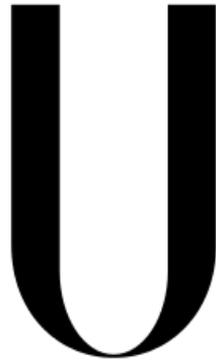


UNIVERSIDADE DE LISBOA
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DEPARTAMENTO DE BIOLOGIA ANIMAL



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**Suppression of nonsense mutations as a therapeutic
approach to treat genetic diseases**

Francesca Manuela Johnson de Sousa Brito

DISSERTAÇÃO

MESTRADO EM BIOLOGIA HUMANA E AMBIENTE

2014

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**Suppression of nonsense mutations as a therapeutic
approach to treat genetic diseases**

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2014

“If I had a world of my own, everything would be nonsense. Nothing would be what it is, because everything would be what it isn't. And contrary wise, what is, it wouldn't be. And what it wouldn't be, it would. You see?”

Lewis Carroll

(English writer, 1832-1898)

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Abstract

Premature termination codons (PTCs or nonsense codons) can arise from various types of mutations in germ or somatic cells. PTCs promote premature translational termination and the induction of nonsense-mediated mRNA decay (NMD). NMD is a surveillance system that prevents the synthesis of C-terminally truncated proteins toxic for the cell. The physiological importance of NMD is manifested by the fact that about one third of genetic disease-associated mutations generate PTCs.

In recent years, a novel therapeutic approach called suppression therapy has been developed that utilizes low molecular weight compounds to induce the translation machinery to recode a PTC into a sense codon. Beta-thalassaemia (β -thalassaemia) is one of the most common genetic diseases worldwide, and nonsense mutations occurring in the beta-globin (β -globin) are among the most frequent mutations associated with β -thalassaemia. Some studies have shown that aminoglycosides, low molecular weight compounds, and non-aminoglycosides can suppress PTCs in cystic fibrosis and Duchenne's muscular dystrophy, but it remains unclear whether β -thalassaemia would also be responsive to a similar drug treatment. Preliminary results obtained in our lab have shown that the aminoglycoside G418 can suppress a nonsense mutation at codon 39 of the human β -globin mRNA, although at low levels in cultured erythroid cells. To investigate if suppression therapy can restore enough β -globin protein to correct the disease manifestations of β -thalassaemia, we tested whether G418 is able to induce efficient levels of suppression in a dose-dependent and time-course manner in HeLa cells transfected with plasmids containing the human β -globin wild type gene (β WT) or the counterpart carrying a nonsense mutation at codon 39 (β 39).

Our results show a slight increase in the levels of mRNA of the β 39 transcript which may indicate a possible effect by the drug. Seen as we were unable to test the effect of the drug on a protein level, in the future, it would be of great interest to evaluate its effect on protein expression levels.

Key-words: Nonsense-mediated mRNA decay (NMD); mRNA translation; genetic diseases; suppression therapy; G418.

Sumário

Algumas das mutações que ocorrem em células somáticas ou na linha germinal podem resultar na formação de codões de terminação prematura da tradução (CTPs ou codões *nonsense*). Um terço das mutações associadas a doenças genéticas resulta na produção de CTPs. Em geral, a introdução dum CTP num transcrito induz a terminação prematura da tradução e a activação do mecanismo de decaimento do mRNA mediado por mutações *nonsense* (nonsense-mediated mRNA decay; NMD). O NMD é um mecanismo de controlo de qualidade que impede a produção de proteínas truncadas na extremidade C-terminal tóxicas para a célula.

No decorrer dos últimos anos, tem sido desenvolvida uma nova terapia, designada como terapia de supressão, que utiliza compostos de baixo peso molecular para induzir a maquinaria de tradução a alterar um CTP para um codão *sense*. O objectivo da terapia de supressão é estimular a capacidade de competição de tRNAs aminoacil com o complexo de terminação para a ligação ao CTP no local A do ribossoma, de forma a suprimir especificamente a terminação em CTPs e não em codões de terminação naturais. Embora a talassémia seja uma das doenças genéticas mais comuns e as mutações *nonsense* no gene da beta-globina (β -globina) estejam muito frequentemente associadas à beta-talassémia (β -talassémia), a utilização da terapia de supressão nestes casos ainda não foi investigada de forma detalhada. Resultados preliminares obtidos pelo nosso laboratório indicam que o aminoglicósido G418 tem a capacidade de suprimir uma mutação *nonsense* no codão 39 do mRNA da β -globina, em células eritróides em cultura. De forma a confirmar o princípio de que a terapia de supressão pode restaurar os níveis de β -globina, testámos se o composto G418 tem a capacidade de induzir níveis significativos de supressão em células HeLa transfectadas com plasmídeos que contêm o gene da β -globina humana normal (β WT) ou o equivalente com mutação *nonsense* no codão 39 (β 39).

Segundo os resultados obtidos, há um ligeiro aumento nos níveis de transcrito β 39 o que pode ser indicador de um possível efeito da droga. Como não nos foi possível verificar o efeito da droga nos níveis de proteína é necessário trabalho futuro para avaliar o seu efeito nos níveis de expressão proteica.

Palavras-chave: Decaimento do mRNA mediado por mutações nonsense (NMD); tradução do mRNA; doenças genéticas; terapia de supressão; G418.

Abbreviations

A	Adenine
A-site	aminoacyl-site
bp	base pairs
CBC	cap binding complex
CBP	cap binding protein
cDNA	complementary deoxyribonucleic acid
C-terminal	carboxyl-terminal
DEAD	Asp-Glu-Ala-Asp motif
DECID	decay inducing complex
DH5 α	strain of <i>Escherichia coli</i>
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
eEF	eukaryotic translation elongation factor
eIF	eukaryotic initiation factor
EJC	exon junction complex
eRF	eukaryotic translation release factor
E-site	exit-site
FBS	foetal bovine serum
G	guanine
GDP	guanosine diphosphate

GTP	guanosine triphosphate
h	hour(s)
HeLa	human cervical cancer cell line
IRES	internal ribosome entry site
MAGOH	mago-nashi homolog
Met	metionin
Met-tRNA _i	methionine-loaded initiator tRNA
min	minute(s)
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein particle
nt	(any) nucleotide
N-terminal	amino-terminus
NMD	nonsense-mediated mRNA decay
ORF	open reading frame
PABP	poly(A)-binding protein
PABPC1	cytoplasmic poly(A)-binding protein 1
PAN	poly(A) nuclease
PBS	phosphate buffered saline
PIN	PiIT N terminus
PCR	polymerase chain reaction
PLB	passive lysis buffer
Poly(A)	poly-adenilate

Pre-mRNA	messenger ribonucleic acid precursor
P-site	peptidyl-site
PTC	premature translation termination codon
Puro	puromycin
RNA	ribonucleic acid
RNase	ribonuclease
RNPS1	RNA binding protein S1
RT	reverse transcription
RT-qPCR	reverse transcription quantitative polymerase chain reaction
sec	second(s)
SMG	suppressor of morphological defects on genitalia
Srm160	SR-related nuclear matrix protein of 160 kDa
SURF	SMG1-UPF1-eRFs complex
T	thymine
tRNA	transfer ribonucleic acid
tRNAi	transfer ribonucleic acid of initiation
U	uracil
uORF	upstream open reading frame
UPF	up-frameshift protein
UTR	untranslated region
WT	wild type
XRN1	5'-3' exoribonuclease 1

β 15	human β -globin transcript with a PTC at codon 15
β 39	human β -globin transcript with a PTC at codon 39
β WT	normal/wild type human β -globin transcript

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I. Introduction

Eukaryotic gene expression pathway involves a series of interconnected steps, from transcription to translation. These steps are integrated with one another to augment the efficiency and fidelity of gene expression. This allows expression of individual genes to be controlled, producing transcripts and eventually detecting and degrading aberrant transcripts (Chang *et al*, 2007; Clancy & Brown, 2008; Nicholson *et al*, 2010; Behm-Ansmant *et al*, 2007). If not detected and degraded, these transcripts can result in the accumulation of potentially harmful truncated proteins (Nicholson *et al*, 2010). Given the complex chain of biochemical reactions involved in transforming the genetic information of an organism into gene products, the overall accuracy of gene expression is quite astonishing (Mühlemann & Lykke-Andersen, 2010). Since mRNAs function primarily as templates for protein synthesis, it is now established that most, if not all, of the steps of the gene expression pathway can be regulated by quality-control mechanisms (or mRNA surveillance mechanisms) both in the nucleus and in the cytoplasm at multiple stages during gene expression. For example, improperly processed mRNAs are degraded by mRNA surveillance mechanisms in the nucleus before they are exported. In the cytoplasm, quality control mechanisms assess the translatability of the mRNA and degrade any that lacks translation termination codons or that has premature translation termination codons (PTCs), thereby preventing the accumulation of potentially toxic protein fragments (Schoenberg & Maquat, 2012; Behm-Ansmant *et al*, 2007; Nicholson *et al*, 2010). Nonsense-mediated mRNA decay (NMD) represents a translation-dependent post-transcriptional process that selectively recognizes and degrades mRNAs whose open reading frame (ORF) is truncated by a PTC. In doing so, NMD protects the cell from accumulating C-terminally truncated proteins with potentially deleterious functions (Mühlemann *et al*, 2008).

NMD acts not only on aberrant mRNAs, but also regulates the expression of naturally occurring transcripts having features that allow them to be recognized as PTC-containing transcripts. In this way, NMD also contributes to the post-transcriptional regulation of gene expression (Behm-Ansmant *et al*, 2007).

I.1. mRNA translation

In order to understand how and when the surveillance mechanisms can act, we need to understand the pathway of gene expression in further detail. One of the most important steps in this pathway is the translation of proteins from messenger RNA (mRNA). Regulation of translation is a mechanism that is used to modulate gene expression in a wide range of biological situations. From early embryonic development to cell differentiation and metabolism, translation regulation is used to fine-tune protein levels in both time and space (Gebauer & Hentze, 2004).

During translation, the sequence of codons on mRNA directs the synthesis of a polypeptide chain. This dynamic process is usually divided into three phases: initiation, elongation and termination (Preiss & Hentze, 2003; Ramakrishnan, 2002). Ribosomes, large ribonucleoprotein particles composed of two subunits in all species, are, along with numerous translation factors, the cellular machines responsible for translating genetic information into polypeptide sequences. During the step-wise movement of an mRNA through the ribosome, amino acids are incorporated into the elongating polypeptide chain. Each subunit has three binding sites for tRNA, designated: the A (aminoacyl), which accepts the incoming aminoacylated tRNA; P (peptidyl), which holds the tRNA with the nascent peptide chain; and E (exit), which holds the deacylated tRNA before it leaves the ribosome. The fidelity of amino acids incorporation depends on base-pair complementarities between sense codons and anticodons of aminoacyl-tRNAs (Korostelev, 2011; Ramakrishnan, 2002). We will discuss the three phases of translation in the following paragraphs.

I.1.1. Translation initiation

The translation of mRNA begins with the formation of the ternary complex composed of eukaryotic initiation factor 2 (eIF2), a hetero-dimer of 3 subunits (α , β and γ), bound to the methionyl-initiator tRNA ($\text{Met-tRNA}_i^{\text{Met}}$), and GTP by the γ subunit (fig 1. A) (Gebauer & Hentze, 2004). Once the ternary complex is assembled and active, it must bind to the 40S ribosomal subunit. This binding is aided by eukaryotic initiation factors 1, 1A, 3 and 5. The structure resulting from the bound of ternary complex to 40S

ribosomal subunit alongside initiation factors 1, 1A, 3 and 5 is designated 43S pre-initiation complex (fig 1. B) (Gebauer & Hentze, 2004; Jackson *et al*, 2010).

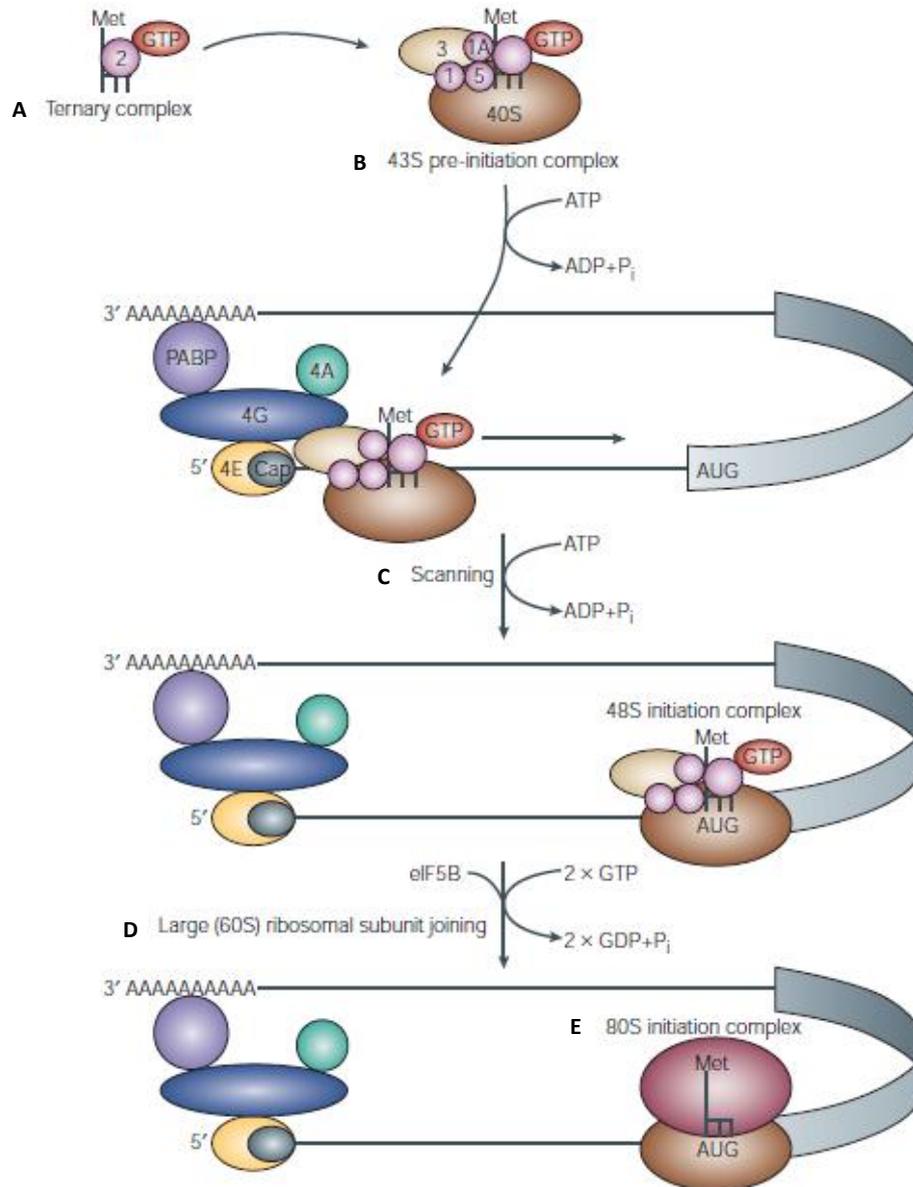


Figure 1: Cap-mediated translation initiation. The methionine-loaded initiator tRNA binds to GTP-coupled eIF2, to yield the ternary complex. This complex then binds to the small (40S) ribosomal subunit, eIF3 and other initiation factors to form the 43S pre-initiation complex. The pre-initiation complex recognizes the mRNA by the binding of eIF3 to the eIF4G subunit of the cap-binding complex. In addition to eIF4G, the cap-binding complex contains eIF4E, which directly binds to the cap, and eIF4A, an RNA helicase that unwinds secondary structure during the subsequent step of scanning. The 43S pre-initiation complex scans the mRNA in a 5' to 3' direction until it identifies the initiator codon AUG. Scanning is assisted by the factors eIF1 and eIF1A. Stable binding of the 43S pre-initiation complex to the AUG codon yields the 48S initiation complex. Subsequent joining of the large (60S) ribosomal subunit results in the formation of the 80S initiation complex. Both AUG recognition and joining of the large ribosomal subunit trigger GTP hydrolysis on eIF2 and eIF5B, respectively. Subsequently, the 80S complex is competent to catalyze the formation of the first peptide bond. P_i: inorganic phosphate (Gebauer & Hentze, 2004).

The 5' cap-proximal region is recognized by eIF4F. This complex comprises the cap-binding protein eIF4E; the DEAD-box RNA helicase eIF4A, which unwinds secondary structures in the 5' untranslated region (5'UTR) so that the 43S complex can bind and scan the mRNA; and eIF4G, which functions as a 'scaffold' that binds eIF4E and eIF4A in order to form eIF4F, the cap-binding complex of translation initiation. The 43S complex then scans the 5'UTR in the 5' to 3' direction, downstream of the cap, until it finds the first AUG in good initiation context (fig 1. C) (scanning model of translation initiation; Kozak, 1989). The 43S complex recognizes the initiation codon through the formation of base pairs between the initiator tRNA and the start codon and formation of 48S initiation complex occurs (Jackson *et al*, 2010; Gebauer & Hentze, 2004). After 48S complex formation and initiation codon recognition, eIF5 promotes the hydrolysis of eIF2-bound GTP and eIF5B, a ribosome-dependent GTPase, and the displacement of eIFs and the joining of the 60S subunit (fig 1. D), leading to the assembly of 80S ribosome (fig 1.E), which is competent to initiate elongation. Although this is the canonical mechanism by which protein synthesis in eukaryotes occurs, there are some alternatives to this translation initiation model which may be cap-dependent (e.g. leaky scanning, reinitiation) or independent such as translation mediated by internal ribosome entry sites (IRESs) or CITEs among some other mechanisms that explain exceptions to this rule (Jackson *et al*, 2010; Gebauer & Hentze, 2004).

I.1.2. Translation Elongation

The end of the initiation process leaves an aminoacylated initiator tRNA in the P site of the ribosome and an empty A site, which serves to start the elongation cycle (Ramakrishnan, 2002). The elongation phase of protein synthesis involves the correct decoding of the mRNA into the amino acid sequence of the encoded polypeptide, involving fewer factors than initiation. A major feature of elongation must therefore be to maintain the accuracy of the process, to ensure errors are not made in synthesising the product (Proud, 1994).

During elongation, amino acids are added sequentially to the growing polypeptide chain, in the order specified by the sequence of the mRNA in frame with the AUG codon. A key factor in this process is eukaryotic elongation factor eEF1A, which is

responsible for delivering the aminoacyl-tRNA to the A-site of the ribosome. The activity of eEF1A is dependent on GTP; the guanine nucleotide-exchange factor eEF1B promotes the regeneration of active eEF1A–GTP complexes. The last elongation factor is eEF2, which is required for the translocation of the peptidyl-tRNA from the A-site of the ribosome to the P-site and the movement of the ribosome along the mRNA. This is also a GTP-dependent process (fig 2) (Abbot & Proud, 2004).

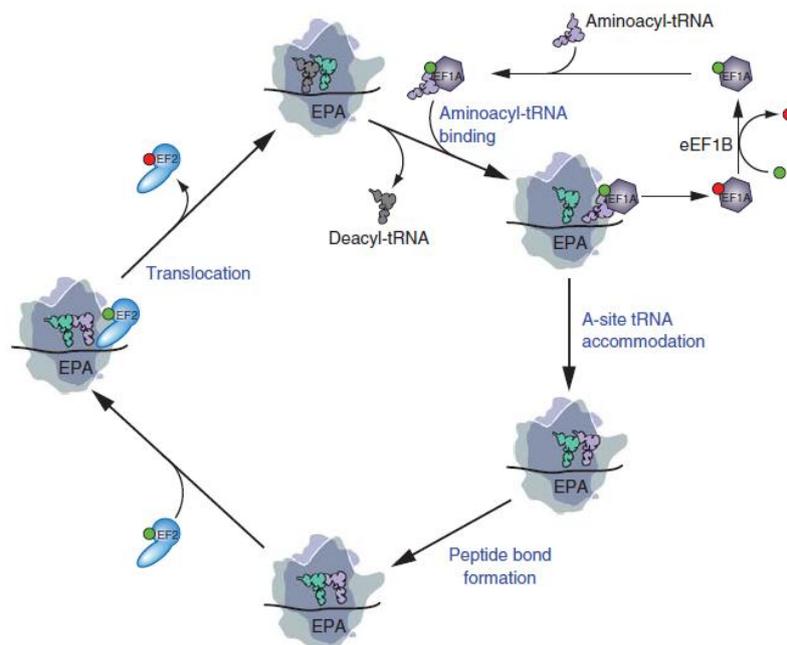


Figure 2: Model of the eukaryotic translation elongation pathway. Starting at the top, an eEF1A-GTP-aminoacyl-tRNA ternary complex binds the aminoacyl-tRNA to the 80S ribosome with the anticodon loop of the tRNA in contact with the mRNA in the A-site of the small subunit. Following release of eEF1A-GDP, the aminoacyl-tRNA is accommodated into the A site, and the eEF1A-GDP is recycled to eEF1A-GTP by the exchange factor eEF1B. Peptide bond formation is accompanied by transition of the A- and P-site tRNAs into hybrid states with the acceptor ends of the tRNAs moving to the P and E sites, respectively. Binding of eEF2-GTP promotes translocation of the tRNAs into the P and E sites, and is followed by release of eEF2-GDP. The ribosome is now ready for the next cycle of elongation with release of the deacylated tRNA from the E site and binding of the appropriate eEF1A-GTP.aminoacyl-tRNA to the A-site. GTP: depicted as a green ball; GDP: depicted as a red ball (Dever & Green, 2012)

The process of elongation is repeated many times, until a stop codon is reached. An additional amino acid is added to the growing polypeptide chain each time the mRNA advances through the ribosome. Once a polypeptide chain of reasonable size is assembled, it begins to emerge from the base of the large subunit (Klug *et al*, 2005).

When a stop codon is recognized by the ribosome, translation terminates, and the nascent protein must be released from the mRNA and ribosome (Clancy & Brown, 2008).

I.1.3. Translation Termination

The final step of translation - termination - takes place when a stop codon, UAG, UAA, or UGA, enters the A-site of the ribosome. For simplicity, termination can be thought of as two distinct steps, stop codon recognition and peptide release. In eukaryotes, translation termination is mediated by eukaryotic release factor 1 (eRF1), which is responsible for stop codon recognition and triggering peptide release, and eRF3, a GTPase that stimulates eRF1-mediated peptide release (Abbot & Proud, 2004). eRF1, in turn, stabilizes binding of GTP to eRF3 so that they form a stable ternary complex (Mitkevich *et al*, 2006; Pisareva *et al*, 2006). The binding of the eRF complex to the ribosome stimulates the cleavage of the bond between the peptide and the tRNA, thus, releasing the newly synthesized peptide and the tRNA from the ribosome, which then dissociates into its subunits. If a stop codon should appear in the middle of an mRNA molecule, the same process occurs, and the polypeptide chain is prematurely terminated (Abbot & Proud, 2004; Klug *et al*, 2005; Dever & Green, 2012; Karijolich & Yu, 2014). Interactions of the eRFs with cellular proteins playing key roles in other gene expression processes may be the reason by which termination activity is adjusted and linked to other events in mRNA translation and NMD (fig 3) (Dever & Green, 2012).

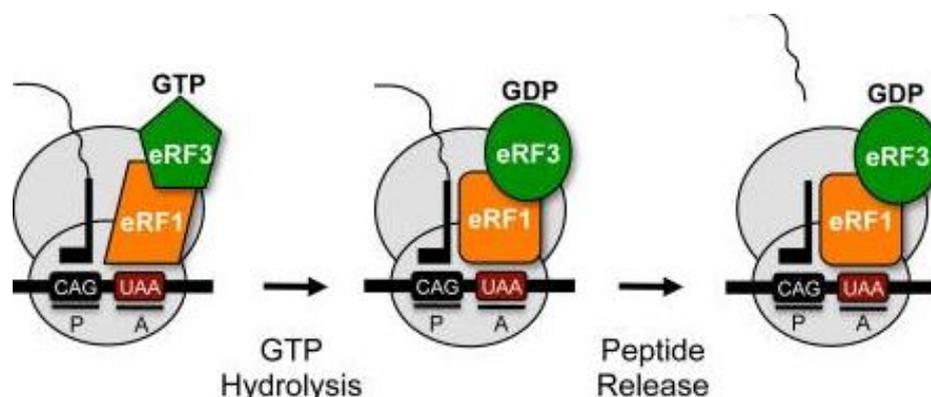


Figure 3: Model of eukaryotic translation termination. A complex comprised of eRF1 and eRF3 mediate translation termination. eRF1 recognizes any of the three stop codons (UAA, UAG, UGA) in the ribosomal A site. GTP hydrolysis by eRF3 assists: 1) stop codon recognition by eRF1, and 2) eRF1 accommodation into the peptidyl transferase center so polypeptide release can occur (Keeling *et al*, 2012).

I.2. Nonsense-mediated mRNA decay - a surveillance mechanism

At the level of mRNA, two important features are monitored by cell quality control mechanisms: first, whether it has the correct set of proteins bound to a particular mRNA; and, secondly, whether the coding potential of the mRNA is intact (Nicholson & Mühlemann, 2010).

There are several quality control mechanisms in mammalian cells such as nonsense-mediated mRNA decay (NMD), no-go mRNA decay and nonstop mRNA decay. Here, we are going to focus on NMD seen as it is the best characterized of the three and the only one studied in any detail from a regulatory perspective (Schoenberg & Maquat, 2012).

NMD is one of the best characterized eukaryotic mRNA quality control mechanisms. NMD targets mRNAs harbouring premature termination (nonsense) codons (PTCs) for degradation. This pathway is important because if PTC-containing messages were allowed to be translated they would produce potentially toxic truncated proteins with potentially deleterious gain-of-function or dominant-negative activity (Nicholson & Mühlemann, 2010; Behm-Ansmant *et al*, 2007; Chang *et al*, 2007; Nicholson *et al*, 2010).

All organisms require the NMD pathway to eliminate transcripts containing PTCs, which arise from inherited or sporadic mutations or alternative splicing, so that normal cellular function is maintained; and to eliminate endogenous error-free transcripts in order to maintain regular levels of transcript and subsequent protein synthesis for development and viability (Chapin *et al*, 2014).

I.2.1. PTC recognition

Even before the identification of the NMD molecular players, a rule for the recognition of PTCs that induce mammalian NMD was postulated. Studies in mammalian systems led to the observation that PTCs, when located more than 50-54 nucleotides upstream the last exon-exon junction, were able to target mRNA for decay, whereas PTCs located downstream of this boundary do not induce NMD. The discovery of the exon junction complex provided a molecular explanation for the empirically detected '50-54 nucleotide boundary' and supports the view that PTC recognition is dependent on the

definition of the exon-exon junctions, suggesting that the process of splicing is implicated in mammalian NMD (Silva & Romão, 2009; Schweingruber *et al*, 2013).

A major issue that is still only partially understood is how NMD machinery distinguishes between premature and normal stop codons. Generation of a PTC can involve as little as a single nucleotide change. How such a subtle alteration can be detected as aberrant has led to several models. In all models, detection is intimately connected to translation termination. Synthesized mRNAs are bound by the cap-binding protein heterodimer CBP80-CBP20, which constitutes the cap-binding complex (CBC), and if derived from intron-containing pre-mRNA, they are also bound by multiprotein assemblies, the EJCs, that presently are known to assemble ~20-24 nts upstream of each exon-exon junction during pre-mRNA splicing. The EJC contains the general splicing activator RNPS1, the RNA export factor Aly/ REF, the shuttling protein Y14, the nuclear matrix-localized serine-arginine-containing protein SRm160, the oncoprotein DEK, and the Y14 binding protein magoh. The interaction of magoh with Y14 may have a role in anchoring the NMD-specific factors UPF3 and UPF2 to the mRNA. Translation termination, which involves eRF1 and eRF3, provides the first signal necessary for activation of NMD. According to the present models, translating ribosomes displace EJCs from the open reading frame (ORF) during the “pioneer” round of translation. If assembly of eRF1-eRF3 at a termination codon occurs ≥ 50 -54 nucleotides (nts) upstream from an exon-exon junction, the footprint of the terminating ribosome is insufficient to physically remove the EJCs (Chang *et al*, 2007; Popp & Maquat, 2013; Inácio *et al*, 2004; Silva & Romão, 2009). The retained EJC(s) can interact with the translation termination complex via bridging interactions between the release complex-associated proteins, UPF1 and SMG-1 (Kashima *et al*, 2005). This bridging interaction has been proposed to trigger accelerated decay (i.e. NMD) of the PTC-containing mRNA (Peixeiro *et al*, 2011).

I.2.2. Molecular events that prepare mRNA for degradation by NMD

Translation termination is triggered by recognition of the stop codon by eRF1 and eRF3. The central feature of the “unified model” of NMD is that the mechanism of

translation termination at a PTC is intrinsically different from translation termination at a “correct” termination codon. In *Saccharomyces cerevisiae*, it has been demonstrated that ribosomes do not efficiently dissociate from the mRNA when stalling at a PTC, presumably because in that special environment they cannot receive the termination-stimulating signal from Poly(A)-binding protein (PABP). Likewise, in flies and human cells, artificial tethering of PABP into close proximity of an otherwise NMD-triggering PTC efficiently suppresses NMD. Based on reported biochemical interactions, this model proposes that the decision of whether NMD is triggered relies on a competition between UPF1 and PABP for binding to eRF3 bound to the terminating ribosome. According to this model, a translation termination event is defined as “correct” if the ribosome stalls close enough from the poly(A) tail to efficiently interact with PABP, which, through yet unknown mechanisms, leads to a fast/efficient polypeptide release and dissociation of the ribosomal subunits. If, in contrast, the spatial distance between the terminating ribosome and the poly(A) tail is too big for this interaction to occur, UPF1 will bind to the ribosome-bound eRF3 instead. Using co-immunoprecipitation analysis, a recent study showed that UPF1, eRF1, and eRF3 are part of a single complex that also contains the UPF1 kinase Suppressor with Morphogenetic effect on Genitalia-1 (SMG1) (Kashima *et al*, 2006). This SMG1 - UPF1 - eRF1 - eRF3 (SURF) complex is proposed to assemble on ribosomes stalled at a stop codon, with eRF1 and eRF3 recruiting unphosphorylated UPF1, which in turn recruits SMG1. At this stage, UPF1 might still be displaced by PABP and NMD would be prevented. However, when this signal is absent, UPF2 and UPF3 will eventually bind UPF1, forming the decay-inducing complex (DECID), which is required for SMG1 to phosphorylate UPF1. UPF1 phosphorylation is believed to definitively commit the mRNA for degradation by NMD, maybe by inducing a conformational change that causes UPF1 to bind the mRNA. Finally, the phosphorylated UPF1 will be bound by the 14-3-3-like phosphoserine-binding domains of SMG5, SMG6 and/or SMG7, ultimately leading to the degradation of the mRNA. Phosphorylation of UPF1 triggers a critical step of translational repression that is required before the messenger ribonucleoprotein particle (mRNP) can be degraded. This step involves an interaction between phosphorylated UPF1 and eIF3 that is part of the 43S ribosomal complex at the initiation codon of an NMD target. This interaction inhibits 60S ribosomal subunit joining to form a translationally

active 80S ribosome and thus further translation initiation events on the mRNP (Chang *et al*, 2007; Mühlemann *et al*, 2008; Schweingruber *et al*, 2013; Popp & Maquat, 2013). Finally, the phosphorylated UPF1 will be bound by the 14-3-3-like phosphoserine-binding domains of SMG5, SMG6 and/or SMG7, ultimately leading to the degradation of the mRNA (Mühlemann *et al*, 2008).

I.2.3. NMD degradation mechanism

Although good progress has been made in the understanding of the PTC-recognition mechanism, little is known about the subsequent degradation of the recognized nonsense mRNA. Current models propose that the factors SMG5, SMG6 and SMG7 are involved in this process through SMG5/SMG7-mediated exonucleolysis and interaction of SMG6 with phospho-UPF1 leads to a SMG6-mediated endonucleolytic cleavage near the aberrant termination site (fig 4) (Nicholson & Mühlemann, 2010; Nicholson *et al*, 2010).

A common concept in metazoan NMD seems to be that phosphorylated UPF1 induces various mRNA decay activities by recruiting decay factors or adaptor proteins for decay complexes through its N- and C-terminal phosphorylation-sites (Schweingruber *et al*, 2013).

During NMD, mRNAs containing a phosphorylated UPF1 are committed to destruction. Subsequent interaction of UPF1 with UPF2 and UPF3 directs SMG1-mediated phosphorylation of UPF1, which in turn recruits SMG5, SMG7 and/or SMG6, which has endonucleolytic activity in its PIN (PiIT N-terminal) domain. Finally, this leads to SMG6-mediated endocleavage near the PTC (Eberle *et al*, 2008). Irreversible endonucleolytic cleavage by SMG6 generates a 5' cleavage product that includes the PTC and a 3' cleavage product that contains the EJC and NMD components. The 5' cleavage product is subject to 3' to 5' decay, possibly by the exosome, or to alternative decay pathways involving degradation from either of the RNA termini (Popp & Maquat, 2013), such as deadenylation-independent, and endonucleolytic cleavage dependent decay (Mühlemann *et al*, 2008).

Irreversible endonucleolytic cleavage by SMG6 generates a 5' cleavage product that includes the PTC and a 3' cleavage product that contains the EJC and NMD components. The 5' cleavage product is subject to 3' to 5' decay, possibly by the exosome. The 3' cleavage product must meanwhile be stripped of its protein components in order to be accessible to nucleases, and this is the job of UPF1. UPF1 activity is normally auto-inhibited by its own N- and C-terminal domains, but when UPF1 binds to UPF2, it undergoes a large conformational change that activates its helicase activity. UPF1 helicase activity disassembles proteins bound to the 3' cleavage product, recycling NMD factors and facilitating 5' to 3' exonucleolytic degradation by the exoribonuclease XRN1 following 5' cap removal by DCP2 initiated by the NMD factors. Phosphorylated UPF1 also recruits SMG5, an adaptor that binds either proline-rich nuclear receptor 2 (PNRC2) or SMG7, each of which in turn recruits activities that result in mRNA decapping followed by 5' to 3' degradation, deadenylation followed by 3' to 5' degradation, or both (Popp & Maquat, 2013; Nagarajan *et al*, 2013). This pathway of deadenylation-dependent RNA decay represents the major decay mechanisms for RNA-turnover in the cytoplasm (Nagarajan *et al*, 2013).

Research carried out in *S. cerevisiae* suggests that nonsense mutated mRNAs are rapidly degraded using the main mRNA-turnover pathway as outlined above with a modification thereof that is typified by deadenylation-independent decapping and XRN1-mediated 5' to 3' exonucleolytic decay. In mammalian cells, studies have shown that nonsense mutated mRNAs can be degraded via the conventional mRNA-turnover pathway, starting with deadenylation, followed by decapping and XRN1-mediated exonucleolytic decay (Nicholson & Mühlemann, 2010; Nicholson *et al*, 2010). Further work is required to determine the relative contributions of the two decay pathways involved in mammalian NMD and to understand what determines which decay route is taken by the different types of mRNAs directed to the NMD pathway (Nicholson & Mühlemann, 2010).

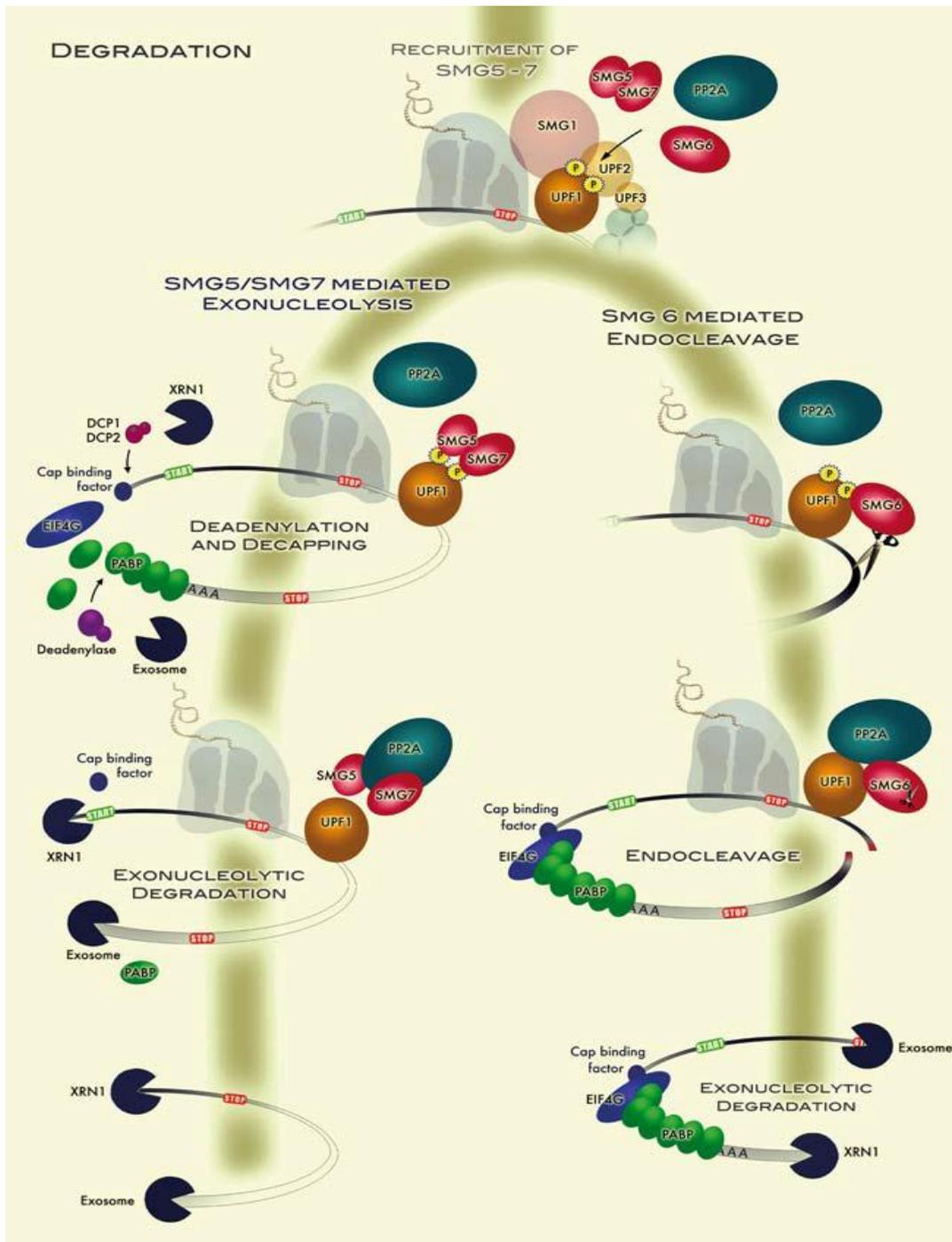


Figure 4: Model for degradation of NMD substrates. The model posits that UPF1-bound mRNAs can be degraded by two different pathways, depending on whether the SMG-5/SMG-7 heterodimer or the endonuclease SMG-6 binds to phosphorylated UPF1. Interaction of SMG-5/SMG-7 with phospho-UPF1 promotes deadenylation followed by decapping and exonucleolytic RNA decay from both ends (left branch). Interaction of SMG-6 with phospho-UPF1 leads to a SMG6-mediated endonucleolytic cleavage near the aberrant termination site, followed by the exonucleolytic degradation of the two RNA fragments from the initial cleavage site (Nicholson *et al*, 2010).

I.2.4. NMD targets

NMD is one of a number of mammalian-cell mRNA decay pathways that is coupled to the translation of its target. Regulators of translation must be considered regulators of NMD (Schoenberg & Maquat, 2012). The discovery that not only the initially identified PTC-containing mRNAs but also many PTC-less mRNAs are targeted by NMD (re-)posed the question, which features render an RNA susceptible to NMD and pointed out our limited understanding of the mechanism of substrate selection (Schweiggruber *et al*, 2013).

Until a few years ago, NMD has been seen merely as a quality control system that rids the cell of faulty mRNAs, but recent studies indicate that NMD represents a much more sophisticated tool serving multiple purposes in gene expression. The population of NMD substrates is not only restricted to faulty transcripts, but also comprises numerous endogenous, physiological transcripts. Among those are: i) mRNAs containing short upstream ORFs (uORFs) where the termination codon of the uORF is likely to be interpreted as PTC, unless the mRNA harbours stabilizing elements nearby; ii) mRNAs encoding selenocysteine-containing proteins where UGA can be recognized as codon for selenocysteine or as PTC, depending on endogenous selenium concentration; iii) mRNAs harbouring introns in their 3' UTR; iv) mRNAs with long 3'UTRs, which, in experimental situations generally make transcripts sensitive to NMD; and v) transposons and retroviruses, likely due to indirect effects of NMD pathway disruption. In addition, pseudo-genes, bicistronic mRNAs, and mRNAs containing signals for programmed frameshifting have been identified as NMD targets in yeast. NMD acts on them because their termination codons can be interpreted as a PTC (Mühlemann *et al*, 2008; Chapin *et al*, 2014).

I.2.5. Implication of NMD in disease

Nonsense mutations account for ~11% of all gene lesions known to cause human inherited disease and ~20% of disease-associated single-base pair substitutions affecting gene coding regions (Mort *et al*, 2008).

There are numerous examples of human diseases associated with mutations that result in PTCs. If translated, the PTC-containing mRNAs would give rise to truncated proteins that have either completely lost their function, are still functional, have acquired dominant-negative function or have gained new functions. As a consequence of these different possibilities, NMD has a double-edged effect on the manifestation of a disease: NMD can act either in a beneficial or in a detrimental way; the former if it prevents the synthesis of toxic truncated proteins and the latter if it prevents the production of proteins with some residual function. Thus, NMD represents a crucial modulator of the clinical outcome of many genetic diseases (Nicholson *et al*, 2010; Nicholson & Mühlemann, 2010).

There are many well-studied examples of human phenotypes resulting from nonsense or frameshift mutations that are modulated by NMD. The phenotypes of genetic diseases are likely to be frequently affected by NMD as PTCs are present in approximately one-third of the human genetic disorders (Khajavi *et al*, 2006).

The majority of PTC-containing disease-associated alleles exert their negative effects due to insufficient production of a functional protein. An example where NMD aggravates the clinical outcome is provided by several disease phenotypes caused by mutations in the dystrophin gene. While most of the truncating mutations in the dystrophin gene are associated with a similar phenotype, the rare truncating mutations that occur near the 3' end of the dystrophin gene can result in extremely variable phenotypes. It has been suggested that all truncated proteins encoded by genes with mutations near the 3' end would in theory be capable of rescuing the Duchene's Muscular Dystrophy phenotype, but when NMD prevents their synthesis, the clinical manifestations of the disease are aggravated. Conversely, NMD has a well-documented beneficial role in the degradation of PTC-containing β -globin mRNA, thereby preventing the synthesis of C-terminally truncated β -globin that would otherwise cause toxic precipitation together with surplus α -globin chains. In a heterozygote context, the second wild-type allele supports almost normal levels of β -globin synthesis, contributing to the correct haemoglobin assembly, which is reflected in the recessive inheritance of this β -thalassaemia type. However, rare NMD-

insensitive PTCs are responsible for the dominant form of β -thalassaemia (Nicholson *et al*, 2010).

I.2.6. β -Thalassaemia as a model disease for studying NMD

β -thalassaemia is one of the most common genetic diseases worldwide (Higgs *et al*, 2012). β -Thalassaemias are a heterogeneous group of inherited human anaemia's characterized by reduced or absent β -globin chain synthesis, resulting in reduced haemoglobin in red blood cells, decreased red blood cells production and anaemia. They are attributed to mutations within or upstream of the β -globin gene. The majority of these mutations are frameshift or nonsense mutations, which are the most prevalent β -globin mutations that cause β -thalassemia, within an exon that have no effect on gene transcription or RNA splicing but result in the premature termination of β -globin mRNA translation (Lim *et al*, 1989; Galanello & Origa, 2010; Peixeiro *et al*, 2011).

The major molecular consequences of stop mutations are the promotion of premature translational termination and NMD. NMD will therefore prevent the production of truncated and faulty proteins, which if failed could result in the synthesis of abnormal proteins that can be toxic to cells through dominant-negative or gain-of-function effects. This nonsense mediated mRNA decay has been found in bacterial, yeast, plant and mammalian cells. It has been proposed that in the human β -globin gene, mutations causing translation premature termination in exons 1 and 2 result in a decrease of the mRNA from the affected allele, causing a 50% reduction of total β -globin chain synthesis in the heterozygote (Romão *et al*, 2000; Salvatori *et al*, 2009a).

In β 39-thalassaemia the CAG (glutamine) codon of the β -globin mRNA is mutated to the UAG stop codon, leading to premature translation termination and to mRNA destabilization through the well-described NMD. Other examples of stop mutations of the β -globin mRNA occur at positions 15, 37 and 127 of the mRNA (Salvatori *et al*, 2009a). In contrast, results obtained by Romão *et al*, 2000 from the study of nonsense-mutated mRNA at codons 5, 15 or 17 indicate that the human β -globin mRNA carrying a nonsense mutation in the 5' half of exon 1 escapes NMD, the AUG-proximity effect.

On the other hand, if a PTC is localized in the last exon of the β -globin gene, the transcripts produced are NMD-insensitive and are translated into truncated proteins with dominant negative effects, as is the case of the stop mutation at position 127 (Mühlemann *et al*, 2008; Salvatori *et al*, 2009a).

The fact that nonsense mutations promote premature translational termination and are the leading cause of up to 30% of inherited diseases, one of them being thalassemia, and given the small size of the β -globin gene and the wide range of nonsense mutations that have been described at this locus make this disease an attractive model for investigating the effects of premature translation termination on mRNA metabolism (Salvatori *et al*, 2009b; Romão *et al*, 2000).

I.2.7. Suppression therapy

For many genetic disorders caused by PTC-generating mutations, there are no effective treatments available. Because NMD plays an important role in modulating the clinical manifestations of such diseases, interfering with NMD represents a promising therapeutic strategy. For those cases where the truncated protein is still functional, inhibiting rapid degradation of the nonsense mRNA would in principle suffice to elevate the protein concentration and ameliorate the condition of patients. However, in most cases, production of the full-length protein would be necessary to restore function, which can be achieved by promoting readthrough of the PTC (Mühlemann *et al*, 2008). When a stop codon enters the ribosomal A-site, the sampling process is initiated just as it does at a sense codon. Near-cognate aminoacyl tRNAs with anticodons that are complementary to two of the three nucleotides of a stop codon can compete with the release factors for A-site binding. Normally, stop codon recognition by the eRF1/3 complex efficiently out-competes near-cognate aminoacyl tRNAs and efficient polypeptide chain release occurs. On occasion, aminoacyl tRNAs that are near-cognate to a stop codon become accommodated in the ribosomal A-site and their amino acid is incorporated into the polypeptide. This process that recodes a stop codon into a sense codon is referred to as a “readthrough” event. PTC readthrough suppresses translation termination and allows translation elongation to continue in the correct reading frame until the normal stop codon is encountered

(Keeling & Bedwell, 2011). Given that the presence of PTCs codons explain one third of all described inherited human diseases (Bhuvanagiri *et al*, 2010), therapeutic strategies aimed at suppressing nonsense codons (so-called nonsense suppression therapies) have the potential to provide a therapeutic benefit for patients with a broad range of genetic diseases (Keeling *et al*, 2014; Keeling & Bedwell, 2011).

The goal of suppression therapy is to enhance the ability of near-cognate aminoacyl tRNAs to compete with the release factor complex for binding PTCs in the ribosomal A site. By increasing the frequency that PTCs are recoded into sense codons, enough full length, functional protein may be restored to provide a therapeutic benefit to patients that carry PTC containing transcripts (Keeling & Bedwell, 2011). In the last few years, it has been demonstrated that drugs can be designed and produced to suppress premature termination, inducing a ribosomal readthrough of PTCs in eukaryotic mRNAs. In order to develop an efficient suppression therapy, aminoglycoside antibiotics, including gentamicin, amikacin, paromomycin, geneticin (G418), lividomycin, tobramycin, and streptomycin have been tested on mRNAs carrying PTCs and shown to suppress disease-causing PTCs in mammalian cells. These drugs bind the decoding centre of the ribosome and decrease the accuracy in the codon-anticodon base-pairing, inducing a ribosomal readthrough of premature termination codons partially restoring protein function to various extents for more than twenty different disease models *in vitro*, and eight different disease models *in vivo* (Mühlemann *et al*, 2008; Salvatori *et al*, 2009a; Salvatori *et al*, 2009b; Keeling & Bedwell, 2011).

However, though these results are promising there are still several obstacles that must be overcome before aminoglycosides can be used long-term in the suppression of nonsense mutations. First, the efficiency of suppressing PTCs is greatly influenced by the identity of the stop codon and the surrounding mRNA sequence. Various aminoglycosides have different abilities to suppress PTCs. This suggests that screening compounds to identify those that best suppress a particular PTC in its natural sequence context is needed. Second, the long-term use of aminoglycosides is limited due to side effects and not all patients respond in the same way to these drugs which could possibly be due to the different efficiencies of NMD, from individual to individual (Keeling & Bedwell, 2011; Welch *et al*, 2007).

I.3. Aims

It has been estimated that about 1.5% of the global population (80 to 90 million people) are carriers of β -thalassemia, with about 60,000 symptomatic individuals born annually, the great majority in the developing world (Galanello & Origa, 2010). The majority of the mutations associated with β -thalassemia are nonsense mutations which result in the premature termination of β -globin mRNA translation and consequently NMD (Galanello & Origa, 2010; Peixeiro *et al*, 2011). Therefore, it would be of great interest that these nonsense mutations in the β -globin gene could be suppressed with the use of drugs such as aminoglycosides. Preliminary results obtained in our lab had shown that the aminoglycoside G418 can suppress a PTC at codon 39 of the human β -globin mRNA, although at low levels in cultured erythroid cells. The aim of the work carried out in this thesis was to further prove that suppression therapy can restore enough β -globin protein and therefore correct the disease manifestations of β -thalassemia. In this regard, it was decided to test whether G418 is able to induce efficient levels of suppression in a dose-dependent and time-course manner in HeLa cells transfected with plasmids containing the wild type (β WT) or β 39 human β -globin genes.

II. Methods

II.1 Plasmid constructs

The plasmids containing β WT (wild type version of the β -globin gene), β 15 (with mutation at codon 15) [CD 15 (TGG \rightarrow TGA)], or β 39 (with mutation at codon 39) [CD 39 (CAG \rightarrow TAG)] human β -globin gene were obtained as previously described in Romão *et al*, 2000. All variants were created within the 428-bp NcoI-BamHI fragment of the β -globin gene template by overlap-extension PCR. Competent *Escherichia coli* were transformed with the plasmid DNA, and transformants were selected on luria-bertani (LB) agar/ampicillin plates. The corresponding plasmid DNAs were purified from overnight cultures of single colonies with the NZYMini prep kit (NZYTech, Portugal) following the manufacturer's instructions. Confirmation of the correct cloned sequences containing the relevant mutation was carried out by automatic sequencing.

II.2 Cell culture and plasmid transfections

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM 1x + GlutaMAX™-I; Gibco® by Life Technologies™, USA) supplemented with 10% (v/v) foetal bovine serum (FBS; Gibco® by Life Technologies™, USA), incubated at 37°C in a humidified atmosphere of 5% CO₂.

Transient transfections were performed using Lipofectamine 2000 Transfection Reagent (Invitrogen® by Life Technologies™, USA), following the manufacturer's instructions, in 35-mm plates containing HeLa cells plated 24h prior to transfection, using 400 ng, of plasmid DNA of each variant (β WT, β 15 and β 39). Twenty-four hours post-transfection, cells were either treated or untreated with G418 (Sigma-Aldrich®, USA). The culture medium was removed and new medium supplemented with 0 μ g/ml, 50 μ g/ml or 200 μ g/ml of G418 (Sigma-Aldrich®, USA) was added. The medium was not changed during the treatment period. Cells were harvested 12h and 24h post treatment with the above mentioned drug by rinsing twice with Phosphate Buffered Saline (PBS) and lysed via solubilisation in Passive Lysis Buffer (PLB; Promega, USA).

II.3 RNA isolation

Total RNA from cultured HeLa cells was isolated using the RNA extraction kit NucleoSpin RNA II (Macherey-Nagel, Germany) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Ambion® by Life Technologies™, USA) and purified by phenol-chloroform extraction.

II.4 Reverse transcription-coupled quantitative PCR (RT-qPCR)

cDNA synthesis was carried out using 2 μ g of total RNA and Reverse Transcriptase (NZYTech, Portugal), according to the manufacturer's instructions. Real-Time quantitative PCR (RT-qPCR) was performed in ABI Prism 7000 Sequence Detection System, using SybrGreen Master Mix (Applied Biosystems® by Life Technologies™, USA). Primers specific for the gene of interest, β -globin (primer forward 5'-GTGGATCCTGAGAACTTCAGGCT-3' and primer reverse 5'-CAGCACACAGACCAGCACGT-3') and for the control, puromycin resistance gene (primer forward 5'-

GGGTCACCGAGCTGCAAGAA-3' and primer reverse 5'-CACACCTTGCCGATGTCGAG-3') were designed. Quantification was performed using the relative standard curve method ($\Delta\Delta C_t$, Applied Biosystems® by Life Technologies™, USA). The following cycling parameters were used: 10 min at 25°C and then 50°C for 30 min and 5 min at 85°C. Technical triplicates from each experiment were assessed in all cases.

II.5. SDS-PAGE and Western blotting

Cells lysates were denatured for 10 minutes at 65°C. Five μ l of SDS sample buffer 5x [Bromophenol blue (0.25%), DTT (dithiothreitol; 0.5 M), Glycerol (50%), SDS (sodium dodecyl sulfate; 10%), Tris-Cl (0.25 M, pH 6.8)] was added to 20 μ l of purified lysates and these were loaded in to a 12% polyacrylamide gel and resolved for 1 hour. After, they were transferred to a PVDF membrane (Bio-Rad, USA) for 1 hour. The membrane was blocked in 5% (w/v) nonfat dry milk for 1 hour and probed using mouse anti- α -tubulin antibody (loading control; Roche, Switzerland) at 1:10 000 dilution and mouse monoclonal anti- β -globin (Santa Cruz Biotechnology, USA) at 1:200 overnight. After incubation with the primary antibody, membranes were washed 3 times in TBS-Tween 20 (Sigma-Aldrich®, USA) 0.05% (v/v) and 0.1% (v/v). Detection was carried out by incubating the membranes for 1 hour with the secondary antibodies, peroxidase-conjugated anti-mouse IgG (Bio-Rad, USA), anti-rabbit IgG (Bio-Rad, USA) antibodies, followed by enhanced chemiluminescence reaction.

Seen as we were unable to detect protein, optimizations had to be made to the protocol. We first altered the dilution of the mouse monoclonal anti- β -globin, the concentration of TBS-Tween 20 was lowered, the washing times were also altered and finally the exposure times were increased. We will further discuss these alterations in point III.2.2.

II.6. Statistical analysis

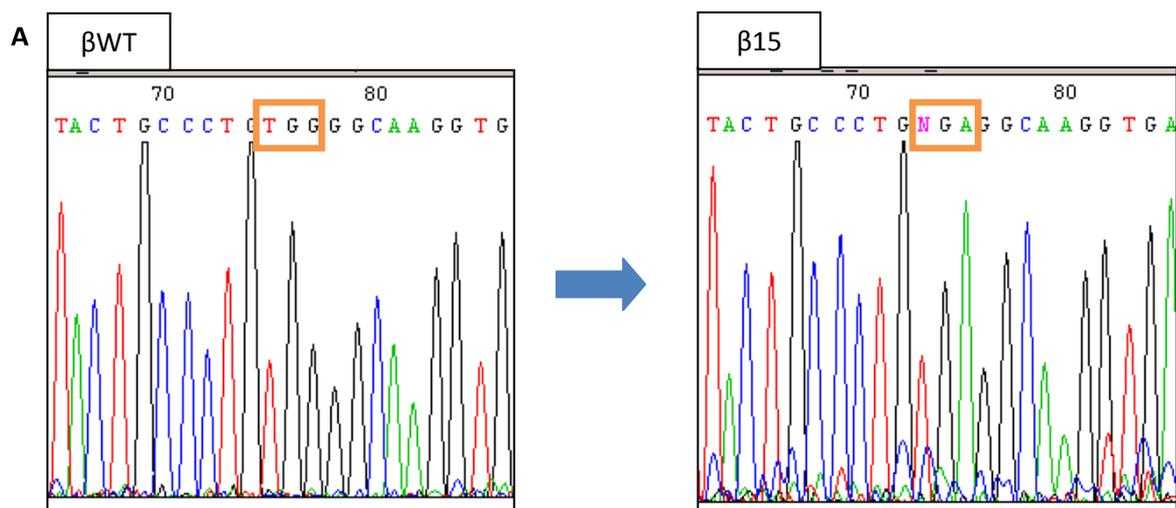
Results are expressed as mean \pm standard deviation of 3 experiments in which the mRNA levels expressed from β 15 and β 39-containing plasmids are normalized to the wild type mRNA levels arbitrarily set to 1. Student's t test was used for estimation of

statistical significance (unpaired, two tails). Significance for statistical analysis was defined as a $p < 0.05$ (Livak & Schmittgen, 2001).

III. Results and Discussion

III.1. Sequence analysis of the plasmid DNAs encoding the β -globin gene

The aim of this study was to prove that suppression therapy by treatment with G418 can restore correct β -globin protein. For this purpose, we chose to transiently express a plasmid encoding the β -globin gene in HeLa cells and check the levels of expression of this gene in the presence or absence of a drug that promotes readthrough of a PTC. For that, we used, alongside the wild type version of the gene, two other versions: one with a nonsense-mutation in codon 15, which is imminent to NMD, due to the AUG-proximity effect (Silva *et al*, 2008); and one version of the gene carrying a nonsense mutation in codon 39, which is typically committed to NMD (Romão *et al*, 2000). In order to check if we were using the plasmids expressing the correct versions of the gene, we sequenced the previously obtained plasmids (Romão *et al*, 2000) to confirm the presence of the mutation in codon 15 and 39 and the correct sequence in wild-type. Sequence analysis of the amplified plasmids encoding the β -globin gene (wild-type, with mutation at codon 15 and with mutation at codon 39) confirmed the presence of a mutation at codon 15 (TGG \rightarrow TGA) or 39 (CAG \rightarrow TAG) (fig 5). In addition, sequence analysis of the β WT gene did not reveal any mutation (fig. 5).



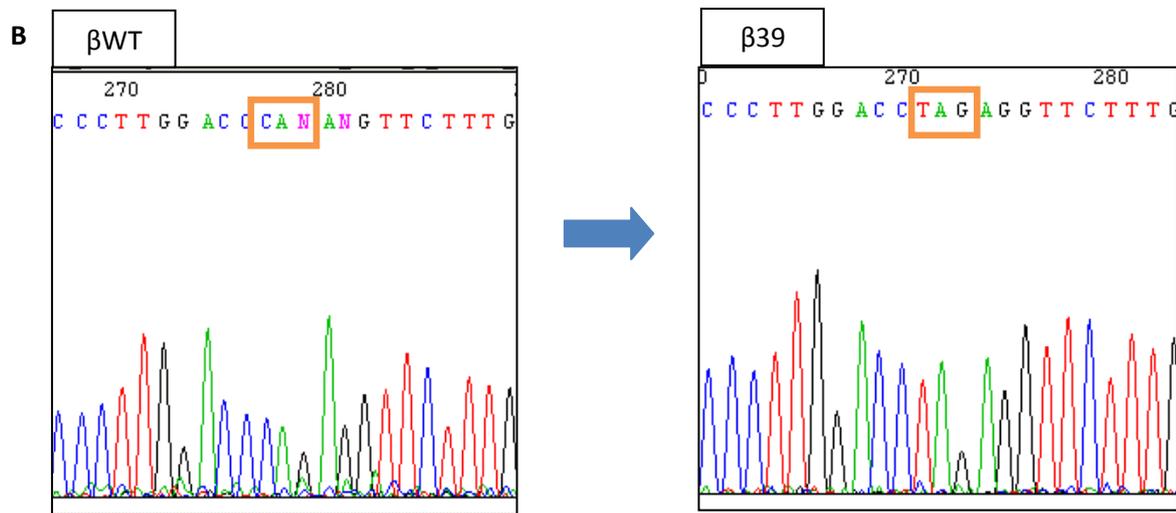


Figure5: Sequencing analysis confirmed the presence of nonsense mutations that originated a stop codon at codons 15 and 39 of the β -globin gene, respectively. The chromatogram of the amplified wild type β -globin gene-containing plasmid DNA is shown on the *left* in order to compare to the sequencing reaction of the amplified plasmid DNA containing the β 15 gene (A) shown on the *right* and the β 39 gene (B) shown also on the *right*. Underlined sequences indicate codon 15 (A) and codon 39 (B). **N** indicates that the algorithm is unable to identify the base. Even though this happens, due to a very low cut-off, it is still possible to identify which base is because the peak is very clear.

III.2. Analysis of β WT, β 15 and β 39 transcripts in transiently transfected HeLa cells

As mentioned above, it has previously been shown that transcripts carrying mutations in exon 1 of the human β -globin gene (namely at codon 15) are expressed at levels approaching those of the wild-type β -globin mRNA in erythroid cells, which means that this AUG-proximal nonsense-mutated gene escapes NMD (Silva *et al*, 2008). On the other hand, those at positions downstream of codon 24, such as β 39 transcripts, are expressed at low levels indicating that they undergo fully efficient decay (Romão *et al*, 2000). As these mutations have been proven to have β -thalassaemia clinical relevance we analysed whether these mutations causing premature translation termination could be suppressed in HeLa cells with the use of aminoglycosides. For that, we transiently transfected HeLa cells with the plasmids carrying the β WT, β 15 or β 39 genes. Twenty-four hours later cells were harvested and protein and RNA were isolated for analysis.

III.2.1. mRNA quantification by RT-qPCR analysis

The relative mRNA levels were quantified by RT-qPCR and normalized to the level of the β WT mRNA (arbitrarily set to 100%) (fig 6). Results show that β 15 relative mRNA level is 70% of the β WT mRNA. In contrast, the level of β 39 mRNA indicates that rapid decay has occurred: 31% of β WT mRNA.

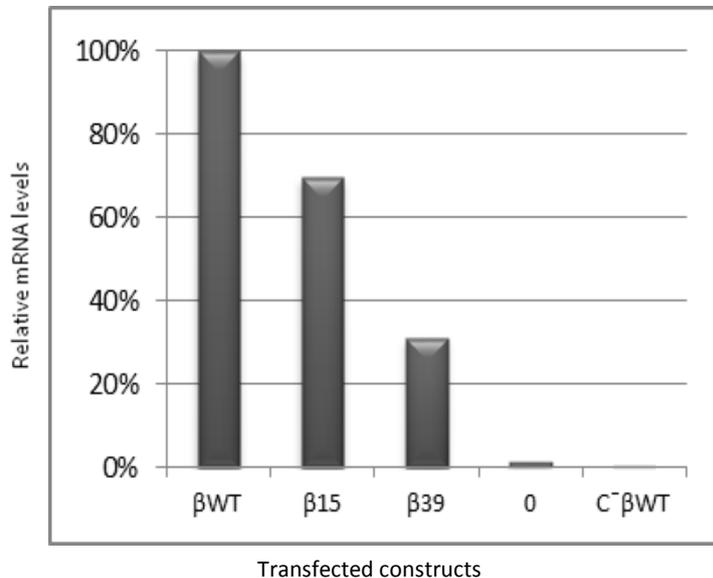


Figure 6: RT-qPCR analysis confirms that the transcript containing the mutation at codon 39 is degraded *via* NMD. (A) Representative RT-qPCR analysis of RNA isolated from untreated HeLa cells transiently transfected with the constructs specified beneath each lane. Resulting mRNA levels were normalized to the expression level of the wild-type mRNA. 0 represents non-transfected HeLa cells which serves as negative control of the transfection efficiency as these cells are not able to express the β -globin gene endogenously. C β WT corresponds to the reverse transcription reaction without the reverse transcriptase enzyme which serves as a control to detect the presence of DNA contamination.

These data were expected as they confirm what was previously reported by Romão *et al*, 2000 and Inácio *et al*, 2004 revealing that the tested β 15 transcript with a PTC located upstream of codon 24 is able to escape NMD, producing mRNA levels similar to that of β WT, whereas the transcript mutated at a position downstream of codon 24, as is the case with β 39, undergoes rapid decay through NMD.

III.2.2. Protein analysis by Western Blot

Western blot analysis of protein samples extracted from the cells transfected with each one of the plasmids was performed in order to evaluate protein levels produced by each transcript (fig 7). As one can observe in figure 5, as far as protein production is concerned, it was unable to detect any production of β -globin protein. This may be as a result of low signal visibility, consequence of low concentration of the antibody or antigen or prolonged washing, and the buffers used may also contribute to the problem. Therefore the conditions in which the Western blot was performed were optimized. Primary antibody dilution was altered from 1:200 to 1:100, increasing its concentration. Incubation time with the primary antibody was increased to intensify the signal. Even though β -globin is not a phosphorylated protein, Bovine Serum Albumine (BSA) was used to see if it produces any significant alteration in the detection of the protein. The stringency of the washing conditions was decreased. Finally, exposure times were increased from 1 s, 30 s, 1 min and 2 min, to 1 s, 1 min, 5 min, 10 min, to achieve an optimum time. After various alterations to our initial protocol (based on manufacturer's instructions) Western blot analysis was performed, yet different conditions were used, we were still unable to detect protein. It was hypothesized that this may be because the conditions in which the cells were kept may somehow cause them some stress inhibiting translation. However, this is not the case since it was able to detect α -tubulin, the loading control, a protein that is produced endogenously. If cells can produce this protein, the translation machinery is working. Also, α -tubulin is cap-dependent translated, so, a priori, no translation factor is missing. It was also hypothesized that the antibody may have not been produced properly. The ideal at this point would be to try a new antibody and if this wouldn't work to try the antibody on a different cell line that produces the β -globin gene endogenously. Due to time and money contingencies, this was not feasible and therefore it was decided to continue the work studying the effect of the drug of choice (G418) only on the mRNA, while trying to understand why the antibody was unable to detect protein. This may possibly indicate some lines of evidence on the effect of the drug at mRNA level and produce some more knowledge regarding this matter.

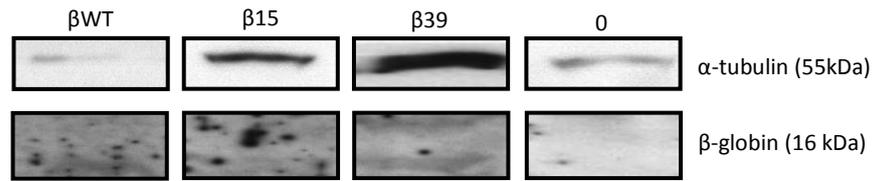
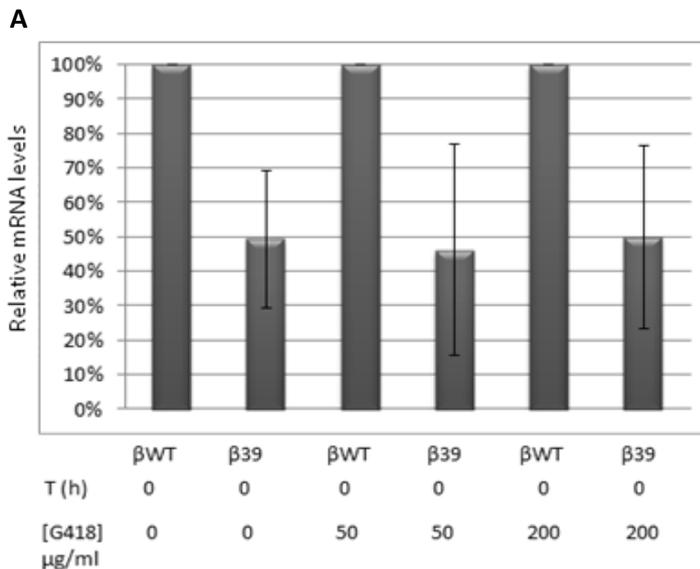


Figure 7: Western blot analysis of cell lysates. The molecular weights of the α -tubulin (55 kDa) and β -globin (16 kDa) proteins are indicated on the right hand side of the autoradiograph. The very faint bands that can be seen in β WT and β 39 are artifacts.

III.3. Analysis of β WT and β 39 transcripts in transiently transfected HeLa cells exposed to geneticin (G418)

We transfected HeLa cells with plasmids expressing β WT and β 39 genes. Twenty-four hours later, cell cultures were either treated or untreated with increasing concentrations (50 μ g/ml or 200 μ g/ml) of G418 and harvested 12 or 24 hours later. The β -globin mRNA levels were quantified by RT-qPCR analysis (fig 8). Results have shown that the relative mRNA level of the β 39 transcript is approximately 40% of the β WT mRNA, which is arbitrarily set to 100% (fig.8A). These data indicate that these nonsense-mutated mRNAs undergo rapid decay, as expected. On the other hand β 39 mRNA levels of G418 treated cells is at similar levels (fig. 8B and C), although there is an increase



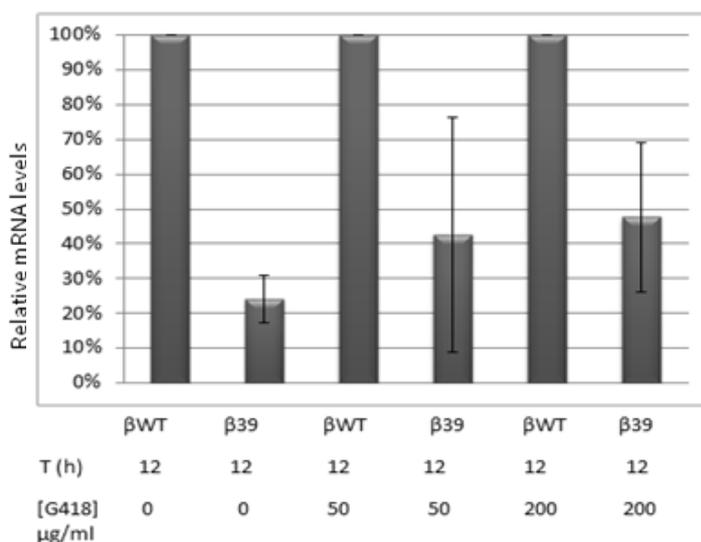
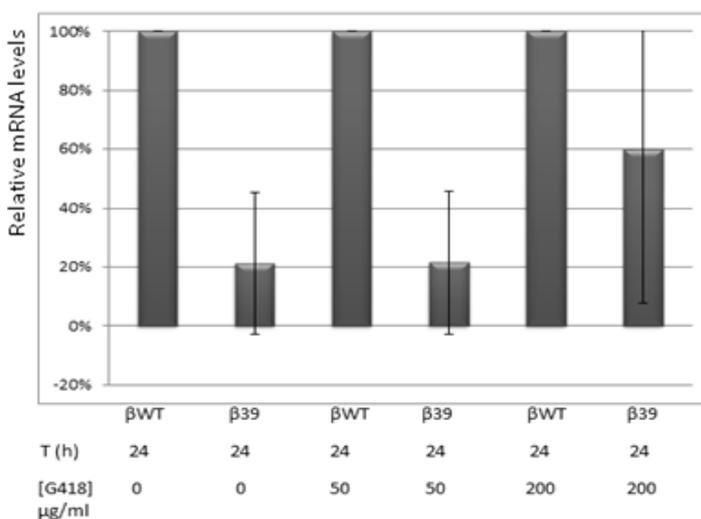
B**C**

Figure 8: RT-qPCR analysis performed on RNA isolated from HeLa cells containing the β WT and β 39 transcripts treated with increasing doses of G418 in a time-course manner. Panel A depicts an untreated control as cells were lysed 24-hours post-transfection and were not treated with any amount of G418. Resulting levels of relative mRNA levels are normalized to the expression level of the wild-type mRNA. Panels B and C depict treated cells with the indicated amounts of G418 for 12 hours and 24 hours, respectively.

of 15% in β 39 mRNA levels in the presence of 50 μ g/ml of G418 and an increase of 25% in the presence of 200 μ g/ml of G418, compared to no treatment conditions (fig. 8 B);

at 24h, there is no change in mRNA levels in the presence of 50 $\mu\text{g}/\text{ml}$ of G418, whereas in the presence of 200 $\mu\text{g}/\text{ml}$ of G418 it is possible to see an increase of 20% compared to control conditions (fig. 8 C). Although we observe an increase in $\beta 39$ mRNA levels upon treatment unfortunately it is not significant. The great standard deviations are due to the fact that the experiments all resulted in very different C_T values. The observed increase may be due to the ability of the aminoglycoside to bind to the decoding centre of the ribosome leading to the reduction of translation fidelity by reducing the proofreading ability of the ribosome and increasing the misincorporation of near-cognate aminoacyl tRNAs into the ribosomal A site at stop codons, resulting in translational misreading at PTCs without premature termination of protein synthesis with concomitant NMD inhibition. However one can not be sure about this seen as it is not possible to see the effects of the drug on protein synthesis. Another possibility could be the ability of the drug to reduce the sensitivity of the NMD machinery to the presence of a PTC, which would be a side-effect of the drug. However it has been shown that this aminoglycoside, and various others, is capable of suppressing disease-causing PTCs in mammalian cells expressing genes carrying these mutations, without altering global protein or mRNA profiles (Keeling & Bedwell, 2011).

IV. Future directions

While the results regarding the steady increase of mRNA levels after treatment with G418 are promising, a previous report describes a promising drug, PTC124 by PTC Therapeutics, Inc., which is able to suppress nonsense mutations by a readthrough activity, as well as, or better than aminoglycosides, at nanomolar concentrations in mammalian cells without toxic side effects. This molecule is administered orally and is expected to be very promising in therapy. PTC124 has no structural similarity to aminoglycosides or other clinically developed drugs and was found to be safer offering therapeutic benefits to many patients (Keeling & Bedwell, 2011; Welch *et al*, 2007; Salvatori *et al*, 2009a). Given that this compound is currently undergoing clinical trials for several diseases (Keeling & Bedwell, 2011) and that thalassemia is a major health problem in developing countries (Salvatori *et al*, 2009a; Galanello & Orita, 2010), it

would be of great interest to test this compound in its ability to suppress PTCs in the β -globin gene in comparison with the effects of G418. It has also been shown that tethering PABPC1 in close proximity of a NMD-competent PTC inhibits NMD (Silva *et al*, 2008; Behm-Ansmant *et al*, 2007). Based on these data we suggest testing if we could increase the efficiency of suppression therapy by transfecting cells with antisense oligonucleotides specific for the β -globin mRNA sequence but with a degenerated poly(A) tail in combination with one of the drugs tested. Each oligonucleotide must have a poly(A) tail long enough to bind at least two PABPC1 molecules which will allow PABPC1 to bind and thus to inhibit NMD. The combination of these two treatments may give a promising and efficient therapy.

V. References

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