Universidade de Aveiro Departamento de Biologia
2010

João Cancela de Amorim Falcão Paredes

Estudo molecular da degeneração e evolução celular induzidas por erros na tradução do mRNA

Molecular study of cell degeneration and evolution induced by mRNA mistranslation

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

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“The known is finite, the unknown infinite; intellectually we stand on an islet in the midst of an illimitable ocean of inexplicability. Our business in every generation is to reclaim a little more land, to add something to the extent and the solidity of our possessions”

Thomas Henry Huxley
(1825 – 1895)

o júri

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palavras-chave

Erros de tradução, código genético, mRNA, degeneração celular, evolução molecular, expressão genética, estabilidade do genoma, stresse oxidativo, controlo da qualidade das proteínas

resumo

As proteínas existentes nas células são produzidas pelo mecanismo de tradução do mRNA, no qual a informação genética contida nos genes é descodificada em cadeias polipeptídicas. O código genético, que define as regras de descodificação do genoma, minimiza os erros de tradução do mRNA, garantindo a síntese de proteínas com elevada fidelidade. Esta é essencial para a estabilidade do proteoma e para a manutenção e funcionamento dos processos celulares. Em condições fisiológicas normais, os erros da tradução do mRNA ocorrem com frequências que variam de 10^{-3} a 10^{-5} erros por codão descodificado. Situações que aumentam este erro basal geralmente estão associadas ao envelhecimento, stresse e a doenças; no entanto, em certos organismos o código genético é traduzido naturalmente com elevado erro, indicando que a síntese de proteínas aberrantes pode de algum modo ser vantajosa.

A fim de estudar a resposta celular aos erros de tradução do mRNA, construímos leveduras que incorporam serina no proteoma em resposta a um codão de leucina, usando a expressão constitutiva de um $tRNA^{Ser}$ mutante. Este fenómeno genético artificial provocou uma forte diminuição da esporulação, da viabilidade e da eficiência de *mating*, afectando imensamente a reprodução sexual da levedura. Observou-se também uma grande heterogeneidade no tamanho e na forma das células e elevada instabilidade genómica, com o aparecimento de populações poliplóides e aneuplóides.

No sentido de clarificar as bases celulares e moleculares daqueles fenótipos e compreender melhor a biologia do erro de tradução do mRNA, construímos também células de levedura que inserem serina em resposta a um codão de leucina de modo indutível e controlado. Utilizaram-se perfis de mRNA total e de mRNA associado a polissomas para elucidar a resposta celular ao erro de tradução do mRNA. Observou-se a indução de genes envolvidos na resposta ao stresse geral, stresse oxidativo e na *unfolded protein response* (UPR). Um aumento significativo de espécies reactivas de oxigénio (ROS) e um forte impacto negativo na capacidade das células pós-mitóticas re-iniciarem o crescimento foram também observados. Este fenótipo de perda de viabilidade celular foi resgatado por *scavangers* de ROS, indicando que o stresse oxidativo é a principal causa de morte celular causada pelos erros de tradução. Este estudo levanta a hipótese de que o stresse oxidativo e a acumulação de ROS, ao invés do colapso súbito do proteoma, são as principais causas da degeneração celular e das doenças humanas associadas aos erros de tradução do genoma.

keywords

mRNA mistranslation, genetic code, cell degeneration, molecular evolution, mRNA profiling, genome stability, oxidative stress, protein quality control

abstract

Proteins are synthesized through the mechanism of translation, which uses the genetic code to transform the nucleic acids based information of the genome into the amino acids based information of the proteome. The genetic code evolved in such a manner that translational errors are kept to a minimum and even when they occur their impact is minimized by similar chemical properties of the amino acids. Protein synthesis fidelity is essential for proteome stability and for functional maintenance of cellular processes. Indeed, under normal physiological conditions, mistranslation occurs at frequencies that range from 10^{-3} to 10^{-5} errors per codon decoded. Situations where this basal error frequency increases are usually associated to aging and disease. However, there are some organisms where genetic code errors occur naturally at high level, suggesting that mRNA mistranslation can somehow be beneficial.

In order to study the cellular response to mRNA mistranslation, we have engineered single codon mistranslation in yeast cells, using constitutive expression of mutant $tRNA^{Ser}$ genes. These mistranslating strains inserted serines at leucine-CUG sites on a proteome wide scale due to competition between the wild type $tRNA^{Leu}$ with the mutant $tRNA^{Ser}$. Such mistranslation event decreased yeast sporulation, viability and mating efficiencies sharply and affected sexual reproduction strongly. High heterogeneity in cell size and shape and high instability in the genome were also observed, with the appearance of some polyploid or aneuploid cell populations.

To further study the cellular and molecular basis of those phenotypes and the biology of mRNA mistranslation, we have also engineered inducible mRNA misreading in yeast and used total mRNA and polysome associated mRNA profiling to determine whether codon misreading affects gene expression. Induced mistranslation up-regulated genes involved in the general stress response, oxidative stress and in the unfolded protein response (UPR). A significant increase in reactive oxygen species (ROS) and a strong negative impact on the capacity of post-mitotic cells to re-initiate growth in fresh media were also observed. This cell viability phenotype was rescued by scavengers of ROS, indicating that oxidative stress is the main cause of cell death caused by mRNA mistranslation. This study provides strong support for the hypothesis that oxidative stress and ROS accumulation, rather than sudden proteome collapse or major proteome disruption, are the main cause of the cellular degeneration observed in human diseases associated mRNA mistranslation.

Contents

List of abbreviations

AAA	ATPase associated activities
APS	ammonium persulphate
ATPase	adenosine triphosphatase
aaRS	aminoacyl-tRNA synthetase
ATP	adenosine 5'-triphosphate
cAMP	cyclic adenosine 5'-monophosphate
cDNA	cyclic deoxyribonucleic acid
Cy3	cyanine 3
Cy5	cyanine 5
DNA	deoxyribonucleic acid
DNase	deoxyribonulease
DNPH	2,4-Dinitrophenylhydrazine
PAGE	polyacrylamide gel electrophoresis
DTT	dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
g (mg, μg, ng)	gram (milligram, microgram, nanogram)
GFP	green fluorescent protein
GTP	guanosine 5'-triphosphate
GDP	guanosine 5'-diphosphate
IRES	internal ribosome entry site
KCl	potassium chloride
kDa	kilodalton
L (mL, μL)	liter (mililiter, microliter)
M (mM, μM)	molar (milimolar, micromolar)
mA	miliampère
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MHC	major histocompatibility complex

mol (mmol, μmol, pmol)	mole (nanomole, micromole, picomole)
mQ	milliQ
mRNA	messenger ribonucleic acid
Na_2HPO_4	disodium hydrogen phosphate
NaH_2PO4	sodium dihydrogen phosphate
OD	optical density
OD_{600}	optical density at 600nm
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
S	Svedberg
SDS	sodium dodecyl sulphate / sodium lauryl sulphate
TEMED	Tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
U	units
UTP	uridine 5'-triphosphate
UTR	untranslated region
V	Volt

Other abbreviations will be explained when used in the text.

A. Introduction

1. Protein synthesis – the process of mRNA translation

In all living organisms, genetic information is stored in deoxyribonucleic acid (DNA) in the form of genes that constitute part of the genome. Genome replication preserves this information from mother to daughter cell. Transcription of this genetic information from DNA into messenger ribonucleic acid (mRNA), and translation of the latter, guaranty that the genome is used as the source of information for protein synthesis. This flow of biologic information from DNA to mRNA and then to proteins is the simplest form of the so-called Central Dogma of Molecular Biology, proposed by Crick (Crick, 1970) (Figure1).

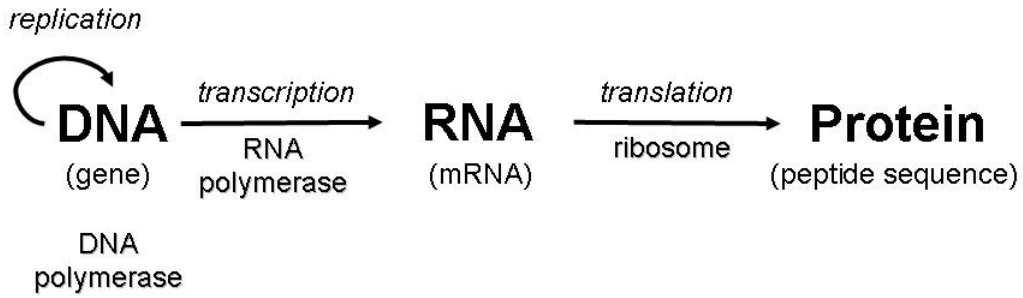


Figure 1. The Central Dogma of Molecular Biology. The genetic information in DNA is preserved by replication of the genome, carried out by DNA polymerase, so that each daughter cell can receive one genome copy at every cell division. In all organisms, transcription of DNA into mRNA is carried out by RNA polymerase and translation of mRNA is carried out by the ribosome.

During translation, ribosomes in conjunction with transfer ribonucleic acids (tRNA), amino acids, translational factors and aminoacyl-tRNA synthetases (aaRS), read the mRNA message and produce protein products according to the instructions written in that message. Ribosomes are supramolecular complexes composed of proteins and ribosomal ribonucleic acid (rRNA) and are used by cells as their protein factories. The bacterial 70S ribosome (S is Svedberg unit for sedimentation velocity) is composed of a small (30S) and a large (50S) subunit. The 30S subunit contains one molecule of 16S rRNA and 21 proteins (S1-S21) and the 50S subunit contains one molecule of 5S rRNA, 23S rRNA plus 34 proteins (L1-L34).

Eukaryotic cytoplasmic ribosomes are larger and contain more rRNAs and proteins than their prokaryotic counterparts (40S small subunit and 60S large subunit), but eukaryotic organellar ribosomes are smaller than prokaryotic ones. All types of ribosomes function on the basis of similar structural and biochemical principles. Translation of mRNAs occurs in three tRNA binding sites, which are located in the 40S (30S) subunits at the inter-subunit interface, namely the aminoacyl site (A site), peptidyl site (P site) and exit site (E site) (Figure 2).

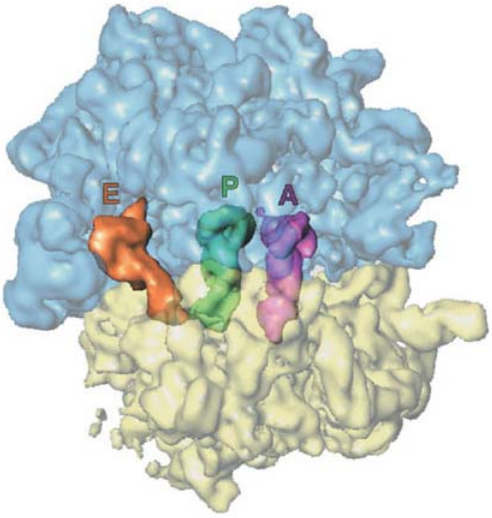


Figure 2. Ribosome structure showing the three tRNA binding sites which are the ribosome domains where mRNA is decoded by tRNAs. The figure shows an image of a pre-translocation ribosome seen by cryo-EM [adapted from (Valle *et al.*, 2003)].

Translation can be divided into 3 steps: initiation, elongation and termination (Figure 3). An extra step can also be included, the ribosome recycling step. In the initiation step in bacteria, the ribosome binds to the mRNA through the Shine-Dalgarno sequence and positions the initiation codon and the methionine initiator tRNA in the peptidyl (P) site. During the elongation phase, the ribosome adds one amino acid at a time to the growing polypeptide chain. Aminoacyl-tRNAs enter in the aminoacyl or acceptor (A) site where decoding takes place. After the tRNA and mRNA are translocated and the next codon moves into the A site, the process is repeated until a stop codon enters de A site. During the termination phase the ribosome releases the newly synthesized polypeptide and then enters the

recycling phase where the ribosomal subunits are dissociated, releasing the mRNA and the deacylated tRNA and setting the stage for another round of initiation. The overall scheme is similar in prokaryotes and eukaryotes, but there are significant differences especially in the eukaryotic translation initiation process [reviewed in (Kapp and Lorsch, 2004)].

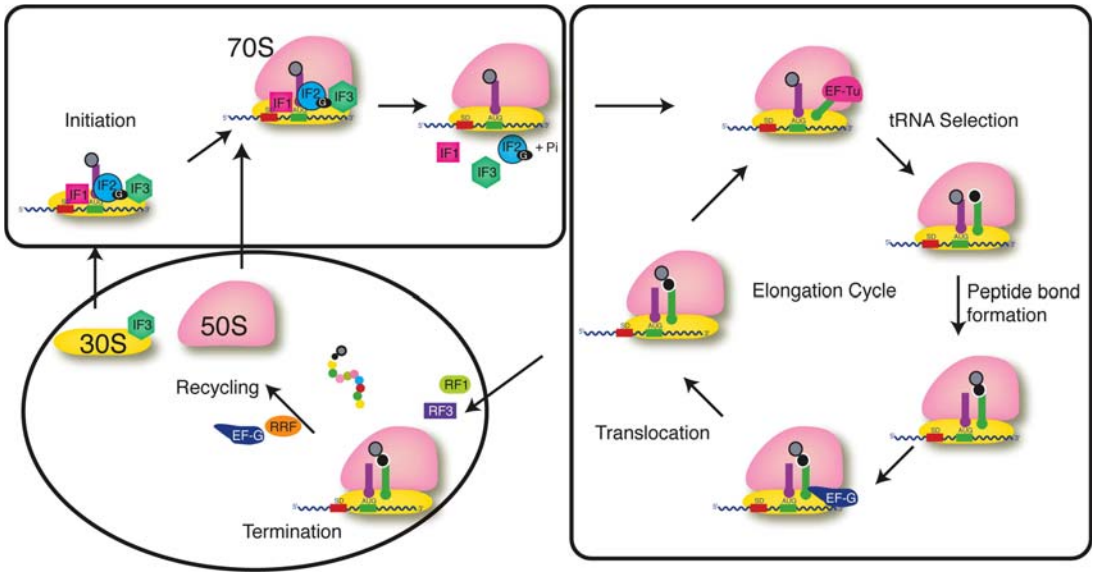


Figure 3. Schematic representation of the major steps in prokaryotic protein synthesis. In the initiation step of protein synthesis the 30S subunit binds mRNA, initiator tRNA, IF1, IF2 and IF3 before binding the 50S subunit. During the elongation cycle, the 70S ribosome binds EF-Tu::GTP::tRNA ternary complex and catalyze bond formation. The mRNA-tRNAs are translocated by EF-G. In the termination step, RF1 or RF2 recognize the stop codons and catalyze the release of the newly complete polypeptide. RF1 and RF2 are removed from the ribosome by RF3. In the recycling step, the ribosome is dissociated into 30S and 50S subunits by the action of RRF, EF-G and IF3 [adapted from (Khade and Joseph, 2010)].

1.1 Initiation

Two events occur as a prelude for protein synthesis: first, aaRSs charge amino acids onto cognate tRNAs. This is a highly specific two-step reaction that starts with activation of the amino acid with AMP, derived from ATP; and then transfer of

the activated amino acids onto the tRNA bound to the aaRS. Another pre-requisite for protein synthesis is the dissociation of the two subunits of the ribosome at the end of each round of translation. In prokaryotes, the initiation Factor 1 (IF1) actively promotes this dissociation by binding to the A site of the small ribosomal subunit (Moazed *et al.*, 1995;Carter *et al.*, 2001). It is helped by IF3 which binds to free 30S subunits and prevents their reassociation with 50S subunits to form a 70S ribosome.

In prokaryotes, the initiation codon is usually AUG, but it can be also GUG, or more rarely, UUG. The initiating aminoacyl-tRNA is modified to N-formyl-methionyl-$tRNA_f^{Met}$ and the modified amino acid (fMet) is the first amino acid to be incorporated into newly synthesized proteins, even in the case where mRNAs use GUG or UUG start codons. fMet is, however, often cleaved by an amino peptidase after from the newly synthesized proteins. The 30S pre-initiation complex is formed by a free 30S ribosomal subunit plus mRNA and fMet-$tRNA_f^{Met}$ plus initiation factor-2 (IF2). Binding between the 30S prokaryotic ribosomal subunit and mRNA depends on base pairing between a short RNA purine-rich sequence (called the Shine-Dalgarno sequence) just 10 bases upstream of the initiation codon and a complementary sequence at the 3'-end of the 16S rRNA (the anti-Shine-Dalgarno). This binding is mediated by IF3, with help from IF1 and IF2. The three initiation factors are bound to the 30S subunit at this stage. IF2 is a GTPase that promotes binding of fMet- $tRNA_f^{Met}$ to the 30S initiation complex. GTP is not hydrolyzed during the binding process. The complete 30S pre-initiation complex contains one 30S ribosomal subunit plus one molecule of mRNA, fMet-$tRNA_f^{Met}$, GTP-IF2, IF1 and IF3. GTP is only hydrolyzed after the 50S ribosomal subunit joins the 30S pre-initiation complex to form the 70S initiation complex. This GTP hydrolysis is carried out by IF2 in conjunction with the 50S ribosomal subunit. The purpose of this hydrolysis is to release IF2 from the ribosome, GDP is then recycled and the IF2-GTP complex can be used for a new round of initiation (Gualerzi and Pon, 1990).

In eukaryotes, 40S ribosomal subunits, together with the ternary complex containing the initiator tRNA (eIF2::GTP::Met-$tRNA_i^{Met}$), locate the start codon by binding to the 5'-cap of mRNAs and by scanning the 5´-untranslated regions (5´-UTRs) until they find the first AUG in a favourable context. The best context contains a purine at position -3 and a G at position +4. In 5-10% of the cases, the ribosomal scanning complex bypass the first AUG codon and continue to scan until they encounter a downstream AUG in a more favourable context. Conversely to prokaryotes, in eukaryotes the methionine bound to the $tRNA_i^{Met}$ is not modified. Also, there is no anti-Shine-Dalgarno sequence in the 18S rRNA of the eukaryotic small ribosomal subunit, which is related to the fact that initiation in prokaryotes and eukaryotes is fundamentally different (Jackson *et al.*, 1995) due to scanning and 5´-cap recognition mechanisms existent in eukaryotes (Kozak, 2002).

The 3 bacterial translation initiation factors are replaced by at least 12 factors in eukaryotes. The eukaryotic initiation factors (eIFs) have the following general functions (Figure 4): eIF2 is involved in binding Met-$tRNA_i^{Met}$ to the ribosome. eIF2B activates eIF2 by replacing its GDP with GTP before each round of translation initiation. eIF3 binds to the 40S ribosomal subunit and inhibits its reassociation with the 60S subunit. eIF5 enhances association between the 43S complex - 40S subunit plus mRNA and Met-$tRNA_i^{Met}$. eIF6 binds to the 60S subunit and blocks its reassociation with the 40S subunit. eIF4F is a 5'-cap-binding protein complex composed of three parts: eIF4E has cap-binding activity and it associates with two other factors, namely eIF4A and eIF4G. eIF4A has RNA helicase activity and unwinds hairpins in the 5'-UTR of eukaryotic mRNAs; its activity is enhanced by eIF4B and, like all other helicases, requires ATP for activity. eIF4G is an adaptor protein capable of binding to a variety of other proteins, including eIF4E (the cap-binding protein), eIF3 (the 40S ribosomal subunit-binding protein) and PAB (a polyA-binding protein). By interacting with these proteins, eIF4G can recruit the 40S subunit to the mRNA and thereby stimulate translation initiation. eIF1 and eIF1A act synergistically to promote formation of a stable 48S complex and apparently dissociate improper complexes between 40S subunits and mRNAs and promote scanning by 48S complexes.

eIF5B is homologous to the prokaryotic factor IF2 as it binds GTP and stimulates association of the two ribosomal subunits in cooperation with eIF5. It also resembles IF2 in using GTP hydrolysis to promote its own dissociation from ribosome, but differs from IF2 as it cannot stimulate the binding of the initiating aminoacyl-tRNA to the small ribosomal subunit. This task is performed by eIF2 in eukaryotes.

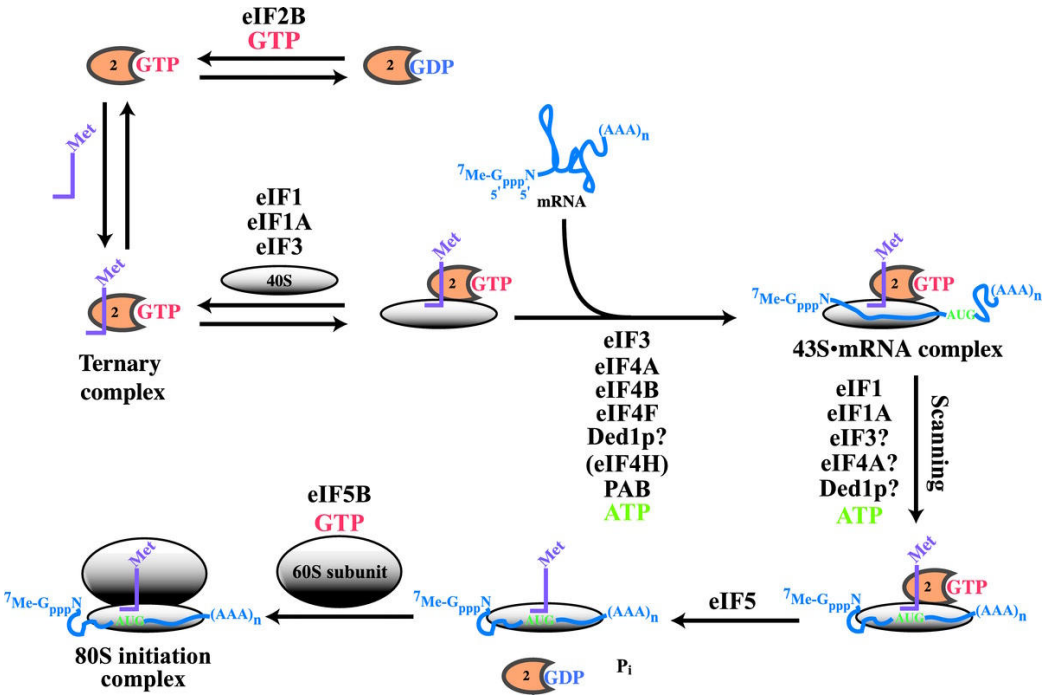


Figure 4. Current model of the steps involved in the process of eukaryotic translation initiation and the roles of the initiation factors. For clarity, the mRNA remains in a linear form and the 5'- and 3'- ends of the mRNA are not interacting. The initiation factors with a question mark (?) were proposed as being involved in the represented processes, but experimental evidence and confirmation are needed. [adapted from (Kapp *et al.*, 2004)].

Prokaryotic mRNAs are short-lived, so control of translation is not common, however, some translational control does occur. mRNA secondary structures can govern translation initiation and some ribosomal proteins can feedback inhibit the translation of their own mRNAs. Eukaryotic mRNA lifetimes are relatively long, so there is more opportunity for translation control than in prokaryotes. The α-subunit

of eIF2 (eIF2α) is phosphorylated and is the favourite target for translational control, but other mechanisms involving other initiation factors also occur.

1.2 Elongation

Conversely to initiation, elongation of translation is highly conserved between prokaryotes and eukaryotes. Elongation takes place in three steps (Figure 5A), namely: i) an aminoacyl-tRNA (aa-tRNA) enters the A site as part of a ternary complex with GTP and the elongation factor 1A (eEF1A; EF-Tu in prokaryotes); after binding of the correct aa-tRNA to the A-site, eEF1A/EF-Tu GTPase activity is activated and eEF1A-GDP/EF-Tu-GDP releases the aa-tRNA into the A site for peptide bond formation; ii) the peptidyl transferase, a ribosomal enzyme, catalyses the formation of the peptide bonds between the peptidyl-tRNA in the ribosomal P site and the newly arrived aa-tRNA in the A site, this lengthens the peptide by one amino acid and shifts it to the A site (Moore and Steitz, 2003); iii) the A-site aa-tRNA is then translocated to the P site and the P site tRNA is translocated to the E site, leaving the A site free to accept a new aa-tRNA. This task is accomplished by elongation factor 2 (eEF2; EF-G in prokaryotes), which hydrolyzes GTP during translocation (Wintermeyer *et al.*, 2001). This cycle is repeated until a stop codon enters the A site and becomes recognized by the release factor machinery.

The protein synthesis machinery achieves accuracy during elongation in a two-step process. First, it gets rid of ternary complexes bearing the wrong aminoacyl-tRNA before GTP hydrolysis occurs. Second, if this screen fails, it can still eliminate the incorrect aminoacyl-tRNA in a proofreading step before the wrong amino acid is incorporated into the growing protein chain (Zaher and Green, 2009a;Zaher and Green, 2009b). The balance between speed and accuracy of translation is delicate. If peptide bond formation goes too fast, incorrect aminoacyl-tRNAs do not have enough time to leave the ribosome, so their amino acids are incorporated into protein. But, if it goes too slowly, proteins are not made fast enough for the organism to grow successfully.

1.3 Termination

As mentioned above, the termination step of translation occurs in response to the presence of a stop codon in the ribosomal A-site (Figure 5B). The end result of this process is the release of the completed polypeptide followed by the hydrolysis of the ester bond that links the polypeptide chain to the P site tRNA. The peptidyl transferase center of the ribosome is believed to catalyze the hydrolysis reaction, in response to the activity of class 1 release factors (RFs in prokaryotes and eRFs in eukaryotes), which decode the stop codons presented in the A site. Class 2 release factors are GTPases that stimulate the activity of class 1 release factors regardless of the nature of the stop codon engaged by the RF.

Prokaryotic translation termination is mediated by three release factors : RF1, RF2 and RF3. RF1 recognizes the termination codons UAA and UAG; RF2 recognizes UAA and UGA. RF3 is the class 2 release factor, a GTP-binding protein that facilitates binding of RF1 and RF2 to the ribosome and is required to eject them from the ribosome following peptidyl-tRNA hydrolysis. In contrast, eukaryotes have only two release factors: one class 1 release factor, eRF1, which recognizes all three termination codons, and one class 2 release factor, eRF3, a ribosome-dependent GTPase that helps eRF1 release the finished polypeptide.

After termination, the ribosomal subunits are recycled so that they can be used in another round of initiation. Ribosomes do not release spontaneously from the mRNA and from the deacylated tRNA, they need help from ribosome release factor (RRF). The latter 3D structure strongly resembles a tRNA, which is a structural pre-requisite for RRF to bind the ribosome A site (Lancaster *et al.*, 2002). RRF works synergistically with EF-G-GTP and IF3 in releasing either the 50S ribosomal subunit or the whole ribosome. The released subunits can then be used in a new round of translation.

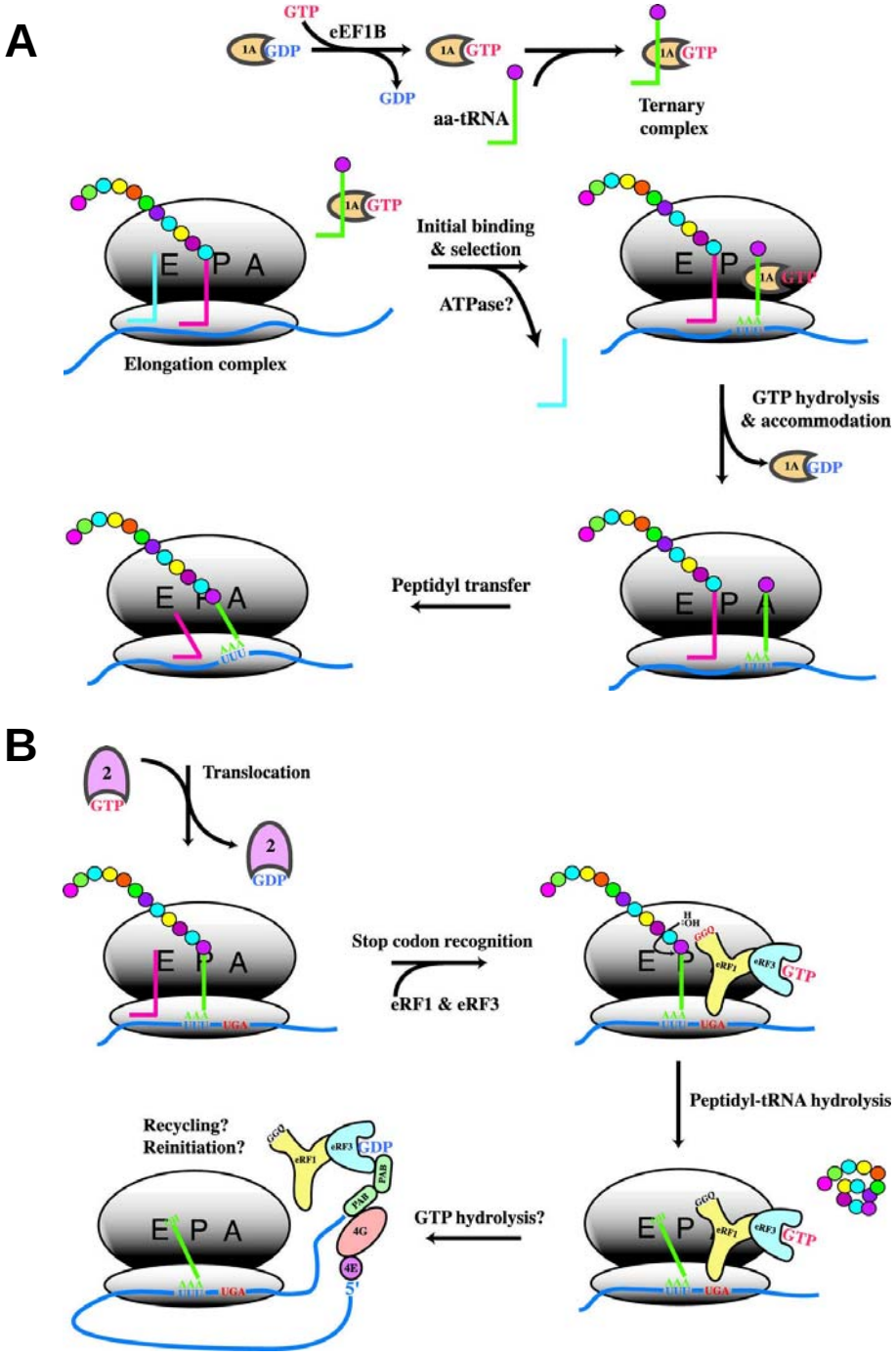


Figure 5. Current models for translation elongation (A) and termination (B) in eukaryotes [(adapted from (Kapp *et al.*, 2004)].

The closed-loop model of translation of eukaryotic mRNAs proposed in the last few years suggests the possibility that termination and recycling may not release the 40S subunit back into the cytoplasm. Instead, the 40S subunit may be shuttled across or over the poly(A)-tail back to the 5'-end of the mRNA via the 5'- and 3'-end-associated factors. In this model, the closed-loop serves to facilitate reinitiation of translation rather than (or in addition to) the first initiation event. This proposal was recently reinforced by the finding that eRF3 and PAB interact with each other, connecting the termination apparatus to the poly(A)-tail (Hoshino *et al.*, 1999).

2. The mRNA translation rules: genetic code

The genetic code is a set of three-base code words, or codons, present in mRNA that instructs the ribosome to incorporate specific amino acids into a polypeptide. It was established in the 1960s and was defined as the rule that governs the transfer of genetic information from nucleic acids to proteins. By establishing a one-to-one correspondence between nucleic acids and proteins, the genetic code allows for stable inheritance of phenotypic variation produced by proteins upon which natural selection acts. The combination in triplets of the 4 ribonucleosides (adenosine or A, uridine or U, guanosine or G, cytidine or C) results in 64 different codons that make the genetic code (Figure 6). This code is non-overlapping, being each base part of only one codon. It is also devoid of gaps, being each base in the coding region of an mRNA part of a codon.

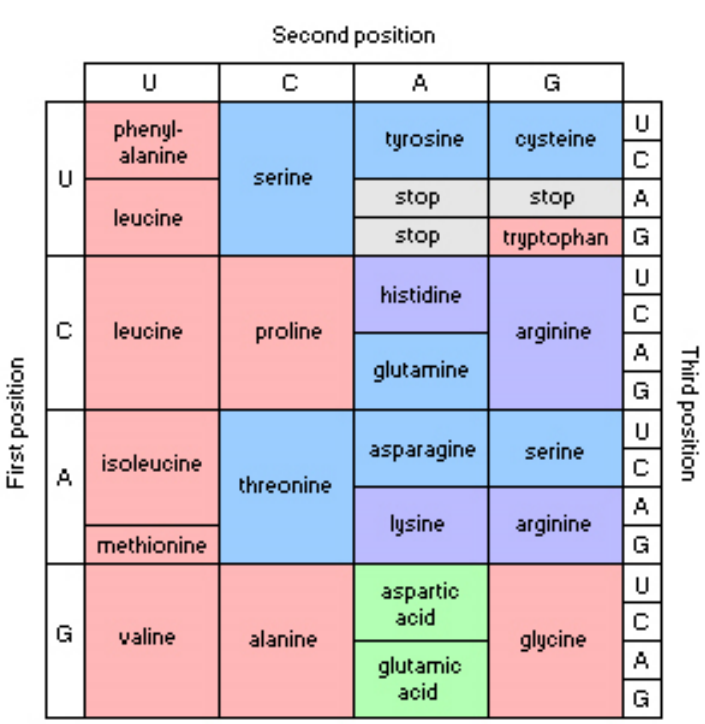


Figure 6. The standard genetic code. The colours in the table indicate the fundamental chemical properties of the side chains of the amino acids. Those in pink are hydrophobic. The others are hydrophilic. Of these, amino acids with polar side chains are coloured in blue, those with acidic side chains in green and those with basic side chains in purple.

The standard genetic code shows considerable order in the assignment of codons within and between amino acids. From the 64 codons, 3 are stop signals and the rest (61) code for the 20 existing amino acids. This means that the code is highly degenerate (Sonneborn, 1965;Zuckerkandl and Pauling, 1965;Woese, 1965b). Some amino acids are specified by only one codon, namely methionine (Met, AUG codon) or tryptophan (Trp, UGG codon), but the remaining amino acids can be specified by more than one codon. Codons that are assigned to the same amino acid are called synonymous codons and in most cases share the first 2 nucleotides, being clustered together, rather than being randomly distributed throughout the code. A group of four synonymous codons is a “family box” or a “four-codon box” and a group of two synonymous codons is a “two-codon set”. Alanine (Ala), glycine (Gly), proline (Pro), threonine (Thr) and valine (Val) are encoded by family boxes of codons, while asparagine (Asn), aspartate (Asp), cysteine (Cys), glutamine (Gln), glutamate (Glu), histidine (His), lysine (Lys), phenylalanine (Phe) and tyrosine (Tyr) are encoded by two-codon sets. Arginine (Arg), leucine (Leu) and serine (Ser) are the exception since they have six synonymous codons (a family box and a two-codon set). Isoleucine (Ile) has three synonymous codons.

The degeneracy of the code appears to be controlled by the GC content of codons. Watson-Crick base pairs between C and G involve three hydrogen bonds, while those between A and U involve only two. Thus, GC base pairs are more stable. All codons in which the doublet (the first two bases) is composed solely of G and C form four-codon boxes, while those in which the doublet is composed solely of A and U form split sets (either two two-codon sets or one three-codon set plus one one-codon set). This pattern might have arisen because all GC doublets bind sufficiently strongly to their cognate tRNA anticodons and do not require the third base pair, while all AU doublets bind weakly and need a third base pair (Lagerkvist, 1978;Lagerkvist, 1980;Lagerkvist, 1981). Mixed doublets form a four-codon box if the second base is a pyrimidine (U or C), but form split boxes if the second base is a purine (A or G). Presumably, the larger purine at the second position reduces binding at the third position, resulting in finer discrimination.

Isoacceptor tRNA species that bind the same amino acid, but recognize different codons can in part explain this degeneracy of the code but not completely, because they would require that all organisms need to have at least 61 tRNAs, one for each sense codon. Indeed, most organisms contain about 60 different tRNAs, but some live with fewer tRNAs than the number of codons. This is explained by the so-called wobble hypothesis – the first two bases of a codon pair correctly with the tRNA anticodon according to Watson-Crick base-pairing rules, but the last base of the codon is allowed to move slightly from its normal position to form a non-Watson–Crick base pair with the anticodon. This wobble phenomenon allows the same aminoacyl-tRNA anticodon to pair with more than one codon, reducing the number of tRNAs required to translate the genetic code. The wobble pairs are G-U (or I-U) and I-A, being I inosine whose structure is similar to that of guanosine.

The distribution of the amino acids over the genetic code table is also biased toward the amino acids polar properties. Codons encoding amino acids with similar chemical properties tend to be related. For example, codons with a U at the second position code for five of the most hydrophobic amino acids (Phe, Leu, Ile, Met and Val) and the six most hydrophilic amino acids (His, Gln, Asn, Lys, Asp and Glu) are encoded by codons with an A at second position. Other connections between codons for amino acids with similar chemical properties can also be observed, in the case of Asp and Glu that share their doublet (Woese, 1965a;Woese, 1965b;Volkenstein, 1966;Woese *et al.*, 1966a;Woese *et al.*, 1966b). This biased codon organization and redundancy may minimize decoding error and the impact of such error on the proteome, suggesting that codon-decoding error was a major contributor to the evolution of the genetic code.

2.1 Genetic code components: tRNAs and aaRSs

2.1.1 Transfer ribonucleic acids (tRNAs)

Transfer RNA (tRNA) was discovered as a small RNA species independent of ribosomes that could be charged with an amino acid and could then pass the amino acid to a growing polypeptide. The “adaptor hypothesis” proposed by Crick (Crick, 1958) predicted the existence of a factor linking codons and amino acids. Indeed, tRNAs have this dual role in the process of translation as they base pair with the codons of the mRNAs and are amino acylated by aminoacyl-tRNA synthetases (aaRS). This amino acid charging activity defines in fact the genetic code as it links anticodons to amino acids, in other words, it links the amino acid alphabet with the nucleic acids alphabet.

tRNAs are grouped in families of isoacceptors, which are tRNA species that are recognized by a single aaRS, but decode different codons. Since their discovery in the early 1970s, up to 5800 different tRNA molecules have been identified in organisms belonging to the three domains of life (Sprinzl and Vassilenko, 2005).

All tRNAs share a common secondary structure represented by a cloverleaf-like structure (Figure 7A) which was predicted by Holley (Holley, 1965). They have four base-paired stems defining three stem-loops – the D loop, the anticodon loop and the T loop – and the acceptor stem with the 3’ single stranded CCA end, to which amino acids are added in the charging step. The number of residues in the stem and loop regions is conserved and can therefore be referenced by a standard number. tRNAs also have a variable or extra region or loop between the anticodon and the T loops, that according to its length, can cluster tRNAs in two families – class I and class II. Class I tRNAs have short variable loops of four or five nucleosides and comprises almost all existing tRNAs, while those of class II have long variable arms of 10 to 24 bases. This last class is formed by leucine and serine tRNAs in eukaryotes and in eubacteria and organelles by leucine, serine and tyrosine tRNAs (Dirheimer *et al.*, 1995).

tRNAs also share a common three-dimensional shape that resembles an inverted L (Figure 7B). This shape maximizes stability by lining-up the base pairs in the D stem with those in the anticodon stem and the base pairs in the T stem with those in the acceptor stem, thus defining two functional domains. The anticodon of the tRNA protrudes from the side of the anticodon loop and is twisted into a shape that readily base-pairs with the corresponding codon in mRNA. The domain that interacts with the mRNA template and the amino acid attachment site are at opposite ends of the tRNA. These distinct structural domains had independent origins. Indeed, they bind to different domains of aaRSs and the T-acceptor minihelix functions as an independent unit which can be recognized and charged by aaRSs and binds to the elongation factor EF-Tu (Schimmel and Ribas de, 1995). This suggests that the T-acceptor minihelix is an ancient structure upon which the early genetic code might have relied upon, whereas the D- and the anticodon arms are late acquisitions (Noller, 1993).

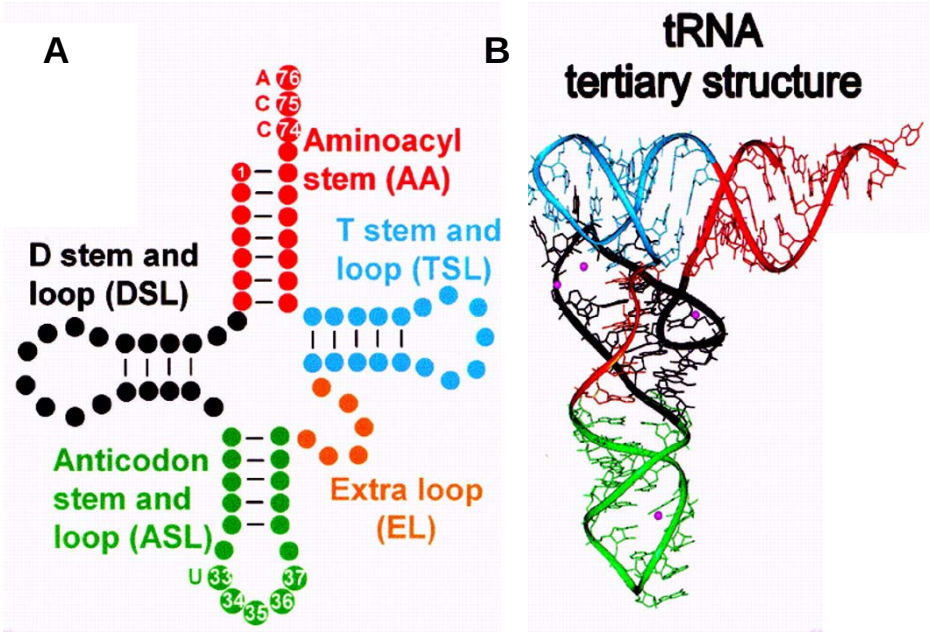


Figure 7. The structure and domains of tRNA. The cloverleaf secondary structure (A) is colour-coded to identify the three-dimensional structure represented in (B). The positions of the invariant U33 and the amino acid accepting 3'-terminus (C74, C75 and A76) are shown [adapted from (Agris, 2004)].

The structural elements of a tRNA that are recognized by each of the 20 aaRSs are usually designated as identity elements and they can be distributed all over the tRNA molecule. In the acceptor region, the first 3 base pairs (1-72, 2-71 and 3-70 pairs) and the unpaired base at position 73 (discriminator base) are important identity elements. Each tRNA family has its own discriminator base and most tRNAs accepting chemically similar amino acids are characterized by an identical, phylogenetically well-conserved residue at this position (Crothers *et al.*, 1972). The anticodon and the variable arm are other regions containing important identity elements for tRNA recognition by aaRS (Figure 8). On the other hand, tRNAs also have negative determinants or anti-determinants that prevent their recognition by non-cognate aaRSs.

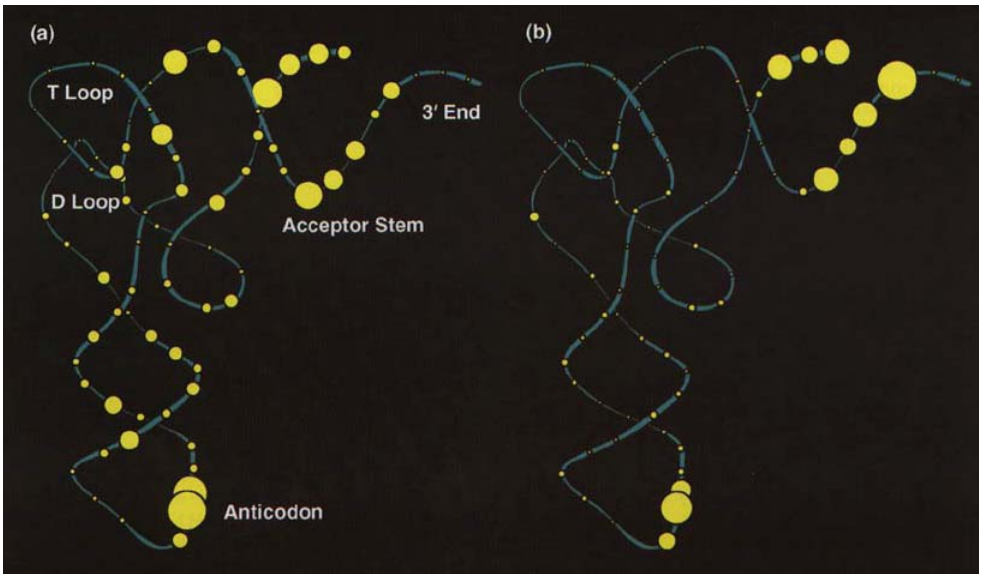


Figure 8. Distribution of computer-predicted (a) and experimentally observed (b) identity elements for *E. coli* tRNA aminoacylation. The tRNA chain is in blue and each nucleotide position is a yellow circle whose diameter is proportional to the fraction of the 20 tRNA acceptor types in *E. coli* for which the nucleotide position is a predicted or observed determinant [adapted from (McClain, 1993)].

Modified nucleosides are also present in tRNA molecules, being this type of nucleic acid the most modified molecule in all kingdoms of life (Woese *et al.*, 1990;Sprinzl *et al.*, 1998). Base modifications are introduced post-transcriptionally and they influence tRNA structure and the efficiency of translation, due to their

direct and indirect involvement in codon recognition and role as determinants of cognate aminoacylation (Bjork, 1995;Agris, 2004). There are more than eighty modified nucleotides in tRNAs and some of them are conserved in the 3 domains of life, namely the dihydrouridine in D-loops or ribothymidine in T-loops (Bjork *et al.*, 1999). The biggest diversity of modifications is found at positions 34 (first anticodon position) and 37 (3' to anticodon position) of the tRNA [reviewed in (Agris, 2004)]. The anticodon region is also the only structural domain that contains hypermodified bases, namely the guanosine derivatives wybutosine (W) and queuosine (Q) (Yokoyama *et al.*, 1985). Methylation and acetylation are evenly distributed over the entire tRNA structure.

The wobble position (position 34) is very sensitive to the presence of modified nucleotides as they affect the decoding properties of tRNAs. For example, the presence of modified U at the wobble position functions either to extend or restrict the decoding properties of tRNAs. Also the presence of inosine (I) is common at that position in some eukaryotic tRNAs allowing base pairing with A, U and C. Queuosine (Q) is a hypermodified nucleoside that replaces G after its excision from the ribophosphate backbone and, with the exception of yeast, Q is found at position 34 of tRNAs and permits base pairing with all four nucleotides at the wobble base position.

Modified bases at position 37 seem to strengthen the base pairing between the last base of the anticodon (position 36) and the first base of the codon, as is the case of isopentenyladenosine (i^6A) in tRNAs that read codons starting with U. However, the most conserved modified residues in position 37 are 1-methylguanosine (m^1G) in tRNAs that decode codons starting with C, and the threonylcarbamoyladenosine (t^6A) in tRNAs that decode codons starting with A (Bjork *et al.*, 1999;Agris, 2004). The existence of these conserved modified residues highlights the important function of base modifications since they appeared early during the evolution of life (Bjork, 1995). Modified nucleosides in the anticodon can modulate the recognition of tRNAs by aaRSs. Modifications at other positions may also act as identity determinants or anti-determinants and

conformational changes of the tRNA induced by modified bases can play an indirect role in the aminoacylation process.

Several tRNAs present in various organisms and cell types have secondary roles that go beyond the traditional tRNA role in protein synthesis. For example, tRNAs can serve as biosynthetic scaffold for several biochemical reactions being important in particular aspects of cellular metabolism in different cells. tRNAs can be important as precursors in the synthesis of other tRNAs, as donors of amino acids for the inter-peptide bridge synthesis of bacterial peptidoglycan, in the degradation pathway of proteins, in the modification of bacterial membrane lipids, in the initiation of reverse transcription in retroviruses, as intermediates in antibiotics synthesis and in the synthesis of tetrapyrroles (Francklyn and Minajigi, 2010). New functions and new mechanisms of action are continuously being discovered as highlighted by the recent findings that tRNAs are able to transform mammalian cells (Marshall *et al.*, 2008) and can work as small interfering RNAs (Maniataki and Mourelatos, 2005;Cole *et al.*, 2009).

2.1.2 Aminoacyl-tRNA synthetases (aaRSs)

Aminoacyl-tRNA synthetases (aaRSs) are the enzymes that establish the genetic code because they catalyze the ligation of amino acids to their cognate tRNAs (aminoacylation reaction). The aaRSs are highly selective for their amino acid and tRNA substrates, and in most cases directly acylate the tRNAs in an ATP-dependent two-step reaction. First, ATP and the amino acid bind to the active site of the aaRS to form an aminoacyl-adenylate and then the amino acid is attached to the tRNA by 3'-esterification [reviewed in (Ibba and Soll, 2000)].

These enzymes are divided in two classes, each class evolved from an independent ancestor. Their catalytic domains are distinct (Eriani *et al.*, 1990;Moras, 1992) and they display significant structural variability in secondary, tertiary and quaternary structures. For example, class I synthetases are

predominantly monomers while class II are obligate homo or heterodimers (Figure 9).

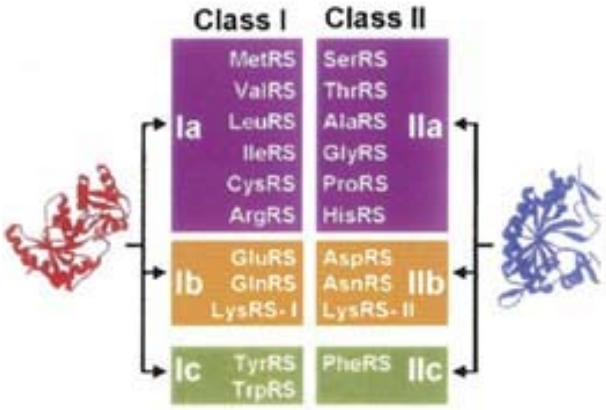


Figure 9. Classes of aminoacyl-tRNA synthetases. The two major classes can be organized into subclasses of enzymes with closely related sequences. Significantly, the subclasses also group tRNA synthetases according to their amino acid chemical types [adapted from(Schimmel, 2008b)].

Class I enzymes aminoacylate the 2'-hydroxyl group of the ribose from the terminal adenosine of the tRNA while class II enzymes aminoacylate the 3'-hydroxyl group, supporting the hypothesis of a common ancestor for synthetases of the same class (Mechulam *et al.*, 1995;Woese *et al.*, 2000). Furthermore, the two synthetase classes also differ in the binding of tRNAs. Class I synthetases have pockets for the acceptor stem and anticodon of their cognate tRNAs and approach the tRNAs from the D loop and acceptor stem minor groove side while class II synthetases also have pockets for the acceptor stem and anticodon, but approach their tRNAs from the opposite side, i.e., the variable arm and major groove of the acceptor stem (Ruff *et al.*, 1991). Interestingly, when the members of the two classes of synthetases are listed according to the subclasses a, b and c (Cusack, 1995), a symmetry emerges, both in terms of the number of members and in terms of the chemical properties of the amino acid. Such symmetry is particularly obvious between the members of subclasses Ib and IIb, as both recognize charged amino acids and their derivates; and between Ic and IIc, that recognize the aromatic amino acids (Moras, 1992;Cusack, 1995;Ribas de and Schimmel, 2001).

The high amino acid selectivity of at least some aminoacyl-tRNA synthetases is controlled by a double-sieve mechanism. The first sieve is a coarse one that excludes amino acids that are too big for the active site. Enzymes accomplish this task with an active site for activation of amino acids that is just big enough to accommodate the cognate amino acid, but not larger amino acids. The second sieve is a fine one that degrades aminoacyl-AMPs that are too small for the active site. The enzyme accomplishes this task with a second active site (the editing site) that admits small aminoacyl-AMPs and hydrolyzes them. The cognate aminoacyl-AMP is too big to fit into the editing site and escapes of being hydrolyzed. Instead, the enzyme transfers the activated amino acid to its cognate tRNA (Nureki *et al.*, 1998).

The aaRSs contribute to other cellular functions in addition to protein synthesis. This is not too surprising because aminoacylation reactions require the capacity to recognize tRNAs as well as small molecules such as amino acids and ATP thus creating important structural plasticity that allows aaRSs to interact with diverse molecules. Besides regulating the expression of their own genes, some aaRSs have also been implicated in translational regulation, amino acid metabolism and intron splicing (Mechulam *et al.*, 1995;Ibba *et al.*, 2000). The catalytic activities for glycyl-, lysyl- and tryptophanyl-tRNA synthetases have been adapted to synthesize diadenosine polyphosphates, which are believed to regulate glucose metabolism (Edgecombe *et al.*, 1997;Verspohl *et al.*, 2003), cell proliferation and death (Nishimura *et al.*, 1997). In higher eukaryotes, aaRSs form macromolecular complexes via multivalent protein-protein interactions (Rho *et al.*, 1999;Lee *et al.*, 2004b). These complexes are involved in the regulation of transcription, translation and in various signalling pathways (Lee *et al.*, 2004b;Park *et al.*, 2005). Also, other aaRSs, like tryptophanyl- and tyrosyl-tRNA synthetases are well-characterized procytokines, but are not generally associated with the complex (Wakasugi and Schimmel, 1999;Wakasugi *et al.*, 2002). Considering the functional versatility of these enzymes and their range of functions it is not surprising that they are associated with various human diseases, namely neuronal diseases, cancer, autoimmune diseases or others, like diabetes (Park *et al.*, 2008). Therefore, the

roles of aaRSs beyond establishing the genetic code and maintaining the fidelity of mRNA translation place them as important regulators of cellular function by coupling translation to cell signalling pathways and biological networks.

2.2 Exceptions to the rules – natural genetic code alterations

When the genetic code was elucidated, the same code structure was found in human cells, in the bacterium *E. coli* and in viruses. This led to the assumption that all organisms used the same genetic code and for this reason it was coined as the universal genetic code. Since any change in the code would be equivalent to introducing mutations throughout the proteome, Crick proposed that the codon assignments were a “frozen accident” that became fixed once proteins played crucial roles in metabolism (Crick, 1967;Crick, 1968). Later, the observation that the vertebrate mitochondrial code differed from the universal code prompted the search for other variants, several of which have now been found in bacterial and in eukaryotic nuclear and mitochondrial systems, showing that the code evolves and is surprisingly flexible. In certain eukaryotic nuclei and mitochondria and even in some prokaryotes, stop codons code for amino acids such as Trp, Glu, Gln, Cys and Tyr and have also been used to expand the genetic code to selenocysteine (Sec) and pyrrolysine (Pyl) (Knight *et al.*, 2001) (Figure 10).

In some organisms, the AUA codon has been reassigned from Ile to Met, the AGA/G (Arg) codons have been reassigned to Ser, Gly or to Stop and the AAA (Lys) codon has been reassigned to Asn. In the mitochondria of several yeast species, including *Saccharomyces cerevisiae*, the CUN (Leu) codon family has been reassigned to Thr. In bacteria, the UGA stop codon has been reassigned to Trp in *Mycoplasma* spp. and *Spiroplasm*a spp. (Knight *et al.*, 2001) and is ambiguously decoded as Stop and Trp in *Bacillus subtilis* (Karow *et al.*, 1998). The A + T rich AUA (Ile) and AGA (Arg) codons are unassigned in the G + C rich genome of *Micrococcus* spp. (75% GC), and the CGG (Arg) codon is unassigned in the A + T rich genome of *Mycoplasma* spp. (25% GC) (Oba *et al.*, 1991;Kano *et*

al., 1993). Different species of ciliates also reassigned stop codons. The UGA stop has been reassigned to Cys in *Euplotes* spp. and is decoded as Cys or Sec in *Euplotes crassus* by two different UGA decoders, namely the $tRNA_{UCA}^{Sec}$ and the $tRNA_{UCA}^{Cys}$ (Turanov *et al.*, 2009). In other ciliates, including the model *Tetrahymena thermophila*, and in the green algae *Acetabularia* spp and *Batophora oerstedii* the UAA and UAG stop codons have been reassigned to Gln (Knight *et al.*, 2001). In several species of the genus *Candida* and *Debaromyces*, Leu CUG codons are decoded as Ser by a novel seryl-$tRNA_{CAG}$ ($tRNA_{CAG}^{Ser}$). This is the only sense-to-sense genetic code alteration known so far in eukaryotic organisms (Santos and Tuite, 1995;Miranda *et al.*, 2006).

The molecular mechanism(s) of evolution of these genetic code alterations are still poorly understood. However, several studies suggest that the genetic code is still evolving despite the strong negative forces working against the fixation of mutations that result in codon mistranslation and reassignment.

Two main theories have been proposed to explain the evolution of the genetic code, namely the Codon Capture and the Ambiguous Intermediate theories (Osawa *et al.*, 1992;Osawa and Jukes, 1995;Schultz and Yarus, 1996). The Codon Capture theory postulates that G + C pressure plays a major role in the evolution of genetic code alterations via its biased effects on codon usage (Osawa *et al.*, 1992). The theory posits that codons can disappear from genomes due to strong G + C or A + T replication pressure, and is supported by the unassignment of the AGA, AUA in *Micrococcus* spp. (75% GC) and CGG in *Mycoplasma* spp. (25% GC). The theory also postulates that such unassigned codons promote reassignment if they reappear in the genome, due to alteration in the DNA replication bias. Their reassignment is mediated by non-cognate tRNAs that misread them (Osawa *et al.*, 1995). However, if such misreading tRNAs do not exist, the re-emerged codons block mRNA decoding and can be toxic (Kowal and Oliver, 1997), but the theory does not provide a mechanism to circumvent such toxicity.

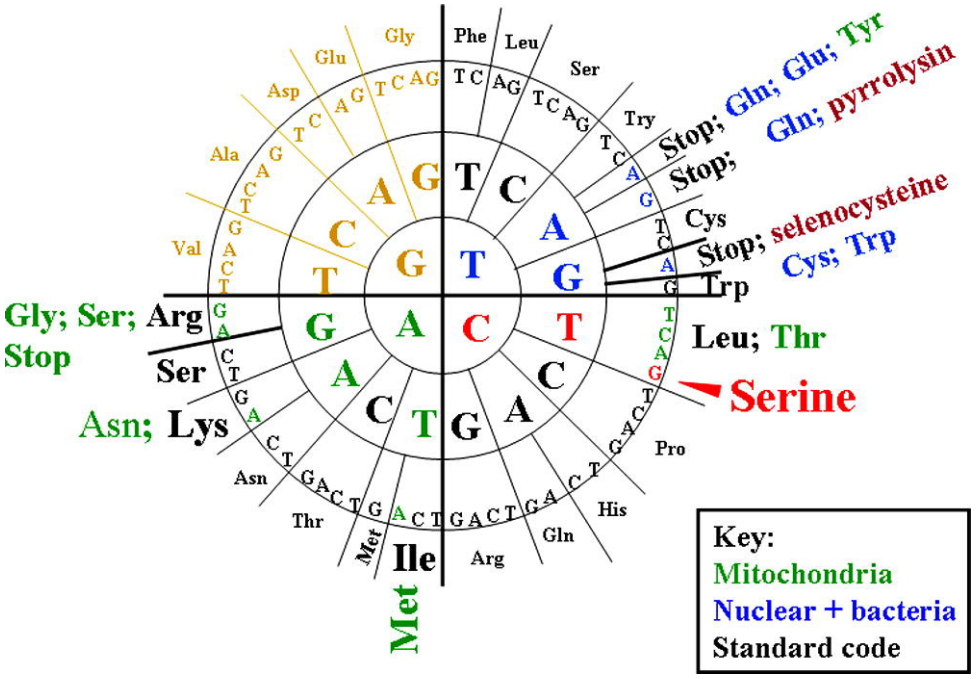


Figure 10. Diagram showing the genetic code alterations discovered so far. To date, 19 genetic code alterations have been discovered in mitochondria (green colour), in bacteria and several unicellular eukaryotes (blue colour). The bacterial and eukaryotic genetic code alterations are a subset of the mitochondrial ones. The diagram indicates that, with exception of the leucine CUN codon family, codon reassignments involve codons of the ANN or UNN types, which suggests that the strength of the first codon–anticodon base pair is important for the evolution of genetic code alterations. The UGA and UAG stop codons are also involved in the expansion of the genetic code to selenocysteine and pyrrolysine, respectively [adapted from (Moura *et al.*, 2010)].

The Codon Capture theory cannot explain reassignment of codons in absence of DNA replication biases or in cases where the usage of the reassigned codon is favoured by such bias. Examples of such exceptions are the reassignment of the UGA stop codon to Trp, the UAA from Stop to Tyr, the UAU from Ile to Met, the AAA from Lys to Asn and the AGA from Arg to Ser, Gly or Stop in A + T rich mitochondria. Also, the reassignment of the entire Leu CUN codon family to Thr in fungal mitochondria or the reassignment of the Leu CUG codon to Ser in some fungal species escape the Codon Capture theory (Knight *et al.*, 2001;Miranda *et al.*, 2006).

These codon reassignments are better explained by the Ambiguous Intermediate theory which postulates that misreading tRNAs can capture codons from their cognate tRNAs through a selection-driven process involving gradual increase of misreading efficiency and subsequent disappearance of cognate tRNAs (Schultz *et al.*, 1996;Knight *et al.*, 2001). The theory does not explain how codon ambiguity is selected, but it is strongly supported by CUG reassignment from Leu to Ser in fungi. The caveat of this theory is that ambiguous mRNA decoding can lead to significant alterations in the proteome and generate potentially growth-inhibiting levels of protein malfunction and misfolding, which in turn, would impact both cell physiology and adaptation. This theory implicates that, contrary to expectation, codon ambiguity provides some kind of selective advantage to drive codon reassignment to completion. It requires also the disappearance or reduction of codon usage to a tolerable minimum caused by ambiguous decoding and in fact, previous studies have already shown that the CTG codons present in the *Candida* clade species are new codons, which evolved recently (Massey *et al.*, 2003). The genetic code change observed in these species altered the decoding rules of CUN codons and altered the usage of CUG codon. CUG codons in *Candida albicans*, for example, almost never align opposite CUG codons in *Saccharomyces cerevisiae*. Instead, CUG serine codons in *C. albicans* align primarily to *S. cerevisia*e codons for serine or other hydrophilic residues and CUG leucine codons in *S. cerevisiae* align primarily to leucine codons in *C. albicans* and to other hydrophobic-residue codons. This suggests that replacement of CUG codons in *Candida* species was completely functional (Butler *et al.*, 2009).

3. mRNA mistranslation – error in protein synthesis

3.1 Basal mRNA translation error rate

Fidelity in the flow of biological information is critical to maintain homeostasis, fitness and survival in all organisms. This fidelity assures the production of stable and functional proteomes. The flow of information from DNA to protein requires that the three polymerization reactions fundamental to life – DNA replication, transcription and translation – proceed with optimized levels of fidelity and speed. Each of these polymerization processes utilizes the complementarity of nucleotides to choose the correct substrate. DNA and RNA polymerases select precursor nucleotide triphosphates (dNTP and NTP) that are complementary to the DNA template for direct incorporation into the growing nucleic acid chain. Similarly, the ribosome selects the cognate aminoacyl-tRNA (aa-tRNA) based on the complementary of its anticodon with the mRNA codon. Both polymerases and the ribosome must distinguish among very similar substrates with small differences in the free energy of binding in order to achieve high level of fidelity [reviewed by (Cochella and Green, 2005)].

Like genome replication, the processes of DNA transcription into RNAs and mRNA translation into proteins are carefully monitored. Of the 3 genetic information processes mRNA translation is the most error-prone and the translational error is similar in eukaryotes and prokaryotes. For example, *Escherichia coli* has a typical replication mutation rate of approximately 10^{-8} – 10^{-9} per base pair (Kunkel and Bebenek, 2000), having sophisticated error correction mechanisms, like editing and repair, while the typical translational missense error rate is in the order of $5x10^{-4}$ (Edelmann and Gallant, 1977). In eukaryotes, DNA replication error rates are even lower, being in the order of 10^{-10} – 10^{-11} (Kunkel *et al.*, 2000). The error rate of transcription *in vivo* in *E. coli* has been estimated at $1.4x10^{-4}$ per nucleotide and thus around $4x10^{-4}$ per codon (Rosenberger and Foskett, 1981;Rosenberger and Hilton, 1983;Ninio, 1991). More recent *in vitro* studies have shown that the

rate of misincorporation of UTP at G sites during transcription is in the order of $2x10^{-6}$ (Kireeva *et al.*, 2008).

Translational error rates *in vivo* are in the order of 10^{-3} – 10^{-4} (one error in every 1000 to 10000 codons translated) (Loftfield and Vanderjagt, 1972;Jakubowski and Goldman, 1992;Stansfield *et al.*, 1998;Farabaugh and Bjork, 1999;Kramer and Farabaugh, 2007). Errors in mRNA translation can arise from incorrect aminoacylation of tRNAs by aaRSs, from selection of the incorrect tRNA by the ribosome, from frameshifting during translation, from premature termination of the process, from stop codon read-through and from ribosome drop off during mRNA decoding. The substitution of one amino acid for another (ambiguous decoding) is the archetypal error in protein synthesis and *in vivo* studies already demonstrated that codon-dependent selection of aminoacyl-tRNA by the ribosome may be the limiting factor in the accuracy of gene expression or at least makes a large contribution to the average mRNA decoding error. The tRNA aminoacylation step is accurate (typical error of 10^{-4} – 10^{-5}) (Francklyn, 2008) owing to enzymatic selectivity mechanisms precisely adapted to the most closely related amino acids for each aminoacyl-tRNA synthetase (for example, the aaRSs "double sieve" editing mechanism already referred above). However, for certain hyperaccurate ribosomal mutants, the fidelity of transcription may limit the overall accuracy of the protein synthesis process (Bouadloun *et al.*, 1983).

Several attempts to quantify misincorporation rates in the mRNA translation process were made over the last few years, but experimental data is only available for less than 5% of all the possible amino acid changes that can occur at each codon and few of these measurements were done in the same organism. *In vitro* studies and *in vivo* studies in *E. coli* or in *S. cerevisiae* showed that the error rate ranges from $5x10^{-6}$ to $1x10^{-3}$ (Edelmann *et al.*, 1977;Ellis and Gallant, 1982;Stansfield *et al.*, 1998;Daviter *et al.*, 2006;Kramer *et al.*, 2007), which means that depending on the codon and the organism that are being studied the values are variable. Translational frameshifting errors are caused by tRNA slippage during mRNA reading and normally result in synthesis of truncated proteins due to

premature translation termination promoted by a shift in reading frame of one or two bases in either 5' or 3' direction (Farabaugh *et al.*, 1999). They occur at a frequency of 10^{-5} in *E. coli* (Curran and Yarus, 1986). Analysis of premature termination in *E. coli* and in *S. cerevisiae* has also shown that this type of error has a frequency in the order of 10^{-4} to 10^{-3} per codon (Arava *et al.*, 2005). Finally, stop codon read-through can result from competition between non-sense suppressor tRNAs and release factors for decoding of the stop codons and result in synthesis of proteins with extended C-termini. In yeast, it happens with a frequency of 10^{-3} per codon (Valente and Kinzy, 2003).

Erroneous protein synthesis is not just the cumulative result of the error rates of replication, transcription and translation, as other kind of disruption can occur in the conversion of an mRNA into a functional protein. These disruptions can be aberrant splicing, faulty post-translational modifications and kinetic misfolding. Some splicing error rates have already been calculated in several organisms, but a big range of values have been obtained for different genes and for different species. Also, the rate of protein folding errors or other post-translational errors, like incorrect proteolytic cleavage or erroneous ubiquitylation, glycosylation or phosphorylation, that also affect the production of functional proteins, remains unknown and poorly understood.

Living organisms cope well with the basal error rate of protein synthesis and there is no apparent evolutionary pressure for the ribosome to be significantly more accurate than 1 error in every 1000 to 10000 codons translated. Maybe this rate of fidelity represents a compromise that optimizes the evolutionary fitness of the organism – probably a significantly more accurate ribosome would slow down protein synthesis and compromise growth and cell division. In eukaryotes, aberrant proteins that are synthesized at that level of error are rapidly degraded by the ubiquitin-proteasome pathway and/or chaperone-mediated autophagy, or refolded by molecular chaperones (Kubota, 2009). These and other quality control systems serve to edit mistakes and generally maintain cellular functionality.

3.2 mRNA mistranslation - negative and positive features

Mistranslation results in the synthesis of aberrant proteins that fold incorrectly, are unstable or are rapidly degraded. This inevitability has negative consequences for homeostasis and physiology. High-level mistranslation caused by aminoglycoside antibiotics (streptomycin) induces massive proteome disruption and sudden cell death; this explains their usage over the years as a therapeutic strategy to combat microbial infections (Balashov and Humayun, 2002;Kohanski *et al.*, 2008). Mistranslation generated by mutations in the editing domain of alanyl-tRNA synthetase (AlaRS) causes severe neurodegeneration and ataxia in mice (Lee *et al.*, 2006). In all cases studied, mistranslation results in increased amounts of unfolded proteins and activates the protein quality control mechanisms (as Unfolded Protein Response) and eventually leads to apoptosis (Nangle *et al.*, 2006;Geslain *et al.*, 2009). In *E. coli*, similar mutations in the editing mechanism of valyl-tRNA synthetase (ValRS) and isoleucyl-tRNA synthetase (IleRS) decreases bacterial growth rate and viability (Nangle *et al.*, 2002;Bacher *et al.*, 2005) and results in the accumulation of defects in proteins of the DNA replication machinery and ultimately in error-prone replication of the genetic material (Bacher and Schimmel, 2007). Therefore, mutations in the ribosome or other factors important for translational fidelity may trigger similar pathologies. These and other observations generalized the idea that translational error is detrimental to life; this is partly true because above a certain mistranslation threshold the proteome is disrupted and cell fitness and viability decrease.

Surprisingly, mistranslation can also be beneficial as it enables organisms under adaptative pressure to sample new landscapes of protein sequences (Shorter and Lindquist, 2005). Direct evidence for this hypothesis comes from studies of the non-Mendelian transmission of the [PSI+] prion trait in the budding yeast *Saccharomyces cerevisiae*. The [PSI+] state is induced by a self-replicating conformation of the termination factor eRF3 (encoded by the gene SUP35) and reduces translation termination efficiency and promotes stop codon read-through (Paushkin *et al.*, 1996). Read-through of stop codons in [PSI+] yeast strains

increases their phenotypic diversity and allows them to better adapt to a variety of challenging environments (True and Lindquist, 2000;True *et al.*, 2004). A functioning prion domain in Sup35 is conserved among yeast species across 100 million years of evolution (Chernoff et al., 2000; Nakayashiki et al., 2001), consistent with the idea that a reduction in translational fidelity can confer a selective advantage. Functionally relevant read-through of stop codons appears to be also used by retroviruses that sequester eRF1 to enhance this process and allow for the expression of key viral factors (Orlova *et al.*, 2003). Other cases where positive aspects of mistranslation were revealed are the CUG misreading in *Candida albicans*, which generates extensive morphological diversity and phenotypic variation (Miranda *et al.*, 2007) and the ambiguous translational decoding caused by mutations in the editing site of IleRS that confers advantages in bacteria, in specific situations of selective pressure. (Pezo *et al.*, 2004;Bacher *et al.*, 2007).

There are also instances where loss of translational fidelity is co-opted to facilitate a regulatory process or to adapt to a specific situation. For example, the level of bacterial termination factor RF2 in the cell is modulated by a frameshifting-regulated feedback loop (Craigen and Caskey, 1986). The production of key gene products in many retroviruses also depends upon similar frameshifting events (Jacks and Varmus, 1985). Studies carried out in HeLa and dendritic cells showed that 30% of newly synthesized proteins are aberrant and are rapidly targeted for degradation through the ubiquitin-proteasome pathway; such defective ribosomal products (DRiPs) are a major source of presentation peptides for the MHC class I system and it is likely that mistranslation plays a critical role in surveillance of cell identity by the immune system (Yewdell *et al.*, 1996;Yewdell *et al.*, 2001). Also, mammalian cells exposed to viral infections, specific receptors or to chemical oxidative stress, increase the misincorporation of methionine (Met) into the proteome due to Met-misacylation of various non-cognate tRNAs. Remarkably, Met is a ROS scavenger and such misacylation may be adaptive as it protects cells from the damage caused by oxidative stress (Netzer *et al.*, 2009). The extent

to which mistranslation contributes to other normal and pathological biological processes is being intensively studied at the moment.

4. Protein quality control systems and protein homeostasis

Proteome quality control systems are essential to protect cells against malformed proteins synthesized as described above and also against physiological situations that increase the concentration of aberrant/damaged proteins in the cell, namely environmental stress. Quality control systems are generalized and act at the level of all macromolecules, namely proteins, RNA and DNA, as well as at the organelle and whole-cell level. They serve to edit mistakes and maintain functionality. Therefore, quality control systems permit a certain level of biological error allowing cellular processes to be a bit messy and slightly unreliable.

The proteome quality control systems (Figure 11) are composed by molecular chaperones, specialized intracellular proteases and accessory factors that supervise protein folding, counteract aggregation and eliminate misfolded and damaged polypeptide chains before they can exert toxic effects. Therefore, the maintenance of functional proteins, their turnover and the removal of damaged and aberrant proteins are the central tasks of proteome quality control systems. Under normal conditions the proteome quality control systems can eliminate defective ribosomal products due to errors in translation or post-translational processes (Schubert *et al.*, 2000) as well as aberrant proteins originating from the load of gene variations present in the respective individual or from damage by covalent protein modifications like those elicited by reactive oxygen species (ROS) (Dunlop *et al.*, 2002). Disturbance of the proteome quality control systems by overloading them with misfolding variant proteins, environmental challenges, or gene variations in protein quality control components may trigger cell degeneration, death and disease.

Proteome quality control systems are highly dynamic, consist of components with often redundant functions and react dynamically to proteome quality needs by increasing or decreasing the transcription and translation of their various components. A number of Protein Quality Control genes are constitutively expressed at high level, namely some chaperones (Hsp70) and proteasome

subunits, while others are expressed at low-level under normal physiological conditions and are strongly induced upon stress. Several factors link the folding and protein degradation machineries and help tuning protein quality control to a diverse array of cellular demands (McClellan *et al.*, 2005). There may be tissue specific differences in the ability to elicit the stress response and tune proteome quality control, which may explain why certain mutations in proteins that are expressed in all tissues only give rise to cellular dysfunction in certain tissues.

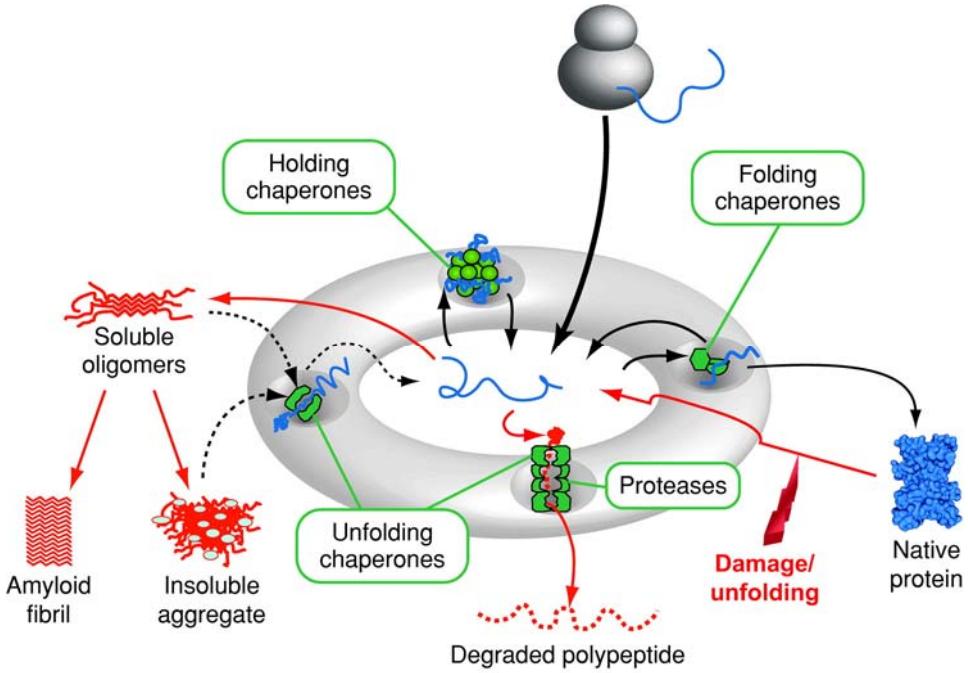


Figure 11. Overview of Proteome Quality Control systems. These systems manage the pool of unfolded and partially folded protein conformations. Folding chaperones promote folding, holding chaperones maintain solubility while unfolding chaperones disaggregate protein aggregates or unfold misfolded proteins and inject them into proteolytic chambers of the Protein Quality Control systems [adapted from (Gregersen *et al.*, 2006)].

4.1 Proteome folding and quality control systems in the cytosol

The Proteome Folding and Quality Control systems of the eukaryotic cytosol comprise a large number of components that constantly assemble and

disassemble with their substrates. Upon emerging from the ribosome, nascent polypeptides are protected by chaperones, such as the nascent-polypeptide-associated complex (NAC), Hsp40, Hsp70, prefoldin and the TCP-1 ring complex (TRiC), and are held in a folding competent state until released from the ribosome. Subsequently, most small proteins complete their folding in the cytosol without assistance whereas a fraction of the cytosolic proteins require further assistance from chaperones, namely Hsp90 and TRiC (Hartl and Hayer-Hartl, 2002). TRiC is the most complex cytosolic chaperone and is composed of a double-ring structure of eight different subunits in each ring forming a large cavity in which the polypeptide is folded to a native or near-native form and later released into the cytosol (Spiess *et al.*, 2004). If the folding to the native structure cannot be completed the chaperones assess whether misfolded conformers should be refolded or degraded by the ubiquitin-proteasome pathway in order to eliminate toxic conformations (McClellan *et al.*, 2005).

Targeting a polypeptide for degradation requires a multistep pathway that covalently attaches ubiquitin monomers to proteins. Ubiquitin is activated by the ubiquitin-activating enzyme (E1) and is transferred to an ubiquitin carrier protein (E2). The E2 enzyme and the polypeptide bind to a specific ubiquitin-protein ligase (E3) and ubiquitin is covalently attached to the substrate. Further steps generate a polyubiquitin chain that targets the polypeptide substrate to the proteasome for degradation (Glickman and Ciechanover, 2002). The integrated system of chaperones with the components of the ubiquitin-proteasome pathway comprise the most important cytosolic Protein Quality Control system. Failure of this system to degrade misfolded proteins may lead to formation of protein aggregates that may accumulate at a single juxtanuclear site called an aggresome or as soluble monomers and oligomers, which later may precipitate into long amyloid fibrils. Aggresomes are large globular deposits formed by retrograde transport of aggregated material along microtubular tracks in a highly ordered transportation system (Kopito, 2000), whereas amyloid fibrils are long protein aggregates with a tube-like core region formed by the inherent properties of circular β-sheet structures (Dobson, 2003). Accumulating data suggest that the fibrillar or

aggresomal deposits may not be the primary toxic agent; rather the inherent pathogenicity may reside in the soluble oligomers since the accumulation of larger aggregates may be a way to minimize the damage caused by cytotoxic aberrant proteins. In mammalian cells, larger aggregates are unspecifically degraded by the lysosomal-autophagic pathway (Levine and Klionsky, 2004).

In yeast several heat-shock proteins perform molecular chaperoning while others are involved in the acquisition of thermotolerance, glycolysis and ubiquitylation of proteins. Chaperoning heat-shock proteins prevent protein aggregation and the accumulation of aberrant proteins, but some of them may also assist in the degradation of stress-damaged proteins by enhancing the flow of substrates through the proteolytic pathways (Craig *et al.*, 1994). The major heat-shock proteins in yeast are Hsp104, essential for acquisition of stress tolerance, the Hsp70 family and the Hsp90 (Hsc83/Hsp83) with chaperone functions and other smaller proteins like Hsp60, Hsp30, Hsp26 and Hsp12, with several different functions.

In relation to the ubiquitin-proteasome system in yeast, like in other eukaryotes, it plays key regulatory roles in numerous aspects of cellular regulation, including metabolic or environmental adaptation, cell differentiation, cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation and endocytosis (Glickman *et al.*, 2002). The 26S proteasome is a multisubunit enzyme complex (molecular mass ~2000kDa) forming a dumbbell-shaped structure that occurs in yeast and in all eukaryotic cells. It is present in the cytosol and in the nucleus (Navon and Ciechanover, 2009). The subunits of the 26S proteasome are distributed between two subassemblies, the core particle (20S particle or CP) and the regulatory particle (19S particle or RP). The core particle houses the proteolytic activities of the proteasome, while the regulatory particle confers ATP-dependence and specificity for ubiquitin-protein conjugates. Almost all proteasomal subunits have already been identified in yeast. The seventeen RP subunits have been characterized from purified yeast proteasomes of which the six Rpt proteins belong to the AAA superfamily (ATPase associated activities). The

remainder of the RP subunits (Rpn proteins) form a heterogeneous group. The important role of the 26S proteasome proteolysis system is underlined by the fact that in yeast most of the genes encoding its components are essential genes. With one exception, individual chromosomal deletions of each of the known yeast 20S proteasome genes are lethal (Hilt and Wolf, 1996), as is chromosomal deletion of several of the 19S cap genes. The 20S core particle in yeast is essentially composed by subunits encoded by the *PRE* and *PUP* genes.

4.2 Proteome folding and quality control systems in the ER

The endoplasmic reticulum (ER) is the first compartment of the secretory pathway. It is engaged with ribosomal protein synthesis, co- and post-translational modification and protein folding. Proteins enter the organelle in an unfolded state and begin to fold co-translationally. The ER lumen contains high concentrations of a specialized set of chaperones and folding enzymes, which assist protein folding in conjunction with post-translational modifications, like signal peptide cleavage, disulfide bond formation and N-linked glycosylation. In this respect, the ER plays a crucial role in the Proteome Quality Control by regulating the transport of proteins from the ER to the Golgi apparatus, as only proteins that have attained their native structure in the ER are exported efficiently (Lee *et al.*, 2004a). The list of such ER folding-assistant proteins includes calnexin, calreticulin, chaperones of the Hsp70 and Hsp90 families (like BiP/Grp78 and the glucose-regulated protein Grp94), the ERp57 protein, thiol-disulphide oxidoreductases and protein disulphide isomerases (like Pdi protein) that catalyzes the formation of disulfide bonds.

The principal function of Proteome Quality Control systems in the ER is the recognition of hydrophobic sequences as well as the content of unpaired cysteine residues, immature glycans and aggregation tendency. Linked to this control point is a mechanism called ER-associated protein degradation (ERAD), which is responsible for the retention of misfolded, unassembled or unmodified non-functional proteins in the ER and their subsequent removal. Protein degradation is

carried out by linking the misfolded protein to ubiquitin after it has been re-translocated into the cytosol through the same ER translocon pore which was used for their protein import. The ubiquitin-marked protein is then recognized and degraded by the 26S proteasome in the cytosol (Tsai *et al.*, 2002;Schroder and Kaufman, 2005;Meusser *et al.*, 2005). Prolonged binding of misfolded proteins hydrophobic patches to either calnexin or the BiP complex targets those polypeptides to the ERAD (Figure 12A).

The fact that accumulation of proteins in the ER influences the synthesis of foldases and chaperones, such as BiP and Pdi by transcriptional activation lead to the conclusion that there must be an intracellular signalling pathway from the ER to the nucleus, called the Unfolded Protein Response (Patil and Walter, 2001;Spear and Ng, 2001). The Unfolded Protein Response pathway (UPR) is activated by a unique mechanism not known in any other signal transduction pathway. The sensor protein Ire1 resides in the ER membrane and possesses both kinase and endonuclease activities. When unfolded proteins accumulate in the ER, Ire1 undergoes auto-phosphorylation and oligomerization and catalyses the cleavage of the mRNA encoding a UPR transcription factor called Hac1 in yeast and Xbp1 in mammalian cells. In this way Ire1 initiates an unconventional intron-splicing event that has been shown in *S. cerevisiae* to be completed by a tRNA ligase. Splicing of yeast *HAC1* mRNA removes a translational block mediated by the intron and enables formation of the activator protein (Figure 12B). In mammalian cells, the unspliced Xbp1 mRNA produces an unstable protein that represses transcription of UPR target genes whereas the spliced mRNA is translated into a potent and stable transcription activator protein. Mammalian cells can also attenuate translation initiation during unfolded protein accumulation in the ER in order to reduce the influx of proteins into the ER. This regulatory pathway is initiated by the ER membrane kinase PERK, which has some similarity with Ire1. PERK decreases translation initiation by phosphorylating the translation initiation factor eIF2α. This mechanism is not known in yeast and PERK orthologues are not found in the genomes of the lower eukaryotes.

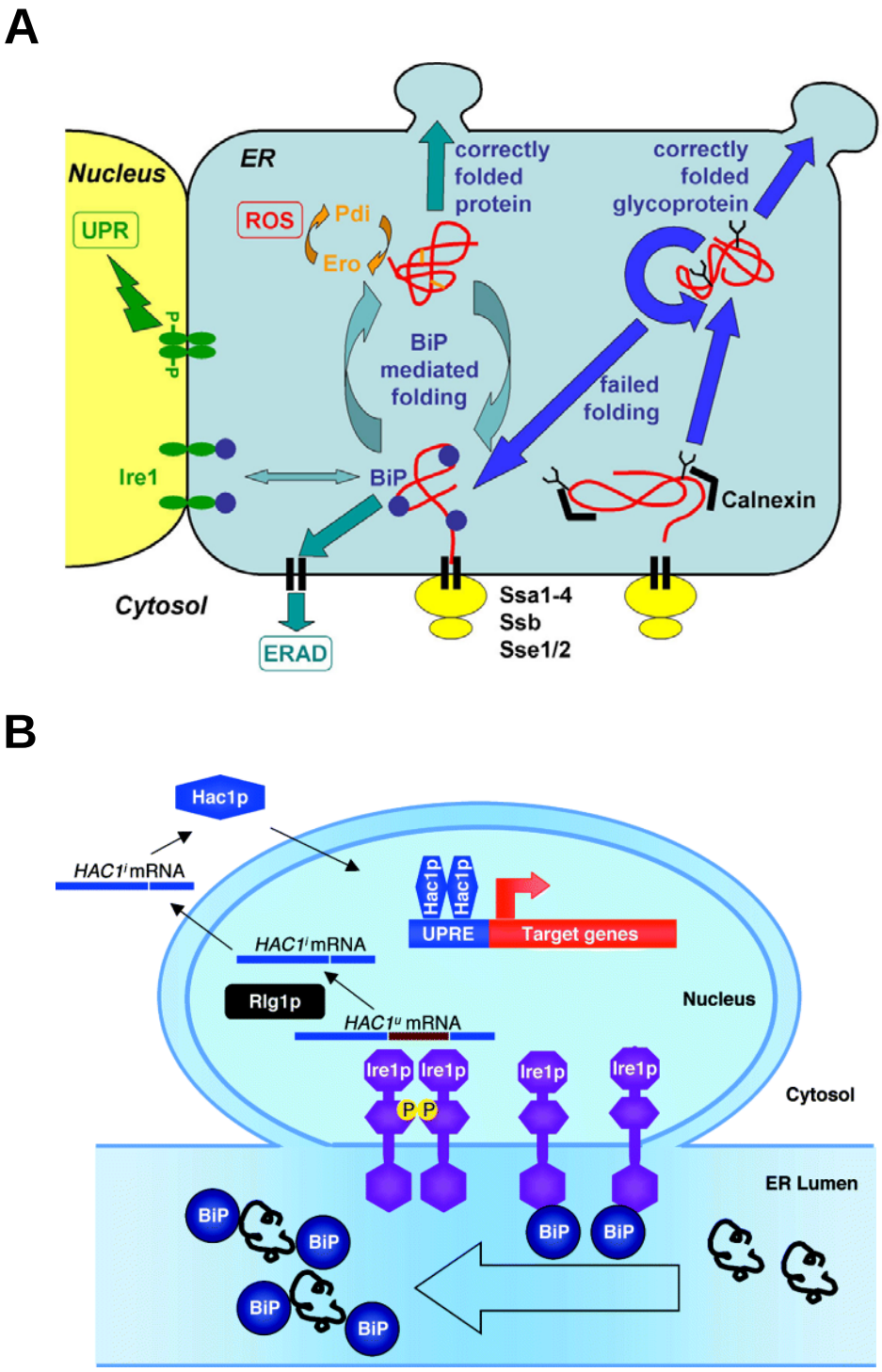


Figure 12. Schematic representation of ER protein folding, quality control, degradation and UPR activation in yeast. (A) Secretory proteins are transported into the ER through the Sec61 translocon complex of the ER membrane either co-translationally or post-translationally. In the latter case, cytosolic chaperones (Ssa1-4, Ssb, Sse1-2) support solubility and prevent aggregation of the polypeptide chains. After translocation into the

ER, nascent polypeptides are bound by BiP and mediated to mature folding in an ATP-dependent cyclic process of release of and binding to BiP. The formation of correct disulfide bonds is mediated in a cycle of Pdi and Ero activity, which may lead to the formation of reactive oxygen species (ROS). Correctly folded protein is released into transport vesicles, while prolonged BiP binding, indicating misfolding, leads to retrograde translocation into the cytosol and proteasomal degradation (ERAD). Nascent glycoproteins are bound by calnexin and mediated to correct folding and processing of the N-glycans. Failed protein folding leads to binding by the BiP complex and targeting to ERAD, while correctly folded and processed glycoproteins are released into transport vesicles. Prolonged binding of BiP to partially misfolded proteins leads to the induction of the unfolded protein response (UPR). (B) In yeast, increased levels of misfolded proteins decrease the relative levels of free BiP chaperones by sequestering them. The occupancy of BiP bound to Ire1 is reduced and promotes its dimerization. Ire1 becomes phosphorylated and the nuclease domain is activated. Through the action of Ire1 and tRNA ligase (Rlg1p), the constitutively expressed *HAC1* transcript encoding the UPR-specific transcription factor is processed, leading to its synthesis. Hac1 translocates into the nucleus, binds to the UPRE target genes and induces their expression [adapted from (Gasser *et al.*, 2008) and (Spear *et al.*, 2001)].

Other types of Proteome Quality Control in the ER often relies on cell specific factors and facilitates secretion of individual proteins or classes of proteins. ER-assisted folding - in competition with ERAD - defines the unique secretory capacity of each tissue. The presence of such tissue-specific Protein Quality Control could provide a general explanation for the development of tissue-specific diseases associated to protein misfolding. A substantial number of cellular proteins are processed and transported through the ER. These include receptors and the ion channels that are expressed on the cell surface, secreted enzymes and hormones, as well as proteins with a specialized function within the organelles of the secretory pathway. Because many of these proteins are essential and indispensable in many physiological processes a variety of disease phenotypes result from impairment of their ER-mediated transport.

4.3 Proteome folding and quality control systems in mitochondria

Mitochondria represent a separate cellular compartment where, in humans, approximately 1500 proteins fold and are degraded (Taylor *et al.*, 2003). Only 13 of the mitochondrial proteins are encoded by the mitochondrial DNA, the bulk is nuclear encoded, synthesized in the cytosol and subsequently imported into the mitochondria. Import of mitochondrial proteins occurs mainly post-translationally in an unfolded conformation through pores in the outer and inner mitochondrial membrane (Wiedemann *et al.*, 2004). Many mitochondrial proteins, especially those of the matrix space, contain amino terminal extensions that counteract premature folding in the cytosol, direct the protein along the mitochondrial import machinery and are cleaved off upon protein arrival in the mitochondrial matrix. Cytosolic molecular chaperones, namely Hsp70 and Hsp90 keep newly synthesized mitochondrial proteins in an unfolded, import-competent conformation (Young *et al.*, 2003). A mitochondrial Hsp70 homolog, the first representative of the mitochondrial Protein Quality Control system, binds to the incoming polypeptide chain and is involved in the translocation process (Wiedemann *et al.*, 2004). The mitochondrial Proteome Quality Control system contains many mammalian and yeast orthologues including molecular chaperones like the mitochondrial Hsp70, the Hsp60/Hsp10 system and a set of proteases with AAA+ domains (resembling the proteasome) that are localized in the matrix or in the inner mitochondrial membrane (Kaser and Langer, 2000).

5. mRNA translation defects, cell degeneration and disease

5.1 Possible degenerative effects caused by mRNA mistranslation

Errors in protein synthesis reduce the organism fitness and these fitness costs can arise by multiple mechanisms. A stable proteome and cellular homeostasis are maintained as long as protein quality control systems work correctly or as long as the basal error of protein synthesis is maintained. However, the failure of these mechanisms or their overloading by increased levels of mistranslation result in accumulation and/or aggregation of aberrant misfolded proteins, causing gradual cell degeneration and possibly death. Protein control quality systems have evolved, as it was already referred in the previous section, to protect cells from unwanted mistranslation products, as well as damaged proteins, all of which may misfold. If the load of misfolded proteins increases, a set of protective response mechanisms induce or up-regulates the expression of chaperones and proteases and reduce protein synthesis rate to alleviate the load (Trotter *et al.*, 2002;Schroder *et al.*, 2005).

In the cases where misfolded proteins are degraded slowly or are aggregation-prone, or if cell stress is intense, long lasting, degradation capacity is compromised, the proteins accumulate and affect a large number of cellular functions. Most of the time, protein synthesis errors lead to loss of function of the proteins, but sometimes they may also produce polypeptides that display a gain of toxic function, generating deleterious effects. For example, the error may confer an alternative or pathological function on an otherwise normal folded protein, or can disrupt folding and the misfolded molecule may start to aggregate and may become cytotoxic. Misfolded proteins can destabilize membranes, decrease quality control of others proteins, induce chronic stress and ultimately increase free radical formation, membrane depolarization and cell death (Stefani and Dobson, 2003;Gidalevitz *et al.*, 2006;Kohanski *et al.*, 2008;Gidalevitz *et al.*, 2009). Cytotoxicity caused by misfolded proteins is being extensively studied as a contributor to several neurodegenerative and other misfolding-associated diseases

(Bucciantini *et al.*, 2002;Lee *et al.*, 2006;Stefani, 2007;Winklhofer *et al.*, 2008). A whole range of damaging mechanisms have already been described in those diseases, including production of ROS and damage to several respiratory complexes (Bruijn *et al.*, 2004). A consequence of oxidative stress is the creation of oxidatively modified proteins that are prone to misfolding and may even block the degradation systems (most notably the ubiquitin-proteasome system) and will create additional oxidative stress, initiating a vicious cycle. To suppress cell damage, due in particular to oxidative stress, cells have a number of defense systems, namely the antioxidant systems (Winyard *et al.*, 2005) and the autophagic system (Levine *et al.*, 2004;Levine and Kroemer, 2008), which detoxify ROS and eliminate damaged cell domains, like dysfunctional mitochondria (Skulachev *et al.*, 2004). When the defense systems fail to sustain cell health, the end point of all cellular dysfunctions is cell death, either as apoptosis or necrosis (Schon and Manfredi, 2003).

5.2 Diseases caused by mistranslation

The list of diseases caused by problems in the mRNA translation process is rapidly growing. There are diseases that result from altered translation of specific mRNAs. Some mutations occurring within the ORF of a specific mRNA or in their 5'-UTRs lead to alterations in the expression of those mRNAs and, therefore, in the expression of the encoded protein (Cazzola and Skoda, 2000;Kozak, 2002;Pickering and Willis, 2005). Diseases like hyperferritinaemia or cataract syndrome, thrombocythaemia and multiple myeloma are caused by defects in the 5'-UTRs of specific mRNAs, which increase their translation efficiency. The loss of IRES-mediated translation of specific mRNAs seems also to be the cause of rare diseases like X-linked Charcot-Marie-Tooth disease (CMT) (Hudder and Werner, 2000) and X-linked dyskeratosis congenita (Ruggero *et al.*, 2003;Yoon *et al.*, 2006). Reduced translational efficiency also triggers some diseases, namely melanomas, where the decrease in the expression of specific tumour suppressor proteins causes deregulation of the cell cycle.

Other protein synthesis related diseases result from mutations that affect translation initiation factors and their regulators. For example, mutations in any of the genes for the eIF2B subunits lead to a severe neurodegenerative disorder termed leukoencephalopathy with vanishing white matter (VWM) or childhood ataxia with central hypomyelination (CACH) (van der Knaap *et al.*, 2002). Also problems in the regulation of translation through eIF2 activity have a role in the development of Wolcott-Rallison syndrome (WRS) (Senee *et al.*, 2004). Diseases caused by mutations that affect cytosolic elongation or termination factors are not so frequent, but mutations in the eEF1A factor cause profound neuromuscular problems in mice (Chambers *et al.*, 1998) and mutations in eRF3 are associated with gastric cancer (Brito *et al.*, 2005).

Few diseases are related with mutations that affect ribosome components and its biogenesis, probably because they are so important for normal cell function that those mutations are lethal during early development. However, some cases of Diamond-Blackfan anaemia (DBA) are due to mutations in ribosomal protein S19 (RPS19) and others (Draptchinskaia *et al.*, 1999;Gazda *et al.*, 2006;Morimoto *et al.*, 2007) and to some bone marrow failure syndromes – X-linked dyskeratosis congenita, cartilage-hair hypoplasia and Shwachman-Diamond disease (Heiss *et al.*, 1998;Ridanpaa *et al.*, 2001;Boocock *et al.*, 2003) – are linked to mutations in genes involved in ribosome biogenesis. Also several tumour suppressors and proto-oncogenes affect ribosome maturation or regulate the activity of various translation factors, thus linking protein synthesis and ribosome biogenesis processes to cancer (Ruggero and Pandolfi, 2003).

Diseases caused by mutations or defects in tRNAs or in their charging enzymes are also known. Several mutations in aminoacyl-tRNA synthetases cause neurological disorders; for example, some types of the peripheral neuropathy Charcot-Marie-Tooth (CMT) are caused by mutations in the GARS gene, which encodes glycyl-tRNA synthetase (GlyRS) and cause its mislocalization in granules within the cell bodies and neurite projections of neuronal cells (Antonellis *et al.*, 2003;Seburn *et al.*, 2006;Xie *et al.*, 2007). Also a mutation that affects the editing

domain of alanyl-tRNA synthetase (AlaRS) in mouse (*sticky* mutation), leads to charging of $tRNA^{Ala}$ with serine and causes ataxia due to apoptotic loss of Purkinje cells. The cerebellum of the *sticky* mouse contains electron-dense structures, which are probably protein inclusions, and shows increased expression of chaperones, indicating accumulation of misfolded proteins (Lee *et al.*, 2006). The specific loss of Purkinje cells is unexplained, but is in line with the fact that diseases characterized by unstable DNA repeats and protein aggregates often affect the cerebellar cortex. Recently, a type of leukoencephalopathy (LBSL) has also been associated to mutations in the mitochondrial aspartyl-tRNA synthetase (AspRS) (Scheper *et al.*, 2007b).

Mutations in mitochondrial tRNAs are also associated with various diseases and disease phenotypes are linked with all mitochondrial tRNAs, except $tRNA^{Met}$ and $tRNA^{Cys}$. The best studied pathological mutation in a mitochondrial tRNA is that related to MELAS, a disease characterized by myopathy, encephalopathy, lactic acidosis, stroke-like episodes and other neurological and non-neurological symptoms (Kirino *et al.*, 2004). The affected tRNA is the mitochondrial $tRNA^{Leu(UUR)}$ and the pathogenicity is caused by the decrease of aminoacylation of the mutant tRNA (Borner *et al.*, 2000;Park *et al.*, 2003). A mutation in the mitochondrial $tRNA^{Lys}$ causes another type of disease, namely the myoclonic epilepsy with ragged-red fibers or MERRF (Shoffner *et al.*, 1990;Borner *et al.*, 2000). Not only mutations in the tRNAs, but also defects in the post-transcriptional modifications of some of them are associated to mitochondrial diseases. Mutations in the enzyme responsible for pseudouridylation of mitochondrial tRNAs are associated with mitochondrial dysfunction, myopathy, lactic acidosis and sideroblastic anaemia (MLASA) (Bykhovskaya *et al.*, 2004). Also a series of other mutations and defects in mitochondrial rRNAs or ribosomal proteins and in mitochondrial translation factors have already been described [for a review, see (Scheper *et al.*, 2007a)].

6. Aims of the study

The main objective of this PhD thesis was to study the cellular responses to mRNA mistranslation using yeast as a model system. For this, I have developed a genetic system to create yeast strains that mistranslate a single codon in a controlled manner. The cellular response to mistranslation was first studied at a phenotypic level and I then went deeper into the molecular and cellular mechanisms of the cellular response to mistranslation. This strategy allowed me to make significant progress on the study of the biology of mistranslation and permitted uncovering a critical link between mistranslation and oxidative stress, which may explain the molecular basis of the human diseases associated to mistranslation.

The main biological questions addressed in this PhD thesis were the following:

(1) What are the phenotypic consequences of mistranslation in yeast? What are the main biological processes affected by mistranslation?

(2) What are the transcriptional and translational responses to mistranslation? What are the mechanisms that cells activate to counteract the deleterious effects of mistranslation?

(3) How does mistranslation cause cell degeneration? Can yeast be a good model system to understand the molecular basis of human diseases caused by mistranslation?

The results obtained and some of the answers and conclusions of this study are described in the following chapters of this thesis.

B. Materials and Methods

1. Materials

1.1 Consumables and Chemicals

Consumables and chemicals were purchased from the following companies, among others: Amersham, AppliChem, Applied Biosystems, BD Biosciences, Beckman Coulter, Bio-Rad, Bioron, Boehringer Mannheim, CalBiochem, Chemicon, Clontech, Dako, Difco, Eppendorf, Esteriplas, Fermentas, Fisher, Fluka, Formedium, Frilabo, GE Healthcare, Gibco, Gilson, Grant, Hybaid, Invitrogen, Merck Biosciences, Millipore, Molecular probes, Nalgene, New England Biolabs, Normax, Nunc, Pierce, Promega, Qiagen, Roche, Sarstedt, Sartorius, Sigma-Aldrich, Stratagene, Vidrolab, VWR, Whatman, Zeiss.

1.2 Equipment

Lab-shakers (Unitron), Incubators (Heraeus), BioLogic LP system (Bio-Rad), ODYSSEY Imager (Li-Cor Biosciences), Gel Doc system (Bio-Rad), AxioImager Z1 microscope and Magnifier (Zeiss), MSM micromanipulator (Singer Instruments), Biofuge fresco (Heraeus), Centrifuges (Eppendorf), Thermocyclers (Eppendorf, Bio-Rad), Thermomixer (Eppendorf), Heating plate (Labnet), Spectrophotometer ND-1000 (Nanodrop), Spectrophotometer DU-530 (Beckman), SW41 Rotor and Ultracentrifuge (Beckman Coulter), micropipettes Pipetman (Gilson), vortex shakers and magnetic stirrers (IKA), Hybridization oven and chambers (Agilent), G2565AA microarray scanner (Agilent), Water baths (Grant, Thermo), Speedvac system DNA120 (Thermo), pH meter (WTW), FastPrep-24 (MP Biomedicals), agarose gel electrophoresis apparatus (Bio-Rad), Power supply units (Bio-Rad), SDS-PAGE and blot apparatus (Bio-Rad, Invitrogen), Ion exchanger Milli-Q (Millipore).

1.3 Commercially available kits

QIAprep Spin Miniprep Kit (Qiagen), GenElute HP Plasmid Maxiprep Kit (Sigma-Aldrich), QIAquick PCR Purification Kit (Qiagen), QIAquick Nucleotide Removal Kit (Qiagen), Oligotex mRNA Mini Kit (Qiagen), DC Protein Assay Kit (Bio-Rad), OxyBlot Protein Oxidation Detection Kit (Millipore).

1.4 Enzymes

Taq polymerase (Bioron), *Pfu* polymerase (Fermentas), SalI and BamHI (Fermentas), Superscript II RT polymerase (Invitrogen), RNAse A (Roche), Proteinase K (Roche), Shrimp Alkalyne Phospatase (USB corporation), T4 DNA ligase (NEB), DNase I (Amersham Biosciences).

1.5 Oligonucleotides

Oligonucleotides were purchased from MWG-Biotech AG (Germany) and were resuspended in ultra pure milliQ (mQ) water to a final concentration of 100μM.

Sequences of oligonucleotides primers (5' - 3') used:

- *HIS3 amplification for integration in his3*

oUA525 (CGTTTTAAGAGCTTGGTGAG)
oUA526 (GAAAAAGCAAAAAGAAAAAAG)

- *URA3 amplification for integration in ura3*

oUA527 (CAAAGAAGGTTAATGTGGCTG)
oUA528 (GCGTCCATCTTTACAGTCC)

- *KanMX4-tRNA cassette amplification for genomic integration in leu2 locus*

oUA243
(CTTGACCGCAGTTAACTGTGGGAATACTCAGGTATCGTAAGATGCAAGAGTTCGCCGGGTTAATTAAGGCGCGC)
oUA244
(GGGGCAGACATTAGAATGGTATATCCTTGAAATATATATATATATATTGCTGTAGTTGAAACACCAAACAAAAGATG)

- *KanMX4 gene amplification for genomic integration in leu2 locus*

oUA243
(CTTGACCGCAGTTAACTGTGGGAATACTCAGGTATCGTAAGATGCAAGAGTTCGCCGGGTTAATTAAGGCGCGC)
oUA611
(GGGGCAGACATTAGAATGGTATATCCTTGAAATATATATATATATATTGCTGGCATGTAATAAAGTCAATCATCTG)

- *Genomic integration of KanMX4 cassettes in leu2 confirmation by PCR*

oUA613 (GTTGGACGAGTCGGAATCG)
oUA614 (GCAAATCTGGAGCAGAACC)

- *Sequencing integrated tRNA*

oUA219 (CTCAATCTCGAGCCCACAGATGATTGAC)
oUA222 (AATTTACCGCGGACTAGTTGAAACACC)

- *Mating type screening by PCR*

oUA531 (AGTCACATCAAGATCGTTTATGG)
oUA532 (GCACGGAATATGGGACTACTTCG)
oUA533 (ACTCCACTTCAAGTAAGAGTTTG)

- *tetO-tRNA amplification and cloning*

oUA650
(TCTTTAGTCGACACTCTATCAATGATAGAGTAACGATACGATGGCCG)

oUA645
(AACTTAGGATCCTGATTGACTTTATTACATGC)

- *KanMX4-tetO-tRNA cassette amplification for integration in leu2 locus*

oUA542 (CAATAGGTGGTTAGCAATCG)
oUA547 (CCTTTACATCCTCCTTTTTCT)

- *Sequencing integrated tetO-tRNA*

oUA645 (AACTTAGGATCCTGATTGACTTTATTACATGC)

- *HAC1 mRNA RT-PCR*

oUA661 (ATGACTGATTTTGAACTAACTAG)
oUA662 (CAATTCAAATGAATTCAAACCTG)

1.6 Plasmids

pUKC707	pRS315 cloned with a *KanMX4* cassette (Santos *et al.*, 1996) (plasmid map in Annexe 1)
pUKC702	pRS315 cloned with a *KanMX4-tRNA*$_{CAG}$ cassette (Santos *et al.*, 1996) (plasmid map in Annexe 1)
pRS305K	plasmid with a multi-cloning site inside a *KanMX4* cassette for genomic integration in *leu2* locus (Taxis and Knop, 2006) (plasmid map in Annexe 1)
pRS305K-tetOtRNA	pRS305K containing a *tetO-tRNA*$_{CAG}$ fragment in its multi-cloning site (cloning made for this work)
pGalTR1	plasmid containing the construction P_{GAL1}-*TetR* (Dingermann *et al.*, 1992) (plasmid map in Annexe 1)

pGL-C1 plasmid containing the *E. coli LacZ* (β-galactosidase) gene for expression in yeast (Santos *et al.*, 1996)

1.7 Strains

Escherichia coli

JM109 genotype *recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB)/F' [traD36, proAB$^+$, lacI^q, lacZ Δ M15]*

Saccharomyces cerevisiae

- Wild-type strains, purchased from EUROSCARF

CEN.PK2 genotype *MATa/MATα; ura3-52/ura3-52; trp1-289/trp1-289; leu2-3_112/leu2-3_112; his3Δ1/his3Δ1; MAL2-8^C/MAL2-8^C; SUC2/SUC2*

BMA64/W303 genotype *MATa/MATα; ura3-52/ura3-52; trp1Δ2/trp1Δ2; leu2-3_112/leu2-3_112; his3-11/his3-11; ade2-1/ade2-1; can1-100/can1-100*

- Modified experimental strains

Strains constructed for this work

Control 1 (sUA3) genotype *MATa/MATα; ura3-52/ura3-52::URA3; trp1-289/trp1-289; leu2-3_112/leu2-3_112::KanMX4; his3Δ1/his3Δ1::HIS3; MAL2-8^C/MAL2-8^C; SUC2/SUC2*

tRNA (sUA5) genotype *MATa/MATα; ura3-52/ura3-52::URA3; trp1-289/trp1-289; leu2-3_112/ leu2-3_112::KanMX4-tRNA$_{CAG}^{Ser}$; his3Δ1/his3Δ1::HIS3; MAL2-8^C/MAL2-8^C; SUC2/SUC2*

Control 2 (sUA10) genotype *MATa/MATα; ura3-52/ura3-52; trp1Δ2/trp1Δ2; leu2-3_112/leu2-3_112::KanMX4; his3-11/his3-11; ade2-1/ade2-1; can1-100/can1-100; pGalTR1*

tetO-tRNA (sUA12) genotype *MATa/MATα; ura3-52/ura3-52; trp1Δ2/trp1Δ2; leu2-3_112/leu2-3_112::KanMX4-tetOtRNA$_{CAG}^{Ser}$; his3-11/his3-11; ade2-1/ade2-1; can1-100/can1-100; pGalTR1*

Strains kindly provided by other laboratories

Hsp104-GFP strain genotype *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; HSP104-GFP*

1.8 Antibodies

anti-eIF2α-P 1st antibody rabbit anti-eIF2α-P (Abcam); dilution 1:1000

anti-eIF2α 1st antibody rabbit anti- total eIF2α (Abcam); dilution 1:1000

anti-DNP 1st antibody rabbit anti-DNP (Millipore); dilution 1:150

anti-rabbit IRDye 690 2nd antibody goat anti-rabbit (Li-Cor), dilution 1:10000

2. Methods

2.1 Standard Methods

Cloning procedures such as restriction, digestion, dephosphorylation of DNA fragments, ligations, and transformation of newly generated vectors in *Escherichia coli* and separation of DNA in agarose gels were done according to Sambrook and Russell, CSHL Press, 2001. Commercial available kits were used according the manufacturer's instructions.

Amplification of genes or DNA fragments was done by standard PCR. Reaction mixes containing 1X Taq buffer, 0.2mM dNTPs, 1pmol/μL of each primer, 2.5mM of $MgCl_2$, 100ng of template DNA and about 0.04U/μL of *Taq* polymerase or *Pfu* polymerase were used. A standard thermocycler programme with a starting incubation temperature of 94ºC during 2 minutes followed by 25 cycles at 94ºC during 30 seconds, primers specific annealing temperature (Tm) during 30 seconds and 72ºC during 2 minutes, ending with a single incubation at 72ºC during 2 minutes, was routinely used. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and run in 1% agarose gels when necessary. To check for the correct integration of the yeast DNA disruption cassettes, colony PCR was performed. The protocol used was based on the protocol described above, but instead of using a DNA template in the reaction, a small amount of cells treated with zymolyase and an increased starting incubation at 94ºC, during about 5 minutes, was used.

2.2 Yeast-specific techniques

2.2.1 Culture of *Saccharomyces cerevisiae*

Yeast strains were cultured at 30ºC in either: rich medium (YPD – 1% yeast extract, 2% Bacto-Peptone and 2% glucose) or minimal medium (MM – 0.67%

yeast nitrogen base, 2% glucose, 0.2% Drop-out mix with all the essential amino acids). Variations of this MM medium were also used, with other types of carbon sources replacing glucose (galactose or glycerol), and with Drop-out mixes, lacking one or two amino acids, for the selection of specific clones in plasmid transformations, for example. Synthetic minimal medium (SD – 0.67% yeast nitrogen base, 2% glucose, 100μg/mL of required amino acids only) was also used. Solid media were always done by addition of 2% agar. All media were sterilised using heat in an autoclave before use.

Whenever needed, geneticin (G418) was used at a concentration of 200mg/L, tetracycline at 40μg/mL and antioxidant compounds, like ascorbate, glutathione and acetyl-L-carnitine, at 80mM, 40mM and 100 mg/mL, respectively. Stress media were also prepared with the following concentrations: cadmium chloride ($CdCl_2$; 100μM) or hydrogen peroxide (H_2O_2; 1.5mM).

2.2.2 Transformation of yeast cells

Transformation of *S. cerevisiae* was carried out using the lithium acetate (LiAc) method (Gietz and Woods, 2002;Gietz and Woods, 2006). Briefly, overnight cultures were diluted in 10mL (for a maximum of 10 transformation reactions) of new and fresh medium at an OD_{600} of 0.05. The cultures were grown at 30ºC, 200rpm shaking, to an OD_{600} of 0.4 – 0.5 and then cells were harvested by centrifugation for 5 minutes at 4000rpm. After washing with 5mL of sterile mQ water, the pellet was resuspended in 500μL of 0.1M LiAc solution. 50μL of cell suspension were transferred onto 1.5mL eppendorf tubes and cells were pelleted by centrifugation at maximum speed, during 15 seconds. The supernatant of each tube was discarded and the transformation reagents were added to the pellet in the following order: 240μL 50% (w/w) PEG, 36μL 1.0M LiAc, 25μL single-stranded carrier DNA (2mg/mL) previously denatured and 50μL of an aqueous solution of the plasmid of interest (containing 0.1 – 1μg of plasmid). Tube were vortexed until a homogeneous suspension was obtained and then heat-shocked at 42ºC, in a

water-bath, between 30 to 40 minutes. Cells were then harvested by centrifugation, at 5000rpm, 1 minute, the supernatant was discarded and the pellet was carefully resuspended in 100 μL of sterile mQ water. Each suspension was plated in selective media plates and incubated at 30ºC, until visible transformant colonies were visible (about 3 – 4 days).

2.2.3 Gene integration by homologous recombination

For integration of DNA fragments in the genome of *S. cerevisiae*, integration cassettes with ends homologous to specific locus of the *S. cerevisiae* genome were amplified by standard PCR. The homologous tails were either inserted in the PCR primers or were already present in the template. Several PCR reactions were mixed (100 – 200μL) and then purified using a commercial PCR purification kit. About 1 – 2μg of the purified integration cassettes were transformed in *S. cerevisiae* with the transformation protocol described above, for integration into the genome by homologous recombination. Positive clones (integrants) were selected in selective medium. Integrations were confirmed by colony PCR and DNA sequencing.

2.2.4 Yeast sporulation, tetrad dissection and mating

Sporulation and tetrad dissection

Overnight cultures were grown to an OD_{600} of about 2.0. 500μL of these cultures were harvested by centrifugation at 5000rpm, 10 seconds, at room temperature. After washing the cells with sterile mQ water, they were resuspended in 2mL of Sporulation medium containing 1% Potassium acetate, 0.1% yeast extract, and 0.05% glucose. After 3 – 5 days at 30ºC, an appropriate volume was placed into a Neubauer counting chamber and the number of cells and asci/tetrads were counted.

100μL of the sporulation culture were also centrifuged and, after removing the supernatant, were treated with 25μL of a zymolyase solution (0.05 mg/mL); the suspension was then incubated at room temperature for 7 – 10 minutes to adequate digestion. Digestion was stopped by adding 250μL of water to the suspension. About 5 – 10μL were then spread thinly in appropriate agar media plates, where the asci were dissected using a MSM micromanipulator (Singer Instruments). Plates were then incubated at 30ºC during 4 – 7 days and the viability of the spores was assessed.

Mating type determination and mating assays

The mating type of the viable spores (haploid cells) was assessed by PCR, as described by Huxley, 1990 (Huxley *et al.*, 1990). This method consists of a colony PCR reaction with 3 different primers, which allowed one to identify the cells mating type according to the length of the PCR product obtained.

Haploid clones (spores) with different mating types (*MATa* and *MAT*α) and with different auxotrophic markers were grown in 2mL cultures at 30ºC to an OD_{600} of about 0.5. Then the number of cells was determined using a Neubauer chamber and the volumes corresponding to 10^6 cells of each culture were pooled into a 1.5mL tube, letting the reaction proceed for 12 hours. Several dilutions of the mating suspensions ($2x10^6$ cells) were made to obtain cell suspensions at densities of $4x10^5$ cells (5x dilution), $2x10^5$ cells (10x dilution) and $1x10^5$ (20x dilution). 10μL of each mating reaction dilution were spotted in a selective plate and incubated for 3 – 4 days at 30ºC, to verify the mating efficiency between the clones.

2.2.5 Preparation of yeast genomic DNA

Genomic DNA extraction was carried out using an adaptation of the protocol described by Hoffman (Hoffman and Winston, 1987). In order to decrease DNA contamination with other nucleic acids or with proteins, 10mL of culture (OD_{600nm}

between 0.5 and 1.0) were harvested by centrifugation and the pellet washed 3 times with sterile mQ water. After a second centrifugation, cells were resuspended in 200μL of Lysis Buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris pH 8.0, 1mM EDTA pH 8.0). 200μL of 25:24:1 Phenol-Chloroform-Isoamyl alcohol and 0.3g of 0.5mm diameter glass beads were added to the suspension and the mix was vortexed during 10 minutes. Then, 200μL of TE (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0) were added and the tubes were centrifuged at the highest speed, during 5 minutes. The upper aqueous phase was transferred to a new tube and the genomic DNA was precipitated with 1mL of 100% ethanol, at room temperature. Genomic DNA was further treated with RNAse A and with Proteinase K, according to these commercial enzymes specific protocols. In the end, genomic DNA was precipitated with 20μL 3.0M Sodium acetate (pH 5.2) and 500μL of 100% cold ethanol, at -80ºC during 1 hour. After centrifugation at highest speed, during 5 minutes, at 4ºC, genomic DNA was dried and resuspended in 200μL of mQ water.

2.3 Preparation of yeast cells for fluorescence microscopy

2.3.1 Nuclear DNA staining with DAPI

Approximately 10^7 cells were pelleted in a microcentrifuge tube (10 seconds pulse) and fixed in 70% ethanol for at least 5 minutes. After that, cells were washed twice with 1X PBS and resuspended in a small volume of 100 – 500ng/mL DAPI (4',6'-diamidino-2-phenylindole). After 10 min, cells were pelleted, resuspended in 1X PBS and observed using a fluorescence microscope with the proper UV filter set.

2.3.2 GFP-expressing yeast cells

Approximately 10^6 cells were harvested by centrifugation with a quick pulse and washed twice in 1X PBS. Then cells were resuspended in PBS and observed using a fluorescence microscope with the proper UV filter set.

2.4 Preparation of yeast cells for flow cytometry analysis

2.4.1 Yeast DNA content analysis

DNA content of cells was determined using flow cytometry as described by Fortuna (Fortuna *et al.*, 2001). Aliquots of 10^7 cells were washed twice in ice-cold water, fixed in 1mL of 70% ethanol and incubated overnight at 4ºC. Fixed cells were then washed with 50mM Sodium citrate buffer (pH 7.5) and resuspended in 750μL of the same buffer. 250μL of 1mg/mL RNase A were added and the suspension was incubated for 1 hour, at 50ºC. Next, 50μL of 20mg/mL Proteinase K were added and the mix was incubated again for 1 hour, at 50ºC. Finally, 20μL of a SYBR Green I working solution were added and samples were stored at 6 – 8ºC in the dark. Before cytometry, 2.7μL of Triton X-100 were mixed in each sample by vortexing. Samples were sonicated and stored on ice until analysis using a flow cytometer (Beckman Coulter) with the proper filters and sets.

2.4.2 Cellular ROS content and membrane integration analysis

ROS levels were quantified by flow cytometry after cell labelling with dihydrorhodamine 123 (DHR123) (Invitrogen). Briefly, approximately 10^7 yeast cells were harvested by centrifugation, washed and resuspended in phosphate-buffered saline buffer (PBS), pH 7.4. DHR123 was added from a 1mg/mL stock solution in ethanol reaching a final concentration of 15μg/mL. Cells were incubated during 90 minutes at 30°C in the dark, washed in PBS and analysed in a flow

cytometer. Cells displaying higher values than a defined threshold of green fluorescence were considered as containing elevated intracellular ROS levels.
Plasma membrane integrity was determined by examining cellular permeability to propidium iodide (PI) (Invitrogen). Cells were incubated with 5μg/mL PI for 15 min and analyzed by flow cytometry. Cells displaying higher values than a defined threshold of red fluorescence were considered as having compromised plasma membrane integrity.

2.5 β-galactosidase activity assay

For routine work, 500μL of exponentially growing yeast cells (OD_{600nm}~0.5) were harvested by centrifugation, washed with sterile mQ water and then resuspended in 800μL of Z-buffer (60mM Na_2HPO_4, 40mM $NaH_2PO_4.2H_2O$, 10mM KCl, 1mM $MgSO_4.7H_2O$, 50mM 2-mercaptoethanol, pH 7.0), 20μL of 0.1% SDS and 50μL of chloroform. Cell suspensions were mixed (vortex) for 30 seconds and incubated in triplicate at 47ºC in a water bath for 10 minutes. This β-gal unfolding step was followed by a refolding step, which was carried out by incubating samples on ice for 30 minutes. Residual β-gal activity was then quantified at 37ºC. For this, the assay tubes (200μL) were incubated for 5 minutes at 37ºC and then 200μL of 4mg/mL o-nitrophenyl-β-D-galactopyranoside (ONPG) substrate were added to each tube and reactions were allowed to proceed for 2 minutes and were then stopped by the addition of 400μL of 1.0M sodium carbonate (Na_2CO_3). β-galactosidase activity was determined by monitoring o-nitrophenol synthesis at 420nm.

2.6 Total RNA extraction

Total RNA was extracted using an acidic hot-phenol based protocol. About 25 OD units (volume of culture X OD_{600nm}) of exponentially growing cells or 100 OD units of stationary phase cells were collected into 50mL tubes and harvested briefly (3

minutes, 4000rpm) in a single step centrifugation. The remaining supernatant was quickly poured-off; the tubes were immediately immersed in liquid nitrogen and stored at -80ºC.

Frozen cells were then taken from the -80ºC freezer and resuspended in 500μL of Acid Phenol-Chloroform 5:1, pH 4.7 (Sigma), warmed up at 65ºC before use. Immediately after, the same volume of TES-buffer (10mM Tris pH 7.5, 10mM EDTA, 0.5% SDS) was added and the tubes were vortexed vigorously for 20 seconds to resuspend the cell pellets. After 1 hour of incubation in a water bath at 65ºC, with 20 seconds vortexing every 10 minutes, the tube content was transferred onto 1.5mL Eppendorf tubes and centrifuged for 20 minutes at 14000rpm at 4ºC. The water-phase was added to a new Eppendorf tube, filled with 500μL of Acid Phenol-Chloroform 5:1, pH 4.7 (Sigma), vortexed for 20 seconds and centrifuged for 10 minutes at 14000rpm at 4ºC. The water-phase from this step was added to a new Eppendorf tube filled with 500μL Chloroform:Isoamyl-alcohol 25:1 (Sigma), vortexed for 20 seconds and centrifuged for 10 minutes at 14000rpm at 4ºC. Again, the water phase was transferred onto a new Eppendorf tube with 50μL of sodium acetate 3M, pH 5.2 and the tube was filled with 100% ethanol (kept at -20ºC) and incubated at -20ºC for approximately 1 hour. After RNA precipitation, tubes were centrifuged for 5 minutes at room temperature, 14000rpm. The liquid was removed carefully to avoid touching the RNA-pellet. The pellet was washed with 500μL of ethanol 80% (kept at -20ºC) and centrifuged for 2 minutes at room temperature, 14000rpm. After removal of all traces of ethanol, the RNA pellet was air dried and dissolved in sterile (mQ) water to a concentration of approximately 10μg/μL. Samples were frozen and kept at -80ºC.

In order to remove possible DNA contamination, total RNA samples were treated with DNase I (Amersham Biosciences), according with the commercial enzyme protocol.

2.7 Polysome analysis

2.7.1 Polysome isolation and profiling by sucrose density gradient centrifugation

Polysomes were isolated as previously described by Arava (Arava *et al.*, 2003), with some modifications. Briefly, for each sample, yeast cultures (80mL) were harvested by centrifugation at 4000rpm, for 4 minutes at 4ºC in the presence of 100μg/mL cyclohexamide to freeze protein synthesis elongation. Cells were then washed twice using 2mL of lysis buffer (20mM Tris-HCl at pH 8.0, 140mM KCl, 1.5mM MgCl2, 0.5mM dithiothreitol, 100μg/mL cyclohexamide, 1mg/mL heparin, 1% Triton X-100), and were then resuspended in 700μL of lysis buffer supplemented with 0.6 volumes of chilled glass beads. Cell lysis was carried out using a vortex and 8 cycles of 30 seconds vortexing and 1 minute cooling on ice. Lysates were transferred to clean microfuge tubes and centrifuged for 5 minutes at 8000rpm at 4ºC. Supernatants were transferred to clean microfuge tubes and 40 units A_{280nm} of sample were loaded onto 11mL 15% – 50% sucrose gradients containing 20mM Tris-HCl at pH 8.0, 140mM KCl, 5mM MgCl2, 0.5mM dithiothreitol, 100μg/mL cyclohexamide, 500μg/mL heparin. Gradients were centrifuged using a SW41 rotor (Beckman-Coulter) at 35000rpm for 2 hours and 45 minutes. Polysomal profiles were visualized by monitoring RNA absorbance at 254nm using a Bio-Rad Biologic LP system adapted for this use. The polysomal fraction of the gradient was recovered into tubes containing 8M guanidine-HCl. In order to obtain sufficient quantity of mRNA for microarray analysis, identical fractions were pooled into a single tube.

2.7.2 Polysomal RNA preparation

After precipitation of the polysomes with ethanol 100%, polysomal RNA was extracted using phenol:chloroform and precipitated, first with 1.5M lithium chloride (LiCl) for removing any residual heparin, and then with 100% ethanol plus 3M

sodium acetate, pH 5.3. mRNA was further isolated using Oligotex beads (Qiagen) and cDNA synthesis was carried out using 3μg of purified mRNA. Labelling and hybridisation were done as described in the next section.

2.8 DNA microarray analysis

2.8.1 Reverse Transcription, cDNA labelling and hybridization

RNA labelling was carried out using the protocols described by van de Peppel (van de *et al.*, 2003), with minor alterations. Reverse transcription (RT) reactions for cDNA synthesis were carried out using 40μg of total RNA or 3μg of purified mRNA, Oligo dT_{12-18} primer (Invitrogen) and Superscript II Reverse Transcriptase (Invitrogen) were used. For labelling, cDNAs were synthesized in presence of aminoallyl-dUTP (Sigma), purified using Microcon-30 (Millipore) columns and were then coupled to Cy3 or Cy5 fluorophores (Amersham). Before hybridization, free dyes were removed using Chromaspin-30 (Clontech) columns, and the efficiency of cDNA synthesis and dye incorporation was measured using a Nanodrop spectrophotometer, determining the full spectrum of absorptions in the 190 – 750nm range and registering the OD values at 260nm, 550nm and 649nm for each sample. Each hybridisation was carried out using 300ng of Cy3- and Cy5-labelled cDNAs and in house made yeast arrays (YAUAv 1.0), for 20 hours at 42ºC, in an Agilent hybridization oven. Slides were scanned using the Agilent G2565AA microarray scanner and raw data was extracted using the QuantArray v3.0 software (PerkinElmer).

2.8.2 Data normalization and analysis

Raw data was normalized using limmaGUI software (Bioconductor R) (Gentleman *et al.*, 2004) and print-tip Lowess normalization within arrays. Using the normalized M values (log2 Ratios) obtained, statistical differences were calculated using the

Significant Analysis of Microarrays (SAM analysis; MeV software (Saeed *et al.*, 2003;Saeed *et al.*, 2006)). Differentially expressed genes were identified for a False Discovery Rate below 0.001 (FDR < 0.001). Functional analysis of expression data obtained was done using Expander software (Shamir *et al.*, 2005) and also YEASTRACT online tool (Teixeira *et al.*, 2006;Monteiro *et al.*, 2008). The raw data for all hybridizations done was submitted to the ArrayExpress database and is available under the accession codes E-MTAB-153, 165 and 166.

2.9 RT-PCR

For RT-PCR, the RT reactions were carried out in the same manner as those used for RNA labelling for microarrays analysis described above, using Oligo dT_{12-18} primer and Superscript II Reverse Transcriptase (Invitrogen). Then cDNAs were denatured at 95ºC for 2 minutes and placed immediately on ice. PCR reactions were carried out using the cDNA preparations as template, specific primers for the required reaction and the adequate thermocycler programme.

2.10 Plating efficiency / cell viability

The plating efficiency of a yeast strain allows for determination of cell viability of a culture since colonies arise from single viable cells (colony forming units or CFUs). To determine plating efficiency, cell density in liquid culture was determined by counting the number of cells using a Neubauer chamber / haemocytometer. Then the dilution factor necessary to dilute cells to 10^3 cells per mL was determined and cells were dispersed by vigorous vortexing and diluted in sterile water. 0.1mL of diluted cells were plated onto an appropriate agar medium and incubated for several days at 30ºC. The colonies were counted and plating efficiency was determined.

2.11 Protein extraction, SDS-PAGE and Western-blot

2.11.1 Protein extraction and preparation

Approximately 50mL of exponentially growing cells (OD_{600nm} between 0.5 and 0.8) were span down and washed 2 times in 1X PBS. Then cells were resuspended in 160μL of lysis buffer. Two types of lysis buffer were used. For protein oxidation/carbonylation detection the lysis buffer had the following composition: 15% glycerol, 2mM EDTA pH 6.8, 1mM Pefablock; for other analysis the lysis buffer was composed of 60mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol. The suspension was kept on ice, transferred to cryo-tubes and 0.35g of glass beads were added. Tubes were placed in the FastPrep and 6 cycles of 30 seconds at speed of 6.5 were performed. After each cycle, tubes were placed on ice for 1-2 minutes. Then, tubes were centrifuged during 5 minutes at 13000 rpm and supernatants were removed into new tubes. This suspension was span down again for 10 minutes at 13000rpm and a clear protein extract was obtained. Protein quantification was carried out using the Bio-Rad DC Protein Assay.

For protein oxidation/carbonylation detection, 20μg of protein in 5μL of buffer were added to 5μL of 12% SDS. Then 10μL of 1X DNPH (prepared from a 10X stock with mQ water) were added to the samples and incubated 15 minutes in a fume hood. Samples to be compared had to be derivatized the same time. After that, 10μL of neutralising solution (Oxyblot kit - Millipore) were added to all samples and these were vortexed. Samples were quickly span down and stored in the refrigerator until running the gel.

For other analysis, about 5μg of extracted proteins were prepared in 15μL 1X SDS gel loading buffer (50mM Tris-HCl pH 6.8, 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heated at 95ºC for 5 minutes, immediately before running the gel.

2.11.2 SDS-PAGE

For protein oxidation analysis, the NuPAGE electrophoresis system (Invitrogen) with 12% NuPAGE Novex Bis-Tris pre-cast gels according to the manufacturer instructions was used. 7μL of each sample were loaded onto the gel and electrophoresis was allowed to proceed at 180V during approximately 1hour and 15 minutes.

For others analysis, SDS-polyacrylamide gels were made with the following components: water, acrylamide/bisacrylamide mix pH 7.0, Tris base, SDS, ammonium persulfate (APS) and TEMED, according to Sambrook and Russell, CSHL Press, 2001. 12% resolving gels and 4% stacking gels were routinely prepared, avoiding air bubbles. After polymerization, combs were removed and the wells were immediately washed with deionised water to remove non-polymerized acrylamide. Gels were mounted in an electrophoresis apparatus and running buffer (25mM Tris base, 250mM glycine pH 8.3, 0.1% SDS) was added to the top and bottom reservoirs. 15μL of each of the samples were loaded into the wells. The gels were run at 80V until the dye front moved into the resolving gel; then the voltage was increased to 150V, until the dye reached the bottom of the gel (about 4 hours).

2.11.3 Western-blot and detection

Detection of oxidized proteins was carried out using western blot analysis; it was used the NuPAGE system according to the manufacturer protocol, during 90 minutes at 30V. Nitrocellulose membranes were used.

For the other analysis, the Bio-Rad wet transferring system was used. Nitrocellulose membrane, six filters with the same size and the cushions were placed in transfer buffer (25mM Tris base, 192mM glycine, 12% methanol). The

transfer system was mounted accordingly and added with transfer buffer. Blotting was allowed to run during 4h at 100mA, at 4ºC.

After blotting, membranes were peeled off from the gels and placed in 5% milk in PBS solution, during 1 hour. Then the membranes were washed in PBS-T (1X PBS, 0.1% Tween) and incubated for 1 hour (or overnight at 4ºC) in an appropriate primary antibody solution in PBS-T. Then, membranes were washed 3 times with PBS-T, during 10 minutes, and incubated in an appropriate secondary antibody solution in PBS-T, during 1 hour and in the dark. In the end, other 3 washes in PBS-T, during 10 minutes were carried out in the dark and the membrane was scanned using the ODYSSEY Infrared Imaging System (Li-Cor Biosciences) with the adequate definitions.

C. Results

1. Impact of constitutive mistranslation on yeast biology

1.1 Overview

Like DNA replicational and transcriptional errors, translational errors are normally viewed as being detrimental to life. There is no doubt that above a certain mistranslation threshold the proteome is disrupted, cell fitness decreases and cell death increases (Nangle *et al.*, 2002;Nangle *et al.*, 2006). But, at least in theory, codon ambiguity can increase proteome diversity by creating statistical populations of proteins, which in turn, may generate phenotypic diversity. The later can be explored by natural selection for developmental, metabolic and regulatory innovation creating a selective pressure to maintain the advantageous phenotypes. The recent discoveries that bacteria and unicellular eukaryotes are far more resistant to mistranslation than anticipated (Ruan *et al.*, 2008), that mischarged tRNAs can participate in mRNA translation (Suzuki *et al.*, 1997;Ambrogelly *et al.*, 2007;Sheppard *et al.*, 2008) and the discovery of genetic code alterations mediated by ambiguous mRNA decoding mechanisms (Schultz *et al.*, 1996;Knight *et al.*, 2001) support the hypothesis that codon ambiguity may be evolutionarily relevant. However, this remains an open question that needs to be clarified experimentally.

Several *Candida* and *Debaromyces* species, the so called CTG clade (Miranda *et al.*, 2006;Butler *et al.*, 2009), redefined the identity of the CUG codon from leucine to serine through ambiguous codon decoding (Kawaguchi *et al.*, 1989;Ohama *et al.*, 1993;Santos *et al.*, 1995;Butler *et al.*, 2009). During the early stages of CUG identity redefinition, decoding ambiguity arose via competition between a cognate $tRNA_{CAG}^{Leu}$ and a mutant $tRNA_{CAG}^{Ser}$ for the decoding of the CUG codon at the ribosome A site (Massey *et al.*, 2003;Santos *et al.*, 2004). The unusual decoding and aminoacylation properties of this mutant $tRNA_{CAG}^{Ser}$ arose from the fact that its anticodon-arm is similar to leucine-tRNAs and contains a leucine anticodon, whereas the remaining part of the molecule is similar to serine-tRNAs, containing a long variable arm and a discriminator base typical of serine tRNAs (G73) (Soma

et al., 1996;Suzuki *et al.*, 1997). This unique tRNA is therefore recognized by both the seryl- and the leucyl-tRNA synthetases (SerRS and LeuRS), producing two distinct isoforms of the $tRNA_{CAG}^{Ser}$, namely a leu-$tRNA_{CAG}^{Ser}$ and a ser-$tRNA_{CAG}^{Ser}$ (Figure 13). In extant species of the CTG clade, these tRNAs compete for translation of CUG codons creating a unique situation of natural CUG ambiguity. The selection and fixation of this natural codon ambiguity over millions of years of fungal evolution can only be explained if it is evolutionarily advantageous, but one does not yet understand how such atypical ambiguity can produce advantageous phenotypes.

This chapter explores the natural ambiguity of the CUG codon existent in the fungal CTG clade species to shed new light on the biology of mistranslation. For this, I have recreated such ambiguity in non-ambiguous *S. cerevisiae* and I have studied the consequences of this codon specific mistranslation event in various aspects of yeast biology. I had two main objectives in mind, namely to understand the negative effects of mistranslation on yeast biology and to identify putative advantageous phenotypes that could be selected by natural selection processes.

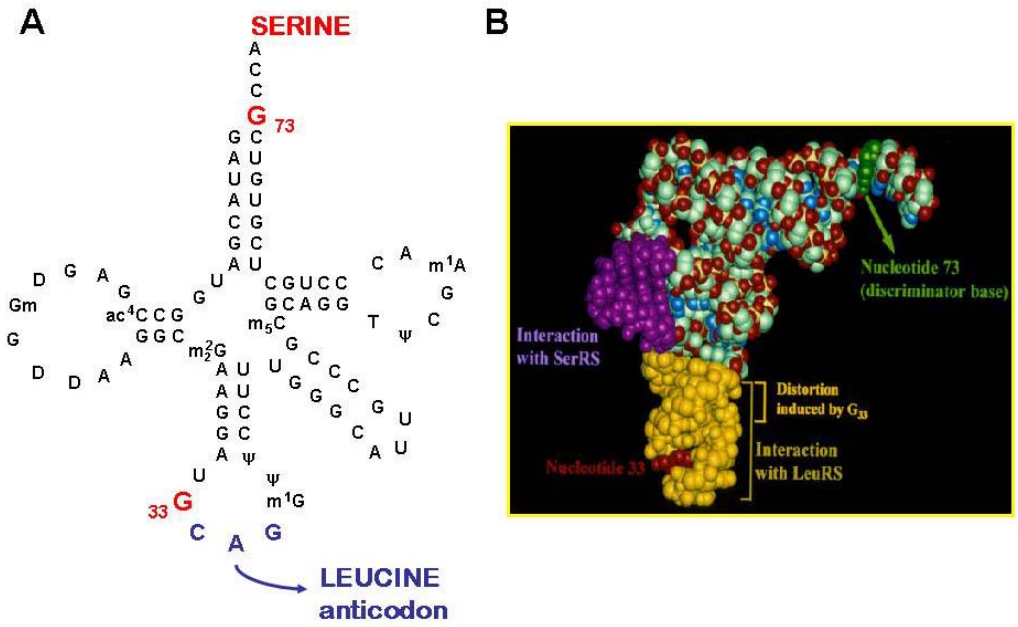


Figure 13. The *Candida albicans* $tRNA_{CAG}^{Ser}$ secondary (A) and tertiary (B) structures. This tRNA is a hybrid molecule containing the body of a serine tRNA and the anticodon arm of a leucine tRNA.

In order to answer the questions mentioned above I have decided to generate CUG ambiguity in *S. cerevisiae* by expressing the *C. albicans* $tRNA_{CAG}^{Ser}$ in the former species. Expression of the $tRNA_{CAG}^{Ser}$ in *S. cerevisiae* creates codon ambiguity due to insertion of leucine by the endogenous cognate $tRNA_{UAG}^{Leu}$ and serine by the mutant $tRNA_{CAG}^{Ser}$ at CUG positions. The impact of this unusual decoding event is global due to the high number of CUG codons present in the *S. cerevisiae* genes (30994 CUG codons spread over 88.8% of the yeast genes). These ambiguous yeast cells allowed me to evaluate the impact of mistranslation on sexual reproduction, namely on mating and spore germination, on the stability of the genome and also on the capacity of the ambiguous yeast to adapt to new environmental conditions.

1.2 Genetic system for expression of a heterologous $tRNA_{CAG}^{Ser}$ in *Saccharomyces cerevisiae*

Previous studies have demonstrated that the *C. albicans* $tRNA_{CAG}^{Ser}$ gene can be expressed in *S. cerevisiae* from single-copy plasmids and that the tRNA is correctly processed and aminoacylated in those cells causing mistranslation (Santos *et al.*, 1996;Silva *et al.*, 2007). The levels of serine misincorporation at CUG codons *in vivo* in diploid *S. cerevisiae* strains transformed with single-copy plasmids carrying the $tRNA_{CAG}^{Ser}$ gene was determined by mass-spectrometry and ranges from 1.4% to 2.3% (Silva *et al.*, 2007), which represents approximately 1400- to 2300-fold increase in decoding error, if one considers the background decoding error in yeast as being in the order of 1 error in every 100000 codons decoded (Stansfield *et al.*, 1998).

The studies mentioned above have also shown that the toxicity generated by misincorporation of serine residues at CUG positions on a proteome wide scale created high population instability in yeast cultures, which was sometimes difficult to circumvent (Santos *et al.*, 1996;Silva *et al.*, 2007). Since such population instability could be related to plasmid instability and in order to minimize this

problem the *C. albicans* $tRNA_{CAG}^{Ser}$ gene was integrated into the genome of the *S. cerevisiae* CEN.PK2 strain. The *KanMX4*-$tRNA_{CAG}^{Ser}$ cassette previously cloned into the pUKC702 plasmid, was amplified by PCR, purified and integrated into the yeast genome at the *leu2* locus by transformation and homologous recombination, as described in the Methods section. Positive clones were selected in YPD+geneticin plates and correct integration was confirmed by PCR and sequencing, using homologous PCR primers. For construction of a control CEN.PK2 strain, DNA cassettes containing only the *KanMX4* gene were amplified, purified and integrated into the *leu2* locus (Figure 14).

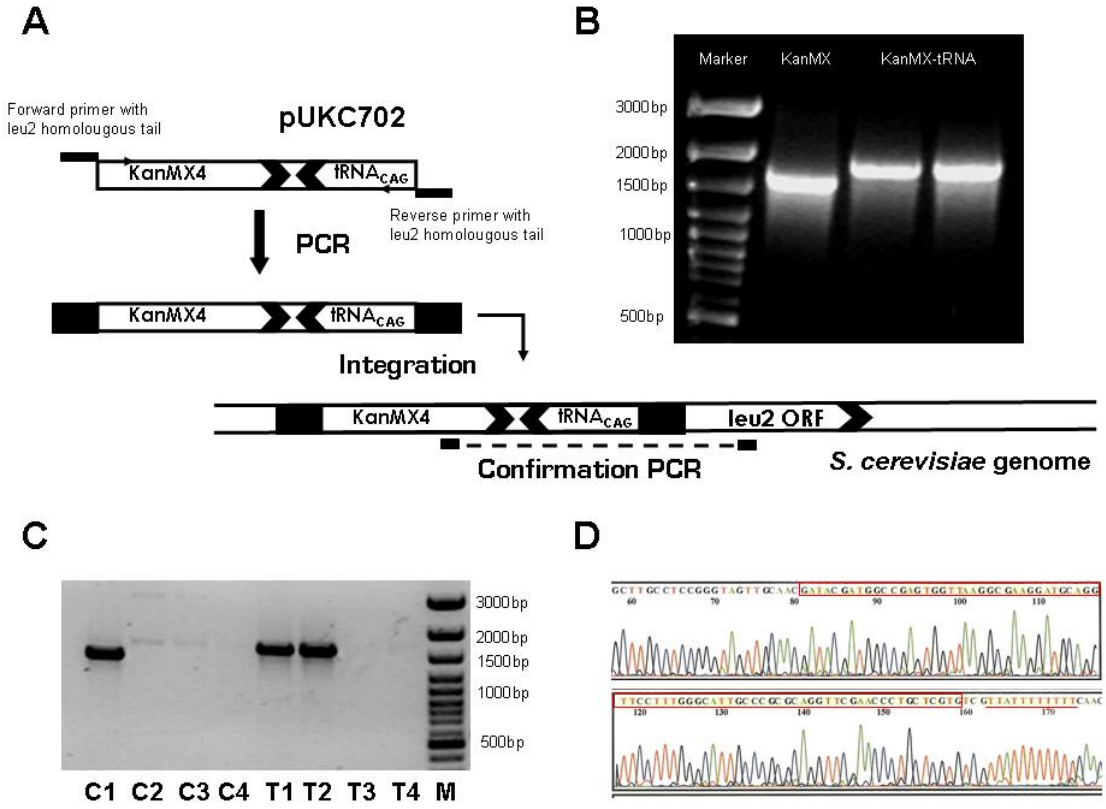


Figure 14. $tRNA_{CAG}$ gene integration into the genome of *S. cerevisiae* CEN.PK2. (A) Scheme of the integration method. (B) Integration cassettes amplified from pUKC702 by PCR. (C) Confirmation of the integration of the tRNA cassette by PCR. (D) Confirmation of the integrated tRNA gene by Sanger DNA sequencing.

After integration and confirmation of the integrated tRNA gene sequence by Sanger DNA sequencing, several CEN.PK2 control and tRNA-bearing clones were

re-grown in selective YPD+geneticin plates and were then frozen in the laboratory strain stock at -80ºC and in YPD medium containing 40% glycerol. The experiments described below were carried out using freshly grown cells obtained from those stocks.

1.3 Effect of mistranslation on yeast sporulation

In order to understand if mRNA mistranslation could affect fundamental processes of yeast biology its impact on the yeast life cycle was studied. The life cycle of *S. cerevisiae* alternates normally between diplophase and haplophase. Both ploidies can exist as stable cultures. In heterothallic strains, haploid cells are of two distinct mating types, a and α. Mating of a and α cells results in a/α diploids which are unable to mate again, but can undergo meiosis (sporulation). The four haploid products resulting from meiosis of a diploid cell (spores) are contained within the wall of the mother cell (the ascus) (Figure 15).

Sporulation is an obligate aerobic process, which is carried out in the absence of an external source of nitrogen. Usually, diploid cells are grown at 30ºC in a rich glucose medium, such as YPD, to stationary phase and then harvested, washed in water and resuspended in sporulation medium (1% Potassium acetate, pH 7.0). Growth of cells to stationary phase is necessary in order to allow cells to adapt to oxidative growth. Efficiency of sporulation is dependent on cell density and also on the change of pH of the sporulation medium from 7.0 to 9.0 during the first 3 - 5 hours of culture. For example, cells maintained in a buffered acetate medium at pH 7.0 are unable to complete sporulation. In the process of sporulation, there is a considerable asynchrony, which reflects different sporulation ability of cells at different stages in cell cycle. Usually newly formed daughter cells sporulate poorly. Sporulation efficiency is also strain-dependent, ranging from 50 to 95% (Haber and Halvorson, 1975).

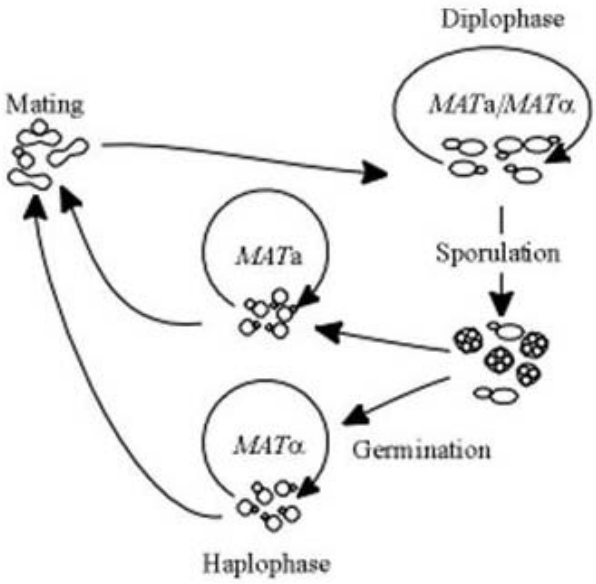


Figure 15. Life cycle of *Saccharomyces cerevisiae* cells. Diploid yeast cells sporulate under conditions of nutrient deficiency. During sporulation, the diploid cell undergoes meiosis yielding four progeny haploid cells, which become encapsulated as spores (or ascospores) within a sac-like structure called an ascus. Because the a and α mating types are under control of a pair of *MATa*/*MATα* heterozygous alleles, each ascus contains two *MATa* and two *MATα* haploid cells. Upon exposure to nutrients, the spores germinate, vegetative growth commences and mating of the *MATa* and *MATα* can occur, originating a new diploid cell, the zygote.

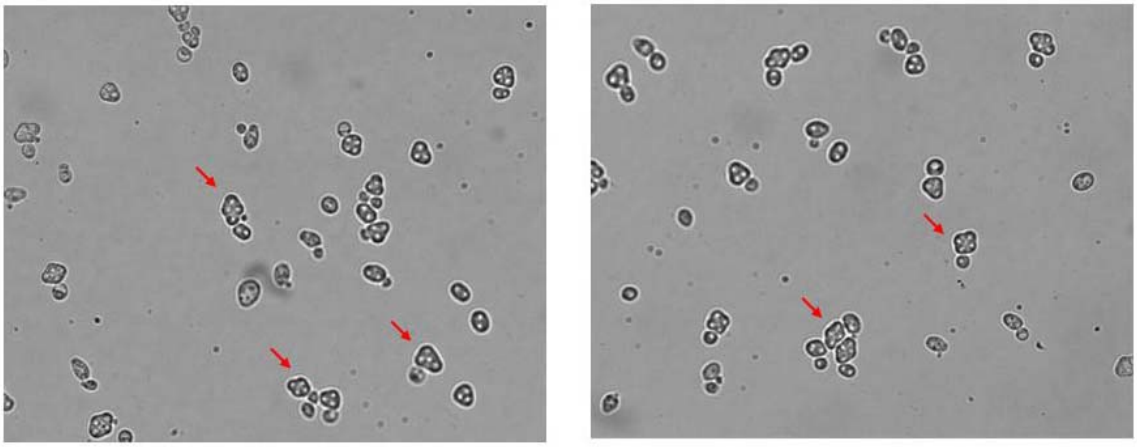


Figure 16. *Saccharomyces cerevisiae* asci observed using a light microscope (400X). Some of the sporulation tetrads are indicated by the red arrows.

There are few morphological phases that one can monitor during sporulation. Indeed, under the light microscope, without staining, only the appearance of refractile ascospores at the end of sporulation can be seen (Figure 16). Stages of meiosis from mononucleate cells to binucleate and tetranucleate can be visualized using staining. These meiotic events can also be followed by several genetic techniques.

Yeast CEN.PK2 cells containing the *KanMX4* (Control) or *KanMX4*-$tRNA_{CAG}^{Ser}$ (tRNA) cassettes integrated in their genome were incubated in sporulation media and the production of sporulation tetrads was followed over several days using a light microscope. The number of asci (tetrads) was counted for each culture after 2, 4, 8 and 11 days of incubation.

Expression of the mutant $tRNA_{CAG}^{Ser}$ in yeast decreased its sporulation efficiency dramatically. An initial qualitative screen showed that in 95% of the cultures of control CEN.PK2 cells tetrad production was abundant, while only 7.5% of yeast clones expressing the $tRNA_{CAG}^{Ser}$ showed abundant sporulation. In the latter case, approximately 65% of the cultures had poor sporulation and 27.5% did not sporulate at all (Figure 17). The quantification of tetrad production in some of the cultures showed a decrease of 20% in sporulation in mistranslating cells relative to control cells.

These results demonstrated clearly that constitutive mistranslation affects meiosis and consequently sporulation efficiency, decreasing obviously not only the proportion of diploid cells that can sporulate, but also the number of sporulation tetrads produced by those cells. These phenotypic results do not explain how mistranslation affects sporulation, however it is reasonable to assume that it may disrupt critical proteins required for the sporulation process itself. In addition, proteins involved in meiosis may also be affected and may become misfolded or even degraded, creating a haplo-insufficiency phenotype that affects the sporulation process. Mistranslation may also cause genome instability, which in turn could block the cell cycle.

A

	Control	tRNA
Abundant sporulation	95%	7.5%
Poor sporulation	-	65%
No sporulation	5%	27.5%
cultures analysed	20	40

B

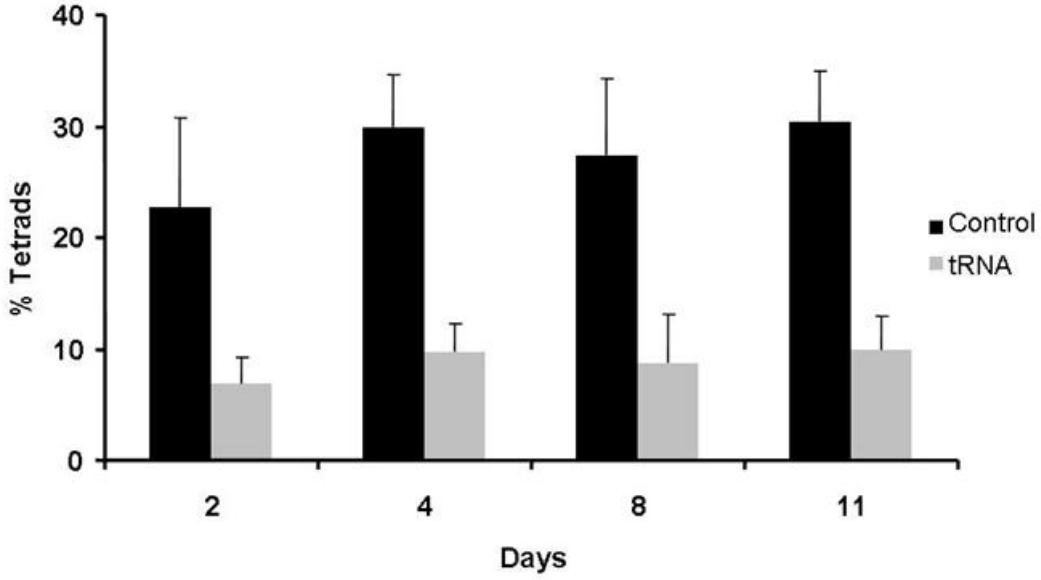


Figure 17. Mistranslation affects yeast sporulation. (A) Percentage of sporulation cultures of control or tRNA expressing cells analysed in clones that showed abundant, poor or no sporulation, after 8 days. (B) Percentage of sporulation tetrads produced by control and tRNA expressing cells, sporulation cultures were analysed after 2, 4, 8 and 11 days of culture.

1.4 Consequences of mistranslation in yeast spore germination

The impact of mistranslation on yeast fertility was also tested by germinating spores of dissected asci. For this, the asci were digested and the spores were separated using the Singer MSM micromanipulator dissecting protocol. A typical

ascus contains four ungerminated ascospores arranged in a tetrahedron shape (tetrads). Under the microscope most asci appear planar and are clearly visible (Figure 16) and after partial digestion and breaking of the tetrad cell wall, spores were separated and allowed to germinated in proper media. The optimal conditions for synchronous germinating spore population required a temperature of 30ºC, glucose in the medium and a pH within the range 5.4 and 8.2; carbon dioxide concentration was not an important factor (Haber *et al.*, 1975).

In selective media (YPD+geneticin), only spores containing the *KanMX4* cassette could germinate. The cassette segregation was 2:2 as only 2 of the 4 spores of each tetrad contained the *KanMX4* cassette conferring resistance to geneticin. 95% of the spores containing the *KanMX4* cassette (control cells) germinated while only 44% of the spores containing the *KanMX4::tRNA* cassette (mistranslating spores) germinated. Of the latter, 42% of the tetrads produced only 1 viable spore and only 23% produced two viable spores (Figure 18). The high percentage of single viable spores indicated that mistranslation had a strong negative impact on the viability of haploid cells (which was not observed in diploid cells), or, alternatively, that it somehow altered the segregation of the *KanMX4::tRNA* cassette from the expected 2:2 to 1:3 (viable:non-viable). The integrity of the tRNA gene present in some viable spores was verified by PCR amplification and direct Sanger DNA sequencing.

The expression of the $tRNA_{CAG}^{Ser}$ decreased spore viability sharply. More than half of the spores did not germinate and those that germinated grew slower than the control spores (Figure 18).

It is not clear why some spores died while others were able to survive, but one possibility is that mistranslation generates population variability or genetic diversity, which may result in differential responses of the quality control systems among members of the cell population. Proteome disruption caused by mistranslation may have also affected proteins that are essential for viability. This

would be however somewhat surprising because yeast housekeeping genes tend to avoid CUG codons (Suzuki *et al.*, 1997).

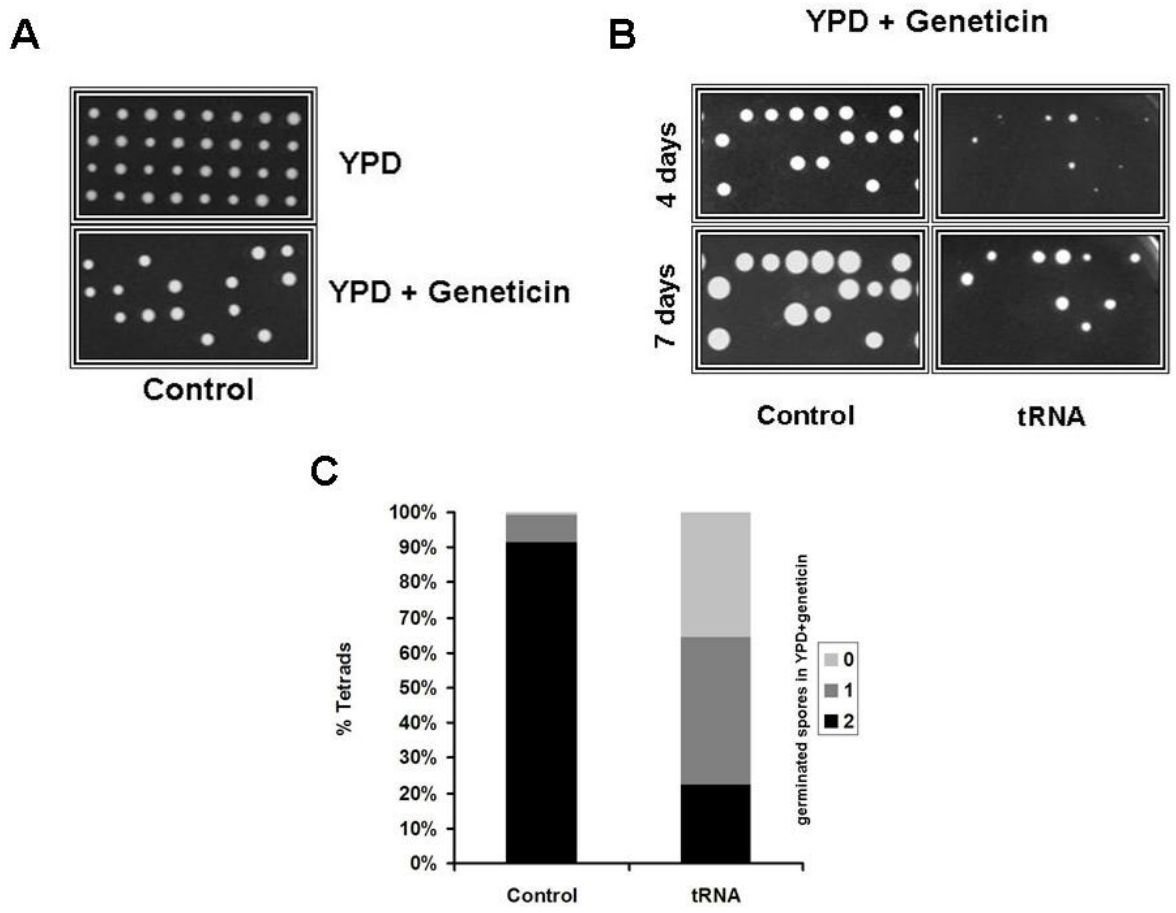


Figure 18. Mistranslation affects yeast spore germination. (A) Percentage of germination of control spores after 3 days of growth at 30ºC in YPD or in YPD+geneticin media. The segregation of the *KanMX4* cassette integrated in the yeast genome followed Mendelian rules (2:2) producing 2 viable and 2 non-viable spores in media containing geneticin. (B) Control and $tRNA_{CAG}^{Ser}$ expressing spores after 4 and 7 days of growth at 30ºC in YPD+geneticin medium. Germination of spores expressing the $tRNA_{CAG}^{Ser}$ was affected and those that germinated grew slower than the control spores. (C) Percentage of tetrads that produced 2, 1 or 0 viable spores in YPD+geneticin medium, showing that mistranslation reduced viability of the spores significantly.

An alternative explanation for those results is that mistranslation may produce toxic misfolded proteins or protein aggregates that may compromise essential biological processes. Since such protein aggregates tend to be variable and have cell-specific consequences (Gidalevitz *et al.*, 2010), they may provide a more likely

explanation for the above-mentioned phenotypes. This is in line with the morphological variability produced by stop codon read-through promoted by the yeast [PSI+] prion, which is also highly variable at the population level (True *et al.*, 2000;True *et al.*, 2004).

1.5 Mistranslation affects mating and zygote viability

In order to have a complete understanding of the impact of mistranslation on yeast sexual reproduction mating and zygote viability were studied. Mating between haploid yeast cells generates zygotes, which typically produce diploid buds by mitosis. Haploid a and α cells starved for 48 hours on buffered solid media usually mate with a frequency of 95% or higher (Sena *et al.*, 1975), allowing one to evaluate the impact of mistranslation on this biological process by monitoring the appearance of diploid cells (a/α) in selective media.

Cells of opposite mating type (a and α) synchronize each other's cell cycles in response to the mating factors produced by the opposite cell type. Once cells progress through the cell cycle, they are unable to mate until the next cell cycle. Conjugation occurs by surface contact of specialized cell projections ('schmoo' formation) on mating cells followed by plasma membrane fusion (Sena *et al.*, 1975).

In order to carry out the mating experiments, the mating type of the spores that germinated as described above were determined by a PCR analysis using three appropriate primers that amplify the DNA present at the *MAT* locus (Huxley *et al.*, 1990) (Figure 19A). When these three oligonucleotides (see Materials and Methods chapter) are used in a single PCR, DNA at *MAT*α generates a 404bp product, whereas DNA at *MATa* generates a 544bp product. Spore auxotrophy for uracil and histidine were also tested, by growing cells in the following media: MM lacking uracil, MM lacking histidine and MM lacking both, uracil and histidine. Only spores displaying auxotrophy for uracil or histidine were chosen for the mating

assays. The genotypes of the spores chosen for the assays were as follows: *MATa*/*URA3*, *MATa*/*HIS3*, *MAT*α/*HIS3* or *MAT*α/*URA3*.

Crosses of haploid cells of different mating type expressing the $tRNA_{CAG}^{Ser}$ (*MATa*/*URA3* or *MATa*/*HIS3* and *MAT*α/*HIS3* or *MAT*α/*URA3*), of these cells expressing the $tRNA_{CAG}^{Ser}$ with control (non-ambiguous) cells and also between control cells only were carried out. The products of mating were spotted and allowed to grow on plates containing MM lacking histidine and uracil for selecting the zygotes of the following genotypes *MATa*/*MAT*α, *URA3*/*HIS3* (Figure 19B). The mating efficiency of the cells expressing the $tRNA_{CAG}^{Ser}$ was always lower than that of control cells (Figure 19B). Also, growth rate and viability of mistranslating zygotes were lower than those of control non-mistranslating zygotes. Therefore, the decreased mating efficiency and the lower viability of zygotes resulting from these crosses showed unequivocally that mistranslation had a major negative impact on the sexual reproduction of yeast, which is a critical aspect of yeast biology.

These mating differences may be explained by gene dosage effects because diploids obtained in the first crosses had two copies of the $tRNA_{CAG}^{Ser}$ gene, while diploids obtained from the second type of crosses had a single copy of this tRNA gene and tRNA copy number determines tRNA abundance (Percudani *et al.*, 1997;Hani and Feldmann, 1998) and consequently mistranslation levels. However, accumulation of mutations or genome alterations/aberrations in mistranslating haploid cells may also have contributed to the lower mating efficiency. In order to understand if mistranslation caused genome instability, the genome of the mistranslating cells was analyzed by flow cytometry, as described in the next section.

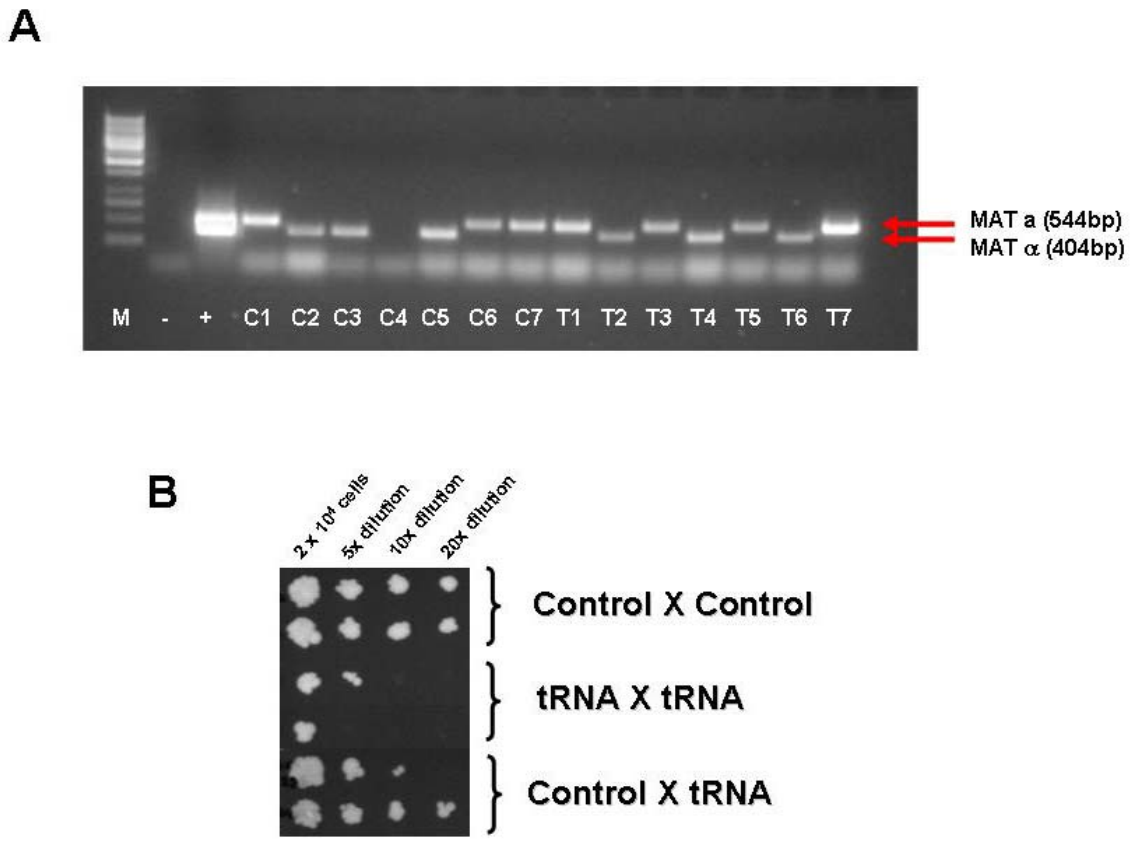


Figure 19. Mistranslation affects yeast sexual reproduction. (A) PCR analysis showing the mating type of control clones and clones expressing the $tRNA_{CAG}^{Ser}$. (B) Mating efficiency of different mating crosses. Haploid control and $tRNA_{CAG}^{Ser}$ expressing cells with opposite mating types were mixed and serial dilutions of the mixtures were plated onto selective media. Control X Control, $tRNA_{CAG}^{Ser}$ X $tRNA_{CAG}^{Ser}$ and Control X $tRNA_{CAG}^{Ser}$ indicate the crosses between the control cells, or between the $tRNA_{CAG}^{Ser}$ cells, or between control and $tRNA_{CAG}^{Ser}$ cells, respectively. The reduced number of diploid zygotes produced by crossing the $tRNA_{CAG}^{Ser}$ cells, or their decreased viability, showed that mating efficiency was also negatively affected by mistranslation.

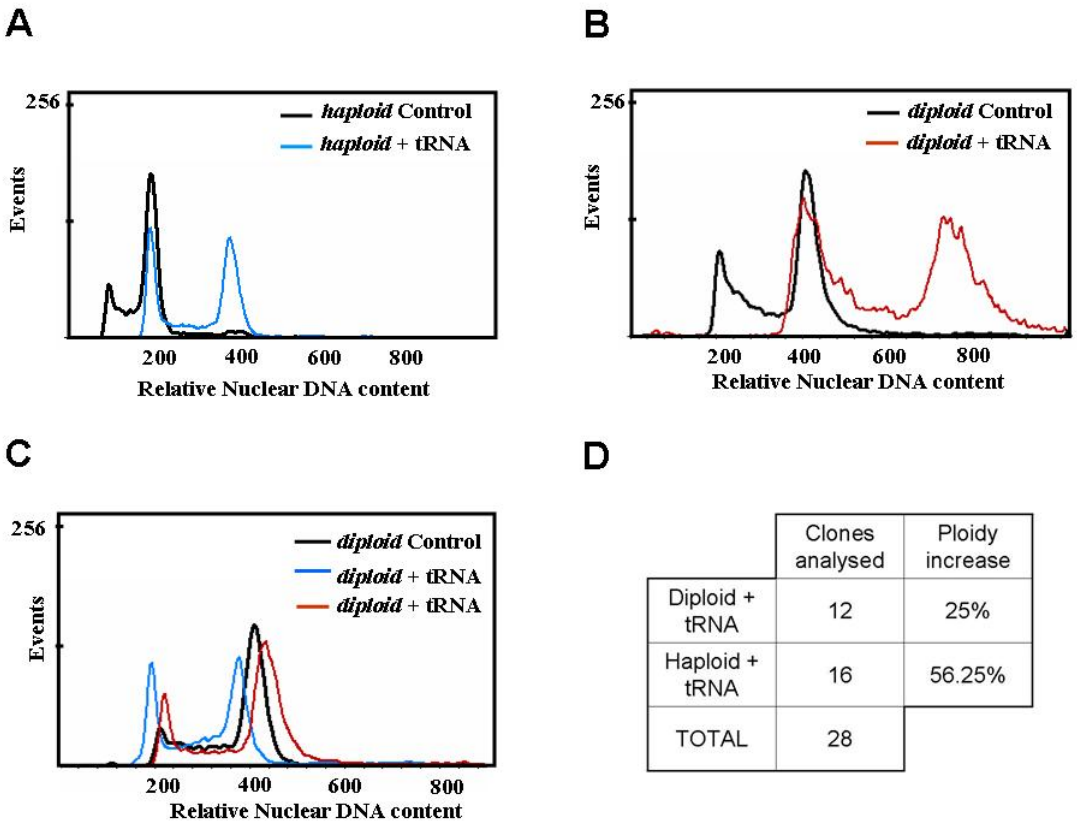
1.6 Mistranslation induces major genome alterations

Flow cytometry and DNA staining can be applied to the study of the yeast cell cycle and to quantify nuclear DNA content in each phase of the cycle. In this analysis, DNA was stained with SYBR Green I, a fluorophore which binds to double-stranded DNA with very high selectivity and has a much higher

fluorescence quantum yield upon DNA binding than do most commonly used fluorophores. This analysis permits differentiating G_0/G_1 and G_2/M cells and provides clear-cut identification of the S-phase. It showed that expression of $tRNA_{CAG}^{Ser}$ increased yeast ploidy, from N to 2N in 56% of the haploid clones analysed and from 2N to 4N in 25% of the diploid clones analysed. In some diploid clones, expression of $tRNA_{CAG}^{Ser}$ also shifted DNA content peaks slightly, suggesting that the cell population contained a significant number of aneuploid cells (Figure 20).

Histograms also showed higher percentage of control cells in the G_2/M phase of the cycle than in the G_0/G_1 phase, while for the $tRNA_{CAG}^{Ser}$ expressing cells there was a higher percentage of cells in G_0/G_1, suggesting that mistranslation affects the entry into the DNA replication phase (S-phase), in other words, it causes growth arrest and blocks cells in the G_0/G_1 phase (Figure 20).

Epifluorescence microscopy analysis of the nuclei of mistranslating cells stained with DAPI showed high percentage of cells containing micronuclei or/and two or more large nuclei. Also, some daughter cells did not have nuclei, suggesting disruption of chromosome segregation or aberrant nuclear division during mitosis (Figure 21). These nuclear morphological alterations in addition to the appearance of polyploid and aneuploid genomes in some clones provided clear indication that constitutive mistranslation had a strong negative impact in the genome structure and stability.



	Clones analysed	Ploidy increase
Diploid + tRNA	12	25%
Haploid + tRNA	16	56.25%
TOTAL	28	

Figure 20. Mistranslation induces ploidy variation. Nuclear DNA content was measured by flow cytometry in control and $tRNA_{CAG}^{Ser}$ expressing cells; expression of the $tRNA_{CAG}^{Ser}$ in *S. cerevisiae* increased cell ploidy both in haploid (A) and diploid (B) clones. Mistranslation also induced discrete shifts in DNA content in some diploid clones (C), indicating either chromosome copy number aneuploidies or whole genome aneuploidy in a fraction of the cell population. Evidences for polyploidy were found in 56 % of the investigated haploid clones and in 25 % of the investigated diploid clones (D).

Expression of $tRNA_{CAG}^{Ser}$ in yeast also generated highly heterogeneous colony and cell morphologies. Colonies of mistranslating cells had major morphological alterations, while control colonies are usually rounded, smooth and homogeneous in shape and size. In the same way, control cells appeared more homogeneous (with an oval/rounded shape) than mistranslating cells under the optical microscope. Indeed, mistranslating cells were seen as a mixture of rounded, oval, elongated or irregular shapes (Figure 21).

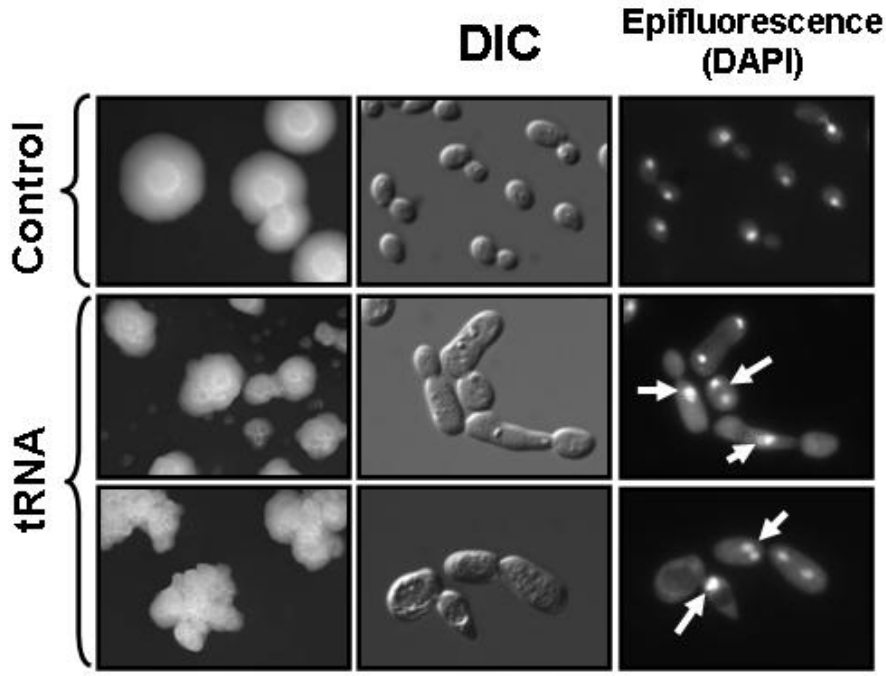


Figure 21. Colony, cell and nuclear morphologies. Heterogeneity in the mistranslating cell population is shown by the variability in colony, cell and bud size and shape. The increase in cell volume is consistent with polyploidization of the ambiguous clones. DAPI staining highlights mistranslating cells with two nuclei or without nucleus, suggesting the presence of polyploid or aneuploid cells probably caused by disruption of chromosome segregation or aberrant nuclear division during mitosis.

Also cell size increased significantly in mistranslating cells, which is consistent with the ploidy increase observed in some of these cells. Increased DNA content normally results in increased cell size (Cavalier-Smith, 1978;Gregory, 2001), although the relationship between ploidy and cell volume varies with environmental conditions and between organisms. For example, in yeast, the volume of diploid cells in rich media is 2.4 times bigger than that of haploid cells at 30ºC, but only 1.1 times bigger if cells are grown at 37ºC (Mable and Otto, 2001). Interestingly, not all features of a cell scale with increased ploidy. For example, kinetochore size and length of the pre-anaphase spindle do not scale with ploidy level, whereas the spindle pole body does (Storchova *et al.*, 2006). Therefore, changes in ploidy upset geometric relationships among key components of the machinery used to segregate chromosomes during meiosis and can probably explain the higher rate of chromosome non-disjunction in the mistranslating cells.

1.7 Mistranslation is advantageous under stress

The results shown above indicate clearly that mistranslation is deleterious, affecting various aspects of yeast biology. This is in line with the disease phenotypes uncovered in both humans and mice, which are associated to mistranslation. However, as mentioned before, codon ambiguity is involved in genetic code alterations and has been selected in the fungal CTG clade (Santos *et al.*, 2004;Miranda *et al.*, 2006;Butler *et al.*, 2009), suggesting that it may also have selective advantages. Previous studies provided some evidence that mistranslating cells are tolerant to various environmental stressors and can grow in the presence of lethal doses of cyclohexamide, arsenite and salt, most likely due to stress cross-protection (Santos *et al.*, 1999).

The studies described above showed that yeast mistranslating cells expressing the $tRNA_{CAG}^{Ser}$ gene grew slower than control cells in rich glucose media (YPD). In order to confirm whether mistranslation could be advantageous under different environmental conditions, the mistranslating cells from above were re-plated in different media, namely in synthetic media (SD) and in Minimal Media (MM) containing $CdCl_2$ or H_2O_2. Remarkably, as the stress increased in the different media the mistranslating cells recovered their growth capacity and were able to grow as fast as the control cells (Figure 22). This supported the hypothesis that, to a certain extent, stress tolerance and stress cross-protection overcome the negative impact of proteome disruption caused by mRNA mistranslation and that under stress conditions, the selective advantage and higher fitness revealed by control cells in rich medium disappeared.

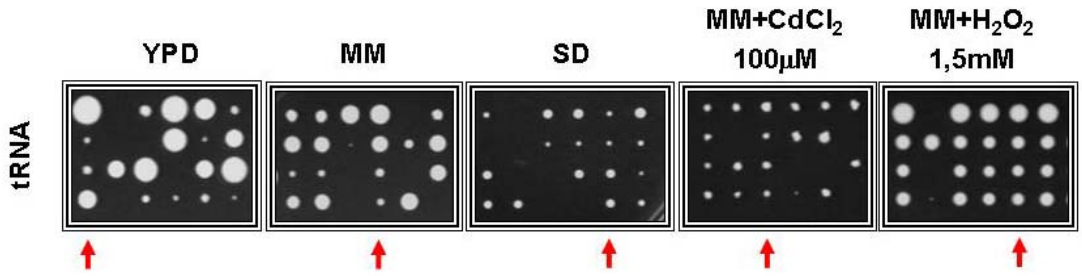


Figure 22. The negative impact of mistranslation on yeast fitness can be overcome by environmental stress. Spores of *S. cerevisiae* expressing the $tRNA_{CAG}^{Ser}$ were grown

for 7 days in agar plates containing rich medium (YPD), synthetic medium (SD) and minimal medium (MM) with 100μM $CdCl_2$ or 1.5mM H_2O_2. Spores expressing the $tRNA_{CAG}^{Ser}$ grew much slower than non-ambiguous spores in rich medium, but recovered growth rate under stress conditions, as shown by similar colony size of control and mistranslating colonies.

1.8 Discussion

Mistranslation generates major proteome chaos and should be eliminated by natural selection. Mistranslation has a major impact on protein primary structure on a proteome-wide scale and one would expect that it creates major degenerative phenotypes and a huge energetic problem due to high protein turnover. In order to understand the cellular response to mistranslation, I started to study it at the phenotypic level, in particular its effect on yeast sexual reproduction, in genome stability and also at cellular and colony morphology and stress resistance levels.

The direct and immediate consequence of mistranslation is the synthesis of aberrant proteins which may misfold, aggregate or be degraded by the ubiquitin-proteasome pathway (Lee *et al.*, 2006;Rochet, 2006). The synthesis of mutant proteins will inevitably up-regulate the proteome quality control systems, namely the molecular chaperones, ubiquitin-proteasome, autophagy, the UPR and the ERAD systems. Accumulation of aggregated and soluble mutant proteins may overload these proteome quality control systems, which may allow for the release of toxic proteins into the cytosol and may explain the decreased viability of the mistranslating cells. In the same manner, mutant proteins with new functions may also be released into the cytosol and generate new phenotypes or phenotypic variation, as is observed in the case of the Hsp90 challenge during stress in *Drosophila melanogaster* or in *Arabidopsis thaliana* (Rutherford and Lindquist, 1998;Queitsch *et al.*, 2002). Also, increased CUG ambiguity in *Candida albicans* increases cell adhesion, secretion of lipases and proteinases and results in a wide variety of colony morphologies (Miranda *et al.*, 2007;Gomes *et al.*, 2007). In yeast, the [PSI^+] prion increases read-through of stop codons, generating proteins with

extended C-termini that accumulate in the cell. These aberrant proteins increase the variety of growth and morphological phenotypes with high selective potential (True *et al.*, 2000).

Furthermore, the ploidy alterations induced by mistranslation have important evolutionary consequences as they alter gene expression, cell size and developmental, physiological, and other unique morphological characteristics, as already described for other several cell types (Galitski *et al.*, 1999). Noteworthy, it is the observation that ploidy shifts are frequently associated to cancer (Storchova and Pellman, 2004;Storchova and Kuffer, 2008) and aneuploidies are associated to drug resistance in the fungus *C. albicans* (Selmecki *et al.*, 2006).

Polyploidy is frequently found in nature; in fact, most wild type yeast strains are polyploid and the polyploidy state can be part of the normal physiology of plants and animals (Otto, 2007), including a few types of human cells. Generally it does not lead to gross defects in the development of an organism or in its physiology (Otto and Whitton, 2000). In addition, duplications of an entire genome have taken place in the evolution of several plants and yeasts and may be a natural event necessary for this process (Wolfe and Shields, 1997;Kellis *et al.*, 2004;Adams and Wendel, 2005). In contrast, aneuploidy frequently causes lethality and has been associated with disease, sterility, and tumour formation. In this case, protein stoichiometry imbalance and reduction in gene dosage of a selected number of genes are the major cause of defects associated with chromosome gains and losses. These cells also replicate and maintain the extra chromosomal material, creating a higher demand on the DNA replication machinery, chromosome maintenance and segregation pathways. In fact, these pathways cause reduced fitness of polyploid yeast strains (Storchova *et al.*, 2006). Because the additional chromosomes in aneuploid cells are active, it is also possible that the transcription and translation machineries become rate limiting, which could also lead to a reduction in organismal fitness. Consistent with this idea is the observation that aneuploid yeast strains exhibit increased sensitivity to conditions that interfere with transcription and protein synthesis (Torres *et al.*, 2007).

Genomic alterations caused by mistranslation may be associated to mutations in key proteins involved in the processes of mitosis and meiosis. Also mutant DNA polymerases and mutant DNA repair enzymes probably may create hypermutagenic clones with high adaptation potential. This hypothesis is supported by the discovery in *E. coli* and other bacteria of a hypermutagenesis phenotype associated with codon ambiguity, the so-called 'translational stress mutagenesis phenotype' (TSM). In bacteria, several types of mistranslation induce spontaneous hypermutagenesis via synthesis of mutant DNA polymerase III or other complexes (Al Mamun *et al.*, 2002;Al Mamun *et al.*, 2006). This increased genome mutation rate generates genetic diversity and, therefore, provides an indirect mechanism for rapid fixation of the advantageous phenotypes linked to mistranslation.

In summary, mistranslation expands the proteome of the cell and generates new phenotypes, creating phenotypic heterogeneity and diversity, including sexual isolation between cells, genomic instability and other morphologic and physiologic alterations. In some cases, selection of advantageous phenotypes creates a positive feedback pressure that maintains the process of mistranslation. This ultimately leads to synthesis of mutant DNA polymerases and DNA repair enzymes and emergence of hypermutagenic clones with an exponential increase in genome mutational load. This accelerates the fixation of advantageous phenotypes and their transmission to progeny and explains the existence of multiple cases where mistranslation has positive outcomes. In order to better understand the phenotypes described above at the cellular and molecular levels and elucidate the cellular response to mistranslation the transcriptome of mistranslating cells was analysed using DNA microarrays. The results of these studies are described in the next chapter.

2. Transcriptional and translational responses to mistranslation

2.1 Overview

Several studies have already been carried out on the cellular responses to mistranslation, however a global picture of the transcriptional and translational responses to mRNA mistranslation is still missing. Protein misfolding caused by misincorporation of the proline analogue azetidine-2-carboxylic acid (AZC) into proteins showed up-regulation of heat-shock factor-regulated genes and selective down-regulation of ribosomal protein genes (Trotter *et al.*, 2002). Interestingly, the general stress response was not activated showing that cells sense the stress imposed by protein misfolding and environmental stress in different manners.

Previous studies on the characterization of the transcriptome and proteome of yeast cells mistranslating constitutively showed that the latter induces a stress response similar to that activated by heat-shock or AZC. However, the general stress response mediated by the transcription factors Msn2 and Msn4 was also up-regulated (Silva *et al.*, 2007). This is in line with other studies showing that mistranslation generates stress cross-protection and increases thermotolerance, and tolerance to oxidative and osmotic stress and also resistance to heavy metals and drugs (Santos *et al.*, 1996;Santos *et al.*, 1999;Silva *et al.*, 2007). Such stress cross-protection arises due to overexpression of molecular chaperones and many other stress genes that protect cells against different stressors. The typical example of such stress cross-protection is the tolerance of yeast grown at 25ºC to the supra-optimal temperature of 40ºC when exposed for a short period of time (15 min) to the intermediate temperature of 37ºC. The latter up-regulates stress genes, in particular molecular chaperones, which are essential for survival and tolerance to the higher temperature of 40ºC. Conversely, direct exposure of cells grown at 25ºC to 40ºC leads to massive cell death (McAlister and Finkelstein, 1980;Miller *et al.*, 1982).

Despite cross-protection and some other similarities, the response triggered by constitutive mistranslation is distinct from that induced by environmental stressors as the former is activated intra-cellularly and is permanent, rather than transient. The transcriptional profiling of yeast cells mistranslating constitutively highlighted some aspects of the cellular mechanisms of adaptation to permanent mistranslation (Silva *et al.*, 2007). However, it did not provide a complete understanding of the cellular responses to mistranslation. In order to get deeper insight into the cellular responses to mistranslation, yeast cells were engineered to mistranslate in a regulated and inducible manner. This new model system of mistranslation allowed one to study not only the long-term effects of mistranslation, but also the short-term changes caused by low-level aberrant protein synthesis. The studies described in this chapter provide a global view of the transcriptome and translatome alterations of those engineered yeast cells exposed to controlled low-level mistranslation, which had little or no impact on yeast fitness. DNA microarrays were used to investigate the immediate and medium term transcriptional and post-transcriptional responses to such regulated mistranslation event.

2.2 Inducible expression of a misreading $tRNA_{CAG}^{Ser}$ in yeast

Transfer RNA genes (tDNAs) have internal promoters (A and B boxes) and are transcribed constitutively by RNA polymerase III (Sharp *et al.*, 1985;Geiduschek and Tocchini-Valentini, 1988). Regulatory elements beyond the A and B boxes are not known and it is assumed that tRNA abundance is mainly determined by tDNA copy number (Percudani *et al.*, 1997;Hani *et al.*, 1998). In order to circumvent this limitation and engineer regulated expression of the mutant misreading $tRNA_{CAG}^{Ser}$ described in the previous chapter, I have taken advantage of the *E. coli* tetracycline repressor-operator (tetR/tetO) system (Dingermann *et al.*, 1992). For this, I have fused the tetO sequence upstream of the $tRNA_{CAG}^{Ser}$ gene and co-expressed the tetR protein and the tRNA in the same yeast cells. Binding of the tetR to the tetO blocked binding of the TFIIIC and TFIIIB general transcription

factors to the tDNA promoter thus preventing its transcription by Pol III. Expression of the tetR gene was driven by the *GAL1* promoter (pGalTR1 plasmid) and the tetR repressor could be expressed in an active form in galactose containing media only. The misreading $tRNA_{CAG}^{Ser}$ gene was amplified by PCR using a 5'-primer that contained the tetO sequence. The tetO-tDNA chimera was designed in such a manner that allowed for insertion of the tetO cis element 3 nucleotides upstream of the mature $tRNA_{CAG}^{Ser}$ sequence. This amplified fragment was cloned in the pRS305K plasmid producing the plasmid pRS305K-tetOtRNA. This plasmid was then used for PCR amplification of DNA cassettes with long tails homologous to the yeast *leu2* locus, containing the *KanMX4* gene and the $tRNA_{CAG}^{Ser}$ gene between them. Control clones contained the *KanMX4* gene only. Correct integrations of these DNA cassettes into the *leu2* locus were checked by colony PCR using specific primers and by Sanger DNA sequencing of the tetO-tRNA artificial gene (Figure 23).

In galactose media, active tetR is produced and binds to the tetO sequence located upstream of the tDNA gene, blocking its transcription as described above. However, in the presence of tetracycline the tetR alters its conformation and the repressor looses its affinity for the tetO leaving the tDNA gene sequence free for TFIIIC, TFIIIB and Pol III binding, thus activating transcription of the tDNA gene. The mature $tRNA_{CAG}^{Ser}$ competes with the endogenous $tRNA_{UAG}^{Leu}$ for CUG codons, misincorporating serine at these leucine codons on a genome wide scale.

This regulated system allowed one to induce the expression of the mutant serine $tRNA_{CAG}^{Ser}$ in exponentially growing cells by adding tetracycline to the growth media, or, alternatively, by growing cells in glucose as the sole carbon source to repress the expression of the tetR protein.

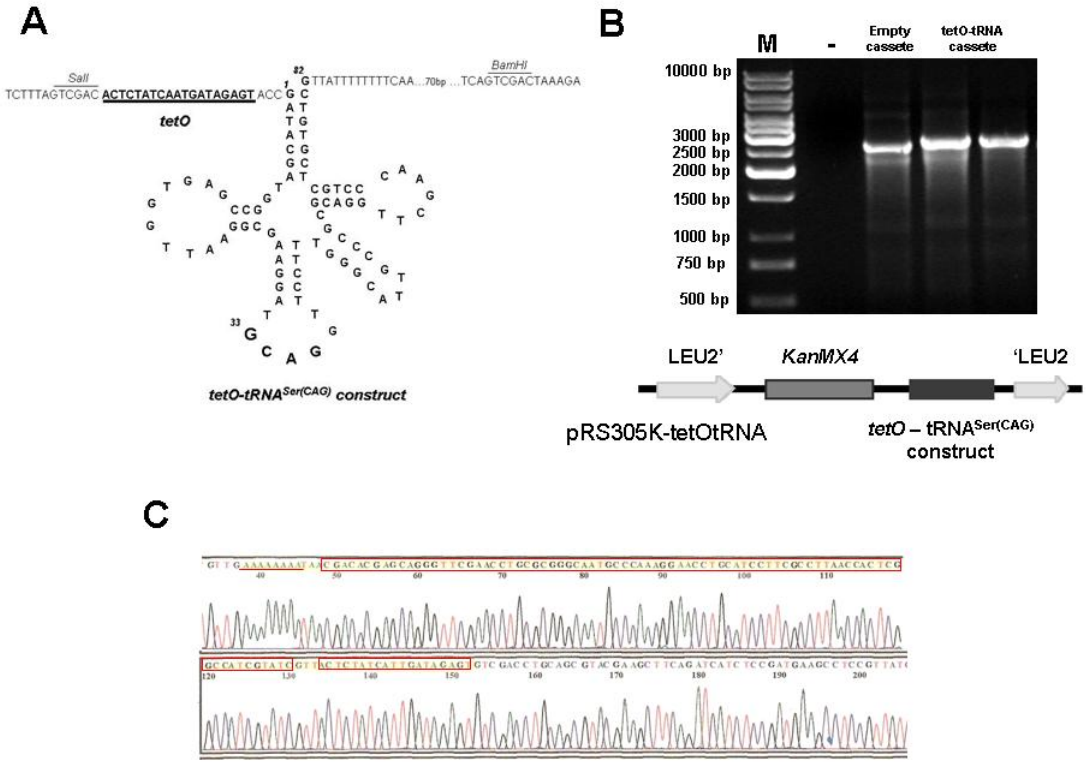


Figure 23. Engineering regulated expression of a heterologous $tRNA_{CAG}^{Ser}$ in yeast. (A) The *C. albicans* $tRNA_{CAG}^{Ser}$ gene was amplified by PCR in order to obtain DNA fragments with *SalI* / *BamHI* restriction sites in their 5'- and 3'-tails and to insert a tetO sequence 3 nucleotides upstream of the 5'-end of the mature $tRNA_{CAG}^{Ser}$. (B) The tetO-tRNA construct was cloned into the pRS305K plasmid producing the pRS305K-tetOtRNA plasmid. Using these plasmids as DNA template for PCR reactions, genome integration cassettes with long tails homologous to the yeast *leu2* locus, containing the *KanMX4* marker gene and the cloned tRNA gene between them, was carried out. Control clones contained the *KanMX4* marker gene only. (C) The sequence of the integrated tetO-tRNA construct was confirmed by Sanger DNA sequencing.

Induction of the $tRNA_{CAG}^{Ser}$ expression and mistranslation was checked initially by monitoring yeast phenotypic alterations, namely the frequency of colony forming units (CFUs), morphology and colony colour. These phenotypes are described in detail below.

2.3 Effect of regulated mistranslation on yeast growth rate

Constitutive mistranslation mediated by expression of the $tRNA_{CAG}^{Ser}$ slows yeast growth rate significantly (50-70%) (Santos *et al.*, 1996;Silva *et al.*, 2007). Surprisingly, one did not detect visible changes in growth rate in various clones analysed upon induction of expression of the $tRNA_{CAG}^{Ser}$ (Figure 24). This suggested that the tRNA gene was expressed in an inactive form, was unstable or was transcribed at very low-level.

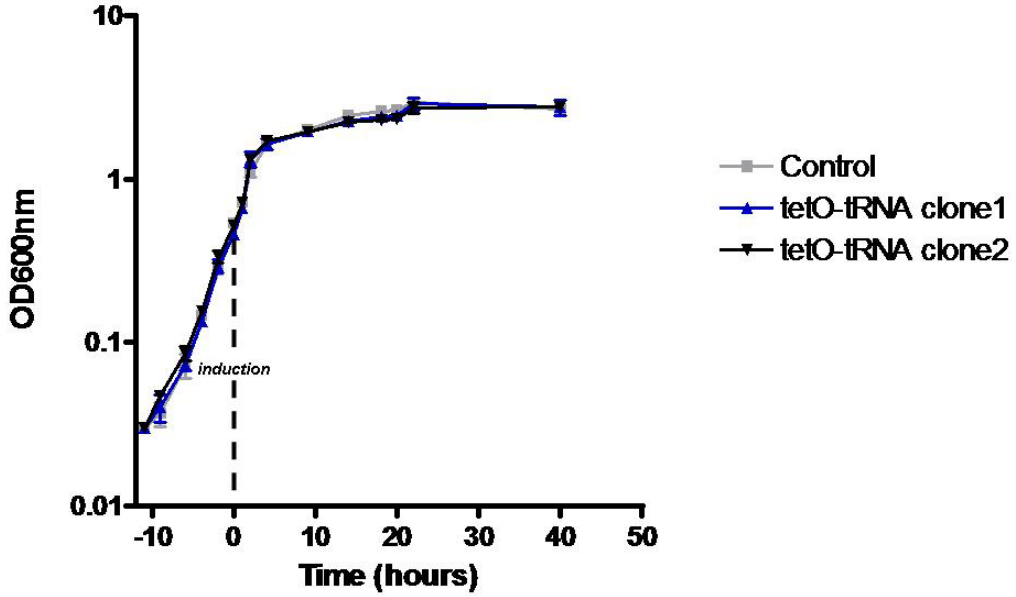


Figure 24. Induction of mistranslation does not affect yeast growth rate. Growth curves of control and tetO-tRNA clones growing in liquid MMgalactose+geneticin medium. Expression of the $tRNA_{CAG}^{Ser}$ was induced by addition of 40μg/mL of tetracycline to the cultures.

In order to clarify why induced mistranslation did not decrease growth rate as expected, the *E. coli LacZ* gene was co-expressed with the mutant serine tRNA. β-galactosidase (β-gal) was used as a reporter system for monitoring serine misincorporation at leucine CUG codons. The *E. coli LacZ* gene contains 54 CUG codons and random serine insertion at these codons generates a combinatorial array of mutant β-gal molecules with altered thermal stability, which could be easily monitored using a thermal denaturation assay (Branscomb and Galas, 1975;Finkelstein and Strausberg, 1983) (see Methods section). This assay

showed a significant decrease in β-gal thermal stability at T40' (25.1%), T90' (35.0%) and T180' (33.0%) indicating that the mutant $tRNA_{CAG}^{Ser}$ was being expressed in an active form and misincorporated serine at leucine-CUG codons (Figure 25). In other words, the neutral effect of mistranslation in yeast growth rate was not due to lack of decoding activity of the mutant serine misreading tRNA. Previous results showed that 2.3% serine misincorporation at CUG positions decreases yeast growth rate by almost 50% (Santos *et al.*, 1996;Silva *et al.*, 2007), thus indicating that the level of CUG misreading generated through this tetR/tetO expression system was far below 2.3%. In our experience, detection of mistranslation below 0.5% of amino acid misincorporation using state of the art mass-spectrometry is very difficult and there is no simple enzymatic method available to detect serine misincorporation at CUG codons in yeast. Therefore, the β-gal values indicated in Figure 25 provide the best estimate of the relative levels of mistranslation present in our engineered yeast cells.

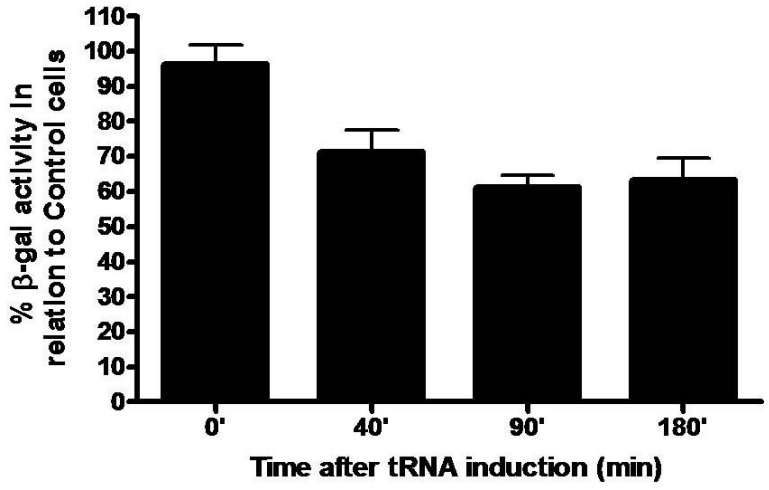


Figure 25. The $tRNA_{CAG}^{Ser}$ is expressed and is functional in yeast. The decoding activity of the serine tRNA was monitored using a β-galactosidase thermal stability assay based on quantification of its activity after thermoinactivation at 47ºC for 10 minutes. Serine misincorporation at the 54 CUG codons present in *E. coli* β-gal destabilizes the protein and provides a highly sensitive enzymatic assay for determination of the decoding activity of the $tRNA_{CAG}^{Ser}$. Control and tetO-tRNA cells of exponential cultures were incubated at 47ºC to denature mistranslated β-galactosidase. The activity of the β-galactosidase fraction that remained functional after incubation for 10 min at 47ºC was determined by incubating cells for 2 min at 37ºC in presence of ONPG.

Considering the strong effect of the $tRNA_{CAG}^{Ser}$ on the activity of β-galactosidase, the lack of a visible effect on yeast growth rate was somewhat surprising. A clearer picture however emerged when the mistranslating cells from above were re-grown in fresh media. In this case there was a clear delay in growth rate (Figure 26). Cells apparently started to divide as fast as control cells during the initial divisions but growth was arrested/delayed significantly after the initial growth phase. Nevertheless, both control and mistranslating cultures reached similar final cell densities indicating that culture viability was not compromised.

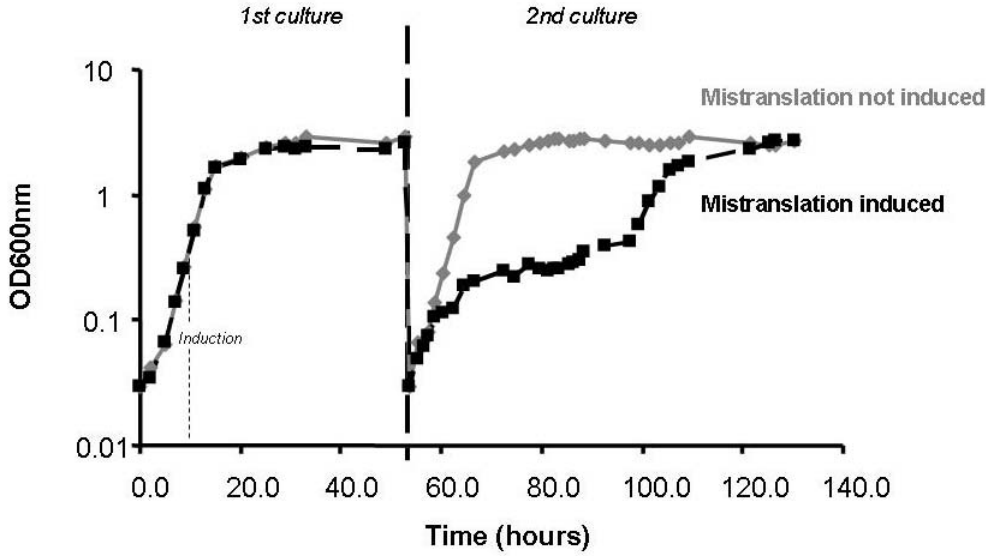


Figure 26. Low-level mistranslation delayed growth rate. The effect of mistranslation was not visible in exponentially growing cells upon induction of expression of the $tRNA_{CAG}^{Ser}$ (first culture), however a clearly visible effect on growth rate was observed when cells from the first culture were re-grown in a fresh culture (second culture). Control and tetO-tRNA clones were grown in liquid MMgalactose+geneticin medium; expression of the $tRNA_{CAG}^{Ser}$ was induced by tetracycline.

The inexistence of a growth phenotype upon induction of mistranslation by the $tRNA_{CAG}^{Ser}$ suggested that the yeast proteome quality control systems were able to cope with the level of aberrant proteins being produced in these cells. These quality control systems involve protein degradation by the ubiquitin-proteasome and autophagy pathways, and also protein refolding by molecular chaperones. It is also likely that misfolded proteins may aggregate, but the accumulation of such

aggregates did not compromise cell viability. In any case, the energetic burden imposed on the cell by high protein turnover and protein refolding should be significant and it is surprising that it did not have a negative impact on fitness. This raises the puzzling question of whether the level of mistranslation induced in the engineered yeast activates the stress response or, conversely, whether the level of aberrant proteins in these cells is so low that the physiological levels of activity of the proteome quality control systems is sufficient to deal with them. This question is addressed below.

2.4 Transcriptional response to mistranslation

The transcriptional response to low-level mistranslation of leucine CUG codons as serine was investigated by mRNA profiling of cells induced with tetracycline at times 0, 40, 60, 90, 120 and 180 minutes (T0', T40', T60', T90', T120' and T180') (Figure 27). For this, total RNAs were extracted from 6 independent cultures and 3 different clones and were reverse transcribed. The corresponding cDNAs were labelled with Cy3 and Cy5 dyes and hybridized to "home-made" yeast arrays (YAUAv 1.0), using a pool of mRNAs extracted from control cells at several time points as a reference RNA sample (See Materials and Methods section).

In a complementary experiment, the transcriptomes of cells adapted to mistranslation (T20h) and control cells at the same time point were also compared (Figure 27) using mRNA samples extracted from 4 independent cultures of two independent clones.

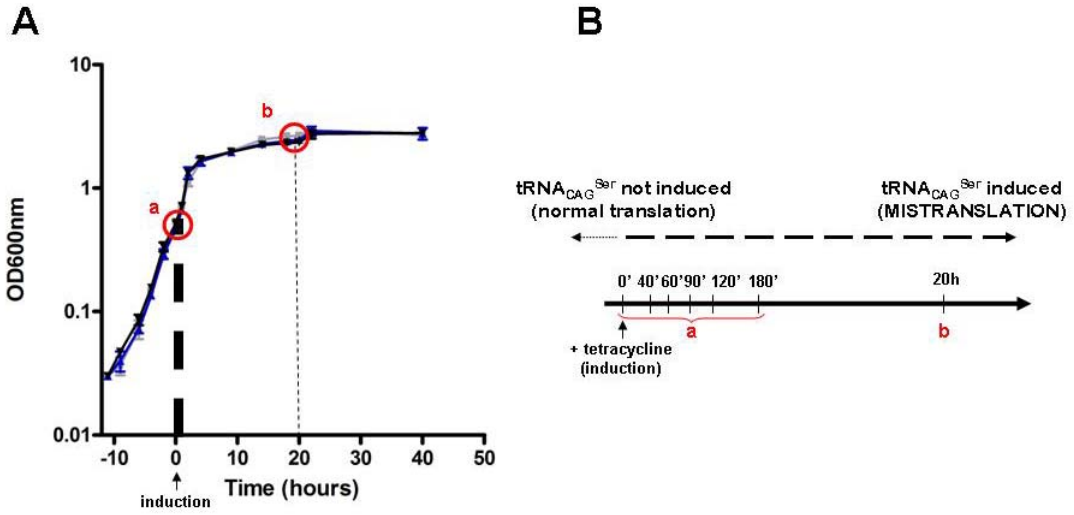


Figure 27. Schematic representation of the RNA extraction time points used in the DNA microarray experiments. (A) Growth curves of control and tetO-tRNA clones already shown in Figure 24. (B) Time points analysed in both a. and b. experiments highlighted in (A).

The mRNA profiling of the mistranslation time-series (T0' to T180') showed significant alteration in gene expression as early as T40' (Figure 28), which was consistent with the decrease in β-gal activity at T40'. The global mRNA profile identified 927 genes whose expression varied more than 1.5 fold between at least 2 time points with the Significance Analysis of Microarrays (SAM) that was carried out; these corresponded to 14.5% of the total number of genes analysed (6392 genes in the array). This global response to mistranslation was similar in many ways to the yeast environmental stress response (ESR) (Gasch *et al.*, 2000;Causton *et al.*, 2001) and suggested that yeast cells sensed aberrant protein synthesis through ESR signalling pathways (see Annexe 2). Indeed, a comparison of the genes that were differentially expressed under both induced mistranslation and environmental stress described in (Gasch *et al.*, 2000) identified 338 genes with similar expression patterns, which corresponded to 39% of the yeast ESR genes (Figure 29 and Annexe 2).

Clustering of the deregulated genes produced six discrete clusters (Figure 28): clusters A and B contain genes that increased their expression during the three hours period of mistranslation analyzed. Genes in cluster A have however higher fold increase that those present in cluster B; cluster C contains genes that were

down-regulated during the first 90 minutes after mistranslation induction, but expression of these genes were up-regulated after 120 and 180 minutes of induction. Cluster D contains genes that were essentially down-regulated after induction of mistranslation and clusters E and F contain genes with heterogeneous expression, but essentially they were down-regulated after mistranslation induction. Cluster F genes seem to have increased their expression immediately after mistranslation, but then expression was down-regulated later on.

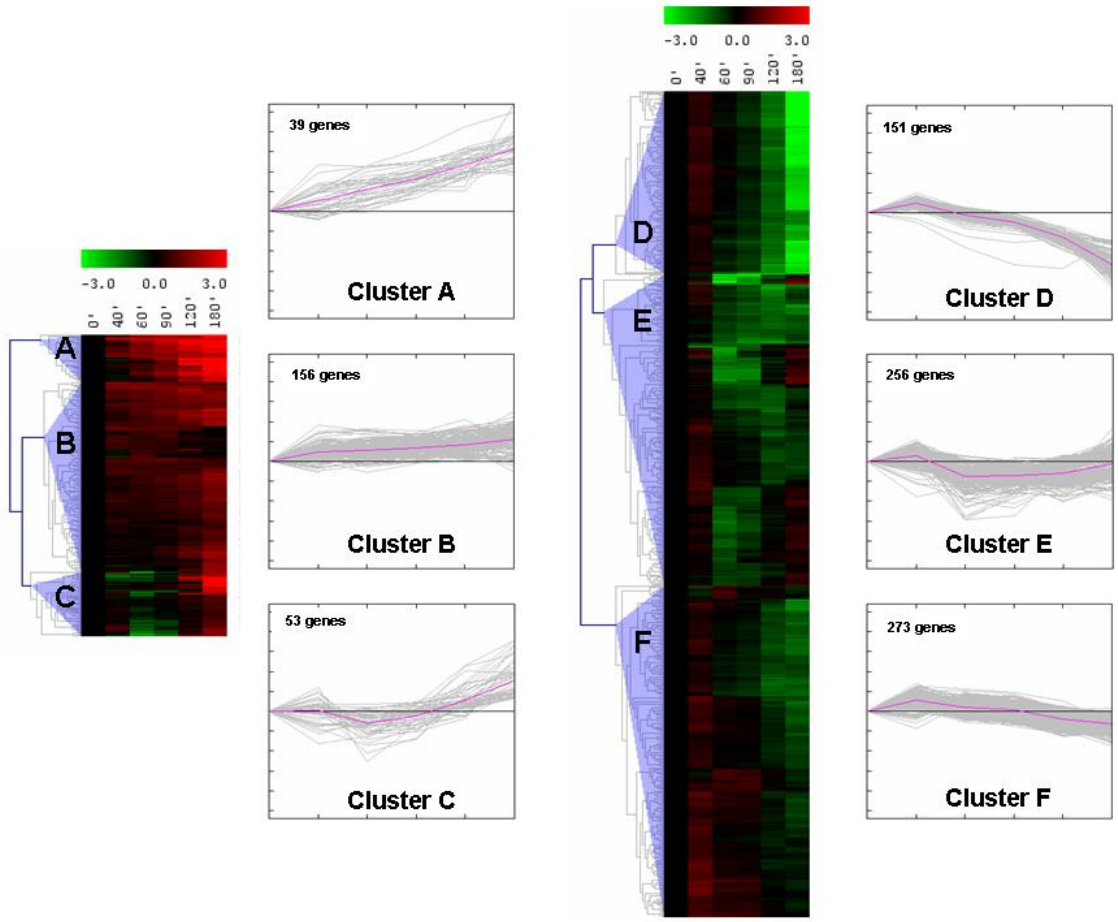


Figure 28. Mistranslation has a strong impact on the yeast transcriptome. Schematic representation of the 6 clusters of genes whose expression was deregulated by mRNA mistranslation. A T0' to T180' time series is represented. Gene expression values are normalised M values (Multiclass SAM analysis, FDR<0.001; only genes with M values corresponding to fold changes lower than -1.5 or higher than 1.5 in at least one time point are shown).

mRNA profiling experiments at T20h identified 1442 genes. Of these, 960 were up-regulated and 482 were down-regulated, according to the SAM analysis carried out. Again, a direct comparison of the genes differentially expressed by mistranslation and environmental stress identified 287 common genes. Of these, 111 were also differentially expressed in the previous time series experiments (T0' to T180'). After these 2 experiments, 514 genes have also been described as deregulated genes in the ESR (almost 60% of the 868 deregulated genes in the ESR). Therefore, the global response to mistranslation, which included short- (T0' up to T180') and long-term (T20h) responses, identified 320 genes that responded to mRNA mistranslation (Figure 29 and Annexe 3).

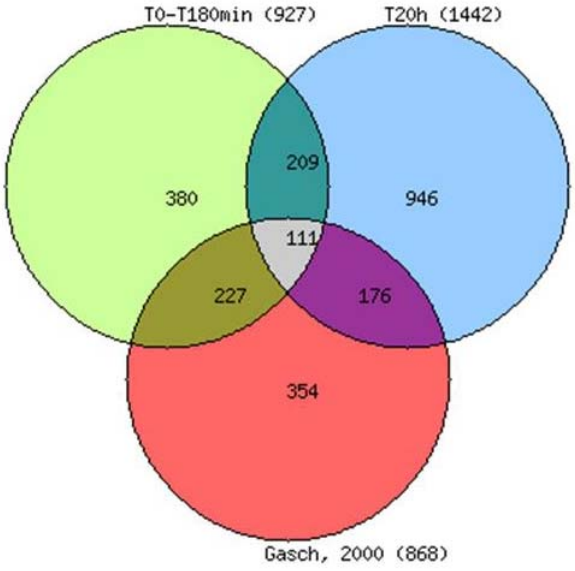


Figure 29. Venn diagram comparing the number of genes whose expression was deregulated by mistranslation. Shown are genes of the 1) T0'-T180' time series experiment, multiclass SAM analysis, FDR<0.001, only genes with M values corresponding to fold changes lower than -1.5 and higher than 1.5 are represented, 2) of the long-term T20h experiment, SAM analysis, FDR<0.001; only genes with M values corresponding to fold changes lower than -1.5 and higher than 1.5 and 3) genes deregulated by the environmental stress response (Gasch *et al.*, 2000).

Functional class analysis of the genes whose expression was altered by low-level mistranslation showed that the up-regulated genes belonged to the stress response, unfolded protein response, redox response, external encapsulating

structure, energy reserve, carbohydrate metabolism and aldehyde and sulfur metabolism (Table 1 and Annexe 4). The down-regulated genes belonged to the protein synthesis, ribosome and rRNA metabolism functional categories (Table 2 and Annexe 4). These data were consistent with other studies already carried out, which showed that mistranslation activates the general stress response and suggested that induced mistranslation affects proteome stability. Cells respond to such proteome destabilization by up-regulating molecular chaperones, the protein degradation and the unfolded protein responses. The redox response suggested that mistranslation also increased oxidative stress, while up-regulation of energy reserve and other metabolic pathways indicated that cells sensed mistranslation through the ESR signalling pathways (Gasch *et al.*, 2000;Causton *et al.*, 2001), which is in line with its strong negative impact on the expression of protein synthesis factors.

Genes encoding molecular chaperones, namely *HSP26*, *HSP30*, *HSP31*, *HSP42*, *HSP104*, *SSA1*, *SSA2* and *SSA4* showed the strongest up-regulation during the time series T0'-T180' (Cluster A and B genes). Some of these genes maintained the up-regulation into the diauxic-shift and stationary phase (T20h). Conversely, the genes encoding proteasome subunits, including several *PRE*, *RPN* and *RPT* genes appeared up-regulated in stationary phase (T20h), but most of them showed normal expression during the time series T0'-T180'. This suggested that molecular chaperones are the primary line of defense against proteome destabilization by mistranslation and that protein degradation becomes relevant at later stages, probably when this chaperone line of defense becomes overloaded or starts to breakdown. This may also be explained by the fact that expression of molecular chaperones and unfolded protein binding genes are activated through the Hsf1 and Msn2/Msn4 transcription factors which respond to the heat-shock and general stress response signalling pathways, while the proteasome subunits are up-regulated through the Rpn4 transcription factor which is activated by the oxidative stress, multidrug resistance and also heat-shock signalling pathways.

Table 1. Significantly enriched functional classes in differentially expressed genes from Clusters A, B, C and T20h up-regulated genes. The enriched classes were obtained using the EXPANDER software tool called TANGO. (corrected p-value cutoff = 0.05)

Significantly enriched Functional Classes	Cluster A		Cluster B		Cluster C		20h	
	Frequency	p-value	Frequency	p-value	Frequency	p-value	Frequency	p-value
aldehyde metabolic process			3.2%	0.031			1.3%	0.003
cellular carbohydrate catabolic process			7.7%	0.001			2.6%	0.001
energy reserve metabolic process			4.5%	0.015			2.1%	0.001
external encapsulating structure			6.4%	0.036				
oxidoreductase activity			14.1%	0.001			9.1%	0.001
peroxidase activity			3.8%	0.001			1.4%	0.001
proteasome complex							3.0%	0.001
protein folding			7.1%	0.002				
regulation of cell redox homeostasis			3.2%	0.001			0.9%	0.002
response to chemical stimulus							8.8%	0.021
response to oxidative stress			7.7%	0.001			3.2%	0.001
response to stress	38.5%	0.001	17.3%	0.019			12.1%	0.001
response to toxin					11.3%	0.001	1.3%	0.030
sulfur metabolic process							3.1%	0.001
sulfur utilization							0.9%	0.001
unfolded protein binding	12.8%	0.014	7.7%	0.001				

Interestingly, genes involved in the cellular responses to toxin and chemical stimulus showed stronger up-regulation at the end of the time series (T120' and T180', Cluster C) and at time T20h. Oxidative stress responsive genes also showed up-regulation during the T0'-T180' time series and at time T20h, but in a different fashion, they started increasing their expression immediately after induction of mistranslation (Cluster B). For example, the oxidative stress *GRX1*, *GRX2*, *HYR1*, *PRX1*, *SOD1*, *SOD2*, *TRX2* and *TSA1* genes were up-regulated up to 5.6 fold. This suggested that mistranslation had a strong connexion with oxidative stress and raised the fascinating possibility that low-level mistranslation causes cell degeneration and death through increased production of reactive oxygen species rather than through generalized proteome collapse. This hypothesis is consistent with the idea that low-level mistranslation only affects a small number of molecules of each protein (most are translated accurately) and, therefore, the major part of the proteome remains functional. This hypothesis is dissected below.

Mistranslation also down-regulated genes encoding translation factors, ribosomal and ribonucleoprotein complex subunits, among others (clusters D-F). For example, cluster-D contains almost all the ribosomal protein genes (*RPS* and *RPL* genes); cluster-E contains genes that are mainly involved in ribosome biogenesis, ribosome assembly, rRNA metabolism and modification. Examples of genes in this cluster are the *NOP* genes, which encode nucleolar proteins, involved in rRNA maturation, processing and modification (Reichow *et al.*, 2007). Several Cluster-F genes are involved in the mitochondrial translation and mitochondrial ribosome biogenesis and organization. Indeed, most of the genes in this cluster encode mitochondrial proteins. This global analysis of the repressed genes indicated that mistranslation had a strong negative impact on both cytoplasmic and mitochondrial protein synthesis, which was consistent with polysome profile alterations detected in mistranslating cells (see below; Figure 31).

Table 2. Significantly enriched functional classes in differentially expressed genes from Clusters D, E, F and T20h down-regulated genes. The enriched classes were obtained with the EXPANDER software tool called TANGO. (corrected p-value cutoff = 0.05)

Significantly enriched Functional Classes	Cluster D		Cluster E		Cluster F		20h	
	Frequency	p-value	Frequency	p-value	Frequency	p-value	Frequency	p-value
biosynthetic process					41.4%	0.001		
intracellular non-membrane-bound organelle			21.2%	0.023			23.0%	0.001
mitochondrial genome maintenance					3.7%	0.001		
mitochondrion					49.1%	0.001		
mitochondrion organization and biogenesis					7.3%	0.001		
regulation of translational fidelity	6.0%	0.001						
ribonucleoprotein complex	90.7%	0.001					13.3%	0.001
ribosome	88.1%	0.001			21.6%	0.001		
ribosome biogenesis and assembly			20.0%	0.001			21.0%	0.001
rRNA metabolic process			13.7%	0.001			12.2%	0.001
rRNA modification			2.4%	0.004			1.9%	0.001
translation	89.4%	0.001			26.7%	0.001		

In order to understand how mistranslation deregulated gene expression and to obtain an integrated view of the signalling networks involved in the response to mistranslation, I have searched for transcription factor binding motifs in the group of differentially expressed genes, using the YEASTRACT algorithm (Teixeira *et al.*, 2006;Monteiro *et al.*, 2008). The top 25 Transcription Factors (TFs) binding motives were scored using the set of genes whose expression was altered during the time series T0'-T180' and T20h and 22 of them were enriched in the time points analysed. In order to determine the significance of the enrichment in the TF binding motives, their genome distribution was included in the analysis. The percentage of differentially expressed genes that were regulated by the 22 TFs analyzed was always higher than that obtained for the entire set of genes (Figure 30). These 22 TFs regulate expression of genes belonging to 5 main functional categories, namely biosynthetic processes (Abf1, Fhl1, Rap1), cell cycle (Swi4), development (Sok2, Ste12, Tec1), ESR (Aft1, Arr1, Cad1, Hsf1, Msn2, Msn4, Pdr1, Pdr3, Rpn4, Yap1) and metabolism (Adr1, Gcn4, Ino4, Met4, Sfp1). If we considered all the TFs in each of these functional categories described by Lee (Lee *et al.*, 2002), we observed that the TFs that were most represented in this list of 22 TFs are related to Biosynthesis (37.5% of all TFs related to Biosynthesis), ESR (31,25% of all TFs related to ESR) and Development (30% of all TFs related to Development).

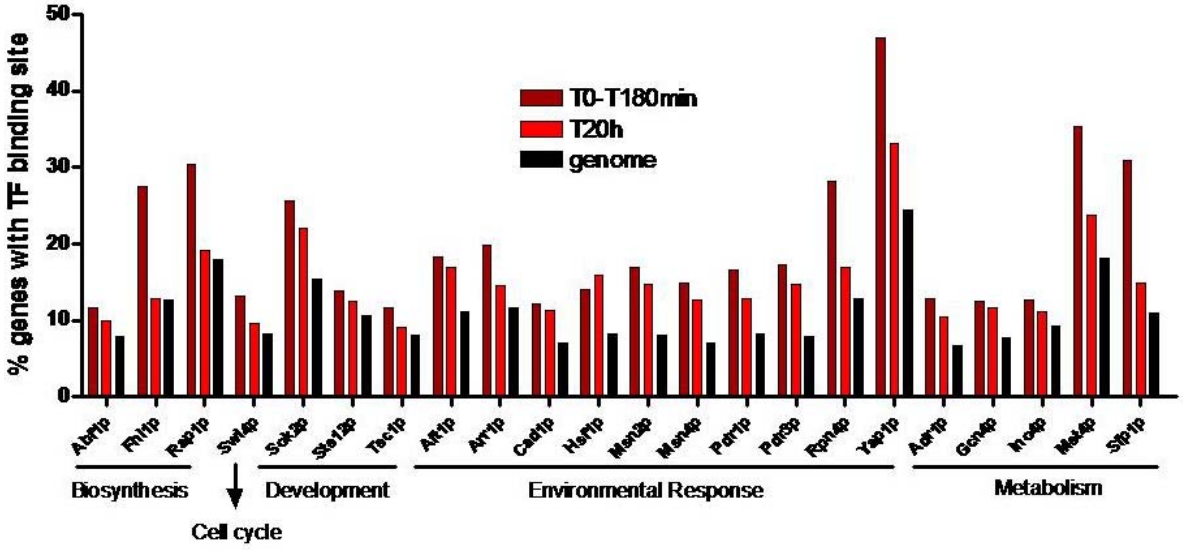


Figure 30. Transcription Factors (TFs) that regulate the cellular response to mistranslation. The 22 TFs shown had enriched binding sites in the set of differentially expressed genes

obtained for the T0'-T180' and T20h experiments. The third bar (black) of each TF corresponds to the yeast genome enrichment of each TF binding site. The functional class classification shown is based in the annotation described by Lee (Lee *et al.*, 2002).

This data was consistent with previous studies showing that mistranslation down-regulates cellular biosynthetic processes, namely protein synthesis and translation, and up-regulates expression of several stress responsive genes, namely protein folding systems, unfolded protein binding genes and genes involved in the response to oxidative stress (Lee *et al.*, 2006;Silva *et al.*, 2007;Kohanski *et al.*, 2008). Indeed, there was a clear enrichment of binding sites for the Yap1 transcription factor which is strongly activated by oxidative stress and also of proteasome subunit genes whose transcription is regulated, at least in part, by Rpn4 (Xie and Varshavsky, 2001). Genes regulated by the Rap1 transcription factor seem also to be highly enriched which is in agreement with the fact of Rap1 regulates expression of a large fraction of ribosomal protein genes and other genes related to translation and growth (Pina *et al.*, 2003).

2.5 Translatome alterations generated by mistranslation

The observation that low-level mistranslation induced the stress response and repressed ribosome processing and assembly prompted one to investigate whether the translatome – fraction of mRNAs that are effectively translated by the ribosome – was somehow affected. For this, the pool of mRNAs associated to polysomes at the time point T90' was analysed and compared to the pool of mRNAs at time point T0'. Polysomes were prepared from 4 independent cultures using sucrose gradients (15 to 50%) and mRNAs were extracted from purified polysomes, as described in methods.

Polysome profiles showed a sharp reduction in the heavier region of the gradient, as early as T40' (Figure 31A), indicating that fewer polysomes were present in the cell and engaged in mRNA translation. In other words, mistranslation induction

had a significant negative impact on translation efficiency, but, surprisingly, there was not a corresponding increase in free 80S, 40S and 60S ribosome subunits, suggesting that mistranslation somehow triggered ribosome degradation. In order to understand if this translational block was being regulated at the level of translation initiation, phosphorylation of the eIF2α factor was analysed by western blot. However, increased phosphorylation of this factor was not detected in samples prepared from cells that mistranslated over 90 minutes post mistranslation induction (Figure 31B). This suggests that mistranslation induces ribosome degradation, probably through ribophagy, and does not block translation initiation through decreased activity of the critical translation initiation factor eIF2α.

For translatome analysis, mRNAs extracted from the polysomes were reverse transcribed and labelled cDNAs were then hybridized onto DNA microarrays, as for the total mRNA profiling described above. A direct comparison of the log2 expression ratios (M values) between the polysomal and total mRNA fractions at mistranslation time T90' showed homodirectional variation between transcription and translation for most genes. In some cases, genes that appeared down-regulated in the total mRNA profile were up-regulated at the translatome level (Figure 32A).

The translatome analysis identified 142 genes with positive variation and 138 genes with negative variation. The former were mainly related to the general stress and oxidative stress responses, to the unfolded protein binding and carbohydrate metabolism, thus confirming that the up-regulated genes identified at the transcriptional level were being translated. A similar result was obtained for the genes with negative fold variation. As before, these genes were involved in ribosome assembly and translation. Finally, 88 genes had negative transcriptional and positive translation values, indicating that they were regulated at translational level by mistranslation. Most of these genes encode proteins whose functions are related to responses to toxin and chemical stimulus and also drug transport. Interestingly, a significant number of genes associated to transposition appeared

in this group and also formed a distinct cluster in the transcriptional profiling (Cluster-E).

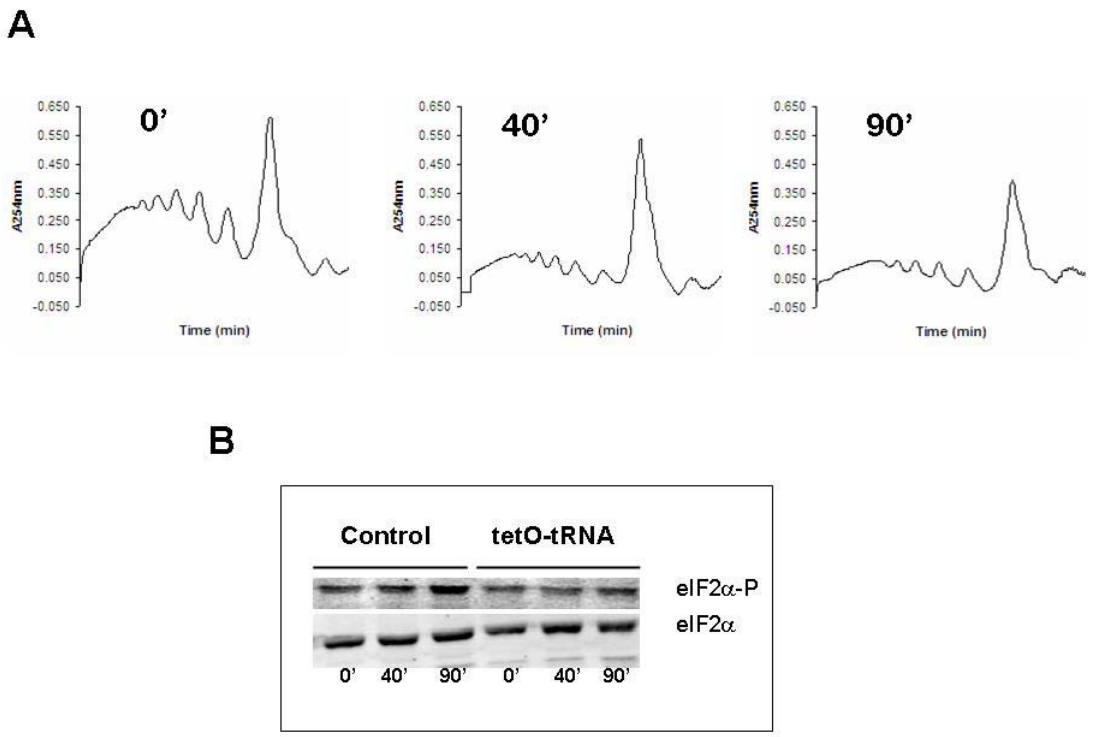
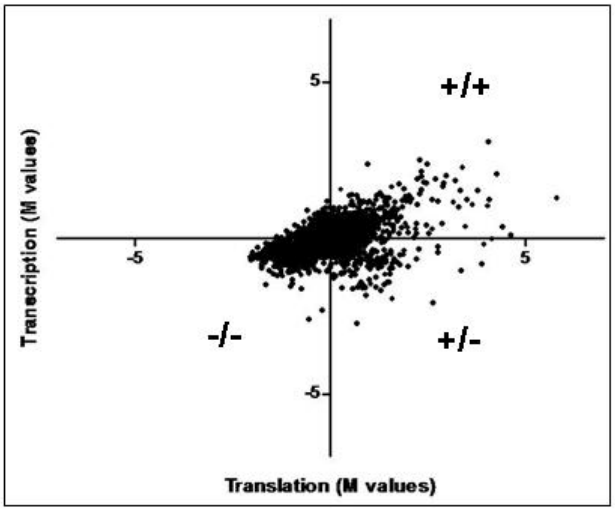


Figure 31. Polysomal profiles (A) and eIF2α phosphorylation analysis (B) of yeast cells at T0' and after mRNA mistranslation induction at T40' and T90'. The polysomal fraction had a sharp reduction after mistranslation induction, indicating that mistranslation had a significant negative impact on translation efficiency. However, increased levels of monosomes or free ribosomal subunits were not observed. Also, increased phosphorylation of the eIF2α factor was not detected in samples prepared from cells that mistranslated over 90 minutes.

Unidirectional changes between transcription and translation are associated to a gene expression phenomenon called potentiation (Preiss *et al.*, 2003;Smirnova *et al.*, 2005;Melamed *et al.*, 2008). Potentiation is not a general phenomenon, it only happens under yeast stress and involves specific groups of genes (Halbeisen and Gerber, 2009). Under cryptic mistranslation, the genes that displayed higher positive potentiation were related to the stress response, namely *HSP30*, *PST1*, *GRX2*, *CTT1*, *TRX2*, *ECM4*, *AHP1*, *ALD3*, *SIP18*, *YGP1*, *DDR2* and *OYE3*. Some of them were related to the response to oxidative stress and maintenance of the cell redox homeostasis, namely *GRX2*, *TRX2*, *AHP1* and *CTT1*. Interestingly,

genes that were poorly represented in the total mRNA profile, but had positive representation in the translatome profile (T90') were also involved in the stress response, namely *FLR1*, *GPX2*, *YDL218W*, *HAC1*, *AAD6*, *AAD10*, *YKL071W*, *SRX1*, *GTT2* and *ATR1* (Figure 32B).

A



B

+/+	poly 90'	90'	-/-	poly 90'	90'	+/-	poly 90'	90'
OYE3	55.8	2.4	RPS18A	-4.1	-1.5	FLR1	6.2	-4.2
ECM4	16.3	2.0	RPS3	-4.1	-1.5	AAD6	14.7	-1.8
HSP30	16.8	2.3	RPL27A	-4.0	-1.5	GTT2	10.3	-2.1
YMR090W	12.5	2.1	RPS9A	-4.1	-1.6	YLR162W	3.0	-3.8
GND2	9.8	2.0	RPS18B	-3.9	-1.6	HAC1	1.6	-6.7
SIP18	11.4	2.3	RPL6A	-3.7	-1.6	PDC6	2.5	-3.2
HSP31	13.8	2.9	RPP0	-3.7	-1.6	GPX2	2.9	-2.7
ALD3	19.1	4.1	RPS5	-3.5	-1.6	YIL168W	4.0	-1.9
YRO2	8.9	2.2	RPL8B	-3.4	-1.6	YCL042W	2.8	-2.7
FMP16	10.0	2.9	RPS13	-3.5	-1.6	YKL071W	4.6	-1.6
TRX2	6.8	2.3	RPL34B	-3.3	-1.5	ATR1	2.6	-2.8
PAI3	6.1	2.3	RPL18B	-3.4	-1.6	SRX1	2.3	-3.0
YGP1	9.2	3.6	RPS7A	-3.4	-1.6	YDL218W	1.9	-3.6
CTT1	7.4	3.2	RPL12B	-3.3	-1.5	AAD10	2.9	-2.2
GRX2	4.7	2.1	YNL300W	-3.3	-1.5	YGL114W	3.5	-1.8
YNL134C	7.5	3.4	RPL26B	-3.2	-1.5	YBL005W-A	2.7	-2.3
PST1	3.5	1.6	RPL13A	-3.0	-1.5	MCH4	3.0	-2.0
DDR2	10.4	4.8	RPL7A	-3.2	-1.6	COB	1.9	-3.1
AHP1	7.9	4.0	RPS22A	-3.2	-1.6	MOH1	2.9	-2.0
PIR3	3.1	1.6	RPL31B	-3.0	-1.5	YFL002W-B	2.1	-2.8

** fold-changes are represented*

Figure 32. Mistranslation induces alterations in total and polysomal mRNA profiles. (A) Graphical representation of the correlation between total mRNA and polysome associated mRNAs. The y-axis represents the M values of the ratio between the total mRNA transcript

fraction at times T90' and T0'. The x-axis represents the M values corresponding to the ratio between the polysome associated transcripts at times T90' and T0'. (B) Table showing the differentially expressed genes present in each of the quadrants of the graph represented in (A) which have higher variation of fold values between the total and the polysome associated mRNAs.

Therefore, with the results obtained with this and with previous experiments, it was possible to show that low-level mistranslation deregulates gene expression at both transcriptional and translational levels, suggesting that its impact on gene expression is stronger than one could anticipate.

2.6 Discussion

Yeast cells remodel part of their transcriptome in response to mRNA mistranslation. Approximately 15% of the yeast genes were differentially expressed 180 minutes after the induction mistranslation. Almost 60% of the genes of the core response to several environmental stressors (Gasch *et al.*, 2000) were identified as differentially expressed in short-term and long-term responses to mRNA mistranslation. This indicates that mRNA mistranslation is a stress situation and cells respond to it as if it were an environmental stress, using similar regulatory pathways. Induced genes were enriched for heat-shock and antioxidant functions, but also for carbohydrate metabolism and energy-generation functions. The repressed genes have growth-related functions, such as translation and ribosome biogenesis, revealing a change of resources from rapid proliferation to stress protection. The other differentially expressed genes in both short and long-term responses to mRNA mistranslation, but not included in the ESR gene list (Gasch *et al.*, 2000), were also enriched in those functional categories. This is in line with the balance between energy-efficient cellular growth and the ability to rapidly respond to environmental changes in most microorganisms (Regenberg *et al.*, 2006;Castrillo *et al.*, 2007;Levy *et al.*, 2007;Brauer *et al.*, 2008).

Several general stress and oxidative stress genes were immediately up-regulated upon induction of mistranslation (Figure 28; Table 1; Annexes 3 and 4), indicating that they are the first line of defense against the proteome disruption caused by mistranslation. Some of these genes respond rapidly to a series of environmental stressors. For example, the multistress response protein gene *DDR2* and the DNA-damage response protein gene *DDR48* are both up-regulated upon induction of mistranslation and respond to several xenobiotic agents, heat-shock and DNA damage chemicals (Treger and McEntee, 1990;Kobayashi *et al.*, 1996). Several chaperones genes were immediately up-regulated upon induction of mistranslation, namely *HSP70* (*SSA1*, *SSA2* and *SSA4*) and *HSP90* (*HSC82*). The main function of Hsp70 Ssa proteins is to bind newly translated proteins and to assist them in proper folding and prevent protein aggregation/misfolding. They are also involved in disassembling aggregates formed by misfolded proteins, translocating selected proteins into the mitochondria and ER, degrading aberrant proteins and regulating the expression of other heat-shock proteins (Hartl, 1996;Bukau and Horwich, 1998;Glover and Lindquist, 1998). Hsp90 family members are required for folding of a specific set of difficult-to-fold proteins as well as for the refolding of denatured proteins back into native conformations. Although most cellular proteins do not require Hsc82 chaperone activity for correct folding under normal conditions, it is required for the activation of many key cellular regulatory and signalling proteins, like kinases and transcription factors (Nathan *et al.*, 1997;Picard, 2002).

The major stress chaperone gene, *HSP104*, was also strongly up-regulated by mistranslation. Unlike most chaperones that prevent proteins from aggregating, Hsp104, in conjunction with the chaperone and co-chaperone Ssa1 and Ydj1 (Hsp40), helps to disassemble protein aggregates that have accumulated due to stress (Parsell *et al.*, 1994;Glover *et al.*, 1998). This indicates that an important consequence of mistranslation is probably the production of protein aggregates. Finally, mistranslation up-regulated the expression of the genes that encode the cytosolic members of the small heat-shock protein (sHSP) family of molecular chaperones (Figure 28; Table 1; Annexes 3 and 4). Hsp26 and Hsp42 protect

yeast against situations of high production of misfolded proteins, as they bind and prevent unfolded substrate proteins from irreversibly forming large protein aggregates (Susek and Lindquist, 1990;Bentley *et al.*, 1992;Haslbeck *et al.*, 2004). Hsp42 functions in both unstressed and stressed cells, while the Hsp26 activity is found only under stress conditions, which probably may explain the higher induction of *HSP26* upon induction of mistranslation.

Interestingly, chaperones present in other cellular compartments (beyond the cytoplasm) were also up-regulated, reinforcing the hypothesis that mistranslation has global effects in the cell. Hsp12 and Hsp30 are two heat-shock proteins localized in the plasma membrane (Seymour and Piper, 1999;Sales *et al.*, 2000) and their genes were strongly up-regulated upon induction of mistranslation. The same was true for Hsp78, the oligomeric mitochondrial matrix chaperone which prevents aggregation of misfolded proteins and re-solubilises protein aggregates (Rottgers *et al.*, 2002) and also for Kar2 (BiP), an Hsp70 chaperone family ATPase involved in protein import into the ER that mediates protein folding in the ER and regulates the unfolded protein response (UPR) via interaction with Ire1 protein (Nishikawa *et al.*, 2001;Kimata *et al.*, 2003).

Other interesting genes up-regulated by mistranslation were *RPN4*, which encodes the transcription factor that stimulates expression of proteasome genes (Xie *et al.*, 2001), *RAD52*, which encodes a protein involved in the repair of DNA double-strand breaks (Symington, 2002) and *TSL1* which encodes a subunit of the trehalose-6-phosphate synthase/phosphatase complex and with other players is involved in trehalose biosynthesis. In yeast, trehalose is a major reserve carbohydrate involved in responses to thermal, osmotic, oxidative and ethanol stresses and prevents aggregation of denatured proteins (Singer and Lindquist, 1998).

The group of oxidative stress responsive genes induced by mistranslation include genes that code for enzymes whose function is to defend cells against ROS production and redox imbalance, enzymes that act directly as ROS detoxifiers,

such as superoxide dismutases (*SOD1* and *SOD2*), catalases (*CTT1* and *CTA1*) and thioredoxin peroxidases (*TSA1*, *AHP1* and *PRX1)* and enzymes that act as redox regulators of protein thiols and contribute to maintain the redox balance of the cell, namely glutaredoxins (*GRX1* and *GRX2*) and thioredoxins (*TRX2*) (Herrero *et al.*, 2008).

An analysis of the transcription factor (TF) binding sites present in the genes up-regulated by mistranslation showed a strong enrichment of TFs associated to the stress response, thus providing strong support for our DNA microarray data. Enrichment of stress-responsive elements (STRE – cis regulatory element with the sequence AGGGGA/T) and of heat-shock responsive elements (HSE - nGAAn) showed that the Msn2/Msn4 and the Hsf1 transcription factors play a fundamental role in the cellular response to mRNA mistranslation, activating the chaperones *HSP26*, *HSP42*, *HSP70 SSA* genes, *HSP104* and others, but also genes encoding glycolytic enzymes, important to accumulate protective compounds such as glycogen and trehalose, and antioxidant enzymes (*CTT1*). In many genes, the STRE and HSE cis-elements appear in the promoter of the same gene, as is the case for *HSP104*, *HSP26* and *HSP42* genes (Amoros and Estruch, 2001;Grably *et al.*, 2002). This enrichment in STRE elements also indicates that this response to mistranslation is in part dependent on protein kinase A (PKA) activity and cAMP levels. Mistranslation seems to decrease cAMP levels and consequently PKA activity, allowing for the localization of Msn2 and Msn4 transcription factors in the nucleus and for subsequent activation of the STRE containing genes (Gorner *et al.*, 1998).

Beside those general and heat-shock responsive genes, yeast cells responded to mRNA mistranslation by activating genes involved in the response to redox state alterations caused by ROS production (Yap1 controlled), in the resistance to drugs and toxic compounds (Pdr1 and Pdr3 controlled) and in proteolysis (Rpn4 controlled). According to our results, Rpn4 activated genes were up-regulated late in response to mistranslation, suggesting that proteolysis is a second line of defense against proteome disruption caused by mistranslation. The *RPN4* gene

has YRE and PDRE cis-elements in its promoter, as well as STRE and HSE elements, suggesting that the expression of the *RPN4* gene is first activated by Msn2/Msn4, Hsf1, Yap1 and Pdr1/Pdr3 signalling pathways and only after this it activates the expression of other proteasome subunits encoding genes, namely *RPT* and other *RPN* genes (Owsianik *et al.*, 2002;Hahn *et al.*, 2006).

The high enrichment of Yap1 binding sites in the differentially expressed genes unveiled an important effect of mistranslation in the oxidative stress response and suggested that mistranslation may increase ROS production. Yap1-mediated pathways are activated by redox sensory mechanisms, which detect changes in the intracellular redox balance, caused by ROS and oxidized thiols. These sensory mechanisms involve redox-sensitive cysteine residues that rapidly sense and transduce the stress signals and activate regulatory proteins; the oxidant receptor Hyr1/Gpx3 protein is the main signal sensor (Delaunay *et al.*, 2002). The gene that encodes for this protein (*HYR1*) is up-regulated (up to 3-fold) by mistranslation (Figure 28).

The gene expression response showed homodirectionality between transcription and translation indicating that the latter potentiates the former. In other words, there is not major post-transcriptional control in gene expression in yeast beyond the potentiation effect observed (both for up- and down-regulated genes). This is consistent with previous studies which showed that translational control of gene expression is weak in yeast (McCarthy, 1998). The translational potentiation observed is also consistent with the translational response induced by environmental stress, namely heat-shock, rapamycin, amino acid starvation or osmotic stress (Preiss *et al.*, 2003;Smirnova *et al.*, 2005;Melamed *et al.*, 2008). However, it is different from the response observed against oxidative stress (H_2O_2 treatment) and butanol (Smirnova *et al.*, 2005;Shenton *et al.*, 2006) which showed a complex translational reprogramming, with great differences between transcriptional and translational levels for most of the yeast genes.

The reduction in the quantity of polysomes observed in mistranslating cells without increase in monomeric ribosome subunits was an interesting result which raises some questions for future studies. The reduction in polysomes, and the strong down-regulation of genes encoding translational factors and ribosome processing and assembly genes, is consistent with the hypothesis that mistranslation has a strong negative impact on the rate of protein synthesis; however increased phosphorylation of eIF2α was not observed. This apparently contradictory result suggests that mistranslation may activate autophagy and that ribosomes are degraded through the autophagy pathway (ribophagy) (Beau *et al.*, 2008); alternatively, mistranslation may induce the formation of P-bodies or stress granules which may sequester both mRNAs and ribosome subunits which would not have been detected in the polysome gradients (Buchan *et al.*, 2008) . This should be further investigated in future studies.

3. Mistranslation causes cell degeneration and death through oxidative stress

3.1 Overview

Recent studies associated mRNA mistranslation with human diseases (Lee *et al.*, 2006;Nangle *et al.*, 2006;Rochet, 2006;Antonellis and Green, 2008). Mistranslation generates mutant proteins that may misfold, aggregate and become toxic, a phenomenon implicated in various neurodegenerative diseases. For example, a mutation in the editing domain of the human valyl-tRNA synthetase that resulted in tRNA mischarging, activated apoptotic markers in cultured cells (like caspase-3) and caused disruption in cell morphology and membrane blebbing (Nangle *et al.*, 2006). In mice, a mutation in the editing site of alanyl-tRNA synthetase (*sticky* mutation) resulted in smaller animals with increased Purkinje cell loss and ataxia. The neurons of these mice accumulated misfolded proteins, up-regulated cytoplasmic molecular chaperones and induced the unfolded protein response (UPR) (Lee *et al.*, 2006). In *E. coli*, similar aaRS mutations had a strong negative impact on cell growth and viability (Nangle *et al.*, 2002;Bacher *et al.*, 2005). To certain extent, these phenotypes are somewhat surprising because the level of mistranslation caused by those mutations is probably low and one would expect that the proteome quality control systems would be able to deal with the aberrant proteins synthesized from such mRNA mistranslation, as it was already described for *E. coli* that survive and tolerate up to 10% of mismade protein (Ruan *et al.*, 2008). Low-level mistranslation should not lead to proteome collapse; therefore, one is left with the intriguing questions of how does mistranslation kill cells and how does it trigger disease development, namely neurodegeneration? It is worth noticing that induced mistranslation in yeast (see previous section) did not compromise growth rate, it only had an observable effect when post-mitotic cells were re-grown in fresh media (Results chapter, section 2; Figures 24 and 26). One possible explanation for this is that mistranslation increases significantly ROS production and accumulation of ROS may cause of cell death and degeneration through their deleterious effects in proteins, DNA, RNA, lipids etc. The strong up-

regulation of oxidative stress genes observed in the total and polysomes associated mRNA profiles support that hypothesis. In this chapter, I took advantage of our yeast model of inducible mistranslation to address the above questions. I had two main concerns in mind, firstly to validate yeast as a model system to study the molecular and cellular basis of the human diseases associated to mistranslation and secondly to obtain the first hints on how this important biological phenomenon causes cell death and degeneration.

3.2 Effects of mistranslation on cell viability

Induction of mistranslation with tetracycline in exponentially growing cells did not affect growth rate, however it did have a major impact on gene expression (see previous section). Also the capacity of post-mitotic cells to re-start growth in fresh media without inducer (expression of the misreading tRNA repressed) was affected severely. In order to understand and quantify this unanticipated phenotype, cell viability was determined at several time-points immediately after induction of mistranslation and compared with the time T0'. Exponentially growing cells were collected immediately before and at time T20', T40', T60', T90', T120', T180' post-induction of mistranslation with tetracycline. Cell viability at each time point was determined in relation to that of time T0' (100%) by replating cells in fresh media without inducer (mistranslation repressed) and counting the number of colony forming units (CFUs). A sharp decrease in the viability of cells after induction of mistranslation was observed: at time T20', 76% of the cells were viable; at T40', only 54%; at times T60' and T90', the percentage of viable cells were 32% and 20% respectively; and at times T120' and T180', only 16% of the cells could form new CFUs (Figure 33). Viability of control cells remained almost constant over the above time series.

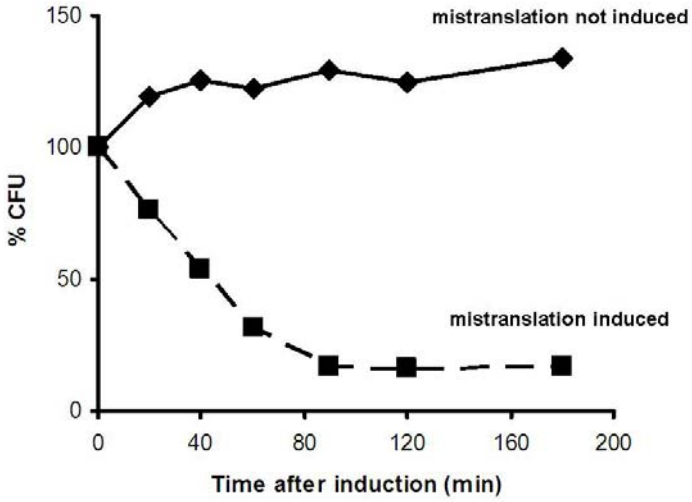


Figure 33. Mistranslation has a strong negative impact on cell viability. The graph shows the decrease in cell viability observed over 180 minutes after induction of mistranslation. The cell viability assay was carried out by plating 100 cells from the culture at each of the time points represented in MMgalactose agar media. The colony forming units (CFU) in each plate were counted after 7 days of incubation at 30ºC. The values plotted correspond to the percentage of colony forming units (% CFU) at each time point relative to the viability of T0' cells (100% viability). Each point is the mean-value for the 5 replicates that have been tested.

The reason for the sudden collapse of cell viability of post-mitotic cells is not clear, however it cannot be related to sudden proteome collapse because in exponential growth phase, when protein synthesis was most active, there was no defect in growth rate (Results chapter, section 2; Figures 24 and 26). Also, protein synthesis rate decreases sharply in stationary phase as only few proteins are synthesized (Werner-Washburne *et al.*, 1996). Furthermore, expression of molecular chaperones increases sharply as cells enter into stationary phase allowing them to cope with misfolded or aged proteins. In other words, lethal accumulation of aberrant proteins in stationary phase, which could lead to proteome collapse, is unlikely. Rather, that loss of viability should be linked to the physiology of the stationary and lag phases or to incapacity of mistranslating cells to re-started growth in fresh media. Interestingly, this is a common phenotype of environmental isolates which show a dramatic loss of viability when re-grown for the first time in the laboratory; a large proportion of environmental isolates are viable, but they are unable to re-start growth in fresh rich media. The reasons for

this phenotype are also unclear (Beuchat, 1992). Since mistranslation induced and up-regulated oxidative stress genes one wondered whether accumulation of ROS could explain the above-mentioned loss of cell viability.

3.3 Mistranslation induces oxidative stress and ROS accumulation

In order to clarify whether the cellular response to oxidative stress was directly correlated with variation in the redox state of the cell and to increased ROS levels, the latter were quantified by flow cytometry using the fluorescent dye dihydrorhodamine 123 (DHR 123). A sharp increase (22%) in ROS accumulation at T20' was observed. At T40' and T60', 47.2% and 61.5% of cells, respectively, produced higher ROS levels (Figure 34) indicating that mistranslation had a strong effect on the accumulation of ROS. This was in line with the strong up-regulation of the oxidative stress genes described previously. Plasma membrane integrity was also determined by examining cellular permeability to propidium iodide (PI) in cells at the same time points, but no decrease in membrane integrity was observed (data not shown).

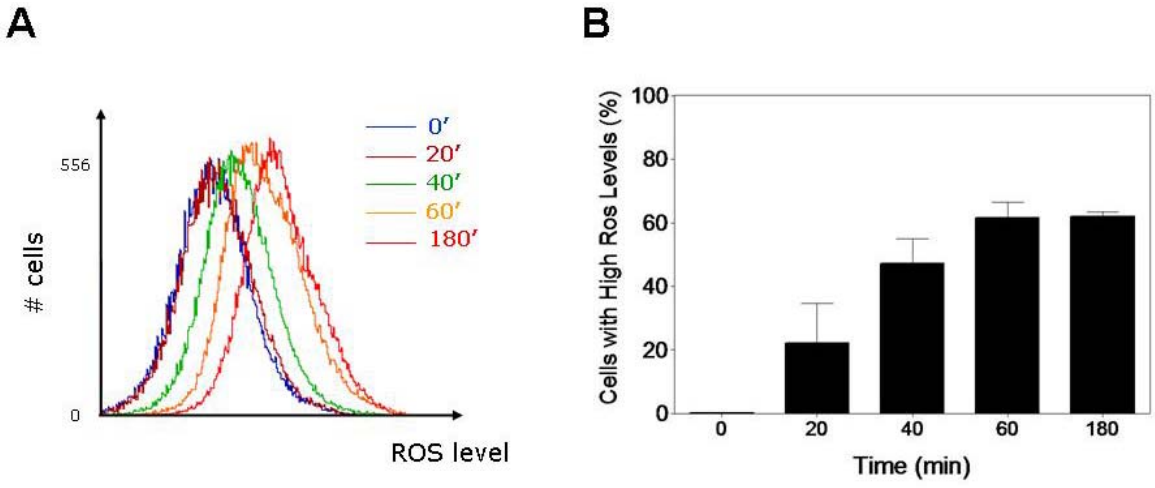


Figure 34. Mistranslation generates ROS. Intracellular ROS accumulation was monitored by flow cytometry of cells incubated with Dihydrorhodamine 123 (DHR123). (A) Overlay of green fluorescence histograms of tetO-tRNA cells analysed at T0', T20', T40', T60' and T180'. (B) Quantification of cells containing high ROS levels. This analysis was carried by harvesting and washing in PBS 10^7 cells at each time point. Cells were incubated in a

solution of 15μg/mL DHR123, during 90 minutes at 30°C in the dark, washed in PBS again and analysed by flow cytometry. Cells displaying higher values than a defined threshold of green fluorescence were considered as containing elevated intracellular ROS levels.

Since ROS and oxidative stress in general are linked to cell degeneration, I investigated whether ROS production was the main cause of cell viability loss observed in mistranslating cells. For this, the above cell viability experiment was repeated in presence of ROS scavengers, namely ascorbate, glutathione and carnitine. Remarkably, ascorbate and glutathione reverted the cell viability phenotype almost completely while carnitine improved the percentage of CFUs from 40.2 to 62.3% at time T60' and from 20.8 to 42.2% at time T180' (Figure 35). Therefore, oxidative stress was the main cause of the cell viability loss induced by mistranslation. This was consistent with the hypothesis that mistranslation does not cause proteome collapse. In other words, the direct effect of mistranslation on the proteome is rather minor when compared to the damage produced by ROS on proteins, lipids, nucleic acids and on other biological molecules.

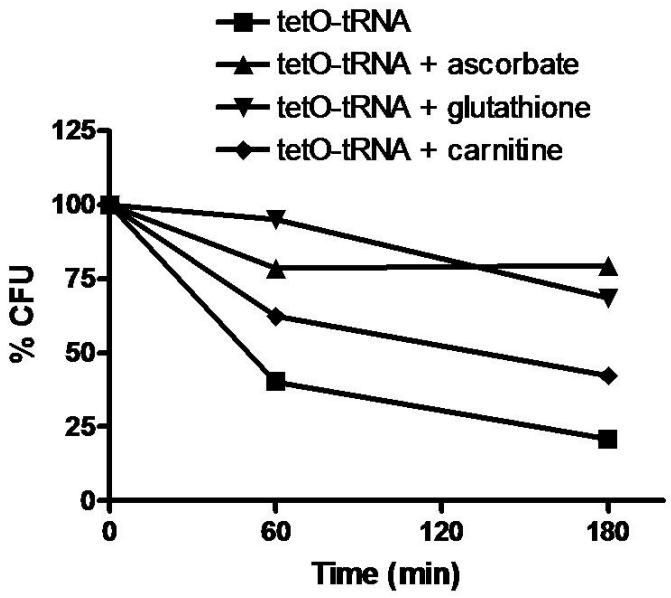


Figure 35. ROS scavengers reverted the viability loss phenotype generated by mistranslation. The graph shows the viability (% of CFUs) of mistranslating clones grown in presence of various ROS scavengers, namely ascorbate 80mM, glutathione 40mM and L-acetyl-

carnitine 100mg/mL. Cell viability was restored almost completely in presence of ascorbate and glutathione, suggesting that ROS accumulation is the main cause of cell death associated to mistranslation Each point in the graph is the mean-value of 4 replicates.

ROS accumulation in cells mistranslating constitutively was also quantified. In this case, only a small increase in ROS accumulation was observed (Figure 36) suggesting that cells were some how able to minimize the effect of mistranslation on ROS production. Interestingly, this explains why cells that mistranslate constitutively did not show a strong loss of viability (unpublished results). Furthermore, transcriptome analysis of constitutively mistranslating cells did not show up-regulation of oxidative stress genes, suggesting once more that ROS are not accumulated in these cells. This surprising difference between inducible (short term, cell not adapted) and constitutive mistranslation (long term, cells adapted) is difficult to explain on the basis of current models of the stress response. However, a recent study may explain this puzzling result. In mammalian cells, various environmental stressors generate ROS and cells respond to this oxidative stress by misacylating many different tRNAs with methionine (Met) (Netzer *et al.*, 2009). The MetRS looses its specificity in presence of ROS through yet unknown mechanisms and aminocylates various non-cognate tRNA species with Met. This apparently catastrophic genetic event is adaptive as Met is a ROS scavenger. In other words, cells increase the level of Met in their proteins through Met misincorporation at many non-cognate codons and “sacrifice” part of their proteome to mitigate ROS damaging activity (Netzer *et al.*, 2009). This raises the possibility that mistranslation activates the Met-misacylation pathway through ROS and that increased Met misincorporation into the proteome may be sufficient to inactivate the ROS generated during constitutive (long-term) mistranslation. If so, cells mistranslating constitutively are well adapted to the oxidative stress generated by mistranslation, explaining the lack of viability loss.

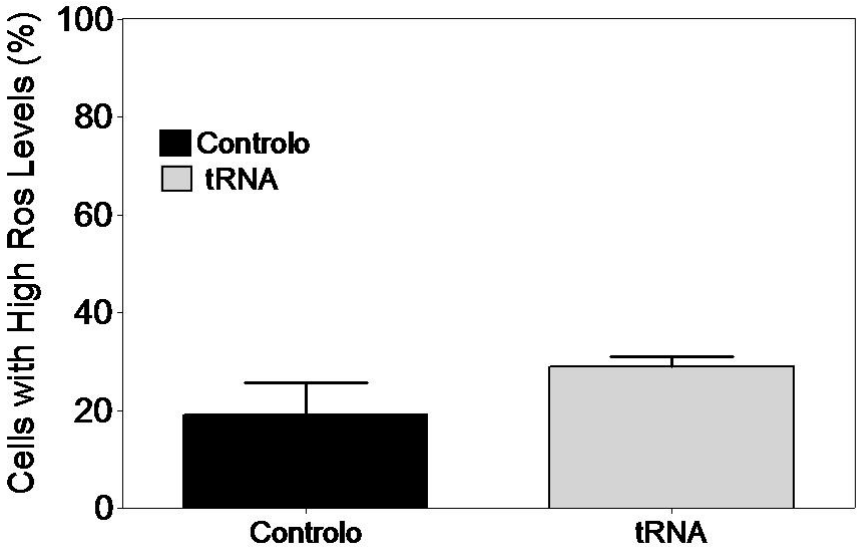


Figure 36. ROS accumulate poorly in cells that mistranslate constitutively. ROS were quantified by flow cytometry using the fluorophore Dihydrorhodamine 123.

The above data prompt the question of the origin of the ROS, that is, how does mistranslation generate ROS? Two different possible sources of ROS have been analysed, namely mitochondrial dysfunction and the unfolded protein response. The results are described below.

3.4 Mitochondrial dysfunction caused by mistranslation

ROS are usually produced by the mitochondrial respiratory chain, by the microsomal/ER metabolism and by phagocytosis (Temple *et al.*, 2005). In order to elucidate whether mistranslation interfered with mitochondrial function the respiratory competence of cells was analyzed. *S. cerevisiae* can survive on fermentative carbon sources and produces energy through glycolysis, that is, in the absence of mitochondrial respiration. As long as glycolysis occurs, respiration incompetent strains - petite mutants lacking mitochondria - remain viable. Yeast can also produce energy through respiration only if grown in non-fermentable carbon sources such as glycerol, lactate or ethanol.

The engineered tetO-tRNA cells grown in galactose media did not express the $tRNA_{CAG}^{Ser}$ due to transcriptional repression by the tetR. In this media, mistranslation was dependent on the addition of tetracycline to the yeast culture or, alternatively, by changing the carbon source of the medium to glucose. This flexible expression system was explored in order to test yeast respiration under mistranslation conditions. For this, tetO-tRNA and control cells growing in galactose were re-plated in fresh galactose, glucose and glycerol media plates and the colony forming units (CFUs) were then counted (Figure 37). Control cells (no mistranslation) showed approximately 100% viability in all media tested, a value that was also observed for the tetO-tRNA cells in non-inducible galactose medium. However, the latter mistranslating cells showed a sharp decrease of viability in inducible glucose and glycerol media. The viability in glucose (fermentative medium) was reduced from 100% to 32% and in glycerol was 8% only, suggesting that mistranslation did affect mitochondrial function. Another manifestation of mitochondrial dysfunction induced by mistranslation was the appearance of white/pink colonies on media that supported mistranslation, namely glucose or galactose+tetracycline media (Figure 38).

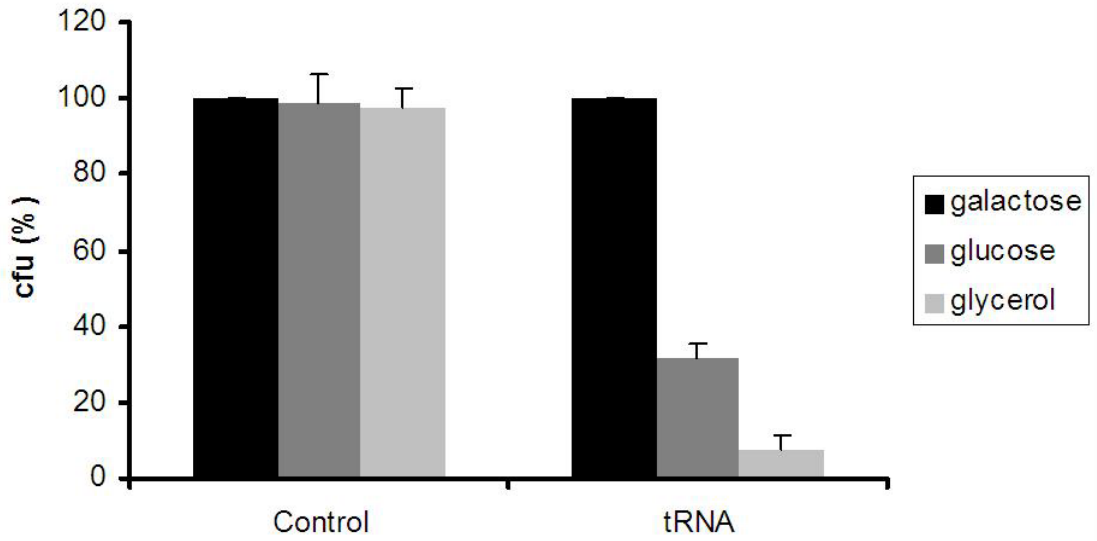


Figure 37. Mistranslation has a stronger negative effect on the viability of cells that produce energy through respiration. Percentage of colony forming units (CFUs) of control and tetO-tRNA cells plated in media containing different carbon sources. Viability in non-inducible galactose media was 100%. Yeast cells produce energy through glycolysis in glucose containing media and through respiration in media containing glycerol. Mistranslation had

a bigger impact on non-fermentable medium, suggesting that mitochondrial function was some how impaired in mistranslating cells.

The yeast strain used in this study was *ade2-1* and formed red colonies on agar plates due to the accumulation of the adenine synthesis intermediate phosphorybosylaminoimidazole (AIR) (Chaudhuri *et al.*, 1997). However, when these *ade2* mutant yeast cells loose mitochondrial function, they form white colonies (Reaume and Tatum, 1949;Jones and Fangman, 1992;Chatterjee and Singh, 2001;Singh *et al.*, 2001). Several studies have already used this colony colour alteration in order to detect the vulnerability of mitochondria to several stress agents and toxic compounds and it has somehow become a method to detect generalized mitochondrial dysfunction and mutation of the mitochondrial genome (Kim *et al.*, 2002). The colony phenotypes obtained with mistranslating cells (Figure 38) suggest that mistranslation causes mitochondrial dysfunction in yeast and probably some mutations in the mitochondrial DNA.

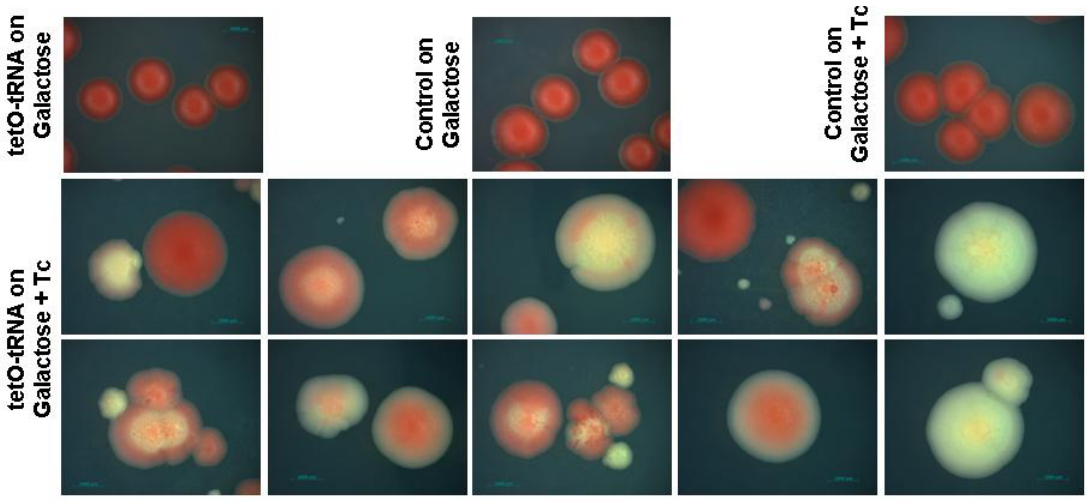


Figure 38. Mitochondrial dysfunction caused by mistranslation could be visualized by the alteration in colony colour from red to white. The yeast strain used in these experiments (BMA64/W303) produced red colonies due to accumulation of a red pigment (top panels), however induction of mistranslation with tetracycline (bottom panels) altered the colour of the colonies from red to pink or white, and several colonies showed white or pink sectoring. This colour alteration was due to mitochondrial dysfunction thus showing that mistranslation has a strong impact in that organelle. The colony colour heterogeneity

shown in the bottom panels indicated that the dysfunction was not homogeneous in the cell population within each colony and between cells of different colonies. This suggested that some cells coped better with mistranslation than others and that the effect of mistranslation was not the same for all the cells in the population.

Alternatively, the white colonies could also have resulted from read-through of the UAA stop codon present in the *ade2-1* gene of these stains. This hypothesis was tested using a stop codon read-through assay based on growth of these cells in media lacking adenine, but such putative nonsense suppression of the *ade2-1* mutation was not detected; none of the mistranslation clones were able to grow in minimal medium lacking adenine (data not shown).

3.5 Mistranslation induces the Unfolded Protein Response

The Unfolded Protein Response (UPR) is activated by accumulation of unfolded/misfolding proteins in the endoplasmic reticulum (ER) and up-regulates several ER-resident molecular chaperones and components of the protein degradation machinery (Spear *et al.*, 2001). Its activation may also generate ROS (Malhotra and Kaufman, 2007).

In yeast, the *KAR2* gene encodes an ATPase with chaperone activity and regulates the UPR by interacting with the Ire1 protein (Okamura *et al.*, 2000;Kimata *et al.*, 2003). This gene was 1.8 fold up-regulated at time T90', 2.3 fold at time T120' and 2.6 fold at time T180' (Figure 39). Also, genes encoding the thiol oxidase Ero1 protein and the multifunctional protein disulfide isomerase Pdi1 protein, which are involved in disulfide bond formation and in the unscrambling of non-native disulfide bonds (*ERO1* and *PDI1*), were up-regulated at times T120'-T180', 1.7-4.6 and 1.8-2.4 fold, respectively (Figure 39).

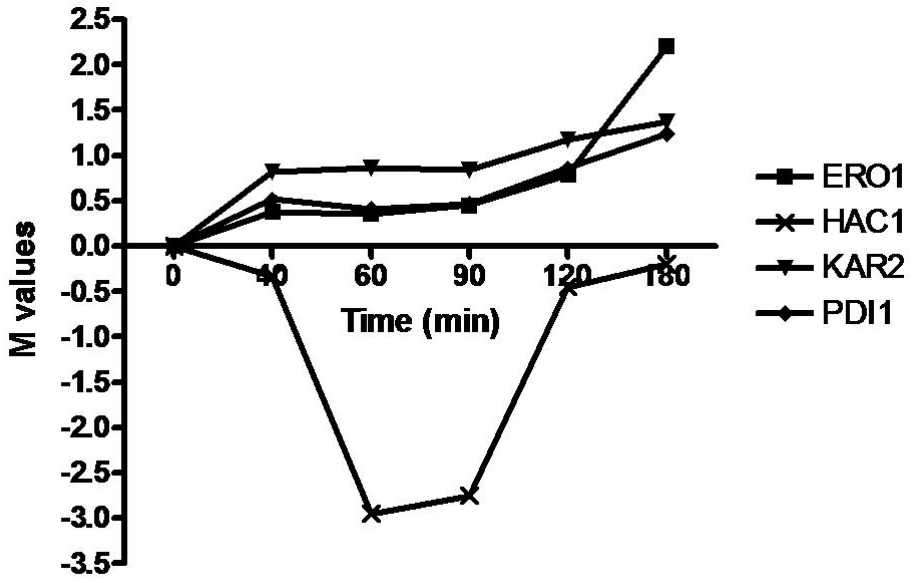


Figure 39. Mistranslation up-regulated the expression of ER genes. Expression profile over the first 180 minutes of mistranslation of several ER stress/UPR related genes showed strong up-regulation. The exception was the transcriptional regulator HAC1 whose expression decreased initially. These genes were differentially expressed according to the DNA microarray analysis described in section 2 of the Results chapter.

Interestingly, the transcription factor Hac1, which regulates expression of the UPR genes through a UPR enhancer (UPRE), was up-regulated 1.6 fold at the translatome level (time T90'), but was down-regulated 6.7 fold in the total mRNA profile. This post-transcriptional regulation of Hac1 expression was consistent with post-transcriptional processing and activation of the *HAC1* mRNA. The *HAC1* gene contains a 252bp intron whose retention in the *HAC1* mRNA renders the latter untranslatable ($HAC1^u$). Splicing of this intron allows for translation of the *HAC1* mRNA (translatable $HAC1^i$) and subsequent activation of the UPR via transcription of ER genes (Spear *et al.*, 2001).

Activation of splicing of the *HAC1* mRNA after induction of mistranslation was verified using RT-PCR. As expected, both the spliced ($HAC1^i$) and unspliced ($HAC1^u$) forms of *HAC1* mRNA were detected at induction time T0', however there was a sharp increase of $HAC1^i$ at time T180', indicating that the UPR was activated by mistranslation (Figure 40).

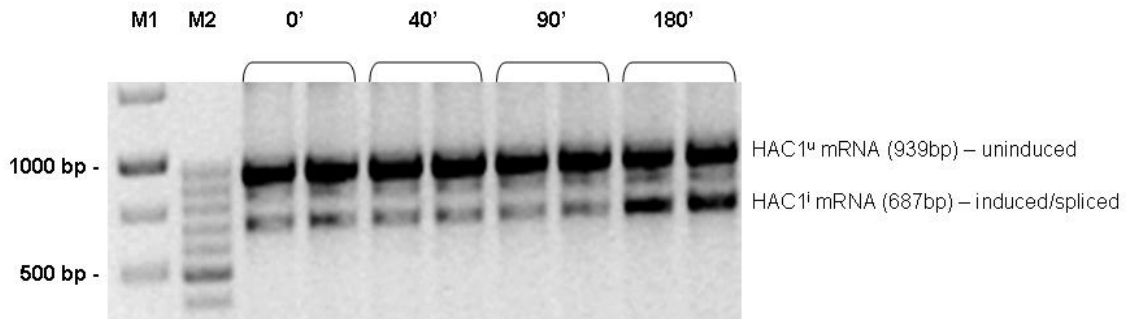


Figure 40. Mistranslation activates the UPR. RT-PCR of *HAC1* mRNA using specific primers to detect the presence (*HAC1*^{u}) or absence (*HAC1*^{i}) of the 252-bp intron in the *HAC1* primary transcript. The DNA fragments were fractionated by electrophoresis on 1% agarose gels.

This result was consistent with increased transcription of UPR genes containing UPREs. The delay of 180 minutes in the activation of the UPR contrasted with the early detection of mistranslation using the β-gal reporter (T40') and suggested that steady state activity of cytosolic proteome quality control systems is sufficiently robust to cope with initial mistranslation. However, above a certain mistranslation threshold those quality control systems become overloaded and proteome quality maintenance requires increased induction of other mechanisms. Alternatively, the UPR response was only activated upon ER overloading with misfolded/aggregated proteins, which in the case of induced mistranslation only happens at time T180'. Activation of the UPR indicates that ROS are also produced in the ER.

3.6 The role of mistranslation in protein oxidation

Error-prone ribosomes increase protein oxidation in *E. coli* (Ballesteros *et al.*, 2001) and treatment of cells with aminoglycosidic antibiotics which cause mistranslation also results in increased levels of protein carbonylation/oxidation (Dukan *et al.*, 2000). Oxidation can impair protein function because oxidized proteins unfold and exposure of their hydrophobic core often results in the formation of toxic aggregates.

Protein carbonylation was analysed in total protein extracts of yeast cells transformed with the pUKC707 (Control) and pUKC702 (tRNA) plasmids and also in extracts of cells expressing the $tRNA_{CAG}^{Ser}$. Carbonyl derivatives can be formed by direct metal catalyzed oxidative attack on the amino-acid side chains of proline, arginine, lysine and threonine. Carbonyl derivatives of lysine, cysteine and histidine can be formed by secondary reactions with reactive carbonyl compounds of carbohydrates (glycoxidation products), lipids and advanced glycation/lipoxidation end products. Different methods have been developed for the detection and quantification of protein carbonyl groups and most of these involve derivatization of the carbonyl group with 2,4-dinitrophenol hydrazine (DNPH) and subsequent immunodetection of the resulting hydrazone, using monoclonal or polyclonal antibodies (Levine, 2002). This method was used for the detection of carbonyl groups in mistranslating yeast, but there was no significant difference between mistranslating and non-mistranslating cells (Figure 41).

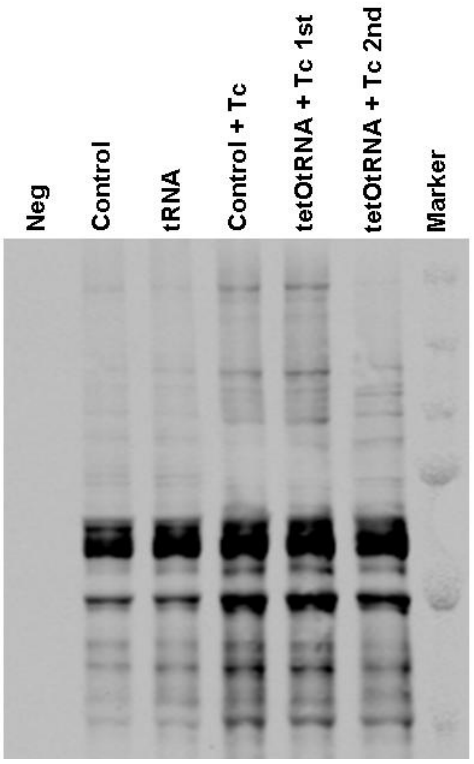


Figure 41. Mistranslation does not induce protein oxidation in yeast. Western blot showing the detection of carbonylated proteins in mistranslating cells. DNPH treated protein extracts were separated in a polyacrylamide gel and blotted onto a nitrocellulose membrane. DNP residues were detected using an anti-DNP antibody. Constitutive and inducible systems of mistranslation were analysed.

In order to confirm or reject the above result (Figure 41) an additional test was carried out. Carbonylated proteins associate with the molecular chaperone Hsp104 forming Hsp104-protein aggregates (Erjavec *et al.*, 2007). Yeast cells expressing an *HSP104-GFP* fusion reporter were transformed with the pUKC707 and pUKC702 ($tRNA_{CAG}^{Ser}$) plasmids and the resulting clones were analyzed by epifluorescence microscopy in order to detect increased *HSP104-GFP* expression (Erjavec *et al.*, 2007). Some mistranslating cells did show a strong increase in GFP fluorescence. However, a significant number of cells did not show increased fluorescence (Figure 42). In total, more 20 to 25% of the mistranslating cells formed protein aggregates than control cells. This suggested that oxidized proteins were only present in some cells of the mistranslating population. It also suggested that a technical rather than a biological problem might have affected the detection of the carbonylated proteins by western blotting. For example, the aggregates may have precipitated during protein extraction and may have been discarded with the cell debris. Lack of time prevents me from confirming these data. In any case, the accumulation of oxidized protein aggregates may explain the loss of cell viability described previously.

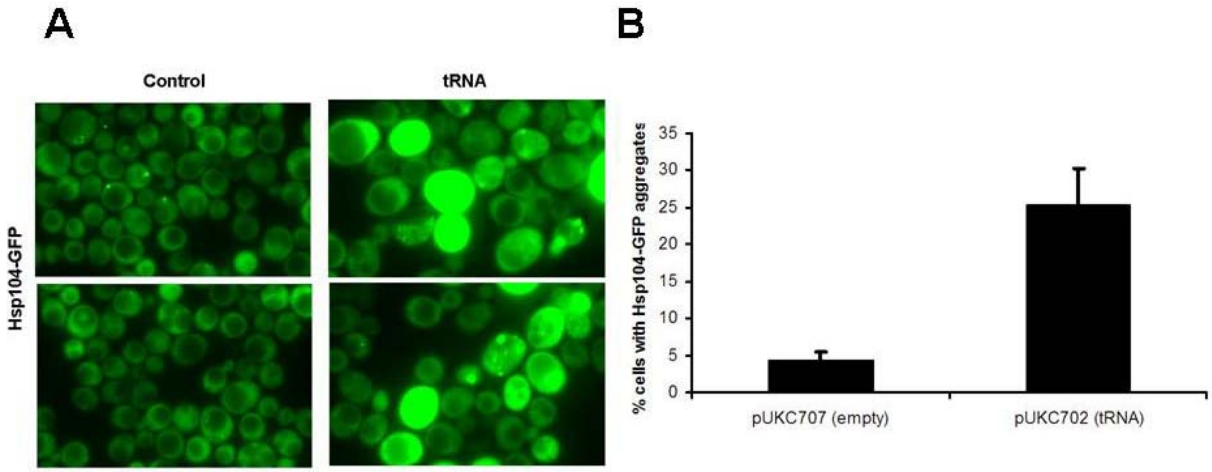


Figure 42. Mistranslation generates protein aggregation in yeast cells. (A) Hsp104-associated aggregates in mistranslating cells were monitored using epifluorescence microscopy of an Hsp104-GFP fusion. (B) Quantification of cells with Hsp104-GFP associated aggregates; 500 cells of control and mistranslating clones were analysed in 5 different images taken with a digital black-and-white camera (Zeiss) coupled to the epifluorescence microscope.

3.7 Discussion

The above data suggest that alteration of redox homeostasis, loss of cell viability, mitochondrial dysfunction and the activation of the unfolded protein response pathway (UPR) are the main causes of cell degeneration induced by mistranslation. It is generally accepted that conditions that disrupt protein folding have a negative impact on cell viability and accumulating evidence suggests that protein misfolding and production of ROS are closely linked events. Recent studies aimed at understanding the interconnection between these 2 mechanisms, specifically in the context of human diseases, showed that oxidative stress and protein misfolding play critical roles in the pathogenesis of neurodegenerative diseases, namely Alzheimer or Parkinson diseases and also in diabetes and atherosclerosis (Malhotra *et al.*, 2007).

In yeast, there is a range of responses to reactive oxygen species (ROS) which are dose-dependent (Temple *et al.*, 2005). At very low doses of ROS, cells adapt to become tolerant to subsequent lethal exposure of ROS. At high ROS doses cells activate various antioxidant functions, including a program of gene expression mediated mainly by the Yap1 and Msn2/Msn4 transcription factors and delay the cell-division cycle. At extreme ROS doses, a significant proportion of cells die initially by apoptosis and then by necrosis. The transcriptome and translatome profiling showed that the cellular response to mistranslation is mediated in part by the Yap1 and Msn2/Msn4 transcription factors which regulate the expression of oxidative and general stress genes (Estruch, 2000;Temple *et al.*, 2005). The results shown in this chapter confirmed the DNA microarray data by showing that mistranslation increased production and accumulation of ROS. The reversion of the cell viability phenotype by ROS scavengers added further support to our hypothesis that ROS are the main cause of cell degeneration associated to mistranslation. These data confirmed our suspicion that low-level mistranslation does not cause cell death through proteome collapse. This is in line with data from previous studies on the environmental stress response, which showed that environmental stressors increase the production of ROS. For example, NaCl,

acetic acid, hypochlorite and glucose in the absence of other nutrients all activate the stress response and increase ROS production and accumulation (Temple *et al.*, 2005). Since most environmental stressors induce protein unfolding, mistranslation mimics to certain extent their deleterious effects because it induces the synthesis of aberrant proteins that also misfold. Therefore, it is not too surprising that the transcriptional factors that regulate the ESR are also involved in the cellular response to mistranslation and, as in the case of the ESR, most of the cell damage is also ROS mediated (Gasch *et al.*, 2000;Causton *et al.*, 2001).

The origin of the ROS generated by mistranslation is also interesting. In yeast, the primary sources of ROS are the mitochondrion and the ER or a combination of both. Mitochondria are the main generators of ROS under physiological conditions. Damage of mitochondrial macromolecules may lead to increased ROS production which further damage mitochondrial components thereby causing a “vicious cycle” of ROS production and damage (Cadenas and Davies, 2000). Mistranslation caused mitochondrial dysfunction in yeast raising the possibility that the high ROS production observed in mistranslating cells is mainly due to malfunction of this essential organelle. On the other hand, 25% of the ROS generated in a cell may result from oxidative protein folding in the ER, where the Ero1 and Pdi1 proteins play an important role in ROS generation (Malhotra *et al.*, 2007). Appropriate disulphide bond formation is essential for proteins to achieve their native conformation and this leads to transfer of two electrons to Pdi1/Ero1. Under aerobic conditions oxygen acts as the terminal electron acceptor of the disulphide bond formation in the ER and formation of ROS has been proposed as a deleterious consequence of oxidative protein folding in the ER (Tu and Weissman, 2004). Therefore, the level of ROS in mistranslating yeast cells also reflects the flux of electrons from reduced sulfhydryls to Pdi1/Ero1 enzymes suggesting that increased oxidative protein folding is occurring in the ER during mistranslation conditions. In yeast, severe stress caused by over-accumulation of misfolded proteins in the ER induces the UPR and elevates ROS production by the protein disulphide-bond machinery and by the mitochondria (Haynes *et al.*, 2004).

Therefore, this is consistent with our hypothesis that in mistranslating yeast cells part of the ROS originates from the ER.

The UPR pathway plays an important role in the stress response since it allows for refolding and degradation of misfolded proteins that accumulate in the ER. This pathway is activated in yeast in response to several environmental stressors and in mammalian cells in response to unfolded proteins in the ER, produced by translational attenuation or apoptosis (Kaufman, 1999;Kaufman, 2004;Wu and Kaufman, 2006). Also interesting is that previous studies have shown that problems affecting the UPR pathway in yeast can lead to autodiploidization events and increased cell size in yeast (Lee *et al.*, 2003), which may also explain the genomic alterations and morphologic variations observed in mistranslating yeast (see Results chapter; section 1).

Since protein folding or refolding in the ER lumen and in the cytosol and protein degradation are energetically demanding processes, mistranslation may stimulate mitochondrial oxidative phosphorylation to increase ATP production, and consequently ROS production may increase as an indirect consequence of the need to increase ATP output. Finally, accumulation of protein aggregates in the cell could stimulate ROS production in metal-dependent reactions, as is the case in some neurodegenerative diseases (Allsop *et al.*, 2008). Whatever the ROS origins, the present study opened up an important new door to understand how mistranslation causes cell degeneration, and eventually human diseases, by establishing a strong link with oxidative stress. The ability of ROS scavengers to revert the cell viability phenotype leaves no doubt that ROS are the main cause of cell degeneration caused by mistranslation. This is in agreement with published data showing that antioxidant therapy is effective in reducing the activation of the UPR, oxidative stress and apoptosis in diseases caused by protein misfolding (Malhotra *et al.*, 2008).

ROS have pleiotropic effects as they damage a wide range of biomolecules, including nucleic acids, proteins, lipids and metabolites. This complicates the

identification of a single molecular process that could explain the cell viability loss. Most likely the viability phenotype is the result of the cumulative effect of global ROS damage and there is not a single molecular process involved. In any case, it will be of paramount importance to clarify whether mistranslation induces protein oxidation and aggregation. Indeed, aging and several diseases, namely Parkinson and Alzheimer diseases, some types of cancer, cataractogenesis, diabetes and sepsis are associated with increased protein oxidation and it will be interesting if mistranslation shares common molecular features with these diseases (Levine, 2002;le-Donne *et al.*, 2003). Also the treatment of *E. coli* cells with aminoglycosidic antibiotics which cause mistranslation and mutations that increase protein synthesis errors both increase protein oxidation (Dukan *et al.*, 2000;Ballesteros *et al.*, 2001). Our results suggest that generalized proteome oxidation/carbonylation does not occur in mistranslating cells, however, increased formation of Hsp104-containing protein aggregates was observed which may contain oxidized proteins (Erjavec *et al.*, 2007). It was not clear why only a subpopulation of cells showed increased formation of Hsp104-protein aggregates. This should also be clarified in future studies.

D. General Discussion

1. General Discussion

1.1 Consequences of mRNA mistranslation

Proteins conformation and folding are essential to life and errors in their synthesis can have catastrophic consequences. Surprisingly, those errors could be beneficial in specific situations. This thesis focused on the role of mRNA mistranslation in cellular degeneration. One of the objectives was to validate yeast as a model system to study the molecular and cellular basis of the human diseases associated to mistranslation. The data leave no doubt that yeast can be used to understand the basic biology of mistranslation diseases, it also confirmed our hypothesis that mistranslation does not cause diseases by inducing proteome collapse, rather it overloads the proteome quality control systems, increases the cellular energetic burden and generates oxidative stress.

That mistranslation is associated to human diseases and to degenerative phenotypes, from bacteria to humans, has been shown by many different studies (Nangle *et al.*, 2002;Bacher *et al.*, 2005;Lee *et al.*, 2006;Nangle *et al.*, 2006;Schimmel, 2008a). The selective advantages generated by mistranslation in microorganisms, where cell death may not be critical, have also been unequivocally demonstrated by various studies (Pezo *et al.*, 2004;Bacher *et al.*, 2007) and the role of mistranslation on protein evolution, on the evolution of genetic code alterations and on the evolution of the genetic code structure have also been thoroughly studied (Schultz *et al.*, 1996;Drummond and Wilke, 2008). Mistranslation also has a major impact on sexual reproduction and, for this reason, may have been an important speciation mechanism, in particular during the early stages of the evolution of life (Woese, 2002). Surprisingly, mistranslation creates selective advantages under stress, most likely due to stress cross protection, indicating that under certain conditions it has the potential to create phenotypes of high adaptation potential. It will be interesting to demonstrate whether it accelerates drug resistance in bacteria, fungi and human cells through both translational stress-induced mutagenesis (Balashov *et al.*, 2002;Al Mamun *et al.*,

2002) or by up-regulating drug efflux pumps, whose expression in yeast is under the control of the Pdr1/Pdr3 transcription factors (Jungwirth and Kuchler, 2006). Taken together, these studies show that the mistranslation phenomenon may be far more important than one would anticipated and one should now develop methodologies to detect it *in vivo* in different cell types under different physiological and pathological conditions in order to evaluate its full biological relevance.

The surprising finding that mistranslation generates phenotypic diversity in both *S. cerevisiae* and *C. albicans* is of paramount evolutionary importance (Miranda *et al.*, 2007;Silva *et al.*, 2007;Gomes *et al.*, 2007). Phenotypic heterogeneity provides a dynamic source of diversity in addition to the diversity derived from genotypic changes, such as genome rearrangements and mutation. Microbial populations benefit from variant subpopulations because these are better equipped to tolerate and adapt to environmental perturbation and allow for the exploration of new ecological niches (Booth, 2002;Sumner and Avery, 2002). In this context, the major problem of “beneficial” mistranslation is the inheritance and fixation of the advantageous phenotypes because mistranslation is stochastic and works at the proteome rather than at the genome level. Advantageous phenotypes can however be selected through positive selection processes, thus providing a mechanism to maintain the molecular source of mistranslation and allowing time for its fixation at the genome level. In this context, mistranslation may speed up genome evolution and TSM generated by mistranslation may provide the molecular mechanism for rapid fixation of the advantageous phenotypes in the population. Also, the genome instability observed in mistranslating yeast cells, which may involve chromosome rearrangements, duplications/deletions of chromosome segments and shifts in ploidy, are also an important mechanism to speed up the fixation of adaptive phenotypes (Riehle *et al.*, 2001;Dunham *et al.*, 2002;Gerstein *et al.*, 2006). Changes in genome structure typically have immediate phenotypic and fitness effects. Changes in ploidy can also result in patho-physiological events and are caused by erroneous cell division or other

mechanisms. Chromosomal instability often leads to aneuploidies (Storchova *et al.*, 2004;Storchova *et al.*, 2008).

The above-mentioned consequences of mistranslation suggest that it should have a strong negative impact on fitness. However, inducible mistranslation did not have an immediate impact on growth rate, rather mistranslating cells grew as fast as control cells and entered in stationary phase without visible alterations in culture density (Results chapter, section 2; Figure 24). Yet, strong alterations on β-galactosidase stability and on total and polysome-associated mRNA profiles were observed (see Results chapter, section 2; Figures 25, 28 and 32), indicating that "hidden" mistranslation had important cellular consequences. Decreased expression of housekeeping and protein synthesis genes and enhanced expression of genes encoding stress proteins, protein degradation processes, repair and detoxification mechanisms, in metabolism and osmolyte production, were detected. These cellular consequences of mistranslation were better understood when the total mRNA profile (transcriptome) was compared with the polysomal mRNA profile (translatome). Most mRNAs changed homodirectionally, indicating a strong coordinated response at both transcriptional and translational levels (Results chapter, section 2; Figure 32). Translational potentiation of gene expression is normally observed under severe stress, while mild stress normally produces a non-correlated response with preferential changes in the translatome profile (Halbeisen *et al.*, 2009). This suggests that cells sense "hidden" mistranslation as a severe stress situation, which is somewhat puzzling because growth rate was not affected. Therefore, it will be interesting to measure simultaneously the alterations in transcriptome, translatome and proteome under mistranslation conditions in order to clarify that apparent paradox.

1.2 The role of protein quality control mechanisms in mistranslation

The gene expression response, both transcriptional and translational, to mRNA mistranslation showed a clear up-regulation of components of the proteome quality

control systems. For example, expression of molecular chaperones, carbohydrate metabolism, proteasome subunits, oxidative stress and UPR genes was up-regulated (Results chapter, section 2; Figure 28 and Table 1). Expression of cytosolic chaperones such as Hsp70 (*SSA* genes), Hsp90 (*HSC82*), Hsp104, Hsp26 and Hsp42 was up-regulated immediately after induction of mistranslation, suggesting that they are the first line of defense against mistranslated proteins (Craig *et al.*, 1994). Hsp70 Ssa proteins stabilize hydrophobic regions of extended polypeptide segments in an ATP dependent manner, Hsp90 is required for the correct folding of a specific set of difficult-to-fold proteins and Hsp104 is crucial for survival of stationary-phase yeast cells and for tolerance to several stresses. Hsp104 alone does not prevent the aggregation of denatured proteins, but in concert with other chaperones can reactivate denatured proteins that have aggregated (Glover *et al.*, 1998). Interestingly, genes encoding the Ssb Hsp70 ribosome-associated chaperones and the mitochondrial Ssc Hsp70 were down-regulated by mistranslation, showing similar expression profile to the other ribosomal and mitochondrial genes. Hsp90 is also implicated in a buffering effect known as canalization (Stearns, 2003). This process ensures that similar traits are produced in an organism despite different genetic backgrounds and environmental perturbation. Hsp90 is known to suppress phenotypic variation under normal conditions, but releases it when functionally compromised. Probably, mistranslation overloads Hsp90 allowing it to release buffered phenotypic diversity; this could explain, at least in part, why mistranslation produces phenotypic diversity.

Overloading of the proteome quality control systems may also explain the accumulation of aggregated proteins that were detected using the Hsp104-GFP reporter system (Results chapter, section 3; Figure 42). One would expect that misfolded proteins become degraded by the ubiquitin-proteasome system or refolded by Hsps, however if these systems are already overloaded then they may escape into the cytosol and may aggregate. More importantly, aberrantly synthesized unfolded proteins can be translocated into other cellular compartments; translocation requires protein unfolding (Simon and Blobel, 1993)

and may contribute to rapid dysfunction of cellular organelles thus accelerating cell degeneration. For example, aberrant proteins may affect the cell wall, plasma membrane, ER and mitochondria if they are allowed to pass the ER quality control system. That the Hsp12, Hsp30, Kar2 or Hsp78 chaperones are present in these cellular compartments (Hsp12 and Hsp30 are present in plasma membrane, Kar2 exists in the ER and Hsp78 is a mitochondrial chaperone) and that their expression was up-regulated by mistranslation supports the hypothesis that the latter does indeed overload the proteome quality control systems. A caveat of this hypothesis is that mistranslating yeast cells are able to increase the expression of components of the stress response when exposed to additional stress (Silva *et al.*, 2007). In other words, mistranslation does not compromise transcriptional responses to environmental stress, suggesting that cells could increase even further the up-regulation of the genes that encode proteins of the proteome quality control systems. This apparent paradox should be clarified in future studies.

The ER Hsp70 chaperone BiP (encoded by *KAR2* in yeast) and other ER-resident proteins such as protein disulfide isomerase (encoded by *PDI1*) or flavoenzyme (encoded by *ERO1*) were also up-regulated by mistranslation through UPR activation (Results chapter, section 3; Figures 39 and 40). BiP prevents the nascent domain of secretory or transmembrane proteins from misfolding, and maintains them in a folding competent status until their synthesis terminates. BiP is not only involved in the translocation of the nascent polypeptides across the ER membrane into the ER lumen, but it is also a key element of the ER-resident quality control mechanism which prevents unfolded proteins from leaving the ER (Dorner and Kaufman, 1994). Other functions associated to BiP are the solubilisation of folding precursors, stabilization of unassembled protein subunits and the redirecting of misfolded polypeptide chains to the cytosol for ubiquitin-labelling and subsequent degradation by the proteasome [ERAD, ER-associated protein degradation (Nishikawa *et al.*, 2001)]. Besides a basal constitutive expression level, BiP transcription is induced by the presence of mutant and misfolded proteins in the ER lumen and by stress effects that result in the accumulation of unfolded proteins (Kozutsumi *et al.*, 1988). Saturation of the

secretory pathway is possible, as extractable levels of free folding assistants BiP and Pdi1 decrease when heterologous proteins are overexpressed in *S. cerevisiae* (Robinson and Wittrup, 1995). Under ER saturation, degradation of secretory proteins increases. ER-assisted protein degradation is a complex process in which misfolded proteins in the ER are redirected to the translocon for retro-translocation to the cytosol, where they are subjected to proteasome degradation. The labelling of substrates destined for degradation by the cytosolic 26S proteasome requires an Ub (ubiquitin) activating enzyme.

Up-regulation of genes encoding proteasome subunits and increased proteasome activity were also detected under mistranslation (Silva *et al.*, 2007). Up-regulation of the *RPN4* gene, which encodes the transcriptional activator of the *RPT* and *RPN* proteasome genes and several other genes related to ubiquitin-proteasome pathway (Xie *et al.*, 2001), was detected immediately after induction of mistranslation. Surprisingly, the activation of a big part of those proteasomal genes (*PRE*, *RPT* and *RPN* genes) was only detected at a later stage. This may suggest that *RPN4* is first induced via other signalling pathways (Msn2/Msn4, Hsf1, Yap1 and Pdr1/Pdr3 regulated signalling pathways) and only after this it will activate other proteasomal genes and establish the programmed proteolysis of unfolded and misfolded proteins in the cell (Owsianik *et al.*, 2002;Hahn *et al.*, 2006).

1.3 Mistranslation is a ROS generator

As mentioned above, mistranslation decreased yeast culture viability - increased cell death – apparently through ROS production. This hypothesis is supported by our finding that the culture viability phenotype could be almost completely reverted by ROS scavengers. This increase in ROS production is consistent with the gene expression response to oxidative stress observed in yeast and with a strong enrichment of YRE cis-elements in the deregulated genes, which mediate activation of transcription through the transcription factor Yap1. The activation of the Yap1 transcription factor requires its translocation from the cytoplasm to the

nucleus and it will be important to investigate using Yap1-GFP fusions whether mistranslation does indeed alter the localization of this oxidative stress regulator. Nuclear accumulation of Yap1 is modulated mainly through changes in its nuclear export signal and it requires a series of redox modifications in a mechanism that involves other sensors of oxidation, namely the peroxiredoxin Hyr1 (Moye-Rowley, 2002). Therefore, studying these mechanisms of Yap1 activation may provide important clues about the cellular response to mistranslation. Activation of Yap1 and other general stress transcription factors, such as Msn2 and Msn4, also explain the stress cross protection induced by mistranslation. Like most environmental stressors, ROS activated stress cross protection mechanisms in yeast whereby exposure of cells to one type of stress protects them against other types of stress (Temple *et al.*, 2005). Indeed, in a previous and in this study, mistranslation also induced stress cross-protection against H_2O_2, cadmium, cyclohexamide, arsenite and NaCl (Santos *et al.*, 1999). This stress cross protection can be explained by the observed up-regulation of general stress and oxidative stress genes, namely chaperones genes (*HSP104*, *HSP26*, *HSP42*, *HSP12*, *HSP30*, *HSP78*, *HSC82*, *HSP10*, *HSP60*, *HSP150*, *SSA1*, *SSA2*, *SSA3* *SSA4*, *SSE1* and *SSE*2), ROS detoxification and redox control genes (*AHP1*, *GRX1*, *GRX2*, *PRX*1, *TRX2*, *SOD1*, *SOD2* and *TSA1*) and other stress genes, like *TSL1*, *DDR2*, *DDR48* and *RAD52*, which also protect yeast against the above mentioned environmental stressors (Davidson and Schiestl, 2001).

The results obtained strongly suggest that ROS are mainly produced by the mitochondria and by the ER, which were both significantly affected by mistranslation (see section 3 of Results chapter). The impact of mistranslation in cellular organelles is mainly due to the cytoplasmic translation of most of the organellar proteins which become exposed to mistranslation. Indeed, the protein subunits of the mitochondrial energy transducing systems are encoded by both nuclear and mitochondrial genes. Subunits of nuclear origin are synthesized in the cytosol and are then imported into the mitochondria in an unfolded conformation, while subunits encoded by the mitochondrial DNA (mtDNA) are synthesized within the mitochondrial matrix (Schwimmer *et al.*, 2006). The nuclear and mitochondrial

genomes coordinate their actions to ensure proper mitochondrial function and one would expect that disruption of the fidelity of synthesis of the nuclear encoded proteins will have an inevitable negative impact on mitochondrial function. In line with this hypothesis are various studies showing that mitochondrial dysfunction and increased ROS production are associated to defective mitochondrial gene expression (Feuermann *et al.*, 2003;Bonawitz *et al.*, 2006). On the other hand, prolonged ER retention of misfolded proteins entails repetitive rounds of oxidative protein folding attempts by foldases such as Pdi1 and consequently results in the generation of ROS. Alleviation of the ER stress is accomplished by the activation of the UPR and subsequent induction of the ERAD quality control system. The activation of the UPR was delayed relative to the induction of mistranslation (Results chapter, section 3; Figure 40) but once activated, the UPR contributed to the accumulation of ROS. Moreover, one cannot exclude the possibility that mistranslation overstrained the ERAD quality control system, which would have resulted in persistent ER stress. This could also have contributed to cell death, as has already been demonstrated in both yeasts and higher eukaryotes (Haynes *et al.*, 2004;Kincaid and Cooper, 2007).

ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins and causing lipid peroxidation. Such oxidative stress is associated with several human pathologies, including cancer, cardiovascular disease, Down's syndrome, Friedreich's ataxia, rheumatoid arthritis, autoimmune diseases and AIDS (Halliwell, 1987). Oxidative damage is emerging as an important factor in mutagenesis, tumorigenesis, ageing and age-related diseases, such as Parkinson's and Alzheimer's diseases (Levine, 2002;le-Donne *et al.*, 2003). Surprisingly, I was unable to detect protein oxidation in mistranslating cells. The hypothesis that oxidized proteins are present only in subpopulations of proteins where protein aggregates were detected with the HSP104-GFP reporter may explain our failure to detect protein oxidation by western blot analysis because the aggregates may have been span down during lysate clearance by centrifugation. Considering the link between ROS, oxidative stress and disease and also the plethora of human diseases caused by mistranslation in the cytoplasm and in the

mitochondria (Scheper *et al.*, 2007a), it will be of paramount importance to clarify experimentally the putative link between mistranslation and protein oxidation in future studies. It will also be interesting to elucidate whether increased ROS production and oxidative stress caused by mRNA mistranslation induces methionine misacylation, as has been recently described in mammalian cells upon exposure to environmental stressors (Netzer *et al.*, 2009). Met-misacylation and misincorporation into the proteome increases up to ten-fold upon exposure of mammalian cells to ROS and oxidative stress. This is apparently an adaptive response since Met is a ROS scavenger.

1.4 Mistranslation as a model system to study protein misfolding and conformational diseases

A large number of diseases are associated to defective protein folding. Although the molecular mechanisms by which the pathologies develop are diverse they can be viewed generically as 'conformational diseases'. Our yeast mistranslation system provided important new clues about the cellular consequences of protein misfolding, degradation and aggregation. The general basis of the so-called conformational diseases is the cellular inability to degrade efficiently the misfolded and damaged proteins whose consequence is the formation of cytotoxic intra- or extracellular oligomers and polymers/aggregates. Interestingly, oxidative stress also contributes to the pathogenesis of many conformational diseases (Butterfield *et al.*, 2001). A cellular condition of oxidative stress develops when the mitochondrial oxidative phosphorylation and the cell's anti-oxidative capacity become overloaded. In these situations reactive oxygen species (ROS) are generated in excessive amounts and damage DNA, RNA, lipids and proteins. Interestingly, misfolded proteins, including β-sheet oligo- and polymers generate oxidative stress (Butterfield *et al.*, 2001) if they are not eliminated by the proteasome degradation system (Bence *et al.*, 2001). In these cases the unfolded protein response is activated and ROS are generated (Imaizumi *et al.*, 2001). Dependent on the strength of the stress insult, cellular responses rescue the cell

from the stress situation, or, alternatively, eliminate it by apoptosis or necrosis (Temple *et al.*, 2005;Perrone *et al.*, 2008). An alternative or synergistic mechanism is related to the exposure of the core of misfolded proteins whose hydrophobic residues may sequester other proteins, such as transcription factors and chaperones, which in turn elicit the stress responses. Of special interest in this context is that misfolded and partly unfolded protein structures may be particularly susceptible to oxidative modifications, which may promote unfolding and thus increase the susceptibility to further modifications that exaggerate the stress responses (Dukan *et al.*, 2000). Despite the fact that the exact mechanisms and order of events may be quite different in the various conformational diseases, the endpoint seems similar: chronic stress and eventual death of the cell.

The balance between the cellular capacity to eliminate misfolded and damaged proteins and the tendency of particular proteins to evade the system is a determining factor in the development and severity of conformational diseases. In healthy and young cells misfolded and damaged proteins are eliminated by the protease factors of the proteome quality-control systems, but if these systems are overwhelmed, as may be the case in cells of patients with inherited defects in the defense systems and in aged cells, aberrant proteins may accumulate and cause the problems discussed above (Soti and Csermely, 2000;Slavotinek and Biesecker, 2001;Macario and Conway de, 2002). In aged cells, the resistance to oxidative stress as well as the capability to induce the activity of the protein quality control systems are decreased and cells may have difficulties in maintaining native protein conformations and elimination of misfolded and damaged proteins (Soti *et al.*, 2000). Although the molecular mechanisms for these disabilities are still poorly defined they may contribute significantly to the pathogenesis of many of the age related conformational diseases.

In the yeast mistranslation model used in this study, the detected conformational stress generated by mistranslation mimicked to certain extent the events that lead to cell degeneration during conformational diseases. The balance between functionality of proteome quality control mechanisms, antioxidant defenses and

production of mistranslated proteins allows cells to survive up to a certain threshold. ROS accumulation and redox imbalance lead to a catastrophic reduction of cell viability and cell subpopulations that were some how more resistant to oxidative stress and other degenerative processes gave rise to new populations of yeast cells with new and highly heterogeneous phenotypes. This raises the intriguing hypothesis that these selected cell subpopulations may have unique phenotypes, namely proliferative and drug resistance traits that may be relevant in the context of cancer and other non-neurodegenerative diseases, while the cell death phenotype may be relevant in the context of neurodegeneration.

2. Conclusions

The main conclusions of the present study are that low-level mistranslation, which does not affect growth rate, induces important gene expression alterations, overloads the proteome quality control systems, increases ROS production, activates the oxidative stress response and has a major negative impact on the viability of post-mitotic cells. These phenotypes are to certain extent surprising because inducible mistranslation had no impact on growth rate of exponentially dividing cells. However, they explain to certain extent why mistranslation causes diseases in adult individuals. Low-level mistranslation is a degenerative process whose effects are long-term rather than immediate. Such degenerative phenotype is most likely caused by ROS, suggesting that mistranslation causes diseases through oxidative stress rather than by proteome collapse. Considering that the cellular responses to mistranslation detected in this study are conserved from yeast to man, yeast is an excellent model system to unravel the basic biology of the mistranslation diseases and probably of the diseases associated to protein misfolding, aggregation and conformational alteration.

This study also allowed us to understand the overall cellular effects of mistranslation and the interconnection between them. This is relevant to understand both disease and evolutionary processes associated to mistranslation. The global view of the pleiotropic effects of mistranslation is summarized in the diagram shown in Figure 43.

Our studies unveiled important new roles of mistranslation in adaptation and evolutionary processes. Mistranslation impaired sexual reproduction in yeast, indicating that it may create genetic isolation and consequently promote speciation and block lateral gene transfer. It also destabilized the genome, increased cell ploidy and generated phenotypic variability, which resulted in highly heterogeneous cell populations. Such phenotypic heterogeneity and variability can be selected ensuring that advantageous traits become fixed and inherited. In this

context, the finding that mistranslation activates stress cross protection and creates selective advantages under stress is of paramount importance.

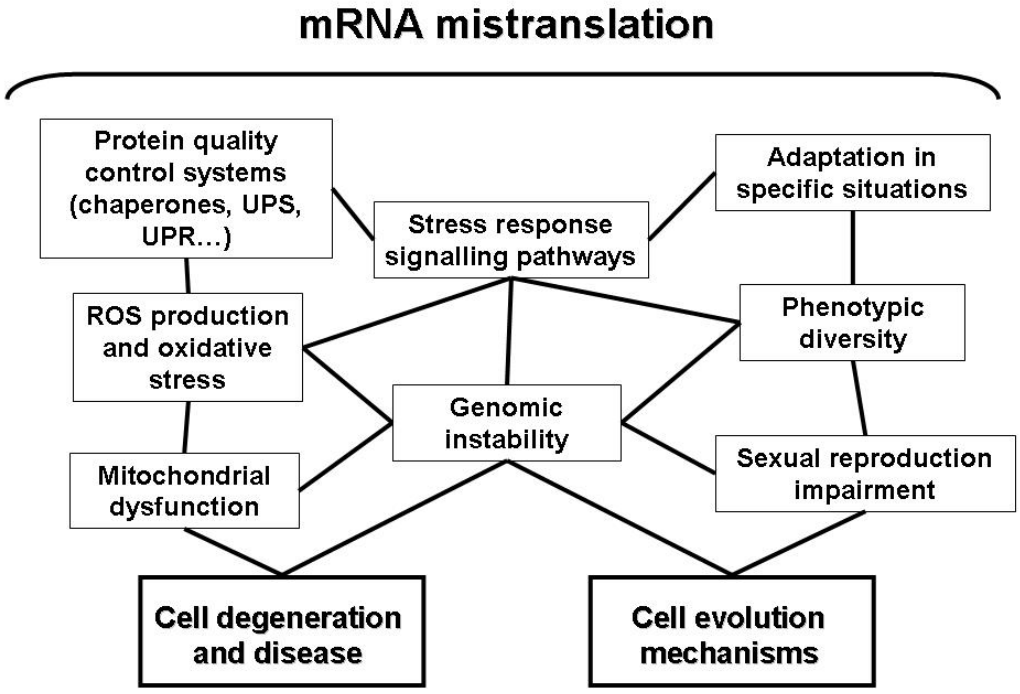


Figure 43. Inter-relationship between the cellular processes affected by mistranslation and their connection with cellular degeneration and evolution.

The homodirectional variation in gene expression observed between total and polysomal mRNA profiles suggests that cells sensed low-level mistranslation as a high stress situation. Overall, mistranslation up-regulated stress response genes and down-regulated protein synthesis genes. This is consistent with the hypothesis that the end point of mistranslation is the synthesis of aberrant proteins that may misfold, become degraded or aggregate creating toxic accumulation of proteinaceous deposits, which are the hallmark of the protein conformational neurodegenerative diseases. This in turn affects the structure and function of cellular organelles increasing ROS production and accumulation. In other words, mistranslation triggers a cascade of cellular events that culminate with cell death or result in a subpopulation of viable cells with unique phenotypic characteristics.

3. Future work

This study raised a number of new biological questions, which should be tackled experimentally in the near future. For example, the genomic alterations induced by mistranslation should be better studied in order to understand the relationship between proteome disruption, oxidative stress, overloading of proteome quality control systems and genome stability. Answering these questions is bound to provide new insight into how mistranslation causes human disease.

The role of oxidative stress in cell degeneration and loss of culture viability should also to be further analysed. Studies with knockout strains harbouring deletions in genes of the oxidative stress response should be carried out and it will be most interesting to induce mistranslation in yeast ρ^0 strains, which lack mitochondria, to evaluate the role of this organelle in ROS production under mistranslation. Also, the role of the *YAP* gene family (*YAP1* to *YAP8*) should be studied in detail to better understand the cellular response to oxidative stress generated by mistranslation.

Mistranslation should also be induced in multicellular organisms in order to evaluate its impact on disease development and to determine whether oxidative stress is a major cause of cell degeneration and death. It would be most interesting to elucidate why mistranslation diseases have a late onset in vertebrates. Could it be that above a certain threshold mistranslation is lethal and prevents embryonic development and, therefore, only certain types of low-level mistranslation are preserved throughout development and during adulthood? If so, could it be that the mistranslation diseases reflect aging related decay of the proteome quality control systems? In other words, could the phenotypes associated to mistranslation only become visible in cells that lost their capacity to maintain fully functional proteome quality control systems? Finally, why are neurons so sensitive to mistranslation? These are fascinating questions that should be tackled in future studies using mouse, *Drosophila melanogaster* or zebrafish models of the mistranslation diseases.

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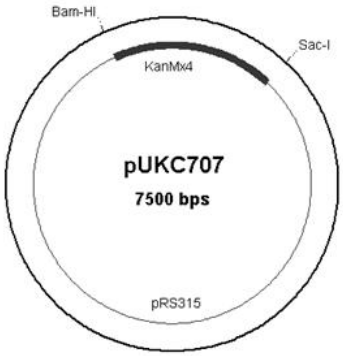
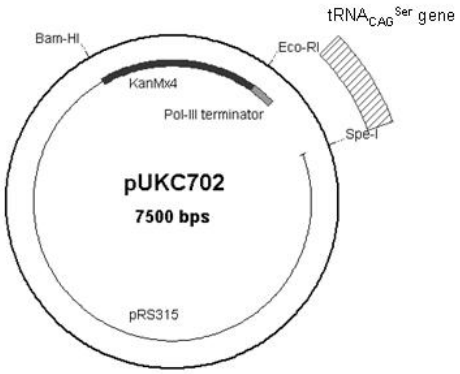
F. Annexes

Annexe 1: maps of the plasmids used in this study

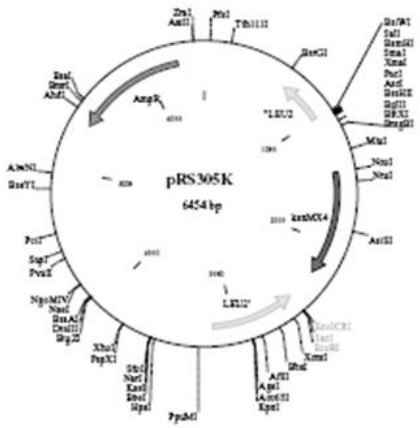
pUKC707



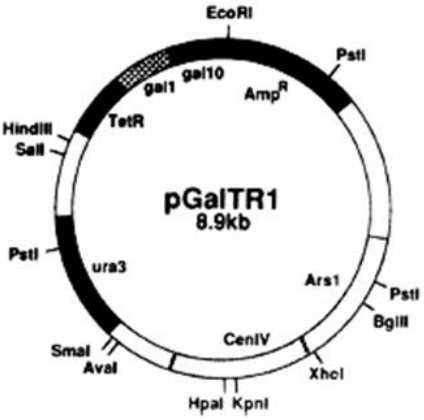
pUKC702



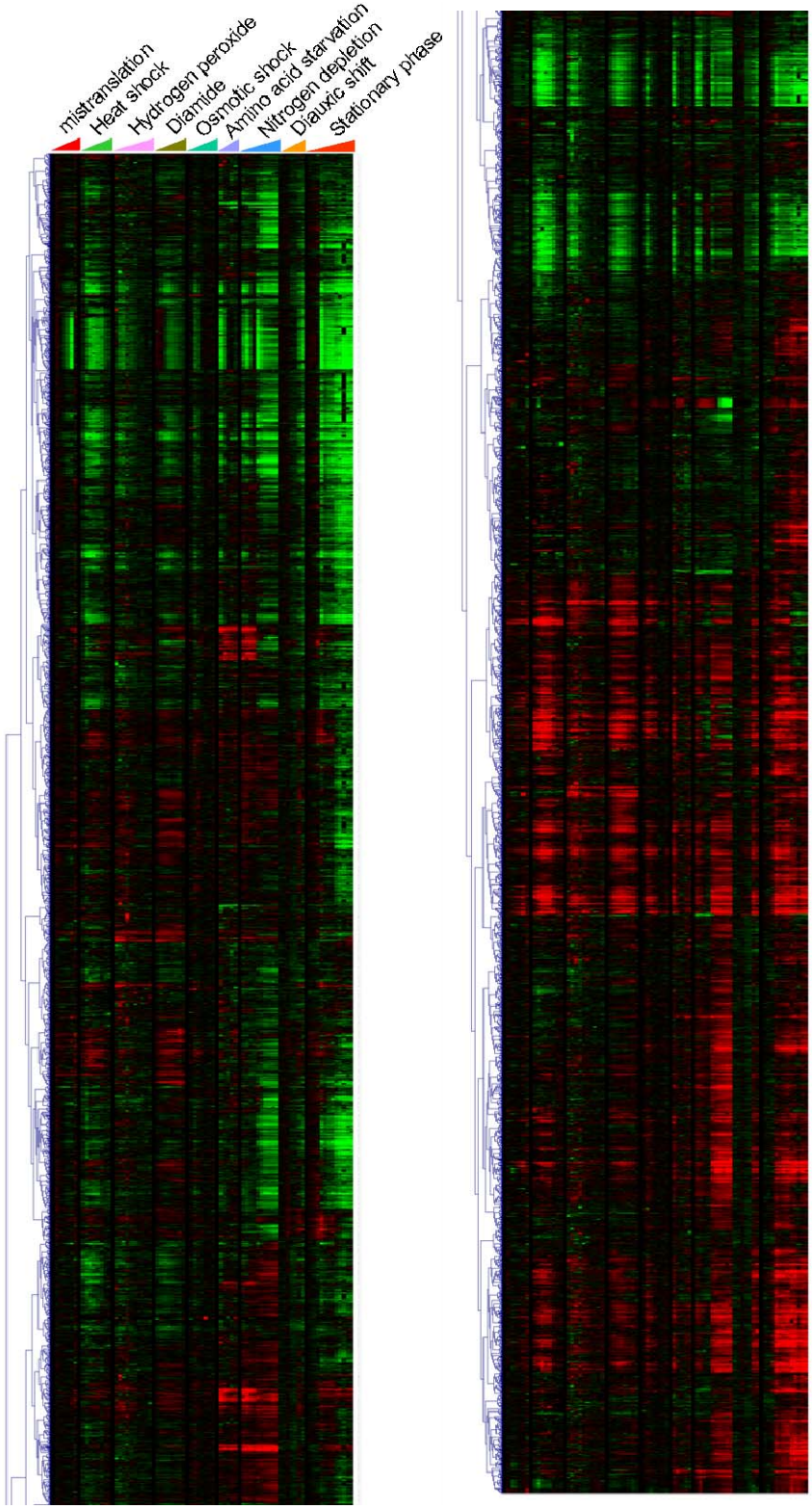
pRS305K



pGalTR1



<u>Annexe 2</u>: heat-map comparing transcriptional responses to mistranslation (this study) and to environmental stressors (Gasch *et al.*, 2000)



Annexe 3: differentially expressed genes in both short- and long-term experiments (T0'-T180' and T20h) and respective cluster according to Figure 28.

AHP1	A	ENO1	B	RPN4	B
ALD3	A	ERG9	B	RPN5	B
BTN2	A	ERV29	B	RPT1	B
DAD3	A	FMP46	B	SED1	B
DCS2	A	GDB1	B	SFA1	B
DDR2	A	GIP2	B	SIS1	B
FMP16	A	GLG1	B	SOD1	B
GND2	A	GLK1	B	SOD2	B
HSP104	A	GPD1	B	SPG5	B
HSP12	A	GPH1	B	SSA1	B
HSP26	A	GPM1	B	SSA2	B
HSP30	A	GRE3	B	SSE1	B
HSP31	A	GRX1	B	SSE2	B
HSP42	A	GRX2	B	THI5	B
HSP78	A	GTT1	B	TIR2	B
MSC1	A	HBN1	B	TPI1	B
OM45	A	HFD1	B	TRX2	B
OYE3	A	HOR7	B	TSA1	B
RTN2	A	HSC82	B	UGP1	B
SIP18	A	HSP10	B	URH1	B
SOL4	A	HSP150	B	YDL124W	B
SSA4	A	HSP60	B	YDL159W-A	B
STF2	A	HXK1	B	YER067W	B
TFS1	A	HYR1	B	YGR146C	B
TSL1	A	KAR2	B	YIF1	B
YGP1	A	MBF1	B	YIL177C	B
YHR087W	A	MDH3	B	YJL225C	B
YJL156W-A	A	MDJ1	B	YJR096W	B
YMR090W	A	MET17	B	YLR064W	B
YNL134C	A	MHT1	B	YMR196W	B
ADE17	B	MRH1	B	YOR059C	B
ALD2	B	MSF1	B	YOR152C	B
APJ1	B	NCE103	B	YPL206C	B
ATG19	B	PDR5	B	ZRT2	B
CDC48	B	PET10	B	AAD14	C
COS3	B	PGM2	B	AAD16	C
CPR1	B	PIC2	B	AAD4	C
CPR6	B	PMP1	B	COS4	C
CUP1-1	B	PMP2	B	FMP23	C
CUP1-2	B	PNC1	B	GRE1	C
CWP2	B	PRB1	B	GRE2	C
CYB2	B	PRX1	B	HXT5	C
DDR48	B	PRY1	B	MNN4	C
ECM15	B	PST2	B	MOH1	C
ECM29	B	RAD52	B	NQM1	C
ELO1	B	RPN2	B	PMA2	C

QNQ1	C	GIN4	E	YHL039W	E
SPG4	C	GUA1	E	YKE4	E
SSA3	C	HEM1	E	YKR018C	E
THI11	C	HXT1	E	YKR075C	E
THI12	C	INO4	E	YLL056C	E
TKL2	C	KAR9	E	YLR256W-A	E
URA10	C	MAE1	E	YLR311C	E
YBR116C	C	MAK21	E	YMR046C	E
YBR147W	C	MLS1	E	YMR206W	E
YBR241C	C	MRK1	E	YMR304C-A	E
YCL042W	C	NHP2	E	YNL144C	E
YKL071W	C	NOC2	E	YNL194C	E
YMR118C	C	NOG1	E	YOR302W	E
YPL276W	C	NOP1	E	YOR309C	E
CBF5	D	NOP10	E	YOR343W-A	E
CDC33	D	NOP2	E	YOR343W-B	E
FET3	D	NOP4	E	YOR387C	E
HXK2	D	NOP53	E	YPR137C-A	E
RPL14B	D	NOP58	E	YPR158W-A	E
RPL3	D	NOP7	E	YPR202W	E
RPL38	D	NSA2	E	ZPS1	E
RPL40A	D	NSR1	E	ACO1	F
RPP1A	D	NUC1	E	ACP1	F
RPP2B	D	PCL5	E	ADH2	F
RPS12	D	PDC6	E	BDH1	F
RPS23A	D	PHO84	E	BOI1	F
RPS25B	D	PRT1	E	CHO2	F
RPS27B	D	ROK1	E	CIK1	F
RPS8A	D	RPM2	E	COQ2	F
SAH1	D	RRP14	E	CPT1	F
SCW10	D	RRP5	E	ERV1	F
SNU13	D	SAP185	E	FAS1	F
SNX3	D	SIK1	E	FAS2	F
TEF4	D	SOK2	E	FCY2	F
YEF3	D	SPB1	E	FMP30	F
ALD6	E	SPG1	E	GAL1	F
AQR1	E	UTP8	E	GAL2	F
ARA2	E	VEL1	E	GDH1	F
ARO10	E	VID24	E	GDS1	F
BAP2	E	YBL005W-A	E	GGA2	F
BLM10	E	YBL028C	E	GIC2	F
BUD22	E	YBL048W	E	HHF1	F
CAK1	E	YBR012W-A	E	HHF2	F
DBP2	E	YBR090C	E	HNM1	F
DBP3	E	YCR100C	E	HTA1	F
EBP2	E	YDR098C-A	E	HTA2	F
ESF1	E	YDR365W-A	E	HYP2	F
FCY22	E	YEL076C	E	KRS1	F
FIT3	E	YER137C-A	E	MMP1	F
FRM2	E	YER159C-A	E	MRPL33	F
FUR1	E	YFL064C	E	PET9	F
GAR1	E	YHB1	E	PSA1	F

PSD1	F	SAM1	F	YDR210C-D	F
RPB5	F	SPC29	F	YHM2	F
RPB8	F	SSC1	F	YLR301W	F
RPL22B	F	SSF2	F	YNR036C	F
RPL41A	F	SUP45	F	YOR248W	F
RPL41B	F	TIF11	F	YOX1	F
RPS30A	F	URA2	F	YPR004C	F
RPS30B	F	VRG4	F	ZPR1	F
RSM27	F	YDR061W	F		

Annexe 4: genes corresponding to the significantly enriched functional classes represented in Tables 1 and 2

- **aldehyde metabolic process**

AAD14 AAD16 AAD4 ALD2 CIT2 GLO1 GLO2 GOR1 HFD1 ICL1 MDH3 MLS1 SFA1

- **cellular carbohydrate catabolic process**

ATH1 CDC19 ENO1 ENO2 FBA1 GDB1 GLK1 GND2 GPH1 GPM1 GRE3 HXK1
MAL12 NTH1 NTH2 PCL7 PGI1 PGK1 PGM2 SOL3 SOL4 SUC2 TDH1 TDH2
TDH3 TKL2 TPI1 ZWF1

- **energy reserve metabolic process**

ATH1 BMH1 BMH2 GDB1 GIP2 GLG1 GLG2 GPH1 GSY1 GSY2 NTH1 NTH2
PCL7 PGM2 PPG1 SHP1 TPS1 TPS2 TPS3 TSL1 UGP1

- **external encapsulating structure**

BGL2 CWP2 HOR7 HPF1 HSP150 PIR1 SED1 SSA1 SSA2 TIR2

- **oxidoreductase activity**

AAD14 AAD16 AAD4 ADH2 ADH5 ADI1 AHP1 ALD2 ALD3 ALD4 ALD6 ARA1 ARA2 ARH1 AYR1
BDH1 BDH2 CTA1 CYB2 CYC7 DOT5 ECM17 ERG11 ERG25 ERG3 ERV1 ETR1 FET3 FMP46
FRE7 FRM2 GCV1 GCV2 GCV3 GND2 GOR1 GPD1 GPX1 GRE2 GRE3 GRX1 GRX2 GRX5
GTT1 GUT2 HBN1 HEM13 HFD1 HIS4 HMX1 HOM6 HYR1 IDP2 JLP1 LOT6 MCR1 MDH3
MET10 MET13 MET16 MTD1 MXR1 NCP1 NDE1 NDI1 OYE3 PDA1 PDI1 POX1 PRX1 QCR7
RNR2 RNR4 SCO1 SER3 SER33 SFA1 SOD1 SOD2 SOR1 TDH1 TDH2 TDH3 TRX1 TRX2
TSA1 TSA2 UGA2 XYL2 ZTA1 ZWF1

- **peroxidase activity**

AHP1 CTA1 DOT5 GPX1 GRX1 GRX2 GTT1 HMX1 HYR1 PRX1 SCO1 TSA1 TSA2

- **proteasome complex**

BLM10 DOA4 ECM29 PRE1 PRE10 PRE2 PRE3 PRE4 PRE5 PRE6 PRE7 PUP1 PUP3 RPN10 RPN12 RPN2 RPN3 RPN4 RPN5 RPN6 RPN9 RPT1 RPT3 RPT5 RPT6 SCL1 UBC4 UBC5 UBP6

- **protein folding**

CPR6 HSC82 HSP10 HSP60 MDJ1 PDI1 SIS1 SSA1 SSA2 SSE1 SSE2

- **regulation of cell redox homeostasis**

AHP1 DOT5 GRX1 GRX2 PRX1 TRX1 TRX2 TSA1 TSA2

- **response to chemical stimulus**

AAD14 AAD16 AAD4 ACO1 ADE1 AFG1 AHP1 ATX1 AZR1 BDS1 CAD1 CDC10 CTA1 CUP1-1 CUP1-2 CUP2 CYS3 DOT5 ECM17 EMI2 ERV1 FET3 FRM2 GAD1 GLO1 GLO2 GOS1 GPX1 GRE2 GRX1 GRX2 GRX5 GSH1 GTS1 HOM3 HOM6 HSP12 HYR1 KAR2 KIN82 LOT6 MAC1 MCR1 MET10 MET14 MET16 MET17 MET22 MET28 MXR1 NCE103 ORM2 OXR1 OYE3 PDR1 PDR10 PDR5 PGD1 PHM6 PRM1 PRX1 RDS2 RPN4 RSB1 RTA1 SCO1 SNF8 SOD1 SOD2 STB5 TPS1 TRX1 TRX2 TSA1 TSA2 UBC5 UGA2 YAP1 YCK1 YDJ1 YGP1 YOR1 YRR1 ZTA1

- **response to oxidative stress**

ACT1 AFG1 AHP1 ATX1 CTA1 DOT5 ERV1 GAD1 GPX1 GRX1 GRX2 GRX5 GSH1 HSP12 HYR1 LOT6 MCR1 MXR1 NCE103 OXR1 PRX1 SCO1 SNQ2 SOD1 SOD2 STB5 TRX1 TRX2 TSA1 TSA2 UGA2 YAP1 ZTA1 ZWF1

- **response to stress**

ACT1 AFG1 AHA1 AHP1 ALD3 ATH1 ATX1 BMH1 BMH2 CAC2 CDC1 CDC2 CTA1 CTT1 DAK1 DAK2 DDR2 DDR48 DOA1 DOG2 DOT5 EAF3 ECO1 ENA1 ERV1 GAD1 GPD1 GPX1 GRE1 GRE2 GRE3 GRX1 GRX2 GRX5 GSH1 HCH1 HOF1 HOR7 HSC82 HSP104 HSP12 HSP26 HSP30 HSP42 HSP78 HTA1 HTA2 HTB1 HYR1 KAR2 LOT6 MCR1 MET22 MLH2 MLH3 MMS2 MNN4 MRK1 MSN1 MXR1 NCE103 NTH1 NTH2 ORM2 OXR1 PAN3 PEP4 PFY1 PHR1 PIM1 PRB1 PRE1 PRE3 PRX1 RAD10 RAD33 RAD51 RAD52 RAD59 RFA1 RFA2 RFC5 RFX1 RPN4 RSC30 RTT109 SCC4 SCO1 SGT2 SIP18 SNQ2 SOD1 SOD2 SSA1 SSA2 SSA3 SSA4 STB5 STF2 TFB3 TIR2 TPP1 TPS1 TPS2 TPS3 TRX1 TRX2 TSA1 TSA2 TSL1 UBC4 UBC5 UBI4 UGA2 UMP1 WSC2 WSC4 XBP1 YAP1 YGP1 ZPR1 ZTA1 ZWF1

- **response to toxin**

AAD14 AAD16 AAD4 AAD6 FRM2 GLO1 GLO2 GRE2 GTT2 MET17 OYE3 PDR5 RSB1 RTA1

- **sulfur metabolic process**

ADI1 BDS1 CYS3 DUG3 ECM17 GLO1 GSH1 GTT1 HOM3 HOM6 JLP1 MET1 MET10 MET13 MET14 MET16 MET17 MET2 MET22 MET28 MET3 MET32 MET6 MHT1 OPT1 SAM1 STR3 TRX1 TRX2 VHR1

- **sulfur utilization**

ECM17 MET1 MET10 MET14 MET16 MET22 MET3 TRX1 TRX2

- **unfolded protein binding**

APJ1 CPR6 HSC82 HSP10 HSP26 HSP31 HSP32 HSP42 HSP78 KAR2 MDJ1 SIS1 SNO4 SSA1 SSA2 SSA4 TSA1

- **biosynthetic process**

ACC1 ACO1 ACP1 ADE6 ADH1 ALA1 ARO4 ATP1 ATP2 CDC21 CDC60 CDS1 CHO2 CLU1 COQ2 COX14 COX15 CPT1 DED81 EFB1 EFT1 EFT2 EPT1 ERG4 FAS1 FAS2 FRS1 FRS2 FUN12 GDH1 HEM12 HYP2 IDH1 ILS1 ILV1 ILV3 ILV5 IMG1 IMG2 KRS1 KTR3 LAG1 MES1 MEU1 MNP1 MRP1 MRP10 MRP13 MRP4 MRP51 MRPL1 MRPL10 MRPL13 MRPL19 MRPL20 MRPL22 MRPL24 MRPL25 MRPL27 MRPL28 MRPL3 MRPL31 MRPL32 MRPL33 MRPL37 MRPL40 MRPL44 MRPL50 MRPL6 MRPL7 MRPL8 MRPS17 MRPS28 MRPS35 MSE1 MSY1 MTO1 NAM9 OPI3 PET123 PRS3 PRS4 PSA1 PSD1 RML2 RPL22B RPL41A RPL41B RPS30A RPS30B RSM22 RSM26 RSM27 RSM28 SAM1 SAM2 SER2 SUP45 SWS2 THS1 TIF1 TIF11 TIF2 TIF35 TIM11 TMA19 TUF1 URA2 URA3 VAS1 VRG4 YAH1 ZUO1

- **intracellular non-membrane-bound organelle**

AIR1 ALB1 ARX1 BBC1 BFR2 BMS1 BRX1 BUD21 CBF1 CBF5 CDC33 CGR1 CIC1 CIN8 DBP3 EBP2 ECM1 EGD1 ENP1 ENP2 ERB1 ESF1 ESF2 FCF2 FYV7 GAR1 GCD11 GCN1 GDE1 HAS1 HCA4 IMP3 KAR1 KRI1 KRR1 LHP1 LSM4 LUG1 MAK16 MAK21 MAK5 MRD1 MRP20 MRPL33 MRPS16 MYO2 NHP2 NIP7 NMD3 NOC2 NOC4 NOG1 NOG2 NOP1 NOP10 NOP13 NOP14 NOP15 NOP16 NOP2 NOP4 NOP53 NOP58 NOP6 NOP7 NOP8 NSA1 NSA2 NSR1 NUG1 PLC1 POP6 PUF6 PXR1 REI1 RIX7 RLP24 RLP7 RMI1 ROK1 ROX1 RPA12 RPA135 RPA14 RPA190 RPA34 RPA49 RPB10 RPB5 RPB8 RPC10 RPF2 RPG1 RPL15B RPL22B RPL3 RPL38 RPP1A RPP2B RPS12 RRB1 RRP12 RRP14 RRP5 RRP9 RSM27 SAS10 SIK1 SLK19 SLX9 SMC2 SNU13 SOF1 SPB1 SPO74 SPR28 SQT1 SSF1 SSF2 STU1 SUI2 TEF4 TIF11 TIF4631 TMA16 TMA23 TRM112 TSR1 UTP10 UTP13 UTP14 UTP18 UTP20 UTP23 UTP4 UTP5 UTP6 UTP8 UTP9 VAR1 YEF3 ZIP1

- **mitochondrial genome maintenance**

ACO1 GGC1 ILV5 MDV1 MGR2 MHR1 MRPL20 MRPL8 RIM2 YHM2

- **mitochondrion**

ACC1 ACO1 ACP1 ADH3 ALA1 ALT1 ATP1 ATP2 CBR1 CDS1 COQ2 COX14 COX15 COX17 COX8 CTF18 CYC1 CYM1 CYT1 DIC1 ECM33 ERV1 FAS1 FAS2 FLX1 FMP30 FMP31 FMP36 FUN12 FUN30 GAS1 GDS1 GGC1 GND1 GNP1 HXT6 HXT7 HYP2 IDH1 ILV1 ILV3 ILV5 IMG1

IMG2 LIP2 LYP1 MAM33 MAS1 MAS2 MBA1 MDM35 MDN1 MDV1 MHR1 MIC17 MNP1 MRP1 MRP10 MRP13 MRP4 MRP51 MRPL1 MRPL10 MRPL13 MRPL19 MRPL20 MRPL22 MRPL24 MRPL25 MRPL27 MRPL28 MRPL3 MRPL31 MRPL32 MRPL33 MRPL37 MRPL40 MRPL44 MRPL50 MRPL6 MRPL7 MRPL8 MRPS17 MRPS28 MRPS35 MRS11 MSB1 MSC6 MSE1 MSY1 MTO1 NAM9 OMS1 OPI3 OXA1 PAM17 PAM18 PET123 PET127 PET9 POL1 POR1 PPA2 PSD1 PUS6 QCR6 QRI5 RIM2 RML2 RSM22 RSM26 RSM27 RSM28 SSC1 SWS2 THS1 TIM11 TIM50 TIM8 TIM9 TMA19 TOM20 TOM22 TOM7 TUF1 URA2 VAS1 VRG4 YAH1 YHM2 YLF2 YME1 YPT31 ZUO1

- **mitochondrion organization and biogenesis**

ACO1 CLU1 GGC1 ILV5 MBA1 MDM35 MDV1 MGR2 MHR1 MRPL20 MRPL8 MRS11 OXA1 POR1 RIM2 TIM8 TIM9 TOM7 YHM2 YME1

- **regulation of translational fidelity**

RPS11A RPS11B RPS2 RPS23A RPS23B RPS9A RPS9B SSB1 SSB2

- **ribonucleoprotein complex**

ALB1 ARX1 ASC1 BUD21 CBF5 CDC33 CWC15 CWC25 DIB1 EGD1 ENP1 ESF2 GAR1 GDE1 IMP3 MAK21 MRPL33 MRPS16 NHP2 NIP7 NMD2 NOC2 NOC4 NOG1 NOP1 NOP10 NOP14 NOP58 NOP7 NSA2 POP6 PRP8 RPL10 RPL11A RPL11B RPL12A RPL12B RPL13A RPL13B RPL14A RPL14B RPL15A RPL16A RPL16B RPL17A RPL17B RPL18A RPL18B RPL19A RPL19B RPL1A RPL1B RPL20A RPL20B RPL21A RPL21B RPL22A RPL22B RPL23A RPL23B RPL24A RPL24B RPL25 RPL26A RPL26B RPL27A RPL27B RPL28 RPL29 RPL2A RPL2B RPL3 RPL30 RPL31A RPL31B RPL32 RPL33A RPL33B RPL34A RPL34B RPL35A RPL35B RPL36A RPL36B RPL37A RPL37B RPL38 RPL39 RPL40A RPL40B RPL42A RPL42B RPL43A RPL43B RPL4A RPL4B RPL5 RPL6A RPL6B RPL7A RPL7B RPL8A RPL8B RPL9A RPL9B RPM2 RPP0 RPP1A RPP1B RPP2A RPP2B RPS0A RPS0B RPS10A RPS10B RPS11A RPS11B RPS12 RPS13 RPS14A RPS14B RPS15 RPS16B RPS17A RPS17B RPS18A RPS18B RPS19A RPS19B RPS1B RPS2 RPS20 RPS21A RPS21B RPS22A RPS22B RPS23A RPS23B RPS24A RPS24B RPS25B RPS26A RPS26B RPS27A RPS27B RPS28A RPS28B RPS29A RPS29B RPS3 RPS31 RPS4A R PS4B RPS5 RPS6A RPS6B RPS7A RPS7B RPS8A RPS8B RPS9A RPS9B RRP14 RRP5 RRP9 RSM27 SAS10 SIK1 SLX9 SNT309 SNU13 SOF1 SQT1 SSB1 SSB2 STM1 TEF4 TIF11 TMA16 TMA23 UPF3 UTP13 UTP14 UTP18 UTP23 UTP4 UTP5 UTP6 UTP8 UTP9 YEF3

- **ribosome**

ARB1 ARD1 ASC1 CDC33 EFB1 EFT1 EFT2 FUN12 HYP2 IMG1 IMG2 LSM12 MNP1 MRP1 MRP10 MRP13 MRP4 MRP51 MRPL1 MRPL10 MRPL13 MRPL19 MRPL20 MRPL22 MRPL24 MRPL25 MRPL27 MRPL28 MRPL3 MRPL31 MRPL32 MRPL33 MRPL37 MRPL40 MRPL44 MRPL50 MRPL6 MRPL7 MRPL8 MRPS17 MRPS28 MRPS35 NAM9 PET123 RML2 RPL10 RPL11A RPL11B RPL12A RPL12B RPL13A RPL13B RPL14A RPL14B RPL15A RPL16A RPL16B RPL17A RPL17B RPL18A RPL18B RPL19A RPL19B RPL1A RPL1B RPL20A RPL20B RPL21A RPL21B RPL22A RPL22B RPL23A RPL23B RPL24A RPL24B RPL25 RPL26A RPL26B RPL27A RPL27B RPL28 RPL29 RPL2A RPL2B RPL3 RPL30 RPL31A RPL31B RPL32 RPL33A RPL33B RPL34A RPL34B RPL35A RPL35B RPL36A RPL36B RPL37A RPL37B RPL38 RPL39 RPL40A RPL40B RPL41A RPL41B RPL42A RPL42B RPL43A RPL43B RPL4A RPL4B RPL5 RPL6A RPL6B RPL7A RPL7B RPL8A RPL8B RPL9A RPL9B RPP0 RPP1A RPP1B RPP2A RPP2B RPS0A RPS0B RPS10A RPS10B RPS11A RPS11B RPS12 RPS13 RPS14A RPS14B RPS15 RPS16B RPS17A RPS17B RPS18A RPS18B RPS19A RPS19B RPS1B RPS2 RPS20 RPS21A RPS21B RPS22A RPS22B RPS23A RPS23B RPS24A RPS24B RPS25B RPS26A RPS26B RPS27A RPS27B RPS28A RPS28B RPS29A RPS29B RPS3 RPS30A RPS30B RPS31 RPS4A RPS4B RPS5 RPS6A RPS6B RPS7A RPS7B RPS8A RPS8B RPS9A RPS9B RSM22 RSM26 RSM27 RSM28 STM1 SWS2 TEF4 TIF1 TIF11 TIF2 TMA108 TMA19 YEF3 ZUO1

- **ribosome biogenesis and assembly**

AAH1 ALB1 ARX1 ATC1 BFR2 BMS1 BRX1 BUD20 BUD21 BUD22 CBF5 CGR1 CIC1 DBP2 DBP3 EBP2 ECM1 ENP1 ENP2 ERB1 ESF1 ESF2 FCF2 FYV7 GAR1 GCD10 HAS1 HCA4 HGH1 HMT1 IMP3 KRI1 KRR1 LIA1 LOC1 LRP1 MAK16 MAK21 MAK5 MIS1 MRD1 MTR2 NCL1 NCS2 NHP2 NIP7 NMD3 NOB1 NOC2 NOC4 NOG1 NOG2 NOP1 NOP10 NOP13 NOP14 NOP15 NOP16 NOP2 NOP4 NOP53 NOP58 NOP6 NOP7 NOP8 NSA1 NSA2 NSR1 NUC1 NUG1 POP6 PPT1 PUF6 PUS1 PXR1 RBG1 REI1 RIX1 RIX7 RLP24 RLP7 RNA1 RNH70 ROK1 RPA12 RPA135 RPA34 RPA49 RPC82 RPF2 RPL3 RRB1 RRP12 RRP14 RRP5 RRP9 SAS10 SDA1 SIK1 SLX9 SNU13 SOF1 SPB1 SQT1 SSF1 SSF2 TIF4631 TMA23 TRM11 TRM8 TRM82 TSR1 T SR2 UTP10 UTP13 UTP14 UTP18 UTP20 UTP23 UTP4 UTP5 UTP6 UTP8 UTP9 VAR1 YEF3

- **rRNA metabolic process**

AIR1 BFR2 BMS1 BUD21 CBF5 CGR1 DBP2 DBP3 EBP2 ENP1 ENP2 ERB1 ESF1 ESF2 FCF2 FYV7 GAR1 HAS1 HCA4 IMP3 KRR1 LRP1 MAK16 MAK5 MRD1 NHP2 NIP7 NOB1 NOC4 NOG1 NOP1 NOP10 NOP14 NOP2 NOP4 NOP53 NOP58 NOP6 NOP7 NOP8 NSA2 NSR1 NUG1 POP6 PXR1 RIX1 RLP7 RNA1 RNH70 ROK1 RPF2 RRP12 RRP5 RRP9 SAS10 SIK1 SLX9 SNU13 SOF1 SPB1 TSR1 TSR2 UTP10 UTP13 UTP14 UTP18 UTP20 UTP23 UTP4 UTP5 UTP6 UTP8 UTP9

- **rRNA modification**

CBF5 IMP3 NHP2 NOP1 NOP10 NOP58 RRP9 SIK1 SPB1

- **translation**

ALA1 ASC1 CDC33 CDC60 CLU1 COX14 DED81 EFB1 EFT1 EFT2 FRS1 FRS2 FUN12 GRS1 HYP2 ILS1 IMG1 IMG2 KRS1 MES1 MNP1 MRP1 MRP10 MRP13 MRP4 MRP51 MRPL1 MRPL10 MRPL13 MRPL19 MRPL20 MRPL22 MRPL24 MRPL25 MRPL27 MRPL28 MRPL3 MRPL31 MRPL32 MRPL33 MRPL37 MRPL40 MRPL44 MRPL50 MRPL6 MRPL7 MRPL8 MRPS17 MRPS28 MRPS35 MSE1 MSY1 MTO1 NAM9 PET123 RML2 RPL10 RPL11A RPL11B RPL12A RPL12B RPL13A RPL13B RPL14A RPL14B RPL15A RPL16A RPL16B RPL17A RPL17B RPL18A RPL18B RPL19A RPL19B RPL1A RPL1B RPL20A RPL20B RPL21A RPL21B RPL22A RPL22B RPL23A RPL23B RPL24A RPL24B RPL25 RPL26A RPL26B RPL27A RPL27B RPL28 RPL29 RPL2A RPL2B RPL3 RPL30 RPL31A RPL31B RPL32 RPL33A RPL33B RPL34A RPL34B RPL35A RPL35B RPL36A RPL36B RPL37A RPL37B RPL38 RPL39 RPL40A RPL40B RPL41A RPL41B RPL42A RPL42B RPL43A RPL43B RPL4A RPL4B RPL5 RPL6A RPL6B RPL7A RPL7B RPL8A RPL8B RPL9A RPL9B RPP0 RPP1A RPP1B RPP2A RPP2B RPS0A RPS0B RPS10A RPS10B RPS11A RPS11B RPS12 RPS13 RPS14A RPS14B RPS15 RPS16B RPS17A RPS17B RPS18A RPS18B RPS19A RPS19B RPS1B RPS2 RPS20 RPS21A RPS21B RPS22A RPS22B RPS23A RPS23B RPS24A RPS24B RPS25B RPS26A RPS26B RPS27A RPS27B RPS28A RPS28B RPS29A RPS29B RPS3 RPS30A RPS30B RPS31 RPS4A RPS4B RPS5 RPS6A RPS6B RPS7A RPS7B RPS8A RPS8B RPS9A RPS9B RSM22 RSM26 RSM27 RSM28 SSB1 SSB2 SUP45 SWS2 TEF4 THS1 TIF1 TIF11 TIF2 TIF35 TMA19 TUF1 VAS1 YEF3 ZUO1