Filipa Isabel Serra e Silva

Mistranslation and telomere stability

Erros de tradução e estabilidade telomérica

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

I would like to dedicate my thesis to my beloved family, especially my parents, brother and grandmother. Last but not least, I would like to dedicate my thesis to my best friend and the love of my life, Alexandre.

o júri / the jury

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Key-words Mistranslation, mRNA, telomere, aging, genome stability.

Abstract The regulation of a stable proteome is crucial for the cell homeostasis. The translation process from the nucleotide sequence of a gene into the aminoacid sequence of a protein is associated with a basal error of 10^{-4} which the cell deals with through quality control mechanisms. The misincorporation of aminoacids into *de novo* synthesized proteins tends to rise when the cell is exposed to stressful conditions. The increase of dysfunctional proteins produced by mistranslation may induce expression of genes related to stress response and genome destabilization. In this work we used yeast as a model to study the impact of high mistranslation rates in telomere stability, since the telomeric and adjacent sub-telomeric regions are key elements on the preservation of genome integrity. Results shown that some types of induced mistranslation may in fact have an impact on telomere length, and also that some proteins' activity, such as Pnc1 and Sir2, is important regarding telomeric DNA stability. These results shed a new light over the importance of controlling mRNA mistranslation rates in eukaryotic cells.

Palavras-chave *Erros de tradução*, ARN mensageiro, telómero, envelhecimento, estabilidade genómica

Resumo

A manutenção de um proteoma estável é crucial para a homeostase celular. Ao processo de tradução da sequência de nucleótidos de um gene para a sequência de aminoácidos de uma proteína está associada uma taxa de erro basal de cerca de 10^{-4}, com a qual a célula lida através de mecanismos de controlo de qualidade das proteínas. A incorporação incorrecta de aminoácidos nas proteínas sintetizadas *de novo* tende a aumentar quando as células estão expostas a condições de stress. Por sua vez, o aumento de proteínas disfuncionais provocado por erros de tradução pode induzir a expressão de genes de resposta ao stress e destabilização do genoma. Neste trabalho, utilizou-se a levedura como modelo para o estudo do impacto de uma elevada taxa de erros de tradução ao nível da estabilidade dos telómeros, uma vez que esta região, juntamente com a região subtelomérica, representa um elemento-chave na preservação da integridade do genoma. Metodologias de engenheria de tRNA foram utilizadas para induzir erros de tradução, sendo a técnica de *Southern blot* escolhida para análise do padrão de migração electroforética de fragmentos de ADN correspondentes à região subtelomérica. Os resultados obtidos demonstraram que os erros de tradução podem, de facto, ter impacto a nível do comprimento dos telómeros, dependendo do tipo de erro de tradução induzido, permitindo ainda confirmar a importância da actividade de proteínas como Pnc1 e Sir2 no controlo da estabilidade do ADN telomérico, lançando uma nova luz sobre a importância do controlo da taxa de erros de tradução nas células eucarióticas.

Contents

List of Abbreviations

PNC1	Pyrazinamidase/nicotinamidase 1
S. cerevisiae	*Saccharomyces cerevisiae*
A site	Acceptor site
aaRS	Aminoacyl-tRNA synthetase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumine
DNA	Deoxyribonucleic acid
E site	Exit site
EDTA	Ethylenediaminetetraacetic acid
eEF	Eukaryotic elongation factor
Estp	Est protein
EtBr	Ethidium bromide
LiAc	Lithium acetate
MM	Minimal medium
MM-leu	Minimal medium minus leucine
mRNA	Messenger RNA
Na	Nicotinic acid
NaAD	Deamido NAD
NAD^+	Nicotinamide adenine dinucleotide

Nam	Nicotinamide
NaMN	Nicotinic acid mononucleotide
OD	Optical density
P site	Peptidyl site
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pnc1p	Pnc1 protein
PRPP	5-phosphoribosyl-1-pyrophosphate
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotation per minute
SDS	Sodium dodecyl sulfate
SIR2	Silent information regulator 2
Sir2p	Sir2 protein
SSC	Saline-sodium citrate
TE	Tris-EDTA
Tna1p)	Nicotinic acid permease
TPE	Telomere Position Effect
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA

Chapter 1

Introduction

1.1 The importance of tRNAs and tRNA synthesizing enzymes in the definition of the genetic code.

1.1.1 The genetic code

The genetic code was revealed in the 1960's as the correspondence between the nucleotide sequence in genomic DNA and the sequence of amino acids in proteins. Combinations of three nucleotides represent a code for one amino acid, in a total of 64 combinations, named codons. Of these, 61 code for the regular 20 amino acids (Figure 1.1). The remaining codons, UAG, UAA and UGA are known as stop codons, since they signal the end of an amino acid sequence and do not code for a specific amino acid [1]. Some amino acids are encoded only by one codon, as triptophan (UGG codon) and methionine (AUG codon). The latter is also known as the start codon, since it signals the beginning of a protein sequence in the genome. The remaining 59 codons code for 18 amino acids, which means that there are amino acids that are assigned by multiple codons and that is why the genetic code is referred to as degenerate. Codons that encode the same amino acid are named synonymous codons and frequently have the same two first nucleotides. A group of two synonymous codons is a 2 codon set while a group of 4 synonymous codons is known as a "family box".

1.1.2 Transfer ribonucleic acids (tRNAs)

Transfer RNAs (tRNAs) are the key elements on the correspondence between genetic code and amino acids. The tRNAs are small RNA sequences, typically composed of 73 to 93 nucleotides and their secondary structure is often represented in a shape that resembles a cloverleaf, with four base-paired stems (Figure 1.2). In this structure, three stem loops are

[1] In some specific circumstances, these stop codons are overridden and may code for two extra amino acids: selenocysteine and pyrrolysine.

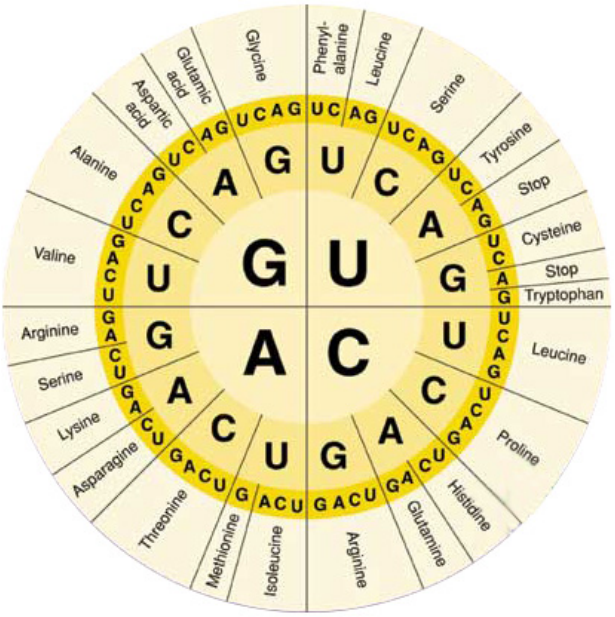

Figure 1.1: The standard genetic code. Adapted from (Budisa, 2006).

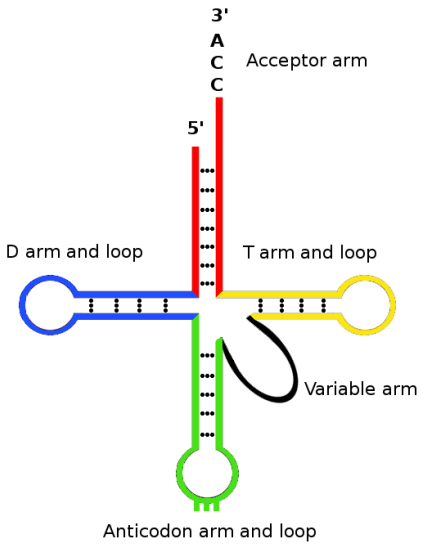

Figure 1.2: Schematic representation of the cloverleaf Secondary structure of tRNA showing its domains in different colors.

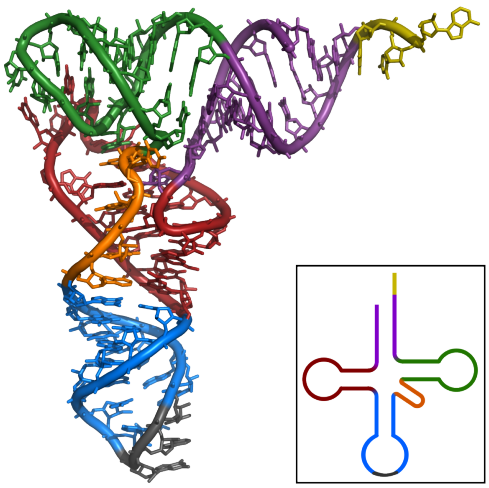

Figure 1.3: Three dimensional structure of tRNA, showing its domains in different colors, violet: acceptor stem; red: D-loop; blue: anticodon loop; orange: variable loop, green: T-loop, yellow: CCA-3' of the acceptor stem; gray: anticodon. The insert scheme shows the tRNA cloverleaf secondary structure, with the corresponding color code for the different domains.

apparent, namely the D-loop, anticodon loop and T-loop. Also in the cloverleaf structure, the remaining base-paired stem, a 3' single stranded CCA sequence, is known as the isoacceptor arm and is the attachment point of the amino acids with which the tRNA is charged. The number of residues present in the stem and stem-loops is conserved among tRNAs, but there is also a variable region between the anticodon and T loops that separates the tRNAs in two classes: Class I, found in Archaea, and Class II, found in Eubacteria and Eukaryotes. Class I tRNAs have shorter variable loops than the Class II tRNAs. The former have normally 4 to 5 bases while the latter have 10 to 24 bases. Leucine and serine tRNAs, which are of particular importance in the context of the present work, are included in Class II tRNAs. The 3D structure of the tRNA has a characteristic shape that resembles an inverted L (Figure 1.3). This conformation maximizes the stability of the structure by aligning the base pairs in the D stem with the ones present in the anticodon stem, as well as the base pairs in the T stem with the ones in the acceptor stem. This conformation defines two functional domains in the tRNA: one that directly interacts with the mRNA, through the anticodon loop, and another where the amino acid is covalently attached. Transfer RNAs are therefore, the key elements on the correspondence between genetic code and amino acids. Noteworthy, tRNAs have other roles in the cell besides mRNA decoding in protein synthesis. They are also involved in the degradation pathway of proteins, and are precursors in the synthesis of other tRNAs (Francklyn and Minajigi, 2010)

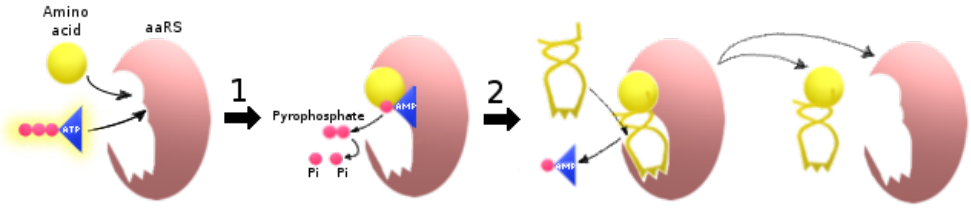

Figure 1.4: Schematic representation of the steps involved in tRNA aminoacylation: (1) aaRS aminoacylation - an aaRS recognizes its cognate amino acid as substrate and catalyzes the activation of the amino acid with adenosyl phosphate from ATP, releasing pyrophosphate; then, the complex binds a cognate tRNA and the aaRS catalyzes the transfer of the aminoacyl group onto the tRNA acceptor stem, releasing cyclic Adenosine monophosphate (AMP) (2); The aminoacylated tRNA leaves the active site of the enzyme leaving it ready for another tRNA aminoacylation round (Budisa, 2006).

1.1.3 Aminoacyl-tRNA synthetases

Aminoacyl tRNA synthetases (aaRSs) are enzymes that play a critical role in the biological process of translation. They catalyze the formation of a covalent bond between tRNAs and their cognated amino acids, to yield aminoacyl-tRNAs. Each aaRS has a corresponding tRNA and its recognition is made by identity elements distributed along the tRNA structure. Each tRNA also has negative determinants to avoid recognition by non-cognate aaRSs. After aminoacylation, the tRNA can enter the ribosome and transfer the attached amino acid to a nascent polypeptide chain, thus participating in the establishment of the genetic code.

The mechanism by which the aaRS acts can be described as a two step reaction: first, the aaRS activates the amino acid that it recognizes as substrate in a reaction involving ATP, forming an aminoacyl adenylate and releasing inorganic pyrophosphate. The complex, formed by the aaRS and the activated amino acid, recognizes its cognate tRNA and then transfers the amino acid onto the tRNA, releasing adenosine monophosphate (AMP) (Figure 1.4). This process is highly regulated both at the charging of the aaRS with the amino acid and at the tRNA aminoacylation in order to ensure translation fidelity. When an aaRS is charged with a non-cognate amino acid, a mechanism of pre-transfer editing is activated and the bond is hydrolyzed before the transfer to the tRNA occurs (Figure 1.5). When this mechanism is not activated, a second editing mechanism takes place, to prevent the incorrect aminoacylation of the tRNA (Cochella and Green, 2005). A third editing mechanism takes place during protein elongation, when a mischarged tRNA is hydrolyzed before it binds to the eukaryotic elongation factor 1A, eEF1A, (post-transfer editing) (Ling

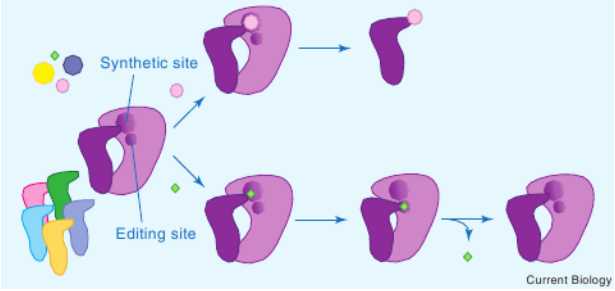

Figure 1.5: Editing mechanism that define the tRNA aminoacylation fidelity. Each aaRS choses its cognate tRNA through a number of specific 'identity elements' (represented by the different tRNA colors). Selection of the cognate amino acid occurs in two steps: first, the synthetic site of the aaRS excludes amino acids that are larger than the cognate one, or that cannot establish sufficient specific interactions (mechanism represented in the top pathway), secondly, if smaller amino acids with some similarity to the cognate one are misincorporated by the synthetic site they are hydrolyzed in a distinct site of the enzyme, the editing site (mechanism represented in the bottom pathway) (Cochella and Green, 2005).

et al., 2009).

The aaRSs maintain their specificity and fidelity by the preferential attachment to their cognate amino acid and by the correct discrimination between the structurally or chemically similar amino acids (Ling et al., 2009). Since the tRNAs cannot self aminoacylate, aaRSs establish, in fact, the genetic code (Donoghue and Luthey-schulten, 2003; Rodin and Rodin, 2008). The aaRSs can be split classified in two major classes, Class I and Class II. With exception of LysRS (Donoghue and Luthey-schulten, 2003), each amino acid is transfered to the tRNA only by one of the two classes of enzymes. Interestingly, each class is specific for 10 amino acids (Rodin and Rodin, 2008) and is very different regarding sequence, secondary and tertiary structures. Also their tRNA recognition is accomplished by symmetrical mechanisms. Class I aaRSs approach the tRNA acceptor arm by its minor groove side while Class II aaRSs recognize the acceptor arm by its major groove side. This fact is consistent with the aaRSs convergent evolution theory which states that both classes were originated from a common DNA sequence, translated both in sense and anti-sense direction (Rodin and Rodin, 2008). The quaternary structure of these two classes also differs, as Class I enzymes are mostly monomers while Class II aaRSs are homo or heterodimers. Both classes can be further divided into three subclasses, namely a, b or c, that provide a better sorting of amino acids as they distinguish amino acids with similar chemical properties (Figure 1.6).

Some aaRSs or 'aaRS-like'-proteins also play an important role in cellular processes not directly related to aminoacylation activity, but rather to the ability of these enzymes to

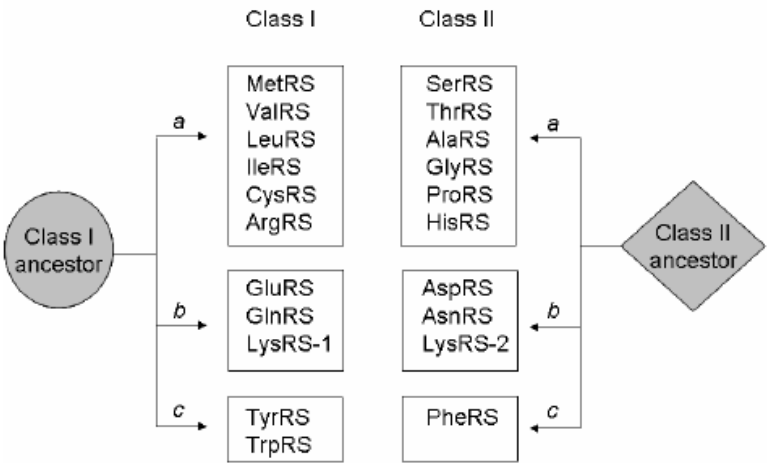

Figure 1.6: The two classes of aminoacyl-tRNA synthetases and their sub-classes (Gomes, 2008).

bind RNA molecules. Some interesting processes in which aaRSs take part are maturation of tRNA precursors, quality control and regulation of translation of their own genes, intron splicing, amino acid metabolism or cell signaling (Brown et al., 2010). These enzymes also seem to be implicated in the process of apoptosis, aging, cancer and human autoimmune diseases (Ibba et al., 2005).

1.2 Translation in Eukaryotes

Translation is the last stage in protein synthesis and can be divided in four major steps: the initiation of the synthesis, elongation of the nascent protein, termination of the synthesis and recycling of translation machinery into a new protein synthesis cycle (Gebauer and Hentze, 2004). The protein synthesis initiation step takes place when the ribosome is stationed at the mRNA's initiation codon (AUG) together with a methionyl tRNA at its peptidyl ('P') site (Figure 1.7). At the beginning of the elongation step, an aminoacyl tRNA enters the acceptor ('A') site (Figure 1.7) of the ribosome and if it is correctly aminoacylated, the codon in the mRNA is decoded and the ribosome synthesizes the peptide bond between the newly incorporated amino acid and the previous one. This process occurs continuously, with the shifting of the mRNA chain and the arrival of the following tRNA, until the elongation complex reaches the stop codon in the mRNA. At this point, the termination stage takes place, with the release of the finished peptide. The final step in translation is the recycling of the ribosome. At this stage, the ribosomal subunits are

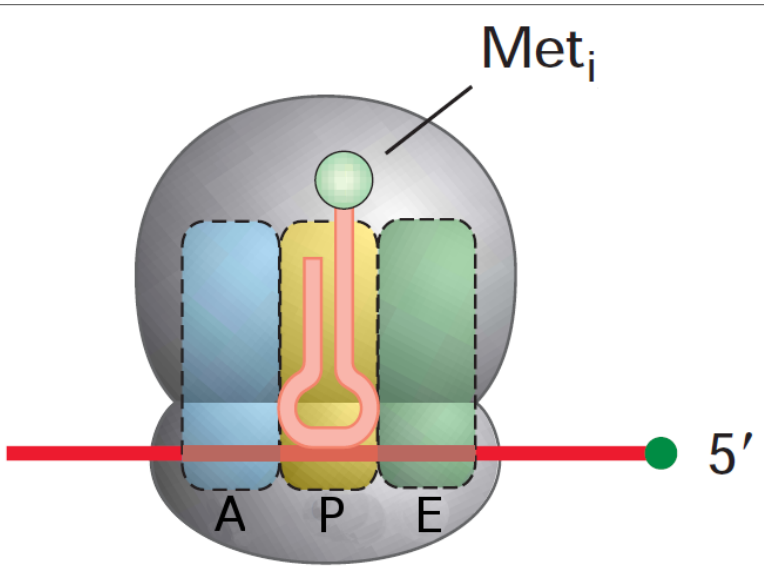

Figure 1.7: The ribosome contains three RNA binding sites named A, P and E. The 'A' site binds the tRNA, aminoacylated with its specific amino acid. The 'P' site binds a peptidyl-tRNA, which is the tRNA bound to the peptide being synthesized. The 'E' site binds a free tRNA before it exits the ribosome, after the aminoacylated transfer to the novel peptide has occurred. Met$_i$ corresponds to the initiator tRNA, that decoded the start Methionine codon. (Adapted from (Lodish et al., 2003))

disassembled and the subunits of the initiation complex are now free and able to resume the process of mRNA translation to a novel peptide.

1.3 Errors in protein synthesis and their consequences

The translation of mRNA sequences into amino acid sequences is a crucial process in the cell and needs to be monitored in order to keep its homeostasis. The fidelity of the translational process assures that a stable and functional proteome is produced. Despite the accuracy of the translational machinery, there is a basal error rate associated to mRNA decoding. In normal conditions the overall translation error is between 10^{-3} and 10^{-4} in yeast (Lee et al., 2006) and this occurs due to an accumulation of errors in the multiple steps involved in the process (Reynolds et al., 2010). If the quality control during protein synthesis is not performed appropriately, the cells tend to be more sensitive to stress and this slows down the cell growth (Reynolds et al., 2010).

Although translation is extremely regulated, there are stages of the process where errors are most prone to occur. These error-prone stages are generally related with processes

of recognition and discrimination of the substrates used in protein synthesis by the machinery involved in the process (Reynolds et al., 2010). In Eukaryotes, errors in protein synthesis can arise at five major steps (Figure 1.8): transcription, splicing, translation, protein folding and post translational modifications (Drummond and Wilke, 2009). During transcription, errors can occur due to polymerase slippage or wrong incorporation of nucleotides. Splicing errors also contribute to this major error cascade, due to exon skipping or unsuccessful removing of introns. Translation errors can result from misincorporated amino acids due, for instance, to incorrect interactions between codon-anticodon (Ling et al., 2009). During translation, other errors, such as incorrect acylation of tRNAs, are prone to occur. Errors can also arise in the ribosome as it can read-trough a stop codon or stop the reading prematurely. A frameshift can also occur, resulting in a change of the coded message in the mRNA. Protein folding errors might happen due to kinetic trapping, spontaneous unfolding or breaking of the folding as a result of an incorrect polypeptide formation. Finally, errors can also arise during post-translational modifications, since proteins can be incorrectly cleaved, wrongly glycolized, phosphorylated or ubiquitinylated (Drummond and Wilke, 2009).

The basal error rate associated with translation might seem irrelevant, but it means that 15% of all produced proteins in a cell will have a misincorporated amino acid (Drummond and Wilke, 2009). The problems arise when the error rates are augmented by induced stress conditions, such as amino acid starvation, heat-shock, exposure to toxics, etc (Parker and Precup, 1986; Silva et al., 2009). The synapse region in neuronal cells provides an interesting overview of a rich and changeable environment due to variations of oxygen and free calcium levels, pH and other highly stressful and dynamic conditions that may favor protein aggregation.

Interestingly, in yeast, (*Saccharomyces cerevisiae*), errors in translation can result in phenotypic diversity and in pre-adaptation to stress (Silva et al., 2007). Moreover, it has been shown by True and Lindquist (True and Lindquist, 2000) that in yeast, the stop-codon read through in [PSI^{+}] (a prion protein) leads to production of new inheritable phenotypes, suggesting that high rates of mistranslation may even trigger genomic changes and, consequently, evolution of the genome.

Errors in translation also play a role in cell degeneration, disease development and aging. Lee et al. (Lee et al., 2006), demonstrated that misfolding proteins accumulated due to mischarging of tRNA in neurons is associated with rising levels of cytoplasmic protein chaperons and activation of stress response mechanism such as the unfolded protein re-

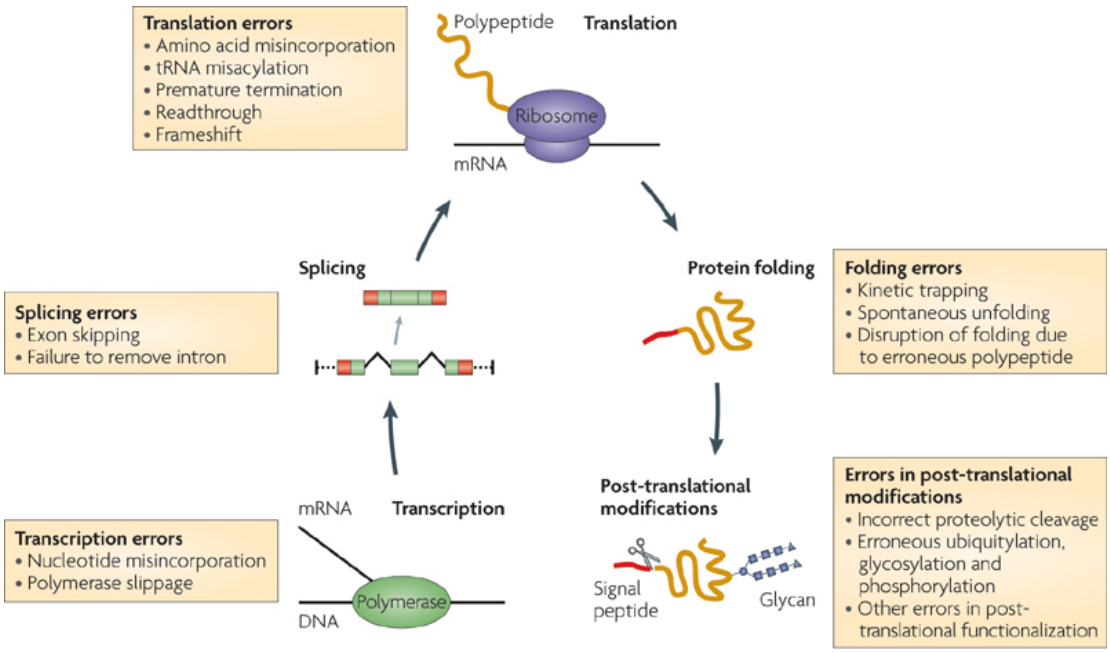

Figure 1.8: Types of errors that occur during the different stages of protein synthesis in Eukaryotes, from transcription to protein post-transcriptional modifications (Drummond and Wilke, 2009)

sponse. These authors also mentioned that missense mutations in the editing domain of an alanyl-tRNA synthetase can cause severe impact in the cell, as in the phenotype described in the 'sticky mouse' model. In this model, a mutant mouse strain develops a sticky secretion on its fur which is accompanied by lack of muscle control, ataxia, loss of Purkinje cells in the cerebellum and, eventually, premature death. Other authors showed that some mutations in mitochondrial tRNAs were shown to lead to neuromuscular, neurodegenerative diseases, hypertension and dyslipidemia (Ramirez-Alvarado et al., 2010).

Translation errors result in the formation of an an heterogeneous pool of proteins. While proteins that are accurately synthesized can be properly folded, the ones that accumulate synthesis errors might have different fates. Some may still be folded in a normal way, with the help of protein quality control mechanisms, while the remaining might be targeted for degradation or accumulate in the cell in protein aggregates (Drummond and Wilke, 2009). When a cell has a high rate of protein synthesis, it cannot afford to have a large pool of wrongly synthesized proteins or protein aggregates. Protein refolding or degradation are very effective mechanisms to rescue cells from the effects of aberrant protein accumulation, as they trigger an enzymatic cascade that cooperates with lysosomes. Ubiquitination plays a crucial role in tagging proteins that will be eliminated by proteasome activity (Malgaroli et al., 2006). However, when aberrant proteins are not removed or refolded, they can compromise cell viability (Tabner et al., 2001). In fact, protein aggregation is strongly related to disorders such as Parkinson or Alzheimer's diseases, frequently associated with cell aging (Drummond and Wilke, 2009).

1.4 Yeast as a model system to study mistranslation

Yeast, is a good model to study cellular processes conserved in Eukaryotes. It has a rapid generation time, it is easy to propagate in both haploid and diploid forms, facilitating genetic analysis and has a highly efficient system of homologous recombination. It is also very easy to transform with plasmids that remain stable in dividing cells. It is also an excellent model to study the consequences of mistranslation, since the mechanisms of proteome quality control and of degradation of proteins are conserved from yeast to man (Morimoto, 2008).

In our laboratory, we have developed a yeast model to study mRNA mistranslation through the introduction of a $tRNA_{CAG}^{Ser}$ from the human pathogen *Candida albicans* that decodes leucine CUG codons as serine (Silva et al., 2007). In this engineered cell system,

CUG codons are being mistranslated as serine at a rate of 2.4% (Silva et al., 2007), which represents a 240 fold increase in mistranslation relative to the typical error of 10^{-4}. Gene expression was monitored in mistranslating cells relatively to control cells, using DNA-microarrays and the results obtained by this technique revealed alterations in the expression of genes belonging to stress response, to carbohydrate and amino-acid metabolism, cell wall structure, protein synthesis and degradation. The stress response gene group showed the most prominent upregulation (34%). Silva et al. (Silva et al., 2007) quantified protein expression in this model system, using radiolabelled ^{35}S-Methionine and 2D-PAGE protein fractioning, observing an up-regulation of 43 proteins belonging to the stress response, as well as up-regulation of proteins involved in degradation pathways. Down-regulation was observed for 34 proteins involved in protein synthesis and amino-acid metabolism. While protein expression quantification data confirmed DNA-microarray data, the fold-induction in both mRNA and protein levels was different for some genes, namely the *PNC1* gene. The mRNA derived from the transcription of this gene was up-regulated by 2.1 fold, while the protein that it encodes, Pnc1p (pyrazinamidase/nicotinamidase 1), showed a surprising increase of 29.5 fold.

PNC1 is an important age-related gene and was also reported to be both necessary and sufficient for lifespan extension by calorie restriction in yeast under low-stress conditions. However, under non-stressing conditions, yeast strains with extra copies of *PNC1* gene lived 70% longer than the wild type (Anderson et al., 2003). Pnc1p converts nicotinamide to nicotinic acid in the NAD^+ salvage pathway (Figure 1.9). Interestingly, nicotinic acid is the precursor of NAD^+, a molecule that is used by the silent information regulator 2 protein (Sir2p). This enzyme is a NAD^+ histone deacetylase that is found in a protein complex with other members of the Sirtuins family, known as the Sir complex. This complex is responsible for chromatin silencing in important repetitive regions of yeast genome, such as telomeres, ribosomal DNA (rDNA) and mating-type *loci*, preventing recombination and fusion of DNA throughout cell division (Gallo et al., 2004; Tollefsbol, 2009). Since Sirtuins actively depend on NAD^+, they become susceptible to fluctuations in concentrations of this particular substrate, thus affecting the extension of chromatin silencing at telomeres and rDNA genes. Sirtuins are also inhibited by high concentrations of nicotinamide (Gallo et al., 2004).

Chromatin silencing has a direct impact on gene expression as it determines the state of activation or repression of certain genes (Tollefsbol, 2009). Genes that are coded in open chromatin (euchromatin) are transcribed and genes that are near condensed chromatin

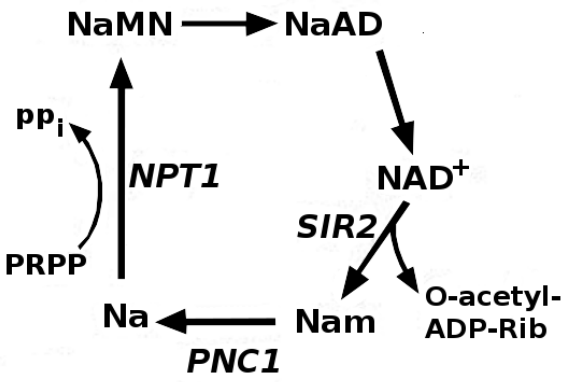

Figure 1.9: In NAD^+ salvage pathway, nicotinamide (Nam) is produced by Sir2p and converted to nicotinic acid mononucleotide (NaMN) by Pnc1p and Nicotinate phosphoribosyltransferase (Npt1p). Npt1p can also utilize nicotinic acid (Na) imported into the cell by the nicotinic acid permease (Tna1p). PRPP: 5-phosphoribosyl-1-pyrophosphate; NaAD: deamido-NAD. Adapted from (Sandmeier et al., 2002)

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(heterochromatin) are not. In yeast, genes are silenced in regions near telomeres, and this is referred to as the telomere position effect (TPE) (Emre et al., 2005). Chromatin silencing at telomeres is mediated by the action of the Sir complex (Figure 1.10), which interacts with the sub-telomeric region, silencing genes by forming a protein cap that protects the telomeric DNA from degradation. Telomeric silent regions, as well as other heterochromatin silenced regions, such as the silent mating type loci and rDNA are rendered inaccessible to the transcription machinery and degrading enzymes (Allis et al., 2007). When the recruitment of Sir complexes is extended to sub-telomeric regions, this can result in the silencing of genes that are found in this particular location, which is rich in stress-response genes.

Experimental evidences obtained in our laboratory (Silva et al., 2009) showed that the over-expression of Pnc1p in mistranslating yeast cells resulted in an increase of Sir2p activity. Using an *URA3* reporter gene integrated at the telomere region, Silva et al. (Silva et al., 2009) were able to show that the increased Sir2p activity in mistranslating yeast was associated to telomere chromatin silencing, since the mistranslating yeast transformants were not able to grow in minimal medium lacking uracil. This phenotype is explained by the silencing of the *URA3* reporter gene present in the telomeric region, therefore confirming the link between the up-regulation of Pnc1p levels of expression increased Sir2p activity and chromatin silencing at telomeres in mistranslating yeast cells. The mistranslating

yeast model suggests that the accumulation of aberrant proteins in the cells ma lead to the activation of mechanisms of genome stabilization such as telomere silencing, which are highly conserved in Eukaryotes (Brachmann et al., 1995).

Telomerase is one of the mechanisms conserved in Eukaryotes that is responsible for maintaining the integrity of telomeres. As the cell ages and divides telomeres become shorter, and ultimately, the decrease in telomere length determines the lifespan of a cell. It has been shown that yeast strains that have mutations in genes required for telomerase function show progressive shortening of telomeric DNA and growth arrest in the G2/M phase after 60–75 generations (Tollefsbol, 2009). It is believed that in yeast cells with shorter telomeres, the pool of Sir protein complexes (see Figure 1.10) that protect the telomeric region, redistributes to other regions of the genome, in what is speculated to be a general process of genome stabilization under stress conditions (Tollefsbol, 2009).

Telomere length is maintained stable due to telomerase activity. Yeast telomerase is composed by the reverse transcriptase catalytic subunit (Est2p), encoded by *EST2* gene, an RNA template (coded by *TLC1* gene), and two additional protein subunits Est1p and Est3p (encoded by *EST1* and *EST3* genes). The telomerase RNA template is the responsible for the repetitive sequences generated with the enzyme activity, which are $C_{1-3}A/TG_{1-3}$. However, it has to be noted that yeast cells have alternate ways to maintain their telomere length. In fact, in critical situations, when telomeres become too short due to loss of telomerase action and the cells become senescent, recombination of the telomeric repetitive sequences may occur. Experimentally, two types of post-senescent survivors can be found, with a distinct telomere fragment pattern: type I survivors show amplification of the Y' elements (repetitive sequences in sub-telomeric region) and have very short TG_{1-3} repetitive tracts on chromosome ends, while type II survivors have a variable pattern of long tracts of TG_{1-3} repeats (Chen et al., 2009).

1.5 Objective of this work

In order to ascertain if the activity of Pnc1p and Sir2p proteins play a role in eventual telomere stabilization, following the lead of the works of Silva et al. (Silva et al., 2009, 2007), this work evaluated the effects of mistranslation in yeast telomeric length. For this, we used a yeast model system available in our laboratory where a constitutive codon misreading is induced, using a mutant tRNA that misreads leucine as serine (Silva et al., 2007).

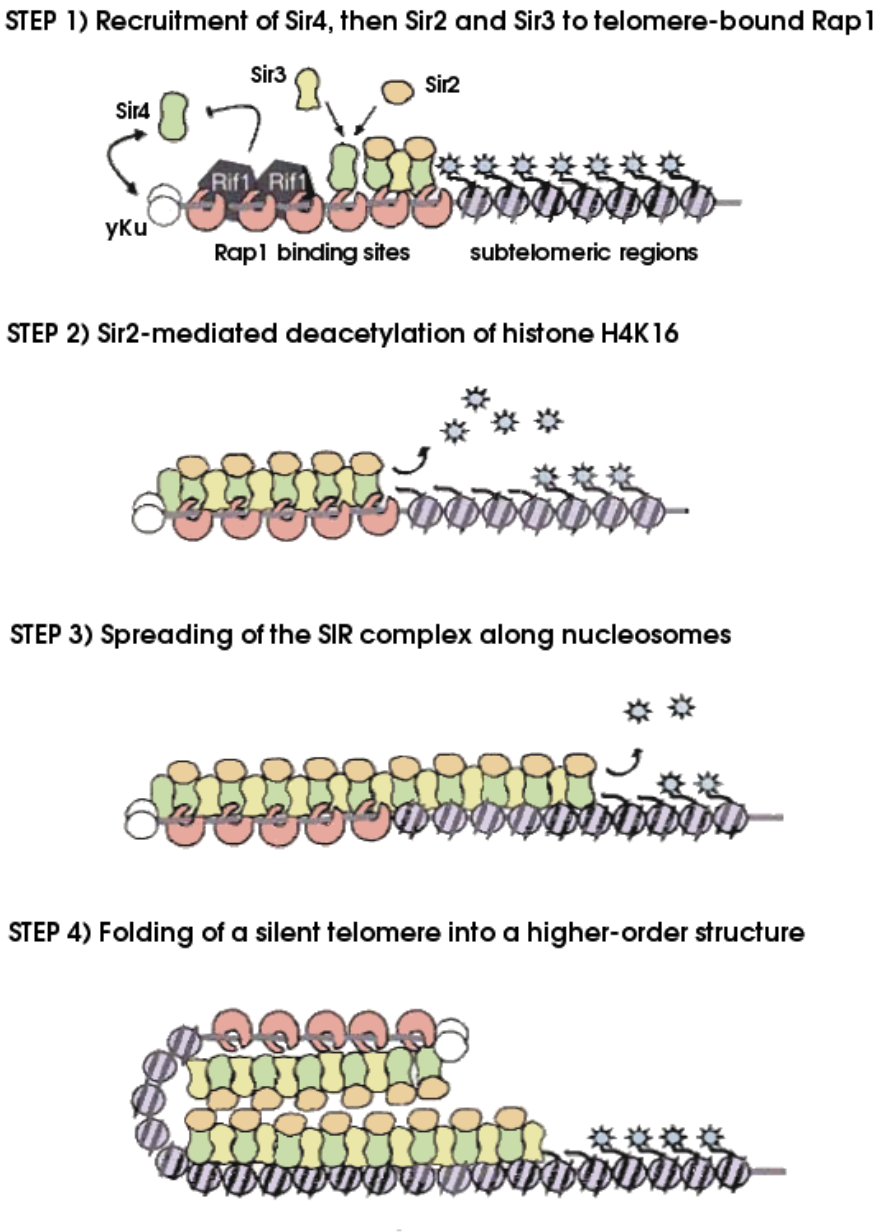

Figure 1.10: The four steps for chromatin silencing at telomeres. Step 1) At telomeres, Rap1 and yKU provide the binding site for Sir4, that is antagonized by Rif1. Sir 3 and Sir2 join Sir4 and establish the Sir complex. Step 2) Sir2 deacetylates lysine residues on the nearby histones, and Step 3) Sir complex spreads along nucleosomes. Step 4) The silent chromatin 'matures' at the end of M phase and creates an inaccessible structure (Allis et al., 2007).

Southern blot analysis was used to probe the length of the repetitive sequences of chromosome ends in which mistranslation was induced in order to evaluate whether or not putative telomere region destabilization could be reflected in altered telomere length.

A wild type strain was tested in parallel to strains of the same genotype where the genes *PNC1* and *SIR2* genes were alternatively deleted. A double deletion mutant, lacking both *PNC1* and *SIR2* genes was also investigated. As a control for telomere region destabilization, the investigation included a mutant yeast strain lacking *EST2* gene, which codes for the catalytic subunit of telomerase, an enzyme responsible for the maintenance of the length of telomeric repetitive regions.

Chapter 2

Materials and Methods

2.1 Materials and Methods

2.1.1 Strains

Haploid *S.cerevisiae* strains used in this study were based on strain BY4742 background and were acquired from EUROSCARF (Table 2.1). Strains were transformed with the plasmids pRS315 (Control), pUKC715 ($tRNA_{CAG}^{Ser}$) as described in (Santos et al., 1996).

Table 2.1: Yeast strains used in this study.

Strain	Abbreviation	Genotype
BY4742	B42	Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0
BY4742Δest2	E	BY4742;Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YLR318w::kanMX4
BY4742Δpnc1	P	BY4742; Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YGL037c::kanMX4
BY4742Δsir2	S	BY4742; Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YDL042c::kanMX4
BY4742Δsir2Δpnc1[a]	SP	BY4742; Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YGL037c: :kanMX4;YDL042c: :natMXthis

[a] Kindly provided by Tatiana Lima Costa, who constructed the double mutant from strain BY4742Δsir2

2.1.2 Growth conditions

Yeast strains were propagated and grown in rich medium (YEPD, 1% Yeast extract, 2% Bacto-Peptone, 2% glucose).

Growth curves were performed in minimal medium (0.2% Drop-out mix containing all the essential amino acids but leucine (MM-leu). Cultures were monitored for optical density (OD) at 600 nm with an initial optical density of 0.1. General growth conditions were 30°C as incubation temperature and 170 rotations per minute. Solid media was used when necessary by adding 2% agar to the above described medium. Media and materials were sterilized before use by autoclaving. The OD measurement made for the growth curves were plotted using a graph $\log(OD_{600})$ as a function of time and a trend line was adjusted to the experimental data, its slop representing the rate of growth of the clones.

2.1.3 Transformation of yeast strains

Transformation of *S. cerevisiae* was accomplished using the lithium acetate (LiAc) method described by Gietz and Woods (Gietz and Woods, 2001). Briefly, overnight cultured cells were diluted into 10 mL of fresh YEPD medium to an initial OD_{600} of 0.1. The culture was incubated at a temperature of 30°C, with 170 rpm agitation for about 5.5 hours until an OD_{600} of 0.4 - 0.5 was reached. Cells were then harvested by centrifugation for 5 minutes at 5220 g, washed in deionized, RNAse free, water and resuspended in 500 µL of 0.1M LiAc solution. A volume of 50 µL of the previous suspension was transferred to Eppendorf tubes and centrifuged in a bench top centrifuge at maximum speed for 15 seconds. The resulting supernatant was rejected and the following reagents were added to the pellet in sequence: 240 µL of 50% (w/w) PEG, 36 µL of 1.0M LiAc, 25 µL of previously denatured single-stranded salmon sperm carrier DNA (2 mg/mL) and 1 µL of plasmid pRS315 (500 µg/µL) or 1 µL of plasmid pUKC715 (500 µg/µL). The mixture was vortexed until complete resuspension of the pellet and submitted to a heat-shock treatment (42°C, for 30 minutes). Cells were harvested by centrifugation for 1 minute at 2300 g, the supernatant was rejected and the pellet resuspended in deionized, RNAse free, water. For selection of the transformed clones, 10 µL of the cell suspension transformed with pRS315 plasmid and 100 µL of the cell suspension transformed with pUKC715 plasmid were plated in solid MM-leu medium and incubated for 3 to 4 days at 30°C, until formation of colonies. Selected colonies were propagated in solid MM-leu medium to obtain a cell stock that was stored at -80°C in MM-leu medium supplemented with 50% glycerol.

2.1.4 Extraction of genomic DNA

Genomic DNA extraction was carried out using an adaptation of the protocol described by Hoffman and colleagues (Hoffman and Winston, 1987). Cells were collected from a cell culture in stationary growth phase (with $0.D._{600}$ between 2.0 and 3.0) by centrifugation for 5 minutes at 5220 g. The pellet was washed twice in deionized, RNAse free, water and resuspended in 200 µL of lysis buffer (10 mM Tris-HCl, 100 mM NaCl - pH 8.0, 1mM Na_2EDTA, 100xTriton 2% and SDS 1%). A volume of 200 µL of 25:24:1 Phenol-Chloroform-Isoamyl alcohol at 4ºC, and 0.3 g of 0.5 mm diameter glass beads were then added to the cell the suspension. The mixture was vortexed for 10 minutes and 200 µL of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) was added. The mixture was centrifuged for 5 minutes at 16000 g in order to isolate the supernatant. DNA in the supernatant was precipitated by addition of 1 mL of absolute ethanol at -20ºC for 3 hours and collected by centrifugation at 16000 g for 5 minutes. The pellet was resuspended in 400 µL of TE. RNA contamination was removed by adding 60 µL of a 1 µg/µL RNAse A solution (Roche) followed by incubation at 37ºC for 5 hours. Protein contamination was removed by adding 10 µL of a 1 µg/µL Proteinase K solution (Roche) followed by incubation at 65ºC for 16 hours. Genomic DNA was precipitated with 50 µL of 3.0 M Sodium acetate (pH 5.0) and 1 mL of cold (-20ºC) absolute ethanol followed by a 24 hour incubation at -20ºC. Finally, DNA was collected by at 16000 g for 5 minutes at 4ºC, and the pellet was dried at room temperature before resuspension in 70 µL of deionized, RNAse free, water. The resulting genomic DNA was quantified using NanoDrop and its integrity was evaluated by electrophoresis (0.8% agarose gel, 80V voltage for 60 minutes).

2.1.5 Southern blot analysis

Prior to southern blot analysis, 8 µg of genomic DNA were digested with 50 units of Xho I restriction enzyme (New England BioLabs, Inc.), overnight, at 37ºC, in a NEBuffer 4 (50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT at a pH of 7,9 at 25ºC), supplemented with 100 µg/mL of Bovine Serum Albumine (BSA) solution. Following the restriction digestion, the enzyme was inactivated by heat, incubating samples at 65ºC for 20 minutes.

In order to study mistranslation effects in telomere length we introduced and optimized Southern blot technique in our laboratory. Briefly, 8 µg of previously digested gDNA were subjected to electrophoresis in a 1% agarose gel (20x15x0.5 cm) running at 50V for 20h. A

molecular marker solution (1 kb ladder, Fermentas) was used to monitor the electrophoresis results. Later the gel was stained in a 2.5 µM ethidium bromide (EtBr) solution.

The migration of the bands was identified with a ruler placed aside the gel and the resulting images were registered with Quantity One software (Bio-rad). The gel was incubated in a denaturing solution (3.0M NaCl, 0.5M Tris-HCl, pH 7.5) for 15 minutes. The gel was then covered with a neutralization solution (1.5M NaCl, 0.5M NaOH) for 5 minutes, with a mild shaking to homogenize the solutions.

DNA was transfered into Hybond N^+ membrane using a vacuum system (VacuGene XL, Pharmacia Biotech). To set the apparatus for transfer, the membrane was soaked in deionized water and placed on to the vacuum platform. The gel was then placed over the membrane and covered with transfer buffer 20xSSC (0.3M Tri-sodium citrate and 3.0M NaCl at a pH of 7.0). After 1.5 hours of transfer, the membrane was rinsed in 2xSSC and left to dry between Whatman 3MM paper. DNA was then immobilized by placing the membrane, with DNA side down and then DNA side up, in a transilluminator (254nm wavelength, 120 kJ). To confirm that the DNA transfer into the membrane was complete, the gel was restained with EtBr 2.5 µM solution.

The probe sequences were detected by promoting hybridization with a radioactive labeled probe based in a specific *S.cerevisiae* Y' element. The sequence of the probe was described by Martin et al. (Martin et al., 2009)(5'- TGT GGG TGT GGT GTG TGG GTG TGG TG -3'). For radioactive labeling 1 µL (10 µM) of the probe was mixed with 30 units of T4 kinase (Takara Bio Inc.), 2 µL of 10xT4 buffer kinase, , 30 µCi of [γ^{32}]ATP and 2 µL of 1M Spermidin in a total reaction volume of 20 µL (deionized, RNAse free water was added to reach the total reaction volume). The mixture was incubated for 1h at 37°C. In order to purify the labeled oligo, 80 µL of deionized, RNAse free, water and 100 µL of Phenol:Chloroform:Isoamylalcohol (25:24:1) were added to the labeling mixture, vortexed, and centrifuged at 16000 g for 5 minutes. The supernatant containing the probe was added to the hybridization solution. The membrane was pre-hybridized 20 mL of hybridization solution (20xSSPE, 50xDenhart solution, 10%SDS) for 3 hours, at 65°C, in a rotating oven at 12 rpm) and then replaced with 20 mL of fresh hybridization solution. The hybridization was carried out at 65°C over night, with 12 rpm rotation.

To remove excess of probe, the membrane was washed twice at room temperature with 50mL of washing solution (20xSSPE, 10%SDS) for 3 minutes, at room temperature and then washed twice at 65°C with 50 mL of washing solution, at 65°C also for 3 minutes. Excess liquid was absorbed from the membrane using Whattman 3MM paper. The membrane

was then concealed in a plastic bag and put against a phosphorous screen in a radiographic cassette (Bio-rad) for 3 days.

To visualize the DNA probed bands, Bio-Rad's Personal Molecular Imager was used with a resolution of 50 µm.

Chapter 3

Results

3.1 Effect of mistranslation in the growth of yeast cells.

After the transformation of the *S.cerevisiae* cells, two clones were selected from strains transfered with the pRS315 plasmid control clones, (C1 and C2) and four clones were selected from strains transformed with pUKC715 plasmid coding for $tRNA_{CAG}{}^{Ser}$ (M1 to M4). Both plasmids had a leucine marker that allowed the transformed cells to grow in selective media MM-Leu. Monitorization of growth was performed for all clones (see Appendix) by measuring the optical density at 600 nm of cells in culture media, from inoculation of the fresh media until the stationary phase was reached. The results displayed in Figure 3.1 represent an example of the obtained growth curves. The control clone, C1, was selected as a representative of a non-mistranslating clone of the BY4742 transformants. The mistranslation clone, M3, was selected to represent mistranslating clones of the BY4742 strain transformants. Comparing both C1 and M3 clones of BY4742, it is clear that the non-mistranslating clone reaches the stationary phase faster than the mistranslating clone. In general, all strains followed the same pattern as the example displayed in Figure 3.1. Control clones with the pRS315 plasmid reached the stationary phase faster than the mistranslating clones, which contained pUKC715 with $tRNA_{CAG}{}^{Ser}$. Growth rates (measured as the rate of OD increase with time) were calculated from the data points obtained at the exponential growth phase and were represented in a bar graphic for comparison (Figure 3.2). In this figure it is apparent that of the tested strains some variability in growth rate is found among clones, specially among mistranslating clones.

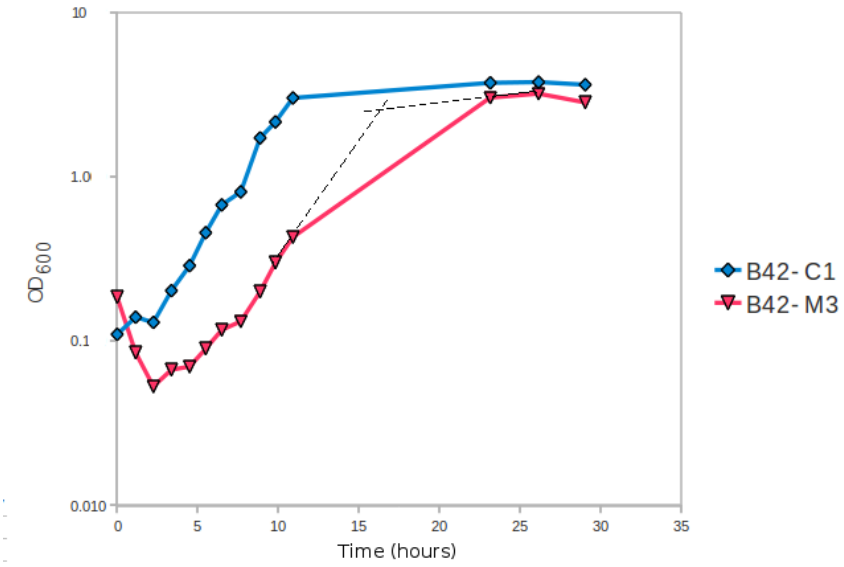

Figure 3.1: Growth curves obtained for a control (C1) and a mistranslating (M3) clone of strain BY4742 (B42). A broken line represents a trend line for exponential and stationary phase growth, from extrapolation of the data points. The control clone reaches the stationary phase faster than the mistranslating clone.

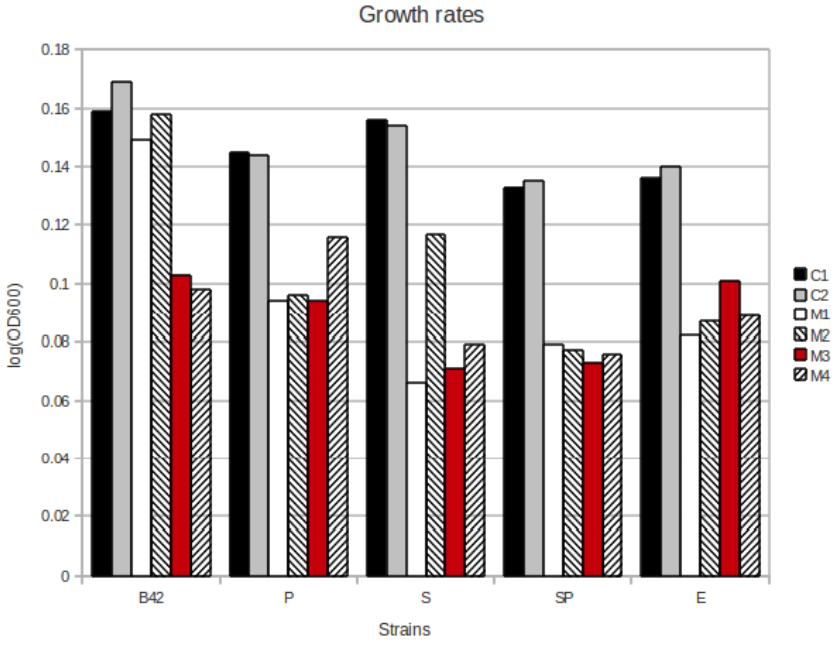

Figure 3.2: Growth rates measured for control and mistranslating clones for all tested strains. Generally, control clones showed a higher growth rate when compared to mistranslating clones.

3.2 Monitorization of telomere length by Southern blot analysis.

Monitorization of telomere fragment length was conducted by Southern blotting. Only one control clone (C1) and one mistranslating clone (M3) were selected from each strain for monitorization.

Prior to Southern blot analysis, genomic DNA was digested with Xho I, an enzyme that specifically recognizes a restriction sequence at the sub-telomeric Y'-element, and separated in an agarose gel. Figure 3.3 shows an example of this separation by electrophoresis. DNA hybridization was performed using a radioactive labeled probe sequence, described by Martin et al. (Martin et al., 2009), that detects Y'-element fragment sequences (around 1.3 kb) present in the sub-telomeric region of yeast chromosomes. In this study, *EST2* gene deletion strain was used as a control since it lacks the gene that encodes Est2 protein (Est2p), the catalytic component of telomerase, therefore rendering the telomeres prone to alterations, particularly at the Y'-element repetitive regions. For direct comparison of telomere fragment length, all tested clones were analyzed in the same electrophoresis gel. Southern blot analysis results can be observed in Figure 3.4.

In the panel A of Figure 3.4, a band pattern belonging to the *EST2* gene deletion strain (E) is readily apparent, as it differs from the other patterns. Samples from this strain show multiple bands with high intensity, in the case of both control (C1) and mistranslating (M3) clones. This band pattern was not observed in the other tested strains, which showed mainly a single strong band with a molecular weight around 1.3 kb, where the Y'-element fragments are expected to appear, as described by Martin et al. (Martin et al., 2009). Panel B of Figure 3.4 B shows a detail of the gel region around the 1.3 kb.

The analysis of the results shown in Figure 3.4 indicate that no significant differences were found between B42 control and mistranslating clones, regarding Y' element fragment length. However, some differences in fragment length can be inferred between B42 clones and those of the deletion strains, specifically for mistranslating clones of these strains. These results are found irrespective of the deletion strain monitored, indicating that mistranslation had an important impact on Y'-element size, only when yeast strains had deletions on *PNC1*, *SIR2* or both genes. In summary, Figure 3.4 shows that mistranslating clones in strains with deleted genes (P, S, SP strains) have decreased length in Y-element fragments, that is to say, shorter telomere lengths. This figure also shows that the E strain, where telomerase activity was impaired, produced a pattern of multiple DNA

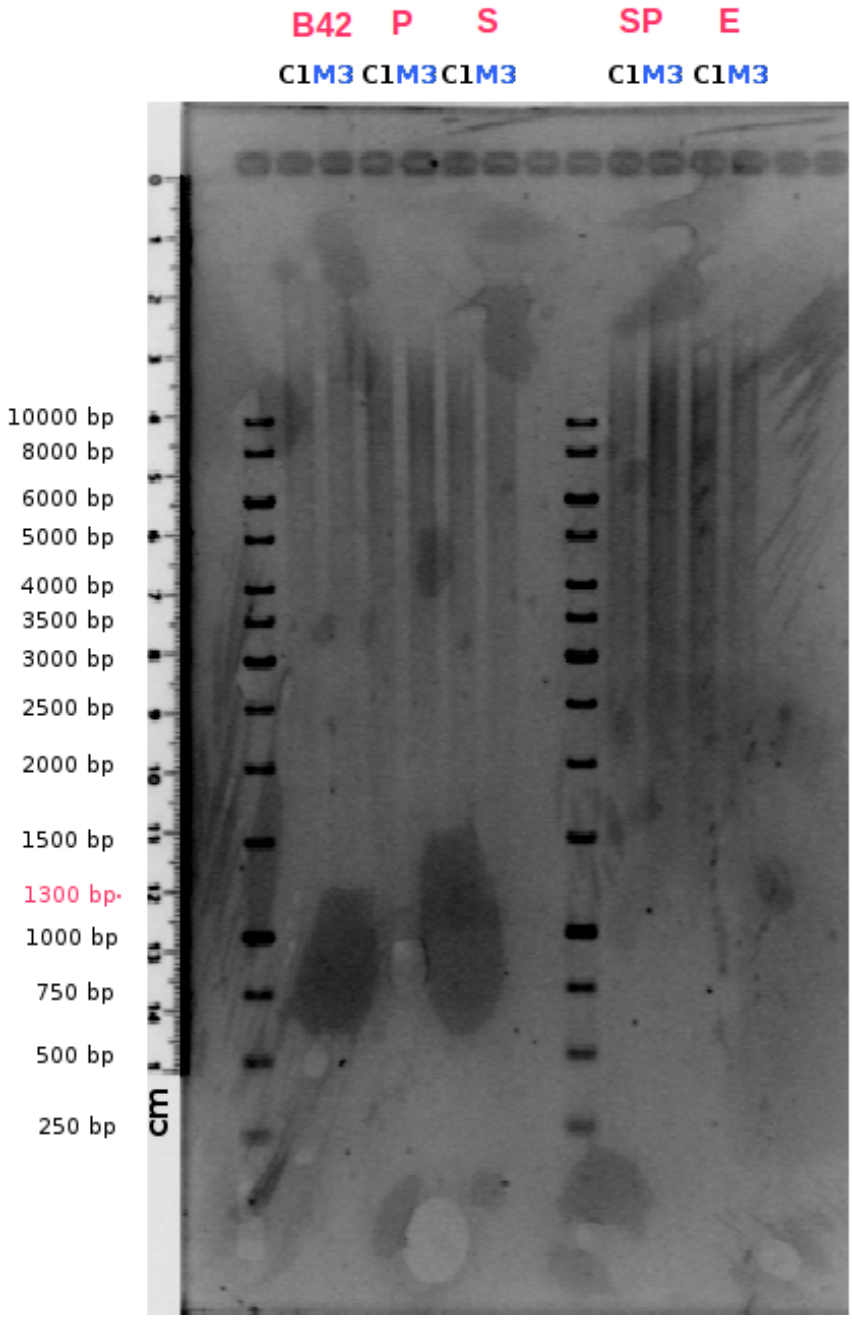

Figure 3.3: Image obtained from an agarose gel electrophoresis where the restriction digestion pattern of the genomic DNA obtained from all the tested strains was compared, following digestion with Xho I. B42 represents the wild type BY4742 strain, while P, S, SP and E represent strains where *PNC1*, *SIR2*, both *PNC1* and *SIR2*, or *EST2* genes were deleted, respectively. Control clones are represented as C1, while mistranslating clones are represented as M3. A molecular weight marker was used in order to calibrate the electrophoretic migration, as indicated by the label on the left of the figure. A ruler was placed aside of the gel for scale reference.

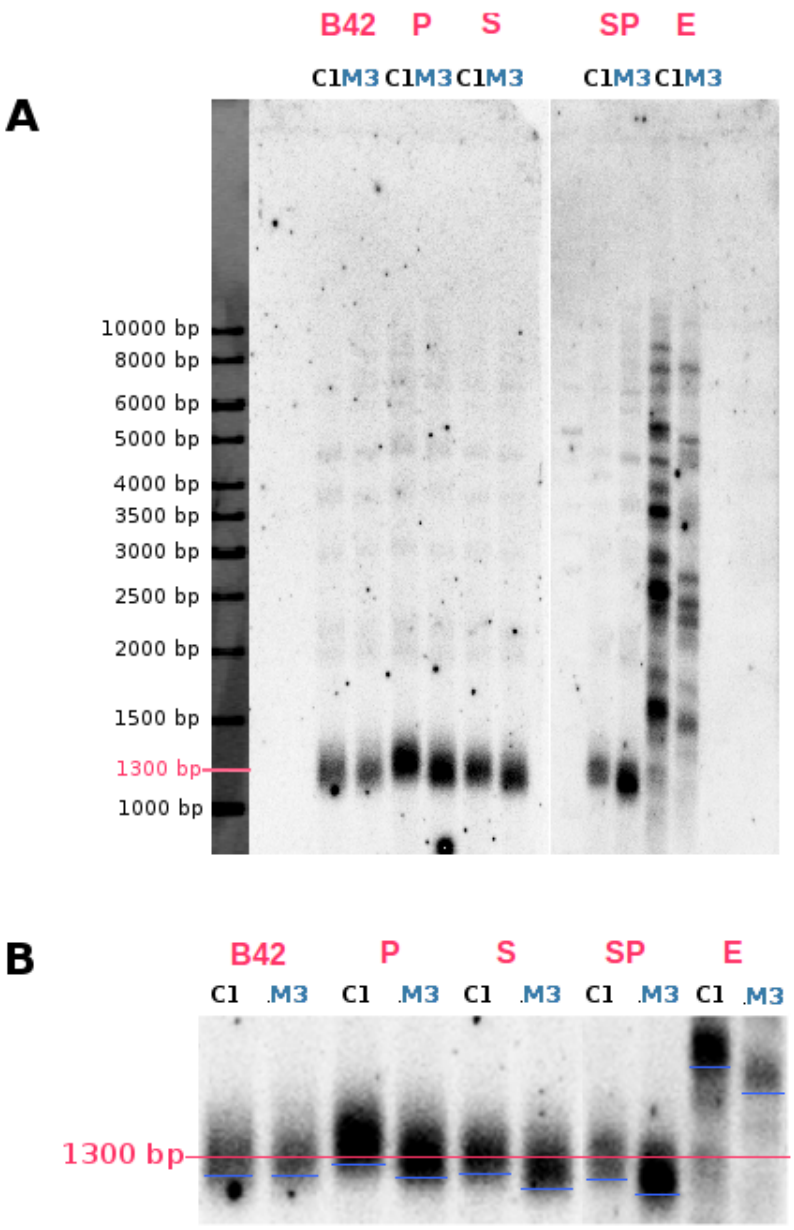

Figure 3.4: Southern blot hybridization patterns of control (C1) and mistranslating (M3) clones using a radiolabeled probe that detects the Y'-element repetitive regions of yeast chromosomes. Strains tested were BY4742 (B42), BY4742Δpnc1 (P), BY4742Δsir2 (S), BY4742Δpnc1Δsir2 (SP) and BY4742Δest2 (E). Panel **A** shows a general view of the blotted membrane, with a molecular weight marker scale on the left, while Panel **B** represents an amplification of the region where DNA fragments with about 1300 bp are found. Lines indicate shifts in the Y'-element fragments between control and mistranslating clones for all strains tested. Mistranslating clones seem to have shorter Y'-element fragments than the control clones in the strains where *PNC1*, *SIR2* or both genes were deleted. E strain, where telomerase activity was impaired, produced a pattern of multiple DNA bands, instead of the expected single fragment around 1300 bp.

bands, instead of the expected single fragment around 1.3 kb. The DNA bands observed for the mistranslating (M3) clone of this strain showed consistently a shift to lower band molecular weights when the positions were compare to these of the control (C1) alone. In spite of the differences in the repetitive fragment length profile observed for strain E, due to lacking of telomerase activity, clones of this strain might experience telomere-telomere recombination events, which can result in longer Y'-element fragments (Teng and Zakian, 1999).

Southern blot analysis indicated that in the case of the wild type strain (BY4742), mistranslation did not result in telomere length alterations. However, in the *PNC1*, *SIR2* and double deletion mutant (*PNC1* and *SIR2* deletion), mistranslation lead to a slight shift to lower molecular weight in the strong band of the Y'-element repetitive regions. This indicates a shortening of telomere length in these strains, confirming the hypothesis that the *PNC1* and *SIR2* genes play a role in the regulation of telomere length in yeast cells subjected to high levels of mistranslation.

These results can be interpreted as a demonstration that the regulation of the levels of Pnc1p and increased activity of Sir2p in mistranslating cells protects them against telomere destabilization. It also raises the question of how high can the levels of stress due to mistranslation be until the *PNC1* gene regulation cannot protect the cells from telomere destabilization. Our laboratory is working with other mistranslating yeast models, where different types of codon misreading are induced, and there is experimental evidence indicating that the level of stress imposed by mistranslation is related to the type of amino acid substitutions imposed on the proteome of the mutant clones. It would be interesting to investigate if telomere stability would be impaired in any of the tested codon misreadings used to cause mistranslation. Therefore, Southern blot analysis of telomere fragment length was conducted using a collection of strains kindly provided by Denisa Mateus and Jorge Paulo. The obtained results are presented in the section 3.3.

3.3 Effect of mistranslation in telomere length as a function of the type of codon misreading

The mistranslating clones listed in (Table 3.1) and investigated in this section, were based on *S. cerevisiae* strain BY4743 (MATa/MATα his3Δ 0/his3Δ 0; leu2Δ /leu2Δ 0; met15Δ 0/MET15; LYS2/lys2Δ 0; ura3Δ 0/ura3Δ 0). Mutant strains were constructed using plasmid pRS315 where the sequence of DNA that decodes the serine UGA codons

($tRNA_{UGA}^{Ser}$) was cloned. This tRNA sequence was then mutated by site directed mutagenesis so that the anticodon sequence would recognize codons of other amino acids. Therefore, the tRNA, conserving all the identity elements of a serine tRNA is aminoacylated with serine but will decode a codon coding for another amino acid. The engineered tRNAs, once introduced in yeast will cause mRNA mistranslation with different types of codon misreading. Investigated strains were listed in Table 3.1, with the strain reflecting the amino acid that is substituted by serine in newly synthesized proteins.

Table 3.1: Yeast strains transformed with plasmid pRS315 where different mutated tRNAs were inserted. Strain designations were based on the corresponding mutated tRNA inserted in the plasmid.

Strain Designation	Inserted tRNA
Ala	$tRNA_{UGC(Ala)}^{Ser}$ [a]
Leu	$tRNA_{CAG(Leu)}^{Ser}$ [a]
Gly	$tRNA_{UCC(Gly)}^{Ser}$ [a]
Thr	$tRNA_{CGU(Thr)}^{Ser}$ [a]
Tyr	$tRNA_{GUA(Tyr)}^{Ser}$ [a]
Val	$tRNA_{CAC(Val)}^{Ser}$ [a]
Arg	$tRNA_{CCU(Arg)}^{Ser}$ [b]
Asn	$tRNA_{AUU(Asn)}^{Ser}$ [b]
Asp	$tRNA_{AUC(Asp)}^{Ser}$ [b]
Cys	$tRNA_{GCA(Cys)}^{Ser}$ [b]
Glu	$tRNA_{CUC(Glu)}^{Ser}$ [b]
His	$tRNA_{AUG(His)}^{Ser}$ [b]
Met	$tRNA_{CAU(Met)}^{Ser}$ [b]
Phe	$tRNA_{AAA(Phe)}^{Ser}$ [b]
Pro	$tRNA_{AGG(Pro)}^{Ser}$ [b]
Trp	$tRNA_{CCA(Trp)}^{Ser}$ [b]

[a] Kindly provided by Denisa Mateus
[b] Kindly provided by Jorge Paulo

Southern blot analysis was conducted as described in the 'Methods' section. The results of the comparison of all fragment electrophoretic migration in all tested strains can be seen in Figures 3.5 and 3.6. As in section 3.2, E strain (Table 2.1) was used as a control for

telomere length alteration. Figure 3.5 shows that all the tested clones transformed with mutant tRNAs presented a strong band near the 1.3 kb region, corresponding to the Y'-element fragments. In Figure 3.5 B it is readily apparent that clones named Gly, Tyr and Val (Table 3.1), where glycine, tyrosine and valine codons were translated as serine, present a gel shift of the fragment detected in this region relatively to the control (wt) strain BY4743 (B43). No alterations relatively to the control strain (B43) were detected in clones where alanine, leucine, and threonine (Ala, Leu, Thr, respectively) were mistranslated as serine (Figure 3.5-B). Figure 3.6 depicts the results obtained for the strains Arg, Asn, Asp, Cys, Glu, His, Met, Phe, Pro and Trp. The Southern blot profiles obtained for the strain BY4743 and strain E, both control and mistranslating strains, were analyzed again for reference. Inspecting the detailed results of the region around 1.3 kb (Figure 3.6), shifts in the Y-element fragment bands are apparent in strains Glu, Phe, Pro and Trp, where glutamic acid, phenylalanine, proline and triptophan were substituted by serine, when compared to BY4743 strain. In summary, Figures 3.5 and 3.6 show decreased length in Y-element fragments, that is to say, shorter telomere lengths, in mistranslating strains where Gly, Tyr, Val Glu, Phe, Pro and Trp were substituted by serine but not in the case of Ala, Leu, Arg, Asn, Asp, Cys, His and Met.

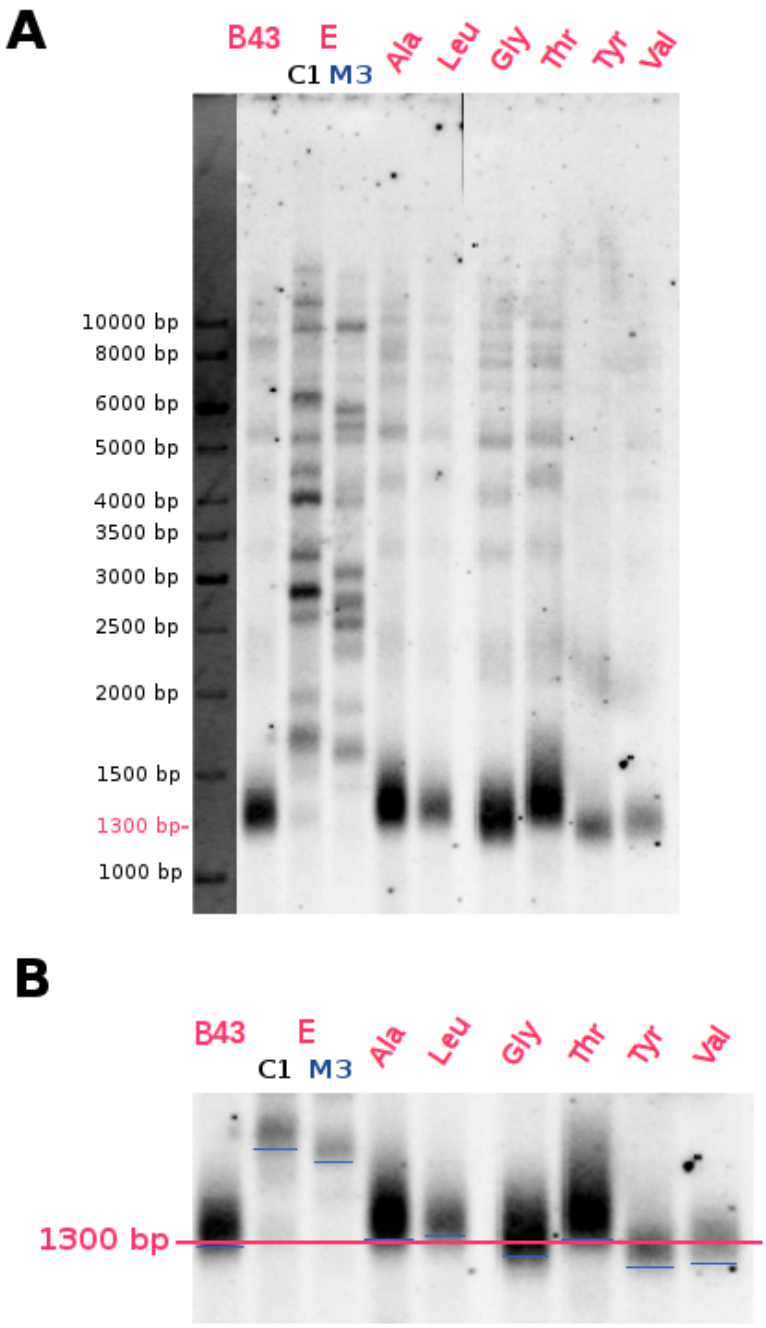

Figure 3.5: Southern blot hybridization patterns of control and mistranslating clones using a radiolabeled probe that detects the Y'-element repetitive regions of yeast chromosomes. Strains tested were Ala, Leu, Gly, Thr, Tyr and Val. Panel **A** shows a general view of the blotted membrane, with a molecular weight marker scale on the left, while Panel **B** represents an amplification of the region where DNA fragments with about 1300 bp are found. Lines indicate shifts in the Y'-element fragments between control and mistranslating clones for all strains tested. Gly, Tyr and Val clones seem to have shorter Y'-element fragments than the control wt clone (B43).

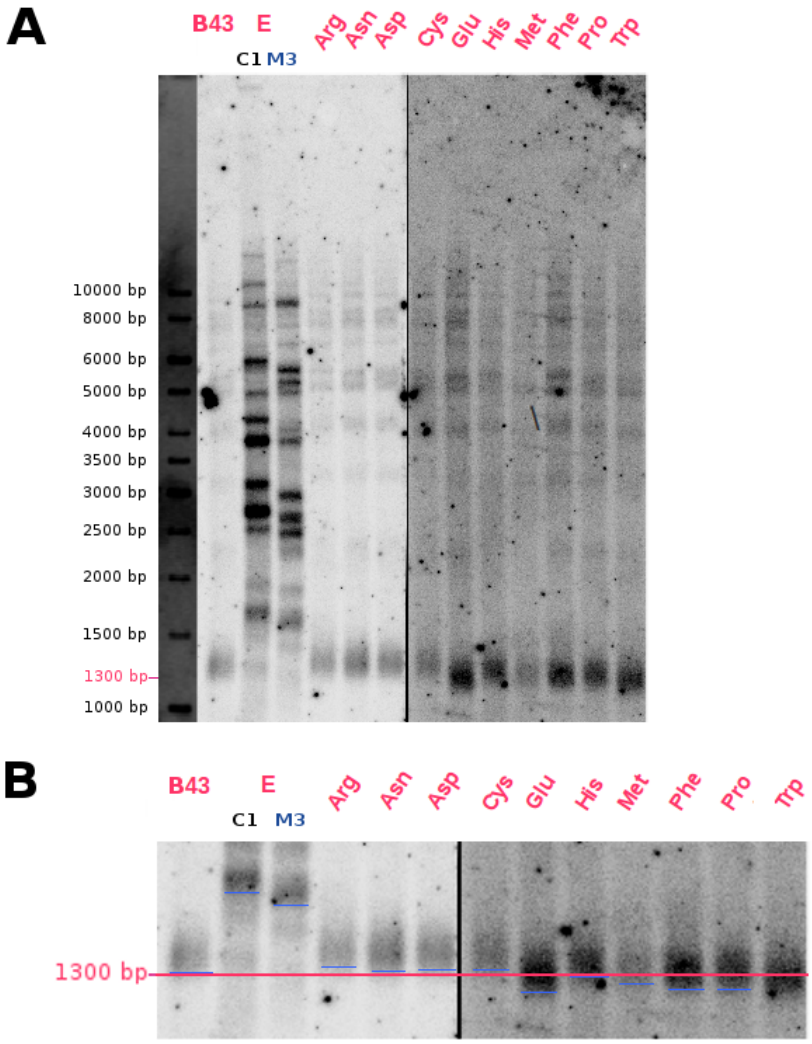

Figure 3.6: Southern blot hybridization patterns of control (wt (B43) and *EST2* deletion strain (E) - control (C1) and mistranslating (M3) clones) and mistranslating clones using a radiolabeled probe that detects the Y'-element repetitive regions of yeast chromosomes. Strains tested were Arg, Asn, Asp, Cys, Glu, His, Met, Phe, Pro and Trp. Panel **A** shows a general view of the blotted membrane, with a molecular weight marker scale on the left, while Panel **B** represents an amplification of the region where DNA fragments with about 1300 bp are found. Lines indicate shifts in the Y'-element fragments between control and mistranslating clones for all strains tested. Glu, Phe, Pro and Trp clones seem to have shorter Y'-element fragments than the control wt clone (B43).

Chapter 4

Discussion

The results obtained in this work show that telomere stability can be affected when mRNA mistranslation is induced in yeast strains, specifically in the cases of mistranslation induced by tRNAs decoding codons of Gly, Tyr, Val, Glu, Phe, Pro and Trp as serine. However, when mistranslation was induced by tRNAs decoding amino acids Ala, Leu, Arg, Asn, Asp, Cys, His and Met, as serine, no evidence for telomere instability was found. These results suggest that the type of codon misreading has different impacts on proteome homeostasis, and perhaps elicits different cellular responses or different levels of the same response. This is a subject still to be investigated.

When strains with deleted *PNC1*, *SIR2*, and both *PNC1* and *SIR2* genes were investigated, mistranslation induced shortening of telomeres. These results confirm the protective role of Pnc1p increased levels in mistranslating cells, proposed by Silva et al. (Silva et al., 2009), particularly strengthening the putative regulation of Sir2p activity and therefore, of the protective role of the Sir complex in th telomere genomic region.

Mistranslating clones obtained for the yeast strain lacking *EST2* gene (E strain), also showed the shifts in the migration pattern of repetitive Y'-element sequences. However, this particular strain also showed a particular band pattern, differing from all the other strains. *EST2* is an essential gene for the correct functioning of telomerase as it codes for its catalytic subunit. In fact, it has been shown that when yeast cells lack a gene required for the telomerase activity, as in the case of the *EST2* deletion strain, they die after 50 to 100 cell divisions. Meanwhile, survivors arising spontaneously in such cultures (McEachern et al., 2000) have one of two distinct patterns (type I or type II) (Teng and Zakian, 1999) of telomeric DNA lengths. The most common of these two patterns is seen in type I survivors reflected as a tandem amplification of Y'-element followed by very short tracts of C_{1-3A}/TG_{1-3} DNA (Teng and Zakian, 1999). Chromosomes in type II survivors were shown to end in very long and heterogeneous-length tracts of C_{1-3A}/TG_{1-3} DNA, with some telomeres having 12 kb or more of C_{1-3A}/TG_{1-3} repeats (Teng and Zakian, 1999). The results obtained for both control and mistranslating clones of strain E are compatible with long, and heterogeneous in length, telomeres, which may be the result

of telomere-telomere recombination. Interestingly, very long and heterogeneous in length telomeres such as those characteristic of type II survivors in *Saccharomyces cerevisiae*, are reminiscent of the telomere alterations found in immortal human cell lines and in tumors that maintain telomeric DNA in the absence of telomerase (Teng and Zakian, 1999).

Overall, from the alterations in telomere length observed in this work for mistranslating strains, we may pose the following question: why are these alterations observed in mistranslating clones, relatively to control clones in the *PNC1*, *SIR2* and double deletion *PNC1* and *SIR2* strains but not for the wild type strain BY4742? The work by Silva et al. (Silva et al., 2009) may help to shed some light in this matter. These authors showed that when a tRNA decoding CUG leucine codons as serine was inserted in yeast cells, there was an increase in the relative abundance of Pnc1p and related this to the increase of activity of Sir2p, a histone deacetylase with particular importance for silencing chromatin at the telomeres. Sir2p is needed in the assembly of the Sir complex (Sir4-Sir3-Sir2) along the telomere TG repeats (Allis et al., 2007). When this complex is formed, the activity of NAD-dependent Sir2p histone deacetylase is stimulated and Sir2p deacetylates histone H4 K16 residue in the near nucleosomes. The Sir complex then spreads along the nucleosomes with Sir3p and Sir4p binding the histone deacetylated H4 tails, and silencing the chromatin (Figure 1.10). Chromatin silencing at telomeres protects this region against degradation and recombination at the repetitive regions. Silva et al. (Silva et al., 2009) observed that mistranslating yeast carrying a *URA3* gene placed at telomeres, were not able to grow in a minimal medium lacking uracil, which means that *URA3* gene was silenced by the enhanced action of Sir2p.

Taking the results from Silva et al. (Silva et al., 2009), it is clear that Pnc1p and Sir2p are important proteins concerning genomic stability through telomere length maintenance. In fact, this work showed that mistranslating yeast strains lacking *SIR2* showed shorter Y'-elements, which meant shorter telomeres. Lacking the activity of Sir2p and when exposed to aggravated stress conditions caused by mistranslation, the cells did not have the capacity to protect the telomeres through chromatin silencing by the Sir complex, which resulted in underproduction of telomere chromatin and in decrease of telomere length, in consequence of instability in these genomic regions.

The mistranslating clones of yeast strain lacking *PNC1* gene also showed a decrease in Y'-element length, which translated into shorter telomeres. This confirms that Pnc1p activity is important in the stabilization of telomeric chromatin, and also strengthens the hypothesis that the regulation of this protein is reflected in intracellular levels of NAD^+,

with consequences in the NAD^+ dependent enzymes, such as Sir2p (Denu, 2007). This may have conditioned Sir2p activity and the role of the Sir complex in protecting the telomere region. As could be expected from these observations, clones lacking both *SIR2* and *PNC1* genes, which could not produce Sir2p nor Pnc1p, also registered alterations in telomere length as consequence of mistranslation, similar to those documented for *SIR2* or *PNC1* mutants. The gene deletions alone did not have an impact on telomere length, since non-mistranslating clones of strains lacking *SIR2*, *PNC1* or both *SIR2* and *PNC1* genes did not show alterations in telomere length. It seems that without the challenges posed by mistranslation, the eventual metabolic alterations caused by the gene deletions did not trigger telomere shortening. This observation may be indicating that yeast cells have alternative mechanisms to compensate for lack of Pnc1p and Sir2p activity in non stressful conditions. However, under the stress caused by mistranslation, Pnc1p and Sir2p activities are needed to protect cells against telomere destabilization. And, indeed, in wild type BY4742 strain, the raise of Pnc1p and Sir2p activity, as shown by Silva et al. (Silva et al., 2009), mistranslation had no impact on telomere length.

The question that may be posed now is if the level of stress induced by mistranslation of leucine CUG codons as serine was not sufficient to make it possible to observe a telomere instability phenotype. It is possible that in a situation where the induced translational error level is higher, this phenotype can be readily observed. It was reported that the levels of intracellular stress may rise when specific amino acids are replaced by others (Jorge Paulo personal communication). Proteins with such substitutions in their polypeptide chain may fold incorrectly or aggregate which may lead to the increase of ROS and cause increase of stress in the cell. To test if specific amino acid misincorporation in the cell aggravated the shorter telomere phenotype, this work also analyzed the telomere regions of strains based in the BY4743 genotype (a diploid strain), where different mutated tRNAs decoded several amino acids. Some of these strains (namely, Gly, Tyr, Val, Glu, Phe, Pro and Trp, where glycine, tyrosine, glutamic acid, phenylalanine, proline and triptophan are being replaced by serine in nascent proteins), showed alterations on telomere length while other amino acid substitutions had no impact on this feature. It is possible that in particular strains, the impact caused by mistranslation was so severe that regulation of Pnc1p and Sir2 would not be sufficient to protect the cells against telomere destabilization. For future work, it might be interesting to compare stress levels in these strains, by monitoring ROS levels, for instance, and see if there is a correlation with the telomere shortening phenotype. It may be interesting to monitor the expression level of *PNC1* and *SIR2*, as well as Pnc1p

and Sir2p levels and enzymatic activity in theses strains. Another interesting subject on which some light needs to be shed is whether or not these particular strains have silenced telomeres, which may be tested using the *URA3* reporter system, as described by Silva et al. (Silva et al., 2009).

Chapter 5

Conclusions and future work

In yeast, mRNA mistranslation seems to have an impact on genome stability, particularly on telomeres. *PNC1* and *SIR2* genes play an important role in protecting the cells against this offense and seem to be involved in the cell stress-response through chromatin silencing at telomeres. However, the specific mechanisms by which these events happen are not clear and further experiments are needed to understand the effect of mistranslation on telomere stability.

In yeast strains on which mistranslation was induced by particular tRNA mutations telomere shortening was observed. It would be interesting to investigate the hypothesis that this is due to Sir proteins extensive delocalization upon mistranslation and why this occurred in some types of codon misreading and not others. Also, codon misreading *per se* might be the cause of the effects of mistranslation depending on specific amino acid substitutions. It would be interesting to characterize the relative abundance of the misread codons in the ORFeome, looking for particular classes of genes or particular cellular functions most affected by specific codon misreading.

It would also be interesting to compare stress levels in strains with different mutated tRNAs and see if there is a correlation with the telomere shortening phenotype. Moreover, the levels of expression of the different tRNAs should be monitored, since differences on their expression levels might cause different degrees of induced mistranslation in the cell. Both subjects are currently being investigated in our laboratory.

Appendix A

Yeast growth curves

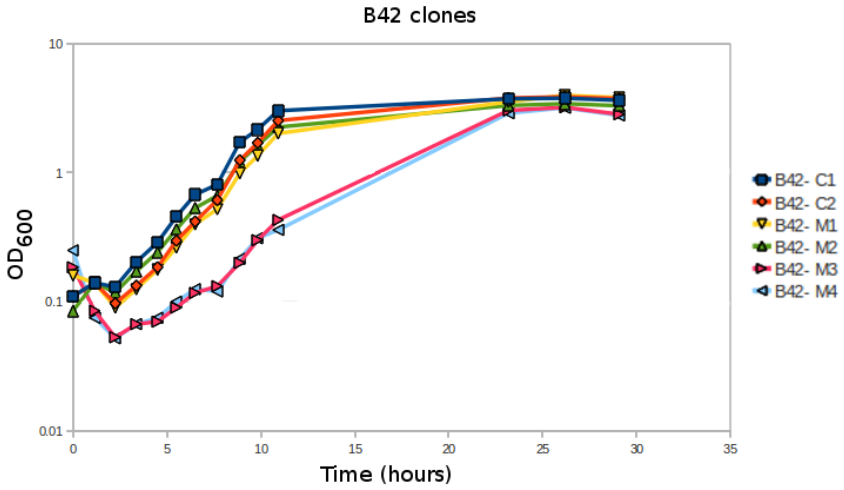

Figure A.1: Growth curves obtained for control (C1 and C2) and a mistranslating (M1 to M4) clones of strain BY4742 (B42). The control clones reach the stationary phase faster than the mistranslating clones.

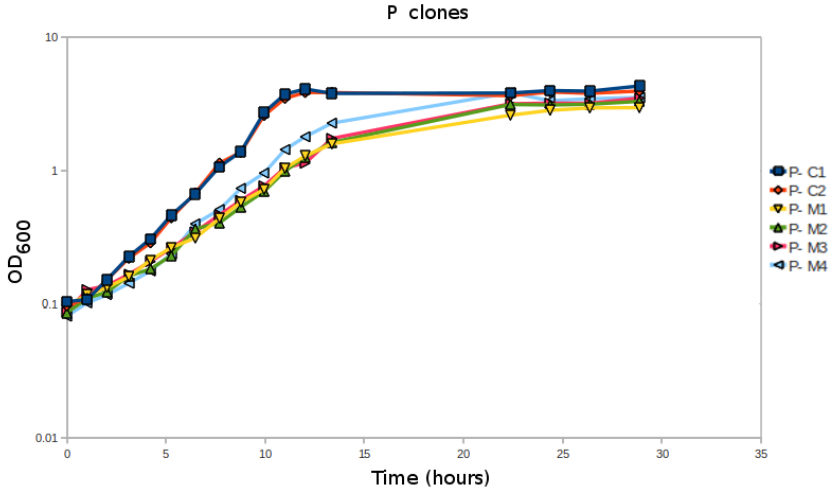

Figure A.2: Growth curves obtained for control (C1 and C2) and a mistranslating (M1 to M4) clones of strain BY4742Δpnc1 (P). The control clones reach the stationary phase faster than the mistranslating clones.

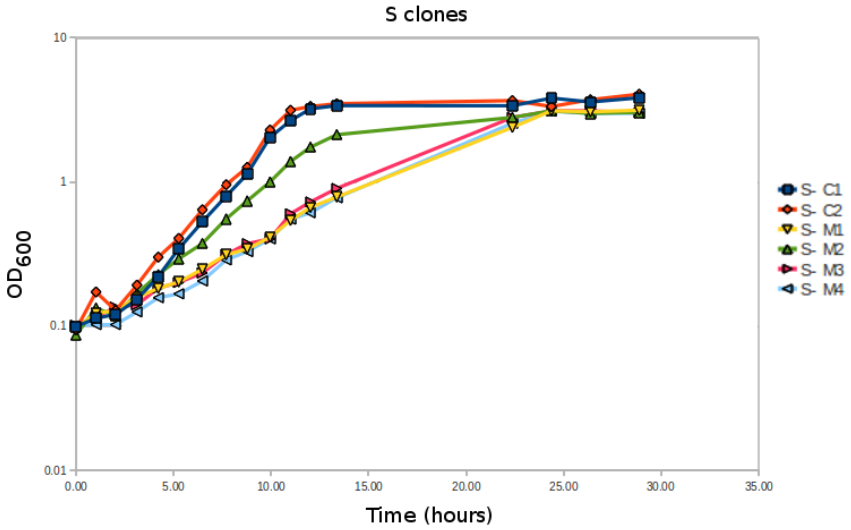

Figure A.3: Growth curves obtained for control (C1 and C2) and a mistranslating (M1 to M4) clones of strain BY4742Δsir2 (S). The control clones reach the stationary phase faster than the mistranslating clones.

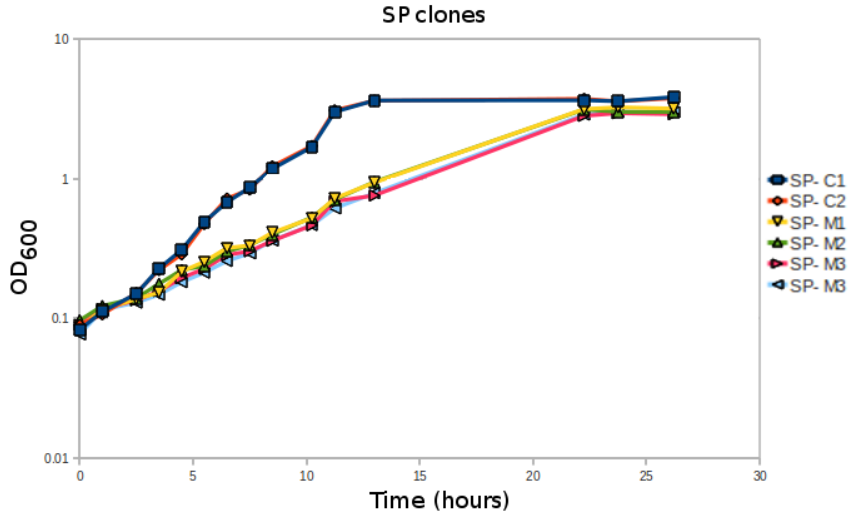

Figure A.4: Growth curves obtained for control (C1 and C2) and a mistranslating (M1 to M4) clones of strain BY4742Δsir2Δpnc1 (SP). The control clones reach the stationary phase faster than the mistranslating clones.

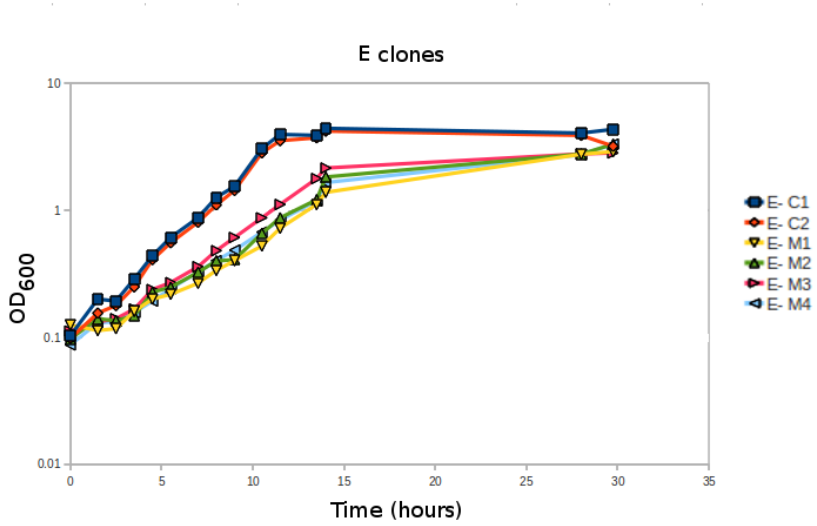

Figure A.5: Growth curves obtained for control (C1 and C2) and a mistranslating (M1 to M4) clones of strain BY4742Δest2 (E). The control clones reach the stationary phase faster than the mistranslating clones.

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