Inhibition of nonsense-mediated mRNA decay by the cytoplasmic poly(A)-binding protein

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

2016

vorgelegt von

Tobias Fatscher

aus Heidelberg

Universität zu Köln

Berichterstatter/in: PD Dr. Niels H. Gehring

Prof. Dr. Karin Schnetz

Tag der mündlichen Prüfung: 05. Juli 2016

Parts of this thesis have been published as:

1) Fatscher T, Boehm V, Weiche B, Gehring NH. The interaction of cytoplasmic poly(A)-binding protein with eukaryotic initiation factor 4G suppresses nonsense-mediated mRNA decay. RNA. 2014; 20(10):1579-92.

2) Fatscher T, Boehm V, Gehring NH. Mechanism, factors, and physiological role of nonsense-mediated mRNA decay. Cell Mol Life Sci. 2015; 72(23):4523-44.

Table of Contents

1 Abstract v
2 Deutsche Zusammenfassung vi
3 Introduction 1
3.1 Poly(A)-binding proteins: A protein family regulating mRNA fate 1
3.1.1 More than one PABP is present in human cells 1
3.1.2 PABPC1 structure and interaction partners 2
3.2 EJC-mediated NMD: deposition, factors, and pathway 4
3.2.1 Characteristics of the EJC 4
3.2.2 Central factors involved in NMD and their interplay 5
3.2.3 Phosphorylation of UPF1 regulates NMD activity 6
3.2.4 Exonucleolytic degradation via SMG5/7 7
3.2.5 Endonucleolytic degradation via SMG6 7
3.3 EJC-independent activation of NMD 10
3.3.1 NMD activation due to a long 3′ UTR 10
3.3.2 The *faux* 3′ UTR model in yeast 11
3.4 The link between translation and NMD 11
3.4.1 Translation initiation 11
3.4.2 Proper translation termination 12
3.4.3 Steps in aberrant translation termination and the link to NMD 14
3.4.4 Active translation is necessary for NMD 16
3.5 Additional NMD targets present in the cell 17
3.6 Physiological implications of NMD 19
3.6.1 Importance of NMD factors on cell viability 19
3.6.2 NMD and genetic disorders 19
3.6.3 Treatment of physiological conditions resulting from NMD 20
3.7 Aim of this thesis 21

4 Results 23
4.1 PABPC1 is able to antagonize long 3′ UTR-mediated NMD 23
4.1.1 Increased reporter abundance is a consequence of PABPC1-mediated NMD suppression 24
4.2 Interaction partners of PABPC1 necessary for NMD suppression 27
4.2.1 The interaction between PABPC1 and eRF3a is not required for NMD suppression 27
4.2.2 PABPC1 needs the interaction with eIF4G to antagonize NMD 30
4.3 PABPC1 does not affect EJC-mediated NMD 31
4.4 eRF3a requires the interaction with PABPC1 for NMD suppression 32
4.5 eIF4G antagonizes NMD independently of PABPC1 35
4.6 PABPC1 acts as a general suppressor of long 3′ UTR-mediated NMD 38
4.7 NMD inhibition by PTBP1 and other proteins known to interact with PABPC1 39
4.8 Peptide-mediated fold-back of the poly(A)-tail stabilizes NMD-targeted mRNA 41
4.8.1 eRF3a and eRF1 suppress NMD by folding back the poly(A)-tail 41
4.8.2 Heterodimer formation of synthetic peptides suppresses NMD by folding back the poly(A)-tail 42
4.9 PAM2 motifs are sufficient to antagonize NMD 44
4.9.1 PABPC1 recruitment via PAM2 motifs efficiently antagonizes NMD 44
4.9.2 Tethering PAM2 motifs has no negative effect on translation of the reporter transcript 46
4.9.3 Not all PAM2 motifs show the expected interaction with PABPC1 47
4.9.4 NMD suppression by PAM2 motifs is a specific tethering effect 48
4.9.5 EJC-factor mediated NMD is antagonized by PAM2 motifs 49
4.9.6 PAM2 motifs suppress NMD of an intron-carrying reporter 51
4.10 PABPC1 suppresses NMD of the β-globin PTC39 mRNA 52
5 Discussion 54

5.1 NMD suppression of long 3′ UTR substrates 55
5.1.1 PABPC1 is a general suppressor of long 3′ UTR mediated NMD 55
5.1.2 Implication of translation termination in NMD suppression by PABPC1 55
5.1.3 Protein-mediated NMD inhibition 58
5.1.4 Peptide-mediated fold-back of the poly(A)-tail 59
5.1.5 PAM2 motifs strongly suppress long 3′ UTR-triggered NMD 60
5.2 EJC-activated NMD is antagonized by PAM2 peptides 62
5.3 Medical implications of NMD inhibition and potential therapeutic approaches 64
5.4 Concluding remarks 66
6 Material and Methods 67
6.1 Molecular biology 67
6.1.1 Mammalian expression plasmids 67
6.2 Molecular cloning 67
6.2.1 Transformation 67
6.2.2 Plasmid preparation 68
6.3 Cell culture 68
6.3.1 Cell lines 68
6.3.2 Plasmid DNA transfection 68
6.4 Immunoprecipitation 69
6.4.1 Transfection of HTO cells 69
6.4.2 Preparation of cell lysates 69
6.4.3 Co-immunoprecipitation 69
6.4.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 70
6.4.5 Western blot analysis 70
6.5 Northern blot analysis 71
6.5.1 Transfection of HTO cells 71
6.5.2 Harvesting cells 71

6.5.3 RNA extraction 71
6.5.4 Northern blotting 72
6.5.5 Protein extraction 73
7 Appendix 75
7.1 List of Antibodies 75
7.2 List of Primers 75
8 References 77
9 Acknowledgement 89
10 Erklärung 90

1 Abstract

The expression of a gene from transcription of the DNA into pre-messenger RNA (pre-mRNA) over translation of messenger RNA (mRNA) into protein is constantly monitored for errors. This quality control is necessary to guarantee successful gene expression. One quality control mechanism important to this thesis is called nonsense-mediated mRNA decay (NMD). NMD is a cellular process that eliminates mRNA transcripts harboring premature translation termination codons (PTCs). Furthermore, NMD is known to regulate certain transcripts with long 3′ UTRs. However, some mRNA transcripts are known to evade NMD. The mechanism of NMD activation has been subjected to many studies whereas NMD evasion or suppression still remains rather elusive. It has previously been shown that the cytoplasmic poly(A)-binding protein (PABPC1) is able to suppress NMD of certain transcripts. In this study I show that PABPC1 is able to suppress NMD of a long 3′ UTR-carrying reporter when tethered immediately downstream of the termination codon. I further am able to show the importance of the interaction between PABPC1 and eIF4G for NMD suppression, whereas the interaction between PABPC1 and eRF3a seems dispensable. These results indicate an involvement of efficient translation termination and potentially ribosome recycling in NMD suppression. I am able to show that if PABPC1 is too far removed from the terminating ribosome NMD is activated. After showing the importance of PABPC1 recruitment directly downstream of a terminating ribosome in NMD suppression, I am further able to demonstrate several different methods by which PABPC1 can be recruited. Fold-back of the poly(A)-tail mediated by two interacting proteins on opposite ends of a 3′ UTR manages to bring PABPC1 bound to the poly(A)-tail into close proximity of the terminating ribosome and therefore suppress NMD. Furthermore, small PAM2 peptides that are known to interact with the MLLE domain of PABPC1 are able to strongly suppress NMD initiated by either a long 3′ UTR or an EJC. I am also able to show the NMD antagonizing power of recruited PABPC1 for the known endogenous NMD target β-globin PTC39, which is responsible for the disease β-thalassemia. This shows the potential medical implications and application of suppressing NMD by recruiting PABPC1 into close proximity of a terminating ribosome.

2 Deutsche Zusammenfassung

Die Expression eines Gens angefangen mit der Transkription der DNA in prä-Messenger RNA (prä-mRNA) über Translation von Messenger RNA (mRNA) in ein Protein ist ein stark überwachter Prozess. Diese Qualitätskontrolle ist notwendig, um fehlerfreie Genexpression zu gewährleisten. Ein wichtiger Mechanismus zur Qualitätssicherung ist Nonsense-mediated mRNA decay (NMD) genannt. NMD ist ein zellulärer Prozess, der mRNA-Transkripte die ein vorzeitiges Stoppcodon (PTC) beherbergen eliminiert. Weiterhin ist bekannt, dass NMD bestimmte Transkripte mit langen 3′ UTRs regulieren kann. Allerdings erkennt NMD nicht alle Transkripte und es sind einige mRNA-Transkripte bekannt die sich NMD entziehen. Der Mechanismus der NMD-Aktivierung wurde bereits vielen Studien unterzogen, während der Mechanismus der NMD Umgehung oder Unterdrückung noch relativ unerforscht ist. Es wurde zuvor schon gezeigt, dass das zytoplasmatische Poly(A)-bindende Protein (PABPC1) imstande ist NMD bestimmter Transkripte zu unterdrücken. In dieser Studie zeige ich, dass PABPC1 in der Lage ist, NMD eines langen 3′ UTR-tragenden Reporters zu unterdrücken, wenn es unmittelbar nach dem Terminationscodon gebunden ist. Ich bin weiter in der Lage, die Bedeutung der Interaktion zwischen PABPC1 und eIF4G in der NMD Unterdrückung zu zeigen, während die Interaktion zwischen PABPC1 und eRF3a hierfür entbehrlich erscheint. Diese Ergebnisse deuten auf eine Beteiligung von effizienter Translationstermination und Ribosom-Recycling in der Unterdrückung von NMD hin. Nachdem ich die Bedeutung der Rekrutierung von PABPC1 direkt hinter einem Terminationscodon in der Unterdrückung von NMD zeigen konnte, war es mir weiterhin möglich verschiedene Methoden zu zeigen, mit denen PABPC1 rekrutiert werden kann. Das Zurückfalten des Poly(A)-Schwanzes durch zwei interagierende Proteine an gegenüberliegenden Enden einer 3′ UTR ermöglicht es PABPC1, welches am Poly(A)-Schwanz gebunden ist, in die Nähe des terminierenden Ribosoms zu bringen und somit NMD zu unterdrücken. Weiterhin zeige ich, dass kleine PAM2 Peptide, die mit der MLLE Domäne von PABPC1 interagieren, in der Lage sind NMD aktiviert durch entweder einer lange 3′ UTR oder eines EJC zu unterdrücken. Die PAM2 Peptide unterdrücken NMD durch die Rekrutierung von PABPC1 in die direkte Nähe des terminierenden Ribosoms. Außerdem bin ich in der Lage NMD des endogenen Transkripts β-Globin PTC39, welches für die Krankheit β-Thalassämie verantwortlich ist, durch die Rekrutierung von PABPC1

zu unterdrücken. Dies zeigt die potentiellen medizinischen Auswirkungen und mögliche Anwendung der NMD Unterdrückung durch PABPC1 auf.

3 Introduction

Eukaryotic gene expression is a complex and highly regulated process. Messenger RNAs (mRNAs) are transcribed from DNA in the nucleus and serve as blueprints for protein synthesis before being degraded. Synthesis of mRNA starts in the nucleus in the form of a pre-mRNA (Moore, 2005; Moore and Proudfoot, 2009). Transcription from DNA to pre-mRNA is undertaken by RNA polymerase II (Pol II). Pre-mRNAs are subjected to co-transcriptional 5′ end capping, splicing, 3′ end cleavage, and polyadenylation before the processed mRNA is exported to the cytoplasm. In the cytoplasm the mature mRNAs are translated into proteins by ribosomes and are eventually degraded. To ensure error-free gene expression, several quality control mechanisms exist in the living cell. One major quality control mechanism is called nonsense-mediated mRNA decay (NMD). NMD is responsible for recognizing and degrading mRNA transcripts with premature termination codons (PTCs) or other aberrant translation termination events. PTCs are known to arise through different mechanisms such as alternative splicing events, transcription errors, or due to mutations on the DNA level (Nicholson et al., 2010). Without NMD the aberrant transcripts could lead to the production of C-terminally truncated proteins with potential dominant-negative effects on the cell. In addition to the quality control function, NMD also regulates many so-called endogenous NMD targets. Thus, NMD is not only responsible for mRNA surveillance but also influences general gene expression.

3.1 Poly(A)-binding proteins: A protein family regulating mRNA fate

Poly(A)-binding proteins (PABPs) are a set of highly conserved proteins among eukaryotes. PABPs are RNA-binding proteins, specifically recognizing the poly(A)-tail of virtually all mRNAs in the cell. PABPs do not feature any catalytic or enzymatic activity themselves. However, they serve as interaction partner to a multitude of other proteins, which highlights the role of PABPs in many different cellular events such as translation, mRNA stability, or quality control.

3.1.1 More than one PABP is present in human cells

Metazoan cells express more than one PABP. Whereas there is more than one cytoplasmic PABP (PABPC), metazoan cells encode a single nuclear PABP (PABPN).

PABPCs and PABPN only share the ability to bind poly(A)-tails of RNAs but have no other common functions and they do not show any structural similarities. The most well researched PABPC is called cytoplasmic poly(A)-binding protein 1 (PABPC1) (Kuhn and Wahle, 2004). Even though PABPC1 is mostly localized in the cytoplasm, it was also found to be able to shuttle into the nucleus. PABPN1 is known to bind to the poly(A)-tail of mRNAs in the nucleus and is required for mRNA export into the cytoplasm (Apponi et al., 2010). Since both PABPC1 and PABPN1 are known to shuttle between the nucleus and the cytoplasm it is difficult to determine the exact time point when PABPC1 replaces PABPN1 on mRNA transcripts. Mammalian cells encode three additional PABPs besides PABPC1 and PABPN1. Two of these PABPs are restricted to the germ line and are called testis-specific PABP (tPABP, PABPC2/PABPC3) and embryonic PABP (ePABP, ePAB). The third PABP is termed PABP4 or inducible PABP (iPABP). These three PABPs share the same domain structure with PABPC1. An additional PABP termed PABPC5 or PABP5 is also found in mammals. However, the function of PABP5 has not been addressed and remains subject of further research (Gray et al., 2015; Kuhn and Wahle, 2004).

3.1.2 PABPC1 structure and interaction partners

PABPC1 is a 636 amino acid long protein with a molecular weight of about 70kDa. The N-terminal region of PABPC1 is comprised of four RNA recognition motifs (RRMs) that are connected by short linker regions. PABPC1 also harbors a C-terminal region connected to the N-terminal region by an unstructured linker region that is proline- and glutamine-rich. PABPC1 has a high binding affinity for poly(A), however it does exhibit a lower binding affinity for poly(U) and poly(G) as well (Kuhn and Wahle, 2004). PABPC1 requires at least 12 adenosines for a high affinity interaction and covers about 25 nucleotides on a saturated stretch of poly(A) (Kuhn and Wahle, 2004). The interaction between PABPC1 and the poly(A)-tail is mediated via the RRMs 1 and 2 of PABPC1 (Deo et al., 1999). The major roles of PABPC1 are in mRNA translation and stability. An additional important characteristic of PABPC1, which is of interest in this work, lies in its ability to affect the nonsense-mediated mRNA decay pathway.

PABPC1 can interact with a multitude of other proteins that can influence mRNA fate. The interaction with other proteins is mediated either by some of the four different RRMs or by the C-terminal region. One such important interaction partner of PABPC1 that binds to the RRMs is eIF4G. eIF4G is part of the eIF4F complex and plays a vital role in mRNA circularization and translation initiation. It has been shown that eIF4G binds to

RRM2 of PABPC1 and this interaction is also believed to enhance the binding affinity of PABPC1 for the poly(A)-tail (Kuhn and Wahle, 2004; Safaee et al., 2012).

Another important interaction partner of PABPC1 is eRF3a, which plays a role in translation termination. eRF3a is known to interact with PABPC1 via a so called PABP-interacting motif 2 (PAM2 motif). The C-terminal region of PABPC1 harbors a specific stretch termed *Mademoiselle* (MLLE) domain, which has been shown to specifically recognize PAM2 motifs of other proteins (Kozlov et al., 2004). The PAM2 motifs comprise only a stretch of about 12 amino acids. Essential amino acids in the PAM2 motif for the interaction with the MLLE domain of PABPC1 are known to be EFxP. These three amino acids are mostly conserved among PAM2 motifs originating from different PABPC1-interacting proteins (Kozlov and Gehring, 2010). The PAM2 motif was first discovered in PABP-interacting protein 1 (Paip1), Paip2, and eRF3a (Kozlov et al., 2001). After the initial discovery of PAM2-containing proteins, many more proteins harboring PAM2 have been found (Albrecht and Lengauer, 2004; Kozlov et al., 2001). Interestingly, even though PAM2-containing proteins interact with PABPC1 in the same way, they play roles in different cellular processes. This also explains why PABPC1, even though not a catalytically active protein itself, can influence so many different aspects of an mRNA. The interaction between PABPC1 and eRF3a is very important as it has been shown to play a role in polypeptide release and ribosome recycling (Hoshino et al., 1999; Kozlov and Gehring, 2010; Uchida et al., 2002). In contrast, other PAM2-containing proteins like PAN3 and TOB1 are known to play a role in deadenylation. PABPC1 is known to recruit the PAN2-PAN3 deadenylase complex directly by interacting with the PAM2-harboring protein PAN3 (Siddiqui et al., 2007). The other major deadenylase complex, CCR4-NOT, can also be recruited by PABPC1. However, this interaction is not mediated directly by PABPC1. The CCR4-NOT complex is recruited to the mRNA through the interaction between TOB1 and PABPC1 via the PAM2 motif and MLLE domain, respectively (Ezzeddine et al., 2007). This shows that PABPC1 can interact with proteins that influence the mRNA fate by, for example, either stabilizing the mRNA or by promoting deadenylation. Furthermore, the PABPC1-interacting protein PAIP2 is a known repressor of translation by interfering with the ability of PABPC1 to interact with the poly(A)-tail (Khaleghpour et al., 2001). It is interesting to note that PABPC1 can interact with a multitude of PAM2-containing proteins, although with only one at a time, which can give rise to potentially contradictory functions. The determinant for an interaction

preference between PABPC1 and the many PAM2-containing proteins is currently unknown.

In summary, PABPC1 is able to interact with many different proteins and plays an important role in multiple cellular processes. It does not influence the fate of an mRNA with direct catalytic activity but does so by acting as a scaffolding protein. Interestingly, PABPC1 can interact with proteins both favoring mRNA stability as well as proteins that will eventually lead to the degradation of the mRNA.

3.2 EJC-mediated NMD: deposition, factors, and pathway

3.2.1 Characteristics of the EJC

Pre-mRNAs undergo several co-transcriptional processing steps in the nucleus. One such processing step important for NMD is splicing. During splicing the introns of a pre-mRNA are excised and the exons are subsequently joined. Furthermore, an exon-junction complex (EJC) is deposited 20 to 24 nucleotides upstream of the splice site. NMD targets mRNAs that harbor a PTC 50 to 55 nucleotides upstream of the final exon-exon junction and fails to do so for many mRNAs carrying a stop codon downstream of this critical border (Nagy and Maquat, 1998; Thermann et al., 1998; Zhang et al., 1998a; Zhang et al., 1998b). The core EJC complex consists of four proteins: the heterodimer MAGOH/Y14, eIF4A3, and Barentsz (BTZ). The EJC remains bound to the mature mRNA during export into the cytoplasm until it is displaced by a translating ribosome (Dostie and Dreyfuss, 2002; Le Hir et al., 2000; Lejeune et al., 2002). Although a translating ribosome is sufficient to remove an EJC from an mRNA, an additional protein named PYM is known to aid in the displacement of the EJC from the mRNA (Gehring et al., 2009). The EJC binds RNA not by recognizing a specific RNA sequence, but does so via the eIF4A3 component of the EJC. eIF4A3 is an ATPase known to strongly bind RNA in an ATP-dependent manner. The reason why the EJC is able to stay on the mRNA during export as well as thereafter, is due to the partial inhibition of the ATPase activity of eIF4A3. This is achieved by the heterodimer Y14 and MAGOH that are able to lock the EJC onto the mRNA by stabilizing a closed formation of eIF4A3 (Andersen et al., 2006; Ballut et al., 2005; Bono et al., 2006; Mishler et al., 2008). Besides the four core EJC components mentioned above, there are several other known EJC factors that are not part of this core complex. One of these so called peripheral EJC components is the RNA-binding protein with serine rich domain 1 (RNPS1). RNPS1 is known to function as a

general co-activator of splicing and is also able to communicate the position of the EJC on the mRNA to other factors of the NMD machinery, which are discussed in more detail later (Le Hir et al., 2000; Lykke-Andersen et al., 2001; Mayeda et al., 1999). Furthermore, the EJC serves as a binding platform for a multitude of other proteins involved in the NMD pathway (Chang et al., 2007).

3.2.2 Central factors involved in NMD and their interplay

There are many factors known to be involved in NMD with more factors most likely still unknown. Of all the factors involved in NMD arguably the most important one is the central NMD factor UPF1 (up-frameshift 1), which is involved in the pivotal stages of the NMD mechanism (Chang et al., 2007). UPF1 was first discovered in yeast more than 30 years ago alongside UPF2 and UPF3 (Cui et al., 1995; Leeds et al., 1991; Leeds et al., 1992). UPF1 has several different functions. It harbors a helicase domain which is able to unwind double stranded nucleic acids in a 5′ - 3′ directionality with the help of ATP hydrolysis. It has been shown that the helicase activity as well as the ability of UPF1 to bind RNA is essential for NMD (Mendell et al., 2002; Weng et al., 1996a, b). The ability of UPF1 to bind RNA directly is, according to the current model, necessary for the assembly of other NMD factors on UPF1. The RNA-binding ability of UPF1 is also mediated by its helicase domain (Bhattacharya et al., 2000; Cheng et al., 2007; Fairman-Williams et al., 2010; Singleton et al., 2007). The first model of UPF1 recruitment to the NMD targeted mRNA proposed that the interaction between UPF1 and the release factor eRF3 (will be discussed in more detail later) at the terminating ribosome were responsible for this recruitment. This suggested that UPF1 recruitment is directly linked to active translation and that UPF1 is bound exclusively in close proximity of a termination codon. The complex formed by this interaction involved an additional protein called SMG1 (discussed in more detail later) and the resulting complex was termed SURF-complex. (Kashima et al., 2006). However, it was more recently shown that UPF1 is able to bind over the whole 3′ UTR of an mRNA. UPF1 was additionally shown to also bind to other regions than the 3′ UTR of an mRNA, but the scanning and translating ribosome is able to displace UPF1. Displaced UPF1 is believed to increasingly bind to the 3′ UTR of a transcript as the ribosome stalls at the termination codon upstream of the 3′ UTR, unable to displace any UPF1 further downstream. This model further proposes that UPF1 occupancy of the 3′ UTR is responsible for NMD activation and not the direct

recruitment of UPF1 via the release factors eRF1 and eRF3 (Hurt et al., 2013; Kurosaki and Maquat, 2013; Zund et al., 2013).

Additional to UPF1, there are two more UPF proteins termed UPF2 and UPF3. Together these proteins form the central set of NMD factors (Cui et al., 1995). An important role of UPF2 is to regulate the helicase activity of UPF1. UPF2 is able to do so by directly interacting with UPF1, causing conformational changes in UPF1, which results in the activation of the UPF1 helicase activity (Chakrabarti et al., 2011; Chamieh et al., 2008). UPF2 is further able to directly interact with UPF3, therefore providing a physical link between UPF1 and UPF3 (Chamieh et al., 2008). It is worth mentioning that higher eukaryotes contain two paralogs of UPF3, namely UPF3a and UPF3b (Lykke-Andersen et al., 2000; Serin et al., 2001). Besides both paralogs displaying some cross-regulation, UPF3b has been shown to be the dominant of the two paralogs (Kunz et al., 2006). UPF3b is able to directly interact with the EJC via a so called EJC-binding motif (EBM) in its C-terminal region (Buchwald et al., 2010; Chamieh et al., 2008; Gehring et al., 2003). This interaction provides a molecular link between UPF1 and the EJC complex and was proposed to play an important role during NMD (Chamieh et al., 2008). However, it has also been shown that UPF3b plays a role in EJC-independent NMD (Metze et al., 2013). As a result, the exact function of UPF3b in NMD remains subject of further research.

3.2.3 Phosphorylation of UPF1 regulates NMD activity

Besides the UPF proteins, the NMD machinery consists of several SMG (suppressor with morphogenetic effect on genitalia) proteins. In humans, the proteins SMG1 and SMG5-7 have been shown to be part of the NMD core machinery (Denning et al., 2001; Ohnishi et al., 2003; Yamashita et al., 2001). Phosphorylation of UPF1 is known to be catalyzed by SMG1, a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family (Denning et al., 2001; Grimson et al., 2004; Yamashita et al., 2001). SMG1 is able to phosphorylate SQ and TQ motifs in the N- and C-terminal regions of UPF1 (Chakrabarti et al., 2014; Page et al., 1999; Yamashita et al., 2001). These phosphorylation sites of UPF1 act as binding platforms for other SMG proteins in the NMD pathway (Fukuhara et al., 2005). Specifically, SMG6 and SMG5/7 are able to interact with phosphorylated UPF1. Furthermore, it is important to note that SMG5 and SMG7 form a heterodimer and SMG5 alone is unable to interact with phosphorylated UPF1. SMG5 is therefore believed to offer additional binding strength between SMG7 and phosphorylated UPF1 (Chakrabarti et al., 2014; Jonas et al., 2013; Obsil and Obsilova, 2011; Okada-Katsuhata

et al., 2012). The SMG proteins are known to activate two different pathways of mRNA degradation: exonucleolytic degradation via SMG5/7 and endonucleolytic degradation via SMG6 (Nicholson and Muhlemann, 2010).

3.2.4 Exonucleolytic degradation via SMG5/7

It has previously been shown that SMG7 is able to position-independently activate degradation of the NMD-targeted mRNA via XRN1 and DCP2 (Unterholzner and Izaurralde, 2004). XRN1 is a known 5′ - 3′ exoribonuclease and DCP2 is part of the decapping complex responsible for the removal of the 5′ cap structure before mRNA degradation (Ghosh and Jacobson, 2010). SMG7 is unable to initiate exonucleolytic degradation by directly recruiting XRN1 or DCP2. Instead, SMG7 interacts with POP2, the catalytic subunit of the CCR4-NOT complex, which is part of the deadenylation machinery in the cell (Loh et al., 2013). This recruitment of the CCR4-NOT complex via SMG7 initiates deadenylation of the transcript, followed by the recruitment of XRN1 and DCP2 as a result of deadenylation. This provides a link between UPF1 and phosphorylation-mediated activation of the SMG5/7-initiated exonuceolytic decay pathway. Furthermore, since SMG5/7 is known to interact and recruit part of the deadenlyation machinery, it is also feasible that the mRNA targeted by NMD can be degraded not only via XRN1-mediated 5′ - 3′ degradation but also in a 3′ - 5′ direction via the exosome.

3.2.5 Endonucleolytic degradation via SMG6

An additional degradation pathway of NMD targets was found when knockdown of the exonucleolytic machinery failed to stabilize NMD-targeted reporter mRNA levels in flies. At the same time, PTC-mediated endonucleolytic cleavage was found to occur on these targets (Gatfield and Izaurralde, 2004). It was then found that SMG6 is responsible for cleaving the target in close proximity of the PTC via its catalytically active PIN domain leading to degradation of the NMD target (Eberle et al., 2009; Gatfield and Izaurralde, 2004; Glavan et al., 2006; Huntzinger et al., 2008). Similar to the heterodimer SMG5/7, SMG6 is recruited via a phospho-dependent interaction with UPF1. Additionally, SMG6 can also interact with UPF1 in a phospho-independent manner (Chakrabarti et al., 2014; Okada-Katsuhata et al., 2012).

Since NMD exhibits two different degradation pathways, it is of interest to examine the interplay between the two pathways. Recent information gathered through high-

throughput sequencing experiments suggests that the SMG6-mediated endocleavage pathway is the major of the two NMD degradation pathways (Lykke-Andersen et al., 2014; Schmidt et al., 2015). Even though SMG6-mediated degradation is most likely the more prominent of the two NMD degradation pathways, there seems to be a connection between SMG6- and SMG5/7-mediated degradation. It has been shown that knocking down either SMG6 or SMG5/7 alone is not enough to sufficiently inhibit NMD. Instead, both pathways need to be impaired simultaneously to achieve complete NMD inhibition (Jonas et al., 2013). These findings seem to indicate that inactivating one NMD pathway will result in the upregulation of the other, thereby ensuring effective NMD even when one pathway is compromised. It is still unknown, however, whether or not both pathways are able to act independently, or are able to affect each other either directly or indirectly through other proteins. For an activation overview of both degradation pathways see Figure 1.

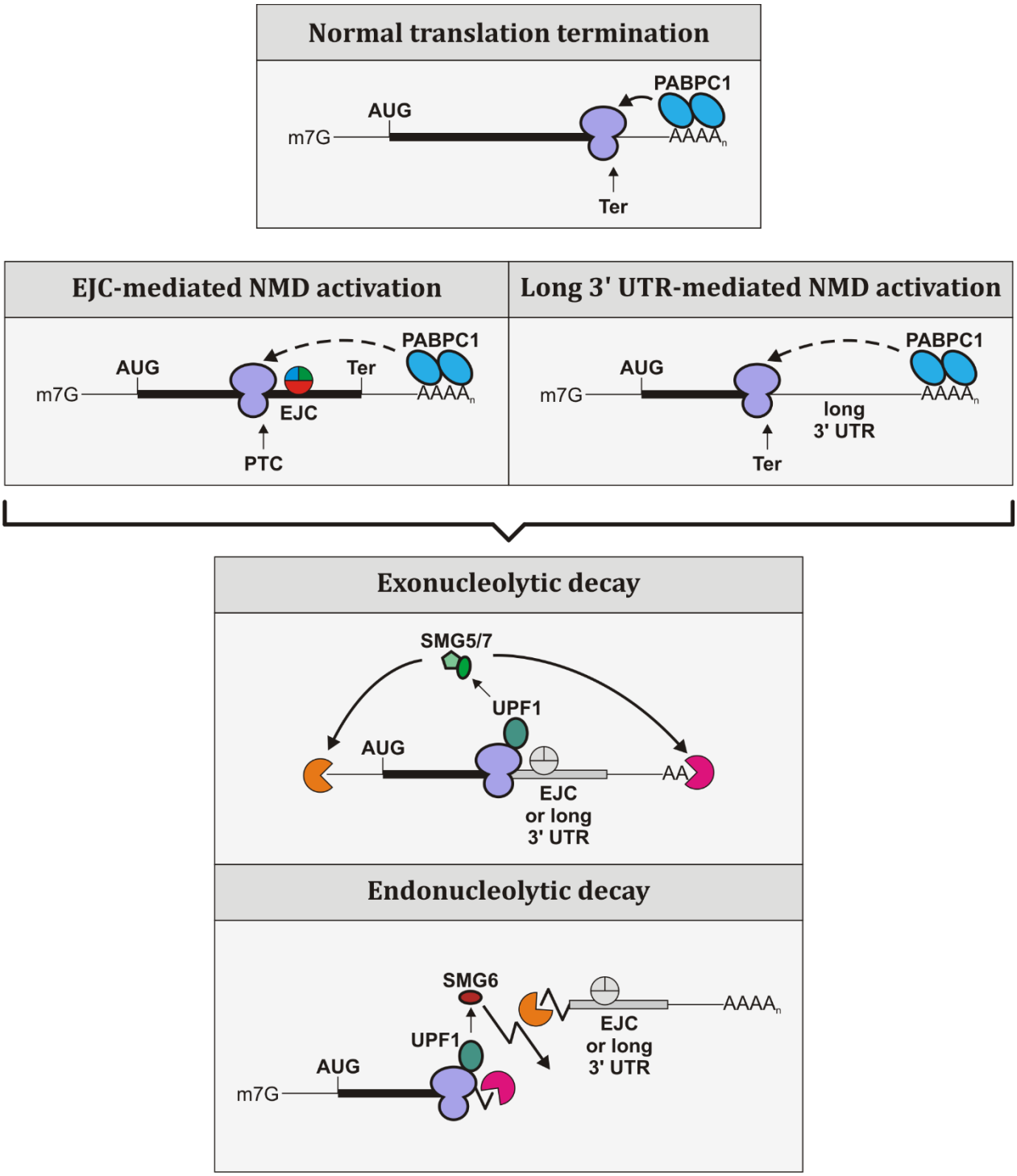

Figure 1: NMD is able to activate two different degradation pathways: exonucleolytic and endonucleolytic.

(Top) During normal translation termination PABPC1 interacts with ribosome-bound proteins to initiate translation termination. **(Middle)** During EJC- or long 3′-UTR-mediated NMD activation the terminating ribosome is too far removed from poly(A)-bound PABPC1, which results in inefficient translation termination and ultimately NMD activation. **(Bottom)** Both exonucleolytic and endonucleolytic decay pathways are activated by UPF1 interacting with the terminating ribosome, followed by SMG5/7- or SMG6-mediated degradation.

3.3 EJC-independent activation of NMD

Critical criteria for NMD activation due to an EJC downstream of a PTC have been discussed in the previous sections. However, adding to the complexity of the NMD mechanism in general is the fact that NMD can also be activated without an EJC downstream of a termination codon. It has been shown that a reporter carrying an intron downstream of a PTC was degraded via NMD even after knocking down the core EJC component eIF4A3. A second experiment in the same study showed that a reporter without an intron downstream of a PTC was unaffected by an eIF4A3 knockdown and still degraded via NMD (Buhler et al., 2006). These results showed that NMD can in fact be activated without an EJC downstream of a stop codon.

3.3.1 NMD activation due to a long 3′ UTR

It has been proposed that the length of a 3′ UTR can be responsible for activation of NMD independently of an EJC downstream of the termination codon (Singh et al., 2008). Complementing these results are a set of reporter constructs harboring the 3′ UTRs of SMG5, UPF3b, or the heterologous GFP coding sequence as 3′ UTRs in separate triosephosphate isomerase (TPI) reporters for which active degradation via NMD has been shown (Boehm et al., 2014). The fact that the 3′ UTRs of the NMD factors SMG5 and UPF3b can elicit NMD indicates a self-regulatory mechanism of NMD. Furthermore, taking into account that a rather arbitrary sequence, in this case the heterologous GFP coding sequence, is actively targeted by NMD suggests that the mere length of a 3′ UTR is the determining factor for whether or not NMD is activated in the absence of an EJC downstream of a termination codon. In contrast, another study has recently shown that not all long 3′ UTRs are actually targeted for degradation via NMD (Toma et al., 2015). This study showed that several reporters contained certain NMD-inhibiting *cis* elements located within the first 200 nucleotides of the 3′ UTR. These *cis* elements were shown to be A/U rich and were able to inhibit NMD in a positional dependent manner. This was made evident by the fact that these 200 nucleotide A/U rich elements were able to suppress NMD when inserted directly downstream of a termination codon of otherwise NMD targeted reporters. Furthermore, there seems to be a second pathway of NMD evasion of long 3′ UTR reporters. Another set of mRNAs showed no NMD inhibiting effects of the first 600 nucleotides of a long 3′ UTR, however the full length 3′ UTR was able to suppress NMD. Taken together these results suggest that there are at least two pathways responsible for NMD inhibition of a long 3′ UTR.

As discussed earlier when describing the central NMD factor UPF1, the position of UPF1 on the 3′ UTR can also influence the fate of an mRNA. Since UPF1 occupation of a 3′ UTR can differ from transcript to transcript, it is feasible why some mRNAs are targeted by NMD whereas others are not. Taken together, these results are an indication that the mere length of a 3′ UTR is not the determining feature for NMD activation.

3.3.2 The *faux* 3′ UTR model in yeast

Another model predating the findings in the previous section is termed the *faux* 3′ UTR model in yeast (Amrani et al., 2004). This model is based on the fact that a transcript harboring a PTC inevitably contains an elongated 3′ UTR. The model states that with increasing length of the 3′ UTR the interaction between the poly(A)-binding protein (in yeast called Pab1) and the terminating ribosome is becoming less efficient. Due to this inefficient or completely absent interaction between the ribosome at the termination codon and Pab1, the translation termination event is believed to be compromised and as a result NMD is activated. Furthermore, tethering Pab1 directly downstream of a PTC so that Pab1 is upstream of the long 3′ UTR is able to antagonize NMD. This is believed to be because of the re-established translation termination due to proximity of Pab1 to the ribosome. Since yeast lack EJCs in general, it is believed that NMD targets in yeast are exclusively recognized based on 3′ UTR length (Muhlrad and Parker, 1999). This model was the first to implicate 3′ UTR length as a result of translation termination in NMD.

3.4 The link between translation and NMD

3.4.1 Translation initiation

To fully understand the link between NMD and translation, it is necessary to take a separate look at translation initiation as well as translation termination events. Translation initiation is the step-wise process in which a ribosome is assembled on a mature mRNA in the cytoplasm in order to start translating the mRNA into protein. Several eukaryotic initiation factors (eIFs) play a pivotal role in the complex process of translation initiation. The first step in translation initiation is the recognition of the 5′ m^7G (methyl-7-guanosine) cap structure on the cytoplasmic mRNA by the eIF4F complex (Jackson et al., 2010). The eIF4F complex is comprised of the cap-binding protein eIF4E, the scaffolding protein eIF4G, and the DEAD-box helicase eIF4A. This complex is essential for translation initiation as it is responsible for the recruitment of

the small ribosomal subunit (Sonenberg and Dever, 2003). Additionally, eIF4G is responsible for the interaction between the cap-bound eIF4F complex and the cytoplasmic poly(A)-binding protein that is bound to the 3′ poly(A)-tail of mRNAs (Imataka et al., 1998). This interaction between 5′-bound eIF4G and 3′-bound PABPC1 was shown to give rise to a circularized form of the mRNA, which is believed to facilitate recycling of terminating ribosomes as well as enhance translation initiation (Amrani et al., 2008; Kahvejian et al., 2005; Wells et al., 1998). Before the complete 80S ribosome can be assembled on the mRNA, the 43S pre-initiation complex (PIC) is assembled on the mRNA by interacting with the eIF4F complex. This 43S PIC consists of several eIFs and the 40S ribosomal subunit (Jackson et al., 2010). An important function of the helicase component eIF4A of the eIF4F complex is believed to be the scanning of the 5′ UTR in a 5′ to 3′ directionality. This action is hypothesized to clear the mRNA 5′ UTR of any unwanted secondary structures or bound proteins to free a space for the 43S PIC-binding to the mRNA (Pestova and Kolupaeva, 2002). Scanning of the 43S PIC commences until an AUG start codon is decoded by the initiator methionine tRNA. After recognition of the start codon, the remaining eIFs are released from the mRNA, the 60S ribosomal subunit is able to join the PIC to form the complete 80S ribosome, and the elongation process is starting (Jackson et al., 2010).

3.4.2 Proper translation termination

Protein synthesis does not only require translation initiation but also efficient translation termination. Translation termination ensures that ribosomes are being freed from the mRNA to start another round of translation as well as the proper release of a finished polypeptide chain. A simplified overview of translation termination can be seen in figure 2. The translating ribosome stops the active translation process when encountering a stop codon (UAA, UAG, and UGA) in the ribosomal A-site. The termination process requires two important proteins: eukaryotic release factor 1 (eRF1) and eukaryotic release factor 3 (eRF3). The eRF3 protein, a GTPase, exists as two paralogs called eRF3a and eRF3b in mammalian cells. It has been shown that in human cells eRF3a is the main factor implicated in translation termination (Chauvin et al., 2005; Hoshino et al., 1998; Hoshino et al., 1989). The direct involvement of eRF3 in translation termination has been shown by eRF3 being required for eRF1 stability in the cell (Chauvin et al., 2005; Janzen and Geballe, 2004). Structurally, eRF1 mimics a tRNA and can therefore decode stop codons by binding in the ribosomal A-site (Chavatte et al.,

2001; Frolova et al., 1999). After binding the ribosomal A-site, eRF1 promotes hydrolysis of the synthesized polypeptide chain via its GGQ motif. This process is supplemented by eRF3 as the hydrolytic activity of eRF1 is stimulated by GTP hydrolysis of eRF3. This stimulation is co-dependent, as eRF3 has an inherently low GTP hydrolyzing activity that is stimulated in turn by the formation of a quaternary complex consisting of GTP, eRF1, eRF3 and the ribosome (Alkalaeva et al., 2006; Frolova et al., 1996; Frolova et al., 1999; Salas-Marco and Bedwell, 2004; Song et al., 2000; Zhouravleva et al., 1995). After the peptide is successfully released, the ribosome remains bound to the mRNA. To free up the termination codon for any approaching upstream ribosomes, the ribosome at the termination codon needs to be removed and potentially recycled for another round of translation. The stalled ribosome is dissociated from the mRNA by the ATP-binding cassette subunit family E member 1 (ABCE1) ATPase. The process of ribosome splitting involves ATP hydrolysis by ABCE1. The exact mechanism by which the ribosome is removed and recycled via ABCE1 is currently unknown. However, several key steps have been elucidated. An important requirement for ribosome splitting by ABCE1 is the interaction with eRF1 after eRF3 dissociates from eRF1 (Pisarev et al., 2010). Additionally, ABCE1 is also known to interact with the ribosome directly (Barthelme et al., 2011; Becker et al., 2012). The process of ribosome removal and recycling is an important mechanism of translation termination as a whole, as it ensures continuous use of the recycled ribosomes as well as an increase in mRNA survivability. An additional factor important for translation termination is PABPC1, which is known to interact with eIF4G (Safaee et al., 2012; Tarun and Sachs, 1996). As mentioned above, this interaction mediates the closed loop formation of the mRNA, which is believed to facilitate ribosome recycling and translation termination in general by bringing the 5′ end and the 3′ end of the mRNA into close proximity (Amrani et al., 2008; Kahvejian et al., 2005; Wells et al., 1998). Additionally, PABPC1 is also known to be a potent interaction partner of eRF3. This interaction plays a vital role in translation termination as it allows for efficient polypeptide release as well as ribosome recycling (Hoshino et al., 1999; Kozlov and Gehring, 2010; Uchida et al., 2002).

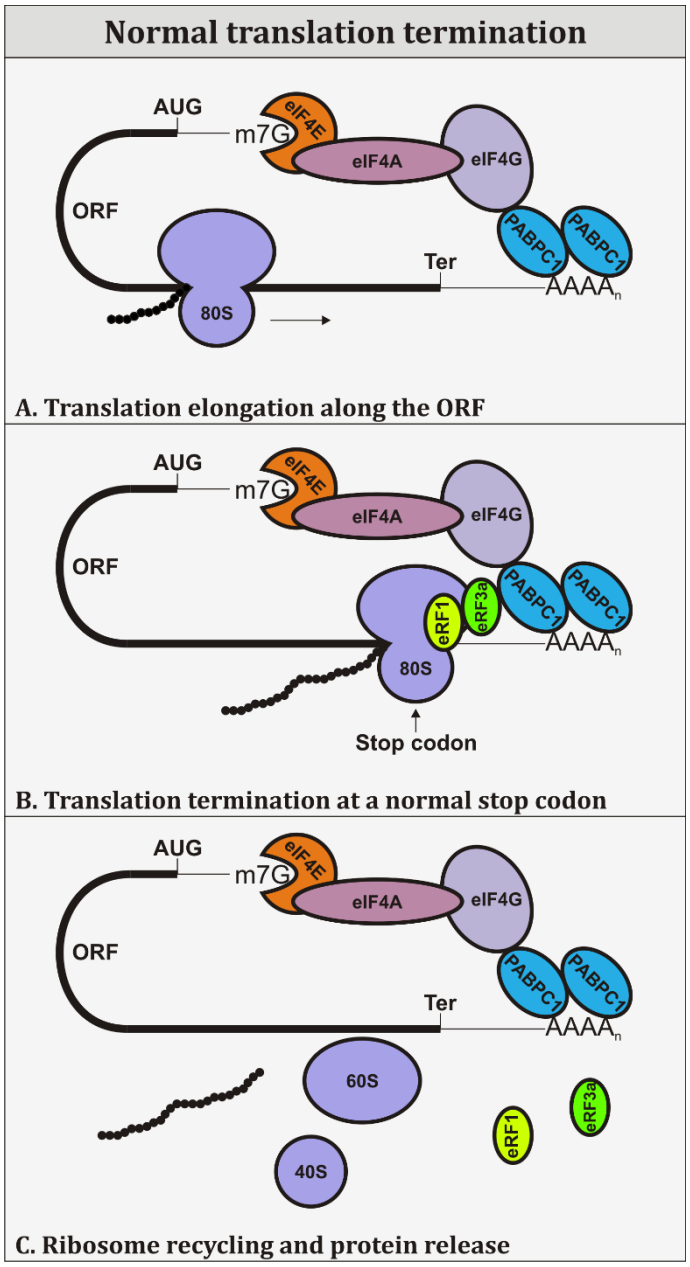

Figure 2: Steps during normal translation termination.

(A) After translation initiation, the ribosomes translate along the ORF until it encounters a termination codon. PABPC1 interacts with eIF4G of the eIF4F complex resulting in a circularized mRNA. **(B)** Once the ribosome stalls at a termination codon it binds the two release factors eRF1 and eRF3a. Poly(A)-bound PABPC1 is able to interact with eRF3a bound to eRF1 at the ribosome, signaling a proper translation termination event. **(C)** As a result of a proper translation termination, the finished protein is released from the ribosome and the latter is removed from the mRNA, opening up the termination codon for any subsequent approaching ribosome.

3.4.3 Steps in aberrant translation termination and the link to NMD

Not all termination events are created equal and although some similarities between normal and aberrant translation termination events exist, there are several significant differences that can lead to the activation of NMD. The most important similarity is that

a PTC, just like a normal termination codon (NTC), enters the A-site of the ribosome. However, the subsequent steps are different. An indication for a significant difference between ribosomes encountering a PTC and an NTC can be seen by ribosomal profiles. Toeprinting assays have shown that there are no signals for ribosomes encountering an NTC unless eRF1 is removed. In stark contrast to this are the toeprinting signals generated for a PTC-carrying mRNA. In this case, typical signals for a stalled ribosome with an occupied A-site are shown independently of eRF1 (Amrani et al., 2004). An additional indication for an aberrant translation termination event is shown by toeprinting assays for the well-known NMD target β-globin NS39, which carries a PTC at amino acid position 39. In this case, toeprinting assays show signals for a stalled ribosome for β-globin NS39, whereas the signals for β-globin wildtype mRNA are normal (Peixeiro et al., 2012). All these results point towards errors during translation termination that ultimately result in the ribosomes being stuck on the mRNA. Without the removal of the ribosome from the mRNA, the degradation of the mRNA is initiated. The exact mechanism by which this degradation is initiated is still unknown. However, a current model suggests a possible mechanism that could be responsible for the degradation of a PTC-carrying transcript (Singh et al., 2008). This model suggests that a stalled ribosome at a PTC is still able to bind the release factors eRF1 and eRF3 in the same manner as when a ribosome encounters an NTC. The major difference, however, is in the interaction partner of eRF3. It has been shown that the central NMD factor UPF1 is able to interact with eRF3, however, the interaction between eRF3 and PABPC1 is normally favored (Kashima et al., 2006; Singh et al., 2008). The fact that UPF1 is able to interact with eRF3, which plays a vital role in translation termination, suggests that there might be a competition between PABPC1 and UPF1 for eRF3 binding. In case of a PTC, just like the *faux* 3′ UTR model in yeast suggests, the ribosome is further removed from the poly(A)-tail and therefore PABPC1. This could lead to an inefficient interaction between PABPC1 and eRF3, which would allow for the less favored interaction between 3′ UTR-populated UPF1 and ribosome-bound eRF3 to occur more readily. In turn, this would allow UPF1 to recruit other NMD factors and eventually initiate NMD. Supporting this competition model is an experiment that shows when increasing amounts of PABPC1 are added to an *in vitro* reaction, the interaction between UPF1 and eRF3 can be prevented (Singh et al., 2008). Furthermore, reporter mRNAs carrying different PTCs at various 5′ and 3′ positions show gradually less efficient NMD depending on the location

of the PTC (Eberle et al., 2008). Interestingly, in this set of data a PTC close to the start codon is able to evade NMD. This observation provides further evidence for the importance of the physical distance of PABPC1 from the terminating ribosome. In case of a PTC close to the start codon, the ribosome would stall close to the translation initiation site. Due to the closed loop form of the mRNA, PABPC1 would be in close proximity of the terminating ribosome due to its interaction with eIF4G. Furthermore, the discussed study uses a reporter with complementary RNA sequences in the 3′ UTR. On the one hand, the complementary sequences are positioned close to the PTC and on the other just upstream of the poly(A)-tail. Due to the complementary sequences interacting with each other, the 3′ UTR folds back the poly(A)-tail. This again brings PABPC1 bound to the poly(A)-tail into close proximity of the terminating ribosome and subsequent interaction with eRF3 can signal a normal translation termination event (Eberle et al., 2008).

These results show a possible model of NMD activation of a PTC by physically enlarging the distance between the terminating ribosome and poly(A)-bound PABPC1. Further research has to be performed to illuminate additional factors and intricacies of this mechanism.

3.4.4 Active translation is necessary for NMD

Early models of NMD stated that NMD was limited to the first or pioneer round of translation. This was believed to be the case because mRNA with cap-binding protein 80 (CBP80) attached was targeted by NMD (Ishigaki et al., 2001). CBP80 is part of the nuclear cap binding complex that is added during transcription and stays on the mRNA until after the mRNA is exported into the cytoplasm and replaced by the eIF4F complex (Izaurralde et al., 1994; Lewis and Izaurralde, 1997; Sonenberg and Dever, 2003). Later studies refuted the notion that NMD is limited to the pioneer round of translation as it was shown, that NMD is also able to degrade mRNAs bound by eIF4E, which is part of the eIF4F complex (Durand and Lykke-Andersen, 2013; Rufener and Muhlemann, 2013). Furthermore, it has been shown that active translation is mandatory for the activation of NMD. Several experiments have highlighted this requirement. For example, structures in the 5′ UTR of an mRNA that are able to block ribosomal translation have been implicated in NMD inhibition (Belgrader et al., 1993). Another set of experiments established that protein synthesis inhibitors like cycloheximide, anisomycin, emetine, pactamycin, and puromycin are able to efficiently block NMD activation of a T-cell receptor mRNA

harboring a PTC (Carter et al., 1995). These examples highlight the need for active translation in order for NMD to be activated on an NMD-targeted mRNA.

3.5 Additional NMD targets present in the cell

There are several other classes of NMD substrates in the cell (Figure 3). One example are mRNAs for so called selenoproteins. Selenoproteins are a class of polypeptides that incorporate the unusual amino acid selenocysteine (Sec). The reason why selenoprotein coding transcripts can be targeted by NMD is because the UAG stop codon triplet can under specific circumstances also encode for Sec. Previous work showed that the stability of selenoprotein transcripts rely on the selenium concentration in the cell. Under low selenium conditions the transcript can be degraded via NMD since not enough Sec is present in the cell to be incorporated into the polypeptide. As a result the UAG codon is treated as a premature termination codon (Bermano et al., 1995; Berry et al., 1993; Hadley and Sunde, 2001; Lee et al., 1989).

However, selenoprotein-coding mRNAs are not the only additional NMD target. Furthermore, transcripts containing an upstream open reading frame (uORF) can be targeted by NMD. A transcript harboring a uORF still contains a regular ORF downstream of the uORF. Since the uORF contains a stop codon, there will still be EJCs present in the regular ORF as the ribosome cannot translate the ORF. In this case, a uORF-containing transcript will be targeted and degraded by the NMD machinery (Mendell et al., 2004). Interestingly however, not all uORF transcript are targeted by NMD (Stockklausner et al., 2006). This indicates that even though a transcript harboring a uORF can be targeted by NMD, a uORF cannot generally be considered as an NMD activator.

Additionally, certain genes can regulate their own expression levels by means of alternative splicing (Sureau et al., 2001). Alternative splicing leads to different mRNA transcript isoforms depending on which splice sites are utilized (Brett et al., 2000). Alternatively spliced pre-mRNAs can harbor a PTC, for example due to intron inclusion, which would result in the degradation of the transcript via NMD (Lewis et al., 2003).

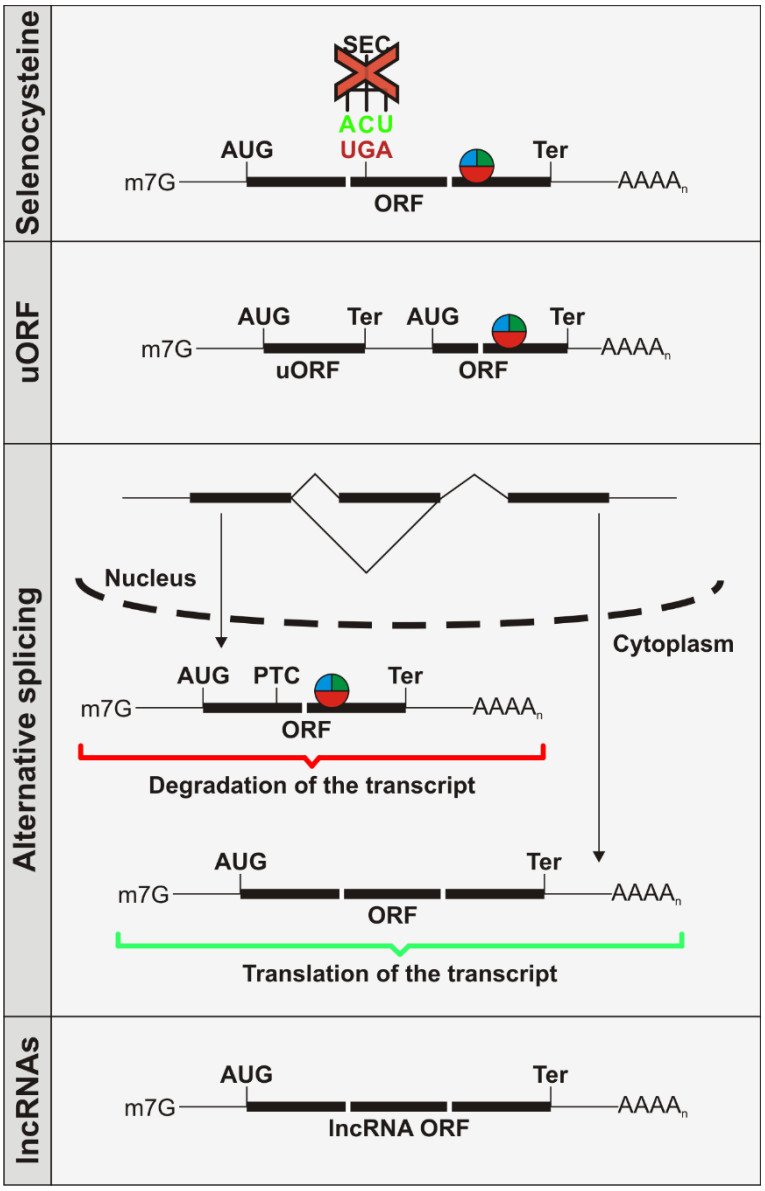

Figure 3: Additional NMD substrates in the cell.

Last but not least, long non-coding RNAs (lncRNAs) can be targeted by NMD as well. As discussed earlier, NMD requires active translation to degrade mRNAs. However, lncRNAs are believed to be untranslated as the name *non-coding* already implies. Therefore, it was surprising to see that the lncRNA growth arrest factor 5 (GAS5) was upregulated under UPF1 knockdown conditions (Tani et al., 2013). Later studies showed, however, that lncRNAs can actually be translated contrary to what was earlier believed (Chew et al., 2013). This could explain why some lncRNAs are targeted by NMD. These examples listed here show that NMD is not only a quality control mechanism in the cell but the role of NMD is more complex and broader than simply quality control.

3.6 Physiological implications of NMD

3.6.1 Importance of NMD factors on cell viability

NMD factors do not only play a vital role in the degradation of faulty mRNA transcripts but several factors also seem to be important for cell viability. It has been shown that multiple NMD factors are essential for embryonic development in mice (Hwang and Maquat, 2011). For example, knocking out the central NMD factor Upf1 in mice causes failure of embryonic development in early stages. After uterine implantation, mouse embryos lacking Upf1 die due to apoptosis. Additionally, homozygous deletion of Upf1 in ES cell lines could not be established and Upf1 null cells have never been observed (Medghalchi et al., 2001). Besides Upf1, Upf2 and Smg1 have also been implicated in cell survivability in mice. Upf2 deficient mice die in utero between embryonic day 3.5 and embryonic day 7.5, whereas Smg1 deficient mice die before embryonic day 12 (McIlwain et al., 2010; Weischenfeldt et al., 2008). Smg6 has also recently been shown to play a role in early development as Smg6 knockout mice have displayed embryonic lethality at the blastocyst stage (Li et al., 2015).

These results show the important functions of several NMD factors besides their role in mRNA degradation during development and on cell viability. This makes studying the function of individual NMD factors difficult as many carry a role in cell survivability and knockout experiments are simply not viable. Using inducible systems where the NMD factors could be depleted at a later stage could help study the function of these proteins.

3.6.2 NMD and genetic disorders

Many inherited genetic disorders as well as cancers are caused by nonsense or frameshift mutations that lead to PTC-carrying transcripts. Over 2400 genetic disorders result from a nonsense mutation. Furthermore, a recent study suggests that about 11% of human genetic disorders are due to nonsense mutations (Culbertson, 1999; Mort et al., 2008; Peltz et al., 2013). NMD is able to modify clinical phenotypes by degrading PTC-containing transcripts. This ensures that the cell does not express any C-terminally truncated proteins with potential dominant negative effects. One example showcasing this protective side of NMD can be seen in erythrocytes. Individuals suffering from the blood disorder called β-thalassemia have in many cases nonsense mutations in the first or second exon of the β-globin gene. These mutations result in a PTC in the β-globin mRNA transcript, which is recognized and therefore degraded by NMD. In heterozygous

individuals with one functional allele of the β-globin gene, the levels of truncated protein are kept very low by NMD and enough functional protein is synthesized from the wild type allele, ensuring that the individuals remain symptom free. Nonsense mutations in the final exon of β-globin are however not recognized by NMD as they lack an EJC downstream of the PTC. As a result, the transcript evades NMD and heterozygous individuals present an intermediate from of the disease called thalassemia intermedia (Hall and Thein, 1994).

Although NMD can certainly be beneficial as seen in the example of β-thalassemia, there are physiological conditions that are worsened by NMD. NMD is not beneficial in cases where a truncated version of the protein would lead to a less severe medical condition of a genetic disorder. A specific example would be Duchenne muscular dystrophy (DMD). A nonsense mutation in the dystrophin gene leads to a PTC-carrying transcript which activates NMD. This leads to a severe form of DMD. However, there are other known mutations in the dystrophin gene that evade NMD and produce a C-terminally truncated version of the protein. This C-terminally truncated protein is known to be partially functional and results in a less severe form of DMD called Becker muscular dystrophy (BMD) (Kerr et al., 2001). These examples show that although NMD has a protective function in the cell, it is not always favorable to activate NMD. In some cases it would be in the patients' interests to subdue NMD in order to increase levels of partially functional C-terminally truncated proteins.

3.6.3 Treatment of physiological conditions resulting from NMD

As described above it can be beneficial to deactivate NMD for certain transcripts to lessen clinical manifestations of a disease. There are two ways in which NMD can either directly or indirectly lead to a more severe form of a genetic disorder: directly, by degrading a transcript leading to an otherwise beneficial truncated protein, or indirectly by transcripts that escape NMD where a dominant negative effect protein will be expressed and active NMD would be beneficial. It would be of interest to generally stabilize a transcript that is targeted by NMD but would produce a favorable medical condition if it escaped NMD. Since blocking the NMD machinery in general could do more harm than good as many dominant-negative proteins could hypothetically be expressed, a potential read-through approach at PTCs is the more favorable approach (Kerem, 2004). Administering a drug that would potentially lead to read-through at a certain PTC could theoretically be used as treatment (Peltz et al., 2013). At first,

aminoglycosides were used to achieve translational read-through. In patients with cystic fibrosis or DMD the use of aminoglycosides was shown to be successful in increasing the respective protein levels. However, the aminoglycosides administered had severe side effects in patients such as renal failure, which limits the usage of aminoglycosides in humans (Kovesi et al., 1998). An alternative drug instead of aminoglycosides was proposed with the drug Ataluren. It has been proposed that Ataluren only selectively promotes read-through and does not affect translation termination at normal stop codons (Welch et al., 2007). The mechanism by which Ataluren acts has recently been challenged and the future of this drug remains to be seen as more clinical trials need to be carried out (Auld et al., 2009; McElroy et al., 2013). Generally, a treatment where specific NMD-targeted transcripts could be altered in a way that they evade NMD, coupled with efficient read-through drugs with minimal side effects could prove to be a favorable approach to treat genetic disorders caused by nonsense mutations.

3.7 Aim of this thesis

Nonsense-mediated mRNA decay plays a pivotal role in mRNA surveillance by targeting aberrant mRNA transcripts. Furthermore, NMD is also able to regulate endogenous mRNAs of non-aberrant nature. The factors involved in NMD as well as the mechanism of activation and the degradation pathways have been subjected to many studies. In contrast, the mechanism by which certain transcripts are able to escape NMD and important proteins involved in this process remain elusive.

One protein that was previously shown to be able to suppress NMD is PABPC1. In this thesis I aim to elucidate the role of PABPC1 during NMD suppression. I further aim to characterize potential important and necessary interaction partners of PABPC1 involved in the suppression of NMD. After identifying other proteins and interactors of PABPC1, I aim to shed light on the potential mechanism by which PABPC1 is able to antagonize NMD.

After elucidating the potential pathway, I further explore several different methods of suppressing NMD by recruiting PABPC1 to the correct position of an NMD-targeted substrate. Since NMD is implicated in regulating the clinical phenotype of many diseases I try to modulate disease associated transcripts with PABPC1 to discuss a potential outlook of NMD suppression via PABPC1 in a medical environment.

Combining these results I try to elucidate a part of the NMD suppression mechanism and factors involved as well as show multiple routes to achieve the antagonizing NMD effect.

4 Results

NMD as an mRNA quality control mechanism targets a multitude of transcripts in the organism. Two of the main NMD target classes are degraded either due to an EJC downstream of a terminating ribosome or due to characteristics of certain 3′ UTRs. The mechanism by which some NMD targets are able to evade NMD is currently not fully understood. It is known that NMD of certain transcripts can be suppressed by PABPC1. Here, I aim to elucidate the role of PABPC1 in NMD suppression and propose a mechanism by which PABPC1 achieves this.

4.1 PABPC1 is able to antagonize long 3′ UTR-mediated NMD

PABPC1 plays an essential role in gene expression. It is required for mRNA circularization, thereby facilitating ribosome recycling, and for suppressing NMD at normal translation termination codons (Behm-Ansmant et al., 2007; Wells et al., 1998). Tandem tethering assays have previously shown that PABPC1 is able to suppress NMD when tethered upstream of an NMD-activating factor (Ivanov et al., 2008). However, the mechanism by which PABPC1 is able to antagonize NMD is not well understood. In light of this, I started investigating NMD suppression of long 3′ UTR-mediated NMD targets. It has previously been shown that the mRNA encoding for the NMD factor SMG5 undergoes NMD due to its 3′ UTR (Singh et al., 2008). To study how PABPC1 can suppress NMD I used a triosephosphate-isomerase (TPI) reporter construct harboring the first 1037 nucleotides of the 3′ UTR of SMG5 downstream of 4MS2 binding sites, which are located directly downstream of the stop codon (Figure 4A). This reporter has been shown to be degraded due to the SMG5 3′ UTR (Boehm et al., 2014). Co-expressing and thereby tethering MS2V5-tagged PABPC1 to the reporter increased the steady-state mRNA levels by a factor of about four compared to the negative control MS2V5-GST (Figure 4B). The stabilization seen in this experiment suggests that PABPC1 is able to antagonize NMD induced by a long 3′ UTR.

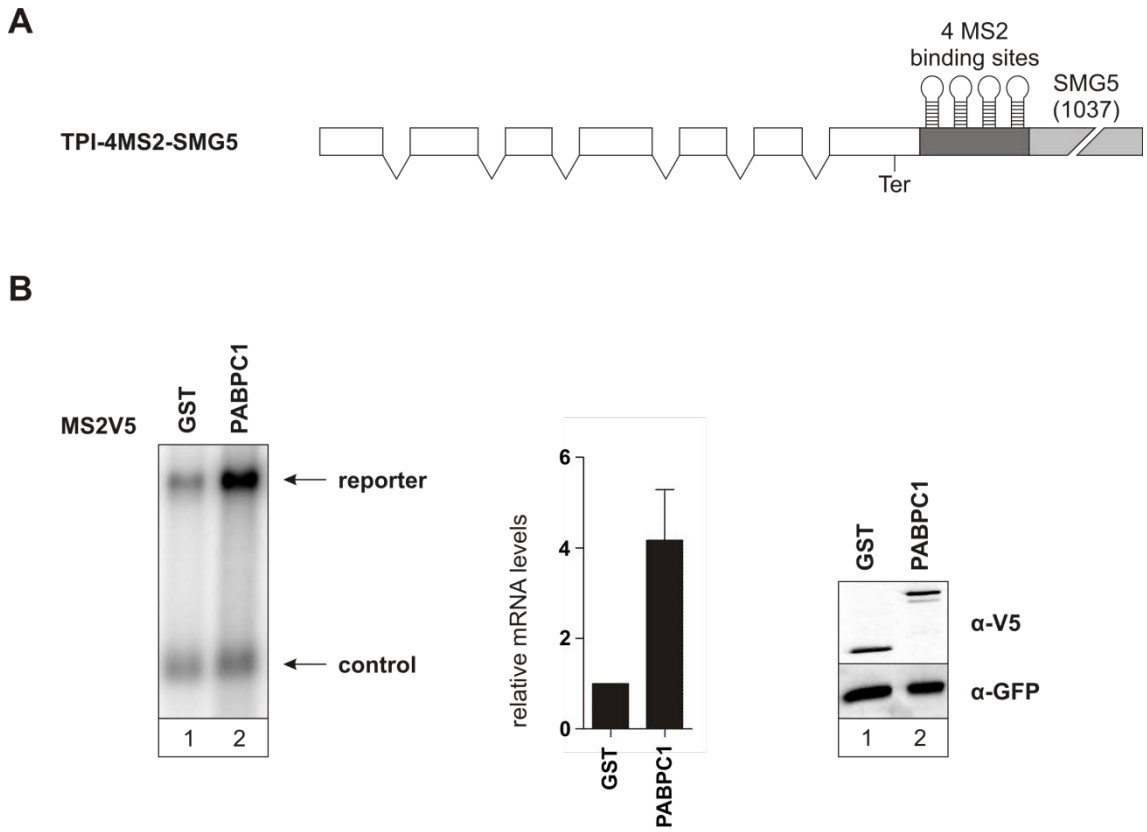

Figure 4: PABPC1 stabilizes an NMD-targeted long 3′ UTR-containing reporter mRNA

(A) Schematic representation of the TPI reporter carrying 4MS2-binding sites upstream of a shortened version of the SMG5 3′ UTR. The TPI ORF is represented as white boxes and introns are shown as lines connecting the boxes. **(B)** Northern blot of total RNA extracted from HeLa cells showing the stabilizing effect of MS2V5-PABPC1 when tethered to the reporter mRNA (left). Quantification of the northern blot with standard deviation represented as black bars (middle). Protein expression was detected by western blotting with anti-V5 and anti-GFP antibody (right).

4.1.1 Increased reporter abundance is a consequence of PABPC1-mediated NMD suppression

As mentioned in the introduction, PABPC1 is known to have a positive effect on general mRNA stability. I made use of two control reporters (Figure 5) to exclude the possibility that the stabilization effect observed when PABPC1 is tethered to the NMD reporter is not a general stabilization effect but instead is a consequence of NMD suppression. To this end, I used a reporter for which the NTC was mutated and a new stop codon appears downstream of the 4MS2 sites (Figure 5). This reporter is called TPI-Δter-4MS2-SMG5. A lack of reporter stabilization was expected since the translating ribosome should displace the tethered PABPC1. Furthermore, the other reporter carried the 4MS2 tethering sites downstream of the SMG5 3′ UTR instead of upstream and was called TPI-

SMG5-4MS2 (Figure 5). Tethering PABPC1 to this reporter should fail to stabilize the transcript as PABPC1 is too far removed from the terminating ribosome.

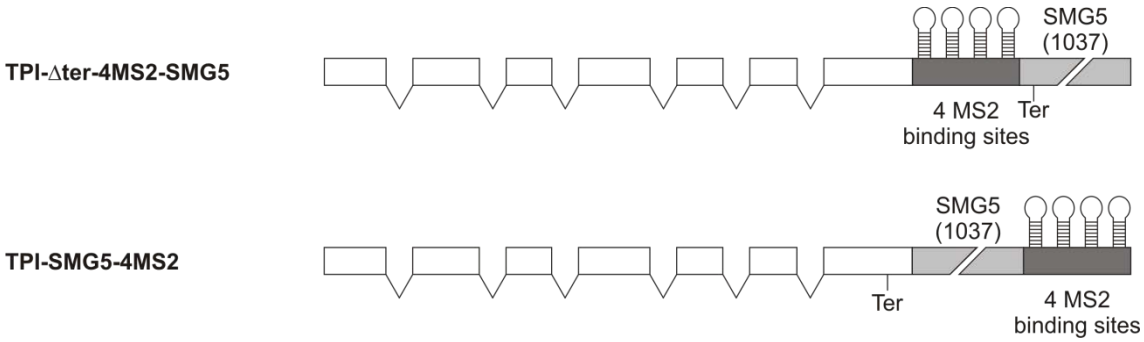

Figure 5: Schematic representation of TPI control reporters

MS2V5-tagged PABPC1 tethered to the TPI-Δter-4MS2-SMG5 reporter failed to antagonize NMD (Figure 6A). The same held true when MS2V5-PABPC1 was tethered to the reporter carrying the 4MS2 sites downstream of the SMG5 3′ UTR (Figure 6B). In both cases the reporter mRNA abundance was unchanged compared to the negative control. I performed an additional experiment to exclude any *trans* effects of PABPC1 on the initially used reporter construct. To this end, I co-expressed FLAG-tagged PABPC1 with the TPI-4MS2-SMG5 reporter (Figure 6C). In this scenario FLAG-PABPC1 should not be directly tethered to the reporter as it carried a FLAG-tag and not an MS2V5-tag and should therefore fail to stabilize the reporter. The results of the northern blot showed that PABPC1 was unable to suppress NMD of the reporter in *trans* and only caused a slight increase in reporter levels (Figure 6C). This slight increase of about 1.5 fold was negligible compared to MS2V5-PABPC1 tethered to the same reporter construct. Western blots for all three experiments showed that PABPC1 is expressed and therefore excluded the lack of PABPC1 expression as the reason for failing to suppress NMD (Figure 6). Combining the results obtained so far shows that PABPC1 is able to antagonize NMD activated by a long 3′ UTR specifically when tethered downstream and in close proximity of terminating ribosomes.

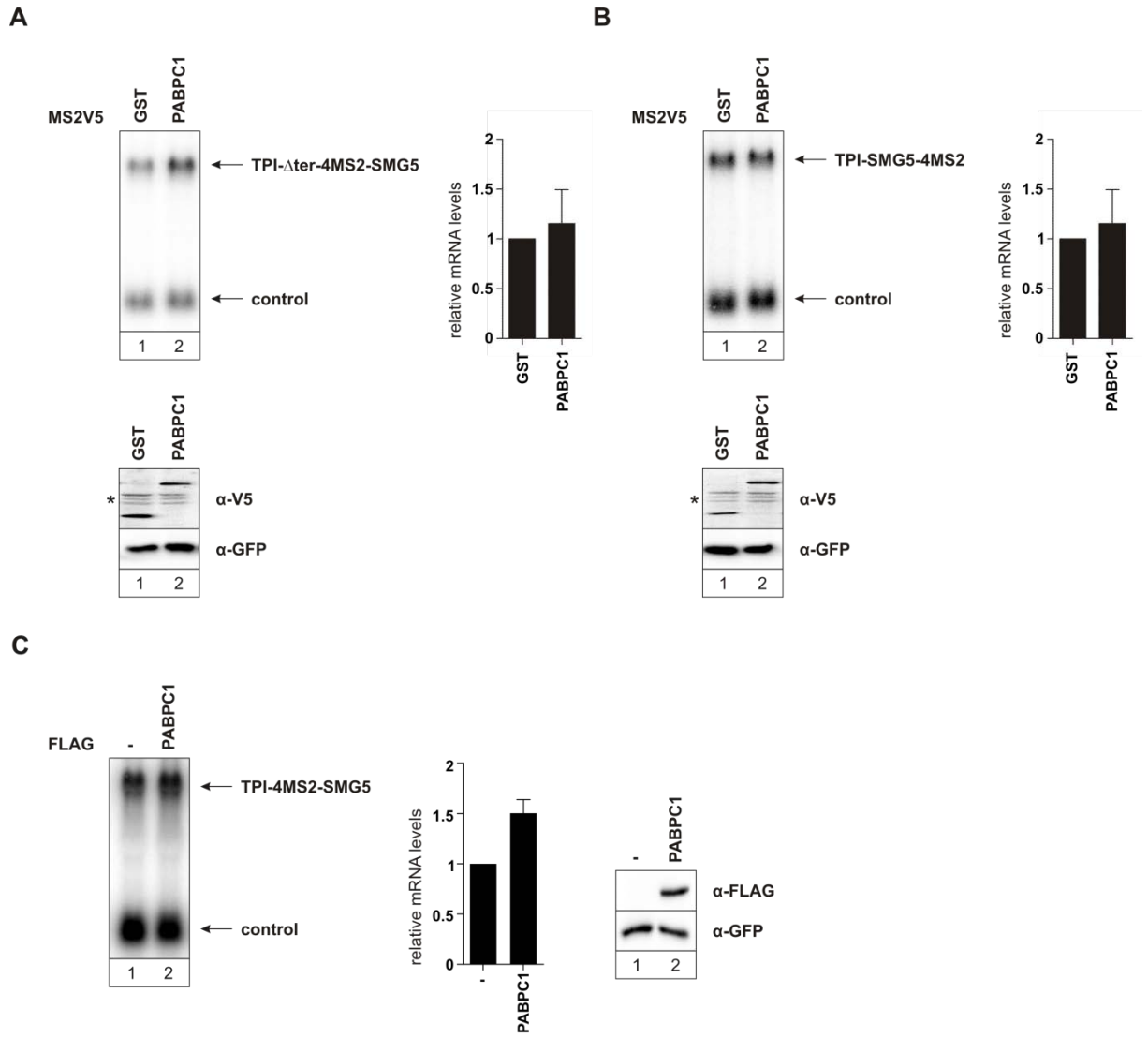

Figure 6: PABPC1 stabilization of a long 3′ UTR-carrying NMD reporter is position and tethering specific

(A) Northern blot showing the stabilizing effect of MS2V5-PABPC1 tethered to the reporter mRNA. Quantification of the northern blot with standard deviation is represented with black bars. Protein expression was detected with anti-V5 and anti-GFP antibodies (Asterisk represents unspecific bands). **(B)** Northern blotting result of tethering MS2V5-PABPC1 to the TPI-SMG5-4MS2 reporter. Quantification and standard deviation is shown with black bars. Protein expression was detected with anti-V5 and anti-GFP antibody. **(C)** Northern blot showing *trans* effects of co-expressed FLAG-PABPC1 alongside the TPI-4MS2-SMG5 reporter. Quantification and standard deviation are represented with black bars. Protein expression was detected using anti-FLAG and anti-GFP antibodies (Asterisk represents unspecific bands).

4.2 Interaction partners of PABPC1 necessary for NMD suppression

4.2.1 The interaction between PABPC1 and eRF3a is not required for NMD suppression

Current data suggest that PABPC1 competes with the central NMD factor UPF1 for eRF3a binding (Singh et al., 2008). It is suggested that UPF1 outcompetes PABPC1 for eRF3a binding when a ribosome is stalling at a termination codon upstream of a long 3′ UTR. This is speculated to be due to the large physical distance between poly(A)-tail bound PABPC1 and ribosome bound eRF3a. To elucidate which domains and interaction partners of PABPC1 play a role in the NMD suppression effect, I created several PABPC1 mutants (Figure 7). It is known that PABPC1 interacts with the PAM2 motifs of eRF3a via its own C-terminal domain called MLLE (*Mademoiselle)* (Kozlov and Gehring, 2010). Therefore, I first made use of two PABPC1 mutants: PABPC1 MLLE^{Mut}, which carries a mutation of the MLLE motif to GAAR, and the PABPC1^{RRM1234} mutant, which lacks the entire C-terminal region (Figure 7).

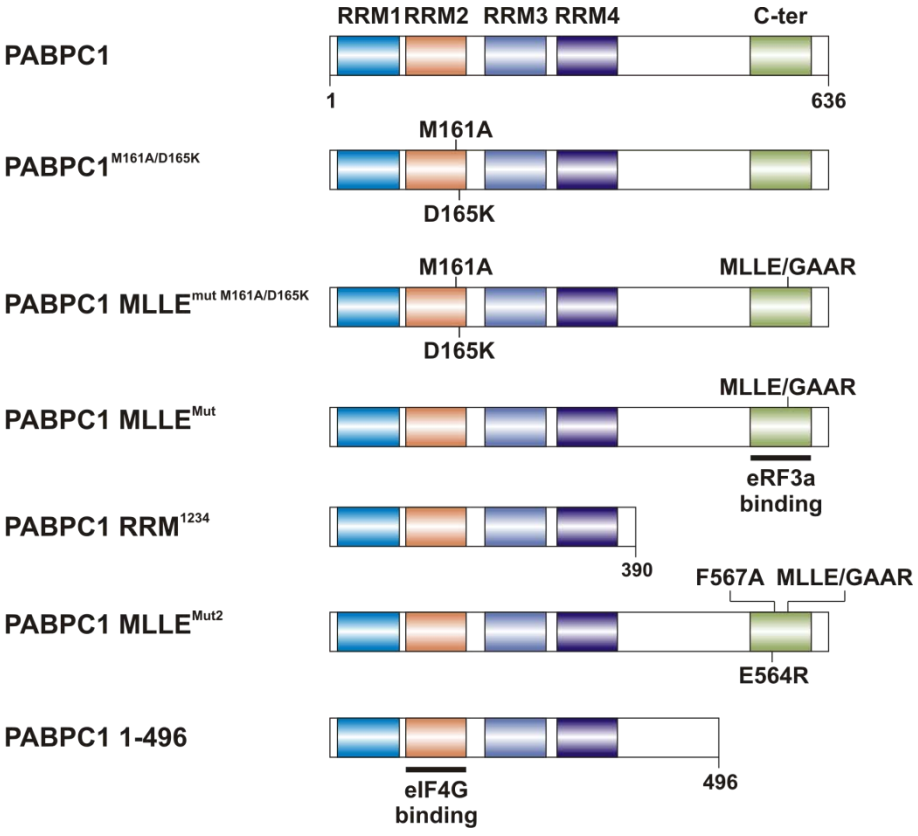

Figure 7: Schematic representation of the PABPC1 mutants used in this work

Surprisingly, tethering these two PABPC1 mutants to the TPI-4MS2-SMG5 reporter mRNA still led to an increase of about 3.5-fold in mRNA abundance (Figure 8A, lanes 3 and 5). Next, I tested two additional PABPC1 mutants with impaired PAM2 interaction. The first mutant contained two additional point mutations (E564R and F567A) that are also known to play a role in PAM2 interaction (Kozlov et al., 2004).

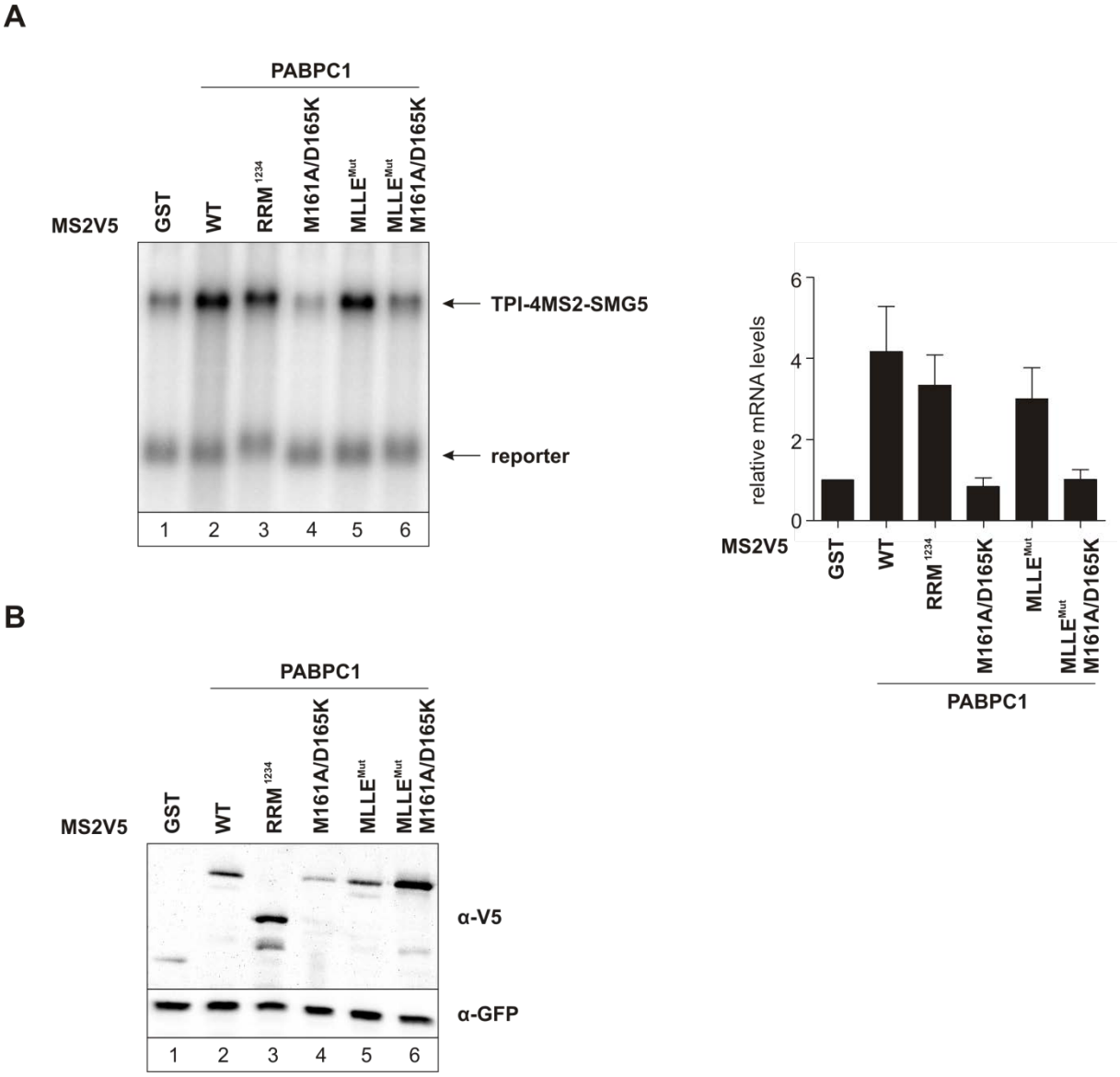

Figure 8: Interaction between PABPC1 and eIF4G necessary to suppress NMD

(A) Northern blot showing the stabilizing effect of the MS2V5-PABPC1 mutants when tethered to the reporter mRNA. Quantification of the northern blot with standard deviation is represented with black bars. **(B)** Protein expression was detected by western blotting with anti-V5 and anti-GFP antibodies.

This mutant was termed PABPC1 MLLE^{Mut2} and the additional two point mutations ensure that the lack of NMD suppression is not due to any residual binding between

PABPC1 and eRF3a due to the lack of these two additional mutations in PABPC1 MLLE^{Mut} (Figure 7). The second additional mutant termed PABPC1$^{1-496}$ is similar to PABPC1 RRM^{1234}, but carries extra C-terminal amino acids (Figure 7). This mutant was used to show that only the lack of the C-terminal region containing the MLLE domain is implicated in the lack of NMD suppression and not the linker region.

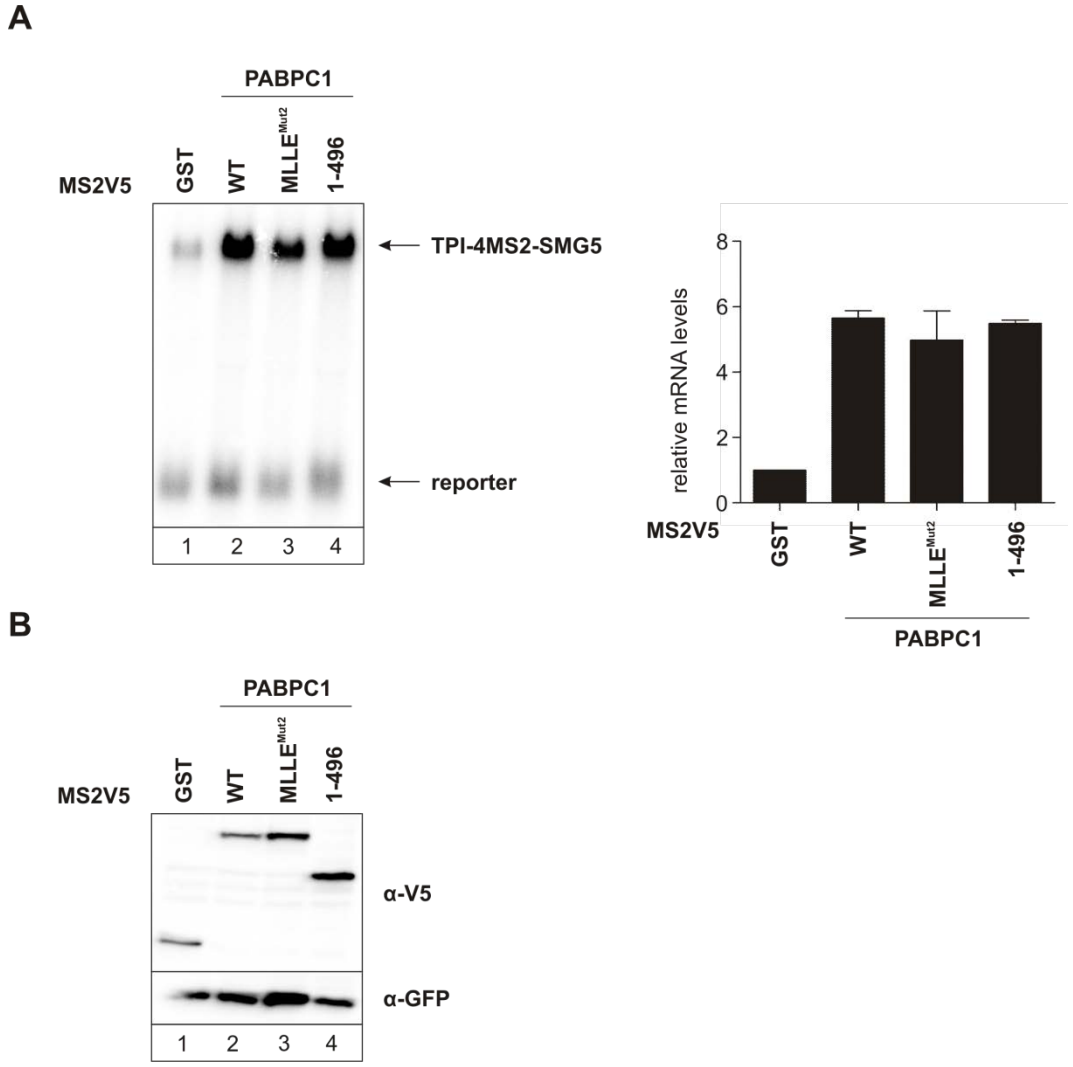

Figure 9: Additional PABPC1 mutants show that the interaction with eRF3a is dispensable for NMD suppression

(A) Northern blot showing stabilization effect of additional PABPC1 mutants tethered to the reporter mRNA. Quantification and standard deviation is shown with black bars. **(B)** Protein expression was detected with anti-V5 and anti-GFP antibodies.

Tethering these mutants to the SMG5 3′ UTR reporter also led to an increase in reporter mRNA abundance similar to PABPC1 wild-type tethering (Figure 9A). Western blots confirmed the expression of the tethered proteins (Figure 8B and figure 9B). These

results were unexpected since PABPC1 mutants, which are unable to interact with eRF3a should not be able to compete with UPF1 for eRF3a binding at the stalling ribosome. This means that no stabilization of the SMG5 3′ UTR carrying reporter mRNA should have been observed, as NMD should have been initiated via the UPF1-eRF3a interaction. However, I clearly observed an increase in reporter mRNA upon tethering of the PABPC1 mutants unable to interact with eRF3a. This suggests that PABPC1 does not require the interaction with eRF3a to suppress NMD of a long 3′ UTR reporter mRNA when tethered downstream of the termination codon. Furthermore, these results also suggest that a direct competition for eRF3a binding between PABPC1 and UPF1 is not responsible for activating or suppressing NMD.

4.2.2 PABPC1 needs the interaction with eIF4G to antagonize NMD

Since I could rule out the interaction between eRF3a and PABPC1 as the determining factor of long 3′ UTR NMD suppression by PABPC1, I looked at the interaction with eIF4G next. The reasoning behind this was that the interaction between eIF4G and PABPC1 plays a pivotal role in mRNA circularization as well as efficient expression of polyadenylated mRNAs (Amrani et al., 2008; Wells et al., 1998). To abolish the interaction between eIF4G and PABPC1 I made use of two point mutations in PABPC1 at amino acid positions 161 and 165 (Figure 7, PABPC1$^{M161A/D165K}$) known to abolish the interaction with eIF4G (Kahvejian et al., 2005; Safaee et al., 2012). Interestingly, tethering the PABPC1$^{M161A/D165K}$ double mutant to the reporter carrying the SMG5 3′ UTR no longer inhibited the degradation of the reporter via NMD (Figure 8A, lane 4). I made use of an additional double mutant of PABPC1 harboring both the MLLE mutation as well as the M161A/D165K double mutations (Figure 7). Co-expressing and therefore tethering this mutant to the SMG5 3′ UTR reporter did not lead to any increase in reporter levels either (Figure 8A, lane 6). These results show that the interaction between PABPC1 and eIF4G is mandatory for PABPC1 to suppress NMD. In conclusion, I have shown here that PABPC1 and UPF1 competition for eRF3a binding does not seem to be a determining factor for NMD suppression. However, the interaction between PABPC1 and eIF4G is essential for NMD suppression by PABPC1. These results potentially implicate mRNA circularization and ribosome recycling in NMD suppression as the PABPC1-eIF4G interaction is known to play a role in both these processes.

4.3 PABPC1 does not affect EJC-mediated NMD

So far I examined the ability of PABPC1 to antagonize NMD of a long 3′ UTR-carrying reporter as well as the importance of the interaction between PABPC1 and both eRF3a and eIF4G in this process. As mentioned earlier, NMD is not only restricted to long 3′ UTR-mediated NMD, but is also activated by the presence of an EJC downstream of the terminating ribosome. To gain further insight into NMD suppression by PABPC1 I next took a closer look at the ability of PABPC1 to antagonize EJC-mediated NMD. To this end, I used two reporter constructs harboring a MINX intron either downstream or upstream of the 4MS2 binding sites of the TPI-4MS2-SMG5 reporter (Figure 10A). The MINX intron is a short synthetic intron carrying adenoviral splice sites. During pre-mRNA processing the MINX intron is spliced and a site-specific EJC is deposited onto the reporter mRNA. The presence of this EJC downstream of the termination codon will lead to the activation of NMD. Strikingly, neither PABPC1 wild-type nor any of the PABPC1 mutants were able to suppress EJC-mediated NMD (Figure 10). When tethering PABPC1 upstream of the MINX intron I observed an increase in mRNA levels of about 1.5 fold (Figure 10B, lane 2). The minimal level of stabilization of the reporter transcript was clearly decreased compared to NMD suppression of the 3′ UTR SMG5 reporter mRNA. Tethering PABPC1 and the PABPC1 mutants to the reporter harboring the MINX intron upstream of the 4MS2 tethering sites did not lead to any increase in reporter mRNA abundance either (Figure 10C). Western blot analysis showed that all MS2V5 fusion proteins were expressed and thus expression problems could be ruled out as an explanation for the decrease in reporter stabilization (Figure 10D). These results indicate that the presence of PABPC1 either upstream of downstream of an intron is unable to antagonize EJC-mediated NMD. PABPC1 seems to play a role in the regulation of long 3′ UTR-containing transcripts but not EJC-targeted mRNAs. From these results I can conclude that PABPC1 is mainly able to regulate NMD of long 3′ UTR-targeted transcripts.

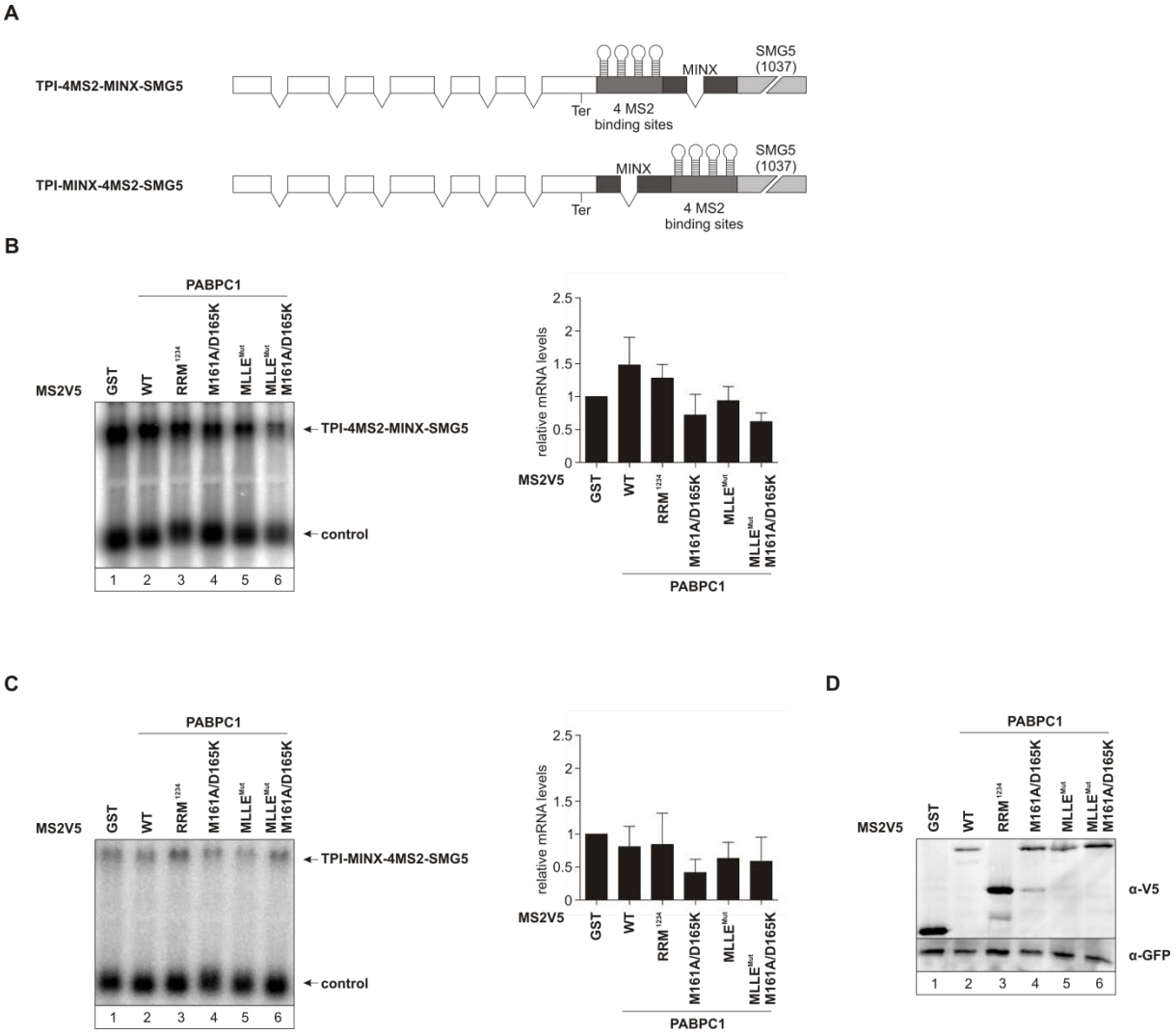

Figure 10: PABPC1 is unable to antagonize EJC-mediated NMD

(A) Schematic representation of MINX-containing reporters. MINX ORF is shown as a dark gray box and the MINX intron as lines connecting the ORF. **(B)** Northern blot showing the effect of the MS2V5-PABPC1 mutants when tethered upstream of an intron. Quantification with standard deviation is represented with black bars. **(C)** Northern blot showing tethering effect of MS2V5-PABPC1 mutants tethered downstream of an intron. Quantification and standard deviation is shown with black bars. **(D)** Protein expression was detected with anti-V5 and anti-GFP antibodies.

4.4 eRF3a requires the interaction with PABPC1 for NMD suppression

So far I have investigated the ability of PABPC1 to suppress NMD. I have shown that PABPC1 requires the interaction with eIF4G but not eRF3a to suppress NMD of a long 3′ UTR reporter. To gain further insight into the role of eRF3a in this mechanism, I tethered eRF3a directly to the TPI-4MS2-SMG5 reporter (Figure 11A). Tethering eRF3a to the NMD-targeted reporter suppressed NMD and increased mRNA reporter abundance by a factor of four (Figure 11A, lane 2). This result is surprising as I showed earlier that

PABPC1 does not require the interaction with eRF3a to suppress NMD. Therefore, to examine the significance of the interaction between eRF3a and PABPC1 in this scenario, I used a point mutant of eRF3a (eRF3a F76A) in the tethering assay. The phenylalanine at position 76 has been shown to be essential for the interaction between eRF3a and PABPC1 (Kononenko et al., 2010; Kozlov and Gehring, 2010; Osawa et al., 2012). When tethering eRF3a F76A to the reporter and comparing the mRNA abundance to tethered eRF3a, I could observe that the eRF3a F76A mutant had lost the ability to suppress NMD (Figure 11A, lane 3). The western blot showed the expression of the eRF3a mutant, making sure that the lack of reporter stabilization is not due to lower expression levels of eRF3a F76A (Figure 11A). Furthermore, I used the two control reporter mRNAs depicted in figure 5 to ensure the specificity of the observed effects. Tethering MS2V5-eRF3a to either the reporter with the termination codon downstream of the 4MS2 sites or with the 4MS2 sites downstream of the SMG5 3′ UTR only marginally increased reporter levels (Figure 11B and C), confirming the specificity of the tethering assay.

These results show that eRF3a can suppress NMD. However, eRF3a needs the interaction with PABPC1 to do so. Combining the results of this section with previous ones points to a linear mechanistic cascade with eRF3a at the terminating ribosome interacting with PABPC1, which in turn interacts with eIF4G. This hypothesis of a linear cascade is strengthened by the individual observation that eRF3a needs to interact with PABPC1, whereas PABPC1 only requires the interaction with eIF4G in order to suppress NMD.

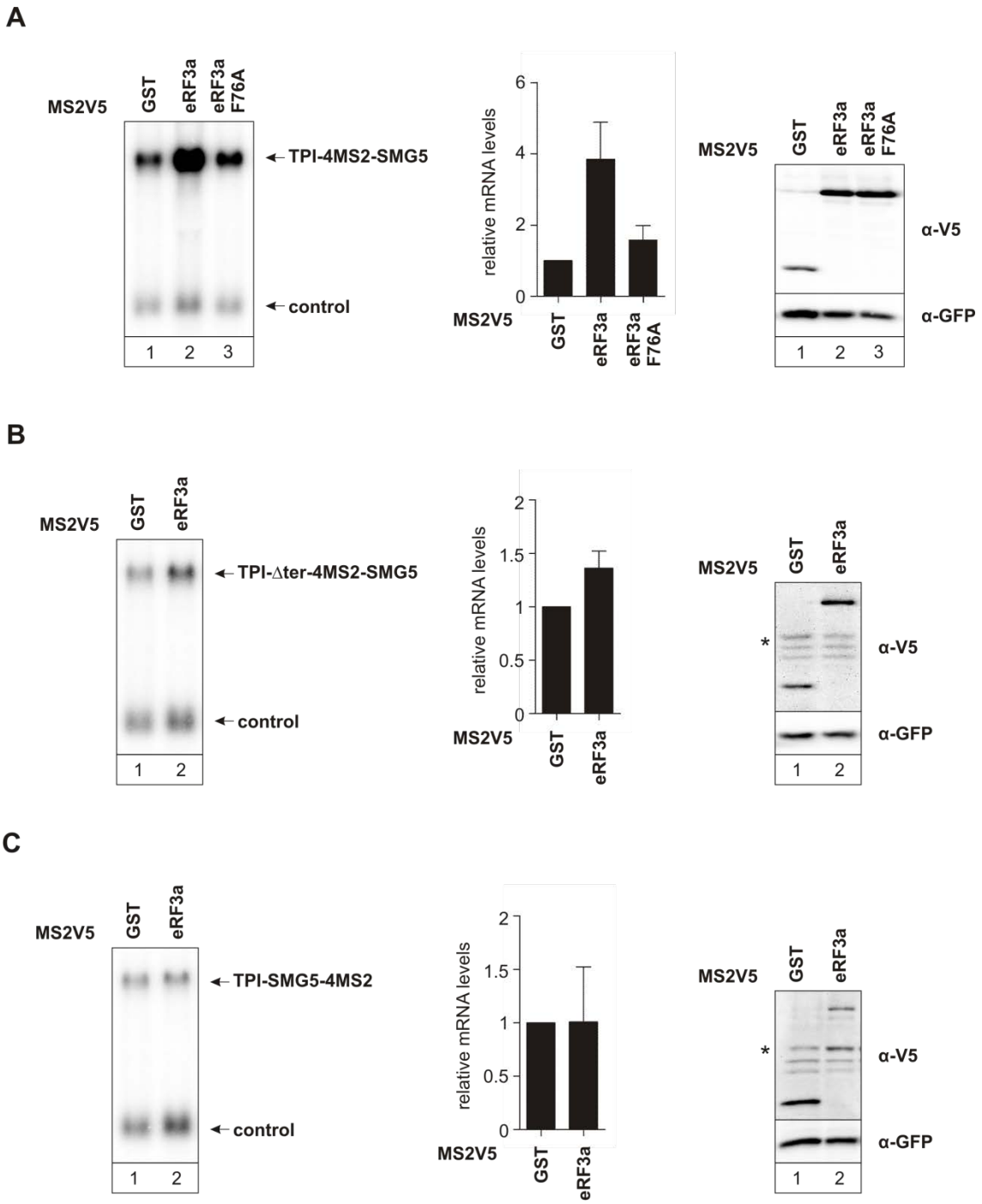

Figure 11: eRF3a requires the interaction with PABPC1 to suppress NMD

(A) Northern blot showing NMD antagonizing effect of MS2V5-eRF3a and MS2V5-eRF3a F76A. Quantification and standard deviation are indicated with black bars. Protein expression has been checked with anti-V5 and anti-GFP antibody. **(B)** Stabilizing effect of MS2V5-eRF3a when tethered to a reporter with the termination codon downstream of the 4MS2 binding sites. Quantification with standard deviation is represented with black bars and protein expression was checked with anti-V5 and anti-GFP antibody (Asterisk represents unspecific bands). **(C)** Northern blot showing stabilization effect of MS2V5-eRF3a on a reporter carrying 4MS2 binding sites downstream of the SMG5 3′ UTR. Quantification and standard deviation are shown with black bars and proteins were detected with anti-V5 and anti-GFP antibodies (Asterisk represents unspecific bands).

4.5 eIF4G antagonizes NMD independently of PABPC1

Up to this point I could establish a linear cascade of protein interactions involved in NMD suppression with PABPC1 at its center. Having looked at eRF3a more specifically in the previous section, I next aimed to investigate the role of eIF4G and the importance of the interaction with PABPC1 further. To this end, I made use of an MS2V5-tagged 83 amino acid truncated version of eIF4G (Figure 12, eIF4G ΔN83). Tethering MS2V5-eIF4G ΔN83 to the TPI-4MS2-SMG5 reporter led to an increase in mRNA reporter abundance by a factor of three (Figure 13A, lane 2). A truncated version of eIF4G where the C-terminal region was shortened, ranging only to amino acid 1089 (Figure 12, eIF4G 84-1089) was still able to suppress NMD of the reporter (Figure 13A, lane 4). To establish the importance of the interaction between eIF4G ΔN83 and PABPC1 in NMD suppression, I made use of an N- and C-terminally truncated version of eIF4G, lacking the region responsible for PABPC1 interaction (Figure 12, eIF4G 206-1089). Surprisingly, tethering MS2V5-eIF4G 206-1089 to the TPI-4MS2-SMG5 reporter still led to an increase in reporter levels, albeit to a lesser degree compared to full length eIF4G (Figure 13A, lane 3). Furthermore, a shortened version of eIF4G carrying a KRERK/AAAAA mutation at position 187-191 (Figure 12, eIF4G 84-1089 KRERK), which was reported to no longer interact with PABPC1 (Wakiyama et al., 2000) was also still able to suppress NMD (Figure 13A, lane 5).

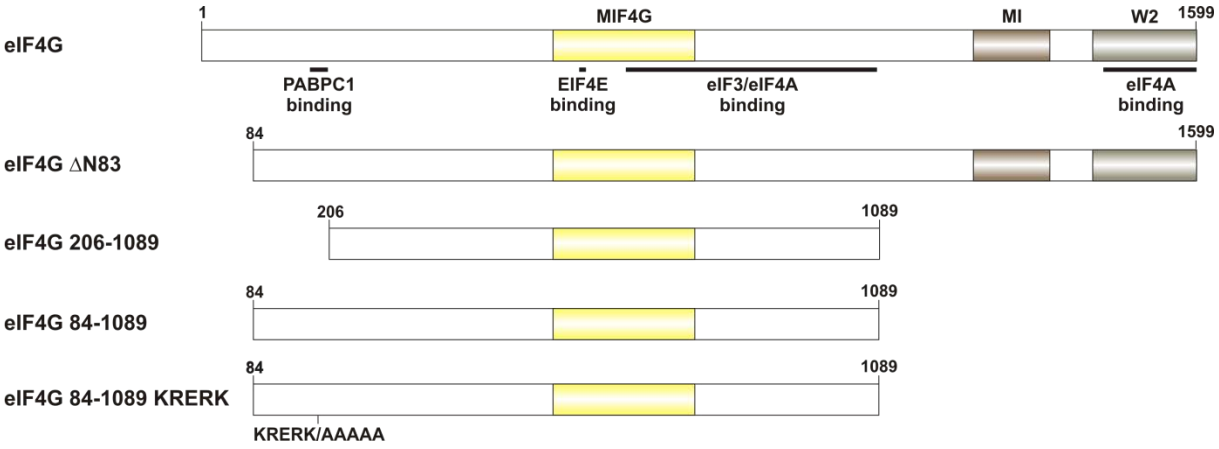

Figure 12: Schematic representation of eIF4G domain structure and mutants

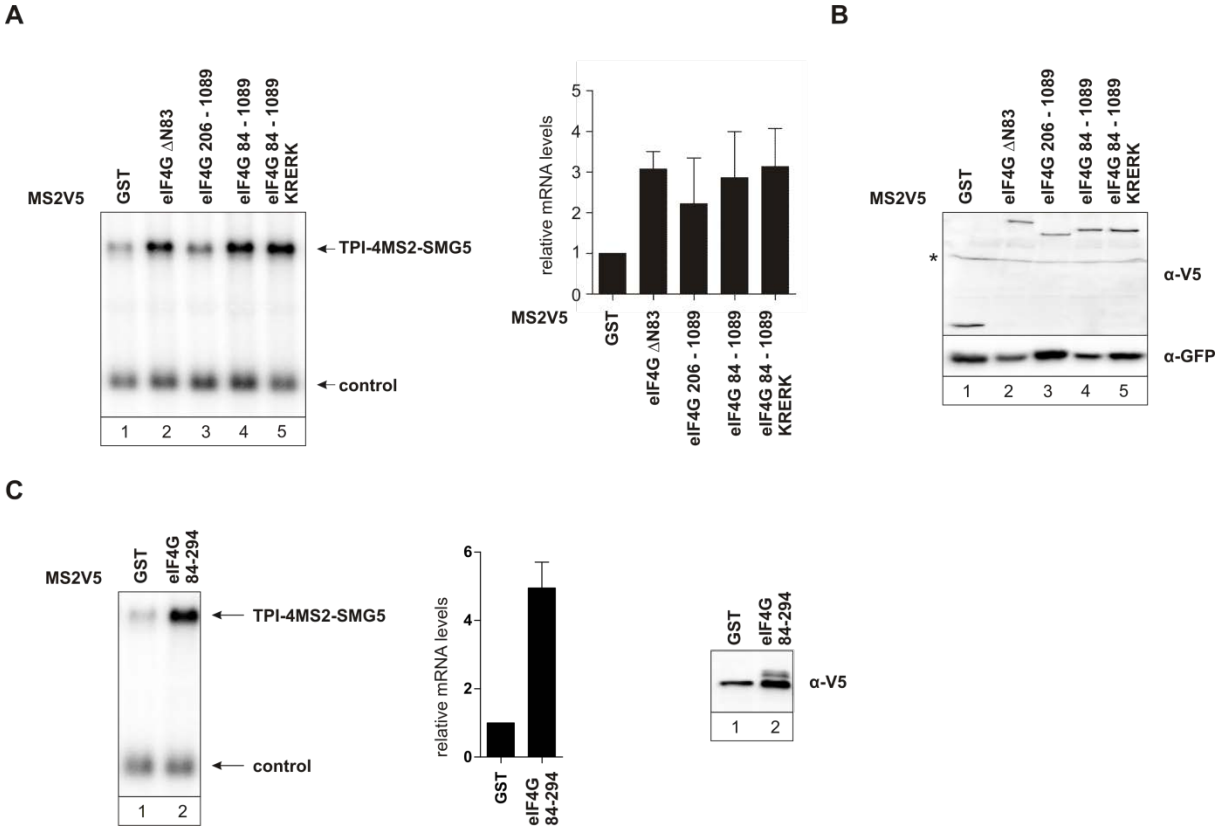

Figure 13: Interaction between eIF4G and PABPC1 is dispensable for NMD suppression

(A) Stabilizing effect of MS2V5-eIF4G mutants when tethered to the TPI-4MS2-SMG5 reporter. Quantification with standard deviation is represented with black bars. **(B)** Western blot showing protein expression visualized with anti-V5 and anti-GFP antibodies (Asterisk represents unspecific bands). **(C)** Stabilization of the TPI-4MS2-SMG5 reporter mRNA when tethering MS2V5-eIF4G 84-294, which is only able to interact with PABPC1. Quantification shows standard deviation and is represented as black bars. Protein expression was shown with anit-V5 antibody.

Furthermore, I tested the specificity of the eIF4G ΔN83 stabilization effect by tethering MS2V5-eIF4G ΔN83 to the reporters depicted in figure 5. Tethering MS2V5-eIF4G ΔN83 to either the TPI-Δter-4MS2-SMG5 or the TPI-SMG-4MS2 reporter did not cause any mRNA reporter stabilization (Figure 14A and B), thereby confirming the specificity of tethering effect in Figure 13A. Moreover, I made use of an additional eIF4G deletion mutant (eIF4G 84-294), which only contains the interaction site for PABPC1. This short form of eIF4G was able to suppress NMD of the TPI-4MS2-SMG5 reporter, which could be seen by a fivefold increase in reporter mRNA (Figure 13C). This result suggests that eIF4G is able to suppress NMD without any other factors but PABPC1, which is made evident by this short version of eIF4G unable to interact with any other protein but PABPC1.

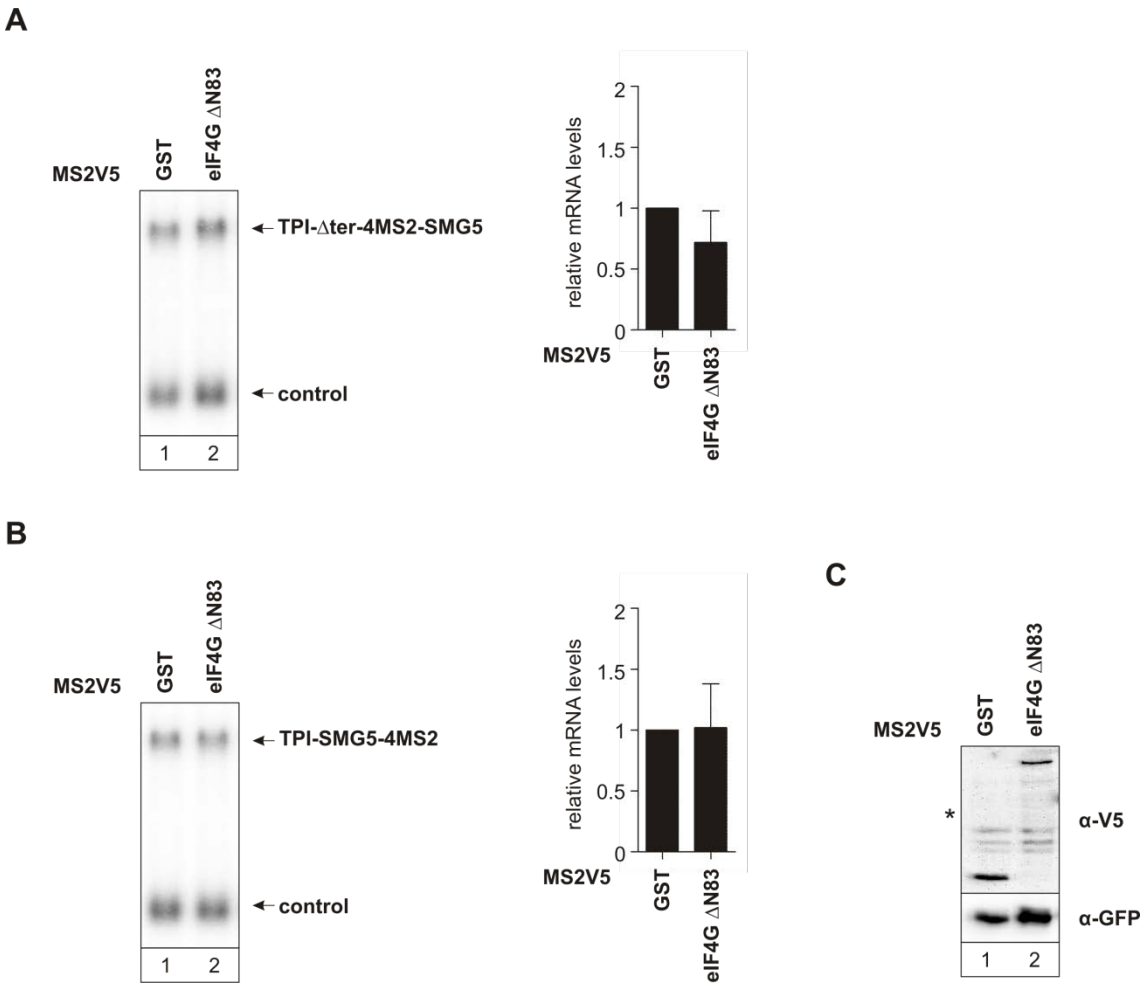

Figure 14: Stabilization effect of eIF4G is specific

(A) Stabilization effect of MS2V5-eIF4G on the TPI-Δter-4MS2-SMG5 reporter. Quantification shows standard deviation and is represented as black bars. **(B)** Northern blot showing stabilization effect of MS2V5-eIF4G on TPI-SMG5-4MS2 reporter. Quantification and standard deviation are shown with black bars. **(C)** Protein expression of eIF4G *(A and B)* was detected with anti-V5 and anti-GFP antibodies (Asterisk represents unspecific bands).

These results show that eIF4G is able to suppress NMD of a long 3′ UTR without interacting with PABPC1. However, the eIF4G deletion mutant lacking the N-terminal PABPC1 binding shows a slightly diminished stabilization effect. I could also show, that eIF4G does not require any other proteins besides PABPC1 as the small fragment from amino acid 84 to 294 is sufficient to suppress NMD. In conclusion, these results indicate that the interaction between eIF4G and PABPC1is not necessary but does favorably enhance NMD suppression by eIF4G.

4.6 PABPC1 acts as a general suppressor of long 3′ UTR-mediated NMD

So far I have established that PABPC1, eRF3a, and eIF4G are able to suppress NMD of a reporter carrying the long 3′ UTR of SMG5 and have shown the dependencies of these proteins amongst each other. Our lab identified several other endogenous long 3′ UTR containing mRNAs that are subjected to NMD (unpublished data). To ensure that PABPC1 is able to suppress other endogenous NMD targets and is not limited to the SMG5 3′ UTR reporter, I used two tethering reporters containing the 3′ UTR of PDRG1 and SURF6 downstream of 4MS2 binding sites (Figure 15A).

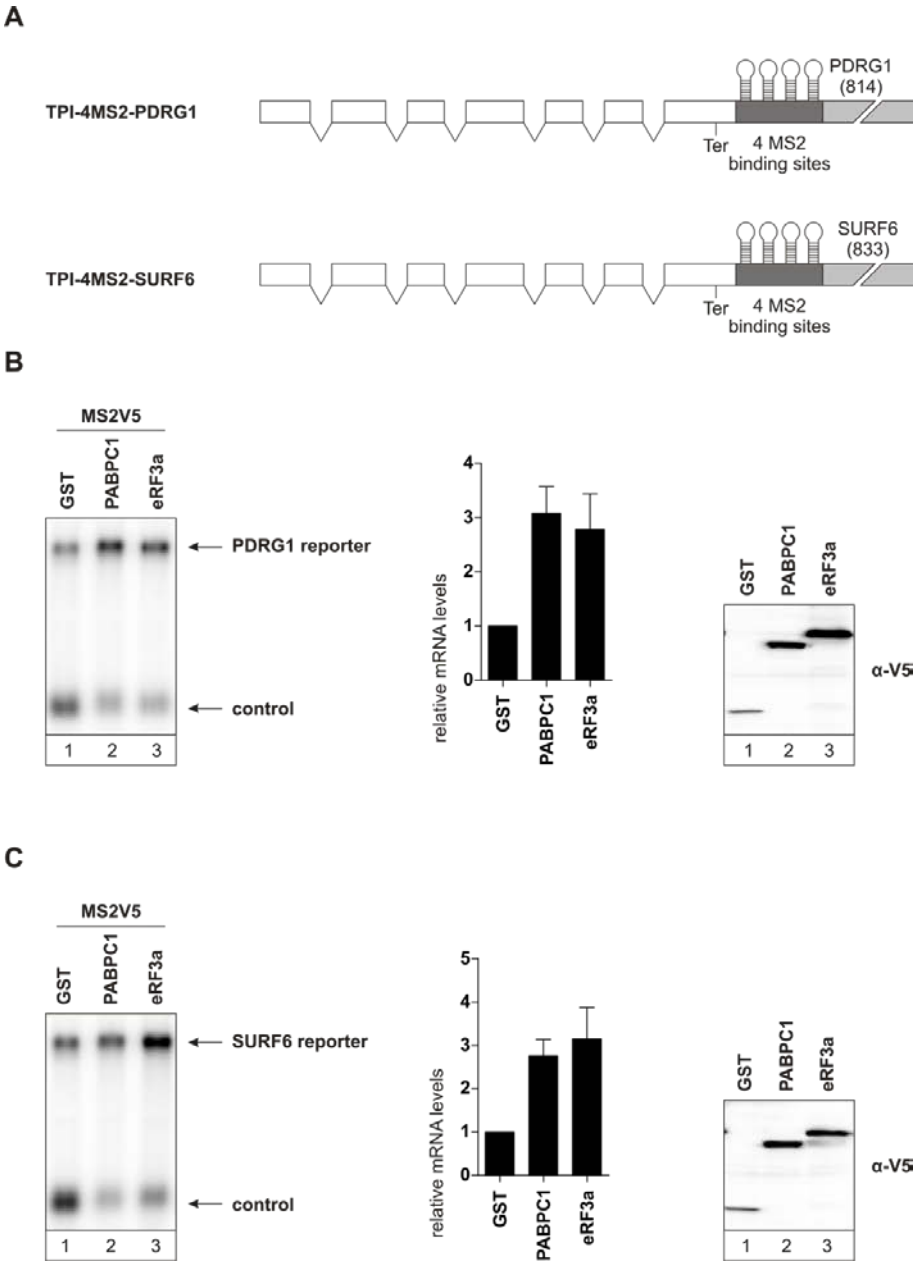

Figure 15: PABPC1 and eRF3a are able to suppress multiple long 3′ UTR-mediated NMD targets

(A) Schematic representation of the two reporters harboring either the PDRG1 or SURF 3′ UTR. **(B)** Stabilizing effect of MS2V5-PABPC1 and MS2V5-eRF3a when tethered to the indicated reporter mRNA. Quantification with standard deviation is represented with black bars and protein expression was checked with anti-V5 antibody. **(C)** Stabilization of the reporter mRNA when tethering MS2V5-PABPC1 and MS2V5-eRF3a to the SURF6 3′ UTR-containing reporter. Quantification shows standard deviation and is represented as black bars. Protein expression was shown with anit-V5 antibody.

As expected, both PABPC1 and eRF3a were able to antagonize degradation via NMD of both reporter constructs (Figure 15B and C). Tethering both proteins to each reporter stabilized the mRNA about three-fold compared to a negative GST-control (Figure 15B and C). These results indicate that PABPC1 and eRF3a are not only able to stabilize a reporter containing the SMG5 3′ UTR but also reporter constructs carrying the 3′ UTRs of PDRG1 and SURF6. All three of these endogenous mRNAs are known to be degraded via NMD due to their 3′ UTR length or composition. This suggests that NMD suppression by both PABPC1 and eRF3a is a general phenomenon that can act on different substrate mRNAs targeted by NMD due to their 3′ UTR.

4.7 NMD inhibition by PTBP1 and other proteins known to interact with PABPC1

Since I established that long 3′ UTR-mediated NMD can be suppressed by PABPC1, I next wanted to test whether or not there are other proteins that can suppress 3′ UTR-mediated NMD. Therefore, I first looked at polypyrimidine tract binding protein 1 (PTBP1). It has previously been reported that PTBP1 is able to protect certain long 3′ UTR-containing mRNAs from degradation via NMD (Ge et al., 2016). It is suggested that PTPB1 bound to the 3′UTR downstream but in close proximity of a termination codon blocks UPF1 from binding and thereby suppresses NMD (Ge et al., 2016). Here, I employed PTBP1 in a tethering assay to suppress NMD of the TPI-4MS2-SMG5 reporter mRNA (Figure 16A). Tethering MS2V5-PTBP1 to this reporter suppressed NMD to the same extent as PABPC1, about 3.5 fold (Figure 16A). Since PTBP1 is no known interaction partner of PABPC1, it seems possible to suppress NMD simply by blocking UPF1 binding in close proximity of the termination codon without the help of PABPC1. Another explanation for the NMD suppression effect by PTBP1 is that one PTBP1 protein is known to bind the mRNA 3′ UTR at several different places simultaneously (Ge et al., 2016). PTBP1 bound downstream of a stop codon could also interact with the 3′ UTR

closer to the poly(A)-tail. This double interaction could potentially compact the mRNA and the poly(A)-tail closer to the stop codon. That way PABPC1 would be in closer proximity to the terminating ribosome and could potentially suppress NMD.

As a next step, I wanted to test whether proteins that are known to indirectly interact with PABPC1 are able to suppress NMD when recruited downstream of a termination codon of an NMD-targeted reporter. To this end I tethered eRF1 to the same reporter (Figure 16B). Eukaryotic release factor 1 does not directly interact with PABPC1 but does so indirectly by interacting with eRF3a instead (Zhouravleva et al., 1995). Tethered eRF1 was able to stabilize the reporter mRNA about seven to eight fold (Figure 16B). Astonishingly, eRF1 is able to strongly suppress NMD, even though the protein does not directly interact with PABPC1. This effect seems rather surprising as the magnitude of increased mRNA levels is much greater compared to tethering PABPC1 alone.

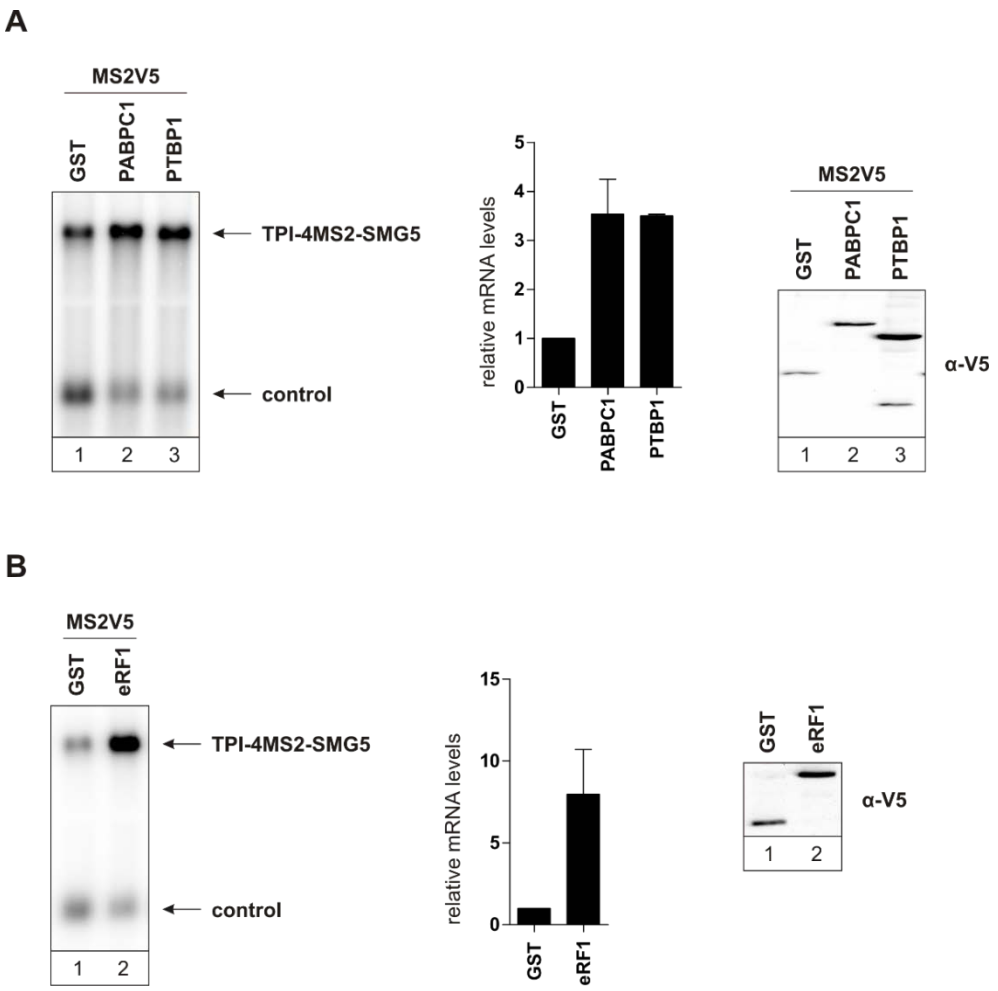

Figure 16: NMD suppression by PTBP1 and eRF1

(A) Northern blot showing NMD suppression of the TPI-4MS2-SMG5 reporter by tethering PTBP1. Quantification showing standard deviation is represented with black bars. Protein expression was tested

for with anti-V5 antibody. **(B)** Northern blot showing antagonizing effect on NMD by tethering eRF1 to the TPI-4MS2-SMG5 reporter. Quantification with standard deviation is represented with black bars and protein expression was checked with anti-V5 antibody.

These results show two additional ways of suppressing NMD. Surprisingly, PTBP1 is able to suppress NMD without the need for PABPC1 as the two proteins are no known interaction partners. However, the mechanism by which PTBP1 suppresses NMD seems to be different from PABPC1 as PTBP1 more likely inhibits UPF1 binding to the transcript, whereas PABPC1 potentially inhibits NMD by efficient translation termination and ribosome recycling. Furthermore, eRF1 is able to inhibit NMD although it only indirectly interacts with PABPC1 via eRF3a. This shows that PABPC1 can suppress NMD even when recruited through indirect interactions.

4.8 Peptide-mediated fold-back of the poly(A)-tail stabilizes NMD-targeted mRNA

Up to this point I have recruited PABPC1 directly downstream of a terminating ribosome, or recruited PABPC1 via a direct or indirect interaction partner to suppress NMD. It has previously been shown that NMD can also be suppressed by employing complementary RNA-mediated fold-back of the poly(A)-tail into close proximity of a termination codon (Eberle et al., 2008). By using this fold-back approach, PABPC1 is brought close to a terminating ribosome and can antagonize NMD. Here, I want to test whether NMD can be suppressed by employing fold-back of the poly(A)-tail mediated by tethered proteins.

4.8.1 eRF3a and eRF1 suppress NMD by folding back the poly(A)-tail

To this end, I used a TPI reporter containing 4MS2 binding sites 5′ of, and 4boxB binding sites 3′ of the SMG5 3′ UTR (Figure 17A). First, I tethered eRF3a F76A and eRF1, two known interaction partners, to this reporter and achieved a stabilization of the mRNA of about 1.5-fold (Figure 17B, lane 3). The F76A point mutant of eRF3a was used to avoid recruitment of PABPC1 via eRF3a itself. The observed effect albeit small is promising that NMD can be suppressed by protein-mediated fold-back of the poly(A)-tail. However, a potential drawback of using two endogenous proteins as mediators for fold-back is that they can interact with other proteins, which might have an effect on NMD suppression.

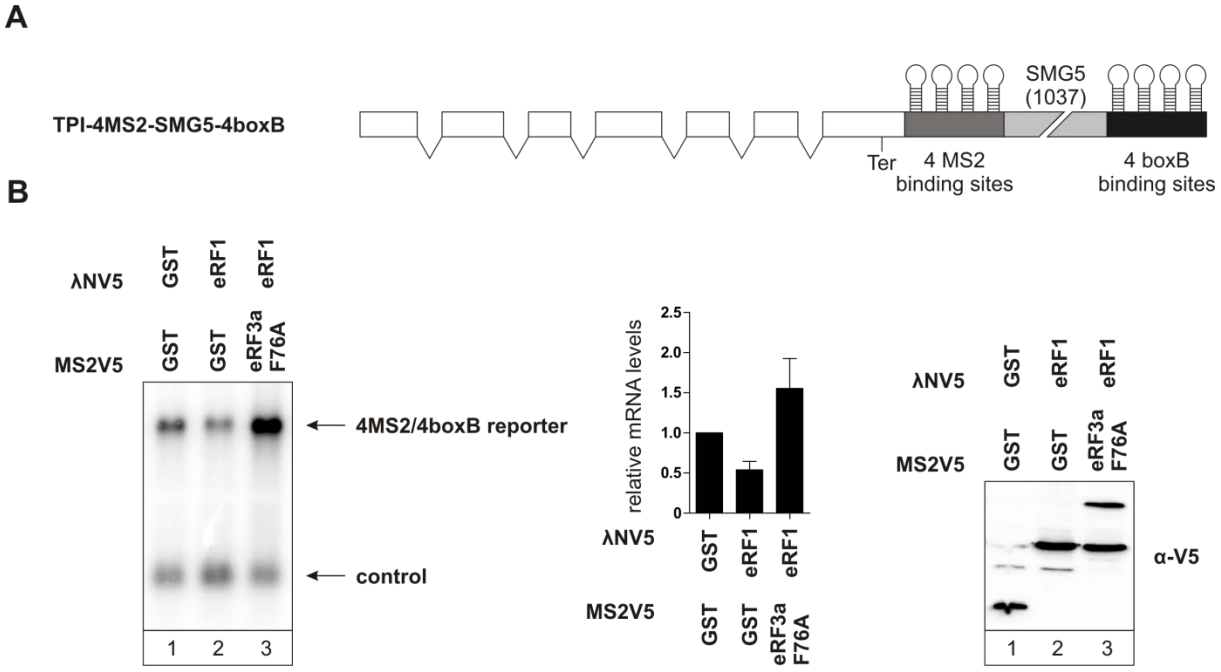

Figure 17: PABPC1-interacting proteins fold back the poly(A)-tail to suppress NMD

(A) Schematic representation of the tandem tethering reporter harboring 4MS2/4boxB tethering sites downstream of TPI. The SMG5 3′ UTR is enclosed by the two tethering sites of the reporter. **(B)** Northern blot showing NMD suppression by using a tandem tethering approach on opposite ends of the 3′ UTR. Quantification with standard deviation is represented with black bars and protein expression was shown with anti-V5 antibody.

4.8.2 Heterodimer formation of synthetic peptides suppresses NMD by folding back the poly(A)-tail

Next, I tethered small, artificial peptides, which are able to interact and therefore fold back the poly(A)-tail in close proximity of the termination codon, 5′ and 3′ of a 3′ UTR. To achieve this, I made us of synthetic 45aa to 50aa coiled-coil peptides called SYNZIPs (Thompson et al., 2012). These SYNZIPs can form heterodimers and can potentially be used to fold back the poly(A)-tail into close proximity of a termination codon. In this study, I made use of SYNZIP 1 (45aa), SYNZIP 2 (50aa), and SYNZIP 14 (47aa). SYNZIP 2 is able to interact with both SYNZIP 1 and 14, whereas SYNZIP 1 and 14 are unable to interact, thereby serving as a negative control (Figure 18B). I employed a tethering reporter with 4PP7 binding sites 5′ of, and 4MS2 binding sites 3′ of the SMG5 3′ UTR (Figure 18A). Tethering SYNZIPs 1 and 2 to the aforementioned reporter mRNA led to an about 3.5 fold increase of mRNA reporter levels (Figure 18C, lanes 4 and 5). The observed effect was specific as SYNZIPS 1 and 14, which do not form a heterodimer, were unable to antagonize degradation of the reporter (Figure 18C, lane 6). These results show that small, artificial peptides are able to suppress NMD by folding the

poly(A)-tail so that PABPC1 is in close proximity of the termination codon. This shows further, that PABPC1 is able to strongly counteract NMD even when recruited indirectly into the vicinity of a termination ribosome.

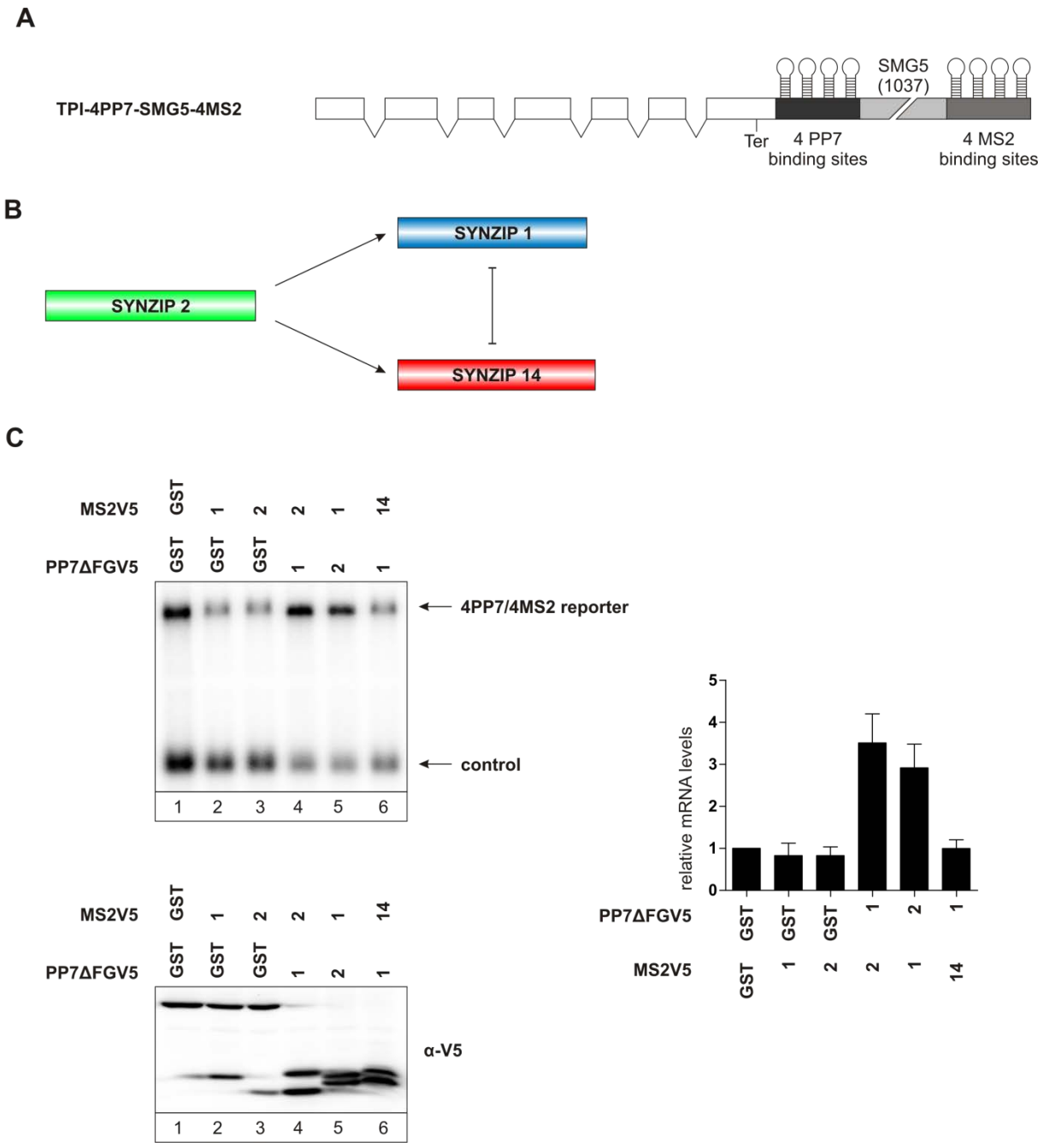

Figure 18: Synthetic SYNZIP peptides antagonize NMD by folding back the poly(A)-tail

(A) Schematic representation of the tandem tethering reporters harboring 4PP7/4MS2 tethering sites downstream of TPI. The SMG5 3′ UTR is enclosed by the two tethering sites. **(B)** Representation of the synthetic SYNZIP proteins and their interaction patterns. **(C)** Northern blot showing NMD suppression by tandem tethering of synthetic SYNZIPs. Quantification is represented as black bars with standard deviation indicated. V5 antibody was used to check protein expression.

4.9 PAM2 motifs are sufficient to antagonize NMD

As mentioned in the introduction, PABPC1 contains a well-conserved 70 amino acid domain termed MLLE in its C-terminal region (Kozlov et al., 2001). It has been shown that this MLLE domain specifically recognizes a small peptide sequence called PAM2 motif (Kozlov et al., 2004). Initially, three different proteins were identified carrying a PAM2 motif: PAIP1 (PABP-interacting protein 1), PAIP2, and eRF3 (Kozlov et al., 2001). Interestingly, eRF3 is different compared to other PAM2-containing proteins since it carries two overlapping PAM2 motifs (Kozlov et al., 2004). Other PAM2-containing proteins have been identified based on sequence homology (Kozlov and Gehring, 2010). Since PABPC1 strongly interacts with proteins harboring a PAM2 motif I made use of isolated PAM2 motifs of several proteins to recruit PABPC1 to an NMD-targeted reporter (Figure 19).

Figure 19: Amino acid make up of PAM2 motifs

Essential amino acids for interaction with PABPC1 are highlighted as gray boxes. Numbers left and right of the amino acid sequences refer to the amino acid position of the PAM2 motif in the respective proteins. The red colored amino acid indicates a mutation.

4.9.1 PABPC1 recruitment via PAM2 motifs efficiently antagonizes NMD

In this experiment I used a FLAG-tagged version of the TPI-4MS2-SMG5 reporter that allows for detection of changed expression levels due to tethered proteins by western blotting. Tethering the MS2V5-tagged overlapping N- and C- terminal eRF3a PAM2 motif to a FLAG-TPI-4MS2-SMG5 reporter mRNA stabilized the reporter about fivefold (Figure 20, lane 2). As a negative control, I used the PAM2 motif of eRF3a F76A, as it should be unable to interact with PABPC1 (Kononenko et al., 2010; Kozlov and Gehring, 2010; Osawa et al., 2012). Tethering MS2V5-eRF3a F76A PAM2 to the reporter mRNA still

stabilized the mRNA, however, to a much smaller extent compared to eRF3a containing both PAM2 motifs (Figure 20, lane 3).

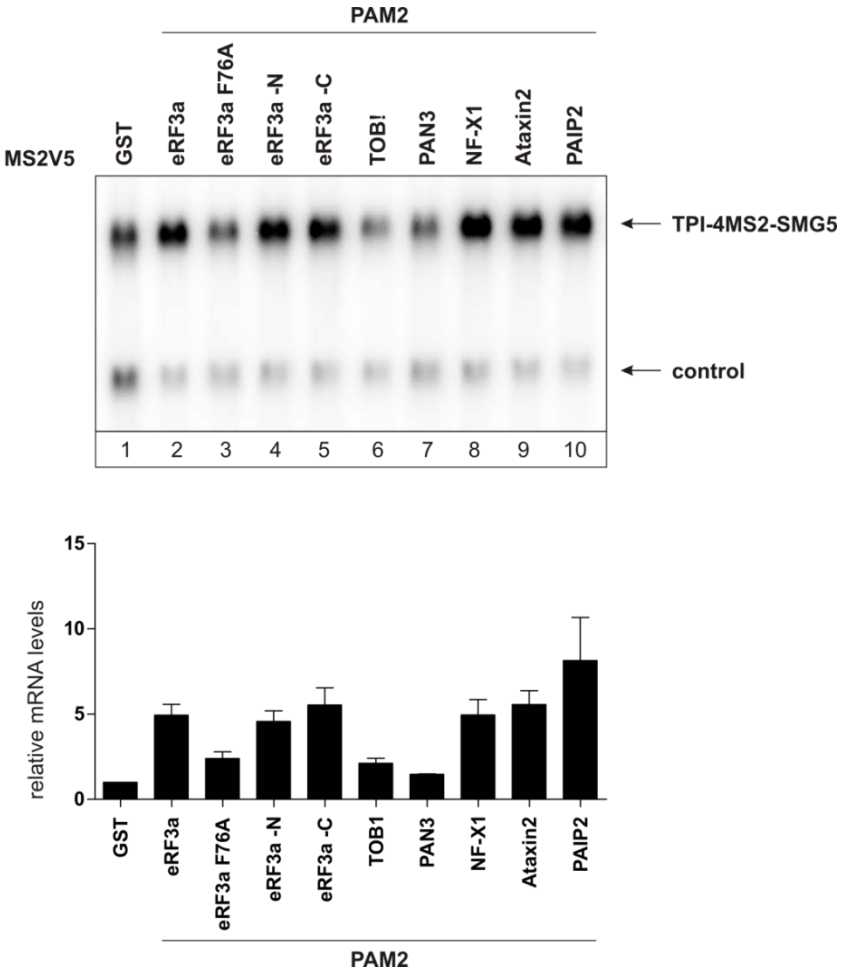

Figure 20: PAM2 motifs are sufficient to suppress 3′ UTR-mediated NMD

Northern blot indicating mRNA abundance after tethering several PAM2 motifs to the reporter. Quantification with standard deviation is represented with black bars.

Similarly, tethering the N- and C-terminal PAM2 motifs of eRF3a separately to the NMD-targeted reporter increased the reporter mRNA levels also about fivefold (Figure 20, lanes 4 and 5). The PAM2 motifs of NF-X1, Ataxin2, and PAIP2 also increased the reporter mRNA levels by five to eight fold upon tethering (Figure 20, lanes 8, 9, and 10). Surprisingly, not all PAM2 motifs behaved as expected. Tethering MS2V5-tagged TOB1 and PAN3 PAM2 motifs to the reporter did not lead to an increase in mRNA levels of about fivefold similar to the other PAM2 motifs. Instead, tethering these two PAM2 motifs led to an increase of about the same magnitude as when tethering the eRF3a F76A PAM2 motif (Figure 20, lanes 6 and 7). This result was surprising since all the

PAM2 motifs share the crucial residues for interaction with PABPC1 (residues highlighted in gray boxes in Figure 19). Interestingly, PAIP2 PAM2 was able to stabilize the reporter mRNA more strongly than the other PAM2 motifs. The reason behind these different stabilization patterns was unknown at the time and subject to further research discussed in section 4.9.3.

4.9.2 Tethering PAM2 motifs has no negative effect on translation of the reporter transcript

Given the strong increase in mRNA levels upon tethering PAM2 motifs to the reporter I wanted to check the effect on translation to ensure that NMD is not inhibited due to the lack of active translation in the system. To this end, I employed the N-terminal FLAG-tag of the TPI reporter to quantify protein expression compared to FLAG-emGFP, which was transfected as control for protein expression (Figure 21).

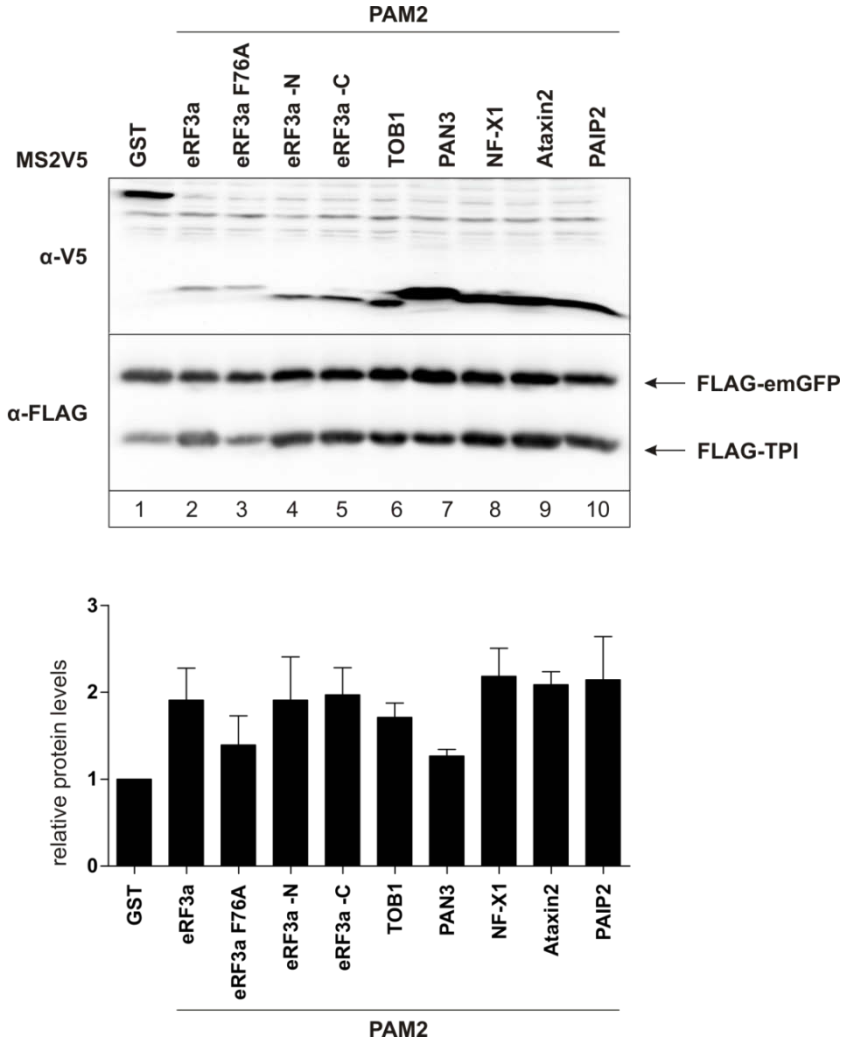

Figure 21: Translation of the reporter is not negatively affected by PAM2 motif tethering

Western blot of PAM2 motifs visualized with a V5 antibody. Anti-FLAG antibody was used to show FLAG-emGFP (transfection control) and FLAG-TPI protein levels. Quantification of protein levels are represented with black bars and standard deviation as indicated.

The protein levels upon tethering were increased by a maximum of two fold for the tethered PAM2 motifs (Figure 21). The PAM2 motifs that lacked a stabilization of the reporter mRNA showed the least change in reporter expression compared to the negative control GST (Figure 21). This result ensures that the expression of the reporter transcript is not negatively altered and the stabilization effect of the PAM2 motifs is in fact due to inhibiting NMD.

4.9.3 Not all PAM2 motifs show the expected interaction with PABPC1

To ensure that the observed increase in reporter mRNA is due to the interaction of the tethered PAM2 motifs with PABPC1 I conducted a co-immunoprecipitation experiment. FLAG-emGFP-tagged versions of the PAM2 motifs used in the tethering assay were transfected and subjected to a FLAG pulldown. I used an antibody against endogenous PABPC1 to check for interactions between PAM2 motifs and PABPC1. The strongest interaction was observed for the overlapping eRF3a PAM2 motifs (Figure 22, lane 2, IP panel).

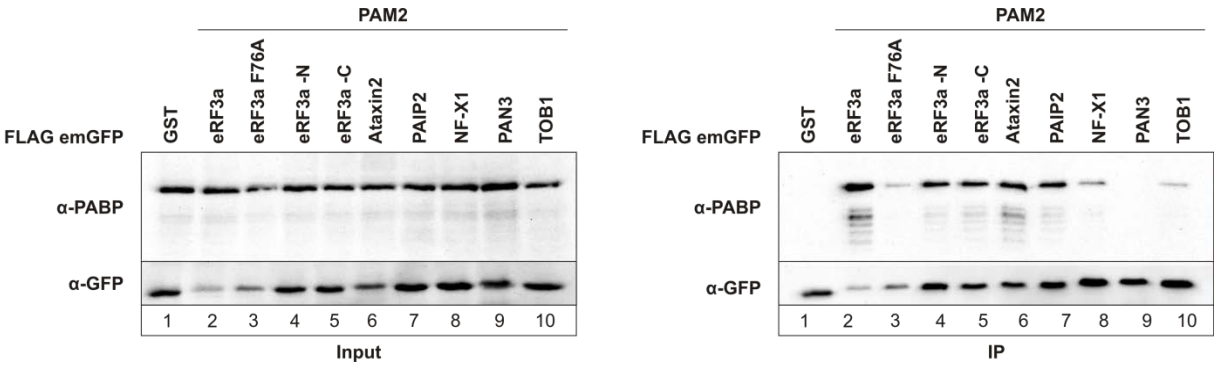

Figure 22: PAM2 motifs show different interaction strength with PABPC1

Co-immunoprecipitation (left: input, right: IP) of FLAG-emGFP and endogenous PABPC1 visualized with anti-PABP antibody. Anti-GFP antibody was used to visualize co-transfected loading control.

As expected, eRF3a F76A PAM2 pulled out significantly less endogenous PABPC1 (Figure 22, lane 3, IP panel). The residual interaction between eRF3a F76A and endogenous PABPC1 can be attributed to the second phenylalanine still present in this mutant. A strong interaction between eRF3a –N PAM2, eRF3a –C PAM2, Ataxin2 PAM2, PAIP2 PAM2, and endogenous PABPC1 was observed (Figure 22, lanes 4, 5, 6, and 7, IP panel).

NF-X1 PAM2 and TOB1 PAM2 pulled out less endogenous PABPC1, whereas PAN3 PAM2 did not interact with endogenous PABPC1 at all (Figure 22, lanes 8, 10, and 9, IP panel). These results explain the only weak NMD-inhibiting effect by the tethered PAM2 motifs of TOB1, PAN3, and the negative control eRF3a F76A PAM2. It is surprising however that some PAM2 motifs are unable to interact with PABPC1 even though the essential amino acids for the interaction with PABPC1 are present.

4.9.4 NMD suppression by PAM2 motifs is a specific tethering effect

To make sure that the observed stabilization effects of the PAM2 motifs are position specific tethering effects, I made use of the control reporters described in figure 5. Additionally, I used a TPI-SMG5 reporter without any tethering sites to test for *trans* effects (Figure 23A).

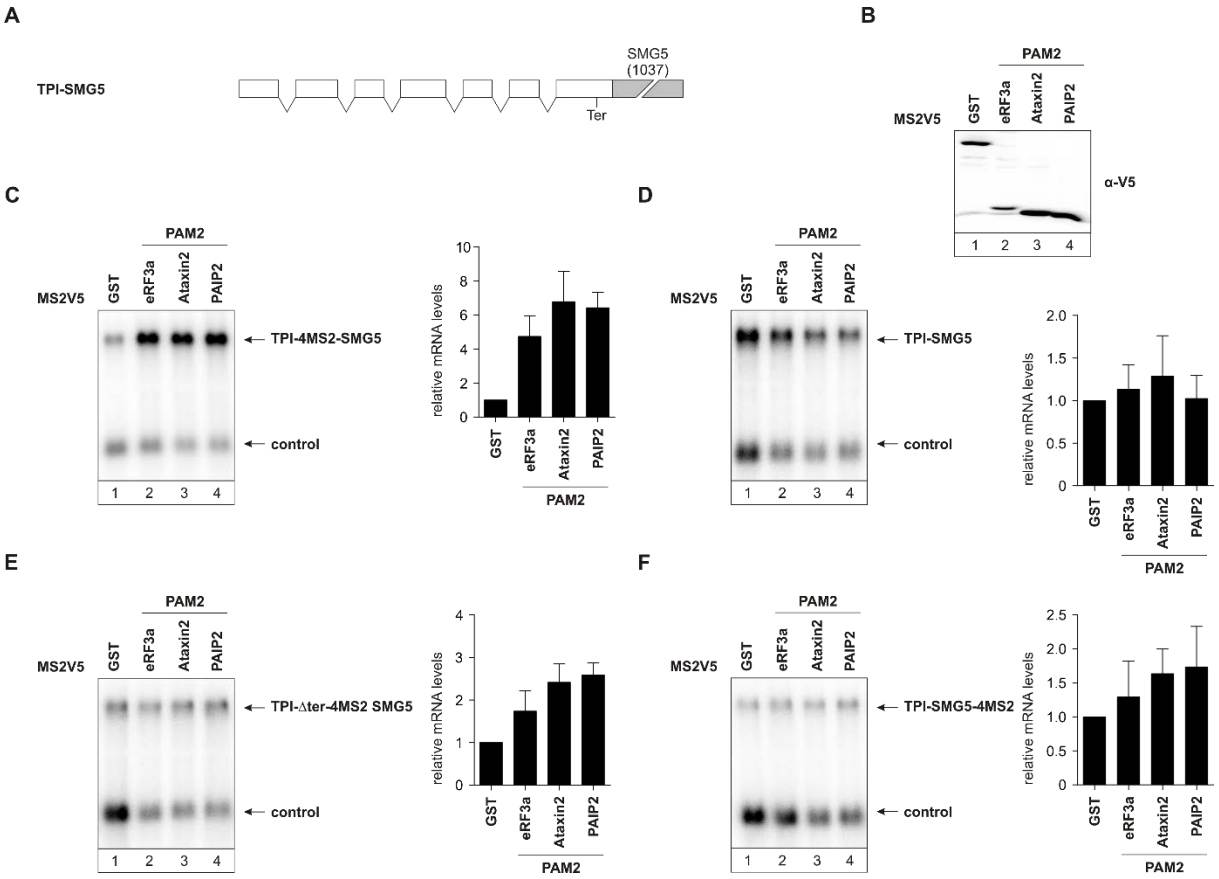

Figure 23: NMD suppression by PAM2 motifs is a specific tethering effect

(A) Additional control reporter mRNA harboring no MS2 sites. **(B)** Protein expression is shown with anti-V5 antibody. **(C, D, E, F)** Northern blot indicating mRNA levels of different reporters upon tethering GST (negative control) and several PAM2 motifs to the reporter. Quantifications with standard deviation are shown as black bars.

For this experiment I only used the PAM2 motifs of eRF3a, Ataxin2, and PAIP2 as these showed the strongest NMD inhibition and these were used in subsequent experiments. First, I tethered the PAM2 motifs to the standard TPI-4MS2-SMG5 tethering reporter for a direct comparison against the negative control reporters (Figure 23C). Tethering eRF3a PAM2 motif to the reporter showed the smallest increase of reporter mRNA abundance by about four fold compared to the negative control (Figure 23C, lane 2). Both the PAM2 motifs of Ataxin2 and PAIP2 showed a stronger stabilization of the reporter transcript by about six fold (Figure 23C, lanes 3 and 4). Expressing the MS2V5-tagged PAM2 motifs alongside a TPI-SMG5 reporter without any tethering sites did not lead to any increase in reporter levels compared to the negative control (Figure 23D). This indicates, that the PAM2 NMD suppression effects are specific tethering effects and the PAM2 motifs do not act in *trans*. The reporter construct for which the termination codon was moved downstream of the 4MS2-binding sites still showed a moderate increase in mRNA abundance of about 1.5 to 2.5 fold upon tethering of the PAM2 motifs (Figure 23E). This increase in mRNA abundance, even though much less compared to the reporter with the termination codon upstream of the 4MS2-binding sites, is unexpected. A possibly explanation for the observed effect could be that the tethered PAM2 motifs might lead to steric blocking of the ribosome by recruiting PABPC1. Less efficient displacements of the tethered proteins from the reporter by the ribosome could lead to the slight increase in mRNA abundance observed here. The final control reporter used here has the 4MS2 binding sites moved downstream of the SMG5 3′ UTR. Tethering the PAM2 motifs to this reporter gave a small increase in reporter levels of about 1.5 fold when tethering Ataxin2 PAM2 and PAIP2 PAM2, and no increase when tethering eRF3a PAM2 (Figure 23F lanes 3, 4, and 2 respectively). The small increase observed for some of the control reporters is significantly less than compared to the TPI-4MS2-SMG5 reporter, showing that the PAM2 tethering effects are specific to tethering and not due to expression of the PAM2 motifs in *trans*.

4.9.5 EJC-factor mediated NMD is antagonized by PAM2 motifs

Previously, I showed that PABPC1 is unable to suppress NMD mediated by an EJC. However, tethering PAM2 motifs gave rise to even more mRNA reporter abundance due to NMD suppression when tethered upstream of the NMD-inducing 3′ UTR of SMG5. Specifically, the PAM2 motif of PAIP2 showed the strongest increase in reporter levels. Since the PAIP2 PAM2 has such a strong effect, I wanted to examine whether the PAM2

motif of PAIP2 is able to antagonize NMD mediated by EJC factors. To this end, I used a β-globin wildtype reporter harboring 4MS2 and 4PP7 tethering sites in tandem (Figure 24A).

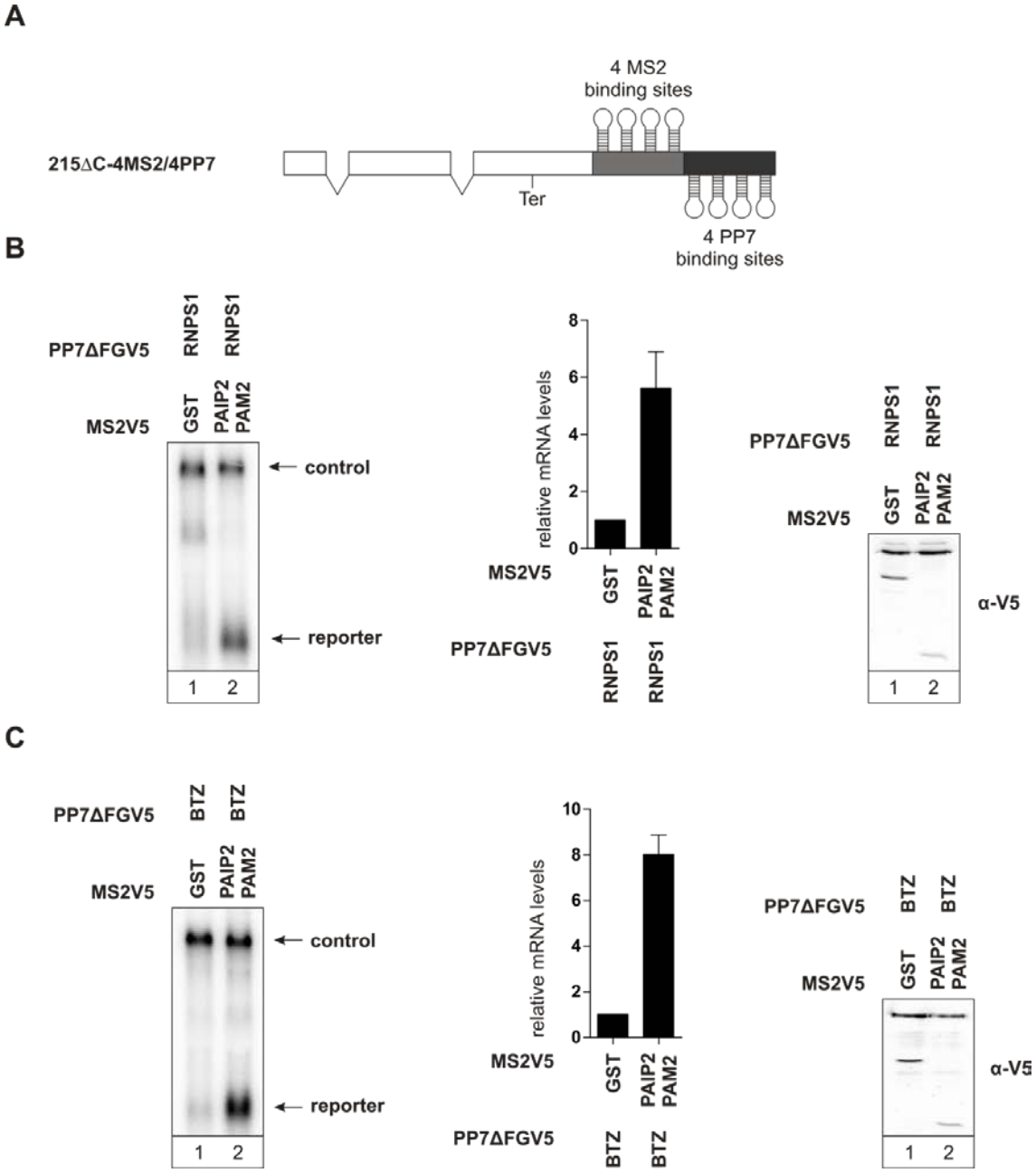

Figure 24: Antagonizing NMD initiated by tethered EJC factors

(A) Schematic of a β-globin reporter mRNA harboring 4MS2 sites upstream of 4PP7 sites. **(B)** Reporter mRNA abundance after tethering RNPS1 downstream of GST (negative control) or PAM2 motif of PAIP2 shown in a northern blot. Quantification with standard deviation is represented with black bars. Protein expression is shown with anti-V5 antibody. **(C)** Levels of reporter mRNA when tethering BTZ downstream of GST (negative control) or downstream of PAIP2-PAM2. Black bars represent quantification with standard deviation and protein expression was checked with anti-V5 antibody.

This reporter allows for the dual tethering of two proteins in a specific orientation. I first made use of an auxiliary component of the EJC termed RNPS1. Tethering MS2V5-PAIP2 PAM2 upstream of PP7ΔFGV5-RNPS1 led to an about six fold increase in reporter mRNA levels (Figure 24B, lane 2). Tethering the PAIP2 PAM2 motif upstream of the EJC core component BTZ gave rise to an even greater stabilization of the reporter by about eight fold (Figure 24C, lane 2). The strong NMD suppression effects observed here were unexpected as tethering PABPC1 directly upstream of the MINX intron did not lead to any NMD suppression. In contrast, I observe here that recruiting PABPC1 indirectly via a PAM2 motif upstream of an EJC factor can substantially increase mRNA reporter levels by antagonizing NMD.

4.9.6 PAM2 motifs suppress NMD of an intron-carrying reporter

In the previous section I explored the ability of PAM2 motifs being able to suppress NMD initiated by the EJC-components RNPS1 and BTZ. I have shown previously that PABPC1 is unable to suppress NMD of a MINX intron-carrying reporter (Figure 10). Since tethering PAM2 motifs upstream of EJC components were able to antagonize NMD, I next wanted to test the ability of PAM2 motifs to suppress EJC-mediated NMD. To this end, I made use of the TPI-4MS2-MINX-SMG5 reporter described in figure 10A. I observed a five to six fold increase in reporter mRNA abundance upon tethering MS2V5-tagged eRF3a PAM2, Ataxin2 PAM2, and PAIP2 PAM2 motifs upstream of the MINX intron (Figure 25).

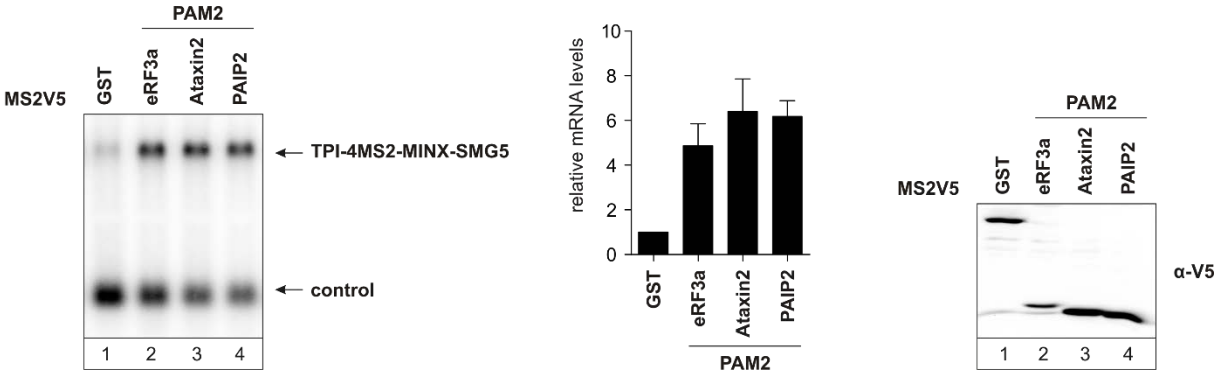

Figure 25: EJC-mediated NMD suppression of PAM2 motifs

Northern blot showing reporter mRNA abundance upon tethering PAM2 motifs upstream of a MINX intron. Quantifications with standard deviation are shown as black bars. Protein expression is shown with anti-V5 antibody.

These results are surprising as tethering PABPC1 directly to the aforementioned reporter does not have any effect. However, recruiting PABPC1 through a small PAM2 motif leads to a significant increase in mRNA abundance. The reason for this discrepancy in the observed effect between tethering PABPC1 directly and recruiting PABPC1 through a PAM2 motif is currently unknown and subject to further research.

4.10 PABPC1 suppresses NMD of the β-globin PTC39 mRNA

Human β-globin carrying a PTC at position 39 has been identified in patients suffering from β-thalassemia and is a well-known NMD target (Baserga and Benz, 1988; Pergolizzi et al., 1981; Thermann et al., 1998). Here, I made use of a reporter carrying 4MS2 binding sites directly after the PTC at position 39. The remainder of the β-globin DNA was cloned after the 4MS2 binding sites (Figure 26A).

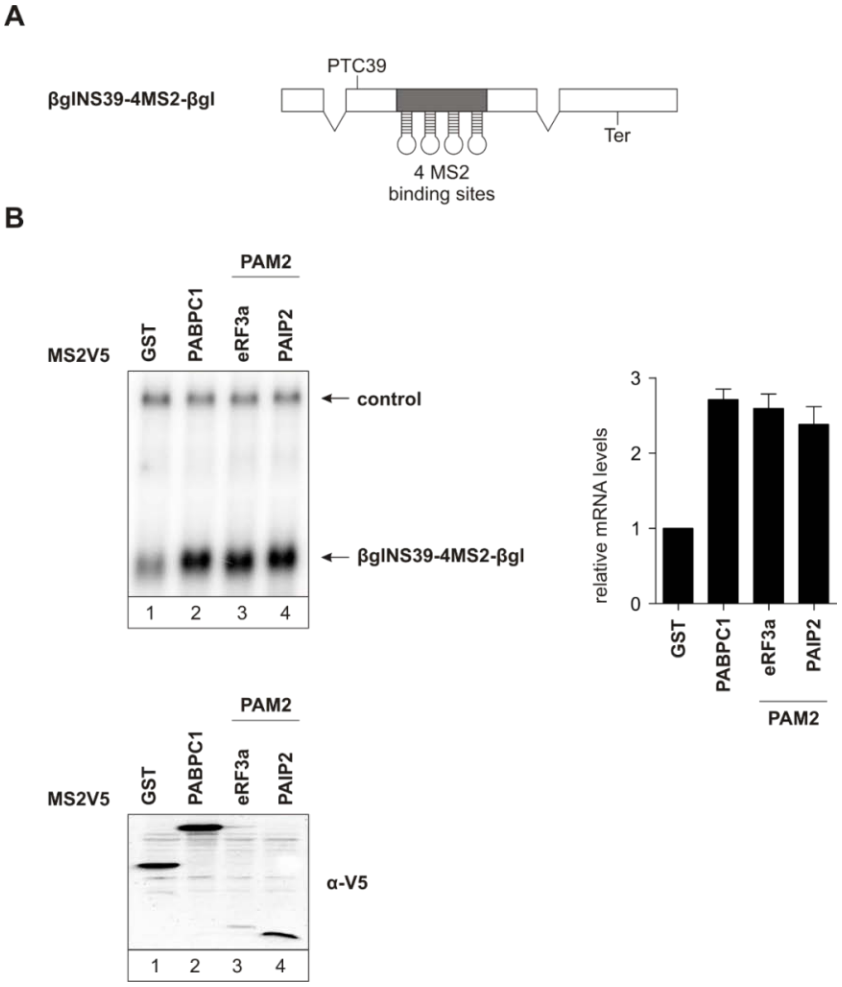

Figure 26: NMD-mediated degradation of β-globin PTC39 is suppressed by tethering PABPC1 and PAM2 motifs immediately downstream of PTC39

(A) Reporter mRNA schematic showing location of 4MS tethering sites immediately downstream of the PTC at position 39 and upstream of the remainder of β-globin. **(B)** Northern blot showing mRNA reporter

levels after tethering PABPC1 and two PAM2 motifs to the reporter. Quantification and standard deviation are shown with black bars. Protein expression was tested with anti-V5 antibody.

This setup allows tethering of NMD suppressing factors in a close to endogenous setting, namely downstream of a PTC where a ribosome stalls and upstream of an EJC that recruits NMD factors, thereby initiating NMD. I observed here that tethering MS2V5-PABPC1 to this reporter led to an increase in mRNA reporter abundance by about three fold (Figure 26B, lane 2). This stabilizing effect is contrary to what I observed previously as PABPC1 was unable to suppress NMD of a reporter carrying a MINX intron. The same magnitude of stabilization was observed when tethering the PAM2 motifs of eRF3a and PAIP2 to the reporter (Figure 26B, lanes 3 and 4). I can show here that PABPC1 either tethered directly or recruited indirectly via PAM2 motifs upstream of an EJC has the capability to suppress NMD of an endogenous NMD target. These results are intriguing as they show that a well-known NMD-target which has a clinical manifestation could potentially be suppressed or inhibited by directly or indirectly recruiting PABPC1 upstream of the EJC.

5 Discussion

Nonsense-mediated mRNA decay has been researched in great detail over the last decades. The molecular process including the activation of NMD as well as the physiological function of this pathway have been subjected to many studies elucidating the intricacies of this quality control mechanism. In contrast, the mechanism by which NMD is suppressed is only poorly understood. It has previously been shown that PABPs are involved in the regulation and suppression of NMD in different organisms.

In yeast, NMD is believed to solely be initiated by the length of the 3′ UTR. The so-called *faux 3′ UTR* model states that a termination codon in yeast is recognized as premature by a specific set of proteins that mark the transcript for degradation by influencing translation termination (Amrani et al., 2004). According to this model, terminating ribosomes at regular stop codons will interact with Pab1 bound to the poly(A)-tail, resulting in proper translation termination. A PTC increases the length of the 3′ UTR, which causes inefficient or slow communication between the terminating ribosome and Pab1. Thus, NMD factors assemble on the transcript, which subsequently initiate the rapid degradation of the mRNA (Amrani et al., 2004; Amrani et al., 2006).

In flies, the *faux 3′ UTR* model holds only partially true. Pabpc1 was shown to suppress transcripts with unusually long 3′ UTRs in flies. Transcripts with regular stop codons can be targeted by NMD when the 3′ UTR length is artificially increased. However, not all unusually long 3′ UTR containing transcripts are targeted by NMD (Behm-Ansmant et al., 2007). This suggests that some 3′ UTRs have certain elements that help them avoid degradation via NMD.

In contrast, human NMD is more complex as it can be activated by multiple signals and degradation is achieved by multiple decay pathways involving exo- and endonucleolytic degradation (Hwang and Maquat, 2011). The two ways in which human NMD can be activated are either EJC-dependent or EJC-independent. The former harbors a stop codon upstream of a spliced intron resulting in the EJC deposition on the mRNA, whereas the latter is activated due to a long 3′ UTR (Schweingruber et al., 2013).

The inhibiting properties of PABPC1 have been studied in other organisms as well as in human cells. However, studying PABPC1 in the latter proofs more difficult as PABPC1 is involved in multiple mechanisms vital to cell survival and can therefore not simply be depleted. Nonetheless, a role for PABPC1 has been described in human NMD

suppression. In this thesis, I propose an updated mechanism on the importance and role of PABPC1 in NMD suppression. Furthermore, I illuminate several different methods by which NMD can be antagonized.

5.1 NMD suppression of long 3′ UTR substrates

5.1.1 PABPC1 is a general suppressor of long 3′ UTR mediated NMD

Other organisms have displayed an NMD-inhibiting function of PABP on unnatural 3′ UTR substrates. Here, I show that PABPC1 is capable of suppressing NMD of a TPI reporter targeted by NMD due to a long 3′ UTR. To this end, I used a shortened version of the SMG5 3′ UTR, which has previously been reported to be subjected to NMD (Boehm et al., 2014; Singh et al., 2008). Tethering PABPC1 directly downstream of the termination codon leads to a stabilization of the reporter mRNA. To ensure that this NMD suppression effect is not limited to the SMG5 3′ UTR but rather a general NMD inhibition effect applicable to all 3′ UTR targeted mRNAs, I made use of two additional 3′ UTRs: PDRG1 and SURF6. Both PDRG1 and SURF6 are endogenous NMD targets, which have been identified in a transcriptome-wide screen and were shown to be degraded by NMD due to their 3′ UTR (unpublished data, Gehring lab). I was able to suppress the degradation of a TPI reporter carrying either the PDRG1 or SURF6 3′ UTR by tethering PABPC1 just downstream of the termination codon. I used several control reporters to confirm the suppression effect is due to directly tethering of PABPC1 downstream of the termination codon and not due to *trans* effects. Furthermore, our lab has used siRNA mediated depletion of UPF1, UPF2, and SMG6 to show that PABPC1 acts as a suppressor in the canonical NMD pathway (Fatscher et al., 2014). Additionally, half-life measurements showed that upon PABPC1-tethering to the NMD-targeted substrates the half-life was increased, indicating efficient NMD suppression (Fatscher et al., 2014). Besides SMG6-mediated endocleavage, future research needs to address whether all other NMD executing pathways, including the SMG5/SMG7 mediated exonucleolytic degradation, are suppressed by PABPC1 as well.

5.1.2 Implication of translation termination in NMD suppression by PABPC1

As mentioned above, it is believed that translation termination is implicated in NMD suppression in yeast. In this thesis, I utilized the essential translation termination factor eRF3a, (Chauvin et al., 2005; Janzen and Geballe, 2004). A current model suggests that

the determining factor whether or not a long 3′ UTR mRNA is targeted by NMD is the competition between UPF1 and PABPC1 for eRF3a-binding at the terminating ribosome (Singh et al., 2008). According to this model, a normal 3′ UTR has a short distance between the terminating ribosome and PABPC1. Therefore, PABPC1 is able to efficiently interact with the ribosome, resulting in normal translation termination. In contrast, a long 3′ UTR allows UPF1 to interact with the terminating ribosome instead due to PABPC1 being further removed from the termination codon and, as a result, activate NMD. To confirm this model, I made use of a PABPC1 mutant unable to interact with eRF3a. Contrary to what the competition model implies, the eRF3a-binding deficient PABPC1 mutant was still able to efficiently suppress NMD just like wildtype PABPC1. This indicates that the competition between UPF1 and PABPC1 for eRF3a-binding is not the sole determinant for NMD activation or inhibition. Interestingly, tethering eRF3a to the NMD-targeted reporter still suppresses degradation. At first, this was surprising in light of the previous results as PABPC1 does not require eRF3a for NMD suppression. To gain further insight into the importance of the eRF3a-PABPC1 interaction in NMD suppression, I made use of an eRF3a point mutant unable to interact with PABPC1. Upon tethering eRF3a F76A to the reporter, eRF3a loses its ability to suppress NMD. This result points to a linear cascade from the terminating ribosome over eRF3a to PABPC1 in order to achieve NMD suppression. Knowing that translation termination is involved in NMD suppression by PABPs in other organisms and mRNA circularization plays a role in translation termination I looked at the potential involvement of eIF4G in antagonizing NMD next. Since eIF4G acts as an intermediator between PABPC1 and the translation initiation complex, resulting in mRNA circularization, I next employed a PABPC1 mutant unable to interact with eIF4G (Amrani et al., 2008; Kahvejian et al., 2005; Wells et al., 1998). Surprisingly, tethering this PABCP1 mutant did no longer suppress NMD. This shows that PABPC1 requires the interaction with eIF4G to antagonize NMD. Since the interaction between PABPC1 and eIF4G is believed to mediate the circularization of the mRNA as well as facilitate ribosome recycling, combining these results points to proper translation termination as well as mRNA circularization as important factors in NMD suppression (Model in figure 27).

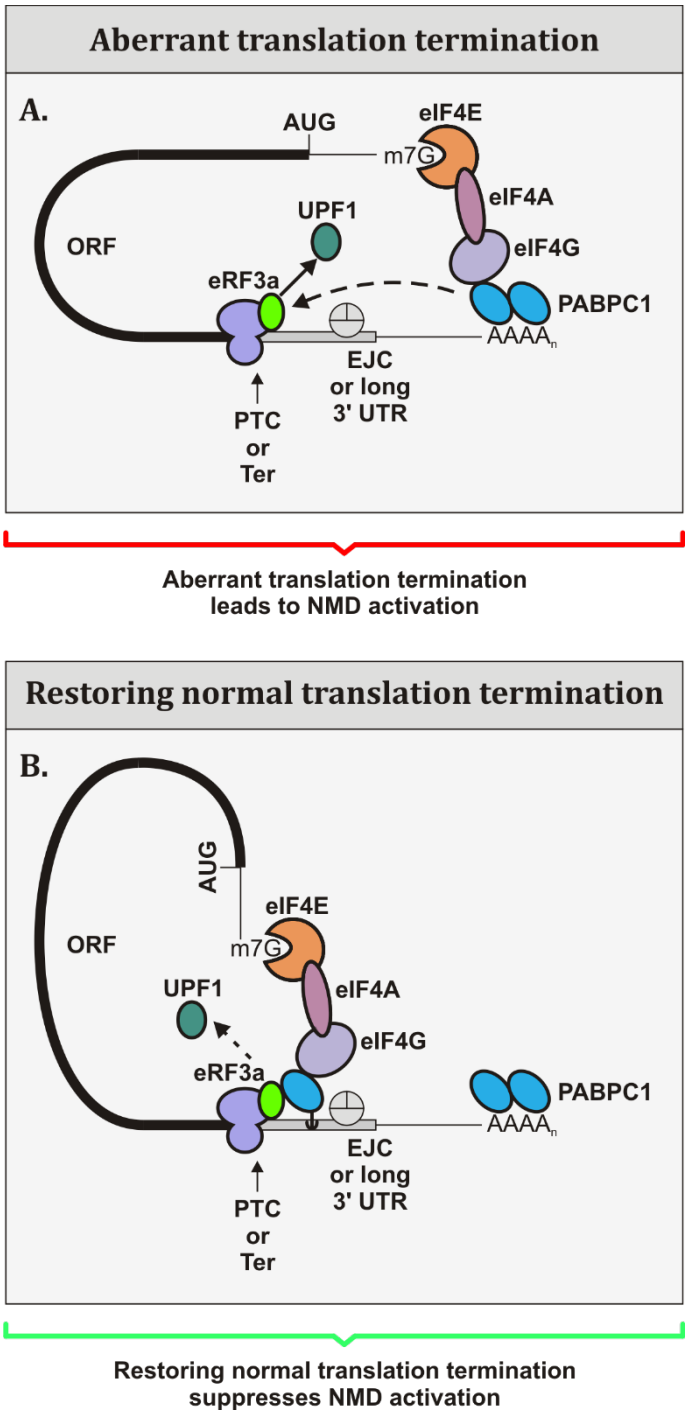

Figure 27: Model of NMD suppression by PABPC1

(A) Due to a large physical distance between PABPC1 and the terminating ribosome resulting from a PTC or a long 3′ UTR, PABPC1 is unable to efficiently interact with the release factors bound to the stalling ribosome. Instead, UPF1 can interact with the release factors, thereby recruiting other NMD factors, which in turn initiate degradation of the transcript. **(B)** In order for PABPC1 to suppress NMD activated because of the large physical distance between poly(A)-bound PABPC1 and the stalling ribosome, PABPC1 needs to be recruited into close proximity of the terminating ribosome. By recruiting PABPC1 immediately downstream of the stalling ribosome, it can interact efficiently with the release factors, thereby promoting proper translation termination and as a result suppress NMD.

I also saw that an eIF4G mutant unable to interact with PABPC1 is still able to suppress NMD, although to a lesser extent. This could be explained due to eIF4G being able to form a closed looped mRNA when tethered directly downstream of the stop codon, thereby circumventing the need of interacting with PABPC1. However, since the mutant unable to interact with PABPC1 showed less increase in mRNA abundance, it seems that the interaction between eIF4G and PABPC1 is favorable for NMD suppression.

5.1.3 Protein-mediated NMD inhibition

Other studies have shown that the mere length of a 3′ UTR is not the determining factor for whether or not NMD is activated (Toma et al., 2015). Instead the composition of the 3′ UTR and potential proteins bound to the 3′ UTR are believed to play a role in whether or not NMD is activated. There seem to be at least two different mechanisms by which some endogenous mRNAs with long 3′ UTRs are able to evade NMD (Toma et al., 2015). Firstly, a 200 nucleotide A/U-rich region immediately downstream of a termination codon has been shown to be responsible for NMD suppression (Toma et al., 2015). This region could potentially be bound by an as of yet unknown protein or proteins, which are either able to block NMD by disrupting the interaction of eRF3a with UPF1, or by potentially interacting with PABPC1 directly. Secondly, some mRNAs with a long 3′ UTR evade NMD but due to a different, unknown mechanism. In this case, the first 600 nucleotides of a long 3′ UTR known to evade NMD were not enough to suppress NMD, indicating an alternative mechanism of NMD suppression (Toma et al., 2015).

An example of a protein bound to the 3′ UTR of some transcripts is PTBP1. This protein has multiple functions in, for example, splicing, 3′ end processing, and mRNA stability (Castelo-Branco et al., 2004; Knoch et al., 2004; Wagner and Garcia-Blanco, 2001). It has been shown that PTBP1 bound closely downstream of a termination codon of a long 3′ UTR-mediated NMD substrate can suppress NMD (Ge et al., 2016). Transcriptome-wide analysis showed that PTBP1 preferentially binds close to termination codons. Ge et al. further identify several endogenous NMD targets protected by PTBP1 from degradation. The publication describes that PTBP1 binds preferentially to CU-rich regions and that a single PTBP1 protein has multiple RNA-interaction sites. Therefore it could be feasible that PTBP1 bound to the mRNA could compact the 3′ UTR by binding stretches of the 3′ UTR further apart. By doing so PABPC1 would be brought into closer proximity of the terminating ribosome, which could lead to NMD suppression. I show here, that tethering PTBP1 downstream of a stop codon, but upstream of the SMG5 3′ UTR strongly

suppresses NMD. The mechanism of compacting the 3′ UTR discussed above could be responsible for NMD suppression by PTBP1 in this scenario. Another possibility would be that PTBP1 antagonizes NMD by blocking UPF1 from binding to the 3′ UTR. This function of PTBP1 goes hand in hand with recent findings about UPF1-mediated NMD activation of long 3′ UTR substrates. It has first been proposed that UPF1 occupancy on a long 3′ UTR is the determining factor of NMD activation. This is supported by the fact that UPF1 populates the 3′ UTR of many transcripts (Hurt et al., 2013; Kurosaki and Maquat, 2013; Zund et al., 2013). However, it has recently been challenged that the mere occupancy of UPF1 on the 3′ UTR is responsible for NMD activation, but instead the phosphorylation state of UPF1 determines if NMD is activated or not (Kurosaki et al., 2014). The degree of phosphorylation and occupancy of UPF1 might differ from transcript to transcript depending on the composition of the 3′ UTR or on other proteins with a stronger binding affinity for the 3′ UTR, thereby prohibiting UPF1 from interacting with the mRNA. This would explain why some long 3′ UTRs are subjected to degradation via NMD whereas others are immune. The exact determinant that regulates phosphorylation of 3′ UTR-bound UPF1 and thereby initiates NMD remains elusive.

5.1.4 Peptide-mediated fold-back of the poly(A)-tail

It has previously been reported that an NMD-targeted reporter harboring a PTC can be stabilized by introducing two complementary RNA stretches 5′ and 3′ of the 3′ UTR (Eberle et al., 2008). The publication shows that the two complementary RNA stretches interact and thereby fold back the poly(A)-tail into close proximity of the termination codon. This effectively brings PABPC1 into close proximity of the terminating ribosome and can suppress NMD. I here show that it is possible to suppress NMD by indirectly recruiting PABPC1 close to a termination codon by peptide-mediated fold-back of the poly(A)-tail. The peptide-mediated fold-back of the poly(A)-tail suggests a possible NMD evasion mechanism in the cell by *cis*-acting proteins. Contrary to a single protein like PTBP1 binding the mRNA multiple times and thereby potentially looping PABPC1 closer to the termination codon, I show that two heterodimer-forming proteins can potentially recruit PABPC1 indirectly. This heterodimer formation has the potential to fold back the poly(A)-tail, thereby bringing PABPC1 into close proximity of the terminating ribosome. Here, I made use of three synthetic peptides to simply fold back the poly(A)-tail and therefore suppressing NMD. Since I used synthetic peptides with no other known interaction partners in the cell, I can show that the folding mechanism is sufficient and

no additional proteins need to be recruited to suppress NMD in this case. Recent studies have also shown evidence for a multitude of previously undiscovered RNA-binding proteins (RBPs) some of which are likely to bind to distinct 3′ UTRs (Baltz et al., 2012; Castello et al., 2012). It is possible that some RBPs are able to form hetero- or homodimers while interacting with opposite ends of a 3′ UTR. This dimer formation could potentially fold the poly(A)-tail in a way that would bring PABPC1 into close proximity of the termination codon. As a proof of principle for this theory I used the two endogenously expressed proteins eRF3a F76A and eRF1 tethered to opposite ends of the TPI-4MS2-SMG5-4boxB reporter to successfully suppress NMD. By making use of the eRF3a F76A mutant unable to interact with PABPC1 directly I ensure that the stabilization effect is in fact due to the looping of the poly(A)-tail and not due to recruiting PABPC1 directly.

5.1.5 PAM2 motifs strongly suppress long 3′ UTR-triggered NMD

Comparing all NMD suppression methods explored in this thesis, the strongest effect was observed when PAM2 motifs were tethered to an NMD reporter construct. Specifically, the 15 amino acid PAM2 motif of PAIP2 showcased the strongest suppression of NMD induced by the SMG5 3′ UTR. The results obtained by tethering PAM2 motifs to the NMD reporter were unexpected. For instance, recruiting PABPC1 via the PAIP2 PAM2 motif to the SMG5 3′ UTR reporter gives a threefold higher mRNA abundance compared to tethering PABPC1 directly. Currently there is no explanation as to why this difference in NMD suppression strength is occurring. The PAM2 motif used is not believed to recruit any other factors besides PABPC1. A possible theory as to why I observe a much stronger stabilization compared to tethered PABPC1 wildtype could be that the MLLE domain of PABPC1 is blocked when PABPC1 is recruited to the reporter via a tethered PAM2 motif. In principle, when PABPC1 is tethered by itself it could theoretically still interact with other PAM2 carrying proteins, which might have an additional effect on PABPC1 or NMD suppression.

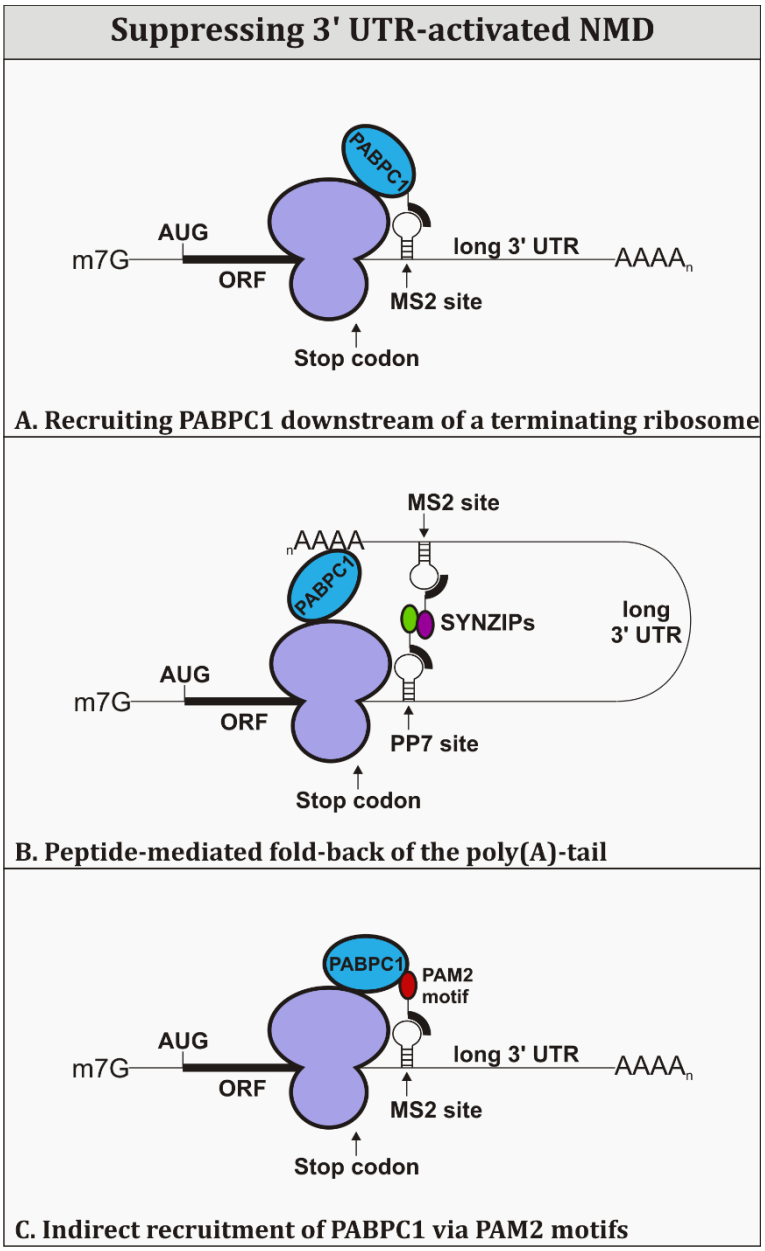

Figure 28: Different approaches to recruit PABPC1 in order to suppress long 3′ UTR-mediated NMD

(A) PABPC1 can suppress NMD activated by a long 3′ UTR when directly recruited downstream of the terminating ribosome. **(B)** Recruiting artificial proteins that can heterodimerize to opposite ends of a long 3′ UTR can mediate fold-back of the poly(A)-tail. As a result, PABPC1 bound to the poly(A)-tail is brought into close proximity of the terminating ribosome and can antagonize NMD. **(C)** Recruiting short PAM2 peptides downstream of a termination codon efficiently brings PABPC1 indirectly close to the termination codon and therefore to the stalling ribosome.

Occupying the MLLE domain of PABPC1 when it is recruited via a PAM2 motif would remove the possibility of other PAM2-carrying proteins to interact with this particular PABPC1. In other words, PABPC1 tethered directly could still interact with a PAM2-

containing protein, which could possibly reduce the NMD-inhibiting effect of PABPC1. It was also surprising to see that not all PAM2 motifs have the same effect on NMD suppression. For example, the TOB1 and PAN3 PAM2 motifs did not stabilize the SMG5 3′ UTR reporter construct. The most likely explanation for this is the weak interaction of these two PAM2 motifs with endogenous PABPC1. This is puzzling as the 15 amino acid long PAM2 motifs of these two proteins contain all three of the crucial amino acids of a PAM2 motif required for the interaction with PABPC1. The reason why these two PAM2 motifs are unable to interact with endogenous PABPC1 is currently not known. Figure 28 shows an overview of the different methods employed in this thesis to suppress NMD activated by a long 3′ UTR.

5.2 EJC-activated NMD is antagonized by PAM2 peptides

So far I could show that PABPC1 can inhibit NMD activated due to a 3′ UTR when recruited to the terminating ribosome by various mechanisms. As mentioned previously however, NMD in humans can also be activated by a 3′ UTR-located EJC. Surprisingly, PABPC1 is unable to suppress NMD activated by a MINX intron when tethered directly downstream or upstream of the intron (Figure 10). I expected PABPC1 to suppress NMD activated by an EJC, as others have previously shown that PABPC1 tethered upstream of the EJC factor Y14 can suppress NMD (Ivanov et al., 2008). The results presented here as well as previously shown results suggest that NMD activated by an EJC seems much stronger compared to NMD activated by a 3′ UTR and cannot be antagonized by PABPC1 (Boehm et al., 2014). The mechanism behind this inability of PABPC1 to suppress EJC-initiated NMD is currently unknown. A possible explanation could be that NMD activated by an EJC employs a different pathway including different additional proteins, which PABPC1 is unable to suppress.

After I was able to show that recruiting PABPC1 via PAM2 motifs is both sufficient and very strong in suppressing NMD activated by a 3′ UTR, I examined the effects of tethered PAM2 motifs on EJC-mediated NMD. To this end, I first made use of the two EJC components RNPS1 and BTZ. In a tandem tethering assay I was able to show that tethering the PAM2 motif of PAIP2 is able to strongly suppress NMD activated by either RNPS1 or BTZ (Figure 24). This result is comparable to the NMD inhibition effect of tethered PABPC1 observed by Ivanov et al. in 2008. Since I was able to suppress NMD initiated by EJC factors by recruiting PABPC1 to the reporter via tethered PAIP2 PAM2, I

next wanted to study the effect a tethered PAM2 motif would have when tethered upstream of a complete EJC. To achieve this, I again employed the TPI-4MS2-MINX-SMG5 reporter described earlier. Tethering the PAM2 motifs that exhibited the strongest suppression effect of a 3′ UTR-targeted reporter, namely eRF3a PAM2, Ataxin2 PAM2, and PAIP2 PAM2, upstream of the MINX intron strongly suppressed NMD of the reporter (Figure 25).

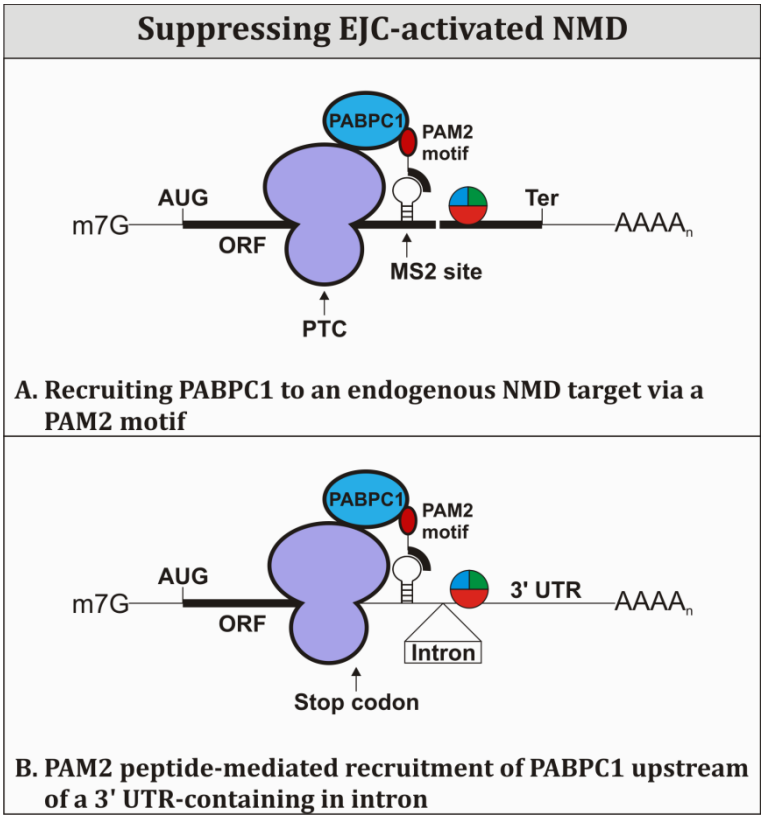

Figure 29: PAM2 peptide-mediated recruitment of PABPC1 is able to suppress EJC-activated NMD
(A) PABPC1 can suppress NMD of an EJC-carrying substrate when recruited via PAM2 peptides directly downstream of the terminating ribosome and upstream of the EJC. **(B)** NMD activated by an EJC deposited due to an intron in the 3′ UTR can be suppressed by PAM2 peptide-mediated recruitment of PABPC1 in between the terminating ribosome and the EJC.

Astoundingly, the EJC deposited during splicing of the MINX intron that inhibited tethered PABPC1 from suppressing NMD is unable to do the same when PABPC1 is recruited via a tethered PAM2 motif. This result indicates that recruiting PABPC1 indirectly is somehow able to more efficiently antagonize EJC-mediated NMD. The exact inhibition mechanism is currently unknown. The fact that PABPC1 is unable to interact with any other PAM2-containing protein in this scenario might be part of the reason.

Since there are many PAM2-containing proteins that can have a multitude of different effects it is feasible that PABPC1 has an as of now unknown interaction partner that suppresses its ability to antagonize EJC-mediated NMD. Nevertheless, the ability to suppress NMD activated by either a 3′ UTR or an EJC opens up the way for potential medical uses which are discussed in the following section. An overview of the EJC-activated NMD substrates suppressed by PAM2 peptide-mediated recruitment of PABPC1 can be seen in figure 29.

5.3 Medical implications of NMD inhibition and potential therapeutic approaches

Many inherited diseases as well as cancers are caused by frameshift or nonsense mutations which lead to transcripts carrying PTCs. A recent study has shown that 11% of all described gene lesions causing human inherited disease are caused by nonsense mutations that additionally account for 20% of disease-associated single-basepair substitutions (Culbertson, 1999; Mort et al., 2008). NMD can act as a beneficial modifier of a medical phenotype by altering the levels of potentially harmful C-terminally truncated proteins (Bhuvanagiri et al., 2010). An example for such a beneficial effect of NMD can be observed for β-thalassemia. This genetic disease is caused by nonsense mutations in the first or second exon of the β-globin gene. NMD acts as a beneficial modifier by degrading transcripts with such a mutation in the cell. As a result, only low levels of the mutant β-globin mRNA is present in the cell and heterozygous individuals are usually asymptomatic because the normal allele produces enough β-globin mRNA. However, NMD is not always protective and active NMD can lead to a more severe form of a disease. In DMD some mutations are known to escape NMD and produce a C-terminally truncated version of the dystrophin protein that is partially functional. These mutations lead to a clinically less severe form of the disease termed Becker muscular dystrophy (Kerr et al., 2001). This is an example of how active NMD leads to a more severe form of the disease as the transcripts evading NMD result in a clinically less severe form of muscular dystrophy.

Here, I made use of a β-globin reporter carrying a PTC at position 39 to address the potential medical implications of suppressing NMD by recruiting PABPC1 to an endogenous NMD target (Figure 26). The reporter carries 4MS2 sites directly downstream of the PTC39 to allow the recruitment of PABPC1 between the PTC and the

downstream EJC. I could show that tethering PABPC1 directly or recruiting PABPC1 via tethered PAM2 motifs results in NMD suppression of the reporter. This shows how NMD of endogenous targets could potentially be suppressed by PABPC1.

Many inherited diseases are caused by frameshift or nonsense mutations leading to transcripts carrying a PTC. These transcripts activate NMD due to one or more EJCs downstream of the termination codon. We have shown here several different methods of inhibiting NMD. These results provide new possibilities for specifically inhibiting the degradation of endogenous NMD targets. It seems that recruiting PABPC1 in close proximity of a termination codon via a small PAM2 peptide is enough to inhibit both 3′ UTR and EJC mediated NMD. The specific mechanism by which PAM2 motifs are able to suppress NMD even more efficiently than directly tethered PABPC1 remains unclear and needs to be subjected to further research.

The methodology described in this work shows how NMD suppression is theoretically feasible by recruiting PABPC1 to an NMD-targeted substrate. However, a limitation of the approach described here is that endogenous NMD targets do not have any tethering sites and PABPC1 can therefore not be recruited this way. To achieve target specific recruiting of PABPC1 to an endogenous NMD substrate, the approach described herein could be combined with a recently described technique of NMD inhibition via antisense oligonucleotides (ASOs) (Nomakuchi et al., 2015). The approach described by Nomakuchi et al. utilizes ASOs specifically targeting a region downstream of a stop codon of an mRNA where an EJC would be deposited on the mRNA. The ASOs bind the mRNA at the same site where an EJC would usually be placed and therefore block the EJC from being deposited onto the mRNA. Due to the absence of the 3′ UTR-located EJC, the stop codon is not recognized as aberrant and the otherwise NMD-targeted reporter is stabilized. Joining ASOs with a small PAM2 peptide would allow specific recruitment of PABPC1 to a PTC, potentially stabilizing the mRNA even further. This approach would open up a new way of specifically stabilizing endogenous mRNAs targeted for degradation by NMD in various genetic diseases.

Stabilizing an NMD substrate in this way would however still lead to only a truncated version of the protein. Since this could potentially cause an even more severe form of a genetic disorder it is not enough to simply inhibit NMD in cases where the truncated protein is known to have a dominant-negative effect. In these cases, translational read-through at a PTC is further needed to translate the mRNA into a full-length protein.

Translational read-through at PTCs could potentially be achieved by using a certain drug that results in read-through and, therefore, could be used to treat diseases (Peltz et al., 2013). One example used to achieve read-through are aminoglycosides (Hermann, 2007). Aminoglycosides used in cell culture to treat cystic fibrosis were successful and were used in clinical trials in patients suffering from cystic fibrosis and DMD (Bedwell et al., 1997; Kovesi et al., 1998). Albeit being successful in principle as the administration of aminoglycosides lead to an increase in protein levels in the patients the widespread usage of the drug is limited due to adverse side-effects after prolonged usage. It is therefore necessary to find other drugs to promote translational read-through without adverse side-effects.

5.4 Concluding remarks

In conclusion, I was able to add to the current model of NMD inhibition by PABPC1. I showed that the competition model for long 3′ UTR-mediated NMD between PABPC1 and UPF1 for eRF3a binding is not the main determining factor for NMD suppression. Instead, I was able to provide evidence that potentially implicates efficient translation termination, ribosome recycling, and circularization of the mRNA in NMD suppression by PABPC1. Furthermore, I showed multiple mechanisms by which NMD of not only a long 3′ UTR-activated reporter but also of an EJC-activated reporter can be suppressed. PABPC1 does not need to be directly recruited downstream of the terminating ribosome but is also able to suppress NMD by indirect recruitment either via other proteins, or by protein-mediated fold-back of the poly(A)-tail. Additionally, short PAM2 peptides are sufficient to strongly suppress NMD by recruiting PABPC1 to the reporter transcript. I discussed possible medical implications of the findings presented here and describe a potential application to target endogenous NMD substrates in the cell without the need for tethering sites. Even though I was able to show the potential involvement of translation termination, ribosome recycling, and mRNA circularization play an important role in NMD inhibition via PABPC1, the exact mechanism by which PABPC1 suppresses NMD is still unknown and subject to future studies.

6 Material and Methods

6.1 Molecular biology

6.1.1 Mammalian expression plasmids

The pCI-neo expression vector (Promega) was modified for protein expression in mammalian cell culture. To this end, pCI-neo contained different N-terminal tags such as FLAG, V5, FLAG-emGFP, MS2V5, PP7ΔFGV5, and λNV5. The plasmid harbored a CMV immediate-early enhancer/promoter region just upstream of the multiple cloning site to activate transgene expression.

6.2 Molecular cloning

The reporter plasmids as well as all deletion and point mutations used in this work were generated by PCR mutagenesis. PCR cleanup of intermediate steps were performed using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Ligations were performed by combining cut vector DNA with insert DNA in a 1:5 ratio. The ligation reaction was kept on ice for 30 minutes before a 2 hour incubation period at RT. All ligation reactions were performed with the T4 DNA ligase (Promega).

6.2.1 Transformation

Newly cloned plasmid DNA was transformed into *E. coli* XL-1 blue bacteria. To this end, 100 µl of chemically competent bacteria was added to the ligation reaction and kept on ice for 30 minutes. The mixture was heat shocked at 42°C for 1 minute and placed on ice for an additional minute. 400 µl LB medium was added to the reaction tube and the samples were pre-grown in a 37°C shaker. After 2 hours, the bacteria were pelleted by centrifugation at 12,000g for 2 minutes and the supernatant was aspirated. The pellet was resuspended in 100 µl LB medium, plated on an ampicillin containing agar plate, and incubated at 37°C overnight.

The same steps were performed for plasmid DNA re-transformation, except that 100 µl bacteria were added to 1 µl plasmid DNA to be re-transformed.

6.2.2 Plasmid preparation

A single bacterial colony from transformations was picked and grown either in 5 ml LB + ampicillin (100 µg/ml) (for minipreps) or in 100 ml LB + ampicillin (100 µg/ml) (for midipreps) at 37°C overnight. The bacteria were pelleted the next day and a mini-prep using the ZR Plasmid Mini-prep kit (Zymo Research) or a midi-prep using the NucleoBond Xtra Midiprep kit (Macherey-Nagel) was performed. All molecular cloning results were verified by Sanger sequencing (GATC Biotech).

6.3 Cell culture

6.3.1 Cell lines

Mammalian cell culture experiments were performed in HeLa Tet-Off (HTO) cells (Clontech). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Gibco), supplemented with 1x penicillin/streptomycin and 9% fetal bovine serum (FBS) at 37°C and 5% CO_2. The cells were passaged every 2-3 days (at approximately 90% confluency) by trypsinization.

6.3.2 Plasmid DNA transfection

Plasmid DNA was transfected using calciumphosphate precipitation. The cells were seeded 24 hours prior to transfection to achieve ~60% confluency on the day of transfection (see the following table for concentrations). The medium was changed 1-2 hours before transfection. The transfection mix was prepared with the plasmid DNA diluted in Ultra Pure water (Biochrom). $CaCl_2$ was added to the diluted plasmid DNA mix for a final concentration of 250 mM. An equal volume of 2x BBS was added to the plasmid DNA/$CaCl_2$ solution and vortexed vigorously. After 15 minutes incubation at room temperature (RT) the transfection mixture was added to the cells. The DMEM medium was changed to following day to remove any calcium-phosphate precipitates. The cells were harvested the following day.

Transfection overview

	6-well plate	6cm dish
Number of cells	240,000	650,000
Volume of medium (ml)	2	4
Volume of H_2O + plasmid DNA (µl)	90	180
Volume of $CaCl_2$ (µl)	10	20
Volume of 2x BBS (µl)	100	200
Total volume (µl)	200	400

2x BBS
50 mM BES (N, N-bis(Hydroxyethyl)-2-Aminoethansolfonate)
1.5 mM Na_2HPO_4
280 mM NaCl
adjust pH to 6.96

6.4 Immunoprecipitation

6.4.1 Transfection of HTO cells

HTO cells were seeded in 6cm dishes and transfected as described in chapter 6.3. 1500 ng plasmid DNA expressing FLAGemGFP-tagged proteins were transfected for endogenous protein pull-down assays. 1500 ng FLAG-tagged proteins were co-transfected with 500 ng plasmid DNA expressing V5-tagged proteins, and 300 ng mVenus plasmid DNA (mVenus used as transfection control) in precipitation assays with non-endogenous proteins. The cells were harvested 48 hours after transfection.

6.4.2 Preparation of cell lysates

Confluent cells were washed once with PBS (Gibco) and scraped off the cell culture dish in 500 µl PBS. The cell suspension was transferred to a 1.5 ml reaction tube (Sarstedt) and centrifuged for five minutes at 2000g. PBS was aspirated, followed by resuspension of the cell pellet in 400 µl IP lysis buffer (1x TBS, 0.5% Triton X-100 (Sigma-Aldrich), 50 µg/ml RNase A (Applichem), protease inhibitor (Sigma-Aldrich, #P2714-1BTL, diluted 1:200)). The lysate was frozen over night at -20°C.

6.4.3 Co-immunoprecipitation

Co-immunoprecipitation experiments were performed with anti-FLAG (M2) magnetic beads (Sigma-Aldrich) to analyze the interaction of proteins extracted from cell lysates. The cell lysates (preparation described in chapter 6.4.2) were centrifuged for 10 minutes at 12,000g. 40 µl of the supernatant was transferred to a new reaction tube

containing 8 µl 6x SDS loading buffer and saved as input. 12 µl anti-FLAG (M2) magnetic beads were washed once with 800 µl IP wash buffer (1x TBS, 0.5% Triton X-100). The remaining supernatant (~360 µl) was added to the washed anti-FLAG (M2) magnetic beads and rotated in an overhead shaker at 4°C for at least 2 hours or overnight. The magnetic beads were washed three times for 5 minutes each with 800 µl IP wash buffer (described above) at 4°C in the overhead shaker. The remaining IP wash buffer was aspirated and the proteins were eluted in 50 µl 1x SDS loading buffer. 15 µl input and 15 µl IP were loaded onto SDS-polyacrylamide gels for further analysis.

6.4.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gels were cast in the BioRad Mini-Protean gel system using 1mm combs with either 10 or 15 wells. The polyacrylamide gel mixes were prepared according to the table below. The gels were loaded with input, IP (described in chapter 6.4.3), and 1.5 µl PageRuler prestained protein ladder (ThermoFisher) and run in 1x SDS-running buffer (25 mM Tris-HCl, 20 mM Glycine, 0.1% SDS) in vertical electrophoresis chambers at 200V.

SDS-PAGE mixes

	Stack gel (4 ml)	**Separating gel (10 ml)**	
Acrylamide concentration	-	10%	12%
MW range (kDa)	-	16-70	14-60
30% Acrylamide mix (37.5:1 Rotiphorese Gel, Roth)	0.67 ml	3.3 ml	4 ml
1.5 M Tris pH 8.8	-	2.5 ml	2.5 ml
1 M Tris pH 6.8	0.5 ml	-	-
H_2O	2.4 ml	4.1 ml	3.4 ml
10% SDS	40 µl	100 µl	100 µl
10% Ammonium Persulfate	80 µl	120 µul	120 µl
TEMED (Roth)	10 µl	14 µl	14 µl
		Volumes are for 2 gels	

6.4.5 Western blot analysis

After SDS-PAGE the gel was blotted on a Hybond-ECL nitrocellulose membrane (GE Healthcare) for 60 to 120 minutes at 11V using the semi-dry TransBlot SD system (BioRad) and 1x transfer buffer. After blotting, the membrane was incubated in 5% blocking solution (5% milk in 1x TBS, 0.2% Tween) for one hour at RT. The membrane was incubated with the

primary antibody (in 1x TBS, 0.2% Tween) at 4°C overnight. The next day the membrane was washed 3x with 1x TBST (1x TBS, 0.2% Tween) for 5 minutes each followed by incubation with an HRP-conjugated secondary antibody (in 1x TBS, 0.2% Tween). The washing step was repeated before visualization with the Western Lightning Plus ECL reagent (PerkinElmer) on the myECL Imager (ThermoScientific).

6.5 Northern blot analysis

6.5.1 Transfection of HTO cells

HTO cells were seeded in 6 well plates and transfected as described in chapter 6.3. I used 800 ng plasmid DNA expressing MS2V5-, PP7ΔFGV5-, or λNV5-tagged proteins. 500 ng plasmid DNA expressing β-globin wild-type or 1500 ng lacZ (serving as internal transfection control) and 300 ng mVenus plasmid DNA (mVenus used as transfection control) were used in each transfection. Additionally, 2000 ng reporter plasmid was transfected in each well. The cells were harvested 48 hours after transfection.

6.5.2 Harvesting cells

Two days after transfection the cells were washed once with 1x PBS and transferred to a fume hood. Here, 1 ml peqGOLD TriFast lysis reagent (Peqlab) was added to each well and incubated for 5 to 10 minutes. Next, the peqGOLD TriFast lysis reagent containing the cell lysates were transferred to new 1.5 ml reaction tubes.

6.5.3 RNA extraction

Total RNA was extracted by first adding 100 µl 1-Bromo-3-chloropropane (Sigma-Aldrich) to the reaction tubes containing the peqGOLD Trifast, followed by vigorous mixing of the contents and a 15 minute incubation period at RT. The tubes were next centrifuged at 12,000g at 4°C for 15 minutes. The aqueous phase (approximately 400 µl) was transferred to a new reaction tube containing 500 µl 2-propanol (Roth) and the remainder frozen at -20°C until being used for protein extraction. The mixture was vortexed and incubated on ice for 15 minutes, followed by centrifugation at 12,000g at 4°C for 15 minutes. The supernatant was discarded and the resulting pellet washed twice with 75% EtOH by centrifuging at 12,000g at 4°C for 10 minutes each. The pellet was air dried and resuspended in 20 µl Ultra Pure H_2O (Biochrom) by shaking the reaction tubes for 15 minutes at 65°C. The resulting RNA-H_2O mixture was stored at -20°C until further use.

6.5.4 Northern blotting

To analyze the RNA, 4 µl of each extracted RNA sample was added to 16 µl northern sample buffer (15 ml formamide (deionized, Roth), 6 ml formaldehyde (37%, Roth), 3 ml 10x MOPS buffer (41,2 g 3-(N-morpholino)propanesulfonic acid in 800 ml autoclaved 100 mM sodium acetate (10,886 g NaOAc), adjust pH 7.0 and add 20 ml autoclaved 0.5 M EDTA pH 8.0, total volume 1 L), 1 drop EtBr) and incubated at 65°C for 15 minutes. After the incubation, 2 µl RNA-loadingbuffer (50% Glycerol, 1 mM EDTA, 0,25% Bromophenolblue) was added to each sample. The samples were loaded onto an agarose gel (2,63 g agarose (SeaKem LE Agarose), 175 ml H_2O, 50 ml 10x MOPS, 25 ml filtered 37% formaldeyhde (Roth)) and run at 33V overnight.

The following day, the gel was washed on a shaker for 15 minutes in H_2O, changing the water once. Next, the gel was incubated in 50 mM NaOH for 5 minutes, followed by 40 minute incubation in 20x SSC (175,32 g Sodium Chloride, 88,2 3g Trisodium-Citrate, pH 7.0, total volume 1 L). The gel was transferred onto a Roti-Nylon 0.2 membrane (Roth) with a capillary transfer for 4 hours. Next, the membrane was UV crosslinked at 0.120 J on both sides. The membrane was then prehybridized for 1 hour in approximately 15 ml Church buffer (0.5 M Na_2HPO_4, 1 mM EDTA, 7% SDS, pH 7.2) at 65°C in a hybridization oven. Afterwards, the radioactively labeled probe (for transcription reaction see table below) was added to the membrane and incubated at 65°C in the hybridization oven over night.

The next day, the membrane was washed twice with wash buffer 1 (2x SSC, 0.1% SDS) and twice with wash buffer 2 (0.2x SSC, 0.1% SDS) for 15 minutes each in the 65°C hybridization oven. The membrane was then air dried for 40 minutes, wrapped in saran wrap, and exposed on a phosphoscreen for 2 – 4 hours. Signals were quantified using a Typhoon FLA 7000 (GE Healthcare) and analyzed with ImageQuant (GE Healthcare).

Transcription reaction for northern probe targeting exon 3 of β-globin

Ingredients	Volume
10x Transcription buffer	2 µl
H_2O (RNase-free)	4 µl
0.1 M DTT	0.5 µl
10 mM ATP	1.2 µl
10 mM CTP	1.2 µl
10 mM UTP	1.2 µl
RNasin (40 U/µl, NEB)	0.7 µl
SP6 RNA-polymerase	1.2 µl
Linearized plasmid DNA (BamHI cut β-globin)	3 µl
α-^{32}P-GTP	5 µl
Incubate 40 minutes at 40°C	
DNaseI (RNase-free, 1 U/µl, NEB)	1,5 µl
Incubate 20 minutes at 37°C	
Add 28 µl H_2O to the reaction and purify the labelled transcripts using Mini Quick-spin columns (Roche). After purification, add 25 µl radioactively labelled transcript to 25 ml Church buffer.	

6.5.5 Protein extraction

The remaining interphase and organic phase saved in chapter 6.5.3 was used to extract proteins. First, 300 µl 100% EtOH was added to the reaction tube and vortexed. The mixture was incubated for 5 minutes at RT. Next, the DNA was pelleted at 2,000g at 4°C for 5 minutes. 500 µl of the supernatant was added to 2 ml reaction tubes containing acetone (Roth), vortexed, and incubated for 10 minutes at RT. The proteins were then pelleted by centrifugation at 12,000g at 4°C for 10 minutes. The supernatant was discarded and the protein pellet was washed twice with 1 ml 0.3 M guanidine hydrochloride in 95% EtOH by incubation for 20 minutes at RT followed by centrifugation at 12,000g at 4°C for 5 minutes. A final washing step was performed with 1 ml 100% EtOH. The pellet was air dried for 30 minutes, followed by resuspension in 200 µl 1% SDS at 35°C and shaking at 800 rpm. 50 µl of the resuspended protein was

mixed with 10 µl 6x SDS-loading buffer and frozen at -20°C. SDS-PAGE and western blotting of the extracted proteins was performed as described in 6.4.4 and 6.4.5 using 18 µl of the extracted proteins.

7 Appendix

7.1 List of Antibodies

Antibody	Company	Species	Dilution
FLAG (F7425)	Sigma	Rabbit	1:3000
V5 (18870)	QED Bioscience	Rabbit	1:3000
GFP (ab290)	Abcam	Rabbit	1:3000
PABPC1 (#4992)	Cell Signaling Technology	Rabbit	1:3000
Rabbit-HRP	Jackson ImmunoResearch	Mouse	1:10000

7.2 List of Primers

Primer	Sequence
globin_2.exon_Xho_as	TTTTCTCGAGCACTTTCTTGCCATGA
globin_2.exon_Xho_se	TTTTCTCGAGGGTGCCTTTAGTGATGGC
globin_3'UTR_Xho_se	TTTTCTCGAGGGTTCCTTTGTTCCCTAAGTC
globin_3'UTR_SalNot_as	TTTTTTTGCGGCCGCCCGGGTCGACATTGCAATGAAAATAAATGTTTTTTATTAG
PABPC1_M161A_as	AATACTTTGCGATCATTTAGGAGTGCTCCATTCATTTTTTCAATAGCT
PABPC1_M161A/D165K_as	CGTCCAACAAATACTTTGCGTTTATTTAGGAGTGCTCCATTCATTTTTTCAATAGCT
PABPC1_1170_Not_as	TTTTTTTGCGGCCGCTTATCGTACACTTGCCATTCTCT
MLLE_mut_se	GCTGGTAAAATCACTGGCGGCGCCGCCCGCATTGATAATTCAGAACTTCTTCAC
PABPC1_580_ter_Not_as	TTTTTTTGCGGCCGCTTAAFCCAGCAAGAGTAGGGTG
PABPC1_1438_as	CGCTGTGTTGACATGACTC
GST_Xho_se	TTTTCTCGAGTCCCCTATACTAGGTTATTGGAA
SYNZIP1_Xho_se	TTTTCTCGAGGGAGGCGGAAATCTGG
SYNZIP1_UAA_Not_as	TTTTTTGCGGCCGCTTATTCTTCAATCTTTTTGCGTAAG
SYNZIP2_Xho_se	TTTTCTCGAGGGCGGAGGCGCCCGT
SYNZIP2_UAA_Not_as	TTTTTTGCGGCCGCTTACTGCTCATGGGACGC
SYNZIP2_Xho_se	TTTTCTCGAGGGTGGAGGCAATGACC
SYNZIP2_UAA_Not_as	TTTTTTGCGGCCGCTTAGGACTGCAGTTCCTGCTT
PTBP1_Xho_se	TTTTCTCGAGATGGACGGCATTGTCC
PTBP1_Not_as	TTTTTTGCGGCCGCCTAGATGGTGGACTTGGAGAA
MS2_se	GCTTCTAACTTTACTCAGTTCGTTC
PP7_se	AAGACCATTGTGCTGAGTGT
eRF3a_PAM2-N_se	TTTTCTCGAGGCCTTCAGCCGGCAACTCAACGTCAACGCCAAGCCCTTCGTGCCCAACGT

eRF3a_PAM2-N_as	TTTTTTGCGGCCGCTTACTCGGCGGCGTGGACGTTGGGCACGAAGGGCTTGGCGTTGACG
eRF3a_PAM2-C_se	TTTTCTCGAGGCCAAGCCCTTCGTGCCCAACGTCCACGCCGCCGAGTTCGTGCCGTCCTT
eRF3a_PAM2-C_as	TTTTTTGCGGCCGCTTACGGGCCCCGCAGGAAGGACGGCACGAACTCGGCGGCGTGGACG
eRF3a-PAM2(both)_se	TTTTCTCGAGGCCTTCAGCCGGCAACTCAACGTCA
eRF3a-PAM2(both)_as	TTTTTTGCGGCCGCTTACGGGCCCCGCAGGAAGGA
eRF3a_F76A_as	GTGGACGTTGGGCACGGCGGGCTTGGCGTTGACG
Tob1_PAM2_se	TTTTCTCGAGCAGAAAACCTCTGCTCTTTCTCCTAATGCCAAGGAATTTATTTTTCCTAA
Tob1_PAM2_as	TTTTTTGCGGCCGCTTATTGACCCTGCATATTAGGAAAAATAAATTCCTTGGCATTAGGA
PAN3_PAM2_se	TTTTCTCGAGAACAATTTACAGACACCAAATCCTACTGCAAGCGAGTTTATTCCTAAAGG
PAN3_PAM2_as	TTTTTTGCGGCCGCTTAGGAGGTTGATCCTCCTTTAGGAATAAACTCGCTTGCAGTAGGA
NFX-1_PAM2_se	TTTTCTCGAGTCAGGTACTTTTAAATTCAATACAGATGCTGCTGAATTCATTCCTCAGGA
NFX-1_PAM2_as	TTTTTTGCGGCCGCTTAAGAATTTTTTTTCTCCTGAGGAATGAATTCAGCAGCATCTGTA
PAIP2_PAM2_se	TTTTCTCGAGGTGGTCAAGAGCAATCTGAATCCAAATGCAAAGGAGTTTGTTCCTGGGGT
PAIP2_PAM2_as	TTTTTTGCGGCCGCTTAATTTCCGTACTTCACCCCAGGAACAAACTCCTTTGCATTTGGA
PAIP2_PAM2_EFxP/AAxA_as	TTTTTTGCGGCCGCTTAATTTCCGTACTTCACCCCGGCAACAGCGGCCTTTGCATTTGGA
PAIP2_PAM2_EFxP/AAxA_se	TTTTCTCGAGGTGGTCAAGAGCAATCTGAATCCAAATGCAAAGGCCGCTGTTGCCGGGGT
Ataxin2_PAM2_as	TTTTTTGCGGCCGCTTATGGCTGAGAGAAGGAACGTGGGTTGAACTCCTTTGCATTGGGA
Ataxin2_PAM2_se	TTTTCTCGAGGTTAGGAAATCAACATTGAATCCCAATGCAAAGGAGTTCAACCCACGTTC
eIF4G_84_Xho_se	TTTTCTCGAGGGATCCCAAGTAATGATGATC
eIF4G_Not_as	TTTTTTTGCGGCCGCTTAGTTGTGGTCAGACTCCTCC
eIF4G_928_Xho_se	TTTTCTCGAGCCATATCGCCTCTCTCCA
eIF4G_3384Not_as	TTTTTTTGCGGCCGCTTATTGAAGGGCTGAGAAGCG
eIF4G_294_Not_as	TTTTTTTGCGGCCGCTTACCCAGGAATAGAGAGGACTG
eIF4G_181-185_KRERK_as	ATCTCGAATTCGGATCGTGGCGGCGGCGGCGGCGGGAGCAATCTGGGGT
eIF4G_DelN205_Xho_se	TTTTCTCGAGGGGGCCCGCACTGCCT
eIF4G_MIF4G(1089)_Not_as	TTTTTTTGCGGCCGCTTATCCAGGTGCAAAGAGCTG
PDRG1_Xho_se	TTTTCTCGAGGTCATCTTGAAAGGATGAGACTC
PDRG1_SalNot_as	TTTTTTTGCGGCCGCCCGGGTCGACGACTTCACGGCAGTTCATAC
SURF6_Xho_se	TTTTCTCGAGCTGGTCTGAGTCTTTCCCA
SURF6_SalNot_as	TTTTTTTGCGGCCGCCCGGGTCGACCCATATGCCACTAAAATTCACC
SMG5_3UTR_Xho_se	TTTTCTCGAGTTGGTTGATACTGACCCCCA
SMG5_3UTR_short_SalNot_as	TTTTTTTGCGGCCGCCCGGGTCGACAGATATTCACCTCAACACTCCA
mVenus_Nhe/Xho_as	TTTTCTCGAGTGATGCGCTAGCCTTGTACAGCTCGTCCATGC
pCI-se2	GGTGTCCACTCCCAGTTCA
pCI-as2	TGCATTCTAGTTGTGGTTTGTC

8 References

Albrecht, M., and Lengauer, T. (2004). Survey on the PABC recognition motif PAM2. Biochem Biophys Res Commun *316*, 129-138.

Alkalaeva, E.Z., Pisarev, A.V., Frolova, L.Y., Kisselev, L.L., and Pestova, T.V. (2006). In vitro reconstitution of eukaryotic translation reveals cooperativity between release factors eRF1 and eRF3. Cell *125*, 1125-1136.

Amrani, N., Ganesan, R., Kervestin, S., Mangus, D.A., Ghosh, S., and Jacobson, A. (2004). A faux 3'-UTR promotes aberrant termination and triggers nonsense-mediated mRNA decay. Nature *432*, 112-118.

Amrani, N., Ghosh, S., Mangus, D.A., and Jacobson, A. (2008). Translation factors promote the formation of two states of the closed-loop mRNP. Nature *453*, 1276-1280.

Amrani, N., Sachs, M.S., and Jacobson, A. (2006). Early nonsense: mRNA decay solves a translational problem. Nature reviews Molecular cell biology *7*, 415-425.

Andersen, C.B., Ballut, L., Johansen, J.S., Chamieh, H., Nielsen, K.H., Oliveira, C.L., Pedersen, J.S., Seraphin, B., Le Hir, H., and Andersen, G.R. (2006). Structure of the exon junction core complex with a trapped DEAD-box ATPase bound to RNA. Science *313*, 1968-1972.

Apponi, L.H., Leung, S.W., Williams, K.R., Valentini, S.R., Corbett, A.H., and Pavlath, G.K. (2010). Loss of nuclear poly(A)-binding protein 1 causes defects in myogenesis and mRNA biogenesis. Hum Mol Genet *19*, 1058-1065.

Auld, D.S., Thorne, N., Maguire, W.F., and Inglese, J. (2009). Mechanism of PTC124 activity in cell-based luciferase assays of nonsense codon suppression. Proceedings of the National Academy of Sciences of the United States of America *106*, 3585-3590.

Ballut, L., Marchadier, B., Baguet, A., Tomasetto, C., Seraphin, B., and Le Hir, H. (2005). The exon junction core complex is locked onto RNA by inhibition of eIF4AIII ATPase activity. Nat Struct Mol Biol *12*, 861-869.

Baltz, A.G., Munschauer, M., Schwanhausser, B., Vasile, A., Murakawa, Y., Schueler, M., Youngs, N., Penfold-Brown, D., Drew, K., Milek, M., *et al.* (2012). The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts. Molecular cell *46*, 674-690.

Barthelme, D., Dinkelaker, S., Albers, S.V., Londei, P., Ermler, U., and Tampe, R. (2011). Ribosome recycling depends on a mechanistic link between the FeS cluster domain and a conformational switch of the twin-ATPase ABCE1. Proceedings of the National Academy of Sciences of the United States of America *108*, 3228-3233.

Baserga, S.J., and Benz, E.J., Jr. (1988). Nonsense mutations in the human beta-globin gene affect mRNA metabolism. Proceedings of the National Academy of Sciences of the United States of America *85*, 2056-2060.

Becker, T., Franckenberg, S., Wickles, S., Shoemaker, C.J., Anger, A.M., Armache, J.P., Sieber, H., Ungewickell, C., Berninghausen, O., Daberkow, I., *et al.*

(2012). Structural basis of highly conserved ribosome recycling in eukaryotes and archaea. Nature *482*, 501-506.

Bedwell, D.M., Kaenjak, A., Benos, D.J., Bebok, Z., Bubien, J.K., Hong, J., Tousson, A., Clancy, J.P., and Sorscher, E.J. (1997). Suppression of a CFTR premature stop mutation in a bronchial epithelial cell line. Nat Med *3*, 1280-1284.

Behm-Ansmant, I., Gatfield, D., Rehwinkel, J., Hilgers, V., and Izaurralde, E. (2007). A conserved role for cytoplasmic poly(A)-binding protein 1 (PABPC1) in nonsense-mediated mRNA decay. The EMBO journal *26*, 1591-1601.

Belgrader, P., Cheng, J., and Maquat, L.E. (1993). Evidence to implicate translation by ribosomes in the mechanism by which nonsense codons reduce the nuclear level of human triosephosphate isomerase mRNA. Proceedings of the National Academy of Sciences of the United States of America *90*, 482-486.

Bermano, G., Nicol, F., Dyer, J.A., Sunde, R.A., Beckett, G.J., Arthur, J.R., and Hesketh, J.E. (1995). Tissue-specific regulation of selenoenzyme gene expression during selenium deficiency in rats. Biochem J *311 (Pt 2)*, 425-430.

Berry, M.J., Banu, L., Harney, J.W., and Larsen, P.R. (1993). Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons. The EMBO journal *12*, 3315-3322.

Bhattacharya, A., Czaplinski, K., Trifillis, P., He, F., Jacobson, A., and Peltz, S.W. (2000). Characterization of the biochemical properties of the human Upf1 gene product that is involved in nonsense-mediated mRNA decay. RNA *6*, 1226-1235.

Bhuvanagiri, M., Schlitter, A.M., Hentze, M.W., and Kulozik, A.E. (2010). NMD: RNA biology meets human genetic medicine. Biochem J *430*, 365-377.

Boehm, V., Haberman, N., Ottens, F., Ule, J., and Gehring, N.H. (2014). 3' UTR length and messenger ribonucleoprotein composition determine endocleavage efficiencies at termination codons. Cell Rep *9*, 555-568.

Bono, F., Ebert, J., Lorentzen, E., and Conti, E. (2006). The crystal structure of the exon junction complex reveals how it maintains a stable grip on mRNA. Cell *126*, 713-725.

Brett, D., Hanke, J., Lehmann, G., Haase, S., Delbruck, S., Krueger, S., Reich, J., and Bork, P. (2000). EST comparison indicates 38% of human mRNAs contain possible alternative splice forms. FEBS letters *474*, 83-86.

Buchwald, G., Ebert, J., Basquin, C., Sauliere, J., Jayachandran, U., Bono, F., Le Hir, H., and Conti, E. (2010). Insights into the recruitment of the NMD machinery from the crystal structure of a core EJC-UPF3b complex. Proceedings of the National Academy of Sciences of the United States of America *107*, 10050-10055.

Buhler, M., Steiner, S., Mohn, F., Paillusson, A., and Muhlemann, O. (2006). EJC-independent degradation of nonsense immunoglobulin-mu mRNA depends on 3' UTR length. Nat Struct Mol Biol *13*, 462-464.

Carter, M.S., Doskow, J., Morris, P., Li, S.L., Nhim, R.P., Sandstedt, S., and Wilkinson, M.F. (1995). A Regulatory

Mechanism That Detects Premature Nonsense Codons in T-Cell Receptor Transcripts in-Vivo Is Reversed by Protein-Synthesis Inhibitors in-Vitro. J Biol Chem *270*, 28995-29003.

Castello, A., Fischer, B., Eichelbaum, K., Horos, R., Beckmann, B.M., Strein, C., Davey, N.E., Humphreys, D.T., Preiss, T., Steinmetz, L.M.*, et al.* (2012). Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. Cell *149*, 1393-1406.

Castelo-Branco, P., Furger, A., Wollerton, M., Smith, C., Moreira, A., and Proudfoot, N. (2004). Polypyrimidine tract binding protein modulates efficiency of polyadenylation. Mol Cell Biol *24*, 4174-4183.

Chakrabarti, S., Bonneau, F., Schussler, S., Eppinger, E., and Conti, E. (2014). Phospho-dependent and phospho-independent interactions of the helicase UPF1 with the NMD factors SMG5-SMG7 and SMG6. Nucleic Acids Res *42*, 9447-9460.

Chakrabarti, S., Jayachandran, U., Bonneau, F., Fiorini, F., Basquin, C., Domcke, S., Le Hir, H., and Conti, E. (2011). Molecular mechanisms for the RNA-dependent ATPase activity of Upf1 and its regulation by Upf2. Molecular cell *41*, 693-703.

Chamieh, H., Ballut, L., Bonneau, F., and Le Hir, H. (2008). NMD factors UPF2 and UPF3 bridge UPF1 to the exon junction complex and stimulate its RNA helicase activity. Nat Struct Mol Biol *15*, 85-93.

Chang, Y.F., Imam, J.S., and Wilkinson, M.F. (2007). The nonsense-mediated decay RNA surveillance pathway. Annual review of biochemistry *76*, 51-74.

Chauvin, C., Salhi, S., Le Goff, C., Viranaicken, W., Diop, D., and Jean-Jean, O. (2005). Involvement of human release factors eRF3a and eRF3b in translation termination and regulation of the termination complex formation. Mol Cell Biol *25*, 5801-5811.

Chavatte, L., Frolova, L., Kisselev, L., and Favre, A. (2001). The polypeptide chain release factor eRF1 specifically contacts the s(4)UGA stop codon located in the A site of eukaryotic ribosomes. Eur J Biochem *268*, 2896-2904.

Cheng, Z., Muhlrad, D., Lim, M.K., Parker, R., and Song, H. (2007). Structural and functional insights into the human Upf1 helicase core. The EMBO journal *26*, 253-264.

Chew, G.L., Pauli, A., Rinn, J.L., Regev, A., Schier, A.F., and Valen, E. (2013). Ribosome profiling reveals resemblance between long non-coding RNAs and 5' leaders of coding RNAs. Development *140*, 2828-2834.

Cui, Y., Hagan, K.W., Zhang, S., and Peltz, S.W. (1995). Identification and characterization of genes that are required for the accelerated degradation of mRNAs containing a premature translational termination codon. Genes Dev *9*, 423-436.

Culbertson, M.R. (1999). RNA surveillance. Unforeseen consequences for gene expression, inherited genetic disorders and cancer. Trends Genet *15*, 74-80.

Denning, G., Jamieson, L., Maquat, L.E., Thompson, E.A., and Fields, A.P. (2001). Cloning of a novel phosphatidylinositol kinase-related kinase: characterization of the human SMG-1 RNA surveillance protein. J Biol Chem *276*, 22709-22714.

Deo, R.C., Bonanno, J.B., Sonenberg, N., and Burley, S.K. (1999). Recognition of polyadenylate RNA by the poly(A)-binding protein. Cell *98*, 835-845.

Dostie, J., and Dreyfuss, G. (2002). Translation is required to remove Y14 from mRNAs in the cytoplasm. Curr Biol *12*, 1060-1067.

Durand, S., and Lykke-Andersen, J. (2013). Nonsense-mediated mRNA decay occurs during eIF4F-dependent translation in human cells. Nat Struct Mol Biol *20*, 702-709.

Eberle, A.B., Lykke-Andersen, S., Muhlemann, O., and Jensen, T.H. (2009). SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. Nat Struct Mol Biol *16*, 49-55.

Eberle, A.B., Stalder, L., Mathys, H., Orozco, R.Z., and Muhlemann, O. (2008). Posttranscriptional gene regulation by spatial rearrangement of the 3' untranslated region. Plos Biol *6*, e92.

Ezzeddine, N., Chang, T.C., Zhu, W., Yamashita, A., Chen, C.Y., Zhong, Z., Yamashita, Y., Zheng, D., and Shyu, A.B. (2007). Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. Mol Cell Biol *27*, 7791-7801.

Fairman-Williams, M.E., Guenther, U.P., and Jankowsky, E. (2010). SF1 and SF2 helicases: family matters. Current opinion in structural biology *20*, 313-324.

Fatscher, T., Boehm, V., Weiche, B., and Gehring, N.H. (2014). The interaction of cytoplasmic poly(A)-binding protein with eukaryotic initiation factor 4G suppresses nonsense-mediated mRNA decay. RNA *20*, 1579-1592.

Frolova, L., LeGoff, X., Zhouravleva, G., Davydova, E., Philippe, M., and Kisselev, L. (1996). Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. Rna-a Publication of the Rna Society *2*, 334-341.

Frolova, L.Y., Tsivkovskii, R.Y., Sivolobova, G.F., Oparina, N.Y., Serpinsky, O.I., Blinov, V.M., Tatkov, S.I., and Kisselev, L.L. (1999). Mutations in the highly conserved GGQ motif of class 1 polypeptide release factors abolish ability of human eRF1 to trigger peptidyl-tRNA hydrolysis. RNA *5*, 1014-1020.

Fukuhara, N., Ebert, J., Unterholzner, L., Lindner, D., Izaurralde, E., and Conti, E. (2005). SMG7 is a 14-3-3-like adaptor in the nonsense-mediated mRNA decay pathway. Molecular cell *17*, 537-547.

Gatfield, D., and Izaurralde, E. (2004). Nonsense-mediated messenger RNA decay is initiated by endonucleolytic cleavage in Drosophila. Nature *429*, 575-578.

Ge, Z., Quek, B.L., Beemon, K.L., and Hogg, J.R. (2016). Polypyrimidine tract binding protein 1 protects mRNAs from recognition by the nonsense-mediated mRNA decay pathway. Elife *5*.

Gehring, N.H., Lamprinaki, S., Kulozik, A.E., and Hentze, M.W. (2009). Disassembly of exon junction complexes by PYM. Cell *137*, 536-548.

Gehring, N.H., Neu-Yilik, G., Schell, T., Hentze, M.W., and Kulozik, A.E. (2003). Y14 and hUpf3b form an NMD-activating complex. Molecular cell *11*, 939-949.

Ghosh, S., and Jacobson, A. (2010). RNA decay modulates gene expression and controls its fidelity. Wiley interdisciplinary reviews RNA *1*, 351-361.

Glavan, F., Behm-Ansmant, I., Izaurralde, E., and Conti, E. (2006). Structures of the PIN domains of SMG6 and SMG5 reveal a nuclease within the mRNA surveillance complex. The EMBO journal *25*, 5117-5125.

Gray, N.K., Hrabalkova, L., Scanlon, J.P., and Smith, R.W. (2015). Poly(A)-binding proteins and mRNA localization: who rules the roost? Biochem Soc Trans *43*, 1277-1284.

Grimson, A., O'Connor, S., Newman, C.L., and Anderson, P. (2004). SMG-1 is a phosphatidylinositol kinase-related protein kinase required for nonsense-mediated mRNA Decay in Caenorhabditis elegans. Mol Cell Biol *24*, 7483-7490.

Hadley, K.B., and Sunde, R.A. (2001). Selenium regulation of thioredoxin reductase activity and mRNA levels in rat liver. J Nutr Biochem *12*, 693-702.

Hall, G.W., and Thein, S. (1994). Nonsense codon mutations in the terminal exon of the beta-globin gene are not associated with a reduction in beta-mRNA accumulation: a mechanism for the phenotype of dominant beta-thalassemia. Blood *83*, 2031-2037.

Hermann, T. (2007). Aminoglycoside antibiotics: old drugs and new therapeutic approaches. Cell Mol Life Sci *64*, 1841-1852.

Hoshino, S., Imai, M., Kobayashi, T., Uchida, N., and Katada, T. (1999). The eukaryotic polypeptide chain releasing factor (eRF3/GSPT) carrying the translation termination signal to the 3'-Poly(A) tail of mRNA. Direct association of erf3/GSPT with polyadenylate-binding protein. J Biol Chem *274*, 16677-16680.

Hoshino, S., Imai, M., Mizutani, M., Kikuchi, Y., Hanaoka, F., Ui, M., and Katada, T. (1998). Molecular cloning of a novel member of the eukaryotic polypeptide chain-releasing factors (eRF) - Its identification as eRF3 interacting with eRF1. J Biol Chem *273*, 22254-22259.

Hoshino, S., Miyazawa, H., Enomoto, T., Hanaoka, F., Kikuchi, Y., Kikuchi, A., and Ui, M. (1989). A Human Homolog of the Yeast Gst1-Gene Codes for a Gtp-Binding Protein and Is Expressed in a Proliferation-Dependent Manner in Mammalian-Cells. Embo Journal *8*, 3807-3814.

Huntzinger, E., Kashima, I., Fauser, M., Sauliere, J., and Izaurralde, E. (2008). SMG6 is the catalytic endonuclease that cleaves mRNAs containing nonsense codons in metazoan. RNA *14*, 2609-2617.

Hurt, J.A., Robertson, A.D., and Burge, C.B. (2013). Global analyses of UPF1 binding and function reveal expanded scope of nonsense-mediated mRNA decay. Genome research *23*, 1636-1650.

Hwang, J., and Maquat, L.E. (2011). Nonsense-mediated mRNA decay (NMD) in animal embryogenesis: to die or not to die, that is the question. Current opinion in genetics & development *21*, 422-430.

Imataka, H., Gradi, A., and Sonenberg, N. (1998). A newly identified N-terminal amino acid sequence of human eIF4G

binds poly(A)-binding protein and functions in poly(A)-dependent translation. The EMBO journal *17*, 7480-7489.

Ishigaki, Y., Li, X., Serin, G., and Maquat, L.E. (2001). Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. Cell *106*, 607-617.

Ivanov, P.V., Gehring, N.H., Kunz, J.B., Hentze, M.W., and Kulozik, A.E. (2008). Interactions between UPF1, eRFs, PABP and the exon junction complex suggest an integrated model for mammalian NMD pathways. The EMBO journal *27*, 736-747.

Izaurralde, E., Lewis, J., McGuigan, C., Jankowska, M., Darzynkiewicz, E., and Mattaj, I.W. (1994). A nuclear cap binding protein complex involved in pre-mRNA splicing. Cell *78*, 657-668.

Jackson, R.J., Hellen, C.U., and Pestova, T.V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nature reviews Molecular cell biology *11*, 113-127.

Janzen, D.M., and Geballe, A.P. (2004). The effect of eukaryotic release factor depletion on translation termination in human cell lines. Nucleic Acids Res *32*, 4491-4502.

Jonas, S., Weichenrieder, O., and Izaurralde, E. (2013). An unusual arrangement of two 14-3-3-like domains in the SMG5-SMG7 heterodimer is required for efficient nonsense-mediated mRNA decay. Genes Dev *27*, 211-225.

Kahvejian, A., Svitkin, Y.V., Sukarieh, R., M'Boutchou, M.N., and Sonenberg, N. (2005). Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes Dev *19*, 104-113.

Kashima, I., Yamashita, A., Izumi, N., Kataoka, N., Morishita, R., Hoshino, S., Ohno, M., Dreyfuss, G., and Ohno, S. (2006). Binding of a novel SMG-1-Upf1-eRF1-eRF3 complex (SURF) to the exon junction complex triggers Upf1 phosphorylation and nonsense-mediated mRNA decay. Genes Dev *20*, 355-367.

Kerem, E. (2004). Pharmacologic therapy for stop mutations: how much CFTR activity is enough? Current opinion in pulmonary medicine *10*, 547-552.

Kerr, T.P., Sewry, C.A., Robb, S.A., and Roberts, R.G. (2001). Long mutant dystrophins and variable phenotypes: evasion of nonsense-mediated decay? Hum Genet *109*, 402-407.

Khaleghpour, K., Svitkin, Y.V., Craig, A.W., DeMaria, C.T., Deo, R.C., Burley, S.K., and Sonenberg, N. (2001). Translational repression by a novel partner of human poly(A) binding protein, Paip2. Molecular cell *7*, 205-216.

Knoch, K.P., Bergert, H., Borgonovo, B., Saeger, H.D., Altkruger, A., Verkade, P., and Solimena, M. (2004). Polypyrimidine tract-binding protein promotes insulin secretory granule biogenesis. Nat Cell Biol *6*, 207-214.

Kononenko, A.V., Mitkevich, V.A., Atkinson, G.C., Tenson, T., Dubovaya, V.I., Frolova, L.Y., Makarov, A.A., and Hauryliuk, V. (2010). GTP-dependent

structural rearrangement of the eRF1:eRF3 complex and eRF3 sequence motifs essential for PABP binding. Nucleic Acids Res *38*, 548-558.

Kovesi, T.A., Swartz, R., and MacDonald, N. (1998). Transient renal failure due to simultaneous ibuprofen and aminoglycoside therapy in children with cystic fibrosis. N Engl J Med *338*, 65-66.

Kozlov, G., De Crescenzo, G., Lim, N.S., Siddiqui, N., Fantus, D., Kahvejian, A., Trempe, J.F., Elias, D., Ekiel, I., Sonenberg, N.*, et al.* (2004). Structural basis of ligand recognition by PABC, a highly specific peptide-binding domain found in poly(A)-binding protein and a HECT ubiquitin ligase. The EMBO journal *23*, 272-281.

Kozlov, G., and Gehring, K. (2010). Molecular basis of eRF3 recognition by the MLLE domain of poly(A)-binding protein. PLoS One *5*, e10169.

Kozlov, G., Trempe, J.F., Khaleghpour, K., Kahvejian, A., Ekiel, I., and Gehring, K. (2001). Structure and function of the C-terminal PABC domain of human poly(A)-binding protein. Proceedings of the National Academy of Sciences of the United States of America *98*, 4409-4413.

Kuhn, U., and Wahle, E. (2004). Structure and function of poly(A) binding proteins. Biochim Biophys Acta *1678*, 67-84.

Kunz, J.B., Neu-Yilik, G., Hentze, M.W., Kulozik, A.E., and Gehring, N.H. (2006). Functions of hUpf3a and hUpf3b in nonsense-mediated mRNA decay and translation. RNA *12*, 1015-1022.

Kurosaki, T., Li, W., Hoque, M., Popp, M.W., Ermolenko, D.N., Tian, B., and Maquat, L.E. (2014). A post-translational regulatory switch on UPF1 controls targeted mRNA degradation. Genes Dev *28*, 1900-1916.

Kurosaki, T., and Maquat, L.E. (2013). Rules that govern UPF1 binding to mRNA 3' UTRs. Proceedings of the National Academy of Sciences of the United States of America *110*, 3357-3362.

Le Hir, H., Izaurralde, E., Maquat, L.E., and Moore, M.J. (2000). The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. The EMBO journal *19*, 6860-6869.

Lee, B.J., Worland, P.J., Davis, J.N., Stadtman, T.C., and Hatfield, D.L. (1989). Identification of a selenocysteyl-tRNA(Ser) in mammalian cells that recognizes the nonsense codon, UGA. The Journal of biological chemistry *264*, 9724-9727.

Leeds, P., Peltz, S.W., Jacobson, A., and Culbertson, M.R. (1991). The product of the yeast UPF1 gene is required for rapid turnover of mRNAs containing a premature translational termination codon. Genes Dev *5*, 2303-2314.

Leeds, P., Wood, J.M., Lee, B.S., and Culbertson, M.R. (1992). Gene products that promote mRNA turnover in Saccharomyces cerevisiae. Mol Cell Biol *12*, 2165-2177.

Lejeune, F., Ishigaki, Y., Li, X., and Maquat, L.E. (2002). The exon junction complex is detected on CBP80-bound but not eIF4E-bound mRNA in mammalian cells: dynamics of mRNP remodeling. The EMBO journal *21*, 3536-3545.

Lewis, B.P., Green, R.E., and Brenner, S.E. (2003). Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. Proceedings of the National Academy of Sciences of the United States of America *100*, 189-192.

Lewis, J.D., and Izaurralde, E. (1997). The role of the cap structure in RNA processing and nuclear export. Eur J Biochem *247*, 461-469.

Li, T., Shi, Y., Wang, P., Guachalla, L.M., Sun, B., Joerss, T., Chen, Y.S., Groth, M., Krueger, A., Platzer, M., *et al.* (2015). Smg6/Est1 licenses embryonic stem cell differentiation via nonsense-mediated mRNA decay. The EMBO journal *34*, 1630-1647.

Loh, B., Jonas, S., and Izaurralde, E. (2013). The SMG5-SMG7 heterodimer directly recruits the CCR4-NOT deadenylase complex to mRNAs containing nonsense codons via interaction with POP2. Genes Dev *27*, 2125-2138.

Lykke-Andersen, J., Shu, M.D., and Steitz, J.A. (2000). Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. Cell *103*, 1121-1131.

Lykke-Andersen, J., Shu, M.D., and Steitz, J.A. (2001). Communication of the position of exon-exon junctions to the mRNA surveillance machinery by the protein RNPS1. Science *293*, 1836-1839.

Lykke-Andersen, S., Chen, Y., Ardal, B.R., Lilje, B., Waage, J., Sandelin, A., and Jensen, T.H. (2014). Human nonsense-mediated RNA decay initiates widely by endonucleolysis and targets snoRNA host genes. Genes Dev *28*, 2498-2517.

Mayeda, A., Badolato, J., Kobayashi, R., Zhang, M.Q., Gardiner, E.M., and Krainer, A.R. (1999). Purification and characterization of human RNPS1: a general activator of pre-mRNA splicing. The EMBO journal *18*, 4560-4570.

McElroy, S.P., Nomura, T., Torrie, L.S., Warbrick, E., Gartner, U., Wood, G., and McLean, W.H. (2013). A lack of premature termination codon read-through efficacy of PTC124 (Ataluren) in a diverse array of reporter assays. Plos Biol *11*, e1001593.

McIlwain, D.R., Pan, Q., Reilly, P.T., Elia, A.J., McCracken, S., Wakeham, A.C., Itie-Youten, A., Blencowe, B.J., and Mak, T.W. (2010). Smg1 is required for embryogenesis and regulates diverse genes via alternative splicing coupled to nonsense-mediated mRNA decay. Proceedings of the National Academy of Sciences of the United States of America *107*, 12186-12191.

Medghalchi, S.M., Frischmeyer, P.A., Mendell, J.T., Kelly, A.G., Lawler, A.M., and Dietz, H.C. (2001). Rent1, a trans-effector of nonsense-mediated mRNA decay, is essential for mammalian embryonic viability. Hum Mol Genet *10*, 99-105.

Mendell, J.T., ap Rhys, C.M., and Dietz, H.C. (2002). Separable roles for rent1/hUpf1 in altered splicing and decay of nonsense transcripts. Science *298*, 419-422.

Mendell, J.T., Sharifi, N.A., Meyers, J.L., Martinez-Murillo, F., and Dietz, H.C. (2004). Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise. Nat Genet *36*, 1073-1078.

Metze, S., Herzog, V.A., Ruepp, M.D., and Muhlemann, O. (2013). Comparison of EJC-enhanced and EJC-independent NMD in human cells reveals two partially redundant degradation pathways. RNA *19*, 1432-1448.

Mishler, D.M., Christ, A.B., and Steitz, J.A. (2008). Flexibility in the site of exon junction complex deposition revealed by functional group and RNA secondary structure alterations in the splicing substrate. RNA *14*, 2657-2670.

Moore, M.J. (2005). From birth to death: the complex lives of eukaryotic mRNAs. Science *309*, 1514-1518.

Moore, M.J., and Proudfoot, N.J. (2009). Pre-mRNA processing reaches back to transcription and ahead to translation. Cell *136*, 688-700.

Mort, M., Ivanov, D., Cooper, D.N., and Chuzhanova, N.A. (2008). A meta-analysis of nonsense mutations causing human genetic disease. Hum Mutat *29*, 1037-1047.

Muhlrad, D., and Parker, R. (1999). Aberrant mRNAs with extended 3' UTRs are substrates for rapid degradation by mRNA surveillance. RNA *5*, 1299-1307.

Nagy, E., and Maquat, L.E. (1998). A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. Trends in Biochemical Sciences *23*, 198-199.

Nicholson, P., and Muhlemann, O. (2010). Cutting the nonsense: the degradation of PTC-containing mRNAs. Biochem Soc Trans *38*, 1615-1620.

Nicholson, P., Yepiskoposyan, H., Metze, S., Zamudio Orozco, R., Kleinschmidt, N., and Muhlemann, O. (2010). Nonsense-mediated mRNA decay in human cells: mechanistic insights, functions beyond quality control and the double-life of NMD factors. Cell Mol Life Sci *67*, 677-700.

Nomakuchi, T.T., Rigo, F., Aznarez, I., and Krainer, A.R. (2015). Antisense oligonucleotide-directed inhibition of nonsense-mediated mRNA decay. Nat Biotechnol.

Obsil, T., and Obsilova, V. (2011). Structural basis of 14-3-3 protein functions. Seminars in cell & developmental biology *22*, 663-672.

Ohnishi, T., Yamashita, A., Kashima, I., Schell, T., Anders, K.R., Grimson, A., Hachiya, T., Hentze, M.W., Anderson, P., and Ohno, S. (2003). Phosphorylation of hUPF1 induces formation of mRNA surveillance complexes containing hSMG-5 and hSMG-7. Molecular cell *12*, 1187-1200.

Okada-Katsuhata, Y., Yamashita, A., Kutsuzawa, K., Izumi, N., Hirahara, F., and Ohno, S. (2012). N- and C-terminal Upf1 phosphorylations create binding platforms for SMG-6 and SMG-5:SMG-7 during NMD. Nucleic Acids Res *40*, 1251-1266.

Osawa, M., Hosoda, N., Nakanishi, T., Uchida, N., Kimura, T., Imai, S., Machiyama, A., Katada, T., Hoshino, S., and Shimada, I. (2012). Biological role of the two overlapping poly(A)-binding protein interacting motifs 2 (PAM2) of eukaryotic releasing factor eRF3 in mRNA decay. RNA *18*, 1957-1967.

Page, M.F., Carr, B., Anders, K.R., Grimson, A., and Anderson, P. (1999). SMG-2 is a phosphorylated protein required for mRNA surveillance in

Caenorhabditis elegans and related to Upf1p of yeast. Mol Cell Biol *19*, 5943-5951.

Peixeiro, I., Inacio, A., Barbosa, C., Silva, A.L., Liebhaber, S.A., and Romao, L. (2012). Interaction of PABPC1 with the translation initiation complex is critical to the NMD resistance of AUG-proximal nonsense mutations. Nucleic Acids Res *40*, 1160-1173.

Peltz, S.W., Morsy, M., Welch, E.M., and Jacobson, A. (2013). Ataluren as an agent for therapeutic nonsense suppression. Annu Rev Med *64*, 407-425.

Pergolizzi, R., Spritz, R.A., Spence, S., Goossens, M., Kan, Y.W., and Bank, A. (1981). Two cloned beta thalassemia genes are associated with amber mutations at codon 39. Nucleic acids research *9*, 7065-7072.

Pestova, T.V., and Kolupaeva, V.G. (2002). The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. Genes Dev *16*, 2906-2922.

Pisarev, A.V., Skabkin, M.A., Pisareva, V.P., Skabkina, O.V., Rakotondrafara, A.M., Hentze, M.W., Hellen, C.U., and Pestova, T.V. (2010). The role of ABCE1 in eukaryotic posttermination ribosomal recycling. Molecular cell *37*, 196-210.

Rufener, S.C., and Muhlemann, O. (2013). eIF4E-bound mRNPs are substrates for nonsense-mediated mRNA decay in mammalian cells. Nat Struct Mol Biol *20*, 710-717.

Safaee, N., Kozlov, G., Noronha, A.M., Xie, J., Wilds, C.J., and Gehring, K. (2012). Interdomain allostery promotes assembly of the poly(A) mRNA complex with PABP and eIF4G. Molecular cell *48*, 375-386.

Salas-Marco, J., and Bedwell, D.M. (2004). GTP hydrolysis by eRF3 facilitates stop codon decoding during eukaryotic translation termination. Mol Cell Biol *24*, 7769-7778.

Schmidt, S.A., Foley, P.L., Jeong, D.H., Rymarquis, L.A., Doyle, F., Tenenbaum, S.A., Belasco, J.G., and Green, P.J. (2015). Identification of SMG6 cleavage sites and a preferred RNA cleavage motif by global analysis of endogenous NMD targets in human cells. Nucleic Acids Res *43*, 309-323.

Schweingruber, C., Rufener, S.C., Zund, D., Yamashita, A., and Muhlemann, O. (2013). Nonsense-mediated mRNA decay - mechanisms of substrate mRNA recognition and degradation in mammalian cells. Biochim Biophys Acta *1829*, 612-623.

Serin, G., Gersappe, A., Black, J.D., Aronoff, R., and Maquat, L.E. (2001). Identification and characterization of human orthologues to Saccharomyces cerevisiae Upf2 protein and Upf3 protein (Caenorhabditis elegans SMG-4). Mol Cell Biol *21*, 209-223.

Siddiqui, N., Mangus, D.A., Chang, T.C., Palermino, J.M., Shyu, A.B., and Gehring, K. (2007). Poly(A) nuclease interacts with the C-terminal domain of polyadenylate-binding protein domain from poly(A)-binding protein. J Biol Chem *282*, 25067-25075.

Singh, G., Rebbapragada, I., and Lykke-Andersen, J. (2008). A competition between stimulators and antagonists of Upf complex recruitment governs human nonsense-mediated mRNA decay. Plos Biol *6*, e111.

Singleton, M.R., Dillingham, M.S., and Wigley, D.B. (2007). Structure and mechanism of helicases and nucleic acid translocases. Annual review of biochemistry *76*, 23-50.

Sonenberg, N., and Dever, T.E. (2003). Eukaryotic translation initiation factors and regulators. Current opinion in structural biology *13*, 56-63.

Song, H., Mugnier, P., Das, A.K., Webb, H.M., Evans, D.R., Tuite, M.F., Hemmings, B.A., and Barford, D. (2000). The crystal structure of human eukaryotic release factor eRF1—mechanism of stop codon recognition and peptidyl-tRNA hydrolysis. Cell *100*, 311-321.

Stockklausner, C., Breit, S., Neu-Yilik, G., Echner, N., Hentze, M.W., Kulozik, A.E., and Gehring, N.H. (2006). The uORF-containing thrombopoietin mRNA escapes nonsense-mediated decay (NMD). Nucleic Acids Res *34*, 2355-2363.

Sureau, A., Gattoni, R., Dooghe, Y., Stevenin, J., and Soret, J. (2001). SC35 autoregulates its expression by promoting splicing events that destabilize its mRNAs. The EMBO journal *20*, 1785-1796.

Tani, H., Torimura, M., and Akimitsu, N. (2013). The RNA degradation pathway regulates the function of GAS5 a non-coding RNA in mammalian cells. PLoS One *8*, e55684.

Tarun, S.Z., Jr., and Sachs, A.B. (1996). Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. The EMBO journal *15*, 7168-7177.

Thermann, R., Neu-Yilik, G., Deters, A., Frede, U., Wehr, K., Hagemeier, C., Hentze, M.W., and Kulozik, A.E. (1998). Binary specification of nonsense codons by splicing and cytoplasmic translation. The EMBO journal *17*, 3484-3494.

Thompson, K.E., Bashor, C.J., Lim, W.A., and Keating, A.E. (2012). SYNZIP protein interaction toolbox: in vitro and in vivo specifications of heterospecific coiled-coil interaction domains. ACS Synth Biol *1*, 118-129.

Toma, K.G., Rebbapragada, I., Durand, S., and Lykke-Andersen, J. (2015). Identification of elements in human long 3' UTRs that inhibit nonsense-mediated decay. RNA *21*, 887-897.

Uchida, N., Hoshino, S., Imataka, H., Sonenberg, N., and Katada, T. (2002). A novel role of the mammalian GSPT/eRF3 associating with poly(A)-binding protein in Cap/Poly(A)-dependent translation. J Biol Chem *277*, 50286-50292.

Unterholzner, L., and Izaurralde, E. (2004). SMG7 acts as a molecular link between mRNA surveillance and mRNA decay. Molecular cell *16*, 587-596.

Wagner, E.J., and Garcia-Blanco, M.A. (2001). Polypyrimidine tract binding protein antagonizes exon definition. Mol Cell Biol *21*, 3281-3288.

Wakiyama, M., Imataka, H., and Sonenberg, N. (2000). Interaction of eIF4G with poly(A)-binding protein stimulates translation and is critical for Xenopus oocyte maturation. Curr Biol *10*, 1147-1150.

Weischenfeldt, J., Damgaard, I., Bryder, D., Theilgaard-Monch, K., Thoren, L.A., Nielsen, F.C., Jacobsen, S.E., Nerlov, C., and Porse, B.T. (2008). NMD is essential for hematopoietic stem and progenitor cells and for eliminating by-

products of programmed DNA rearrangements. Genes Dev *22*, 1381-1396.

Welch, E.M., Barton, E.R., Zhuo, J., Tomizawa, Y., Friesen, W.J., Trifillis, P., Paushkin, S., Patel, M., Trotta, C.R., Hwang, S.*, et al.* (2007). PTC124 targets genetic disorders caused by nonsense mutations. Nature *447*, 87-91.

Wells, S.E., Hillner, P.E., Vale, R.D., and Sachs, A.B. (1998). Circularization of mRNA by eukaryotic translation initiation factors. Molecular cell *2*, 135-140.

Weng, Y., Czaplinski, K., and Peltz, S.W. (1996a). Genetic and biochemical characterization of mutations in the ATPase and helicase regions of the Upf1 protein. Mol Cell Biol *16*, 5477-5490.

Weng, Y., Czaplinski, K., and Peltz, S.W. (1996b). Identification and characterization of mutations in the UPF1 gene that affect nonsense suppression and the formation of the Upf protein complex but not mRNA turnover. Mol Cell Biol *16*, 5491-5506.

Yamashita, A., Ohnishi, T., Kashima, I., Taya, Y., and Ohno, S. (2001). Human SMG-1, a novel phosphatidylinositol 3-kinase-related protein kinase, associates with components of the mRNA surveillance complex and is involved in the regulation of nonsense-mediated mRNA decay. Genes Dev *15*, 2215-2228.

Zhang, J., Sun, X., Qian, Y., and LaDuca, J.P. (1998a). At least one intron is required for the nonsense-mediated decay of triosephosphate isomerase mRNA: a possible link between nuclear splicing and cytoplasmic translation. Mol Cell Biol.

Zhang, J., Sun, X.L., Qian, Y.M., and Maquat, L.E. (1998b). Intron function in the nonsense-mediated decay of beta-globin mRNA: Indications that pre-mRNA splicing in the nucleus can influence mRNA translation in the cytoplasm. RNA *4*, 801-815.

Zhouravleva, G., Frolova, L., Le Goff, X., Le Guellec, R., Inge-Vechtomov, S., Kisselev, L., and Philippe, M. (1995). Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. The EMBO journal *14*, 4065-4072.

Zund, D., Gruber, A.R., Zavolan, M., and Muhlemann, O. (2013). Translation-dependent displacement of UPF1 from coding sequences causes its enrichment in 3' UTRs. Nat Struct Mol Biol *20*, 936-943.

9 Acknowledgement

I foremost would like to thank Dr. Niels Gehring for giving me the opportunity to conduct my PhD thesis work in his lab. His guidance and support both professionally and personally throughout my entire stay in his lab are very much appreciated.

I want to express my thanks to Prof. Dr. Karin Schnetz for taking the time and interest to be the second reviewer for this thesis and Prof. Dr. Ulrich Baumann to agree to chair my defense.

Many thanks go to all present and past members of the Gehring lab who were all supportive throughout my time here and made the daily life in the group even more enjoyable. Thank you for the great times both in the lab and the countless activities outside the lab. You have made the past six years fly by way too fast.

I also want to thank my friends both here in Cologne and in Heidelberg for their friendship and support throughout the years. Specifically, I want to thank Anna Lucia Ramirez for her constant support and encouragement; Volker Böhm and Simon Schumacher for their good friendship that made my life in Cologne so much more fun; and Tobias Schäfer, Florian Pitschmann, and Michael Bilinski for the great times we shared from the early days of high school continuing until today.

I want to express a very special thank you to my family who always supported me in every way they could. I specifically want to thank my parents. I appreciate the constant advice and support and am thankful for everything they have done for me over the years. This thesis would not have been possible in this way without their help.

10 Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von (Name des anleitenden Dozenten oder der anleitenden Dozentin) betreut worden.

Unterschrift