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The role of mRNA translation on nonsense-mediated decay inhibition of transcripts carrying a short open reading frame

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“I like nonsense, it wakes up the brain cells.”

Theodor Seuss Geiseil

(American writer, 1904-1991)

Prefácio

O trabalho de investigação descrito na presente tese de Doutoramento foi realizado na Unidade de Investigação e Desenvolvimento do Departamento de Genética do Instituto Nacional de Saúde Dr. Ricardo Jorge, sob a orientação da Doutora Luísa Romão Loison e co-orientação da Professora Doutora Margarida Amaral, membro da Faculdade de Ciências da Universidade de Lisboa.

Este estudo teve como objectivo principal identificar o mecanismo molecular subjacente à inibição do mecanismo de *nonsense-mediated mRNA decay* (NMD) previamente descrita para transcritos portadores de um codão de terminação prematuro localizado na proximidade do codão de iniciação. Este trabalho contribuiu para a identificação de novos determinantes implicados na indução do mecanismo de NMD e na tradução.

Em conformidade com o disposto no nº 5 do artigo 41º do Regulamento dos Estudos Pós-Graduados da Universidade de Lisboa, deliberação nº 93/2006, publicado em Diário da República, 2ª série – Nº 209 – 30 de Outubro de 2006, esta tese apresenta-se em língua inglesa e inclui um resumo em português com mais de 1200 palavras (ver Resumo).

De acordo com o nº 1 do mesmo artigo do referido Regulamento, na elaboração desta tese foi aproveitado o resultado do trabalho de colaboração já publicado numa revista de circulação internacional, estando a minha contribuição pessoal devidamente indicada:

Peixeiro I, Inácio A, Barbosa C, Silva AL, Liebhaber SA and Romão L. (2011) Interaction of PABPC1 with the translation initiation complex is critical to the NMD resistance of AUG-proximal nonsense mutations. Nucleic Acids Research doi:10.1093/nar/gkr820.

No âmbito do trabalho realizado para a obtenção desta dissertação foi ainda publicado um artigo de revisão numa revista de circulação internacional:

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Resumo

O mecanismo de decaimento do mRNA mediado por mutações *nonsense*, ou *nonsense-mediated mRNA decay* (NMD), é um processo de controlo de qualidade da expressão génica que permite a eliminação de transcritos portadores de codões de terminação da tradução prematura (CTPs). A eliminação dos transcritos anómalos permite reduzir a produção de proteínas truncadas, protegendo a célula dos efeitos dominantes negativos resultantes da acumulação de péptidos potencialmente tóxicos (Behm-Ansmant & Izaurralde, 2006; Chang et al, 2007). Este mecanismo desempenha não só uma função como modulador de doenças genéticas, mas também uma importante função na regulação de transcritos fisiológicos envolvidos em vários processos celulares (McGlincy et al, 2010; Mendell et al, 2004; Rehwinkel et al, 2006).

Nos mamíferos, durante o processo de *splicing*, é depositado um complexo proteico, cerca de 20-24 nucleótidos (nt) a montante de cada junção exão-exão, designado complexo junção exão-exão (*exon junction complex* – EJC) (Le Hir et al, 2001). Estes complexos permanecem associados ao mRNA durante o seu transporte para o citoplasma e são removidos pelo ribossoma durante a primeira ronda de tradução (Ishigaki et al, 2001; Lejeune et al, 2002; Maquat, 2004). Assim, admite-se que, no caso de existir um CTP situado a 50-55 nt a montante da última junção exão-exão, o transcrito permanecerá associado a pelo menos um EJC, que poderá induzir a activação do mecanismo de NMD. A relação entre os EJCs e o NMD é mediada por três factores UPF1, UPF2 e UPF3, considerados os principais efetores do processo de NMD. A interacção das proteínas UPF2 e UPF3, componentes do EJC, com a proteína UPF1, que é recrutada para o complexo de terminação pelos factores de terminação da tradução eRF1 e eRF3, resulta na activação do mecanismo de NMD (Kashima et al, 2006; Le Hir et al, 2001). Por outro lado, quando o codão de terminação se localiza depois da última junção exão-exão, o ribossoma atinge o codão de terminação depois de remover todos os EJCs, que não poderão, assim, desencadear a degradação do transcrito (Maquat, 2004).

No decorrer do últimos anos, vários estudos têm vindo a evidenciar o envolvimento da proteína citoplasmática 1 de ligação à cauda poli(A), PABPC1, no processo de discriminação entre codões de terminação normais e prematuros. A proteína PABPC1

compete com a UPF1 pela interacção com o complexo de terminação e estimula a eficiência da terminação da tradução. De facto, existem evidências experimentais de que a proximidade da PABPC1 ao codão de terminação é suficiente para inibir o mecanismo de NMD, mesmo na presença de um EJC a jusante (Eberle et al, 2008; Ivanov et al, 2008; Silva et al, 2008; Singh et al, 2008). Desta forma, quanto maior a distância entre o codão de terminação prematuro e a cauda poli(A), maior será a probabilidade de desencadear o NMD, e a presença de um EJC a jusante poderá funcionar como um potenciador da degradação do transcrito (Stalder & Muhlemann, 2008).

De acordo com este modelo, a localização de um codão de terminação prematuro na proximidade do codão de iniciação (AUG) será suficiente para activar o NMD. No entanto, verificou-se que os transcritos da β-globina humana portadores de CTPs no início do primeiro exão constituem uma excepção a esta regra, uma vez que apresentam uma estabilidade semelhante à do transcrito normal (Romao et al, 2000). A análise funcional destes transcritos permitiu concluir que a resistência ao mecanismo de NMD não é uma consequência da ocorrência de re-iniciação da tradução e é independente quer da identidade do transcrito quer do contexto da sequência (Inacio et al, 2004; Silva et al, 2006). De facto, estudos anteriores indicam que a proximidade do codão de terminação ao AUG é o principal determinante na inibição do NMD (Silva et al, 2006; Silva et al, 2008). Com o objectivo de compreender o mecanismo molecular subjacente ao efeito da proximidade do CTP ao AUG foi proposto um modelo que postula que a PABPC1, por se encontrar na vizinhança do AUG iniciador durante o processo de tradução de uma grelha de leitura curta, poderá estar implicada na resistência ao NMD dos transcritos portadores de um CTP próximo do codão de iniciação (Silva et al, 2008; Silva & Romao, 2009). Este modelo baseia-se na capacidade da PABPC1 interagir com a cauda poli(A), na extremidade 3’ do mRNA, e em simultâneo com o factor de iniciação eIF4G, associado à extremidade 5’ do transcrito. Esta interacção entre as duas extremidades dos mRNAs durante a tradução resulta na aquisição de uma conformação circular (Wells et al, 1998). As evidências experimentais que indicam que alguns dos factores de iniciação da tradução envolvidos no processo de *scanning* permanecem associados ao ribossoma durante os primeiros passos do alongamento da tradução (Poyry et al, 2004) levaram-nos a sugerir que a PABPC1 poderia ser trazida para a vizinhança do AUG em associação com o complexo de iniciação. Assim, no caso de um

evento de terminação demasiadamente prematuro, a PABPC1 manter-se-ia numa posição favorável para interagir com o complexo de terminação e estimular a eficiência da terminação da tradução, com a consequente supressão do NMD (Silva & Romao, 2009).

O trabalho apresentado nesta dissertação focou-se na identificação dos determinantes da resistência ao NMD de transcritos portadores de um CTP na proximidade do AUG, contribuindo, assim, para a compreensão do mecanismo de discriminação de um codão prematuro e do mecanismo geral da tradução.

Com o objectivo de confirmar o papel da PABPC1 na inibição do NMD de transcritos portadores de um CTP localizado na proximidade do codão de iniciação, analisámos o efeito da ausência da interacção PABPC1-eRF3 na estabilidade destes transcritos. Mediante a inibição específica da expressão da proteína PABPC1 endógena por interferência de RNA e expressão de uma proteína mutante PABPC1 com uma deleção na região C-terminal (domínio responsável pela interacção com eRF3), observámos uma desestabilização dos mRNAs portadores de CTP na proximidade do AUG. Este resultado foi corroborado pela análise da estabilidade destes transcritos em condições de inibição da expressão da eRF3 endógena e expressão de uma proteína mutante eRF3 sem a região de interacção com PABPC1 (domínio N-terminal). Estes resultados sugerem que, à semelhança do que acontece nos casos de codões de terminação na parte 3' do último exão, quando o CTP se encontra posicionado na vizinhança do AUG, a PABPC1 interage com o complexo de terminação, impedindo a interacção deste complexo com a UPF1, com a consequente inibição do NMD.

Adicionalmente, demonstrámos que a resistência ao NMD dos transcritos portadores de um CTP próximo ao AUG pode ser reduzida pela inibição da interacção entre a PABPC1 e o factor do complexo de iniciação eIF4G resultante da sobreexpressão da proteína PAIP2. De facto, foi possível reverter o efeito destabilizador da sobreexpressão desta proteína mediante a inibição da expressão da UPF1, o principal mediador do mecanismo de NMD.

O factor de iniciação da tradução eIF3 é um complexo multiproteico que interage com o ribossoma e a proteína eIF4G, funcionando como um elo de ligação entre os dois durante o processo de *scanning* (Hinnebusch, 2006; LeFebvre et al, 2006). O facto deste complexo ter a capacidade de se manter associado ao ribossoma durante os primeiros

passos de elongação (Pestova, 2007) levou-nos a questionar se algumas subunidades do eIF3 poderiam estar envolvidas no posicionamento da PABPC1 na vizinhança do CTP próximo do AUG. Os nossos resultados demonstraram não só que as subunidades eIF3F e eIF3h são essências para a interacção do complexo eIF3 com o factor eIF4G e o ribossoma, respetivamente, mas também para a supressão do NMD de transcritos portadores de um CTP na proximidade do codão de iniciação.

Por outro lado, uma vez que existem evidências experimentais em plantas que demonstram que a subunidade eIF3h é essencial para a re-iniciação da tradução num codão AUG a jusante do codão de terminação de pequenas grelhas de leitura a jusante (*upstream open reading frame* – uORF) da ORF principal; a destabilização dos transcritos em estudo em células com uma expressão reduzida da eIF3h poderia ser um resultado da incapacidade do ribossoma re-iniciar a tradução num AUG a jusante do CTP. No entanto, o estudo do efeito da eIF3h na re-iniciação da tradução após a tradução da uORF da eritropoietina humana demonstrou que a eficiência da re-iniciação da tradução não é afectada em condições de inibição da expressão da eIF3h.

Em resumo, o trabalho desenvolvido nesta dissertação contribuiu para a elucidação da base molecular subjacente ao efeito da proximidade do AUG na inibição do NMD de transcritos portadores de um CTP próximo ao codão de iniciação. Os resultados obtidos demonstram que esta resistência é dependente da interacção da PABPC1 com o complexo de iniciação da tradução, através do eIF4G e das subunidades eIF3f e eIF3h, que, no caso de um CTP localizado na proximidade do AUG iniciador, posiciona a PABPC1 numa situação favorável para interagir com o complexo de terminação, através do factor de terminação eRF3, com a consequente supressão do NMD. Deste modo, o trabalho aqui apresentado, que inclui resultados já publicados num artigo científico e outros que serão submetidos em breve para publicação, contribui para a caracterização dos determinantes da discriminação de um codão prematuro e activação/inibição do mecanismo de NMD. A elucidação da função de factores de iniciação da tradução no efeito supressor de NMD resultante da proximidade do AUG ao CTP contribui também para a compreensão de conceitos da tradução, nomeadamente para o caso de pequenas grelhas de leitura.

Palavras-chave

mRNA; β-globina humana; codão de terminação prematuro; decaimento do mRNA mediado por codões *nonsense*; controlo de qualidade do mRNA; tradução

Abstract

Nonsense-mediated mRNA decay (NMD) is a surveillance pathway that recognizes and rapidly degrades mRNAs containing premature termination codons (PTC). The unified model for NMD, proposes that the decision of NMD triggering is the outcome of the competition between the cytoplasmatic poly(A)-binding protein 1 (PABPC1) and the NMD effector UPF1 for the termination complex. Consequently, PTCs located far, in a linear sense, from the poly(A) tail and associated PABPC1 in mRNAs containing residual downstream exon junction complexes (EJCs) are expected to elicit NMD. Nevertheless, it has been reported that mRNAs containing PTCs in close proximity to the translation initiation codon (AUG-proximal PTCs) can substantially evade NMD. The work described in this thesis was focused on the mechanistic basis for this NMD resistance, exemplified by human β-globin transcripts carrying an AUG-proximal PTC. We demonstrated that translation termination at an AUG-proximal PTC lacks the ribosome stalling that is evident in an NMD-sensitive PTC. In fact, we have shown that the establishment of an efficient translation termination reaction at the AUG-proximal PTC is dependent on PABPC1 interaction with the initiation factor eIF4G and with the release factor eRF3 at the terminating ribosome. These interactions underlie critical 3'-5' linkage of translation initiation with efficient termination at the AUG-proximal PTC and contribute to an NMD-resistant PTC definition at an early phase of translation elongation. Furthermore, we provide strong evidence that the eukaryotic initiation factor 3 is involved in delivering eIF4G-associated PABPC1 into the vicinity of the AUG-proximal PTC through eIF3h and eIF3f subunits. These data support the "AUG-proximity effect" as a major inhibitor of the NMD pathway.

The work presented here corroborates a role for PABPC1 on NMD evasion of AUG-proximal transcripts and provides further insights into the mechanistic details of PTC definition and translation initiation.

Keywords

mRNA; human β-globin; premature termination codon; nonsense-mediated mRNA decay; mRNA surveillance; translation

Abbreviations

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
A	adenosine
Aa	aminoacid
Ab	antibody
ADP	adenosine diphosphate
A-site	aminoacyl-site
ATP	adenosine triphosphate
bp	base pairs
BRCA1	breast cancer protein 1
BTZ	Barentz
C	cytidine
CBC	cap binding complex
CBP	cap binding protein
cDNA	mRNA-complementary DNA
CH	cysteine- and histidine-rich domains
CHX	cyclohexamide
C-terminal	carboxyl-terminal
DCP	decapping-protein
DEAD	Asp-Glu-Ala-Asp motif
DECID	decay-inducing complex
DHX34	DEAH box polypeptide 34
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyrybonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DSE	downstream sequence element
DTT	dithiothreitol
EDTA	ethylenediaminotetraacetic acid
eEF	eukaryotic translation elongation factor
eIF	eukaryotic translation initiation factor
EJC	exon junction complex
EPO	erythropoietin
eRF	eukaryotic translation release factor
E-site	exit-site
G	guanosine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCN2	general control non-derepressible-2 protein
GDP	guanosine diphosphate
GFP	green-fluorescent protein
GTP	guanosine triphosphate
HDAC	histone deacetylase 1
Hrp1p	yeast heterogeneous ribonucleoprotein 1

Ig	immunoglobulin
IP	immunoprecipitation
IRES	internal ribosome entry site
LUC	luciferase
m7G	7-methylguanosine
MAGOH	mago-nashi homolog
Met	methionine
Met-tRNAi	methionine-loaded initiator tRNA
MIF4G	middle of eIF4G-like domain
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein particle
mTOR	mammalian target of rapamycin
NLS	nuclear localization signals
NMD	nonsense-mediated mRNA decay
NP40	nonidet-P40
nt	nucleotide
N-terminal	amino-terminus
ORF	open reading frame
p53	tumour protein 53
PABP	poly(A)-binding protein
PABPC1	cytoplasmic poly(A)-binding protein 1
PABPN	nuclear poly(A)-binding protein
PAGE	polyacrilamide gel electrophoresis
PAIP2	poly(A)-binding protein-interacting protein 2
PAN	poly(A) nuclease
PARN	poly(A) ribonuclease
P-bodies	processing bodies
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PERK	PKR-like endoplasmic reticulum kinase
Pi	inorganic phosphate
PIC	pre-initiation complex
PIKK	phosphatidylinositol 3-kinase-related protein
PIN	PilT N terminus
PKR	protein kinase activated by double-stranded RNAs
Poly(A)	poly-adenilate
PP2A	protein phosphatase 2a
Pre-mRNA	messenger ribonucleic acid precursor
P-site	peptidyl-site
PTC	premature translation termination codon
Puro^r	puromycin resistance
PVDF	polyvinylidene difluoride
REF	RNA and export factor binding protein
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RNPS1	RNA binding protein S1

RPA	ribonuclease protection assay
rpS6	ribosomal protein small subunit 6
RRL	rabbit reticulocyte lysate
RRM	RNA recognition motif
RT	reverse-transcription
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SDS	sodium dodecyl sulphate
Ser	serine
siRNA	short interfering RNA
SKI	superkiller
SMG	suppressor of morphological defects on genitalia
sORF	short open reading frame
Srm160	SR-related nuclear matrix protein of 160 kDa
STAU1	staufen-1
SURF	SMG1-UPF1-eRFs complex
T	thimidine
TCR	T-cell receptor
TERRA	telomeric repeat-containing RNA
Tet	tetracycline
TPI	triosephosphate isomerase
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
U	uridine
uORF	upstream open reading frame
UPF	up-frameshift protein
UTR	untranslated region
WT	wild type
WT1	Wilms tumour protein 1
XRN1	5’-3’ exoribonuclease 1
β15	human β-globin transcript with PTC at codon 15
β39	human β-globin transcript with PTC at codon 39
βN	normal human β-globin transcript (also termed βWT)

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Chapter I

General Introduction

I.1. mRNA metabolism and quality control

Eukaryotic gene expression involves a sequence of biochemical processes that initiates with transcription of the genetic material into messenger RNAs (mRNAs) followed by protein synthesis and that, ultimately, culminates in mRNA and protein degradation. The key intermediate, mRNA precursor, is produced by DNA transcription in the nucleus. The pre-mRNA is then subjected to removal of introns (splicing) and addition of the cap structure and the poly(A) tail, and this mature mRNA is transported into the cytoplasm where translation occurs. Throughout this process, mRNA molecules are in dynamic association with RNA-binding proteins constituting the messenger ribonucleoprotein particle (mRNP) (Dreyfuss et al, 2002; Isken & Maquat, 2007; Moore, 2005). The mRNP content influences the mRNA fate by mediating its cellular localization, translation and decay, responding to cellular signal networks (Moore, 2005). This post-transcriptional regulatory mechanism allows the cells to rapidly adapt to changes in their environment by altering the patterns of gene expression (Silva & Romao, 2009). A tight control of such a mechanism is therefore essential to guarantee cell survival. Thus, to ensure the quality and fidelity of RNA metabolism and function, cells have evolved quality control mechanisms that preferentially degrade aberrant transcripts or nonfunctional RNAs (Doma & Parker, 2007). These quality control mechanisms, also called mRNA surveillance pathways, operate in the nucleus, where improperly processed mRNAs are degraded before export; and in the cytoplasm, where the quality control mechanisms assess the translatability of the mRNA and degrade those that lack translation termination codons or that carry premature translation termination codons (Behm-Ansmant et al, 2007b). In fact, the quality-control mechanisms do not need to recognize specific defective features of an RNA or RNP but, instead, can function on a broad range of defects affecting the rate of a given step in metabolism or function (Doma & Parker, 2007). Moreover, the function of these mRNA surveillance pathways extends to the post-transcriptional regulation of gene expression, since they act not only in aberrant mRNAs, but also regulate the expression of naturally occurring transcripts that present features recognized by the surveillance machinery (Behm-Ansmant et al, 2007b).

I.1.1. mRNA Translation

Translation regulation of existing mRNAs allows for a spatial and temporal fine-tuning of levels of the encoded proteins, allowing the maintenance of cellular homeostasis (Gebauer & Hentze, 2004; Sonenberg & Hinnebusch, 2009). The translation process can be divided into four stages - initiation, elongation, termination and ribosome recycling – each of which requires a particular set of conditions and factors (Figure I.1) (Sonenberg & Hinnebusch, 2009). Translation initiation is the rate-limiting step and requires the function of several eukaryotic translation initiation factors (eIFs) (Livingstone et al, 2010). This complex event involves the assembly of elongation-competent 80S ribosomes at the initiation codon (AUG). On most 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' untranslated region (5'UTR) for an AUG codon. The 43S pre-initiation complex comprises the small (40S) ribosomal subunit, the eIFs 3, 1, 1A and 5, and a ternary complex, which consists of the methionine-loaded initiator tRNA (Met-$tRNA_i^{Met}$) and eIF2 coupled to GTP (Gebauer & Hentze, 2004). Binding of this complex to the mRNA requires the cooperative action of eIF4F and eIF4B or eIF4H, which unwind the 5'UTR of the mRNA allowing ribosomal attachment. eIF4F is composed by eIF4E protein, that binds the m^7G cap structure, the DEAD-box RNA helicase eIF4A and by eIF4G, which functions as a scaffold protein by interacting with eIF4E, eIF4A, eIF3 and with the cytoplasmic poly(A)-binding protein 1 (PABPC1) (Jackson et al, 2010). The simultaneous interaction of eIF4E and PABP with eIF4G is believed to circularize the mRNA, bringing the 3'UTR in close proximity to the 5'end of the mRNA (Wells et al, 1998). The functional connection between the mRNA ends during translation is highlighted by the fact that most known regulatory sequences are found within the 3'UTR (Gebauer & Hentze, 2004).

After loading to the mRNA, 43S complex scans downstream to the initiation codon. The scanning process is assisted by eIF4A, eIF4G, eIF4B and possibly by eIF3 (Jackson et al, 2010; Pestova & Kolupaeva, 2002). eIF3 plays a key role in recruiting the 43S pre-initiation complex to the mRNA and, at least in mammals, also forms a bridge to the mRNA by interacting with eIF4G (Hinnebusch, 2006; Pestova, 2007).

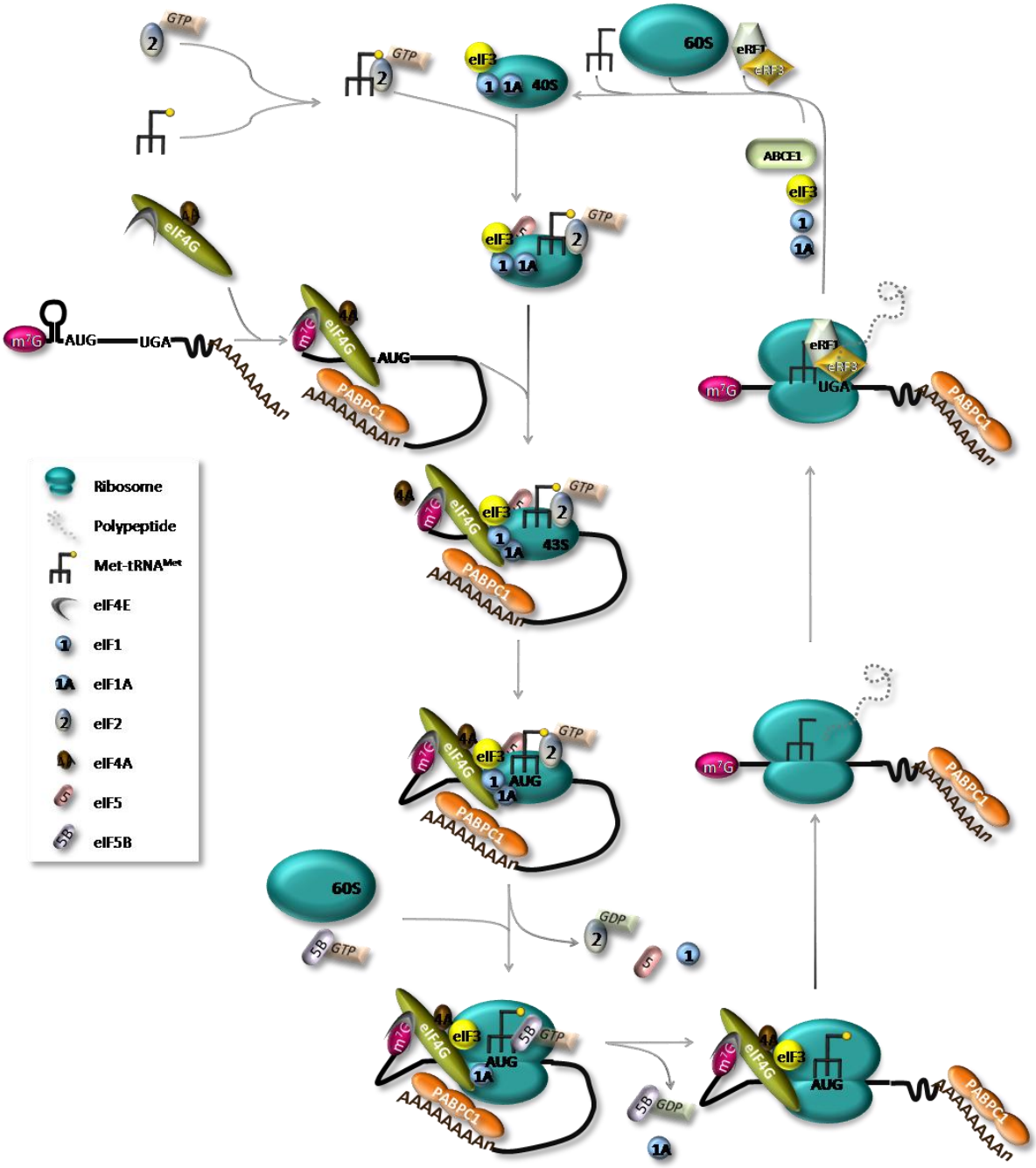

Figure I.1. The canonical pathway of eukaryotic translation. The ternary complex, comprising eIF2-GTP-Met-tRNAi^{Met}, binds the 40S ribosomal subunit together with eIFs 1, 1A, 3 and 5, creating the 43S pre-initiation complex. eIF4G functions as a scaffold protein by interacting with other components of the eIF4F complex, eIF4E and eIF4A, and with the poly(A)-binding protein PABPC1. This interaction is presumed to circularize the mRNA. The eIF4F and eIF4B or eIF4H, will then unwind the 5' UTR of the mRNA allowing 43S complex attachment and 5' to 3' scanning by the 43S complex. After recognition of the AUG initiation codon and 48S initiation complex formation, eIF1 is displaced and eIF5 mediates the hydrolysis of eIF2-bound GTP. Joining of the 60S ribosomal subunit will cause the displacement eIF2-GDP and other eIFs mediated by eIF5B and the assembly of 80S elongation-competent ribosomes induces the release of eIF1A and eIF5B. After translation termination, recycling generates separated ribosomal subunits. Although not represented, the potential interaction of PABPC1 with the release factor eRF3 may also result in formation of a closed-loop structure during translation termination.

eIF1 has a crucial role in the selection of initiation codons, allowing scanning 43S complexes to discriminate against codon-anticodon mismatches and preventing premature eIF5-induced hydrolysis of eIF2-GTP and Pi release (Holcik & Pestova, 2007). eIF1A also regulates start codon selection promoting continued scanning at non-AUG codons or by arresting scanning and promoting eIF1 release at AUG codons (Sonenberg & Hinnebusch, 2009). The Met-tRNA$_i^{Met}$ is anchored to the 43S complex by the GTP-bound form of eIF2, and a perfect match with an AUG start codon [usually the first AUG triplet in an optimum context – GCC(A/G)CCAUGG, with a purine at the -3 and a G at the +4 positions (relative to the A of the AUG codon, which is designated +1) (Kozak, 1991)] triggers the arrest of scanning and irreversible hydrolysis of GTP in the eIF2-GTP-Met-tRNAi ternary complex. This step, during which the ribosome becomes committed to initiation at that codon, is catalyzed by eIF5, an eIF2-specific GTPase-activating protein (Gebauer & Hentze, 2004; Jackson et al, 2010). With the release of eIF2-GDP and other eIFs, mediated by eIF5B, the large (60S) subunit joins to form an 80S initiation complex ready to accept the appropriate aminoacyl-tRNA into the A- (aminoacyl) site and synthesize the first peptide bond (Pestova, 2007). Because eIF3 binds mainly to the solvent side of the 40S subunit, the dissociation of this factor is not essential for subunit joining and may thus be delayed (Szamecz et al, 2008; Valasek et al, 2002).

During the elongation stage, amino acids are added sequentially to the growing polypeptide chain (Abbott & Proud, 2004). The ribosome has three tRNA-binding sites: the A-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 key factor in this process is the elongation factor (eEF) 1A which delivers cognate tRNA to the A-site, upon hydrolysis of GTP. The nascent peptide chain is then transferred to the amino acid of the A-site aminoacyl-tRNA. The translocation of peptidyl-tRNA from A- to P- and of deacylated tRNA from P- to E- sites is then promoted by eEF2; this process is also dependent on GTP hydrolysis (Abbott & Proud, 2004; Pisarev et al, 2007).

Termination of translation is triggered by peptide release factors eRF1 and eRF3. When a stop codon enters the A-site, eRF1 induces the hydrolysis of the ester bond of the P-site peptidyl-tRNA (Pestova, 2007). The function of eRF3 is to ensure the rapid and efficient hydrolysis of the peptidyl-tRNA by eRF1 (Alkalaeva et al, 2006). It is suggested that eRF1,

eRF3 and GTP bind the pre-termination complexes as a ternary complex. However, the mechanism of post-termination dissociation of the release factors is unknown (Pisarev et al, 2007).

After translation termination, the mRNA and P-site deacylated tRNA remain associated with ribosomes in post-termination complexes. eIFs 3, 1, 1A and 3j cooperatively dissociate such complexes into free 60S subunits and mRNA- and tRNA-bound 40S subunits (Pisarev et al, 2007). According to the model proposed by Pisarev et al. (2007), eIF1 promotes dissociation of P-site deacylated and eIF3j mediates release of mRNA. Ribosomal recycling might influence post-termination events such as reinitiation and possibly mRNA decay pathways.

Reinitiation may occur after translation of an upstream open reading frame (uORF), if the 40S ribosomal subunit remains bound to the mRNA molecule upon translation termination. Following translation-termination and the consequent release of the 60S ribosomal subunit, the 40S subunit may resume scanning downstream from the uORF, exhibiting a conditional loss of reinitiation competence. The *de novo* recruitment of the eIF2-GTP-Met-$tRNA_i^{Met}$ ternary complex, that will allow recognizing the AUG of the main ORF, determines the acquirement of full reinitiation competence (Kozak, 1987; Kozak, 2001). The reinitiation efficiency depends on *i) cis*-acting mRNA features; *ii)* the time required for the uORF translation, which is determined by the relative length of the uORF and the translation elongation rate; and *iii)* the translation initiation factors involved in the translation initiation event (Kozak, 2001; Poyry et al, 2004). It has been hypothesized that the eIFs involved in resuming scanning remain at least transiently associated with the elongating ribosome, and increasing the uORF length or the ribosome transit time increases the odds of these factors dissociate (Kozak, 2001). Corroborating this hypothesis, in yeast, eIF3 remains 80S-bound for several rounds of elongation and enhances the reinitiation competence of post-termination 40S ribosomes (Szamecz et al, 2008).

I.1.2. Translation regulation

Global control of protein synthesis is mostly exerted at the stage of translation initiation. The mechanisms regulating initiation may impact on the eIFs or ribosomes, affecting

scanning-dependent mechanisms; or may impact directly on the mRNA, through sequence-specific RNA-binding proteins or microRNAs.

The best well-known examples of regulation mechanisms impacting on the eIFs are the control of the availability of active eIF2 and eIF4F by reversible protein phosphorylation. Phosphorylation of the α subunit of eIF2 at Ser51 blocks the GTP-exchange reaction by reducing the dissociation rate of eIF2 from eIF2B (Gebauer & Hentze, 2004). There are four mammalian protein kinases that phosphorylate eIF2α at Ser51 decreasing global translation: haem-regulated kinase, which is stimulated by haem depletion; PKR (protein kinase activated by double-stranded RNAs), which is activated by viral infection; GCN2 (general control non-derepressible-2), which is activated by amino-acid starvation; and PERK (PKR-like endoplasmic reticulum kinase), which is activated under circumstances of endoplasmic reticulum stress (Jackson et al, 2010). The availability of the cap-binding protein eIF4E is also used to regulate general translation rates. When hypophosphorylated, a eIF4E-binding protein (4E-BP) binds eIF4E competitively displacing eIF4G; but phosphorylation of the 4E-BP at multiple sites (mainly by mTOR) releases eIF4E for assimilation into eIF4F, thus promoting translation initiation (Gebauer & Hentze, 2004). The phosphorylation of several other eIFs (eIF1, eIF2β, several eIF3 subunits, eIF4G, eIF4B, eIF4H, eIF5 and eIF5B) and of ribosomal protein 6 (RPS6) increasing under conditions in which translation is activated has also been described. However, there is no concrete evidence that these phosphorylation events are the cause of such activation (Jackson et al, 2010).

Regulation of translation initiation through the mRNA itself, by a sequence-specific RNA-binding protein, is exclusive of the mRNAs that contain the relevant RNA sequence motif and is generally inhibitory, with the activation of such mRNAs requiring the inactivation or the degradation of the inhibitory protein. The regulation by specific 5'UTR-protein interactions is surprisingly rare, being the ferritin mRNAs the only well-studied example. In contrast, there are several cases, most of them important in development, of control by 3'UTR-protein interactions (Jackson et al, 2010).

While the mentioned protein-RNA interactions usually result in decreased translation, the binding of PABPC1 to the poly(A) tail stimulates translation (Borman et al, 2000). This stimulatory effect appears to result mainly from the ability of PABPC1 to interact with eIF4G, mediating mRNA circularization by linking the cap and the poly(A) tail

(Imataka et al, 1998; Wells et al, 1998). By interacting with eIF4G, PABPC1 enhances eIF4F binding to the cap of the mRNA (Kahvejian et al, 2005) and, therefore, stimulates mRNA binding do the 43S pre-initiation complex (Pestova, 2007). In fact, an *in vitro* study showed that the formation of the closed-loop occurs before or during the 48S complex assembly, independently of a prior termination event (Amrani et al, 2008). The formation of a second closed-loop mRNP, comprising PABPC1, eIF4G, eIF4E, eIF3 and the termination factors eRF1 and eRF3, was also described during the subunit joining to form the 80S ribosome (Amrani et al, 2008). The formation of such a circular conformation could facilitate reinitiation by post-termination ribosomes. Moreover, PABP also stimulates 60S subunit joining (Kahvejian et al, 2005) and is required to block the inhibitory effect of RNA helicases on subunit joining factor eIF5B (Searfoss et al, 2001).

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

The best characterized mRNA quality control system in eukaryotes is nonsense-mediated mRNA decay (NMD). This surveillance mechanism detects and ensures the rapid degradation of faulty mRNAs harboring premature translation termination codons (PTCs) that would otherwise result in the synthesis of C-terminally truncated proteins. Hence, the quality control function of NMD relies in protecting the cell from the deleterious dominant-negative or gain-of-function effects of these truncated proteins (Behm-Ansmant & Izaurralde, 2006; Chang et al, 2007).

I.2.1. Aberrant and natural NMD targets

Cells produce a large number of faulty mRNAs harboring PTCs that are recognized and rapidly eliminated by NMD. PTC-harboring transcripts can arise at the DNA level as the result of germline or somatic alterations (direct nonsense-mutations; frame-shifting deletions and insertions that can lead to PTCs downstream of the shift; mutations within either an intron or an exon that results in inaccurate intron removal from the pre-mRNA and create an intron-derived PTC or a frameshift) or at the RNA level due to errors in transcription or pre-mRNA processing (Kuzmiak & Maquat, 2006; Stalder & Muhlemann,

2008). Other types of faulty mRNAs are transcripts from non-functional pseudogenes, endogenous retroviral and transposon RNAs or mRNA-like non-protein coding RNAs from intergenic regions (Mendell et al, 2004; Nicholson et al, 2010; Rehwinkel et al, 2006). By targeting these transcripts for decay, NMD exerts a role in reducing genomic noise.

In addition, PTCs can also be generated by non-faulty regulated processes of the mRNA metabolism, such as somatic rearrangements in the DNA, alternative splicing or utilization of alternative AUG initiation sites that lead to a shift in the main ORF (Holbrook et al, 2004; Kuzmiak & Maquat, 2006). In fact, unproductive alternative splicing is believed to constitute the major source of PTC-containing mRNAs in mammals, since bioinformatic approaches studies suggested that about one-third of the alternatively spliced human mRNAs contain a PTC that is targeted for NMD (Lewis et al, 2003); and, at least for same cases, it constitutes a form of autoregulation of the expression of the canonical protein-encoding isoforms, as the protein products of these genes are responsible for NMD triggering (Neu-Yilik & Kulozik, 2008). Thus, besides ensuring the degradation of faulty transcripts, NMD also has a crucial role in regulating gene expression. Indeed, genome-wide screens in NMD-deficient budding yeast, fruitfly and human cells have revealed that NMD controls the abundance of 3% - 10% of the transcriptome (He et al, 2003; Mendell et al, 2004; Rehwinkel et al, 2005; Rehwinkel et al, 2006). Examples of natural NMD targets include transcripts containing a regulatory ORF that resides upstream of the primary downstream ORF (uORFs); introns in the 3' UTR; programmed frameshifts; and long 3' UTRs (Kuzmiak & Maquat, 2006; Nicholson et al, 2010). Notably, mRNAs containing UGA triplets that direct selenocysteine incorporation can also elicit NMD, since the UGA codon can be interpreted as a PTC when the selenium concentration in the cell is low [reviwed in (Bhuvanagiri et al, 2010; Nicholson et al, 2010)]. The process of maturation of the immune system is other example of the physiological role of NMD. This programmed somatic rearrangements and hypermutations generate PTCs at a high frequency (Kuzmiak & Maquat, 2006; Nicholson et al, 2010).

Remarkably, many of the identified natural NMD targets encode proteins that could be grouped in clusters acting in related pathways (Neu-Yilik et al, 2004; Weischenfeldt et al, 2005). Such mRNAs are involved in a variety of cellular processes such as stress

responses, hematopoietic stem cell development, regulation of alternative splice forms, genomic stability, cell-cycle, telomere length maintenance and embryonic development (Bhuvanagiri et al, 2010; Stalder & Muhlemann, 2008).

I.2.2. Nonsense-mediated mRNA decay implications in disease

The biological and medical significance of the NMD pathway is evidenced by the fact that approximately 30% of all inherited genetic disorders are due to PTCs, and in many of these cases, NMD influences the severity of the clinical phenotype (Holbrook et al, 2004; Stalder & Muhlemann, 2008). In most cases, NMD has a beneficial effect by eliminating transcripts harboring PTCs that would otherwise give rise to truncated proteins either with a complete loss of function or with a dominant-negative function leading to toxicity (Bhuvanagiri et al, 2010). The benefits that can be achieved by the elimination of mRNAs encoding these faulty proteins are clearly illustrated in β-thalassemia (Frischmeyer & Dietz, 1999). β-Thalassemia is characterized by the absence or reduction in the synthesis of β-globin polypeptide chains, one of the hemoglobin subunits (Weatherall, 2000). This disorder exemplifies the phenotypic impact of the polar effect of PTCs at different positions between the same β-globin gene (Holbrook et al, 2004). If a PTC is located at a position that activates NMD, the disease results in a recessive mode of inheritance with the heterozygous being asymptomatic (Figure I.2). In this case the defective β-globin transcript is degraded by NMD resulting in a limited synthesis of the truncated product. The excess of free α-globin, as well as the limited amount of truncated β-globin protein (if translated) are proteolytically degraded. Indeed, it has been shown that these defects result in very low amounts of β-globin chain production from the affected allele causing a reduction about 50% of total β-globin chain synthesis in the heterozygote. This produces the clinically asymptomatic phenotype of β-thalassemia trait (also known as thalassemia minor), as heterozygotes usually synthesize enough β-globin from the remaining normal allele to support near-normal haemoglobin levels (Holbrook et al, 2004; Peixeiro et al, 2011b). If the PTC is located at a position that does not induce NMD, at less than 55 nucleotides (nts) upstream the last exon-exon junction or at the 5’ part of exon 3, the amount of truncated protein that is synthesized might be small enough to be efficiently degraded

by the proteolytic system of the red blood cell, together with the excess of free α-globin chains. In this case, the heterozygotes are also asymptomatic and the disease still results in a recessive mode of inheritance (Figure I.2). In contrast, if the PTC is located further downstream at exon 3 of the β-globin mRNA, NMD is not triggered; instead, substantial amounts of mutant β-globin mRNA are present in the patients, encoding for translation products that are long enough to overburden the cellular proteolytic system (Figure I.2). Under these conditions, accumulation of the truncated products, as well as free α-globin chains in excess, act in a dominant negative manner, leading to deleterious effects on the cell, as the nonfunctional globin chains cause toxic precipitation of insoluble globins (Holbrook et al, 2004; Neu-Yilik & Kulozik, 2008; Peixeiro et al, 2011b). This condition is related to a symptomatic form of the disease in heterozygotes named "thalassemia intermedia" with a dominant mode of inheritance (Hall & Thein, 1994).

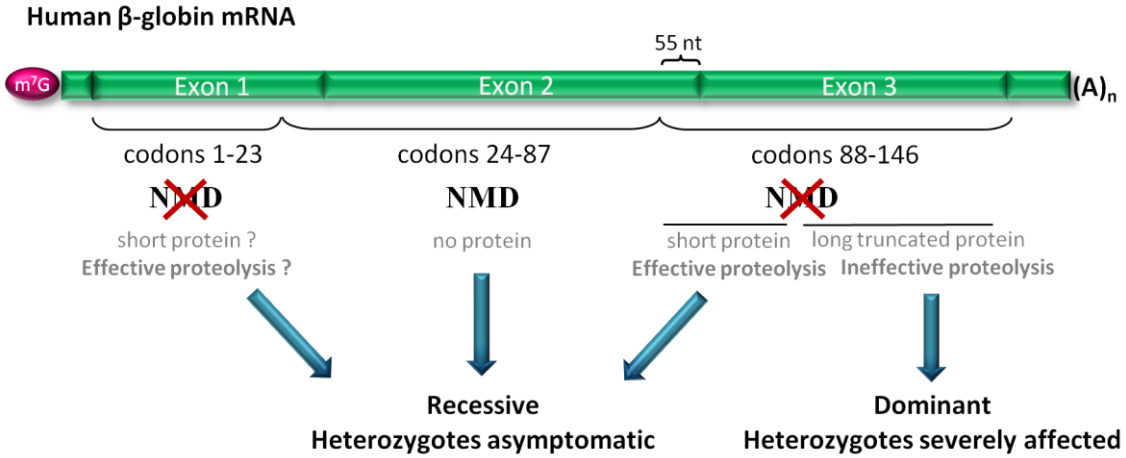

Figure I.2. Nonsense-mediated mRNA decay modulates the disease phenotype. The example here presented shows the human β-globin mRNA. AUG-proximal PTCs do not trigger NMD but heterozygotes are asymptomatic, as the translated short β-globin peptides, along with the β-globin chains in excess, are effectively degraded. If the PTC is located downstream of codon 23 and more than 55 nucleotides (nt) upstream of the last exon-exon junction, the corresponding transcript is targeted for NMD and heterozygotes are also protected from thalassemia. In contrast, transcripts bearing PTCs located less than 55 nt upstream of the last exon-exon junction, or in the 5'-part of exon 3, escape NMD, resulting in the production of truncated proteins that are small enough to be efficiently degraded, along with the α-globin chains in excess, and heterozygotes are also asymptomatic for β-thalassemia. However, if the PTC is located further downstream, the encoded truncated nonfunctional β-globin proteins overwhelm the cellular proteolytic system and cause toxic precipitation of insoluble globin chains. Heterozygotes with these mutations are affected with dominantly inherited β-thalassemia intermedia.

Other examples of the protective impact of NMD and of its disease-modulator function are retinal degeneration, von Willebrand disease, myotonia congenital and factor X

deficiency (Behm-Ansmant et al, 2007b; Khajavi et al, 2006). A potential influence of NMD in cancer has also been suggested. In fact, transcripts of several mutant forms of the tumor suppressor proteins breast cancer 1 (BRCA1), p53 and Wilms tumor (WT1) have been shown to be eliminated by NMD. The targeting for degradation of these nonsense-transcripts that would convert the tumor suppressors into dominant-negative oncoproteins, protects the heterozygous carriers from developing cancer (Behm-Ansmant et al, 2007b; Bhuvanagiri et al, 2010).

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 proteins, leading to haploinsufficiency (Khajavi et al, 2006). Examples of the detrimental effect of NMD are cystic fibrosis, Hurler syndrome and X-linked nephrogenic diabetes insipidus (Neu-Yilik & Kulozik, 2008). The clinical picture of protein deficiency induced by NMD is also clearly illustrated in Duchenne muscular dystrophy (DMD), where the rare truncating mutations that occur near the 3' end of dystrophin gene result in variable mild phenotypes while the 5' PTCs are associated with a severe form of DMD and fail to rescue the phenotype as a consequence of NMD triggering (Khajavi et al, 2006). In these cases, where NMD has such a detrimental effect, therapeutics that specifically suppresses premature translation termination by promoting translational readthrough would be clinically useful (Holbrook et al, 2004; Kuzmiak & Maquat, 2006; Neu-Yilik & Kulozik, 2008). The use of aminoglycosides as a therapeutic approach has been tested in the treatment of the above mentioned genetic disorders. The aminoglycoside antibiotics bind to the decoding center of the ribosome and reduce the accuracy requirements for the codon-anticodon pairing, promoting the incorporation of an amino-acid at the stop codon (Eustice & Wilhelm, 1984), generating functionally active, although missense-mutated, proteins. This therapeutic strategy was tested by using gentamicin in clinical trials performed in patients with DMD, Becker muscular dystrophy (Wagner et al, 2001) and cystic fibrosis (Clancy et al, 2001), showing that these antibiotics can promote *in vivo* readthrough of nonsense mutations and lead to the expression of full-length proteins. However, the variability in the response to the aminoglycoside treatment, the side-effects (such as kidney damage), and the fact that these suppressing agents may lead to a general loss in the fidelity of translation termination by promoting nonspecific

readthrough of normal termination codons, limited the use of its application (Bhuvanagiri et al, 2010; Frischmeyer & Dietz, 1999).

Recently, PTC124, a new drug similar to gentamicin, has been shown to effectively promote the selective readthrough of nonsense codons without interfering with translation termination at the normal stop codon. Unlike aminoglycosides, PTC124 is efficient at very low dosages and its side effects appear to be rare and mild (Welch et al, 2007), which makes this new drug a promising clinical application for the treatment of PTC-associated genetic disorders.

The elimination of the PTC-carrying portion of a nonsense-mutated transcript constitutes another viable approach for the treatment of NMD-associated diseases. One example of these repairing strategies is the use of antisense oligoribonucleotides to redirect splicing avoiding the creation of PTCs and it was first implemented to correct aberrant splicing of the β-globin gene (Dominski & Kole, 1993). The major drawback of this strategy relates to the lack a proper delivery system, issues of transfection efficiency and undesired side effects (Bhuvanagiri et al, 2010).

Biological evidence suggests that individuals with similar genetic mutations may exhibit different phenotypic severities as a result of variability in NMD efficiency (Kerr et al, 2001). This points out the modulation of NMD itself as another potential therapeutic strategy, by stimulation or inhibition of this decay pathway, rather than modulating recognition of PTCs. Even though this could benefit cases where the truncated proteins retain a certain level of function, the variety of functions of NMD and its cofactors in RNA surveillance and gene expression makes very challenging to identify a strategy to attack NMD systemically without undesired side-effects (Bhuvanagiri et al, 2010; Holbrook et al, 2004). Therefore, a balance between the potential side-effects and efficacy of these drugs will have to be found for therapeutic strategies targeting the physiological NMD function.

I.2.3. Molecular basis of the NMD pathway

NMD was first described in 1979 through the observation that in *Saccharomyces cerevisiae* (*S. cerevisiae*) nonsense mutations in the *Ura3* resulted in reduced amount of the affected transcript (Losson & Lacroute, 1979) and that the absence of human β-

globin expression in β^0-thalassemic patients was due to the presence of PTCs within the coding sequence of the transcripts (Chang & Kan, 1979). Subsequent studies revealed NMD as a well phylogenetic conserved surveillance mechanism by documenting that the fast degradation of nonsense-mutated organisms is common to other organisms such as *Caenorhabditis elegans* (*C. elegans*) (Pulak & Anderson, 1993), *Drosophila melanogaster* (*D. melanogaster*) (Brogna, 1999) and plants (Jofuku et al, 1989; Voelker et al, 1990).

Three key steps constitute the NMD pathway: *i)* the identification of the PTC; *ii)* the assembly of the surveillance complex on the mRNA and *iii)* the degradation of the NMD target. The mechanism by which premature stop codons are recognized and discriminated from natural stop codons is translation-dependent and leads to the recruitment of NMD *trans*-acting factors. Once assembled, the surveillance complex interacts with eukaryotic translation termination factors, eRF1 and eRF3, and triggers the fast degradation of the aberrant mRNA (Behm-Ansmant et al, 2007b; Conti & Izaurralde, 2005).

The NMD machinery comprises three *trans*-acting factors designated up-frameshift (UPF) proteins and seven proteins designated SMGs (for suppressor of morphological defects on genitalia). These effectors were first identified through genetic screens in *S. cerevisiae* and *C. elegans* and were later identified in other organisms by homology searches (Amrani et al, 2006b; Cali et al, 1999; Conti & Izaurralde, 2005; Hodgkin et al, 1989; Leeds et al, 1991; Leeds et al, 1992; Pulak & Anderson, 1993). The UPF1, UPF2 and UPF3 yeast factors, homologous of *C. elegans* SMG2, SMG3 and SMG4, respectively, are essential for NMD and orthologues are present in all eukaryotes. The SMG1, SMG5, SMG6 and SMG7 are conserved in almost all high eukaryotes, with the exception of *D. melanogaster*, which contains no clear orthologue for SMG7. Homologues of SMG1, SMG5 and SMG6 as regulators of NMD are not present in *S. cerevisiae* (Behm-Ansmant et al, 2007b; Muhlemann, 2008; Neu-Yilik & Kulozik, 2008; Nicholson et al, 2010). In human cells, the NMD pathway comprises the factors UPF1, UPF2 and UPF3A and UPF3B and the factors SMG1, SMG5, SMG6 and SMG7 (Culbertson & Leeds, 2003). However, additional NMD effectors are likely to exist. Indeed, recently, new NMD factors have been described: NAG and DHX34, the human homologous of *C. elegans* SMGL-1 and SMGL-2 (Longman et al, 2007); SMG8 and SMG9, which were shown to regulate the

SMG1 kinase activity (Yamashita et al, 2009) and RUVBL1 and RUVBL2, which also regulate SMG1 (Izumi et al, 2010).

I.2.3.1. UPFs: the core of the NMD machinery

UPF1 is the most conserved NMD factor and appears to be a key factor of this surveillance pathway in all organisms (Culbertson & Leeds, 2003). UPF1 is an RNA helicase and nucleic-acid-dependent ATPase. The conserved central region of this complex phosphoprotein harbors two cysteine-rich zinc-fingers and seven group I helicase motifs, two of which are responsible for the ATPase activity. The ATPase activity is essential for NMD in yeast and humans and is linked to the ATP-dependent 5' to 3' helicase activity (Bhattacharya et al, 2000; Chang et al, 2007). In spite of being primarily a cytoplasmic protein, UPF1 has been shown to shuttle between the nucleus and the cytoplasm (Mendell et al, 2002). UPF1 interacts with UPF2 through its N-terminal cysteine- and histidine-rich domains (CH), which forms three zinc-binding motifs arranged in two tandem modules (Kadlec et al, 2006). UPF1 also interacts with the termination factors eRF1 and eRF3 (Czaplinski et al, 1998; Ivanov et al, 2008) and immunoprecipitation studies revealed an association of UPF1 with SMG1, eRF1 and eRF3 that constitutes the SURF complex (Kashima et al, 2006). In higher eukaryotes, UPF1 activity is regulated by phosphorylation/dephosphorylation cycles (Ohnishi et al, 2003). Phosphorylation of its serine-glutamine C-terminal motifs is catalyzed by SMG1 and requires UPF2 and UPF3 (Grimson et al, 2004; Kashima et al, 2006; Page et al, 1999; Yamashita et al, 2001). Nevertheless, recent studies have provided evidence for the existence of UPF2-independent and UPF3-independent NMD pathways (Chan et al, 2007; Gehring et al, 2005; Ivanov et al, 2008). SMG5, SMG6 and SMG7 mediate the dephosphorylation of phospho-UPF1 by the protein phosphatase 2A (PP2A) (Anders et al, 2003; Chiu et al, 2003; Ohnishi et al, 2003). In coimmunoprecipitation studies, UFP1 has been found to interact with a variety of other NMD factors, such as exon-junction complex (EJC) components, but also with other proteins with no obvious role in this decay mechanism, such as the initiation factor eIF3, through eIF3e subunit (Isken et al, 2008; Morris et al, 2007). Moreover, a recent study showed that the cap binding protein CBP80 at the 5' mRNA end chaperones SMG1 and UPF1 to an EJC, thus promoting NMD

by augmenting UPF1-UPF2 (presumably EJC-bound UPF2) interaction (Hwang et al, 2011).

UPF2 is also a phosphoprotein and functions as bridge between UPF1 and UPF3 (Chiu et al, 2003; Lykke-Andersen et al, 2000; Mendell et al, 2000). The interaction of UPF2 with UPF3 involves the highly conserved residues in the last three middle of eIF4G-like (MIF4G) domains of UPF2 and the β-sheet surface of the RNA-binding domain of UPF3B (Kadlec et al, 2004). Recently, a structural study determined the precise interaction of the C-terminal region of UPF2 with the CH domain of UPF1 (Clerici et al, 2009). UPF2 interacts with UPF1 mainly through its C-terminal region (amino acids 1084-1272) but the amino acids 94-133 from the N-terminal domain also contribute to this interaction (Serin et al, 2001). It is not yet clear if the UPF2- and eRF3-binding sites in UPF1 overlap, but the bipartite nature of UPF2-binding may allow to UPF2 and eRF3 simultaneously bind UPF1 in an initial interaction (Clerici et al, 2009). Albeit the presence of nuclear localization signals in the N-terminal domain of UPF2, this protein localizes predominantly in the cytoplasm. Besides the function in bridging UPF1 to UPF3, UPF2 also plays a role in UPF1 phosphorylation (Wittmann et al, 2006). Indeed, immunoprecipitation assays showed that UPF2 directly binds SMG1 and is essential for the association of SMG1 with the mRNP (Kashima et al, 2006).

The human genome encodes two UPF3 genes, designated UPF3A (on chromosome 13) and UPF3B (also known as UPF3X, as it is localized on the X chromosome) (Lykke-Andersen et al, 2000; Serin et al, 2001). Splice variants generated by both genes have been described. UPF3AL and UPF3AS retain or skip exon 4, respectively, and UPF3B isoforms are generated by alternatively splicing exon 8 (Lykke-Andersen et al, 2000; Serin et al, 2001). All UPF3 isoforms, components of the EJC (Le Hir et al, 2001), shuttle between the nucleus and the cytoplasm, although their predominantly location resides in the nucleus (Lykke-Andersen et al, 2000). The N-terminal RNA-binding domain of these proteins is responsible for the interaction with UPF2 and is absent in the UPF3AS isoform (Kadlec et al, 2004). The C-terminal domains, involved in the assemble of the complex containing the EJC proteins Y14 and MAGOH (Gehring et al, 2003), from UPF3A and UPF3B are considerably divergent. This divergence explains the weaker ability of UPF3A, compared to UPF3B, to trigger NMD (Kunz et al, 2006). Evidence that UPF3A is an NMD factor regulated by UPF3B and UPF2 has emerged recently (Chan et al, 2009).

Chan and colleagues (2009) showed that, as a consequence of competitive binding of UPF3A and UPF3B for UPF2, when UPF3B levels are low, UPF3A is able to bind UPF2. Conversely, when UPF3B levels are high, less UPF3A molecules can bind UPF2, which results in decreased UPF3A levels, since UPF3A alone is intrinsically unstable. The UPF3A proteins appear to specify two UPF1-containing complexes, one containing phospho-UPF1, UPF2, UPF3B and UPF3AL, and the other containing phospho-UPF1, UPF3B, SMG5, SMG7 and UPF3AS (Ohnishi et al, 2003). The exchange between the two UPF3A isoforms might represent the switch between a phosphorylation and a pre-dephosphorylation UPF1-complex, based on the hypothesis that phospho-UPF1 recruits the SMG5-SMG7 complex that together with PP2A and UPF3AS induce dephosphorylation of UPF1 (Anders et al, 2003; Ohnishi et al, 2003). Moreover, a role for these isoforms in translation termination at the PTC, instead of in the degradation phase of NMD, is likely since there is evidence that UPF3A proteins interact with a UPF1-eRF1-ERF3 complex even when the binding of UPF2, UPF3B and EJC-components to UPF1 is abrogated (Kashima et al, 2006).

I.2.3.2. SMGs - determining the phosphorylation status of UPF1

As above mentioned, SMG1 and SMG5-7 control the phosphorylation status of UPF1 (Wilkinson, 2003). SMG1 is a protein kinase that phosphorylates specifically serine and threonine residues and belongs to the phosphatidylinositol 3-kinase-related protein (PIKK) family (Yamashita et al, 2001). A recent report showed that the novel identified NMD factors SMG8 and SMG9 assemble a complex with SMG1 and suppress SMG1 kinase activity in this isolated complex. SMG8 protein is further required to the recruitment of SMG1 to form the SURF complex (Yamashita et al, 2009) and SMG9 indirectly regulates SMG1 through the recruitment of SMG8 to the N-terminal region of SMG1 (Arias-Palomo et al, 2011). In addition to the SURF complex, SMG1 presence on other multiprotein complexes was revealed by immunoprecipitations assays. SMG1 interacts with the EJC components eIF4A, Y14 and MAGOH and with the NMD factors UPF1, UPF2, UPF3B and SMG7 (Kashima et al, 2006).

C. elegans mutants for SMG5, SMG6 or SMG7 accumulate hyperphosphorylated SMG2 suggesting that these proteins are responsible for UPF1 dephosphorylation. SMG5, SMG6 and SMG7 share a common 14-3-3-like domain, which is usually associated with

protein-protein interactions. This domain in SMG7 is thought to promote the dephosphorylation of UPF1 by binding phospho-UPF1 (Fukuhara et al, 2005) and also contributes to an interaction with the 14-3-3-like domain of SMG5 (Anders et al, 2003; Unterholzner & Izaurralde, 2004). Tethering of SMG7 to a reporter mRNA is sufficient to trigger decay even in the absence of a PTC and the NMD factors UPF1, SMG5 and SMG6, indicating that SMG7 functions at a late step in NMD (Unterholzner & Izaurralde, 2004).
SMG5 also interacts with phospho-UPF1 and PP2A, which might give PP2A specificity for UPF1 (Anders et al, 2003). SMG5 and SMG6 contain a C-terminal PIN (for PilT N terminus) domain which characteristically exhibits nuclease activity. However, only the SMG6 has the triad of acidic residues for the RNase H activity (Glavan et al, 2006). Actually, studies in *D. melanogaster*, zebrafish and human cells showed that the endonuclease activity of SMG6 can initiate cleavage of NMD targets (Eberle et al, 2009; Gatfield & Izaurralde, 2004; Huntzinger et al, 2008). The requirement of the 14-3-3-like domain of SMG6 in NMD was recently revealed by a study suggesting that the N-terminal domain of this protein is required for association with other NMD factors and also mediates binding to the EJC (Kashima et al, 2010). Accordingly, SMG6 was shown to co-purify with PP2A, SMG1, UPF1, UPF2 and UPF3B (Chiu et al, 2003). As the N-terminal domain of SMG6 contains conserved motifs related to a C-terminal motif present in UPF3B that is responsible for binding to the EJC (Buchwald et al, 2010; Gehring et al, 2003), UPF3B may block SMG6 recruitment to mRNAs lacking PTCs. However, the assembly of the surveillance complex with phospho-UPF1 on mRNAs harboring PTCs could result in the replacement of UPF3B for the shorter isoform UPF3AS with low affinity to the EJC. Such a rearrangement would confer specificity in SMG6 recruitment to the EJC of PTC-containing transcripts (Kashima et al, 2010).

I.2.3.3. Splicing and the EJC

Even earlier than the discovery of NMD effectors, studies in mammalian cells revealed that PTCs located more than 50-54 nucleotides (nt) upstream the last exon-exon junction were able to target the mRNA for rapid decay (Nagy & Maquat, 1998). This evidence implicates splicing in mammalian NMD by supporting the view that PTC recognition is dependent on the definition of exon-exon junctions.

In metazoans, during pre-mRNA splicing, the exon junction complex (EJC) assembles 20-24 nt upstream of each exon-exon junction (Le Hir et al, 2000a). The EJC is formed by the assembly of four conserved core proteins - Y14, MAGOH, eIF4AIII and Barentz (BTZ) – that associate with the mRNA in the nucleus and travels with the mRNP to the cytoplasm (Bono et al, 2006). The EJC is a dynamic structure, and therefore, additional EJC factors (RNPS1, Pinin, SRm160, UAP56, TAP, the mRNA export-related proteins Aly/REF, the RNA-binding protein PYM) 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 (Chang et al, 2007; Le Hir et al, 2001; Le Hir et al, 2000a). It was recently shown that EJC deposition is a sequential process coordinated by splicing, involving a pre-EJC as an intermediate. The pre-EJC, which consists of eIF4AIII and Y14-MAGOH heterodimer, is assembled before exon ligation and provides a binding platform for peripheral EJC components (Gehring et al, 2009).

The EJC is involved in translation and mRNA transport and turnover (Nott et al, 2004; Tange et al, 2004); and the identification of UPF2 and UPF3 as EJC components suggested a role for this complex also in NMD (Le Hir et al, 2000a). UPF3b associates with the EJC in the nucleus by interacting with the complex eIF4AIII:Y14-MAGOH (Buchwald et al, 2010). The NMD-competent core EJC is accomplished by the association of BTZ, which most likely occurs after nuclear export (Gehring et al, 2009). Supporting a function for EJC in this surveillance mechanism, the artificially tethering of RNPS1, Y14, MAGOH and PYM at more than 50 nt downstream the native stop codon of β-globin transcripts resulted in NMD triggering (Bono et al, 2004; Gehring et al, 2003; Lykke-Andersen et al, 2001). Moreover, the knockdown of Y14, eIF4AIII or BTZ by RNA interference was shown to impair NMD activation of PTC-harboring mRNAs (Gehring et al, 2003; Palacios et al, 2004). In contrast, studies with β-hexosaminidase, Igμ and hybrid mouse/human β-globin transcripts revealed that NMD could be triggered without the requirement of an EJC downstream the PTC (Buhler et al, 2006; Rajavel & Neufeld, 2001; Zhang et al, 1998b). Additionally, wild-type Igμ minigene, β-globin or TPI transcripts with 3'UTRs extended in length were targeted by the NMD pathway (Buhler et al, 2006; Eberle et al, 2008). Noteworthy, the EJC is dispensable for NMD in *C. elegans* and *D. melanogaster* (Gatfield et al, 2003; Longman et al, 2007). Therefore, the EJC may have

evolved to facilitate efficient recognition and subsequent degradation of nonsense transcripts in organisms that produce such a large number of aberrant transcripts by alternative splicing (Stalder & Muhlemann, 2008).

Once in the cytoplasm, it is though that during the first round of translation the EJCs located on the mRNA are displaced by the elongating ribosomes (Lejeune et al, 2002). This process involves the EJC disassembly factor PYM, which interacts with Y14-MAGOH while associated with the ribosome (Gehring et al, 2009).

I.2.4. PTC definition

The mechanism of discrimination between a premature from a natural termination codon has been one of the central questions regarding the NMD mechanism. As above mentioned, the involvement of a splicing-dependent signal in NMD activation seems to be restricted to mammalian cells. Indeed, yeast lacks orthologues for most of the EJC components and PTC definition in this organism is independent of exon-exon junctions (Culbertson & Leeds, 2003). Likewise, NMD in *C. elegans* and *D. melanogaster* can be triggered by transcripts generated from intronless genes (Gatfield et al, 2003; Longman et al, 2007). Moreover, the EJC components Y14 and eIF4AIII are not essential for NMD in *C. elegans* (Longman et al, 2007).

Nevertheless, in mammalian tissue culture, the splicing-dependent signal was established as a *trans*-acting component of the NMD pathway (Le Hir et al, 2000a) since it facilitates recognition of alternatively spliced transcripts as NMD substrates (Zhang et al, 1998b). In fact, the involvement of splicing and the EJC in NMD led to a formulation of a model for PTC definition where the EJCs would function as a “mark” to discriminate PTCs from natural termination codons. It is assumed that during the first round of translation the elongating ribosomes have the ability to displace the EJC that were assembled during splicing. Hence, if a premature termination codon is located more than 50-54 nt upstream of the last exon-exon junction, at least one EJC will remain associated with the transcript when the ribosome reaches the PTC. The presence of an EJC downstream the terminating ribosome would allow the interplay between UPF2 and/or UPF3b at the EJC with the SURF complex at the ribosome, culminating in NMD triggering. Contrary to the above proposed model, as previously mentioned, several

studies demonstrated that PTCs can activate NMD in the absence of downstream EJCs (Buhler et al, 2006; Matsuda et al, 2007). However, a lower extent of mRNA decay is observed for transcripts without EJCs when compared to the decay of NMD targets harboring an EJC in the 3'UTR. The presence of the EJC downstream of the termination codon may, thus, function as a potent NMD enhancer (Stalder & Muhlemann, 2008).

I.2.4.1. Pioneer round of translation

The 5'end of mRNAs acquires a modified guanine residue during transcription that binds to the nuclear cap-binding protein (CBP) complex CBP80-CBP20 (CBC) that supports the pioneer round of translation (Ishigaki et al, 2001). The cap is then replaced by eIF4E that directs subsequent rounds of translation. Spliced CBC-bound mRNAs differ from eIF4E-bound mRNAs in being associated with one or more EJCs. This feature suggests a role for CBC in nonsense-mediated mRNA decay. Indeed, it has been proposed that NMD occurs exclusively during the pioneer round of translation and that transcripts bound to eIF4E are NMD-insensitive (Chiu et al, 2004; Ishigaki et al, 2001; Lejeune et al, 2002). Additionally, CBC-bound complexes contain the nuclear poly(A) binding protein 1 (PABPN1) (Chiu et al, 2004; Kashima et al, 2006). The distinguished feature between the pioneer round of translation complexes and the steady-state translation initiation complexes contribute to the specialized function of each complex. Nonetheless, there are likely to be more similarities than differences between the two mRNPs: both include PABPC1, eIF2α, eIF3, eIF4G, eRF1 and eRF3 (Chiu et al, 2004; Hosoda et al, 2006; Isken et al, 2008; Kashima et al, 2006; Lejeune et al, 2002) and both assemble into polysomes (Lejeune et al, 2002; Sato & Maquat, 2009). Although translation contributes to remodeling the pioneer round of translation complexes by augmenting the displacement of EJC components, the replacement of CBC by eIF4E is promoted by the binding of the nuclear transport receptor importin β (Sato & Maquat, 2009).

In support for a role of CBC in NMD, the recently shown interaction of CBP80 with UPF1 promotes NMD in two sequential steps (Hwang et al, 2011). First CBP80 chaperones SMG1-UPF1 to eRF1-eRF3 at the PTC, promoting SURF complex assembly; and subsequently CBP80 physically joins the distal-EJC while still chaperoning SMG1-UPF1, which results in SMG1-mediated UPF1 phosphorylation (Hwang et al, 2011) (Figure I.3).

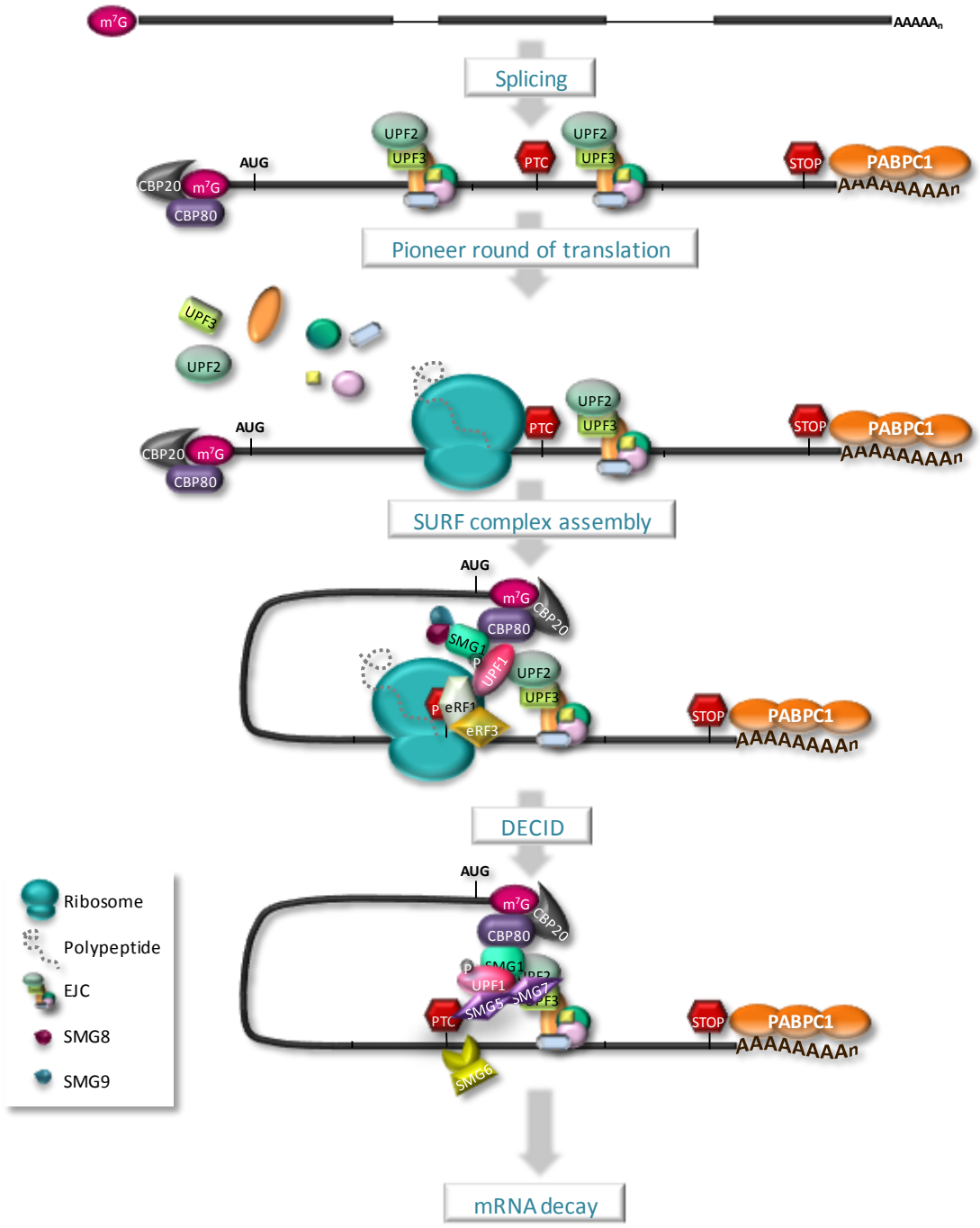

Figure I.3. Nonsense-mediated mRNA decay occurs during the pioneer round of translation. During splicing, exon junction complexes (EJCs) assemble 20-24 nucleotides (nt) upstream of exon-exon junctions. The translating ribosome displaces the EJCs during the pioneer round of translation. If a PTC is located more than 50-54 nt upstream from the last EJC, the terminating complex is able to interact with NMD factors EJC-associated. The CBP80 protein at the mRNA 5'end can bridge UPF1-SMG-1 to eRF1-eRF3 at the PTC, leading to the formation of the SURF complex. The interaction of UPF1 with EJC-bound UPF2 induces the SMG1-mediated phosphorylation of UPF1 and dissociation of eRF1-eRF3. Phosphorylated UPF1 recruits SMG-7 and SMG-5 proteins and EJC recruits SMG-6, triggering the decay of the PTC-containing transcript.

Phosphorylated-UPF1 binds eIF3 of the 43S preinitiation complex positioned at the translation initiation codon and inhibits 60S subunit joining, thereby eliciting translation repression (Isken et al, 2008).

Nevertheless, it was recently reported a substantial stabilization of transcripts containing elements that direct readthrough at less than 1% efficiency, indicating that the decision to the decay covers many translation termination events and that mammalian NMD may target to decay mRNAs that have proceeded beyond the first round of translation (Hogg & Goff, 2010).

The decision of whether an mRNA will be target or not for degradation by the NMD pathway is made when the ribosome is poised at the termination codon. During normal translation termination, when the stop codon enters the A-site, it is recognized by the termination factor eRF1, which forms a complex with eRF3 that involves the C-terminal domains of both proteins. eRF3, through its N-terminal domain, also interacts with the C-terminal domain of PABPC1 (Cosson et al, 2002a; Hoshino et al, 1999; Kozlov et al, 2001) and this interaction results in stimulation of proper and efficient translation termination (Mangus et al, 2003).

I.2.4.2. Proper *versus* aberrant termination

Early data revealed that, in yeast, the key NMD factor Upf1p also interacts with the termination 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 coimmunoprecipitation studies, as mentioned above, that UPF1 interacts with SMG1 and the release factors eRF1 and eRF3 to form the complex SURF, thus implicating UPF1 in translation termination (Kashima et al, 2006). The fact that SMG1 and UPF1 interact with eRF1-eRF3 as well as 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 (Figure I.3). However, the same study showed that SURF is formed independently of its interaction with the EJC complex, which supports that the 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-SMG9 - will interact with UPF1 and the termination factors to

form the SURF complex at the termination site. SMG1-UPF1 will then interact with the 3' EJC forming the DECID complex which will trigger SMG1-mediated UPF1 phosphorylation (Kashima et al, 2006; Yamashita et al, 2009; Yamashita et al, 2005; Yamashita et al, 2001). This phosphorylation precludes additional ribosome loading, through interaction of phospho-UPF1 with eIF3, and mRNP remodeling resulting in a transcript physically accessible to degradation activities (Isken et al, 2008). The SMG1-mediated UPF1 phosphorylation creates binding platforms for SMG6 and the complex SMG5-SMG7. The complex SMG5-SMG7 will then promote the dissociation of the ribosome from DECID, and the binding of SMG6 will promote UPF1 dissociation from the mRNA (Okada-Katsuhata et al, 2011) (Figure I.3).

I.2.4.3. Alternative branches

Rather than a single linear RNA surveillance pathway as originally thought, previous studies suggests that NMD consists of several alternative branches, regulating different subsets of transcripts. Gehring and colleagues provided the first evidence for two independent branches of the NMD pathway. Tethering assays suggested the existence of a UPF2-independent branch that requires EJC components Y14, MAGOH, eIF4AIII and UPF3B, and is insensitive to UPF2 depletion; and of a UPF2-dependent branch that requires RNPS1 and UPF2, but is not affected by other EJC components depletion (Gehring et al, 2005). Both branches involve UPF3B and are UPF1-dependent. Furthermore, a UPF3-independent branch was identified in a study where TCR-β nonsense transcripts fate was unaffected by depletion of either of the UPF3 paralogs (Chan et al, 2007). Another branch of the NMD pathway may be EJC-independent, as it has been reported that, in some instances, PTC-harboring transcripts can undergo NMD even in the absence of a downstream EJC (Buhler et al, 2006).

Although the mentioned alternative branches of the NMD pathway vary in their dependence on the cofactors UPF2, UPF3b and EJC components, the requirement of UPF1 is common to all regardless of the NMD entry point (Buhler et al, 2006; Chan et al, 2007; Gehring et al, 2005). The existence of these alternative branches may influence NMD efficiency and may allow for specific regulation of subsets of cellular NMD targets by allowing the discrimination of different NMD-competent mRNPs using different cofactors.

I.2.4.4. An evolutionary conserved model with different second signals

Emerging data suggests that mammalian NMD can occur in the absence of splicing and downstream EJCs and that a presence of a 3'UTR intron is not sufficient to trigger this surveillance mechanism. This challenges the requirement of splicing in mammalian NMD activation suggesting that the EJC has evolved as a specialized enhancer in this organism and that the mechanism of PTC definition can be more closely conserved in all eukaryotes than originally thought. Remarkably, downstream sequence elements (DSEs), that function as a binding platform for the NMD-enhancing factor Hrp1p, were identified in yeast, but these elements remain poorly defined (Peltz et al, 1993; Ruiz-Echevarria et al, 1998).

Despite the variations in PTC definition among the different organisms, there is a common NMD-eliciting feature that relies in the length of the 3'UTR: the smaller the physical distance between the PTC and the poly(A) tail the lesser the sensitivity of the transcript to NMD (Rehwinkel et al, 2006; Silva & Romao, 2009). Consistently, an alternative model for PTC definition invoking a "faux 3'UTR" was proposed in yeast. This model postulates that the intrinsically aberrant nature of termination at a PTC functions as a determinant for NMD activation, while the right positioning of the natural termination codon results in proper translation termination, because the terminating ribosome is able to interact with 3'UTR proteins (Amrani et al, 2004). Supporting the "faux 3'UTR" model, toeprinting analysis revealed that ribosomes that encounter a PTC fail to be released (Amrani et al, 2004). Moreover, the deletion of the coding sequence between the PTC and the normal 3'UTR, as well as the tethering of Pab1p in the vicinity of the PTC, abolished NMD (Amrani et al, 2004). In accordance, yeast 3'UTRs are relatively short and similar in length and abnormally extended 3'UTRs are NMD substrates (Muhlrad & Parker, 1999). However, the same is not true for higher eukaryotes, as in these organisms 3'UTRs can vary in length and transcripts with abnormally extended 3'UTRs are not NMD targets (Behm-Ansmant et al, 2007a; Rehwinkel et al, 2005). Even so, increasing the 3'UTR length directs the transcripts to NMD in *D. melanogaster* and tethering of PABPC1 downstream of a PTC also abolishes NMD in this organism (Behm-Ansmant et al, 2007a). Similarly, in humans the artificial extension of the 3'UTRs of β-globin, TPI and Igμ transcripts lacking a PTC resulted in NMD activation (Eberle et al, 2008; Singh et al, 2008) and tethering of PABPC1 in the

vicinity of a PTC suppresses NMD, even in the presence of a downstream EJC (Eberle et al, 2008; Kashima et al, 2006; Silva et al, 2008; Singh et al, 2008). In addition, spatial rearrangements in the 3'UTR increased the stabilization of nonsense transcripts, suggesting that the physical distance rather than the number of nucleotides between the termination codon and the poly(A) tail is a crucial determinant for PTC definition (Eberle et al, 2008). Therefore, the inhibitory effect of PABPC1 on NMD as a result of stimulation of proper translation termination, as the "faux 3'UTR" model proposes, might be extended to all species. In fact, depletion of PABPC1 increases the level of readthrough at all three stop codons (Ivanov et al, 2008). Surprisingly, the knockdown of UPF1 results in a decrease of readthrough level, indicating that UPF1 and PABPC1 have antagonistic effects on translation termination efficiency that can be reflected in their respective stimulatory and inhibitory effects on NMD (Ivanov & Anderson, 2010). Furthermore, it was shown *in vitro* that PABPC1 competes with UPF1 for interaction with eRF3, which is consistent with NMD being impaired when the termination codon is located in proximity of PABPC1 (Singh et al, 2008). Thus, when the terminating ribosome stalls at a termination codon distant from PABPC1, UPF1 is able to bind eRF3; on the other hand, the proximity of the termination codon to PABPC1 allows this protein to interact with eRF3 in detriment of UPF1, enhancing efficient translation termination.

While there is considerable support to the "faux 3'UTR" model, it was observed that, in yeast, NMD occurs in deadenylated transcripts or in mutant strains lacking PABP (Meaux et al, 2008). Additionally, a variety of studies shows that *cis*-acting elements, such as long 3'UTRs or EJCs located downstream of the termination codon, are sufficient to trigger NMD, suggesting that PTC definition is not likely to rely exclusively on PABPC relative position to the PTC. 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'UTR" model and proposes that the discrimination between a normal and a premature termination event is the outcome of the combination of antagonistic signals (Muhlemann, 2008; Shyu et al, 2008; Silva & Romao, 2009; Singh et al, 2008). 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 (Figure I.4). If PAPBC1 is favorably located to interact with the terminating ribosome - through eRF3 - a normal termination event will occur, impairing

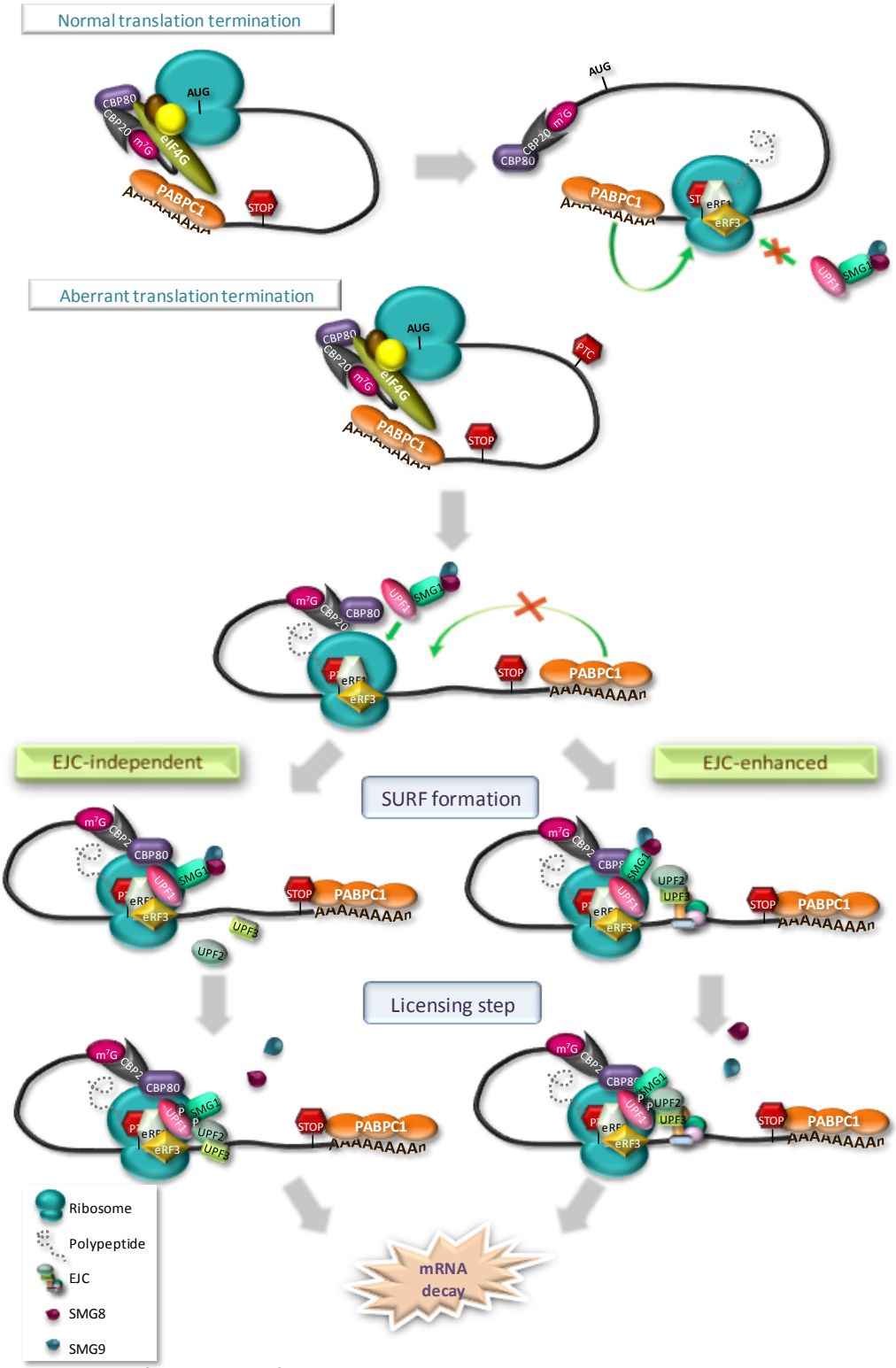

Figure I.4. The unified model for nonsense-mediated mRNA decay. The model proposes that normal translation termination involves PABPC1 interaction with the termination complex, via eRF3, inhibiting UPF1 binding to eRF3. PABPC1 interaction with eRF3 promotes efficient translation termination. However, when the premature stop codon (PTC) is located far from the poly(A) tail, PABPC1 fails to interact with the termination complex, and the ribosome stalls for a prolonged period of time, allowing UPF1 to interact with eRF3. After SURF formation, UPF2 and/or UPF3 stimulate SMG1-mediated UPF1 phosphorylation. At this point, the presence of a downstream EJC will function as a strong NMD enhancer, since the proximity of UPF2 and/or UPF3 at the EJC will facilitate the interaction with UPF1, and the recruitment of the UPF proteins is likely to take longer in the absence of an EJC. Phosphorylation of UPF1 will irreversibly commit the mRNA to decay.

the association of UPF1, thus repressing NMD triggering, even in the presence of downstream EJCs. Conversely, the failure of PABPC1 (and/or other associated 3'UTR elements) to interact with the terminating complex will favor the association of dephosphorylated UPF1, together with SMG1, to the terminating complex, forming the SURF complex. Then, a second signal, such as UPF1 interaction with UPF2 and/or UPF3b that induces SMG1-mediated UPF1 phosphorylation, will trigger NMD, with the presence of downstream EJCs functioning, in some cases, as a secondary NMD enhancer (Figure I.4).

An alternative model called the "ribosome-release" model was recently proposed, but remains to be experimentally tested (Brogna & Wen, 2009). This model hypothesizes that NMD would be a consequence of the release of post-termination ribosomes from a region of the mRNA that normally would be translated. Therefore, in the case of a premature termination event, a long region of the mRNA would not be traversed by ribosomes subunits which would result in mRNA destabilization. UPF1 would have an active role, by interacting with eIF3 to stimulate ribosome release.

So far, it is not possible to unambiguously predict the importance of each mentioned mRNP feature in NMD-triggering. It seems that neither a long 3'UTR or a downstream EJC are absolutely required for NMD, instead a combination of several features is likely to be involved in the mechanism of PTC definition, with the only definite requirement for NMD being the translation-dependent recruitment of UPF1 to the mRNA (Nicholson et al, 2010).

I.2.4.5. AUG-proximal PTCs unexpectedly evade NMD

Even though a nonsense codon at position +1 with respect to the initiation AUG codon is sufficient to trigger NMD (Lew et al, 1998), there are several reports of PTCs close to the 5' end of the first exon that fail to elicit NMD (Asselta et al, 2001; Boyer et al, 2005; Buisson et al, 2006; Buzina & Shulman, 1999; Danckwardt et al, 2002; Denecke et al, 2004; Harries et al, 2004; McLean et al, 2003; Paulsen et al, 2006; Perrin-Vidoz et al, 2002; Rolfini & Cabrini, 1993; Romao et al, 2000; Tournier et al, 2004; Zhang & Maquat, 1997). In fact, our lab reported that human β-globin transcripts harboring a naturally occurring nonsense mutation at codon 15 accumulate to levels comparable to those of wild-type (Romao et al, 2000). Similarly, PTCs generated at codons 5 and 17 were also

shown to fail to trigger NMD on these transcripts (Romao et al, 2000). For some AUG-proximal PTCs, the NMD-resistance is explained by translation reinitiation, since the ribosome that initiates at an in-frame AUG downstream of the early PTC removes the remaining EJCs from the mRNA and terminates translation at the normal termination codon, culminating in the production of an N-terminal truncated protein (Perrin-Vidoz et al, 2002; Zhang & Maquat, 1997). However, translation reinitiation does not fully explain the NMD-resistance observed for some AUG-proximal PTCs. For instance, in Igμ transcripts bearing PTCs located at codons 3 or 32, expressed in hybridoma cells, translation reinitiation occurs at Met100 with an efficiency of 4% of the normal rate (Buzina & Shulman, 1999); still, no detectable protein products corresponding to translation reinitiation at Met100 were found in HeLa cells expressing Igμ transcripts with a nonsense codon in position 32 (Buhler et al, 2004). Likewise, the NMD-resistance of β-globin transcripts bearing a PTC at position 15 is not attributable to translation reinitiation since blocking potential reinitiation sites does not result in NMD-typical mRNA levels (Inacio et al, 2004; Silva et al, 2006). Moreover, abnormal splicing and impaired translation have also been shown not to be the determinants of this resistance to NMD (Inacio et al, 2004; Silva et al, 2006). Indeed, our lab has shown that, in these transcripts, NMD inhibition reflects the proximity of the PTC to the translation initiation codon (Inacio et al, 2004; Silva et al, 2006). This “AUG-proximity effect”, which is independent of sequence context and transcript identity and appears to be a general attribute of mammalian NMD (Silva et al, 2006), may reflect the nature of the early termination event. In the case of such a premature termination event, the translation round might be brief enough to maintain PABPC1 in close proximity to the AUG codon, as a consequence of mRNA circularization. This favorable location might allow PABPC1 to interact with the translation termination complex by the time the ribosome reaches the stop codon, superimposing its inhibitory effect on NMD (Silva et al, 2008; Silva & Romao, 2009) (Figure I.5).

Nonetheless, a recent study (Neu-Yilik et al, 2011) reported that translation reinitiation is the determinant of the NMD-resistance of β-globin AUG-proximal nonsense-mutated transcripts. This inconsistency with our lab previous findings might be explained by differences in the experimental settings, since in the study of Neu-Yilik et al. (2011), the natural β-globin exon 1 was transformed into a functional exon 2 by the introduction of

an intron into the 5'UTR, which resulted in down-modulation of the expression of nonsense-mutated transcripts, when compared to the normal β-globin transcript.

Figure I.5. Model for the AUG-proximity effect on NMD-evasion of transcripts harboring an AUG-proximal PTC. During cap-mediated translation initiation, PABPC1 interacts with the initiation factor eIF4G. It has been proposed that the resulting configuration brings PABPC1 into the vicinity of the AUG initiation codon as a consequence of 43S scanning. The maintenance of eIF4G-PABPC1 association with the 40S during the initial phase of translation elongation brings it into close contact with an AUG-proximal PTC in a transcript where the ORF is quite short. This proximity to the PTC allows PABPC1 to interact with the release factor eRF3 at the termination complex, thus impairing the association of UPF1 to the ribonucleoprotein complex, resulting in efficient translation termination and inhibition of NMD.

I.2.5. Degradation of NMD targets

Another still unresolved question about NMD relates with the exact mechanism of degradation of the recognized nonsense transcripts – does it utilizes the major mRNA-degradation pathway, or is it initiated by special nucleases? The bulk mRNA-turnover is usually initiated by deadenylation. While in yeast the complex formed by the carbon catabolite repressor 4 (Ccr4) and its associated factor Caf1p is responsible for the removal of the poly(A) tail, in mammals there are two different complexes involved in this reaction. The poly(A) nuclease (PAN) 2 and 3 complex initiates poly(A) tail shortening and the complex CCR4-CAF1 removes the remaining adenines (Nicholson & Muhlemann, 2010). After deadenylation, mRNAs can be degraded by two general pathways: *i)* in one pathway the decapping enzyme complex DCP1-DCP2 hydrolyses the 5'cap and the exonuclease XRN1 promotes 5' → 3' decay; *ii)* in the other pathway, deadenylation is followed by 3' → 5' degradation by the exosome (Meyer et al, 2004). Studies in *S. cerevisiae* suggest that the degradation phase of NMD bypasses

deadenylation before decapping (Muhlrad & Parker, 1994). However, PTC-dependent accelerated deadenylation has also been reported and the initiation of NMD in this organism by decapping or deadenylation seems to be dependent on the relative position of the PTC to the cap and poly(A) tail (Cao & Parker, 2003; Mitchell & Tollervey, 2003). In contrast, in *D. melanogaster*, where there is no SMG7 ortholog, nonsense mRNAs are degraded by endonucleolytic cleavage within the vicinity of the PTC. The resulting 5' and 3' intermediates are then rapidly degraded by XRN1 and the exosome, respectively (Gatfield et al, 2003).

In mammals, available data indicates that nonsense transcripts degradation initiates with deadenylation followed by decapping and XRN1-mediated decay (Chen & Shyu, 2003; Couttet & Grange, 2004; Lejeune et al, 2003). Consistently, UPF proteins are able to form a complex with XRN1, XRN2 and some exosome proteins (Lejeune et al, 2003). Recently, the proline-rich nuclear receptor co-regulatory protein2 (PNRC2) was shown to be involved in promoting degradation of NMD targets. As this protein interacts with phospho-UPF1 and DCP1, it might provide a link between UPF1-bound mRNAs and the decapping enzymes (Cho et al, 2009).

Nonetheless, emerging data has revealed that degradation of human nonsense transcripts can be initiated by endonucleolytic cleavage in the vicinity of the PTC (Eberle et al, 2009). As above mentioned, the PIN domain of SMG6 exhibits the endonucleolytic activity responsible for initiating NMD in human cells as in *D. melanogaster* (Eberle et al, 2009). Therefore, mammalian NMD seems to consist of two independent routes for RNA decay: an endonucleotic SMG6-dependent route, and other decapping-dependent exonucleolytic route. In either case, the resulting RNA fragments are subjected to exonucleolytic decay (Muhlemann & Lykke-Andersen, 2010b; Nicholson & Muhlemann, 2010) (Figure I.6). The determinants of which pathway to be activated remain unclear. It has been shown that SMG7, when tethered to a reporter mRNA, activated mRNA decay in a DCP2- and XRN1-dependent manner, but independently of SMG6. Since SMG5 and SMG7 co-localize in processing bodies (P-bodies) while SMG6 forms separate foci (Unterholzner & Izaurralde, 2004); it is possible that the binding of SMG5/7 or SMG6 to phospho-UPF1 represents a branching point. Even so, the relative contribution of each pathway remains to be clarified.

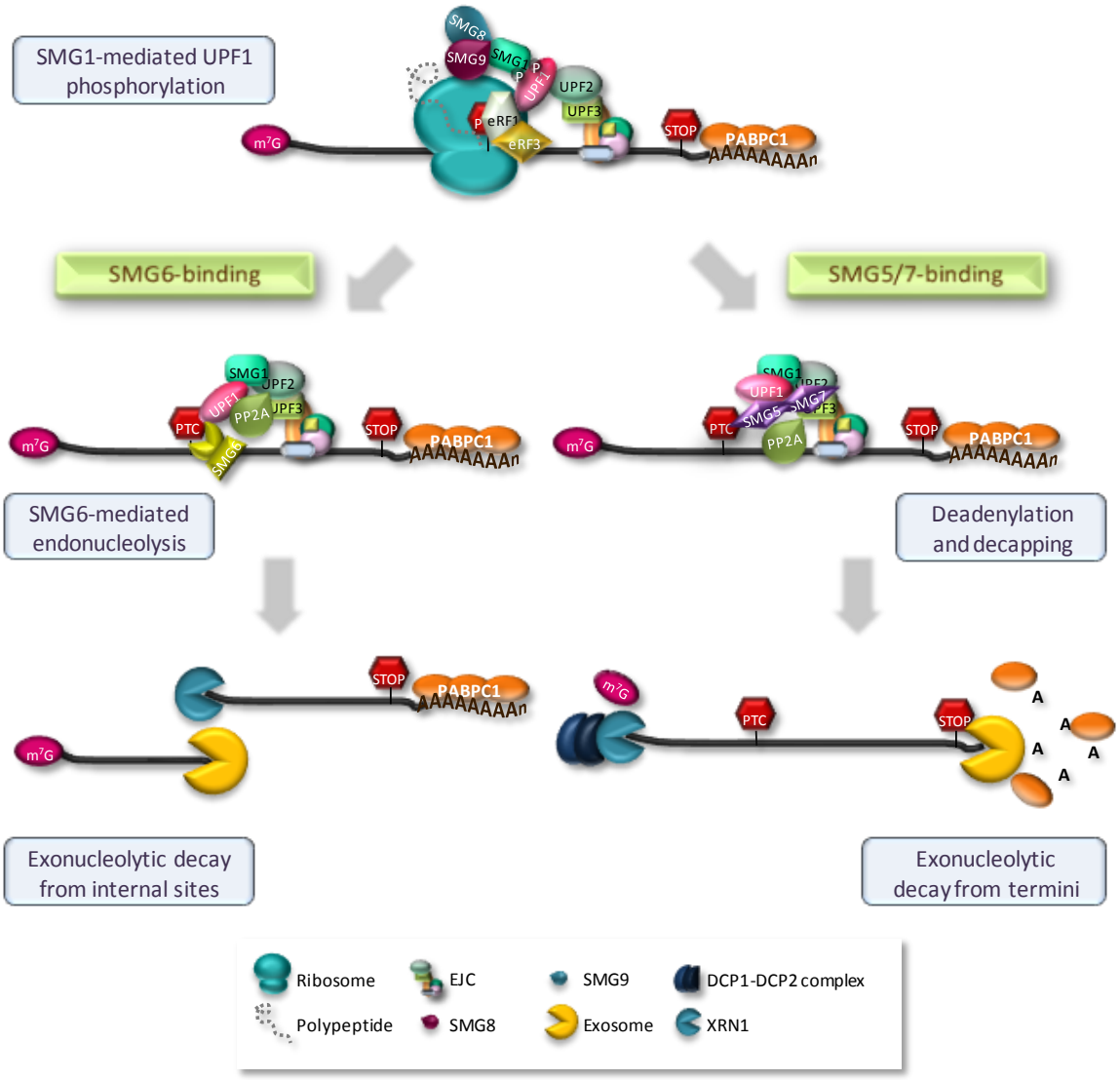

Figure I.6. Decay pathways of human NMD. SMG1-mediated UPF1 phosphorylation induces a conformational change in UPF1 that results in an increased affinity for the mRNA and irreversibly marks the mRNA for degradation. Phosphorylated UPF1 can either interact with SMG6 or with the heterodimer SMG5-SMG7. Both routes will lead to PP2A recruitment and UPF1 dephosphorylation. SMG6 is an endonuclease and, upon UPF1 binding, will cleave the mRNA in the vicinity of the premature termination codon. The resulting decay intermediates will be rapidly degraded 5' → 3' by XRN1 and 3' → 5' by the exosome. On the other hand, UPF1 binding to SMG5-SMG7 results in the recruitment of deadenylases. Deadenylation is followed by the removal of the cap structure by the DCP1/DCP2 decapping complex and the transcripts will be degraded 5' → 3' by XRN1 and 3' → 5' by the exosome.

I.2.6. Feedback regulatory mechanisms

Given that NMD plays a role in gene regulation and mRNA surveillance, the perturbation of this surveillance pathway could have catastrophic consequences due to the variation in transcripts profiles and to the production of potentially toxic truncated proteins

(Muhlemann et al, 2008). A buffering system that confers robustness to the NMD mechanism would be essential to protect against this deleterious consequences. Indeed, evidence for such homeostatic pathway acting on NMD has emerged from reports showing that UPF3a is stabilized in response to loss of its paralog UPF3b (Chan et al, 2009) and that UPF1 depletion stabilizes SMG5 encoding mRNA (Mendell et al, 2004; Singh et al, 2008).

Consistently, Huang and colleagues recently identified a large number of NMD factors that are upregulated upon NMD perturbation. This NMD regulation consists of a series of negative feedback regulatory loops. Analysis of the UPF and SMG mRNA half-lifes in UPF1- and SMG1-depleted cells suggests that mRNAs encoding UPF1 and a subset of other NMD factors are direct NMD targets (Huang et al, 2011). Moreover, the same study showed that overexpression of SMG1, SMG5 and SMG6 augments NMD, suggesting that these three factors are rate limiting for NMD. Another recent study of transcriptome profiling in human UPF1-, SMG6- or SMG7-depleted cells revealed that most mRNAs coding for NMD factors are NMD-sensitive, all possess long 3'UTRs and some of them harbor uORFs (Yepiskoposyan et al, 2011). In addition, the long 3'UTRs of UPF1, SMG5 and SMG7 mRNAs were shown to constitute the main NMD-inducing features of these mRNAs (Yepiskoposyan et al, 2011).

The NMD feedback regulation is a highly directed response that is mediated by different branches of the NMD pathway, probably because, in some cases, NMD may selectively increase the levels of NMD factors of the same branch of the perturbed factor, maximizing NMD buffering, or because the upregulation of partially redundant factors will bolster NMD. As cell types differ in their NMD factor profile and sensitivity, there are cell-type specific differences in rate-limiting and redundant NMD factors. Therefore, this buffering system provides versatile and subtle feedback control to confer robustness to NMD, by fine-tuning the level of NMD factors in order to achieve optimal NMD activity (Huang et al, 2011). This mechanism may be conserved in eukaryotes, as recent studies show that SMG5 and SMG6 in *D. melanogaster* (Rehwinkel et al, 2005) and SMG7 and UPF3 in *Arabidopsis thaliana* (Riehs et al, 2008; Saul et al, 2009) are upregulated in response to NMD depletion.

I.2.7. NMD effectors: other functions & new players

NMD factors either alone or in groups participate in additional cellular pathways and functions that range from other RNA surveillance pathways over functions in genome stability, telomere maintenance and translation. So far, it is not known if these other functions are always independent from NMD or if they converge in functional networks (Neu-Yilik & Kulozik, 2008).

I.2.7.1. NMD factors and genome stability

The first link between NMD factors and telomere maintenance arose from studies in yeast showing that Upf1p, Upf2p and Upf3p mutations led to telomere shortening (Lew et al, 1998). In addition, SMG5, SMG6 and SMG7 were found to be homologous of hEST1B, hEST1A and hEST1C, respectively. These proteins are orthologues of yeast Est1p protein, which is involved in telomerase regulation and promotes telomere elongation. Human SMG6 overexpression leads to progressive telomere shortening and SMG5 and SMG6 were found to associate with telomerase activity in human cell extracts (Reichenbach et al, 2003). Surprisingly, UPF1, UPF2, SMG1 and SMG6 were found to be enriched at telomeric chromatin fractions, and they negatively regulate the association of the recently discovered set of telomeric repeat-containing RNAs (TERRAs) with chromatin (Azzalin et al, 2007). This data suggests that the NMD factors link TERRA regulation with telomere maintenance, and thus, with genome stability.

SMG1 is a member of the PIK family and, like ATM and ATR kinases, it functions in genome surveillance. Both SMG1 and ATM phosphorylate the cell-cycle checkpoint protein p53, and SMG1 depletion leads to spontaneous DNA damage and increased sensitivity to ionizing radiation (Brumbaugh et al, 2004). Moreover, UPF1 also has a role in DNA replication: UPF1 depletion results in early S-phase arrest and induces an ATR-dependent DNA-damage response (Azzalin & Lingner, 2006). The identification of UPF1 as the DNA polymerase δ -associated helicase corroborates its function in DNA repair (Carastro et al, 2002) that is separable and likely independent of its role in NMD, since ATR depletion impairs chromatin loading of UPF1 without interfering with the NMD pathway (Brumbaugh et al, 2004).

I.2.7.2. Functions in other mRNA turnover pathways

In addition to its role in guiding NMD targets for decay, UPF1 also serves in other mRNA turnover pathways. Yeast two-hybrid analysis and GST-pull down assays showed that Staufen-1 (STAU1), which plays a role in mRNA decay, interacts with UPF1. Tethering of STAU1 to a reporter transcript results in UPF1-recruitment and induces translation-dependent STAU1-mediated mRNA decay (SMD) without involving other NMD effectors (Kim et al, 2005). UPF1 has also been reported to play a role in replication-dependent histone-mRNA degradation (Kaygun & Marzluff, 2005), however its function in histone mRNA metabolism remains unclear (Nicholson et al, 2010).

I.2.7.3. NMD factors and translation

There are several studies indicating a role for the UPF proteins in translation. Analysis of premature translation in yeast cell-free extracts demonstrated that a significant fraction of ribosomes terminating translation at a PTC reinitiated at proximal AUG codons in a UPF1-dependent fashion (Amrani et al, 2004). Furthermore, the NMD effectors UPF1, UPF2, UPF3A, UPF3B, MAGOH, Y14 and RNPS1 promote polysome association and increase translation when tethered within the ORF of a reporter mRNA (Kunz et al, 2006; Nott et al, 2004). Fascinatingly, the NMD and the translation functions of UPF3A and UPF3B require different protein domains and interacting factors. Indeed, the UPF3 proteins require Y14/MAGOH/eIF4IIIA/BTZ to trigger NMD while their activity in enhancing translation is independent of these EJC components (Kunz et al, 2006), even though Y14 and MAGOH also stimulate translation (Nott et al, 2004).

Whilst increasing the rate of translation, some NMD factors also appear to stimulate the efficiency of translation termination as, in yeast, codon readthrough is enhanced when any of the three upframeshift proteins is inactive or absent (Wilkinson, 2005). Nevertheless, a recent study demonstrated that the apparent loss of termination fidelity seems to be a indirect consequence of the NMD-sensitive ALR1 mRNA that results in increased intracellular Mg^{2+} levels (Johansson & Jacobson, 2010).

Several data have implicated possible roles for the UPF proteins in the regulation of translation termination, repression and initiation, both in yeast and in mammals, which seem to be independent of their activity on mRNA decay (Czaplinski et al, 1998; Isken et al, 2008; Ivanov et al, 2008; Muhlrad & Parker, 1999; Wang et al, 2001). In fact, an

hyperphosphorylated mutant of UPF1 that is impaired for NMD, repressed translation of a reporter mRNA in rabbit retyculocyte lysates (Isken et al, 2008). Consistently, data from a ribosome reutilization assay showing that the elimination of any of the UPF proteins led to reduced availability of ribosomes for translation initiation, suggest that UPF1 regulates initiation efficiency of ribosomes entering a new cycle of translation (Ghosh et al, 2010). Furthermore, toeprinting assays revealed that extracts lacking UPF1 had fewer 80S ribosomes at the AUG, while exhibiting similar 40S recruitment to the wild-type extracts, indicating that UPF1 loss affects subunit joining (Ghosh et al, 2010).

At the same time, as new functions for NMD factors are revealed, it becomes more apparent that factors involved in translation play multiple roles that may link translation initiation with termination and/or NMD (Amrani et al, 2008). eIF3, together with eIF1, eIF1A and the eIF3j subunit, has been implicated in recycling by stimulating the *in vitro* dissociation of post-termination 80S complexes, and remains bound to 40S subunits after this process (Pisarev et al, 2007). Moreover, eIF3e depletion impairs NMD in a manner similar to UPF1 depletion (Morris et al, 2007) and interacts with phospho-UPF1, triggering translation repression (Isken et al, 2008). It has also been shown that, at elevated temperatures, yeast strains harboring the temperature-sensitive prt1-1 mutation (in eIF3) lose the ability to initiate translation and promote NMD (Welch & Jacobson, 1999). The role of eIF3 in recycling may, thus, be linked to its role in NMD, for example, in the resolution of the SURF/post-termination complex.

I.2.8. Human β-globin as a model system for studying NMD

The human β-globin gene has two introns and three exons that are flanked by 5' and 3' UTRs (Huisman, 1993). Thus, after the two introns are spliced out, two EJCs might be assembled on the transcript (Le Hir et al, 2000a; Le Hir et al, 2000b). A β-globin NMD-behaviour corroborating the "50-55 nt boundary rule" has been broadly described, suggesting that the EJC functions as an enhancer in β-globin NMD. Indeed, the first indications regarding whether a β-globin PTC elicits NMD, concerning its position relatively to the downstream intron, were obtained by Kulozik and Maquat labs (Thermann et al, 1998; Zhang et al, 1998b). These 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, 60-61, 75, and 82) elicit NMD. In contrast, mRNAs bearing 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 and 141) are all NMD-resistant. This illustrates a clear boundary between 48 and 66 nt upstream of the last exon-exon junction. Although the onset of NMD requires a minimal distance between the nonsense mutation and the final exon-exon junction, on β-globin transcripts, the requirement for a maximal distance appears to be considerably less crucial. Indeed, a β-globin mRNA with a nonsense mutation at codon 39 (Baserga & Benz, 1988) remains NMD competent, even when the distance between the PTC and the 3' exon-exon junction is increased from the normal 180 nt up to 654 nt (Neu-Yilik et al, 2001). This is a much longer distance than the one required for NMD in the triosephosphate isomerase (TPI) transcripts (Ruiz-Echevarria et al, 1998; Sun & Maquat, 2000; Zhang et al, 1998a). On the other hand, an intronless human β-globin gene, carrying a nonsense mutation at codon 39, generates an mRNA that lacks the EJCs and, consequently, is immune to NMD (Neu-Yilik et al, 2001). This is supported by the fact that naturally intronless mRNAs containing PTCs are not usually degraded by NMD (Maquat & Carmichael, 2001). Furthermore, the insertion of an intron more than 50 nt downstream of the native stop codon, redefines this codon as premature and triggers this mRNA to NMD (Thermann et al, 1998).

Nevertheless, exceptions to the "50-55 nt boundary rule" have also been reported for the human β-globin mRNAs. As above mentioned, our lab showed that β-globin transcripts containing naturally occurring nonsense mutations in the 5' region of exon 1 accumulate to levels similar to those of normal β-globin transcripts, being, unexpectedly according to their position, NMD-resistant (Romao et al, 2000). Furthermore, NMD was found to be triggered in hybrid mouse-human β-globin transcripts (carrying nonsense mutations at codons 21-22, 39 or 60-61) in the absence of the last intron (Zhang et al, 1998b) and therefore, in the absence of downstream EJCs. Even so, Neu-Yilik and colleagues have shown that splicing is an indispensable component of the β-globin NMD pathway (Neu-Yilik et al, 2001). Additionally, a study using the IVS1 +5 G→A thalassemic β-globin gene as a model system, showed that the splicing of these pre-mRNAs at a cryptic site, generates a mature transcript carrying a PTC at codon 30 (located more than 55 nt 5' of the final exon-exon junction) that is immune to NMD (Danckwardt et al, 2002). Although the mechanism underlying this NMD-behavior was not clarified, it was

demonstrated that neither abnormalities of splicing nor translation reinitiation at downstream AUG codons is the cause for the NMD resistance of this transcript (Danckwardt et al, 2002).

I.3. Aims

In summary, there are several examples of PTC-containing transcripts, expected to contain residual EJCs, that escape NMD even when the PTC is located, in a linear sense, quite far from the poly(A) tail and PABP. Previous studies of AUG-proximal PTC mRNAs revealed that its observed NMD resistance does not reflect downstream translation reinitiation or extension of ribosome elongation 3' of the PTC and is instead a direct effect of the termination event being located in close proximity to the AUG. This effect constitutes a general attribute of mammalian NMD that is independent of sequence context and independent of the 5'UTR length. The aim of the work presented in this thesis was to clarify the mechanistic basis for the NMD resistance of mRNAs carrying AUG-proximal PTCs and expand the current models for NMD and translation. Therefore, the following objectives were established:

- To confirm the inhibitory effect of PABPC1 on the NMD commitment of AUG-proximal nonsense-mutated transcripts.
- To evaluate the biochemical mechanism by which PABPC1 controls ribosomal termination and how it may alter the recruitment of UPF1 to a terminating ribosome at an AUG-proximal PTC.
- To investigate the molecular basis by which translation initiation factors are involved in the NMD inhibition of mRNAs harboring an AUG-proximal PTC.

Chapter II

Interaction of PABPC1 with the translation initiation complex is critical to the NMD-resistance of AUG-proximal nonsense mutations

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Author's note

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II.1. Abstract

Nonsense-mediated mRNA decay (NMD) is a surveillance pathway that recognizes and rapidly degrades mRNAs containing premature termination codons (PTC). The strength of the NMD response appears to reflect multiple determinants on a target mRNA. We have previously reported that mRNAs containing PTCs in close proximity to the translation initiation codon (AUG-proximal PTCs) can substantially evade NMD. Here, we explore the mechanistic basis for this NMD resistance. We demonstrate that translation termination at an AUG-proximal PTC lacks the ribosome stalling that is evident in an NMD-sensitive PTC. This difference is associated with demonstrated interactions of the cytoplasmic poly(A)-binding protein 1, PABPC1, with the cap-binding complex subunit, eIF4G, and the 40S recruitment factor eIF3 as well as the ribosome release factor, eRF3. These interactions, in combination, underlie critical 3'-5' linkage of translation initiation with efficient termination at the AUG-proximal PTC and contribute to an NMD-resistant PTC definition at an early phase of translation elongation.

II.2. Introduction

Nonsense-mediated decay (NMD) targets mRNAs harboring premature translation-termination codons (PTCs) for rapid decay. This surveillance pathway limits the synthesis of potentially deleterious C-terminally truncated proteins encoded by mutant mRNAs (Muhlemann & Lykke-Andersen, 2010a; Nicholson et al, 2010; Rebbapragada & Lykke-Andersen, 2009; Silva & Romao, 2009). As such, NMD serves as an important modifier of many genetic disorders (Behm-Ansmant & Izaurralde, 2006; Holbrook et al, 2004; Peixeiro et al, 2011b). The NMD pathway also functions as an important determinant of wild-type gene expression with approximately 10% of the mammalian transcriptome impacted by components of the NMD apparatus (McGlincy et al, 2010; Mendell et al, 2004; Rehwinkel et al, 2006). A comprehensive description of the determinants and mechanisms of NMD is therefore central to the understanding of both normal and mutant gene expression.

A substantial body of evidence supports a role for the supramolecular exon junction complexes (EJC) in triggering NMD. The EJC is deposited 20-24 nucleotides (nts)

upstream of each exon-exon junction during transcript splicing in the nucleus (Le Hir et al, 2001). These complexes are subsequently displaced from the mature mRNA in the cytoplasm by the elongating ribosome (Maquat, 2004) during the first round of translation ["pioneer round of translation"; (Ishigaki et al, 2001; Lejeune et al, 2002)]. If a PTC is located more than 50-54 nts 5' to the last exon-exon junction, one or more EJCs will remain beyond the reach of the elongating ribosome and will be retained on the mRNA. The retained EJC(s) can interact with the translation termination complex *via* bridging interactions between the release complex associated proteins, UPF1 and SMG1 (Kashima et al, 2006) and the EJC-associated factors, UPF2-UPF3 (Le Hir et al, 2001). This bridging interaction has been proposed to trigger accelerated decay (i.e., NMD) of the PTC-containing mRNA.

While multiple reports support a role for the EJC-release complex interaction in NMD, an accumulating body of data indicates that additional determinants may play a significant role in this surveillance pathway. Converging lines of evidence from studies in *D. melanogaster*, *C. elegans*, and *S. cerevisiae* reveal that NMD can be triggered independently of transcript splicing and EJC deposition (Conti & Izaurralde, 2005; Rehwinkel et al, 2006). These studies further reveal that 3' untranslated region (UTR) length and the proximity of the PTC to the cytoplasmic poly(A)-binding protein 1 (PABPC1) may constitute critical determinants of NMD. Studies in yeast support these findings by demonstrating that correct positioning of the termination codon relative to PABPC1 are necessary for efficient termination and NMD resistance (Amrani et al, 2004). Recent studies in mammalian cell culture support the conclusion that the strength of the NMD response is inversely related to the distance between the PTC and PABPC1 (Eberle et al, 2008; Singh et al, 2008); shortening this distance by tethering PABPC1 in close proximity to an otherwise NMD-sensitive PTC suppresses NMD, even in the presence of a downstream EJC (Eberle et al, 2008; Ivanov et al, 2008; Silva et al, 2008; Singh et al, 2008). In further support of this model, it has been demonstrated *in vitro* that PABPC1 can competitively block the association of UPF1 with eRF3 (Singh et al, 2008) with a corresponding blunting of UPF1 actions and the NMD response (Singh et al, 2008). Thus the impact of PABPC1 on NMD appears to reflect its ability to interact with, and alter the impact of, supramolecular interactions at the translation termination complex.

We have previously reported that mRNAs containing PTCs in close proximity to the translation initiation AUG codon (AUG-proximal PTCs) escape NMD. This was initially surprising as these mRNAs would be expected to contain residual EJCs and in addition would situate the PTC quite far, in a linear sense, from the poly(A) tail and PABP (Romao et al, 2000). Detailed analyses of AUG-proximal PTC mRNAs revealed that its observed NMD resistance did not reflect downstream translation reinitiation or extension of ribosome elongation 3' of the PTC and was instead a direct effect of the termination event being located in close proximity to the AUG (Inacio et al, 2004; Silva et al, 2006). Based on these studies, and on the observation by others that PABPC1 is able to bind simultaneously to the cap-binding complex subunit eIF4G and to the poly(A) tail, ["closed-loop" mRNP configuration (Wells et al, 1998)], we have proposed a model in which the short open reading frame on an AUG-proximal nonsense-mutated mRNA, situates PABPC1 and its associated cap-binding complex, in close proximity to the PTC. This proximity would allow PABPC1 to alter the structure and/or function of the translation termination complex with a consequent inhibitory effect on NMD (Silva et al, 2008; Silva & Romao, 2009).

In the present report we further focus on the mechanism of NMD-resistance of AUG-proximal PTCs. These studies support the model in which PABPC1 is brought into close proximity with an AUG-proximal PTC *via* interactions with the translation initiation complexes. This proximity of PABP to the AUG-proximal PTC allows PABP to interact with eRF3 with a consequent enhancement of the release reaction and repression of the NMD response.

II.3. Materials and Methods

II.3.1. Plasmid constructs

The wild type β-globin gene (βN), as well as the human β-globin variants β15 and β39 were cloned into the pTRE2pur vector (BD Biosciences) as previously described (Silva et al, 2006). The pDEST26-PABPC1delC construct was obtained by deleting the DNA fragment encoding the C-terminal portion of PABPC1 from pDEST26-PABPC1 plasmid (IOH13850-pDEST26; RZPD). To accomplish this deletion, the coding sequence of PABPC1, excluding the C-terminal domain (NM 002568, nucleotides 2140-2394,

corresponding to C-terminal 85 amino acids), was amplified by PCR from pDEST26-PABPC1 using a pair of primers, one with an AgeI linker (primers #1 and #2; Table II.1). After HindIII digestion, the PCR DNA product was inserted into HindIII/AgeI sites of pDEST26-PABPC1. The pUHD-eRF3 plasmid was a generous gift of Marla J. Berry. eRF3 cDNA sequence was then subloned into pcDNA3.1 (Invitrogen). To obtain pcDNA-eRF3delN, the coding sequence of eRF3, excluding the N-terminal domain (X17644, nucleotides 1-648, corresponding to N-terminal 138 amino acids), was amplified by PCR from pcDNAeRF3 using a pair of primers, one with an EcoRI linker and another with an EcoRV linker (primers #3 and #4; Table II.1) and inserted into EcoRI/EcorV sites on pcDNA3.1/hygro+ (Invitrogen). The plasmid encoding PAIP2, pDEST26-PAIP2 (o834B1117-pDEST26; RZPD) was purchased from BD Bioscience.

Table II.1. DNA oligonucleotides used in the current work.

Primer	Sequence (5' → 3')
#1	GTGGAAAATCCAAAGGATTTGG
#2	ACCGGTCAAAGGTTCCTGACCTTGTAC
#3	CCGGAATTCGCCATGGAACTTTCAGAACC
#4	AAAGATATCTTAGTCTTTCTCTGGAACCAG
#5	GTGGATCCTGAGAACTTCAGGCT
#6	CAGCACACAGACCAGCACGT
#7	CGCAACCTCCCCTTCTACG
#8	GGTGACGGTGAAGCCGAG
#9	CTGCTCATTGCAGGCCAGAT
#10	GAGCCTGGGCCATGAAGAG
#11	CACCCAGTCATTTTGGCCTC
#12	CGACAGTTCCCAACAGGGTC
#13	GGACAAGCATGGTTTTAGGCA
#14	TGCTGCTCCTGAGTAATTCCC
#15	ATGGCGCAGACGCAGGG
#16	CCGCACTAGGCTGGAACATC
#17	FAM-GCAATGAAAATAAATGTTTTTTATT
#18	FAM-CCCTTGAGGTTGTCCAGGT
#19	CTACCCTTGGACCTAGAGGTTCTTTGAGTC
#20	GACTCAAAGAACCTCTAGGTCCAAGGGTAG

The *in vitro* transcription DNA templates used for toeprinting assays (β15 and β39), were cloned into the pSP64 vector (Promega) carrying the β-globin cDNA (Shakin & Liebhaber, 1986). The β15 and β39 cDNAs carry a nonsense mutation at codon 15 (TGG → TAG) or codon 39 (CAG → TAG), respectively. These mutations were created by site-directed

mutagenesis using the QuickChange™Site-directed Mutagenesis Kit (Stratagene) and mutagenic primers #19 and #20 and the plasmid with the βN cDNA as template.

II.3.2. Cell culture, plasmid and siRNA transfection

HeLa cells, stably expressing the tet transactivator (HeLa/tTA) (Kong et al, 2003), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. siRNA duplexes (Table II.2) were designed as 19-mers with 3'-dTdT overhangs and purchased from Thermo. Transfections of cells with siRNAs were carried out using Lipofectamine 2000 Transfection Reagent (Invitrogen), following the manufacturer's instructions, in 35-mm plates using 200 pmol of siRNA oligonucleotides and 4 µl of transfection reagent. Twenty four hours later, cells were transfected with an additional 50 pmol of siRNAs along with 300 ng of the test construct DNA and 1700 ng of pEGFP vector (BD Biosciences) to control for transfection efficiency. Where specified, 1700 ng of pDEST26-PABPC1, pDEST26-PABPC1delC, pcDNA-eRF3delN or 1000 ng of pDEST26-PAIP2 [since in the study of Khalegpour et al. (Khaleghpour et al, 2001) 5000ng of PAIP2 DNA resulted in a decrease of about 30% of the reporter translation in HeLa cells] were co-transfected with 300 ng of the test β-globin construct DNA.

II.3.3. RNA isolation

Total RNA from transfected cells was prepared using the Nucleospin RNA extraction II (Marcherey-Nagel) following the manufacturer's instructions.

II.3.4. Reverse transcription-coupled quantitative PCR (RT-qPCR)

Synthesis of cDNA was carried out using 2 µg of total RNA and Superscript II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Real-Time PCR was performed in ABI Prism 7000 Sequence Detection System, using SybrGreen Master Mix (Applied Biosystems). Primers specific for β-globin (primers #5 and #6, Table 1) and for puromycin (primers #7 and #8, Table 1) were designed using the ABI Primer Express software. Quantification was performed using the relative standard curve method (ΔΔCt, Applied Biosystems). The following cycling parameters were used: 10 min at 95°C and 40

cycles of 15 sec at 95°C and 1 min at 60°C. Technical triplicates from 3-4 independent experiments were assessed in all cases.

Table II.2. Sequences of the siRNAs used in the current work.

siRNA	Sequence (5' → 3')
PABPC11	UCACUGGCAUGUUGUUGGA
PAB3UTR	UUGAUCAGGGACCAUGAAA
eRF3a1	GAGGAACAGUCAUUGUGUG
eRF3a2	UCCUCUCAAGAGGAACAGU
UPF1a	AAGAUGCAGUUCCGCUCCAUU
UPF1b	GCAGCCACAUUGUAAAUCAUU
eIF3h1	ACUGCCCAAGGAUCUCUCU
eIF3h2	GAUCGGCUUGAAAUUACCA
eIF3f1	GUGAAGGAGAAAUGGGUUU
eIF3f2	AUACGCGUACUACGACACU
eIF3e1	CCAGGGAUGGUAGGAUGCU
eIF3e2	UGCAGAAUUGGGAUGCAGC
Luc	CGUACGCGGAAUACUUCGA

II.3.5. Semi-quantitative RT-PCR

1500 ng of total mRNA were reverse-transcribed (RT) with Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's standard protocol and using 250 ng of Random Primers (Invitrogen) in a final volume of 20 µl. The PCR reactions for eIF3h, eIF3f or eIF3e and histone deacetylase 1 (HDAC1) cDNAs were performed in parallel at similar conditions: 3 µl of the RT product was amplified in a 50 µl reaction volume using 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 15 pmol of each primer (primers #9 and #10 for eIF3h, primers #11 and #12 for eIF3f, primers #13 and #14 for eIF3e and primers #15 and #16 for HDAC1; Table II.1), 0.75 U of Amplitaq (Promega), and 1X PCR buffer (Promega). Thermocycler conditions were 95°C for 4 min followed by 26 cycles of 95°C for 45 sec, 56°C for 45 sec, and 72°C for 45 sec followed by a final extension of 72°C for 10 min. Ten-microliter aliquots from each RT-PCR sample were analyzed by electrophoresis on 1.8% agarose gels.

II.3.6. SDS-PAGE and Western blotting

Protein lysates were resolved, according to standard protocols, in 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were probed using mouse

monoclonal anti-α-tubulin (Sigma) at 1:10,000 dilution, goat polyclonal anti-hUPF1 (Bethyl Labs) at 1:500 dilution, rabbit polyclonal anti-PABPC1 N-terminal domain (Cell Signaling) at 1:500 dilution, rabbit polyclonal anti-eRF3 (Abcam) at 1:500 dilution, goat polyclonal anti-PAIP2 (Santa Cruz) at 1:250 dilution or anti-GFP (Abcam) at 1:5000 dilution. Detection was carried out using secondary peroxidase-conjugated anti-mouse IgG (Bio-Rad), anti-rabbit IgG (Bio-Rad) or anti-goat IgG (Sigma) antibodies followed by chemiluminescence.

II.3.7. *In vitro* transcription and capping

The pSP64 plasmids carrying the βN, β15 or β39 cDNA containing a $(T)_{30}$-tail were linearized with HindIII and purified. Capped and polyadenylated RNA was synthesized with SP6 RNA polymerase using the DNA linearized templates and the mMessage mMachine kit (Ambion), according to the supplier's protocol. The RNA samples were treated with RNase-free DNase I (Ambion), purified by phenol:chloroform extraction and the yield of RNA quantified.

II.3.8. Toeprinting assay

The #17 or #18 primers (Table II.1) were synthesized and end labeled with 5'-FAM (Invitrogen). Twenty picomoles of primer and 200 ng of capped and polyadenylated mRNA were combined in 50 mM Tris-HCl, pH 7.5, heated to 68^{o}C for 2 min and cooled to 37^{o}C for 8 min and immediately added to the translation mixture, comprising 60% rabbit reticulocyte lysate (Promega), 28 mM AcK, 180 µM $MgCl_2$, 102 µg/ml creatine phosphokinase (Sigma), 6 mM creatine phosphate (Sigma), 24 mM KCl, 15 mM Hepes, 27 µM amino-acid mixture (Promega) and 0.6 U/µl RNase inhibitor (Invitrogene). Where appropriated, 500 µg/mL cycloheximide (Sigma) was added before the reaction. The reactions were incubated at 30^{o}C for 20 min. For the primer extension stage, 4 µl of the translation reaction were supplemented with 50 mM Tris-HCl, pH 7.5, 40 mM KCl, 3.5 mM $MgCl_2$, 5mM DTT, 0.8 mM each dNTP, 500 µg/mL cycloheximide, 1.5 U/µl ribonuclease inhibitor, and 5 U/µl Superscript II reverse transcriptase (Invitrogen) in a final volume of 20 µl and placed at 37^{o}C for 10 min. Primer extension products were extracted with phenol, and the ethanol-precipitated pellets were resuspended in 3 µl of

Hi-Di Formamide (Applied Biosystems) and brought to a total volume of 12 µl aliquot with Hi-Di Formamide, which included 0.5 µl ROX 500 or 2500 size standard (Applied Biosystems). The products were separated by electrophoresis using standard GeneScan conditions and analyzed using the GeneMapper Software – version 3.7 (Applied Biosystems).

II.4. Results

II.4.1. Translation termination at an AUG-proximal PTC obviates ribosome stalling characteristic of NMD-sensitive PTCs.

Given the demonstrated linkage between NMD and defects in translation termination (Amrani et al, 2006a; Amrani et al, 2004; Singh et al, 2008), we hypothesized that translation termination at an NMD-resistant AUG-proximal PTC might be more efficient than that at a more distal NMD-sensitive PTC. This possibility was explored by an *in vitro* "toeprinting" analysis. We compared the residency of terminating ribosomes at the termination codon of the wild-type β-globin mRNA (βN), at a NMD resistant "AUG-proximal" stop codon (β15), and at a more distal NMD sensitive PTC (β39). Capped, polyadenylated βN, β15, and β39 mRNAs were each incubated in translationally-active rabbit reticulocyte lysate (RRL) followed by primer extension using a fluorescently labeled primer (#17 primer; Figure II.1A and Table II.1) and analysis of reverse transcription products by capillary electrophoresis (Gould et al, 2005; Sachs et al, 2002) (Figure II.1B). Parallel controls included incubation of the mRNAs in RRL containing cycloheximide (CHX) and incubation in buffers in the absence of RRL. CHX at the levels used, blocks peptidyl transferase and should therefore ablate toeprinting signals corresponding to the terminating ribosome and accentuate signals corresponding to ribosomes blocked at the initiation AUG codon ("AUG toeprint"). mRNA incubated in the absence of RRL was used to distinguish genuine ribosome-generated toeprints (TP) from primer extension "short-stops" due to mRNA secondary structures.

The toeprinting analyses of βN, β15 and β39 mRNAs under each of the three incubation conditions are summarized in figure II.1B. The predominant peak at 626 nts observed on each of the three mRNAs represents the full-length transcripts. A second peak corresponding to the initiation AUG toeprint (at 555 nts, AUG TP) is seen for all three

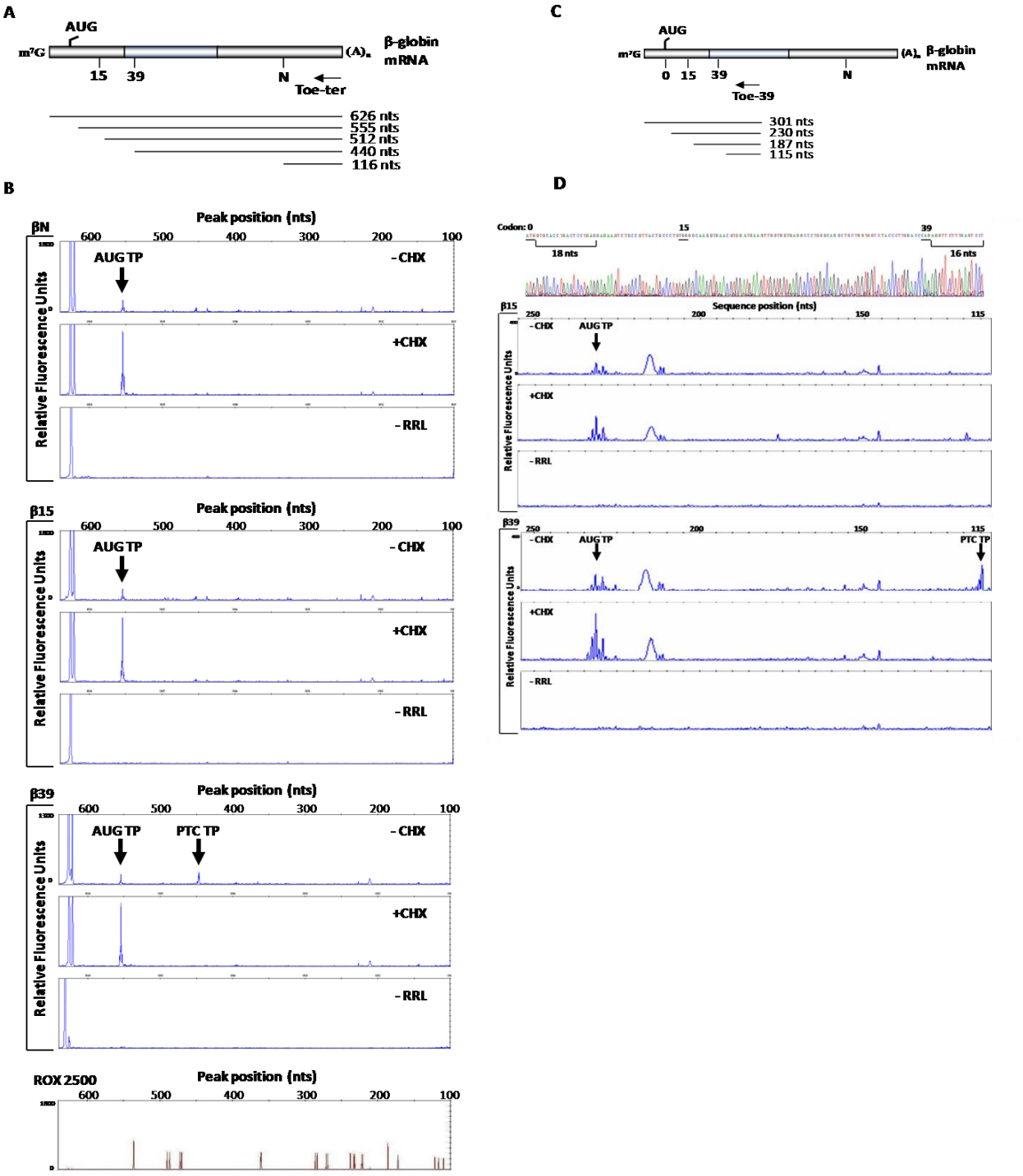

Figure II.1. Translation termination at the AUG-proximal β15 PTC occurs in the absence of the ribosome stalling evident at the more distal β39 PTC. (A) Diagram of the human β-globin mRNA showing positions of initiation and termination codons [native (N) or premature (at position 15 or 39)]. The arrow indicates the position and orientation of the #17 primer (Table 1) used in the primer-extension (toeprinting) assays. The lines below the mRNA diagram represent the length (in nucleotides; nts) of the synthesized cDNA from the #17 primer to the 5'-end of the mRNA, the initiation AUG codon, or to the βN, β15, and β39 translation termination codons. **(B)** Electropherograms of βN, β15 and β39 toeprint assays using the fluorescently labeled #17 primer. The toeprint reactions were performed in the presence or absence of cycloheximide (+CHX or -CHX, respectively). A control reaction was carried out in the absence of added rabbit reticulocyte lysate (-RRL). Primer extension products were suspended in a mix containing

ROX 2500 molecular weight marker (Applied Biosystems) and subjected to capillary electrophoresis. Size standard peaks are shown below and sequence position is indicated in nts. The toeprint peaks at 626 nts represent the full length transcript; the peaks at 620 nts correspond to the cap-binding complex-bound full length transcript; the peaks at 555 nts map 18 nts downstream the AUG codon (AUG TP). The peak at 440 nts maps to a position 16 nts downstream the β39 stop codon (PTC TP). **(C)** The arrow below the mRNA diagram represents position and orientation of #18 primer (Table 1) used in the second, high resolution toeprinting assays. The lines below the mRNA diagram represent the length in nts of the primer-extended cDNA to the 5'-end of the mRNA, to the initiation AUG, as well as to the translation termination codons at positions 15 or 39. **(D)** Electropherograms of β15 and β39 toeprint assays using the fluorescently labeled #18 primer. A parallel sequencing reaction on the βN cDNA, performed with the same primer is shown on the top. Underlined sequences indicate codon position: 0 (AUG), 15, and 39. The toeprint reactions were performed in the presence or absence of cycloheximide (+CHX or -CHX, respectively). A control reaction lacking rabbit reticulocyte lysate (-RRL) is shown. Primer extension and sequencing products were resuspended in a mix containing ROX 500 (Applied Biosystems) molecular weight standard and separated by capillary electrophoresis. Sequence position is indicated in nts. Peaks at 230 nts correspond to the initiation AUG toeprint that maps 18 nts downstream the AUG codon. The peak at 115 nts, present uniquely in the β39 analysis, maps 16 nts downstream of the PTC.

mRNAs (βN, β15 and β39 mRNAs) and in each case it is appropriately enhanced by CHX. A toeprint at 440 nts is specifically observed on the β39 mRNA incubated in the RRL. This band, mapping to a position 16 nts 3' to codon 39, is ablated in the presence of CHX and is not generated in the -RRL incubation. The position of this toeprint is consistent with the presence of a stalled ribosome at the β39 termination codon (PTC TP; Figure II.1B). The parallel analysis of the βN and β15 mRNAs failed to reveal and extension product of 116 or 512 nts that could be ascribed to ribosome stalling at the normal stop codon or at codon 15, respectively.

To confirm and extend the initial toeprinting study, a re-analysis of the β15 and β39 transcripts was performed with a primer positioned closer to the PTCs to achieve higher resolution (#18 primer; Figure II.1C and Table II.1). The β15 and β39 mRNAs both generate a peak at 230 nts (Figure II.1D) that is accentuated in the presence of CHX (AUG TP; Figure II.1D). This product maps to a position 18 nts 3' the AUG codon, as confirmed by direct sequencing with the same primer (Figure II.1D). Additional CHX-independent peaks, observed for both transcripts in the presence but not in the absence of RRL most likely represent mRNA regions where mRNA binding proteins or structures impede cDNA synthesis. The analysis of the β39 mRNA generated an additional peak at 115 nts that was not seen in the presence of CHX; this signal corresponds to a toeprint at 16 nts downstream the PTC at codon 39 (PTC TP; Figure II.1D). No corresponding band was observed immediately 3' to codon 15 of the β15 mRNA. It is unlikely that the absence of a toeprint corresponding to the β15 PTC is due to inefficient translation

because these transcripts show a normal initiator AUG toeprint that responds positively to CHX (Figure II.1B and D). Together, these *in vitro* studies reveal aberrant ribosome termination/release at the β39 PTC that is not seen at the β15 PTC.

II.4.2. PABPC1 plays an essential role in establishing NMD-resistance of an AUG-proximal nonsense-mutated mRNA

Prior studies have demonstrated that PABPC1 can associate simultaneously with the cap-associated eIF4G and the poly(A) tail (Wells et al, 1998). This dual binding has the potential to bridge the 3' and 5' termini of an mRNA resulting in a circularized or "closed loop" conformation (Wells et al, 1998). Such a conformation would bring PABPC1 into close proximity with 5'-terminal translation complexes. In related studies, PABPC1 has been shown to inhibit NMD when located close to the PTC (Eberle et al, 2008; Ivanov et al, 2008; Silva et al, 2008; Singh et al, 2008) and this effect is dependent on its C-terminal domain (Silva et al, 2008). With these observations in mind, we hypothesized that PABPC1 might be brought into the vicinity of the AUG-proximal PTC during cap-dependent translation by binding to and tracking with the initiation complex. This would allow it to interact with eRF3 and repress NMD. To test this model, HeLa cells were transfected with a siRNA targeting PABPC1. Twenty four hours later, these cells were transiently co-transfected with a plasmid encoding PABPC1 lacking the 85 residues of the C-terminal segment critical to eRF3 interactions (Hoshino et al, 1999), (PABPdelC, see Materials and Methods) and with plasmid encoding βN, β15, or β39 mRNAs (Figure II.2A). Levels of endogenous PABPC1 and exogenous PABPdelC proteins were monitored by Western blot (Figure II.2B; lanes 4-6) and the impact of altering the levels and structure of the PABPC1 on the expression of the various β-globin mRNAs was monitored by quantitative RT-PCR (RT-qPCR) (Figure II.2C). The studies were controlled by a parallel study in cells treated with a control (Luciferase; LUC) siRNA (Figure II.2B; lanes 1-3). The data revealed that expression of the C-terminally-deleted PABPC1 in PABPC1-depleted cells (Figure II.2B; Lanes 4-6) results in a significant decrease on β15 mRNA (from 103% to 70% of βN). In contrast, the same alteration in PABP constitution fails to alter β39 mRNA expression levels (Figure II.2C). These observations are consistent with the model that NMD-evasion of β15 transcripts is dependent on PABPC1, and specifically on a function mediated by its C-terminal domain. Efforts at

testing the single effect of endogenous PABPC1 depletion on the NMD-resistance of β15 transcripts failed because HeLa cells became detached from plates after the second transfection. The apparent toxicity of the siRNA-mediated PABPC1 depletion suggests that the knock down of this critical factor was effective (unpublished data).

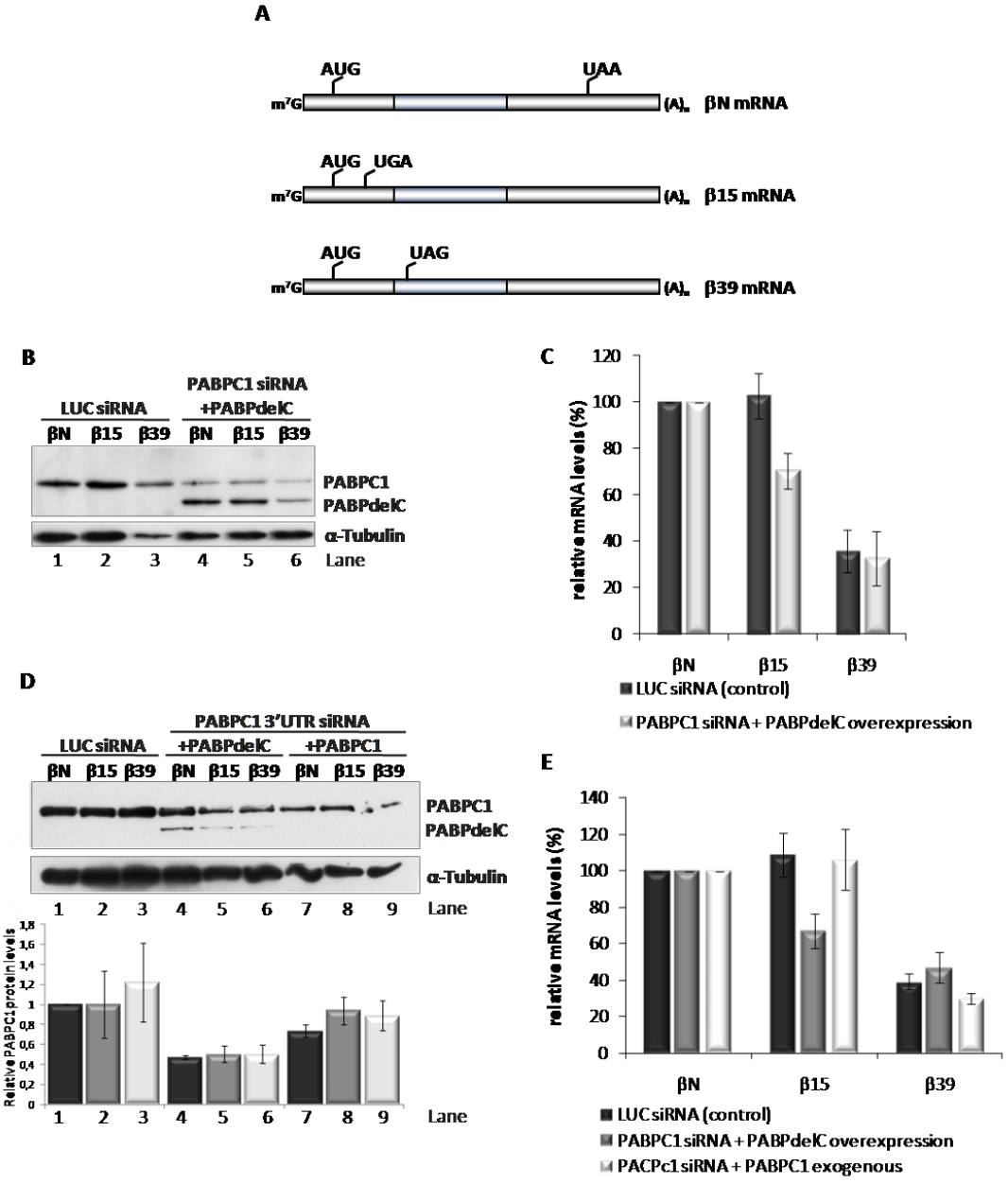

Figure II.2. PABPC1 plays an essential role in NMD-resistance of an AUG-proximal PTC. (A) Diagram representing βN, the NMD-resistant β-globin mRNA with an AUG-proximal nonsense mutation at codon 15 (UGA; β15) and the NMD-sensitive β39 mRNA. The positions of initiation (AUG) and termination (native and premature) codons are indicated. **(B)** Representative Western blot analysis of HeLa cells extracts transfected with human PABPC1 siRNA (lanes 4-6) or with a control Luciferase siRNA target (LUC siRNA; lanes 1-3). Twenty-four hours after siRNA treatment, cells were transfected with the β-globin reporter constructs (βN, β15 or β39) with or without a plasmid expressing PABPdelC mutant protein (pDESTPABPC1delC plasmid); lanes 4-6 or 1-3, respectively. Twenty-four hours post transfection, protein and RNA were isolated from the cells for analysis. Immunoblotting to confirm PABPC1 knockdown was carried out with anti-PABPC1 (specific for the N-terminal domain) and with anti-α-tubulin antibodies as a

loading control (lanes 4-6 *versus* lanes 1-3). Identification of each band is on the right. **(C)** Depletion of endogenous PABPC1 in conjunction with expression of exogenous PABPdelC protein represses β15 mRNA levels. Relative β-globin mRNA levels under control conditions (LUC siRNA-treated HeLa cells; dark bars) and at conditions of PABPdelC expression in PABPC1-depleted HeLa cells (PABPC1 siRNA + PABPdelC overexpression; light bars), normalized to the levels of puromycin resistance mRNA ($Puro^r$; plasmids carrying the reporter β-globin gene also contain the $Puro^r$ gene), were determined by quantitative RT-qPCR and compared to the corresponding βN mRNA levels (defined as 100%). Average and standard deviation (SD) of three independent experiments corresponding to three independent transfections are shown in the histogram. **(D)** Representative Western blot analysis of HeLa cells extracts transfected with control Luciferase siRNA (LUC siRNA; lanes 1-3) or with siRNA targeting the human PABPC1 3'UTR (PABPC1 3'UTR siRNA; lanes 4-9). After siRNA treatment, cells were transfected with the plasmids expressing βN, β15 or β39 mRNAs (lanes 1-3) or co-transfected with these plasmids in combination with a plasmid expressing PABPdelC mutant protein [pDESTPABPC1delC plasmid as above in *(B)*; lanes 4-6], or with a plasmid expressing wild-type PABPC1 (encoded by an mRNA with an heterologous 3'UTR resistant to the PABPC1 siRNA; lanes 7-9). Protein levels present in the cell extracts were analyzed by Western blot for PABPC1 and α-tubulin (loading control) as in *(B)*, to monitor endogenous PABPC1 knockdown (lanes 4-9 *versus* lanes 1-3) and exogenous expression of mutant PABPdelC protein (lanes 4-6) or PABPC1 expression rescue (lanes 7-9). Identification of each band is indicated to the right of the gel image. The histogram below the immunoblot shows the average and standard deviation values of three independent experiments for quantification of relative PABPC1 protein levels. **(E)** Expression of wild type PABPC, but not PABPdelC, restores β15 expression in PABPC1 depleted cells. Relative β-globin mRNA levels for the control conditions (LUC siRNA-treated cells; dark bars), at conditions of PABPdelC expression in PABPC1-depleted HeLa cells (PABPC1 siRNA + PABPdelC overexpression; light bars), and at conditions of PABPC1 rescue (PABPC1 siRNA-treated cells plus expression of exogenous PABPC1; light bars) were determined by RT-qPCR and compared to the corresponding βN mRNA levels as in *(C)*. Average values and SD of three independent experiments corresponding to three independent transfections are shown in the histogram. All values are represented as a percentage (%) of the corresponding βN mRNA (defined as 100%).

The preceding experiment was repeated by directly comparing the impact of the native PABPC1 versus the PABPdelC mutant in the cells depleted of endogenous PABPC1 (Figure II.2D and F). HeLa cells were transfected with a siRNA targeting the 3'UTR region of PABPC1 encoding mRNA (PAB3UTR; Table II.2).

HeLa cells were transfected with a siRNA targeting the 3'UTR region of PABPC1 encoding mRNA (PAB3UTR; Table II.2). Twenty four hours later, the PABPC1-depleted HeLa cells were co-transfected with plasmids encoding the β-globin reporters and with a plasmid encoding PABPdelC protein (Figure II.2D; lanes 4-6) or encoding exogenous PABPC1 (with a 3'UTR resistant to PABPC1-directed siRNA, Figure II.2D; lanes 7-9). Levels of β-globin mRNAs were measured by RT-qPCR (Figure II.2E). As before, we observed that depletion of PABPC1, followed by expression of PABPdelC (Figure II.2D; lanes 4-6 *versus* lanes 1-3), decreased β15 mRNA levels while the corresponding levels of the β39 mRNA levels remained unaltered (Figure II.2F). In contrast, repletion of the cells with wild type PABPC1 from about 40-50% to approximately 75-95% of the normal protein levels (Figure II.2D; lanes 7-9 *versus* lanes 4-6) fully restored β15 mRNA levels so that they were comparable to βN mRNA levels, while β39 mRNA remained at low levels (Figure

II.2E). Together, these results demonstrate that the NMD-resistance of the β15 RNA is dependent on the action of PABPC1 and in particular on the function of its C-terminal domain.

II.4.3. Interaction of PABPC1 with the ribosome release factor, eRF3, is critical to NMD-evasion by an AUG-proximal nonsense-mutated mRNA

Prior studies have revealed that PABPC1 can bind to eRF3 (Cosson et al, 2002a; Czaplinski et al, 1998; Hoshino et al, 1999; Kashima et al, 2006; Kozlov et al, 2001; Mangus et al, 2003; Uchida et al, 2002). This binding blocks UPF1 binding to the release complex. PABPC1 is thus able to antagonize the UPF1-eRF3 interaction involved in NMD (Singh et al, 2008). PABPC1 interacts with the N-terminal domain of eRF3 (Cosson et al, 2002a; Cosson et al, 2002b), while UPF1 interacts with the non-overlapping eRF3 GTPase domain (Ivanov et al, 2008). To further test the model that NMD-resistance of the AUG-proximal nonsense-mutated transcripts relies on the PABPC1-eRF3 interaction, we depleted HeLa cells of eRF3 by siRNA treatment and subsequently co-transfected these cells with plasmids bearing the β-globin reporters genes (βN, β15 or β39) and with a plasmid encoding an eRF3 mutant protein lacking its N-terminal domain [residues 1 to 183 (eRF3delN)]. Importantly, this deletion does not impair eRF3-eRF1 or eRF3-UPF1 interactions (Ivanov et al, 2008; Uchida et al, 2002). eRF3 levels as monitored by Western blot were depleted to about 25% of wild-type levels (Figure II.3A, lanes 4-6 *versus* lanes 1-3). Under these eRF3-depleted conditions, the expression eRF3delN resulted in a significant repression of β15 mRNAs to 55% of βN mRNA levels, while levels of β39 transcript were not appreciably altered (Figure II.3B). These results, along with those summarized in figure II.2, lead us to conclude that an AUG-proximal nonsense-mutated mRNA can evade NMD through a mechanism that is dependent on the interaction of PABPC1 with eRF3.

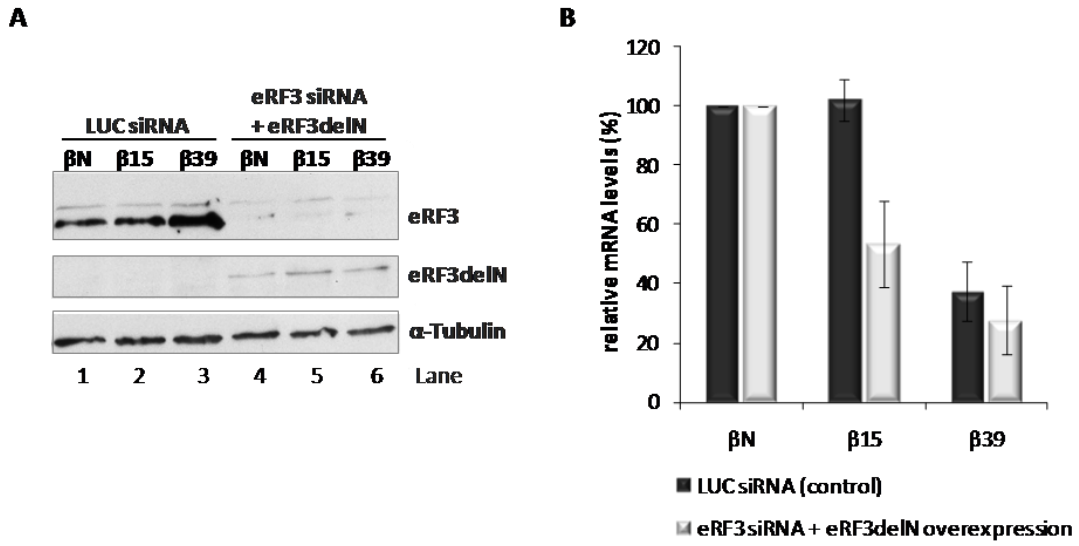

Figure II.3. The N-terminal domain of eRF3 is critical to NMD-evasion by an AUG-proximal nonsense-mutated mRNA. (A) Western blot analysis of HeLa cells extracts transfected with human eRF3 siRNA (lanes 4-6) or with a control Luciferase siRNA target (LUC siRNA; lanes 1-3). After siRNA treatment, cells were transfected with plasmids expressing βN, β15, or β39 mRNAs with or without a plasmid expressing eRF3delN mutant protein (pcDNAeRF3delN plasmid); lanes 4-6 or 1-3, respectively. Twenty-four hours post transfection, protein and RNA were isolated from the cells. Immunoblotting was carried out with anti-eRF3 to monitor endogenous eRF3 knockdown (lanes 4-6 *versus* lanes 1-3) and expression of mutant eRF3delN protein (lanes 4-6). Detection of α-tubulin served as a loading control. Identification of each band is on the right. **(B)** Depletion of endogenous eRF3 in combination with expression of exogenous eRF3delN represses β15 mRNA levels. Relative levels of β-globin mRNA under control conditions (LUC siRNA-treated cells; dark bars) and in eRF3-depleted cells expressing exogenous eRF3delN protein (eRF3 siRNA + eRF3delN overexpression; light bars), normalized to the levels of Puro^r mRNA expressed from the β-globin plasmids, were determined by quantitative RT-PCR and compared to the corresponding βN mRNA levels (defined as 100%). Average and standard deviation values of four independent experiments corresponding to three independent transfections are shown in the histogram.

II.4.4. Inhibition of PABPC1 function by PAIP2 sensitizes an AUG-proximal nonsense-mutated mRNA to NMD

The closed-loop configuration of a capped mRNA is dependent on the PABPC1-eIF4G interaction. The PABP-interacting protein 2, PAIP2, competitively blocks this interaction *via* direct binding to PABPC1 (Karim et al, 2006). We therefore reasoned that over-expression of PAIP2 might sensitize an AUG-proximal nonsense-mutated mRNA to NMD by inhibition of the closed loop conformation. To ensure that the PAIP2 over-expression was not having a secondary effect on NMD efficiency *via* a repression of translation, we limited our studies to low concentrations of PAIP2 DNA (see Materials and Methods). HeLa cells were transiently co-transfected with the plasmids encoding βN, β15, or β39 mRNAs along with the plasmid encoding PAIP2. PAIP2 protein levels were monitored by Western blot (Figure II.4A; lanes 1-3 versus lanes 4-9) and levels of β-globin mRNAs were

quantified by RT-qPCR (Figure II.4B). PAIP2 expression induced a substantial decrease in β15 mRNA levels from 117% to 53% of the corresponding βN mRNA levels, without altering the expression of the β39 mRNA (Figure II.4B).

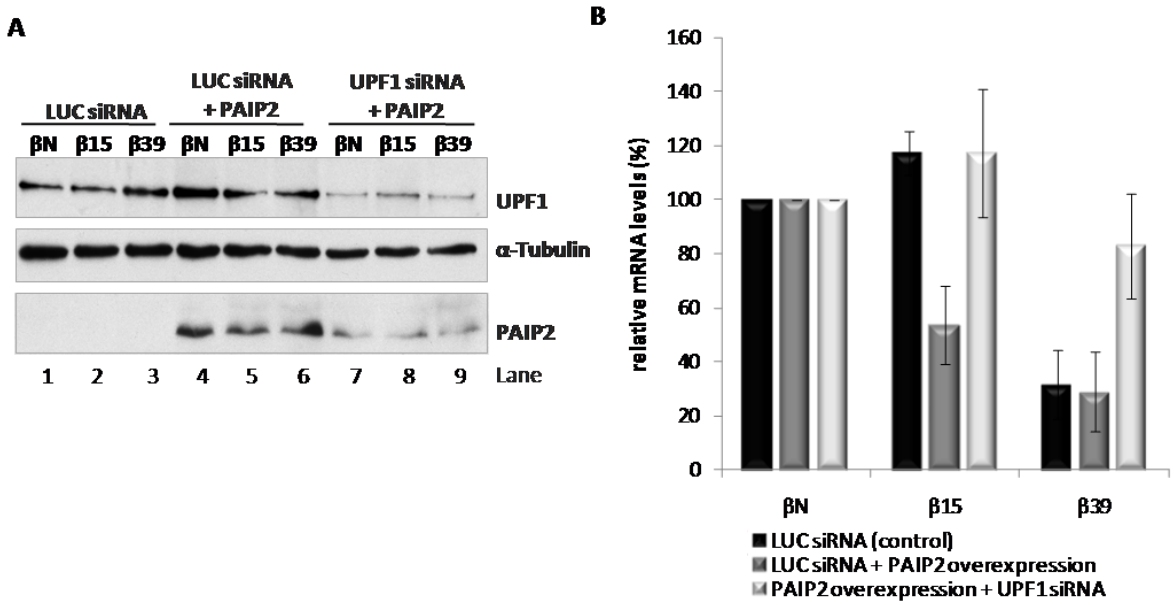

Figure II.4. Over-expression of PAIP2 induces NMD sensitivity in the β15 nonsense-mutated mRNA. (A) Western blot analysis of HeLa cells treated with siRNAs specific to Luciferase (LUC) (lanes 1-6), or siRNA targeting UPF1 (lanes 7-9). After siRNA treatment, cells were transfected with plasmids expressing βN, β15, or β39 mRNAs with or without a plasmid expressing PAIP2 protein (pDEST26PAIP2 plasmid); lanes 4-9 or 1-3, respectively. Twenty-four hours post transfection, protein and RNA were isolated from the cells for analysis. Immunoblotting was carried out with anti-UPF1, anti-PAIP2, and anti-α-tubulin antibodies. Detection of α-tubulin served as a loading control. Identification of each band is indicated to the right of the gel image. **(B)** Overexpression of PAIP2 represses β15 mRNA levels in a UPF1-sensitive manner. Relative β-globin mRNA levels under control conditions (dark bars), PAIP2 overexpression (dark grey bars), and in conditions of PAIP2 overexpression co-existing with UPF1 depletion (light bars) are shown. All values determined by RT-qPCR are normalized to the mRNA levels of Puro^r, and compared to the corresponding βN mRNA levels. Average values and standard deviation of four independent experiments corresponding to four independent transfections are shown. All values are represented as a percentage (%) of the corresponding βN mRNA (defined as 100%).

To confirm that the decrease of β15 mRNA levels seen in the PAIP2 treated cells reflected NMD, the experiment was repeated in UPF1-depleted HeLa cells. The UPF1 depletion resulted in the expected increase in β39 mRNA and, in addition, reversed the repressive effect of the PAIP2 over-expression on β15 mRNA (Figure II.4A and B). These data lead us to conclude that an AUG-proximal nonsense-mutated mRNA can be sensitized to NMD by blocking the interaction of PABPC1 with eIF4G.

II.4.5. The eIF3 subunits that tether the 40S ribosomal subunit to eIF4G are required for NMD-resistance of an AUG-proximal nonsense-mutated mRNA

Interactions between mRNA 5' end and the poly(A) tail are thought to be established during the initial round of translation. These interactions involve interactions of PABPC1 with the initiation factor eIF4G. eIF4G can simultaneously interact with PABPC1 and with the 40S recruitment factor, eIF3 (Amrani et al, 2008). Mammalian eIF3 is composed of 13 subunits designated eIF3a to eIF3m (Masutani et al, 2007). The structure and organization of this multisubunit has not been complete elucidated, although crystallography, mass spectrometry and immunoprecipitation studies allows the prediction of an interaction map of the free eIF3 as it is represented in Figure II.5A [adapted from references (Masutani et al, 2007; Wei et al, 2004; Zhou et al, 2008)]. The eIF3-eIF4G interaction is established *via* eIF3e and eIF3f subunits (LeFebvre et al, 2006; Masutani et al, 2007). This interaction of eIF4G with eIF3 has the potential to retain the eIF4G-bound PABPC1 on the scanning 40S ribosome and on the 80S ribosome during the early phase of elongation. Besides, in plants, the eIF3h subunit helps to prevent the permanent loss of reinitiation competence (Roy et al, 2010), suggesting that this subunit might also be involved in tethering 40S to eIF4G. Such an activity could blunt NMD of an AUG-proximal nonsense-mutated mRNA by bringing the PABPC1 into close proximity with the PTC. To test this hypothesis, we assessed the impact of depleting eIF3 subunits on the expression of an AUG-proximal PTC mRNA. Efficient knock-down was confirmed at the mRNA level for each respective eIF3 subunit mRNA, by normalization to mRNA levels of the histone deacetylase 1 (HDAC1) (Figure II.5B, C and D). HDAC1 mRNA was chosen as an internal control for these analyses since it is constitutively expressed (de Ruijter et al, 2003). We further demonstrated that under the experimental conditions used in the study, these depletions did not alter cellular translation as assessed by GFP reporter expression (Figure II.5E; lanes 3-5 *versus* lane 2 and 1, respectively).

We next evaluated the effect of individually depleting each of the three eIF3 subunits on NMD. Plasmids encoding the β15, β39, and βN mRNAs were each transiently expressed in eIF3h-, eIF3f- or eIF3e-depleted HeLa cells. β-globin mRNA levels, quantified by RT-qPCR, revealed that eIF3h depletion (Figure II.5F; lanes 4-6 *versus* lanes 1-3) results in a decrease of β15 mRNA level from 113% to 79% of the corresponding normal control βN,

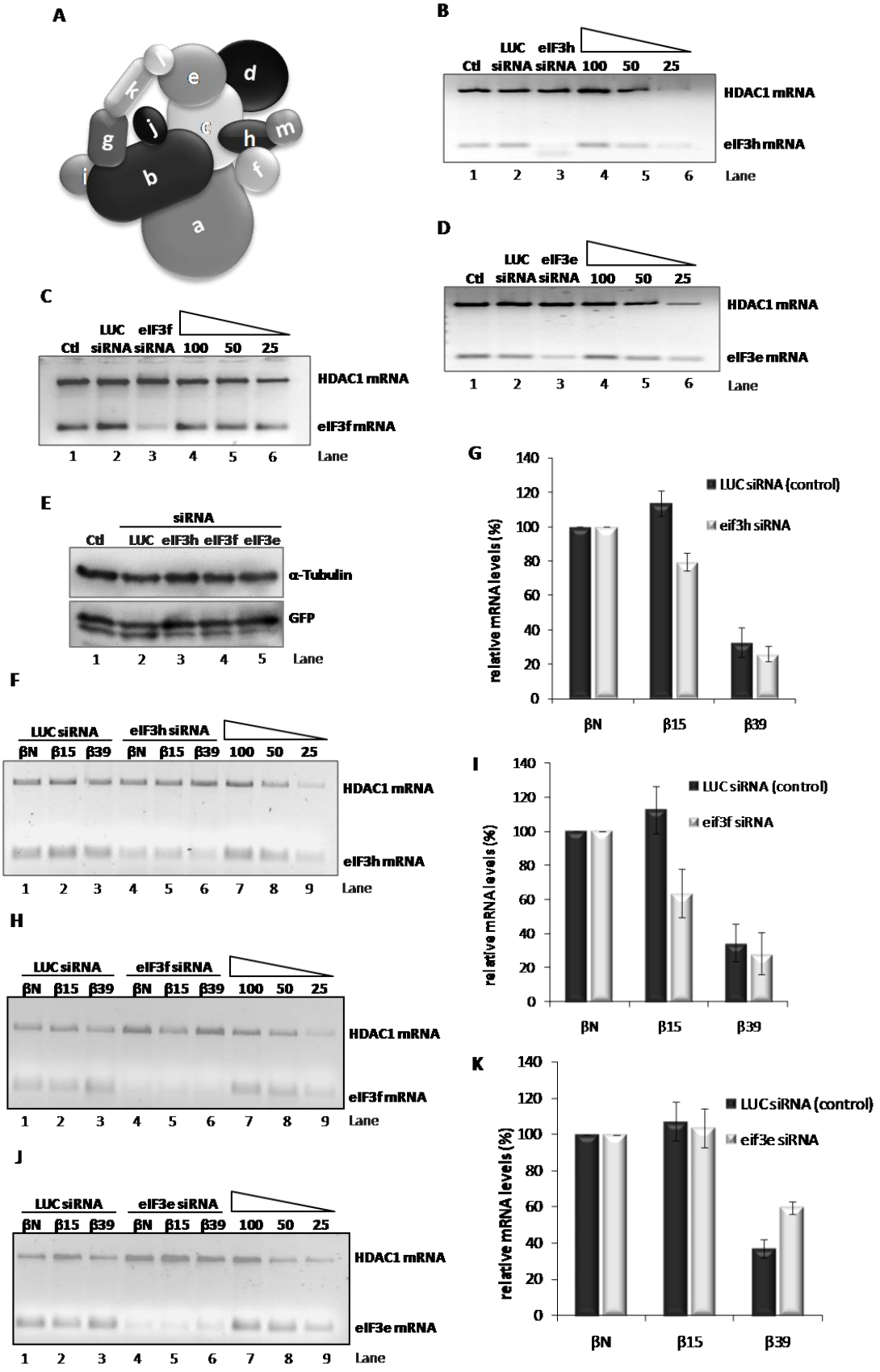

Figure II.5. Legend on the next page.

Figure II.5. The translation initiation factors eIF3h and eIF3f are required for the NMD-resistant phenotype of the β15 nonsense-mutated mRNA. (A) Representation of the predicted organization of the mammalian eIF3 subunits [adapted from references (Masutani et al, 2007; Wei et al, 2004; Zhou et al, 2008)]. Free eIF3 complex can be assigned into three stable modules. One module consists of eIF3a, b, g and i, and it interacts with a second module composed by eIF3c, d, e, l and k. eIF3b functions as a scaffold protein connecting eIF3a, c, i and g subunits. The third subcomplex is composed by eIF3f, h and m. Subunits eIF3f and m bind to the subcomplex eIF3 c:d:e:l:k through subunit eIF3h. The remaining subunit eIF3j, a labile subunit, attaches to the complex via eIF3b. **(B), (C)** and **(D)** Representative RT-PCR analyses of RNAs extracted from untreated (Ctl; lane 1), Luciferase (LUC; lane 2) or eIF3h, eIF3f and eIF3e (lane 3) siRNAs-treated HeLa cells, respectively, in panels *(B)*, *(C)* and *(D)*. RT-PCRs were carried out with eIF3h, eIF3f or eIF3e mRNA specific primers to monitor endogenous eIF3h, eIF3f or eIF3e knockdown, respectively (lanes 3 *versus* lanes 1-2). The eIF3h, eIF3f and eIF3e mRNA levels were normalized to those of histone deacetylase 1 (HDAC1) mRNA level. In each panel, the right three lanes correspond to serial dilutions of RNA, demonstrating semiquantitative conditions used for RT-PCR. **(E)** Representative Western blot analysis of HeLa cells extracts, untreated (Ctl, lane 1) or treated with Luciferase (LUC; lane 2), eIF3h (lane 3), eIF3f (lane 4) or eIF3e (lane 5) specific siRNAs (see Material and Methods). After siRNA treatment, cells were transiently transfected with the pEGFP plasmid (BD Biosciences) expressing green fluorescent protein (GFP). Protein lysates were analyzed by immunoblotting using anti-GFP and anti-α-tubulin (loading control) antibodies to monitor GFP protein expression. Identification of each band is indicated to the right of the gel image. **(F)**, **(H)** and **(J)** Semiquantitative RT-PCR analyses of RNAs extracted from HeLa cells transfected with a Luciferase (LUC) siRNA target (lanes 1-3) or human eIF3h, eIF3f or eIF3e siRNAs, respectively, at panels *(F)*, *(H)* or *(J)* (lanes 4-6 of each panel), using the same experimental settings as in *(B)*. Twenty-four hours after siRNA-treatment, cells were transfected with plasmids expressing βN, β15 or β39 mRNAs. Twenty-four hours after constructs transfection, total RNA was isolated from the cells. RT-PCR was carried out as in *(B)* with eIF3h, eIF3f or eIF3e mRNA specific primers, respectively, to monitor endogenous eIF3h, eIF3f or eIF3e knockdown (lanes 4-6 *versus* lanes 1-3). **(G)** Knockdown of eIF3h represses β15 mRNA levels. Relative β-globin mRNA levels for the control conditions (LUC siRNA; dark bars) and at conditions of eIF3h depletion (eIF3h siRNA; light bars), normalized to the levels of puromycin resistance mRNA encoded from the β-globin gene plasmid, were determined by quantitative RT-PCR (RT-qPCR) and compared to the corresponding βN mRNA levels (defined as 100%). **(I)** Knockdown of eIF3f represses β15 mRNA levels. Relative β-globin mRNA levels for the control conditions (LUC siRNA; dark bars) and at conditions of eIF3f depletion (eIF3f siRNA; light bars), normalized to the levels of puromycin resistance mRNA were determined by RT-qPCR and compared to the corresponding βN mRNA levels (defined as 100%). **(K)** Knockdown of eIF3e fails to repress β15 mRNA expression. Relative β-globin mRNA levels for the control conditions (LUC siRNA; dark bars) and at conditions of eIF3e depletion (eIF3e siRNA; light bars), normalized to the levels of puromycin resistance mRNA were determined by RT-qPCR as in *(G)* and compared to the corresponding βN mRNA levels (defined as 100%). *(G)*, *(I)* and *(K)* histograms show average and standard deviation values of three independent experiments corresponding to three independent transfections.

without a corresponding effect on the β39 mRNA (Figure II.5G). A similar selective repression of β15 mRNA was observed in eIF3f-depleted cells (Figure II.5H and I). In contrast, depletion of eIF3e (Figure II.5J) had no appreciable effect on β15 NMD-resistant transcript levels (Figure II.5K). Instead, this depletion results in an increase in β39 mRNA levels [from 37% to 59% of βN; (Figure II.5K)]. This later result is consistent with prior observations that the eIF3e subunit may be required, at least in part, for NMD-commitment (Morris et al, 2007). The destabilizing effect of eIF3h and eIF3f depletions on the β15 mRNA, together with the suggestion that eIF3 remains bound to the translating ribosome for a few peptide bonds (Hinnebusch, 2006; Park et al, 2001)

are consistent with the model where eIF3h and eIF3f have a role in bringing PABPC1 in association with eIF4G into the vicinity of the PTC of an mRNA with a short open reading frame. A schematic of this model is shown in Figure II.6.

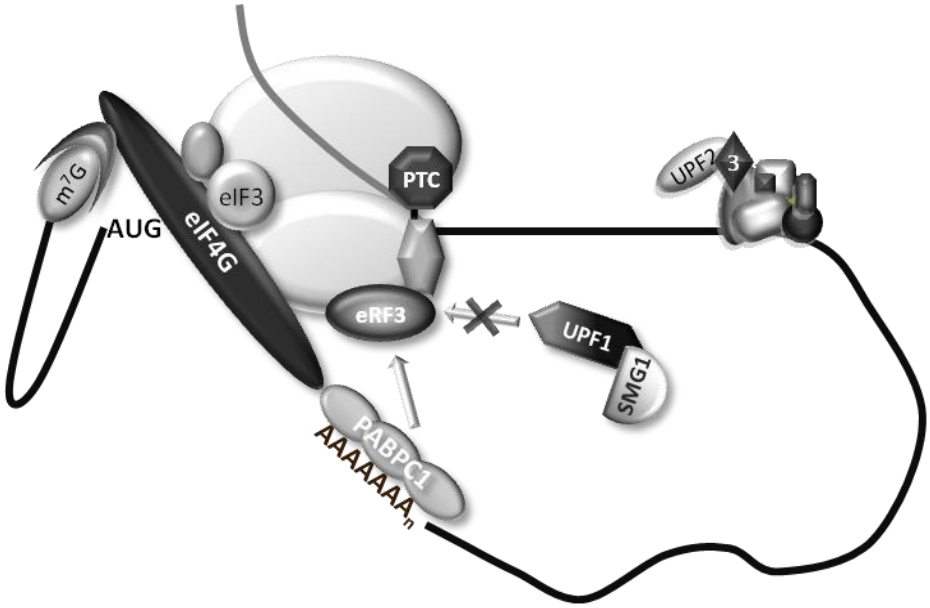

Figure II.6. A model for NMD-resistance of AUG-proximal nonsense-mutated mRNAs. The current and prior data supports the model shown in this figure. During cap-mediated translation initiation, PABPC1 interacts with the initiation factor eIF4G. This interaction indirectly tethers PABPC1 to the 40S ribosomal subunit *via* the interaction of eIF4G with eIF3 subunits. The resulting configuration brings PABPC1 into in the vicinity of the AUG initiation codon as a consequence of 43S scanning and the maintenance of eIF4G-PABPC1 association with the 40S during the initial phase of translation elongation brings it into close contact with an AUG-proximal PTC in a transcript where the ORF is quite short. This proximity to the PTC allows PABPC1 to interact with the release factor eRF3 at the termination complex, thus impairing the association of UPF1 to the ribonucleoprotein complex, resulting in efficient translation termination and inhibition of NMD.

II.5. Discussion

We have previously reported that mammalian mRNAs bearing AUG-proximal PTCs evade NMD (Romao et al, 2000; Silva et al, 2006). This immunity to NMD is independent of sequence context of the open reading frame and PTC, independent of transcript identity, and appears to be a general attribute of the mammalian NMD pathway (Silva et al, 2006). Extensive mapping studies, using the human β-globin gene as a primary model, have revealed that this effect reflects a close proximity of a PTC to the translation initiation codon (Inacio et al, 2004; Silva et al, 2006). In the case of the β-globin mRNA, PTCs that occur up to 23 codons into the open reading frame effectively evade NMD while mRNAs with PTCs at a greater distance from the AUG are targeted for decay. We

have further demonstrated that NMD evasion by AUG-proximal PTCs does not, as a rule, reflect defects in transcript splicing, impaired translation, or reinitiation of translation 3' to the PTC (Inacio et al, 2004; Silva et al, 2006). Thus, the mechanism underlying the NMD resistance of the AUG-proximal nonsense-mutated mRNA remains to be more clearly delineated.

The potential role of translation reinitiation in NMD resistance of AUG-proximal PTCs has been the subject of a recent report. In that study (Neu-Yilik et al, 2011) the NMD-resistance of β-globin AUG-proximal nonsense-mutated mRNAs was attributed to translation reinitiation 3' to the PTC. It may be of importance, however, to note that the interpretation of these studies is complicated by the use of a β-globin gene in which exon 1 was transformed into a functional exon 2 by the introduction of an exogenous intron into the 5'UTR. The expression of this recombinant β-globin gene, even lacking a PTC, was repressed when compared to the normal β-globin transcript. In contrast, we have found using globin genes with native structures, that even in cases in which we do detect some level of reinitiation 3' to the AUG-proximal PTC, site-specific elimination of the reinitiation sites fails to fully restore NMD-sensitivity (Silva et al, 2006). Thus, while a contribution of translation reinitiation in NMD-evasion cannot be completely ruled out in any particular circumstance, our detailed analyses of the human β-globin gene lead us to conclude that AUG-proximity is a major inhibitor of the NMD pathway and that other mechanisms must be considered.

The present data points to a major role for PABPC1 in protecting mRNAs harboring AUG-proximal PTCs from the NMD surveillance pathway (Silva et al, 2008; Silva & Romao, 2009). A critical contribution of interactions between PABPC1 and components of translation initiation complexes is highlighted by the dependence of the NMD evasion on interactions of PABPC1 with the eIF4G and most probably of eIF4G with eIF3 subunits. The data further reveal that these interactions are linked to the establishment of an efficient translation termination reaction at the AUG-proximal PTC. Toeprinting analysis of capped and polyadenylated human β-globin transcripts reveals substantial ribosome stalling at the NMD sensitive β39 stop codon that is absent at the termination codon of the β15 mRNA, as well as at the stop codon of the normal transcript (Figure II.1). The results of these studies lead us to conclude the interactions of PABPC1 with the preinitiation of the mRNA specifically blunt the NMD response on an AUG-proximal

nonsense-mutated mRNA? Our current observations lead us to propose that this repositioned PABPC1 can enhance the efficiency of the translation termination at the AUG-proximal PTC and in so doing represses NMD. It has been demonstrated by others that PABPC1 can compete with UPF1 for binding to eRF3 with a consequent antagonistic effect on NMD response (Ivanov et al, 2008; Singh et al, 2008). Recruitment of PABPC1 to the 5' end of the mRNA, with retention of ribosome association during the early phase of elongation, would position PABPC1 in close proximity to an early PTC. An impact on the termination reaction is supported by our prior demonstration that AUG-proximal nonsense-mutated transcripts fail to bind UPF1 as efficiently as NMD-sensitive transcripts (Silva et al, 2008). The interactions that support the repositioning of PABPC1 close to an AUG-proximal PTC are further defined by the *in vitro* observation that interactions of PABPC1 with eIF4G, eIF4E and eIF3, and the termination factors eRF1 and eRF3 can form a closed-loop structure during translation of short mRNAs (Amrani et al, 2008). The proposed role for PABPC1 in blunting NMD on AUG-proximal nonsense-mutated mRNAs is also consistent with the observation by ourselves and others of an inverse relationship between the distance among the PTC and an artificially tethered PABPC1 and the strength of NMD (Rebbapragada & Lykke-Andersen, 2009; Silva & Romao, 2009). It is worth to note that the destabilization of transcripts bearing AUG-proximal PTCs obtained by the impairment of PABPC1-eRF3 interaction corroborates the importance of the AUG-proximity effect in NMD-evasion of such transcripts.

In the case of the AUG-proximal nonsense-mutated mRNA, our data supports the conclusion that the PABPC1-eIF4G interaction enables PABPC1 to travel with the eIF4F/43S complex, as it scans from the cap to the AUG. The dependence of the AUG-proximity effect on the function of eIF3 (Figure II.5), which bridges 43S complex to eIF4G (Hinnebusch, 2006), further supports this model. Thus, we provide evidence that eIF4G and eIF3 initiation factors are both implicated in PABPC1 inhibitory effect on NMD. This model is further supported by the observation that the interruption of the PABPC1-eIF4G interaction by over-expression of PAIP2 leads to NMD of transcripts bearing an AUG-proximal PTC (Figure II.4). The observations that eIF3 can remain bound to the translating ribosome during the initial phase of elongation (Hinnebusch, 2006; Park et al, 2001) and that it can function as the link between the eIF4F-bound mRNA (through eIF4G) and the 40S ribosomal subunit (LeFebvre et al, 2006), supports the notion that

eIF3 subunits might be involved in the delivery of eIF4G-associated PABPC1 to the vicinity of the AUG-proximal PTC. The strength of the NMD effect decay would then be determined by the physical distance between PABPC1 at the AUG vicinity and the PTC; the closer this distance, the more effectively PABPC1 could interact with eRF3, diminishing the binding of UPF1, with the consequent enhancement of translation termination and blunting of the NMD response (Figure II.6).

Considering that mRNA circularization (Kahvejian et al, 2001; Wells et al, 1998) and PABPC1 function as NMD inhibitor when located in the PTC proximity (Amrani et al, 2004; Eberle et al, 2008; Ivanov et al, 2008; Silva et al, 2008; Singh et al, 2008) are common features in all eukaryotes, the model for the AUG-proximity effect that we propose here might be also a general attribute for these organisms. Here, we show that the human eIF3h and eIF3f subunits, which are not conserved in *Saccharomyces cerevisiae*, have a role in bringing PABPC1 in association with eIF4G into the vicinity of an AUG-proximal PTC. While the individual functions of the different mammalian subunits are not yet well established, many of the eIF3 subunits are thought to be conserved (Abbott & Proud, 2004; Hinnebusch, 2006; Masutani et al, 2007; Wei et al, 2004; Zhou et al, 2008). Indeed, the six subunits that constitute the yeast eIF3 - eIF3a, b, c, g, i and j - are conserved in mammalian (Asano et al, 1998; Hinnebusch, 2000; Hinnebusch, 2006). However, mammalian eIF3 has evolved to include additional subunits, which are likely to function as specific regulatory factors and some extra structural motifs provide the capacity to mediate extra protein-protein or protein-RNA interactions. Accordingly, eIF3e possesses a PCI (proteosome-COP9-initiation factor) domain, and eIF3f and eIF3h have a MPN (Mpr1-Pad1-N-terminal) domain. PCI or MPN domains are found in components of large protein complexes and have been implicated in protein-protein interactions (Hinnebusch, 2000). In fact, several results have indicated that the non-core subunits of the mammalian eIF3 regulate specific mRNAs (Choudhuri et al, 2010; Guo et al, 2000; Ray et al, 2008). Also, one attractive hypothesis is that the presence of a particular non-core subunit, e.g., eIF3h in the eIF3 protein complex, may in turn govern the efficiency with which the 43S preinitiation complex is able to scan through the 5'-UTR of a particular set of mRNAs (Kim et al, 2007). In addition, the non-core subunits may serve to stabilize the eIF3 complex (Masutani et al, 2007) and/or may have redundant functions (Choudhuri et al, 2010). It has been shown that mammalian

subunits eIF3f:h:m constitute a stable module that is located on the periphery of the complex and is not involved in interactions between other subunits (Zhou et al, 2008). Also, it is known that subunit eIF3h is essential for binding the trimer f:h:m to the core eIF3c subunit (Zhou et al, 2008). Based on these data, the possibility exists that in yeast, the model for the AUG-proximity effect may be dependent on the eIF3c, which may not evolved in order to become as specialized as its mammalian ortholog and thus it performs different biological functions, among which, those attributable to the mammalian eIF3f and h subunits. The relevance of the mammalian non-conserved eIF3f and h subunits in bridging eIF4G in association with PABPC1 to the ribosome during the process of translation initiation, which seem to be maintained during the first steps of elongation, might be linked to the need of a tighter regulation of translation in higher eukaryotes.

In conclusion, the present data supports a role for PABPC1 and associated translation initiation factors in NMD evasion of AUG-proximal nonsense-mutated transcripts. Future efforts addressing the biochemical mechanism by which PABPC1 is involved in cap-dependent translation initiation and how it participates in the eIF4F/43S scanning will contribute to our understanding of mRNA translation.

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II.7. Acknowledgments

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Chapter III

Further insights into the role of eIF3f and eIF3h non-conserved subunits on translational regulation

Isabel Peixeiro, Cristina Barbosa and Luísa Romão

Author's note

Some of the presented data in this chapter were obtained by the other indicated author. Cristina Barbosa was responsible for cloning the luciferase reporter plasmid constructs and contributed for the luciferase assays.

III.1. Abstract

The mammalian eIF3 is the most complex initiation factor and it comprises 13 non-identical subunits. eIF3h and eIF3f subunits are non-conserved and seem to function in translational regulation. Although in plants eIF3h plays a role in translation reinitiation, and eIF3f is able to interact with the mammalian mTOR pathway, the function of these subunits in the eIF3 complex is still unclear. A role for these subunits in bridging eIF4G and PABPC1 to the ribosome during translation has been recently shown in HeLa cells. Here we explore the role of eIF3f and eIF3h subunits in translational regulation. Supporting the previously published data, we demonstrate that eIF3h and eIF3f depletion in HeLa cells affect the association of the eIF3 complex with the ribosome and eIF4G, respectively. Consistently, eIF3f silencing by RNA interference augments CBP80 association with eIF3-mRNA complexes. Surprisingly, eIF3h does not seem to be involved in translation reinitiation downstream of a short uORF in human cells. From these observations, we propose that eIF3f and eIF3h are involved in the interaction of eIF4G with the ribosome and that, at least the eIF3f subunit, is required for the transition from the first round of translation to subsequent rounds of translation. These data further support a function of these non-conserved subunits in translational regulation in higher eukaryotes.

III.2. Introduction

The efficiency and accuracy of the initiation phase of translation is ensured by numerous proteins designated eukaryotic initiation factors (eIFs). The first step comprises the recruitment of the eIF2-GTP-Met-tRNA$_i^{Met}$ ternary complex to the small ribosomal subunit (40S), producing the 43S pre-initiation complex (PIC). This process is mediated by the multiprotein complex eIF3 together with eIFs 1, 1A and 5. The mRNA 5' capped-end binds the eukaryotic initiation factor 4F (eIF4F) complex, composed of the cap-binding protein eIF4E, as well as eIF4A and eIF4G, which then recruits the 43S pre-initiation scanning complex (Holcik & Pestova, 2007; Jackson et al, 2010; Sonenberg & Hinnebusch, 2009). The 3' end of the mRNA also participates in this process. In fact,

PABPC1 is able to bind simultaneously to the cap-associated eIF4G and to the poly(A) tail (Wells et al, 1998) and it has been described a formation of a closed-loop mRNP during translation in yeast extracts, that involves PABP, the initiation factors eIF4G, eIF4E and eIF3 (Amrani et al, 2008). The formed 48S PIC scans the mRNA searching usually for the first AUG codon. When an AUG codon is recognized by the anticodon of Met-tRNA$_i^{Met}$, in a process involving the concerted action of eIFs 1, 1A, 2 and 5, eIF5 induces irreversible hydrolysis of GTP in the eIF2-GTP-Met-tRNAi ternary complex. The Met-tRNA$_i^{Met}$ is then released to the P-site and the large (60S) subunit joins to form an 80S initiation complex ready to accept the appropriate aminoacyl-tRNA into the A-site to synthesize the first peptide bond (Pestova, 2007). Subunit joining is catalyzed by eIF5B (Pestova & Hellen, 2000) and is thought to mediate the release of eIF2-GDP and other eIFs. Because eIF3 binds mainly to the solvent side of the 40S subunit, the dissociation of this factor is not essential for subunit joining and may thus be delayed (Jackson et al, 2010).

eIF3 is the most complex initiation factor and it comprises 13 non-identical subunits designated from eIF3a to eIF3m in mammalian cells (Unbehaun et al, 2004). In *Saccharomyces cerevisiae*, eIF3 only comprises the orthologues of the four core essential subunits eIF3a, b, c, and g and one noncore subunit eiF3j (Hinnebusch, 2006). The real composition of the mammalian eIF3 core and the interactions of the different eIF3 subunits are not yet well established. Nevertheless, experiments to reconstitute the human eIF3 *in vitro* suggested that the functional core comprises subunits eIF3a, b, c, e, f and h (Masutani et al, 2007), while a recent study based on tandem mass spectrometry and solution disruption assays identified the formation of three stable modules: eIF3(a:b:i:g) resembling the yeast eIF3 core, eIF3(c:d:e:l:k) and eIF3(f:h:m) (Zhou et al, 2008). This multiprotein complex clearly plays a central role in translation initiation including promoting the binding of the ternary complex and other eIFs to the 40S subunit, subsequent mRNA recruitment and scanning for AUG recognition. In fact, eIF3 functions as a bridge of the 40S ribosomal subunit to the mRNA by interacting with eIF4G (Hinnebusch, 2006).

In addition to its functions in general translation, eIF3 was also implicated in translation reinitiation. Translation reinitiation is a mechanism for up- or downregulation of the expression of regulatory proteins such as transcription factors and proto-oncogenes in response to environmental stimuli (Kozak, 2005). Reinitiation may occur after translation

of an upstream open reading frame (uORF), if the 40S ribosomal subunit remains bound to the mRNA molecule upon translation termination. Following translation termination and the consequent release of the 60S ribosomal subunit, the 40S subunit may resume scanning downstream from the uORF. The *de novo* recruitment of the eIF2-GTP-Met-tRNAi ternary complex, that will allow recognizing the AUG of the main ORF, determines the acquirement of full reinitiation competence (Kozak, 1987; Kozak, 2001). The reinitiation efficiency depends on *i) cis*-acting mRNA features; *ii)* the time required for the uORF translation, which is determined by the relative length of the uORF and the translation elongation rate; and *iii)* the translation initiation factors involved in the translation initiation event (Kozak, 2001; Poyry et al, 2004). It is thought that the eIFs involved in resuming scanning remain at least transiently associated with the elongating ribosome, and increasing the uORF length or the ribosome transit time increases the likelihood of these factors to dissociate (Kozak, 2001). Supporting this idea, in yeast, eIF3 remains 80S-bound for several rounds of elongation and enhances the reinitiation competence of post-termination 40S ribosomes (Szamecz et al, 2008). Moreover, studies in yeast have implicated eIF3a and g subunits in this process (Cuchalova et al, 2010; Szamecz et al, 2008; Valasek et al, 2002), while experiments in plants point for a role of eIF3h subunit in promoting reinitiation competence during translation of mRNAs harboring uORFs (Roy et al, 2010).

The role of eIF3 subunits in translation reinitiation further supports the hypothesis that some subunits of this multiprotein complex are involved in translation regulation rather than directly participating in translation initiation. Consistently, mammalian eIF3e subunit, which directly binds eIF4G (LeFebvre et al, 2006), is required for nonsense-mediated mRNA decay (NMD) (Morris et al, 2007; Peixeiro et al, 2011a). Nonsense-mediated mRNA decay is a translation-dependent mRNA surveillance mechanism that degrades transcripts bearing premature translation termination codons (PTCs). The current model for mammalian NMD proposes that the discrimination between a normal and a premature termination event is the outcome of the combination of antagonistic signals at the translation termination event: the decision of NMD triggering will be the result from the competition between the poly(A)-binding protein 1 (PABPC1) and the NMD factor UPF1 for the termination complex (Nicholson et al, 2010).

We have previously reported that eIF4G and eIF3 initiation factors are both implicated in PABPC1 inhibitory effect on NMD. Our data demonstrate that human eIF3h and eIF3f subunits are involved in the NMD-resistance of mRNAs containing PTCs in close proximity to the translation initiation AUG codon (AUG-proximal PTCs), suggesting that these non-conserved subunits have a role in bringing PABPC1 in association with eIF4G into the vicinity of an AUG-proximal PTC (Peixeiro et al, 2011a). Here we aim to provide further insights into eIF3h and eIF3f subunits regulatory functions. Our data indicates that eIF3h and eIF3f depletion affect the association of the eIF3 complex with the ribosome and eIF4G. Surprisingly, eIF3h is not involved in translation reinitiation after a short uORF. These data support a role for eIF3h and eIF3f subunits in bridging eIF4G-associated PABPC1 to the ribosome, by interacting both with the 40S ribosomal subunit and eIF4G.

III.3. Materials and Methods

III.3.1. Plasmid constructs

The pGEMβN-MS2 vector, that carries the binding site for the MS2 phage coat protein, was obtained by reverse-transcription (RT) of βN-MS2 mRNA, after RNA extraction from HeLa cells expressing the βN-MS2 plasmid previously described (Silva et al, 2008), with Superscript II (Invitrogen) according to the manufacturer's standard protocol and using 2 pmol of reverse primer #1 and 0.5 mM espermidin. The RT product was amplified by polymerase chain reaction (PCR) using primers #2 and #3 (Table III.1), and then subjected to a second PCR amplification with primers with HindIII and XbaI linkers (#4 and #5, respectively, Table III.1) and inserted into XbaI/HindIII sites of pGEM plasmid (Promega). To obtain pGEMβNins5'UTR-MS2 construct, the β^{WT}ins5'UTR plasmid (Silva et al, 2006) was amplified by PCR using one primer with a SmaI linker (#6) and primer #7 (Table III.1) containing a NcoI site. The obtained fragment was cloned into NcoI/SmaI sites of pGEMβN-MS2.

The plasmid encoding firefly luciferase (pGL2hCMV) was obtained by insertion of hCMV promoter sequence from pcDNA3.1/hygro+ BglII/HindIII digested, into BglII/HindIII sites of pGL2-enhancer (Promega). The puORF^{EPO}Luc construct was obtained by inserting the erythropoietin (EPO) uORF sequence upstream of the luciferase ORF in the pGL2hCMV

plasmid. This was by overlap-extension PCR with flanking primers #8 (with a HindIII linker) and #9 and overlapping primers #10 and #11 (Table III.1), and the EPO gene and the luciferase ORF in pGL2hCMV plasmid as templates. The obtained fragment was cloned into HindIII/XbaI sites of pGL2hCMV. The pGL2uORF(TTG)^{EPO} variant, carrying a mutation at the uORF AUG codon (ATG → TTG), was obtained by overlap-extension PCR with flanking primers #8 and #9 and overlapping primers #12 and #13, and the pGL2uORF^{EPO} as template and the resultant primer was cloned into HindIII/XbaI. The pRL-TK plasmid encoding *Renilla* luciferase was a kind gift of Margarida Gama-Carvalho.

Table III.1. DNA oligonucleotides used in the current work.

Primer	Sequence (5' → 3')
#1	AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$
#2	AAGCAGTGGTATCAACGCAGA
#3	ACATTTGCTTCTGACACAAC
#4	GCCAAGCTTGTATCAACGCAGAGTACTTT
#5	TGCTCTAGAACATTTGCTTCTGACACAAC
#6	TCCCCCGGGACATTTGCTTCTGACACAACTG
#7	TGCACCATGGTGTCTGTTTG
#8	CCCAAGCTTCCCGGAGCCGGACCGG
#9	CGTACGTGATGTTCACCTC
#10	GCCAGGCGCGGAGATGGAAGACGCCAAAAACATAAAG
#11	CTTTATGTTTTTGGCGTCTTCCATCTCCGCGCCTGGC
#12	CTTCCCGGGTTGAGGGC
#13	GCCCTGAACCCGGGAAG
#14	CTGCTCATTGCAGGCCAGAT
#15	GAGCCTGGGCCATGAAGAG
#16	CACCCAGTCATTTTGGCCTC
#17	CGACAGTTCCCAACAGGGTC
#18	GGACAAGCATGGTTTTAGGCA
#19	TGCTGCTCCTGAGTAATTCCC
#20	ATGGCGCAGACGCAGGG
#21	CCGCACTAGGCTGGAACATC
#22	AAACTCTCAAGGATCTTTCC
#23	TTGGACTTAGGGAACAAAGG
#24	ACATTTGCTTCTGACACAAC
#25	TGCCACACTGAGTGAGCTG

III.3.2. Cell culture, plasmid and siRNA transfection

HeLa cells, stably expressing the tet transactivator (HeLa/tTA) (Kong et al, 2003), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The short RNA interference (siRNA) duplexes (Table III.2) were designed as 19-

mers with 3'-dTdT overhangs and purchased from Thermo. For the Luciferase assay transfections of cells with siRNAs were carried out using Lipofectamine 2000 Transfection Reagent (Invitrogen), following the manufacturer's instructions, in 35-mm plates using 200 pmol of siRNA oligonucleotides and 4µl of transfection reagent. Twenty four hours later, 750 ng of pGL2hCMV, pGL2uORF^{EPO} or pGL2uORF(TTG)^{EPO} were co-transfected with 750ng of the pRL-TK plasmid. For immunoprecipitations, HeLa cells were seeded in 60-mm dishes and transfected 20 hours later using 400 pmol of siRNA oligonucleotides and 8 µl of Lipofectamine 2000 reagent, according to the supplier's protocol.

Table III.2. Sequences of the siRNAs used in the current work.

siRNA	Sequence (5' → 3')
eIF3h1	ACUGCCCAAGGAUCUCUCU
eIF3h2	GAUCGGCUUGAAAUUACCA
eIF3f1	GUGAAGGAGAAAUGGGUUU
eIF3f2	AUACGCGUACUACGACACU
eIF3e1	CCAGGGAUGGUAGGAUGCU
eIF3e2	UGCAGAAUUGGGAUGCAGC
GFP	GGCUACGUCCAGGAGCGCAC

III.3.3. RNA isolation

Total RNA from transfected cells was prepared using the Nucleospin RNA extraction II (Marcherey-Nagel) following the manufacturer's instructions.

III.3.4. Semi-quantitative RT-PCR

1500 ng of total mRNA were reverse-transcribed with Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's standard protocol and using 250 ng of Random Primers (Invitrogen) in a final volume of 20 µl. The PCR reactions for eIF3h, eIF3f or eIF3e and histone deacetylase 1 (HDAC1) cDNAs were performed in parallel at similar conditions: 3 µl of the RT product was amplified in a 50-µl reaction volume using 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 15 pmol of each primer (primers #14 and #15 for eIF3h, primers #16 and #17 for eIF3f, primers #18 and #19 for eIF3e and primers #20 and #21 for HDAC1; Table III.1), 0.75 U of Amplitaq (Promega), and 1X PCR buffer (Promega). Thermocycler conditions were 95°C for 4 min followed by 26 cycles of 95°C

for 45 sec, 56°C for 45 sec, and 72°C for 45 sec followed by a final extension of 72°C for 10 min. Ten-microliter aliquots from each RT-PCR sample were analyzed by electrophoresis on 1.8% agarose gels.

III.3.5. Dual luciferase assay

Co-transfected HeLa cells were lysed with Passive lysis buffer (Promega) and luminescence was measured in Lucy 2 Luminometer (Anthos Labtec) with the Dual Luciferase Assay System (Promega) according to the manufacturer's indications.

III.3.6. *In vitro* transcription and capping

The pGEMβN-MS2 and pGEMβNins5'UTR-MS2 plasmids carrying the βN-MS2 cDNA containing a $(T)_{30}$-tail were linearized with HindIII and purified. Polyadenylated RNA was synthesized with T7 RNA polymerase using the DNA linearized templates and the Ribomax large scale kit (Promega), according to the supplier's protocol, with or without cap addition. The RNA samples were treated with RNase-free DNase I (Ambion), purified by phenol:chloroform extraction and the yield of RNA quantified.

III.3.7. Immunoprecipitation

HeLa cells cultured in 60-mm dishes and treated with siRNAs were collected 72h after transfection. Cells were lysed in 300 µl of NP40 buffer [500mM Tris-HCl, pH 7.4; 100 mM NaCl; 10mM $MgCl_2$; 0.1% (v/v) Nonidet P-40; 100 U of RNase inhibitor per ml (Invitrogen); protease inhibitor mixture (Sigma)]. Total lysates were cleared by centrifugation at 2,500g for 5 min. A 40 µl aliquot of total lysate (Pre-IP) was saved for RNA and protein analysis (a 20 µl sample was used for RNA extraction as described above, prior to semi-quantitative RT-PCR analysis; 4 µl of 5x SDS loading buffer was added to the remaining 20 µl sample for Western-blot analysis). The lysates were incubated for 2 hours at 4^oC with rabbit polyclonal anti-eIF3F (Abcam) or rabbit polyclonal anti-eIF3H (Cell Signaling) at a 1:100 dilution. An 80 µl volume of protein G-agarose beads (Roche) were then added and the samples were incubated for 1 hour at 4^oC, washed 4 times with excess NP40 buffer and ressuspended in 30 µl of 2x SDS sample buffer.

III.3.8. SDS-PAGE and Western blotting

Protein lysates were resolved, according to standard protocols, in 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were probed using mouse polyclonal anti-PCNA (Abcam) at 1:10,000 dilution (as a loading control), goat polyclonal anti-hUPF1 (Bethyl Labs) at 1:500 dilution; rabbit polyclonal anti-PABPC1 (Cell Signaling) at 1:500 dilution, rabbit monoclonal anti-eIF3H (Cell Signaling), rabbit polyclonal anti-CPB80 (generous gift from E. Izaurralde), rabbit polyclonal anti-eIF4G (Cell Signaling) or mouse monoclonal anti-RPS6 (Cell Signaling) at 1:250 dilution. Detection was carried out using secondary peroxidase-conjugated anti-mouse IgG (Bio-Rad), anti-rabbit IgG (Bio-Rad) or anti-goat IgG (Sigma) antibodies followed by chemiluminescence.

III.3.9. GST-Pull down assays

Recombinant GST-MS2 was expressed by transforming DH5α with pGEX-MS2 plasmid. 250 ml of LB media supplemented with 50 μg/ml ampicillin were inoculated with 5 ml of overnight culture. At OD_{600} ≈ 0.9, expression was induced with 0.1 mM IPTG for 3h and cells were pellet. Cell pellet was resuspended in 2 ml of lysis buffer [50 mM TrisHCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and protease inhibitor mixture (Sigma)] , sonicated for 4x 30 sec bursts, and centrifugated at 6,000rpm for 30min at 4^{o}C. Supernatant was bound to 1ml of glutathione-agarose beads (Sigma) overnight at 4^{o}C, washed 4 times with excess PBS-T and resuspendend in PBS (1:1).

Transcripts lacking the poly(A) tail were obtained by hybridization of uncapped GEMβNins5'UTR mRNA with 500ng of oligo$(dT)_{18}$ (Promega), followed by RNase H (Promega) digestion (4.0 U per 100 μl of total volume) in RNase H buffer (20 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT). The undeadenylated RNAs were purified by phenol:chloroform extraction.

300 ng of each substrate for *in vitro* pull-down assays were first heated to 65^{o}C for 5 min and cooled to 37^{o}C for 5 min and immediately added to the translation mixture, comprising 25 μl of supplemented untreated rabbit reticulocyte lysate [URRL (Promega) containing 0.6 M AcK, 5 mM $MgCl_2$, 1 mg/ml creatine phosphokinase (Sigma), 100 mM creatine phosphate (Sigma), 0.2 mM amino-acid mixture (Promega), 2.5 mM DTT, 0.25

mg/ml *E. coli* tRNA, 0.02 mM hemin, 0.6 U/µl RNase inhibitor (Invitrogene) and 0.5 g/L cyclohexamide (CHX)]. The reactions were incubated at 30°C for 10 min. For RNase H digestion, the translation mixture was supplemented with 500 mg of ssDNA probe (5'CCCACGTTCTTCGGG3'), 10 µl of RNase H Buffer 10x and 4.0 U of RNase H (Promega) in a final volume of 100 µl and incubated for 2h at 37°C. After the addition of 200 µl RSB-T (10 mM TrisHCl pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5 v/v Triton X-100) and 25 µl of GST-MS2 agarose beads and nutation for 3h at 4°C, the beads were washed 6 times with excess RSB-T and resuspended in elution buffer (100 mM TrisHCl pH 7.0, 150 mM NaCl, 12.5 mM EDTA, 1% SDS) and heated at 95°C for 3min. RNA samples were then purified by phenol:chloroform extraction and reversed-transcribed with Superscript II (Invitrogen) according to the manufacturer's standard protocol and using 2 pmol of primer #22 for the 5'UTR fragment or #23 for the 3'UTR fragment. The RT products were amplified by PCR using primers #22 and #24 for the 5'UTR fragment and primers #23 and # 25 for the 3'UTR fragment.

III.4. Results

III.4.1. mRNA acquires a closed-loop conformation during *in vitro* translation in rabbit reticulocyte lysate

Mammalian PABPC1 directly binds eIF4G (Imataka et al, 1998). We thus hypothesized that, as in yeast (Amrani et al, 2008), this interaction may mediate the formation of a closed-loop mRNP in mammalian extracts. To test this hypothesis, we employed an *in vitro* pull-down assay. We synthesized transcripts that included three binding sites for the bacteriophage MS2 coat protein at the 3' end of the βN capped and with a 30nt poly(A) tail or βN uncapped and deadenylated (Figure III.1A). These mRNAs were subjected to *in vitro* translation in supplemented URRL containing CHX to block peptidyl transferase, thus enriching the sample with stalled ribosomes at the AUG initiation codon. These mRNAs were cleaved by RNase H upstream of the AUG codon (Figure III.1A, ssDNA probe), the 3' fragments were selected by binding to recombinant GST fused to MS2 coat protein (GST-MS2) and the pellet was assayed for the presence of the 5' fragment (Figure III.1B). Efficient co-selection of the 5' fragment was dependent of an intact cap structure and poly(A) tail. This result suggest that the mRNA acquires a closed-

loop structure during *in vitro* translation due to 5' – 3' ends interactions that is maintained after ribosome scanning and is dependent of the presence of an intact poly(A) tail.

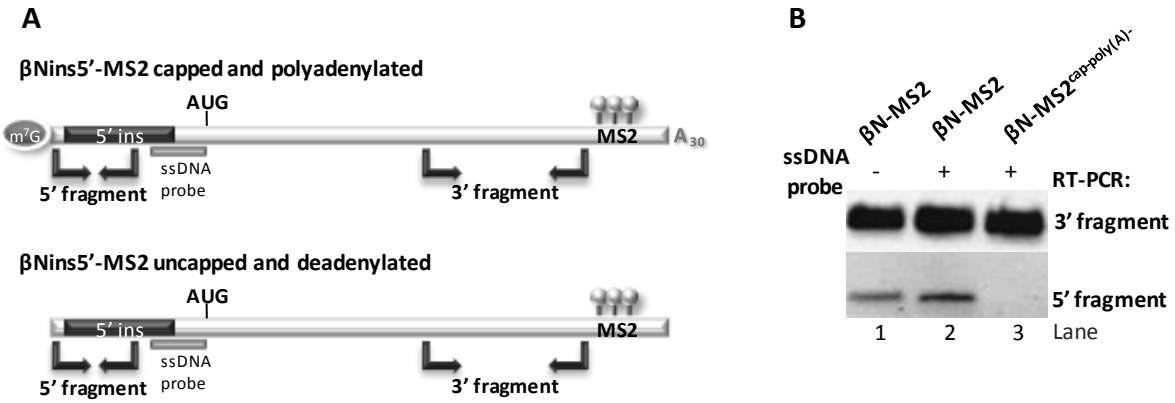

Figure III.1. 5'-3' mRNA ends interaction during translation in rabbit reticulocyte lysates. (A) Schematic representation of the human β-globin mRNAs assayed in the GST-pull down showing positions of the MS2-binding site, of the 5' spacer insertion, and complementarity to the oligonucleotide used for RNase H cleavage. The arrows indicate the position and orientation of the primers used for RT-PCR. **(B)** Pannels show the pellet of the 5' and 3' fragment as indicated. Samples in lanes 2 and 3 were treated with the oligonucleotide and subjected to RNase H cleavage prior to the GST-pull down.

III.4.2. eIF3f silencing affects eIF3h expression

Since eIF3h and eIF3f subunits interact with each other and assemble a subcomplex of eIF3 by also interacting with the subunit eIF3m (Zhou et al, 2008), we investigated the effect of individually depleting eIF3h and eIF3f subunits on eIF3h proteins levels. HeLa cells were eIF3h- or eIF3f-depleted by RNAi and, two days later, protein lysates were analyzed by Western blot. Both eIF3h and eIF3f knockdown resulted in decreased eIF3h protein levels (Figure III.2A and B). The incubation with the anti-eIF3h antibody revealed two distinct bands, that may correspond to eIF3h isoforms 3 (UniProt O15372) and eIF3h isoform 8 (UniProt E5RJT0), according to the molecular weight of ≈ 40 kDa and ≈ 28 kDa, respectively. To confirm that eIF3h destabilization observed in eIF3f-depleted cells was specific of eIF3f depletion and not an outcome of eIF3 complex destabilization, protein lysates from eIF3e-depleted HeLa cells were also analyzed by Western blot. The incubation with anti-eIF3h antibody revealed that eIF3e depletion does not significantly affect the amount of eIF3h subunit (Figure III.2C). Efficient knockdown was confirmed at the mRNA level for eIF3h and eIF3f subunits mRNA, by normalization to mRNA levels of

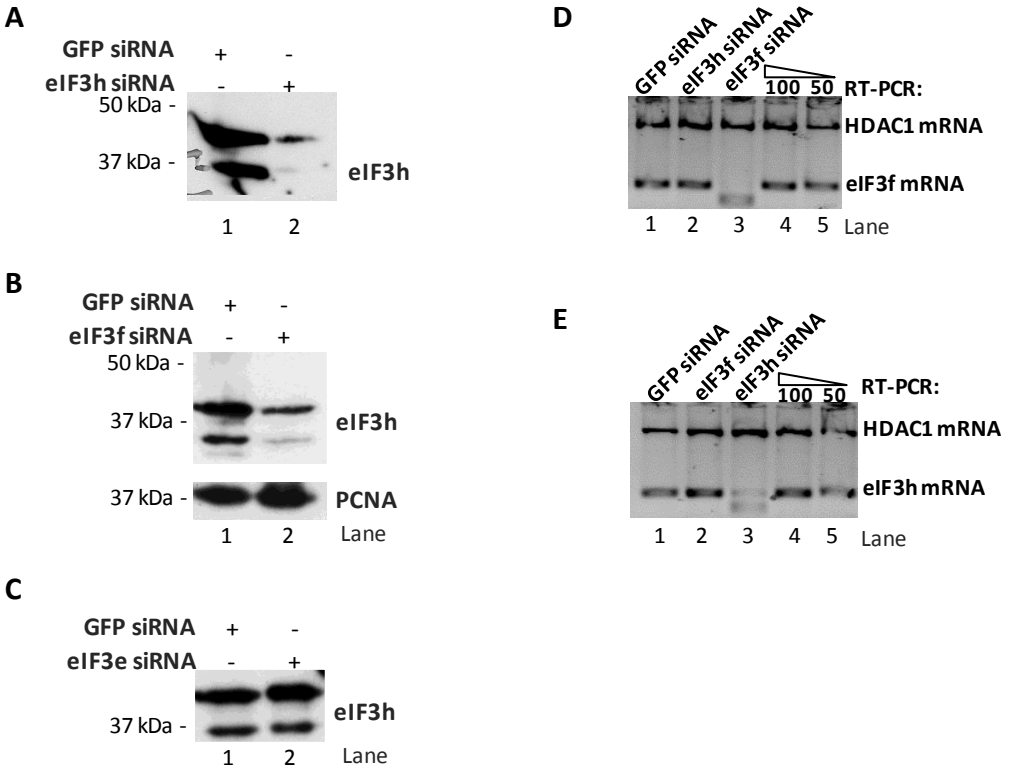

Figure III.2. Knocking down eIF3f subunit destabilizes eIF3h protein. (A) Western blot analysis of HeLa cells extracts transfected with human eIF3h siRNA (lane 2) or with a control GFP siRNA target (lane 1). Two days post transfection, protein and RNA were isolated from the cells. Immunoblotting was carried out with anti-eIF3h antibody to monitor endogenous eIF3h knockdown (lane 2 *versus* lanes 1). Identification of each band is on the right. **(B)** Western blot analysis of HeLa cells extracts transfected with human eIF3f siRNA (lane 2) or with a control GFP siRNA target (lane 1) as in *(B)*. Immunoblotting was carried out with anti-eIF3h antibody to monitor endogenous eIF3h (lane 2 *versus* lanes 1). Detection of PCNA served as a loading control. Identification of each band is on the right. **(C)** Western blot analysis of HeLa cells extracts transfected with human eIF3e siRNA (lane 2) or with a control GFP siRNA target (lane 1) as in *(B)*. Immunoblotting was carried out with anti-eIF3h antibody to monitor endogenous eIF3h (lane 2 *versus* lanes 1). Identification of each band is on the right. **(D)** Representative RT-PCR analyses of RNAs extracted from GFP (lane 1), eIF3f (lane 2) and eIF3h (lane 3) siRNAs-treated HeLa cells. RT-PCRs were carried out with eIF3h mRNA specific primers to monitor endogenous eIF3h expression (lanes 2 and 3 *versus* lanes 1). The eIF3h mRNA levels were normalized to those of histone deacetylase 1 (HDAC1) mRNA level. The right two lanes correspond to serial dilutions of RNA, demonstrating semiquantitative conditions used for RT-PCR. **(E)** Representative RT-PCR analyses of RNAs extracted from GFP (lane 1), eIF3h (lane 2) and eIF3f (lane 3) siRNAs-treated HeLa cells. RT-PCRs were carried out with eIF3f mRNA specific primers to monitor endogenous eIF3f expression (lanes 2 and 3 *versus* lanes 1). The eIF3h mRNA levels were normalized to those of HDAC1 mRNA level as in *(D)*.

the histone deacetylase 1 (HDAC1) (Figure III.2D and E). The reduced expression of eIF3h in eIF3f-depleted cells occurs at the protein level, given that semiquantitative RT-PCR analysis revealed that eIF3f knockdown has no effect on eIF3h mRNA levels (Figure III.2E, lane 2). These results suggest that eIF3f stabilizes eIF3h protein.

III.4.3. eIF3f depletion decreases eIF4G-eIF3 interaction and increases CBP80-mRNA association

It has been suggested that eIF3f subunit plays a role in translation regulation by directly binding mTOR, which controls the association of eIF4G with eIF3 (Harris et al, 2006). Our previous findings showing that eIF3f subunit is required for the NMD-resistance of AUG-proximal nonsense-mutated mRNAs (Peixeiro et al, 2011a) also support a possible function of eIF3f in mediating eIF4G (and associated PABPC1) interaction with eIF3-bound ribosome.

To address eIF3f role in eIF4G-eIF3 interaction, HeLa cells were treated with control or eIF3f-specific siRNAs and total lysates were generated two days later. Relative to the Pre-IP, 6.5-fold more amount of each lysate (GFP-depleted or eIF3f-depleted cells lysates) was separately immunoprecipitated using anti-eIF3h antibody and protein G-agarose beads or protein G-agarose beads alone to control for nonspecific immunoprecipitation (IP). Western blot analysis using eIF4G1 antibody revealed that eIF3h immunoprecipitated eIF4G (Figure III.3, top panel) in a RNA-independent manner. Even though we were unable to detect full-length eIF4G in eIF3f-depleted cells, eIF3h specifically immunoprecipitated two proteins (of ≈ 130 kDa and ≈ 110 kDa) in eIF3f siRNA-treated cells detected with the anti-eIF4G antibody, even after RNase treatment. These bands might correspond to eIF4G cleavage fragments, as the ones that result from caspase-mediated proteolytic cleavage in apoptotic cells (Marissen & Lloyd, 1998).

Most capped mRNAs are subjected to NMD almost immediately after transcription, when they are in transit to the cytoplasm and still bound by the cap binding complex (CBC) CBP80-CBP20 (Chiu et al, 2004). The CBC supports the pioneer round of translation, and the cap is then replaced by eIF4E that directs subsequent rounds of translation (Ishigaki et al, 2001). Since eIF3f depletion targets AUG-proximal nonsense-mutated mRNAs for decay, we investigated whether eIF3f silencing could increase CBP80 association with complexes containing eIF3. Immunoblotting using anti-CBP80 antibody shows an increased RNA-dependent association of CBP80 with eIF3h containing complexes in eIF3f-depleted cells (Figure III.3, lane 5 and 7 *vs* lane 4 and 6). Together these results suggest that eIF3f subunit is involved in eIF3-eIF4G interaction and that it might be required for the transition from the pioneer round of translation to subsequent rounds of translation.

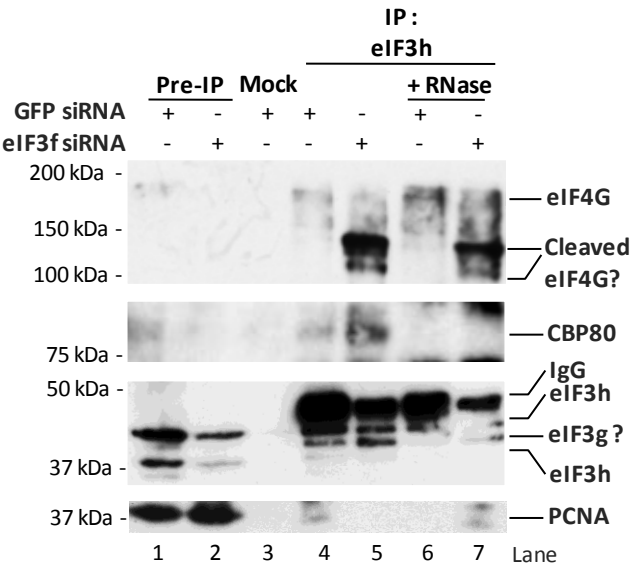

Figure III.3. eIF3f depletion decreases eIF4G-eIF3 interaction and increases CBP80-mRNA association. Lysates of HeLa cell transfected with control (GFP) siRNA (lane 1) or siRNAs targeting eIF3f (lane 2) were immunoprecipitated (IP) with an antibody to eIF3h (lanes 4-7), which was omitted in lane 3. Lysates were analyzed for the presence of eIF4G and CBP80 by immunoblotting. Lanes 1 and 2 correspond to aliquots of cell lysates prior to IP. Efficiency of RNAi and protein loading was controlled by the detection of eIF3f and PCNA proteins, respectively. Identification of each band is on the right.

It is worth notice that eIF3h antibody specifically immunoprecipitated a third protein detected with the anti-eIF3h antibody that may be the eIF3g subunit (Figure III.3), according to its molecular weight, but a MALDI-TOF/mass spectrophotometry analysis would be necessary to correctly identify the detected protein.

III.4.4. eIF3h is involved in eIF3 interaction with the 40S ribosomal subunit

It has been described that eIF3h subunit helps to prevent the permanent loss of reinitiation competence (Roy et al, 2010), suggesting that this subunit might also be involved in tethering 40S to eIF4G, as this interaction is essential for translation reinitiation to occur. To assess if eIF3h is involved in the association of the eIF3 complex to the 40S ribosomal subunit, HeLa cells were treated with control (GFP) siRNAs or with eIF3h-specific siRNAs and total lysates were generated two days later. Relative to the Pre-IP, 6.5-fold more amount of each lysate (LUC-depleted or eIF3h-depleted cells lysates) were separately immunoprecipitated using anti-eIF3f antibody or with protein G-agarose beads alone to control for nonspecific immunoprecipitation. Western blot analysis using anti-eIF3h antibody detected eIF3h subunit. eIF3f specifically immunoprecipitated the ribosomal protein S6 (component of the 40S ribosomal

subunit), even after RNase treatment (Figure III.4, bottom panel). However, this association was decreased in eIF3h-depleted cells (Figure III.4). This observation indicates that eIF3h regulates the interaction of the eIF3 complex with the 40S ribosomal protein.

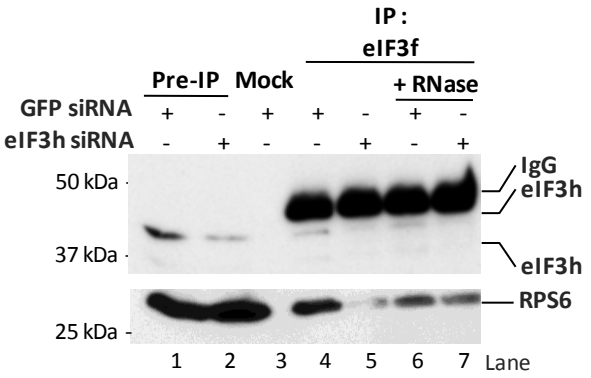

Figure III.4. eIF3h depletion decreases eIF3 interaction with the 40S ribosomal subunit. Lysates of HeLa cell transfected with control (GFP) siRNA (lane 1) or siRNAs targeting eIF3h (lane 2) were immunoprecipitated (IP) with an antibody to eIF3f (lanes 4-7), which was omitted in lane 3, and analyzed for the presence of RSP6 by immunoblotting (bottom panel). Lanes 1 and 2 correspond to aliquots of cell lysates prior to immunoprecipitation. Efficiency of RNAi was controlled by the detection of eIF3h. Identification of each band is on the right.

III.4.5. Efficiency of translation reinitiation downstream the erythropoietin uORF is not affected by the knockdown of eIF3 non-conserved subunits

Erythropoietin (EPO) is a glycoprotein synthesized and released mainly from the kidney, which has a key role in hematopoiesis. The human EPO mRNA presents a 5' leader with 181 nucleotides containing a 14-codon-uORF, 22 nucleotides upstream the main ORF. Preliminary data from our lab suggest that the EPO uORF can repress expression of the main ORF in about 70% (unpublished data). In addition our data indicate that reinitiation and, in less extend, leaky-scanning are responsible for translation of the main ORF (unpublished data). Since eIF3h subunit promotes reinitiation competence during translation of mRNAs bearing uORFs in plants (Roy et al, 2010) we decided to test if eIF3h also plays a role in reinitiation efficiency in mammals. We prepared constructs encoding the firefly luciferase (FLuc) reporter fused to the 5'leader sequence of the EPO gene containing the intact uORF ($uORF^{EPO}$) or the uORF with a mutated initiation codon [$uORF(TTG)^{EPO}$)] (Figure III.5A). HeLa cells were treated with eIF3h-siRNAs and subsequently co-transfected with the reporter plasmids and with the pRL-TK plasmid encoding *Renilla* luciferase (RLuc). Efficient knockdown was confirmed at the mRNA level for each respective eIF3 subunit mRNA, by normalization to mRNA levels of the histone

deacetylase 1 (HDAC1) (Figure III.5B). In transient expression the EPO uORF repressed luciferase activity by about 3-fold in control conditions (GFP siRNA) and by about 3.5-fold in eIF3h-depleted cells (Figure III.5C). These results failed to reveal a significant role for eIF3h in regulation of translation reinitiation efficiency. The same experiment was done for eIF3f- and eIF3e-depleted cells (Figure III.5D and III.5E, respectively). Similar results were obtained in control cells and in eIF3f- or eIF3e-depleted cells (Figure III.5F) suggesting that these subunits are not involved in translation reinitiation efficiency downstream of the EPO uORF.

III.5. Discussion

Our findings suggest that eIF3h and eIF3f subunits are involved in the association of the eIF3 complex to the ribosome and eIF4G. Even though it has been attributed a role for these subunits in translation regulation, the functional contribution of these subunits in the eIF3 complex has not been defined. Orthologues for eIF3h and eIF3f subunits are not found in *S.cerevisae*; however, in *S. pombe*, eIF3f is essential for cell viability and general translation (Zhou et al, 2005) and eIF3h physically associates with the 40S ribosomal particles but is not indispensable for global protein synthesis (Ray et al, 2008). eIF3f and eIF3h both contain a MPN (Mpr1-Pad1-N-terminal) domain. MPN domains are found in components of large protein complexes and have been implicated in protein-protein interactions (Hinnebusch, 2000) supporting a possible interaction of these proteins with other initiation factor complexes. In addition, the non-core subunits may serve to stabilize the eIF3 complex (Masutani et al, 2007). In accordance, our findings suggest that eIF3f stabilizes eIF3h subunit, since the knockdown of eIF3f resulted in reduced eIF3h protein levels (Figure III.2).

eIF3f subunit was reported to bind mTOR, which controls the association of eIF3 and eIF4G (Harris et al, 2006). Besides eIF4G, one of the best characterized targets of mTOR is the eIF4E binding protein (4EBP1). Hypophosphorylated 4EBP1 inhibits cap-dependent translation by blocking eIF4E binding to eIF4G (Gingras et al, 1999a). Interestingly, eIF3f depletion reduced the amount of intact eIF4G associated with the eIF3 complex by apparently leading to an increase in the amount of eIF4G cleavage fragments (Figure III.3). An attractive explanation for this observation is that when mTOR-eIF3f interaction

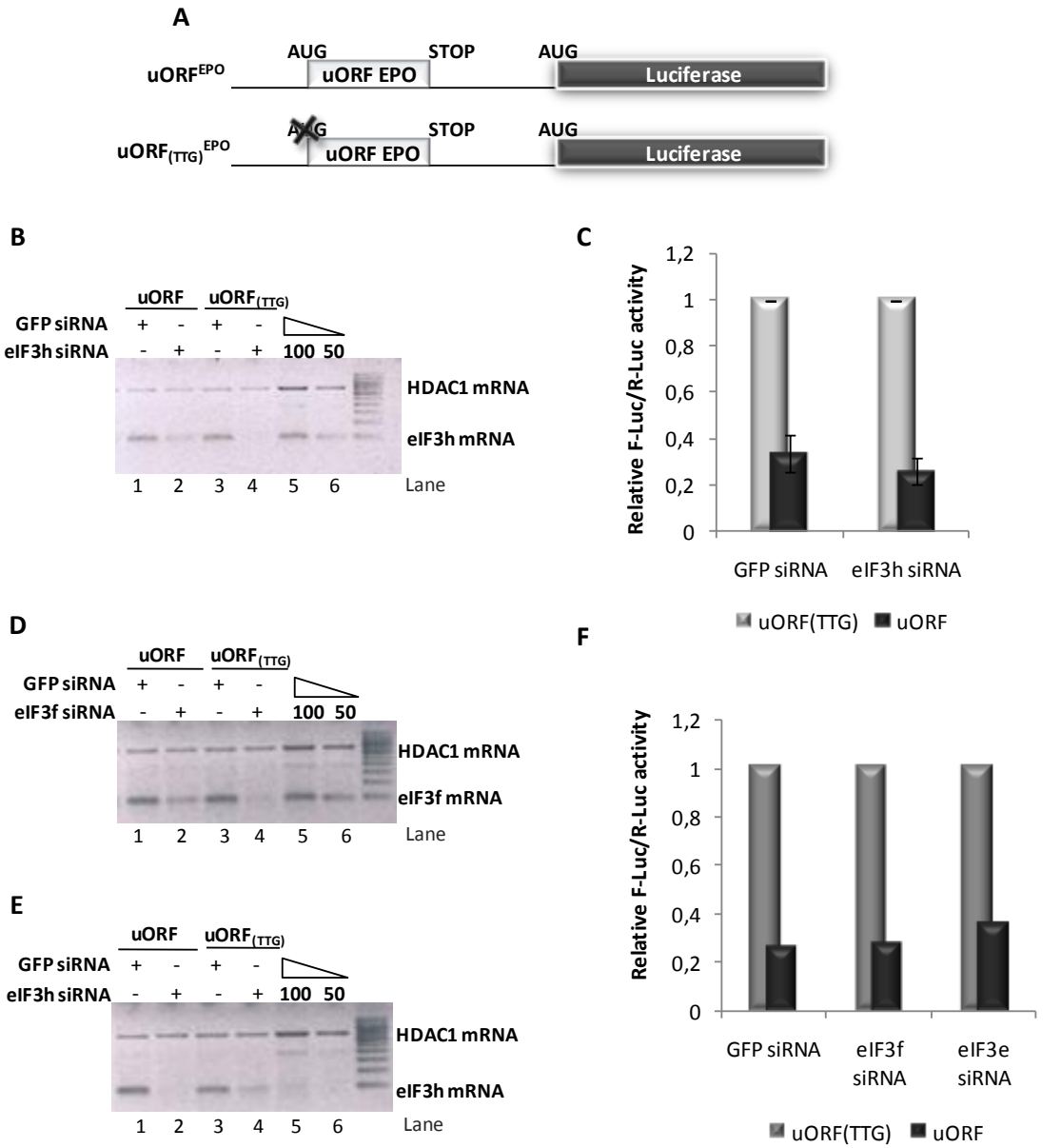

Figure III.5. Efficiency of translation reinitiation downstream the erythropoietin uORF is not affected by eIF3h, eIF3f or eIF3e depletion. (A) Schematic representation of the the firefly luciferase reporter coding region fused to the original (uORF) or the mutated ($uORF_{(TTG)}$) 5'leader sequence of the erythropoietin (EPO) gene. **(B)**, **(D)** and **(E)** Representative RT-PCR analyses of RNAs extracted from GFP (GFP; lane 1 and 3), or eIF3h, eIF3f and eIF3e (lane 2 and 4) siRNAs-treated HeLa cells, respectively, in panels *(B)*, *(D)* and *(E)*. RT-PCRs were carried out with eIF3h, eIF3f or eIF3e mRNA specific primers to monitor endogenous eIF3h, eIF3f or eIF3e knockdown, respectively (lanes 2 and 4 *versus* lanes 1 and 3). The eIF3h, eIF3f and eIF3e mRNA levels were normalized to those of HDAC1 mRNA level. In each panel, the right two lanes correspond to serial dilutions of RNA, demonstrating semiquantitative conditions used for RT-PCR. **(C)** Effect of eIF3h depletion on reinitiation downstream from EPO uORF. HeLa cells treated with a control (GFP) siRNA or eIF3h-specific siRNAs were transiently co-transfected with the uORF-LUC or $uORF_{(TTG)}$-LUC reporters and with a plasmid encoding *Renilla* luciferase. Luciferase activity was measured 20 hours later. Firefly luciferase activity values were normalized to renilla luciferase activity for transfection efficiency. Average values and standard deviation of three independent experiments are shown. **(F)** Effect of eIF3f and eIF3e depletion on reinitiation downstream the EPO uORF. HeLa cells treated with a GFP siRNA or eIF3f- or eIF3e-specific siRNAs were transiently co-transfected with the uORF-LUC or $uORF_{(TTG)}$-LUC reporters and with a plasmid encoding *Renilla* luciferase. Luciferase activity was measured as in *(C)*.

is impaired, hypophosphorylated 4EBP1 can tightly associate with eIF4E, inhibiting eIF4E binding to eIF4G, thus decreasing eIF4F bound to the 5' cap region of the mRNA (Gingras et al, 1999b). Moreover, the accumulation of eIF4G cleavage fragments could disrupt the closed-loop mRNA conformation (Amrani et al, 2008; Wells et al, 1998) acquired during translation (Figure III.1). The possible disruption of 5'-3' mRNA ends interaction in the absence of eIF3f would support a role for eIF3f subunit in bridging eIF4G and associated PABPC1 to the ribosome as it was previously suggested (Peixeiro et al, 2011a). Our results showing that eIF3f silencing augments CBP80 association with the mRNA-bound eIF3 complex also suggest a function for eIF3f subunit in promoting the transition from the pioneer round of translation to subsequent rounds of steady-state translation (Figure III.3). Indeed, it has been suggested that the composition of eIF3 is likely to be dynamic, and that a specific subcomplex (including eIF3e subunit) would be implicated in the pioneer round of translation, while other subcomplex would be specific for steady-state translation (Morris et al, 2007). Additionally, the absence of eIF3f-mTOR interaction that can result in impaired eIF4E binding to the capped mRNA could also explain the arrest in the pioneer round of translation. Further research will be needed to determine if the observed eIF4G cleavage and the increased CBP80-eIF3-mRNA association upon eIF3f depletion are outcomes of inhibiting the mTOR signaling pathway.

Several studies have implicated the non-core subunits of the mammalian eIF3 in regulation of specific mRNAs (Choudhuri et al, 2010; Guo et al, 2000; Ray et al, 2008). It has been proposed that the presence of a particular non-core subunit, e.g., eIF3h in the eIF3 protein complex, may in turn govern the efficiency with which the 43S preinitiation complex is able to scan through the 5'-UTR of a particular set of mRNAs in plants (Kim et al, 2007). Consistently, we confirmed that human eIF3h subunit is involved in the eIF3 binding to the 40S ribosomal subunit (Figure III.4), suggesting a potential role for eIF3h in scanning processivity by the 40S, resumption of scanning or reinitiation downstream of a uORF. With the expectation that, as in plants (Roy et al, 2010), mammalian eIF3h could be part of a larger functional module responsible for efficient translation reinitiation after uORF translation, we sought to investigate the effect of eIF3h depletion on translation reinitiation after a short uORF. Surprisingly, eIF3h silencing had no significant effect on repression of translation of the main ORF after EPO uORF

translation (Figure III.5). These data did not reproduce the eIF3h effect observed in *A. thaliana*, where eIF3h assists the ribosome to retain competence for reinitiation during translation of an uORF by enhancing efficient resume of scanning (Roy et al, 2010). Nevertheless, computational modeling of mutant eIF3h plants indicated that in the wild type, 50% loss of reinitiation competence was estimated at 58 nucleotides (nt) of translated uORF, while in the mutant 50% loss of competence was estimated at 22 nt (Roy et al, 2010). Moreover, reinitiation after the short uORF of the AtbZip11 leader of 18 codons was predicted to be only moderately eIF3h-dependent (Roy et al, 2010). In the light of the length of the EPO uORF (14 codons), translation reinitiation at the main ORF is not likely to be eIF3h-dependent because the uORF is not long enough to make reinitiation highly inefficient in eIF3h-depleted cells. Another possible explanation for the failure to detect a significant eIF3h-dependence of translation reinitiation during translation of the EPO uORF, might rely in the length of the intercistronic spacer sequence (of only 22 nt in the EPO gene) since translation reinitiation is less efficient when the distance between the uORF termination codon and the main ORF initiation codon is too short (Kozak, 1987). Additional research is required to determine if eIF3h facilitates post-initiation retention of eIF3 on the ribosome, thereby facilitating resumed scanning and translation reinitiation at a downstream AUG.

In conclusion, our findings support a function for the non-conserved eIF3f and eIF3h subunits in mediating the interaction of the eIF3 complex with eIF4G and with the ribosome, thereby contributing for translation regulation.

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Chapter IV

General Discussion

IV.1. General Discussion

NMD is a translation-dependent mRNA quality control mechanism that, in several cases, also exhibits a dependence on splicing. The “unified model” for NMD proposes that the decision of NMD triggering is the outcome of the competition between PABPC1 and UPF1 for the termination complex (Muhlemann, 2008; Shyu et al, 2008; Silva & Romao, 2009; Singh et al, 2008). Consequently, PTCs located far, in a linear sense, from the poly(A) tail and associated PABPC1, in mRNAs containing residual downstream EJCs, are expected to elicit NMD. However, our lab has reported that mammalian mRNAs bearing AUG-proximal PTCs evade NMD (Romao et al, 2000; Silva et al, 2006). This immunity to NMD was proven to be independent of sequence context of the open reading frame and independent of transcript identity (Silva et al, 2006). Previous studies, using the human β-globin gene as a primary model, have revealed that this effect appears to be a general attribute of the mammalian NMD pathway and reflects a close proximity of a PTC to the translation initiation codon (Inacio et al, 2004; Silva et al, 2006). Additional studies demonstrated that this “AUG-proximity effect” does not, as a rule, reflect defects in transcript splicing, impaired translation, or translation reinitiation downstream the PTC (Inacio et al, 2004; Silva et al, 2006).

Based on the emerging inhibitory role of PABPC1 on NMD, we have proposed a model in which the short open reading frame on an AUG-proximal nonsense-mutated mRNA situates PABPC1 into the vicinity of the PTC during cap-dependent translation. This proximity would allow PABPC1 to alter the structure and/or function of the translation termination complex with a consequent inhibitory effect on NMD (Silva et al, 2008; Silva & Romao, 2009).

The aim of the work presented in this doctoral thesis was to clarify the mechanistic basis of the “AUG-proximity effect”. Results point to a major role for PABPC1 in protecting mRNAs harboring AUG-proximal PTCs from the NMD surveillance pathway (Silva et al, 2008; Silva & Romao, 2009). In fact, NMD-resistance of AUG-proximal nonsense-mutated transcripts is dependent on PABPC1-eIF4G interaction that allows the establishment of an efficient translation termination reaction at the AUG-proximal PTC by enhancing PABPC1 interaction with the termination complex. The present data further supports the notion that eIF3f and eIF3h subunits might be involved in delivering

eIF4G-associated PABPC1 to the vicinity of the AUG-proximal PTC. Indeed, our results indicate that eIF3f and eIF3h subunits are involved in the eIF3 complex association to the ribosome and to eIF4G, respectively.

While, in the light of the "unified NMD model", it is straightforward to conceptualize the NMD resistance of mRNAs harboring PTCs in the 3' part of the terminal exon; the NMD evasion of AUG-proximal nonsense-mutated mRNAs is not so clear. For some AUG-proximal PTCs translation reinitiation has been pointed out as the cause for the observed NMD-resistance (Buisson et al, 2006; Buzina & Shulman, 1999; Howard et al, 2004; Paulsen et al, 2006; Perrin-Vidoz et al, 2002; Zhang & Maquat, 1997). Even though, for most of these cases it was not addressed whether or not preventing reinitiation was sufficient to fully restore NMD triggering (Buisson et al, 2006; Howard et al, 2004; Paulsen et al, 2006).

For the case of β-globin, the elimination of potential downstream reinitiation codons revealed that precluding translation reinitiation has no effect on NMD inhibition (Inacio et al, 2004). However, our findings are in conflict with a recent report (Neu-Yilik et al, 2011) suggesting that the NMD-resistance of β-globin AUG-proximal nonsense-mutated mRNAs was attributed to translation reinitiation 3' to the PTC. It may be of importance, though, to note that this study uses a β-globin gene in which exon 1 was transformed into a functional exon 2 by the introduction of an exogenous intron into the 5'UTR, leading to the downregulation of this recombinant β-globin gene, even lacking a PTC. In contrast, our studies based on globin genes with native structures show that, even in cases in which we detect some level of reinitiation downstream the PTC, the elimination of the reinitiation sites fails to fully restore NMD-sensitivity (Silva et al, 2006). Therefore, while a contribution of translation reinitiation in NMD-evasion cannot be completely ruled out, our previous data demonstrate that the presence of a short ORF (sORF) is, by itself, sufficient to circumvent NMD, being the AUG-proximity a major inhibitor of the NMD pathway.

Considering that, in mammalian systems, when the 5' proximal AUG initiation codon is followed by a short ORF (sORF), a fraction of the ribosomes have the potential to resume scanning after translation termination at the sORF stop codon and are likely to reinitiate at a downstream AUG (Kozak, 1987; Kozak, 2001); it is tempting to assume that translation reinitiation and translation of a sORF are related mechanisms. Nonetheless,

the process of translation reinitiation does not seem to be sufficient to explain the overall NMD-resistance (Buzina & Shulman, 1999; Silva et al, 2006; Zhang & Maquat, 1997). Thus, one might conjecture that translation reinitiation may be, as the observed NMD-resistance, a consequence of the process of translation of a sORF. What is the mechanism basis for a sORF translation that can lead to NMD-inhibition and/or translation reinitiation?

As translation reinitiation efficiency is dependent on initiation factors and translation time, it has been proposed that some initiation factors remain transiently bound to the elongating ribosome, and increasing the ORF length would increase the likelihood of the eIFs to dissociate (see *Chapter I.1.1. mRNA translation*). In fact, while ribosome subunit joining causes the dissociation of some initiation factors, the dissociation of the eIFs that bind mainly to the solvent part of the 40S subunit, like eIF3 and eIF4G, may be delayed (Szamecz et al, 2008; Valasek et al, 2002).

Additionally, it has been described in yeast that PABPC1 is able to bind simultaneously to the mRNA poly(A) tail and to eIF4F complex component eIF4G (Amrani et al, 2008; Wells et al, 1998). Our findings further support the formation of a closed-loop mRNA structure dependent on an intact poly(A) tail in mammalian extracts (Figure III.1). This closed-loop conformation is also likely to be formed during the pioneer round of translation, as CBP80-associated mRNPs were shown to contain both eIF4G and PABPC1 (Chiu et al, 2004). Thus, the recruitment of PABPC1 to the 5' end of the mRNA, with retention of ribosome association during the early phase of elongation, would position PABPC1 in close proximity to an early PTC.

Strong evidence that the positioning of the PABPC1 at the 5' end of the mRNA specifically blunts the NMD response on an AUG-proximal nonsense-mutated mRNA arose from PABPC1 role in stimulating efficient translation termination (Ivanov et al, 2008) and on inhibiting triggering of NMD mechanism (Amrani et al, 2006a; Behm-Ansmant et al, 2007a; Ivanov et al, 2008; Silva et al, 2008; Singh et al, 2008). Consistently, toeprinting analysis revealed that the translation termination at an AUG-proximal PTC obviates ribosome stalling characteristic of NMD-sensitive PTCs (Figure II.1) leading us to propose that this repositioned PABPC1 can enhance the efficiency of the translation termination at the AUG-proximal PTC and, in so doing, represses NMD. The destabilization of transcripts bearing AUG-proximal PTCs obtained by the

impairment of PABPC1-eRF3 interaction (Figure II.2 and II.3) further supports the importance of the AUG-proximity effect in NMD-resistance of such transcripts. Importantly, eIF4G function in PABPC1 inhibitory effect on NMD was corroborated by the NMD eliciting of AUG-proximal nonsense-mutated mRNAs due to abrogation of PABPC1-eIF4G interaction (Figure II.4).

The present data also shed new light on the question of how PABPC1-eIF4G interaction enables PABPC1 to travel with the eIF4F/43S complex, as it scans from the cap to the AUG. The function of eIF3 as the link between eIF4G and the ribosome (Hinnebusch, 2006; LeFebvre et al, 2006) as well as its ability to remain bound to the elongating ribosome by binding to the solvent portion of the 40S subunit (Szamecz et al, 2008) supports the hypothesis that eIF3 subunits might be involved in bringing eIF4G-associated PABPC1 to the vicinity of the AUG-proximal PTC. Indeed, our results suggest that eIF3h and eIF3f subunits are required not only for the interaction of eIF3 complex with the ribosome and with eIF4G, respectively (Figures III.3 and III.4), but also for the NMD-resistance of transcripts harboring an AUG-proximal PTC (Figure II.5). Furthermore, eIF3f silencing increases CBP80 association with the mRNA-bound eIF3 complex also suggesting a possible arrest in the pioneer round of translation (Figure III.3).

As it has been demonstrated that the eIF3h subunit promotes reinitiation competence during translation of uORFs in plants (Roy et al, 2010), one could argue that the observed destabilization of AUG-proximal nonsense-mutated transcripts upon eIF3h depletion could reflect a loss of reinitiation competence. However, this is unlikely because eIF3h (as well as eIF3f and eIF3e) depletion failed to significantly affect translation reinitiation efficiency after translation of a 14 nt ORF (Figure III.5). In accordance, in plants the reinitiation competence after translation of an 18 nt ORF was predicted to be only moderately eIF3h-dependent (Roy et al, 2010).

Considering that the mRNA closed-loop (Kahvejian et al, 2001; Wells et al, 1998) and PABPC1 function as an NMD inhibitor are common features to all eukaryotes (Amrani et al, 2004; Eberle et al, 2008; Ivanov et al, 2008; Silva et al, 2008; Singh et al, 2008), we can envision that the proposed AUG-proximity effect might be a general attribute for these organisms. Although *S. cerevisiae* lacks orthologues for eIF3h and eIF3f subunits, the model for the AUG-proximity effect may be dependent on a different eIF3 subunit while in higher eukaryotes, new non-core eIF3 subunits may have evolved to function as

specialized translation regulators. Indeed, the mammalian eIF3e subunit, that also lacks an ortholog in budding yeast, is required for NMD [Figure II.5 and (Morris et al, 2007)]. Thus, the relevance of the mammalian non-conserved eIF3f and h subunits in bridging eIF4G in association with PABPC1 to the ribosome during the process of translation initiation might be related to the requirement of a tighter regulation of translation in higher eukaryotes. With the ability of PABPC1 to travel with the elongating ribosome, the physical distance between the initiation codon and the PTC would then determine the strength of PABPC1 inhibitory effect: the closer this distance, the more likely PABPC1 would remain associated with eIF3-bound eIF4G and more effectively could interact with eRF3, diminishing the binding of UPF1, with the consequent enhancement of translation termination and blunting of the NMD response.

IV.2. Conclusions and future directions

Even though the NMD mechanism has been subject of intensive investigation for several years, the molecular basis underlying this surveillance pathway is not yet fully understood. According to the unified NMD model, the physical distance between the termination codon and the poly(A) tail is a crucial determinant for PTC definition, as PABPC1 and UPF1 compete for eRF3-binding.

The work presented in this doctoral thesis corroborates PABPC1 function in PTC definition. Indeed, our results support the AUG-proximity effect as a major determinant of NMD inhibition, providing a role for PABPC1 also in the NMD-resistance of 5' proximal PTCs, due to 5'-3' mRNA ends interaction during translation initiation. Furthermore, the data indicate that eIF3 subunits might be involved in the delivery of eIF4G-associated PABPC1 to the vicinity of the AUG-proximal PTC. The strength of the NMD effect decay would then be determined by the physical distance between PABPC1 at the AUG vicinity and the PTC; the closer this distance, the more effectively PABPC1 could interact with eRF3, diminishing the binding of UPF1 and circumventing the full activity of the NMD pathway. Finally, our findings support the model that the NMD-resistance of AUG-proximal nonsense-mutated transcripts is related to translation of a sORF. The intrinsically nature of a sORF may allow the maintenance of the elongating ribosome

interaction with initiation factors, namely eIF4G and eIF3, while increasing the ORF length increases the likelihood of disengaging these transient associations.

The results here presented can provide the basis for new lines of mechanistic research, which may expand the current models of NMD and translation in eukaryotes. One important aspect of future investigation will be to address the particular features of a sORF translation and what is the critical size that allows PABPC1 to interact with the terminating complex by participating in the eIF4F/43S scanning. As the maintenance of eIFs-ribosome interaction is also a requirement for translation reinitiation downstream a uORF, determining the parameters of sORFs PTC definition may be critical for the elucidation of the mechanistic details of translation regulation by uORFs.

Chapter V

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