Universidade de Aveiro Departamento de Biologia
2015

Ana Patrícia Figueiredo Fidalgo

The contribution of tRNA pool deregulation in the acquisition of a malignant phenotype

A contribuição da desregulação da *pool* de tRNA na aquisição do fenótipo maligno

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Ana Raquel Santos Calhôa Mano Soares, Investigadora de pós-doutoramento do Departamento de Biologia da Universidade de Aveiro.

À Tua memória.

o júri

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agradecimentos

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palavras-chave

Síntese proteica, *pool* de tRNAs, *codon usage*, stress proteotóxico, vias de controlo de qualidade proteica, tumorigénese.

resumo

Os tecidos humanos exibem diferentes padrões de expressão de tRNAs que se correlacionam com o *codon usage* de genes altamente expressos. Isto pode representar um controlo ao nível da tradução tendo em conta que os tRNAs são intervenientes importantes durante a síntese proteica. Nas células cancerígenas é observada a desregulação de componentes do processo de tradução, sendo que elevados níveis de tRNAs específicos estão correlacionados com a expressão preferencial de genes relacionados com o cancro. Assim, a desregulação da *pool* de tRNAs pode aumentar a eficiência de tradução desses genes, promovendo a transformação maligna.

O processo tumorigénico é acompanhado por aumento do conteúdo proteico celular. A evasão à apoptose, a instabilidade do genoma e as mutações frequentes são também observadas em células cancerígenas. Isto pode promover a acumulação de proteínas mutantes que desencadeia o *stress* proteotóxico e a produção elevada de HSPs, de forma a contrariar a instabilidade proteica. Para além disso, elevados níveis de proteínas incorretamente enoveladas, derivadas da elevada taxa de síntese proteica, induzem outras vias de controlo de qualidade proteica que auxiliam a ação dos chaperones: a resposta das proteínas não enoveladas e o sistema de degradação associado ao retículo endoplasmático.

Este estudo teve como objetivo avaliar a influência da desregulação da *pool* de tRNAs na aquisição do fenótipo maligno e a contribuição das vias de controlo de qualidade proteica na transformação celular. Globalmente, os resultados mostraram que a desregulação da *pool* de tRNAs induzida pela sobre expressão do $tRNA^{Ser}$ leva à aquisição de um fenótipo intermédio entre as células normais e as células cancerígenas. Assim, concluímos que esta desregulação pode representar um promotor da aquisição da malignidade celular.

keywords

Protein synthesis, tRNA pool, codon usage, proteotoxic stress, protein quality control pathways, tumorigenesis.

abstract

Human tissues display different tRNA expression patterns correlated with the codon usage of highly-expressed genes, which may represent a form of translational control since tRNAs are critical players during protein synthesis. In cancer cells, misregulation of the components of translational machinery is observed, namely elevated levels of tRNAs in a specific fashion, correlated with a preferential expression of cancer-related genes. Therefore, tRNA pool deregulation may enhance the translational efficiency of these genes, promoting the malignant transformation.

The tumorigenic process is accompanied by increased cellular protein load. Evasion of apoptosis, genome instability and frequent mutations are also observed in cancer cells. This may promote accumulation of mutated proteins that leads to proteotoxic stress and high production of HSPs, in order to counteract protein instability. Furthermore, high levels of misfolded proteins derived from the high rate of protein synthesis induce other protein quality control pathways to support the action of chaperones: the unfolded protein response and the endoplasmic reticulum-associated degradation system.

This study aimed to evaluate the influence of tRNA pool deregulation in the acquisition of a malignant phenotype and the contribution of protein quality control pathways in cell transformation. In general, the results showed that the deregulation of the tRNA pool prompted by $tRNA^{Ser}$ overexpression leads to the acquisition of an intermediary phenotype between normal cells and cancer cells. Therefore, we concluded that this deregulation may be a driven force for cellular malignancy.

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5’ UTR - 5’ Untranslated Region

A
A – Adenosine
aaRS – aminoacyl tRNA synthetase
aa-tRNA – aminoacylated tRNA
ALK - Anaplastic Lymphoma Kinase
AMP – Adenosine Monophosphate
ATF4 – Activating Transcription Factor 4
ATF6 – Activating Transcription Factor 6
ATP – Adenosine Triphosphate

B
BiP – Binding immunoglobulin Protein
bp –base pair

C
C – Cytosine
CMA – Chaperones-Mediated Autophagy
cMET - mesenchymal-epithelial transition factor
CSC – Cancer Stem Cell

E
EGFR - epidermal growth factor receptor
eIF – eukaryotic Initiation Factor
eRF - eukaryotic Release Factor
EML - Echinoderm Microtubule-associated protein-Like 4
EMT - Epithelial-Mesenchymal Transition
ER – Endoplasmic Reticulum
ERAD – Endoplasmic Reticulum Associated Degradation

G
G – Guanosine
GAP – GTPase Activating Protein
GDP – Guanosine-5’-Diphosphate
GTP – Guanosine-5’-Triphosphate
GPR78 - G Protein-Coupled Receptor 78

H
HSF1 – Heat Shock Factor 1
HSP – Heat Shock Protein
HSR –Heat Shock Response

I
IRE1α – Inositol Requiring kinase 1
IRES- Internal Robosome Entry Sites
ITAF – IRES Trans-Acting Factor

K
K-ras - Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

L
Leu – Leucine

M
MAPK – Mitogen-Activated Protein Kinase
Met - Metionine
mRNA – messenger RNA
mTOR – mammalian Target Of Rapamycin

N
NSCLC – Non-Small Cell Lung Cancer

O
ORF – Open Reading Frame

P
PABP – Poly(A) Binding Protein
PERK – PKR-like Endoplasmic Reticulum Kinase
PI3K - phosphoinositide 3-kinase
PIC – Preinitiation Complex
PKR – Protein Kinase RNA-activated
PPi – Pyrophosphate
PTC – Peptidyl Transferase Center

R
RB – Retinoblastoma protein
ROS – Reactive Oxygen Species
rRNA – ribosomal RNA

S
SCLC - Small Cell Lung Cancer
Ser – Serine
SUnSET - Surface Sensing of Translation

T
TFIIIB - RNA polymerase III-specific transcription factor
tRF – tRNA derived Fragment
tRNA – transfer RNA
Tyr – Tyrosine

U
U – Uridine
UPR – Unfolded Protein Response
UPS – Ubiquitin-Proteasome System

V
VCP – Valosin Containing Protein
VEGF - Vascular Endothelial Growth Factor

X
XBP1 - X-box binding protein 1

CHAPTER 1

V. Introduction

1.1.The Genetic Code

The central dogma of molecular biology states that the genetic information is coded in DNA molecules confined to the nucleus, which are transcribed in messenger RNA (mRNA) molecules that, in turn, will originate proteins in a process called translation. Translation is the last step of gene expression and it occurs according with the rules established by the genetic code, proposed by F. Crick in 1968.[1,2]

The mRNA is composed by codons that are non-overlapping nucleotide triplets, among adenosine (A), guanosine (G), cytosine (C) and uridine (U). Despite the 64 possible combinations between the ribonucleotides only 20 amino acids are coded, demonstrating the degenerative character of the genetic code (Figure 1).[3] The number of possible codons for each amino acid is variable, known as synonymous codons, but only one exists to start the translation of all proteins, the start codon methionine (AUG) and 3 stop codons (UAA, UAG, UGA) in eukaryotes to ensure the translation termination.[3–5] However, the universal genetic code is flexible since deviations to the canonical genetic code are described both in eukaryotes and prokaryotes, suggesting that it is still evolving.[6]

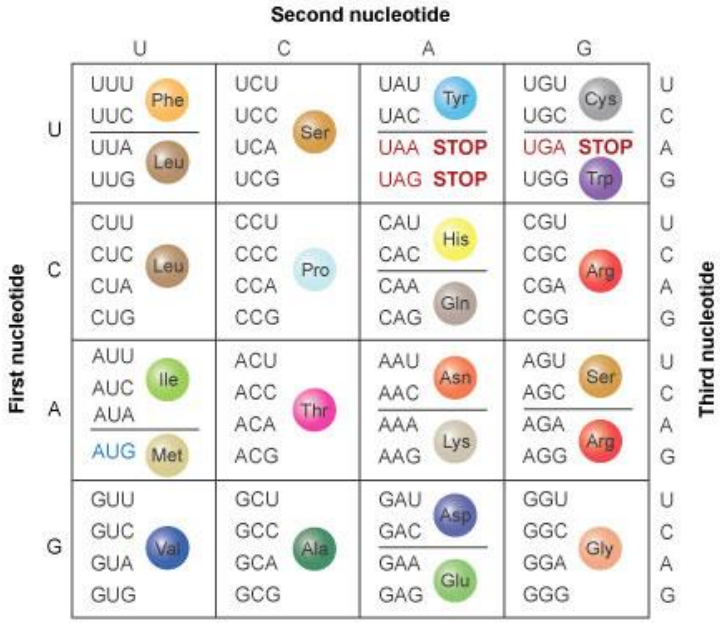

Figure 1. **The genetic code**. Clancy, S. and Brown, W. 2008

Amino acids are transported by transfer RNAs (tRNAs) that recognize the corresponding codon through interactions with its anticodon. The interaction between the first and second position of mRNA with tRNA is based on the canonical Watson-Crick pairing rules, by which an A or a G (purines) pairs an U or a C (pyrimidines), respectively. Furthermore, in 1996, F. Crick proposed that the third position, also known as the wobble position, could pair with the perfectly matched tRNA anticodon or adopt a non-canonical interaction to allow the same tRNA to recognize more than one codon, giving rise to the "Wobble Hypothesis".[7,8]

1.2.Codon Usage

The genetic code degeneracy allows a choice between different codons for the same amino acid in the transcriptome, which will affect the efficiency and accuracy of translation. This is known as codon usage. The codon usage has a close relation with the cellular availability in tRNAs (tRNA pool), that is regulated at several levels such as transcription, posttranscriptional processing, amino acid loading and degradation. The balance between these two factors affects protein production levels and the cellular fitness in a global view.[9–11] Non-optimal codon usage derived from poor correlation between codon usage and the tRNA pool may result in incorrect allocation of resources, namely the increase of ribosomes sequestration due to translation speed decrease, reducing the global cellular fitness.[12–14]

Highly expressed genes are often codon optimized to match the tRNA pool so they can be translated more efficiently. These genes are under a higher pressure for translational efficiency and accuracy, in particular the speed by which they are recognized by the ribosome and the corresponding tRNA selection. Thus, codon usage has a crucial role in modelling gene expression and can increase the expression of a gene more than 1000-fold. Moreover, alternative nucleotide sequences arising from synonymous codons may have a direct influence in protein folding and stability of secondary structures.[9–12,15]

The transcriptome's codon usage and the cellular tRNA pool are dynamic and adapted to biological conditions and tissue requirements, allowing different translation patterns accordingly with the cellular microenvironment.[12,15] Recently, Gingold *et al.* suggested that cells' content in tRNAs and the respective codons in the transcriptome have approximately

the same gene copy numbers and that changes in tRNA's basal levels are required to restore the balance between codon usage and tRNA pool when the first is altered.[15]

1.3.Eukaryotic Translation

Translation is the process whereby information encoded in mRNAs is converted in polypeptide chains. This process is carried out in the core of ribosomes, where the interaction between mRNA codons and acylated-tRNA anticodons is allowed.[16] Ribosomes are ribonucleoproteins, complexes with several proteins and ribosomal RNAs (rRNAs), comprising a large and a small subunit, known as the 60S and 40S in respect with its sedimentation rate. In their inactive state, ribosomal subunits are apart and they only form complexes in the presence of mRNA transcripts that need to be translated, acquiring distinct functions. The small subunit offers the suitable environment for interaction between tRNAs and mRNA codons, while in the large subunit occurs the formation of peptide bonds between the recently added amino acids.[16]

The ribosome moves along the mRNA transcript by reading the nucleotide triplets from its 5' end to 3' end, giving rise to the polypeptide chain from the N-terminal to the C-terminal. The ribosome has one binding site for mRNA chains and three binding sites for tRNAs, which are the A-site, the P-site and the E-site, allowing amino acid adding in three major steps: tRNA binding, peptide bond formation and large and small subunits translocation, in a processes fully described below.[16,17]

a. Initiation

The initiation of translation requires several eukaryotic initiation factors (eIFs) and their isoforms. Generally in eukaryotes and for most cellular mRNAs, initiation occurs in a CAP-dependent manner, with the start codon AUG being recognized through scanning of the mRNA transcript.[18,19] The ternary complex is the major player in the scanning process and is fundamental in CAP-dependent translation, comprising the eIF2-GTP (guanosine-5'-triphosphate) and the initiator $tRNA_i^{Met}$.[20–23] The α-subunit of eIF2 (eIF2α) is crucial for translation regulation during initiation. Its phosphorylation at Ser51 prevents GDP (guanosine-5'-diphosphate) recycling triggered by eIF2B, hindering the interaction of eIF2 with the additional ternary complex components and consequent inhibition of protein

synthesis. This inhibition usually occurs during stress conditions to reduce protein synthesis rate and to enhance the translation of mRNAs that allow the adaptation to stress and the recovery of translation.[23]

Anyhow, translation initiation only occurs in the presence of the 43S preinitiation complex (PIC) that comprises the ternary complex, the 40S ribosomal subunit and the factors eIF3, eIF1, eIF1A and probably eIF5. The mRNA 5' CAP is recognized by the multimeric eIF4F complex, composed by eIF4E, the CAP-binding protein stabilized by the ATP-dependent RNA helicase eIF4A, and the eIF4G, a scaffold protein that links the mRNA and the ribosome using the eIF3. This complex unwinds the structures in the 5' untranslated region (5' UTR) and, together with eIF3 and poly(A) binding protein (PABP), attaches the 3'-poly(A) tail allowing the PIC to scan mRNAs for the initiation codon.[20–23] When the initiation codon, localized in a favorable context as Kozak sequence, is recognized in the P-site by the anticodon of the Met-$tRNA_i^{Met}$ in the ternary complex, the scanning is arrested and GTP is irreversible hydrolyzed by the GTPase-activating protein (GAP) eIF5, consequentially releasing the eIF2-GDP and other eIFs. At this point, the eIF5B-GTP promotes the association between the 60S ribosomal subunit and the complex formed by the 40S small subunit, the initiation aminoacyl-tRNA and the mRNA chain. When GTP is hydrolyzed, the eIF5B is released from the ribosome, dictating the final of the initiation.[21–23]

However, CAP-independent translation represents an alternative to CAP-dependent translation both under normal and stress conditions, such as endoplasmic reticulum (ER) stress, hypoxia, nutrient deprivation, mitosis and cell differentiation and it is used not only by eukaryotic cells but also by virus that infect them as a strategy to express their mRNAs. At stress conditions, CAP-dependent translation is compromised leading to a significant increase in cellular Internal Ribosome Entry Sites (IRES)-mediated translation that recruits both canonical and non-canonical initiation factors, IRES trans-acting factors (ITAFs) and 40S ribosomal subunits.[24,25]

b.Elongation

Translation elongation is conserved among Eukarya, Bacteria and Archaea and it follows the initiation process.[21] In the beginning of elongation the P-site is occupied by the Met-$tRNA_i^{Met}$ and the A-site is empty. Then, the aminoacyl-tRNA whose anticodon complements

the second codon, or the cognate aminoacyl-tRNA, is added to the A-site due to the formation of another tertiary complex comprising the cognate aa-tRNA and the eEF1A-GTP. The interaction between this tRNA and the mRNA codon involves conformational changes in the decoding center of the 40S ribosomal subunit and the GTP hydrolysis ensures the presence of the cognate tRNA. If the complementarity is assured, the eEF1A-GTPase becomes activated. Then, eEF1A-GDP is released and the aminoacyl tRNA is accommodated in the A-site.[17,21,26,27]

The next step is the peptide bond formation between Met and the recently added amino acid, in the peptidyl transferase center (PTC) located in the large ribosomal subunit. The Met accommodated in the P-site moves to the A-site of this center and a peptide bond can be made with the second amino acid still attached to its cognate tRNA, in a peptidyl transferase induced reaction. Then, the P-site is occupied with a deacylated tRNA connecting the mRNA in the small subunit and the 3' CCA in the E-site of the large subunit. Also, the peptidyl tRNA is in a hybrid state as its acceptor arm is in the P-site and the anticodon arm forming a mRNA-tRNA duplex in the A-site of the small subunit.[21,26,27] The eEF2-GTP is required to restore the canonical positions in the A and P-sites, in a process named translocation. GTP hydrolysis turns the ribosome free to move temporarily three codons towards the mRNA 3' end. Posttraslocation state accounts for a deacylated tRNA in the E-site, a peptidyl-tRNA in the P-site and a new codon in the A-site to be decoded. At this point, a new codon can be read and this cyclic mechanism is repeated until a stop codon is detected.[21,27]

c. Termination

When the ribosome detects a stop codon in the A-site a signal to finish the translation and to release the polypeptide chain is recognized. Stop codon recognition can be made by the ribosome, by external factors that interact with the ribosome and by the combined action of both.[17,28] This signal activates the eukaryotic release factor 1 (eRF1), a class I release factor that binds the ribosome at the A-site. It is responsible for high-fidelity stop codon recognition and peptidyl-tRNA hydrolysis, promoting the addition of a water molecule to the peptide chain.[27] eRF1 may also open a channel that triggers the incoming of water molecules into the ribosome; engender conformational changes in the ribosome so the water molecule can reach the active center; activate the water molecule and the ester bond so peptidyl-tRNA hydrolysis can occur.[21] This step destroys the attachment of the carboxyl end

to the ribosome, and the recently synthetized polypeptide is finally released. eRF1 activity and connection with the ribosome is supported by a class II release factor, the eRF3-GTPase whose hydrolysis removes eRF1 from the ribosome.[17,21,28]

d.Recycling

The ribosomal subunits have to be recycled after the synthesis of a newly polypeptide chain, so another translational cycle could be performed. Therefore, the mRNA chain and the deacylated-tRNA have to be released as well as the ribosomal subunits have to be separated in a process called recycling, a mechanism that lacks fully explanation in the eukaryotic model.[27]

Nevertheless, partial dissociation of ribosomes is also observed in a mechanism termed reinitiation. In these cases, the ribosome translates further Open-Reading Frames (ORFs) in the same mRNA without complete recycling of the ribosomal machinery. In this way, translation of the same ORF can be done, through incomplete recycling potentially taking place in the stop codon, allowing scanning along the 3' UTR and triggering the 40S transference to the 5' UTR. Although interactions involved in this process are not completely known, there is a potential hypothesis based on the role of PABP, eRF3, eIF4E and eIF4G in a mechanism that potentiate a close proximity between the mRNA 5' and 3' ends.[21,27]

The eIF3 has an active role in the recycling process in higher eukaryotic translation, since it directly binds to and induces conformational changes in the 40S small subunit surface avoiding its reconnection with the 60S large subunit until a new mRNA emerge to be translated.[20,21,27]

1.4.Transfer RNAs

Transfer RNAs (tRNAs) are universally conserved small and ubiquitous RNA molecules that are the interface between the genetic information encoded in mRNA transcripts and the proteins. [29,30] The secondary structure of tRNAs (Figure 2) has a cloverleaf shape derived from a single nucleotide chain with 73 to 90 nucleotides in length and 4 domains organized in unpaired and paired regions: acceptor arm, D arm, anticodon arm and TΨC arm. The acceptor arm has a 3' CCA tail that links covalently the cognate amino acid, and the anticodon arm has a three nucleotide sequence in the unpaired region, the anticodon loop,

that interacts with the mRNA codon. Unpaired regions also mold loops in the D and the TΨC arms and an unpaired region between the anticodon and the TΨC arms represents the variable arm, also known as extra arm. [16,29–31] The size of the variable arm can range from 3 to 21 bases and dictates tRNA grouping in class I or II. The first includes the majority of tRNA molecules and is characterized by small extra arms, while class II tRNAs, comprising Leucine (Leu), Serine (Ser) and Tyrosine (Tyr), have larger variable arms and a R_{13}-R_{22} base pair (bp) in the D-loop.[16,32]

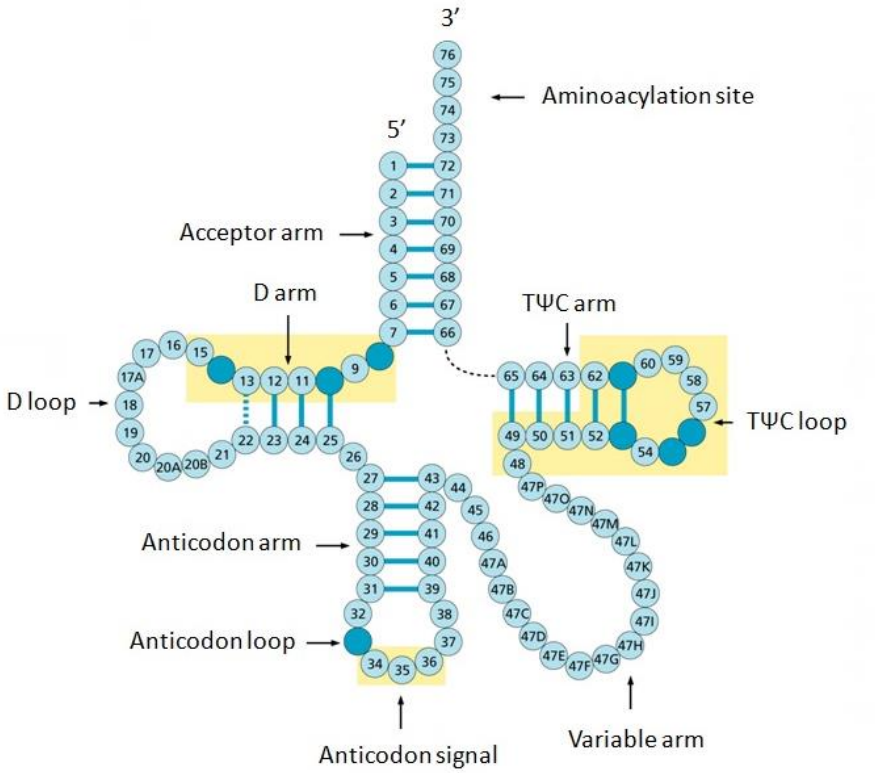

Figure 2. **tRNA secondary structure**. Adapted from Zvelebil, M. and Baum, J. O. 2007

Functional and mature tRNAs acquire a L-shape tertiary structure through the stacking between acceptor stem and TΨC arm and between D-arm and anticodon arm, both stacks forming a continuous A-helix. Thus, functional tRNAs are characterized by an amino acid accepting branch where cognate amino acid covalently links an adenosine in 3' CCA tail and an anticodon branch that connects with the codons in mRNAs. This highly stable structure is obtained from the interaction between conserved and semi conserved nucleotides in the tRNA chain and it is substantially altered according to its functional state.[29–31,33]

tRNAs are codified by 506 genes [Genomic tRNA Database] that are transcribed by the RNA polymerase III, whose activation is regulated by cellular nutrient availability and other environmental features. To become functional, tRNA transcripts should be processed to get the standard length and be posttranscriptionally modified (Figure 3). Still in the nucleus, the ribonucleoprotein RNase P performs endonucleolytic cleavage of the 5' ladder sequence in precursor tRNA. Then, endonucleases (such as RNase E and RNase III) cleave the 3' trailer extensional sequence and exonucleases (such as RNase T and RNase PH) cleave the residual trailer sequence. In some cases where CCA tail is absent, it is added by the CCA-adding enzyme following the discriminator base N_{73}. Next, the splicing of remaining introns occurs, as well as modification of several nucleotide residues.

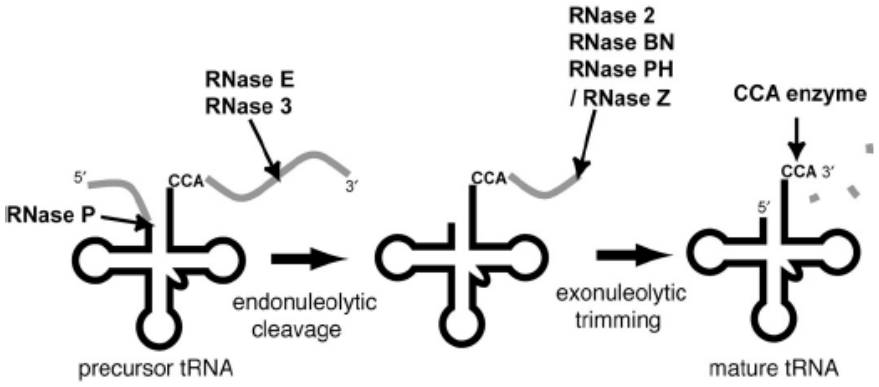

Figure 3. **tRNA processing**. Nakanishi, K and Nureki, O. 2005

These posttranscriptional modifications are divided in two types, according to its target: modifications in the loops of D and TΨC arms have the purpose to stabilize the tertiary L-shape structure, while modifications in the anticodon loop promote precise codon pairing and accurate recognition by the cognate aminoacyl tRNA synthetase (aaRS).[33,34]

In spite of tRNAs being mainly associated with its role during translation, these molecules also participate actively in other cellular functions, both in prokaryotic and eukaryotic cells. Uncharged tRNAs act as signaling molecules that activate cellular stress responses in the presence of nutritional stress, to stimulate the expression of genes related to amino acid synthesis and their uptake and to aminoacyl-tRNA synthetases, thus contributing to cell survival.[29,31] Additionally, tRNAs are mediators in non-ribosomal processes, as the peptide bond formation between peptidoglycans in bacterial cell wall biosynthesis and in the modification of phospholipids that form the cell membrane.[35] These molecules can also label

proteins for degradation, participate in the regulation of apoptosis and in antibiotic biosynthesis.[31,36]

Recently, tRNA-derived fragments (tRFs), resulting from tRNA degradation, were also found to be functional forms that can actively participate in regulation of gene expression at translational level under stress conditions and in gene silencing.[31,37,38]

Since tRNAs can have a variety of functions, it is not surprising that their biosynthesis occurs under several control pathways, which recognize and degrade misfolded or hypomodified forms. Also, tRNAs account for more than 15% of total RNA amount and comprise one of the most abundant transcripts in the cells.[29]

1.5.Aminoacyl-tRNA Synthetases

Aminoacyl-tRNA synthetases (aaRSs) are a highly conserved enzyme family that catalyzes tRNA aminoacylation with the cognate amino acid. The aminoacylation reaction occurs in two steps, illustrated in Figure 4. From the first step results a stable complex between the active site of aaRS and the cognate amino acid, in an ATP dependent reaction. In this way, a firmly bounded aminoacyl-adenylate is formed, releasing a pyrophosphate (PPi). In the second step, the tRNA binds the amino acid by the 3' end adenosine in the acceptor stem, releasing AMP and esterifying the tRNA at 3' end.[39]

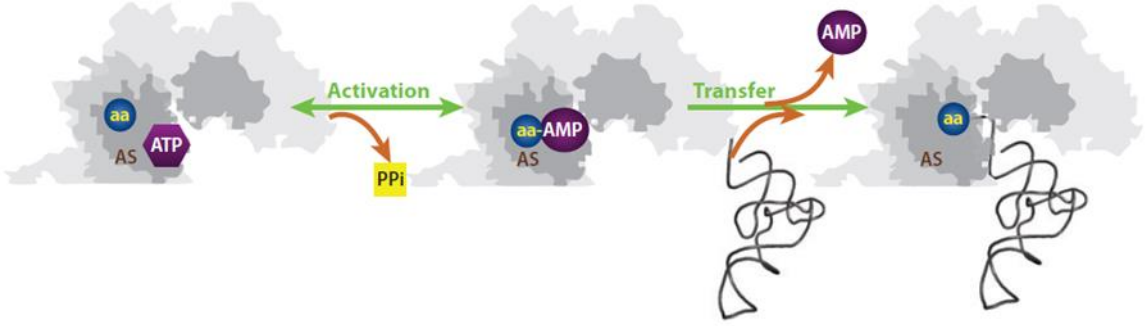

Figure 4. Schematic representation of **aminoacylation reaction**. AS, active site; aa, amino acid; PPi, pyrophosphate. Jiqiang Ling, J. *et al*. 2009

The recognition of tRNAs by its cognate aaRSs is based on the interaction with specific elements, as the discriminator base N_{73}, the acceptor stem and the anticodon region. Less often, interactions with elements in the extra arm, the D stem, the core of the tRNA tertiary

structure and the phosphate backbone or with modified nucleotides and the wobble base can occur.[39]

To avoid inaccuracy derived from tRNA charging with the incorrect amino acid, aaRSs have an editing capacity encoded by a different active site. The double-sieve model suggests that the editing capacity allows aaRSs to discriminate amino acids with similar properties, and it can occur before or after amino acid transference to the tRNA, the pretransfer editing and posttransfer editing, respectively. Pretransfer editing is based on the hydrolysis of amino acids incorrectly activated before transference to the tRNA, while posttransfer editing requires the interaction between the tRNA 3' CCA tail with the aaRS editing site. Trans editing was recently proposed and relies on the capacity of other proteins to recognize and hydrolyze incorrectly charged tRNAs.[40–42] Moreover, the editing capacity is wider and it can act through discrimination between tRNAs by direct interaction, recognition of the acceptor stem nucleotide sequence and by contact with key nucleotides in the tRNA.[17,41] This editing capacity is essential to avoid protein synthesis errors and its disturbance can be associated with various pathologies, which severity varies inversely with the editing capacity.[40]

1.6.Protein Folding and Misfolding

Proteins are the most structurally and functionally complex molecules, thus its activation requires more than its translation. After leaving the ribosome, the polypeptide chain has to be modified to get its unique three-dimensional conformation. Protein-modifying enzymes bind small-molecule cofactors essential for protein activity or assemble proteins with other protein subunits.[17,43]

The information necessary for these modifications is encoded by the amino acid sequence itself and its biochemical properties. The interaction between amino acids from different regions of the polypeptide occurs by weak non-covalent bonds that taken together determine the protein folding stability. Also, folding is influenced by the hydrophobic character of the side chains, as the non-polar tend to cluster in the interior of the molecules forming a hydrophobic core, while polar amino acids are exposed and form hydrogen bonds with other molecules. These interactions result in a three dimensional structure that lacks the conformation of lowest free energy, without losing the flexibility to interact with other molecules that may influence protein function.[16,17,43]

Incorrect folding results in misfolded and nonfunctional proteins characterized by exposed hydrophobic amino acids. Alterations in cell homeostasis caused by stochastic fluctuations, destabilizing mutations, stress conditions or metabolic alterations resulting from processes such as cancer and aging, trigger wrong interactions, unfolding or denaturing of proteins and inhibit interactions between subunits of larger protein complexes. These alterations can be dangerous for the cell because misfolded/unfolded proteins can aggregate in toxic forms and give rise to several human diseases. Thereby, cells account with several mechanisms to maintain the stability of the proteome, ensuring misfolded proteins refolding, degradation or sequestration.[17,44–46]

1.7.Proteotoxic Stress and Protein Quality Control Pathways

The endoplasmic reticulum (ER) is the cellular compartment responsible for structural maturation of one-third of all eukaryotic proteins. Proteins remaining in the cytoplasm and mitochondria are under constant vigilance of chaperones responsible for its maturation. Proteins matured in the ER, when leaving this compartment, no longer need the assistance of chaperones, meaning that only correctly folded proteins leave the ER. In this way, highly sophisticated and robust quality control systems are required to avoid release of aberrant proteins to perform their functions.[43,47] However, as a result of errors during transcription and translation, aberrant proteins are produced and its accumulation leads to homeostatic perturbations and proteotoxic stress.[2,48,49]

To counteract the proteotoxic stress, several quality control responses are activated to promote the refolding of misfolded proteins or, if not possible, its degradation. The activation of these pathways aims to restore homeostasis and to avoid apoptosis induction. Homeostasis is achieved by upregulation of the ER folding capacity, increasing chaperones availability and foldases as well as ER size; through downregulation of biosynthetic load, inhibiting protein synthesis at transcriptional and translational levels; and increasing elimination of unfolded proteins through upregulation of Endoplasmic Reticulum Associated Degradation (ERAD) protein clearance mechanism.[2,45–47,49] It is estimated that 30% to 70% of proteins do not pass the quality control mechanisms and end up being degraded.[50]

If cells are unable to activate these control pathways or if these pathways become overloaded, waste of nutrients occurs and misfolded and nonfunctional proteins that can aggregate in toxic forms are produced, dictating cell death as the final fate.[2]

a. Chaperones and Heat Shock Response

Molecular chaperones are proteins that assist noncovalent folding of newly synthesized polypeptides, assembly of protein subunits, preventing or reversing incorrect folding. This large protein family, first discovered in 1978 by Laskey *et al.*, recognizes and binds reactive surfaces exposed in the client protein, avoiding incorrect interactions. Still, chaperones assist oligomeric structures synthesis in the transport of proteins through membranes, by preventing the three-dimensional folding that can be a hitch in the membrane crossing, maintaining the protein in an unfolded and more flexible status. Chaperones can also signal the protein for degradation.[16,46,51]

A significant number of chaperones are designated heat shock proteins (HSPs), term that derives from its overexpression in cells exposed to higher temperatures. While chaperones have the folding as their major role during normal metabolism, stress conditions induce higher levels of HSPs that assist the refolding and repair of damaged proteins, to prevent protein denaturation and aggregation. HSPs derive from the transcription of distinct gene families and they are classified according to their approximate relative molecular mass in five main HSP families: Hsp90, Hsp70, Hsp60, small HSPs and large HSPs. The genes encoding chaperones have three classes regulated differently: constitutively expressed chaperones, during growth and development; constitutively and induced chaperones and strictly induced chaperones.[51–54]

Regarding their action, HSPs can be classified, not exclusively, in holding and folding proteins. The first group comprises HSP70 and HSP90 families and they recognize and bind exposed hydrophobic domains in unfolded polypeptides. They can act during mRNA translation to prevent premature self-association of nascent polypeptide chains; during heat shock response through interaction with totally or partially unfolded proteins and constitutively to bind unstable tertiary structures. In this process a large complex is formed, comprising five core proteins that accounts for Hsp90, the scaffold protein Hop, the p23 protein as mediator of substrate selection and the complex HSP70/HSP40 to mediate the interaction between HSP90 and the client protein. These complexes interact with many

accessory proteins, also known as co-chaperones, to facilitate substrate selection and cycles of association and dissociation from the client protein.[16,44,54]

Folding activity can be performed individually by Hsp70, Hsp40 or co-chaperone GrpE or in a chaperonin system comprising a large oligomeric assembly that lodges unfolded proteins and promotes a suitable environment for their folding. Also, Hsp90 together with Hsp70 acts directly with specific classes of proteins involved in signal transduction, thereby maintaining the target in an appropriate function until it is stabilized through the interaction with other components of the pathway.[16,44,54]

The transcription of HSPs is dependent on heat shock factor 1 (HSF1) transcription factor that binds the promoters of *HSP* genes, inducing HSP mRNA transcription. Activation of HSF1 is still under investigation but some explanations were proposed. HSF1 is inhibited by its products, which in stress conditions may be sequestrated in protein aggregates, thus de-repressing HSF1. Another mechanism for HSF1 activation relies on the heat-induced binding to large non-coding RNAs, which are known to be involved in the regulation of a wide range of genes, complexed with eEF1A. Besides, during stress conditions, a rapid phosphorylation of serine 326 in the HSF1 occurs, correlated with the onset of *HSF* gene transcription, suggesting that this serine has a role in stress response regulation prompted by HSF1: the heat shock response (HSR).[52,53]

The HSR is a cellular mechanism activated in the presence of a wide range of stress conditions that triggers protein inactivation. This response is based on a combination of events called thermotolerance, a condition directly related with the coordinated synthesis of HSPs. [44,52,53]

b. Unfolded Protein Response

The UPR relies on the activation of different but complementary signal pathways triggered by three ER transmembrane proteins: the inositol requiring kinase 1 (IRE1α), the double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) and the activating transcription factor 6 (ATF6), schematically represented in Figure 5. During homeostatic conditions these proteins are inactivated by formation of stable complexes with the ER stress sensor GPR78, also known as binding immunoglobulin protein (BiP). However, presence of unfolded proteins leads to a competition for BiP, derepressing the signal pathways.[45,55,56]

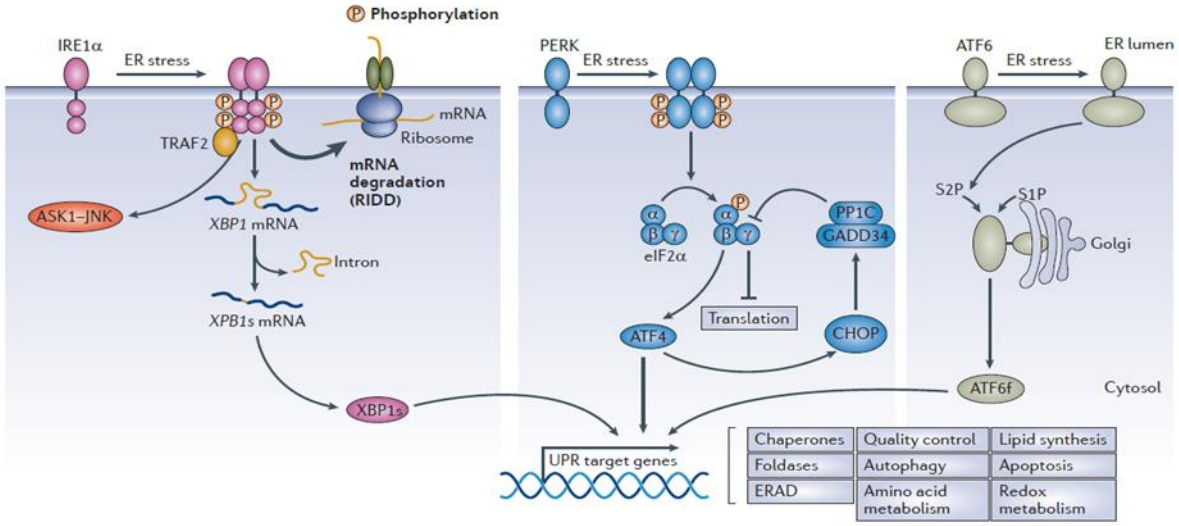

Figure 5. Schematic representation of the **unfolded protein response**. Adapted from Claudio Hetz *et al.* 2013

When IRE1α is dissociated from BiP, it undergoes dimerization and autophosphorylation, activating its endonuclease activity for mRNA processing. IRE1α activates the X-box binding protein 1 (XBP1), through splicing of its mRNA transcript. When activated, XBP1 upregulates the expression of genes encoding proteins that have roles in protein folding, insurance of its quality and ERAD activation.[45,55–57]

After being released from BiP, PERK becomes activated through dimerization and autophosphorylation, activating its kinase activity. PERK activation prompts global protein synthesis inhibition through inhibitory phosphorylation of eIF2α, to reduce the influx of newly synthetized proteins into the ER. The downregulation of translation upregulates the activating transcription factor 4 (ATF4) that promotes cell survival since it regulates genes involved in oxidative stress, amino acid synthesis, protein folding and cell differentiation. [45,55–57]

Finally, ATF6 is released from BiP and translocated to the Golgi compartment. There, ATF6 is cleaved by the site-1 and site-2 proteases releasing its N-terminal fragment, the cytoplasmic domain, which acts as a transcription factor. In the nucleus, ATF6 fragment promotes upregulation of UPR target genes, such as XBP1, and ERAD associated proteins.[45,55–57]

c. Endoplasmic Reticulum Associated Degradation Response

ERAD is fundamental to eliminate misfolded proteins, maintaining the equilibrium between protein synthesis and degradation, in a demand to prevent proteotoxicity, ER stress and subsequent apoptosis.[57]

The ERAD response promotes degradation of aberrant proteins by the Ubiquitin-Proteasome System (UPS), the major eukaryotic proteolytic pathway. This process is initiated with the recognition of substrate and its translocation across the ER lipid bilayer to the cytoplasm. In the cytoplasm, proteins are polyubiquitinated, the signal for degradation by the 26S proteasome subunit.[46,58]

d. Autophagy

Autophagy is an evolutionarily conserved protein and organelle degradation mechanism with a complex molecular background, missing full explanation. Autophagy can be characterized into three different types: macroautophagy, microautophagy and chaperones-mediated autophagy (CMA). All end up with the delivery of the target, the cargo, into the lysosome where it occurs protein unfolding and degradation by the action of proteases.[59]

Regarding the protein quality control, the autophagic process represents an important mechanism to eliminate aggregation prone proteins. It functions as a backup of ERAD since its impairment leads to an upregulation of autophagy, as a strategy to recognize and degrade protein aggregates.[46,60] Also, autophagy has a role during stress caused by nutrient and oxygen deprivation, and biosynthetic and homeostatic functions. Examples are the degradation of mitochondria, known as mitophagy, as an approach to control their reliability and to avoid the production of reactive oxygen species (ROS), or the degradation of peroxisomes that are no longer needed, the pexophagy. [59,61]

1.8.Tumorigenesis

The tumorigenic process starts with a normal cell that becomes transformed by acquisition of autonomous proliferation features driven by accumulation of genetic alterations, leading to its immortalization. Acquisition of malignancy is the result of tumour progression rather than transformation. During tumour progression, cancer cells lose their original shape and polarity. Finally, they acquire the capacity to invade nearby tissues and metastasize to distant locations through the blood or lymphatic system.[62,63]

Variability is an intrinsic characteristic of tumors that promotes functional and phenotypic heterogeneity in tumors established in the same organ, known as intertumoral heterogeneity, and even within individual tumors, the intratumoral heterogeneity.[62] The intertumoral heterogeneity is associated with different genetic and epigenetic mutations, distinct cells in the origin of tumor, different molecular profiling characterization, different expression of specific markers and different morphological features.[62]

Intratumoral heterogeneity can be explained by the cancer stem cell (CSC) and the clonal-evolution models.[62] The first proposes that the tumor has its origin in normal self-renewing stem cell or downstream progenitor with limited or no self-renewal. Considering that the expansion of the progenitor cell may originate downstream cells which accumulate different mutations, CSCs can originate different clones within the same tumor, thus contributing to its heterogeneity.[64,65] In turn, the clonal evolution model suggests that a single cell accumulates mutations that are hereditarily transmitted through successive generations and the most advantageous for the tumor are selected through natural selection.[64] The genetic and epigenetic changes which provide the cell with the more aggressive, invasive and drug-resistant phenotype are those prevailing. However, these models cannot be applied in a mutually exclusive characterization since all the events interact to define the tumor histopathology and behavior.[62,64,65]

In a global view, cancer cells can be characterized based on traits that enable them with distinct but complementary capabilities. These features allow tumor growth and metastatic dissemination and they are known as the hallmarks of cancer (Figure 6). The acquisition and development of these hallmarks are possible due to genome instability, product of several random and particular genetic mutations and chromosomal rearrangements, and the inflammatory state of established or premalignant lesions that is supported by immunologic cells.[66]

Figure 6. The **hallmarks of cancer** and enabling characteristics. Adapted from Hanahan, D. and Weinberg, R, 2011.

Cancer cells present deregulation of growth-promoting signals, leading to sustained proliferative signaling. Moreover, cancer cells are also able to evade growth suppressors that could inhibit its proliferation.[66,67]

The programmed cell death is essential to prevent cancer development. The most relevant player in responses to stress, damage and regulation of apoptosis is the p53. In cancer cells, mutations in *TP53* gene or in its upstream or downstream effectors are frequent, allowing apoptosis evasion, invasion, metastasis, proliferation and cell survival.[66,68,69]

Proliferation of normal cells is limited by a number of successive division cycles in a process controlled by the size of telomeres, which triggers cell death when they become too short to protect the chromosomes. In cancer cells, the DNA telomerase is highly expressed, opposing to what happens in normal cells, which is correlated with resistance to senescence and acquisition of replicative immortality.[66]

Cancer cells are also associated with the capacity to induce angiogenesis, in a process triggered by deregulated proangiogenic signals that give rise to aberrant neovascularization. Considering that tumor microenvironment is characterized by hypoxia, nutrient deprivation and low pH, angiogenesis may be induced as an alternative to obtain nutrients and oxygen and to discard metabolic wastes.[66]

Lastly, malignant cells have the capacity to invade, to avoid apoptosis and to disseminate, in a process called metastization, closely related to the regulatory program "epithelial-

mesenchymal transition" (EMT). The reprogramming of energy metabolism and the capacity of tumor cells to avoid immune surveillance are now emerging as new hallmarks of cancer, as major evidences are rising to prove its crucial role in cancer development.[66,70]

1.9.Lung Cancer and Non-Small Cell Lung Cancer

Cancer is the leading cause of death in the world with a continuous increase in the number of cases, particularly in developing countries, estimated at over 20 million per year at 2025.[71] Lung cancer represents the main cancer related death and it is associated to cigarettes active smoking, followed by passive smoking and occupational exposure to chemical and physical carcinogenic agents as nickel, asbestos, arsenic, radiation and air pollution. Susceptibility to lung cancer is also allied with individual inherent susceptibility to these agents and familiar history of lung cancer, without overlooking lifestyle factors as diet and physical inactivity, established diagnosis of acquire lung diseases and HIV related infections.[72] The stage and degree of tobacco epidemic have a close relationship with countries socioeconomic development. Countries that had its smoking peak in the middle of 20th century are currently detecting a decrease in lung cancer rates; instead, countries where tobacco consumption epidemic was established recently are handling increasing rates. Yet, as the rate of smokers decreases it is observed a greater frequency of lung cancer among passive smokers. Another bequest of socioeconomic development is the increase in smoking habit among women, resulting in 50% of cancer related death in this gender and 80% in men.[72–74]

Lung cancer is classified in two major subtypes based on histological features and response to conventional therapies: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the last accounting for 85% of all lung cancers. SCLC type can be also divided in classical small cell carcinoma, large cell neuroendocrine and combined, while NSCLC comprises adenocarcinoma, squamous cell carcinoma and large cell carcinoma.[74–76]

Lung cancer has a 5-years survival rate of 15%, as result of late diagnosis of advanced tumors.[74,75] Diagnosis of lung cancer is achieved by analysis of complete medical history and physical examination that can reveal suggestive signs and symptoms of lung disease, such as alterations in expectoration quantity, amount and presence of blood, shortness of

breathing, wheezing, chest pain and frequent respiratory infections. Then the physician should proceed with more conclusive and chest located tests as radiography and also computed tomography and magnetic resonance imaging, which can identify metastatic episodes and support a differential diagnosis. Adenocarcinoma and large cell carcinoma have preferential localization in the periphery of lung, lining the small airways while squamous cell carcinoma and SCLC have their origin in epithelial cells that line the larger airways in the central area of chest. At the cellular level, cytological analysis can be made after expectoration collection, being enough to diagnose 80% of lung tumors. To improve diagnostic capacity and precision, a bronchoscopy and fine-needle biopsy of lung and metastatic lesion or lymph node may be done.[75,77]

NSCLC has a complex molecular character underlying his pathogenesis that is not fully understood. However, it is crucial to understand and characterize diagnostic and prognostic biomarkers and therapeutic targets so worry rates associated to lung cancer can be weakened.[78] The epidermal growth factor receptor (EGFR) is responsible for the activation of phosphoinositide 3-kinase (PI3K)/AKT and RAS/RAF/MAPK pathways that ultimately lead to the active transcription of genes involved in cell survival, proliferation, angiogenesis, invasion and metastasis. Mutations in *EGFR* are presented in 10% of NSCLC cases and are closely related with tobacco consumption, being inversely proportional to the degree of smoking.[76,79] Mutations on the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-*ras*) gene are present in 20-30% of NSCLCs, with influence on cell proliferation and apoptosis via MAPK and on cell survival responses via PI3K. These represents 90% of all *ras* mutations in adenocarcinomas and they are common in smokers and patients that were exposed to asbestos, being more prevalent in women.[76,79,80]

Despite those factors are markedly involved in the carcinogenic process of NSCLC, others have been identified. The vascular endothelial growth factor (VEGF) is highly expressed in NCSLC and it is critical in physiologic and pathologic angiogenesis, through promotion of survival and tumor growth. Its overexpression relates to poor prognosis.[76] Similarly, overexpression, amplification or gain-of-function of the mesenchymal-epithelial transition factor (c-MET) are evident in lung adenocarcinoma, participating in tumor growth, differentiation and metastasis and thus contributing to a poor prognosis.[77,81,82]

The rearrangement of the anaplastic lymphoma kinase (*ALK*) gene derived from the fusion with the echinoderm microtubule-associated protein-like 4 (*EML4*) gene was

classified as a gain of fusion mutation. *EML4-ALK* fusion was identified in 2% to 7% of NCSLC, in the absence of *KRAS* and *EGFR* mutations, with a positive influence in neoplastic transformation and cell survival.[76,83]

1.10. tRNA Pool Deregulation in Cancer

The deregulation of protein synthesis machinery and tRNA pool, in particular, are observed in a wide range of tumors, suggesting that misregulation of translation components are involved in malignant transformation.[84]

Pavon-Eternod *et al*. observed a significant overexpression of tRNAs in breast cancer cell lines and tumor tissues when compared with non-cancer-derived breast epithelial cell lines and normal breast tissues.[85] In particular, $tRNA^{Ser}$, $tRNA^{Tyr}$ and $tRNA^{Thr}$ were the most expressed and its overexpression seems to favor the codon usage of cancer-related genes, important in tumor initiation and progression, but not the house-keeping genes and cell-line specific genes. Also, the overexpressed tRNAs carry polar amino acids that are targets of protein kinases and phosphatases, demonstrating a possible mechanism for potentiating posttranslational regulation of proteins involved in signal transduction.[85] Notably, the overexpression of tRNAs in cancer cells is accompanied by altered metabolic activity and unregulated growth.[86]

Differential expression of tRNAs regulates the efficiency of translation through the codon usage of specific genes and it is possible that during active cell growth there is a correlation between the tRNA pool and the codon usage of highly translated genes.[85] So, deregulation of the tRNA pool in cancer may be responsible for the quantitative and qualitative alterations in protein expression, since this deregulation leads to preferential expression of key proteins in tumor progression and development, such as growth factors, cell-cycle promoters and oncoproteins, particularly c-Myc and VEGF, the last known to be upregulated in NSCLC tumors.[76,85,86]

The deregulation of RNA polymerase III is also observed in several tumors, explained by the releasing of pol III-specific transcription factor TFIIIB from the inhibitory effect provided by retinoblastoma protein (RB) and p53, which are often mutated in cancer cells.[84,85] Also, TFIIIB is activated by the upregulation of c-Myc and mutations in *KRAS*, alterations observed in NSCLC cells.[80,87]

Cellular stress leads to an alteration in the codon usage of demanded genes and so the request for tRNAs by certain codons increases and the availability in tRNAs for other codons decreases. It is also known that codons that became preferentially expressed under stress conditions corresponded to tRNAs that are represented by the lowest gene copy number.[15]

In another study, Pavon-Eternod *et al*. verified that induction of the elongator $tRNA_e^{Met}$ does not alter significantly its levels but punctual changes in levels of several other tRNAs not induced experimentally are observed. This can be explained by a regulatory feedback mechanism related with overexpression of $tRNA_i^{Met}$.[86]

Nonetheless, the knowledge available about effects derived from overexpression of tRNAs and the mechanisms of response from cells to these perturbations is very limited yet.[86]

1.11. Proteotoxic Stress in Cancer

The process of malignant transformation is accompanied by increased protein load. Likewise, evasion of apoptosis, genome instability and frequent mutations are observed in cancer cells, which may promote accumulation of mutated proteins and sustained activation of proliferation signals.[88] Qualitative and quantitative alterations of the proteome induce cellular proteotoxic stress that is faced by the induction of HSPs as an attempt to repair and refold aberrant products. In cancer cells, an increase in HSF1 levels is observed through a process not yet understood but that may be related with increased transcription and translation as well as epigenetic regulation.[53,88]

The relation between increased chaperoning capacity and tumorigenesis can be explained by the "addiction to chaperones" hypothesis. This assumption is based on the increased requirement for chaperoning the larger protein load due to rising in mRNA translation derived from highly metabolic and proliferative rates and the polyploidy observed in several cancer cells. The instability created in the proteome is supported by chaperones in an addictive process so high protein expression and gene mutations can support tumor growth and progression. Interestingly, in studies where the Hsp90 chaperone is inhibited downregulation of several oncogenes is observed, probably because the instability of mutant and aberrant proteins is not counteracted and they are eliminated by quality control pathways.[53]

Moreover, HSPs activity in cancer cells supported by HSF1 seems to affect a wide range of pathways that are essential to sustain the malignant phenotype. Evidences exist to prove their role in the acquisition of cancer hallmarks, excluding the evasion of growth suppressors where the role of chaperones is still inconclusive.[53] Hsp90 seems to be the main chaperone

in tumorigenesis, acting to maintain the active conformation of mutant and signal proteins as an approach for faster information flux in response to extracellular signals, essential to development and cell renewal.[53,54] Hsp90 and Hsp70 overexpression in cancer allows to stabilize precancerous proteins, such as growth factor receptors, survival-signaling kinases, oncogenes and mutated proteins, supported by Hsp27 that is known to be upregulated in many cancers.[53,89] In the NSCLC context, low levels of HSP90 are correlated with better prognosis and pharmaceutical inhibitors of Hsp90 also impair EGFR activity.[90]

Cancer cells grow in an environmental context that differs from normal cells as it is hypoxic and with lower pH and nutrient availability, as a result of low vascularization. The higher tumor growth rate outdoes new blood supply, although in an inefficiently manner because the synthesis of new vessels is aberrant and the blood flow dynamics are altered.[57,91] The low nutritional availability directly influences protein glycosylation and ATP production, which triggers synthesis of misfolded proteins, an event also supported by the lack of oxygen that is crucial for disulfide bond formations in proper protein folding. These perturbations lead to ER stress with consequent activation of quality control pathways, at which cancer cells can adapt and so escape to apoptosis.[57,92]

Cancer cells have high activation of the UPR and take advantage from activation of characteristic signal pathways, in a chronic manner. BiP is known to be increased in aggressive malignant forms, including lung cancer and it seems to promote cell survival, tumor progression, metastasis and resistance to therapeutics.[57,93] This is possible since overexpression of BiP has a crucial role in pro-survival and cytoprotective responses in malignant cells, by diverse mechanisms such as inactivation of caspase-7.[57,94]

Despite its role in tumorigenesis is not entirely explained, IRE1α inhibition appears to prompt reduction in tumor growth, in angiogenesis and in blood perfusion of tumor. It is known that IRE1α induces cellular proliferation through XBP1 splicing and the deletion of *Xbp1* increases sensitivity to hypoxia-induced cell death and consequent decrease in tumor formation capacity, since it is a major factor in the adaptive response to ER stress, solid tumor growth and survival under hypoxic conditions. The activation of such related factors promotes induction of proangiogenic factors like VEGF, highlighting its role in angiogenesis and proposing it as possible therapeutic target.[57,92]

PERK can have an active role in tumor growth and proliferation through limitation of oxidative stress derived from ATF4 activation, which also stimulates transcription of pro-

survival genes and upregulation of adhesion proteins, VEGF and type 1 collagen inducible protein, important factors for angiogenesis.[92,95] Furthermore, ATF4 expression triggers cell survival by negative regulation of genes related with cellular senescence.[57] Moreover, the upregulation of the autophagosomal membrane LC3B by ATF4 allows lysosomal degradation of unnecessary cellular components and thereby cell survival. In a global view, inhibition of PERK remarkably reduces cells adaptation and survival under hypoxic conditions.[57] Recently, Fan *et al.* detected an active form of ATF4 phosphorylated at Ser 245 highly expressed in NSCLC tumors that may contribute to its progression, particularly in metastasis, since it promotes adaptive response to ER stress and triggers stress-induced angiogenesis. However, the mechanism involved in its upregulation is still unclear.[57,95]

Although few studies have been conducted, it is know that ATF6 is required to malignant transformation. Its functionally active form improves tumor survival by activating the mammalian target of rapamycin (mTOR) signaling pathway, which has been associated with malignant transformation, taking into account its influence in cell growth and proliferation.[59,97] Particularly in NSCLC, the mTOR activation, AKT-dependent or independent, has been correlated with the proliferative character of these cancer cells.[82,100]

Regarding the ERAD system, its regulation is also visible in cancer cells as a way to evade apoptosis and to reduce the accumulation of misfolded proteins that may be toxic for the cell. The valosin containing protein (VCP) controls ubiquitin mediated degradation of misfolded proteins and it is involved in protein folding, cell cycle control and apoptosis and it is known to be upregulated in several tumors. [57] Valle *et al.* demonstrated that the inhibition of VCP in cells from a NSCLC suppresses tumor growth and induces apoptosis *in vitro* and in xenograft murine models.[97]

Autophagy can act both as tumor suppressive and as oncogenic player. As tumor suppressive, autophagy intervenes in cell cycle arrestment, strives for maintenance of genome and organelle integrity and promotes inhibition of necrosis and inflammation. On the other hand, autophagy promotes cell survival under conditions of metabolic stress, through elimination of damaged mitochondria, ROS and protein aggregates, which cause DNA damage and activation of tumorigenesis.[59,98]

VI. Aim of Study

The deregulation of tRNA pool has been reported in different tumors and correlated with the expression of cancer-related genes.[85,86] However, a major question remains: is tRNA pool deregulation a cause or a consequence in the acquisition of a malignant phenotype?

In order to contribute to the clarification of this issue, this study proposed to evaluate the influence of tRNA deregulation in the acquisition of a malignant phenotype and the contribution of protein quality control pathways in cell transformation. Therefore, the following cellular mechanisms were evaluated:

- Phenotypic profiling;
- Transformation ability *in vitro*;
- Induction of proteotoxic stress;
- Activation of protein quality control mechanisms;
- Insoluble protein expression profile.

CHAPTER 2

VII. Experimental Design

To assess the effects of tRNA overexpression for the acquisition of a malignant phenotype, a comparative study was performed between a normal human cell line derived from the bronchial epithelium (BEAS-2B cell line) and a NSCLC cell line (NCI-H460 cell line) derived from a large cell lung carcinoma.

Pavon-Eternod *et al*. observed a particular overexpression of $tRNA^{Ser}$ in breast cancer cell lines and breast tumor tissues.[85] Therefore, BEAS-2B cells were stably transfected with a pIRES2-DsRED plasmid containing $tRNA^{Ser}$ to induce $tRNA^{Ser}$ overexpression and so promote deregulation of the cellular tRNA pool. This transfection created BEAS-2B $tRNA^{Ser}$ cell line. Also, BEAS-2B cells and NCI-H460 cells were stably transfected with the empty pIRES2-DsRED plasmid, given rise to the BEAS-2B Mock cell line and NCI-H460 Mock cell line, respectively. BEAS-2B Mock cell line is the control in the assays performed.

VIII. Material and Methods

2.1.pIRES2-DsRED plasmid

To induce overexpression of $tRNA^{Ser}$ in a stable cell line we used the pIRES2 DsRed-Express2 vector, a bicistronic expression vector (Figure 7) that allows simultaneously the expression of the $tRNA^{Ser}$ coding sequence and the Ds-Red Express2 fluorescent protein, useful to identify cells expressing the $tRNA^{Ser}$ gene through fluorescence microscopy. Also, this vector contains a kanamycin/neomycin resistance gene, suitable for selection of *Escherichia Coli* (*E. Coli*) competent cells with the plasmid using kanamycin, and for the selection of stably transfected mammalian cells using geneticin (G418) (Formedium^{Tm}). Previously co-workers inserted the coding sequence for the $tRNA^{Ser}$ using EcoRI and XhoI restriction enzymes into the vector. A pIRES2 DsRed-Express2 vector without the $tRNA^{Ser}$ coding sequence was used to normalize the effect of plasmid insertion.

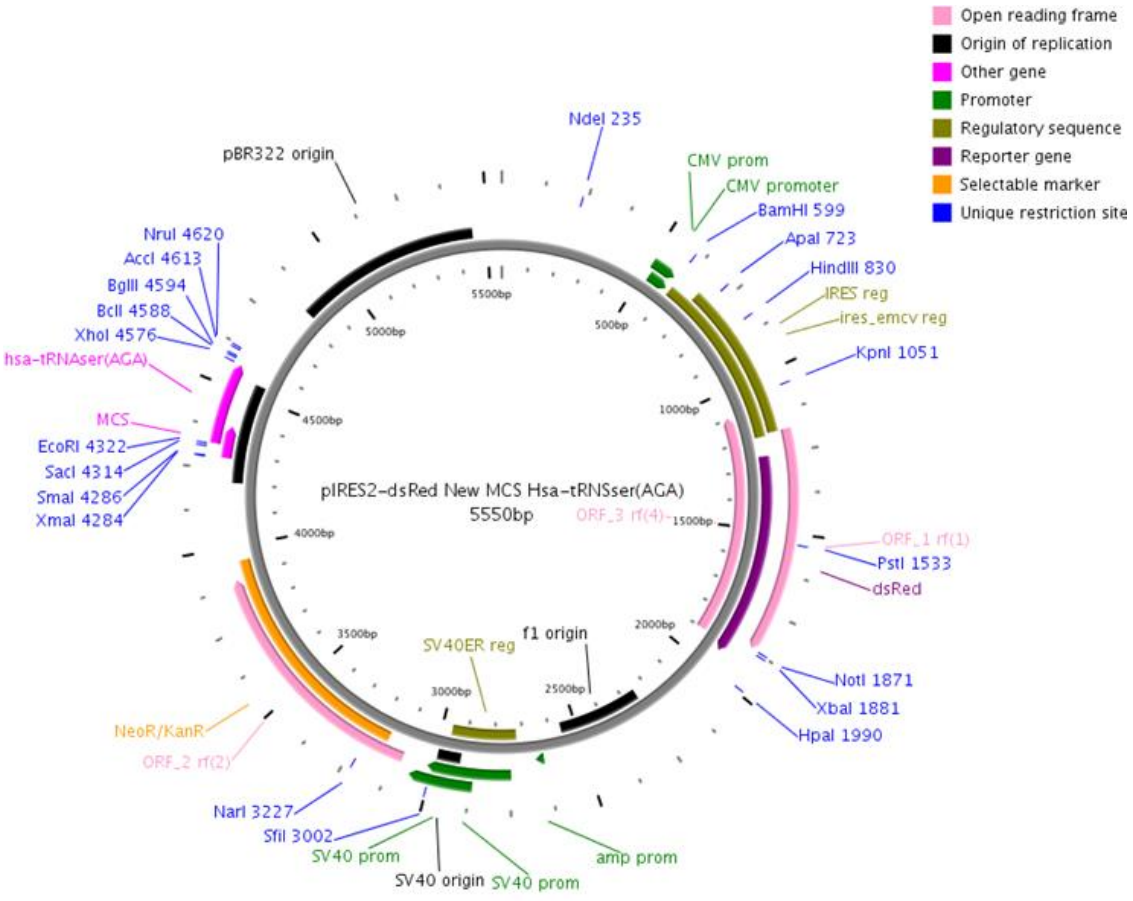

Figure 7. **pIRES-DsRed containg the $tRNA^{Ser(AGA)}$** coding sequence.

2.2.*Escherichia Coli* Competent Cells

E. Coli cells were grown to obtain enough amount of plasmids to create our cell lines. Previously, co-workers transformed these cells with the pIRES2-DsRED containing the $tRNA^{Ser}$ and the empty pIRES2-DsRED plasmid and stored at -20ºC. To cultivate the *E. Coli* cells, Lysogeny Broth (LB) medium was supplemented with kanamycin. After homogenization, 20µl of transformed *E. Coli* cells were added to the medium and maintained overnight in a shaking incubator at 37ºC.

2.3.Extraction and Quantification of pDNA

Plasmid DNA (pDNA) from transformed *E. Coli* was extracted with MiniPrep Nzytech Kit, according with the manufacturer's instructions. After extraction, the pDNA concentration was quantified with the NanoDrop spectrophotometry (ThermoScientific). The system was calibrated using 1.5µl of miliQ H_2O and purity values were calculated through the ratios 260nm/280nm and 260nm/230nm.

2.4.Polymerase Chain Reaction

To verify if the *E. Coli* selected carried our plasmid without alterations, we amplified the tRNA region of our plasmid by PCR using 50ng of pDNA. The reagents were acquire to Invitrogen (Thermo Scientific) and primers' sequences are represented in Table I. Note that primers are the same for the amplification of pIRES2-DsRED containing the $tRNA^{Ser}$ and the empty pIRES2-DsRED plasmid since the flaking regions are equivalent. The PCR reaction comprised 35 cycles and occurred in the MyCycler™ Thermal Cycler (Bio-Rad).

Table I**. Primers** used and respective sequences

Primer	**Sequence**
Primer Forward	CAATACGCCCGCGTTTCTT
Primer Reverse	TTATCCAAAAAGGATCTTCACCTAGA

2.5.Agarose Gel Electrophoresis

PCR amplification products were analyzed by performing an agarose gel electrophoresis, a technique that allows the separation of DNA fragments, according to their molecular size, under an electric field.[99] Five µl of PCR products were run in 1% agarose with 0.01% ethidium bromide (EtBr) at 80V and in an electrophoretic thank containing 1X Tris-Acetate-EDTA (TAE) (GRiSP). After electrophoresis, gels were scanned in the UV-Transilluminator (Bio-Rad) and results were visualized with Quantity One 4.2.1 software.

2.6.Purification of PCR products

PCR purification was performed using the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's instructions. After the purification, DNA concentration was evaluated in the NanoDrop, as described in 2.3.

2.7.DNA Sequencing

To confirm the nucleotide sequences of the pIRES2-DsRED containing the $tRNA^{Ser}$ and the empty plasmid, purified PCR products were prepared to be sequenced by GATC Biotech. For that, microtubes with 5µl of 80ng/µl of amplified DNA were prepared for each sample as well as microtubes with the forward and reverse primers with 5µl of 5µM, according to the recommendations of the LIGHTRUN sequencing kit. Results were analyzed using the FinchTV v.04 software (Geospiza Inc).

2.8.Cell Culture

BEAS-2B cell line was kindly provided by Professor Maria Carmen Alpoim, from IBILI, University of Coimbra and NCI-H460 cell line was obtained from IPATIMUP's Cell Bank. BEAS-2B cells were cultured in LHC-9 medium (Gibco, Life Technologies) supplemented with 1% of Penicillin-Streptomycin (Pen/Strep) (Gibco, Life Technologies). NCI-H460 cells were cultured in RPMI 1640 medium (Gibco, Life Technologies), supplemented with 10% Fetal Bovine Serum (FBS) (Sigma) and 1% Pen/Strep. Cells were maintained in an incubator at 37ºC with 5% CO_2 and 95% relative humidity.

To execute the following procedures, cells were detached using trypsin (Sigma). Then, BEAS-2B cells were resuspended in trypsin neutralizer solution [0.5% FBS in 1X Phosphate Buffered Saline (PBS)] while NCI-H460 cells were resuspended in complete RPMI 1640 medium. Cells were then centrifuged at room temperature (RT) and ressuspended in complete medium or 1X PBS, depending on the following procedures.

2.9.Lipotransfection

Lipotransfection is a procedure that uses cationic lipids formulations to deliver the foreign genetic material into eukaryotic cells. [100]

Transfection protocols using Lipofectamine® 3000 Transfection Kit (Invitrogen), for BEAS-2B and NCI-H460, were carefully optimized to reach close to 100% transfection efficiency. 1.0 x 10^5 BEAS-2B cells and 5.0 x 10^4 NCI-H460 cells were plated in MW24 plates and after 48h cells were transfected with small alterations to manufacturer's protocol. BEAS-2B were transfected using 1µg of plasmid DNA and 1µL of Lipofectamine® 3000, whereas NCI-H460 were transfected using 1.5µg of plasmid DNA and 0.75µL of Lipofectamine® 3000. In this transfection, while a set of BEAS-2B cells were transfected with the pIRES2-DsRED containing the $tRNA^{Ser}$ another set of BEAS-2B cells were transfected with the empty pIRES2-DsRED plasmid. NCI-H460 cells were transfected with the empty pIRES2-DsRED plasmid.

Since the goal was the integration of the plasmid in the genome and the expansion of these cells, cell lines were on culture for three weeks and under the selection of G418 in a concentration of 200µg/ml in BEAS-2B cells culture and 800µg/ml in NCI-H460 cells culture (concentrations previously optimized through a death curve).

Despite this methodology allowed the creation of a stable NCI-H460 Mock cell line, BEAS-2B Mock cell line or BEAS-2B $tRNA^{Ser}$ cell line were not successfully created since these cells lost the plasmid during selection and died.

2.10. Electroporation

As an alternative to create stable BEAS-2B derived cell lines, we used electroporation, which is a technique that relies in the use of electric pulses to transiently alter the cell membrane permeability, allowing the DNA to enter the cell.[100]

To perform the electroporation, BEAS-2B cells were seeded in 100mm dishes and cultured until 70-90% of confluence was reached. Cells were detached as described in 4.8 and the pellet was resuspended in Hepes Buffered Saline (HBS) solution (Appendix) to improve the transfection efficiency. Then, 4mm electroporation cuvettes were prepared with 10µg of plasmid and 0.5ml of cell suspension was added, mixing carefully. For each sample, two conditions were tested differing in the voltage applied, 230V and 260V, both with capacitance of 1500µF and resistance of 125Ω. This step was performed using ECM Electro Cell Manipulator (BTX, Harvard Apparatus). Immediately after the electroporation, 1ml of LHC-9 culture medium was added, homogenizing carefully, and the mixture was transferred to 60mm dishes, already prepared with 3ml of LHC-9 culture medium.

The stable cell lines BEAS-2B Mock and BEAS-2B $tRNA^{Ser}$ were obtained by selection with G418 in a concentration of 200µg/ml for three weeks, as in the lipotransfection.

2.11. Fluorescence Microscopy

pIRES2-DsRED plasmid codifies for the red fluorescent protein DsRed-Express2, which allows to validate its integration in the cell genome through fluorescence microscopy. For that, a coated coverslip (Corning™) was placed in the well or culture dish of each culture cell line in the moment of transfection, so the cells could adhere and grown into it. 48h later, the culture medium was removed and the well/culture dish was washed ten times with 1X PBS. Then, enough volume of Hoechst dye (1µg/ml) was added to the coverslip and incubated during 15min at RT, protected from the light. The coverslip was washed five times with 1X PBS, the excess was removed and it was transferred to a microscope slide containing the Fluoroshield mounting medium (Sigma), leaving to dry for 15min. Fluorescence was detected in the Zeiss MC80 Axioplan 2 Light microscope with the filter set HE38. Photographs were taken using an AxionCam HRc camera.

2.12. Extraction and Quantification of gDNA

To ensure the plasmid did not acquire mutations when integrated in the genome, gDNA was extracted to be sequenced as described in 2.7. For that, it was used the NZY Tissue gDNA Isolation Kit, following the recommended instructions, and gDNA concentration was quantified in the NanoDrop, as described in 2.3.

2.13. Cellular Viability Assay

To perform this assay 1.5 x 10^5 cells/well of BEAS-2B Mock cells, BEAS-2B $tRNA^{Ser}$ cells and NCI-H460 Mock cells were seeded in a 24-well plate. After two days in culture, cells were detached and equal volumes of cell suspension and trypan blue were mixed. Finally, cell viability (%) was obtained by counting the live and death cells using a $TC10^{Tm}$ Automated Cell Counter (Bio-Rad). This assay was performed with triplicates and repeated three times.

2.14. Cellular Proliferation Assay

To evaluate cellular proliferation, 5.0 x 10^4 cells/well of BEAS-2B Mock cells, BEAS-2B $tRNA^{Ser}$ cells and NCI-H460 Mock cells were seeded into four 24-well treated culture plates, so cell counting could be performed before the first cellular division (0h) and over the next three days (24h, 48h, 72h). Cells were detached and equal volumes of cell suspensions and trypan blue were mixed and viable cells were counted in a Neubauer chamber at each time point. The procedure accounted with triplicates and it was repeated three times.

2.15. Anchorage-Dependent Colony Formation Assay

To assess the tumorigenic ability of our cell lines *in vitro* we performed an anchorage-dependent colony formation assay. This assay requires well individualized cells, so suspensions of 300 cells of BEAS-2B Mock cells, BEAS-2B $tRNA^{Ser}$ cells and NCI-H460 Mock cells were seeded in 60mm dishes and maintained on culture during two weeks. After, the colonies were fixed using ice cooled methanol and maintained at -30ºC during 30min. Methanol was removed and a solution of 0.1% crystal violet in methanol was added and the plates were laid on stirring at least for 30min. When colonies were stained, each well was washed with H_2O milliQ to remove excess dye and the colonies were counted. This assay was performed with triplicates and repeated four times.

2.16. Total Protein Extraction

To obtain total protein from the three cell lines, BEAS-2B Mock cells, BEAS-2B $tRNA^{Ser}$ cells and NCI-H460 Mock cells were seeded on 60mm dishes and maintained on culture until they reached about 90% confluence. At that time, the culture medium was removed and the plates were washed with 1X PBS. Cells were detached and pelleted as described in 2.8.

The next step was to lyse cellular membranes to release their content, by resuspending the pellet in protein lysis buffer (Appendix) (volume was adjusted regarding the size of the cell pellet) and incubating during 30min. From this point, sample manipulation should be performed on ice to avoid proteases activity. During the incubation with protein lysis buffer, each sample was sonicated in a Branson Sonifier S-250A (Fisher Scientific) and centrifuged at 4ºC and 16000*g* for 30min in a Centrifuge 5415R (Eppendorf®). Finally, the supernatants were collected and the samples were concentrated in a DNA 120 SpeedVac System (Thermo Scientific) until a volume of 50µl was reached.

Protein quantification was assessed through the Pierce BCA Protein Kit (Thermo Scientific), according to the recommendations of the manufacturer. Absorbance at the 575nm wavelength was obtained using a microplate reader (iMark™ Microplate Reader, Bio-Rad) and results were analyzed in the Microplate Manager Software v6.3 (Bio-Rad Laboratories, Inc.).

2.17. Western Blot

Western blot is a technique that separates proteins based on its molecular weight under an electric field, which are then transferred to a solid supported and identified using specific and labeled antibodies.[101] Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which allows proteins to migrate according to their molecular weight. Accordingly to the molecular weight of proteins in study, 8% and 10% polyacrylamide gels (Appendix) were prepared.

Protein quantity was optimized for each protein studied (ranging from 30µg to 60µg of protein) and protein samples preparation required addition of 6X SDS Protein Loading Buffer (Appendix). Proteins were denatured at 95ºC for 5min in the Thermomixer Comfort (Eppendorf®). The denatured proteins were then loaded into gels in an electrophoretic thank

filled with 1X running buffer (Appendix), under a voltage of 80V when running in the stacking gel and 100V during migration in the running gel. After their separation, proteins were transferred from gels to 0.2µm nitrocellulose membranes (GE Healthcare Life Sciences) in a transference system filled with cold transfer buffer (Appendix) under the constant voltage of 100V, for 1h30min at 4ºC. As a transference quality control, the membranes were stained with 0.1% (m/v) Ponceau S (Sigma-Aldrich) in 5% (m/v) acetic acid during 1min at RT and excess dye was removed with distilled H2O (dH2O). Staining was removed by washing the membranes with Tris-Buffered Saline – Tween (TBS-T) (Appendix).

To avoid antibody unspecific hybridization, membranes were incubated with blocking solution [5% BSA in TBS-T] for 1h at RT and then washed three times with TBS-T for 5 min each. At this point, the membranes were ready for hybridization with the primary antibodies: anti-Hsp27, anti-Hsp70, anti-Hsp90α, anti-BiP and anti-ubiquitin (StressMarq, Biosciences Inc.) prepared in blocking solution with 1:1000 dilution factor. Their hybridization occurred overnight at 4ºC. After the incubation, membranes were washed three times with TBS-T for 5 min each.

The secondary antibodies goat anti-mouse IgG and goat anti-rabbit IgG (Odyssey, LI-COR) diluted 1:10000 in blocking solution were incubated by 2h at RT, protected from the light. Anti-β tubulin (StressMarq, Biosciences Inc.) was used as internal control and its hybridization occurred for 2h at RT and also 2h with the secondary antibody goat anti-mouse IgG. Primary antibodies and the correspondent secondary antibodies are represented in Table II.

After secondary antibody hybridization, membranes were washed two times with TBS-T for 5min each and 15min with TBS and scanned in the Odyssey Infrared Imaging System (LI-COR, Biosciences Inc.). Data was obtained with the software Odyssey v3.0.16 (LI-COR, Biosciences Inc.).

For multiple probing, membranes were incubated with stripping solution (Appendix) at RT, long enough to dissociate the antibodies from the membrane.

Table II. **Primary antibodies** and respective **secondary antibodies**

Primary antibody	Secondary antibody
Anti-Hsp27	Goat anti-mouse IgG
Anti-Hsp70	
Anti-Hsp90α	
Anti-ubiquitin	
Anti-β tubulin	
Anti-BiP	Goat anti-rabbit IgG

2.18. Proteasome activity assay

Proteasome activity can be determined by measuring the intensity of fluorescence derived from the cleavage of a labeled peptide (Suc-LLVY-AMC), a substrate for enzymes with chemotrypsin-like activity.[102]

The three different cell lines were seeded in 60mm dishes and maintained in culture until 90% of confluence was reached. At this point, culture medium was removed and cells were washed with PBS. Then, proteasome assay lysis buffer (Appendix) was added to the culture plates placed on ice and, after an incubation of 5min at RT, cells were scrapped and collected to 2ml microcentifuge tubes. Total protein extraction was performed as described in 2.16.

Protein quantification was obtained through the Bradford method, Bradford reagent was obtained from Bio-Rad and absorbance was read in the absorbance microplate reader (iMark™ Microplate Reader, Bio-Rad).

To assess proteasome activity, 20µg of protein were incubated with proteasome activity buffer (Appendix) in a black 96 multiwell plate (Costar™) to avoid light interferences, platting 6 wells for each sample. To eliminate unspecific proteasome activity, the proteasome inhibitor MG132 (Sigma) was added to three wells per sample. The fluorescence emitted from the cleavage of Suc-LLVY-AMC (Sigma) was accessed in the fluorometer system of Synergy 2 (BioTek®), for one hour with reads every 5min, using 360nm wavelength for excitation and 460nm for emission. Results were analyzed in the Gen5™ v.1.11.5 software (BioTek). Each experiment was performed with triplicates and repeated seven times.

2.19.SUnSET Method

As a strategy to obtain a similar number of cells to perform the assay, 5.0 x 10^5 cells of BEAS-2B Mock cells and BEAS-2B $tRNA^{Ser}$ cells and 1.0 x 10^5 cells of NCI-H460 Mock cells were seeded in 100mm dishes and maintained in culture for three days. 10µg/ml puromycin in 1X PBS was added to the culture medium of each culture dish, in a volume corresponding to 10% of culture medium. After 10 min incubation at 37ºC with 5% CO_2, the culture medium was removed and replaced by fresh culture medium. Cells were once again placed in the incubator for 50 min, after which the culture medium was removed and 1X PBS was added to wash the culture plate. Cells were detached and pelleted as described in 2.8.

Total protein extraction was performed using proteasome assay lysis buffer and as described in 2.16 and protein quantification was achieved by the Bradford method, described in 2.18.

Incorporation of puromycin was detected by immunoblotting, so samples were prepared to SDS-PAGE. 100µg of total protein was loaded into 10% polyacrylamide gels, separated and transferred to nitrocellulose membranes. For the detection of puromycin, membranes incubated with anti-puromycin clone 12D10 primary antibody (1:5000 in blocking solution) overnight at 4ºC and with goat anti-mouse IgG secondary antibody by 2h at RT, protected from the light. Anti-β tubulin was used as internal control and secondary antibody fluorescence detection occurred as described in 2.17.

2.20.Insoluble Protein Fraction

BEAS-2B Mock cells, BEAS-2B $tRNA^{Ser}$ cells and NCI-H460 Mock cells were seeded on 60mm dishes and maintained on culture until about 90% confluence was reached. Cells were detached and pelleted, as described before, and suspensions with 5.0 x 10^6 cells were aliquoted after total cell counting in a Neubauer chamber.

To perform protein extraction, cellular pellets were resuspended in protein lysis buffer and maintained on ice for 30min. Meanwhile, samples were sonicated in a Branson Sonifier S-250A (Fisher Scientific). Then, samples were centrifuged for 15min at 2300*g* and at 4ºC in a Centrifuge 5415R (Eppendorf®). Supernatants were collected and five µl were kept apart to quantify total protein with the BCA method. The supernatant was centrifuged for

20min at 16000*g*, 4ºC. At this point, the supernatant (the soluble protein fraction) was separated from the pellet (the insoluble fraction). The pellet was ressuspended in protein lysis buffer and 10% Triton X-100. Another centrifugation was performed for 20min at 16000*g* and at 4ºC. To finish, the supernatant was discarded and the pellet was resuspended in 50 µl of protein lysis buffer.

Relative expression of insoluble protein fraction was obtained though SDS-PAGE, as described in 4.6. Samples were solubilized with 2% urea SDS loading buffer (Appendix), denatured at 95ºC in the Thermomixer Comfort (Eppendorf®) and loaded into 10% polyacrylamide gels. Then, gels were stained with Coomassie Brilliant Blue (Appendix) during 2h and the excess was removed with distaining solution (Appendix). Gels were scanned in the Odyssey Infrared Imaging System (LI-COR, Biosciences Inc.) and data was obtained with the software Odyssey v3.0.16 (LI-COR, Biosciences Inc.). This assay was performed with triplicates and repeated three times.

2.21. Statistical analysis

Statistical analysis was performed in the GraphPad Prim® v6.01 software, applying the one-way ANOVA test with Tukey post-test for all the experiments except for proliferation assay, which was analyzed with two-way ANOVA test with Tukey post-test.

CHAPTER 3

IX. Results

3.1.Stable cell lines construction

To obtain the three stable cell lines two different approaches were applied. BEAS-2B Mock cell line and BEAS-2B $tRNA^{Ser}$ cell line were obtained through electroporation while NCI-H460 Mock cell line was obtained by lipotransfection.

The pIRES2-DsRED plasmid contains the coding sequence for the DsRed Express2 fluorescent protein, therefore 48h after transfection it was possible to detect the integration of plasmid in cells through fluorescent microscopy and images of non- and transfected BEAS-2B are shown in Figure 8 as well images of non- and transfected NCI-H460 cells are shown in Figure 9.

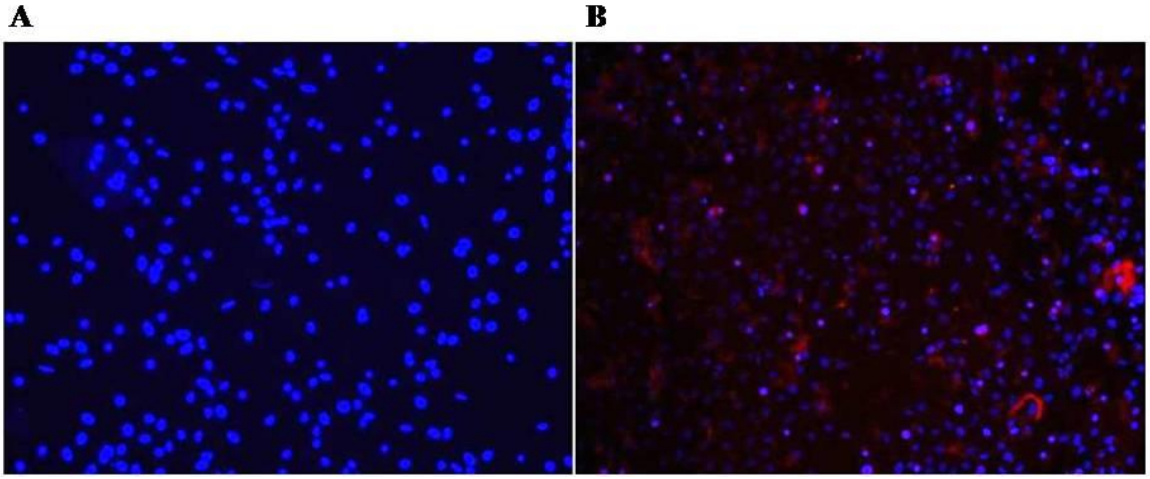

Figure 8. **A**. **BEAS-2B cells non-transfected** and stained with Hoechst dye (20x). **B**. **BEAS-2B cells transfected** with plasmid pIRES2-DsRED and stained with Hoechst dye (20x).

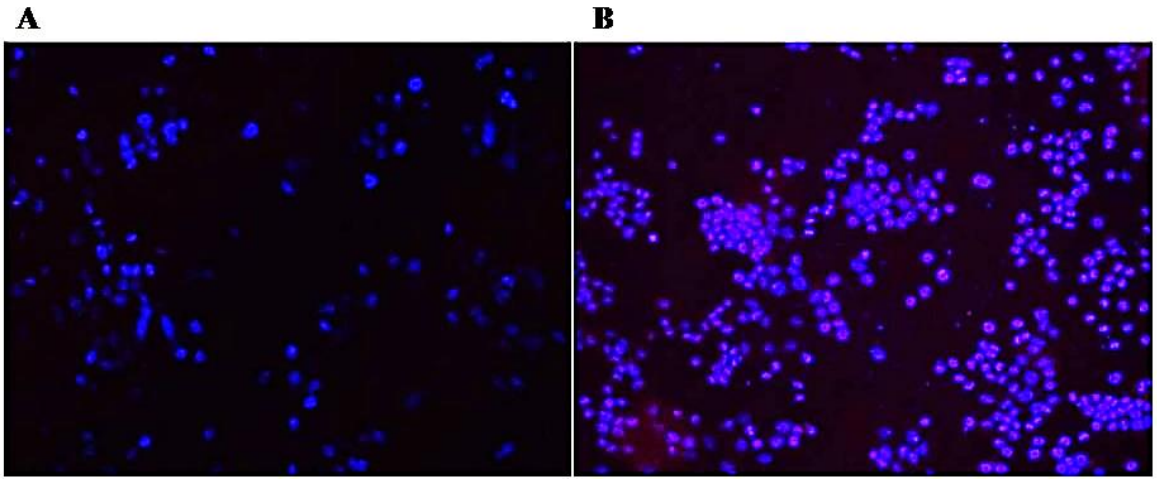

Figure 9. **A**. **NCI-H460 cells non-transfected** and stained with Hoechst dye (20x). **B**. **NCI-H460 cells transfected** with the empty pIRES2-DsRED plasmid and stained with Hoechst dye (20x).

To ensure that stable cell lines retained the plasmid in their genomes a PCR was performed and its products were sequenced to check if no mutations occurred. Results from PCR (Figure 10) confirm the genome's integration of pIRES2-DsRED plasmid containing the $tRNA^{Ser}$ in BEAS-2B $tRNA^{Ser}$ cell line and the empty pIRES2-DsRED plasmid in BEAS-2B Mock cell line and NCI-H460 Mock cell line. Also, the results from sequencing (Figure 11) ensure the absence of mutations.

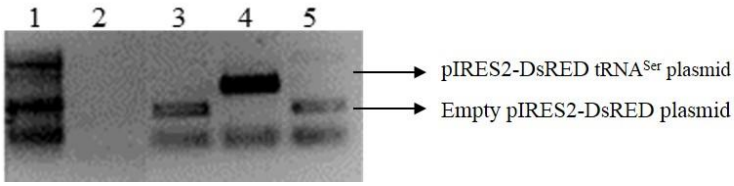

Figure 10. **pIRES2-DsRED plasmid PCR amplification.** Lane 1: ladder; Lane 2: negative control; Lane 3: BEAS-2B Mock cells; Lane 4: BEAS-$tRNA^{Ser}$ cells; Lane 5: NCI-H460 Mock cells.

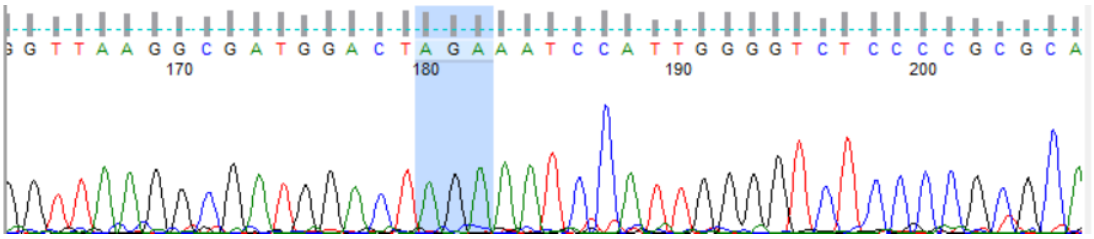

Figure 11. **Sequencing of BEAS-2B $tRNA^{Ser}$ cells** PCR products. In the highlighted zone is the $tRNA^{Ser(AGA)}$ anticodon.

3.2. Phenotypic Profilling

a. Cellular Viability

Cellular viability was assessed by determining the number of viable cells applying the Trypan Blue dye exclusion test. This test is based on the assumption that live cells have intact membranes that exclude particular dyes, such as trypan blue. So, nonviable cells present blue stained cytoplasm while viable cells have no staining.[103] The percentage (%) of viability resulted from the ratio between the number of live cells and the total number of cells and it is represented in Figure 12.

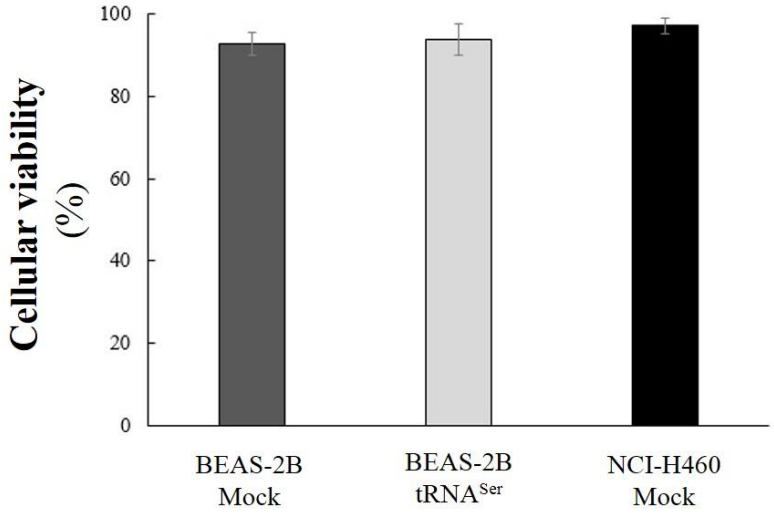

Figure 12. Effect of pIRES2-DsRED plasmid on **cell viability**. No differences are observed. Graphic represents mean ± SD of three independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test ($p > 0.05$).

b. Cellular Proliferation

Cellular proliferation was accessed to verify if deregulation of tRNA pool promotes alterations in the proliferative capacity of BEAS-2B $tRNA^{Ser}$ cells. To do so, cells from the three cell lines were counted in a Neubauer chamber excluding nonviable cells, according to the trypan blue exclusion principle. The first counting was done before their doubling, at 0h, and over the next three days: after 24h, 48h and 72h. Results are represented in Figure 13.

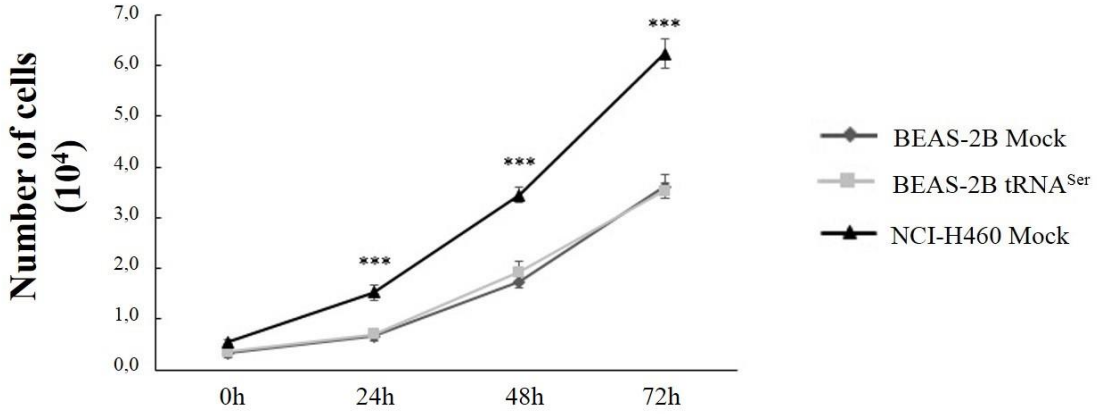

Figure 13. **Proliferation capacity** of the three cell lines. NCI-H460 Mock cell line has higher proliferation capacity than BEAS-2B tRNA^{Ser} cell line and the control. No differences are observed between BEAS-2B Mock and BEAS-2B tRNA^{Ser} cell lines. Graphic represents mean ± SEM of three independent experiments. Statistical analysis was performed using the Two-Way ANOVA with Tukey's post-test (***$p < 0.001$).

3.3.Tranformation Ability

The anchorage-dependent colony formation assay was done to access the transformation ability of each cell line through their capacity to form colonies consisting in 50 cells, at least[104] (Figure 14).

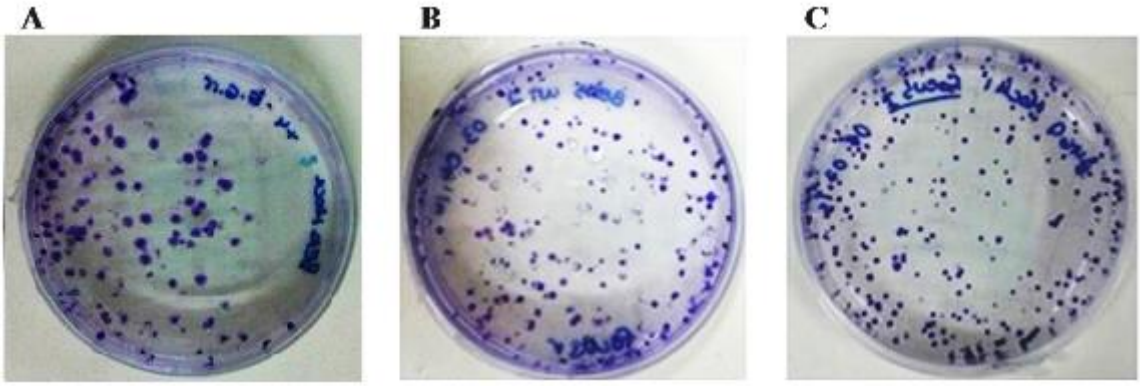

Figure 14. **Anchorage-dependent colony formation assay**. Cells were seeded at low density to assure their individualization and were maintained in culture for two weeks. Then colonies were fixed, stained and counted. **A**. BEAS-2B Mock cells; **B**. BEAS-2B tRNA^{Ser} cells; **C**. NCI-H460 Mock cells.
Image represents results from one independent experiment.

This assay was performed to characterize the unlimited reproductive capacity of BEAS-2B tRNA^{Ser} cells and compare it to cancer cells colony formation capacity. Results are shown in Figure 15.

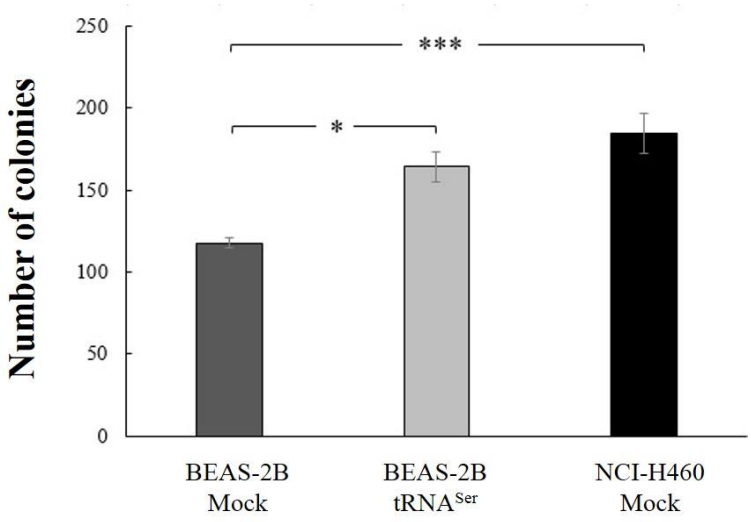

Figure 15. Evaluation of **transformation ability *in vitro***, based on the number of colonies. Both BEAS-2B tRNA^{Ser} and NCI-H460 Mock cells showed higher tumorigenic capacity when compared with the control. No statistical significant differences were observed between BEAS-2B tRNA^{Ser} cell line and NCI-H460 Mock cell line. Graphic represents mean ± SEM of four independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test (*$p < 0.05$; ***$p < 0.001$).

3.4.Study of Proteotoxic Stress Induction and Activation of Protein Quality Control Pathways

Despite the protein quality control systems, errors still occur during protein synthesis, which can prompt proteotoxic stress resulting from the toxic character of aberrant proteins that became nonfunctional and aggregate.[49] Proteotoxic stress and quality control pathways activation were evaluated in this study by assessing chaperones availability, protein synthesis, activation of UPR and UPS systems and insoluble protein fraction evaluation. These mechanisms were chosen based on previous results by co-workers indicating their alteration when facing proteotoxic stress conditions.

a. Expression of chaperones

The western blot assay was performed to assess the expression of Hsp90α, Hsp70 and the small Hsp27 (Figure 16) and the results from their relative expression are shown in Figure 17.

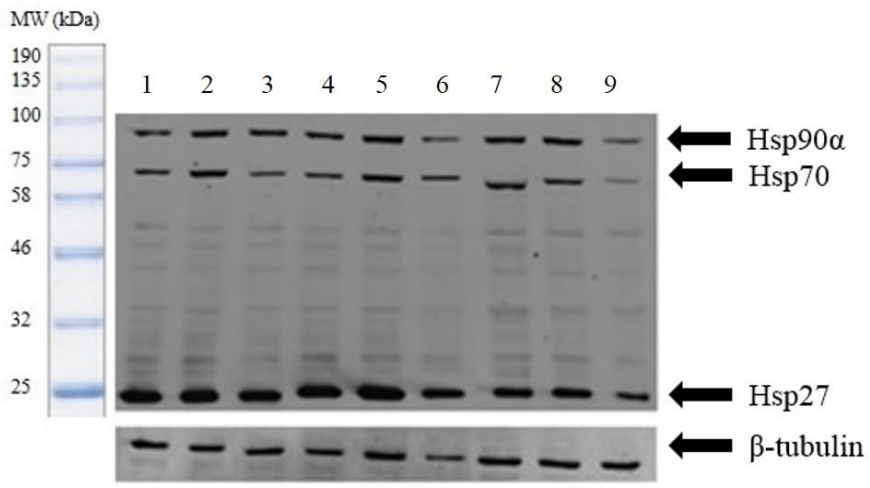

Figure 16. **Expression of Hsp90α, Hsp70 and Hsp27**. β-tubulin represents the internal control. Total protein was extracted from BEAS-2B Mock cells (lanes 1, 2 and 3), BEAS-2B $tRNA^{Ser}$ (lanes 4, 5 and 6) and NCI-H460 Mock cells (lanes 7, 8 and 9). 10% polyacrylamide gels were loaded with 50µg of total protein. Image represents results from one independent experiment.

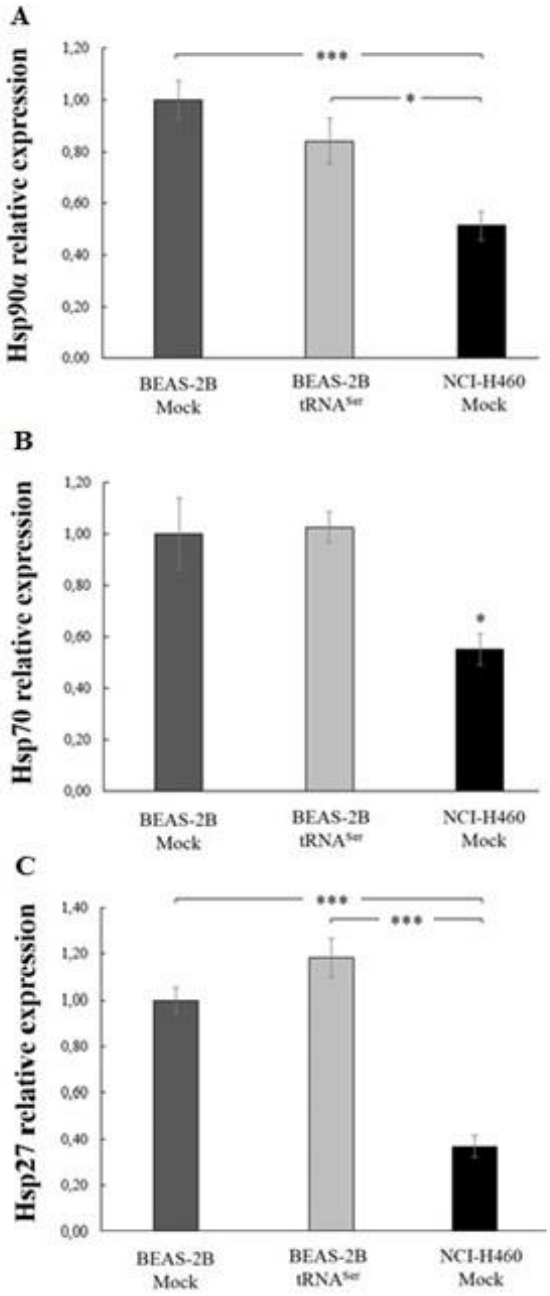

Figure 17. **A. Relative expression of Hsp90α**. Statistical analysis indicates decreased expression of Hsp90α in NCI-H460 Mock cells comparing with control and BEAS-2B $tRNA^{Ser}$ cells. No differences in the expression of Hsp90α are observed between control and BEAS-2B $tRNA^{Ser}$. Graphic represents mean ± SEM of eight independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test (*$p < 0.05$; ***$p < 0.001$). **B. Relative expression of Hsp70**. Statistical analysis indicates decreased expression of Hsp70 in NCI-H460 Mock cells comparing with control and BEAS-2B $tRNA^{Ser}$ cells. No differences are observed between control and BEAS-2B $tRNA^{Ser}$. Graphic represents mean ± SEM of three independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test (*$p < 0.05$). **C. Relative expression of Hsp27** Statistical analysis indicates decreased expression of Hsp27 in NCI-H460 Mock cells comparing with control and BEAS-2B $tRNA^{Ser}$. No differences are observed between control and BEAS-2B $tRNA^{Ser}$. Graphic represents mean ± SEM of four independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test (***$p < 0.001$).
β-tubulin was used as internal control and data was normalized to the control in all experiments.

b. Protein Synthesis Rate

Almost all types of stress prompt reduced translation at global levels as a strategy to decrease energy costs and production of proteins that could be prejudicial in the demand against cellular stress. At the same time, synthesis of proteins that support cell survival under stress is favored.[23]

The Surface Sensing of Translation (SUnSET) method represents a strategy to evaluate the protein synthesis rate. It is based on the incorporation of puromycin, an analog of aminoacyl tRNAs, into nascent polypeptide chains, inhibiting its elongation. Thus, puromycin incorporation directly infers about *in vitro* translation rate.[105] Detection of puromycin was done by western blot and results are represented in Figure 18.

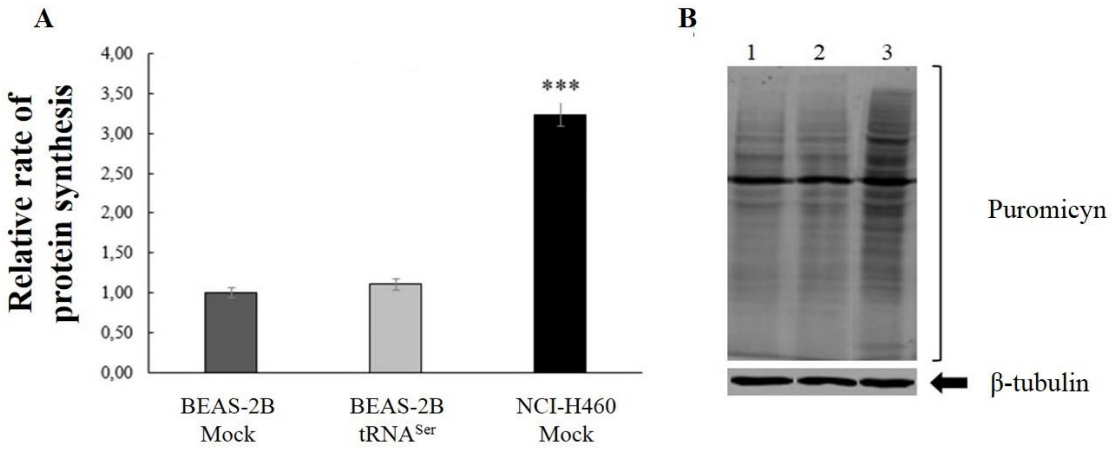

Figure 18. **A**. **Relative rate of protein synthesis.** β-tubulin was used as internal control and data was normalized to the control. Statistical analysis indicates increased relative rate of protein synthesis in NCI-H460 Mock cells compared with control and BEAS-2B $tRNA^{Ser}$. No differences are observed between control and BEAS-$tRNA^{Ser}$ cells. Graphic represents mean ± SEM of four independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test (***$p < 0.001$).
B. **Expression of puromycin**. β-tubulin represents the internal control. Total protein was extracted from BEAS-2B Mock cells (lane 1), BEAS-2B $tRNA^{Ser}$ cells (lane 2) and NCI-H460 Mock cells (lane 3). 10% polyacrylamide gels were loaded with 100µg of total protein. Image represents results from one independent experiment.

c. Unfolded Protein Response Activation

The stress sensor BiP is the main player of the UPR since it recognizes and binds unfolded proteins during ER stress, aiming to restore its conformational structure. This leads to BiP dissociation from IRE1α, PERK and ATF6 which become activated and up-regulates genes that encode proteins involved in protein folding, insurance of its quality and ERAD activation.[57]

Relative expression of BiP in the three cell lines was assessed by western blot and results are observed in Figure 19.

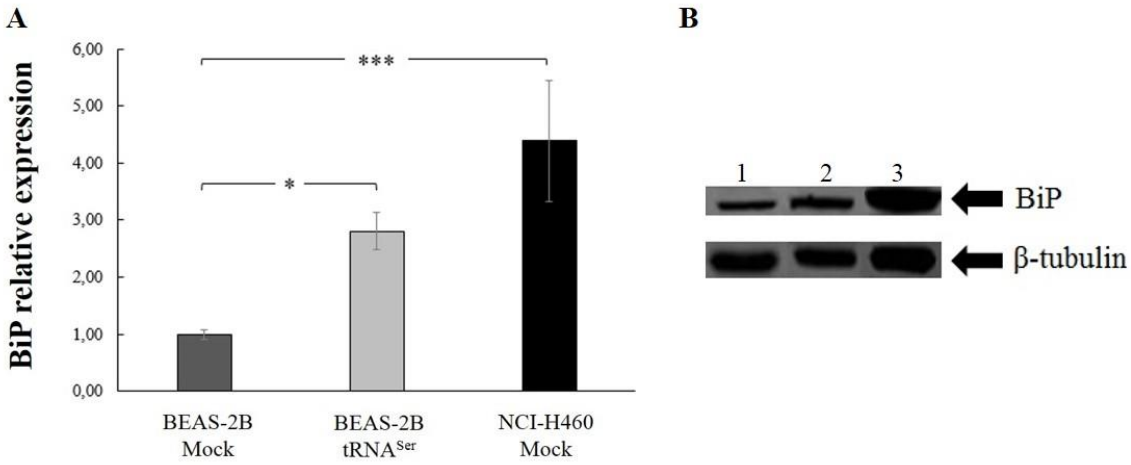

Figure 19. **A. Relative expression of BiP**. β-tubulin was used as internal control and data was normalized to the control. Statistical analysis indicates increased relative expression of BiP in BEAS-2B $tRNA^{Ser}$ cells and NCI-H460 Mock cells compared to the control. No differences are observed between BEAS-$tRNA^{Ser}$ cells and NCI-H460 Mock cells. Graphic represents mean ± SEM of five independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test (*$p < 0.05$); ***$p < 0.001$).
B. Expression of BiP. β-tubulin represents the internal control. Total protein was extracted from BEAS-2B Mock cells (lane 1), BEAS-2B $tRNA^{Ser}$ cells (lane 2) and NCI-H460 Mock cells (lanes 3). 8% polyacrylamide gels were loaded with 60µg of total protein. Image represents results from one independent experiment.

d. Ubiquitin-Proteasome System Activation

The UPS is an important system to retain the equilibrium between protein synthesis and protein destruction, controlling proteins' turn over and maintaining the homeostasis.[57] The 26S proteasome is an ATP-dependent proteolytic complex consisting in two subunits, the 20S proteolytic core and the 19S ATP-dependent regulatory cap, which degrades polyubiquitinated polypeptide chains to ensure the elimination of damaged or no longer essential proteins.[102]

Ubiquitin is involved in the regulation of proteolysis as well as other biological functions, namely DNA repair, autophagy and signal transduction. The polyubiquitination of proteins allows protein unfolding and degradation in the 26S proteasome.[106,107] The relative protein ubiquitination was assessed by western blot and results are shown in Figure 20.

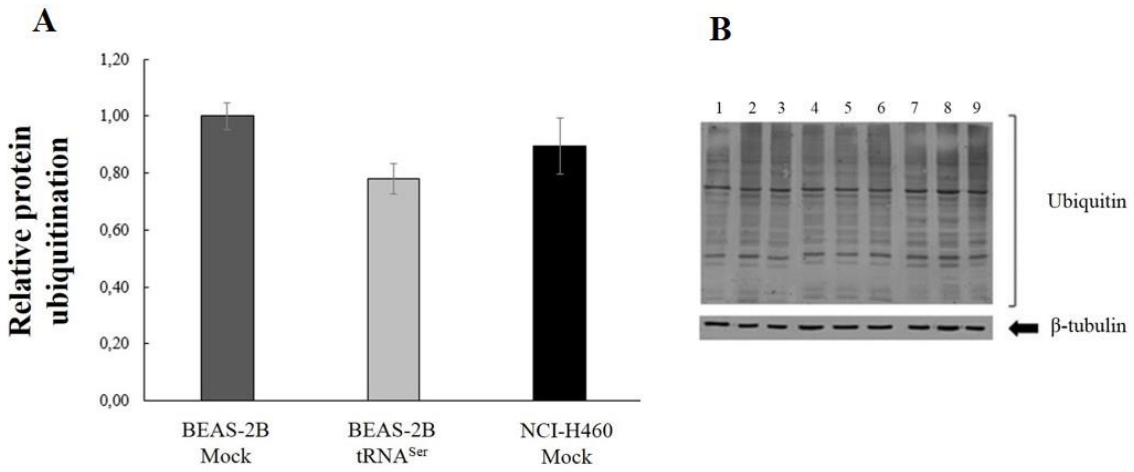

Figure 20. **A**. **Relative protein ubiquitination.** β-tubulin was used as internal control and data was normalized to the control. No differences are observed between control, BEAS-2B $tRNA^{Ser}$ cells and NCI-H460 Mock cells. Graphic represents mean ± SEM of five independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test. **B**. **Expression of ubiquitin.** β-tubulin represents the internal control. Total protein was extracted from BEAS-2B Mock cells (lanes 1, 2 and 3), BEAS-2B $tRNA^{Ser}$ cells (lanes 4, 5 and 6) and NCI-H460 Mock cells (lanes 7, 8 and 9). 10% polyacrylamide gels were loaded with 50µg of total protein. Image represents results from one independent experiment.

Proteasome activity was determined by measuring the intensity of fluorescence derived from the cleavage of a labeled peptide (Suc-LLVY-AMC), a substrate for enzymes with chymotrypsin-like activity[102] and results are represented in Figure 21.

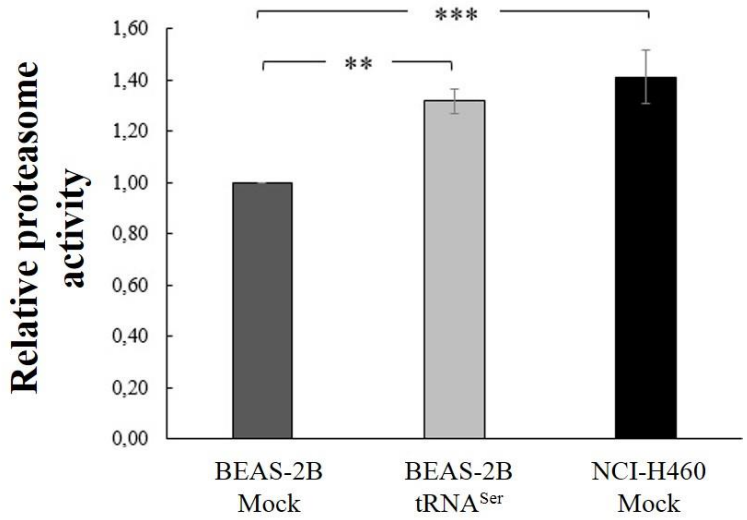

Figure 21. **Relative proteasome activity.** Statistical analysis indicate increase in the relative proteasome activity in BEAS-2B $tRNA^{Ser}$ cells compared with the control as well as in NCI-H460 Mock cells. No differences are observed between BEAS-2B $tRNA^{Ser}$ cells and NCI-H460 Mock cells. Graphic represents mean ± SEM of seven independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test (**$p < 0.01$; ***$p < 0.001$).

e. Insoluble protein fraction

To verify if alterations exist in protein insoluble profiles, we analyzed insoluble protein fractions through SDS-PAGE and results are represented in Figure 22.

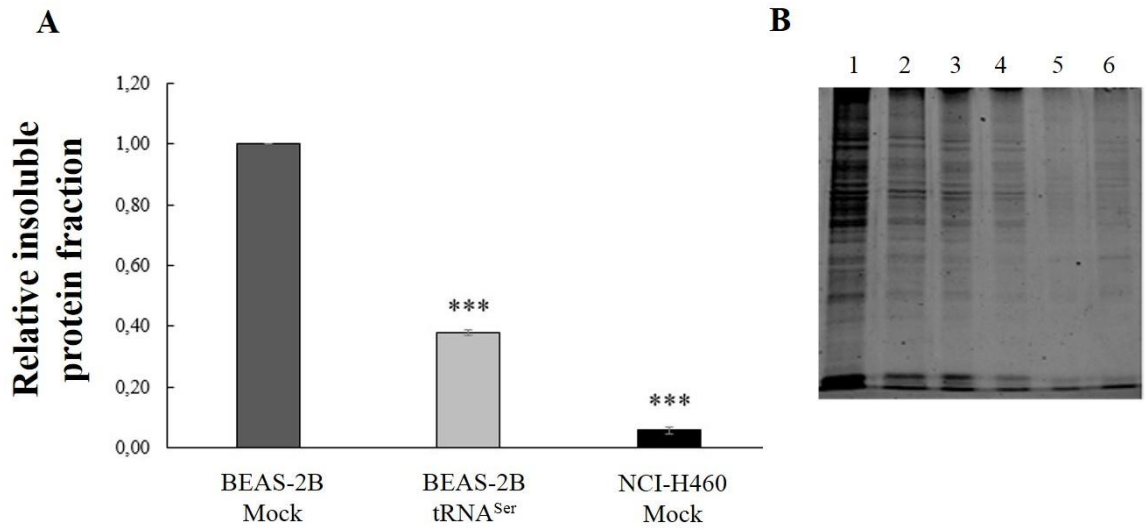

Figure 22. **A**. **Relative insoluble protein expression**. Data was normalized with the total protein concentration and to the control. Statistical analysis indicates decrease of relative insoluble protein fraction in BEAS-2B $tRNA^{Ser}$ cells and in NCI-H460 Mock cells. Graphic represents mean ± SEM of three independent experiments. Statistical analysis was performed using the One-Way ANOVA with Tukey's post-test (***$p < 0.001$). **B**. **Expression of insoluble protein fraction**. Insoluble protein fraction was extracted from BEAS-2B Mock cells (lanes 1 and 2), BEAS-2B $tRNA^{Ser}$ cells (lanes 3 and 4) and NCI-H460 Mock cells (lanes 5 and 6). 10% polyacrylamide gels were loaded with 20µl of insoluble protein fraction. Image represents results from one independent experiment.

X. Discussion

Differential expression of tRNAs may represent a mechanism of translational control through the codon usage of specific genes.[85,108] Between human tissues there are differences in individual tRNA expression correlated with the codon usage of highly-expressed tissue specific genes.[85] The deregulation of tRNA pool and mutations in tRNA genes are associated with human diseases.[109] Also, deregulation of the translational machinery is common in cancer cells and it has been proposed as a trigger for the acquisition of the malignant phenotype.[84] Elevated levels of tRNAs have already been documented in breast cancer in a specific fashion since there is a preferential overexpression of tRNAs accordingly with the chemical properties of their cognate amino acids.[85]

To date, data about the effects of tRNA overexpression and how cells respond to it are very limited. In this work, a model to study the effects of $tRNA^{Ser}$ overexpression in BEAS-2B cells was successfully generated, since results from PCR and sequencing insure the plasmids' integration and stability, and the absence of differences in cellular viability between the three cell lines excluded the possibility of plasmid toxicity. Therefore, a comparative analysis between the effects derived from $tRNA^{Ser}$ overexpression and the acquisition of a malignant phenotype *in vitro* was possible. BEAS-2B cell line and NCI-H460 cell line were already used to infer about the acquisition of malignant features by other researchers.[110]

Nevertheless, it should be noted that the profile of tRNA pool in cells with induced tRNA overexpression is dependent on different factors, such as tRNA identity, cells' genetic background and the site of its integration in the genome.[86] Also, it is still unknown the role of individual tRNAs in different cell types and their effect in the transcriptome and the cellular physiology and how they are affected by these two factors.[11]

Cellular Proliferation

In normal cells the production and release of growth-promoting signals is tightly controlled so cells can enter and progress through the cell cycle in conditions that favor homeostasis. However, deregulation of growth and proliferation promoting signals is obvious in cancer cells, which leads to unregulated cell cycle divisions and aberrant cell proliferation.[66] Proliferation promoting signals are related with other biological properties

such as cell growth, firmly expressed by the bigger cell size, and the energy metabolism, which is typically altered in cancer cells.[66] In this context, our study demonstrated higher proliferation capacity in NCI-H460 cells when compared with BEAS-2B derived cell lines, as early as 24h.

When comparing BEAS-2B $tRNA^{Ser}$ cells to the control no differences are observed in their proliferative capacity. However, Pavon-Eternod *et al.* verified that induced overexpression of $tRNA_i^{Met}$ in normal breast cells was enough to prompt increase in proliferation capacity but overexpression of other tRNAs failed to do so.[86] Withal, the same research group verified that the different range of doubling times between normal and cancer breast cells has no correlation with mitochondrial and nuclear global tRNA content.[85]

Transformation Ability

The transformation ability of cells with induced tRNA overexpression in BEAS-2B $tRNA^{Ser}$ cells is equivalent to that observed in NCI-H460 Mock cells suggesting that overexpressing $tRNA^{Ser}$ is enough to prompt the acquisition of transformation ability *in vitro* in a level comparable with cancer cells. This represents further evidence for the hypothesis that deregulation of translational machinery can be a driving force for cellular transformation.[111] Similarly, De Marco *et al.* observed that BEAS-2B cells carrying a mutation on AKT1 do not show alterations in proliferation capacity in complete medium but increased tumorigenic capacity demonstrated by higher transformation ability *in vitro* and induction of tumor formation *in vivo*.[112]

However, it should be noted that assessment of transformation ability was carried out *in vitro* conditions that do not mimic *in situ* microenvironment, like cell-to-cell interactions, oxygen and nutrient availability and pH levels. Thus, it is not possible to ensure the behavior of these cells *in vivo* and so *in vivo* assays should be performed to evaluate the influence of tRNA pool deregulation in the acquisition of tumorigenic capacity.

Proteotoxic Stress Induction and Activation of Protein Quality Control Pathways

A study performed by Stoletzki and Eyre-Walke, provided evidences that codon usage in *E. Coli* is adapted to highly expressed genes to reduce the energy costs derived from missense errors that can lead to non- or misfunctional proteins production.[113] In multicellular eukaryotes the quantification of gene expression and tRNA abundance are difficult due to

their variation between tissues and developmental stages.[114] However, it was observed a positive correlation for the adaptation of codon usage to higher expressed genes, supporting the hypothesis that the equilibrium between tRNA pool and codon usage may increase translation accuracy and efficiency in a demand to reduce mistranslation.[114–116] Thus, we cannot exclude the possibility of misfolded protein synthesis derived from tRNA pool perturbation by overexpression of $tRNA^{Ser}$.

Expression of Chaperones

Surprisingly, the results of HSPs relative expression do not agree with the "addiction to chaperones" hypothesis, which dictates that high levels of chaperones are required to stabilize the increased protein load containing aberrant proteins, characteristic of cancer cells.[53] In our study, a downregulation of Hsp90α, Hsp70 and Hsp27 is clear in NCI-H460 Mock cells but no differences were observed in their expression in BEAS-2B derived cell lines.

Recently, Gallegos Ruiz *et al.* performed an integrated genome wide screening to analyze resected tumor samples from NSCLC patients and described a deletion on chromosome 14. This deletion was presented in 44% of samples and correlated with overall survival, comparing with NSCLC patients with normal gene dosage at the same locus.[90] The study revealed that this deletion only affects the expression of *HSPAA1* and the consequent lower levels of Hsp90α seems to have a crucial role to promote sensitivity to therapeutics, probably derived from the more unstable status of oncoproteins when Hsp90α is expressed at lower levels.[90] A tendency for lower levels of Hsp90α in BEAS-2B $tRNA^{Ser}$ cells is also observed but further studies are required to unveil the regulation of *HSPAA1* in BEAS-2B $tRNA^{Ser}$.

Despite the contribution of Hsp70 to achieve the malignant phenotype, a protective role in lung cancer development is also supported by high levels of this chaperone. Overexpression of Hsp70/Hsc70 can sequester mutant p53 and reduce the inhibition of wild-type p53 due to association with mutant p53. In this way, wild-type p53 is free to perform its antiproliferative activity, suggesting that high levels of Hsp70 can be detrimental to maintenance of tumorigenesis and can be related with survival advantage.[117,118] Note that mutations in *TP53* are present in 50% of NSCLC cancers.[119]

Despite Hsp27 being normally overexpressed amongst different tumors[89], Huang Qi *et al.* did not observe differences in its expression in tissue samples from patients with NSCLC, suggesting that the high levels of Hsp27 detected in other tumors are related with the different functions of Hsp27 in different cell types.[120] Alain Michils *et al.* did not observed differences in Hsp27 expression NSCLC samples as well, instead, they detected low levels of Hsp27 in a small set of samples.[89]

Nonetheless, even in cells from normal lung different HSP expression patterns are detected.[118] In a study performed by Marcel Bonay *et al.* it was observed that only a set of normal human lung cells were positive for Hsp90α and Hsp70, meaning that only a subset of cells in lung expresses high levels of HSPs. Similarly, they suggest that high levels of inducible Hsp70 and Hsp90 reflect the differentiated states of bronchial epithelial cells, since they are required to specific functions according to the physiological state.[121]

Considering the dynamic expression of HSPs in the different types of normal and cancer cells and even in different physiological and differentiation states, other cell lines from normal lung and cancer lung tissues should be tested to better understand the behavior of HSPs in normal and lung cancer cells and in the presence of an inducible tRNA overexpression. Regarding the cells in study, NCI-H460 cell line was obtained from pleural effusions of patients with large cell carcinoma, which is commonly located in the periphery of lung, while BEAS-2B cell line has its origin in bronchus' epithelium. The different origins of cells can also have influence in the unexpected results and derail the normalization with the control.

Still, it should be noted that in this study the total protein was obtained from cell cultures *in vitro.* The model *in vitro* abolishes the great influence of microenvironment of cancer cells, particularly the hypoxia, the low pH and nutrient availability, which are important sources of proteotoxic stress.[57]

Protein Synthesis Rate

The protein synthesis rate is closely related with the protein and DNA content and the cell size.[122] Accordingly, the results from protein synthesis rate show that deregulation of tRNA pool does not promote alterations in protein synthesis rate *in vitro,* but a clear increase in protein synthesis rate is observed in NCI-H460 Mock cells The tumorigenic process is accompanied by increased protein load related with overexpression of oncogenes and

polyploidy.[53] Particularly, higher levels of anti-apoptotic and pro-mitotic proteins are common in cancer cells that are accompanied by overexpression of eIF2α.[123] Increased levels of this factor allows the cell to maintain the increased metabolism and to progress in the cell cycle. At the same time, higher rates of protein synthesis allows to outdo metabolic consequences of stress conditions intrinsic to cancer cells, namely environmental stressors and free radicals.[123] Thus, it should be interesting to quantify the eIF2α expression and its phosphorylation status to explore the mechanisms underlying the protein synthesis deregulation in NCI-H460 Mock cells.

Unfolded Protein Response Activation

Cancer cells are known to be under ER stress, triggered by the particular features of the microenvironment, thus elevated BiP levels in cancer cells are required to activate pro-survival and cytoprotective responses to counteract this chronic stress. BiP may interact directly with apoptotic pathway intermediates and block caspase activation, leading to apoptosis inhibition and cell survival, being overexpressed in malignant forms of cancer such as lung cancer.[57,124] *Qi Wang et al.* detected an overexpression of BiP in cancer lung tissues comparing with normal lung tissues, without differences between types of lung cancer despite their intrinsic morphological and molecular heterogeneity.[125] Similarly, in our study a clear increase in BiP expression is observed in NCI-H460 Mock cells and BEAS-2B $tRNA^{Ser}$ cells, which adopted an intermediate phenotype. Notably, there are evidences for an increase in BiP expression correlated with the tumor stage, suggesting that elevated expression of BiP can be a feature of lung cancer which is correlated with its origin and progression.[125]

Therefore, it seems that tRNA pool deregulation by overexpression of a unique tRNA is enough to activate the UPR response. However, the activation of the different branches of UPR should be analyzed to understand if the activation of UPR in BEAS-2B $tRNA^{Ser}$ occurs similarly to UPR activation and modulation in cancer cells.

Ubiquitin-Proteasome System Activation

UPS is the major eukaryotic proteolytic pathway responsible for the elimination of most of the soluble misfolded proteins.[46] Upregulation of UPS system is evident in cancer cells as a strategy to reduce the accumulation of proteins and so to evade apoptosis. Also, proteasome

is responsible for degradation of cell cycle regulatory proteins, allowing cells to bypass cell cycle checkpoints. Inhibition of 26S proteasome activity has been studied as a therapeutic in lung cancer.[126,127]

Similarly to the comparative study between cell lysates from lung carcinomas and cell lysates from non-cancerous tissues performed by Alain Michils *et al*[89], no differences were observed in protein ubiquitination between the different cell lines in our study but apparently proteins with higher molecular size are more ubiquitinated. Considering its involvement in basic cellular processes whose perturbation leads to malignant transformation, these results were not expected.[89] Nonetheless, results from proteasome activity show that deregulation of tRNA pool is enough to boost the proteasome activity at similar levels observed in NCI-H460 Mock cells, suggesting an increasing demand to eliminate aberrant proteins.

Therefore, results suggest that UPR and UPS quality control pathways were activated in response to proteome instability in BEAS-2B $tRNA^{Ser}$. Since no alteration in size, proliferation capacity and in protein synthesis rate are observed, contrarily to NCI-H460 Mock cells, it seems that deregulation of tRNA pool derived from $tRNA^{Ser}$ overexpression does not trigger an increase in protein load, but it rather affects proteins that are being produced as well as translation speed and accuracy of those proteins.

In cancer cells the activation of these pathways is chronic and cancer cells can adapt to it and use to their advantage the cytoprotective benefits of its activation to survive and progress.[57]

Insoluble Protein Fraction

The analysis of insoluble protein fractions indicates a progressive reduction of insoluble proteins from control cells to NCI-H460 Mock cells suggesting that induced $tRNA^{Ser}$ overexpression leads to alterations in protein expression profiles. Probably, these alterations are advantageous to the acquisition of the malignant phenotype, since it is visible that BEAS-2B $tRNA^{Ser}$ cells adopted an intermediate phenotype.

Cancer cells have quantitative and qualitative alterations in protein expression, with a preferential expression of key proteins in tumor progression and development, such as growth factors, cell-cycle promoters and oncoproteins, particularly c-Myc and VEGF, the last known to be upregulated in NSCLC tumors.[76,111] This alterations in insoluble protein

expression in BEAS-2B $tRNA^{Ser}$ and in NCI-H460 Mock cells can be associated with those alterations in gene expression profiles due to tRNA pool deregulation. This reduction in the insoluble protein content can also be explained by a reduction in protein aggregates, whose toxic character is incompatible with the higher metabolism of cancer cells, which upregulate autophagy to their elemination.

XI. Conclusion and Future Work Suggestions

In general, cells overexpressing the $tRNA^{Ser}$ seem to acquire an intermediate phenotype between control and cancer cells, thus we can conclude that tRNA pool deregulation by a unique tRNA overexpression is enough to the acquisition of an intermediated behavior suggestive of progressive acquisition of the malignant phenotype. The activation of protein quality control pathways show that $tRNA^{Ser}$ overexpression arouse interferences in the stability of the proteome but more studies should be performed to confirm and unveil its origin, namely through mass spectrometry analysis.

To better understand the mechanism of UPR activation the IRE1α, PERK and ATF6 branches should be assessed by western blot, allowing to unveil if the modulation of UPR in BEAS-2B $tRNA^{Ser}$ cells is equivalent to that observed in NCI-H460 Mock cells.

Autophagy is essential to eliminate protein aggregates and so its study would allow to understand if the alterations in insoluble protein fractions are mainly a byproduct of gene expression patterns alterations or an increase in autophagy derived from accumulation of aberrant proteins. To do so, western blot could be applied.

Considering the evidences that cancer cells express higher levels of tRNAs and that induction of an unique tRNA isoacceptor leads to other tRNAs overexpression[86], through the tRNA microarray technology we could confirm the $tRNA^{Ser}$ overexpression in BEAS-2B $tRNA^{Ser}$ cell line and assess other perturbations in the tRNA pool. Likewise, patters of tRNA pool in NCI-H460 Mock cell line would be obtained, allowing to perform a correlation with the tRNA patterns in BEAS-2B $tRNA^{Ser}$ cells. Besides, the application of cDNA microarray technology would be useful to confirm alterations in gene expression patterns in BEAS-2B $tRNA^{Ser}$ cells and to establish a correlation with gene expression profiles in NCI-H460 Mock cells. Thereby, a correlation between tRNA patterns and the codon usage of specific genes in BEAS-2B $tRNA^{Ser}$ would be possible and we could understand if tRNA pool deregulation is a trigger to preferential expression of cancer-related genes.

This study shows that deregulation of the translational machinery is involved in the acquisition of the malignant phenotype and it represents a step for the development of a screnning mechanism for the characterization of malignancy through a simple tRNA pattern analysis.

XII. References

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XIII. Appendix

1X PBS (1L)	
Reagent	Mass (g)
NaCl	0.10
KCl	0.20
Na_2HPO_4	1.44
KH_2PO_4	0.24
In 1L dH_2O	
Adjust pH to 7.4	

HBS (1L)	
Reagent	Mass (g)
Hepes	0.25
NaCl	0.40
KCl	0.0185
Na2HPO4	0.005
D(+) glucose	0.054
In 1L dH_2O	
Adjust pH to 7.14	

Protein lysis buffer	
Reagent	Volume (µl)
ELB	4645
DTT (1M)	5
NaF (1M)	5
EDTA (0.5M)	20
EGTA (0.1M)	50
Na_3VO_4 (0.1M)	50
For 5 aliquots of 955µl	
Adjust pH to 7.14	
1M PMSF	25
Complete Mini EDTA-free protease inhibitor cocktail tablets	20
Add to aliquots immediately before use	

2 Polyacrylamide gels			
Reagents	Running gel		Stacking gel
	10%	8%	4%
H_2O miliQ	3.6ml	3.8ml	3.465ml
Tris-HCl 1.5M pH 8.8	3.75ml	3.960ml	-------
Tris-HCl 0.625M pH 6.8	-------	-------	1.0ml
40% Acrylamide/bis-acrylamide solution, (29:1)	2.5ml	2.1ml	500µl
10% SDS	100µl	100µl	50µl
10% APS	100µl	50µl	50µl
TEMED	10µl	10µl	10µl

6X SDS Protein Loading Buffer	
Reagent	
Tris-HCl	375 mM
SDS	9%
Glycerol	50%
Bromophenol Blue	0.03%

10X Running buffer	
Reagent	Mass (g)
Glycine	144
Tris	30.2
SDS	10

In 1L dH_2O

Dilute 10X to use

Transfer Buffer	
Reagent	
Tris Base	3.03g
Glycine	14.4g
Methanol	200ml
dH_2O	800ml

TBS 10X	
Reagent	Mass (g)
NaCl	80
KCl	2
Tris base	30

In 1L dH_2O

Dilute 10X to use

Stripping solution	
Reagent	
Glycine	1.5g
SDS	0.1g
Tween-20	1ml

In 100ml dH_2O

Adjust pH to 2.2

Proteasome assay lysis buffer	Proteasome activity buffer
0.5M EDTA	500mM EDTA
200mM Tris-HCl pH8	100mM Tris-HCl pH8
40% Glycerol	1mM Suc
H_2O miliQ	------
1M DTT	------
1M ATP	1M ATP

In 10ml solution

Add immediately before use

2% Urea SDS loading buffer
Reagent
8M urea
2% SDS
50mM DTT
50mM Tris pH7.4
A trace of bromophenol

Coomassie Brilliant Blue
Reagent
0.25% (w/v) Comassie Brilliant Blue R in ethanol
dH_2O
Acetic acid
[5:5:1]

Distaining solution
Reagent
Ethanol
dH_2O
Acetic acid
[5:5:1]