Universidade de Aveiro Departamento de Biologia
Ano 2014

Mónica Rodrigues Barbosa

Evolution of antifungal drug resistance in *Candida albicans*

Evolução de resistência a drogas em *Candida albicans*

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Evolução de resistência a drogas em *Candida albicans*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Ana Rita Macedo Bezerra, investigadora em Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro e Co-Orientação do Doutor Manuel António da Silva Santos, Professor associado do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

C.albicans, tRNA, evolução experimental, erros de tradução, resistência a drogas.

resumo

O fungo patogénico *Candida albicans* tem a particularidade de possuir um tRNA ($tRNA_{CAG}^{Ser}$) híbrido que é aminoacilado pelas sintetases SerRS e LeuRS. Esta característica é responsável pela ambiguidade do codão CUG que é decodificado como Ser (97%) e como Leu (3%). Estudos anteriores demonstraram que o nível de ambiguidade é variável dependendo da condição de crescimento do fungo e revelaram que estipes com maior erro de tradução crescem melhor na presença de azoís, particularmente em meio com fluconazol.

Para analisar os efeitos dos erros de tradução na aquisição de resistência a drogas, construímos três estirpes de *C. albicans* (T0, T1 e T2) com níveis crescentes de incorporação de Leucina, inserindo uma cópia (T1) ou duas cópias (T2) do gene $tDNA_{CAG}^{Leu}$. Estas estirpes foram evoluídas experimentalmente na presença e na ausência de fluconazol de acordo com o protocolo da EUCAST. O nível de ambiguidade destas estirpes foi avaliado durante o período da evolução usando um sistema reporter baseado na proteína fluorescente GFP. Finalmente foi também avaliada o tipo de resistência adquirida pelas estirpes ambíguas (resistência genética ou fenotípica).

Os dados experimentais sugerem que a aquisição de resistência depende da droga e da percentagem do erro de tradução. As estirpes com maior erro de tradução (T2) adquirem mais resistência (256 µg/ml) e mais rapidamente sendo que mutações no gene ERG11 poderão ser responsáveis por essa resistência. Esta estirpe apresentou também um decréscimo da taxa de erro de tradução em ambas as evoluções (com e sem droga), resultado da deleção de uma das duas cópias do gene $tDNA_{CAG}^{Leu}$. Este resultado reforça a instabilidade genética da estirpe T2.

keywords *C. albicans*, tRNA, experimental evolution, mistranslation, drug resistance.

abstract

The human pathogen *Candida albicans* is characterized by the presence of a hybrid tRNA ($tRNA_{CAG}^{Ser}$) that can be aminoacylated by both LeuRS and SerRS. This tRNA is responsible for the ambiguity of the CUG codon that can be decoded as serine (97%) and Leucine (3%). Previous studies showed that the level of ambiguity is variable depending on the growth condition of the fungus. These studies also revealed that strains mistranslating at a higher level grew better in the presence of azoles, particularly in the presence of fluconazole.

To analyse the effect of translation errors on the acquisition of drug resistance, three strains of *C. albicans* were used (T0, T1 and T2). These strains have increasing levels of Leu misincorporation and were constructed by inserting a copy (T1) or two copies (T2) of a $tDNA_{CAG}^{Leu}$ gene. These strains were evolved experimentally in the presence and absence of the antifungal agent according to the approved EUCAST protocol. The level of ambiguity of all strains was measured along the evolution experiment, using a reporter system based on the green fluorescent protein (GFP). Finally, the type of acquired resistance was also evaluated (genetic or phenotypic resistance).

Experimental data suggest that the acquisition of resistance is dependent on the drug and the percentage of mistranslation. Strains with increased mistranslation (T2) acquired more resistance (256 µg/ml) and faster than the control T0. Mutations in ERG11 gene might be responsible for this resistance. Also, T2 strain showed a decrease in the mistranslation level during both evolution experiments (with and without drug) as a consequence of the deletion of one of the two copies of the mutant $tDNA_{CAG}^{Leu}$ gene. This result further highlights the genetic instability of strain T2.

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aaRSs - Aminoacyl-tRNA synthetases

AIDS - Acquired immune deficiency syndrome

A-site -Aminoacyl site

ATP - Adenosine 5‟-triphosphate

C.albicans - *Candida albicans*

C. neoformans- Cryptococcus neoformans

CO_2 - Carbon dioxide

$CuSO_4$ - Copper sulfate

CMT - Charcot-Marie-Tooth

14DM - 14α-demethylase

DNA - Deoxyribonucleic acid

E.coli - Escherichia coli

eIF - Eukaryotic initiation factors

E-site - Exit site

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

GDP - Guanosine 5‟-diphosphate

GTP - Guanosine 5‟-triphosphate

HIV - Human Immunodeficiency Virus

H_2O_2 – Hydrogen peroxide

HSP - Heat shock protein

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

LOH - Loss of heterozygosity

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

MIC - Minimun inibitory concentration

mRNA - Mensseger ribonucleic acid

MTL - Mating-type-like

OD - Optical density

P-site - Peptidyl site

PCR - Polymerase chain reaction

RNA - Ribonucleic acid

ROS - Reactive oxygen species

Rpm - Revolutions per minute

RPS10 - Ribosomal protein S10

S: Svedberg

S.cerevisiae - *Saccharomyces cerevisiae*

SDS - Sodium dodecyl sulphate

SNP - Single-nucleotide polymorphism

Sp. and spp. - species and "several species"

tRNA - Transfer ribonucleic acid

yEGFP - Yeast-enhanced green fluorescent protein

Other abbreviations will be explained when used in the text.

I. Introduction

1. Protein Synthesis – the Process of mRNA Translation

All living systems store and transmit the genetic information to their offspring through the deoxyribonucleic acid (DNA). The functional parts of the DNA are called genes and they constitute the genome along with other elements. Transcription of this genetic information from DNA into messenger ribonucleic acid (mRNA) and its subsequent translation assures that the genome is used as the source of information for protein synthesis. This process in which the information in genes flows into proteins constitutes the Central Dogma of Molecular Biology, proposed by Crick [(1)].

Decoding the genetic information contained in the mRNA into protein occurs in the ribosome, during the process of translation. This is achieved by pairing each amino acid-specific codon from the mRNA with a complementary sequence, the anticodon triplet of the transfer RNA (tRNA). The tRNA thus connects the worlds of nucleic acids and proteins, as it binds a specific amino acid corresponding to the anticodon. The enzymes that charge the tRNAs with the respective amino acids are the aminoacyl-tRNA synthetases (aaRSs) [(2, 3)].

Translation can be divided into three steps: initiation, elongation and termination. In eukaryotes, the initiation step needs at least ten proteins, which are nominated eIFs (eukaryotic initiation factors). The proteins eIF-1, eIF-1A, and eIF-3 bind to the 40S ribosomal subunit, and eIF-2 (in a complex with GTP) associates with the initiator methionyl tRNA. The mRNA is recognized and transported to the ribosome via interactions with the eIF-4 group of factors. Another factor, eIF-4G, binds to both eIF-4E and to a poly-A binding protein (PABP) associated with the poly-A tail at the 3’ end of the mRNA. After, the 5’ and 3’ ends of mRNAs are recognized by eIFs. The initiation factors eIF-4E and

eIF-4G, in association with eIF-4A and eIF-4B, then bring the mRNA to the 40S ribosomal subunit, with eIF-4G interacting with eIF-3. The 40S ribosomal subunit is then bound to methionyl tRNA and eIFs, and this complex scans the mRNA until it recognizes the AUG initiation codon. After the AUG codon is reached, eIF-5 triggers the hydrolysis of GTP bound to eIF-2. Initiation factors are then released and the 80S initiation complex of eukaryotic cells is established, by junction of 60S and the 40S subunits (Figure 1) [4, 5].

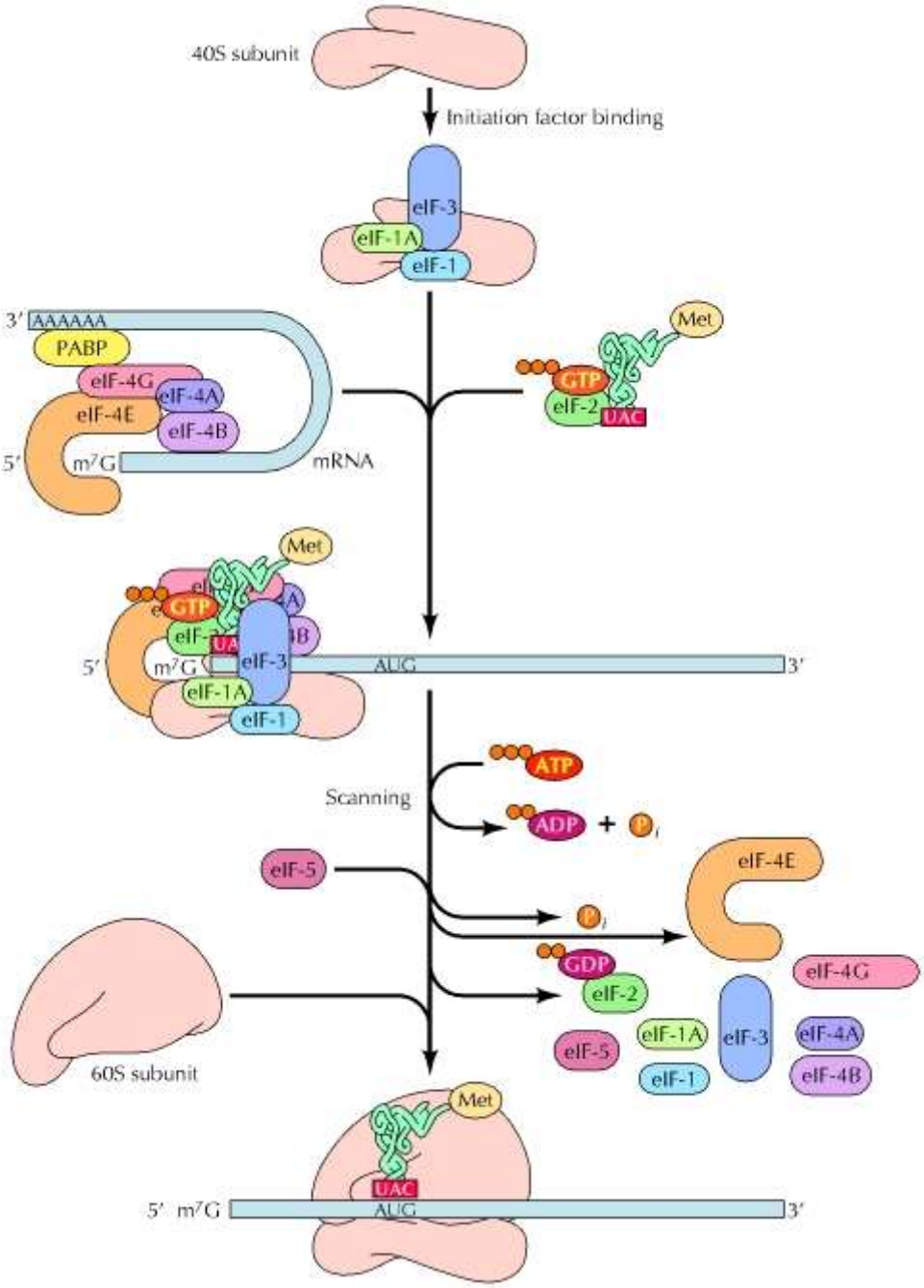


Figure 1 - Initiation of translation in eukaryotic cells. Initiation factors eIF-3, eIF-1, and eIF-1A bind to the 40S ribosomal subunit. The initiator methionyl tRNA is brought to the ribosome by eIF-2 (complexed to GTP), and the mRNA by eIF-4E (which binds to the 5´ cap), eIF-4G (which binds to both eIF-4E at the 5' cap and PABP at the 3' poly-A tail), eIF-4A, and eIF-4B. The ribosome then scans mRNA until recognize the AUG initiation codon. After that eIF-5 triggers the hydrolysis of GTP, followed by the release of the initiation factors. The 60S ribosomal subunit then joins the 40S complex (Adapted from reference 6).

Translation proceeds by elongation of the polypeptide chain. The mechanism of elongation in prokaryotic and eukaryotic cells is very similar. The ribosome has three sites for tRNA binding, designated the P (peptidyl), A (aminoacyl), and E (exit) sites. The initiator methionyl tRNA occupies the P site in the ribosome, and the A site is ready to receive an aminoacyl-tRNA by pairing with the second codon of the mRNA. The aminoacyl tRNA is followed to the ribosome by an elongation factor, eEF-1α in eukaryotes, which is in a complex with GTP. When the correct aminoacyl tRNA is inserted into the A site of the ribosome, the GTP is hydrolyzed to GDP, and the elongation factor bound to GDP is released (Figure 2). Next, a peptide bond is formed between the initiator methionyl tRNA at the P site and the second aminoacyl tRNA at the A site. This reaction is catalyzed by the large ribosomal subunit. The result is the transfer of methionine to the aminoacyl tRNA at the A site of the ribosome, forming a peptidyl tRNA at this position and leaving the uncharged initiator tRNA at the P site. The next step in elongation is translocation, which requires another elongation factor, eEF-2 in eukaryotes, and is again coupled to GTP hydrolysis. During translocation, the ribosome moves three nucleotides along the mRNA, positioning the next codon in an empty A site. This step translocates the peptidyl tRNA from the A site to the P site, and the uncharged tRNA from the P site to the E site. The ribosome is then left with a peptidyl tRNA bound at the P site, and an empty A site. The binding of a new aminoacyl tRNA to the A site then induces the release of the uncharged tRNA from the E site, leaving the ribosome ready for insertion of the next amino acid in the growing polypeptide chain [(6)].

The end of translation is signalled by the presence of one of three codons (UAA, UAG, or UGA) called stop codons at the A site. These are recognized by a release factor rather than by a tRNA. In eukaryotic cells the eRF-1 recognizes all three termination codons. The release factors bind to a termination codon at the A site and stimulate hydrolysis of the bond between the tRNA and the polypeptide chain at the P site, resulting in release of the completed polypeptide from the ribosome, followed by the dissociation of tRNA and mRNA from the ribosome. The ribosome separates into the large and small subunits, which can assemble on another mRNA molecule to begin a new round of protein synthesis [6].

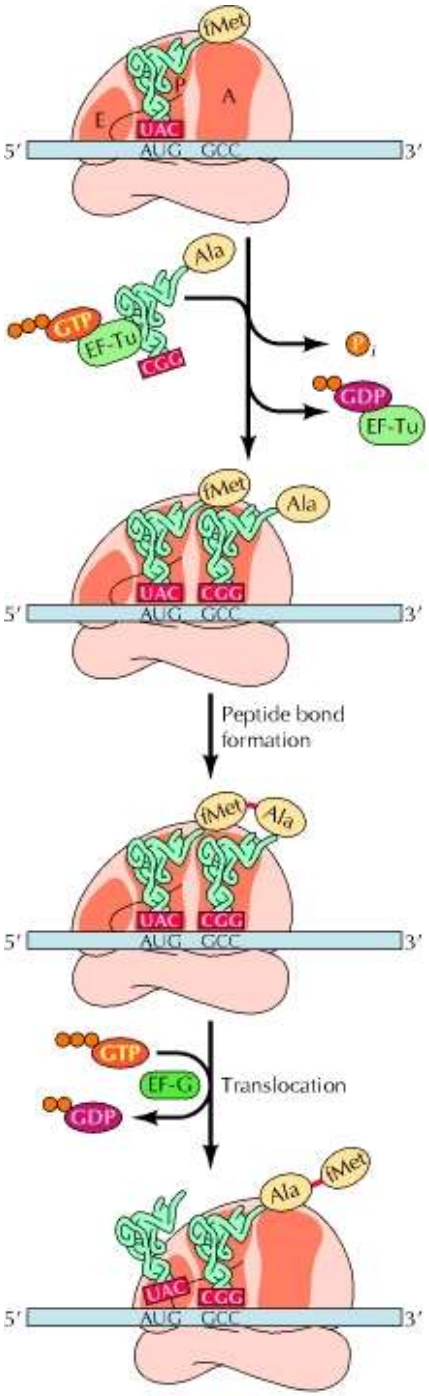


Figure 2 - The elongation step in translation process. The ribosome has three tRNA-binding sites, designated P (peptidyl), A (aminoacyl), and E (exit). The initiating methionyl tRNA is positioned in the P site, leaving an empty A site. The second aminoacyl tRNA is then brought to the A site by EF-Tu (complexed with GTP). Following GTP hydrolysis, EF-Tu (complexed with GDP) leaves the ribosome, with second aminoacyl tRNA inserted into the A site. Forms a peptide bond, resulting in the transfer of methionine to the aminoacyl tRNA at the A site. The ribosome then moves three nucleotides along the mRNA and result in a translocation of the peptidyl tRNA to the P site and the uncharged tRNA to the E site, leaving an empty A site ready for addition of the next amino acid. EF-Tu is the designations used for the bacterial elongation factors, in eucaryotes, is called EF-1 (Adapted from reference 6).

1.1. mRNA Mistranslation – Error in Protein Synthesis

The Central Dogma of Molecular Biology describes the ways in which information flows. First DNA replication (DNA to DNA), and then transcription (DNA to RNA), and finally translation (RNA to protein) [(1)] proceed with optimized levels of fidelity and speed. Of the three processes mRNA translation is more likely to experience errors and error rates are similar in eukaryotes and prokaryotes [(7)]. The overall error rate of protein synthesis has been measured to be in the order of 10^{-3} – 10^{-4} (one error in every 1000 to 10000 codons translated) [(8-10)].

Fidelity of translation mostly relies on the accuracy of tRNA aminoacylation and mRNA decoding by the ribosome. Mischarging of tRNAs may be caused by the failure of aaRSs to recognize their cognate tRNAs or by the activation of incorrectly bound amino acids. To minimize mischarging, some aaRSs have editing mechanisms that discard chemically similar amino acids from their active sites [(11)]. The aaRSs use editing mechanisms that can occur before or after the transfer of the amino acids to the tRNA, namely pre-transfer editing (the hydrolysis of mischarged aminoacyl adenylates) or post-transfer editing (the hydrolysis of mischarged aminoacyl-tRNAs) , respectively [(10)]. Pre-transfer editing can be either tRNA dependent or tRNA independent and occurs through a range of mechanisms that promote hydrolysis of the labile non-cognate (two

molecules matched not according to the rules of the genetic code) aminoacyl adenylate species, leading to the release of 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 [12].

Codon decoding errors are mainly caused by misreading of sense codons (missenseerrors) and nonsense codons (nonsense errors) by near cognate or noncognate tRNAs. The result is the synthesis of mutant proteins. Other causes for synthesis of mutant proteins are the loss of the reading frame (frameshifting), that alters the reading frame to the -1 or to the +1 frames by tRNA splippage during elongation, or by the premature ribosome drop off from the mRNA (processivity errors). Together, these errors result in premature translation termination and synthesis of truncated polypeptide [11, 13, 14].

Although the presence of mutant proteins should be highly detrimental, organisms survive well with a protein synthesis error rate of 1 error in every 1000 to 10000 codons translated. This suggests that apparently there is not an evolutionary pressure for the ribosome to be more accurate. It is argued that this rate of fidelity represents a compromise between speed and accuracy that optimizes the fitness of the organism. In order to maintain cellular functionality eukaryotes use numerous quality control systems to deal with aberrant proteins, namely degradation by the ubiquitin-proteasome pathway, the chaperone mediated autophagy, or refolding by molecular chaperones [15].

1.2. mRNA Mistranslation – Negative and Positive Features

Generally, the occurrence of mistranslation is considered deleterious and infrequent, but the observations of organisms that decode some codons ambiguously and the discovery of a compensatory increase in mistranslation frequency to combat environmental stress have changed the way that translational errors are viewed in decoding [16].

In fact, some advantages of mistranslation are well described. For example, the non-methionyl tRNAs that can be methionylated by methionyl-tRNA synthetases from *E. coli,* yeast*,* and mammals, under conditions of oxidative stress. Because methionine may spontaneously react with reactive oxygen species (ROS) that are formed under oxidative stress, methionine residues mistranslated at non-methionyl codons may contribute to decrease the presence of ROS, that are later reduced by methionine sulfoxide reductases [17].

Another notable example is the CUG codon of *C. albicans*. This pathogen reassigned the CUG codon from leucine (Leu) to Serine (Ser) trough CUG decoding ambiguity, which resulted in proteomic and phenotypic diversity. Ser and Leu are incorporated in the *C. albicans* proteome with efficiencies of 97% and 3%, respectively, and it can tolerate up to 28% of Leu misincorporation without visible effects on growth rate [18, 19]. The resulting proteomic and phenotypic diversity may make this opportunistic pathogen a ‘‘moving target’’ for the host’s adaptive immune system [20].

In *S. cerevisiae,* ambiguity of the CUG codon does not generate the high phenotypic and morphological diversity observed in *C. albicans*, however it can increase the tolerance to many environmental agents and permits growth in the presence of lethal doses of cicloheximide, arsinite, cadmium, and salt [21].

In another example, *Mycoplasma spp.* have error-prone LeuRS, PheRS and ThrRS with mutations and deletions in their editing domains [22]. The mischarging of tRNAs by these AARSs increases mistranslation and leads to the production of statistical proteins with Phe/Tyr, Leu/Met and Leu/Val substitutions. The high frequency of mistranslation may provide a mechanism

for the remarkable phenotypic plasticity of *Mycoplasma* that in turn allow it to escape host defences [22].

Recently, Javid and colleagues reported that mycobacteria substitute glutamate for glutamine and aspartate for asparagine at high rates under specific growth conditions. Increasing the substitution rate results in phenotypic resistance to rifampicin, whereas decreasing mistranslation produces increased susceptibility to the antibiotic [23].

On the other hand, several mutations in aaRSs cause human neurological disorders, including the peripheral neuropathy CMT (Charcot–Marie–Tooth). Mutations in GARS, which encodes glycyl-tRNA synthetase, are linked to CMT disease type 2 because they affect the localization of glycyl-tRNA synthetase in granules within the cell bodies and neurite projections of neuronal cells, which results in a slowly progressive axonal polyneuropathy [24, 25].

Another example of the negative effects of incorrect translation is described by Lee and collagues. The mouse sticky mutation affects the editing domain of alanyl-tRNA synthetase, leading to charging of $tRNA^{Ala}$ with serine. The global misincorporation of serine at alanine codons results in the accumulation of intracellular misfolded proteins, protein aggregation and ubiquitination. Together, these phenomena lead to rapid loss of Purkinje cells and mouse premature death [25, 26].

2. The Genetic Code

The genetic code was first described by Crick in 1966 derived from studies in *E.coli* [27]. It englobes a set of rules by which information encoded within mRNA is translated into proteins by living cells. It postulated that there are four ribonucleotide base combinations C (cytosine), G (guanine), A (adenine) and U (uracil), organized into nucleotide-triplets (codons), that must be employed to encode the 20 standard amino acids used by living cells to build proteins. 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 (UAA, UAG and UGA codons). Therefore, it is clear that some amino acids are decoded by more than one codon, which makes the genetic code degenerate (Figure 3) [28, 29]. These codons are named “synonymous codons”, except methionine (Met) and tryptophan (Trp), which are decoded by only one codon, AUG codon and UGG codon, respectively [30]. One codon, AUG, has a double role because it specifies initiation of protein synthesis in addition to being a normal Met (methionine) codon [28].

The emergence of two news amino acids, designated as the 21st and 22nd amino acids, nominated selenocysteine (Sec) [31] and pyrrolysine (Pyl) [32], respectively, confirms the expansion of the genetic code and its flexibility. In this case, some organisms translate the UGA stop codon as Sec and UAG stop codon as Pyl [31, 32].

The discovery that several organisms and organelles developed an alternative genetic code sets aside the hypothesis that the genetic code is frozen and universal. This also suggests that the evolution of alternative genetic codes may have physiological significance and may play an important role in the evolution of the species [21].

UUU [F] Phe	UCU [S] Ser	UAU [Y] Tyr	UGU [C] Cys
UUC [F] Phe	UCC [S] Ser	UAC [Y] Tyr	UGC [C] Cys
UUA [L] Leu	UCA [S] Ser	UAA [ ] Ter	UGA [ ] Ter
UUG [L] Leu	UCG [S] Ser	UAG [ ] Ter	UGG [W] Trp
CUU [L] Leu	CCU [P] Pro	CAU [H] His	CGU [R] Arg
CUC [L] Leu	CCC [P] Pro	CAC [H] His	CGC [R] Arg
CUA [L] Leu	CCA [P] Pro	CAA [Q] Gln	CGA [R] Arg
CUG [L] Leu	CCG [P] Pro	CAG [Q] Gln	CGG [R] Arg
AUU [I] Ile	ACU [T] Thr	AAU [N] Asn	AGU [S] Ser
AUC [I] Ile	ACC [T] Thr	AAC [N] Asn	AGC [S] Ser
AUA [I] Ile	ACA [T] Thr	AAA [K] Lys	AGA [R] Arg
AUG [M] Met	ACG [T] Thr	AAG [K] Lys	AGG [R] Arg
GUU [V] Val	GCU [A] Ala	GAU [D] Asp	GGU [G] Gly
GUC [V] Val	GCC [A] Ala	GAC [D] Asp	GGC [G] Gly
GUA [V] Val	GCA [A] Ala	GAA [E] Glu	GGA [G] Gly
GUG [V] Val	GCG [A] Ala	GAG [E] Glu	GGG [G] Gly

Figure 3 - The standard genetic code. The codon series are shaded in accordance with the polar requirement scale values, which is a measure of an amino acid's hydrophobicity: the greater hydrophobicity the darker the shading (the stop codons are shaded black) (Adapted from reference 29).

2.1. Natural Genetic Code Alterations

After the discovery of codon reassignments in human mitochondrial genes [(33)], a variety of other deviations from the standard genetic code in bacteria, archaea, eukaryotic nuclear genomes and, especially, organellar genomes have been reported [(34)].

The mitochondrial genetic code seems to be particularly prone to change (with the exception of plants). For example, in the mitochondria of most organisms, the standard UGA stop codon codes for tryptophan, while the isoleucine-AUA codon codes for methionine [(35)]. In the mitochondrial genomes of platyhelminths and echinoderms, the standard AAA-lys codon has been reassigned to asparagine [(36)]. Among all mitochondrial reassignments, the arginine AGA/G codon pair is particularly interesting because it codes for different amino acids in different organisms. For example, AGA /G codes for termination in vertebrates, glycine in tunicates and serine in arthropods, echinoderms, molluscs, nematodes and platyhelminths [(37)]. Also noteworthy is the mitochondrial genetic code of yeast in which UGA codes for tryptophan, AUA codes for methionine and not isoleucine, and the entire standard leucine-CUN codon family codes for threonine [(38)].

In bacteria, genetic code changes have been found in *Micrococcus*, *Mycoplasma* and *Spiroplasma* [(38)]. These genetic code changes involve reassignment of the UGA stop codon to tryptophan in *Mycoplasma* and *Spiroplasma* and the apparent non-assignment of the CGG arginine codon in *Mycoplasma* [(38)]. In *Micrococcus*, the arginine AGA and the isoleucine AUA codons also appear to be unassigned (they do not appear to code for any amino acid) [(38)].

In eukaryotes, the UAG/A and UGA codons have been reassigned to glutamine and cysteine in ciliates, acetabularia and euplots, respectively [38], while the CUG codon has been reassigned to serine in some species of the genus *Candida* [39, 40].

2.2. The *Candida* spp. genetic code

A unique genetic code change is the reassignment of the CUG codon from leucine to serine in *C. albicans* and several other *Candida* species (41). This alteration to the standard genetic code is mediated through ambiguous codon decoding by a novel tRNA (figure 4), the Ser-tRNA^{CAG}, that is charged with both leucine (~3%) and serine (~97%) [42].

This unique tRNA has identity elements for both the seryl-tRNA synthetase (SerRS) and the leucyl-tRNA synthetase (LeuRS), which allows the charging with both Ser and Leu. The SerRS recognizes the $(GC)_3$ helix of the extra-loop and the discriminator base of the tRNA (G_{73}) while the LeuRS recognizes A_{35} and m^1G_{37} in the anticodon-loop [43]. Also, the novel ser-tRNA has a guanosine at position 33, a position that is almost always occupied by a pyrimidine or, more specifically, a uridine in all others serine tRNAs. U-33 is critical for the correct turn of the phosphate backbone and assembling of the anticodon bases [43]. Despite this fact, studies have shown that G-33 has an important role in the reassignment mechanism because it lowers the decoding efficiency of the ser-tRNA$_{CAG}$ and minimizes mischarging of the ser-tRNA$_{CAG}$ with leucine [34, 43, 44].

rRNA and tRNA molecular phylogeny provides clear evidences that the Ser-tRNA$_{CAG}$ appeared approximately 272 ± 25 million years ago [45]. The Ser-tRNA$_{CAG}$ was able to compete with the natural Leu-tRNA$_{CAG}$ decoder for the CUG codon, thus generating an ambiguous CUG codon. This enabled CUG reassignment through selection of the mutant Ser-tRNA and elimination of the cognate Leu-tRNA. The Ser-tRNA$_{CAG}$ was maintained in the lineage that

originated the genus *Candida*, but was lost in the lineage leading to the genus *Saccharomyces*. This separation occurred 170 million years ago, indicating that the yeast ancestor was ambiguous for at least 100 million years. CUG codon ambiguity imposed strong selection against old CUG codons, which mutated to UUG and UUA codons and this resulted in low CUG codon usage [45]. Currently in the genus *Candida*, some species still have CUG ambiguity (as in the case of *C. albicans*), while others have achieved complete reassignment of the CUG codon (such as *C. cylindracea*) [46-48].

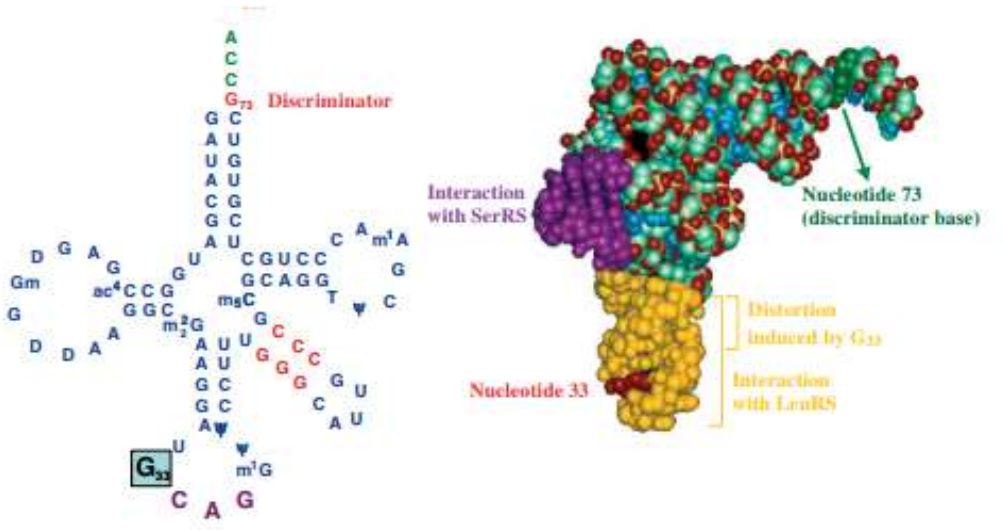


Figure 4 - Secondary structure of the *Candida albicans* $tRNA_{CAG}^{Ser}$. This unique tRNA has identity elements for both the seryl-tRNA synthetase (SerRS) and the leucyl-tRNA synthetase (LeuRS) and it is charged with both Ser and Leu. The SerRS recognizes the $(GC)_3$ helix of the extra-loop and the discriminator base of the tRNA (G_{73}). The LeuRS recognizes A_{35} and m^1G_{37} in the anticodon-loop. The G_{33} distorts the anticodon-arm of the tRNA and lowers the efficiency of leucylation of the tRNA (Adapted from reference 46).

2.3. Mistranslation in *Candida albicans*

Under laboratory conditions, serine and leucine are incorporated into the *C. albicans* proteome with efficiencies of 97% and 3%, respectively, but such CUG ambiguity increases up to ~5% under stress [18]. *C. albicans* tolerates up to 28% leucine misincorporation before mistranslation becomes noticeably detrimental

to growth, suggesting that CUG ambiguity may fluctuate between 3% to 28%, depending on environmental conditions [18].

Remarkably, CUG ambiguity increases secretion of lipases and proteases and cell adhesion and spawns a wide variety colony morphologies that reflect changes in the cell wall influencing fungal recognition by human immune cells [20, 49, 50]. Recently, *C. albicans* recombinant strains were constructed by engineering Ser-tRNAs. This approach resulted in a series of strains mistranslating from 20% up to 99% of leucine at CUG positions. These highly ambiguous strains grew faster than the control in the presence of the oxidative stressors menadione and H_2O_2, in media supplemented with protein misfolding agents (urea and guanidine hydrochloride) and in media containing SDS and $CuSO_4$ [20]. Also, the work from Bezerra and colleagues showed that strains with more leucine misincorporation grow faster than the control when they are in the presence of azoles, which is an indication of the fitness benefit that this alteration conveys [20]. This result is of great importance because the ability of a pathogen with an altered genotype to survive in the presence and/or absence of drug greatly influences the degree to which that genotype will proliferate in the population and the degree to which it can be targeted if the drug regimen is changed [20, 50].

Genome re-sequencing of the recombinant strains showed that increasing CUG ambiguity leads to rapid genome evolution through mutation and loss of heterozygosity (LOH) relative to the control strain of *C. albicans*. There was a near-complete LOH in a 300-kb region on chromosome V and the entire chromosome R in all strains with a level of leucine misincorporation higher than 50%. Although some genes within this region have no known function, some of the known genes are involved in the regulation of biological processes, organelle organization, stress response, filamentous growth, pathogenesis, conjugation and antifungal drug resistance [20]. In fact, LOH in the left arm of chromosome 5 is well described as a mechanism used by *C. albicans* to

increase resistance to fluconazole because it contains TAC1, the transcription factor that control expression of efflux pumps [51].

In the part of the genome not affected by LOH, it was observed an increasing number of heterozygote SNPs for strains with increasing Ser-for-Leu misincorporation. For example, the filamentous growth regulators (FGRs) FGR23, FGR6-4, and FGR6-1 had more than three mutations, and members of the ALS family of cell surface glyco-proteins, namely ALS4 and ALS9, had two nonsynonymous mutations [20, 52]. Since filamentation and adhesion are two of the most important aspects of the host-pathogen interaction, it is crucial to study the relevance of mistranslation to these features.

3. *Candida albicans* biology

3.1. Characteristics

Candida albicans is a dimorphic fungus that exists as a commensal of warm-blooded animals including humans. It colonizes mucosal surfaces of the oral and vaginal cavities and the digestive tract and is also able to cause a variety of infections, depending on the nature of the host defect [53].

One of the most noteworthy features of this organism is its ability to grow with three distinct morphologies: the yeast, pseudohypha and hyphal forms. The unicellular yeast form is an ovoid-shaped budding yeast and the two distinct filamentous forms are elongated ellipsoid cells with constrictions at the septa (pseudohyphae) or parallel-walled true hyphae (Figure 5). *C. albicans* cells are also of two types, namely white and opaque cells, formed during switching and they may also form structures named chlamydospores, which are thick-walled spore-like structures [54, 55].

This pathogen was first described in 1839 as 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 [48, 56, 57]. For many years, it was thought that *C. albicans* was an obligate diploid (2N) but recently tetraploid and haploid cells have been detected [58, 59].

To date, no complete sexual cycle has been observed in *C. albicans*. The described cycle for *C. albicans* is termed the parasexual cycle. In this case, mating of diploid cells is followed by mitosis and concerted chromosome loss instead of meiosis [60]. Diploid cells (2n) of *C. albicans* are typically heterozygous at the mating type locus (MTL) but cells may lose their heterozygosity by loss of one copy of Chromosome 5, eliminating the a or α allele, to create α/α or a/a diploid strains (2n, opaque cells). When diploid a/a and α/α cells mate, they form tetraploid aa/αα cells (4n, white cells) which undergo mitosis and non-meiotic reduction in the number of chromosomes to return to diploid a/α cells (2n, white cells). The parasexual cycle is completed with no recognized meiosis (Figure 6) [60]. Apparently, haploid cells also form via a non-meiotic reduction in chromosome number, similar to that seen in the tetraploid-to-diploid transition [58]. The existence of semi-stable, non-diploid *C. albicans* cell types highlights the flexibility of the *C. albicans* genome [58]. Furthermore, cases of aneuploidy (cells with an abnormal chromosome number) are especially common in fluconazole resistant strains [49].

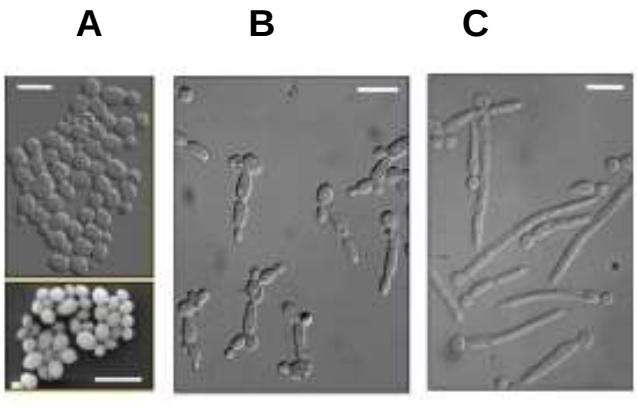


Figure 5 - Distinct morphologies of *C. albicans*, including yeast (A), pseudohyphae (B) and hyphae (C) (Adapted from reference 83).

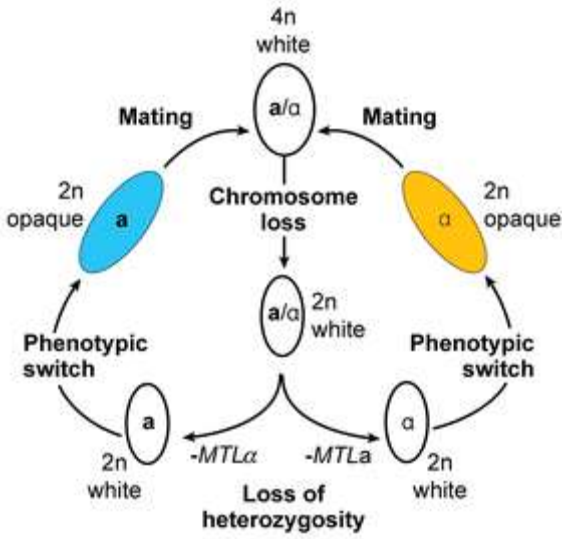


Figure 6 - Parasexual cycle of *C. albicans*. Relatively to the MTL locus of *C. albicans*, diploid cells (2n) are a or α opaque cells, that mate efficiently to create tetraploid (4n) a/a/α/α cells. These cells can lose chromosomes to return to the diploid (2n) state (Adapted from reference 60).

3.2. Epidemiology

C. albicans and a few other *Candida* species live in the oral cavity of up to 75% of the population. This colonization normally remains benign in healthy persons. However, individuals that are immunocompromised can often suffer from persistent infections of the oral cavity, denominated oral candidiasis [(54)]. These are mostly caused by *C. albicans* and can affect the oropharynx and the esophagus of individuals with dysfunctions of the immune system. The major risk factors for developing oral candidiasis are HIV and the wearing of dentures and extremes of age [(61)].

Studies estimated that approximately 75% of all women develop at least once in their lifetime vulvovaginal candidiasis, with 40–50% suffering at least one additional episode of infection. A small percentage of women (5–8%) suffer from at least four recurrent episodes per year. Predisposing factors for this type of infection are diabetes mellitus, the prolonged use of antibiotics, oral contraception, pregnancy and hormone therapy [(61)].

Superficial *C. albicans* infections are non-lethal, but on the other hand, systemic candidiasis is associated with a high mortality rate, even with first line antifungal therapy. Risk factors leading to disseminated systemic candidiasis comprise neutropenia and damage of the gastrointestinal mucosa. Others risk factors include the use of central venous catheters, which allow direct access of the fungus to the bloodstream, the application of broad-spectrum antifungals, which enable fungal overgrowth, and gastrointestinal surgery, which disrupts mucosal barriers [(54)].

3.3. Pathogenicity

The notable capacity of *C. albicans* to infect diverse host niches is sustained by a wide range of virulence factors and fitness attributes. Among the most important are the morphological transition between yeast and hyphal forms, the expression of adhesins on the cell surface, thigmotropism, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes. Furthermore, fitness attributes include rapid adaptation to fluctuations in environmental pH, metabolic flexibility and strong stress response machineries (Figure 7) [(54)].

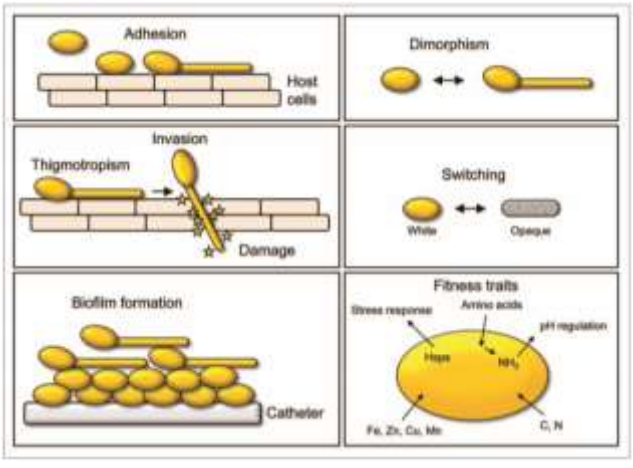


Figure 7 - *C. albicans* pathogenic mechanisms. Cells adhere to the host cell surface by the expression of adhesins. Contact to host cells triggers the yeast-to-hypha transition and directed growth via thigmotropism. Adhesion, physical forces and secretion of fungal hydrolases facilitate the mechanism of invasion. Switching has been proposed to influence antigenicity and biofilm formation of *C. albicans*. (Adapted from reference 54).

3.3.1. Polymorphism

Several environmental signals can affect the morphology of *C. albicans* cells. For example, at low pH (< 6) *C. albicans* cells predominantly grow in the yeast form, while at a high pH (> 7) hyphal growth is induced [62]. In fact, a number of other conditions, including starvation, the presence of serum or N-acetylglucosamine, temperature and levels of CO_2 stimulate the formation of hyphae [63].

Morphogenesis has also been shown to be regulated by quorum sensing, a mechanism used by *C. albicans* cells for communication. Due to quorum sensing, high cell densities (> 10^7 cells ml^{-1}) produce farnesol that signals yeast growth, while low cell densities (< 10^7 cells ml^{-1}) lead to hyphal formation [64].

The transition between yeast and hyphal growth forms is termed dimorphism and it has been proposed that both growth forms are important for pathogenicity. The hyphal form has been shown to be more invasive than the yeast form, but the smaller yeast form is believed to represent the form primarily involved in dissemination [54, 65, 66].

3.3.2. Adhesins

C. albicans has a specialized set of proteins (adhesins) that mediate adherence to other *C. albicans* cells, to abiotic surfaces and to host cells [54]. The best studied *C. albicans* adhesins are the agglutinin-like sequence (ALS) proteins. The *ALS* genes encode glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins and of the eight Als proteins, the hypha-associated adhesin Als3 is required for viable hypha formation and it particularly important for adhesion [67]. *ALS3* gene expression is upregulated during infection of oral epithelial cells *in vitro* and during *in vivo* vaginal infection [68]. Another important adhesin of *C. albicans* is Hwp1, which is localized on the cell surface as a hypha-associated GPI-linked protein [54]. Interestingly, these *ALS* and HWP1 genes are rich in

CUG codons and misincorporation of leucine at these codons may strongly contribute to increased adhesion [50].

Morphology-independent proteins can also contribute to adhesion. These include GPI-linked proteins such as Eap1, Iff4 and Ecm33, non-covalent wall-associated proteins Mp65 (a putative β-glucanase) and Phr1 (a β-1,3 glucanosyl transferase), cell-surface associated proteases (Sap9 and Sap10) and the integrin-like surface protein Int1 [69].

3.3.3. Biofilm

An additional important virulence factor of *C. albicans* is its capability to form biofilms on abiotic or biotic surfaces. Catheters, dentures (abiotic) and mucosal cell surfaces (biotic) are the most common substrates. Biofilms form in a chronological process including adherence of yeast cells to the substrate, followed by proliferation of these yeast cells and formation of hyphae in the upper part of the biofilm. Finally, there is accumulation of extracellular matrix material and dispersion of yeast cells from the biofilm complex [54].

Several transcription factors control biofilm formation and these include Bcr1, Tec1 and Efg1 [70]. For the dispersion phase in the *C. albicans* biofilm formation process, it was recently identified a key regulator, the major heat shock protein Hsp90, which is also required for antifungal drug resistance [71].

3.3.4. Contact sensing and thismotropism

An important environmental factor that triggers hypha and biofilm formation in *C. albicans* is contact sensing. After the contact with a surface, such as agar or mucosal surfaces, yeast cells switch to the hyphal form and can then invade into the substratum [72]. Likewise, contact to different solid surfaces induces the formation of biofilms. For example, on surfaces with particular topologies (such as the presence of ridges) directional hyphal growth (thigmotropism) may occur

[54]. Recently, studies demonstrated that thigmotropism of *C. albicans* hyphae is regulated by extracellular calcium uptake through the calcium channels Cch1, Mid1 and Fig1 [73].

3.3.5. Secreted hydrolases

Following the adhesion to host cell surfaces and hyphal growth, *C. albicans* cells can secrete hydrolases. These are thought to facilitate active penetration into the host cells [74]. Also, secreted hydrolases are thought to increase the efficiency of acquisition of extracellular nutrients [75]. Three different classes of secreted hydrolases are expressed by *C.albicans*: proteases, phospholipases and lipases [54].

4. Antifungal drugs and resistance

4.1. Mechanisms of action of antifungal drugs

Treatment of *Candida albicans* infections is restricted to the classes and number of antifungal drugs available. The major classes of currently available antifungal drugs are the azoles, polyenes and echinocandins (Figure 9) [76].

The azoles affect the biosynthesis of ergosterol that constitutes the major sterol of the *C. albicans* plasma membrane. The azoles mechanism of action consists of inhibiting the enzyme sterol 14-α-demethylase (14DM), a cytochrome P-450 enzyme [77] encoded by ERG11 gene. This is a key enzyme in the ergosterol biosynthesis pathway because it catalyzes the oxidative removal of the 14-α-methyl group from lanosterol. Azoles enter the cell by facilitated diffusion and then bind to the heme group located in the active site of 14DM. The interference with the active site of the enzyme causes a block in the production of ergosterol and the accumulation of a toxic sterol produced by an enzyme encoded by *ERG3* [78, 79]. This toxic sterol exerts severe membrane stress on the cell, which can lead to its death (Figure 8A).

The triazole fluconazole is the most widely used drug to treat *Candida* infections because its target has no homologues with similar functions in humans [80]. The downside of this drug is that it acts in a fungistatic rather than fungicidal manner. Consequently, there is not the complete eradication of the fungi in treatment of oropharyngeal candidiasis [80]. Also, prolonged and repeated treatment of candidiasis with azoles has resulted in an increasing frequency of therapy failures caused by the development of fluconazole-resistant *C. albicans* strains [81].

Unlike azoles, polyenes are fungicidal drugs with both hydrophobic and hydrophilic sides. They strongly bind to ergosterol in order to produce drug-lipid complexes, which insert into the fungal cell membrane to form a membrane-spanning channel [82]. This allows some ions, namely potassium, to leave the cell, which destroys the proton gradient (Figure 8B) [82].
The polyene amphotericin B is effective against systemic fungal disease and has *in vitro* and *in vivo* activities against several *Candida* species as well as *C. neoformans* and *Aspergillus* species [83]. The main limitation of their use is host toxicity, probably as a consequence of the structural similarities between ergosterol and cholesterol in the mammalian cell membrane [83, 84].

The most recent drugs to be used are the echinocandins caspofungin, micafungin and anidulafungin. The echinocandins are large lipopeptides that act as non-competitive inhibitors of the (1,3)-β-D-glucan synthase, an enzyme involved in fungal cell wall synthesis [85]. The inhibition of this enzyme results in the loss of cell wall integrity, which causes severe cell wall stress on the fungal cell (Figure 8C). The activity of the echinocandins is generally fungicidal against yeast but fungistatic against fungus. For example, it has been reported a notable antifungal activity against *Candida* and *Aspergillus* but the echinocandins show no antifungal activity against *C. neoformans* [83, 84].

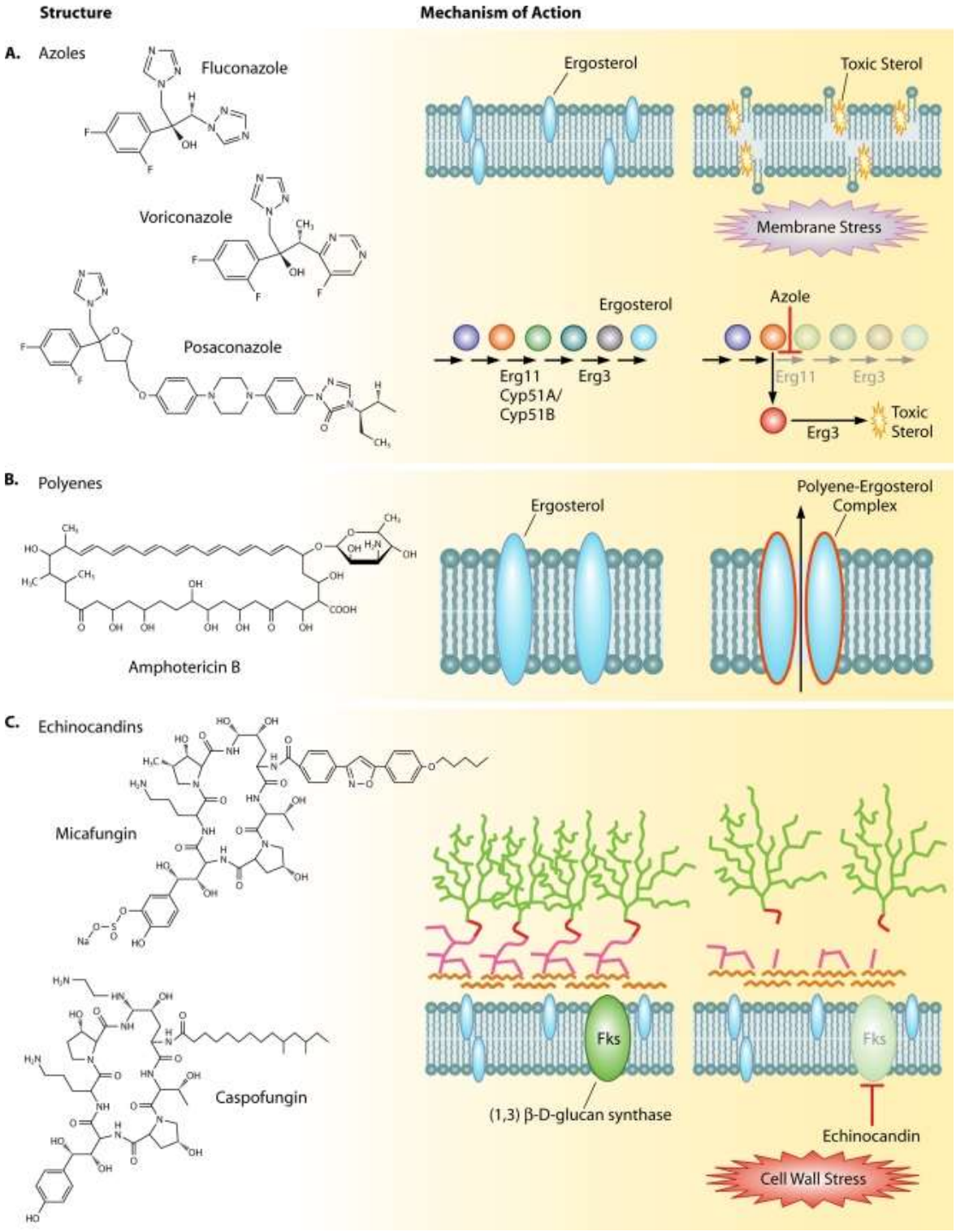


Figure 8 - Mechanisms of action of antifungical drugs. (A) The azoles function by targeting the ergosterol biosynthetic enzyme lanosterol demethylase, encoded by ERG11, causing the accumulation of a toxic sterol. (B) The polyenes are amphipathic drugs that function by binding to ergosterol to create drug-lipid complexes, to form a membrane-spanning channel, causing an osmotic cellular lysis. (C) The echinocandins act as noncompetitive inhibitors of (1,3)-β-D-glucan synthase, encoded by FKS1, causing a loss of cell wall integrity. (Adapted from reference 83)

4.2. Mechanisms of drug resistance

C. albicans can exploit several cellular stress responses to tolerate exposure to the azoles and can also acquire high levels of resistance by multiple distinct mechanisms. These mechanisms are based in the alteration of the target enzyme, up-regulation of multidrug transporters and cellular stress responses (Figure 9) [20, 83, 84, 86].

The alteration of the target enzyme, Erg11, is one mechanism of triazole resistance. At least 12 mutations in Erg11 have been associated with triazole resistance because the majority of these mutations alter the target, which hinders binding of the azole to its target [87-89]. Upregulation of *ERG11* has also been associated with azole resistance [90]. An intriguing mechanism involving genomic alterations that amplify the copy number of *ERG11* was recently identified in azole-resistant strains [91, 92]. These alterations include mitotic recombination, gene conversion, and the formation of isochromosome 5 that confer resistance by increasing the gene dosage of *ERG11* and the gene encoding the transcription factor Tac1 that regulates the expression of multidrug transporters [51, 91, 92].

The second mechanism of azole resistance is the upregulation of multidrug transporters. For example, constitutive upregulation of Mdr1, which is a transporter that belongs to the major facilitator superfamily, confers resistance to fluconazole [93]. Several studies also discovered the identity of the key transcription factor regulating the multidrug transporter (MRR1). They also revealed the associated mutations causing hyper-activation of this transcription factor leading to constitutive *MDR1* overexpression [94]. Constitutive upregulation of the members of the *A*TP-*b*inding *c*assette (ABC) transporter superfamily CDR1 and CDR2 is the most-frequently encountered azole resistance mechanism in clinical isolates [93]. Multiple regulatory elements have been identified along with the key transcription factor regulating the expression of these transporters, TAC1 [95, 96].

In addition to the two mechanisms that minimize the impact of the drug on the cell, other mechanisms are related to cellular stress responses [84]. One well-characterized mechanism that falls into this category involves the loss of function of Erg3 in the ergosterol biosynthetic pathway. This blocks the accumulation of the toxic sterol intermediates when Erg11 is inhibited by the azoles [78, 79]. Some resistance mechanisms have a dual role by minimizing the impact of the drug on the cell and enabling the cell to cope with stress. For example, the transcription factors MRR1 and TAC1 regulate targets in addition to the efflux pumps mentioned above. MRR1 regulates oxidoreductases, which may help prevent drug-induced cell damage resulting from the generation of toxic molecules [97]. TAC1 also regulates a glutathione peroxidase, a sphingosine kinase and a phospholipid flippase, which have important roles in oxidative stress response and lipid metabolism [96].

Calcineurin is part of another pathway mediating crucial responses to antifungal drugs [98, 99]. This molecule mediates essential responses to diverse stresses, including exposure to serum [100], alkaline pH [101] and agents inducing cell membrane stress [102]. One downstream effector of calcineurin is the transcription factor Crz1 that has been associated with azole resistance in *C. albicans* by several recent studies [103]. Crz1 is dephosphorylated by calcineurin. Once it is dephosphorylated, Crz1 translocates to the nucleus where it activates the expression of a set of genes involved in signalling pathways, ion/small-molecule transport, vesicular trafficking and particularly cell wall integrity [104].

Another regulator of cellular signalling that plays a key role in azole resistance is the molecular chaperone Hsp90 [105, 106]. Hsp90 interacts with the catalytic subunit of calcineurin and it is required for its function [107]. In fact, inhibition of Hsp90 with the metabolites geldanamycin and radicicol (they bind to the ATP binding pocket of Hsp90 [108]) prevents the rapid emergence of drug resistance in *C. albicans* in a laboratory experiment [109]. Hsp90 inhibitors also reduce the resistance of Erg3 loss-of-function mutants and clinical isolates that have acquired resistance by multiple mechanisms [109, 110].

The stress responses that mediate azole resistance also include the conserved cyclic AMP (cAMP)-protein kinase A (PKA) signaling pathway [111, 112]. The production of cAMP is regulated by adenylate cyclase and cyclase-associated proteins and the deletion of these proteins in *C. albicans* confers hypersensitivity to azoles that is in part reversed by the addition of cAMP to the medium [113].

Finally, an interesting way in which signalling pathways can mediate azole resistance is the formation of fungal biofilms [114, 115]. The mechanisms of azole resistance in *C. albicans* biofilms are probably multifactorial, including upregulation of multidrug transporters, reduced drug diffusion, reduced growth rate and alterations of the plasma membrane and cell wall [114]. It is now clear that multiple signalling pathways are a prerequisite to sense environmental cues and activate cascades of events that eventually leads to biofilm-mediated azole resistance [84].

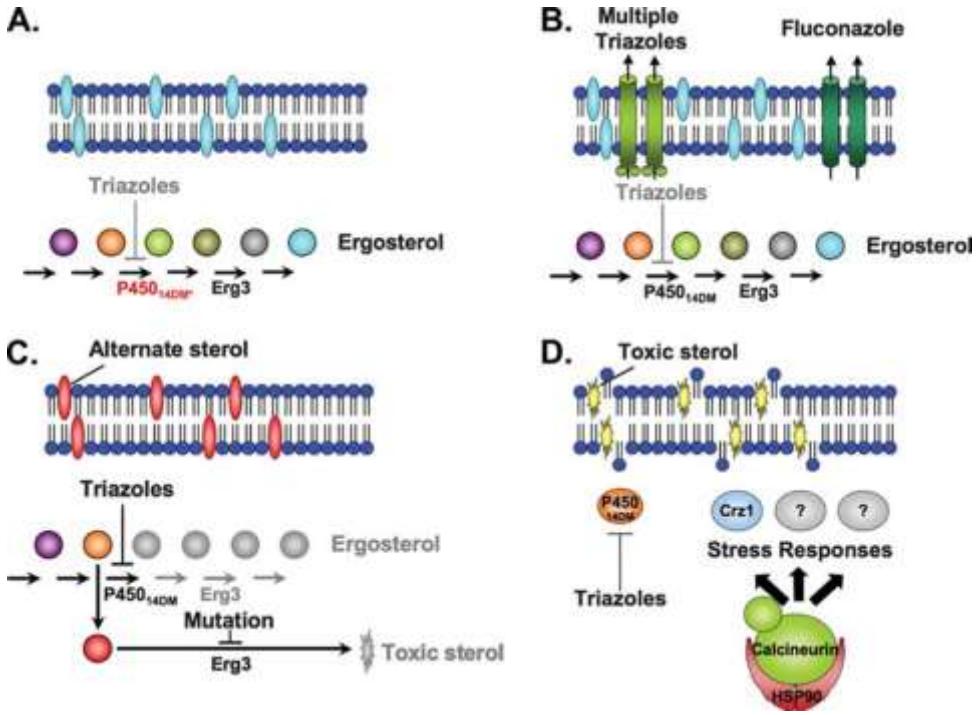


Figure 9 - *C.albicans* azole resistance mechanisms. (A) Mutation or upregulation of the target of the azoles, cytochrome $P450_{14DM}$ (also known as lanosterol 14α-demethylase). **(B)** Constitutive upregulation members of the ABC transporter family of multidrug efflux pumps and upregulation of a major facilitat transporter. **(C)** Loss-of-function mutation of Erg3. **(D)** The molecular chaperone Hsp90 stabiliz calcineurin, thereby enabling calcineurin-dependent stress responses that are required for triazole toleranc Multiple downstream effectors of calcineurin mediate cellular responses to azoles, including t transcription factor Crz1 (Adapted from reference 84).

Relatively to polyenes, the main problem is the severe host toxicity rather than drug resistance [116]. Cases of resistance to amphotericin B have been reported in *C. albicans* but the majority of amphotericin B resistance is related with reduced ergosterol content in the cell membrane. In most cases this is caused by defective Erg3 function (Figure 10) [117]. Resistance to polyenes was also observed for patients previously treated with azoles because long-term fluconazole therapy causes defects in the C-5,6-desaturase, which blocks the synthesis of ergosterol and leads to the accumulation of an alternate sterol in the membrane [118].

The relation between amphotericin B resistance and cellular signaling is poorly understood. This may be related to the fact that amphotericin B is fungicidal against *Candida* species and causes leakage of cytoplasmic contents [119]. Until now, both key regulators of stress responses, Hsp90 and calcineurin, have not been directly implicated in resistance to amphotericin B [120].
Similar to what happens to the azoles, *C. albicans* biofilms show resistance to treatment with polyenes and this has been attributed to the presence of persister cells within the population [121].

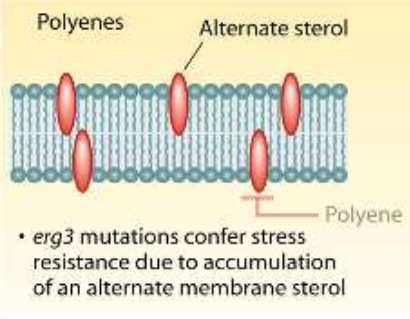


Figure 10 – Mechanism of resistance to polyenes. Reduced ergosterol content due to the mutations in in Erg3 leading to the accumulation of an alternated sterol in membrane (Adapted from reference 83).

The clinical use of echinocandins is fairly recent, but cases of resistance to this treatment have also been reported [122]. The target of the echinocandins is the (1,3)-β-d-glucan synthase, that is a complex enzyme composed of two subunits, Fks1 and Rho1 (Figure 11). The catalytic subunit is encoded by *FKS1* and the regulatory subunit is encoded by *RHO1* [123, 124]. Usually, this type of resistance is mediated by mutations in the *FSK1* gene, particularly in two regions, the Hotspot 1 (HS1) and Hotspot 2 (HS2). The region around Ser645 (within HS1) is considered to be the major contributor for echinocandins resistance [125, 126].

Unlike the azoles, multidrug efflux transporters play a small role in echinocandin resistance. Studies showed that the overexpression of the ABC transporter Cdr2 confers a moderate increase in caspofungin resistance [127].

On the other hand, there is a relation between cellular stress responses and the ability to survive to the stress employed by the echinocandins. The inhibition of the synthesis of (1,3)-β-d-glucan by echinocandins causes loss of cell wall integrity and induces severe cell wall stress [126]. Rho1, that is a positive regulator of (1,3)-β-d-glucan synthase, also coordinates the protein kinase C (PKC) cell wall integrity signaling pathway that is responsible for remodeling the cell wall [128, 129]. Recent works also demonstrate the involvement of calcineurin signaling in echinocandin resistance. The calcineurin-dependent transcription factor Crz1 is required for activation of the expression of several chitin synthases in response to stress conditions [97]. The upregulation of this enzyme elevates chitin content in the cell wall and make cells less susceptible [130].

The biofilm cellular state does not seem to confer resistance to echinocandins [114]. When *C. albicans* cells grow under high concentration of echinocandins, the clinical isolates grow better compared with planktonic counterparts. This can be related to increased (1,3)-β-d-glucan content in *C. albicans* cell walls from biofilms compared to the (1,3)-β-d-glucan content in planktonic cells [131].

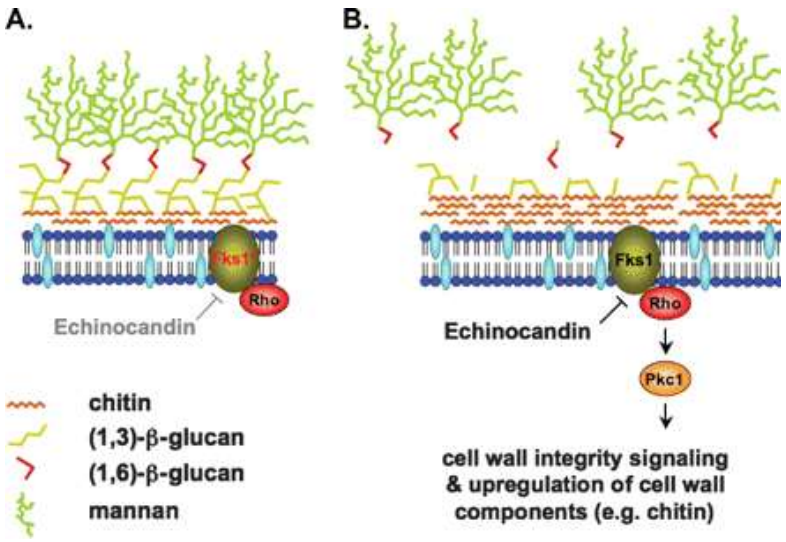


Figure 11 – C. *albicans* echonocandin resistance. Mutations in the (1,3)-β-d-glucan synthase subu encoded by FSK1 confers resistance by minimizing the impact of the drug on the cell **(A)**. Rho1 is a positi regulator of glucan synthase and contributes to tolerance through stress responses **(B)** (Adapted frc reference 84).

5. Objectives

Pathogenic yeasts from the genus *Candida* can cause serious infections in humans, particularly in immunocompromised patients. In recent years, resistance to frequently administered antifungal drugs is increasing [(132)].

Phenotypic variability and genomic plasticity are considered driving forces during evolution of drug resistance [(133)] and considering the high phenotypically diversity in ambiguous *C. albicans* strains we asked if tRNA mistranslation in this pathogen favours adaptation under antifungal drug treatment. Fluconazole was chosen for this project because it is the most widely used antifungal in clinics nowadays. The specific objectives addressed in this thesis were the following:

1. Analyze the effect of mistranslation on the acquisition of resistance to fluconazole.

2. Measure mistranslation levels during long-term evolution of drug resistance.

3. Identify molecular determinants by which resistance evolves in mistranslating strains.

II. Material and Methods

1. Strains and growth conditions

Candida albicans SN148 (arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434iro1Δ::imm434/iro1Δ::imm434) [(134)] was grown at 30°C in YPD (2% glucose; 1% yeast extract, and 1% peptone). *C. albicans* strains T0, T1 and T2 were grown in minimal medium lacking uridine (0.67% yeast nitrogen base without amino acids, 2% glucose, 2% agar and 100 µg/ml of the required amino acids). Cells were grown aerobically at 30°C unless indicated otherwise. All *C. albicans* strains used in this study are listed in Table 1 below.

Table 1- *C. albicans* strains used in this study.

Strains	Genotype	% mistranslation
T0	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 RPS1/rps1Δ::pUA709 (URA3)	1.45
T1	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 RPS1/rps1Δ::pUA702 (URA3, Sc tLCAG)	20.61
T2	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 RPS1/rps1Δ::pUA706 (URA3, Sc tLCAG, Sc tLCAG)	67.29

2. Experimental evolution with fluconazole

9 clones of each of the ambiguous strains T0, T1 and T2 were inoculated in RPMI 1x media (Sigma) in a 96-well format plate and incubated at 37ºC (Figure 12). An aliquot of stationary phase cultures was transferred to fresh media every 2 days using a multichannel pipette. Each passage the fluconazole

(Sigma Aldrich) concentration of the liquid growth medium was adjusted to a concentration corresponding to twice the last measured MIC (minimum inhibitory concentration). Passages were pursued until clones were growing in liquid media containing a fluconazole concentration of 256 µg/ml. The same experimental evolution approach was used for approximately 200 generations in liquid media without the drug. In both situations, all clones from each passage were frozen with 40% glycerol.

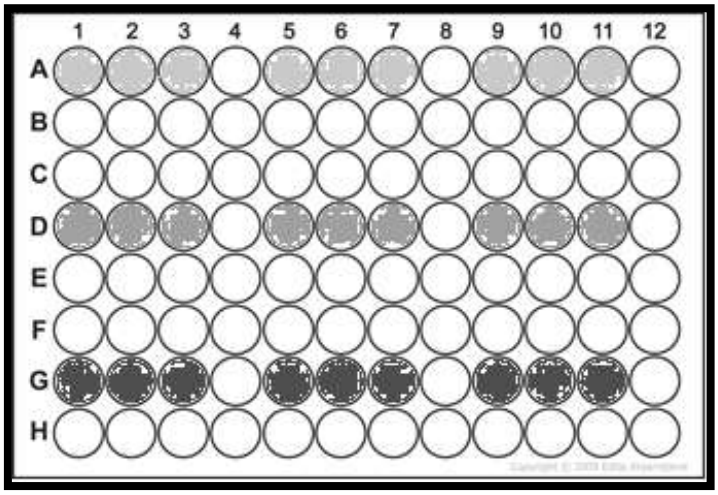


Figure 12 - 96-well plate used in evolution with fluconazole. The A correspond to the T0 strain, the D correspond to T1 strain and G correspond to the T2 strain. The 1, 2, 3, 5, 6, 7, 9, 10, 11 spots correspond to the nine different clones of each strain that were growing in liquid media containing fluconazole at twice the last measured MIC. The A4, A8, A12**,** D4, D8, D12, G4, G8, G12 correspond to controls spots containing only RMPI media.

3. Determination of broth dilution MICs

Resistance to antifungal drugs was monitored at each passage using the European Committee on Antimicrobial Susceptibility Testing (EUCAST protocol) [(135)] with alterations, as described below. A 96-well plate was prepared with a broth dilution series of fluconazole (0.125 – 256 µg/ml range) and control wells without drug (Figure 13). The plate was inoculated with a standardised number of cells from each clone of each strain ($2{,}5x10^4$ cells counted using the TC10^{tm}

Automated cell Counter from BioRad). The microdilution plate was incubated without agitation at 37ºC for 24 ± 2 h. After this period the absorbance of the plate at 600 nm was recorded using the iMARK^{tm} Microplate Reader. Failure to reach an absorbance of 0.2 after 48 h constituted a failed test. The value of the MIC for each passage was the lowest fluconazole concentration giving rise to an inhibition of growth of ±50% of that of the drug free control.

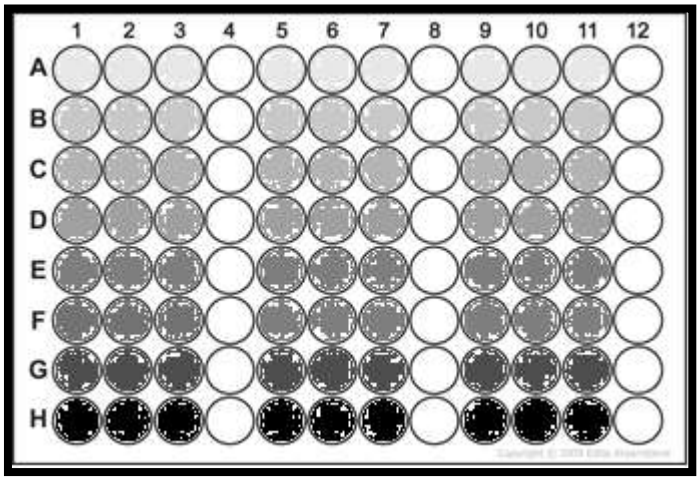


Figure 13 - 96-well plate used to determine broth MICs. The 1, 2, 3 spots were inoculated with one clone of the T0 strain; 5, 6, 7 with one clone of the T1 strain and 9, 10, 11 with to one clone of the T2 strain. The 4, 8, 12 spots correspond to controls containing media without drug. A, B, C, D, E, F, G, H correspond to different and crescent fluconazole.concentrations.

4. Quantification of leucine misincorporation

The levels of mistranslation during the evolution were assessed using a reporter system based on the yeast enhanced fluorescent protein (yEGFP). This protein permits quantification of mistranslation by fluorescence microscopy using a method described by Bezerra and colleagues [20]. The reporter is based on a "gain of function" mutation at the yEGFP gene. The reporter has no activity when faithfully translated but if the reporter is expressed in cells mistranslating the mutated codon, a small portion of the reporter molecules are functional. The

degree of activity detected (fluorescence) when compared to the wild-type functional protein allows the quantification of the mistranslation rate.

*yE*GFP expression was visualized in *C. albicans* cells at the beginning, middle and final points of the experimental evolution. Clones were grown overnight in liquid medium at an OD_{600} of 2.0-2.5 and aliquots were spotted onto microscope slides. Fluorescence was detected using a Zeiss MC80 Axioplan 2 light microscope, equipped for epifuorescence microscopy with the filter set HE38. Photographs were taken using an AxioCam HRc camera and images were analysed using ImageJ software. Mean fluorescence intensities (± standard deviation) were quantified in individual *C. albicans* cells containing the reporter *yE*GFP Leu-UUA_{201} (positive control), Ser-UCU_{201} (negative control) and Ser/Leu-CUG_{201} (reporter). yEGFP fluorescence (intensity/pixel) was determined for at least 1000 cells in each case.

5. DNA extraction

The DNA extraction was performed for each clone of all strains. Genomic DNA extraction was carried out using an adaptation of the protocol described by Hoffman [(136)]. 1mL of culture (OD_{600nm} between 0.5 and 1.0) was harvested by centrifugation. After centrifugation, cells were resuspended in 0.1 ml of Solution I (1M sorbitol, 0.1M Na_2EDTA, pH±7.5). 5 µl of a 10 mg/ml solution of lyticase 100T was added and the suspension was incubated for 1 hour at 37ºC.

After incubation, the mixture was centrifuged in a microfuge for 1 minute a 10 000 rpm. The supernatant was discarded and cells were resuspended in 0.1 ml of solution II (50 mM Tris-Cl (pH±7.4), 20 mM Na_2EDTA). After, 0.001 ml of 10% SDS was added and stirred well. The mixture was incubated for 30 minutes at 65ºC. 0.004 ml of 5M potassium acetate was added and the microfuge tube was placed in ice for 1 hour. After the incubation, the suspension was centrifuged in a microfuge for 5 minutes. The supernatant was transferred to a fresh microfuge tube and2V of 100% ethanol kept at -30ºC was added along with 0.1V of 5M NaCl. The mixture was precipitated for 2 hours at -

30ºC. After precipitation, the mixture was centrifuged in microfuge for 10 minutes. The supernatant was pour off and the pellet air-dried. The pellet was then ressuspended in 0.3 ml of H_2O miliQ and the sample was stored at -30ºC.

6. PCR

Amplification of the RPS10 locus fragment from each clone was performed according to current polymerase chain reaction protocols. In an eppendorf, 1 µl of DNA sample (± 100ng/ml) was added to 14 µl of the PCR mix. The mix for each reaction was composed of 1.5 µl Dream Taq Buffer 10x, 0.3 µl 10mM dNTP's, 0.3 µl of 10 mM oUA2058 primer, 0.3 µl of 10 mM primer oUA1554, 0.075 µl of Dream Taq 5U / µl and 11.525 µl of H_2O miliQ.

Amplification of ERG11 from each clone was performed according to current polymerase chain reaction protocols. In an eppendorf, 1 µl of DNA sample (± 100ng/ml) was added to 14 µl of the PCR mix. The mix for each reaction was composed of 1.5 µl Dream Taq Buffer 10x, 0.3 µl 10mM dNTP's, 0.3 µl of 10 mM OUA2060 primer, 0.3 µl of 10 mM primer OUA2061, 10 mM OUA2062 primer, 10 mM OUA2063 primer, 10 mM OUA2064 primer, 10 mM OUA2065 primer, 0.075 µl of Dream Taq 5U / µl and 11.525 µl of H_2O miliQ. All primers used are listed in Table 2 below.
PCRs were performed in the My Cicler^{TM} thermal-cycler (BIO RAD). An initial step of denaturation has lasted 3 minutes at 95 ° C. The heat-denaturation step has lasted 30 seconds at 95ºC, the annealing step has lasted 30 seconds at 59ºC, and the extension step has lasted 1 minute at 72°C. The second, third and fourth steps were performed for 30-35 cycles. The last step was one cycle of 5 minute at 72ºC. The PCR product was subjected to a gel electrophoresis for quality check.

Table 2 - Sequence of primmers used.

Primmer	Sequence
OUA1554	5'-CGTATTCACTTAATCCCACAC-3'
OUA2058	3'-CGGATAACAATTTCACACA-5'
OUA2060	5'-GACAAAGAAAGGGAATTCAATC-3'
OUA2061	3'-CCAAATAAAGATCTTGAAGCAG-5'
OUA2062	5'-TGCCAATGTTATGAAAACTC-3'
OUA2063	3'-GAGAAACTAAAACATAATGAC-5'
OUA2064	5'-ATCAACCGATTCCGCCTCAC-3'
OUA2065	3′-ATCAACCGATTCCGCCTCAC-5′

7. Purification of DNA

After DNA extraction and amplification, the RPS10 fragment was purified using the Qiagen PCR Purification Kit. 5 volumes of Buffer PB and 10 µl sodium acetate were added to each sample. The mixture was then passed through a column of the kit and centrifuged up to 1 minute at maximum speed. The column was washed with 750 µl of Buffer PE, and centrifuged up to 1 minute at maximum speed. The column was centrifuged once again to remove residues. The column was placed in an eppendorf and 35 µl of H_2O milliQ was added, and the column was centrifuged for 1 minute at maximum speed. The remaining DNA was quantified using the NanoDrop equipment.

Approximately 2µg of DNA was sent to StabVida for sequencing using the Sanger method.

III. Results

1. Analyze the effect of mistranslation on the acquisition of resistance to fluconazole.

Candida albicans is one of the leading causes of hospital-acquired bloodstream infections [137, 138]. Resistance to frequently administered antifungal drugs is increasing, particularly fluconazole that is the most widely used antifungal in clinics nowadays [49]. These facts highlight the importance of understanding the mechanisms behind the acquisition of resistance [83].

Candida pathogens are unique in their natural ability to mistranslate at high level. The CUG codon is decoded as both leucine (3%) and serine (97%) due to a single tRNA species ($tRNA_{CAG}^{Ser}$) that is the substrate for two tRNA synthetases, namely the seryl and leucyl-tRNA synthetases [42, 44]. Exposure of *C. albicans* to oxidative, pH stress and antifungals increases Leu misincorporation levels from 3% to 15%, suggesting that mistranslation may be part of the pathogen response to immune cells and antifungals [18]. The ability of this pathogen to tolerate high levels of mistranslation was further demonstrated when Bezerra and colleagues constructed a series of recombinant strains that were able to incorporate from 20% up to 99% of leucine at CUG positions [20]. These strains were constructed by engineering Ser-tRNAs that were able to decode the CUG codon and expressing them in *C. albicans* SN148 strain. This manipulation revealed that the high misincorporating strains, namely T2 with 67% of ambiguity at CUGs, displayed remarkable phenotypic diversity, highly variable colony and cell morphologies and increased tolerance to antifungals [20].

These results raised the question of whether tRNA mistranslation could favour adaptation and subsequent acquisition of resistance under antifungal drug treatment. Therefore, in order to analyze the effect of mistranslation on the

acquisition of fluconazole resistance, we measured adaptation to inhibitory concentrations of the drug in replicated experimental populations founded from three drug-sensitive strains of *C. albicans* and reared over approximately 200 generations. We took advantage of the mistranslating strains constructed by Bezerra and colleagues, specifically strains T0, T1 and T2 that are characterized by different levels of leucine misincorporation at serine CUGs: 1.5%, 20% and 67%, respectively [20]. The experimental evolution protocol that we used to investigate the emergence of drug resistance in *C. albicans* was similar to that used in studies of adaptation in large populations of bacteria [139, 140] and other fungi [141].

Nine clones of each of the strains T0, T1 and T2 were grown in liquid media with fluconazole, and nine other clones were propagated without drug. Clones were inoculated in RPMI media in a 96-well format plate. Each passage, the fluconazole concentration of the liquid growth medium was adjusted to a concentration corresponding to twice the last measured MIC. Passages were pursued until clones were growing in liquid media containing a fluconazole concentration of 256 µg/ml. Resistance to antifungal drugs was monitored at each passage using the European Committee on Antimicrobial Susceptibility Testing (EUCAST protocol) [135]. The value of the MIC for each passage was the lowest fluconazole concentration giving rise to an inhibition of growth of ±50% compared to the growth in the drug free control.

Results showed that in the absence of fluconazole, every clone of each strain (T0, T1 and T2) preserved the sensitivity levels throughout evolution (MIC~ 0.125 µg/ml) (Figure 14). This result suggests that mistranslation by itself (without the pressure factor of the presence of the drug) does not lead to acquisition of resistance to fluconazole.

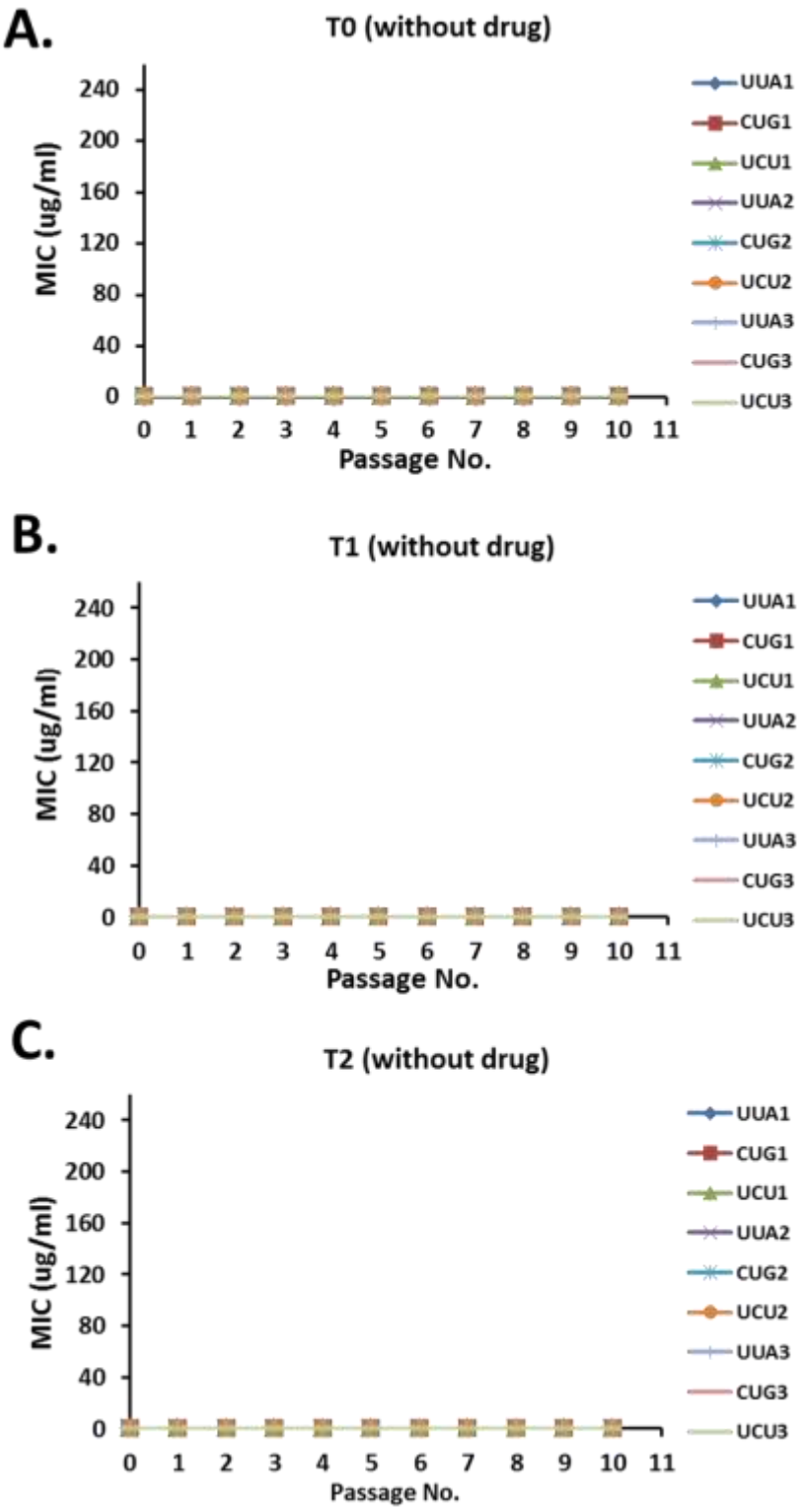

A.
T0 (without drug)
MIC (ug/ml)
240
200
160
120
80
40
0
0 1 2 3 4 5 6 7 8 9 10 11
Passage No.
UUA1
CUG1
UCU1
UUA2
CUG2
UCU2
UUA3
CUG3
UCU3
B.
T1 (without drug)
MIC (ug/ml)
240
200
160
120
80
40
0
0 1 2 3 4 5 6 7 8 9 10 11
Passage No.
UUA1
CUG1
UCU1
UUA2
CUG2
UCU2
UUA3
CUG3
UCU3
C.
T2 (without drug)
MIC (ug/ml)
240
200
160
120
80
40
0
0 1 2 3 4 5 6 7 8 9 10 11
Passage No.
UUA1
CUG1
UCU1
UUA2
CUG2
UCU2
UUA3
CUG3
UCU3

Figure 14 - Experimental evolution of 9 clones of each strain T0, T1 and T2 without fluconazole. Cultures were grown at 37ºC in RPMI media. The MIC values were determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST protocol) and revealed the lowest fluconazole concentration giving rise to an inhibition of growth of ±50%. The MIC values for strains T0 (A), T1 (B) and T2 (C) are the same during the 10 passages (MIC~0.125µg/ml).

The results of the experimental evolution with fluconazole showed that among the 9 clones of strain T0, two clones (UCU2 and UUA2) achieved the highest level of fluconazole resistance (MIC~256 µg/ml) and retained this level to generation 200 (Figure 16A). One population (UCU1) achieved an intermediate level of resistance (MIC~ 32 µg/ml) and then showed a decrease (MIC~ 4 µg/ml). The remaining evolved populations kept their MIC at levels between 0.5 µg/ml and 8 µg/ml (Figure 15A).

Among the 9 clones of the strain T1, two clones achieved the highest level of fluconazole resistance (MIC~ 256 µg/ml) but one of the clones did not maintain the resistance. In the following passage, it immediately decreased its MIC to 120 µg/ml (Figure 16B). Clone UUA1 obtained a final MIC of 32 µg/ml but the majority of the evolved populations achieved a MIC of 16 µg/ml and retained this level until generation 200 (Figure 15B).

Finally, among the 9 clones of strain T2, five clones achieved the highest level of fluconazole resistance (MIC~ 256µg/ml) and all of them retained this level until generation 200 (Figure 15C). One population (UCU1) achieved an intermediate level of resistance (MIC~ 32 µg/ml) and then showed a decrease (MIC~ 2 µg/ml). The remaining evolved populations kept their MIC at 2 µg/ml (Figure 15C).

Thus, strains with more ambiguity, T1 and T2, obtained higher levels of resistance in relation to T0 strain. Moreover, the T2 strain acquired more resistance, quicker and more stable compared to clones of the strains T0 and T1 (Figure 15). Also, it is noteworthy that in passage 5 occurred the breakpoint

for the three strains (T0, T1 and T2). After that, there was a noticeable increase of resistance in each of the strains tested.

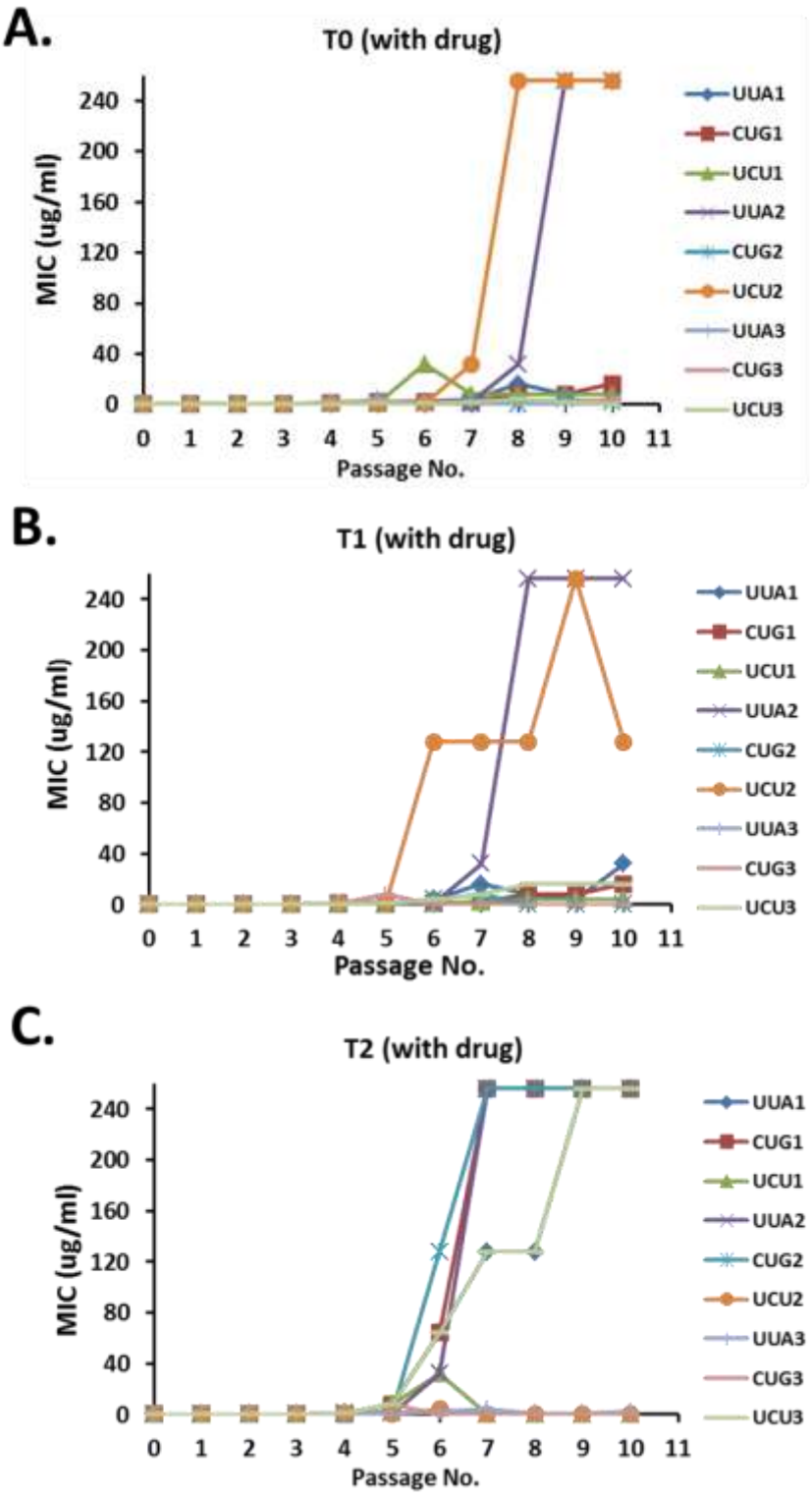


Figure 15 - Experimental evolution of 9 clones of each strain T0, T1 and T2 with fluconazole. Cultures were grown at 37ºC in RPMI media. The MIC values were determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST protocol) and revealed the lowest fluconazole concentration giving rise to an inhibition of growth of ±50%. The MIC values for strain T0 revealed that 2 clones reached the MIC~256µg/ml (A), the MIC values for strain T1 revealed that 2 clones reached the MIC~256µg/ml (B) and the MIC values for strain T2 revealed that 5 clones reached the MIC~256µg/ml (C).

2. Measure mistranslation levels during long-term evolution of drug resistance.

To verify if throughout the experimental evolution there were changes in the ambiguity levels of the strains (T0, T1 and T2), we used a reporter system based on the yeast enhanced fluorescent protein (yEGFP). This reporter was first described in Bezerra *et al.* [20] and allows the quantification of mistranslation through fluorescence microscopy.

The reporter is based on a "gain of function" mutation at the *yE*GFP gene. Typically, there is a UUA-Leu codon at position 201of this gene and the cognate $tRNA^{Leu}$ inserts leucine. The leucine insertion at this critical position actives GFP and there is the release of fluorescence. When this codon was mutated to a UCU-Ser codon at position 201, the corresponding cognate tRNA inserted serine. The incorporation of serine inactived GFP and no release of fluorescence was observed. In this situation, the residual fluorescence is the auto-fluorescence of the cell. Finally, when the 201 position was mutated to the ambiguous CUG codon, there could be decoding by the endogenous $tRNA^{Ser}_{CAG}$ and the mutant $tRNA^{Leu}_{CAG}$. Consequently, the observed fluorescence is proportional to the insertion of leucine at that specific position (Figure 16) [20].

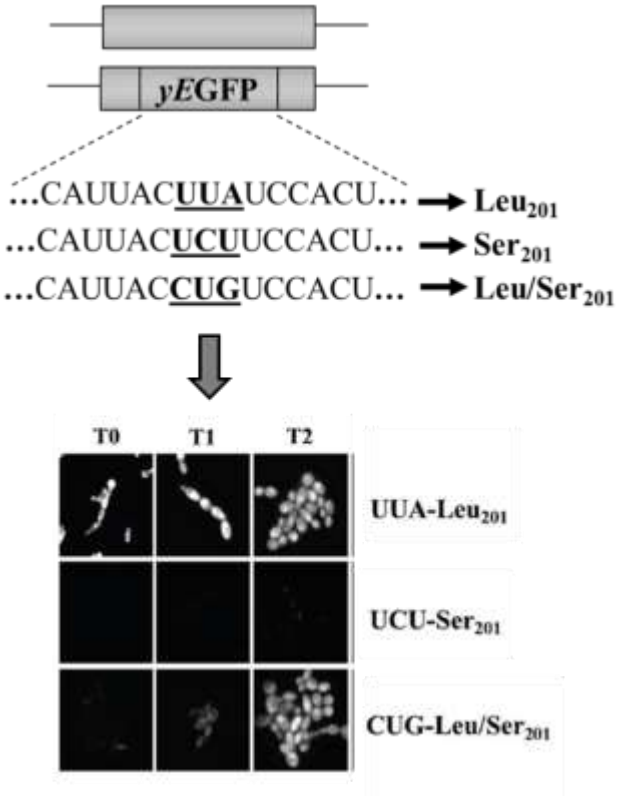


Figure 16 - **Fluorescent reporter system to quantify leucine insertion at CUG positions *in vivo*.** The system was based on the yeast-enhanced green fluorescent protein (*yE*GFP) gene. The leucine UUA codon at position 201 was mutated to the ambiguous CUG codon and to a UCU serine codon. Cell fluorescence was determined using a microscope and images were processed using ImageJ software. The level of fluorescence was directly proportional to the amount of leucine incorporated at the CUG-201 (Adapted from reference 20).

In our study, we used nine clones of each of the ambiguous strains T0, T1 and T2 where increased levels of incorporation of Leu were generated by inserting a copy (T1) or two copies (T2) of a mutant $tRNA^{Leu}_{CAG}$ gene. Among the nine clones of each strain (T0, T1 and T2), three function as positive controls (containing the UUA-Leu codon at position 201 of the *yE*GFP gene), three were negative controls (containing the UCU-Ser codon at position 201 of the *yE*GFP gene) and the other three were reporter clones (containing the CUG codon at position 201) (Figure 17).

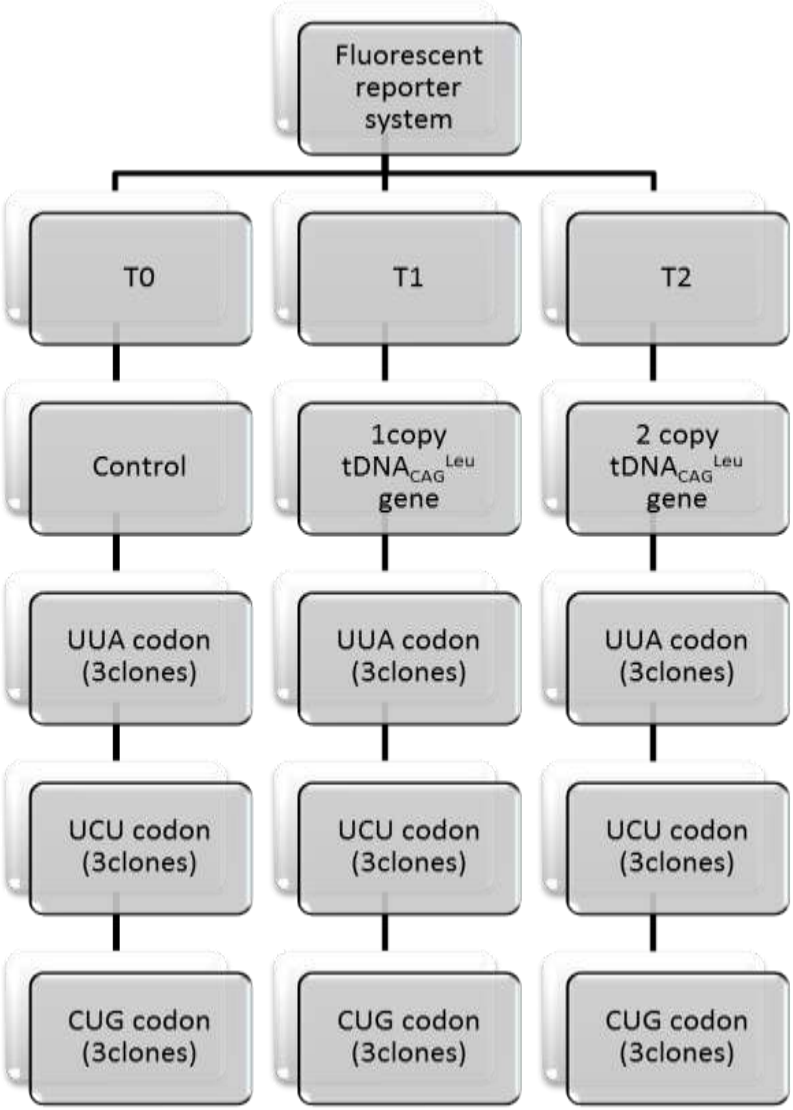


Figure 17 – Strains of *C. albicans* used in experimental evolution of resistance to fluconazole. Three strains (T0, T1 and T2) were tested and each strain had 9 different clones. Among the 9 clones evolved, there were 3 positive controls (UUA_{201} codon), 3 negative controls (UCU_{201} codon) and 3 reporter clones (CUG_{201} codon).

Clones carrying the different versions of the reporter were grown overnight in liquid media and fluorescence was measured by epifluorescence microscopy at the beginning of the evolution, in the breakpoint passage and in the final stage of the experiment. Photographs were taken and absolute GFP fluorescence intensities were quantified for individual yeast cells using ImageJ software. In each case, mean fluorescence intensities (± standard deviation) were calculated for at least 1000 individual cells. After obtaining the values of intensity of fluorescence of clones UUA_{201} (positive control) and Ser/Leu-

CUG_{201} (reporter), the value of fluorescence of the clones Ser-UCU_{201} (negative control) was subtracted and the ambiguity level was obtained as a ratio between CUG_{201} and UUA_{201} (Figure 18). All the others absolute values of fluorescence for each version of the reporter system and final calculation of the percentage of mistranslation are in the section annexes (Figure 25-34 in annex).

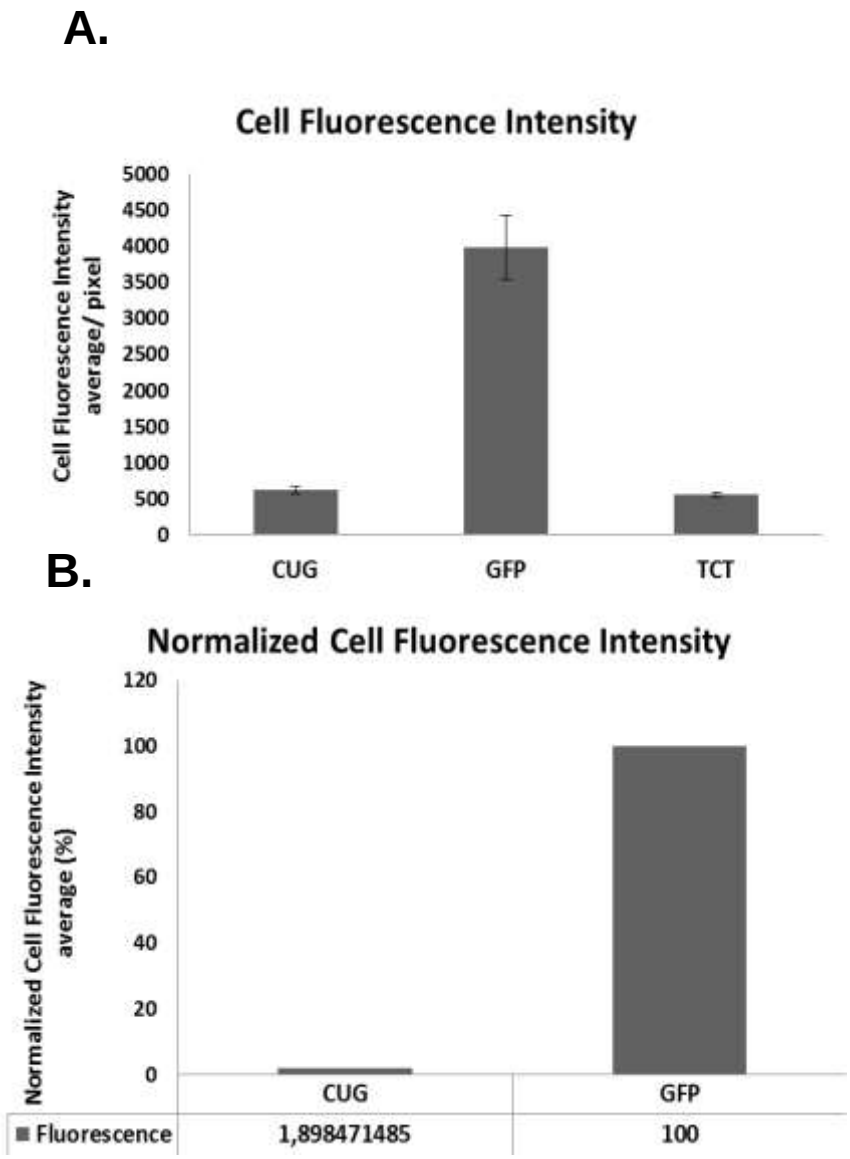


Figure 18 – Example of absolute values of fluorescence for each version of the reporter system and final calculation of the percentage of mistranslation of the strain T0 at the passage 5 of evolution without fluconazole (B). Absolute values of each clone (A).

As exemplified in Figure 18, it was possible to obtain the percentage of mistranslation in each strain at different stages of the evolution experiment with and without fluconazole (Figure 19, 20 and 21).

Results show that the values of mistranslation in strain T0 over the entire evolution experiment remain similar. Also, results are identical to the clones that grow with drug and without drug (Figure 19). In the beginning of evolution, T0 strain had ~1.5% of ambiguity and this value was maintained in passage 5. In the end of the evolution, strain T0 showed 0.1% of leucine misincorporation in both cases, with and without drug (Figure 19).

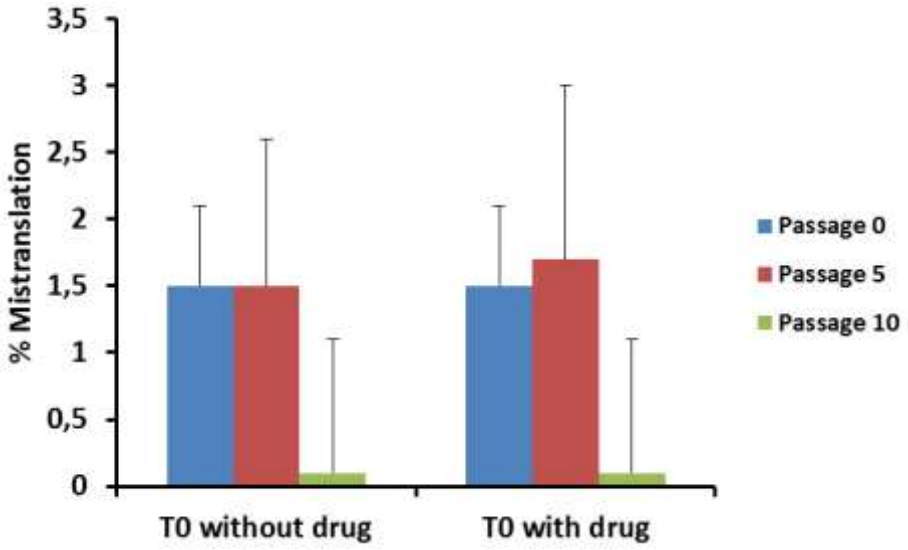


Figure 19 – Percentage of mistranslation of strain T0 in passages 0, 5 and 10. In media without drug, the percentage of mistranslation ranges from 1.45 ± 0.6%, 1.46 ± 1.1 % and 0.1 ± 1.1 %, referent to passage 0, 5 and 10, respectively. In media with drug, the percentage of mistranslation ranges from 1.45± 0.6%, 1.67± 1.3% and 0,1± 1%, referent to passage 0, 5 and 10, respectively. The statistical test, t-test, demonstrated that there is no statistical significance.

A different trend was observed for the strain T1 (Figure 20). In this case, in the beginning of evolution T1 strain had ~20% of ambiguity and this value was maintained in passage 5 and 10 of the evolution with fluconazole. In the

evolution without drug, levels of leucine misincorporation were slightly increased in passage5 and 10 (~30%). Although there was this notorious increase, this result was not statistically significant (Figure 20).

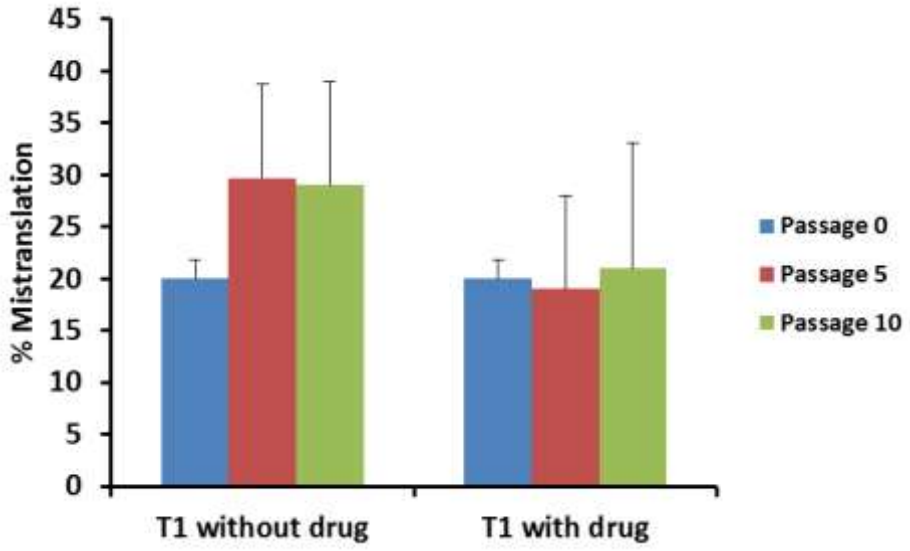


Figure 20 - Percentage of mistranslation of strain T1 in passage 0, 5 and 10. In media without drug, the percentage of mistranslation ranges from 20.61± 1.8%, 29.56± 9% and 29.02± 10%, referent to passage 0, 5 and 10, respectively. In media with drug, the percentage of mistranslation ranges from 20.61± 1.8%, 18.88± 9% and 21.05± 12%, referent to passage 0, 5 and 10, respectively. The statistical test, t-test, demonstrated that there no statistical significance.

In the case of strain T2, it was observed a clear decrease in the ambiguity level over the evolution process with and without drug (Figure 21). In the beginning of evolution, T2 strain had ~67% of ambiguity and this value drastically decreased in passage 5 (5.27% without drug and 18.76% with drug). This is likely due to a deletion of a fragment or part of the second copy of the $tRNA_{CAG}^{Leu}$ gene inserted at the RPS10 locus (results in section 2.1 below) in all clones of the strain T2. At passage 10, it was not possible to quantify the level of leucine misincorporation in both media with and without drug. The presence of cellular aggregates and the high level of auto-fluorescence of the cells that grew in media with drug, made it impossible to quantify the fluorescence (Figure 21).

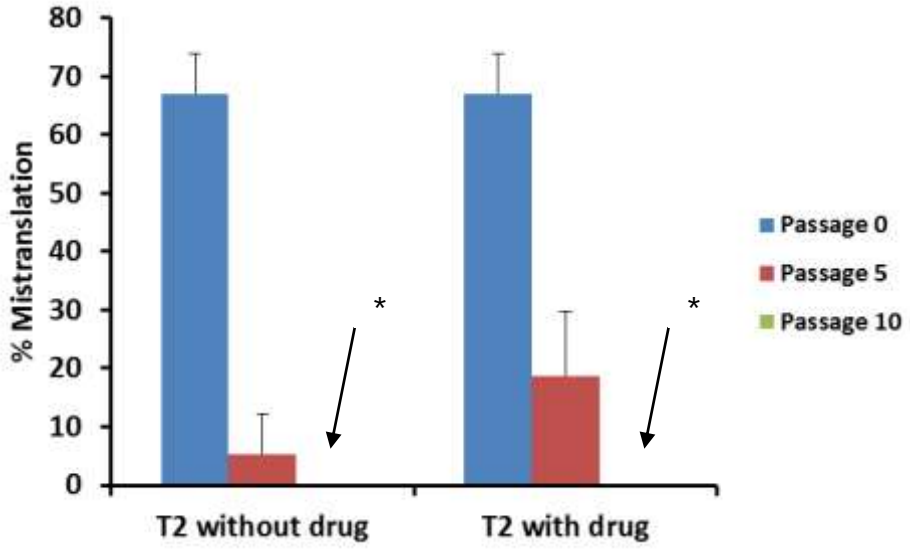


Figure 21 - Percentage of mistranslation of strain T2 in passages 0, 5 and 10. In media without drug, the percentage of mistranslation ranges from 67.29± 6.8% to 5.27± 7%, referent to passage 0 and 5, respectively. In media with drug, the percentage of mistranslation ranges from 67.29± 6.8% to 18.76± 11% referent to passage 0 and 5, respectively. The values for passage 10 (*) were impossible to quantify correctly.

2.1. Sequencing of the insertion at RP10 locus

As mentioned above, strain T2 showed a sharp decrease in mistranslation, as soon as passage 5, in both evolutions with and without drug. This decrease rendered a value similar to that of T1 strain (~20%), which prompted us to confirm that there was not cross-contamination of the more resistant strain T2 with a less resistant strain. In order to do that, we analyzed a fragment of the RPS10 locus of all clones to confirm the presence of the two insertions of the $tRNA_{CAG}^{Leu}$ gene (T2). If there is one insertion of $tRNA_{CAG}^{Leu}$ gene, clones belonged to strain T1 and if there was no insertion of the $tRNA_{CAG}^{Leu}$ gene, clones belonged to strain T0.

A portion of the RPS10 locus of all evolved clones was amplified from genomic DNA using standard PCR techniques. The amplified fragment was purified and

sequenced using the services of Stavida. Results revealed a deletion of a fragment containing the second $tRNA_{CAG}^{Leu}$ inserted on the RPS10 locus in all clones of the strain T2 evolved with fluconazole (Figure 22A). To confirm when this deletion may have occurred and if the presence of the drug was the trigger for the deletion, we also sequenced clones of strain T2 at passage 5 of the evolution without fluconazole. Results demonstrate that there was a deletion in clones evolved without fluconazole at passage 5 (Figure 22B).

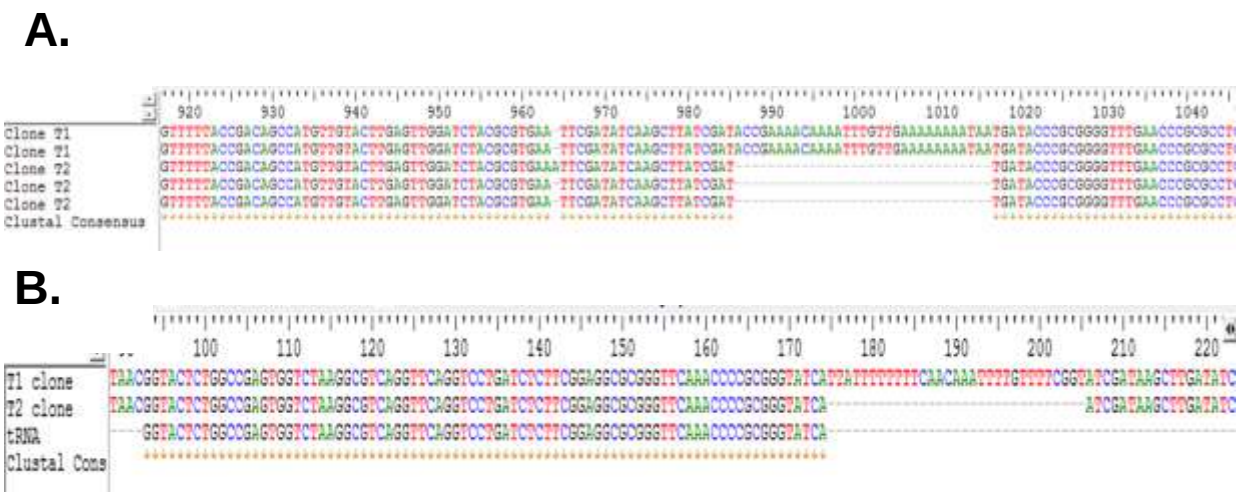


Figure 22 - Result of sequencing of RPS10 locus. Sequence of three clones of the strain T2 and two of the strain T1 belonging to passage 10 of the evolution with fluconazole (A). Sequence of one clone of the strain T2 at passage 5 of the evolution without fluconazole (B).

3. Identify molecular determinants by which resistance evolves in mistranslating strains

The increased fluconazole resistance observed in the strains with higher level of mistranslation may arise from transient physiological changes or more stable genetic changes. To verify if the increased drug resistance was stable, clones of strain T2 that presented a MIC of 256 µg/ml at passage 10 were subjected to the consecutive transfer in fresh media without fluconazole for three passages. After this, MICs were again measured and compared to the MICs of the original

mutants. Notably, MIC values of all the clones tested did not decrease following the nonselective serial transfer and always remained higher than the MIC values of the parental strain, suggesting that the drug resistance is stable for a period of at least three passages and is likely due to a genetic change rather than a phenotypic resistance.

Several mechanisms of resistance to fluconazole have been described. They can be separated in three categories: alteration of drug target, upregulation of drug transporters and cellular stress responses [83, 84]. One of the most studied mechanisms concerns the mutation of the drug target ERG11, which decreases the affinity of azole the binding. In fact, the three-dimensional structure of *C. albicans* Erg11 was modeled, and residues important for its interaction with the azoles were predicted (Figure 23) [142]. At least 12 different point mutations clustering in 3 “hot-spots” in *ERG11* gene have been associated with azole resistance in clinical isolates [143].

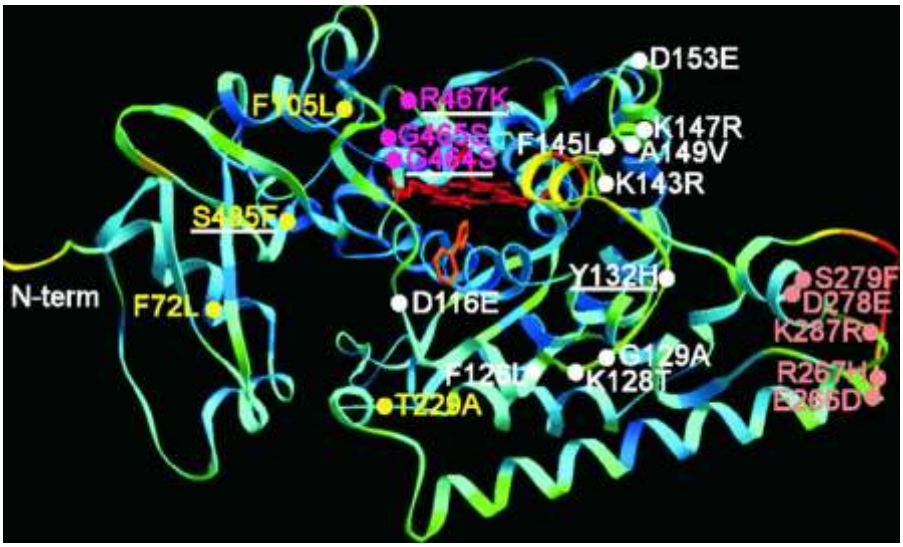


Figure 23 - Mapping of *C. albicans* mutations in azole-resistant isolates onto Erg11 structure. Red and yellow colors correspond to the most dynamic regions of MTCYP51. Numbering of residues in the figure is according to *C. albicans*. (Adapted from reference 132).

In order to verify if there are mutations in any of those “spots” in the ERG11 gene of our resistant evolved clones, we amplified ERG11from genomic DNA

using standard PCR techniques. The amplified fragment was purified and sequenced using the services of Stavida. Results showed that all clones of strain T2 with a MIC of 256 µg/ml at passage 10 have 2 specific mutations, the K128T and F105L (Figure 24). These are 2 mutations that are known to cause azole resistance in clinical strains. Clones that were not resistant to fluconazole did not show these mutations. Also, a few other resistant clones had the mutations I471T and G464S (data not show) but these were not present in all clones of the T2 strain.

Figure 24 - Sequencing of the ERG11 gene. Sequencing of the ERG11 gene from resistant clones of strain T2 grown in media with fluconazole showed the K128T and F105L mutations.

IV. Discussion

Relatively few experimental evolution projects have been performed with eukaryotic pathogens. Yet *Candida albicans* is the most prevalent fungal pathogen of humans and is commonly treated with fluconazole because of its low toxicity, low cost and oral availability. The acquisition of drug resistance is an evolutionary process and the prolonged use of fungistatic antifungal therapies increases the incidence of acquired antifungal drug resistance [79, 144]. One series of studies by Cowen *et al.* tackled this challenge by following adaptation by the pathogenic fungus [145] *Candida albicans* to inhibitory concentrations of the antimicrobial drug fluconazole. Six independent populations were exposed to either steady or increasing concentrations of the antibiotic, depending on the level of the most recently measured minimum inhibitory concentration (MIC). Six additional populations were passaged in the absence of antibiotic; these served as controls for adaptation to the laboratory environment in general and not the antibiotic. As in the previous studies, each of these populations was founded by a single clone with no capacity for genetic exchange between individuals, so all variation arose *de novo* by mutation during the experiment. This stochastic variation arising uniquely within each population allowed the authors to study how chance affects the evolution of azole resistance in *C. albicans* [145].

The evolution of drug resistance depends on phenotypic variability, and the ultimate source of that variability has been considered to be mutations that alter gene expression or protein activities [145]. Recent studies indicate that copy number variation (CNV), including short segmental CNV and whole chromosome aneuploidy, are important contributors to genetic variation leading to drug resistance [146, 147].

A previous study of Bezerra *et al* showed that strains with more Leu misincorporation (strain T2 with 67,29% ± 8,83%) grew better in media with

fluconazole and itraconazole [20], which suggested that that CUG ambiguity is relevant to evolution of antifungal drug resistance, particularly in the case of azoles. In the same work was possible to show that in strain T2, genes with a higher number of CUGs accumulated more SNPs. The study of Bezerra and coworkers also showed that highly ambiguous strains had loss of heterozygosity, which can arise via chromosome missegregation and/or via recombinations events, namely in chromosome R, which contains the genes coding for rDNA, and in chromossome 5, which contains genes that are involved in the regulation of biological process, namely stress response and antifungal drug resistance [20, 145]. This suggested that mistranslation is accompanied by an increased frequency of genomic changes that can interfere with the response to drug treatment in *C. albicans*.

Also, Selmecki *et al* demonstrated in their studies that chromosomal rearrangements, like aneuploidy, confer drug resistance. As an example is the i(5L), an isochromosome composed of two identical arms (Chr5L) flanking centrometer [49]. *TAC1* is located is located ~48 kb from the centromere on the left arm of chromosome 5 (Chr 5) and it is a transcription factor that activates *CDR1* and *CDR2* expression [95, 148]. *ERG11* is also located at this chromosome, ~150 kb from the left telomere [49].

Others studies suggest that codon ambiguity leads to adaptation under stress conditions (increase the fitness) like in the presence of drugs and the process of adaptation results from the development of mechanisms such metabolic alterations, activations of chaperons and signaling transduction cascades, that also contribute to drug tolerance [84].

In this experimental study, we were to check the role of tRNA mistranslation on the acquisition of resistance through evolution in media with and without fluconazole, based on the determination of MIC. The MIC of an antifungal agent is an important indicator of drug susceptibility [144].

It was observed that all strains (T0, T1 and T2), that grew in the absence of fluconazole, kept the sensitivity levels throughout evolution (MIC~ 0.125µg/ml) (Figure 14). Thus, the presence of drug is fundamental to the acquisition of resistance. The results of the experimental evolution with fluconazole showed that among the 9 clones of strain T0, two clones achieved the highest level of fluconazole resistance (MIC~ 256µg/ml). The same occurred with strain T1 where two clones achieved the highest level of fluconazole resistance (MIC~ 256µg/ml) and other clones achieved an intermediate level of resistance. Among the 9 clones tested of strain T2, five clones achieved the highest level of fluconazole resistance (MIC~ 256µg/ml). So strains with more ambiguity, T1 and T2, obtained more resistance in relation to T0 strain, and the T2 strain acquires more resistance, quicker and more stable compared to the strain T1 (Figure 15). In this study, it is also shown that passage 5 is the turning point for the three strains (T0, T1 and T2) in the presence of drug. After this passage, strains gradually acquired more resistance.

During a period of drug treatment, reversible resistance often develops such that when selection is relaxed resistance levels can drop [91]. The acquisition of unstable resistance has been documented in clinical isolates from patients treated with azole antifungals [149]. In our study, MIC values of all the clones tested did not decrease following the nonselective serial transfer and always remained higher than the MIC values of the parental strain, suggesting that the drug resistance is stable for a period of at least three passages and is likely due to a genetic change rather than phenotypic resistance.

The work of Lamb and Sanglard demonstrated that *C. albicans* drug tolerance involves mutations in the antifungal target gene, ERG11, which encodes for enzyme lanosterol 14-α demethylase. These mutations usually block drug binding [87, 89]. Studies of Morschhãuser and de Micheli [94, 150] revealed that overexpression of multiple drug resistance genes such as ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS) membrane transporters can also be responsible for removing the drug of the cell. Other

studies showed that some enzymes involved in the ergosterol biosynthesis pathways, such as Erg3, have been associated with azole resistance in *C. albicans* clinical isolates [151, 152]. In this work, we have analyzed one mechanism of resistance, the alteration of the target enzyme. The results suggest that the resistance acquired by T2 clones may be due to 2 mutations found in Erg11, precisely the K128T and F105L alterations (Figure 24). We have analysed this mechanism because several studies mentioned that at least 12 different point mutations in *ERG11* gene have been associated with azole resistance in clinical isolates [88, 143]. In a future work it would be interesting to verify possible other mechanisms involved in resistance, such mutations in ERG3 and upregulation of multidrug transporters.

Relative to the mistranslation level, it appears that throughout evolution with and without drug, T0 and T1 strains maintained the levels of ambiguity identical to the original values. The results showed that the values of mistranslation in strain T0 over the different passages were similar with a minimum of 0.1% and maximum of 1.67% (Figure 19). The same happened for the strain T1 over the evolution with a minimum of 18.49% and maximum of 29.56% (Figure 20). Results were identical among clones that grew with drug and without drug. In the case of strain T2, it was observed a clear decrease in the ambiguity level over the passages with a minimum of 5.27% and maximum of 67% (Figure 21). This occurred even without drug, suggesting that 67% of ambiguity is not within the physiological range tolerated by *C. albicans* cells. Indeed, previous studies showed a variation between 1.5% and 28% in ambiguity under environmental stress, with minimal decrease in growth rate [18].

The genomic instability of high mistranslating strains may also explain the decrease in ambiguity level. This decrease may be due to a deletion of a fragment of the RPS10 locus containing the second $tRNA_{CAG}^{Leu}$ (Figure 22A). Thus, the strain T2 that was supposed to have two insertions of the $tRNA_{CAG}^{Leu}$ gene has only one insertion of the mutant gene. Therefore, strain T2 acquired a typical behavior of strain T1, hence the decrease of mistranslation. In addition, it

was possible to verify that this deletion had already occurred at the turning point of the evolution, the passage 5. Results demonstrated the appearance of the deletion in both evolution experiments, with and without fluconazole (Figure 22B). So, this deletion does not depend of the presence of drug, but it is rather due to the evolution process itself.

With this experimental work it was possible to verify that genetic code ambiguity may provide an initial step for the acquisition of resistance but then becomes intolerable, which in turn results in the decrease of ambiguity levels.

V. Conclusions and future perspectives

Genetic code ambiguity and the pressure exerted by the presence of the drug deliver a powerful mechanism to induce resistance. Our data suggest that the acquisition of resistance is dependent on the drug and the percentage of mistranslation. Results also demonstrated that strains with increased mistranslation (T2) acquired more resistance and faster than the control. Mutations in ERG11 gene might be part of the mechanism of resistance. Also, T2 strain showed a decrease in mistranslation level during both evolution experiments (with and without drug) as a consequence of the deletion of one of the two copies of the mutant $tRNA_{CAG}^{Leu}$ gene. This further highlights the genetic instability of strain T2. It will be interesting to identify more molecular determinants involved in the acquisition of resistance, namely the increased expression of pumps genes and mutations in *ERG 3* gene, as well as sequencing of the whole genome of each clone.

VI. Bibliography

1. Crick F. Central dogma of molecular biology. Nature. 1970;227(5258):561-3. Epub 1970/08/08.
2. Silva RMMd. Molecular reconstruction of a genetic code alteration: University of Aveiro 2005.
3. Lodish H BA, Zipursky SL, et al. Molecular Cell Biology. New York: W. H. Freeman; 2000.
4. Preiss T, M WH. Starting the protein synthesis machine: eukaryotic translation initiation. BioEssays : news and reviews in molecular, cellular and developmental biology. 2003;25(12):1201-11. Epub 2003/11/25.
5. Kapp LD, Kolitz SE, Lorsch JR. Yeast initiator tRNA identity elements cooperate to influence multiple steps of translation initiation. RNA. 2006;12(5):751-64. Epub 2006/03/28.
6. GM C. The Cell: A Molecular Approach. 2nd edition ed. Sunderland (MA)2000.
7. Zaher HS, Green R. Fidelity at the molecular level: lessons from protein synthesis. Cell. 2009;136(4):746-62. Epub 2009/02/26.
8. Kramer EB, Farabaugh PJ. The frequency of translational misreading errors in E. coli is largely determined by tRNA competition. RNA. 2007;13(1):87-96. Epub 2006/11/11.
9. Loftfield RB, Vanderjagt D. The frequency of errors in protein biosynthesis. The Biochemical journal. 1972;128(5):1353-6. Epub 1972/08/01.
10. Jakubowski H, Goldman E. Editing of errors in selection of amino acids for protein synthesis. Microbiological reviews. 1992;56(3):412-29. Epub 1992/09/01.
11. Reynolds NM, Lazazzera BA, Ibba M. Cellular mechanisms that control mistranslation. Nature reviews Microbiology. 2010;8(12):849-56. Epub 2010/11/17.
12. Gott JM, Emeson RB. Functions and mechanisms of RNA editing. Annual review of genetics. 2000;34:499-531. Epub 2000/11/28.
13. Moura GR, Carreto LC, Santos MA. Genetic code ambiguity: an unexpected source of proteome innovation and phenotypic diversity. Current opinion in microbiology. 2009;12(6):631-7. Epub 2009/10/27.
14. Farabaugh PJ, Bjork GR. How translational accuracy influences reading frame maintenance. The EMBO journal. 1999;18(6):1427-34. Epub 1999/03/17.
15. Kubota H. Quality control against misfolded proteins in the cytosol: a network for cell survival. Journal of biochemistry. 2009;146(5):609-16. Epub 2009/09/10.
16. Moghal A, Mohler K, Ibba M. Mistranslation of the genetic code. FEBS letters. 2014. Epub 2014/09/16.
17. Pan T. Adaptive translation as a mechanism of stress response and adaptation. Annual review of genetics. 2013;47:121-37. Epub 2013/08/31.
18. Gomes AC, Miranda I, Silva RM, Moura GR, Thomas B, Akoulitchev A, et al. A genetic code alteration generates a proteome of high diversity in the human pathogen Candida albicans. Genome biology. 2007;8(10):R206. Epub 2007/10/06.
19. Miranda I, Rocha R, Santos MC, Mateus DD, Moura GR, Carreto L, et al. A genetic code alteration is a phenotype diversity generator in the human pathogen Candida albicans. PloS one. 2007;2(10):e996. Epub 2007/10/04.
20. Bezerra AR, Simoes J, Lee W, Rung J, Weil T, Gut IG, et al. Reversion of a fungal genetic code alteration links proteome instability with genomic and phenotypic diversification. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(27):11079-84. Epub 2013/06/19.
21. Santos MA, Cheesman C, Costa V, Moradas-Ferreira P, Tuite MF. Selective advantages created by codon ambiguity allowed for the evolution of an alternative genetic code in Candida spp. Molecular microbiology. 1999;31(3):937-47. Epub 1999/02/27.
22. Li L, Boniecki MT, Jaffe JD, Imai BS, Yau PM, Luthey-Schulten ZA, et al. Naturally occurring aminoacyl-tRNA synthetases editing-domain mutations that cause mistranslation in Mycoplasma

parasites. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(23):9378-83. Epub 2011/05/25.
23. Javid B, Sorrentino F, Toosky M, Zheng W, Pinkham JT, Jain N, et al. Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(3):1132-7. Epub 2014/01/08.
24. Antonellis A, Ellsworth RE, Sambuughin N, Puls I, Abel A, Lee-Lin SQ, et al. Glycyl tRNA synthetase mutations in Charcot-Marie-Tooth disease type 2D and distal spinal muscular atrophy type V. American journal of human genetics. 2003;72(5):1293-9. Epub 2003/04/12.
25. Scheper GC, van der Knaap MS, Proud CG. Translation matters: protein synthesis defects in inherited disease. Nature reviews Genetics. 2007;8(9):711-23. Epub 2007/08/08.
26. Lee JW, Beebe K, Nangle LA, Jang J, Longo-Guess CM, Cook SA, et al. Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration. Nature. 2006;443(7107):50-5. Epub 2006/08/15.
27. Crick FH. The origin of the genetic code. Journal of molecular biology. 1968;38(3):367-79. Epub 1968/12/01.
28. Crick FH. The genetic code--yesterday, today, and tomorrow. Cold Spring Harbor symposia on quantitative biology. 1966;31:1-9. Epub 1966/01/01.
29. Koonin EV, Novozhilov AS. Origin and evolution of the genetic code: the universal enigma. IUBMB life. 2009;61(2):99-111. Epub 2009/01/02.
30. Osawa S, Jukes TH, Watanabe K, Muto A. Recent evidence for evolution of the genetic code. Microbiological reviews. 1992;56(1):229-64. Epub 1992/03/01.
31. Chambers I, Frampton J, Goldfarb P, Affara N, McBain W, Harrison PR. The structure of the mouse glutathione peroxidase gene: the selenocysteine in the active site is encoded by the 'termination' codon, TGA. The EMBO journal. 1986;5(6):1221-7. Epub 1986/06/01.
32. Hao B, Gong W, Ferguson TK, James CM, Krzycki JA, Chan MK. A new UAG-encoded residue in the structure of a methanogen methyltransferase. Science. 2002;296(5572):1462-6. Epub 2002/05/25.
33. Barrell BG, Bankier AT, Drouin J. A different genetic code in human mitochondria. Nature. 1979;282(5735):189-94. Epub 1979/11/08.
34. Santos MA, Ueda T, Watanabe K, Tuite MF. The non-standard genetic code of Candida spp.: an evolving genetic code or a novel mechanism for adaptation? Molecular microbiology. 1997;26(3):423-31. Epub 1997/12/24.
35. Osawa S, Ohama T, Jukes TH, Watanabe K, Yokoyama S. Evolution of the mitochondrial genetic code. II. Reassignment of codon AUA from isoleucine to methionine. Journal of molecular evolution. 1989;29(5):373-80. Epub 1989/11/01.
36. Ohama T, Osawa S, Watanabe K, Jukes TH. Evolution of the mitochondrial genetic code. IV. AAA as an asparagine codon in some animal mitochondria. Journal of molecular evolution. 1990;30(4):329-32. Epub 1990/04/01.
37. Osawa S, Ohama T, Jukes TH, Watanabe K. Evolution of the mitochondrial genetic code. I. Origin of AGR serine and stop codons in metazoan mitochondria. Journal of molecular evolution. 1989;29(3):202-7. Epub 1989/09/01.
38. Osawa S. Evolution of genetic code. New york: Oxford University Press. 1995.
39. Ohama T, Suzuki T, Mori M, Osawa S, Ueda T, Watanabe K, et al. Non-universal decoding of the leucine codon CUG in several Candida species. Nucleic acids research. 1993;21(17):4039-45. Epub 1993/08/25.
40. Ueda T, Suzuki T, Yokogawa T, Nishikawa K, Watanabe K. Unique structure of new serine tRNAs responsible for decoding leucine codon CUG in various Candida species and their putative ancestral tRNA genes. Biochimie. 1994;76(12):1217-22. Epub 1994/01/01.
41. Tuite MF, Santos MA. Codon reassignment in Candida species: an evolutionary conundrum. Biochimie. 1996;78(11-12):993-9. Epub 1996/01/01.
42. Santos MA, Keith G, Tuite MF. Non-standard translational events in Candida albicans mediated by an unusual seryl-tRNA with a 5'-CAG-3' (leucine) anticodon. The EMBO journal. 1993;12(2):607-16. Epub 1993/02/01.
43. Mateus DD, Paredes JA, Espanol Y, Ribas de Pouplana L, Moura GR, Santos MA. Molecular reconstruction of a fungal genetic code alteration. RNA biology. 2013;10(6):969-80. Epub 2013/04/27.
44. Perreau VM, Keith G, Holmes WM, Przykorska A, Santos MA, Tuite MF. The Candida albicans CUG-decoding ser-tRNA has an atypical anticodon stem-loop structure. Journal of molecular biology. 1999;293(5):1039-53. Epub 1999/11/05.

45. Massey SE, Moura G, Beltrao P, Almeida R, Garey JR, Tuite MF, et al. Comparative evolutionary genomics unveils the molecular mechanism of reassignment of the CTG codon in Candida spp. Genome research. 2003;13(4):544-57. Epub 2003/04/03.
46. Miranda I, Silva R, Santos MA. Evolution of the genetic code in yeasts. Yeast. 2006;23(3):203-13. Epub 2006/02/25.
47. Moura GR, Paredes JA, Santos MA. Development of the genetic code: insights from a fungal codon reassignment. FEBS letters. 2010;584(2):334-41. Epub 2009/11/28.
48. Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, Munro CA, et al. Evolution of pathogenicity and sexual reproduction in eight Candida genomes. Nature. 2009;459(7247):657-62. Epub 2009/05/26.
49. Selmecki AM, Dulmage K, Cowen LE, Anderson JB, Berman J. Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance. PLoS genetics. 2009;5(10):e1000705. Epub 2009/10/31.
50. Miranda I, Silva-Dias A, Rocha R, Teixeira-Santos R, Coelho C, Goncalves T, et al. Candida albicans CUG mistranslation is a mechanism to create cell surface variation. mBio. 2013;4(4). Epub 2013/06/27.
51. Coste A, Selmecki A, Forche A, Diogo D, Bougnoux ME, d'Enfert C, et al. Genotypic evolution of azole resistance mechanisms in sequential Candida albicans isolates. Eukaryotic cell. 2007;6(10):1889-904. Epub 2007/08/19.
52. Forche A, Abbey D, Pisithkul T, Weinzierl MA, Ringstrom T, Bruck D, et al. Stress alters rates and types of loss of heterozygosity in Candida albicans. mBio. 2011;2(4). Epub 2011/07/28.
53. Molero G, Diez-Orejas R, Navarro-Garcia F, Monteoliva L, Pla J, Gil C, et al. Candida albicans: genetics, dimorphism and pathogenicity. International microbiology : the official journal of the Spanish Society for Microbiology. 1998;1(2):95-106. Epub 2000/12/08.
54. Mayer FL, Wilson D, Hube B. Candida albicans pathogenicity mechanisms. Virulence. 2013;4(2):119-28. Epub 2013/01/11.
55. Hameed S, Fatima Z. Novel Regulatory Mechanisms of Pathogenicity and Virulence to Combat MDR in Candida albicans. International journal of microbiology. 2013;2013:240209. Epub 2013/10/29.
56. Kim J, Sudbery P. Candida albicans, a major human fungal pathogen. J Microbiol. 2011;49(2):171-7. Epub 2011/05/04.
57. Braun BR, van Het Hoog M, d'Enfert C, Martchenko M, Dungan J, Kuo A, et al. A human-curated annotation of the Candida albicans genome. PLoS genetics. 2005;1(1):36-57. Epub 2005/08/17.
58. Harrison BD, Hashemi J, Bibi M, Pulver R, Bavli D, Nahmias Y, et al. A tetraploid intermediate precedes aneuploid formation in yeasts exposed to fluconazole. PLoS biology. 2014;12(3):e1001815. Epub 2014/03/20.
59. Hickman MA, Zeng G, Forche A, Hirakawa MP, Abbey D, Harrison BD, et al. The 'obligate diploid' Candida albicans forms mating-competent haploids. Nature. 2013;494(7435):55-9. Epub 2013/02/01.
60. Noble SM, Johnson AD. Genetics of Candida albicans, a diploid human fungal pathogen. Annual review of genetics. 2007;41:193-211. Epub 2007/07/07.
61. Pappas PG, Kauffman C, Andes D, Jr. BD, TF C, Eduards JE Jr. ea. Clinical practice guidelines for the manegement of candidiasis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2009;48:503-35.
62. Odds FC, Webster CE, Mayuranathan P, Simmons PD. Candida concentrations in the vagina and their association with signs and symptoms of vaginal candidosis. Journal of medical and veterinary mycology : bi-monthly publication of the International Society for Human and Animal Mycology. 1988;26(5):277-83. Epub 1988/01/01.
63. Sudbery PE. Growth of Candida albicans hyphae. Nature reviews Microbiology. 2011;9(10):737-48. Epub 2011/08/17.
64. Albuquerque P, Casadevall A. Quorum sensing in fungi--a review. Medical mycology. 2012;50(4):337-45. Epub 2012/01/25.
65. Hostetter MK. Linkage of adhesion, morphogenesis, and virulence in Candida albicans. The Journal of laboratory and clinical medicine. 1998;132(4):258-63. Epub 1998/10/30.
66. Jacobsen ID, Wilson D, Wachtler B, Brunke S, Naglik JR, Hube B. Candida albicans dimorphism as a therapeutic target. Expert review of anti-infective therapy. 2012;10(1):85-93. Epub 2011/12/14.
67. Leng P, Lee PR, Wu H, Brown AJ. Efg1, a morphogenetic regulator in Candida albicans, is a sequence-specific DNA binding protein. Journal of bacteriology. 2001;183(13):4090-3. Epub 2001/06/08.

68. Naglik JR, Moyes DL, Wachtler B, Hube B. Candida albicans interactions with epithelial cells and mucosal immunity. Microbes and infection / Institut Pasteur. 2011;13(12-13):963-76. Epub 2011/08/02.
69. Zhu W, Filler SG. Interactions of Candida albicans with epithelial cells. Cellular microbiology. 2010;12(3):273-82. Epub 2009/11/19.
70. Fanning S, Mitchell AP. Fungal biofilms. PLoS pathogens. 2012;8(4):e1002585. Epub 2012/04/13.
71. Robbins N, Uppuluri P, Nett J, Rajendran R, Ramage G, Lopez-Ribot JL, et al. Hsp90 governs dispersion and drug resistance of fungal biofilms. PLoS pathogens. 2011;7(9):e1002257. Epub 2011/09/21.
72. Kumamoto CA. Molecular mechanisms of mechanosensing and their roles in fungal contact sensing. Nature reviews Microbiology. 2008;6(9):667-73. Epub 2008/08/06.
73. Brand A, Shanks S, Duncan VM, Yang M, Mackenzie K, Gow NA. Hyphal orientation of Candida albicans is regulated by a calcium-dependent mechanism. Current biology : CB. 2007;17(4):347-52. Epub 2007/02/06.
74. Wachtler B, Citiulo F, Jablonowski N, Forster S, Dalle F, Schaller M, et al. Candida albicans-epithelial interactions: dissecting the roles of active penetration, induced endocytosis and host factors on the infection process. PloS one. 2012;7(5):e36952. Epub 2012/05/19.
75. Naglik JR, Challacombe SJ, Hube B. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiology and molecular biology reviews : MMBR. 2003;67(3):400-28, table of contents. Epub 2003/09/11.
76. Bitar I, Khalaf RA, Harastani H, Tokajian S. Identification, typing, antifungal resistance profile, and biofilm formation of Candida albicans isolates from lebanese hospital patients. BioMed research international. 2014;2014:931372. Epub 2014/07/02.
77. Lupetti A, Danesi R, Campa M, Del Tacca M, Kelly S. Molecular basis of resistance to azole antifungals. Trends in molecular medicine. 2002;8(2):76-81. Epub 2002/01/30.
78. Anderson JB. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. Nature reviews Microbiology. 2005;3(7):547-56. Epub 2005/06/15.
79. Cowen LE. The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype. Nature reviews Microbiology. 2008;6(3):187-98. Epub 2008/02/05.
80. Rex JH, Rinaldi MG, Pfaller MA. Resistance of Candida species to fluconazole. Antimicrobial agents and chemotherapy. 1995;39(1):1-8. Epub 1995/01/01.
81. Morschhauser J. The genetic basis of fluconazole resistance development in Candida albicans. Biochimica et biophysica acta. 2002;1587(2-3):240-8. Epub 2002/06/27.
82. Ostrosky-Zeichner L, Casadevall A, Galgiani JN, Odds FC, Rex JH. An insight into the antifungal pipeline: selected new molecules and beyond. Nature reviews Drug discovery. 2010;9(9):719-27. Epub 2010/08/21.
83. Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiology and molecular biology reviews : MMBR. 2011;75(2):213-67. Epub 2011/06/08.
84. Cowen LE, Steinbach WJ. Stress, drugs, and evolution: the role of cellular signaling in fungal drug resistance. Eukaryotic cell. 2008;7(5):747-64. Epub 2008/04/01.
85. Denning DW. Echinocandin antifungal drugs. Lancet. 2003;362(9390):1142-51. Epub 2003/10/11.
86. Spampinato C, Leonardi D. Candida infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. BioMed research international. 2013;2013:204237. Epub 2013/07/24.
87. Lamb DC, Kelly DE, Schunck WH, Shyadehi AZ, Akhtar M, Lowe DJ, et al. The mutation T315A in Candida albicans sterol 14alpha-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. The Journal of biological chemistry. 1997;272(9):5682-8. Epub 1997/02/28.
88. Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, et al. Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in Candida albicans. Microbiology. 1999;145 (Pt 10):2701-13. Epub 1999/10/28.
89. Sanglard D, Ischer F, Koymans L, Bille J. Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant Candida albicans clinical isolates contribute to resistance to azole antifungal agents. Antimicrobial agents and chemotherapy. 1998;42(2):241-53. Epub 1998/04/04.

90. Du W, Coaker M, Sobel JD, Akins RA. Shuttle vectors for Candida albicans: control of plasmid copy number and elevated expression of cloned genes. Current genetics. 2004;45(6):390-8. Epub 2004/03/23.
91. Selmecki A, Forche A, Berman J. Aneuploidy and isochromosome formation in drug-resistant Candida albicans. Science. 2006;313(5785):367-70. Epub 2006/07/22.
92. Selmecki A, Gerami-Nejad M, Paulson C, Forche A, Berman J. An isochromosome confers drug resistance in vivo by amplification of two genes, ERG11 and TAC1. Molecular microbiology. 2008;68(3):624-41. Epub 2008/03/28.
93. Perea S, Lopez-Ribot JL, Kirkpatrick WR, McAtee RK, Santillan RA, Martinez M, et al. Prevalence of molecular mechanisms of resistance to azole antifungal agents in Candida albicans strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrobial agents and chemotherapy. 2001;45(10):2676-84. Epub 2001/09/15.
94. Morschhauser J, Barker KS, Liu TT, Bla BWJ, Homayouni R, Rogers PD. The transcription factor Mrr1p controls expression of the MDR1 efflux pump and mediates multidrug resistance in Candida albicans. PLoS pathogens. 2007;3(11):e164. Epub 2007/11/07.
95. Coste A, Turner V, Ischer F, Morschhauser J, Forche A, Selmecki A, et al. A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in Candida albicans. Genetics. 2006;172(4):2139-56. Epub 2006/02/03.
96. Liu TT, Znaidi S, Barker KS, Xu L, Homayouni R, Saidane S, et al. Genome-wide expression and location analyses of the Candida albicans Tac1p regulon. Eukaryotic cell. 2007;6(11):2122-38. Epub 2007/10/02.
97. Munro CA, Selvaggini S, de Bruijn I, Walker L, Lenardon MD, Gerssen B, et al. The PKC, HOG and Ca2+ signalling pathways co-ordinately regulate chitin synthesis in Candida albicans. Molecular microbiology. 2007;63(5):1399-413. Epub 2007/02/17.
98. Fox DS, Heitman J. Good fungi gone bad: the corruption of calcineurin. BioEssays : news and reviews in molecular, cellular and developmental biology. 2002;24(10):894-903. Epub 2002/09/27.
99. Steinbach WJ, Reedy JL, Cramer RA, Jr., Perfect JR, Heitman J. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. Nature reviews Microbiology. 2007;5(6):418-30. Epub 2007/05/17.
100. Blankenship JR, Heitman J. Calcineurin is required for Candida albicans to survive calcium stress in serum. Infection and immunity. 2005;73(9):5767-74. Epub 2005/08/23.
101. Kullas AL, Martin SJ, Davis D. Adaptation to environmental pH: integrating the Rim101 and calcineurin signal transduction pathways. Molecular microbiology. 2007;66(4):858-71. Epub 2007/10/12.
102. Onyewu C, Blankenship JR, Del Poeta M, Heitman J. Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against Candida albicans, Candida glabrata, and Candida krusei. Antimicrobial agents and chemotherapy. 2003;47(3):956-64. Epub 2003/02/27.
103. Onyewu C, Wormley FL, Jr., Perfect JR, Heitman J. The calcineurin target, Crz1, functions in azole tolerance but is not required for virulence of Candida albicans. Infection and immunity. 2004;72(12):7330-3. Epub 2004/11/24.
104. Karababa M, Valentino E, Pardini G, Coste AT, Bille J, Sanglard D. CRZ1, a target of the calcineurin pathway in Candida albicans. Molecular microbiology. 2006;59(5):1429-51. Epub 2006/02/14.
105. Pearl LH, Prodromou C. Structure and mechanism of the Hsp90 molecular chaperone machinery. Annual review of biochemistry. 2006;75:271-94. Epub 2006/06/08.
106. Zhao R, Houry WA. Hsp90: a chaperone for protein folding and gene regulation. Biochemistry and cell biology = Biochimie et biologie cellulaire. 2005;83(6):703-10. Epub 2005/12/08.
107. Imai J, Yahara I. Role of HSP90 in salt stress tolerance via stabilization and regulation of calcineurin. Molecular and cellular biology. 2000;20(24):9262-70. Epub 2000/11/30.
108. Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(18):8324-8. Epub 1994/08/30.
109. Cowen LE, Lindquist S. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. Science. 2005;309(5744):2185-9. Epub 2005/10/01.
110. Cowen LE, Carpenter AE, Matangkasombut O, Fink GR, Lindquist S. Genetic architecture of Hsp90-dependent drug resistance. Eukaryotic cell. 2006;5(12):2184-8. Epub 2006/10/24.
111. Pan X, Heitman J. Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in Saccharomyces cerevisiae. Molecular and cellular biology. 1999;19(7):4874-87. Epub 1999/06/22.

112. Robertson LS, Fink GR. The three yeast A kinases have specific signaling functions in pseudohyphal growth. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(23):13783-7. Epub 1998/11/13.
113. Jain P, Akula I, Edlind T. Cyclic AMP signaling pathway modulates susceptibility of candida species and Saccharomyces cerevisiae to antifungal azoles and other sterol biosynthesis inhibitors. Antimicrobial agents and chemotherapy. 2003;47(10):3195-201. Epub 2003/09/25.
114. d'Enfert C. Biofilms and their role in the resistance of pathogenic Candida to antifungal agents. Current drug targets. 2006;7(4):465-70. Epub 2006/04/14.
115. Nobile CJ, Mitchell AP. Genetics and genomics of Candida albicans biofilm formation. Cellular microbiology. 2006;8(9):1382-91. Epub 2006/07/20.
116. Ellis D. Amphotericin B: spectrum and resistance. The Journal of antimicrobial chemotherapy. 2002;49 Suppl 1:7-10. Epub 2002/01/22.
117. Nolte FS, Parkinson T, Falconer DJ, Dix S, Williams J, Gilmore C, et al. Isolation and characterization of fluconazole- and amphotericin B-resistant Candida albicans from blood of two patients with leukemia. Antimicrobial agents and chemotherapy. 1997;41(1):196-9. Epub 1997/01/01.
118. Kelly SL, Lamb DC, Kelly DE, Manning NJ, Loeffler J, Hebart H, et al. Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol delta5,6-desaturation. FEBS letters. 1997;400(1):80-2. Epub 1997/01/02.
119. Brajtburg J, Powderly WG, Kobayashi GS, Medoff G. Amphotericin B: current understanding of mechanisms of action. Antimicrobial agents and chemotherapy. 1990;34(2):183-8. Epub 1990/02/01.
120. Matthews RC, Rigg G, Hodgetts S, Carter T, Chapman C, Gregory C, et al. Preclinical assessment of the efficacy of mycograb, a human recombinant antibody against fungal HSP90. Antimicrobial agents and chemotherapy. 2003;47(7):2208-16. Epub 2003/06/25.
121. LaFleur MD, Kumamoto CA, Lewis K. Candida albicans biofilms produce antifungal-tolerant persister cells. Antimicrobial agents and chemotherapy. 2006;50(11):3839-46. Epub 2006/08/23.
122. Laverdiere M, Lalonde RG, Baril JG, Sheppard DC, Park S, Perlin DS. Progressive loss of echinocandin activity following prolonged use for treatment of Candida albicans oesophagitis. The Journal of antimicrobial chemotherapy. 2006;57(4):705-8. Epub 2006/02/09.
123. Schimoler-O'Rourke R, Renault S, Mo W, Selitrennikoff CP. Neurospora crassa FKS protein binds to the (1,3)beta-glucan synthase substrate, UDP-glucose. Current microbiology. 2003;46(6):408-12. Epub 2003/05/07.
124. Qadota H, Python CP, Inoue SB, Arisawa M, Anraku Y, Zheng Y, et al. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. Science. 1996;272(5259):279-81. Epub 1996/04/12.
125. Park S, Kelly R, Kahn JN, Robles J, Hsu MJ, Register E, et al. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical Candida sp. isolates. Antimicrobial agents and chemotherapy. 2005;49(8):3264-73. Epub 2005/07/29.
126. Perlin DS. Resistance to echinocandin-class antifungal drugs. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy. 2007;10(3):121-30. Epub 2007/06/16.
127. Schuetzer-Muehlbauer M, Willinger B, Krapf G, Enzinger S, Presterl E, Kuchler K. The Candida albicans Cdr2p ATP-binding cassette (ABC) transporter confers resistance to caspofungin. Molecular microbiology. 2003;48(1):225-35. Epub 2003/03/27.
128. Lesage G, Bussey H. Cell wall assembly in Saccharomyces cerevisiae. Microbiology and molecular biology reviews : MMBR. 2006;70(2):317-43. Epub 2006/06/09.
129. Levin DE. Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiology and molecular biology reviews : MMBR. 2005;69(2):262-91. Epub 2005/06/10.
130. Wiederhold NP, Kontoyiannis DP, Prince RA, Lewis RE. Attenuation of the activity of caspofungin at high concentrations against candida albicans: possible role of cell wall integrity and calcineurin pathways. Antimicrobial agents and chemotherapy. 2005;49(12):5146-8. Epub 2005/11/24.
131. Nett J, Lincoln L, Marchillo K, Massey R, Holoyda K, Hoff B, et al. Putative role of beta-1,3 glucans in Candida albicans biofilm resistance. Antimicrobial agents and chemotherapy. 2007;51(2):510-20. Epub 2006/11/30.
132. Mishra NN, Prasad T, Sharma N, Payasi A, Prasad R, Gupta DK, et al. Pathogenicity and drug resistance in Candida albicans and other yeast species. A review. Acta microbiologica et immunologica Hungarica. 2007;54(3):201-35. Epub 2007/09/28.
133. Selmecki A, Forche A, Berman J. Genomic plasticity of the human fungal pathogen Candida albicans. Eukaryotic cell. 2010;9(7):991-1008. Epub 2010/05/25.

134. Noble SM, Johnson AD. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen Candida albicans. Eukaryotic cell. 2005;4(2):298-309. Epub 2005/02/11.
135. EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2008;14(4):398-405. Epub 2008/01/15.
136. Hoffman CS, Winston F. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. Gene. 1987;57(2-3):267-72. Epub 1987/01/01.
137. Odds FC, Bougnoux ME, Shaw DJ, Bain JM, Davidson AD, Diogo D, et al. Molecular phylogenetics of Candida albicans. Eukaryotic cell. 2007;6(6):1041-52. Epub 2007/04/10.
138. Pfaller MA, Diekema DJ. Epidemiology of invasive mycoses in North America. Critical reviews in microbiology. 2010;36(1):1-53. Epub 2010/01/22.
139. Lenski RE, Simpson SC, Nguyen TT. Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. Journal of bacteriology. 1994;176(11):3140-7. Epub 1994/06/01.
140. Travisano M, Mongold JA, Bennett AF, Lenski RE. Experimental tests of the roles of adaptation, chance, and history in evolution. Science. 1995;267(5194):87-90. Epub 1995/01/06.
141. Zeyl C, Bell G. The advantage of sex in evolving yeast populations. Nature. 1997;388(6641):465-8. Epub 1997/07/31.
142. Podust LM, Poulos TL, Waterman MR. Crystal structure of cytochrome P450 14alpha -sterol demethylase (CYP51) from Mycobacterium tuberculosis in complex with azole inhibitors. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(6):3068-73. Epub 2001/03/15.
143. Alvarez-Rueda N, Fleury A, Morio F, Pagniez F, Gastinel L, Le Pape P. Amino acid substitutions at the major insertion loop of Candida albicans sterol 14alpha-demethylase are involved in fluconazole resistance. PloS one. 2011;6(6):e21239. Epub 2011/06/24.
144. White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clinical microbiology reviews. 1998;11(2):382-402. Epub 1998/06/20.
145. Cowen LE, Sanglard D, Calabrese D, Sirjusingh C, Anderson JB, Kohn LM. Evolution of drug resistance in experimental populations of Candida albicans. Journal of bacteriology. 2000;182(6):1515-22. Epub 2000/02/29.
146. Duesberg P, Stindl R, Hehlmann R. Explaining the high mutation rates of cancer cells to drug and multidrug resistance by chromosome reassortments that are catalyzed by aneuploidy. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(26):14295-300. Epub 2000/12/20.
147. Duesberg P, Stindl R, Hehlmann R. Origin of multidrug resistance in cells with and without multidrug resistance genes: chromosome reassortments catalyzed by aneuploidy. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(20):11283-8. Epub 2001/09/13.
148. Coste AT, Karababa M, Ischer F, Bille J, Sanglard D. TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of Candida albicans ABC transporters CDR1 and CDR2. Eukaryotic cell. 2004;3(6):1639-52. Epub 2004/12/14.
149. Marr KA, White TC, van Burik JA, Bowden RA. Development of fluconazole resistance in Candida albicans causing disseminated infection in a patient undergoing marrow transplantation. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1997;25(4):908-10. Epub 1997/11/14.
150. de Micheli M, Bille J, Schueller C, Sanglard D. A common drug-responsive element mediates the upregulation of the Candida albicans ABC transporters CDR1 and CDR2, two genes involved in antifungal drug resistance. Molecular microbiology. 2002;43(5):1197-214. Epub 2002/03/29.
151. Vale-Silva LA CA, Ischer F, Parker JE, Kelly SL, Pinto E, Sanglard D. Azole resistance by loss of function of the sterol $\Delta^{5,6}$-desaturase gene (ERG3) in Candida albicans does not necessarily decrease virulence. Antimicrobial agents and chemotherapy. 2012;56(4):1960-8.
152. Eddouzi J, Parker JE, Vale-Silva LA, Coste A, Ischer F, Kelly S, et al. Molecular mechanisms of drug resistance in clinical Candida species isolated from Tunisian hospitals. Antimicrobial agents and chemotherapy. 2013;57(7):3182-93. Epub 2013/05/01.

VII. Annexes

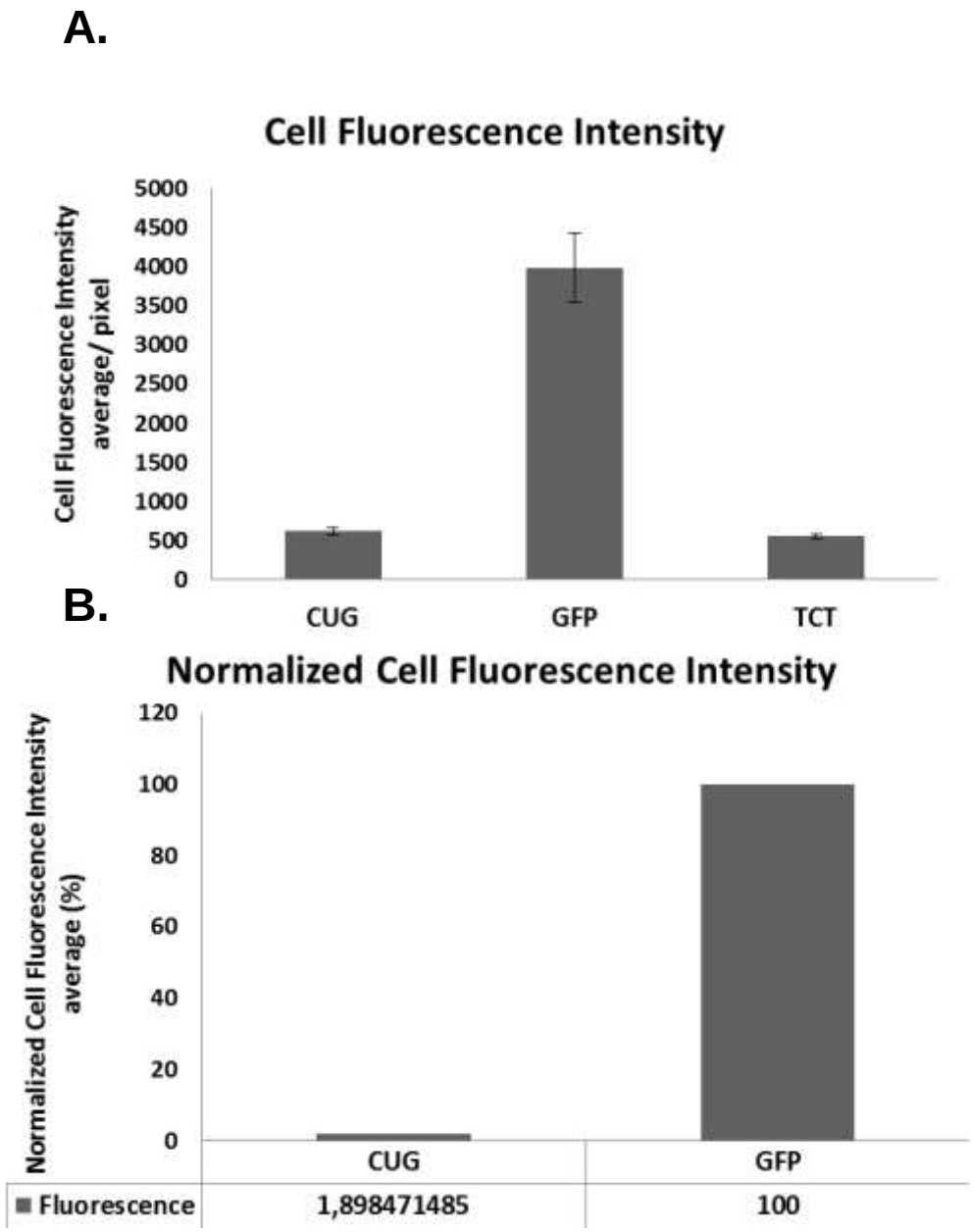
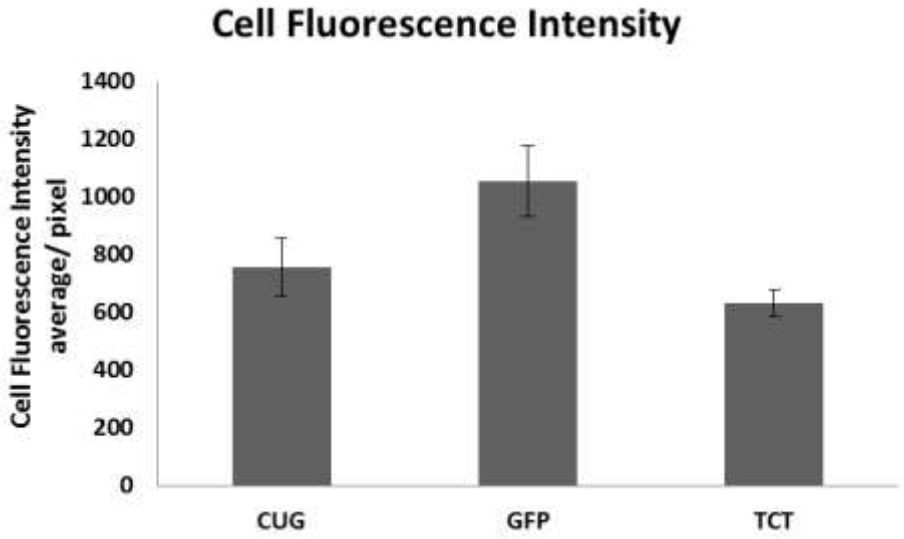


Figure 25 - Percentage of mistranlation of the strain T0 of the 5 passage grew without fluconazole (B). Absolut values of each clone (A).

A.



B.

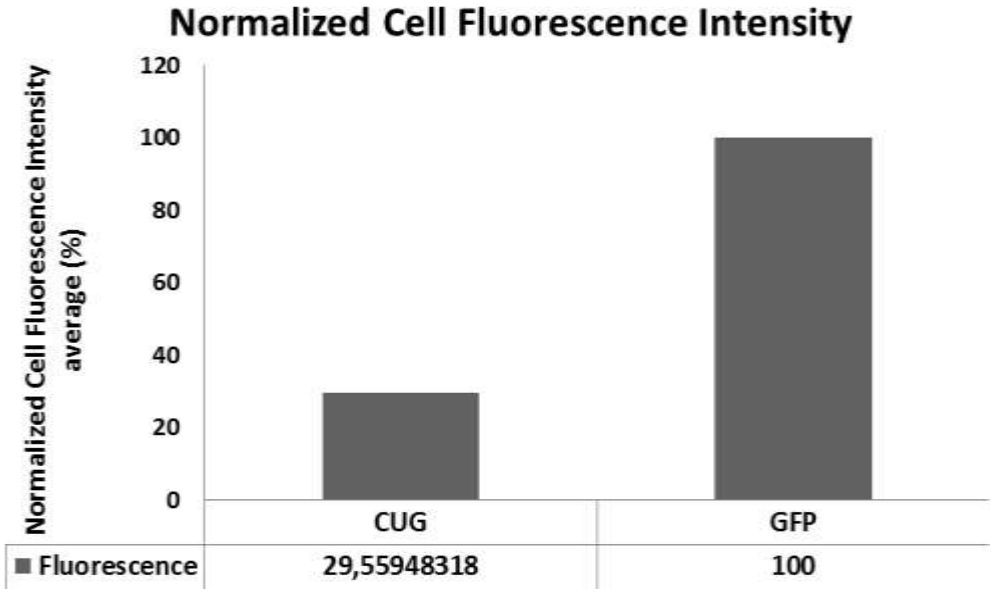
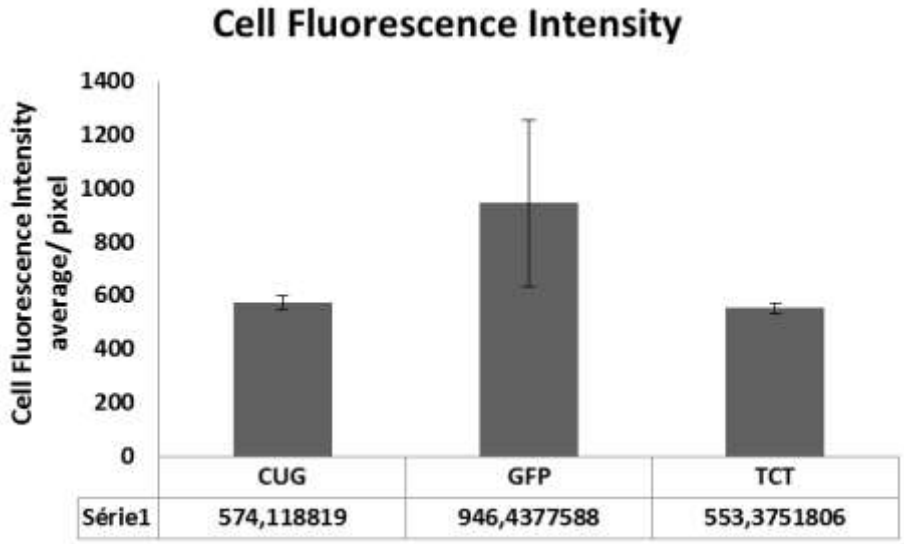


Figure 26 - Percentage of mistranlation of the strain T1 of the 5 passage grew without fluconazole (B). Absolut values of each clone (A).

A.



B.

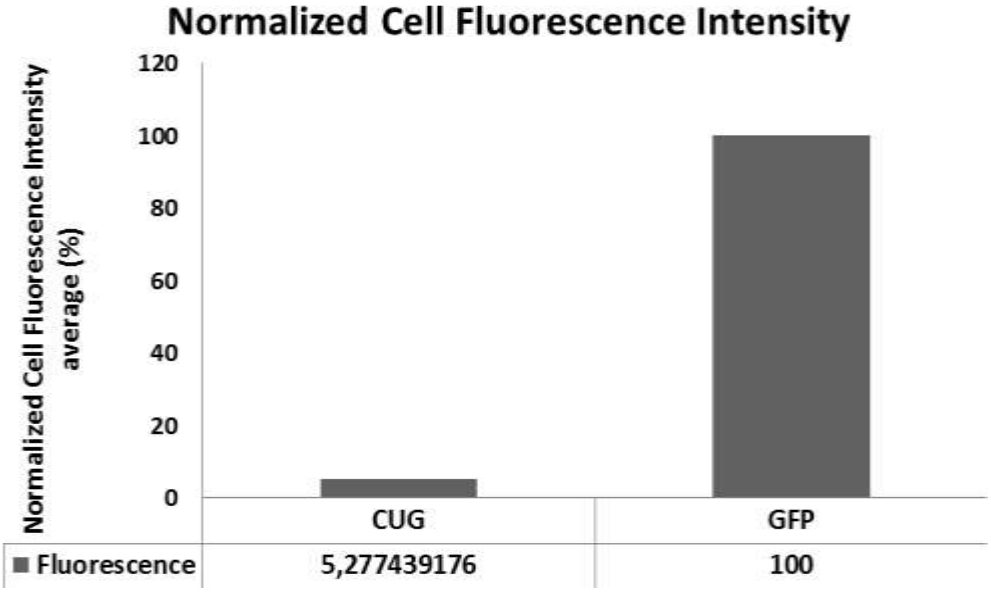


Figure 27 - Percentage of mistranlation of the strain T2 of the 5 passage grew without fluconazole (B). Absolut values of each clone (A).

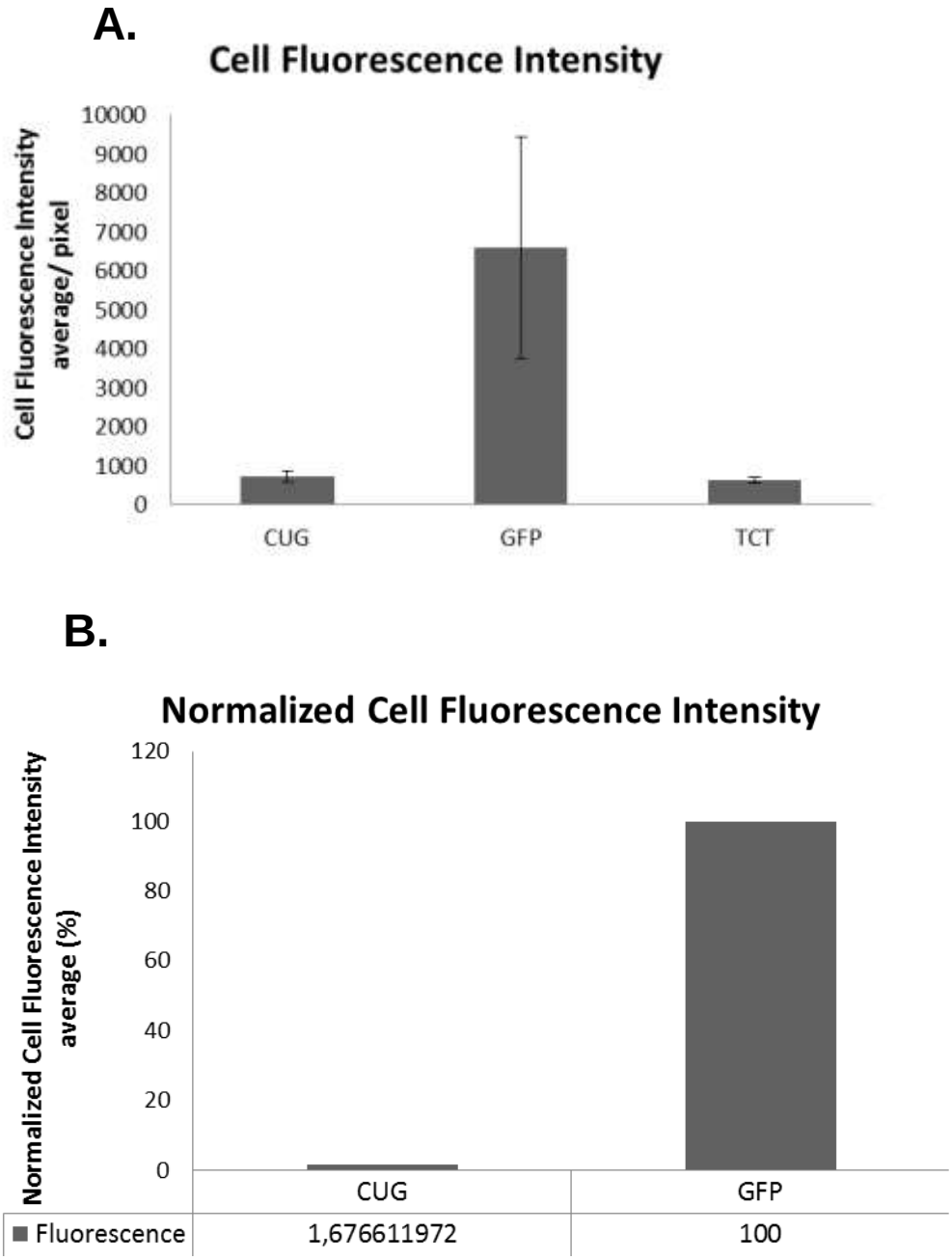
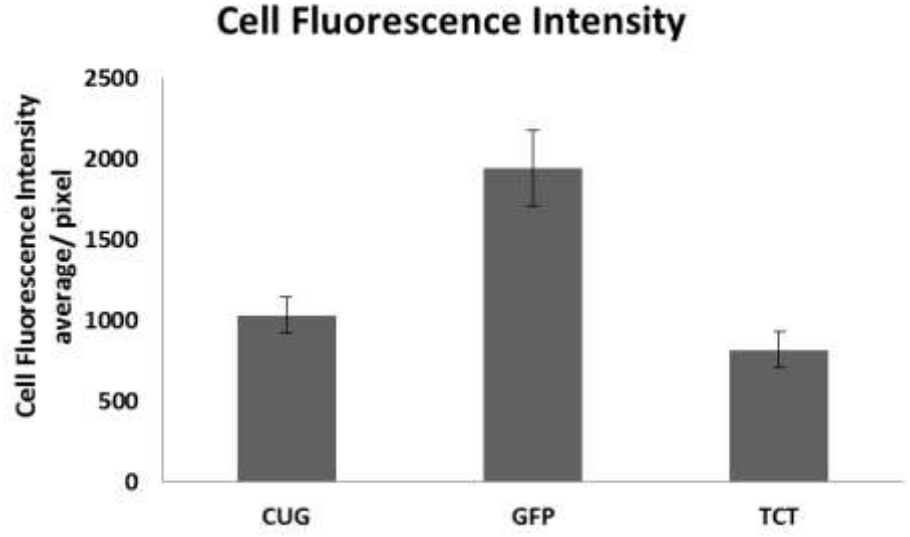


Figure 28 - Percentage of mistranlation of the strain T0 of the 5 passage grew with fluconazole (B). Absolut values of each clone (A).

A.



B.

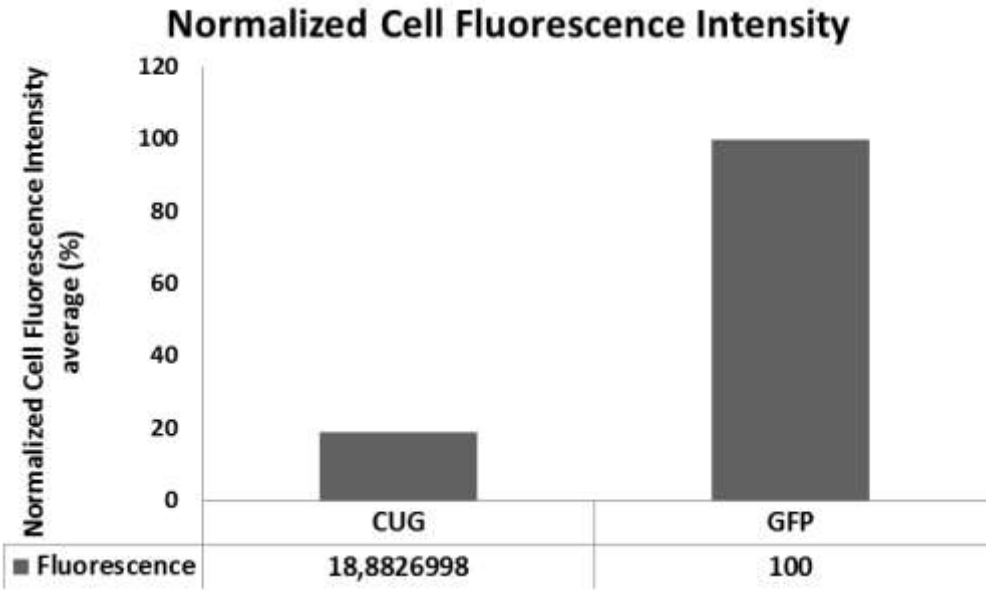


Figure 29 - Percentage of mistranlation of the strain T1 of the 5 passage grew with fluconazole (B). Absolut values of each clone (A).

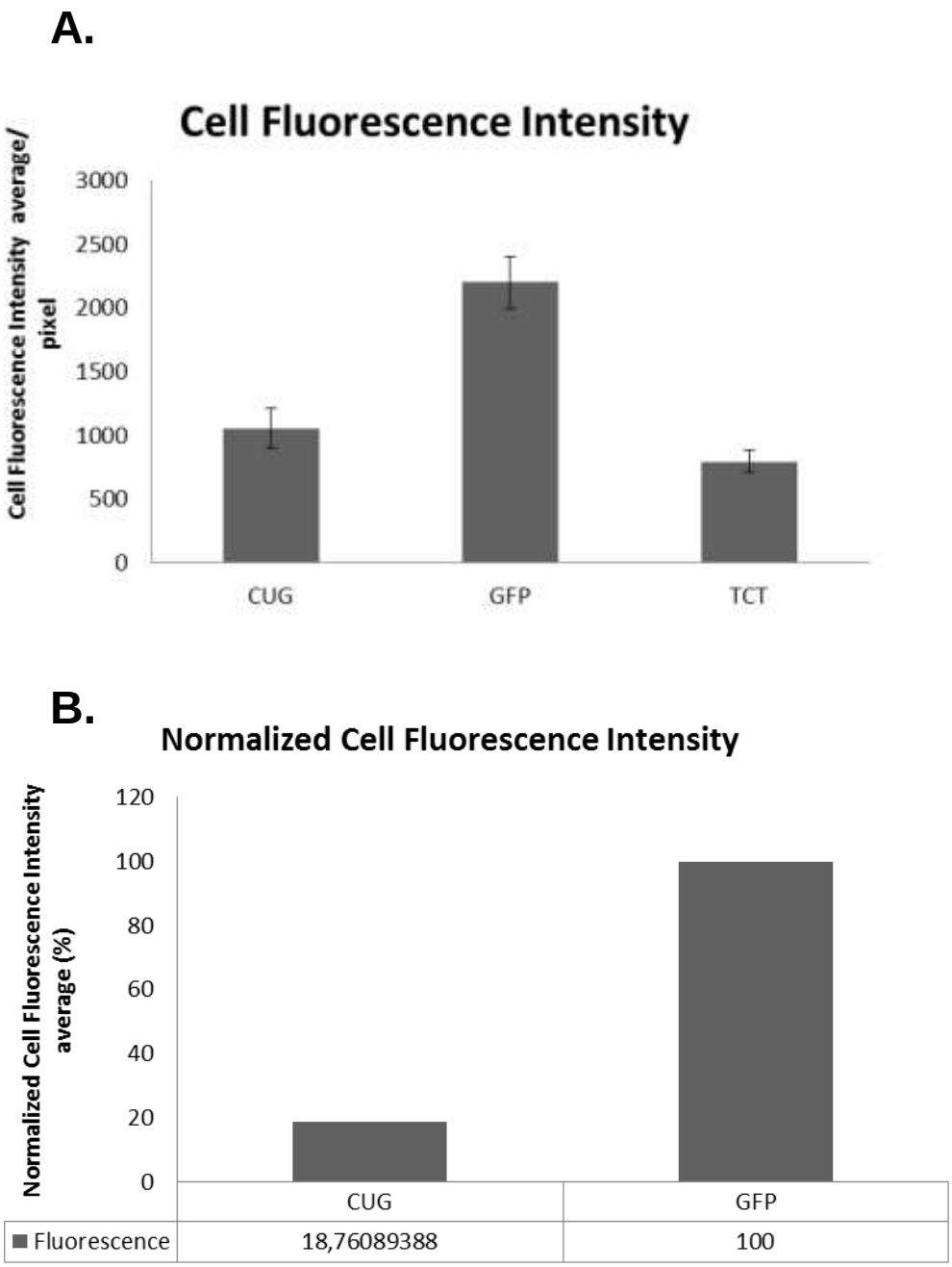


Figure 30 - Percentage of mistranlation of the strain T2 of the 5 passage grew with fluconazole (B). Absolut values of each clone (A).

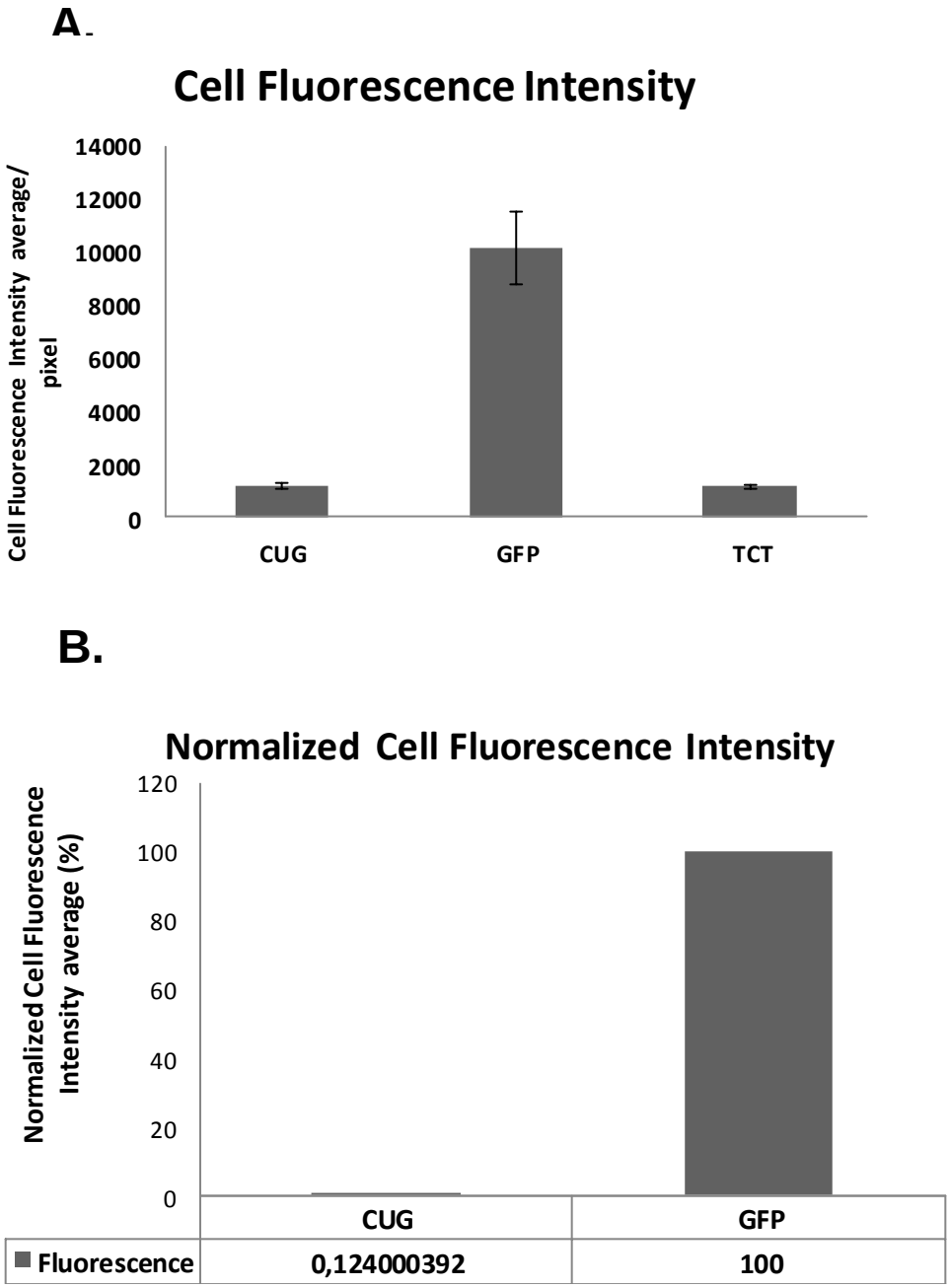
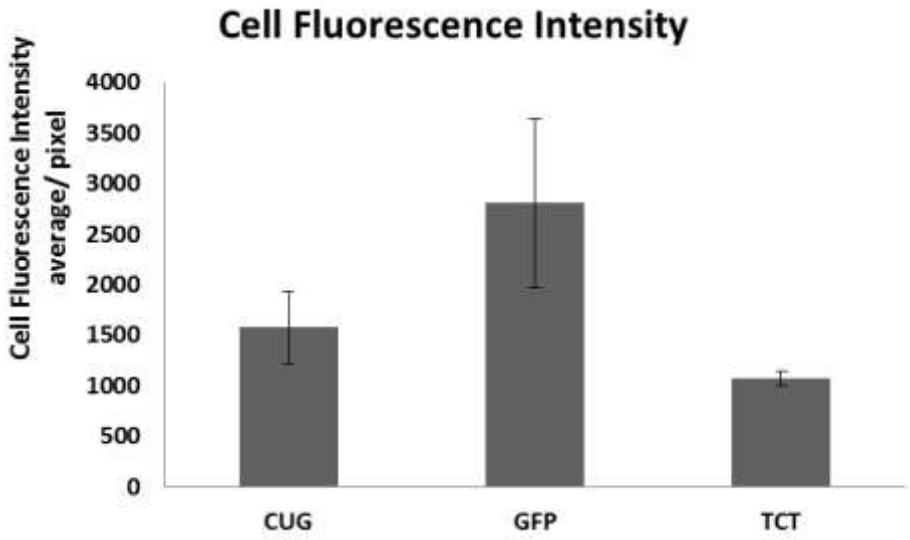


Figure 31 - Percentage of mistranlation of the strain T0 of the 10 passage grew without fluconazole (B). Absolut values of each clone (A).

A.



B.

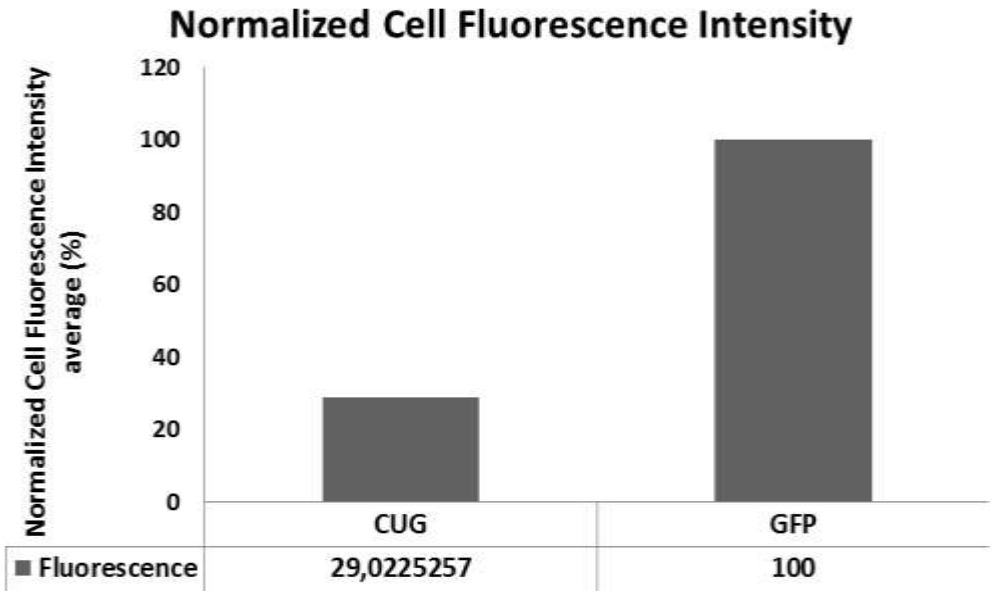


Figure 32 - Percentage of mistranlation of the strain T1 of the 10 passage grew without fluconazole (B). Absolut values of each clone (A).

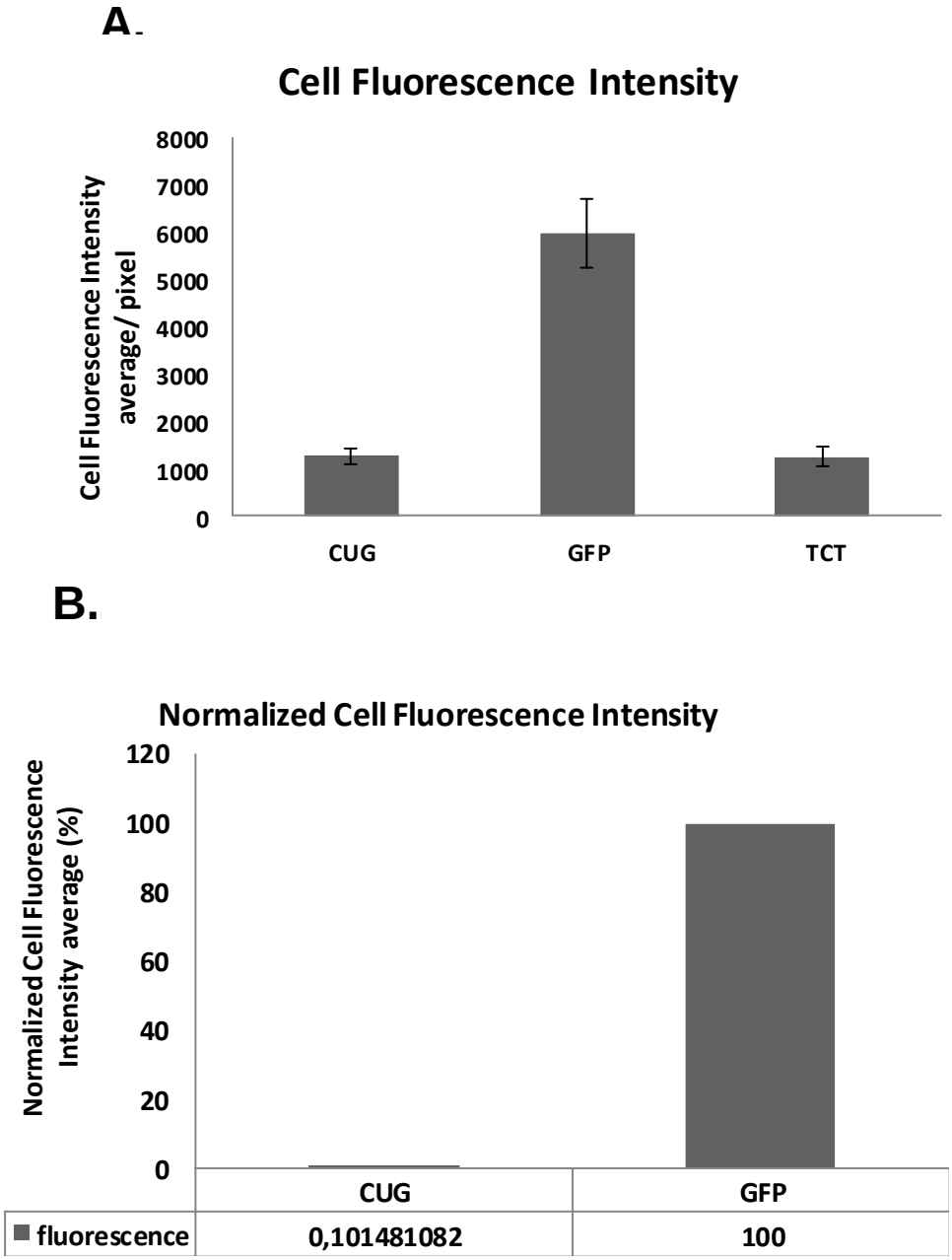


Figure 33 - Percentage of mistranlation of the strain T0 of the 10 passage grew with fluconazole (B). Absolut values of each clone (A).

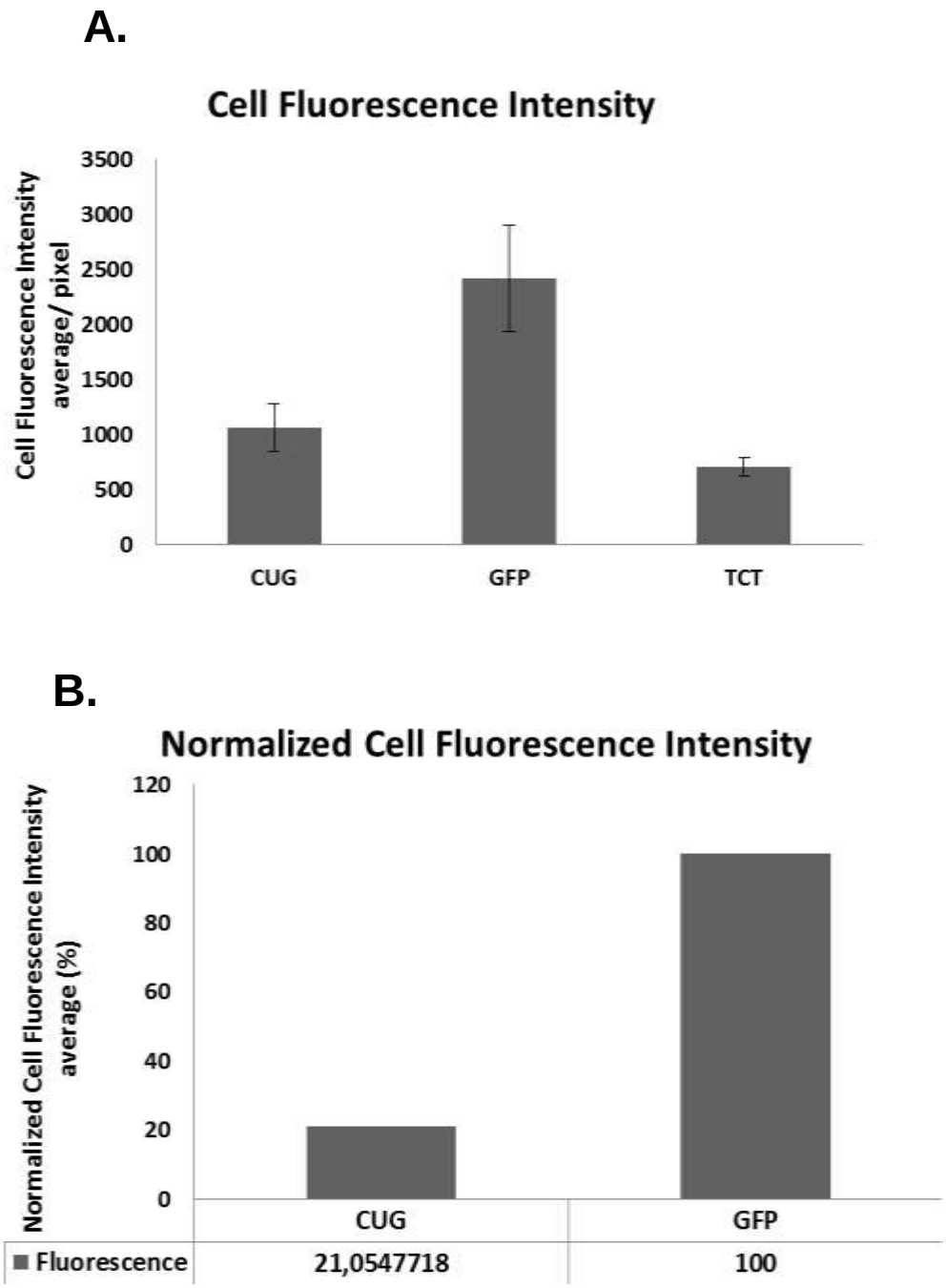


Figure 34 - Percentage of mistranlation of the strain T1 of the 10 passage grew with fluconazole (B). Absolut values of each clone (A).