The CCR4-NOT deadenylation complex functions in the Nonsense Mediated mRNA Decay pathway.

DISSERTATION

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Belinda Loh

aus Singapore

Tübingen

2014

Tag der mündlichen Qualifikation: 25.09.2014

Dekan: Prof. Dr. Wolfgang Rosenstiel

1. Berichterstatter: Prof. Dr. Ralf Jansen

2. Berichterstatter: Prof. Dr. Elisa Izaurralde

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This thesis represents a milestone in my life that has been incredibly productive and immensely challenging in almost every way possible. This memorable and life-changing experience would not have been possible without the influences of many people whom I wish to thank...

First and foremost, I would like to express my deepest gratitude to Prof. Dr. Elisa Izaurralde for giving me the opportunity to study under her direct supervision in one of the world's most prestigious institutes. I am extremely grateful for her support, patience and guidance in every aspect of my training. I am thankful for her optimism at times when I had none. Thank you for the high expectations that have constantly pushed me towards excellence and for the rewarding experience in your lab.

I would like to thank Prof. Dr. Ralf Jansen at the Eberhard Karls Universität Tübingen for supervising this thesis and for taking care of my scientific development during this PhD.

I am also extremely grateful to the other members of my thesis advisory committee, Dr. Fulvia Bono and Dr. Remco Sprangers for their interest and insightful discussions.

I wish to thank the International PhD program coordinator Dagmar Sigurdardottir for her support and advice in many aspects of the PhD and beyond.

Friday seminars were not always met with a full attendance; however, I am thankful to those who attended and contributed to the discussions, especially Dr. Wolfram Antonin and Dr. Gáspár Jékely, both of whom have never failed to challenge me when it came to my turn to present my work.

I would like to thank all past and present members of Department II at the MPI for Developmental Biology for the stimulating environment, many of whom I have had the privilege to collaborate with for various projects. I wish to thank Dr. Isao

Kashima for introducing me to the lab, for his supervision and brief mentorship at the start of my PhD. Special thanks go to the PhD students (both past and present) for their support both within and outside of the lab, especially Valentina Ahl, Andreas Boland, Joerg E. Braun and Anna Schneider. I am eternally grateful to Dr. Eric Huntzinger and Duygu Kuzuoğlu-Öztürk for saving my sanity on many occasions and for being around despite me being difficult at times. I would like to thank those surrounding my bench not only for the useful and creative scientific discussions but also for the friendship and encouragements, namely Dr. Dipankar Bhandari, Dr. Chang Chung-Te, Dr. Praveen Bawankar and Dr. Moritz Thran. I would also like to acknowledge Dr. Catia Igreja for the insightful discussions and for always having ideas. Many thanks to Dr. Heike Budde for taking care of the plasmid databank and to the technicians in particular Sigrun Helms and Maria Fauser for keeping the lab organized and looking out for our welfare.

My heartfelt thanks go to my friends, Julia Hildebrandt, Vladimír Polívka and Linda Ebertsch for their support and providing necessary distractions.

I would like to thank my parents and my sisters for their understanding when I was unable to fly home when they wanted me to. I send my greatest thanks to them for their patience and tolerance during my absence and my notorious lack of communication. Having said this, I am extremely thankful for the development of Skype and Google's android that have recently helped bridge some of these barriers.

And last but certainly not least, I would like to thank my husband, Sebastian Leptihn for his love and care during my PhD. He was not as patient as I would have liked him to be but it was, in some way, necessary to protect me from over-working myself. Thank you for taking care of the household when I was barely around to do it. And thank you for taking care of me when I was doing a terrible job at it.

1A. SUMMARY

Quality control mechanisms have evolved to guard the cell against defects in gene expression. The nonsense mediated mRNA decay (NMD) pathway is one of the most well studied surveillance mechanisms. It detects and triggers degradation of aberrant mRNAs that contain pre-mature termination codons (PTCs), preventing the accumulation of truncated polypeptides that are potentially deleterious to the cell. PTC recognition in eukaryotes results in the assembly of a surveillance complex on the mRNA, which triggers the degradation of the PTC-containing mRNA. The surveillance complex consists of the Up-frameshift (UPF) proteins (1 to 3) and in metazoa, the Suppressor with morphological effects on genitalia (SMG) proteins (1, 5 to 9). UPF1 undergoes a phosphorylation/dephosphorylation cycle that is a key event driving NMD. Upon PTC recognition, UPF1 is phosphorylated by the SMG1 kinase, initiating mRNA decay through the recruitment of the 14-3-3 domain-containing proteins SMG5, SMG6 and SMG7. SMG6 was shown to possess endonuclease activity that cleaves the target mRNA in the vicinity of the PTC. On the other hand, SMG5 and SMG7 form a heterodimer and recruit general cellular decay enzymes. SMG5 was shown to recruit the decapping enzyme DCP2 and its co-factors through PNRC2. SMG7 was shown to decay mRNA efficiently through its Proline-rich C-terminus (PC) region, which is necessary and sufficient for this activity. However, which decay factors are recruited to the target mRNA has remained unknown.

The major part of my doctoral work focused on elucidating the significance of the SMG5:SMG7 heterodimer formation in NMD and understanding how SMG7 elicits decay of a NMD-targeted mRNA. Here, I could show that SMG5:SMG7 heterodimer formation is necessary for functional NMD and that SMG5 and SMG7 use distinct mechanisms to degrade NMD-targeted mRNA. SMG5 promotes decapping independently of deadenylation while SMG7 promotes deadenylation-dependent decapping through a direct interaction with POP2, a catalytic subunit of the CCR4-NOT deadenylase complex. This interaction is specific, as SMG7 did not bind to CAF1, a paralog of POP2. I could further demonstrate that POP2 contributes to

NMD target degradation in human cells and that the SMG7-POP2 interaction was critical for NMD in cells depleted of SMG6. This indicated that SMG6 and SMG7 act redundantly to degrade NMD targets. Taken together, my work demonstrated how NMD employs diverse and partially redundant decay mechanisms to ensure that aberrant mRNAs are efficiently degraded.

The CCR4-NOT deadenylase complex is the major component involved in the first step of cellular mRNA degradation. This complex catalyzes the removal of the poly(A) tail from the 3'-end of the mRNA, hence causing translational repression and committing the mRNA to degradation. The core components of the CCR4-NOT complex consist minimally of two modules, i.e. the NOT module that includes NOT1, NOT2 and NOT3, and the catalytic module that involves two deadenlyases, CCR4 and POP2/CAF1 bound to NOT1. In human cells, additional components have been identified, namely, CAF40/CNOT9, CNOT10 and CNOT11. These components are conserved in *Drosophila* cells, however the role of these proteins and how they are integrated into the complex remained unknown. Thus, the next part of my studies addressed the molecular characterization of the CCR4-NOT complex in *Drosophila* cells. In this part of my work, I could show that NOT10 binds directly to NOT11 and forms a novel module of the CCR4-NOT complex. This module docks directly on the N-terminus of NOT1 that was hence named the NOT10/11 Binding Domain (NOT10/11 BD). This direct interaction to NOT1 was mediated by NOT11 and is conserved in human cells.

1B. ZUSAMMENFASSUNG

Um defekter Genexpression vorzubeugen, haben sich im Laufe der Evolution Qualitätskontrollmechanismen entwickelt. Der am besten untersuchte Überwachungsmechanismus ist der sogenannte *nonsense-mediated mRNA decay* (NMD) Signalweg. Fehlerhafte mRNAs mit vorzeitigen Stopp-Codons (abgekürzt PTC; englisch: *pre-mature termination codon*) werden vom NMD erkannt und abgebaut, was die Anhäufung von verkürzten Polypeptiden mit potentiell schädlicher Wirkung verhindert. Nach der Erkennung eines PTC in Eukaryoten folgt die Anlagerung eines Überwachungskomplexes, was schließlich zum Abbau der PTC-enthaltenden mRNA führt. Der Komplex besteht aus den *Up-frameshift* (UPF) Proteinen (1, 2 und 3) sowie den Metazoa-spezifischen *suppressor with morphological effects on genitalia* (SMG) Proteinen (1 und 5 bis 9). UPF1 unterliegt einem Phosphorylierung/De- Phosphorylierungszyklus, was den NMD reguliert. Nach der Erkennung eines PTC wird UPF1 durch die SMG1 Kinase phosphoryliert, wodurch die 14-3-3 Proteine SMG5, SMG6 und SMG7 rekrutiert werden, woraufhin der mRNA Abbau beginnt. Einerseits kommt es zum Ziel-mRNA Abbau durch SMG6, welches als Endonuklease die RNA in unmittelbarer Nähe zum PTC spaltet. Andererseits bildet SMG5 und SMG7 ein Heterodimer aus, was zur Rekrutierung genereller mRNA Degradationsfaktoren führt. So konnte gezeigt werden, dass durch SMG7 das *decapping* Enzym DCP2 zusammen mit seinen Kofaktoren über die Bindung zu PNRC2 rekrutiert wird. Zudem wurde gezeigt, dass der Prolin-reiche C-Terminus von SMG7 essentiell und ausreichend für die Degradation von mRNA ist. Dennoch bleibt unklar, welche Faktoren der Degradationsmaschinerie letztlich an der mRNA rekrutiert werden.

Im Hauptteil dieser Doktorarbeit wurde die Frage nach der Relevanz der Ausbildung des SMG5:SMG7 Heterodimers innerhalb des NMD gestellt. Zudem sollte untersucht werden, wie SMG7 letztlich den Abbau der NMD Ziel-mRNA verursacht. Es konnte gezeigt werden, dass die Ausbildung des SMG5:SMG7 Heterodimers für die NMD-Funktion benötigt wird und dass beide Proteine unterschiedliche Mechanismen zum Abbau der NMD Ziel-mRNA verwenden. SMG5

verursacht *decapping* welches unabhängig von Deadenylierung ist, wohingegen SMG7 zu *decapping* in einem Deadenylierungs-abhängigen Prozess führt. Letzteres wird über die Interaktion mit POP2, einer katalytischen Untereinheit des CCR4-NOT Komplexes, vermittelt. Dass diese Interaktion spezifisch ist, wurde dadurch bestätigt, dass SMG7 nicht an CAF1, einem Paralog von POP2 bindet. Die in dieser Arbeit gewonnenen Daten zeigen, dass POP2 zum NMD-abhängigen Abbau von Ziel-RNAs in humanen Zellen beiträgt, wobei die Interaktion mit SMG7 absolut notwendig ist, wenn die Zellen von SMG6 depletiert wurden. Dies deutet daraufhin, dass SMG6 und SMG7 beim Abbau von NMD Ziel-mRNAs redundante Funktionen haben. Zusammenfassend zeigen die Ergebnisse dieser Arbeit, dass der NMD Prozess verschiedene und teils redundante Mechanismen verwendet, um sicherzustellen, dass fehlerhafte mRNA Moleküle effizient abgebaut werden.

Der CCR4-NOT Deadenylase-Komplex ist die Hauptkomponente für den ersten Schritt des mRNA Abbaus. Durch die enzymatische Abspaltung des Poly-(A) Schwanzes am 3' Ende der RNA verhindert der Komplex die Translation und initiiert den Abbau der mRNA. Die Kernkomponenten des Komplexes bestehen aus mindestens zwei Modulen: dem NOT Modul welches NOT1, NOT2 und NOT3 beinhaltet und dem katalytischen Modul, das aus den an NOT1 gebunden zwei Deadenylasen CCR4 und POP2/CAF1 besteht. In humanen Zellen wurden weitere Faktoren identifiziert: CAF40/CNOT9, CNOT10 sowie CNOT11. Diese Komponenten sind in Drosophila konserviert, jedoch blieb die Funktion dieser Proteine und ihre Rolle im Komplex bislang unbekannt. Daher befassten sich die weiteren Untersuchungen im Rahmen dieser Arbeit mit der molekularen Charakterisierung des CCR4-NOT Komplexes in Drosophila. In diesem Teil der Arbeit wurde gezeigt, dass NOT10 an NOT11 bindet und somit ein neues Modul des CCR4-NOT Komplexes ausbildet. Dieses Modul interagiert direkt mit dem N-Terminus von NOT1 und wurde daher als NOT10/11-bindende Domäne bezeichnet (NOT10/11 BD). Diese direkte Interaktion von NOT1 und dem Modul wurde durch NOT11 vermittelt und ist in humanen Zellen konserviert.

2. INTRODUCTION

Translation and mRNA degradation are interdependent mechanisms that are important for regulated gene expression. In eukaryotic cells, the connections between messenger RNA (mRNA) translation and decay are most evident in quality control mechanisms such as the Non-Stop Decay (NSD), the No-Go Decay (NGD) and the Nonsense Mediated Decay (NMD) pathways where a defect that stalls translation triggers mRNA decay. Hence, to fully appreciate the studies conducted in this thesis, I will first briefly introduce the steps in eukaryotic protein synthesis, followed by an overview of the mechanisms involved in mRNA degradation and finally, I will discuss the main pathways involved in cellular quality control.

2.1. Translation.

Protein production is a multistep process that is essentially divided into three steps: initiation, elongation and termination. Translation initiation is a highly regulated process that involves the binding of the 43S pre-initiation complex (comprising of the 40S ribosomal subunit, the eIF2-GTP-Met-tRNA$^{Met}_i$ ternary complex, eukaryotic initiation factor eIF3, eIF1, eIF1A and eIF5) to the 5' end of the mRNA (Jackson et al, 2010). Translation is stimulated by the 5' 7-methyl-guanosine (m^7G) cap structure and the 3' poly(A) tail that help to circularize the mRNA. Circularization is mediated by interactions between eIF4G (the scaffold protein of the eIF4F complex) that binds the 5' cap structure, and the poly(A) binding proteins (PABP) that bind and coat the poly(A) tail of the mRNA (Preiss & M, 2003). The 43S pre-initiation complex is recruited to the 5'-end of the mRNA that is kept free from any secondary structures by eIF4A, eIF4B and eIF4F (Jackson et al, 2010). The 43S complex then scans the 5'-UTR for the initiation codon in a 5'→3' direction. Upon recognition of the start codon, the scanning complex switches to a "closed" conformation that leads to eIF5-mediated hydrolysis of eIF2-bound GTP (Jackson et al, 2010). This results in the displacement of eIF2-GDP and other factors (eIF1, eIF3, eIF4B and eIF5) and allows the 60S ribosomal subunit to
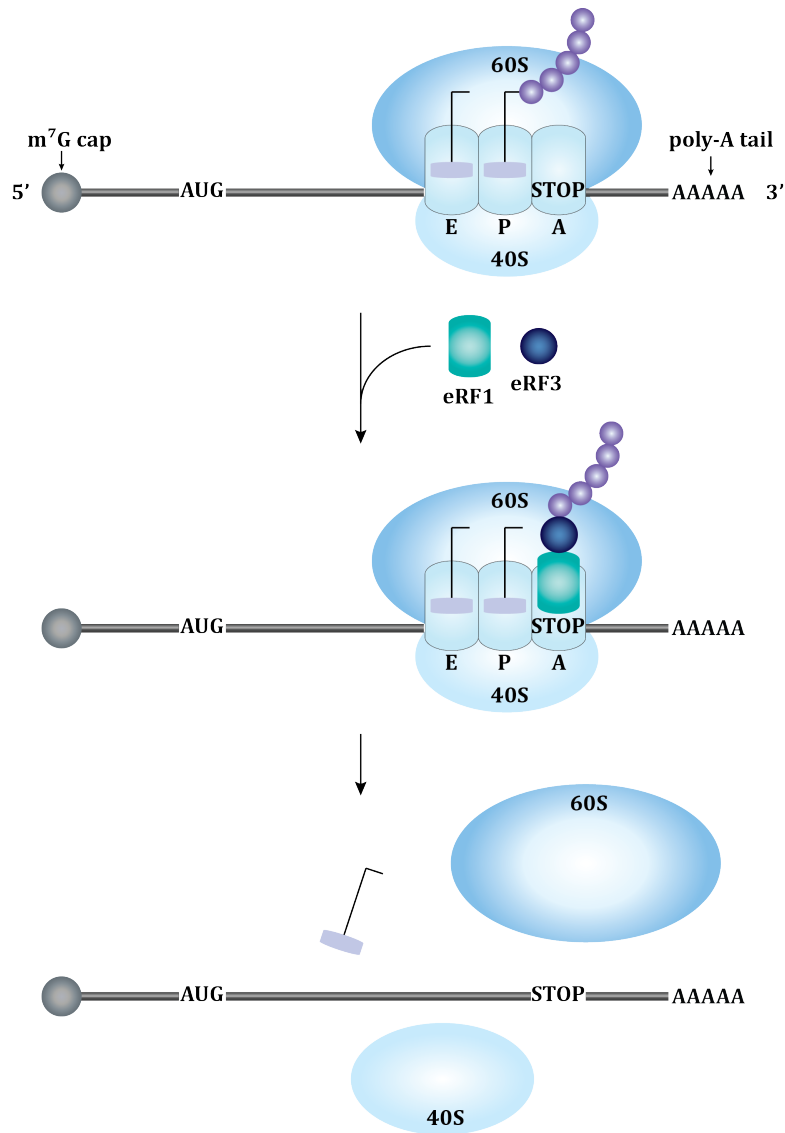
join the 40S ribosomal subunit, forming the 80S ribosome that provides three binding sites for tRNAs: the aminoacyl site (A-site), the peptidyl site (P-site) and the exit site (E-site) (Figure 1) (Jackson et al, 2010; Lafontaine & Tollervey, 2001). Upon the formation of the 80S ribosome, the Met-tRNA base-pairs with the initiation codon in the P-site to start translation elongation.

Translation elongation is facilitated by two factors, eukaryotic elongation factor eEF1A that delivers the aminoacyl-tRNA to the A-site of the ribosome in a GTP-dependent manner, and eEF2 that translocates the tRNAs and associated mRNA from their positions in the A-site and P-site to the P-site and E-site respectively, and releases previously bound E-site tRNA (Steitz, 2008).

Translation termination is catalysed by release factors (RF) upon the identification of one of the three stop codons (UGA, UAG and UAA). In eukaryotes, the release factors eRF1 and eRF3 are recruited to terminate translation in a cooperative manner (Figure 1). eRF1 is responsible for recognizing the stop codon by mimicking tRNAs and basepairing with the stop codon (Baierlein & Krebber, 2010). By binding the stop codon in the ribosomal A-site, eRF1 induces peptide release by hydrolysing the polypeptide from the peptidyl-tRNA in the P-site of the ribosome (Pisareva et al, 2011). Hydrolysis of the peptidyl-tRNA occurs in the peptidyl transferase center of the 60S ribosomal subunit however hydrolysis by eRF1 alone is very inefficient (Jackson et al, 2012). To accelerate stop codon recognition and peptide release, eRF1 binds directly to the GTPase, eRF3 (Baierlein & Krebber, 2010). eRF3 is required to bind both the 80S ribosome and eRF1 to stimulate GTP hydrolysis and this in turn, induces conformational changes in eRF1 to trigger peptidyl-tRNA hydrolysis (Frolova et al, 1996; Jackson et al, 2012). Hence, there is a mutual interdependence of eRF1 and eRF3 in terminating translation that requires their direct interaction (Jackson et al, 2012).

Figure 1: Translation Termination.

The 80S ribosome is formed by the 60S ribosomal subunit joining the 40S subunit. The interface between the 60S and 40S subunits provides the three tRNA-binding sites: A, P and E sites. mRNA is represented in a linear configuration for simplicity. However, as mentioned in the text, eukaryotic mRNAs are held in circular conformations by interactions between eIF4G and PABP (not shown). Translation is terminated when the ribosome reaches the end of the coding region and a stop codon enters the A-site. The string of purple spheres represents a polypeptide chain. ⌐: tRNA. eRF: eukaryotic release factors.

2.2. General mRNA decay.

In eukaryotes, degradation of bulk mRNA begins with deadenylation, the removal of the poly(A) tail at the 3'-end of the mRNA (Doidge et al, 2012b; Wahle & Winkler, 2013). In metazoa, deadenylation is catalyzed by the concerted action of two complexes. The Poly(A)-Specific nuclease PAN2 associated with PAN3, starts the shortening of the poly(A) tail that is then taken over and completed by the CCR4-NOT complex (Figure 2) (Yamashita et al, 2005).
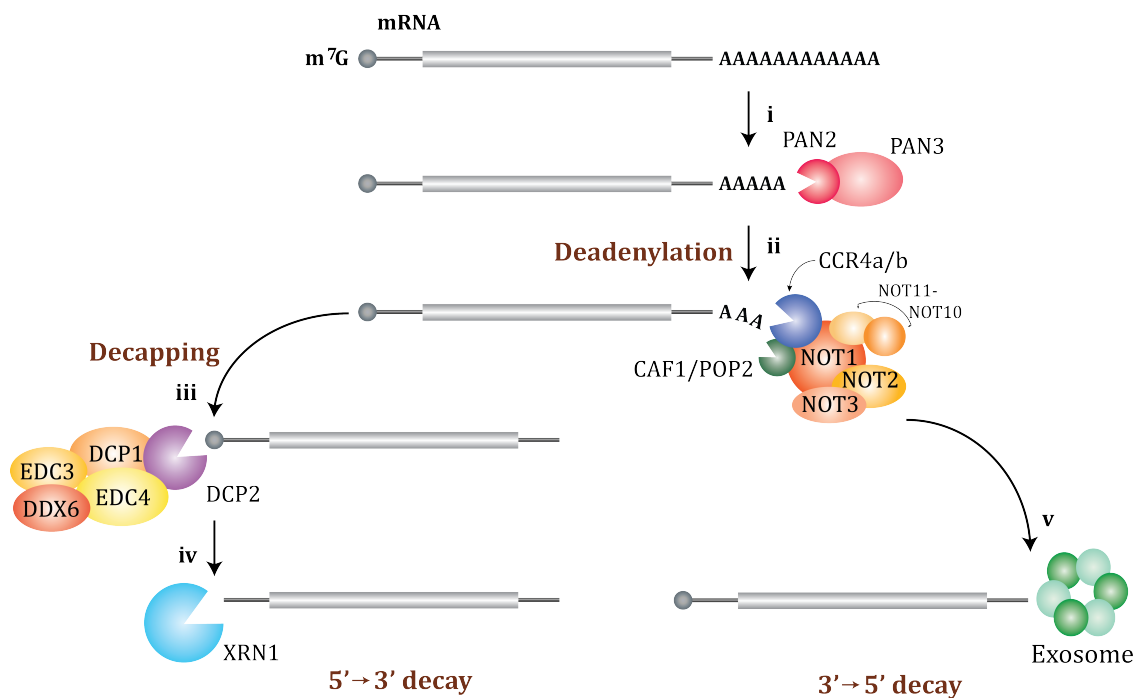
Figure 2: General mRNA decay.
i., **ii**: The first step of cellular mRNA decay is deadenylation. Two separate deadenylase complexes catalyse the poly(A) tail removal; first the PAN2-PAN3 complex (i) followed by the CCR4-NOT complex (ii). The deadenylated mRNA can then undergo one out of two pathways for complete degradation. **iii**: In the 5'→ 3' decay pathway, the decapping enzyme DCP2 and its associated co-factors are recruited to remove the 5' cap structure. **iv**: The uncapped mRNA is then completely degraded from the 5'-end to the 3'-end by the exonuclease XRN1. **v**: In the 3'→ 5' decay pathway, the deadenylated mRNA is fully degraded from the 3'-end by the exosome complex.

The CCR4-NOT complex is a multiprotein complex that is evolutionarily conserved among eukaryotes (Albert et al, 2000; Basquin et al, 2012; Lau et al, 2009; Temme et al, 2010). It contains two exonucleases, CCR4a (or its paralog CCR4b) and CAF1 (or its paralog POP2). Additional components of the complex include NOT1, NOT2, NOT3, NOT9 (also known as CAF40, Rcd1 or RQCD1), NOT10, NOT11 (also known as C2ORF29) and TAB182 (Lau et al, 2009). The composition of the CCR4-NOT complex differs between species. In yeast, NOT4 has been identified as a stable subunit but not in *Drosophila melanogaster* (*Dm*) and human cells (Bawankar et al, 2013; Collart & Panasenko, 2012). Additionally, NOT5 has been identified as a NOT3 paralog in yeast while in metazoans only one gene encodes NOT3 (also known as NOT3/5) (Bawankar et al, 2013; Collart & Panasenko, 2012). Species-specific subunits have also been identified: CAF130 is a yeast-specific subunit without a metazoan counterpart while TAB182 was identified as a human-specific subunit with no ortholog found in yeast (Collart & Panasenko, 2012; Lau et al, 2009).

In the context of deadenylation, it should be noted that another adenosine specific 3' → 5' exonuclease found in human cells is the Poly(A) ribonuclease or PARN. This enzyme is conserved in eukaryotes although it has been shown to be lacking in yeast and *D. melanogaster* and is dispensable in *C. elegans* (Parker & Song, 2004). Like CAF1, PARN belongs to the DEDD class of exonucleases but it also binds to the 5'-cap structure for efficient deadenylation (Godwin et al, 2013). It has been shown to function in small nucleolar RNA maturation, mRNA decay following DNA damage and translational repression (Parker & Song, 2004).

Following deadenylation, mRNA degradation can proceed by two alternative pathways. In one pathway, decapping may proceed directly after deadenylation. The LSm1-7-PatLI complex mediates the transition between deadenylation and decapping (Haas et al, 2010; Ozgur et al, 2010; Tharun, 2009). DCP2 is the enzyme responsible for removing the mRNA cap structure. Its activity is stimulated by additional co-activators including DCP1, EDC3 (enhancer of mRNA decapping 3), EDC4 (also known as Ge-1 or Hedls) and the DEAD-box helicase DDX6 (also known as

RCK or p54 in vertebrates and Me31B in *D. melanogester*). Cap removal exposes the mRNA to the exonuclease XRN1 that degrades the target mRNA in the 5' → 3' direction (Parker & Song, 2004). Alternatively, deadenylated mRNAs can be degraded by a protein complex called the exosome (Parker & Song, 2004). The decapping scavenger enzyme, DCPS, then hydrolyzes the residual cap structure for complete degradation (Liu et al, 2002).

2.3. Quality Control Mechanisms.

Eukaryotic cells have evolved mRNA quality control processes that are aimed at preserving the accuracy of functional protein production. These processes play an important role in detecting and degrading faulty mRNA transcripts as any errors in the protein could have detrimental effects on the cell and organism. In the following subsections, I will introduce and discuss the main mRNA quality control mechanisms found in eukaryotes, however, putting greater emphasis on the nonsense mediated mRNA decay pathway (NMD).

2.3.1. Cytoplasmic Quality Control.

In the cytoplasm, there is an important set of quality control pathways that specialize in monitoring protein synthesis co-translationally. These pathways prevent the production of truncated polypeptides that may be potentially toxic. In eukaryotes, there are at least three types of mRNA defects that can stall a translating ribosome and activate quality control mechanisms.

2.3.1.1. Non-Stop Decay Pathway.

The first type of defect activates the NSD (Non-Stop Decay) pathway when the translating ribosome reaches the 3'-end of the mRNA and does not encounter a termination codon (Frischmeyer et al, 2002; Lykke-Andersen & Bennett, 2014). The lack of a stop codon could result from premature polyadenylation that occurs within the coding region or when transcription is terminated prematurely (Frischmeyer et al, 2002). NSD has been extensively studied in yeast and has been shown to require the eRF3-homologous factors, Superkiller (Ski) 7 and Hbs1, and

the eRF1-related factor Dom34 (Frischmeyer et al, 2002; Tsuboi et al, 2012; van Hoof et al, 2002). Ski7 plays a central role in triggering mRNA degradation, as it is associated with the cytoplasmic exosome in the SKI complex (Hoshino, 2012). The depletion of Ski7 in yeast cells resulted in stabilization of target mRNAs, showing the requirement of the Ski complex in NSD (van Hoof et al, 2002). Ski7 was hypothesized to target defective mRNA by binding the A-site of stalled ribosomes and recruit the exosome for mRNA decay (van Hoof et al, 2002). In other studies, Hbs1 and Dom34 were shown to function as a complex and together with Rli1, a ribosome recycling ATPase, promote the disassembly of stalled ribosomes at the 3'-end of the aberrant mRNA (Pisareva et al, 2011; Shoemaker et al, 2010; Tsuboi et al, 2012). This might therefore serve as the requirement to allow access of the exosome to the 3'-end of mRNA targets (Tsuboi et al, 2012). However, the connection between Ski7 and Hbs1-Dom34 remains elusive.

2.3.1.2. No-Go Decay Pathway.

The second type of mRNA defect causes a translating ribosome to stall during elongation and activates the NGD (No-Go Decay) pathway. One such defect is the presence of a stable hairpin structure in the mRNA (Doma & Parker, 2006). Additionally, rare codons and polylysine-codon tracts have been shown to activate NGD (Tsuboi et al, 2012). NGD requires the Hbs1-Dom34 complex, as deletion of either component results in stabilization of NGD substrates (Doma & Parker, 2006). Genetic studies propose that the Hbs1-Dom34 complex dissociates the stalled ribosome and stimulates endonucleolytic cleavage close to the ribosome stall site (Doma & Parker, 2006; Tsuboi et al, 2012). This is then followed by subsequent degradation of the 5' fragment mRNA by the exosome (Tsuboi et al, 2012). Despite this, the identity of the endonuclease remains elusive. Additionally, it remains unclear if the endonucleolytic cleavage is a result of direct recruitment or nuclease activation by Hbs1-Dom34 or whether it is a consequence of ribosome disassembly by Hbs1-Dom34. The mechanism underlying the NGD pathway has mainly been examined in yeast. NGD has also been observed in *Drosophila* and is presumed to function in metazoans however little is known about the NGD

pathway in multicellular organisms (Lykke-Andersen & Bennett, 2014; Passos et al, 2009).

2.3.1.3. Nonsense Mediated mRNA Decay (NMD) Pathway.

Finally, the third type of mRNA defect is PTCs (Premature Termination Codons) that will lead to the activation of the NMD (Nonsense Mediated mRNA Decay) pathway. There are multiple ways by which a PTC might arise in an mRNA. The most common source of PTCs is thought to be due to alternative splicing events since genome wide studies have detected alternative splicing events for 60-70% of human pre-mRNAs and 45% of these alternatively spliced mRNAs produce at least one mRNA isoform that might be degraded by NMD (Johnson et al, 2003; Lewis et al, 2003; Schweingruber et al, 2013). Additionally, the programmed DNA rearrangements that T-cell receptors and immunoglobulin genes require to generate diversity in antigen receptors generate a PTC approximately two out of every three occurrences (Isken & Maquat, 2007; Li & Wilkinson, 1998). PTCs can also arise from other sources, such as transcription errors, homologous recombination and mutations in the genome.

2.3.2. The Nonsense Mediated mRNA Decay Pathway Detailed.

The NMD pathway is the most extensively studied quality control mechanism in eukaryotes. NMD plays a significant role in modulating clinical manifestations in genetic diseases (Bhuvanagiri et al, 2010; Nicholson et al, 2010). It is estimated that 30% of known disease-associated mutations result from PTC-containing mRNAs (Muhlemann et al, 2008). However besides PTCs, NMD has also been shown to regulate the abundance of 5-10% of naturally occurring transcripts (Kervestin & Jacobson, 2012). Several features that have been identified to trigger NMD include upstream ORFs (uORFs), long 3'UTRs, introns in the 3'UTR and selenoprotein mRNAs (Chan et al, 2007; Isken & Maquat, 2007; Mendell et al, 2004).

2.3.2.1. PTC detection.

NMD can essentially be divided into two phases, i.e the detection of the PTC followed by the degradation of the aberrant mRNA. PTC detection can be further divided into splicing independent or dependent mechanisms. In invertebrates, NMD occurs independently of splicing. There are several models that have been proposed to understand how a PTC is distinguished from normal stop codons (Behm-Ansmant et al, 2007b; Isken & Maquat, 2007). One model proposes that NMD activation requires the loosely defined downstream elements (DSEs) in addition to a premature nonsense codon (Amrani et al, 2006; Isken & Maquat, 2007). In yeast, it was proposed that DSEs located within 150 nucleotides of the PTC activates NMD and NMD effectors are recruited through a DSE-binding protein, Hrp1, the only one characterised to date (Amrani et al, 2006; Gonzalez et al, 2000; Wang et al, 2006). Alternative models have suggested that generic features of mRNA, such as the poly(A) tail or the length of the 3'UTR can provide positional information to discriminate PTCs from natural stop codons (Behm-Ansmant et al, 2007b; Isken & Maquat, 2007; Muhlrad & Parker, 1999).

Another proposed model is the *faux*-3'UTR model. According to this model, natural 3'UTRs are marked by a specific set of proteins that influence translation termination. Termination at a natural stop codon is efficient due to the interactions formed between the translating ribosome and the proteins that mark the 3'UTR. However in the presence of a PTC, translation termination conducted by the eRF1 and eRF3 complex is inefficient as the stop codon is flanked by a "faux 3'UTR" and the appropriate interactions between the translating ribosome and proteins marking the 3'UTR cannot be established (Amrani et al, 2004; Behm-Ansmant et al, 2007b; Kervestin et al, 2012). One such factor is the cytoplasmic poly(A)-binding protein (PABP). Translation termination occurs efficiently when it is located close to PABP, and antagonizes the formation of the NMD complex. In contrast, translation that terminates in a distal location from PABP is inefficient and hence, results in NMD complex formation and subsequent mRNA degradation (Amrani et al, 2004; Amrani et al, 2006; Behm-Ansmant et al, 2007a).

In vertebrates, PTC recognition relies on splicing. A destabilizing PTC triggers NMD when it is located more than 50-55 nucleotides upstream of an exon-exon boundary (Amrani et al, 2006; Behm-Ansmant et al, 2007b; Lejeune & Maquat, 2005). The exon-exon boundary is marked by a multiprotein complex called the exon junction complex (EJC), which is deposited 20-24 nucleotides upstream of the splice junction during splicing (Behm-Ansmant et al, 2007b; Le Hir et al, 2001). The EJC provides positional information to distinguish a natural termination codon from a PTC. The EJC core components include the MAGOH/Y14 heterodimer, the RNA helicase eIF4A3 and MLN51 (also known as Barentsz, BTZ and CASC3), and functions as a scaffold that is used by additional factors for dynamic interactions (Bono & Gehring, 2011). During translation, in the absence of a PTC, the EJC components are displaced by the ribosome as it traverses the mRNA (Behm-Ansmant & Izaurralde, 2006). However, in the presence of a PTC, the translating ribosome terminates translation and communicates with the EJC to trigger NMD as further detailed below.

2.3.2.2. Activation of NMD in vertebrates.

NMD makes use of highly conserved effectors called Up-frameshift (UPF) 1, UPF2 and UPF3. In multi-cellular organisms, additional effectors required for NMD are the Suppressor of morphological effect on genitalia (SMG) 1, SMG5, SMG6, SMG7, SMG8 and SMG9.

Human UPF3 is a nuclear-cytoplasmic shuttling protein. It is mainly localized in the nucleus and in splicing-dependent NMD, UPF3 is thought to bind the EJC during splicing prior to export into the cytoplasm (Behm-Ansmant & Izaurralde, 2006; Kim et al, 2001; Lykke-Andersen et al, 2000). In human cells, UPF2 is localized in the cytoplasm and is recruited to the mRNA by binding to UPF3 (Figure 3I) (Lykke-Andersen et al, 2000; Melero et al, 2012; Mendell et al, 2000; Serin et al, 2001). However, when the ribosome encounters a PTC, translation terminates at the stop codon by eRF1 and eRF3. This results in the recruitment of UPF1 and the SMG1 complex (SMG1C), thus forming the SURF (SMG1-UPF1-eRF1-eRF3) complex that irreversibly commits the target mRNA for degradation (Figure 3II) (Kashima et al,

2006). As translation is terminated prematurely, UPF2 and UPF3 along downstream mRNA sequences are not removed, allowing UPF1 to bind the UPF2:UPF3 complex (Figure 3III). Within the SURF complex, UPF1 is mainly unphosphorylated and binding to the UPF2:UPF3 complex stimulates UPF1 phosphorylation by the phosphatidylinositol 3 kinase-related protein kinase, SMG1 (Figure 3III) (Kashima et al, 2006).

In metazoa, the phosphorylation/dephosphorylation cycle of UPF1 is a critical event that drives NMD, suggesting that the kinase activity of SMG1 must be suppressed until the SURF complex associates with the UPF2:UPF3:EJC complex. SMG8 and SMG9 are two novel regulators of SMG1 activity found in all multi-cellular organisms except plants (Arias-Palomo et al, 2011; Fernandez et al, 2011; Yamashita et al, 2009). Based on structural and biochemical data, it has been suggested that a preassembled SMG8:SMG9 complex is recruited to SMG1 via SMG9, forming the SMG1C (Fernandez et al, 2011). SMG8 suppresses the kinase activity of SMG1 and recruits the inactive SMG1C to the PTC recognition complex (Yamashita et al, 2009). SMG9 mediates binding between SMG1 and SMG8 and enhances the formation of SMG1C (Arias-Palomo et al, 2011; Fernandez et al, 2011). By inducing large-scale conformational changes, SMG8 and SMG9 regulate and help to tune the kinase activity of SMG1 to the requirements of the NMD machinery (Arias-Palomo et al, 2011).

SMG1 phosphorylates UPF1 at several serine/threonine-glutamine (S/TQ) motifs in the N- and C-terminus *in vitro* (Chakrabarti et al, 2014; Okada-Katsuhata et al, 2012; Yamashita et al, 2001). Of the multiple phosphorylation sites detected, functional significance has only been found for three sites as binding platforms for SMG6 and the SMG5:SMG7 heterodimer (Chakrabarti et al, 2014; Okada-Katsuhata et al, 2012). Due to the different locations, it was additionally shown that SMG6 and SMG5:SMG7 can bind UPF1 simultaneously, i.e SMG6 uses its 14-3-3-like domain to bind phospho-T28 at the N-terminal region of UPF1 (Figure 3IV) while SMG7 contains phosphoserine residues in its N-terminus to bind phospho-S1096 at the C-terminus of UPF1 (Figure 3V) (Okada-Katsuhata et al, 2012). While SMG5

has been suggested to bind the N-terminus of UPF1 (Ohnishi et al, 2003), a recent study proposed that SMG5 binds phospho-S1116 at the C-terminus of UPF1 (Chakrabarti et al, 2014). In addition to biochemical data, this recent study had also observed that the distance between Ser1096 and S1116 fits the distance between the positions of the phosphoserine binding sites in the back-to-back orientation of the SMG5:SMG7 heterodimer, making the C-terminus of UPF1 the region for SMG5 binding (Chakrabarti et al, 2014; Jonas et al, 2013).
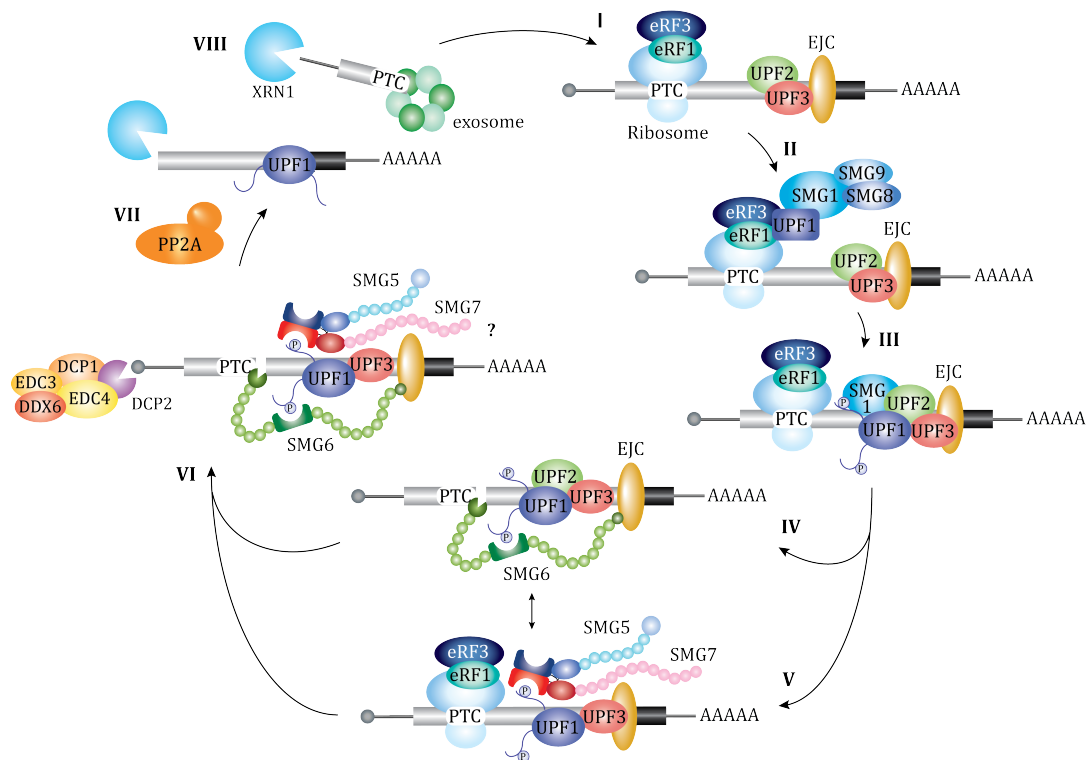
Figure 3: Model of key NMD events in vertebrates.

Each step in the model is indicated by roman numerals and detailed in the text. DCP: mRNA decapping enzyme. DDX: DEAD Box helicase. EDC: Enhancer of mRNA decapping. EJC: Exon junction complex. eRF: eukaryotic release factor. PP2A: Protein phosphatase 2A. PTC: Premature termination codon. UPF: Up-frameshift. SMG: Suppressor of morphological effect on genitalia. XRN1: 5'→3' Exoribonuclease.

In addition to binding phosphorylated UPF1, the recruitment of SMG6 may also be assisted by its EJC-binding motifs (EBM) (Kashima et al, 2010). A close examination of the N-terminus revealed that SMG6 contains two EBMs that have

been shown to be necessary and sufficient for direct binding to the EJC (Figure IV). Even though UPF3 binds the EJC with higher affinity, studies have revealed that SMG6 has the capacity to compete with UPF3 for binding to the same surface on the EJC (Kashima et al, 2010). Complementation assays have shown that the SMG6-EJC interaction is necessary in NMD (Kashima et al, 2010). However, the role this interaction plays in NMD is still unclear.

UPF1 dephosphorylation is facilitated by SMG5, SMG6 and SMG7 (Figure 3VII). Genetic studies in *C. elegans* have shown that inhibiting UPF1 dephosphorylation by depleting SMG5, SMG6 or SMG7, inhibits NMD (Page et al, 1999). This demonstrated that the phosphorylation/dephosphorylation cycle of UPF1 is a key event driving NMD. The protein phosphatase 2A (PP2A) has been shown to be responsible for UPF1 dephosphorylation (Anders et al, 2003; Ohnishi et al, 2003). The recruitment of PP2A is mediated by interactions with SMG5 in both *C. elegans* as well as in human cells (Anders et al, 2003; Ohnishi et al, 2003). While SMG6 seems to influence UPF1 dephosphorylation, it is still unclear whether it directly recruits a phosphatase.

2.3.2.3. Degradation of NMD targets.

A key feature in NMD is the degradation of targeted mRNA to ensure that C-terminally truncated proteins are not generated. Besides dephosphorylating UPF1, the 14-3-3 domain containing proteins SMG5, SMG6 and SMG7 induce mRNA decay. SMG6 possesses nuclease activity as it contains a PIN domain at its C-terminus that cleaves the target mRNA at the vicinity of the PTC (Figure 3VI) (Eberle et al, 2009; Glavan et al, 2006; Huntzinger et al, 2008). The resulting mRNA fragments are then degraded by general cellular exonucleases, XRN1 and the exosome complex (Gatfield & Izaurralde, 2004). SMG5 also contains a PIN domain at its C-terminus, however its catalytic centre lacks the conserved Aspartic acid residues that confer nuclease activity, hence rendering SMG5 inactive (Glavan et al, 2006).

In addition to the endonuclease activity of SMG6, NMD has also been shown to use other decay pathways such as deadenylation and decapping to degrade NMD targets (Chen & Shyu, 2003; Couttet & Grange, 2004; Lejeune et al, 2003; Lykke-Andersen, 2002). Human UPF1 was shown to recruit the decapping enzyme, DCP2 and decapping activators DCP1 and the proline-rich nuclear receptor coactivator 2 (PNRC2) (Cho et al, 2009; Lai et al, 2012; Lejeune et al, 2003; Lykke-Andersen, 2002). Because PNRC2 binds UPF1 and DCP1 directly, PNRC2 was proposed to link NMD to the decapping complex (Cho et al, 2013; Lai et al, 2012; Loh et al, 2013). In addition, PNRC2 was shown to form a complex with SMG5 independently of SMG7, suggesting that there are two SMG5 containing complexes *in vivo*, i.e SMG5:SMG7 and SMG5:PNRC2 (Cho et al, 2013; Schweingruber et al, 2013). These studies also showed that the degradative activity of SMG5 is independent of SMG7 but dependent on UPF1 and PNRC2. This suggested that SMG5 functions independently of SMG7 and that the SMG5:PNRC2 interaction is dominant over the SMG5:SMG7 interaction in triggering NMD-targeted mRNA decay (Cho et al, 2013). SMG7 alone does not have any nuclease activity but has been shown to degrade mRNA efficiently using its Proline-rich C-terminus (PC) region, which is necessary and sufficient for this activity (Unterholzner & Izaurralde, 2004). It was suggested that SMG7-targeted degradation involves the decapping enzyme DCP2 and the exonuclease XRN1 (Unterholzner & Izaurralde, 2004). However, whether SMG7 directly recruits decay factors and whether the PC region is relevant for NMD remained unclear until I started my PhD.

3. OBJECTIVES

3.1. How are premature termination codon-containing mRNAs degraded by the SMG5-SMG7 heterodimer and what role does the Proline-rich C-terminus of SMG7 play in the nonsense mediated mRNA decay pathway?

Despite our extensive understanding of NMD, there were still many unanswered questions in the field, some of which I wanted to address during my PhD study. In 2004, work from our lab showed that SMG7 mediates mRNA decay through its Proline-rich C-terminus (PC) (Unterholzner & Izaurralde, 2004). Although the use of the 5' → 3' mRNA decay pathway was implicated for this function, a direct recruitment of decay factors had not been identified. Furthermore, whether the PC region of SMG7 was required for NMD function was unknown. To complicate matters, previous studies showed that both SMG5 and SMG7 mediate mRNA decay independently (Cho et al, 2013). While SMG5 requires UPF1 and PNRC2 for degradation, direct interactions had not been shown and whether SMG5 functions independently of SMG7 remained unclear. In addition, an unresolved controversial subject was whether SMG5 function was predominant over SMG7 in NMD. Therefore, in this context, the aims of my PhD were the following:

1. Determine the effects of SMG5 and SMG7 in NMD when they are incapable of forming a heterodimer.

2. Determine if SMG5 and SMG7 make use of distinct decay pathways to degrade target mRNA.

3. Identify decay factors that interact with the PC region of SMG7 and determine whether these decay factors are responsible for SMG7-mediated mRNA decay.

4. Determine if the PC region of SMG7 is required for functional NMD.

3.2. How do co-factors of the CCR4-NOT complex assemble on the NOT1 scaffold protein?

The composition of the CCR4-NOT complex has been shown to differ between yeast and metazoans. Proteomics analyses identified components of the human CCR4-NOT complex and defined the core components (Lau et al, 2009). Previous interaction studies have shown that the primary function of CNOT1 was to act as a scaffold protein for the docking of other subunits (Albert et al, 2000; Bai et al, 1999; Ito et al, 2011; Lau et al, 2009). However, whether this is conserved across species was unclear. Additionally, detailed molecular mapping between the subunits of the CCR4-NOT complex was lacking in the field. During my PhD, preliminary data showed that NOT10 interacts with NOT11 and this module binds to NOT1. However, whether any of these interactions were direct was yet unknown and the function of these novel subunits in deadenylation and mRNA decay was still unclear. Hence, in this section of my PhD, the aim was to locate direct interactions and determine how the NOT10-NOT11 module docks onto NOT1.

4. RESULTS & DISCUSSION

4.1 The nonsense mediated mRNA decay pathway requires the SMG5-SMG7 heterodimer and SMG6 for efficient target degradation.

This section briefly summarizes my findings in relation to what is currently known in the field, with the aim of understanding how NMD efficiently degrades targeted mRNA. My PhD focused on the metazoan effectors SMG5, SMG6 and SMG7 and how they function together to ensure robust degradation of aberrant mRNA. In addition, I dissected SMG7 to understand how it promotes mRNA decay and a direct connection was found linking NMD to deadenylation and the 5'→3' mRNA decay mechanism. SMG7 was then shown to function redundantly with SMG6 and is thus essential for functional NMD in the absence of SMG6.

The work summarized in this section was published by Loh et. al. (2013) and is attached for detailed experimental data and procedures.

4.1.1. SMG5-SMG7 heterodimerization is required for functional NMD.

The metazoan effectors SMG5, SMG6 and SMG7 are related proteins that share similar domain organization (Figure 4). It has long been established that SMG5 and SMG7 form a heterodimer and that the 14-3-3-like domains at the N-terminus of both proteins mediate this dimer formation (Anders et al, 2003; Fukuhara et al, 2005; Jonas et al, 2013; Ohnishi et al, 2003).

Previous work from our lab showed that a SMG5 mutant that does not dimerize with SMG7 is unable to rescue NMD in SMG5-depleted HeLa cells (Jonas et al, 2013). Similarly, a SMG7 mutant that fails to dimerize with SMG5 is incapable of rescuing NMD in SMG7-depleted cells (Jonas et al, 2013). However, studies from others have proposed that SMG5 is able to function independently of SMG7 by forming a complex with PNRC2 (Cho et al, 2013). Hence, to further investigate the

need for SMG5:SMG7 heterodimerization in NMD, I tested additional mutants of monomeric SMG5 or SMG7 to rescue NMD in complementation assays.
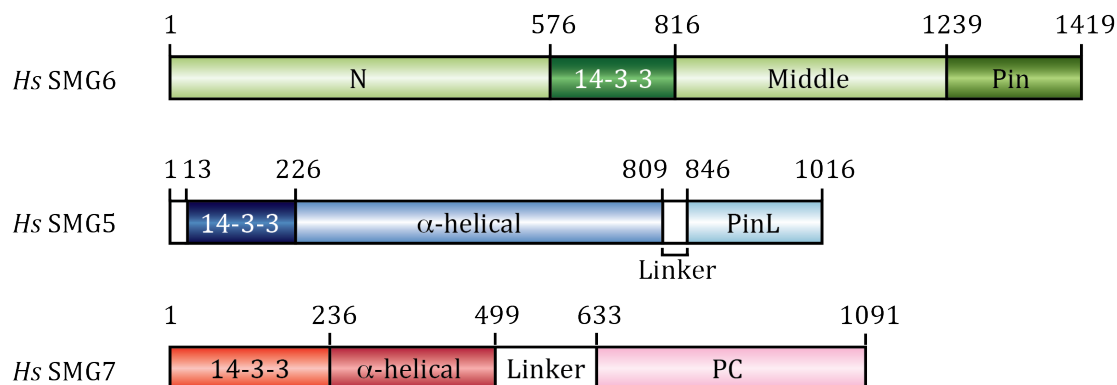
Figure 4: Schematic representation of *Hs* SMG5, 6 and 7 illustrating their domain organization.

The three proteins contain a 14-3-3-like domain (14-3-3); for SMG5 and SMG7 this is located at the N-terminus while in SMG6, the 14-3-3-like domain is located in the middle region. In SMG5 and SMG7, an α–helical domain (α-helical) and a short linker (Linker) follow the 14-3-3-like domain. At the C-terminus of SMG5 and SMG6, lies the Pin domain as described before in the introduction. The Pin domain in SMG6 confers nuclease activity to the protein while SMG5 has a Pin-Like domain (PinL), which lacks the necessary residues for catalytic activity. SMG7 does not contain a Pin domain but contains a Proline-rich C-terminus (PC) that is necessary for mRNA degradation. The numbers above the schematic diagrams indicate the amino acid residue numbers.

Mutations that would definitively disrupt SMG5:SMG7 binding were designed based on the knowledge we had gained from the SMG5:SMG7 crystal structure obtained in our lab (Jonas et al, 2013). These mutants were used to examine NMD function in human cells. Briefly, an shRNA that targets the SMG5 or SMG7 ORF was used to deplete endogenous SMG5 or SMG7 from HeLa cells that stably expressed a well-characterized NMD reporter, which is based on the β-globin gene (Thermann et al, 1998). Previous studies have shown that the endonucleotlytic activity of SMG6 is partially redundant to the mode of decay triggered by the SMG5:SMG7 complex (Jonas et al, 2013; Luke et al, 2007; Metze et al, 2013). Therefore the experiments I conducted in HeLa cells were co-depleted of SMG6 and SMG5 or

SMG7 to provide a broader dynamic range to the complementation assay. shRNA-resistant forms of SMG5 and its mutants were then tested for their ability to complement the SMG5 and SMG6 co-depletion. Hypothetically, if SMG5 could function independently of SMG7, the SMG5 mutants that fail to bind SMG7 would still be able to rescue NMD. However, the results showed that SMG5 alone could not rescue NMD while the wild-type heterodimer-forming SMG5 retained its ability to rescue NMD.

In the reciprocal experiment, I co-depleted SMG6 and SMG7 in HeLa cells and tested shRNA-resistant forms of SMG7 mutants that fail to interact with SMG5 for their ability to rescue NMD. Here I showed that SMG7 alone lost its ability to rescue NMD while dimer-forming SMG7 could rescue NMD. This provides one line of evidence to show that SMG5:SMG7 heterodimerization is necessary for NMD function in human cells, which agrees with previous observations made in our lab (Jonas et al, 2013). Additional evidence from various other sources further indicates that SMG5 and SMG7 function as a complex in NMD (Anders et al, 2003; Ohnishi et al, 2003). One such indication comes from experiments showing that SMG5 and SMG7 form a highly stable heterodimer both *in vivo* and *in vitro* (Anders et al, 2003; Jonas et al, 2013). In fact, efficient binding to phosphorylated UPF1 requires SMG5:SMG7 heterodimerization as well (Chakrabarti et al, 2014). Furthermore, co-depleting SMG5 and SMG7 does not intensify the effects of individual depletions, indicating that SMG5 and SMG7 probably act together as a complex on the same target mRNA (Jonas et al, 2013; Metze et al, 2013). In contrast, co-depleting SMG6 and SMG5 or SMG7 results in a synergistic inhibition of NMD, suggesting that SMG6 has partially redundant functions with the SMG5:SMG7 complex in NMD (Jonas et al, 2013; Luke et al, 2007; Metze et al, 2013). Therefore, these observations support the notion that SMG5:SMG7 heterodimer formation is necessary for functional NMD.

4.1.2. SMG5 and SMG7 use distinct mechanisms to degrade target mRNA.

SMG5 has an inactive Pin domain and lacks any enzymatic activity (Glavan et al, 2006). Nevertheless, tethering SMG5 to a reporter mRNA results in degradation of the reporter mRNA, indicating that SMG5 can induce decay (Lykke-Andersen et al, 2000). However, the decay mechanism used by SMG5 was still unclear. A previous study showed that SMG5 is active in tethering assays by triggering decapping of the mRNA and that its activity was dependent on UPF1 and the decapping factor PNRC2 but independent of SMG7 (Cho et al, 2013). However, work from our lab showed that SMG5 only promotes decay when co-expressed with SMG7 (Jonas et al, 2013; Unterholzner & Izaurralde, 2004). Hence to address this apparent contradiction, I made use of the MS2- based tethering system to test whether a SMG5 mutant that fails to bind SMG7 but should have retained its ability to bind PNRC2 and UPF1, can still degrade targeted mRNA.

Briefly, to conduct this experiment, SMG5 and its mutants were cloned to have an N-terminal MS2 tag followed by a Hemagglutinin (HA) tag for detection. I then transiently transfected a mixture of three plasmids into human HEK293T cells: one encoded for the MS2-HA fusion protein, another was a reporter plasmid that encoded for the β-globin gene with six copies of the high affinity binding site for the MS2 viral coat protein in its 3'UTR, and a transfection control plasmid that encodes for the β-globin gene with the GAPDH ORF and 3'UTR inserted at its 3'end (Figure 5). RNA was extracted from the cells two days post-transfection and analyzed by Northern blotting.

Unlike the complementation assay that relies on the presence of a PTC in the β-globin gene to activate NMD and trigger degradation of the reporter mRNA, the tethering assay relies on the MS2 tag to artificially recruit SMG5 or its mutants to the 3' UTR of the β-globin reporter mRNA that contains six copies of the MS2 binding site to trigger decay. As expected, wild type MS2-SMG5 induced the decay of the β-globin reporter that contains six MS2-binding sites at its 3'-UTR while the transfection control was unaffected, showing that there were no non-specific effects induced by SMG5. However, when a SMG5 mutant that fails to bind SMG7

was tested, its decay activity was not completely abolished. This shows that SMG5 activity is only partially dependent on SMG7 and indicates that SMG5 utilizes an alternative mechanism for mRNA decay. The possibility that decapping is the alternative decay mechanism and relies on UPF1 and PNRC2 to trigger decay cannot be excluded. However, the interaction between PNRC2 and SMG5 could not be recapitulated under the conditions used in our lab, questioning whether this interaction is transient or indirect, perhaps through UPF1.
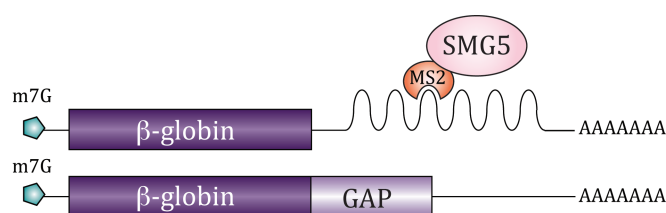

Figure 5: Schematic representation of the reporter mRNAs used in the tethering assay.
Both reporters encode the β-globin gene. The top schematic shows six MS2 binding sites in the 3'UTR that form secondary hairpin structures. The MS2 fusion protein (MS2-SMG5) is artificially recruited to the reporter mRNA due to the high affinity binding between the MS2 protein and the six binding sites. The bottom schematic shows the control mRNA that lacks the six MS2 binding sites but instead, contains a fragment of the GAPDH ORF and 3'UTR after the β-globin gene.

SMG7 does not posses any nuclease activity but previous work showed that SMG7 mediates mRNA decay (Unterholzner & Izaurralde, 2004). Therefore, to investigate the region of SMG7 that is responsible for this decay, I made use of the MS2-based tethering system. I tested the activity of the N-terminus of SMG7 that includes the 14-3-3-like domain and α-helical domain, and the activity of the Proline-rich C-terminus (PC region) (Figure 7A). Consistent with past studies, the results I had obtained showed that the PC region of SMG7 was required and sufficient for mRNA decay (Unterholzner & Izaurralde, 2004). Since SMG7 uses its N-terminus to bind SMG5 and UPF1, the tethering results also indicated that SMG7 induces decay independently of SMG5 and UPF1 and thus, through a different mechanism.

Past studies suggested that this alternative mechanism induced by SMG7 is dependent on the decapping enzyme, DCP2 and the 5'→3' exonuclease, XRN1 (Unterholzner & Izaurralde, 2004). However, whether this was a direct recruitment of decapping factors or a consequence of deadenylation was yet unknown. Hence, I made use of a dominant negative approach to provide more insight into SMG7 function. The idea behind this assay was to over-express a catalytically inactive mutant of DCP2 to displace the endogenous DCP2 from decapping complexes and render decapping dysfunctional in human cells (Figure 6). If the 5'→3' mRNA decay pathway is employed and deadenylation occurs while decapping is dysfunctional, an accumulation of deadenylated mRNA will be detected by Northern blotting as a faster migrating species.

Using this approach together with the MS2-tethering assay, I showed that SMG7 relies on decapping activity for decay. In the presence of SMG7, the β-globin-six MS2 binding sites reporter mRNA that accumulated in cells in which decapping was blocked, migrated faster than the control. This indicates that the mRNA is shorter and possibly deadenylated. To confirm this, I treated the accumulated mRNA with the RNaseH enzyme that catalyzes RNA cleavage in a DNA/RNA duplex, in the presence of oligo dT. In the absence of SMG7, the poly(A) tail of the reporter mRNA was base-paired with an oligo dT and subjected to RNaseH cleavage, forming the faster-migrating reporter mRNA that is deadenylated. However, in the presence of SMG7 or the PC region of SMG7, the fast migrating form of mRNA did not change mobility, suggesting that it was indeed deadenylated and indicated that the PC region of SMG7 induced decay by triggering deadenylation prior to decapping. Conversely, when I tested SMG5 using the dominant negative approach, the reporter mRNA accumulated in a polydenylated form. This implied that SMG5 could mediate mRNA decay through decapping in the absence of deadenylation.
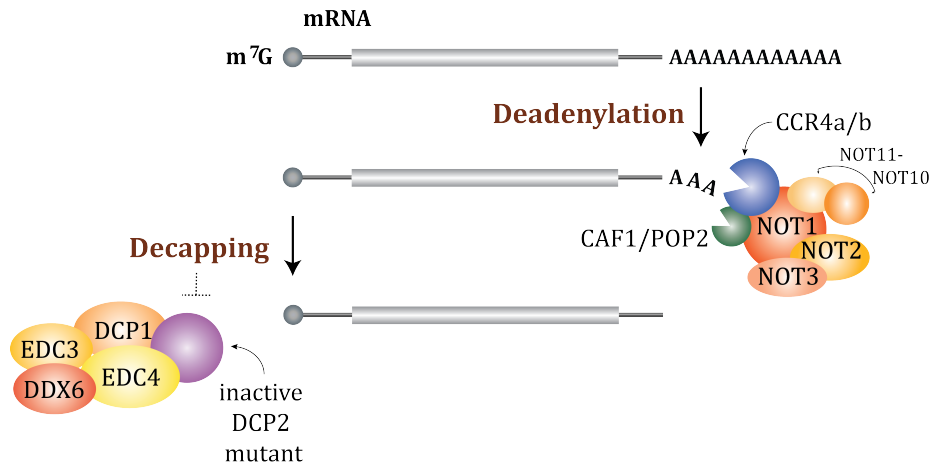
Figure 6: Illustration of the principle behind the dominant negative experiments in human cells.
The CCR4-NOT complex is the major machinery that deadenylates mRNA. The components of the CCR4-NOT complex shown in this figure are the two exonucleases CCR4a/b and CAF1/POP2 and the other core subunits NOT1, NOT2, NOT3, NOT10 and NOT11. The decapping step is blocked by over-expressing a catalytically inactive DCP2 mutant that then replaces the endogenous DCP2 enzyme in decapping complexes. The components of the decapping complex shown in this figure are DCP1, EDC3, EDC4 and DDX6.

In conclusion, SMG5 and SMG7 mediate mRNA decay through distinct mechanisms where SMG7 triggers deadenylation-dependent decapping and SMG5 can trigger decapping independently of deadenylation. My data cannot exclude the possibility that SMG5 functions independently of SMG7 *in vivo* as this could be required under specific conditions or could be target specific. However, SMG5 and PNRC2 do not form a stable complex like SMG5 and SMG7 do. The previous study that had proposed the SMG5:PNRC2 model observed that PNRC2 depletion suppressed SMG5 activity in tethering assays. This observation could be explained by the inhibition of decapping upon PNRC2 depletion as PNRC2 has previously been shown to stimulate decapping activity (Lai et al, 2012). Therefore, this would agree with the observations made from experiments I had conducted using a catalytically inactive form of DCP2 to inhibit decapping, partially abolishing SMG5 activity.

4.1.3. SMG7 recruits the CCR4-NOT complex via direct interactions with POP2.

Having determined that SMG7 mediates deadenylation, I next wanted to identify potential interaction partners of SMG7. To this end, I made use of a global proteomics approach called the Tandem Affinity Purification (TAP) method to identify any interactions between SMG7 and mRNA decay components. I expressed the PC region of SMG7 (Figure 7A) as a fusion protein with a Protein A tag, a Precission Protease cleavage site followed by a Streptavidin binding peptide (SBP) tag in HEK293T cells. The cells were then harvested and the cell lysates were subjected to two rounds of affinity purification using the Protein A tag and the SBP tag consecutively. The proteins still bound to the PC region of SMG7 were then identified by mass spectrometry. Remarkably, all ten subunits of the CCR4-NOT complex were identified. Furthermore, these were the only decay components identified as PARN or the PAN2-PAN3 complex was absent, thus showing specificity of the interactions.
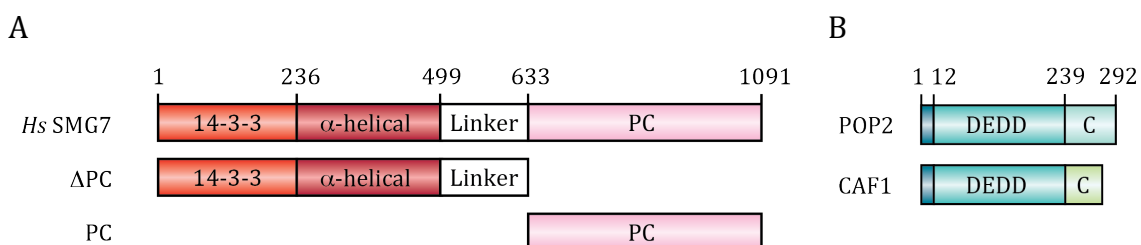
Figure 7: Schematic representation of protein domain organisation.
A) SMG7 and the truncated mutants tested during this study. ΔPC: The N-terminus of SMG7 that consists of the 14-3-3-like domain, α-helical domain and linker region. PC: Proline rich C-terminus.
B) POP2 and its paralog CAF1. DEDD: the single domain that harbours the DEDD nuclease motif responsible for catalytic activity. C: C-terminus that confers most diversity between the paralogs. Numbers above indicate the amino acid numbers.

In order to validate the interactions found, I co-expressed SBP-tagged full length SMG7 together with each individual component of the CCR4-NOT complex and performed SBP-pulldown assays. Strikingly, under the conditions used, I could only

confirm an interaction between SMG7 and CNOT8, also called POP2, one of the catalytic subunits of the CCR4-NOT complex. This result indicated that the SBP pulldown assays detect binary interactions. Furthermore, the other subunits were not identified possibly due to the lack of endogenous POP2 to bridge the interaction between over-expressed SMG7 and the other subunits. I further validated this interaction by conducting SMG7 pulldown assays to identify endogenously expressed POP2 using POP2-specific antibodies. Additionally, these experiments indicate that this interaction is a protein:protein interaction and is not mediated by RNA as lysates were treated with RNaseA prior to the SBP pull-down.

4.1.4. SMG7 discriminates POP2 from CAF1 through their catalytic domains

In human cells, two paralog proteins exist POP2 and CAF1; they are single domain proteins that adopt an RNase-D like fold and share an overall sequence identity of 74% (Figure 7B) (Daugeron et al, 2001; Petit et al, 2012). Hence it was surprising that SMG7 interacted with POP2 but not CAF1 in the SBP pull-down assays. To determine the basis for this discrimination, GST-pulldown experiments were conducted using *E. coli* expressed recombinant GST-tagged POP2 or CAF1. The PC region of SMG7 was expressed *in vitro* in wheat germ extract and radioactively labelled by incorporating ^{35}S Met to the reaction. As observed before, the GST-pulldown experiments showed that the PC region of SMG7 binds to POP2 with much greater affinity than CAF1, demonstrating a preference in binding partner. Previous studies have shown that POP2 and CAF1 are mutually exclusive components of the CCR4-NOT complex (Lau et al, 2009). This revealed the co-existence of different variants of the CCR4-NOT complex depending on the deadenylase subunit that is incorporated into the complex. While the parameters that govern the recruitment of each variant of the CCR4-NOT complex are unknown, recent studies in neural development have shown distinct expression patterns of the complex subunits (Chen et al, 2011). For example, mouse CAF1 is uniformly expressed during brain development while the expression levels of POP2 were shown to be highest during embryonic stages and decreases thereafter

(Chen et al, 2011). Such differences in protein expression and the possibility that subunits may be expressed in a tissue- or cell-type specific manner add a layer of complexity to NMD function, suggesting that perhaps mRNA decay induced by SMG7 may have some specificity for transcripts expressed during various cellular physiological states. Nevertheless, the finding that SMG7 preferentially recruits POP2 over CAF1 provides an unprecedented example of how substrate specificity can be achieved for alternative CCR4-NOT complexes.

To understand the selectivity of SMG7, I examined the paralogs, POP2 and CAF1, in greater detail. The main disparity between POP2 and CAF1 lies in the short C-terminus of the proteins (Figure 7B). Hence, I hypothesized that the C-terminal extensions were the binding regions for SMG7. To address this hypothesis, I made use of *E. coli* expressed recombinant GST-tagged deletion constructs to conduct GST-pulldown experiments. The results showed that the PC region of SMG7 interacted with the catalytic domain of POP2 with the same intensity regardless of whether the flanking sequences were present or not. The catalytic domain of POP2 is known to interact with the anti-proliferative protein TOB and other components of the CCR4-NOT complex namely, CNOT1 and CCR4 simultaneously without overlapping binding surfaces (Horiuchi et al, 2009; Lau et al, 2009; Petit et al, 2012; Temme et al, 2010). In fact, structural studies have revealed the critical residues that mediate the various interactions in the catalytic domain. Since SMG7 binds the catalytic domain as well, I determined whether SMG7 binding displaces any of the other interaction partners of POP2. Using immunoprecipitation experiments, I showed that POP2 point mutants which failed to bind CNOT1, CCR4 and TOB had no effect on SMG7 binding. This indicates that the binding surface of SMG7 on POP2 does not overlap with any previously characterized surfaces required for CNOT1 or CCR4a/b binding. This finding hence agrees with the TAP tag results that had identified all components of the CCR4-NOT complex and supports the idea that SMG7 does not bind to free POP2 in the cell but recruits the whole deadenylation complex.

In addition, SMG7 was found not to compete with the antiproliferative protein TOB for binding surface, indicating that POP2 can bind both TOB and SMG7 simultaneously. Previous studies have demonstrated that complex formation between TOB and POP2/CAF1, is crucial for exhibiting a strong antiproliferative activity (Doidge et al, 2012a; Horiuchi et al, 2009). Hence, the interaction between SMG7 and POP2 may not only bridge NMD to deadenylation and mRNA decay, but may also suggest a link to cell growth repression through TOB. In a wider context, NMD is believed to provide a protective role against cancer by triggering the degradation of mRNAs encoding toxic truncated proteins (Karam et al, 2013). The finding that SMG7 could bind POP2 in the presence of TOB could therefore provide an additional preventive measure against tumour growth.

4.1.5. Functional POP2 is required for SMG7 function and NMD.

Having identified the interacting partner of SMG7, I wanted to investigate the significance of POP2 function in SMG7-mediated decay. First, I conducted SMG7 tethering in POP2 depleted cells. If POP2 was the factor responsible for SMG7-mediated decay, its absence would result in the reporter mRNA being stabilized in the polyadenylated form. However, despite trying different siRNA target sequences in isolation or in combination, my attempts to efficiently deplete endogenous POP2 in human cells proved unsuccessful for the proposed experiment. The most efficient depletion I had accomplished was a 50% reduction. As POP2 is an active deadenylase and has been shown to be the primary catalytic component of the CCR4-NOT complex, a more efficient depletion (<10%) would have been needed to observe any effect in the tethering assay (Bawankar et al, 2013). Hence, the dominant negative approach was used as described before (Figure 6) with the exception of over-expressing a catalytically inactive form of POP2.

Eukaryotic CAF1 and POP2 are relatively well studied and the residues responsible for its catalytic activity have been defined (Bianchin et al, 2005; Daugeron et al, 2001). Hence, designing and constructing a catalytically inactive POP2 mutant was straightforward. SMG7 or PC tethering while over-expressing the POP2 mutant

resulted in stabilization of the reporter mRNA, showing that the POP2 mutant inhibited degradation of the target mRNA by SMG7 and the PC region. However, unlike the results from the DCP2 mutant, the reporter mRNA accumulated in the polyadenylated form. This demonstrates that POP2 not only serves as a bridge to link SMG7 to the 5'→3' mRNA decay pathway but its nuclease activity is also required for SMG7-mediated decay.

Next, I wanted to examine the significance of POP2 and the 5'→3' mRNA decay pathway to target degradation in NMD. Hence, catalytically inactive mutants of DCP2 and POP2 were expressed in HeLa cells that stably express the β-globin NMD reporters. The results from examining the β-globin mRNA levels showed stabilization of the NMD reporters in both cases, however the mobility of the bands detected indicated that deadenylation occurred prior to decapping. In the presence of the DCP2 mutant, the band detected was broad, indicating that a population of the accumulated mRNA was deadenylated. In contrast, addition of the POP2 mutant resulted in a sharp band, suggesting that the accumulated mRNA was polyadenylated. This indicated the important role that deadenylation plays in NMD, which agrees with a previous study where blocking deadenylation by expressing the RNA-binding proteins UNR or NSAP1 resulted in stabilization of PTC-containing reporter (Chen & Shyu, 2003). In addition, the amount of stabilized mRNA observed in these dominant negative experiments (two-fold increase) were comparable to those obtained when SMG5 or SMG7 (two-fold increase) were depleted (Jonas et al, 2013; Loh et al, 2013). Hence, this demonstrated the significant contribution of deadenylation-dependent decapping to target mRNA decay in NMD.

4.1.6. The PC region of SMG7 is required for NMD in the absence of SMG6.

One of the open questions in the field that has never been addressed prior to my PhD was whether the PC region of SMG7 is important for functional NMD. Data from our lab and others have shown that the N-terminus of SMG7 is responsible for binding to UPF1 and recruiting SMG7 to the surveillance complex (Fukuhara et

al, 2005; Ohnishi et al, 2003). Also, through its N-terminus, SMG7 forms a heterodimer with SMG5 that is required for the dephosphorylation of UPF1, a key event that drives NMD (Anders et al, 2003; Ohnishi et al, 2003; Page et al, 1999). Hence, to determine if NMD is functional without the active PC region of SMG7, a SMG7 mutant lacking the PC region was examined for its ability to rescue NMD in complementation experiments. Briefly, I made use of an shRNA that targets the SMG7 ORF to deplete endogenous SMG7 in HeLa cells, which constitutively express the β-globin NMD reporter. At the same time, I over-expressed shRNA-resistant versions of SMG7 to complement the SMG7 depletion. Unexpectedly, the mutant was sufficient to restore NMD, concluding that the PC region of SMG7 was not required to degrade the PTC containing β-globin reporter mRNA. However, as mentioned before, SMG6 is able to partially compensate for SMG7 deficiency (Jonas et al, 2013; Metze et al, 2013). Hence, by repeating the experiments in cells co-depleted of SMG6 and SMG7, I showed that the N-terminus alone failed to rescue NMD and therefore infers that functional NMD requires the PC region of SMG7 in the absence of SMG6.

4.1.7. The role of SMG7 in insects, plants and yeast.

The SMG proteins were first identified in *C. elegans* as suppressors of nonsense mutations in a wide variety of genes, controlling overall body shape and sex determination (Hodgkin et al, 1989). Even though NMD is not essential in nematodes, loss-of-function alleles of *smg* genes were shown to eliminate NMD, thus preventing the degradation of mRNA containing nonsense mutations (Cali et al, 1999; Pulak & Anderson, 1993). Since their first identification, SMG5, SMG6 and SMG7 have been found to be highly conserved in most multi-cellular organisms (Behm-Ansmant et al, 2007b). During my PhD, I showed that NMD in human cells relies on deadenylation-dependent decapping mediated by SMG7, a process that is essential for NMD in the absence of SMG6. In contrast to the reports from Cho et. al. (2013) who showed that SMG5 functions independently of SMG7, my studies demonstrated the necessity for dimer formation between the effectors SMG5 and

SMG7 for a functional NMD mechanism. This dimerization has also been previously observed by Jonas et al. (2013).

While SMG7 plays an important role in mammalian cells, a SMG7 homolog has not been identified in *Drosophila*. This raises questions as to how NMD targets are degraded in this organism. Depletion experiments have determined that *Dm* SMG5 and *Dm* SMG6 are essential for NMD (Gatfield et al, 2003), and rely on the endonucleolytic activity of SMG6 for NMD-targeted mRNA decay (Gatfield & Izaurralde, 2004; Huntzinger et al, 2008). Like *Hs* SMG5, the Pin domain in the C-terminus of *Dm* SMG5 adopts a Pin-like fold (Glavan et al, 2006). However its active site is impaired, as the residues required for activity are not conserved. Therefore the question arises: what is the role of SMG5 in *Drosophila* cells? Previous work suggests that NMD substrates in *Drosophila* are unlikely to be degraded by decapping or deadenylation (Gatfield & Izaurralde, 2004). Hence the possibility that *Dm* SMG5 fulfils the role of *Hs* SMG7 by initiating deadenylation-dependent decapping is unlikely. Previous work have also demonstrated that *Hs* SMG5:SMG7 heterodimerization is required for efficient binding to phosphorylated *Hs* UPF1, which initiates the irreversible degradation process (Chakrabarti et al, 2014; Jonas et al, 2013; Ohnishi et al, 2003). If SMG7 does not exist in *Drosophila*, how is *Dm* SMG5 recruited to the NMD complex? In *C. elegans* and metazoa, SMG5 has been shown to recruit the protein phosphatase 2A required for UPF1 dephosphorylation (Anders et al, 2003; Ohnishi et al, 2003). However, evidence to support this role in *Drosophila* cells is lacking. In light of these open questions, biochemical characterization of *Dm* SMG5 is required to fully understand how NMD functions in the absence of SMG7 in *Drosophila*.

While *Drosophila* only has SMG5 and SMG6, in plants SMG7 is the only SMG protein found. In rice (Oryza sativa) a single gene encodes SMG7 while in other plants, SMG7 is duplicated several times in the genome (Benkovics et al, 2011). *Arabidopsis thaliana* contains two SMG7 homologs (known as SMG7 and SMG7-like, SMG7L) while the grapevine *Vitis vinifera* has been found to contain three homologs, two copies of SMG7 and one copy of SMG7L (Benkovics et al, 2011;

Riehs et al, 2008). It has been shown that of the two SMG7 genes found in plants, only SMG7 but not SMG7L, has conserved its function in NMD (Benkovics et al, 2011; Riehs et al, 2008). This was determined by observing the accumulation of PTC-containing transcripts in plants deficient of SMG7. Additionally, tethering *V. vinifera* SMG7 to a reporter mRNA resulted in rapid decay of the target, demonstrating conservation of SMG7 function in NMD-target degradation (Benkovics et al, 2011). Nevertheless, the mechanism that SMG7 uses to degrade NMD target transcripts in plants has not been well studied, leaving open the question of whether SMG7 employs deadenylation-dependent decapping for mRNA decay in plants. In addition, it is still unclear what the function of SMG7L is and whether it has gained a new function through evolution. In *Arabidopsis*, SMG7 has been described to play an additional role in regulating the meiotic cell cycle (Riehs et al, 2008). *Arabidopsis* plants depleted of SMG7 are fully sterile, as the anaphase-telophase transition in the second meiotic division cannot be completed (Riehs et al, 2008).

SMG7 homologs were identified in plants by sequence homology to the budding yeast Est1p (Ever shorter telomeres 1), which is a subunit of the telomerase holoenzyme (Riehs et al, 2008). In yeast, there are three orthologs of Est1p named Est1A, Est1B and Est1C, which have been found to be homologous to metazoan SMG6, SMG5 and SMG7, respectively. However, whether the Est1p proteins function in NMD is still unclear. Yet it is notable that *Hs* SMG6 may have conserved an additional function distinct from NMD in telomere regulation (Reichenbach et al, 2003). In yeast, two paralog proteins exist, Est1p and Ebs1p and depletion of either protein leads to telomere shortening (Luke et al, 2007). Sequence analysis suggests that Ebs1p has a similar domain organisation to *Hs* SMG7 and depletion experiments have shown Ebs1p to be a member of the NMD machinery in yeast (Luke et al, 2007). However, this raises more questions than it answers. How does Ebs1p contribute to NMD and is it recruited to the NMD complex? In yeast, UPF1 has been shown to recruit the decapping enzyme DCP2 and decapping co-factors EDC3 and Pat1 to achieve complete degradation of NMD-target mRNAs (He & Jacobson, 1995; Swisher & Parker, 2011). Also, while in human cells

phosphorylated UPF1 is required to recruit the SMG proteins, there is a lack of evidence to show that there is a similar requirement in yeast. In fact, it is still unclear whether yeast UPF1 undergoes phosphorylation as no apparent functional homolog of the SMG1 kinase has been identified in this organism. Indeed, the precise mechanism of Ebs1p function in NMD is unclear and would hence require further investigation.

4.1.8. Degradation mechanisms employed by the NMD pathway.

NMD in vertebrates employs multiple decay mechanisms to ensure targeted mRNAs are efficiently degraded. These mechanisms include the endonucleolytic activity of SMG6 that cleaves the aberrant mRNA in the vicinity of the PTC (Eberle et al, 2009; Gatfield & Izaurralde, 2004; Huntzinger et al, 2008), and the 5'→3' mRNA decay that encompasses deadenylation and decapping (Figure 8) (Chen & Shyu, 2003; Lejeune et al, 2003). Studies to show the engagement of deadenylation and decapping in NMD date back over ten years and yet, evidence for direct recruitment of the nucleases responsible for these effects have been lacking (Chen & Shyu, 2003; Lejeune et al, 2003; Lykke-Andersen, 2002). While SMG5 and SMG7 do not possess any nuclease activity, experimental evidence have suggested that the SMG5:SMG7 heterodimer is mainly responsible for recruiting the general mRNA decay enzymes (Unterholzner & Izaurralde, 2004). Recent studies have presented PNRC2 as the missing link between NMD effector SMG5 and decapping co-factor DCP1a (Cho et al, 2013; Cho et al, 2009). In contrast to this, the results obtained during my doctoral studies suggest that SMG5 and PNRC2 do not form a stable complex. Yet, the possibility that SMG5 may function independently of SMG7 under specific conditions or for specific targets cannot be excluded. Furthermore, SMG5 function relies on decapping as a catalytically inactive DCP2 mutant prevents SMG5-mediated decay. So does SMG5 directly recruit another decapping factor or indirectly through an unidentified protein? While decapping is normally coupled to and may occur as a consequence of deadenylation, UPF1 has previously been shown to recruit the decapping factors DCP1, DCP2 and PNRC2 to NMD targets independently of deadenylation (Cho et al, 2009; Lai et al, 2012; Lejeune et

al, 2003; Lykke-Andersen, 2002). Hence, it is possible that SMG5 mediates decay indirectly through UPF1 however further studies would be required to fully elucidate the mechanism behind the decay activity of SMG5.
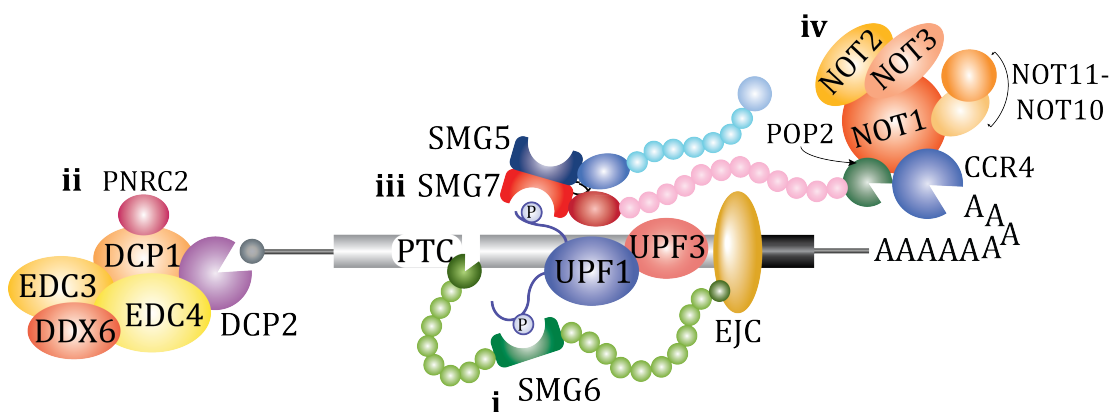
Figure 8: Model of NMD-targeted mRNA degradation.
NMD makes use of various redundant decay pathways to ensure robust mRNA target degradation. Phosphorylation of UPF1 triggers mRNA decay that is mediated by SMG6 and SMG5:SMG7. **i**. SMG6 uses its endonuclease activity to cleave the aberrant mRNA at the vicinity of the PTC while SMG5:SMG7 recruits general mRNA decay enzymes. **ii**. The decapping complex is recruited by SMG5 through PNRC2 (the interaction is not shown), which binds to the decapping co-activator DCP1. **iii**. SMG7 on the other hand, initiates deadenylation of target mRNA by recruiting the CCR4-NOT complex via the deadenylase, POP2. **iv**. The CCR4-NOT complex is composed of several modules that dock with the scaffold protein NOT1 that are required for deadenylation. The three main modules identified are the NOT module (includes NOT2 and NOT3), the catalytic module (comprised of CCR4a/b and POP2/CAF1) and the NOT10/NOT11 module, that I also studied during my PhD.

In trying to understand how SMG7 mediates deadenylation, I identified a direct link between the PC region of SMG7 and the deadenylase, POP2 (Figure 8). This interaction mediates the recruitment of the major cytoplasmic deadenylase complex in eukaryotes, the CCR4-NOT complex to the NMD target (Figure 8). In addition, I showed that SMG7 could distinguish between the two POP2 paralogs (i.e. POP2 and CAF1) with a preference for binding to POP2 rather than CAF1, hence providing a novel example that demonstrates selectivity and specificity for

variants of the CCR4-NOT complex. However, many questions still go unanswered regarding the SMG7-POP2 interaction. One such question is to identify specific residues that mediate this interaction. In my preliminary experiments, simple N- or C- terminal deletions of the SMG7-PC region could not delineate this interaction to a more specific region of the protein. It appears that the responsible individual residues could be distributed throughout this region. Given the possibility to obtain sufficient amounts of recombinant SMG7, nuclear magnetic resonance spectrometry (NMR) could be used to explore this interaction further. It observes the behaviour of the atomic nuclei of the protein in an applied magnetic field, allowing the study of protein structure and dynamics in solution. NMR would also be able to provide details such as affinity measurements, stoichiometry and kinetics of the interaction (Zuiderweg, 2002). Another attractive method to identify the interaction sites would be to chemically cross-link SMG7 to POP2 and then analyze the complex by mass spectrometry. Chemical cross-linking stabilizes weak transient interactions to allow many types of measurements and analysis to be performed including NMR spectrometry, X-ray crystallography and mass spectrometry (Chavez et al, 2011). Indeed, other studies have successfully used this method to examine protein-protein interactions *in vivo* and *in vitro* (Chavez et al, 2011; Dihazi & Sinz, 2003; Seebacher et al, 2006; Zhang et al, 2009; Zhang et al, 2008). These experiments could provide an initial insight into the residues that participate in the interaction for subsequent *in vivo* studies.

Another interesting question is whether post-translational modifications are required to mediate SMG7-POP2 binding. Post-translational modifications play important roles in regulating gene expression and profoundly affect protein properties and protein-protein interactions. In NMD, an important point of regulation can be observed in the phosphorylation of UPF1. Previous studies have shown that UPF1 is also recruited to mRNAs that are not targeted for degradation by NMD (Hogg & Goff, 2010; Hurt et al, 2013; Kurosaki & Maquat, 2013; Zund et al, 2013). Therefore, a tight regulation on the phosphorylation state of UPF1 is crucial to prevent the untimely recruitment of decay factors and irreversible degradation. In a similar context, POP2 is an active exonuclease and post-translational

modifications on SMG7 might be required to stimulate and perhaps enhance its interaction with POP2. Mass spectrometry (MS) is a powerful tool for detecting post-translational modifications (Mann & Jensen, 2003). Hence, making use of the MS results I had obtained from the TAP tag purification, we identified potential phosphorylation sites on the PC region of SMG7. Unfortunately, preliminary experiments making use of phosphomimetic mutations showed no change in the SMG7-POP2 interaction. Nevertheless, the residues tested were most probably high-abundant modifications and a detailed map of phosphorylation sites would require biochemical enrichment for phosphorylation prior to the MS analysis. At the same time, phorsphorylation may not be the post-translational modification required for SMG7-POP2 interaction. Hence a more systematic approach would be the use of post-translational modification inhibitors first, to determine the type of modification necessary for the interaction prior to MS analysis.

In human cells, SMG7 localizes to distinct cytoplasmic foci known as processing bodies (also called P-bodies) (Unterholzner & Izaurralde, 2004). P-bodies are dynamic structures that are characterised by high local concentrations of mRNA decay factors including decapping factors, the 5'→3' exonuclease XRN1, components of the CCR4-NOT deadenylation complex and factors involved in the miRNA pathway (Eulalio et al, 2007a; Stalder & Muhlemann, 2009). They have been found in both yeast and human cells and are thought to be sites for mRNA turnover and storage (Stalder & Muhlemann, 2009). In yeast, NMD factors UPF1, UPF2 and UPF3 accumulate in P-bodies when decapping is inhibited (Sheth & Parker, 2006). Additionally, UPF1 was shown to target PTC-containing transcripts to P-bodies in yeast (Sheth & Parker, 2006). However, it has been shown that the formation of P-bodies is not essential for functional NMD in *Drosophila* cells (Eulalio et al, 2007b; Rehwinkel et al, 2005). In human cells, SMG5 and UPF1 localise to P-bodies together with SMG7 when over-expressed, while SMG6 does not localise to the foci (Durand et al, 2007; Fukuhara et al, 2005; Unterholzner & Izaurralde, 2004). Hence, another question would be whether P-bodies are required for SMG7-mediated decay. A previous study in human cells showed that disrupting P-bodies by depleting the decapping factor Ge-1 (also known as EDC4)

had no effect on the mRNA levels of PTC containing mRNA or the abundance of endogenous NMD substrates (Stalder & Muhlemann, 2009). Thus, degradation of NMD-target transcripts by SMG7 might not require P-body formation. However, does SMG7 interact with POP2 in P-bodies? One method that could potentially address this question would be the use of GFP-fragment reassembly (Wilson et al, 2004). This technique involves the independent expression of fusions with the N- or C-terminal fragments of green fluorescent protein (GFP) (Wilson et al, 2004). Upon protein-protein interaction, both halves of GFP come close enough to form the complete GFP protein and can be detected visually. However, as GFP requires structural integrity to emit fluorescence, one potential limitation could be that the fusion proteins hinder proper folding and lead to a false negative result. Another potential technique could be to use FRET (Förster resonance energy transfer) that makes use of energy transfer between two fluorophores in nanometer proximity. By using donor and acceptor fluorescent protein fusions of SMG7 and POP2 (for example GFP-SMG7 and red-FP-POP2), the molecular interaction of the proteins can be determined in the cytosol and in P-bodies.

Despite the interesting questions outlined above, my results show that NMD in human cells makes use of multiple redundant activities to ensure complete degradation of target mRNA. The NMD pathway uses (a) the endonucleolytic activity of SMG6 and (b) the 5'→3' decay pathway that includes the deadenylase activity of the CCR4-NOT complex and the decapping activity of DCP2, through the SMG5:SMG7 heterodimer.

4.2. NOT10 and NOT11 form a novel module on NOT1 in the CCR4-NOT complex.

This section summarizes some of the work that contributed to defining the novel NOT10:NOT11 module of the CCR4-NOT deadenylase complex. It focuses on the complex in the model organism *Drosophila melanogaster* (*Dm*), to demonstrate the highly conserved nature of this complex and hence through its evolutionary preservation, to emphasize its importance in mRNA turnover.

The work summarized in this section was published by Bawankar et al. (2013). The publication is attached for detailed experimental data and procedures.

NOT10 and NOT11 were first identified as subunits of the human CCR4-NOT complex in a global proteomics analysis (Lau et al, 2009). The NOT10 protein is considered a bona fide subunit of the CCR4-NOT complex as it has been systematically identified in previous studies (Gavin et al, 2002; Lau et al, 2009; Morita et al, 2007). However, prior to my PhD work, NOT11 had only been reported in one previous study in human cells and therefore lacked confirmatory data (Lau et al, 2009). Thus, we aimed to determine whether the NOT10 and NOT11 proteins are truly subunits of the CCR4-NOT complex in *Drosophila* cells and to investigate their function. In our study, immunoprecipitation experiments from both *Drosophila* and human cells showed that NOT10 strongly interacted with NOT11. However this does not show that the interactions are direct as interactions mediated by other proteins can still be detected in immunoprecipitation experiments. Hence, I cloned and co-expressed MBP-tagged NOT10 and GST-tagged NOT11 in *E. coli*. GST-pulldown experiments showed that NOT11 binds directly and specifically to NOT10 as GST alone did not interact with NOT10. Conversely, in MBP pull-down experiments, recombinant NOT10 binds NOT11 in a direct and specific manner as the MBP tag alone did not bind NOT10. Therefore, I showed that *Drosophila* NOT10 and NOT11 interact directly to form a novel module of the CCR4-NOT complex. Two independent studies further support

my data that NOT10 and NOT11 are conserved subunits of the CCR4-NOT complex. One of these studies made use of mass spectrometry to analyze NOT1 immunoprecipitates from *Drosophila* cells (Temme et al, 2010). In their analysis, while the *Drosophila* NOT10 homolog (CG18616) was identified, the authors had concluded that the NOT11 homolog (CG13567) was not. However careful inspection of the complete list of interactors identified CG13567 but with only 2 peptides and could hence explain its exclusion from the final list of interactors (Temme et al, 2010). Another study by Mauxion et. al. that was conducted concurrently with ours had also validated the direct interaction between human NOT10 and NOT11 using yeast-2-hybrid analysis (Mauxion et al, 2013).

Previous studies of the CCR4-NOT complex have identified NOT1 as a scaffold protein, providing binding sites for other subunits to assemble (Albert et al, 2000; Chen et al, 2001; Ito et al, 2011; Lau et al, 2009; Maillet et al, 2000). Based on sequence alignments and secondary structure predictions, NOT1 was divided into three main regions: N-terminal (NOT1-N), Middle (NOT1-M) and C-terminal (NOT1-C) (Figure 9). NOT1-N was predicted to be entirely α-helical while NOT1-M contains two domains, a MIF4G domain that was named as such due to its structural similarity to other MIF4G domains, and the recently named CAF40/CNOT9 binding domain (CN9BD) that, as its name suggests, mediates binding to CAF40 (NOT9) (Basquin et al, 2012; Chen et al, 2014; Petit et al, 2012). Finally, NOT1-C contains a conserved NOT1 superfamily homology domain.

To determine whether NOT1 serves as a scaffold protein in *Drosophila* cells as well, we tested NOT1 with the core components of the CCR4-NOT complex. Using immunoprecipitation experiments, we confirmed that POP2 binds the MIF4G domain of NOT1-M, which agrees with the interactions found in yeast and human cells (Basquin et al, 2012; Petit et al, 2012). Also in yeast, CAF40 (also known as NOT9) was reported to bind NOT1 (Chen et al, 2001). Hence using immunoprecipitation assays, we showed that this interaction is conserved in *Drosophila* cells. In fact, the NOT1 domain that mediates this interaction was further shown to be CN9BD that, at the time of this study, was known as the

domain of unknown function, DUF3819, located C-terminal to the MIF4G domain in NOT1-M (Figure 9). In agreement with other studies, we also showed that the C-terminus of NOT1 binds to the C-termini of NOT2 and NOT3 in *Drosophila* cells (Bai et al, 1999; Ito et al, 2011; Lau et al, 2009).
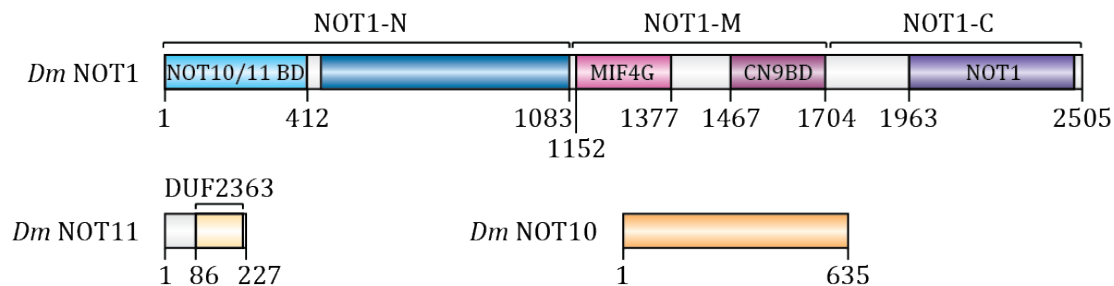

Figure 9: Domain organisation of *Dm* NOT1, *Dm* NOT10 and *Dm* NOT11.
Dm NOT1 forms a platform for the attachment of different modules. NOT1-N: The N-terminus provides a docking site for the new NOT10:NOT11 module and is hence named the NOT10/11 binding domain (NOT10/11 BD). NOT1-M: The mid region contains a MIF4G domain that binds the catalytic subunits of the CCR4-NOT complex, POP2/CAF1, and CCR4a/b. C-terminal to the MIF4G domain is the CAF40/CNOT9 binding domain (CN9BD). NOT1-C: The C-terminus contains the conserved NOT1 domain (NOT1) that is required for binding to NOT2 and NOT3. *Dm* NOT11 contains a single conserved domain, DUF2363 that mediates binding to the NOT10/11 BD of NOT1. *Dm* NOT10 has not been detected to contain a putative conserved domain. Numbers under the schematics correspond to amino acid residue numbers.

Finally, the *Dm* NOT10:NOT11 module described before was shown to interact with NOT1-N in immunoprecipitation assays. However, there was a discrepancy between the results obtained with *Drosophila* or human homologs that required resolution. In *Drosophila* cells, NOT1 was shown to bind NOT10 but not NOT11. However in yeast-2-hybrid assays, human NOT1 was reported to bind NOT11 instead of NOT10 (Figure 10) (Mauxion et al, 2013). This raised a contradiction because the proteins are well conserved between the two organisms indicating that their interactions should have been conserved as well. Hence to resolve this inconsistency and to show that binding to NOT1 is direct, I performed pull-down experiments as described before. Two different lengths of the NOT1-N (amino

acids 1 to 1083 and 1 to 412) were cloned with a GST tag and co-expressed in *E. coli* with MBP-tagged NOT10 or NOT11. Using glutathione beads to pull on GST, I was able to show that the *Dm* NOT1 N-terminus binds directly to NOT11 and not NOT10.
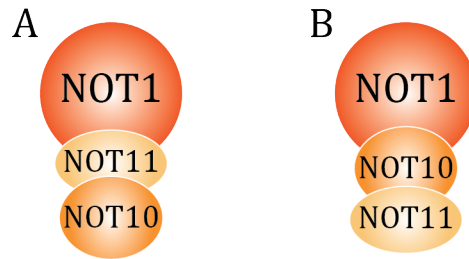
Figure 10: Models of the interaction dilemma in NOT1 and the NOT10:NOT11 module between the human and *Drosophila* homologs.

A: Proposed model for the interaction between *Hs* NOT1 and the *Hs* NOT10: *Hs* NOT11 module where *Hs* NOT11 mediates the interaction. The model was based on observations made in yeast-two-hybrid assays.

B: Proposed model for the interaction between *Dm* NOT1 and the *Dm* NOT10: *Dm* NOT11 module where *Dm* NOT10 mediates the interaction. The model was based on observations made in immunoprecipitation assays from *Drosophila* cell lysates.

Conversely, pulldown experiments using the MBP tag showed that NOT11 binds directly to the N-terminus of NOT1. Furthermore, both fragments of NOT1-N were able to bind NOT11. Hence, from these results I was able to draw two conclusions: Firstly, the N-terminal 412 amino acids of NOT1 were necessary and sufficient for binding to NOT11. Secondly, NOT11 mediates direct binding of the NOT10:NOT11 module onto the NOT1 scaffold. With these results, why was NOT10 identified as the mediating factor between NOT1 and the NOT10:NOT11 module in *Drosophila* cells? A possible explanation could be that in *Drosophila* cells, the endogenous NOT11 is present in excess. To conduct the immunoprecipitation experiments, NOT1 was co-expressed with either NOT10 or NOT11. In the presence of over-expressed NOT10, the endogenous NOT11 could bridge the interactions between NOT10 and NOT1, and hence this interaction could be detected. However, in experiments where NOT11 is over-expressed with NOT1, endogenous NOT11

could have competed with over-expressed tagged NOT11 for binding to NOT1, resulting in no interaction being identified between NOT1 and NOT11. To determine if this explanation is plausible, antibodies against the endogenous NOT11 could be used to detect for the presence of NOT11 in the immunoprecipitation assays. However, as an antibody was not available at the time of the study, this question was not addressed.

The CCR4-NOT complex plays a direct role in mRNA degradation and is the key component responsible for deadenylation (Daugeron et al, 2001; Ito et al, 2011; Temme et al, 2004; Temme et al, 2010). The core components including NOT1, NOT2, NOT3, CCR4 and POP2 have been shown to efficiently degrade mRNA targets through the tethering assay (Chekulaeva et al, 2011; Piao et al, 2010). With the identification of the novel NOT10:NOT11 module of the CCR4-NOT complex, we asked whether NOT10 or NOT11 can promote mRNA decay. Using *Drosophila* cells, Praveen Bawankar expressed NOT10 or NOT11 with the N-peptide from the bacteriophage λ (referred to as the λN-tag) together with a reporter plasmid that contains the firefly luciferase ORF and five BoxB elements in its 3'UTR. Due to the high affinity binding between the N-peptide and the BoxB elements, the λN-tag tethers NOT10 or NOT11 to the reporter mRNA. With this assay, NOT10 and NOT11 were shown to promote degradation of the reporter mRNA, however not as efficiently as the other subunits of the complex despite being expressed at similar levels. This decay activity was specific since reporter mRNAs that do not contain BoxB elements were unaffected. As NOT10 and NOT11 lack their own nuclease activity, the results suggest that tethering any subunit of the complex leads to the recruitment of the other components of the complex through direct or indirect protein-protein interactions, causing mRNA decay. This agrees with tethering results from other non-catalytically active components of the complex, such as NOT1, NOT2 and NOT3 (Bawankar et al, 2013; Chekulaeva et al, 2011). With this in mind, these results may not fully represent the contribution of the NOT10:NOT11 module to mRNA degradation. Hence, we decided to examine the activity of isolated protein domains of NOT1. Since the interaction studies revealed that NOT1-N is sufficient for binding to the NOT10:NOT11 module, the NOT1-N

fragment was tested in the λN-based tethering assay. These results showed that NOT1-N alone was inactive in degrading the reporter mRNA, and deleting the NOT1-N region did not significantly affect the activity of the full length NOT1 protein. In contrast, NOT1-M elicits mRNA decay and a single point mutation that disrupts POP2 binding abolishes mRNA decay activity (Bawankar et al, 2013; Petit et al, 2012). This indicates that the NOT10:NOT11 module may not participate in mRNA degradation. In the concurrent study by Mauxion et. al., NOT11 was depleted in human cells to disrupt the formation of the NOT10:NOT11 module on the CCR4-NOT complex. The poly(A) tail length of a reporter mRNA was then visualised to determine deadenylation rate. The results suggested that, in contrast to NOT1 or POP2 depletion, the reduction of NOT11 had no significant effect on the rate of deadenylation (Mauxion et al, 2013). Consistently, these results imply that the function of the NOT10:NOT11 module remains elusive and requires further investigation. Nevertheless, given the central role of the CCR4-NOT complex in regulating gene expression, there is little doubt that the function(s) of the conserved NOT10:NOT11 module will be revealed in time.

The CCR4-NOT complex is essential for regulating mRNA expression in eukaryotes. Besides directing irreversible degradation for mRNA targets, the CCR4-NOT complex plays multiple roles in a wide range of cellular processes including transcription, ubiquitination and translational repression (Bartlam & Yamamoto, 2010; Collart & Panasenko, 2012). Even though some subunits are highly conserved, the composition of the CCR4-NOT complex differs across species as yeast-specific and metazoa-specific subunits have been described (Collart & Panasenko, 2012; Lau et al, 2009; Temme et al, 2010). Hence, characterizing the components and the assembly of the CCR4-NOT complex in various organisms promises to advance our understanding of its diverse functional roles. In the course of my studies, I demonstrated the direct interactions involved between two newly identified components of the CCR4-NOT complex in *Drosophila* cells, NOT10 and NOT11. Additionally, I showed that the novel NOT10:NOT11 module anchors directly onto the N-terminus of the NOT1 scaffold protein through NOT11. While the function of this novel module remains unclear, our data provides the basis for

future studies aimed to understand the functional diversity of the CCR4-NOT complex.

5. AUTHOR'S CONTRIBUTIONS TO PUBLICATIONS

5.1. NOT10 and NOT11 form a novel module that binds NOT1.

Bawankar, P., **Loh, B.**, Wohlbold, L., Schmidt, S. & Izaurralde, E. 2013. NOT10 and C2orf29/NOT11 form a conserved module of the CCR4-NOT complex that docks onto the NOT1 N-terminal domain. *RNA Biol* 10: 228–244.

B. Loh contributed to this publication by defining the direct interactions between NOT10, NOT11 and NOT1. She cloned and optimized protein expressions for NOT10, NOT11 and fragments of NOT1 in *E. coli*. She also planned and optimized conditions for the pull-down experiments. She executed the final experiments and interpreted the results. B. Loh prepared figures and contributed to the writing of the manuscript.

5.2. SMG7 mediates deadenylation-dependent decapping through direct interactions with POP2.

Loh, B., Jonas, S. & 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: 2125-38.

B. Loh designed and constructed all SMG7 and POP2 clones (wild-type and mutants). She discovered and characterized the interaction between SMG7 and POP2 (except in Figure 5H). All functional studies including tethering assays, complementation assays (except Figure 1C-E) and RNaseH assays were planned, executed, optimized and analyzed by B. Loh. She provided figures, interpreted the results and contributed to the writing of the manuscript.

5.3. DCP2 activation by a conserved loop in DCP1 occurs on EDC4.

Chang, CT., Bercovich, N., **Loh, B.**, Jonas, S. & Izaurralde, E. 2014. The activation of the decapping enzyme DCP2 by DCP1 occurs on the EDC4 scaffold and involves a conserved loop in DCP1. *Nucleic Acids Res* 42: 5217-5233.

B. Loh supported this publication by confirming the results from immunoprecipitation experiments. She planned and executed additional immunoprecipitation experiments to test the interaction between DCP1 mutants

and PNRC2. She conducted dominant negative experiments with DCP2 mutants in SMG7-tethering assays. She analyzed data, prepared figures and contributed to the writing of the manuscript.

5.4. Structures of CNOT1-DDX6 and CNOT1-CNOT9 complexes.

Chen, Y.*, Boland, A.*, Kuzuoğlu-Öztürk, D.*, Bawankar, P., **Loh, B.**, Chang, CT., Weichenrieder, O., Izaurralde, E. 2014. A DDX6-CNOT1 Complex and W-Binding Pockets in CNOT9 Reveal Direct Links between miRNA Target Recognition and Silencing. *Mol Cell* 54(5):737-750.

In this publication, B. Loh contributed to the study of the DDX6:CNOT1 interaction in silencing experiments. She constructed the DDX6 mutants to abolish binding with CNOT1. She planned and optimized the conditions for DDX6 knock-down and complementation assays. She analyzed and interpreted the results from these experiments. B. Loh also conducted immunoprecipitation experiments with CNOT1 mutants to validate the DDX6:CNOT1 interface and to verify that the mutations tested had no effect on protein structure. B. Loh provided figures and contributed to writing the manuscript.

5.5. Structure of the PAN2:PAN3 complex.

Jonas, S.*, Christie, M.*, Peter, D., Bhandari, D., **Loh, B.**, Huntzinger, E., Weichenrieder, O. & Izaurralde, E. 2014. An asymmetric PAN3 dimer recruits a single PAN2 exonuclease to mediate mRNA deadenylation and decay. *Nat Struct Mol Biol* 21:599-608.

B. Loh contributed to this study by planning and conducting all immunoprecipitation experiments in *Drosophila* S2 cells. She prepared all immunoprecipitation data (both human and *Drosophila* experiments) for figures and contributed to the writing of the manuscript.

6. REFERENCES

Albert TK, Lemaire M, van Berkum NL, Gentz R, Collart MA, Timmers HT (2000) Isolation and characterization of human orthologs of yeast CCR4-NOT complex subunits. *Nucleic acids research* **28:** 809-817

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

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

Anders KR, Grimson A, Anderson P (2003) SMG-5, required for C.elegans nonsense-mediated mRNA decay, associates with SMG-2 and protein phosphatase 2A. *The EMBO journal* **22:** 641-650

Arias-Palomo E, Yamashita A, Fernandez IS, Nunez-Ramirez R, Bamba Y, Izumi N, Ohno S, Llorca O (2011) The nonsense-mediated mRNA decay SMG-1 kinase is regulated by large-scale conformational changes controlled by SMG-8. *Genes Dev* **25:** 153-164

Bai Y, Salvadore C, Chiang YC, Collart MA, Liu HY, Denis CL (1999) The CCR4 and CAF1 proteins of the CCR4-NOT complex are physically and functionally separated from NOT2, NOT4, and NOT5. *Mol Cell Biol* **19:** 6642-6651

Baierlein C, Krebber H (2010) Translation termination: new factors and insights. *RNA biology* **7:** 548-550

Bartlam M, Yamamoto T (2010) The structural basis for deadenylation by the CCR4-NOT complex. *Protein & cell* **1:** 443-452

Basquin J, Roudko VV, Rode M, Basquin C, Seraphin B, Conti E (2012) Architecture of the nuclease module of the yeast Ccr4-not complex: the Not1-Caf1-Ccr4 interaction. *Molecular cell* **48:** 207-218

Bawankar P, Loh B, Wohlbold L, Schmidt S, Izaurralde E (2013) NOT10 and C2orf29/NOT11 form a conserved module of the CCR4-NOT complex that docks onto the NOT1 N-terminal domain. *RNA biology* **10:** 228-244

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

Behm-Ansmant I, Izaurralde E (2006) Quality control of gene expression: a stepwise assembly pathway for the surveillance complex that triggers nonsense-mediated mRNA decay. *Genes Dev* **20:** 391-398

6. References

Behm-Ansmant I, Kashima I, Rehwinkel J, Sauliere J, Wittkopp N, Izaurralde E (2007b) mRNA quality control: an ancient machinery recognizes and degrades mRNAs with nonsense codons. *FEBS letters* **581:** 2845-2853

Benkovics AH, Nyiko T, Merai Z, Silhavy D, Bisztray GD (2011) Functional analysis of the grapevine paralogs of the SMG7 NMD factor using a heterolog VIGS-based gene depletion-complementation system. *Plant molecular biology* **75:** 277-290

Bhuvanagiri M, Schlitter AM, Hentze MW, Kulozik AE (2010) NMD: RNA biology meets human genetic medicine. *Biochem J* **430:** 365-377

Bianchin C, Mauxion F, Sentis S, Seraphin B, Corbo L (2005) Conservation of the deadenylase activity of proteins of the Caf1 family in human. *RNA* **11:** 487-494

Bono F, Gehring NH (2011) Assembly, disassembly and recycling: The dynamics of exon junction complexes. *RNA biology* **8:** 24-29

Cali BM, Kuchma SL, Latham J, Anderson P (1999) smg-7 is required for mRNA surveillance in Caenorhabditis elegans. *Genetics* **151:** 605-616

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

Chan WK, Huang L, Gudikote JP, Chang YF, Imam JS, MacLean JA, 2nd, Wilkinson MF (2007) An alternative branch of the nonsense-mediated decay pathway. *The EMBO journal* **26:** 1820-1830

Chavez JD, Liu NL, Bruce JE (2011) Quantification of protein-protein interactions with chemical cross-linking and mass spectrometry. *Journal of proteome research* **10:** 1528-1537

Chekulaeva M, Mathys H, Zipprich JT, Attig J, Colic M, Parker R, Filipowicz W (2011) miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. *Nature structural & molecular biology* **18:** 1218-1226

Chen C, Ito K, Takahashi A, Wang G, Suzuki T, Nakazawa T, Yamamoto T, Yokoyama K (2011) Distinct expression patterns of the subunits of the CCR4-NOT deadenylase complex during neural development. *Biochemical and biophysical research communications* **411:** 360-364

Chen CY, Shyu AB (2003) Rapid deadenylation triggered by a nonsense codon precedes decay of the RNA body in a mammalian cytoplasmic nonsense-mediated decay pathway. *Mol Cell Biol* **23:** 4805-4813

6. References

Chen J, Rappsilber J, Chiang YC, Russell P, Mann M, Denis CL (2001) Purification and characterization of the 1.0 MDa CCR4-NOT complex identifies two novel components of the complex. *Journal of molecular biology* **314:** 683-694

Chen Y, Boland A, Kuzuoglu-Ozturk D, Bawankar P, Loh B, Chang CT, Weichenrieder O, Izaurralde E (2014) A DDX6-CNOT1 Complex and W-Binding Pockets in CNOT9 Reveal Direct Links between miRNA Target Recognition and Silencing. *Molecular cell* **54:** 737-750

Cho H, Han S, Choe J, Park SG, Choi SS, Kim YK (2013) SMG5-PNRC2 is functionally dominant compared with SMG5-SMG7 in mammalian nonsense-mediated mRNA decay. *Nucleic acids research* **41:** 1319-1328

Cho H, Kim KM, Kim YK (2009) Human proline-rich nuclear receptor coregulatory protein 2 mediates an interaction between mRNA surveillance machinery and decapping complex. *Molecular cell* **33:** 75-86

Collart MA, Panasenko OO (2012) The Ccr4--not complex. *Gene* **492:** 42-53

Couttet P, Grange T (2004) Premature termination codons enhance mRNA decapping in human cells. *Nucleic acids research* **32:** 488-494

Daugeron MC, Mauxion F, Seraphin B (2001) The yeast POP2 gene encodes a nuclease involved in mRNA deadenylation. *Nucleic acids research* **29:** 2448-2455

Dihazi GH, Sinz A (2003) Mapping low-resolution three-dimensional protein structures using chemical cross-linking and Fourier transform ion-cyclotron resonance mass spectrometry. *Rapid communications in mass spectrometry : RCM* **17:** 2005-2014

Doidge R, Mittal S, Aslam A, Winkler GS (2012a) The anti-proliferative activity of BTG/TOB proteins is mediated via the Caf1a (CNOT7) and Caf1b (CNOT8) deadenylase subunits of the Ccr4-not complex. *PloS one* **7:** e51331

Doidge R, Mittal S, Aslam A, Winkler GS (2012b) Deadenylation of cytoplasmic mRNA by the mammalian Ccr4-Not complex. *Biochemical Society transactions* **40:** 896-901

Doma MK, Parker R (2006) Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation. *Nature* **440:** 561-564

Durand S, Cougot N, Mahuteau-Betzer F, Nguyen CH, Grierson DS, Bertrand E, Tazi J, Lejeune F (2007) Inhibition of nonsense-mediated mRNA decay (NMD) by a new chemical molecule reveals the dynamic of NMD factors in P-bodies. *The Journal of cell biology* **178:** 1145-1160

6. References

Eberle AB, Lykke-Andersen S, Muhlemann O, Jensen TH (2009) SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nature structural & molecular biology* **16:** 49-55

Eulalio A, Behm-Ansmant I, Izaurralde E (2007a) P bodies: at the crossroads of post-transcriptional pathways. *Nature reviews Molecular cell biology* **8:** 9-22

Eulalio A, Behm-Ansmant I, Schweizer D, Izaurralde E (2007b) P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. *Mol Cell Biol* **27:** 3970-3981

Fernandez IS, Yamashita A, Arias-Palomo E, Bamba Y, Bartolome RA, Canales MA, Teixido J, Ohno S, Llorca O (2011) Characterization of SMG-9, an essential component of the nonsense-mediated mRNA decay SMG1C complex. *Nucleic acids research* **39:** 347-358

Frischmeyer PA, van Hoof A, O'Donnell K, Guerrerio AL, Parker R, Dietz HC (2002) An mRNA surveillance mechanism that eliminates transcripts lacking termination codons. *Science* **295:** 2258-2261

Frolova L, Le Goff X, Zhouravleva G, Davydova E, Philippe M, Kisselev L (1996) Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. *RNA* **2:** 334-341

Fukuhara N, Ebert J, Unterholzner L, Lindner D, Izaurralde E, 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, Izaurralde E (2004) Nonsense-mediated messenger RNA decay is initiated by endonucleolytic cleavage in Drosophila. *Nature* **429:** 575-578

Gatfield D, Unterholzner L, Ciccarelli FD, Bork P, Izaurralde E (2003) Nonsense-mediated mRNA decay in Drosophila: at the intersection of the yeast and mammalian pathways. *The EMBO journal* **22:** 3960-3970

Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, Remor M, Hofert C, Schelder M, Brajenovic M, Ruffner H, Merino A, Klein K, Hudak M, Dickson D, Rudi T, Gnau V, Bauch A, Bastuck S, Huhse B, Leutwein C, Heurtier MA, Copley RR, Edelmann A, Querfurth E, Rybin V, Drewes G, Raida M, Bouwmeester T, Bork P, Seraphin B, Kuster B, Neubauer G, Superti-Furga G (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **415:** 141-147

Glavan F, Behm-Ansmant I, Izaurralde E, 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

6. References

Godwin AR, Kojima S, Green CB, Wilusz J (2013) Kiss your tail goodbye: the role of PARN, Nocturnin, and Angel deadenylases in mRNA biology. *Biochimica et biophysica acta* **1829:** 571-579

Gonzalez CI, Ruiz-Echevarria MJ, Vasudevan S, Henry MF, Peltz SW (2000) The yeast hnRNP-like protein Hrp1/Nab4 marks a transcript for nonsense-mediated mRNA decay. *Molecular cell* **5:** 489-499

Haas G, Braun JE, Igreja C, Tritschler F, Nishihara T, Izaurralde E (2010) HPat provides a link between deadenylation and decapping in metazoa. *The Journal of cell biology* **189:** 289-302

He F, Jacobson A (1995) Identification of a novel component of the nonsense-mediated mRNA decay pathway by use of an interacting protein screen. *Genes & Development* **9:** 437-454

Hodgkin J, Papp A, Pulak R, Ambros V, Anderson P (1989) A new kind of informational suppression in the nematode Caenorhabditis elegans. *Genetics* **123:** 301-313

Hogg JR, Goff SP (2010) Upf1 senses 3'UTR length to potentiate mRNA decay. *Cell* **143:** 379-389

Horiuchi M, Takeuchi K, Noda N, Muroya N, Suzuki T, Nakamura T, Kawamura-Tsuzuku J, Takahasi K, Yamamoto T, Inagaki F (2009) Structural basis for the antiproliferative activity of the Tob-hCaf1 complex. *The Journal of biological chemistry* **284:** 13244-13255

Hoshino S (2012) Mechanism of the initiation of mRNA decay: role of eRF3 family G proteins. *Wiley interdisciplinary reviews RNA* **3:** 743-757

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

Hurt JA, Robertson AD, Burge CB (2013) Global analyses of UPF1 binding and function reveal expanded scope of nonsense-mediated mRNA decay. *Genome research* **23:** 1636-1650

Isken O, Maquat LE (2007) Quality control of eukaryotic mRNA: safeguarding cells from abnormal mRNA function. *Genes Dev* **21:** 1833-1856

Ito K, Takahashi A, Morita M, Suzuki T, Yamamoto T (2011) The role of the CNOT1 subunit of the CCR4-NOT complex in mRNA deadenylation and cell viability. *Protein & cell* **2:** 755-763

Jackson RJ, Hellen CU, Pestova TV (2010) The mechanism of eukaryotic translation initiation and principles of its regulation. *Nature reviews Molecular cell biology* **11:** 113-127

Jackson RJ, Hellen CU, Pestova TV (2012) Termination and post-termination events in eukaryotic translation. *Advances in protein chemistry and structural biology* **86:** 45-93

Johnson JM, Castle J, Garrett-Engele P, Kan Z, Loerch PM, Armour CD, Santos R, Schadt EE, Stoughton R, Shoemaker DD (2003) Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science* **302:** 2141-2144

Jonas S, Weichenrieder O, 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

Karam R, Wengrod J, Gardner LB, Wilkinson MF (2013) Regulation of nonsense-mediated mRNA decay: implications for physiology and disease. *Biochimica et biophysica acta* **1829:** 624-633

Kashima I, Jonas S, Jayachandran U, Buchwald G, Conti E, Lupas AN, Izaurralde E (2010) SMG6 interacts with the exon junction complex via two conserved EJC-binding motifs (EBMs) required for nonsense-mediated mRNA decay. *Genes Dev* **24:** 2440-2450

Kashima I, Yamashita A, Izumi N, Kataoka N, Morishita R, Hoshino S, Ohno M, Dreyfuss G, 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

Kervestin S, Jacobson A (2012) NMD: a multifaceted response to premature translational termination. *Nature reviews Molecular cell biology* **13:** 700-712

Kervestin S, Li C, Buckingham R, Jacobson A (2012) Testing the faux-UTR model for NMD: analysis of Upf1p and Pab1p competition for binding to eRF3/Sup35p. *Biochimie* **94:** 1560-1571

Kim VN, Kataoka N, Dreyfuss G (2001) Role of the nonsense-mediated decay factor hUpf3 in the splicing-dependent exon-exon junction complex. *Science* **293:** 1832-1836

Kurosaki T, Maquat LE (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

6. References

Lafontaine DL, Tollervey D (2001) The function and synthesis of ribosomes. *Nature reviews Molecular cell biology* **2:** 514-520

Lai T, Cho H, Liu Z, Bowler MW, Piao S, Parker R, Kim YK, Song H (2012) Structural basis of the PNRC2-mediated link between mrna surveillance and decapping. *Structure* **20:** 2025-2037

Lau NC, Kolkman A, van Schaik FM, Mulder KW, Pijnappel WW, Heck AJ, Timmers HT (2009) Human Ccr4-Not complexes contain variable deadenylase subunits. *Biochem J* **422:** 443-453

Le Hir H, Gatfield D, Izaurralde E, Moore MJ (2001) The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. *The EMBO journal* **20:** 4987-4997

Lejeune F, Li X, Maquat LE (2003) Nonsense-mediated mRNA decay in mammalian cells involves decapping, deadenylating, and exonucleolytic activities. *Molecular cell* **12:** 675-687

Lejeune F, Maquat LE (2005) Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells. *Current opinion in cell biology* **17:** 309-315

Lewis BP, Green RE, Brenner SE (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

Li S, Wilkinson MF (1998) Nonsense surveillance in lymphocytes? *Immunity* **8:** 135-141

Liu H, Rodgers ND, Jiao X, Kiledjian M (2002) The scavenger mRNA decapping enzyme DcpS is a member of the HIT family of pyrophosphatases. *The EMBO journal* **21:** 4699-4708

Loh B, Jonas S, 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

Luke B, Azzalin CM, Hug N, Deplazes A, Peter M, Lingner J (2007) Saccharomyces cerevisiae Ebs1p is a putative ortholog of human Smg7 and promotes nonsense-mediated mRNA decay. *Nucleic acids research* **35:** 7688-7697

Lykke-Andersen J (2002) Identification of a Human Decapping Complex Associated with hUpf Proteins in Nonsense-Mediated Decay. *Molecular and Cellular Biology* **22:** 8114-8121

6. References

Lykke-Andersen J, Bennett EJ (2014) Protecting the proteome: Eukaryotic cotranslational quality control pathways. *The Journal of cell biology* **204:** 467-476

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

Maillet L, Tu C, Hong YK, Shuster EO, Collart MA (2000) The essential function of Not1 lies within the Ccr4-Not complex. *Journal of molecular biology* **303:** 131-143

Mann M, Jensen ON (2003) Proteomic analysis of post-translational modifications. *Nature biotechnology* **21:** 255-261

Mauxion F, Preve B, Seraphin B (2013) C2ORF29/CNOT11 and CNOT10 form a new module of the CCR4-NOT complex. *RNA biology* **10:** 267-276

Melero R, Buchwald G, Castano R, Raabe M, Gil D, Lazaro M, Urlaub H, Conti E, Llorca O (2012) The cryo-EM structure of the UPF-EJC complex shows UPF1 poised toward the RNA 3' end. *Nature structural & molecular biology* **19:** 498-505, S491-492

Mendell JT, Medghalchi SM, Lake RG, Noensie EN, Dietz HC (2000) Novel Upf2p orthologues suggest a functional link between translation initiation and nonsense surveillance complexes. *Mol Cell Biol* **20:** 8944-8957

Mendell JT, Sharifi NA, Meyers JL, Martinez-Murillo F, Dietz HC (2004) Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise. *Nature genetics* **36:** 1073-1078

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

Morita M, Suzuki T, Nakamura T, Yokoyama K, Miyasaka T, Yamamoto T (2007) Depletion of mammalian CCR4b deadenylase triggers elevation of the p27Kip1 mRNA level and impairs cell growth. *Mol Cell Biol* **27:** 4980-4990

Muhlemann O, Eberle AB, Stalder L, Zamudio Orozco R (2008) Recognition and elimination of nonsense mRNA. *Biochimica et biophysica acta* **1779:** 538-549

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

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

Ohnishi T, Yamashita A, Kashima I, Schell T, Anders KR, Grimson A, Hachiya T, Hentze MW, Anderson P, 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, Ohno S (2012) N- and C-terminal Upf1 phosphorylations create binding platforms for SMG-6 and SMG-5:SMG-7 during NMD. *Nucleic acids research* **40:** 1251-1266

Ozgur S, Chekulaeva M, Stoecklin G (2010) Human Pat1b connects deadenylation with mRNA decapping and controls the assembly of processing bodies. *Mol Cell Biol* **30:** 4308-4323

Page MF, Carr B, Anders KR, Grimson A, 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

Parker R, Song H (2004) The enzymes and control of eukaryotic mRNA turnover. *Nature structural & molecular biology* **11:** 121-127

Passos DO, Doma MK, Shoemaker CJ, Muhlrad D, Green R, Weissman J, Hollien J, Parker R (2009) Analysis of Dom34 and its function in no-go decay. *Molecular biology of the cell* **20:** 3025-3032

Petit AP, Wohlbold L, Bawankar P, Huntzinger E, Schmidt S, Izaurralde E, Weichenrieder O (2012) The structural basis for the interaction between the CAF1 nuclease and the NOT1 scaffold of the human CCR4-NOT deadenylase complex. *Nucleic acids research* **40:** 11058-11072

Piao X, Zhang X, Wu L, Belasco JG (2010) CCR4-NOT deadenylates mRNA associated with RNA-induced silencing complexes in human cells. *Mol Cell Biol* **30:** 1486-1494

Pisareva VP, Skabkin MA, Hellen CU, Pestova TV, Pisarev AV (2011) Dissociation by Pelota, Hbs1 and ABCE1 of mammalian vacant 80S ribosomes and stalled elongation complexes. *The EMBO journal* **30:** 1804-1817

Preiss T, M WH (2003) Starting the protein synthesis machine: eukaryotic translation initiation. *BioEssays : news and reviews in molecular, cellular and developmental biology* **25:** 1201-1211

Pulak R, Anderson P (1993) mRNA surveillance by the Caenorhabditis elegans smg genes. *Genes Dev* **7:** 1885-1897

6. References

Rehwinkel J, Behm-Ansmant I, Gatfield D, Izaurralde E (2005) A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. *RNA* **11:** 1640-1647

Reichenbach P, Hoss M, Azzalin CM, Nabholz M, Bucher P, Lingner J (2003) A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. *Current biology : CB* **13:** 568-574

Riehs N, Akimcheva S, Puizina J, Bulankova P, Idol RA, Siroky J, Schleiffer A, Schweizer D, Shippen DE, Riha K (2008) Arabidopsis SMG7 protein is required for exit from meiosis. *Journal of cell science* **121:** 2208-2216

Schweingruber C, Rufener SC, Zund D, Yamashita A, Muhlemann O (2013) Nonsense-mediated mRNA decay - mechanisms of substrate mRNA recognition and degradation in mammalian cells. *Biochimica et biophysica acta* **1829:** 612-623

Seebacher J, Mallick P, Zhang N, Eddes JS, Aebersold R, Gelb MH (2006) Protein cross-linking analysis using mass spectrometry, isotope-coded cross-linkers, and integrated computational data processing. *Journal of proteome research* **5:** 2270-2282

Serin G, Gersappe A, Black JD, Aronoff R, Maquat LE (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

Sheth U, Parker R (2006) Targeting of aberrant mRNAs to cytoplasmic processing bodies. *Cell* **125:** 1095-1109

Shoemaker CJ, Eyler DE, Green R (2010) Dom34:Hbs1 promotes subunit dissociation and peptidyl-tRNA drop-off to initiate no-go decay. *Science* **330:** 369-372

Stalder L, Muhlemann O (2009) Processing bodies are not required for mammalian nonsense-mediated mRNA decay. *RNA* **15:** 1265-1273

Steitz TA (2008) A structural understanding of the dynamic ribosome machine. *Nature reviews Molecular cell biology* **9:** 242-253

Swisher KD, Parker R (2011) Interactions between Upf1 and the decapping factors Edc3 and Pat1 in Saccharomyces cerevisiae. *PloS one* **6:** e26547

Temme C, Zaessinger S, Meyer S, Simonelig M, Wahle E (2004) A complex containing the CCR4 and CAF1 proteins is involved in mRNA deadenylation in Drosophila. *The EMBO journal* **23:** 2862-2871

6. References

Temme C, Zhang L, Kremmer E, Ihling C, Chartier A, Sinz A, Simonelig M, Wahle E (2010) Subunits of the Drosophila CCR4-NOT complex and their roles in mRNA deadenylation. *RNA* **16:** 1356-1370

Tharun S (2009) Lsm1-7-Pat1 complex: a link between 3' and 5'-ends in mRNA decay? *RNA biology* **6:** 228-232

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

Tsuboi T, Kuroha K, Kudo K, Makino S, Inoue E, Kashima I, Inada T (2012) Dom34:hbs1 plays a general role in quality-control systems by dissociation of a stalled ribosome at the 3' end of aberrant mRNA. *Molecular cell* **46:** 518-529

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

van Hoof A, Frischmeyer PA, Dietz HC, Parker R (2002) Exosome-mediated recognition and degradation of mRNAs lacking a termination codon. *Science* **295:** 2262-2264

Wahle E, Winkler GS (2013) RNA decay machines: deadenylation by the Ccr4-not and Pan2-Pan3 complexes. *Biochimica et biophysica acta* **1829:** 561-570

Wang W, Cajigas IJ, Peltz SW, Wilkinson MF, Gonzalez CI (2006) Role for Upf2p phosphorylation in Saccharomyces cerevisiae nonsense-mediated mRNA decay. *Mol Cell Biol* **26:** 3390-3400

Wilson CG, Magliery TJ, Regan L (2004) Detecting protein-protein interactions with GFP-fragment reassembly. *Nature methods* **1:** 255-262

Yamashita A, Chang TC, Yamashita Y, Zhu W, Zhong Z, Chen CY, Shyu AB (2005) Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover. *Nature structural & molecular biology* **12:** 1054-1063

Yamashita A, Izumi N, Kashima I, Ohnishi T, Saari B, Katsuhata Y, Muramatsu R, Morita T, Iwamatsu A, Hachiya T, Kurata R, Hirano H, Anderson P, Ohno S (2009) SMG-8 and SMG-9, two novel subunits of the SMG-1 complex, regulate remodeling of the mRNA surveillance complex during nonsense-mediated mRNA decay. *Genes Dev* **23:** 1091-1105

Yamashita A, Ohnishi T, Kashima I, Taya Y, 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

6. References

Zhang H, Tang X, Munske GR, Tolic N, Anderson GA, Bruce JE (2009) Identification of protein-protein interactions and topologies in living cells with chemical cross-linking and mass spectrometry. *Molecular & cellular proteomics : MCP* **8:** 409-420

Zhang H, Tang X, Munske GR, Zakharova N, Yang L, Zheng C, Wolff MA, Tolic N, Anderson GA, Shi L, Marshall MJ, Fredrickson JK, Bruce JE (2008) In vivo identification of the outer membrane protein OmcA-MtrC interaction network in Shewanella oneidensis MR-1 cells using novel hydrophobic chemical cross-linkers. *Journal of proteome research* **7:** 1712-1720

Zuiderweg ER (2002) Mapping protein-protein interactions in solution by NMR spectroscopy. *Biochemistry* **41:** 1-7

Zund D, Gruber AR, Zavolan M, Muhlemann O (2013) Translation-dependent displacement of UPF1 from coding sequences causes its enrichment in 3' UTRs. *Nature structural & molecular biology* **20:** 936-943

7. ABBREVIATIONS

A-site	Aminoacyl-site
BTZ	Barentsz
CAF	CCR4-associated factor
CASC3	Cancer susceptibility candidate 3
CCR4	Carbon catabolite repression 4
C. elegans	*Caenorhabditis elegans*
CN9BD	CAF40/CNOT9 binding domain
DCP	mRNA decapping enzyme
DDX	DEAD box helicase
Dm	*Drosophila melanogaster*
Dom34	Duplication of multilocus region 34
DSE	Downstream elements
DUF	Domain of unknown function
E. coli	*Escherichia coli*
EDC	enhancer of mRNA decapping
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
EJC	Exon junction complex
E-site	Exit-site
Est1	Ever shorter telomeres 1
FRET	Förster resonance energy transfer
GFP	Green fluorescent protein
GTP	Guanosine-5'-triphosphate
GST	Glutathione S-transferase
HA	Hemagglutinin
Hbs1	Hsp70 subfamily B suppressor 1
HEK293T	Human embryonic kidney 293T cells
HeLa	Henrietta Lacks
Hrp1	Heterogeneous nuclear ribonucleoprotein 1
LSm	U6 snRNA associated Sm-like protein

MAGOH	Mago nashi homolog
MBP	Maltose binding protein
Me31B	Maternal expression at 31B
Met	Methionine
m^{7}G	5' 7-methyl-guanosine
MIF4G	Middle domain of eukaryotic initiation factor 4G
MLN	Metastatic lymph node gene 51 protein
mRNAs	messenger ribonucleic acid
mRNPs	messenger ribonucleoprotein particles
MS	Mass spectrometry
NGD	No-Go decay
NMD	Nonsense mediated mRNA decay
NMR	Nuclear magnetic resonance spectrometry
NOT	CCR4-NOT transcription complex
NSAP1	NS1-associated protein 1
NSD	Non-stop decay
ORF	Open reading frame
PABP	Poly(A) binding protein
PAN	Poly(A) specific nuclease
PARN	Poly(A) ribonuclease
PatL1	Protein PAT1 homolog
PC	Proline-rich C-terminus
PIN	PilT N-terminus
PNRC2	Proline-rich nuclear receptor coactivator 2
POP2	Poly(A) ribonuclease 2
PP2A	Protein phosphatase 2A
P-site	Peptidyl-site
PTC	Pre-mature termination codon
RF	Release factor
Rli1	RNaseL inhibitor
SBP	Streptavidin binding peptide
shRNA	Short-hairpin RNA

siRNA	Small interfering RNA
Ski	Superkiller
SMG	Suppressor with morphological effects on genitalia
SMG1C	SMG1 complex
SURF	SMG1-UPF1-eRF1-eRF3
TAB182	Tankyrase-binding protein 182
TAP	Tandem affinity purification
tRNA	transfer ribonucleic acid
TOB	Transducer of erbB-2
UNR	N-ras upstream gene protein
uORF	upstream open reading frame
UPF	Up-Frameshift
UTR	Untranslated region
XRN1	5'->3' Exoribonuclease 1

8. APPENDIX

8.1. List of publications

8.1.1. Discussed publications

1. The SMG5-SMG7 heterodimer directly recruits the CCR4-NOT deadenylase complex to mRNAs containing nonsense codons via interaction with POP2.
    Loh, B., Jonas, S. & Izaurralde, E.
    Genes Dev. 2013 Oct;27(19):2125-38.

2. NOT10 and C2orf29/NOT11 form a conserved module of the CCR4-NOT complex that docks onto the NOT1 N-terminal domain.
    Bawankar, P., Loh, B., Wohlbold, L., Schmidt, S. & Izaurralde, E.
    RNA Biol. 2013 Feb;10(2):228-44.

8.1.2. Additional publications (during PhD)

3. The activation of the decapping enzyme DCP2 by DCP1 occurs on the EDC4 scaffold and involves a conserved loop in DCP1.
    Chang, CT., Bercovich, N., Loh, B., Jonas, S. & Izaurralde, E.
    Nucleic Acids Res. 2014 Apr;42(8):5217-5233.

4. A DDX6-CNOT1 Complex and W-Binding Pockets in CNOT9 Reveal Direct Links between miRNA Target Recognition and Silencing.
    Chen, Y.*, Boland, A.*, Kuzuoğlu-Öztürk D.*, Bawankar, P., Loh, B., Chang, CT., Weichenrieder, O. & Izaurralde, E.
    Mol Cell. 2014 Jun 5;54(5):737-50.*equal contributions

5. An asymmetric PAN3 dimer recruits a single PAN2 exonuclease to mediate mRNA deadenylation and decay.
    Jonas, S.*, Christie, M.*, Peter, D., Bhandari, D., Loh, B., Huntzinger, E., Weichenrieder, O. & Izaurralde, E.
    Nat Struct Mol Biol. 2014 Jun 21:599-608. *equal contributions

8.1.3. Additional publications (prior PhD)

6. Gyrase B inhibitor impairs HIV-1 replication by targeting Hsp90 and the capsid protein.
    Vozzolo, L., <u>Loh, B.</u>, Gane, PJ., Tribak, M., Zhou, L., Anderson, I., Nyakatura, E., Jenner, RG., Selwood, D. & Fassati, A.
    J Biol Chem. 2010 Dec;285:9314-28.

7. Inhibition of HIV-1 replication by isoxazolidine and isoxazole sulfonamides.
    <u>Loh, B.</u>, Vozzolo, L., Mok, J., Lee, C., Fitzmaurice, R., Caddick, S. & Fassati, A.
    Chem Biol Drug Des. 2009 May;75(5): 461-74.

8. SARM: A novel Toll-like receptor adaptor, is functionally conserved from arthropod to human.
    <u>Loh, B.</u>, Wang, XW., Bui, THH., Luan, XL., Ho, B. & Ding, JL.
    Mol Immunol. 2008 Mar;45(6):1732-42.

8.2. Curriculum Vitae

Belinda <u>Loh</u>

PERSONAL INFORMATION

Date of Birth:	10th Dec 1981
Nationality:	Singaporean
Address:	Department of Biochemistry
	Max Planck Institute for Developmental Biology
	Spemannstrasse 35, 72076 Tübingen, Germany
E-mail address:	belinda.loh@tuebingen.mpg.de

EDUCATION

Oct 2010 - present	**University of Tübingen**
	Max Planck Institute for Developmental Biology
	Group of Prof. Dr. Elisa Izaurralde
	Dissertation: The nonsense mediated mRNA decay is directly linked to deadenylation via SMG7 and the deadenylase POP2.
Jul 2002 - Jun 2004	**University of Queensland (Brisbane, Australia)**
	Bachelor of Biotechnology (Second Upper Class Honours) Honours score: 79.9% (H1: 80%); Third Year GPA: 5.25
	Honours Dissertation: Characterization of the K-*ras* pseudogene.
Jul 2000 - Jun 2002	**Singapore Polytechnic (Singapore)**
	Diploma in Biotechnology – General Biotechnology
	Final Year Project Dissertation: Replacement of Fish meal with Soyabean meal in the Diets for *Barbus tetrazona*.

1998 – 1999	**Serangoon Junior College (Singapore)**
	Singapore-Cambridge GCE 'A' Level certification (Science)

CONFERENCE PARTICIPATIONS

June 2013	18th Annual Meeting of the RNA Society, Davos, ***Poster***
April 2013	Meeting of the DFG network FOR855 on "Cytoplasmic regulation of gene expression", University of Cologne, ***Speaker***
September 2012	Translational Control Meeting, Cold Spring Harbour Laboratories, ***Poster***
July 2012	TIPP Retreat 2012, The Tübingen International PhD Program and the International Max Planck Research School, ***Speaker***
June 2011	TIPP Retreat 2011, The Tübingen International PhD Program and the International Max Planck Research School, ***Poster***

The SMG5–SMG7 heterodimer directly recruits the CCR4–NOT deadenylase complex to mRNAs containing nonsense codons via interaction with POP2

Belinda Loh, Stefanie Jonas and Elisa Izaurralde

Supplemental Material	http://genesdev.cshlp.org/genesdev/suppl/2013/10/10/27.19.2125.DC1.html
References	This article cites 58 articles, 29 of which can be accessed free at: http://genesdev.cshlp.org/content/27/19/2125.full.html#ref-list-1
Open Access	Freely available online through the *Genes & Development* Open Access option.
Creative Commons License	This article, published in *Genes & Development*, is available under a Creative Commons License (Attribution-NonCommercial 3.0 Unported), as described at http://creativecommons.org/licenses/by-nc/3.0/.
Email Alerting Service	Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or **click here.**

The SMG5–SMG7 heterodimer directly recruits the CCR4–NOT deadenylase complex to mRNAs containing nonsense codons via interaction with POP2

Belinda Loh, Stefanie Jonas, and Elisa Izaurralde[1]

Department of Biochemistry, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany

Nonsense-mediated mRNA decay (NMD) is a eukaryotic quality control mechanism that detects aberrant mRNAs containing nonsense codons and induces their rapid degradation. This degradation is mediated by SMG6, an NMD-specific endonuclease, as well as the SMG5 and SMG7 proteins, which recruit general mRNA decay enzymes. However, it remains unknown which specific decay factors are recruited and whether this recruitment is direct. Here, we show that SMG7 binds directly to POP2, a catalytic subunit of the CCR4–NOT deadenylase complex, and elicits deadenylation-dependent decapping and 5′-to-3′ decay of NMD targets. Accordingly, a catalytically inactive POP2 mutant partially suppresses NMD in human cells. The SMG7–POP2 interaction is critical for NMD in cells depleted of SMG6, indicating that SMG7 and SMG6 act redundantly to promote the degradation of NMD targets. We further show that UPF1 provides multiple binding sites for decapping factors. These data unveil a missing direct physical link between NMD and the general mRNA decay machinery and indicate that NMD employs diverse and partially redundant mechanisms to ensure robust degradation of aberrant mRNAs.

The nonsense-mediated mRNA decay (NMD) pathway rids the cell of aberrant mRNAs that have acquired premature translation termination codons (PTCs, or nonsense codons) as a result of mutations or inaccuracies in gene expression that inadvertently generate PTCs. Through this action, NMD prevents the accumulation of truncated proteins, which can be toxic to the cell (Kervestin and Jacobson 2012). In addition to eliminating aberrant mRNAs, NMD post-transcriptionally regulates the abundance of 5%–10% of naturally occurring transcripts that exhibit features recognized by the NMD machinery (Kervestin and Jacobson 2012).

In vertebrates, stop codons trigger efficient NMD when they are located at least 50–55 nucleotides (nt) upstream of an exon–exon boundary, which is marked by the exon junction complex (EJC) (Nagy and Maquat 1998; Le Hir et al. 2000). Alternatively, stop codons can trigger NMD when other features of the mRNA (e.g., long 3′ untranslated regions [UTRs]) may prevent efficient translation

termination by interfering with the interaction of the cytoplasmic poly(A)-binding protein (PABPC) with the eukaryotic release factors (eRFs; "faux 3′ UTR") (Amrani et al. 2004; Behm-Ansmant et al. 2007; Ivanov et al. 2008; Silva et al. 2008; Singh et al. 2008; Eberle et al. 2009). Aberrant translation termination at a PTC causes the assembly of a "surveillance complex" on the mRNA, which subsequently triggers mRNA degradation (Muhlemann and Lykke-Andersen 2010).

The surveillance complex consists of the evolutionarily conserved proteins UPF1, UPF2, and UPF3. Additional NMD factors include the kinase SMG1, which phosphorylates UPF1, and SMG5, SMG6, and SMG7, which trigger UPF1 dephosphorylation in metazoans (Kervestin and Jacobson 2012; Yamashita 2013).

Current models of NMD suggest that UPF1 and SMG1 are recruited by ribosomes prematurely terminating translation through interactions with eRF1 and eRF3 (Czaplinski et al. 1998; Kashima et al. 2006). UPF1 is phosphorylated by SMG1 when it interacts with UPF2 and/or UPF3, which

Loh et al.

are generally bound to downstream EJCs (Yamashita 2013). Phosphorylated UPF1 subsequently recruits SMG5, SMG6, and SMG7 to the mRNA (Anders et al. 2003; Chiu et al. 2003; Ohnishi et al. 2003; Okada-Katsuhata et al. 2012). SMG5, SMG6, and SMG7 are three related proteins that bind phosphorylated UPF1 through 14-3-3-like domains and trigger mRNA target degradation, UPF1 dephosphorylation, and the recycling of NMD factors to initiate new rounds of NMD (Ohnishi et al. 2003; Fukuhara et al. 2005; Franks et al. 2010; Okada-Katsuhata et al. 2012; Jonas et al. 2013).

The degradation of NMD targets is known to involve both endonucleolytic and exonucleolytic activities in vertebrates (Muhlemann and Lykke-Andersen 2010). Endonucleolytic degradation is catalyzed by SMG6, which cleaves the mRNA target in the vicinity of the PTC (Gatfield and Izaurralde 2004; Glavan et al. 2006; Huntzinger et al. 2008; Eberle et al. 2009). Exonucleolytic degradation is catalyzed by general mRNA decay factors that promote deadenylation followed by either 3′-to-5′ degradation or decapping and 5′-to-3′ decay (Chen and Shyu 2003; Lejeune et al. 2003; Couttet and Grange 2004; Yamashita et al. 2005). Moreover, deadenylation-independent decapping and 5′-to-3′ degradation also contribute to NMD (Chen and Shyu 2003; Lejeune et al. 2003; Couttet and Grange 2004).

Several lines of evidence indicate that decay enzymes are recruited to NMD targets via interactions with UPF1, SMG5, and/or SMG7. For example, human UPF1 interacts with the decapping enzyme DCP2 and the decapping activators DCP1 and PNRC2 (Lykke-Andersen 2002; Lejeune et al. 2003; Fenger-Grøn et al. 2005; Isken et al. 2008; Cho et al. 2009, 2013; Lai et al. 2012). Because PNRC2 binds directly to DCP1 and UPF1, it has been proposed to bridge the interaction between the surveillance and decapping complexes (Lai et al. 2012; Cho et al. 2013).

Additionally, both SMG5 and SMG7 induce the degradation of bound mRNA in tethering assays (Unterholzner and Izaurralde 2004; Cho et al. 2013). The degradative activity of SMG7 resides in its C-terminal proline-rich region (termed the PC region) and involves the decapping enzyme DCP2 and the 5′-to-3′ exonuclease XRN1 (Unterholzner and Izaurralde 2004). Although SMG5 heterodimerizes with SMG7, the degradative activity of SMG5 in tethering assays is independent of SMG7 and requires UPF1 and PNRC2 instead (Cho et al. 2013). This observation led to the hypothesis that SMG5 acts in NMD independently of SMG7 by interacting with PNRC2 and that the SMG5–PNRC2 interaction dominates over the SMG5–SMG7 interaction (Cho et al. 2013).

Despite the wealth of available information, key questions regarding NMD target degradation remain unanswered. In particular, the identity of the decay factors that are directly recruited to NMD targets remains unknown, along with whether this recruitment is mediated by UPF1, SMG5, and/or SMG7. Moreover, it remains unclear whether the interactions of UPF1 with DCP2 and DCP1 are direct or mediated by PNRC2 and to what extent these interactions contribute to NMD. Similarly, whether the SMG7 PC region interacts directly with decay factors and

the relevance of the PC region for NMD have not been addressed. Finally, little is known about the mechanism by which tethered SMG5 triggers mRNA degradation, and it remains unclear whether SMG5 functions in two alternative complexes containing either PNRC2 or SMG7.

To address these questions, we investigated the mechanism of NMD target degradation and the interplay between SMG5, SMG6, and SMG7 in human cells. Here, we show that the SMG7 PC region directly binds POP2, a catalytic subunit of the CCR4–NOT deadenylase complex, and thereby promotes deadenylation and subsequent decapping of NMD targets. The SMG7 PC region is required for NMD in cells depleted of SMG6, indicating that SMG7 and SMG6 act redundantly to promote NMD target degradation. Furthermore, our results do not confirm a role for the SMG5–PNRC2 interaction in NMD but rather indicate that SMG5–SMG7 heterodimerization is critical for NMD. Finally, we show that DCP2 and PNRC2 interact independently with UPF1, perhaps facilitating the decapping of NMD targets. Our data reveal that the surveillance complex has multiple and redundant activities to ensure robust target degradation: It induces endonucleolytic cleavage by SMG6, recruits decapping factors through UPF1, and promotes deadenylation-dependent decay through the direct recruitment of the CCR4–NOT complex by SMG7.

Results

### SMG5 requires interaction with SMG7 to function in NMD

In our previous work, we demonstrated that a SMG5 mutant that does not heterodimerize with SMG7 fails to rescue NMD in human cells depleted of endogenous SMG5, indicating that SMG5 requires interaction with SMG7 to act in NMD (Jonas et al. 2013). In contrast, Cho et al. (2013) reported that SMG5 can function with PNRC2 independently of SMG7. To investigate whether monomeric SMG5 plays a role in NMD, we generated additional SMG5 mutants to disrupt the interaction with SMG7. Based on the structure of the SMG5–SMG7 heterodimer (Jonas et al. 2013), we deleted a SMG5 loop between helices α4 and α5 (loop L4). A symmetrical loop in SMG7 is disordered in the structure of monomeric SMG7 and folds onto the SMG5 surface upon binding, contributing several residues to the heterodimer interface (Supplemental Fig. S1; Fukuhara et al. 2005; Jonas et al. 2013). Therefore, deletion of loop L4 (ΔL4) is predicted to disrupt the interaction between SMG5 and SMG7 without affecting the fold of the SMG5 N-terminal domain. We also combined this deletion with the previously described G120E mutation (Jonas et al. 2013). The SMG5 residue G120 is at the center of the interface with SMG7 and allows for a close packing of the two proteins in the heterodimer (Supplemental Fig. S1; Jonas et al. 2013).

V5-SBP (streptavidin-binding peptide)-tagged wild-type or mutant SMG5 proteins were expressed in human HEK293T cells and examined for their ability to interact with SMG7. Consistent with previous structural studies,

the mutations and deletions in SMG5 abolished binding to either endogenous or HA-tagged SMG7 (Fig. 1A,B). The mutations also abolished the interaction with endogenous and phosphorylated UPF1 (Fig. 1B), as previously reported (Jonas et al. 2013). However, the interaction with overexpressed UPF1 was not affected (Supplemental Fig. S2A,B), consistent with the observation that monomeric SMG5 interacts with UPF1 when the two proteins are overexpressed (~100-fold) in human cells (Ohnishi et al. 2003; Jonas et al. 2013).

Because the mutations strongly impair SMG5–SMG7 heterodimerization, we next examined the effect of these mutations in NMD using a complementation assay in human cells. Briefly, an shRNA targeting the ORF of the *smg5* mRNA was used to deplete endogenous SMG5 in cell lines constitutively expressing a well-characterized NMD reporter based on the β-globin gene (Thermann et al. 1998). Because SMG6 can partially compensate for the absence of SMG5 (Jonas et al. 2013; Metze et al. 2013), we depleted SMG6 in combination with SMG5. SMG5 and SMG6 codepletion resulted in a 12-fold increase in the level of β-globin PTC mRNA (Fig. 1C,D). This codepletion did not affect the expression of the wild-type β-globin reporter (Supplemental Fig. S2C). The SMG5 and SMG6 protein levels were reduced to ~25% of their control levels by the shRNA (Supplemental Fig. S2D,E), whereas
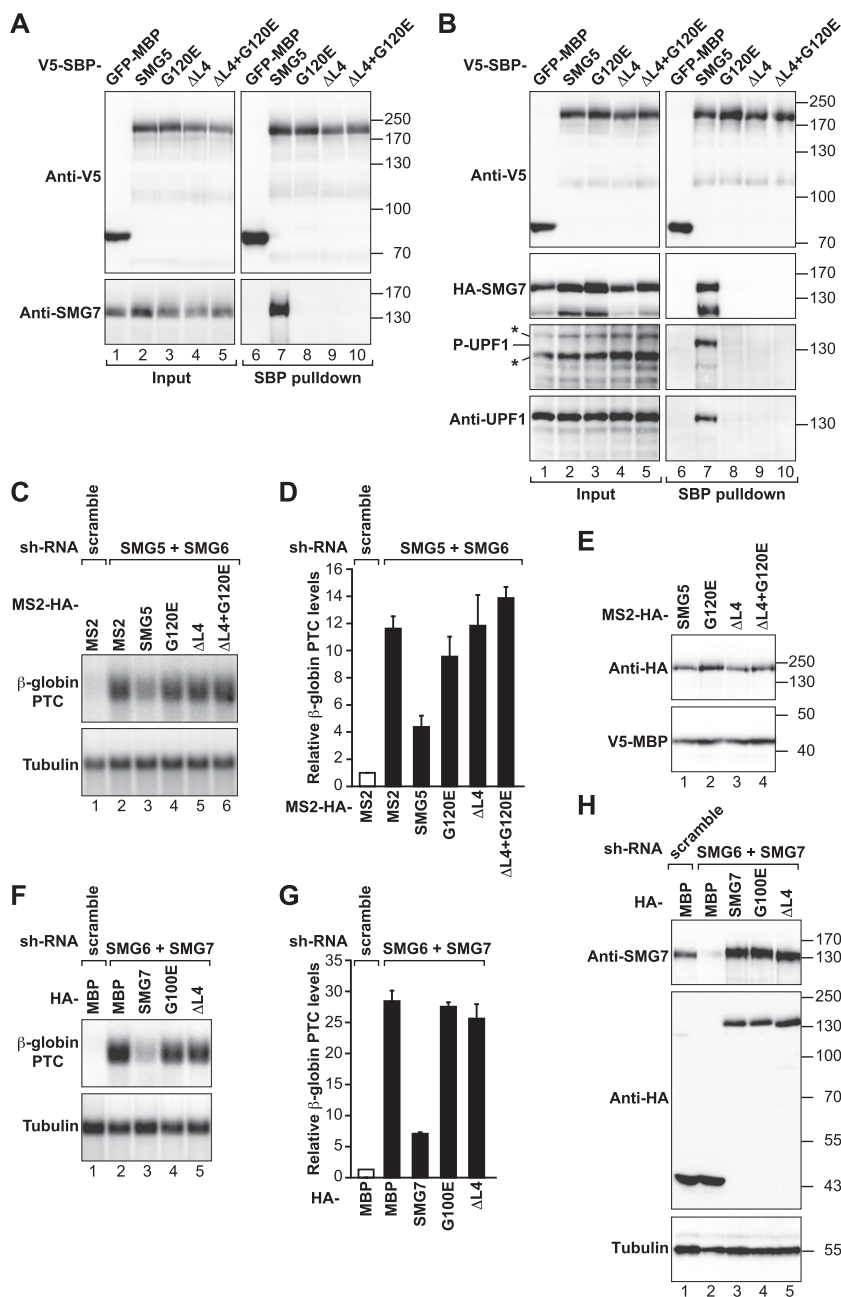
Figure 1. SMG5–SMG7 heterodimerization is required for NMD. (*A*) Interaction of SMG5-V5-SBP-MBP (wild type or mutants) with endogenous SMG7 in HEK293T cells. A V5-SBP-tagged GFP-MBP fusion served as a negative control. Inputs (1%) and bound fractions (5%) were analyzed by Western blotting. Samples were treated with RNase A before the pull-down. (*B*) Interaction of SMG5-V5-SBP-MBP (wild type or mutants) with endogenous and phosphorylated UPF1 (P-UPF1) and HA-SMG7 in cell lysates treated with RNase A. Inputs (1%) and bound fractions (2% for the SBP and HA-tagged proteins and 30% for UPF1) were analyzed by Western blotting. Asterisks indicate phosphorylated proteins (distinct from UPF1) recognized by the phospho-(Ser/Thr) ATM/ATR substrate antibody in input samples. The identity of phosphorylated UPF1 in the pull-down was confirmed by reprobing the membrane using anti-UPF1 antibodies. (*C–E*) HeLa cell lines expressing the β-globin NMD reporter (containing a PTC) were transfected with plasmids expressing the indicated shRNAs. Plasmids expressing shRNA-resistant versions of MS2-HA-SMG5 (wild type or mutants) were included in the transfection mixtures as indicated. MS2-HA served as a negative control. (*C*) The levels of the PTC-containing β-globin reporter were analyzed by Northern blotting, normalized to those of endogenous β-tubulin mRNA, and set to 1 in control cells (i.e., cells expressing MS2-HA and treated with a scrambled shRNA). *D* shows mean values ± standard deviations obtained in three independent experiments. (*E*) Expression of MS2-HA-SMG5 (wild type or mutants) in the complementation assay. V5-SBP-MBP served as a transfection control. (*F,G*) A complementation assay as described in *C* and *D* was performed in cells codepleted of SMG7 and SMG6 and transfected with plasmids expressing shRNA-resistant HA-SMG7 (wild type or mutants). (*H*) Expression of HA-SMG7 (wild type or mutants) in the complementation assay shown in *F* and *G*.

Loh et al.

the SMG7 levels remained unchanged (Supplemental Fig. S2F).

In codepleted cells, we then examined the ability of shRNA-resistant versions of SMG5 to rescue NMD. If SMG5 acts independently of SMG7, mutations that disrupt its interaction with SMG7 should not compromise its ability to rescue NMD. However, as shown in Figure 1, C and D, the aforementioned SMG5 mutants were either strongly impaired or inactive in rescuing NMD, whereas wild-type SMG5 did rescue NMD (although not completely because SMG6 is codepleted) (Fig. 1C,D). The levels of the wild-type reporter remained unchanged (Supplemental Fig. S2C). All SMG5 proteins were expressed at similar levels (Fig. 1E). These levels were approximately twofold higher than the levels of endogenous SMG5 in control cells (Supplemental Fig. S2G).

To further validate our conclusions, we generated the reciprocal mutations in SMG7. We observed that a SMG7 protein lacking loop L4 did not interact with SMG5 and failed to complement NMD in cells codepleted of SMG6 and SMG7 (Fig. 1F,G; Supplemental Fig. S3A). As a control, a previously described G100E substitution (corresponding to the SMG5 G120E mutant) abolished SMG5 binding and abrogated SMG7's ability to rescue NMD in complementation assays, as reported before (Fig. 1F,G; Supplemental Fig. S3A; Jonas et al. 2013). Importantly, deletion of loop L4 or the G100E substitution did not prevent SMG7 from interacting with UPF1 (Supplemental Fig. S3B), indicating that these mutations do not disrupt the protein fold. The SMG6 and SMG7 protein

levels were reduced to ~25% and 10% of their control levels in depleted cells (Supplemental Fig. S3C,D), whereas the SMG5 levels remained unchanged (Supplemental Fig. S3E). SMG7 wild-type and mutants were expressed at levels comparable with endogenous SMG7 (Fig. 1H). We conclude that SMG5 and SMG7 function as a complex in NMD.

SMG5 does not stably associate with PNRC2

The observation that SMG5 requires interaction with SMG7 to function in NMD contrasts with the suggestion that SMG5 functions with PNRC2 and independently of SMG7 (Cho et al. 2013). One possible explanation for these results is that the mutations in SMG5 that disrupt its binding to SMG7 also disrupt PNRC2 binding. Because the SMG5 residues involved in the interaction with PNRC2 have not been defined, we tested the interaction of wild-type or mutant SMG5 with PNRC2. In contrast to a previous study (Cho et al. 2013), we could not detect an interaction between V5-SBP-SMG5 and HA-PNRC2 in SBP pull-down assays (data not shown).

To clarify this discrepancy, we next tested the interaction of PNRC2 with endogenous SMG5, SMG7, and UPF1. V5-SBP-tagged PNRC2 did not interact with endogenous SMG5 or SMG7 at detectable levels (Fig. 2A, lane 4). Under the same conditions, PNRC2 exhibited a weak (above background) association with endogenous UPF1 and efficiently pulled down HA-DCP1, which was included as a positive control (Figure 2A, lane 4; Lai et al.
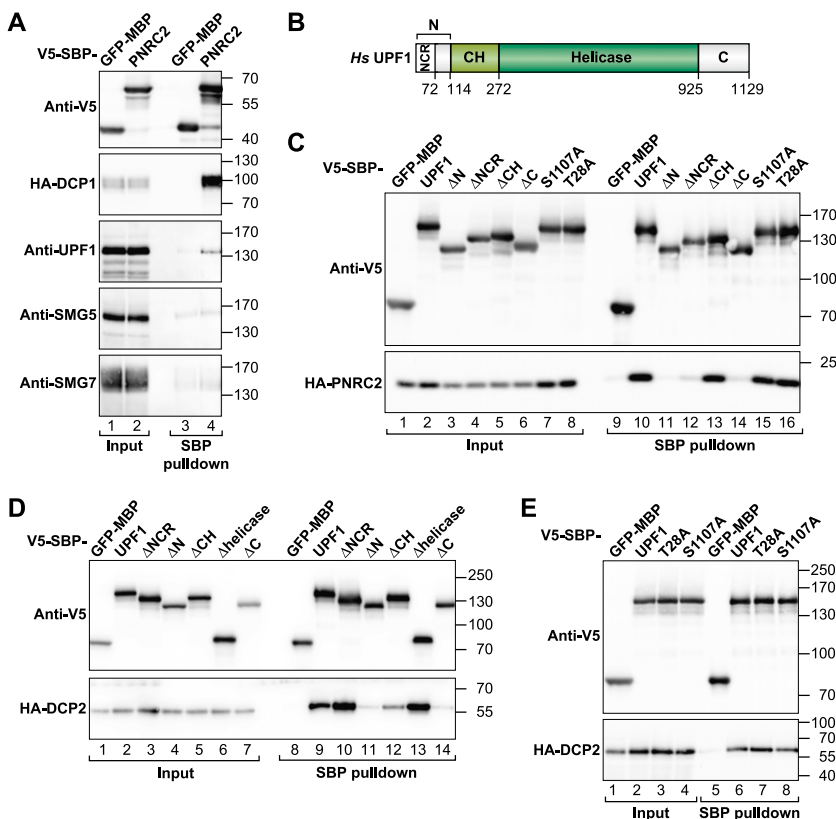
Figure 2. UPF1 interacts with decapping factors. (A) V5-SBP-MBP-tagged PNRC2 does not interact with endogenous SMG5 or SMG7 but binds HA-DCP1 and weakly interacts with endogenous UPF1. V5-SBP-MBP served as a negative control. Inputs (1%) and bound fractions (8% for V5-tagged proteins, 15% for HA-tagged proteins, and 35% for UPF1, SMG5, and SMG7) were analyzed by Western blotting. (B) The domain architecture of UPF1. (NCR) N-terminal conserved region; (N) N-terminal tail; (C) C-terminal tail. The globular cysteine–histidine (CH) and helicase domains are indicated. (C–E) Interaction of V5-SBP-UPF1 (wild type or mutants) with HA-PNRC2 (C) and HA-DCP2 (D,E). V5-SBP-GFP-MBP served as a negative control.

2012). Note that these pull-downs were performed in the presence of okadaic acid, which inhibits UPF1 dephosphorylation and has been reported to enhance its binding to PNRC2 and DCP1 (Cho et al. 2009; Lai et al. 2012). From these results, we conclude that SMG5 may interact with PNRC2 only transiently or indirectly, possibly through UPF1.

UPF1 interacts with DCP2 in a PNRC2-independent manner

UPF1 interacts with PNRC2 through its N-terminal and C-terminal unstructured tails (Cho et al. 2009). UPF1 also interacts with DCP2 and DCP1 (Lykke-Andersen 2002; Fenger-Grøn et al. 2005; Isken et al. 2008), but the domains involved in these interactions have not been defined, and it remains unclear whether these interactions are mediated by PNRC2. Therefore, we tested which domains of UPF1 are required for DCP2 binding.

The UPF1 protein consists of an N-terminal cysteine–histidine (CH) regulatory domain, which provides a binding site for UPF2, and a central helicase domain (Fig. 2B; Cheng et al. 2007; Clerici et al. 2009). These structured domains of UPF1 are flanked by N-terminal and C-terminal tails (~100 residues) containing several serine/threonine–glutamine (S/T-Q) motifs that are phosphorylated by SMG1 and that serve as binding sites for SMG6 and the SMG5–SMG7 complex (Ohnishi et al. 2003; Okada-Katsuhata et al. 2011). SMG6 and SMG7 have been shown to bind phosphorylated T28 and S1096 (corresponding to S1107 in UPF1 isoform 1), respectively (Okada-Katsuhata et al. 2012).

We observed that both the N-terminal and C-terminal tails of UPF1 are required for PNRC2 binding (Fig. 2C, ΔN and ΔC), in agreement with Cho et al. (2009). Interestingly, deletion of the N-terminal conserved region (ΔNCR) (Ohnishi et al. 2003) abrogated PNRC2 binding (Fig. 2C, lane 12). In contrast, the CH and helicase domains were dispensable (Fig. 2C; data not shown). Additionally, alanine substitutions of residues T28 and S1107 did not prevent PNRC2 binding (Fig. 2C, lanes 15,16), suggesting that UPF1 interacts with PNRC2 independently of SMG6 and SMG7.

Next, we tested the aforementioned UPF1 deletion mutants for their ability to interact with DCP2. Similar to the results obtained with PNRC2, deletion of the UPF1 N-terminal or C-terminal tails abolished DCP2 binding (Fig. 2D, lanes 11,14). Importantly, deletion of the NCR region was ineffectual despite the fact that this deletion abrogated PNRC2 binding (Fig. 2, C vs. D). This result indicates that DCP2 interacts with UPF1 independently of PNRC2. Additionally, deletion of the helicase domain as well as the T28A and S1107A mutations was ineffectual (Figs. 2D,E). We conclude that UPF1 provides multiple and independent binding sites for decapping factors.

SMG7 but not SMG5 triggers degradation of bound mRNAs independently of additional NMD factors

Using an MS2-based tethering system, Cho et al. (2013) reported that SMG5 is active in tethering assays and that

this activity requires UPF1 and PNRC2 but is independent of SMG7. In contrast, using a λN-based reporter assay, we observed that tethered SMG5 only promotes mRNA decay when it is coexpressed with SMG7 (Unterholzner and Izaurralde 2004; Jonas et al. 2013). To address this apparent discrepancy, we compared the activity of tethered SMG5 and SMG7 using the MS2 tethering system (Lykke Andersen et al. 2000).

Tethered MS2-SMG5 promoted the degradation of a β-globin reporter containing six copies of the high-affinity binding site for the MS2 viral coat protein in its 3′ UTR (6xMS2bs) (Fig. 3A–C), as reported by Cho et al. (2013). SMG5 activity was partially dependent on SMG7 because the aforementioned mutations that prevent binding to SMG7 reduced, but did not abolish, SMG5 activity in tethering assays, although the mutant proteins were expressed at levels similar to wild-type levels (Fig. 3A–C). The residual activity of the SMG5 mutants is most likely due to their interactions with UPF1 because UPF1 depletion suppresses the activity of tethered MS2-SMG5 (Cho et al. 2013). The reason for the partial different results obtained with the MS2 and λN reporters was not further investigated.

SMG7 consists of an N-terminal 14-3-3-like domain, a middle α-helical domain, and a PC region (Fig 3D). Using the MS2 system, we observed that the SMG7 PC region is both necessary and sufficient to promote the degradation of the mRNA reporter (Fig. 3E–G). In contrast, the SMG7 N-terminal region (denoted ΔPC mutant) was inactive, consistent with our previous studies (Unterholzner and Izaurralde 2004; Jonas et al. 2013). Thus, although the SMG7 N-terminal region interacts with both SMG5 and UPF1, this region does not lead to mRNA degradation in isolation. In contrast, the PC region, which does not interact with SMG5 or UPF1 (Anders et al. 2003; Jonas et al. 2013), is sufficient to cause degradation. Our results, together with the observation that SMG7 activity in tethering assays is not affected by UPF1 or PNRC2 depletion (Cho et al. 2013), indicate that SMG7 causes mRNA degradation independently of UPF1, SMG5, and PNRC2. We conclude that SMG7 recruits decay enzymes independently of any other NMD factor, whereas SMG5 degradative activity depends on SMG7 and UPF1.

The SMG7 PC region promotes deadenylation-dependent decapping

SMG7-mediated degradation is inhibited in cells depleted of the decapping enzyme DCP2 or the 5′-to-3′ exonuclease XRN1 (Unterholzner and Izaurralde 2004). However, it remains unknown whether decapping is dependent on prior deadenylation and whether SMG5 uses a similar mechanism to degrade bound mRNAs. If deadenylation precedes decapping and 5′-to-3′ mRNA degradation, then deadenylated mRNA decay intermediates are expected to accumulate in cells in which decapping is inhibited. Consistent with this expectation, degradation of the β-globin-6xMS2bs reporter by full-length SMG7 or the PC fragment was inhibited in cells overexpressing a

Loh et al.
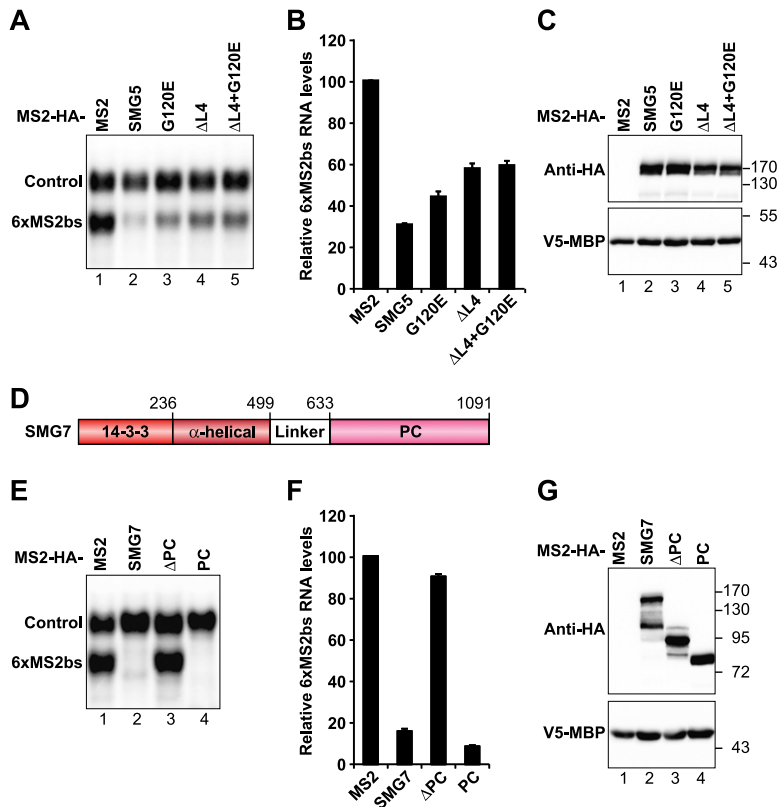
Figure 3. SMG5 and SMG7 degrade bound mRNAs. (A–C,E–G) Human HEK293T cells were transfected with a mixture of three plasmids: one expressing β-globin-6xMS2bs mRNA, another expressing MS2-HA or the indicated MS2-HA-tagged proteins, and a third expressing a transfection control containing the β-globin gene fused to the GAPDH 3′ UTR but lacking MS2-binding sites (β-globin-GAP; Control). β-Globin-6xMS2bs mRNA levels were normalized to those of the control mRNA. The normalized values of the β-globin-6xMS2bs mRNA levels were set to 100 in the presence of MS2-HA. Mean values ± standard deviations from three independent experiments are shown in B and F. A and E show Northern blots of representative RNA samples. (C,G) Western blot analysis showing equivalent expression of the MS2-HA-tagged proteins used in the corresponding tethering assays. (D) The domain architecture of SMG7 consists of an N-terminal 14-3-3-like domain, a middle α-helical domain, and a C-terminal PC region.

catalytically inactive DCP2 mutant (DCP2 Mut; E148Q) (Fig. 4A–C). The reporter accumulated in a fast-migrating form, corresponding to the deadenylated decay intermediate (A_0). Indeed, the mobility of the fast-migrating form did not change after oligo(dT)-directed RNase H cleavage (Fig. 4D). Thus, the PC region of SMG7 elicits mRNA decay by triggering deadenylation and then decapping.

Notably, although overexpression of the DCP2 inactive mutant also inhibited SMG5-mediated decay in tethering assays, the reporter accumulated in the polyadenylated form (Fig. 4E,F). These results indicate that tethered SMG5 triggers decay by a mechanism other than SMG7, as SMG5-mediated decay involves decapping in the absence of deadenylation.

In summary, our results confirm that the SMG5 activity in tethering assays can be independent of SMG7, although in complementation assays, SMG5 strictly depends on SMG7. The simplest hypothesis that explains these observations is that in complementation assays, SMG5 requires interaction with SMG7 to be recruited to NMD targets, a step that is bypassed in tethering assays (see the Discussion).

The SMG7 PC region interacts with POP2

Having established that the PC region of SMG7 promotes deadenylation-dependent decapping, we next sought to identify potential interaction partners. To achieve this, we performed tandem affinity purification (TAP) (Rigaut et al. 1999) in human HEK293T cells using a TAP tag

consisting of protein A, a PreScission protease cleavage site, and the SBP. Remarkably, the TAP-tagged SMG7 PC region copurified with the entire CCR4–NOT deadenylase complex, which consists of 10 subunits (Supplemental Table S1). No other decay factors were identified as potential binding partners (Supplemental Table S1).

To validate the interaction between SMG7 and the CCR4–NOT complex and identify the subunits of this complex that may interact directly with the SMG7 PC region, we expressed the individual subunits together with SBP-tagged SMG7 in HEK293T cells and performed pull-down assays. The core of the human CCR4–NOT complex consists of six subunits (CNOT1, CNOT2, CNOT3, CNOT9, CNOT10, and CNOT11) and two catalytically active subunits (CCR4 and CAF1). In human cells, there are two alternative paralogs for each catalytic subunit: CCR4a or CCR4b (also known as CNOT6 or CNOT6-L) and CAF1 or POP2 (also known as CNOT7 or CNOT8) (Lau et al. 2009).

Remarkably, only POP2 (CNOT8) interacted with SMG7 in pull-down assays (Fig. 5A; Supplemental Fig. S4). This result is in contrast to the TAP tag selection, in which the endogenous CCR4–NOT complex associates with SMG7. The simplest explanation for these results is that in SBP pull-down assays, the isolated subunits are overexpressed and may not be efficiently incorporated into endogenous complexes. Accordingly, SMG7 did not interact with the human-specific subunit TAB182 (Supplemental Fig. S4; Lau et al. 2009). These results indicate that the SBP pull-down assays using transiently expressed proteins detected
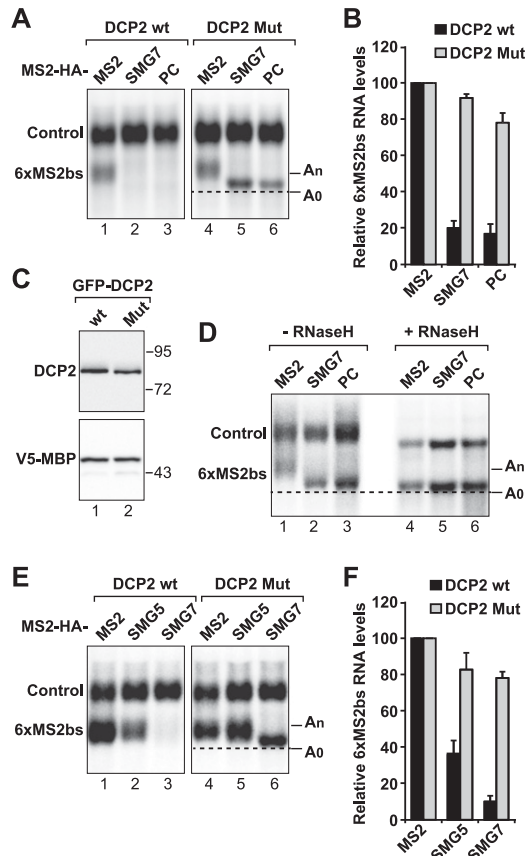
Figure 4. SMG5 and SMG7 use distinct mechanisms to degrade bound mRNA in tethering assays. (*A–F*) Tethering assays using the β-globin-6xMS2bs reporter were performed as described in Figure 3, with the exception that plasmids expressing wild-type DCP2 or a catalytically inactive mutant (Mut; E148Q) were included in the transfection mixtures as indicated. *A* and *E* show Northern blots of representative RNA samples. β-Globin-6xMS2bs mRNA levels were normalized to those of the control β-globin-GAP mRNA. These normalized values were set to 100 in the presence of MS2-HA. Mean values ± standard deviations from three independent experiments are shown in *B* and *F*. Black and gray bars show the values obtained in cells expressing DCP2 wild type (wt) and mutants (Mut), respectively. (*C*) Western blot analysis showing equivalent expression of the DCP2 proteins. (*D*) Samples corresponding to lanes *4–6* of *A* were treated with RNase H in the presence of oligo(dT).

binary interactions, although the SBP-tagged SMG7 was expressed at levels comparable with endogenous SMG7 (Supplemental Fig. S5A). Furthermore, SBP-tagged SMG7 did not interact with the subunits of the PAN2–PAN3 deadenylase complex (Supplemental Fig. S4) or with PNRC2 (Fig. 2A; Supplemental Table S1), underscoring the specificity of the interaction with POP2. Importantly, the interaction of SMG7 with POP2 was insensitive to RNase A treatment, suggesting that this interaction is not mediated by RNA (Fig. 5A).

To further validate the SMG7–POP2 interaction, we pulled down SMG7 and identified endogenous CAF1/POP2 in the bound fractions using an antibody that does not discriminate between POP2 and CAF1 (Fig. 5B).

Collectively, these results indicate that SMG7 associates with the CCR4–NOT complex through interactions with POP2.
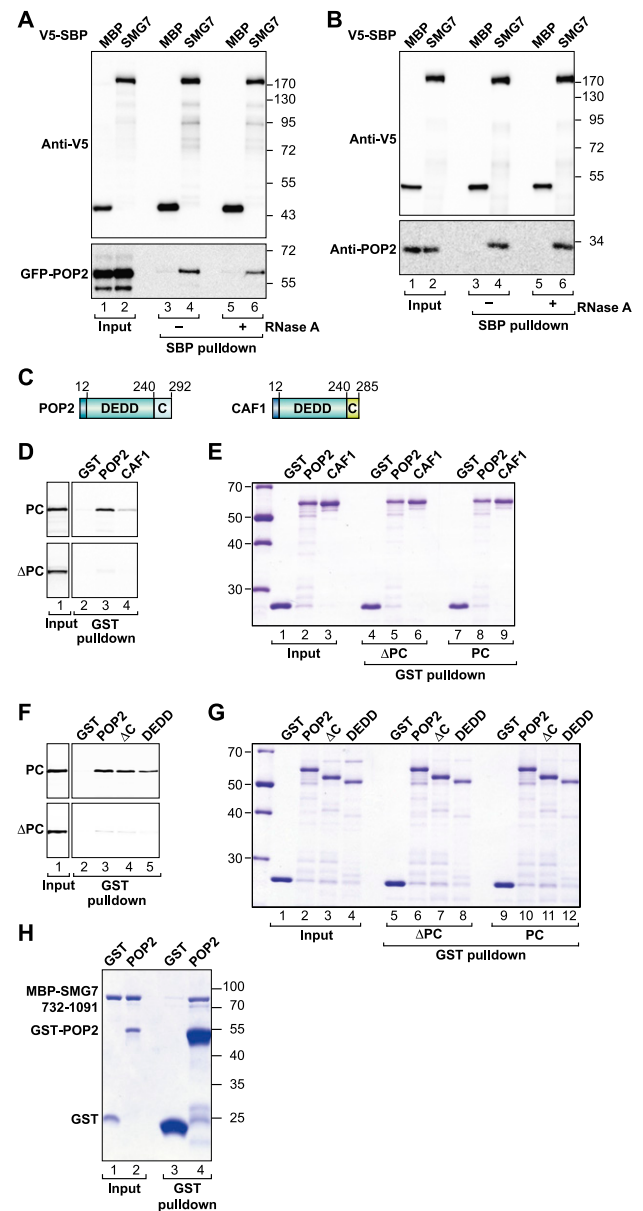
Figure 5. SMG7 interacts directly with POP2. (*A,B*) Interaction of SMG7-V5-SBP-MBP with GFP-POP2 (*A*) or endogenous POP2/CAF1 (*B*) in the absence or presence of RNase A. A V5-SBP-MBP served as a negative control. (*C*) The domain architectures of POP2 and CAF1. POP2 and CAF1 contain a central catalytic domain of the DEDD family exhibiting 84% identity. The C-terminal extensions are more divergent in sequence and length. (*D–G*) GST-tagged CAF1 or POP2 (wild type or mutants) or GST was used to pull down [^{35}S]-methionine-labeled in vitro translated SMG7 PC and ΔPC fragments. (*D,F*) Inputs (1%) and bound fractions (16%) were analyzed by 10% SDS-PAGE followed by fluorography. The corresponding Coomassie-stained gels are shown in *E* and *G*. (*H*) GST, GST-POP2, and MBP-SMG7 were expressed in *E. coli* and purified. The purified proteins were mixed in a 1:1 ratio (inputs), pulled down using glutathione agarose beads, and analyzed by SDS-PAGE followed by Coomassie staining.

Loh et al.

SMG7 discriminates between the catalytic domains of POP2 and CAF1

POP2 and CAF1 are one-domain proteins that adopt an RNase D-like fold (Daugeron et al. 2001) and exhibit 74% overall identity with each other (Fig. 5C). The main divergence between these two proteins is in their short C-terminal extensions. Given the high identity between these paralogs, it is surprising that SMG7 interacts with POP2 but not with CAF1. To investigate the basis for this selectivity, we performed GST (glutathione S transferase) pull-down assays with recombinant POP2 and CAF1 proteins expressed in *Escherichia coli* as GST fusions. The SMG7 PC fragment was translated in vitro in wheat germ extracts to minimize potential indirect interactions mediated by other proteins. We observed that the PC fragment preferentially interacted with GST-POP2 and exhibited a much weaker interaction with GST-CAF1 (Fig. 5D,E). The interaction was specific because the PC fragment was not pulled down with GST alone (Fig. 5D,E). Furthermore, a SMG7 mutant lacking the PC region (ΔPC) did not interact with any of the proteins tested at detectable levels (Fig. 5D,E).

We next investigated whether binding to the SMG7 PC region required the POP2 C-terminal extension, which is the sequence exhibiting the greatest divergence between POP2 and CAF1. Unexpectedly, the PC region interacted with the POP2 catalytic domain and did not require N-terminal or C-terminal extensions (Fig. 5F,G). We conclude that SMG7 has an exquisite preference for the catalytic domain of POP2 despite the fact that this domain is 84% identical to the equivalent domain in CAF1.

The SMG7 PC region interacts directly with POP2

To investigate whether the POP2–SMG7 interaction is direct, we expressed and purified the SMG7 PC fragment in *E. coli* fused to maltose-binding protein (MBP). GST-POP2 pulled down MBP-SMG7-PC, demonstrating that the interaction is indeed direct (Fig. 5H, lane 4).

The catalytic domains of CAF1/POP2 interact with CNOT1, CCR4, and the anti-proliferative protein TOB simultaneously, with nonoverlapping binding surfaces. Structural studies identified critical surface residues that disrupt CAF1/POP2 binding to CNOT1, CCR4, and TOB when mutated (Supplemental Table S2). Therefore, we tested these mutants for binding to SMG7. We observed that POP2 mutants that do not bind CNOT1, CCR4a,b, or TOB still interacted with SMG7 (Supplemental Fig. S5B,C). Together, these results indicate that the binding surface of SMG7 on POP2 does not overlap with the previously characterized binding surfaces for CNOT1, CCR4a,b, or TOB. Accordingly, SMG7 copurifies with the entire CCR4–NOT complex and thus does not compete with CNOT1 and CCR4 for binding to POP2.

A POP2 catalytically inactive mutant suppresses SMG7-mediated mRNA decay and NMD

To further establish the role of POP2 in SMG7-mediated decay, we used a tethering assay and overexpressed a cata-

lytically inactive POP2 mutant (POP2 Mut; D40A,E42A). The POP2 mutant inhibited the degradation of the reporter by SMG7 or the PC region (Fig. 6A–C). In contrast to the results obtained with the DCP2 mutant (Fig. 4A), the POP2 mutant led to the accumulation of the mRNA reporter in the polyadenylated form, as expected (Fig. 6A).
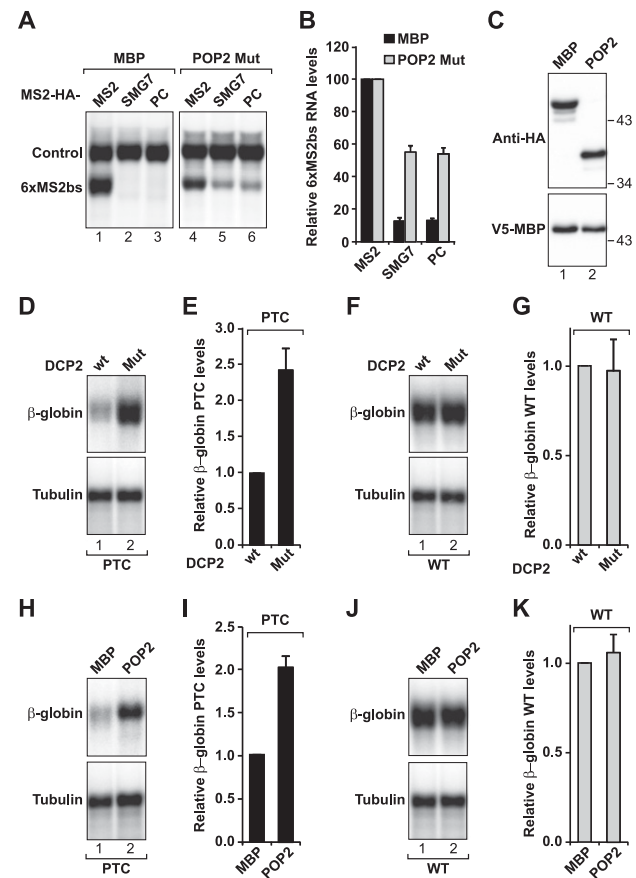
Figure 6. A POP2 catalytically inactive mutant inhibits SMG7-mediated decay and NMD in a dominant-negative manner. (*A–C*) Tethering assays using the β-globin-6xMS2bs reporter were performed as described in Figure 3, with the exception that plasmids expressing HA-MBP or a HA-POP2 catalytically inactive mutant (Mut; D40A,E42A) were included in the transfection mixtures as indicated. *A* presents a Northern blot of representative RNA samples. The β-globin-6xMS2bs mRNA levels were normalized to those of the control β-globin-GAP mRNA. These normalized values were set to 100 in the presence of MS2-HA. Mean values ± standard deviations of three independent experiments are shown in *B*. Black and gray bars represent the values obtained in cells expressing MBP and the POP2 mutant, respectively. (*C*) Western blot analysis demonstrating equivalent expression of the transfected proteins. (*D–K*) HeLa cells constitutively expressing a wild-type (WT) or PTC-containing β-globin reporter were transfected with plasmids expressing DCP2 (wild type or mutant) (*D–G*) or MBP and POP2 mutant (*H–K*). *D, F, H,* and *J* show Northern blots of representative RNA samples. The levels of wild-type or PTC-containing β-globin reporters were normalized to those of β-tubulin mRNA and set to 1 in cells expressing DCP2 wild type (*E,G*) or MBP (*I,K*). Mean values ± standard deviations of three independent experiments are shown in *E, G, I,* and *K*.

To investigate the contribution of the 5′-to-3′ mRNA decay pathway and POP2 to NMD target degradation, we tested whether the catalytically inactive DCP2 and POP2 mutants suppressed NMD. We observed that the levels of the PTC-containing reporter increased 2.5-fold and two-fold in cells overexpressing DCP2 and POP2 mutants, respectively (Fig. 6D–K). In the presence of the DCP2 mutant, the reporter migrated as a broad band (Fig. 6D, lane 2), suggesting that a fraction of the mRNA is de-adenylated. In contrast, in cells expressing the POP2 mutant, it migrated as a sharp band (Fig. 6H, lane 2), suggesting that it is fully polyadenylated in this case. The levels of the wild-type reporter did not change (Fig. 6F,G,J,K). The twofold increase observed in PTC mRNA levels when deadenylation or decapping is blocked is in the same range as the effects observed in cells depleted of SMG5 or SMG7 (see Fig. 7; Jonas et al. 2013).

The SMG7 PC region functions redundantly with SMG6

To investigate the functional relevance of the SMG7 PC region for NMD target degradation, we performed com-

plementation assays, as described above. First, cells were depleted of endogenous SMG7 and reconstituted with shRNA-resistant versions of wild-type SMG7 or the SMG7-ΔPC mutant. SMG7 depletion caused a 2.5-fold increase in the PTC-containing β-globin reporter levels (Fig. 7A,B). Reintroduction of SMG7 restored NMD (Fig. 7A,B). Unexpectedly, we found that the SMG7-ΔPC mutant was also fully competent in restoring NMD (Fig. 7A,B). The expression levels of the corresponding wild-type β-globin mRNA were unaltered (Fig. 7C,D). Endogenous SMG7 protein levels were reduced below 12% by the shRNA expression (Fig. 7E). The shRNA-resistant versions of SMG7 wild-type and ΔPC were expressed at similar levels (Fig. 7F, lanes 2,3); these levels were comparable with the expression of endogenous SMG7 in control cells (Supplemental Fig. S5D). We conclude that the PC region of SMG7 is not required for the degradation of the β-globin PTC mRNA.

Because SMG6 can compensate, at least in part, for the lack of SMG7, we next repeated the complementation assay in cells codepleted of SMG6 and SMG7 (Fig. 7G). We observed that the codepletion of both proteins resulted
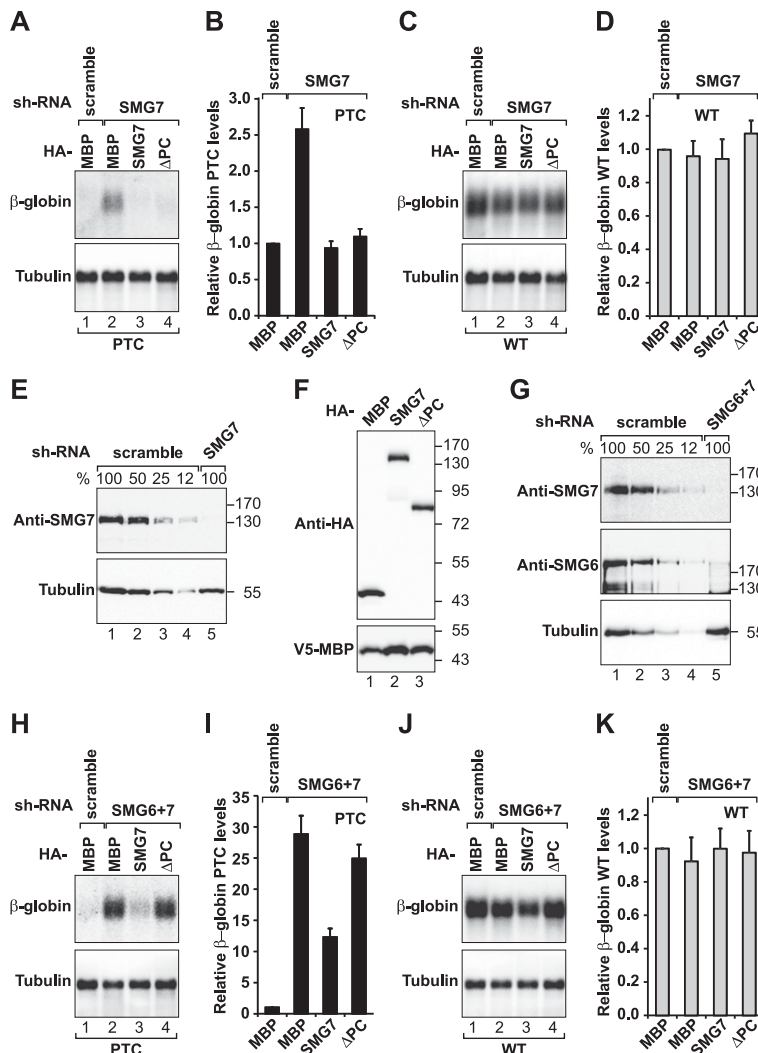
Figure 7. The SMG7 PC region is required for NMD in the absence of SMG6. (A–F) HeLa cell lines expressing the β-globin reporter (wild type [WT] or with PTC) were transfected with plasmids expressing the indicated shRNAs and shRNA-resistant forms of SMG7 full-length or ΔPC. (A,C) The levels of the PTC-containing or wild-type β-globin reporters were analyzed by Northern blot, normalized to those of endogenous β-tubulin mRNA, and set to 1 in control cells (i.e., cells treated with a scrambled shRNA and expressing HA-MBP). B and D show the mean values ± standard deviations obtained in three independent experiments. (E) Western blot showing the efficiency of SMG7 depletion. (F) Expression levels of transiently expressed HA-tagged SMG7 proteins. (G–K) A complementation assay with plasmids expressing an shRNA-resistant version of HA-SMG7 (wild type or ΔPC) was performed in cells codepleted of SMG6 and SMG7. (G) Western blot showing the efficiency of SMG6 and SMG7 codepletion. Northern blot analyses of representative RNA samples are shown in H and J. The relative levels of PTC-containing or wild-type β-globin reporters in three independent experiments are shown in I and K.

in a synergistic inhibition of NMD, leading to a 30-fold increase in the β-globin PTC reporter levels, as previously reported (Luke et al. 2007; Jonas et al. 2013; Metze et al. 2013). This effect was prevented by the expression of a shRNA-resistant version of SMG7, which partially restored NMD (Fig. 7H,I). In contrast, the ability of the ΔPC mutant to rescue NMD was strongly impaired, indicating that the PC region of SMG7 is required for NMD in the absence of the SMG6 endonuclease. The expression levels of the wild-type β-globin mRNA remain unchanged (Fig. 7J,K).

Discussion

NMD targets are degraded by multiple mechanisms in vertebrates. These mechanisms include endonucleolytic cleavage by SMG6, deadenylation-dependent decapping, accelerated decapping of partially deadenylated mRNAs, and exosome-mediated degradation (Muhlemann and Lykke-Andersen 2010). Despite this wealth of information, the direct physical interactions that link the surveillance complex to the general mRNA decay machinery have not been described. In this study, we demonstrate that SMG7 directly recruits the CCR4–NOT deadenylase complex to NMD targets and promotes deadenylation-dependent decapping. We further show that this recruitment is mediated by direct interactions between the PC region of SMG7 and POP2, a catalytic subunit of the CCR4–NOT complex, the major cytoplasmic deadenylase complex in eukaryotes. The ability of SMG7 to discriminate between POP2 and CAF1, which are mutually exclusive subunits of the CCR4–NOT complex, provides an unprecedented example for how substrate specificity for alternative CCR4–NOT complexes can be achieved.

SMG5 requires heterodimerization with SMG7 to function in NMD

SMG5 and SMG7 heterodimerize through interactions mediated by their 14-3-3-like domains. Several lines of evidence indicate that they function as a complex in NMD (Anders et al. 2003; Ohnishi et al. 2003; Okada-Katsuhata et al. 2012; Jonas et al. 2013). First, they form highly stable heterodimers in vivo and in vitro (Jonas et al. 2013). Second, SMG5 or SMG7 mutants that do not interact with one another do not rescue NMD in cells depleted of SMG5 or SMG7, respectively (Fig. 1; Jonas et al. 2013). Furthermore, codepletion of SMG5 and SMG7 does not exacerbate the effects of the individual depletions, suggesting an epistatic interaction. In contrast, codepletion of SMG6 with either SMG5 or SMG7 results in a synergistic inhibition of NMD (Luke et al. 2007; Jonas et al. 2013; Metze et al. 2013), suggesting that the SMG5–SMG7 complex acts redundantly with SMG6 to promote the degradation of NMD targets.

In contrast to these results, which were obtained in complementation assays using a genuine NMD target, SMG5 mutants that do not interact with SMG7 can nevertheless trigger mRNA decay in tethering assays,

although less efficiently than wild-type SMG5 (Fig. 1). SMG5 activity in tethering assays is suppressed by PNRC2 or UPF1 depletions (Cho et al. 2013). These observations were interpreted as evidence that SMG5 acts in NMD independently of SMG7 through its interaction with PNRC2 and that the SMG5–PNRC2 interaction is dominant over the SMG5–SMG7 interaction (Cho et al. 2013). Our study challenges this view and shows that although MS2-based tethering assays bypass the requirement for SMG5–SMG7 interaction, in complementation assays, SMG5 requires interaction with SMG7 to function in NMD.

Although we cannot rule out the possibility that SMG5 acts independently of SMG7 under some circumstances or for some specific targets, we found that SMG5 does not form a stable complex with PNRC2. Thus, the observation that PNRC2 depletion suppresses SMG5-mediated degradation in tethering assays could simply be explained as a general inhibition of decapping without invoking the formation of a complex with SMG5. Similarly, a catalytically inactive DCP2 mutant prevents SMG5-mediated decay (Fig. 4) without directly interacting with SMG5. In other words, it appears possible that PNRC2 is a decapping factor rather than a bona fide NMD factor. Remarkably, *Drosophila melanogaster* lacks both SMG7 (Gatfield et al. 2003) and PNRC2, raising the question of whether alternative SMG5 partners are present in this organism.

How does SMG5 trigger decay in tethering assays? The activity of SMG5 depends on UPF1 (Cho et al. 2013), which in turn interacts with decapping factors, suggesting that mRNA degradation is caused by UPF1. The dependence of SMG5 on UPF1 in tethering assays contrasts with the sequential recruitment of NMD factors to bona fide NMD targets in which UPF1 acts upstream of SMG5 and suggests that tethering assays bypass this sequential assembly and may not faithfully recapitulate NMD. Thus, although tethering assays are a powerful tool for studying the activity of proteins in isolation, they are bypassing important recruitment and assembly steps. Therefore, conclusions drawn from these assays require additional insight and validation in complementation assays.

Role of decapping in NMD

Decapping is normally coupled to deadenylation and may occur by default as a consequence of this coupling. However, the observation that UPF1 interacts with decapping factors suggests that these factors could be recruited to NMD targets independently of deadenylation. Indeed, UPF1 interacts with DCP1, DCP2, and PNRC2, and these interactions are enhanced by UPF1 phosphorylation (Fig. 2; Lykke-Andersen 2002; Lejeune et al. 2003; Fenger-Grøn et al. 2005; Isken et al. 2008; Cho et al. 2009; Lai et al. 2012). While PNRC2 was proposed to bridge the interactions between UPF1 and the decapping complex, in this study, we show that DCP2 interacts with UPF1 independently of PNRC2. Notably, UPF1 also interacts with DCP2 and the decapping factors Edc3 and Pat1 in yeast

(He and Jacobson 2001; Swisher and Parker 2011) despite the fact that yeast lacks PNRC2.

How do the multiple and potentially redundant interactions of UPF1 with decapping factors contribute to the degradation of NMD targets? UPF1 may simply increase the local concentration of decapping factors on NMD targets, facilitating their degradation, and contribute to the accelerated decapping observed for bona fide NMD targets (Supplemental Fig. S6; Couttet and Grange 2004). Nevertheless, decapping factors could also be indirectly recruited to NMD targets by SMG7 through the CCR4–NOT complex.

An important challenge for future studies is the elucidation of the molecular basis for the interaction of UPF1 with decapping factors and its regulation by phosphorylation. One particularly intriguing observation is that both the N-terminal and C-terminal unstructured tails are required for PNRC2 and DCP2 binding (Fig. 2; Cho et al. 2009; Lai et al. 2012). One possible explanation is that in the context of full-length UPF1, these extensions are in close proximity and cooperate to form a common binding site. For example, the UPF1 C-terminal tail interacts with the helicase domain (Fiorini et al. 2013). However, the observation that deletion of the helicase domain is not detrimental for decapping factor binding (Fig. 2) suggests that a predefined three-dimensional conformation of these extensions is not required for binding. Alternatively, the N-terminal and C-terminal tails may provide low-affinity binding sites for decapping factors, in which case both will be required for full binding affinity because of additive/avidity effects.

In yeast, decapping plays a major role in the degradation of NMD targets, which are rapidly decapped without any requirement for prior deadenylation (Muhlrad and Parker 1994; Bond et al. 2001; He et al. 2003). A minor pathway involving accelerated deadenylation followed by exosome-mediated 3′-to-5′ decay is also observed, particularly when decapping is impaired (Cao and Parker 2003; Mitchell and Tollervey 2003; Takahashi et al. 2003). Thus, it appears that the basic mechanisms of NMD target degradation have been maintained throughout evolution. However, in metazoans, additional mechanisms for recruiting decay factors and degrading mRNA targets have been appended through the acquisition of SMG6, a specific NMD endonuclease, and the SMG5–SMG7 complex, which directly interacts with the CCR4–NOT complex (Supplemental Fig. S6). As a consequence, tighter regulation of mRNA target degradation is possible in these organisms. Indeed, SMG6 and the SMG5–SMG7 complex are only recruited to NMD targets upon UPF1 phosphorylation, enabling the sequential assembly of the surveillance complex and step-wise recruitment of decay factors. In this context, it is important to note that UPF1 also associates with mRNAs that are not destined for degradation by NMD (Hogg and Goff 2010; Hurt et al. 2013; Kurosaki and Maquat 2013; Zünd et al. 2013), and thus a tight regulation of UPF1 phosphorylation and the subsequent recruitment of decay factors are required to prevent unscheduled degradation. In contrast, in yeast, UPF1 is not phosphorylated and is thought to recruit decay factors to the target mRNA (He and Jacobson 2001; Swisher and Parker 2011). Thus, serendipitous UPF1 binding might lead to undesired degradation of normal mRNAs in yeast, which is unlikely to occur in metazoan.

In summary, together with previously published data (Muhlemann and Lykke-Andersen 2010), our results indicate that the surveillance complex disposes of multiple and redundant activities to ensure robust target degradation: It uses the endonucleolytic activity of SMG6, the deadenylase activity of the CCR4–NOT complex through direct interaction with SMG7, and the decapping activity of DCP2 through interactions with UPF1 and/or SMG7.

Materials and methods

DNA constructs

Plasmids for the expression of SMG5 and SMG7 and deadenylation factors have been described previously (Braun et al. 2011; Jonas et al. 2013). SMG7 ΔPC carries a deletion of amino acids 633–1091. For the TAP tag selection, SMG7 cDNA encoding the PC region (residues 633–1091) was inserted into the pCMV-TAPtagN-PrP-SBP vector using XhoI and Acc651 restriction sites. The TAP tag consists of protein A, a PreScission protease cleavage site, and the SBP. For in vitro translation, cDNAs encoding SMG7 fragments (PC and ΔPC) were cloned into XhoI–NotI sites of the pClneo-λNHA vector. For the expression of SMG5 and SMG7 with MS2-HA tags, the corresponding cDNAs were inserted into the XhoI–NotI sites of pCN-MS2 (Lykke-Andersen et al. 2000), which was modified by the insertion of an HA tag C-terminal to the MS2. SMG5 and SMG7 mutants were generated by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) and the appropriate oligonucleotide sequences. Mutants used in this study are described in Supplemental Table S2. To express PNRC2 with an N-terminal V5-SBP-MBP tag, the cDNA was inserted into the XhoI–NotI restriction sites of the pCIneo-V5-SBP-MBP vector. Plasmids for expression of recombinant proteins in *E. coli* are described in the Supplemental Material.

TAP tag purification

For the TAP tag purification, HEK293T cells were grown in 145-mm dishes and transfected with 30 μg of plasmid per dish using the calcium phosphate method. Cells were harvested 48 h post-transfection and lysed on ice in lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1% Triton-X-100, 1 mM EDTA, 0.5 mM DTT, 10% glycerol with 2.5 mL per 84×10^6 cells) supplemented with Complete protease inhibitor (Roche). Cell lysates were spun at 18,000g for 15 min at 4°C. Cleared lysates were treated with 50 μg/mL RNase A (Qiagen) for 30 min at 4°C and then spun at 18,000g for 10 min at 4°C. Supernatants were pooled together and then incubated with 60 μL of IgG sepharose beads (50% slurry; GE Healthcare) for 1 h at 4°C with gentle mixing followed by an overnight digestion with 1 μg/μL PreScission protease at 4°C. The IgG beads were removed by centrifugation, and the supernatant was incubated with 70 μL of streptavidin beads (50% slurry; GE Healthcare) for 2 h at 4°C. The beads were washed seven times with lysis buffer, and the proteins were eluted with 50 μL of NuPage LDS sample buffer (Invitrogen). Samples were analyzed by mass spectrometry.

Loh et al.

Coimmunoprecipitation, pull-down assays, and Western blotting

For pull-down and coimmunoprecipitation assays, HEK293T cells were grown in 10-cm plates and transfected using the calcium phosphate method, except for the experiments shown in Figure 2A, which used Lipofectamine 2000 according to the manufacturer's protocol. Cells were transfected with 24 µg of total plasmid DNA. Two days after transfection, cells were lysed for 15 min on ice in NET lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1% Triton-X-100, 10% glycerol, 2.5 mM MgCl$_2$, 1 mM DTT) supplemented with protease inhibitor (Roche) and 200 µg/mL RNase A (Qiagen). Cell lysates were spun at 16,000g for 15 min at 4°C. For SBP-tagged proteins, the cleared lysate was rotated for 30 min at 4°C with 25 µL of streptavidin sepharose (GE Healthcare). The beads were washed three times with NET buffer. Bound proteins were eluted with 100 µL of protein sample buffer and analyzed by Western blotting. For the PNRC2 and UPF1 pull-downs, cells were incubated with 50 nM okadaic acid for 4 h before harvest, and NET buffer supplemented with PhosSTOP phosphatase inhibitor (Roche) was used for lysis and washing. Coimmunoprecipitations using anti-GFP antibodies and antibodies used in this study are described in the Supplemental Material and Supplemental Table S3, respectively. All Western blots were developed with the ECL Western blotting detection system (GE Healthcare) as recommended by the manufacturer.

Protein expression, purification, and pull-down assays

BL21 star cells (Invitrogen) harboring plasmids encoding GST, GST-POP2, or MBP-SMG7 732–1091 were grown at 37°C in LB medium until reaching OD$_{600}$ = 0.4. Protein expression was induced with IPTG, and all proteins were expressed overnight at 20°C. After harvest, cells were resuspended in lysis buffer (50 mM HEPES at pH 7.5, 200 mM NaCl, 1 mM DTT) supplemented with EDTA-free protease inhibitor (Roche), 1 mg/mL lysozyme, and 5 µg/mL DNase I and then lysed by sonication. The cleared lysates were incubated for 1 h with 5 mL of pre-equilibrated amylose resin (New England Biolabs) or glutathione agarose beads (Macherey-Nagel). The beads were washed with lysis buffer, and the proteins were eluted after a 15-min incubation with lysis buffer containing 25 mM maltose or L-glutathione. The proteins were subsequently purified over a gel filtration column (Superdex 200, GE Healthcare) and stored at −80°C after flash-freezing in liquid nitrogen. Pull-down assays were performed as described in the Supplemental Material.

Tethering and complementation assays

For tethering assays, HEK293T cells were cultured in six-well plates and transiently transfected with a mixture of three plasmids: 0.5 µg of the control plasmid (β-globin-GAP), 0.5 µg of the plasmid encoding the β-globin-6xMS2bs, and various amounts of the pCN-MS2-HA plasmid for the expression of MS2-HA fusion proteins. The amount of total DNA was adjusted to 3 µg with the pcDNA3 plasmid. When indicated, the transfection mixtures also contained plasmids encoding GFP-DCP2 (wild type or mutant), HA-MBP, or catalytically inactive HA-POP2 mutant. Complementation assays were performed as previously described (Jonas et al. 2013). Transfection mixtures contained 0.4 µg of plasmid pSUPERpuro expressing shRNAs. Total RNA was isolated using TriFast (Peqlab) and analyzed as described previously (Huntzinger et al. 2008). β-Tubulin was used as normalization control.

References

Amrani N, Ganesan R, Kervestin S, Mangus DA, Ghosh S, Jacobson A. 2004. A faux 3′-UTR promotes aberrant termination and triggers nonsense-mediated mRNA decay. *Nature* **432:** 112–118.

Anders KR, Grimson A, Anderson P. 2003. SMG-5, required for *C. elegans* nonsense-mediated mRNA decay, associates with SMG-2 and protein phosphatase 2A. *EMBO J* **22:** 641–650.

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

Bond AT, Mangus DA, He F, Jacobson A. 2001. Absence of Dbp2p alters both nonsense-mediated mRNA decay and rRNA processing. *Mol Cell Biol* **21:** 7366–7379.

Braun JE, Huntzinger E, Fauser M, Izaurralde E. 2011. GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets. *Mol Cell* **44:** 120–133.

Cao D, Parker R. 2003. Computational modeling and experimental analysis of nonsense-mediated decay in yeast. *Cell* **113:** 533–545.

Chen CY, Shyu AB. 2003. Rapid deadenylation triggered by a nonsense codon precedes decay of the RNA body in a mammalian cytoplasmic nonsense-mediated decay pathway. *Mol Cell Biol* **23:** 4805–4813.

Cheng Z, Muhlrad D, Lim MK, Parker R, Song H. 2007. Structural and functional insights into the human Upf1 helicase core. *EMBO J* **26:** 253–264.

Chiu SY, Serin G, Ohara O, Maquat LE. 2003. Characterization of human Smg5/7a, a protein with similarities to *Caenorhabditis elegans* SMG5 and SMG7 that functions in the dephosphorylation of Upf1. *RNA* **9:** 77–87.

Cho H, Kim KM, Kim YK. 2009. Human proline-rich nuclear receptor coregulatory protein 2 mediates an interaction between mRNA surveillance machinery and decapping complex. *Mol Cell* **33:** 75–86.

Cho H, Han S, Choe J, Park SG, Choi SS, Kim YK. 2013. SMG5–PNRC2 is functionally dominant compared with SMG5–SMG7 in mammalian nonsense-mediated mRNA decay. *Nucleic Acids Res* **41:** 1319–1328.

Clerici M, Mourao A, Gutsche I, Gehring NH, Hentze MW, Kulozik A, Kadlec J, Sattler M, Cusack S. 2009. Unusual bipartite mode of interaction between the nonsense-mediated decay factors, UPF1 and UPF2. *EMBO J* **28:** 2293–2306.

Couttet P, Grange T. 2004. Premature termination codons enhance mRNA decapping in human cells. *Nucleic Acids Res* **32:** 488–494.

Czaplinski K, Ruiz-Echevarria MJ, Paushkin SV, Han X, Weng Y, Perlick HA, Dietz HC, Ter-Avanesyan MD, Peltz SW. 1998.

The surveillance complex interacts with the translation release factors to enhance termination and degrade aberrant mRNAs. *Genes Dev* **12:** 1665–1677.

Daugeron MC, Mauxion F, Seraphin B. 2001. The yeast POP2 gene encodes a nuclease involved in mRNA deadenylation. *Nucleic Acids Res* **29:** 2448–2455.

Eberle AB, Lykke-Andersen S, Mühlemann O, Jensen TH. 2009. SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nat Struct Mol Biol* **16:** 49–55.

Fenger-Grøn M, Fillman C, Norrild B, Lykke-Andersen J. 2005. Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping. *Mol Cell* **20:** 905–915.

Fiorini F, Boudvillain M, Le Hir H. 2013. Tight intramolecular regulation of the human Upf1 helicase by its N- and C-terminal domains. *Nucleic Acids Res* **41:** 2404–2415.

Franks TM, Singh G, Lykke-Andersen J. 2010. Upf1 ATPase-dependent mRNP disassembly is required for completion of nonsense- mediated mRNA decay. *Cell* **143:** 938–950.

Fukuhara N, Ebert J, Unterholzner L, Lindner D, Izaurralde E, Conti E. 2005. SMG7 is a 14-3-3-like adaptor in the nonsense-mediated mRNA decay pathway. *Mol Cell* **18:** 537–547.

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

Gatfield D, Unterholzner L, Ciccarelli FD, Bork P, Izaurralde E. 2003. Nonsense-mediated mRNA decay in *Drosophila*: At the intersection of the yeast and mammalian pathways. *EMBO J* **22:** 3960–3970.

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

He F, Jacobson A. 2001. Upf1p, Nmd2p, and Upf3p regulate the decapping and exonucleolytic degradation of both nonsense-containing mRNAs and wild-type mRNAs. *Mol Cell Biol* **21:** 1515–1530.

He F, Li X, Spatrick P, Casillo R, Dong S, Jacobson A. 2003. Genome-wide analysis of mRNAs regulated by the nonsense-mediated and 5′ to 3′ mRNA decay pathways in yeast. *Mol Cell* **12:** 1439–1452.

Hogg JR, Goff SP. 2010. Upf1 senses 3′UTR length to potentiate mRNA decay. *Cell* **143:** 379–389.

Huntzinger E, Kashima I, Fauser M, Saulière J, Izaurralde E. 2008. SMG6 is the catalytic endonuclease that cleaves mRNAs containing nonsense codons in metazoa. *RNA* **14:** 2609–2617.

Hurt JA, Robertson AD, Burge CB. 2013. Global analyses of UPF1 binding and function reveal expanded scope of nonsense-mediated mRNA decay. *Genome Res* **23:** 1636–1650.

Isken O, Kim YK, Hosoda N, Mayeur GL, Hershey JW, Maquat LE. 2008. Upf1 phosphorylation triggers translational repression during nonsense-mediated mRNA decay. *Cell* **133:** 314–327.

Ivanov PV, Gehring NH, Kunz JB, Hentze MW, Kulozik AE. 2008. Interactions between UPF1, eRFs, PABP and the exon junction complex suggest an integrated model for mammalian NMD pathways. *EMBO J* **27:** 736–747.

Jonas S, Weichenrieder O, 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.

Kashima I, Yamashita A, Izumi N, Kataoka N, Morishita R, Hoshino S, Ohno M, Dreyfuss G, 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.

Kervestin S, Jacobson A. 2012. NMD: A multifaceted response to premature translational termination. *Nat Rev Mol Cell Biol* **13:** 700–712.

Kurosaki T, Maquat LE. 2013. Rules that govern UPF1 binding to mRNA 3′ UTRs. *Proc Natl Acad Sci* **110:** 3357–3362.

Lai T, Cho H, Liu Z, Bowler MW, Piao S, Parker R, Kim YK, Song H. 2012. Structural basis of the PNRC2-mediated link between mRNA surveillance and decapping. *Structure* **20:** 2025–2037.

Lau NC, Kolkman A, van Schaik FM, Mulder KW, Pijnappel WW, Heck AJ, Timmers HT. 2009. Human Ccr4–Not complexes contain variable deadenylase subunits. *Biochem J* **422:** 443–453.

Le Hir H, Izaurralde E, Maquat LE, Moore MJ. 2000. The spliceosome deposits multiple proteins 20–24 nucleotides upstream of mRNA exon–exon junctions. *EMBO J* **19:** 6860–6869.

Lejeune F, Li X, Maquat LE. 2003. Nonsense-mediated mRNA decay in mammalian cells involves decapping, deadenylating, and exonucleolytic activities. *Mol Cell* **12:** 675–687.

Luke B, Azzalin CM, Hug N, Deplazes A, Peter M, Lingner J. 2007. *Saccharomyces cerevisiae* Ebs1p is a putative ortholog of human Smg7 and promotes nonsense-mediated mRNA decay. *Nucleic Acids Res* **35:** 7688–7697.

Lykke-Andersen J. 2002. Identification of a human decapping complex associated with hUpf proteins in nonsense-mediated decay. *Mol Cell Biol* **22:** 8114–8121.

Lykke-Andersen J, Shu MD, Steitz JA. 2000. Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. *Cell* **103:** 1121–1131.

Metze S, Herzog VA, Ruepp M-D, Mühlemann O. 2013. Comparison of EJC-enhanced and EJC-independent NMD in human cells reveals two partially redundant degradation pathways. *RNA* **19:** 1432–1448.

Mitchell P, Tollervey D. 2003. An NMD pathway in yeast involving accelerated deadenylation and exosome-mediated 3′ → 5′ degradation. *Mol Cell* **11:** 1405–1413.

Muhlemann O, Lykke-Andersen J. 2010. How and where are nonsense mRNAs degraded in mammalian cells? *RNA Biol* **7:** 28–32.

Muhlrad D, Parker R. 1994. Premature translational termination triggers mRNA decapping. *Nature* **370:** 578–581.

Nagy E, Maquat LE. 1998. A rule for termination-codon position within intron-containing genes: When nonsense affects RNA abundance. *Trends Biochem Sci* **23:** 198–199.

Ohnishi T, Yamashita A, Kashima I, Schell T, Anders KR, Grimson A, Hachiya T, Hentze MW, Anderson P, Ohno S. 2003. Phosphorylation of hUPF1 induces formation of mRNA surveillance complexes containing hSMG-5 and hSMG-7. *Mol Cell* **12:** 1187–1200.

Okada-Katsuhata Y, Yamashita A, Kutsuzawa K, Izumi N, Hirahara F, 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.

Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Séraphin B. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* **17:** 1030–1032.

Silva AL, Ribeiro P, Inacio A, Liebhaber SA, Romao L. 2008. Proximity of the poly(A)-binding protein to a premature termination codon inhibits mammalian nonsense-mediated mRNA decay. *RNA* **14:** 563–576.

Singh G, Rebbapragada I, Lykke-Andersen J. 2008. A competition between stimulators and antagonists of Upf complex

recruitment governs human nonsense-mediated mRNA decay. *PLoS Biol* **6:** e111.

Swisher KD, Parker R. 2011. Interactions between Upf1 and the decapping factors Edc3 and Pat1 in *Saccharomyces cerevisiae*. *PLoS ONE* **6:** e26547.

Takahashi S, Araki Y, Sakuno T, Katada T. 2003. Interaction between Ski7p and Upf1p is required for nonsense-mediated 3′-to-5′ mRNA decay in yeast. *EMBO J* **22:** 3951–3959.

Thermann R, Neu-Yilik G, Deters A, Frede U, Wehr K, Hagemeier C, Hentze MW, Kulozik AE. 1998. Binary specification of nonsense codons by splicing and cytoplasmic translation. *EMBO J* **17:** 3484–3494.

Unterholzner L, Izaurralde E. 2004. SMG7 acts as a molecular link between mRNA surveillance and mRNA decay. *Mol Cell* **16:** 587–596.

Yamashita A. 2013. Role of SMG-1-mediated Upf1 phosphorylation in mammalian nonsense-mediated mRNA decay. *Genes Cells* **18:** 161–175.

Yamashita A, Chang TC, Yamashita Y, Zhu W, Zhong Z, Chen CY, Shyu AB. 2005. Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover. *Nat Struct Mol Biol* **12:** 1054–1063.

Zünd D, Gruber AR, Zavolan M, Mühlemann O. 2013. Translation-dependent displacement of UPF1 from coding sequences causes its enrichment in 3′ UTRs. *Nat Struct Mol Biol* **20:** 936–943.

Supplemental Material

The SMG5-SMG7 heterodimer directly recruits the CCR4-NOT deadenylase

complex to mRNAs containing nonsense codons via interaction with POP2

Belinda Loh, Stefanie Jonas and Elisa Izaurralde

Supplemental Materials and Methods

DNA constructs

To express the SMG7 PC fragment in *E. coli*, the corresponding DNA (residues 732–1091) was synthesized and codon usage optimized for expression in bacteria. The cDNA was cloned into the *Xho*I-*Nhe*I restriction sites of a pnYC-NpM vector (Diebold et al. 2011) with an N-terminal MBP-tag followed by a HRV3C cleavage site. CAF1, POP2 and POP2 catalytic domain (amino acids 12–239) were amplified from human cDNA and cloned into the *Xho*I-*BamH*I sites of a pnEA-NvG vector with an N-terminal GST-tag followed by a TEV cleavage site.

Coimmunoprecipitation assays

For coimmunoprecipitation assays using anti-GFP antibodies (Supplemental Fig. S5B,C), HEK293T cells were grown in 10 cm plates and transfected using the calcium phosphate method. Two days after transfection, cells were lysed for 15 min on ice in NET lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton-X-100, 10% glycerol, 2.5 mM MgCl$_2$, 1 mM DTT) supplemented with protease inhibitor (Roche) and 200 μg/ml RNase A (Qiagen). Cell lysates were spun at 16,000 $\times g$ for 15 min at 4°C. Rabbit polyclonal anti-GFP antibodies were added to the supernatants (dilution 1:1,000) and samples were incubated for 1 hr at 4°C. Then, 25 μl of GammaBind G Sepharose (GE Healthcare) was added, and the mixtures were rotated for an additional hour at 4°C. The beads were washed three times with NET buffer. Bound proteins were eluted with 100 μl of protein sample buffer and analyzed by western blotting.

Pulldown assays

For the experiment shown in Figure 5H, 10 µM MBP-SMG7 732–1091 was mixed with 10 µM GST or GST-POP2 in interaction buffer (50 mM HEPES (pH 7.5), 200 mM NaCl, 2 mM DTT) with 22 µl of glutathione agarose beads (final volume 100 µl). After 1 h incubation at 4°C, the beads were washed three times with 0.5 ml of interaction buffer. Bound proteins were eluted with 32 µl of interaction buffer supplemented with 25 mM L-glutathione. For analysis 0.5 µl of the input and 20 µl of the elution were separated on an SDS-PAGE gradient gel (Any-kD, Bio-Rad).

To perform the GST pulldown assays shown in Figure 5D-G, cleared lysates from *E. coli* cells (100 ml cultures) expressing GST, GST-POP2 or GST-CAF1 were incubated with 25 µl of Protino glutathione agarose beads in NET buffer supplemented with 1 mM DTT and protease inhibitor for 1 hr at 4°C. Beads were washed three times with 1 ml of NET buffer. The beads coated with GST-tagged proteins were then incubated with [^{35}S]-methionine-labeled *in vitro* translated proteins in 150 µl of NET buffer (see above) supplemented with 1mM DTT and 0.1 µg/µl BSA. The beads were washed three times with 1 ml of NET buffer and eluted with 40 µl of SDS-PAGE loading buffer. The proteins were analyzed by 10% SDS-PAGE. [^{35}S]-methionine-labeled proteins were detected by fluorography using Amplify (NAMP100, GE Healthcare) according to the manufacturers' instructions. [^{35}S]-methionine-labeled PC and ΔPC fragments were expressed using the TNT T7-coupled Wheat Germ System (Promega, L4140) and [^{35}S]-methionine (EasyTag Perkin Elmer, NEG709A) according to manufacturer's instructions. Before the pulldown, the *in vitro* translated proteins were supplemented with 2.5 mM CaCl$_2$ and treated with micrococcal nuclease.

Supplemental reference

Diebold ML, Fribourg S, Koch M, Metzger T, Romier C. 2011. Deciphering correct strategies for multiprotein complex assembly by co-expression: Application to complexes as large as the histone octamer. *J Struct Biol* **175:** 178–188.
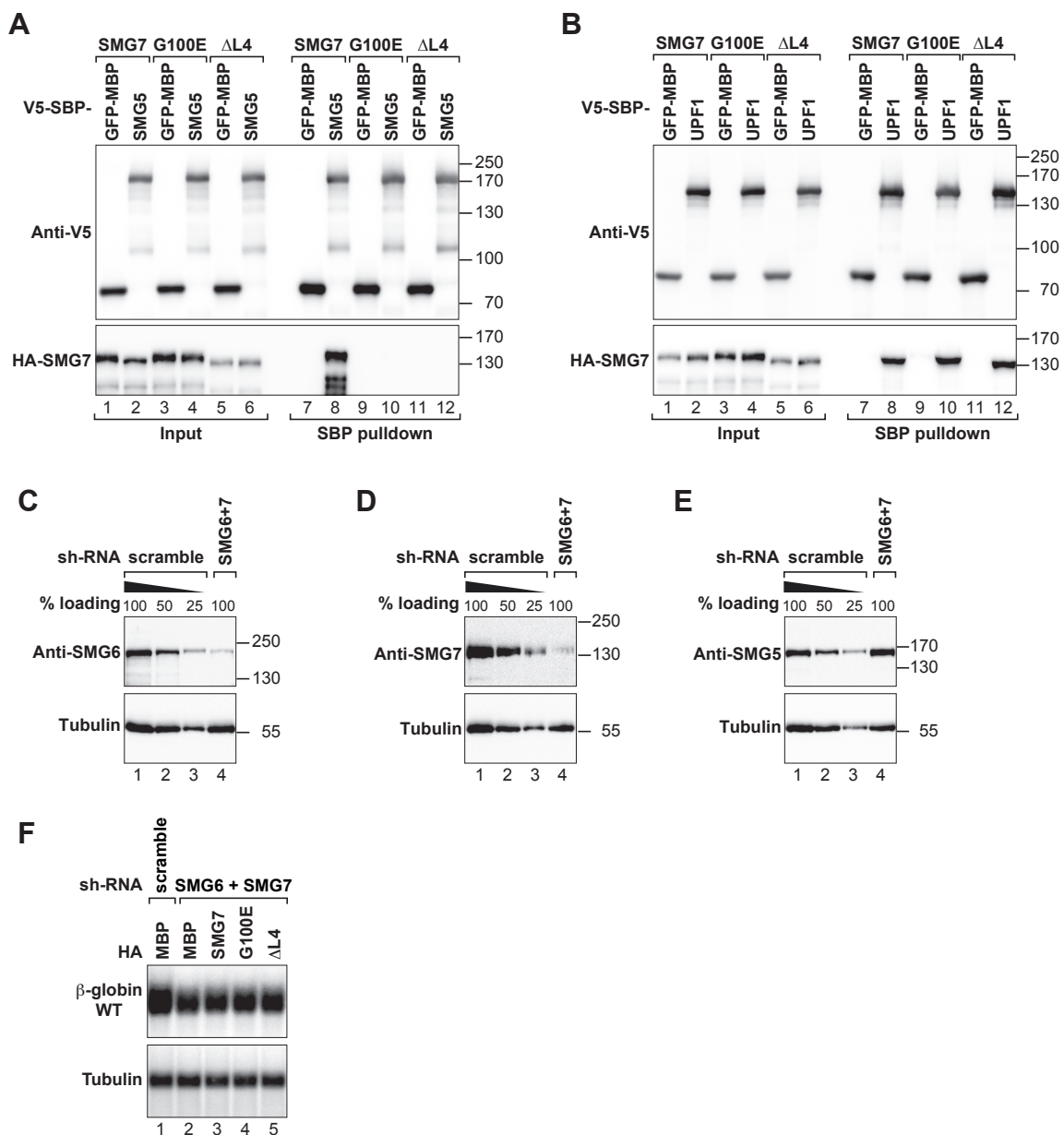
Figure S1
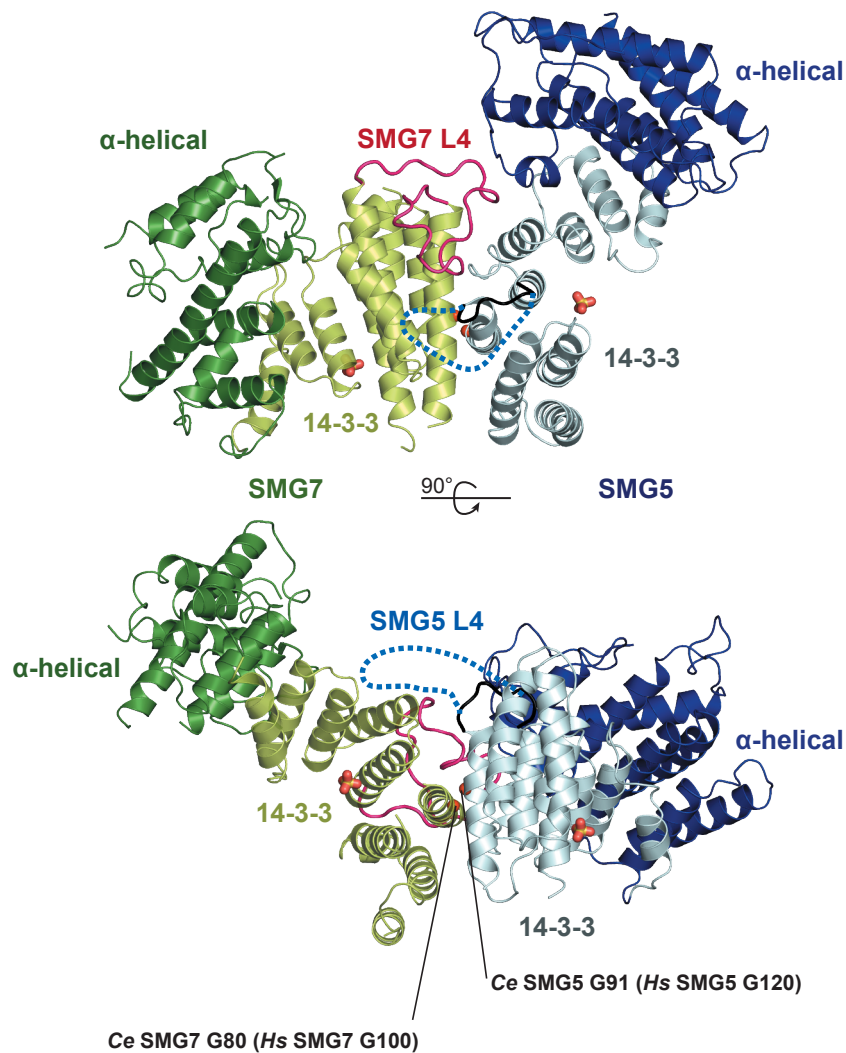
Supplemental Figure S1. Structure of the *C. elegans* (*Ce*) SMG5-SMG7 heterodimer highlighting the position of loop L4. Both proteins have a homologous domain organization resulting in a pseudosymmetric heterodimer. The 14-3-3 domain of SMG7 is shown in light green, the α-helical domain in dark green, while the equivalent domains of SMG5 are coloured light blue and dark blue, respectively. *Ce* SMG7 has a long loop L4, shown in red, which binds the concave surface of SMG5. The equivalent loop L4 in SMG5 (shown in black) is short in the *C. elegans* protein but long in the human SMG5, suggesting that it might bind the concave surface of human SMG7 in a similar manner as loop L4 of SMG7 binds to SMG5 (dotted blue line). Consistent with this hypothesis, deletion of loop L4 in human SMG5 abolishes binding to SMG7, indicating that this loop contributes to the interface. The positions of the phosphate-binding sites in the 14-3-3-like domains are marked by sulfate ions. The glycine residues at the center of the heterodimer interface are label as red dots.

Figure S2
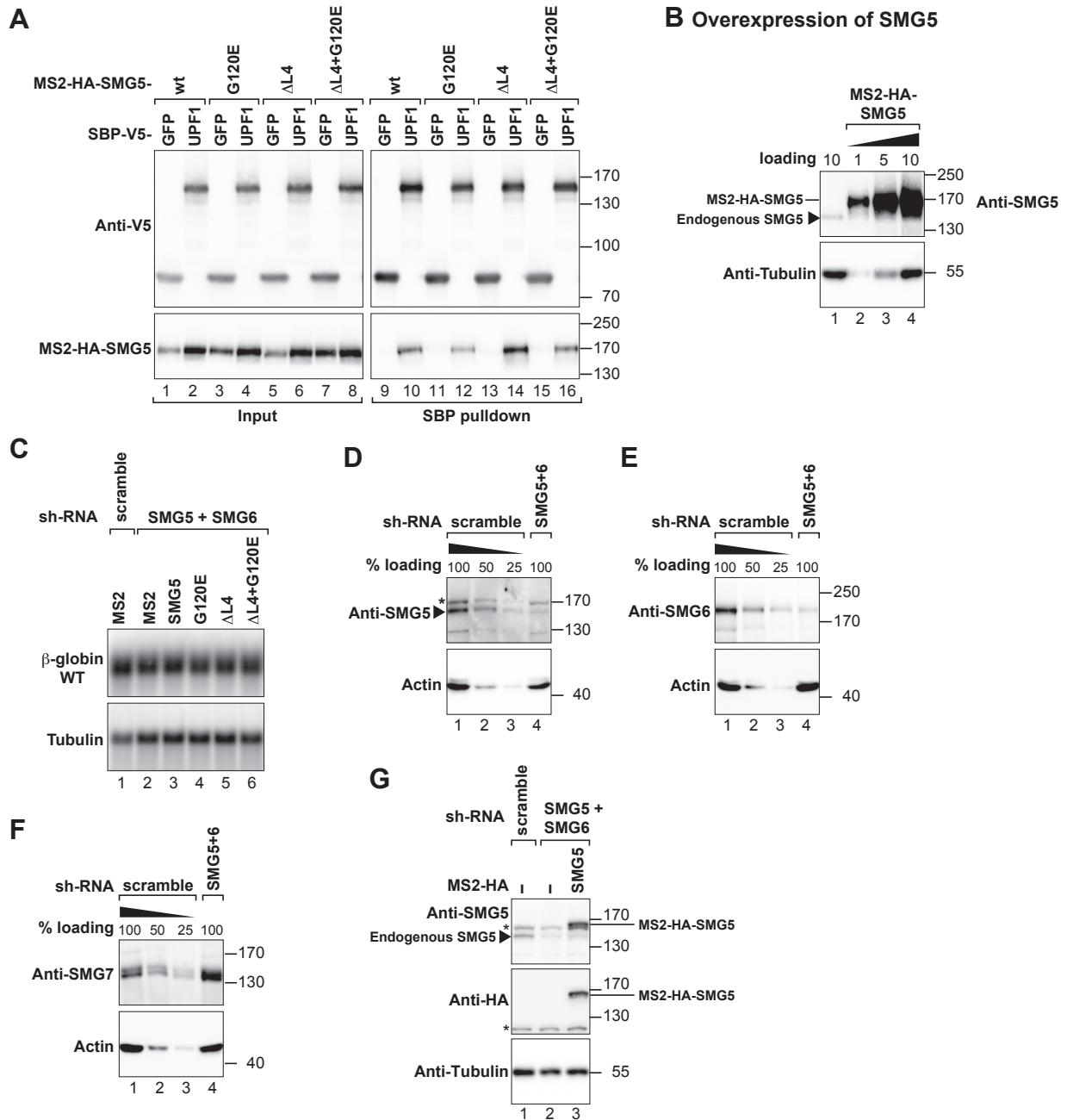


Supplemental Figure S2. SMG5 requires interaction with SMG7 to act in NMD. (*A*) Interaction of MS2-HA tagged SMG5 with overexpressed UPF1. V5-SBP-tagged UPF1 was coexpressed with MS2-HA-tagged SMG5 (wild-type or mutants) in HEK293T cells. A V5-SBP-tagged GFP-MBP fusion served as a negative control. Proteins were pulled down from cell lysates using streptavidin beads. Inputs (1%) and bound fractions (2% for UPF1 and 30% for SMG5) were analyzed by western blotting. (*B*) Samples from the pulldown assay shown in panel (A) were analyzed by western blot using anti-SMG5 antibodies. MS2-HA tagged SMG5 was expressed at levels approximately 100-fold higher than endogenous SMG5. (*C*) In a complementation assay, the wild type β-globin reporter is not affected by SMG5 mutants. This assay was performed in parallel to the assay with the PTC-containing reporter shown in Fig. 1C,D. (*D,E*) Western blots showing the efficiency of the SMG5 and SMG6 depletions in samples corresponding to the experiments shown in Figure 1C,D. (*F*) Expression levels of endogenous SMG7 in cells codepleted of SMG5 and SMG6. (*G*) Expression of MS2-HA-tagged SMG5 in the complementation assay shown in Fig. 1C,D. In the top panel the western blot was probed with anti-SMG5 antibodies to test the expression of the tagged protein (lane 3) relative to endogenous SMG5 in control cells (lane 1). In the lower panel the western blot was probed with anti-HA antibodies. Asterisks indicate crossreactivity of the antibodies.

Figure S3

Supplemental Figure S3. SMG7 requires interaction with SMG5 to act in NMD. (*A,B*) Interaction of HA-tagged SMG7 (wild-type or mutant) with overexpressed SMG5-V5-SBP-MBP (A) or overexpressed V5-SBP-UPF1. A V5-SBP-tagged GFP-MBP fusion served as a negative control. Inputs (1%) and bound fractions (2.5% for SMG5, 1% UPF1, 2% and 15% HA-SMG7 in panels A and B respectively) were analyzed by western blotting. (*C,D*) Western blots showing the efficiency of the SMG6 and SMG7 depletions in samples corresponding to the experiments shown in Fig. 1F,G. (*E*) Expression levels of endogenous SMG5 in cells codepleted of SMG7 and SMG6. (*F*) In a complementation assay, the wild type β-globin reporter is not affected by the SMG7 mutants. This assay was performed in parallel to the assay with the PTC-containing reporter shown in Figure 1F,G.

Figure S4
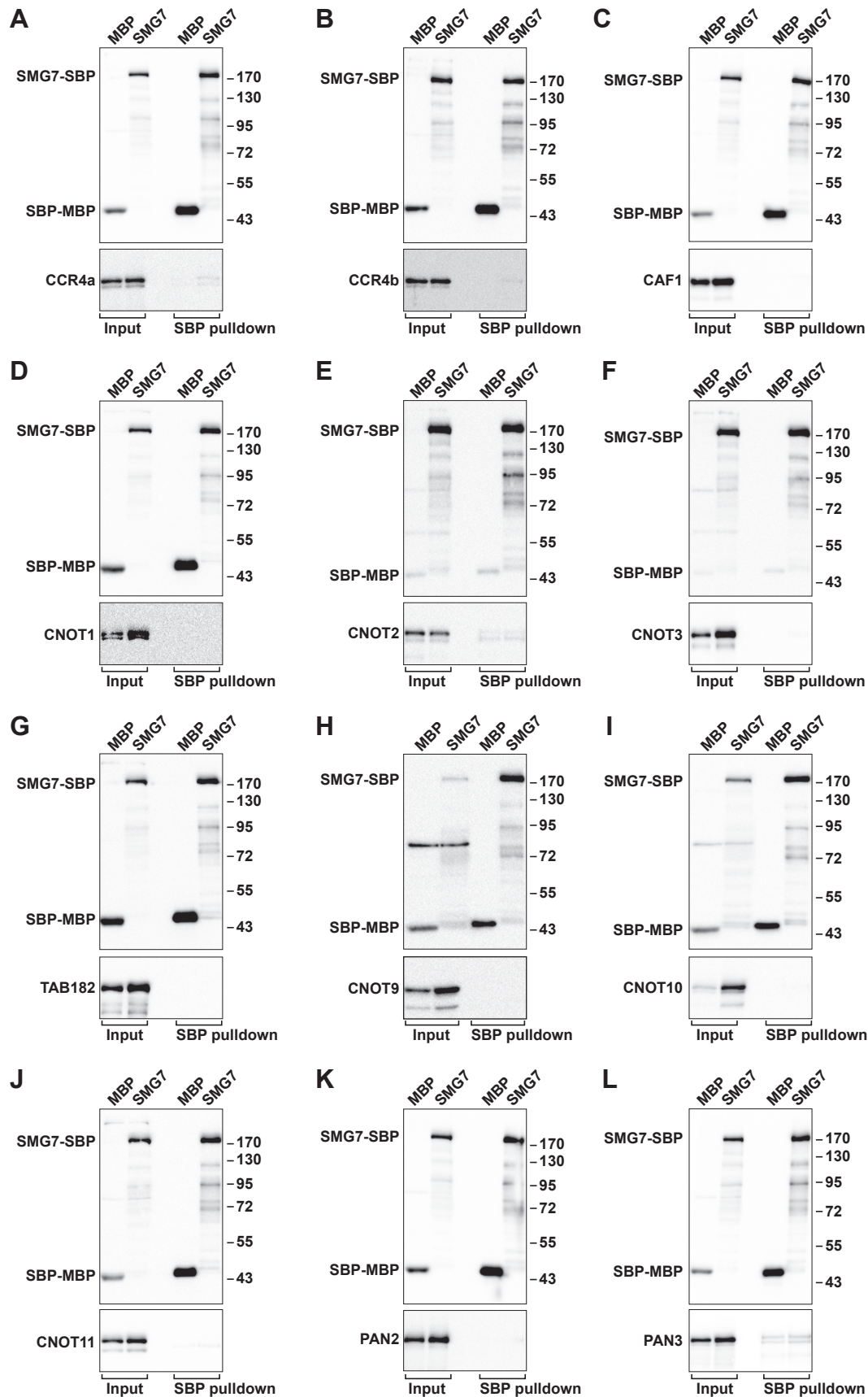
Supplemental Figure S4. SMG7 interacts with POP2. Interaction of SMG7-V5-SBP-MBP with GFP-tagged subunits of the CCR4-NOT complex, PAN2 and PAN3 in HEK293T cells. A V5-SBP-tagged MBP fusion served as a negative control. Inputs (1%) and bound fractions (30%) were analyzed by western blotting. Samples were treated with RNase A before the pulldown. The corresponding pulldown with POP2 is shown in Fig. 5A.

Figure S5
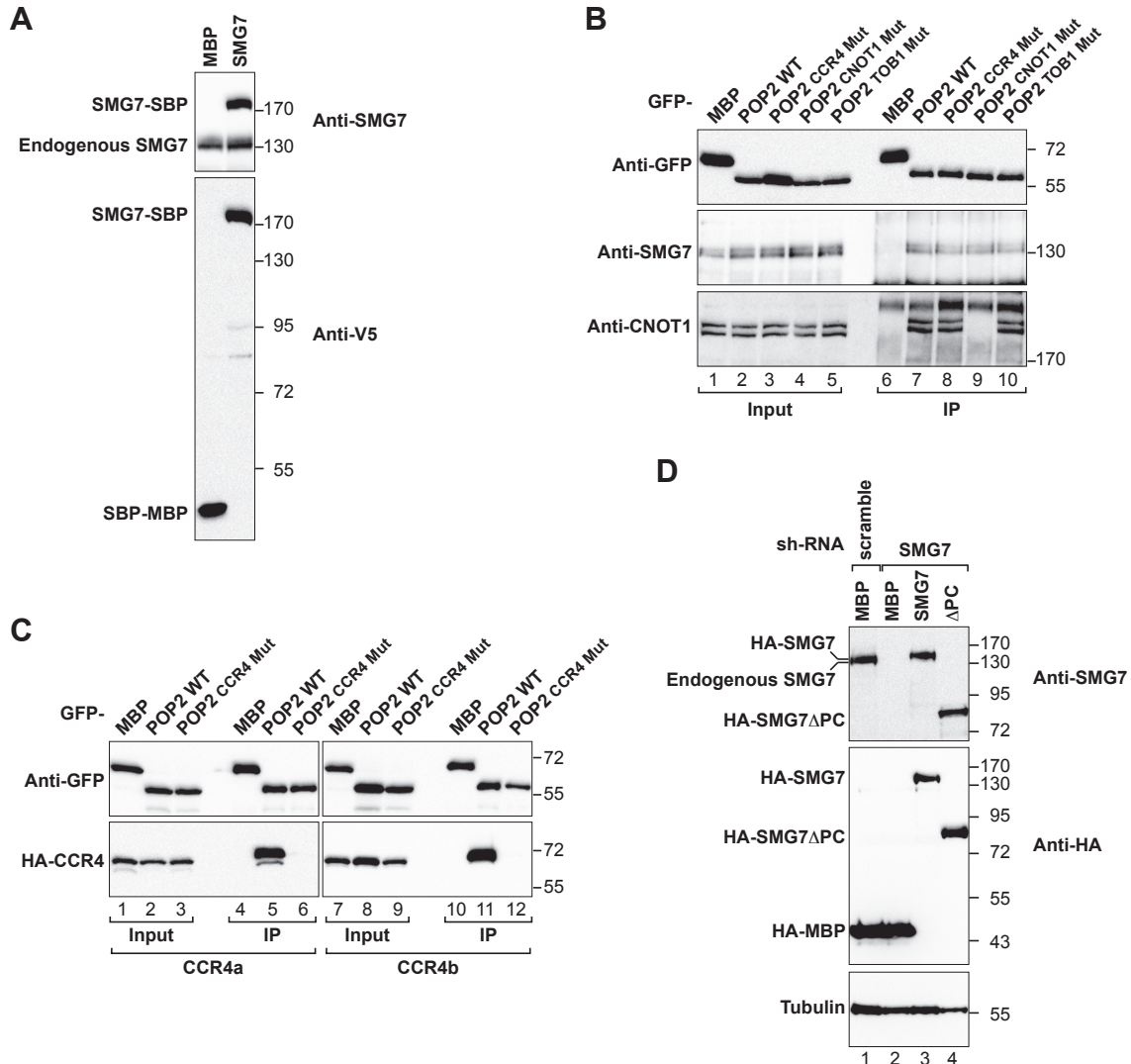
Supplemental Figure S5. (*A*) Western blot of samples from a SBP-pulldown assay (corresponding to Fig. 5A) showing that SBP-tagged SMG7 was expressed at levels comparable to endogenous SMG7. (*B*,*C*) SMG7 binding to POP2 is not affected by mutations that disrupt POP2 binding to CNOT1, CCR4a,b or TOB. The interaction of GFP-POP2 (wild-type or mutants) with endogenous SMG7 and CNOT1 (B) or HA-tagged CCR4a,b (C). A GFP-tagged MBP fusion served as a negative control. Inputs (1%) and bound fractions (10% for GFP tagged proteins, 20% for HA-CCR4a,b and endogenous CNOT1 and 30% for SMG7) were analyzed by western blotting. Samples were treated with RNase A before immunoprecipitation. (*D*) Western blot of samples corresponding to the complementation assay shown in Fig. 7A–D. The expression levels of HA-tagged SMG7 and SMG7ΔPC (lanes 3 and 4) were comparable to the levels of endogenous SMG7 in control cells (expressing MBP and treated with a scrambled shRNA, lane 1).

Figure S6
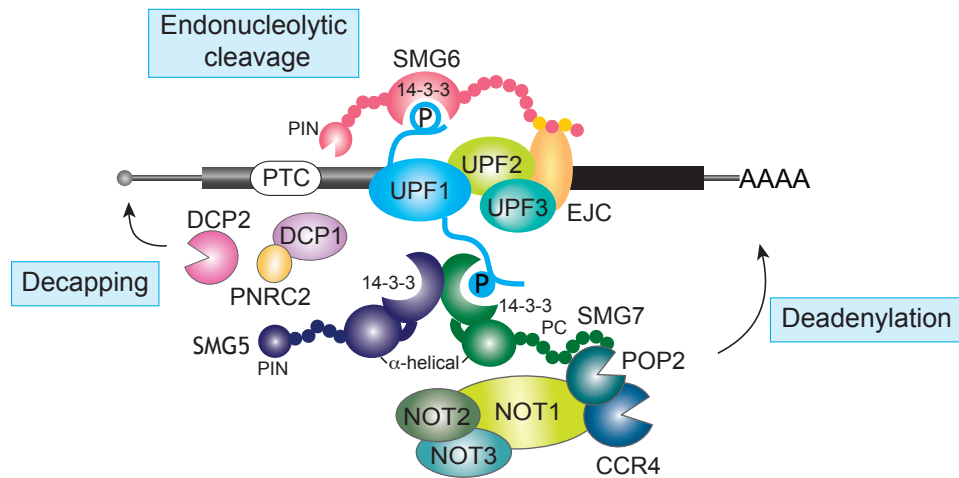
Supplemental Figure S6. Model for the degradation of NMD targets. UPF1 is recruited to PTC-containing mRNAs and phosphorylated when it interacts with UPF2 and/or UPF3, which are generally bound to downstream EJCs (exon junction complexes). Phosphorylated UPF1 recruits SMG6 and the SMG5-SMG7 heterodimer. The interaction is mediated by phosphory-lated residues (P) in the N- and C-terminal tails of UPF1, which are recognized by the 14-3-3-like domains in SMG6 and SMG7. SMG5 is recruited through SMG7 or may contact UPF1 directly. SMG6 has a PIN domain that cleaves the target in the vicinity of the PTC (premature termination codon). SMG7 recruits the CCR4-NOT deadenylase complex through interactions between its C-terminal PC region and POP2. Deadenylated mRNAs are normally decapped by the decapping enzyme DCP2 and degraded. Therefore, decapping can occur as a conse-quence of deadenylation. Additionally, our data shows that UPF1 interacts with DCP2 and PNRC2, although it is not known whether these interactions are direct. These interactions may increase the local concentration of decapping factors on NMD targets and might initiate decapping independently of deadenylation. Thus the surveillance complex deploys multiple and redundant activities to ensure robust target degradation..

Supplemental Table S1. Proteins copurifying with the SMG7 PC region.

Protein	Number of peptides	Sequence Coverage (%)	Protein	Number of peptides	Sequence Coverage (%)
CCR4-NOT complex			**Splicing & RNA Processing**		
CNOT1	209	30.8	HNRNPM	242	46.3
CNOT3	43	11.7	HNRNPH1	31	19.9
CNOT2	77	26.9	HNRNPC	3	6.9
CNOT7	18	33.7	HNRNPF	14	19
CNOT10	37	14.3	HNRNPD	13	11.3
CNOT6	4	11.1	DDX5	16	13.4
CNOT8	24	27.4	DDX17	3	8.8
CNOT6L	27	12.8	EFTUD2	9	5.2
CNOT11	11	17.6	RBM4	3	8.2
CNOT9	60	41.8	RBM7	2	7.5
tRNA			RALY	2	6.5
FARSA	6	7.7	**Transcription & Translation**		
TRNAU1AP	3	10.1	EEF1A1	73	34.8
Cell Cycle & Differentiation			RUVBL2	62	41.3
IRS4	30	6.4	RUVBL1	44	44.1
HAUS5	25	10.9	MATR3	17	8.6
MAGED1	9	6	GCN1L1	25	9.4
GAS7	3	1.5	RBM14	19	12.9
CDC2	5	18.2	AKAP8L	19	14.1
CDC42	3	4.2	TRIM28	8	10.8
HOOK1	7	7	EIF4A1	4	9.1
CDC23	3	5.5	FUBP3	11	12.6
CSNK1A1	3	5.8	POLR2B	12	5.7
Nuclear Pore			TRIM27	3	7.6
SEC13	6	14.7	**Nuclear Transport**		
NUP93	14	13.4	SEC16A	49	11.6
DNA Repair			RAN	4	18.5
RNF219	27	14.5	MDN1	32	6
DDB1	12	6.4	FAM120A	8	5.1
Signal Transduction			**Endocytosis**		
IQGAP2	48	16.4	CLINT1	6	8.4
YWHAZ	2	12.6	HAX1	4	16.4
Cell Adhesion			**Host-Viral Interaction**		
LGALS3BP	14	10.9	VIM	23	36.1
PKP2	8	7.9	YWHAE	8	29.4
Coenzyme A Biosynthesis			**Apoptosis**		
PANK4	11	9.7	KIAA1967	7	6.3

Protein	Number of peptides	Sequence Coverage (%)
Motor Proteins		
MYO1D	16	11.3
MYL6	19	28.6
DYNLL1	2	24.7
MYH9	123	35.7
MYH10	47	26.8
MYL12B	6	12.2
Glycolysis		
LDHB	3	12.3
Additional proteins		
P4HA1	21	23
SLFN11	37	13.1
P4HA2	8	10.1
IQGAP3	19	7.3
PCBP1	9	23.3
CPVL	8	9.9
IQCB1	4	5.5
YTHDF2	11	7.4
MLF2	4	8.5

Protein	Number of peptides	Sequence Coverage (%)
Ribosomal		
RPL7	6	8.9
RPS3	80	65.8
RPL11	54	35.4
RPS15A	12	14.6
RPS13	2	12.6
PSMD3	12	18.9
RPS11	31	33.5
RPLP0	5	13.2
RPL13	3	9.5
RPL30	5	31.3
RPL22	4	30.5
RPS25	2	15.2
RPL12	4	24.2
RPS18	5	21.1
RPS19	4	17.9
RPL28	6	18.9
Chaperones		
HSPA9	286	47.7
CCT1	19	23.4
BAG5	15	20.5

Supplemental Table S2. Protein mutants used in this study.

Name	SMG5
G120E	G120E
ΔL4	Δ133–155
ΔL4+G120E	Δ133–155 + G120E

Name	SMG7
ΔPC	1–633
PC	633–1091
MBP-SMG7 732-1091	732–1091
G100E	*G100E*
ΔL4	residues 112–141 replaced by GSSG

Name	UPF1
ΔN	Δ1–114
ΔNCR	Δ1–72
ΔCH	Δ119–272
ΔC	Δ926–1129
S1107A	S1107A
T28A	T28A
Δhelicase	Δ272–925

Name	DCP2
Mut	E148Q

Name	POP2
ΔC	1–240
DEDD	12–240
Mut	D40A, E42A
CCR4 Mut	C67E, L71E[1]
NOT1 Mut	E137K, M140R, T141Y[2]
TOB1 Mut	K203A[3]

[1]Basquin J, Roudko VV, Rode M, Basquin C, Séraphin B, Conti E. 2012. Architecture of the nuclease module of the yeast Ccr4-not complex: the Not1-Caf1-Ccr4 interaction. *Mol Cell* **48:** 207–218.

[2]Petit AP, Wohlbold L, Bawankar P, Huntzinger E, Schmidt S, Izaurralde E, Weichenrieder O. 2012. The structural basis for the interaction between CAF1 nuclease and the NOT1 scaffold of the human CCR4-NOT deadenylase complex. *Nucleic Acids Res* **40:** 11058–11072.

[3]Horiuchi M, Takeuchi K, Noda N, Muroya N, Suzuki T, Nakamura T, Kawamura-Tsuzuku J, Takahasi K, Yamamoto T, Inagaki F. 2009. Structural basis for the antiproliferative activity of the Tob–hCaf1 complex. *J Biol Chem* **284:** 13244–13255.

Supplemental Table S3. Antibodies used in this study.

Antibody	Source	Catalog Number	Dilution	Monoclonal/ Polyclonal
Anti-V5	AbD Serotec	MCA1360GA	1:5,000	Monoclonal
Anti-HA-HRP	Roche	12 013 819 001	1:5,000	Monoclonal
Anti-GFP (for Western blotting)	Roche	11 814 460 001	1:3,000	Monoclonal
Anti-tubulin	Sigma aldrich	T6199	1:5,000	Monoclonal
Anti-UPF1	Bethyl Laboratories	A301-902A	1:3,000	Rabbit Polyclonal
Phospho(Ser/Thr) ATM/ATR Substrate (4F7) Rabbit mAb	Cell Signalling Technology	2909	1:2,000	Monoclonal
Anti-POP2/CAF1	Abnova	H00029883	1:3,000	Monoclonal
Anti-mouse-HRP	GE Healthcare	NA931V	1:10,000	Polyclonal
Anti-rabbit-HRP	GE Healthcare	NA934V	1:10,000	Polyclonal
Anti-SMG5	In house		1:3,000	Rabbit polyclonal
Anti-SMG7	In house		1:3,000	Rabbit polyclonal
Anti-SMG6	Dr. Shigeo Ohno		1:3,000	Rabbit polyclonal

NOT10 and C2orf29/NOT11 form a conserved module of the CCR4-NOT complex that docks onto the NOT1 N-terminal domain

Praveen Bawankar, Belinda Loh, Lara Wohlbold, Steffen Schmidt and Elisa Izaurralde*

Department of Biochemistry; Max Planck Institute for Developmental Biology; Tübingen, Germany

The CCR4-NOT complex plays a crucial role in post-transcriptional mRNA regulation in eukaryotes. This complex catalyzes the removal of mRNA poly(A) tails, thereby repressing translation and committing an mRNA to degradation. The conserved core of the complex is assembled by the interaction of at least two modules: the NOT module, which minimally consists of NOT1, NOT2 and NOT3, and a catalytic module comprising two deadenylases, CCR4 and POP2/CAF1. Additional complex subunits include CAF40 and two newly identified human subunits, NOT10 and C2orf29. The role of the NOT10 and C2orf29 subunits and how they are integrated into the complex are unknown. Here, we show that the *Drosophila melanogaster* NOT10 and C2orf29 orthologs form a complex that interacts with the N-terminal domain of NOT1 through C2orf29. These interactions are conserved in human cells, indicating that NOT10 and C2orf29 define a conserved module of the CCR4-NOT complex. We further investigated the assembly of the *D. melanogaster* CCR4-NOT complex, and demonstrate that the conserved armadillo repeat domain of CAF40 interacts with a region of NOT1, comprising a domain of unknown function, DUF3819. Using tethering assays, we show that each subunit of the CCR4-NOT complex causes translational repression of an unadenylated mRNA reporter and deadenylation and degradation of a polyadenylated reporter. Therefore, the recruitment of a single subunit of the complex to an mRNA target induces the assembly of the complete CCR4-NOT complex, resulting in a similar regulatory outcome.

Introduction

In eukaryotes, the removal of mRNA poly(A) tails by deadenylases, a process known as deadenylation, represses translation and generally commits the mRNA to degradation. Therefore, deadenylation provides a major mechanism for the post-transcriptional regulation of mRNA expression.[1] Eukaryotic mRNAs are deadenylated by the consecutive action of two cytoplasmic deadenylase complexes.[2,3] The PAN2-PAN3 complex is involved in the early phase of deadenylation and shortens mRNA poly(A) tails in a distributive manner.[4,5] The second, more rapid phase of deadenylation is catalyzed by the CCR4-NOT complex.[5,6] The CCR4-NOT complex is sufficient for mRNA deadenylation in the absence of the PAN2-PAN3 complex.[5-7]

In addition to its general role in bulk mRNA deadenylation, the CCR4-NOT complex and associated proteins have been implicated in a broad range of biological processes, including transcription initiation and elongation, ubiquitination and protein modification.[3,8] Furthermore, this complex plays a key role in the post-transcriptional regulation of specific mRNAs, to which it is recruited via interactions with sequence-specific RNA-binding proteins. These RNA-binding proteins include Pumilio, Nanos, Bicaudal-C and Smaug, which regulate the temporal and

spatial expression of mRNA targets during *Drosophila melanogaster* (*Dm*) oogenesis and embryogenesis.[1,8] Recently, it was shown that GW182 family proteins, which are required for miRNA-mediated silencing in animal cells, directly interact with NOT1 and recruit the CCR4-NOT complex to miRNA targets.[9-11]

The conserved core of the CCR4-NOT complex consists of at least five subunits: NOT1, NOT2, NOT3 and two catalytically active subunits, CCR4a (or its paralog CCR4b) and POP2 (or its paralog CAF1).[3,8] The catalytic subunits interact to form a catalytic module, which is recruited to the complex through interactions between POP2/CAF1 and a central MIF4G (middle domain of eukaryotic initiation factor 4G) domain in NOT1.[12-18] Additional complex subunits include CAF40 (also known as NOT9, Rcd1 or RQCD1), CAF130, NOT4, NOT5, NOT10, C2orf29 and TAB182.[3,8] CAF130 is a yeast-specific subunit with no metazoan counterpart.[8] Although NOT4 is conserved and is an integral yeast CCR4-NOT complex subunit, it is not stably associated with the *D. melanogaster* (*Dm*) and human complexes.[17,18] NOT5 is a yeast NOT3 paralog; however, in contrast with yeast, there is only one gene that encodes a NOT3/NOT5 ortholog in metazoans, termed NOT3 (or NOT3/5).[8] Finally, NOT10, C2orf29 and TAB182 were identified as subunits of the human CCR4-NOT complex, and these factors lack orthologs in
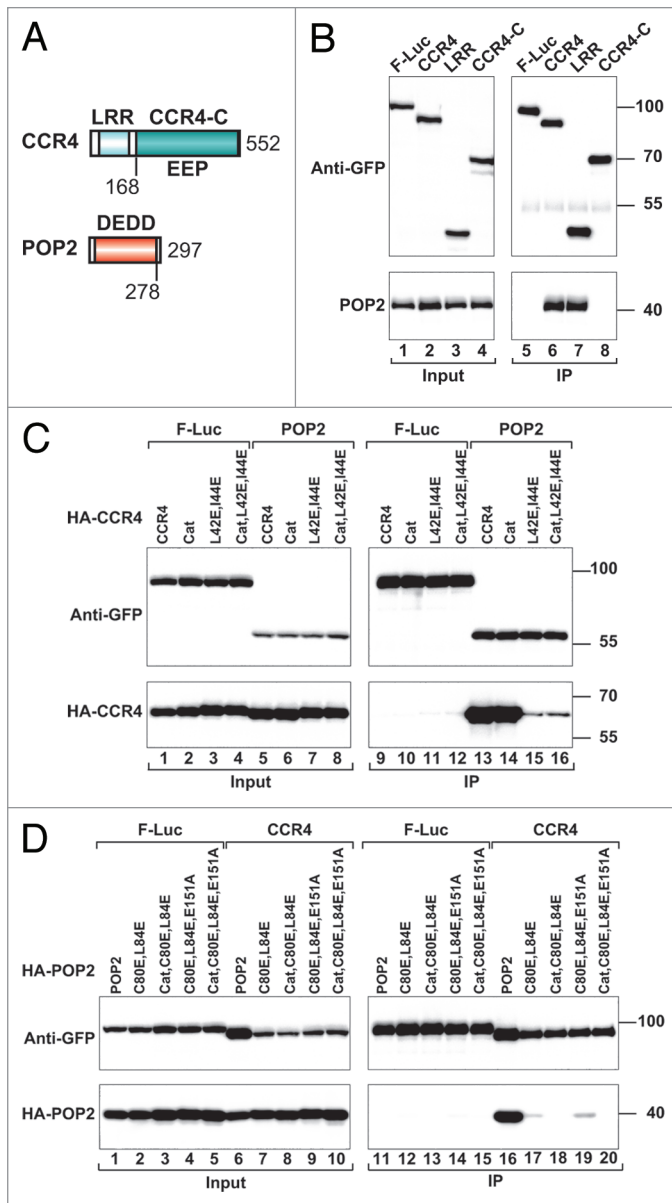
Figure 1. *Dm* CCR4 interacts with POP2. (**A**) Domain organization of *Dm* CCR4 and POP2. CCR4 contains a LRR domain and a catalytic EEP-nuclease domain (CCR4-C). POP2 consists of a single RNase D-like DEDD family catalytic domain. The numbers beneath the protein outline represent the amino acid position at the fragment/domain boundaries. (**B**) S2 cells were co-transfected with plasmids expressing GFP-tagged CCR4 (full-length or fragments) and HA-tagged POP2. GFP-tagged firefly luciferase (F-Luc) served as a negative control. Cell lysates were immunoprecipitated with polyclonal anti-GFP antibodies. Inputs (1%) and immunoprecipitates (5% GFP tagged proteins or 30% HA-tagged proteins) were subjected to western blotting using anti-GFP and anti-HA antibodies. (**C**) Interaction between GFP-tagged POP2 and the indicated CCR4 mutants. (**D**) Interaction between GFP-tagged CCR4 and the indicated POP2 mutants.

yeast.[18-20] Unlike TAB182, NOT10 and C2orf29 are conserved in metazoans, and NOT10 copurify with the *Dm* CCR4-NOT complex.[17] These observations point to differences in the composition of the CCR4-NOT complexes across species.

Studies of the interaction between the subunits of the CCR4-NOT complex have indicated that NOT1 acts as a scaffold for the assembly of the complex, providing binding sites for both the catalytic module and the CAF40, CAF130 and NOT2-NOT3/5 subunits.[18,21-28] However, how the NOT10 and C2orf29 subunits are incorporated into the CCR4-NOT complex and the precise function of these subunits are unknown.

The differences in the composition of the CCR4-NOT complexes in yeast and metazoans and the role of the metazoan CCR4-NOT complex in post-transcriptional regulatory mechanisms such as the miRNA pathway, which has no counterpart in fungi, highlight the importance of studying the assembly and function of this complex in multicellular eukaryotes. In this study, we characterized the assembly of the CCR4-NOT complex in *D. melanogaster* cells. We confirmed that the C-terminal regions of NOT2 and NOT3, which contain a highly conserved NOT-box domain, interact and dock onto the NOT1 C-terminal domain. We also defined the CAF40-binding site on NOT1 and demonstrated that it overlaps with a domain of unknown function (DUF3819) that is located between the binding sites for the catalytic module and the NOT2-NOT3 module. We further show that the *Dm* CNOT10 and C2orf29 orthologs CG18616 and CG13567, respectively, interact, thereby defining a new module of the CCR4-NOT complex. This module is recruited to the CCR4-NOT complex via an interaction with the NOT1 N-terminal domain and C2orf29. Similar results were obtained for the human CNOT10 and C2orf29 proteins in human cells. Finally, our functional studies demonstrate that all subunits in the CCR4-NOT complex trigger the degradation of a polyadenylated reporter in tethering assays and repress translation when tethered to a reporter lacking a poly(A) tail. These observations indicate that each subunit has the ability to recruit the remaining subunits of the complex to an RNA target, repressing its expression through a common mechanism.

Results

Assembly of the catalytic module of the *Dm* CCR4-NOT complex. To elucidate the assembly of the CCR4-NOT complex in metazoans, we investigated the interactions between the subunits of the CCR4-NOT complex in *D. melanogaster* Schneider cells (S2 cells). CCR4 consists of an N-terminal leucine-rich repeat (LRR) domain and a C-terminal catalytic domain (CCR4-C), and belongs to the endonuclease-exonuclease-phosphatase (EEP) enzyme family (**Fig. 1A**).[29,30] Yeast CCR4 has been shown to interact with POP2 through its LRR domain.[12-15,21,23] POP2 is a one-domain protein that adopts an RNase D-like fold and belongs to the DEDD nuclease family (**Fig. 1A**).[15,16,31,32] POP2 interacts with NOT1 and the CCR4 LRR domain, thereby bridging the interaction between CCR4 and NOT1.[12-16,18,21-23,26,27,33]
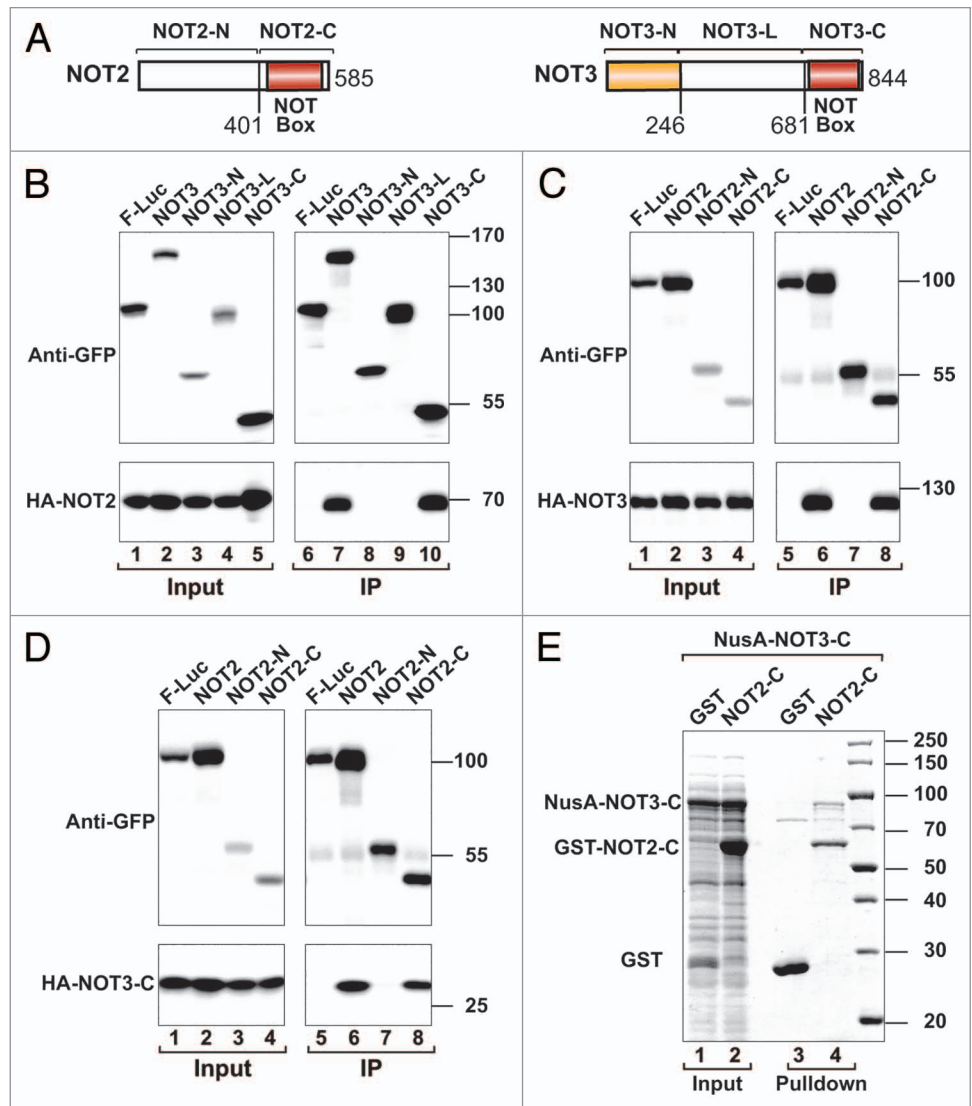
In immunoprecipitation assays using S2 cell lysates, we confirmed that *Dm* CCR4 and POP2 interact and that this interaction is mediated by the CCR4 LRR domain. Indeed, GFP-tagged *Dm* CCR4 and the isolated LRR domain co-immunoprecipitated HA-tagged POP2 (**Fig. 1B**, lanes 6 and 7). The CCR4 catalytic

domain had no detectable interaction with POP2 (**Fig. 1B**, lane 8).

The crystal structure of the *S. cerevisiae* POP2 protein (also known as CAF1) in complex with CCR4 identified critical interface residues that mediate the interaction between the two proteins.[15] Together with conservation of the protein folds, the conservation of these residues[15,16,29-32] suggests that POP2 and CCR4 interact in a similar manner in all eukaryotes. Therefore, based on the structure of the *S. cerevisiae* POP2-CCR4 complex,[15] we designed mutations in *Dm* POP2 and CCR4 to disrupt their interaction. A double L42E,I44E mutation on the CCR4 LRR domain strongly reduced its interaction with POP2 (**Fig. 1C**, lane 15), as has been described for *S. cerevisiae* CCR4.[15] In contrast, a catalytically inactive CCR4 mutant (Cat: D412A,N414A) interacted with POP2 as efficiently as wild-type (**Fig. 1C**, lane 14). Accordingly, mutation of catalytic residues did not exacerbate the effect of the double L42E,I44E mutation (**Fig. 1C**, lane 16). Conversely, substitution of POP2 residues C80 and L84 (corresponding to *S. cerevisiae* POP2 residues A215 and F219, respectively) with glutamic acid strongly impaired binding to CCR4 (**Fig. 1D**, lane 17). These results indicate that the POP2-CCR4 interface is conserved.

NOT2 and NOT3 proteins interact through their C-terminal regions comprising the NOT-boxes. NOT2 and NOT3 are related proteins that share a conserved C-terminal motif termed the NOT-box (**Fig. 2A**).[34] The sequence identity between the *Dm* NOT2 and NOT3 NOT-boxes is 28%.[34] In addition to the NOT-box, NOT2 contains a less-conserved N-terminal extension, which is rich in glycine (22.6%) and serine (12.4%) residues and is predicted to be unstructured (NOT2-N; **Fig. 2A**). NOT3 contains a highly conserved N-terminal domain (NOT3-N) that is predicted to be primarily α-helical, which is connected to the NOT-box by a linker region (NOT3-L) that is rich in serine (16.4%) and glutamine (11.8%) residues (**Fig. 2A**).

To define the NOT2 and NOT3 regions that are required for their interaction, we performed co-immunoprecipitation assays using S2 cells. We observed that GFP-tagged NOT3

co-immunoprecipitated HA-tagged NOT2 and that this interaction was mediated by a NOT3 C-terminal fragment comprising the NOT-box (**Fig. 2B**, lanes 7 and 10). Conversely, GFP-tagged NOT2 co-immunoprecipitated full-length NOT3 and NOT3-C, and these interactions were also mediated by the NOT2 C-terminal region containing the NOT-box (**Fig. 2C and D**, lanes 8), which is in agreement with previous studies.[18,21,27,28] The NOT2-NOT3 interaction is direct because a GST (glutathione S-transferase)-tagged NOT2 C-terminal fragment (GST-NOT2-C) pulled down the C-terminal NOT3 fragment, which was expressed in *Escherichia coli* with a NusA tag (**Fig. 2E**,

Figure 2. NOT2 and NOT3 interact via their C-terminal regions, which contain NOT-boxes. (**A**) NOT2 and NOT3 share a conserved C-terminal motif termed the NOT-box. NOT2 contains a less conserved N-terminal extension that is predicted to be unstructured (NOT2-N). NOT3 contains a highly conserved N-terminal domain (NOT3-N) that is connected to the NOT-box by a linker region (NOT3-L). (**B**) S2 cells co-expressing GFP-tagged NOT3 (full-length or fragments) and HA-tagged NOT2 were lysed 3 d after transfection. Cell lysates were immunoprecipitated using polyclonal anti-GFP antibodies and analyzed as described in **Figure 1**. (**C and D**) The interaction between GFP-tagged NOT2 (full-length or fragments) and HA-tagged NOT3 or NOT3-C was analyzed as described in **Figure 1**. (**E**) Interaction between recombinant NusA-tagged NOT3-C and GST-tagged NOT2-C. GST was used as a negative control.

Figure 3. NOT1 interacts with the catalytic module and with the NOT2-NOT3 complex. (**A**) NOT1 domain organization. NOT1 consists of an N-terminal (NOT1-N), middle (NOT1-M) and C-terminal (NOT1-C) region. The NOT1-N region contains a metazoan-specific α-helical domain (residues 1–412) that interacts with NOT10 and NOT11 (NOT10/11-binding domain (NOT10/11-BD), this study). The NOT1-M region comprises a MIF4G domain that interacts with POP2[15,16] and a domain of unknown function (DUF3819) that interacts with CAF40 (this study). The NOT1-C region harbors a conserved NOT1 domain. (**B**) S2 cells were co-transfected with plasmids expressing GFP-tagged NOT1 and HA-tagged deadenylase subunits as indicated. GFP-tagged firefly luciferase served as a negative control. Cell lysates were immunoprecipitated using polyclonal anti-GFP antibodies. Inputs and immunoprecipitates were analyzed as described in **Figure 1**. (**C**) Interaction of POP2 with the NOT1-M region and the MIF4G domain. (**D and E**) The interaction of GFP-NOT1 (full-length or the indicated fragments) with HA-tagged NOT2 and NOT3 was analyzed as described in **Figure 1**.

lane 4). We conclude that the NOT2 and NOT3 C-terminal regions containing the NOT-boxes mediate the assembly of the NOT2-NOT3 complex.

Binding of POP2-CCR4 and NOT2-NOT3 complexes to the NOT1 scaffold. Studies of the interaction of the CCR4-NOT complex subunits indicate that NOT1 serves as a scaffold protein, providing binding sites for the catalytic module and the additional subunits of the complex (**Fig. 3A**).[18,21-28] Therefore, we investigated the interaction of NOT1 with the conserved core components of the CCR4-NOT complex. We observed that GFP-tagged NOT1 co-immunoprecipitated HA-tagged NOT2,

NOT3, POP2 and CCR4 (**Fig. 3B**), which is in agreement with the hypothesis that NOT1 serves as a scaffold protein.

To define the NOT1 domains involved in binding to the core complex subunits, we performed co-immunoprecipitation assays using a series of NOT1 deletion mutants. Sequence comparison, secondary structure predictions and available structural information indicate that NOT1 consists of three main regions: N-terminal (NOT1-N), Middle (NOT1-M) and C-terminal (NOT1-C) (**Fig. 3A**). The NOT1-N is predicted to be entirely α-helical and structural information is available for a *S. cerevisiae* N-terminal region corresponding to *Dm* NOT1 residues

415–1,373.[15] The NOT1-M region contains an N-terminal MIF4G domain that directly interacts with POP2[15,16] and a DUF3819 domain of unknown function (**Fig. 3A**). The NOT1-C region contains a conserved NOT1 homology domain (**Fig. 3A**).

First, we confirmed that the NOT1-M region interacts with POP2 via the MIF4G domain as was recently shown for human and yeast POP2 (**Fig. 3C**, lanes 8 and 10).[15,16] We also investigated the NOT1 interaction with NOT2 and NOT3 and observed that these interactions were mediated by the NOT1-C domain (**Fig. 3D and E**, lanes 12), which is in agreement with previous studies.[18,22-24]

NOT2 and NOT3 interact with NOT1 via their C-terminal regions, which contain the NOT-boxes. We next defined the NOT2 and NOT3 regions required for NOT1-C binding. We observed that the regions mediating NOT2-NOT3 interaction were also required for NOT1-C binding. Indeed, NOT2 and NOT3 co-immunoprecipitated NOT1-C through their C-terminal regions (**Fig. 4A and B**). Previous studies have indicated that the interaction between NOT1 and NOT3 is mediated by NOT2.[18,21,22,24,28] To more precisely define the NOT2 sequences that interact with NOT1 and NOT3, we generated a series of C-terminal fragments containing the NOT-box (residues 444–566) and increasing N-terminal extensions. However, only the NOT2 fragment 402–585 interacted with NOT1-C and NOT3-C as efficiently as full-length NOT2 (**Fig. 4C and D**, lane 12 vs. 10), whereas the 436–585 fragment failed to interact with NOT1-C and exhibited residual NOT3 binding (**Fig. 4C and D**, lane 13). These results indicate that the NOT2 NOT-box is not sufficient for NOT1 and NOT3 binding and that residues 402–436 (upstream of the NOT-box) are required for the interaction with NOT1 and NOT3. To determine whether these residues were sufficient for NOT1 binding, we performed co-immunoprecipitation assays using NOT2 fragments comprising residues 1–436 and 402–436 (fused to GFP). We observed that these fragments did not interact with NOT1 (**Fig. 4E**, lanes 7 and 8). Therefore, both the NOT2 NOT-box and the upstream N-terminal sequences (residues 402–436) are required for NOT1 binding.

CAF40 interacts with a middle region of NOT1. CAF40 contains a highly conserved domain comprising six armadillo repeats (**Fig. 5A**).[35] In yeast, CAF40 interacts with NOT1; however, the domains mediating the NOT1 and CAF40 interaction have not been precisely defined.[25] In immunoprecipitation assays, we confirmed that CAF40 preferentially associates with NOT1 (**Fig. 5B**, lane 8), specifically, the NOT1-M region (**Fig. 5C**, lane 9). These results indicate that CAF40 binds NOT1 independently of NOT2 and NOT3, which interact with the NOT1-C fragment. A more detailed analysis indicates that CAF40 interacts with the C-terminal portion of the NOT1-M fragment (residues 1,387–1,717) comprising the DUF3819 domain and not with the MIF4G domain, which interacts with POP2[15,16] (**Fig. 5D**, lane 14). Moreover, CAF40 interacts with a NOT1-M fragment harboring a mutation that abolishes its interaction with POP2 (K1277A)[16] (**Fig. 5D**, lane 16). These results indicate that CAF40 binds NOT1 independently of POP2. Additionally, CAF40 did not detectably interact with NOT10 and C2orf29 (see below).

The interaction between CAF40 and NOT1 fragment 1,387–1,717 was mediated by the highly conserved armadillo repeat domain in CAF40 (**Fig. 5E**, lane 6).

NOT10 and C2orf29 (NOT11) form a conserved module of the CCR4-NOT complex. Human CNOT10 and C2orf29 were originally identified as subunits of the human CCR4-NOT complex.[18-20] These proteins are conserved in *D. melanogaster* (NOT10 and CG13567, respectively) and NOT10 co-purify with the *Dm* CCR4-NOT complex.[17] Both proteins are predicted to be primarily α-helical. CG13567 contains a conserved domain of unknown function named DUF2363 (**Fig. 6A**). In the accompanying manuscript by Mauxion et al.,[36] human C2orf29 is shown to be a bona fide subunit of the human CCR4-NOT complex and is termed CNOT11. In accordance with the accompanying manuscript,[36] we will refer to *Dm* CG13657 as *Dm* NOT11 hereafter.

In immunoprecipitation assays, we observed that NOT10 strongly interacted with NOT11, suggesting that they form a complex (**Fig. 6B**, lane 7). NOT10 also interacted with itself (**Fig. 6B**, lane 6). Conversely, NOT11 strongly interacted with NOT10 (**Fig. 6C**, lane 6).

To investigate whether the NOT10-NOT11 interaction was direct, we coexpressed the proteins in *E. coli*. We observed that MBP(maltose binding protein)-tagged NOT10 co-purified with GST-tagged NOT11, but not with GST, on glutathione agarose beads (**Fig. 6D**, lane 6 vs. 4). Conversely, GST-NOT11 copurified with MBP-NOT10 but not with MBP on amylose resin (**Fig. 6D**, lane 9 vs. 8). Furthermore, the expression levels of MBP-NOT10 increased by coexpression with GST-NOT11. We conclude that NOT10 and NOT11 directly interact and form a new module of the CCR4-NOT complex.

The NOT10-NOT11 complex interacts with the N-terminal NOT1 domain. To investigate how the NOT10-NOT11 complex interacts with the CCR4-NOT complex, we performed immunoprecipitation assays in S2 cells. We observed that NOT1 and NOT10 interact with NOT11 (**Fig. 7A**, lanes 10 and 11, respectively). The NOT1-NOT11 interaction was detectable only when NOT11 was used as bait (**Fig. 7A** and data not shown) and was enhanced in S2 cells in which NOT10 was also coexpressed (**Fig. 7A**, lane 12 vs. 10), suggesting that NOT10 and NOT11 interact with NOT1 as a complex. Further analysis revealed that NOT10 interacts with the NOT1-N region (**Fig. 7B**, lane 8 and **Fig. 7C**, lane 10) but not with the NOT1-M or NOT1-C fragments (**Fig. 7B**, lanes 9 and 10 and **Fig. 7C**, lane 11). Moreover, the 412 N-terminal-most residues of NOT1 were sufficient for binding to NOT10 (**Fig. 7C**, lane 12), although the NOT1 fragment 416–1,148 retained residual binding (**Fig. 7C**, lane 14).

To investigate which protein in the NOT10-NOT11 complex directly interacts with, NOT1 we co-expressed GST-tagged NOT1 N-terminal fragments (1–412 or 1–1,083) with MBP-tagged NOT10 or NOT11 in *E. coli* and performed pull-down assays. We found that NOT11, but not NOT10, interacted with the NOT1 N-terminal fragments (**Figs. 7D and E**, lanes 6 and 9; and data not shown), indicating that NOT11 is the subunit that docks the NOT10-NOT11 complex onto the NOT1-N domain. This result was unexpected because in immunoprecipitation
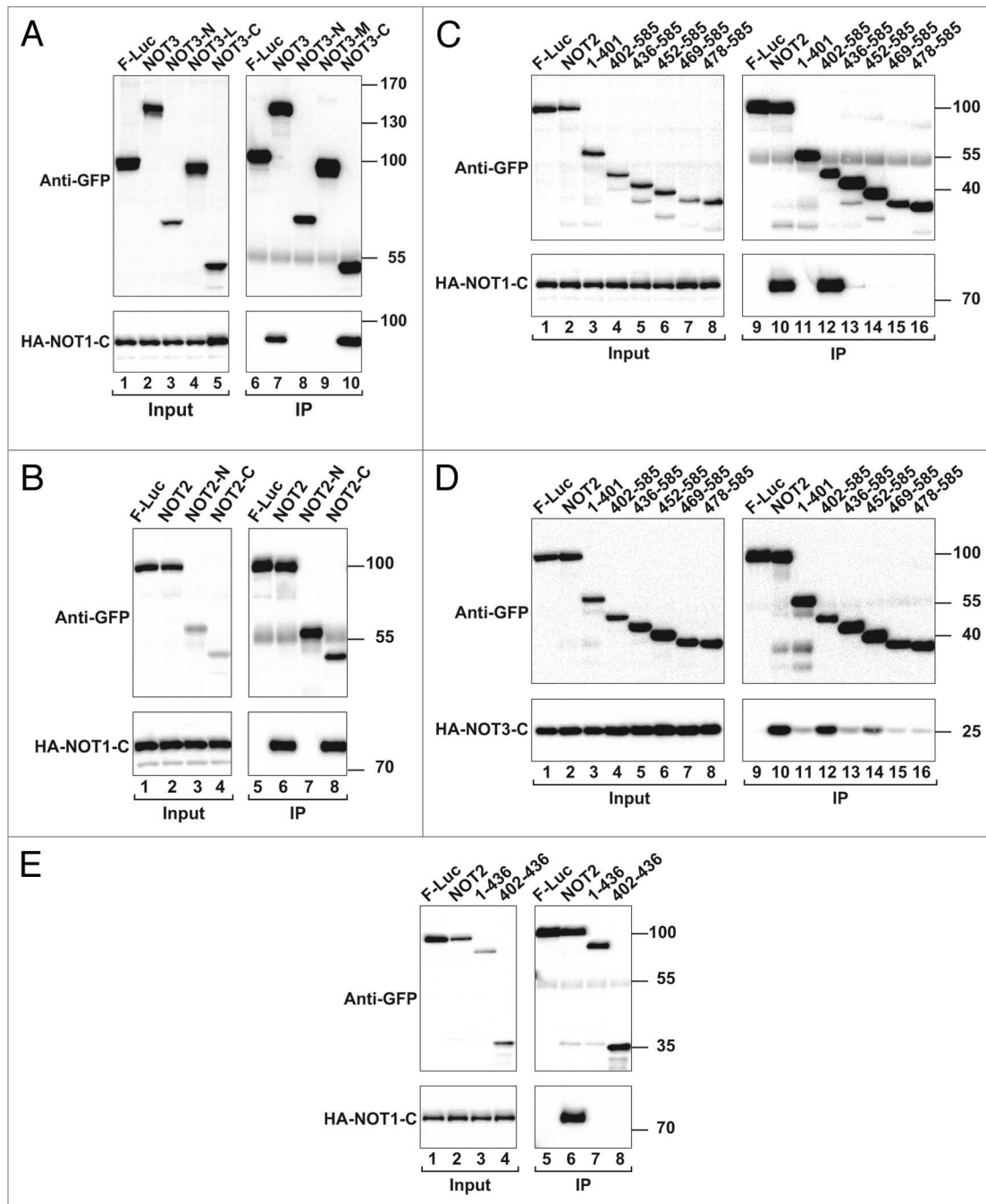
Figure 4. The NOT2 NOT-box is not sufficient for binding to NOT1-C and NOT3-C. (**A and B**) Interaction between GFP-tagged NOT3 and NOT2 (full-length or fragments) and HA-tagged NOT1-C. (**C–E**) Interaction between GFP-tagged NOT2 (full-length or fragments) and HA-tagged NOT1-C and NOT3-C.

assays, NOT10 interacted with NOT1 more efficiently and enhanced NOT11 binding (**Fig. 7A**). One possible explanation for this observation is that NOT11 is present in excess in S2 cells. Therefore, overexpressed NOT10 efficiently binds endogenous NOT11 and NOT1, whereas overexpressed NOT11 does not efficiently compete with endogenous NOT11 for NOT1 binding unless NOT10 is also overexpressed. This possibility also suggests that NOT10 facilitates the interaction between NOT11 and NOT1, either by contacting NOT1 directly or by stabilizing

the NOT11 fold. Together, our results indicate that NOT10 and NOT11 form a complex that docks onto the NOT1 scaffold via interactions with NOT11 and the N-terminal NOT1 domain.

Human CNOT10 and CNOT11 form a complex that interacts with the CNOT1 N-terminal domain. To investigate whether the interactions between NOT1, NOT10 and NOT11 are conserved in humans, we performed immunoprecipitation assays of the human orthologs in human HEK293 cells (**Fig. 8A**). As observed for the *D. melanogaster* proteins, human CNOT10
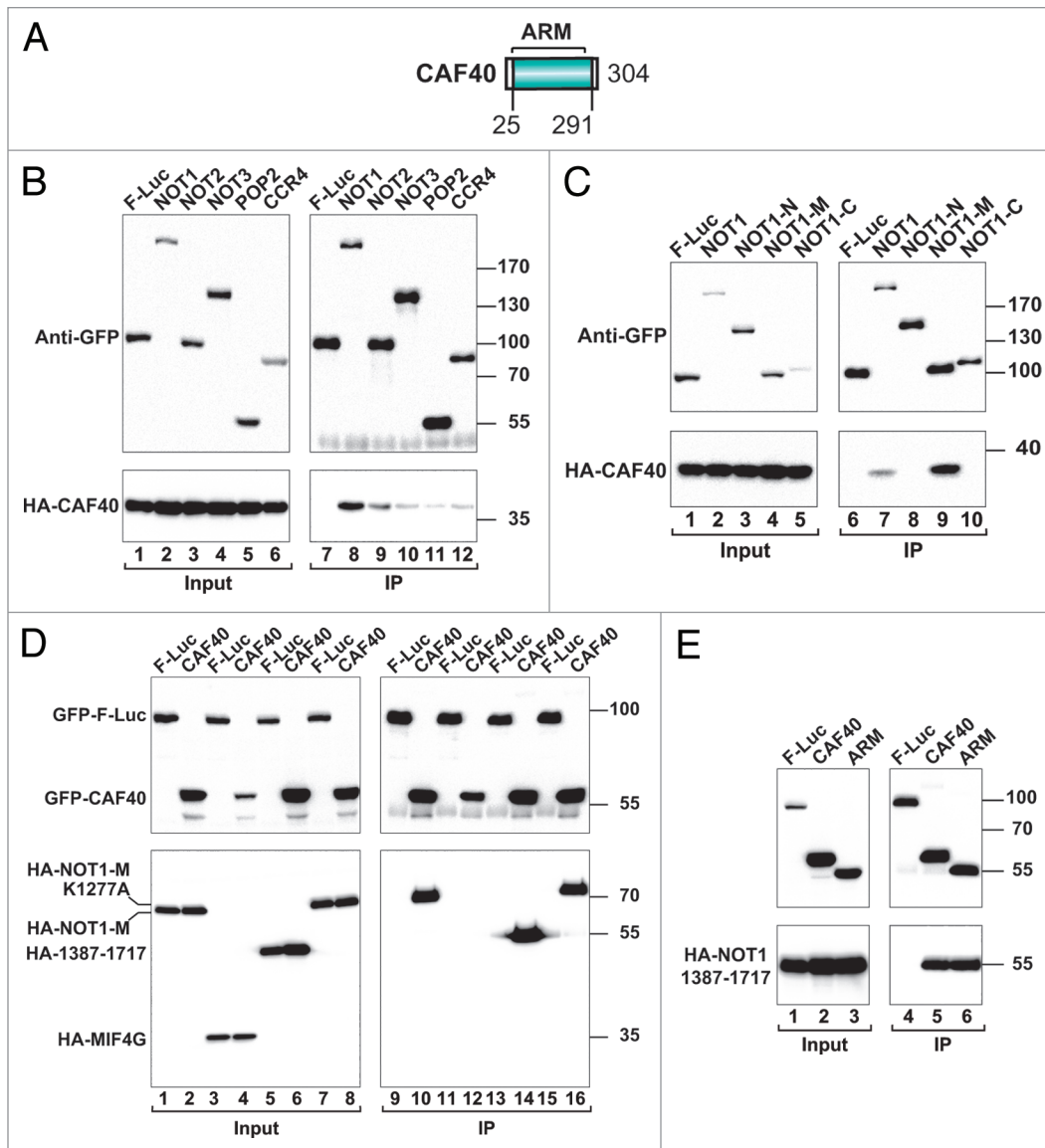
Figure 5. CAF40 interacts with a NOT1 fragment comprising the DUF3819 domain. (**A**) CAF40 contains a highly conserved domain comprising six armadillo repeats (ARM). (**B**) S2 cells were transiently transfected with expression vectors encoding GFP-tagged subunits of the CCR4-NOT complex and HA-tagged CAF40. Cell lysates were immunoprecipitated using anti-GFP antibodies and analyzed as described in **Figure 1**. GFP-F-Luc served as a negative control. (**C**) Interaction between GFP-tagged NOT1 (full-length or fragments) and HA-tagged CAF40. (**D**) Interaction between GFP-tagged CAF40 and the indicated HA-tagged NOT1 fragments. (**E**) Interaction between GFP-tagged CAF40 (full-length or the armadillo repeat domain) and a HA-tagged NOT1 fragment comprising the DUF3819 domain.

interacted with CNOT1 and CNOT11 (**Fig. 8B**, lanes 9 and 14, respectively). More specifically, CNOT10 interacted with the N-terminal domain of CNOT1 (CNOT1-N) but not with the CNOT1-M or CNOT1-C domains (**Fig. 8B**, lanes 10–12). The interaction between CNOT11 and CNOT1 was weak and was enhanced when CNOT10 was co-expressed as observed in S2 cells (**Fig. 8C**, lane 6 vs. 8). In contrast to the *D. melanogaster* proteins, the 302 N-terminal-most residues of CNOT1 were not sufficient for CNOT10 binding (**Fig. 8B**, lane 13).

Human CNOT11 consists of a highly conserved C-terminal DUF2363 domain (residues 260–496; **Fig. 8A**) and a less conserved N-terminal region that is absent in *Dm* NOT11. Because

the interaction of CNOT11 with CNOT10 is conserved, we speculated that this interaction is most likely mediated by the most conserved region of CNOT11, the DUF2363 domain. Accordingly, we observed that a CNOT11 fragment comprising the DUF2363 domain interacted with CNOT10 as efficiently as full-length CNOT11 did (**Fig. 8D**, lanes 6 vs. 5). We conclude that CNOT10 and CNOT11 form a complex that interacts with CNOT1 in both human and *D. melanogaster* cells.

NOT10 and NOT11 promote the degradation of bound mRNAs. Tethering of the CCR4-NOT complex subunits promotes the degradation of polyadenylated mRNA targets.[10,37] These effects have been shown for NOT1, NOT2, NOT3, CCR4
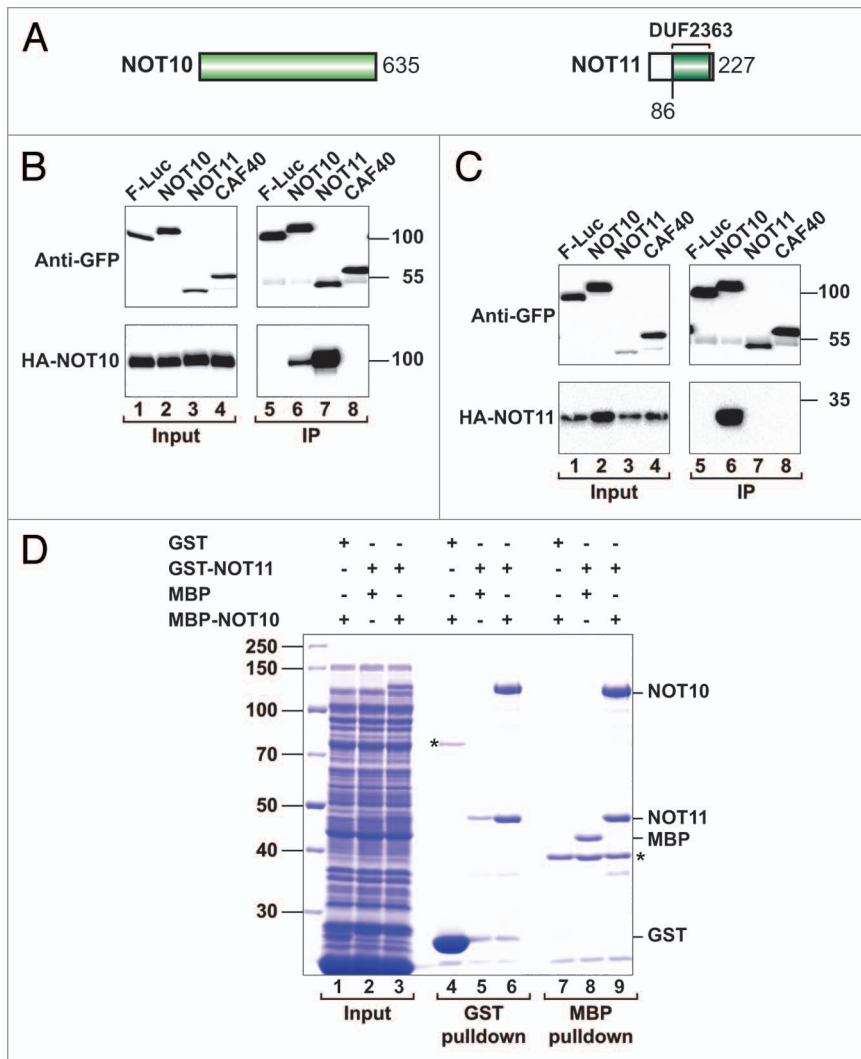
Figure 6. NOT10 directly interacts with NOT11. (**A**) NOT10 and NOT11 (also known as CG13567 or C2orf29) are predicted to be primarily α-helical. NOT11 contains a conserved domain of unknown function named DUF2363. (**B and C**) S2 cells were transiently transfected with expression vectors encoding GFP-tagged NOT10, NOT11 or CAF40 and HA-tagged NOT10 (**B**) or NOT11 (**C**). Cell lysates were immunoprecipitated with anti-GFP antibodies and analyzed as described in **Figure 1**. (**D**) The interaction between recombinant GST-tagged NOT11 and an MBP fusion of NOT10 was analyzed by SDS-PAGE followed by Coomassie blue staining. The pull-downs were performed using glutathione agarose beads (lanes 4–6) or amylose resin (lanes 7–9). Input samples (1%) and bound fractions (50%) were analyzed on SDS PAGE. The asterisk indicates a contaminant protein that copurified with GST (lane 4) or bound to the amylose resin (lanes 7–9).

CCR4-NOT subunits in a tethering assay. For this assay, CCR4-NOT complex subunits were expressed with an N-terminal tag derived from the N protein of the bacteriophage λ (λN tag) to enable tethering to a firefly luciferase (F-Luc) reporter.[38] The F-Luc reporter contained five Box B hairpins (5BoxB) inserted in the 3' UTR;[38] which bind the λN tag with high affinity and recruit CCR4-NOT complex subunits to the F-Luc-5BoxB mRNA. We observed that all of the CCR4-NOT subunits repressed the expression of the F-Luc-5BoxB mRNA reporter (**Fig. 9A**). Northern blot analyses revealed that all of the subunits also reduced the abundance of the F-Luc-5BoxB mRNA to different extents (**Fig. 9B**). CAF40 was a potent trigger of mRNA degradation, whereas although active, NOT10 and NOT11 promoted F-Luc mRNA degradation less efficiently (**Fig. 9B**). All subunits were expressed at comparable levels and had no effect on an F-Luc reporter lacking the BoxB hairpins (**Fig. 9C, D and G**).

To identify the CCR4-NOT complex subunits that can promote translational repression, we used an F-Luc-5BoxB reporter whose 3' end is generated by a self-cleaving hammerhead ribozyme (F-Luc-5BoxB-HhR) and lacks a poly(A) tail.[39] In agreement with previous studies,[10,37] tethering of the λN-tagged NOT1, NOT2, NOT3, CCR4 and POP2 subunits reduced luciferase activity without affecting mRNA abundance (**Fig. 9E and F**).[10,37] CAF40 was a potent translational repressor when expressed at comparable levels, whereas NOT10 was inactive at the concentration tested (**Fig. 9E**). These results indicate that the tethering of any subunit of the CCR4-NOT complex leads to the recruitment of additional subunits via direct and indirect protein-protein interactions (except NOT10 at this concentration), resulting in the translational repression of unadenylated reporters or the deadenylation and degradation of polyadenylated mRNAs.

Multiple NOT1 domains promote target degradation. Given that all of the CCR4-NOT complex subunits have the ability to recruit the complete complex, tethering assays using full-length proteins do not reveal the contribution of the individual subunits to translational repression and mRNA degradation. Therefore, we examined the activity of isolated protein domains. We observed that the NOT1-N fragment, which interacts with NOT10 and NOT11, was inactive in tethering assays, irrespective of the poly(A) tail (**Fig. 10A–F**). Accordingly, deletion of the NOT1-N region did not significantly affect the activity of the NOT1 protein in tethering assays (**Fig. 10A–F**). NOT1

and POP2.[10,37] These data suggest that tethered complex subunits recruit the catalytic module to promote the deadenylation and degradation of mRNAs. Additionally, the CCR4-NOT complex subunits repress the translation of mRNA reporters lacking poly(A) tails in tethering assays, indicating that the CCR4-NOT complex has the ability to repress translation in the absence of deadenylation.[10,37] The identity of the CCR4-NOT subunits mediating translational repression and the mechanism of this repression are unknown.

To gain insight into the role of NOT10 and NOT11, we compared the activity of these proteins with that of additional

Figure 7. The NOT10-NOT11 complex interacts with the NOT1 N-terminal domain. (**A**) S2 cells were co-transfected with a mixture of two plasmids: one expressing GFP-NOT11 and one expressing HA-NOT1. In addition, where indicated, the transfection mixtures contained a third plasmid expressing HA-NOT10 (lanes 2, 3, 5 and 6). Cell lysates were immunoprecipitated using polyclonal anti-GFP antibodies. The inputs and immunoprecipitates were analyzed by western blotting as described in **Figure 1**. (**B and C**) Interaction between GFP-tagged NOT1 (full-length or fragments) and HA-tagged NOT10. (**D and E**) The interaction between recombinant GST-tagged NOT1 (1–1083 or 1–412) and an MBP fusion of NOT11 was analyzed by SDS-PAGE followed by Coomassie blue staining, as described in **Figure 6D**.
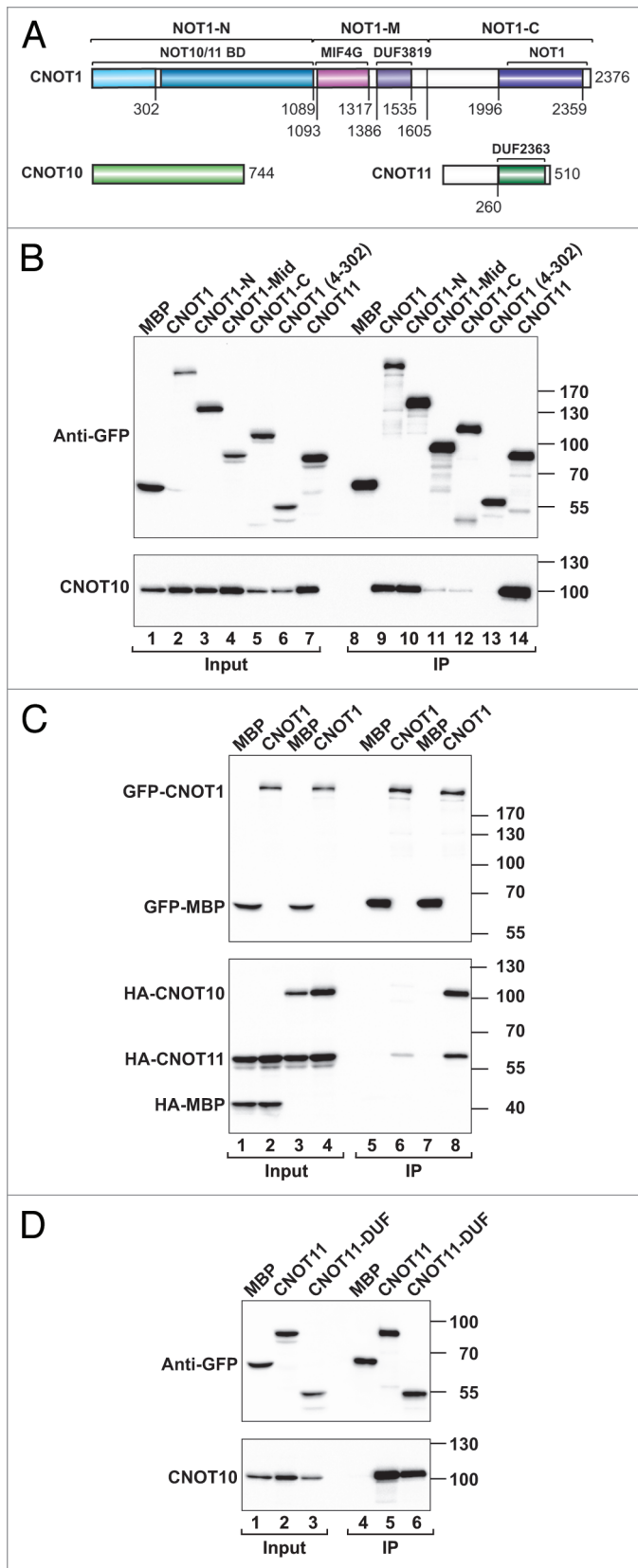
Figure 8. The NOT10-NOT11 complex is conserved in human cells. (**A**) Human CNOT1 consists of an N-terminal (NOT1-N), middle (NOT1-M) and C-terminal (NOT1-C) region. The POP2-binding domain adopts an MIF4G fold and is termed the CNOT1 MIF4G domain.[15,16] The CNOT1-C region harbors a conserved NOT1 homology domain. (**B**) GFP-tagged CNOT1 (full-length or fragments) or GFP-CNOT11 were co-expressed with HA-tagged CNOT10 in HEK293 cells. The GFP-tagged proteins were immunoprecipitated from RNase A-treated cell lysates using anti-GFP antibodies. GFP-tagged MBP served as a negative control. Inputs (1.25% for the GFP-tagged proteins or 0.5% for HA-tagged proteins) and immunoprecipitates (10% for the GFP-tagged proteins or 25% for HA-tagged proteins) were analyzed by western blotting. (**C**) HEK293 cells were cotransfected with a mixture of two plasmids: one expressing GFP-CNOT1 and one expressing HA-CNOT11. In addition, where indicated, the transfection mixtures contained a third plasmid expressing HA-CNOT10 (lanes 3, 4, 7 and 8). Cell lysates were immunoprecipitated using polyclonal anti-GFP antibodies. The inputs and immunoprecipitates were analyzed by western blotting as described in **B**. (**D**) Interaction of GFP-tagged CNOT11 (full-length or DUF2363 domain) with HA-tagged CNOT10 in HEK293 cells.

mRNA regulation. In contrast, the NOT1-M and NOT1-C fragments elicited translational repression and mRNA degradation (**Fig. 10A–F**). The activity of the NOT1-M fragment was reduced by a single amino acid substitution (K1277A), which disrupts POP2 binding[16] (**Fig. 10H**), indicating that the activity of the NOT1-M fragment is primarily mediated by the catalytic module but not by CAF40. However, the K1277A mutation had only a minor effect in the context of full-length NOT1 (**Fig. 10I**), indicating that the interaction with POP2 contributes but is not strictly required for NOT1 to promote translational repression and mRNA degradation in tethering assays.

The catalytic module requires interaction with the NOT module for full activity. We next tested the activity of POP2 and CCR4 mutants in tethering assays. A catalytically inactive POP2 mutant (Cat, D53A+E55A) promoted mRNA degradation as reported previously.[10,37] In contrast, a POP2 mutant (E151A)[16] that does not interact with NOT1 was impaired in tethering assays (**Fig. 11A**). Similarly, mutations that disrupt the interaction with CCR4 (C80E,L84E) impaired POP2 activity in tethering assays (**Fig. 11A**). POP2 activity was abolished when the mutations that disrupt NOT1 and CCR4 binding were combined (**Fig. 11A**). The effect of these mutations was independent of whether POP2 was catalytically active or inert (**Fig. 11A**). These results indicate that wild-type POP2 requires interaction with NOT1 and CCR4 for full activity and that the catalytic activity of POP2 is not sufficient to trigger degradation of the reporter in tethering assays. All POP2 mutants were expressed at comparable levels (**Fig. 11B**).

For CCR4, we observed that the isolated LRR, but not the catalytic domain (CCR4-C), was active in tethering assays at the concentrations tested (**Fig. 11C**). Accordingly, a catalytically inactive CCR4 mutant also promoted target degradation (**Fig. 11C**, Cat: D412A,N414A). This mutant still interacted with POP2 (**Fig. 1C**, lane 14). The activity of CCR4 or the isolated LRR was abolished by mutations that disrupt POP2 binding (L42E,I44E) despite the fact that these mutants were expressed at levels comparable to the wild-type (**Fig. 11D**). Therefore, as shown for POP2, the catalytic activity of CCR4 is not sufficient

fragments were expressed at comparable levels (**Fig. 10G**). This result suggests that the NOT1-N region and, thus, the NOT10-NOT11 complex may not participate in post-transcriptional
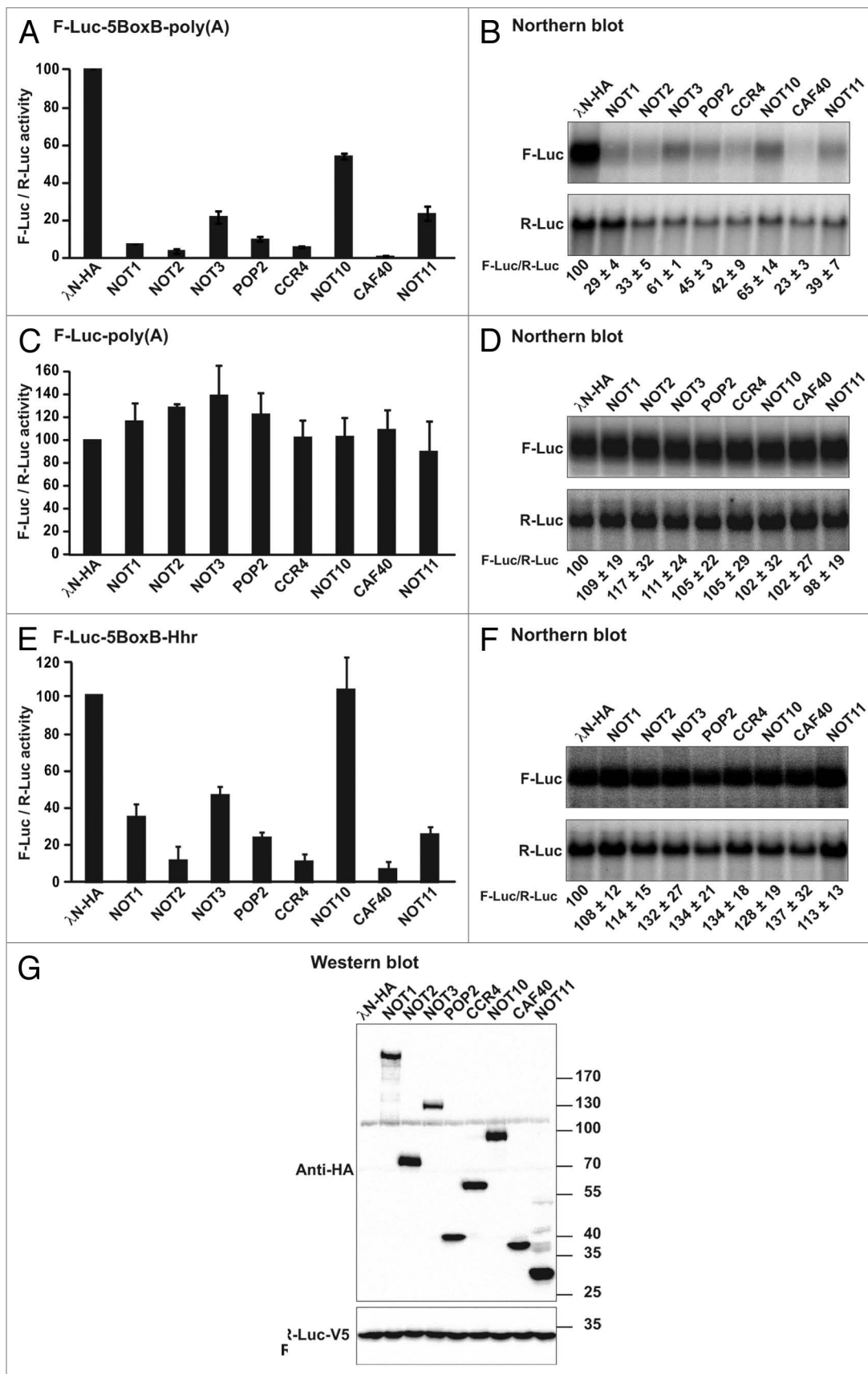
Figure 9. For figure legend, see page 239.

to trigger degradation of the reporter in tethering assays at the concentration tested. However, it is important to note that at higher concentrations, the catalytic domain was active.

Importantly, the POP2 and CCR4 mutants that promoted degradation of the polyadenylated reporter also promoted translational repression of the unadenylated reporter (**Fig. S1**), indicating that these activities are interconnected. Furthermore, at the concentration tested, the proteins had no effect on a reporter lacking the BoxB hairpins, indicating that the effects are specific (**Fig. S1**).

The NOT2 N-terminal domain promotes the translational repression and degradation of bound mRNAs. We also analyzed the activity of NOT2 and NOT3 fragments. For NOT2, we observed that the N-terminal extension and the C-terminal region were active in tethering assays (**Fig. 12A and B**). In contrast, for NOT3, only the C-terminal region, which interacts with NOT2, was active (**Fig. 12C and D**), suggesting that NOT3 promotes mRNA degradation through its interaction with NOT2, which, in turn, interacts with the remainder of the CCR4-NOT complex. As shown for POP2 and CCR4, the NOT2 and NOT3 fragments that promoted degradation of the polyadenylated reporter also promoted translational repression of the unadenylated reporter but had no effect on a reporter lacking the BoxB hairpins (**Fig. S1**).

The observation that the isolated NOT2 N-terminal region promotes target degradation was unexpected because this region exhibited no detectable interaction with the core complex subunits, suggesting that this region interacts with unidentified protein partners. Our observations indicate that in contrast to the full-length proteins, only a subset of protein domains can cause translational repression and mRNA degradation independently (e.g., NOT2-N, NOT2-C, NOT1-M and NOT1-C). Further studies are required to determine whether these domains have intrinsic activity or interact indirectly with additional complex subunits or with unknown partners to regulate mRNA expression. In particular, structural studies of the interaction of subunits of the complex are needed to provide information on how to specifically disrupt these interactions, which is of critical importance to evaluate the contribution of the individual subunits to mRNA degradation and translational repression.
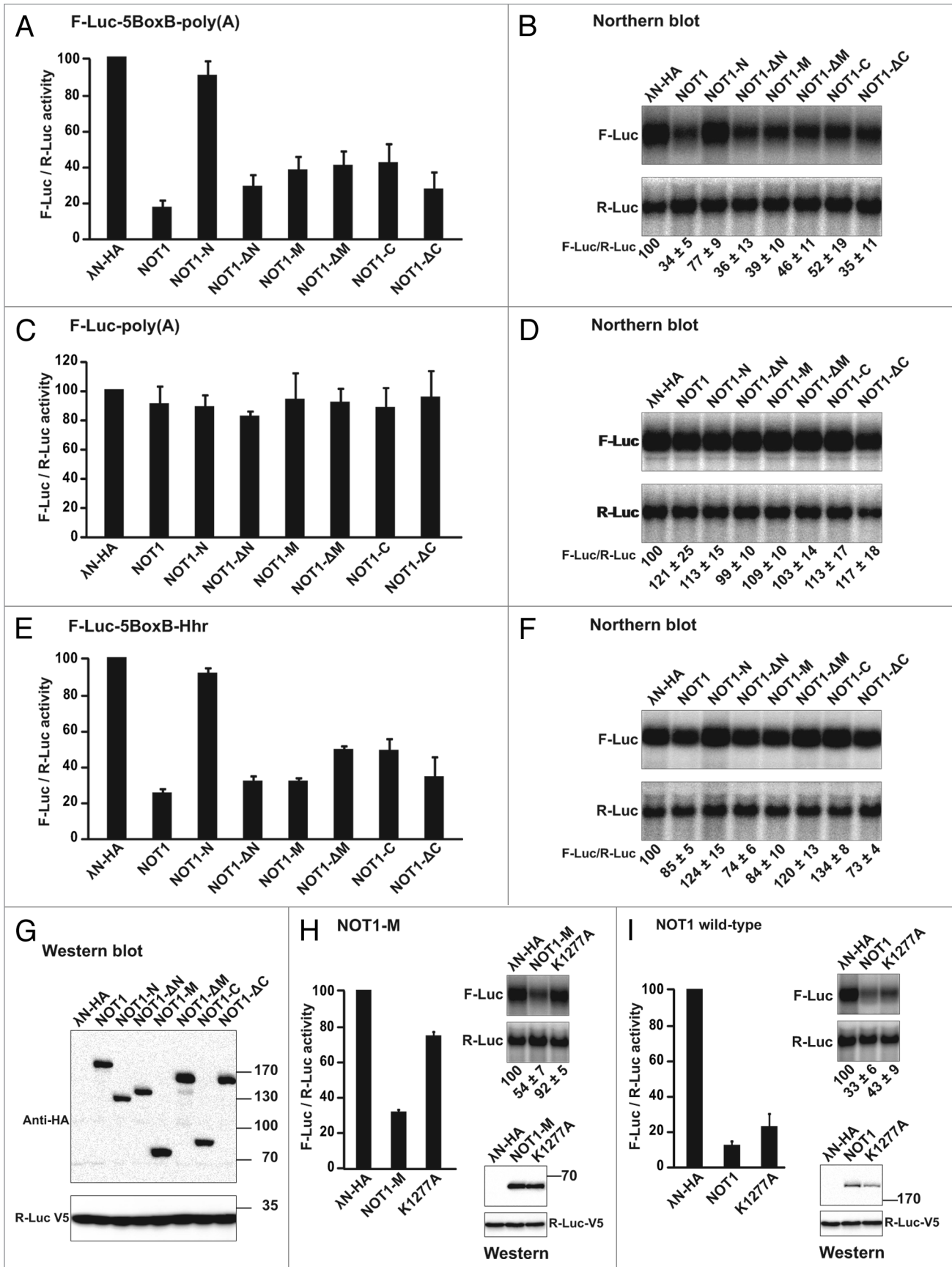
Discussion

The CCR4-NOT complex is a master regulator of mRNA expression. It promotes translational repression, which can occur even in the absence of deadenylation, and can direct the irreversible degradation of mRNA targets.[3,8,10,37] In addition to its role in mRNA regulation, the CCR4-NOT complex has been implicated in a wide range of cellular processes, including transcription, ubiquitination, DNA repair and protein modification.[8] Although most of the complex subunits are conserved among eukaryotes, yeast-specific and metazoan-specific subunits have been described, indicating that the complex composition differs across species. Therefore, the study of this multifunctional complex in diverse organisms is relevant and promises to further our understanding of its diverse molecular functions. In this study, we characterized the *Dm* CCR4-NOT complex. We confirmed and extended the interactions that have been described in other species and defined the domains mediating the NOT1-CAF40 interaction (**Fig. 13**). We further demonstrated that NOT10 and NOT11 interact and dock onto the N-terminal NOT1 domain through NOT11 (**Fig. 13**) in both *D. melanogaster* and human cells. Similar results are presented in the accompanying manuscript describing interactions between human CNOT10, CNOT11 and CNOT1.[36] We conclude that NOT10 and NOT11 form a conserved module of the CCR4-NOT complex. Finally, our analysis of the protein domains that mediate the interactions between the subunits and play a role in mRNA degradation provides a foundation for future studies aimed at understanding how the complex assembles and regulates the expression of target mRNAs.

Materials and Methods

Co-immunoprecipitation assays in *D. melanogaster* and human cells. The plasmids encoding the deadenylase subunits for expression in *D. melanogaster* S2 cells are described in **Table S1**. Plasmids encoding *Dm* NOT10 (CG18616), *Dm* C2orf29 (CG13567) and *Dm* CAF40 (CG14213) were generated by inserting the corresponding cDNAs into the pAc5.1-EGFP and pAc5.1-λNHA vectors using the following restriction sites: *EcoRV-XbaI* (NOT10) and HindIII-*XbaI* (CG13567 and CAF40). Co-immunoprecipitation assays using S2 cells were performed as previously described.[9] S2 cells were grown in 6-well dishes, transfected using Effectene (Qiagen) transfection reagent and harvested 3 d after transfection. The transfection mixtures contained a total of 2–5 μg of plasmid, including both HA-tagged and GFP-tagged proteins. A plasmid expressing GFP-F-Luc served as a negative control. HA and GFP-tagged proteins were detected using HRP-conjugated monoclonal anti-HA (Roche 3F10; 1:5,000) and anti-GFP antibodies (Roche, catalog number 11814460001; 1:2,000), respectively. All western blots were developed using the ECL western blotting detection

Figure 10. For figure legend, see page 241.

system (GE Healthcare) as recommended by the manufacturer. Co-immunoprecipitation assays in human HEK293 cells were performed as described previously.[16] Plasmids expressing deadenylase subunits in human cells were described previously.[9] Plasmids encoding GFP or HA-tagged human CNOT10 were generated by inserting the CNOT10 cDNA (clone on15275; Kazusa DNA Research Institute) into the pEGFP-C1 and pλN-HA-C1 vectors, respectively, using the BamHI and *XhoI* restriction sites. Plasmids encoding GFP or HA-tagged human CNOT11 (full-length or fragment 260–496) were generated by inserting the CNOT11 cDNA into the pT7-EGFP-C1 and pλN-HA-C1 vectors, respectively, using the BamHI and KpnI (CNOT11 full-length) or the BamHI and *XhoI* (CNOT11 260–496) restriction sites.

In vitro pull-down assays. To express the *Dm* NOT2-C fragment in *E. coli*, the corresponding cDNA was cloned into the pnEA-NvG vector,[40] resulting in an N-terminal TEV protease-cleavable GST-tagged NOT2-C protein (**Table S1**). *Dm* NOT3-C was cloned into the pETM-60 plasmid and resulted in a vector encoding N-terminal NusA-tagged proteins. To express the *Dm* NOT1 fragments 1–412 and 1–1,083 in *E. coli*, the corresponding cDNAs were cloned into the pnEA-NvG and pnEA-NpG vectors, respectively,[40] resulting in N-terminal GST fusion proteins. For co-expression, the full-length *Dm* NOT10 cDNA was cloned into pnYC-NpHM, and the full-length *Dm* NOT11 cDNA was cloned into both pnYC-NpHM and pnEA-NpG vectors,[40] resulting in N-terminal MBP or GST fusion proteins, respectively. GST-NOT11 or GST-NOT1 was co-expressed with MBP-NOT10 or MBP-NOT11 in *E. coli* BL21 cells at 20°C overnight. Cells were resuspended in lysis buffer [50 mM Tris-Cl (pH 7.5), 200 mM NaCl, 2 mM MgCl$_2$, 2 mM ATP, 1 mM DTT] supplemented with lysozyme (1 mg/ml), DNase I (5 μg/ml) and protease inhibitors. Cell lysates were incubated on ice for 10 min, lysed by sonication and cleared by centrifugation. Cleared lysates were incubated with 50 μl (50% slurry) of Protino Glutathione Agarose 4B beads (Macherey Nagel) or 50 μl (50% slurry) of amylose resin (New England BioLabs) for 1 h at 4°C with gentle rotation. Beads were washed three times with lysis buffer. The

bound proteins were eluted with 40 μl of sample buffer [50 mM TRIS-HCl (pH 6.8), 2% SDS, 10% (v/v) glycerol, 100 mM DTT and 0.05% bromophenol blue] and separated on an 11% SDS-PAGE.

Tethering assays in S2 cells. For the λN-tethering assay, S2 cells were grown in 6-well dishes and transfected using Effectene transfection reagent (Qiagen). The transfection mixtures contained the following plasmids: 0.1 μg of reporter plasmid (F-Luc-5BoxB, F-Luc or F-Luc-5BoxB-Hhr), 0.4 μg of pAc5.1-R-Luc as a transfection control and various quantities of pAc5.1λN-HA constructs encoding the CCR4-NOT subunits that were adjusted to obtain comparable protein expression levels as follows: 1,000 ng for the λN-HA control, NOT1 and NOT11; 100 ng for NOT10 and CAF40; 70 ng for CCR4; 30 ng for NOT2 and 5 ng for NOT3 and POP2. When necessary, the total amount of transfected DNA was adjusted to 1.5 μg using the pAc5.1A plasmid lacking an insert. Firefly and *Renilla* luciferase activities were measured three days after transfection using the Dual-Luciferase Reporter Assay System (Promega). Total RNA was isolated using TriFast (Peqlab Biotechnologies) and analyzed as previously described.[38]

Supplemental Materials

Supplemental materials may be found here:
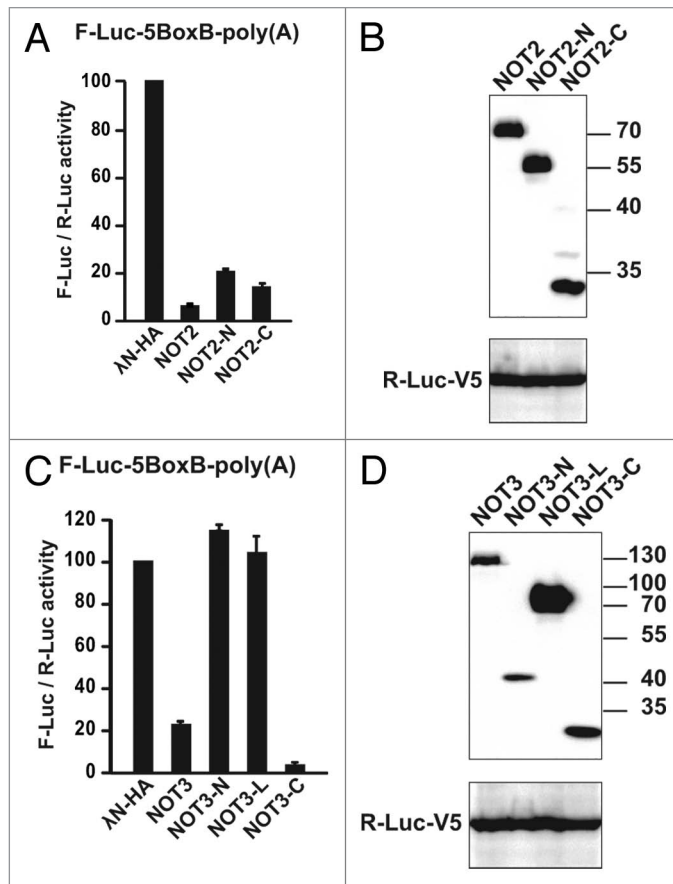www.landesbioscience.com/journals/rnabiology/article/23018

References

1. Weill L, Belloc E, Bava FA, Méndez R. Translational control by changes in poly(A) tail length: recycling mRNAs. Nat Struct Mol Biol 2012; 19:577-85; PMID:22664985; http://dx.doi.org/10.1038/nsmb.2311.

2. Houseley J, Tollervey D. The many pathways of RNA degradation. Cell 2009; 136:763-76; PMID:19239894; http://dx.doi.org/10.1016/j.cell.2009.01.019.

3. Bartlam M, Yamamoto T. The structural basis for deadenylation by the CCR4-NOT complex. Protein Cell 2010; 1:443-52; PMID:21203959; http://dx.doi.org/10.1007/s13238-010-0060-8.

4. Brown CE, Sachs AB. Poly(A) tail length control in Saccharomyces cerevisiae occurs by message-specific deadenylation. Mol Cell Biol 1998; 18:6548-59; PMID:9774670.

5. Yamashita A, Chang TC, Yamashita Y, Zhu W, Zhong Z, Chen CY, et al. Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover. Nat Struct Mol Biol 2005; 12:1054-63; PMID:16284618; http://dx.doi.org/10.1038/nsmb1016.

6. Tucker M, Valencia-Sanchez MA, Staples RR, Chen J, Denis CL, Parker R. The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in Saccharomyces cerevisiae. Cell 2001; 104:377-86; PMID:11239395; http://dx.doi.org/10.1016/S0092-8674(01)00225-2.

7. Brown CE, Tarun SZ Jr., Boeck R, Sachs AB. PAN3 encodes a subunit of the Pab1p-dependent poly(A) nuclease in Saccharomyces cerevisiae. Mol Cell Biol 1996; 16:5744-53; PMID:8816488.

8. Collart MA, Panasenko OO. The Ccr4--not complex. Gene 2012; 492:42-53; PMID:22027279; http://dx.doi.org/10.1016/j.gene.2011.09.033.

9. Braun JE, Huntzinger E, Fauser M, Izaurralde E. GW182 proteins directly recruit cytoplasmic dead-enylase complexes to miRNA targets. Mol Cell 2011; 44:120-33; PMID:21981923; http://dx.doi.org/10.1016/j.molcel.2011.09.007.

10. Chekulaeva M, Mathys H, Zipprich JT, Attig J, Colic M, Parker R, et al. miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. Nat Struct Mol Biol 2011; 18:1218-26; PMID:21984184; http://dx.doi.org/10.1038/nsmb.2166.

11. Fabian MR, Cieplak MK, Frank F, Morita M, Green J, Srikumar T, et al. miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT. Nat Struct Mol Biol 2011; 18:1211-7; PMID:21984185; http://dx.doi.org/10.1038/nsmb.2149.

12. Draper MP, Liu HY, Nelsbach AH, Mosley SP, Denis CL. CCR4 is a glucose-regulated transcription factor whose leucine-rich repeat binds several proteins important for placing CCR4 in its proper promoter context. Mol Cell Biol 1994; 14:4522-31; PMID:8007957.

13. Dupressoir A, Morel AP, Barbot W, Loireau MP, Corbo L, Heidmann T. Identification of four families of yCCR4- and Mg2+-dependent endonuclease-related proteins in higher eukaryotes, and characterization of orthologs of yCCR4 with a conserved leucine-rich repeat essential for hCAF1/hPOP2 binding. BMC Genomics 2001; 2:9; PMID:11747467; http://dx.doi.org/10.1186/1471-2164-2-9.

14. Clark LB, Viswanathan P, Quigley G, Chiang YC, McMahon JS, Yao G, et al. Systematic mutagenesis of the leucine-rich repeat (LRR) domain of CCR4 reveals specific sites for binding to CAF1 and a separate critical role for the LRR in CCR4 deadenylase activity. J Biol Chem 2004; 279:13616-23; PMID:14734555; http://dx.doi.org/10.1074/jbc.M313202200.

15. Basquin J, Roudko VV, Rode M, Basquin C, Séraphin B, Conti E. Architecture of the nuclease module of the yeast ccr4-not complex: the not1-caf1-ccr4 interaction. Mol Cell 2012; 48:207-18; PMID:22959269; http://dx.doi.org/10.1016/j.molcel.2012.08.014.

16. Petit AP, Wohlbold L, Bawankar P, Huntzinger E, Schmidt S, Izaurralde E, et al. The structural basis for the interaction between the CAF1 nuclease and the NOT1 scaffold of the human CCR4-NOT dead-enylase complex. Nucleic Acids Res 2012; 40:11058-72; PMID:22977175; http://dx.doi.org/10.1093/nar/gks883.

17. Temme C, Zhang L, Kremmer E, Ihling C, Chartier A, Sinz A, et al. Subunits of the Drosophila CCR4-NOT complex and their roles in mRNA deadenylation. RNA 2010; 16:1356-70; PMID:20504953; http://dx.doi.org/10.1261/rna.2145110.

18. Lau NC, Kolkman A, van Schaik FM, Mulder KW, Pijnappel WW, Heck AJ, et al. Human Ccr4-Not complexes contain variable deadenylase subunits. Biochem J 2009; 422:443-53; PMID:19558367; http://dx.doi.org/10.1042/BJ20090500.

Figure 11. The catalytic module requires interaction with the NOT module for full activity in tethering assays. (**A and C**) Tethering assays were performed as described in **Figure 9A** with POP2 or CCR4 (wild-type or mutants). Firefly luciferase activity was normalized to that of *Renilla* luciferase and set to 100 in the cells expressing the λN-HA peptide. (**B and D**) Western blot showing the expression level of the protein fragments and mutants tested.

Figure 12. The NOT2-N-terminal domain is active in tethering assays. (**A and C**) Tethering assays were performed as described in **Figure 9A** with the indicated NOT2 and NOT3 fragments. Firefly luciferase activity was normalized to that of *Renilla* luciferase and set to 100 in the cells expressing the λN-HA peptide. (**B and D**) Western blot showing the expression level of the protein fragments tested.

19. Morita M, Suzuki T, Nakamura T, Yokoyama K, Miyasaka T, Yamamoto T. Depletion of mammalian CCR4b deadenylase triggers elevation of the p27Kip1 mRNA level and impairs cell growth. Mol Cell Biol 2007; 27:4980-90; PMID:17452450; http://dx.doi.org/10.1128/MCB.02304-06.

20. Miyasaka T, Morita M, Ito K, Suzuki T, Fukuda H, Takeda S, et al. Interaction of antiproliferative protein Tob with the CCR4-NOT deadenylase complex. Cancer Sci 2008; 99:755-61; PMID:18377426; http://dx.doi.org/10.1111/j.1349-7006.2008.00746.x.

21. Liu HY, Badarinarayana V, Audino DC, Rappsilber J, Mann M, Denis CL. The NOT proteins are part of the CCR4 transcriptional complex and affect gene expression both positively and negatively. EMBO J 1998; 17:1096-106; PMID:9463387; http://dx.doi.org/10.1093/emboj/17.4.1096.

22. Bai Y, Salvadore C, Chiang YC, Collart MA, Liu HY, Denis CL. The CCR4 and CAF1 proteins of the CCR4-NOT complex are physically and functionally separated from NOT2, NOT4, and NOT5. Mol Cell Biol 1999; 19:6642-51; PMID:10490603.

23. Albert TK, Lemaire M, van Berkum NL, Gentz R, Collart MA, Timmers HT. Isolation and characterization of human orthologs of yeast CCR4-NOT complex subunits. Nucleic Acids Res 2000; 28:809-17; PMID:10637334; http://dx.doi.org/10.1093/nar/28.3.809.

24. Maillet L, Tu C, Hong YK, Shuster EO, Collart MA. The essential function of Not1 lies within the Ccr4-Not complex. J Mol Biol 2000; 303:131-43; PMID:11023781; http://dx.doi.org/10.1006/jmbi.2000.4131.

25. Chen J, Rappsilber J, Chiang YC, Russell P, Mann M, Denis CL. Purification and characterization of the 1.0 MDa CCR4-NOT complex identifies two novel components of the complex. J Mol Biol 2001; 314:683-94; PMID:11733989; http://dx.doi.org/10.1006/jmbi.2001.5162.

26. Russell P, Benson JD, Denis CL. Characterization of mutations in NOT2 indicates that it plays an important role in maintaining the integrity of the CCR4-NOT complex. J Mol Biol 2002; 322:27-39; PMID:12215412; http://dx.doi.org/10.1016/S0022-2836(02)00707-6.

27. Winkler GS, Mulder KW, Bardwell VJ, Kalkhoven E, Timmers HT. Human Ccr4-Not complex is a ligand-dependent repressor of nuclear receptor-mediated transcription. EMBO J 2006; 25:3089-99; PMID:16778766; http://dx.doi.org/10.1038/sj.emboj.7601194.

28. Ito K, Takahashi A, Morita M, Suzuki T, Yamamoto T. The role of the CNOT1 subunit of the CCR4-NOT complex in mRNA deadenylation and cell viability. Protein Cell 2011; 2:755-63; PMID:21976065; http://dx.doi.org/10.1007/s13238-011-1092-4.

29. Dlaki M. Functionally unrelated signalling proteins contain a fold similar to Mg2+-dependent endonucleases. Trends Biochem Sci 2000; 25:272-3; PMID:10838565; http://dx.doi.org/10.1016/S0968-0004(00)01582-6.

30. Wang H, Morita M, Yang X, Suzuki T, Yang W, Wang J, et al. Crystal structure of the human CNOT6L nuclease domain reveals strict poly(A) substrate specificity. EMBO J 2010; 29:2566-76; PMID:20628353; http://dx.doi.org/10.1038/emboj.2010.152.

31. Daugeron MC, Mauxion F, Séraphin B. The yeast POP2 gene encodes a nuclease involved in mRNA deadenylation. Nucleic Acids Res 2001; 29:2448-55; PMID:11410650; http://dx.doi.org/10.1093/nar/29.12.2448.

32. Thore S, Mauxion F, Séraphin B, Suck D. X-ray structure and activity of the yeast Pop2 protein: a nuclease subunit of the mRNA deadenylase complex. EMBO Rep 2003; 4:1150-5; PMID:14618157; http://dx.doi.org/10.1038/sj.embor.7400020.

33. Sandler H, Kreth J, Timmers HT, Stoecklin G. Not1 mediates recruitment of the deadenylase Caf1 to mRNAs targeted for degradation by tristetraprolin. Nucleic Acids Res 2011; 39:4373-86; PMID:21278420; http://dx.doi.org/10.1093/nar/gkr011.

34. Zwartjes CG, Jayne S, van den Berg DL, Timmers HT. Repression of promoter activity by CNOT2, a subunit of the transcription regulatory Ccr4-not complex. J Biol Chem 2004; 279:10848-54; PMID:14707134; http://dx.doi.org/10.1074/jbc.M311747200.

35. Garces RG, Gillon W, Pai EF. Atomic model of human Rcd-1 reveals an armadillo-like-repeat protein with in vitro nucleic acid binding properties. Protein Sci 2007; 16:176-88; PMID:17189474; http://dx.doi.org/10.1110/ps.062600507.

36. Mauxion F, Preve B, Séraphin B. C2ORF29/CNOT11 and CNOT10 form a new module of the CCR4-NOT complex. RNA Biol 2012; In press.

37. Cooke A, Prigge A, Wickens M. Translational repression by deadenylases. J Biol Chem 2010; 285:28506-13; PMID:20634287; http://dx.doi.org/10.1074/jbc.M110.150763.

38. De Gregorio E, Preiss T, Hentze MW. Translation driven by an eIF4G core domain in vivo. EMBO J 1999; 18:4865-74; PMID:10469664; http://dx.doi.org/10.1093/emboj/18.17.4865.

39. Eulalio A, Huntzinger E, Izaurralde E. GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nat Struct Mol Biol 2008; 15:346-53; PMID:18345015; http://dx.doi.org/10.1038/nsmb.1405.

40. Diebold ML, Fribourg S, Koch M, Metzger T, Romier C. Deciphering correct strategies for multiprotein complex assembly by co-expression: application to complexes as large as the histone octamer. J Struct Biol 2011; 175:178-88; PMID:21320604; http://dx.doi.org/10.1016/j.jsb.2011.02.001.
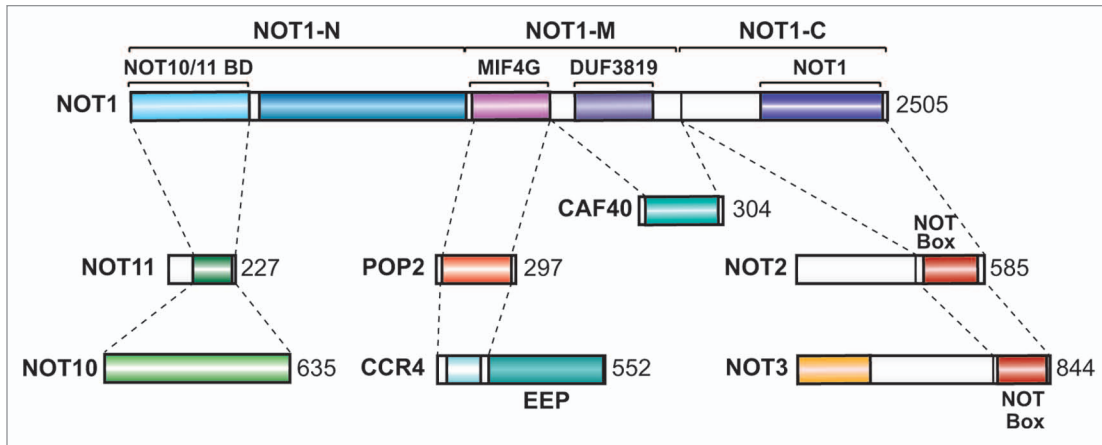
Figure 13. Diagram summarizing the interactions described in this study.

Supplemental Material to:

Praveen Bawankar, Belinda Loh, Lara Wohlbold, Steffen Schmidt and Elisa Izaurralde

NOT10 and C2orf29/NOT11 form a conserved module of the CCR4-NOT complex that docks onto the NOT1 N-terminal domain
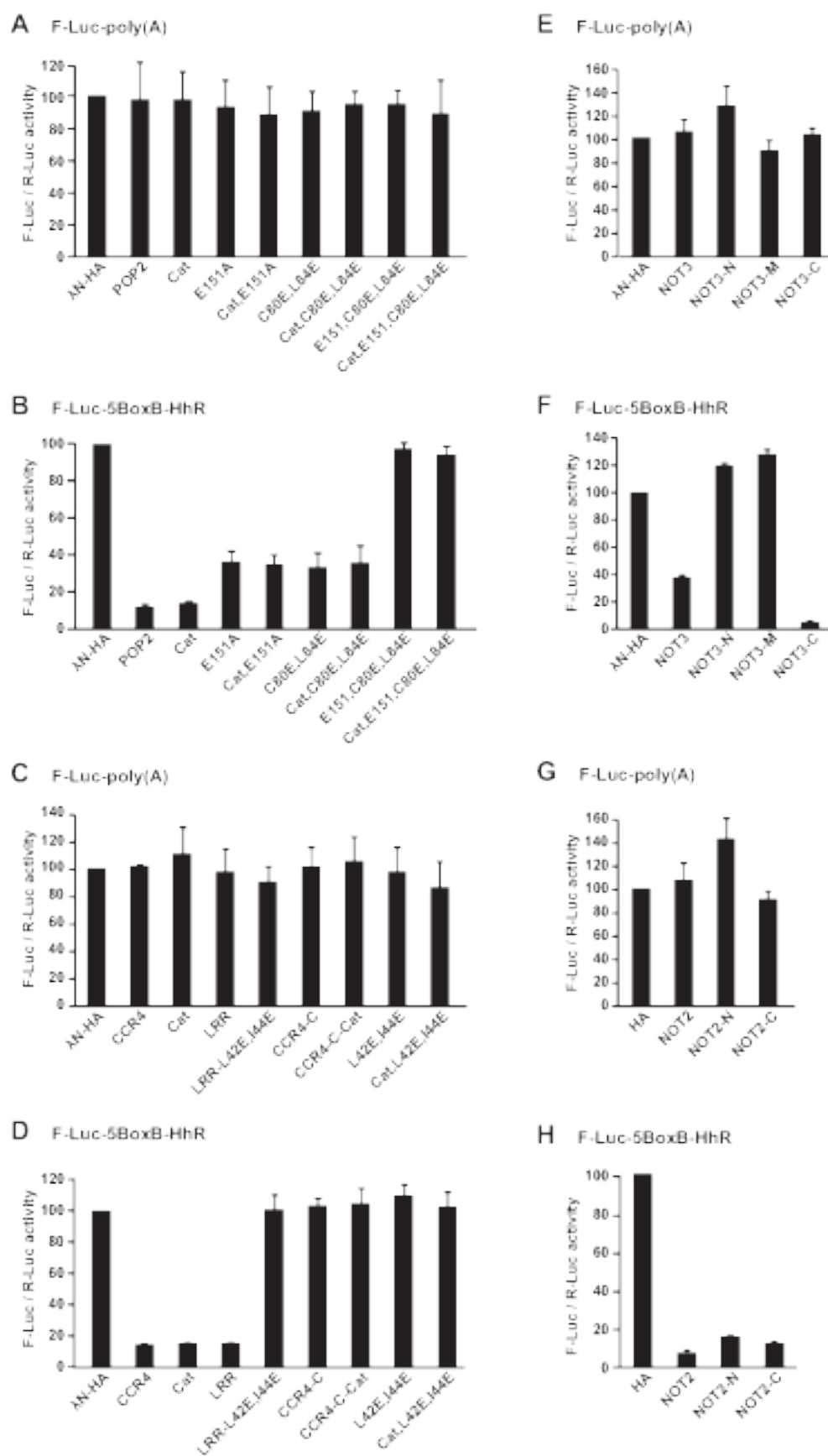
Figure S1. (A–H) Tethering assays using the F-Luc reporter lacking the Box B hairpins (F-Luc-poly(A)) or the unadenylated (F-Luc-BoxB-HhR) reporter were performed as described in Fig. 9 with the indicated proteins.

Table S1. Plasmids used in this study.

Protein (alternative names) / ORF length in amino acids / accession	Plasmid	Protein	Cloning sites
CCR4/CG31137/552/NM_170129.2	pAc5.1B-EGFP-DmCCR4	CCR4	HindIII-XbaI
	pAc5.1B-EGFP-DmCCR4 1-166	LRR	EcoRI-NotI
	pAc5.1B-EGFP-DmCCR4 168-552	CCR4-C	HindIII-XbaI
	pAc5.1B-λN-HA-DmCCR4	CCR4	HindIII-XbaI
	pAc5.1B-λN-HA-DmCCR4 1-166	LRR	EcoRI-NotI
	pAc5.1B-λN-HA-DmCCR4 168-552	CCR4-C	HindIII-XbaI
	pAc5.1B-λN-HA-CCR4 168-552 D412A,N414A	CCR4-C-Cat	HindIII-XbaI
	pAc5.1B-λN-HA-DmCCR4-1-166 L42E,I44E	LRR L42E,I44E	HindIII-XbaI
	pAc5.1B-λN-HA-CCR4 D412A/N414A	Cat	HindIII-XbaI
	pAc5.1B-λN-HA-CCR4 168-552 D412A,N414A	CCR4-C-cat	HindIII-XbaI
	pAc5.1B-λN-HA-DmCCR4 L42E,I44E	CCR4 L42E,I44E	HindIII-XbaI
	pAc5.1B-λN-HA-DmCCR4-L42E,I44E,D412A,N414A	CCR4 Cat,L42E,I44E	HindIII-XbaI
POP2/CG5684-Pa/297/NM_140281.2	pAc5.1B-λN-HA-DmPOP2a	POP2	EcoRV-NotI
	pAc5.1B-EGFP-DmPOP2a	POP2	EcoRV-NotI
	pAc5.1B-λN-HA-DmPOP2a	POP2	EcoRV-NotI
	pAc5.1B-λN-HA-DmPOP2a D53A,E55A	Cat	EcoR-NotI
	pAc5.1B-λN-HA-DmPOP2a E151A	POP2 E151A	EcoRV-NotI
	pAc5.1B-λN-HA-DmPOP2a-D53A,E55A,E151A	Cat,E151A	EcoRV-NotI
	pAc5.1B-λN-HA-DmPOP2a C80E,L84E	C80E,L84E	EcoRV-NotI
	pAc5.1B-λN-HA-DmPOP2a D53A,E55A,C80E,L84E	Cat,C80E,L84E	EcoRV-NotI
	pAc5.1B-λN-HA-DmPOP2a E151,C80E,L84E	C80E,L84E,E151A	EcoRV-NotI
	pAc5.1B-λN-HA-DmPOP2a D53A,E55A,E151,C80E,L84E	Cat,C80E,L84E,E151A	EcoRV-NotI
NOT3.5/CG8426/844aa/NM_136332.2	pAc5.1B-EGFP-DmNNOT3	NOT3	HindIII-NotI
	pAc5.1B-EGFP-DmNOT3 1-246	NOT3-N	HindIII-NotI
	pAc5.1B-EGFP-DmNOT3 239-681	NOT3-L	HindIII-EcoRI
	pAc5.1B-EGFP-DmNOT3 677-844	NOT3-C	HindIII-NotI
	pAc5.1B-λN-HA-DmNOT3	NOT3	HindIII- NotI
	pAc5.1B-λN-HA-DmNOT3 677-844	NOT3-C	HindIII-NotI
	pETM-60-DmNOT3 677-844	NOT3-C	NdeI-XhoI
NOT2/CG2161, Rga/585aa/NM_169080	pAc5.1B-λN-HA-DmNOT2	NOT2	HindIII-XbaI

	pAc5.1B-EGFP-DmNOT2	NOT2	HindIII-XbaI
	pAc5.1B-EGFP-DmNOT2 1-401	NOT2-N	HindIII-Not1
	pAc5.1B-EGFP-DmNOT2 402-585	NOT2-C	HindIIIXbaI
	pnEA-NvG-DmNOT2 402-585	GST-NOT2-C	NdeI-XhoI
	pnEA-NvM-DmNOT2 402-585	MBP-NOT2-C	NdeI-XhoI
	pAc5.1B-EGFP-DmNOT2 436-585	436-585	HindIII-XbaI
	pAc5.1B-EGFP-DmNOT2 452-585	452-585	HindIII-XbaI
	pAc5.1B-EGFP-DmNOT2 469-585	469-585	HindIII-XbaI
	pAc5.1B-EGFP-DmNOT2 478-585	478-585	HindIII-XbaI
	pAc5.1B-EGFP-DmNOT2 1-436	1-436	HindIII-XbaI
	pAc5.1B-EGFP-DmNOT2 402-436	402-436	HindIII-XbaI
NOT1/ CG34407/2505aa/NM_001103772.2.	pAc5.1B-EGFP-DmNOT1	NOT1	PacI- XbaI
	pAc5.1B-λN-HA-DmNOT1	NOT1	PacI-HpaI
	pAc5.1B-EGFP-DmNOT1 1-1148	NOT1-N	EcoRI-XbaI
	pAc5.1B-EGFP-DmNOT1 1147-1717	NOT1-M	NheI-XbaI
	pAc5.1B-EGFP-DmNOT1 1147-2505	NOT1-M+C	HindIII-XbaI
	pAc5.1B-EGFP-DmNOT1 1710-2505	NOT1-C	NheI-XbaI
	pAc5.1B-λN-HA-DmNOT1 1147-1717	NOT1-M	HindIII-XbaI
	pAc5.1B-λN-HA-DmNOT1 1387-1717	NOT1 1387-1717	HindIII-XbaI
	pAc5.1B-λN-HA-DmNOT1 1147-1386	MIF4G	HindIII-XbaI
	pAc5.1B-λN-HA-DmNOT1 1710-2505	NOT1-C	HindIII-XbaI
	pAc5.1B-λN-HA-DmNOT1 1147-1717 K1277A	M-K1277A	HindIII-XbaI
	pAc5.1B-EGFP-DmNOT1 1-412	1-412	EcoRI-XbaI
	pAc5.1B-EGFP-DmNOT1 416-1148	416-1148	EcoRI-XbaI
	pAc5.1B-EGFP-DmNOT1 1-226	1-226	EcoRI-XbaI
	pAc5.1B-λNHA-DmNOT1 Δ1-1148	NOT1-ΔN	HpaI-PacI
	pAc5.1B-λNHA-DmNOT1 Δ1147-1717	NOT1-ΔM	PacI-XbaI
	pAc-5.1B-λNHA-DmNOT1 Δ1-1717	NOT1-C	HpaI-PacI
	pAc-5.1B-λNHA-DmNOT1 Δ1710-2505	NOT1-ΔC	PacI-XbaI
	pAc5.1B-λN-HA-DmNOT1 K1277A	K1277A	PacI-XbaI
	pnEA-NpG-DmNOT1 1-1083	GST-NOT1 1-1083	XhoI-XbaI
	pnEA-NvG-DmNOT1 1-412	GST-NOT1 1-412	BamHI-XbaI
CAF40/CG14213/304aa/NM_167675.1	pAc5.1B-λN-HA-DmCAF40	CAF40	HindIII-XbaI

	pAc5.1B-EGFP CAF40 25-291	CAF40 25-291	HindIII-XbaI
	pAc5.1B-EGFP-DmCAF40	CAF40	HindIII-XbaI
NOT10/CG18616/635aa/NM_141958.3	pAc5.1B-λN-HA-DmNOT10	NOT10	EcoRV-XbaI
	pAc5.1B-EGFP-DmNOT10	NOT10	EcoRV-XbaI
	pnEA-NpM-DmNOT10	MBP-NOT10	XhoI-XbaI
	pnYC-NpHM-DmNOT10	MBP-NOT10	KpnI-XbaI
C2orf29/CG13567/227aa/NM_138030	pAc5.1B-λN-HA-DmC2orf29	NOT11	HindIII-XbaI
	pAc5.1B-EGFP-DmC2orf29	NOT11	HindIII-XbaI
	pnEA-NpG-DmC2orf29	GST-NOT11	XhoI-BamHI
	pnYC-NpHM-DmC2orf29	MBP-NOT11	KpnI-XbaI