-PKA signaling (cyclic AMP-protein kinase A) pathway that regulates hyphal growth (78–80) and responds to stimuli like temperature (79), CO_2 (81) or farnesol (80). Genotoxic stress also stimulates hyphal growth, as in the case of inhibition of DNA synthesis by hydroxyurea or aphidicolin and damage to DNA by UV light or methyl methanesulfonate (82). This genotoxic stress works by affecting the checkpoint kinase Rad53 and preventing the cell cycle to progress naturally (82,83), allowing for the hyphal growth. Other stressors like nitrogen starvation (84) can also influence hyphal growth to allow cells to forage nutrients in distant locations.

Nosocomial bloodstream infections by *C.albicans* are mainly caused by the formation of biofilms in implanted medical devices such as catheters and dental fixtures (85–87). The formation of these biofilms is an important aspect of *C.albicans* virulence since biofilms are usually drug-resistant leading to better fitness and infection capacity (86). The formation of biofilm can be broken down into three different stages: 1) attachment and the colonization of a surface, 2) growth and proliferation of yeast cells and formation of a basal layer, 3) morphological switch and growth of pseudohyphae and hyphae with the production of extracellular hydrolases (86,88). Yeast cells removed from the biofilm are more virulent, and are resistant to killing by neutrophils and do not trigger production of ROS leading to more effective infections(Figure 1.7) (89).

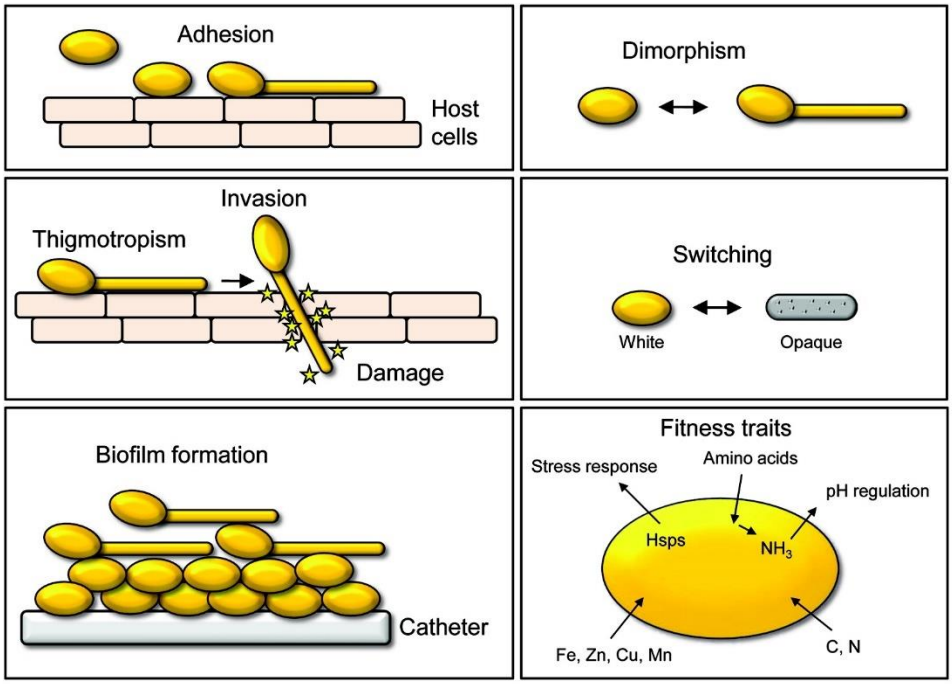

Figure 1.6: **An overview of selected C. albicans pathogenicity mechanisms**. C.albicans cells adhere to host cell surfaces by the expression of adhesins. Contact to host cells triggers the yeast-to-hypha transition(dimorphism) and growth via thigmotropism. Adhesion, physical forces, and secretion of fungal hydrolases has been proposed to help the second mechanism of invasion. The attachment of yeast cells to abiotic (e.g., catheters) or biotic (host cells) surfaces can give rise to the formation of biofilms Phenotypic switching has been shown to influence antigenicity and biofilm formation of C. albicans. Several fitness traits influences the fungal pathogenicity, namely a robust stress response mediated by heat shock proteins (Hsps), excretion of ammonia (NH3) metabolic flexibility and uptake of different compounds as carbon (C) and nitrogen (N) sources; and uptake of essential trace metals, e.g., iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn). Adapted from(89)

Candida albicans genetic code and codon mistranslation

C.albicans and other *Candida* spp. have a unique genetic code due to a change of identity of the CUG leucine codon to serine (90). These *Candida* spp. belong to the CTG clade (91) (Figure 1.8), which diverged from the nearest phylogenetic group about 178 ± 19 million years ago (92). The change of identity of the CUG leucine codon to serine evolved during 272 ± 25 million years through a mutant Ser tRNA that acquired a 5'-CAG-3' Leu anticodon,

this ambiguous codon decoding mechanism affected around 26,000 to 30,000 CUG codons present in about 50% of its genes (40,93,94). At the beginning of the CUG reassignment, the $tRNA_{CAG}^{Ser}$ was able to compete with the $tRNA_{CAG}^{Leu}$ for the CUG codons leading to an incorporation of both amino acids and CUG ambiguity. For reasons not yet known the $tRNA_{CAG}^{Leu}$ disappeared and the mutant serine was able to totally reassign the CUG codon (93). *Candida albicans* uses a dedicated $tRNA_{CAG}^{Ser}$ for CUG codons and a single $tRNA_{CAG}^{Leu}$ for the codons CUC, CUA, and CUU(91).

In *C.albicans* the CUG codon is ambiguously translated as Serine (95% to 97%) and Leucine (3% to 5%) by the mutant serine tRNA ($tRNA_{CAG}^{Ser}$), which is recognized by both the Seryl and the Leucyl-tRNA synthetase (40,41,95). This is due to the hybrid nature of the $tRNA_{CAG}^{Ser}$ that includes identity elements for both leucyl and seryl tRNA synthetases. The anticodon arm is characteristic of leucine tRNA and contain two elements recognized by the LeuRS in the anticodon loop A_{35} and m^1G_{37}. And the serine tRNA identity elements can be found in the variable arm and the acceptor stem, the identity elements recognized by the SerRS are the G_{73} and the 3 C-G pairs in the variable arm (95,96). Another modification induces a long-range distortion of the top of the anticodon stem of the $tRNA_{CAG}^{Ser}$ leading to lower decoding and leucylation efficiencies, this modification is a guanine (G) instead of a critical uridine (U) in the position 33 immediately adjacent to the 5' side of its 5'-CAG-3' anticodon (95–97).

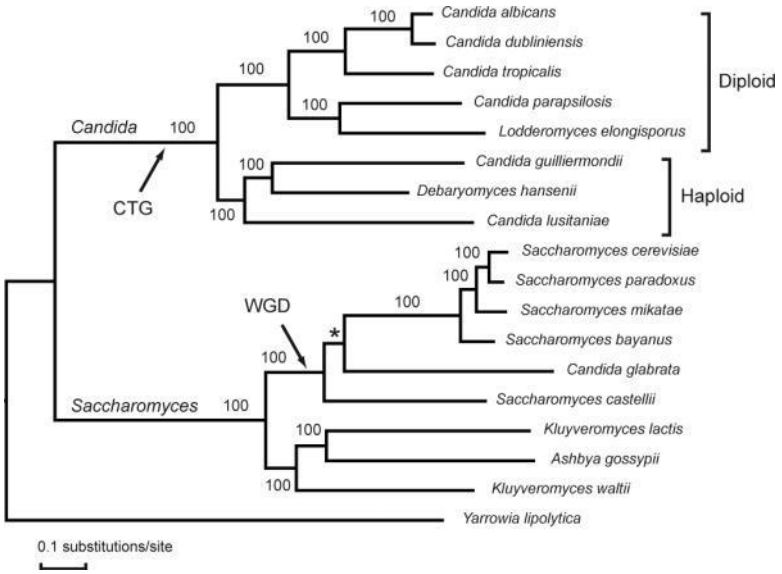

Figure 1.7: **Phylogeny of sequenced Candida and Saccharomyces clade species.** Representation of the CTG clade and Saccharomyces and Candida families. Adapted from(91)

When exposed to stress the mistranslation levels tend to increase depending on the type and severity of the conditions; temperature, pH and oxidative stress, have a strong effect in mistranslation. At 30ºC 2,96% of Leu is misincorporated while cells grown at 37ºC misincorporate 3,9% of Leu and cells grown at pH 4.0 misincorporated 4,03% of Leu (94). Other studies showed that a *C.albicans* mutant strain with 28% of leucine incorporation increased its adherence and cell surface hydrophobicity while also reducing the susceptibility to macrophage phagocytosis (41), another study showed that when exposed to antibiotics like fluconazole *C. albicans* strains with high mistranslation level (22,5% of mistranslation) are more tolerant and resistant to drugs (98). CUG ambiguity induces the stress response and leads to pre-adaptation to adverse conditions which in turn allows *C. albicans* to colonize new ecological niches (99). Also, high CUG ambiguity tolerance was demonstrated in a study were *C. albicans* strains misincorporated up to 98.46% of Leucine at CUG codons. The misincorporating strains grew faster than the controls when presented to oxidative stress and protein misfolding agents, while also being resistant to antifungal drugs (42). Gene expression profiling in cells expressing G_{33} and U_{33} $tRNA_{CAG}^{Ser}$ showed deregulated genes involved in the stress response, carbohydrate and amino-acid metabolism, cell wall structure, protein synthesis, and degradation.

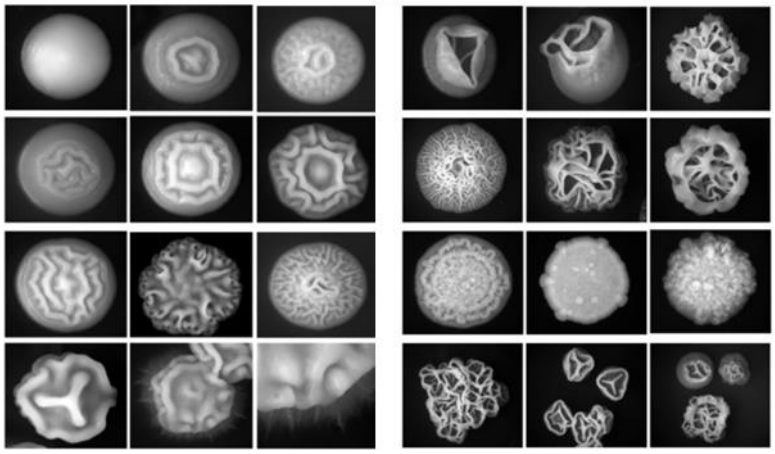

Figure 1.8: **Phenotypic diversity produced by mistranslating C. albicans strains**. The visual differences in colony morphology phenotypes include smooth, ring, wrinkled, and hyphae. Adapted from(42)

CUG is prevalent in genes that are associated with the membrane and the cell wall and the reported ambiguity can enhance virulence by creating cell surface variation which in turn leads to an increased adhesion and also reduced susceptibility to macrophage phagocytosis

(40,41,100). Also, since CUG are prevalent in genes of the *C. albicans* cell membrane and wall higher mistranslation levels impact on colony morphology variability (Figure 1.9) and eventually on the recognition by the immune system (42).

When exposed to stress conditions *C. albicans* cells produce proteins involved in the stress response. The mechanism to produce these proteins is closely related with CUG ambiguity and mistranslation (96). When mistranslation is induced a novel set of stress proteins is produced and often cross-protection to other stresses not present is obtained (99)

In *C. albicans* about 20 genes are involved in the stress response, which is a very low number when compared with the 220 genes of *S. cerevisiae* and 400 genes of *C. glabrata* (101,102). This major difference in the number of stress response of genes can be explained by the fine-tuning of *C. albicans* to the stable environment of host niches, in other words, *C.albicans* is not normally exposed to large environmental changes like free living yeasts (101,103).

The stress response in *C. albicans* is mainly regulated by Hog1 SAPK (stress-activated protein kinase) and by Cap1 (candida AP-1-like transcription factor) pathways (101,102). Hog 1 is responsible for the regulation of many core stress genes in response to osmotic and heavy metal stressors and the deletion of Hog 1 leads to lack of the stress response and significant alterations in the transcriptome(102) .Strains without functional Hog 1 showed complete avirulence when tested in mice models (104). Cap1 is involved in the oxidative stress response and bud-hypha transitions and filamentous growth and its deletion produces avirulent strains (105).

Working Hypothesis and objectives

Previous studies performed in the host laboratory showed that *C. albicans* has two acylated forms of the $tRNA_{CAG}^{Ser}$; the Ser-$tRNA_{CAG}^{Ser}$ and the Leu-$tRNA_{CAG}^{Ser}$ which compete for the CUG codon leading to incorporation of 3% of leucine and 97% of serine in the proteome. The misincorporation of leucine is dynamic and highly sensitive to physiological stress. Since the $tRNA_{CAG}^{Ser}$ has identity elements for both the LeuRS and the SerRS we raised the hypothesis that Leu and Ser misincorporation is dependent on the competition between LeuRS and SerRS for the hybrid tRNA. In other words, understanding the mechanism that controls the expression of the LeuRS and the SerRS is crucial to elucidate how CUG ambiguity is controlled *in vivo* In order to test our hypothesis, we carried out the following experiments:

1. Quantify SerRS and LeuRS expression levels in several different physiological conditions
2. Correlate the LeuRS/SerRS levels with Leu/Ser misincorporation levels
3. Identification of potential transcription factors that control LeuRS and SerRS expression

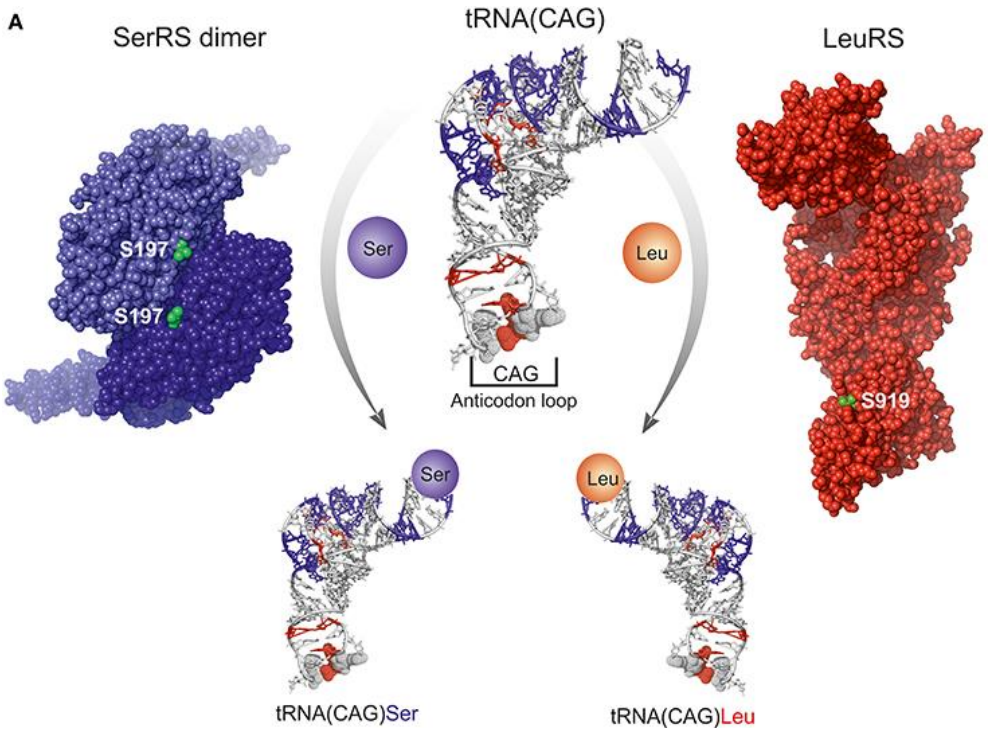

Figure1.9 ***Schematic representation of the competition between the seryl- (SerRS), and leucyl- (LeuRS) tRNA synthetases for C. albicans tRNA (CAG)*** *Taken from*(106)

Chapter II - Materials and Methods

Strains and Growth conditions

Strains

Escherichia coli strain JM109 (*recA1 SupE44 endA1 hsdR17 gyrA96 relA1 thi* Δ*[Lac-proAB] F'[traD36 proAB-lacI lacZΔM15*) was used as a host for all DNA manipulations. *Candida albicans* strain SN152 (arg4Δ/ arg4Δ leu2Δ/ leu2Δ his1Δ/ his1Δ URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434) was used by Homann *et al* (107) to construct the transcription factor (TF) deletion library used in this study. *C. albicans* strains were selected using the web resource (http://pathoyeastract.org/calbicans/index.php) (108) by searching TF binding sites in The Candida Genome Database (CGD, http://www.candidagenome.org)(109) for CDC60 + 1000 bp upstream and SES1 + 1000bp upstream, all strains are described in Table 2.1.

Table 2.1: List of potential strains used in the study.

TF	Strain	Gene description	ORF
SN152	Control strain	Wild Type strain	
22	Brg1	Transcription factor; recruits Hda1 to hypha-specific promoters	19.4056
37	Tye7	bHLH transcription factor; control of glycolysis	19.4941
42	Zcf29	Zn (II)2Cys6 transcription factor	19.5133
56	Wor2	Zn (II)2Cys6 transcription factor	19.5992
59	Ace2	Transcription factor; similar to S. cerevisiae Ace2 and Swi5	19.6124

65	Mac1	Copper fist transcription factor; regulator of CTR1 copper transporter;	19.7068
69	Mrr1	Putative Zn(II)2Cys6 transcription factor; regulator of MDR1 transcription	19.7372
77	Upc2	Zn2-Cys6 transcript factor; regulator of ergosterol biosynthetic genes and sterol uptake	19.391
80	Hap43	CCAAT-binding factor-dependent transcription factor;	19.681
83	Skn7	Predicted to be a response regulator protein in a phosphorelay signal transduction pathway	19.971
93	Hap5	Component of CCAAT-binding transcription factor	19.1973
95	Ndt80	Ortholog of Ndt80; meiosis-specific transcription factor	19.2119
107	Mig1	C2H2 transcription factor	19.4318
115	Tec1	TEA/ATTS transcription factor	19.5908
125	Nrg1	Transcription factor/repressor, effects both Tup1 dependent and independent regulation	19.7150
126	Rim101	Transcription factor	19.7247
156	Efg1	bHLH transcription factor	19.610
158	Rap1	Transcription factor; binds telomeres and regulatory sequences in DNA	19.1773
Not present in the list	Hmo1	HMG-box transcription factor	19.6645
Not present in the list	Tbf1	Essential transcription factor; induces ribosomal protein genes and the rDNA locus	19.801

Growth conditions

E. coli growth conditions

E. coli cells were grown at a constant temperature of 37ºC using LB broth medium (1% peptone from casein, 0,5% yeast extract, 1% sodium chloride; Formedium) or using LB broth medium with 5% agar. To perform *E. coli* selection LB broth medium was supplemented with the antibiotic ampicillin (75mg/ml; Sigma-Aldrich).

C. albicans growth conditions

C. albicans cells were grown at a constant temperature of 30ºC using YPD medium (2% glucose, 1% yeast extract, 1% peptone; Formedium), and for the *C.albicans* cell from the KO and TF strains, carrying the plasmids pUA563, pUA564, pUA567, pUA568 and pUA569, were grown using minimal medium(MM) without the amino acid arginine (0,67% bacto yeast nitrogen base without amino acids, 2% glucose, and 0,2% drop out mixtures off all required amino acids except arginine; Formedium).

Primers

Primers used in this study were purchased from IDT® (*Integrated DNA Technologies*) and were diluted in milli-Q water to a final stock concentration of 100 μM. The primers were designed with *OligoCalc* software to check their properties (http://www.basic.northwestern.edu/biotools/oligocalc.html) (110). All primers used in this study are listed in table 2.2.

Table 2.2: List of used primers.

Primer name	Function	Primer sequence (5'-3')	Melting temperature ºC
oUA 1554	Confirmation of plasmid integration at RP10 locus	CGTATTCACTTAATCCCACAC	51
oUA 1555	Confirmation of plasmid integration at RP10 locus	CCAATTGGTGATGGTCC	50.5

Plasmid construction

Choice of transcription factors

In order to select the transcription factors for this study, a bioinformatic analysis was performed. This bioinformatic approach focused on the identification of the TF binding sites for the LeuRS (CDC60) and SerRS(SES1) start codon plus 1000 bp upstream. For this, the two DNA sequences were submitted to the online tool motif finder "Find TF Binding Site (s)" from the PATHOYEASTRACT database (http://pathoyeastract.org/calbicans/index.php) (108). This tool retrieved a list of motif sequences and the corresponding transcription factors that bind to them. This bioinformatic study produced the results shown in table 2.1 and helped reducing the results from the initial 166 mutants in the deletion library to only 20. This list of 20 TFs was reduced to 6 TFs by comparing with previous studies developed in the host laboratory and removal of TFs already studied. The 6 transcription factors studied are described in table 2.3. The TF 37 and TF 125 were removed during the study due to of the difficulties encountered in the insertion of plasmid DNA into the genome.

Table 2.3:List of selected transcription factors

TF	ORF	Gene description	Gene Name	Name S. *cerevisiae*	Name *C. albicans*	SerRS	LeuRS
SN152		Control Strain					
79	19.517	Putative CCAAT-binding transcription factor; required for resistance to rapamycin	Heme Activator Protein	*HAP3*	*HAP31*	*X*	
8	19.2730	Has domain(s) with predicted zinc ion binding activity	Regulator of PHR1	*RPH1*	*RPH2*		*X*
37	19.4941	bHLH transcription factor; control of glycolysis;	Ty-mediated Expression	*TYE7/PGM2*	*TYE7/PGM2*		*X*
59	19.6124	Transcription factor; similar to S. cerevisiae Ace2 and Swi5	Activator of CUP1 Expression	*ACE2*	*ACE2*	*X*	*X*
69	19.7372	Putative Zn (II)2Cys6 transcription factor;	Heme Activator Protein	*HAP1*	*MRR1*		*X*
125	19.7150	Transcription factor/repressor;	Negative Regulator of Glucose-repressed genes	*NRG1*	*NRG1*		*X*

156	19.610	bHLH transcription factor; required for white-phase cell type	Exit from G1	*EFG1*	*EFG1*		*X*
158	19.1773	Transcription factor; binds telomeres and regulatory sequences in DNA	Repressor Activator Protein	*RAP1*	*RAP1*		*X*

Plasmids for LeuRS/SerRS quantification

In order to access the levels of the SerRS and LeuRS expression, a plasmid containing a reporter system was previously constructed. Plasmid pUA563 contains the reporter for determination of SerRS expression while pUA564 contains the reporter for determination of LeuRS expression. Both reporters are based on the integrative vector CIp20 (111) and contain the yeast-enhanced green fluorescent protein (*yE*GFP) gene. In the plasmid pUA 563 the open reading frame of *yE*GFP is fused to the promoter of the SerRS (SES1) gene, so that fluorescence signal is proportional to the SES1 expression. In pUA564, the open reading frame of *yE*GFP was fused to the promoter of the LeuRS (CDC60) gene. These reporters also contain the mCherry gene associated with the actin promoter (ACT1), which is used as an internal control. These plasmids also contained arginine (ARG4 gene) as the selective marker and were used to transform the TF knockout collection and the control strain listed in table 2.4.

Table 2.4:List of plasmids used for LeuRS/SerRS quantification

Plasmid	Description
pUA 563	Plasmid containing the *yE*GFP reporter system associated with the SES1 promoter, allowing the determination of SerRS expression; used to transform WT and TF KO strains with ARG4 as a selective marker
pUA 564	Plasmid containing the *yE*GFP reporter system associated with the CD60 promoter, allowing the determination of LeuRS expression; used to transform WT and TF KO strains with ARG4 as a selective marker

Plasmids for quantification of Leu misincorporation

In order to quantify Leucine misincorporation in the proteins of the mutant strains a gain of function fluorescent reporter system based on *y*EGFP was used (112). This reporter system is based in the report system described by Bezerra *et al*. (42) , consisting in three different versions of the *y*EGFP assembled in three different plasmids that were previously built by the host laboratory (pUA 553, pUA 554, pUA555). The plasmid pUA 553 contains the *y*EGFP gene with WT TTA-leucine at position 201 and functions as a positive control for misincorporation. The plasmid 554 contains the CUG codon at position 201, producing stable GFP when Leu is incorporated, only. The plasmid pUA 555 containss TCT-serine at position 201 and produces an inactive GFP; a negative control. All plasmid contains URA3 as the selective marker. Since the strains from the TF deletion library were modified using the URA3 selective it was replaced with ARG4 in all plasmids, leading to the pUA 567, pUA 568 and pUA 569. The plasmids used are listed in table 2.5.

Table 2.5: List of plasmids used to quantify Leu misincorporation

Plasmid	Description
pUA553	Plasmid containing the *yE*GFP reporter system with a WT TTA-leucine at position 201; URA3 gene as selective marker
pUA554	Plasmid containing the *yE*GFP reporter system with a CTG ambiguous codon at position 201; URA3 gene as selective marker
pUA555	Plasmid containing the *yE*GFP reporter system with a TCT-serine codon at position 201; URA3 gene as selective marker
pUA567	Plasmid derived from pUA 553 containing an ARG4 gene as a selective marker inserted between *SpeI* and *NotI* restriction sites

pUA568	Plasmid derived from pUA 554 containing an ARG4 gene as a selective marker inserted between *SpeI* and *NotI* restriction sites
pUA569	Plasmid derived from pUA 555 containing an ARG4 gene as a selective marker inserted between *SpeI* and *NotI* restriction sites

E. coli competent cells preparation

E.coli competent JM109 cells were prepared using the TFB method (113). Firstly, 200 µl of cells from an overnight culture were incubated in 5 ml of LB medium at 37º C and at 180 rpm until an OD_{550} of 0,3. Approximately 4 ml of the above culture were inoculated in 100 ml of LB medium and incubated at 37º C ,180 rpm until an OD_{550} of 0,3. After that, the cells were collected in two 50 ml falcons and stored for 5 minutes on ice. The two falcons were then centrifuged at 2500 rpm for 5 minutes at 4º C. Then the supernatant was discarded and each pellet was resuspended in 20ml of cold TFBI solution (0.03 mM CH_3CO_2K, 0.08 mM $RbCl_2$, 0.013 mM $CaCl_2$, 0.08 mM $MnCl_2$, 15.4% glycerol, pH 5.8). The two falcons were centrifuged at 2500 rpm, 4ºC, for 5 minutes, the supernatant was discarded and the two pellets were resuspended in a cold solution of TFBII (0.01 mM MOPS Na, 0.01 mM $CaCl_2$, 0.008 mM $RbCl_2$, 13.4% glycerol, pH 6.5). The two falcons were incubated on ice for 5 minutes, and then distributed in aliquots of 200 µl and frozen at -80ºC.

Transformation of *E. coli*

The *E. coli* transformation was prepared using the Sambrook's SOC method (113). 20 µl of ligation reactions were prepared using 1:0 to 1:5 of vector to insert ratios. In addition, the reaction contained 1 µl of DNA Ligase (5 U/µl) (*Thermo Scientific*), 2 µl of 10x DNA Ligase Buffer (*Thermo Scientific*) and milli-Q water to complete the volume. Then the Tubes were incubated at 20º C for 4 h, followed by an inactivation of the enzyme by incubating for 10 min at 65º C. Then the ligation reactions were added to 200 µl aliquots of *E. coli* JM109 competent cells and mixed without vortexing. The mixed reagents were then incubated on ice for 30 min followed by a heat shock of 90 sec at 42º C with an immediate incubation of

2 min on ice. Next 800 µl of SOC medium (2 g of tryptone, 0.5 g of yeast extract and 0.05 g of NaCl were weighted, and 1 ml of KCl 250 mM and 20 ml of glucose 1 M were added, for preparation of 100 ml at pH 7) were added to the mixture which was then incubated for 1 h at 37º C with 180 rpm. Each reaction tube was centrifuged for 1 min at 2500 rpm, and their supernatant was removed until the solution volume was 50 µl. The pellet was then homogenized and spread on plates of LB medium with 75 µg/ml ampicillin (*Sigma-Aldrich*). The plates were then incubated overnight at 37º C.

Plasmidic DNA purification from *E. coli*

DNA purification from *E. coli* used the "NZYMiniprep" kit (*Nzytech*). Firstly, *E. coli* cells were grown overnight in 5 ml of LB medium (*Formedium*) with ampicillin (75 µg/ml; *Sigma-Aldrich*). Then each tube was centrifuged for 30 sec at 4000 rpm and the supernatant was discarded. Next, the pellet was resuspended in 250 µl of Buffer A1 by vortexing, and 250 µl of Buffer A2 was added and mixed gently. The mixture was then incubated for a maximum of 4 min at room temperature and centrifuged for 10 min. 2 ml collecting tubes with an "NZYTech" spin column inside which supernatant was loaded were centrifuged for 1 min at 13000 rpm. After that, the flow-through was discarded and 500 µl of AY Buffer previously heated to 50º C was added to the spin column and centrifuged for 1 min at 13000 rpm. Next, the flow through was discarded and 400 µl of Buffer A4 was added to the spin column and centrifuged for 1 min at 13000 rpm. After that, the flow through was discarded and the spin column was centrifuged again for 2 min at 13000 rpm. The last step was the addition of 50 µl of Buffer AE in the spin column inside a microcentrifuge tube and incubated for 1 min at room temperature and centrifuged for 1 min at 13000 rpm. After the purification, NanoDrop™ was used to quantify the yield of the purified DNA. Purified plasmids were stored at -20º C.

Manipulation of *C. albicans* strains

Transformation of *C. albicans*

Before starting the transformation protocol, plasmids were linearized with StuI (*Thermo Technologies)* during 4 h at 37º C. The transformation of *C. albicans* cells was done using an improved lithium method with minor modifications (114). *C. albicans* cells were grown overnight at 30º C in 10 ml YPD medium. Then the cultures were diluted to obtain an OD_{600}=0,3 and grown for an additional 4 h at 30º C with 180 rpm in 5 ml of YPD medium until OD_{600}=1-1,2. Next, the cells were centrifuged at 4000 rpm during 5 min the supernatant was discarded and the pellets resuspended in 150 µl of LiAc-solution (10% of LiAc 1 M, 10% TE buffer 10x, 80% of milliQ-water). Then, 200 µl of *C. albicans* cells were transferred to a micro centrifuge tube and 5 µl of the plasmid DNA previously treated with StuI (*Thermo Technologies)* and 100 µg of carrier single strand DNA (1 mg/ml salmon sperm previously heated at 95ºC for 5 min and immediately cooled in ice) were added. 600 µl of PEG/LiAc-solution (50% (w/v) polyethyleneglycol, 50% LiAc) was added to the transformation mix and briefly vortexed. The transformation mixture was then incubated for 4 h or overnight at 30º C with 180 rpm, followed by a heat shock of 15 min at 44º C and an incubation on ice for 2 min. The mixture was centrifuged at 4000 rpm for 15 sec, the supernatant was discarded and the pellet was resuspended in 230 µl of minimal medium. Aliquots of 50 to 100 µl were spread on the selective plates and incubated at 30º C for 4 to 5 days.

DNA extraction from *C. albicans*

DNA extraction was performed using two different methods. The first method was an adaptation of the Lyticase method developed by Hoffman and Winston (115), and the second one was an extraction method for PCR-based applications (116).

In the first method, cells were incubated overnight in 5 ml of minimal medium at 30º C and 180 rpm. After that, the cells are centrifuged for 5 min at 2000 rpm and the supernatant discarded. The cells are resuspended in 500 µl of Solution I (Sorbitol 1 M; EDTA-Na_2 0,1

M; pH 7,4) and transferred to a new centrifuge tube along with 5 µl of Lyticase enzyme (*Sigma-Aldrich)*, and a new incubation was performed at 37º C during 60 min. Next, the tubes were centrifuged for 1 min at 2000 rpm and the supernatant discarded. Cells were resuspended in 500 µl of Solution II (Tris-Hcl 5 mM; EDTA-Na_2 20 mM; pH 7,4) and 50 µl of SDS was added and the tubes vortexed. Next, the cells were incubated for 30 min at 65º C. 200 µl of Potassium Acetate 5 M was added and the tubes were incubated on ice for 1 h. The samples were centrifuged for 5 min at 2000 rpm and 600 µl of supernatant was transferred to a new centrifuge tube. Then, 2 Volume of ethanol 100% and 0,1 Volume of NaCl 5 M was added to the mixture, which was incubated for at least 2 h at -30º C. After that, the mixture was centrifuged for 5 min at 13000 rpm and its supernatant discarded. Finally, the samples were air dried and resuspended in 100 µl of milliQ-water. Later the DNA concentration was assessed using the NanoDrop™.

In the second method, 100 µl of 200 mM of LiAC-SDS(1%) solution were added to a microcentrifuge tube and one yeast colony from the plate was resuspended in this solution. The mixture was vortexed and incubated at 70º C for 5 min. 300 µl of ethanol 100% was added to the sample and briefly vortexed to induce DNA precipitation. Then, the samples were centrifuged at 13000 rpm for 3 min and the supernatant was discarded. 500 µl of ethanol 70% was added to the sample and centrifuged at 13000 rpm for 2 min. The supernatant was discarded and the samples were air dried. Finally, the samples were centrifuged at 13000 rpm for 2 min and the supernatant was transferred to a new centrifuge tube. Later the DNA concentration was assessed using NanoDrop™.

Integration confirmation

To confirm plasmid integration in the *C. albicans* gnome a polymerase chain reaction (PCR) followed by an electrophoresis were performed. Since the RP10 locus was the target of the inserted plasmids this locus was amplified by PCR. To perform the PCR several reagents were added: 1,5 µl Dream Taq Buffer, 0,075 µl of Dream Taq polymerase, 0,3 µl dNTP mix (*Fermentas*), 0,3 µl of each specific primer (oUA 1554 and oUA 1555), 2 µl of *C. albicans* DNA and milli-Q water to a final volume of 15 µl. The PCR reaction was performed in *My Cycler™* thermal-cycler (*BIO RAD*) and consisted of a denaturation step at 95º C for 3 min, 30 cycles of denaturation at 95º C for 30 sec, annealing temperature of 48º

C for 1 min, extension at 72º C for 1 min and a final extension step at 72º C for 5 min. Finally, the PCR products were used to perform an electrophoresis on a 1 % agarose gel, and later visualized using ChemiDoc™ XR+ (*BIO RAD*).

Western Blot analysis

Protein extraction

In order to extract protein from *C. albicans* to perform the western blot analysis first, strains are grown overnight at 30º C with 180 rpm in 10 ml of adequate medium. Cells were then collected at OD_{600}=1 and centrifuged at 4000 rpm for 5 min at room temperature. After that, the supernatant was discarded and the cells were incubated at -80º C for at least 1 h. 300 µl of lysis buffer (0,41 ml of milli-Q water; 0,5 ml of PBS pH 7,4; 2 µl of EDTA 0,5 M; 57,5 µl of glycerol 87%; 10 µl of PMSF 100 mM; 20 µl of protease inhibitor Roche 25x) and 1 volume of glass beads were used to resuspend the cells. Cells were disrupted using PRECELLYS 24 TISSUE HOMOGENIZER (*Bertin instruments*) passing through 2 cycles of 25 sec at 6500 rpm, followed by 2 min on ice. Next, the cells were centrifuged at 5000 rpm for 10 min at 4º C. The supernatant was transferred to a new tube and centrifuged for 10 min at 4º C and 13000 rpm. Finally, the supernatant was transferred to a new tube and stored at -80º C until further use.

Protein quantification

Protein samples were quantified using the, a Pierce® BCA Protein Assay kit (*Thermo Scientific*). Samples were diluted 100 times with milli-Q water and vortexed. Then, a 50 to 1 reaction of the BCA reagent A was mixed with the BCA reagent B. Afterwards, 10 µl of both the protein standards and the diluted samples were arranged in duplicate in a 96 wells microplate and then the reaction of the BCA reagent A and B was added. Finally, the microplate was incubated for 30 min at 37º C and the absorbance was measured using an iMark™ Microplate Absorbance Reader (*Bio-Rad*).

Protein Electrophoresis and transfer

The SDS gels were composed of 15% Resolving lower gel (5,8 ml of milli-Q water; 4 ml of Tris-HCL 1,5 M pH 8.0; 6 ml of Bis-Acrylamide 29:1; 160 µl of SDS 10%; 160 µl of APS 10%; 16 µl of TEMED) and 4% Stacking Upper gel (3,94 ml of milli-Q water; 1,5 ml of Tris-HCL 0,625 M pH 6.8; 1,5ml of Bis-Acrylamide 29:1; 60 µl of SDS 10%; 60 µl of APS 10%; 6 µl of TEMED). 20 µg of protein with 4 µl of loading buffer 6x were incubated for 5 min at 95º C. Afterward, the samples were loaded on the gels along with 4 µl of the protein marker (*Nzytech*). Gels were run at 80 V until the dye front moved into the resolving gels, then the voltage was increased to run at 130 V until the dye reached the bottom part, after that the gel was removed and hydrated with distilled water. The gels were transferred to a nitrocellulose membrane using a Trans-Blot® Turbo™ Transfer System, twelve filters of the same size and the nitrocellulose membrane were placed in 1x transfer buffer (*BIO-RAD*). The transfer occurred at 25v for 10 min. After the transfer nitrocellulose membranes were placed in a container with 5% BSA-TBST for 1 h. The membranes were then washed 3 times with TBS-T (1X TBS, 0.1% Tween20) and then incubated for 2 h with the primary antibody (1:3000 LeuRs; SerRS). The membranes were then washed 3 times for 15 min with TBS-T and incubated overnight with the secondary antibody (antirabbit 1:10000). Finally, the membranes were washed 3 more times with TBS-T and the membranes were observed using Odyssey Infrared Imaging System (*Li-Cor Biosciences*).

Stress conditions

In order to evaluate the expression of SerRS and LeuRS, 3 clones from each of the transformed and control strains were incubated at a different temperatures and media compositions. Strains were grown overnight in different physiological conditions, as shown in Table 2.6 and were fixed onto microscope slides and analyzed by epifluorescence microscopy.

Table 2.6: Physiological conditions tested

Assay	Stress factor	Growth temperature
Control	None	30º C
Temperature	37º C	37º C
Osmotic stress	0,2 M	30º C
pH	pH 6	30º C

Epifluorescence Microscopy

To access the LeuRS and SerRS expression levels, yEGFP and mCherry expression was observed using epifluorescence microscopy. Fluorescence was detected using a Zeiss AxioImager Z1 microscope equipped with filter sets 38 HE GFP and 63 HE mRFP. Photographs were taken using Axiocam HRm camera. Images were analyzed using ImageJ software. LeuRs and SerRs expression were calculated in at least 300 cells carrying plasmid pUA 564 and plasmid pUA 563 respectively. In order to calculate the expression of LeuRS in pUA 564, the mean of the GFP (controlled by the LeuRS promoter) was divided by the mean mCherry intensity. To calculate the expression of SerRS in pUA563, the mean of the GFP (controlled by SerRS promoter) was divided by the mean mCherry intensity, this allowed the indirect quantification of the synthetase.

To quantify leucine incorporation at CUG sites, mean GFP fluorescence was divided by the mean mCherry intensity. GFP quantification was performed in at least 300 cells carrying each version of the reporter: pUA56, pUA568, pUA569. Values obtained using the negative control strain were subtracted to the values of the reporter and positive control strains and values from the reporter strain were divided by the values of the positive strain, as follows.

$$\text{Leucine incorporation at CUG codons} = \frac{[\text{GFPCUG (Reporter)} / \text{mCherry(Reporter)}] - [\text{GFPUCU (Negative)} / \text{mCherry (Negative)}]}{[\text{GFPUUA (Positive)} / \text{mCherry(Positive)}] - [\text{GFPUCU (Negative)} / \text{mCherry (Negative)}]}$$

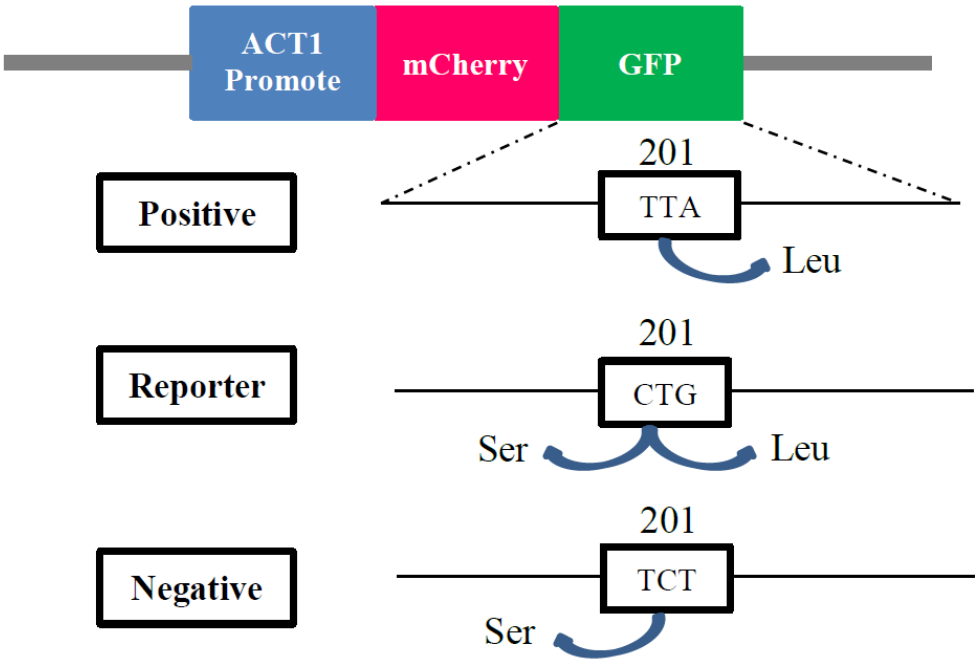

Figure 1.10 **This reporter system is based on the yeast enhanced green fluorescent protein (yEGFP) described by Bezerra el al. (76).** The Leu-TTA codon at position 201 was mutated to the ambiguous CTG codon (reporter) and to the Ser TCT codon (negative control). The positive version was assembled into pU567, the reporter was integrated in the pUA568 and the negative control was assembled in the plasmid pUA569.Taken from (117)

Statistical Analysis

All statistical analyses were performed using the GraphPad Prism software version 8.0 for windows. Data represent the mean (± standard deviation - s.d.) of three clones tested for each strain. Statistical comparisons between the deletion strains and the control WT strain were carried out using one-way ANOVA followed by a Dunnett comparison test with 95% interval against the control (****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$). Heat maps were created using the MeV software version 4.8.1 for windows.

Chapter III – Results

SerRS and LeuRS expression and CUG ambiguity

Overview

Candida albicans has the ability to decode ambiguously the CUG codon as leucine(3%) and serine (97%), using a hybrid $tRNA_{CAG}^{Ser}$ that has identity elements for both the leucyl and seryl tRNA synthetases(94,95). Decoding of CUG requires, therefore, that the Leu-$tRNA_{CAG}^{Leu}$ and the Ser-$tRNA_{CAG}^{Ser}$ compete at the ribosome A-site for the CUG codons.The incorporation of 3% of Leu and 97% of Ser at protein CUG sites indicates that SerRS charges the $tRNA_{CAG}^{Ser}$ more efficiently than the LeuRS. However, recent studies indicate that LeuRS charging can increase rather dramatically under stress(94), suggesting that the activity and/or expression of both synthetases is regulated in response to environmental challenges. To better understand if such regulation occurs at the transcription level, we used a fluorescent reporter system fused to the promoters of the SerRS and LeuRS genes to quantify their transcriptional activity. Western blotting techniques were also used to quantify LeuRS and SerRS levels and determine possible variation in the LeuRS/SerRS ratio in different physiological conditions and in different genetic backgrounds.

SerRS and LeuRS expression in different physiological conditions

The chimeric reporter system mentioned above was constructed by fusing the Yeast-enhanced GFP (*y*EGFP)(112) open reading frame to the promoter of the SerRS gene (SES1) and to the promoter of the LeuRS gene (CDC60). The mCherry gene was also fused to the actin promoter (ACT1) to normalize protein expression levels.

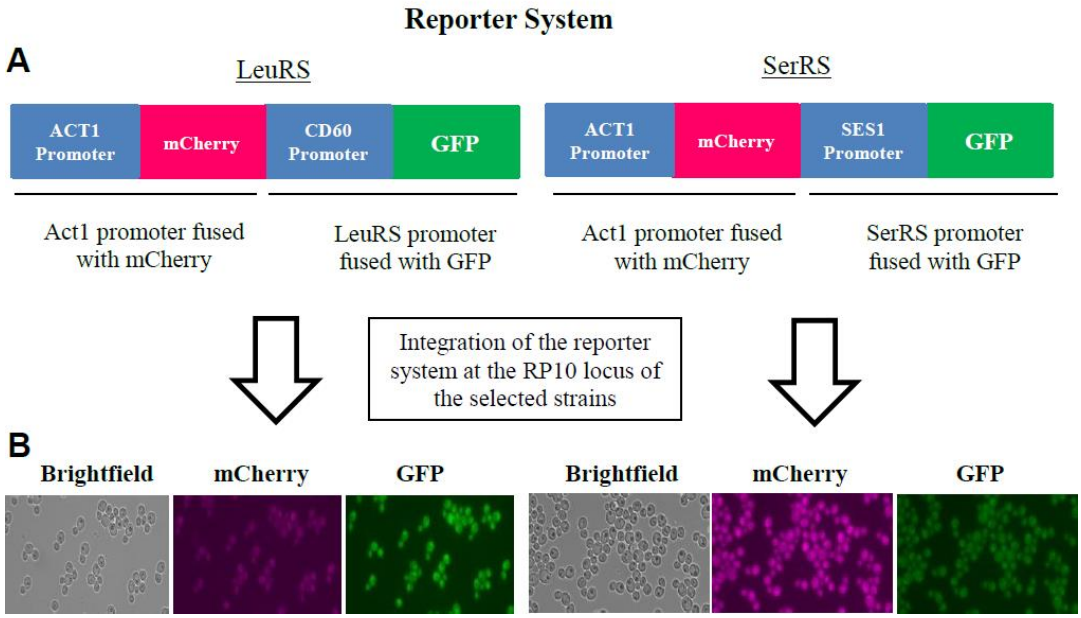

Figure 11 **Schematic representation of the reporter systems used to quantify LeuRS and SerRS expression**. A) These reporter systems are based on yEGFP, where the open reading frame of GFP was fused to the promoters of the SerRS and LeuRS genes (SES1 and CDC60). Fluorescence is proportional to the transcriptome activity of the promoters. A mCherry fluorophore tuned with actin promoter (ACT1) was used as internal control. LeuRS and SerRS reporters were assembled into pUA564 and pUA563 respectively. B) Fluorescence and brightfield images of *C. albicans* cells obtained by epifluorescence microscopy (magnification: 630x) with the appropriate filters, using an AxionCam HRc camera (Zeiss).Taken from (117)

These reporters were integrated into the RP10 locus of *C. albicans* cells which were grown at different temperatures and media compositions, that are potentially relevant during infection. *C. albicans* cells were grown in hypotonic media at pH 7, and 30ºC (non-stress conditions), and then at pH 6 hypotonic media at 30ºC, pH 7 and 0,2M of sorbitol hypotnic media at 30ºC and pH7 and hypotonic media at 37ºC.

In this assay, the mCherry gene fused to the actin promoter was used as internal control, to normalize the data. The ratio of the mCherry normalized GFP values of the CDC60/SES1 promoters was calculated for all strains according the equation 2. It was defined that the ratio LeuRS/SerRS of the WT strain was 1. Thus, strains having a value greater than 1 had higher CDC60 promoter activity, or lower activity of the SES1 promoter (or both), relative to WT. Strains showing values < 1, have lower CDC60 or higher SES1 promoters activity.

Equation 2-Calculation of the Promoters Expression Ratio

$$Ratio = \left(\frac{GFP\ of\ CDC60}{mcherry\ of\ CDC60}\right) / \left(\frac{GFP\ of\ Ses1}{mcherry\ of\ Ses1}\right)$$

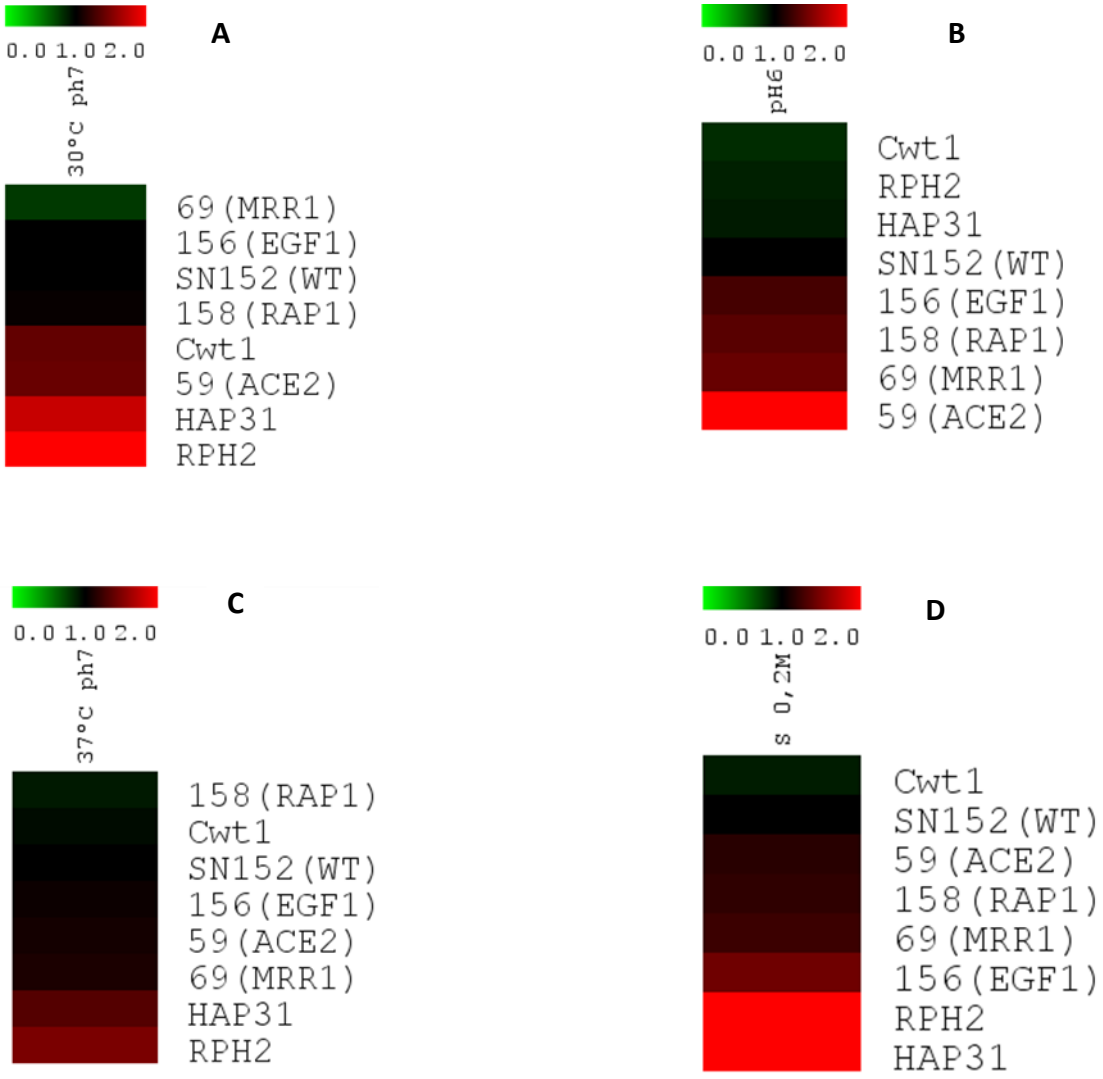

Figure 12 **Heat maps representing the ratio of LeuRS/SerRS expression in different physiological conditions.** Data extracted from the TF KO collection and WT strain SN152 at control conditions **(A)**, pH6 **(B)** at 37ºC **(C)** and sorbitol 0.2M **(D).** The values of each KO strain were normalized to the WT strain.

In control conditions the strains lacking the EGF1 and MRR1 TFs where the only that showed a promotor activity levels below the WT strain. At pH6 and 0,2M sorbitol(hypotonic) all the mutant strains showed a promoter activity value higher when compared with the WT. At 37ºC the RAP1 KO TF strain was the only one that showed lower promoter activity relative to the WT strain.

In the deletion strain lacking the TF ACE2 the LeuRS/SerRS ratio increased in all conditions, since ACE2 has binding sites in the promoters of both LeuRS and SerRS these results suggest that the strain ACE2 could activate the promotor of LeuRS or repress the SerRS promoter. The strain with KO in the MRR1 TF showed higher LeuRS/SerRS ratio at 37ºC, pH 6 and 0,2M, suggesting that the LeuRS is overexpressed in these conditions, or, alternatively, that these conditions favor LeuRS expression relative to the SerRS expression. The strain RAP1 showed higher LeuRS/SerRS ratio at 30ºC, pH6 and 0,2M, and lower ratio at 37ºC.The strain EGF1 showed higher ratio at 37ºC, pH 6 and 0,2M.

This data was compared with the data for the Δ HAP31, Δ RPH2 and Δ CTW1 strains obtained from a previous work done in the host laboratory by Edgar Lopes (unpublished results), which were used as positive controls. The HAP31 binds to the SES1 promoter and was a positive control for the SerRS expression. RPH2 binds to the CDC60 promoter and was used as a LeuRS expression positive control. The CTW1 TF has no binding motifs in the CDC60 and SES1 prmoters and was used as a negative control. The strains Δ HAP31 and Δ RPH2 showed higher promoter activity levels than the WT strain in all the physiological conditions tested except at pH6 where the promoter levels were lower than the WT strain. The results showed that the RPH2 TF is a positive regulator of LeuRS and a negative regulator at pH6. The HAP31 TF data showed that it is a repressor of the SES1 promoter, but this situation changes at pH6 where it works as an activator.

The above studies were complemented with western blot analysis to exclude possible post-transcriptional regulatory control of the SerRS and LeuRS expression. The analysis was performed with polyclonal antibodies against each synthetase. Relative protein abundance was determined at 30ºC, pH7 in media lacking sorbitol; pH 6 without sorbitol at 30ºC; pH 7 and 0,2M of sorbitol at 30ºC and pH7 in media lacking sorbitol at 37ºC (table 2.6).

A ratio >1 relative to WT strain, determined in the same conditions, indicate that the TF being analyzed activated the LeuRS promoter. While a LeuRS/SerRS ratio <1 would indicate that the deletion TF repressed the LeuRS expression or activated the SerRS promoter.

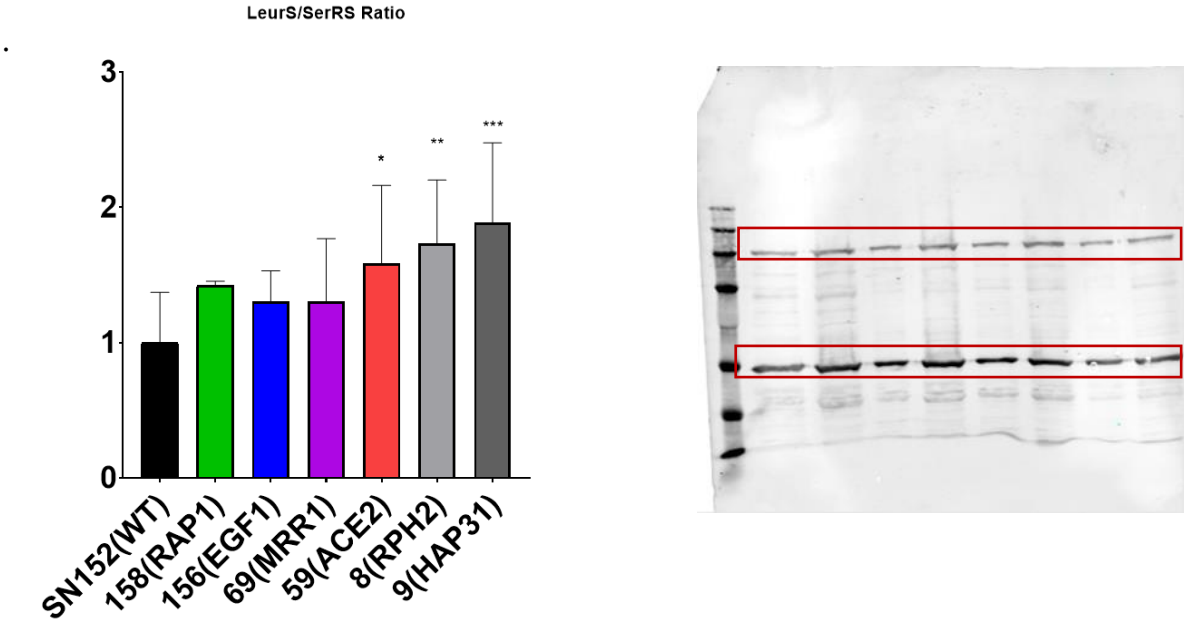

Figure 13 **A) Results of the western blot assays of the LeuRS and SerRS relative abundance B) Western blot of the LeuRS and SerRS using polyclonal antibodies against both enzymes** (***p<0.001, **p<0.01, *p<0.05 vs control).

The western blot data showed that all the strains tested had a LeuRS/SerRS ratio >1, however statistically different results were only found for ACE2, RPH2 and (Figure1.12). These results suggest that these 3 strains have higher LeuRS, than SerRS levels. Since ACE2 binds to both promoters this could mean that this TFis a repressor of the LeuRS or activator of the SerRS. Since RPH2 binds to the LeuRS promotor it is a repressor of the LeuRS while HAP31 is an activator of SerRS.

Leu misincorporation in the TF knock-outs

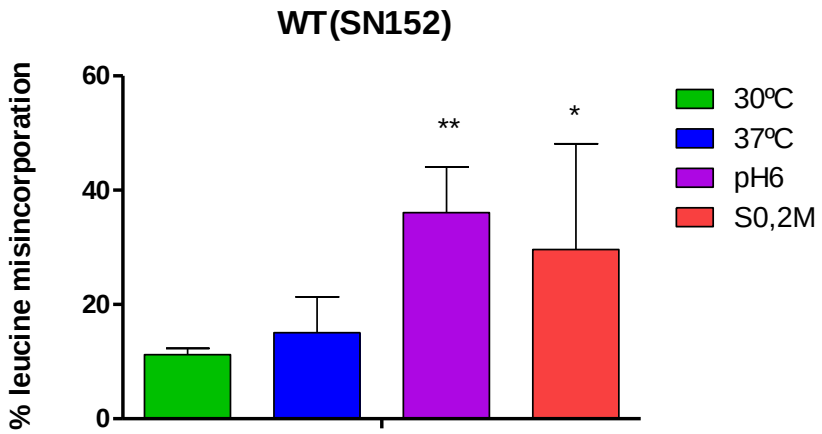

Figure 14 **Leucine misincorporation at protein CUG sites in the WT strain in various physiological conditions**(30ºC, pH7 in media lacking sorbitol ; pH 6 without sorbitol at 30ºC; pH 7 and 0,2M of sorbitol at 30ºC and pH7 in media lacking sorbitol at 37ºC) (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05).

To confirm whether the changes in LeuRS/SerRS ratio observed in different strains and in different physiological conditions had an impact on Leu misincorporation levels at protein CUG sites, we have used the CUG mistranslation reporter system to measure the levels of Leu at those sites (see methods). Figure 1.18 shows levels of Leu misincorporation in WT cells grown at 30ºC, pH7; in media lacking sorbitol at 30ºC (table 2.4), pH 6 without sorbitol at 30ºC, pH 7 and 0,2M of sorbitol at 30ºC and pH7 in media lacking sorbitol at 37ºC. The data showed increase in misincorporation in all the conditions tested but only when grown at pH 6 without sorbitol at 30ºC, pH 7 and 0,2M of sorbitol at 30ºC showed a significant difference in misincorporation.

The results of the 5 strains were compared when possible with the HAP31 and RAPH2 positive control strains that were previously studied at the host laboratory by Edgar Lopes (unpublished work).

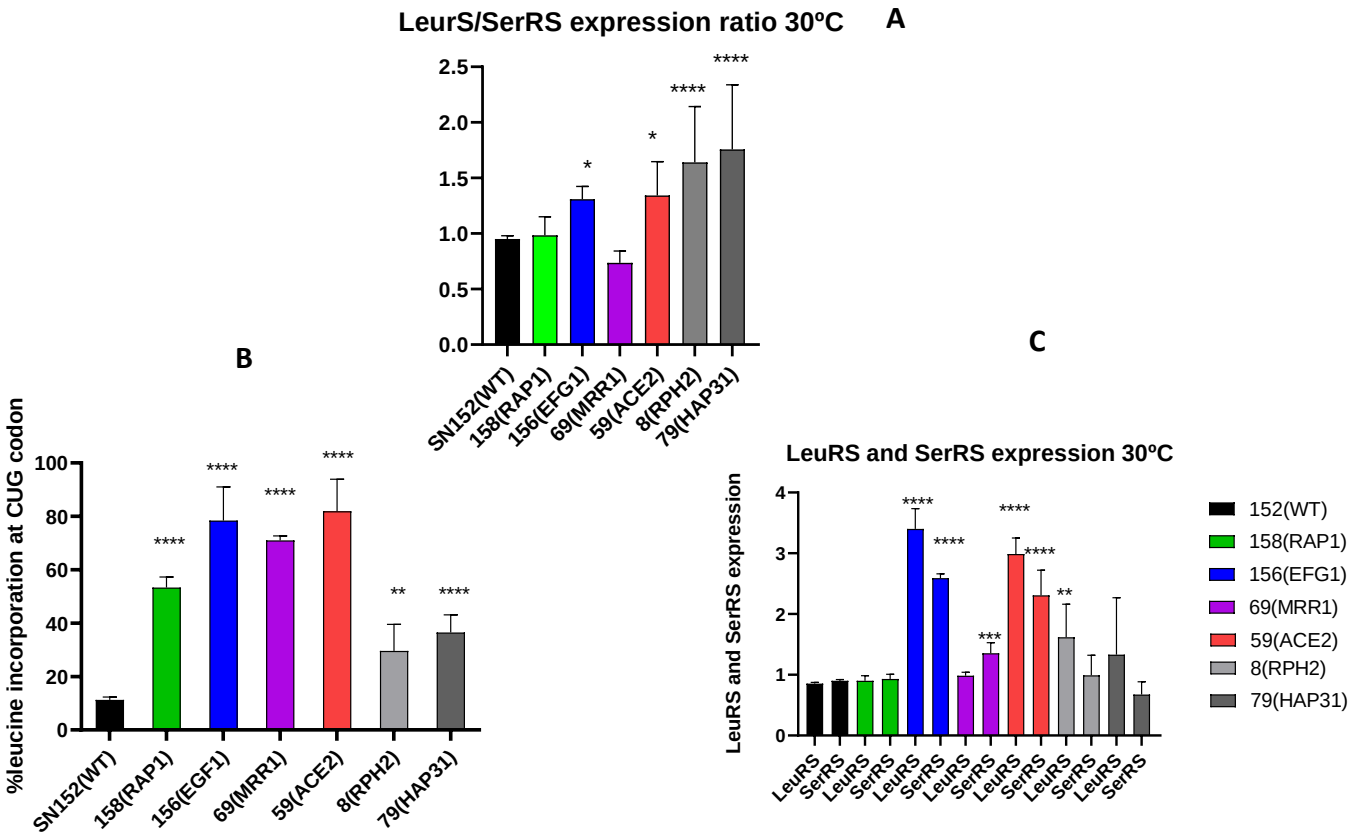

Figure 15 **LeuRS/SerRS expression ratio and Leu misincorporation in WT SN152 strain and in the strains harboring deletions of *RAP1, EFG1, MRR1, ACE2, RPH2 and HAP31* genes , grown under control conditions.**
A) LeuRS/SerRS expression ratio, **(B)** leucine misincorporation levels at CUG sites, **(C)** LeuRS and SerRS expression Data was collected from at least 300 cells of 3 different clones from each strain. (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 vs control).

WT cells (SN152) grown at pH 7, without sorbitol, at 30ºC misincorporate 12,23 ± 1,12% (Figure 1.14 A) of leucine at CUG sites, which is higher than previously described (3%). The reason for this difference is not known but it may be related to problems encountered during image analysis. The other strains showed higher leucine incorporation levels than the WT. In this condition only EFG1 ACE2, RPH2 and HAP31 strains displayed a higher LeuRS/SerRS ratio relative to the WT (Figure 1.14 A). The levels of Leu incorporation in the ACE2 and EFG1 strains were higher than in the other strains and this is correlated with the LeuRS/SerRS and LeuRS/SerRS expression ratios. Therefore, these data indicatet that deletion of the EFG1, ACE2, RPH2 and HAP31 genes results in the downregulation of the LeuRS.

The RPH2 and HAP31 strains showed a higher LeuRS/SerRS expression ratios relative WT, yet only the RPH2 strain had LeuRS levels significantly higher than the WT (>1). The changes observed for the HAP31 were not significant.

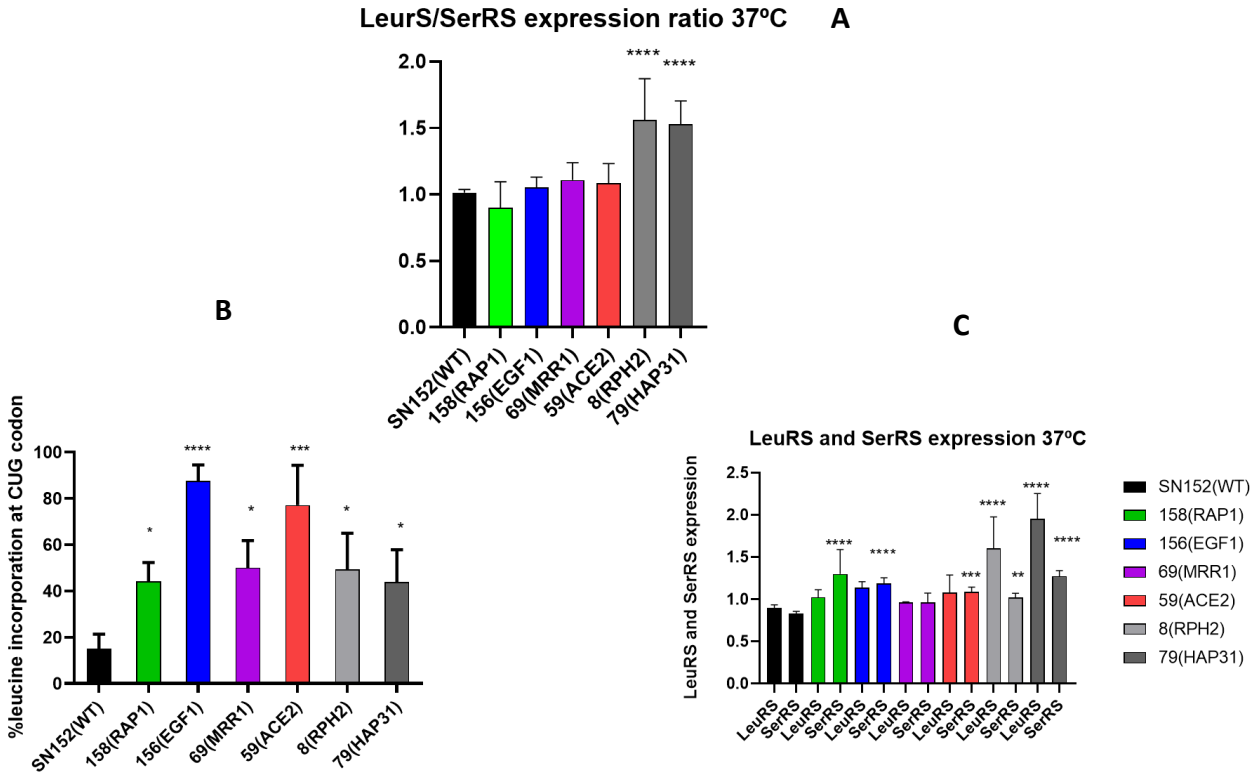

Figure 16 **LeuRS/SerRS expression ratio and Leu misincorporation in WT SN152 strain and in the strains harboring deletions of RAP1, EFG1, MRR1, ACE2, RPH2 and HAP31 genes, grown at 37ºC. A)** LeuRS/SerRS expression ratio, **(B)** leucine misincorporation levels at CUG sites, **(C)** LeuRS and SerRS expression Data was collected from at least 300 cells of 3 different clones from each strain. (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 vs control).

The data showed that WT cells grown at pH7 and hypotonic media at 37ºC misincorporate 15,05 ±6,28% of leucine which is a higher when compared with the control conditions (12,23 ± 1,12%). All KO strains misincorporated higher levels of leucine incorporation at CUG codon sites (Figure 1.15 B). Only the RAPH2 and HAP31 strains showed increase in the LeuRS/SerRS ratios level relative to WT, probably due to upregulation of LeuRS expression, suggesting that these TF repress LeuRS transcription in this physiological condition (Figure 1.15 B, C).

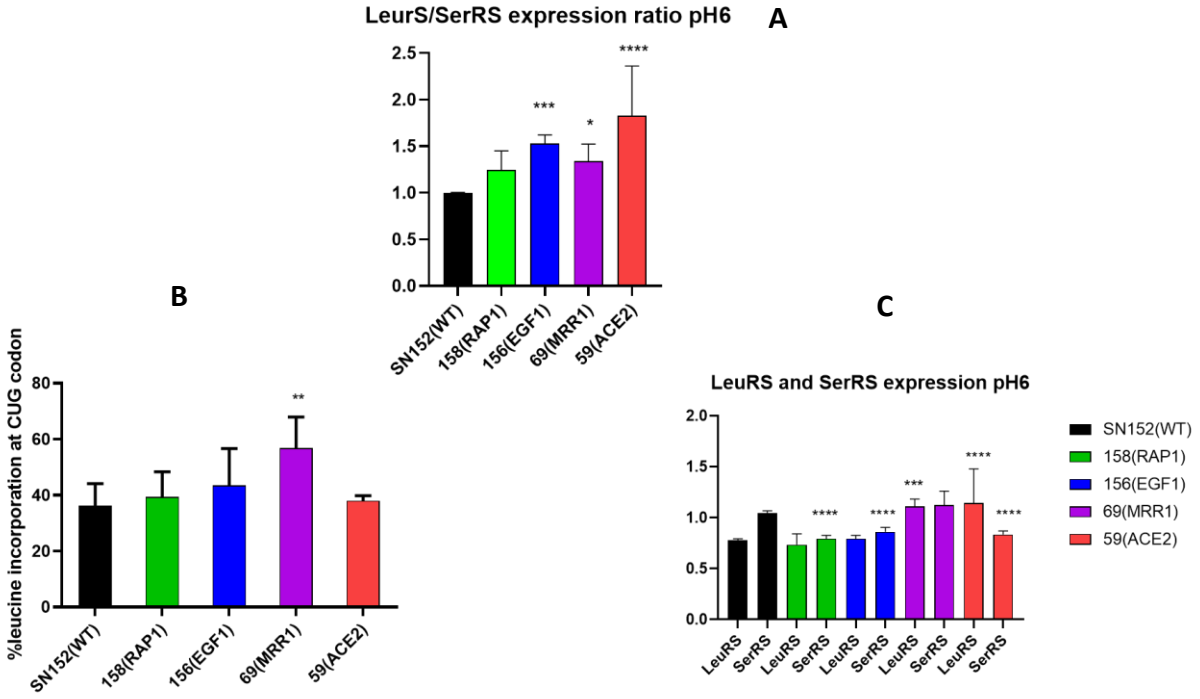

Figure 17 **LeuRS/SerRS expression ratio and Leu misincorporation in WT SN152 strain and in the strains harboring deletions of RAP*1, EFG1, MRR1, and ACE2 genes*, grown at pH6.**
A) LeuRS/SerRS expression ratio, **(B)** leucine misincorporation levels at CUG sites, **(C)** LeuRS and SerRS expression levels. Data was collected from at least 300 cells of 3 different clones from each strain. (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 vs control).

WT cells grown at pH6, in hypotonic media, at 30ºC misincorporated 36,10±7,97% of Leucine at CUG codon sites which is higher relative to control conditions (12,23 ± 1,12%). (Figure 1.16 B). EFG1, MRR1 and ACE2 strains showed a higher LeuRS/SerRS ratio, although only ACE2 and MRR1 strains showed upregulation of LeuRS, suggesting that under these conditions these TFs repress of LeuRS expression (Figure 1.16 A, C). The ACE2 and WT strains incorporated similar levels of Leu, however leucine misincorporation value is already high, in the WT strain and it is likely that Leucine levels are correlated with LeuRS/SerRS ratio and LeuRS/SerRS expression. EFG1 strain misincorporated higher levels of leucine (43.42±13,17%) and also had higher LeuRS/SerRS ratio, but there were no changes in the levels of LeuRS and SerRS expression, suggesting that the higher percentage of Leucine misincorporation do not result in alteration of SerRS and LeuRS (Figure 1.16 C). In other words, post-transcriptional mechanisms of gene expression control may also play a role in the regulation of CUG ambiguity.

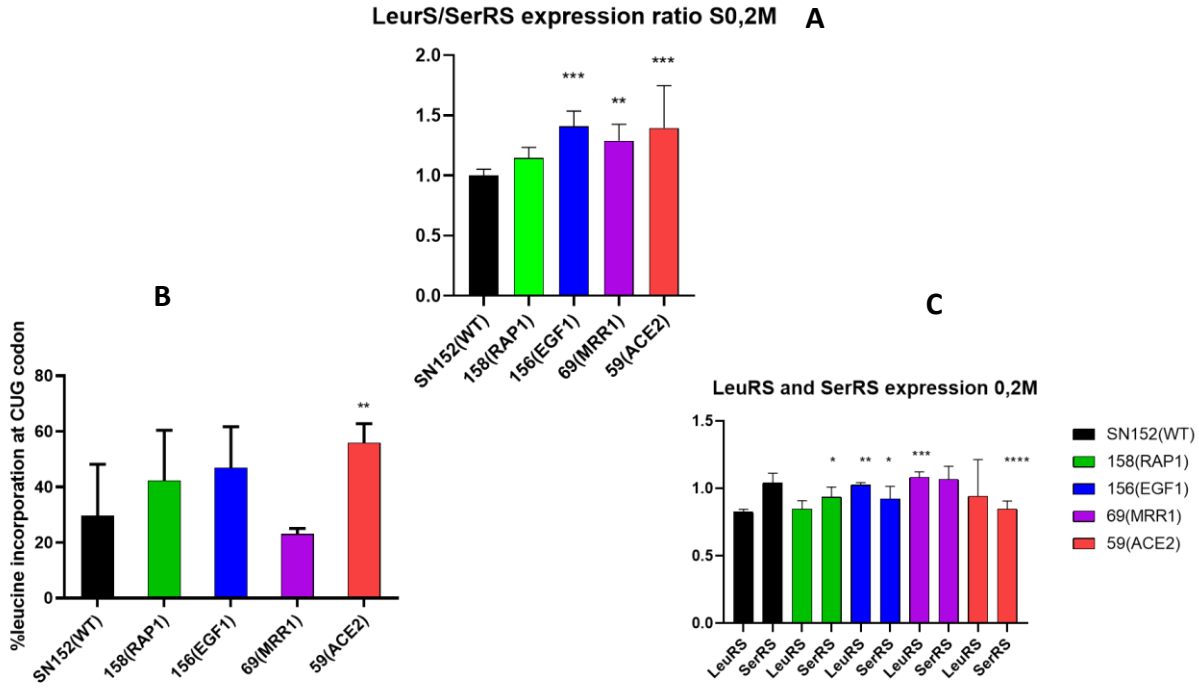

Figure 18 **LeuRS/SerRS expression ratio and Leu misincorporation in WT SN152 strain and in the strains harboring deletions of RAP*1, EFG1, MRR1, and ACE2 genes*, grown under osmotic stress 0,2M(hypotonic)**
A) LeuRS/SerRS expression ratio, **(B)** leucine misincorporation levels at CUG sites, **(C)** LeuRS and SerRS expression Data was collected from at least 300 cells of 3 different clones from each strain. (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 vs control).

WT cells grown under osmotic stress of (0,2M of sorbitol), at 30ºC showed 29,65±18,49% of Leu misincorporation at CUG (Figure 1.17 B), this value is higher than in the control conditions (12,23 ± 1,12%). All the mutant strains except RAP1 showed a slightly higher LeuRS/SerRS ratio. The leucine misincorporation levels were not significantly different from the WT in all but the ACE2 strain. This is not a surprising result since 0,2M sorbitol is a mild stress which is not expected to impose major physiological alterations to the cell. In the EGF1 strain the higher LeuRS/SerRS ratio is due to an increase in the LeuRS expression suggesting that EFG1 is likely a repressor of LeuRS expression in these growth conditions. (Figure 1.17 A, C).

Therefore, our data strongly suggest that EFG1, MRR1 and ACE2 TFs are putative regulators of LeuRS and SerRS expression. EFG1 is likely a repressor of LeuRS expression, in particular under control conditions or in presence of 0,2M sorbitol. MRR1 is likely a repressor of LeuRS expression at 30ºC, pH6, and in presence of 0,2M sorbitol. ACE2 may

be a LeuRS repressor at 30ºC, pH6 and an activator of SerRS expression, in particular in control conditions and in presence of 0,2M of sorbitol.

Chapter IV-Discussion

Discussion:

The objective of this thesis was to understand how leucine is regulated in *C. albicans*. We tested if the expression of SerRS and LeuRS was correlated with the leucine misincorporation in different physiological conditions using strains harboring deletions in 4 transcription factors, a fluorescence reporter system and western blot analysis. A significant variation in the ratio of LeuRS/SerRS was observed between the WT and KO strains, in normal conditions and in the stress conditions tested. By analyzing the data obtained in our study especially the expression of the synthetases we can reach he conclusion that the increase in the LeuRS/SerRS levels is due to an increase in the LeuRS levels or decrease in the SerRS levels.

To access the regulatory mechanism of the LeuRS and SerRS expression we used a collection of TF strains previously reported by Homann *et al*(107) . The selection of the TFs for our study was done using bioinformatics approaches that identified TFs that may bind to specific regulatory elements present in the promoters of both LeuRS and SerRS genes.The LeuRS/SerRS ratio determined to provide information about the relative levels of both synthetases, which is relevant for leucine misincorporation since it depends on the cellular stoichiometry of these enzymes. The TF data showed significant variation in TF regulatory behavior in the different physiological conditions tested. This is expected since transcription factors play different activation and repression roles in response to environmental changes.(118,119). Under control conditions (30ºC) the KO strains behaved rather similarly with a general increase in LeuRS expression. In other words, most of the TFs tested repress LeuRS expression in normal physiological situations. Another explanation for this increase is that the deletion of some TFs could be stressful for *C. albicans* cells and consequently activate other pathways that lead to increase leucine misincorporation. Similar results were obtained at pH6, 0,2M sorbitol, but the ACE2 KO strain suggest that the ACE2 TF is a LeuRS repressor and a SerRS activator. When the KO strains were grown at 37ºC surprisingly no major differences were observed relative to the WT.

The cellular roles of the TFs

RAP1 is a transcription factor involved in the binding of telomeres and regulatory sequences in DNA and is a repressor of hyphal formation under yeast-favoring conditions(120–122). Our data indicate that it does not regulate LeuRS or SerRS expression in the conditions tested.

EFG1 is a bHLH transcription factor that is required for white-phase cell type switching and has a role in hyphal growth, cell adhesion, biofilm formation and virulence (123–126). Mutants lacking EFG1 lack a potential PKA phosphorylation site and are defective in filament formation, meaning that EGF1 could affect PKA (127).EFG1 is also a negative regulator of the target genes of the transcription factor ACE2(128). The strain EFG! KO grown in control conditions and in presence of 0,2M sorbitol showed increase LeuRS expression, suggesting that this TF is a negative regulator of the LeuRS in these growth conditions.

MRR1 is a Putative Zn(II)2Cys6 transcription factor involved in multi drug resistance(129–131). When MRR1 is mutated induces overexpression of the MDR1 gene, which is responsible resistance to Fluconazole, Cerulenin and Brefeldin A(129). Tin our experimental conditions MRR1 behaved as a LeuRS repressor at pH6 and in presence of 0,2M sorbitol.

ACE2 is a transcription factor that regulates morphogenesis, cell separation and virulence (132–135).ACE2 regulates the expression of CHT3, DSE1, and SCW11 and several genes involved in the biosynthesis of cell wall proteins. ACE2 enters the nucleus at the end of mitosis, but is exported from the nucleus of the mother cell to the nucleus of the daughter cell(133).When ACE2 is inactivated in *C. glabrata* a phenotype of hypervirulence arises (136). Our ACE2 data shows that it is a putative repressor of LeuRS in the control conditions at pH6, and a activator of SerRS when grown at pH6 and in presence of 0,2M sorbitol. Our bioinformatics analysis confirmed that ACE2 can bind to the promoters of both SerRS and LeuRS genes.

The Leu misincorporation levels observed in our study were abnormally high when compared with the levels obtained by Gomes *et al*.(94). In control conditions, we obtained 12,23% of misincorporation, while Gomes *et al* (94) obtained 2.96%. While when grown at 37ºC we obtained a value of Leu misincorporation of 15,06% and Gomes *et al* (94) obtained 3.9%. When grown at pH6 we obtained 43,42% and in presence of 0,2M sorbitol we obtained 29,65% of misincorporation. We do not have a good explanation for these results, but they may be related to the fact that different methodologies ere used in both studies. In any case, such differences should not interfere with the conclusions of our study since we used relative rather than absolute values in our comparative analysis.

In control conditions (30ºC) all the strains had significant higher levels of leucine misincorporation than the WT strain suggesting that all TFs are putative regulators of LeuRS and SerRS expression. Similar results were obtained at 37ºC, however in this case the LeuRS/SerRS obtained at 37ºC, however in this case the LeuRS/SerRS oratios, LeuRS and SerRS expression levels did not show significant variations. At pH 6 only the strain MRR1 showed a statistically significant level of Leucine misincorporation however this data is not corroborated by LeuRS/SerRS ratio obtained previously and need to be confirmed in future studies. When grown in presence of 0,2M sorbitol only the strain ACE2 showed a significant difference of leucine misincorporation when compared with the WT strain, which is not surprising since 0,2M sorbitol is a mild stress.

The western blot analysis showed that only ACE2 regulate the LeuRS and SerRS expression at 30ºC, suggesting that the expression of this enzyme may also be regulated at the post-transcriptional level.

We summarize the data obtained in one study in table 2.7. This summary shows that EFG1, MRR1 and ACE2 are likely repressors of LeuRS expression, but Ace2 is likely an activator of SerRS at pH6 and in presence of 0,2M sorbitol.

Table 2.7- Summary of the results

	30ºC		37ºC		pH6		S 0,2M	
	LeuRS	SerRS	LeuRS	SerRS	LeuRS	SerRS	LeuRS	SerRS
SN152	Control	Control	Control	Control	Control	Control	Control	Control
158(RAP1)								
156(EFG1)	Repressor						Repressor	
69(MRR1)					Repressor		Repressor	
59(ACE2)	Repressor				Repressor	Activator		Activator

Future perspectives

Future experiments should clarify the function of our transcription factors by confirming if the EFG1,MRR1 and ACE2 TF bind to the promoters of the SerRS and LeuRS genes, using Chromatin Immunoprecipitation with next generation sequencing (ChIP-seq)(137).

It will also be important to clarify the reason for higher levels of leucine misincorporation at CUG sites detected in our study relative to previous studies, using flow cytometry instead of fluorescence microscopy. Flow cytometry will allow for the analysis of a much higher number of cells overcoming possible biases in cell counting by microscopy.

Chapter V-References

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