Universidade de Aveiro Departamento de Biologia
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Ana Raquel Dias Pereira Guedes

O papel das ribonucleases SMG6 e PM/Scl100 em mecanismos de degradação do RNA mensageiro

The role of SMG6 and PM/Scl100 ribonucleases in messenger RNA degradation mechanisms

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The role of SMG6 and PM/Scl100 ribonucleases in messenger RNA degradation mechanisms

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Luís Manuel Souto de Miranda, Professor Auxiliar Convidado do Departamento de Biologia da Universidade de Aveiro, e da Doutora Luísa Maria Ferreira Romão Loison, Investigadora Principal com Habilitação do Departamento de Genética Humana do Instituto Nacional de Saúde Doutor Ricardo Jorge, em Lisboa

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palavras-chave

RNA mensageiro (mRNA); decaimento do mRNA mediado por mutações *nonsense* (NMD); decaimento do mRNA *nonstop* (NSD); SMG6; PM/Scl100.

resumo

A expressão génica em eucariotas envolve uma série de etapas interligadas, desde a transcrição do material genético até à síntese da proteína correspondente, nas quais os RNAs mensageiros (mRNAs) são os intermediários cruciais. Embora a panóplia de eventos que ocorrem ao longo de todo o processo permita que a produção proteica seja controlada a vários níveis, também torna a expressão génica vulnerável a erros. As células eucarióticas desenvolveram mecanismos elaborados de controlo de qualidade do mRNA que reconhecem e degradam transcritos anómalos. Dois exemplos destes mecanismos são o decaimento do mRNA mediado por mutações *nonsense* (NMD), que detecta mRNAs com codões de terminação da tradução prematuros (PTCs), e o decaimento do mRNA *nonstop* (NSD), que elimina mRNAs que não possuem codões de terminação da tradução em fase na grelha de leitura. A SMG6 e a PM/Scl100 são ambas ribonucleases já implicadas em vias de degradação de mRNAs. Um dos mecanismos propostos para o NMD em mamíferos envolve a clivagem endonucleolítica dos transcritos na proximidade do PTC, catalizada pela SMG6. Por outro lado, o exossoma humano, que inclui a subunidade catalítica PM/Scl100, já foi associado não só a mecanismos de vigilância do mRNA, mas também ao *turnover* do mRNA. No entanto, questões relativas à especificidade ou indispensabilidade destas enzimas nos mecanismos nos quais participam ainda não têm resposta. O presente trabalho teve como objectivo explorar o papel das ribonucleases SMG6 e PM/Scl100 na degradação de mRNAs normais ou sensíveis ao NSD e ao NMD. Os resultados obtidos apontam para o envolvimento da SMG6, não só no NMD, mas também no NSD e no *turnover* do mRNA. Para além disso, sugerem também que a SMG6 desempenha um papel indirecto na degradação de alvos do NMD. A PM/Scl100 também parece intervir no NMD, no NSD e no *turnover* do mRNA; no entanto, os resultados aqui apresentados sugerem que a principal contribuição para a degradação 3'→5' de transcritos que desencadeiam o NMD é oferecida por outras exoribonucleases.

keywords

messenger RNA (mRNA); nonsense-mediated mRNA decay (NMD); nonstop mRNA decay (NSD); SMG6; PM/Scl100.

abstract

Eukaryotic gene expression comprises a series of interconnected steps, from transcription to protein synthesis, in which messenger RNAs (mRNAs) are the key intermediates. While the multitude of events that take place throughout the whole process allows for the production of proteins to be controlled at many levels, ensuring maximum efficiency and fidelity, it also makes gene expression susceptible to errors. Eukaryotic cells have developed intricate mRNA quality control mechanisms that recognize and degrade aberrant transcripts. Two examples of these mechanisms are the nonsense-mediated mRNA decay (NMD), which targets mRNAs with premature translation termination codons (PTCs), and the nonstop mRNA decay (NSD), which eliminates mRNAs lacking any in-frame translation termination codons. SMG6 and PM/Scl100 are both ribonucleases which have been implicated in mRNA degradation pathways. One of the mechanisms proposed for mammalian NMD involves an endonucleolytic cleavage of transcripts in the vicinity of the PTC catalyzed by SMG6. On the other hand, the human exosome, which includes the catalytic subunit PM/Scl100, has been associated not only with mRNA surveillance mechanisms, but also with normal mRNA turnover. However, questions relative to the specificity or indispensability of these enzymes in the pathways in which they participate have not yet been answered. The present work aimed to explore the role of SMG6 and PM/Scl100 ribonucleases in the degradation of normal or NSD- and NMD-sensitive mRNAs. The results obtained point to the involvement of SMG6, not only in NMD, but also in NSD and normal mRNA turnover. Moreover, they suggest that SMG6 plays an indirect role on the degradation of NMD targets. PM/Scl100 also appears to intervene in NMD, NSD and normal mRNA turnover; however, the results herein presented suggest that the main contribution to NMD-eliciting transcripts 3'→5' degradation may be offered by other exoribonucleases.

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ARE	AU-rich element
A-site	aminoacyl-site
β15	human β-globin gene with PTC at codon 15
β26	human β-globin gene with PTC at codon 26
β39	human β-globin gene with PTC at codon 39
βNS	human β-globin gene lacking an in-frame stop codon
βWT	wild-type human β-globin gene
C-terminal	carboxyl-terminal
DECID	decay inducing complex
DIS3L	DIS3-like
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
eIF	eukaryotic translation initiation factor
EJC	exon junction complex
eRF	eukaryotic translation release factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate
LB	Luria-Bertani
LUC	luciferase
m7G	7-methylguanosine
mRNA	messenger ribonucleic acid
NMD	nonsense-mediated mRNA decay
NP40	nonidet P-40
NSD	nonstop mRNA decay
nt	nucleotide
N-terminal	amino-terminal

ORF	open reading frame
PABPC1	cytoplasmic poly(A)-binding protein 1
PCR	polymerase chain reaction
PIN	PilT N-terminus
PNRC2	proline-rich nuclear receptor co-regulatory protein 2
Poly(A)	poly-adenylate
PP2A	protein phosphatase 2A
Pre-mRNA	messenger ribonucleic acid precursor
P-site	peptidyl-site
PTC	premature translation termination codon
PVDF	polyvinylidene difluoride
qPCR	quantitative PCR
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
Rrp	ribosomal RNA-processing protein
RT	reverse transcription
RT-PCR	reverse transcription coupled polymerase chain reaction
RT-qPCR	reverse transcription coupled quantitative polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	short interfering RNA
SMG	suppressor with morphogenetic effects on genitalia
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SOC	super optimal broth with catabolite repression
SURF	SMG1-UPF1-eRFs complex
tRNA	transfer ribonucleic acid
UPF	up-frameshift
UTR	untranslated region
WT	wild-type
XRN1	5’-3’ exoribonuclease 1

1. Introduction

Eukaryotic gene expression comprises a series of interconnected steps, with messenger RNAs (mRNAs) being the crucial intermediates throughout the whole process. Genetic information encoded in DNA is transcribed into a mRNA precursor (pre-mRNA), which undergoes several modifications, including splicing, 5'-end capping and 3'-end polyadenylation. The mature mRNA is then exported to the cytoplasm where it is translated to protein and, finally, degraded (Behm-Ansmant et al., 2007; Chang, Imam & Wilkinson, 2007).

While the multitude of events that take place during gene expression allows for the production of proteins to be controlled at many levels, ensuring maximum efficiency and fidelity, it also makes the process susceptible to errors. Eukaryotic cells have developed intricate nuclear and cytoplasmic mRNA quality control mechanisms that target and degrade aberrant transcripts (Fasken & Corbett, 2005; Ana Luísa Silva & Romão, 2009). For instance, improperly processed mRNAs are degraded by surveillance mechanisms in the nucleus before they are exported (Fasken & Corbett, 2009). In the cytoplasm, the quality control machinery assesses the translatability of the mRNA and eliminates any that lack translation termination codons (nonstop mRNA decay, NSD) or that have premature translation termination codons (nonsense-mediated mRNA decay, NMD), preventing the accumulation of potentially deleterious proteins (Fasken & Corbett, 2005).

mRNA quality control pathways act not only on anomalous mRNAs, but also regulate the expression of naturally occurring transcripts having certain features recognized by the surveillance machinery. In this way, surveillance pathways also contribute to the post-transcriptional regulation of gene expression (Rehwinkel, Raes & Izaurralde, 2006).

1.1. Nonsense-mediated mRNA decay

NMD was first identified almost forty years ago, when it was observed that the presence of nonsense codons in the coding region of *URA3* mRNA in *Saccharomyces cerevisiae* and β-globin mRNA in a β-thalassemic patient was correlated with decreased abundance of the affected transcripts, rather than production of truncated proteins (Chang & Kan, 1979; Losson & Lacroute, 1979). Degradation of mRNAs harbouring premature translation termination codons (PTCs) was subsequently reported in many other organisms, and it is believed to occur in most if not all eukaryotes. Although important aspects of the NMD mechanism can differ between species, the core process and machinery seem to be conserved (Atkinson, Baldauf & Hauryliuk, 2008; Culbertson & Leeds, 2003). Thus, NMD can be defined as an evolutionary conserved post-transcriptional quality control mechanism that identifies and rapidly induces the degradation not only of faulty mRNAs containing PTCs, but also of several physiological transcripts.

1.1.1. Origin of premature translation termination codons

PTCs can arise from various types of mutations in germ or somatic cells. Genetic events that result in a PTC include: single base pair substitutions that convert a sense codon in a PTC, commonly known as nonsense mutations; insertion or deletion mutations that change the ribosomal reading frame (designated as frameshift mutations), causing translating ribosomes to encounter a PTC; mutations that give rise to mRNA splicing defects, causing retention of an intron that alters the reading frame and leads translating ribosomes to encounter a PTC, or causing retention of an intron that contains an in-frame PTC but does not alter the distal ribosomal reading frame (Chang, Imam & Wilkinson, 2007; Mühlemann et al., 2008; Silva & Romão, 2009). Overall, these different classes of PTCs are estimated to be associated with one third of all genetic disorders, including several types of cancer (Frischmeyer & Dietz, 1999).

The introduction of a PTC on a transcript can have two important consequences on gene expression. First, a PTC will induce mRNA translation termination before synthesis of

a full-length polypeptide is completed, leading to production of truncated proteins that are often nonfunctional and/or harmful. In addition, PTC containing mRNAs are also frequently unstable, resulting in a serious reduction in steady-state mRNA abundance. Together, these two PTC-induced events may affect the level of functional protein produced to such an extent that a severe disease state results (Keeling & Bedwell, 2011).

1.1.2. Premature translation termination codons recognition in mammals

The initial step in the NMD pathway is the recognition of a termination codon as premature, which, in mammals, depends on its location relative to certain downstream sequence elements, the exon-exon junctions, formed after pre-mRNA splicing. In fact, while natural stop codons are typically located within terminal exons, NMD eliciting nonsense codons usually lie more than 50-55 nucleotides upstream of the last exon-exon junction. On the other hand, if nonsense codons are located downstream of the 50-55 nucleotides boundary, they are not recognized as premature and NMD is not activated (Figure 1) (Maquat, 2004; Nagy & Maquat, 1998). The molecular basis of this "50-55 nucleotides boundary" empirical rule is not yet fully understood, but the prevailing model for mammalian NMD attempts to clarify the relationship between PTC location relative to this boundary and NMD activation.

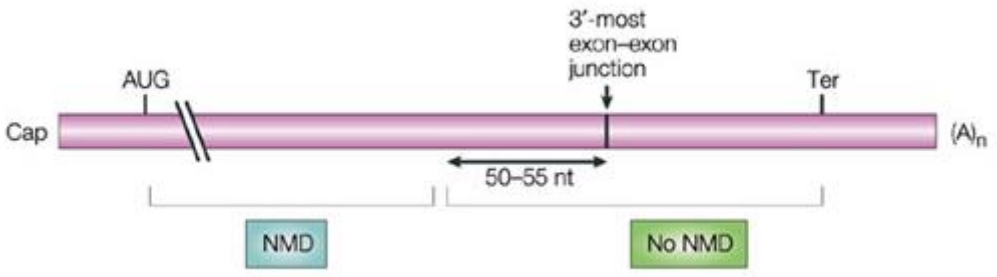


Figure 1. Illustration of the "50-55 nucleotides boundary" rule of NMD. A PTC located more than 50-55 nucleotides upstream of the last exon-exon junction is able to elicit NMD, whereas a PTC that is located downstream of this boundary fails to elicit NMD. The normal termination codon (Ter) usually resides within the terminal exon. Only the last exon–exon junction within a generic mammalian mRNA is shown. *In* Maquat, 2004.

1.1.3. NMD dependence on splicing and translation

It has been observed, in mammals, that nonsense mutations do not elicit NMD when they occur in transcripts generated by genes that naturally lack any introns (Brocke et al., 2002; Maquat & Li, 2001). In addition, translation repression has been demonstrated to repress NMD (Maquat, 2004). Splicing and translation must therefore be required in order to induce NMD activation.

During pre-mRNA splicing, multiprotein complexes named exon junction complexes (EJCs) are deposited 20-24 nucleotides upstream of exon-exon junctions and assist in mRNA export to cytoplasm (Le Hir et al., 2000). These complexes are subsequently displaced from the mature mRNA by the elongating ribosome during the pioneer round of translation (Ishigaki et al., 2001; Lejeune et al., 2002; Maquat, Tarn & Isken, 2010).

Translation termination, in eukaryotes, is mediated by eukaryotic release factors eRF1 and eRF3 (Figure 2). When a stop codon (UAA, UAG or UGA) enters the A-site of the ribosome, since there are no cognate aminoacyl-transfer RNAs (aminoacyl-tRNAs) for stop codons, the ribosome stalls. eRF1 adopts a structure that resembles that of tRNA and recognizes the termination codon through its N-terminus, while the C-terminus forms a complex with the GTPase eRF3. This interaction triggers GTP hydrolysis, inducing a conformational change in eRF1 that allows it to move closer to the P-site and stimulate cleavage of the ester bond of the peptidyl-tRNA, promoting the release of the newly synthesized polypeptide chain (Keeling & Bedwell, 2011; Keeling et al., 2012; Song et al., 2000).

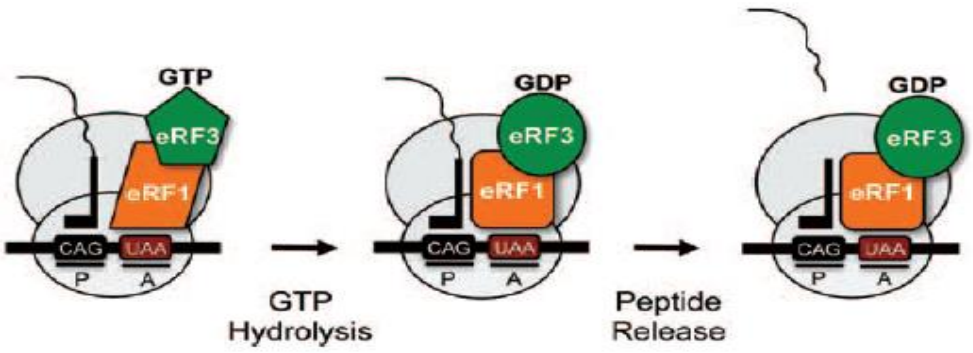


Figure 2. Eukaryotic translation termination. In eukaryotes, translation termination is mediated by eRF1 and eRF3. Stop codon recognition by eRF1 in the ribosomal A-site is followed by GTP hydrolysis by eRF3, which facilitates polypeptide chain release. *In* Keeling et al., 2012.

The bridge linking splicing and translation to NMD activation is established by three key players, the up-frameshift proteins UPF1, UPF2 and UPF3 [in mammals, UPF3 has two variants, UPF3/UPF3a and UPF3X/UPF3b, which derive from different genes (Serin et al., 2001)]. UPF factors directly mediate NMD. In fact, inhibition of their expression is enough to suppress NMD (Singh & Lykke-Andersen, 2003). Moreover, tethering of any of these proteins to the 3' UTR of a transcript causes a natural stop codon to be perceived as a premature one, leading to NMD activation (Lykke-Andersen, Shu & Steitz, 2000). It has been observed that UPF2 and UPF3 associate with EJCs, and this happens in different subcellular compartments: while UPF3 binds to the EJC in the nucleus, during pre-mRNA splicing, UPF2 seems to interact with the complex in the perinuclear region, possibly during mRNA export to the cytoplasm (Serin et al., 2001). Immediately after export, transcripts are subject to a pioneer round of translation and, if a PTC resides more than 50-55 nucleotides upstream of an exon-exon junction that is bound by an EJC, UPF1 is recruited to the terminating ribosome, where it interacts with eRF3 and also with UPF2 and UPF3, both bound to the EJC, as will be discussed in more detail in the next section (Wang et al., 2001).

1.1.4. NMD mechanism

As previously mentioned, EJCs are displaced from the mature mRNA by the elongating ribosome during the pioneer round of translation. However, if a PTC is located more than 50-55 nucleotides upstream of the last exon-exon junction, the ribosome will stall at the PTC before it reaches the subsequent EJC(s), which will be retained on the mRNA. In these circumstances, NMD is activated. In contrast, if the PTC resides less than 50-55 nucleotides upstream of the last exon-exon junction of a transcript, the ribosome is able to remove all EJCs and NMD is not triggered (Maquat, 2004; Singh & Lykke-Andersen, 2003).

The pioneer round of translation occurs when transcripts are still associated with the cap-binding complex (CBC), a heterodimer consisting of cap-binding protein 80 (CBP80) and CBP20, which binds to the 7-methylguanosine cap on the 5'-end of pre-mRNAs in the

nucleus. CBC is later replaced by its cytoplasmic counterpart, the eukaryotic translation initiation factor 4E (eIF4E), which directs steady-state rounds of translation (Ishigaki et al., 2001; Maquat et al., 2010). CBC has been documented to "chaperone" UPF1 to the terminating ribosome, located at the PTC, helping to form what is termed the SURF complex, which consists of the serine/threonine kinase SMG1, UPF1 and the heterodimer eRF1-eRF3 (Figure 3) (Hwang et al., 2010; Kashima et al., 2006).

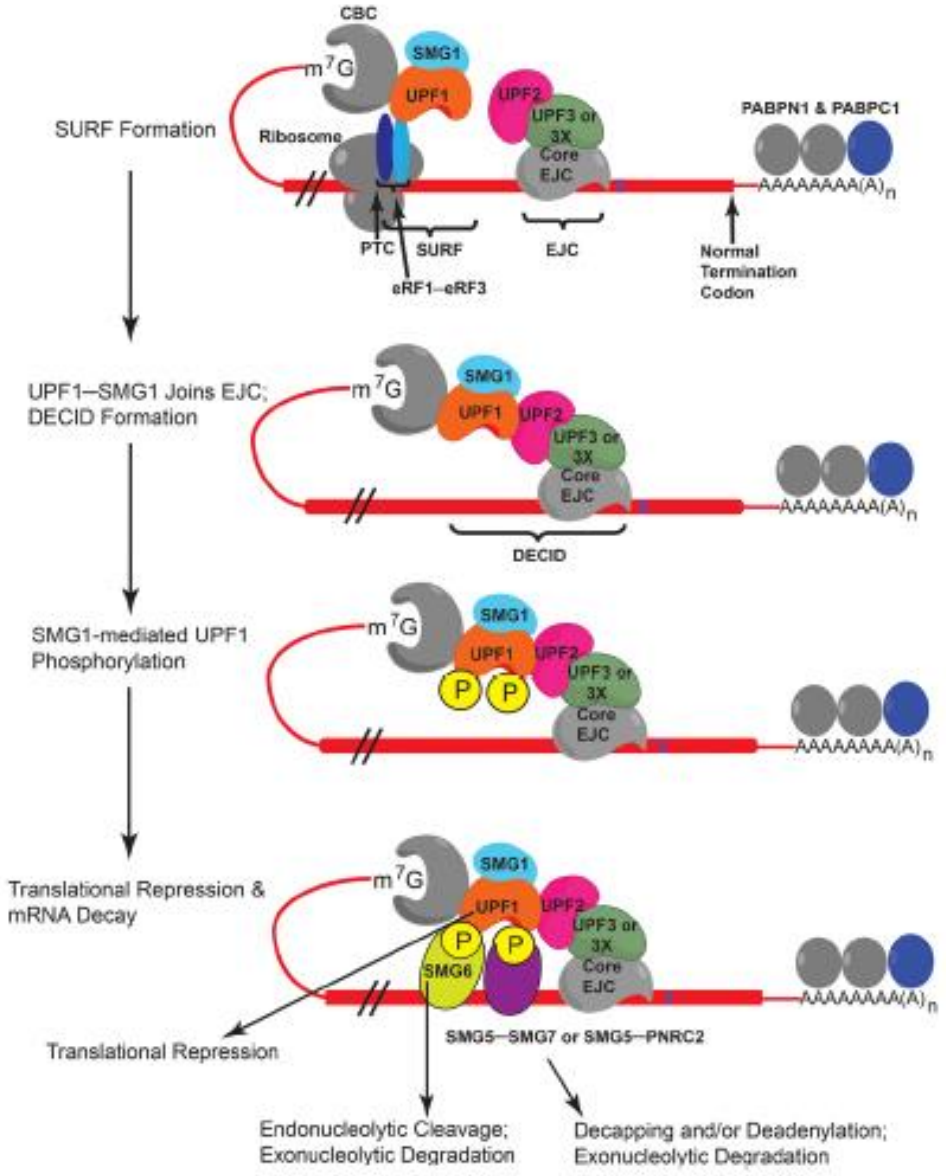


Figure 3. The NMD pathway. During the pioneer round of translation, if a PTC is located more than 50-55 nucleotides upstream of the last exon-exon junction, UPF1 and SMG1 are escorted by CBC to the eRF3 component of the eRF1-eRF3 heterodimer, positioned at the PTC, resulting in the assembly of the SURF complex. UPF1 then interacts with UPF2, which is bound to the EJC via UPF3, resulting in the formation of the DECID complex. In this configuration, SMG1 phosphorylates UPF1, which induces translation repression and leads to recruitment of SMG6, SMG5-SMG7 or SMG5-PNRC2 and, ultimately, to mRNA degradation. *In* Popp & Maquat, 2014.

In a mechanism that may involve “reeling in” of the mRNA spanning the PTC and the nearest EJC, UPF1 contacts UPF2, which is bound to the EJC via UPF3, resulting in the formation of the decay inducing complex (DECID) (Shigeoka et al., 2012; Yamashita et al., 2009). This promotes SMG1-mediated phosphorylation of UPF1 at multiple serine and threonine residues located within N- and C-terminal portions of UPF1, increasing its affinity for RNA and inducing the dissociation of eRF1-eRF3. Hyperphosphorylation of UPF1 is a prerequisite for degradation of PTC-bearing mRNAs and functions to recruit additional SMG factors, specifically the endonuclease SMG6 and the heterodimer SMG5-SMG7, as well as proline-rich nuclear receptor co-regulatory protein 2 (PNRC2) (Cho, Kim & Kim, 2009; Okada-Katsuhata et al., 2012).

Recruitment of SMG6 to PTC-containing templates causes one or more endonucleolytic cleavages between the PTC and the EJC, generating intermediates with unprotected ends that predispose them to degradation in the 3’→5’ direction by the exosome and in the 5’→3’ direction by the exonuclease XRN1. Alternatively, in the case of SMG5-SMG7 or SMG5-PNRC2 complexes, they further recruit deadenylation complexes PAN2/PAN3 and CCR4/CAF1 and the decapping heterodimer DCP1/DCP2 resulting, once again, in mRNA with unprotected ends and subsequent degradation in both directions (Eberle et al., 2009; Fukuhara et al., 2005; Huntzinger et al., 2008; Lejeune, Li & Maquat, 2003; Unterholzner & Izaurralde, 2004). What determines which of these degradation mechanisms predominates for a given transcript remains to be elucidated. SMG5-SMG7 recruitment also functions to mobilize protein phosphatase 2A (PP2A), which dephosphorylates UPF1, recycling it for further rounds of NMD (Anders, Grimson & Anderson, 2003; Chiu et al., 2003; Ohnishi et al., 2003).

Another consequence of UPF1 phosphorylation is translational repression, which has long been known to be a prerequisite for degradation in the context of NMD. Hyperphosphorylated UPF1 acts to limit the amount of translation products that could arise from PTC-containing mRNAs. It does so by interacting directly with the eukaryotic initiation factor eIF3, inhibiting the conversion of the pre-initiation complex to a translationally competent form (Isken et al., 2008). Nonetheless, the fate of the encoded truncated polypeptides, which may be toxic, exhibiting dominant-negative or gain-of-function effects

that result in disease, must be addressed by the cell. In addition to degrading PTC-bearing mRNAs, cells may be able to mediate destruction of truncated polypeptides that form as a result of the pioneer round of translation of those transcripts (Popp & Maquat, 2014). In yeast, several reports suggest that UPF1 is itself an E3 ubiquitin ligase and, therefore, may be involved in the attachment of an ubiquitin moiety onto proteins that are destined for proteasome-mediated destruction (Kuroha, Tatematsu & Inada, 2009; Takahashi et al., 2008). However, since apparently not all truncated proteins are degraded, this notion bears much further investigation in mammalian cells (Anczuków et al., 2008).

The current model for NMD states that this surveillance mechanism acts only during the pioneer round of translation, before CBC replacement with eIF4E, making the identification of PTCs and subsequent transcripts degradation unlikely to occur during steady-state rounds of translation, since EJCs and associated UPF proteins have already been removed from mRNA. This idea is supported by multiple lines of evidence: when NMD is activated, mRNA is still associated with CBC and, moreover, this complex increases NMD efficiency (Hwang et al., 2010; Ishigaki et al., 2001; Lejeune et al., 2002); short interfering RNA (siRNA) mediated depletion of CBP80 inhibits NMD (Hosoda et al., 2005); UPF2 and UPF3 are detected interacting with mRNA when it is still associated with CBC but not with eIF4E (Chiu et al., 2004; Lejeune et al., 2002). However, it has been recently suggested that mammalian NMD may not be restricted to CBC-bound transcripts. It was observed that UPF1 co-precipitates with eIF4E in an RNA-dependent manner, indicating that NMD could be a regulated event that can be activated on substrates already engaged in translation, according to the cell needs (Durand & Lykke-Andersen, 2013; Rufener & Mühlemann, 2013). Thus, although eIF4E-bound templates can be targeted for NMD, it may simply be that, in most cases, mRNAs are destroyed sufficiently fast that the cap has yet to be remodeled (Popp & Maquat, 2014). In fact, it has been shown that the pioneer round of translation might even begin before the whole mRNA molecule and associated proteins are exported to the cytoplasm, with the ribosome functioning as a proofreader, triggering NMD and hence eliminating faulty transcripts early on, before they are committed to the eIF4E-associated bulk translation (Maquat, 2004; Rufener & Mühlemann, 2013).

1.1.5. Exceptions to the EJC-dependent NMD model

Even though most PTC-bearing transcripts are degraded according to the depicted EJC-dependent NMD model, some exceptions have been reported. For instance, β-globin transcripts possessing a PTC near the initiation codon AUG fail to trigger NMD, despite the existence of downstream EJCs (Inácio et al., 2004; Romão et al., 2000). These exceptions suggest that additional determinants may be involved. Indeed, there is evidence that, similar to what happens in yeast, the decision of whether NMD is to be triggered or not, relies upon competition between UPF1 and cytoplasmic poly(A)-binding protein 1 (PABPC1) for binding to eRF3 on the terminating ribosome (Peixeiro et al., 2011; Singh, Rebbapragada & Lykke-Andersen, 2008).

Following mRNA export to the cytoplasm, it has been suggested that CBC interacts with the eukaryotic translation initiation factor 4G (eIF4G), promoting the recruitment of the small ribosomal subunit 40S to begin scanning along the 5' untranslated region (UTR) for a start codon (AUG). Once the start codon is identified, the large ribosomal subunit 60S is engaged to form a competent complex for protein synthesis, initiating the pioneer round of translation (Lejeune, Ranganathan & Maquat, 2004). Besides interacting with CBC, eIF4G also binds PABPC1 (a protein associated with the 3' poly(A) tail of mRNA), bringing the 3' UTR closer to the 5'UTR, which results in mRNA circularization (Wells et al., 1998). Under these conditions, if PABPC1 is in close proximity to the PTC, which is the case when the PTC lies near the initiation codon, it interacts with eRF3, preventing UPF1 from binding to it, thus repressing NMD and stimulating proper translation termination; on the contrary, when the PTC is distant from PABPC1, the interaction with eRF3 is less efficient, allowing for UPF1 to bind to eRF3 and NMD to proceed (Ivanov et al., 2008; Silva et al. 2008; Uchida et al., 2002).

1.1.6. Physiological NMD targets

In addition to eliminating faulty transcripts, NMD has been implicated in the regulation of a broad section of the transcriptome, degrading many non-mutated

transcripts. In fact, several microarray studies comparing the mRNA levels of normal cells with NMD-deficient cells in *Saccharomyces cerevisiae, Drosophila melanogaster and Homo sapiens* revealed that NMD directly and indirectly controls the abundance of 3–10% of the transcriptome in the respective cells (Mendell et al., 2004).

For some classes of transcripts, such as RNA-binding proteins (in particular splicing factors) and the NMD factors themselves, NMD provides an autoregulatory loop that ensures homeostatic control of the genes encoding these transcripts (Huang et al., 2011; Yepiskoposyan et al., 2011).

Abnormally long 3' UTRs, for example, may signal NMD-mediated destruction, but how this precisely fits within the better understood EJC-mediated framework, aside from these abnormally long 3' UTRs binding unusually high levels of UPF1, remains to be determined (Hogg & Goff, 2010; Kurosaki & Maquat, 2013; Popp & Maquat, 2014). Other transcripts that possess certain features which can render them NMD-sensitive include: mRNAs harbouring introns in their 3' UTR; transcripts containing regulatory ORFs that reside upstream of the primary ORF (Barbosa, Peixeiro & Romão, 2013); mRNAs containing UGA triplets, which code for selenocysteine when the endogenous selenium concentration is high, but may be perceived as PTCs when selenium is scarce (Moriarty, Reddy & Maquat, 1998).

Physiological NMD targets are involved in a variety of cellular processes, such as stress responses, stem cell differentiation, genomic stability, cell cycle, telomere length maintenance and embryonic development, potentially allowing NMD to modulate protein synthesis according to cellular needs (Holstein, Clark & Lydall, 2014; Martins et al., 2012; Rehwinkel, Raes & Izaurralde 2006).

1.1.7. NMD significance in human disease

NMD relevance as a protective surveillance mechanism is highlighted by the fact that one third of all inherited genetic disorders are associated with PTCs (Holbrook, Neu-Yilik & Hentze, 2016). Transcripts derived from mutant alleles are degraded by the NMD pathway, normally leading to a recessive mode of inheritance, as the wild-type protein is

able to compensate, at least in part, for the absence of the nonsense-containing transcript. If the nonsense transcript evades NMD, resulting in the production of a truncated protein which can exert deleterious effects, this may lead to a dominant genetic defect (Behm-Ansmant et al., 2007). This is illustrated by β-thalassemia, a disorder characterized by reduced or absent β-globin chain synthesis, resulting in reduced hemoglobin in red blood cells, which may present itself in a mild, recessive phenotype, or as a dominantly inherited disease due to the accumulation and toxic precipitation of insoluble α-globin chains. A similar protective role of NMD for heterozygotes with NMD-competent PTCs has been suggested for other genetic diseases, including factor X deficiency and von Willebrand disease, both blood clotting disorders, and retinal degeneration (Holbrook et al., 2004).

Mutations in tumour-suppressor genes are common steps in the development and progression of cancer. As with inherited genetic conditions, NMD appears to provide protection against expression of mutated, truncated tumour-suppressor peptides. For example, NMD has been shown to degrade PTC-containing transcripts arising from the *BRCA1*, *TP53* and *WT1* genes. If not eliminated by NMD, the mutated transcripts would give rise to dominant-negative oncoproteins. Indeed, the expression of the corresponding truncated proteins from intronless cDNAs encoding unspliced mRNAs that are NMD-incompetent has been demonstrated to increase tumourigenicity, cell survival and resistance to chemotherapy (Behm-Ansmant et al., 2007; Holbrook et al., 2004).

While in some cases, the resulting truncated protein might be nonfunctional and easily degraded by proteolysis without harmful effects, in other cases, such as cystic fibrosis and Duchenne muscular dystrophy, NMD can contribute to a disease phenotype when it inhibits expression of partially functional proteins. The loss of the protein product is more deleterious to the cell than the expression of the truncated protein would be, and so interventions to prevent degradation of transcripts containing PTCs may be therapeutically useful (Behm-Ansmant et al., 2007; Holbrook et al., 2004; Holbrook, Neu-Yilik & Hentze, 2016).

1.1.8. β-Thalassemia as a disease model for studying NMD

The prototypical genetic condition illustrating the protective effects of NMD is β-thalassemia. β-Thalassemia syndromes are a group of inherited genetic disorders characterized by reduced (β^+) or absent (β^0) synthesis of the beta chains of hemoglobin, with phenotypes ranging from severe anemia to clinically asymptomatic individuals (Galanello & Origa, 2010).

β-Thalassemias are generally caused by nonsense or frameshift mutations in the β-globin gene on chromosome 11 (Galanello & Origa, 2010). The severity and inheritance pattern of the disease depend on the location of the mutation in the gene (Figure 4).

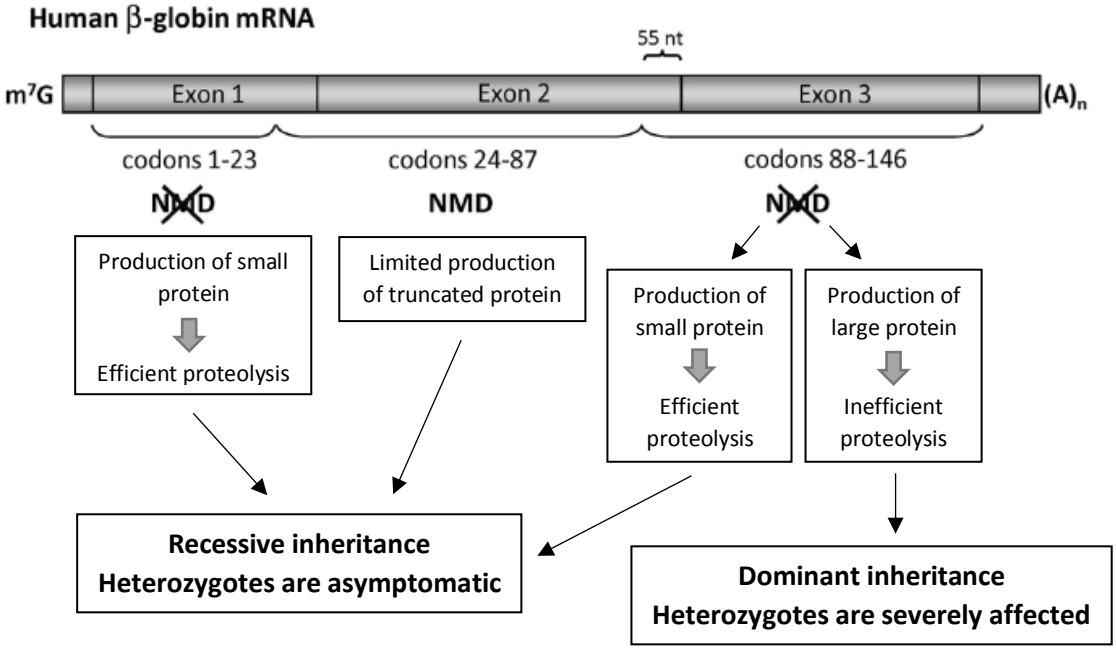


Figure 4. Representation of the location-dependent effects of nonsense mutations in the inheritance pattern and clinical severity of β-thalassemia. PTCs in close proximity to the initiation codon or located less than 55 nucleotides (nt) upstream of the last exon-exon junction and in the 5'-part of exon 3 do not trigger NMD but any translated protein is still small enough to be effectively degraded by proteolysis. If the PTC is located downstream of codon 23 and more than 55 nt upstream of the last exon-exon junction, the transcript is targeted for NMD and production of truncated protein is limited. Both cases are associated with a recessive mode of inheritance. Rare forms of dominant β-thalassemia are caused by transcripts bearing PTCs within the final region of the last exon, which give rise to nonfunctional protein that is too large to be efficiently eliminated and precipitates in toxic inclusion bodies. *Adapted from* Peixeiro, Silva & Romão, 2011.

In the common recessive form of β-thalassemia caused by NMD-competent PTCs, defective β-globin mRNA is degraded by NMD and, therefore, synthesis of truncated

protein is limited. Free α-globin, which is toxic to the cell, is then present in excess and is degraded proteolytically. Thus, the quantity of tetrameric hemoglobin is insufficient, causing severe anemia in affected homozygotes. In contrast, heterozygous carriers of a single NMD-competent PTC generally produce enough β-globin from the normal allele to maintain sufficient amounts of tetrameric hemoglobin, and they are clinically healthy. The recessive form of β-thalassemia is also associated with NMD-insensitive PTCs in close proximity to the start codon, as well as those located less than 55 nucleotides upstream of the last exon-exon junction and in the 5'-part of exon 3; although, in these cases, truncated β-globin is produced, it is small enough to be efficiently eliminated by proteolysis. Rare forms of dominant β-thalassemia are caused by NMD-incompetent PTCs within the final region of the last exon of the β-globin gene. These PTCs give rise to truncated β-globin protein that is too large to be efficiently degraded and precipitates in toxic inclusion bodies (Holbrook et al., 2016; Peixeiro, Silva & Romão, 2011).

1.2. Nonstop mRNA decay

In eukaryotes, transcripts that do not possess in-frame stop codons are targeted for rapid degradation by the nonstop mRNA decay mechanism. Nonstop transcripts can arise in different circumstances: when transcription aborts, when polyadenylation occurs prematurely or through point mutations that disrupt the stop codon. It is also possible that, as occurs with several nonsense transcripts, certain nonstop mRNAs are prone to decay in order to maintain the levels of these transcripts under certain/normal physiological conditions (Klauer & van Hoof, 2012).

NSD factors involve another eRF3 family member, the Ski7 GTPase, and the auxiliary Ski complex composed of Ski2, Ski3 and Ski8 proteins. In the proposed model for the NSD mechanism in yeast, instead of terminating, the ribosome continues to translate the 3' UTR and even the poly(A) tail of the mRNA, adding a poly-lysine tail to the newly synthesized protein (Figure 5). The ribosome then stalls and is recognized by Ski7 through its C-terminal domain. The poly-lysine tail may provide the tag for recognition of the stalled ribosome. Ski7 associates with the Ski complex and the exosome through its N-terminal domain,

triggering fast 3'→5' degradation of the transcript. Thus, NSD is translation-dependent and is accomplished by the 3'→5' exonucleolytic action of the exosome, without the need for prior deadenylation (Atkinson et al., 2008; Klauer & van Hoof, 2012; Saito, Hosoda & Hoshino, 2013). Moreover, NSD does not require NMD factors, including UPF1 (Saito, Hosoda & Hoshino, 2013).

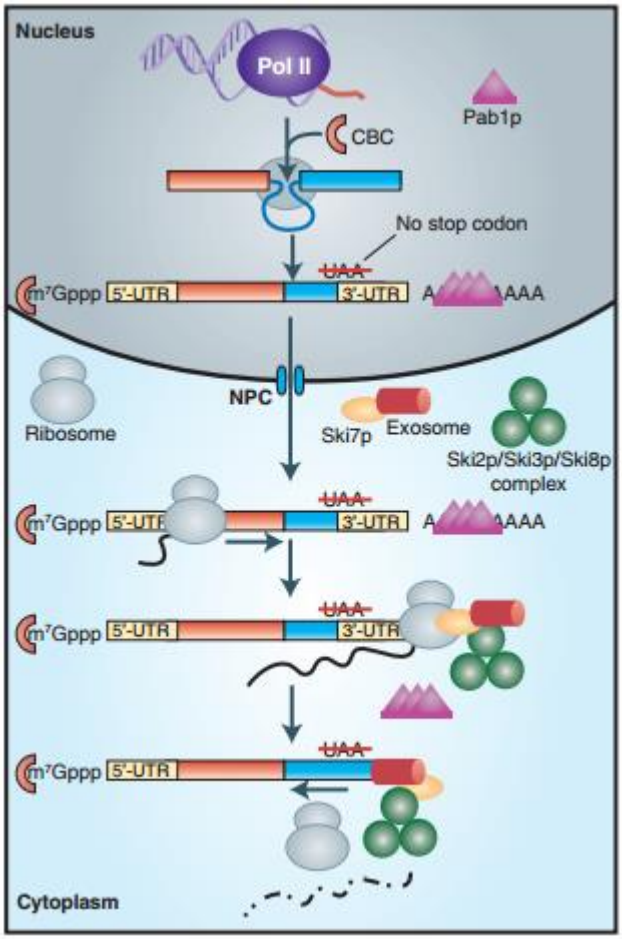


Figure 5. The NSD pathway. After splicing and export of the nonstop mRNA, the transcript is translated by the ribosome. Without a termination codon, the elongating ribosome proceeds through the poly(A) tail and stalls at the 3' end of the mRNA. The now empty ribosomal A-site is recognized by Ski7, which results in the recruitment of the exosome and the Ski2–Ski3–Ski8 complex, and dissociation of the ribosome. The nonstop mRNA is then degraded from the 3' end by the exosome and the protein product is destroyed. *In* Fasken & Corbett, 2005.

The components involved in the yeast NSD mechanism are conserved in mammals except for the key regulator Ski7. This protein is not found in mammalian cells, and even the existence of the NSD mechanism itself has been questioned (Saito, Hosoda & Hoshino, 2013). A study using a GFP-based reporter in HeLa cells showed that the steady-state

amount of nonstop mRNA was not significantly reduced relative to the wild-type transcript, and no significant difference was observed between the stability of the wild-type and nonstop mRNAs (Akimitsu, Tanaka & Pelletier, 2007). Moreover, even in clinical cases, inconsistent findings have been described. In Diamond-Blackfan anemia, levels of mutant RPS19 (ribosomal protein S19) mRNA lacking a stop codon were low compared with wild-type mRNA from a healthy donor (Chatr-aryamontri et al., 2004). In contrast, in a mitochondrial neurogastrointestinal encephalopathy patient, nonstop TYMP mRNA was stable and its levels were similar to those of wild-type mRNA (Torres-Torronteras et al., 2011). A study from 2013 suggested that, in mammals, instead of Ski7, NSD requires another member of the eRF3 family of G proteins, Hbs1, as well as its binding partner Dom34. Both proteins are also involved in the no-go decay pathway, which targets mRNAs with a structural propensity to cause ribosome stalling (Saito, Hosoda & Hoshino, 2013).

1.3. The eukaryotic exosome

The exosome is an evolutionarily conserved multiprotein complex which was first identified in *Saccharomyces cerevisiae* for its function in 5.8S ribosomal RNA processing in the nucleolus (Mitchell et al., 1997). It was later found to contribute to most cellular processes in eukaryotes involving RNA degradation from the 3' end, in both the nucleus and the cytoplasm (Schmid & Jensen, 2008).

1.3.1. Functions of the exosome

Studies in yeast have revealed a nuclear and nucleolar function of the exosome in the maturation of many small RNAs (e.g. small nuclear RNAs and small nucleolar RNAs) (Staals et al., 2010). The nuclear exosome also plays a role in the maturation of rRNAs and tRNAs, as well as in the degradation of RNA processing by-products (Tomecki et al., 2010). Besides being responsible for the rapid elimination of defective nuclear precursors of many types of RNA, including pre-mRNAs, pre-tRNAs and pre-rRNAs, the nuclear exosome has been implicated in the control of expression levels of some mRNAs (Staals et al., 2010). In

the cytoplasm, the exosome is involved not only in the “normal” turnover of mRNA, but also in specific mRNA decay pathways, including NMD and NSD (van Dijk, Schilders & Pruijn, 2007; van Hoof et al., 2002). In human cells, it plays a role in the regulated degradation of unstable transcripts containing AU-rich elements (AREs) in their 3’ UTRs. AREs are present in many mRNAs that encode proteins for which transient expression is important, including growth factors and proto-oncogenes. In addition, the cytoplasmic exosome degrades mRNAs cleaved by Argonaute proteins during RNA interference. Finally, different classes of noncoding transcripts, including yeast cryptic unstable transcripts (CUTs), antisense RNAs and human promoter-upstream transcripts (PROMPTs) are also targeted by the exosome (Schmid & Jensen, 2008; Tomecki et al., 2010).

How the eukaryotic exosome can identify such a variety of substrates and reliably distinguish between the different classes of RNAs is still the subject of intense research, however, it is assumed that its specificity is, at least partly, achieved through the interaction with accessory factors such as nuclear complexes TRAMP and Nrd1/Nab3/Sen1, as well as the cytoplasmic Ski7 GTPase/Ski complex (Araki et al., 2001; Lacava et al., 2005; Vasiljeva & Buratowski, 2006).

1.3.2. Structure of the exosome

The *S. cerevisiae* exosome is a multimeric complex with approximately 400 kDa, consisting of a catalytically inert core of nine subunits, six of which (Rrp41, Rrp42, Rrp43, Rrp45, Rrp46 and Mtr3) form a ring structure and contain domains homologous to the bacterial phosphorolytic ribonuclease RNase PH. The remaining three core subunits (Rrp40, Csl4 and Rrp4) harbour RNA-binding domains and are positioned on top of the RNase PH-like ring (Liu, Greimann & Lima, 2006; Tomecki et al., 2010). The catalytic activity is obtained from the ubiquitously present endo-/exo-nuclease Dis3 and the nucleus-restricted 3’→5’ exonuclease Rrp6. In human cells, the exosome complex has diversified, as it is found in at least three compartment-specific isoforms: a nucleolar, a nucleoplasmic, and a cytoplasmic one (Figure 5) (Tomecki et al., 2010).

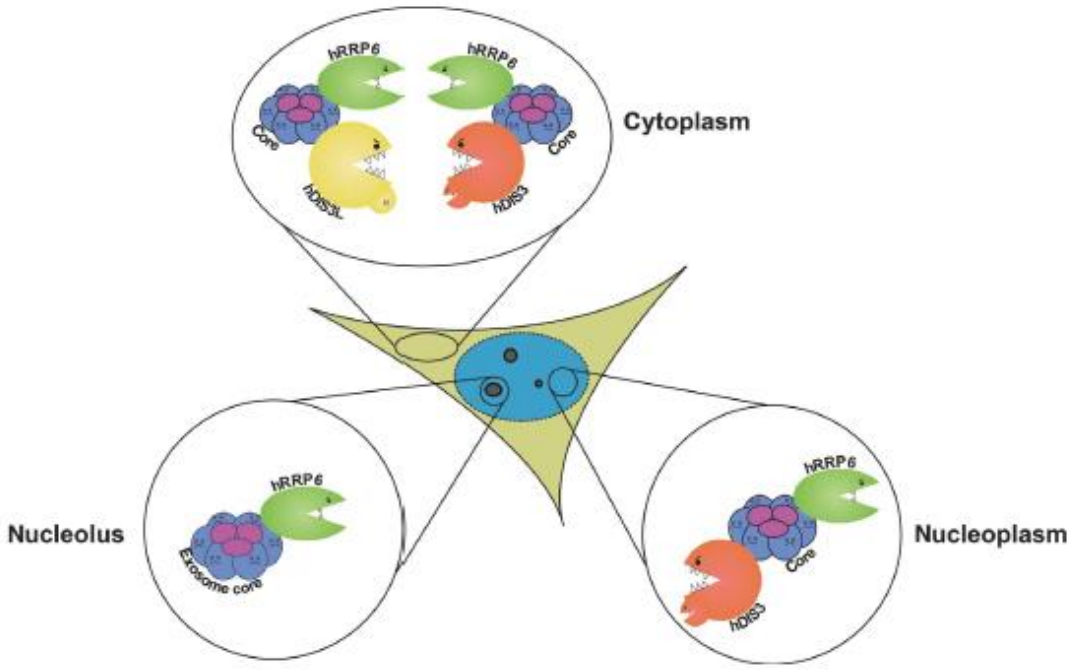


Figure 6. Compartment-specific human exosome isoforms. The catalytically inert exosome core associates in the nucleus with hDIS3 and the ubiquitously present hRRP6 (PM/Scl100). In the nucleolus, the exosome core primarily binds hRRP6. In the cytoplasm, the core exosome preferentially associates with hDIS3L; hDIS3 and hRRP6 are also present in the cytoplasm, but in lower amounts than in the nucleus/nucleolus. *In* Tomecki et al., 2010.

Dis3 is the largest subunity of the exosome and is indispensable for cellular viability. It is predominantly present in the nucleus, but it is also found in the cytoplasm. It harbours a catalytic ribonuclease II-like (RNB) domain, responsible for the processive 3'→5' exonucleolytic activity, supported by three RNA binding domains (CSD1, CSD2 and S1). Dis3 also displays endonucleolytic activity that derives from the PilT N-terminus (PIN) domain (Staals et al., 2010; Tomecki et al., 2010). In vivo evidence indicates that cooperation between endo- and exonucleolytic activities of Dis3 is crucial for efficient degradation and/or processing of several natural exosome substrates (Schaeffer et al., 2009). In addition to its catalytic activity, the PIN domain, together with the upstream-located zinc-binding CR3 motif, is responsible for the association of Dis3 with the exosome core (Schaeffer et al., 2012). In humans, three Dis3 isoforms have been identified: DIS3, DIS3L, and DIS3L2. DIS3 is enriched in the nucleoplasm, and DIS3L and DIS3L2 are exclusively cytoplasmic (Malecki et al., 2013). DIS3L does not possess endonucleolytic activity because its PIN domain is rendered inactive by mutated residues; however, this protein still

associates with the exosome core (Staals et al., 2010; Tomecki et al., 2010). DIS3L2 does not harbour a PIN domain, therefore it is also only endowed with exonucleolytic activity and, contrary to DIS3L, does not interact with the exosome core (Lubas et al., 2013; Malecki et al., 2013).

Rrp6 (designated PM/Scl-100 in humans), an enzyme belonging to the RNase D family of hydrolytic exoribonucleases, also associates with the exosome core and contributes to its activity (Staals et al., 2010). Rrp6 has been reported to reside exclusively in the nucleus of yeast cells, whereas in human cells, PM/Scl-100 was found in the nucleus, nucleolus and cytoplasm (Lejeune et al., 2003; Staals et al., 2010). Depletion of Rrp6 from cells has been shown to give rise to phenotypes notoriously different from when core exosome subunits are depleted, suggesting that its function is at least partially uncoupled to that of the core exosome (Staals et al., 2010; van Dijk et al., 2007).

Recent studies found that Dis3 and Rrp6 have both shared and distinct roles in the degradation of several RNAs. For example, Rrp6 is largely responsible for snRNA processing whereas both Rrp6 and Dis3 seem to be involved in the degradation of unspliced pre-mRNAs (Fox et al., 2015).

2. Aims

SMG6 and PM/Scl100 are both ribonucleases which have been implicated in RNA degradation pathways. One of the mechanisms proposed for mammalian NMD involves an endonucleolytic cleavage of transcripts in the vicinity of the PTC catalyzed by SMG6. On the other hand, the human exosome, which includes the catalytic subunit PM/Scl100, has been associated not only with mRNA surveillance mechanisms, but also with normal mRNA turnover. However, questions relative to the specificity or indispensability of these enzymes in the pathways in which they participate have not yet been answered. For instance, is SMG6 ribonucleolytic action specific for NMD-sensitive mRNAs or is it possible that this ribonuclease is involved in the degradation of other transcripts? Since DIS3 has been appointed as the major enzyme responsible for the exosome ribonucleolytic activity (Tomecki et al., 2010), does absence of PM/Scl100 have any remarkable effect on mRNA degradation pathways efficiency?

In order to address these issues, the role of SMG6 and PM/Scl100 ribonucleases in the degradation of normal or NSD- and NMD-sensitive mRNAs was investigated. HeLa cells were depleted of SMG6 or PM/Scl100, using RNA interference, and transiently transfected with a plasmid expressing the human wild-type β-globin gene (βWT) or the same gene carrying a nonsense mutation at positions 15, 26, or 39 (β15, β26 or β39 genes, respectively) or encoding an mRNA that lacks any termination codon (βNS). The impact of SMG6 or PM/Scl100 depletion on β-globin mRNA levels, as well as on endogenous transcripts that have already been reported to be regulated by NMD, was assessed by reverse transcription coupled quantitative polymerase chain reaction (RT-qPCR).

3. Methods

3.1. Amplification of expression vectors

The wild type β-globin gene (βWT), as well as the β-globin variants β15, β26, β39 and βNS, were subcloned into a pTRE2pur vector (Clontech) as previously described (Pereira et al., 2015).

Ten microliters of NZY5α competent *E. coli* cells (NZYTech), together with 1 µl of each pTRE2purβ-globin variant, were incubated on ice for 30 minutes. Cells were then heat-shocked for 40 seconds in a 42°C water bath and placed again on ice for at least 2 minutes. Following addition of 90 µl of super optimal broth with catabolite repression (SOC) medium, cells were shaken at 300 rpm (37°) for 1 hour. One hundred microliters of putatively transformed cells were spread on LB agar/ampicillin plates and incubated overnight at 37°C. Single colonies were selected and allowed to grow in 4 ml of liquid Luria-Bertani (LB)/ampicillin overnight at 220 rpm (37°C). Plasmid DNA was purified with the NZYMiniprep kit (NZYTech), according to the manufacturer's protocol.

3.2. Dye-terminator sequencing

Confirmation of purified plasmid DNA sequences was carried out by automated sequencing. DNA samples were subjected to amplification reactions with four different primers (Thermo Scientific; Table 1 – primers #1 to #4), each encompassing a different region of the β-globin gene and, in the case of primers #1 and #4, the upstream and downstream plasmid regions, respectively. The reaction mixture contained 300 ng of plasmid DNA, 1 µl of one of the primers mentioned above at a concentration of 2 µM, 1 µl of BigDye® Master Mix (Applied Biosystems® by Life Technologies™) and double-distilled water to a final volume of 10 µl. The amplification program consisted of an initial denaturation at 96°C for 45 seconds, followed by 25 cycles of denaturation at 96°C for 20 seconds, annealing at 55°C for 5 seconds and extension at 60°C for 4 minutes. Samples

were then sent to the Technology and Innovation Unit of the Human Genetics Department of National Health Institute Doutor Ricardo Jorge, where they were subjected to dye-terminator sequencing.

Table 1. Primers used in the current work

Primer	Target	Orientation	Sequence 5'→3'
#1	β-globin gene	Reverse	CCTTGATACCAACCTGCCCA
#2	β-globin gene	Forward	ACATTTGCTTCTGACACAAC
#3	β-globin gene	Reverse	TTGCCTTAACCCAGAAATTATCACT
#4	β-globin gene	Forward	CCTAATCTCTTTCTTTCAGGGCAAT
#5	SMG6 cDNA	Forward	GACACCAACGGCTTCATTGA
#6	SMG6 cDNA	Reverse	CAGGCCGTCCAGCTCATT
#7	PM/Scl100 cDNA	Forward	AGAGAGAGCGAGCAACAAGC
#8	PM/Scl100 cDNA	Reverse	TCCAGCAAAAGCCTTGAAGT
#9	GAPDH cDNA	Forward	CCATGAGAAGTATGACAACAGCC
#10	GAPDH cDNA	Reverse	GGGTGCTAAGCAGTTGGTG
#11	β-globin cDNA	Forward	GTGGATCCTGAGAACTTCAGGC
#12	β-globin cDNA	Reverse	CAGCACACAGACCAGCACGT
#13	GADD45A cDNA	Forward	GGAGGAATTCTCGGCTGGAG
#14	GADD45A cDNA	Reverse	CGTTATCGGGGTCGACGTT
#15	SLC7A11 cDNA	Forward	GGGCATGTCTCTGACCATCT
#16	SLC7A11 cDNA	Reverse	TCCCAATTCAGCATAAGACAAA

3.3. Cell culture, plasmid and siRNA transfection

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM (1x) + GlutaMAX™-I; Gibco® by Life Technologies™) supplemented with 10% (v/v) fetal bovine serum (Gibco® by Life Technologies™), at 37°C in a humidified atmosphere of 5% CO_2.

Transfection of cells with siRNAs (Table 2) were carried out when cells had a confluence of 30-40%, using 200 pmol of siRNA oligonucleotides and 4 µl of Lipofectamine® 2000 Transfection Reagent (Invitrogen® by Life Technologies™), according to the manufacturer's instructions. Twenty-four hours later, a supplemental 50 pmol siRNA transfection was performed, along with the transfection of 500 ng of each pTRE2purβ-globin variant. siRNAs were designed as 19-mers with 3'-dTdT overhangs and were purchased from Thermo Scientific. Twenty-four hours later, cells were harvested for RNA and protein expression analysis.

To perform the βWT, β39, GADD45A and SLC7A11 mRNAs half-life analyses, 24 hours after the transfection with the corresponding plasmids, HeLa cells were treated with 60 µM of the adenosine analogue 5,6-dichloro-1-3-D-ribofuranosylbenzimidazole (DRB; Sigma-Aldrich®) to inhibit transcription and harvested at different time points for further RT-qPCR analysis.

Table 2. siRNAs used in the current work

siRNA	**Orientation**	**Sequence 5'→3'**
SMG6	Sense	AAGCCAGUGAUACAGCGAA
SMG6	Antisense	UUCGCUGUAUCACUGGCUU
PM/Scl100	Sense	GCUGCAGCAGAACAGGCCA
PM/Scl100	Antisense	UGGCCUGUUCUGCUGCAGC
UPF1	Sense	AAGAUGCAGUUCCGCUCCAUU
UPF1	Antisense	AAUGGAGCGGAACUGCAUCUU
Luciferase	Sense	UCGAAGUAUUCCGCGUACG
Luciferase	Antisense	CGUACGCGGAAUACUUCGA

3.4. Isolation of total RNA and protein lysates

Total RNA and protein extracts were obtained from lysis of transfected cells. Briefly, cells were washed with 1 ml of phosphate buffer saline and lysed with 100 µl of NP40 buffer

[50 mM Tris-HCl pH=7.5, 10 mM MgCl2, 100 mM NaCl, 10% (v/v) glycerol and 1% (v/v) Nonidet P-40 (Roche)]. Total lysates were centrifuged at maximum speed for 2 minutes. Twenty microliters of the supernatant were collected in tubes with 5 µl 5x SDS sample buffer [200 mM Tris-HCl pH=6.8, 25% (v/v) glycerol, 25% sodium dodecyl sulfate (w/v; Sigma-Aldrich®), 525 mM dithiothreitol and 0.25% (v/v) bromophenol blue], for protein analysis, and total RNA was extracted from remaining volume with the Nucleospin® RNA kit (Macherey-Nagel), following the manufacturer's protocol. In the cases where samples were not to be subjected to protein analysis, RNA was isolated directly from total lysates.

3.5. Immunoblotting

Protein lysates were denatured at 95ºC for 10 minutes. Then, they were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using a 10% acrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), according to standard protocols. Nonspecific sites were blocked using a solution of 5% (w/v) bovine serum albumin (Sigma-Aldrich®) in tris-buffered saline with 0.1% (v/v) Tween® 20 (Sigma-Aldrich®), at room temperature, for 1 hour. Membranes were probed overnight at 4°C with rabbit polyclonal UPF1 antibody (Cell Signaling) diluted to 1:250 in blocking solution and mouse monoclonal α-tubulin antibody (Sigma-Aldrich®) diluted to 1:50000 in blocking solution. Detection was carried out by incubating membranes for 1 hour at room temperature with secondary peroxidase-conjugated anti-rabbit IgG (Bio-Rad) diluted to 1:3000 in blocking solution and anti-mouse IgG (Bio-Rad) diluted to 1:4000 in blocking solution, followed by enhanced chemiluminescence. Film bands density was analyzed using ImageJ (National Institutes of Health).

3.6. Reverse transcription (RT)

cDNA was prepared by incubating 1 µg of isolated total mRNA with 1 µL of dNTPs mix (10 mM; Bioline), 250 ng of random primers (Invitrogen® by Life Technologies™) and double-distilled water to a final volume of 16 µL, during 5 min at 65°C. Then, 2 µL of reaction

buffer (10x; NZYTech), 4 U of ribonuclease inhibitor (NZYTech), 100 U of reverse transcriptase (NZYTech) and double-distilled water were added, to a final volume of 20 µL. The mixture was incubated in a thermocycler at 25°C for 10 minutes, 50°C for 50 min and 85°C for 5 min.

3.7. Semi-quantitative PCR

The PCR reactions for SMG6, PM/Scl100 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNAs were performed with 3 µl of the diluted RT product in a 50 µl reaction volume containing 1 µl of dNTPs mix (10 mM; Bioline), 15 pmol of each primer (Thermo Scientific; Table 1 – primers #5 to #10), 0.75 U of DreamTaq DNA polimerase (Thermo Scientific), and 5 µl of DreamTaq green buffer (10x; 20 mM $MgCl_2$; Thermo Scientific). Thermocycler conditions for each reaction are described in Table 3. Ten microliter aliquotes from each sample were analyzed by electrophoresis on 2% agarose gels. Gel bands density was analyzed using ImageJ (National Institutes of Health).

Table 3. Semi-quantitative PCR conditions for SMG6, PM/Scl100 and GAPDH cDNAs

SMG6			**PM/Scl100**			**GAPDH**		
Temp.	**Time**	**Number of cycles**	**Temp.**	**Time**	**Number of cycles**	**Temp.**	**Time**	**Number of cycles**
95°C	5 min	1	95°C	5 min	1	95°C	10 min	1
94°C	30 s	35	94°C	30 s	30	95°C	45 s	25
60°C	60 s		52°C	60 s		62°C	30 s	
72°C	90 s		72°C	90 s		72°C	45 s	
72°C	5 min	1	72°C	5 min	1	72°C	10 min	1

3.8. Quantitative PCR

Quantitative PCR was performed in Applied Biosystems® 7500 Real-Time PCR System, using SYBR® Green PCR Master Mix (Applied Biosystems® by Life Technologies™). The relative expression levels of β-globin, GADD45A and SLC7A11 mRNAs were normalized to endogenous GAPDH mRNA and calculated using the comparative C_t method ($\Delta\Delta C_t$, Applied Biosystems® by Life Technologies™). The amplification efficiencies of the β-globin, GADD45A and SLC7A11 targets and GAPDH reference amplicons were determined for each assay by dilution series. Primers used are indicated in Table 1 (primers #9 to #16) and were purchased from Thermo Scientific. Amplifications were carried out under the following conditions: an initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 62°C for 30 seconds. Technical triplicates from each independent experiment were assessed in all cases.

3.9. Statistical analysis

When appropriate, Student's unpaired, two-tailed t-test was used for estimation of statistical significance. Significance for statistical analysis was defined as $p<0.05$. Results are expressed as mean ± standard deviation from three or four independent experiments.

4. Results

4.1. The role of PM/Scl100 in mRNA degradation

4.1.1. PM/Scl100 depletion increases both NMD-competent and incompetent human β-globin mRNAs levels, as well as those of the NSD-sensitive β-globin transcript

The exosome has been implicated not only in normal mRNA turnover, but also in mRNA surveillance pathways. Aiming to assess the contribution of the PM/Scl100 subunity to these mRNA degradation mechanisms, HeLa cells were treated with siRNAs targeting PM/Scl100 or, as a transfection control, luciferase (LUC). Twenty-four hours later, cells were transfected with a plasmid expressing the human βWT gene or the β15, β26, β39 or βNS variants. Levels of endogenous PM/Scl100 mRNA were monitored by reverse transcription coupled semi-quantitative PCR (RT-PCR) (Figure 6) and the impact of PM/Scl100 depletion on β-globin mRNA levels measured by RT-qPCR (Figure 7).

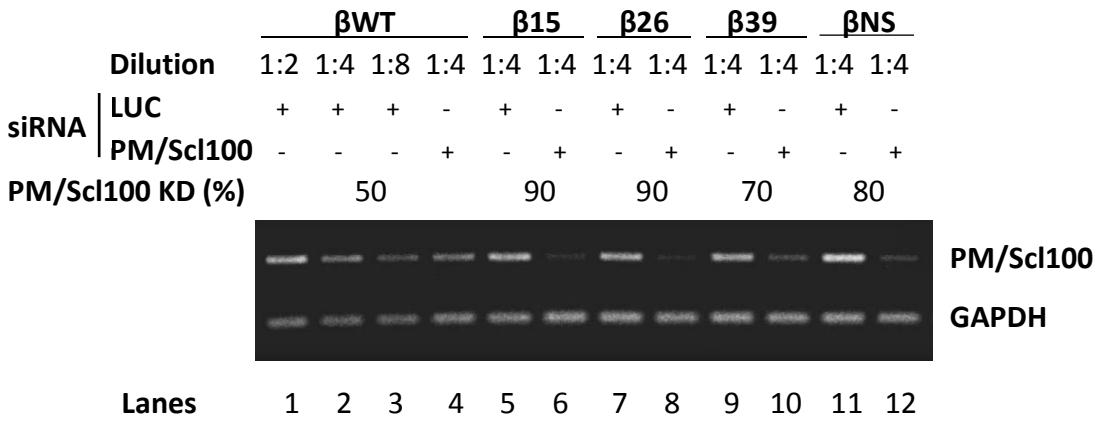


Figure 7. Representative semi-quantitative RT-PCR analysis of mRNAs extracted from luciferase [(+LUC siRNA); lanes 1-3, 5, 7, 9 and 11] or PM/Scl100 [(+PM/Scl100 siRNA); lanes 4, 6, 8, 10 and 12] siRNA-treated HeLa cells transiently transfected with plasmids expressing βWT, β15, β26, β39 or βNS mRNAs. Semi-quantitative PCRs were carried out with human PM/Scl100 mRNA specific primers to monitor its endogenous levels or with human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA specific primers as an internal standard. Lanes 1 to 3 correspond to serial dilutions of cDNA, demonstrating semi-quantitative conditions used for RT-PCR. An estimative of the percentage of achieved PM/Scl100 knockdown (KD) for each sample is indicated.

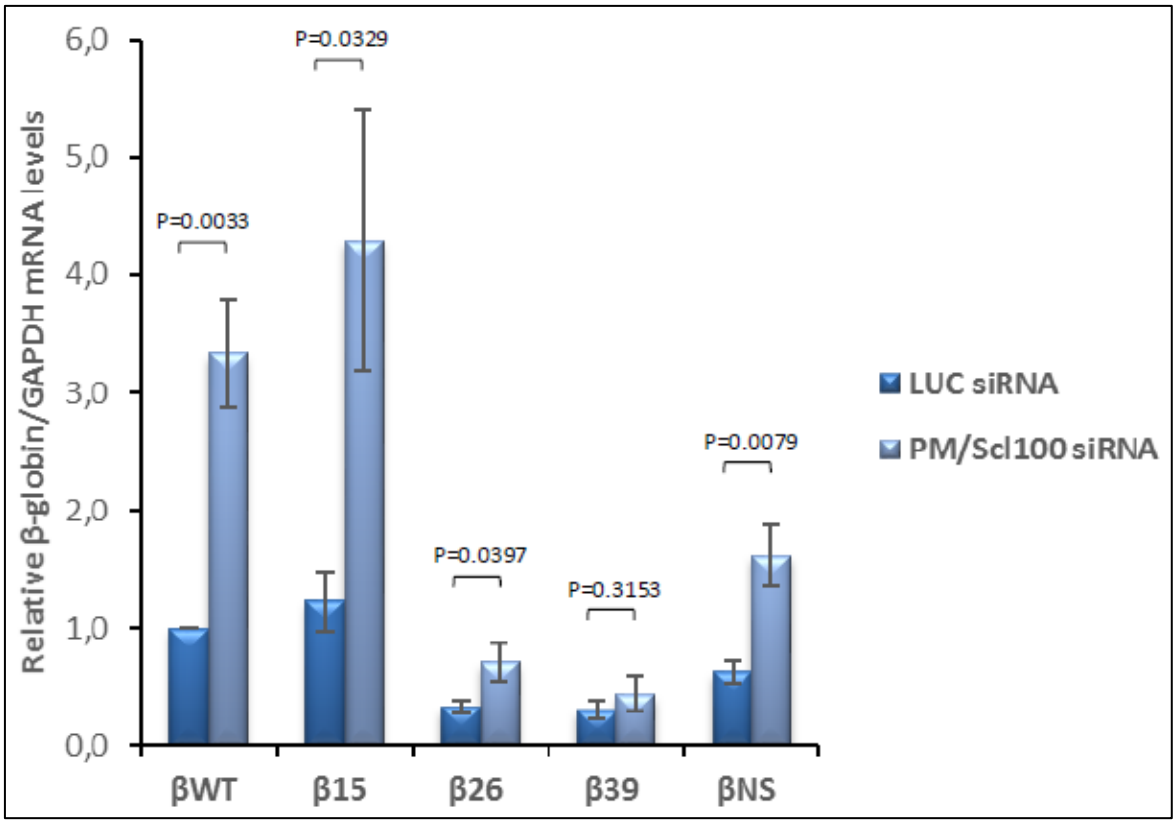


Figure 8. PM/Scl100 depletion increases both NMD-competent and incompetent human β-globin mRNAs levels, as well as those of the NSD-sensitive β-globin transcript. RT-qPCR analysis was carried out on mRNAs extracted from luciferase (LUC) or PM/Scl100 siRNA-treated HeLa cells transiently transfected with plasmids expressing βWT, β15, β26, β39 or βNS mRNAs, using specific primers for the human β-globin and for the GAPDH mRNAs. Quantification was performed by the relative standard curve method. Histogram represents fold-change of each sample relative to the control mRNA levels (βWT at LUC siRNA-treated cells), arbitrarily set to 1. All values are normalized internally to GAPDH mRNA levels. Standard deviations (n=4) and p-values from Student's t-tests are shown.

As expected, under control conditions, β26, β39 and βNS mRNAs are present at lower levels than βWT mRNA – at about 33%, 30% and 62% of the βWT mRNA level (arbitrarily set to 100%), respectively, as a result of these mRNAs being subjected to the degradation mechanisms inherent to NMD, in the cases of β26 and β39 (Romão et al., 2000), and to NSD, in the case of βNS (Saito, Hosoda & Hoshino, 2013). This decrease in mRNA levels is not observed for the β15 variant, which is in accordance with the finding that this transcript is able to escape NMD due to the PTC being in close proximity to the initiation codon (Inácio et al., 2004).

On the other hand, in conditions of PM/Scl100 knockdown, either the normal βWT transcript or the β15, β26, β39 and βNS variants show an increase in levels to about 334%, 430%, 71%, 44% and 162%, respectively. This difference is statistically significant ($p<0.05$)

for all but one of the variants – the β39 transcript (p=0.3153). Nonetheless, these results show that PM/Scl100 plays a role in the degradation of both NMD and NSD targets, as well as transcripts that undergo normal degradation. Moreover, albeit the increase of β26 and β39 transcript levels in conditions of PM/Scl100 knockdown, the normal mRNA levels are not fully recovered, which points to the involvement of additional 3'→5' ribonucleases in the degradation of transcripts targeted by NMD.

4.1.2. PM/Scl100 knockdown increases SLC7A11 mRNA levels, but decreases those of GADD45A mRNA

In an attempt to obtain further confirmation of PM/Scl100 involvement in NMD, the expression levels of two physiological transcripts already reported to be NMD-sensitive – SLC7A11 and GADD45A (Martin & Gardner, 2014; Nelson et al., 2016) – were also evaluated. SLC7A11 encodes a subunit of a heteromeric, sodium-independent cystine/glutamate transport system known as the xCT system. In human cancer cell lines, cystine uptake is largely mediated by this system. Once inside the cell, cystine is reduced to cysteine, the limiting amino acid in glutathione synthesis. Glutathione is thought to mediate cellular detoxification pathways, which contribute to tumourigenesis and chemotherapy resistance. In fact, SLC7A11 depletion has been shown to increase the potency of several anticancer drugs (Huang et al., 2005; Liu et al., 2007). GADD45A codes for the growth arrest and DNA-damage inducible 45 alpha protein, which mediates the activation of the p38/JNK stress response pathway via the MTK1/MEKK4 kinase, triggering a signaling cascade that promotes apoptosis. In physiological conditions, this gene is thought to be down-regulated by NMD. In the presence of a viral infection, NMD is suppressed by trans-acting factors encoded by viruses and the resulting accumulation of GADD45A elicits a stress response and subsequent cell death (Nelson et al., 2016).

To test whether SLC7A11 and GADD45A mRNAs are sensitive to PM/Scl100, along with the two groups of HeLa cells treated with either LUC or PM/Scl100 siRNAs, a third group was treated with UPF1 siRNAs. Since UPF1 is a key element in the NMD mechanism, knockdown of the corresponding mRNA aimed to provide a positive control for these

experiments. UPF1 knockdown efficiency was assessed by Western blot (Figure 8). The impact of both PM/Scl100 and UPF1 depletion on SLC7A11 and GADD45A mRNA levels was measured by RT-qPCR (Figures 9 and 10).

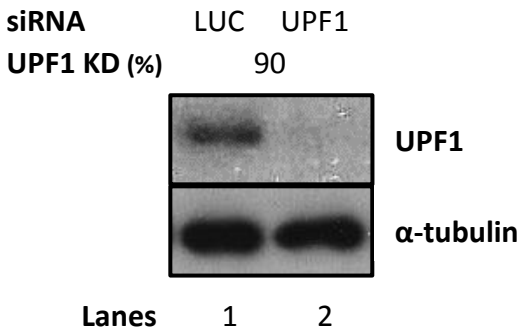


Figure 9. Western blotting analysis of protein samples obtained from HeLa cells treated with either luciferase (LUC; lane 1) or UPF1 (lane 2) siRNAs. Anti-UPF1 and anti-α-tubulin (control) antibodies were used as indicated. An estimative of the percentage of achieved UPF1 knockdown (KD) is indicated.

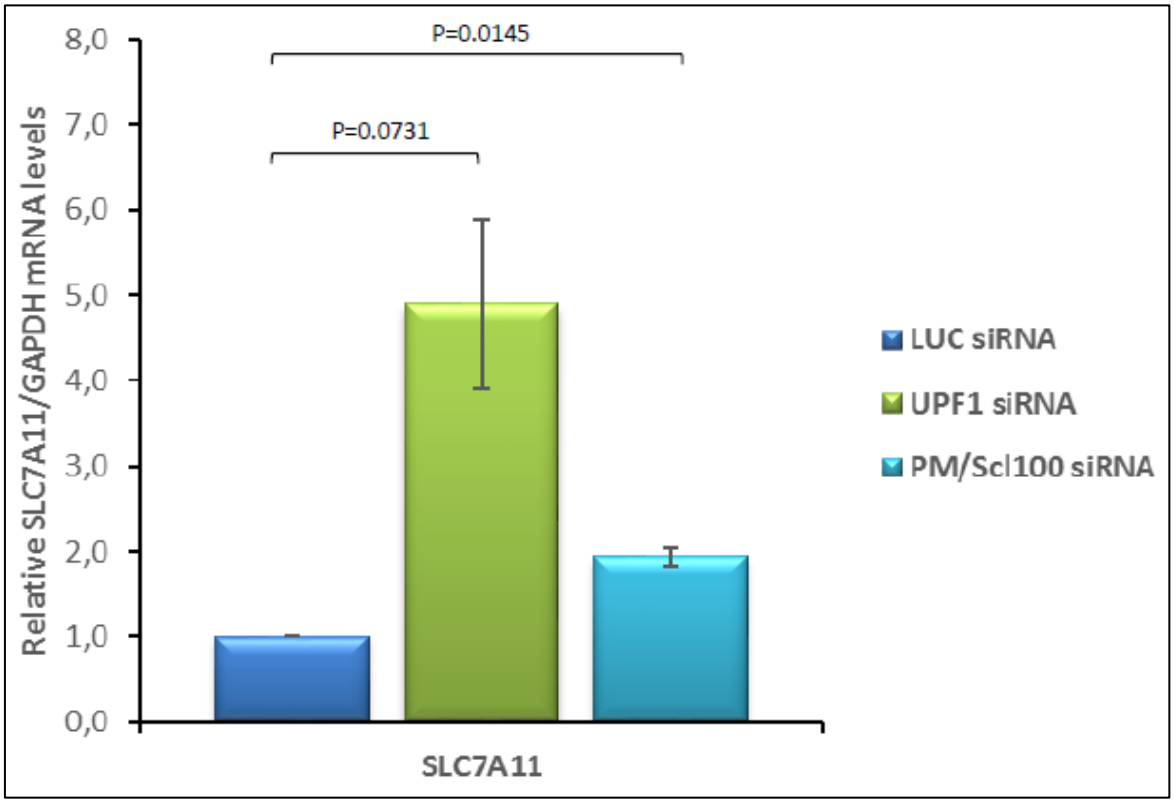


Figure 10. PM/Scl100 knockdown increases SLC7A11 mRNA levels. RT-qPCR analysis was carried out on mRNAs extracted from luciferase (LUC), UPF1 or PM/Scl100 siRNA-treated HeLa cells, **u**sing specific primers for the SLC7A11 and for the GAPDH mRNAs. Quantification was performed by the relative standard curve method. Histogram represents fold-change of each sample relative to the control mRNA levels (SLC7A11 at LUC siRNA-treated cells), arbitrarily set to 1. All values are normalized internally to GAPDH mRNA levels. Standard deviations (n=3) and p-values from Student's t-tests are shown.

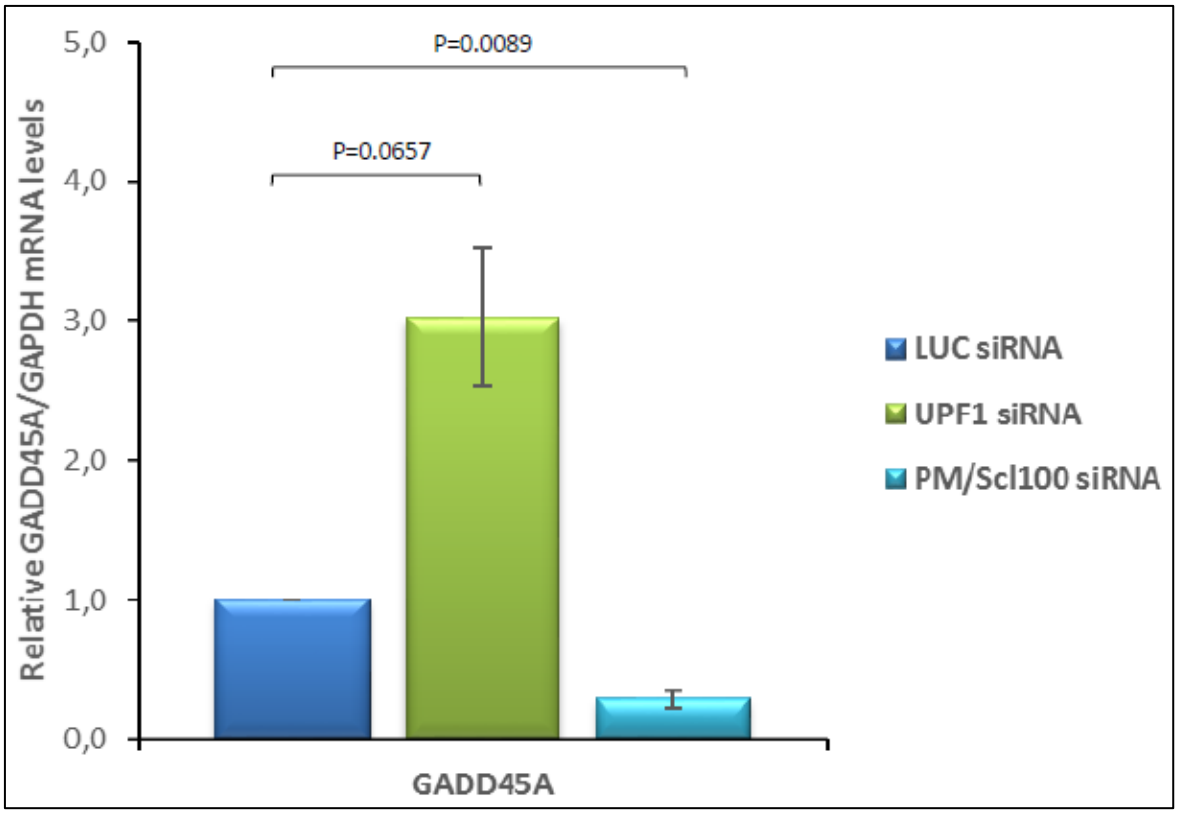


Figure 11. PM/Scl100 knockdown decreases GADD45A mRNA levels. RT-qPCR analysis was carried out on mRNAs extracted from luciferase (LUC), UPF1 or PM/Scl100 siRNA-treated HeLa cells, using specific primers for the GADD45A and for the GAPDH mRNAs. Quantification was performed by the relative standard curve method. Histogram represents fold-change of each sample relative to the control mRNA levels (GADD45A at LUC siRNA-treated cells), arbitrarily set to 1. All values are normalized internally to GAPDH mRNA levels. Standard deviations (n=3) and p-values from Student's t-tests are shown.

As expected for both SLC7A11 and GADD45A mRNAs, when UPF1 is depleted, their levels greatly increase to 490 and 302%, respectively, albeit without statistical significance (p=0.0731 and p=0.0657, respectively). Under conditions of PM/Scl100 knockdown, SLC7A11 mRNA levels almost double, and this increase is statistical significant (p=0.0145), which contributes to corroborate PM/Scl100 involvement in the NMD pathway. However, with respect to GADD45A, its mRNA levels do not follow the same pattern, as they show a dramatic decrease to 29%. Moreover, this decline appears to be statistically significant (p=0.0089).

4.2. The role of SMG6 in mRNA degradation

4.2.1. SMG6 depletion increases both NMD-competent and incompetent human β-globin mRNAs levels, as well as those of the NSD-sensitive β-globin transcript

In order to test if SMG6 ribonucleolytic activity is limited to NMD-eliciting mRNAs, HeLa cells were treated with SMG6 or LUC siRNAs and, 24 hours later, transiently transfected with each one of the plasmids encoding the βWT, β15, β26, β39 or βNS reporter transcripts. Levels of endogenous SMG6 mRNA were monitored by RT-PCR (Figure 11) and the impact of SMG6 depletion on β-globin mRNA levels assessed by RT-qPCR (Figure 12).

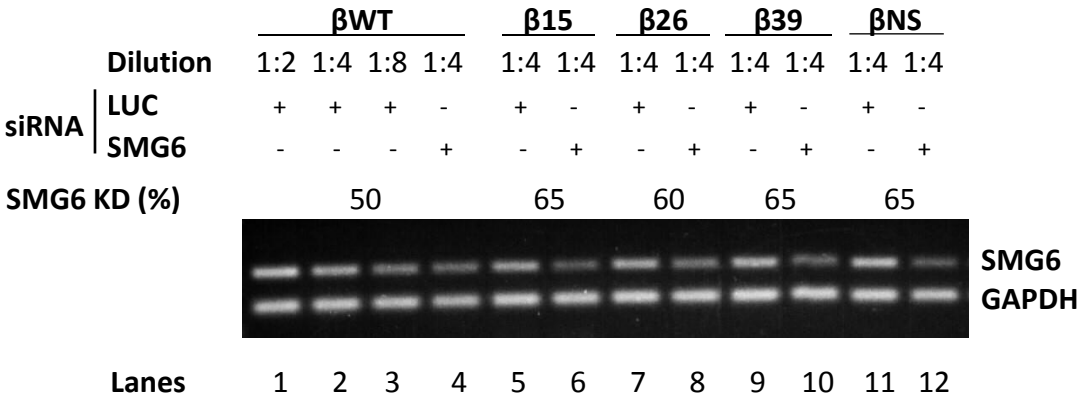


Figure 12. Representative semi-quantitative RT-PCR analysis of mRNAs extracted from luciferase [(+LUC siRNA); lanes 1-3, 5, 7, 9 and 11] or SMG6 [(+SMG6 siRNA); lanes 4, 6, 8, 10 and 12] siRNA-treated HeLa cells transiently transfected with plasmids expressing βWT, β15, β26, β39 or βNS mRNAs. Semi-quantitative PCRs were carried out with human SMG6 mRNA specific primers to evaluate endogenous SMG6 expression or with GAPDH mRNA specific primers as an internal standard. Lanes 1 to 3 correspond to serial dilutions of cDNA, demonstrating semi-quantitative conditions used for RT-PCR. An estimative of the percentage of achieved SMG6 knockdown (KD) for each sample is indicated.

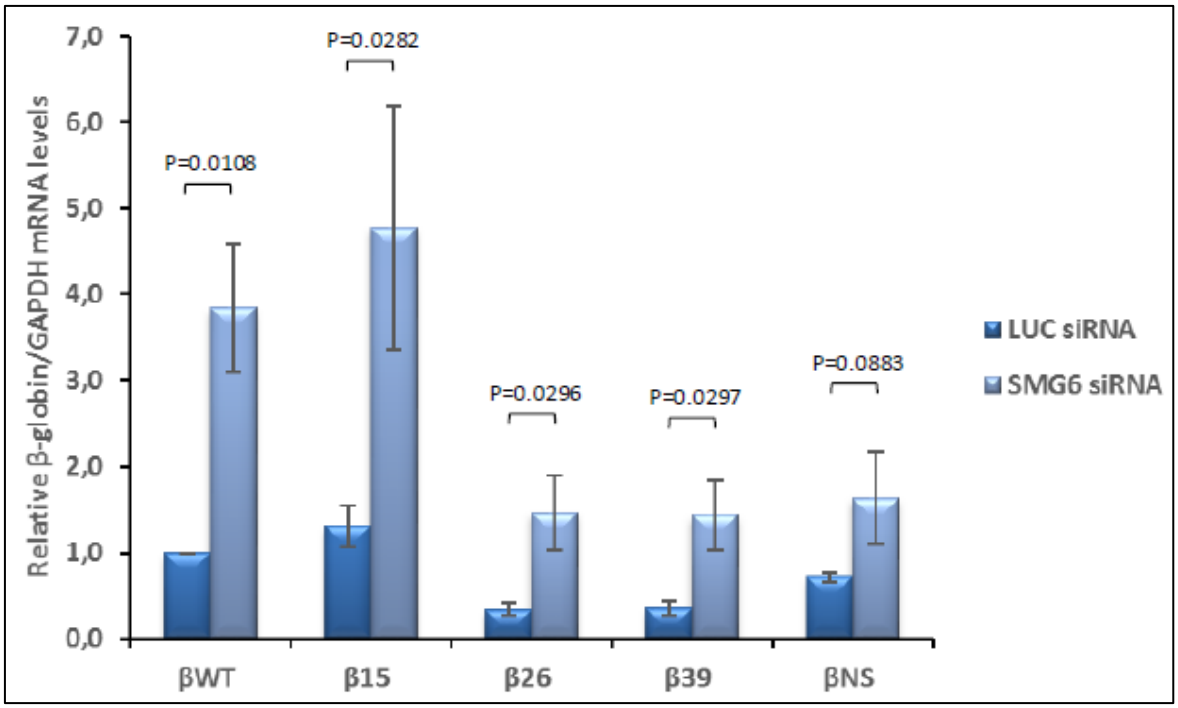


Figure 13. SMG6 depletion increases both NMD-competent and incompetent human β-globin mRNAs levels, as well as those of the NSD-sensitive β-globin transcript. RT-qPCR analysis was carried out on mRNAs extracted from luciferase (LUC) or SMG6 siRNA-treated HeLa cells transiently transfected with plasmids expressing βWT, β15, β26, β39 or βNS mRNAs, using specific primers for the human β-globin and for the GAPDH mRNAs. Quantification was performed by the relative standard curve method. Histogram represents fold-change of each sample relative to the control mRNA levels (βWT at LUC siRNA-treated cells), arbitrarily set to 1. All values are normalized internally to GAPDH mRNA levels. Standard deviations (n=4) and p-values from Student's t-tests are shown.

As expected, under control conditions, β26, β39 and βNS mRNAs are present at lower levels than βWT mRNA – at about 34%, 36% and 72% of the βWT mRNA level, respectively, as they are targeted by NMD (Romão et al., 2000) and NSD (Saito, Hosoda & Hoshino, 2013) degradation pathways. Once again, this decrease in mRNA levels is not observed for the β15 variant, due to this transcript being able to evade NMD (Inácio et al., 2004).

Surprisingly, SMG6 knockdown affects the levels of all analyzed transcripts. Both NMD-insensitive βWT and β15 and NMD-sensitive β26 and β39 mRNA levels are increased, with statistical significance, to about 385%, 478%, 147% and 144%, respectively. Regarding the βNS variant, it follows the same trend - its levels increase from 72 to 164%; however, this increase is not statistically significant (p=0.0883). These findings point to a potential function of SMG6 in other cytoplasmic mRNA degradation pathways, besides NMD.

4.2.2. SMG6 knockdown increases SLC7A11 mRNA levels, but decreases those of GADD45A mRNA

To investigate the generality of SMG6 function in NMD, the expression levels of SLC7A11 and GADD45A mRNAs were measured, under conditions of SMG6 depletion. Its impact on SLC7A11 and GADD45A mRNA levels was evaluated by RT-qPCR (Figures 13 and 14).

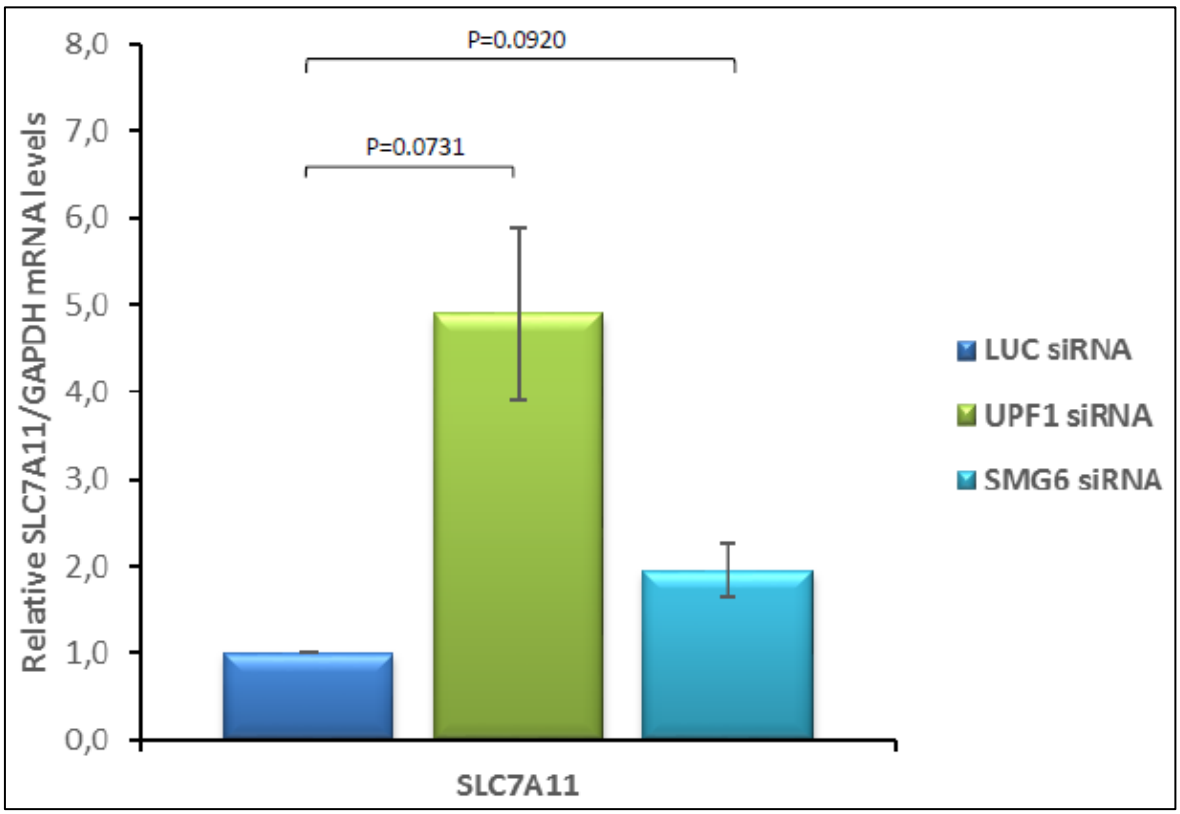


Figure 14. SMG6 knockdown increases SLC7A11 mRNA levels. RT-qPCR analysis was carried out on mRNAs extracted from luciferase (LUC), UPF1 or SMG siRNA-treated HeLa cells, using specific primers for the SLC7A11 and for the GAPDH mRNAs. Quantification was performed by the relative standard curve method. Histogram represents fold-change of each sample relative to the control mRNA levels (SLC7A11 at LUC siRNA-treated cells), arbitrarily set to 1. All values are normalized internally to GAPDH mRNA levels. Standard deviations (n=3) and p-values from Student's t-tests are shown.

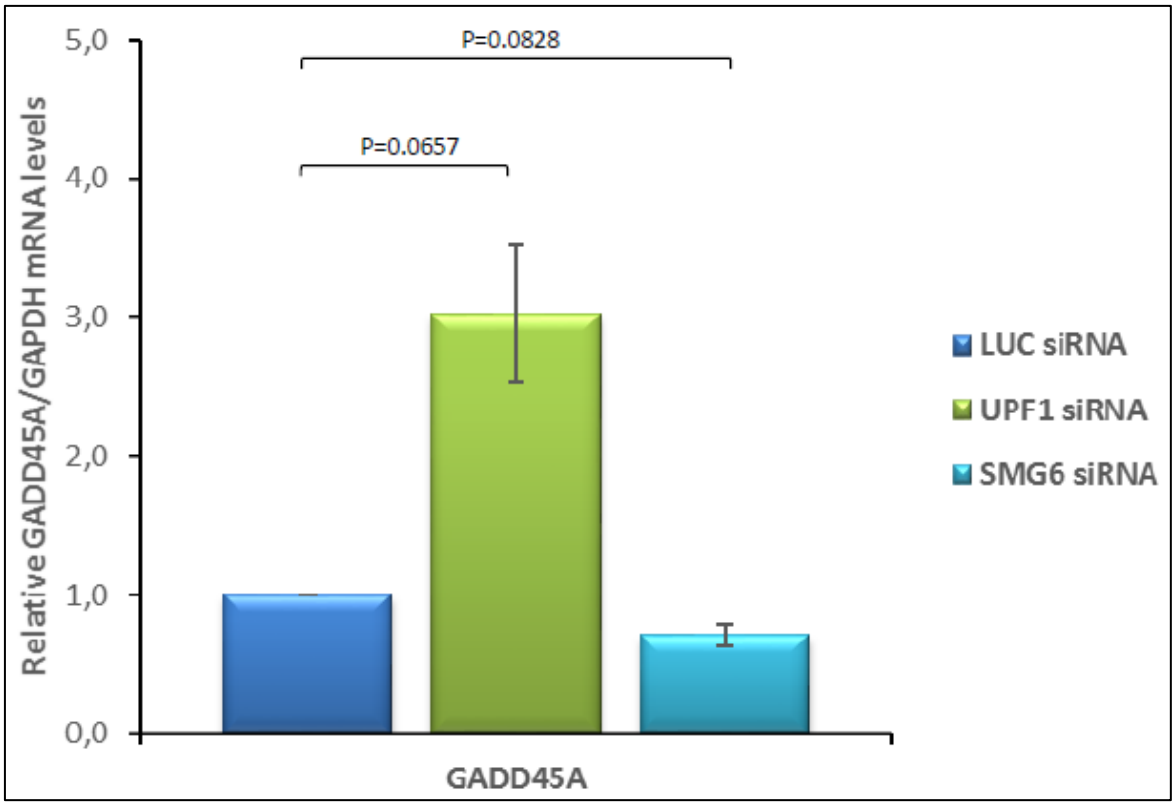


Figure 15. SMG6 knockdown decreases GADD45A mRNA levels. RT-qPCR analysis was carried out on mRNAs extracted from luciferase (LUC), UPF1 or SMG siRNA-treated HeLa cells, using specific primers for the GADD45A and for the GAPDH mRNAs. Quantification was performed by the relative standard curve method. Histogram represents fold-change of each sample relative to the control mRNA levels (GADD45A at LUC siRNA-treated cells), arbitrarily set to 1. All values are normalized internally to GAPDH mRNA levels. Standard deviations (n=3) and p-values from Student's t-tests are shown.

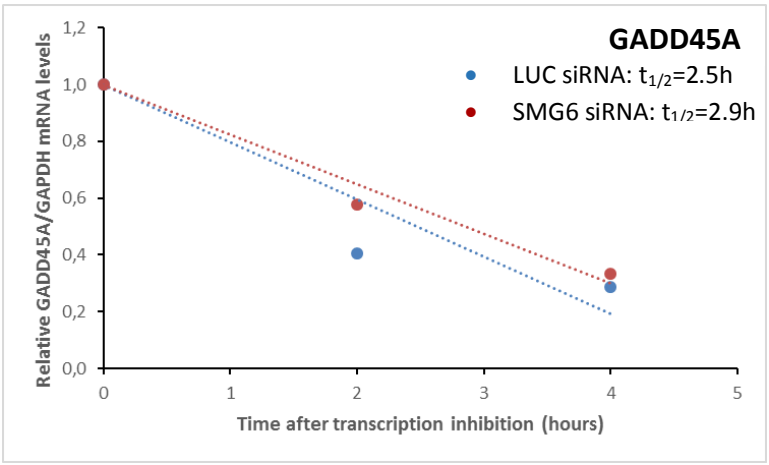
These results demonstrate that SMG6 knockdown induces an increase in SLC7A11 mRNA levels to 195%, pointing to a potential function of SMG6 in the degradation of this NMD target. However, the rise in SLC7A11 mRNA levels is not statistical significant (p=0.0920). On the other hand, GADD45A mRNA levels show a decrease to 71% when SMG6 is depleted. This decline does not present statistical significance (p=0.0828).

4.2.3. SMG6 depletion does not directly affect neither GADD45A nor SLC7A11 mRNAs stability

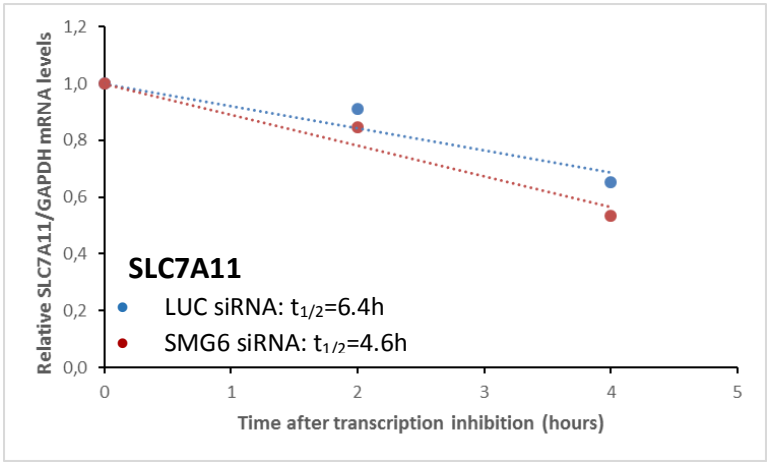
In light of the results presented herein, and as a mean to confirm them, it was decided to test whether SMG6 knockdown is directly affecting the stability of mRNA targets. To accomplish that, the half-lives of βWT and β39 transcripts, as well as those of

GADD45A and SLC7A11 mRNAs, were intended to be compared in LUC and SMG6-depleted HeLa cells. Transcription of these transcripts was inhibited through cell treatment with DRB at a concentration of 60 µM, and total RNA was extracted at different time points after transcription inhibition for further quantification by RT-qPCR.

Regarding the βWT and β39 transcripts, it was not observed any major difference in their levels through time, which was probably due to the DRB concentration used not being sufficient to successfully inhibit transcription. For the GADD45A and SLC7A11 mRNAs, it was possible to establish the corresponding decay rates (Fig. 15 A and B).



A



B

Figure 16. SMG6 depletion does not directly affect neither GADD45A nor SLC7A11 mRNAs stability. RT-qPCR analysis was carried out on mRNAs isolated from HeLa cells transfected with luciferase (LUC) or SMG siRNAs, at various time points, following treatment with 5,6-dichloro-1-3-D-ribofuranosylbenzimidazole (DRB) for transcription inhibition. GADD45A (A) and SLC7A11 (B) mRNA levels were assessed using specific primers for the GADD45A or SLC7A11 mRNAs and for the GAPDH mRNA. Each time point value was expressed as a ratio of target:GAPDH mRNA and normalized to expression levels prior DRB treatment (time 0 = 1.0). Linear regression analysis was performed by standard techniques. The half-lives ($t_{1/2}$) of the mRNAs are indicated. The values presented here correspond to only one experiment (n=1).

As GADD45A mRNA is known to be down-regulated by NMD, it presents a fast decay rate under control conditions, with a half-life of 2.5 hours. In accordance with the results previously obtained, SMG6 knockdown does not seem to majorly affect this value. SLC7A11 mRNA shows a half-life of 6.4 hours, under control conditions, a value that is notably higher than what has already been reported in literature (Martin & Gardner, 2014). Surprisingly, when SMG6 is depleted, SLC7A11 mRNA stability decreases to 4.6 hours. These data point to an indirect involvement of SMG6 in SLC7A11 mRNA degradation.

5. Discussion

The objective of the current work was to investigate the role of the ribonucleases SMG6 and PM/Scl100 in the mechanisms inherent to NMD, NSD or normal mRNA turnover. SMG6 has been implicated only in NMD, being responsible for the endonucleolytic cleavage of transcripts in the vicinity of the PTC. PM/Scl100 is one of the catalytic subunits of the human exosome and preferentially accumulates in the nucleus and nucleolus, but is also present, in lower amounts, in the cytoplasm. The exosome core is highly conserved, but targets of its catalytic subunits remain elusive and no specific exosome subunit has been directly related to surveillance mechanisms.

SMG6 and PM/Scl100 function in the aforementioned mechanisms was explored via RNA interference experiments. HeLa cells were depleted of SMG6, PM/Scl100 or luciferase (as a control) and transiently transfected with a plasmid expressing the human wild-type β-globin gene (βWT) or the same gene carrying a PTC at codon 15, 26, or 39 (β15, β26 or β39 genes, respectively) or lacking any termination codon (βNS). The impact of SMG6 or PM/Scl100 depletion on β-globin mRNA levels was assessed by RT-qPCR. The same impact was also assessed on two endogenous NMD targets, SLC7A11 and GADD45A. In these last two cases, a fourth group of HeLa cells was depleted of UPF1, with the purpose of serving as a positive control for these experiments. Knockdown efficiencies were measured either by semi-quantitative RT-PCR or Western blot. Because mRNA quantification by RT-PCR may not be representative of the actual protein levels in the cell, as a result of the protein having high stability so that it is not required constant translation from the corresponding mRNA, the Western blot technique is more trustworthy to estimate knockdown efficiencies. However, as there were no primary antibodies for SMG6 and PM/Scl100 available in the lab, semi-quantitative RT-PCR was the method chosen to monitor their endogenous levels.

Results obtained from RT-qPCR analysis show that both PM/Scl100 and SMG6 knockdowns correlate with an increase in all of the reporter transcripts. In the case of PM/Scl100 depletion, this increase is statistically significant ($p<0.05$) for all but the β39 mRNA (Figure 7). Moreover, this and the β26 mRNA present the smaller increases (from 30 to 44%, for the β39 mRNA, and from 33 to 71%, for the β26 mRNA), which do not allow

normal mRNA levels to be recovered. Taken together, these data suggest that, while PM/Scl100 plays a major role in the normal turnover of mRNA or in the degradation of transcripts targeted by NSD, the main contribution to NMD-eliciting transcripts 3'→5' degradation may be offered by the other catalytic subunits of the exosome, DIS3 and DIS3L1, and by the exosome-independent ribonuclease DIS3L2. Regarding SMG6 knockdown, the statistical significant increase in both NMD-competent β26 (from 34 to 147%) and β39 (from 36 to 144%) mRNA levels corroborates the involvement of this endoribonuclease in the NMD pathway (Figure 12). A statistical significant increase is also observed for the NMD-insensitive βWT (from 100 to 385%) and β15 (from 131 to 478%) transcripts, which implicate SMG6 in the normal turnover of mRNA. The βNS variant follows the same trend, with its levels increasing from 72 to 164%, albeit with no statistically significance (p=0.0883). Therefore, the potential participation of SMG6 in the NSD pathway requires confirmation through the execution of more experiments. The achievement of higher SMG6 knockdown efficiencies may also contribute to increase even more the βNS mRNA levels.

In order to confirm and generalize PM/Scl100 and SMG6 function in NMD, the expression levels of two physiological transcripts already reported to be targeted by this mechanism – SLC7A11 and GADD45A – were also evaluated. Either PM/Scl100 or SMG6 depletion is associated with an increase in SLC7A11 mRNA levels (to 194 and 195%, respectively) (Figures 9 and 13). However, this change is only statistically significant in the case of PM/Scl100, which contribute to assert the previously obtained results that implicate this exoribonuclease in the NMD pathway. Intervention of SMG6 in the degradation of SLC7A11 mRNA requires confirmation through the realization of more experiments, aiming to reduce the high standard deviation obtained for SLC7A11 mRNA levels when SMG6 is depleted, relative to when PM/Scl100 is depleted. Concerning GADD45A mRNA, its levels decrease to 29 or 71% with the knockdown of either PM/Scl100 or SMG6, respectively, which is an unexpected finding (Figures 10 and 14). However, as the decline observed when SMG6 is depleted does not present statistical significance (p=0.0828), further repetition of these experiments is needed before ruling SMG6 out of the GADD45A degradation mechanism. As for the decline registered when PM/Scl100 is

depleted, besides being much more accentuated than when SMG6 is depleted, it is statistically significant (p=0.0089). The decrease in GADD45A mRNA levels associated with PM/Scl100 knockdown could be caused by indirect effects, for instance, a disruption in the degradation by the exosome of mRNAs encoding other ribonucleases that, in turn, are involved in GADD45A mRNA degradation.

GADD45A mRNA is also reported to be downregulated by NMD, and so the fast decay rate presented by this transcript, which has a half-life of 2.5 hours, is in accordance with what was expected (Figure 15 A). In line with the previous observation that GADD45A mRNA levels did not increase when SMG6 was depleted, this transcript half-life was not majorly altered in conditions of SMG6 knockdown. As for SLC7A11 mRNA, it shows a half-life considerably higher than what has already been described in literature, with a value of 6.4 hours. This may be due to the DRB concentration used not being sufficient to successfully inhibit transcription, similar to what happened with the βWT and β39 variants. Surprisingly, when SMG6 is depleted, SLC7A11 mRNA stability decreases to 4.6 hours. These data suggest that SMG6 is not directly involved in SLC7A11 mRNA degradation. However, before drawing any conclusions, it is imperative to increase the number of experiments, not only to confirm the high SLC7A11 mRNA half-life under control conditions, but also to clarify whether SMG6 is directly or indirectly involved in the degradation of this transcript.

6. Final considerations and future directions

Overall, the findings herein presented point to the involvement of both PM/Scl100 and SMG6 in mRNA surveillance mechanisms, namely NMD and NSD, but also in normal transcript degradation. However, some of the results obtained still require the number of experiments performed to be increased, in order to accomplish statistical significance and effectively allow more credible conclusions to be drawn.

To guarantee that the function of PM/Scl100 and SMG6 in the aforementioned mechanisms is a general attribute observed for different targets and in different tissues, the experiments described could be performed using other model genes, besides the β-globin gene, and other cell lines, besides HeLa. Moreover, to clarify the mixed results obtained from the evaluation of SLC7A11 and GADD45A mRNAs levels when PM/Scl100 or SMG6 are depleted, other endogenous NMD targets should be tested. Examples of these transcripts are the HFE mRNA, whose corresponding gene, when mutated, might be involved in hereditary hemochromatosis, a disease of iron metabolism that is characterized by an excessive intestinal iron absorption, which culminates in organ damage (Martins et al., 2012), and the SMG5 mRNA, whose autoregulatory degradation by NMD ensures homeostatic control of the respective gene (Huang et al., 2011; Yepiskoposyan et al., 2011).

In order to confirm that other 3'→5' ribonucleases, aside from PM/Scl100, are involved in the degradation of NMD-eliciting transcripts, co-depletion of PM/Scl100 and DIS3, DIS3L1 or DIS3L2 could be performed and results compared to those of PM/Scl100 knockdown experiments already presented. Co-depletion experiments of SMG6 and other NMD effectors, such as SMG7, could also contribute to shed some light on the potential competition between the different NMD mechanisms.

Finally, the half-life determination experiments should be repeated, not only for the GADD45A and SLC7A11 mRNAs, but also for the β-globin variants. DRB concentration to be used should be increased, in order to more successfully inhibit transcription and, consequently, obtain results with higher fidelity.

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