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CONTRIBUTION OF TRNA MODIFICATIONS FOR PROTEOSTASIS IN HUMAN CELLS

RELEVÂNCIA DAS MODIFICAÇÕES DE TRNA NA PROTEOSTASE EM CÉLULAS HUMANAS

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Ana Raquel Santos Calhôa Mano Soares do Departamento de Ciências Médicas da Universidade de Aveiro

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“Go as far as you can see.
When you get there, you’ll be able to see further.”

Thomas Carlyle

O Júri

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palavras-chave

RNA de transferência, enzimas modificadoras de tRNA, proteostase, agregação proteica

resumo

Mutações nas moléculas de RNA de transferência (tRNAs), moléculas chave no processo de tradução, levam à perda da precisão da tradução do RNA mensageiro (mRNA). Isto traduz-se em alterações na taxa de síntese proteica, misfolding de proteínas e acumulação de proteínas agregadas que, por sua vez, levam a stress proteotóxico e ativação da UPR, algumas das características de doenças conformacionais de proteínas. Para serem totalmente ativos, os tRNA sofrem modificações pós-transcricionais catalisadas por diferentes enzimas modificadoras de tRNA. A desregulação de algumas modificações de tRNA e de algumas das enzimas modificadoras foram já correlacionadas com alterações ao nível da fidelidade e eficiência da tradução, especialmente em leveduras. No entanto, um estudo exaustivo sobre o impacto de todas as modificações e enzimas modificadoras de tRNA em mamíferos não foi ainda realizado.

Para identificar quais as enzimas modificadoras essenciais para a manutenção da proteostase, a nossa equipa desenvolveu um sistema repórter de agregação de proteínas (pcDNA3.1 Hsp27-GFP) que nos permite detetar in vivo a nível celular a produção de agregados proteicos. Após o estabelecimento de uma linha celular estável a expressar este repórter, foi realizado um screening fenotípico que identificou as enzimas ELP1, ELP3, ELP6, URM1 e TRMT2A como essenciais para a proteostase. Verificou-se que a via UPS foi ativada na ausência das enzimas ELP1, ELP3, ELP6 e análises de proteómica de células com silenciamento da ELP3 revelaram que as vias mais alteradas dizem respeito à tradução e transcrição, entre outras, que estão geralmente desreguladas em doenças neurológicas, como a Esclerose Lateral Amiotrófica. Os resultados desta tese identificam e demonstram que um grupo particular de enzimas modificadoras de tRNA afetam a proteostase em células humanas e que as mesmas podem ser novos alvos terapêuticos para doenças conformacionais.

keywords

Transfer RNA, tRNA modifying enzymes, proteostasis, protein aggregation

abstract

Mutations in transfer RNAs (tRNA), key molecules in the translation process, lead to loss of accuracy of messenger RNA (mRNA) translation. This may induce changes in protein synthesis rate, protein misfolding and accumulation of aggregated proteins which, in turn, leads to proteotoxic stress and UPR activation, some of the characteristics of conformational protein diseases. To be fully active, tRNA molecules undergo post-transcriptional modifications catalyzed by different tRNA modifying enzymes. Deregulation of some modifications of tRNAs and some of the modifying enzymes has already been correlated with changes in translation fidelity and efficiency, especially in yeast. However, a comprehensive study on the impact of all tRNA-modifying enzymes and modifications in mammals has not been performed yet.

In order to identify the key modifying enzymes for proteostasis preservation, our team developed a protein aggregation reporter system (pcDNA3.1 Hsp27-GFP) that allows the detection of the production of protein aggregates in vivo at the cellular level. After establishing a stable cell line expressing this reporter, a phenotypic screening was performed, identifying ELP1, ELP3, ELP6, URM1 and TRMT2A enzymes as essential for proteostasis. The UPS pathway was activated in the absence of the ELP1, ELP3, ELP6 enzymes and proteomic analyzes of ELP3 silencing cells revealed that the most altered pathways are concerned to transcription and translation processes, which are generally dysregulated in neurological diseases, such as Amyotrophic Lateral Sclerosis. In this thesis, the proteostasis relevant tRNA modifying enzymes are identified and characterized in human cells. This group of enzymes may represent promising therapeutic targets for conformational diseases.

Declaração

Declaro que este trabalho é inteiramente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como claramente identificadas as citações dessas obras. Não contém, por isso, qualquer tipo de plágio, quer de textos publicados, quer de trabalhos académicos, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos.

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I. Abbreviations list

Abbreviation	Definition
Aa	Amino acid
AARSs	Aminoacyl-tRNA synthetases
Ac^4C_m	N^4-acetyl-2'-O-methylcytidine
AD	Alzheimer’s disease
ADATs	Adenosine deaminases acting on tRNAs
ALKBH8	Methyltransferase Alkylation repair homolog 8
ALP	Autophagy-lysosomal pathway
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
ARID	Autosomal recessive intellectual disorder
ASL	Anticodon stem loop
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
A-to-I	Adenosine to Inosine
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
BECTS	Benign epilepsy with centrotemporal spikes
BiP	Binding immunoglobulin protein
BSA	Bovine serum albumin
bZIP	Basic Leucine Zipper
CMA	Chaperone-mediated autophagy
COSMIC	Catalogue of Somatic Mutations in Cancer
DMEM	Dulbecco’s modified eagle medium
DUBs	Deubiquitinases
E1	Activating Ubiquitin Enzyme
E2	Conjugating Ubiquitin Enzyme
E3	Ligase Ubiquitin Enzyme
EEG	Electroencephalography
eIF2α	Eukaryotic translation initiation factor 2α
eIF2α-P	Phosphorylated eukaryotic translation initiation factor 2α
ELP	Elongator protein
ELP1	Elongator protein 1 homolog
ELP2	Elongator protein 2 homolog
ELP3	Elongator protein 3 homolog
ELP4	Elongator protein 4 homolog
ELP5	Elongator protein 5 homolog

ELP6	Elongator protein 6 homolog
FBS	Fetal bovine serum
FD	Familial Dysautonomia
GFP	Green fluorescent protein
G_m	2'-O-methylguanosine
HAT	Histone acetyltransferase
HSP27	Heat-shock protein 27
I^6A37	N6 isopentenyl adenine 37
IPTases	Isopentenyl-transferases
IRE1	Inositol-requiring enzyme 1
JNK	c-jun NH(2)-terminal kinase
KAT	Lysine acetyltransferase
LB	Loading buffer
M^1A	1-methyladenosine
M^1G	1-methylguanosine
$M^2{}_2G_m$	N^2,N^2,2'-O-trimethylguanosine
Mcm^5	5-methoxycarbonylmethyl
Mcm^5s^2U	5-methoxycarbonylmethyl-2-thiouridine
Mcm^5Um	5-methoxycarbonylmethyl-2'-O-methyluridine
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	Myoclonic epilepsy with ragged-red fibers
MLASA	Mitochondrial myopathy, lactic acidosis and sideroblastic anemia
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
Ncm^5	5-carbamoylmethyl
OECD	Organization for Economic Co-operation and Development
PBS	Phosphate-buffered saline
PD	Parkinson’s disease
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PFA	paraformaldehyde solution
PN	Proteostasis network
PS	Protein synthesis
Q	Queuosine
RT	Room temperature
S^2T	2-thioribothymidine
SAM	S-adenosylmethionine
siRNA	Small interference RNA

TGT	tRNA guanine transglycosylase
TRDMT1	tRNA aspartic acid Methyltransferase 1
TRMT1	tRNA methyltransferase 1
TRMT2A	tRNA (uracil-5-)-methyltransferase homolog A
TRMT5	tRNA (guanine(37)-N1)-methyltransferase
TRMT61A	tRNA Methyltransferase 61A
tRNA	Transfer RNA
URM1	Ubiquitin related modifier 1
WR	Working reagent
yW	Wybutosine
τm5s2U	5-taurinomethyl-2-thiouridine
Ψ	Pseudouridine

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CHAPTER 1.

Introduction

1.1. Proteostasis in aging

Over the years, the advance of the scientific knowledge and the availability of drugs and therapies has allowed the population to live longer. Although this increase of life expectancy is a positive factor, age-related diseases such as neurological disorders and cancer are emerging with aging (1), which is described as the "persistent decline in the age-specific fitness components of an organism due to internal physiological degeneration" (2).

One of the hallmarks of aging and age-related diseases is the loss of proteostasis - protein homeostasis - characterized by a progressive decline in the ability of cells to maintain protein quality control, accompanied by the appearance of protein aggregates in several tissues. Proteostasis is maintained by processes such as protein synthesis, folding, aggregation, disaggregation and degradation, comprised in the proteostasis network (PN) (3,4). In short, the PN comprises mechanisms that are responsible for the stabilization of correctly folded proteins, particularly the heat-shock family of proteins, and mechanisms to restore the structure of misfolded proteins or to remove and degrade them by proteasomes or lysosomes in order to prevent them from accumulating in the cell (5). Moreover, when this unfolded, misfolded or aggregated proteins are chronically expressed, they contribute to the development of some age-related pathologies, such as Alzheimer's and Parkinson's disease, among others (1).

In Portugal, according to the Organization for Economic Co-operation and Development (OECD) statistics, between the years 2000 and 2016, people dying every year from Alzheimer's disease (AD) had an increase of 43,8% and from Parkinson's disease (PD) this value was about 62.9%. In the United States of America, in the same period, the data was very similar: from AD the increase of deaths per year was approximately 55.2% and from PD was 44.0%. This massive growth warns us of the urgency of understanding the underlying mechanisms of these diseases and finding new therapies not only to provide better quality of life for patients but also to prevent these diseases from developing and progressing rapidly.

1.2. Protein conformational diseases

In 1997, Carrel and Lomas came up with the concept of conformational disease to characterize the similar mechanism for the development of neurodegenerative disorders (Huntington's, Alzheimer's and Parkinson's disease, Prion encephalopathies), cystic fibrosis, diabetes type II and systemic amyloidosis. This discovery was largely due to the fact that the onset and progression of the disease was caused by protein misfolding and conformational change. The conformational change can promote the disease in two ways, by causing a gain of a toxic activity or taunting the absence of biological function of the native folded protein (6,7). Each disease is associated to a specific protein or group of

proteins that could be unfolded, misfolded and/or aggregated and the accumulated protein deposits are pathological hallmarks for the corresponding disease (Figure 1), however the causative agents and toxicity mechanisms are still not very well understood (8).

There are two characteristics shared by the majority of neurodegenerative disorders, which are the presence of amyloid-like misfolded protein deposits and the impairment of neuronal function (9). Moreover, even though each disorder involves the amyloidogenic aggregation of distinct proteins, the biophysical and structural properties of amyloid fibrils are identical (10). Evidences suggest that the development of amyloid fibrils is related to the loss of protein function, a toxic gain of function or a functional reversible amyloid assembly (11,12). In the majority of these disorders, the formation of the amyloid structures is correlated with cell death but it is unclear if amyloid is cell pathogenic in a direct way or if it is the transition from a native folded protein to amyloid that triggers the toxic event (13).

The mechanisms that lead to the formation of particular protein aggregates are still unclear. It is known that post-translational modifications of proteins play a role in protein stability and structure, but loss of mRNA translation accuracy leads to amino acid misincorporations in nascent polypeptides that in turn may also lead to protein misfolding and accumulation of aggregated proteins (14,15). Among the various molecules involved in the translation process, transfer RNAs (tRNAs) stand out as one of the most relevant as they are the effector molecules of translation, whose function will be explored in more detail in the next topics.

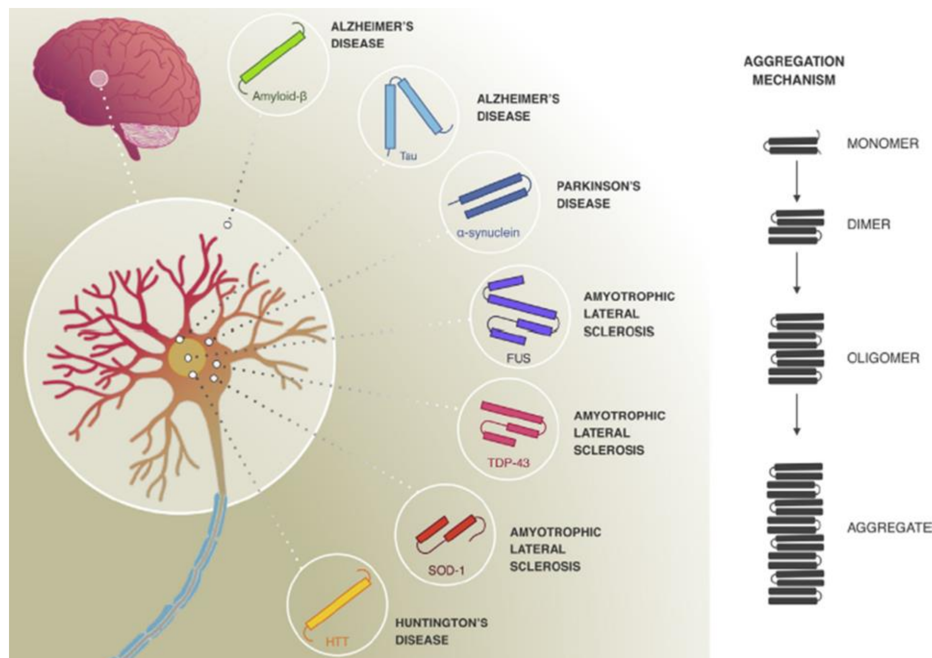

Figure 1: Protein aggregation in neurodegenerative diseases. In several protein misfolding diseases, native unfolded monomers form cross β-sheet assemblies, evolving into oligomers to form highly ordered fibrillary aggregates. This process produces insoluble protein deposits and it is associated with neurodegeneration (13).

1.3. tRNA – The “bridge” molecule between mRNA and proteins

tRNA is a key adaptor molecule, responsible for the conversion from RNA to protein, translating the genetic code by matching a codon in a messenger RNA (mRNA) with the amino acid (aa) it codes for during translation within the ribosome (16). Different types of tRNAs are floating around in a cell, each one with its own anticodon and corresponding amino acid; once inside the ribosomes, tRNAs bind to mRNA codons through complementarity with their anticodons, allowing the delivery of the amino acids to be added to the protein chain. Ribosomes possesses three slots for tRNAs: A, P and E site and tRNAs move through the A site to the P site and then to the E site as they deliver amino acids during translation (17).

Mature tRNAs are made from a single strand RNA of approximately 70-100 nucleotides long with a conserved three-dimensional shape that arises mostly from the interaction between bases in different regions of the RNA sequence, giving rise to the secondary “clover leaf” structure (Figure 2). This base pairing results in three double-stranded regions and stem-loops - the D loop, the Anticodon loop and the T loop – in an open-ended stem (amino acid attachment site) formed by the pairing of the 5’ and 3’ ends and it also has a vestigial projection called variable loop that it is between the Anticodon loop and the T-loop, like (Figure 2. The D-loop has this name because it has a D-hidro-uridine in position 16 and is responsible to bond to the aminoacyl tRNA synthetase. The T-loop or GTPCG (Thiamine-Pseudouridine(ψ)) loop is the ribosome recognition site and facilitates the connection of the amino acid to the A site. The Anticodon stem loop (ASL) is comprised between the positions 34, 35 and 36 of the tRNA and binds to complementary bases of the mRNA (18–20).

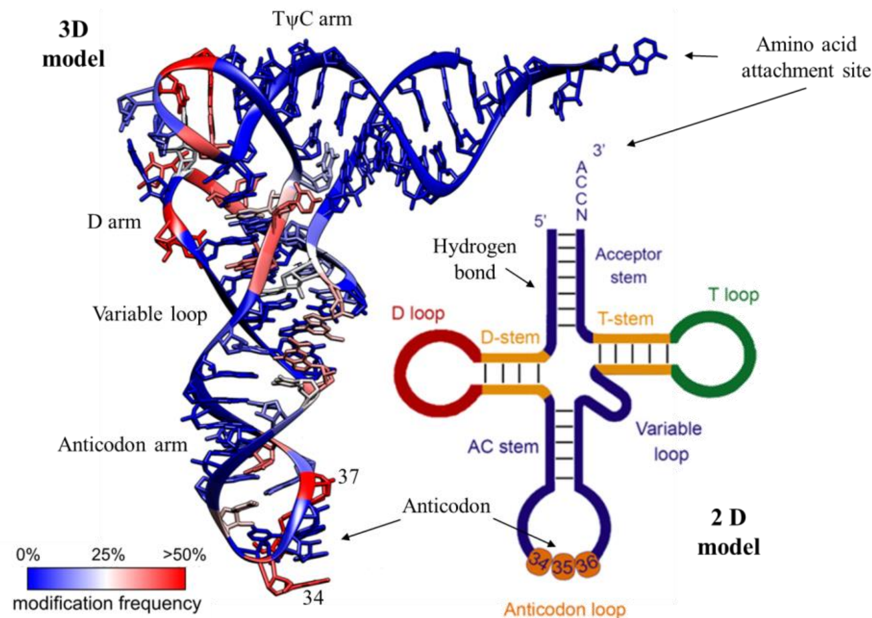

Figure 2: Crystal structure of the $tRNA^{Phe}$ from *S. cerevisiae* (3D model) and its modification frequency (Adapted from (38)) with the correspondent 2D “clover leaf” model and the described identity elements of tRNA. The Acceptor stem (7bp), the D-stem (3-4bp) and the anticodon (AC) stem (5bp). The variable (V) region (4-23 nt) and the D-loop (4-12nt) confers some variety in the tRNA length, however, the anticodon in the anticodon loop is always numbered 34-36 and the CCA tail at the 3’-terminus is numbers 74-76. The 3’-CCA triplet is added post-transcriptionally by a CCA-adding enzyme. Adapted from (31).

The right amino acid binds to the right tRNA due to aminoacyl-tRNA synthetases; for each amino acid there is a different synthetase enzyme that recognizes only that amino acid and its correspondent tRNA. The cognate amino acid is only charged at the 3'-end after maturation of the tRNA. Through their anticodon loop, that contains the three nucleotide base sequence, tRNAs pair specifically with the codons in mRNAs during translation. Position 34 of tRNAs can wobble, meaning that it can pair with different nucleotides of the third position of mRNA triplets via non Watson-Crick interactions, giving some flexibility to the genetic code and allowing some tRNAs to decode different sets of codons for the same amino acid and even some codons to be recognized by more than one anticodon sequence (Figure 3A) (21,22).

To explain this possibility, Francis Crick proposed the Wobble Hypothesis, that elucidates how tRNAs recognize and read more than one codon, clarifying why cells have only 40 distinct tRNA species for 64 codons. In summary, the first two bases pair between tRNA nucleosides 36 and 35 and the coding triplet in mRNA are canonical (A-U; C-G or *vice versa*) base-pairings. The third base pairing (position 34 of tRNA) can also be canonical but to enlarge tRNA recognition of codons in protein synthesis, there are unconventional and non-canonical base pairings in the third base of the codon-anticodon triplet (18,23). The genetic code is illustrated in Figure 3B as well as its codon degeneracy.

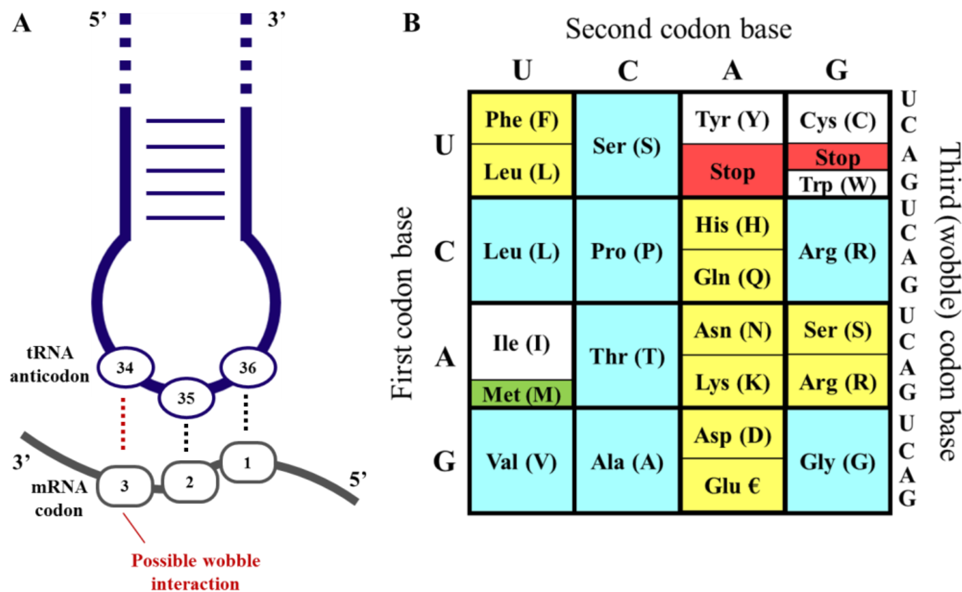

Figure 3: Representation of codon-anticodon interaction and Wobble Hypothesis. A) Interaction of the anticodon bases (34–36) of a tRNA with the corresponding bases of the mRNA codons (3, 2, 1). A wobble interaction is possible between codon base 3 and anticodon base 34. The latter is frequently modified and directs the wobble interactions with the third codon base. B) The standard genetic code is illustrated as a simple decoding table; 2-fold degenerate codon boxes are colored yellow; 4-fold degenerate boxes are blue; special boxes are colored white. The start codon is colored green and stop codons are colored red. Adapted from (38).

The main function of tRNAs is to participate in protein synthesis as a key component of translation. However, this molecule participates in other processes such as

inhibition of apoptosis via complexation of cytochrome c, amino acyl addition to membrane lipids, HIV-1 priming, antibiotics target and biosynthesis and can originate tRNA derived fragments (24).

1.4. tRNA Modifications

tRNAs are heavily chemically modified to maintain their proper structure, stability and function. In fact, tRNAs are the RNA molecules bearing, the largest number of post-transcriptional modifications. Their chemical diversity is, in this way, significantly augmented. Nucleoside modifications become an integral part of the process that ensures that tRNAs accomplish the deciphering of the genetic material. In fact, near 10% of the genes in the genome code for enzymes that are involved in tRNA modification (25), highlighting the relevance of such modifications for correct tRNA function. Figure 4 describes the essential roles of these modifications for tRNA function in the different compartments of the cell. Some modified nucleosides are present in a specific group of tRNA species while others are found in most tRNAs and for these nucleoside modifications to occur, one or more enzymes and enzymatic steps may be involved (26,27).

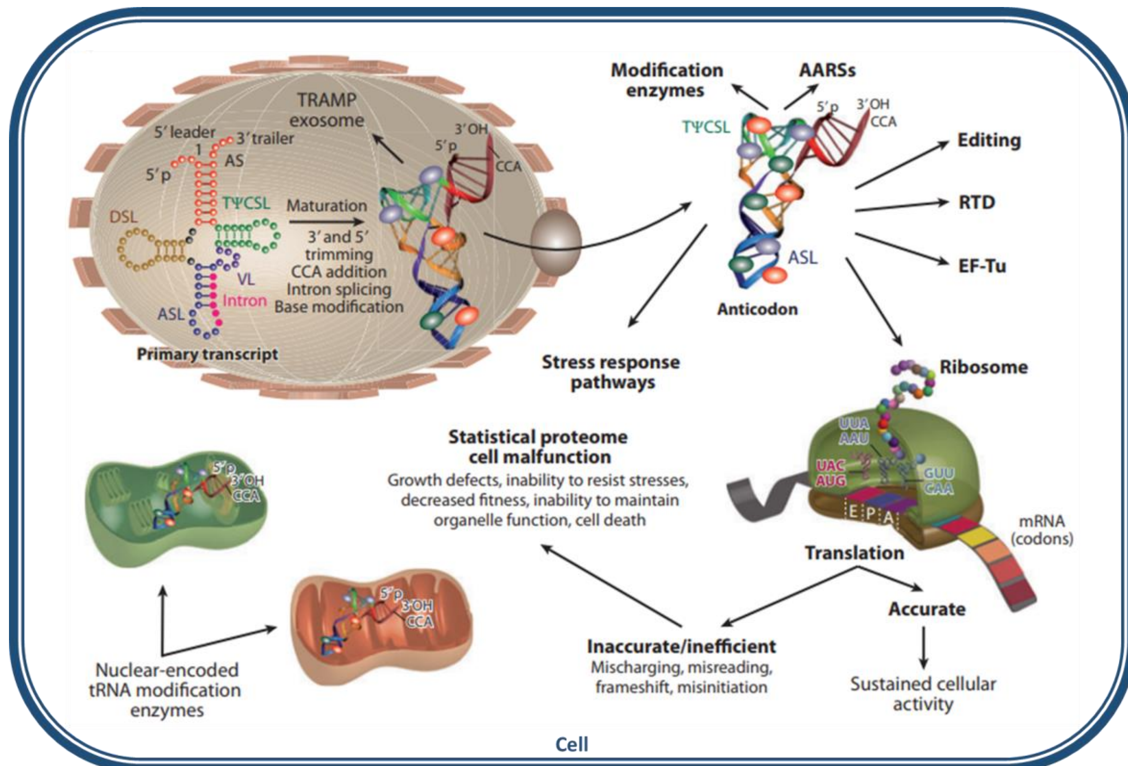

Figure 4: Essential roles of nucleosides modifications for tRNA function. Nucleoside modifications have structural roles (e.g. correct formation of the tRNA L shape), are involved in tRNA interactions with translation machinery players (e.g. modification enzymes, ribosomes, mRNA codons, translation factor, aminoacyl-tRNA synthetases (AARSs), editing and RNA-degradation systems). Modifications can be introduced on tRNA substrates in the nucleus, cytoplasm or organelles (25).
Abbreviations: AARS, aminoacyl-tRNA synthetase; ASL, anticodon stem loop; DSL, D stem loop; EF-Tu, Elongator factor Tu; mRNA, messenger RNA; RTD, rapid tRNA decay pathway; TψCSL, TψC stem loop; TRAMP, Trf4/Air2/Mtr4p polyadenylation complex; tRNA, transfer RNA; VL, variable stem loop.

A total of 93 different tRNA modifications with their correspondent type and location have been identified and listed in the RNA Modification and MODOMICS databases, 51 of them belonging to Eukarya kingdom (http://mods.rna.albany.edu/) (28). The lack of a phenotype associated to tRNA modifications makes it difficult to classify their biological significance and function. However, the advance of technology allowed to describe numerous modification defects as well as their related phenotypes, which bring us insights of their biological roles (29). There are already tRNA modifications associated to human diseases whose chemical structures are illustrated in Figure 5. There has been an attempt to clarify some of the functions of these modifications and they are usually based on three principles: many modifications are in or around the anticodon loop, affecting translation and cellular growth; many modifications in the main body of tRNA molecules affect their folding and stability and some modifications at several positions affects specifically tRNA identity (30). Concrete examples of such functions are the stabilization of codon-anticodon interactions, increased tRNA capability to decode multiple synonymous mRNA codons, rapid tRNA response to environmental challenges like stress, increased protein synthesis fidelity, prevention of frame shift mutations, and codon reading tuning (24).

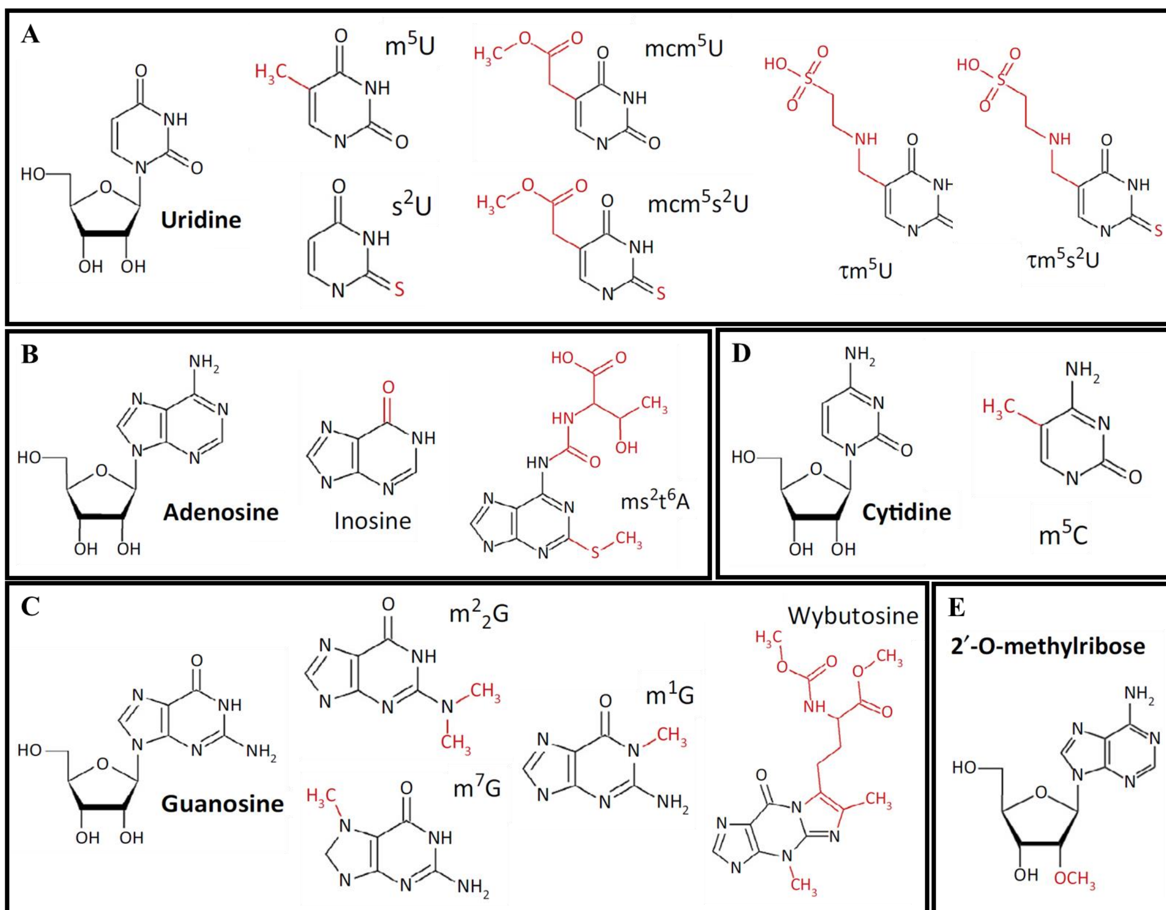

Figure 5: Chemical structures of tRNA modifications linked to human diseases. Unmodified nucleosides are depicted in black. Atomic changes for each chemical structure upon modification are shown in red. Nucleobase (A-D) and ribose (E) modifications are shown. Adapted from (22).

The understanding of the diversity of the cellular functions of tRNAs has increased in the last years due to the development of new technologies, as well as due to the understanding of the mechanisms by which their expression is coordinated in specific tissues, making it a dynamic regulator of the stress response. tRNA mutated genes have been linked to several human pathologies, suggesting that its different abundance in certain tissues modulates the effect of such pathologies and their associated phenotypes, since tRNA diversity modulates the proteome depending on the tissue and the cell, even in the same genome (31).

1.4.1. Modifications at several positions of tRNAs

One of the simplest and most frequent modifications found in tRNAs is methylation that can occur in all positions of the target nucleotide; this type of modification destabilizes Watson-Crick interactions leading to massive structural changes in the global tRNA fold (32,33). For example, 1-methyladenosine at position 9 (m^1A9) in human mitochondrial $tRNA^{Lys}$ shifts the structural equilibrium from an alternative hairpin structure to the functional cloverleaf structure, while Pseudouridine (ψ) at positions 32 and 39 shape the anticodon stem loop (34). Other modifications like N^4-acetyl-2'-O-methylcytidine (ac^4C_m), 2'-O-methylguanosine (Gm) and N^2,N^2,2'-O-trimethylguanosine ($m^2{}_2G_m$) are involved in thermal stabilization of tRNA; this is relevant, for example, in case of hyperthermophiles that modify more extensively their tRNAs when growing at higher temperatures: survival at high temperature of *Thermus thermophilus* is dependent on the formation of 2-thioribothymidine (s^2T) (35,36). Hypo modification of tRNAs usually causes their targeting for degradation, meaning that another role of tRNA modifications is to prevent tRNAs from entering into specific degradation pathways (22).

1.4.2. Modifications in or around the anticodon loop

Modifications of bases occurring at the wobble position in the anticodon or immediately next to the anticodon triplet frequently influence the decoding capacities of tRNAs by restricting and/or improving the codon-anticodon interactions, which can affect the behavior of tRNAs during gene translation and the maintenance of the reading frame (22,37). Modifications in the anticodon loop also have structural functions, since they reinforce a defined loop structure, necessary for a stable codon-anticodon interaction (38).

In addition to having a great diversity of hypermodified nucleotides at positions 34 and 37, modifications at position 34 are usually associated with decoding capacity because base modifications at this position are generally necessary for codon-anticodon wobbling to occur (39). However, these modifications may also prevent translational frameshifting and may even be required for amino acylation (40). Examples of wobble modifications include uridine (U) 34 modifications like the incorporation of methyl, hydroxyl and thiol groups, and adenosine (A) 34 modifications such as adenosine-to-inosine (A-to-I) editing,

as described in Table 1 (30,41). Modifications at position 37 help to stabilize codon-anticodon interactions by providing base-stacking interactions , preventing translational frameshifting (25).

Table 1: Prominent modifications in the tRNA anticodon loop. Adapted from (39).

Modification	Characteristics
Queuosine (Q)	Occurs with GUN anticodons (N represents any nucleotide); mediated by the tRNA-guanine transglycosylase (TGT) complex; changes in Q abundance correlated with stress tolerance, cell proliferation and tumor growth (42,43).
Inosine	Post-transcriptional modification found in tRNAs residues 34, 37 and 57; results from a deamination reaction of adenines that is catalyzed by adenosine deaminases acting on tRNAs (ADATs); Adenine-to-inosine (A-to-I) editing allows the enlargement of the decoding capability of individual tRNAs and the limitation of the tRNA species number for codon-anticodon recognition; I34 hypomodification associated with myositis and missense mutation in ADAT3 gene associated with intellectual disability (44).
5-methoxycarbonyl methyl-2 thiouridine (mcm^5)	Elongator (ELP) complex is needed; U34 base of cytoplasmic tRNA carries mcm^5 or 5-carbamoylmethyl (ncm^5) modifications; U34 modification associated with the enhancement of the translation efficiency and fidelity; cells lacking U34 modifications exhibit hallmarks of proteotoxic stress, like protein aggregation (45).
Wybutosine (yW)	G37 is methylated to form 1-methylguanosine (m^1G); m^1G is the first step for Wybutosine formation; Presence of yW provides base-stacking interactions of the tRNA anticodon with the A-site codon, playing a key function in reading frame maintenance since they prevent the propensity for ribosome sliding on phenylalanine codons (UUU and UUC) (46).
Threonyl-carbamoyl-adenosine	tRNA isopentenyl-transferases (IPTases) introduce an isopentenyl group onto N6 of adenine at position 37 (i^6A37); i^6A37 promotes translational efficiency and fidelity at cognate codons but decreases fidelity at non-cognate codons (47); mistranslation of several proteins due to the lack of such modifications associated to glucose intolerance and type 2 diabetes (48).
5-methylcytosine (m^5C)	Positions 38, 48 and 49 are the most commonly modified; m^5C protects tRNAs against endonucleolytic cleavage, conserving the stable levels of substrate tRNA and helping protein translation and differentiation; C38 tRNA methylation contributes to tRNA stability and translation accuracy (49,50).

1.4.3. Modifications in the main body of tRNAs

Some modifications outside the anticodon loop are important for the structure or stability of the tRNA and may also regulate the speed and fidelity of translation (51,52). There are cases suggesting that the loss of certain single modifications can be compensated by the presence of others, signifying the existence of some functional redundancy among certain tRNA modifications, contributing to the lack of phenotypes of single mutants, particularly in yeast (53).

Modifications taking place in the main body of tRNAs usually have a structural and stabilizing role in these molecules. For example, pseudouridine leads the sugar conformation of the nucleobase into the C3'-endo which will cause an increase in binding affinity and strengthens the tRNA structure while dihydrouridines are important to maintain a flexible tRNA structure and some others serve as identity elements for tRNAs (e.g., aminoacyl tRNA synthetase recognition) (20,25).

1.4.4. tRNA modifications in human diseases

The first tRNA mutation linked to a human disease was only discovered in 1990, although the participation of tRNA in the translation process has already been known since 1950s (54). Nowadays, disorders associated with defects in tRNA modifications can be divided, in a more extensive way, in X-linked intellectual disability, familial dysautonomia, type II Diabetes, mitochondrial disorders (MELAS - Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes, MERRF - Myoclonic epilepsy with ragged-red fibers), Infantile hypertrophic cardiomyopathy, respiratory defects, myopathies, encephalopathies and MLASA (myoclonic epilepsy, myopathy, lactic acidosis and sideroblastic anaemia) (24). Examples of such modifications and associated diseases are described in Table 2.

In the early 1970s it was suggested that alterations in modification levels would be involved in modulating the expression of specific proteins, occasionally leading to observable phenotypes (e.g. MELAS is caused by the elimination of the naturally occurring taurine modification by a mutation in mitochondrial $tRNA^{Leu}_{UUR}$) (55).

The existence of some cases in which mutants of tRNA modifying enzymes genes exhibit lethal or serious pleiotropic phenotypes raises questions such as: Does the absence of modifications in tRNA per se is causing these phenotypes due to a translation defect? If this is the case, it is crucial to identify the protein(s) affected by the translation defects. On the other hand, could the absence of the modification in a molecule other than tRNA (another RNA, protein) be causing the phenotypes? Did modifying enzymes own functions that are not associated with tRNA modifications?

Table 2: Human disorders and associated tRNA modifications. Adapted from (22).

Disease Category	Disease	Affecter tRNA modification	Gene involved
Neurological	Intellectual disability	2'O-methylribose	FTSJ1[a]
		m^2_2G	TRM1
		m^5C	NSUN2
		m^7G	WDR4[b]
		A-to-I editing	ADAT3
	Familial dysautonomia	mcm^5s^2U	IKBKAP (ELP1)
	Amyotrophic lateral sclerosis		ELP3
	Rolandic epilepsy		ELP4
Cardiac	Noonan-like syndrome[c]	m^5C	NSUN2
Respiratory	Bronchial asthma	mcm^5s^2U	IKBKAP
Cancer	Breast	m^5U	TRMT2A
	Urothelial	mcm^5U	HABH8 (HALKBH8)
	Epigenetic cancer treatment	m^5C	DNMT2
Metabolic	Type 2 diabetes	ms^2t^6A	CDKAL1
Mitochondrial-linked	MELAS	τm^5U	mt $tRNA^{Leu}$(UAA)
	MERRF	τm^5s^2U	mt $tRNA^{Lys}$(UUU)
	MLASA	ψ	PUS1
	Infantile liver failure	s^2U	MTU1 (TRMU)

Abbreviations: mt, mitochondrial; m^2_2G, N2,N2-dimethyl guanosine; m^5C, 5-methylcytosine; m^7G, 7-methylguanosine; mcm^5s^2U, 5-methoxycarbonylmethyl-2-thiouridine; m^5U, 5-methyl uridine; mcm^5U, 5-methoxycarbonylmethyl uridine; ms^2t^6A, 2-methylthio-N6-threonyl carbamoyladenosine; τm^5U, 5-taurinomethyluridine; τm^5s^2U, 5-taurinomethyl-2-thiouridine; ψ, pseudouridine; s^2U, 2-thiouridine.

[a]Linked to chromosome X

[b]Might be involved in Down's syndrome (no direct link to the disease has been shown)

[c]Heart problems are one of the main features of the disease, but it is also characterized by specific morphological phenotypes (widely set eyes, low set ears, webbed neck and chest deformity) and mental retardation in some cases.

For example, the urmylation pathway is a tRNA modification pathway with two functions with different cellular mechanisms: The C-terminal glycine thiocarboxylated ubiquitin-related modifier 1 (URM1) acts as a sulfur donor for thiolation of U34 and as a protein modifier when it is under oxidative stress (in yeast and humans), which means that defects in URM1 have pleiotropic phenotypes (56,57).

Another interesting cases are *elp*-defective mutants whose defects are redundant with those of URM1. The Elongator complex is composed by six different protein subunits – Elongator protein (ELP) 1 to 6- and has a role in transcription as an H3 and H4 histone acetylase. Beyond this, it also participates in diverse cellular processes such as transcriptional elongation, formation of modified wobble uridines in tRNAs, polarized exocytosis and telomeric gene silencing (58). In humans there are already several mutations in Elongator subunits genes linked to diverse pathologies concerning neurodegeneration, pinpointing a role of this complex in neurodevelopment and in protection against neurodegeneration (59,60). Figure 6 comprises data collected from several studies concerning defects in tRNA modifications associated with human diseases such as cancer, neurological disorders, Type II Diabetes and mitochondrial-linked disorders (61).

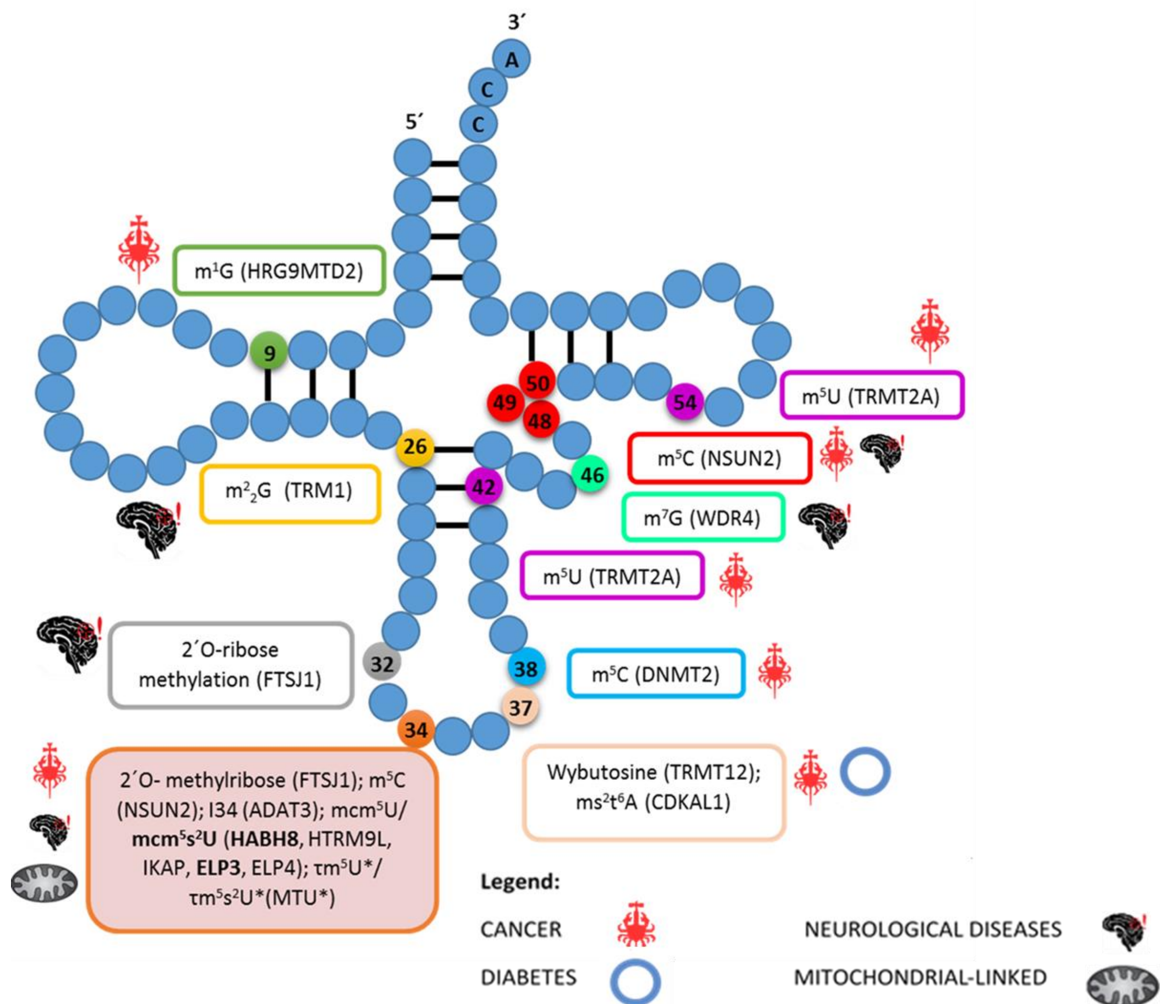

Figure 6: Representation of the tRNA secondary structure with the respective tRNA modifications and associated tRNA modifying enzymes (in parenthesis) that have been linked to human pathologies – indicated near to each box. Connecting lines between RNA residues represents base pairing. Asterisks indicate modifications that are only found in mitochondrial tRNAs. Adapted from (61, 109).

1.5. Elongator complex

Originally, the Elongator complex was described as a promoter of elongation of RNA polymerase II transcription (62) as well as other functions in different compartments of the cell (Figure 7B), with the acetyltransferase domain of ELP3 required in all of them (63). However, more recent studies have shown that its main cellular function is to promote the formation of 5-carbamoylmethyl (ncm^5) and 5-methoxycarbonylmethyl (mcm^5) side-chains on wobble uridines, once the conserved six-subunit elongator complex is crucial in the first step of the formation of ncm^5 and mcm^5 side chains, that frequently contain uridines at the wobble position in eukaryotic cytoplasmic tRNAs (64,65).

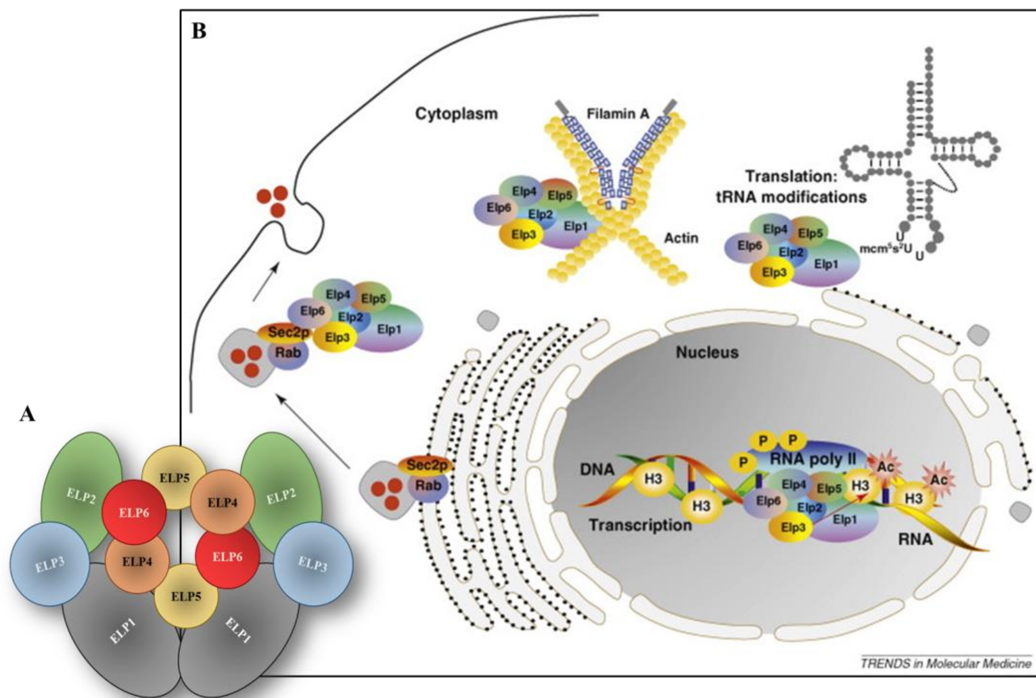

Figure 7: Structure of the Elongator complex and its biological roles. A) Elongator complex structure; B) Elongator complex functions. Is involved in protein translation fidelity by regulating tRNA modifications in the cytoplasm. In the nucleus, Elongator is associated with hyper phosphorylated RNA polymerase II and is required for histone H3 acetylation across the transcribed regions of multiple genes, including candidates coding for proteins involved in cell motility. It is also a filamin A-binding protein in the cytoplasm that regulates actin cytoskeleton organization. An interaction between ELP1 and Sec2p, a regulator of Rab activation, also negatively regulates exocytosis. Adapted from (**63).**

As we can see in Figure7A, the Elongator complex is composed by six ELP proteins that form two distinct sub-complexes, a symmetric dimer composed by ELP1-ELP2-ELP3 and an heterohexameric ring composed by ELP4-ELP5-ELP6 that is bound to one of the two ELP1-ELP3 sub-complexes (66,67). All six ELP proteins are necessary for tRNA modifications, however, it is thought that the catalytic activity have its place in the ELP3 protein, since it contains both a lysine acetyltransferase (KAT) domain (earlier called a histone acetyltransferase (HAT)) and a radical S-adenosylmethionine (SAM)

domain (64,68,69). This notion can be supported by the fact that only homologues of ELP3 and none of the other subunits are found in most archaea and some bacteria.

It was already possible to associate several mutations in genes for Elongator subunits to several human diseases, as is the case of Familial dysautonomia (FD), an autosomal recessive neurodegenerative disorder that is caused by a point mutation in IKBKAP (ELP1) gene which changes a splice site in one of the introns, leading to the skipping on an exon and the generation of frameshift and therefore a premature translation termination codon. FD patients show decreased levels of ELP1 protein, reduced levels of mcm^5s^2U in tRNA in brain tissue and fibroblast cell lines (70–72). Other mutations in genes for other Elongator subunits have been associated with other diseases. For example, allelic variants of ELP3 have shown strong association with axonal biology and neuronal degeneration in Amyotrophic lateral sclerosis (ALS), a progressive motor neuron disease that results in death from respiratory failure. Motor neurons are highly vulnerable to stress because they have an elevated threshold for activating heat-shock proteins (Hsps) and since ELP3 regulates Hsps expression (specifically HSP70) by histones (H3 and H4) acetylation, this ability of ELP3 to increase the Hsp70 transcription could explain the association between elevated levels of ELP3 expression and motor neuron protection from degeneration. In fact, ELP3 knockdown in zebrafish resulted in an anomalous splitting of neurons with no associated morphological abnormalities, two loss of function ELP3 mutations in *Drosophila* conferred irregular photoreceptor axonal targeting and synaptic development and, in humans, risk-associated ELP3 alleles were correlated with lower brain ELP3 expression (73–75). ALS pathogenesis is modified by ELP3 through the control and regulation of mcm^5s^2U wobble uridine tRNA modifications, affecting the aggregation of vulnerable proteins, disturbing muscle enervation, disease onset and survival in the mutant SOD1 mouse model. Evidences of such correlation lies on the fact that lowering ELP3 levels reduces mcm^5s^2U levels and increasing of ELP3 expression restore the levels of this modification, attenuating mutant SOD1 aggregation (76).

Evidences correlated ELP4 to Rolandic epilepsy, the most common form of human epilepsy that affects children between 3 and 12 years old. One of the plausible explanations is the abolishment of the Elongator function in the central nervous system due to ELP4 mutations, since they affect cell motility and actin cytoskeleton genes and/or proteins during development. Although this association exists, no causative variant was already identified, but there are evidences suggesting that a non-coding mutation in ELP4 impairs brain-specific elongator-mediated interaction of genes that are implicated in brain development, which results in a predisposition to seizures (77). In the human genome, the gene locus of ELP4 is close to the brain-derived neurotrophic factor (BDNF) and recent reports demonstrate that ELP4 and BDNF genes act together, affecting cell motility, migration and adhesion of neurons, which may implicate them in the pathogenesis of benign epilepsy with centrotemporal spikes (BECTS) (78,79).

1.6. Mechanisms associated with proteostasis deregulation

Once tRNAs are a key element of the translation machinery, it is clear that their synthesis, posttranscriptional modifications and degradation is highly regulated and integrated into the cellular response circuit. In fact, the levels of many tRNA modifications are dependent of the rate of growth, oxygen levels and even the presence of vitamins and metals, reinforcing the potential role of tRNA modifications in the cellular response to environmental stimuli (80,81).

Although it is not yet fully understood how these modifications cause such diverse phenotypes and diseases, the deregulation of the proteostasis caused by changes in the tRNA decoding efficiency is a common feature of many of these diseases. Usually, the rate of protein synthesis decreases, cell growth and survival are affected and erroneous proteins aggregate, compromising the PN (5). This way, a great number of human disorders are considered proteostasis network disruptions, regardless of whether they arise from the chronic expression of instable mutant proteins or from mutations in PN genes (82).

There are numerous signaling pathways controlling the PN, responsible for the improvement of the capability of the proteostasis network to enable and maintain cellular protein folding and function in spite of adverse challenges (83). When facing errors in protein folding, endoplasmic reticulum (ER) becomes stressed and this will activate a set of signaling pathways that will be described below (84). This PN regulation is represented in Figure 8. Activation of genes coding for folding enzymes, trafficking components, chaperone proteins or components of the degradation process will increase the cell's folding capacity and will allow the degradation of misfolded and aggregated proteins, relieving the stress of the cell and restore ER homeostasis (85).

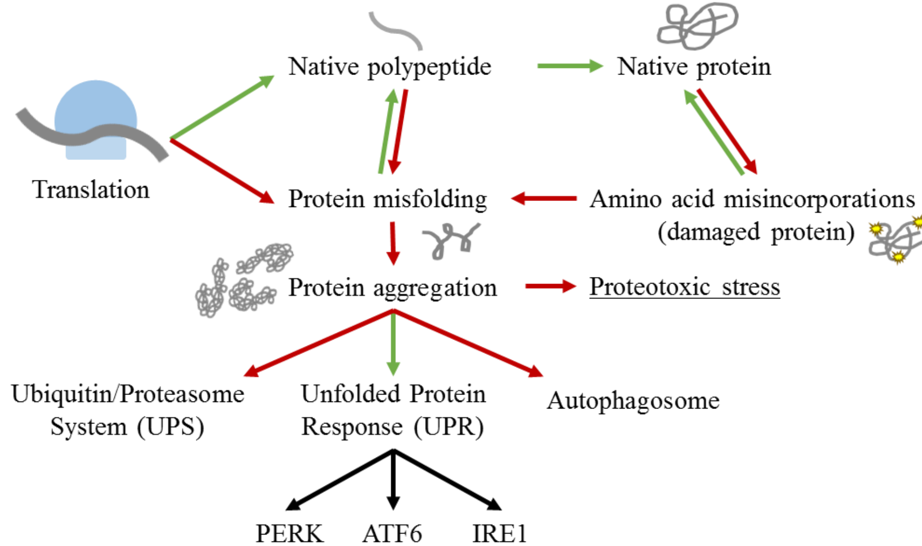

Figure 8: Representation of protein fates in the Proteostasis Network. Green lines represent the normal course of a functional protein since their synthesis. Red arrows and lines represent mRNA translation accuracy lost, stress or mutations, triggering the production of non-functional proteins, causing proteotoxic stress to cells and the consequential degradation pathways.

1.6.1. Unfolded Protein Response (UPR)

Conditions like changes in intralumental calcium, nutrient deprivation, mutations, pathogen infection and aging disturb the protein folding in the ER lumen, threatening cell's viability. To solve this problem, cells developed mechanisms to guarantee that misfolded proteins are discarded. The endoplasmic reticulum (ER) is the site where proteins that follow the secretory pathway enter and where the folding and assembling into subunit complex occur before they transit to the Golgi compartment. The accumulation of unfolded proteins in the ER leads to its stress, triggering the unfolded protein response (UPR), an adaptive signaling cascade activated by three ER-localized transmembrane signal transducers (86,87), described in Table 3.

Table 3: ER transmembrane receptors. Adapted from (87).

ER transmembrane receptors	Description
PERK: The pancreatic endoplasmic reticulum-resident kinase pathway	Serine/threonine kinase that phosphorylates the translation initiation factor eIF2α (eIF2α-P), causing the reduction of the protein synthesis rate, releasing calcium from the ER and further activate pro-apoptotic pathways. Elevated levels of phosphorylated eIF2α indicate that the protein synthesis rate is reduced (88).
ATF6: The activating transcription factor 6	Basic Leucine zipper (bZIP) transcription factor that after being cleaved by local proteases will be responsible to activate the transcription of UPR-responsive genes (89).
IRE1: Inositol-requiring enzyme 1	Bi-functional protein required for UPR and also with a site-specific endoribonuclease (RNase) activity, important for RNA degradation to reduce protein synthesis (90).

Both PERK, ATF6 and IRE1 luminal domains are bound to the protein chaperone Binding immunoglobulin Protein (BiP), the main regulator of UPR, in non-stressed cells; when in stress, there is an accumulation of unfolded proteins that will bind to BiP, sequestering it and promoting its release from the UPR sensors (91,92). When it is localized in ER lumen but when it's overexpressed it may become detectable on the cell surface (93). BiP expression is correlated with cancer, cell proliferation and histological grade (94). The three branches of the UPR, as well as their signaling cascades, are illustrated in Figure 9.

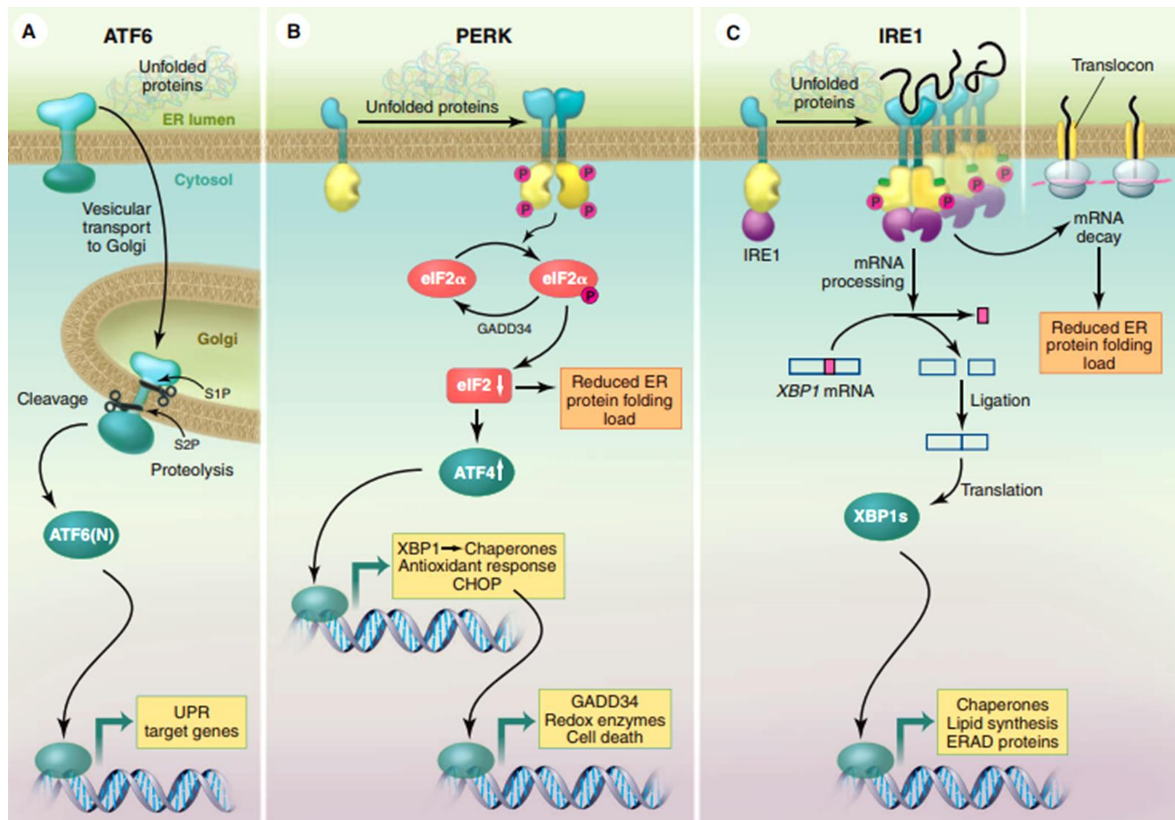

Figure 9: The three divisions of the UPR (A to C). Three families of signal transducers (ATF6, PERK, and IRE1) sense the protein-folding conditions in the ER lumen and transmit that information, resulting in production of bZIP transcription regulators that enter the nucleus to drive transcription of UPR target genes. Each pathway uses a different mechanism of signal transduction: ATF6 by regulated proteolysis, PERK by translational control, and IRE1 by nonconventional mRNA splicing. In addition to the transcriptional responses that largely serve to increase the protein-folding capacity in the ER, both PERK and IRE1 reduce the ER folding load by down-tuning translation and degrading ER bound mRNAs, respectively (92).

1.6.2. Ubiquitin/Proteasome System (UPS)

The discovery and characterization of the Ubiquitin/Proteasome pathway was made by Avram Hershko, Aaron Ciechanover and Irwin Rose and was worthy of a Novel Prize in 2004, demonstrating its importance (95). Ubiquitin molecule participates in the targeting of substrates in the most important protein degradation pathways – the proteasome, the lysosome and the autophagosome (95).

Ubiquitin is a member of a family of structural conserved proteins with 76 amino acids that can conjugate to other proteins/substrates by one process denominated ubiquitination that involves three steps carried out by three classes of enzymes: The Ubiquitin activating enzyme (E1) belongs to the initial activation step, forming a thio-ester bond with ubiquitin allowing the succeeding binding of ubiquitin to Ubiquitin conjugating enzyme (E2) – intermediate step - where the carboxy-terminus of ubiquitin forms a bond with the ε-amino group of a lysine residue on the substrate; The final step, facilitated by

the Ubiquitin ligase enzyme (E3) is where ubiquitin reaches its last destination of the substrate amino group and it is the identity of E3 that provides substrate specificity (96).

After protein ubiquitination, they are targeted to the 26S proteasome for degradation or will suffer changes in their location or activity. The proteasome is an abundant complex found in the cytosol and nucleus of cells with an empty cylindrical structure composed of a central barrel-shaped core (20S) particle with many proteolytic sites and a regulatory (19S) particle at either or both of its ends that rules access to the core, selecting, preparing and translocating the substrates into the 20S core for degradation. The latter has three associated deubiquitinating enzymes (DUBs) – POH1/PSMD14, USP14 and UCH37 – with the major function of recover the ubiquitin from substrate protein in order to preserve the cellular ubiquitin pool (95). When the polypeptide substrate goes to the central chamber of the 20S particle it is cleaved by the six proteolytic sites resulting in small peptides, that are quickly digested by the cytosolic endopeptidases and aminopeptidases into constituent amino acids and then metabolized or reused to synthesize new proteins (97,98).

The UPS involves two stages, namely the conjugation of the multiple ubiquitin molecules with the substrate protein, marking them and the degradation of these proteins that are transported to the proteasome (99,100). Ubiquitin-mediated proteolysis also regulates other cellular processes, such as apoptosis, DNA transcription and repair, differentiation and development, immune response, ribosome biogenesis, viral infection and so on (101). Figure 10 demonstrates UPS pathway under normal and pathological conditions compared with autophagy-lysosomal pathway (ALP).

1.6.3. Autophagy-lysosomal pathway (ALP)

Autophagy means “self-eating” and it is a vital catabolic pathway responsible for cytoplasmic components degradation inside the lysosome that plays a key function in the quality control of the cell and also serves as an indispensable cytoprotective response to pathological stresses that occur during diseases (102). In eukaryotic cells, it comprises three main intracellular pathways - macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) – that are mechanistically different but whose final destination is the lysosome (103).

The intracellular components degradation involves a multi-step process consisting of substrate recognition, its delivery to lysosomes, consequent degradation and following recycling of the breakdown products. Figure 10 demonstrate in a simple way this pathway under normal and pathological conditions, comparing to UPS pathway. During macroautophagy, the substrate is confiscated inside the autophagosomes, a double-membrane vesicle formed by intact organelles such as mitochondria and portions of the cytosol, for its delivery to lysosomes or endosomes through vesicular fusion, forming an autolysosome. Mammalian target of rapamycin (mTOR) complex is the central, but not exclusive, player of the machinery components involved in autophagosome formation and,

under normal nutrient conditions, active mTOR phosphorylates ULK1 and sequesters it in a complex, inhibiting autophagy (104).

In microautophagy, the cargo is internalized by single-membrane vesicles that are formed through invaginations in the surface of lysosomes (105). In the case of chaperone-mediated autophagy, there is no need for vesicles since unfolded, soluble proteins are identified by a cytosolic chaperone that delivers them to lysosomes for their internalization through a translocation complex formed by the multimerization of the CMA receptor protein LAMP-2A (106).

AMP-activated protein kinase (AMPK) is the main positive regulator of autophagy that is activated by a high ratio of AMP to ATP (107). mTOR and AMPK also control cell growth and metabolism, coupling these processes to autophagy and malfunctions of this catabolic pathway have been associated with a wide range of human diseases, arising mostly from its role in quality control of the proteome and the maintenance of the proteostasis. For example, when autophagy is disrupted in post-mitotic tissues, such as neurons, it leads to altered proteins accumulation and its activation is part of the cellular response to stress (102).

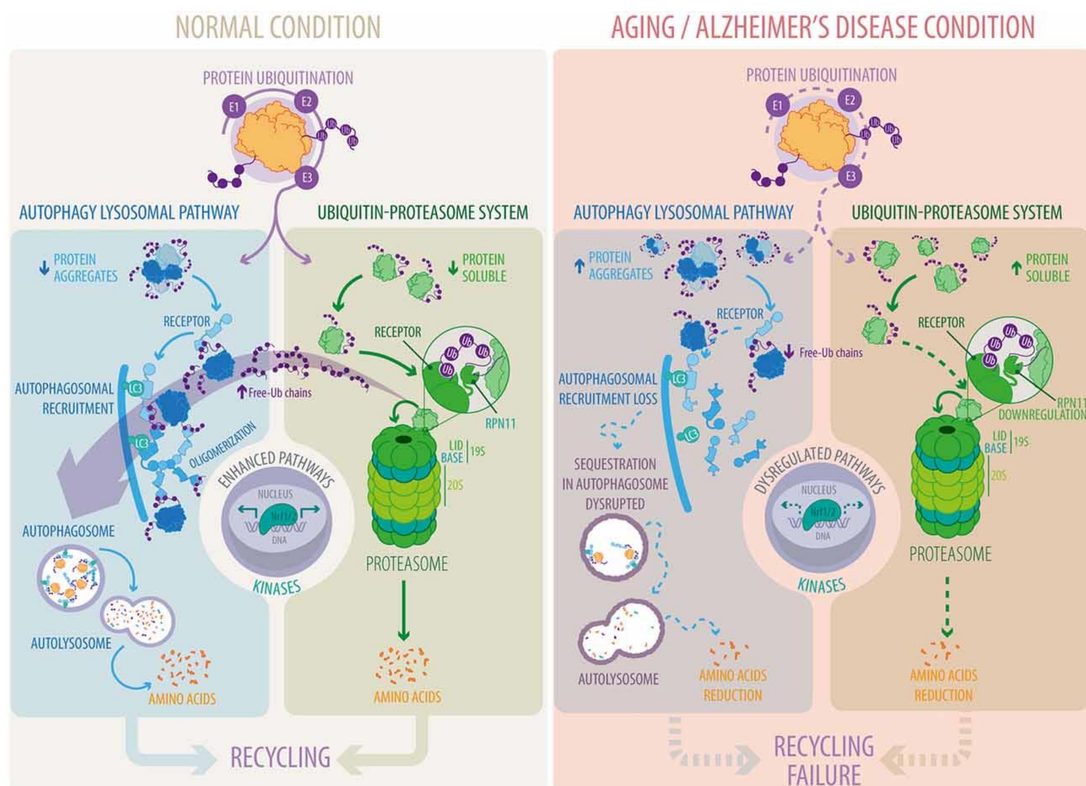

Figure 10: Autophagy-lysosomal pathway (ALP) and ubiquitin-proteasome system (UPS) pathways under normal and pathological conditions. Proteins are tagged with ubiquitin conjugates through a sequential enzymatic mechanism involving three classes of enzymes (E1, E2, E3). Under normal conditions, ubiquitylated substrates are recognized by ubiquitin receptors present in ALP and UPS pathways and efficiently eliminated. In the UPS, substrates are subsequently deubiquitylated, a key step for substrate degradation and amino acid recycling. Free-Ub chains formed promote ALP function. Ubiquitin receptors in the ALP form oligomers to facilitate substrate recognition and autophagosomal recruitment. Under aging and Alzheimer's disease conditions there is a decrease in the function of the ALP and the UPS that reduces substrate degradation and amino acid recycling (100)

1.7. Hela cells and the Aggregation Reporter System

Hela cells are human cervical cancer cells that became very important and helpful to medical research because they grow rapidly when in the right medium (with the appropriate nutrients and conditions) and with proper space. Compared to normal cells, they multiply and grow quickly and are extremely resilient and, under the right conditions, they form an immortal cell line, dividing indefinitely (108).

To monitor protein aggregation in human cells, our team has previously developed a fluorescence-based sensor assay that consists in HeLa stable cell line expressing a HSP27:GFP chimeric reporter protein characterized by the fusion of the heat-shock protein 27 (HSP27) with a fluorescent protein, in this case, the Green Fluorescent Protein (GFP), as schematized in Figure 11. HSP27 (HSPB1) is a human small heat shock protein and represent the first line of defense in proteostasis, being activated by diverse triggers such as temperature, pH or post-translational modifications. HSP27 is recruited by misfolded proteins and binds to them in an ATP-independent manner, forming an HSP27-substrate complex which allows the refolding by the larger ATP-dependent chaperones (HSP70 and HSP90) or leads the proteins to degradation (109). If there are misfolding proteins, the GFP fluorescence is re-localized to foci. This cell line was used to perform fluorescence-based genetic screenings. Based on the small interfering RNA (siRNA) technology, our group performed experiments where the expression of the human tRNA modifying enzymes was knocked-down and observed the consequences of their absence for protein misfolding. The stable HeLa HSP27:GFP cell line can provide valuable information to identify modulators of proteostasis, identify compounds that lead to proteostasis deregulation and find novel targets that modulate protein aggregation (110).

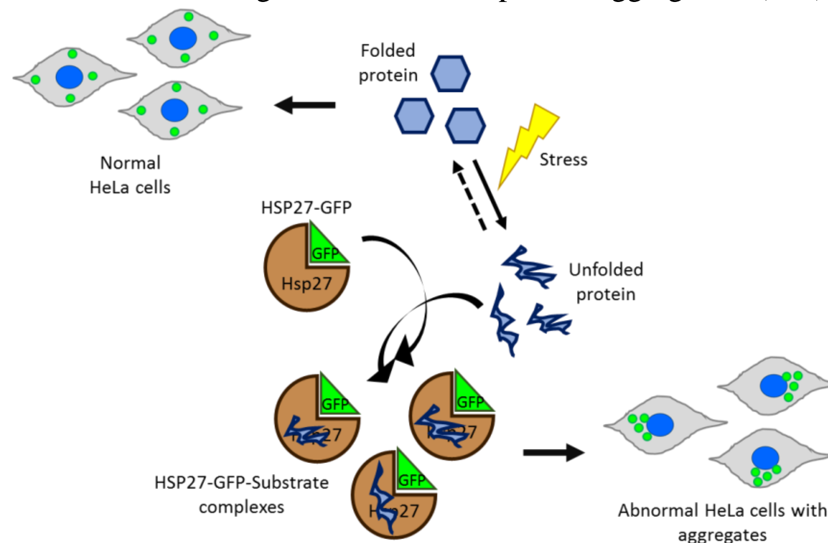

Figure 11: Schematic representation of the functioning of the stable cell line expressing the HSP27:GFP reporter.

1.8. Motivations and Aim of the study

Evidences show that a growing number of tRNA modifying enzymes are implicated in diseases where proteostasis is affected, specifically neurological disorders, cancer and mitochondrial-linked diseases. However, the implication of deregulation of tRNA modifying enzymes to human diseases is not very clear and the role of these enzymes in proteostasis deregulation, proteotoxic stress and protein aggregation as the causal mechanism of disease has not been fully experimentally demonstrated in mammalian cells.

Our hypothesis is that the deregulation of tRNA modifying enzymes results in protein aggregation and consequent activation of UPR, characteristic of protein conformational diseases.

To test this hypothesis and making use of the fluorescence-image based siRNA screen developed by our team in the past, the main goal is to identify human tRNA modifying enzymes involved in proteostasis, pinpoint the most relevant for protein aggregation and elucidate which protein quality control pathways are affected.

ELP3 was identified in this study as the most relevant tRNA modifying enzyme for proteostasis in human cells, highly involved in protein aggregation, with alterations at the level of protein synthesis and increased ubiquitination.

This data reinforces the viability of our initial hypothesis and additional tests are now undergoing to further disclose the molecular consequences of ELP3 deregulation and validate it as a therapeutic target for conformational disorders.

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CHAPTER 2.

Metodology

2.1. Cell Culture

A stable HeLa cell line expressing HSP27-GFP was cultured in Dulbecco's Modified Eagle Medium (DMEM) complemented with 10% of Fetal Bovine Serum (FBS) and 1% of Pen-Strep-Glut (a combination of the antibiotics penicillin and streptomycin and the amino acid glutamine). These cells were maintained in culture in a culture chamber at 37ºC with 5% of CO_2 and 95% of humidity. Except for fluorescence and proteostat assays, cells were detached from plates using TrypLe Express (ThermoFisher Scientific) and incubated 5 minutes at 37ºC. The subsequent cell suspension was centrifuged for 3 minutes at 3000 rpm at Room Temperature (RT), the supernatant was discarded and the pellet was ressuspended in fresh medium. To perform reverse transfection with siRNAs, cells were counted in an optical microscope using a Neubauer chamber, diluting 2µL of suspended cells with 18µL of trypan blue.

2.2. Reverse transfection with siRNAs

SiGenome SMARTpool human siRNA targeting different RNA modifying enzymes (Table 5) were obtained from Dharmacon (Thermo scientific) and reverse transfected in triplicate into the stable HSP27-GFP HeLa cell line in 24 well plates, as exemplified in figure 12.

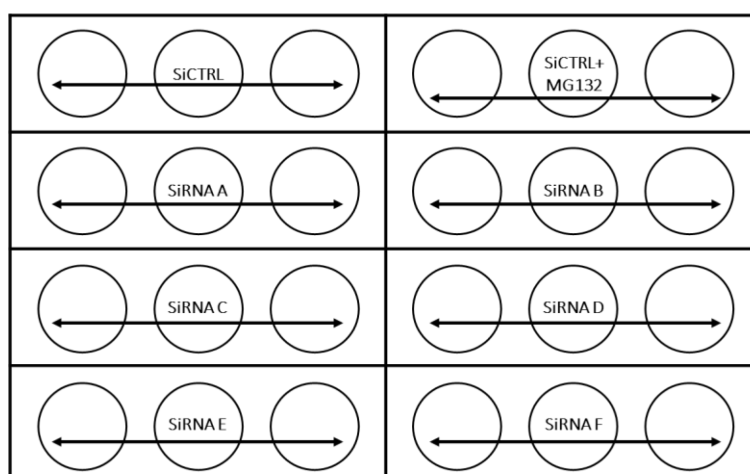

Figure 12: Schematic representation of a 24 well plate with the disposition of the SiRNAs.

In each well 12µL of 500nM siRNA duplex and 88µL of Opti-MEM were added, followed by the addition and mixing of 1µL/well of the mix of Lipofectamine RNAimax and an incubation period of 30 minutes. After this time, 500µL of $2x10^4$ cells solution was added to each well and incubated for 72 hours at 37ºC in a CO_2 incubator (Solutions preparation described in Table 4).

Table 4: Reverse transfection solutions

siRNA aliquots	Lipofectamine mix	Cells solution
CiVi = CfVf (=) $5.4x10^4$ x Vi = $500x10^3$ x 100µL (=) Vi = 2.5µL of SiRNA stock + 97.5µL of TE	(0.5µL lipofectamine + 0.5µL Opti-MEM) x Number of wells	CiVi = CfVf (=) $5.4x10^4$ x Vi = $2x10^4$ x 2.4mL (=) Vi = 889µL of cells + 2.399mL of DMEM without antibiotics

For experiments with the positive control condition MG132 (carbobenzoxy-Leu-Leu-leucinal), 5µM of MG132 was added to cells 16h before collecting the pellets. For protein synthesis study by SUnSET, 15 min before collecting the pellets, cells were incubated with puromycin (10µg/mL) and after that time the collection and protein extraction processes were the same.

Table 5: Human tRNA modifying enzymes tested

Human tRNA modifying enzyme	Modification
IKBKAP (Elp1)	mcm^5U_{34}, mcm^5s2U_{34}, ncm^5U_{34}, ncm^5Um_{34}
Elp2	mcm^5U_{34}, $mcm^5s^2U_{34}$, ncm^5U_{34}, ncm^5Um_{34}
Elp3	mcm^5U_{34}, mcm^5s2U_{34}, ncm^5U_{34}, ncm^5Um_{34}
Elp4	mcm^5U_{34}, mcm^5s2U_{34}, ncm^5U_{34}, ncm^5Um_{34}
Elp5 (Orf81)	mcm^5U_{34}, $mcm^5s^2U_{34}$, ncm^5U_{34}, ncm^5Um_{34}
Elp6 (Orf75)	mcm^5U_{34}, $mcm^5s^2U_{34}$, ncm^5U_{34}, ncm^5Um_{34}
TRMT1	$m^{2,2}G_{26}$
URM1	$mcm^5s^2U_{34}$
TRMT61A	m^1A
TRMT2A	m^5U_{54}
TRMT5	m^1G_{37}, m^1I_{37}, yW_{37}
ALKBH8 (TRM9)	mcm^5U_{34}, $mcm^5s^2U_{34}$
TRDMT1	m^5C_{34}

2.3. Total protein extraction and quantification

During all the procedures, the cells remained on ice to avoid the proteases activity. Extracts were prepared by sonication of the pellets in 100µL of Empigen lysis buffer (ELB) (Table 6) for 2 cycles, at a 60% frequency during 15 seconds each and centrifuged at 200G during 20 minutes at 4ºC. After centrifugation, supernatants were kept for the following stage of total protein quantification.

Total protein quantification was performed using Pierce™ Bovine Serum Albumin (BCA) Protein Assay Kit (Thermo Scientific) following the manufacturer's instructions, using a 20x dilution for our samples. The absorvance was read at 575nm using Microplate Manager 6 Bio-Rad Software. The concentration values provided by the software were in µg/mL, so the conversion to µg/µL was made by multiplying each value by 20 (20x dilution) and division by 1000 (mL to µL).

Table 6: Empigen lysis buffer (ELB) reagents and preparation.

ELB (10mL)	Complete ELB (10mL)
- Triton X-100: 50µL - Hepes (1M, pH 7): 500µL - NaCl (5M): 500µL - H2O: 8.95mL	- ELB: 9.29mL - DTT (1M): 10µL - Naf (1M): 10µL - EDTA (0.5M): 40µL - EGTA (100mM, pH 8): 100µL - Na3VO4 (100mM): 100µL - *Roche 50x - *PMSF (40nM)
Lysis buffer was prepared without Roche 50x and PMSF (*) and aliquots of 955µL were made. When needed, it was added to each aliquot 20µL Roche 50x and 25µL PMSF.	

For SDS-PAGE of total protein extracts, the volume equivalent to 10µg of total protein was diluted in MilliQ Water to a final volume of 10µL, 3µL of Loading Buffer (LB) 6x were added and samples were denaturated at 95ºC during 5 minutes. For SDS-PAGE of total protein extracts for western blot analysis, the volume equivalent to 30µg of total protein was diluted in MilliQ Water to a final volume of 25µL, 5µL of LB 6x were added and samples were denaturated at 95ºC during 5 minutes.

For proteomic labeling and analysis, 100µg of total protein extract of SiCtrl and SiElp3 transfected cells were send to I3S. Dr. Hugo Osório perform a quantitative mass spectrometry using iTRAQ and used the software Proteome Discoverer to analyse the data. The data show the up- and down-regulated genes when compared to control.

2.4. Insoluble protein fraction extraction

To isolate the insoluble protein fraction, the volume equivalent to 50µg of total protein was diluted in ELB to a final volume of 100 µL. Samples were centrifuged during 20 minutes, 16000G at 4ºC. Once the insoluble proteins were deposited, the supernatant was discarded. Pellets were washed with lysis buffer (80µL complete ELB + 20µL NP40 10%), sonicated 2 x 20 seconds and centrifuged at 16000G, 20 minutes at 4ºC. The supernatant was discarded and pellets were washed with lysis buffer with SDS 1% (95µL complete ELB + 5µL SDS 20%) and centrifuged at 16000G, 20 minutes at 4ºC. The supernatant was discarded and 25µL of complete ELB + 5µL Loading Buffer 6x were added to each pellet, ressuspended, denaturated at 95ºC during 5 minutes and run in SDS-PAGE.

2.5. Polyacrylamide gel electrophoresis (SDS-PAGE)

The glass plates were placed in the casting apparatus. The resolving gel was made by adding the components in the order they appear in the Table 7. The resolving solution was pippeted carefully between the casting plates, leaving about 2cm empty and then filled with water to allow the gel to polimerize properly. The polimerization process takes about 30min to be completed.

The stacking solution was made by the same principles that the lower gel. The water that is on top of the resolving gel was discarded and the upper gel was pippeted. Carefully, the spacers ensuring that no bubbles were formed. The polimerization takes about 20 min to be completed.

After this process, the gel was ready to get into the electrophoresis chamber allong with the running buffer. After the denaturation of samples at 95ºC for 5 minutes, they were loaded carefully. The protein marker was loaded in the first well, followed by the samples in the remaining wells.

Table 7: Reagents for 2 protein gels

	Resolving – lower – gel (10%)	Stacking – upper – gel (4%)
H_2O	3.6 mL	3.464 mL
Tris HCl	pH 8.8 3.75 mL	pH 6.8 1mL
Acrylamide 29.1	2.5 mL	500µL
SDS 10%	100 µL	50 µL
APS 10%	100 µL	50 µL
TEMED	10µL	10µL

Gels containing total protein fraction and insoluble protein fraction were stained with Bluesafe (NZYTech) for about 1h and revealed with Odissey® and gels containing the samples for western blotting tests were transfered to Trans-blot® Turbo™ mini-size stacks (nitrocelulose membranes) from Bio-Rad.

2.6. Western Blotting

Samples to perform western blott were at a concentration of 30µg/µL and were loaded in a polyacrylamide gel. After electrophoresis was complete, the gel was transfered to a membrane for antibody staining and detection

2.6.1. Trans-Blot® Turbo^{Tm} Blotting System (Bio-Rad) adapted protocol

Transfer buffer was prepared for the transfer cell and immersion of filter papers, gel and membrane. In the transfer cell, the nitrocelulose membrane and gel were placed between eight buffer-soaked filter papers, overlapped and the roler was used to remove any air trapped between layers. The cell was connected to the power supply, the appropriate protocol was seted – 25V/1300mA,7min – and the transfer was started. When the transfer ended, the membrane was blocked during 1h at RT in a 50mL falcon with 5mL of BSA5% (in case of ATF6 membrane, it was 5mL of BSA2%) to completely block unoccupied binding sites that can lead to high background.

2.6.2. Immunodetection

After blocking the membranes, they were incubated overnight for 16h with the primary antibodys – Ubiquitin, eiF2α-P and ATF6-α (Table 8). Next day, membranes were washed 3 times with TBS-T for 5 minutes each and then incubated with the secondary antibody for 1h. After this time, membranes were washed 2 times with TBS-T for 5 minutes each, 1 time with TBS for 5 minutes and revealed with Odissey®. After revelation, membranes were incubated with ß-tubulin for 1h, then washed 3 times with TBS-T for 5 minutes and incubated with the correspondent secondary antibody for 1h. They were washed 2 times with TBS-T for 5 minutes and 1 time with TBS for 5 minutes and revealed with Odissey®.

Membranes with total protein extracts of cells incubated with puromycin during transfection for protein synthesis study were incubated overnight with anti-puromycin and incubated with anti-mouse for 1h and revealed.

Table 8: Antibodies used for Western Blot Analysis

Primary Antibody	Host	Secondary Antibody (1:10000)
Anti-ATF6 (α) (1:400) Stressgen	Mouse	Anti-Mouse IRDye®800CW LI-COR
Anti-eif2α (1:1000) Cell signaling	Rabbit	Anti-Rabbit IRDye®680LT LI-COR
Anti-eif2α-P (1:4000) Abcam	Rabbit	Anti-Rabbit IRDye®680LT LI-COR
Anti-puromycin (1:25000) Sigma Aldrich	Mouse	Anti-Mouse IRDye®800CW LI-COR
Anti-Ubiquitin (1:1000) Sigma Aldrich	Mouse	Anti-Mouse IRDye®800CW LI-COR
Anti-β-tubulin (1:1000) Invitrogen	Mouse	Anti-Mouse IRDye®800CW LI-COR

2.7. Proteasome Activity Analysis

This protocol was optimized by Doctor Sofia Varanda, from our RNA lab.

In order to evaluate the proteasome activity of Elongator complex enzymes, in a 6-well plate, $2x10^5$ cells/well were platted. After a period of 48h, cells were washed with PBS and resuspended in 100µL of lysis buffer (1mM EDTA, 10mM Tris-HCl pH 7.5, 20% glycerol, 4mM DTT, 2mM ATP). Cells were sonicated with a probe sonicator in 5 pulses of 5 seconds and centrifuged at 13000rpm for 10min at 4ºC. The supernatant was diluted (1:20) and protein content was quantified using Bradford method (Sigma-Aldrich). 20µg of protein were incubated in Lysis Buffer with the substrate suc-LLVY-MCA (50µM) (Sigma-Aldrich) in the presence or absence of the proteasome inhibitor MG132 (10µM) (Sigma-Aldrich) in a medium containing 1mM EDTA, 10mM Tris-HCl pH 7.5; 2mM ATP for a final volume of 100µL).

Substrate degradation was monitored every 5 minutes during 1h at 37ºC in a fluorescence-luminescence detector Synergy™ HT Multi-Mode Microplate Reader (Biotek) and set to excitation (380nm) and emission (460 nm) wavelengths. Specific proteasome activity was determined by subtracting the values for each sample without MG132 to the values with MG132. The final activity of proteasome was calculated as fluorescence emission at 0min subtracter from fluorescence after 1h, relative to control (Mock).

2.8. Statistical analysis

Statistical analysis were performed using GraphPad Prism® v.8.0.0 software, using Student's t-test (paired t-test). The results were presented as mean values of the number of experiments. The gene-annotation enrichment analysis and gene-disease association of the data provided by Hugo Osório from I3S was made using Cytoscape Clue-Go and DAVID Bioinformatics Resources 6.8.

2.9. CRISP-R

This recent technology will allow us to modify the HeLa cells genome with great precision, efficiency and flexibility in order to obtain a stable KO Elp3 cell line, making usage of a plasmid for expression of the Cas9 nuclease protein and a synthetic single guide RNA designed for our target gene of interest. The protocol used was Dharmacon™ Edit-R™ CRISPR-Cas9 gene engineering with Cas9 nuclease expression plasmids and synthetic guide RNAs and all steps of this protocol were performed in the flow cell culture hood. After the selection of three to five synthetic guide RNAs for ELP3 we started the transfection. In brief, $1x10^5$ cells/well of HeLa cells were plated in a 24 well plate and incubated at 37ºC overnight with three different co-transfected samples, described in Table 9.

Table 9: Recommended Samples for a gene engineering co-transfection experiment.

Table 10: Sample name	Purpose
Cas9 Nuclease Expression plasmid only with a Non-targeting control synthetic guide RNA	Negative control: Expression of Cas9 nuclease without targeting RNAs
Cas9 Nuclease Expression plasmid with gene-specific synthetic guide RNA	Gene engineering sample: Expression of Cas9 nuclease programmed by RNAs for targeted double-strand break in gene of interest
Untreated	No treatment control sample: Confirmation of cell viability

A solution of 100ng/µL Cas9 plasmid with Tris buffer was prepared as well as the 2µM synthetic guide RNA transfection complex. The samples were prepared in 1.5mL tubes for a final 25nM concentration of the guide RNA and 1µg/well of Cas9 expression plasmid. After that, a 60µg/mL of DharmaFECT Duo working reagent was prepared with serum-free medium and incubated for 5 minutes at RT. After this time, 50µL of DharmaFECT Duo working solution was added to each sample tube, resulting in 3µg/well final DharmaFECT Duo concentration, except to the untransfected control that contain only serum-free medium. Each tube contained the total volume of 100µL and after a gentle mixing were incubated for 20 minutes at RT. The transfection medium was then prepared by adding 400µL of antibiotic-free complete medium to each sample, reaching a total volume of 500µL. The medium of the cells of the 24 well plate was removed and replaced by the 500µL of the appropriate transfection medium to each well, as shown in Table 10.

Table 10: Preparing transfection samples for gene editing experiment in a 24-well plate format. All values are in µL. For replicates, sufficient sample volumes should be prepared, accounting the number of replicated and the pipetting errors.

Table 11: Sample name	Serum-free medium	Working guide RNA solution (2µM)	Working Cas9 plasmid solution (100ng/µL)	Working DharmaFECT Duo solution (60µg/mL)	Growth medium	Total volume per 24 well
Negative control	35	5	10	50	400	500
Gene engineering sample	35	5	10	50	400	500
Untreated	100	0	0	0	400	500

Cells were then incubated at 37ºC in 5%CO_2 for 72 hours before proceeding with gene editing analysis.

CHAPTER 3.

Results

3.1. Transfection

The tRNA modifying enzymes studied on this thesis were the ones selected as the best hits after performing the phenotypic screening (unpublished data), namely ELP1, ELP2, ELP3, ELP4, ELP5, ELP6, TRMT1, URM1, TRMT61A, TRMT2A, TRMT5, ALKBH8 and TRDMT1 (Table 11).

Table 11: Characterization of the two groups of enzymes studied.

Table 12: Group of enzymes	Tested enzymes
ELP Family	ELP1
	ELP2
	ELP3
	ELP4
	ELP5
	ELP6
Other Enzymes	TRMT1
	URM1
	TRMT61A
	TRMT2A
	TRMT5
	ALKBH8
	TRDMT1

3.2. Insoluble fraction

To confirm the screening results, the insoluble protein fraction of cells transfected with siRNAs against the enzymes described in the previous topic were isolated and analyzed by SDS-PAGE. An increase in the percentage of insoluble fraction was observed in all the tested enzymes, although some of them were not statistically significant. Cells incubated with MG132, a proteasome inhibitor, were used as a positive control.

From the initial screenings, not all ELPs showed increased formation of protein aggregates. However, these enzymes belong to a complex, working together and for that reason, we decided that test all the ELP subunits as that there could be changes that were due to the association between some subunits rather that to a subunit by itself.

The isolation of insoluble proteins was performed 72 hours after reverse transfection with the selected siRNAs, to ensure the highest knock-down level. After total protein extraction, quantification and subsequent insoluble fraction isolation, samples were loaded into SDS-PAGE and after running, gels were observed on the Odyssey IR scanner.

Concerning to ELP family, an increase in insoluble protein fraction in the absence of each one of the complex enzymes was observed, however, the standard deviations are high and only siELP1 (P=0.0291), siELP2 (P=0.238) and siELP3 (P=0.0125) are statistically significant when compared to siCtrl (Figure 13A). In this case, siCtrl+Mg132 represents the positive control, as it is a proteasome inhibitor that inhibits degradation of misfolded proteins that ultimately accumulate in cells.

Regarding to the Other enzymes, it is possible to note an increase in insoluble protein fraction in the absence of almost every tRNA modifying enzymes, however, only siTRMT1 (P=0.0026), siURM1 (P=0.0011) and siALKBH8 (P=0.0160) are statistically significant when compared to siCtrl (Figure 13B).

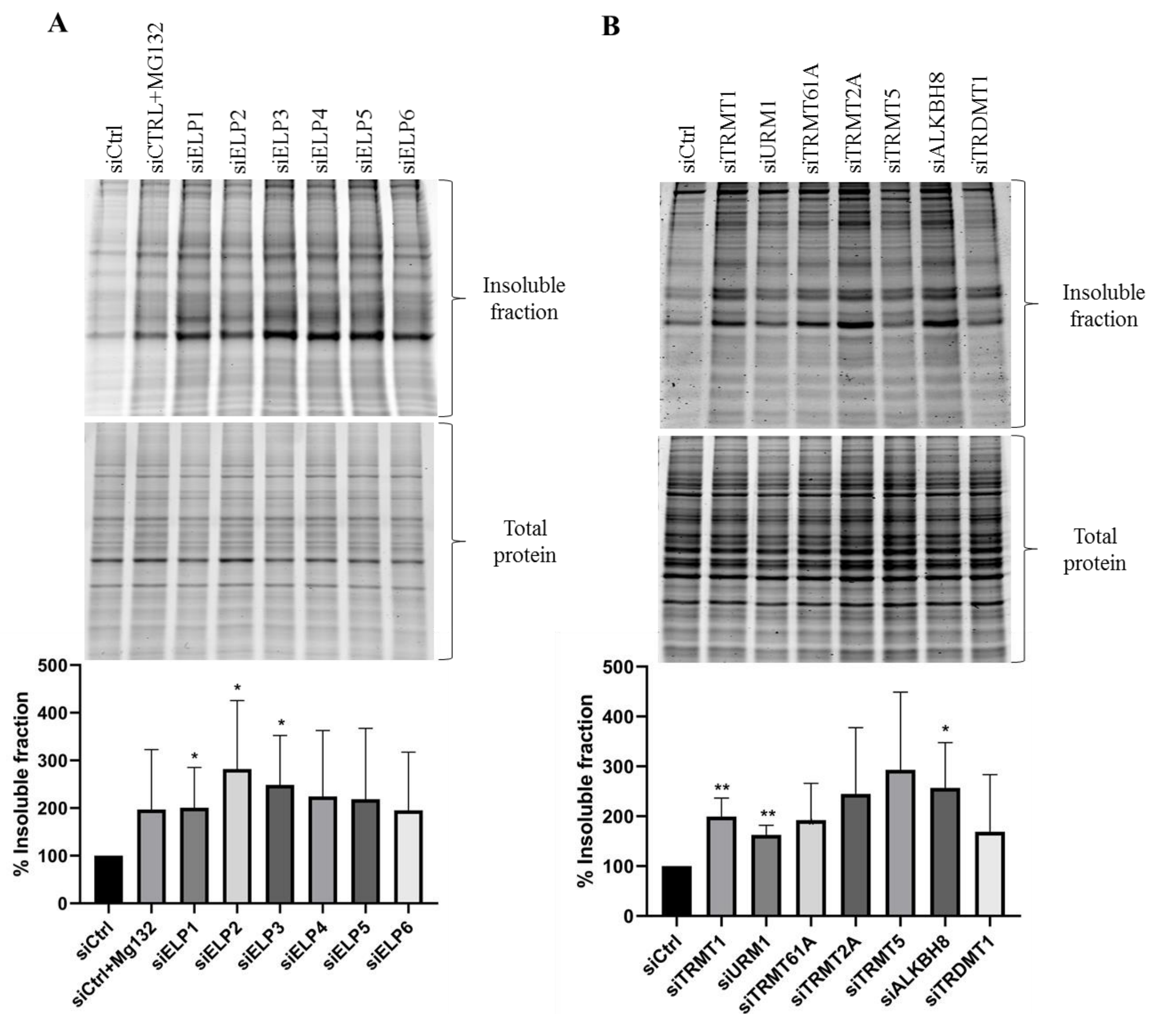

Figure 13: Analysis of the insoluble fraction of transfected cells. SDS-PAGE of insoluble protein fraction and total protein for each transfection condition and the relative amount (%) of insoluble fraction (ratio of insoluble/total) in transfected cells compared with the control condition (siCtrl). A) ELP Family: a statistically significant increase in insoluble fraction in siELP1 (P=0.0291), siELP2 (P=0.0238) and siELP3 (P=0.0125) knockdown cells is observed (Data analysis was done using Student's t-test (N=5)). B) Other enzymes: a statistically significant increase in insoluble fraction in siTRMT1 (P=0.0026), siURM1 (P=0.0011) and siALKBH8 (P=0.0160) knockdown cells is observed (Data analysis was done using Student's t-test (N=4)).

3.3. Western Blotting

After confirming that the silencing of some tRNA modifying enzymes led to an increase of the insoluble fraction, we were able to verify whether increased misfolded proteins induced specific cell responses. For this, the UPS and UPR protein quality control pathways were tested by western blots, testing the anti-ubiquitin antibody which correspond to the UPS pathway and the antibodies anti-eIF2α-P, anti-total eIF2α and anti-ATF6 corresponding to the UPR pathway. All values were normalized for total protein fraction, as we realized that knock down of particular enzymes affected the expression of housekeeping genes, such as tubulin and GADPH.

The following data is presented as follows: images data are divided in two groups: A) tRNA modifying enzymes referring to the ELP Family (ELP1-6) and B) other tRNA modifying enzymes (TRMT1, URM1, TRMT61A, TRMT2A, TRMT5, ALKBH8, TRDMT1). In each group, western blotting bands images are described, with the respective molecular weights, followed by the graphic with statistical analysis obtained with GraphPad Prism 8.0.0.

3.3.1. Ubiquitin-Proteasome System Pathway Activation

Ubiquitin protein recognizes misfolded proteins, binds to them for their degradation by proteasome and before their degradation, the ubiquitin is released and recycled. The anti-ubiquitin antibody marks both mono and poly-ubiquitinated proteins in the total extract. Once again, MG-132 was used as a positive control and the total protein extract was used as a normalizer.

After quantification of the total protein extract and ubiquitin bands, all values were normalized for total protein extract. In Figure 14A it is evident that the band representing siCtrl+Mg132 is ubiquitin abundant, consistent with the fact that Mg132 is a proteasome inhibitor and that ubiquitin molecules remain bound to misfolded proteins, instead of being released. We observe that the % of ubiquitin is increased relative to SiCtrl with statistical significance in siELP1 (P=0.0280), siELP3 (P=0,0122) and siELP6 (P=0.0087) cells. Concerning to the other enzymes, it is possible to observe that the % of ubiquitination is statistically significative increased relative to siCtrl in siTRMT61A (P=0.0175), siTRMT5 (P=0.0119) and siTRDMT1 (P=0.0375) (Figure 14B).

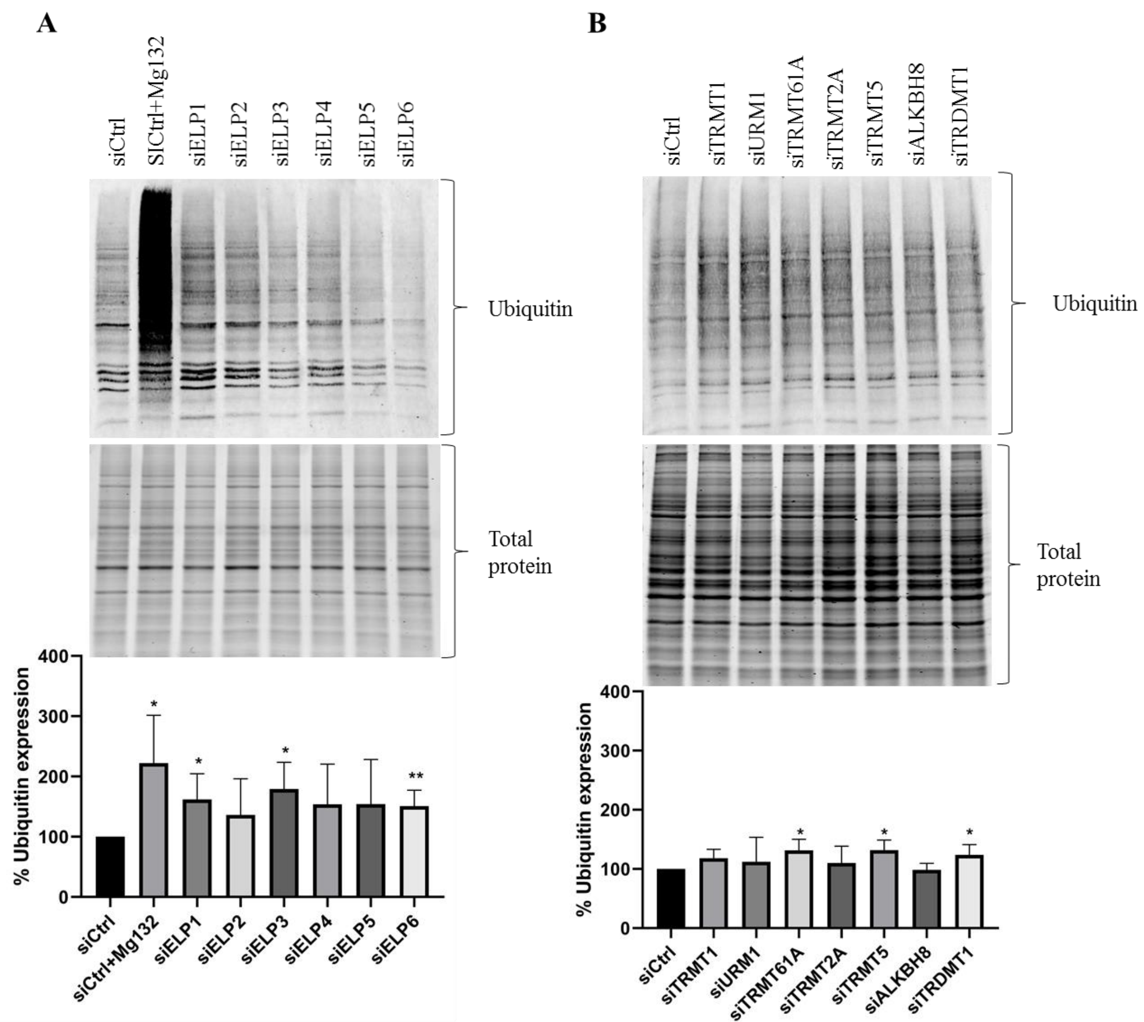

Figure 14: Analysis of protein ubiquitination. The respective western blot bands are described; Graphic of the ubiquitin expression percentage in Hela HSP27-GFP transfected cells. A) ELP family: statistically significant changes were observed concerning to the control in siELP1 (P=0.0280), siELP3 (P=0,0122) and siELP6 (P=0.0087); (Data analysis was done using Student's t-test (N=4)) B) Other enzymes: statistically significant changes were observed concerning to the control in siTRMT61A (P=0.0175), siTRMT5 (P=0.0119) and siTRDMT1 (P=0.0375); (Data analysis was done using Student's t-test (N=4)).

3.3.2. Unfolded Protein Response Activation

The endoplasmic reticulum chaperone protein BiP is the major target of the ER response, namely UPR, and is an important regulator of this pathway. When cell faces stress situations, BiP will dissociate from the other transducers (PERK, ATF6 and IRE1) and will activate their UPR pathways.

Phosphorylation of the translation initiation factor eIF2α by PERK is an indicator of protein synthesis inhibition. As knock down of particular tRNA modifying enzymes leads to an increase in misfolded proteins that can elicit cellular stress responses, we evaluated the levels of eIF2α phosphorylated (eIF2α-P). In Figure 15A, concerning to ELP family, statistically significant changes were observed when we silence ELP1 (P=0.0028),

ELP3 (P=0.0305), ELP5 (P=0.0174) and ELP6 (P=0.0015), compared to the control. Observing Figure 15B, only the siTRMT1 (P=0.0045) and siURM1 (P=0.0018), after normalization with total protein extract, presents statistically significant changes when compared to siCtrl.

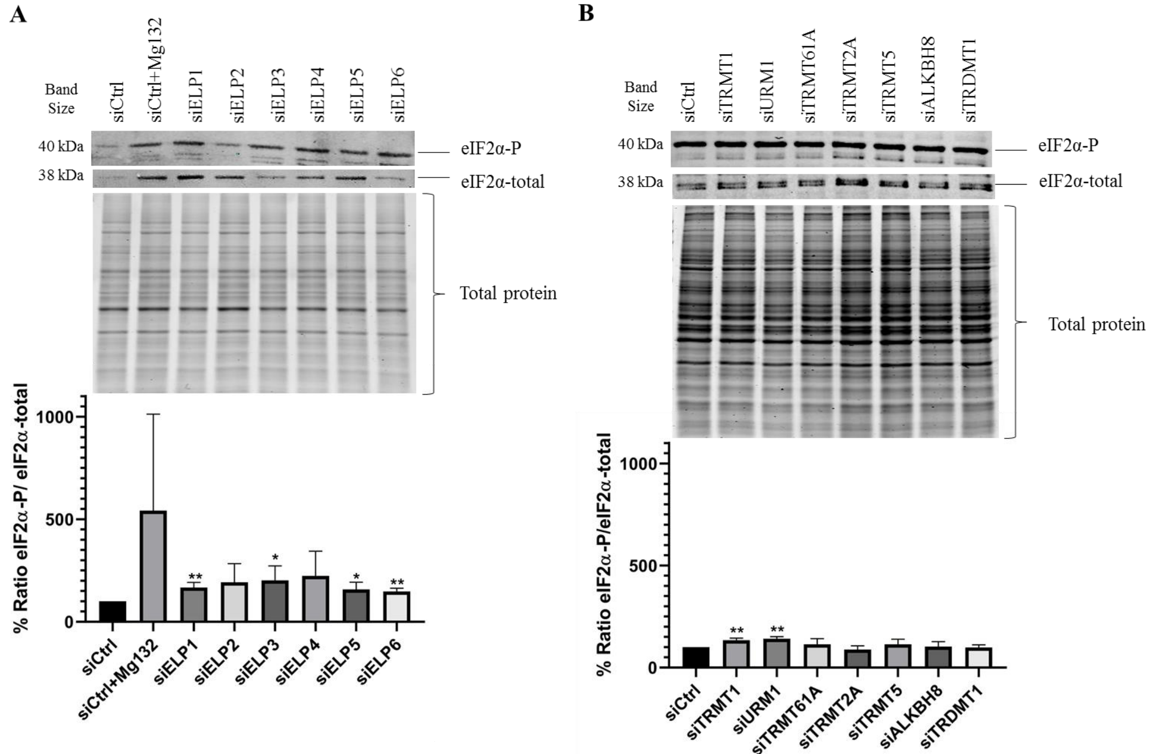

Figure 15: Analysis of total eIF2α and phosphorylated eIF2α expression. The respective western blot bands, eIF2α-total, eIF2α-P and the endogenous control – total protein – are described; Graphic of % of ratio between eIF2α-P and eIF2α-total in Hela HSP27-GFP transfected cells. A) ELP Family: statistically significant changes were observed relative to the control in siELP1 (P=0.0028), siELP3 (P=0.0305), siELP5 (P=0.0174) and siELP6 (P=0.0015) (Data analysis was done using Student's t-test (N=3)). B) Other enzymes: statistically significant changes were observed relative to the control in siTRMT1 (P=0.0045) and siURM1 (P=0.0018) (Data analysis was done using Student's t-test (N=3)).

ATF6 factor is cleaved when cell faces proteotoxic stress. The cleaved fraction will induce chaperones and enzymes transcription that will be responsible for proper protein folding, helping cells to survive to proteotoxicity.

A statistically significant increase in cleaved ATF6 expression was observed when ELP6 was knocked down (P=0.0003), compared to Control cells (Figure 16A). Almost every cells transfected with siRNAs presents statistically significative decrease in cleaved ATF6 when compared to the control, namely the siTRMT1 (P=0.0004), siTRMT61A (P=0.0061), siTRMT2A (P<0.0001), siTRMT5 (P=0.0020), with the exception of siTRDMT1, where a statistically increase in ATF6 is observed, siTRDMT1 (P=0.0276) (Figure 16B). However, there was only two qualified replicates to perform the quantification, normalization and statistical analyses needed. For that reason and despite the P values and respective standard deviations, more replicates are needed to draw final conclusions.

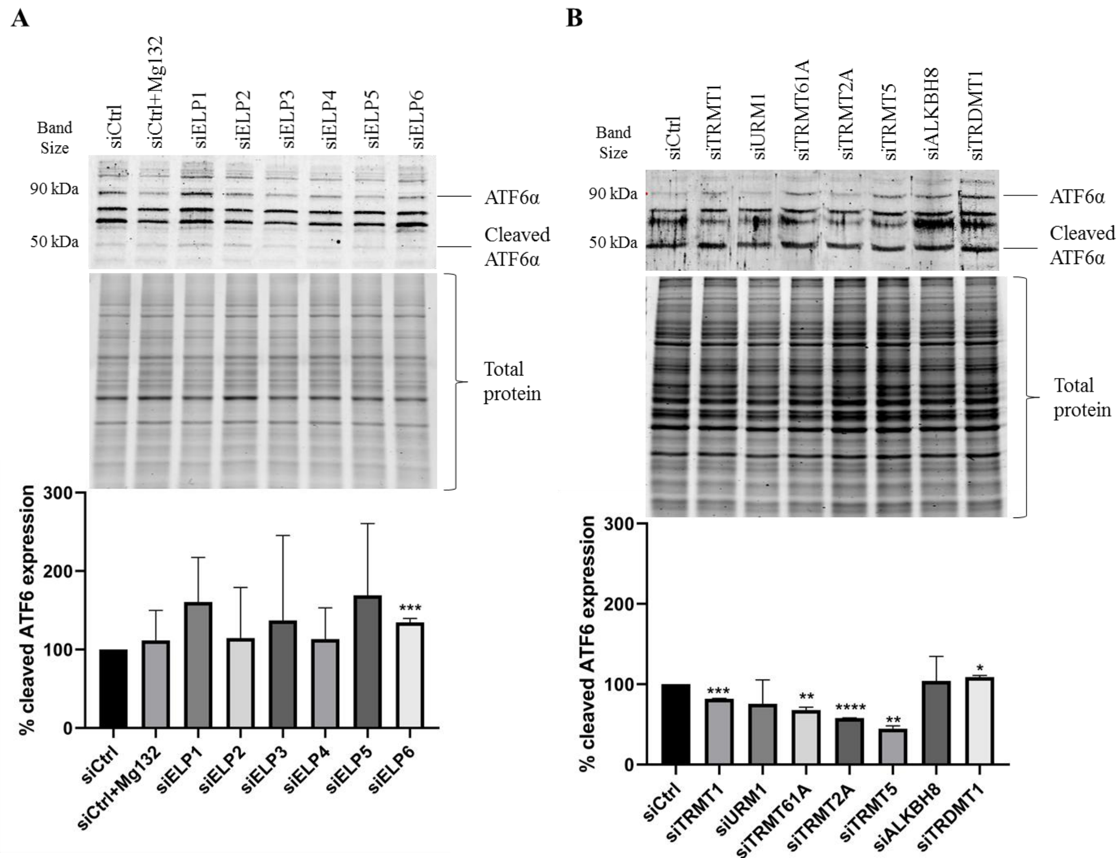

Figure 16: Analysis of ATF6 factor expression. The respective western blot bands are described with the respective graphic of percentage of the ATF6 factor cleaved in HeLa cells with Hsp27-GFP reporter. A) ELP Family: statistically significant changes were observed relative to control in siELP6 (P=0.0003); (Data analysis was done using Student's t-test (N=3)). B) Other enzymes: although there are statistically significant changes relative to control in siTRMT1 (P=0.0004), siTRMT61A (P=0.0061), siTRMT2A (P<0.0001), siTRMT5 (P=0.0020) and siTRDMT1 (P=0.0276), there are not enough replicates in this case (Data analysis was done using Student's t-test (N=2)).

3.4. Protein Synthesis rate

As phosphorylation of eIF2α is an indicator of decreased protein synthesis rate, we tested this in four conditions that showed a statistically significant increase in eIF2α-P, namely siELP1, siELP3, siELP6 and siURM1, and in one condition where there were no differences in eIF2α levels, namely siTRMT2A. We tested three ELP proteins that catalyze the mcm^{5}U modification at U34 and have been implicated in different neurological disorders, URM1 that is essential for the thiolation of U34 after the mcm^{5}U modification and TRMT2A that catalyzes methylations at positions 42 and 54 of tRNAs, outside the anticodon. These two enzymes have not been correlated so far to a particular disease, but our data indicates that they are indeed crucial for proteostasis maintenance in human cells.

The protein synthesis evaluation is based on the puromycin incorporation by the cells. Puromycin is an inhibitor of protein synthesis and elevated levels of puromycin incorporation in cells indicates increased protein synthesis.

Our results, represented in Figure 17, show that there is a decrease in puromycin incorporation when we knockdown the tRNA modifying enzymes ELP3 (P=0.0072), ELP6 (P=0.0001) and URM1 (0.0002), indicating that the protein synthesis rate is decreased, which correlates with the eIF2α-P results obtained previously.

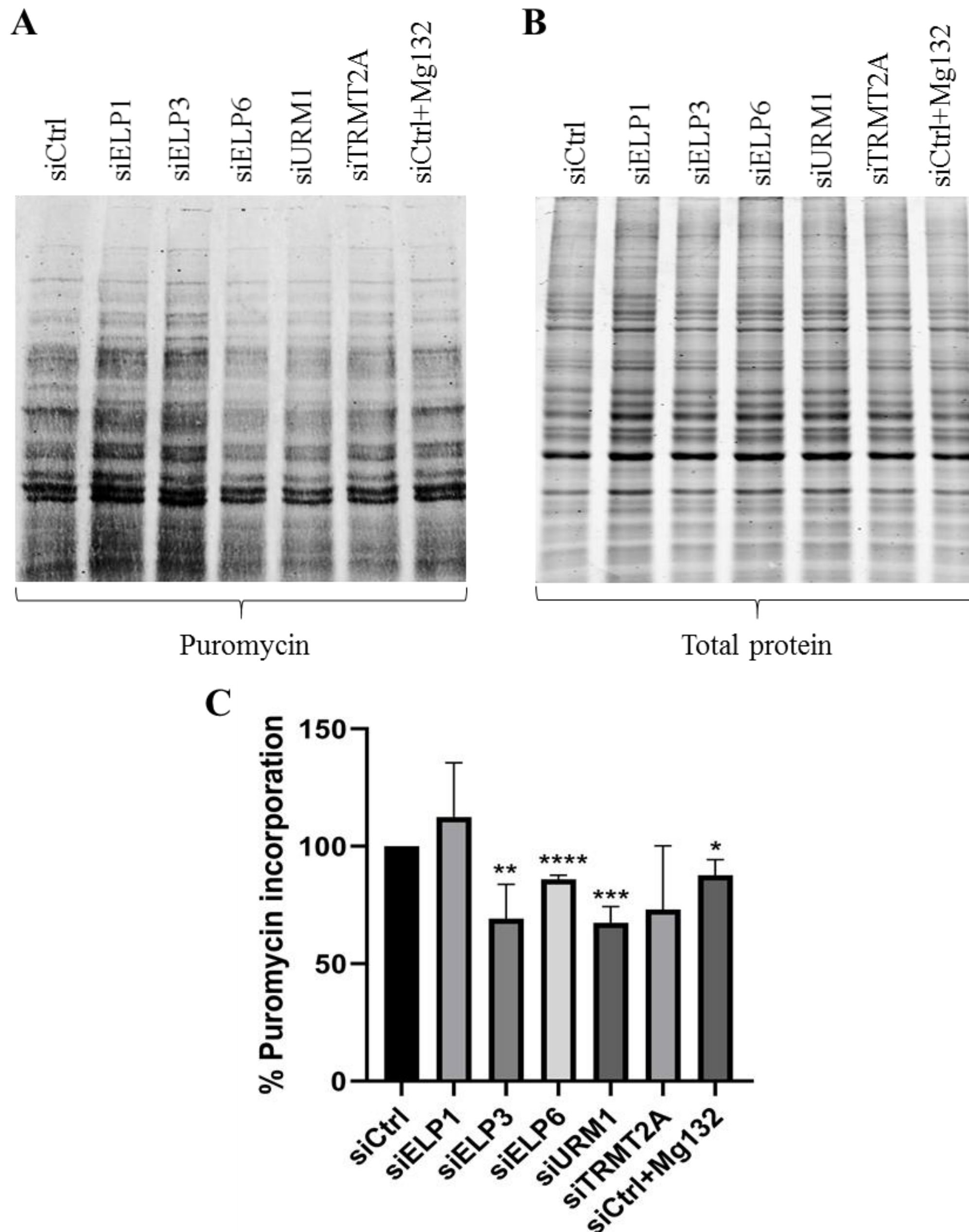

Figure 17: Analysis of protein synthesis of transfected cells with the respective bands described; A) Western Blot analysis of puromycin incorporation; B) SDS-PAGE of total protein for each transfection condition; C) Relative amount of puromycin incorporation in transfected cells compared with the control condition (siCtrl). Statistical significant changes were observed in siELP3 (P=0.0072), siELP6 (P<0.0001), siURM1 (P=0.0002) knockdown cells and in siCtrl+Mg132 positive control (Data analysis was done using Student's t-test; $p < 0.0001$ was considered highly significant (N=3).

3.5. Proteasome Activity

Since ubiquitin and proteasome are players in the UPS pathway and considering the results obtained with regard to the percentage of ubiquitination when we silenced some tRNA modifying enzymes, we next evaluated whether there were changes in relative proteasome activity in cells transfected with the different siRNAs of the ELP Family. Although there are only two biological replicates (N=2), it was already possible to observe a decrease in the relative proteasome activity in siELP1 (P=0.0402), siELP2 (P=0.0025) and siELP3 (P=0.0003) when compared to siCtrl.

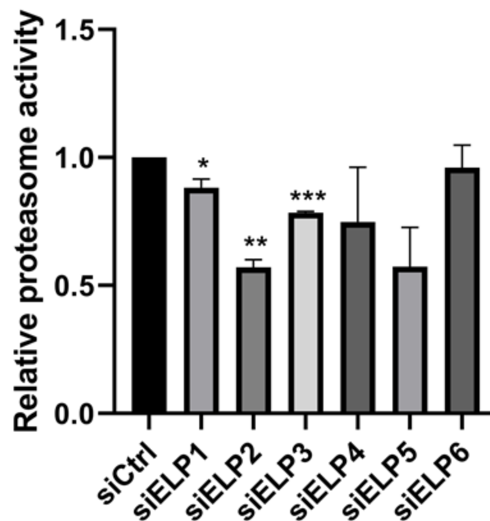

Figure 18: Relative proteasome activity in transfected cells compared with the control condition (siCtrl). Statistical significant changes were observed in siELP1 (P=0.0402), siELP2 (P=0.0025) and siELP3 (P=0.0003) but there are not enough replicates in this case (Data analysis was done using Student's t-test (N=2)).

3.6. ELP3 proteomics

As ELP3 was correlated with increased levels of misfolded levels and decreased protein synthesis rate and proteasome activity, we decided to study the effects of its absence at the proteome level to further understand the pathways affected by the lack of ELP3. For that, we performed mass spectrometry of total protein extracts by iTRAQ, at I3S and identified the up and down-regulated proteins.

Based on peptide mass spectrometry analysis, transfected SiElp3 cells were compared to transfected SiCtrl cells and the up- and down-regulated proteins were

considered biological relevant when the ration between the condition and the control cells was higher than 1.5 or lower than 0.5, respectively.

The list of genes obtained for each one of these conditions was analyzed in Cytoscape, with the App ClueGo in order to understand in which processes these genes were involved and confirmed with DAVID bioinformatics resources 6.8 online platform.

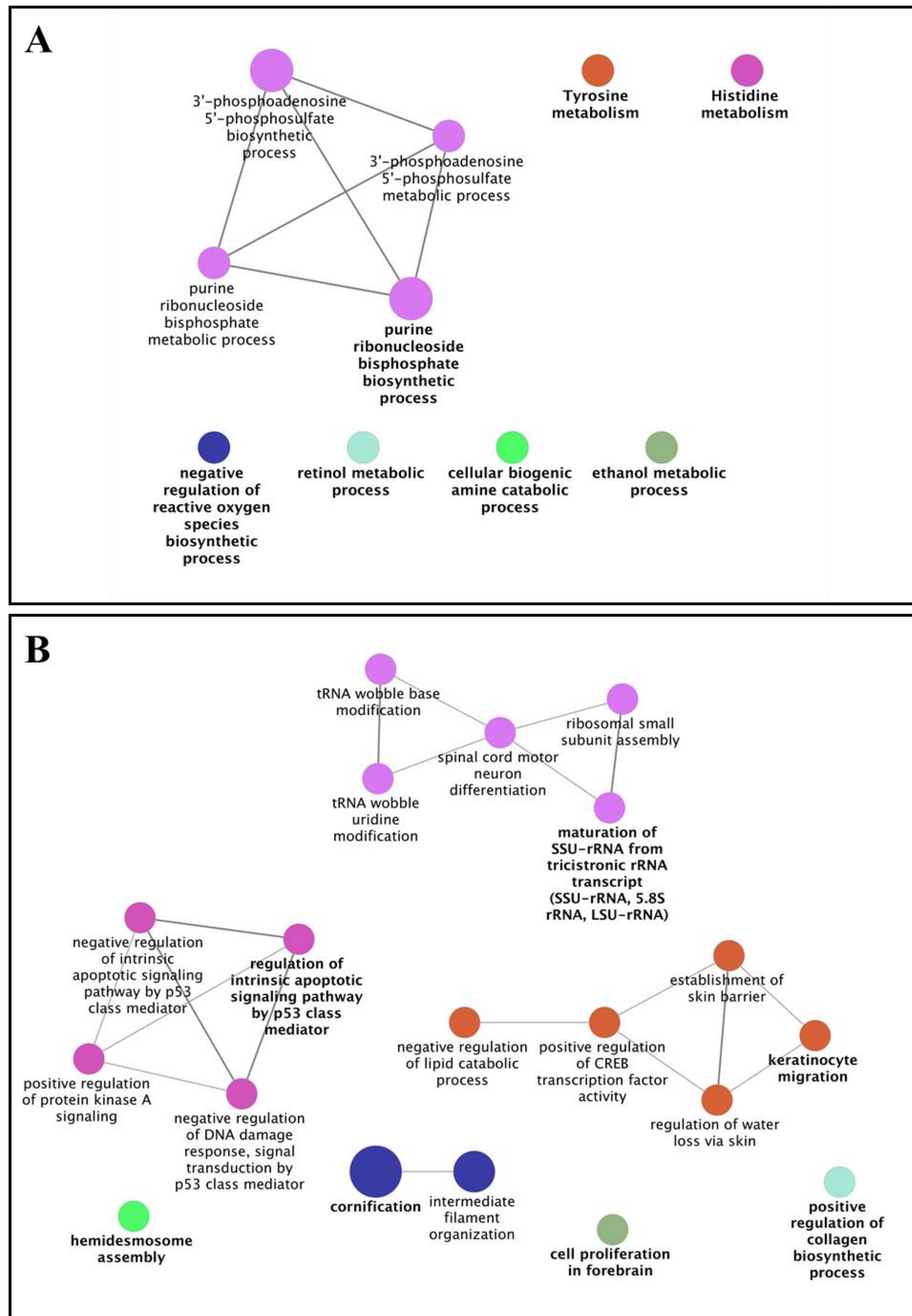

Figure 19: Molecular interaction networks and biological pathways of the genes up-regulated (A) and down-regulated (B) after Elp3 knockdown on HeLa cells (Chart obtained with Cytoscape - ClueGo).

3.7. Summary

Table 12: Summary of the achieved results of our tests with the correspondent tRNA modified residue. The asterisks (*) represent the statistically significant results regarding to the silencing of each tRNA modifying enzyme in each condition; the hyphen (-) represent the absence of results and the red colored cells represent the tRNA modifying enzymes that were not tested for the condition in question.

Enzyme	tRNA residue	Insoluble Fraction	Ubiquitin	eIF2α-P	ATF6α	Puromycin incorporation	Proteasome activity
ELP1	34	↑ *	↑ *	↑ **	-	-	↓ *
ELP2		↑ *	-	-	-		↓ **
ELP3		↑ *	↑ *	↑ *	-	↓ **	↓ ***
ELP4		-	-	-	-		-
ELP5		-	-	↑ *	-		-
ELP6		-	↑ **	↑ **	↑ ***	↓ ****	-
TRMT1	26	↑ **	-	↑ **	↓ ***		
URM1	34	↑ **	-	↑ **	-	↓ ***	
TRMT61A	58	-	↑ *	-	↓ **		
TRMT2A	42, 54	-	-	-	↓ ****	-	
TRMT5	37	-	↑ *	-	↓ **		
ALKBH8	34	↑ *	-	-	-		
TRDMT1	38	-	↑ *	-	↑ *		

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CHAPTER 4.

Discussion

4.1. tRNA modifying enzymes that affect proteostasis

4.1.1. Elongator Complex

Over the years it has been shown that the Elongator complex promotes the formation of ncm^5 and mcm^5 side-chains at wobble uridines in a variety of organisms, including humans (72,111) and that its inactivation is responsible for the appearance of different phenotypes, suggesting that the Elongator complex has numerous distinct functions (112). Despite this diversity of functions, it is believed that its primary cellular function is to promote tRNA modifications and the phenotypes that arise after its inactivation are likely a consequence of the tRNA modification defect (113). There are evidences that the pathological mechanisms responsible for the origin and progression of some neurological disorders might result from decreased Elongator activity, as is the case of some allelic variants of ELP3 responsible for ELP3 reduced expression levels associated with several cases of sporadic ALS (73). Numerous human diseases have been associated with several mutations in genes for the elongator subunits, since defects in this complex affect diverse cellular pathways, as it is the case of histone acetylation, exocytosis, telomeric gene silencing and transcriptional elongation. However, it is recognized now that the phenotypes associated with Elongator defects arise from the absence of mcm^5s^2U formation at position 34 of tRNAs (22,30). Elongator also influence α-tubulin acetylation in neurons, which suggests that this complex may play a role in other neurological disorders due to abnormal microtubule-dependent intracellular trafficking but this has not been directly verified yet (63).

Higher amounts of insoluble protein fraction present in transfected cells are correlated with elevated levels of protein aggregation. Facing raised levels of protein aggregation, the cell activates the UPS pathway, where ubiquitin will link to the aggregates, transporting them to the proteasome to be degraded, which causes ubiquitin levels to also increase. In addition to UPS, the UPR may also be activated, activating for example the PERK pathway that will phosphorylate the eIF2α, increasing its levels. In this study, only the conditions with ELP1 and ELP3 knockdown compared to the control presented statistical significant increased levels of the insoluble protein fraction, ubiquitin levels and phosphorylated eIF2α (Figure 13A, 14A and 15A) and ELP6 knockdown cells with increased levels of ubiquitin and eIF2α-P (Figure 14A and 15A), indicating that knockdown of these tRNA modifying enzymes triggers a stress response in cells. There are several studies confirming our findings. For instance, the silencing of ELP3 in melanoma cells resulted in the accumulation of endogenous protein aggregates (114). These tRNA modifying enzymes were already associated with some disorders: Tissue-specific expression of splicing mutations in the human IKBKAP (ELP1) gene, coding IKAP, were already identified as causative of Familial dysautonomia (FD), sometimes called Riley-Day syndrome, in individuals from the Ashkenazi Jewish population, in which the most prevalent mutation found in homozygosity is a single nucleotide change leading to exon

skipping and consequently an aberrant truncated protein (115). FD patients, characterized by reduced levels of ELP1, have also decreased levels of ELP3, once ELP3 is unsteady without ELP1 (116). Concerning to Elp3, it was already shown that the knockdown of ELP3 in zebrafish give rise to motor axonal abnormalities, a determinant factor in ALS disorder, and risk-associated ELP3 alleles related to lower brain ELP3 expression strongly suggests that this Elongator subunit is implicated in axonal biology and is a risk gene to neuronal degeneration (73). As ELP1 and ELP3 knockdown led to the accumulation of protein aggregates, as shown in this thesis, and as it is known, these enzymes are involved in FD and ALS development, respectively, we can speculate that the lack ELP1 and ELP3 activity in each disease is correlated with proteostasis impairments, that are characteristic of these diseases. This study validates that defects in these enzymes lead to proteostasis impairments, but additional experiments are needed to conclude whether these defects arise from ribosome pausing, altered translation fidelity or both. Besides, the deletion of ELP3 in cortical stem cells causes ER stress, activating the UPR response and autophagy in consequence of the decreasing codon translation rates (117).

With respect to the remaining subunits of the Elongator complex, there are already mutations described as being associated with certain disorders, namely, an autosomal recessive mutation in ELP2 is related to non-syndromic mental retardation (118) and the *ELP4* gene is associated with the pathogenesis of Rolandic epilepsy, affecting children between 3 and 12 years old, but the specific mutation still needs to be determined. Mutations in this gene may also partially abolish Elongator functions in the central nervous system regarding to cell motility and actin cytoskeleton genes and proteins during development (77). ELP5 and ELP6 are crucial for Elongator integrity since they bind ELP4 to ELP3 and, in addition, as integral subunits of the Elongator complex, they are key players for migration, invasion and tumorigenesis of melanoma cells (119). A recent study reported a point mutation in the gene encoding ELP6 in a mutant mouse for cerebellar ataxia causing Purkinje neuron degeneration and an ataxia-like phenotype, negatively affecting the Elongator activity and resulting in protein misfolding and aggregation that further induces ER-stress and succeeding apoptosis (120).

Our data suggest that among all ELP subunits, the knockdown of ELP1, ELP3 and ELP6 are the most relevant for protein aggregation, affecting proteostasis and the consequent triggering of degradation pathways. Although the knockdown of ELP2 and ELP5 had some impact on proteostasis, with increased insoluble protein fraction and increased eIF2α-P levels respectively, no other effects were observed. Moreover, no differences in any of the pathways tested were observed after knocking down ELP4. This raises the possibility that not all ELP subunits play a central part in tRNA modification and that perhaps lack of these specific subunits can be compensated by other components still to be uncovered. One cannot also discard the hypothesis that the strong effects observed in the absence of ELP3 may be due to other non-canonical functions of this enzyme, besides tRNA modification, particularly because it is the catalytical unit of the Elongator complex involved in transcriptional elongation. In fact, proteomic analysis of cells with ELP3 knock down revealed that down-regulated proteins participate in fundamental processes of protein

synthesis, such as nucleotide binding, RNA-binding and splicing, mRNA processing and splicing, translation and ribosomal constitution, with enrichment scores above 1.5, reinforcing the higher influence of this Elongator subunit both in transcriptional and translational processes. It will be important to also identify changes in the insoluble fractions of ELP3 knock down cells to understand which proteins are more prone to become insoluble and in which processes they are involved.

As mentioned above, several studies associated ELP1 with FD and some ELP3 variants with ALS, suggesting that the Elongator-dependent pathways may be deregulated in several neurological disorders (121) and that the deregulation of these enzymes have an impact in the cell's function. The study presented here complements the emergent agreement that perturbations in the Elongator complex subunits contribute to a variety of neurodevelopmental disorders but the mechanism by which specific mutations cause different neuropathologies remains to be elucidated and additional experiments are needed. LC-MS analysis of modified nucleotides and ribosome profiling will be crucial to correlate altered ELP3 levels with tRNA hypomodification and codon specific ribosome stalling, respectively.

4.1.2. Other Enzymes

Besides ELP proteins, there are other enzymes that modify tRNA, including at position 34, led to increased protein aggregation when silenced and that are involved in some disorders, including cancer. Of such tRNA modifying enzymes, the ones that presented more statistically significant changes were TRMT1 and URM1, whose knockdown caused an increase in the insoluble protein fraction, in the levels of phosphorylated eIF2α-P (Figure 13B and 15B) and in the case of Trmt1, also an accentuated decrease of ATF6α-cleaved values (Figure 16B). This data indicates that the knockdown of these enzymes causes protein aggregation, inducing ER stress in cells, which in turn activates the UPR pathway, specifically the PERK path. Concerning to TRMT1, the increased levels of eIF2α-P and decreased levels of cleaved ATF6α might be explained by the fact that the multiple steps involved in trafficking of ATF6 from ER to Golgi and lately into the nucleus makes this pathway slower than the PERK pathway (122).

TRMT1 is a tRNA modifying enzyme that acts as a dimethyltransferase, modifying a guanine residue in position 26 of tRNAs. A frameshift mutation identified in *TRMT1* gene resulting in an allelic variant within the functional domain of this enzyme, responsible for the enzymatic activity of the protein, leads to a premature termination codon affecting the splicing of all TRMT1 splice variants possibly undergoing degradation mediated by mRNA decay, categorize this gene as a candidate for autosomal recessive intellectual disorder (ARID), a neurodevelopmental disorder characterized by low IQ, in two Iranian families (123).

The deletion of the *URM1* genes leads to multiple phenotypic expressions, similar to the deletions of the ELP complex (124). In order to understand the mechanism by which

URM1 modifies the U34 nucleoside and why reduced URM1 levels in HeLa cells cause severe cytokinesis defects resulting in an enlarged multinucleated cells accumulation, a study followed a proteomic approach and the results showed an enzymatic activity linking URM1 to a tRNA modification pathway, in which URM1 was activated to produce a thiocarboxylated intermediate that donates sulfur in tRNA thiolation reactions (125). This mechanism was already used by prokaryotic sulfur carriers, reinforcing the high level of conservation of this ubiquitin-related modifier (126). Other studies relative to this enzyme deficiency shows that, in *S. cerevisiae*, it causes rapamycin sensitivity, invasive growth defects and pseudohyphal development (56,127) suggesting a particular role in nutrient sensing. YIL008W defective strains also presents sensitive to temperature and under oxidative stress they become hypersensitive, signifying that URM1 also play a role in stress tolerance (128). In *Drosophila melanogaster*, the complete loss of Urm1 causes embryonic lethality, it's deficiency causes oxidative stress tolerance and in addition it activates, directly or indirectly, the JNK (c-Jun NH(2)-terminal kinase) pathway (129). In human cells, when levels of URM1 were reduced, there was an increase of hypomodified $tRNAs^{LYS(UUU)}$ (125). Taking this into account, the results from our western blots analysis corresponded in a certain way to what we were expecting, since the statistical significant change was relative to the increasing of the insoluble protein fraction and the phosphorylation of eIF2α (Figure 13B and 15B), meaning that protein aggregation is occurring when we knockout this enzyme and that the PERK pathway is activated. In agreement with the last screening results where URM1 was a positive hit, this tRNA modifying enzyme was included in our protein synthesis study since it plays an essential role for the next step of ELP complex modifications, the thiolation, and it modifies the wobble position.

According to Catalogue of Somatic Mutations in Cancer (COSMIC), approximately 70% of Trmt61 mutations are due to missense substitutions. Trmt61A is localized in mitochondria and catalyzes the N_1-methyladenine (M^1A) formation at position 58 in methionyl-tRNA initiator, important for tRNA structure and stability, and to data it was not correlated with human diseases (130). This tRNA modifying enzyme presented an increase in the ubiquitin levels and a decrease in the cleaved ATF6α.

TRMT2A methylates the C5-position of uridine at positions 42 and 54 and was already linked to some HER-2 positive breast cancer where is overexpressed and it was also associated with an increased risk of recurrence (131). Despite of the only two replicates of the cleaved-ATF6α levels assessment, the silencing of this enzyme caused a significant decrease of this transcription factor, with a P value <0.0001 (Figure 16B). TRMT5 catalyzes the formation of m^1G37 in mitochondrial tRNA and individuals with possibly pathogenic TRMT5 variants express mitochondrial respiratory-chain deficiencies, however, the loss of m^1G37 does not impact, apparently, the tRNA stability (132). ALKBH8 modifies tRNA at position 34 (mcm^5Um) and contains a methyltransferase domain and an RNA binding motif. A study conducted in mice showed that this enzyme is induced in response to Reactive Oxygen Species (ROS) revealing an increasing stop codon recoding and mcm^5Um tRNA modifications, demonstrating a regulator role of the response

to oxidative stress and DNA damage prevention, which may indicate that AlkBH8 is linked to pathologies (133). In our study, its silencing only caused an increase in insoluble protein fraction and no impact was observed at the UPS and UPR level. It was quite unexpected as it catalyzes the same modification that ELP proteins. However, this may also indicate that lack of ALKBH8 is compensated by the Elongator complex. TRDMT1 is a methyltransferase that methylates position 38 of tRNAs. Knockdown experiments in Zebrafish show an induction of lethal differentiation defects in the retina, liver and brain (4) and Drosophila mutants exhibited a reduction of viability in stress situations (3). Studies in mice revealed that removing the *TRDMT1* gene changes the levels of various genes involved in cardiac hypertrophy, suggesting that, by mediation of RNA Polymerase II transcriptional activity, it is important to limit the differentiation and growing of cardiac tissue (134).

4.2. Knock down of tRNA modifying enzymes impair protein synthesis and proteasome activity in human cells

As mentioned earlier, puromycin is a protein synthesis inhibitor, so assessing the protein synthesis rate using this method indicates that elevated levels of puromycin incorporation implies an increased protein synthesis rate. Between the tested enzymes, ELP3, ELP6 and URM1 knock-down lead to decreased incorporation of puromycin (Figure 17), indicating a decrease in protein synthesis rate. This is consistent with what was previously shown in yeast, namely that the lack of some tRNA modifications, particularly defective wobble uridine tRNA modifications, is associated with a decrease in protein synthesis rate, impairing the proteasome leading to protein aggregation increase (45). Also, ELP3 was implicated in the impairment of codon translation speed and concomitant triggering of UPR via activation of the PERK-eIF2α-Atf4 signaling in cortical neurons (117).

The decrease in the proteasome activity is an aging hallmark. This can be triggered by the impairment of the heat-shock response or endoplasmic reticulum stress, leading to the aggregation of erroneous proteins (135). At this point, we only have two replicates concerning to proteasome activity assessment, however, it was possible to observe that the knockdown of the tRNA modifying enzymes ELP1, ELP2 and ELP3 caused a significant decrease in proteasome activity (Figure 18). This is consistent with some reports in which the proteasomal dysfunction is associated with neurodegeneration, where the inclusion bodies of AD, for example, contain elevated levels of ubiquitin and a decreased proteasomal activity can boost the neurodegenerative phenotype (136,137). In fact, some papers already emphasized an UPS loss in neurodegenerative disorders such as Parkinson's and Alzheimer's disease (138).

A connection between the deprived activity of the Elongator complex in ELP3 conditional knockout mice and the UPR-pathway weakening was previously stablished, relating it with an incapacity to produce neurons during corticogenesis (117). Another study shown that faulty wobble uridine tRNA modification results in slower codon

translation rates, harming the proteasome and consequently increasing protein aggregation (45).

Considering all the data obtained so far, the contribution of ELP3 to tRNA modifications and proteostasis maintenance is undeniable, reinforcing the need to deepen the knowledge about this enzyme and characterize the affected proteins.

4.4. Ongoing work

CRISPR/Cas9 system is a genome editing technology adapted from a naturally occurring genome in bacteria: when viruses invade the bacteria, it arrests snips of its DNA and uses them to create DNA segments, identified as CRISPR arrays, allowing the bacteria to remember the virus and if they attack again, the bacteria will produce RNA segments from the CRISPR arrays to target the DNA of the virus and, making use of Cas9 or a similar enzyme, they cut the DNA apart and the virus is disabled (139,140). In the lab, this system works similarly, where the scientist create a small piece of RNA with a short-guide sequence that binds to a specific target sequence of DNA in a genome and once that DNA is cut, we use the cell’s own DNA repair machinery to add or delete pieces of genetic material (141).

Our main goal in making use of this technology is to develop a stable ELP3 KO cell line, however, this project is still at an early stage. This cell line will allow to have cells that are constantly in misfolding conditions, which will allow to perform long term studies to characterize the cellular pathways affected, which cannot be performed with transient siRNA transfections. Moreover, this may constitute a promising protein aggregation model to perform drug screenings and identify relevant molecules to revert protein misfolding.

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CHAPTER 5.

Concluding remarks

5.1. Conclusion and future perspectives

As a matter of time and logistics, we were not able to test all the enzymes for all the alterations in the different pathways, so during the study we had to select according to those who presented the most promising results in the different studies. Amongst all the tRNA modifying enzymes tested, the contribution of Elongator complex for tRNA modifications is undeniable and reinforces the fact that modifications in residue 34 of tRNA are the ones causing more changes to the proteostasis network of the cell. However, LC-MS studies of tRNA modifications are ongoing to prove that tRNA modifying enzyme silencing leads to particular tRNA hypomodification, so this connection can be experimentally proven in our cells. The Elp3 knockdown in HeLa cells was the one causing more alterations in proteostasis, concerning to UPS and UPR activation, as well as protein synthesis and proteasome activity impairment, consistent to what is described in the literature.

Knock-down of ELP3 in HeLa cells led to deregulation of proteins mainly involved in translation, transcription and ribosome processes, so it would be interesting to analyze the composition of aggregates in the insoluble protein fraction to determine whether they are also enriched for translation and transcription factors and ribosomal subunits and complement it with ribosome profiling.

The involvement of tRNA modification enzymes in such a diversity of essential mechanisms to protein synthesis and homeostasis makes them a promising therapeutically target, especially in the neurodegenerative disorders field.

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“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”
Marie Curie