

Differences and similarities between YTHDF-mediated and microRNA-guided gene silencing



D i s s e r t a t i o n

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der Fakultät für Biologie und Vorklinische Medizin

der Universität Regensburg

vorgelegt von Simone Larivera

aus Termoli (Italien)

Regensburg, December 2023

Das Promotionsgesuch wurde eingereicht am:
14.12.2023

Die Arbeit wurde angeleitet von:
Prof. Dr. Gunter Meister

Unterschrift:

*“Viaggiare, è proprio utile, fa lavorare l’immaginazione. Tutto il resto è delusione e fatica. Il viaggio
che ci è dato è interamente immaginario. Ecco la sua forza.
Va dalla vita alla morte. Uomini, bestie, città e cose, è tutto inventato. È un romanzo, nient’altro che
una storia fittizia. Lo dice Littré, lui non sbaglia mai.
E poi in ogni caso tutti possono fare altrettanto. Basta chiudere gli occhi.
È dall’altra parte della vita.”*

Viaggio al termine della notte, Louis-Ferdinand Céline

Abstract

Post-transcriptional gene silencing is a fundamental process that is mainly mediated by AGO proteins and by the CCR4-NOT deadenylase complex. AGO proteins associate with small noncoding RNAs, which guide them to recognize specific target RNAs. In case of perfect pairing between the miRNA and the target mRNA, AGO proteins possess endonucleolytic activity and can cleave their targets. In case of partial overlap, they recruit auxiliary factors like TNRC6A-B-C proteins, which function as a flexible scaffold between AGO proteins and downstream effectors like the CCR4-NOT complex. The CCR4-NOT complex is the major deadenylase complex in the cell and plays a key role in mRNA degradation by catalyzing the removal of poly(A) tails, a process also known as deadenylation. For bulk mRNAs, the deadenylation process is followed by translational repression, and decapping, through the recruitment of the decapping complex via several accessory proteins, like the helicase DDX6, and target degradation through the major cytoplasmic nuclease 5'-to-3' exoribonuclease 1 (XRN1). While AGO proteins are guided to their specific targets by around 1000 miRNA genes (Kim et al., 2021), in the human genome there are over 2000 RNA Binding Proteins (RBPs) that recognize specific sequence motifs or structural elements, and for most of them, their impact on microRNA-mediated decay is still unknown (Corley et al., 2020). Moreover, specific modifications of the mRNA, like m⁶A, can regulate gene expression by recruiting the CCR4-NOT complex through the YTH domain-containing protein YTHDF2. By systematically comparing and analyzing the different interacting partners of YTHDF2, we found out that not only YTHDF2 did not interact with the miRISC complex, but also exhibited a weak *in vivo* interaction with the CCR4-NOT complex and an RNA-dependent interaction with the cytoplasmic poly(A) binding protein 1 (PABPC1). Moreover, we demonstrated through tethering experiments that TNRC6A-B-C proteins depletion did not increase YTHDF2 activity, thereby excluding a possible competitive effect for the CCR4-NOT complex. Furthermore, we found out that YTHDF2-mediated mRNA decay through the CCR4-NOT complex relies on the interaction between NOT6 and NOT7. Additionally, we not only showed that, unlike AGO2, YTHDF2 activity was not affected by DDX6 depletion, but also that only a small pool of the YTHDF2 tethered 5BoxB beta-globin reporter undergoes 5'-3' decay in XRN1 K.O. cells. Finally, steady-state full-length mRNA sequencing (FLAM-Seq) act at profiling the poly(A) tail in the triple knockdown (KD) of YTHDF and TNRC6 paralogs, showed a shortening of the poly(A) tail, almost comparable to the steady state poly(A) tail profiling of XRN1 depleted HEK 293T cells, which is correlated with an increase in mRNA stability. These findings indicate that m⁶A, and AGO auxiliary factors have different effects on miRNA-targeting, paving the way for new studies on how different RBPs affect CCR4-NOT complex recruitment and miRNA-targeted deadenylation and in general 5'-3'mRNA decay.

Zusammenfassung

Die posttranskriptionelle Gen-Stummschaltung ist ein grundlegender Prozess, der hauptsächlich durch AGO-Proteine und den CCR4-NOT-Deadenylase-Komplex vermittelt wird. AGO-Proteine verbinden sich mit kleinen nichtkodierenden RNAs, die ihnen dabei helfen, spezifische Ziel-RNAs zu erkennen. Bei perfekter Paarung zwischen miRNA und Ziel-mRNA besitzen AGO-Proteine endonukleolytische Aktivität und können ihre Ziele spalten. Bei teilweiser Überlappung rekrutieren sie Hilfsfaktoren wie TNRC6A-B-C-Proteine, die als flexibles Gerüst zwischen AGO-Proteinen und nachgeschalteten Effektoren wie dem CCR4-NOT-Komplex fungieren. Der CCR4-NOT-Komplex ist der wichtigste Deadenylasekomplex in der Zelle und spielt eine Schlüsselrolle beim mRNA-Abbau, indem er die Entfernung von Poly(A)-Schwänzen katalysiert, ein Prozess, der auch als Deadenylierung bekannt ist. Bei Bulk-mRNAs folgt auf den Deadenylierungsprozess eine translationale Repression und Decapping durch die Rekrutierung des Decapping-Komplexes über mehrere akzessorische Proteine, wie die Helikase DDX6, und den Zielabbau durch die wichtigste zytoplasmatische Nuklease 5'-to-3'-Exoribonuklease 1 (XRN1). Während AGO-Proteine von rund 1000 miRNA-Genen zu ihren spezifischen Zielen geleitet werden (Kim et al., 2021), gibt es im menschlichen Genom über 2000 RNA-Bindungsproteine (RBPs), die spezifische Sequenzmotive oder Strukturelemente erkennen, und zwar für die meisten davon ihr Einfluss auf den microRNA-vermittelten Zerfall ist noch unbekannt (Corley et al., 2020). Darüber hinaus können auch spezifische Modifikationen der mRNA, wie m6A, die Genexpression regulieren, indem sie den CCR4-NOT-Komplex über das YTH-Domänen enthaltende Protein YTHDF2 rekrutieren. Durch den systematischen Vergleich und die Analyse der verschiedenen Interaktionspartner von YTHDF2 fanden wir heraus, dass YTHDF2 nicht nur nicht mit dem miRISC-Komplex interagiert, sondern auch eine schwache In-vivo-Interaktion mit dem CCR4-NOT-Komplex und eine RNA-abhängige Interaktion mit dem Zytoplasma aufwies Poly(A)-bindendes Protein 1 (PABPC1). Darüber hinaus haben wir durch Tethering-Experimente gezeigt, dass der Abbau der TNRC6A-B-C-Proteine die YTHDF2-Aktivität nicht erhöhte, wodurch ein möglicher Konkurrenzeffekt für den CCR4-NOT-Komplex ausgeschlossen wurde. Darüber hinaus haben wir herausgefunden, dass der YTHDF2-vermittelte mRNA-Zerfall durch den CCR4-NOT-Komplex auf der Interaktion zwischen NOT6 und NOT7 beruht. Darüber hinaus haben wir nicht nur gezeigt, dass die YTHDF2-Aktivität im Gegensatz zu AGO2 nicht durch die DDX6-Depletion beeinträchtigt wurde, sondern auch, dass nur ein kleiner Pool des an YTHDF2 gebundenen 5BoxB-Beta-Globin-Reporters einen 5'-3'-Zerfall in XRN1 K.O. erfährt. Zellen. Schließlich dient die Steady-State-mRNA-Sequenzierung voller Länge (FLAM-Seq) zur Profilierung des Poly(A)-Schwanzes beim Triple Knockdown (KD) von YTHDF- und TNRC6-Paralogs und zeigte eine nahezu vergleichbare Verkürzung des Poly(A)-Schwanzes zum Steady-State-Poly(A)-Schwanzprofil von XRN1-depletierten HEK 293T-Zellen, was mit einer

Erhöhung der mRNA-Stabilität korreliert. Diese Ergebnisse deuten darauf hin, dass m⁶A-, und AGO-Hilfsfaktoren unterschiedliche Auswirkungen auf das miRNA-Targeting haben, was den Weg für neue Studien darüber ebnet, wie verschiedene RBPs die Rekrutierung des CCR4-NOT-Komplexes und die miRNA-zielgerichtete Deadenylierung sowie allgemein 5'-3'-mRNA beeinflussen Verfall.

Publications and Presentations

Parts of this thesis constitute two manuscripts in preparation:

Larivera S¹, Legnini I², Aigner D², Raisch T³, Lehmann G¹, Rajewsky G² & Meister G¹: Differences and similarities between YTHDF-mediated and microRNA-guided gene silencing

¹ Biochemistry Center Regensburg (BZR), Laboratory for RNA Biology, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany

² Laboratory for Systems Biology of Gene Regulatory Elements, Berlin Institute for Medical Systems Biology, Max-Delbrück Center for Molecular Medicine, Berlin, Germany

³ Department of Structural Biochemistry, Max Planck Institute of Molecular Physiology, Dortmund, Germany

Ho-Xuan H^{1,5}, Larivera S¹, De la Cruz-Thea B², Lehmann G¹, Schabenberger F³, Strauß D³, Feederle R⁴, Stolz A⁵, Bruckmann A¹, Kretz M³, M Musri M² & Meister G¹: Autophagy contributes to YTHDF protein clearance during contact inhibition

¹ Biochemistry Center Regensburg (BZR), Laboratory for RNA Biology, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany

² Instituto de Investigación Médica Mercedes y Martín Ferreyra, Consejo Nacional de Investigaciones Científicas y Técnicas (INIMEC-CONICET), Universidad Nacional de Córdoba, Cátedra de Genética, Departamento de Fisiología, Facultad de Ciencias Exactas Físicas y Naturales (FCEFN), Universidad Nacional de Córdoba, Córdoba, Argentina.

³ Regensburg Center for Biochemistry (RCB), Laboratory for long non-coding RNA and tissue homeostasis, University of Regensburg, Regensburg, Germany

⁴ Helmholtz Zentrum München, German Research Center for Environmental Health GmbH, Institute for Diabetes and Obesity, Monoclonal Antibody Core Facility and Research Group, 85764 Neuherberg, Germany

⁵ Buchmann Institute for Molecular Life Sciences, Goethe University Frankfurt, Frankfurt am Main, Germany.

Additionally, I contributed to the following Preview & Review:

Larivera S¹, Meister G¹. Domain confusion 2: m⁶A-independent role of YTHDC2. Mol Cell. 2022 May 5;82(9):1608-1609. doi: 10.1016/j.molcel.2022.04.012. PMID: 35523125.

¹ Biochemistry Center Regensburg (BZR), Laboratory for RNA Biology, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany

Larivera S¹, Neumeier J¹, Meister G¹. Post-transcriptional gene silencing in a dynamic RNP world. Biol Chem. 2023 Sep 25. doi: 10.1515/hsz-2023-0203. Epub ahead of print. PMID: 37739934.

¹ Biochemistry Center Regensburg (BZR), Laboratory for RNA Biology, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany

Parts of this thesis have been presented at national and international conferences:

Larivera S¹, Legnini I², Aigner D², Raisch T³, Lehmann G¹, Rajewsky G² & Meister G¹: Differences and similarities between AGO2- and YTHDF2-mediated gene regulation

- Poster presentation 15th and 16th Microsymposium on small RNAs, May 2019 & April 2022, Vienna, Austria.
- Poster presentation 73rd Mosbacher Kolloquium, March-April 2022, Mosbach, Germany.
- Poster presentation SFB960 Conference, RNP Dynamics, July 2022, Regensburg, Germany.
- Oral presentation 1st Meeting on Argonaute Proteins August 2022, Regensburg, Germany.
- Poster presentation Keystone Symposia on Molecular and Cellular Biology Protein-RNA Interactions, January-February 2023, Vancouver, Canada.

Contents

Abstract.....	4
Zusammenfassung.....	5
Publications and Presentations.....	7
1 Introduction	12
1.1 Gene expression in eukaryotes.....	12
1.1.1 Transcription.....	12
1.1.2 Capping	12
1.1.3 Splicing.....	12
1.1.4 The role of the EJC in splicing and mRNA surveillance.....	14
1.1.5 Cleavage and Polyadenylation of the pre-mRNA.....	14
1.1.6 The EJC-mediated mRNA export.....	17
1.1.7 mRNA Translation.....	17
1.1.8 Translation Initiation.....	19
1.1.9 Translation Elongation	20
1.1.10 Translation Termination, Ribosome Recycling, and Ribosome Quality Control	20
1.1.11 Eukaryotic mRNA Surveillance pathways	21
1.2 mRNA decay.....	21
1.3 Recruitment of the CCR4-NOT complex by RBPs	23
1.3.1 The CCR4-NOT complex is a protein hub for RBPs.....	23
1.3.2 The importance of Intrinsically Disordered Regions (IDRs) in RBPs recruiting the CCR4-NOT complex	27
1.4 PABPC role in deadenylation.....	28
1.5 Components and mechanisms of human miRNA-guided gene silencing.....	29
1.5.1 Molecular architecture of AGO proteins.....	31
1.5.2 miRNA-guided gene regulation processes	32
1.5.3 GW182 Proteins: Essential players of mRNA Fate and Gene Regulation.....	34
1.6 Regulation of miRNA-mediated gene silencing.....	35

1.6.1	Regulation of miRNA stability.....	35
1.6.2	Regulation of Argonaute proteins by phosphorylation	37
1.6.3	Influence of RBPs on miRNA-guided gene silencing.....	38
1.7	m ⁶ A RNA modification	41
1.7.1	m ⁶ A Writers, Erasers, and Readers.....	41
1.7.2	The role of YTHDF proteins in mRNA metabolism.....	43
2	Aim of the thesis.....	46
3	Results	47
3.1	Analysis of the AGO2 interactome	47
3.1.1	Investigation of AGO-containing mRNPs through sucrose gradient fractionation	47
3.1.2	YTHDF2 interacts <i>in vitro</i> with the CCR4-NOT complex.....	49
3.1.3	SLiM motifs in YTHDF2 are essential for optimal binding to the CCR4-NOT complex.....	50
3.1.4	YTHDF proteins interact weakly with the CCR4-NOT complex.....	53
3.1.5	YTHDF proteins do not interact with AGO2	54
3.1.6	YTHDF proteins interact with PABPC1 in an RNA-dependent way	54
3.2	Functional analysis of YTHDF proteins	56
3.2.1	YTHDF proteins do not degrade as efficiently as AGO2	56
3.2.2	TNRC6 proteins depletion does not enhance YTHDF2-mediated mRNA degradation.....	57
3.2.3	YTHDF2 requires the CCR4-NOT complex to mediate mRNA deadenylation.....	59
3.2.4	YTHDF proteins absence does not impair AGO2 activity.....	61
3.2.5	YTHDF2 tethered BGG-5BoxB reporter does not retain the cap	63
3.2.6	DDX6 absence does not affect YTHDF2-mediated mRNA degradation	64
3.2.7	Distinct effects of AGO2 and YTHDF2 tethering on the accumulation of BGG-5BoxB reporter intermediates in the absence of XRN1	66
3.2.8	Tethering of both single molecules of AGO2 and YTHDF2 is not sufficient to elicit mRNA degradation	69
3.3	Impact of YTHDF proteins on translation.....	72
3.4	YTHDF and TNRC6 proteins level regulation.....	74

3.4.1	YTHDF proteins are degraded in contact-inhibited cells.....	74
3.4.2	TNRC6 proteins are degraded in contact-inhibited cells.....	75
3.6.3	YTHDF protein degradation is not dependent on transcriptional or translational regulation.....	76
3.5	Analysis of YTHDF and TNRC6 proteins depletion on steady-state poly(A) tail length.....	79
3.5.1	Full-length mRNA sequencing is the ideal sequencing to study the effects of YTHDF, TNRC6, and XRN1 proteins depletion on the poly(A) tail length	79
3.5.2	FLAM-Seq analysis of steady state mRNAs revealed a shortening of poly(A) tail upon depletion of YTHDF, TNRC6, and XRN1 proteins	80
3.5.3	Depletion of YTHDF and XRN1 proteins induced a pronounced shortening of poly(A) tail length for transcripts with median tail length higher than 150 nucleotides.	85
3.5.4	Depletion of TNRC6 proteins induced a lengthening of poly(A) tail length for transcripts with median tail length lower than 150 nucleotides	87
3.5.5	Validation of FLAM-Seq targets.....	89
3.5.6	Impact of poly(A) tail shortening on mRNA stability	94
3.6	Poly(A) tail shortening is associated with mRNA stability	94
3.6.1	Bulk RNA-Seq analysis from FLAM-Seq counts of YTHDF1-3, YTHDF1-2-3 depleted HEK 293T cells, showed a moderate level of mRNA stabilization for transcripts with short poly(A) tail.....	95
3.6.2	Bulk RNA-Seq analysis from FLAM-Seq counts of TNRC6ABC depleted HEK 293T cells, show a mild level of mRNA stabilization for transcripts with short poly(A) tail.....	98
3.6.3	Bulk RNA-Seq analysis from FLAM-Seq counts in HEK 293T XRN1 K.O. cells showed a clear correlation between poly(A) tail shortening and mRNA stability	99
3.6.4	RNA-Seq and Ribosome Profiling analysis of HEK 293T XRN1 K.O. cells, revealed increased mRNA stability and decreased translation efficiency for targets with short poly(A) tail.....	101
3.6.5	Bulk RNA-Seq analysis from YTHDF1-3 and YTHDF1-2-3 depleted XRN1 K.O. cells showed a moderate correlation between poly(A) tail shortening and mRNA stability ..	104
4	Discussion	108
4.1	Different way of CCR4-NOT complex recruitment by AGO2 and YTHDF2.....	108
4.2	Comparison between miRNA- and YTHDF2- mediated mRNA decay	112
4.3	The role of YTHDF proteins on translation regulation	113

4.4	YTHDF and TNRC6 proteins regulation	114
4.5	YTHDF proteins depletion causes a shortening of the poly(A) tail and stabilization of specific target mRNAs	115
5	Materials and Methods	117
5.1	Materials.....	117
5.1.1	Chemicals and enzymes.....	117
5.1.2	Instruments, software and algorithms.....	117
5.2	Kits and Solutions.....	119
5.2.1	Kits	119
5.2.2	Solutions and buffers	120
5.3	Consumables	121
5.4	Oligonucleotides	121
5.5	Vectors and plasmids.....	127
5.5.1	Vectors	127
5.5.2	Plasmids	128
5.6	Antibodies.....	131
5.7	Cell Lines and Bacteria strains.....	133
5.8	Methods	134
5.8.1	DNA-based methods.....	134
5.8.2	RNA-based methods.....	140
5.8.3	Protein-based methods.....	143
5.8.4	Cell culture	146
6	Figures and Tables	149
7	List of Abbreviations.....	153
8	Bibliography	157
9	Acknowledgments	167

1 Introduction

1.1 Gene expression in eukaryotes

1.1.1 Transcription

Eukaryotic gene expression regulation is a multi-step process that is highly controlled and subject to regulation across multiple levels. In eukaryotic cells, gene expression begins in the nucleus, where RNA polymerase II (RNA pol II) transcribes the nucleotide information present in DNA into a primary transcript known as precursor-messenger RNA (pre-mRNA). This pre-mRNA undergoes co-transcriptional processing, involving steps like 5' RNA capping, pre-mRNA splicing, and 3' RNA polyadenylation (Heck and Wilusz 2018).

1.1.2 Capping

During the early stages of mRNA maturation, a 7-methylguanosine (m7G) cap structure is added to the unbound 5'-end of the RNA molecule. This process requires three enzymatic reactions.

In the first step, the gamma-phosphate of the 5'-terminal nucleotide is removed by the RNA triphosphatase. In a second step, the guanylyltransferase (Ceg1 in yeast) hydrolyzes GTP to GMP by removing the gamma and beta phosphates and transfers the GMP on the dephosphorylated 5'-terminus of the pre-mRNA in an unusual 5'-5'-triphosphate linkage. In the last step, the Guanine-N7-methyltransferase adds a methyl group to the N7 position of the guanine base (Ramanathan, Robb and Chan 2016). The cap structure is vital for cap-dependent translation initiation, protecting the mRNA's 5'-end from exonuclease degradation and facilitating the recruitment of pre-mRNA splicing, 3'-polyA addition, and nuclear export proteins (Galloway and Cowling 2019).

1.1.3 Splicing

With a few exceptions, mammalian genes are interrupted by interspersed non-coding RNA sequences (introns), and to generate a functional message from the DNA template, mRNA splicing must occur. Splicing involves the removal of introns, from the primary RNA transcript to form a contiguous stretch of coding sequences called exons (Nick J. Proudfoot, Andre Furger and Dye 2002). This intricate process relies on the recognition of consensus sequences on the introns, defining the intron-exon borders and recognized by a ribonucleoprotein (RNP) complex called the spliceosome (Baralle and Baralle 2018, Shi 2017)

The spliceosome is formed by five small nuclear ribonucleoproteins (snRNPs) particles named U1, U2, U4, U5, and U6, connected with a large number of additional proteins (Elisa Izaurrealde, Joe Lewis et al. 1994, Joe D. Lewis, Görlich and Mattaj 1996, Wahl, Will and Luhrmann 2009). In addition, to have an efficient co-transcriptional spliceosomal assembly, a direct interaction of the

Cap Binding Complex (CBC) with the protein components of the U4/U6.U5 tri-snRNP is required (Elisa Izaurralde, Janusz Stepinski et al. 1992, Elisa Izaurralde, Joe Lewis et al. 1994, Joe D. Lewis, Görlich and Mattaj 1996, Pabis, Neufeld et al. 2013). The CBC has an important function in splicing since not only enhances the interaction of the U1 snRNPs with the 5' splice site (5' ss) but also regulates its displacement during spliceosome assembly and therefore influences U6 snRNP binding to the 5' ss (Flaherty SM 1997 Oct 28).

During the splicing process, the spliceosome plays a crucial role in accurately identifying the boundaries between introns and exons within the pre-mRNA molecule. This recognition is achieved through the interaction of specific sequence elements, like the 5' splice site and the 3' splice site (3' ss) (**Figure 1.1**).

The 5' ss typically contains the consensus sequence "GU" dinucleotide at the 5' end of the intron and serves as the initial docking site for the spliceosome, in particular by the U1 snRNP.

At the other end of the intron, the 3' splice site contains three conserved sequence elements: the branch point site, the polypyrimidine tract, and the terminal "AG" dinucleotide at the very final 3' end of the intron. The branch point site is present within 20-50 nucleotides upstream of the 3' splice site and is recognized by the splicing factor 1 protein (SF1) (Black 2003).

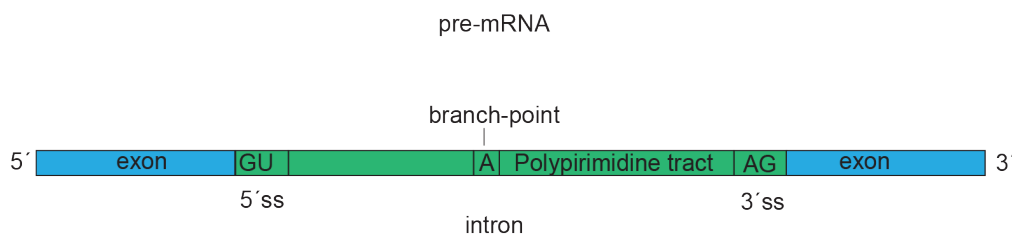


Figure 1.1: Intron Exon Boundary in pre-mRNA. 5' splice site (5' ss) and the 3' splice site (3' ss).

The branch point site is followed by a region rich in pyrimidine bases, which terminates in the "AG" dinucleotide. This region is bound by the U2 auxiliary factor (U2AF) complex, which helps to recruit additional components of the spliceosome and proceed with the splicing process.

After defining the intron ends, the U2 snRNP displaces SF1 and binds itself to the branch point site. Subsequently, the U4/U6.U5 tri-snRNP is recruited to the other two snRNPs to form a large and complex intermediate of the spliceosome, which positions the 5' end and the branch point in proximity. After numerous rearrangements and protein exchanges, and the removal of the U1 and U4 snRNPs, the 2'OH of the branch point site performs a nucleophilic attack at the 5' splice site of the pre-mRNA forming a lariat-shaped RNA molecule. At this point, the 3' OH end of the released 5' exon attacks the phosphodiester bond at the first nucleotide of the exon after the 3' splice site {Glavan, 2006 #182}. As a result, the two exons are covalently bound, and the lariat containing the intron is released (Herzel, Ottoz et al. 2017).

1.1.4 The role of the EJC in splicing and mRNA surveillance

After splicing, proteins of the exon junction complex (EJC) marking the site of each splicing event, are added and, four in particular, constitute the core of the EJC: eIF4A3 (eukaryotic initiation factor 4A3), MAGOH, Y14 (also known as RNA-binding motif 8A) and MLN51 (metastatic lymph node 51, also known as CASC3) (Ballut, Marchadier et al. 2005, Christian B. F. Andersen, Lionel Ballut et al.).

One of the core components of the EJC, the eIF4A3 helicase forms a stable complex with CWC22 (complexed with CEF1 22; also named NCM or Nucampholin in *D. Melanogaster*) a core factor of the spliceosome. CWC22 prevents eIF4A3 binding to both RNA and MAGOH-Y14. Once the splicing is completed, eIF4A3 is recruited to the active spliceosome and is thereafter integrated into the EJC (Alexandrov, Colognori et al. 2012, Steckelberg, Boehm et al. 2012).

Furthermore, this EJC core forms a binding platform for several proteins which can associate during splicing and mRNA export or even in the cytoplasm where the complex stays with mRNAs until translation occurs (Herve Le Hir, David Gatfield et al. 2001). In the cytoplasm, the EJC also functions as a surveillance complex for one of the most conserved cytoplasmic quality control mechanisms called Nonsense-mediated mRNA decay (NMD) that aims to identify mRNAs harboring a premature termination codon (PTC) (Kurosaki, Popp and Maquat 2019).

1.1.5 Cleavage and Polyadenylation of the pre-mRNA

Before being transported into the cytoplasm as an export-competent mRNP, pre-mRNAs have to undergo extensive processing. 3' end processing of eukaryotic mRNAs involves a cleavage step followed by the addition of 150-200 adenosine residues (only ~60 nt in *S. cerevisiae*), also called poly(A) tail, close to the 3' end of the pre-mRNAs.

This step is termed polyadenylation and is required for nuclear export, prevents transcript degradation, and stimulates many steps of translation (Eckmann, Rammelt and Wahle 2011).

Eukaryotic pre-mRNAs contain sequence elements such as the polyadenylation signal, which consists of a highly conserved A(A/U)UAAA element located 10–30 nucleotides upstream of the cleavage/polyadenylation site (poly(A) site) and a more variable U/GU-rich downstream element (DSE) ~30 nucleotides downstream of the poly(A) site (Jean-Christophe Dantonel, Kanneganti G. K. Murthy et al. , Jing Zhao, Hyman and Moore) (**Figure 1.2**).

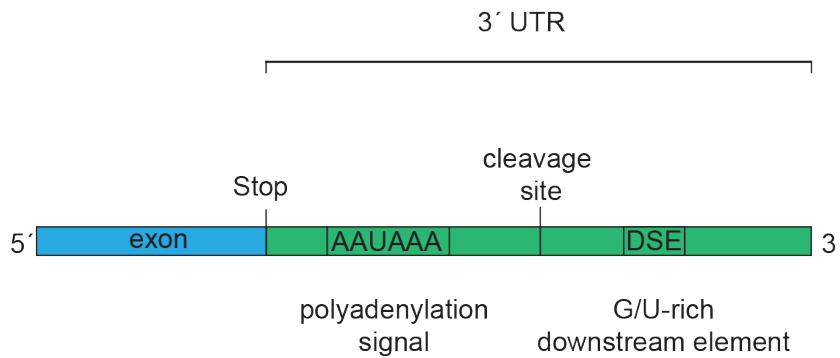


Figure 1.2: Sequence elements required for the efficient cleavage and polyadenylation of the pre-mRNA. Spatial organization of the sequence elements in the 3'UTR of mammalian pre-mRNA. The cleavage site is flanked by the polyadenylation signal and the G/U-riche downstream elements (DSE).

These sequence elements are important for the correct positioning and recognition of the cleavage site by specific protein complexes like the cleavage and polyadenylation specificity factor (CPSF), and the cleavage stimulation factor (CstF) complex.

Additional core factors include the poly(A) polymerase (PAP), the nuclear poly(A) binding protein (PABPN1), symplekine, and the mammalian cleavage factor I (CFI) and II (CFII) (Neve J. et al., 2017). The major multisubunit complex that mediates the cleavage of the 3' untranslated region (UTR) is the CPSF. This complex contains five core subunits and one of them, CPSF160, directly binds to the polyadenylation site of the pre-mRNA. Subsequently, another component, CPSF73, recognizes the cleavage site and cleaves the pre-mRNA (Clerici, Faini et al. 2017). The Cstf complex instead, is required for the binding to the DSE element and consists of three subunits. The Cstf-64 subunit (CSTF2) is the subunit binding the DSE, whereas the other two components, Cstf-50 (CSTF1) and Cstf-77 (CSTF3), are required for CPSF-CstF cooperative RNA binding during the assembly process (Ana Casañal 2017, Casanal, Kumar et al. 2017) **(Figure 1.3 A)**. CFI and CFII are essential for the stabilization of CPSF binding to the pre-mRNA and interact with the pre-mRNA immediately upstream of the polyadenylation signal. Lastly, Symplekin serves as a scaffold for recruiting other factors to the cleavage and polyadenylation complex (Takagaki and Manley 2000) (Neve, Patel et al. 2017).

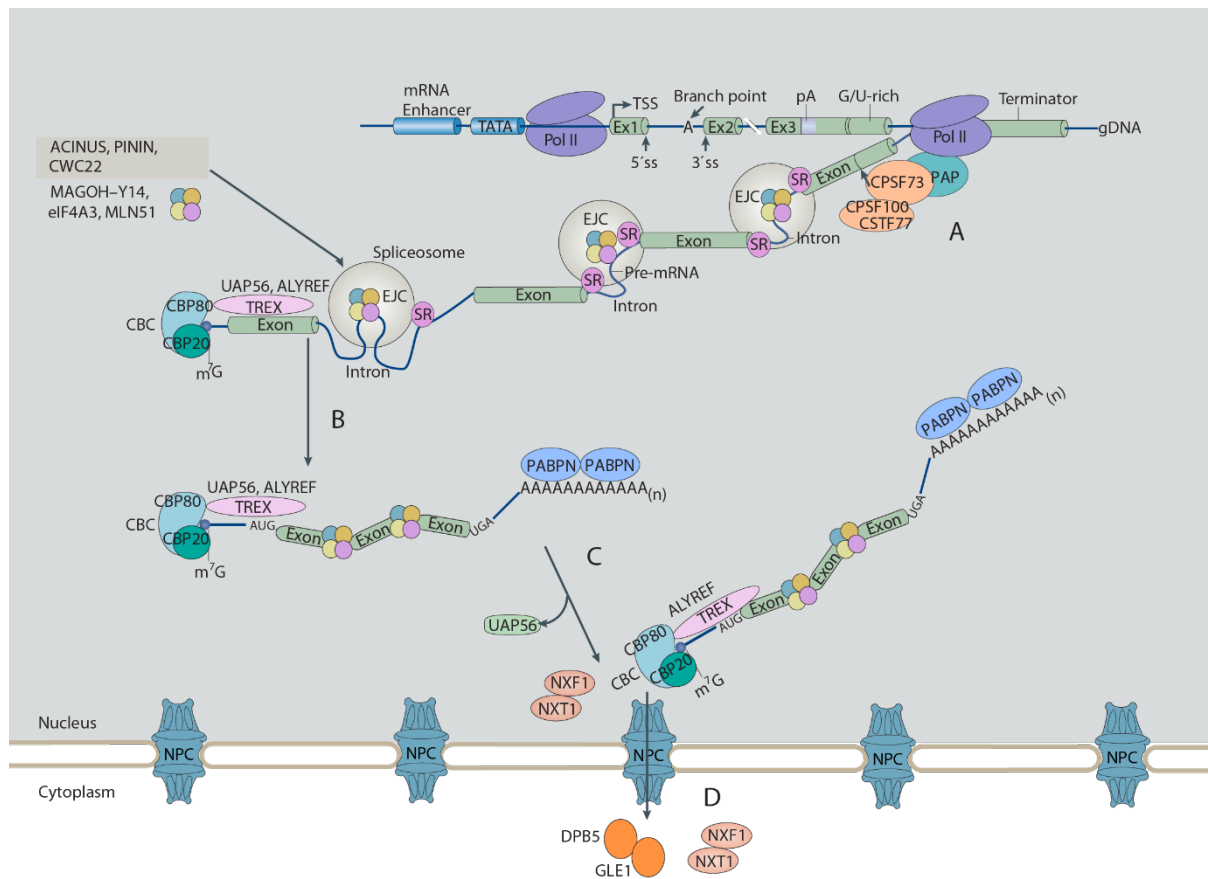


Figure 1.3: Synopsis of eukaryotic gene expression in the nucleus. Gene expression begins in the nucleus, RNA pol II transcribes the nucleotide information present in DNA into a pre-mRNA. This pre-mRNA undergoes co-transcriptional processing, involving steps like 5' RNA capping (not shown), pre-mRNA splicing, and 3' RNA polyadenylation. **(A)** Before being transported into the cytoplasm as an export-competent mRNP, pre-mRNAs undergo 3' end processing which involves a cleavage step followed by the addition of 150-200 adenosine residues. **(B)** During splicing, introns are removed and exons are joined together by the spliceosome which directly interacts with the CBC. After splicing, proteins of the EJC marking the site of each splicing event, are added. The CBC binds directly to the ALY/REF subunit of the human TREX complex. **(C)** Once the mRNP is ready to be exported UAP56 departure ensures the recruitment of NXF1 and NXT1 by ALYREF which work as general mRNA export receptors for carrying the mRNPs, through the NPCs. **(D)** Once in the cytoplasm, the mRNPs require a termination step to be released from their nuclear receptors. Here, Dbp5 ATP-dependent RNA helicase activity is triggered by Gle1 allowing the final release of the mature transcript.

Following cleavage, the A(A/U)UAAA-bound CPSF anchors PAP to the pre-mRNA to carry out polyadenylation helped by PABPN1, which avoids the dissociation of the poly(A) tail from the enzyme after the addition of each new nucleotide (Kuhn and Wahle 2004). As the tail grows, additional PABPN1 molecules are bound. When the poly(A) tail exceeds a critical length of ~150-200 adenosine residues, additional PABPN1 molecules can no longer be held in the spherical RNA-protein complex, and the contact between PAP and CPSF is disrupted (Eckmann, Rammelt and Wahle 2011).

1.1.6 The EJC-mediated mRNA export

After the last step of maturation is finished, thanks to the addition of the poly(A) tail, the mRNA is ready to be exported into the cytoplasm. Early pioneering discoveries led to the conclusion that the EJC was the connecting piece between the splicing event and export in metazoan. The EJC core constitutes a binding platform for more than a dozen factors, called EJC peripheral factors. Among those peripheral factors, we can find UAP56 (also known as DDX39B) and Aly/REF export factor (ALYREF; also known as THOC4) which are conserved factors that are part of the TREX (transcription export) complex that bridges pre-mRNA synthesis and export of the mRNA to the cytoplasm (Joao P. Rodrigues, Michaela Rode et al. , Katahira 2012) (**Figure 1.3 B**). UAP56 is loosely connected with purified EJCs (Tange, Shibuya et al. 2005) hinting that it may dissociate from the EJC immediately after its release from the spliceosomes.

Nevertheless, UAP56 departure might be required for the ensuing recruitment of nuclear export factor 1 (NXF1) and NTF2-related export protein 1 (NXT1) (also known as TAP-p15 complex) by ALYREF and/or by RNPS1 that work as general mRNA export receptors for carrying the messenger ribonucleoproteins (mRNPs), through the Nuclear pore complexes (NPCs) (Segref, Sharma et al. 1997, Herve Le Hir, David Gatfield et al. 2001, Gatfield and Izaurralde 2002) (**Figure 1.3 C**). However, an illuminating study from (Cheng, Dufu et al. 2006) showed that the human TREX complex is recruited in a splicing-dependent and cap-dependent manner at the very 5' end of the mRNA through the CBP80 subunit of the CBC. By binding directly the ALY/REF subunit of the human TREX complex it was revealed that the entire TREX complex does not bind at every exon-exon junction but at the very first exon. Once in the cytoplasm, the mRNPs require a termination step in order to be released from their nuclear receptors. Dbp5 is an ATP-dependent RNA helicase, which is involved in mRNA export and its helicase activity can be triggered by Gle1, an essential export factor that is also asymmetrically located at the cytoplasmic side of the nuclear pore (Lund and Guthrie 2005, Hodge, Tran et al. 2011). This local activation could generate directionality either by preventing the mRNP from sliding back from the cytoplasm to the nucleus or by applying a unidirectional force on the protruding 5' end of the mRNA, allowing the final release of the mature transcript (**Figure 1.3 D**).

1.1.7 mRNA Translation

In the cytoplasm, the CBC and the poly(A) binding protein nuclear (PABPN) dissociate from the mRNA molecule and are replaced by the poly(A) binding protein cytoplasmic PABPC1 and the eukaryotic initiation factor (eIF) 4E, along with several other eIFs (**Figure 1.4 A**). This process is essential for promoting ribosomal recruitment and mRNA translation, a process where each

consecutive nucleotide triplet of the mRNA, called codon, is decoded into a specific amino acid (AA) (Jackson, Hellen and Pestova 2010, Shirokikh and Preiss 2018).

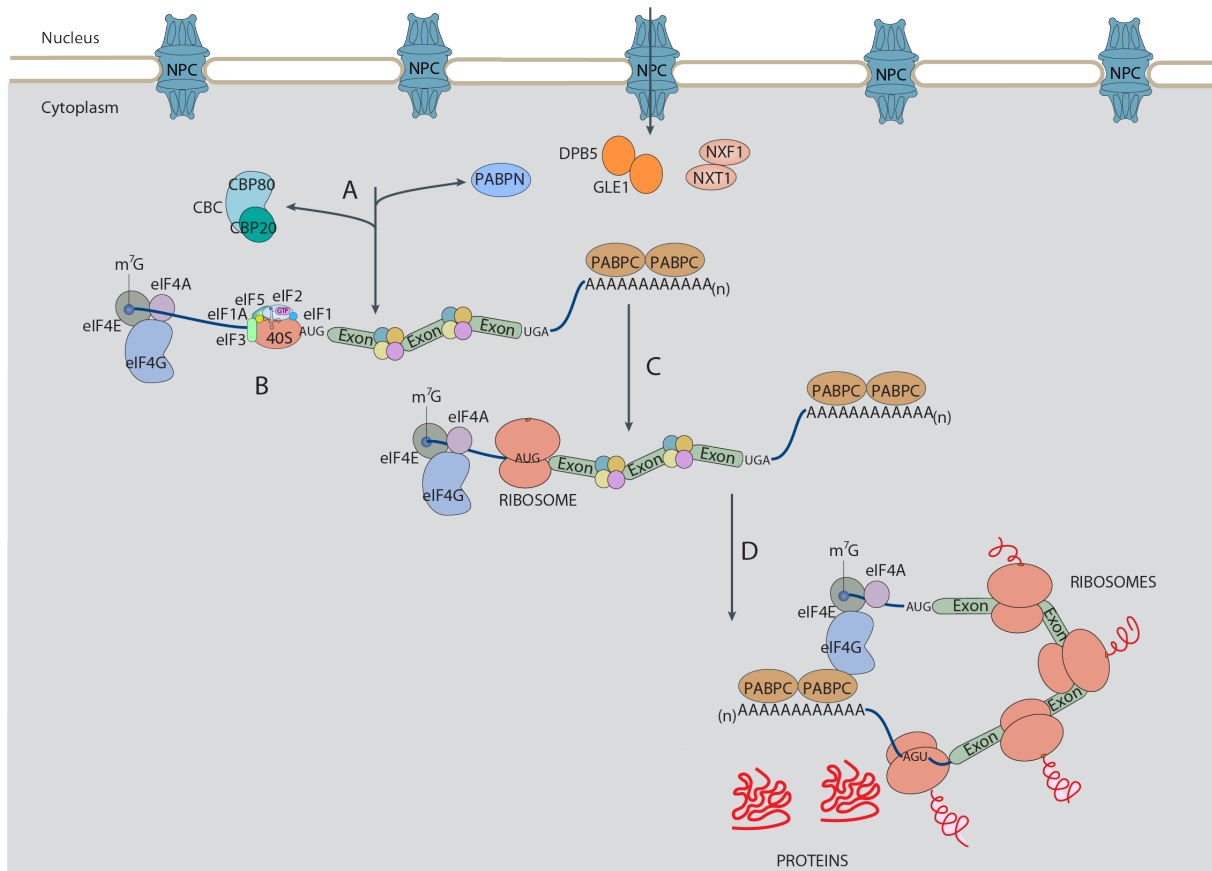


Figure 1.4: Synopsis of eukaryotic gene expression in the cytoplasm. (A-B) In the cytoplasm, the CBC and the PABPN dissociate from the mRNA molecule and are replaced by the PABPC1 and eIF4E, along with several other eIFs. **(C-D)** The interaction of eIF4E-eIF4G on the 5' end in conjunction with eIF3 (not shown) and the PAB bound to the 3' poly(A) tail, loads the mRNA onto the 43S complex to form the 80S complex. The 80S complex then starts scanning down the mRNA in a 5' to 3' direction, looking for the initiation codon. When the 43S complex recognizes the first AUG codon, the codon-anticodon base pairing takes place between the initiation codon and the initiator tRNA in the ternary complex. After GTP hydrolysis by eIF2 (not shown), the 60S subunit joins to form the elongation-competent 80S initiation complex (Hinnebusch 2017). At the end of the initiation process of translation, there is a complete ribosome linked to the mRNA in correspondence of an AUG starting codon. During each cycle of the elongation phase of translation, a loaded tRNA with anticodon complementarity to the mRNA codons releases the corresponding amino acid to the growing polypeptide chain and translocates between the tRNA binding sites on the 80S ribosome. Translation termination happens when a stop codon (UAA/UAG/UGA) sequence is found in the A site of the ribosome. The protein is produced and the cycle can start again.

mRNA translation occurs in a structure called the ribosome. Ribosomes are ribonucleoprotein (RNP) composed of four ribosomal RNAs (18S, 5S, 5.8S, and 25S (yeast)/28S humans rRNAs) and 80 ribosomal proteins. These constitute the scaffold for a complex machinery, the 80S ribosome that is formed by two subunits, the small 40S and the large 60S subunits (Shirokikh and Preiss 2018). At the ribosome's functional core, ribosomal RNAs (rRNAs) provide a structural scaffold and catalytic activity for the critical steps of decoding the mRNA and facilitating amino acid

polymerization. Ribosomes interact with transfer RNAs (tRNAs) that decode the mRNA and deliver the amino acids to synthesize new polypeptides. The genome encodes about 300 different cytoplasmic tRNAs, mitochondrial DNA encodes another 22, and there is at least one tRNA species for each of the 20 amino acids. tRNA molecules are characterized by a cloverleaf-like secondary structure of 76 to 93 nucleotides that end with CCA trinucleotides at their 3'-ends, with the 2'- or 3'-OH of the terminal adenosine that conveys the AA specific for the corresponding mRNA codon, forming an aminoacyl-tRNA (aa-tRNA) (Graifer and Karpova 2015, Schimmel 2018) (Yusupova and Yusupov 2017). Once an amino acid is stably attached to the tRNA, its position in a growing polypeptide chain is defined by the codon-anticodon interactions on the mRNA (Schimmel 2018). The ribosome contains three sites that host the tRNAs during translation: an aminoacyl-tRNA site (A Site), where the nucleotide triplets of the mRNA are decoded by the aa-tRNA, a peptidyl site (P site), where the growing peptide with the tRNA associated is located, and an exit site (E site), where the uncharged tRNA is released (Steitz 2008, Pena, Hurt and Panse 2017, Yusupova and Yusupov 2017). The combined actions of the ribosomes, tRNAs, mRNAs, and associated proteins define the molecular mechanism of protein synthesis, which can be identified by four main stages: initiation, elongation, termination, and ribosome recycling (Shirokikh and Preiss 2018).

1.1.8 Translation Initiation

The first step in the initiation of cap-dependent translation is the assembly of the eIF2-GTP-Met-tRNA_i initiator (tRNA_i) ternary complex. The tRNA_i plays a critical role in the cell since it reads the start codon and carries the initial aa (methionine). After its formation, the ternary complex binds to the small (40S) ribosomal subunit. The resulting complex is called the 43S complex. After that, the initiation phase requires the cap-binding complex eIF4F, formed by the cap-binding protein eIF4E, the DEAD-box RNA helicase eIF4A, and the scaffolding protein eIF4G (Jackson, Hellen and Pestova 2010). It assembles on the 5'-cap of the mRNA and unwinds structures found in the 5'UTR. This process is achieved through the ATP-dependent action of eIF4A.

The interaction of eIF4E-eIF4G on the 5' end in conjunction with eIF3 and the poly(A) binding protein (PAB) bound to the 3'-poly(A) tail, loads the mRNA onto the 43S complex from the 48S complex (Sandra E. Wells, Paul E. Hillner et al., Amrani, Sachs and Jacobson 2006, Chen and Shyu 2011) (**Figure 1.4 B**). The 48S complex then starts scanning down the mRNA in a 5' to 3' direction, looking for the initiation codon. When the 43S complex recognizes the first AUG codon that is embedded in a specific sequence context, the codon-anticodon base pairing takes place between the initiation codon and the tRNA_i in the ternary complex (Kapp and Lorsch 2004).

This stimulates GTP hydrolysis by eIF2, a reaction facilitated by the GTPase-activating protein (GAP) eIF5, and in turn, eIF2-GDP releases the Met-tRNA_i into the P site of the 40S subunit and dissociates from it. Finally, eIF5B-GTP binds the 48S complex and, after its hydrolysis to GDP,

mediates the 60S subunit joining to form the elongation-competent 80S initiation complex (Hinnebusch 2017). At the end of the initiation process of translation, there is a complete ribosome linked to the mRNA in correspondence of an AUG starting codon (**Figure 1.4 C**).

1.1.9 Translation Elongation

During each cycle of the elongation phase of translation, a loaded tRNA with anticodon complementarity to the mRNA codons releases the corresponding amino acid to the growing polypeptide chain and translocates between the tRNA binding sites on the 80S ribosome (Dever, Dinman and Green 2018). The whole process is dependent on eukaryotic elongating factors (eEFs). Briefly, after the initiator tRNAⁱ Met bound to the P site of a ribosome is in position at an AUG start codon, a new aminoacyl-tRNA carrying the anticodon related to the next nucleotide triplets of the mRNA binds to the A site on the ribosome. At the end of the cycle, the deacylated tRNA shifts from the P site to the E site and the ribosome translocates to recognize the next codon on the mRNA (Steitz 2008).

1.1.10 Translation Termination, Ribosome Recycling, and Ribosome Quality Control

Translation termination happens when a stop codon (UAA/UAG/UGA) sequence is found in the A site of the ribosome (**Figure 1.4 D**). Firstly, during the termination step, release factors (eRFs) are recruited on site and the mature polypeptide is freed from the last tRNA. Subsequently, with the help of the ABC-family ATPase ABCE1 factor, the mRNA- and tRNA-bound 40S dissociate from the 60S, and the ribosomal subunits are recycled (Pisarev, Skabkin et al. 2010, Pisareva, Skabkin et al. 2011, Shoemaker and Green 2011). It is important to highlight that not every translational step always works efficiently and, as the ribosome elongates along an ORF, different factors that slow its decoding process have to be taken into account.

For example, different consecutive amino acids can stall the ribosome, either because of minimal kinetic reactions coming from the different nature of the amino acids, or from sterically difficult conformations of the nascent peptide that impair the passing through the exit tunnel site of the ribosome. Sometimes, ribosomal stalling can occur when mRNA sequences predominantly contain codons that correspond to tRNAs with low abundance. In particular cases, this stalling is due to a slow peptide bond formation like poly-proline or poly-lysine stretches. Furthermore, mRNA secondary structures such as stem-loops or pseudoknots can also affect the processivity of the ribosome (Doma and Parker 2006, Kuroha, Akamatsu et al. 2010, Letzring, Dean and Grayhack 2010, Letzring, Wolf et al. 2013, Han, Shichino et al. 2020, Matsuo, Tesina et al. 2020). In these situations, the ribosome can either rescue or halt translation until the proper factor or tRNA is conveyed. Alternatively, if the stalling is not solved, translation is interrupted, ribosomes are recycled and the protein gets degraded (Schuller and Green 2018).

In particular, when ribosomes stall within an ORF, the upstream ribosome clashes with the stalled ribosome (Simms, Yan and Zaher 2017), producing a unique interface that activates the entire cascade of ribosome associated quality control (RQC) (Juszkiewicz, Chandrasekaran et al. 2018, Ikeuchi, Tesina et al. 2019). Recent studies indicate that the interface of a di-ribosome collision is recognized by the ubiquitin ligase ZNF598 (Hel2 in yeast). Its ubiquitinating activity is essential for triggering quality control pathways that immediately arrest the translation of faulty transcripts, solve the stall and recycle or optionally favor ribosomal clearance (Juszkiewicz, Chandrasekaran et al. 2018). Ribosomal stalling is subsequently followed by endonucleolytic cleavage, after which mRNA degradation takes place through XRN1-mediated 5'-3' degradation and exosome-mediated 3'-5' degradation.

1.1.11 Eukaryotic mRNA Surveillance pathways

In the cell, there are three predominant pathways for co-translational mRNA surveillance: nonsense-mediated decay (NMD), no-go decay (NGD), and nonstop decay (NSD). Nonsense-mediated mRNA decay (NMD) specifically recognizes mRNAs having a premature stop codon, no-go decay (NGD) targets mRNAs having a wide range of potential structurally stalling elements (e.g stem-loops) and nonstop mRNA decay (NSD) targets transcripts lacking a termination codon. While these pathways do exhibit some level of specificity, it appears that they function cooperatively in facilitating the cell's ability to clear out faulty mRNA species. This concerted effort plays a pivotal role in detecting and responding to perturbations in protein homeostasis stress on a ribosomal level (Lykke-Andersen and Bennett 2014, Karousis and Muhlemann 2019, D'Orazio and Green 2021).

1.2 mRNA decay

How is the mRNA turnover elicited in the cell? Two major mRNA decay pathways determine the fate of eukaryotic mRNAs: the 5'-to-3' and the 3'-to-5' pathway. Both of them require the shortening of the poly(A) tail, a process also known as deadenylation. Poly(A) tails are removed by specific exonucleases that act mainly on adenosines (deadenylases). Two major protein complexes account for the bulk eukaryotic deadenylation activities in the cytoplasm: PAN2-PAN3 (**Figure 1.5A**) and CCR4-NOT.

The CCR4-NOT complex is the major deadenylase complex in the cell and 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 the two deadenylases CCR4, which is an EEP-type exonuclease and CAF1, which is DEDD-type exonuclease. CCR4 and CAF1 have two paralogs in human, (CCR4a/b and NOT6/6L, CAF1a/NO7 and CAF1b/NOT8). Other subunits include NOT9 (Caf40 in budding yeast) and two newly

identified subunits, NOT10 and NOT11 (Morgan Tucker, Marco A. Valencia-Sanchez et al. 2001, Bartlam and Yamamoto 2010, Boland, Chen et al. 2013, Mauxion, Preve and Seraphin 2013, Temme, Simonelig and Wahle 2014, Jonas and Izaurralde 2015, Collart 2016, Chen, Khazina et al. 2021, Passmore and Collier 2022, Raisch and Valkov 2022). In addition, NOT4 subunit can associate with the complex and induce protein degradation thanks to its E3 ubiquitin ligase activity (Albert TK, Hanzawa H et al. 2002).

The shortening of the poly(A) tail causes the dissociation of PABP and the destabilization of the mRNA (Collart 2016, Heck and Wilusz 2018). What we know at the moment regarding the processivity of the CCR4-NOT complex is that CCR4 engages in PABPC displacement from the poly(A) tail while CAF1 is the deadenylase responsible for trimming the unprotected poly(A) (Yi, Park et al. 2018) (**Figure 1.5 B-C**).

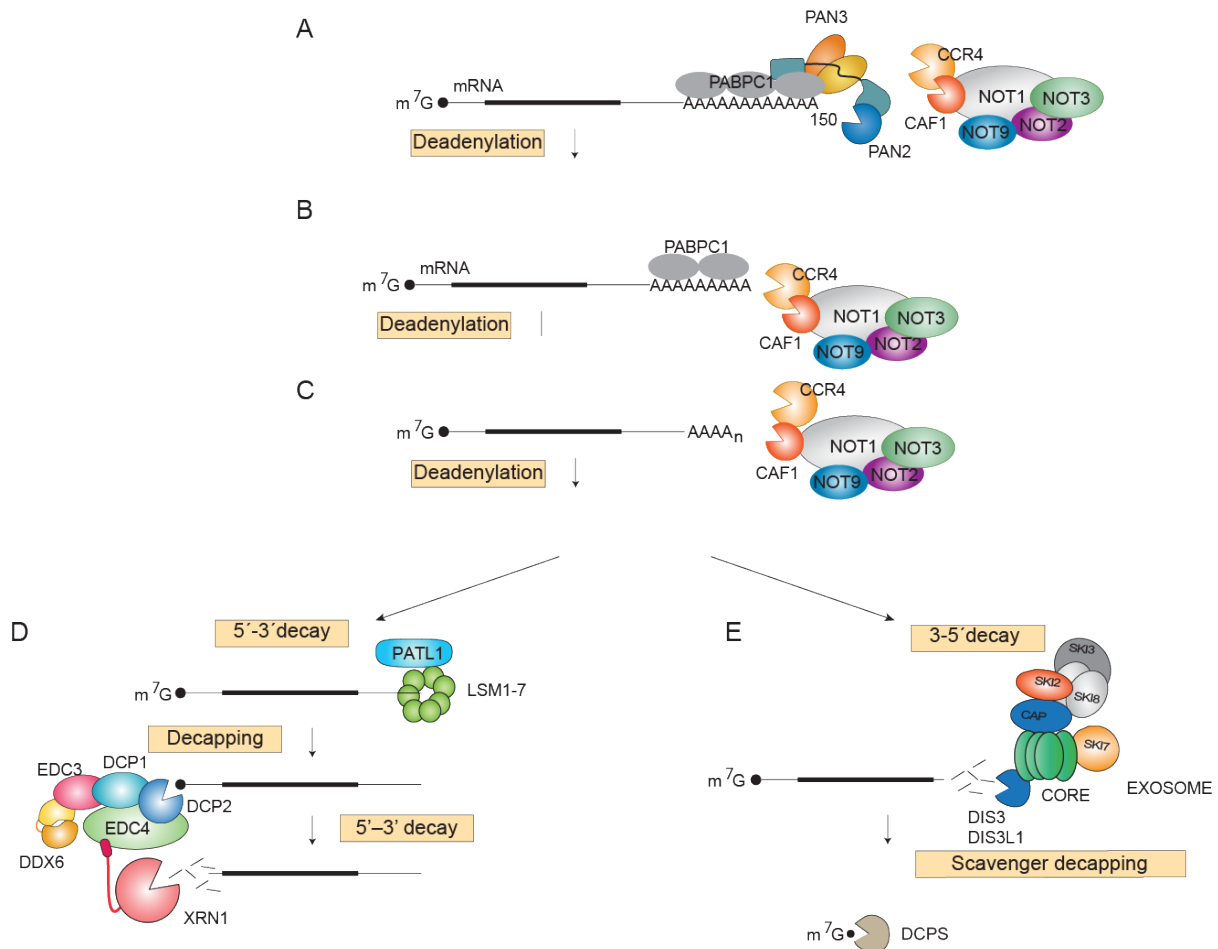


Figure 1.5: Mechanisms of bulk mRNA degradation (Figure adapted and modified from NL Garneau et al., 2007).

Schematic representation of the two major mRNA decay mechanisms in the cell, both beginning with the deadenylation of the mRNA at the 3'-end. Deadenylation is a two-step process, initiated by the PAN2-PAN3 complex, which catalyzes the removal of a short adenosine stretch from the 3'-end. Subsequently, the CCR4-NOT complex assumes responsibility for comprehensive deadenylation. Following the elimination of the poly(A) tail, mRNA degradation can proceed through two divergent mechanisms, each acting in opposite directions: 5' to 3' or 3' to 5'. The first pathway necessitates mRNA

decapping, a process executed by the DCP2 enzyme, in collaboration with auxiliary proteins such as LSM1-7, PATL1, and DDX6. Subsequent degradation is initiated at the 5' end, orchestrated by the XRN1 exonuclease. In contrast, the second mechanism requires the involvement of the exosome complex, which initiates degradation from the mRNA's 3' end. The culmination of this process involves the hydrolysis of the mRNA's cap structure, catalyzed by the DCPS enzyme.

After deadenylation, the pathways of mRNA decays separate: in the 5'-to-3' decay, several accessory proteins are recruited to the mRNA and are needed for efficient decapping.

These accessory proteins include enhancers of mRNA decapping (EDCs) such as sm-like protein 14 (LSM14), LSM15, the LSM1-7 complex, the PAT1 homolog protein 1 (PATL1), and the RNA helicase DEAD-box 6 (DDX6) (Jonas and Izaurralde 2013) (**Figure 1.5 D**). The process of decapping is primarily orchestrated by the decapping enzyme subunit 2 (DCP2), in complex with DCP1. DCP2 belongs to the Nudix hydrolase superfamily and uses its enzymatic activity to hydrolyze the 5' cap to a m⁷GDP and a mRNA with a 5' monophosphate end (Arribas-Layton, Wu et al. 2013) (Valkov, Muthukumar et al. 2016, Grudzien-Nogalska and Kiledjian 2017, Valkov, Jonas and Weichenrieder 2017, Wurm and Sprangers 2019, Kreml, Lazzaretti and Sprangers 2023). At this point, the DCP2 decapping enzyme in complex with activating co-factors removes the 5' cap so that the unprotected mRNA can be degraded by the 5'-to-3' exoribonuclease 1 XRN1 (**Figure 1.5 D**). Another 3'-to-5' exoribonuclease, PARN (poly(A)-specific ribonuclease), has been shown to carry out deadenylation on a specific small group of mRNAs but the context where it functions still has to be determined (Yamashita, Chang et al. 2005).

Alternatively, in the 3'-to-5' pathway, the mRNA is degraded directly from the 3' end by the multi-subunit protein complex called exosome, followed by DCPS (decapping scavenger enzyme) action, a protein that catalyzes the cleavage of the residual cap structure (**Figure 1.5 E**) (Garneau, Wilusz and Wilusz 2007, Fuchs, Wurm et al. 2020).

1.3 Recruitment of the CCR4-NOT complex by RBPs

It is well known that specific sequences (very often present in the 3'UTR) are recognized and bound by different RNA binding proteins (RBPs) that in turn can recruit deadenylases to specific transcripts. This recruitment leads to target-specific deadenylation. RBPs are characterized by RNA binding domains (RBDs), which bind to specific sequence motifs or structural elements (Sasse, Lavery et al. 2018, Corley, Burns and Yeo 2020, Katsantoni, van Nimwegen and Zavolan 2023).

1.3.1 The CCR4-NOT complex is a protein hub for RBPs

Many RBDs typically bind rather short sequence motifs and RBPs often possess multiple RBDs, which strongly increase binding specificity due to combined and thus more complex binding motifs (Hennig and Sattler 2015). In the case of the CCR4-NOT complex recruitment, some

proteins, like BTG2/Tob and SMG7 (Stupfler, Birck et al. 2016, Loh, Jonas and Izaurralde 2013), can directly interact with the nucleases themselves.

However, most other factors interact with specific regions of NOT1 and CAF40, which are the main docking sites for RBPs. Among the most known proteins that are able to recruit the major deadenylase complex to their target mRNAs are members of the Nanos protein family, Roquin, Cup, NOT4, 4E-T, Bicaudal-C, HELZ, YTHDF2 and PUM1/2. They all use the C-terminal domain of NOT1 as a binding site. The second main docking site for recruitment is through the small CAF40 subunit, which is preferentially bound by known RBPs as TTP (ZFP36), Roquin, Bam, NOT4, RNF219, and TNRC6A-B-C proteins (**Figure 1.6**).

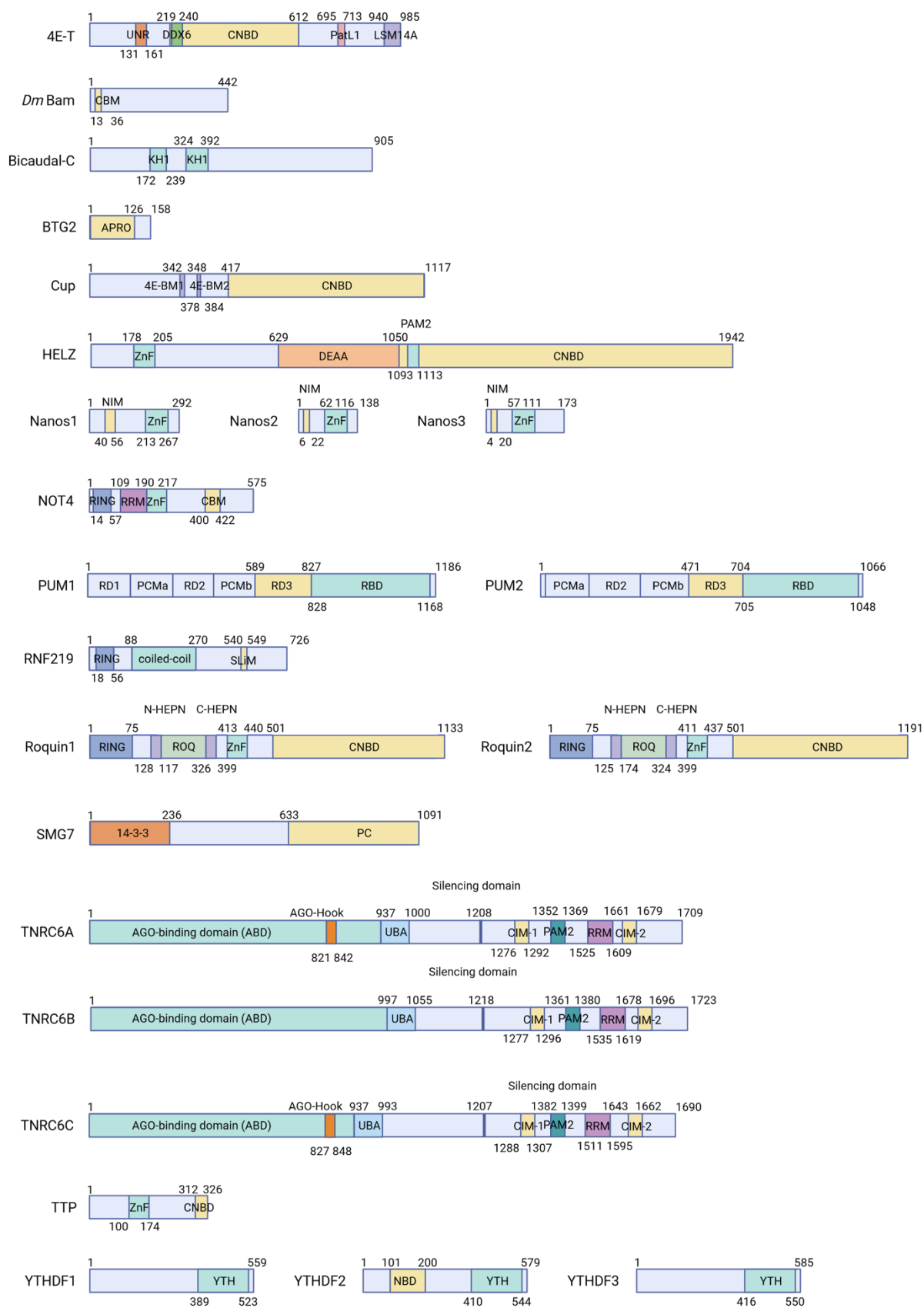


Figure 1.6: Schematic representation of the domain organization of all the mentioned RBPs that are known to recruit the CCR4-NOT complex. In yellow are highlighted all the domains, which interact with the CCR4-NOT complex.

(Dm) *Drosophila melanogaster*; CNOT Binding Domain [CNBD]; canonical eIF4E-binding motif [C]; noncanonical eIF4E-binding motif [NC]; UNR-interacting domain [UNR]; DDX6-interacting domain [DDX6]; PatL1-interacting domain [PatL1]; Lsm14A-interacting domain [Lsm14A]; CAF40-binding motif [CBM]; K Homology (KH) domain [KH]; AntiPROliferative domain [APRO]; eIF4E-binding motifs [4E-BM1 and 4E-BM2]; RNA Recognition Motif [RRM]; Zinc finger domain [Znf]; putative helicase (Asp, Glu, Ala, Ala) domain [DEAA]; NOT module binding region [NBR]; Nanos effector domain [NED]; NOT1-interacting motif [NIM]; Repression domains [RD]; Pumilio conserved motifs [PCMa and PCMb]; RNA-binding domain [RBD]; short linear motif [SLiM]; RING-finger E3 ubiquitin ligase domain [RING]; ROQ RNA-binding domain [ROQ]; 14-3-3-like domain [14-3-3]; Proline-rich C-terminus [PC] region; Argonaute-binding domain [ABD]; CCR4-NOT interacting motif [CIM]; an ubiquitin associated domain [UBA]; a PABP-interacting motif 2 [PAM2]; YT521-B homology domain [YTH]; NOT1 Binding Domain [NBD]; Created with BioRender.com. (Huntzinger, Braun et al. 2010, Chekulaeva, Mathys et al. 2011, Fabian, Cieplak et al. 2011, Igreja and Izaurralde 2011, Huntzinger, Kuzuoglu-Ozturk et al. 2013, Leppek, Schott et al. 2013, Bhandari, Raisch et al. 2014, Poetz, Corbo et al. 2021).

To give some examples in more detail, tristetraprolin (TTP) directly interacts with the CCR4-NOT complex to induce degradation of target mRNAs, like cytokines, which contain AU-rich elements (AREs) in their 3'-UTRs (Sandler, Kreth et al. 2011, Fabian, Frank et al. 2013)).

Other RBPs that lead to mRNA silencing through deadenylation are members of the highly conserved Pumilio protein family (PUM/Puf proteins, reviewed in (Nishanth & Simon, 2020)). Pumilio proteins contact RNA substrates through a conserved domain, referred to as the PUM-homology domain or PUM-HD (Zamore, Williamson et al., 1997).

This domain contains eight rather short repeats each contacting one base of the RNA binding motif (Wang, McLachlan et al., 2002). Pumilio proteins have been implicated in processes as diverse as embryonic development (Lehmann 1987, Nüsslein-Volhard C 1987), innate immunity or general cellular pathways such as ribosome biogenesis for example (Goldstrohm, Hall and McKenney 2018). Among many other mechanisms that have been reported, some Pumilio proteins can recruit the CCR4-NOT complex by direct interaction with NOT1 and NOT2 to induce deadenylation-dependent RNA degradation (Arvola, Weidmann et al. 2017, Arvola, Chang et al. 2020, Enwerem, Elrod et al. 2021). Another RBP that can recruit the CCR4-NOT complex is Nanos (NOS). Similarly to Pumilio proteins, NOS is important for development in various organisms. NOS proteins use a specialized Zn finger motif to contact its substrate RNA (Curtis, Treiber et al. 1997). NOS can also directly interact with the CCR4-NOT complex through interaction with the NOT module and recruit it to specific target mRNAs (Bhandari, Raisch et al. 2014, Raisch, Bhandari et al. 2016).

Moreover, PUM and NOS have been found together in one repressive complex and since they contact the CCR4-NOT at different sites, these concerted interactions could increase the affinity of the CCR4-NOT complex to specific RNAs. In *Drosophila*, NOS can enhance PUM-mediated repression (Asaoka-Taguchi 1999), which would be consistent with such a model. A very important example are GW182/TNRC6 proteins which are fundamental factors in microRNA (miRNA) mediated gene silencing.

Although not being RBPs, these proteins interact with the miRISC (miRNA-induced silencing) complex and promote deadenylation, translational repression, and mRNA degradation through the recruitment of the CCR4-NOT and PAN2-PAN3 complexes (Braun, Huntzinger et al. 2011, Chekulaeva, Mathys et al. 2011, Fabian, Cieplak et al. 2011). Besides RBPs and miRNAs, also specific mRNA modifications like m6A, can regulate gene expression by recruiting effector proteins like the YTH (YT521-B homology) family proteins, particularly YTHDF2. It has been shown that YTHDF2 can recruit the CCR4-NOT complex and influence the stability of m6A-marked mRNAs (Du, Zhao et al. 2016) (Wang, Lu et al. 2014). Notably, recent findings in yeast, have shown that codon usage is one of the major determinants of mRNA stability and that mRNA sequence is responsible for almost 60% of mRNA half-life variations compared to 5.5% of 3'UTR paving the way for a new layer of complexity in mRNA metabolism (Passmore and Collier 2022), which won't be focused and discuss further in this dissertation.

1.3.2 The importance of Intrinsically Disordered Regions (IDRs) in RBPs recruiting the CCR4-NOT complex

What do all the RBPs that recruit the CCR4-NOT complex have in common? In addition to an RNA binding module, most RBPs accommodate Intrinsically Disordered Regions (IDRs), which are protein regions that do not adopt a stable or well-defined three-dimensional structure under physiological conditions. These regions lack hydrophobic amino acids and mainly contain charged residues, which exhibit high flexibility. A subclass of IDR includes the so-called Short linear motifs (SLiMs), short regions of less than ten amino acids that mediate low-affinity, transient interactions with peptide binding domains.

Despite their small size, SLiMs are involved in a diverse array of functions, including protein complex assembly, substrate recruitment, protein stability control, subcellular localization, and upon binding onto the surface of the partner's defined domains, inducing secondary structures. Recently, the hypothesis that SLiMs play key roles in protein evolution has gained interest, as they offer a mechanism by which a protein can gain important regulatory functions with the change of only a few amino acids (van der Lee, Buljan et al. 2014, Calabretta and Richard 2015, Davey, Cyert and Moses 2015).

Most RBPs contain SLiMs within their IDRs that enable them to interact with specific RNA sequences or other proteins involved in RNA regulation. Roquin, for example, contains a SLiM that interacts with the NOT9 protein to regulate mRNA stability. TNRC6ABC can recruit the CCR4-NOT complex through several tryptophan residues which are inserted into tandem hydrophobic pockets exposed at the convex surface of NOT9 (Chen, Boland et al. 2014).

Pumilio and YTHDF2 also contain multiple SLiMs that bind to regulatory elements in target mRNAs but, so far, how they structurally interact with NOT1 is not known. Similarly, the

components of the CCR4-NOT complex also contain such elements, suggesting that a dense network of IDR interactions facilitates the coordination of gene regulatory pathways and promotes the formation of biomolecular condensates like P-bodies. Although we are still in the early stages of understanding the intricacies of gene regulation within the context of messenger ribonucleoproteins (mRNPs), it is becoming increasingly evident that these dynamic structures are essential for maintaining normal cellular function. Any disruption or dysfunction in these processes can lead to various diseases and developmental anomalies.

1.4 PABPC role in deadenylation

One of the key steps in the initial phase of mRNA decay is poly(A) tail shortening by deadenylases like the PAN2-PAN3 and the CCR4-NOT complexes. In the cytoplasm, PABPC1 covers the poly(A) tail, and its presence is critical for promoting translation initiation and termination, recycling of ribosomes, and mRNA stability (Mangus 2003). PABPC1 contains four RRM motifs (1–4), a proline-rich unstructured linker, and a MLLE domain, formed by a conserved KITGMLLE motif (Huntzinger et al., 2010). The MLLE domain recognizes a conserved motif termed PABP-interacting motif 2 (PAM2), which was first identified in the translational regulators Paip1 and Paip2 (PABP-interacting proteins 1 and 2), and in other RBPs involved in mRNA decay (Huntzinger et al., 2013). Early depletion experiments have shown that yeast PABPC (Pab1p) functions as an inhibitor of mRNA decapping, and in its absence, the rates of deadenylation are affected (Caponigro G 1995). Additionally, Pab1p was found to stimulate the yeast Pan2 deadenylase (Boeck, Tarun et al. 1996) and to inhibit Ccr4p deadenylase activity, suggesting that the dissociation of Pab1p from the poly(A) tail may be rate-limiting for deadenylation *in vivo* (Morgan Tucker, Marco A. Valencia-Sanchez et al. 2001). Furthermore, PABP binds TNRC6 proteins. This association is thought on one hand to be essential for juxtaposing the poly(A) tail against the miRISC-associated deadenylase complex, on the other hand, to compete with eIF4G binding and interfere with eIF4G-PABP cap-dependent translation (Fabian et al., 2009, Zekri et al., 2009).

Only recently though, the relationship between PABPC and deadenylases in *human* has been uncovered. It has been shown that the PAN2-PAN3 complex recognizes the PABPC1 oligomerization interface and therefore acts mainly on poly(A) RNA long enough to accommodate multiple PABPC1 (Schäfer I. B. et al., 2019).

Regarding the CCR4-NOT complex, CAF1 is responsible for trimming PABPC-free poly(A) tails, while CCR4 removes PABPC-bound poly(A) tails, protecting transcripts from terminal uridylation and early decay (Webster, Chen et al. 2018, Yi, Park et al. 2018, Webster, Stowell and Passmore 2019). It was also found that PABPC not only plays a critical role in regulating the abundance of a specific class of mRNAs containing short UTRs but also protects stable transcripts from

undergoing deadenylation-independent decay by inhibiting the LSM1-7 complex (Kajjo, Sharma et al. 2022) making PABPC as the last standing signal before committing an mRNA to decay.

1.5 Components and mechanisms of human miRNA-guided gene silencing

In the intricate landscape of mRNA regulation and turnover, miRNAs are a key component since they silence gene expression by repressing translation and accelerating target mRNA degradation. MiRNAs are small regulatory RNAs that are present in almost all eukaryotes (Bartel 2018) (Treiber, Treiber and Meister 2019). They are encoded in the genome as individual genes or as a cluster and some of them are even embedded in introns of protein-coding genes. MiRNAs are transcribed by RNA pol II to capped and polyadenylated primary transcripts (Cai, Hagedorn and Cullen 2004) (Lee, Kim et al. 2004, Treiber, Treiber and Meister 2019). In the nucleus, the microprocessor complex composed of the RNase III enzyme Drosha and its double-stranded (ds) RNA binding partner DGCR8 process such transcripts to hairpins often referred to as miRNA precursors or pre-miRNAs (Denli 2004) (Han, Lee et al. 2004) (Landthaler, Yalcin and Tuschl 2004) (Lee 2003). The pre-miRNA products are transported to the cytoplasm by the export factor Exportin5 in an RNA-GTP dependent manner (Yi, Qin et al. 2003, Bohnsack, Czaplinski and Gorlich 2004, Lund E 2004). In the cytoplasm, the RNase III enzyme Dicer cleaves the pre-miRNA and produces a short dsRNA of about 20-24 nucleotides (nts) in length (Grishok A 2001, Ketting, Fischer et al. 2001). In human, Dicer, with the help of the dsRNA binding TRBP (protein transactivation response element RNA-binding protein), loads the strand with the less stably paired 5' end directly to a member of the Argonaute (AGO) protein family to form the RNA-induced silencing complex (RISC) (**Figure 1.7**) (Khvorova A 2003, Schwarz DS 2003, Noland, Ma and Doudna 2011). In addition, heat shock proteins like Hsc70 and Hsp90 are required for the loading step, probably to stabilize the open conformation of AGO (Iwasaki, Kobayashi et al. 2010, Miyoshi, Takeuchi et al. 2010, Willkomm, Jakob et al. 2022).

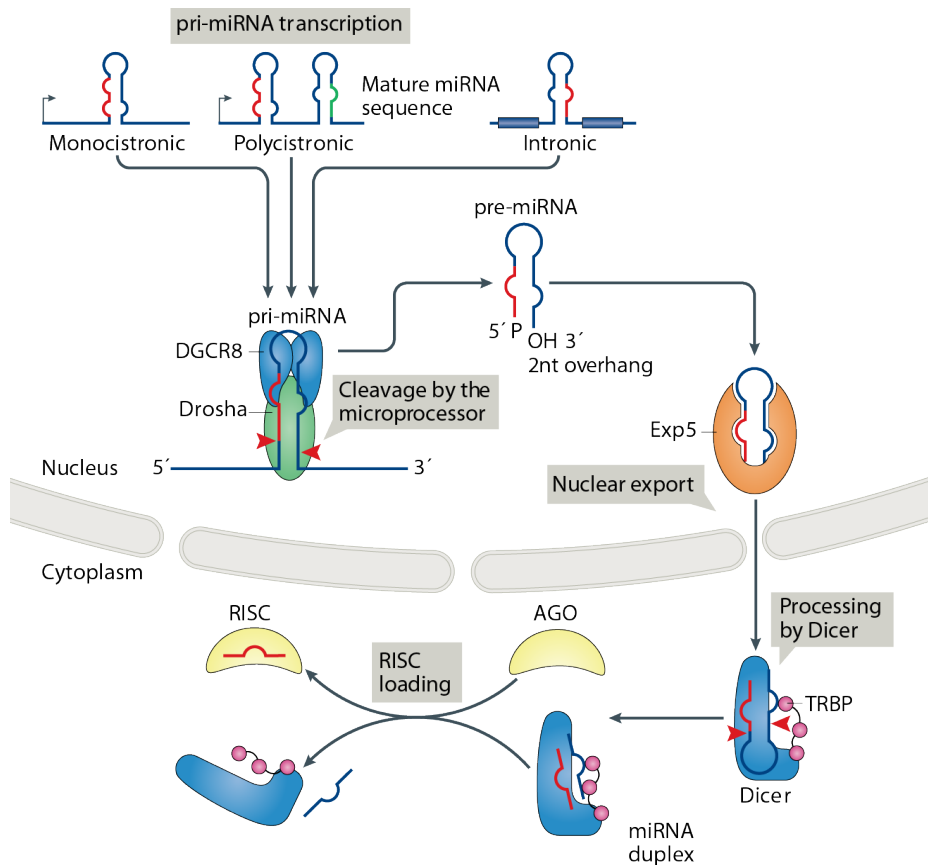


Figure 1.7: Canonical microRNA biogenesis. (Adapted from Treiber et al., 2019). MiRNAs are transcribed by RNA polymerase II to capped and polyadenylated primary transcripts. In the nucleus, the microprocessor complex composed of the RNase III enzyme Drosha and its double-stranded (ds) RNA binding partner DGCR8 process such transcripts to hairpins often referred to as miRNA precursors or pre-. The pre-miRNA products are transported to the cytoplasm by the export factor Exportin5. In the cytoplasm, the RNase III enzyme Dicer cleaves the pre-miRNA and produces a short dsRNA of about 20-24 nucleotides (nts) in length. In human, Dicer, with the help of the dsRNA binding protein TRBP, loads the strand with the less stably paired 5' end directly to a member of the Argonaute (AGO) protein family to form the RNA-induced silencing complex (RISC).

MiRNA containing AGO proteins are AGO1, 2, 3, 4, and a miRNA-carrying RISC is often referred to as miRISC. AGO proteins are highly conserved between species and many organisms present multiple copies of the family (e.g. *H. sapiens* 8, *C. elegans* 27, *A. thaliana* 10, *D. melanogaster* 5) (Meister 2013). These proteins can interact directly with small RNAs and are classified according to the class of small RNAs that they bind (Peters and Meister 2007, Hutvagner and Simard 2008). The eukaryotic Argonaute family can be divided into AGO proteins and PIWI proteins. While the expression of the PIWI subfamily of Argonaute proteins seems to be limited to the germline, where they bind to PIWI Interacting RNAs (piRNAs) and inhibit the expression of mobile genetic elements (Siomi, Sato et al. 2011), the subfamily AGO is ubiquitously expressed and interacts with short interfering RNAs or miRNAs to mediate post-transcriptional gene silencing processes (Meister G. 2004, Carthew and Sontheimer 2009). Notably, only a subset of AGO proteins has

endoribonuclease activities and can be called "slicers" (Meister, Landthaler et al. 2004) (Liu J 2004). In *Human*, AGO2 is the only slicer enzyme guided by small RNAs such as miRNAs or siRNAs (Meister, Landthaler et al. 2004) (Liu J 2004) and deep-sequencing analysis has shown that specific miRNA subpopulations might preferentially bind to distinct AGO proteins and that AGO identity impacts the length of the bound miRNA. On the other hand, it has also been reported that miRNAs exhibit non-specific binding to AGO clade proteins (Dueck, Ziegler et al. 2012).

1.5.1 Molecular architecture of AGO proteins

Structurally, Argonaute proteins consist of several domains, including an amino-terminal (N), a PAZ (Piwi-Argonaute-Zwille) domain, a MID (middle) domain, and a PIWI (P-element-induced wimpy testes domain) (Jinek and Doudna 2009) (**Figure 1.8**). The N-terminus of mammalian AGO proteins shows some degree of variation. The N-terminus of AGO2, for example, consists of two clusters of residues (44-48 aa and 134-166 aa), which are essential for its complete catalytic activity. If these amino acids are mutated, AGO2 is incapable of initiating RISC activation and mRNA cleavage. These residues are located close to the catalytic center in the PIWI domain and therefore play a crucial role in ensuring the correct positioning of the guide-target complex (Hauptmann, Dueck et al. 2013). The PAZ domain provides a specific pocket that accommodates the 3' end of the small RNA. The 5' end of the guide strand of the small RNA instead, is anchored to the MID domain by stacking its base against the residue Y529, which additionally recognizes the first nucleotide of the guide strand through hydrogen bonding and van der Waals interactions (Schirle et al., 2012).

The PIWI domain adopts an RNaseH fold and in its catalytic center harbors two metal ions required for RNA cleavage. The metal ions, like Manganese or Magnesium, are coordinated by aspartate and histidine residues located within the PIWI domain at position D512, D571, and E578, which has to relocate during binding to form the catalytic triad (Yuan, Pei et al. 2005). In addition to its structural domains, AGO2 also contains a flexible linker region that connects the MID and PIWI domains (Yang, Jakymiw et al. 2004, Liu, Valencia-Sanchez et al. 2005, Yuan, Pei et al. 2005, Frank, Sonenberg and Nagar 2010, Elkayam, Kuhn et al. 2012, Schirle N. T. 2012, Schirle N. T. 2014) (**Figure 1.8**). It has been proposed that also AGO3 might act similarly to AGO2 (Meister, Landthaler et al. 2004, Park, Sim et al. 2020). However, even though the catalytic tetrad is conserved in AGO3, it has been shown that an AGO3-specific insertion at the N-terminus inactivates it when loaded with a 21 nt long RNA (Hauptmann, Dueck et al. 2013, Nakanishi, Ascano et al. 2013) (Schurmann, Trabuco et al. 2013). Interestingly, a recent work has shown that AGO3 can accommodate smaller RNA species (referred to as tinyRNAs), which can serve as guides for AGO3-mediated cleavage of complementary RNAs (Park, Sim et al. 2020). The remaining AGO1 and AGO4 are deficient in crucial catalytic residues therefore, the only way for them to become

catalytically active is by replacing these domains with their AGO2 equivalents as showed in detail for AGO4 (Hauptmann, Dueck et al. 2013, Nakanishi, Ascano et al. 2013).

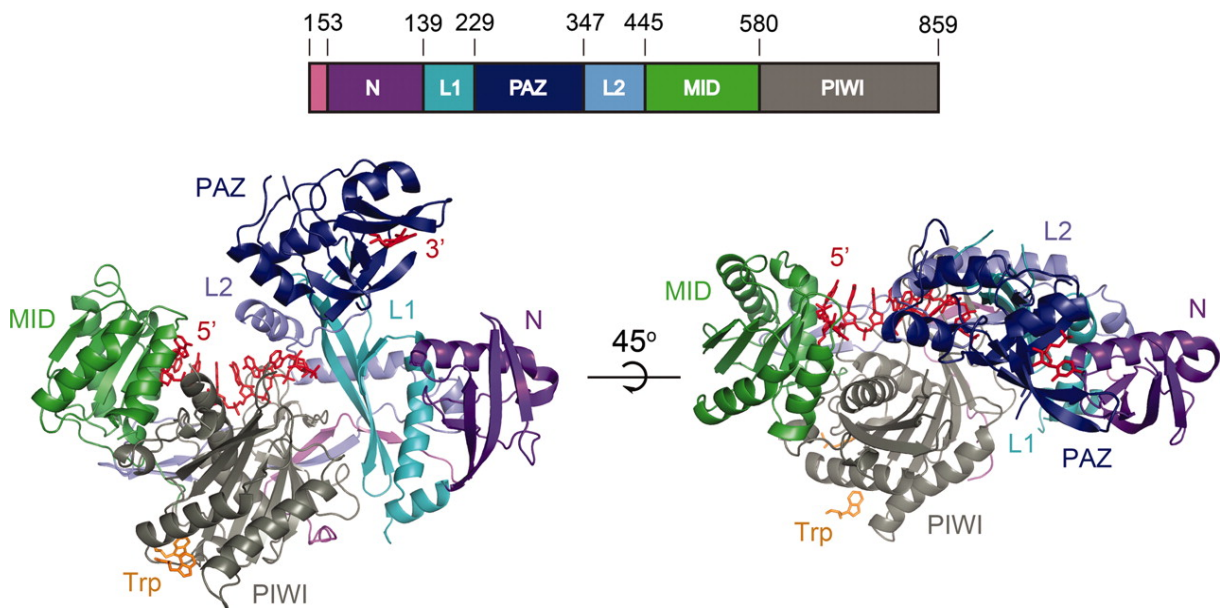


Figure 1.8: Structure of human AGO2 (Schirle et al., 2012). (Top): Representation of the primary sequence of AGO2. (Bottom): Front and top views of *human* AGO2, highlighting the N domain in purple, PAZ domain in navy, MID domain in green, PIWI domain in gray, and linkers L1 in teal and L2 in blue. A generic guide RNA is shown in red, spanning nucleotides 1 to 8 and 21. Tryptophan molecules are depicted in orange, binding to hydrophobic pockets within the PIWI domain.

1.5.2 miRNA-guided gene regulation processes

Small RNAs direct AGO proteins to their target RNAs, and AGOs act as scaffolds for associated proteins that assist in the key processes of gene silencing. Briefly, in the case of pairing with partially complementary sequences often localized in the 3' untranslated region (3'UTR) of the mRNA, the mature miRISC mediates the post-transcriptional silencing of the target mRNA. Perfect complementarity between targets and miRNA induces instead the cleavage of the mRNAs by catalytically active AGOs (Ameres and Zamore 2013, Meister 2013, Jonas and Izaurralde 2015, Bartel 2018). This process is rare in mammals but is frequently found in plants. In mammals, on mRNA targets which are only partially complementary to the miRNA, AGO proteins recruit the GW182 protein family which plays a central part in this process and are among the most extensively studied AGO partners. Through this mechanism, the mRNA is silenced by a combination of translational repression and mRNA degradation (Jonas and Izaurralde 2015). GW182 proteins function as a bridge between AGO proteins and downstream effector complexes, such as the cytoplasmic PAN2–PAN3 and CCR4–NOT deadenylase complexes (Braun, Huntzinger et al. 2011, Fabian, Cieplak et al. 2011). Subsequent deadenylation leads to recruitment of the decapping complex to the 5' cap (Rehwinkel, Behm-Ansmant et al. 2005). Decapped mRNA targets

are rapidly degraded by the 5'-3' exoribonuclease XRN1 (Braun, Truffault et al. 2012) (**Figure 1.9**).

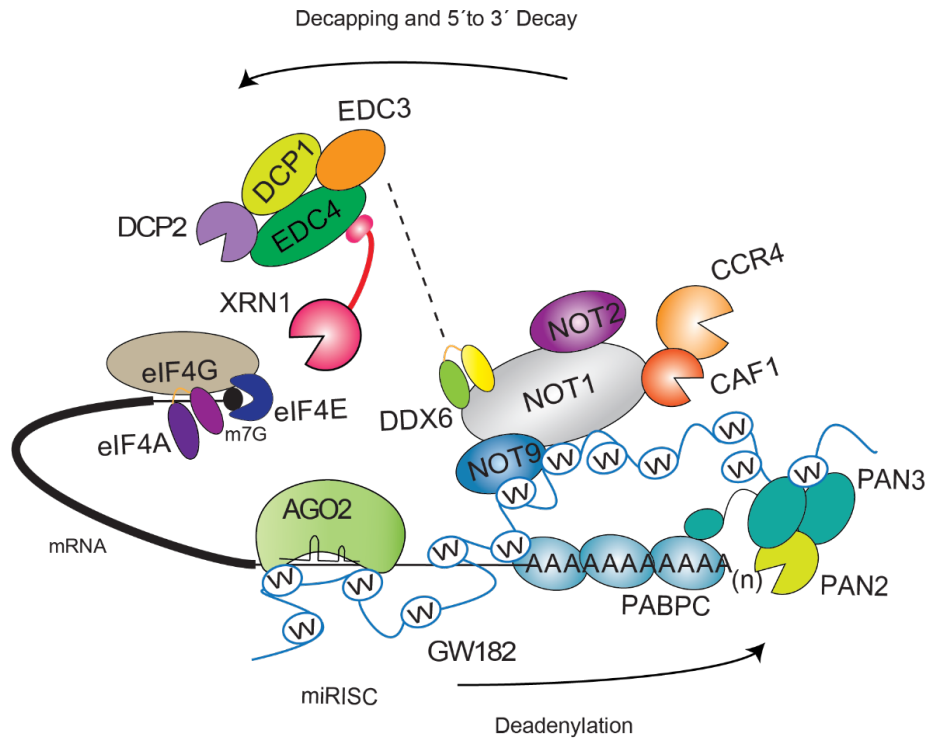


Figure 1.9: Schematic overview of miRNA-mediated gene regulation: a miRNA is loaded into an AGO protein and guides it to its target mRNA. A TNRC6 protein is then recruited and favors the binding of further downstream factors like the PAN2-PAN3 and CCR4-NOT deadenylase complexes as well as the PABPC proteins. For degradation of the target, the mRNA is first deadenylated by the PAN2-PAN3 and the CCR4-NOT complexes and then decapped by the decapping complex. The decapped mRNA is then subjected to 5' to 3' decay by the exonuclease XRN1 which is connected to the decapping enhancer EDC4 via a short linear motif in its very C-terminal end (modified from (Jonas and Izaurralde 2015)).

In addition, the consequent recruitment of DDX6 by NOT1 induces translational repression, mediated by different factors such as eIF4E-binding proteins (4E-BPs), like eIF4E-transporter protein (4E-T) for example, which compete with eIF4G for eIF4E binding, and the eIF4E-homologous protein (4EHP), which together with different RBPs, can impair translation by competing with eIF4E to bind the cap structure (Bartel 2018, Ozgur, Basquin et al. 2015, Peter, Igreja et al. 2015, Chapat, Jafarnejad et al. 2017, Peter, Ruscica et al. 2019, Ruscica, Bawankar et al. 2019, Rasch, Weber et al. 2020, Peter, Weber et al. 2017) (**Figure 1.10**).

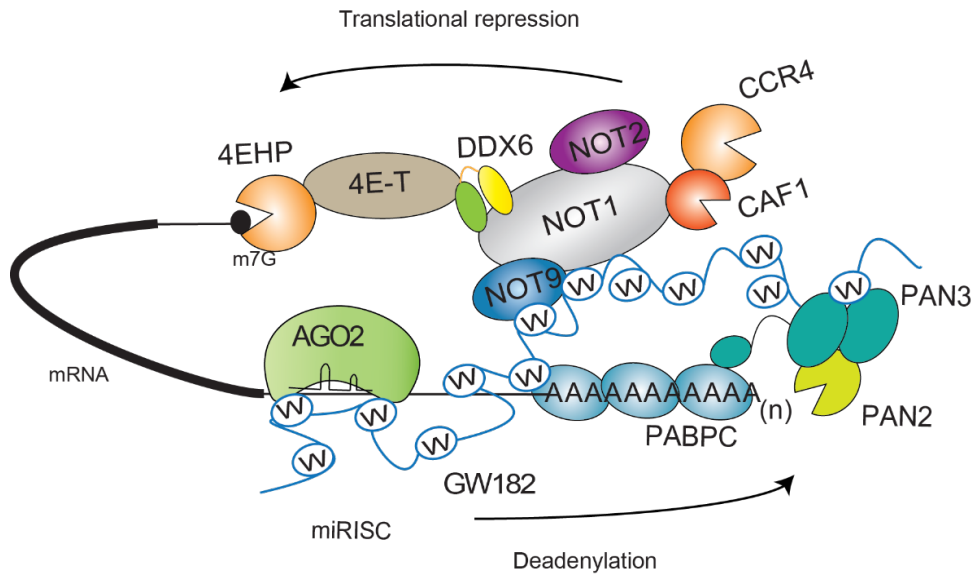


Figure 1.10: Example of one of the possible mechanisms for miRNA-mediated translational repression: a miRNA is loaded into an AGO protein and guides it to its target mRNA. A TNRC6 protein is then recruited and favors the binding of further downstream factors like the PAN2-PAN3 and CCR4-NOT deadenylase complexes as well as the PABPC proteins. For translational repression of the target, the helicase DDX6 bound to the CCR4-NOT complex recruits 4EHP to the miRNA target mRNA through the CCR4-NOT/DDX6/4E-T axis (adapted from Chapat et al., 2017).

Another key player in translation repression is the Grb10-interacting GYF (glycine-tyrosine-phenylalanine) protein 2, or GIGYF2 also known as TNRC15. GIGYF2 has been demonstrated to interact with TNRC6/GW182, AGO, and the CCR4-NOT complex in human cell lines (Morita, Ler et al. 2012, Ruscica, Bawankar et al. 2019, Weber, Chung et al. 2020) and, like TNRC6, is a W-rich protein harboring several Q-repeat regions. Alongside DDX6 and 4EHP, GIGYF2 forms a repressive complex that inhibits translation initiation (Morita, Ler et al. 2012, Ozgur, Basquin et al. 2015, Ruscica, Bawankar et al. 2019, Weber, Chung et al. 2020). However, the precise mechanistic and temporal contributions of these interactions to miRNA-mediated translational repression, and the effects on the canonical pathway of miRNA-mediated mRNA degradation, require further investigation.

1.5.3 GW182 Proteins: Essential players of mRNA Fate and Gene Regulation

The GW182 protein family is exclusive to metazoans. In vertebrates and certain insect species, this family includes three paralogous proteins known as trinucleotide repeat-containing 6 (TNRC6): TNRC6A (also referred to as GW182), TNRC6B, and TNRC6C, while in *D. melanogaster* exists as a single family member, GW182 (Eulalio, Helms et al. 2009). TNRC6 proteins are large scaffold proteins of about 180 kDa in size, which are present in biomolecular condensates known as cytoplasmic P-bodies. They are characterized by two functional domains: an amino-terminal AGO-binding domain (ABD) and a carboxy-terminal silencing domain (SD) (Lazzaretti et al., 2009). These functional domains are primarily predicted to lack a defined structure and are distinguished by the presence of multiple tryptophan (W)-containing motifs, frequently flanked

by glycine residues (GW, WG, or GWG repeats), hence the name GW proteins. In addition, the SD domain also contains an RNA recognition motif (RRM) and a PABP interacting motif 2 (PAM2) (Eulalio, Huntzinger and Izaurralde 2008, Eulalio, Huntzinger et al. 2009, Eulalio, Tritschler and Izaurralde 2009, Huntzinger, Braun et al. 2010) which connect TNRC6 proteins to the poly(A) tail. Structural and functional studies on human TNRC6C-PABPC1 complex also revealed that this interaction is important to promote miRNA-mediated gene silencing (Huntzinger, Braun et al. 2010, Jinek, Fabian et al. 2010). The tryptophan-containing residues facilitate binding to AGO proteins and various subunits of deadenylase complexes, including PAN3, NOT1, and NOT9 (also known as CAF40). This binding occurs through the insertion of their tryptophan residues into hydrophobic pockets located on the surface of these binding partners (Eystathiou, Jakymiw et al. 2003, Meister, Landthaler et al. 2004, Yang, Jakymiw et al. 2004, Rehwinkel, Behm-Ansmant et al. 2005, Behm-Ansmant, Rehwinkel et al. 2006, Eulalio, Huntzinger and Izaurralde 2008, Eulalio, Helms et al. 2009, Braun, Huntzinger et al. 2011, Chekulaeva, Mathys et al. 2011, Fabian, Cieplak et al. 2011, Huntzinger, Kuzuoglu-Ozturk et al. 2013, Christie, Boland et al. 2013, Sheu-Gruttadauria and MacRae 2018). Thanks to this predominant way of binding, TNRC6 proteins can interact in a redundant manner and contribute to increase the affinity of the interaction with its partners. TNRC6 can bind to multiple AGO proteins, for example, and therefore enhance the silencing effect, suggesting biochemical cooperativity, i.e. binding to one target site enhances the affinity to the second binding site, of AGO protein that are bound to different target sites (Briskin, Wang and Bartel 2020). However, it is also conceivable that TNRC6 proteins link AGO proteins on different target mRNAs and thus recruit such silenced mRNPs to the condensates even though P-bodies are not essential for miRNA-guided gene silencing (Eulalio, Behm-Ansmant et al. 2007).

1.6 Regulation of miRNA-mediated gene silencing

MiRNA-guided gene regulation plays a key role in almost all cellular processes; therefore, its regulation is essential. As additional evidence of their functional relevance, miRNAs and their targets often are also evolutionary conserved (Brennecke, Stark et al. 2005). Consequently, several steps of regulation are necessary to ensure a correct modulation of miRNA activity.

1.6.1 Regulation of miRNA stability

MiRNAs are generally thought to be stable (Ruegger and Grosshans 2012); however, certain developmental transitions and cellular stimuli can lead to reductions in the levels of specific miRNAs. For example, miRNAs exhibit notably diminished stability in neuronal contexts (Rajasethupathy, Fiumara et al. 2009, Sethi and Lukiw 2009, Krol, Loedige and Filipowicz 2010) during specific phases of the cell cycle (Rissland, Hong and Bartel 2011), and in response to viral infections (Buck, Perot et al. 2010, Cazalla D 2010, Rissland, Hong and Bartel 2011, Marcinowski, Tanguy et al. 2012, Lee, Song et al. 2013). This is in line with the concept that miRNAs are rapidly and spatially controlled to modulate gene expression (Sambandan S 2017). Therefore, adapting

the transcriptome in response to particular stimuli necessitates the remodeling of miRNA activity, and regulating miRNA biogenesis and functions can modulate these changes. As the first level of regulation, miRNA biogenesis can be tuned at the transcriptional level, altering the expression of specific miRNAs (Treiber, Treiber and Meister 2019). Second, the efficiency of miRNA targeting can be influenced by the degree of complementarity between the miRNA and the target mRNA, as well as by the accessibility of the target site on the mRNA occupied by RBPs (Fabian and Sonenberg 2012). Third, miRNAs can also be degraded. However, how specific miRNAs are distinguished and degraded is not fully understood. Only recently, it has been realized that target RNAs with a high degree of complementarity to the miRNA induce miRNA degradation, a process commonly referred to as target-directed miRNA degradation (TDMD). The identification of endogenous mammalian transcripts, such as *Cyrano*, *Nrep*, and *Serpine1*, that act as natural triggers for TDMD, regulating the abundance of miR-7, miR-29b, and miR-30b/c, respectively, established TDMD as a general mechanism to regulate the abundance of specific miRNA *in vivo* (Bitetti, Mallory et al. 2018, Ghini, Rubolino et al. 2018, Kleaveland, Shi et al. 2018). Multiple findings have also unveiled the involvement of a cullin–RING ubiquitin ligase (CRL) complex, featuring the substrate adapter protein ZSWIM8, as a crucial mediator of TDMD. The extensive complementarity between miRNAs and TDMD-inducing targets induces a structural change in AGO, which is recognized by the ZSWIM8 complex (Shi CY, Kingston ER et al. 2020, Han, LaVigne et al. 2020). Consequently, AGO undergoes ubiquitylation, leading to its degradation by the proteasome (**Figure 1.11**). This degradation releases the miRNA, making it susceptible to the action of cytoplasmic nucleases. When engaged with TDMD-inducing targets, the extended base pairing of the miRNA's 3' end also prompts its release from the AGO PAZ domain (Sheu-Gruttadauria, Pawlica et al. 2019, Willkomm, Jakob et al. 2022). This facilitates subsequent processes like tailing (addition of nontemplated nucleotides) by polymerases or trimming (removal of nucleotides) by exoribonucleases. Notably, TDMD using ZSWIM8 has deep evolutionary roots, as the loss of ZSWIM8 orthologs in worms and flies stabilizes specific miRNAs. In fact, TDMD has been shown to be crucial in *D. melanogaster*, as disrupting this pathway leads to embryonic lethality, as demonstrated by (Kingston, Blodgett and Bartel 2022).

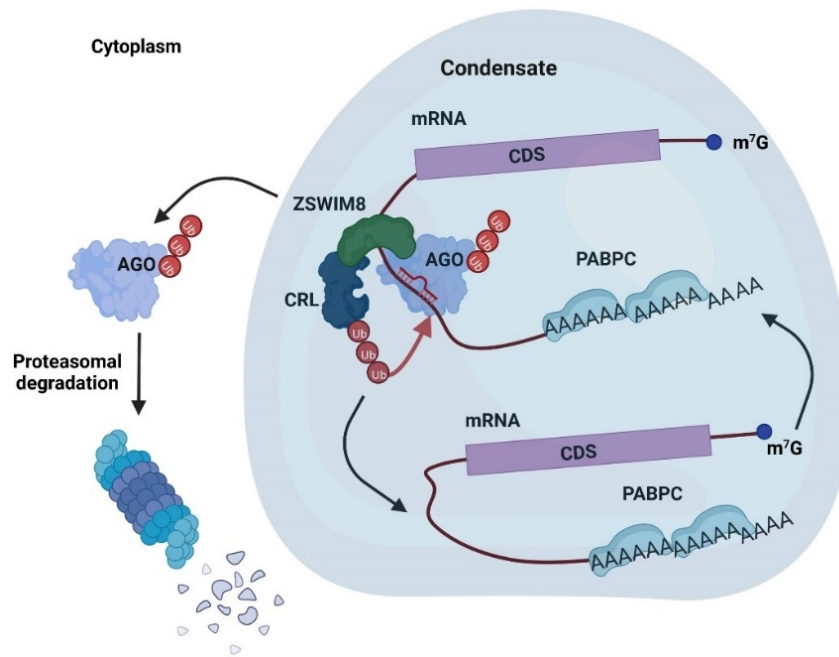


Figure 1.11: Summary of the miRNA-mediated mRNA decay in the cell and the different layers of regulations carried out on and by AGO and TNRC6 proteins. Extensive pairing in the 3' end of the miRNA induces AGO structural changes recognized by the ZSWIM8 ubiquitin ligase which in turn polyubiquitinates AGO, causing protein degradation and possible recycling of the mRNA. Coding sequence [CDS] Created with BioRender.com.

1.6.2 Regulation of Argonaute proteins by phosphorylation

AGO proteins play a central role in miRNA-mediated mRNA decay, therefore, regulating AGO is also one of the possibilities to regulate miRNA-guided silencing (Gebert and MacRae 2019). One common way to regulate proteins is through post-translational modifications (PTMs) and several modifications at distinct sites have been characterized for AGO proteins. It has been shown that AGO proteins undergo PTMs on several residues (Gebert and MacRae 2019). Phosphorylation is the best characterized AGO PTM and it mainly occurs at three main sites: at the L2 linker at Serine in position 387 and Tyrosine 393 (Shen, Xia et al. 2013, Yang, Haase et al. 2014); at the miRNA 5'-end binding region in the MID domain on Tyrosine 529 (Rudel, Wang et al. 2011). Finally, AGO2 is phosphorylated on the exposed surface of the PIWI domain, on a highly conserved residue also referred to as S824-S834 cluster (S824-S834). Specifically, different studies showed that upon target binding, *human* AGO2 undergoes a continual phosphorylation and dephosphorylation cycle by CSNK1A1 kinase, and at least another additional one like casein kinase 2 (CK2) in *C. elegans*, and the ANKRD52-PPP6C dephosphatase complex on S824-S834 cluster (Golden, Chen et al. 2017, Quevillon Huberdeau, Zeitler et al. 2017, Shah, Neumeier et al. 2023). As the phosphorylation of AGO2 in the cluster hinders target RNA binding, the dephosphorylation by ANKRD52-PPP6C enables AGO2 to engage new targets. Constant rounds of phosphorylation and dephosphorylation in these residues are necessary for efficient miRNA activity because AGO2 is then able to engage new targets and maintain a sufficient pool of active protein for efficient

miRNA-mediated repression. However, it remains to be established in which context in time AGO needs to be phosphorylated and which is the fate of hyper-phosphorylated AGO proteins (**Figure 1.12**).

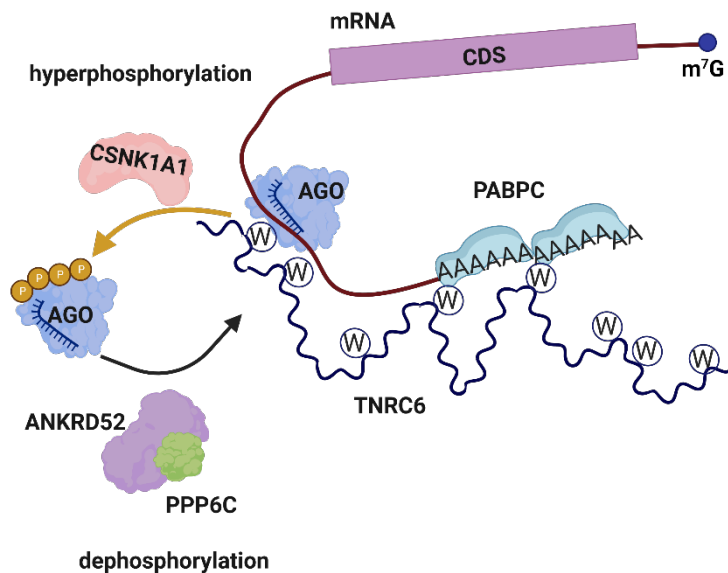
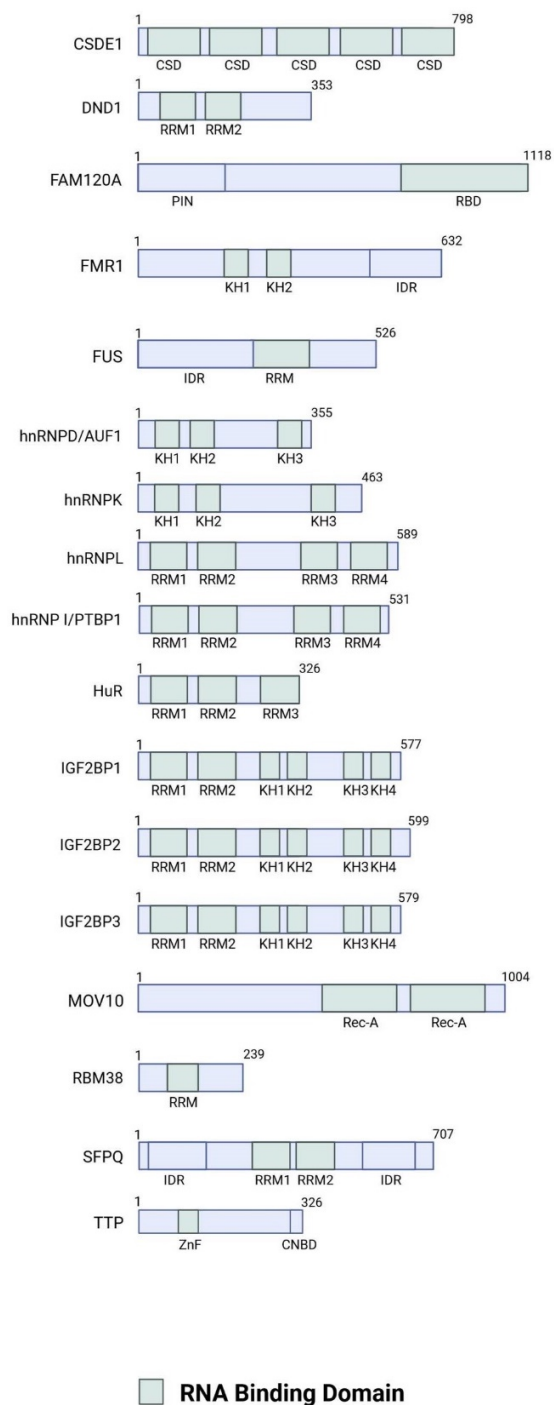


Figure 1.12: AGO phosphorylation cycle to regulate target binding. Once incorporated into repressive mRNPs, AGO undergoes a process of hyper-phosphorylation, particularly at the 824:34 cluster. This hyper-phosphorylation results in the addition of negatively charged phosphates, which, in turn, induce the mRNA to detach from the AGO proteins, effectively releasing the mRNA target. It is worth noting that phosphorylation could also serve as a signal triggering mRNA decay (modified from Larivera et al. 2023). Created with BioRender.com.

1.6.3 Influence of RBPs on miRNA-guided gene silencing

AGO proteins are an intriguing class of RBPs that require miRNAs to recognize and regulate target mRNAs. However, other RBPs operate without the aid of a guide molecule and directly bind substrate RNAs. While AGO proteins are guided to their specific targets by around 1000 miRNA genes (Kim, Kim et al. 2021), in the human genome there are over 2000 RBPs with RNA binding domains (RBDs) that recognize specific sequence motifs or structural elements (Katsantoni, van Nimwegen and Zavolan 2023, Sasse, Laverty et al. 2018, Corley, Burns and Yeo 2020). These RBDs typically interact with short sequence motifs, but many RBPs possess multiple combined RBDs that create more complex and specific binding motifs (Hennig and Sattler 2015). Similar to miRNAs, the repertoire of target mRNAs for RBPs is often not well understood. Interestingly, different RNA binding proteins have been found to affect miRNA-mediated gene silencing. (**Figure 1.13**).



RNA Binding Motifs

U-rich regions

5'-GGGAGGG-3'

5'-ACUK-3' and 5'-WGGA-3' (K=G/U, and W=A/U)

G-C rich elements

G quadruplexes

5'-GGGGG-3'

A-U rich elements (AREs)

C rich regions, 5'-CCCUUCCC-3'

G-A rich elements, 5'-CACACACA-3'

Polypyrimidine tract, 5'-UUCCUUUC-3'

A-U rich elements (AREs)

CAUH (H = A, U, or C)

G-C rich elements

U rich elements

5'-CUGU-3', 5'-CUGUA-3'

A-U rich elements (AREs)

Figure 1.13: Domain organization of RBPs that have been reported to have either miRNA-targeting enhancing or miRNA suppressing functions. Cold Shock Domain [CSD]; RNA Recognition Motif [RRM]; PiLT N terminus domain [PIN]; RNA-binding domain [RBD]; K Homology (KH) domain [KH]; Intrinsically Disordered Region [IDR]; Recombination-A domain [Rec-A]; Repression domains [RD]; CNOT Binding Domain [CNBD]; Created with BioRender.com.

Direct functional and mechanistic connections between miRNA- and RBP-guided gene silencing have been reported (**Figure 1.14**). Several scenarios can be envisioned of how RBPs could influence miRNA-guided silencing as part of an mRNP. First, some RBPs can interact with miRISC and either stimulate or inhibit the binding of the complex to the target mRNA. HuR, an AU-rich element (AREs) binding RBP, can oligomerize on specific target mRNAs leading to dissociation of miRISC and loss of P-body localization, or actively recruit miRISC to distinct mRNAs suggesting that the complex interplay within an mRNP might be key for the regulatory outcome (Bhattacharyya, Habermacher et al. 2006, Tominaga, Srikantan et al. 2011, Kundu, Fabian et al. 2012, Young, Moore et al. 2012, Zhuang, Rao et al. 2013).

Second, and perhaps more prevalent than previously recognized, RBPs can modulate local RNA secondary structures. These effects can be either positive or negative depending on whether a miRNA binding site is liberated from or included in a structure initiated by RBP binding. For instance, PUM1 binding to the 3' UTR of the mRNA of the p27 tumor suppressor increases accessibility of miRISC carrying miR-221 and miR-222 (Kedde, van Kouwenhove et al. 2010), while the RBP Dnd1 blocks target site access (Kedde, Strasser et al. 2007). The protein Roquin is also able to recruit the CCR4-NOT complex through the recognition of stem-loop structures in the 3'UTR of a target mRNA. One could speculate that its binding could facilitate or even enhance miRNA-mediated mRNA decay by exposing binding sites present in secondary structures in the 3' UTR (Sgromo, Raisch et al. 2017, Raisch and Valkov 2022). Another example of an mRNA structure-modulating RBP is FMRP, which in complex with the RNA helicase MOV10 can affect the accessibility of miRNA target sites in neurons (Gregersen, Schueler et al. 2014).

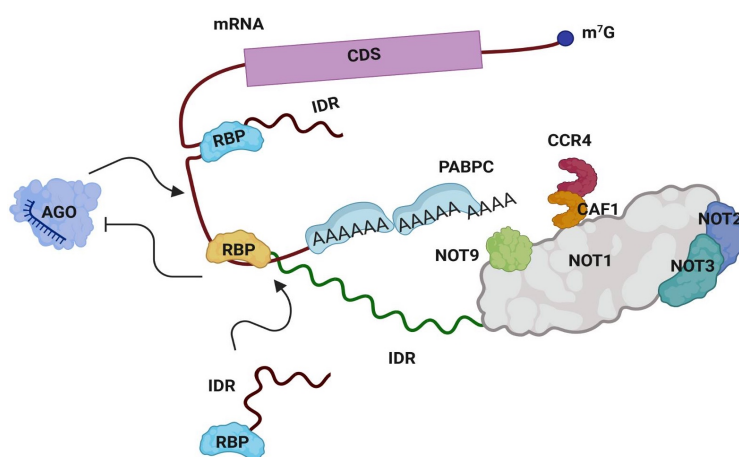


Figure 1.14: Synergistic or competitive effects of RNA binding proteins (RBPs) on miRNA-mediated mRNA decay. Such RBPs could either recruit or inhibit the binding of miRISC to a specific target mRNA. Some of them can directly contact the CCR4-NOT complex for deadenylation. RBPs typically contain IDRs which engage in RNA or protein interactions. Created with BioRender.com.

1.7 m⁶A RNA modification

Besides RBPs and miRNAs, also specific mRNA modifications can regulate gene expression through the recruitment of deadenylases such as the CCR4-NOT complex. Methylation of the amino group at the N6 position of the adenine nucleobase (m⁶A) has emerged as one of the most abundant internal post-transcriptional RNA modifications in eukaryotes (Meyer, Saletore et al. 2012).

1.7.1 m⁶A Writers, Erasers, and Readers

In mammalian cells, m⁶A is co-transcriptionally added in the nucleus by the “writer” methyltransferase complex METTL3/METTL14/WTAP (Liu, Yue et al. 2014, Ke, Pandya-Jones et al. 2017), and can be removed by demethylases such as ALKBH5 and in a less extent by FTO (Jia, Fu et al. 2011, Zheng, Dahl et al. 2013), also known as “erasers” (**Figure 1.15**). This modification is typically found clustered around the stop codon and within the 3'UTR. Transcriptome analyses of human cells and mouse tissues have revealed that m⁶A modification is often enriched within a GG(m⁶A)C sequence motif of RNAs (Fu, Dominissini et al. 2014). Based on recent findings, it is becoming more evident that m⁶A modification is widespread throughout the whole mRNA and its placement is dependent on the EJC.

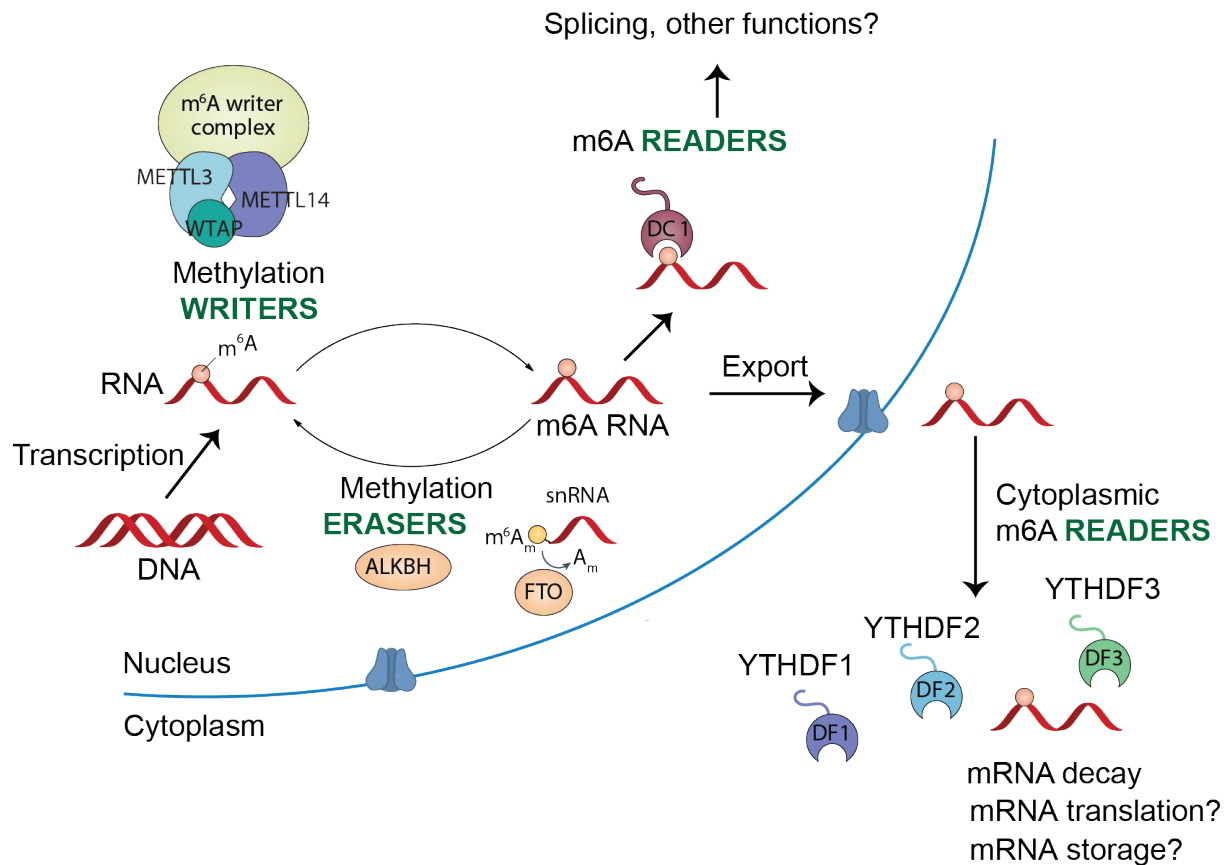


Figure 1.15: Schematic representation of the life cycle of m⁶A RNA methylation in the cell (Adapted from Yue et al., 2015). In mammalian cells, m⁶A is co-transcriptionally added in the nucleus by the “writer” methyltransferase complex METTL3/METTL14/WTAP, and can be removed by demethylases such as ALKBH5 and in a lesser extent by FTO, also known as “erasers”. Accumulating evidence suggests that m⁶A regulates the stability, processing, or translation of the modified RNA through the recruitment of specific proteins known as “readers”. Distinct m⁶A readers exist and can be divided into nuclear and cytoplasmic ones. Among the different nuclear m⁶A readers, we find YTHDC1 and HNRNPA2B1. Among the cytoplasmic m⁶A readers, we find IGF2BP1-2-3, the 3'-5' RNA helicase YTHDC2 and the YTHDF proteins.

The EJC, which is deposited close to a splice junction after splicing, occupies the CDS. Therefore, the DRACH motifs within the CDS are not accessible. Motifs that are not protected by the EJC are accessible and found often close to the 3' end (Uzonyi, Slobodin and Schwartz 2022, Yang, Triboulet et al. 2022). Exceptions are also observed and it is possible that specific sites of distinct mRNAs can escape protection and can still be modified (Slobodin, Han et al. 2017). Accumulating evidence suggest that m⁶A regulates the stability, processing, or translation of the modified RNA through the recruitment of specific proteins known as “readers” (Wang et al., 2014). Distinct m⁶A readers exists and can be divided into nuclear and cytoplasmic ones. Among the different nuclear m⁶A readers, we find the YTH domain-containing protein 1 (YTHDC1) and the Heterogeneous nuclear ribonucleoproteins A2B1 (HNRNPA2B1). Among the cytoplasmic m⁶A readers, we find

Insulin-like growth factor 2 mRNA-binding protein 1-2-3 (IGF2BP1-2-3), the 3'-5' RNA helicase YTHDC2 and the YTHDF proteins (He and He 2021).

The YTHDF proteins are cytoplasmic readers of m⁶A that selectively recognize and bind the consensus GG(m⁶A)C sequence through a highly conserved YTH domain. The *Human* YTHDF family includes three members, YTHDF1-2-3, each of which comprises a YTH domain and a less conserved amino-terminal region. The crystal structure of the YTH domain revealed an aromatic cage that specifically recognizes and stabilizes the m⁶A through stacking interactions (Zhang, Theler et al. 2010). The amino-terminal region instead, contains Q/P/G rich, low complexity domains and is responsible for the activity of these proteins. How they mechanistically act will be described in the next chapter.

1.7.2 The role of YTHDF proteins in mRNA metabolism

Early findings on m⁶A modification showed that mRNAs with lower m⁶A methylation levels had longer half-life (Sommer S. et al., 1978). Only recently, it has been found that among the various m⁶A reader proteins, YTHDF2 selectively bind to m⁶A-modified RNA and regulate RNA decay in a methylation-dependent manner by recruiting the CCR4-NOT complex through its CNOT1 subunit (Wang, Lu et al. 2014) (**Figure 1.16**). However, YTHDF2 mutant lacking this small region can still cause some degree of deadenylation of the target mRNA (Du, Zhao et al. 2016). Since YTHDF2 was the only reader found to recruit the CCR4-NOT complex despite the ~60% amino acid identity and 70% amino acid similarity with the other paralogues (Zaccara and Jaffrey 2020) it is very likely that the complexity resides in the unstructured N-terminal region and the regulation comes at different time points of specific cells. In fact, YTHDF2 is the only paralog that is able to recruit not only the endonuclease complexes RNase P/MRP, contributing to the degradation of mRNAs and circRNAs (Park, Ha et al. 2019), but also to recruit UPF1 and induce decapping of m⁶A-marked mRNAs (Boo, Ha et al. 2022). It's puzzling how a cytoplasmic protein like YTHDF2 can interact with complexes, which are not only present in the cytoplasm but also in the nucleus and nucleolus. Therefore, future detailed studies are needed to unravel not only the mechanisms of these interactions but how and the context in which they happen.

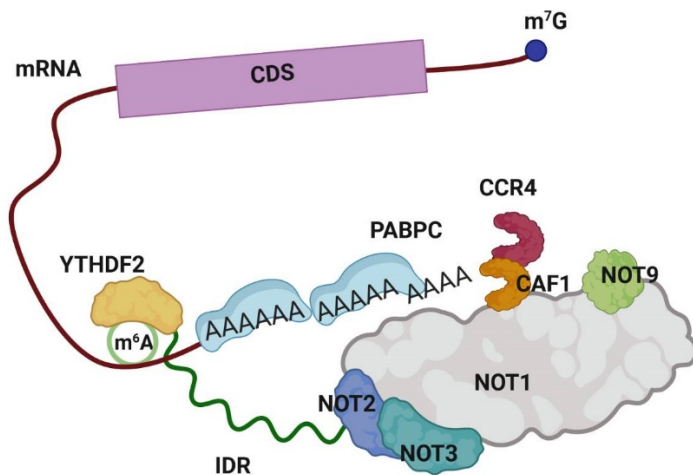


Figure 1.16: YTHDF2 induced mRNA deadenylation. YTHDF2 selectively binds to m⁶A-modified RNA and regulates mRNA decay in a methylation-dependent manner by recruiting the CCR4-NOT complex through its CNOT1 subunit. Created with BioRender.com.

The other m⁶A reader protein, YTHDF1, the m⁶A writer protein, METTL3, and even the m⁶A alone have been shown to promote the translation of modified mRNAs, further highlighting the diverse roles of m⁶A and its associated proteins in RNA metabolism (Meyer, Patil et al. 2015, Wang, Zhao et al. 2015, Lin, Choe et al. 2016). Finally, YTHDF3 has been shown to play a dual role in the regulation of m⁶A-marked mRNAs by facilitating both mRNA translation and mRNA decay. Specifically, YTHDF3 is involved in mRNA decay through the recruitment of the PAN2/PAN3 deadenylase complex (Shi, Wang et al. 2017, Liu, Gao et al. 2020). Interestingly, another member of the YTH domain family proteins, YTHDC2, is also able to interact with XRN1 and induce the degradation of m⁶A-marked mRNAs. Although YTHDC2 binds U-rich motifs instead, it functions independently of m⁶A but through its unusual DExD helicase domain during spermatogenesis in mice and fish (Wojtas, Pandey et al. 2017). Despite their structure and sequence similarity, the question of whether these various proteins act redundantly has been a topic of ongoing research. Only recently, it has been shown that YTHDF proteins directly influence mRNA stability rather than translation efficiency through a significant dosage-dependent redundancy (Lasman, Krupalnik et al. 2020). Indeed, knockout studies in mice have demonstrated a significant level of redundancy among the three Ythdf paralogs, particularly in early developmental stages. Notably, Ythdf2 appears to be the sole Ythdf reader necessary for proper gametogenesis, and its expression pattern differs from that of Ythdf1 and Ythdf3. Furthermore, when all three readers were knocked out, compensatory effects were observed between Ythdf1 and Ythdf3, but not for Ythdf2, which required at least one additional Ythdf reader to prevent embryonic lethality (Lasman, Krupalnik et al. 2020). Nevertheless, it is still possible that some degree of difference in activity between YTHDF proteins exists in a specific cellular context. Lastly, regarding their general involvement in

mRNA stability pathways, few is known. Previous BioID experiments and mass spectrometry analysis of AGO protein interactions in miRNA-containing and miRNA-depleted cells, also showed that only YTHDF2 can interact with AGO2. These findings suggest that there might be further crosstalks between the two pathways besides the common recruitment of the CCR4-NOT complex (Frohn, Eberl et al. 2012, Youn, Dunham et al. 2018). Therefore, further studies are to clarify the involvement of YTHDF proteins not only on miRNA-mediated mRNA decay but also, more in general, on mRNA metabolism.

2 Aim of the thesis

Post-transcriptional gene regulation is a complex and dynamic process that involves the orchestrated action of factors as diverse as miRNA, RBPs, or m⁶A mRNA modifications. Nevertheless, much remains to be learned about the timing and the specific roles and interactions of these various factors.

This thesis aims to study the m⁶A binding proteins YTHDF1-2-3, the miRNA pathway proteins TNRC6A-B-C function, and the pathways in which they are involved. Moreover, this thesis aims at identifying the common and diverging principles of these different pathways and the mechanisms governing their protein degradation within specific contexts.

Finally, this work aims to uncover the possible enhancing or suppressing actions of these factors on the same target mRNA and the consequent effects on the steady-state poly(A) tail of target mRNAs.

3 Results

3.1 Analysis of the AGO2 interactome

The miRNA-mediated mRNA decay pathway has been deeply studied in the past but our understanding of the additional interactions that AGO proteins can establish particularly with RBPs that associate with the same mRNA remains limited.

3.1.1 Investigation of AGO-containing mRNPs through sucrose gradient fractionation

Previous BioID experiments and mass spectrometry analysis of AGO protein interactions in miRNA-containing and miRNA-depleted cells, showed that AGO2 can interact with YTHDF2 suggesting possible crosstalk between the two pathways besides the common recruitment of the CCR4-NOT complex (Frohn, Eberl et al. 2012, Du, Zhao et al. 2016, Youn, Dunham et al. 2018).

Here we employed a sedimentation gradient approach, followed by western blot analysis to characterize AGO2 macromolecular complexes. Lysates were treated with or without RNase A (Figure 3.1 and Figure 3.2) to assess whether the sedimentation pattern was dependent on mRNA.

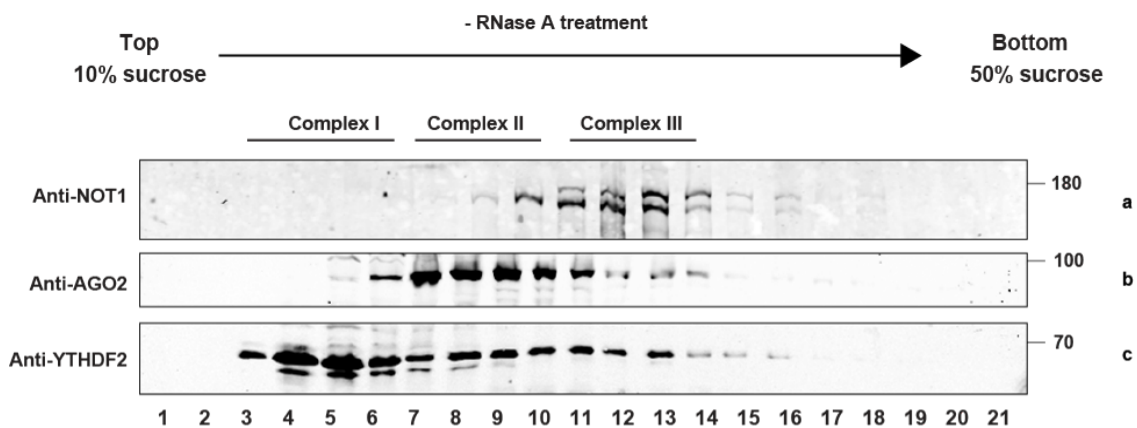


Figure 3.1: Human AGO2 associates with YTHDF2 in sedimentation gradient approach in the absence of RNase A treatment. Lysates from wild-type HEK 293T cells were separated by sucrose density centrifugation under conditions that allow the separation of mRNPs. Endogenous proteins were analyzed using specific antibodies. Lysates shown in the lower panel were treated with 100 µg/ml RNase A before centrifugation. The size markers (kDa) are shown on the right of the panel. The arrow indicates AGO2. (a) is referred to NOT1 sedimentation gradient, (b) is referred to AGO2 sedimentation gradient, (c) is referred to YTHDF2 sedimentation gradient.

In gradients where the lysates were not treated with RNase A, probing with antibodies against YTHDF2 showed that it migrated in both low and high sucrose density fractions. A portion of

YTHDF2 was found in both low fractions, which we refer to as complex I (**Figure 3.1 lanes 3-6, c**), and high molecular weight fractions overlapping with AGO2, which we refer to as complex II (**Figure 3.1 lanes 7-10, compare b and c**). In addition, a small portion of both YTHDF2 and AGO2 was found in higher molecular weight fractions that also contained NOT1, which we refer to as complex III (**Figure 3.1 fractions 11-14, compare a-b, and a-c**).

To assess whether the presence of RNA could affect NOT1, AGO2 or YTHDF2 distribution, we treated the lysates with RNase A and performed again a sedimentation gradient and western blot experiments (**Figure 3.2**).

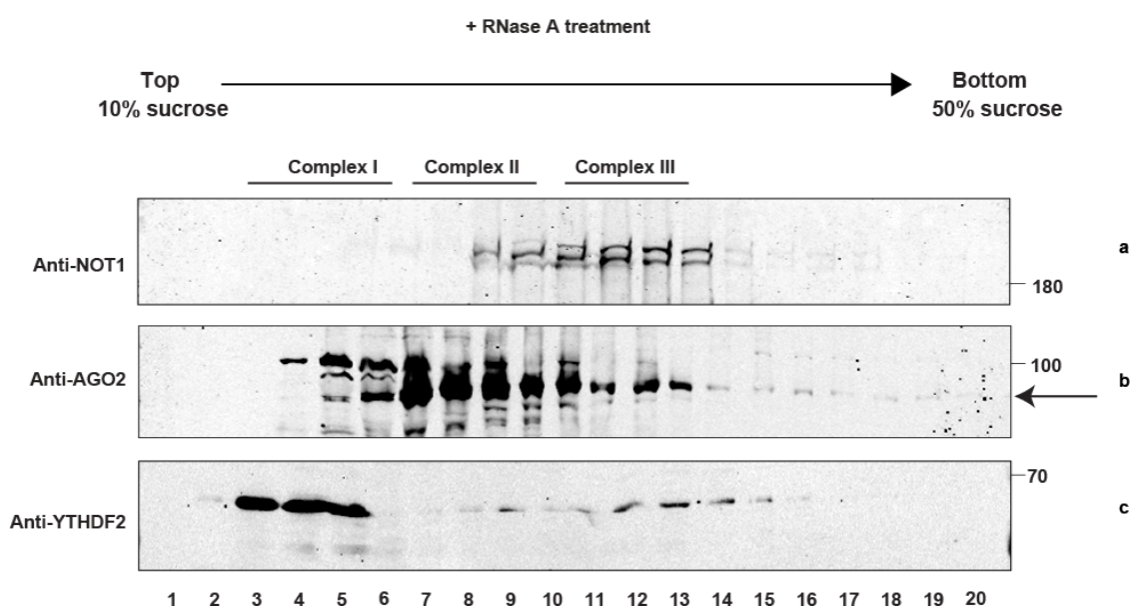


Figure 3.2: Human AGO2 associates with YTHDF2 in sedimentation gradient approach in lysates treated with RNase A. Lysates from wild-type HEK 293T cells were separated by sucrose density centrifugation under conditions that allow the separation of mRNPs. Endogenous proteins were analyzed using specific antibodies. Lysates shown here were not treated with RNase A before centrifugation. The size markers (kDa) are shown on the right of the panel. The arrow indicates AGO2. (a) is referred to NOT1 sedimentation gradient, (b) is referred to AGO2 sedimentation gradient, (c) is referred to YTHDF2 sedimentation gradient.

Probing with antibody against NOT1 showed that its sedimentation pattern did not change in higher molecular weight fractions (**Figure 3.2 fractions 9-14, Complex II- III, a**).

AGO2 sedimentation pattern was also not particularly affected (**Figure 3.2 fractions 7-10 Complex II, and fractions 11-14, Complex III, b**). YTHDF2 on the other hand, showed a clear shift in low sucrose density fractions, complex I (**Figure 3.2 lanes 3-6, c**), with only a small portion of YTHDF2 found in high fractions (**Figure 3.2 lanes 11-14, c**). Together, our data show that NOT1 and AGO2 do not associate with RNA, while YTHDF2 complexes II and III are sensitive to RNase A treatment.

3.1.2 YTHDF2 interacts *in vitro* with the CCR4-NOT complex

It has been previously shown that YTHDF2 interacts with the CCR4-NOT complex (Wang et al., 2014, Du et al., 2016). To discriminate between direct and indirect interactions with different subunits of the CCR4-NOT complex, we performed pulldown assays *in vitro* using purified recombinant YTHDF2 100 amino acids (100aa). This fragment has been proven to be important for YTHDF2 association with the SH domain of NOT1 (Du et al., 2016). Initially, we expressed in *E.coli* the 100aa of YTHDF2, tagged with a Maltose binding protein (MBP). We then performed an MBP *in vitro* pulldown to test the ability of MBP-YTHDF2 100aa to bind the different subunits of the CCR4-NOT complex (**Figure 3.3 A**).

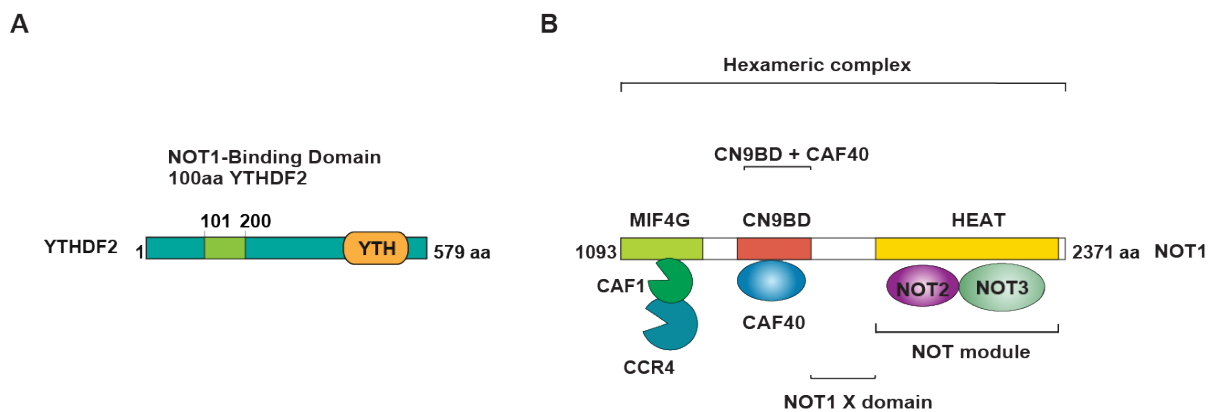


Figure 3.3: Schematic representation of the CCR4-NOT complex and subcomplex modules. (A) Purified 100aa of YTHDF2 associate with the SH domain of NOT1 (Du et al., 2016) tagged with a Maltose binding protein (MBP). YTH (YT521-B homology domain). **(B)** Schematic overview of the hexameric human CCR4-NOT complex used for *in vitro* interaction studies. The hexameric subcomplex is composed of NOT1 (residues E1093-E2371), CAF1, CCR4, CAF40 (residues R19-E285), NOT2 (residues T344-F540), and NOT3 (residues L607-Q753). The CAF1-CCR4 module contains the NOT1 MIF4G-like domain, CAF1 (dark green), and CCR4 (blue). The CN9BD and CAF40 module consists of CAF40 (light blue; residues R19-E285) bound to the CAF40-binding coiled-coil domain (red; residues V1351-L1588). The adjacent NOT1 X domain consists of residues D1607-S1815, while the NOT module consists of NOT1 (in yellow, residues H1833-M2361), NOT2 (residues M350-F540; purple), and NOT3 (residues L607-E748; light green).

The CCR4-NOT complex is the major deadenylase complex in the cell and 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 the two deadenylases CCR4 and CAF1. Other subunits include NOT9 (Caf40 in budding yeast), NOT10 and NOT11 (Morgan Tucker, Marco A. Valencia-Sanchez et al. 2001, Bartlam and Yamamoto 2010, Boland, Chen et al. 2013, Mauxion, Preve and Seraphin 2013, Temme, Simonelig and Wahle 2014, Jonas and Izaurralde 2015, Collart 2016, Chen, Khazina et al. 2021, Passmore and Collier 2022, Raisch and Valkov 2022). To elucidate which subunits of the hexameric subcomplex were

involved in the interaction with 100aa of YTHDF2, we analyzed the binding to the CAF1-CCR4/NOT1-middle domain of eukaryotic initiation factor 4G (MIF4G) domains, the CAF40 module (CAF40/CN9BD), the subsequent NOT1 X domain, and the NOT module (comprising the NOT1 SHD and the C-terminal regions of NOT2 and NOT3) (**Figure 3.3 B**). Dr. Tobias Raisch, provided the purified components of the CCR4-NOT complex.

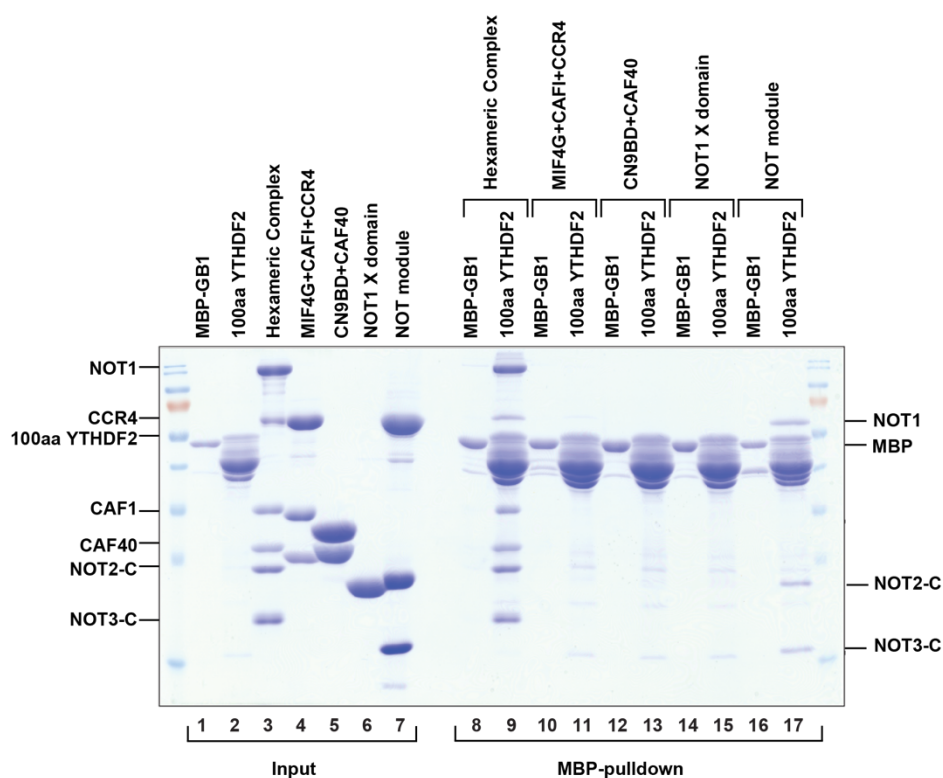


Figure 3.4: YTHDF2 interacts *in vitro* with the CNOT module and the CCR4-NOT hexameric complex. An MBP-pulldown assay showing the interaction of MBP-GB1-tagged 100aa of YTHDF2 with recombinant purified CCR4-NOT modules. MBP-GB1 tag served as a negative control. The respective starting materials (“input”) and pull-down samples were analyzed by SDS-PAGE and Coomassie blue staining.

MBP-tagged 100aa of YTHDF2 pulled down the hexameric subcomplex (**Figure 3.4 lane 9**), and the NOT module (NOT1/2/3) (**Figure 3.4 lane 17**). No interaction was found with the other modules of the CCR4-NOT complex (**Figure 3.4 lanes 11, 13, 15**). We concluded that human YTHDF2 100aa fragment directly binds the NOT module and the hexameric complex and no other subcomplex modules of the CCR4-NOT complex.

3.1.3 SLiM motifs in YTHDF2 are essential for optimal binding to the CCR4-NOT complex

After confirming the *in vitro* interaction between the 100aa region of YTHDF2, referred to as NOT1 Binding Domain (NBD), and the CCR4-NOT complex, our focus shifted to precisely mapping the binding site on YTHDF2. We aimed to understand the molecular principles governing the recruitment of the CCR4-NOT complex by YTHDF2. To achieve this, we utilized the provided

Docker script of the AlphaFold-Multimer model (Jumper, Evans et al. 2021). Subsequently, we retrieved the prediction with the highest confidence rank and we visualized the outcome using PyMOL (**Figure 3.5**).

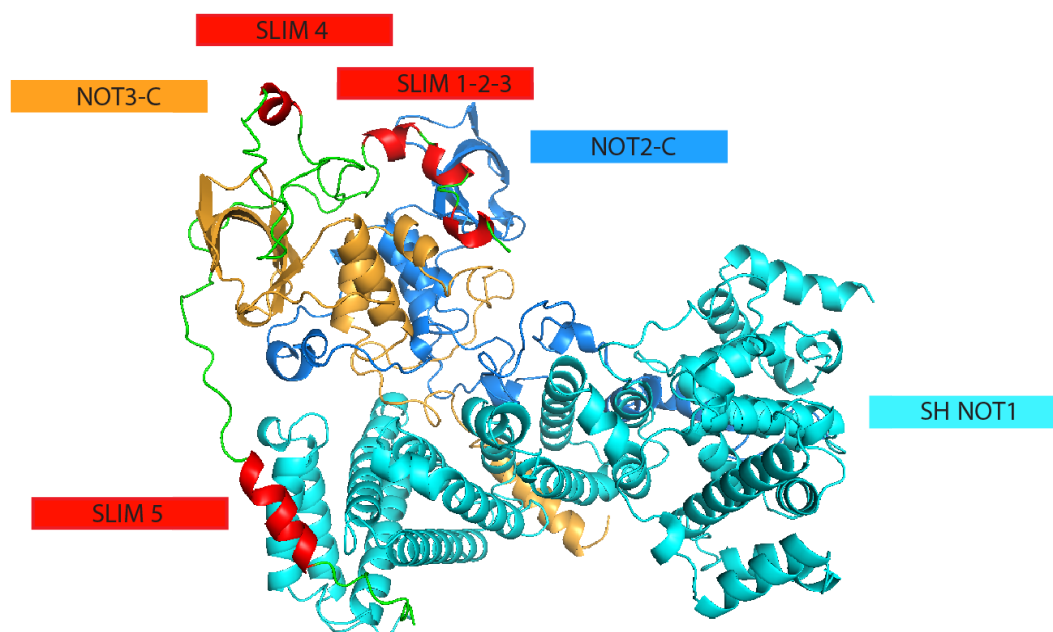


Figure 3.5: Alphafold prediction of the interaction between the 100aa present at the N-terminal of YTHDF2 NBD and the NOT module of the CCR4-NOT complex. The 100 aa YTHDF2 peptide NBD (green unstructured with red alpha-helices) is bound to the NOT module. NOT2-C (dark blue) and NOT3-C (orange) form a heterodimer through their NOT-box domains. The NOT1-C contains the NOT1 superfamily homology (SH) domain.

Based on these structural predictions, we divided the NBD of YTHDF2 into three fragments (**Figure 3.6, fragment 1-2-3**) to assess the importance of the predicted SLiM motifs identified, and tested them in MBP pulldown assays *in vitro*.

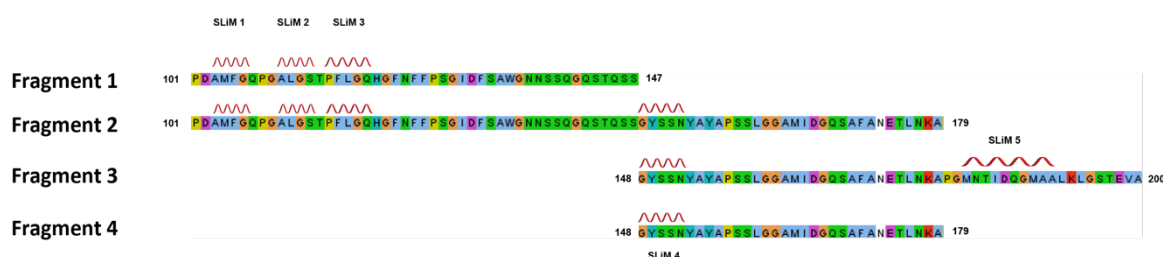


Figure 3.6: Schematic overview of the fragments division of the 100 amino acids of YTHDF2 used for *in vitro* interaction studies. Schematic overview of the hexameric human CCR4-NOT complex used for *in vitro* interaction studies. Fragment 1 includes the first 3 SLiM motifs (SLiM 1-2-3) (residues P101-S147), fragment 2 includes an additional SLiM motif (SLiM 4) (residues P101-A179). Fragment 3 includes SLiM 4 and SLiM 5 (residues G148-A200). Fragment 4 contains only the SLiM 4 (residues G148-A179).

The first fragment contained three SLiM motifs (SLiM 1-2-3), which were supposed to bind at the interface of the NOT2-C-NOT3-C heterodimer and partially delve into the connector sequence of NOT2, which is an extended N-terminal sequence that envelops around the NOT-box domains. Fragment 2 covered the whole fragment 1, and contained another predicted SLiM motif (SLiM 4). According to the structural model, fragment 2, like fragment 1, was suggested to interact only with the NOT2-C-NOT3-C heterodimer. Finally, fragment 3, contained the main predicted alpha helix at the very C-terminus of the NBD, which should be responsible for the binding to the SH domain of NOT1 (SLiM 5), and the SLiM4. Remarkably, in MBP *in vitro* pulldown assay, fragments 2 and 3 displayed reduced binding to the hexameric complex (**Figure 3.7 A lanes 9, 10**), while the binding with fragment 1 was strongly impaired (**Figure 3.7 A lane 8**).

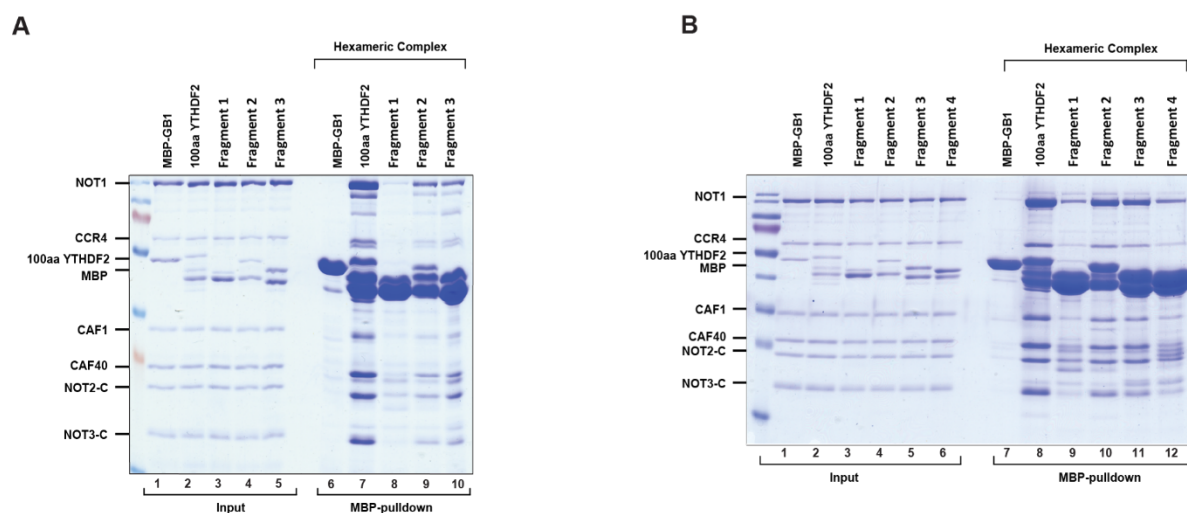


Figure 3.7: Different SLiM motifs in the YTHDF2 CNBD are essential for an optimal binding to the CCR4-NOT complex. (A–B) MBP pull-down assays with MBP tagged 100aa YTHDF2 and the purified hexameric complex. (A) To further define individual interactions, 100aa YTHDF2 was subdivided into the Fragment1, Fragment 2, and Fragment 3 regions. MBP-tagged 100aa YTHDF2 or MBP-tagged 100aa YTHDF2 fragments were used to pull down the hexameric complex. MBP-GB1 (Streptococcal protein G) alone served as a negative control. The respective starting materials (“input”) and pull-down samples were analyzed by SDS-PAGE and Coomassie blue staining. (B) The same experiment was repeated including Fragment 4 which further carried a deletion of a predicted alpha helix and main docking site into the SH domain of NOT1.

This suggests not only a potential functional redundancy between fragments 2 and 3 but also the presence of multiple binding sites for the hexameric complex within the NBD of YTHDF2 as predicted by AlphaFold. Overall, the C-terminal part of the NBD of YTHDF2 seemed to be essential for the binding but it needed the three SLiM motifs in the N-terminal part to enhance binding. We then replicated the experiment with a novel fragment, fragment 4, which uniquely lacked the predicted alpha helix at the C-terminal domain of the NBD of YTHDF2 (**Figure 3.6, fragment 4**). Indeed, deletion of the alpha helix drastically reduced the binding to the hexameric complex

(**Figure 3.7 B lane 12**) hinting that it is an important docking site into NOT1. It is important to mention that the other bands present in fragment 1 and 4 pulldowns (**Figure 3.7 B lanes 9 and 12**), were not present in the input and might therefore indicate that some degradation of the YTHDF2 fragment happens during the pulldown.

3.1.4 YTHDF proteins interact weakly with the CCR4-NOT complex

To test whether the *in vitro* interaction was also reproducible *in vivo* we overexpressed YTHDF1-2-3 proteins tagged with GFP in human HEK 293T cells and immunoprecipitated the GFP tagged proteins using anti-GFP antibody coupled beads followed by western blot analysis. All samples were lysed in the presence of RNase A to assess the direct protein-protein interactions. GFP-Firefly Luciferase (F-LUC) was used as a negative control while GFP-NOT2 was used as a positive control.

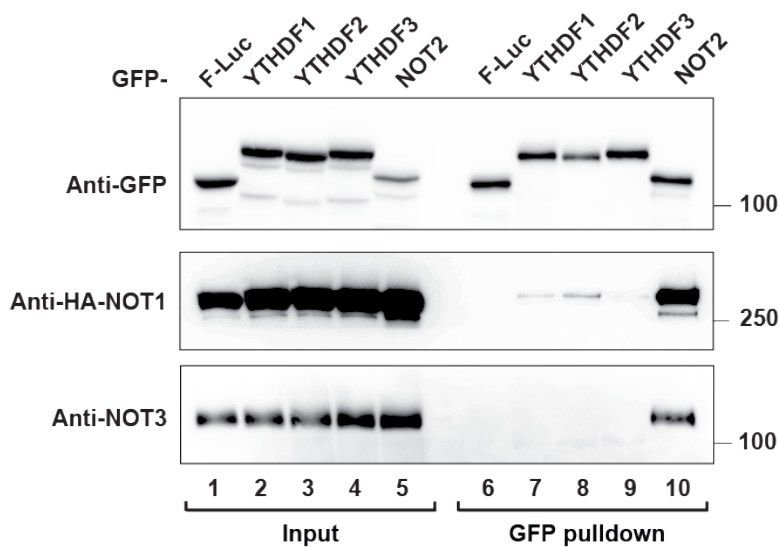


Figure 3.8: YTHDF proteins interact weakly with the CCR4-NOT complex and do not interact with AGO2. YTHDF proteins interact weakly *in vivo* with the NOT1 subunit of the CCR4-NOT complex. The interaction of GFP-tagged YTHDF proteins with overexpressed HA-NOT1 and endogenous NOT3. The proteins were immunoprecipitated using anti-GFP antibodies. GFP-F-Luc served as a negative control. Inputs (10%) and bound fractions (IP; 30%) were analyzed by western blotting using the indicated antibodies. The size markers (kDa) are shown on the right of the panel.

Immunoprecipitation experiments showed a very weak interaction between YTHDF1 and YTHDF2 and NOT1 in HEK 293T cells compared to the positive control NOT2 (**Figure 3.8 lanes 7 and 8**), and to the *in vitro* pulldown. No interaction was found between YTHDF proteins and NOT3 (**Figure 3.8 lanes 7, 8, 9**) contrary to what is shown in the *in vitro* pulldown for YTHDF2 (**Figure 3.4 lane 17**). We concluded that the interaction between YTHDF2 and the CCR4-NOT complex might be transient and might therefore be more difficult to capture *in vivo*.

3.1.5 YTHDF proteins do not interact with AGO2

In addition to its interaction with the CCR4-NOT complex, both BioID experiments and mass spectrometry analysis revealed that YTHDF2 can also interact with AGO2, suggesting a specific function in the process of miRNA-mediated mRNA decay and possible crosstalk between the two pathways (Frohn et al., 2012; Youn et al., 2018). We therefore wanted to verify these data since no interaction between YTHDF2 and AGO2 was reported *in vivo* before.

We overexpressed the FLAG-HA tagged version of all the YTHDF proteins in HEK 293T cells and performed an α -FLAG IP. In this case, we changed the immunoprecipitation approach since GFP is a rather big tag (~27 kDa) and therefore we wanted to rule out a possible cross-reaction with other binding partners. All samples were lysed in the presence of RNase A to study direct protein-protein interactions (**Figure 3.9**).

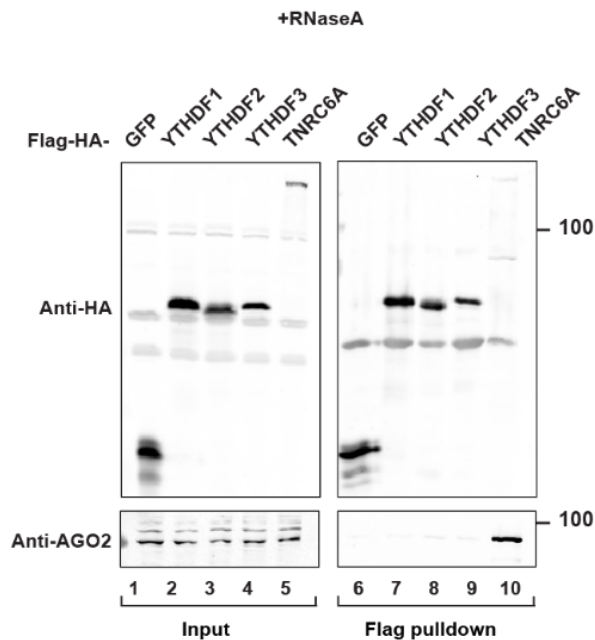


Figure 3.9: YTHDF proteins do not interact with AGO2. Flag pulldown assay in HEK293T cells expressing Flag-HA-tagged YTHDF1, YTHDF2, YTHDF3, and TNRC6A. A Flag-HA-tagged GFP served as a negative control. The presence of endogenous AGO2 in the bound fractions was analyzed by western blotting using a specific antibody. The inputs (1.5%) and bound fractions (45% for the Flag-HA tagged proteins) were analyzed by western blotting. The size markers (kDa) are shown on the right of the panel.

The Western blot analysis clearly showed that YTHDF proteins do not interact with AGO2 if compared to the positive control TNRC6A (**Figure 3.9 compare lanes 7-9 with lane 10**). Therefore, there is no observed binding between YTHDF proteins and AGO2 *in vivo* in HEK 293T cells.

3.1.6 YTHDF proteins interact with PABPC1 in an RNA-dependent way

Having established that YTHDF2 does not interact with AGO2 and TNRC6 proteins (data not shown), but can recruit the CCR4-NOT complex *in vitro* and *in vivo*, we next wondered whether

YTHDF proteins, like TNRC6 proteins, are also able to bind the PABPC1. Since the YTHDF proteins have a large unstructured N-terminal domain, we hypothesized that they might anchor on PABPC1 to stabilize their interaction with the CCR4-NOT complex.

We overexpressed the FLAG-HA tagged version of all the YTHDF proteins and performed α -Flag IP experiments in HEK 293T cells. All samples were lysed in the absence of RNase A (**Figure 3.10**). Flag-HA-GFP was used as a negative control and Flag-HA-TNRC6B as positive control.

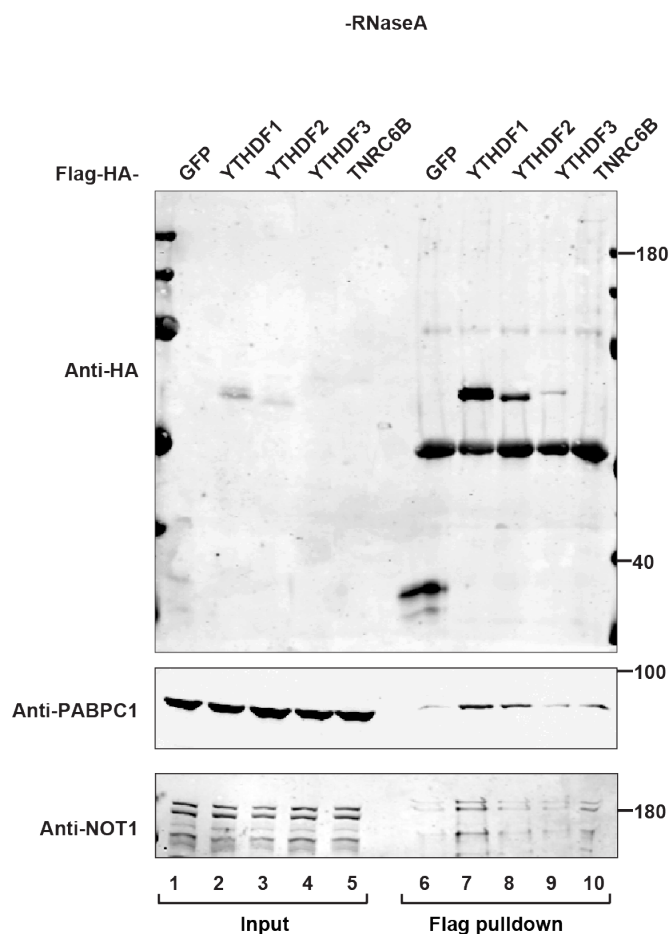


Figure 3.10: The interaction of Flag-HA-tagged YTHDF proteins with endogenous PABPC1. YTHDF proteins interact in a RNA dependent way with the PABPC1. Flag pulldown assay in HEK293T cells expressing Flag-HA-tagged YTHDF1, YTHDF2, YTHDF3, and TNRC6B. A Flag-HA-tagged GFP served as a negative control. The presence of endogenous PABPC1 and NOT1 in the bound fractions was analyzed by western blotting using a specific antibody. The inputs (1.5%) and bound fractions (45% for the Flag-HA tagged proteins) were analyzed by western blotting. The size markers (kDa) are shown on the right of the panel.

As shown in (**Figure 3.10 lanes 7-9**), YTHDF proteins bound PABPC1 in an RNA-dependent way and in a comparable way to TNRC6B. The interaction with NOT1 served as a further positive control. Whether this association is also direct and essential to elicit mRNA deadenylation remains to be established and it is subject to later experiments.

3.2 Functional analysis of YTHDF proteins

To study the activity of YTHDF proteins, we took advantage of the Lambda N-Box B system. This system derives from the bacteriophage lambda, where the interaction of boxB RNA with the RNA-binding domain of the N protein (N peptide) (Lazinski, Grzadzielska and Das 1989) allows the formation of a transcriptional anti-termination complex which enables the RNA polymerase to read through the two termination sites so that particular genes of the bacteriophage are transcribed (Barik, Gosh et al. 1987). It has been thoroughly used in RNA biology to physically bring the protein of interest to an artificial RNA and study the effects of this recruitment. To test their activity, a tethering reporter assay was employed in human HEK 293T cells. Unless stated differently, all the proteins under study were fused to the lambda-N peptide and an HA (hemagglutinin) tag, and they were tethered to a β -GLOBIN reporter (BGG-5BoxB) that contained 5 BoxB binding sites in the 3' untranslated region (UTR). A truncated version of the GAPDH gene fused to Beta globin (BGG-GAP) was co-transfected and used as an internal control since it differed in size from the BGG-5BoxB (**Figure 3.11**).

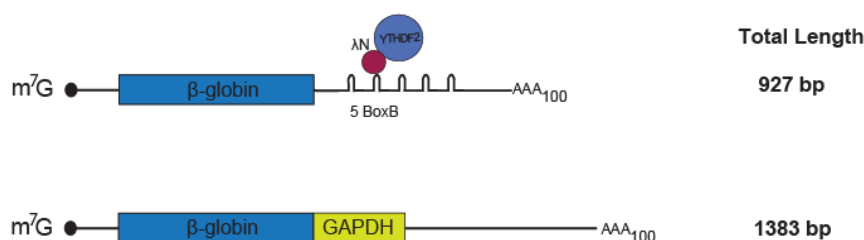


Figure 3.11: β -GLOBIN reporters used in this study. (BGG) β -GLOBIN. The BGG-GAP reporter contains a truncated version of the GAPDH (GAP) gene to distinguish it from the BGG-5BoxB reporter by size (Lykke-Andersen et al. 2000). The BGG-5BoxB reporter contains 5 BoxB binding sites in the 3' UTR. (bp) base pairs.

3.2.1 YTHDF proteins do not degrade as efficiently as AGO2

Since the *in vivo* IP experiments proved that the interaction between YTHDF2 and the CCR4-NOT was not so strong, we conducted a tethering assay followed by northern blot experiments to investigate how this interaction might affect mRNA levels. Furthermore, this investigation involved a direct comparison between the degradation pathway mediated by YTHDF2 and the well-studied pathway involving AGO2. We overexpressed a Lambda N-HA version of AGO2 as a positive control, a Lambda N-HA only vector as a negative control, and a Lambda N-HA version of YTHDF1-2-3 (**Figure 3.12 A**), all tethered to the β -GLOBIN 5 BoxB reporter (BGG-5BoxB). A truncated version of the GAPDH gene fused to Beta globin (BGG-GAP) was co-transfected and used as an internal control since it differed in size from the BGG-5BoxB.

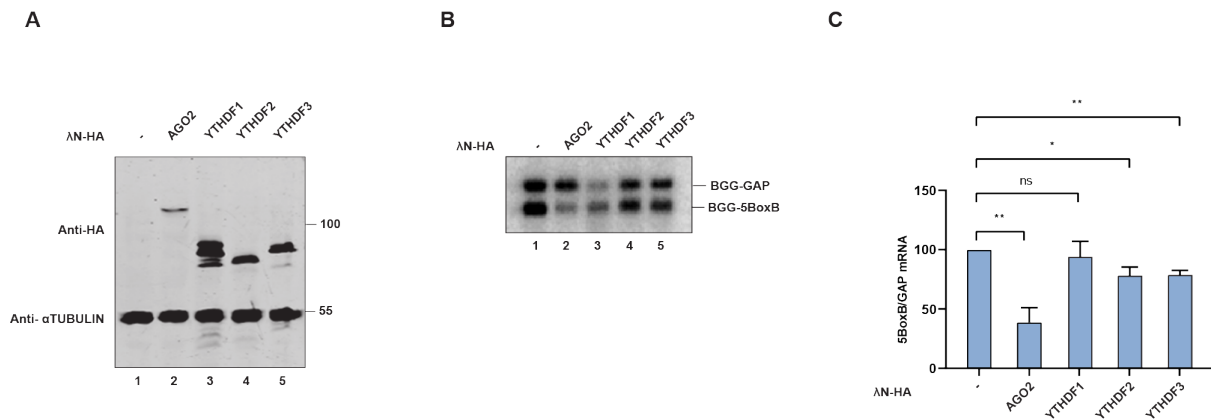


Figure 3.12: YTHDF proteins do not degrade as efficiently as AGO2. **(A)** Western blot showing the expression levels of the tethered proteins HA, YTHDF1, 2, 3 AGO2 tagged with the Lambda-N peptide. A-TUBULIN was used as a loading control. **(B)** Northern blot analysis of a tethering assay using the BGG-5BoxB reporter and Lambda-N tagged HA, YTHDF1, 2, 3, and AGO2 proteins in HEK 293T cells. A plasmid expressing BGG-GAP served as a transfection control and lacked the 5BoxB binding sites. **(C)** BGG-5BoxB mRNA levels determined by Northern blotting were normalized to those of BGG-GAP and set to 1 in cells expressing Lambda-N-HA. Mean values $\pm$ standard deviation (SD) are shown ($n = 3$). (*) $P < 0.05$, two-way ANOVA-test. BGG-5BoxB mRNA levels were normalized to that of the BGG-GAPDH mRNA. For each experiment the normalized values of BGG-5BoxB mRNA levels were set to 100 in cells transfected with the empty vector.

The results obtained from the Northern Blot analysis of the tethering assay experiments demonstrated a noticeable disparity in mRNA degradation efficiency between AGO2 and the YTHDF proteins (**compare lane 2 with lanes 3, 4, 5 in Figure 3.12 B**). Specifically, the degradation process mediated by AGO2 was highly efficient, whereas YTHDF1, YTHDF2, and YTHDF3 displayed weaker effects on mRNA stability (**Figure 3.12 C**). These findings emphasize the differential efficiency of AGO2 and the YTHDF proteins in regulating mRNA expression and degradation pathways.

3.2.2 TNRC6 proteins depletion does not enhance YTHDF2-mediated mRNA degradation

As previously mentioned, TNRC6 proteins play a crucial role in repressing target mRNAs through the miRNA pathway. Since both TNRC6 and YTHDF2 recruit the CCR4-NOT complex, we wondered whether depletion of TNRC6 proteins would increase the efficiency of the YTHDF2-mediated degradation pathway, as the AGO2-mediated mRNA decay would be impaired and therefore the CCR4-NOT complex be more available for binding. We conducted a tethering experiment overexpressing Lambda N-HA, Lambda N-HA AGO2, and Lambda N-HA-YTHDF2 in HEK 293T cells where TNRC6 protein expression was depleted via knockdown experiments (**Figure 3.13 A**).

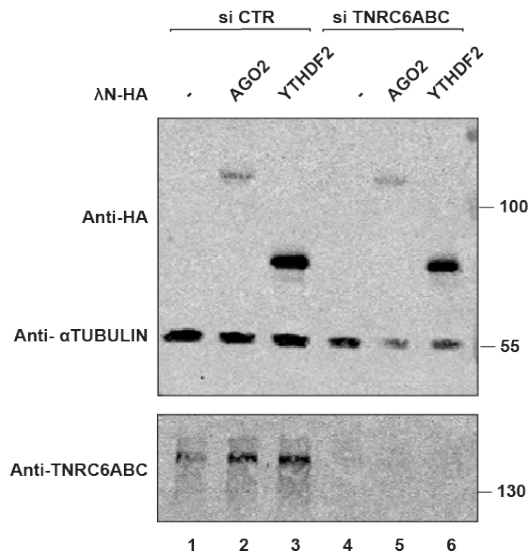
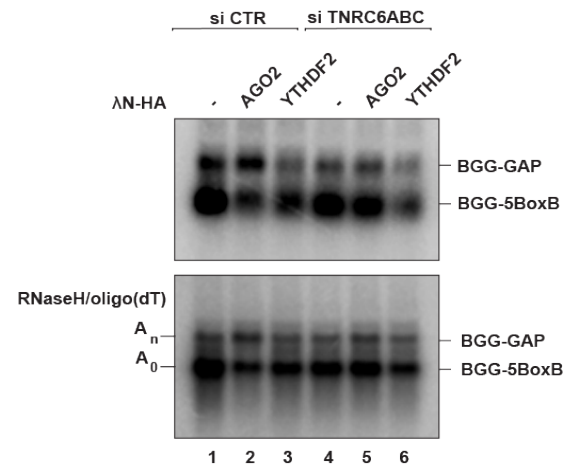
A**B**

Figure 3.13: TNRC6 proteins do not affect YTHDF2-mediated mRNA degradation. **(A)** Western blot showing the expression levels of the tethered proteins HA, AGO2, and YTHDF2 tagged with the Lambda-N peptide. A-TUBULIN was used as loading control. Specific in-house antibody 11C12 recognized all three paralogs of TNRC6 and was used to detect the efficiency of the knockdown. **(B)** Northern blot analysis of a tethering assay using the BGG-5BoxB reporter and Lambda-N tagged HA, AGO2 and YTHDF2 proteins in HEK 293T cells. A plasmid expressing BGG-GAP served as a transfection control and lacked the 5BoxB binding sites. RNA samples isolated from cells expressing Lambda-N tagged HA, AGO2, and YTHDF2 proteins BGG-5BoxB, and BGG-GAP were treated with oligo(dT)15 in the presence of RNase H and analyzed by Northern blot. Note that upon RNase H treatment the poly(A) tails of the BGG reporters are removed in the presence of oligo(dT).

As observed in the Northern Blot analysis (**Figure 3.13 B**), upon TNRC6 proteins knockdown, YTHDF2 degradation activity was not enhanced. On the contrary, AGO2 degradation was impaired as summarized in the quantification chart in **Figure 3.14**.

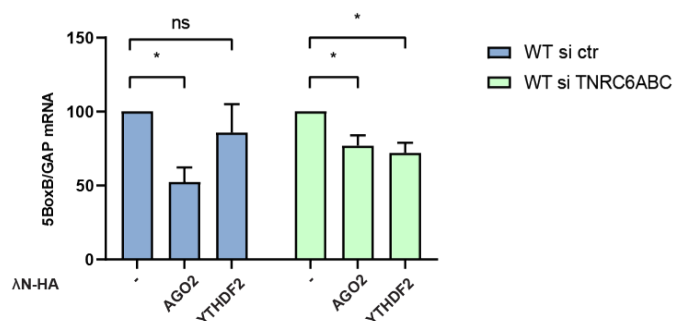


Figure 3.14: TNRC6 proteins do not affect YTHDF2-mediated mRNA degradation. BGG-5BoxB mRNA levels determined by Northern blotting were normalized to those of BGG-GAP and set to 1 in cells expressing Lambda-N-HA. Mean values $\pm$ standard deviation (SD) are shown ($n = 3$). (*) $P < 0.05$, two-way ANOVA-test. BGG-5BoxB mRNA levels were normalized to that of the BGG-GAPDH mRNA. For each experiment the normalized values of BGG-5BoxB mRNA levels were set to 100 in cells transfected with the empty vector.

Since AGO2-mediated mRNA decay involves deadenylation processes, we then proceeded by treating the RNA samples with ribonuclease H (RNase H) and oligo(dT). In this way, we wanted to verify if there was a shift in electrophoretic mobility upon removal of the poly(A)-tail by oligo(dT)-targeted RNase H cleavage and if this shift was affected upon TNRC6 proteins depletion.

Upon northern blot analysis, RNase H and oligo(dT) treatment did not reveal any difference between the deadenylated BGG-5BoxB reporter accumulated as a band with increased mobility (A0), compared to a polyadenylated (An) mRNA, and the negative control, upon tethering of AGO2 and YTHDF2 (**Figure 3.13 B lower panel compare lanes 5 and 2 and lanes 6 and 3**). These data suggest that the absence of TNRC6 proteins likely prevents any recruitment of the CCR4-NOT and the PAN2/PAN3 deadenylase complexes by AGO2 and highlights that YTHDF2-mediated mRNA regulation is TNRC6 independent.

3.2.3 YTHDF2 requires the CCR4-NOT complex to mediate mRNA deadenylation

The CCR4-NOT complex is the major deadenylase complex in the cell and is responsible for the shortening of the poly(A) tails of most mRNAs. It functions as a direct or indirect binding platform for additional factors and enzymes that contribute to mRNA degradation, including AGO2 and YTHDF2. To compare the requirement of the CCR4-NOT complex in AGO2-mediated and YTHDF2-mediated mRNA deadenylation, we inhibited the deadenylase activity of the complex by depleting the catalytic components of the CCR4-NOT complex, NOT6, and NOT7 via siPOOLS. We then overexpressed the Lambda-N-HA, Lambda-N-HA-AGO2, or Lambda-N-HA-YTHDF2 encoding plasmids in HEK 293T cells (**Figure 3.15 A**).

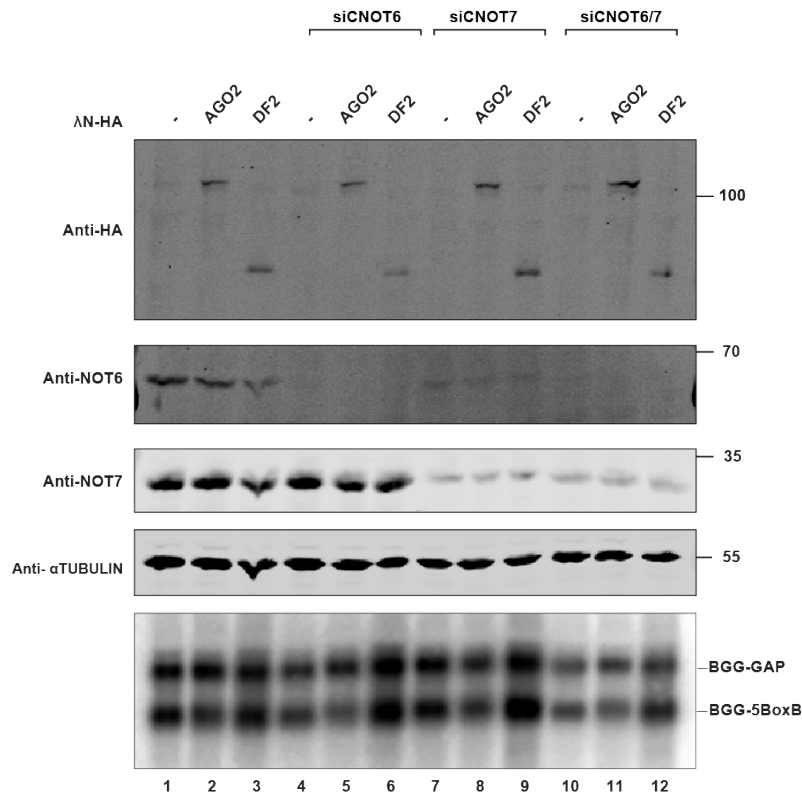


Figure 3.15: YTHDF2 requires the CCR4-NOT complex to mediate mRNA deadenylation. (Upper panel) Western blot showing the expression levels of the tethered proteins HA, AGO2, and YTHDF2 tagged with the Lambda-N peptide in HEK 293T WT cells. Specific NOT6 and NOT7 antibodies were used to detect the efficiency of the knockdown. A-TUBULIN was used as loading control. **(Lower panel)** Northern blot analysis of a tethering assay using the BGG-5BoxB reporter and Lambda-N tagged HA, AGO2, and YTHDF2 proteins in NOT6, NOT7, and NOT6/7 depleted cells. A plasmid expressing BGG-GAP served as a transfection control and lacked the 5BoxB binding sites.

Upon Northern blot analysis, we observed that while the depletion of NOT6 partially blocked mRNA deadenylation mediated by AGO2, it completely abolished YTHDF2-mediated mRNA degradation of the BGG-5BoxB reporter (**Figure 3.15 compare lanes 5 and 6 and Figure 3.16**). On the other hand, the depletion of NOT7, which is responsible for NOT6 binding and recruitment, completely impaired both AGO2- and YTHDF2-mediated mRNA degradation (**Figure 3.15 compare lanes 8 and 9 and Figure 3.16**).

Finally, the depletion of both NOT6 and NOT7 resulted in a similar outcome to the depletion of NOT7 alone (**Figure 3.15 compare lanes 11 and 12 with lanes 8 and 9; Figure 3.16**).

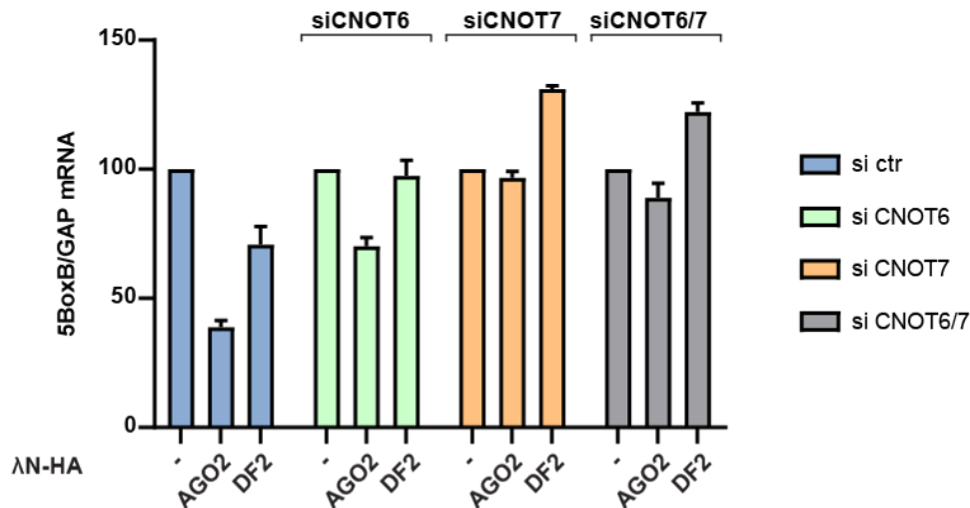


Figure 3.16: YTHDF2 requires the CCR4-NOT complex to mediate mRNA deadenylation. BGG-5BoxB mRNA levels determined by Northern blotting were normalized to those of BGG-GAP and set to 1 in cells expressing Lambda-N-HA. Mean values $\pm$ standard deviation (SD) are shown ($n = 2$). BGG-5BoxB mRNA levels were normalized to that of the BGG-GAPDH mRNA. For each experiment the normalized values of BGG-5BoxB mRNA levels were set to 100 in cells transfected with the empty vector.

The tethering of YTHDF2 to the BGG-5BoxB reporter exhibited a minor variation in the knockdown of NOT7 and the double knockdown of NOT6/7, possibly due to variability in loading. Finally, these data suggest that, while AGO2-mediated mRNA decay is at least partially dependent on NOT6, YTHDF2-mediated mRNA decay is highly dependent on the NOT6-NOT7 interaction. Moreover, these data confirmed that NOT7, with its role in NOT6 binding and recruitment, plays a crucial role in the regulation of mRNA degradation mediated by both AGO2 and YTHDF2.

3.2.4 YTHDF proteins absence does not impair AGO2 activity

So far, our results have pointed out some major differences between AGO2-mediated and YTHDF2-mediated mRNA decay. As both proteins can potentially bind to the same mRNA or compete for the same factors, like the CCR4-NOT complex, we explored the possibility that YTHDF2 could influence the ability of AGO2 to mediate mRNA degradation by competing with TNRC6 proteins for binding to the deadenylase complex. Therefore, we knockdown first YTHDF1-3 and then YTHDF1-2-3, to assess if YTHDF2 has a greater impact compared to its paralogues, and tethered Lambda-N-HA and Lambda-N-HA-AGO2 to the BGG-5BoxB reporter (**Figure 3.17 A**).

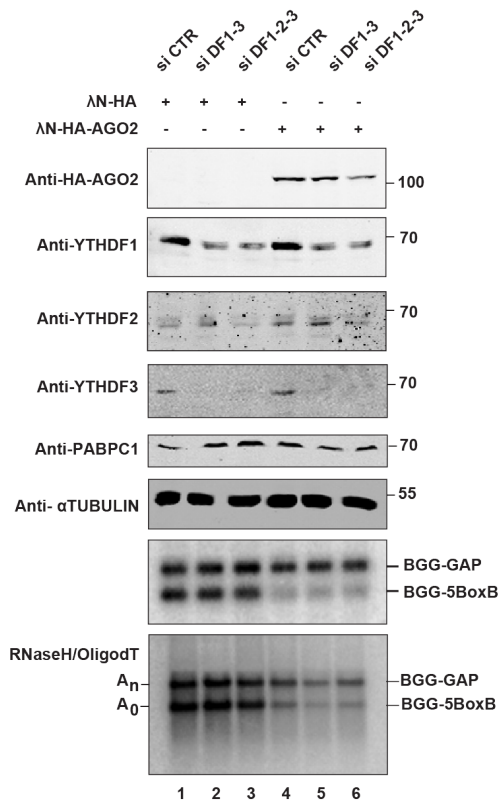
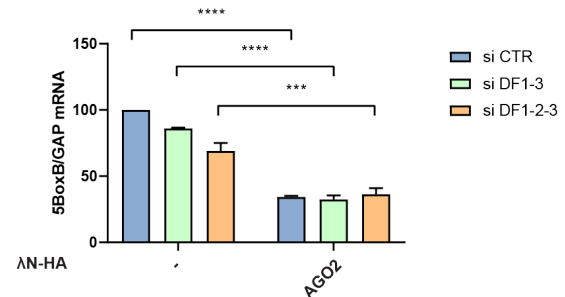
A**B**

Figure 3.17: YTHDF proteins absence does not impair AGO2 activity. (A) In the upper part, western blot showing the expression levels of the tethered proteins HA, AGO2, and YTHDF2 tagged with the Lambda-N peptide in HEK 293T WT cells. Specific antibody recognized YTHDF1, 2, and 3 were used to detect the efficiency of the knockdown. α-TUBULIN was used as a loading control. In the lower part, northern blot analysis of a tethering assay using the BGG-5BoxB reporter and Lambda-N tagged HA, AGO2 and YTHDF2 proteins in HEK 293T WT cells. A plasmid expressing BGG-GAP served as a transfection control and lacked the 5BoxB binding sites. RNA samples isolated from the experiment were treated with oligo(dT)15 in the presence of RNase H and analyzed by Northern blot. Note that upon RNase H treatment the poly(A) tails of the BGG reporters are removed in the presence of oligo(dT). **(B)** BGG-5BoxB mRNA levels determined by Northern blotting were normalized to those of BGG-GAP and set to 1 in cells expressing Lambda-N-HA. Mean values ± standard deviation (SD) are shown (n = 2). (*) P < 0.05, two-way ANOVA-test. BGG-5BoxB mRNA levels were normalized to that of the BGG-GAPDH mRNA. For each experiment the normalized values of BGG-5BoxB mRNA levels were set to 100 in cells transfected with the empty vector.

Notably, the cytoplasmic poly(A) binding protein (PABPC1) was not affected by the knockdown of YTHDF proteins, highlighting that the BGG-5BoxB reporter mRNA and all the other poly(A) transcripts in the cell were stably associated with it. As shown in **(Figure 3.17 A and B)**, AGO2 activity was unaffected upon YTHDF proteins knockdown, suggesting that if two distinct pathways exist, they do not compete with each other. The internal control BGG-GAP showed a slight variation in the triple knockdown of YTHDF1-2-3, possibly due to variability in loading,

We then treated the RNA samples with RNase H and oligo(dT) to verify if there was a shift in electrophoretic mobility of the BGG-5BoxB reporter mRNA upon removal of the poly(A) tail. In this way, we wanted to verify whether YTHDF proteins were essential to elicit some intermediate steps of deadenylation during AGO2-mediated mRNA decay.

Upon northern blot analysis, RNase H and oligo(dT) treatment revealed that upon tethering of AGO2, no deadenylated BGG-5BoxB reporter accumulated as a band with increased mobility (A0) compared to a polyadenylated (An) mRNA and to the control, neither in the double knockdown of YTHDF1-3 nor in the triple knockdown of YTHDF1-2-3. These data confirmed once again that the depletion of YTHDF proteins does not affect AGO2-mediated mRNA deadenylation and decay (**Figure 3.17 A lower panel**).

3.2.5 YTHDF2 tethered BGG-5BoxB reporter does not retain the cap

In the 5′–3′ mRNA degradation pathway, the deadenylation process is followed by decapping and ultimately 5′–3′ exonucleolytic degradation by XRN1. The dependency of YTHDF2 to the CCR4-NOT complex led us to postulate that the subsequent step likely involves the decapping process as in the general 5′–3′ mRNA degradation pathway.

To study the degree of decapping in WT cells, we utilized the Terminator nuclease, a processive 5′–3′ exonuclease that digests RNA with a 5′-monophosphate end, which is generated when mRNAs are decapped.

With this assay, we wanted not only to verify whether the BGG-5BoxB reporter retained its cap upon YTHDF2 tethering, but also discriminate whether YTHDF2, after deadenylation, initiates mRNA degradation via a 3′–5′ exonucleolytic pathway or if YTHDF2 tethering results in cap loss, through a 5′–3′ exonucleolytic pathway. Therefore, after overexpressing a Lambda N-HA version of AGO2 as a positive control, a Lambda N-HA only vector as a negative control, and a Lambda N-HA version of YTHDF2, we treated the RNA samples with the Terminator nuclease. Upon northern blot analysis, the terminator nuclease treated samples did not show any differences in the degradation pattern of the BGG-5BoxB reporter when bound to either Lambda-N-HA, Lambda-N-HA-AGO2, or Lambda-N-HA-YTHDF2 (**Figure 3.18 A**), nor did show any differences in the degradation pattern of the BGG-GAP mRNA if compared with untreated samples.

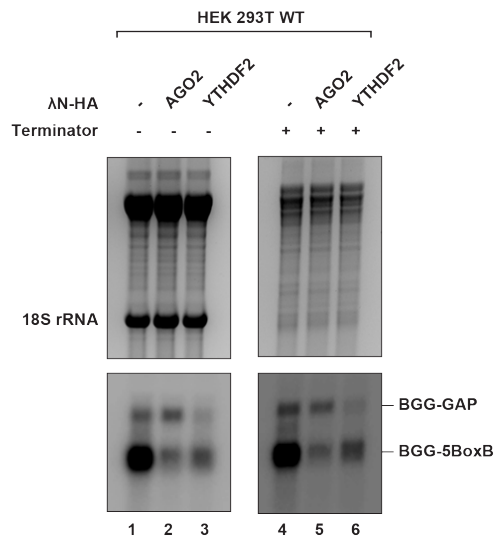
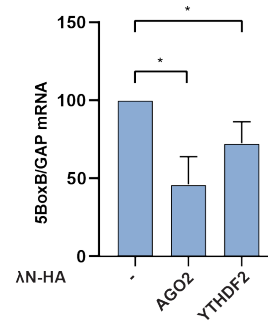
A**B**

Figure 3.18: YTHDF2-mediated mRNA degradation does not occur through the 3′–5′ exonucleolytic pathway.

(A) Northern blot analysis of a tethering assay using the BGG-5BoxB reporter and Lambda-N tagged HA, AGO2 and YTHDF2 proteins in HEK 293T WT cells. A plasmid expressing BGG-GAP served as a transfection control and lacked the 5BoxB binding sites. RNA samples isolated from cells expressing Lambda-N tagged HA, AGO2, and YTHDF2 proteins BGG-5BoxB, and BGG-GAP were incubated with Terminator 5′-phosphate-dependent exonuclease and analyzed by Northern blotting. 18S ribosomal RNA (rRNA) served as uncapped RNA control. **(B)** BGG-5BoxB mRNA levels determined by Northern blotting were normalized to those of BGG-GAP and set to 1 in cells expressing Lambda-N-HA. Mean values $\pm$ standard deviation (SD) are shown ($n = 3$). (*) $P < 0.05$, two-way ANOVA-test. BGG-5BoxB mRNA levels were normalized to that of the BGG-GAPDH mRNA. For each experiment the normalized values of BGG-5BoxB mRNA levels were set to 100 in cells transfected with the empty vector.

Based on our observations, it is evident that upon tethering, a pool of capped transcripts remains available. This is due to the fact that proteins studied do not achieve 100% degradation efficiency as previously investigated. In contrast, the uncapped 18S rRNA, which functions as positive control, was fully degraded upon the addition of the Terminator nuclease. We concluded that both AGO2 and YTHDF2 do not have a substantial impact on the decapping of the reporter mRNA other than the one that already caused the decay of the BGG-5BoxB reporter in the untreated samples **(Figure 3.18 B)**. In addition, these results might indicate that both AGO2- and YTHDF2-mediated mRNA degradation of the BGG-5BoxB reporter does not proceed through 3′–5′ exonucleolytic degradation after deadenylation.

3.2.6 DDX6 absence does not affect YTHDF2-mediated mRNA degradation

To further study the involvement of YTHDF2 in 5′–3′ mediated mRNA decay, we conducted tethering assay experiments to examine the impact of DDX6 depletion on the stability of the BGG-

5BoxB reporter. DDX6 interacts with the CCR4-NOT complex and recruits additional components of the decapping complex physically linking deadenylation to decapping (Jonas and Izaurralde 2016). Mutations studies which disrupted the DDX6–CNOT1 interaction, proved to impair miRISC-mediated gene silencing in human cells (Chen et al., 2014, Rouya et al., 2014), therefore we hypothesized that DDX6 absence could potentially affect YTHDF2 activity.

We depleted DDX6 through siPOOLS in HEK 293T WT cells while overexpressing the Lambda-N-HA, Lambda-N-HA-AGO2, or Lambda-N-HA-YTHDF2 encoding plasmids (**Figure 3.19 A**).

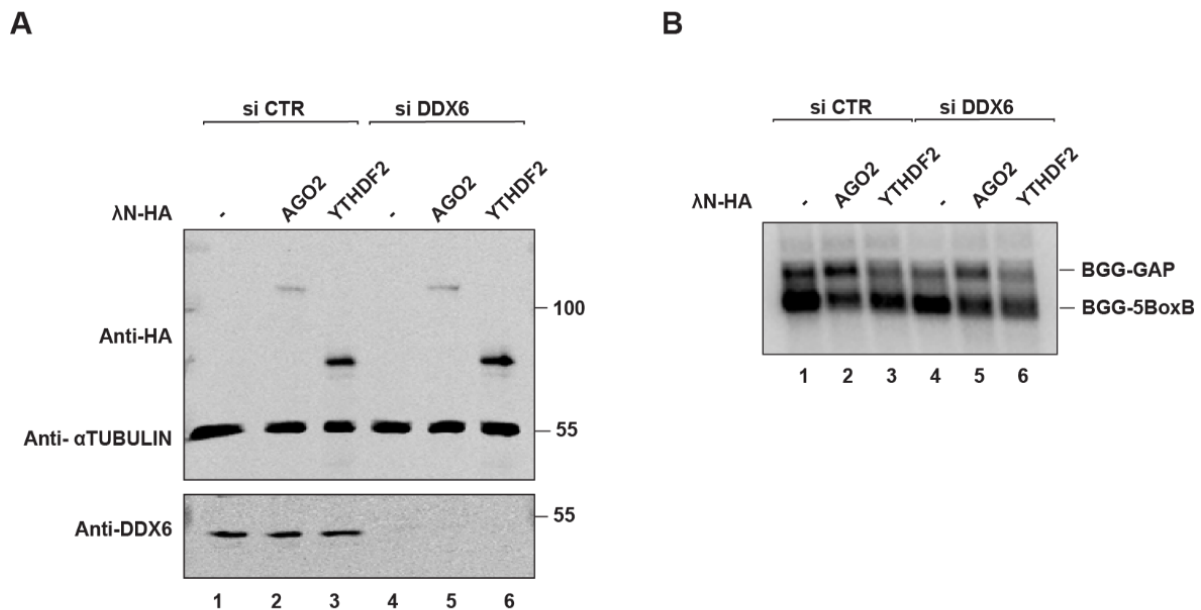


Figure 3.19: DDX6 absence does not affect YTHDF2-mediated mRNA degradation. (A) Western blot showing the expression levels of the tethered proteins HA, AGO2, and YTHDF2 tagged with the Lambda-N peptide in HEK 293T WT cells. Specific antibody recognized DDX6 and was used to detect the efficiency of the knockdown. A-TUBULIN was used as a loading control. (B) Northern blot analysis of a tethering assay using the BGG-5BoxB reporter and Lambda-N tagged HA, AGO2, and YTHDF2 proteins in HEK 293T WT cells. A plasmid expressing BGG-GAP served as a transfection control and lacked the 5BoxB binding sites.

As observed in the Northern Blot analysis, AGO2-mediated RNA degradation of the BGG-5BoxB reporter showed decreased activity when DDX6 was depleted (**Figure 3.19 B compare lane 5 with lane 2**). This effect would probably be even higher if more amount of protein was transfected (**Figure 3.19 A lanes 2 and 5**). On the contrary, the ability of YTHDF2 to degrade the BGG-5BoxB reporter remained unaffected in DDX6 depletion (**Figure 3.19 B compare lane 6 with lane 3**). . The difference in DDX6 impact on the two pathways was clear when looking at the quantification results (**Figure 3.20**) confirming that there is no involvement of DDX6 in the YTHDF2-mediated pathway on a mRNA level as also observed in IP experiments (data not shown).

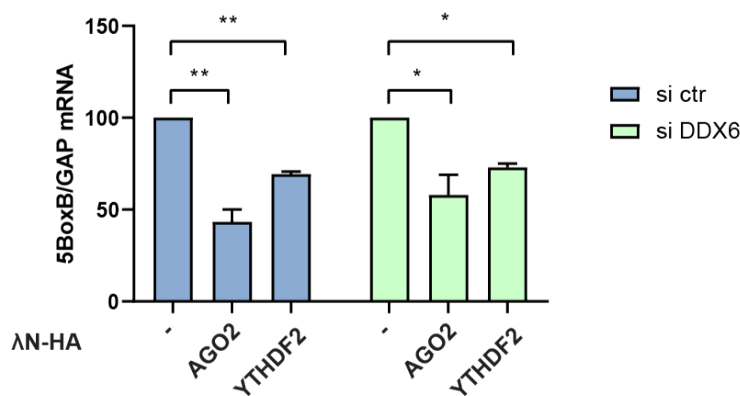


Figure 3.20: DDX6 absence does not affect YTHDF2-mediated mRNA degradation. BGG-5BoxB mRNA levels determined by Northern blotting were normalized to those of BGG-GAP and set to 1 in cells expressing Lambda-N-HA. Mean values $\pm$ standard deviation (SD) are shown (n = 3). (*) P < 0.05, two-way ANOVA-test. BGG-5BoxB mRNA levels were normalized to that of the BGG-GAPDH mRNA. For each experiment the normalized values of BGG-5BoxB mRNA levels were set to 100 in cells transfected with the empty vector.

It remains to be established whether another decapping activator, like PATL1 or LSM14 for example, might connect deadenylation and decapping for YTHDF2-mediated mRNA decay.

3.2.7 Distinct effects of AGO2 and YTHDF2 tethering on the accumulation of BGG-5BoxB reporter intermediates in the absence of XRN1

Since no cap was retained in the previous terminator assay experiments, we investigated the involvement of 5'-3'-mediated mRNA decay in YTHDF2-mediated gene-silencing. To this end, we took advantage of XRN1-null cells provided by Dr. Ramona Weber (Chang et al., 2019). By tethering YTHDF2 in XRN1-null cells, we expected an accumulation of a deadenylated and fast running form of the BGG-5BoxB reporter, since deadenylation CCR4-NOT-mediated can still happen but 5'-3' exonucleolytic mRNA degradation via XRN1 is impaired. We conducted a tethering experiment overexpressing Lambda N-HA, Lambda N-HA AGO2, and Lambda N-HA-YTHDF2 in both HEK 293T and XRN1 K.O. cells (**Figure 3.21 A**).

Tethering of AGO2 in XRN1-null cells led to the accumulation of a fast migrating deadenylated and decapped BGG-5BoxB reporter compared with the tethering in HEK 293T WT cells (**Figure 3.21 B compare lane 5 with lane 2**) and to the negative control, consistent with impairment of the 5'-3' exonucleolytic degradation in the absence of XRN1.

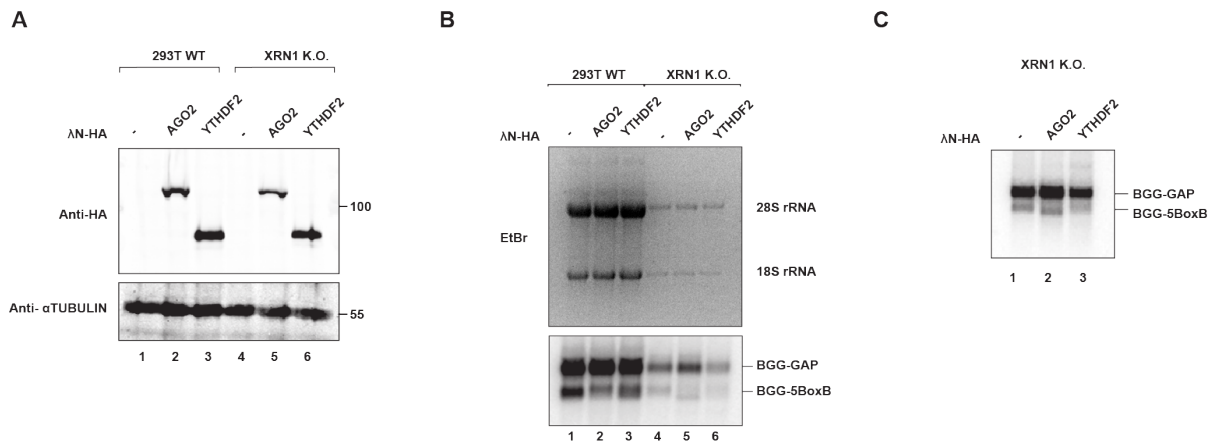


Figure 3.21: Distinct effects of AGO2 and YTHDF2 Tethering on the accumulation of BGG-5BoxB reporter intermediates in the absence of XRN1. (A) Western blot showing the expression levels of the tethered proteins HA, AGO2, and YTHDF2 tagged with the Lambda-N peptide in both HEK 293T WT and XRN1 K.O. cells. A-TUBULIN was used as a loading control. (B) Northern blot analysis of a tethering assay using the BGG-5BoxB reporter and Lambda-N tagged HA, AGO2, and YTHDF2 proteins in HEK 293T WT and XRN1 K.O. cells. A plasmid expressing BGG-GAP served as a transfection control and lacked the 5BoxB binding sites. (C) Northern blot analysis of a tethering assay using the BGG-5BoxB reporter and Lambda-N tagged HA, AGO2, and YTHDF2 proteins in XRN1 K.O. cells. A plasmid expressing BGG-GAP served as a transfection control and lacked the 5BoxB binding sites.

Tethering of YTHDF2 to the BGG-5BoxB reporter in XRN1 K.O. cells instead, did not show any visible difference compared to tethering in HEK 293T WT cells, as shown in the northern blot analysis (**Figure 3.21 B compare lane 6 with lane 3**). Furthermore, upon longer exposure of the northern blot membrane, no visible difference in migration pattern through the gel was observed when comparing YTHDF2 tethering with the tethering of AGO2 in XRN1 K.O. HEK 293T cells (**Figure 3.21 C compare lane 3 with lane 2**). Quantification results, on the other hand, revealed a decrease in YTHDF2 activity by ~10% (**Figure 3.22**).

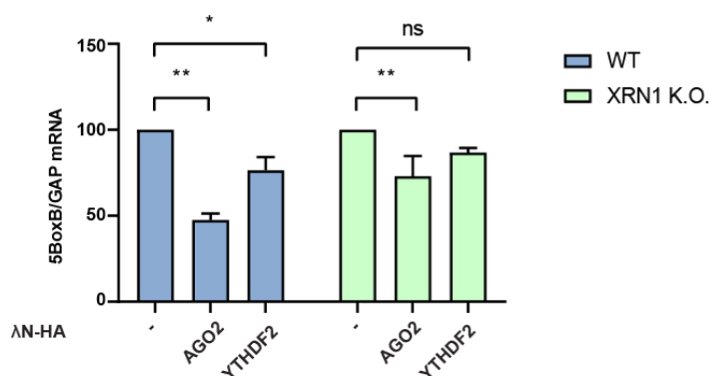


Figure 3.22: Loss of XRN1 leads to an accumulation of reporter intermediates upon AGO2 tethering and mildly upon YTHDF2. BGG-5BoxB mRNA levels determined by Northern blotting were normalized to those of BGG-GAP and set to 1 in cells expressing Lambda-N-HA. Mean values $\pm$ standard deviation (SD) are shown ($n = 3$). (*) $P < 0.05$, two-tailed.

way ANOVA-test. BGG-5BoxB mRNA levels were normalized to that of the BGG-GAPDH mRNA. For each experiment the normalized values of BGG-5BoxB mRNA levels were set to 100 in cells transfected with the empty vector.

Therefore, we treated the RNA samples with RNase H and oligo(dT) to verify if there was a shift in electrophoretic mobility upon removal of the poly(A) tail. Moreover, we wanted to validate if this shift was affected upon tethering of YTHDF2 compared to the tethering of AGO2 in XRN1 depleted cells and, to which extent the 5'-3' mediated mRNA decay was involved YTHDF2-guided gene silencing.

Upon northern blot analysis, RNase H and oligo(dT) treatment revealed that upon tethering of AGO2, the deadenylated BGG-5BoxB reporter mainly accumulated as a band with increased mobility (A₀) compared to a polyadenylated (A_n) mRNA and to the negative control (**Figure 3.23 compare lanes 3 and 4, and lanes 3 and 1**). On the contrary, upon tethering of YTHDF2, we observed that only a small pool of the BGG-5BoxB reporter accumulated as a band with increased mobility (A₀) after RNase H and oligo(dT) treatment, while most of it accumulated in a slow-migrating and polyadenylated (A_n) form (**Figure 3.23 lanes 5 and 6, and lanes 5 and 3**).

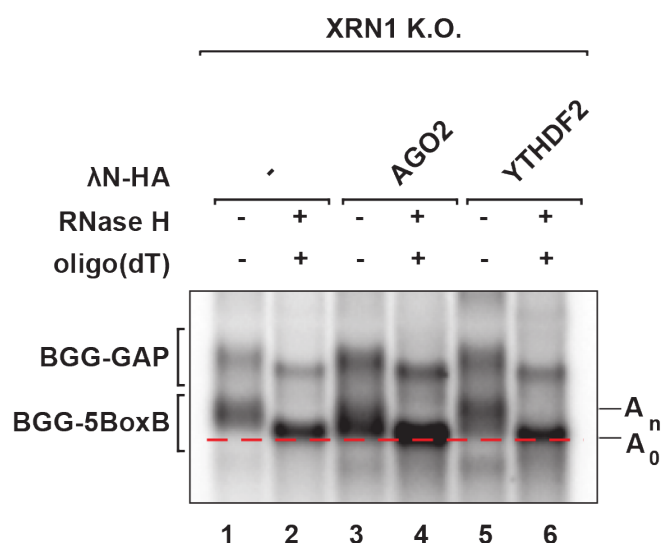


Figure 3.23: Accumulation of fast migrating band in XRN1 K.O. upon YTHDF2 tethering. RNA samples isolated from cells expressing Lambda-N tagged HA, AGO2, and YTHDF2 proteins BGG-5BoxB, and BGG-GAP were treated with oligo(dT)₁₅ in the presence of RNase H and analyzed by Northern blot. Note that upon RNase H treatment the poly(A) tails of the BGG reporters are removed in the presence of oligo(dT). A₀, deadenylated and A_n, polyadenylated reporter mRNAs. The red dashed line indicates the migration of the band with increased mobility (A₀).

These data suggested that only a small subset of the BGG-5BoxB reporter undergoes 5'-3' decay upon tethering of YTHDF2, in contrast to the more prominent effect observed upon AGO2 tethering. This discrepancy hints at the possibility of YTHDF2 mediating mRNA degradation

through a distinct pathway, paving the way for further investigation into the specific mechanisms involved.

3.2.8 Tethering of both single molecules of AGO2 and YTHDF2 is not sufficient to elicit mRNA degradation

Both AGO2 and YTHDF2 bind indirectly or directly to the CCR4-NOT complex. Early studies showed that 67% of 3' UTRs that contain m⁶A peaks also contain at least one TargetScan-predicted miRNA-binding site (Meyer D. K et al., 2012). Therefore, we wanted to study the possibility for AGO2 and YTHDF2 to have synergistic or antagonistic effects upon binding the same reporter mRNA. To initiate the investigation, we designed a new reporter harboring both a BoxB and an MS2 binding sites which allowed us to bring to the same construct, at the same time, two different proteins of interest. MS2 is a 19-nucleotide viral (bacteriophage) RNA sequence present as a hairpin loop structure at the ribosomal binding site of the MS2 replicase mRNA. This hairpin loop can be recognized with high specificity and affinity by the bacteriophage capsid RNA-binding protein MS2 and enables the encapsidation of the genome (Keryer-Bibens, Barreau and Osborne 2012). Therefore, by fusing the proteins of interest with an MS2 tag, we were able, as for the Lambda-N and the BoxB element, to physically bring the studied proteins to the newly designed reporter (**Figure 3.24**).

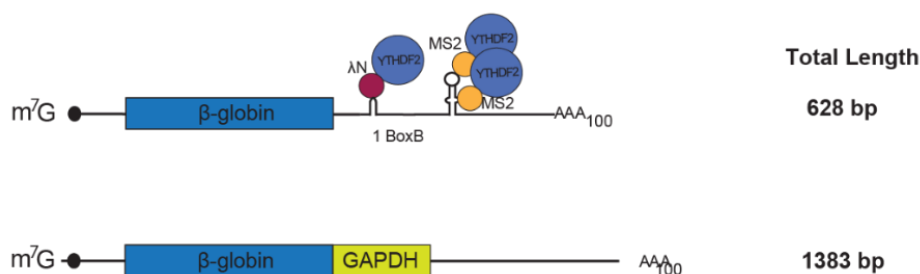


Figure 3.24: β-GLOBIN reporters used in this study. (BGG) β-GLOBIN. The BGG-GAP reporter contains a truncated version of the GAPDH (GAP) gene to distinguish it from the BGG-1BoxB and 1MS2 reporter by size (Lykke-Andersen et al. 2000). The BGG-5BoxB reporter contains 1 BoxB and 1MS2 binding sites in the 3' UTR.

Taking advantage of these two tethering systems, we then conducted a tethering assay followed by northern blot experiments. Specifically, we first assessed the ability of a distinct tag to elicit mRNA degradation by overexpressing a Lambda N-HA version of AGO2 and YTHDF2 for the 1 BoxB site. Lambda N-HA only vector was used as a negative control.

Then, we overexpressed an MS2-HA version of AGO2 and YTHDF2 for the MS2 binding site. An MS2-HA only vector was used as a negative control. We carried on by combining the MS2-HA version of the constructs, with the overexpression of the Lambda-N-HA plasmid alone, the

Lambda-N-HA-AGO2 plasmid, and the Lambda-N-HA-YTHDF2 plasmid. A truncated version of the GAPDH gene fused to Beta globin (BGG-GAP) was co-transfected and used as an internal control since it differed in size from the BGG-1BoxB-1MS2. It is important to state that in the tethering systems based on these two bacteriophage proteins, the lambda N peptide binds to cognate RNA sequence as a monomer whereas the MS2 coat protein as a dimer. Therefore, by overexpressing the proteins of interest in the combination previously described, we studied not only the degradation effects that the AGO2 and YTHDF2 might have combined, but also the differential effects in terms of protein molecules recruited on the BGG-1BoxB-1MS2 reporter (**Figure 3.25**).

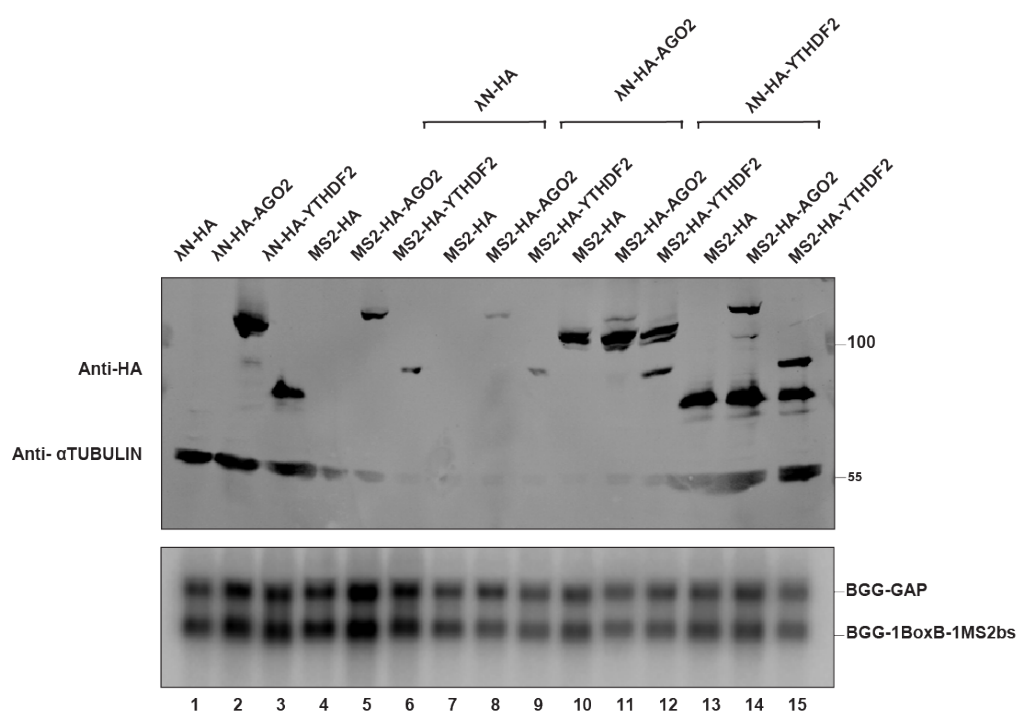


Figure 3.25: Tethering of both single molecules of AGO2 and YTHDF2 is not sufficient to elicit mRNA degradation. In the upper part, a western blot analysis showing the expression levels of the tethered proteins HA, AGO2, and YTHDF2 tagged with the Lambda-N peptide and MS2-peptide in HEK 293T WT cells. A-TUBULIN was used as a loading control. In the lower part, a northern blot analysis showing the tethering assay of both Lambda-N and MS2-tagged HA, AGO2, and YTHDF2 overexpressed proteins to the BGG-1BoxB and 1MS2 reporter in HEK 293T WT cells. A plasmid expressing BGG-GAP served as a transfection control and lacked the 1BoxB and MS2 binding sites.

Upon northern blot analysis, we did not notice any differences between the tethering of the Lambda N version of AGO2 and YTHDF2, and the tethering of the MS2 version of AGO2 and YTHDF2 (**Figure 3.25 compare lanes 2 and 3 with lanes 5 and 6**), suggesting that either one or two molecules of AGO2, or YTHDF2, are not sufficient to elicit the degradation of the BGG-1BoxB-1MS2 reporter.

We then overexpressed the MS2-HA alone, the MS2-HA version of AGO2, and the MS2-HA version of YTHDF2, in combination with the overexpression of the Lambda-N-HA alone (**Figure 3.25 lanes 7-9**), the Lambda-N-HA version of AGO2 (**Figure 3.25 lanes 10-12**), and Lambda-N-HA version of YTHDF2 (**Figure 3.25 lanes 13-15**). The overexpressed MS2-HA plasmid in combination with the overexpression of the Lambda-N-HA plasmid served as a negative control (**Figure 3.25 lane 7**). Upon northern blot analysis (**Figure 3.25**), and quantification (**Figure 3.26**), we did not observe any significant difference upon tethering of the MS2-HA tagged version of AGO2 and YTHDF2 with the Lambda-N-HA empty plasmid, as expected (**Figure 3.25 lanes 7-9, and Figure 3.26**). Moreover, when did not see any significant difference when we overexpressed the Lambda-N-HA version of AGO2 with the MS2-HA version of AGO2, and YTHDF2 (**Figure 3.25 compare lanes 10-12 with lane 7, and Figure 3.26**). No difference was also observed when we overexpressed the MS2-HA tagged version of AGO2, and YTHDF2 in combination with the Lambda-N-HA version of YTHDF2 (**Figure 3.25 compare lanes 13-15 with lane 7, and Figure 3.26**).

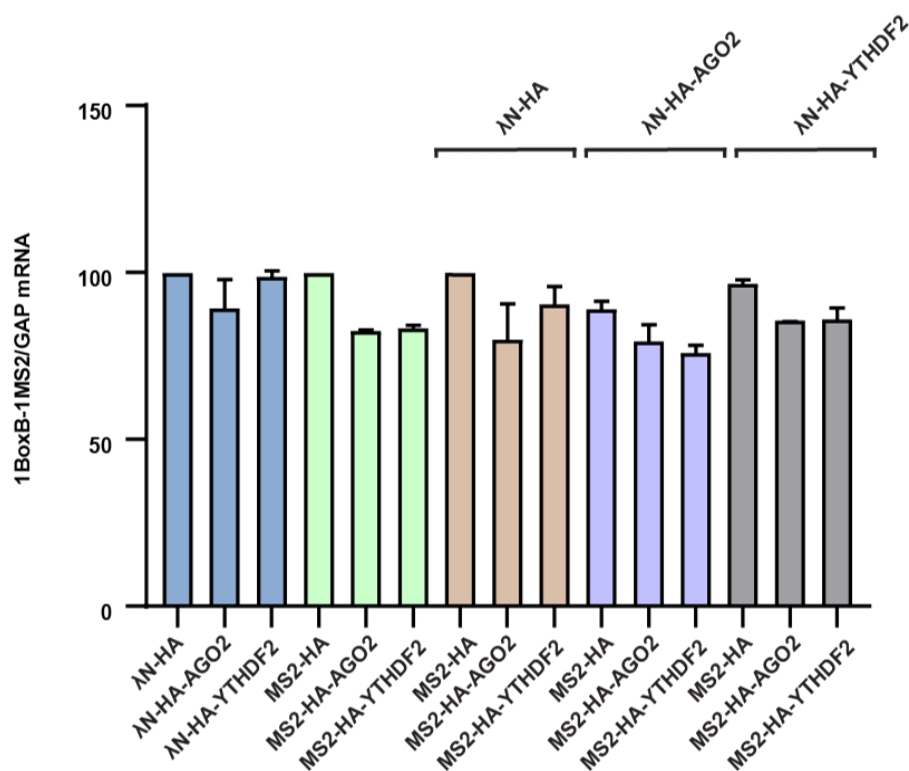


Figure 3.26: Tethering of both single molecules of AGO2 and YTHDF2 is not sufficient to elicit mRNA degradation. BGG-1BoxB-1MS2 mRNA levels determined by Northern blotting were normalized to those of BGG-GAP and set to 1 in cells expressing Lambda-N-HA. Mean values $\pm$ standard deviation (SD) are shown ($n = 2$). BGG-1BoxB-1MS2 mRNA levels were normalized to that of the BGG-GAPDH mRNA. For each experiment the normalized values of BGG-5BoxB mRNA levels were set to 100 in cells transfected with the empty vector. In case of double transfection, the normalized values of BGG-1BoxB-1MS2 mRNA levels were set to 100 in cells transfected with double empty vectors.

This experiment confirmed that the extent of reporter degradation is proportional to the number of B-box hairpins and MS2 sites present in the 3' UTR as it has already been shown for AGO2 (Doench et al. 2003; Pillai RS et al., 2004; Bartel 2004; Doench and Sharp 2004). Hence, a new reporter with multiple binding sites is needed to verify the interplay of AGO2 and YTHDF2 upon binding the same mRNA.

3.3 Impact of YTHDF proteins on translation

Although the tethering experiments indicated that YTHDF paralogs trigger mRNA degradation, there is still a possibility that these proteins could also affect mRNA translation. Both YTHDF1 and YTHDF3 have been reported to be enhancers of mRNA translation (Wang et al., 2015, Shi et al., 2017). To investigate this possibility, we conducted double and triple knockdown experiments targeting YTHDF proteins, followed by polysome profiling (**Figure 3.27**).

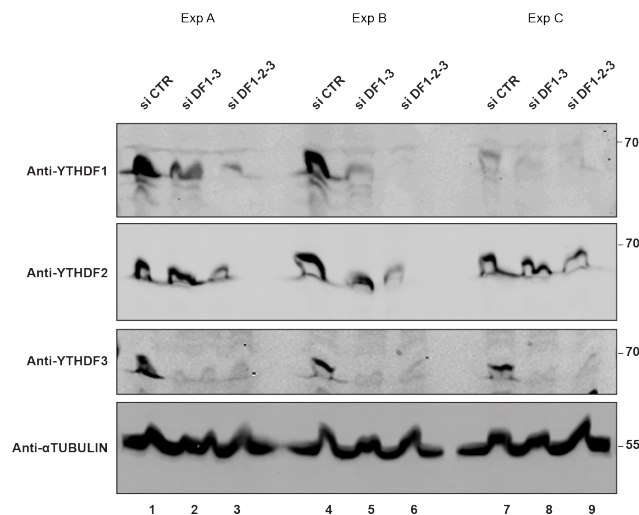


Figure 3.27: YTHDF proteins have distinct functions in translation regulation. Western blot showing siRNA-mediated depletion of YTHDF1, YTHDF2, and YTHDF3 in HEK 293T cells. α -TUBULIN served as a loading control. Exp A, B, and C indicate the analyzed biological triplicates.

During polysome profiling, cell lysates were separated on sucrose density gradients by ultracentrifugation. The gradients were then analyzed using an ultraviolet-visible spectrophotometer to measure the absorbance at various wavelengths. If the YTHDF paralogs were involved in regulating mRNA translation, we would expect to observe changes in absorbance in specific fractions of the polysome profile, such as the 40S, 60S, 80S, or polysome fractions. Interestingly, in the double knockdown of YTHDF1-3, we observed a decrease in absorbance in the polysome fractions compared to the control, suggesting a potential impairment in translation efficiency upon YTHDF1-3 depletion (**Figure 3.28, Exp A, B, C**).

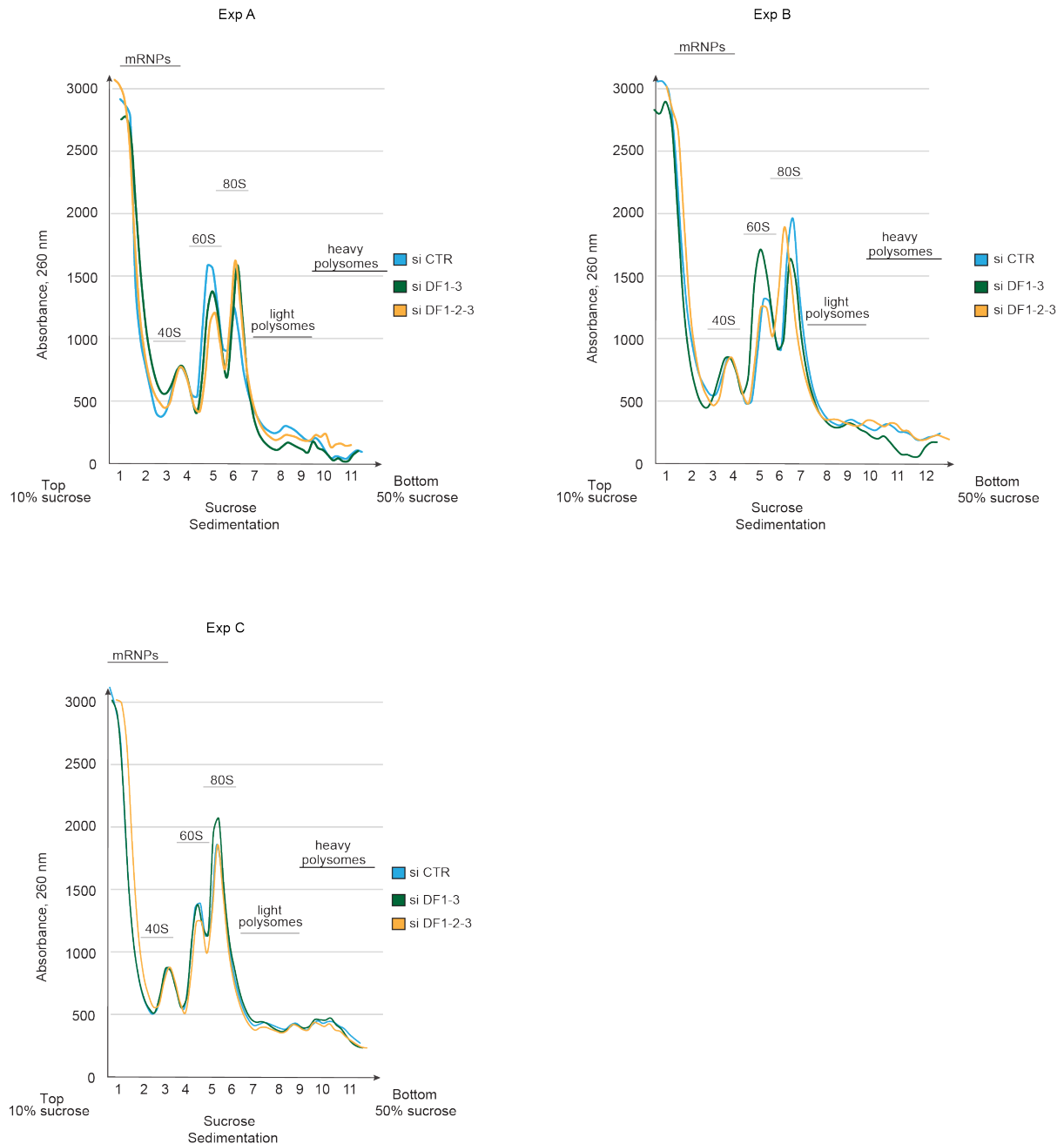


Figure 3.28: YTHDF proteins have distinct functions in translation regulation. UV absorbance profile at 260 nm of scramble siRNA (control, light blue) and YTHDF1-3-depleted (si DF1-3, dark green) and YTHDF1-2-3-depleted (si DF1-2-3, orange) HEK 293T cell extracts after polysome sedimentation in a sucrose gradient. mRNPs, 40S and 60S subunits, 80S monosomes, and light and heavy polysome peaks, are indicated. Exp A, B, C indicate the three biological replicates performed.

Additionally, we observed an increase in 80S absorbance in two out of three replicates, indicating a potential accumulation of ribosomes in the translation initiation state. Surprisingly, upon triple knockdown of YTHDF1-2-3 proteins, we did not observe significant effects in most of the analyzed fractions, suggesting a lack of substantial impact on translation. However, a mild reduction was observed in the absorbance peak indicating the 60S fraction (**Figure 3.28, Exp A, B, C**). These

findings suggest that the depletion of all three YTHDF paralogs may have a minor influence on the 60S subunit association with ribosomes or other translation-related processes.

It is important to mention that we cannot completely rule out the possibility that this effect might be more pronounced for mRNAs marked with m⁶A modification and that the knockdown of these proteins could have secondary effects, potentially destabilizing proteins involved in translational control, which could contribute to the observed changes in translation efficiency. Nevertheless, these findings not only suggest a potential role of YTHDF1-3 proteins in regulating translation initiation or ribosome assembly but also highlight an intriguing observation: the absorbance spectra upon YTHDF1-3 depletion differs from that of the triple knockdown of YTHDF1-2-3. This divergence in absorbance patterns may signify the involvement of yet uncharacterized mechanisms and further emphasizes the potentially diverse roles that these proteins, and in particular YTHDF2 alone, may play in the regulation of translation.

3.4 YTHDF and TNRC6 proteins level regulation

So far, we reported the effect that YTHDF proteins have on mRNA metabolism but little is known about YTHDF proteins regulation itself. Very recently, it has been discovered that YTHDF2 expression diminishes as cells progress through the cell cycle in HeLa cells. More in detail, YTHDF2 protein stability is dependent on cyclin-dependent kinase 1 (CDK1) activity and its expression is regulated by E3 ubiquitin ligase complexes which has in Cullin 1 (CUL1), Cullin 4A (CUL4A), damaged DNA-binding protein 1 (DDB1), and S-phase kinase-associated protein 2 (SKP2) the main components (Fei, Zou et al., 2020). Regarding AGO2, we know that unloaded AGO proteins are unstable and rapidly degraded by the proteasome (Smibert, Yang et al., 2013). In addition, it has been found that target RNAs with a high degree of complementarity to the miRNA induce miRNA degradation through TDMD, where a ubiquitin ligase complex centered on ZSWIM8 was identified to ubiquitinate AGO proteins and initiate degradation by the proteasome. Considering the intriguing parallels between these mechanisms involving ubiquitin ligase complexes, their similar localization (data not shown), and the same recruitment of the CCR4-NOT deadenylase complex, we next wondered whether also a certain degree of regulatory functions might exist. Therefore, we investigated if AGO2-associated proteins like TNRC6A-B-C, shared similar regulation to YTHDF1-2-3 proteins.

3.4.1 YTHDF proteins are degraded in contact-inhibited cells

Numerous tissue culture cells exhibit a phenomenon known as contact inhibition, wherein they cease dividing once they become densely packed. However, this behavior is frequently disrupted in cancer cells, like our working system HEK 293T cells, or in other cell lines like HeLa, HCT116, Sw620, and HT29 cells, leading to uncontrolled proliferation (McClatchey & Yap, 2012). Building

on this information, we examined the protein levels of YTHDF1-2-3 in these cell lines through western blot analysis. We demonstrated that their expression levels remained elevated when we compared low-density (LD), a condition reached when there is about 30% confluency, with high density (HD), a condition reached 1-2 days after 100% confluence (**Figure 3.29 A, data generated by Dr. Hung-Ho Xuan**). Consequently, we deduced that YTHDF proteins do not undergo reduction when the regulatory mechanisms of the cell cycle are disrupted due to contact inhibition loss. This finding could hold significance for tumor advancement, aligning with observations that YTHDF proteins possess oncogenic potential across various cancer types (Chang, Shi et al., 2020; Dixit, Prager et al., 2021; Liu, Wei et al., 2020b; Nishizawa, Konno et al., 2018; Paris, Morgan et al., 2019).

On the contrary, when examining YTHDF protein expression in MRC5, C643, RPE1, and A549 cell lines, and also Mouse NIH-3T3, and Rat NRK cells, which are known to undergo contact inhibition, a consistent finding emerged: all three proteins were significantly downregulated in HD compared to LD conditions (**Figure 3.29 B, data generated by Dr. Hung-Ho Xuan**).

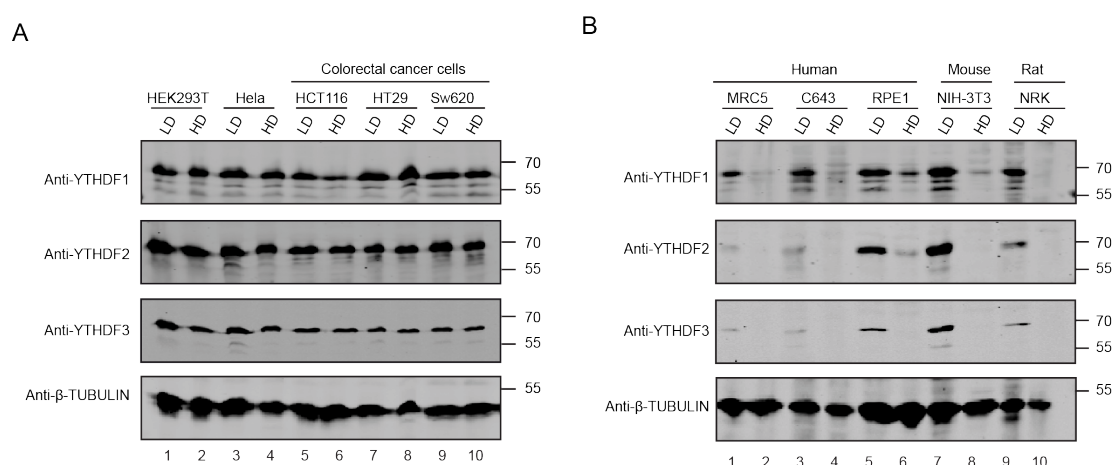


Figure 3.29: Changes in protein expression of YTHDF proteins in low and high density. (A) Western blot of YTHDF proteins at LD and HD in non-contact-inhibited cells, including HEK293T, Hela, and three colorectal cancer cell lines (HCT116, HT29, Sw620). Western blots were performed using cells seeded at LD (about 30% confluence) and HD (1-2 days after 100% confluence). Beta-α-TUBULIN (b-tub) served as a loading control. **(B)** Western blot of YTHDF proteins at LD and HD in contact-inhibited cells, including human cell lines (MRC5, C643, RPE1), murine cell NIH-3T3, and rat cell NRK. All cells were seeded at LD (about 30% confluence) and HD (1-2 days after 100% confluence) and then collected for western blot analysis. Beta-α-TUBULIN (β-tub) served as a loading control.

3.4.2 TNRC6 proteins are degraded in contact-inhibited cells

We then decided to verify if also TNRC6A-B-C protein levels were affected in contact inhibited cell lines by comparing MRC5 LD with HD conditions. Through western blot analysis, we were able to demonstrate that TNRC6A-B-C protein levels were reduced upon contact inhibition (**Figure 3.30 A**) while their mRNA levels remained unaffected (**Figure 3.30 B**).

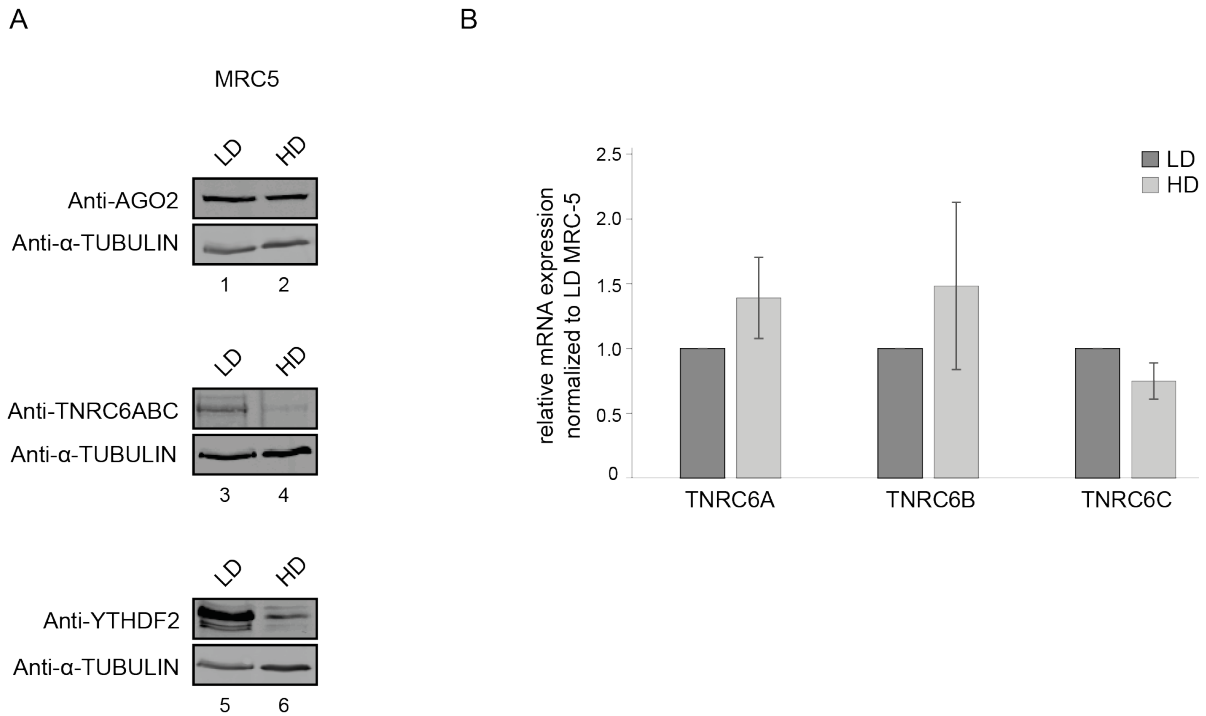


Figure 3.30: Changes in protein expression of TNRC6 proteins in low and high density. (A) Western blot of TNRC6 and AGO2 proteins at LD and HD in MRC5 contact-inhibited cells. All cells were seeded at LD (about 30% confluence) and HD (1-2 days after 100% confluence) and then collected for western blot analysis. Alpha- α -TUBULIN served as a loading control. **(B)** Relative expression of TNRC6 A-B-C mRNAs in MRC5 cells. The expression was normalized over LD condition.

Interestingly, AGO2 expression did not change, suggesting that only proteins directly in contact with the CCR4-NOT complex or proteins with a higher degree of unstructured domains might be regulated in this way **(data generated by Julia Neumeier)**.

3.6.3 YTHDF protein degradation is not dependent on transcriptional or translational regulation

YTHDF proteins are downregulated when contact-inhibited cells reach high density but there might be several reasons behind their degradation. To understand in more detail the mechanisms, we first examined whether the regulation of YTHDF protein levels occurs on a transcriptional level. Taking MRC5 cells as an example, we tested the expression of YTHDF1-2-3 mRNAs in LD and HD cells. RNA from MRC5-LD and MRC5-HD was extracted and analyzed by qPCR **(Figure 3.31 A, data generated by Dr. Hung-Ho Xuan)**. As a positive control, we found robust downregulation of Ki67, which served as a proliferative cell marker. Surprisingly, YTHDF1-2-3 mRNA levels remained unchanged when high density was reached. Similar results were obtained when Northern blots on poly(A)⁺ RNA were performed **(Figure 3.31 B, data generated by Dr. Hung-Ho Xuan)**.

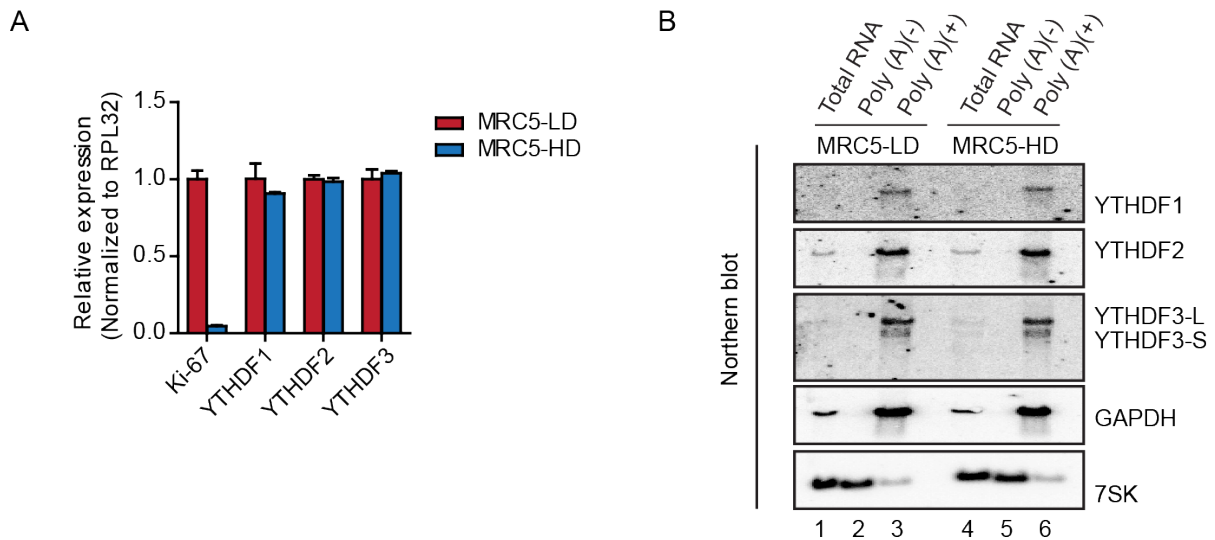


Figure 3.31: YTHDF protein degradation is not dependent on transcriptional regulation. (A) Relative expression of YTHDF proteins in MRC5 cells by qPCR analysis. The MRC5 cells were seeded at LD and HD and collected. Ki67 served as a proliferation marker, while RPL32 served as the control. **(B)** Northern blot analysis of YTHDF transcripts in LD and HD MRC5 cells (MRC5-LD and MRC5-HD). Total RNA from MRC5 was collected for fractionation of poly (A)+ RNA and poly(A)- RNA. RNA was separated on 1% MOPS/Agarose gel for northern blot analysis using specific probes for YTHDF transcripts. GAPDH served as a positive control of poly (A)+ RNA, while 7SK served as a positive control of poly (A)- RNA.

These findings demonstrated that the reduction of YTHDF1-3 expression upon contact inhibition was not caused by transcriptional regulation.

Since the regulation seemed to be the same in all the cells exhibiting contact inhibition phenotypes, including A549 (data not shown), we conducted polysome profiling in A549 cells to confirm whether the overall decline in YTHDF1-2-3 protein levels resulted from a decrease in translation **(Figure 3.32 A-B)**.

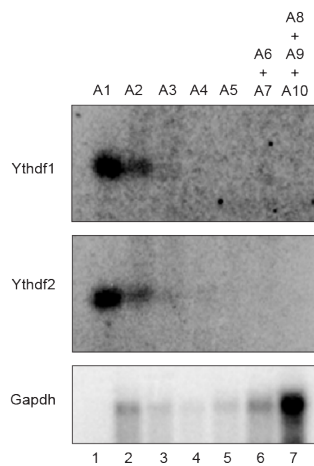
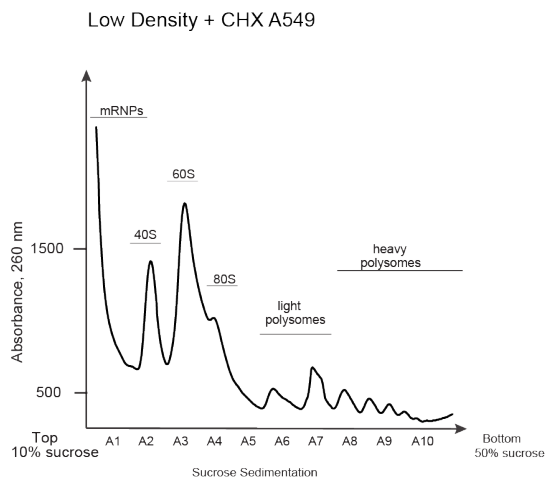
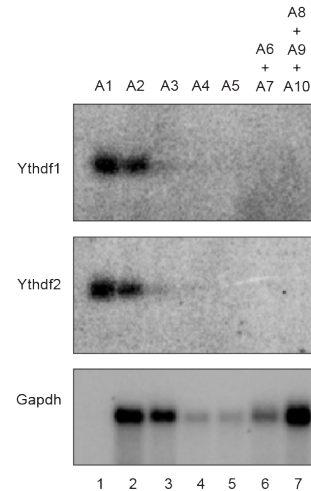
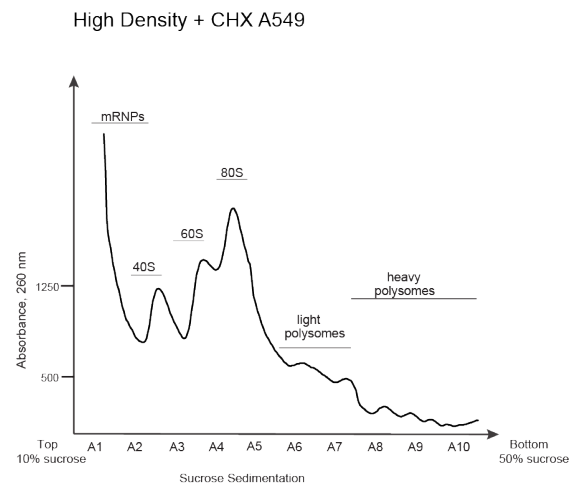
A**B**

Figure 3.32: YTHDF1-2 mRNAs expression remained unchanged under both LD and HD conditions in polysome profiling analysis. (Top panel): UV absorbance profile at 260. A549 cell extracts after polysome sedimentation in a sucrose gradient. mRNPs, 40S and 60S subunits, 80S monosomes, and light and heavy polysome peaks are indicated. (Bottom panel): Northern blot assay of polysome profiling fractions of LD (A) and HD (B) A549 cell extracts. The A549 cells were seeded at LD and HD and collected. For YTHDF mRNA levels in low-density and high-density, total RNA from A549 was extracted from each fraction and RNA was separated on 1% MOPS/Agarose gel for northern blot analysis using specific probes for YTHDF transcripts. GAPDH served as a positive control for high translation.

After extracting RNA from different sucrose gradient fractions, running the RNA on a 1% agarose gel, and performing Northern Blot assay, we noticed that endogenous Ythdf1 and Ythdf2 mRNAs levels within the polysome profile remained consistent and showed no shift from the free mRNPs fractions (**Figure 3.32 A-B compare lanes 1, 2 and 3**). This distribution indicated that the translation rates of these proteins remained unchanged under both LD and HD (**Figure 3.32 A-B**) and that the reduced YTHDF1-2 protein levels were due to a different level of regulation.

3.5 Analysis of YTHDF and TNRC6 proteins depletion on steady-state poly(A) tail length

Given the recruitment of the CCR4-NOT complex by both YTHDF and TNRC6 proteins, it is logical to expect that depleting these proteins would have repercussions on the overall length of poly(A) tails. To investigate the effects of YTHDF, TNRC6, and XRN1 proteins depletion, on the poly(A) tail and their related mRNAs, in collaboration with Rajewsky lab in Berlin we utilized full-length poly(A) and mRNA sequencing (FLAM-Seq) (Legnini et al., 2019).

3.5.1 Full-length mRNA sequencing is the ideal sequencing to study the effects of YTHDF, TNRC6, and XRN1 proteins depletion on the poly(A) tail length

The FLAM-Seq is a rapid and simple method for high-throughput and high-quality sequencing of entire mRNAs. To have a global idea about the effects on the poly(A) tail we decided to sequence different conditions. Among the conditions included in the sequencing we had both double knockdown of YTHDF1-3 (si DF1-3) and triple knockdown of YTHDF1-2-3 (si DF1-2-3), as well as triple knockdown of TNRC6A-B-C (si TNRC6ABC) and XRN1-depleted HEK 293T cells. Additionally, we incorporated the double knockdown of YTHDF1-3 and the triple knockdown of YTHDF1-2-3 in HEK 293T XRN1 K.O. to examine the impact of YTHDF proteins depletion on endonuclease cleaved transcripts (**Figure 3.33**).

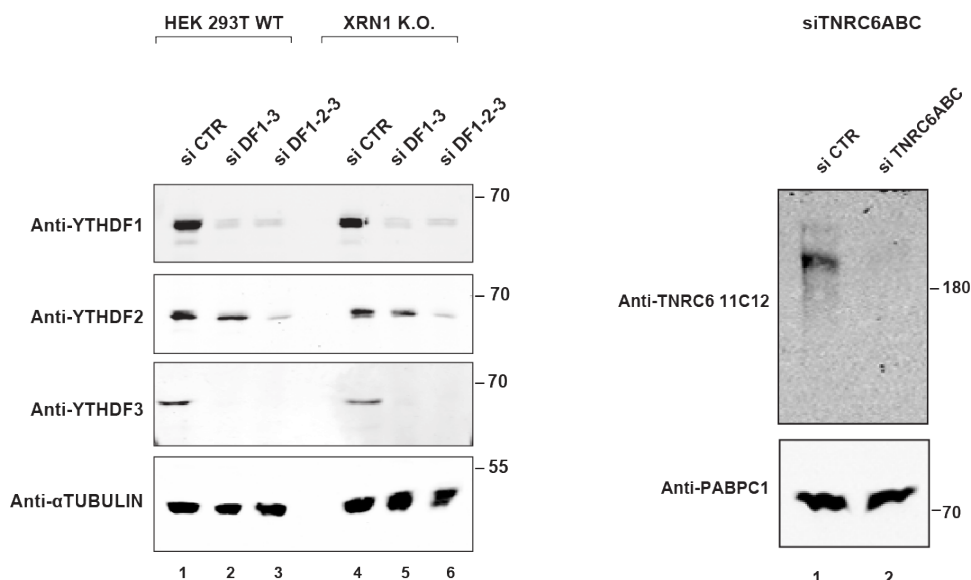


Figure 3.33: Knockdown validation for FLAM-Seq experiments. Western blot showing siRNA-mediated depletion of YTHDF1, YTHDF2, and YTHDF3 (**A**) and TNRC6ABC (**B**) in HEK 293T cells. Specific in-house antibody 11C12 recognized all three paralogs of TNRC6 and was used to detect the efficiency of the knockdown. Alpha-TUBULIN and PABPC1 served as loading controls.

Furthermore, we examined the levels of steady-state total mRNA. Briefly, the FLAM-Seq library was sequenced by using Single Molecular Real-Time (SMRT) sequencing in zero-mode waveguides (ZMW) with the PacBio Sequel II. In this method, the polymerase is fixed to the bottom of the well and allows the DNA strand to progress through the ZMW. The SMRT sequencing allows having real-time imaging since the DNA polymerase incorporates fluorescently tagged nucleotides that are synthesized along with individual DNA template molecules. The sequencing reaction ends when the template and polymerase dissociate. It is important to notice that the transcriptome sequencing taken into consideration for this analysis derived solely from the FLAM-Seq, which relied on a 3' guanosine-inosine tailing for template cDNA synthesis. Consequently, this approach might introduce a bias favoring the initiation of cDNA synthesis at the poly(A) tail, which may impact the representation of mRNA sequences, particularly at the 3' end. On the other hand, PacBio instrument allows to sequence as average read length, approximately 2 kb, with some reads which may reach over 20 kb. Since in this way the reads of many mRNAs can span over complex or repetitive regions with a single continuous read, we were also able to study the effects on endonuclease-mediated cleaved mRNAs as well as exon usage of different mRNAs. Finally, by sequencing directly single molecules, the errors that can occur during the PCR amplification steps are minimized, resulting in a very accurate base calling. In conclusion, all the previous characteristics and advantages made the FLAM-Seq in combination with the PacBio Sequencing, the perfect tool to analyze precisely the effects of YTHDF1-2-3, TNRC6A-B-C, and XRN1 depletion on the poly(A) tail level.

3.5.2 FLAM-Seq analysis of steady state mRNAs revealed a shortening of poly(A) tail upon depletion of YTHDF, TNRC6, and XRN1 proteins

To validate the precision of our approach, we examined the differences in poly(A) tail length across all sequenced samples. The differential poly(A) distributions were assessed per-gene basis (as defined by the median length of all unique molecular identifiers (UMIs) associated with a particular gene) (**Figure 3.34**). Interestingly, these observed poly(A) length distributions exhibited distinct characteristics unique to each biological sample (**Plot in Figure 3.34 generated by Dr. Ivano Legnini**).

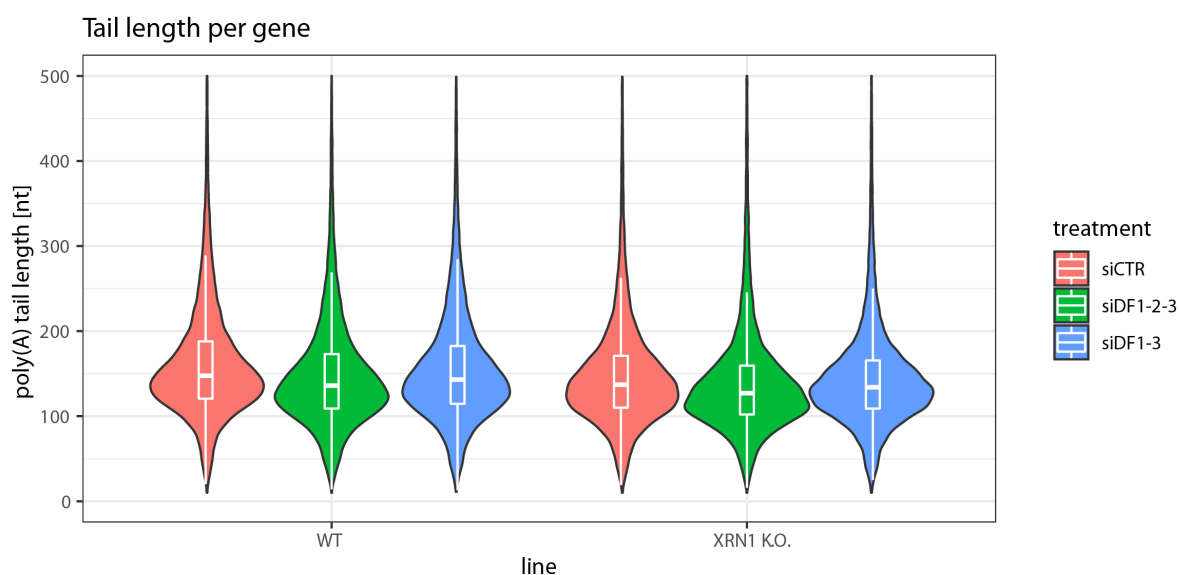


Figure 3.34: Violin plot of poly(A) length distribution per gene across samples. Distribution per gene among the different conditions mentioned in Figure 3.57 A. The violin plots represent the total distribution of the poly(A) tail among the three different biological replicates. The violin shape represents the data's probability density, and a box plot inside shows median, quartiles, and outliers. Additionally, the line in the middle represents the median value of three biological replicates.

By looking at the median length of all UMIs associated with a particular gene we noticed a decrease in median poly(A) tail length upon depletion of YTHDF (in HEK 293T WT and XRN1 K.O.), and XRN1 proteins when compared to the si CTR (**Figure 3.34**). The difference in poly(A) tail upon depletion of YTHDF proteins was even clearer by looking at the density plot in **Figure 3.35** and comparable to the XRN1 K.O. condition (**Figure 3.36**).

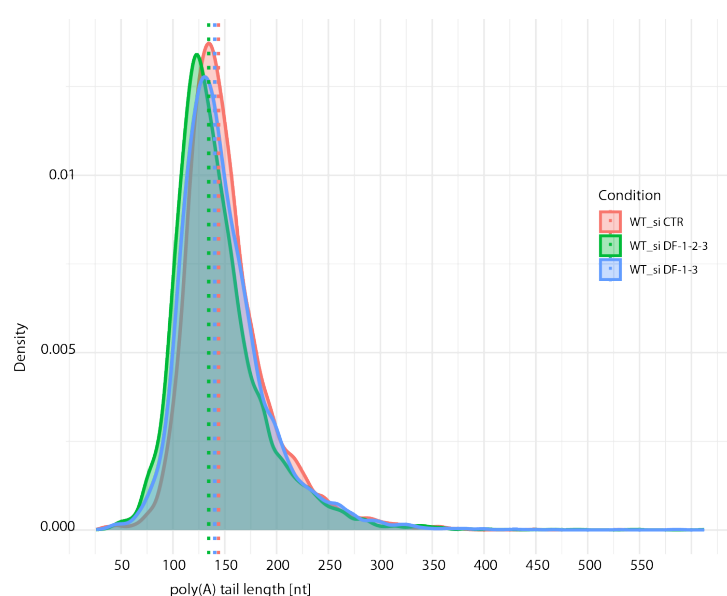


Figure 3.35: Density plot of poly(A) length distribution per gene across samples in HEK 293T sictr, siDF1-3 and siDF1-2-3 conditions. Density plot showing the distribution per gene in HEK 293T si CTR, siDF1-3, and siDF1-2-3. The

x-axis represents poly(A) tail length values, while the y-axis represents the estimated probability density. The shaded area represents regions of higher density, providing insights into the concentration of data points. Peaks in the plot indicate modes or clusters within the distribution. The vertical dotted line shows the median poly(A) tail for each mentioned condition.

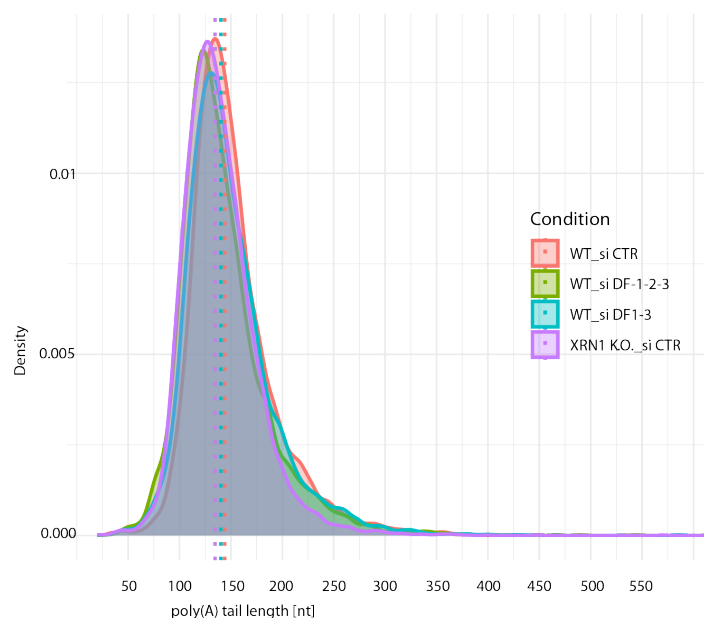


Figure 3.36: Density plot of poly(A) length distribution per gene across samples in HEK 293T si CTR, siDF1-3 and siDF1-2-3 and XRN1 K.O. conditions. Density plot showing the distribution per gene in HEK 293T si CTR, siDF1-3, siDF1-2-3, and XRN1 K.O. (light purple). The x-axis represents poly(A) tail length values, while the y-axis represents the estimated probability density. The shaded area represents regions of higher density, providing insights into the concentration of data points. Peaks in the plot indicate modes or clusters within the distribution. The vertical dotted line shows the median poly(A) tail for each mentioned condition.

Notably, the impact of poly(A) tail shortening was more pronounced when all YTHDF paralogs were depleted, as opposed to the double depletion of YTHDF1-3. It is worth mentioning that the highest effect on poly(A) shortening was observed when YTHDF1-2-3 proteins were depleted in XRN1 K.O. hinting that the whole RNA metabolism of the cell might be completely disrupted without these factors or that they act together to finely tune the poly(A) tail length (**Figure 3.37**).

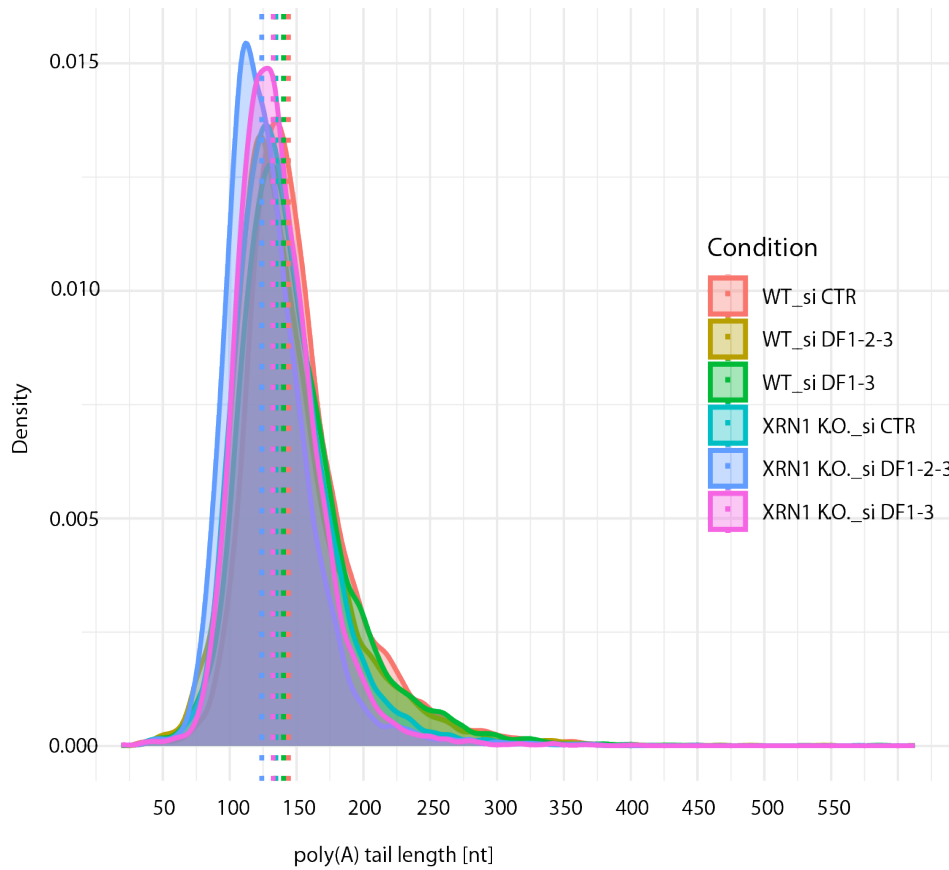


Figure 3.37: Density plot of poly(A) length distribution per gene across samples in HEK 293T si CTR, siDF1-3 and siDF1-2-3 and HEK 293T XRN1 K.O. si CTR, siDF1-3 and siDF1-2-3 conditions. Density plot showing the distribution per gene among the different conditions mentioned above. The x-axis represents poly(A) tail length values, while the y-axis represents the estimated probability density. The shaded area represents regions of higher density, providing insights into the concentration of data points. Peaks in the plot indicate modes or clusters within the distribution. The vertical dotted line shows the median poly(A) tail for each mentioned condition.

Surprisingly, we did not observe any global effect on the steady-state poly(A) tail length when we depleted the cell of all TNRC6 proteins (**Figure 3.38**).

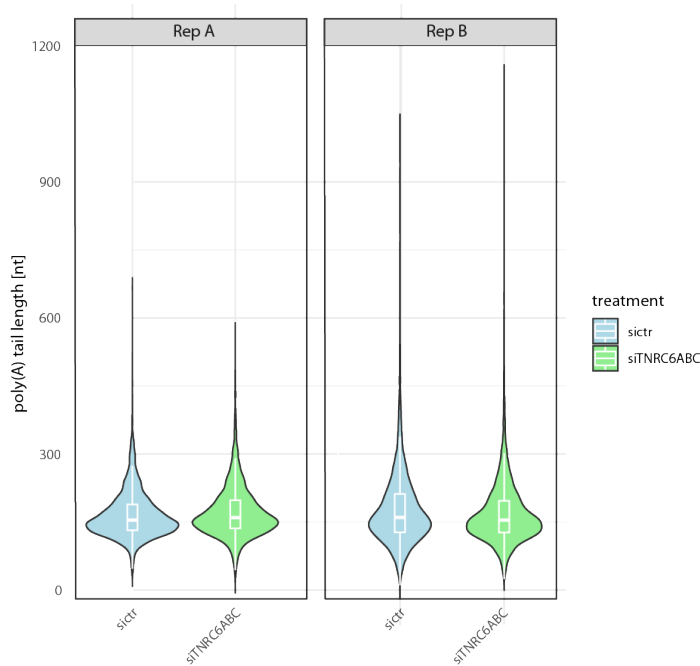


Figure 3.38: Violin plot of poly(A) length distribution per gene across samples. Distribution per gene among the different conditions mentioned in Figure 3.33 B. Rep A and B indicate the two different biological replicates.

Also looking at the density plot we did not notice any significant effect on the global poly(A) tail (Figure 3.39) upon depletion of TNRC6 proteins. This finding though, was again in contrast with our expectation, as TNRC6 proteins, like YTHDF proteins, recruit the CCR4-NOT complex and, therefore their absence should result in an increase in median poly(A) tail length distribution.

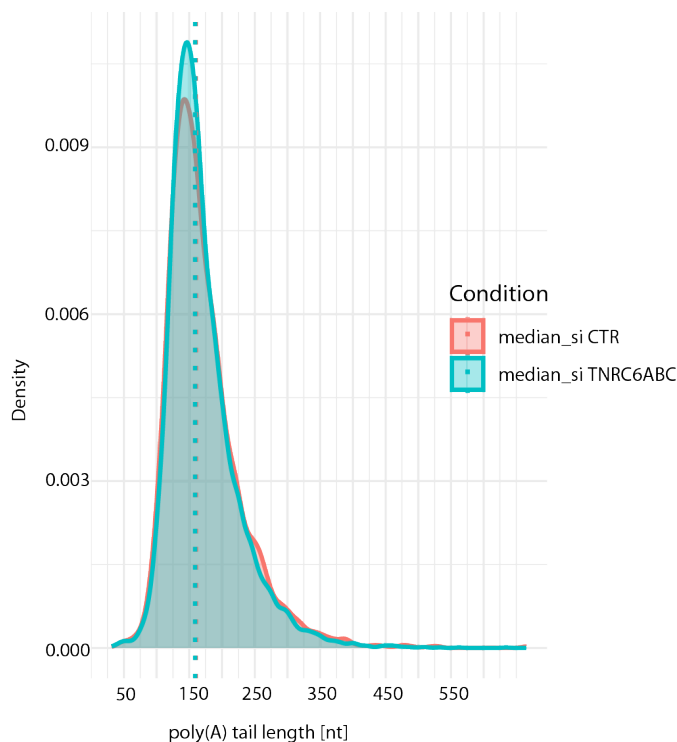


Figure 3.39: Density plot of poly(A) length distribution per gene across samples in HEK 293T si CTR and siTNRC6A-B-C conditions. Density plot showing the distribution per gene among the different conditions mentioned above. The x-axis represents poly(A) tail length values, while the y-axis represents the estimated probability density.

The shaded area represents regions of higher density, providing insights into the concentration of data points. Peaks in the plot indicate modes or clusters within the distribution. The vertical dotted line shows the median poly(A) tail for each mentioned condition.

3.5.3 Depletion of YTHDF and XRN1 proteins induced a pronounced shortening of poly(A) tail length for transcripts with median tail length higher than 150 nucleotides

It has been recently proved that the degradation rate of transcripts with short tails prevents the steady accumulation of such mRNAs (Eisen et al., 2019). Therefore, we assessed the distinctive poly(A) distributions at the level of specific genes by examining the changes in median length of all UMIs associated per gene with poly(A) tail lower than 150 nt (**Figure 3.40**). In this way we wanted to verify if there is a length limit where the poly(A) tail is more sensitive to the deadenylation dynamics of the transcripts when YTHDF proteins are depleted.

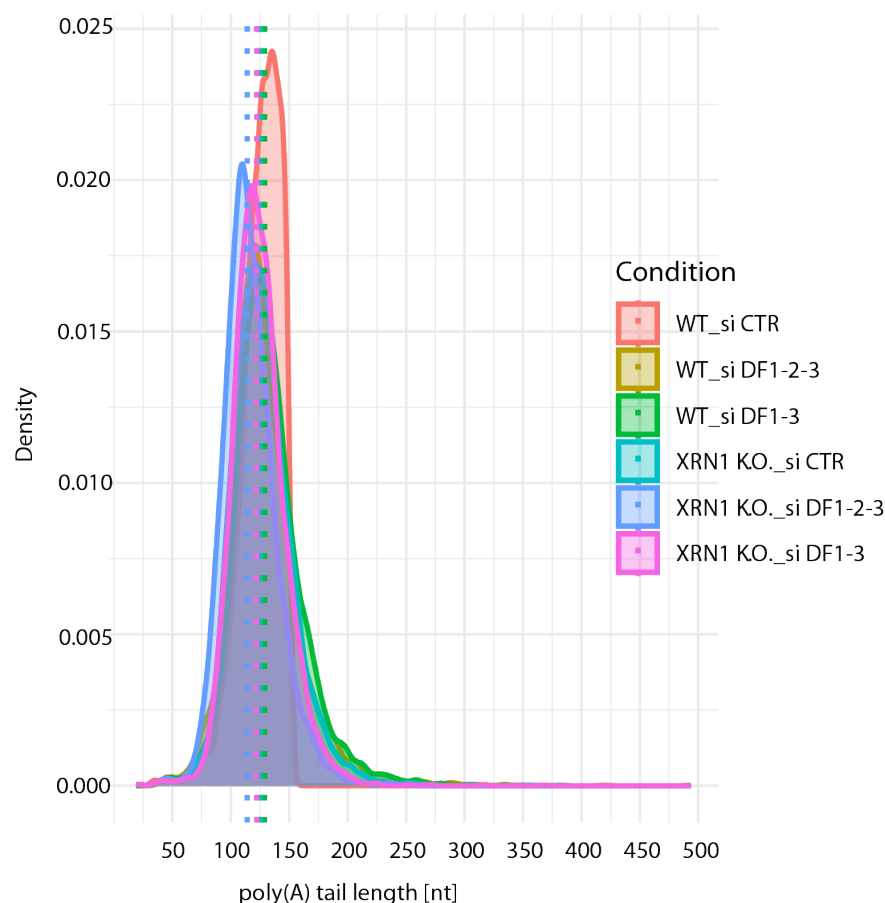


Figure 3.40: Depletion of YTHDF and XRN1 proteins also induced a shortening of poly(A) tail length for transcripts with a median tail length lower than 150 nucleotides. Density plot distribution per gene among the different conditions for transcripts with poly(A) higher than 150 nt. The x-axis represents poly(A) tail length values, while the y-axis represents the estimated probability density. The shaded area represents regions of higher density,

providing insights into the concentration of data points. Peaks in the plot indicate modes or clusters within the distribution. The vertical dotted line shows the median poly(A) tail for each mentioned condition.

The density plot in **(Figure 3.40)** showed that the double knockdown of YTHDF1-3 resulted in a decrease in poly(A) tail length in both HEK 293T WT and HEK 293T XRN1 K.O. samples compared to the si CTR control. Furthermore, the knockdown of YTHDF1-2-3 in both WT and XRN1 K.O. also exhibited a reduction in poly(A) tail length when compared to the si CTR control (median tail length of WT si CTR is represented under the median tail length of WT siDF1-3). The most significant shortening of poly(A) tail length was observed in the triple knockdown of YTHDF1-2-3 in XRN1 K.O. conditions.

Since no difference was observed for transcripts with poly(A) tail lower than 150 nt, compared to the global poly(A) tail profiling, we analyzed the different effects of YTHDF depletion in both WT and XRN1 K.O. for genes with poly(A) tail higher than 150 nt.

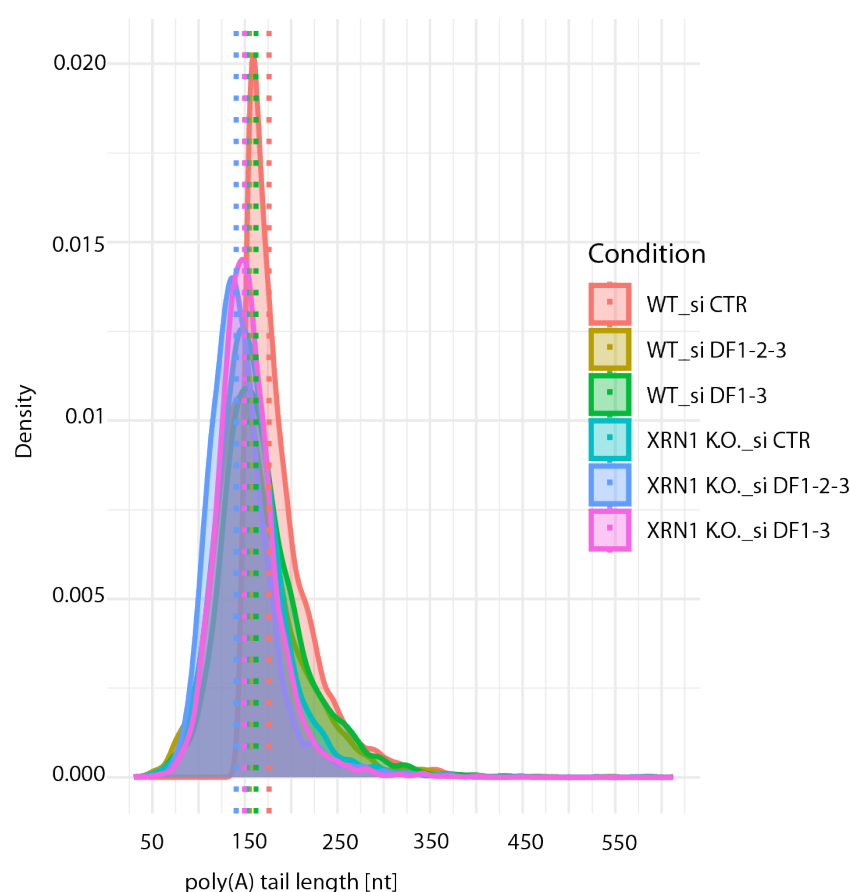


Figure 3.41: Depletion of YTHDF and XRN1 proteins induced a more pronounced shortening of poly(A) tail length for transcripts with a median tail length higher than 150 nucleotides. Density plot distribution per gene among the different conditions for transcripts with poly(A) higher than 150 nt. The x-axis represents poly(A) tail length values, while the y-axis represents the estimated probability density. The shaded area represents regions of higher

density, providing insights into the concentration of data points. Peaks in the plot indicate modes or clusters within the distribution. The vertical dotted line shows the median poly(A) tail for each mentioned condition.

As shown in **(Figure 3.41)**, we did not observe any change in poly(A) tail length distribution compared to the global distribution. Also in this case, the double knockdown of YTHDF1-3 already caused a significant decrease of poly(A) tail HEK 293T WT and in HEK 293T XRN1 K.O. compared to the si CTR. Along the same line, upon knockdown of YTHDF1-2-3 in both HEK 293T WT and HEK 293T XRN1 K.O., a significant reduction in poly(A) tail length was immediately apparent when compared to si CTR, and even greater than the double YTHDF1-3 knockdown. Notably, the most pronounced shortening of poly(A) tail length was observed in the triple knockdown of YTHDF1-2-3 in HEK 293T XRN1 K.O.

In conclusion, the depletion of YTHDF paralogs in both HEK 293T WT and XRN1 K.O. samples resulted in a consistent shortening of the poly(A) tail length, independently of the tail length.

3.5.4 Depletion of TNRC6 proteins induced a lengthening of poly(A) tail length for transcripts with median tail length lower than 150 nucleotides

Since we did not observe any particular global change on the poly(A) tail upon TNRC6A-B-C kd, as for YTHDF proteins, we decided to verify the distinctive poly(A) distributions at the level of specific genes, by examining the median length per gene with poly(A) tail lower than 150 nt **(Figure 3.42)**. Also in this case, we wanted to verify if there is a length limit where the poly(A) tail is more sensitive to the deadenylation dynamics of the transcripts when TNRC6 proteins are depleted. As no overall difference was observed in the global poly(A) tail distribution, we anticipated similar results when examining the median length per gene with a poly(A) tail lower than 150 nt.

Surprisingly, the density plot showed a significant increase in the median poly(A) tail length compared to si CTR HEK 293T WT condition in TNRC6A-B-C depleted HEK 293T cells **(Figure 3.42)**. This finding followed our expectations since the CCR4-NOT complex recruitment is impaired in the absence of TNRC6 proteins and therefore the median poly(A) tail length should increase. However, it remains to be established whether these targets also exhibit an increase in mRNA stability and if these results are the product of differences in distribution since the pool of transcripts is less deep.

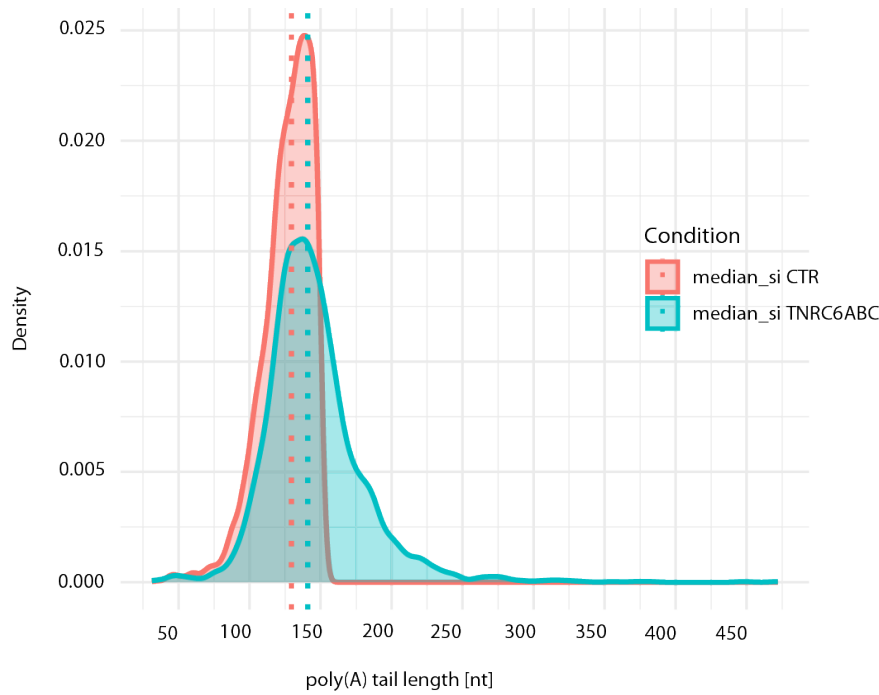


Figure 3.42: Depletion of TNRC6 proteins induces a lengthening of poly(A) tail length for transcripts with median tail length lower than 150 nucleotides. Density plot distribution per gene among the different conditions for transcripts with poly(A) higher than 150 nt. The x-axis represents poly(A) tail length values, while the y-axis represents the estimated probability density. The shaded areas represent regions of higher density, providing insights into the concentration of data points. Peaks in the plot indicate modes or clusters within the distribution. The vertical dotted line shows the median poly(A) tail for each mentioned condition.

By examining the median length per gene with poly(A) tail higher than 150 nt (**Figure 3.43**), we again observed a significant decrease of poly(A) tail compared to si CTR HEK 293T WT condition. In conclusion, our investigation into the impact of TNRC6A-B-C depletion on poly(A) tail dynamics revealed intriguing findings.

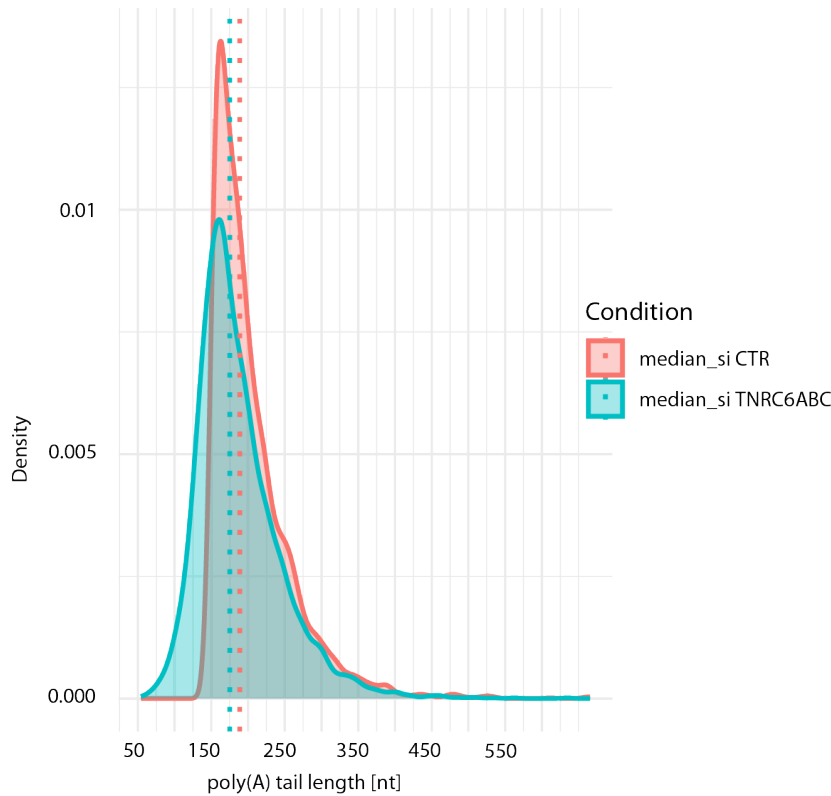


Figure 3.43: Depletion of TNRC6 proteins induced a shortening of poly(A) tail length for transcripts with a median tail length higher than 150 nucleotides. Density plot distribution per gene among the different conditions for transcripts with poly(A) higher than 150 nt. The x-axis represents poly(A) tail length values, while the y-axis represents the estimated probability density. The shaded areas represent regions of higher density, providing insights into the concentration of data points. Peaks in the plot indicate modes or clusters within the distribution. The vertical dotted line shows the median poly(A) tail for each mentioned condition.

While a global change in poly(A) tail length was not observed (**Figure 3.38, Figure 3.39**), a closer examination at the level of specific genes uncovered different variations. The significant increase in median poly(A) tail length in TNRC6A-B-C depleted HEK 293T cells, aligns with our expectations, while the observed significant decrease in poly(A) tail length for genes with tails higher than 150 nt, aligns with what seen for YTHDF and XRN1 proteins depletion. Which one of these differences is also related to mRNA stability remains to be established.

3.5.5 Validation of FLAM-Seq targets

To further assess the accuracy of our poly(A) tail length measurements, we first considered mitochondrial mRNAs, which have a well-defined 50 nt tail length, as previously measured by other methods (Temperley et al., 2010; Legnini et al., 2019). We found that our data is in agreement with published datasets. We observed that the poly(A) tail length of mitochondrial

genes did not differ between the considered conditions (**Figure 3.44**) (Plot generated by Dr. Ivano Legnini).

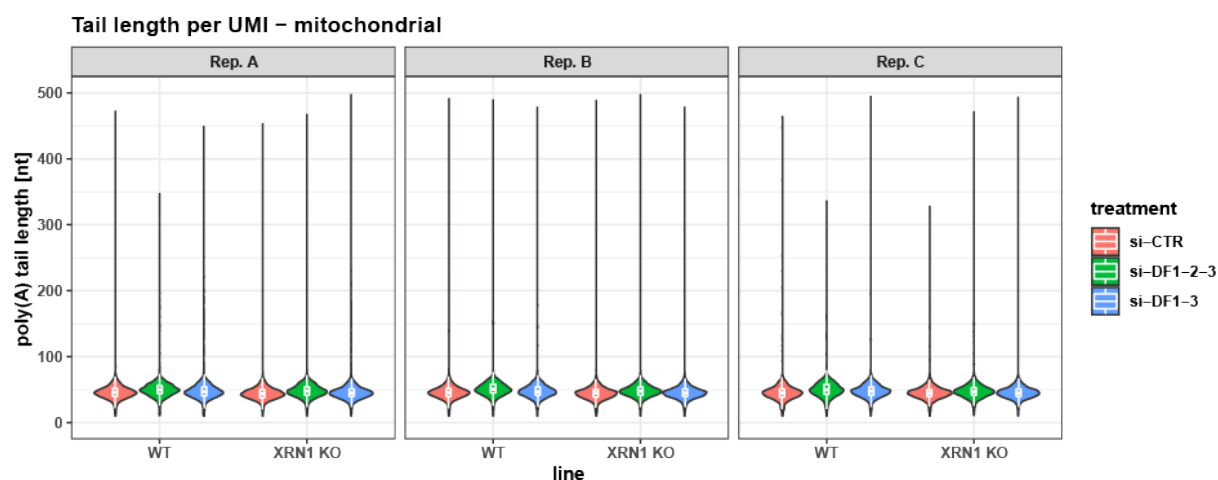


Figure 3.44: Depletion of YTHDF and XRN1 proteins did not change the poly(A) tail length of mitochondrial transcripts. Distribution per UMI among the different conditions for mitochondrial transcripts. Rep A, B, C indicate the three different biological replicates. The violin shape represents the data's probability density, and a box plot inside shows median, quartiles, and outliers. Additionally, the line in the middle of the boxplot represents the median value of three biological replicates.

We then proceeded to validate our sequencing results by performing poly(A) tail length assay and extension Poly(A) Test (ePAT) assay on some of the genes that showed the highest variation between the YTHDF depletion experiments (**Figure 3.45**). We used GAPDH as a control since in our sequencing data we did not observe any major variation in the poly(A) tail length.

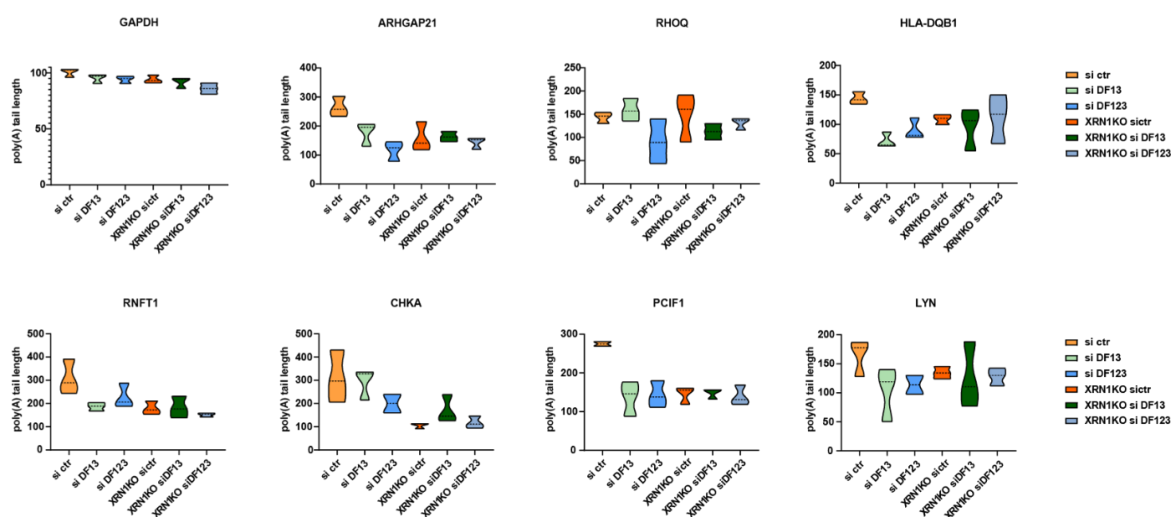


Figure 3.45: Most variant poly(A) tail length transcripts after FLAM-Seq sequencing used for validation. Distribution per gene among the different studied conditions for mitochondrial transcripts. The violin shape represents

the data's probability density, and a box plot inside shows median, quartiles, and outliers. Additionally, the dotted line in the middle represents the median value of three biological replicates. GAPDH was used as a negative control.

For poly(A) tail length assay experiments, after performing kd experiments recapitulating the sequencing conditions (**Figure 3.46 A**), we used the same 3' guanosine-inosine tailing approach for template cDNA synthesis (from Thermo Fisher Poly(A) Tail-Length Assay Kit), to do not diverge from the FLAM-Seq library preparation.

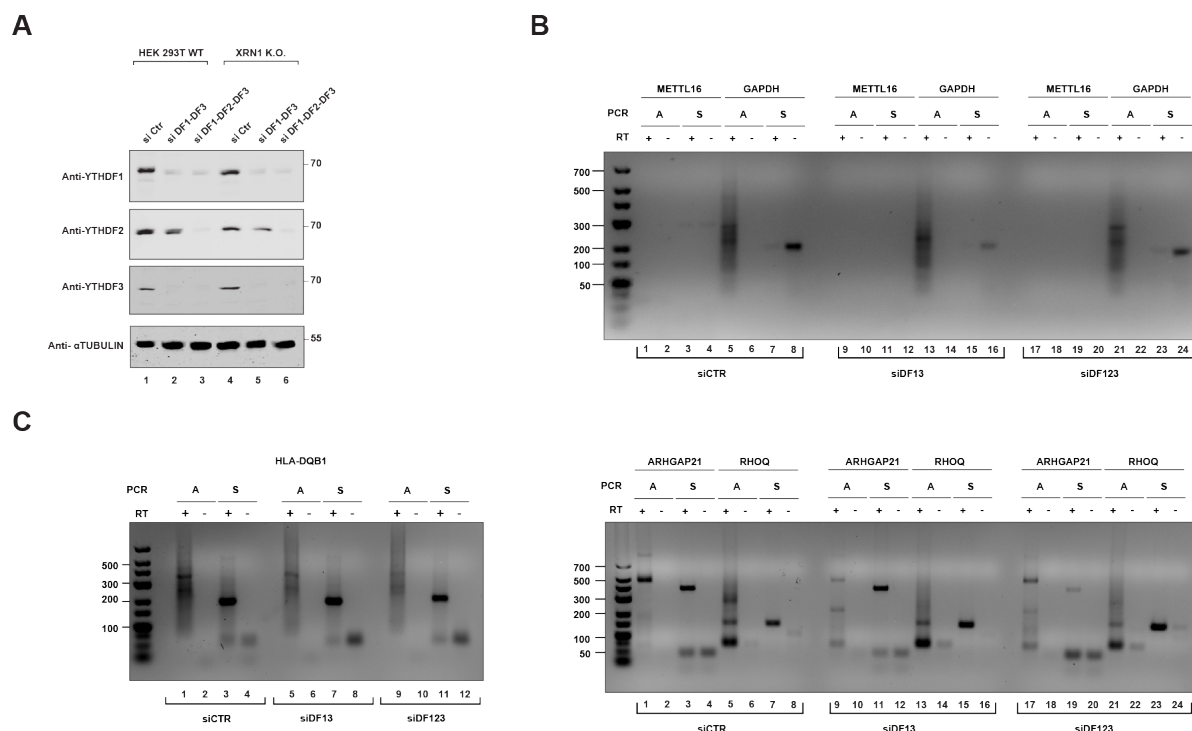


Figure 3.46: FLAM-Seq targets validation through Poly(A) tail length assay. (A) Western blot showing siRNA-mediated depletion of YTHDF1, YTHDF2, and YTHDF3 in HEK 293T and XRN1 K.O. cells. A-TUBULIN served as a loading control. (B-C) Poly(A) tail lengths were measured for METTL16, GAPDH, HLA-DQB1, ARHGAP21, and RHOQ mRNAs. Total RNAs were extracted from HEK 293T cells 72 hours after knockdown experiments, and poly(A) tail lengths were measured by use of a poly(A) tail length assay kit. 1 microgram (ug) of total RNA and 4 µl of diluted RT samples were used in G/I Tailing and PCR reactions, respectively. The recommended two-step PCR program was used. One half of each PCR reaction (12.5 µl) was analyzed on 2.5% agarose-TAE gel stained with ethidium bromide. RT (+); No RT Negative Control (-); poly(A) tail PCR (A); gene-specific PCR (S); and Gene ruler mix DNA Ladder.

Afterwards, PCR amplification products were generated by using two different primer pairs. A gene-specific forward and reverse primers set, with the forward primer designed to pair upstream of the polyadenylation site (e.g. the 3'-UTR), were used to verify the expression of the target (S). For the second set of primers, we used a gene-specific forward primer and the universal reverse primer provided with the kit to amplify a product that included the poly(A) tails of the gene-of-interest (A). In addition, templates were amplified without retrotranscriptase as a further control (RT -). The length of the poly(A) tail was estimated by removing the length of the sequence

upstream of the polyadenylation site to the final length. As shown in **(Figure 3.46 B, C)**, except for METTL16 which we were not able to amplify, the length of the poly(A) tail in the different conditions recapitulated exactly the shortening of the poly(A) tail observed in the FLAM-Seq data **(compare lane 5 with lanes 13 and 21 for GAPDH, compare lane 1 with lanes 5 and 9 for HLA-DQB1 in Figure 3.46 C; compare lane 1 with lanes 9 and 17 for ARHGAP21 and lane 5 with lane 13 and 21 for RHOQ in Figure 3.46 C).**

To further corroborate these findings, we repeated the kd experiments **(Figure 3.47)** and performed a Klenow-mediated extension to measure the poly(A) tail through the ePAT assay.

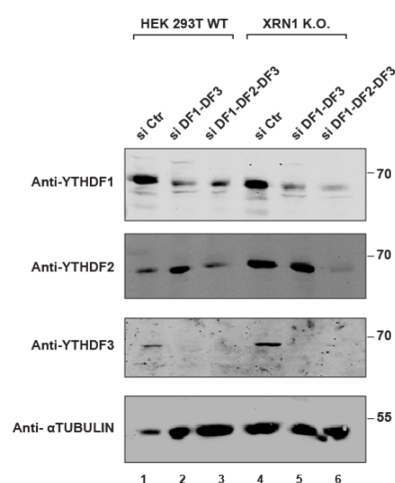


Figure 3.47: FLAM-Seq targets validation through ePAT. (A) Western blot showing siRNA-mediated depletion of YTHDF1, YTHDF2, and YTHDF3 in HEK 293T and XRN1 K.O. cells. A-TUBULIN served as a loading control.

Briefly, as shown in **Figure 3.48** in the first step of the ePAT, an oligo-(dT) stretch was annealed to the adenylated RNA **(Figure 3.48 a).**

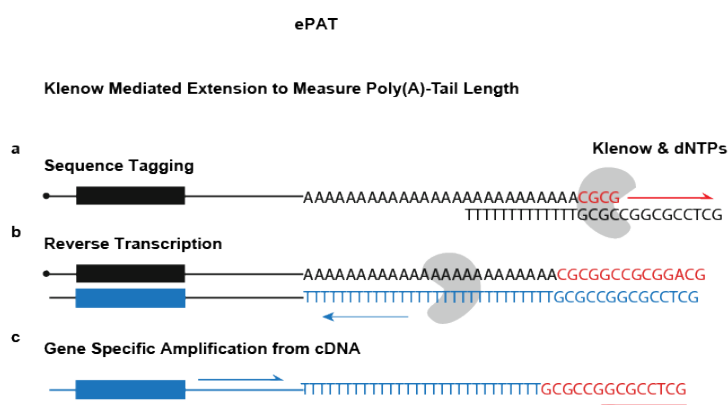


Figure 3.48: Schematic illustration of the ePAT assay. In the first step, a DNA oligo was annealed to the adenylated RNA via an oligo-(dT) stretch. The addition of Klenow polymerase and dNTPs to the reaction resulted in the extension of the template at the 3' terminus of the poly(A) tract to fill in the recessed end. The second step was performed at 55°C to ensure that the primer used for extension would remain annealed to end-extended molecules and not internal

poly(A) tracts. In the last step, a PCR amplification of cDNA using a gene-specific primer (blue arrow in c) and a universal reverse primer (red arrow in c) resulted in the amplification of the poly(A)-tails in the sample.

Afterwards, the Klenow polymerase and dNTPs were added to the reaction allowing a template extension of the 3' terminus of the poly(A) (**Figure 3.48 b**). The second step of the assay was performed at 55°C to make sure that only the primer used for extension remained annealed to end-extended molecules. For the last step, as for the poly(A) tail length assay, a PCR amplification of cDNA, using ^{32}P radioactively labeled nucleotides, was performed. A gene-specific primer and a universal reverse primer annealed to the nucleotides added by the Klenow polymerase (**Figure 3.48 c**). As expected, and previously confirmed by poly(A) tail length assay and FLAM-Seq, we managed to validate with the ePAT assay most of the poly(A) tail length of some of the most variant transcripts showing a decrease in poly(A) tail length in the triple knockdown of YTHDF1-2-3 (**Figure 3.49, compare lanes 1 with lanes 2 and 3 of RHOQ, CHKA, and HLA-DQB1**) compared to si CTR and si YTHDF1-3 conditions in HEK 293T WT cells.

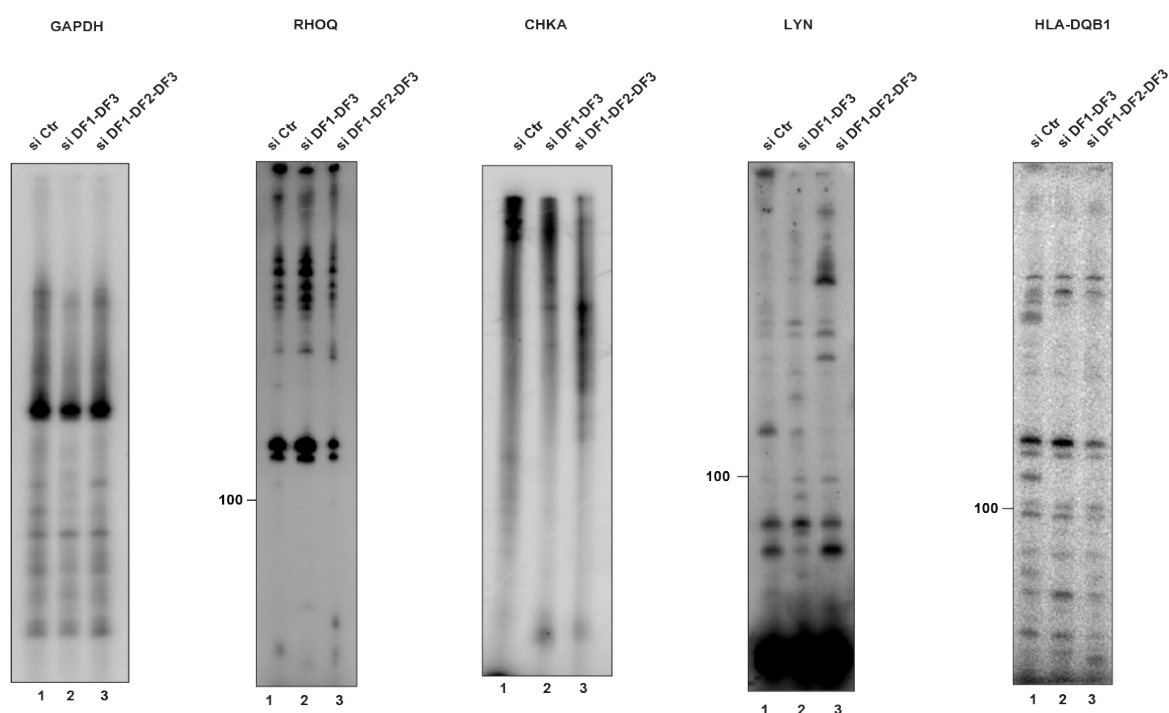


Figure 3.49: ePAT validation of FLAM-Seq targets. Poly(A) tail length measurement of GAPDH, RHOQ, CHKA, LYN, and HLA-DQB1 targets through ePAT assay in si CTR, siYTHDF1-3 (siDF1-DF3) and siYTHDF1-2-3 (siDF1-DF2-DF3) conditions. The PCR reaction (12.5 μl) was analyzed on 6% polyacrylamide gel with ^{32}P radioactive dCTP and dATP nucleotides.

Therefore, thanks to two different validation approaches, we could conclude that our sequencing results were experimentally reproducible. Finally, we were able to provide a very accurate dataset of steady-state poly(A) tail length measurements in HEK 293T cells depleted of YTHDF proteins.

3.5.6 Impact of poly(A) tail shortening on mRNA stability

To verify the impact of poly(A) shortening on transcript stability, we decided to measure the mRNA levels of some of the validated targets via qPCR. Therefore, we proceeded by depleting via siPOOLS YTHDF1-3 and YTHDF1-2-3 in HEK 293T cells. Then we extracted the RNA, synthesized the cDNA via oligod(T), and measured mRNA levels through qPCR. Although the standard deviation of the biological replicates indicated a high variability among the measurements, we noticed a tendency of the targets to increase their mRNA stability in the absence of YTHDF proteins (**Figure 3.50 A-B**).

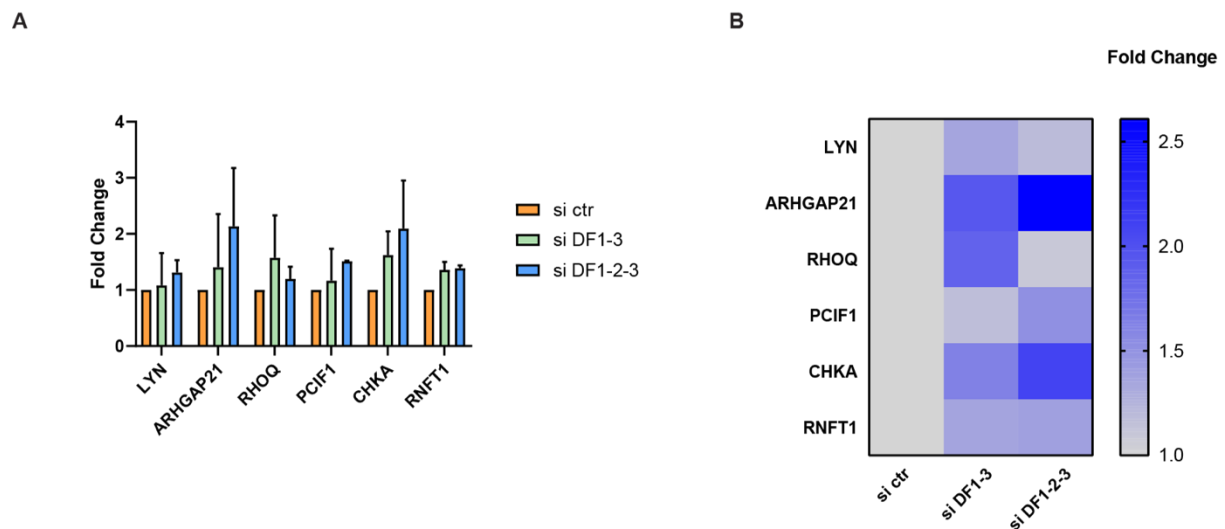


Figure 3.50: Impact of poly(A) tail shortening on mRNA stability. mRNA levels of validated targets which exhibited poly(A) tail shortening were tested through qPCR experiments. (A) Transcript levels of LYN, ARHGAP21, RHOQ, PCIF1, CHKA, RNFT1 were determined in si CTR, si YTHDF1-3 (si DF1-3), si YTHDF1-2-3 (si DF1-2-3) in HEK 293T WT cells. Transcript levels were normalized to GAPDH mRNA. Shown are the normalized expression ratios $\pm$ SD for three independent experiments. **(B)** Heatmap representation of qPCR experiments.

In conclusion, these data pointed to a general shortening of the poly(A) tail in the absence of the YTHDF proteins, which in turn causes a stabilization of the target mRNAs. Whether the shortening stops because the deadenylation by the CCR4-NOT complex cannot proceed further, remains to be established.

3.6 Poly(A) tail shortening is associated with mRNA stability

Next, we wanted to assess the regulatory role of the poly(A) tail by associating the differences in poly(A) tail length with the quantified transcript levels deriving from the FLAM-Seq counts. The counts represent the number of times a particular sequence (e.g., a gene) is observed in our sequencing experiment. To do this, we used the DESeq2 package in R, a widely used tool for differential gene expression. As first thing, we calculated with DESeq2 the size factor estimation to ensure that the counts were comparable across the different conditions, by adjusting the read

counts to the differences in sequencing depth or library across the samples. Then we calculated the mean-dispersion using the normalized counts, which reflected the biological and technical variability for each considered gene. We next applied a stabilizing transformation to the normalized counts by regularized logarithm (rlog) transformation. In this way, we stabilized the variance across genes in different samples by making the variance more similar for genes that show close mean expression levels. We then proceeded by modeling the relationship between the mean and the variance of gene expression counts following the negative binomial distribution. After that, we assumed that the mean and the variance were linked through a dispersion parameter. Then, based on the estimated dispersion values we performed a statistical test, usually DESeq2 uses a Wald test, to identify genes that were differentially expressed compared to the reference sample (si CTR in this case). To ensure the integrity of the analysis, we excluded sample C from the dataset as its inclusion did not yield any statistically significant p-value. At this step, from the p-value and log-fold change given for each gene obtained from replicate A and B, we were able to find all the significantly varied genes across the conditions. Finally, using ggplot2 and pheatmap packages in R, we were able to visualize our data with heatmaps and volcano plots. Pearson's correlation test was used to measure the degree to which two variables are linearly related.

3.6.1 Bulk RNA-Seq analysis from FLAM-Seq counts of YTHDF1-3, YTHDF1-2-3 depleted HEK 293T cells, showed a moderate level of mRNA stabilization for transcripts with short poly(A) tail

To begin, we wanted to gain more insight into YTHDF mRNA targets. Therefore, we investigated how the cellular transcriptome was affected in the absence of YTHDF1-3 and YTHDF1-2-3 proteins, compared to si CTR, and correlated the mRNA expression with the change in poly(A) tail. Thus, initially, after performing DESeq2 analysis, we generated a heatmap displaying the top fifty variant genes by comparing the transcriptomes of si CTR with those of siDF1-3 and siDF1-2-3.

It is essential to note that the most variant genes were those with the highest variability or dispersion across the samples, but their significance was determined separately through statistical testing. As shown in **Figure 3.51**, the reliability of the assay was confirmed since YTHDF2 was present among the most variant genes, and its mRNA expression was reduced exclusively in the siDF1-2-3 condition, while remaining mostly unchanged in siDF1-3 and si CTR.

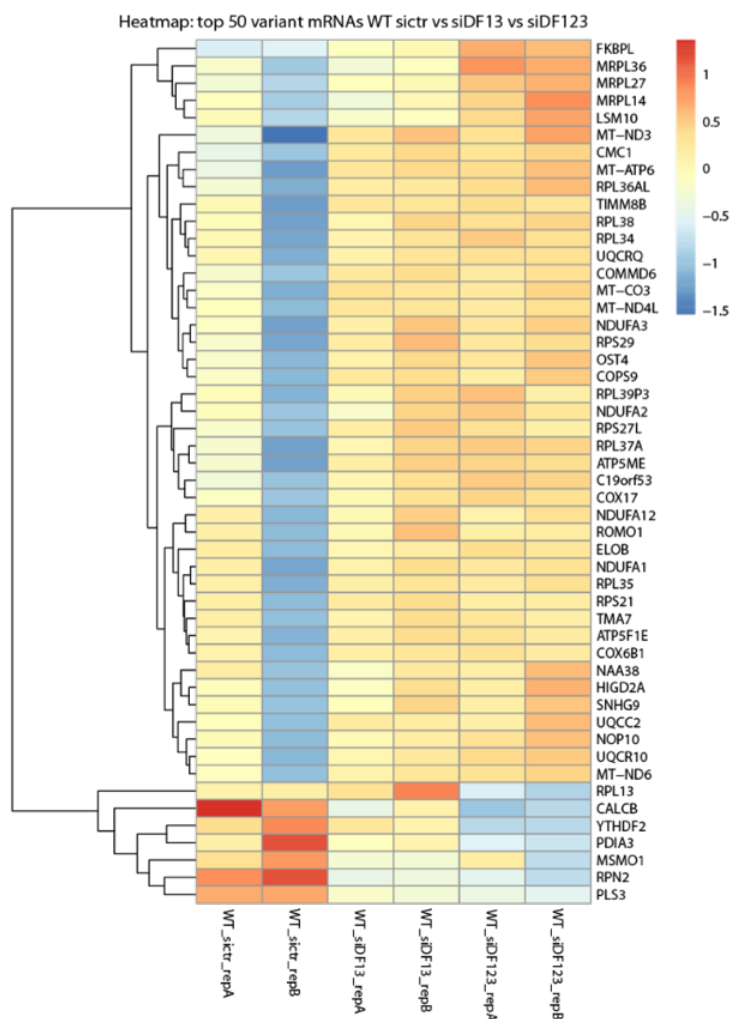


Figure 3.51: Identification of most variant mRNAs from FLAM-Seq read counts. Heatmap of most variant differentially expressed mRNAs in siYTHDF1-3 (siDF1-3) and siYTHDF1-2-3 (siDF1-2-3) relative to si CTR in HEK 293T cells.

Remarkably, since YTHDF proteins were previously suggested to exhibit redundancy (Lasman, Krupalnik et al. 2020) and, since YTHDF2 is the sole paralogue interacting with the CCR4-NOT complex, our observations revealed elevated mRNA expression levels for the majority of genes, when YTHDF2 was knocked down in addition to YTHDF1-3 (**Figure 3.51**). These findings further supported the notion that YTHDF2 might play a central role as the primary functional protein among the cytoplasmic YTHDF proteins. Unfortunately, due to the utilization of FLAM-Seq long reads analysis, only reads from the most abundant genes were accurately counted. Consequently, merely ~2% of the genes exhibited a p-value lower than 0.05 in the siDF1-3 and siDF1-2-3 conditions relative to si CTR WT cells. Nevertheless, using the ggplot2 package in R, we were able to plot all the significant differentially expressed genes and correlate their mRNA levels with the difference in tail length (**Figure 3.52 A and B**).

3.6.2 Bulk RNA-Seq analysis from FLAM-Seq counts of TNRC6ABC depleted HEK 293T cells, show a mild level of mRNA stabilization for transcripts with short poly(A) tail

We then investigated how the cellular transcriptome was affected in the absence of TNRC6ABC proteins compared to si CTR, and correlated the mRNA expression with the change in poly(A) tail. After performing DESeq2 analysis, we generated a heatmap displaying the top fifty variant genes by comparing the transcriptomes of si CTR with those of siTNRC6ABC. The reliability of the assay was confirmed since TNRC6A levels were present among the most variant genes and its mRNA expression was reduced exclusively in the triple knockdown conditions when compared to si CTR (Figure 3.53 A).

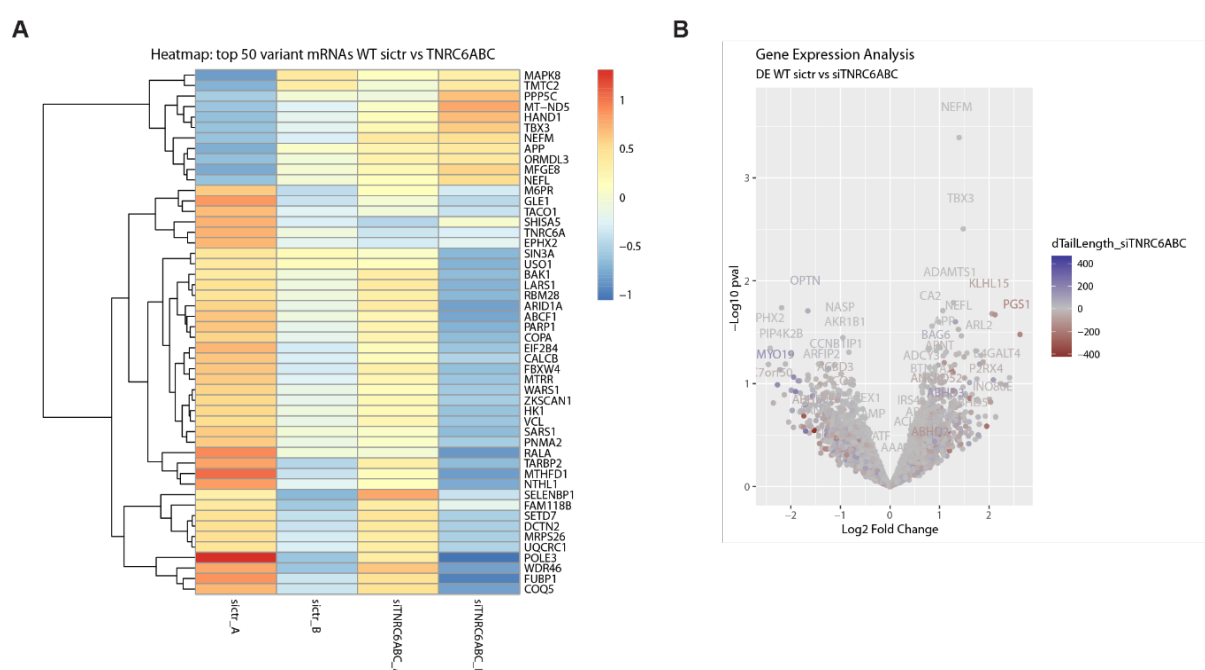


Figure 3.53: Identification of most variant mRNAs from FLAM-Seq read counts. (A) Heatmap of most variant differentially expressed mRNAs in si TNRC6ABC (siTNRC6ABC) relative to si CTR in HEK 293T cells. Gene expression analysis from FLAM-Seq counts. **(B)** Volcano plot depicting the differentially expressed genes in siTNRC6ABC condition relative to si CTR in HEK 293T cells. Red dots represent genes expressed with a shorter poly(A) tail length relative to si CTR, while blue dots represent genes with longer poly(A) tail length relative to si CTR. The Y-axis denotes $-\log_{10} P$ values adjusted while the X-axis shows $\log_2$ fold change values. The Volcano plot was generated using ggplot2 3.4.2 in R.

After calculating the differential expression with DESeq2 package in R, we identified ~50 genes which exhibited a p-value lower than 0.05 in the siTNRC6ABC conditions relative to si CTR WT cells. Naturally, we would have expected a higher number of variable genes, but the differences between the biological replicates did not allow us to have strong statistical power in the analysis. Nevertheless, using the ggplot2 package in R, we were able also in this case to plot all the significant differentially expressed genes, and correlate their mRNA levels with the difference in tail length (Figure 3.53 B). After examining the volcano plots, it became evident that few targets

showed significant enrichment in log2 fold change compared to si CTR, and these targets also exhibited a shortening of the poly(A) tail. However, even though to a lesser extent, we also noticed a shortening of the poly(A) tail for different groups of genes with decreased mRNA stability. This observation was in line with what was previously observed in the absence of YTHDF proteins. Therefore, a positive control was needed to shed light about the poly(A) tail shortening in the studied conditions.

3.6.3 Bulk RNA-Seq analysis from FLAM-Seq counts in HEK 293T XRN1 K.O. cells showed a clear correlation between poly(A) tail shortening and mRNA stability

Given the debatable association between mRNA levels, turnover, and translational efficiency with the poly(A) tail length, we sought to study the relationship between these features using a positive control like XRN1 K.O. cell line. As reported in a few recent publications, the median poly(A) tail length negatively correlated with gene expression, and typically, highly expressed targets displayed short poly(A) tail (SA Lima et al., 2017; Legnini et al., 2019; TJ Eisen et al., 2020) but, so far the effects of specific proteins depletion, like XRN1, on poly(A) tail length it has not been deeply explored. After retrieving the FLAM-Seq counts for XRN1 K.O. samples, we estimated the variance-mean dependence in count data and tested for differential expression using DESeq2. We further proceeded by generating a heatmap displaying the top fifty variant genes by comparing the transcriptomes of si CTR with those of XRN1 depleted HEK 293T cells and finally, we plotted all the significant differentially expressed genes, correlating their mRNA levels with the difference in poly(A) tail length (**Figure 3.54 A**).

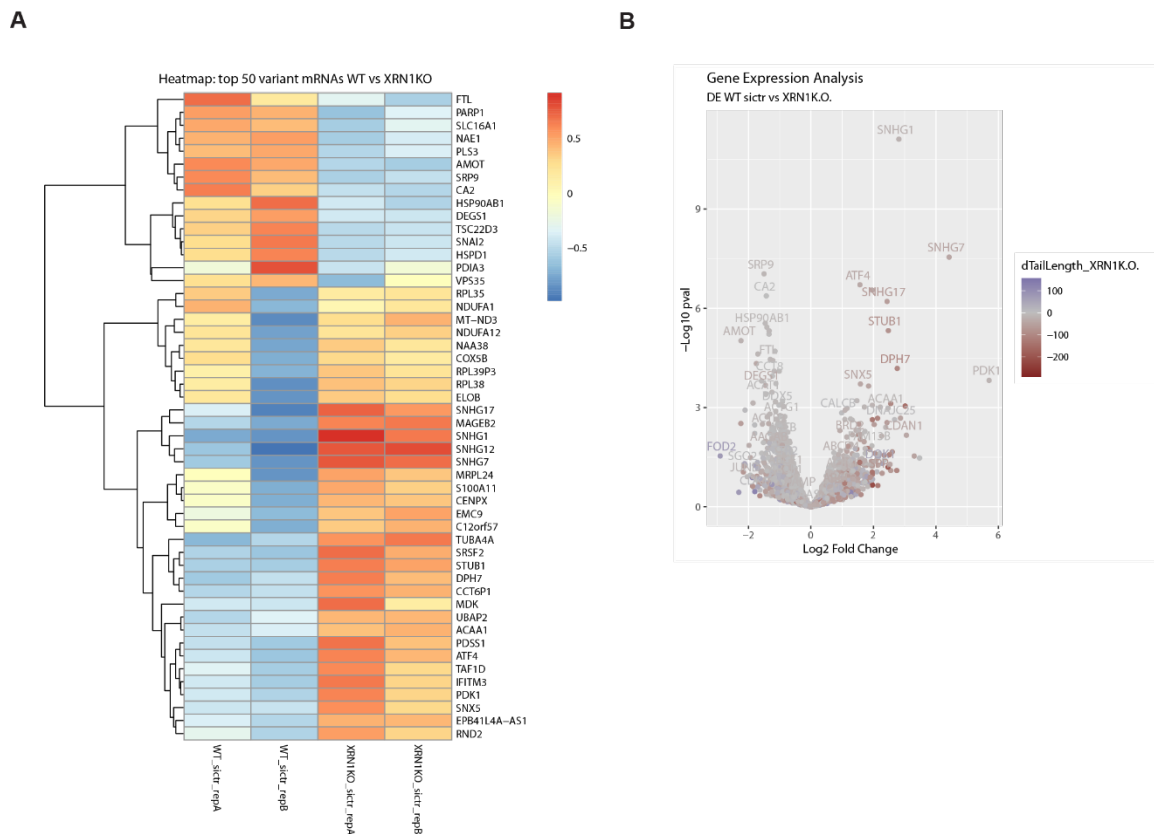


Figure 3.54: Identification of most variant mRNAs from FLAM-Seq read counts. (A) Heatmap of most variant differentially expressed mRNAs in XRN1 K.O. relative to si CTR in HEK 293T cells. Gene expression analysis from FLAM-Seq counts. **(B)** Volcano plot depicting the differentially expressed genes in XRN1 K.O. condition relative to si CTR in HEK 293T cells. Red dots represent genes expressed with a shorter poly(A) tail length relative to si CTR, while blue dots represent genes with longer poly(A) tail length relative to si CTR. The Y-axis denotes $-\log_{10}$ P values adjusted while the X-axis shows $\log_2$ fold change values. The Volcano plot was generated using ggplot2 3.4.2 in R.

As shown in the volcano plot in **Figure 3.54 B**, all the observed most variant genes showed an increase in mRNA expression associated with a shortening of poly(A) tail giving us for the first time a clear association between mRNA abundance and shortening of the poly(A) tail. Our result suggested that steady-state poly(A) tail length is a major determinant of mRNA stability for certain targets undergoing 5'-3' exoribonucleolytic RNA decay. This was also confirmed by Pearson's correlation test. We observed a strong negative correlation between the difference in tail length and increased mRNA stability in XRN1 K.O. condition relative to si CTR in HEK 293T WT if we considered only the statistically significant genes (**corr XRN1 K.O./si CTR = -0.4798904**), indicating that the shorter the poly(A) tail the higher the mRNA levels for these specific targets (**Figure 3.55**).

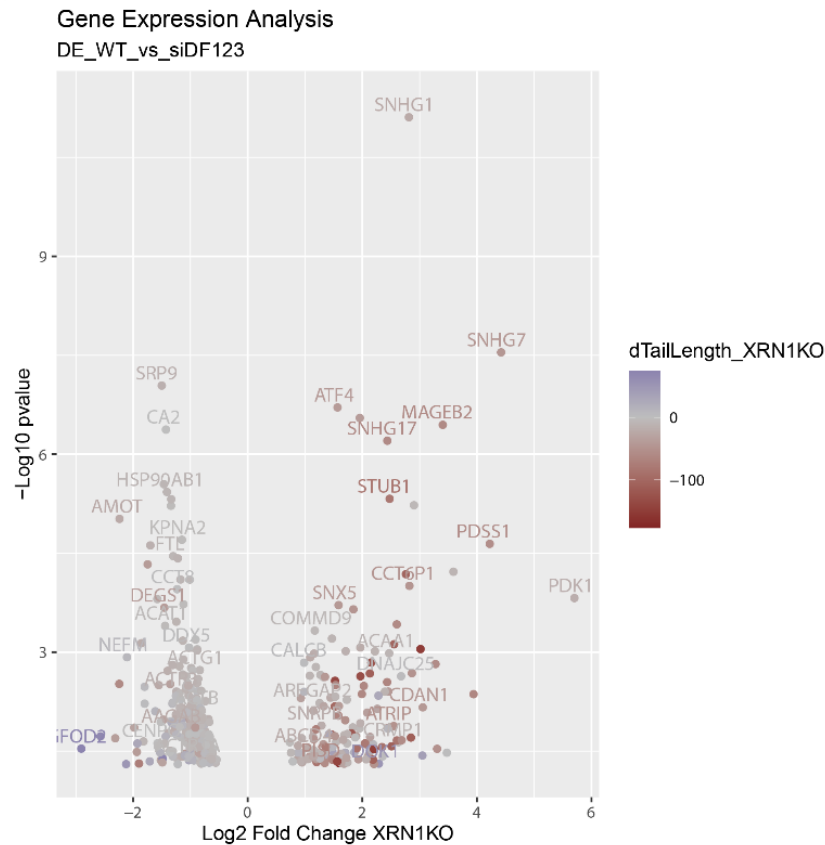


Figure 3.55: Identification of most variant mRNAs from FLAM-Seq read counts. Volcano plot depicting only the statistically significant and differentially expressed genes in XRN1 K.O. condition relative to si CTR in HEK 293T cells. Red dots represent genes expressed with a shorter poly(A) tail length relative to si CTR, while blue dots represent genes with longer poly(A) tail length relative to si CTR. The Y-axis denotes $-\log_{10}$ P values adjusted while the X-axis shows $\log_2$ fold change values. The Volcano plot was generated using ggplot2 3.4.2 in R.

3.6.4 RNA-Seq and Ribosome Profiling analysis of HEK 293T XRN1 K.O. cells, revealed increased mRNA stability and decreased translation efficiency for targets with short poly(A) tail

We then wondered how the mRNA expression and the translome changed in the absence of XRN1. To this end, we referred to the dataset of RNA-Seq and ribosome profiling (Ribo-Seq) already generated in the same XRN1 K.O. HEK 293T cells in use (Chang et al., 2019). Previous differential gene expression analysis revealed that 2906 mRNAs were significantly upregulated in the XRN1 K.O. cell line while 598 transcripts displayed significantly reduced translation efficiency (TE). Notably, just 102 transcripts were enhanced in their TE. By plotting the difference in poly(A) tail in relation to mRNA abundance (**Figure 3.56**), we observed that the majority of target mRNAs had a shorter poly(A) tail in XRN1 K.O. compared to si CTR, confirming what we've already noticed by analyzing the FLAM-Seq counts (**corr XRN1 K.O./si CTR = -0.1396963; Pearson's coefficient for only pvalue < 0.05 targets in XRN1 K.O./si CTR = -0.1608774**).

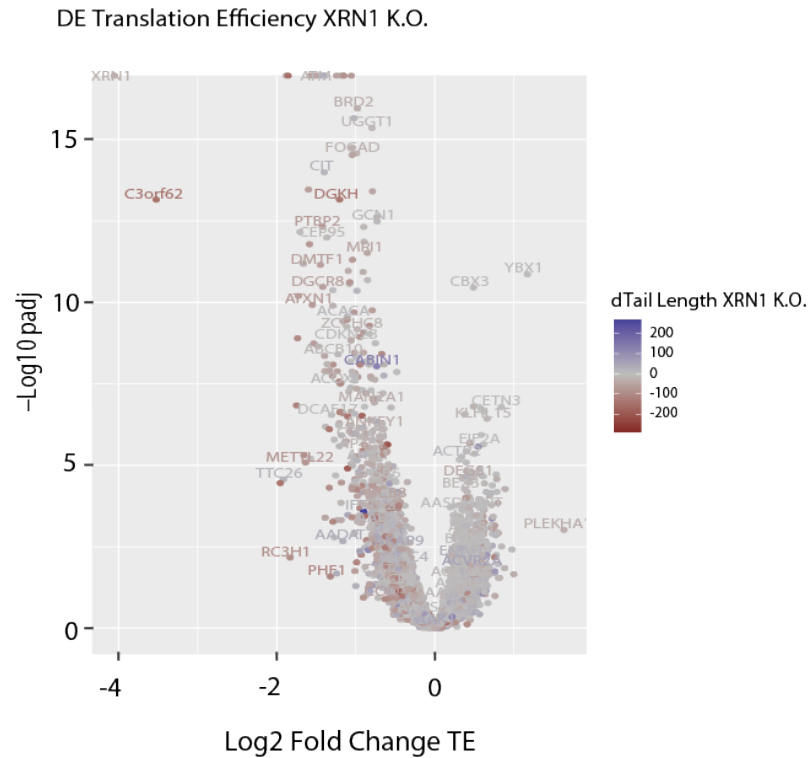


Figure 3.57: Comparative analysis of Translation Efficiency (TE) and poly(A) tail length in XRN1 K.O. HEK 293T cells. Volcano plot depicting the differentially expressed genes in XRN1 K.O. condition relative to si CTR in HEK 293T cells. Red dots represent genes expressed with a shorter poly(A) tail length relative to si CTR, while blue dots represent genes with longer poly(A) tail length relative to si CTR. The Y-axis denotes $-\log_{10}$ P values adjusted while the X-axis shows $\log_2$ fold change values in Translation Efficiency. The Volcano plot was generated using ggplot2 3.4.2 in R.

By plotting the difference in poly(A) tail in relation to TE, we observed that the majority of targets with reduced translation efficiency had also a shorter poly(A) tail in XRN1 K.O. compared to si CTR (**corr XRN1 K.O./si CTR = 0.1865449; Pearson's coefficient for only TE padj < 0.05 targets in XRN1 K.O./si CTR = 0.287161**). Gathering all the observations, we could finally state that mRNAs with reduced poly(A) tail were deadenylated, most likely decapped but could not be neither degraded nor translated in HEK 293T XRN1 K.O. cells. Additionally, it seemed quite obvious that without XRN1 the deadenylation process could not proceed further, highlighting how the 5'-3' decay is an orchestrated mechanism that needs all its components to function. To confirm and corroborate these sequencing findings, we decided to validate the most abundant targets by a biochemical approach. Therefore, we extracted the RNA from HEK 293T WT and XRN1 depleted cells, we synthesized cDNA via oligod(T) pairing and performed qPCR experiments. As shown in **(Figure 3.58 A)**, for all the targets having a decreased TE, we observed an increased mRNA stability compared to WT, confirming the RNA-Seq results.

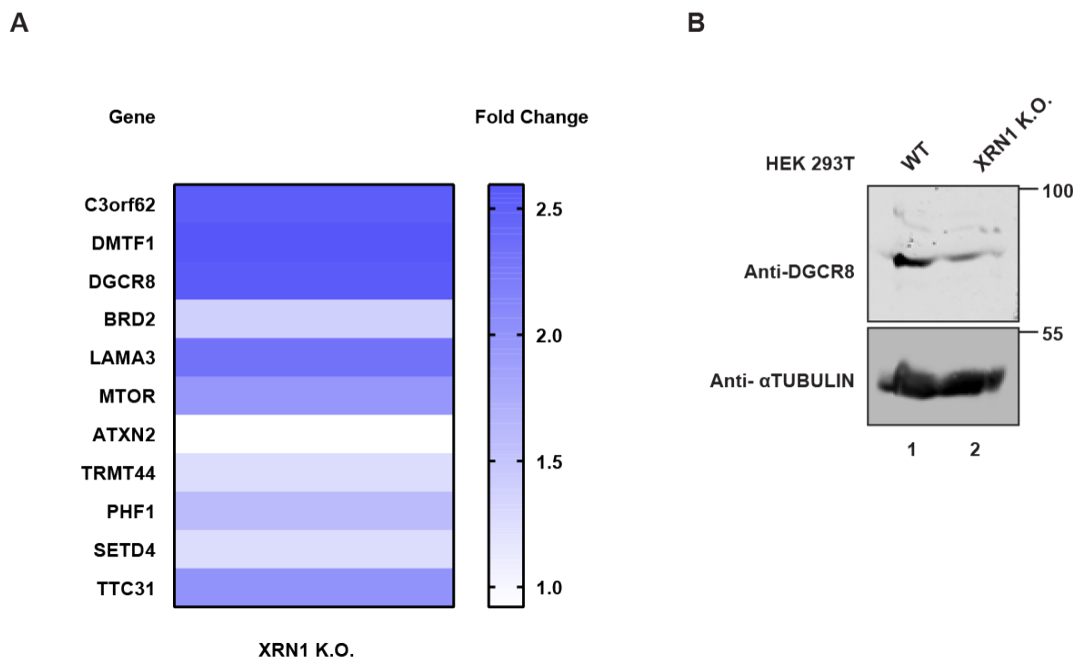


Figure 3.58: Comparative analysis of Translation Efficiency (TE) and poly(A) tail length in XRN1 K.O. HEK 293T cells. (A) Heatmap representation of qPCR experiments. **(B)** Western blot showing reduced protein levels of DGCR8 in HEK 293T XRN1 K.O. cells compared to HEK 293T WT cells. A-TUBULIN was used as a loading control.

Lastly, in order to verify the reduced translation levels, we lysed pellets deriving from HEK 293T WT and HEK 293T XRN1 K.O. cells with NP40 lysing buffer, loaded 40ug on a 10% SDS gel, and performed western blot experiments. After the detection of DGCR8 (**Figure 3.58 B**) protein levels, we were finally able to state that in XRN1 K.O., targets undergoing 5'-3' exoribonucleolytic RNA decay showed a shortening of poly(A) tail which induced increased mRNA stability and decreased translational expression.

3.6.5 Bulk RNA-Seq analysis from YTHDF1-3 and YTHDF1-2-3 depleted XRN1 K.O. cells showed a moderate correlation between poly(A) tail shortening and mRNA stability

Building upon the established link between mRNA abundance and shortened poly(A) tail length evident in the XRN1 K.O. dataset, our attention focused on the observation that depletion of YTHDF proteins in XRN1K.O. cells, yielded a further pronounced reduction in median poly(A) tail length. Therefore, after retrieving the FLAM-Seq counts, we estimated the variance-mean dependence in count data and tested for differential expression using DESeq2. We further proceeded by generating a heatmap displaying the top fifty variant genes by comparing the transcriptomes of XRN1 K.O. si CTR with those of depleted YTHDF1-3 and YTHDF1-2-3 in HEK 293T XRN1 K.O. cells. Finally, we plotted all the significant differentially expressed genes, correlating their mRNA levels with the difference in poly(A) tail length. The reliability of the assay was confirmed since YTHDF2 was present among the most variant genes, and its mRNA

expression was reduced exclusively in the XRN1 K.O. siDF1-2-3 condition, while remaining mostly unchanged in XRN1 K.O. siDF1-3 and XRN1 K.O. si CTR (**Figure 3.59**).

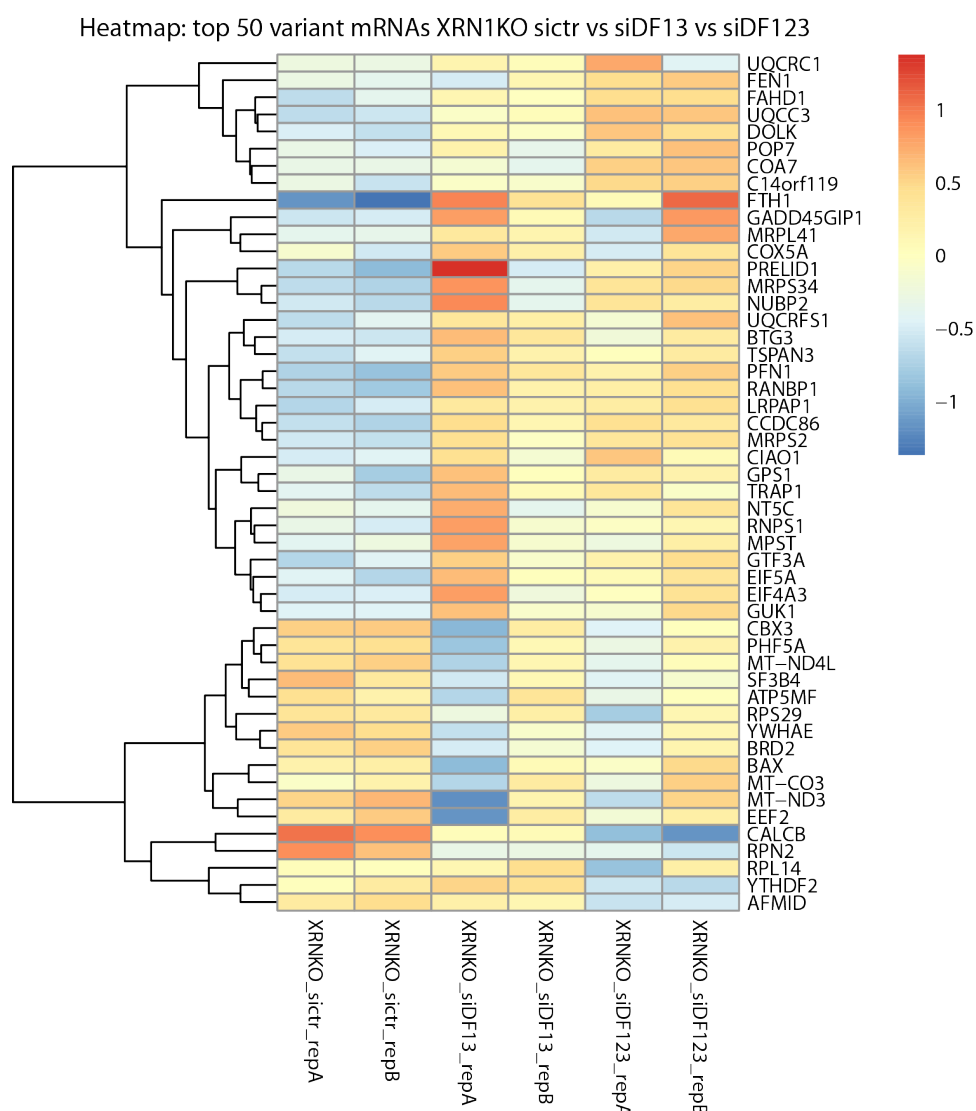


Figure 3.59: Identification of most variant mRNAs from FLAM-Seq read counts. Heatmap of most variant differentially expressed mRNAs in XRN1 K.O. depleted YTHDF1-3 (siDF1-3) and YTHDF1-2-3 (siDF1-2-3) relative to si CTR in HEK 293T XRN1 K.O. cells. Gene expression analysis from FLAM-Seq counts.

Remarkably, as observed of si DF1-3 and siDF1-2-3 in HEK 293T WT, we observed elevated mRNA expression levels for the majority of genes, when YTHDF2 was knocked down in addition to YTHDF1-3 (**Figure 3.59**). Moreover, we also observed for a small pool of genes a decrease in mRNA expression levels when YTHDF2 was knockdown in addition to YTHDF1 and YTHDF3. These findings support once again the notion that YTHDF2 might play a central role as the primary functional protein among the cytoplasmic YTHDF proteins.

We then explored the relationship between poly(A) tail shortening and mRNA abundance in the aforementioned conditions (**Figure 3.60 and Figure 3.61**).

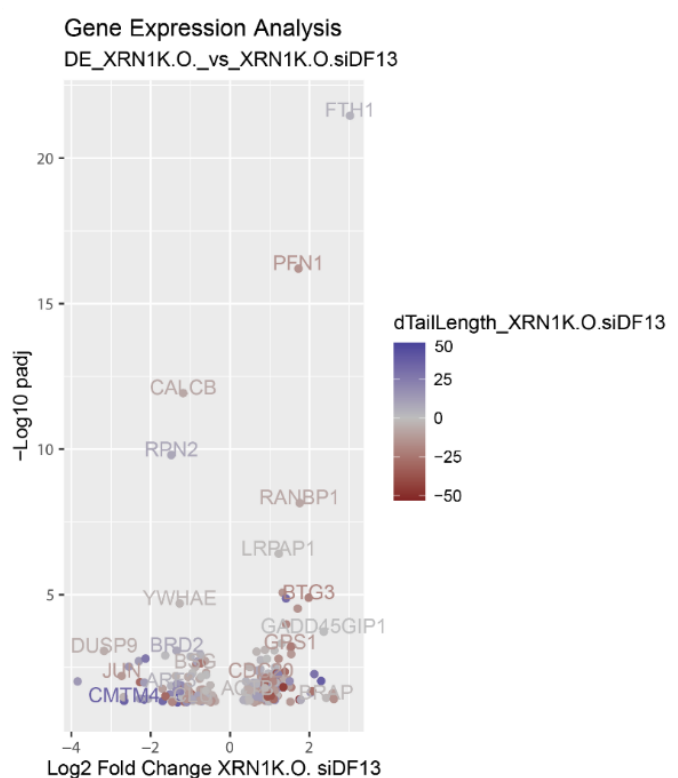


Figure 3.60: Volcano plot depicting the differentially expressed genes in XRN1 K.O. depleted YTHDF1-3 (siDF1-3) condition relative to si CTR in HEK 293T XRN1 K.O. cells. Red dots represent genes expressed with a shorter poly(A) tail length relative to XRN1 K.O. si CTR, while blue dots represent genes with longer poly(A) tail length relative to si CTR. The Y-axis denotes $-\log_{10}$ P values adjusted while the X-axis shows $\log_2$ fold change values. The Volcano plot was generated using ggplot2 3.4.2 in R.

After examining the volcano plots, it became evident that a substantial number of targets showed significant enrichment in $\log_2$ fold change in siDF1-3 and siDF1-2-3 conditions compared to XRN1 K.O. si CTR, and these targets also exhibited a shortening of the poly(A) tail.

This was also confirmed by Pearson's correlation test. We observed a slightly to moderate negative correlation between the difference in tail length and increased mRNA stability for the statistically significant genes that change the expression in siDF1-3 and siDF1-2-3 conditions (**corr siDF1-3/si CTR = -0.1890168; corr siDF123/si CTR = -0.3019524**). However, we also noticed a minor correlation for a distinct group of genes, where the poly(A) tail shortening was observed despite no significant enrichment in mRNA levels or even a decrease in mRNA stability.

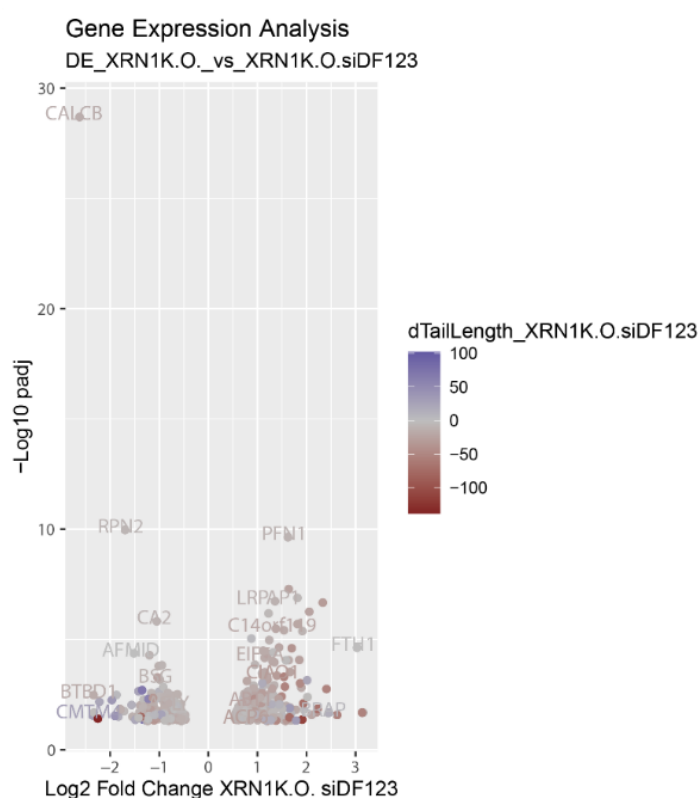


Figure 3.61: Volcano plot depicting the differentially expressed genes in XRN1 K.O. depleted YTHDF1-2-3 (siDF1-2-3) condition relative to si CTR in HEK 293T XRN1 K.O. cells. Red dots represent genes expressed with a shorter poly(A) tail length relative to XRN1 K.O. si CTR, while blue dots represent genes with longer poly(A) tail length relative to si CTR. The Y-axis denotes $-\log_{10}$ P values adjusted while the X-axis shows $\log_2$ fold change values. The Volcano plot was generated using ggplot2 3.4.2 in R.

Taken together, these results pointed out to an additional role of YTHDF proteins in the shortening of the poly(A) tail, with YTHDF2 playing a major role. We could speculate that YTHDF proteins might be involved in the fine tuning of the deadenylation process since in their absence, in XRN1 K.O. cells, the poly(A) tail of specific targets gets reduced further. Whether these effects are due to secondary effects caused by the absence of the YTHDF proteins still remains to be established.

4 Discussion

AGO proteins are known for their interactions with several protein partners, playing a pivotal role within miRNA-mediated silencing pathways. These crucial functions are orchestrated by at least two distinct functional complexes: the RISC-loading complex (RLC) and the miRNA-induced silencing complex (miRISC). In the RLC, which consists of AGO2, DICER, and TRBP, AGO gets loaded with miRNA. This loading process is assisted by auxiliary factors like HSC70/HSP90 chaperone proteins (Pare JM et al., 2009; Johnston et al. 2010). Conversely, the miRISC complex incorporates TNRC6A-B-C proteins (also referred to as GW182 proteins), which orchestrate the posttranscriptional repression of target mRNAs (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011). The TNRC6 proteins are the core components of the miRISC complex and serve as key scaffold proteins for inducing the shortening of the poly(A) tail through the recruitment of the PAN2-PAN3 and the CCR4-NOT deadenylase complexes, which in turn leads to translational repression and subsequent mRNA destabilization. Within various cell types, prior investigations have unveiled a multitude of other AGO protein partners (Meister et al., 2005; Höck et al. 2007; Landthaler et al., 2008; Frohn et al., 2012; Li et al., 2014; Schopp et al., 2017) but few have been fully characterized. Furthermore, the fate of an mRNA upon binding of different proteins remains unclear. Here, we reveal new insights into the miRNA-mediated mRNA decay. By deeply characterizing different RBPs like YTHDF1-2-3, as well as TNRC6A-B-C, and their regulatory effects on AGO2, our results extended the network of possible interactions around miRNA-mediated mRNA decay by revealing YTHDF silencing activities in additional pathways alongside AGO2. A potential mechanism for cross-regulation among these pathways could arise from their common reliance on the CCR4-NOT complex. This shared dependency might enable AGO proteins to modulate the activities of other RNA-silencing pathways by sequestering the deadenylation machinery and thereby facilitating targeted degradation of specific mRNA targets, as shown for UPF1 and TRIM71 (Welte et al., 2023). Additionally, both TNRC6 and YTHDF proteins as well as components of the CCR4-NOT complex are largely unstructured and might be responsible for connecting and coordinating gene regulatory pathways or even the formation of biomolecular condensates such as cytoplasmic granules and P-bodies.

4.1 Different way of CCR4-NOT complex recruitment by AGO2 and YTHDF2

After ruling out a possible direct YTHDF2 involvement in miRNA-mediated mRNA decay through IP experiments that demonstrated no YTHDF2 binding to AGO2 (**Figure 3.9**) and TNRC6 proteins (data not shown), our focus shifted to a comparative analysis of the mechanisms of CCR4-NOT complex recruitment. We know that AGO2 relies on auxiliary proteins such as TNRC6, whereas YTHDF2 contacts directly the CCR4-NOT complex (Figure 3.8). Human AGO2 is formed by four

distinct domains: the amino-terminal domain, the PIWI-AGO-ZWILLE (PAZ) domain, the MID domain, and the PIWI domain. Human AGO2 accommodates the W-binding pockets, which represent binding sites for the TNRC6 proteins, on the surface of its PIWI domain at the opposite site of the miRNA-binding surface (**Figure 4.1**).

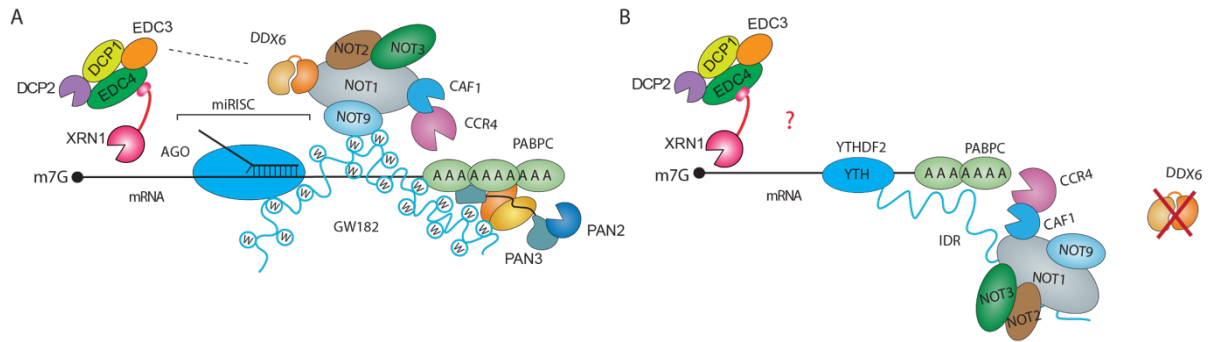


Figure 4.1: Comparison between AGO2- (A) and YTHDF2- (B) mediated mRNA decay. (A) a miRNA is loaded into an AGO protein and guides it to its target mRNA. A TNRC6 protein is then recruited and favors the binding of further downstream factors like the PAN2-PAN3 and CCR4-NOT deadenylase complexes as well as the PABPC proteins. For degradation of the target, the mRNA is first deadenylated by the PAN2-PAN3 and the CCR4-NOT complexes and then decapped by the decapping complex. The decapped mRNA is then subjected to 5' to 3' decay by the exonuclease XRN1 which is connected to the decapping enhancer EDC4 via a short linear motif in its very C-terminal end (modified from (Jonas and Izaurralde 2015)). (B) YTHDF2 selectively bind to m6A-modified RNA and regulate RNA decay in a methylation-dependent manner by recruiting the CCR4-NOT complex through its NOT1 subunit. Unlike AGO2-mediated mRNA decay, DDX6 is not needed for YTHDF2-mediated mRNA degradation. In addition, only a small pool of YTHDF2 targets seem to be subjected to 5' to 3' decay via XRN1.

The configuration of the AGO2 W-binding pockets is designed to fit successive W residues, as long as they maintain a minimum separation of 8 to 10 residues (equivalent to 20 to 25 Å) between them. In this way, the coordinate and consecutive binding of proximal W residues contributes to the affinity of the interaction. In addition, at least two W repeats are required by the NOT9 subunit and the CNOT9 binding domain of NOT1, for the CCR4-NOT complex to interact with TNRC6 proteins (**Figure 4.2 A** and **Figure 4.3 A**). Additionally, NOT1 mutants which no longer interact with NOT9, are still capable of binding to GW182 proteins, indicating that there are further anchoring sites within the CCR4-NOT complex (Chen, Y. et al., 2014; Mathys, H. et al., 2014). Finally, TNRC6 proteins are also able to interact with the cytoplasmic poly(A) binding protein PABPC (Fabian et al., 2009; Huntzinger et al., 2013). PABPC consists of four N-terminal RRM repeats, an unstructured proline-rich linker and a conserved C-terminal domain called MLLE, which interacts with the C-terminal part of TNRC6 proteins called PABP-interacting motif 2 (PAM2) (Jinek et al., 2010). Mutations at the binding interface impair mRNA deadenylation in mammalian cell extracts, showing that these anchoring points are essential for miRNA-mediated

gene silencing. Lastly, TNRC6 proteins also recruit the PAN2-PAN3 deadenylase complex through a tryptophan-binding pocket at the dimer interface of PAN3. Since PAN2-PAN3 complex recognizes the PABPC1 oligomerization interface, it mainly acts on long poly(A) tails RNA suggesting that its affinity progressively decreases with the trimming of the poly (A) tail (Passmore and Collier 2022).

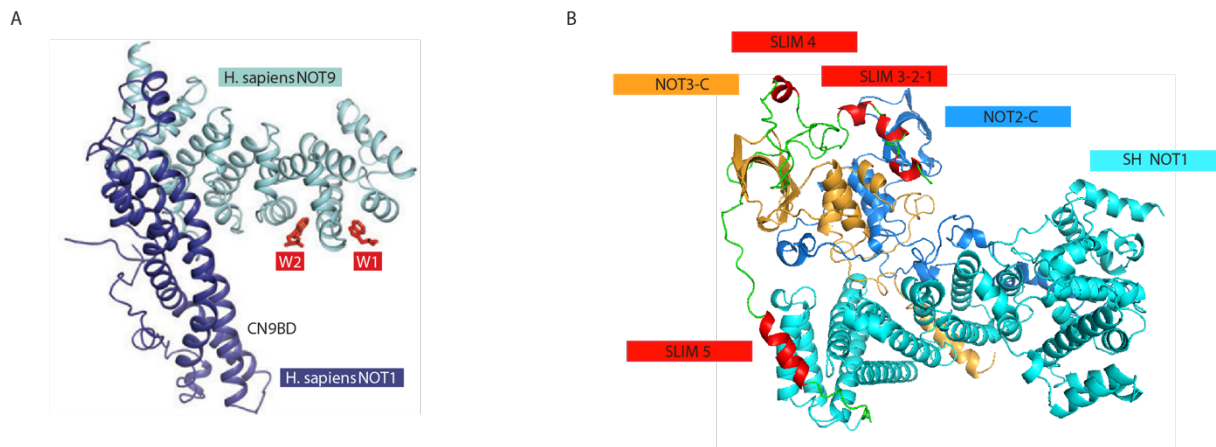


Figure 4.2: Comparison between the W-binding pockets of TNRC6 and the YTHDF2 SLiM5 binding to SH domain of NOT1. (A) Crystal structure of Hs CNOT9 binding domain (CN9BD) bound to the NOT9 subunit (PDB code: 4CRV) displays two binding pockets for tryptophan (W) on NOT9 subunit (Jonas S, Izaurralde E. 2016). **(B)** AlphaFold structural prediction of YTHDF2 SLiM5 and the SH domain of NOT1. Residues showing the pockets are represented as sticks and partially labelled for a better description.

YTHDF2 instead, is divided in a structured YTH domain at the C-terminus, which recognizes the m⁶A marked mRNAs, and a rather long IDR in its N-terminus domain (Li, F. et al., 2014). It has been previously shown that a specific 101-200aa region in the IDR of YTHDF2 interacts with the SH domain of NOT1 (Du et al., 2016) and, as we saw, it is also able to recruit PABPC, in an RNA dependent way. Whether this interaction is also direct and happens through a conserved residues present in the PAM2 motif remains to be established. Interestingly, recent findings revealed that *A. thaliana* YTH domain proteins, particularly ECT2, contain two short Intrinsically Disordered Region (IDR) elements. These IDR elements are formed by a 19-amino acids region, including a tyrosine-rich motif that is conserved in both plant and metazoan YTHDF proteins, which are necessary for binding to the major cytoplasmic poly(A)-binding proteins PAB2, PAB4, and PAB8 (Due Tankmar M et al., 2023).

After *in vitro* mapping the region of YTHDF2 that elicited repression and the modular contact on the CCR4-NOT complex, structural predictions allowed us to identify five different SLiM domains that directly bound not only the SH domain of NOT1 subunit, as thought, but the whole CNOT module of the CCR4-NOT deadenylase complex (**Figure 4.2 B and Figure 4.3 B**). Detailed *in vitro* pulldowns and structure analysis revealed that there are two main anchoring point: the SLiM

motifs 1-2-3, which lays at the interface of the NOT2-C-NOT3-C box domains, and the SLiM motif 5, which seems to be essential *in vitro* for the interaction with the SH domain of NOT1. Focusing more on the NOT1 anchoring, we identified, through structural prediction, the SLiM motif 5 as a main docking point for the interaction with the SH domain of NOT1.

In the specific, while the methionine 82 (M82) polarized sulfur atom engages in electrostatic interactions with Lysine's positive charge at position 34 (K34) and their hydrophobic regions contribute to van der Waals interactions, the Methionine 89 (M89) residues engages in strong hydrophobic interactions with the nonpolar portions of the Valine 40 (V40) side chains (**Figure 4.3 B**). Moreover, the hydrogen bonding between serine's hydroxyl group at position 37 (S37) of the SLiM 5 and isoleucine's carbonyl oxygen at position 85 (I85) on the SH domain, plus the hydrophobic interactions, make sure that YTHDF2 anchors itself to the CCR4-NOT deadenylase complex. In conclusion, it seemed clear, at least *in vitro*, that the cooperative binding of YTHDF2 relies on the presence of multiple SLiM motifs in its IDR, whereas TNRC6 proteins recruit the CCR4-NOT complex through a series of W residues. Mutation analysis followed by IP experiments will be essential to clarify if *in vivo* these residues are determinant for the CCR4-NOT complex recruitment.

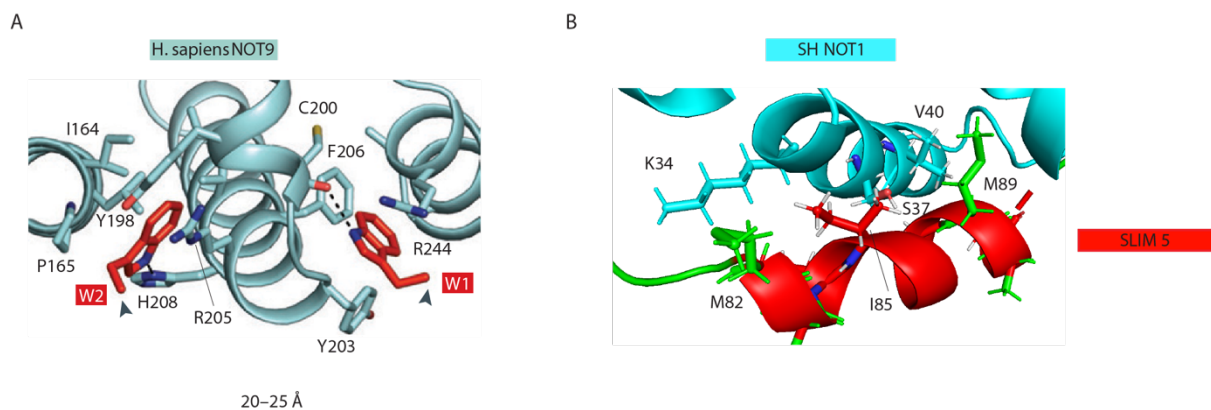


Figure 4.3: Close-up view of the W-binding pockets of TNRC6 and YTHDF2 SLiM5 binding to SH domain of NOT1 is shown. (A) Crystal structure of *Hs* CNOT9 binding domain (CN9BD) bound to the NOT9 subunit (PDB code: 4CRV) displays two binding pockets for tryptophan (W) on NOT9 subunit (Jonas S, Izaurralde E. 2016). **(B)** AlphaFold structural prediction of YTHDF2 SLiM5 and the SH domain of NOT1. Residues showing the pockets are represented as sticks and partially labelled for a better description. The minimal distance between the two W residues is indicated.

Furthermore, both TNRC6 and the *S. pombe* orthologue of YTHDF2, Mmi1, were shown to stimulate the activity of the deadenylases *in vitro*, thereby functioning as a deadenylase co-stimulator (Fabian et al., 2011, Stowell et al., 2016). In fact, TNRC6 proteins seem to stimulate the deadenylation process by using two distinct CCR4-NOT interaction motifs (CIMs) 1294-1300 (CIM-1) and 1647-1660 (CIM-2) which probably assist the deadenylase complex in folding event. On the other hand, Mmi1 seemed to accelerate the deadenylation in a sequence specific manner for determinant of selective removal (DSR) DSR-containing RNAs. This specialized removal

mechanism prevents the buildup of mRNAs specific to meiosis while the cell is undergoing mitosis in fission yeast (Yamashita A. et al., 2011). Also in this case, is tempting to speculate that the IDR of Mmi1 might help to stabilize the CCR4-NOT complex conformation but a clear structural characterization is needed. At the same time, how the human YTHDF2 would enhance the deadenylation process after recruiting the CCR4-NOT complex on m⁶A marked mRNA still have to be tested and clarified, as has to be clarified if also YTHDF proteins can recruit the PAN2-PAN3 complex since they also harbor key tryptophan residues that might mediate the binding to the PAN3 dimer interface.

4.2 Comparison between miRNA- and YTHDF2- mediated mRNA decay

Since the CCR4-NOT complex is crucial for miRNA- and YTHDF2-mediated mRNA decay, we aimed to compare the different efficiencies of the two pathways in mRNA degradation, as well as to investigate the potential sharing of upstream and downstream effectors. Through tethering assay experiments, we discovered that the recruitment of single YTHDF1-2-3 proteins to a reporter mRNA induced a certain level of mRNA degradation (~22% of the BGG-5BoxB reporter was degraded). However, the efficiency of degradation was less than half compared to AGO2 activity (~55% of the BGG-5BoxB reporter was degraded) (**Figure 3.12**). Furthermore, while the initiation of deadenylation by the CCR4-NOT complex upon AGO2 recruitment, along with subsequent mRNA degradation steps such as deadenylation, decapping, and XRN1-catalyzed 5'-to-3' mRNA degradation, is relatively well understood, little was known about the subsequent steps following the recruitment of the CCR4-NOT complex by YTHDF2. Therefore, another significant observation in this work is that through single depletions of components in the miRNA-mediated silencing pathway, such as TNRC6A-B-C, NOT6, NOT7, as well as DDX6, and XRN1 proteins, we were able to precisely pinpoint most of the steps involved in YTHDF2-mediated RNA degradation. First, we demonstrated that TNRC6 and YTHDF proteins do not compete with each other for CCR4-NOT complex recruitment, as their absence did not enhance the activity of YTHDF2-mediated and AGO2-mediated mRNA decay (**Figure 3.13, Figure 3.17**). Second, we found that while AGO2-mediated mRNA decay is at least partially dependent on NOT6, YTHDF2-mediated mRNA decay relies completely on the NOT7-NOT6 interaction (**Figure 3.15**).

In addition, our evaluation through terminator assays revealed that upon AGO2 and YTHDF2 tethering, the entire residual reporter mRNA retained the cap and was degraded with the same extent as the untreated samples. These data indicated that AGO2- and YTHDF2-mediated mRNA degradation most likely do not proceed through 3'-5' exonucleolytic degradation after deadenylation in HEK 293T cells (**Figure 3.18**). Tethering assay experiments in exosome component depleted context are necessary to validate these findings.

Third, we observed that the absence of DDX6 did not affect YTHDF2 activity in the tethering assay (**Figure 3.19**). DDX6 directly interacts with the NOT1 subunit of the CCR4-NOT complex and links deadenylation to decapping by binding to EDC3 upon AGO2 binding. It might be that YTHDF2 interacts with other untested decapping activators, like PATL1, LSM14,15, LSM1-7 for example, which might connect deadenylation and decapping for YTHDF2-mediated mRNA decay.

In addition, our data suggested that only a small subset of the BGG-5BoxB reporter undergoes 5'-3' decay upon tethering of YTHDF2, in contrast to the more prominent effect observed upon AGO2 tethering. This discrepancy hints at the possibility of YTHDF2 mediating mRNA degradation through a distinct pathway.

Lastly, we used a BGG-1BoxB-1MS2 reporter that allowed the recruitment of both single YTHDF2 and AGO2 molecules, to study a shared CCR4-NOT complex binding condition to the same reporter mRNA. Since no degradation was observed, we could conclude that the extent of reporter degradation is proportional to the number of B-box hairpins and MS2 sites present in the 3' UTR. This highlights the importance of having multiple binding sites to achieve a sufficient level of mRNA degradation for both YTHDF2 and AGO2 (**Figure 3.25**). Hence, a new reporter with multiple binding sites is needed to verify the interplay of AGO2 and YTHDF2 upon binding the same mRNA. Consequently, no synergistic outcome was observed.

In conclusion, variations in CCR4-NOT complex and TNRC6 recruitment could explain why different RBPs can have different effects on mRNA levels even if they exhibit similar effects on deadenylation. Therefore, exploring the differences between AGO2-mediated and YTHDF2-mediated mRNA decay, and characterizing the different associated proteins allowed us to better understand not only the contributions of YTHDF proteins but also the roles of all the other RBPs involved in mRNA metabolism. Whether YTHDF2 goes through endonucleolytic cleavage before or after deadenylation is still an open possibility, since it has been proposed that YTHDF2 might elicit m⁶A-mediated endonucleolytic mRNA cleavage via recruitment of the RNaseP/MRP complex through HRSP12 (Park, Ha et al. 2019). Ideally, tethering assay of YTHDF2 in XRN1 K.O. cells after depletion of POP1, the catalytic component of the RNaseP/MRP complex, would help us shed the light about the YTHDF2-mediated endonucleolytic pathway.

4.3 The role of YTHDF proteins on translation regulation

Because all three YTHDF proteins might redundantly mediate mRNA degradation, we considered the possibility that they may also regulate mRNA translation. Depletion studies of YTHDF proteins followed by polysome profiling analysis revealed that YTHDF1 and YTHDF3 absence not only caused a decrease in global translation, but also a potential accumulation of ribosomes in the translation initiation state (**Figure 3.28**). Surprisingly, the triple knockdown of YTHDF1-2-3 proteins, did not cause any significant effect on global translation. These findings highlight that

only YTHDF1 and YTHDF3 might work together to promote translation in HEK 293T cells, while the depletion of all three YTHDF paralogs possibly have a minor influence on translation-related processes. We would speculate that, for example, even though YTHDF proteins might act redundantly and together to enhance mRNA decay, there is some kind of specificity involving YTHDF2, which could have a defined and separate role compared to YTHDF1 and YTHDF3. Finally, it would be interesting to compare the effects on global translation caused by YTHDF protein depletion with the ones in absence of TNRC6 proteins. Such a comparison could provide valuable insights into the distinct impacts these two pathways have on mRNA metabolism and the interplay between mRNA translation, decay regulation, and overall cellular mRNA homeostasis.

4.4 YTHDF and TNRC6 proteins regulation

During contact inhibition of proliferation, cells sense their neighboring environments via cell-cell contacts to control cell growth and proliferation (Levine, Becker et al., 1965). This process is bypassed during embryonic development, tissue regeneration or wound healing, for example. Cancer cells also evolve ways of escaping such growth restrictions (McClatchey & Yap, 2012). Here, we found that the YTHDF and TNRC6 proteins are specifically degraded when cells reach high density and undergo contact inhibition (**Figure 3.29, Figure 3.30**). Moreover, we found that YTHDF proteins are downregulated during keratinocyte differentiation (data not shown), consistent with a previous report showing that YTHDF1 and YTHDF3 are downregulated upon cardiomyocyte differentiation (Wang, Zhang et al., 2021). In cancer cells, on the other hand, for both YTHDF and TNRC6 proteins (data not shown for TNRC6A-B-C) we did not observe reduced expression levels when cells reached high density (**Figure 3.29, Figure 3.30**). Since cancer cells circumvent contact inhibition, a common mechanism aiming at regulating YTHDF and TNRC6 proteins expression during cell growth restrictions is conceivable. Moreover, we demonstrated that this dependency did not rely on transcriptional activity or translational control (**Figure 3.31**). It was shown before that YTHDF2 is regulated during cell cycle progression by ubiquitination (Fei et al., 2020). This study showed that the proteolysis of YTHDF2 is mediated by Cullin 1 (CUL1), Cullin 4A (CUL4A), damaged DNA-binding protein 1 (DDB1), and S-phase kinase-associated protein 2 (SKP2) as part of the components of E3 ubiquitin ligase complexes. However, whether these complexes also contribute to the turnover of YTHDF1-2-3 during contact inhibition and whether TNRC6A-B-C are also regulated by the proteasome during the cell cycle, needs to be further investigated. Lastly, it is important to highlight that AGO2 protein expression did not change upon contact inhibition, hinting that mainly proteins containing IDRs might be regulated by the proteasome and that AGO2 is regulated in a different way.

4.5 YTHDF proteins depletion causes a shortening of the poly(A) tail and stabilization of specific target mRNAs

To observe the dynamics of endogenous mRNA tail lengths, we conducted FLAM-Seq analysis (Legnini, Alles et al. 2019) under steady-state conditions using three independently passaged HEK 293T WT and HEK 293T XRN1 K.O. cell lines where YTHDF1-3 and YTHDF1-2-3 proteins were depleted, and two independently passaged HEK 293T cell lines where TNRC6A-B-C proteins were efficiently knocked down (**Figure 3.33**). Given that FLAM-Seq employs oligo(dT) capture, it exhibits a 3'-directional bias. Moreover, due to long-read sequencing, our results might have overrepresented highly abundant genes, limiting the transcriptome analysis to a subset of transcripts. Nonetheless, the analysis of median poly(A) tail lengths for numerous individual transcripts revealed a reduction in poly(A) tail length under steady-state conditions when YTHDF proteins were absent, with a more pronounced decrease in the triple YTHDF1-2-3 proteins knockdown compared to YTHDF1-3 knockdown in HEK 293T WT and in HEK XRN1 K.O. cells (**Figure 3.37**). Surprisingly, the depletion of TNRC6 proteins led to almost no difference in global median poly(A) tail length (**Figure 3.39**). By inhibiting CCR4-NOT complex recruitment, we expected that the median poly(A) tail of target mRNAs would be longer in both YTHDF1-2-3 and TNRC6A-B-C depletion.

Since it has been recently proved that the degradation rate of transcripts with short tails prevents the steady accumulation of such mRNAs, we then verified the distinctive poly(A) distributions per gene with poly(A) tail lower than 150 nt. Depletion of YTHDF proteins led to a global poly(A) shortening independently of the length of the poly(A) tail (**Figure 3.40, Figure 3.41**).

In the case of TNRC6 depletion, we observed an accumulation of transcripts with longer poly(A) tail compared to the control (**Figure 3.42**) which is what we would have expected. On the other hand, for targets with median poly(A) tail longer than 150 nt we noticed a global median poly(A) tail shortening upon TNRC6 proteins depletion (**Figure 3.43**).

To interpret these results, we have to consider that miRNAs cause not only an acceleration of deadenylation but also a faster degradation of short poly(A) tailed mRNAs, hence, it might be that only the transcripts that are undergoing miRNA-mediated mRNA degradation, and then with decreased poly(A) tail, can be affected by TNRC6 proteins depletion. At the same time, it is also conceivable that what we measured is not the real representation of what happens after depleting TNRC6 proteins since we have only end points measurements.

Collectively, these findings were unexpected, as we initially anticipated an increase in poly(A) tail length in the absence of CCR4-NOT recruitment. To comprehend this unusual outcome, we must consider though the broader mRNA decay process in the cell. It is known that the relationship between deadenylation rate and decay operates at two levels: first, mRNAs with higher

deadenylation rates quickly reach a minimal length that is no longer able to accommodate PABPC proteins, thus marking them for decay.

Secondly, mRNAs that have already undergone rapid deadenylation, decay faster upon reaching this minimal tail length. This process is crucial to ensure rapid degradation of non-essential mRNAs. Moreover, alongside deadenylation, various factors contribute to mRNA decay, working synergistically to ensure efficient clearance (Eisen et al., 2020). Among these factors, we have decapping enhancers, the decapping complex, uridylyases, m⁶A modification and RBPs, which coordinate and localize mRNA molecules to facilitate effective degradation (Jonas and Izaurralde 2015, Ozgur, Basquin et al. 2015).

Now considering that microRNAs, RBPs and other features like m⁶A binding proteins help recruit and enhance the CCR4-NOT deadenylation complex, the shortening of the poly(A) tail until a certain point in a YTHDF1-2-3 depleted context, can be seen as if only the initial step of deadenylation gets carried out. In this context, the lack of YTHDF proteins appears to hinder the further progression of deadenylation, potentially due to impaired processivity of the CCR4-NOT complex. If this interpretation holds true, it suggests that YTHDF proteins, specifically YTHDF1-2-3, are involved in either facilitating or inhibiting the full process of deadenylation and processivity of the CCR4-NOT complex. In fact, also XRN1 impairs the CCR4-NOT complex activity via the low-complexity 'C-terminal interacting region' (CIR) and block CAF1-dependent mRNA deadenylation (Chang, Muthukumar et al. 2019). This was also confirmed by the further shortening of the global poly(A) tail in YTHDF1-3 and YTHDF1-2-3 knockdown in XRN1 K.O. cells (**Figure 3.37**).

Regarding the relationship between poly(A) tail and mRNA stability, by focusing and comparing the mRNA expression in the context of double YTHDF1-3 and triple kd of YTHDF1-2-3 proteins, in HEK 293T WT and in HEK 293T XRN1 K.O. cells, with HEK 293T XRN1 K.O. cells, where we know that mRNAs get shorter poly(A) tail and exhibit an increased stability, we noticed a moderate negative correlation between shortening of the poly(A) tail and mRNA abundance. This observation was verified not only by differential expression analysis from the FLAM-Seq analysis read counts, but also from qPCR measurements of validated targets. Therefore, we could conclude that poly(A) shortened mRNAs tend to have an increase in mRNA stability in YTHDF depleted proteins conditions. Further experiments involving *in vitro* deadenylation assays, RNA-Seq and poly(A) tail profiling over time will shed the light on how YTHDF and TNRC6 proteins influence the CCR4-NOT complex activity, how these intricate pathways act together in order to ensure an efficient deadenylation process, and which are the specific targets that undergo these two different pathways.

5 Materials and Methods

5.1 Materials

5.1.1 Chemicals and enzymes

Unless stated differently, all chemicals used were purchased from Sigma-Aldrich (St. Louis, USA), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Thermo Fisher Scientific (Waltham, USA) and AppliChem GmbH (Darmstadt, Germany).

Cell culture reagents were purchased from Sigma-Aldrich (St. Louis, USA). Enzymes were obtained from Thermo Fisher Scientific (Waltham, USA). Radiochemicals were purchased from Hartmann Analytic GmbH (Braunschweig, Germany).

5.1.2 Instruments, software and algorithms

5.1.2.1 Instruments

All instruments used for the experiments are listed in Table 1

Table 1: List of the used instruments

Instrument	Company
2720 Thermal Cycler	Applied Biosystems (Foster City, USA)
ÄKTApurifier	GE Healthcare (Chicago, USA)
Avanti J-20 XP Centrifuge	Beckman Coulter (Krefeld, Germany)
Centrifuge 5415D	Eppendorf (Hamburg, Germany)
CFX96™Real-Time System	Bio-Rad (Hercules, USA)
Geiger Counter LB123 EG&G	Berthold (Bad Wildbad, Germany)
GeneAmp PCR System 9700	Applied Biosystems (Foster City, USA)
GSTrap™ HP Columns	Cytiva (Marlborough, Massachusetts, USA)
HeraCell 240i CO2 Incubator	Thermo Fisher Scientific (Waltham, USA)
HeraSafe KS	Thermo Fisher Scientific (Waltham, USA)
HisTrap HP Columns, 1 x 5 ml	Cytiva (Marlborough, Massachusetts, USA)
Hybridization oven T 5042	Heraeus (Hanau, Germany)
Illustra MicroSpin G-25 Columns	Cytiva (Marlborough, Massachusetts, USA)
Incubator Model B6200	Heraeus (Hanau, Germany)
Leica TCS SP8 MP	Leica Microsystems (Wetzlar, Germany)
LSM 710, AxioObserver microscope	Zeiss (Oberkochen, Germany)
Magnetic stirrer MR 300	Heidolph (Schwabach, Germany)
Megafuge 40R	Thermo Fisher Scientific (Waltham, USA)
Milli-Q PLUS	Millipore (Billerica, USA)
Model 583 Gel Dryer	Bio-Rad (Hercules, USA)
Nanodrop® spectrophotometer ND-100	Thermo Fisher Scientific (Waltham, USA)
Odyssey Infrared Imaging System	LI-COR Biosciences (Lincoln, USA)

peqSTAR Thermocycler	PeqLab (Erlangen, Germany)
Personal Molecular Imager (Phosphoimager)	Bio-Rad (Hercules, USA)
Polymax 2040	Heidolph (Schwabach, Germany)
Power Supply EV233	Consort (Turnhout, Belgium)
Quantum ST4	PeqLab (Erlangen, Germany)
Screen Eraser-K	Bio-Rad (Hercules, USA)
siFractor	siTOOLS (Munich, Germany)
Superdex® 200 10/300 GL Columns	Cytiva (Marlborough, Massachusetts, USA)
Thermomixer compact	Eppendorf (Hamburg, Germany)
Trans-Blot SD	Bio-Rad (Hercules, USA)
UV Stratalinker 2400	Stratagene (Santa Clara, USA)
Vortexer REAX top	Heidolph (Schwabach, Germany)
Wet-blot	Bio-Rad (Hercules, USA)

5.1.2.2 Software and algorithms

All instruments used for the experiments are listed in Table 2

Table 2: List of the used software and algorithms

Resource	Source	Identifier/Developer
Adobe Illustrator	CS5	Adobe
Adobe Photoshop	CS5	Adobe
Alphafold		DeepMind
BioMaRT	Version 2.28.0	Huber W
Biorender		BioRender.com
ChimeraX	Version ChimeraX-1.5	Thomas Ferrin
CHOPCHOP		Labun et al., 2016, 2019; Montague et al., 2014
Clustal Omega		EMBL-EBI
DESeq2	Version 3.16	Michael Love
DexSeq	Version 1.46.0	Simon Anders and Alejandro Reyes
EnrichR		Avi Ma'ayan
Ensembl		NCBI
ggplot2	Version 3.4.2	Hadley Wickham
Integrative Genomics Viewer (IGV)		Robinson et al., 2011; Thorvaldsdóttir et al., 2013
Jalview		University of Dundee
NCBI database		NCBI, RRID: SCR_006472
Odyssey	LI-COR	Odyssey CLx, RRID: SCR_014579
Pheatmap	Version 1.0.12	Raivo Kolde
PyMOL		PyMOL, RRID: SCR_000305
Quantity One Software	BioRad	Quantity One 1-D Analysis Software, RRID: SCR_014280

R	Version R 4.2. 0.	R Core Team, Ross Ihaka and Robert Gentleman
RCSB Protein Data Bank (PDB)	RCSB PDB), RRID: SCR_012820	RCSB Protein Data Bank (PDB)
RStudio	Version R 4.2. 0.	Posit, PBC
Treefam		EMBL-EBI
Tidyverse	Version 2.0.0	Hadley Wickham
UCSC Genome Browser		Jim Kent
Uniprot		UniProtKB, RRID: SCR_004426
Zen10 Microscope Software	Zeiss Zen 3.6 (Blue edition)	Zeiss

5.2 Kits and Solutions

5.2.1 Kits

The kits used for the experiments are listed in Table 3

Table 3: List of the used kits

Kit	Company
First Strand cDNA Synthesis Kit	Thermo Fisher Scientific (Waltham, USA)
Lipofectamine™ 2000	Thermo Fisher Scientific (Waltham, USA)
Lipofectamine™ RNAiMAX	Thermo Fisher Scientific (Waltham, USA)
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel (Düren)
NucleoSpin® Plasmid	Macherey-Nagel (Düren)
NucleoSpin® RNA	Macherey-Nagel (Düren)
NucleoSpin® Xtra Midi	Macherey-Nagel (Düren)
NucleoSpin®-Extract	Macherey-Nagel (Düren)
Passive Lysis 5X Buffer	Promega Corporation (Fitchburg, USA)
pGEM®-T Easy Vector System	Promega Corporation
Phusion™ High-Fidelity DNA Polymerase	Thermo Fisher Scientific (Waltham, USA)
Roti®-Phenol/Chloroform/Isoamyl alcohol (25:24:1), pH 4.5-5	Carl Roth GmbH + Co. KG
Roti®-Quant (5X)	Carl Roth GmbH + Co. KG
SequaGel® UreaGel™ System	National Diagnostics Inc., (Atlanta, USA)
SsoFast™ EvaGreen® Supermix	Bio-Rad Laboratories Inc. (Hercules, USA)
SuperScript™ III First Strand Synthesis Super Mix	Thermo Fisher Scientific (Waltham, USA)
TRIzol Reagent	Thermo Fisher Scientific (Waltham, USA)

5.2.2 Solutions and buffers

The used solutions and buffers are listed in Table 4

Table 4: List of the used solutions and buffers

Buffer or solution	Compounds
10x native Tris-borat buffer (TBE)	450 mM Tris base, 450 mM boric acid
2x Hepes	274 mM NaCl, 54.6 mM Hepes, 1.5 mM Na ₂ HPO ₄
2x RNA Loading dye denaturing gel (short RNAs)	90% (v/v) Formamide, 0,025% (w/v) Xylencyanol, 0,025% (w/v) Bromphenolblue, in 1 x TBE
50x Denhardtts solution	1% Albumin Fraction V, 1% Polyvenylpyrrolidon K30, 1% Ficoll 400
5x SDS loading dye	250 mM Tris pH 6.8, 50 % Glycerol, 10 % SDS, 0.01 % Bromophenol Blue, 250 mM DTT
Blue Juice	30 % Glycerol, 0.05% Bromphenol Blue
Coomassie destaining solution	10 % Acetic acid, 30 % EtOH
Coomassie staining solution	10 % NaOAc, 30 % EtOH, 0.25 % Coomassie brilliant blue
dNTPs radioactive PCR mix	dTTP 100 mM, dGTP 100 mM, dATP 10 mM, dCTP 10 mM
EMSA sample buffer	10 % glycerol, 20 mM Tris pH 7.5, 100 mM NaCl, 5 mM MgCl
Gel filtration buffer	50 mM Hepes pH 7.4, 2 mM DTT, 200 mM NaCl
GST-Trap Buffer 1	1X PBS, 1M NaCl
GST-Trap Buffer 2	20mM (GSH(L)) Glutathione, 50mM Tris pH8, 1X PBS
Hybridization solution	5xSSC, 20 mM NaPi, pH 7.2, 7 % SDS, 1 x Denhardtts solution
His-Trap Solution A	50 mM Natriumphosphate buffer pH 8.0, 300 mM NaCl, 10 mM Imidazol
His-Trap Solution B	50 mM Natriumphosphate buffer pH 8.0, 300 mM NaCl, 500 mM Imidazol
MOPS buffer (10x)	20 mM MOPS, 2 mM NaAc, 1 mM EDTA
MOPS RNA running buffer (1x)	1x MOPS buffer, 2% Formaldehyde
Northern blot hybridization solution	5 x SSC, 20 mM NaPi pH 7.2, 7 % SDS, 1x Denhardtts Solution
Northern blot wash solution 1	5xSSC, 1% SDS
Northern blot wash solution 2	2xSSC, 1% SDS
Northern blot wash solution 3	2x SSC, 0.1% SDS
Northern blot wash solution 4	0.5x SSC, 0.1% SDS
Northern blot wash solution 5	0.1x SSC, 0.1% SDS
NP-40 lysis buffer	150 mM KCl, 25 mM Tris pH 7.5, 2 mM EDTA, 1 mM NaF, 0.5 % NP-40, 1mM DTT, 1mM AEBSF
NP-40 wash buffer	300 mM NaCl, 50 mM Tris pH 7.5, 1 mM NaF, 0.01 % NP-40, 5 mM MgCl ₂
RNA Loading buffer (2x) for long RNAs	45 % Formamide, 1x MOPS, 5.92 % Formaldehyde, 20 % Blue Juice
Saline sodium citrate buffer (20x SSC)	3 M NaCl, 0.3 M NaCitrate pH 7.0
SDS running buffer	200 mM Glycine, 25 mM Tris pH 7.5, 25 mM SDS
TBS	10 mM Tris pH 7.5, 150 mM NaCl

TBS-T	10 mM Tris pH 7.5, 150 mM NaCl, 0.05 % Tween 20
Towbin buffer	38.6 mM Glycine, 48 mM Tris, 0.0037 % SDS, 20 % Methanol
Tris/Borate/EDTA buffer (1x TBE)	89 mM Tris, 89 mM Boric acid, 2 mM EDTA

5.3 Consumables

All consumables used for the experiments are listed in Table 5

Table 5: List of consumables

Consumable	Company
Amersham Hybond ECL Membrane	GE Healthcare (Buckinghamshire, UK)
Amersham Hybond-N Membrane	GE Healthcare (Buckinghamshire, UK)
CAWO X-Ray Cassette	CAWO (Schrobenhausen, Germany)
Molecular Imager FX Imaging Screen-K	Bio-Rad (Hercules, USA)
Plastic Pipettes, Pipet tips and reaction tubes	Sarstedt (Nuembrecht, Germany)
Reaction tubes (1.5 ml and 2 ml)	Eppendorf (Hamburg, Germany)
Tubes (15 mL and 50 mL)	Sarstedt (Nuembrecht, Germany)
Tissue culture dishes	Sarstedt (Nuembrecht)
Whatman® Cellulose Filter Paper	GE Healthcare (Buckinghamshire, UK)
X-Ray Cassette	CAWO (Schrobenhausen, Germany)

5.4 Oligonucleotides

5.4.1.1 DNA Oligonucleotides

The used oligonucleotides are listed in Table 6, Table 8, Table 10.

Table 6: List of primer used for sequencing.

Primer	Sequence
Bgh Rv	TAGAAGGCACAGTCGAGG
DF2seq_mid_rev	GTGAATATCGTCCTGAGAGTAG
FW_df2_pet	GTTCCCTTTTAACTTTCTT
LP14rev (Multibac)	TCATTTTATGTTTCAGGTCAGGG
LP6for (MultiBac/pFL)	AAATGATAACCATCTCGCAAATAAA
pCS2 seq rev	GTTGTGGTTTGTCCAAACTCATC
pGEX for	GGGCTGGCAAGCCACGTTTGGTG
pGEX rev	GGAGCTGCATGTGTCAGAG
petdf2_finalrev2	TATGAGAAACGCCAAGAGGAA
petFWseq	TTTGTTAGCAGCCGGATCTC
petFWseqREVCOMP	GAGATCCGGCTGCTAACAAA
petupstreamREV	ATGCGTCCGGCGTAGA
pX_rev	CTATTGACGTCAATGGGCGG
sp6-FW	ATTTAGGTGACACTATAGAA
VP5-FW	CCCACTGCTTACTGGCTTATCG
VP5-REV	CTCACCCCAACAGCTGGCCCTC

Table 7: List of the used primer for cloning.

Primer	Sequence
5BB_RLUC_XhoI_FW	TTATTCTCGAGAAGTCCAACACTAAACTGGGGATTCTGG
5BB_RLUC_NotI_Rev	TTATTGCGGCCGCTCGAGATAATATCCTCGATAGGGCCCTTCT
BamHI_Fw_TEV_Ago2	TTATTGGATCCGAAAACCTGTATTTTCAGGGAATGTACTCGGGAGCCGGCCCCGC
CNOT3_g1 RNA_FW	CACCGGCGGACAAGCGCAAACCTCCA
CNOT3_g1 RNA_RV	AAACTGGAGTTTTCGCTTGTCCGCC
CNOT3_g2 RNA_FW	CACCGCTTTTCTTTCTGTTTCGCGT
CNOT3_g2 RNA_RV	AAACACGCGAACCAGAAAAGAAAAGC
CNOT10_g1 RNA_FW	CACCGAGCTTCAATCAGACATTCTGA
CNOT10_g1 RNA_RV	AAACTCGAATGTCTGATTGAAGCTC
CNOT10_g2 RNA_FW	CACCGAAGGCACAGGTCAGTCCTCT
CNOT10_g2 RNA_RV	AAACAGAGGACTGACCTGTGCCTTC
CNOT11_g1 RNA_FW	CACCGGGTCAAGCTCATCTGCCCCG
CNOT11_g1 RNA_RV	AAACCGGGCAGGATGAGCTTGACCC
CNOT11_g1 RNA_FW	CACCGATCACAGAAGCTTTAGTCAG
CNOT11_g1 RNA_RV	AAACCTGACTAAAGCTTCTGTGATC
DCP2_E148Q_FW	TGCTGCTAGAGAGGTCTTTGAACAAACTGGTTTTGATATCAAAGACTATA
DCP2_E148Q_RV	TATAGTCTTTGATATCAAAACCAGTTTGTTCAAAGACCTCTCTAGCAGCA
fam120a g1 Fw	CACCGCGGTGTAGAAGCCGCGTAGAGG
fam120a g1 Rev	AAACCTCTACGGCGGCTTCTACACCGC
fam120a g2 Fw	CACCGAGCACCTCAGGTGCGACGGGGGG
fam120a g2 Rev	AAACCCCCCGTCGCACCTGAGGTGCTC
Fam120aFW_D49A	CCGCTGCGCCTGTGTTGGCAGCCGACAACCTGCCTGCACCGCCTCTAC
Fam120amut FW_D188A	GGCTTGGTTGCGTATGACTCTGCATATGCACTGTGCAACATCCCCTACTAT
Fam120amut_Rv_D188A	ATAGTAGGGGATGTTGCACAGTGCATATGCAGAGTCATACGCAACCAAGCC
Fam120amut_RV_D49A	GTAGAGGCGGTGCAGGCAGTTGTTCGGCTGCCACCAGCAGGCGCAGCGG
Flag_HA_BamHI_Rev_EDC3	TTATTGGATCCCTAAGCAGAGTGCAGTGGGAT
Flag_HA_BamHI_Rev_FTSJ1	TTATTGGATCCTTAAGGTGAACAACCTCATTTTC
Flag_HA_BamHI_Rev_Trm13	TTATTGGATCCTTAAGCAGTTGTTTCTGGTG
Flag_HA_NotI_FW_EDC3	TTATTGCGGCCGCTGAGTACAGATTGGCTGGGA
Flag_HA_NotI_FW_FTSJ1	TTATTGCGGCCGCTGAGTACAGTCAAAGGACA
Flag_HA_NotI_FW_Trm13	TTATTGCGGCCGCTGAGTACAGTCAAAGGACA
FRT0_fam120aF_bamhl	TTATTGGATCCATGGGCGTGCAGGGCTTCCA
Fw_xhoI_slic_hmga2	GAAGGGCCCTATCGAGGATATTATCTCGAGCTACTAATAGTTTGTGATC
HOXD10_3utr_NotI_Rev	TTATTGCGGCCGCTGAACTCATTTCCAGAGTTTA
HOXD10_3utr_XbaI_FW	TTATATCTAGAGTCTGAGGCCGGTCTGAGGCC
MS2_AGO2_NotI_Rev	TTAATGCGGCCGCTCAAGCAAAGTACATGGT
MS2_AGO2_XbaI_Fw	TTTATTCTAGATACTCGGGAGCCGGCCCC
nha_df1_ECORI_fw	TTATGAATTCTCGGCCACCAGCGTGGACAC
nha_df1_NotI_Rev	TTATGCGGCCGCTCATTTGTTTTCGACTCTG
nha_df2_ECORI_fw	TTATGAATTCTCGGCCAGCAGCTCTTG
nha_df2_NotI_Rev	TTATGCGGCCGCTTATTTCCACGACCTTGAC

nha_df3_ECORI_fw	TTATGAATTCTCAGCCACTAGCGTGGATCA
nha_df3_NotI_Rev	TTATGCGGCCGCTTATTGTTTGTCTATTTCT
nha_FAM_Fw_XbaI	TTATTTCTAGAGGCGTGCAGGGCTTCCAGGAC
nha_FAM_REV_NotI	TAATTGCGGCCGCTTATTGCTTGGCAACTTCATG
NHA_NotI_Rev_EDC3	TTATTGCGGCCGCTAAGCAGAGTGCAGTGGGAT
NHA_NotI_Rev_FTSJ1	TTATTGCGGCCGCTTAAGGTGAACAACTCATTTT
NHA_NotI_Rev_Trm13	TTATTGCGGCCGCTTAAGCAGTTGTTTCTGGTG
Nha_SalI_FW_fam120a	TTATTGTCGACATGGGCGTGCAGGGCTTCCA
NHA_XbaI_FW_EDC3	TTATATCTAGAATGGCTACAGATTGGCTGGGA
NHA_XbaI_FW_FTSJ1	TTATATCTAGAATGGGACGGACGTCAAAGGACA
NHA_XbaI_FW_Trm13	TTATATCTAGAATGGCGACCTCCGCGACG
PAN2_mut_D1087A_Fw	GTTTGTGGGTCATGGCCTGCAGAAGGCATTCCGGGTCGTCAACCTGATG
PAN2_mut_D1087A_Rv	CATCAGGTTGACGACCCGGAATGCCTTCTGCAGGCCATGACCCACAAAC
PAN2_mut_D1139A_Fw	GAAACCCATGACAGTATTGAGGCAGCCCGCACAGCCCTTCAGCTG
PAN2_mut_D1139A_Rv	CAGCTGAAGGGCTGTGCGGGCTGCCTCAATACTGTCATGGGTTTC
pcdna5_fh120a_FBamHI	TTATTGGATCCATGGACTACAAGGACGACGAT
pcdna5_fh120a_RNotI	TTATTGCGGCCGCTTACTCTTCTTTATTTAAGACAGC
pcs2-HRSP12-Fw_FseI	TATTGGCCGCCCATGTCGTCCTTGATCAGAAGG
pcs2-HRSP12-rv_AscI	TTATGGCGCGCCTTATAGTGATGCCGTTGTCAG
pET_SalI_DF1_rev	TTATTGTCGACTTGTTTGTTTCGACTCTGCCG
pET_SalI_DF3_rev	TTATTGTCGACTTGTTTGTTTCTATTTCTCTC
pet_YTHDF2_FW	GTCGACTATAATTTTCAACACCTGCT
pet_YTHDF2_REV	GGATCCGGCCGGGTTTTCATCATTAAGAGC
pet_YTHDF2_REV2	GGATCCATGGGCCGGGTTTTCATCATTAAGAGC
pETBamHI_DF3	TTATTGGATCCTCAGCCACTAGCGTGGATCAG
pETBamHIDF1Final	TTATTGGATCCTCGGCCACCAGCGTGGACACC
POP2catmut_BamHI_Rv	TTATTGGATCCTCACTGCTGCATGTTGTTGAT
POP2catmut_NotI_Fw	TTATTAGCGGCCGCTGCAGCACTTGTGGAGAAT
RLUC_5BB_XhoI_FW	TTATTCTCGAGAAGTCCAACTACTAACTGGGGATTTCCTGG
RLUC_5BB_NotI_Rev	TTATTGCGGCCGCTCGAGATAATATCCTCGATAGGGCCCTTCT
Rv_xbaI_slic_hmg2	ACACTATAGAATAGGGCCCTCTAGAACTGATACACAATGAAAAGAGAGAG
SalI_Rev_TEV_Ago2	TTATTGTCGACTCAAGCAAAGTACATGGTGCGCA
TRIM71_NotI_Rev	TTAATGCGGCCGCTTAGAAGACGAGGATTTCGATT
TRIM71_XbaI_Fw	TTTATTCTAGAGCTTCGTTCCCCGAGACC
VP5_BamHI_DCP2_Rv	TTATGGATCCTCAAAGGTCCAAGATTTTCAT
VP5_fam120a_FW_NotI	TTATTGCGGCCGCTGGGCGTGCAGGGCTTCCA
VP5_NotI_DCP2_FW	TTATAGCGGCCGCTGGAGACCAAACGGGTGGAG
YTHDF1_F383A_PAM2_FW	GCTGCTCACAGCTACAACCCGAAAGAGGCAGAGTGGAATCTGAAAAGCGGG
YTHDF1_F383A_PAM2_Rev	CCCGCTTTTCAGATTCCACTCTGCCTCTTTCGGGTGTAGCTGTGAGCAGC
YTHDF1 g1 Fw	CACCGTAAGGAAATCCAATGGACGG
YTHDF1 g1 Rev	AAACCCGTCCATTGGATTTCTTTAC
YTHDF1 g2 Fw	CACCGAGAGCCCACCGTCTTGACGG
YTHDF1 g2 Rev	AAACCCGTCAAGACGGTGGGCTCTC

YTHDF2_F403A_PAM2_FW	TCCATTAATAACTATAACCCCAAAGATGCAGACTGGAATCTGAAACATGGC
YTHDF2_F403A_PAM2_Rev	GCCATGTTTCAGATTCCAGTCTGCATCTTTGGGGTTATAGTTATTAATGGA
YTHDF2_mut_frag4_FW	TTTGCCAATGAGACCCTCAATAAGGCTAGCAATGTTCCAAAAGTTGTAGGTTCT
YTHDF2_mut_frag4_Rev	AGAACCTACAACCTTTTGAACATTGCTAGCCTTATTGAGGGTCTCATTGGCAAA
YTHDF2 g1 Fw	CACCGGTCCATTACTAGTAACATCG
YTHDF2 g1 Rev	AAACCGATGTTACTAGTAATGGACC
YTHDF2-K571R-FW	AAGAGGAAGAAGAAAGTGTAGGAAGGAACGTCAAGGTCGTGG
YTHDF2-K571R-REV	CCACGACCTTGACGTTCCCTTCCTAACACTTTCTTCTTCTCTT
YTHDF2-S487A-FW	CAACACATGTGCAGGTGTGTGGGCCCAGGACAAATGGAAGGGTCGTT
YTHDF2-S487A-REV	CAACACATGTGCAGGTGTGTGGGCCCAGGACAAATGGAAGGGTCGTT
YTHDF2-S487E-FW	CAACACATGTGCAGGTGTGTGGGAGCAGGACAAATGGAAGGGTCGTT
YTHDF2-S487E-REV	AACGACCCTTCCATTGTCTGCTCCACACACCTGCACATGTGTTG
YTHDF2-W432A-FW	TCCATTAAGTATAATATTGCATGCAGCACAGAGCATGGTAAC
YTHDF2-W432A-REV	GTTACCATGCTCTGTGCTGCATGCAATATTATACTTAATGGA
YTHDF2-W486A-FW	ACATGTGCAGGTGTGGCATCCCAGGACAAATGG
YTHDF2-W486A-REV	CCATTTGTCTGGGATGCCACACCTGCACATGT
YTHDF2-Y429A-FW	CGATATTCACCGTTCCATTAAGGCTAATATTTGGTGCAGCACAGAGC
YTHDF2-Y429A-REV	GCTCTGTGCTGCACCAAATATTAGCCTTAATGGAACGGTGAATATCG
YTHDF2-Y429E-FW	CGATATTCACCGTTCCATTAAGGAAAATATTTGGTGCAGCACAGAGC
YTHDF2-Y429E-REV	GCTCTGTGCTGCACCAAATATTTTCCTTAATGGAACGGTGAATATCG
YTHDF2-Y429F-FW	ATATTCACCGTTCCATTAAGTTTAATATTTGGTGCAGCACAGA
YTHDF2-Y429F-REV	TCTGTGCTGCACCAAATATTAACTTAATGGAACGGTGAATAT
YTHDF3_F409A_PAM2_FW	GCCATAAACAACCTATAATCCCAAAGACGCAGATTGGAATCTGAAGAATGGA
YTHDF3_F409A_PAM2_Rev	TCCATTCTTCAGATTCCAATCTGCGTCTTTGGGATTATAGTTGTTTATGGC
YTHDF2_FW_fragment1_XhoI	TTATTCTCGAGGATGCAATGTTTGGGCAACCAG
YTHDF2_Rev_fragment1_BamHI	TTATT GGATCCCAATTGCTTAAGTTAAGAGCTCTGAGTAG
YTHDF2_FW_fragment2_XhoI	TTATTCTCGAGGATGCAATGTTTGGGCAACCA
YTHDF2_Rev_fragment2_BamHI	TTATTGGATCCCAATTGCTTAAGTTAAGCCTTATTGAGGGTCTC
YTHDF2_FW_fragment3_XhoI	TTATTCTCGAGATGGGATATAGTAGCAATTATGCT
YTHDF2_Rev_fragment3_BamHI	TTATTGGATCCCAATTGCTTAAGTAATGCAACTTCTGTGCTACCCAA
YTHDF2_FW_fragment4_XhoI	TTATTCTCGAGGGATATAGTAGCAATTATGCTT
YTHDF2_Rev_fragment4_BamHI	TTATTGGATCCCAATTGCTTAAGTTAAGCCTTATTGAGGGTCTCATTGG
YTHDF3 g1 Fw	CACCGTGGGTAGCTCCTCGTAACAG
YTHDF3 g1 Rev	AAACCTGTTACGAGGAGCTACCCAC

Table 8: List of the used primer for qPCR.

Primer	Sequence
actin-F-RTqPCR	ATTGGCAATGAGCGGTTC
actin-R-RTqPCR	GGATGCCACAGGACTCCAT
ATXN1_FW_qpcr	AGATCGACTCCAGCACCGTAGA

ATXN1_Rev_qpcr	CTCTACCAAACTTCAACGCTGAC
ATXN2_FW_qpcr	GCTGTTGCCATGCCTATTCCAG
ATXN2_Rev_qpcr	GCAGGAGAGTTCTGCCTCTGAT
BRD2_FW_qpcr	CGGCTTATGTTCTCCAACGCTA
BRD2_Rev_qpcr	GGCAGTAGAGACTGGTAAAGGC
c3orf62_FW_qpcr	GGCACCTGAAAGAGAGTCTTGG
c3orf62_Rev_qpcr	GTGGCATGTTTGAGTCAGCCTTC
CAD_FW_qpcr	TAGTCCTTGGCTCTGGCGTCTA
CAD_Rev_qpcr	TAGTCGGTGCTGACTGTCTCTG
cDOPEY2-Div Fw	AGGGATCCTCTTCCGTTTACT
cDOPEY2-Div Rv	AGGGTGAGTGGATAATCTGGAG
cFAM20B Fw	GACACAATGCAGAGGTAGCAG
cFAM20B Rev	GAGCTGGTGGTGATGGTTAG
CHKA_qPCR_FW	GAGGACGAGTTCCACATCAGTG
CHKA_qPCR_RV	ATACAGCCGCAGGAGCACTTTC
cirNRIP1-Div Fw	GCTCAGAGCTTGGAGACAGAC
cirNRIP1-Div Rv	GGCTGTGTTTCTCCCAAATGTTAAA
cMATR Fw	CCATCTGTGCTCTATATGTG
cMATR Rev	CCCTGTGAGTCCCTACTGAG
cSLC45A4 Fw	GGCAGGGAGTTCTGTTACGC
cSLC45A4 Rev	ACAGGTGGTCCGCTGGTAAT
cZCCHC7 Fw	GGTGATAATGTGGAAAGCTG
cZCCHC7 Rev	ACTCAGTTCACTAGATGACTC
DGCR8_FW_qpcr	GGAAATCCGAGGTCTGCATCCT
DGCR8_Rev_qpcr	GAGGCACCAAAGGCTCACTTG
DGKH_FW_qpcr	ATGGATGACCCGACAGTTCACC
DGKH_Rev_qpcr	ATCTGGAGACCGAGGTGCAGTT
DMTF1_FW_qpcr	CGCTTGGAAGATAATACAGCCATC
DMTF1_Rev_qpcr	CTGAGTCAACGGTAGCAGGAGA
F8A1_fw_qpcr	GTTTGCGTCTGGGGAGGAAT
F8A1_rev_qpcr	TG GTAACGTT CAGCCAACGA
GAPDH-F	AATGGAAATCCCATCACCATCT
GAPDH-R	CGCCCCACTTGATTTTGG
HAND1_fw_qpcr	GGAGTCCGCAGAAGGGTTAAA
HAND1_rev_qpcr	CGGGCAAGGCTGAAAATGAG
Hoxd10_3utr_qpcr_FW	CAGAGGCCAGGATTGGAGAGG
Hoxd10_3utr_qpcr_Rev	GGTTTTGTTTCCGCTTTCCCA
LAMA3_FW_qpcr	TAGAGGAAGCCTCTGACACAGG
LAMA3_Rev_qpcr	CCGATAGTATCCAGGGCTACAAC
MAP3K7_3utr_qpcr_FW	CAGCAGAAACGACAAGGCAC
MAP3K7_3utr_qpcr_Rev	GGCATTCAGAACACGCCAAA
MTOR_FW_qpcr	AGCATCGGATGCTTAGGAGTGG
MTOR_Rev_qpcr	CAGCCAGTCATCTTTGGAGACC

NCOR2_3utr_qpcr_FW	AAAGCAGGATGACCACGCA
NCOR2_3utr_qpcr_Rev	CCATCTGTCCAGAGCGCTAA
NFICI_fw_qpcr	GACCTGTACCTGGCCTACTTTG
NFICI_Rev_qpcr	CACACCTGACGTGACAAAGCTC
PCIF1_qPCR_FW	CTCTGCCTTTGAGAGGTTCCCTG
PCIF1_qPCR_RV	AGCACTCGAAGCTGACGCCAAA
PHF1_FW_qpcr	GGTCCAGTTTGGAGGATGATTTCGC
PHF1_Rev_qpcr	ATGGCGACACTTCTCACAGCTG
RC3H1_FW_qpcr	CTTCACCAACCTTGCCCTCTAC
RC3H1_Rev_qpcr	ATGTAGGAGCCGATGGTGTCAC
RNFT1_qPCR_FW	TGGAGTCTTGGGATACTGCTGG
RNFT1_qPCR_RV	TCTTGCTGGCAGCCACTCCATA
SDHAP3_FW_qpcr	GCCTCGGTACATGGTGCCAATC
SDHAP3_Rev_qpcr	CAGATTCTTCCCCAGCGTTTGG
SERBP1_FW_qpcr	ACATCCATAATCAGCCACCA
SERBP1_Rev_qpcr	AAGCTGTCTGCTGCCCAAAT
SETD4_FW_qpcr	CACCACGGACACAGTGATTTCGA
SETD4_Rev_qpcr	AGGCTTCCAAAGAGATCGGTGC
SLC7A5 qpcr FW	TTCTTCAACTGGCTCTGCGT
SLC7A5 qpcr Rev	GGACGGTCGTGGAGAAGATG
ST6GALNAC6_fw_qpcr	CATTTCGTGGTTGAGCACAGG
ST6GALNAC6_rev_qpcr	CCGCTGGCTGCAGTAGTT
TRMT44_FW_qpcr	AAGAAGTGGGCTTCACCTGTGG
TRMT44_Rev_qpcr	CTGAGTCCTCTTTTCATCCACGG
TTC31_FW_qpcr	ACAACAGAGCCAGGAACTGGCA
TTC31_Rev_qpcr	AACCGCTCATGGCAGAAGGAAC

Table 9: List of the used primer for Northern Blot.

Primer	Sequence
BGG-FW	ATGGTGCACCTGACTCCTGAG
BGG-Rev	TTAGTGATACTTGTGGGCCAGGG
<i>Hs</i> F-Luc FW	AGGCCCGGCGCCATTCTATC
<i>Hs</i> F-Luc Rev	CTTTCGGCCCTTCTTGGCC
<i>Hs</i> R-Luc FW	ATGGCTTCCAAGGTGTACGAC
<i>Hs</i> R-Luc Rev	TTACTGCTCGTTCTTCAGCAC
DF1_NB_3utr_RV	GCTGTTCAGTGGGCAACAGATCT
DF2_NB_3utr_RV	ACAAGAACAGAACATTTCTTAGG
DF3_NB_3utr_RV	AATGTTCTAATATAATTTAGAT
RMRP_FW	GTTCGTGCTGAAGGCCTGTA
RMRP_REV_	CAGCCGCGCTGAGAATGAGC
RPPH1_FW_	ATAGGGCGGAGGGAAGCTCA
RPPH1_REV_	AATGGGCGGAGGAGAGTAGT

Table 10: List of the used primer for ePAT.

Primer	Sequence
ARHGAP21_REV_spe	AATTGGGTATGCACACAGGCAT
ARHGAP21_FW_pA_tail	TTACATTGAATAAACGAATGC
CELSR3_REV_spe	AAGTTTATTCCCAAAACAGA
CELSR3_FW_pA_tail	GTCTGTGCAGACACCTCCTC
HLADQB1_FW_pA_tail	ATGCTCAGTGCATTGGCCTTAGAATAT
HLADQB1_REV_spe	TTATTGCTGGTTATGCTTTCT
LYN_FW_pA_tail	GTGGAAGAGAGATCGTAGTT
LYN_REV_spe	AAGCGAAGCCAATCTATTCC
METTL16_FW_pA_tail	TATGAAATTAAATCTTGGTGA
METTL16_REV_spe	AATCTGGGTAGCAACTGGGTA
NAGLU_FW_pA_tail	TAGATTGCCACCACTGGGCCTT
NAGLU_REV_spe	TTTAAGTGGAAGAAAACAGTAC
Oligo(dT)_anchor	GCGAGCTCCGCGGCCGCGTTTTTTTTTTTTT
Oligo_dT_18mer	TTTTTTTTTTTTTTTTTTTT
RHOQ_FW_pA_tail	AGAAATGCCAAAAATGAAATGTTAAAAA
RHOQ_REV_spe	TTAACAGTCTTCAAAGCCAGT
SLC25A30_FW_pA_tail	TCGTTGTCAGGGTTCTGGCT
SLC25A30_REV_spe	AACAAGGCTTATGCCACATA
STARD9_FW_pA_tail	GCTCAGTTCCAGAACTTACCTC
STARD9_REV_pA_tail	TAAAGTACACAGAGGACTTTTA
YEATS2_FW_pA_tail	TACCTGAACTGCTATAAGCA
YEATS2_REV_spe	ATACACATTATGATACACGT

5.4.1.2 RNA Oligonucleotides

siPools were designed by and ordered at siTools Biotech (Martinsried, Germany). Following siTools were used during this study: siNegC, siDDX6, siPOP1, siRPP25, siTNRC6A, siTNRC6B, siTNRC6C, siYTHDF1, siYTHDF2, siYTHDF3.

5.5 Vectors and plasmids

5.5.1 Vectors

The used vectors for molecular cloning are listed in Table 11.

Table 11: list of the used vectors

Vector	Resistance	Source	Identifier
pcDNA	Ampicillin	AG Meister	N/A
pCINeo	Ampicillin	AG Meister	N/A
pCMV	Ampicillin	University of Dundee	N/A

pCS2	Ampicillin	AG Meister	N/A
pEGFP	Ampicillin	Izaurralde lab	N/A
pGEX-4T1	Ampicillin	AG Meister	N/A
pet2A	Ampicillin	AG Meister	N/A
pT7	Kanamycin	Izaurralde lab	N/A
pTBG	Ampicillin	Jinbiao Ma ³⁵	N/A
VP5	Ampicillin	AG Meister	N/A

5.5.2 Plasmids

All used plasmids for the experiments are listed in Table 12.

Table 12: list of the used plasmids

Plasmid	Insert	Vector	Cloning enzymes	Identifier
pCDNA-HsPOP1	POP1	pcDNA	XhoI/XbaI	Addgene Cat #53968
pCDNA-HsRPP25	RPP25	pcDNA	XhoI/XbaI	Addgene Cat #87194
pCDNA-MS2-HA		pcDNA		Izaurralde lab
pCDNA-MS2-HA-HsAGO2	AGO2	pcDNA	XbaI/NotI	Izaurralde lab
pCDNA-MS2-HA-HsTRIM71	TRIM71	pcDNA	XbaI/NotI	This study
pCIneo-Rluc	R-Luciferase	pCIneo		Izaurralde lab
pCIneo-Rluc-5BoxB	Renilla-Luciferase	pCIneo		Izaurralde lab
pCIneo-Rluc-6XMS2bs-5BoxB	Renilla-Luciferase	pCIneo		This Study
pCIneo-λNHA		pCIneo		AG. Meister
pCIneo-λNHA-HsAGO2	AGO2	pCIneo		AG. Meister
pCIneo-λNHA-HsFAM120A	FAM120A	pCIneo		This study
pCIneo-λNHA-HsNOT1	NOT1	pCIneo		Izaurralde lab
pCIneo-λNHA-HsTRIM71	TRIM71	pCIneo	XbaI/NotI	This study
pCIneo-λNHA-HsYTHDF1	YTHDF1	pCIneo	EcoRI/NotI	This study
pCIneo-λNHA-HsYTHDF2	YTHDF2	pCIneo	EcoRI/NotI	This study
pCIneo-λNHA-HsYTHDF3	YTHDF3	pCIneo	EcoRI/NotI	This study
pCIneo-Rluc-HsHOXD10-3'utr	HoxD10 3'UTR	pCIneo	NotI/XbaI	This study
pCMV5-FLAG-FAM120A	FAM120A	pCMV5		University of Dundee
pCS2-GFP	GFP	pCS2	FseI/XbaI	AG. Meister

pCS2-HsYTHDF2	YTHDF2	pCS2	FseI/XbaI	This study
pCS2-HsYTHDF2-K281R	YTHDF2-K281R	pCS2	FseI/XbaI	This study
pCS2-HsYTHDF2-K571R	YTHDF2-K571R	pCS2	FseI/XbaI	This study
pCS2-HsYTHDF2-S487A	YTHDF2-S487A	pCS2	FseI/XbaI	This study
pCS2-HsYTHDF2-S487E	YTHDF2-S487E	pCS2	FseI/XbaI	This study
pCS2-HsYTHDF2-W432A-W486A	YTHDF2-W432A-W486A	pCS2	FseI/XbaI	This study
pCS2-HsYTHDF2-W486A	YTHDF2-W486A	pCS2	FseI/XbaI	This study
pCS2-HsYTHDF2-Y429A	YTHDF2-Y429A	pCS2	FseI/XbaI	This study
pCS2-HsYTHDF2-Y429E	YTHDF2-Y429E	pCS2	FseI/XbaI	This study
pCS2-HsYTHDF2-Y429F	YTHDF2-Y429F	pCS2	FseI/XbaI	This study
pCS2-HsYTHDFW432A	YTHDF2-W432A	pCS2	FseI/XbaI	This study
pEGFP-N3-FLuc	Firefly-Luciferase	pCIneo		Izaurrealde lab
pet-HsYTHDF2-Full length	YTHDF2	pet2a	BamHI/SalI	This study
pet-HsYTHDF2-K571R	YTHDF2-K571R	pet2a	BamHI/SalI	This study
pet-HsYTHDF2-S487A	YTHDF2-S487A	pet2a	BamHI/SalI	This study
pet-HsYTHDF2-S487E	YTHDF2-S487E	pet2a	BamHI/SalI	This study
pet-HsYTHDF2-W432A-W486A	YTHDF2-W432A-W486A	pet2a	BamHI/SalI	This study
pet-HsYTHDF2-Y429A	YTHDF2-Y429A	pet2a	BamHI/SalI	This study
pet-HsYTHDF2-Y429E	YTHDF2-Y429E	pet2a	BamHI/SalI	This study
pet-HsYTHDF2-Y429F	YTHDF2-Y429F	pet2a	BamHI/SalI	This study
pGEX-4T1-FAM120A-PIN	FAM120A-PIN	pGEX-4T1	BamHI/SalI	This study
pGEX-4T1-FAM120A-PIND188A	FAM120A-PIN-D188A	pGEX-4T1	BamHI/SalI	This study
pGEX-4T1-FAM120A-PIND49A	FAM120A-PIN-D49A	pGEX-4T1	BamHI/SalI	This study
pGEX-4T1-FAM120A-PIND49A-D188A	FAM120A-PIN-D49A-D188A	pGEX-4T1	BamHI/SalI	This study
pnEA-NpM-MCS1-YTHDF2-100aa-GB1	YTHDF2-100aa-	pnEA-NpM	XhoI/BamHI	This study
pnEA-NpM-MCS1-YTHDF2-100aa-Fragment 1	YTHDF2-100aa-Fragment 1	pnEA-NpM	XhoI/BamHI	This study
pnEA-NpM-MCS1-YTHDF2-100aa-Fragment 2	YTHDF2-100aa-Fragment 2	pnEA-NpM	XhoI/BamHI	This study
pnEA-NpM-MCS1-YTHDF2-100aa-Fragment 3	YTHDF2-100aa-Fragment 3	pnEA-NpM	XhoI/BamHI	This study
pnEA-NpM-MCS1-YTHDF2-100aa-Fragment 4	YTHDF2-100aa-Fragment 4	pnEA-NpM	XhoI/BamHI	This study
pSpCas9(BB)-2A-Puro V2.0-pX459-FAM120A-gRNA1	FAM120A-gRNA1	pSpCas9(BB)-2A-Puro V2.0-pX459	BbsI	This study
pSpCas9(BB)-2A-Puro V2.0-pX459-FAM120A-gRNA2	FAM120A-gRNA2	pSpCas9(BB)-2A-Puro V2.0-pX459	BbsI	This study

pSpCas9(BB)-2A-Puro pX459-YTHDF1-gRNA1	V2.0-	YTHDF2-gRNA1	pSpCas9(BB)- 2A-Puro V2.0-pX459	BbsI	This study
pSpCas9(BB)-2A-Puro pX459-YTHDF1-gRNA2	V2.0-	YTHDF2-gRNA2	pSpCas9(BB)- 2A-Puro V2.0-pX459	BbsI	This study
pSpCas9(BB)-2A-Puro pX459-YTHDF2-gRNA1	V2.0-	YTHDF2-gRNA1	pSpCas9(BB)- 2A-Puro V2.0-pX459	BbsI	This study
pSpCas9(BB)-2A-Puro pX459-YTHDF3-gRNA1	V2.0-	YTHDF2-gRNA1	pSpCas9(BB)- 2A-Puro V2.0-pX459	BbsI	This study
pSpCas9(BB)-2A-Puro pX459-YTHDF3-gRNA2	V2.0-	YTHDF2-gRNA2	pSpCas9(BB)- 2A-Puro V2.0-pX459	BbsI	This study
pT7-EGFP-C1-HsNOT10		NOT10	pT7	-	Izaurrealde lab
pT7-EGFP-C1-HsYTHDF1		YTHDF1	pT7	-	Izaurrealde lab
pT7-EGFP-C1-HsYTHDF2		YTHDF2	pT7	-	Izaurrealde lab
pT7-EGFP-C1-HsYTHDF3		YTHDF3	pT7	-	Izaurrealde lab
pT7-MS2-HA-HsAGO2		AGO2	pcDNA	XhoI/BamHI	This study
pT7-MS2-HA-HsTRIM71		TRIM71	pcDNA	XhoI/BamHI	This study
pT7-MS2-HA-HsYTHDF1		YTHDF1	pcDNA	XhoI/BamHI	Izaurrealde lab
pT7-MS2-HA-HsYTHDF2		YTHDF2	pcDNA	XhoI/BamHI	Izaurrealde lab
pT7-MS2-HA-HsYTHDF3		YTHDF3	pcDNA	XhoI/BamHI	Izaurrealde lab
pTBG- β -globin-1BoxB-1MS2		β -globin, 1BoxB-1MS2	pTBG	NotI/XbaI	This study
pTBG- β -globin-5BoxB		β -globin, 5BoxB	pTBG	NotI/XbaI	This study
pTBG- β -globin-GAPDH		β -globin, GAPDH	pTBG	From J.Lykke- Andersen et al.,	Izaurrealde lab
pTBG- β -globin-5BoxB-HMGA2- without-let7bs		HMGA2-without-let7bs	pTBG		This study
pTBG- β -globin-SON		β -globin, GAPDH, SON	pTBG		from Du et. al.,35
pTBG- β -globin-SON $\Delta\Delta$ mut		β -globin, SON $\Delta\Delta$ mut	pTBG		from Du et. al.,35
VP5-Empty		-	VP5		AG. Meister
VP5-GFP		GFP	VP5		AG. Meister
VP5-HsANKRD17		ANKRD17	VP5	BamHI/NotI	AG. Meister
VP5-HsCAF1		CAF1	VP5	BamHI/NotI	This study
VP5-HsCAF1-D40A-E42A		CAF1-D40A-E42A	VP5	BamHI/NotI	This study
VP5-HsCSTF1		CSTF1	VP5		AG. Meister
VP5-HsCSTF3		CSTF3	VP5		AG. Meister

VP5-HsDCP2	DCP2	VP5		This study
VP5-HsDCP2-E148Q	DCP2-E148Q	VP5	BamHI/NotI	This study
VP5-HsHRSP12	HRSP12	VP5	BamHI/NotI	This study
VP5-HsTNRC6A	TNRC6A	VP5		AG. Meister
VP5-HsTNRC6B	TNRC6B	VP5		AG. Meister
VP5-HsTNRC6C	TNRC6C	VP5		AG. Meister
VP5-HsUPF1	UPF1	VP5	BamHI/NotI	AG. Meister
VP5-HsYTHDF1	YTHDF1	VP5	BamHI/NotI	AG. Meister
VP5-HsYTHDF2	YTHDF2	VP5	BamHI/NotI	AG. Meister
VP5-HsYTHDF2-K281R	YTHDF2-K281R	VP5	BamHI/NotI	This study
VP5-HsYTHDF2-K571R	YTHDF2-K571R	VP5	BamHI/NotI	This study
VP5-HsYTHDF2-S487A	YTHDF2-S487A	VP5	BamHI/NotI	This study
VP5-HsYTHDF2-S487E	YTHDF2-S487E	VP5	BamHI/NotI	This study
VP5-HsYTHDF2-W432A	YTHDF2-W432A	VP5	BamHI/NotI	This study
VP5-HsYTHDF2-W432A-W486A	YTHDF2-W432A-W486A	VP5	BamHI/NotI	This study
VP5-HsYTHDF2-W486A	YTHDF2-W486A	VP5	BamHI/NotI	This study
VP5-HsYTHDF2-Y429A	YTHDF2-Y429A	VP5	BamHI/NotI	This study
VP5-HsYTHDF2-Y429E	YTHDF2-Y429E	VP5	BamHI/NotI	This study
VP5-HsYTHDF3	YTHDF3	VP5	BamHI/NotI	AG. Meister
VP5-HsYTHDF2-Y429F	YTHDF2-Y429F	VP5	BamHI/NotI	This study

5.6 Antibodies

The used antibodies are listed in Table 13.

Table 13: list of used antibodies

Antibody	Source	Dilution	Identifier
Alexa 488 α -mouse	Goat	1:400	Thermo Fisher Scientific, RRID:AB_2536161 (Waltham, Massachusetts, USA)
Alexa 594 α -rabbit	Goat	1:400	Thermo Fisher Scientific, RRID:AB_2534079 (Waltham, Massachusetts, USA)
Anti-AGO2	rat	1:100	Clone 11A9 (In house Antibody)

Anti-AGO2	rabbit	1:200	Proteintech Cat# 66720-1-Ig, RRID:AB_2882071 (Chicago, USA)
Anti-ARHGAP21	rabbit	1:1000	Proteintech Cat# 22183-1-AP, RRID:AB_2879018 (Chicago, USA)
Anti-β-α-TUBULIN	rabbit	1:1000	Abcam Cat# ab6046 (Cambridge, UK)
Anti-β-actin	mouse	1:10000	Abcam, Cat# ab6276, RRID: AB_2223210 (Cambridge, UK)
Anti-c-Myc	rabbit	1:1000	Sigma Aldrich, Cat #C3956 (Munich, Germany)
Anti-CNOT6 (E1L8F)	rabbit	1:1000	Cell Signaling Technology, #13415 (Danvers, Massachusetts, USA)
Anti-CNOT7 (D3M6O)	rabbit	1:1000	Cell Signaling Technology, #86665 (Danvers, Massachusetts, USA)
Anti-DDX6	rabbit	1:1000	Proteintech 14632-1-AP (Chicago, USA)
Anti-DGCR8	rabbit	1:10	Clone 1C9 (In house Antibody)
Anti-EDC4	rabbit	1:400	(In house Antibody from Tiana Hanelt)
Anti-FAM120A	rabbit	1:1000	Bethyl Catalog # A303-889A
Anti-FLAGM2	mouse	1:1000	Sigma Aldrich Cat# F3165, RRID: AB_259529 (Munich, Germany)
anti-GAPDH	mouse	1:500	GeneTex Cat# GTX627408, RRID: AB_11174761 (Irvine, USA)
Anti-GFP	mouse	1:1000	Merck, #11814460001 (Darmstadt, Germany)
Anti-HA	mouse	1:1000	Covance Cat# MMS-101P, RRID: AB_2314672 (Princeton, USA)
Anti-HSP90	rabbit	1:500	Epitomics, #1492-1, Abcam (Cambridge, UK)
Anti-HRSP12	rabbit	1:1000	Proteintech 12930-1-AP (Chicago, USA)
Anti-NOT1	rabbit	1:1000	In house (From Izaurralde lab)
Anti-POP1	rabbit	1:1000	Proteintech 12029-1-AP (Chicago, USA)
anti-YTHDF1	rabbit	1:200	Proteintech 17479-1-AP (Chicago, USA)
anti-YTHDF2	rabbit	1:1000	Proteintech 24744-1-AP (Chicago, USA)
anti-YTHDF2	mouse	1:1000	Clone 32A11 (In house Antibody)
anti-YTHDF3	mouse	1:250	Santa Cruz Biotechnology sc-377119 (Dallas, USA)
Anti-TNRC6A/GW182	rabbit	1:1000	Bethyl Catalog # A302-330A
Anti-TNRC6ABC	rat	1:5	Clone 7A9 (In house Antibody)
Anti-TNRC6ABC	rat	1:5	Clone 11C12 (In house Antibody)
anti-mouse 680	goat	1:10000	Li-Cor Biosciences, #926-32220 (Lincoln, USA)
anti-rabbit 680	goat	1:10000	Li-Cor Biosciences, #926-32221 (Lincoln, USA)
anti-rat 680	goat	1:10000	Li-Cor Biosciences, #926-68076 (Lincoln, USA)
anti-mouse 800	goat	1:10000	Li-Cor Biosciences, # 926-32219 (Lincoln, USA)
anti-rabbit 800	goat	1:10000	Li-Cor Biosciences, #926-32211 (Lincoln, USA)
anti-rat 800	goat	1:10000	Li-Cor Biosciences, # 926-32219 (Lincoln, USA)

5.7 Cell Lines and Bacteria strains

All cultured eukaryotic and prokaryotic cell lines are listed in Table 14, Table 15.

Table 14: list of all eukaryotic cultured cell lines

Cell line	Specification	Source
A549	Human adenocarcinomic alveolar basal epithelial cells	ATCC Cat# A549, RRID: CVCL_0023
C643	Human Thyroidea Carcinoma cells	ATCC Cat# CLS, RRID:CVCL_5969
C643 YTHDF2 K.O.	Human Thyroidea Carcinoma cells	This study
C643 METTL3 K.O.	Human Thyroidea Carcinoma cells	(Ho-Xuan et al., 2020)
HEK 293T	Human embryonic kidney cells	From Izaurralde lab
HEK 293T siDDX6	Human embryonic kidney cells	This study
Flp-In-T-REx	Human embryonic kidney cells	CVCL_U427
Flp-In-T-REx FAM120A K.O.	Human embryonic kidney cells	This study
HEK 293T triple siTNRC6ABC	Human embryonic kidney cells	This study
HEK 293T YTHDF1 K.O.	Human embryonic kidney cells	This study
HEK 293T double YTHDF1-3 K.O.	Human embryonic kidney cells	This study
HEK 293T double siYTHDF1-3	Human embryonic kidney cells	This study
HEK 293T triple siYTHDF1-2-3	Human embryonic kidney cells	This study
HEK 293T XRN1 K.O.	Human embryonic kidney cells	From Izaurralde lab
HEK 293T XRN1 K.O. double siYTHDF1-3	Human embryonic kidney cells	This study
HEK 293T XRN1 K.O. triple siYTHDF1-2-3	Human embryonic kidney cells	This study
HeLa	Human cervical cancer cells	ATCC Cat# CCL-2, RRID: CVCL_0030

Table 15: list of all prokaryotic cultured cell lines

Cell line	Source	Identifier
<i>Escherichia coli</i> Rosetta BL21(DE3) competent cells	Novagen	Cat#70954-3
<i>Escherichia coli</i> Rosetta XL1 blue competent cells	This paper	N/A

For all cloning experiments competent *Escherichia coli* (*E. coli*) XL1 blue (Genotype: *F-recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lacF'*[*proAB lacI qZAM15 Tn10 (TetR)*]) cells were used.

For protein expression *Escherichia coli* (*E. coli*) BL21(DE3) (Genotype: *E. coli* str. B F⁻ ompT gal dcm lon hsdSB(rB-mB-) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB+*]K-12(λS)) cells were used.

5.8 Methods

5.8.1 DNA-based methods

5.8.1.1 Molecular cloning

The aim of molecular cloning was the generation of new plasmid constructs for the expression of recombinant proteins in *E. Coli* or transient expression of the constructs in above mentioned cell lines. The first step includes the amplification of the desired inserts from complementary DNA (cDNA) or plasmids using polymerase chain reaction (PCR). Phusion DNA-polymerase was used for all the reactions. The cycling conditions and composition of the reactions are shown in Table 16 and Table 17. As template either 5 µl cDNA or 100 ng plasmid-DNA was used for one reaction. The annealing temperature was adjusted to the melting temperature of the primers and calculated or determined by temperature gradient PCR.

Table 16: Composition of a standard 50 µl PCR reaction

Component	Volume
Template	X
HF-buffer (5x)	10 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
dNTPs mix (10mM)	1 µl
DMSO (100%)	1.2 µl
Phusion polymerase	0.5µl
H ₂ O	to 50 µl

Table 17: Cycling conditions of a standard PCR reaction

Temperature	Time
98°C	30 s
98°C	10 s
X	30 s
72°C	15-30 s per kb
72°C	5-10 min
4°C	∞

35x

Afterwards the reaction products were separated on an agarose gel and bands of correct size were cut out of the gel. The DNA-fragments were purified using the NucleoSpin® Gel and PCR Clean-up kit according to the manufacturer's protocol. Afterwards, the purified PCR-product was digested using the restriction enzymes required for the ligation into the desired vector. The PCR fragments were digested for 30 min at 37 °C and the reaction was subsequently purified with the

NucleoSpin® Gel and PCR Clean-up kit according to the manufacturer's protocol. The vector was digested 30 min at 37°C, afterwards separated on an 1 % agarose gel and subsequently purified using the NucleoSpin® Gel and PCR Clean-up kit according to the manufacturer's protocol. The reaction composition for the restriction digest of PCR fragments or plasmids are shown in Table 18.

Table 18: Composition of restriction digestion reactions

Component	PCR fragment digestion
DNA	X µl eluate from purification
Restriction enzymes	2 µl each
NEB Cut Smart (10x)	4 µl
H ₂ O	to 40 µl

By using the same restriction enzymes for both PCR products and digested plasmids, it was possible to insert the PCR amplicons into the desired vectors using the T4 DNA-Ligase. The composition of the ligation reaction is shown in Table 19. The ligation was incubated for at least 1 h at room temperature (RT). Afterwards, 10 µl of the reaction was transformed into *E. Coli* XL1 blue cells. More in detail, 50 µl of the cell suspension was added to the ligation reaction. After mixing the cells carefully, they were incubated together with the DNA for 10-15 min on ice. Next, the heat shock was performed by incubating the cells at 42 °C for 45 seconds. Before plating, the cells were cooled on ice for 5 - 10 min. The transformation reaction was plated on a LB-Agar + the antibiotic resistance present in the vector, and incubated overnight (o.n.) at 37 °C. In case of no successful clones the rest of the reaction (10 µl), which was incubated at RT in the meanwhile was used for a second transformation following the previously described protocol. The following day, from every plate several colonies were picked and inoculated in 5 ml LB-Ampicillin medium. The cultures were incubated o.n. at 37 °C on a shaker. Afterwards, the plasmid DNA was isolated using the NucleoSpin® Plasmid Kit and 500 ng were used for an analytical test digest as shown in Table 20. DNA from positive clones was sequenced. Afterwards, in order to obtain higher yields, the plasmid was transformed as previously described and 250 ml LB-Ampicillin medium were inoculated with the positive clone. Finally, a MIDI plasmid isolation using the NucleoSpin® Xtra Midi Kit was performed.

Table 19: Composition of a T4 DNA Ligation reaction

Component	Volume
Vector	2 µl
Insert	2 µl, 4 µl or 6 µl
10x T4-ligase buffer	2 µl
T4 Ligase	1 µl
H ₂ O	to 20 µl

Table 20: Compositions of a test digest reaction

Component	Volume
DNA	2 µl
Restriction enzymes	2 µl each
Fast Digest buffer (10x)	4 µl
H ₂ O	to 40 µl

5.8.1.2 Mutagenesis PCR

To mutate single amino acid residues a quick change PCR reaction was performed. The reaction mix and the conditions used are shown in Table 21 and in Table 22. After the reaction was complete, 1 µl of DpnI was added to the mix to digest the template plasmid and the reaction was incubated at 37 °C for 4 – 5 h. Following that, 10 µl of the reaction was transformed into *E. Coli* XL1 blue cells. From here the protocol for molecular cloning was followed with the difference that no test digestion was performed and the plasmids were sequenced immediately after the MINI preparation.

Table 21: Composition of a quick change PCR reaction

Component	Amount
5x HF buffer	10 µl
dNTPs mix	1 µl
FW primer (5 µM)	1 µl
REV primer (5 µM)	1 µl
Template	200 ng
Phusion polymerase	0.5 µl
H ₂ O	to 50 µl

Table 22: Cycling conditions of a mutagenesis PCR

Temperature	Time	
98 °C	30 s	18x
98 °C	10 s	
56 °C	30 s	
68 °C	16 min	
68 °C	30 min	
4°C	∞	

5.8.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate nucleic acid (DNA or RNA molecules) according to their size, for visualization and purification purposes. Gels with 1 – 2 % agarose were generally used. To prepare a 1 % agarose gel, 1 g of agarose was dissolved in 1x TBE via heating. After cooling the solution for a few minutes, ethidium bromide (EtBr) was added to a final concentration of approximately 0.2-0.5 µg/mL. Then, the gel was poured into a gel chamber. As running buffer 1x TBE was used. The Samples were prepared through adding an appropriate amount of 6x

TriTrack DNA Loading Dye (Thermo Fisher Scientific). The gels were run at 120 V for 40 min. Afterwards the gels were imaged by the Quantum ST4 (PeqLab) through UV light.

5.8.1.4 Sanger sequencing

For sequencing 500 ng plasmid was diluted in 5 μ l H₂O. Additionally, 5 μ l of the primer concentrated 5 μ M was added. The samples were sequenced by Eurofins Genomics.

5.8.1.5 Reverse transcription quantitative PCR

The reverse transcription quantitative PCR (RT-qPCR) was used to determine RNA levels in various samples. Firstly, cDNA was synthesized from the extracted RNA. Thereafter a qPCR reaction was performed.

5.8.1.6 cDNA Synthesis

For cDNA synthesis, the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used. As input either 1 μ g RNA extracted from HEK293T cells using the NucleoSpin® RNA kit (Macherey-Nagel) or 5 μ l of eluted RNA extracted using the TRIzol reagent (see **Error! Reference source not found.**) were used. cDNA synthesis can be performed using either total RNA or poly(A)⁺-selected RNA primed with oligo(dT), random primers, or a gene-specific primer. As primer oligo-dT₁₈ was used. Subsequently, the manufacturer's protocol was followed and the final volume was diluted in a 1:1 ratio with H₂O.

5.8.1.7 Quantitative PCR (RT-qPCR)

For qPCR reaction, newly synthesized cDNA (see previous chapter) was used as template. For each condition, three technical replicates were prepared and for the qPCR, 96 well plates with 50 μ l well capacity were used. The reactions were prepared in 20 μ l total volume, a master mix as listed in Table 23 was prepared for every target and 18 μ l were placed in each well. Subsequently, 2 μ l of cDNA were added to complete the reaction. Thereafter, the plate was briefly centrifuged and then the qPCR was performed using the CFX96™ Real-Time System (Bio-Rad).

Table 23: Composition of one qPCR master mix reaction

Component	Volume
Takyon™ 2x mix	8 μ l
Primer Fw 10 μ M	1 μ l
Primer Rev 10 μ M	1 μ l
H ₂ O	10 μ l

5.8.1.8 Poly A tail length assay (kit)

The poly(A) tail length assay was performed using a poly(A) tail length assay kit (Affymetrix, Cleveland, OH) according to the manufacturer's protocol. G/I tailing was performed according to Table 24 in a total volume of 20 μ l. The reaction mix was incubated at 37°C for 60 min. Next, 2 μ l 10 $\times$ tail stop solution was added to stop the G/I tailing reaction.

Table 24: Composition of G/I tailing mix reaction

Component	Volume
Total RNA sample, 1 μ g (0.1 – 2 μ g)	Up to 14 μ l
5X Tail buffer mix	4 μ l
10X tail enzyme mix	2 μ l
H ₂ O	To 20 μ l

For reverse transcription, 5 μ l of G/I-tailed RNA sample, 4 μ l 5 $\times$ RT buffer mix, and 2 μ l RT enzyme mix were mixed in a total of 20 μ l and incubated at 44°C for 60 min and 92°C for 10 min, after which the mix was held at 4°C. The negative reverse transcriptase control was produced by using the same mix excluding the 2 μ l RT enzyme mix (Table 25)

Table 25: Composition of RT mix

Component	RT +	RT – (control)
G/I-tailed RNA sample	5 μ l	5 μ l
5X Tail buffer mix	4 μ l	4 μ l
10X tail enzyme mix	2 μ l	-
H ₂ O	9 μ l	11 μ l

The reverse transcription sample was diluted with water 1:1 to a final volume of 40 μ l, and then 2 μ l diluted RT sample was used in a 25- μ l PCR mixture containing 5 μ l 5 $\times$ PCR buffer mix, 1 μ l 10 μ M universal PCR reverse primer, 1 μ l 10 μ M gene-specific forward primer, and 1.25 units HotStart-IT Taq DNA polymerase (Table 26).

Table 26: PCR mix

Component	RT + Tail PCR	RT – Tail PCR	RT + Specific PCR	RT – Specific PCR
Diluted RT sample	Up to 5 μ l	Up to 5 μ l	Up to 5 μ l	Up to 5 μ l
5X PCR buffer mix	5 μ l	5 μ l	5 μ l	5 μ l
10 μ M Gene-specific PCR Forward Primer	1 μ l	1 μ l	1 μ l	1 μ l
10 μ M Universal PCR Reverse Primer	1 μ l	1 μ l	-	-

10 µM Gene-specific PCR Reverse Primer	-	-	1 µl	1 µl
1.25units/µl HotStart-IT® Taq DNA Polymerase	1 µl	1 µl	1 µl	1 µl
H ₂ O, Nuclease-Free	To 25 µl	To 25 µl	To 25 µl	To 25 µl

PCR conditions are described in Table 27

Table 27: PCR conditions

Temperature	Time	35x
94°C	2 min	
94°C	10 s	
58°C	30 s	
72°C	30 s	
72°C	5 min	
4°C	∞	

Five microliters of PCR product was resolved in a 2% agarose gel and stained with ethidium bromide. The primers used can be found at Table 8.

5.8.1.9 Extension Poly(A) Test (ePAT) assay

To measure poly(A) tail lengths of multiple individual mRNAs, extension Poly(A) Test (ePAT) assay was performed. Briefly, 1–3 µg of total RNA was first mixed with 1 µl of oligo(dT) anchor primers [200 ng/ml, final concentration], incubated at 80°C for 5 min and subsequently ramped down to RT (24°C). Once reached RT, 11 µl of the reverse transcriptase mix were added and after brief vortexing and spinning down, the solution was placed at 25°C for 1 hr (Table 28) (PAT anchor 5'-GCGAGCTCCGCGGCCGCGTTTTTTTTTTTTT).

Table 28: Reverse transcriptase mix

Component	Volume
5x AMV reverse transcriptase buffer	4 µl
DTT (0.1 M)	1 µl
H ₂ O	3 µl
dNTPs mix (10 mM each)	1 µl
Klenow Polymerase	1 µl
RNase inhibitor (40 U/ml)	1 µl

At the end of the incubation, the mix was put at 80°C for 10 min and then at 55°C for 1 min. Finally, while at 55°C, 2 µl of AMV reverse transcriptase (10U/ µl) were added and the mix was incubated at 55°C for 1 hour and heat inactivated at 80°C for 10 min. The cDNA is then diluted in a 1:6 ratio in 120ul of H₂O and 5ul of the dilution were used for HOT-PCR amplification and analysis (Table 29).

Table 29: HOT- PCR mix e-PAT

Component	Volume
Template e-PAT cDNA	5 µl
10X PCR buffer mix (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton X-100, 15 mM MgCl ₂)	2.5 µl
dNTPs mix (10 mM each)	0.5 µl
[α - ³² P] dCTP	5 µl
[α - ³² P] dATP	5 µl
25 pmol/µl oligo(dT) anchor	0.5 µl
25 pmol/µl message specific primer	0.5 µl
Taq DNA Polymerase	1 µl
H ₂ O, Nuclease-Free	To 25 µl

PCR conditions are described in Table 27.

The cDNA products were diluted through an addition of an appropriate amount of 6x TriTrack DNA Loading Dye (Thermo Fisher Scientific). Finally, poly(A) tracts were resolved by denaturing (urea) 6%. The gel was dried and exposed to a phosphor screen for an appropriate time. The screen was scanned by the Personal Molecular Imager System (Bio-Rad).

5.8.2 RNA-based methods

5.8.2.1 RNA extraction

For RNA isolation from whole cell extracts or immunoprecipitation reactions, the TRIzol reagent was used. First, to the sample containing an appropriate amount of TRIzol. Chloroform was added in a 1:5 ratio. Following that, the samples were mixed thoroughly and centrifuged for 15 min at 4 °C and 20870 xg. Afterwards, the clear phase was taken and 1:1 Isopropanol was added. After vigorously mixing the tubes, 1 µl of Glycogen was added and the RNA was precipitated at -20 °C over night. The next day, the samples were centrifuged for 15 min at 4 °C and 20870 xg. Hereafter, the supernatant was removed and 700 µl of 70% Ethanol were added. Subsequently, the samples were centrifuged for 30 min at 4 °C and 20870 xg and the supernatant was carefully removed. Afterwards, the RNA was dried at RT for 10 min and finally dissolved in 30 µl H₂O for northern blot or 20 µl for RT-qPCR. The samples were used for northern blot or cDNA synthesis and the rest was stored at -20 °C.

5.8.2.2 RNase H digestion

10 µg of RNA were incubated with 3 µl of RNase H 5 U/µl (New England BioLabs) and 6 µM of oligo(dT) 15-mer (100 µM) in 100 µl H₂O for 1 h at 37°C and subsequently purified by phenol-chloroform extraction. The RNase H-treated RNA samples were then analyzed via Northern blotting.

5.8.2.3 Terminator assay

10 µg of RNA were treated with 1 µL of Terminator 5'-phosphate-dependent exonuclease 1 U/µL (Epicentre) in 20 µL of H₂O for 1 h at 37°C and later purified by phenol-chloroform extraction and analyzed via Northern blotting.

5.8.2.4 RNA Immunoprecipitation protocol

The first steps will be described in Chapter 6.8.3.2.1 and 6.8.3.2.2. After these steps, from the cell lysate, 25 µl input samples were collected as western blot and northern blot input. To the western blot input 6 µl of 5x SDS-LD was added, to the northern blot input 75 µl TRizol was added. The remaining lysate was incubated with the pre-coupled beads (see 0) and incubated for 3 h on a spinning wheel at 4 °C. Afterwards, the lysate was centrifuged for 2 min at 1000 xg and 4°C and the supernatant was removed. The IP was washed three times with 1 ml ice cold IP wash buffer. At the last wash step, the resuspended beads were split into two different tubes with 20% of the lysate (200 µl) taken as protein sample and 80% (800 µl) taken for RNA extraction. After centrifuging for 2 min at 1000 xg, at 4°C, the supernatant was removed, and the protein sample was eluted in 30 µl SDS-LD, the RNA sample was eluted by adding 500 µl TRizol.

5.8.2.5 Northern blot

5.8.2.5.1 Northern blot for long RNAs

5.8.2.5.2 MOPS agarose gel electrophoresis

For a capillary northern blot, a MOPS gel containing 1% (w/v) agarose and 2% (w/v) formaldehyde was prepared. The agarose was first dissolved in water, via heating and then 1/10 MOPS buffer (10X) and 1/50 Formaldehyde (37%) were added. The gel was poured into a prepared chamber. After polymerization, the running chamber was filled with 1x MOPS RNA running buffer. From the 30 µl RNA following an isolation using the TRizol reagent (see **Error! Reference source not found.**), 15 µl were taken for one northern blot experiment. The RNA samples were mix 1:1 with the RNA loading dye, denatured for 10' at 65°C and placed immediately on ice. Before loading 2.3 ul EtBr (0.4 mg/ml) were added to the mix. The gel was run at 90 V until the dye moved approximately 1cm above the end (4 – 6 hours).

5.8.2.5.3 Capillary northern blot

Following separation, the RNA is transferred from the gel onto a blotting membrane by capillary transfer. Capillary transfer utilizes a stack of dry paper towels to draw the transfer solution through a wick of blotting paper (such as Whatman 3MM), the gel, the membrane and into the dry towels. Specifically, two long Whatman paper were soaked with 20x SSC and put on a glass plate with both ends reaching into the buffer reservoir. Followed by the inverted gel, the Amersham

Hybond-N nitrocellulose membrane and three pieces Whatman paper were placed on it. Finally, a stack of towels papers and an additional weight of approximately 1 kg were put on top of the blot. The blot was left for 20 h at RT. After transfer is completed, the membrane should be treated to immobilize the nucleic acid, otherwise there is a loss in signal intensity. Therefore, the RNA was crosslinked to the membrane using UV Stratalinker at 254 nm and the transfer was validated under UV light. Afterwards, the membrane was stored at -20°C or immediately incubated with hybridization solution.

5.8.2.5.4 Probe synthesis

To synthesize cDNA probes, radioactively labeled [α -³²P] dCTP and [α -³²P] dATP were used. In Table 30 is listed how the reaction mix was prepared. Afterwards, a PCR (see 5.8.1) with 96 cycles was performed. Finally, the probes were purified using the Illustra MicroSpin G-25 Columns (GE Healthcare) according to the manufacturer's protocol.

Table 30: Composition of a cDNA labeling PCR

Component	Volume
5x HF-buffer	10 μ l
dNTP radioactive PCR mix	1 μ l
[α - ³² P] dCTP	5 μ l
[α - ³² P] dATP	5 μ l
reverse primer (10 μ M)	1 μ l
template (approx. 100 ng)	X
Phusion Polymerase	0.5 μ l
H ₂ O	Up to 50 μ l

5.8.2.5.5 Northern blot hybridization, washing and detecting

The membrane was prehybridized in approx. 30 ml hybridization solution at 65°C. Then the freshly labeled probe was added into the solution and the membrane was incubated o.n. at 65 °C. Afterwards, the membrane was washed briefly with wash solution three and afterwards for 30 min at 65 °C with the solutions three, four and five. After the last wash, the membrane was dried and exposed to a phosphor screen for an appropriate time. Finally, the screen was scanned by the Personal Molecular Imager System (Bio-Rad).

5.8.2.6 Polysome profiling

On Day 1, cells were seeded on a 15cm dish after performing RNAi (in this case, the siPOOLs used were against NegC, YTHDF1-3, YTHDF1-2-3 and TNRC6A-B-C) according to siPOOLs manufacturer protocol or according to high and low density which determines cell's contact

inhibition (in this case no knockdown experiments were performed). 72 hours post knockdown, the medium was changed with medium containing CHX 100ug/ml. To another plate, Puromycin 1mM was used as a control. The cells were incubated for 5 minutes at 37°C, then trypsinized for 5 minutes at 37°C, washed with 1X ice-cold PBS and spun down. The supernatant was removed and the cells were lysed in 1000ul of NET or NP40 Lysis buffer supplemented with 1mM DTT, AEBSF or PIC, 2ul of DNaseI (in 1ml), and 16ul of Ribolock (in 1ml). The lysate was then incubated for 15 minutes on ice, then spun for 15 minutes at 16000g and 4°C. (NET Buffer: 50mM TrisHCl pH7.5, 150mM NaCl, 0.1% TritonX 100, 1mM EDTA, 10% Glycerol). The supernatant was transferred to a new tube and loaded on a 10-50% sucrose gradient (11 steps long sucrose, sucrose solution: TrisHCl pH 7.5 50mM, 150mM KCl, 5mM MgCl₂, + Add fresh CHX 100ug/ml and DTT 1mM). RNA concentration was then measured at the Nanodrop and between 300-500ug of the lysate were loaded. SW40T1 rotor at 35000rpm for 3 hours at 4°C was used and the fractions collected with the Äkta purifier fractionator siFractor.

5.8.3 Protein-based methods

5.8.3.1 Generation of whole cell extracts from mammalian cells

5.8.3.1.1 Harvesting cells from 15 cm confluent cell culture plates

In order to harvest mammalian cells from confluent cell culture plates, the medium was removed and the cells were washed once with 5 ml ice cold 1X PBS. Afterwards, the PBS was removed completely by pipetting. Hereafter, 1000 µl IP-lysis buffer was added and the cells were scratched off the plates. The suspension was transferred to 1.5 ml tubes and incubated for 15 min on ice. After the lysis, the tubes were centrifuged for 15 min at 20870 *xg* at 4 °C. The supernatant containing the cell extract was used for further experiments.

5.8.3.1.2 Harvesting cells from 6 well plates

In order to harvest cells from 6 well cell culture plates, the medium was removed and the cells were washed once with 1 ml ice cold 1X PBS by pipetting. The suspension was collected in 1.5 ml tubes and then centrifuged for 10 min 3000 *xg* at 4°C. The pellet was used for further experiments or snap frozen in liquid nitrogen and stored at - 80°C.

5.8.3.2 Immunoprecipitation (IP)

5.8.3.2.1 Beads preparation

Specific monoclonal antibodies were coupled to the protein G sepharose beads. For each 15 cm plate, approximately 30 µl beads were used. They were washed three times with ice cold 1X PBS (2 min, 1000 *xg*, 4°C) in order to move the ethanol. Then 1 ml 1X PBS and 10 µg antibodies were added to the beads and incubated o.n. at 4°C on a spinning wheel. The next day they were washed

again three times with ice cold IP-wash buffer, if pre-coupled Flag-M2 beads were used, they only were washed this way. After centrifuging (2 min, 1000 *xg*, 4°C) the beads and removing the supernatant carefully they were equilibrated for the IP with additional two rounds of washes with IP lysis buffer.

5.8.3.2.2 Immunoprecipitation protocol

From 1 ml cell lysate, 10 % was collected as input and 25 µl of 5x SDS loading dye (SDS-LD) was added. After collecting the input, the total lysate can be either treated with RNase A or incubated directly with the beads. Treatment with RNase A ensures the specific endoribonucleolytic degradation of single stranded RNAs at C and U residues and is needed to analyze protein-protein interactions. The remaining lysate, were then incubated with the pre-coupled beads for 3 h on a spinning wheel at 4°C. Afterwards, the lysate was centrifuged for 2 min at 1000 *xg* and 4°C and the supernatant was removed. The IP was washed three times with 1 ml ice cold IP wash buffer. At the last wash step the supernatant was removed carefully and the proteins were eluted in 5x SDS-LD. *In vitro* Pulldown Assays

The CCR4-NOT complex domains were kindly received from Tobias Raisch and purified according to Raisch et al., 2019. Concerning the MBP-99aa of YTHDF2, all purification steps were performed on ice in lysis buffer (10mM Hepes pH 7.5, 300mM NaCl, supplemented with fresh 1mM DTT, 1 mg/ml Lysozyme, 5 µg/ml DNaseI, 1:1000 Protase Inhibitor Cocktail). The bacterial lysates were incubated with amylose resin for 1 hour and eluted in lysis buffer supplemented with 30 mM maltose. MBP pulldown assays were performed in a final volume of 500 µl of Lysis buffer. Sonication was performed with 3 cycles of 30 seconds intervals on ice, with Duty 70-80% and Output 3. In order to clear the lysate, the supernatant was centrifuged for 45 min at 20000 *xg* and 4 °C. As last step before chromatography analysis, the lysate was filtrated through a 0.45 µm filter. After clearing, 300uL of amylose resin were added in each tube after equilibration with 1X PBS (2X) and lysis buffer (1X) and MBP only was used as negative control. The proteins were then incubated for 1 hour at 4°C. After that, the beads were spun down at 2000g for 5 minutes at room temperature, washed 3X with lysis buffer, the buffer was removed and fresh buffer in the volume of 500 uL was added. At this point the following concentration of the CCR4-NOT complex components were added (Table 31):

Table 31: Concentration of CCR4-NOT complex components used in the *in vitro* pulldown

Concentration	Components
20uL of 11.5 mg/mL	Hexameric complex
9uL of 12.2 mg/mL	CNOT Module
14 uL of 5.8 mg/mL	MIF4G+CAFI+CCR4

4.4 uL of 7.9 mg/mL	NOT1 X domain
1.6 uL of 30.5 mg/mL	CN9BD+CAF40

The purified proteins were incubated with the MBP-99aa of YTHDF2 fragment for 1 hour at 4°C. The beads were then washed four times with 1X PBS buffer and eluted in elution buffer (PBS supplemented with 30 mM maltose) on ice for 30 minutes by gently tapping the tube every 5 minutes. In the final step, 5uL of 5X SDS Loading Dye were added to the eluate and 100uL of 2X SDS Loading dye were added to the beads. The interaction was then analyzed on a 8% SDS-PAGE followed by coomassie staining.

5.8.3.3 Analyzing proteins using SDS polyacrylamide gel electrophoresis

5.8.3.3.1 Separation of proteins using SDS-PAGE

For the separation of proteins, 10% SDS-polyacrylamide gels were used. Table 32 shows the composition of SDS separation and stacking gels. Every sample contained an appropriate amount of 5x SDS loading buffer and was heated for 5 min at 95°C before loading. The SDS-PAGE was run in 1x SDS running buffer. Big gels were run for 2.5 h at 200 V, minigels were run 1 h at 140 V. Afterwards, the separated proteins were analyzed by either western blot or coomassie staining.

Table 32: Composition of 10 % SDS polyacrylamide gels

Stacking gel	125 mM Tris pH 6.8, 0.1% SDS, 0.15% TEMED, 5% Acrylamid/Bis-Acrylamid (37.5:1), 0.05% APS
Separation gel	380 mM Tris pH 8.8, 0.1% SDS, 0.15% TEMED, 10% Acrylamid/Bis-Acrylamid (37.5:1), 0.05% APS

5.8.3.3.2 Coomassie staining of SDS-PAGE gels

For coomassie staining, the gel was washed briefly with water and then stained with coomassie staining solution for 30 min. afterwards the gel was destained in coomassie destining solution until clear bands were visible and the background signal vanished. The gels were digitalized by scanning with the Odyssey Infrared Imaging System (LI-COR Biosciences).

5.8.3.4 Western Blot

Three Whatman paper were soaked with 1x Tobwin blotting buffer and placed on the positive electrode of a Trans-Blot SD device. On top of them, an Amersham Hybond-ECL membrane (GE Healthcare), activated in 1x Tobwin blotting buffer was placed, followed by the gel and another three soaked Whatman papers. The semi-dry blot runs at 2 mA per cm² of the gel size, for 100

min or 1 min per kDa of estimated protein size. For wet-blot transfer, the sandwich was run at 55V for 3hours at 4°C with a magnetic stirrer. After blotting, the membrane was blocked for at least 30 min in 5% milk in TBS-T. Afterwards, the primary antibody, diluted in blocking solution, was added to the membrane and incubated o.n. at 4°C. Then, the membrane was washed three times for 5 min in TBS-T. After the 3 washing steps, the secondary antibody was added to the membrane and incubated for 1 h at RT in absence of light. Then the membrane was washed three times 5 min in TBS-T and scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences).

5.8.4 Cell culture

5.8.4.1 Cultivation of all described cells

All used cell lines were cultivated at 37 °C in an incubator with air atmosphere and additional 5 % CO₂. DMEM medium containing 10 % FBS (Fetal bovine serum) and 1 % Penicillin/Streptavidin was used. Cells were passaged once they had reached confluency above 90 %.

5.8.4.2 Transfection

5.8.4.2.1 Transfection using RNAi Max Reagent

Lipofectamine RNAi Max reagent was used for transfection reactions of siRNAs (Table 33).

Table 33: precise volumes used for single wells and for plate transfections.

plate type	final volume	Optimem for siPOOL dilution (μl)	siPOOL pre-dilution stock 0,05 or 0,15 μM (μl)	Optimem for RNAiMax dilution (μl)	RNAiMax (μl)	transfection mix/ well (μl)	cell seeding number	cell susp. volume
6 well	2 ml	210	40	246	4	500	0.3 * 10 ⁶	1.5 ml
15 cm	20 ml	2500	40	2500	40	5000	5.1 * 10 ⁶	15 ml

Per reaction two dilutions were created. More in details: for a 6 well plate, 40 μl of siPOOLS in a pre 0.15μM concentration were added to 210 μl of Optimem. A second reactions is then made with 4 μl of RNAi Max added to 210 μl of Optimem. After 5 min incubation at RT, the two mixes were combined and incubated for further 5 min. The final reaction was added to the plate prior cell's seeding. For 15 cm plate the volume is higher but the mix preparations were carried the same way, see Table 33.

5.8.4.2.2 Transfection using the Lipofectamine 2000 Reagent

Per well, 0.8*10⁶ cells were seeded and transfected the next day. Therefore, for every well, the same appropriate amount of plasmid DNA was diluted in 50 μl opti-MEM medium. In another tube, for every transfection 2 μl Lipofectamine was added to 50 μl opti-MEM medium and incubated for

5 min at RT. Afterwards, the two tubes were mixed and incubated for 20 min at RT. Thereafter, 500 µl of the mix were added to every well and the cells were incubated at 37 °C over night. Then, the next day the medium was changed to DMEM and the cells were used for further experiments.

5.8.4.2.3 Transfection using the calcium phosphate method

For the calcium phosphate transfection, for every 15 cm plate and plasmid, 10 µg DNA and 123 µl of 2 M CaCl₂ were diluted in a total volume of 1 ml H₂O in a reaction tube. Afterwards, 1 ml 2x HEPES was added dropwise into the solution during vortexing and incubated for 5 min at RT. The next day the cells were supplied with new medium. Finally, 48 h after transfection, the cells were harvested and used for further experiments.

5.8.4.3 Generation of CRISPR/Cas9 Knock out cell lines

For generation of CRISPR-cas9 Knockout cell lines, HEK293T cell were used. Per well, $0.8 \cdot 10^6$ cells were seeded and transfected the next day with with 1 µg of the respective guide RNAs (for YTHDF2 and FAM120A) inserted into the pSpCas9(BB)-2A-Puro V2.0 -pX459 plasmid using lipofectamine 2000. 24hr post-transfection, the medium was removed and replaced with complete medium containing 6 µg/ml puromycin for two days selection. After that cells were resuspended and reseeded for another round of transfection and a second round of puromycin selection. After two days from the second transfection, the cells were then distributed in a single colony on a 24 well plate and let grow for 2 weeks. At the end of the two weeks the expanded single colonies were tested for knockout by genomic PCR and western blotting.

5.8.4.4 Immunofluorescence

A 24-well format cell culture was prepared with coverslips. Each coverslip-well was washed with 70% EtOH and once with 1X PBS before incubation with 200 µl Gelatin type B (Sigma-Aldrich) solution (1:10 in 1X PBS) at 37 °C. After 2 hours, residual gelatin type B was removed and HEK293T in a concentration of 15000-20000 cells/well in 500 µl were added for growth over night at 37°C in medium supplemented with 10 % FBS (Fetal bovine serum) and 1 % Penicillin/Streptavidin.

Table 34: Buffers used for Immunofluorescence.

Fixation solution	1:10 (10 x stock solution PBS + 37 % formaldehyde)
Stopping solution	1X PBS + 100 mM Glycin
Permeabilization solution	1X PBS + 0,2 % TritonX-100
Blocking solution	1X PBS + 0,05 % TritonX-100 + 3 % BSA

Wash buffer	1X PBS + 0,05% Tween 20 + 1 % BSA
-------------	-----------------------------------

Next day, cells were fixed with ice cold fixation solution (500ul) and incubated for 5 – 10 min at 37°C. The fixation solution was then removed and 1ml of stopping solution was added. The cells were placed at 37°C for 5 min. The cells were then washed with 1x PBS and permeabilized for 15 min with the permeabilization buffer. Afterwards, the cells were washed 4 times with the wash buffer and blocked with the blocking solution for 30 min. Buffers compositions are listed in Table 34. At this point the primary antibodies, diluted in blocking solution, for 1h at RT. The cells were then washed 4 times with the washing solution and secondary antibodies (Alexa Fluor® system) diluted in blocking solution, were added. The cells were incubated for 1h at RT. Finally, the cells were washed again for 4X before being mounted on a microscopy slide after applying 8 µl of ProLong™ Gold with DAPI (Life Technologies) and dried for at least 10 hours in the dark. Confocal images were detected and analyzed on a LSM710 microscopy system (Zeiss), measuring DAPI with an individual 405 nm laser, Alexa Fluor® 488 using an argon laser (488 nm) and Alexa Fluor® 555 with a DPSS laser (561 nm).

5.8.4.5 Tethering assay

HEK293T cells were seeded in 6 well plates (0.8×10^6 cells per well) and transfected 24 hours later using Lipofectamine 2000 (Invitrogen). The backbone of the reporter coding for β -GLOBIN were described previously (Lykke-Andersen et al. 2000; Lazzaretti et al. 2009) and kindly given from Izaurralde lab. These reporters was modified in order to contain five BoxB (β -GLOBIN-5xboxB) binding sites in the 3' UTR. The transfection mixtures contained 0.5 µg BGG-5xBoxB, 0.25 µg BGG-GAP and 0.2 µg of λ N-HA or λ N-HA-AGO2 or 1 µg of λ N-HA-YTHDF1 or λ N-HA-YTHDF2 or λ N-HA-YTHDF3. mRNA deadenylation was blocked by overexpression of 0.5 µg of the VP5-NOT8 catalytic inactive mutant (VP5-HA-NOT8*) or 0.5 µg VP5-HA as control. To interfere with mRNA decapping, 1 µg of the plasmid expressing catalytic inactive DCP2 (VP5-DCP2*), or VP5-HA as control, were expressed in HEK293T cells. The cells transfected with the BGG reporters were collected 24 hours post transfection resuspended with 1mL 1X PBS and divided for 1/4 for protein expression control and 3/4 for RNA extraction. Total RNA was isolated either with TRIzol (Thermo Scientific) or with NucleoSpin® RNA kit from Macherey-Nagel and analyzed via MOPS based northern blot with ³²P-labeled cDNA probes complementary to the full open reading frames of the reporters. Band intensities were quantified with QuantityOne from non-saturated images obtained with Personal Molecular Imager (Phosphoimager) (Biorad).

6 Figures and Tables

Figure 1.1: Intron Exon Boundary in pre-mRNA.....	13
Figure 1.2: Sequence elements required for the efficient cleavage and polyadenylation of the pre-mRNA.....	15
Figure 1.3: Synopsis of eukaryotic gene expression in the nucleus.....	16
Figure 1.4: Synopsis of eukaryotic gene expression in the cytoplasm.....	18
Figure 1.5: Mechanisms of bulk mRNA degradation (Figure adapted and modified from NL Garneau et al., 2007).	22
Figure 1.6: Schematic representation of the domain organization of all the mentioned RBPs that are known to recruit the CCR4-NOT complex.....	25
Figure 1.7: Canonical microRNA biogenesis. (Adapted from Treiber et al., 2019).....	30
Figure 1.8: Structure of human AGO2 (Schirle et al., 2012).....	32
Figure 1.9: Schematic overview of miRNA-mediated gene regulation	33
Figure 1.10: Example of one of the possible mechanisms for miRNA-mediated translational repression:	34
Figure 1.11: Summary of the miRNA-mediated mRNA decay in the cell and the different layers of regulations carried out on and by AGO and TNRC6 proteins.	37
Figure 1.12: AGO phosphorylation cycle to regulate target binding.....	38
Figure 1.13: Domain organization of RBPs that have been reported to have either miRNA-targeting enhancing or miRNA suppressing functions.....	39
Figure 1.14: Synergistic or competitive effects of RNA binding proteins (RBPs) on miRNA-mediated mRNA decay.....	40
Figure 1.15: Schematic representation of the life cycle of m ⁶ A RNA methylation in the cell (Adapted from Yue et al., 2015).....	42
Figure 1.16: YTHDF2 induced mRNA deadenylation.	44
Figure 3.1: Human AGO2 associates with YTHDF2 in sedimentation gradient approach in the absence of RNase A treatment	47
Figure 3.2: Human AGO2 associates with YTHDF2 in sedimentation gradient approach in lysates treated with RNase A.	48
Figure 3.3: Schematic representation of the CCR4-NOT complex and subcomplex modules.....	49
Figure 3.4: YTHDF2 interacts <i>in vitro</i> with the CNOT module and the CCR4-NOT hexameric complex.	50
Figure 3.5: Alphafold prediction of the interaction between the 100aa present at the N-terminal of YTHDF2 NBD and the NOT module of the CCR4-NOT complex.	51
Figure 3.6: Schematic overview of the fragments division of the 100 amino acids of YTHDF2 used for <i>in vitro</i> interaction studies.	51
Figure 3.7: Different SLIM motifs in the YTHDF2 CNBD are essential for an optimal binding to the CCR4-NOT complex.	52
Figure 3.8: YTHDF proteins interact weakly with the CCR4-NOT complex and do not interact with AGO2.....	53
Figure 3.9: YTHDF proteins do not interact with AGO2.	54
Figure 3.10: The interaction of Flag-HA-tagged YTHDF proteins with endogenous PABPC1.	55
Figure 3.11: β -GLOBIN reporters used in this study.....	56
Figure 3.12: YTHDF proteins do not degrade as efficiently as AGO2.	57
Figure 3.13: TNRC6 proteins do not affect YTHDF2-mediated mRNA degradation.	58

Figure 3.14: TNRC6 proteins do not affect YTHDF2-mediated mRNA degradation.	58
Figure 3.15: YTHDF2 requires the CCR4-NOT complex to mediate mRNA deadenylation.....	60
Figure 3.16: YTHDF2 requires the CCR4-NOT complex to mediate mRNA deadenylation.....	61
Figure 3.17: YTHDF proteins absence does not impair AGO2 activity.....	62
Figure 3.18: YTHDF2-mediated mRNA degradation does not occur through the 3'-5' exonucleolytic pathway.....	64
Figure 3.19: DDX6 absence does not affect YTHDF2-mediated mRNA degradation.	65
Figure 3.20: DDX6 absence does not affect YTHDF2-mediated mRNA degradation.	66
Figure 3.21: Distinct effects of AGO2 and YTHDF2 Tethering on the accumulation of BGG-5BoxB reporter intermediates in the absence of XRN1.	67
Figure 3.22: Loss of XRN1 leads to an accumulation of reporter intermediates upon AGO2 tethering and mildly upon YTHDF2.....	67
Figure 3.23: Accumulation of fast migrating band in XRN1 K.O. upon YTHDF2 tethering.	68
Figure 3.24: β -GLOBIN reporters used in this study.....	69
Figure 3.25: Tethering of both single molecules of AGO2 and YTHDF2 is not sufficient to elicit mRNA degradation.	70
Figure 3.26: Tethering of both single molecules of AGO2 and YTHDF2 is not sufficient to elicit mRNA degradation.	71
Figure 3.27: YTHDF proteins have distinct functions in translation regulation.....	72
Figure 3.28: YTHDF proteins have distinct functions in translation regulation.....	73
Figure 3.29: Changes in protein expression of YTHDF proteins in low and high density.	75
Figure 3.30: Changes in protein expression of TNRC6 proteins in low and high density.	76
Figure 3.31: YTHDF protein degradation is not dependent on transcriptional regulation.	77
Figure 3.32: YTHDF1-2 mRNAs expression remained unchanged under both LD and HD conditions in polysome profiling analysis.	78
Figure 3.33: Knockdown validation for FLAM-Seq experiments.....	79
Figure 3.34: Violin plot of poly(A) length distribution per gene across samples.....	81
Figure 3.35: Density plot of poly(A) length distribution per gene across samples in HEK 293T sictr, siDF1-3 and siDF1- 2-3 conditions.	81
Figure 3.36: Density plot of poly(A) length distribution per gene across samples in HEK 293T si CTR, siDF1-3 and siDF1-2-3 and XRN1 K.O. conditions.	82
Figure 3.37: Density plot of poly(A) length distribution per gene across samples in HEK 293T si CTR, siDF1-3 and siDF1-2-3 and HEK 293T XRN1 K.O. si CTR, siDF1-3 and siDF1-2-3 conditions.....	83
Figure 3.38: Violin plot of poly(A) length distribution per gene across samples.....	84
Figure 3.39: Density plot of poly(A) length distribution per gene across samples in HEK 293T si CTR and siTNRC6A-B- C conditions.	84
Figure 3.40: Depletion of YTHDF and XRN1 proteins also induced a shortening of poly(A) tail length for transcripts with a median tail length lower than 150 nucleotides.....	85
Figure 3.41: Depletion of YTHDF and XRN1 proteins induced a more pronounced shortening of poly(A) tail length for transcripts with a median tail length higher than 150 nucleotides.	86
Figure 3.42: Depletion of TNRC6 proteins induces a lengthening of poly(A) tail length for transcripts with median tail length lower than 150 nucleotides.	88
Figure 3.43: Depletion of TNRC6 proteins induced a shortening of poly(A) tail length for transcripts with a median tail length higher than 150 nucleotides.....	89

Figure 3.44: Depletion of YTHDF and XRN1 proteins did not change the poly(A) tail length of mitochondrial transcripts.	90
Figure 3.45: Most variant poly(A) tail length transcripts after FLAM-Seq sequencing used for validation.....	90
Figure 3.46: FLAM-Seq targets validation through Poly(A) tail length assay.	91
Figure 3.47: FLAM-Seq targets validation through ePAT.	92
Figure 3.48: Schematic illustration of the ePAT assay.	92
Figure 3.49: ePAT validation of FLAM-Seq targets.....	93
Figure 3.50: Impact of poly(A) tail shortening on mRNA stability. mRNA levels of validated targets which exhibited poly(A) tail shortening were tested through qPCR experiments.	94
Figure 3.51: Identification of most variant mRNAs from FLAM-Seq read counts.....	96
Figure 3.52: Gene expression analysis from FLAM-Seq counts. Volcano plot depicting the differentially expressed genes in siYTHDF1-3 (siDF1-3) (A) and siYTHDF1-2-3 (siDF1-2-3) conditions (B) relative to si CTR in HEK 293T cells.....	97
Figure 3.53: Identification of most variant mRNAs from FLAM-Seq read counts.....	98
Figure 3.54: Identification of most variant mRNAs from FLAM-Seq read counts.....	100
Figure 3.55: Identification of most variant mRNAs from FLAM-Seq read counts.....	101
Figure 3.56: Comparative analysis of mRNA abundance and poly(A) tail length in XRN1 K.O. HEK 293T cells.	102
Figure 3.57: Comparative analysis of Translation Efficiency (TE) and poly(A) tail length in XRN1 K.O. HEK 293T cells.	103
Figure 3.58: Comparative analysis of Translation Efficiency (TE) and poly(A) tail length in XRN1 K.O. HEK 293T cells.	104
Figure 3.59: Identification of most variant mRNAs from FLAM-Seq read counts.....	105
Figure 3.60: Volcano plot depicting the differentially expressed genes in XRN1 K.O. depleted YTHDF1-3 (siDF1-3) condition relative to si CTR in HEK 293T XRN1 K.O. cells.....	106
Figure 3.61: Volcano plot depicting the differentially expressed genes in XRN1 K.O. depleted YTHDF1-2-3 (siDF1-2-3) condition relative to si CTR in HEK 293T XRN1 K.O. cells.....	107
Figure 4.1: Comparison between AGO2- (A) and YTHDF2- (B) mediated mRNA decay.....	109
Figure 4.2: Comparison between the W-binding pockets of TNRC6 and the YTHDF2 SLIM5 binding to SH domain of NOT1.	110
Figure 4.3: Close-up view of the W-binding pockets of TNRC6 and YTHDF2 SLIM5 binding to SH domain of NOT1 is shown.	111
Table 1: List of the used instruments	117
Table 2: List of the used software and algorithms.....	118
Table 3: List of the used kits	119
Table 4: List of the used solutions and buffers.....	120
Table 5: List of consumables	121
Table 6: List of primer used for sequencing.....	121
Table 7: List of the used primer for cloning.....	121
Table 8: List of the used primer for qPCR.....	124
Table 9: List of the used primer for Northern Blot.....	126

Table 10: List of the used primer for ePAT.	127
Table 11: list of the used vectors.....	127
Table 12: list of the used plasmids.....	128
Table 13: list of used antibodies	131
Table 14: list of all eukaryotic cultured cell lines.....	133
Table 15: list of all prokaryotic cultured cell lines.....	133
Table 16: Composition of a standard 50 µl PCR reaction	134
Table 17: Cycling conditions of a standard PCR reaction	134
Table 18: Composition of restriction digestion reactions	135
Table 19: Composition of a T4 DNA Ligation reaction.....	135
Table 20: Compositions of a test digest reaction.....	136
Table 21: Composition of a quick change PCR reaction	136
Table 22: Cycling conditions of a mutagenesis PCR.....	136
Table 23: Composition of one qPCR master mix reaction	137
Table 24: Composition of G/I tailing mix reaction.....	138
Table 25: Composition of RT mix	138
Table 26: PCR mix	138
Table 27: PCR conditions	139
Table 28: Reverse transcriptase mix	139
Table 29: HOT- PCR mix e-PAT	139
Table 39: Composition of a cDNA labeling PCR.....	142
Table 43: Concentration of CCR4-NOT complex components used in the <i>in vitro</i> pulldown.....	144
Table 44: Composition of 10 % SDS polyacrylamide gels	145
Table 47: precise volumes used for single wells and for plate transfections.....	146
Table 48: Buffers used for Immunofluorescence.	147

7 List of Abbreviations

Units were abbreviated according to the International System of Units. Chemical names not indicated below were abbreviated according to the IUPAC (International Union of Pure and Applied Chemistry) recommendations. Protein names not indicated below were abbreviated according to the UNIPROT database.

4E-BM	eIF4E-binding motifs
4E-BPs	eIF4E-binding proteins
A	Adenine
Ala	Alanine
Asp	Aspartic acid
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>
AA	Amino acid
aa-tRNA	Aminoacyl-tRNA
ABD	AGO-binding domain
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
Amp	Ampicillin
APRO	AntiPROliferative domain
APS	Ammonium persulphate
AREs	AU-rich elements
ATP	Adenosine triphosphate
Bis	N,N'-methylene bisacrylamide
Bp	Base pair(s)
BSA	Bovine serum albumin
C	Cytosine
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
Cas9	CRISPR-associated protein 9
CBC	Cap binding complex
CBM	CAF40-binding motif
cDNA	Complementary DNA
CDS	Coding sequence
CIM	CCR4-NOT interacting motif
circRNA	Circular RNA
CNBD	CNOT Binding Domain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRL	Cullin-RING ubiquitin ligase
CSD	Cold Shock Domain
CTD	C-terminal domain
C-terminal	Carboxyl terminal
cv	Column volume
D	Aspartic acid
<i>D. melanogaster</i>	<i>Drosophila melanogaster</i>
DCPS	Decapping scavenger enzyme
DMEM	Dulbecco's Modified Eagle's Medium

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ds	Double-stranded
DSE	Downstream sequence element
DTT	Dithiothreitol
E	Glutamic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDC	1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimid
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
EJC	Exon junction complex
et al.	And others
EtBr	Ethidium bromide
FBS	Fetal bovie serum
G	Guanine
GAP	GTPase-activating protein
GFP	Green fuorescent protein
Gly	Glycine
Glu	Glutamic acid
GMP	Guanosine monophosphate
GSH	Glutathione
GST	Glutahione-S-transferase
GTP	Guanosine triphosphate
GYF	Glycine-tyrosine-phenylalanine
<i>H. sapiens</i>	<i>Homo sapiens</i>
HA	Hemagglutinin
HEK 293T	Human embryonic kidney 293T
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High pressure liquid chromatography
IDRs	Intrinsically Disordered Regions
IF	Immunofluorescence
IP	Immunoprecipitation
IPTG	Isopropyl -D-1-thiogalactopyranoside
Kana	Kanamycin
Kb	Kilobase
kDa	Kilodalton
KH	K Homology (KH) domain
K.O.	Knock Out
LB	Lysogeny broth
LC	Liquid chromatography
LLPS	liquid liquid phase separation
lncRNA	Long non-coding RNA
m6A	N-6-methyl-adenosine
m7G	7-methylguanosine
mESC	Mouse embryonic stem cells
MID	Middle

miRNA	MicroRNA
MOPS	3-morpholinopropane-1-sulfonic acid
mRNA	MRNA messenger RNA
mRNP	Messenger ribonucleoprotein
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NBD	NOT1 Binding Domain
NBR	NOT module binding region
NCBI	National center for biotechnology information
ncRNA	Non-coding RNA
NED	Nanos effector domain
NGD	No-go decay
NIM	NOT1-interacting motif
NMD	Nonsense-mediated mRNA decay
NPCs	Nuclear pore complexes
NSD	Nonstop decay
nts	nucleotides(s)
N-terminal	Amino terminal
o.n.	Over night
OH	Hydroxide
ORF	Open reading frame
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PAM2	PABP interacting motif 2
PAZ	PIWI-Argonaute-Zwille
P-bodies	Processing bodies
PBS	Phosphate-buffered saline
PBS (-T)	Phosphate-buffered saline (containing Tween 20)
PC	Proline-rich C-terminus
PCM	Pumilio conserved motifs
PCR	Polymerase chain reaction
PIN	PiLT N terminus domain
piRNA	PIWI-interacting RNA
PIWI	P-element-induced wimpy testes
PNK	Polynucleotide kinase
pre-miRNA	Precursor miRNA
pre-mRNA	Precursor-messenger RNA
pri-miRNA	Primary miRNA
PTC	Premature termination codon
PTM	Post-translational modification
qPCR	Quantitative PCR
RBDs	RNA binding domains
RBPs	RNA binding proteins
RD	Repression domains
Rec-A	Recombination-A domain
RING	RING-finger E3 ubiquitin ligase domain
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid

RNAi	RNA interference
RNase A	Ribonuclease A
RNase H	Ribonuclease H
RNP	Ribonucleoprotein
Rpm	Revolutions per minute
RRM	RNA recognition motif
rRNA	Ribosomal RNA
RT	Room temperature
<i>S.c</i>	<i>Saccharomyces cerevisiae</i>
SAM	S-adenosyl-methionine
SD	Silencing domain
SDS	Sodium dodecyl sulphate
SG	Stress granule
SH domain	Superfamily homology domain
siRNA	Small interfering RNA
SLiMs	Short linear motifs
snRNA	Small nuclear RNA
ss	Single-stranded
SSC	saline-sodium citrate buffer
T	Thymine
TBE	Tris/Borate/EDTA
TBS	Tris-buffered saline (containing Tween 20)
TBS-T	Tris buffered saline + 0.05 % Tween
TDMD	Target-directed miRNA degradation
TEMED	tetramethylethylenediamine
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
tRNA	transfer RNA
tRNAi	tRNA initiator
U	Uracil
UNR	UNR-interacting domain
UTR	Untranslated region
UV	Ultraviolet (light)
W	Tryptophan
Y	Tyrosine
YTH	YT521-B homology
Znf	Zinc finger domain

8 Bibliography

- Albert TK, Hanzawa H, Legtenberg YI, de Ruwe MJ, van den Heuvel FA, Collart MA, Boelens R and T. HT. (2002). "Identification of a ubiquitin-protein ligase subunit within the CCR4-NOT transcription repressor complex." *EMBO J Feb* **1;21(3):355-64**.
- Alexandrov, A., D. Colognori, M. D. Shu and J. A. Steitz (2012). "Human spliceosomal protein CWC22 plays a role in coupling splicing to exon junction complex deposition and nonsense-mediated decay." *Proc Natl Acad Sci U S A* **109(52): 21313-21318**.
- Ameres, S. L. and P. D. Zamore (2013). "Diversifying microRNA sequence and function." *Nat Rev Mol Cell Biol* **14(8): 475-488**.
- Amrani, N., M. S. Sachs and A. Jacobson (2006). "Early nonsense: mRNA decay solves a translational problem." *Nat Rev Mol Cell Biol* **7(6): 415-425**.
- Ana Casañal, A. K., 1* Chris H. Hill,1 Ashley D. Easter,1 Paul Emsley,1 Gianluca Degliesposti,1 Yuliya Gordiyenko,1,2 Balaji Santhanam,1 Jana Wolf,1 Katrin Wiederhold,1 Gillian L. Dornan,1 Mark Skehel,1 Carol V. Robinson,2 Lori A. Passmore (2017). "Architecture of eukaryotic mRNA 3'-end processing machinery."
- Arribas-Layton, M., D. Wu, J. Lykke-Andersen and H. Song (2013). "Structural and functional control of the eukaryotic mRNA decapping machinery." *Biochim Biophys Acta* **1829(6-7): 580-589**.
- Arvola, R. M., C. T. Chang, J. P. Buytendorp, Y. Leviansky, E. Valkov, P. L. Freddolino and A. C. Goldstrohm (2020). "Unique repression domains of Pumilio utilize deadenylation and decapping factors to accelerate destruction of target mRNAs." *Nucleic Acids Res* **48(4): 1843-1871**.
- Arvola, R. M., C. A. Weidmann, T. M. Tanaka Hall and A. C. Goldstrohm (2017). "Combinatorial control of messenger RNAs by Pumilio, Nanos and Brain Tumor Proteins." *RNA Biol* **14(11): 1445-1456**.
- Asaoka-Taguchi, M., Yamada, M., Nakamura (1999). "Maternal Pumilio acts together with Nanos in germline development in Drosophila embryos." *Nat Cell Biol* **1, 431-437**.
- Ballut, L., B. Marchadier, A. Baguet, C. Tomasetto, B. Seraphin and H. Le Hir (2005). "The exon junction core complex is locked onto RNA by inhibition of eIF4AIII ATPase activity." *Nat Struct Mol Biol* **12(10): 861-869**.
- Baralle, M. and F. E. Baralle (2018). "The splicing code." *Biosystems* **164: 39-48**.
- Barik, S., B. Gosh, D. Lazinski, W. Whalen and D. A. (1987). "An antitermination protein engages the elongating transcription apparatus at a promoter-proximal recognition site." *Cell Sep* **11;50(6):885-99**.
- Bartel, D. P. (2018). "Metazoan MicroRNAs." *Cell* **173(1): 20-51**.
- Bartlam, M. and T. Yamamoto (2010). "The structural basis for deadenylation by the CCR4-NOT complex." *Protein Cell* **1(5): 443-452**.
- Behm-Ansmant, I., J. Rehwinkel, T. Doerks, A. Stark, P. Bork and E. Izaurralde (2006). "mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes." *Genes Dev* **20(14): 1885-1898**.
- Bhandari, D., T. Raisch, O. Weichenrieder, S. Jonas and E. Izaurralde (2014). "Structural basis for the Nanos-mediated recruitment of the CCR4-NOT complex and translational repression." *Genes Dev* **28(8): 888-901**.
- Bhattacharyya, S. N., R. Habermacher, U. Martine, E. I. Closs and W. Filipowicz (2006). "Relief of microRNA-mediated translational repression in human cells subjected to stress." *Cell* **125(6): 1111-1124**.
- Bitetti, A., A. C. Mallory, E. Golini, C. Carrieri, H. Carreño Gutiérrez, E. Perlas, Y. A. Pérez-Rico, G. P. Tocchini-Valentini, A. J. Enright, W. H. J. Norton, S. Mandillo, D. O'Carroll and A. Shkumatava (2018). "MicroRNA degradation by a conserved target RNA regulates animal behavior." *Nature Structural & Molecular Biology* **25(3): 244-251**.
- Black, D. L. (2003). "Mechanisms of alternative pre-messenger RNA splicing." *Annu Rev Biochem* **72: 291-336**.
- Boeck, R., S. Tarun, Jr., M. Rieger, J. A. Deardorff, S. Muller-Auer and A. B. Sachs (1996). "The yeast Pan2 protein is required for poly(A)-binding protein-stimulated poly(A)-nuclease activity." *J Biol Chem* **271(1): 432-438**.
- Bohnsack, M. T., K. Czapinski and D. Gorlich (2004). "Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs." *RNA* **10(2): 185-191**.
- Boland, A., Y. Chen, T. Raisch, S. Jonas, D. Kuzuoglu-Ozturk, L. Wohlbold, O. Weichenrieder and E. Izaurralde (2013). "Structure and assembly of the NOT module of the human CCR4-NOT complex." *Nat Struct Mol Biol* **20(11): 1289-1297**.
- Boo, S. H., H. Ha, Y. Lee, M. K. Shin, S. Lee and Y. K. Kim (2022). "UPF1 promotes rapid degradation of m(6)A-containing RNAs." *Cell Rep* **39(8): 110861**.
- Braun, J. E., E. Huntzinger, M. Fauser and E. Izaurralde (2011). "GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets." *Mol Cell* **44(1): 120-133**.
- Braun, J. E., V. Truffault, A. Boland, E. Huntzinger, C. T. Chang, G. Haas, O. Weichenrieder, M. Coles and E. Izaurralde (2012). "A direct interaction between DCP1 and XRN1 couples mRNA decapping to 5' exonucleolytic degradation." *Nat Struct Mol Biol* **19(12): 1324-1331**.
- Brennecke, J., A. Stark, R. B. Russell and S. M. Cohen (2005). "Principles of microRNA-target recognition." *PLoS Biol* **3(3): e85**.

Briskin, D., P. Y. Wang and D. P. Bartel (2020). "The biochemical basis for the cooperative action of microRNAs." *Proc Natl Acad Sci U S A* **117**(30): 17764-17774.

Buck, A. H., J. Perot, M. A. Chisholm, D. S. Kumar, L. Tuddenham, V. Cognat, L. Marcinowski, L. Dolken and S. Pfeffer (2010). "Post-transcriptional regulation of miR-27 in murine cytomegalovirus infection." *RNA* **16**(2): 307-315.

Cai, X., C. H. Hagedorn and B. R. Cullen (2004). "Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs." *RNA* **10**(12): 1957-1966.

Calabretta, S. and S. Richard (2015). "Emerging Roles of Disordered Sequences in RNA-Binding Proteins." *Trends Biochem Sci* **40**(11): 662-672.

Caponigro G, P. R. (1995). "Multiple functions for the poly(A)binding protein in mRNA decapping and deadenylation in yeast." *Genes Dev* **9**(19):2421-32.

Carthew, R. W. and E. J. Sontheimer (2009). "Origins and Mechanisms of miRNAs and siRNAs." *Cell* **136**(4): 642-655.

Casanal, A., A. Kumar, C. H. Hill, A. D. Easter, P. Emsley, G. Degliesposti, Y. Gordiyenko, B. Santhanam, J. Wolf, K. Wiederhold, G. L. Dornan, M. Skehel, C. V. Robinson and L. A. Passmore (2017). "Architecture of eukaryotic mRNA 3'-end processing machinery." *Science* **358**(6366): 1056-1059.

Cazalla D, Y. T., Steitz JA. (2010). "Down-regulation of a host microRNA by a Herpesvirus saimiri noncoding RNA. ." *Science*.

Chang, C. T., S. Muthukumar, R. Weber, Y. Leviansky, Y. Chen, D. Bhandari, C. Igreja, L. Wohlbold, E. Valkov and E. Izaurralde (2019). "A low-complexity region in human XRN1 directly recruits deadenylation and decapping factors in 5'-3' messenger RNA decay." *Nucleic Acids Res* **47**(17): 9282-9295.

Chapat, C., S. M. Jafarnejad, E. Matta-Camacho, G. G. Hesketh, I. A. Gelbart, J. Attig, C. G. Gkogkas, T. Alain, N. Stern-Ginossar, M. R. Fabian, A. C. Gingras, T. F. Duchaine and N. Sonenberg (2017). "Cap-binding protein 4EHP effects translation silencing by microRNAs." *Proc Natl Acad Sci U S A* **114**(21): 5425-5430.

Chekulaeva, M., H. Mathys, J. T. Zipprich, J. Attig, M. Colic, R. Parker and W. Filipowicz (2011). "miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs." *Nat Struct Mol Biol* **18**(11): 1218-1226.

Chen, C. Y. and A. B. Shyu (2011). "Mechanisms of deadenylation-dependent decay." *Wiley Interdiscip Rev RNA* **2**(2): 167-183.

Chen, Y., A. Boland, D. Kuzuoglu-Ozturk, P. Bawankar, B. Loh, C. T. Chang, O. Weichenrieder and E. Izaurralde (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.

Chen, Y., E. Khazina, E. Izaurralde and O. Weichenrieder (2021). "Crystal structure and functional properties of the human CCR4-CAF1 deadenylase complex." *Nucleic Acids Res* **49**(11): 6489-6510.

Cheng, H., K. Dufu, C. S. Lee, J. L. Hsu, A. Dias and R. Reed (2006). "Human mRNA export machinery recruited to the 5' end of mRNA." *Cell* **127**(7): 1389-1400.

Christian B. F. Andersen, Lionel Ballut, Jesper S. Johansen, Hala Chamieh, Klaus H. Nielsen, Cristiano L. P. Oliveira, Jan Skov Pedersen, Bertrand Séraphin, Hervé Le Hir and G. R. Andersen (2006). "Structure of the Exon Junction Core Complex with a Trapped DEAD-Box ATPase Bound to RNA."

Christie, M., A. Boland, E. Huntzinger, O. Weichenrieder and E. Izaurralde (2013). "Structure of the PAN3 pseudokinase reveals the basis for interactions with the PAN2 deadenylase and the GW182 proteins." *Mol Cell* **51**(3): 360-373.

Clerici, M., M. Faini, R. Aebersold and M. Jinek (2017). "Structural insights into the assembly and polyA signal recognition mechanism of the human CPSF complex." *Elife* **6**.

Collart, M. A. (2016). "The Ccr4-Not complex is a key regulator of eukaryotic gene expression." *Wiley Interdiscip Rev RNA* **7**(4): 438-454.

Conti, E. and E. Izaurralde (2005). "Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species." *Curr Opin Cell Biol* **17**(3): 316-325.

Corley, M., M. C. Burns and G. W. Yeo (2020). "How RNA-Binding Proteins Interact with RNA: Molecules and Mechanisms." *Mol Cell* **78**(1): 9-29.

Curtis, D., D. K. Treiber, F. Tao, P. D. Zamore, J. R. Williamson and R. Lehmann (1997). "A CCHC metal-binding domain in Nanos is essential for translational regulation." *EMBO J* **16**(4): 834-843.

D'Orazio, K. N. and R. Green (2021). "Ribosome states signal RNA quality control." *Mol Cell* **81**(7): 1372-1383.

Davey, N. E., M. S. Cyert and A. M. Moses (2015). "Short linear motifs - ex nihilo evolution of protein regulation." *Cell Commun Signal* **13**: 43.

Denli, A., Tops, B., Plasterk, R. et al. (2004). "Processing of primary microRNAs by the Microprocessor complex." *Nature* **432**, 231-235.

Dever, T. E., J. D. Dinman and R. Green (2018). "Translation Elongation and Recoding in Eukaryotes." *Cold Spring Harb Perspect Biol* **10**(8).

Doma, M. K. and R. Parker (2006). "Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation." *Nature* **440**(7083): 561-564.

Du, H., Y. Zhao, J. He, Y. Zhang, H. Xi, M. Liu, J. Ma and L. Wu (2016). "YTHDF2 destabilizes m(6)A-containing RNA through direct recruitment of the CCR4-NOT deadenylase complex." *Nat Commun* **7**: 12626.

Dueck, A., C. Ziegler, A. Eichner, E. Berezikov and G. Meister (2012). "microRNAs associated with the different human Argonaute proteins." *Nucleic Acids Res* **40**(19): 9850-9862.

Due Tankmar M, Reichel M, Arribas-Hernández L, Brodersen P. A YTHDF-PABP interaction is required for m⁶A-mediated organogenesis in plants. *EMBO Rep*. 2023 Dec 6;**24**(12):e57741. doi: 10.15252/embr.202357741. Epub 2023 Nov 27. PMID: 38009565; PMCID: PMC10702811.

Eckmann, C. R., C. Rammelt and E. Wahle (2011). "Control of poly(A) tail length." *Wiley Interdiscip Rev RNA* **2**(3): 348-361.

Eisen, T. J., S. W. Eichhorn, A. O. Subtelny and D. P. Bartel (2020). "MicroRNAs Cause Accelerated Decay of Short-Tailed Target mRNAs." *Mol Cell* **77**(4): 775-785 e778.

Elisa Izaurralde, Janusz Stepinski, Edward Darzynkiewicz and a. I. W. Mattaj* (1992). "Izaurralde E, Stepinski J, Darzynkiewicz E, Mattaj I W." *J Cell Biol Sep*; **118**(6):1287-95.

Elisa Izaurralde, Joe Lewis, Marzena Jankowska, Caroline McGuigan, Edward Darzynkiewicz and I. W. Mattaj" (1994). "A nuclear cap binding protein complex involved in pre-mRNA splicing " *cell* **Aug 26**; **78**(4):657-68.

Elkayam, E., C. D. Kuhn, A. Tocilj, A. D. Haase, E. M. Greene, G. J. Hannon and L. Joshua-Tor (2012). "The structure of human argonaute-2 in complex with miR-20a." *Cell* **150**(1): 100-110.

Enwerem, III, N. D. Elrod, C. T. Chang, A. Lin, P. Ji, J. A. Bohn, Y. Leviansky, E. J. Wagner, E. Valkov and A. C. Goldstrohm (2021). "Human Pumilio proteins directly bind the CCR4-NOT deadenylase complex to regulate the transcriptome." *RNA* **27**(4): 445-464.

Eulalio, A., I. Behm-Ansmant, D. Schweizer and E. Izaurralde (2007). "P-body formation is a consequence, not the cause, of RNA-mediated gene silencing." *Mol Cell Biol* **27**(11): 3970-3981.

Eulalio, A., S. Helms, C. Fritsch, M. Fauser and E. Izaurralde (2009). "A C-terminal silencing domain in GW182 is essential for miRNA function." *RNA* **15**(6): 1067-1077.

Eulalio, A., E. Huntzinger and E. Izaurralde (2008). "GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay." *Nat Struct Mol Biol* **15**(4): 346-353.

Eulalio, A., E. Huntzinger, T. Nishihara, J. Rehwinkel, M. Fauser and E. Izaurralde (2009). "Deadenylation is a widespread effect of miRNA regulation." *RNA* **15**(1): 21-32.

Eulalio, A., F. Tritschler and E. Izaurralde (2009). "The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing." *RNA* **15**(8): 1433-1442.

Eystathioy, T., A. Jakymiw, E. K. Chan, B. Seraphin, N. Cougot and M. J. Fritzler (2003). "The GW182 protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies." *RNA* **9**(10): 1171-1173.

Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, Svitkin YV, Rivas F, Jinek M, Wohlschlegel J, Doudna JA, Chen CY, Shyu AB, Yates JR 3rd, Hannon GJ, Filipowicz W, Duchaine TF, Sonenberg N. Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Mol Cell*. 2009 Sep 24;**35**(6):868-80. doi: 10.1016/j.molcel.2009.08.004. Epub 2009 Aug 27. PMID: 19716330; PMCID: PMC2803087.

Fabian, M. R., M. K. Cieplak, F. Frank, M. Morita, J. Green, T. Srikumar, B. Nagar, T. Yamamoto, B. Raught, T. F. Duchaine and N. Sonenberg (2011). "miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT." *Nat Struct Mol Biol* **18**(11): 1211-1217.

Fabian, M. R., F. Frank, C. Rouya, N. Siddiqui, W. S. Lai, A. Karetnikov, P. J. Blackshear, B. Nagar and N. Sonenberg (2013). "Structural basis for the recruitment of the human CCR4-NOT deadenylase complex by tristetraprolin." *Nat Struct Mol Biol* **20**(6): 735-739.

Fabian, M. R. and N. Sonenberg (2012). "The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC." *Nat Struct Mol Biol* **19**(6): 586-593.

Flaherty SM, F. P., Izaurralde E, Mattaj IW, Gilmartin GM. (1997 Oct 28). "Participation of the nuclear cap binding complex in pre mrna 3 processing." *Proc Natl Acad Sci U S A* **94**(22):11893-8.

Frank, F., N. Sonenberg and B. Nagar (2010). "Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2." *Nature* **465**(7299): 818-822.

Frohn, A., H. C. Eberl, J. Stohr, E. Glasmacher, S. Rudel, V. Heissmeyer, M. Mann and G. Meister (2012). "Dicer-dependent and -independent Argonaute2 protein interaction networks in mammalian cells." *Mol Cell Proteomics* **11**(11): 1442-1456.

Fu, Y., D. Dominissini, G. Rechavi and C. He (2014). "Gene expression regulation mediated through reversible m(6)A RNA methylation." *Nat Rev Genet* **15**(5): 293-306.

Fuchs, A. L., J. P. Wurm, A. Neu and R. Sprangers (2020). "Molecular basis of the selective processing of short mRNA substrates by the DcpS mRNA decapping enzyme." *Proc Natl Acad Sci U S A* **117**(32): 19237-19244.

Galloway, A. and V. H. Cowling (2019). "mRNA cap regulation in mammalian cell function and fate." *Biochim Biophys Acta Gene Regul Mech* **1862**(3): 270-279.

Garneau, N. L., J. Wilusz and C. J. Wilusz (2007). "The highways and byways of mRNA decay." *Nat Rev Mol Cell Biol* **8**(2): 113-126.

Gatfield, D. and E. Izaurralde (2002). "REF1/Aly and the additional exon junction complex proteins are dispensable for nuclear mRNA export." *J Cell Biol* **159**(4): 579-588.

Gebert, L. F. R. and I. J. MacRae (2019). "Regulation of microRNA function in animals." *Nat Rev Mol Cell Biol* **20**(1): 21-37.

Ghini, F., C. Rubolino, M. Climent, I. Simeone, M. J. Marzi and F. Nicassio (2018). "Endogenous transcripts control miRNA levels and activity in mammalian cells by target-directed miRNA degradation." *Nat Commun* **9**(1): 3119.

Glavan, F., I. Behm-Ansmant, E. Izaurralde and E. Conti (2006). "Structures of the PIN domains of SMG6 and SMG5 reveal a nuclease within the mRNA surveillance complex." *EMBO J* **25**(21): 5117-5125.

Golden, R. J., B. Chen, T. Li, J. Braun, H. Manjunath, X. Chen, J. Wu, V. Schmid, T. C. Chang, F. Kopp, A. Ramirez-Martinez, V. S. Tagliabracci, Z. J. Chen, Y. Xie and J. T. Mendell (2017). "An Argonaute phosphorylation cycle promotes microRNA-mediated silencing." *Nature* **542**(7640): 197-202.

Goldstrohm, A. C., T. M. T. Hall and K. M. McKenney (2018). "Post-transcriptional Regulatory Functions of Mammalian Pumilio Proteins." *Trends Genet* **34**(12): 972-990.

Graifer, D. and G. Karpova (2015). "Interaction of tRNA with eukaryotic ribosome." *Int J Mol Sci* **16**(4): 7173-7194.

Gregersen, L. H., M. Schueler, M. Munschauer, G. Mastrobuoni, W. Chen, S. Kempa, C. Dieterich and M. Landthaler (2014). "MOV10 Is a 5' to 3' RNA helicase contributing to UPF1 mRNA target degradation by translocation along 3' UTRs." *Mol Cell* **54**(4): 573-585.

Grishok A, P. A., Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC (2001). "Genes and Mechanisms Related to RNA Interference Regulate Expression of the Small Temporal RNAs that Control *C. elegans* Developmental Timing." *Cell* **Jul 13;106(1):23-34**.

Grudzien-Nogalska, E. and M. Kiledjian (2017). "New insights into decapping enzymes and selective mRNA decay." *Wiley Interdiscip Rev RNA* **8**(1).

Han, J., C. A. LaVigne, B. T. Jones, H. Zhang, F. Gillett and J. T. Mendell (2020). "A ubiquitin ligase mediates target-directed microRNA decay independently of tailing and trimming." *Science* **370**(6523).

Han, J., Y. Lee, K. H. Yeom, Y. K. Kim, H. Jin and V. N. Kim (2004). "The Drosha-DGCR8 complex in primary microRNA processing." *Genes Dev* **18**(24): 3016-3027.

Han, P., Y. Shichino, T. Schneider-Poetsch, M. Mito, S. Hashimoto, T. Udagawa, K. Kohno, M. Yoshida, Y. Mishima, T. Inada and S. Iwasaki (2020). "Genome-wide Survey of Ribosome Collision." *Cell Rep* **31**(5): 107610.

Hauptmann, J., A. Dueck, S. Harlander, J. Pfaff, R. Merkl and G. Meister (2013). "Turning catalytically inactive human Argonaute proteins into active slicer enzymes." *Nat Struct Mol Biol* **20**(7): 814-817.

He, P. C. and C. He (2021). "m(6) A RNA methylation: from mechanisms to therapeutic potential." *EMBO J* **40**(3): e105977.

Heck, A. M. and J. Wilusz (2018). "The Interplay between the RNA Decay and Translation Machinery in Eukaryotes." *Cold Spring Harb Perspect Biol* **10**(5).

Hennig, J. and M. Sattler (2015). "Deciphering the protein-RNA recognition code: combining large-scale quantitative methods with structural biology." *Bioessays* **37**(8): 899-908.

Herve Le Hir, David Gatfield, E. Izaurralde and a. M. J. Moore (2001). "The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay." *EMBO J* **Sep 3;20(17):4987-97**.

Herzel, L., D. S. M. Ottoz, T. Alpert and K. M. Neugebauer (2017). "Splicing and transcription touch base: co-transcriptional spliceosome assembly and function." *Nat Rev Mol Cell Biol* **18**(10): 637-650.

Hinnebusch, A. G. (2017). "Structural Insights into the Mechanism of Scanning and Start Codon Recognition in Eukaryotic Translation Initiation." *Trends Biochem Sci* **42**(8): 589-611.

Hodge, C. A., E. J. Tran, K. N. Noble, A. R. Alcazar-Roman, R. Ben-Yishay, J. J. Scarcelli, A. W. Folkmann, Y. Shav-Tal, S. R. Wentz and C. N. Cole (2011). "The Dbp5 cycle at the nuclear pore complex during mRNA export I: dbp5 mutants with defects in RNA binding and ATP hydrolysis define key steps for Nup159 and Gle1." *Genes Dev* **25**(10): 1052-1064.

Huntzinger, E., J. E. Braun, S. Heimstadt, L. Zekri and E. Izaurralde (2010). "Two PABPC1-binding sites in GW182 proteins promote miRNA-mediated gene silencing." *EMBO J* **29**(24): 4146-4160.

Huntzinger, E., D. Kuzuoglu-Ozturk, J. E. Braun, A. Eulalio, L. Wohlbold and E. Izaurralde (2013). "The interactions of GW182 proteins with PABP and deadenylases are required for both translational repression and degradation of miRNA targets." *Nucleic Acids Res* **41**(2): 978-994.

Hutvagner, G. and M. J. Simard (2008). "Argonaute proteins: key players in RNA silencing." *Nat Rev Mol Cell Biol* **9**(1): 22-32.

Igreja, C. and E. Izaurralde (2011). "CUP promotes deadenylation and inhibits decapping of mRNA targets." *Genes Dev* **25**(18): 1955-1967.

Ikeuchi, K., P. Tesina, Y. Matsuo, T. Sugiyama, J. Cheng, Y. Saeki, K. Tanaka, T. Becker, R. Beckmann and T. Inada (2019). "Collided ribosomes form a unique structural interface to induce Hel2-driven quality control pathways." *EMBO J* **38**(5).

Iwasaki, S., M. Kobayashi, M. Yoda, Y. Sakaguchi, S. Katsuma, T. Suzuki and Y. Tomari (2010). "Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes." *Mol Cell* **39**(2): 292-299.

Jackson, R. J., C. U. Hellen and T. V. Pestova (2010). "The mechanism of eukaryotic translation initiation and principles of its regulation." *Nat Rev Mol Cell Biol* **11**(2): 113-127.

Jean-Christophe Dantonel, Kanneganti G. K. Murthy, J. L. Manley and L. Tora (1997). "Transcription factor TFIID recruits factor CPSF for formation of 3' end of mRNA".

Jia, G., Y. Fu, X. Zhao, Q. Dai, G. Zheng, Y. Yang, C. Yi, T. Lindahl, T. Pan, Y. G. Yang and C. He (2011). "N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO." *Nat Chem Biol* **7**(12): 885-887.

Jinek, M. and J. A. Doudna (2009). "A three-dimensional view of the molecular machinery of RNA interference." *Nature* **457**(7228): 405-412.

Jinek, M., M. R. Fabian, S. M. Coyle, N. Sonenberg and J. A. Doudna (2010). "Structural insights into the human GW182-PABC interaction in microRNA-mediated deadenylation." *Nat Struct Mol Biol* **17**(2): 238-240.

Jing Zhao, L. Hyman and a. C. Moore (1999). "Formation of mRNA 3' ends in eukaryotes mechanism, regulation, and interrelationships with other steps in mRNA synthesis".

Joao P. Rodrigues, Michaela Rode, David Gatfield, Benjamin J. Blencowe, Maria Carmo-Fonseca and a. E. Izaurralde (2001). "REF proteins mediate the export of spliced and unspliced mRNAs from the nucleus.pdf."

Joe D. Lewis, D. Görlich and a. I. W. Mattaj (1996). "A yeast cap binding protein complex (yCBC) acts at an early step in pre-mRNA splicing." *Nucleic Acids Res* **Sep 1;24(17):3332-6**.

Jonas, S. and E. Izaurralde (2013). "The role of disordered protein regions in the assembly of decapping complexes and RNP granules." *Genes Dev* **27**(24): 2628-2641.

Jonas, S. and E. Izaurralde (2015). "Towards a molecular understanding of microRNA-mediated gene silencing." *Nat Rev Genet* **16**(7): 421-433.

Jumper, J., R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Zidek, A. Potapenko, A. Bridgland, C. Meyer, S. A. A. Kohl, A. J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A. W. Senior, K. Kavukcuoglu, P. Kohli and D. Hassabis (2021). "Highly accurate protein structure prediction with AlphaFold." *Nature* **596**(7873): 583-589.

Juszkiewicz, S., V. Chandrasekaran, Z. Lin, S. Kraatz, V. Ramakrishnan and R. S. Hegde (2018). "ZNF598 Is a Quality Control Sensor of Collided Ribosomes." *Mol Cell* **72**(3): 469-481 e467.

Kajjo, S., S. Sharma, S. Chen, W. R. Brothers, M. Cott, B. Hasaj, P. Jovanovic, O. Larsson and M. R. Fabian (2022). "PABP prevents the untimely decay of select mRNA populations in human cells." *EMBO J* **41**(6): e108650.

Kapp, L. D. and J. R. Lorsch (2004). "The molecular mechanics of eukaryotic translation." *Annu Rev Biochem* **73**: 657-704.

Karousis, E. D. and O. Muhlemann (2019). "Nonsense-Mediated mRNA Decay Begins Where Translation Ends." *Cold Spring Harb Perspect Biol* **11**(2).

Katahira, J. (2012). "mRNA export and the TREX complex." *Biochim Biophys Acta* **1819**(6): 507-513.

Katsantoni, M., E. van Nimwegen and M. Zavolan (2023). "Improved analysis of (e)CLIP data with RCRUNCH yields a compendium of RNA-binding protein binding sites and motifs." *Genome Biol* **24**(1): 77.

Ke, S., A. Pandya-Jones, Y. Saito, J. J. Fak, C. B. Vagbo, S. Geula, J. H. Hanna, D. L. Black, J. E. Darnell, Jr. and R. B. Darnell (2017). "m(6)A mRNA modifications are deposited in nascent pre-mRNA and are not required for splicing but do specify cytoplasmic turnover." *Genes Dev* **31**(10): 990-1006.

Kedde, M., M. J. Strasser, B. Boldajipour, J. A. Oude Vrielink, K. Slanchev, C. le Sage, R. Nagel, P. M. Voorhoeve, J. van Duijse, U. A. Orom, A. H. Lund, A. Perrakis, E. Raz and R. Agami (2007). "RNA-binding protein Dnd1 inhibits microRNA access to target mRNA." *Cell* **131**(7): 1273-1286.

Kedde, M., M. van Kouwenhove, W. Zwart, J. A. Oude Vrielink, R. Elkon and R. Agami (2010). "A Pumilio-induced RNA structure switch in p27-3' UTR controls miR-221 and miR-222 accessibility." *Nat Cell Biol* **12**(10): 1014-1020.

Kelly, T. J., H. I. Suzuki, J. R. Zamudio, M. Suzuki and P. A. Sharp (2019). "Sequestration of microRNA-mediated target repression by the Ago2-associated RNA-binding protein FAM120A." *RNA* **25**(10): 1291-1297.

Keryer-Bibens, C., C. Barreau and H. B. Osborne (2012). "Tethering of proteins to RNAs by bacteriophage proteins." *Biology of the Cell* **100**(2): 125-138.

Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon and R. H. Plasterk (2001). "Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*." *Genes Dev* **15**(20): 2654-2659.

Khvorova A, R. A., Jayasena SD. (2003). "Functional siRNAs and miRNAs exhibit strand bias. ." *Cell* **Oct 17;115(2):209-16**.

Kim, S., S. Kim, H. R. Chang, D. Kim, J. Park, N. Son, J. Park, M. Yoon, G. Chae, Y. K. Kim, V. N. Kim, Y. K. Kim, J. W. Nam, C. Shin and D. Baek (2021). "The regulatory impact of RNA-binding proteins on microRNA targeting." *Nat Commun* **12**(1): 5057.

Kingston, E. R., L. W. Blodgett and D. P. Bartel (2022). "Endogenous transcripts direct microRNA degradation in *Drosophila*, and this targeted degradation is required for proper embryonic development." *Mol Cell* **82**(20): 3872-3884 e3879.

Kleaveland, B., C. Y. Shi, J. Stefano and D. P. Bartel (2018). "A Network of Noncoding Regulatory RNAs Acts in the Mammalian Brain." *Cell* **174**(2): 350-362 e317.

Kobayashi, Y., K. Suzuki, H. Kobayashi, S. Ohashi, K. Koike, P. Macchi, M. Kiebler and K. Anzai (2008). "C9orf10 protein, a novel protein component of Puralpha-containing mRNA-protein particles (Puralpha-mRNPs): characterization of developmental and regional expressions in the mouse brain." *J Histochem Cytochem* **56**(8): 723-731.

Krempl, C., D. Lazzaretti and R. Sprangers (2023). "A structural biology view on the enzymes involved in eukaryotic mRNA turnover." *Biol Chem*.

Krol, J., I. Loedige and W. Filipowicz (2010). "The widespread regulation of microRNA biogenesis, function and decay." *Nat Rev Genet* **11**(9): 597-610.

Kuhn, U. and E. Wahle (2004). "Structure and function of poly(A) binding proteins." *Biochim Biophys Acta* **1678**(2-3): 67-84.

Kundu, P., M. R. Fabian, N. Sonenberg, S. N. Bhattacharyya and W. Filipowicz (2012). "HuR protein attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA." *Nucleic Acids Res* **40**(11): 5088-5100.

Kuroha, K., M. Akamatsu, L. Dimitrova, T. Ito, Y. Kato, K. Shirahige and T. Inada (2010). "Receptor for activated C kinase 1 stimulates nascent polypeptide-dependent translation arrest." *EMBO Rep* **11**(12): 956-961.

Kurosaki, T., M. W. Popp and L. E. Maquat (2019). "Quality and quantity control of gene expression by nonsense-mediated mRNA decay." *Nat Rev Mol Cell Biol* **20**(7): 406-420.

Landthaler, M., A. Yalcin and T. Tuschl (2004). "The human DiGeorge syndrome critical region gene 8 and its D. melanogaster homolog are required for miRNA biogenesis." *Curr Biol* **14**(23): 2162-2167.

Lasman, L., V. Krupalnik, S. Viukov, N. Mor, A. Aguilera-Castrejon, D. Schneir, J. Bayerl, O. Mizrahi, S. Peles, S. Tawil, S. Sathe, A. Nachshon, T. Shani, M. Zerbib, I. Kilimnik, S. Aigner, A. Shankar, J. R. Mueller, S. Schwartz, N. Stern-Ginossar, G. W. Yeo, S. Geula, N. Novershtern and J. H. Hanna (2020). "Context-dependent functional compensation between Ythdf m(6)A reader proteins." *Genes Dev* **34**(19-20): 1373-1391.

Lazinski, D., E. Grzadzińska and A. Das (1989). "Sequence-Specific Recognition of RNA Hairpins by Bacteriophage Antiterminators Requires a Conserved Arginine-Rich Motif." *Cell* **59**(1):207-18.

Lazaretti D, Tournier I, Izaurrealde E. The C-terminal domains of human TNRC6A, TNRC6B, and TNRC6C silence bound transcripts independently of Argonaute proteins. *RNA*. 2009 Jun;**15**(6):1059-66. doi: 10.1261/rna.1606309. Epub 2009 Apr 21. PMID: 19383768; PMCID: PMC2685519.

Lee, S., J. Song, S. Kim, J. Kim, Y. Hong, Y. Kim, D. Kim, D. Baek and K. Ahn (2013). "Selective degradation of host MicroRNAs by an intergenic HCMV noncoding RNA accelerates virus production." *Cell Host Microbe* **13**(6): 678-690.

Lee, Y., Ahn, C., Han, J. et al. (2003). "The nuclear RNase III Drosha initiates microRNA processing." *Nature* **425**, 415-419

Lee, Y., M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek and V. N. Kim (2004). "MicroRNA genes are transcribed by RNA polymerase II." *EMBO J* **23**(20): 4051-4060.

Legnini, I., J. Alles, N. Karaikos, S. Ayoub and N. Rajewsky (2019). "FLAM-seq: full-length mRNA sequencing reveals principles of poly(A) tail length control." *Nat Methods* **16**(9): 879-886.

Lehmann, R., Nüsslein-Volhard, C (1987). "Involvement of the pumilio gene in the transport of an abdominal signal in the Drosophila embryo." *Nature* **329**, 167-170 (1987).

Leppeck, K., J. Schott, S. Reitter, F. Poetz, M. C. Hammond and G. Stoecklin (2013). "Roquin promotes constitutive mRNA decay via a conserved class of stem-loop recognition motifs." *Cell* **153**(4): 869-881.

Letzring, D. P., K. M. Dean and E. J. Grayhack (2010). "Control of translation efficiency in yeast by codon-anticodon interactions." *RNA* **16**(12): 2516-2528.

Letzring, D. P., A. S. Wolf, C. E. Brule and E. J. Grayhack (2013). "Translation of CGA codon repeats in yeast involves quality control components and ribosomal protein L1." *RNA* **19**(9): 1208-1217.

Lin, S., J. Choe, P. Du, R. Triboulet and R. I. Gregory (2016). "The m(6)A Methyltransferase METTL3 Promotes Translation in Human Cancer Cells." *Mol Cell* **62**(3): 335-345.

Liu, J. C. M., Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ. (2004). "Argonaute2 is the catalytic engine of mammalian RNAi." *Science* **305**(5689):1437-41.

Liu, J., M. Gao, S. Xu, Y. Chen, K. Wu, H. Liu, J. Wang, X. Yang, J. Wang, W. Liu, X. Bao and J. Chen (2020). "YTHDF2/3 Are Required for Somatic Reprogramming through Different RNA Deadenylation Pathways." *Cell Reports* **32**(10).

Liu, J., M. A. Valencia-Sanchez, G. J. Hannon and R. Parker (2005). "MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies." *Nat Cell Biol* **7**(7): 719-723.

Liu, J., Y. Yue, D. Han, X. Wang, Y. Fu, L. Zhang, G. Jia, M. Yu, Z. Lu, X. Deng, Q. Dai, W. Chen and C. He (2014). "A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation." *Nat Chem Biol* **10**(2): 93-95.

Lobel, J. H., R. W. Tibble and J. D. Gross (2019). "Pat1 activates late steps in mRNA decay by multiple mechanisms."

Loh, B., S. Jonas and E. Izaurralde (2013). "The SMG5-SMG7 heterodimer directly recruits the CCR4-NOT deadenylase complex to mRNAs containing nonsense codons via interaction with POP2." *Genes Dev* **27**(19): 2125-2138.

Lund E, G. S., Calado A, Dahlberg JE, Kutay U. (2004). "Nuclear export of microRNA precursors." *Science*.

Lund, M. K. and C. Guthrie (2005). "The DEAD-box protein Dbp5p is required to dissociate Mex67p from exported mRNPs at the nuclear rim." *Mol Cell* **20**(4): 645-651.

Lykke-Andersen, J. and E. J. Bennett (2014). "Protecting the proteome: Eukaryotic cotranslational quality control pathways." *J Cell Biol* **204**(4): 467-476.

Mangus, D. A., Evans, M.C. & Jacobson, A. (2003). "Poly(A)-binding proteins multifunctional scaffolds for the post-transcriptional control of gene expression." *Genome Biol*.

Marcinowski, L., M. Tanguy, A. Krmpotic, B. Radle, V. J. Lisnic, L. Tuddenham, B. Chane-Woon-Ming, Z. Ruzsics, F. Erhard, C. Benkartek, M. Babic, R. Zimmer, J. Trgovcich, U. H. Koszinowski, S. Jonjic, S. Pfeffer and L. Dolken (2012). "Degradation of cellular mir-27 by a novel, highly abundant viral transcript is important for efficient virus replication in vivo." *PLoS Pathog* **8**(2): e1002510.

Matsuo, Y., P. Tesina, S. Nakajima, M. Mizuno, A. Endo, R. Buschauer, J. Cheng, O. Shounai, K. Ikeuchi, Y. Saeki, T. Becker, R. Beckmann and T. Inada (2020). "RQT complex dissociates ribosomes collided on endogenous RQC substrate SDD1." *Nat Struct Mol Biol* **27**(4): 323-332.

Mauxion, F., B. Preve and B. Seraphin (2013). "C2ORF29/CNOT11 and CNOT10 form a new module of the CCR4-NOT complex." *RNA Biol* **10**(2): 267-276.

Meister, G. (2013). "Argonaute proteins: functional insights and emerging roles." *Nat Rev Genet* **14**(7): 447-459.

Meister, G., M. Landthaler, Y. Dorsett and T. Tuschl (2004). "Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing." *RNA* **10**(3): 544-550.

Meister, G., M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng and T. Tuschl (2004). "Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs." *Mol Cell* **15**(2): 185-197.

Meister G., T. T. (2004). "<Mechanisms of gene silencing by double-stranded RNA.pdf>."

Meyer, K. D., D. P. Patil, J. Zhou, A. Zinoviev, M. A. Skabkin, O. Elemento, T. V. Pestova, S. B. Qian and S. R. Jaffrey (2015). "5' UTR m(6)A Promotes Cap-Independent Translation." *Cell* **163**(4): 999-1010.

Meyer, K. D., Y. Saletore, P. Zumbo, O. Elemento, C. E. Mason and S. R. Jaffrey (2012). "Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons." *Cell* **149**(7): 1635-1646.

Miyoshi, T., A. Takeuchi, H. Siomi and M. C. Siomi (2010). "A direct role for Hsp90 in pre-RISC formation in *Drosophila*." *Nat Struct Mol Biol* **17**(8): 1024-1026.

Morgan Tucker, Marco A. Valencia-Sanchez, J. C. Robin R. Staples, Clyde L. Denis and a. R. Parker (2001). "The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in *Saccharomyces cerevisiae*."

Morita, M., L. W. Ler, M. R. Fabian, N. Siddiqui, M. Mullin, V. C. Henderson, T. Alain, B. D. Fonseca, G. Karashchuk, C. F. Bennett, T. Kabuta, S. Higashi, O. Larsson, I. Topisirovic, R. J. Smith, A. C. Gingras and N. Sonenberg (2012). "A novel 4EHP-GIGYF2 translational repressor complex is essential for mammalian development." *Mol Cell Biol* **32**(17): 3585-3593.

Nakanishi, K., M. Ascano, T. Gogakos, S. Ishibe-Murakami, A. A. Serganov, D. Briskin, P. Morozov, T. Tuschl and D. J. Patel (2013). "Eukaryote-specific insertion elements control human ARGONAUTE slicer activity." *Cell Rep* **3**(6): 1893-1900.

Neve, J., R. Patel, Z. Wang, A. Louey and A. M. Furger (2017). "Cleavage and polyadenylation: Ending the message expands gene regulation." *RNA Biol* **14**(7): 865-890.

Nick J. Proudfoot, Andre Furger and a. M. J. Dye (2002). "Integrating mRNA Processing with Transcription."

Noland, C. L., E. Ma and J. A. Doudna (2011). "siRNA repositioning for guide strand selection by human Dicer complexes." *Mol Cell* **43**(1): 110-121.

Nüsslein-Volhard C, F. H., Lehmann R. (1987). "Determination of anteroposterior polarity in *Drosophila*." *Science* **238**(4834):1675-81.

Ozgun, S., J. Basquin, A. Kamenska, W. Filipowicz, N. Standart and E. Conti (2015). "Structure of a Human 4E-T/DDX6/CNOT1 Complex Reveals the Different Interplay of DDX6-Binding Proteins with the CCR4-NOT Complex." *Cell Rep* **13**(4): 703-711.

Pabis, M., N. Neufeld, M. C. Steiner, T. Bojic, Y. Shav-Tal and K. M. Neugebauer (2013). "The nuclear cap-binding complex interacts with the U4/U6.U5 tri-snRNP and promotes spliceosome assembly in mammalian cells." *RNA* **19**(8): 1054-1063.

Park, M. S., G. Sim, A. C. Kehling and K. Nakanishi (2020). "Human Argonaute2 and Argonaute3 are catalytically activated by different lengths of guide RNA." *Proc Natl Acad Sci U S A* **117**(46): 28576-28578.

Park, O. H., H. Ha, Y. Lee, S. H. Boo, D. H. Kwon, H. K. Song and Y. K. Kim (2019). "Endoribonucleolytic Cleavage of m(6)A-Containing RNAs by RNase P/MRP Complex." *Mol Cell* **74**(3): 494-507 e498.

Passmore, L. A. and J. Collier (2022). "Roles of mRNA poly(A) tails in regulation of eukaryotic gene expression." *Nat Rev Mol Cell Biol* **23**(2): 93-106.

Pena, C., E. Hurt and V. G. Panse (2017). "Eukaryotic ribosome assembly, transport and quality control." *Nat Struct Mol Biol* **24**(9): 689-699.

Peter, D., C. Igreja, R. Weber, L. Wohlbold, C. Weiler, L. Ebertsch, O. Weichenrieder and E. Izaurralde (2015). "Molecular architecture of 4E-BP translational inhibitors bound to eIF4E." *Mol Cell* **57**(6): 1074-1087.

Peter, D., V. Ruscica, P. Bawankar, R. Weber, S. Helms, E. Valkov, C. Igreja and E. Izaurralde (2019). "Molecular basis for GIGYF-Me31B complex assembly in 4EHP-mediated translational repression." *Genes Dev* **33**(19-20): 1355-1360.

Peter, D., R. Weber, F. Sandmeir, L. Wohlbold, S. Helms, P. Bawankar, E. Valkov, C. Igreja and E. Izaurralde (2017). "GIGYF1/2 proteins use auxiliary sequences to selectively bind to 4EHP and repress target mRNA expression." *Genes Dev* **31**(11): 1147-1161.

Peters, L. and G. Meister (2007). "Argonaute proteins: mediators of RNA silencing." *Mol Cell* **26**(5): 611-623.

Pisarev, A. V., M. A. Skabkin, V. P. Pisareva, O. V. Skabkina, A. M. Rakotondrafara, M. W. Hentze, C. U. Hellen and T. V. Pestova (2010). "The role of ABCE1 in eukaryotic posttermination ribosomal recycling." *Mol Cell* **37**(2): 196-210.

Pisareva, V. P., M. A. Skabkin, C. U. Hellen, T. V. Pestova and A. V. Pisarev (2011). "Dissociation by Pelota, Hbs1 and ABCE1 of mammalian vacant 80S ribosomes and stalled elongation complexes." *EMBO J* **30**(9): 1804-1817.

Poetz, F., J. Corbo, Y. Leviansky, A. Spiegelhalter, D. Lindner, V. Magg, S. Lebedeva, J. Schweiggert, J. Schott, E. Valkov and G. Stoecklin (2021). "RNF219 attenuates global mRNA decay through inhibition of CCR4-NOT complex-mediated deadenylation." *Nat Commun* **12**(1): 7175.

Quevillon Huberdeau, M., D. M. Zeitler, J. Hauptmann, A. Bruckmann, L. Fressigne, J. Danner, S. Piquet, N. Strieder, J. C. Engelmann, G. Jannot, R. Deutzmann, M. J. Simard and G. Meister (2017). "Phosphorylation of Argonaute proteins affects mRNA binding and is essential for microRNA-guided gene silencing in vivo." *EMBO J* **36**(14): 2088-2106.

Raisch, T., D. Bhandari, K. Sabath, S. Helms, E. Valkov, O. Weichenrieder and E. Izaurralde (2016). "Distinct modes of recruitment of the CCR4-NOT complex by Drosophila and vertebrate Nanos." *EMBO J* **35**(9): 974-990.

Raisch, T. and E. Valkov (2022). "Regulation of the multisubunit CCR4-NOT deadenylase in the initiation of mRNA degradation." *Curr Opin Struct Biol* **77**: 102460.

Rajasethupathy, P., F. Fiumara, R. Sheridan, D. Betel, S. V. Puthanveetil, J. J. Russo, C. Sander, T. Tuschl and E. Kandel (2009). "Characterization of small RNAs in Aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB." *Neuron* **63**(6): 803-817.

Ramanathan, A., G. B. Robb and S. H. Chan (2016). "mRNA capping: biological functions and applications." *Nucleic Acids Res* **44**(16): 7511-7526.

Rasch, F., R. Weber, E. Izaurralde and C. Igreja (2020). "4E-T-bound mRNAs are stored in a silenced and deadenylated form." *Genes Dev* **34**(11-12): 847-860.

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

Rissland, O. S., S. J. Hong and D. P. Bartel (2011). "MicroRNA destabilization enables dynamic regulation of the miR-16 family in response to cell-cycle changes." *Mol Cell* **43**(6): 993-1004.

Rudel, S., Y. Wang, R. Lenobel, R. Korner, H. H. Hsiao, H. Urlaub, D. Patel and G. Meister (2011). "Phosphorylation of human Argonaute proteins affects small RNA binding." *Nucleic Acids Res* **39**(6): 2330-2343.

Ruegger, S. and H. Grosshans (2012). "MicroRNA turnover: when, how, and why." *Trends Biochem Sci* **37**(10): 436-446.

Ruscica, V., P. Bawankar, D. Peter, S. Helms, C. Igreja and E. Izaurralde (2019). "Direct role for the Drosophila GIGYF protein in 4EHP-mediated mRNA repression." *Nucleic Acids Res* **47**(13): 7035-7048.

Sambandan S, A. G., Kochen L, Rinne J, Kahlstatt J, Glock C, Tushev G, Alvarez-Castelao B, Heckel A, Schuman EM (2017). "Activity-dependent spatially localized miRNA maturation in neuronal dendrites." *Science* **355**(6325):634-637.

Sandler, H., J. Kreth, H. T. Timmers and G. Stoecklin (2011). "Not1 mediates recruitment of the deadenylase Caf1 to mRNAs targeted for degradation by tristetraprolin." *Nucleic Acids Res* **39**(10): 4373-4386.

Sandra E. Wells, Paul E. Hillner, Ronald D. Vale and a. A. B. Sachs (1998). "Circularization of mRNA by Eukaryotic Translation Initiation Factors."

Sasse, A., K. U. Lavery, T. R. Hughes and Q. D. Morris (2018). "Motif models for RNA-binding proteins." *Curr Opin Struct Biol* **53**: 115-123.

Schimmel, P. (2018). "The emerging complexity of the tRNA world: mammalian tRNAs beyond protein synthesis." *Nat Rev Mol Cell Biol* **19**(1): 45-58.

Schirle N. T., I. J. M. (2012). "The crystal structure of human Argonaute2 " *Science*.

Schirle N. T., J. S.-G., Ian J. MacRae (2014). "Structural basis for microRNA targeting." *Science*.

Schneider, C., E. Leung, J. Brown and D. Tollervy (2009). "The N-terminal PIN domain of the exosome subunit Rrp44 harbors endonuclease activity and tethers Rrp44 to the yeast core exosome." *Nucleic Acids Res* **37**(4): 1127-1140.

Schuller, A. P. and R. Green (2018). "Roadblocks and resolutions in eukaryotic translation." *Nat Rev Mol Cell Biol* **19**(8): 526-541.

Schurmann, N., L. G. Trabuco, C. Bender, R. B. Russell and D. Grimm (2013). "Molecular dissection of human Argonaute proteins by DNA shuffling." *Nat Struct Mol Biol* **20**(7): 818-826.

Schwarz DS, H. G., Du T, Xu Z, Aronin N, Zamore PD. (2003). "Asymmetry in the assembly of the RNAi enzyme complex." *Cell* **115**(2):199-208.

Segref, A., K. Sharma, V. Doye, A. Hellwig, J. Huber, R. Luhrmann and E. Hurt (1997). "Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores." *EMBO J* **16**(11): 3256-3271.

Sethi, P. and W. J. Lukiw (2009). "Micro-RNA abundance and stability in human brain: specific alterations in Alzheimer's disease temporal lobe neocortex." *Neurosci Lett* **459**(2): 100-104.

Sgromo, A., T. Raisch, P. Bawankar, D. Bhandari, Y. Chen, D. Kuzuoglu-Ozturk, O. Weichenrieder and E. Izaurralde (2017). "A CAF40-binding motif facilitates recruitment of the CCR4-NOT complex to mRNAs targeted by Drosophila Roquin." *Nat Commun* **8**: 14307.

Shah, V. N., J. Neumeier, M. Q. Huberdeau, D. M. Zeitler, A. Bruckmann, G. Meister and M. J. Simard (2023). "Casein kinase 1 and 2 phosphorylate Argonaute proteins to regulate miRNA-mediated gene silencing." *EMBO Rep* **24**(11): e57250.

Shen, J., W. Xia, Y. B. Khotskaya, L. Huo, K. Nakanishi, S. O. Lim, Y. Du, Y. Wang, W. C. Chang, C. H. Chen, J. L. Hsu, Y. Wu, Y. C. Lam, B. P. James, X. Liu, C. G. Liu, D. J. Patel and M. C. Hung (2013). "EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2." *Nature* **497**(7449): 383-387.

Sheu-Gruttadauria, J. and I. J. MacRae (2018). "Phase Transitions in the Assembly and Function of Human miRISC." *Cell* **173**(4): 946-957 e916.

Sheu-Gruttadauria, J., P. Pawlica, S. M. Klum, S. Wang, T. A. Yario, N. T. Schirle Oakdale, J. A. Steitz and I. J. MacRae (2019). "Structural Basis for Target-Directed MicroRNA Degradation." *Mol Cell* **75**(6): 1243-1255 e1247.

Shi CY, Kingston ER, Kleaveland B, Lin DH, Stubna MW and B. DP (2020). "The ZSWIM8 ubiquitin ligase mediates target-directed microRNA degradation".

Shi, H., X. Wang, Z. Lu, B. S. Zhao, H. Ma, P. J. Hsu, C. Liu and C. He (2017). "YTHDF3 facilitates translation and decay of N(6)-methyladenosine-modified RNA." *Cell Res* **27**(3): 315-328.

Shi, Y. (2017). "Mechanistic insights into precursor messenger RNA splicing by the spliceosome." *Nat Rev Mol Cell Biol* **18**(11): 655-670.

Shirokikh, N. E. and T. Preiss (2018). "Translation initiation by cap-dependent ribosome recruitment: Recent insights and open questions." *Wiley Interdiscip Rev RNA* **9**(4): e1473.

Shoemaker, C. J. and R. Green (2011). "Kinetic analysis reveals the ordered coupling of translation termination and ribosome recycling in yeast." *Proc Natl Acad Sci U S A* **108**(51): E1392-1398.

Simms, C. L., L. L. Yan and H. S. Zaher (2017). "Ribosome Collision Is Critical for Quality Control during No-Go Decay." *Mol Cell* **68**(2): 361-373 e365.

Siomi, M. C., K. Sato, D. Pezic and A. A. Aravin (2011). "PIWI-interacting small RNAs: the vanguard of genome defence." *Nat Rev Mol Cell Biol* **12**(4): 246-258.

Slobodin, B., R. Han, V. Calderone, J. Vrieling, F. Loayza-Puch, R. Elkon and R. Agami (2017). "Transcription Impacts the Efficiency of mRNA Translation via Co-transcriptional N6-adenosine Methylation." *Cell* **169**(2): 326-337 e312.

Steckelberg, A.-L., V. Boehm, Agnieszka M. Gromadzka and Niels H. Gehring (2012). "CWC22 Connects Pre-mRNA Splicing and Exon Junction Complex Assembly." *Cell Reports* **2**(3): 454-461.

Steitz, T. A. (2008). "A structural understanding of the dynamic ribosome machine." *Nat Rev Mol Cell Biol* **9**(3): 242-253.

Stupfler, B., C. Birck, B. Seraphin and F. Mauxion (2016). "BTG2 bridges PABPC1 RNA-binding domains and CAF1 deadenylase to control cell proliferation." *Nat Commun* **7**: 10811.

Takagaki, Y. and J. L. Manley (2000). "Complex protein interactions within the human polyadenylation machinery identify a novel component." *Mol Cell Biol* **20**(5): 1515-1525.

Tange, T., T. Shibuya, M. Jurica and M. Moore J. (2005). "Biochemical analysis of the EJC reveals two new factors and a stable tetrameric protein core." *RNA*.

Temme, C., M. Simonelig and E. Wahle (2014). "Deadenylation of mRNA by the CCR4-NOT complex in Drosophila: molecular and developmental aspects." *Front Genet* **5**: 143.

Tominaga, K., S. Srikantan, E. K. Lee, S. S. Subaran, J. L. Martindale, K. Abdelmohsen and M. Gorospe (2011). "Competitive regulation of nucleolin expression by HuR and miR-494." *Mol Cell Biol* **31**(20): 4219-4231.

Treiber, T., N. Treiber and G. Meister (2019). "Regulation of microRNA biogenesis and its crosstalk with other cellular pathways." *Nat Rev Mol Cell Biol* **20**(1): 5-20.

Uzonyi, A., B. Slobodin and S. Schwartz (2022). "Exon-intron architecture determines mRNA stability by dictating m6A deposition."

Valkov, E., S. Jonas and O. Weichenrieder (2017). "Mille viae in eukaryotic mRNA decapping." *Curr Opin Struct Biol* **47**: 40-51.

Valkov, E., S. Muthukumar, C. T. Chang, S. Jonas, O. Weichenrieder and E. Izaurralde (2016). "Structure of the Dcp2-Dcp1 mRNA-decapping complex in the activated conformation." *Nat Struct Mol Biol* **23**(6): 574-579.

van der Lee, R., M. Buljan, B. Lang, R. J. Weatheritt, G. W. Daughdrill, A. K. Dunker, M. Fuxreiter, J. Gough, J. Gsponer, D. T. Jones, P. M. Kim, R. W. Kriwacki, C. J. Oldfield, R. V. Pappu, P. Tompa, V. N. Uversky, P. E. Wright and M. M. Babu (2014). "Classification of intrinsically disordered regions and proteins." *Chem Rev* **114**(13): 6589-6631.

Wahl, M. C., C. L. Will and R. Luhrmann (2009). "The spliceosome: design principles of a dynamic RNP machine." *Cell* **136**(4): 701-718.

Wang, X., Z. Lu, A. Gomez, G. C. Hon, Y. Yue, D. Han, Y. Fu, M. Parisien, Q. Dai, G. Jia, B. Ren, T. Pan and C. He (2014). "N6-methyladenosine-dependent regulation of messenger RNA stability." *Nature* **505**(7481): 117-120.

Wang, X., B. S. Zhao, I. A. Roundtree, Z. Lu, D. Han, H. Ma, X. Weng, K. Chen, H. Shi and C. He (2015). "N(6)-methyladenosine Modulates Messenger RNA Translation Efficiency." *Cell* **161**(6): 1388-1399.

Weber, R., M. Y. Chung, C. Keskeny, U. Zinnall, M. Landthaler, E. Valkov, E. Izaurralde and C. Igreja (2020). "4EHP and GIGYF1/2 Mediate Translation-Coupled Messenger RNA Decay." *Cell Rep* **33**(2): 108262.

Webster, M. W., Y. H. Chen, J. A. W. Stowell, N. Alhusaini, T. Sweet, B. R. Graveley, J. Collier and L. A. Passmore (2018). "mRNA Deadenylation Is Coupled to Translation Rates by the Differential Activities of Ccr4-Not Nucleases." *Mol Cell* **70**(6): 1089-1100 e1088.

Webster, M. W., J. A. Stowell and L. A. Passmore (2019). "RNA-binding proteins distinguish between similar sequence motifs to promote targeted deadenylation by Ccr4-Not." *Elife* **8**.

Welte, T., A. Goulois, M. B. Stadler, D. Hess, C. Soneson, A. Neagu, C. Azzi, M. J. Wisser, J. Seebacher, I. Schmidt, D. Estoppey, F. Nigsch, J. Reece-Hoyes, D. Hoepfner and H. Grosshans (2023). "Convergence of multiple RNA-silencing pathways on GW182/TNRC6." *Mol Cell* **83**(14): 2478-2492 e2478.

Willkomm, S., L. Jakob, K. Kramm, V. Graus, J. Neumeier, G. Meister and D. Grohmann (2022). "Single-molecule FRET uncovers hidden conformations and dynamics of human Argonaute 2." *Nat Commun* **13**(1): 3825.

Wojtas, M. N., R. R. Pandey, M. Mendel, D. Homolka, R. Sachidanandam and R. S. Pillai (2017). "Regulation of m(6)A Transcripts by the 3'→5' RNA Helicase YTHDC2 Is Essential for a Successful Meiotic Program in the Mammalian Germline." *Mol Cell* **68**(2): 374-387 e312.

Wurm, J. P. and R. Sprangers (2019). "Dcp2: an mRNA decapping enzyme that adopts many different shapes and forms." *Curr Opin Struct Biol* **59**: 115-123.

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

Yang, M., A. D. Haase, F. K. Huang, G. Coulis, K. D. Rivera, B. C. Dickinson, C. J. Chang, D. J. Pappin, T. A. Neubert, G. J. Hannon, B. Boivin and N. K. Tonks (2014). "Dephosphorylation of tyrosine 393 in argonaute 2 by protein tyrosine phosphatase 1B regulates gene silencing in oncogenic RAS-induced senescence." *Mol Cell* **55**(5): 782-790.

Yang, X., R. Triboulet, Q. Liu, E. Sendinc and R. I. Gregory (2022). "Exon junction complex shapes the m6A epitranscriptome." *Nature Communications* **13**(1).

Yang, Z., A. Jakymiw, M. R. Wood, T. Eystathioy, R. L. Rubin, M. J. Fritzler and E. K. Chan (2004). "GW182 is critical for the stability of GW bodies expressed during the cell cycle and cell proliferation." *J Cell Sci* **117**(Pt 23): 5567-5578.

Yi, H., J. Park, M. Ha, J. Lim, H. Chang and V. N. Kim (2018). "PABP Cooperates with the CCR4-NOT Complex to Promote mRNA Deadenylation and Block Precocious Decay." *Mol Cell* **70**(6): 1081-1088 e1085.

Yi, R., Y. Qin, I. G. Macara and B. R. Cullen (2003). "Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs." *Genes Dev* **17**(24): 3011-3016.

Youn, J. Y., W. H. Dunham, S. J. Hong, J. D. R. Knight, M. Bashkurov, G. I. Chen, H. Bagci, B. Rathod, G. MacLeod, S. W. M. Eng, S. Angers, Q. Morris, M. Fabian, J. F. Cote and A. C. Gingras (2018). "High-Density Proximity Mapping Reveals the Subcellular Organization of mRNA-Associated Granules and Bodies." *Mol Cell* **69**(3): 517-532 e511.

Young, L. E., A. E. Moore, L. Sokol, N. Meisner-Kober and D. A. Dixon (2012). "The mRNA stability factor HuR inhibits microRNA-16 targeting of COX-2." *Mol Cancer Res* **10**(1): 167-180.

Yuan, Y. R., Y. Pei, J. B. Ma, V. Kuryavyy, M. Zhadina, G. Meister, H. Y. Chen, Z. Dauter, T. Tuschl and D. J. Patel (2005). "Crystal structure of A. aeolicus argonaute, a site-specific DNA-guided endoribonuclease, provides insights into RISC-mediated mRNA cleavage." *Mol Cell* **19**(3): 405-419.

Yusupova, G. and M. Yusupov (2017). "Crystal structure of eukaryotic ribosome and its complexes with inhibitors." *Philos Trans R Soc Lond B Biol Sci* **372**(1716).

Zaccara, S. and S. R. Jaffrey (2020). "A Unified Model for the Function of YTHDF Proteins in Regulating m(6)A-Modified mRNA." *Cell* **181**(7): 1582-1595 e1518.

Zekri L, Huntzinger E, Heimstädt S, Izaurralde E. The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. *Mol Cell Biol*. 2009 Dec;**29**(23):6220-31. doi: 10.1128/MCB.01081-09. Epub 2009 Sep 21. PMID: 19797087; PMCID: PMC2786699.

Zhang, Z., D. Theler, K. H. Kaminska, M. Hiller, P. de la Grange, R. Pudimat, I. Rafalska, B. Heinrich, J. M. Bujnicki, F. H. Allain and S. Stamm (2010). "The YTH domain is a novel RNA binding domain." *J Biol Chem* **285**(19): 14701-14710.

Zheng, G., J. A. Dahl, Y. Niu, P. Fedorcsak, C. M. Huang, C. J. Li, C. B. Vagbo, Y. Shi, W. L. Wang, S. H. Song, Z. Lu, R. P. Bosmans, Q. Dai, Y. J. Hao, X. Yang, W. M. Zhao, W. M. Tong, X. J. Wang, F. Bogdan, K. Furu, Y. Fu, G. Jia, X. Zhao, J. Liu, H. E. Krokan, A. Klungland, Y. G. Yang and C. He (2013). "ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility." *Mol Cell* **49**(1): 18-29.

Zhuang, R., J. N. Rao, T. Zou, L. Liu, L. Xiao, S. Cao, N. Z. Hansraj, M. Gorospe and J. Y. Wang (2013). "miR-195 competes with HuR to modulate stim1 mRNA stability and regulate cell migration." *Nucleic Acids Res* **41**(16): 7905-7919.

9 Acknowledgments

I am thankful and grateful for all the people that accompanied me during this long journey.

First, I want to thank Gunter. Thank you for allowing me to work for and with you, for challenging and supporting me through my PhD. Thank you for the endless discussions about my project and life in general, politics included. Thank you for showing me how a scientist should think, behave and act and for teaching me how a project has to be carried out. Without your guidance, I would have never had the chance to improve myself and be the scientist that I am now.

I would like to express my sincere gratitude to Prof. Dr. Reinhard Sterner for being the chairperson and to Prof Dr. Remco Sprangers, Prof. Dr. Klauss Grasser for being members of my examination committee and evaluating my thesis. Thank you for your time and availability. Thanks also to Prof. Dr. Thomas Dresselhaus for being my Substitute examiner.

I would like to thank the members of my thesis advisory committee Prof. Dr Markus Landthaler and Prof. Dr Gernot Längst.

I would like to thank my collaborators Dr. Ivano Legnini, Dr. Tobias Raisch, Dr. Hung-Ho Xuan, Julia Neumeier, Gerhard Lehmann, Denise Aigner and Prof. Dr. Nikolaus Rajewsky, whose contribution significantly enriched the research meaning presented in this thesis. A special thanks, goes to Ivano. Thank you for your time, ideas, for the numerous discussions. Thank you Gerhard for your time, patience and the endless R coding sessions. Thank you Hung and Julia for the infinite talks and ideas and the help in the lab. Lastly, I would also like to thank Simon Holzinger for all the R support and help.

Big thanks also to Carolin Apfel and Kinga Ay for assisting me throughout my PhD as a RIEGeL member.

In addition, I would like to thank:

Birgit Clemens for the moral and the exceptional administrative and personal support, which made my time here easy, productive and enjoyable.

Prof. Dr. Markus Kretz and Prof. Dr Jan Medenbach for the countless discussions and guidance during my time here.

All the former and current members of Biochemie I for the amazing times in and out of the lab, in and out of the pubs, for all the kicker matches and for the support during these years. I would have never managed without you: Tiana Hanelt, Alice Gryselin, Dr. Eva Schöller, Dr. Claudia Latini, Michaela Bauer, Brent Cuveele, Andreas Meindl, Dr. Franziska Weichmann, Philipp Becker, Kevin Heizler, Julia Neumeier, Florian Heiss.

I would also like to thanks all the other members of Biochemie I for all the help and the fantastic working atmosphere: Dr. Balagopal Pai, Elisabetta Scarfiello, Carla Till, Julian Eichlinger, Mathias Gradl, Dr. Daniela Zeitler, Dr. Johannes Danner, Dr. Daniele Hasler, Markus Romberger, Dr. Johannes Graf, Dr. Sonja Hombach, Fabian Schabenberger, Carolin Molthof, Eva Morgenstern, Veronika Graus, Dr. Thomas and Nora Treiber, Dr. Astrid Bruckmann, Matthias Gruber, Eduard Hochmuth, Dr. Patricia Luckner, Dr. Erman Kocak, Divya Kandala, Dr. Benjamin De la Cruz-Thea, Daniela Strauß, Bianca Forstl, Julia Daiß, Matthias Breindl, Mona Höcherl, Tirza Brandl.

Many thanks to Norbert Eichner, Corinna Friederich, Sigrun Ammon and Robert Hett, which provided an amazing support and a wonderful company for my staying in the lab.

This PhD was a very very long path, which started in Tübingen in January 2017.

I was only a young person, and if I am also at this point in my life, I have to thank the many other people that accompanied me during my first years in Germany.

First, I want to thank Prof. Dr. Elisa Izaurralde who was the first person to believe in me as a young scientist. Thanks for your mentoring, guidance, strength and life example. I will never forget your last Christmas speech to the lab.

A special thanks goes to Dr. Dipankar Bhandari and Dr. Catia Igreja for raising me as a scientist. Thanks for taking care of me and for all the time and patience that you had. Without your support, I would have been lost.

Special thanks also to Dr. Eugene Valkov for the constant guidance throughout my PhD and for several scientific and life advices and for all the trust that always had in me.

Thanks to Prof. Dr. Ralf-Peter Jansen and Dr. John Weir for being part of my Thesis advisory committee.

I would like to thank all the former and current members of Department II at the MPI for all the good times inside and outside the lab: Dr. Ramona Weber, Dr. Csilla Keskeny, Dr. Vincenzo Ruscica, Dr. Felix Räscher, Dr. Michelle Noble, Dr. Yevgen Levitsky, Dr. Alina Stein, Dr. Aoife Haney, Dr. Annamaria Sgromo, Dr. Sowndarya Muthukumar, Dr. Maria Fernandez, Dr. Leon Kleeman.

Thanks to all the other people of the Department II and at the MPI that provided me help and nice working atmosphere: Dr. Chung-Te Chang, Dr. Elena Khazina, Dr. Ying Chen, Dr. Praveen Bawankar, Kevin Sabath, Felix Sandmeier, Min-Yi Chung, Dr. Stefan Grüner, Dr. Daniel Peter, Ayshwarya Seenivasan, Dr. Lara Wohlbold, Dr. Oliver Weichenrieder, Stefanie Becker, Catrin Weiler, Gabriele Wagner, Nadine Weiss, Max Widmann, Marie Palaj, Sigrun Helms, Maria Fauser, Maria Götz, Sibylle Patheiger and Dr. Sarah Danes.

I want to thank also all my friends from Tübingen and Regensburg that accompanied me and supported me during this time: Mario, MJ, Bilal, Giambattista, Albane, Magda, Cristina, Sergio, Ricardo, Guadalupe, Daniela.

I want to thank Danija Krieg for the encouragement, understanding and all the support and patience.

Infine vorrei ringraziare tutti i miei amici e tutta la mia famiglia per essermi stati sempre vicini. Un grazie particolare ai miei genitori per il supporto costante, l'affetto, e per avermi insegnato ad affrontare qualsiasi situazione. Grazie alle mie sorelle Rebecca ed Elisa per essere state sempre presenti.

The Meister Lab

