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Analysis of the Intranuclear Life of Nonsense Transcripts

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To my parents
and
to my sister

Preface

This thesis is the outgrowth of my four year research training, carried out at Departamento de Genética Humana of the Instituto Nacional de Saúde Dr. Ricardo Jorge, seeking a Ph.D. degree in the Doctoral Program in Biology at Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa.

This Ph.D. thesis provides new insights to a eukaryotic cellular surveillance mechanism, the nonsense-mediated mRNA decay (NMD), which detects and rapidly degrades messenger RNAs harbouring premature translation-termination codons (PTCs). Although mammalian NMD depends on translation, and requires recognition of the PTC by the cytoplasmic ribosomes, the work presented here shows that the presence of a PTC can also affect the nuclear metabolism of the abnormal transcripts. Therefore, this study poses as an additional support to the emerging view that elimination of transcripts harbouring PTCs might comprise several NMD-related surveillance pathways, acting both in the nucleus and in the cytoplasm of mammalian cells.

Part of the results from this Ph.D. thesis were published in a peer-reviewed international scientific journal:

Ana Morgado, Fátima Almeida, Alexandre Teixeira, Ana Luísa Silva, Luísa Romão (2012) Unspliced precursors of NMD-sensitive β-globin transcripts exhibit decreased steady-state levels in erythroid cells. *PLoS ONE* 7(6): e38505. (doi: 10.1371/journal.pone.0038505)

Many individuals and institutions helped me to bring my Ph.D. project to fruition.

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Abstract

Nonsense-mediated mRNA decay (NMD) is a quality control mechanism that detects and rapidly degrades mRNAs carrying premature translation-termination codons (PTCs). Mammalian NMD depends on both splicing and translation, and requires recognition of the premature stop codon by the cytoplasmic ribosomes. Surprisingly, some published data have suggested that nonsense codons may also affect the nuclear metabolism of the nonsense-mutated transcripts. Therefore, we hypothesized that human β-globin transcripts sensitive to NMD could have a singular subcellular localization and processing state in mammalian cells nuclei. To determine if PTCs could influence nuclear events, we have established mouse erythroleukemia (MEL) cell lines stably transfected with wild-type or PTC-containing human β-globin genes. Subsequently, we analyzed the accumulation of NMD-competent β-globin transcripts *versus* wild-type counterparts using two different approaches: visualization of transcripts localization by fluorescence *in situ* hybridization (FISH); and quantification of pre-mRNA steady-state levels by ribonuclease protection assays (RPA) and reverse transcription-coupled quantitative polymerase chain reaction (RT-qPCR).

FISH analysis shows that MEL cells stably expressing PTC-containing β-globin transcripts present a marked tendency to display an abnormal speckled-like pattern of localization in the nucleus. However, in addition to the presence of the PTC, other effectors may act on the β-globin transcripts localization, as some wild-type β-globin MEL cells presented this abnormal FISH phenotype as well. On the other hand, our analyses by RPA and RT-qPCR clearly show that β--globin pre-mRNAs carrying NMD-competent PTCs, but not those containing a NMD-resistant PTC, exhibit a significant decrease in their steady-state levels relatively to the wild-type or to a missense-mutated β-globin pre-mRNA. Conversely, in non-erythroid HeLa cells, human β-globin pre-mRNAs carrying NMD-competent PTCs accumulate at normal levels. Half-life analysis of these pre-mRNAs in MEL cells demonstrate that their low steady-state levels do not reflect significantly lower pre-mRNA stabilities when compared to the normal control. Furthermore, our results also provide evidence that the relative splicing efficiencies of intron 1 and 2 are unaffected.

In conclusion, our set of data highlights potential nuclear pathways that induce a selective downregulation of PTC-containing β-globin pre-mRNA in MEL cells, albeit not affecting their stability or splicing effectiveness. These specialized nuclear pathways, which may act in concert with the general NMD mechanism, might discriminate the NMD-sensitive transcripts as abnormal in a promoter- and/or cell line-specific manner, probably to obtain optimal NMD activity.

Keywords: nonsense-mediated mRNA decay (NMD); mRNA metabolism; nuclear mRNA surveillance; human β-globin pre-mRNA; mouse erythroleukemia (MEL) cells.

Resumo

O mecanismo de decaimento do mRNA mediado por codões *nonsense* (*nonsense-mediated mRNA decay*, NMD) constitui uma via de controlo de qualidade celular que permite a detecção e rápida degradação de transcritos portadores de codões de terminação da tradução prematuros (CTPs). Nas células de mamíferos, o mecanismo de NMD depende dos processos de *splicing* e tradução, ocorrendo o reconhecimento do CTP no citoplasma durante a tradução dos mRNAs processados. Trabalhos publicados por vários autores sugerem que os codões *nonsense* podem afectar igualmente o metabolismo nuclear dos transcritos portadores destas alterações. Desta forma, postulámos a hipótese de que transcritos do gene da β-globina humana sensíveis ao NMD podem apresentar uma localização subcelular e um estado de processamento característicos, no núcleo de células de mamíferos. Para determinar se os CTPs podem influenciar eventos nucleares, foram estabelecidas linhas celulares eritroleucémicas de ratinho (MEL) estavelmente transfectadas com o gene normal da β-globina humana ou com variantes portadoras de CTPs. A localização celular dos respectivos transcritos da β-globina foi analisada através de FISH (*fluorescence in situ hybridization*), e os níveis do pre-mRNA foram quantificados através de ensaios de RPA (*ribonuclease protection assays*) e RT-qPCR (*reverse transcription-coupled quantitative polymerase chain reaction*).

A análise por FISH mostrou que linhas celulares MEL que expressam transcritos da β-globina contendo CTPs apresentam frequentemente um padrão de distribuição anómalo no núcleo. No entanto, para além da presença do codão *nonsense*, outros factores podem afectar a localização dos transcritos da β-globina, visto que este fenótipo foi igualmente observado em alguns clones de células MEL expressando o gene normal da β-globina humana. Por outro lado, a análise quantitativa efectuada nestas linhas demonstra claramente que os níveis de pre-mRNA da β--globina portador de CTPs, sensíveis ao NMD, são significativamente inferiores aos dos transcritos normais, não sendo, no entanto, este efeito observado na linha celular não eritróide HeLa. Curiosamente, a análise dos tempos de meia-vida destes pre-mRNAs demonstram que os níveis reduzidos destes transcritos não reflectem uma estabilidade significativamente reduzida em comparação com o controlo normal. Adicionalmente, os resultados obtidos fornecem evidências de que as eficiências de *splicing* dos intrões não são afectadas.

Em conclusão, o trabalho aqui apresentado evidencia potenciais mecanismos que induzem uma redução selectiva do pre-mRNA da β-globina humana portador de mutações *nonsense* em células eritroleucémicas, sem no entanto, afectar a sua estabilidade ou eficiência de *splicing*. Estes mecanismos nucleares poderão funcionar combinadamente com o mecanismo geral de NMD, actuando especificamente em determinados tecidos e/ou genes, visando um controlo de qualidade celular mais eficaz dos transcritos sensíveis ao NMD.

Palavras-chave: mecanismo de decaimento do mRNA mediado por mutações *nonsense*, metabolismo de mRNA, controlo de qualidade do mRNA no núcleo, pre-mRNA da β-globina humana; células eritroleucémicas de ratinho.

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List of Abbreviations, Acronyms and Symbols

A	adenine
Air1	Arginine methyltransferase-interacting RING finger protein 1
Air2	Arginine methyltransferase-interacting RING finger protein 2
A-site	aminoacyl-site
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pairs
BRCA1	breast cancer 1
BTZ	Barentz
C	cytosine
CAF1	CCR4-associative factor 1
CBC	cap-binding complex
CBP	cap-binding protein
CCR4	carbon catabolite repressor 4
cDNA	mRNA-complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CTD	C-terminal domain
C-terminal	carboxyl-terminal
CTP	cytidine triphosphate
Dbr1	debranching 1
DCP	decapping protein
DCP1	decapping enzyme 1
DCP2	decapping enzyme 2
dCTP	deoxycytosine triphosphate
Dis3	homolog of Schizosacharomyces pombe dis3 (chromosome disjunction)
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Dom34	duplication of multilocus region 34
EDTA	ethylenediaminetetraacetic acid
eEF	eukaryotic translation elongation factor
eIF	eukaryotic translation initiation factor
EJC	exon junction complex
eRF	eukaryotic translation release factor
Esc1	establishes silent chromatin 1

ESE	exonic splicing enhancer
E-site	exit-site
ESS	exonic splicing silencer
F	faraday
FISH	fluorescence *in situ* hybridization
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPx1	glutathione peroxidase 1
GTP	guanosine 5'-triphosphate
GTPase	guanosine triphosphatase
Hba-a1	hemoglobin alpha, adult chain 1
HBB	hemoglobin, beta
Hbs1	Hsp70 subfamily B suppressor
HeLa	line of human epithelial cells derived from a cervical carcinoma
HIV-1	human immunodeficiency virus type 1
hnRNP	heterogeneous nuclear ribonucleoprotein
Hrp1	heterogeneous ribonucleoprotein 1
HS	hypersensitive site
Ig	immunoglobulin
IRES	internal ribosome entry site
kb	kilobase
LCR	locus control region
m^7G	7-methylguanosine
MAGOH	mago-nashi homolog
MEL	mouse erythroleukemia
Met	methionine
Met-$tRNA_i$	methionine-loaded initiator transfer RNA
Mex67	messenger RNA export factor of 67 kDa
Mlp	myosin-like protein
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
Mtr2	mRNA transport 2
Mtr4	mRNA transport 4
MVM	minute virus of mouse
Nab2	nuclear polyadenylated RNA-binding 2
NaCl	sodium chloride
NAS	nonsense-mediated altered splicing
NGD	no-go decay
NIPS	nonsense codon-induced partitioning shift

NLS	nuclear localization signal
NMD	nonsense-mediated mRNA decay
NMTGS	nonsense-mediated transcriptional gene silencing
NMUP	nonsense-mediated upregulation of pre-mRNA
NPC	nuclear pore complex
nt	nucleotides
N-terminal	amino-terminus
Nup60	nuclear pore 60
NXF1	nuclear RNA export factor 1
ORF	open reading frame
P	probability
Pab2	poly(A)-binding protein 2
PABP	poly(A)-binding protein
PABPC1	poly(A)-binding protein, cytoplasmic 1
PABPN1	poly(A)-binding protein, nuclear 1
PAN	poly(A) nuclease
PAN2	PABP-dependent poly(A) nuclease 2
PAN3	PABP-dependent poly(A) nuclease 3
PAP	poly(A) polymerase
Pap2	poly(A) polymerase 2
P-bodies	processing bodies
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PM/Scl-100	polymyositis/scleroderma-100
Pml39	pre-mRNA leakage 39
Poly(A)	polyadenylate
PP2A	protein phosphatase 2A
pre-mRNA	messenger ribonucleic acid precursor
Prp43	pre-mRNA processing 43
P-site	peptidyl-site
PTC	premature translation-termination codon
puro	puromycin
Rai1	Rat1p interacting protein
Rat1	ribonucleic acid trafficking 1
REF	RNA and export factor-binding protein
RNA	ribonucleic acid
RNA Pol II	RNA polymerase II
RNAi	RNA interference

RNase	ribonuclease
RNPS1	RNA-binding protein S1
RPA	ribonuclease protection assay
RPMI	Roswell Park Memorial Institute
Rrp44	ribosomal RNA processing 44
Rrp6	ribosomal RNA processing 6
RT	reverse transcription
RT-qPCR	reverse transcription-coupled quantitative polymerase chain reaction
SC-35	splicing component of 35 kD
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
Ski7	superkiller 7
SMG	suppressor of morphological defects on genitalia
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SR	serine/arginine-rich domain
SSC	saline-sodium citrate buffer
Sub2	suppressor of Brr1-1 2
SURF	SMG1-UPF1-eRF1-eRF3 complex
Swt1	synthetically lethal with trex 1
T	thymidine
THO	suppressor of the transcriptional defects of *Hpr1*Δ by overexpression
Thy1	thymus cell antigen 1, theta
TPI	triosephosphate isomerase
TRAMP	Trf4/Trf5-Air1/Air2-Mtr4 polyadenylation complex
Trf4	Topoisomerase one-related function 4
Trf5	Topoisomerase one-related function 5
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris hydrochloride
tRNA	transfer ribonucleic acid
U	uracil
UAP56	56-kDa U2AF-associated protein
uORF	upstream open reading frame
UPF	up-frameshift
UTP	uridine 5’-triphosphate
UTR	untranslated region
V	volt
v/v	volume per volume
w/v	weight per volume

WT	wild-type
Xrn1	exoribonuclease 1
XRN2	exoribonuclease 2
Yra1	yeast RNA annealing protein 1
α	alpha
α-^{32}P	alpha phosphorus-32
β	beta
β127	human β-globin gene with nonsense mutation at codon 127
β26	human β-globin gene with nonsense mutation at codon 26
β39	human β-globin gene with nonsense mutation at codon 39
β39missense	human β-globin gene with missense mutation at codon 39
β62	human β-globin gene with nonsense mutation at codon 62
βWT	wild-type human β-globin gene
µ	micro

CHAPTER I. General Introduction

I.1. mRNA Biogenesis and Quality Control

I.1.1. Overview of the gene expression steps

Gene expression comprises a series of interconnected events, in which genes are transcribed into messenger RNA (mRNA) and then translated into protein. In eukaryotic cells, a nuclear envelope separates DNA from the protein synthesis machinery, partitioning transcription from translation. During transcription, which occurs in the nucleus, the resulting mRNA precursors (pre-mRNAs) undergo several covalent chemical modifications, including 5'-end capping, splicing, and 3'-end cleavage and polyadenylation. These mRNA processing events take place with the aid of several specific factors (Orphanides and Reinberg, 2002; Moore, 2005; Moore and Proudfoot, 2009). Mature mRNAs are then exported to the cytoplasm, where they can be translated to protein, and ultimately, degraded (Reed, 2003; Dimaano and Ullman, 2004) (Figure I.1).

Eukaryotic mRNA precursors are synthesized by RNA polymerase II (RNA Pol II) in a process comprised of three stages: initiation, elongation and termination. Transcription starts with the assembly of the pre-initiation complex at the promoter of genes. This complex consists of RNA Pol II and several auxiliary proteins known as transcription factors, which recognize and bind to consensus sequences of the promoter located upstream of the start site for transcription (Proudfoot et al., 2002; Luna et al., 2008; Hocine et al., 2010). Also, the activity of the promoters may be greatly increased by enhancer sequences, which can act over distances of several kilobases, located either upstream or downstream of the gene. Transcription factors recruit and position RNA Pol II near the transcription start site and, subsequently, elongation occurs after transition to an RNA Pol II elongation complex. This switch is associated with alterations of chromatin structure and changes of the RNA Pol II C-terminal domain (CTD) phosphorylation state (Hocine et al., 2010). Also, productive transcriptional elongation is tightly coupled to mRNA processing events such as 5'-capping and splicing (Luna et al., 2008; Hocine et al., 2010). RNA Pol II proceeds through the remainder of the gene until conserved polyadenylation signals direct cleavage and polyadenylation at the 3' end of the nascent transcript and transcription termination occurs (Luna et al., 2008).

Capping takes place shortly after transcription initiation in which a 7-methylguanosine (m^7G) cap is added to the 5' end of the emergent transcript (Luna et al., 2008; Hocine et al., 2010). The cap structure helps to confer stability to the transcript, protecting both pre-mRNA and mRNA from cellular 5' to 3' exonucleases. In addition, the cap serves as binding site for two important factors: the cap-binding complex (CBC) in the nucleus; and the eukaryotic translation initiation factor (eIF) 4E in the cytoplasm (Neugebauer, 2002). CBC comprises two subunits, CBP80 and CBP20 (CBP, cap-binding protein), and is required for the subsequent steps of splicing, export, and first round of translation, after which is replaced by eIF4E (Neugebauer, 2002; Luna et al., 2008; Hocine et al., 2010).

In the pre-mRNA processing step of splicing, noncoding intervening sequences, or introns, are removed and coding sequences, or exons, are spliced together by a two-step transesterification reaction catalysed by the spliceosome (Figure I.1). This complex consists of small nuclear ribonucleoproteins (snRNPs) comprising five small nuclear RNAs (snRNAs) - U1, U2, U4, U5 and U6 - in conjunction with a large number of additional proteins, like the Sm ribonucleoproteins, which assembles onto the pre-mRNA (Neugebauer, 2002; Soller, 2006). Formation of the spliceosome complex at particular splice junctions relies on certain sequences, including the 5' splice site, the branch point, a variable stretch of pyrimidines termed polypyrimidine tract, and the 3' splice site (Neugebauer, 2002; Luna et al., 2008; Hocine et al., 2010). Additionally, in higher eukaryotes, flanking pre-mRNA regulatory elements, namely intronic and exonic splicing enhancers or silencers, bind *trans*-acting splicing factors that enhance or repress snRNP recruitment to splice sites. Generally, exonic splicing enhancers (ESEs) are bound by serine/arginine-rich (SR) proteins, whereas exonic splicing silencers (ESSs) are bound by heterogeneous nuclear ribonucleoproteins (hnRNPs) (Hocine et al., 2010). Hence, splice site selection results from the cumulative effect of multiple factors.

Processing at the 3' end of the pre-mRNA involves a cleavage step downstream a conserved polyadenylation signal (AAUAAA sequence). Endonucleolytic cleavage yields a free 3'-hydroxyl group to which a string of adenylic acid residues [poly(A) tail] is added by an enzyme called poly(A) polymerase (PAP). Generally, processive polyadenylation by PAP complexes is followed by rapid decoration of the poly(A) tail by poly(A)-binding proteins (PABPs), which protects the transcripts from 3' to 5' exonucleolytic degradation (Neugebauer, 2002; Hosoda et al., 2006; Luna et al., 2008).

In addition to processing, nascent transcripts must be loaded with specific RNA-binding proteins to form messenger ribonucleoproteins (mRNPs). Several mRNP assembly factors, including the THO complex, mRNA export factors, namely the RNA helicase Sub2p (*Saccharomyces cerevisiae*)/UAP56 (human) and RNA-binding protein Yra1/ALY, the mRNA export receptor, Mex67:Mtr2/NXF1:p15, and hnRNPs are required for the mRNA to be packaged into properly formed mRNPs for export (Moore, 2005; Luna et al., 2008; Rougemaille et al., 2008b; Hocine et al., 2010). These mRNPs are targeted to the nuclear pore complexes (NPCs), which comprise large channels inserted in the nuclear membrane that mediate macromolecular traffic between the nucleus and cytoplasm (Björk and Wieslander, 2011). Afterwards, mRNPs are exported through the NPCs to the cytoplasm, where they undergo remodelling and dissociation to release the mRNAs for protein synthesis (Reed, 2003; Dimaano and Ullman, 2004). Translation of mRNA into protein takes place on large ribonucleoprotein complexes known as ribosomes and is mechanistically similar to transcription (Orphanides and Reinberg, 2002; Kapp and Lorsch, 2004). Starts with the localization of the initiation or start codon (AUG) by eukaryotic translation initiation factors together with ribosome subunits, and further evolves along the mRNA to elongation and termination phases (Orphanides and Reinberg, 2002; Kapp and Lorsch, 2004).

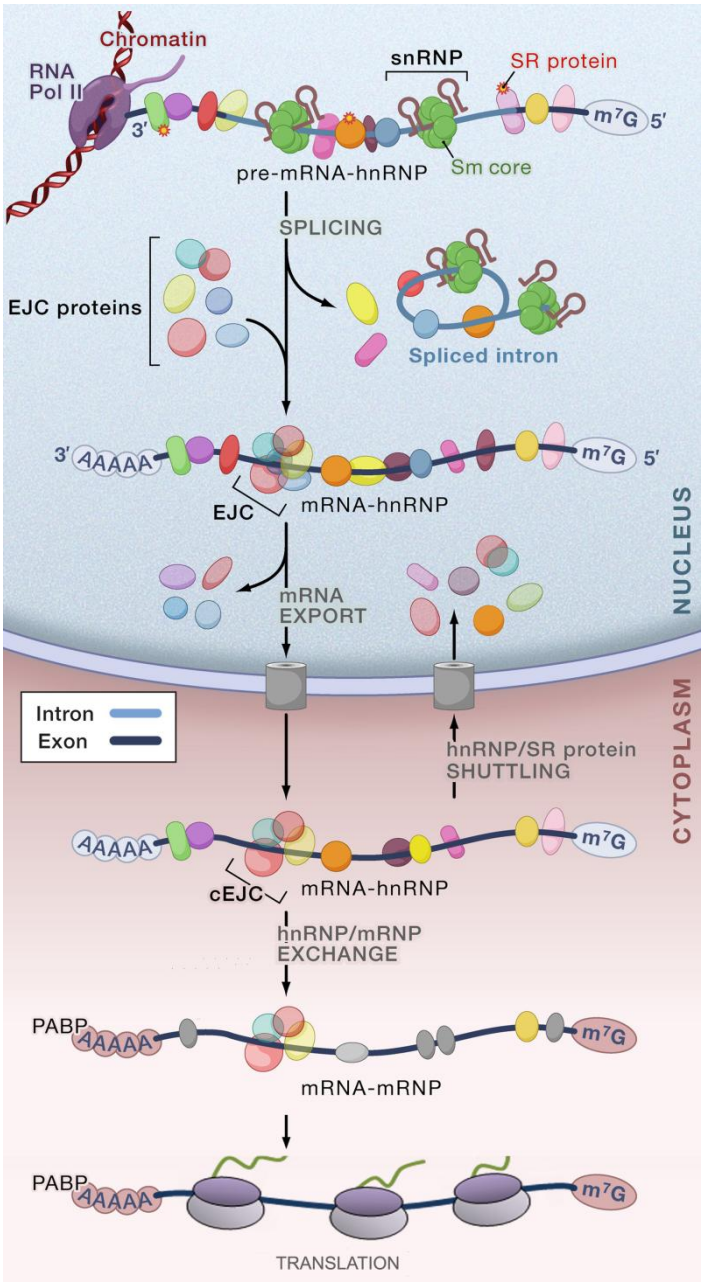

Figure I.1 Overview of the gene expression steps. The nascent transcript (pre-mRNA), capped with a 7-methylguanosine (m^7G) cap, is bound by heterogeneous nuclear ribonucleoproteins (hnRNPs) and SR (serine/arginine-rich domain-containing) proteins. Small nuclear ribonucleoprotein particles (snRNPs) with an extremely stable Sm core make up the spliceosome, which bind to the splice sites at the 5' and 3' ends of introns. The spliceosome catalyzes the removal of introns, which are excised as lariats and subsequently debranched and degraded, and the ligation of the flaking exons. The exon junction complex (EJC) assembles on spliced mRNA about 20 to 25 nucleotides upstream of joined exons, followed by the export of the mRNA to the cytoplasm. Many of the RNA-binding proteins shuttle between the nucleus and the cytoplasm. The poly(A)-binding protein (PABP) binds to the poly(A) tail of cytoplasmic mRNAs. cEJC indicates the remaining stable EJC on mRNA in the cytoplasm, which is removed by translating ribosomes. *Figure adapted from Cooper et al. (2009)*.

Despite the fact that RNA processing and translation occurs in different compartments, several views portray gene expression as an organization of events physically and/or functionally connected, in which different steps are dependent or influenced by one another (Orphanides and Reinberg, 2002). For instance, the RNA polymerase II transcription machinery plays an active role in recruiting the cellular apparatus that caps and processes the nascent RNA transcript. On the other hand, the 5'-cap structure and the poly(A) tail are required to stabilize the mRNA and also play an essential function in translation initiation and termination (Proudfoot et al., 2002). Moreover, several evidences supports that pre-mRNA splicing and mRNP remodelling for export occur co-transcriptionally (Luna et al., 2008; Perales and Bentley, 2009). Likewise, pre-mRNA splicing promotes transcription elongation and is required for efficient export of the processed mRNA into the cytoplasm. Furthermore, the splicing event can also link the nuclear history of the mRNA to its cytoplasmic fate. During splicing, a set of proteins called exon junction complex (EJC) is deposited close to the splice sites (Le Hir et al., 2001b). Once bound, EJCs travel with the mRNA to the cytoplasm where most are removed as a consequence of the translation process (Figure I.1). Prior to their displacement, however, they can act as effectors of almost every aspect of mRNA metabolism, including subcellular mRNA localization, mRNA translational efficiency and mRNA decay (Kuersten and Goodwin, 2005; Moore and Proudfoot, 2009).

I.1.2. mRNA quality control

Considering the multitude of events involved in RNA biogenesis, as well as the series of transitions of RNAs between different complexes of proteins and subcellular compartments, the process of gene expression is susceptible to mistakes. For instance, errors in transcription, nuclear pre-mRNA processing or mRNP maturation can originate abnormal mRNAs which may be translated into deleterious proteins or impair mRNA metabolism and potentially lead to disease (Fasken and Corbett, 2005; Doma and Parker, 2007). In addition, mutations in genes can also give rise to aberrant mRNAs. To prevent these abnormal mRNAs from producing harmful proteins or effects, eukaryotic cells have developed multiple nuclear and cytoplasmic mRNA quality control mechanisms which recognize and degrade aberrant mRNAs (Doma and Parker, 2007; Isken and Maquat, 2007; Schmid and Jensen, 2008a; Fasken and Corbett, 2009; Houseley and Tollervey, 2009). These surveillance pathways generally intervene whether the mRNP production and transport is affected or if mRNP translation is disrupted (Mühlemann and Jensen, 2012). Hence, abnormal mRNAs are directly or indirectly recognized by means of specific factors, which in turn recruit ribonucleases that rapidly degrade the targeted transcripts (Houseley and Tollervey, 2009). Therefore, eukaryotic RNA processing steps and quality control mechanisms are deeply interconnected in order to ensure the fidelity of gene expression. Several events of mRNA processing have been described as checkpoints for mRNA quality control mechanisms, which include mRNA capping, splicing, 3'-end formation and mRNP nuclear exit, in the nucleus, or interaction with ribosomes in the cytoplasm (Isken and Maquat, 2007; Schmid and Jensen, 2008a; Mühlemann and Jensen, 2012).

I.1.2.1. mRNA quality control in the nucleus

Multiple surveillance pathways appear to be active on eukaryotic mRNA within the nucleus, which have been described mostly in the yeast *Saccharomyces cerevisiae* and are likely to be conserved in mammals and other higher eukaryotes (Doma and Parker, 2007; Schmid and Jensen, 2008a; Fasken and Corbett, 2009; Mühlemann and Jensen, 2012). Although the underlying molecular mechanisms are yet to be fully understood, generally, nuclear quality control systems either lead to rapid degradation of aberrant mRNAs or to retention of the targeted RNAs in a nuclear subdomain, in order to trigger subsequent processing or degradation. Specifically, different pathways have been described: (i) rapid RNA degradation in the nucleus, (ii) export to the cytoplasm for degradation, (iii) retention in transcription site-associated foci, (iv) retention at the nuclear pore, and (v) transcriptional downregulation of the genes from which abnormal RNAs are being produced (Doma and Parker, 2007; Schmid and Jensen, 2008a; Fasken and Corbett, 2009). Interestingly, these quality control mechanisms are not triggered exclusively by a single checkpoint or mRNA-processing event (Table I.1).

I.1.2.1.1. Nuclear degradation

This nuclear quality control mechanism targets aberrant transcripts for degradation *via* a few conserved RNA-degrading enzymes or ribonucleases: endonucleases that cut RNA internally, 5' to 3' exonucleases that hydrolyze RNA from the 5' end, and 3' to 5' exonucleases that degrade RNA from the 3' end (Houseley and Tollervey, 2009). In the nucleus, quality control is carried out by the exosome, which comprises an evolutionary conserved multiprotein complex containing two active 3' to 5' exonucleases, Dis3p (also referred to as Rrp44p) and Rrp6p (PM/Scl-100 in humans) (Houseley and Tollervey, 2009; Lykke-Andersen et al., 2011). The nuclear exosome is involved in the degradation of most nuclear RNAs, although Rat1p (XRN2 in humans), a 5' to 3' exonuclease, may also affect nuclear degradation (Doma and Parker, 2007; Schmid and Jensen, 2008a; Fasken and Corbett, 2009). For instance, yeast transcripts failing to receive a proper 5'--cap structure are selectively degraded by Rat1p, which stimulates the activity of the decapping endonuclease Rai1, within a quality control process occurring during transcription elongation (Kim et al., 2004b; West et al., 2004; Jiao et al., 2010; Jimeno-González et al., 2010). Degradation by the 5' to 3' exonuclease Rat1p was also reported to target abnormal transcripts originated from impaired splicing or 3' splice site mutations in yeast, even though the major degradation pathway for these transcripts involved 3' to 5' decay mediated by several exosome components, including Rrp44p and Rrp6p (Bousquet-Antonelli et al., 2000).

Table I.1 Nuclear mRNA quality control.

Organism	*Defect*	*Consequences of quality control*
Yeast	**Capping**	Nuclear decapping and 5' to 3' degradation by Rat1p (Jiao et al., 2010; Jimeno-González et al., 2010)
Yeast	**Splicing:** trapped lariat intermediate	Debranching, export, and cytoplasmic 5' to 3' decay by Xrn1p (Hilleren and Parker, 2003; Mayas et al., 2010) Nuclear decapping and 5' to 3' degradation by Rat1p (Bousquet-Antonelli et al., 2000) Rrp6p and/or core exosome-dependent nuclear degradation (Bousquet-Antonelli et al., 2000)
Yeast	**Splicing:** blocked spliceosome formation / first catalytic step or not recognized by spliceosome	Nuclear decapping and 5' to 3' degradation by Rat1p (Bousquet-Antonelli et al., 2000) Rrp6p and/or core exosome-dependent nuclear degradation (Bousquet-Antonelli et al., 2000; Lemieux et al., 2011) Retention in the nucleus by Mlp proteins near or at the nuclear pore complex (Galy et al., 2004; Palancade et al., 2005; Sommer and Nehrbass, 2005; Schmid and Jensen, 2008a; Fasken and Corbett, 2009; Dieppois and Stutz, 2010)
Yeast	**mRNP assembly:** THO complex / Sub2p / Yra1p mutants **3'-end processing**	Rrp6p and/or core exosome-dependent nuclear degradation and retention near the transcription site (Burkard and Butler, 2000; Hilleren et al., 2001; Jensen et al., 2001; Libri et al., 2002; Torchet et al., 2002; Thomsen et al., 2003; Rougemaille et al., 2007; Assenholt et al., 2008; Saguez et al., 2008) Retention in the nucleus by Mlp proteins near or at the nuclear pore complex (Palancade et al., 2005; Sommer and Nehrbass, 2005; Vinciguerra et al., 2005; Schmid and Jensen, 2008a; Fasken and Corbett, 2009) Transcriptional downregulation (Jensen et al., 2004; Vinciguerra et al., 2005)
Fruit fly	**Splicing:** not recognized by spliceosome	Rrp6p and/or core exosome-dependent nuclear retention near the transcription site (Eberle et al., 2010) Transcriptional downregulation (Eberle et al., 2010)
Mammals	**Splicing:** not recognized by spliceosome absence of introns in a gene that normally contains introns	Accelerated nuclear degradation dependent on 3' poly(A) tail (Conrad et al., 2006)
Mammals	**3'-end processing** **Splicing:** not recognized by spliceossome	Rrp6 and/or core exosome-dependent nuclear retention of RNA near or at the transcription site (Custódio et al., 1999; de Almeida et al., 2010) Transcriptional downregulation (Furger et al., 2002; Damgaard et al., 2008)

Adapted from Doma and Parker (2007).

Several studies suggest that the nuclear exosome is associated with the elongating RNA polymerase II in active genes and functions together with a set of cofactors, which recognize aberrant RNAs by structure or sequence or are loaded onto abnormal mRNAs during defective mRNA processing, and subsequently stimulate the exosome to rapidly degrade such transcripts (Vanacova and Stefl, 2007; Schmid and Jensen, 2008b; Houseley and Tollervey, 2009). There are also evidence that polyadenylation can represent a path to nuclear RNA decay mediated by the exosome. For instance, defects in mRNP assembly may lead to improperly polyadenylated mRNAs with short poly(A) tails, which are recognized and degraded in an Rrp6p-dependent manner (Doma and Parker, 2007; Schmid and Jensen, 2008a; Fasken and Corbett, 2009). Degradative polyadenylation has been mainly associated with the activity of a nuclear exosome cofactor called TRAMP complex, comprised of a noncanonical poly(A) polymerase (Trf4 or Trf5), an RNA-binding protein (Air1 or Air2), and an RNA helicase (Mtr4), which adds short poly(A) tails to aberrant or unstable transcripts, forming a favourable substrate for rapid RNA degradation by the exosome (LaCava et al., 2005; Wyers et al., 2005; Vanacova and Stefl, 2007). Accordingly, inactivation of the TRAMP complex or exosome activities leads to the accumulation of abnormal RNAs originated from impaired splicing or 3'-end processing (Bousquet-Antonelli et al., 2000; Burkard and Butler, 2000; Torchet et al., 2002). In addition, yeast cells harbouring mutations in the THO complex and in the associated RNA helicase Sub2p, which are involved in mRNP assembly and transcription elongation, present rapidly degradation of several abnormal mRNAs *via* a mechanism requiring Rrp6p and the TRAMP complex poly(A) polymerase Trf4 (also known as Pap2) (Libri et al., 2002; Rougemaille et al., 2007; Assenholt et al., 2008; Saguez et al., 2008).

Interestingly, although polyadenylation-mediated exosome degradation *via* the TRAMP complex has been widely associated with nuclear RNA quality control, recent evidences suggest that the nuclear exosome can also target transcripts polyadenylated by canonical poly(A) polymerases. A pre-mRNA nuclear decay pathway, targeting specific polyadenylated intron-containing transcripts in *Schizosaccharomyces pombe* yeast, was shown to involve the nuclear poly(A)-binding protein Pab2 (PABPN1 in humans) and the nuclear exosome subunit Rrp6p (Lemieux et al., 2011).

I.1.2.1.2. Export to the cytoplasm for degradation

Abnormal mRNAs can also be originated during splicing, either because the transcript has escaped the splicing machinery or it has suffered a miss-splicing event, namely the use of an incorrect splice site due to spliceosome disassembly or escape from the proofreading activities promoted by the spliceosome (Egecioglu and Chanfreau, 2011). The escape of unspliced mRNAs from the splicing machinery is common for transcripts containing splicing signal mutations, although it was also reported in endogenous mRNAs containing suboptimal splicing signals (Legrain and Rosbash, 1989; Hilleren and Parker, 2003; Sayani et al., 2008; Mayas et al., 2010). After escaping from the spliceosome or avoiding recognition, the unspliced transcripts can remain in the nucleus or get exported to the cytoplasm (Doma and Parker, 2007).

Unspliced transcripts that have escaped to the cytoplasm could eventually undergo translation,

which would result in the production of non-functional or truncated proteins. Specifically, retention of intronic sequences likely result in the presence of a premature translation-termination codon (PTC) within the transcript. Therefore, unspliced pre-mRNAs are common substrates for the nonsense-mediated mRNA decay (NMD) in the cytoplasm, an surveillance mechanism that relies on translation and targets PTC-containing transcripts for rapid degradation in the cytoplasm (Isken and Maquat, 2007; Nicholson and Mühlemann, 2010). However, another RNA quality pathway targeting yeast aberrant unspliced precursors or splicing intermediates, which undergo export into the cytoplasm for 5' to 3' digestion by Xrn1p, was described to occur independently from NMD (Hilleren and Parker, 2003). This pathway involving aberrantly processed nuclear transcripts is currently poorly understood. The splicing ATPase Prp43p is required for the release of lariat intermediates from the spliceosome and their export into the cytoplasm, where they are subjected to degradation with the aid of the debranching enzyme Dbr1p (Hilleren and Parker, 2003; Mayas et al., 2010).

I.1.2.1.3. Retention in transcription site-associated foci

Another quality control mechanism retains aberrant RNAs within the nucleus. For instance, mRNAs bearing defects in splicing or 3'-end processing are retained in the nucleus of both yeast *Saccharomyces cerevisiae* and fruit fly *Drosophila melanogaster* cells (Hilleren et al., 2001; Jensen et al., 2001; Eberle et al., 2010). Several data suggests that these aberrant RNAs are retained at the transcription site through a process involving the nuclear exosome and Rrp6 (Hilleren et al., 2001; Libri et al., 2002; Thomsen et al., 2003; Rougemaille et al., 2007; Eberle et al., 2010). In addition, other studies linked this pathway with the nuclear surveillance of mRNP assembly. In *Saccharomyces cerevisiae* THO/Sub2 mutants, a fraction of transcripts escapes degradation and is retained in transcription-site-associated foci in an Rrp6-dependent manner (Jensen et al., 2001; Libri et al., 2002; Rougemaille et al., 2007). This could represent a surveillance mechanism that prevents defectively processed or assembled transcripts from being exported to the cytoplasm by tethering them near the gene template. However, the underlying molecular mechanisms remain to be fully characterized. Transcript retention could be due to delayed release of the transcripts from the RNA Pol II complex, active tethering of mRNP to chromatin, or independent binding of gene loci and mRNPs to the same subnuclear domain, namely the NPC (Schmid and Jensen, 2008a). The nuclear exosome and Rrp6p may act either directly, by interacting with the aberrant mRNA and RNA Pol II or chromatin, or indirectly, by shortening the poly(A) tails of nascent transcripts, which would force their retention since appropriate poly(A) tails, presumably coupled to PABPs, are required for expedient release of processed transcripts from sites of transcription (Schmid and Jensen, 2008a). Subsequently, aberrant mRNAs or mRNPs stalled at or near the gene loci could be properly processed instead of being readily degraded by the exosome (Schmid and Jensen, 2008a; Fasken and Corbett, 2009).

I.1.2.1.4. Retention at the nuclear pore complex

The perinuclear quality control mechanism plays a role in recognizing and concentrating correctly processed mRNAs at the nuclear pore for efficient export (Green et al., 2003; Sommer and Nehrbass, 2005). Retention of unspliced RNAs and malformed mRNPs at or near the nuclear pore complex, in order to prevent their escape to the cytoplasm, has been mostly described in *Saccharomyces cerevisiae* (Green et al., 2003; Dziembowski et al., 2004; Galy et al., 2004; Palancade et al., 2005; Vinciguerra et al., 2005; Lewis et al., 2007). This nuclear quality control mechanism comprises several nuclear proteins which retain abnormal mRNAs and mRNPs and potentially degrade them (Sommer and Nehrbass, 2005; Schmid and Jensen, 2008a; Fasken and Corbett, 2009). For instance, the nuclear pore-associated proteins Mlp1p, Mlp2p (Trp in vertebrates) and Pml39p proteins were described as major players in sorting aberrant mRNAs for retention (Galy et al., 2004; Fasken and Corbett, 2009). Additional NPC-associated proteins are involved, such as Esc1p and Nup60p proteins, probably indirectly because they are important for NPC assembly and Mpl1p anchoring, respectively (Fasken and Corbett, 2009). Furthermore, mRNPs may directly interact with Mlp1p *via* the poly(A)-binding protein Nab2, which is implicated in mRNA export and poly(A) tail length control (Fasken and Corbett, 2009; Dieppois and Stutz, 2010). The underlying mechanism still requires further characterization, however it is though that Mlp1p, Mlp2p and Pml39p could recognize and retain abnormal RNAs or RNPs by interacting with mRNA splicing and mRNP assembly factors (Galy et al., 2004). Subsequently, a ribonuclease would be required to rapidly degrade these mRNAs before they can escape this perinuclear surveillance mechanism and reach the cytoplasm (Fasken and Corbett, 2009). Swt1, a *Saccharomyces cerevisiae* endoribonuclease, was identified as a potential player in the degradation of aberrant mRNAs at the nuclear pore. The mRNA products of Swt1 cleavage might then be subjected to further degradation by 5' to 3' exonucleases and the exosome (Fasken and Corbett, 2009; Dieppois and Stutz, 2010). As above mentioned, defects in mRNP assembly can also trigger exosome-dependent accumulation of the mRNA in association with the site of transcription. This linkage of the defective mRNP to the transcription site has been hypothesized to direct the entire locus towards the NPCs (Rougemaille et al., 2008a). Notably, Mlp proteins are present only in sections of the nuclear envelope adjacent to chromatin (Galy et al., 2004), suggesting that they may contact nascent transcripts, thereby linking mRNA synthesis to export and exerting surveillance very early on during mRNP formation (Rougemaille et al., 2008b; Schmid and Jensen, 2008a; Fasken and Corbett, 2009; Dieppois and Stutz, 2010).

I.1.2.1.5. Transcriptional downregulation

Several authors reported that the nuclear pore-associated proteins, Mlp1 and Mlp2, also play a critical role in a nuclear quality control mechanism that possibly feeds back on transcription in *Saccharomyces cerevisiae* (Schmid and Jensen, 2008a). Indeed, the Mlp proteins were found to mediate the retention of intronless mRNP complexes assembled with a mutant Yra1, a

transcription-coupled mRNA export protein, which triggered the transcriptional downregulation of a subset of genes (Vinciguerra et al., 2005). Conversely, the artificial decrease of transcription levels was shown to rescue the effects induced by quality control (i.e., nuclear retention) of mRNP assembly mutants or 3'-end processing mutants (Jensen et al., 2004). Interestingly, a study in *Drosophila melanogaster* cells reported that aberrant RNAs harbouring splice site mutations, which undergo retention at the transcription site involving the nuclear exosome and Rrp6, also show transcriptional impairment of the corresponding gene due to chromatin modifications (Eberle et al., 2010). In overall, a nuclear quality control pathway acting on transcription may be part of a cellular response to provide a favourable environment for proper mRNP formation (Schmid and Jensen, 2008a; Mühlemann and Jensen, 2012).

I.1.2.1.6. Nuclear mRNA quality control in mammals

In higher eukaryotes, and specifically in mammals, research on nuclear mRNA quality control mechanisms is still scarce. Although the high conservation of complexes involved in RNA surveillance, namely the exosome and TRAMP complexes, strongly predicts the existence of similar pathways across species, a specific role of these complexes during mRNA quality control in mammals has yet to be established (Anderson and Wang, 2009; Lykke-Andersen et al., 2011). In addition, some differences could reflect distinct functional properties of the components of surveillance machineries. For instance, Rrp6p (PM/Scl-100) associates only with the nuclear exosome in yeast, whereas in human cells it is present both in the nucleus and in the cytoplasm (Lykke-Andersen et al., 2011).

Another aspect to take into account is the gene expression control and regulation in the context of the nuclear structure. Yeast genes diffuse rapidly throughout the nucleus, and gene expression can be regulated and quality controlled by contacts with components of the NPCs at the nuclear periphery, as mentioned in the previous subchapters. In contrast, although mammalian genes may also interact with the nuclear pores, proportionally, fewer peripheral contacts occur since the nuclear volume is much greater (Cook, 2010). In fact, in mammalian cell nuclei, it is though that active chromatin is unfolded and forms loops of various lengths which may be found inside chromosome territories or at the surface of these territories. Active transcription might occur at the interface between chromatin and interchromatin. Nascent pre-mRNPs become accessible from the interchromatin space, and upon termination of transcription or processing, mRNPs are directly delivered into the interchromatin space (Björk and Wieslander, 2011). The diffusion of mRNPs, however, might be restricted by the chromatin organization and structures inside the interchromatin. Most active higher eukaryotic transcription units reportedly cluster in intranuclear domains, called transcription factories, which may also contain mRNA processing and quality control factors (Cook, 2010). Indeed, mammalian cell nucleus is compartmentalized into interchromatin granule clusters or non-membranous subnuclear domains, namely nucleolus, Cajal bodies, paraspeckles, or nuclear speckles, which regulate key nuclear functions (Mao et al., 2011). For instance, nuclear speckles (also known as SC-35 domains) contain pre-mRNA

processing factors, non-coding RNAs, snRNPs and many of their constituents work in concert to coordinate multiple steps of gene expression, including transcription, pre-mRNA processing and mRNA export (Misteli and Spector, 1998; Smith et al., 1999; Mao et al., 2011). It has been observed that within the interchromatin space, mRNPs move unrestricted in and out of interchromatin granule clusters, suggesting that, in general, mRNPs do not accumulate at specific regions for specific processing or modification steps (Molenaar et al., 2004; Politz et al., 2006). Nonetheless, specific spliced and unspliced RNAs have been described to colocalize at the periphery of these domains (Smith et al., 1999; Shopland et al., 2002; Handwerger and Gall, 2006).

In addition to the increased intricacy of the physical and/or functional organization of gene expression, widespread usage of alternative splice and polyadenylation sites can further complexify mRNP assembly in mammals (Kim et al., 2004a). Such increased complexity is probably met by more elaborate quality control mechanisms, which may also function in the regulation of mRNA metabolism (Schmid and Jensen, 2008a; Mühlemann and Jensen, 2012).

Nevertheless, several evidences support that some nuclear RNA quality control pathways might be conserved from yeast to higher eukaryotes. As in yeast, splicing defective transcripts can be subjected to rapid RNA decay in mammalian nuclei. Human β-globin transcripts harbouring 5' or 3' splice site mutations, or intronless polyadenylated β-globin cRNA, are more rapidly degraded in the nucleus of human cells and accumulate when degradation is inhibited (Conrad et al., 2006). In mammalian cells, defects in mRNA splicing and 3'-end formation can also cause accumulation of aberrant transcripts near the transcription site. For instance, fluorescence *in situ* hybridization (FISH) experiments were performed to visualize the release of wild-type and mutated β-globin RNAs from their DNA template in stably transfected mouse erythroleukemia (MEL) cells (Custódio et al., 1999). In both conditions, β-globin RNAs visualized by FISH accumulate in a single nuclear focus at or near the site of human β-globin gene transcription, probably corresponding to nascent RNA. More importantly, after transcription shut-off the wild-type RNAs are released, whereas pre--mRNAs defective in either splicing or 3'-end formation remain associated with the gene template (Custódio et al., 1999). This supported the concept that efficient pre-mRNA processing is crucial and rate limiting for the release of transcripts from the site of transcription. Using the same reporter gene in MEL cells, a later study show that the CTD of RNA Pol II is directly involved in the retention of mRNAs at the transcription site in a splicing- and cleavage-independent manner (Custódio et al., 2007). Notably, both components of the EJC and the nuclear exosome--associated factor PM/Scl-100 (mammalian ortholog of Rrp6p) were recruited by the retained β--globin mRNAs (Custódio et al., 2007). More recently, another study provided further evidence that a nuclear RNA quality control pathway targets aberrant mRNAs at the transcription site in mammalian cells. Human β-globin transcripts harbouring splicing and 3'-end cleavage defects, stably expressed in human cells, are reported to be retained near the site of transcription in association with RNA Pol II in a Rrp6p (PM/Scl-100)-dependent manner (de Almeida et al., 2010).

Transcriptional downregulation of genes bearing 5' splice site mutations, from which abnormal RNAs are being produced, is also described in mammalian cells. For example, human cells transfected with HIV-1 and β-globin genes containing altered splice donor sequences present impaired transcription of the corresponding nascent transcripts (Furger et al., 2002; Damgaard et al., 2008).

I.1.2.2. mRNA quality control in the cytoplasm

After leaving the nucleus, mammalian mRNPs may be further subjected to several RNA quality control mechanisms in the cytoplasm (Figure I.2). A general feature of these mechanisms comprises the discrimination of aberrant mRNAs from normal mRNAs by adaptor proteins, which interact with the translation apparatus and direct the aberrant mRNAs into a degradation pathway. The main event that triggers the rapid destruction of abnormal mRNAs in the cytoplasm is the failure of ribosomes to terminate translation correctly (Moore, 2005; Doma and Parker, 2007; Isken and Maquat, 2007). For instance, mRNAs with ribosomes stalled at stable secondary structures, or at a stretch of rare codons, are targeted for endonucleolytic cleavage in a surveillance pathway named no-go mRNA decay (NGD) (Doma and Parker, 2006). Aberrant mRNAs lacking an in-frame termination codon, such that translation continues to the end of the poly(A) tail, are targeted for degradation by the cytoplasmic exosome through the action of Ski7p – a paralog of the eukaryotic translation elongation factor (eEF) 1A – and the eukaryotic translation release factor (eRF) 3, within a quality control mechanism referred to as nonstop mRNA decay (NSD) (Frischmeyer et al., 2002; Vasudevan et al., 2002; van Hoof et al., 2002). Recent evidences suggest that NSD and NGD processes are conserved from yeast to mammals and mechanistically related. For instance, translation of the poly(A) tail of a nonstop mRNA generates a polylysine chain that was reported to stall ribosomes by clogging their exit tunnels, which originates a no-go situation (Bengtson and Joazeiro, 2010). Moreover, both NSD and NGD are promoted by two eRF-mimicking proteins: the eRF1 paralog Pelota (Dom34p in yeast) and the eRF3 homologous GTPase Hbs1, which presumably interact with the stalled ribosome (Doma and Parker, 2007; Passos et al., 2009; Bengtson and Joazeiro, 2010; Shoemaker et al., 2010; Pisareva et al., 2011). Another cytoplasmic surveillance pathway, called ribosome extension--mediated mRNA decay pathway (REMD), targets mRNAs when ribosomes inappropriately carry out translation throughout the 3' untranslated region (3' UTR) until another termination codon is encountered within this region. This quality control mechanism was reported to be specific to erythroid cells, triggering the destabilization of human α-globin mRNAs containing an antitermination mutation (Kong and Liebhaber, 2007). Finally, one of the best characterized surveillance mechanism is the nonsense-mediated mRNA decay (NMD), which targets aberrant transcripts containing a premature termination codon for decapping and 5' to 3' degradation by XRN1, endonucleolytic cleavage, and deadenylation and 3' to 5' degradation by the exosome (Isken and Maquat, 2007; Brogna and Wen, 2009; Rebbapragada and Lykke-Andersen, 2009; Nicholson and Mühlemann, 2010). NMD represents a striking example of the extensive coupling

between nuclear and cytoplasmic events in the eukaryotic gene expression process. Transcripts harbouring PTCs are distinguished from normal mRNAs in a process involving specific conserved factors, the UPF (up-frameshit) proteins, and their interactions with both the EJC, deposited during splicing, and the translation termination complex, triggering rapid decay of the aberrant mRNAs (Kashima et al., 2006).

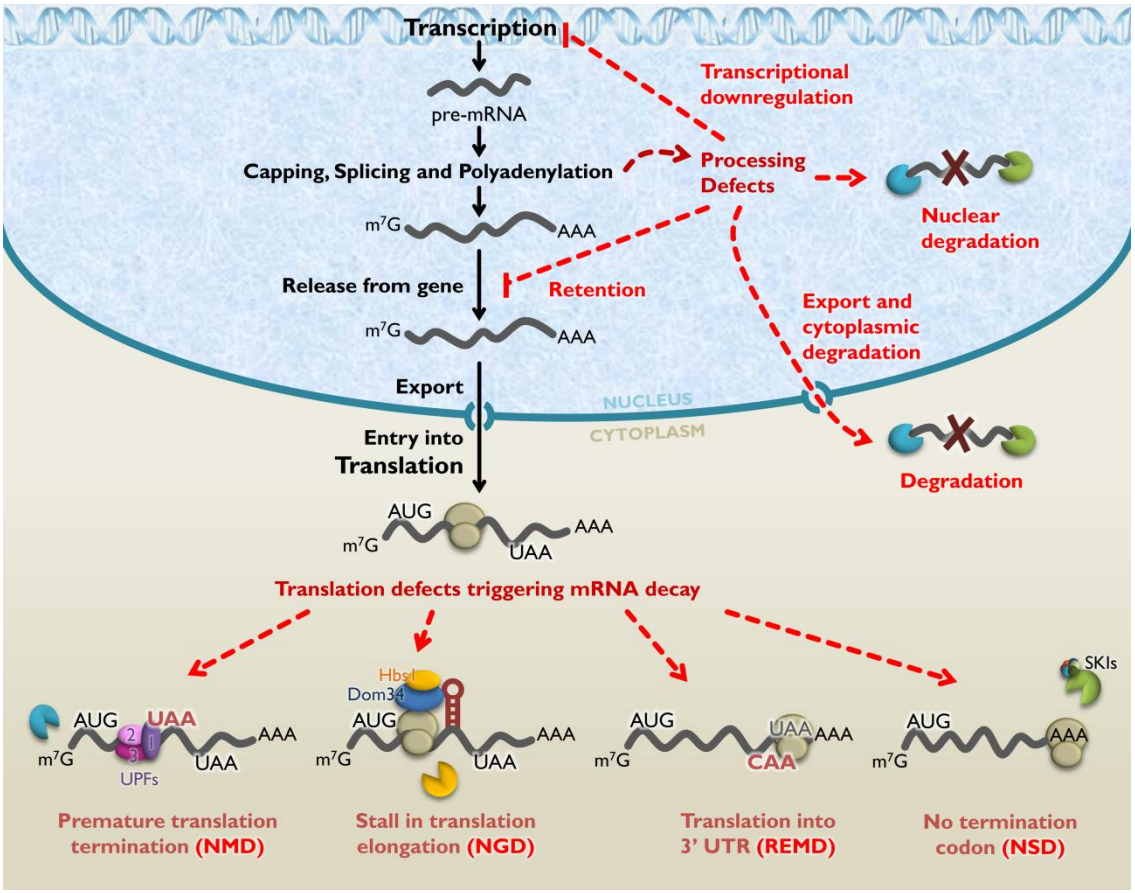

Figure I.2 mRNA quality control systems in eukaryotes. The figure depicts some of the known RNA quality control pathways for aberrant mRNA in eukaryotic cells. Nuclear mRNA quality control mechanisms are summarized in Table I.1. Quality control of cytoplasmic mRNAs include: nonsense--mediated mRNA decay (NMD), triggered by premature translation termination upon recruitment of UPF proteins to the termination complex and resulting in rapid deadenylation, decapping, exonucleolytic decay and endonuclease cleavages; No-go decay (NGD), which results from strong stalls in translation elongation and recruitment of Hbs1p and Dom34p to the stalled elongating ribosome, and involves endonucleolytic cleavage and subsequent exonucleolytic decay; Ribosome extension-mediated decay (REMD), triggered by translation beyond the normal stop codon, into the 3' UTR, and resulting in accelerated deadenylation and decay; and Nonstop decay (NSD), which targets transcripts lacking stop codons and involves the recruitment of exosome by Ski7p and subsequent rapid 3' to 5' degradation. *Adapted from Doma and Parker (2007)*.

I.2. Nonsense-mediated mRNA Decay

I.2.1. NMD targets and functions

Transcripts containing PTCs can arise from different biological processes in germ or somatic cells (Mühlemann et al., 2008). Namely, inherited genetic lesions or errors during replication can cause single base pair substitutions that change a sense codon to an in-frame PTC, commonly known as nonsense mutations. Also, insertion or deletion mutations can alter the ribosomal reading frame causing translating ribosomes to encounter a PTC, or introduce an in-frame PTC. Programmed somatic cell DNA rearrangements and hypermutations that occur in TCR (T-cell receptor) and Ig (immunoglobulin) genes in lymphocytes often generate frameshift mutations and downstream PTCs, which are also targeted to NMD (Li and Wilkinson, 1998). At the RNA level, PTCs can be generated by transcriptional errors and by abnormal pre-mRNA processing. For instance, mutations that alter splicing signals can produce PTCs, frequently due to the retention of intronic sequences containing in-frame nonsense codons (Mendell and Dietz, 2001; Holbrook et al., 2004; Egecioglu and Chanfreau, 2011). Moreover, it is estimated that approximately 60 to 70% of human pre-mRNAs undergo alternative splicing and, among these, 35% are predicted to have at least one splice variant that is expected to be targeted by NMD (Lewis et al., 2003; McGlincy and Smith, 2008).

PTCs can originate two outcomes on gene expression. Firstly, a PTC will terminate mRNA translation prior to completion of a full-length polypeptide, leading to the production of truncated proteins that are often non-functional and/or unstable. Secondly, mRNAs harbouring PTCs are also frequently unstable as they undergo rapid degradation *via* NMD, resulting in a drastic reduction of steady-state mRNA abundance (Maquat, 1995). Therefore, by downregulating mRNAs bearing nonsense codons, NMD prevents the synthesis of C-terminally truncated proteins potentially toxic for the cell (Frischmeyer and Dietz, 1999; Khajavi et al., 2006). As about one third of all known disease-causing mutations originate a nonsense codon, NMD may function as a significant modulator of genetic disease phenotypes in humans (Frischmeyer and Dietz, 1999; Khajavi et al., 2006; Bhuvanagiri et al., 2010; Nicholson et al., 2010).

Moreover, many physiological mRNAs have been recently described as NMD substrates, suggesting an additional role for NMD as a posttranscriptional regulator of gene expression. Several studies in eukaryotes suggest that NMD reduces genomic noise by targeting transcripts from non-functional pseudogenes, transcripts encoded by transposable elements or long terminal repeats, mRNAs containing small upstream open reading frames (uORFs), and transcripts that escaped from nuclear retention. Additional disclosed NMD substrates include transcripts harbouring UGA codons encoding selenocysteine, mRNAs with abnormally extended 3' UTRs, and mRNAs with extra out-of-frame AUGs near the correct AUG (Mendell et al., 2004; Rehwinkel et al., 2006; Bhuvanagiri et al., 2010; Nicholson et al., 2010). Notably, NMD or NMD factors were

also found to play a role in regulating the expression of RNA transcripts involved in several biological processes, namely, stress responses, haemopoietic stem cell development, regulation of alternative splice forms, chromosome structure and function, cell-cycle progression, and embryonic development (Neu-Yilik et al., 2004; Rehwinkel et al., 2006; Bhuvanagiri et al., 2010; Gardner, 2010). NMD therefore could affect a large proportion of the transcriptome, which highlights the importance of this posttranscriptional mechanism in the quality control and regulation of eukaryotic gene expression.

I.2.1.1. NMD implications in disease

The biological and medical significance of the NMD pathway is pointed up by the fact that approximately 30% of all inherited genetic disorders are due to PTCs, as above mentioned, and in many of these cases, NMD influences the severity of the clinical phenotype (Holbrook et al., 2004; Stalder and Mühlemann, 2008). The majority of nonsense-associated diseases are caused either by insufficient levels of functional proteins that can result from degradation of the PTC-containing mRNAs by NMD or by the inability of the abnormally PTC-containing mRNAs, which are able to escape NMD, to generate full-length functional proteins (Lejeune and Maquat, 2005). Nevertheless, NMD can have beneficial effects by eliminating transcripts harbouring PTCs that would otherwise originate C-terminally truncated proteins, either with a complete loss of function or with a dominant-negative function leading to toxicity (Bhuvanagiri et al., 2010). An example of such beneficial effects on disease phenotype is β-thalassemia (Frischmeyer and Dietz, 1999; Holbrook et al., 2004). Beta-thalassemia is a hereditary form of anemia, characterized by the absence or reduction in the synthesis of β-globin polypeptide chains, one of the hemoglobin subunits (Weatherall, 2000). Hemoglobin comprises a tetrameric complex required for oxygen transport, composed of two α- and two β-globin subunits (Huisman, 1993). Defective β-globin production leads to subunit imbalance with an excess of the complementary α-globin chain and subsequent lack of functional hemoglobin in the cell (Weatherall, 2000). This condition can emerge due to the presence of PTCs in the β-globin transcripts (Huisman, 1993). If a PTC is located at a position that activates NMD, the production of truncated proteins is reduced and the possible deleterious effects due to their accumulation are minimized. Indeed, the excess of free α--globin, as well as the limited amount of truncated β-globin protein that can be produced, are proteolytic degraded. As a result, individuals carrying only one affected allele present a clinically asymptomatic phenotype of β-thalassemia trait, whose mode of inheritance is recessive, also known as “thalassemia minor” (Hall and Thein, 1994; Kugler et al., 1995; Holbrook et al., 2004). On the other hand, if a PTC is located at a position that does not induce NMD, such as nonsense mutations in the third exon of the β-globin gene, substantial amounts of abnormal β-globin mRNAs are translated into truncated non-functional β-globin chains, which may overburden the cellular proteolytic system. Subsequent accumulation of these truncated products can often act in a dominant negative manner, leading to deleterious effects on the cell. This condition is correlated with a symptomatic form of the β-thalassemia trait in heterozygotes, named “thalassemia

intermedia", presenting a dominant mode of inheritance (Hall and Thein, 1994; Holbrook et al., 2004; Neu-Yilik and Kulozik, 2008).

There are several other conditions where NMD exerts a protective impact and acts as a modulator of the disease phenotype. Examples of such conditions are the Marfan syndrome, retinal degeneration, von Willebrand disease, myotonia congenita and factor X deficiency (Frischmeyer and Dietz, 1999; Khajavi et al., 2006; Bhuvanagiri et al., 2010). A potential influence of NMD in cancer has also been suggested. In fact, transcripts of several mutant forms of the tumour suppressor proteins genes breast cancer 1 (BRCA1), TP53 and Wilms tumour (WT1) have been shown to be eliminated by NMD. The targeting for degradation of these PTC-containing transcripts, that would convert the tumour suppressors into dominant-negative oncoproteins, protects the heterozygous carriers from developing cancer (Holbrook et al., 2004).

However, it is important to note that NMD can also aggravate the disease phenotype by eliminating mRNAs that would otherwise support the synthesis of partially functional truncated proteins, leading to haploinsufficiency (Khajavi et al., 2006). Examples of such detrimental effect of NMD are Duchenne muscular dystrophy (DMD), cystic fibrosis, Hurler syndrome and X-linked nephrogenic diabetes insipidus (Holbrook et al., 2004). The aggravated clinical picture of protein deficiency induced by NMD is clearly illustrated in DMD, where NMD-insensitive PTCs located near the 3' end of the dystrophin gene result in variable mild phenotypes, whereas PTCs sensitive to NMD are associated with a severe form of DMD (Khajavi et al., 2006). In addition, detrimental effects of the NMD activation were also reported in cancer conditions, namely in hereditary diffuse gastric cancer (HDGC) associated with PTC-causing mutations within the cadherin-1 (CHD1) gene (Bhuvanagiri et al., 2010). In these cases, where NMD has a detrimental effect on the disease phenotype, therapeutics that specifically modulates NMD would be clinically useful (Holbrook et al., 2004; Kuzmiak and Maquat, 2006; Bhuvanagiri et al., 2010). In the last decade, a therapeutic approach named suppression therapy has been developed that utilizes low molecular weight compounds to induce the translation machinery to recode a PTC into a sense codon. Suppression of translation termination at a nonsense codon enables translation elongation to proceed in the correct reading frame, which allows the production of a full-length protein and restore its function (Keeling and Bedwell, 2011).

I.2.2. Molecular basis of the NMD pathway in mammals

NMD has been extensively studied for decades in yeast, worms, fruit fly, plants and mammals, and several models have been proposed depicting different aspects of the NMD pathway, such as nonsense codon recognition or subcellular localization, amongst others (Isken and Maquat, 2007; Brogna and Wen, 2009; Rebbapragada and Lykke-Andersen, 2009; Nicholson and Mühlemann, 2010). In the overall, NMD starts with the recognition and discrimination of the PTC from the natural stop codon within a process dependent on mRNA translation and on highly conserved *trans*-acting factors, namely the UPF1-3 proteins. Subsequently, the molecular players of the

surveillance complex assemble and interact to trigger NMD. UPF proteins form the core complex of the NMD machinery, linking premature translation termination to rapid mRNA degradation *via* specific pathways of decay.

From early studies concerning the destabilization of mRNAs containing PTCs, several evidences suggested that NMD occurs during translation (Maquat, 2004). In relation to mammalian NMD, pharmacological inhibitors of translation, hairpin structures in the 5' UTR (that prevent translational initiation), and expression of suppressor tRNAs (which allow read-through of PTCs), were shown to inhibit NMD and stabilize mammalian transcripts harbouring PTCs (Urlaub et al., 1989; Nishimoto et al., 1991; Belgrader et al., 1993; Cheng et al., 1994). Furthermore, when translation initiation was prevented by the insertion of either a stem-loop structure or an iron--responsive element into the 5' UTR of mammalian transcripts, NMD was as well abrogated (Belgrader et al., 1993; Thermann et al., 1998). Given the important role of translation on NMD pathway, a more detailed description of its mechanism is provided next.

I.2.2.1. Overview of the translation mechanism

The translation process comprises three phases – initiation, elongation and termination. Translation initiation requires at least 11 initiation factors and occurs in two stages: formation of the 48S initiation complex at the initiation or start codon of mRNA, and its joining with a 60S ribosomal subunit, which results in the assembly of a competent 80S ribosome so it proceeds with elongation (Pestova et al., 2007; Sonenberg and Hinnebusch, 2009). On most mammalian mRNAs, the start codon is identified by a scanning mechanism, where the 43S pre-initiation complex binds to the mRNA near the 5' end and scans the 5' UTR for an AUG codon. The 43S pre-initiation complex comprises the small (40S) ribosomal subunit, the initiation factors eIF1, eIF1A, eIF3 and eIF5, together with the so-called ternary complex. The ternary complex consists of the methionine-loaded initiator transfer RNA (Met-tRNA$_i$), which will recognize the initiation AUG codon, and eIF2 coupled to GTP (Gebauer and Hentze, 2004) (Figure I.3, steps 1 and 2). Binding of the 43S pre-initiation complex to the mRNA requires the cooperative action of eIF4F and eIF4B or eIF4H, which unwind the 5' UTR of the mRNA to allow ribosomal attachment. eIF4F is composed by eIF4E (or CBP80:20), that physically binds the m^{7}G cap structure, eIF4A and by eIF4G, which functions as a scaffold protein promoting the assembly of the several factors involved in initiation (Gebauer and Hentze, 2004; Pestova et al., 2007). eIF4G interacts simultaneously with eIF4E, eIF4A, eIF3, and with the 3' end-associated cytoplasmic poly(A)--binding protein (PABPC1) (Jackson et al., 2010) (Figure I.3, step 3). The eIF4G-mediated interaction between eIF4E and PABP is thought to circularize the mRNA, bringing the 3' UTR in close proximity to the 5' end of the mRNA (Wells et al., 1998; Gebauer and Hentze, 2004). The unwinding of the 5' UTR by the ATP-dependent helicase eIF4A, enables binding of the 40S ribosomal subunit. Concomitantly, association of eIF1, eIF1A and eIF3 to the 40S subunit facilitates the binding of the ternary complex eIF2-GTP-Met-tRNA$_i$ (Pestova et al., 2007; Jackson

et al., 2010) (Figure I.3, step 4). The resulting 43S pre-initiation complex can now land next to the cap and scan the mRNA, in a 5' to 3' direction, until encountering the most 5'-proximal AUG start codon in a Kozak consensus sequence, where it forms a 48S initiation complex (Pestova et al., 2007) (Figure I.3, steps 5 and 6). Once the anticodon of the Met-tRNA$_i$ has engaged the start codon, eIF5 triggers the eIF2-bound GTP hydrolysis, resulting in the release of eIF2-GDP and probably of other 40S-bound initiation factors (Gebauer and Hentze, 2004; Jackson et al., 2010). Finally, eIF5B catalyzes the joining of 60S ribosomal subunit (Figure I.3, steps 7 and 8). This event results in the assembly of an 80S ribosome at the initiation codon, and elongation can start to synthesize the polypeptide (Pestova et al., 2007).

In the elongation stage of translation, amino acids are added sequentially to the growing polypeptide chain (Abbott and Proud, 2004). The ribosome has three tRNA-binding sites through which the tRNA substrates progress in a stepwise fashion: the A-(aminoacyl) site, which accepts the incoming aminoacyl-tRNA, the P-(peptidyl) site, which holds the tRNA with the nascent peptide chain, and the E-(exit) site that holds the deacylated tRNA before it leaves the ribosome (Proud, 1994). The elongation process depends on the factor eEF1A, which mediates the delivery of the aminoacyl-tRNA to the A-site of the ribosome where decoding takes place. Following a proofreading step to confirm the proper codon-anticodon interaction, the correct (cognate) aminoacyl-tRNA becomes accommodated into the A-site. The ribosome then catalyses peptide bond formation between the aminoacyl-tRNA and the peptidyl-tRNA bound in the adjacent P-site, resulting in the transfer of the peptide chain to the A-site tRNA. Subsequently, eEF2 catalyses translocation of the peptidyl-tRNA and mRNA from the A- to the P-site, and translocation of the deacylated tRNA from the P- to the E-site. The ribosome is moved along the mRNA such that the next codon is positioned in the A-site, and the elongation process is repeated (Abbott and Proud, 2004; Kapp and Lorsch, 2004).

Translation termination occurs when a termination or stop codon (UAA, UGA or UAG) is encountered in the ribosomal A-site. Subsequently, the finished polypeptide is released from the ribosome upon the hydrolysis of the bond linking the polypeptide chain to the P-site tRNA (Kapp and Lorsch, 2004; Pestova et al., 2007). The peptidyl transferase center of the ribosome is thought to catalyse this hydrolysis reaction, in response to the activity of the eukaryotic release factor eRF1, which recognizes all three stop codons when they are present in the A-site of the stalling ribosome (Frolova et al., 1994, 1996; Zhouravleva et al., 1995). eRF1 forms a complex with the C-terminus of eRF3, which is a GTPase that stimulates the activity of eRF1 in both stop codon recognition and polypeptide release from the ribosome (Zhouravleva et al., 1995; Frolova et al., 1996; Kisselev et al., 2003; Jacobson, 2005). Meanwhile, the N-terminus of eRF3 interacts with the C-terminal domain of PABPC1, which is believed to catalyse proper and efficient ribosome release and translation termination (Kozlov et al., 2001; Hosoda et al., 2003; Mangus et al., 2003). In the final step of translation, the ribosomal subunits are dissociated, releasing the mRNA and deacylated tRNA and setting the stage for other rounds of translation (Kapp and Lorsch, 2004).

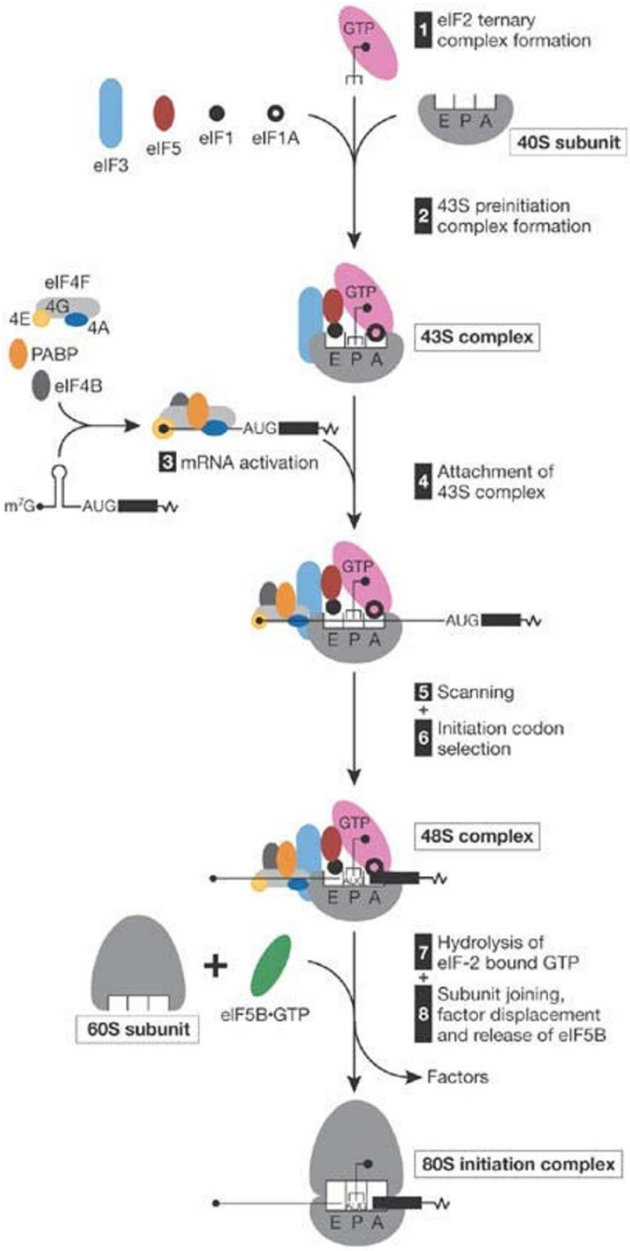

Figure I.3 Simplified model of the translation initiation mechanism. eIF2-GTP/Met-tRNAi^{Met} ternary complex, eIF3, eIF1, eIF1A, eIF5 and a 40S subunit form a 43S pre-initiation complex, which initially binds to the 5' end region of mRNA. The secondary structure of the mRNA is unwound in an ATP--dependent manner by the cooperative action of eIF4A, eIF4B, eIF4F and poly(A)-binding protein (PABP). Subsequently, this complex scans downstream to the initiation codon, where it forms a 48S initiation complex with an established codon-anticodon interaction. Joining of a 60S subunit is mediated by eIF5, which induces hydrolysis of eIF2-bound GTP and partial dissociation of eIF2-GDP from a 40S subunit, and eIF5B, which promotes subunit joining and dissociation of other factors. PABP bound to the 3'-poly(A) tail and recycling of eIF2-GDP by eIF2B are not shown. *Adapted from Holcik and Pestova (2007)*.

The mechanism of translation in mammals therefore congregates the cellular machinery mandatory for the recognition of termination codons, whether or not they are premature. The decision of whether an mRNA will be committed for rapid degradation by the NMD pathway occurs when the ribosome is poised at the termination codon. Hence, the discrimination of PTCs imperatively requires interplay between the translation termination apparatus and specific NMD factors within the premature termination site. Indeed, several studies revealed that the essential NMD factor UPF1 directly interacts with the eRF1:3 complex, providing a link between translation and mRNA quality control (Czaplinski et al., 1998; Bhattacharya et al., 2000; Wang et al., 2001).

I.2.2.2. Essential NMD factors

Trans-acting factors involved in NMD were initially identified in *Saccharomyces cerevisiae* (UPF1, UPF2 and UPF3) and in *Caenorhabditis elegans* (SMG1, SMG5, SMG6 and SMG7, called SMGs for suppressor of morphological defects on genitalia) (Culbertson et al., 1980; Hodgkin et al., 1989; Leeds et al., 1991, 1992; Pulak and Anderson, 1993; He and Jacobson, 1995; Cali et al., 1999; Grimson et al., 2004). The human orthologs were later identified based on sequence similarities (Applequist et al., 1997; Lykke-Andersen et al., 2000; Denning et al., 2001; Yamashita et al., 2001; Anders et al., 2003; Chiu et al., 2003; Ohnishi et al., 2003).

I.2.2.2.1. UPF proteins

The UPF proteins constitute the core NMD machinery, where UPF1 is essential for NMD and hence is the most conserved UPF factor (Culbertson and Leeds, 2003; Conti and Izaurralde, 2005). UPF1 comprises a phosphoprotein with nucleic acid-dependent ATPase and RNA helicase activity (Bhattacharya et al., 2000). In spite of being primarily a cytoplasmic protein, UPF1 has been shown to shuttle between the nucleus and the cytoplasm (Mendell et al., 2002). Similarly to UPF1, UPF2 is also a phosphoprotein and generally acts as an adapter molecule that bridges UPF1 and UPF3 to elicit NMD (Mendell et al., 2000; Kadlec et al., 2004; Kashima et al., 2006; Wittmann et al., 2006). UPF3 is the least conserved of the UPF proteins: mammals have two UPF3 isoforms called UPF3A and UPF3B (also known as UPF3 and UPF3X in humans), whereas *Saccharomyces cerevisiae* and *Caenorhabditis elegans* have only one isoform (Lykke-Andersen et al., 2000; Serin et al., 2001). Both UPF3 proteins are characterized by a conserved domain with some similarity to an RNA recognition motif (RRM) (Lykke-Andersen et al., 2000). Although this kind of domain is commonly involved in RNA binding, neither UPF3A nor UPF3B were shown to bind directly to RNA (Kadlec et al., 2004). Instead, the N-terminal RNA-binding domain of these proteins is responsible for the interaction with UPF2 (Kadlec et al., 2004). In addition, UPF3 proteins are components of the EJC deposited on mRNA during splicing (Le Hir et al., 2001b). Notably, the C-terminal domains from UPF3A and UPF3B, which are involved in the assembly of the complex containing the EJC proteins Y14 and MAGOH (Gehring et al., 2003), are considerably divergent. This divergence explains the weaker ability of UPF3A, compared to

UPF3B, to trigger NMD (Lykke-Andersen et al., 2000; Kunz et al., 2006).

Several observations indicate that UPF1, UPF2 and UPF3 proteins form a trimeric complex (Lykke-Andersen et al., 2000; Serin et al., 2001), and that association of UPF2 and UPF3 to UPF1 stimulates the ATPase and RNA helicase activities of UPF1, triggering NMD (Chamieh et al., 2008). Strikingly, the UPF proteins show a distinct subcellular distribution: UPF1 is primarily distributed throughout the cytoplasm, UPF2 is concentrated in the perinuclear area, whereas UPF3 is predominantly nuclear and shuttles between the nucleus and the cytoplasm (Lykke-Andersen et al., 2000; Mendell et al., 2000; Serin et al., 2001). These findings suggest that UPF3 and UPF2 join the EJC in different subcellular compartments: UPF3A/B is loaded onto mRNAs in the nucleus during splicing *via* interaction with components of the EJC; UPF2 may join the complex soon after cytoplasmic export is initiated; later, at translation termination, UPF1 is thought to be recruited to the termination complex where it binds to the EJC-associated NMD factors, establishing the surveillance complex that induces NMD (Lykke-Andersen et al., 2000). Moreover, UPF1 is recruited to the mRNA *via* the interaction with eRFs and function as a molecular bridge between the terminating ribosome and the downstream EJC-associated UPF2 and UPF3 (Kashima et al., 2006). This association would form an active NMD-complex that triggers rapid mRNA decay (Kashima et al., 2006; Chamieh et al., 2008).

I.2.2.2.2. SMG proteins

According to data obtained in human cells, regulation of the phosphorylation/dephosphorylation state of UPF1, which contributes to the remodelling of the mRNA surveillance complex, is essential for NMD in mammals (Ohnishi et al., 2003). The phosphorylation and dephosphorylation of UPF proteins is mediated by the SMG proteins, whose depletion was found to inhibit NMD (Cali et al., 1999; Chiu et al., 2003; Unterholzner and Izaurralde, 2004). SMG1 is a kinase that catalyses the phosphorylation of UPF1 (Denning et al., 2001; Yamashita et al., 2001; Grimson et al., 2004). Furthermore, SMG5, SMG6 and SMG7 are non-redundant proteins that are involved in the dephosphorylation of UPF1 (Anders et al., 2003; Chiu et al., 2003; Fukuhara et al., 2005). These SMG proteins promote the dephosphorylation of UPF1 by recruiting protein phosphatases such as PP2A (protein phosphatase 2A), which is thought to be the factor responsible for UPF1 dephosphorylation (Chiu et al., 2003; Ohnishi et al., 2003; Conti and Izaurralde, 2005). Two additional proteins, SMG8 and SMG9, have also been shown to bind SMG1 (Yamashita et al., 2009). Both of them suppress SMG1 kinase activity and are components of the NMD-inducing complex – a complex containing the NMD factors, SMG1, UPF1, and the eukaryotic release factors eRF1 and eRF3 (called SURF complex) (Kashima et al., 2006; Yamashita et al., 2009). SMG8 also seems to play an important role in the interaction between the SURF complex and the EJC (Yamashita et al., 2009).

I.2.2.3. Splicing and the NMD rule

A key question in the NMD field is how an mRNA with a PTC is discriminated from an mRNA with a normal stop codon. In mammalian cells, even before the identification of the NMD molecular players, several evidences pointed towards the splicing dependence of NMD. For instance, studies of NMD involving nonsense-mutated β-globin and triosephosphate isomerase (TPI) mRNAs in human cells indicated that NMD might target nucleus-associated mRNAs, whereas the stability of cytoplasmic mRNAs with or without PTCs remain unaltered (Baserga and Benz, 1992; Cheng and Maquat, 1993). Furthermore, nucleus-associated NMD in human TPI mRNA was reported to take place after splicing (Belgrader et al., 1994; Cheng et al., 1994). Subsequently, several reports provided evidence that PTCs only trigger NMD if they are upstream of a functional and spliced intron, and located more than 50 to 54 nucleotides (nt) upstream of the 3'-most intron (Carter et al., 1996; Zhang and Maquat, 1996; Li and Wilkinson, 1998; Nagy and Maquat, 1998; Thermann et al., 1998; Zhang et al., 1998). These findings demonstrated that PTC recognition is dependent on the definition of exon-exon junctions, suggesting the crucial role of mRNA splicing in mammalian NMD. A general rule for the identification of PTCs that trigger NMD was then postulated: if PTCs are located more than 50 to 54 nt upstream of the 3' most exon-exon junction, the mRNA will be subjected to NMD, whereas PTCs located downstream of this region will not be targeted (Figure I.4). It was concluded that the splicing event could leave a landmark on the mRNA, which would later enable the NMD machinery to assess the position of the PTC relatively to the splice junctions.

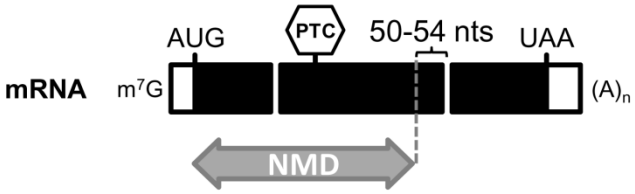

Figure I.4 Splicing and the boundary rule for PTC definition in mammals. Splicing is mediated by the spliceosome and through this process non-coding introns are excised and exons are joined to produce the mature, translatable form of mRNA. In mammals, it has been long observed that premature termination codon (PTC) definition abides by a rule, which links splicing to the mechanism of nonsense--mediated mRNA decay (NMD): only PTCs located more than 50 to 54 nucleotides upstream the last exon--exon junction target mRNA for decay. White boxes: untranslated regions; black boxes: exons; white bars: exon-exon junctions; m^7G: 7-methylguanosine; AUG: initiation codon; UAA: stop codon; $(A)_n$: poly(A) tail.

This hypothesis was later substantiated by the discovery that pre-mRNA splicing deposits multisubunit protein complexes, termed EJCs, about 20 to 25 nucleotides upstream of each exon-exon junction, (Le Hir et al., 2000a, 2000b, 2001b). Assembled EJC comprises at least four conserved core proteins, MAGOH, Y14, eIF4AIII and MLN51 (also known as Barentsz, BTZ), which associate with the mRNA in the nucleus and travels with the mRNP to the cytoplasm (Bono et al., 2006). Additionally, more than a dozen proteins has been identified as peripheral

components of the EJC, including the splicing-related proteins RNPS1, Srm160, Pinin and UAP56, the mRNA export-related proteins ALY/REF and TAP, and mRNA localization factors (Tange et al., 2004). As the EJC is a dynamic structure, these additional factors might associate with the mRNA more transiently, either by assembling in the nucleus but dissociating before mRNA export, or by only binding the EJC during the subsequent processes of mRNA metabolism in the cytoplasm (Le Hir et al., 2000a, 2000b, 2001a, 2001b; Palacios et al., 2004; Tange et al., 2004; Chang et al., 2007b). The EJC assembly appears to be a stepwise process coordinated by splicing, involving a pre-EJC as an intermediate. The pre-EJC, comprising eIF4AIII, which is thought to constitute the RNA-anchoring factor of the EJC (Shibuya et al., 2004), and a Y14:MAGOH heterodimer, is assembled before exon ligation and provides a binding platform for other peripheral EJC components that join later after the release from the spliceosome (Gehring et al., 2009a). On the other hand, the EJC disassembles as it is removed from the mRNAs during the first round of translation by the elongating ribosomes (Dostie and Dreyfuss, 2002; Lejeune et al., 2002; Bono and Gehring, 2011). During this process, the ribosome-associated EJC cofactor PYM mediates EJC disassembly and contributes to the recycling of its components (Gehring et al., 2009b; Bono and Gehring, 2011).

The EJC functions in various post-transcriptional processes, namely translation, mRNA transport and turnover (Nott et al., 2004; Tange et al., 2004), and also provides a direct link between splicing and NMD. Several studies reported that depletion of MAGOH, BTZ and eIF4AIII in mammalian cells leads to defects in NMD (Gehring et al., 2003; Ferraiuolo et al., 2004; Palacios et al., 2004; Shibuya et al., 2004). More importantly, EJC was found to serve as the anchor site for NMD factors UPF2 and UPF3 (Kim et al., 2001; Le Hir et al., 2001b; Tange et al., 2004). A conserved domain in UPF3 associates with Y14 and this interaction is essential for NMD, since NMD proteins UPF2 and UPF3 are thought to bridge the EJC and the translation post-termination complex *via* UPF1 (Gehring et al., 2003; Singh et al., 2007). The interaction between the EJC and UPF1 activates the ATPase-dependent helicase activity of UPF1 and induces the formation of the SURF complex (Kashima et al., 2006; Chamieh et al., 2008).

I.2.3. Models for PTC definition

A key question in the NMD field is how a PTC-containing mRNA is discriminated from an mRNA harbouring a normal stop codon. Several studies pointed up that mammalian NMD is a posttranscriptional surveillance mechanism dependent on splicing and translation, involving a wide range of *trans*-acting factors which recognize PTC-containing transcripts by detecting the presence of RNA-binding proteins downstream of the PTC (Isken and Maquat, 2007; Stalder and Mühlemann, 2008; Brogna and Wen, 2009; Rebbapragda and Lykke-Andersen, 2009; Nicholson *et al*, 2010).

According to the present model for mammalian NMD, this surveillance pathway requires the splicing-dependent deposition of the EJCs that assemble 20 to 25 nt upstream of each exon-exon

junction (Le Hir et al., 2000a; Maquat, 2004). The EJCs, which also serve as anchoring point for the NMD factors UPF2 and UPF3, function as “marks” to discriminate PTCs from natural termination codons (Kim et al., 2001; Le Hir et al., 2001b). Moreover, it has been proposed that PTC recognition and NMD activation occurs during the first round of translation, also called the “pioneer round of translation” (Ishigaki et al., 2001). This model also assumes that during this initial round the elongating ribosomes displace the assembled EJCs from the transcripts (Dostie and Dreyfuss, 2002; Lejeune et al., 2002; Singh and Lykke-Andersen, 2003). If translation termination occurs at a PTC located more than 50 to 54 nt upstream the last exon-exon junction, the ribosome will fail to displace distal EJC(s) associated with the transcript. The retention of one or more competent EJCs will allow the interplay between this complex and the terminating ribosome. When the ribosome reaches the PTC, the translation release factors eRF1 and eRF3 associate with UPF1 and SMG1 (forming the SURF complex). After recognition of the termination codon, SMG1 and UPF1 then interact with the UPF2:UPF3 proteins associated with a downstream retained EJC, and a surveillance complex is assembled (Behm-Ansmant and Izaurralde, 2006). This event results in SMG1-mediated UPF1 phosphorylation and marks the mRNA as PTC-containing (Le Hir et al., 2001b; Gehring et al., 2003; Tange et al., 2004; Kashima et al., 2006; Singh et al., 2007). Subsequently, phosphorylated UPF1 promotes translational repression and elicits rapid mRNA decay, e.g. NMD (Behm-Ansmant and Izaurralde, 2006; Kashima et al., 2006; Isken et al., 2008). On the other hand, if the PTC is located less than 50 to 54 nt upstream or located downstream relatively to the last exon-exon junction, the terminating ribosome reaches the stop codon having displaced all the EJCs present in the transcript (Singh and Lykke-Andersen, 2003). Consequently, NMD is not triggered, a normal termination event occurs and transcripts undergo multiple rounds of translation (Maquat, 2004). Generally, native stop codons are not followed by a downstream EJC and when this occurs, the EJC is usually located less than 50 nt downstream of the stop codon (Nagy and Maquat, 1998). Nevertheless, there are some cases in which an NMD-competent EJC is positioned in the 3’ UTR. Some of these cases represent the so-called NMD-natural targets in which the NMD pathway can play a crucial role in regulating gene expression (Neu-Yilik et al., 2004).

I.2.3.1. The role of the pioneer round of translation

Several mRNP remodelling events occur during the first time that mRNA passes through the ribosome. As mentioned above, elongating ribosomes displace EJCs from the exon-exon junctions during the pioneer round of translation. Subsequently, the nuclear cap-binding complex CBP80:CBP20 bound at the 5’-cap structure is replaced by the eIF4E that directs steady-state rounds of translation (Ishigaki et al., 2001; Maquat, 2004; Isken et al., 2008). Therefore, spliced CBC-bound mRNAs differ from the eIF4E-bound mRNAs in being associated with one or more EJCs. In addition, at the mRNA 3’ end, nuclear poly(A)-binding protein PABPN1 appears to associated only with CBP80-bound mRNAs, whereas cytoplasmic PABPC1 is present in both eIF4E- and CBP80-bound transcripts (Chiu et al., 2004; Kashima et al., 2006). It is unclear when

PABPC1 joins the poly(A) tail during mRNA maturation, however, both shuttling nuclear and cytoplasmic PABPs can be present on the same mRNA molecule in the cytoplasm (Chiu et al., 2004). Nevertheless, the pioneer round of translation promotes the replacement of PABPN1 by PABPC1, which decorates the poly(A) tail of eIF4E-bound mRNAs (Sato and Maquat, 2009). Despite these differences on mRNP composition, there are likely more similarities than differences between CBC-bound and eIF4E-bound mRNAs. Both of these mRNPs include the same translation-associated factors, namely PABPC1, eIF2, eIF3, eIF4A, eIF4B, eIF4G, eRF1 and eRF3 (Lejeune et al., 2002; Chiu et al., 2004; Hosoda et al., 2006; Kashima et al., 2006; Isken et al., 2008), and both support protein synthesis assembling into polysomes (Lejeune et al., 2002; Sato and Maquat, 2009). This indicates that the steady-state translation machinery can as well mediate the translation of CBC-bound transcripts. However, their purpose is different: translation of CBC-bound mRNAs provides a way to RNA quality control; whereas translation of eIF4E-bound mRNAs generates the bulk of cellular proteins (Ishigaki et al., 2001; Maquat, 2004; Isken and Maquat, 2008).

Several studies indicate that NMD is triggered while newly processed mRNA is still bound to the CBC (Ishigaki et al., 2001; Chiu et al., 2004; Hosoda et al., 2005). Indeed, it has been proposed that NMD occurs exclusively during the pioneer round of translation and that transcripts bound to eIF4E are NMD-insensitive (Ishigaki et al., 2001; Lejeune et al., 2002; Chiu et al., 2004; Hosoda et al., 2005). The CBC plays a critical role in NMD, not only because it comprises the mRNP that harbours EJCs, but also, because CBP80 interacts directly with the essential NMD factor UPF1 and promotes the interaction between UPF1 and UPF2 (Hosoda et al., 2005). Recently, it was suggested that interaction of CBP80 with UPF1 promotes NMD in two sequential steps (Hwang and Maquat, 2011). Firstly, CBP80 chaperones the association of SMG1-UPF1 with eRF1-eRF3 at a PTC to form the SURF complex. Secondly, CBP80 physically joins the downstream EJC while still chaperoning SMG1-UPF1, which results in SMG1-mediated UPF1 phosphorylation. Subsequently, phosphorylated UPF1 promotes translational repression and rapid mRNA degradation by NMD (Behm-Ansmant and Izaurralde, 2006; Kashima et al., 2006; Isken et al., 2008) (Figure I.5, A).

I.2.3.2. Surveillance complex assembly and NMD triggering

The decision of whether an mRNA will be targeted or not for degradation by the NMD pathway is made when the ribosome is poised at the termination codon. Early data revealed that, in yeast, the key NMD factor Upf1p also interacts with the translation termination-associated factors eRF1 and eRF3 (Czaplinski et al., 1998) and evidence for this interaction in mammalian cells arose from *in vitro* experiments (Wang et al., 2001). Indeed, it was shown by co-immunoprecipitation analysis in human cell extracts that UPF1 interacts with SMG1 and the release factors eRF1 and eRF3 to form the SURF complex, thus implicating UPF1 in translation termination (Kashima et al., 2006). The fact that SMG1 and UPF1 interact with eRF1:eRF3 as well with the EJC components

suggests the formation of a decay-inducing complex (DECID) that is thought to trigger UPF1 phosphorylation and dissociation of the release factors. However, the same study showed that SURF is formed independently of its interaction with the EJC, which supports that SURF assembles on the terminating ribosome before it interacts with UPF2:EJC complex (Kashima et al., 2006). Therefore, and according to current NMD models, when the ribosome is poised at a PTC located upstream of an EJC, SMG1, chaperoned by SMG8 and SMG9, will interact with UPF1 and translation termination factors to form the SMG1:UPF1:eRF1:eRF3 complex (SURF), which associates with the ribosome on the mRNP (Yamashita et al., 2005b, 2009). The association of ribosome:SURF with the distal EJC forms the DECID complex, which induces SMG1-mediated UPF1 phosphorylation, distinguishing a PTC from a normal termination codon (Yamashita et al., 2001, 2005b, 2009; Kashima et al., 2006). UPF1 phosphorylation precludes additional ribosome loading, as phosphorylated UPF1 binds to the eIF3 constituent of the 43S pre-initiation complex that is poised at the translation initiation codon and inhibits 60S subunit joining, thereby eliciting translational repression (Isken et al., 2008) (Figure I.5, A).

SMG1-mediated UPF1 phosphorylation also promotes mRNP remodelling which is essential for NMD, since it results in a PTC-containing mRNA physically accessible to degradation activities (Ohnishi et al., 2003; Behm-Ansmant and Izaurralde, 2006; Kashima et al., 2006). Messenger RNP remodelling involves the disassembly of the DECID complex (ribosome:SURF:EJC) mediated by the sequential phosphorylation and dephosphorylation of UPF1. SMG1-mediated UPF1 phosphorylation creates binding platforms for the SMG5, SMG6 and SMG7 factors that are involved in the dephosphorylation of UPF1, probably through the recruitment of phosphatase PP2A (Anders et al., 2003; Chiu et al., 2003; Ohnishi et al., 2003; Kashima et al., 2006). The majority of SMG5 and SMG7 forms a complex which binds to phosphorylated UPF1 and therefore induces UPF2 dissociation from UPF1 (Ohnishi et al., 2003). This SMG5:SMG7 complex will then promote the dissociation of the ribosome and eRFs from the DECID complex, whereas the binding of SMG6 will promote UPF1 dissociation from the mRNA (Okada-Katsuhata et al., 2012). Therefore, sequential phosphorylation and dephosphorylation of UPF1 by SMG1 and PP2A, respectively, contribute to recycling of the ribosome, release factors and NMD factors. In addition, the phospho-specific binding of SMG6 and SMG7 to UPF1 is thought to be required for NMD triggering, since SMG6 and SMG7 might mediate the recruitment of the mRNA decay machinery. SMG6 have been shown to promote endonucleolytic cleavage of PTC-containing mRNAs, as catalytic inactive SMG6 fails to support NMD in mammalian cells (Glavan et al., 2006; Huntzinger et al., 2008; Eberle et al., 2009b). On the other hand, tethering of SMG7 at either the 3'- or 5'-UTR of mRNAs induces mRNA decay dependent on decapping and 5' to 3' exonucleolytic activities (Unterholzner and Izaurralde, 2004) (Figure I.5, A).

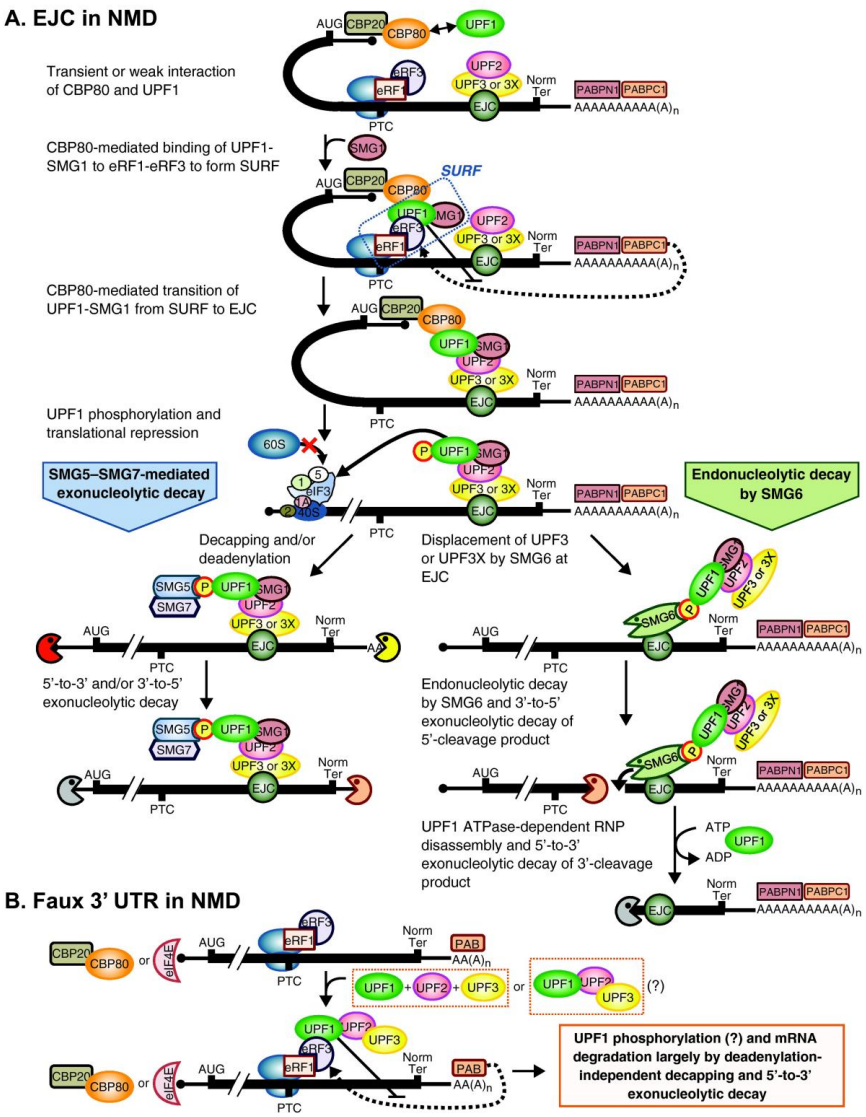

Figure I.5 Mammalian NMD models. (A) EJC function in NMD. NMD is a consequence of PTC recognition during the pioneer round of translation of newly synthesized mRNA bound by the cap-binding complex comprised of the CBP80:CBP20 heterodimer. As mRNA derived from splicing, at least one exon--exon junction complex (EJC) is situated about 20 to 25 nucleotides upstream of such a junction. The direct, although weak or transient interaction of CBP80 with the essential NMD factor UPF1 promotes at least two steps during NMD. Firstly, promotes the joining of UPF1 and its kinase SMG1 to eRF1 and eRF3 at a PTC to form the SURF complex. During NMD, this step is thought to compete effectively with joining of the poly(A)--binding protein C1 (PABPC1) to eRF3, the latter of which is specified as a dotted line. Secondly, CBP80 promotes the joining of UPF1 and SMG1, presumably from SURF, to a downstream EJC, which leads to UPF1 phosphorylation by SMG1. Phosphorylated UPF1 binds to the eIF3 constituent of the 43S pre-initiation complex that is poised at the translation initiation codon and inhibits 60S subunit joining, thereby eliciting translational repression. SMG5 and SMG7 form a complex with phosphorylated UPF1, as does SMG6. It is thought that SMG5:SMG7-mediated NMD leads to deadenylation and/or decapping followed, respectively, by exosome-mediated 3' to 5' and XRN1-mediated 5' to 3' exonucleolytic activities. An alternative or additional mRNA degradation pathway involves SMG6, whose binding to hyperphosphorylated UPF1 leads to SMG6-mediated endonucleolytic cleavage of the NMD substrate, resulting into 5'- and 3'-cleavage products. Activation of the RNA-dependent ATPase activity of UPF1 subsequently results in the XRN1--mediated 5' to 3' decay of the 3' fragment. **(B)** Faux 3' UTR model in NMD. When a ribosome reaches a PTC that is situated abnormally upstream of the poly(A) tail, UPF1 can effectively compete with PABPC1 for association with eRF3 so as to trigger NMD. *Adapted from Hwang and Maquat (2011).*

I.2.3.3. Alternative NMD pathways in mammals

Although the conserved core of the NMD machinery has been considered to rely on the trimeric complex formed by UPF1, UPF2 and UPF3, some studies indicate that NMD consists of several alternative branches, regulating different subsets of transcripts. The existence of an UPF2--independent branch of the NMD pathway was first suggested when, using tethering assays, UPF3B mutants lacking the domain of interaction with UPF2 were shown to be still able to elicit NMD (Gehring et al., 2003). Further studies provided evidence that EJCs might have two alternative compositions, both capable of elicit the NMD pathway in human cells. Tethering assays suggested the existence of a UPF2-independent branch that requires the EJC components Y14, MAGOH, eIF4AIII and UPF3B, and is insensitive to RNPS1 and UPF2 depletion; and of a UPF2-dependent branch that requires RNPS1 and UPF2, but is not affected by the depletion of other EJC components (Gehring et al., 2005). Still, both branches involve UPF3B and are UPF1-dependent. Furthermore, a UPF3-independent branch was identified in a study where the fate of PTC-containing TCR-β mRNAs was unaffected by depletion of UPF3B, UPF3A or both (Chan et al., 2007).

Although the above mentioned alternative branches of the NMD pathway diverge in their requirement for UPF2, UPF3B and EJC components, the dependence on UPF1 is a shared feature. The existence of these alternative branches possibly influence the NMD efficiency and may reflect transcript- or cell type-specific processes for the regulation of subsets of cellular NMD targets (Huang et al., 2011).

Strikingly, the NMD model for PTC recognition involving the EJC has been challenged by reports that NMD occurs even in the absence of a downstream EJC in mammals (Zhang et al., 1998; Bühler et al., 2006; Eberle et al., 2008; Singh et al., 2008). An alternative model for PTC recognition invokes a “faux 3’ UTR” (Figure I.5, B).

I.2.3.4. An evolutionary conserved 3’ UTR model for PTC recognition

The faux 3’ UTR model postulates that rapid degradation of mRNA *via* NMD is triggered by the intrinsically aberrant nature of premature translation termination at a PTC, which impairs the interaction between the terminating ribosome and factors associated with the poly(A) tail that promote proper translation termination (Amrani et al., 2004, 2006). Therefore, PTC definition of this EJC-independent NMD pathway seems rather to rely on the 3’ UTR length and on the distance between the PTC and the poly(A) tail – comprising the so-called faux 3’ UTR. Consistent with the faux 3’ UTR model for PTC recognition, deletions that eliminate most of the sequence downstream of a PTC abolish NMD (Bühler et al., 2006). Furthermore, mRNAs with a long 3’ UTR were identified as being NMD substrates in *Saccharomyces cerevisiae*, *Caenorhabditis elegans, Drosophila melanogaster*, *Arabidopsis thaliana* and humans (Muhlrad and Parker, 1999; Gatfield

et al., 2003; Kertész et al., 2006; Behm-Ansmant et al., 2007a; Longman et al., 2007; Eberle et al., 2008; Singh et al., 2008). On the other hand, NMD of PTC-containing reporter transcripts expressed in humans cells was suppressed by bringing the poly(A) tail into the vicinity of the PTC, showing that the physical distance between a PTC and the poly(A) tail is a crucial determinant for NMD (Eberle et al., 2008). Notably, several studies in *Drosophila melanogaster* and humans demonstrated that tethering PABPC1 downstream of, but close to, a PTC rescues the stability of the mRNA (Amrani et al., 2004; Behm-Ansmant et al., 2007a; Silva et al., 2008). Based on these findings, also previously observed in yeast, a conserved model for PTC recognition that extends the faux 3' UTR model to all species has been proposed (Stalder and Mühlemann, 2008; Brogna and Wen, 2009). At the heart of this EJC-independent NMD model there is a kinetic competition between PABPC1 and the NMD factor UPF1 for a mutually exclusive interaction with eRF3 (Ivanov et al., 2008; Singh et al., 2008) (Figure I.5, A). According to this model, the eRF3--PABPC1 interaction is required for proper mRNA translation termination (Hoshino et al., 1999; Cosson et al., 2002; Mangus et al., 2003). Consistent with this hypothesis, mammalian cells lacking PABPC1 exhibit an increased read-through of termination codons (Ivanov et al., 2008). Proper spacing between the stop codon and the poly(A) tail is considered determinant for the eRF3-PABPC1 interaction to occur, as the release factors are placed in close proximity to PABPC1. On the other hand, at an mRNA containing a PTC, the stop codon is not in the appropriate position and, as a result, the longer 3' UTR might not be adequate to bring PABPC1 into the proximity of the eRF3 bound at the termination codon. In addition, ribosomes that terminate prematurely are released less efficiently or too slowly compared with those encountering normal stop codons, increasing the probability of congregation of NMD components and thus leading to rapid degradation of the mRNA (Amrani et al., 2004, 2006; Nicholson et al., 2010). Hence, the larger the distance between eRF3 at the termination site and PABPC1 at the poly(A) tail, the less efficient the eRF3-PABPC1 interaction is, increasing the probability of the competing eRF3-UPF1 interaction to occur, which induces NMD. In support if this hypothesis, if PABPC1 is absent, UPF1 has been shown to more readily interact with the translation termination factors eRF1 and eRF3 (Singh et al., 2008). However, the importance of EJCs in the recognition of PTCs is not excluded by this model, since the EJC may function as a NMD enhancer, acting as a barrier between the mRNA poly(A)-binding proteins and the termination complex (Figure I.5, A).

While there is considerable support to the faux 3' UTR model, a variety of studies suggests that PTC recognition is not likely to rely exclusively on the position of PABPC1 relatively to the PTC (Rehwinkel et al., 2005; Behm-Ansmant et al., 2007b). Instead, additional molecular signals might influence the nature of the termination event. The unified 3' UTR model integrates elements from both the "downstream marker" model and the "faux 3' model" and proposes that the discrimination between normal and premature termination events is the outcome of the combination of antagonistic signals (Shyu et al., 2008; Singh et al., 2008; Stalder and Mühlemann, 2008; Brogna and Wen, 2009; Silva and Romão, 2009). At a translation termination event, the decision of NMD triggering will be the result from the competition between PABPC1 and UPF1 for the termination complex. If PAPBC1 is favourably located to interact with the terminating ribosome, mediated by

eRF3, a normal termination event will occur, impairing the association of UPF1, thus repressing NMD triggering, even in the presence of distal EJCs. In opposition, the failure of PABPC1 to interact with the terminating complex will favour the association of dephosphorylated UPF1, together with SMG1, with the terminating complex and the assembly of the SURF complex. Then, a second signal, such as the UPF1 interaction with UPF2 and/or UPF3 that promotes SMG1--mediated UPF1 phosphorylation, will induce NMD. In addition, the EJCs located downstream of a stop codon may have evolved as a specialized secondary NMD enhancer. UPF2 and UPF3, comprised on the "faux 3' UTR"-bound EJC, are ideally positioned for readily interact with ribosome-bound UPF1 and SMG1. As a consequence, the time window between the binding of UPF1 to the terminating ribosome and its SMG1-mediated phosphorylation would be shortened, and thus the competition between PABP and UPF1 for binding to the stalled ribosome would tilt towards NMD (Stalder and Mühlemann, 2008).

I.2.4. Pathways of mRNA decay associated with mammalian NMD

Regular mRNA turnover is associated with two major cytoplasmic pathways of decay that are thought to be conserved from yeast to mammals (Meyer et al., 2004; Garneau et al., 2007). Both pathways require a rate-limiting first step that consists in the shortening of the poly(A) tail. In mammals there are two different complexes involved in the deadenylation process: the poly(A) nuclease (PAN) 2 and 3 complex initiates poly(A) tail shortening; and the complex CCR4:CAF1 removes the remaining adenines. After deadenylation, mRNAs can be degraded by two general pathways: (i) removal of the protective 5'-cap structure by the DCP1:DCP2 decapping enzyme complex and subsequent degradation by XRN1, the major cytoplasmic 5' to 3' exonuclease; and (ii) deadenylated mRNAs are subjected to 3' to 5' exonucleolytic degradation mediated by the exosome (Meyer et al., 2004; Garneau et al., 2007). Notably, the mRNA decay process associated with NMD was also found to utilize enzymes and co-activators involved in normal mRNA decay. Nonetheless, some differences amongst the decay pathways of PTC--containing and normal mRNAs have already been recognized (Mühlemann and Lykke-Andersen, 2010; Nicholson and Mühlemann, 2010).

In mammals, available data indicates that degradation of nonsense transcripts involves both decapping followed by 5' to 3' exonucleolytic activity as well as accelerated deadenylation and subsequent 3' to 5' exonucleolytic degradation (Chen and Shyu, 2003; Lejeune et al., 2003; Couttet and Grange, 2004; Yamashita et al., 2005a). In fact, NMD targets showed enhanced deadenylation relatively to normal mRNAs, and accumulated as full-length transcripts upon depletion of enzymes involved in decapping, deadenylation, or in 5' to 3' or 3' to 5' exonucleolytic degradation (Chen and Shyu, 2003; Lejeune et al., 2003; Couttet and Grange, 2004; Yamashita et al., 2005a). Depletion of DCP2 decapping protein, of XRN1, and of exosome--associated RRP6/PM-Scl100 increased PTC-containing mRNA abundance and reduced their

decay rate (Lejeune et al., 2003). With regards to deadenylation, depletion, or overexpression of inactive mutants, of the CCR4:CAF1 and PAN2:PAN3 deadenylase complexes were shown to inhibit NMD (Yamashita et al., 2005a). Several reports also support that the NMD pathway is able to promote decapping and deadenylation. For instance, UPF1 was shown to interact with the decapping complex and copurify with the poly(A) ribonuclease PARN (Lykke-Andersen, 2002; Lejeune et al., 2003; Fenger-Grøn et al., 2005; Cho et al., 2009). In addition, the proline-rich nuclear receptor co-regulatory protein 2 (PNRC2) was shown to be involved in inducing degradation of NMD targets. As this protein interacts with phosphorylated UPF1 and DCP1, it may provide a link between UPF1-bound mRNAs and the decapping enzymes (Cho et al., 2009). Furthermore, all three NMD factors UPF1, UPF2 and UPF3B were found to coimmunoprecipitate with the 5' to 3' exonucleases XRN1 and XRN2, as well as with several exosome components, including PM/Scl-100 (Lejeune et al., 2003).

Nonetheless, emerging data has revealed that degradation of PTC-containing transcripts can be initiated by endonucleolytic cleavage near the PTC in human cells (Huntzinger et al., 2008; Eberle et al., 2009b). Upon depletion of XRN1, the presence of polyadenylated 3' fragments of nonsense mRNA was detected. The accumulation of these 3' fragments resulting from endocleavage was shown to be dependent on UPF1 and unaffected by depletion of DCP2 (Eberle et al., 2009b). Moreover, endonucleolytic cleavage was demonstrated to occur without any apparent sequence preference and dependent of the PTC position (Eberle et al., 2009b). Further data, involving in vivo depletion and reconstitution experiments, indicate that SMG6 is the factor responsible for the endonucleolytic cleavage of NMD targets in mammals (Eberle et al., 2009b). Notably, the analysis of PTC-containing β-globin transcripts expressed in erythroid cells suggest an additional decay pathway. It was shown that decay products of these mRNAs were polyadenylated but lacked sequences from the 5' end of the full-length transcript (Lim et al., 1989, 1992; Lim and Maquat, 1992). Later studies suggest that nonsense β-globin mRNA undergoes endonucleolytic cleavage by a PMR-like enzyme, however, these cleavages occur preferentially on UG dinucleotides, and independently of the PTC position (Stevens et al., 2002; Bremer et al., 2003). These results suggest that nonsense β-globin mRNAs in erythroid cells are subjected to a specialized decay pathway that may occur simultaneously or in parallel with the UPF1- and SMG6-dependent cleavage near the PTC (Maquat, 2004).

In the overall, rapid decay of NMD targets in mammalian cells appears to involve either a conventional mRNA turnover pathway, starting with deadenylation and/or decapping, or a degradation pathway initiated by SMG6-dependent endonucleolytic cleavage. In both cases, the resulting RNA fragments undergo exonucleolytic degradation from the unprotected ends (Mühlemann and Lykke-Andersen, 2010; Nicholson and Mühlemann, 2010). A recent model for degradation of NMD substrates assumes that UPF1-bound mRNAs can be committed to two different decay pathways, depending on whether the SMG5:SMG7 complex or the endonuclease SMG6 binds to phosphorylated UPF1 (Mühlemann and Lykke-Andersen, 2010; Nicholson and Mühlemann, 2010; Nicholson et al., 2010). Interaction of SMG5:SMG7 with phospho-UPF1

induces deadenylation followed by decapping and exonucleolytic decay from both ends. On the other hand, binding of SMG6 to phospho-UPF1 leads to SMG6-mediated endonucleolytic cleavage near the PTC, and subsequent exonucleolytic degradation of the two remaining RNA fragments. Nevertheless, the determinants of which pathway to be activated as well as the relative contributions of each pathway to nonsense-mediated mRNA decay remain unclear.

I.2.5. Subcellular localization of NMD

While in yeast the NMD appears to be a cytoplasmic event, the subcellular location of NMD in mammalian cells is less clear. Whether the PTC recognition and NMD are nuclear, nucleus--associated or cytoplasmic events remains a matter of debate (Wilkinson and Shyu, 2002; Maquat, 2004; Chang et al., 2007b; Mühlemann et al., 2008; Mühlemann and Lykke-Andersen, 2010). Since PTC recognition is tightly coupled to translation, it was expected that NMD must occur in the cytoplasm. However, most examined mammalian nonsense mRNAs are found to be degraded when still physically attached to the nucleus. Observations based on subcellular fractionation assays show that transcripts harbouring PTCs present reduced steady-state levels not only in the cytoplasm, but also in the nuclear fraction of mammalian cells (Maquat, 1995). For instance, decay of nuclear-associated mRNAs was observed in transcripts coding for β-globin (Baserga and Benz, 1992; Kugler et al., 1995; Carter et al., 1996; Thermann et al., 1998; Zhang et al., 1998), dihydrofolate reductase (DHFR) (Urlaub et al., 1989), adenine phosphoribosyl--transferase (APRT) (Kessler and Chasin, 1996), TPI (Belgrader et al., 1993, 1994; Cheng and Maquat, 1993; Cheng et al., 1994; Zhang and Maquat, 1996), and TCR-β (Carter et al., 1995, 1996; Li et al., 1997). An altered efficiency of transcription has never been attributed to a PTC, as transcription rates have been measured using nuclear run-on for wild-type and nonsense genes encoding for human TPI (Cheng and Maquat, 1993) and DHFR (Urlaub et al., 1989). These findings suggest that mammalian NMD takes place either in the nucleus, or during or immediately after nuclear export while the mRNA is still associated with the nucleus.

The model for a nuclear translation-dependent mammalian NMD is supported by some studies (Li et al., 1997; Iborra et al., 2001; Brogna et al., 2002; Wilkinson and Shyu, 2002; Dahlberg and Lund, 2004). Reports presenting evidences for translation within the nucleus of HeLa cells (Iborra et al., 2001), the confinement of NMD to CBC-bound mRNA (Ishigaki et al., 2001), the detection of translation factors and ribosomal proteins in the nuclear compartments (Iborra et al., 2004), combined with the observation that the inhibition of mRNA export does not affect the downregulation of PTC-containing TCR-β transcripts in the nuclear fraction of mammalian cells (Bühler et al., 2002), further invigorated the hypothesis of intranuclear NMD. On the other hand, there are also several lines of evidence arguing against this hypothesis (Thermann et al., 1998; Bohnsack et al., 2002; Nathanson et al., 2003; Dahlberg and Lund, 2004). One later study reported that overexpression in the cytoplasm of dominant negative peptides of UPF proteins, designed to inhibit the interactions between them, specifically inhibit NMD in human cells (Singh

et al., 2007). Conversely, the same dominant negative peptides does not inhibit NMD when they were confined to the nucleus by the introduction of a nuclear localization signal (NLS), suggesting that most of NMD occurs in the cytoplasm of mammalian cells (Singh et al., 2007).

Other evidence suggests that the coding potential of a pre-mRNA can influence splicing decisions, inducing either exon skipping or intron retention, or affect transcription (Mühlemann et al., 2008) (see next section). While nonsense-mediated perturbation of splicing may result *via* a different pathway from NMD, it suggests the ability to recognize a termination codon in the nucleus. Multiple models have been proposed that attempt to reconcile an apparent role for the nucleus with the evidence that conventional translation in the cytoplasm is essential for mammalian NMD (Maquat, 1995; Frischmeyer and Dietz, 1999). The cytonuclear feedback model proposes that the identification of nonsense mRNA during cytoplasmic translation signals degradation of nascent nuclear transcripts derived from that allele. Although not directly supportable or refutable, the machinery and mechanism for signal transduction remain largely hypothetical. The favoured model at this time, termed the co-translational export model, posits that PTC recognition by the cytoplasmic translation machinery can trigger NMD before a nonsense mRNA has completely transited the nuclear pore. Thus, PTC-containing mRNAs could co-purify with the nuclear fraction even though NMD occurs in the cytoplasm (Maquat, 2004). In support to this possibility, visualization by electron microscopy provides evidence that the Balbiani ring granule, a large RNP particle of the dipteran *Chironomus tentans,* is exported from the nucleus to the cytoplasm 5'-end-first and becomes associated with cytoplasmic ribosomes before the 3' end transits the nuclear pore (Mehlin et al., 1992; Visa et al., 1996a, 1996b). Consistent with the interpretation that decay is limited to newly synthesized mRNA, studies using either total or cytoplasmic cell fractions report that the presence of a PTC decreases the steady-state of nuclear mRNA, but does not significantly alter the half-life of cytoplasmic RNA of TPI (Cheng and Maquat, 1993), DHFR (Urlaub et al., 1989) and β-globin in non-erythroid cells (Baserga and Benz, 1992), in spite of the low steady-state levels of the nonsense mRNAs. Nevertheless, it was also shown that nucleus-associated NMD of β-globin transcripts depends on cytoplasmic translation (Thermann et al., 1998). In fact, the insertion of a hairpin-forming iron-responsive element (IRE) into the 5' UTR of these transcripts results in translation inhibition as a consequence of iron regulatory protein (IRP) binding. Since IRP localization is restricted to the cytoplasm, the binding of IRP to the IRE will specifically inhibit cytoplasmic translation of IRE-containing transcripts. Hence, it was shown that, by precluding cytoplasmic translation, the activation of NMD on nonsense IRE-containing mRNAs is also prevented (Thermann et al., 1998).

In addition, the co-translational export model also defends that when the NMD process occurs in association with the nucleus, those mRNAs that escape to the cytoplasm, acquire immunity to further NMD degradation (Cheng and Maquat, 1993; Stephenson and Maquat, 1996). In fact, the abundance of nonsense NMD-competent transcripts was shown to be comparable in nuclear and cytoplasmic cell fractions, indicating that the decay takes place prior to the release of the mRNA into the cytoplasm (Maquat, 2004). Still in agreement with this model, newly processed CBC-

-bound mRNA would undergo the pioneer round of translation as it exits the nuclear pore (Ishigaki et al., 2001) and this would allow PTCs to be detected by the cytoplasmic translation machinery, while the mRNA is still nucleus-associated. The subsequent exchange of CBP80:CBP20 for the eukaryotic initiation factor eIF4E, which supports the cytoplasmic steady-state translation, would disqualify an eIF4E-bound mRNA as a potential NMD target. As eIF4E-bound mRNA is known to be derived from CBP80-bound mRNA, the reduced level of eIF4E-bound mRNA results from the degradation of CBP80-bound mRNA (Lejeune et al., 2002).

Although most mammalian mRNAs seem to be subjected to NMD while they are associated with the nucleus, subcellular fractionation studies in mammalian cells also show that some mRNAs are degraded by NMD after they are released into the cytoplasm. Examples of such cytoplasmic-NMD are β-globin mRNAs expressed in erythroid cells and glutathione peroxidase 1 transcripts (GPx1). Indeed, the decay of those PTC-containing transcripts was shown to be restricted to the cytoplasmic cell fraction (Lim et al., 1992; Moriarty et al., 1998). Notably, the cytoplasmic NMD of GPx1, similarly to what has been described to nucleus-associated NMD, seems to occur while the mRNA is still associated with CBP80:CBP20 and the same loss of susceptibility to NMD is observed when the cap binding complex is replaced by eIF4E (Ishigaki et al., 2001). This suggests that transcripts that undergo cytoplasmic NMD are also subjected to a pioneer round of translation as observed for nucleus-associated NMD.

The matter concerning the cellular localization of NMD became even more complex with the discovery of mRNA-processing bodies (P-bodies) (Mühlemann and Lykke-Andersen, 2010; Nicholson et al., 2010). P-bodies are discrete and highly dynamic cytoplasmic granules present in eukaryotic cells which seem to represent important sites for translational repression, mRNA silencing, mRNA surveillance and degradation (Cougot et al., 2004; Fillman and Lykke-Andersen, 2005; Teixeira et al., 2005; Durand et al., 2007; Eulalio et al., 2007a; Parker and Sheth, 2007; Franks and Lykke-Andersen, 2008). Notably, although none of the factors involved in 3' to 5' decay was shown to localize in P-bodies, these structures are enriched for components of the decapping and 5' to 3' degradation machinery, such as DCP1:DCP2, decapping activators and the 5' to 3' exonuclease XRN1 (Eulalio et al., 2007a; Franks and Lykke-Andersen, 2008). Mammalian P-bodies also contain the deadenylases CCR4:CAF1 and PAN2:PAN3 (Zheng et al., 2008). The association of an mRNP with the P-body seems to comprise at least two steps: (i) the mRNP release from translation ribosomes; and (ii) association of the mRNP with factors that enable its aggregation with other translationally repressed mRNPs (Eulalio et al., 2007a; Parker and Sheth, 2007; Franks and Lykke-Andersen, 2008).

In addition to being a site of mRNA degradation, several lines of evidence support that cytoplasmic P-bodies may constitute a site for mammalian NMD to occur. In fact, it has been found that NMD factors UPF1, UPF2, UPF3, SMG5 and SMG7 are localised in P-bodies under certain conditions (Unterholzner and Izaurralde, 2004; Bruno and Wilkinson, 2006; Sheth and Parker, 2006; Durand et al., 2007; Cho et al., 2009). Nonetheless, despite the fact that yeast NMD was shown to occur in P-bodies, this might not be the case for mammalian NMD. For instance,

NMD is not affected in *Drosophila melanogaster* and human cells by depletion of specific factors essential for the formation of microscopically visible P-bodies (Eulalio et al., 2007b; Stalder and Mühlemann, 2009). These results indicate that NMD does not imperatively require the presence of cytoplasmic P-bodies. Although mRNPs targeted to NMD might have the ability to multimerize with other repressed mRNPs into P-bodies, several lines of evidence support that this is not critical for the degradation of NMD targets (Sheth and Parker, 2006; Durand et al., 2007; Eulalio et al., 2007b; Stalder and Mühlemann, 2009). Moreover, SMG6 does not colocalize with P-bodies (Unterholzner and Izaurralde, 2004), which is consistent with the view that the majority of nonsense mRNA degradation in mammalian cells might occur outside of P-bodies wherever NMD is initiated by a decapping-independent SMG6-mediated endonucleolytic cleavage (Eberle et al., 2009b). On the other hand, this observation does not exclude the possibility that NMD may occur to some extent in cytoplasmic P-bodies, conciliating the above-mentioned colocalization of several NMD factors and a nonsense reporter transcript with P-bodies under certain conditions (Unterholzner and Izaurralde, 2004; Durand et al., 2007; Cho et al., 2009).

In summary, the subcellular localization of mammalian NMD remains an issue for future study. The conflicting evidences collected so far suggest that the NMD pathway may not be restricted to a particular location in the mammalian cell, but instead, one fraction of NMD could occur in the nucleus, or in association with the nucleus, while another could take place in the cytoplasm.

I.2.5.1. Nuclear aspects of NMD

PTCs have been shown to elicit not only rapid decay of mRNA but also other responses within the nucleus of mammalian cells. Regardless of the controversy about nuclear- *versus* cytoplasmic--associated NMD, several evidences indicate that the presence of PTC in a transcript affects nuclear processes such as mRNA export, pre-mRNA splicing and transcription.

I.2.5.1.1. Nonsense-codon induced partitioning shift

One example of nuclear responses to PTCs was reported in a study describing a dramatic shift in the ratio of TCR-β reporter mRNAs in the nuclear and cytoplasmic fraction of HeLa cells, resulting in few PTC-containing transcripts escaping to the cytoplasmic fraction (Bhalla et al., 2009). This so-called nonsense-codon induced partitioning shift (NIPS) is specifically triggered by recognition of a disrupted reading frame, as missense mutations do not elicit it and it depends on translation (Bhalla et al., 2009). Furthermore, NIPS was shown to be dependent on the NMD factors UPF1 and eIF4AIII but UPF3B-independent. The underlying mechanism for this pathway still remains to be fully characterized, however, NIPS seems to result from retention of PTC-containing mRNAs in either the nuclear membrane or the nucleoplasm and not from nucleus-associated NMD (Bhalla et al., 2009).

I.2.5.1.2. Nonsense-associated altered splicing

Another nuclear response to PTCs might involve an increase in the level of alternatively spliced transcripts that skip the frame-disrupting mutation. In this quality control pathway, the translational reading frame influences splice site choice in a way that favours translation termination at the normally used stop codon (Dietz and Kendzior, 1994; Gersappe and Pintel, 1999; Gersappe et al., 1999; Mendell et al., 2002; Wang et al., 2002a, 2002b; Chang et al., 2007a). Whether splicing is altered specifically as a consequence of the open reading frame truncation, or if the PTC-causing mutation affects splicing directly, remains a crucial question about this nonsense-associated altered splicing (NAS) pathway (Maquat, 2002). Actually, two distinct branches have been proposed for NAS. In class-I NAS, alternative splicing is triggered by mutations that disrupt ESEs, which comprise short generate sequence elements that are bound by SR proteins (Valentine, 1998; Cartegni et al., 2002). ESEs are thought to improve splicing by recruiting SR proteins, which in turn recruit spliceosome components (Blencowe, 2000; Graveley, 2000; Cartegni et al., 2002; Zheng, 2008). Whenever mutations disrupt an ESE, SR protein binding is impaired, which lowers the inclusion frequency of the exon harbouring that ESE. NAS is triggered if alternative splice sites are available to compete successfully with the normal splice sites for the spliceosomal apparatus. Because ESEs can be disrupted not only by nonsense, but also by missense and silent mutations, a hallmark of an exonic site susceptible to class-I NAS is that all three types of mutations have the potential to upregulate the alternatively spliced transcript (Cartegni et al., 2002). Known examples of class-I NAS come from the BRCA1, fibrillin-1, sodium channel modifier 1 (SCNM1), cystic fibrosis transmembrane regulator (CFTR), Ig-μ and TCR-β genes (Liu et al., 2001; Caputi et al., 2002; Buchner et al., 2003; Pagani et al., 2003; Bühler and Mühlemann, 2005; Mohn et al., 2005; Chang et al., 2007a).

In class-II NAS, upregulation of alternatively spliced mRNA is elicited by disruption of the reading frame due to nonsense and frameshift mutations. Several lines of evidence support the existence of class-II NAS. For instance, nonsense mutations, but not missense mutations at corresponding positions, elicit upregulation of alternatively spliced transcripts from several genes, including parvovirus minute virus of mice (MVM), fibrillin-1, and TCR-β (Dietz et al., 1993; Dietz and Kendzior, 1994; Gersappe and Pintel, 1999; Gersappe et al., 1999; Maquat, 2002; Mendell et al., 2002; Wang et al., 2002a, 2002b). Moreover, upregulation of the alternatively spliced transcripts is dependent on translation, as it is reversed by suppressor tRNAs, stem loops introduced before the translation initiation start site, or by mutating the start AUG or adjacent Kozak consensus sequences (Gersappe et al., 1999; Wang et al., 2002a, 2002b). A later study provided further evidence that TCR-β transcripts can be subjected to both classes of NAS and that the consequences of reading frame-disrupting mutations, e.g. PTCs, depend on context, namely PTC position as well as splice site strength (Chang et al., 2007a). In addition, requirement for UPF1 is a distinction criteria for class-II NAS although other NMD factors do not appear to be required (Mendell et al., 2002; Chang et al., 2007a). Interestingly, specific NMD-inactivating amino acid substitutions in UPF1 did still support frame-dependent NAS, indicating genetically separable

functions of UPF1 in these two processes (Mendell et al., 2002). NAS and NMD therefore seem to be distinct but not mutually exclusive mechanisms, triggered by the same signal. That recognition of a PTC in an mRNA could generate a signal that alters splice site selection on its own pre-mRNA species is intriguing, and the underlying mechanism remains to be elucidated.

I.2.5.1.3. Nonsense-mediated upregulation of pre-mRNA

Several evidence support that disruption of the mRNA reading frame can have direct effects on intranuclear mRNA metabolism. A pathway that shares some characteristics with NAS, involving nonsense-mediated upregulation of pre-mRNA (NMUP) when frame disruption is sensed, has also been described in MVM, Ig-κ, Ig-μ and TCR-β genes (Naeger et al., 1992; Lozano et al., 1994; Aoufouchi et al., 1996; Gersappe and Pintel, 1999; Gersappe et al., 1999; Mühlemann et al., 2001). For instance, PTC-generating mutations introduced at various locations in MVM viral genome increase the levels of precursor MVM mRNA retaining one of the introns (Naeger et al., 1992; Gersappe and Pintel, 1999; Gersappe et al., 1999). Somatic mutations introduced in Ig-κ gene during normal B-cell development lead to the generation of PTCs and increased levels of Ig-κ pre-mRNA by a mechanism that appears to involve inhibited RNA splicing (Lozano et al., 1994; Aoufouchi et al., 1996). Finally, another important example of NMUP reports that nonsense mutations within transcripts derived from the endogenous Ig-μ gene in mouse hybridoma cells results in an increase in the level of the corresponding pre-mRNA. This accumulation is evident at or near the site of transcription as visualized by FISH, in a way that is not attributable to increased rates of transcription as determined by nuclear run-on assays (Mühlemann et al., 2001). Comparison of variant plasma cell lines with different nonsense mutations in a common Ig-μ gene reveals that disruption of reading frame correlates with upregulation of Ig-μ pre-mRNA (Mühlemann et al., 2001). This effect is independent of PTC position, as the same Ig-μ introns are retained, regardless of the location of the nonsense or frameshift mutations tested (Mühlemann et al., 2001). However, a subsequent study revealed that the level of endogenous Ig-μ pre-mRNA varies in plasma cell lines obtained from different sources, raising the question of whether frame--disrupting mutations actually increase Ig-μ pre-mRNA levels (Lytle and Steitz, 2004). Nevertheless, analysis of TCR-β reporter genes stably expressed in HeLa cells show that alleles containing PTCs, but not those containing a missense mutation or a frameshift followed by frame correcting mutations that prevent the generation of PTCs, exhibit elevated levels of pre-mRNA, which accumulates at or near the site of transcription as well (Mühlemann et al., 2001).

A later study performed with TCR-β constructs expressed in human cells proposed that frame disruption is not responsible for NMUP, as the TCR-β sequences conferring the NMUP response are shown to be densely packed with ESEs (Imam et al., 2010). Therefore, nonsense and frameshift mutations may, instead, disrupt ESEs resulting in mRNA splicing inhibition and subsequent pre-mRNA upregulation. Furthermore, several lines of evidence support that TCR-β NMUP is both frame- and translation-independent, as well as UPF1-independent, and hence does not involves PTC recognition (Imam et al., 2010). However, the possibility that a nonsense

mutation may, in some instances, induce pre-mRNA NMUP as a result of the disruption of reading frame lives on. The NMUP response formerly described by Mühlemann *et al* (2001), induced by nonsense mutations in the Ig-μ and TCR-β genes, could still be reading frame-dependent. Some of those mutations are positioned far away from the retained introns present in the upregulated pre-mRNA, and, hence, unlikely disrupt ESEs since their effectiveness decreases as their distance from a given intron increases (Mühlemann et al., 2001; Imam et al., 2010). As in NAS, divergent observations regarding the nature of the PTC effect on pre-mRNA levels might reflect the existence of both frame-dependent and -independent NMUP pathways that either compete or mask each other (Imam et al., 2010). These data are consistent with the cytonuclear feedback model for NMD. This apparently PTC-specific interference of splicing could be induced by a cytonuclear feedback signal triggered by PTC recognition during translation in the cytoplasm. But the nature of this putative signal and whether it would be allele-specific or also target coexpressed PTC-containing transcripts is currently unknown.

I.2.5.1.4. Nonsense-mediated transcriptional gene silencing

An unexpected PTC-dependent nuclear effect was discovered in Ig-μ reporter genes expressed in human cells (Bühler et al., 2005). Integration of Ig-μ minigenes constructs into HeLa cells, derived from those mouse hybridoma Ig-μ genes which showed NMUP at the transcription site (Mühlemann et al., 2001), lead to a PTC-specific, UPF1- and translation-dependent inhibition of the transcription of these minigenes. Similarly to Ig-μ minigenes, when Ig-γ minigenes were stably transfected in HeLa cells, transcription of PTC-containing genes was silenced (Bühler et al., 2005). This nonsense-mediated transcriptional gene silencing (NMTGS) is PTC-specifically accompanied by changes in the chromatin structure, which is an important determinant for the transcriptional activity, manifested by the loss of typical marks for transcriptionally active euchromatin (acetylated histone H3) and a simultaneous accumulation of inactive heterochromatin marks (methylated histone H3-K9), revealed by chromatin immunoprecipitation (ChIP) assays (Bühler et al., 2005). Consistently, NMTGS can be reversed by treating the cells with inhibitors of histone deacetylases, which promote hypoacetylation and transcriptional silencing (Bühler et al., 2005). The intriguing question whether recognition of a PTC during mRNA translation could generate a signal that feeds back and specifically alters transcription on its own cognate gene was also addressed. As transcriptional silencing of genes *via* chromatin remodelling might involve components of the RNA interference (RNAi) system and is triggered by small double-stranded RNA molecules, interfering with this system might affect NMTGS (Bühler et al., 2005). Notably, Ig-μ NMTGS is inhibited by overexpression of the double-stranded small interfering RNAs (siRNAs) ribonuclease 3'hExo, which counteracts RNAi, and indicates that siRNAs are involved in a signalling pathway that links cytoplasmic translation back to transcription in the nucleus (Bühler et al., 2005). Consistently, overexpression of 3´hExo affects NMTGS but does not interfere with NMD (Bühler et al., 2005). However, Ig-μ specific siRNAs have not been detected so far (Bühler et al., 2005; Stalder and Mühlemann, 2007; de Turris et al., 2011). A

subsequent study regarding Ig-μ NMTGS in HeLa cells shows that this pathway is translation- and UPF1-dependent, which suggests that NMTGS branches from the NMD pathway after translation of the PTC-containing mRNA and UPF1-dependent PTC recognition (Stalder and Mühlemann, 2007).

Notably, NMTGS was only observed with PTC-containing Ig-μ and Ig-γ minigenes expressed in human cells, but not with other classical NMD reporter genes as TCR-β, β-globin and GPx1 (Bühler et al., 2005). On this basis, it was hypothesized that NMTGS might be an Ig-specific quality control pathway important to silence expression of non-productively rearranged heavy chain alleles in B cells and potentially other specialized lineages. However, no evidence for a reduced transcription rate of PTC-harbouring Ig-μ alleles was observed previously in mouse hybridoma cells (Mühlemann et al., 2001). Furthermore, investigation of clonal lines of immortalized murine pro-B cells did not reveal a difference of the transcriptional state between the productively and the non-productively rearranged Ig-μ allele (Eberle et al., 2009a). Another possibility is that the transcriptional gene silencing response elicited by PTCs depends on the differentiation stage of B lymphocytes (Eberle et al., 2009a). Until different NMD targets are tested on several cell lineages with different developmental or functional specificities, the biological significance of NMGTS remains elusive.

Nevertheless, on the basis of the previous studies, it is tempting to speculate that NMUP and NMGTS consist of nuclear responses dependent on the recognition of a PTC and thus comprise an NMD-related function at the site of transcription. A recent study shed some light upon the components and underlying mechanisms of these pathways (de Turris et al., 2011). To investigate if mRNA reading frame is able to influence events at or near the site of transcription, a combination of FISH, live-cell imaging and ChIP analysis of human U2OS cells stably transfected with Ig-μ minigenes constructs bearing NMD-sensitive PTCs was performed (de Turris et al., 2011). Several lines of evidence show that a mechanism dependent on the NMD factors UPF1 and SMG6 elicits specific retention of a fraction of the PTC-containing transcripts, which accumulate as unspliced RNA at the site of transcription (de Turris et al., 2011). Also, UPF1 and SMG6 can physically associate with the Ig-μ minigene encoding chromatin of both wild-type and PTC-bearing variants, invigorating the possibility of their role in recognizing and preventing PTC--containing pre-mRNA from subsequent splicing and export by retaining them at the transcription site (de Turris et al., 2011). In addition, depletion of UPF1, and not of SMG6, increase specifically the transcription elongation rate of the wild-type mini Ig-μ RNA, suggesting an additional role of UPF1 in the Ig-μ transcriptional regulation (de Turris et al., 2011). The authors speculate that the presence of UPF1 on the chromatin could reduce the transcription elongation rate *via* an unknown mechanism prior to PTC recognition. Subsequently, PTC recognition could trigger UPF1 phosphorylation/dephosphorylation and recruit other factors that would reduce the transcription rate. This might explain why an increase in transcription elongation was not observed with PTC--containing Ig-μ after depletion of UPF1 (de Turris et al., 2011). Nevertheless, consistent with earlier reports of NMUP (Mühlemann et al., 2001) and NMTGS (Bühler et al., 2005), this study

shows that the transcriptional and post-transcriptional dynamics of the PTC-containing Ig-μ transcripts at the transcription site are affected, suggesting that a PTC-containing transcript can be discriminated during its synthesis and/or processing.

In summary, several studies regarding the nuclear aspects of NMD imply that NMD or NMD--associated quality control pathways may act in the nucleus, as well as post-transcriptionally in the cytoplasm to collectively reduce the abundance of PTC-containing mRNAs in mammalian cells. In addition, these reports suggested the existence of a reading frame scanning mechanism in the nucleus or, alternatively, of a cytonuclear feedback mechanism triggered by PTC recognition *via* NMD during cytoplasmic translation that acts on nuclear events. On the other hand, the above-mentioned nuclear responses to PTCs might be restricted to a particular subset of NMD targets. In addition, other studies strongly argue against nuclear NMD (see section I.2.5). Therefore, only additional studies using different NMD targets and cell lines can resolve this issue, namely using the classic NMD reporter gene such as human β-globin expressed in erythroid cells, which is a more physiological context than non-erythroid cells.

I.3. Human β-globin as a Model System for Studying NMD

The human β-globin gene spans about 1601 base pairs (bp) and encodes 146 amino acids. This stretch of 1.6 kilobases (kb) comprises two intervening non-coding introns and three coding exons that are flanked by 5' and 3' UTRs (Huisman, 1993). The β-globin gene contains a promoter that includes three erythroid-specific positive *cis*-acting elements, located at 28 to 105 bp upstream of the transcription initiation site, and two erythroid-specific enhancer elements found in intron 2 and 3' of the β-globin gene, which spans about 600 to 900 bp downstream of the poly (A) site (Antoniou, 1991; Thein, 1998).

After the two β-globin introns are spliced out, two EJCs can be assembled on the transcript, each one located 20 to 25 nt from each exon-exon junction (Le Hir et al., 2000a, 2000b). In conformity with the β-globin mRNA structural context, an NMD-behaviour corroborating the "50 to 54 nt boundary rule" has been broadly described for these transcripts. Several authors showed that β--globin nonsense mutations located in the 3' region of exon 1 (at codon 26) and within the 5' two--thirds of exon 2 (at codons 36, 39, 60-61, 62, 75, and 82) were all able to elicit NMD (Thermann et al., 1998; Zhang et al., 1998; Romão et al., 2000; Inácio et al., 2004). In contrast, mRNAs harbouring PTCs towards the 3' end of exon 2 (at codons 88, 91, 95, 98, 101, and 103) and those with PTCs in exon 3 (at codons 106, 107, 114, 121, 127 and 141) are all NMD-resistant (Hall and Thein, 1994; Thermann et al., 1998; Zhang et al., 1998; Romão et al., 2000). Nevertheless, unusual NMD behaviours concerning PTC definition in β-globin transcripts have been also described (Zhang et al., 1998; Romão et al., 2000; Neu-Yilik et al., 2001; Danckwardt et al., 2002). For instance, our lab reported that human β-globin transcripts bearing nonsense mutations in the 5' region of exon 1 (at codons 5, 15, and 17) accumulate to levels similar to those of wild-

-type β-globin transcripts, comprising, unexpectedly according to their position, exceptions to the "50 to 54 nt boundary rule". Abnormal splicing and impaired translation have been shown not to be the determinants of this resistance to NMD; instead, it might reflect the nature of the premature termination events (Inácio et al., 2004; Silva et al., 2006). Indeed, β-globin NMD exceptions might be explained by the proximity of the PTC to the AUG codon, which has been called as the "AUG--proximity effect", or translation reinitiation of β-globin AUG-proximal nonsense-mutated transcripts. For some AUG-proximal PTCs, translation reinitiation might explain their NMD--resistance, since the ribosomes that initiate at an in-frame UAG downstream of the early PTC removes the remaining EJCs from the mRNA and terminates translation at the normal stop codon, which might result in the production of N-terminal truncated proteins (Zhang and Maquat, 1997; Perrin-Vidoz et al., 2002; Neu-Yilik et al., 2011). On the other hand, the "AUG-proximity effect", which is independent of sequence context and transcript identity, also appears to be a general attribute of mammalian NMD (Silva et al., 2006). In the case of a premature termination event at a AUG-proximal stop codon, translation might be brief enough to maintain PAPBC1, bound at the poly(A) tail, in close proximity to the AUG codon *via* its interaction with translation initiation factors, as a result of mRNA circularization (Peixeiro et al., 2012). This favourable location might allow PABPC1 to interact with the translation termination complex by the time the ribosome reaches the β-globin AUG-proximal PTC, superimposing its inhibitory effect on NMD and enabling efficient translation termination to occur (Silva et al., 2008; Silva and Romão, 2009; Peixeiro et al., 2012).

Human β-globin gene has been extensively used as a classical NMD reporter in several cell lines, contributing to the elucidation of different features of the NMD mechanism. For instance, the β--globin transcript containing the naturally occurring nonsense mutation at codon 39 (β39) have been widely tested in different experimental systems, using both erythroid and non-erythroid cells, and consistently shows low steady-state levels (Baserga and Benz, 1992; Zhang et al., 1998; Romão et al., 2000; Danckwardt et al., 2002; Gardner, 2008; Wang et al., 2011). Moreover, several authors have directly associated the downregulation of β39 mRNA with decreased mRNA decay rates and high NMD efficiency, which further establishes the β39 construct as an ordinary control for NMD competence (Thermann et al., 1998; Inácio et al., 2004; Silva et al., 2006, 2008; Eberle et al., 2009b).

A known experimental system in erythroid cells consists of human β-globin constructs stably transfected into MEL cells under the control of β-globin promoter and locus control region (LCR) sequences. The β-globin LCR is composed of four erythroid-specific DNase I hypersensitive sites (HS), HS1-4, that are made up of clusters of binding sites for transcription activators (Grosveld et al., 1987; Forrester et al., 1989; Li et al., 2002). Human β-globin genes linked with LCR components reproducibly express at physiological high levels, which are directly proportional to transgene copy number and position-independent relatively to the site of integration in the cell genome, during the induced terminal differentiation of MEL cells (Blom van Assendelft et al., 1989; Talbot et al., 1989; Collis et al., 1990; Antoniou, 1991). Even though β-globin transgenes are integrated at random ectopic sites within the host cell genome as a tandem array, they are

transcribed and processed at normal levels as surrounded by a natural chromatin context (Blom van Assendelft et al., 1989; Talbot et al., 1989; Collis et al., 1990; Antoniou, 1991; Milot et al., 1996).

I.4. Aim of Study

In summary, NMD is a cellular surveillance mechanism that detects and rapidly degrades mRNAs carrying premature translation-termination codons. Although several models for mammalian NMD have been proposed, the prevailing view is that this mechanism requires recognition of the PTC by the elongating ribosomes during translation in the cytoplasm. Strikingly, some published data have suggested that PTCs may also affect the nuclear metabolism of the nonsense-mutated transcripts, disturbing nuclear events such as pre-mRNA splicing and transcription. Indeed, several nuclear quality control pathways have been described to target aberrant RNAs and RNPs for nuclear degradation or retention within nuclear domains as well as for transcriptional downregulation. Hence, the aim of the work presented in this thesis is to elucidate whether the presence of a PTC in a classical NMD reporter gene can influence nuclear events of the corresponding transcript in mammalian cells. Thus, it was hypothesized that human β-globin transcripts containing a PTC, sensitive to NMD, would have a singular nuclear localization and processing status in erythroid cells. To test this hypothesis, the following tasks were established:

- To assess whether the presence of a PTC, sensitive to NMD, affects the intranuclear localization of β-globin transcripts in erythroid cells;
- To ascertain whether or not the presence of a NMD-sensitive PTC affects the steady-state levels of the β-globin pre-mRNA in erythroid cells;
- To evaluate the biochemical mechanism by which a PTC could affect the steady-state levels of the corresponding β-globin pre-mRNA;
- To verify whether the PTC effect on the steady-state level of β-globin pre-mRNA is cell-type specific.

CHAPTER II. Materials and Methods

II.1. Gene Constructs

Plasmids containing the human β-globin gene (HBB, Gene ID:3043[1]) were derived from p158.2 (Romão et al., 2000), which comprises a 4.1-kb HpaI/XbaI genomic fragment encoding the entire 1.6-kb gene along with 0.8 kb of the 3' flanking region and 1.7 kb of the 5' flanking sequence including the promoter, adjacent to a 1.9-kb KpnI/PvuII DNA fragment of the human β-globin LCR DNase I hypersensitive site 2 (HS-2). Variant β-globin genes carrying the β26 (codon 26 GAG→TAG), β39 (codon 39 CAG→TAG), β62 (codon 62 GCT→TAG) or β127 (codon 127 CAG→TAG) mutations were obtained as previously described (Romão et al., 2000; Inácio et al., 2004). The β39missense gene variant was originated from the wild-type (βWT) human β-globin construct by the introduction of a CAG→GAG missense mutation at codon 39 *via* site-directed mutagenesis, using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) with the specific primers 5'-GGT CTA CCC TTG GAC CGA GAG GTT CTT TGA GTC-3' and 5'-GAC TCA AAG AAC CTC TCG GTC CAA GGG TAG ACC-3'. The pTRE2pur vectors (Clontech) encoding the βWT or β39 genes under the control of a cytomegalovirus promoter and a puromycin resistance gene were cloned as described by Silva et al. (2006). In brief, 1.6-kb human β-globin genes along with 0.2 kb of the 3' flanking region were subcloned into the ClaI/BspLU11I sites of pTRE2pur vector by PCR amplification of the 1.8-kb fragment, using primers with linkers for ClaI and BspLU11I.

II.2. Cell culture, Stable Transfection and Drug Treatments

II.2.1. MEL cells

Mouse erythroleukemia C88 cells (Deisseroth and Hendrick, 1978) were cultured in RPMI medium with Glutamax-I (Life Technologies), supplemented with 10% (v/v) fetal bovine serum at 37°C and 5% CO_2. Stable transfection of MEL cells was carried out as previously described (Inácio et al., 2004). Briefly, aliquots of $3x10^7$ MEL cells/mL were transferred to electroporation chambers (Bio-Rad) with 50 µg of SalI linearized p158.2-βWT or its derivatives, mixed with 2 µg of PvuII linearized pGK-puro (plasmid encoding a puromycin resistance gene) to obtain βWT, β26, β39, β39missense, β62 or β127 cell lines. Electroporations were performed in a Cell-Porator (Power Pac 300; Bio-Rad) apparatus at the following settings: high ohms, 250 V and 960 µF. Each MEL cell pool was expanded in selective medium by adding puromycin (Sigma-Aldrich) to 2.5 µg/mL and single-cell clones were established by the limiting dilution method.

Erythroid differentiation was induced in equal amounts of MEL cells by adding 2% (v/v) dimethyl

[1] Entrez Gene database (http://www.ncbi.nlm.nih.gov/gene) at the National Center for Biotechnology Information (NCBI), National Library of Medicine (NLM), National Institutes of Health (NIH), USA.

sulfoxide (DMSO) to the culture media during 4 days. For pre-mRNA half-life determination experiments, transcription of reporter β-globin genes was inhibited by addition of actinomycin D (Sigma-Aldrich) to a final concentration of 5 μg/mL, after induction of erythroid differentiation. Total RNA was isolated 0, 15 and 30 minutes (min) after transcription arrest.

II.2.2. HeLa cells

HeLa cells (ATCC CCL-2) were grown in DMEM medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum. Transfection with the pTRE2pur-βWT or pTRE2pur-β39 plasmids and subsequent cell selection with puromycin were performed as previously described (Silva et al., 2008). Stable transfections were performed using Lipofectamine 2000 Transfection Reagent (Life Technologies) in 35-mm plates using 2 μg of each β-globin encoding plasmid, following the instructions of the manufacturer. Stably transfected HeLa cell pools were subsequently selected using 1.5 μg/mL puromycin (Sigma-Aldrich).

II.3. Transgene Integrity and Copy Number Analysis

The structure of the transgene in each MEL cell clone was determined by Southern blotting of genomic DNA from transfected MEL cell lines, isolated by the standard phenol:chloroform method and digested with EcoNI and KpnI. Digested DNAs were agarose gel-fractioned and transferred by Southern blotting onto Hybond N+ membranes (GE Healthcare). Blots were hybridized with human β-globin DNA probes labeled by the Multiprime DNA Labeling Kit (Amersham) using [α-^{32}P] dCTP. The probe template consisted of a 768-bp EcoRI-PstI human β-globin gene fragment. Hybridization reactions, washing and exposure were carried out following the manufacturer's instructions (GE Healthcare).

To determine the transgene copy number of MEL cell clones, the human β-globin transgene copy number was compared with that of an endogenous diploid reference, the murine thymus cell antigen 1 gene (Thy1; Gene ID:21838), by quantitative polymerase chain reaction (PCR), performed in an ABI Prism 7000 Sequence Detection System, using SYBR Green Master Mix (Life Technologies). Quantification was performed by relative quantification using the standard curve method with serial dilutions of a plasmid carrying one copy of β-globin and Thy1 gene sequences. The forward and reverse primers for the β-globin gene were 5'-GAT CTG TCC ACT CCT GAT GC-3' and 5'-AGC TTG TCA CAG TGC AGC TC-3'; producing a 149-bp amplicon. For the amplification of the Thy1 gene, primers were 5'-GGT CAA GTG TGG CGG CAT A-3' and 5'--GAA ATG AAG TCC AGG GCT TGG-3', generating a 99-bp PCR product. Amplifications were performed under the following conditions: 95°C for 10 minutes (min); and 95°C for 15 seconds (sec), 60°C for 1 min (40 cycles). After the quantitative PCR reaction, a dissociation curve analysis was performed to discard any non-specific product amplification.

II.4. RNA Fluorescence *In Situ* Hybridization

II.4.1. RNA probes preparation

To detect the human β-globin transcripts subcellular localization by fluorescence *in situ* hybridization, two different digoxigenin-labelled riboprobes were generated by *in vitro* transcription of plasmids containing DNA fragments from the human β-globin gene. The βtotal probe comprises the full-length 1.6-kb β-globin transcriptional unit, which was amplified by PCR using primers 5'--ACA TTT GCT TCT GAC ACA ACT G-3' and 5'-GCA ATG AAA ATA AAT GTT TTT TAT-3'. The βintron2 probe includes a PCR-generated fragment, comprising nucleotides 464 to 815 of the β--globin intron 2, amplified using primers 5'-CAT ATT GAC CAA ATC AGG G-3' and 5'-GCA AAA GGG CCT AGC TTG G-3'. The βtotal or βintron2 fragments were inserted into the cloning site of pCR2.1-TOPO (Life Technologies) in antisense orientation relative to the T7 polymerase promoter sequence. Each vector was linearized and transcribed in the presence of digoxigenin-11-UTP (Roche) using the Maxiscript T7 Kit (Life Technologies) under standard conditions. Final concentration of ATP, GTP and CTP was 1 mM each; final concentration of UTP was 0.65 mM and that of digoxigenin-11-UTP was 0.35 mM. After the transcription reaction, the generated riboprobes were purified by lithium chloride-precipitation and resuspended in 10 mM EDTA solution. The size of the probes was then shortened to about 200 nucleotides by alkaline hydrolysis. RNA probes were dissolved in carbonate buffer (60 mM Na_2CO_3; 40 mM $NaHCO_3$; pH 10.2) and incubated at 60°C for 25 min (βintron2) or 40 min (βtotal). Hydrolysed riboprobes were ethanol-precipitated after addition of glycogen (Life Technologies), 3 M sodium acetate, pH 5.2 (Life Technologies) and vanadyl ribonucleoside complexes (VRC, Sigma-Aldrich). Finally, riboprobes were resupended in diethyl pyrocarbonate (DEPC)-treated water and stored at -80°C.

II.4.2. Preparation, fixation and pre-treatment of MEL cells

Induced or uninduced stably transfected MEL cell clones and untransfected MEL cells were allowed to adhere onto 10x10-mm glass coverslips coated with 0.01% poly-L-lysine in phosphate buffered saline (PBS) solution (Sigma-Aldrich). MEL cells were fixed in 3.7% paraformaldehyde in PBS at room temperature for 10 min, and rinsed three times in PBS for 5 min each. Subsequently, MEL cells were permeabilized in 0.5% TritonX-100 (Sigma-Aldrich) in PBS supplemented with 2 mM VRC for 10 min, and washed three times 5 min in PBS with 2 mM VRC. MEL cells were then incubated in prehybridization buffer [2x saline-sodium citrate buffer (SSC); 0.01% Tween20 (Sigma-Aldrich); 2 mM VRC; pH 7.5] for 5 min at 50°C.

II.4.3. Hybridization

RNA probes were ethanol-precipitated with 1 µg/µL *Escherichia coli* tRNA (Sigma-Aldrich), 250 ng/µL herring sperm DNA (Sigma-Aldrich) and diluted in hybridization buffer [60% formamide; 2x SSC; 50 mM sodium phosphate, pH 7.0; 10% dextran sulphate; pH 7.5] to a final concentration of about 3 ng/µL. Immediately prior to the hybridization, riboprobes were denatured by heating the mixture at 80°C for 2 min. Hybridization mix was placed on each coverslip and incubated overnight at 50°C in a moist chamber.

II.4.4. Posthybridization washes

Following hybridization, MEL cells were washed in a 50% formamide, 2xSSC, 0.01% Tween20 solution at 50°C for three times 5 min each, equilibrated in NTE buffer (0.5 M NaCl, 10 mM Tris--HCl, pH 7.5; 1 mM EDTA), incubated with 20 µg/mL RNase A (Sigma-Aldrich) in NTE buffer for 30 min at 37°C, and washed in NTE twice for 5 min each. Subsequently, cells were washed with 2x SSC, 0.01% Tween20 buffer three times for 5 min each.

II.4.5. Immunodetection

For the detection of the digoxigenin-labelled β-globin riboprobes, MEL cells were incubated in 4x SSC, 0.1% Tween20, 0.1% bovine serum albumin (BSA, Sigma-Aldrich), and 0.2% gelatin (Sigma-Aldrich) for 5 min at 37°C. Each coverslip was then incubated at 37°C for 30 min with Cy3-conjugated mouse anti-digoxigenin antibody (diluted 1:250, Jackson IR Labs). Afterwards, MEL cells were washed in 4xSSC, 0.1% Tween20 at 37°C for three times 5 min each.

For the combined immunofluorescence of splicing factor SC-35 and β-globin RNA FISH, MEL cells were incubated with 0.1% Tween20 and 0.1% BSA in PBS for 5 min at room temperature. Each coverslip was incubated for 2 hours (h) with mouse anti-SC-35 primary antibody (diluted 1:100, Sigma-Aldrich) and washed afterwards with 0.1% Tween, 0.1% BSA in PBS three times for 5 min each. Subsequently, coverslips were incubated with AlexaFluor488-coupled goat anti--mouse secondary antibody (diluted 1:200, Life Technologies) and rhodamine-conjugated sheep anti-digoxigenin antibody (diluted 1:200, Roche) for 1 h. MEL cells were then washed again with 0.1% Tween20 and 0.1% BSA in PBS for three times 5 min each.

Following the immunodetection washing steps, MEL cells were rinsed in PBS at room temperature, fixed with 3.7% paraformaldehyde in PBS for 10 min and washed twice in PBS. MEL cells were next incubated with the DNA counterstain 4',6-diamidino-2-phenylindole (DAPI, Sigma--Aldrich) 2 µg/mL in PBS for 5 min and rinsed twice in PBS. Coverslips were mounted in a microscopy glass with Vectashield mounting medium (Vector Labs).

II.4.6. Microscopy

MEL cells were visualized on a Leica TCS SPE confocal laser scanning microscope using an ACS APO 63.0x1.30 Oil objective. Confocal microscopy was performed using 405 nm diode, 488 nm argon ion and 532 nm helium-neon lasers to excite DAPI (blue), AlexaFluor 488 (green), and Cy3 or rhodamine (red) fluorescence, respectively. Images were acquired in series of 10 to 40 optical sections through cell material (consecutive sections were separated by 0.5-0.7 µm), deconvolved and projected in two dimensions using the Leica Application Suite software.

II.5. RNA Isolation

Total RNA from MEL and HeLa cells was extracted using the RNeasy Total Kit (Qiagen) following the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Life Technologies) and purified by phenol:chloroform extraction. Potential DNA contamination of the isolated total RNA was discarded by PCR using specific primers for the human β-globin gene promoter region and exon 1. The primers sequences were 5'-TAA GCC AGT GCC AGA AGA G-3' and 5'-ACC ACC AAC TTC ATC CAC G-3', and amplifications were carried out under the following conditions: 95°C for 5 min; 95°C for 1 min, 56°C for 1 min, 72°C for 30 sec (30 cycles); and 72°C for 5 min.

II.6. Ribonuclease Protection Assays

The used ribonuclease protection assay (RPA) probes were generated by *in vitro* transcription of plasmids containing DNA fragments from human β-globin intron 1 and exon 2 (positions +178--486) (McCracken et al., 1997), β-globin intron 2, β-globin intron 2 and exon 3 (positions +1307--1496) (McCracken et al., 1997), murine α-globin intron 1 and exon 2 (Hba-a1, Gene ID: 15122; positions +556-800) (Liebhaber et al., 1996) or murine glyceraldehyde 3-phosphate dehydrogenase exons 5-8 (GAPDH, Gene ID:14433) cloned in pTRI-GAPDH (Life Technologies). The template for the βintron2 probe is a 352-bp PCR-generated fragment comprising nucleotides 464 to 815 of the β-globin intron 2, which was inserted into the cloning site of pCR2.1-TOPO (Life Technologies). Each transcription vector was linearized and transcribed in the presence of [α-^{32}P] CTP (Perkin Elmer) using a Maxiscript T7/SP6 Kit (Life Technologies) under standard conditions. Ribonuclease protection assays were performed using 5 to 12 µg of total RNA as previously described (Inácio et al., 2004). In brief, RNA was added to hybridization buffer (40 mM PIPES, pH 6.4; 1 mM EDTA, pH 8.0; 0.4 M NaCl; 80% formamide) supplemented with probes. Samples were heated at 95°C for 5 min, incubated overnight at 50°C, and digested for 30 min at room temperature in RNase assay buffer (300 mM NaCl; 10 mM Tris-HCl, pH 7.5; 5 mM EDTA, pH 8.0) containing 1 µL of RNase Cocktail (Life Technologies). Digestions were terminated by addition of 2 mg/mL Proteinase K (Life Technologies) in a 10% SDS solution (Life technologies) to each sample followed by incubation during 20 min at 37°C. RNA was extracted, precipitated, dissolved

in loading buffer, and resolved onto a 5 or 8% acrylamide 8 M urea gel. Radioactivity in bands of interest was quantified by phosphorimaging using a Typhoon Imager 8600 (GE Healthcare). The human β-globin pre-mRNA and mRNA hybridization signals from the MEL cell clones and pools were normalized to the respective endogenous control mRNA signal and compared with the reference βWT counterparts. In MEL clones, β-globin expression levels were also normalized to the transgene copy number.

II.7. Reverse Transcription-coupled Quantitative PCR

First-strand cDNA was synthesized from 1 µg of total RNA using the SuperScript II Reverse Transcriptase (Life Technologies) according to the manufacturer's instructions. From all MEL and HeLa pools cDNA, a single full-length product was amplified using primers specific for the human β-globin 5'- and 3'-UTRs and sequenced. Real-Time PCR was performed with the ABI7000 Sequence Detection System (Life Technologies) using SYBR Green PCR Master Mix (Life Technologies). The relative expression levels of the β-globin mRNA and pre-mRNA were normalized to the endogenous GAPDH mRNA in MEL cells, or to the internal control puromycin resistance mRNA in HeLa cells, and calculated using the comparative C_t method ($2^{-\Delta\Delta Ct}$) (Pfaffl, 2001). The C_t values of variant β-globin mRNA and pre-mRNA amplicons were compared to the respective βWT counterpart and normalized with the reference amplicon C_t value. The amplification efficiencies of the β-globin target and the GAPDH or puromycin reference amplicons were determined for each assay by dilution series. To check for DNA contamination, quantitative PCR without reverse transcription was also performed for all samples. The forward and reverse primers for the human β-globin mRNA were 5'-GTG GAT CCT GAG AAC TTC AGG CT-3' and 5'--CAG CAC ACA GAC CAG CAC GT-3'; for β-globin intron 1 pre-mRNA were 5'- GCA CTG ACT CTC TCT GCC TAT TGG T-3' and 5'-GGG TTG CCC ATA ACA GCA TCA GGA-3'; and for β--globin intron 2 pre-mRNA were 5'-CTG GCT CAC CTG GAC AAC CTC AAG G-3' and 5'-AGC GTC CCA TAG ACT CAC CCT-3'. The primers for the murine GAPDH mRNA were 5'-ATC ACC ATC TTC CAG GAG CGA-3' and 5'-AGC CTT CTC CAT GGT GGT GAA-3', and for the puromycin resistance mRNA were 5'-CGC AAC CTC CCC TTC TAC G-3' and 5'-GGT GAC GGT GAA GCC GAG-3'. Amplifications were carried out under the following conditions: 95°C for 10 min; and 95°C for 15 sec, 62°C for 30 sec (40 cycles). After the quantitative PCR reaction, a dissociation curve analysis was performed to discard any non-specific product amplification.

II.8. 3'-Rapid Amplification of cDNA Ends

First-strand cDNA synthesis was performed on 3 µg of total RNA from each MEL and HeLa cell pool using the SMART RACE DNA Amplification kit (Clontech), according to the manufacturer's instructions. The 3'-rapid amplification of cDNA ends (RACE) PCR covering the entire β-globin mRNA was performed with the synthesized cDNAs using primers 5'-ACA TTT GCT TCT GAC ACA ACT G-3' and Nested Universal Primer A Mix (Clontech). After initial denaturation for 5 min

at 95°C, cDNA amplification was carried out for 28 cycles using AmpliTaq polymerase (Roche) and 1 min at 95°C, 1 min at 58°C, 1min at 72°C as cycling conditions. The products were subjected to electrophoresis in a 1% agarose gel and sequenced.

II.9. Statistical Analysis

Results are expressed as mean ± standard deviation from at least three independent experiments. Student's two-tailed t-test was used for estimation of statistical significance. Significance for statistical analysis was defined as a $P < 0.05$.

CHAPTER III. Results

In the present study, our aim is to investigate whether the nuclear metabolism of nonsense--mutated β-globin transcripts expressed in erythroid cells is altered due to the presence of a PTC sensitive to NMD. Therefore, an experimental system consisting of human β-globin genes stably transfected into MEL cells was established. In order to address the issue whether a PTC can affect the nuclear localization of β-globin pre-mRNAs, FISH and confocal microscopy were used to examine the subcellular localization of either wild-type (βWT) or nonsense mutated at codon 39 (β39) β-globin RNAs stably expressed in MEL cells. Regarding the matter whether a PTC can affect the processing of PTC-containing β-globin mRNA β39 relatively to the wild-type counterpart, direct measurement of β-globin pre-mRNA and mRNA steady-state levels was performed using two different quantitative approaches: ribonuclease protection assays and reverse-transcription coupled quantitative PCR (RT-qPCR). Furthermore, to investigate whether the PTC effect on β-globin pre-mRNA levels is reading frame-dependent, i.e. due to PTC recognition *via* the NMD pathway, transcripts bearing missense mutations at the same position of the nonsense mutation at codon 39 (β39missense) or PTCs insensitive to NMD at codon 127 (β127) were also tested. In addition, to assess whether the effect on the pre-mRNA levels is PTC-specific or dependent on the PTC position, the steady-state levels of β-globin transcripts bearing NMD-sensitive PTCs at different locations (codon 26, β26; or codon 62, β62) were quantified. To assess if the presence of a PTC could induce decreased stability of nonsense-mutated β-globin pre-mRNAs, the half-lives of β39 and βWT pre-mRNA levels were assessed by RT-qPCR quantification following transcription inhibition by actinomycin D. Moreover, in order to verify whether the PTC effect is cell type-specific, β-globin RNA levels expressed in HeLa cells stably transfected with either a β-globin construct bearing a PTC at codon 39, or a βWT construct, were also quantified. Finally, alterations on β-globin mRNA splicing due to reading frame-independent effects were surveyed by using RT-qPCR, to determine the level of the pre-mRNA containing either intron 1 or intron 2. In addition, to dismiss alterations in the splice site choice, β-globin mRNAs were analysed by 3'-rapid amplification of cDNA ends followed by sequencing.

The results presented in this thesis in section III.3 - *Analysis of the nonsense-mutated β-globin transcripts processing status in erythroid cells* - were published in a peer-reviewed international scientific journal: Ana Morgado, Fátima Almeida, Alexandre Teixeira, Ana Luísa Silva, Luísa Romão (2012) Unspliced precursors of NMD-sensitive β-globin transcripts exhibit decreased steady-state levels in erythroid cells. *PLoS ONE* 7(6): e38505.

III.1. Establishment of Mouse Erythroleukemia Cell Lines with Stably Integrated Human β-globin Genes

With the aim to investigate if the presence of a nonsense codon in a transcript could affect its nuclear metabolism, in this study, we generated stably transfected MEL cell lines expressing the wild-type human β-globin gene, or a β-globin gene variant carrying a nonsense mutation at codon 39 (β39, CAG→UAG), which is a well-characterized β-globin NMD substrate in erythroid as well

as in non-erythroid cells (Thermann et al., 1998; Zhang et al., 1998; Romão et al., 2000; Neu-Yilik et al., 2011). Each human β-globin gene was cloned into the p158.2 vector, as previously described, where it is expressed under the transcriptional control of the corresponding promoter and the HS-2 enhancer element of the LCR (Romão et al., 2000). To select cell line clones for further studies, the integration of the intact human β-globin gene in the murine genome was verified by Southern blot analysis (Figure III.1, A). From the different MEL cell lines analysed, we have chosen, for further analyses, clones showing a single band of hybridization corresponding in size to the restriction segment of the transgene (βWT #146, βWT #147, βWT #154, βWT #156, βWT #166, β39 #241, β39 #249, β39 #252, β39 #268, and β39 #271). In these selected clones, accurate evaluation of the human β-globin transgene copy number was performed by quantitative PCR using the endogenous diploid Thy1 gene as a copy number reference. Quantitative PCR results from these clones show that the stable insertion of β-globin genes generated MEL cell lines presenting a wide range of variability in the copy number of the transgene (4 to 50 copies) (Figure III.1, B).

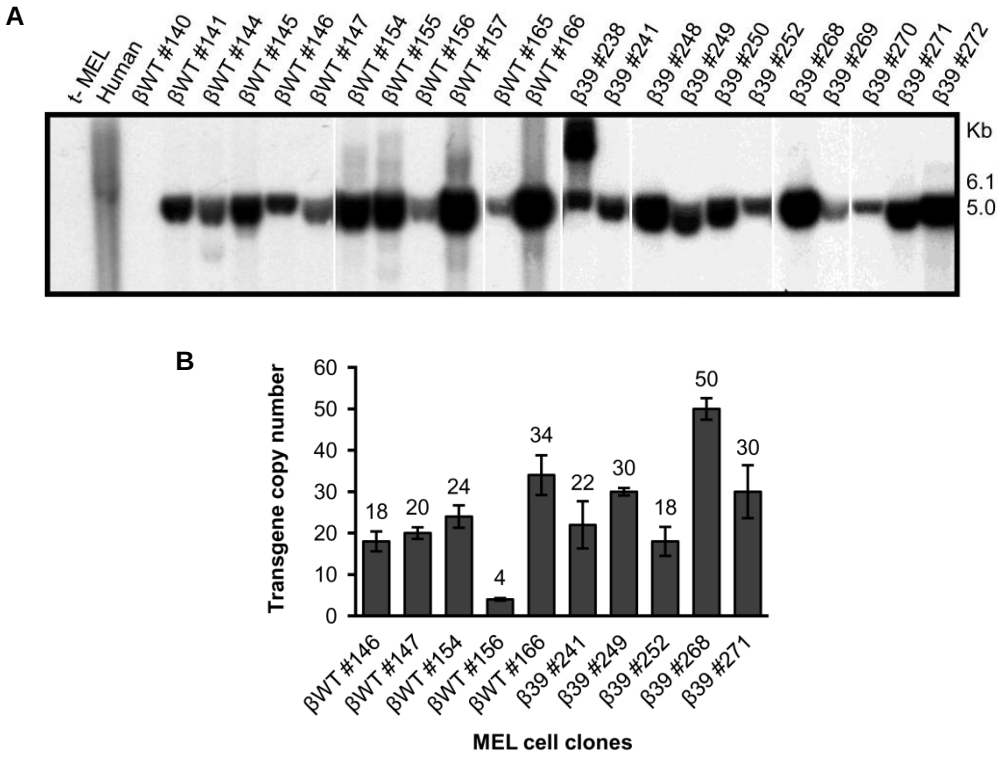

Figure III.1 Human β-globin transgene integrity and copy number analysis in stably transfected MEL cell clones. (A) Representative Southern blot analysis of DNA from MEL cell clones stably transfected with wild-type (βWT) or nonsense-mutated (β39; CAG→UAG) human β-globin gene constructs. Genomic DNA was extracted from MEL cells transfected with a β-globin construct as specified above each lane, where each number indicates an independent cell clone. Untransfected MEL (t- MEL) and human genomic DNA were used as negative and positive controls, respectively. DNA was digested with EcoNI plus KpnI enzymes and blots were hybridized with a [α-^{32}P]dCTP-labelled probe derived from the human β-globin gene that recognizes a 5.0 kb fragment integrated in the murine genome or a 6.1 kb fragment in the human genomic DNA. **(B)** Transgene copy number for each selected MEL cell clone was determined by quantitative PCR using primers specific for human β-globin gene and the endogenous murine Thy1 gene. Quantification was performed by the relative standard curve method. Chart shows the mean ± standard deviation qPCR data from three independent experiments.

III.2. Subcellular Localization of Human β-globin Transcripts Bearing a Nonsense Codon in Erythroid Cells

III.2.1. The subcellular localization of β-globin depends on the presence of a nonsense codon and transgene copy number

In order to analyse, by FISH, the subcellular localization of β-globin transcripts stably expressed in MEL cells, we selected three independent clones from each βWT and β39 MEL cell lines, harbouring 18 to 34 copies of the transgene (Table III.1). To obtain high-level expression of the human β-globin genes, MEL cells were induced to undergo terminal erythroid differentiation by addition of DMSO to the culture medium for 4 days (Antoniou, 1991). FISH analysis was also performed on the parental untransfected (t-) MEL C88 cell line. Uninduced and induced MEL cells were fixed, permeabilized, and hybridized under non-denaturing conditions with digoxigenin--labelled RNA probes. These riboprobes were produced using the full-length transcribed sequence of the human β-globin gene (βtotal, see Figure III.2, A). The sites of hybridization were detected using Cy3-coupled anti-digoxigenin antibodies and visualized by confocal microscopy.

Table III.1 Human β-globin transgene copy number in MEL cell clones selected for study.

Transgene copy number for each MEL cell clone stably transfected with wild-type (βWT) or nonsense mutation-containing (β39) β-globin genes was determined by quantitative PCR. Shown here are the mean transgene copy numbers for each MEL cell clone ± standard deviations from three independent experiments.

MEL cell clone	*Transgene copy number*
βWT #146	18 ± 2
βWT #154	24 ± 3
βWT #166	34 ± 5
β39 #252	18 ± 3
β39 #249	30 ± 1
β39 #271	30 ± 6

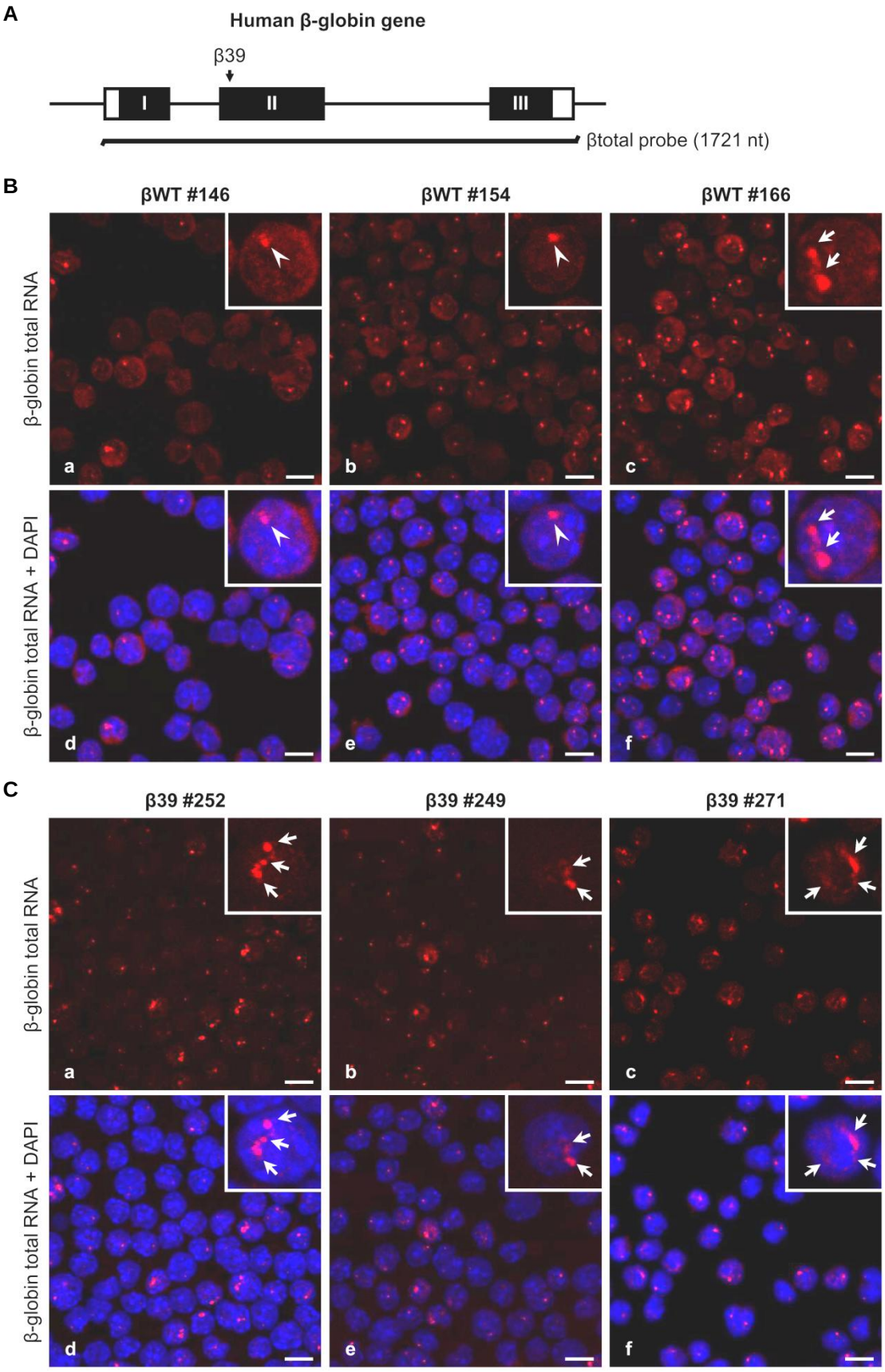

Figure III.2 Beta-globin transcripts subcellular localization depends on the presence of a PTC and probably on the transgene copy number. (A) Schematic representation of the human β-

-globin genes stably transfected in MEL cell lines. The black and white rectangles and lines depict β-globin exons, untranslated sequences and introns, respectively. The vertical arrow represents the position of the nonsense mutation (CAG→UAG) at codon 39 (β39). Localization and extent in nucleotides (nt) of the FISH probe comprising the full-length human β-globin transcript (βtotal) is shown below. **(B and C)** Representative images of fluorescence *in situ* visualization of β-globin total RNA stably expressed in MEL cells. Wild-type (βWT) and mutant (β39) β-globin MEL cell clones were fixed, permeabilized and hybridized to digoxigenin--labelled genomic βtotal riboprobes. MEL cells were stained with Cy3-coupled anti-digoxigenin antibody to visualize the sites of hybridization of the RNA probe (a-c, red staining) and DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI) dye (blue staining). (d-f) depict the superimposition of red and blue images. Shown here are deconvolved optical sections projected onto a single plane ($z > 10$, $\Delta z = 0.7$ µm). **(B)** In βWT MEL cells β-globin localization shows an accumulation of RNA probably at the site of transcription (red, arrowhead) and additional staining in the cytoplasm. In βWT MEL #166, β-globin RNA--FISH also results in the appearance of additional intranuclear foci (red, c; arrows). **(C)** β39 MEL cells show nuclear localization of β-globin RNA with a speckled-like pattern and no staining in the cytoplasm (red, arrows). Cells were induced to undergo erythroid differentiation for 4 days. The scale bar represents 10 µm.

The βtotal FISH probe enabled the simultaneous staining of β-globin pre-mRNA and mRNA in MEL cells (red staining, Figure III.2, B and C). In addition, labelling of total DNA with DAPI allowed the distinction between intranuclear and cytoplasmic localization of the human β-globin RNA (blue staining, Figure III.2, B and C). FISH analysis shows that induced βWT MEL cell clones (#146, #154 and #166) contain a fluorescent focus in the nucleus, probably corresponding to β-globin transcripts accumulation near the transcription site, and additional cytoplasmic staining (Figure III.2, B). Interestingly, βWT #166 cells also present smaller spots surrounding the brighter nuclear focus (Figure III.2, B, c). On the other hand, induced β39 MEL cell clones (#252, #249 and #271) present no cytoplasmic accumulation, as expected for mRNA committed to rapid decay, and show discrete hybridization sites throughout the nucleoplasm with a spotty-like pattern (Figure III.2, C). Observation of sequential confocal optical sections confirmed that PTC-containing β-globin RNA is detected as multiple foci within the nucleus of all β39 MEL cell clones. Finally, uninduced stably transfected MEL cells and untransfected MEL cells are devoid of any significant hybridization signals (data not shown), which confirms the specificity of the βtotal FISH probe.

As readily apprehended in Figure III.2, stably transfected MEL cells present a heterogeneous staining of the β-globin transcripts in the nucleus. Therefore, qualitative analysis was performed in a large quantity of cells from each MEL cell clone. Multiple optical sections of randomly selected fields were projected onto a single plane and each cell was assigned to a category of intranuclear hybridization signal pattern: speckled foci, single focus or without staining. FISH results for βWT or β39 MEL cell clones were subsequently shown as percentage of cells with a particular hybridization signal pattern (Figure III.3). βWT MEL clones present 72 to 83% of cells with β--globin RNA accumulated in a nuclear focus, whereas 47 to 54% of cells from β39 MEL clones show this hybridization pattern. Regarding the speckled-like pattern, all three β39 MEL cell clones present 9 to 46% of cells with speckled-like foci, while only one βWT MEL clone, #166, presents cells within this hybridization signal category. Interestingly, βWT#166 MEL cell clone contains the highest transgene copy number (34 copies). This might indicate that the speckled-like nuclear localization of β-globin transcripts might be associated with the transgene copy number. However, all three β39 MEL cell clones present a speckled-like FISH pattern in a subset of cells, regardless

of transgene copy number (18-30 copies), which suggests that the presence of the PTC presence, *per se,* affects the intranuclear localization of the β-globin transcripts in MEL cells.

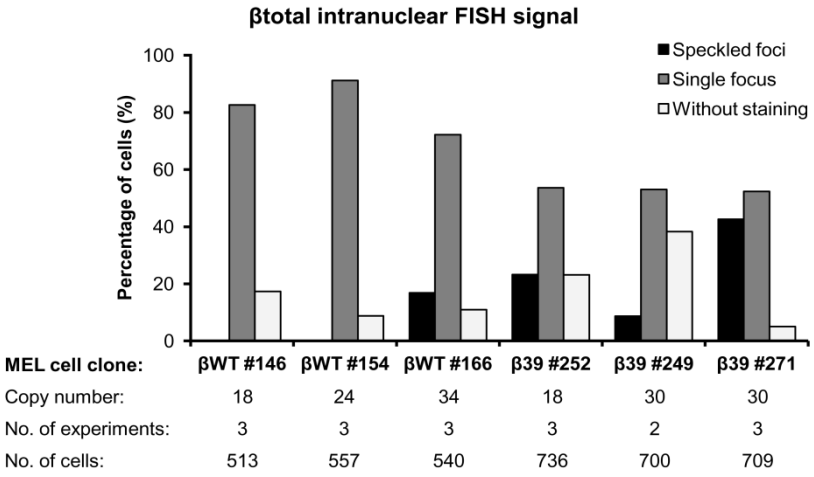

Figure III.3 Summary of fluorescence *in situ* hybridization results for human β-globin total RNA intranuclear localization in MEL cell lines. Chart displays the percentage of cells within the following categories of βtotal probe intranuclear hybridization patterns: speckled foci, single focus and without staining, for each βWT and β39 MEL cell clone. Transgene copy number, number of FISH experiments and number of analysed cells are indicated below. No.: number.

III.2.2. Beta-globin transcripts presenting a nuclear speckled-like FISH pattern do not colocalize with the SC-35 domains

Next, we aimed to characterize the localization of β-globin transcripts, in the context of nuclear structure. While single nuclear focus have already been described as corresponding to transcript accumulation sites near the site of transcription (Lawrence et al., 1989; Dirks et al., 1995; Jolly et al., 1998; Custódio et al., 1999; Mühlemann et al., 2001), the structural basis of dispersed β--globin transcripts foci within the nucleus is unclear. These multiple foci might consist of posttranscriptional RNA tracks accumulating in a variety of intranuclear compartments. One of such compartments might be the SC-35 domains or nuclear speckles, which are enriched in snRNPs and many other transcription- and splicing-related proteins (see section I.1.2.1.6). Therefore, as a preliminary investigation of the structural basis of the β-globin speckled-like nuclear accumulations, we examined the colocalization of β-globin transcripts relatively to the SC--35 domains in two stably transfected MEL cell clones: βWT #154 (with a single nuclear focus) and β39 #271 (with speckled-like foci).

The formerly described FISH procedure using βtotal riboprobe (red staining, Figure III.4, A and D), combined with SC-35 immunofluorescence staining using mouse anti-SC-35 and anti-mouse--AlexaFluor488 antibodies (green staining, Figure III.4, B and E), was used to study the localization of β-globin total RNA relatively to the nuclear speckles domain. Superimposition of the red and green images shows no colocalization of the SC-35 domains with the β-globin transcripts distributed throughout the nucleoplasm in multiple foci (Figure III.4, F). Hence, nuclear PTC--containing β39 nonsense transcripts in MEL cells might localize near a subnuclear domain other than the SC-35 domains.

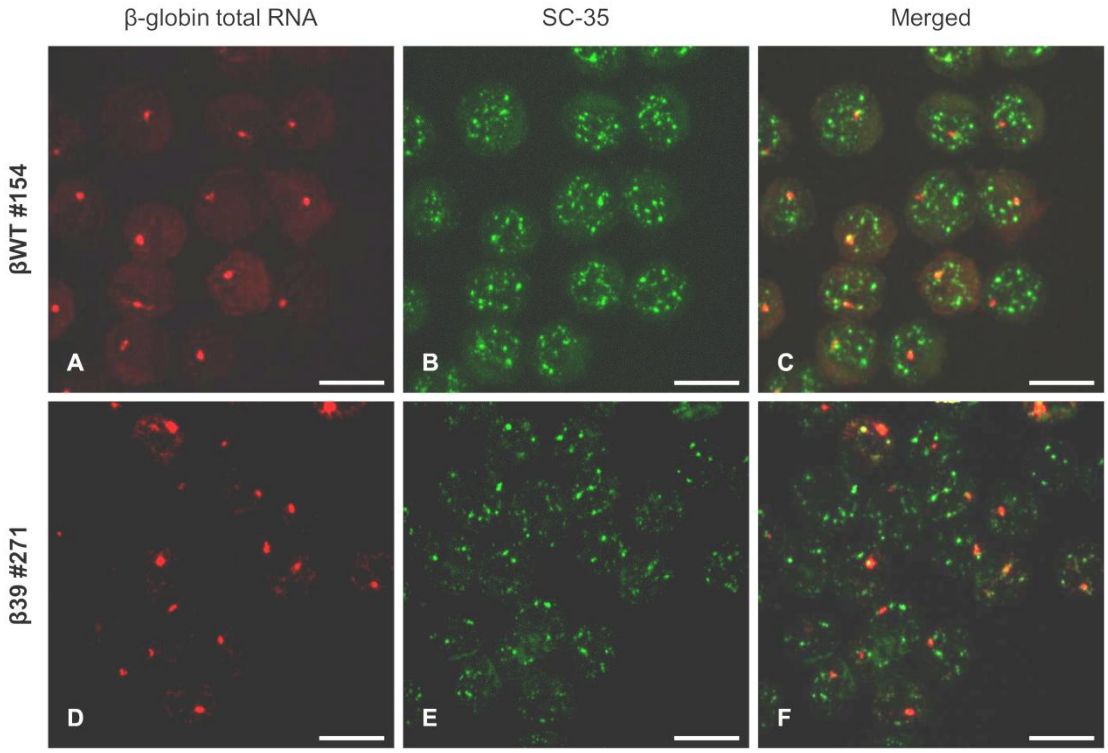

Figure III.4 Beta-globin transcripts presenting a nuclear speckled-like pattern do not colocalize with the SC-35 domains. Representative images from colocalization studies of intranuclear β-globin total RNA and SC-35 domains in MEL cells. Wild-type (βWT) and nonsense codon-bearing (β39) β--globin transcripts stably expressed in MEL cell clones were hybridized to βtotal probe labelled with digoxigenin. MEL cells were stained with anti-digoxigenin-Cy3 antibody, and mouse anti-SC-35 plus anti--mouse-AlexaFluor488 antibodies to visualize the hybridization sites of the RNA probe (red; A, D) and SC-35 localization (green; B, E), respectively. Shown here are deconvolved optical sections projected onto a single plane ($z > 15$, $\Delta z = 0.5$ µm). C and F depict the superimposition of red and green images. Beta39 MEL cells show no colocalization of nuclear β-globin RNA with a speckled-like localization and the SC-35 domains (F). MEL cells were induced to undergo erythroid differentiation for 4 days. The scale bar represents 10 µm.

III.2.3. Beta-globin speckled-like intranuclear RNA localization is due, at least in part, to unspliced transcripts

To assess whether intranuclear-accumulating β-globin RNA species contained unspliced precursors, fluorescence *in situ* visualization was performed using an intronic FISH probe. A digoxigenin-labelled riboprobe was produced using the human β-globin intron 2 sequence (βintron2, Figure III.5, A), specifically hybridizing to unspliced β-globin transcripts. Subsequently, the same set of βWT and β39 MEL cell clones and untransfected MEL cells was analysed by FISH analysis as formerly described.

The analysis with the β-globin intron 2 FISH probe yielded similar results to those obtained with the βtotal probe (Figure III.2 versus Figure III.5). Indeed, FISH results shows that induced βWT MEL cell clones (#146, #154 and #166) present a single nuclear focus, probably corresponding to the transcription site (Figure III.5, B). No additional staining in the cytoplasm was observed, confirming that βintron2 probe only hybridize to unprocessed β-globin RNA. As before, βWT #166 cells present a nuclear focus with additional satellite dots (Figure III.5, B, c). Furthermore, induced β39 MEL cell clones #252, #249 and #271 show a speckled-like hybridization pattern for the PTC--bearing β-globin pre-mRNA (Figure III.5, C). Uninduced stably transfected MEL cells and untransfected MEL cells were devoid of any significant hybridization signals. In addition, as negative controls for the immunocytochemical staining protocol, induced MEL cell clones were also tested without βintron2 probe or without anti-digoxigenin-Cy3 antibody and showed no fluorescent signal (data not shown).

Qualitative analysis of β-globin hybridization signals using the βintron2 probe for each clone of the βWT and β39 MEL cell lines was performed as described before, allowing the display of FISH results for many cells (Figure III.6). All β39 MEL cell clones present intranuclear speckled-like hybridization patterns for the β-globin unspliced RNA in a subset of cells (from 1 to 28%), whereas only one βWT MEL cell clone, #166, show 14% of cells within this signal category. FISH results using the βintron2 probe indicate that at least the unspliced β-globin RNA presents a speckled-like intranuclear localization in MEL cells. As previously shown for total β-globin RNA, transcript localization might be influenced by transgene copy number. However, the presence of a PTC, *per se*, is able to affect the nuclear pattern of transcript accumulation.

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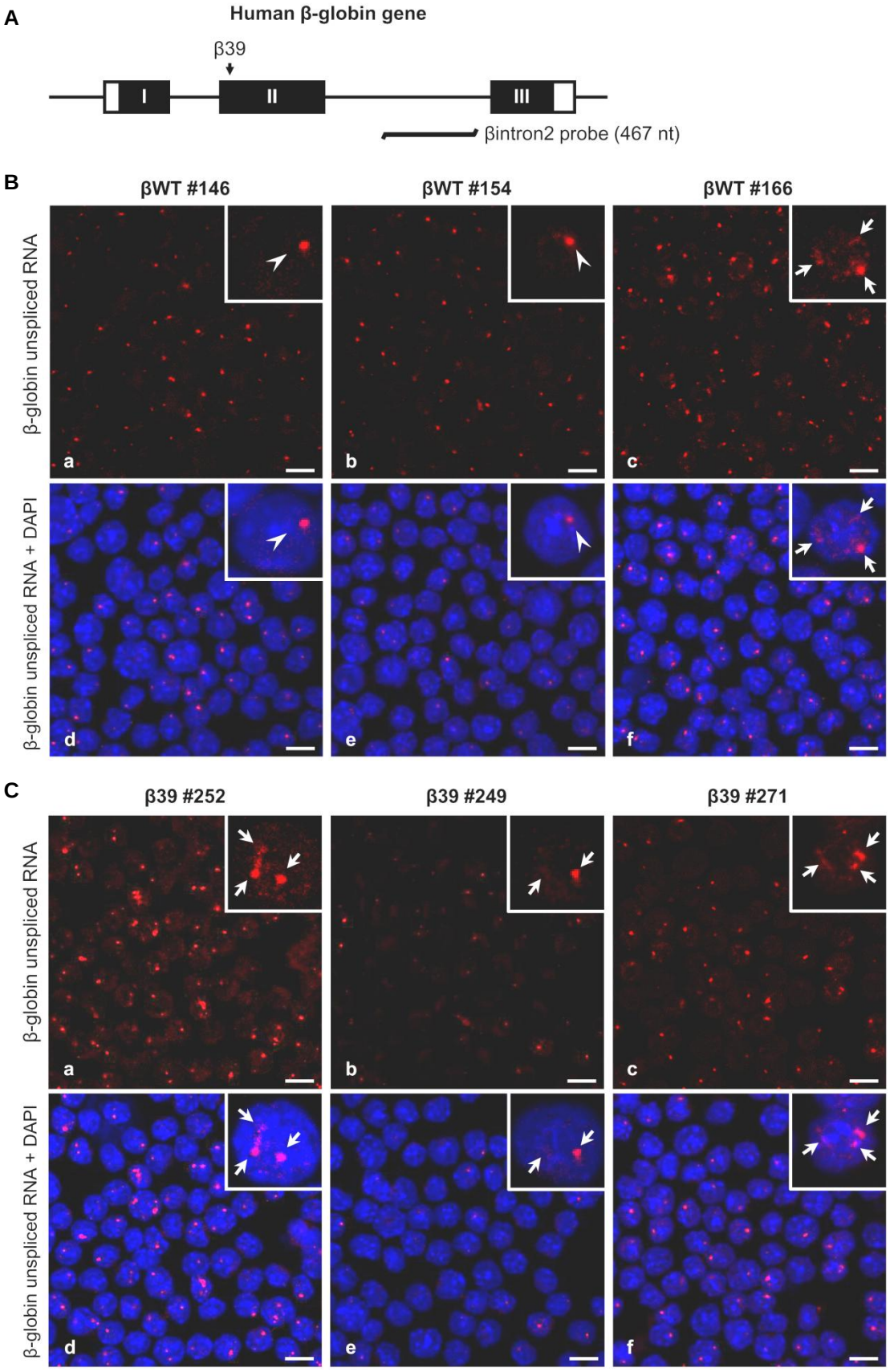
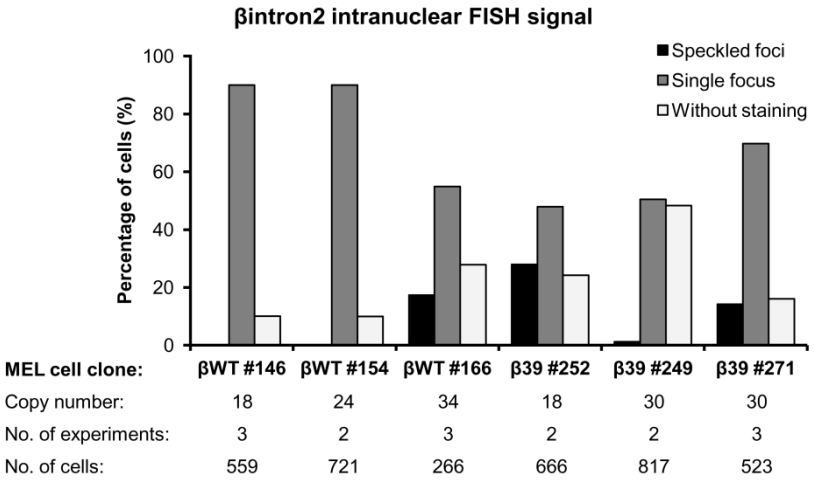


Figure III.5 Beta-globin speckled-like intranuclear RNA localization is due, at least in part, to unspliced transcripts expressed in MEL cell lines. (A) Schematic representation of the human

β-globin genes stably transfected in MEL cell lines. The black and white rectangles and lines depict β-globin exons, untranslated sequences and introns, respectively. The vertical arrow represents the position of the nonsense mutation (CAG→UAG) at codon 39 (β39). Localization and extent in nucleotides (nt) of the FISH probe comprising the intron 2 human β-globin transcript (βintron2) is shown below. **(B and C)** Representative images of fluorescence *in situ* visualization of β-globin intron 2 in MEL cell clones stably transfected with wild-type and nonsense-mutated β-globin genes. Wild-type (βWT) and mutant (β39) β-globin MEL cell clones were fixed, permeabilized and hybridized to digoxigenin-labelled intronic βintron2 riboprobes. MEL cells were stained with Cy3-coupled anti-digoxigenin antibody to visualize the sites of hybridization of the RNA probe (red staining, a-c) and DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI) dye (blue staining). (d-f) depict the superimposition of red and blue images. Shown here are deconvolved optical sections projected onto a single plane ($z > 10$, $\Delta z = 0.7$ µm). **(B)** In βWT MEL cells β-globin localization shows an accumulation of unspliced RNA probably at the site of transcription (red, arrowhead). In βWT MEL #166, β-globin unspliced RNA-FISH also results in the appearance of additional intranuclear foci (red, c; arrows). **(C)** β39 MEL cells show nuclear localization of β-globin unspliced RNA with a speckled-like pattern (red, arrows). MEL cells were induced to undergo erythroid differentiation for 4 days. The scale bar represents 10 µm.



MEL cell clone:	βWT #146	βWT #154	βWT #166	β39 #252	β39 #249	β39 #271
Copy number:	18	24	34	18	30	30
No. of experiments:	3	2	3	2	2	3
No. of cells:	559	721	266	666	817	523

Figure III.6 Summary of fluorescence *in situ* hybridization results for β-globin unspliced RNA intranuclear localization in MEL cell lines. Chart displays the percentage of cells within the following categories of βintron2 probe intranuclear hybridization patterns: speckled foci, single focus and without staining, for each βWT and β39 MEL cell clone. Transgene copy number, number of FISH experiments and number of analysed cells are indicated below. No.: number.

III.3. Analysis of the Nonsense-mutated β-globin Transcripts Processing Status in Erythroid Cells

III.3.1. Human β-globin pre-mRNAs carrying a nonsense mutation accumulate at low levels

To assess the effect of the PTC on the nuclear metabolism of the β-globin transcripts, we next compared the steady-state expression levels of the βWT and NMD-sensitive nonsense codon--bearing β39 genes, using the same set of MEL cell clones previously analysed by FISH, and an additional β39 MEL cell clone harbouring 22 ± 6 copies of the transgene (β39 #241). The selected MEL cell clones were induced to undergo erythroid differentiation by DMSO and the respective total RNA was extracted for quantification of human β-globin mRNA and pre-mRNA steady-state levels by ribonuclease protection assays (RPA).

Using a [α-^{32}P] CTP-labelled riboprobe spanning β-globin intron 1 and exon 2 sequences (Figure III.7, A), the pre-mRNA as well as the processed mRNA from total RNA were simultaneously detected and quantified (Figure III.7, B). The hybridization signals of both β-globin spliced and unspliced transcripts from all MEL cell clones were normalized to the murine α-globin mRNA signal produced by the respective riboprobe, and estimated as a percentage of the normalized value for the βWT #146 clone (arbitrarily considered 100%). Our results show that the β39 MEL cell clones exhibit reduced β-globin mRNA levels, in agreement with rapid decay by NMD, as expected (Thermann et al., 1998; Zhang et al., 1998; Romão et al., 2000; Neu-Yilik et al., 2011) (Figure III.7, C). Remarkably, all β39 MEL cell clones display a significant 3- to 14-fold reduction in the pre-mRNA steady-state levels relatively to the reference βWT #146 pre-mRNA level, and relatively to the pre-mRNA level from the corresponding βWT clone with equivalent transgene copy number (Figure III.7, D). These results suggest that the presence of a NMD-sensitive nonsense codon can affect the metabolism of the unspliced β-globin transcripts in MEL cells nuclei, independently of the transgene copy number.

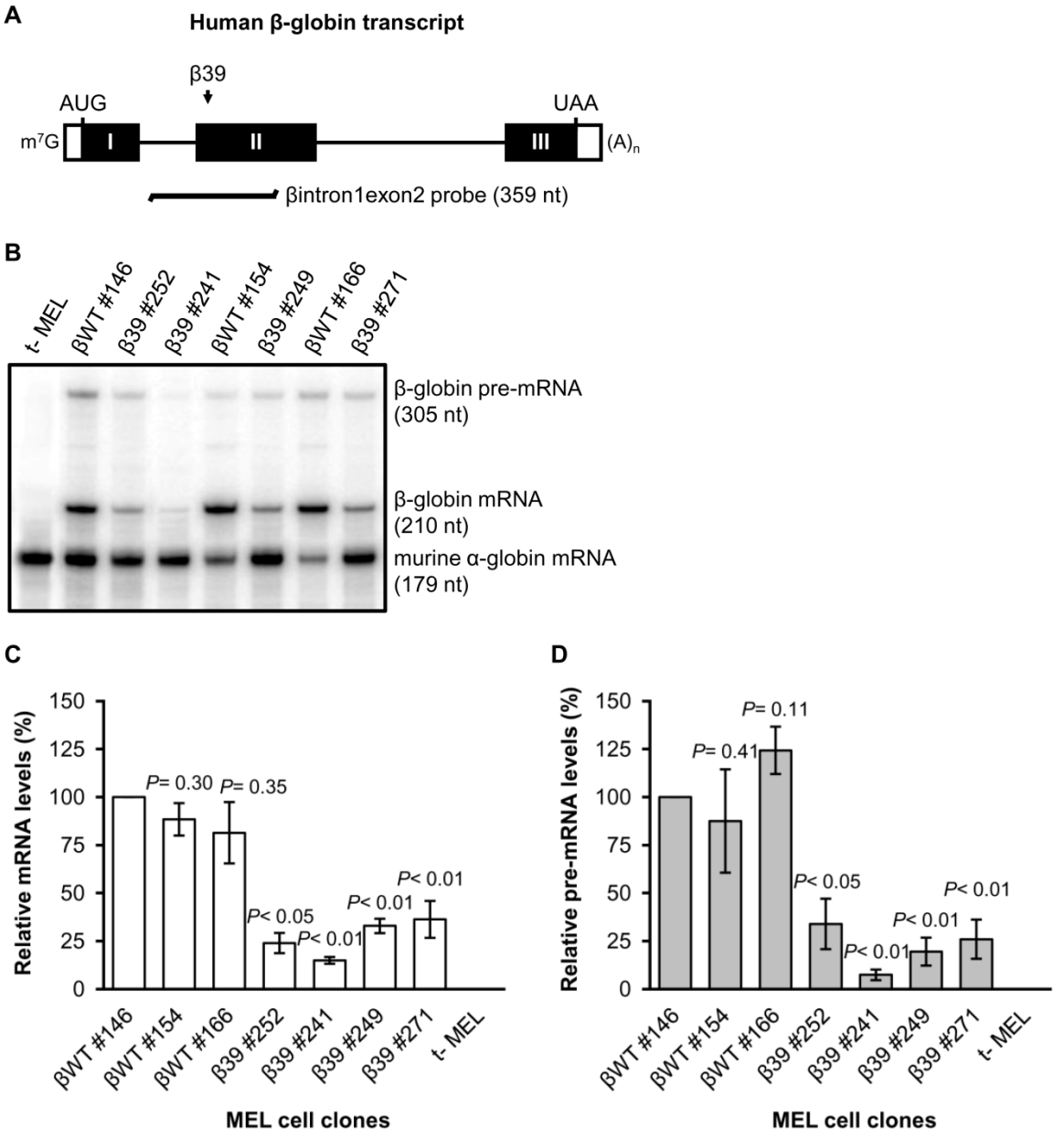


Figure III.7 Human β-globin pre-mRNAs carrying a nonsense mutation accumulate at low levels in MEL cells. (A) Schematic representation of the test human β-globin constructs stably expressed in MEL cell lines. The black and white rectangles and lines depict exons, untranslated sequences and introns, respectively. The vertical small arrow represents the position of the nonsense mutation (CAG→UAG) at codon 39 (β39). Position of initiation (AUG) and termination (UAA) codons, as well as cap structure (m^7G) and poly(A) tail [(A)n] are also represented. Localization and length in nucleotides (nt) of the probe comprising intron 1-exon 2 sequences (βintron1exon2 probe) for detection and quantification of the human β-globin RNA by ribonuclease protection assays (RPA) is shown below the diagram. **(B)** MEL cells were stably transfected with a test human β-globin construct as specified in each lane, where each number indicates an independent MEL cell clone. After erythroid differentiation induction, steady-state total RNA from either transfected or untransfected (t-) MEL cells was isolated and analysed by RPA using specific probes for human β- and mouse α-globin transcripts (see Materials and Methods). The protected bands corresponding to the human β-globin pre-mRNA and mRNA and mouse α-globin mRNA are shown on the right, and the corresponding intensities were quantified by phosphorimaging. The level of mRNA and pre-mRNA from each β-globin allele was normalized to the level of endogenous mouse α-globin in order to control for RNA recovery and erythroid differentiation induction. Normalized values were then calculated as the percentage of wild-type β-globin (βWT) mRNA from cell line #146 (arbitrary defined as 100%). The values exposed on the

graphs **(C)** and **(D)** are representative of three independent experiments, and are plotted for each construct showing the mean value and standard deviations. Statistical analysis was performed using the Student's t--test (unpaired, two-tailed).

III.3.2. The low levels of the β39 pre-mRNAs are PTC--specific

In order to discard a pleiotropic effect of the β39 nonsense mutation that, for example, could disrupt an exonic splicing regulatory element surrounding codon 39, we generated MEL cell pools stably expressing a β-globin construct bearing a different mutation at codon 39 – a missense mutation (β39missense; see Materials and Methods). After erythroid cell differentiation induction, the mRNA levels were determined by RPA, as before, using probes comprising part of the human β-globin intron 2 and exon 3 or murine α-globin mRNA sequences. Results were compared to those of MEL cell pools stably expressing the βWT or β39 genes (Figure III.8, A-C). Our data show that the β39missense mRNA level accumulates at about 72% of the βWT mRNA, while β39 mRNAs accumulate at about 9% of the normal control (Figure III.9, C). As expected, these results show that the missense mutation at codon 39 does not significantly affect the corresponding steady-state mRNA accumulation level (P=0.12).

In parallel, β39missense pre-mRNA levels were also quantified by RPA using a probe specific for the second intron (βintron2 probe; Figure III.8, D), whose intensity was normalized with the murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA signal generated by the respective riboprobe protection and compared to the βWT and β39 controls (Figure III.8, E and F). These analyses revealed that the β39missense pre-mRNA accumulates at about 68% of the βWT pre-mRNA (P=0.12), while β39 unspliced mRNA accumulates at about 40% of the normal (P<0.01), showing that, contrary to what occurs with the β39 pre-mRNA, the β39missense pre--mRNA is not significantly decreased. Taken together, these results clearly show that the steady--state decreased levels of β39 pre-mRNA are not due to a pleiotropic effect of the mutation at position 39, but, instead, they seem to be PTC-specific.

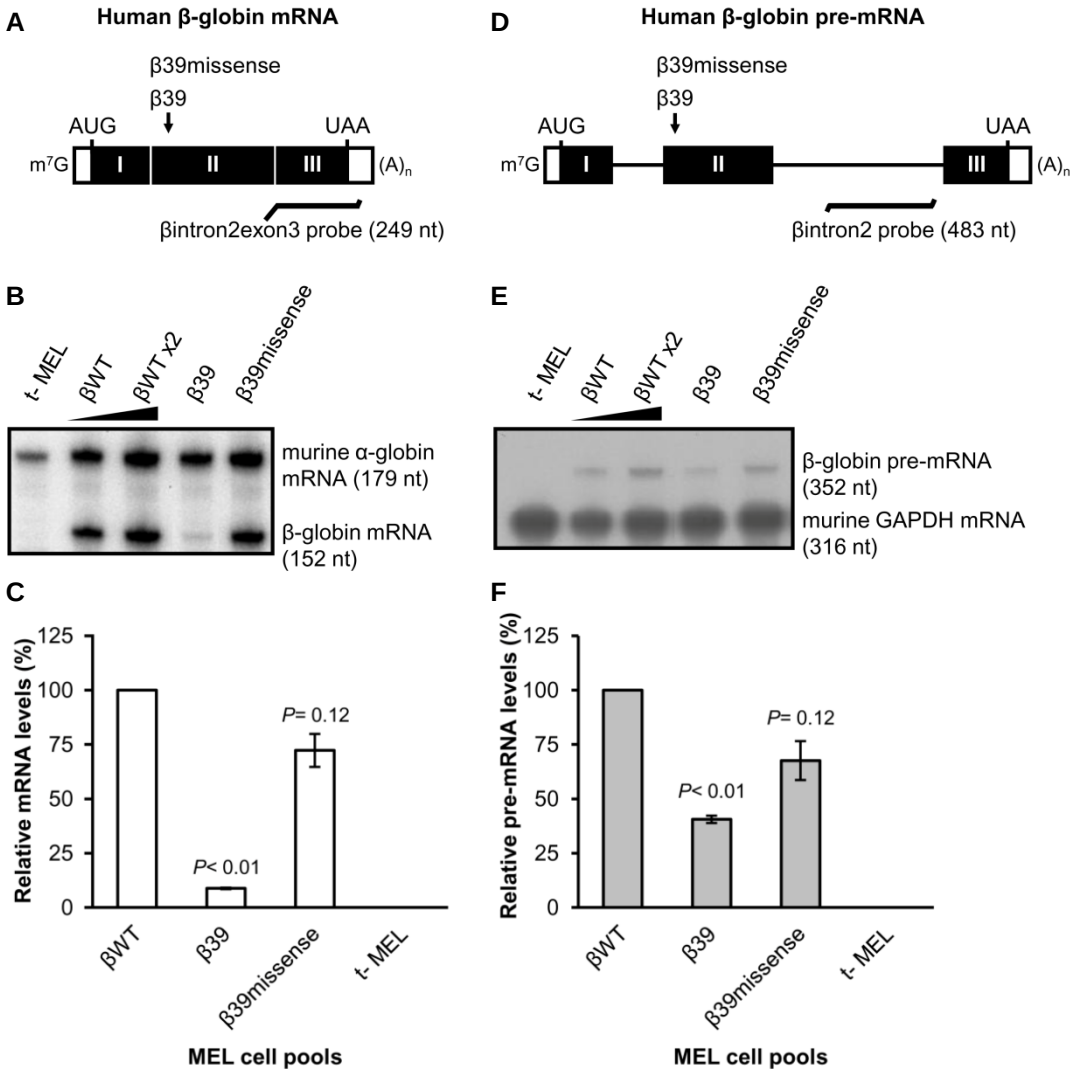


Figure III.8 The low levels of the β39 pre-mRNA are not due to the disruption of a regulatory element encompassing codon 39. **(A)** Schematic representation of the test human β-globin mRNA stably expressed in MEL cell pools. The black and white rectangles depict exons and untranslated regions, respectively. The vertical small arrow represents the position of the nonsense (CAG→UAG) or missense (CAG→GAG) mutation at codon 39 (β39 and β39missense respectively). Position of initiation (AUG) and termination (UAA) codons, as well as cap structure (m^7G) and poly(A) tail [(A)n] are also represented. Localization and length in nucleotides (nt) of the probe comprising intron 2-exon 3 sequences (βintron2exon3 probe) for detection and quantification of the human β-globin RNA by ribonuclease protection assays (RPA) is shown below the diagram. **(B)** MEL cells were stably transfected with a test human β-globin construct as specified above each lane. A 2-fold RNA sample (βWT x2) from MEL cells transfected with the βWT gene was also assayed to demonstrate that the experimental RPA was carried out in probe excess. After erythroid differentiation induction, steady-state total RNA from either transfected or untransfected (t-) MEL cells was isolated and analysed by RPA using specific probes for human β- and mouse α-globin mRNAs (see Materials and Methods). The protected bands corresponding to the human β -globin and mouse α-globin mRNAs are shown on the right, and the corresponding intensities were quantified by phosphorimaging. The level of mRNA from each β-globin allele was normalized to the level of endogenous mouse α-globin in order to control for RNA recovery and erythroid differentiation induction. Normalized values were then calculated as the percentage of wild-type β-globin mRNA. **(C)** The percentage mRNA values were plotted for each construct, and standard deviations from three independent experiments are shown. Statistical analysis was performed using Student's t-test (unpaired, two-tailed). **(D)** Schematic representation of the test human β--globin pre-mRNA stably expressed in MEL cell pools as in (A). Localization and length in nucleotides (nt) of the probe comprising part of intron 2 (βintron2 probe) for detection and quantification of the human β-globin

pre-mRNA by RPA is shown below the diagram. **(E)** After erythroid differentiation induction, steady-state total RNA from either transfected or untransfected (t-) MEL cells was isolated and analysed by RPA using specific probes for human β-globin pre-mRNA and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (see Materials and Methods). The corresponding protected bands are shown on the right, and their intensities were quantified by phosphorimaging as in (B). **(F)** The percentage pre-mRNA values were plotted for each construct, and standard deviations from three independent experiments are shown, as in (C).

III.3.3. The decreased β-globin pre-mRNA levels are specific for transcripts carrying NMD-sensitive nonsense codons

Considering the formerly observed downregulation of unspliced β-globin transcripts carrying a nonsense mutation at codon 39, we next asked whether this effect occurs in other transcripts carrying a different PTC. We thus established two different MEL cell pools stably expressing the human β-globin gene carrying a NMD-sensitive nonsense mutation at codon 26 (exon 1; β26) or at codon 62 (exon 2; β62) (Inácio et al., 2004). The β26 and β62 mRNAs were previously found to accumulate at reduced steady-state levels when compared to the wild-type β-globin mRNA in erythroid and non-erythroid cells (Romão et al., 2000; Inácio et al., 2004). These transcripts are NMD-sensitive because the respective PTCs are located more than 50 to 54 nt upstream to the 3'-most exon-exon junction and when the ribosome reaches the PTC, the terminating complex can interact with the downstream EJC *via* UPF1 (Inácio et al., 2004; Silva and Romão, 2009). Regarding β62 MEL cell pools, we were able to isolate two independent MEL cell pools (β62 #1 and β62 #2). After erythroid cell differentiation induction, the transgene mRNA levels were determined by RPA as before, using probes comprising part of the human β-globin intron 2 and exon 3 or murine α-globin mRNA sequences, and results were compared to those of MEL cell pools stably expressing the βWT or β39 genes (Figure III.9, A and B).

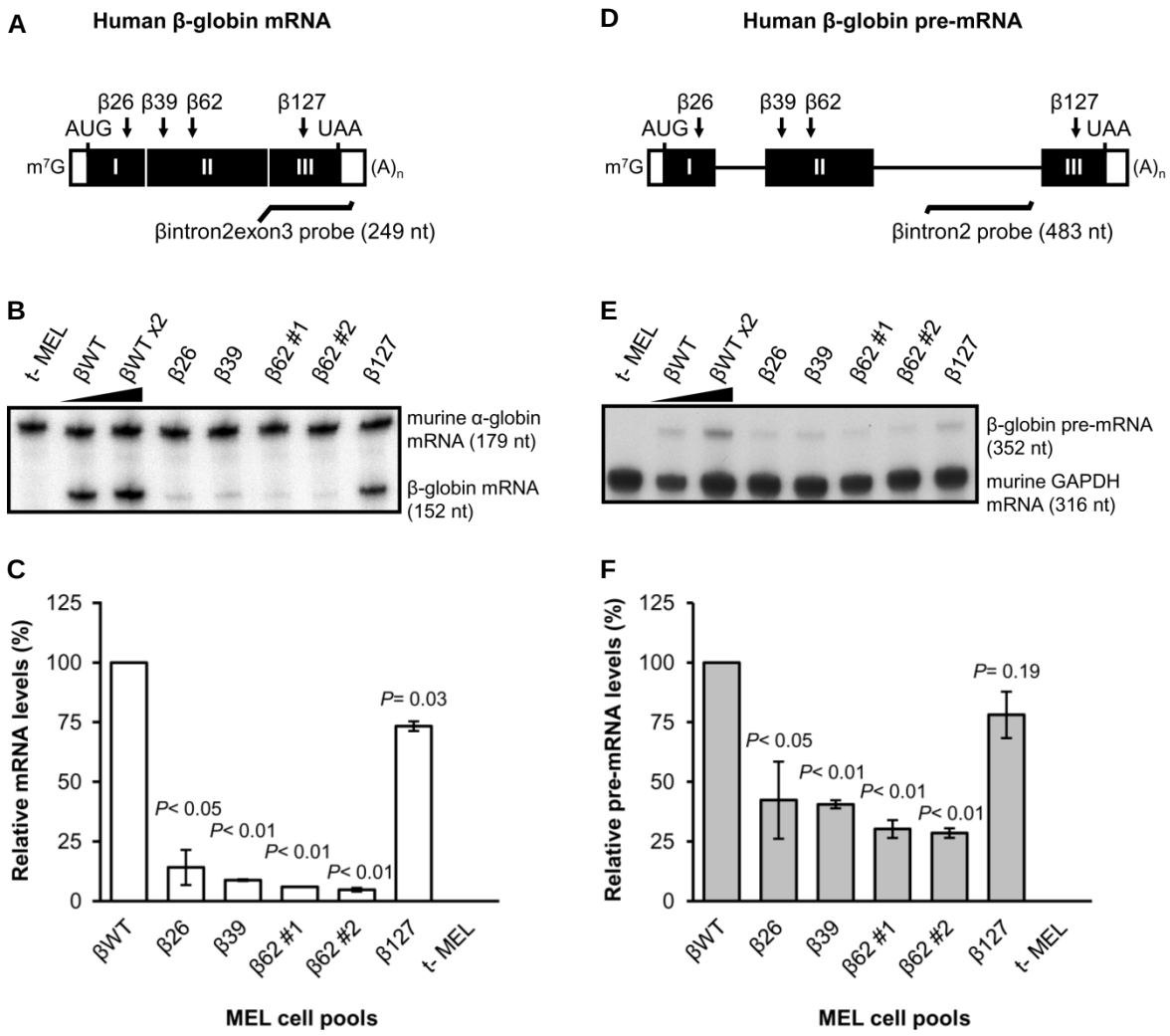


Figure III.9 The decreased β-globin pre-mRNA levels are specific for transcripts carrying NMD-competent nonsense mutations. **(A)** Schematic representation of the test human β-globin mRNA stably expressed in MEL cell pools. The black and white rectangles depict exons and untranslated regions, respectively. The vertical small arrows represent the position of the nonsense mutations at codon 26 (GAG→UAG; β26), 39 (CAG→UAG; β39), 62 (GCU→UAG; β62) or 127 (CAG→UAG; β127). Position of initiation (AUG) and termination (UAA) codons, as well as cap structure (m^7G) and poly(A) tail [(A)n] are also represented. Localization and length in nucleotides (nt) of the probe comprising intron 2-exon 3 sequences (βintron2exon3 probe) for detection and quantification of the human β-globin RNA by ribonuclease protection assays (RPA) is shown below the diagram. **(B)** MEL cells were stably transfected with a test human β-globin construct as specified above each lane. A 2-fold RNA sample (βWT x2) from MEL cells transfected with the βWT gene was also assayed to demonstrate that the experimental RPA was carried out in probe excess. After erythroid differentiation induction, steady-state total RNA from either transfected or untransfected (t-) MEL cells was isolated and analysed by RPA using specific probes for human β- and mouse α-globin mRNAs (see Materials and Methods). The protected bands corresponding to the human β-globin and mouse α-globin mRNAs are shown on the right, and the corresponding intensities were quantified by phosphorimaging. The level of mRNA from each β-globin allele was normalized to the level of endogenous mouse α-globin in order to control for RNA recovery and erythroid differentiation induction. Normalized values were then calculated as the percentage of wild-type β-globin mRNA. **(C)** The percentage mRNA values were plotted for each construct, and standard deviations from three independent experiments are shown. Statistical analysis was performed using Student's t-test (unpaired, two-tailed). **(D)** Schematic representation of the test human β-globin pre-mRNA stably expressed in MEL cell pools as in (A). Localization and length in nucleotides (nt) of the probe comprising part of intron 2 (βintron2 probe) for detection and quantification of the human β-globin pre-mRNA by RPA is shown below the diagram. **(E)** After

erythroid differentiation induction, steady-state total RNA from either transfected or untransfected (t-) MEL cells was isolated and analysed by RPA using specific probes for human β-globin pre-mRNA and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (see Materials and Methods). The corresponding protected bands are shown on the right, and their intensities were quantified by phosphorimaging as in (B). **(F)** The percentage pre-mRNA values were plotted for each construct, and standard deviations from three independent experiments are shown, as in (C). Statistical analysis was performed using Student's t-test (unpaired, two-tailed).

According to our previously published data (Inácio et al., 2004), our results show that β26 and β62 mRNA levels of the corresponding MEL cell pools are strongly downregulated relatively to the βWT mRNA levels, presenting levels similar to those observed in the β39 MEL cell pools, meaning that they are induced to rapid decay, as expected (Figure III.9, B and C). These results indicate that under our experimental conditions, the cellular NMD machinery is functional.

At these experimental conditions, the pre-mRNA levels of the β26 and β62 MEL cell pools were quantified using a probe specific for the second intron (βintron2 probe; Figure III.11D), whose intensity was normalized with the murine GAPDH mRNA signal generated by the respective riboprobe protection (Figure III.9, E and F), as before. RPA analysis revealed that the β26 and β62 pre-mRNA steady-state levels are at about 40% and 30% of the normal control, respectively. These levels are significantly lower relatively to the βWT pre-mRNA ($P<0.05$ and $P<0.01$, respectively for β26 and β62), being comparable to that of β39 pre-mRNA (Figure III.9, E and F). These results clearly demonstrate that the reduced nonsense pre-mRNA levels phenotype in MEL cells is independent of the position of the PTC.

Knowing that the reduced nonsense pre-mRNA levels phenotype is PTC-specific, and independent of the PTC position, we next asked if it depends on NMD, e.g. PTC recognition. Thus, we also established a pool of MEL cells stably expressing the human β-globin gene carrying a nonsense mutation at codon 127 located at the 3'-most exon (β127) that does not induce NMD, as it is located downstream of the 3'-most exon-exon junction (Hall and Thein, 1994; Thermann et al., 1998; Zhang et al., 1998; Romão et al., 2000). The mRNA and pre-mRNA levels were quantified as before. Results show that β127 mRNA steady-state levels are at about 73% of the normal control (Figure III.9, B and C), showing that this transcript is not efficiently degraded by the NMD pathway, as expected. In parallel, β127 pre-mRNA levels were also quantified and compared to those of the normal control. Our data show that β127 pre-mRNA accumulates at about 78% of the βWT pre-mRNA (Figure III.9, E and F), being this difference not significant ($P=0.19$). Together, this full set of data shows that the decreased β-globin pre-mRNA levels phenotype is specific for transcripts carrying a NMD-sensitive nonsense codon, and dependent upon their recognition.

III.3.4. The presence of an NMD-sensitive nonsense codon does not affect the relative rates of removal of introns 1 and 2 in the human β-globin pre-mRNAs

In order to test to what extent the presence of the nonsense codon affects the relative amount of intron 1 *versus* intron 2 containing β-globin pre-mRNAs, we analysed the β39 and β62 transcripts stably expressed in differentiated MEL cell pools and results were compared to those of the βWT, β127 and β39missense control transcripts. This analysis was carried out by reverse transcription--coupled quantitative PCR assays to specifically quantify the amount of either intron 1 or intron 2 containing human β-globin pre-mRNAs (Figure III.10). Thus, pre-mRNA quantification was carried out with two sets of primers specific for the human β-globin intron 1 and intron 2 pre--mRNA sequence, respectively, using a set of primers specific for the murine GAPDH mRNA as an internal control (Figure III.10, C and D). As a control, RT-qPCR was also performed with a set of specific primers to quantify processed mRNA, to show that, under these experimental conditions, the PTCs at position 39 or 62 are able to induce a strong downregulation of the steady-state levels as expected for mRNAs typically committed to NMD, while levels of mRNA bearing a PTC at the 3'-most exon (β127) are not significantly different from the normal control (Figure III.10, A and B). The quantitative PCR efficiency for all amplicons was found to be similar and near to 100%. Control reactions using total RNA samples from untransfected MEL cells, confirmed that unspecific amplification of the murine β-globin transcripts was negligible. In agreement with the previously obtained RPA data, RT-qPCR analysis of the intron 2-containing pre-mRNA steady-state levels shows a significant 2.3 to 3.8-fold reduction of the β39 and β62 unspliced RNAs relatively to the βWT pre-mRNA (P<0.01) (Figure III.10, D). On the other hand, β127 and β39missense unspliced transcripts exhibit similar levels, which are not significantly different from the normal control (P=0.12 and P=0.08, respectively). Additionally, in each case, both β-globin intron 1 and intron 2 containing pre-mRNAs yielded very similar expression levels (P>0.05). Therefore, the presence of the NMD-sensitive nonsense codons does not differentially affect the rates of removal of intron 1 and 2, and, thus, splicing efficiency in transcripts bearing NMD-competent nonsense codons seems to be normal.

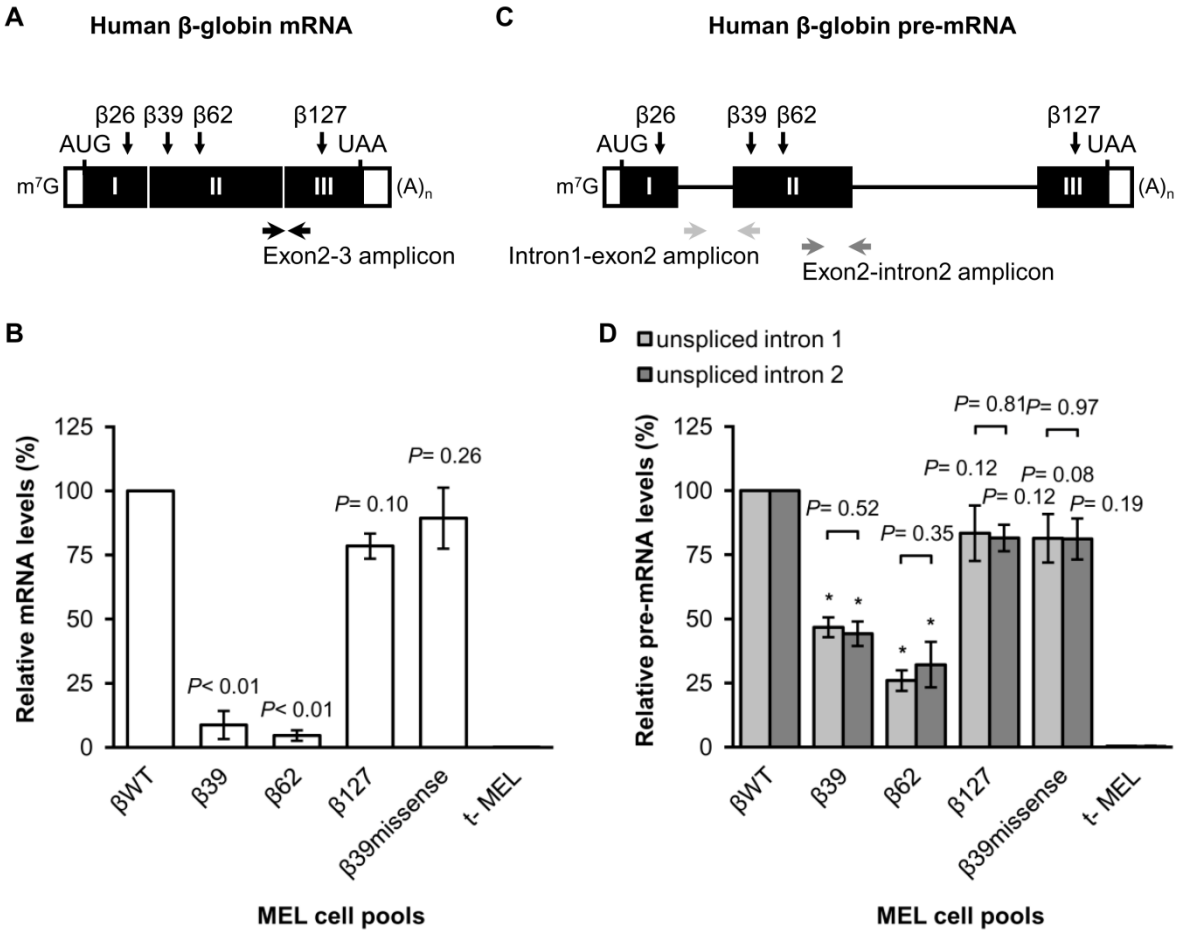


Figure III.10 The presence of the nonsense codon equally decreases the abundance of intron 1 *versus* intron 2 containing human β-globin pre-mRNAs. (A) Schematic representation of the studied human β-globin mRNAs as in Figures III.8 A and III.9 A. The pair of arrows represents the coordinates of the amplicon obtained in the quantitative PCR (qPCR) reactions: exon2-3 amplicon. **(B)** MEL cells were stably transfected with a test human β-globin construct as specified below the histogram. After erythroid differentiation induction, steady-state total RNA from either transfected or untransfected (t-) MEL cells was isolated and analysed by reverse transcription-coupled quantitative PCR (RT-qPCR), with specific primers for the human β-globin mRNA, as shown in (A). For each case, human β-globin mRNA levels were determined by normalization to the level of murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, using the comparative C_t method, and compared to the wild-type control. The percentage mRNA values were plotted for each construct and the histogram shows the mean and standard deviations from three independent experiments. Statistical analysis was performed using Student's t-test (unpaired, two-tailed). **(C)** Schematic representation of the human β-globin pre-mRNA, as in Figures III.8 D and III.9 D. The two pairs of arrows represent the coordinates of both amplicons obtained in the qPCR reactions: intron1--exon2 and exon2-intron2 amplicons. **(D)** Human β-globin pre-mRNA quantification was performed by RT--qPCR as in (B), but using specific primers for intron 1 or intron 2 containing human β-globin unspliced RNA. Levels of each human β-globin pre-mRNA variant were determined by normalization to the level of murine GAPDH mRNA, using the comparative C_t method, and compared to the wild-type control. The histogram shows the mean and standard deviations from three independent experiments. Statistical analysis was performed as in (B).

III.3.5. The reduced steady-state pre-mRNA level of NMD--sensitive transcripts does not reflect differential decay rates

As the steady-state level of any unspliced transcript depends on the balance between the rate of its transcription and splicing and/or degradation, we next asked if the low steady-state pre-mRNA levels of the NMD-sensitive transcripts indeed reflect increased decay rates rather than changes at the transcriptional level. Thus, we determined the decay kinetics of the β39 pre-mRNA relatively to that of the wild-type control pre-mRNA stably expressed in MEL cells. For this purpose, we treated the erythroid differentiated βWT and β39 MEL cell pools with actinomycin D to inhibit RNA synthesis. Total RNA was isolated at three time points after actinomycin D treatment. As before, the amount of unspliced human β-globin transcripts was determined by RT--qPCR. Results show that the βWT pre-mRNA has an average half-life of 32 min (Figure III.11). The presence of the PTC at position 39 does not significantly accelerate the decay of the reporter pre-mRNA as it results in a half-life of 28 min (*P*=0.50) (Figure III.11). Although βWT and β39 pre--mRNAs are not similarly abundant, the β39 turns at similar rates of those of βWT pre-mRNA. Our results suggest that low steady-state pre-mRNA levels of NMD-sensitive transcripts might be due to changes at the transcriptional level.

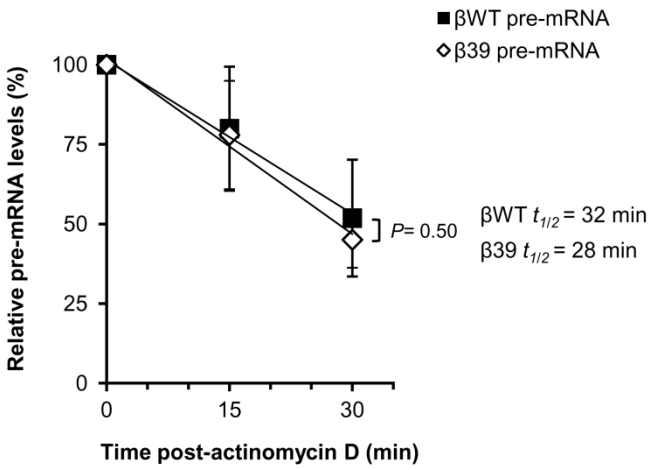


Figure III.11 The half-life of a pre-mRNA carrying an NMD-sensitive PTC is not significantly different from that of the wild-type control pre-mRNA. To determine the pre-mRNA decay kinetics, erythroid differentiated MEL cell pools stably expressing the βWT or the β39 transgenes were incubated with 5μg/mL of actinomycin D. Total RNA was extracted at the indicated times (0, 15 and 30 min) after actinomycin treatment. Relative pre-mRNA levels were measured by RT-qPCR, as described before. For that, the amount of human β-globin pre-mRNA was normalized against the amount of murine GAPDH mRNA and then re-normalized to the initial time point value (time 0 = 100%). Each point represents the mean and standard error mean from three independent experiments. Linear regression analysis was performed by standard techniques and the difference between slopes was assessed by Student's t-test. The half-lives ($t_{1/2}$) of the pre-mRNAs are indicated.

III.3.6. The NMD-competent PTC effect on β-globin pre--mRNA abundance exhibits promoter and/or cell line specificity

To assess whether the reduced nonsense pre-mRNA levels phenotype is cell line-specific, we next analyzed the abundance of βWT and β39 pre-mRNAs in non-erythroid cells. Thus, HeLa cells were stably transfected with the βWT or β39 genes, which were previously cloned into the pTRE2 vector, behind the human cytomegalovirus promoter (see Materials and Methods). The corresponding stably expressed spliced and unspliced human β-globin transcript levels were quantified by RT-qPCR analyses as before (Figure III.12). Although the PTC-bearing β-globin mRNA steady-state level is downregulated (Figure III.12, A), as expected for a transcript typically committed to NMD (Thermann et al., 1998; Zhang et al., 1998; Romão et al., 2000; Neu-Yilik et al., 2011), the corresponding β39 unspliced RNA steady-state level is neither lower nor significantly different relatively to the βWT control (P>0.05) (Figure III.12, B).

MEL and HeLa cells might require different splicing regulators for β-globin RNA processing. Thus, the nonsense mutation at codon 39 could affect a particular splicing factor binding sequence which might lead to processing defects in the reporter transcript. This could explain the discrepancies between the two cell lines. Thus, we carried out 3' rapid amplification of cDNA ends experiments using primers that amplify the full-length human β-globin processed RNA to analyze the integrity of the transcripts (Figure III.13, A). This study was conducted for all constructs expressed in MEL or HeLa cell pools. As expected, all cDNAs generated a product of 681 bp (Figure III.13, B). Furthermore, sequencing analyses of these fragments did not reveal any abnormal splicing event (data not shown). Thus, these results demonstrate normal splicing patterns for all the analyzed transcripts. Therefore, from this full set of data, we can conclude that the decreased β-globin unspliced RNA levels observed in MEL cells due to the presence of a NMD-sensitive nonsense codon is a cell line-specific effect. In addition, as reporter genes are expressed in MEL and HeLa cells under the control of different promoters, a promoter-specific effect cannot be excluded.

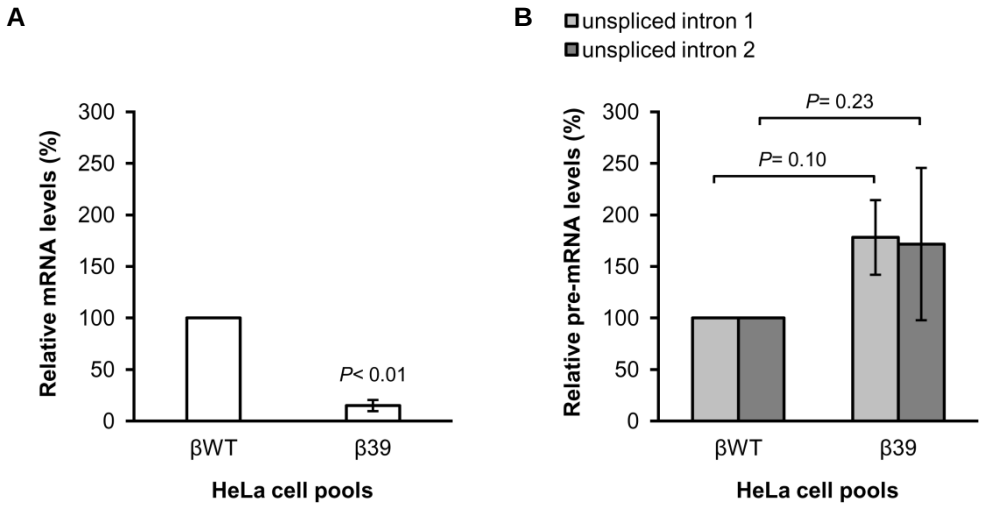


Figure III.12 The nonsense codon effect on the β-globin pre-mRNA abundance exhibits cell line specificity. (A) HeLa cells were stably transfected with the βWT or β39 constructs as indicated below the histogram. Total RNA was isolated and βWT and β39 steady-state mRNA levels were quantified by reverse transcription-coupled quantitative PCR (RT-qPCR) using specific primers for the human β-globin processed mRNA, as in Figure III.10. For each case, human β-globin mRNA levels were determined by normalization to the level of the puromycin resistance mRNA, using the comparative C_t method, and compared to the wild-type control. The histogram shows the mean and standard deviations from three independent experiments. Statistical analysis was performed using Student's t-test (unpaired, two-tailed). **(B)** Total RNA was also analysed by RT-qPCR with specific primers for the human β-globin pre-mRNA, as in Figure III.10. For each case, intron 1 and intron 2 containing human β-globin pre-mRNA levels were determined as in (A). The percentage pre-mRNA values were plotted for each construct and the histogram shows the mean and standard deviations from three independent experiments. Statistical analysis was performed as in (A).

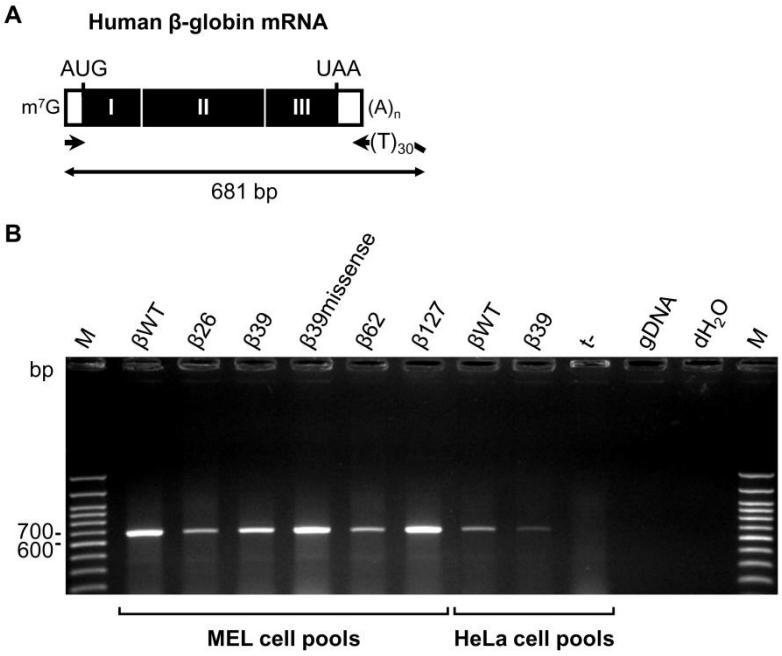


Figure III.13 The structures of the reporter mRNAs indicate that the corresponding transcripts are normally spliced. (A) Schematic representation of the human β-globin mRNA as in Figure III.10A. The small arrows represent primers localization for reverse transcription and PCR reactions. Reverse primer contains a 30 nt poly(T) tail as well as a degenerated sequence. Below, the full-length of the processed mRNA is also indicated. **(B)** Representative ethidium bromide-stained agarose gel with the structural analysis of the human β-globin mRNAs stably expressed in MEL or HeLa cells, as indicated below the gel. The identity of each construct is indicated above the respective lane. RNA from untransfected (t-) cells, human genomic DNA (gDNA) and water (dH_2O) were used as negative controls. The molecular weight marker (M) is the 100 bp DNA ladder (Life Technologies).

CHAPTER IV. Discussion

In the work present here, we addressed the subcellular localization and processing state of stably expressed human β-globin transcripts, bearing nonsense codons, and compared it to wild-type β--globin transcripts. To assess whether the presence of a NMD-sensitive PTC could affect intranuclear events, we developed FISH analysis which enabled the visualization of β-globin RNA in stably transfected MEL cell lines. On the other hand, RPA and RT-qPCR analysis were used for quantification of both spliced and unspliced β-globin transcripts. *In situ* localization studies of β--globin RNA in stably transfected MEL cell clones reproduced features formerly described by other authors regarding the accumulation of newly sinthethized transcripts. Firstly, as depicted in Figures III.2 and III.5, RNA-FISH signals are heterogeneous among cells from the same MEL cell clone. This observation is in accordance with previous reports concerning FISH studies in stably transfected cells. Although each MEL cell line comprises isogenic clones stably transfected with β-globin genes, the inherent asynchrony of the cell cultures as well as the epigenetic position effect variegation of the transgene enhancer contribute to expression variation and silencing in a subset of cells (Fraser et al., 1993; Milot et al., 1996; Custódio et al., 1999; Mühlemann et al., 2001). Secondly, MEL cells stably expressing the βWT gene showed a single nuclear focus. This signal was shown to correspond to a locus internal to the nucleus (as shown by DNA labelling with DAPI and RNA-FISH analysis through multiple focal planes). This FISH phenotype was already described by several authors as well and most likely corresponds to β-globin transcripts localization at the site of transcription, since their accumulation is highest at its site of synthesis (Lawrence et al., 1989; Dirks et al., 1995; Jolly et al., 1998; Custódio et al., 1999; Mühlemann et al., 2001). Finally, as observed by other authors, βWT MEL cells also showed additional staining in the cytoplasm, corresponding to processed mRNA accumulation (Custódio et al., 1999). On the other hand, β39 MEL cells showed no cytoplasmic staining, as expected to transcripts committed to rapid decay by NMD, which probably results in β39 mRNA accumulation to levels below the FISH sensibility (Mühlemann et al., 2001).

Interestingly, FISH staining of nonsense-mutated β-globin RNA using either full-length or intronic FISH probes, resulted in a nuclear speckled-like pattern within a significant subset of cells from all three β39 MEL cell lines (see Figures III.2 and III.5). These β-globin transcripts bearing a PTC show an abnormal intranuclear FISH signal, and combining the observations from the full-length and intronic FISH probes, it suggests that most of the accumulated transcripts are unspliced, as reported by de Turris *et al* (2011). Therefore, the presence of a PTC within a β-globin gene can indeed influence nuclear events in erythroid cells, affecting the nuclear accumulation of the corresponding pre-mRNA. On the other hand, the nuclear speckled-like pattern implies that, at least, unspliced PTC-bearing β-globin transcripts are released from the site of transcription and accumulate at other sites throughout the nucleoplasm. This is surprising, since it would be expected the pre-mRNA to be processed before its release from the transcription site. In the mammalian nucleus, pre-mRNA associates co-transcriptionally with mRNPs. Also, it has been generally accepted that the subsequent processing steps and release of the transcripts from the transcription machinery, and hence from the gene locus, are interconnected events (Luna et al., 2008; Moore and Proudfoot, 2009). Moreover, several reports have shown by FISH analysis that

either splicing defective β-globin transcripts expressed in MEL cells, or PTC-containing Ig-μ pre--mRNAs expressed in human cells are retained at the site of transcription (Custódio et al., 1999; de Turris et al., 2011).

In order to better understand the intranuclear fate of the β39 transcript, the assessment of the relationship of the RNA localization to nuclear structure would be enlightening. As SC-35 domains are pre-mRNA metabolic factors reservoirs enriched in polyadenylated RNAs, which have been implicated in post-splicing steps before export (Smith et al., 2007; Mao et al., 2011), colocalization of β-globin RNA with these nuclear bodies was assessed. Beta-globin hybridization and SC-35 immunodetection overlapping signals in deconvolved optical sections of β39 MEL cells, showed that the β39 transcripts might accumulate in other nuclear domain rather than the SC-35 domains (see Figure III.4). Another important experiment would be to assess the relative localization of β39 transcripts to the transcription site, by performing double hybridizations where the β-globin RNA and the transgene DNA could be simultaneously visualized. However, further experiments regarding the β39 RNA localization and sound interpretation of FISH results were precluded by an important control: although all β39 MEL cell clones present a marked tendency irrespective of copy number, the highest transgene copy number βWT MEL clone #166 also showed additional staining throughout the nucleoplasm (Figures III.2 and III.5). Therefore, it seems that the speckled-like FISH signal for β-globin transcripts might depend not only on PTC presence but also on transgene copy number. One can speculate that higher transgene copy numbers and hence, higher β-globin expression levels could lead to a threshold where the processing apparatus is impaired, resulting in an abnormal β-globin RNA accumulation within the nucleus. Conversely, as all β39 MEL cell lines show a speckled-like FISH pattern for β-globin pre-mRNA irrespectively from their transgene copy number, disruption of nuclear events could also be enhanced by the presence of the PTC. However, the “threshold hypothesis” for copy number--dependent disruption of nuclear events might be unlikely or hindered by the inherent expression variegation amongst subsets of cells, given that the three β39 MEL cell lines show variable percentages of cells with β-globin transcripts accumulated in speckled foci, not proportional to the transgene copy number (see Figures III.3 and III.6). Therefore, another possible interpretation could rely on the experimental model itself. MEL cells were transfected with constructs carrying human beta-globin genes under the control of the native promoter and LCR sequences, enabling a copy number-dependent and position-independent transgene expression in MEL cells, as previously described by others (Blom van Assendelft et al., 1989; Forrester et al., 1989; Talbot et al., 1989). Although the MEL/LCR system can be used to compare expression levels between different stably transfected MEL cell clones, the stochastic nature of the number of integration events and site of integration in the genome of the host cells can generate an undesired side--effect on FISH-based studies: genomic integration of the transgene might lead to the inactivation of endogenous genes, such as genes required for RNA processing or localization. This might in part explain the heterogeneity in the speckled-like pattern observed amongst the MEL cell clones with a similar copy number for the same transgene. In addition to the insertion sites, the variegation of transgene copy number hinders a definitive conclusion about the intranuclear RNA

localization of PTC-bearing β-globin transcripts using FISH in our experimental model.

An ideal alternative experimental system to circumvent possible copy number and site of integration side-effects on the FISH analysis would be creating a MEL cell line using the Flp-In system (Life Technologies). This system enables the integration and expression of a single copy of the transgene at a specific genomic location and involves the introduction of an Flp Recombination Target (FRT) site into the genome of the selected cell line. Subsequently, an expression vector containing the reporter gene is integrated into the genome *via* Flp recombinase--mediated DNA recombination at the FRT site. However, single-copy β-globin expression levels often fall below the detection limits of the RNA-FISH standard experimental conditions. High--sensitive live-cell imaging approaches could further elucidate the dynamics of nuclear events in response to the presence of a PTC on a transcript, as shown by de Turris et al. (2011).

To assess whether the presence of the PTC could affect the pre-mRNA levels of β-globin, we next analysed the pre-mRNA and mRNA steady-state levels of the selected βWT and β39 MEL cell clones. RPA analysis showed that all β39 clones, as expected for NMD sensitive transcripts, displayed a 2.5 to 5.5-fold reduction in their mRNA steady-state levels relatively to the expression levels of the βWT (Figure III.7). Regarding the pre-mRNA levels of the β39 transcripts, RPA analysis also showed a marked decrease of at least 60% relatively to the βWT counterparts in all MEL cell clones, irrespective of transgene copy number. This downregulation of the pre-mRNA containing a PTC was not expected, since it has been generally accepted that NMD occurs upon processed transcripts in the cytoplasm (or during transit to the cytoplasm). As PTC recognition is dependent on splicing and translation, the precursor mRNA steady-state levels should not be affected. The observed downregulation of β-globin pre-mRNA therefore poses additional evidence that PTCs can also influence nuclear events.

RPA quantification of β-globin pre-mRNA and mRNA levels in MEL cell clones also provide essential information towards the interpretation of the FISH results. The observed FISH phenotype of a speckled-like pattern for β-globin transcripts could indicate a disruption in processing events due to overexpression of β-globin transgenes as mentioned-above, which could lead to impairment of several events, including the NMD mechanism. Quantification of the β39 mRNA relative to the βWT mRNA for all MEL cell clones showed that the PTC-bearing processed transcripts are downregulated to levels expected to transcripts committed to NMD. Therefore, the MEL cell lines comprising the LCR/β-globin experimental system is suited to study the relative quantitative differences between stably expressed βWT and β39 genes, as already described by other authors (Antoniou, 1991; Romão et al., 2000). Regarding the speckled-like pattern accumulation of β39 unspliced transcripts throughout the nucleoplasm of β39 MEL clones, one should expect that the corresponding pre-mRNA levels would be higher relatively to the βWT MEL clones, which present a single focus. In fact, previous work from Mühlemann et al. (2001) linked brighter single nuclear focus with higher pre-mRNA steady-state levels for PTC-bearing transcripts relatively to the wild-type counterparts due to retention at the transcription site. However, in this work, RPA quantification shows that β39 pre-mRNA levels are lower than the

ones of the βWT pre-mRNA. This indicates that the speckled-like pattern for the β-globin transcripts might not correspond to retention and concomitant quantitative accumulation of unspliced transcripts at a nuclear domain. Instead, this suggests that β-globin transcripts are released from the site of transcription and distribute to precise locations within the nucleus. On other words, the majority of β-globin pre-mRNA molecules, present within the nucleus, preferentially localize into distinct nuclear loci instead of diffusing into the nucleoplasm, enabling their visualization by FISH analysis. Accordingly, the speckled-like FISH pattern might not represent sites of retention and concomitant accumulation of unspliced β-globin transcripts. Probably, these sites represent reservoirs where β-globin pre-mRNA continuously enter and leave, resulting in steady-state levels visible within the FISH sensitivity limits. Although detectable by FISH analysis, PTC-bearing β-globin pre-mRNAs present lower steady-state levels relatively to the βWT pre-mRNAs. On the other hand, as RPA quantification was performed using total RNA extracted from cell cultures of each MEL cell line, it cannot be discriminated whether the subset of cells with β-globin speckled-like FISH pattern present increased or decreased levels of β39 pre--mRNA relatively to the βWT counterparts. Indeed, as described for the Ig-μ reporter genes expressed in human cells, PTCs can either elicit retention and subsequent accumulation of the pre-mRNA by the NMUP pathway or transcriptional downregulation *via* NMGTS (Mühlemann et al., 2001; Bühler et al., 2005; Stalder and Mühlemann, 2007; de Turris et al., 2011).

As stated before, the presence of multiple transgene copies and integration sites may adversely affect the levels of transgene expression and could also explain the observed variation in β39 gene expression amongst MEL cell clones. Bearing this in mind, MEL cell pools stably transfected with the βWT and β39 genes were next generated. These MEL pools contain a large number of clones, in which any position or number of genomic integration events is averaged, resulting in a smaller variegation in β-globin expression among different pools due to the transgene integration side-effects. Adding up to βWT and β39 MEL pools, MEL pools stably expressing constructs bearing different PTCs sensitive to NMD (β26 and β62), as well as constructs with a missense mutation in codon 39 (β39missense) or a PTC insensitive to NMD (β127), were also established. Whilst β39missense and β127 mRNAs showed a slight variation relatively to the βWT mRNA levels (Figures III.8 and III.9), β26, β39 and β62 mRNA steady-state levels were found to be strongly downregulated (Figure III.9). Regarding the pre-mRNA steady-state levels in stably transfected MEL cell pools, those expressing NMD-sensitive PTCs presented significantly decreased steady-state levels relatively to βWT counterparts, irrespective of PTC position (Figure III.9). In addition, both RPA and RT-qPCR assays are concordant and show that β39missense and β127 pre-mRNAs are expressed at much higher levels than β39 and β62 pre-mRNAs (Figure III.10). This indicates that decreased pre-mRNA levels are specific for transcripts carrying a NMD-sensitive nonsense codon. Therefore, this work presents evidence that the presence of a PTC, which is recognized *via* NMD, elicits a reading frame-dependent nuclear response, resulting in the downregulation of β-globin pre-mRNAs in erythroid cells.

Several nuclear RNA metabolism events could account for the decreased levels of PTC-bearing β-globin pre-mRNA, namely an abnormal rate of transcription, splicing or degradation of the nascent precursors. For instance, mammalian nuclear RNA surveillance pathways that rapidly degrade aberrant pre-mRNAs have been reported (Doma and Parker, 2007; Schmid and Jensen, 2008a; Fasken and Corbett, 2009). However, pre-mRNAs containing nonsense codons were never described as substrates for rapid nuclear degradation. Indeed, Lim et al. (1992) compared the half-life of a β-globin pre-mRNA carrying a frameshift mutation that introduces an inframe PTC between codons 60 and 61, relatively to wild-type β-globin pre-mRNA, expressed in transgenic mice erythroid cells (Lim et al., 1992). These authors described a similar half-life for both transcripts. Our results are consistent with this observation, as we found no significant differences between the half-lives of β-globin pre-mRNA bearing a PTC at codon 39 and the normal β-globin pre-mRNA, expressed in stably transfected MEL cells (see Figure III.11). On the other hand, transcripts with processing defects are the most common substrates for nuclear RNA quality control (Doma and Parker, 2007; Schmid and Jensen, 2008a; Fasken and Corbett, 2009), being inefficient splicing a cause for decay (Bousquet-Antonelli et al., 2000; Lemieux et al., 2011). This evidence directs us to the hypothesis that the observed decreased steady-state levels of the β--globin pre-mRNAs carrying a NMD-sensitive PTC could be due to an effect on pre-mRNA splicing. Some studies have suggested that PTCs can affect the splicing process directly, either by inhibiting splicing or by regulating splice site selection (Carter et al., 1996; Li et al., 1997; Lejeune and Maquat, 2005). However, in many cases, these effects may result from the disruption of an exonic splicing enhancer by the mutation that also generates the nonsense codon (Shiga et al., 1997; Liu et al., 2001; Imam et al., 2010). Moreover, several studies conducted in β-globin, TPI, APRT or Ig-μ genes did not found evidences for differences in the splicing or 3'-end formation rates in transcripts bearing nonsense codons comparatively to the wild-type (Maquat et al., 1981; Baserga and Benz, 1992; Lim et al., 1992; Cheng and Maquat, 1993; Kessler and Chasin, 1996; Lytle and Steitz, 2004). Furthermore, neither Maquat et al. (1981), Lim et al. (1992), nor Inácio et al. (2004) observed any abnormal splicing rate or pattern for the β-globin transcripts bearing PTCs in erythroid cells (Maquat et al., 1981; Lim et al., 1992; Inácio et al., 2004). Indeed, the results obtained by RT-qPCR of total RNA extracted from the MEL cell pools are in accordance with the above-mentioned reports, as removal efficiency of the β-globin intron 1 *versus* intron 2 does not seem to be affected and the structure of the processed mRNAs is normal (Figures III.10 and III.13).

Therefore, another possible interpretation of our data is that the reduced pre-mRNA steady-state levels of the NMD-sensitive transcripts results from impaired transcription. A number of studies examining the abundance of PTC-containing pre-mRNAs relatively to the wild-type counterparts in different genes, including β-globin, have not detected reduced steady-state levels or transcriptional alterations (Maquat et al., 1981; Urlaub et al., 1989; Baserga and Benz, 1992; Lim et al., 1992; Cheng and Maquat, 1993; Kessler and Chasin, 1996). In what concerns the β-globin pre-mRNA steady-state levels in erythroid cells, the sensitivity of the assays based on S1 nuclease mapping and RNA blotting could explain the discrepancy with our results. Another issue

to address is the promoter- and/or cell line-specificity of the reduced steady-state levels of the β--globin pre-mRNA effect. Since β-globin genes assemble into transcriptionally silent heterochromatin in HeLa cells (Maquat and Kinniburgh, 1985), we used HeLa cells stably expressing cytomegalovirus promoter-driven βWT and β39 constructs and observed no decrease of the steady-state level of pre-mRNAs bearing a NMD-sensitive PTC relatively to the βWT pre--mRNA (Figure III.12). An alternative interpretation of the difference between MEL and HeLa cells is that β-globin splicing in these cell lines might involve different splicing-associated factors and hence the nonsense mutation at codon 39 could disrupt a binding sequence for a cell line-specific splicing factor. For instance, disruption of an erythroid-specific exonic splicing enhancer by the PTC could cause exon skipping and hence, result in an apparent downregulation of the pre--mRNA since it would impair the amplification of RT-qPCR fragments comprising both intronic and exonic sequences of β-globin (see Figure III.10). However, we checked the integrity of all studied β-globin transcripts by 3'-RACE and sequencing and found no evidence for exon skipping or alterations in the splice site choice. Indeed, results show that these nonsense mutations do not introduce any splicing defect in the reporter transcripts both in MEL and HeLa cell pools (Figure III.13). These findings raised the possibility that a promoter-specific effect is responsible for the β39 pre-mRNA downregulation in MEL cells, as these cells were transfected with β-globin constructs driven by their native promoters which comprise binding-sequences for erythroid--specific transcription factors, which are not found in HeLa cells. In fact, Enssle et al (1993) demonstrated that the nature of the promoter can dictate the fate of the β-globin transcripts (Enssle et al., 1993). Nonetheless, Bühler et al (2005) analysed HeLa cells stably transfected with the βWT and β39 genes driven by the β-globin promoter, and found no evidence for transcriptional gene silencing induced by the PTC (Bühler et al., 2005). In contrast to this observation, Baserga and Benz (1992), using a AF8 cell line derived from baby hamster kidney cells stably transfected with human β-globin genes under the control of their native promoters, did find by nuclear run-off transcription assays that β39 mRNA transcription initiation rate is 57% of that of βWT mRNA (Baserga and Benz, 1992). On that ground, our estimation that the observed decreased steady-state levels of the β-globin pre-mRNA bearing nonsense codons, in MEL cells, are due to a reduced transcription rate induced by the PTC in a promoter- and/or cell line-specific manner is in accordance. Our results therefore underscore that the nonsense-mediated transcriptional gene silencing pathway might not be restricted to the Ig-μ nonsense-containing genes (Bühler et al., 2005; Stalder and Mühlemann, 2007). Beta-globin transcripts bearing PTCs might also be targeted for transcriptional downregulation when expressed in erythroid cells by a PTC recognition- and/or NMD-dependent mechanism similar to NMTGS, which would explain the observed decrease in the nonsense-mutated pre-mRNA steady-state levels.

More recently, it has been shown that the regulatory effect of NMD on gene expression of many normal mRNAs is exerted in a cell type-specific and developmentally-regulated manner, which supports the idea that the NMD surveillance mechanism may have tissue-specific characteristics (Huang et al., 2011). Specialized nuclear pathways for regulation of the NMD-competent transcripts may act in concert with the general NMD pathway to help making it more efficient in

cell types where specific transcripts are expressed at very high levels. This reality may have driven the erythroid cells to evolve very efficient mechanisms to recognize and downregulate nonsense-mutated globin RNAs. Different sets of data are indeed in conformity with the occurrence of tissue-specific distinctive NMD features/branches. For instance, it has been reported that nonsense codons decrease the abundance of mRNAs by reducing the half-life of cytoplasmic human β-globin mRNAs in erythroid cells (Lim and Maquat, 1992), whereas the presence of a nonsense codon also reduces the nuclear β-globin mRNA half-life in non-erythroid cells (Humphries et al., 1984; Maquat and Kinniburgh, 1985; Baserga and Benz, 1992; Kugler et al., 1995). Furthermore, along with a strong downregulation of β-globin nonsense mRNAs, erythroid cells generate detectable β-globin decay intermediates (Lim et al., 1989, 1992; Lim and Maquat, 1992; Stevens et al., 2002), possibly resulting from cell type-specific endo- and exonucleolytic activities that may act concomitantly with the typical degradation pathways of NMD. Moreover, a cell-type specific mRNA surveillance pathway, the ribosome extension-mediated decay, was already described in MEL cells, which is dependent on translation and results in low levels of abnormal human α-globin transcripts containing an anti-termination mutation (Kong and Liebhaber, 2007).

As tissue-specific idiosyncrasies might not provide major contributions to the overall elucidation of the NMD mechanism, they could be crucial to understand the pathophysiology of some diseases induced by nonsense mutations. In more specialized and differentiated cells, while NMD is still holding the major role, supporting mechanisms may come into the spotlight in the RNA quality control screen for transcripts bearing nonsense codons.

CHAPTER V. Conclusions and Perspectives

The main purpose of this study was to assess whether the presence of a PTC can influence nuclear events in erythroid cells. Therefore, the subcellular localization and processing status of β-globin transcripts bearing PTCs *versus* wild-type β-globin transcripts were analysed in stably transfected MEL cells. Firstly, FISH analysis shows that, at least, β-globin unspliced transcripts committed to NMD present a marked tendency to localize throughout the nucleoplasm with an abnormal speckled-like pattern. Although this result underscores that a PTC affects the nuclear localization of β-globin transcripts in erythroid cells, its presence might not be the only determinant for abnormal localization of β-globin transcripts, at least under our experimental conditions. Therefore, further studies are required to elucidate the underlying mechanisms involved in abnormal localization of PTC-bearing β-globin transcripts expressed in erythroid cells.

Secondly, by using two different quantitative approaches, we were able to show that β-globin transcripts containing a PTC present reduced steady-state levels. Moreover, we demonstrate that this effect depends on the presence of a NMD-sensitive PTC, regardless of its position. Half-life determinations of these pre-mRNAs in MEL cells demonstrate that their low steady-state levels do not reflect significantly lower pre-mRNA stabilities when compared to the wild-type control. Furthermore, our results also provide evidence that the relative splicing rates of the introns neighbouring a PTC-containing exon are similar and no splicing defects are elicited by the PTC. We show that only those β-globin transcripts bearing a PTC, which is recognized *via* NMD, are specifically discriminated as abnormal during their nuclear metabolism. Moreover, we provide evidence that PTC-bearing β-globin pre-mRNAs are downregulated probably due to alterations at the transcriptional level, in a promoter and/or cell line-specific manner. Along with other reports, our study provides further arguments for one of the remaining debates in the field of NMD: whether PTCs can be recognized and affect events in the nucleus of mammalian cells.

Our set of data highlights potential specialized nuclear pathways for regulation of the NMD--competent transcripts that may collaborate with the general NMD mechanism, probably to achieve optimal NMD activity. One possibility is that such mechanisms may act in concert with the cytoplasmic mRNA surveillance pathway after mRNA translation, eliciting a feedback to the nucleus and triggering the PTC-bearing unspliced β-globin transcripts downregulation. In order to address this issue, impairment of the NMD pathway by RNAi-mediated knockdown of essential NMD factors would provide clearer data about the NMD input on nuclear events. Another aspect that must be clarified is whether transcriptional impairment is induced by the PTC and determines the reduction of nonsense pre-mRNA steady-state levels. Therefore, further studies should include the assessment of transcription rates and RNA polymerase II density for nonsense codon--containing human β-globin gene variants in MEL cells. Undoubtedly, these remaining open questions need to be explored to unravel the underlying mechanism of the PTC effect on nuclear events.

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