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General and microRNA-mediated mRNA degradation occurs on
ribosome complexes in *Drosophila* cells

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2 Abstract

Post-transcriptional processes such as mRNA degradation, mRNA surveillance, translation, translation repression, and miRNA or siRNA-mediated gene silencing play crucial roles in the regulation of gene expression. They are highly regulated and targeted by many factors. These processes not only share several protein factors but also appear physically connected. It is well known that translation and mRNA degradation are tightly coupled. While their mechanisms are well established, it is still not completely clear where in the cell mRNA degradation occurs. The close connection between translation and degradation suggests the possibility for mRNA degradation to take place on the ribosome.

Therefore, our aim in this study was to investigate the possibility of mRNA degradation on the ribosome in *Drosophila* cells as a higher Eukaryote.

At first, we used polysome profilesto demonstrate the co-migration of HPat, NOT2, and AGO1 with ribosome complexes. These factors were chosen and tested as representatives of the decapping, deadenylation and miRNA complex. Later we have established ribosome affinity purification and we could show the co-purification of numerous deadenylation and decapping factors with ribosome complexes. Interestingly, both AGO1 and GW182, key factors of the miRNA mediated mRNA degradation pathway, were also associated with ribosome complexes. However, the copurification of factors failed in the presence of limiting amounts of RNases. This suggests that the association of factors rather with mRNA than with ribosomes. The next step was to show whether the mRNA-degradation intermediates could be detected on the ribosome complexes and how abundant is the degradation on the ribosomes. We have isolated decapped mRNA-degradation intermediates from ribosome complexes and we have performed high-throughput sequence analysis. Approximately 12.000 mRNAs or 93% of the decapped mRNA fragments were detected at the same relative abundance on the ribosome complexes as in the cell lysate. This showed the great abundance of mRNA degradation on the ribosome.

In summary, our data strongly indicate the association of most mRNAs as well as mRNA targeted by miRNAs with the ribosomes during their degradation in *Drosophila* cells. A better understanding of the mechanism and location of both general and miRNA-mediated degradation of mRNAs will help us improve our understanding of the kinetics of mRNA degradation and its regulation andalso of various cellular processes, such as development, differentiation or how biological systems with all its complexity respond to various external stimuli to overcome the stressful situation and keep its homeostasis.

3 Zusammenfassung

Post-transkriptionelle Prozesse wie mRNA-Abbau, mRNA-Kontrolle, Proteinsynthese und ihre Repression sowie die miRNA- oder siRNA-vermittelte Genkontrolle spielen eine entscheidende Rolle bei der Regulation der Genexpression. Die Prozesse sind durch viele verschiedene Faktoren stark reguliert. Neben der Verwendung einiger gemeinsamer Proteinfaktoren scheinen die Prozesse auch physisch eng aneinander gekoppelt zu sein. Dies ist beispielsweise für die Proteinsynthese und den Abbau von mRNA bekannt. Während deren Mechanismen gut etabliert sind, ist es noch nicht klar wo in der Zelle der mRNA-Abbau lokalisiert ist. Die enge Koppelung von Proteinsynthese und mRNA-Abbau legt die Möglichkeit des Abbaues von mRNAs auf dem Ribosom nahe.

Daher war es unser Ziel die Möglichkeit eines mRNA-Abbaus auf dem Ribosom in Drosophila-Zellen, als einen Vertreter höherer Eukaryoten, zu untersuchen.

Zuerst verwendeten wir Polysomprofile um die Ko-Migration von HPat, NOT2 und AGO1 mit Polysomen nachzuweisen. Diese Faktoren wurden ausgewählt, da sie Bestandteile in den Komplexen für das Decapping, der Deadenylierung oder die Regulation durch miRNAs sind. In weiterer Folge haben wir die Methode der Ribosomen-Affinitätsreinigung etabliert. Damit konnten wir nachweisen, dass zahlreiche Deadenylierungs- und Decapping Faktoren mit Ribosomenkomplexen assoziiert sind. Interessanterweise waren sowohl AGO1 als auch GW182, zwei Schlüsselfaktoren des miRNA-vermittelten mRNA-Abbauweges, ebenfalls mit Ribosomenkomplexen assoziiert. Wenn die Assoziation von Faktoren mit Ribosomenkomplexen in Gegenwart von RNasen getestet wurde, kam es zu keiner gemeinsamen Aufreinigung. Dies legt nahe, dass die Assoziation der Proteinfaktoren am wahrscheinlichsten mit mRNA und nicht mit Ribosomen erfolgt. Der nächste Schritt bestand darin zu zeigen, dass Abbauzwischenprodukte von mRNAs an den Ribosomenkomplexen nachgewiesen werden können. Es ist uns gelungen mRNA-Abbau-Intermediate aus Ribosomenkomplexen zu isolieren und mittels Hochdurchsatz-Sequenzanalyse zu analysieren. Ungefähr 12 000 mRNAs oder 93% der entkappten mRNA-Fragmente wurden mit der gleichen relativen Häufigkeit auf den Ribosomenkomplexen wie im Zelllysate nachgewiesen. Dies zeigt, dass nicht nur einzelne mRNAs, sondern die große Mehrzahl der mRNAs an den Ribosomen abgebaut wird.

Zusammenfassend weisen unsere Daten auf die Assoziation der meisten mRNAs mit den Ribosomen während ihres Abbaues in Drosophila-Zellen hin. Ein besseres Verständnis des Mechanismus und der Lage von generellem und miRNA-vermitteltem RNA-Abbau kann nicht nur beim Verständnis der Kinetik und Regulation des mRNA-Abbaus helfen, sondern auch beim Verständnis verschiedener zellulärer Prozesse, wie zum Beispiel Entwicklung, Differenzierung oder wie biologische Systeme mit all ihrer

Komplexität auf externe Stimuli reagieren um stressvolle Situationen zu meistern und zurück zur Homöostase zu finden.

4 Introduction

Messenger RNA (mRNA) conveys the genetic information from the nucleus to the cytoplasm, where it serves as a template for translation into a protein product. The life of mRNA begins with a 'birth' - transcription and keeps on through splicing, polyadenylation, and export from the nucleus (Moore, 2005). Finally, once in the cytoplasm, the fate of the mRNA depends on how well it is translated and where it is placed or stored subcellular (Besse and Ephrussi, 2008). Since all mRNAs are targeted for degradation at the end, the processes of translation and mRNA degradation play important roles in modulating the gene expression and thereby protein production. Both processes are highly regulated and targeted by many factors to enable a cell to respond and adapt to various environmental and physiological changes rapidly and efficiently. Deregulation of these processes has been implicated in different diseases. The molecular mechanisms are becoming increasingly evident, and it is well established that translation and mRNA degradation are tightly coupled but where the degradation is taking place and how it relates to translation is still topic to elucidate. During my thesis, I was working on understanding the interconnection between mRNA translation and mRNA degradation and the possibility of mRNA decay occurring co-translationally on the ribosome complexes.

4.1 mRNA cycle - from 'birth' to 'death.'

Transcription is the 'birth' of RNA in which a particular segment of DNA is copied by the enzyme RNA polymerase giving rise to the primary transcript (pre-mRNA). Pre-mRNA undergoes a series of modifications (e.g., 5' capping, splicing, 3' polyadenylation) before becoming mature mRNA that is taken across from the nucleus to the cytoplasm to be translated into a functional protein (Moore, 2005). Mature mRNA is built of the 5' untranslated region (5' UTR), coding sequence (CDS) and 3' untranslated region (3' UTR) with their modifications and diverse functional elements depicted in Figure 1 (Firoz et al., 2011). One of the most significant modifications is a m⁷G (7-methyl-guanosine) cap on 5' termini and poly(A) tail on 3' termini. These modifications and functional cis-elements in UTRs interact with different RNA-binding proteins (RBPs) and influence or coordinate some regulatory aspects of gene expression, such as splicing forms, translation rates or mRNA stability (Firoz et al., 2011).

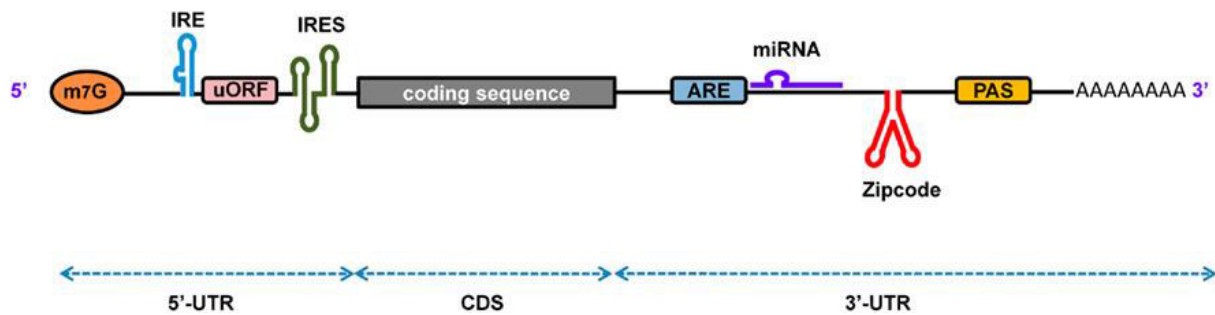


Figure 1 The structure of eukaryotic protein-coding mRNA. Mature mRNA consists of 5' UTR (untranslated region), CSD (coding sequence) and 3' UTR (untranslated region) together with different modifications and cis-elements: m⁷G – 7-methyl-guanosine cap, IRE – iron-responsive element, uORF – upstream open reading frame, IRES – internal ribosome entry site, ARE – AU-rich element, PAS – poly(A) signal (Figure derived from Firoz et al., *Frontiers in Plant Science*, 2011).

Once mRNA is mature, it associates with various proteins building mRNA-protein complexes (mRNPs). These mRNPs are in dynamic equilibrium with the translational pool, shifting rapidly between translation, storage or degradation depending on various cellular processes and adjusting to the needs of the cells at the moment. After a couple of cycles of translation, mRNA may undergo degradation or may be stored in P-bodies or stress granules (Decker and Parker, 2012). For many years it was thought that degradation happens only upon termination of translation when ribosomes drop off from the mRNA. In that case, mRNA depleted of ribosomes is translocated in P-bodies, where most of the factors of degradation machinery are accumulating (Franks and Lykke-Andersen, 2008; Cougot et al., 2004) and where mRNA is finally degraded. However, nowadays there is more and more evidence that mRNA translation and mRNA degradation are linked both in time and space and that mRNA degradation can happen co-translationally while mRNA is still bound to the actively translating ribosomes without the necessity to be transferred to P-bodies. Besides that, the functional role of P-bodies in degradation remains still poorly understood.

After giving a summary of molecular mechanisms of both mRNA translation and mRNA degradation I will focus more on examples of the intimate interconnection between mRNA translation and mRNA degradation which could indicate the possibility of co-translational mRNA degradation and consequently exclusion of P-bodies as the single place for mRNA degradation.

4.1.1 Eukaryotic mRNA translation

mRNA translation is a cyclic process consisting of initiation, elongation, termination, and recycling. The translation initiation is the most highly regulated phase of the translation cycle. An aim is to assemble an 80S ribosome out of the small (40S), and the large (60S) subunits with the initiator methionyl-tRNA (Met-tRNA_i) positioned over the start (AUG) codon of the mRNA prepared for elongation (Reviewed in Aitken and Lorsch, 2012). Such a complex and multistep process is needed to be regulated and

coordinated by at least 12 proteins called eukaryotic initiation factors (eIFs), composed of up to 24 distinct polypeptides (Reviewed in Aitken and Lorsch, 2012).

At the beginning of initiation of translation, the ternary complex (TC) is formed out of eIF2, GTP and the initiator tRNA (Figure 2) (Kolitzand Lorsch, 2010). The ternary complex is recruited to the 40S subunit to form the 43S pre-initiation complex (PIC) thanks to factors eIFs 1, 1A, 3 and 5 (Asano et al., 2001; Algire et al., 2002; Majumdar et al., 2003; Kolupaeva et al., 2005). Meanwhile, eIF4 factors (E, G, A, B) bind to the 5' m⁷G cap of mRNA, whereas poly(A) binding protein (PABP) binds to the poly(A) tail, to form an activated mRNP. An activated mRNP could be recruited to the pre-initiation complex (Sonenberg and Hinnebusch, 2009; Jackson et al., 2010). Upon binding at the 5' end of the activated mRNP, the PIC can start scanning of 5' UTR in a 5'-to-3' direction in an ATP-dependent manner to locate the start codon (AUG) (Berthelot et al., 2004; Hinnebusch, 2011). Start codon recognition triggers eIF1 release and conversion of eIF2 to its GDP-bound state, resulting in an arrest of the scanning process. (Maag et al., 2005; Algire et al., 2005). eIF2•GDP and eIF5 dissociate, clearing the way for eIF5B to mediate joining of the 60S subunit. (Pestova et al., 2000; Acker et al., 2006). Upon joining of 60S subunit, GTP is hydrolyzed by eIF5B and followed by dissociation of factors eIF5B, eIF1A, and eIF3 (Acker et al., 2009). After these steps, 80S initiation complex (IC) is formed and competent to elongate. During elongation, the sequence between the 'start' and 'stop' codon is translated from mRNA into an amino acid sequence—releasing the synthesized protein from the ribosomes. Once translation machinery reaches the stop codon (UAA, UGA or UAG) the termination of translation is triggered. During termination series of coupled events centered around PABP result in the continued recruitment of translation release factor eRF1–eRF3, followed by the hiring of deadenylase complexes (Frolova et al., 1994) and will be explained later in more details in chapter 4.1.2.2.1.

One of the essential characteristics of actively translating mRNA is its circularization provided thanks to the scaffold protein eIF4G (*Wells et al., 1998*). eIF4G is bringing together the 5' and 3' end of the same mRNA via interaction with 5' cap-binding protein (eIF4E) and poly(A) binding protein (PABP) (Figure 2). This 'closed-loop mRNP' stimulates initiation of translation and permits coupling of translation termination and recycling events with subsequent rounds of initiation on the same mRNA (Tarun and Sachs, 1996; Uchida et al., 2002).

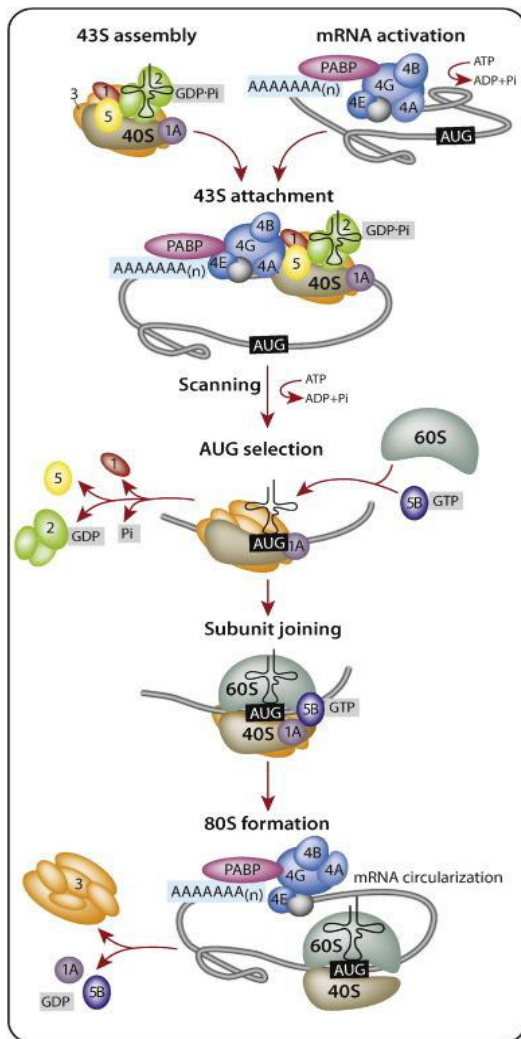


Figure 2 Initiation of translation in Eukaryotes. 43S assembly starts with formation of ternary complex (eIF2, GTP, and tRNA_i) which recruits the 40S subunits thanks to the factors eIFs 1, 1A, 3 and 5. mRNA is activated by binding of eIF4 factors (E, G, A, B) to the 5' cap and by binding of PABP to the poly(A) tail. After recruitment of 43S to activated mRNP, it starts AUG selection using ATP energy. Recognition of start codon causes the release of factors eIF1, eIF5 and eIF2•GDP with Pi allowing the joining of the 60S subunit with the help of eIF5B. Due to the hydrolysis of GTP by eIF5B, the rest of factors (eIF5B, eIF1A, and eIF3) are leaving, and the 80S can start to elongate (Figure derived from Aitken and Lorsch, *Nature Structural & Molecular Biology*, 2012).

However, some mRNAs undergo a cap-independent translation. Though this process is more common in viral genomes cap-independent translation might also happen with cellular mRNAs. The ribosome binds directly to an internal ribosome entry site (IRES) within 5'UTR and bypasses the canonical cap-dependent initiation of translation (Pelletier and Sonenberg, 1988).

4.1.2 Eukaryotic mRNA decay pathways

When the cell does not need particular mRNA anymore, mRNA can be stored temporarily and recycled to polysomes upon request or degraded and thus removed completely (Decker and Parker, 2012). About the storage of mRNA will be written in chapter 4.3, and in this chapter, we will focus on mRNA degradation.

Many components are involved in degradation processes, and some of them are common for both translation and degradation. For example, initiation factor eIF4E which binds to the 5' m⁷G cap of mRNA and poly(A)binding protein (PABP) which binds to the poly(A) tail are not only essential for mRNA circularization and initiation of translation but at the same time, they are also preventing mRNA

from degradation. eIF4E together with a scaffold protein eIF4G protects the 5' cap of mRNA from the 5'-3' exonucleases. On the other end poly(A)-binding protein (PABP) by binding to the poly(A) tail protects transcript from exonucleases attacking the 3' ends of the mRNA. It is enough that at least one of these two structures – the PABP-poly(A) tail, or the eIF4E-m⁷G cap is removed or that mRNA is cleaved internally by endonucleases, and the mRNA decay may be initiated (Garneau et al., 2007).

In Eukaryotes, there are two main degradation pathways. One is general mRNA decay pathway for bulk mRNAs - deadenylation-dependent mRNA decay, which is initiated by poly(A) tail shortening and can lead to either 5'-to-3' exonucleolytic or the 3'-to-5' exonucleolytic decay (Figure 3A) (Houseley and Tollervey, 2009). The second degradation pathway is characteristic for several exceptions when mRNA decay is happening due to aberrant mRNA translation (Houseley and Tollervey, 2009). They are also known as surveillance pathways, and they include nonsense-mediated decay (NMD), non-stop decay (NSD) and no-go decay (NGD) (Figure 3B).

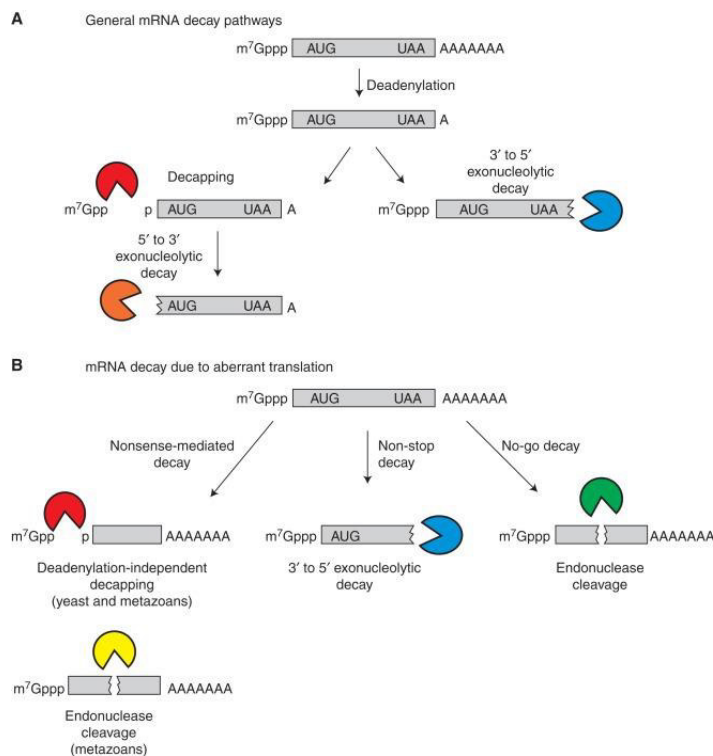


Figure 3 Eukaryotic mRNA decay pathways. A) General mRNA decay pathway - deadenylation-dependent that can proceed with 5'-to-3' decay or 3'-to-5' decay.

B) Specialized decay pathways for degradation of translationally aberrant mRNAs. Non-sense mediated decay (NMD), non-stop decay (NSD) and no-go decay (NGD) (Figure derived from Decker and Parker, Cold Spring Harb Perspect Biol, 2012).

4.1.2.1 General mRNA decay pathways – deadenylation-dependent mRNA decay

General mRNA degradation which is known for bulk mRNAs initiates with the deadenylation as the first and major step (Figure 4).

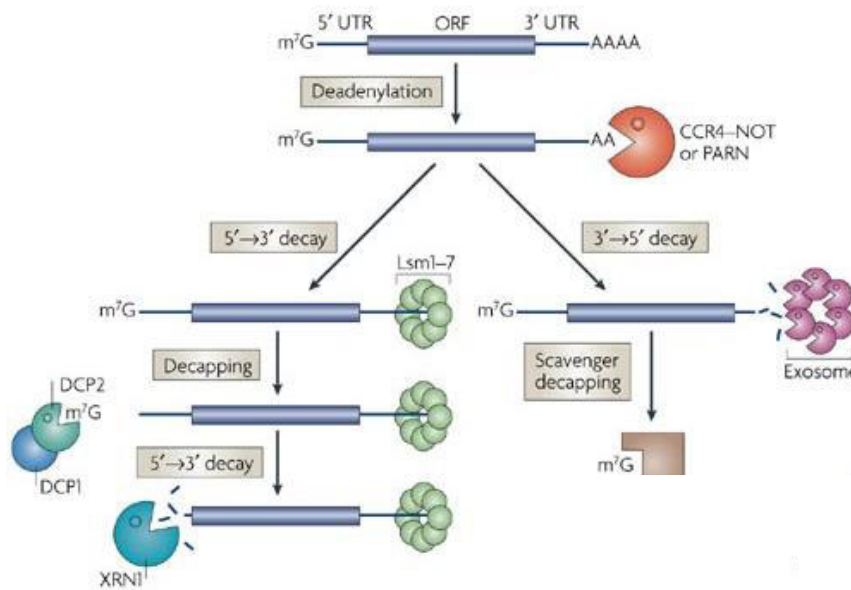


Figure 4 General mRNA decay pathway: deadenylation-dependent mRNA decay. Degradation starts with removal of poly(A) tail and can proceed with 5'-to-3' decay in which 5' m⁷G cap is removed during decapping and after that decapped mRNA body is susceptible to 5'-3' exoribonuclease XRN1. The alternative pathway is 3'-to-5' decay during which a mRNA is degraded by 3'-5' exoribonuclease complex known as exosome and the 5' cap is removed at the end (Figure derived from Garneau et al., Nat Rev Mol Cell Biol, 2007).

Deadenylation is an often rate-limiting step in mRNA degradation as the first and primary step triggering mRNA decay in Eukaryotic cells (Chen and Shyu, 2011). It is unique since it is reversible – transcripts that bear the correct signal can be re-adenylated and return to polysomes (Garneau et al., 2007). But once the cell decides to destroy a mRNA, degradation proceeds with one of two routes: degradation in the 5'-to-3' direction which is predominantly occurring or degradation in the 3'-to-5' direction which is not such a frequent event (Figure 4) (Garneau et al., 2007).

Deadenylation is triggered by recruitment of deadenylase complex PAN2-PAN3, due to its direct interaction with PABP (Siddiqui et al., 2007). Alternatively, deadenylation is triggered by recruitment of deadenylase complex CCR4-NOT via TOB1/TOB2 proteins (Funakoshi, 2007; Ezzeddine et al., 2007) during the general deadenylation or deadenylation can be triggered thanks to cis-acting destabilizing elements or mutations. Cis-acting destabilizing elements together with their binding complexes can recruit deadenylation machinery and promote deadenylation known as targeted deadenylation and will be discussed later in more details (Wahle and Winkler, 2013). Deadenylation occurs in two phases. The first step is carried out by PAN2-PAN3 complex which is a PABP-dependent poly(A) nuclease and is trimming the poly(A) tails of nascent mRNAs to the standard lengths of 60-80 nucleotides (Wahle and Winkler, 2013). PAN2 - PAN3 (PABP-dependent Poly(A) Nuclease) consists of two proteins – PAN2 and PAN3. PAN2 is the first identified in yeast but is conserved across the Eukaryotes. The structure of PAN2 is shown in Figure 5. PAN2 belongs to DEDD/RNaseD family (Death Effector Domain Containing)

of 3′-5′ exonucleases. The same type of domain is found in the CAF1/POP2 exonuclease subunit of the CCR4-NOT complex (Zuo and Deutscher, 2001). It is dependent on magnesium, requires a 3′-OH group and the product of the reaction is 5′-AMP (Lowell et al., 1992). PAN3 is the interacting partner of PAN2. Its structure is also shown in Figure 5. Besides region involved in interaction with PAN2, PAN3 contains unstructured region named PABP-interacting motif 2 (PAM2) that binds to the C-terminal domain of poly(A) binding proteins (PABPs) (Siddiqui et al., 2007). This interaction is vital for recruiting PAN2-PAN3 to the mRNAs (Wolf and Passmore, 2014). Mutations in Pab1p in yeast has the same phenotype as PAN2 and PAN3 deletion strains – longer poly(A) tails indicating that PAN2-PAN3 is active only due to stimulation by poly(A): PABP complex (Sachs and Deardorff, 1992).

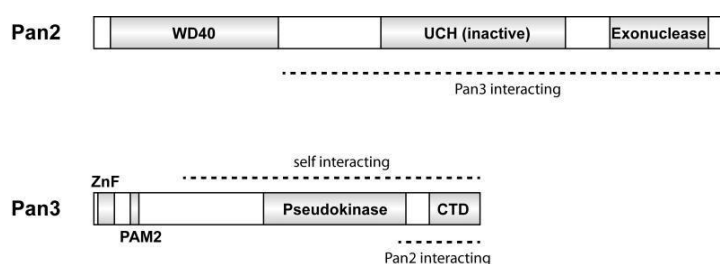


Figure 5 Domain structures of PAN2 and PAN3. The domain structures of PAN2 and PAN3 are depicted as gray rectangles. PAN2 consists of WD40, ubiquitin C-terminal hydrolase (UCH) and exonuclease domain, while PAN3 consists of zinc finger (ZnF), PABP interacting motif2 (PAM2), pseudokinase and C-terminal domain (CTD). Regions involved in protein-protein interactions are indicated by dot line (Figure derived from Wolf and Passmore, Biochemical Society Transactions, 2014).

PAN2-PAN3 carries out the first shortening and is not efficient in removal of the terminal 20-25 nucleotides of the tail since it is PABP-dependent, and once PABP leaves the mRNA, PAN2-PAN3 is not active anymore (Lowell et al., 1992). Therefore, the deadenylation is taken over by another deadenylase complex CCR4-NOT (Carbon Catabolite Repressor 4 - Negative On TATA). The CCR4-NOT complex is the primary deadenylase which unlike PAN2-PAN3 is inhibited by PABPs (Tucker et al., 2001). In *Drosophila* it consists of two exonucleases CCR4 and POP2/CAF1 (Ccr4p-Associated Factor 1) together with NOT1, NOT2, NOT3, NOT4, NOT10, NOT11 and CAF40 (Temme et al., 2014). The domain structure of CCR4-NOT complex and interactions between proteins are shown in Figure 6.

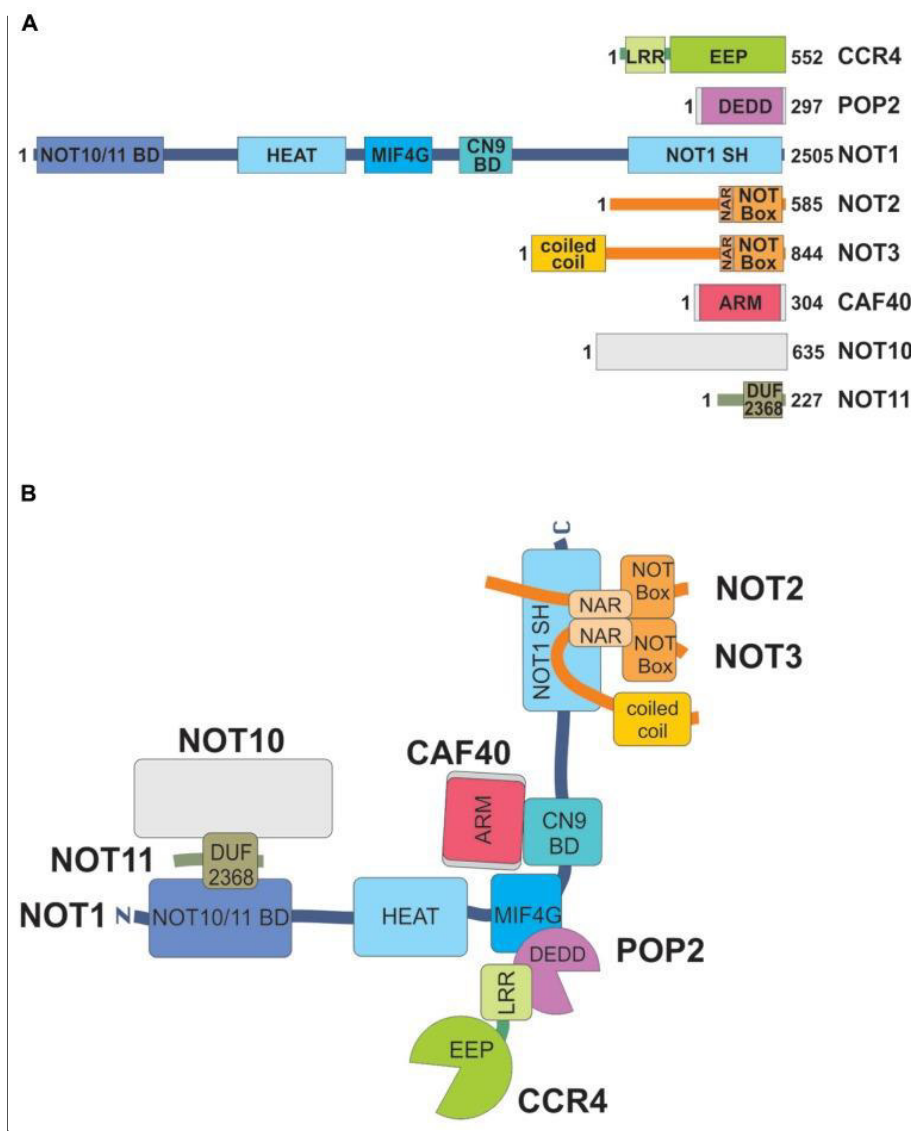


Figure 6 *Drosophila* CCR4-NOT complex and their subunits, domains, and interactions. **A)** Domain structures of subunits. The size of each polypeptide (in amino acids) is indicated on the right, but this can vary due to alternative splicing. The structured domain is depicted as a rectangle. CCR4 contains a leucine-rich repeat (LRR) and an exonuclease-endonuclease-phosphatase (EEP) domain. POP2/CAF1 consists of a single nuclease domain of the DEDD class. NOT1 includes a NOT10/NOT11 binding domain, HEAT repeats, a middle-of-4G (MIF4G) domain, and a CAF40/CNOT9 binding domain (CN9BD) in the middle and conserved NOT1 superfamily homology (NOT1 SH) domain. Both NOT2 and NOT3 contain a C-terminal NOT box preceded by a NOT1

anchor region (NAR). Besides it, NOT3 also has a coiled-coil domain at N-terminus. CAF40 consists of armadillo (ARM) repeats. NOT10 has no known or predicted domain. NOT11 contains a domain of a still unknown function. **B)** Interactions between the subunits of the CCR4-NOT complex. NOT1 as a scaffold protein which reacts directly or indirectly with the other CCR-NOT complex subunits (Figure derived from Wahle and Winkler, *Biochim Biophys Acta*, 2013).

The CCR4 is one of two catalytic subunits of the complex with poly(A) specific 3'-5' exonuclease activity. The protein is a member of the exonuclease-endonuclease-phosphatase (EEP) family of enzymes, which like the DEDD enzymes, catalyzes the hydrolysis of phosphate ester bonds by a two-metal-ion mechanism (Tucker et al., 2002; Chen et al., 2002). POP2/CAF1 is the second catalytic subunit of the complex which has the only one domain DEDD. DEDD domain shows similarities with a domain of all the other proteins belonging to the DEDD/RNaseD family (Zuo and Deutscher, 2001) with magnesium dependent 3'-5' exonucleases activity (Andersen et al., 2009) releasing 5' AMP (Bianchin et al., 2005).

NOT1 is a large L-shaped protein which serves as the central scaffold to anchor all the other components of the CCR4-NOT complex (Wahle and Winkler, 2013). Interestingly, tethering of any of the subunits of the complex to a reporter mRNA was enough to destabilize and repress the translation of that mRNA. Presumably, any subunit, when tethered to the mRNA, can recruit the entire complex, including the polypeptides involved in mRNA destabilization and translation repression (Bawankar et al., 2013).

The CAF40 and NOT modules have no catalytic activity and have been implicated in mediating interactions with RNA-associated proteins which recruit the CCR4-NOT complex to their targets (Sgromo et al., 2018). Some of them are the GW182 family, tristetraprolin (TTP), the germline determinant Nanos, the human Roquin1 and Roquin2 proteins that will be explained in detail in next chapters.

Both PAN2-PAN3 and CCR4-NOT deadenylase complexes are active in cytoplasmic mRNA deadenylation, thus they are predominantly localized in the cytoplasm (Cougot et al., 2004). Moreover, they have been identified as components of P-bodies, cytoplasmic foci enriched in mRNA decapping and the other degradation factors (Cougot et al., 2004; Teixeira et al., 2007) which will be discussed in chapter 4.3.

4.1.2.1.1 General mRNA decay pathways – deadenylation-dependent mRNA decay: the 5'-to-3' degradation

Once the deadenylation is finished the degradation is irreversible and proceeds either with 5'-to-3' or 3'-to-5' deadenylation-dependent mRNA decay. The 5'-to-3' decay keeps on with the removal of 5' m⁷G cap in a process called decapping by a decapping holoenzyme. The decapping holoenzyme is composed of the catalytic DCP2 subunit and the coactivator DCP1. Decapping results into m⁷GDP and 5' monophosphorylated mRNA body (Figure 7) (Garneau et al., 2007).

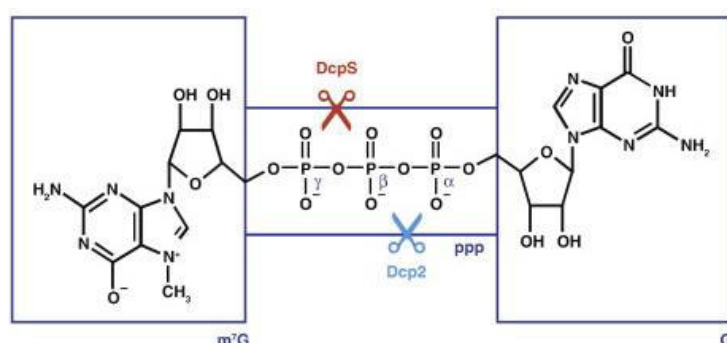


Figure 7 Decapping by DCP2 and DcpS. DCP2 hydrolyzes the m⁷GpppN releasing m⁷GDP and 5'ppN mRNA body (Lykke-Andersen, 2002) whereas DcpS is releasing m⁷GMP and 5'ppN mRNA body (Milac et al., 2014) (Figure derived from Milac et al., Biochim Biophys Acta, 2014).

The decapping enzyme DCP2 is a member of Nudix family of pyrophosphatases. It catalyzes the hydrolysis of the cap structure in a divalent ion-dependent reaction, releasing m^7GDP and a 5' monophosphorylated mRNA body (Lykke-Andersen, 2002). Like other Nudix-domain pyrophosphatases, the catalytic center of DCP2 contains three conserved glutamate residues. They coordinate a divalent cation responsible for activation of a water molecule for cap hydrolysis (She et al., 2006). It is flanked on both sides by two additional conserved regions known as Box A and Box B (Figure 8). Box A is inside of regulatory domain and has been implicated in facilitating the interaction of DCP2 with decapping activator DCP1 while Box B has been shown to be essential for RNA binding (Reviewed in Arribas-Layton et al., 2012).

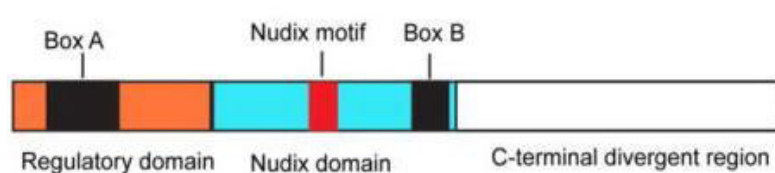


Figure 8 Domain organization of DCP2 enzyme. DCP2 consists of a regulatory domain with Box A, of Nudix domain with Nudix motif and Box B, and of a C-terminal divergent region (Figure derived from Arribas-Layton M. et al., *Biochimica et Biophysica Acta*, 2012)

Purified recombinant DCP2 is not able to efficiently hydrolyze unmethylated cap or free GTP and shows weak activity on a short m^7GpppN - capped oligonucleotides (LaGrandeur and Parker, 1998). That suggests that DCP2 must contact the mRNA in two ways to promote efficient mRNA decapping: firstly, through the m^7GpppN cap and secondly through the contacts with the rest of the 5' end of the mRNA. RNA substrate is binding in a channel on the surface of DCP2. The cap structure is binding in the active site, while the body of the mRNA is wrapping across the Nudix domain and a channel along the Box B. For efficient binding to DCP2 the minimum length of the RNA substrate was predicted to be 12 nucleotides (She et al., 2008). The activity increases significantly when the length of the RNA is increased from around 20–50 to around 100 nucleotides suggesting the existence of an RNA-binding site on DCP2 that is separated from the active site. Deletion analysis has identified such a site on the C-terminal side of the Nudix domain (Piccirillo et al., 2003). The requirement of DCP1-DCP2 for free cap structure and a longer RNA as a substrate suggests that DCP1-DCP2 is avoiding accidental decapping of actively translating mRNAs which already have the translation initiation complex assembled on itself (Coller and Parker, 2004). Structural and kinetics studies indicated that decapping is controlled by the conformational change between the open and closed forms of DCP2 enzyme. DCP1 and the RNA substrate induce the closed form of the enzyme and accelerate the catalytic step of decapping. In addition, the interaction between DCP2 and DCP1, recruitment, and activity of DCP2 are

regulated by various decapping activators and inhibitors (Jonas and Izaurralde, 2013). These factors involve enhancers of decapping 3 - EDC3, like Sm14A - LSm14A (Tral in *Drosophila*, RAP55 in vertebrate and Scd6 in *Saccharomyces cerevisiae*), Pat (Pat1p in *Saccharomyces cerevisiae*, HPat in *Drosophila melanogaster* and PatL1 in humans), the LSm1-7 complex, the RNA helicase DDX6 (Dhh1 in yeast, Me31b in *Drosophila melanogaster* and DDX6/RCK/p54 in mammals), and the species-specific activators Edc1, Edc2 and EDC4/Ge1 (Hedls in human). Their domain structure is shown in Figure 9.

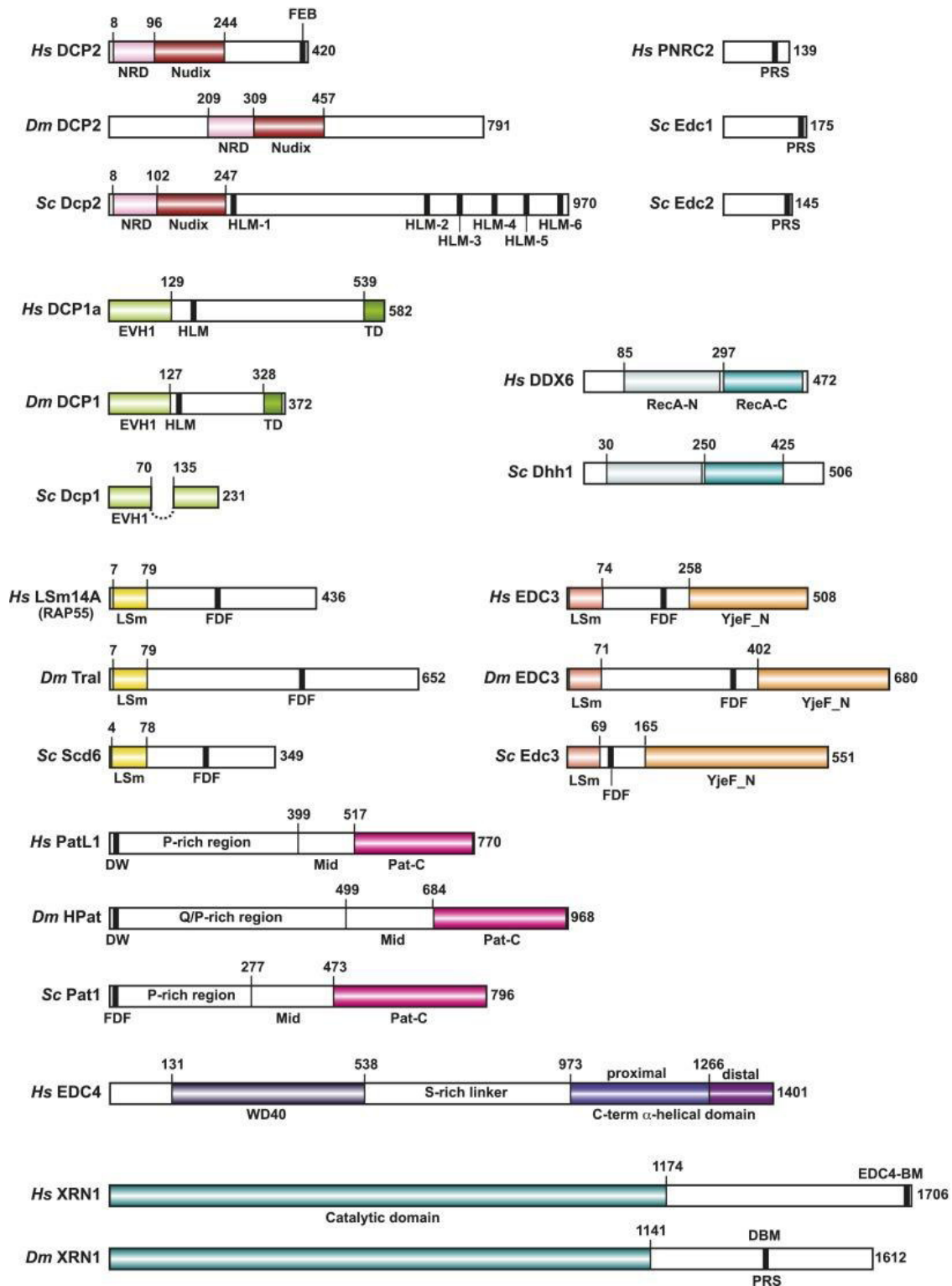


Figure 9 Domain organization of decapping factors. *Sc* (*Saccharomyces cerevisiae*), *Dm* (*Drosophila melanogaster*), *Hs* (*Homo sapiens*). The structured domains are presented as a colorful rectangle while white boxes are disordered regions. The numbers above are numbers of amino-acids. (Figure derived from Jonas and Izaurralde, Genes Dev, 2013).

The exact molecular mechanism how these factors stimulate decapping *in vivo* is still not well known. Decapping activators can be divided in few groups. Some of the decapping activators may promote decapping by inhibiting translation and promoting mRNP rearrangements. These rearrangements increase the accessibility of DCP2 to the mRNA cap structure *in vivo* or *in vitro* which will be discussed later. This group consists of decapping activators like Pat1 (Pat1p/HPat/PatL1), Dhh1 (Dhh1p/Me31b/DDX6), and Scd6 (Tral/LSm14A) (Reviewed in Arribas-Layton et al., 2012). The second group of decapping activators is helping in a transition from deadenylation to decapping and Pat1 also belongs here but also Lsm1-7 complex (Reviewed in Arribas-Layton et al., 2012). The third set of decapping activators is promoting assembling of a larger decapping complex and here are Dcp1, Scd6 and Dhh1 and especially Edc3 and Pat1 which might play a scaffolding role since they interact and recruit many components of decapping machinery (Decker and Parker, 2012). DCP1, Edc1, Edc2, and EDC3 directly enhance decapping by stimulating DCP2 catalytic activity (Decker and Parker, 2012). Meanwhile, EDC4 behaves as a scaffold protein which has a role in indirect activation of DCP2 by bringing DCP1 close to DCP2 (Chang et al., 2014). In human cells, EDC4 binds XRN1 as well and thus may serve to couple DCP2 activity with 5'-to-3' mRNA degradation by XRN1 (Chang et al., 2014) (Figure 10).

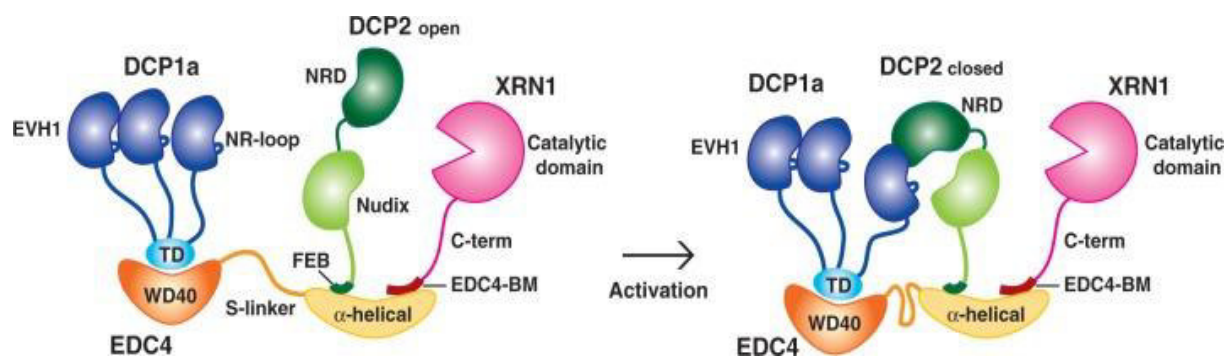


Figure 10 EDC4 as a scaffold protein of decapping complex. N-terminal WD40 domain of EDC4 has binding sites for DCP1 trimers, while the C-terminal α -helical domain of EDC4 binds DCP2 and XRN1. The DCP1 EVH1 domain can interact with the DCP2 NRD. This interaction promotes the active, closed DCP2 confirmation by a mechanism that includes the NR-loop in the DCP1 EVH1 domain. Since the XRN1 binds to the same EDC4 scaffold, it is ensured that decapped mRNAs are near XRN1. DCP1 trimerization is essential for binding to EDC4 and DCP2, although it is still not evident how (Figure derived from Chang et al., Nucleic Acids Res, 2014).

Upon removal of 5' cap mRNA body is susceptible to the very processive XRN1 exoribonuclease which is hydrolyzing mRNA body and finalizing the degradation in 5'-to-3' direction (Jones et al., 2012). The family is typically represented by one cytoplasmic enzyme (XRN1/PACMAN or XRN4) and one or more nuclear enzymes (XRN2/RAT1 and XRN3) (Parker and Song, 2004). Domain structure is shown in Figure 9. The structure of the active site limits the type of RNA that can be degraded to RNA with an exposed 5' monophosphate. Larger structures such as the m⁷GpppN cap or triphosphorylated RNAs do not fit into the primary pocket. This prevents capped mRNA to be degraded before the right timing. C-

terminal half of the protein may act as a scaffold for other proteins involved in 5'-to-3' degradation (Nagarajan et al., 2013).

4.1.2.1.2 General mRNA decay pathways – deadenylation-dependent mRNA decay: 3'-to-5' degradation

As seen in Figure 4 after deadenylation the alternative pathway is degradation in 3'-to-5' direction. In that case unprotected 3' ends of mRNA is attacked by a large complex of 3'-5' exonuclease known as the exosome complex (Garneau et al., 2007). The exosome complex can be cytoplasmic or nuclear, but both have a ring-shaped architecture of the RNA degradation machinery which is structurally conserved throughout bacteria, archaea, yeast, and humans (Lorentzen et al., 2008). Six subunits (Rrp41, Rrp42, Rrp43, Rrp45, Rrp46, and Mtr3 in eukaryotes) possess the RNase PH fold that is characteristic of phosphorolytic RNases and forms a hexameric ring structure around a central channel (Malet et al., 2010). Three more core subunits Rrp4, Rrp40, and Csl4, which contain S1 and KH putative nucleic-acid-binding domains are binding to one side of this hexamer giving rise to Exo9 (core) (Figure 11) (Malet et al., 2010). Unlike archaea, in both the yeast and the human complexes, the phosphorolytic active sites of all the exosome RNase PH subunits have accumulated mutations, which have resulted in the nine subunits complex inactive as a nuclease (Dziembowski et al., 2007). Instead, the RNase activity is mediated by a tenth subunit—hydrolytic protein Rrp44 with both exoribonuclease and endoribonuclease activity—which, in yeast and human, stably associates with Exo9 (core) to form the active ten-subunit complex (Exo10⁴⁴) in the cytoplasm (Malet et al., 2010). In the nucleus of yeast, the exosome core and Rrp44 interact additionally with Rrp6, a distributive 3'-5' exoribonuclease forming Exo11^{44/6}. In human cells also appears to be a nucleolar exosome composed of the core and Rrp6 (Januszyk and Lima, 2014). The exosome complex which has phosphorolytic enzyme activity are using inorganic phosphate to cleave the phosphodiester bonds - releasing nucleotide diphosphates while the exosome complex with hydrolytic enzymes uses water to hydrolyze these bonds - releasing nucleotide monophosphates.

Essential complex for recruitment of exosome to mRNAs is Ski (super killer) complex. It consists of at least three primary proteins, the RNA helicase Ski 2 and the proteins Ski3 and Ski8. In yeast, Ski7, which contains a GTPase-like protein guides the RNA molecules to the exosome complex to be degraded (Wang et al., 2005).

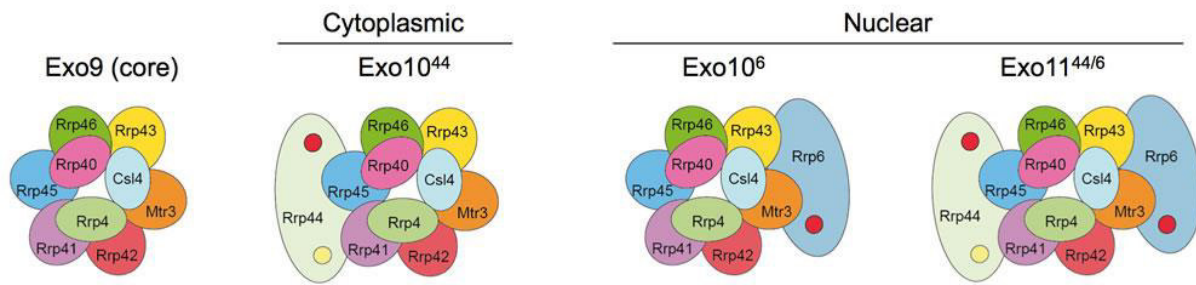


Figure 11 The Eukaryotic exosomes. From the left to the right - the core with nine subunit (Exo9), then cytoplasmic exosome complex with additional Rrp44 subunit with both exo- and endo-ribonuclease activity (red and yellow dot respectively) (Exo10⁴⁴), nuclear exosome complexes with Rrp6 (Exo10⁶) or with both Rrp44 and Rrp6 (Exo11^{44/6}) (Figure derived from Januszyk and Lima, Current Opinion in Structural Biology, 2014).

Upon cleavage of mRNA body into ribonucleotides by exosome starting from 3' end to the 5' end, the m⁷GpppN cap is removed at the very end by the second type of decapping enzyme known as the scavenger decapping enzyme (DcpS) (Coller and Parker, 2004). DcpS enzymes are dimers belonging to the Histidine Triad (HIT) superfamily of pyrophosphatases. Because of DcpS-mediated hydrolysis, the cap (m⁷GpppN) is turned into 7-methylguanosine monophosphate (m⁷GMP) and nucleoside diphosphate (NDP) (Figure 7) (Milac et al., 2014). Although the structure of DcpS does not exclude the possibility of long binding mRNAs, the biochemical data showed that DcpS does not cleave the cap from the large mRNA molecules (Liu et al., 2002; Wang et al., 2002). The inability to cleave long mRNAs may be biologically essential to prevent DcpS from premature decapping of mRNAs which are not targeted for degradation.

4.1.2.2 mRNA decay due to aberrant translation

As mentioned in paragraph 4.1.2 and shown in Figure 3 beside general mRNA decay for bulk mRNAs some aberrant mRNAs are undergoing the decay known as surveillance pathway. It prevents mRNAs to be translated into aberrant protein thereby inhibiting the synthesis of truncated and potentially harmful proteins. Depending on a type of mistakes in mRNA there are three different mechanisms of decay: nonsense-mediated decay (NMD) (Popp and Maquat, 2013; Lykke-Andersen and Jensen, 2015), non-stop decay (NSD) (van Hoof, 2002) and no-go decay (NGD) (Doma and Parker, 2006). These processes were first to show the close link between mRNA translation and mRNA decay, and therefore they will be described in more details.

4.1.2.2.1 mRNA decay due to aberrant translation: Nonsense-mediated mRNA decay (NMD)

Nonsense-mediated mRNA decay (NMD) pathway is the translation-dependent pathway which recognizes and eliminates mRNAs with translation premature termination codon (PTC) positioned more than 50–55 nucleotides upstream of the last exon-exon junction (Nagy and Maquat, 1998). It can

also recognize the mRNAs which have a long 3' UTR (Lewis et al., 2003). PTC can be introduced because of genetic or somatic mutations, mistakes during transcription, or errors during splicing. The defective polymer both mRNAs and proteins must be first recognized, subsequently labeled for degradation, and finally destroyed (Popp and Maquat, 2013). Detection of PTC is intimately connected to translation termination during an initial pioneer round of translation (Maquat et al., 2010).

As seen from the Figure 12 A during the efficient translation termination one of the termination codons (TC) (UAA, UGA or UAG) is in physical proximity to both 3' poly(A) tail and 5' 7-methylguanosine (m⁷GpppN) cap of the mRNA. It is due to the circularization of mRNA provided by interaction of proteins PABPC and eIF4G. Once the terminated ribosome reaches the TC the eukaryotic release factor complex eRF1–eRF3-GTP recognizes the TC and interacts with the terminated ribosome (Frolova et al., 1994). In a normal situation, when the TC is proximal to the 3' and the 5' end, mRNA circularization also has a role in preventing ATP-dependent RNA helicase up-frame shift 1 (UPF1) from interacting with eRF3-GTP. In this case, UPF1 only binds transiently and nonspecifically to the mRNA allowing to the termination efficiently to end (Kervestin and Jacobson, 2012). Terminated ribosome and eRF1 stimulate hydrolysis of GTP by eRF3, this triggers eRF1 rearrangements that induce nascent protein to leave and eRF3-GDP to dissociate (Frolova et al., 1996). After eRF3-GDP dissociation, Rli1 (RNase L inhibitor 1) binds to the ribosome causing ribosome subunit dissociation and eRF1 leaves (Barthelme and Dinkelaker, 2011).

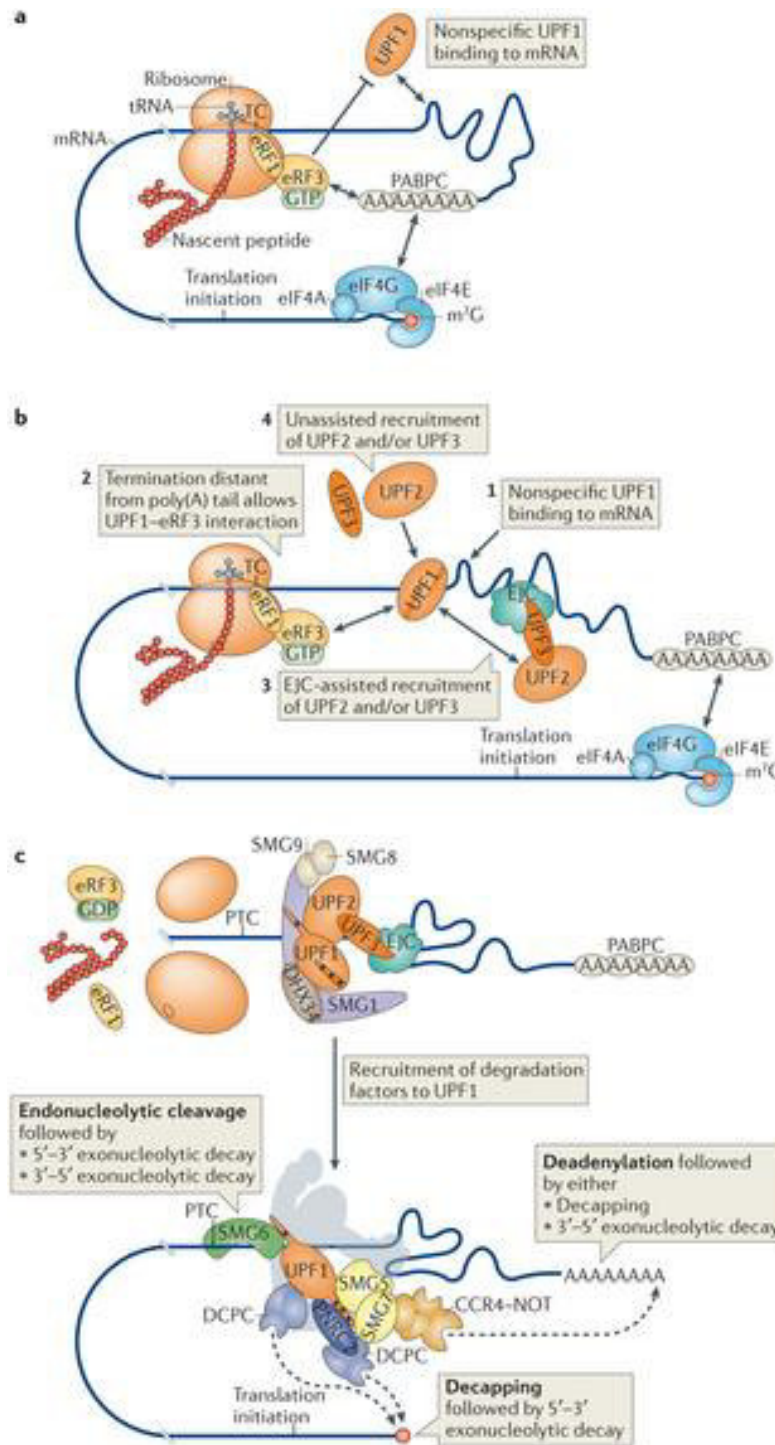


Figure 12 Regular and nonsense-mediated mRNA decay (NMD)-activating translation termination.

A) Efficient termination of translation. PABPC-eIF4G interaction prevents stable binding of UPF1 to mRNA. The ribosome reaches the TC recognized by eRF1-eRF3-GTP and induces the multi-steps process which leads to disassembling of ribosome subunits and nascent protein.

B) Premature termination of translation. Since TC is distant from 3' and 5' termini, UPF1 binds to mRNA and associates with eRF3 and together with EJC recruits UPF2 and UPF3.

C) NMD activation. After UPF1, UPF2 and UPF3 NMD complex recruits SMG1, SMG8, SMG9, DHX34, the ribosome subunits disassemble and NMD complex initiates endonucleolytic cleavage, deadenylation, and decapping (Figure derived from Lykke-Andersen and Jensen, Nature Reviews Molecular Cell Biology, 2015).

In the case of PTC (Figure 12B) terminated ribosome will associate with eRF1-eRF3-GTP but 50 – 55 nucleotides upstream of exon-exon junction where the exon junction complex (EJC) is assembled. Therefore, ribosome cannot physically move on due to EJC. Also, in this case, eRF3 is too far from both 5' and 3' termini and can not block anymore the association of UPF1 with eRF3 and their interaction can trigger the NMD. UPF1 and EJC will now recruit the other well-conserved proteins like UPF2 and

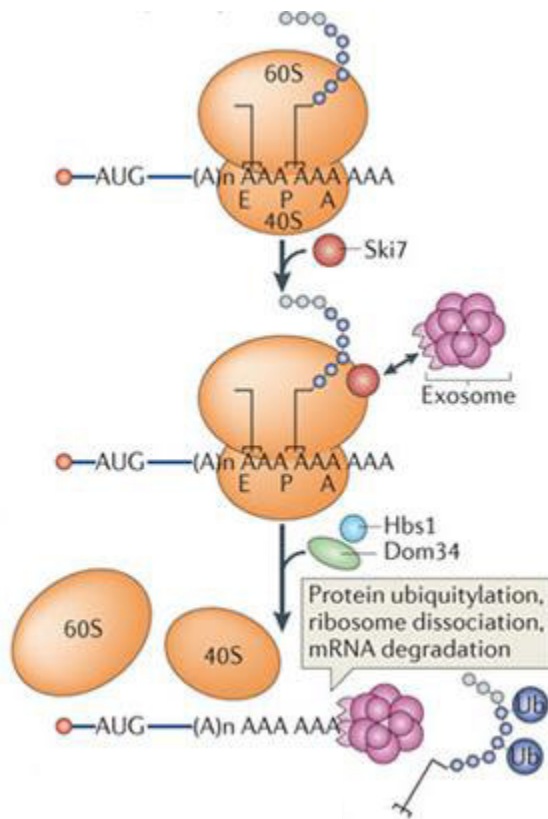
UPF3, suppressor of morphogenetic effect on genitalia 1 (SMG1), SMG5–SMG7 dimer, SMG8, SMG9, DEAH box polypeptide 34 (DHX34) (Lykke-Andersen and Jensen, 2015). At this point, translation may terminate, ultimately leading to the dissociation of the individual ribosomal subunits, the release of factors and the nascent protein. At the same time, SMG1 phosphorylates residues in the amino- and carboxy-terminal domains of UPF1 (Yamashita et al., 2009). Phosphorylated UPF1 allows the recruitment (Figure 12C) of factors that initiate mRNA degradation by endonucleolytic cleavage done by SMG6 (Gatfield and Izaurralde, 2004; Eberle et al., 2009). Also, phosphorylated UPF1 allows deadenylation by CCR4–NOT deadenylase complex recruited via the SMG5–SMG7 dimer (Loh et al., 2013) and/or decapping done by the decapping complex recruited via Pro-rich nuclear receptor co-activator 2 (PNRC2) (Lai et al., 2012). Endonucleolytic cleavage, deadenylation, and decapping are followed by complete mRNA degradation by general cellular 5′-to-3′ and 3′-to-5′ exonucleolytic activities (Garneau et al., 2007). Interestingly, it seems that UPF1 is a mediator between translation termination and decay activator and can be considered to have a dual role as translation repressors and a decay activator.

4.1.2.2.2 mRNA decay due to aberrant translation: Non-stop decay (NSD)

The translational-dependent decay pathways that are induced by stalling the ribosomes are non-stop decay (NSD) and no-go decay (NGD). As a result of mutations, some mRNAs lack the stop codon (UAA, UGA or UAG) thus ribosome is stalled inside the poly(A) tail in 3′ end of mRNA and mRNA is translated into nascent protein with a poly-Lys extension (Figure 13A). This triggers non-stop decay (NSD) by recruiting the super killer 7 (Ski7)–exosome complex leading to ribosome dissociation, release of the nascent protein and exosome-mediated mRNA degradation in 3′-to-5′ direction (Graille and Séraphin, 2012). In *S. cerevisiae* in the absence of Ski7, a second pathway - the 5′-to-3′ mRNA decay pathway can mediate NSD. It seems to be due to the removal of PABP by the translating ribosome. Removal of PABP has, as a result, the reduction of the translational efficiency but also renders transcripts susceptible to decapping, connecting mRNA translation and mRNA decay (Inada and Aiba, 2005).

4.1.2.2.3 mRNA decay due to aberrant translation: No-go decay (NGD)

The ribosome can stall due to faulty mRNAs that have a very stable stem-loop near the stalling site, a damaged base within the ribosomal A-site or an in-frame poly(A) stretch (at least 18 adenine residues) (Figure 13B). These mRNAs are degraded by the no-go decay (NGD) pathway in which such mRNAs are endonucleolytically cleaved by Dom34 and Hbs1 and afterward degraded by the 5′-3′ exoribonuclease XRN1 and by exosome in 3′-to-5′ direction (Doma and Parker, 2006). The nascent peptide is degraded by Ltn1–Not4-mediated proteasomal degradation (Graille and Séraphin, 2012).

A) Non-stop decayⁱ

B) No-go decay

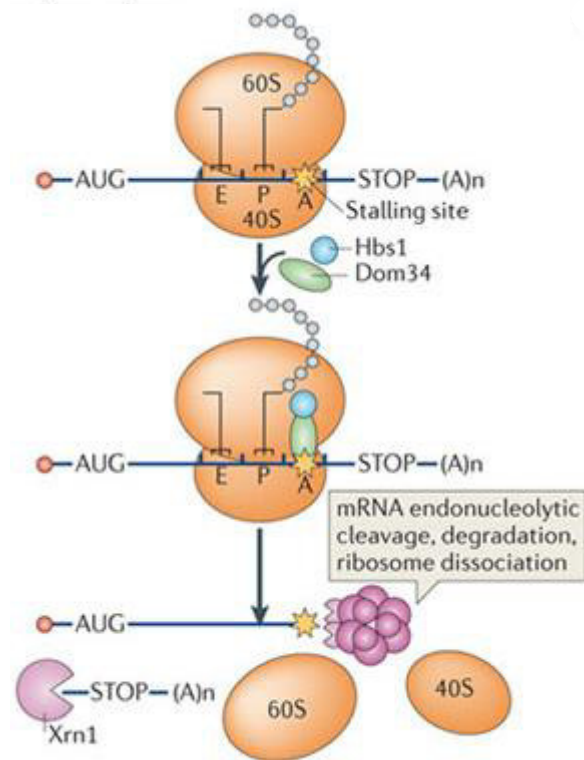
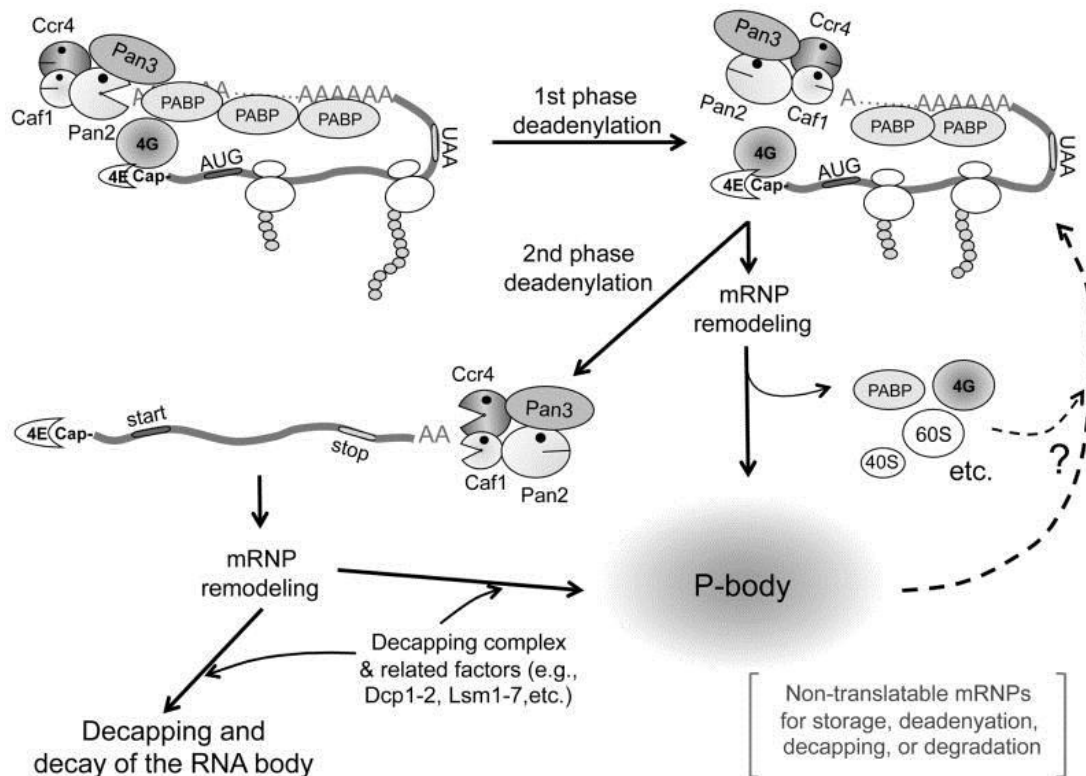


Figure 13 mRNA decay due to the stalling of the ribosome. A) In the case of non-stop decay, the translating ribosome is stalled in poly(A) tail triggering the recruitment of Ski7-exosome complex which starts the degradation of aberrant mRNA in 3'-to-5' direction. **B)** When the ribosome is stalled, no-go decay is triggering the endonucleolytic cleavage by Hbs1 and Dom34 (Figure derived from Graille and Séraphin, Nature Reviews Molecular Cell Biology, 2012).

4.1.3 P bodies and stress granule

According to many observations, it has been believed that mRNA degradation occurs only after ribosome dissociation, in ribosome-free areas known as P-bodies (processing bodies, also referred to as GW182 or DCP bodies). mRNA degradation factors are accumulating there (Franks and Lykke-Andersen, 2008; 2006; Ingelfinger et al., 2002; Sheth and Parker, 2003). As we saw above mRNA bound to polysomes is undergoing rounds of translation initiation, elongation, and termination to produce polypeptides. In response to defects in any of phases of translation or to make sure not to make translation ad infinitum, mRNA recruits deadenylase complexes PAN2-PAN3 and CCR4-NOT that are responsible for two-steps deadenylation (Wahle and Winkler, 2013; Lowell et al., 1992; Tucker et al., 2001). After deadenylation factors for efficient translation such as PABP, eIF4G and ribosomes may dissociate from the mRNPs. It would allow mRNPs to reversibly associate with P-bodies where



In addition to non-translated mRNAs, P-bodies contain the conserved group of proteins that participate in mRNA decay and translation repression. These proteins involve the decapping enzyme complex DCP1/DCP2, the decapping activators Edc3 and the Lsm1-7 complex. Besides it includes factors that function both as repressors of translation and as activators for decapping as Dhh1/RCK/p54, Pat1 and Scd6/RAP55 and the 5'-3' exonuclease Xrn1, but also the Ccr4/Pop2/Not deadenylase complex (reviewed in Anderson and Kedersha, 2006; Eulalio et al., 2007a; Parker and Sheth, 2007). Interestingly, metazoan P-bodies contain additional factors including proteins and miRNAs involved in the miRNA repression pathway (Eulalio et al., 2008). Beside general mRNA decay machinery, P-bodies can also contain mRNAs and proteins engaged in NMD (Sheth and Parker, 2006; reviewed in Franks and Lykke-Anderson, 2008). Translation initiation factors and ribosomal proteins are excluded from P-bodies except for eIF4E in mammalian P-bodies. However, since P-bodies also contain eIF4E-T, which inhibits eIF4E function, EIF4E is anyway bound to repressed mRNA (Ferraiuolo et al., 2005).

The formation of P-bodies is expected to have the specific roles of increasing the local concentration of decay factors within granules and their depletion from the bulk cytosol. They are self-assembling structures, but the mechanism of assembling itself is still unknown though it has been hypothesized that mechanism involves intermolecular contacts between disordered protein regions of proteins involved in decapping (Jonas and Izaurralde, 2013). For example, in budding yeast, the decapping stimulator Edc3 and carboxy-terminal domain of Lsm4, subunit of the Lsm1-7 complex play important roles in assembling mRNPs into P bodies. Most probably it is provided by promoting intermolecular contacts between mRNPs constructed with decapping complexes (Decker et al., 2007).

The presence of the mRNA decay machinery and mRNA decay intermediates in P-bodies is consistent with the hypothesis that mRNA degradation can occur in these structures (Sheth and Parker, 2003). Few other observations can additionally suggest that as well. Namely, once translation initiation is inhibited by drugs, stresses, or mutations the mRNA is dropping off from polysomes which results in increased mRNAs in P-bodies and stress granules (Kedersha et al., 2005; Teixeira et al., 2005; Anderson and Kedersha, 2006). On the other hand, trapping mRNAs in polysomes by blocking translation elongation decreases P-bodies and stress granules even during the continued stress (Cougot et al., 2004; Teixeira et al., 2005; Mollet et al., 2008).

Collectively the observations forehead mentioned could support the hypothesis that mRNA degradation only happens upon dropping off from the ribosomes and accumulating of mRNAs in P-bodies. However, recently this model was challenged by several observations. For example, it was shown that release of mRNAs from polysomes is not enough to trigger P-body assembly: polysome-free mRNAs must enter repression and/or decapping pathways to nucleate P bodies. Thus, even though P-body components play significant roles in mRNA silencing and decay, gathering into P bodies is not necessary for function but is instead a consequence of their activity (Eulalio et al., 2007b). When the aggregation of mRNPs into microscopically visible P-bodies was inhibited by generating genetic mutations, decapping was still happening, showing that P-bodies are not required for mRNA decapping in yeast (Decker et al., 2007). P-bodies were also not needed for translation repression during the stress in both yeast and mammals (Decker et al., 2007; Buchan et al., 2008) nor for the mRNA stability during the stress in yeast (Buchan et al., 2008). Also, in metazoans, depletion of microscopically visible P-bodies does not seem to affect miRNA-mediated repression, the decay of messages containing ARE elements, or the decay of transcripts subjected to NMD (Chu and Rana, 2006; Stoecklin et al., 2006; Eulalio et al., 2007b).

Thus, it seems that P-bodies are not the only place for mRNA silencing and decay. That gave us a hint for the hypothesis that mRNA degradation could occur even without P-bodies while mRNA is still

associated with the ribosomes thus co-translationally. Furthermore, we have found the numerous observations showing the close interconnection between mRNA translation and mRNA degradation that was encouraging our assumption. They will be presented in the chapter 4.2.

4.2 Eukaryotic translation and mRNA degradation are closely linked

The intimate link between mRNA translation and degradation could be noticed in surveillance pathways but also in general mRNA degradation.

4.2.1 The link between Eukaryotic translation and surveillance pathways

As we saw in previous chapters (4.1.2.2.1, 4.1.2.2.2 and 4.1.2.2.3) all three types of surveillance pathways (NMD, NSD, and NGD) specialized for degradation of aberrant mRNAs are translationally-dependent, thus in close relation with translation. If a translation is stopped during the elongation step (NGD) or the termination step (NMD and NSD), the decay machinery will be recruited to repress the translation and to degrade aberrant mRNA. Evidence for polysome-associated NMD are deriving from yeast, where decapped NMD substrates are detected in the polyribosome fraction (Hu et al., 2010). The finding that translational repression is obligatory for NMD in mammals also indicates that at least the first stages of NMD take place on polysomes (Isken et al., 2008).

4.2.2 The link between Eukaryotic translation and general mRNA degradation pathway

Not only surveillance pathways, but it seems that general degradation pathway has also been interconnected with translation in many ways. One of the first evidence of linkage between translation and degradation is the direct and both ways dependence between the level of translation and the stability of mRNA. More protein may be produced from mRNA that has higher stability (Yu and Russell, 2001). Conversely, well-translated mRNAs have longer half-lives, while poorly translated mRNAs are relatively unstable.

Two mRNA structures have the leading role in connecting translation and degradation - the poly(A) tail and the m⁷G cap. As described above (chapter 4.1.1), the poly(A) tail and the m⁷G cap structure together with their binding proteins (PABP and eIF4E) are enhancing translation initiation by forming a closed loop. Also, PABP and eIF4E have a remarkable role in the regulation of mRNA decay by protecting the poly(A) tail and the m⁷G cap from exonuclease attacks (Sachs, 2000). So deadenylation and removal of PABP or decapping and removal of eIF4E can impact a mRNA by reducing its translatability and stimulating its degradation (Chen and Shyu, 2013). Thus, the dual role of poly(A) tail - PABP and of m⁷G cap - eIF4E in both translation and mRNA decay suggests the intimate link between translation and mRNA decay. Poly(A) tail and m⁷G cap could be presented as a competition point for

translation machinery and decay machinery. Depending on protein complexes that are assembled around the poly(A) tail and m⁷G cap the mRNA will be actively translated or repressed and/or degraded.

Since deadenylation and decapping serve as an important control point for both degradation and translation, it is not surprising that regulation of deadenylation and decapping provides the critical roles in regulating eukaryotic gene expression during different biological processes, such as embryogenesis, cell growth, and cell differentiation (Chen and Shyu, 2013).

4.2.2.1 Regulation of PABP and deadenylation: the link between translation and mRNA degradation

In this chapter, we will see how PABP and poly(A) tail have a dual role in both translation and degradation and how their regulators can modulate at the same time both translation and degradation suggesting the intimate connection between these two processes.

The mRNA translation is stimulated by binding of PABP to poly(A) tail (Reviewed in Peltz et al., 1992). Whereas the translational repression and/or mRNA degradation are triggered upon removal of PABP and poly(A) tail. The regulators that stimulate removal of PABP and poly(A) tail are making the mRNA less translated and more degradable. The poly(A) tail can be removed by general mRNA deadenylation or by targeted mRNA deadenylation (Reviewed in Wahle and Winkler, 2013). mRNA which is undergoing targeted mRNA deadenylation have specific cis-sequences that can be recognized by sequence-specific RNA-binding proteins or by the microRNA machinery that afterward recruits CCR4–NOT deadenylation complex and induces repression of translation and promotion of mRNA decay (Reviewed in Wahle and Winkler, 2013). These mRNAs that recruit deadenylase complexes better than the others are less efficiently translated and less stable (Reviewed in Goldstrohm et al., 2008). There are several examples of targeted recruitment of CCR4–NOT deadenylase mediated via sequence-specific RNA elements and their RNA-binding proteins. These sequence-specific RNA elements and their RNA-binding proteins are called regulators. They are called dual regulators since they are translation repressors and degradation activators at the same time. By accelerating the recruitment of CCR4–NOT deadenylase they affect both translation and decay by causing the translation repression and degradation activation. Therefore, in the next chapters will be presented few examples of targeted CCR4–NOT recruitments facilitated by dual factors. Dual factors might be the direct link between translation and degradation.

4.2.2.1.1 PUF family as a dual regulator: a translation repressor and a degradation activator

Members of PUF family are important dual regulators in controlling both the translation and mRNA stability, therefore taking part in modulation of mRNA expression. They perform by binding to the 3' UTR and acting combinatorial with the other regulatory proteins enhancing turnover or repressing translation (Wickens et al., 2002). One of the most common proteins of PUF family is Mpt5 RNA binding protein which is involved in inducing translation repression and degradation of several mRNAs, including the *HO* mRNA in *S. cerevisiae* (Goldstrohm et al., 2006). Repression by Mpt5 requires its binding to a particular sequence element in the 3' UTR of the target mRNA and recruitment of the Ccr4–Not complex via interactions with the Pop2/Caf1 subunit followed by mRNA degradation (Goldstrohm et al., 2007). *Drosophila* has similar RNA binding protein as Mpt5 called Pumilio. The interactions between PUF family proteins and CAF1 homologs seem to be conserved in *C. elegans* and human, suggesting that PUF proteins may have similar roles in translation repression and mRNA degradation in other eukaryotes as well (Goldstrohm et al., 2006).

4.2.2.1.2 ARE elements as dual regulators: a translation repressor and a degradation activator

A second example of regulated recruitment of the CCR4–NOT complex which causes both translation repression and acceleration of mRNA degradation is provided by ARE-mediated decay acting as dual regulator. AREs (Adenylate/uridylate-Rich Elements) are defined as a short region with frequent adenine and uridine bases within 3' UTR of mRNA (Chen and Shyu, 1995). They are present in many mRNAs with short half-lives, including the transcripts encoding the cytokine tumor necrosis factor (TNF) α or the inducible transcription factors c-Fos and c-Myc (Chen and Shyu, 1995; Barreau et al., 2005; Shaw et al., 1986). The ARE elements are recognized by the zinc-finger-containing Tristetraprolin (TTP) protein. A short peptide motif at C-terminus of TTP interacts with NOT1 which stimulates recruitment of the whole CCR4–NOT complex and therefore promotes translation repression and mRNA degradation (Sandler et al., 2011). TIS11 is the *Drosophila* ortholog of mammalian TTP and mechanism of ARE-mediated decay seems conserved between *Drosophila* and mammals (Brooks et al., 2013; Fabian et al., 2013).

4.2.2.1.3 Smaug protein as a dual regulator: translation repressor and degradation activator

The third example of targeted recruitment of CCR4–NOT components that induces repression of translation and acceleration of mRNA degradation is happening during the early embryonic development of *D. melanogaster* when maternal mRNAs must be degraded fastly. The most important RNA-binding regulator is Smaug which strictly regulates the degradation of two maternal mRNAs: nanos and Hsp83. Smaug has an affinity for sequences in the 3' UTR of the *nanos* mRNA called SRE

(Smaug Recognition Element) and in the meantime has the affinity for the CCR4–NOT complex. Upon binding of Smaug to the 3' UTR of *nanos* the CCR4–NOT deadenylase is recruited to the *nanos* mRNA resulting in its fast degradation (Semotok et al., 2005; Jeske et al., 2006; Zaessinger et al., 2006). Smaug can also interact with CUP protein in embryos (Nelson et al., 2004) and since CUP interacts with CCR4–NOT, it is believed that CUP additionally facilitates tethering of deadenylase complex to *nanos* mRNA (Igreja and Izaurralde, 2011).

Interestingly, *nanos* mRNA is also regulated by two piRNAs (Piwi-interacting RNAs), small non-coding, 23-30 nt long RNAs, deriving from transposable elements. They are binding within 3' UTR of *nanos* mRNA by complementarity and can recruit Argonaute proteins Aubergine and Argonaute 3 as well as Smaug and CCR4. It triggers deadenylation and leads to repression and degradation of *nanos* mRNA (Rouget et al., 2010).

4.2.2.1.4 TOB/BTG proteins as dual regulators: translation repressor and degradation activator

BTG/TOB factors are a family of antiproliferative proteins whose expression is altered in numerous cancers. TOB/BGT family could be involved in regulation of mRNA expression in cell differentiation, development, and apoptosis. While *Drosophila* has only the single TOB protein, humans have family consists of six members. Expression of TOB proteins increases the rate of deadenylation, but the exact mechanism is still to elucidate. However, it may be that TOB is interacting with specific RNA-binding protein and thus recruits the CCR4–NOT through interaction with CAF1 subunit of deadenylase complex (Mauxion et al., 2009; Doidge et al., 2012; Ezzeddine et al., 2012). The two mammalian TOB and single *Drosophila* TOB have the conserved PAM2 (PABP interacting motif 2) that allows binding to PABP and thus recruitment of CCR4–NOT deadenylase resulting in translation repression and mRNA degradation.

4.2.2.1.5 Hsp70 3' UTR as a dual regulator: translation repressor and degradation activator

Hsp70 is a heat shock protein, 70kDa, well-conserved, involved in critical cellular processes, as the folding of proteins and protecting cells from the stress (Tavaria et al., 1996). Upon heat shock, the transcription of *Hsp70* mRNA is increased tremendously, but as soon as the cell is returned to normal growth temperature, the *Hsp70* mRNA must be removed quickly. Half-life is very short due to rapid deadenylation and degradation. It is known that fast deadenylation is induced by 3' UTR (Boenisch et al., 2007) which in this case plays dual role in repressing translation and triggering decay. But specific cis-element has not been identified yet as well as the protein factors that might be involved.

4.2.2.1.6 Roquin as a dual regulator: translation repressor and degradation activator

Roquin proteins *Hs* Roquin1 and *Hs* Roquin2 are implicated in the prevention of autoimmunity. The ROQ domain of *Hs* Roquin1 and Roquin2 recognizes specific stem-loop structures in the 3'-untranslated region (UTR) of target mRNAs. These targets include mRNAs encoding regulators of inflammation such as the inducible T-cell costimulator, the costimulatory receptor Ox40, neuropilin-1, interleukin-6, interferon γ (IFN- γ) and the tumor necrosis factor- α (TNF- α). *Hs* Roquin1 and Roquin2 use their C-terminal extensions to directly recruit the CCR4-NOT complex to mRNA targets, promoting the degradation of these mRNAs. *Drosophila melanogaster* (*Dm*) Roquin also interacts with the CCR4-NOT complex through their C-terminal regions. The C-terminal region of *Dm* Roquin contains multiple motifs that mediate CCR4-NOT binding. One motif binds to the CAF40 subunit of the CCR4-NOT complex (Sgromo et al., 2017).

4.2.2.1.7 Bam as a dual regulator: translation repressor and degradation activator

Drosophila melanogaster Bag-of-marbles (Bam) promotes germline stem cell (GSC) differentiation by repressing the expression of mRNAs encoding stem cell maintenance factors. Bam regulates the expression of its mRNA targets by recruiting the CCR4-NOT complex through interaction with CAF40 and which causes mRNA decay and translational repression (Sgromo et al., 2018).

4.2.2.1.8 miRNAs as dual regulators: translation repressor and degradation activator

One of the most important examples of a connection between translation, repression, and degradation is miRNA-mediated repression and/or degradation which is the result of targeted recruitment of CCR4-NOT components where miRNAs play a role as dual regulators of translation repression and decay and will be explained in more details.

miRNAs are a conserved class of small non-coding RNAs approximately 21 nucleotides long which are assembling into RNP called miRNP or miRNA-induced silencing complex (miRISC) (Bushati et al., 2007; Kloosterman and Plasterk, 2006). One of the most important components of miRISC are proteins of Argonaut (AGO) family present in all metazoans and plants (Peters and Meister, 2007; Tolia and Joshua-Tor, 2007). Mammals contain four AGO proteins, from AGO1 to AGO4. AGO2 has endonucleolytic activity and together with small interfering RNAs (siRNAs) which are perfectly complementary to their mRNA targets are involved in RNA interference (RNAi) (Peters and Meister, 2007; Tolia and Joshua-Tor, 2007). *D. melanogaster* has only two AGO proteins, AGO1 and AGO2. AGO2 is specialized for RNAi while AGO1 is included in miRNA pathway. *C. elegans* expresses even 27 AGO

proteins, but only RDE1 is involved in RNAi while ALG1, and ALG2 are involved in miRNA pathway (Peters and Meister, 2007; Tolia and Joshua-Tor, 2007).

Structural and biochemical analysis of archaeal, bacterial, yeast and human AGO proteins have shown bi-lobed architecture. One lobe contains N-terminal and PIWI-AGO-ZWILLE (PAZ) domains, and the other lobe includes the MID and PIWI domains (Ipsaro and Joshua-Tor, 2015). Domain structure of human AGO2, as a representative of the family, is shown in Figure 15. The PAZ domain makes a binding pocket for the 3'-hydroxyl group of small RNAs (either miRNA or siRNA) while the PIWI domain makes a binding pocket for 5'-phosphate group of small RNAs. Small RNA and its mRNA target bind in a cleft between the two lobes (Ipsaro and Joshua-Tor, 2015). PIWI domain has RNaseH fold that gives endonucleolytic activity to some AGO proteins (like in the case of AGO2 in *D. melanogaster* and human) so that they mainly function in RNAi (Ipsaro and Joshua-Tor, 2015).

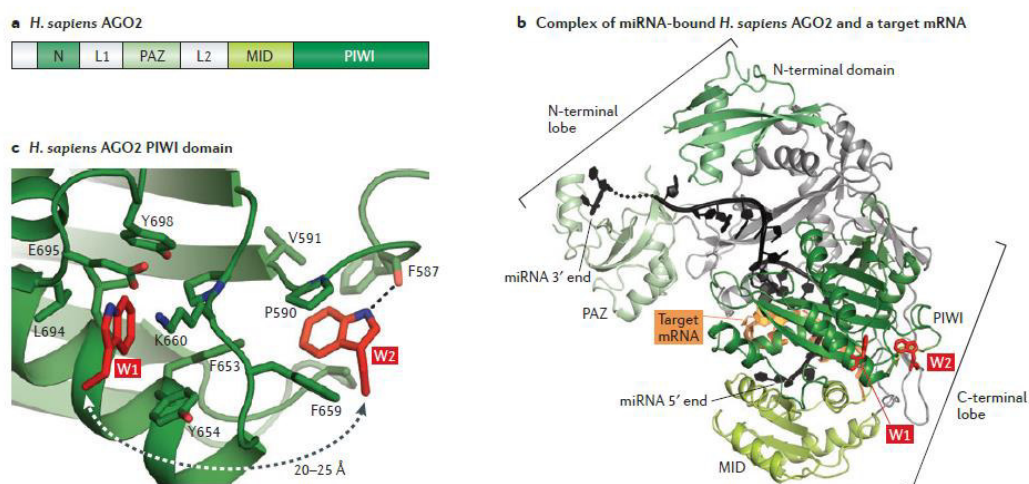


Figure 15 Domain structure of *Homo sapiens* AGO2. AGO proteins have four domains, N, PAZ, MID and PIWI connected with linkers L1 and L2. (Figure derived from Jonas and Izaurralde, Nat Rev Genet, 2015).

Beside proteins from AGO family miRISC complex contains very often additional proteins which are regulatory factors or effectors conducting inhibitory function of miRNPs (Peters and Meister, 2007). One of the most common effector proteins is GW182. This protein is present in all metazoans, but interestingly they are not found in fungi nor plants (Rehwinkel et al., 2005). In vertebrates and some insects, the family contains three paralogues, named trinucleotide repeat-containing 6 (TNRC6) proteins: TNRC6A (GW182), TNRC6B and TNRC6C. In *D. melanogaster* there is only one-member GW182, and in *C. elegans* there are two highly different members, AIN-1 and AIN-2 (Behm-Ansmant et al., 2006; Eulalio et al., 2009) (Figure 16). The proteins are made of two domains: an amino-terminal AGO-binding domain (ABD) and a carboxy-terminal silencing domain (SD) (Huntzinger and Izaurralde,

2011; Fabian and Sonenberg, 2012). These functional domains are mostly unstructured and contain multiple tryptophan (W)-containing motifs, often flanked by glycine residues (GW, WG or GWG repeats) after which the family got the name (Huntzinger and Izaurralde, 2011; Fabian and Sonenberg, 2012).

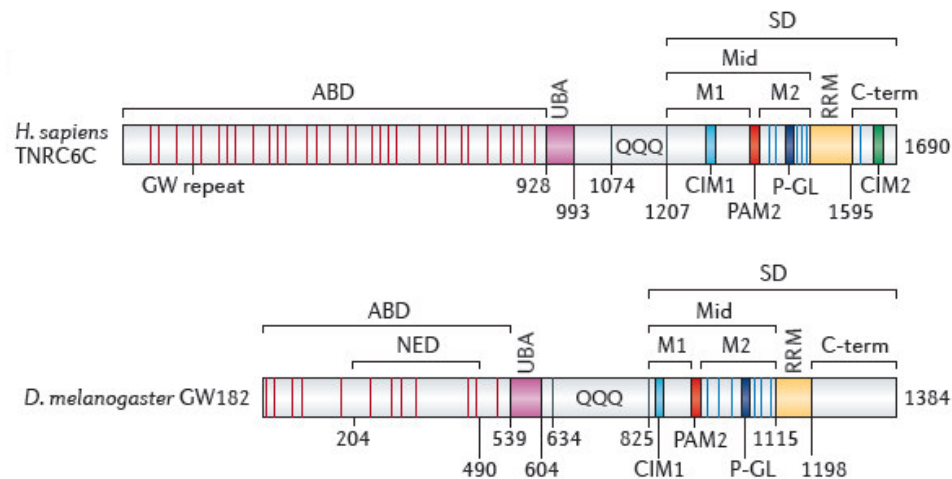


Figure 16 Domain structure of GW182. Amino terminal domain (ABD) and carboxy-terminal silencing domain (SD) with GW repeats in *H. sapiens* and *D. melanogaster* (Figure derived from Jonas and Izaurralde, Nat Rev Genet, 2015).

miRNAs bind predominantly within 3' UTR of mRNA targets with partially complementary base pairing and induce silencing by translation repression and/or by mRNA decay (Huntzinger and Izaurralde, 2011; Fabian and Sonenberg, 2012). The mechanism, timing and the relative contribution of translation repression and decay to overall silencing mediated by miRNAs are still topics of numerous debates.

Genome-wide measurements of the effects on protein and mRNA levels, combined with ribosome profiling experiments, have demonstrated that translational repression comes first, at early time points, but results are weak (6-26%). mRNA deadenylation or full degradation is having a dominant impact (66-90%) of the miRNA-mediated silencing observed in endogenous targets in cultured mammalian cells (Selbach et al., 2008; Baek et al., 2008; Guo et al., 2010). Lot of research should be still done to shed light on miRNA-mediated silencing, but the existing data collected so far can be reconciled by a linear model. The linear model proposes that silencing begins with repression of translation, potentially triggered (or enhanced) by deadenylation, followed by general mRNA decay to consolidate the more transient translational repression (Djuranovic et al., 2011; Meijer et al., 2013). However, the magnitude of both translational repression and mRNA decay induced by miRNA binding varies significantly between miRNA targets, depending on characteristics and features of the 5'UTR, CDS, and 3'UTR (Cottrell et al., 2017).

Many studies are done to explain the mechanism how miRISC complex induces repression of translation and decay of mRNA target. Upon binding of miRNA, AGO protein recruits GW182, and now once that miRISC is assembled it induces translation repression. The most recent model how miRISC is causing the translation repression is that miRISC is interfering with translation initiation step. It inhibits assembling or activity of components of eIF4F initiation complex by targeting eIF4E (Mathonnet et al., 2007; Zdanowicz et al., 2009), eIF4G (Ryu et al., 2013) or eIF4A initiation factor (Meijer et al., 2013; Fukaya et al., 2014; Fukao et al., 2014) (Figure 17).

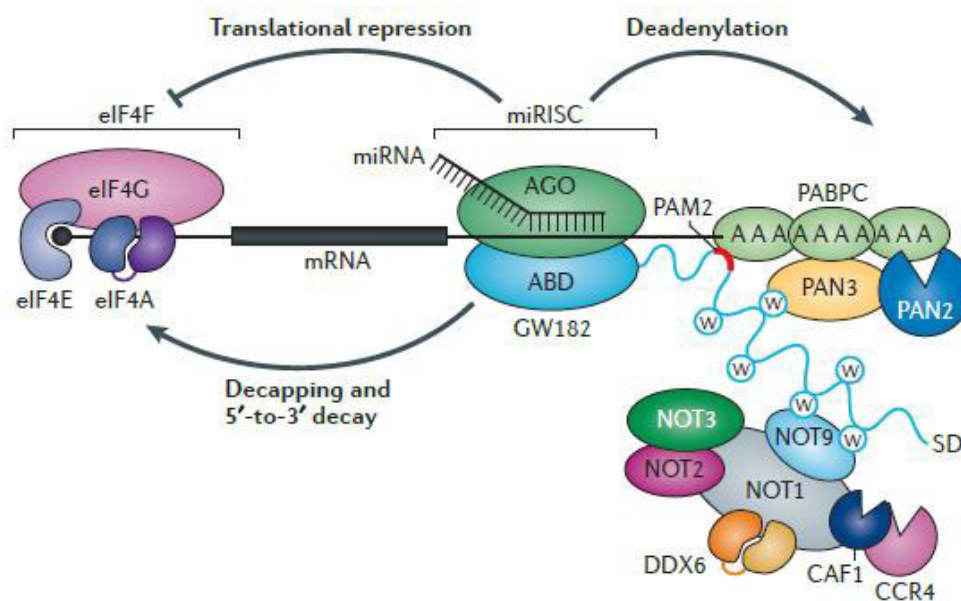


Figure 17 Model of miRNA-mediated gene silencing in animals. Upon binding of miRNA to a partially complementary sequence within 3' UTR of mRNA target, together with AGO and GW182, it makes miRISC complex. It can further induce repression of translation initiation by interacting with initiation factors and/or can cause decay by recruiting general mRNA decay machinery, first deadenylase complex and then decapping complex leading to 5'-to-3' degradation pathway (Figure derived from Jonas and Izaurralde, Nat Rev Genet, 2015).

Experiments in human and *D. melanogaster* cell-free extract showed that miRNAs induce the release of RNA helicases, either of eIF4A1 or eIF4A2 so that secondary structure within mRNA 5' UTR region is unable to unwind. Therefore, 43S pre-initiation complex is not allowed to scan the 5' UTR towards the start codon (Jackson et al., 2010). In another study with *D. melanogaster* cell-free extract when GW182 was tethered to reporter mRNAs the eIF4A was replaced, as well as cap-binding protein eIF4E (Fukaya et al., 2014). However, it is not always the case that eIF4A is forced to leave. In one study was showed that miRISC even specifically recruits eIF4A2 via NOT1 but then the NOT1-eIF4A2 interaction made a roadblock for the 43S pre-initiation complex to scan 5' UTR towards the start codon and thereby the translation initiation was repressed (Meijer et al., 2013). From these studies could be concluded that

miRNAs can make silent only mRNAs that are translated in a cap-dependent manner and need eIF4A for scanning of 5' UTR. Consistent with this conclusion was the observation that mRNAs with unstructured 5' UTR, which do not require eIF4A activity for translation initiation, were immune to miRNA-mediated repression (Meijer et al., 2013). Also, several studies have shown that miRNAs fail to repress translation initiated by IRESs which bypass scanning. It was noticed in hepatitis C virus, cricket paralysis virus and the *D. melanogaster reaper gene* (Ricci et al., 2013; Pillai et al., 2005; Meijer et al., 2013; Fukaya et al., 2014; Fukao et al., 2014). However, miRNAs do repress translation of mRNAs driven by IRESs that require eIF4A activity, like those in encephalomyocarditis virus (EMCV) (Meijer et al., 2013; Fukao et al., 2014) and poliovirus (Ricci et al., 2013). But since there are studies which are still controversial, it must be further investigated to set up the final model how miRNAs mediate translation repression.

Much more is known about mechanisms how miRNAs induce mRNA decay. GW182 works as a flexible scaffold to make a bridge between AGO and the general mRNA decay machinery (Figure 17). *Drosophila* GW182, like its mammalian orthologs, interacts with the cytoplasmic poly(A) binding protein, PABPC (Fabian et al., 2009; Zekri et al., 2009; Huntzinger et al., 2010; Jinek et al., 2010). Two models are explaining how this interaction can induce decay and therefore silencing. The first one is suggesting that PABP serves as a platform to increase affinity and specificity of miRISC to bind polyadenylated mRNA which accelerates its degradation. This model is supported by the fact that AGO proteins bind more efficiently polyadenylated mRNAs in vitro (Moretti et al., 2012). Also, polyadenylated mRNAs are more efficiently repressed in human and *D. melanogaster* cells (Zekri et al., 2013; Walters et al., 2010; Beilharz et al., 2009). The second model suggests that binding of GW182 to PABP interferes with a regular role of PABP. Since PABP stimulates the translation by providing circularization, thereby binding of GW182 to PABP directly leads to repression of translation (Fabian et al., 2009; Zekri et al., 2009; Huntzinger et al., 2010; Beilharz et al., 2009). However, there are controversial observations showing that silencing is happening even without the contribution of PABP. For example, PABP depletion does not prevent silencing in *D. melanogaster* cell-free extracts or zebrafish embryos (Fukaya and Tomari, 2011; Mishima et al., 2012). And even more convincing is that miRNA can silence reporters lacking poly(A) tail and PABP in human and *D. melanogaster* cells (Fukaya and Tomari, 2011; Beilharz et al., 2009; Mishima et al., 2012). It suggests that PABP may enhance decay and silencing but is not the prerequisites for miRISC activity, at least in some cellular contexts.

GW182 tryptophan-containing motifs regions also bind and recruit PAN3 subunit of PAN2-PAN3 deadenylase complex and/or the NOT1 and NOT9 subunits of CCR4–NOT deadenylase complex which may trigger the deadenylation. The role of the GW182 proteins in recruiting the deadenylase complexes is conserved in *Drosophila*, *C. elegans*, and human (Chekulaeva et al., 2011; Fabian et al.,

2012). Upon recruitment of CCR4-NOT complex, NOT1 subunit recruits DDX6 to miRNA target (Chen et al., 2014; Mathys et al., 2014; Jonas and Izaurralde, 2013). DDX6 has a dual role (in more details in chapter 4.2.2.2.2); as a translation repressor, DDX6 interacts with the eIF4E transporter (eIF4E-T). eIF4E-T binds the eIF4E and causes disruption of the eIF4E-eIF4G which consequently inhibits translation initiation (Kamenska et al., 2013). As a decapping activator, DDX6 by interacting with EDC3 can recruit the whole decapping machinery and thus induce decapping which further enhances silencing of miRNA target (Tritschler et al., 2009). Thus, upon miRNA, the target is led from translation repression through deadenylation and decapping to the complete 5'-to-3' degradation.

Though lot is known about miRNAs, still the further studies are needed to be done since most of the studies about silencing are done mostly with reporters genes and not so many with the endogenous genes. This would elucidate the exact mechanism, timing and the contribution of translation repression and mRNA decay to the silencing induced by miRNA. But the apparent fact is that miRNA affects both translation and decay suggesting the firm link between these two processes.

4.2.2.2 Regulation of decapping: the link between translation and mRNA degradation

As we could see in previous chapter PABP, poly(A) tail, deadenylation, and their regulators have an important role in the regulation of mRNA turnover by affecting both mRNA translation and mRNA decay, showing the link between translation and degradation. The same is for the 5' cap, eIF4E, decapping and their regulators. They are another vital factors which are regulating mRNA turnover through competition between translation and decapping machinery. Numerous regulators are involved in regulation of decapping. Most of these regulators can have a dual role affecting both translation and decapping process at the same time indicating the intimate link between mRNA translation and mRNA decay. This link can be on the level of translation initiation, elongation or termination. In following chapters, we will see few examples of regulators which are at the same time inhibitors of translation (initiation, elongation or termination) and activators of decapping and therefore a link between translation and degradation.

4.2.2.2.1 Dual regulators: the inhibitors of translation that are the stimulators of decapping

In many cases when translation initiation was inhibited or defective then the decapping rate was increased (Schwartz and Parker, 1999; Teixeira et al., 2005). For instance, yeast strains that are defective for translation initiation, after knocking out any of translation initiation factors members of the cap-binding eIF4F complex (eIF4E, eIF4G or eIF4A), are showing an increase of both deadenylation and decapping rates. It suggests that decay may be controlled primarily by the translational status of mRNA (Schwartz and Parker, 1999). Conversely, mutations or conditions which stimulate translation

by stabilizing mRNAs within the polysome pool are leading to a decrease in mRNA decapping (Sheth and Parker, 2003; Teixeira et al., 2005).

eIF4E has a dual role as a regulator for both translation initiation and decapping. As a part of eIF4F, eIF4E primarily stimulates translation. However, eIF4E is at the same time an inhibitor for decapping due to its ability to bind the 5' cap structure which physically prevents binding of decapping machinery and reduces the rate of decapping in vitro (Schwartz and Parker, 2000; Ramirez et al., 2002).

Besides having a role in stimulation of the translation, PABP was also shown to inhibit decapping in yeast (Coller et al., 1998; Muhlrads et al., 1994; Caponigro and Parker, 1995). Diminished decapping might be due to increased occupancy of the cap by eIF4E. PABP and eIF4E both bind eIF4G at nearby sites (Tarun and Sachs, 1996; Tarun et al., 1997) so that PABP increases the affinity of eIF4E for the cap (Wei et al., 1998) and in that way PABP inhibits decapping indirectly.

The control of decapping by initiation factors is explained by few hypotheses. One hypothesis is that the rate of decapping is controlled by a steric competition for the cap structure among the translation initiation machinery and the decapping machinery. It was additionally confirmed when a temperature-sensitive allele of eIF4E suppressed the decapping defect of a *DCP1-1* mutant, which argues that removal of eIF4E from the cap is required prior the decapping (Schwartz and Parker, 2000).

However, several observations suggest that the interrelationship between translation initiation and decapping may be more complex than steric competition for the cap and that also changes during the initiation of translation in steps downstream of the cap may affect decapping rate and mRNA stability. For example, mutations in the eIF3 translation initiation complex, which acts in the translation initiation process but downstream of the cytoplasmic cap-binding complex, also promote faster mRNA degradation (Kapp and Lorsch, 2004). Another example is inhibition of translational initiation by inserting the stable secondary structure in 5' UTR of the mRNA-like stem-loop structure so that scanning ribosome is inhibited, and it was also leading to faster rates of mRNA decapping (Hatfield et al., 1996; Coller and Parker, 2005). In addition, when chimeric mRNA was made by changing 5' UTR of *PGK1* mRNA, which has a short half live and low translation efficiency, with 5' UTR of *MFA2* mRNA, which is known to have a long half-life and generous translation, the translation and stability of chimeric mRNA was increased (LaGrandeur and Parker, 1999). And finally creating a poor start AUG context also was leading to decreased translation efficiency but faster rates of mRNA decapping. And finally, when the nucleotides around the stop codon were changed in *PGK1* mRNA it showed less ability in translation but at the same time decay were increased (LaGrandeur and Parker, 1999).

Thus, the link between translation initiation and decapping is prominent but it seems to be more complex than just steric competition for the cap since the events during translation initiation that are downstream of the cap can also affect the decapping and stability of mRNA.

Interestingly, not only steps during the initiation of translation can influence the decapping of mRNAs. Namely, inhibition of translation elongation significantly decreases the rate of decapping (Coller and Parker, 2004). We have already seen that in the case of NGD when the ribosome is stalled during elongation, decay is promoted (chapter 4.1.2.2.3). Recently, genome-wide RNA decay analysis revealed that stable mRNAs are enriched in optimal codon for translation, whereas unstable mRNAs contain predominately non-optimal codons. Substitution of optimal codons with synonymous, non-optimal codons results in dramatic mRNA destabilization, whereas the converse change significantly increases stability. It seems that codon optimality impacts ribosome translocation, connecting the processes of translation elongation and decay through codon optimality. Thanks to this mechanism, it is possible to finely tune levels of mRNAs and, ultimately, proteins (Presnyak et al., 2015).

The observations above have shown that there is not only direct and physical competition between translation and decapping factors. Might be that inhibiting or just slowing down translation after initiation steps give time to multiple mRNP rearrangement events to happen. It involves the disassociation of the translation initiation factors from the mRNA and recruitment of the decapping activators onto the mRNA to provide the decapping machinery to gain access to the cap (Schwartz and Parker, 1999; 2000; Tharun and Parker, 2001).

4.2.2.2.2 Dual regulators: The decapping activators that are translation inhibitors

The next example suggesting intertwining of mRNA translation and mRNA decay is a phenomenon that some mRNA decapping activators are translation inhibitors at the same time.

Decapping activators Dhh1/Rck - a DEAD-box helicase, Pat1, Edc1-4, Scd6/Rap55, and the Lsm1-7 complex appear to play one of the most important roles at the intersection of translation initiation, deadenylation, translational inhibition and mRNA decapping. A particular role has Dhh1, Pat1, and Scd6 which are promoting decapping by inhibiting translation initiation (reviewed in Weston and Sommerville, 2006; Marnef and Standart, 2010). Though decapping activators they are also shown to have a role as translation inhibitors (Coller and Parker, 2005; Nissan et al., 2010) and will be described in more details in next two chapters.

4.2.2.2.1 Pat1p

The involvement of Pat1p protein (Pat1p in *S. cerevisiae*, HPat in *D. melanogaster* and PatL1 in humans) in many post-transcriptional steps can be seen in Figure 18.

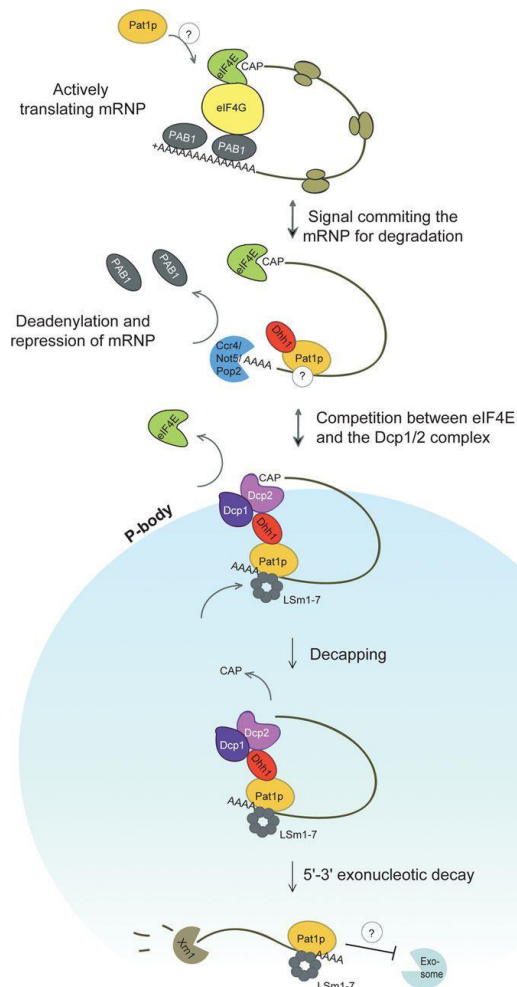


Figure 18 Pat1p protein in translation initiation, translation repression, and mRNA decay.

Pat1p seems to have, still unknown, role in the initiation of translation by interacting with initiation factors. Upon signal for degradation, Pat1p is involved in translation repression together with Dhh1. After deadenylation Pat1p interacts with LSm1-7 complex and connects deadenylation and decapping by recruiting decapping machinery and later Xrn1 5'-3' exoribonuclease, directing mRNA degradation from 5'-to-3' direction and preventing degradation by exosome in 3'-to-5' direction. (Figure derived from Marnef and Standart, Biochem Soc Trans, 2010).

Pat1p is thought to associate with actively translating mRNP and even perhaps to act at the translation initiation steps since it interacts with the initiation factors eIF4E, eIF4G, and PABP, in an RNA-dependent manner (Tharun and Parker, 2001). Moreover, a fraction of Pat1p was found to be associated with polysomes (Bonnerot et al., 2000) with the 40-48S ribosomal subunit (Wyers et al., 2000), confirming its role in the translation initiation. Once mRNA gets the signal for degradation, Pat1p represses the mRNA translation. Pat1p together with Dhh1p function as a general translational repressor since the translation is no longer repressed in the *pat1Δ/dhh1pΔ* mutant upon glucose deprivation or amino-acid starvation in yeast (Coller and Parker, 2005). Conversely, over-expression of Pat1p or Dhh1p, but not the other decapping activators as DCP1p or Edc3p or enzyme Dcp2p, dramatically reduces polysomes. It also causes relocation of the *PGK1*, *RPL41a*, *CYH2*, *COX17*, and

TIF51aCYH2 mRNAs from heavy polysomes to the lighter mRNP region of the density gradient, reduces the rate of translation as indicated by a decrease in [^{35}S] methionine incorporation, and finally inhibits cell growth (Coller and Parker, 2005). Pat1p or Dhh1p functions independently of the cap structure and can also repress translation from a broad spectrum of IRES elements (Coller and Parker, 2005). Investigating the mechanism how Pat1 suppresses the initiation of translation it appears to stop translation before the arrangement of a 48S preinitiation complex (Coller and Parker, 2005; Nissan et al., 2010) either directly or via the other protein partners which later triggers the deadenylation of the mRNA. Pat1p can also interact with CCR4–NOT complex providing recruitment of Pat1p to mRNAs undergoing deadenylation (Haas et al., 2010; Ozgur et al., 2010). Afterward, Pat1 associates with LSm1–7 complex which binds preferentially at the 3′ end of oligoadenylate mRNAs rather than polyadenylated mRNAs (Tharun and Parker, 2001; Chowdhury et al., 2007; 2008; Tharun et al., 2009). In this way the LSm1–7–Pat1 complex is preventing 3′ end trimming of oligoadenylate mRNAs by exosome thus directing it to the 5′-to-3′ decay pathway (Chowdhury et al., 2007; He and Parker, 2001). The association between Pat and decapping factors promotes the formation of decapping complexes in *cis*, handing over deadenylated mRNAs to decapping and 5′-to-3′ mRNA degradation. Once the cap is cleaved, transcripts are digested by Xrn1. The LSm1–7–Pat1 complex seems to remain bound on the mRNA while the 5′-to-3′ exonucleolytic digestion occurs (Tharun and Parker, 2001), through LSm1–7 complex-Xrn1 interaction (Bouveret et al., 2000; Tharun et al., 2000; Fromont-Racine et al., 2000). Thus, it seems Pat1 proteins act as a scaffold or remodeling protein involved in the shifting from actively translating mRNPs to silent mRNPs. It also links deadenylation and decapping by sequentially binding different factors involved in translational repression, deadenylation and decay (Figure 19).

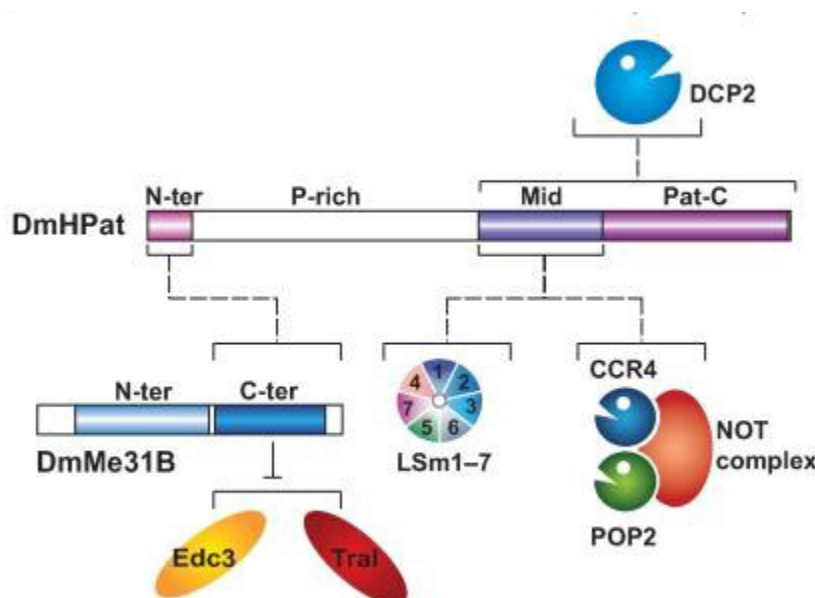


Figure 19 HPat as a scaffold protein.

HPat provides a connection between deadenylation and decapping in metazoan, interacting in a coordinated fashion with the CCR4–NOT complex, the LSm1–7 ring, and decapping factors and thereby allowing movement between following steps along the mRNA decay pathway. (Figure derived from Haas et al., J Cell Biol, 2010).

Moreover, HPat was shown to interact with GW182, which is one of a miRNP component, (Jäger and Dorner, 2010). Thus, HPat seems to have an additional role in miRNA function, and indeed, it has been evaluated by microarray analysis that it regulates 15% of AGO1mRNA targets (Eulalio et al., 2007).

4.2.2.2.2 Dhh1p

Dhh1p (Dhh1p in *S. cerevisiae*, Me31b in *D. melanogaster* and DDX6 in humans) together with Pat1p functions both as a general translational repressor (4.2.2.3.1) and as a decapping activator (Coller and Parker, 2005). As a translation inhibitor, Dhh1p reduces the level of translation directly, even in the absence of an active decapping pathway (Carroll et al., 2011; Sweet et al., 2012). It has been proposed that Dhh1p helicase represses translation by acting on translational initiation step (Coller and Parker, 2005). However, more recently it was observed that tethered Dhh1p could slow down translational elongation/termination in yeast (Sweet et al., 2012). One theory is that Dhh1 directly impacts the function of the 40S ribosomal subunit itself when it is free or involved in an actual translation with two different consequences (Figure 20). The interaction between Dhh1 and free 40S subunits could influence translation at early steps and manifests as an initiation block. It might be happening both in vitro and during Dhh1 over-expression in cells (Coller and Parker, 2005). In another case when Dhh1p interacts with 40S subunits in the context of an actively translating mRNA, Dhh1 interaction might affect ribosome movement on mRNA which implies a role for Dhh1 in inhibiting translation either during elongation, termination, or ribosome recycling. Repressed polyribosomal mRNA can then either be decapped or stored depending on the biological conditions and needs (Presnyak and Coller, 2013).

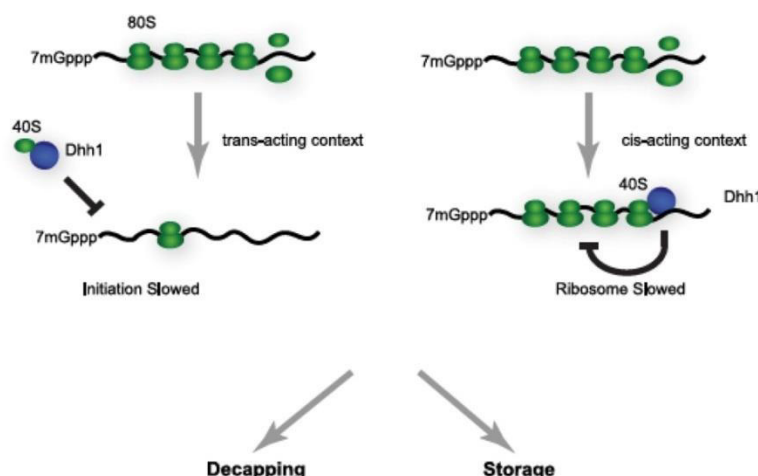


Figure 20 A hypothesized mechanism of Dhh1 as a translation repressor. It is hypothesized that Dhh1 helicase may function directly on 40S ribosomal subunits. If Dhh1 binds free 40S subunits, the consequence would be repression of translation at initiation (depicted on the left side of the figure). If Dhh1 helicase acts on assembled polyribosomes *in vivo*, this will lead to repression of translation at a late, post-initiation step (depicted on the right side of the figure). Both scenarios would result in a storage and/or decapping (Figure derived from Presnyak and Coller, Biochim

Biophys Acta - Gene Regul Mech, 2013).

The role of Dhh1p as an activator of decapping has been proposed to be an indirect consequence of the inhibition of translation (Presnyak and Collier, 2013). However, DDX6 interacts directly with the decapping factors EDC3 and Pat and indirectly with the catalytic core of the decapping complex formed by the decapping enzyme DCP2 and its coactivator DCP1 (Jonas and Izaurralde, 2013). Thus, DDX6 could play a direct role in decapping by promoting recruitment of decapping complexes to the mRNA target. Also, it was shown that CNOT1 MIF4G domain interacts preferentially with DDX6. This indicates that the CNOT1-DDX6 association could provide a direct physical link between deadenylation and decapping, even in particular cases when CCR4-NOT complex is recruited via PUF regulatory proteins (Goldstrohm et al., 2008) or via miRNA complex (Chen et al., 2014).

4.3 Hypothesis of co-translational mRNA degradation

As a summary of all examples of interconnections between translation and degradation, first, we saw how surveillance pathways had been connected to translation (Chapter 4.2.1). All three types of surveillance pathways (NMD, NSD, and NGD) specialized for degradation of aberrant mRNAs are translationally-dependent, thus in close relation with translation (Chapters 4.1.2.2.1, 4.1.2.2.2 and 4.1.2.2.3).

Then we also saw that general mRNA decay is also linked to translation (Chapter 4.2.2). Many regulators of deadenylation (Chapter 4.2.2.1) or decapping (Chapter 4.2.2.2) are having dual roles in both the translation and the decay. Factors which are regulating specific mRNAs: PUF proteins (Chapter 4.2.2.1.1), AREs (Chapter 4.2.2.1.2), Smaug, nanos, CUP (Chapter 4.2.2.1.3), TOB (Chapter 4.2.2.1.4), Hsp70 elements (Chapter 4.2.2.1.5), miRNAs (Chapter 4.2.2.1.6) are all both translation repressors and accelerators of decay. Later we saw how inhibitors of translation are the decapping activators (Chapter 4.2.2.2.1) and another way around (Chapter 4.2.2.2.2) explaining in more detail Pat1p (Chapter 4.2.2.2.2.1) and Dhh1p (Chapter 4.2.2.2.2.2) which are known as general repressors but also decapping activators.

Also, it seems that P-bodies are not the only place for mRNA silencing and decay (Chapter 4.1.3).

Finally, Hu et al. (Hu et al., 2009; 2010) have shown decapped degradation intermediates of several mRNAs, including *CYH2*, *ADH1*, and *RPL41a* mRNAs, and the mRNA reporter *PGK1* on active ribosomes. Also, in yeast XRN1 and mRNA degradation intermediates can be detected in polysome fractions (Mangus and Jacobson, 1999; Hsu and Stevens, 1993).

All these findings taken together could indicate that degradation at least in yeast and for some mRNAs can occur while they are still associated with ribosomes, supporting the hypothesis of co-translational mRNA degradation.

In this study, we investigated the possibility of mRNA degradation on the ribosome in the higher eukaryote *Drosophila*. Using ribosome affinity purification, we could demonstrate the co-purification of mRNA degradation factors and factors of the miRNA pathway with ribosome complexes. Furthermore, we analyzed decapped intermediates of mRNA degradation from ribosome complexes by high-throughput sequencing. Interestingly, we detected a significant fraction of the decapped transcriptome on the ribosome. Together these observations strongly argue for the ribosomes as a site of mRNA degradation in eukaryotic cells.

5 Materials

5.1 Chemicals

100 x Penicillin-Streptomycin Glutamine	GIBCO
200 mM L-Glutamine	GIBCO
2-Aminomethanol (Ethanolamine)	Sigma-Aldrich
2-Propanol	VWR
4-Iodophenylboronic acid	Sigma-Aldrich
Express Five SFM Medium	GIBCO
Acetic Acid 100%	VWR
Acrylamide: Methylene-Bisacrylamide 40% 37.5/1	Gerbu
Agar Bacteriology grade	Applichem
Agarose	Sigma
Ammonium Chloride	Applichem
Ammonium persulphate	Sigma-Aldrich
Ampicillin sodium salt	Applichem
Avidin	IBA(BioTAGnology)
Brilliant Blue R-250	Sigma-Aldrich
Bromophenol Blue	Sigma-Aldrich
BSA	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Canamycin	Sigma-Aldrich
Chloramphenicol	Sigma-Aldrich

Copper sulfate (CuSO ₄)	Merck
Cycloheximide	Sigma-Aldrich
D-Biotin	IBA (BioTAGnology)
Diethylpyrocarbonate (DEPC)	Roth
di-Sodium Hydrogen Phosphate	Merck
Dithiothreitol (DTT)	Gerbu
DMSO (Dimethylsulfoxide)	Fluka
dNTP bundle (4 x 100 mM dATP, dCTP, dGTP, dTTP)	Jena Bioscience
EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'tetraacetic acid	Applichem
Ethanol	Merck
Ethidium bromide (1% solution in water)	Merck
Ethylenediaminetetraacetic acid (EDTA)	Applichem
Fetal Bovine Serum	Sigma-Aldrich
Formamide	Merck
Formaldehyde solution 37 % in H ₂ O, 10-15 % Methanol	Sigma-Aldrich
Fluorinert	Isco
Glycerol	Sigma
Glycine	Gerbu
Glycogen	PEQLAB
Heparin sodium salt from porcine intestinal mucosa	Sigma Aldrich
HEPES-KOH pH 8.0	AppliChem
Hydrochloric acid fuming 37 %	Merck

Hydrogen peroxide 30 %	Sigma-Aldrich
Hygromycin B	PAA
Igepal CA-630	Sigma-Aldrich
Imidazole	Sigma-Aldrich
IPTG (Isopropyl- β -D-thiogalactopyranoside)	Applichem
Low melting agarose	Sigma
Luminol	Fluka
Magnesium Chloride	Applichem
Manganese Chloride	Merck
Methanol	Applichem
MOPS 3-(N-Morpholinopropanesulfonic acid)	Gerbu
NTP bundle (4 x 100 mM ATP, CTP, GTP, UTP)	Jena Bioscience
Phenol water saturated	Applichem
Phenol water saturated: Chloroform: Isoamylalcohol	Applichem
Potassium acetate	Gerbu
Potassium Chloride	Fluka
Potassium Dihydrogenphosphate-anhydrous	Gerbu
Potassium hydroxide pellets	Roth
Rubidiumchloride	Applichem
Schneiders <i>Drosophila</i> Medium Modified	Lonza
Skim Milk Powder	Gerbu
Sodium acetate	Merck
Sodium azide	Merck

Sodium Chloride	Gerbu
Sodium hydroxide pellets	Applichem
Sodium Tetraborate decahydrate	Fluka
Sodium deoxycholate (DOC)	Sigma-Aldrich
Sodiumdodecylsulfate (SDS)	Applichem
Spermidine	Sigma-Aldrich
Sucrose	Gerbu
Tetramethylethylenediamine (TEMED)	Fluka
TRI Reagent RNA Isolation Reagent	Sigma-Aldrich
Tris base	Sigma-Aldrich
Trichloroacetic acid (TCA)	Merck
Tris HCl	Gerbu
Triton X-100	Sigma-Aldrich
Trypton	Fluka
Tween 20	Merck
Ultra Pure Agarose	Invitrogen
Urea	Applichem
Ribonucleoside Vanadyl Complex (S1402S)	NEB
Xylene Cyanol FF	Sigma-Aldrich
Yeast Extract	Formedium

5.2 Buffers and solutions

10 x Leampli (GTS)

1.92 M Glycine

250 mM Tris base

1% SDS

10 x Transfer buffer

250 mM Tris base

1.92 M Glycine

10 x PBS

1.37 M NaCl

27 mM KCl

81 mM Na₂HPO₄

14.7 mM KH₂PO₄

50 x TAE buffer

2M Tris base

0.05 M EDTA

1 M Acetic acid

Tris buffers

The corresponding amount of Tris base was dissolved in 500 ml ddH₂O, and the pH was adjusted to a desired value by adding concentrated HCl.

1.5 M Tris/HCl pH 8.7

1 M Tris/HCl pH 6.8

TE buffer

10 mM Tris/HCl (pH 8.0)

1mM EDTA

0.5 M EDTA (0.5L)

186 g EDTA (Na-salt) (FW 372.24) was dissolved in 450 ml ddH₂O or mQH₂O (RNase free DEPC treated), and the pH was adjusted to pH 8.0 with NaOH.

10 M NaOH and 10 M KOH

NaOH and KOH pellets were added progressively to mQH₂O under the hood.

Improved ECL working solution

100 mM Tris/HCl pH 8.7

2 mM 4-IPBA

1.25 mM Luminol

Detection solution

6 µl H₂O₂ (3%) was added to 1 ml working solution just before use.

Coomassie Staining Solution

45% MeOH

10% AcOH

5 g Brilliant Blue R-250

Protein Gel Destaining Solution

25 % Isopropanol

10 % AcOH

5 x DNA Agarose Dye

20 % Ficoll 400

1 mM EDTA

0.1 % SDS

0.05 % Bromophenol Blue

RNA Loading (Formamide) Dye

95 % formamide

18 mM EDTA

0.025 % SDS

0.025 % bromophenol blue

0.025 % xylene cyanol FF

0.1 % ethidium bromide

2 x Protein Sample buffer

100 mM Tris-HCl pH 6.8

4 % w/v SDS

20 % Glycerol

0.2 M DTT

0.05 % bromophenol blue

6 x Protein Sample buffer

1.2 g SDS

2.1 ml ddH₂O

4.7 ml 100 % Glycerol

1.2 ml 0.5 M Tris-HCl pH 6.8

Warm it and stir with a magnet. When it is dissolved add 0.93 g DTT. After dissolving make a one ml aliquots and store at -20°C.

10x MOPS running buffer (pH 7.0)

200 mM MOPS

80 mM Sodium acetate

10 mM EDTA

Protease Inhibitor (Roche Ultra complete cocktail tablets EDTA free)

1 tablet was dissolved in 2 ml mQH₂O DEPC treated. Aliquots were stored at -20°C. This was a 25 x stock.

DEPC treated water

1 ml DEPC was added to 1L of mQH₂O to be treated and shaken vigorously to bring the DEPC into solution. The solution was incubated for 12 hours at 37°C and autoclaved twice to remove any traces of DEPC.

5.3 Molecular weight size markers**DNA size marker**

100bp	NEB
-------	-----

1kb	NEB
-----	-----

5 µl per lane (0.5µg) were loaded onto the gel as a size standard.

RNA size marker

100 bp	Thermo Fisher Scientific
--------	--------------------------

4 µl per lane were loaded onto the gel as a size standard.

Protein size marker

Prestained Protein MarkerP7708	NEB
Protein MarkerP7702	NEB

5 µl of P7708 were loaded per lane for western blot analysis and 5 µl of P7702 were used for gels stained with Coomassie.

5.4 Resins

MyOne Streptavidin T1/C1 Dynabeads	Life Technologies
Ni-NTA Agarose	Qiagen
Agencourt RNAClean XP system	Beckman Coulter
Agencourt AMPure XP system	Beckman Coulter
MicroSpin S-300 HR column	GE Healthcare

5.5 Membranes

Hybond-P PVDF Membrane	GE healthcare
Hybond- LFP Membrane (Low-fluorescent PVDF)	GE healthcare
PVDF FL Membrane	Peqlab

5.6 Antibiotics

Ampicillin (100 mg/ml), dissolved in mQH₂O and filtered (0.22 µM)

Carbenicillin (100 mg/ml), dissolved in mQH₂O and filtered (0.22 µM)

Chloramphenicol (34 mg/ml), dissolved in Ethanol abs.

Kanamycin (50 mg/ml), dissolved in mQH₂O and filtered (0.22 µM)

5.7 Enzymes

T7 Polymerase	purified in the lab
GoTaq® DNA Polymerase (5 U/µl)	Promega
Expand High Fidelity Enzyme mix	Roche
M-MLV Reverse Transcriptase (200 U/µl)	Promega
RQ RNase-Free DNase I (1 U/µl)	Promega
Turbo DNase (2 U/µl)	Life Technologies
Micrococcal nuclease (2000 gel U/µl = 200 U/µl)	NEB
GatewayR BP II Clonase Enzyme Mix	Invitrogen
GatewayR LR II Clonase Enzyme Mix	Invitrogen
Proteinase K (2 U/µl)	Invitrogen
RiboLock™ RNase Inhibitor (40 U/µl)	Fermentas
Restrictionenzymes	NEB
Terminator	
5'-Phosphate-Dependent Exonuclease (1 U/µl)	Epicentre
SuperScript III Reverse Transcriptase (200 U/µl)	Invitrogen
T4 RNA ligase 1 (ssRNA ligase) (10 U/µl)	NEB
Antarctic phosphatase (5 U/µl)	NEB
T4 RNA ligase 2, truncated KQ (200 U/µl)	NEB
Tabacco Acid Pyrophosphate (TAP) (10 U/µl)	Epicentre

Second Strand synthesis Enzyme mix	NEBNext Module
End repair enzyme	NEBNext Module

5.8 Kits

Wizard SV Gel and PCR Clean-Up System	Promega
PureYield™ Plasmid Miniprep System	Promega
Plasmid Plus Midi kit	Qiagen
RNeasy® Mini kit	Qiagen
RNA 6000 Pico kit	Agilent Technologies
GoTaq® qPCR Master mix	Promega
LongAmp® Taq 2 x Master Mix	NEB
Effectene Transfection Reagent	Qiagen
Bio-Rad Protein Assay (500-0006)	Biorad
ReliaPrep™ RNA Cell Miniprep system	Promega
Ribo-Zero kit (human/mouse/rat)	Epicentre
MinElute kit	Qiagen
PeqGold total RNA kit	Peqlab

5.9 Media for *Drosophila* S2 cells

S2 complete medium (125 ml)

111.25 ml S2 *Drosophila* Schneider's media (Lonza)

12.5 ml 10% fetal bovine serum (FBS, heat inactivated)

1.25 ml 100 x penicillin/streptomycin/glutamine

Medium is sterile filtered (0.22 µm)

Express Five serum free (SFM) medium for dsRNA treatment (1.09 L)

1 L Five Express Medium (GIBCO)

90 ml Glutamine 200 mM

Express Five medium 10% FBS for induction and dsRNA treatment (125 ml)

111.25 ml 5 Express S2 medium serum free (GIBCO)

12.5 ml 10 % fetal bovine serum (FBS, heat inactivated)

1.25 ml 100 x penicillin/streptomycin/glutamine

Medium is sterile filtered (0.22 µm)

5.10 Media for Bacteria

Luria-Betrani (LB) Media (1 L)

10 g Trypton

5 g Yeast extract

10 g NaCl

5.11 Antibodies

5.11.1 Primary antibodies

Name	Antigen	Company	Produced in	Working dilution in 0.05% T-PBS
Anti-HA 12CA5	Hemagglutinin (YYPVPDYA)	Roche	Mouse hybridoma clone 12CA5	1:3000
Anit-tubulin	Tubulin	Sigma	Mouse hybridoma clone DM1A	1:1000
Anti-AGO1	AGO1	Pineda	rabbit	1:1000
Anti-HPat	HPat	Pineda	rabbit	1:3000
Anti-GW182	GW182	Pineda	rabbit	1:1000
Anti-eGFP	eGFP	Pineda	rabbit	1:10000

Anti-RpL10Ab	RpL10Ab	Pineda	rabbit	1:10000
Anti-RpS3	RpS3	Pineda	rabbit	1:10000
Anti-PABPC	PABPC	Pineda	rabbit	1:1000
Anti-NOT1	Gift from E. Whale (Halle, Germany)		Mouse hybridoma clone	1:1000
Anti-NOT2	Gift from E. Whale (Halle, Germany)		Rabbit	1:1000
Anti-eIF4E	Gift from E. Izaurralde (Tübingen, Germany)		Rabbit	1:1000
Anti-Me31B	Gift from E. Izaurralde (Tübingen, Germany)		Rabbit	1:1000
Anti-EDC4	Gift from E. Izaurralde (Tübingen, Germany)		Rat	1:2000
Anti-XRN1	Gift from S. Newbury (Brighton, UK)		Rabbit	1:2000

5.11.2 Secondary antibodies

Name	Antigen	Conjugated with	Company	Produced in	Working dilution in 0.05 % T-PBS
Anti-rabbit	Rabbit IgG Fc fragment	Peroxidase	Jackson Immuno Research	Goat	1:10000
Anti-mouse	Mouse IgG Fc fragment	Peroxidase	Jackson Immuno Research	Goat	1:10000

Anti-rabbit	Rabbit (H+L)	IgG	Alexa fluor 680	Life Technologies	Goat	1:10000
Anti-rabbit	Rabbit (H+L)	IgG	IRDye800CW	LI-COR	Goat	1:10000

5.12 Plasmids

Plasmids were named according to the initials of the researchers (SD, Silke Dorner, and EJ, Elisabeth Jäger). All cDNAs were verified by sequencing.

DONOR VECTOR	
pDONR221	chapter 5.13 (Invitrogen)

ENTRY VECTOR		
Vector Name	Donor Vector	cDNA (full length)
pSD1	pDONR221	RpL10Ab (CG7283)
pSD2	pDONR221	RpS3 (CG6779)
pSD3	pDONR221	PABPC (CG5119)
pSD4	pDONR221	BirA (<i>E. coli</i>)

DESTINATION VECTOR	
Vector	Vector map
pDEST17	chapter 5.13, (The <i>Drosophila</i> Gateway Vector Collection)
pMK33/pMthY	chapter 5.13, Invitrogen

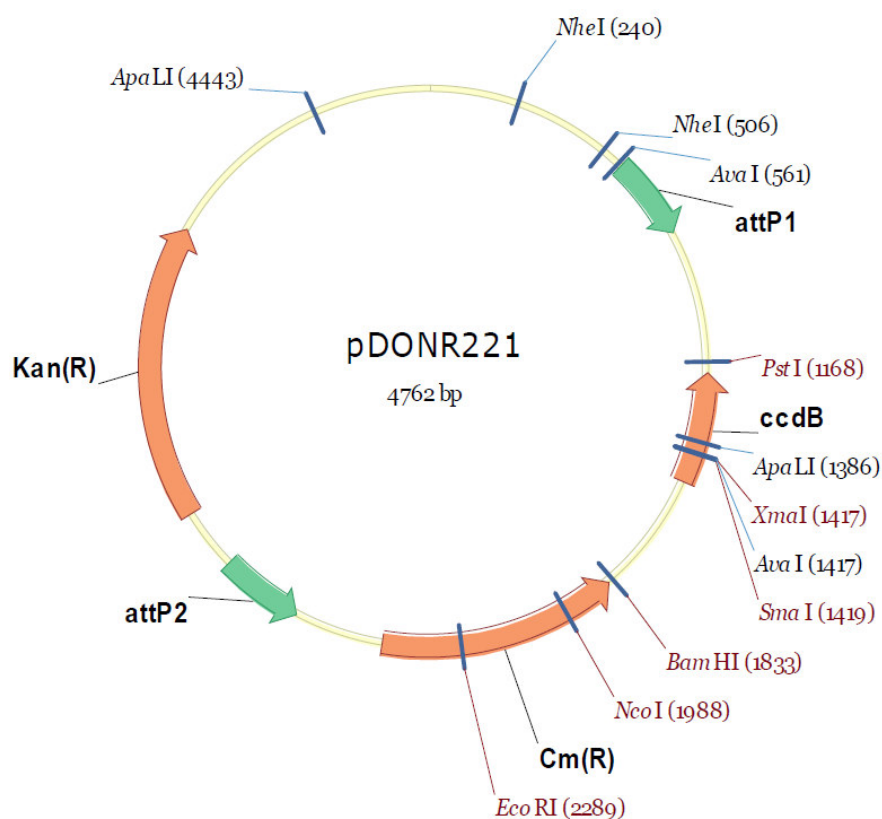
EXPRESSION VECTORS used for recombinant protein expression in <i>E. coli</i> BL21 RIPL cells			
Vector name	Entry vector	Destination Vector	Expressed protein
pSD5	pSD1	pDEST17	His-RpL10Ab (1-539 aa)
pSD6	pSD2	pDEST17	His-RpLS3 (1-489 aa)
pSD7	pSD3	pDEST17	His-PABPC (1-522 aa)

EXPRESSION VECTORS used for stable transfection of <i>Drosophila</i> S2 cells			
Vector name	Entry vector	Destination Vector	Expressed protein
pSD462	pSD1	BLRP-GFP- pMK33/pMTHy*	BLRP-GFP-RpL10Ab
pSD449	pSD4	3 x Flag- pMK33/pMTHy	3 x Flag-BirA

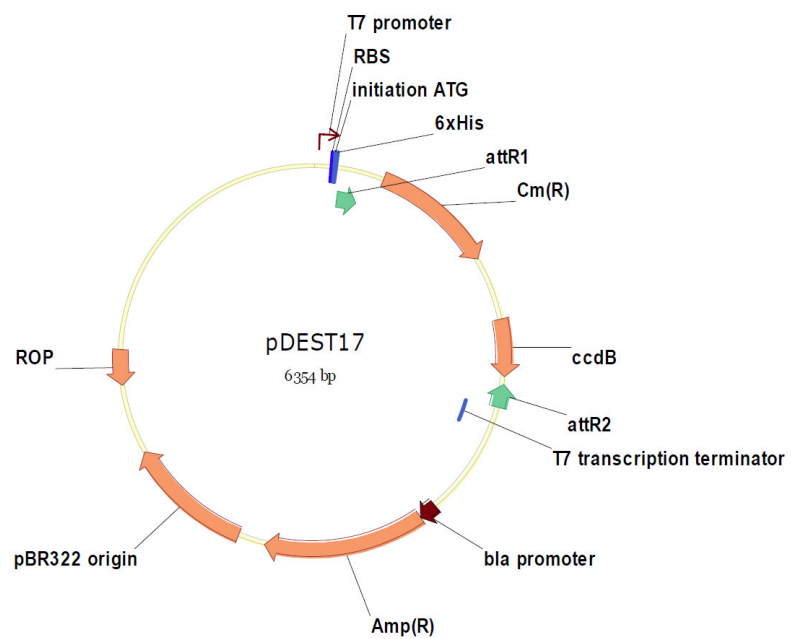
*The 23-amino-acid *E. coli* BirA Biotin Ligase Recognition Peptide (BLRP; MASSLRQILDSQKMEWRSNAGGS (de Boer et al., 2003) was cloned downstream of the metallothionein promoter into the pMK33/pMTHy backbone (Koelle et al., 1991), followed in frame by a GFP-coding sequence and a gateway cassette (Life Technology).

5.13 Plasmid maps

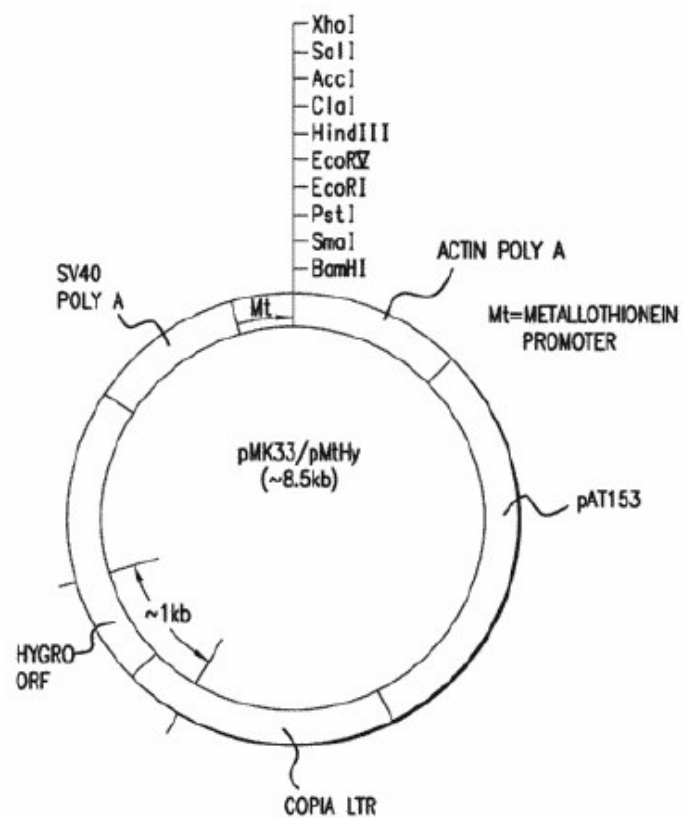
Donor vector: pDONR221 (Invitrogen)



Destination vector: pDEST17 (Invitrogen)



Destination vector: pMK33/pMtHy (Invitrogen)



5.14 Primers

5.14.1 Oligos for T7 transcription of dsRNA

Oligo Name	Template	Primer Sequence 5'-3'
SD299 (forward)	XRN1 cDNA	TAATACGACTCACTATAGGGAGATGAACTGATCGAGGAACTGTGCC
SD307 (reverse)	XRN1 cDNA	TAATACGACTCACTATAGGGAGACCAGCTGGCGCTTGCG
SD310 (forward)	AGO1 pSD469	TAATACGACTCACTATAGGGAGACATTA AAAAGCTGACCGATATGC
SD311 (reverse)	AGO1 pSD469	TAATACGACTCACTATAGGGAGATTGACGTTGATCTTCAGACACAG
SD921 (forward)	MBP pEJ113	TAATACGACTCACTATAGGGAGAAATGAAAATCGAAGAAGGTAAAC
SD922 (reverse)	MBP pEJ113	TAATACGACTCACTATAGGGAGACTAGTCTTTCAGGGCTTC

5.14.2 Oligos for qPCR analysis

Oligo Name	Target transcript	Primer Sequence 5'-3'
SD937 (forward)	RNA Linker	CTCTTTCCTACACGACGCTCTTCC
SD670 (forward)	FLuc CDS	AGAGATACGCCCTGGTTCCT
SD671 (reverse)	FLuc CDS	ATAAATAACGCGCCCAACAC
SD938 (reverse)	FLuc Linker	CTCTCCAGCGGTTCCATCCTCT
SD955	FBgn0039857 linker	GCCCTTTTGGCCGACTTGG

(reverse)		
SD958 (reverse)	FBgn0003279 linker	CCTGGCGTTGCCTAAGCTCAT
SD952 (reverse)	FBgn0000556 linker	GAATCGACGTGTCCGATCACGAC
SD940 (reverse)	FBgn0046696 linker	CACCTTGATACTTTCTATCCTAGGCCC

5.14.3 LNA oligos added to Ribo-Zero kit during rRNA depletion

Oligo Name	Target	Primer Sequence 5'-3'
SD768	5.8S rRNA	GT+TCATGTGT+CCTGCAGTT+CAC
SD769	28S rRNA	GG+CAATGTCCT+TGAATTGG+ATC
SD770	28S rRNA	CC+TTGCCTGTT+ACAAAAGT+CGT

+ in front of the nucleotide stands for LNA nucleotide

5.14.4 Oligos for library preparation

Oligo Name	Primer Sequencing 5'-3'
SD698 Solexa 5' RNA linker	rArCrArCrUrCrUrUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrN
SD697 3' pre-adenylated DNA linker	5rApp/NNNNAGATCGGAAGAGCACACGTCT/3ddC
SD802 Adapter Illumina	5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTC/ideoxyU/ACA CTCTTTCCCTACACGACGCTCTTCCGATC*T -3'
SD759 TruSeq Universal	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
SD760 TruSeq_IDX_2	CAAGCAGAAGACGGCATACGAGAT ACATCG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
SD761 TruSeq_IDX_4	CAAGCAGAAGACGGCATACGAGAT TGGTCA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
SD762 TruSeq_IDX_5	CAAGCAGAAGACGGCATACGAGAT CACTGT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
SD763 TruSeq_IDX_7	CAAGCAGAAGACGGCATACGAGAT GATCTG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
SD764 TruSeq_IDX_16	CAAGCAGAAGACGGCATACGAGAT GGACGG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

SD765 TruSeq_IDX_19	CAAGCAGAAGACGGCATACGAGAT TTTCAC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
SD766 TruSeq_RT	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

5.15 Stable *Drosophila* S2 cell lines

Name	Plasmid	µg DNA transfected
45	pSD462 (BLRP-GFP-RpL10Ab)	200 ng
	pSD543 (FLuc-N-Csy4BoxB)	100 ng
	pSD114 (RL)	200 ng
58	pSD462 (BLRP-GFP-RpL10Ab)	200 ng
	pSD449 (FLAG-BirA)	22 ng
	pSD543 (FLuc-N-Csy4BoxB)	100 ng
	pSD114 (RL)	200 ng
	pMK	200 ng

5.16 Bacteria strains

Bacteria Name	Company	Genotype
DH5α TM -T1R	Invitrogen	F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 tonA (confers resistance to phage T1)
BL21-CodonPlus (DE3)- RIPL strain	Stratagene	E. coli B F- ompT hsdS (rB- mB-) dcm+ Tetr gal λ(DE3) endA Hte [argU proL Camr] [argU ileY leuW Strep/Specr

6 Methods

6.1 DNA Methods

6.1.1 Plasmid generation using the gateway recombination Technology

The plasmids were generated using the Gateway recombination cloning technology. DNA fragments with flanking recombination sites termed attB1 and attB2 were generated by PCR. They were used for further recombination with a donor vector containing attP sites catalyzed by BP Clonase II. It was giving rise to an entry vector with attL sites and a by-product of attR sites. Once the gene cassette was in a gateway entry clone, it was transferred into different destination plasmids containing attR recombination sequences, a specific promoter and an epitope tag of interest. Once the DNA fragment of the donor vector was recombined into the destination vector by the LR Clonase II, the resulting expression vector was ready to be used for protein expression analysis.

6.1.1.1 PCR fragment amplification for BP recombination into pDONR221

Primer design:

The forward and reverse primers were flanked by either attB1 (GGGGACAAGTTTGTACAAAAAAGCAGGCTTC) or attB2 (GGGGACCACTTTGTACAAGAAAGCTGGGTC) recombination sites. The recombination sequences were followed by gene-specific sequences ranging from 18-20nt.

To amplify the DNA fragment of interest with high yield and fidelity, the Expand High Fidelity Plus PCR System (Roche) was used.

PCR reaction (final volume 50 µl):

0.75 µl 100 µM forward primer

0.75 µl 100 µM reverse primer

5 µl 5x Expand HiFiPlus reaction buffer

1 µl dNTPs (10 mM each)

0.5 µl Expand HiFiPlus enzyme (5 U/µl) (Roche)

5-500 ng genomic DNA or 100 pg-10 ng plasmid DNA

The final volume was adjusted with mqH₂O to 50 µl.

Thermocycling conditions:

Step	Time	Temperature	Number of cycles
Initial denaturation	2 min	94°C	1
Denaturation	30 sec	94°C	25
Annealing	30 sec	50°C - 60°C*	
Elongation	30 sec - 5 min**	72°C	
Final elongation	5 min	72°C	1
Cooling	∞	4°C	1

* Optimal annealing temperature was depending on the melting temperature of the primers.

** Elongation time depended on the length of the PCR product to be amplified (1 min per kb).

Upon amplification PCR products were loaded onto a DNA agarose gel and the DNA band of correct size was cut from the gel. DNA purification was performed with the Wizard SV Gel and PCR Clean-Up System (Promega) according to manufacturer's protocol. The purified PCR fragment was then used for recombination into the donor vector pDONR221 (chapter 5.13).

6.1.1.2 Entry vector generation

The BP recombination reaction was performed with an *attB*-flanked PCR product and an *attP*-containing donor vector to generate an entry clone with the *attL*-flanked gene of interest.

BP Recombination Reaction (final volume 5 µl):

75 ng attB-PCR product

75 ng pDONR221 vector

1 µl BP Clonase II enzyme mix (Invitrogen)

The final volume was adjusted with TE buffer, pH 8.0 to 5 µl.

The reaction was incubated for 1 h at RT, and the recombination was inactivated by a proteinase K digestion. 1 µl of proteinase K (2 U/µl) was added to the reaction mix and incubated at 37°C for 10 min. After this, the reaction mix was ready to use for transformation into DH5α™-T1R competent cells. The appropriate antibiotic-resistant clone was grown for small-scale plasmid purification (chapter 6.1.4). All entry vectors were confirmed by sequencing.

6.1.1.3 Expression vector generation

To generate an expression clone, an LR recombination reaction was performed with an *attL*-containing entry clone and an *attR*-containing destination vector (chapter 5.13). In this study, expression vectors were used for protein analysis in *Drosophila* S2 cells or *E. coli* (BL21-CodonPlus (DE3)-RIPL strain).

LR Recombination Reaction (final volume 5 µl):

75 ng Donor Vector

75 ng Destination Vector

1 µl LR Clonase II enzyme (Invitrogen)

The final volume was adjusted with TE buffer, pH 8.0 to 5 µl.

The reaction was incubated for at least 1 h at RT. Afterwards the recombination was inactivated by a proteinase K digest. 1 µl of proteinase K (2 U/µl) was added to the reaction mix and incubated at 37°C for 10 min. The reaction mix was then ready to use for transformation into DH5α™-T1R competent cells. The appropriate antibiotic-resistant clone was then grown, and expression plasmids were further used for protein expression in *Drosophila* S2 cells or *E. coli* (BL21-CodonPlus (DE3)-RIPL strain).

6.1.2 Preparation of RbCl competent cells

A single colony of the selected bacteria strain (BL21-(DE3)-RIPL) was inoculated in 4 ml LB_{CAM34} and grown overnight at 37°C. 1.5 ml of an overnight culture was diluted 1/200 by adding to 300 ml LB_{CAM34} medium. The cells were grown to log phase (O.D.₆₀₀=0.5) and harvested at 5,000 x g for 5 min at 4°C. The cell pellet was resuspended in 100 ml ice-cold RF-1 buffer and kept on ice for 1-2 h. The cells were spun 5 min at 5,000 x g, at 4°C, resuspended in 6 ml ice-cold RF-2 buffer and incubated on ice for 30 min. 50 µl aliquots of the bacteria suspension were prepared, flash frozen in liquid N₂ and stored at -80°C.

RF-1 buffer:

100mM RbCl

50mM MnCl₂

30mM KOAc

10mM CaCl₂

15% Glycerol

The buffer was adjusted with acetic acid to pH 5.8, sterile filtered (0.22µm) and stored at 4°C.

RF-2 buffer:

10 mM RbCl

10 mM MOPS

75 mM CaCl₂

15% Glycerol

The buffer was adjusted with 1 M NaOH to pH 6.8, sterile filtered (0.22µm) and stored at 4°C.

6.1.3 Transformation

Competent bacteria strain of interest (BL21-(DE3)-RIPL or DH5α™-T1R) was slowly thawed on ice. Meanwhile, the DNA plasmid was cooled on ice in a sterile 1.5 ml reaction tube. For transforming with a recombination reaction, 50 µl of competent cells were used while to transform with a purified plasmid only 25 µl of competent cells were used. The respective number of bacteria was added to the DNA and incubated for 20-30 min on ice. The bacteria were then heat shocked for 1 min at 42°C and cooled on ice for 1-2 min. The bacteria transformed with a plasmid of interest carrying a carbenicillin resistance were directly plated on the respective media and grown for 12 h at 37°C. For canamycin, ampicillin and chloramphenicol resistant strain selection, 500 µl LB media were added to the transformation mix, and the bacteria were grown for 1 h while shaking at 37°C. 100 µl of the culture was then plated on the respective media and grown for 12h at 37°C.

6.1.4 Plasmid purification

For isolation of small amount of DNA plasmid was used the PureYield™ Plasmid Miniprep System (Promega) according to the manufacturer's protocol. Plasmid DNA of larger quantity and higher purity was isolated with the QIAGEN Plasmid Plus Midi kit (Qiagen).

6.1.5 DNA agarose gel electrophoresis

Agarose was dissolved in 1 x TAE buffer to a final concentration of 1 – 2%, heated in the microwave to dissolve and let to cool down to ~60°C. After addition of 0.05% ethidium bromide, the gel was poured into a gel casting tray (12 x 14 cm) to solidify. DNA or dsRNA samples were supplemented with 5 x DNA dye and loaded onto the gel together with DNA size marker. The gel was running in 1 x TAE buffer in a DNA electrophoresis chamber (submarine (horizontal) gel tank PerfectBlue™ MiniL, Peqlab) at constant 140 V for 45 min. The nucleic acids were visualized by UV transillumination with the Alpha Imager gel documentation systems.

6.2 RNA Methods

6.2.1 Isolation of total RNA

Total RNA was isolated from the cells using Trizol or from cell lysate/beads using RNeasy® Mini kit (Qiagen).

6.2.1.1 Isolation of total RNA from cells using Trizol

10-20 x 10⁶ cells were harvested at 1000 x rpm for 5 min at 4°C. The cell pellet was washed with ice-cold 1 x PBS and spun at 1000 x rpm for 5 min at 4°C and resuspended in 1 ml Trizol and incubated for 5 min at RT. 200 µl chloroform was added, vigorously mixed by hand for 15 sec and incubated for 2-3 min at RT. The suspension was centrifuged for 15 min at 12 000 x g at 4°C. The supernatant was carefully transferred to a fresh tube containing 1 volume (~500 µl) isopropanol supplemented with 1 µl of Glycogen (10 mg/ml). After 10 min incubation at RT, the RNA was precipitated for 10 min at 12 000 x g at 4°C. The RNA pellet was washed with 1 ml ice-cold 75% EtOH and centrifuged at 7 500 x g for 5 min at 4°C. The pellet was briefly air dried and resuspended in 42.5 µl mQH₂O.

6.2.1.2 Isolation of total RNA from cell lysate/beads

Isolation of total RNA from lysate or beads was done using RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol including an on-column DNase treatment. RNA was eluted with 42.5 µl mQH₂O.

6.2.2 DNase digestion

6.2.2.1 DNase I digestion

DNase I digestion reaction (final volume 50 µl):

42.5 µl RNA from the previous step

5 µl 10 x RQ Reaction buffer (Promega)

0.5 µl RiboLock™ RNase Inhibitor (40 U/µl) (Fermentas)

2 µl RQ-RNase-free DNase I (1 U/µl) (Promega)

The mixture was incubated for 30 min at 37°C.

6.2.2.2 Purification of RNA

To purify the RNA, the volume was adjusted to 200 µl by adding 150 µl mQH₂O and extracted with 1 volume phenol/chloroform. The solution was briefly vortexed and centrifuged for 3 min at 16,000 x g

at RT. This extraction step was repeated with 1 volume of chloroform. The supernatant was carefully taken, and RNA was precipitated with 2.5 volume 100% ethanol and 1/10 volume 5 M NH₄OAc for at least 20 min at -20°C. The mixture was centrifuged for 15 min at maximum speed at 4°C to pellet the RNA. The supernatant was discarded, and the RNA pellet was washed with 75% ice-cold EtOH and spun for 5 min at 7500 x g at 4°C. RNA pellet was air dried for 5 min at RT and resuspended in 42.5 µl mQH₂O.

6.2.2.3 Turbo DNase digestion

Turbo DNase I digestion reaction (final volume 50µl):

42.5 µl RNA from the previous step

5 µl 10 x Turbo DNase Reaction buffer (Life Technologies)

0.5 µl RiboLock™ RNase Inhibitor (40 U/µl) (Fermentas)

2 µl Turbo DNase (2 U/µl) (Life Technologies)

The mixture was incubated for 30 min at 37°C, and afterward, additional 2 µl of Turbo DNase (2 U/µl)(Life Technologies) was added and incubated for another 30 min at 37°C.

6.2.2.4 Purification of RNA

RNA was purified by using ReliaPrep™ RNA Cell Miniprep system (Promega) according to the manufacturer's protocol including an on-column DNase treatment.

6.2.3 Denaturing agarose gel – RNA/Formaldehyde

Gel preparation

All steps were done in RNase-free working conditions, therefore, to ensure it the MiniL 12 x 14 cm agarose gel chamber was rinsed with 1% SDS and mQH₂O. 1.4 g agarose and 86 ml mQH₂O (RNase-free) were weighed in and heated in a microwave to dissolve. The melted agarose was cooled down to ~60°C, supplemented with 12 ml of 10 x MOPS running buffer and 22 ml 37% formaldehyde. The gel was solidified in the chamber.

Sample preparation

The RNA sample (2-4 µg/lane) was added with at least equivolume RNA loading (formamide) dye and heated for 15 min at 75°C. The samples are chilled on ice and loaded onto the gel.

Gel run

The gel run was performed in 1 x MOPS running buffer in an electrophoresis chamber (submarine (horizontal) gel tank PerfectBlue™ MiniL, Peqlab) at 120 V in the fume hood for about 1.5-2 hours at RT.

Addition of ethidium bromide to the RNA loading dye allowed visualization of the RNA by the UV transilluminator.

6.2.4 RNA 6 000 Pico assay

The integrity of RNA was determined using Agilent RNA 6000 Pico kit (Agilent Technologies) according to the manufacturer's protocol and using 2100 Expert software for eukaryotic total RNA on Agilent 2100 Bioanalyzer instrument.

6.2.5 Preparation of dsRNA for soaking in *Drosophila* S2 cells

6.2.5.1 PCR reaction, template preparation

Primer design:

Both forward and reverse primers were beginning with the T7 promoter: 5'-TAATACGACTCACTATAGGG- 3'. The T7 promoter sequence was followed by gene-specific sequences (5.14.1). Ideally, gene-specific sequences were usually designed to have an annealing temperature between 55°C and 60°C to standardize the PCR reaction. The PCR products were more than 500 bp in length, mostly around 700 bp.

PCR reaction (final volume 100 µl):

1.5 µl 100 µM forward primer

1.5 µl 100 µM reverse primer

10 µl 10 x Green GoTaq® reaction buffer (Promega)

2 µl dNTP mix (10 mM each dNTP)

Template 20 ng plasmid DNA or 0.2 µg genomic DNA

1 µl GoTaq® DNA polymerase (5 U/µl) (Promega)

The reaction was filled up to 100 µl with ddH₂O.

Thermocycling conditions:

Step	Time	Temperature	Number of cycles
Initial denaturation	2 min	94°C	1
Denaturation	30 sec	94°C	40
Annealing	30 sec	55°C	
Elongation	1 min	72°C	

Final elongation	5 min	72°C	1
Cooling	∞	4°C	1

Cleanup of PCR product:

The PCR product was purified from agarose gel with the Promega Wizard® SV Gel and PCR Clean-up purification kit according to manufacturer's protocol except that the membrane wash solution was substituted with 80% ethanol. The DNA concentration was determined by measuring the absorbance at 260 nm by the NanoDrop 1000 spectrophotometer (Peqlab).

6.2.5.2 T7 transcription reaction

This protocol was derived from Milligan and Uhlenbeck (Milligan et al., 1987) with modifications optimized for dsRNA preparation according to Ambion.

T7 reaction (final volume 60 µl):

6 µl 10 x T7 buffer

18 µl NTP mix (25 mM each NTP)

3 µl 0.1 M DTT

2 µl T7 Polymerase (purified in the lab)

1 µg DNA (PCR)

The reaction was filled up to 60 µl with mQH₂O and incubated at 37°C usually overnight (6 hours minimum).

10 x T7 buffer:

400 mM Tris pH 7.9

30 mM spermidine

% Triton X-100

360 mM MgCl₂

Inorganic pyrophosphate (PPi) built up during the transcription was precipitated with magnesium ions.

6.2.5.3 Purification of dsRNA

A 1/10 volume of 0.5 M EDTA pH 8.0 was added to the transcription reaction mix. All Mg-PPi had to go into solution before the cleanup procedure. Cleaning up was done using peqGold total RNA kit (Peqlab) according to the manufacturer's protocol. The RNA was eluted with 100 µl mQH₂O.

Annealing of dsRNA

The RNA was incubated at 75°C for 15 min followed by slow coolingdown for at least 15 min at RT. The concentration of the dsRNA was estimated on a 2% agarose gel (chapter 2.1.5). 1, 2 and 3 µl of a 1/10 dilution of dsRNA were loaded next to 1, 2 and 3 µl of PCR product (c=250 ng/µl).

6.2.6 Two-Step RT-PCR

6.2.6.1 First-Strand cDNA synthesis

In a typical procedure between 0.5µg and 1 µg total RNA was used with 125 ng-250 ng random hexamers primers 5'-d(NNNNNN)-3'(Fermentas).

RT reaction:

1 µg total RNA and 250 ng random hexamer primers (1 µl of a 250 ng/µl stock) were filled up with mQH₂O up to 10 µl. The mixture was incubated for 5 min at 70°C and quickly cooled on ice for 5 min. Meanwhile, the 15 µl RT-Master mix was prepared and added.

RT-Master mix per reaction (15µl):

5 µl M-MLV RT 5 x Reaction buffer (Promega)

1.25 µl dNTP mix (10 mM each dNTP)

1 µl M-MLV RT (200 U/µl) (Promega)

7.75 µl mQH₂O

The samples were incubated for 10 min at RT followed by additional 50 min incubation at 42°C. The samples were heat-inactivated for 15 min at 75°C. The cDNA was then used as a template for amplification by PCR or qPCR. In parallel was running the sample without M-MLV-RT enzyme (-RT).

6.2.6.2 Second-Strand cDNA synthesis and PCR amplification

1 µl or 2 µl of first-strand cDNA reaction was used in the subsequent PCR amplification. For quantitative analysis, the cDNA was amplified by qPCR.

PCR reaction (final volume 25µl):

0.4 µl 100 µM forward primer

0.4 µl 100 µM reverse primer

5 µl 10 x Green GoTaq® reaction buffer (Promega)

1 µl 25 mM MgCl₂

0.5 µl dNTP mix (10 mM each dNTP)

1-2µl template (first-strand cDNA synthesis)

0.125 μ l GoTaq® DNA Polymerase (5 U/ μ l) (Promega)

The reaction volume was adjusted to 25 μ l with mQH₂O.

Thermocycling conditions:

Step	Time	Temperature	Number of cycles
Initial denaturation	2 min	94°C	1
Denaturation	30 sec	94°C	30 - 40
Annealing	30 sec	55 - 68°C*	
Elongation	30 sec	72°C	
Final elongation	5 min	72°C	1
Cooling	∞	4°C	1

*Optimal annealing temperature depended on the melting temperature of the primers.

10 μ l of PCR reaction were loaded on a 1-2% agarose gel.

6.2.6.3 Second-Strand cDNA synthesis and qPCR amplification

To set up conditions, each primer pair used in the qPCR assay was tested for efficient amplification during qPCR analysis. For this purpose, cDNA template started from 1 μ g of total RNA from *Drosophila* S2 cells was diluted as 1/10 and 1/100. 1 μ l of undiluted and 1 μ l of each dilution was transferred into a 96-well plate. Duplicates of each template dilution were required to obtain the greater confidence in the data. Also, one water sample for each primer pair was analyzed as a negative control. 19 μ l of a qPCR master mix including primers of a final concentration of 0.3 μ M each was added to the pre-laid template. The 96-well plate was sealed, briefly centrifuged and put into the instrument (StepOnePlus™ Real-time PCR system, Applied Biosystems). The run was performed with standard qPCR cycling conditions.

qPCR Master mix (for 1 x 96-well) (final volume 19 μ l):

1.2 μ l 5 μ M forward primer

1.2 μ l 5 μ M reverse primer

10 μ l 2 x GoTaq® qPCR Master mix (Promega)

0.2 μ l CXR Reference Dye

The volume was adjusted with mQH₂O to 19 μ l.

Standard qPCR Cycling Conditions:

Step	Time	Temperature	Number of cycles
Hot-Start activation	10 min	95°C	1
Denaturation	15 sec	95°C	40
Annealing/Extension	1 min	60°C	
Melting curve	15 sec	95°C	1
Final elongation	1 min	60°C	1
Cooling	15 sec	95°C	1

The obtained data were analyzed using StepOnePlus™ software v2.3.

6.2.6.4 qPCR run on transcripts

Quantitative PCR analysis was performed to measure selected transcript levels. Samples from first cDNA strand synthesis (chapter 6.2.6.1) were used as a template. For each transcript of interest, the qPCR conditions were set up. The $\Delta\Delta C_T$ threshold cycle method was used to assess relative changes in different transcripts (Livak and Schmittgen, 2001). First C_T values were adjusted to baseline $C_T=0.5$. Afterward, the C_T value for the RNA from the gene of interest was divided by the C_T value of the RNA from an internal reference. This normalization permitted comparison of expression of a gene of interest among different samples. Equations used for quantification:

$$\Delta C_T (\text{sample 1}) = C_T (\text{gene of interest}) - C_T (\text{reference})$$

$$\Delta C_T (\text{sample 2}) = C_T (\text{gene of interest}) - C_T (\text{reference})$$

$$\Delta\Delta C_T = \Delta C_T (\text{sample 1}) - \Delta C_T (\text{sample 2})$$

$$2^{-\Delta\Delta C_T} = \text{relative fold difference between sample 1 and sample 2}$$

6.3 Protein Methods

6.3.1 Protein purification for antibody production

6.3.1.1 Recombinant protein expression in BL21 RIPL cells

BL21 RIPL cells were transformed with pSD5 (His-RpL10Ab 1-539 aa), pSD6 (His-RpS3 1-489 aa) or pSD7 (His-PABPC 1-522 aa) (chapter 5.12). Single colonies were picked, inoculated in 20 ml LB supplemented with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) and grown overnight at 37°C. The overnight

culture was diluted in 1 L of LB medium (100 µg/ml ampicillin, 34 µg/ml chloramphenicol) and cells were grown to an OD₆₀₀ of approximately 0.5 before induction. The induction was done with 1 mM IPTG for 3 h and harvested 5 min at 5,000 x g. Cell pellets were frozen at -20°C overnight.

6.3.1.2 Purification of His-tagged proteins by Ni-NTA agarose under denaturing conditions

Purification of pSD5 (His-Rpl10Ab 1-538 aa), pSD6 (His-RpS3 1-489 aa) or pSD7 (His-PABPC 1-522 aa)

The pellet of 1 L induced cell culture was thawed on ice and resuspended in 20 ml lysis buffer. The cell lysate was split into 4 x 50 ml falcon tubes and sonicated 4 x for 15 sec 9 cycles 97% on ice. The cell lysate was cleared for 20 min at 10,000 x g at RT. Clarified lysate was added to 4 ml pre-equilibrated Ni-NTA agarose beads (Qiagen) and overhead rotated for 30 min at RT. Pre-equilibration was done two times with three bed volume mQH₂O and once with three bed volume lysis buffer with spinning at 1000 rpm for 5 min at RT in between. After rotation, the beads were collected at 1000 rpm for 5 min at RT and washed 3 x with 3 column volumes of washing buffer I by spinning at 1000 rpm for 5 min at RT. After third wash, the beads were resuspended in 6 ml Washing buffer II and transferred into a Glass Econo-Column Chromatography Column (Biorad 737-4152). The column was washed with three-bed volumes with Wash buffer II. The protein was eluted with 3 volumes of elution buffer and fractions were collected. Two fractions of 1.5 ml, 8 fractions of 0.5 ml and two of 1.5 ml each. A1/100 volume of the fractions were supplemented with 2 x protein sample buffer and analyzed by PAGE followed by Coomassie staining. Fractions of the highest and purest protein amount were collected and transferred into a slide-AlyzerR dialysis cassette G2 (Thermo Scientific 7000 - 20,000 MWCO depending on the size of purified protein). The cassette was dialyzed against 1 L of dialysis buffer for three times. First, the dialysis buffer was changed after overnight dialysis and then twice after 2 hours. The protein sample was collected, and protein concentration was determined by OD measurement (ABS280), and Bradford assay (Bio-Rad Protein Assay (500-0006)). The protein sample was flash frozen in liquid N₂ and stored at -80°C.

Lysis buffer

100 mM Na₂HPO₄

10 mM Tris base

8 M Urea

10 mM Imidazole

Adjust pH to 8.0 with 10 M NaOH.

Wash buffer I

100 mM Na₂HPO₄

10 mM Tris base

8 M Urea

20 mM Imidazole

Adjust pH to 8.0 with 10 M NaOH.

Wash buffer II

100 mM Na₂HPO₄

10 mM Tris base

8 M Urea

30 mM Imidazole

Adjust pH to 8.0 with 10 M NaOH.

Elution buffer

100 mM Na₂HPO₄

10 mM Tris base

8 M Urea

250 mM Imidazole

Adjust pH to 8.0 with 10 M NaOH.

Dialysis buffer

1 x PBS

4 M Urea

5% Glycerol

Cool down to 4°C before use.

6.3.1.3 Antibody production by PINEDA company

Polyclonal antibodies against RpL10Ab, RpS3, and PABPC were produced in rabbits. His-epitope tagged proteins fragments (His-RpL10Ab 1-539aa, His-RpS3 1-489aa, and His-PABPC 1-522aa) were expressed in BL21 RIPL cells and purified under denaturing conditions with a minimal yield of 0.5 mg/ml. The proteins were tested for their stability at 40°C for 24 h, and 0.5 mg of antigen was sent for immunization of the selected animals. After three months of immunization, the serum was tested by Western blot analysis.

6.3.2 Western blot

6.3.2.1 SDS-Polyacrylamide gel electrophoresis (PAGE)

Depending on the sample amount, denaturing protein electrophoresis was either performed in mini or large polyacrylamide gels. Mini gels (1 mm thick, 9 cm height, 8 cm wide) were run in Mini Hoefer electrophoresis unit and gel casting chambers with constant voltage 100 V in stacking gel, and 120 V in separating gel. Large gels (1.5 mm thick, 14.5 cm height, 14.5 cm wide) were run in C.B.S. Scientific, dual adjustable slab gel kit with 120 V in stacking gel, and 140 V in separating gel. Gels were run in 1 x

Laemmli (GTS) buffer at indicated constant voltage. Depending on the size of the protein of interest, the gels contained different percentage of acrylamide. Generally, proteins larger than 100 kDa were separated on 5 - 7.5% polyacrylamide gels, whereas smaller proteins were analyzed by 10 - 12% polyacrylamide gels.

Separating Gel 5% - 12% (final volume 15ml):

	5%	6%	7.5%	10%	12%
ddH ₂ O	9.0 ml	8.7 ml	8.286 ml	7.275 ml	6.435 ml
1.5 M Tris/HCl pH 8.7	3.75 ml	3.75 ml	3.75 ml	3.75 ml	3.75 ml
10% SDS	150 µl	150 µl	150 µl	150 µl	150 µl
40% AA/bisAA (37.5/1)	1.9 ml	2.25 ml	2.813 ml	3.75 ml	4.5 ml
10% APS	150 µl	150 µl	150 µl	150 µl	150 µl
TEMED	15 µl	15 µl	15 µl	15 µl	15 µl

Stacking Gel 4% (final volume 7.5 ml):

	4%
ddH ₂ O	0.94 ml
1 M Tris/HCl pH 6.8	3.75 ml
10% SDS	75 µl
40% AA/bisAA (37.5/1)	750 µl
10% APS	75 µl
TEMED	7.5 µl

6.3.2.2 Transfer

After the gel electrophoresis, the separating gel was transferred into cold 1 x transfer buffer. For mini gels was used Miniblot Cassette System while for large gels was used Biorad Trans-Blot electrophoretic transfer cell system. The membrane was pre-wetted for 10 s in 100% methanol and subsequently used

for western blot assembly. A pre-wetted fiber pad was placed onto the blotting cassette. Three layers of Whatmann papers were added and the gel was placed on top of them. The pre-wetted membrane was transferred onto the gel. Air bubbles between the gel and the membrane were removed and three layers of Whatmann papers were added. The sandwich was completed by addition of a fiber pad on top of the gel sandwich. The cassette was then transferred into the transfer unit. The transfer unit was filled with 2 L of pre-cooled transfer buffer. Addition of a stir bar guaranteed an equal ion distribution in the tank. The proteins were transferred for 90 min at constant 100 V onto the membrane or O/N at constant 30 V. Generally, the Hybond-P PVDF membrane was used. However, quantitative western blot analyses were performed with the Hybond-LFP membrane (Low-fluorescent PVDF). After transferring the sandwich was disassembled and the membranes were blocked in 5% Milk T-PBS for at least one hour.

T-PBS:

1 x PBS

0.05% Tween 20

6.3.2.3 Antibody incubation

The milk was removed, and the membrane was rinsed briefly with T-PBS. The primary antibody working solution was added to the membrane and incubated for 1 h at 4°C. After the first antibody incubation, the membrane was washed 3 x with T-PBS for 10 min each. After 1 h incubation with the secondary antibody working solution at 4°C, the membrane was again washed with T-PBS for 10 min each. In case of using fluorescently labeled secondary antibodies, the membrane was protected from the light.

6.3.2.4 Detection

Chemiluminescent detection:

The membrane was transferred to a glass plate, and 1 x detection solution (working solution + 6 µl H₂O₂ per ml) was spread over the membrane for one minute. The membrane was placed between two overhead foils in a film cassette. The membrane was then exposed to Fuji medical X-Ray film. The films were developed by the AGFA Curix 60 film processor.

Fluorescent detection:

The Hybond-LFP membrane was scanned on the Odyssey CLx (LI-COR) Imaging System at 700nm or 800 nm channel. The signal intensities were analyzed and quantified using ImageStudio software (LI-COR) 2.1 Version.

6.3.3 Analysis of polysomes by sedimentation in sucrose gradients

The protocol describes a method for separation of ribosomal components and polysomes by sucrose gradient centrifugation and detection of protein components co-purifying with polysomes by Western blot analysis. The polysome analysis was done in cytoplasmic extracts of stable cell line 58 generated from *Drosophila* S2 cells overexpressing BLRP-GFP-RpL10Ab fusion protein and 3 x Flag-tagged BirA co-expressed (5.15). During the experiment were used the conditions to keep polysomes intact. All glass, plastic wares and reagent that have been used were RNase-free and buffers were made with DEPC treated mQH2O.

Growing cells

On day zero the cells of stable cell line 58 were counted and plated in Falcon™ Nontreated Tissue Culture Flask with a final concentration $1-2 \times 10^6$ /ml in final volume of 16 ml Express Five medium 10% FBS. The stable cell line was induced with 0.5 mM CuSO₄ for three days. The third day the cells were harvested and counted. Their concentration was around $5-6 \times 10^6$ /ml. To experiment were taken around 100 Mio cells per gradient.

Polysome fractionation

The cycloheximide was added with a final concentration of 0.1 mg/ml to the growth medium, and the cells were incubated for 10 min at 25°C. The cycloheximide was added to inhibit nascent protein synthesis by preventing the translocation of tRNA on 80S ribosomes preventing the ribosomes from completing translation cycle and therefore “freezing” ribosome subunits on the mRNA. All the next steps were done on ice and with buffers supplemented with cycloheximide and MgCl₂ to keep the ribosomes intact. After cycloheximide incubation, the cells were transferred to 15 ml tubes and spun at 1000 x rpm for 5 min at 4°C (Labofuge 400R - Heraeus). The tubes were put on the ice, and all manipulations were continued on ice. The cell pellet was resuspended in 1.5 ml of ice-cold 1 x PBS supplemented with 0.1 mg/ml cycloheximide, and quickly spun for 8 sec in a table-top centrifuge. The cells were lysed in 500 µl of lysis buffer A on ice for 10 min. The lysate was clarified by centrifugation at 16 000 x g for 10 min at 4°C, and the supernatant was carefully collected. The 50 µl out of 500 µl was taken as an input for Western blot analysis and 50 µl of 2 x Protein Sample buffer was added.

Optionally the cytoplasmic extract was sent to ribosome pelleting. In this case the cytoplasmic extract was loaded into pre-cooled ultracentrifugation tubes (Thickwall, Polycarbonate centrifuge tubes, 1 ml, 11 x 34 mm, Beckman) to pellet ribosomes by ultracentrifugation for 1 hour at 100 000 x g (53 000 rpm) at 4°C using rotor TLA 100.2 (Beckman Coulter Optima™ MAX Ultracentrifuge 130 000 RPM). The

ribosome pellet was gently resuspended by pipetting in 500 µl lysis buffer A. The 50 µl out of 500 µl was taken as a pellet for Western blot analysis, and 50 µl of 2 x SDS Protein Sample buffer were added. The rest of the cell lysate or resuspended ribosome pellet, 450 µl out of 500 µl, was layered on top of a linear 15% to 45% sucrose gradient in Buffer B. The tubes for centrifugation were put in rotor bucket and they were placed in the SW 40 Ti rotor (Beckman) and centrifuged for 2 hours at 36 000 x rpm at 4°C. When centrifugation was finished buckets were taken off and kept on ice until fractions from each tube were collected. During the fractionation using Teledyne ISCO Gradient Fractionation system, the absorbance at 254 nm was recorded. The data were extracted and presented in Excel.

Lysis Buffer A (final volume 1 ml):

20 µl 1 M HEPES-KOH, pH 8.0 - 20mM
 37.5 µl 4 M NH₄Cl – 150 mM
 5 µl 1 M MgCl₂ – 5 mM
 2 µl 1 M DTT – 2 mM
 10 µl 10 mg/ml heparin sodium salt – 0.1 mg/ml
 100 µl 10% NP-40 (Igepal CA-630) – 1%
 40 µl 25 x Complete ULTRA Protease inhibitor EDTA-free – 1x
 12.5 µl 40 U/µl Ribolock RNase (Fermentas) – 0.5 U/µl
 100 µl 10 mg/ml Cycloheximide – 1 mg/ml
 673 µl mQH₂O to fill up to 1ml

15% and 45% sucrose in Buffer B (final volume 1 ml):

20 µl 1 M HEPES-KOH, pH 8.0 - 20mM
 37.5 µl 4 M NH₄Cl – 150 mM
 10 µl 1 M MgCl₂ – 10 mM
 1 µl 1 M DTT – 1 mM
 100 µl 10 mg/ml heparin sodium salt – 1 mg/ml
 12.5 µl 40 U/µl Ribolock RNase (Fermentas) – 0.5 U/ µl
 10 µl 10 mg/ml Cycloheximide – 0.1 mg/ml
 250 µl or 750 µl 60% Sucrose – 15% or 45%
 559 µl mQH₂O or 59 µl mQH₂O to fill up to 1 ml.

Preparation of gradient

Gradients were prepared in ultracentrifuge tubes (14 x 95 mm) for SW 40 Ti rotor. It was first loaded 6 ml of 15% sucrose solution in buffer B in the tube and then gently was inserted the tip of a blunt end stainless steel needle to the bottom of the tube and slowly released 6 ml of the 45% sucrose solution in buffer B to displace 6 ml of 15% sucrose solution upwards in the tube. Afterward, the tube was closed with a BioComp cap, short one. During cap insertion, it was important not to have air bubbles trapped inside the tube since free-floating bubbles have a deleterious effect on gradient formation. The capped tubes were put into the Gradient Master. The gradient was made according to manufacturer's instructions choosing the program for SW 40 Ti rotor, short caps and 15%-45% sucrose gradient. When the gradient formation was finished, the caps were carefully removed, and the tubes were transferred to rotor swing buckets and kept at 4°C. Gradients were stable for at least 12 hours.

6.3.4 Ribosome affinity purification

Growing the cells

The cells were growing as described in polysome analysis (chapter 6.3.3) just that for ribosome affinity purification beside using stable cell line 58 (overexpressing BLRP-GFP-RpL10Ab fusion protein and 3 x Flag-tagged BirA co-expressed) generated from Drosophila S2 (chapter 5.15) were also used stable cell line 45 (overexpressing tagged RpL10Ab but without FLAG-BirA)(chapter 5.15) as a negative control. To experiment, 10 Mio cells per sample were taken. All glass, plastic wares and reagent that have been used were RNase-free and buffers were made with DEPC treated mQH₂O.

Pulldown procedure

The cells were treated as described above for polysome analysis (chapter 6.3.3). First, they were incubated in cycloheximide that was added to the growth medium to a final concentration of 0.1 mg/ml. After 10 min incubation at 25°C, the cells were harvested at 1000 x rpm for 5 min at 4°C. All the other steps were done on the ice. The cell pellet was resuspended with ice-cold 1 x PBS supplemented with 0.1 mg/ml cycloheximide. Cells (10×10^6) were lysed in 250 µl ice-cold lysis buffer C and incubated for 10 min on ice. The lysate was clarified for 10 min at 16 000 x g at 4°C. 30 µl out of 250 µl of supernatant was taken as input for Western blot analysis after adding 30 µl 2 x Protein Sample buffer. The rest of supernatant was transferred into a pre-cooled TLA100 tube (Thickwall, Polycarbonate, 230 µL, 7 x 21 mm). The supernatant was centrifuged for one hour at 52,000 x rpm at 4°C in a Beckman Optima TLX Ultracentrifuge (Rotor TLA 100). The 30 µl out of 250 µl of supernatant was taken as Supernatant for Western blot analysis after adding 30 µl 2 x Protein Sample buffer. The rest of

supernatant was discarded, and the ribosome pellet was resuspended gently in 250 µl of lysis buffer C. 30 µl out of 250 µl of resuspended pellet was taken as a pellet for Western blot analysis after adding 30 µl 2 x Protein Sample buffer. The rest of resuspended pellet, 220 µl, was incubated with 100 µl of MyOne streptavidin T1 Dynabeads (Life Technologies). The 100 µl of a slurry of Dynabeads had been prewashed three times with 1 x PBS and resuspended in 100 µl of Lysate buffer C. The ribosome pellet was incubated with beads for 30 min at 4°C by overhead rotation. Afterward, the beads were washed three times with 250 µl of washing buffer D and one time with 1 x PBS supplemented with 0.1 mg/ml Cycloheximide and 2 mM MgCl₂. The washing was done at 4°C. The tubes were put on a magnetic stand for 2 min and the supernatant was carefully removed, washing buffer was added and the tubes were shaken for 10 sec by hands, spun for 5 sec and put again to magnetic stand. After last washing the beads were resuspended in 25 µl 2 x SDS Protein Sample buffer, heated for 2 min at 95°C, vortexed for 30 sec and ready for Western blot analysis as Pulldown sample. Samples of Input, Supernatant, Pellet, and Pulldown were separated on an SDS-polyacrylamide gel and analyzed by Western blot analysis as described in section 6.3.2.4.

Lysis buffer C (final volume 1 ml):

20 µl 1 M HEPES-KOH, pH 8.0 - 20 mM

12.5 µl 4 M NH₄Cl – 50 mM

5 µl 1 M MgCl₂ – 5 mM

2 µl 1 M DTT – 2 mM

10 µl 10 mg/ml heparin sodium salt – 0.1 mg/ml

100 µl 10% NP-40 (Igepal CA-630) – 1%

40 µl 25 x Complete ULTRA Protease inhibitor EDTA-free – 1 x

12.5 µl 40 U/µl Ribolock RNase (Fermentas) – 0.5 U/µl

100 µl 10 mg/ml Cycloheximide – 1 mg/ml

698 µl mQH₂O to fill it up to 1ml.

Washing buffer D (final volume 1 ml):

50 µl 1 M HEPES-KOH, pH 8.0 - 50 mM

75 µl 4 M NH₄Cl – 300 mM

5 µl 1 M MgCl₂ – 5 mM

2 µl 1 M DTT – 2 mM

50 µl 10% NP-40 (Igepal CA-630) – 0.5%

10µl 10 mg/ml Cycloheximide – 0.1 mg/ml

808 µl mQH₂O to fill it up to 1ml.

6.3.5 Ribosome pelleting with Micrococcal nuclease treatment

The stable cell line 58 (5.13) was growing and treated as described above for polysome analysis (chapter 6.3.3) or ribosome affinity purification (chapter 6.3.4) just that 10 Mio of cells were lysed in 250 µl lysis buffer C with reduced amount of RiboLock – 20 U/ml and without heparin but with 2 Kunitz units of micrococcal nuclease and 5 mM CaCl₂. The cells were lysed for 15 min on ice. The nuclease digestion was quenched by adding EGTA to final concentration 6.25 mM. The lysate was clarified for 10 min at 16 000 x g at 4°C. 50 µl out of 250 µl of supernatant was taken as input for Western blot analysis after adding 50 µl 2 x Protein Sample buffer. The rest of the supernatant was layered on top of 550 µl 1.1 M sucrose cushion in buffer B with 20 U/ml Ribolock and without heparin in pre-cooled ultracentrifugation tubes (Thickwall, Polycarbonate centrifuge tubes, 1 ml, 11 x 34 mm, Beckman). The ribosomes were pelleted by ultracentrifugation for 1 hour at 100 000 x g (53 000 x rpm) at 4°C using rotor TLA 100.2 (Beckman Coulter Optima™ MAX Ultracentrifuge 130 000 RPM). The ribosome pellet was gently resuspended by pipetting in 200 µl lysis buffer C with 20 U/ml Ribolock and without heparin. At the end, 200 µl 2 x SDS Protein Sample buffer was added and input and pellet samples were separated on an SDS-polyacrylamide gel and analyzed by Western blot analysis as described in section 6.3.2.4.

6.4 Library preparation and sequencing

6.4.1 Decapped library

Cell growing and double-strand mediated RNA interference

The cells co-expressing the BLRP-GFP-RpL10Ab fusion protein, and 3 x Flag-tagged BirA and its control without 3 x Flag-tagged BirA protein were treated with dsXRN1 to induce XRN1 knockdown as described later in chapter 6.5.3.4.

Ribosome affinity purification

After 9 days ribosome affinity purification was done as in chapter 6.3.4 just that 40 Mio cells of stablecell lines were used per sample and every other next step was scaled up 4 times. The cells were lysed in 1 ml of Lysis buffer C, after lysate clarification 100 µl (10%) was taken as input (IN). The rest of cell extract, 900 µl (90%) was loaded in a pre-cooled ultracentrifugation tubes (Thickwall,

Polycarbonate centrifuge tubes, 1 ml, 11 x 34 mm, Beckman). The ribosomes were pelleted by ultracentrifugation for 1 hour at 100 000 x g (53 000 x rpm) at 4°C using rotor TLA 100.2 (Beckman Coulter Optima™ MAX Ultracentrifuge 130 000 RPM). The ribosome pellet was gently resuspended by pipetting in 1ml lysis buffer C and incubated with 400 µl of MyOne streptavidin T1 Dynabeads (Life Technologies). Afterward, the beads were washed three times with 1 ml of washing buffer D and one time with 1 x PBS supplemented with 0.1 mg/ml Cycloheximide and 2 mM MgCl₂. The beads were resuspended in 1 ml of RLT buffer from Qiagen.

RNA isolation

RNA was isolated from input (IN) samples and from the beads after ribosome pulldown (PD) using RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol including an on-column DNase treatment. The RNA was further treated with DNases as described in chapter 6.2.2. The RNA concentration was determined by measuring the absorbance at 260 nm by the NanoDrop 1000 spectrophotometer (PepLab), and the RNA integrity was determined using an RNA 6000 Pico kit as described in chapter 6.2.4. The yield of total RNA isolated from the beads was calculated as a percentage of the total RNA used for the pulldown experiment - (number of micrograms of RNA from the beads) / (9 x number of micrograms of input RNA) x 100.

Library preparation

Three biological replicates were pooled for preparation of one library. Two independent libraries were prepared and analyzed by Illumina sequencing.

rRNA depletion

20 µg total RNA of input and pulldown was used per sample. Total RNA was subjected to two rounds of rRNA depletion using a Ribo-Zero kit (Human/Mouse/Rat, Epicentre) according to the manufacturer's protocol. Since the manufacturer's protocol suggest using 5 µg of total RNA per one rRNA depletion around 20 µg of total RNA was divided in three reactions so that 6.67 µg of total RNA was undergoing rRNA depletion by kit and by additional depletion with three biotinylated oligonucleotides against 5.8S rRNA and 28S rRNA (LNA – SD768, SD769, SD770). After rRNA depletion with a kit but before RNA precipitation to rRNA-depleted sample was added 1 pmol of each LNA and the RNA was treated as in the manufacturer's protocol for the kit just in this case 20 µl of MyOneStreptavidin C1 Dynabeads (Life Technologies) was used to remove biotinylated LNA –rRNA complex. rRNA-depleted RNA was purified by ethanol precipitation, three reaction tubes were pooled in one, and additional round of rRNA depletion was done by using kit together with three LNA oligonucleotides. The final RNA-rRNA depleted was eluted with 13 µl mQH₂O for the next step.

5' linker ligation (final volume 20µl):

13 µl RNA-rRNA depleted from the previous step

1 µl T4 10 x Reaction buffer (NEB)

2 µl 10 mM ATP

50 pmol Solexa 5' RNA linker (SD698) (chapter 5.14.4)

2 µl T4 RNA ligase 1 (ssRNA ligase) (10 U/µl) (NEB)

mQH₂O up to 20 µl final volume.

Incubate the reaction for one hour at 37°C and stop the reaction at 65°C for 10 min.

RNA was purified using an Agencourt RNAClean XP system (Beckman Coulter) to remove unligated 5' RNA linker according to manufacturer's protocol adjusting the volume of samples before purification to 50 µl and using 1.8 x volume of beads (90µl). The purification was done twice just that after the first time the RNA was eluted with 50 µl of mQH₂O and after the second time with 10 µl of mQH₂O.

Fragmentation (final volume 20 µl):

10µl of RNA sample from the previous step

8 µl 5 x SuperScript III buffer (Invitrogen) – 2 x

2 µl 100 mM DTT.

The reaction was incubated at 94°C for 5 min. The conditions were adjusted to have fragments ~200bp long.

Dephosphorylation (final volume 50µl):

20 µl fragmented RNA from the previous step

5 µl 10 x Antarctic phosphatase buffer (NEB)

0.5 µl Ribolock RNase (40 U/µl) (Fermentas)

1 µl Antarctic phosphatase (5 U/µl) (NEB)

mQH₂O up to 50 µl.

Incubate the reaction for one hour at 37°C.

The RNA was purified with Phenol/Chloroform extraction as described in chapter 6.2.2.2. The RNA was resuspended in 12 µl mQH₂O.

3' linker ligation (final volume 20.3 µl):

12 µl RNA from the previous step

50 pmol 3' pre-adenylated linker (SD697) (chapter 5.14.4)

2 µl 10 x T4 RNA Ligase 2, truncated KQ buffer (NEB)

2 µl PEG 8000

2 µl T4 RNA Ligase 2, truncated KQ (200 U/µl) (NEB)

0.5 µl Ribolock RNase (40 U/µl) (Fermentas).

Incubate the reaction for one hour at 37°C.

RNA was purified using an Agencourt RNAClean XP system (Beckman Coulter) as in the previous step, two times and in the last step, RNA was eluted in 23 µl mQH₂O.

The first cDNA synthesis – reverse transcriptase (final volume 40 µl):

23 µl RNA from the previous step

50 pmol True_Seq RT primer (SD 766) (chapter 5.14.4)

Incubated for 2 min at 70°C and afterward added the RT mix:

2 µl dNTP (10 mM each dNTP)

8 µl 5 x SuperScript III Reaction buffer (Invitrogen)

4 µl 100 mM DTT

0.5 µl Ribolock (40 U/µl) (Fermentas)

1 µl SuperScript III Reverse Transcriptase (200 U/µl) (Invitrogen)

The reaction was incubated for one hour at 50°C and stopped by incubating for 15 min at 70°C.

cDNA/RNA was purified using an Agencourt RNAClean XP system (Beckman Coulter) as in previous steps, two times and in the last step cDNA/RNA was eluted with 14.8 µl mQH₂O.

Second Strand synthesis (final volume 20 µl):

2 µl 10 x Second Strand Reaction buffer (NEB)

1 µl dNTP (10 mM each dATP, dCTP, dGTP and **dUTP**)

50 pmol TruSeq Universal primer (SD 759) (chapter 5.14.4)

0.2 µl RNaseH (5 U/µl) (NEB)

1 µl DNA polymerase I (10 U/µl) (NEB)

14.8 µl cDNA/RNA from the previous step.

The reaction was incubated for 2.5 hours at 16°C.

cDNA/DNA duplex was purified using an AMPure XP system (Beckman Coulter) triple purification/size selection according to manufacturer's protocol. During the first round it was used one volume (20 µl) AMPure XP beads, and DNA was eluted with 10 µl TE buffer. In the second round, it was used 1.4 volume (14µl) beads and eluted with 10 µl TE buffer. During the third round was done size selection using 0.7 volume (7 µl) with 0.5 volume fresh beads. The DNA was eluted with 30 µl mQH2O.

PCR (final volume 90µl):

30µl cDNA/DNA from the previous step

45 µl LongAmp® Taq 2 x Master Mix (NEB)

250 pmolTruSeq Universalforward primer (SD 759) (chapter 5.14.4)

250 pmolTruSeq_IDX_-- (2, 4, 5, 7, 16 or 19) reverse primer (SD 760-765)

mQH2O up to 90 µl.

Termocycling conditions:

Step	Time	Temperature	Number of cycles
Initial denaturation	30 sec	94°C	1
Denaturation	15 sec	94°C	12
Annealing	30 sec	62°C	
Elongation	30 sec	70°C	
Final elongation	5 min	70°C	1
Cooling	∞	4°C	1

PCR products were running on 1.5% low melting point agarose at 80 V for 2 hours, and fragments from 200 bp to 700bp were purified from the gel using MiniElute Qiagen system according to manufacturer's protocol.

The libraries were multiplexed in three lanes (see Table 2) and subjected to 100-nt paired-end Illumina sequencing, resulting in 120 million to 180 million reads per lane. Sequencing was performed at the Campus Science Support Facility (CSF; NGS Unit). Demultiplexing yielded 25 million to 52 million reads per sample (see Table 2 for the exact numbers of each sample).

Library analysis

Sequencing and index primers were removed from the demultiplexed samples with the cutadapt (v1.4) tool (Martin, 2011), and mapping against the Berkeley Drosophila Genome Project BDGPv5 reference genome (www.fruitfly.org) was performed with the segemehl (v0.1.7) program (Hoffmann et al., 2009, Hoffmann et al., 2014). In a postprocessing step, the ViennaNGS toolbox (Wolfinger et al., 2015) was applied to extract all uniquely mappable reads for the follow-up differential gene expression (DGE) analysis. The htseq-count utility from the HTSeq Python library (Anders et al., 2015) was used to determine read count numbers for each sample. DGE analysis under different conditions was finally performed with the DESeq package (Anders and Huber, 2010).

6.4.2 Expression library preparation

Cell growing and double-strand mediated RNA interference

The cells co-expressing the BLRP-GFP-RpL10Ab fusion protein and 3 x Flag-tagged BirA were treated with dsXRN1 or dsAGO1 to induce XRN1 and AGO1 knockdown respectively as in the chapter 6.5.3.4.

Isolation of total RNA

The 10 Mio cells were incubated in 0.1 mg/ml final concentration of Cycloheximide for 10 min at room temperature (24°C). They were spun at 1000 rpm for 10 min at 4°C and washed with 1 x PBS supplemented with Cycloheximide. Lysis of cells was done in 175 µl of Lysis buffer C on ice for 10 min. Cell extract was clarified by centrifugation at 16 000 x g for 10 min at 4°C and the total RNA was isolated from the supernatant using RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol including an on-column DNase treatment. The RNA was further treated with DNases as described in chapter 6.2.2. The RNA concentration was determined by measuring the absorbance at 260 nm by the NanoDrop 1000 spectrophotometer (Pqlab), and the RNA integrity was determined using an RNA 6000 Pico kit as described in chapter 6.2.4. In the case of the sample without Cycloheximide, it was treated the same way just that Cycloheximide was omitted from each step.

Library preparation

Three biological replicates were pooled for preparation of one library. Two independent libraries were prepared and analyzed by Illumina sequencing.

rRNA depletion

10 µg of total RNA was used per sample. rRNA was depleted as in section 6.4.1 and rRNA depleted RNA was eluted with 5.35 µl mQH2O.

Fragmentation (final volume 10.5µl):

5.35 µl rRNA depleted RNA

4.1 µl 5 x SuperScript III Reaction buffer (Invitrogen)

1.05 µl 100 mM DTT

The reaction was incubated for 5 min at 94°C. The conditions were adjusted to have fragments ~200bp long.

First cDNA strand synthesis – reverse transcriptase (final volume 20µl):

9.5 µl fragmented RNA from the previous step

2 µl Random primer (0.5µg/µl) (Promega)

0.5 µl Ribolock RNase (40 U/µl) (Fermentas)

The reaction was incubated for 1 min at 50°C. Upon incubation the RT mix was added:

5.25 µl mQH₂O

1 µl 100 mM DTT

1.25 µl dNTP (10 mM each dNTP)

0.5 µl SuperScript III Reverse Transcriptase (200 U/µl) (Invitrogen).

The reaction was incubated for 10 min at 20°C and then for 50 min at 50°C.

cDNA/RNA was purified using an Agencourt RNAClean XP system (Beckman Coulter) according to the manufacturer's protocol using 1.8 volume of beads (36µl). The cDNA/RNA was eluted with 20 µl mQH₂O.

Second strand synthesis, end repair, dA-tailing and adaptor ligation was done according to NEBNext module.

Second strand synthesis (final volume 80µl):

20 µl cDNA/RNA from the previous step

46 µl mQH₂O

2 µl Random primer (0.5µg/µl) (Promega)

8 µl 10 x Second Strand Synthesis buffer (NEBNext Module)

4 µl Second strand Synthesis mix (NEBNext)

The reaction was incubated for 2.5 hours at 16°C. Double strand DNA (dsDNA) was purified using 1.8 volume (144µl) of AMPure XP beads according to the manufacturer's protocol. DNA was eluted with 50 µl mQH₂O.

End repair (final volume 100µl):

50 µl dsDNA from the previous step

35 µl mQH₂O

10 µl 10 x End Repair Reaction buffer (NEBNext)

5 µl End Repair enzyme (NEBNext)

The reaction was incubated for 30 min at 20°C. dsDNA was purified using 1.8 volume (180 µl) of AMPure XP beads according to the manufacturer's protocol and eluted with 42 µl mQH₂O.

dA-tailing (final volume 50µl):

42 µl dsDNA with blunt ends from the previous step

5 µl 10 x dA-tailing Reaction buffer (NEBNext Module)

3 µl Klenow fragment (3'→5') (NEBNext Module).

The reaction was incubated for 30 min at 37°C. dsDNA was purified using 1.8 volume (90 µl) of AMPure XP beads according to the manufacturer's protocol and eluted with 36.5 µl mQH₂O.

Ligation of Adaptors (final volume 50 µl):

36.5 µl dsDNA with repaired ends from the previous step

10 µl 5 x Quick Ligation Reaction buffer (NEBNext Module)

15 pmol annealed Adapter Illumina (SD802) (chapter 5.14.1)

2 µl T4 DNA ligase (2000 U/µl) (NEB)

0.5 µl 1M DTT.

The reaction was incubated for 20 min at room temperature (20 - 25°C). Upon the incubation 3 µl USERTM enzyme (1 U/µl) (NEB) was added and the reaction was incubated at 37°C for 15 min.

DNA with ligated adaptors was purified using an AMPure XP system (Beckman Coulter) triple purification/size selection according to manufacturer's protocol and in section 6.3.5.1. DNA was eluted in 23µl mQH₂O.

PCR (final volume 50 µl):

23 µl DNA from the previous step

25 µl LongAmp® Taq 2 x Master Mix (NEB)

25 pmolTruSeq Universal forward primer (SD759) (chapter 5.14.4)

25 pmolTruSeq_IDX (2, 4, 5, 7, 16 or 19)reverse primer (SD760-765)

mQH2O up to 50 µl.

Termocycling conditions:

Step	Time	Temperature	Number of cycles
Initial denaturation	10 sec	98°C	1
Denaturation	10 sec	98°C	10
Annealing	30 sec	65°C	
Elongation	30 sec	72°C	
Final elongation	5 min	72°C	1
Cooling	∞	4°C	1

PCR products were running on 1.5% low melting point agarose at 80 V for 2 hours, and fragments from 200 to 700bp were purified from the gel using MiniElute Qiagen system according to manufacturer's protocol.

The libraries were multiplexed in three lanes (see Table 2) and subjected to 100-nt paired-end Illumina sequencing, resulting in 120 million to 180 million reads per lane. Sequencing was performed at the Campus Science Support Facility (CSF; NGS Unit). Demultiplexing yielded 25 million to 52 million reads per sample (see Table 2 for the exact numbers of each sample).

The library analysis was done as in chapter 6.4.1.

6.4.3 Control libraries

Cell growing and double-strand mediated RNA interference

To show the importance of XRN1 knock down the controlled libraries were made. The cells co-expressing the BLRP-GFP-RpL10Ab fusion protein and 3 x Flag-tagged BirA were treated with dsXRN1 or dsMBP to induce XRN1 knockdown or to use as a control (chapter 6.5.3.4).

RNA isolation and rRNA depletion

Total RNA was isolated by Trizol as described in Chapter 6.2.2.1 and DNase digestion and purification were done as in Chapter 6.2.2, first was done DNase digestion by DNase I and then DNase digestion by Turbo DNase. The RNA concentration was determined by measuring the absorbance at 260 nm by the NanoDrop 1000 spectrophotometer (PepLab), and the RNA integrity was determined using an RNA 6000 Pico kit as described in chapter 6.2.4. 20 µg total RNA was depleted of rRNA as described in chapter 6.4.1. For Tobacco acid pyrophosphate (TAP)-treated libraries, rRNA-depleted RNA was treated with a TAP.

TAP treatment (final volume 20 µl):

2 µg rRNA-depleted RNA

1 µl TAP (10 U/µl) (Epicentre)

2 µl 10 x TAP buffer

mQH₂O up to 20 µl.

The reaction was incubated at 37°C for 3 h.

For Terminator treated control libraries, rRNA-depleted RNA was treated with 5'-phosphate-dependent exonuclease (Terminator).

Terminator treatment (final volume 20 µl):

2 µg rRNA-depleted RNA

1 µl Terminator (10 U/µl) (Epicentre)

2 µl 10 x Terminator buffer

mQH₂O up to 20 µl.

The reaction was incubated at 37°C for 3 h.

RNA was purified through MicroSpin S-300 HR column (GE Healthcare) according to manufacturer's protocol. RNA was eluted in 25 µl mQH₂O.

5' linker ligation (final volume 35 µl):

25 µl RNA from the previous step

3.5 µl 10 x T4 RNA buffer (NEB)

3.5 µl 10 mM ATP

70 pmol 5' RNA linker (SD698) (chapter 5.14.1)

2 µl T4 RNA ss ligase (10.000 U/ml) (NEB)

The reaction was incubated at 37°C for 1 h and stopped at 65°C for 15 min. Again, RNA was purified through MicroSpin S-300 HR column (GE Healthcare) as above just that RNA was eluted in 40 µl mQH₂O.

First cDNA strand synthesis – reverse transcriptase (final volume 20µl):

40 µl RNA from the previous step

1 µl Random primer (0.5 µg/µl) (Promega)

The reaction was incubated for 5 min at 70°C. Upon incubation, the samples were put on the ice, and the RT mix was added:

12 µl 5 x M - MLV - RT buffer (Promega)

3 µl dNTP (10 mM each dNTP)

2 µl x M - MLV - Reverse Transcriptase (200 U/µl) (Promega)

mQH₂O up to 19 µl.

The reaction was incubated for 10 min at 25°C and then for 50 min at 42°C. The reaction was stopped with 70°C for 15 min.

After reverse transcriptase PCR or qPCR was done as described in chapters 6.2.6.2 and 6.2.6.3 using primers from chapter 5.14.2.

6.5 Cell culture

6.5.1 *Drosophila* S2 cell growing conditions

Drosophila S2 cells were cultivated in S2 complete medium at 25°C in flasks.

Standard volumes used for culture flasks are listed:

5 ml T25 flask

15 ml T75 flask

50 ml T175 flask

Cells were kept at a density of $1-2 \times 10^6$ cells/ml. The end of logarithmic cell growth was reached at a cell density of $\sim 10 \times 10^6$ cells/ml. Cells were split and transferred to new culture flask every third to the fourth day.

6.5.2 Transfection of *Drosophila* S2 cells using Effectene (Qiagen)

Cells were seeded at a cell density of 3.5×10^6 cells per 6-well in S2 media. After ~ 30 min the cells were settled, and the media was replaced by 1.5 ml of S2 complete medium. Up to 1 μ g of DNA was prepared in a sterile Eppendorf tube for the addition of the Effectene transfection reagent (Qiagen). Under a sterile hood, 150 μ l of EC buffer and 8 μ l of Enhancer were added to the DNA and mixed well. After 5 min incubation 25 μ l of Effectene was added to the reaction mix and incubated for 10-15 min. The transfection mix was supplemented with 600 μ l S2 complete medium and drop-wise added to the cells in the 6 well. The 6 well-plate was sealed with parafilm and incubated at 25°C. The Enhancer: DNA ratio always had to be kept constant. The cells were harvested 3 days after transfection. Cells were either transferred into a selective medium for polyclonal stable cell line generation or were used in protein analysis.

6.5.3 Selection of polyclonal stable *Drosophila* S2 cell lines using hygromycin

S2 cells were transfected with a cloned gene of interest integrated into pMK33/pMT (hygromycin resistance and inducible promoter) (chapter 5.13). Cells were seeded in a 6 well plate at a density of 3×10^6 cells and were transfected with 0.5 μ g up to 1 μ g of plasmid DNA. In case of cells co-expressing BLRP-GFP-RpL10Ab fusion protein and 3 x Flag-BirAligase the plasmids SD462 and SD499 (Chapter 5.12) were a mix in 19:1 ration and cotransfected as described in Mito et al., 2005. The cells were grown for 3 days without selection. One 6 well of non-transfected cells were kept as a control for subsequent selection. After 3 days the cells of 1 x 6 well were harvested and washed once in regular growth medium supplemented with 300 μ g/ml hygromycin. The cells were resuspended in 5 ml of a selective medium and were transferred to a T25 flask. After 3-4 weeks in selective medium the cells started growing and were expanded in the T75 flask.

6.5.3.1 Protein expression check

The selected cells were seeded at a density of 3×10^6 cells per 6 well and were induced with 0.5 mM CuSO₄ for 3 days. For each transfected cell line, two 6 wells were seeded, one was induced, and the

other one served as an uninduced reference sample. After 3 days the cells were harvested 5 min at 1000 x rpm and resuspended in 100 µl PBS. The cell suspension was supplemented with 100 µl 2 x protein sample buffer and boiled at 95°C for 5 min. 10 µl to 20 µl of the protein samples were loaded on a gel and analyzed by Western blot.

6.5.3.2 Freezing of polyclonal stable *Drosophila* S2 cell lines

A healthy cell culture at approximately 5×10^6 cells/ml (mid-exponential growth) was collected by centrifugation (5 min 1000 x rpm). The cells were resuspended in freezing medium (S2 complete medium, 150 µg/ml hygromycin, and 15% DMSO) to give a final concentration of approximately 2×10^7 cells/ml. The cells were carefully resuspended, and 1 ml of cell suspension was transferred into a cryovial. The vial was tightly sealed and put into a pre-cooled (4°C) Stratacooler. The cooler was kept for 2 h at -20°C and afterward transferred into a -80°C freezer. After 24 h at -80°C the vials were stored at -150°C. After one to two days one vial was thawed to check for sterility and viability of the cells. If cells were very sensitive, the freezing medium was supplemented with up to 50% conditioned media (supernatant of the collected cells).

6.5.3.3 Thawing of polyclonal stable *Drosophila* S2 cell lines

5 ml of S2 complete medium was transferred into a 15 ml falcon tube. The cryovial was taken from the -150°C freezer and quickly thawed at 37°C. The cell suspension was added to the 5 ml media and incubated for 30 min at RT. The cells were harvested at 100 x g for 5 min and resuspended in 5 ml fresh selective medium (150 µg/ml hygromycin). The 5 ml cells suspension was kept in a T25 flask. After 24 h at 25°C, the cells were harvested again at 100 x g for 5 min. The supernatant was removed, and 5 ml fresh selective medium was added. The cells should grow normally and be maintained according to the general cell culture procedure.

6.5.3.4 Double-stranded RNA mediated interference in *Drosophila* S2 cells

At day 0 cells were harvested at 1000 x rpm for 5 min and washed 2 x with 1 x PBS (3 ml for $\sim 5 \times 10^6$ cells). After the last wash, the cells were resuspended in Express Five SFM S2 medium. The cells were counted and diluted to 1.5×10^6 cells/ml. 1 ml of cell suspension was plated in a 6 well, and 30 µg of dsRNA were added to the cells. After 1 h incubation at 25°C in serum-free medium, 2 ml of Express Five media supplemented with 10% FBS was added. The 6 well plate was sealed with parafilm. At day 4 the cells were either harvested (AGO1 knockdown) or treated again with dsRNA. For 9 days dsRNA treatment (XRN1 knockdown), on day 4 the cells were washed 2 x with 1 x PBS and resuspended in Express Five SFM S2 medium. The cells were counted and diluted to 1.5×10^6 cells/ml. 1 ml of the cell

dilution was transferred to a 6 well, and 30 µg of dsRNA was added to the cells. The cells were starved in serum-free medium for 1 h at 25°C. After one-hour incubation 2 ml of Express Five media supplemented with 10% FBS was added to the cells and the 6 well plate was sealed with parafilm. Stable cells lines coexpressing the BLRP-GFP-RpL10Ab fusion protein and 3 x Flag-tagged BirA were induced with 0.5 mM CuSO₄ 3 days before harvesting.

7 Aim

mRNA degradation and mRNA translation are well-known regulators of gene expression. Mechanistically both processes are well described but their interconnection is still topic of research. Since many examples were indicating the close relation of these two processes both in time and space, our aim was to investigate if this relation is happening on the ribosomes. Co-translational mRNA degradation is bringing new possibilities of gene expression providing more efficient adaptation of the organism during the changeable environment conditions or having key role in developmental processes. Therefore, our study was focused on mRNA degradation on the ribosomes in *Drosophila* cells.

8. Results

8.1 Sedimentation of NOT2, HPat, and AGO1 with polysomes performing polysome profiling

As a first step to test whether the degradation is happening on the ribosomes was to perform polysome profile and look for the sedimentation of degradation factors with ribosomes (Method described in Chapter 6.3.3). The cell lysate was laid down and separated on 15-45% sucrose gradient. Absorbance was measured at 254nm during the fractionation. Input and fractions were analyzed by Western blot (Figure 1). We have chosen to check one representative of deadenylation machinery, NOT2, one of decapping machinery, HPat, and one factor of miRNA complex, AGO1, thus we have used anti-NOT2 (lanes 1-12), anti-HPat (lanes 13-24), and antiAGO1 (lanes 25-36) antibodies for Western blot analysis. As we can see from the Figure 1, all three factors have shown co-sedimentation not only with light fractions containing ribosomes (lanes 3-11) but also with heavy fractions containing polysomes (lanes 13-21). As a negative control we have used cytoplasmic protein tubulin and as seen from the figure tubulin is present only in the lightest fraction of the gradient and not associated neither with ribosomes nor polysomes (lanes 37-48). To make sure that the ribosomes are sedimenting with sucrose gradient fractions we have probed the Western blots against RpL10Ab and RpS protein which is component of large and small ribosomal subunit, respectively. Polysome analysis is the method based on size separation still co-sedimentation of degradation factors with polysomes detected with this method was the first prerequisite for mRNA degradation on the ribosome in *Drosophila* cells (Antic et al., 2015).

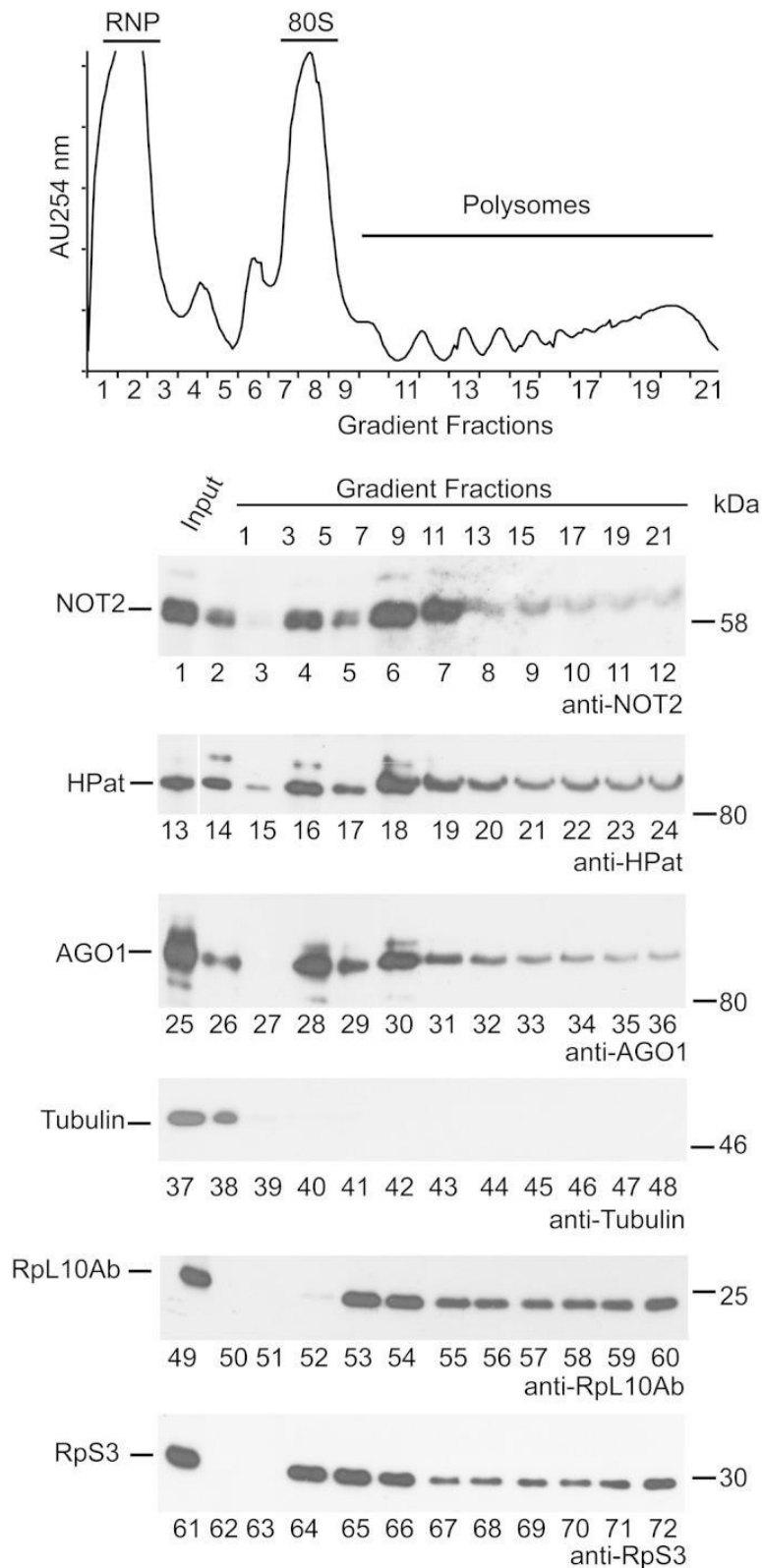


Figure 1 Sedimentation of NOT2, HPat, and AGO1 with polysomes.

The cells were lysed, and the cell lysate was separated in 15-45% sucrose gradient. The fractions were collected and analyzed by Western blot using the antibodies against endogenous NOT2, HPat, AGO1, Tubulin, Rpl10Ab and Rps3 proteins. During the fractionation, absorbance was measured at 254 nm. The amounts of input and fractions loaded on SDS-polyacrylamide gels: for NOT2, HPat, AGO1 - 0.1% input, 1 μ l of fractions 1 and 3, 20 μ l of fractions 5 and 7, 60 μ l of fractions 9-21; for tubulin - 0.5% input and for the fractions 1-21 the same amount as for NOT2, HPat, and AGO1; and for Rpl10AB and Rps3 - 0.03% input, 1 μ l of fractions 1 and 3, 10 μ l of fractions 5-21. The experiment was done in at least three biological replicates (Figure published in Antic et al., 2015).

8.2 Setting up ribosome affinity purification in *Drosophila* cells

Though polysome profile analysis has shown co-sedimentation of degradation factors with polysomes, we were not able to make the firm statement. Namely, polysome profiling is based on size-dependent separation, and we could not exclude that P-bodies or pseudo-polysomes as heavy particles did not co-sediment with polysomes and showed us the false positive results. Still, we had a definite hint that directed us to start establishing the ribosome affinity purification in *Drosophila* cells as a highly specific method for isolating the ribosome complexes and testing copurification of degradation factors with them (Method described in Chapter 6.3.4). A similar method was established in mammalian cell and flies (Heiman et al., 2008; Thomas et al., 2012). The idea behind was to tag one of the ribosomal proteins with biotin and use highly specific and strong biotin-streptavidin link to purify the ribosomes and test by Western blot whether mRNA degradation factors copurify with ribosomes complexes. Thus, we have developed stable cell line coexpressing the N-terminal fusion protein BLRP-GFP-RpL10Ab and the *E. Coli* BirA ligase (Mito et al., 2005; de Boer et al., 2003). BLRP is an abbreviation for Biotin Ligase Recognition Protein, and this sequence is recognized and biotinylated by enzyme BirA ligase. Biotinylation allows using the streptavidin beads to isolate the ribosome. First crucial steps were to check whether the tagged protein is expressed and incorporated into actively translating ribosomes and does it impair the usual ribosome activity. Therefore, we have performed the polysome profile as described in section 8.1 just that in this case, we did pre-purification step which means that cell lysate was first ultracentrifuged and S100 pellet was resuspended and separated on 15-45% sucrose gradient. And as we can see from Figure 2.A, the GFP-RpL10Ab is not only highly expressed (lane 1) but also present in mRNPs, the 80S and polysomes fractions (lanes 2-13) without impairing translation activity.

When the lysate was treated with a limited amount of micrococcal nuclease, it disrupted the polysomes (Figure 2B) since mild nuclease degraded the unprotected mRNA between ribosomes while the ribosomes stayed intact. This caused the shift of ribosomes from heavy fractions to lighter (lanes 15-16 for endogenous RpL10Ab and lanes 28-39 for endogenous RpS3). The same pattern is seen for tagged-RpL10Ab (lanes 2-13). We could conclude that tagged-RpL10Ab is not entirely but still incorporated in actively translated polysomes and does not impact the usual behavior of ribosomes.

Next task was to test whether biotinylation is efficient and whether the biotin-tagged protein is accessible to streptavidin binding. Therefore, S100 pellet was resuspended and was exposed to streptavidin beads (Figure 2C). Because of intensive biotin-streptavidin affinity biotinylated BLRP-GFP-RpL10Ab showed the massive shift in the SDS-polyacrylamide gel (lane 4) comparing to the control cell line which was expressing BLRP-GFP-RpL10Ab but without BirA ligase (lane 8). Therefore, ribosomal

protein was not biotinylated and thus was not shifted upon addition of streptavidin beads (Antic et al., 2015).

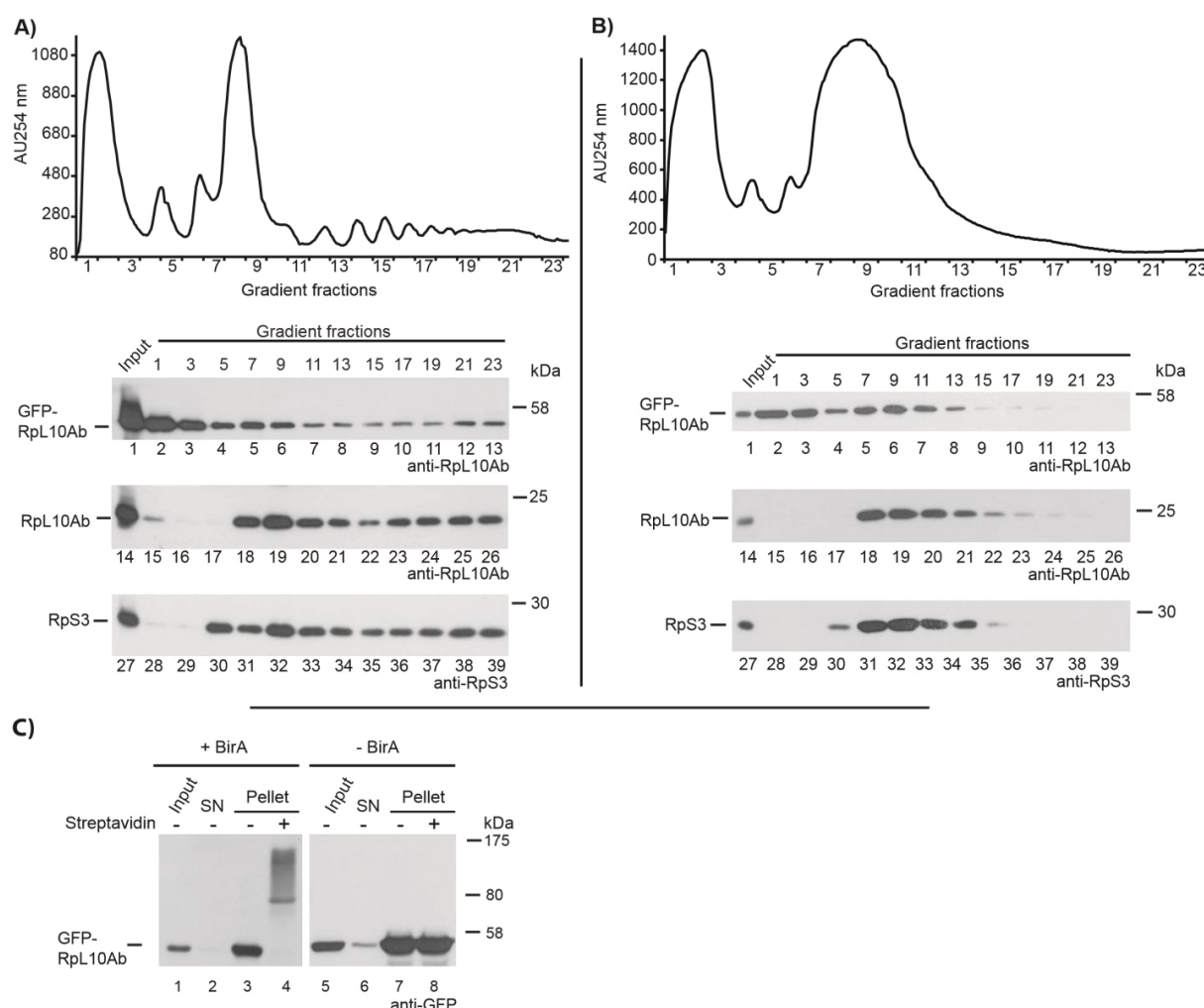


Figure 2 A, B) Polysome profile of cell lysate expressing BRLP-GFP-RpL10Ab and BirA protein without and with micrococcal treatment. The cell lysate was pre-purified by ultracentrifugation S100, and the ribosome pellet was resuspended in buffer and separated on 15-45% linear sucrose gradient. During the fractionation, the absorption at 254 nm (AU 254 nm) was recorded. In case of B) the pellet was resuspended in buffer with the micrococcal enzyme. The amounts of input and fractions loaded on SDS-polyacrylamide gels: for input 0.4% of total cell lysate and 10 μ l for every second fraction. Antibodies anti-RpL10Ab (lanes 1 - 26) or anti-RpS3 (lanes 27 - 39) were used for Western blot analysis. **C) Streptavidin shift of biotinylated GFP-RpL10Ab.** The cell lysate was pre-purified by ultracentrifugation S100, and the ribosome pellet was resuspended in SDS sample buffer, heated, cooled down to RT and 4 μ g Streptavidin was added. Cells co-expressing BirA ligase (+ BirA) were compared to cells without BirA ligase (- BirA) The amounts of input, supernatant loaded on SDS-polyacrylamide gels: input and supernatant after ribosome pelleting 2.3% (3.5%), ribosome pellet 25% (38%). Antibodies anti-GFP were used for Western blot analysis. The experiments were done in at least three biological replicates (Figure published in Antic et al., 2015).

After we have proved that GFP-RpL10Ab protein was biotinylated and accessible to the streptavidin we have performed the pulldown. Upon lysis of cells and S100 pelleting, the pellet was resuspended in lysis buffer and exposed to streptavidin beads. Biotin-streptavidin interaction provided the isolation

of biotinylated-GFP-RpL10Ab and eluate was analyzed by Western blot analysis (Figure 3A, lane 13). As a negative control for specificity was used cell line expressing tagged GFP-RpL10Ab but without BirA ligase (Figure 3A, lane 14). Since we wanted to quantify the efficiency and specificity of pulldown, we loaded linear amount of ribosome pellet of both + BirA ligase cell line (Figure 3A, lanes 5-8) and - BirA ligase cell line (Figure 3A, lanes 9-12), which in this case represented the input. For accurate quantification, it was important that the signal intensity of input is linear, and that signal intensity of pulldown is in the range (Figure 3B), so we could use linear regression analysis. The efficiency of pulldown was calculated by normalizing the signal intensity of pulldown with signal intensity of ribosome pellet of + BirA cells while specificity was calculated by normalizing the signal intensity of pulldown with signal intensity of ribosome pellet of - BirA cells. The results were plotted on a graph (Figure 3C). $27.3\% \pm 3.5\%$ of GFP-RpL10Ab protein from ribosome pellet was isolated by streptavidin beads and present in pulldown eluate in + BirA ligase cells, while less than 1% of the GFP-RpL10Ab protein was eluted in - BirA cells, showing good both efficiency and specificity of pulldown (Antic et al., 2015).

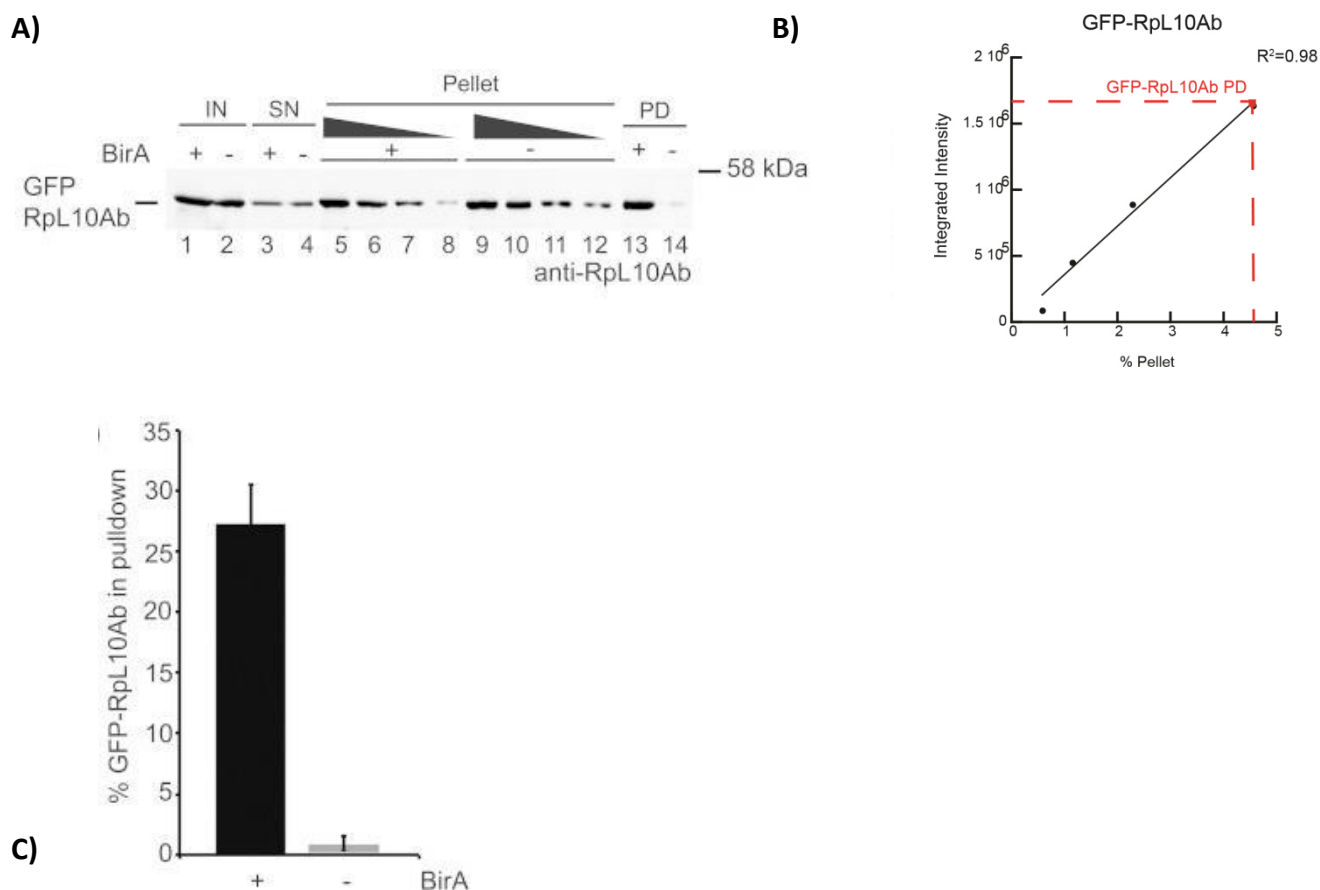


Figure 3 Ribosome affinity purification - efficiency and specificity. **A)** Cells expressing GFP-RpL10Ab with BirA (+BirA) or control cells without BirA (- BirA) were lysed, and ribosome complexes were pelleted and purified using streptavidin-coated beads. Lysate (Input (IN)), supernatant (SN), pellet and pulldown (PD) were analyzed by Western blot analysis using anti-RpL10Ab antibody. Loading: IN 4.9% lysate in lanes 1 and 2, SN 4.9% in lanes 3 and 4, decreasing amount of pellet 4.6% in lanes 5 and 9, 2.3% in lanes 6 and 10, 1.1% in lanes 7 and 11, 0.6% in lanes 8 and 12, PD 20% in lanes 13 and 14. **B)** Quantitative analysis of GFP-RpL10Ab from figure A) was done measuring signal intensity of resuspended ribosome pellet by Odyssey 2.1 (Li-Cor) instrument, plotting it against the percentage of resuspended ribosome pellet and analyzing by linear regression method. **C)** The amount of GFP-RpL10Ab in both + BirA and - BirA was presented as a percentage of the total amount of GFP-RpL10Ab in the resuspended pellet, and it is plotted on the graph. The bars represent the mean values, and the error bars represent the standard deviations from at least three independent experiments (Figure published in Antic et al., 2015).

8.3. Ribosome affinity purification - copurification of the component of mRNA degradation machinery

After setting up the efficient and specific ribosome affinity purification, we have been testing copurification with ribosome of several degradation factors including factors of miRNA mediated degradation complex and general degradation factors (Figure 4). We have analyzed input after lysis the cells (In), supernatant (SN) after S100 ribosome pelleting which we found as crucial pre-purification step, ribosome pellet, and pulldown. Firstly, to make sure that efficiency and specificity of ribosome affinity purification were accomplished we have probed Western blot with anti-GFP antibodies (lanes 17-24). Secondly, it was essential to check whether we have isolated the whole ribosome complex, the

80S and not only big 60S subunit. Therefore, we have probed Western blot with anti-RpS3 antibodies, a ribosomal protein of small 40S subunit (lanes 25-32). Next step was to probe Western blot with anti-tubulin antibodies. Since tubulin is cytoplasmic protein, it served as a negative control that was not expected to be present in ribosome pellet nor pulldown (lanes 33-40). And finally, as a positive control, we have used an initiation factor of translation eIF4E that is expected to be present at actively translating ribosomes (lanes 9-16). After having checked all controls, we have tested copurification of degradation factors one by one. As we can see from Figure 4, both effector components of miRNA-mediated degradation complex are copurifying nicely with ribosomes (Figure 4A and B, lanes 1-8). In those cases, we have probed Western blots with anti-AGO1 and anti-GW182 antibodies, respectively. Next, we have analyzed the Western blots by probing them with anti-NOT1 and anti-NOT2 antibodies, and as we can see at Figure 4B, lanes 9-16 and lanes 17-24 both NOT1 and NOT2, components of deadenylase complex copurified with ribosomes complexes. Later we have tested copurification of several decapping activators. Western blots were probed with anti-HPat, anti-EDC4 and anti-Me31b antibodies. All three factors copurified efficiently and specifically with ribosomes (Figure 4B, lanes 25-32, lanes 33-40, and lanes 41-48). Unlike EDC4, HPat and Me31B did not pellet entirely and they partially stayed in the supernatant. We have also tested copurification of exoribonuclease XRN1 which was also shown to be actively involved in decapping (Braun et al., 2012). XRN1 was also still present in supernatant but also copurifying with ribosomes in decent extent (Figure 4B, lanes 49-56). At the end we have analyzed Western blot by probing with anti-PABPC antibodies, cytoplasmic poly(A)-binding protein, which in one hand could also serve as a positive control as eIF4E. It strongly copurified with ribosomes (Figure 4B, lanes 57-64) (Antic et al., 2015).

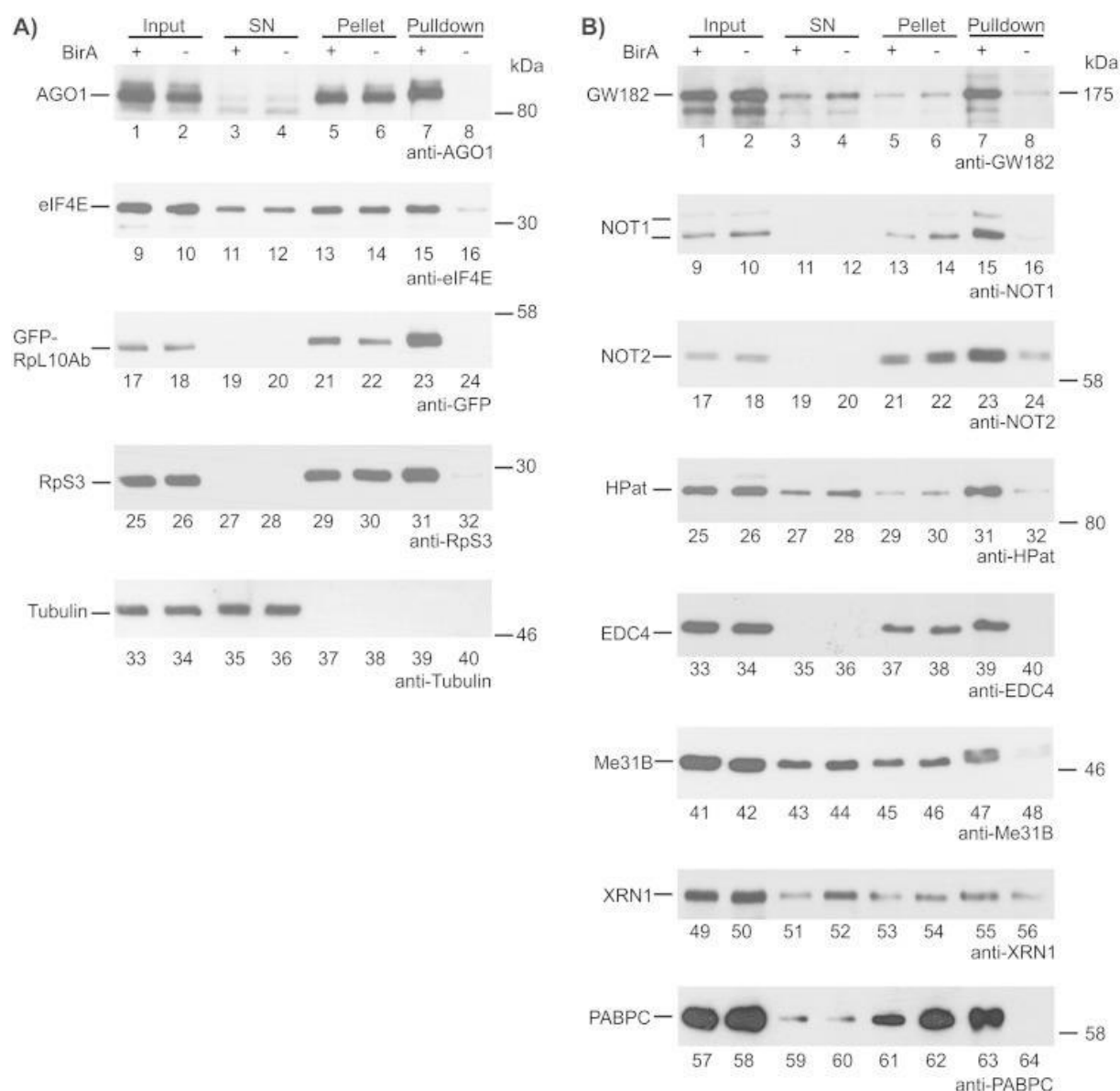


Figure 4 Ribosome affinity purification-copurification of mRNA degradation machinery with ribosome complexes. A) AGO1 and controls, B) GW182, NOT1, NOT2, HPat, EDC4, Me31b, XRN1, and PABPC. Cells expressing GFP-RpL10Ab with BirA (+ BirA) or control cells without BirA (- BirA) were lysed, and ribosome complexes were pelleted and purified using streptavidin-coated beads. Lysate (Input (IN)), supernatant (SN), pellet and pulldown (PD) were analysed by Western blot analysis using anti-AGO1, anti-eIF4E, anti-GFP, anti-RpS3, or anti-tubulin antibodies A) or an anti GW182, anti NOT1, anti-NOT2, anti-HPat, anti-EDC4, anti-Me31b, anti-XRN1, or anti PABPC antibodies B). The amount of IN, SN, and pellet loaded was calculated as a percentage of the total amount of lysate used for pulldown, the amount of pulldown loaded was calculated as a percentage of the total elute. Sample loaded in figure A) for anti-AGO1 probing: IN, SN, pellet 1%, PD 32%; for anti-eIF4E: IN, SN, pellet 0.6%, PD 14%; for anti-GFP and anti-RpS3 and anti-tubulin: IN, SN, pellet 0.4%, PD 4%. Sample loaded in figure B) for anti-GW182 and anti-HPat probing: IN, SN, pellet 0.4%, PD 36%; for anti-NOT1: IN, SN, pellet 1.2%, PD 50%; for anti-NOT2: IN, SN, pellet 0.5%, PD 50%; for anti-EDC4: IN, SN, pellet 0.6%, PD 84%; for anti-Me31b: IN, SN, pellet 1.3%, PD 40%; for anti-XRN1: IN, SN, pellet 1.2%, PD 50%; and for anti-PABPC: IN, SN, pellet 0.2%, PD 30%. The experiments were done in at least three biological replicates (Figure published in Antic et al., 2015).

The ribosome affinity purification was done in the presence of antibiotic Cycloheximide which was stabilizing the ribosomes on the mRNA and thus was helping to increase the efficiency of pulldown.

Therefore, we performed the same experiment just without Cycloheximide. The effectiveness of pulldown and copurification of degradation factors was slightly decreased, but still, factors were copurifying with ribosomes (Figure 5) (Antic et al., 2015).

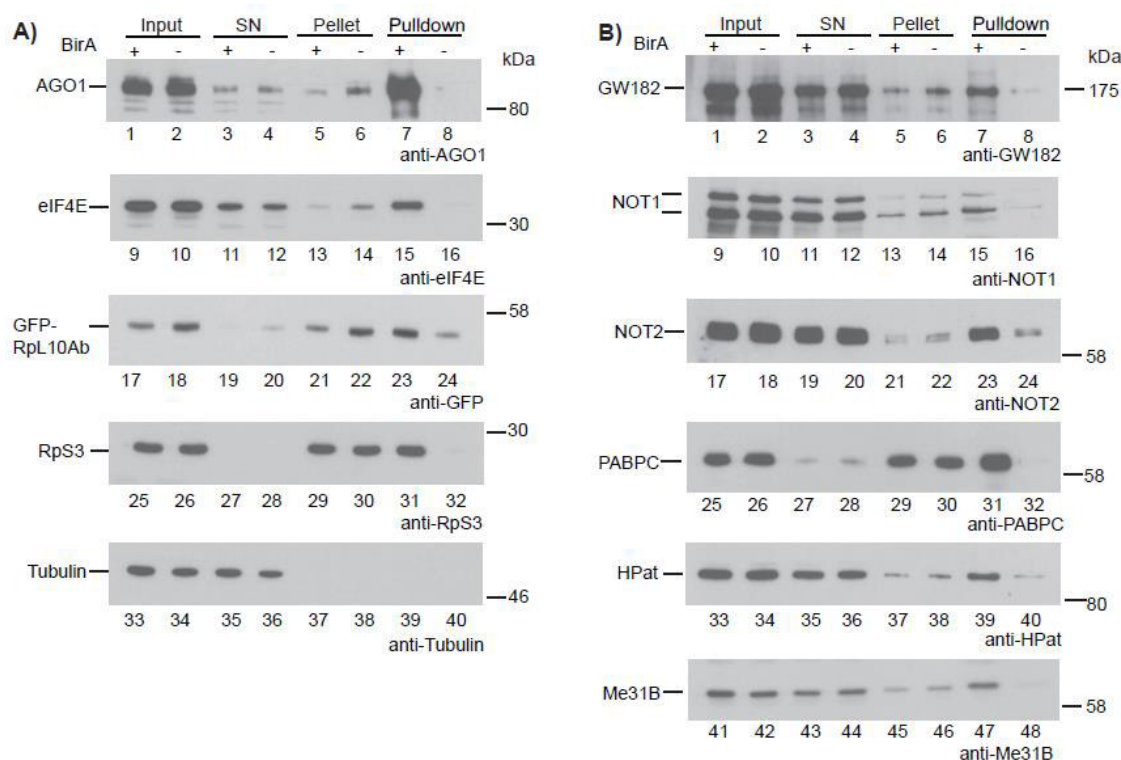


Figure 5 Ribosome affinity purification-copurification of mRNA degradation machinery with ribosome complexes without cycloheximide. A) AGO1 and controls, B) GW182, NOT1, NOT2, PABPC, HPat, and Me31b. Cells expressing GFP-RpL10Ab with BirA (+ BirA) or control cells without BirA (- BirA) were lysed, and ribosome complexes were pelleted and purified using streptavidin-coated beads. Lysate (Input (IN)), supernatant (SN), pellet and pulldown (PD) were analysed by Western blot analysis using anti-AGO1, anti-eIF4E, anti-GFP, anti-RpS3, or anti-tubulin antibodies (A) or an anti-GW182, anti-NOT1, anti-NOT2, anti-PABPC, anti-HPat and anti-Me31b antibodies (B). The amount of IN, SN, and pellet loaded was calculated as a percentage of the total amount of lysate used for pulldown, the amount of pulldown loaded was calculated as a percentage of the total elute. Sample loaded in figure A) for anti-AGO1 probing: IN, SN, pellet 0.4%, PD 32%; for anti-eIF4E: IN, SN, pellet 0.6%, PD 14%; for anti-GFP and anti-RpS3 and anti-tubulin: IN, SN, pellet 0.4 %, PD 4 %. Sample loaded in figure B) for anti-GW182, anti-NOT2 and anti-HPat probing: IN, SN, pellet 0.4%, PD 32%; for anti-NOT1 and anti-Me31b: IN, SN, pellet 1.2%, PD 40%; for anti-PABPC: IN, SN, pellet 1.2%, PD 40% (Figure published in Antic et al., 2015).

8.4 mRNA dependence of association of miRNA components and mRNA degradation factors with ribosomes

Next question that we wanted to answer was whether the components of miRNA effector complex and mRNA degradation factors are associated with mRNA or with ribosome complexes. To test that we have used the same conditions as in Figure 2B). We have treated cell lysate with a limited amount

of micrococcal nuclease, which was not too harsh to digest rRNA and destroy ribosomes, but efficient enough to digest unprotected mRNA between ribosomes, causing polysome shift to 80S peak (Figure 2B), lanes 18-26 and lanes 31-39). To check copurification in these conditions, we have analyzed S100 pellet by Western blot analysis without and in the presence of a micrococcal nuclease (Method described in Chapter 6.3.5). As seen at Figure 6. ribosome complexes are intact, both subunits are pelleting in both conditions without or with a nuclease (Figure 6, lanes 3 and 4 for RpL10Ab from big 60S subunit and lanes 7 and 8 for RpS3 from small 40 subunit). As expected eIF4E and PABPC which are bound to the mRNA are not copurifying with ribosomes in the presence of nuclease that can be seen by comparing lanes 11 and 12 for eIF4E and lanes 15 and 16 for PABPC. All analyzed factors have shown mRNA dependence since upon nuclease treatment they did not show copurification with ribosomes anymore. Among tested factors were AGO1 (lanes 19 and 20) as miRNA effector component, NOT2 (lanes 23 and 24) as a component of deadenylase complex, Me31b (lanes 27 and 28), HPat (lanes 31 and 32), EDC4 (lanes 35 and 36) as decapping activators and XRN1 (lanes 39 and 40) as an exoribonuclease (Antic et al., 2015).

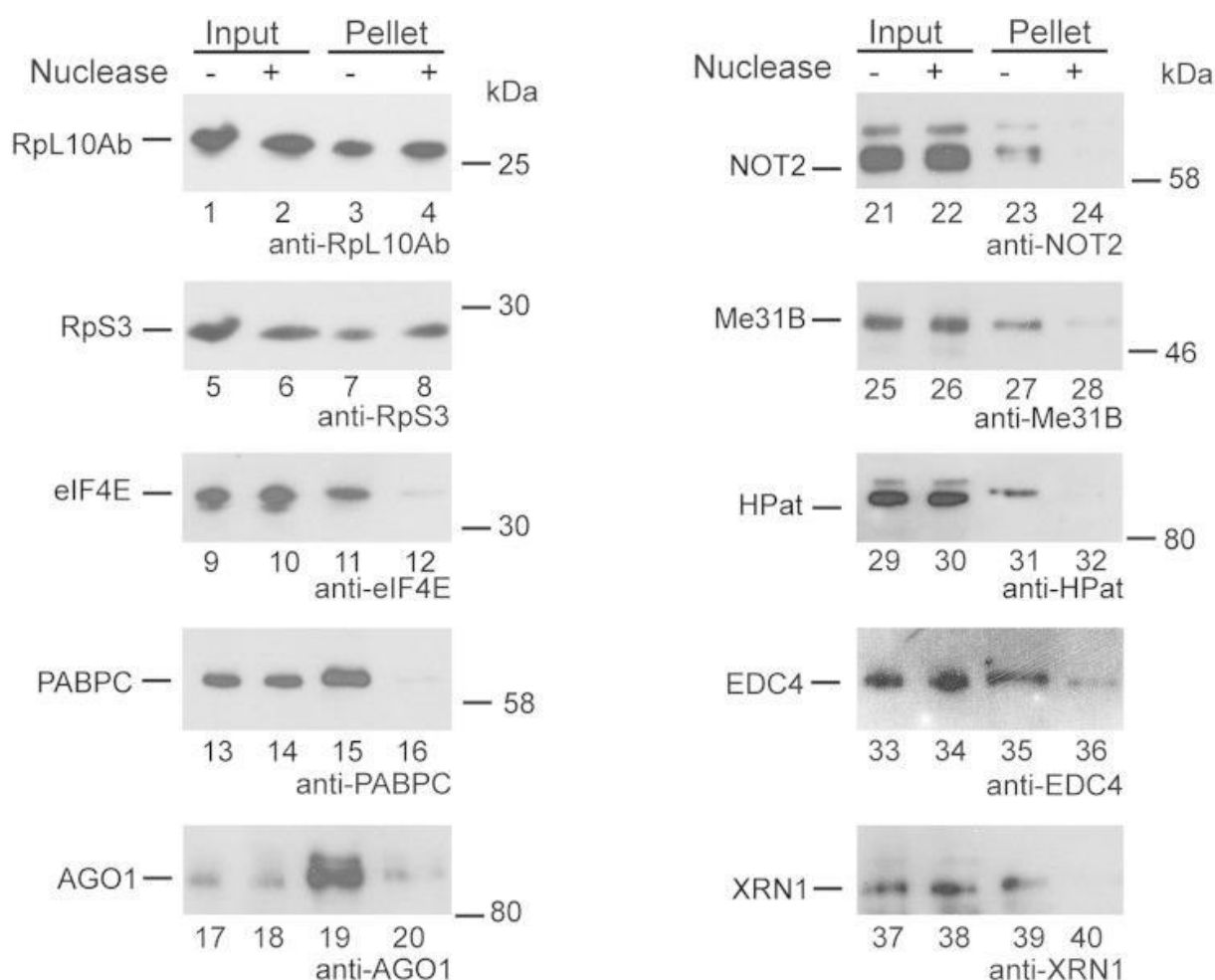


Figure 6 mRNA dependence of association of miRNA components and mRNA degradation factors with ribosomes. Cells expressing GFP-RpL10Ab with BirA were lysed in the presence of a micrococcal nuclease (lanes +) or without (lanes -), and ribosome complexes were pelleted by 100S ultracentrifugation. Cell lysate (IN) and ribosome pellet were analyzed by Western blot analysis using antibodies. Sample loaded for anti-RpL10Ab and anti-RpS3: IN and pellet 0.8%; for anti-eIF4E: IN 0.3%, pellet 2.5%; for anti-PABPC: IN 0.5%, pellet 3.8%; for anti-AGO1: IN 0.1%, pellet 7.5%; for anti-NOT2: IN 0.5%, pellet 4%; for anti-Me31b: IN 0.1%, pellet 1.5%; for anti-HPat: IN 0.5%, pellet 10%; for anti-EDC4: IN 0.3%, pellet 15%; for anti-XRN1: IN 0.5%, pellet 5% (Figure published in Antic et al., 2015).

8.5 Decapped mRNA degradation intermediates on the ribosomes

Not only that miRNA effector components and mRNA degradation factors were found associated with ribosomes we also identified mRNA degradation intermediates as decapped mRNAs copurifying with ribosome complexes. The idea behind the detection of decapped mRNA intermediates was based on ligation of 5' RNA linker to 5' monophosphate decapped mRNA which allows specific amplification of mRNAs lacking cap structure. The setting up of the method is presented as a cartoon in Figure 7. Namely, hydrolysis of the m7G cap structure by the decapping complex gives rise 7-methyl-GDP and 5' monophosphate mRNA. Unlike capped mRNA decapped - 5' monophosphate mRNA is capable of ligating 5' RNA linker using T4 RNA ligase. Since the vast majority of total RNAs takes rRNA which also contains 5' monophosphate, rRNA was removed at the very beginning. Upon 5' RNA linker ligation mRNA was fragmented and afterward 3' linker was ligated and thus only fragments with both 5' and 3' linker were amplified in RNA-seq libraries (Antic et al., 2015).

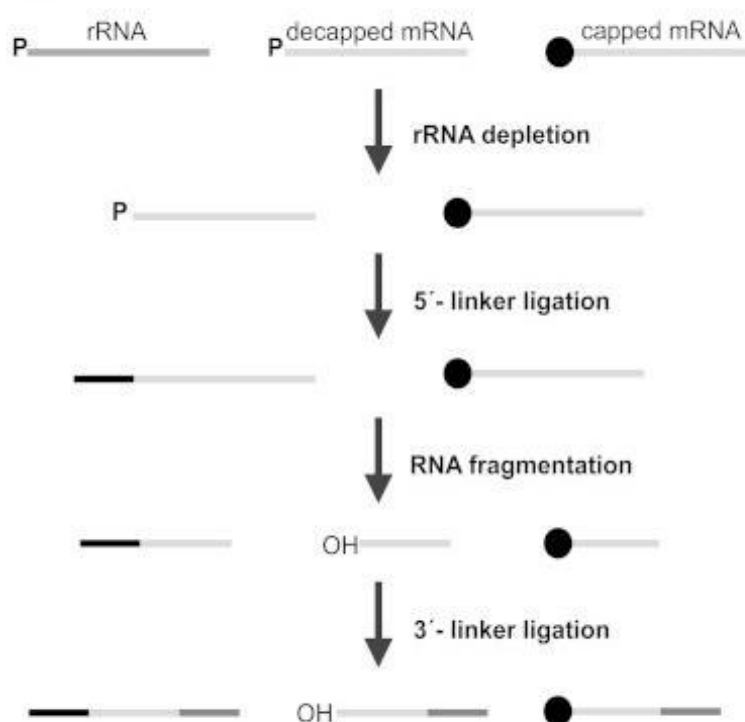


Figure 7 Cartoon is presenting the first steps of a decapped RNA-seq library preparation. Total RNA was depleted from rRNA, and afterward 5' RNA linker was ligated to 5' monophosphate decapped RNA. The next step, RNA fragmentation, was followed by 3' linker ligation. 5' and 3' linker ligation provided that only RNA with both linker sequences get amplified in the RNA-seq library (Figure published in Antic et al., 2015).

Since decapped mRNA is very susceptible and degraded very fast by XRN1 exoribonuclease to make decapped mRNA stable and detectable we have performed XRN1 depletion by knocking it down using dsXRN1 (Figure 8A), lane 4) (Method described in Chapter 6.5.3.4). After achieving XRN1 knockdown, we have checked whether miRNA-mediated and general mRNA degradation factors are still copurifying with ribosomes. We have picked up just one representative from each group, and as we can see from Western blot analysis at Figure 8B) AGO1 (lane 7), NOT2 (lane 15) and HPat (lane 23) are still associated with ribosomes complexes (Antic et al., 2015).

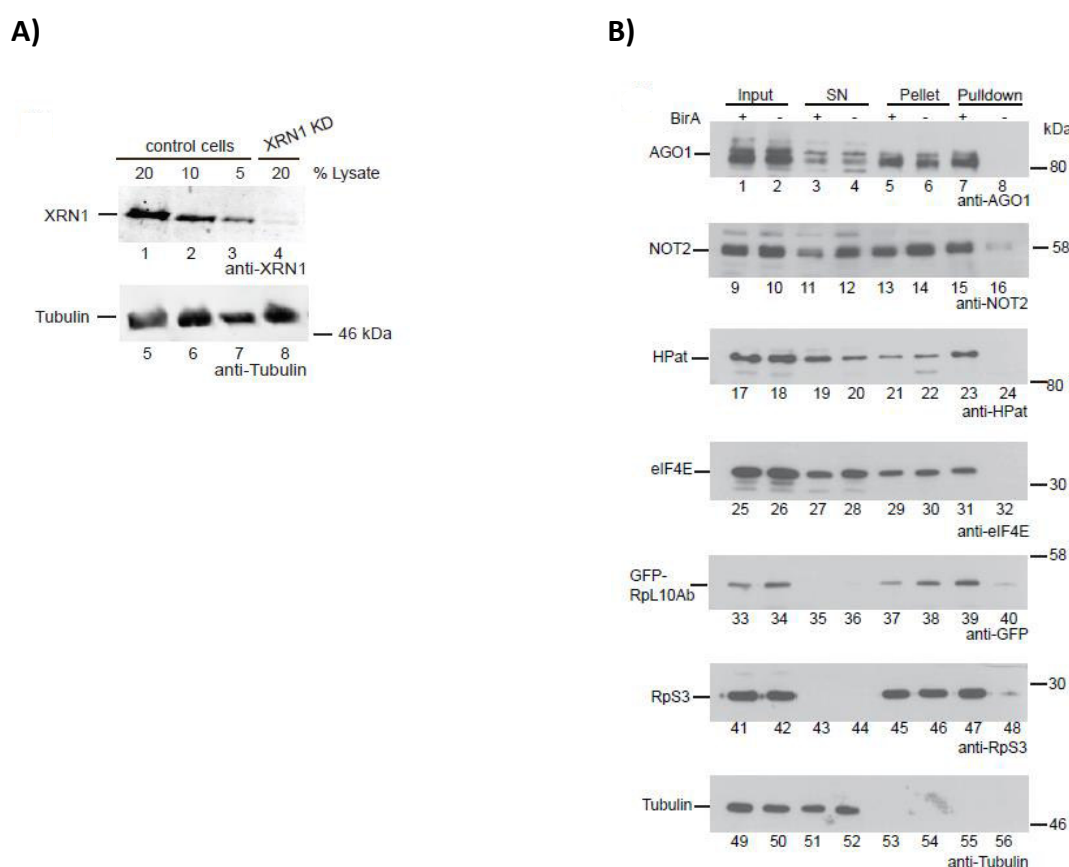


Figure 8 Ribosome affinity purification-copurification of mRNA degradation machinery with ribosome complexes in XRN1 knockdown cells. A) XRN1 knock down. **B)** AGO1, NOT2, HPat, and controls. The XRN1 knockdown efficiency was analyzed by Western blot analysis using anti-XRN1 antibody. 12 Mio of control cells and 12 Mio of cells treated with dsXRN1 were lysed and loaded on the SDS-PAGE gel with percentage as indicated (lanes 1-3, lanes 5-7 for control cells and lanes 4, 8 for cells treated with dsXRN1). Tubulin was used as a loading control (lanes 5-8). B) Cells expressing GFP-RpL10Ab were treated with dsXRN1, and ribosome affinity purification was done as in Figure 4. The ribosome complexes were analyzed by Western blot analysis using anti-AGO1 antibodies (lanes 1-8), anti-NOT2 (lanes 9-16), anti-HPat (lanes 17-24), anti-eIF4E (lanes 25-32), anti-GFP (lanes 33-40), anti-RpS3 (lanes 41-48), anti-tubulin (lanes 49-56). Sample loaded for anti-AGO1, NOT2 and HPat: IN, SN,

Pellet 0.4%, PD 20%; for anti-eIF4E: IN, SN, Pellet 0.6%, PD 12%; for anti-GFP, RpS3, tubulin: IN, SN, Pellet 0.4%, PD 4% (Figure published in Antic et al., 2015).

Before finally the libraries were prepared, we did one more control experiment where we have tested our method and the importance of XRN1 knockdown. Our cells beside over-expressing GFP-RpL10Ab and BirA ligase were also expressing Firefly luciferase reporter who allowed us to measure its signal in PCR reaction (Figure 9). First, we have used control cells (without XRN1 knockdown) and cells with XRN1 knockdown (Method described in chapter 6.4.3). After isolation of total RNA from both type of cells, rRNA was removed and 5' RNA linker ligated. Next step, reverse transcription was done by using random hexamers. The last step was PCR or qPCR using two primers pairs, the first pair was binding 5' RNA linker and 5' end of Firefly luciferase thus amplifying only cDNAs with ligated 5' RNA linker (linker PCR) (lanes 1-7). The second pair of primers was amplifying firefly luciferase coding sequence (CDS PCR) and served as a loading control (lanes 8-14). As we can see from Figure 9 the 5' RNA linker ligated product can be detected only in XRN1 knockdown cells, while not in cells without XRN1 knockdown (lanes 2 and 5). At the same time for both type of cells the CDS firefly luciferase was detected (lanes 9 and 12). As expected, when T4 RNA ligase was omitted the signal was not detected in both cells (lanes 3 and 7), while the CDS firefly luciferase signal was still detected (lanes 10 and 14). As a positive control, before ligation, we have treated the mRNA with tobacco acid pyrophosphatase (TAP). 5' cap is the substrate for TAP, which cleaves pyrophosphate bond in the cap giving rise the 5'monophosphate RNA, exactly as it is happening during the decapping making the substrate for T4 RNA ligase. Thereby, we could detect signal for 5' RNA linker ligated product in both cells (lanes 1 and 4). And as a negative control, before T4 RNA ligation, we have treated mRNA with exoribonuclease (Terminator) which degrades 5' monophosphate RNA, decapped RNA, and thereby no signal detected for this PCR reaction (lane 6) even in XRN1 knockdown cells. This experiment has shown both the specificity of our method for decapped, 5' monophosphorylated RNAs and the importance of XRN1 knockdown (Antic et al., 2015).

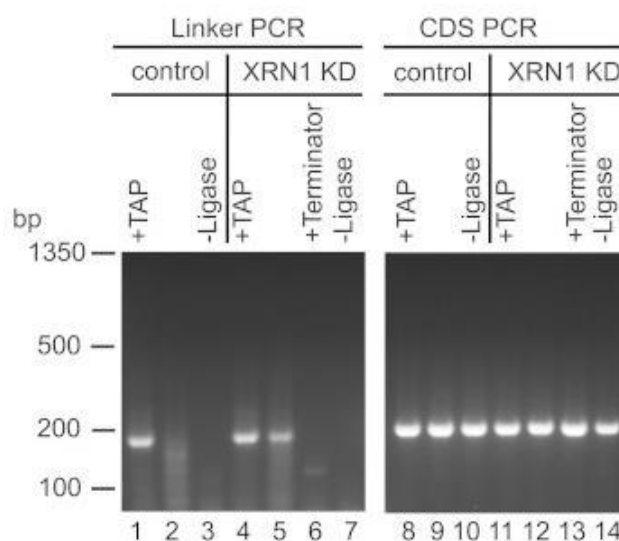


Figure 9 Ethidium bromide-stained agarose gel with RT-PCR fragments detecting firefly luciferase mRNA. On the left part of the figure we have identified linker-ligated firefly luciferase mRNA - Linker PCR (lanes 1 to 7) while on the right part we have detected total firefly luciferase mRNA - CDS PCR (lanes 8 to 14). Lines 1 to 3 and 8 to 10 are from control cells and lines 4 to 7 and lines 11 to 14 are from XRN1 knockdown cells. In all samples, total RNA was depleted from rRNA. In control reactions RNA afterward was treated with TAP (lanes 1, 4, 8 and 11), or with Terminator exoribonuclease (lanes 6 and 13). Whereas in lanes 3, 7, 10, and 14, RNA ligase was omitted in the RNA linker ligation (Figure published in Antic et al., 2015).

To prove that we had mRNA and comparable amount of cDNA in all samples we have performed qPCR for all libraries from Figure 9 using linker and CDS Firefly luciferase primers. As read from mean Ct values for CDS PCR the amount of mRNA Firefly luciferase was comparable and not detected signal in Linker PCR for - Ligase (lanes 3 and 7), + Terminator (lane 6) and the most importantly in control library in control cells (lane 2) was not due to lower amount of mRNA. Additionally, we have performed qPCR analysis also of the library of pulldown sample of XRN1 knockdown cells and as seen in Table 1 the Ct values for linker and CDS PCR products have demonstrated the similar amounts of mRNA in input and pulldown samples. Thus, in summary, we have confirmed that XRN1 knockdown is essential to stabilize the decapped intermediates, next to that RNA linker ligation is specific for 5' monophosphorylated RNAs and that we have a comparable amount of mRNA in input and pulldown (Antic et al., 2015).

		Linker PCR		CDS PCR	
		mean Ct	S.D.	mean Ct	S.D.
Control cells	+ TAP	20.78	0.01	16.86	0.04
	control library	n.d. *		16.56	0.03
	- Ligase	n.d. *		16.18	0.06
XRN1 KD cells	+ TAP	20.61	0.06	18.69	0.05
	control library	21.37	0.07	15.87	0.05
	+ Terminator	n.d. *		15.38	0.05
	- Ligase	n.d. *		16.77	0.03
	Pull-down library	20.84	0.06	14.95	0.04

Table 1 qPCR using linker or CDS specific PCR primer pairs for firefly luciferase. The table shows mean Ct values and standard deviations for one of the technical triplicates of one biological replicate. All libraries were prepared from RNAs of cell lysates except for the sample in the last row, which was made from RNA of a pulldown sample (Table published in Antic et al., 2015).

After setting up and testing linker ligation method, we have prepared libraries for high-throughput sequencing to check whether decapped mRNA degradation intermediates copurify with ribosomes and how bulk is this process in the whole transcriptome. For this purpose, total RNA was isolated from XRN1 knockdown cells expressing GFP-RpL10Ab and BirA ligase from cell lysate-input and from the streptavidin beads after ribosome pulldown (Method described in Chapter 6.4.1). $14.6\% \pm 1.3\%$ of RNA was isolated from the beads comparing with the total amount of RNA used in pulldown (mean $\pm$ standard deviation calculated for three biological replicates). To test specificity, we have isolated RNA from cells expressing GFP-RpL10Ab but not BirA ligase, and then only $0.9\% \pm 0.2\%$ of total RNA could have been isolated from the beads after pulldown, showing low background and reliable specificity. To exclude the possibility that XRN1 knockdown affects the efficiency or specificity of pulldown we have isolated RNA from cells treated with dsRNA against maltose binding protein (MBP). The amount of RNA isolated from beads after pulldown from cells expressing GFP-RpL10Ab and BirA ligase was similar as for XRN1 knockdown cells, $14.0\% \pm 1.9\%$, as well as for control cells without expressing BirA $0.9\% \pm 0.2\%$. Since efficiency and specificity of pulldown were good enough, we have prepared RNA libraries by isolating the RNA from the cell lysate and pulldown from cells expressing GFP-RpL10Ab and BirA ligase, treated with dsXRN1. The libraries were analyzed by high-throughput sequencing. The outcome is present in Table 2, a D1-decapped library of input and a D2-decapped library of pulldown (Antic et al., 2015).

description	ID	reads	fragments uniquely mappable fragments	
Input, decapped RNA library, Cx, XRN1 KD	D1_R1	44135632	88271264	42671521
Input, decapped RNA library, Cx, XRN1 KD	D1_R2	51493481	102986962	52018286
PD, decapped RNA library, Cx, XRN1 KD	D2_R1	44509336	89018672	41054296
PD, decapped RNA library, Cx, XRN1 KD	D2_R2	45242052	90484104	49338684
Input, expression library	E3_R1	46425731	92851462	32786090
Input, expression library	E3_R2	27788186	55576372	21770389
Input, expression library, Cx	E4_R1	45777608	91555216	27581734
Input, expression library, Cx	E4_R2	27534596	55069192	18228535
Input, expression library, Cx, XRN1 KD	E6_R1	38762083	77524166	28056810
Input, expression library, Cx, XRN1 KD	E6_R2	24586667	49173334	21905718
Input, expression library, Cx, XRN1 KD	E6_R3	28347126	56694252	21866679
Input, expression library, AGO1 KD	E7_R1	36946386	73892772	21512518
Input, expression library, AGO1 KD	E7_R2	26177650	52355300	16090914

Table 2 A number of reads per sample and mapping summary. Samples were multiplexed into three lanes (identified by different colors) and subjected to 100nt paired-end Illumina sequencing. The numbers in column 'reads' give the total number of demultiplexed mates for each sample. Column 'fragments' lists the numbers for corresponding fragments (i.e., both reads and mates). Cx = Cycloheximide added during purification, XRN1 KD = XRN1 knockdown cells, AGO1 KD = AGO1 knockdown cells (Table published in Antic et al., 2015).

When D1-input and D2-pulldown library were compared, most of all detected transcripts, about 12,000 (93%) had the same abundance of both input and pulldown. Only 680 (5%) of all transcripts had a lower abundance on the ribosome, and just 210 (2%) were enriched on the ribosome. All those transcripts are listed in Table S1. And the percentages of all three groups were presented as a pie chart at Figure 10.



Figure 10 Pie charts depicting the percentages of all detected transcript species with an abundance of decapped mRNA degradation fragments on ribosome complexes that was unchanged, lower, or higher compared to that in cell lysates in XRN1 knockdown cells (Figure published in Antic et al., 2015).

As it was expected the transcripts enriched on the ribosomes were protein-coding genes, whereas the transcripts with lower abundance on the ribosome were mostly non-coding genes, and only 40% were

protein-coding genes. Among lower abundant non-coding transcripts, we could identify RNase MRP RNA which is a product of polymerase III with nucleolar localization in *Drosophila* (Schneider et al., 2010). As a transcript of polymerase III with 5'phosphate it was clear that is a suitable substrate for RNA linker ligation, and since localized in nucleolar it was not a surprise to be less abundant on the ribosome. For our work that was very important since it has shown the specificity and reliability of our method (Antic et al., 2015).

To prove our RNA-seq data we have randomly picked up few transcripts that had an equal abundance of input and pulldown (FBgn0039857, FBgn0003279, FBgn0000556). In addition, we have chosen RNase MRP RNA (FBgn0046696) that was significantly reduced in pulldown (-5.09 log₂ - fold change) and performed qPCR analysis using primers for 5' RNA linker and 5' end of the transcripts. As a template, we have used cDNA from control libraries with XRN1 depletion used in Figure 9. Mean Ct values with a standard deviation of three biological replicates of both input and pulldown are presented in Table 3.

	Input		Pull-down	
	mean Ct	S.D.	mean Ct	S.D.
FBgn0039857	23.57	0.06	22.71	0.03
FBgn0003279	26.32	0.07	24.66	0.05
FBgn0000556	21.38	0.10	21.65	0.06
FBgn0046696	18.52	0.02	24.01	0.08

Table 3 Linker qPCR for endogenous genes, mean Ct values and standard deviations of a technical triplicate of a biological replicate (Table published in Antic et al., 2015).

As an internal control for normalization, we have chosen one of the transcripts, so we used Ct values of FBgn0039857 to get relative abundances - dCt values of transcripts in input and pulldown samples. To compare relative abundances of transcripts in input and pulldown was used ddCt method (Livak and Schmittgen, 2001) (Table 4). And when the relative abundances of input and pulldown were compared with obtained RNA-seq analysis for FBgn0003279, FBgn0000556 and RNase MRP RNA (FBgn0046696) they were similar. Relative abundance of input and pulldown of both FBgn0003279 and FBgn0000556 were similar while relative abundance of RNase MRP RNA (FBgn0046696) was decreased in pulldown as much as according to RNA-seq analysis. Therefore, we could recapitulate the results gained by RNA-seq method for 3 randomly picked transcripts (Antic et al., 2015).

		dCt Mean	dCt S.D.	ddCt	ddCt S.D.	log2 fold	S.D.
FBgn0039857	Input	0					
	Pulldown	0					
FBgn0003279	Input	2.62	0.16	0			
	Pulldown	1.90	0.09	-0.72	0.18	0.72	0.18
FBgn0000556	Input	-2.03	0.20	0			
	Pulldown	-1.20	0.18	0.83	0.27	-0.83	0.27
FBgn0046696, RNase MRP RNA	Input	-5.09	0.11	0			
	Pulldown	1.72	0.18	6.81	0.21	-6.81	0.21
Firefly luciferase	Input	-1.77	0.15	0			
	Pulldown	-1.62	0.14	0.14	0.20	-0.14	0.20

Table 4 Linker - qPCR analysis for various transcripts using the ddCt Method described by Livak and Schmittgen, 2001. For each library, dCt values were calculated relative to FBgn0039857 and used for the mean value of the biological replicates. The mean dCt values were used to compare input and pull-down libraries calculating the ddCt values. ddCt was further used to calculate the fold and the log2 fold change. The error was carried through to the final relative change using standard propagation of error methods (Table published in Antic et al., 2015).

To make sure we have detected a significant number of transcripts in our decapping libraries we have prepared expression library from total RNA of input (E6 library in Table 2) and we compared it with decapped library D1 (Method described in Chapter 6.4.2). Only 21 transcripts were not detected in D1 whereas were detected in E6 which means we have recognized most RNA transcripts in our decapped library D1.

Also, we have tested whether XRN1 knockdown or Cycloheximide treatment that we have used in our experiment for stabilization of RNA degradation intermediates had an impact on detected transcripts' levels. We have prepared and compared expression libraries with and without XRNA1 knockdown and compared them (libraries E6 vs. E4 Table 2). Only 77 transcripts were differently expressed in those two libraries (Table S2). While when we prepared and compared libraries with and without Cycloheximide (libraries E3 vs. E4 Table 2), we could detect no significant difference at transcripts' levels. Therefore, XRN1 knockdown nor Cycloheximide could not affect our decapping analysis (Antic et al., 2015).

Next, we were curious to see if among transcripts that we have detected in decapping libraries we could also have miRNA targets and are their abundances changed in cell lysate compared to ribosome pulldown. To answer this question first we had to identify potential miRNA targets. Thus, we prepared and compared expression libraries with and without AGO1 knockdown (E3 vs. E7, Table 2) (Method described in Chapter 6.4.2). The AGO1 knockdown efficiency was tested by Western blot analysis using antiAGO1 antibodies (Figure 11) (Method described in chapter 6.5.3.4).

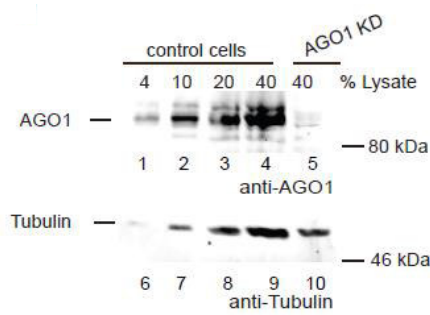


Figure 11. AGO1 knock down. The AGO1 knockdown efficiency was analyzed by Western blot analysis using anti-AGO1 antibody. 12 Mio of control cells and 4 Mio of cells treated with dsAGO1 were lysed and loaded on the SDS-PAGE gel with percentage as indicated (lanes 1-4, lanes 6-9 for control cells and lanes 5, 10 for cells treated with dsAGO1. Tubulin was used as a loading control (lanes 6-10) (Figure published in Antic et al., 2015).

We have identified 1115 transcripts that were upregulated in AGO1 knockdown cells (Table S3) (Antic et al., 2015). To verify potential miRNA targets we have compared them to the list of miRNA targets known and described by Hong et al., 2009 and 256 out of our 1115 were overlapping. Once we have identified potential miRNA targets, we wanted to see if they were detected in our decapping libraries D1 and D2. Interestingly 1110 out of 1115 could have been discovered in decapped libraries. Mostly, 89% had the same abundance in input cell lysate as in ribosome pulldown, while only 5% was higher and 6% was lower in the ribosome pulldown (Figure 12) (Antic et al., 2015).



Figure 12. Pie charts depicting the percentages of potential mRNA transcripts targets of miRNAs species with an abundance of decapped mRNA degradation fragments on ribosome complexes that was unchanged, lower, or higher compared to that in cell lysates in XRN1 knockdown cells (Figure published in Antic et al., 2015).

Thus, we could conclude that not only general mRNA degradation but also degradation of miRNA targets is taking place on the ribosomes complexes.

Table S1: Comparison of decapped mRNAs in input to ribosome PD samples (D1 vs. D2). The table is divided in two parts: Part I lists the 211 transcripts which are enriched on the ribosome (padj < 0.1, log2fold change > 0.58). Transcripts highlighted in blue are upregulated in AGO1 knockdown cells (libraries E7). Part II lists all 675 transcripts that were lower abundant on the ribosome than in input (padj

< 0.1, log2fold change < -0.58) (Table published in Antic et al., 2015).

Gene Name: Flybase ID	Base mean Input	Base mean Pull-down	log2FoldChange
Part I. Transcripts with higher abundance on the ribosome			
FBgn0053286	1	12	4,313
FBgn0036972	1	11	4,159
FBgn0261086	5	23	2,133
FBgn0035410	24	72	1,573
FBgn0034095	35	84	1,276
FBgn0030407	19	46	1,259
FBgn0050484	27	62	1,211
FBgn0025833	24	54	1,144
FBgn0030266	322	704	1,128
FBgn0039663	59	123	1,056
FBgn0051462	73	150	1,047
FBgn0003977	225	455	1,019
FBgn0031050	89	180	1,016
FBgn0030720	201	394	0,976
FBgn0026616	103	201	0,962
FBgn0039883	46	90	0,962
FBgn0030809	1020	1949	0,934
FBgn0023458	1206	2278	0,917
FBgn0031321	43	80	0,909
FBgn0250788	2369	4442	0,907
FBgn0034975	502	940	0,905
FBgn0261015+	3081	5765	0,904
FBgn0011722	11832	22036	0,897
FBgn0264089	1428	2613	0,872
FBgn0261922	1127	2056	0,867
FBgn0031107	549	997	0,861
FBgn0029167	495	898	0,860
FBgn0020616	524	949	0,857
FBgn0034976	238	430	0,853
FBgn0050069	727	1305	0,844
FBgn0037344	1961	3508	0,839
FBgn0036985	1786	3185	0,834
FBgn0085451	582	1037	0,833
FBgn0033246	2556	4553	0,833
FBgn0259736	64	113	0,832
FBgn0261563	434	771	0,830
FBgn0040234	147	258	0,819
FBgn0030486	362	638	0,816
FBgn0023530	112	196	0,811
FBgn0027571	11071	19349	0,805
FBgn0263968	670	1169	0,802
FBgn0029157	426	742	0,800
FBgn0004406	182	318	0,799
FBgn0031078	908	1577	0,797
FBgn0029688	1075	1863	0,793

FBgn0026441	300	517	0,786
FBgn0036448	1368	2356	0,784
FBgn0030674	2897	4984	0,783
FBgn0039508	442	757	0,774
FBgn0052311	449	764	0,767
FBgn0036668	994	1685	0,761
FBgn0052017	84	143	0,761
FBgn0032679	830	1404	0,757
FBgn0086697	317	535	0,756
FBgn0042131+	4788	8066	0,753
FBgn0033155	286	481	0,752
FBgn0250754	3414	5747	0,751
FBgn0259685	69	116	0,751
FBgn0085412	559	941	0,751
FBgn0262619	157	264	0,751
FBgn0038303	264	444	0,749
FBgn0061200	1217	2044	0,749
FBgn0011335	89	149	0,748
FBgn0036254	178	298	0,745
FBgn0031052	338	564	0,740
FBgn0016080+	580	968	0,739
FBgn0036386	93	155	0,738
FBgn0011230	8384	13966	0,736
FBgn0262167	391	651	0,736
FBgn0026679	184	305	0,733
FBgn0259240	1909	3172	0,732
FBgn0035237	288	479	0,732
FBgn0038578	164	273	0,731
FBgn0259234	210	348	0,729
FBgn0032208	3204	5306	0,728
FBgn0032499	139	229	0,726
FBgn0039633	768	1269	0,726
FBgn0034087	861	1423	0,724
FBgn0019886	771	1270	0,720
FBgn0003137	9539	15694	0,718
FBgn0053087	3599	5912	0,716
FBgn0016081	1993	3273	0,716
FBgn0030087	665	1089	0,713
FBgn0019968	749	1226	0,711
FBgn0032231	178	290	0,710
FBgn0030466	365	596	0,709
FBgn0010434	2816	4597	0,707
FBgn0005634	5013	8166	0,704
FBgn0040233+	662	1077	0,702
FBgn0000140	560	911	0,702
FBgn0016075	9946	16172	0,701
FBgn0000299	17803	28937	0,701
FBgn0023172	2211	3593	0,701

FBgn0261260	2415	3921	0,699
FBgn0039153	140	228	0,699
FBgn0250789+	6813	11038	0,696
FBgn0262127	1165	1885	0,694
FBgn0027783	371	600	0,694
FBgn0038827	173	278	0,688
FBgn0033698	612	986	0,688
FBgn0030434	98	157	0,687
FBgn0039644	753	1211	0,684
FBgn0025725	1371	2202	0,684
FBgn0259789	233	374	0,683
FBgn0002909+	341	546	0,680
FBgn0036004	1099	1761	0,680
FBgn0026181	706	1130	0,678
FBgn0260442	3460	5527	0,676
FBgn0002526	31516	50298	0,674
FBgn0263601	584	930	0,673
FBgn0086901	2469	3931	0,671
FBgn0028552	194	309	0,670
FBgn0036686	529	842	0,669
FBgn0039214	1510	2399	0,668
FBgn0065080+	1684	2674	0,667
FBgn0038473	378	599	0,666
FBgn0002466	1034	1640	0,665
FBgn0030274	185	293	0,664
FBgn0003268	1168	1850	0,664
FBgn0032723	346	547	0,662
FBgn0010051	985	1558	0,661
FBgn0053196	3149	4975	0,660
FBgn0001324	686	1083	0,658
FBgn0002528	7618	12022	0,658
FBgn0261710	1100	1735	0,658
FBgn0026876	208	328	0,657
FBgn0030662	295	465	0,657
FBgn0026702	97	153	0,656
FBgn0031327	513	807	0,655
FBgn0003464	599	942	0,655
FBgn0039016	472	744	0,654
FBgn0036480	243	382	0,653
FBgn0050183	135	213	0,652
FBgn0032904	203	319	0,652
FBgn0003189	510	801	0,650
FBgn0039419	158	248	0,649
FBgn0030687	247	388	0,649
FBgn0000578	2665	4175	0,648
FBgn0261934	1018	1594	0,648
FBgn0016641	407	636	0,642
FBgn0005596	293	456	0,640

FBgn0001341	256	398	0,637
FBgn0004924	1337	2079	0,637
FBgn0010909	5334	8294	0,637
FBgn0004449	15999	24872	0,637
FBgn0013984	2568	3991	0,636
FBgn0015589	1506	2339	0,635
FBgn0260397	230	356	0,635
FBgn0004647	854	1324	0,633
FBgn0260935	199	309	0,632
FBgn0030121	144	223	0,632
FBgn0087008	1074	1662	0,631
FBgn0010235	798	1235	0,630
FBgn0263392	6730	10413	0,630
FBgn0262160	2535	3919	0,628
FBgn0003048	1437	2218	0,626
FBgn0030501	302	466	0,625
FBgn0015402	267	411	0,624
FBgn0021818	776	1196	0,623
FBgn0037249	2840	4374	0,623
FBgn0263599	1273	1961	0,623
FBgn0038889	251	387	0,623
FBgn0083959	910	1399	0,621
FBgn0025802	740	1137	0,621
FBgn0038108	2036	3131	0,621
FBgn0000052	482	741	0,620
FBgn0261397	808	1241	0,620
FBgn0037580	724	1111	0,618
FBgn0052011	166	255	0,618
FBgn0261283	4211	6461	0,618
FBgn0036398	3485	5343	0,617
FBgn0031759	1642	2516	0,616
FBgn0034503	130	200	0,613
FBgn0260789	321	490	0,612
FBgn0039466	543	828	0,610
FBgn0085229	461	703	0,609
FBgn0053208	1225	1869	0,609
FBgn0026430	299	456	0,608
FBgn0085365	238	363	0,608
FBgn0000257	268	408	0,607
FBgn0003862	1370	2086	0,607
FBgn0261823	787	1196	0,604
FBgn0025864	1124	1707	0,604
FBgn0003520	859	1303	0,601
FBgn0035411+	726	1099	0,599
FBgn0035617	691	1046	0,599
FBgn0013765	1441	2182	0,599
FBgn0027508	308	466	0,599
FBgn0030403	250	378	0,598

FBgn0261553	958	1448	0,597
FBgn0034987	793	1197	0,595
FBgn0035420	1527	2304	0,594
FBgn0000414	188	283	0,594
FBgn0010355	1103	1664	0,594
FBgn0026751	229	346	0,593
FBgn0004367	244	368	0,593
FBgn0036514	161	243	0,592
FBgn0027794	142	214	0,592
FBgn0015615	690	1040	0,591
FBgn0002431	3001	4518	0,590
FBgn0050085	790	1190	0,590
FBgn0033454	204	306	0,590
FBgn0001965	520	782	0,588
FBgn0011785	865	1299	0,588
FBgn0025724	3183	4782	0, 587
FBgn0037098	807	1212	0, 587
FBgn0005386	496	745	0,586
FBgn0035713	1524	2288	0,586
FBgn0003308	222	333	0,585
FBgn0039727	743	1114	0,585
FBgn0038415	0	9	Inf
Gene Name: Flybase ID	Base mean Input	Base mean Pull-down	log2FoldChange
Part	II. Transcripts	with lower abundance	on the ribosome
FBgn0011956	3831	12	-8,305
FBgn0065100	66862	365	-7,515
FBgn0083044	34985	227	-7,270
FBgn0011955	2945	21	-7,108
FBgn0263468	18663	173	-6,752
FBgn0011877	2491	31	-6,349
FBgn0011962	75	1	-6,261
FBgn0011958	269	4	-6,164
FBgn0083020	5900	84	-6,141
FBgn0082987	10571	157	-6,070
FBgn0012004	27	0	-6,035
FBgn0046696	91419	1459	-5,969
FBgn0263488	409002	7154	-5,837
FBgn0011957	20	0	-5,603
FBgn0052785	19	0	-5,504
FBgn0083014	12205	285	-5,420
FBgn0086668	47034	1119	-5,393
FBgn0083023	11146	274	-5,345
FBgn0011906	21	1	-5,226
FBgn0065058	774781	20697	-5,226
FBgn0051471	15	0	-5,185
FBgn0011921	45	1	-5,171
FBgn0065098	194192	5702	-5,090

FBgn0012000	38	1	-5,065
FBgn0011980	19	1	-5,042
FBgn0011993	13	0	-5,003
FBgn0011849	401	13	-4,982
FBgn0051494	43	1	-4,951
FBgn0083041	62995	2044	-4,946
FBgn0039109	194941	6439	-4,920
FBgn0050238	134	5	-4,781
FBgn0011963	22	1	-4,707
FBgn0035630	2108104	81860	-4,687
FBgn0083009	11641	455	-4,677
FBgn0052481	208	8	-4,646
FBgn0053535	69	3	-4,640
FBgn0052359	38	2	-4,638
FBgn0040286	31974	1289	-4,632
FBgn0011956	3831	12	-8,305
FBgn0065100	66862	365	-7,515
FBgn0083044	34985	227	-7,270
FBgn0011955	2945	21	-7,108
FBgn0263468	18663	173	-6,752
FBgn0011877	2491	31	-6,349
FBgn0011962	75	1	-6,261
FBgn0011958	269	4	-6,164
FBgn0083020	5900	84	-6,141
FBgn0082987	10571	157	-6,070
FBgn0012004	27	0	-6,035
FBgn0046696	91419	1459	-5,969
FBgn0263488	409002	7154	-5,837
FBgn0011957	20	0	-5,603
FBgn0052785	19	0	-5,504
FBgn0083014	12205	285	-5,420
FBgn0086668	47034	1119	-5,393
FBgn0083023	11146	274	-5,345
FBgn0011906	21	1	-5,226
FBgn0065058	774781	20697	-5,226
FBgn0051471	15	0	-5,185
FBgn0011921	45	1	-5,171
FBgn0065098	194192	5702	-5,090
FBgn0012000	38	1	-5,065
FBgn0011980	19	1	-5,042
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FBgn0011849	401	13	-4,982
FBgn0051494	43	1	-4,951
FBgn0083041	62995	2044	-4,946
FBgn0039109	194941	6439	-4,920
FBgn0050238	134	5	-4,781
FBgn0011963	22	1	-4,707
FBgn0035630	2108104	81860	-4,687

FBgn0083009	11641	455	-4,677
FBgn0052481	208	8	-4,646
FBgn0053535	69	3	-4,640
FBgn0052359	38	2	-4,638
FBgn0040286	31974	1289	-4,632
FBgn0083026	3619	152	-4,576
FBgn0052200	302	13	-4,542
FBgn0025118	93	4	-4,526
FBgn0050316	263	12	-4,508
FBgn0263544+	94355	4149	-4,507
FBgn0083042	6145	279	-4,459
FBgn0052480	224	10	-4,443
FBgn0086672	58354	2688	-4,440
FBgn0052520	12	1	-4,434
FBgn0011984	54	3	-4,422
FBgn0263474	1534	73	-4,387
FBgn0050223	102	5	-4,360
FBgn0012002	67	3	-4,333
FBgn0086658	21140	1114	-4,246
FBgn0083031	10977	585	-4,230
FBgn0012001	39	2	-4,216
FBgn0052328	44	2	-4,212
FBgn0051939	21	1	-4,206
FBgn0263469	12851	697	-4,205
FBgn0082971	62416	3416	-4,191
FBgn0052324	15	1	-4,185
FBgn0050198	37	2	-4,176
FBgn0083045	17110	962	-4,152
FBgn0011850	180	10	-4,121
FBgn0086666	47802	2753	-4,118
FBgn0011848	309	18	-4,111
FBgn0011961	170	10	-4,100
FBgn0011959	83	5	-4,094
FBgn0083054	2714	159	-4,089
FBgn0028889	19	1	-4,088
FBgn0086602	124350	7378	-4,075
FBgn0025882	2055	123	-4,062
FBgn0050240	246	15	-4,016
FBgn0011851	132	8	-4,011
FBgn0051282	38	2	-3,996
FBgn0082925	9596	602	-3,995
FBgn0011999	51	3	-3,988
FBgn0029994	116	7	-3,945
FBgn0263477	196735	12850	-3,936
FBgn0082967	10315	688	-3,906
FBgn0086601	812	54	-3,901
FBgn0082981	15235	1027	-3,891
FBgn0083046	114833	7756	-3,888

FBgn0083040	10683	724	-3,884
FBgn0052456	49	3	-3,876
FBgn0012003	57	4	-3,873
FBgn0011944	88	6	-3,867
FBgn0016792	197344	13609	-3,858
FBgn0052791+	447448	30873	-3,857
FBgn0065099	61925	4280	-3,855
FBgn0082941	56	4	-3,847
FBgn0085374	74	5	-3,844
FBgn0050201	558	39	-3,842
FBgn0263463	790	55	-3,834
FBgn0082968	460	32	-3,834
FBgn0085364	357371	25104	-3,831
FBgn0051383	16	1	-3,831
FBgn0011864	73	5	-3,824
FBgn0263487	170343	12115	-3,814
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FBgn0004185	5412	392	-3,786
FBgn0083003	30819	2278	-3,758
FBgn0029897	189138	14025	-3,753
FBgn0052546	137	10	-3,736
FBgn0083013	12514	950	-3,719
FBgn0011994	48	4	-3,706
FBgn0051248	45	3	-3,705
FBgn0050229	75	6	-3,701
FBgn0051578	41	3	-3,685
FBgn0263460	23585	1841	-3,679
FBgn0263478	128	10	-3,668
FBgn0086079	387	31	-3,656
FBgn0052449	24	2	-3,642
FBgn0011981	14	1	-3,632
FBgn0086062	202	16	-3,629
FBgn0058469	12209	992	-3,621
FBgn0052173	530	43	-3,617
FBgn0050218	154	13	-3,603
FBgn0041717	2764	228	-3,602
FBgn0050250	141	12	-3,600
FBgn0031934+	19	2	-3,593
FBgn0050452	164	14	-3,588
FBgn0082973	577047	48161	-3,583
FBgn0060291	3706	314	-3,561
FBgn0011928	46	4	-3,560
FBgn0052518	187	16	-3,552
FBgn0083029	6971	597	-3,545
FBgn0028911	52	4	-3,542
FBgn0082945	7958	686	-3,536
FBgn0051337	130	11	-3,535
FBgn0065064	257893	22328	-3,530

FBgn0051577	19	2	-3,527
FBgn0052330	251	22	-3,524
FBgn0050299	35	3	-3,522
FBgn0065066	10628	935	-3,507
FBgn0082966	21017	1850	-3,506
FBgn0050251	260	23	-3,505
FBgn0003295	329	29	-3,491
FBgn0050202	22	2	-3,484
FBgn0082980	5288	478	-3,467
FBgn0052460	159	15	-3,452
FBgn0011871	237	22	-3,450
FBgn0052362	35	3	-3,440
FBgn0082972	26530	2476	-3,421
FBgn0051279	42	4	-3,399
FBgn0000003	93002	8835	-3,396
FBgn0261504	162799	15532	-3,390
FBgn0004189	270	26	-3,388
FBgn0011865	117	11	-3,384
FBgn0263459	3324	319	-3,381
FBgn0052358	128	12	-3,380
FBgn0050241	107	10	-3,377
FBgn0086662	12523	1206	-3,376
FBgn0086063	186	18	-3,367
FBgn0086661	215049	20942	-3,360
FBgn0028912	33	3	-3,358
FBgn0082957	170032	16825	-3,337
FBgn0050208	25	3	-3,328
FBgn0050200	11	1	-3,325
FBgn0036824	215727	21559	-3,323
FBgn0051191	27195	2730	-3,316
FBgn0033344	113	11	-3,311
FBgn0011862	99	10	-3,310
FBgn0082927	162	16	-3,301
FBgn0051023	18	2	-3,295
FBgn0063386	460	48	-3,272
FBgn0053504	96	10	-3,271
FBgn0051940	17	2	-3,271
FBgn0011870	104	11	-3,261
FBgn0051971	497	52	-3,259
FBgn0011894	528	55	-3,253
FBgn0019660	2711	285	-3,250
FBgn0034484	108	11	-3,239
FBgn0050249	198	21	-3,239
FBgn0083030	2483	264	-3,233
FBgn0086066+	965065	103259	-3,224
FBgn0263466	1909	204	-3,224
FBgn0082944	2941	318	-3,209
FBgn0263472	1004	111	-3,178

FBgn0028981	26	3	-3,151
FBgn0011936	15	2	-3,150
FBgn0051215	17	2	-3,142
FBgn0065070	21549	2451	-3,136
FBgn0052320+	850	98	-3,111
FBgn0011998	24	3	-3,098
FBgn0083004	5006	586	-3,096
FBgn0004183	7728	922	-3,068
FBgn0011868	253	30	-3,066
FBgn0012026	223	27	-3,064
FBgn0050449	244	29	-3,047
FBgn0083053	1432	173	-3,047
FBgn0086027	1986	241	-3,045
FBgn0050406	89	11	-3,038
FBgn0082994	263911	32126	-3,038
FBgn0052287	96	12	-3,037
FBgn0035254	3047	372	-3,035
FBgn0086664	8718	1065	-3,033
FBgn0011867	154	19	-3,010
FBgn0083028	33493	4160	-3,009
FBgn0028910	51	6	-2,988
FBgn0011859	109	14	-2,975
FBgn0032859	70645	9067	-2,962
FBgn0011872	286	37	-2,957
FBgn0052303	77	10	-2,957
FBgn0263465	7511	976	-2,944
FBgn0051518	192	25	-2,941
FBgn0011855	79	10	-2,939
FBgn0083038	3113	412	-2,917
FBgn0011869	155	21	-2,915
FBgn0086051	363	48	-2,908
FBgn0012024	26	3	-2,905
FBgn0011985	18	2	-2,890
FBgn0011878	159	22	-2,887
FBgn0052420	27	4	-2,884
FBgn0083010	3580	487	-2,878
FBgn0086024	12070	1649	-2,872
FBgn0083024	59	8	-2,870
FBgn0052127	303	42	-2,867
FBgn0011861	490	68	-2,851
FBgn0083025	2718	378	-2,844
FBgn0003936	27	4	-2,841
FBgn0263839	2102	296	-2,830
FBgn0082960	2733	391	-2,804
FBgn0050235	19	3	-2,800
FBgn0051491	325	47	-2,782
FBgn0263462	6392	935	-2,774
FBgn0011910	1403	206	-2,769

FBgn0086028	711	105	-2,757
FBgn0051242	46	7	-2,754
FBgn0033926	17742	2655	-2,740
FBgn0050454	140	21	-2,728
FBgn0050239	255	38	-2,727
FBgn0083036	15752	2380	-2,726
FBgn0083007	161	25	-2,715
FBgn0003916	72291	11068	-2,707
FBgn0082958	1744	268	-2,700
FBgn0082962	4690	731	-2,683
FBgn0004190	1058	165	-2,681
FBgn0052288	182	29	-2,668
FBgn0050220	199	32	-2,660
FBgn0264000	39	6	-2,629
FBgn0039600	23463	3809	-2,623
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FBgn0086048	3509	582	-2,592
FBgn0001215	251923	42006	-2,584
FBgn0011860	192	32	-2,573
FBgn0082999	2508	423	-2,567
FBgn0086075	492	84	-2,545
FBgn0051580	225	39	-2,544
FBgn0052421	19	3	-2,542
FBgn0082961	843	145	-2,542
FBgn0011863	389	67	-2,538
FBgn0051228	80	14	-2,531
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FBgn0082928	480	84	-2,514
FBgn0011897	3666	645	-2,507
FBgn0083987	22500	3963	-2,505
FBgn0026592	22531	3979	-2,502
FBgn0053539	17	3	-2,497
FBgn0050209	48	8	-2,496
FBgn0086056+	300855	53526	-2,491
FBgn0263470	59875	10719	-2,482
FBgn0052826	28	5	-2,481
FBgn0082959	33033	5925	-2,479
FBgn0082946	1280	232	-2,463
FBgn0011977	20	4	-2,461
FBgn0082952	701	129	-2,442
FBgn0083005	3225	595	-2,437
FBgn0086600	97250	18013	-2,433
FBgn0052286	62	12	-2,431
FBgn0033636	23735	4421	-2,425
FBgn0003935	589	111	-2,415
FBgn0035121	75099	14100	-2,413
FBgn0082976	24807	4777	-2,376
FBgn0263018	590	114	-2,374

FBgn0263475	1083	210	-2,364
FBgn0011987	46	9	-2,350
FBgn0085322	28	6	-2,346
FBgn0083008	196	39	-2,344
FBgn0083055	15250	3017	-2,338
FBgn0053276	120	24	-2,334
FBgn0086061	8568	1711	-2,324
FBgn0263482	1045	210	-2,319
FBgn0082995	7377	1482	-2,315
FBgn0011909	312	63	-2,306
FBgn0086078	559	113	-2,306
FBgn0051333	123	25	-2,303
FBgn0083001	189461	38666	-2,293
FBgn0036108	21	4	-2,290
FBgn0034743	61094	12560	-2,282
FBgn0020518	1009	211	-2,256
FBgn0015129	67	14	-2,252
FBgn0003931	18316	3849	-2,251
FBgn0083021	270	57	-2,235
FBgn0011888	2176	463	-2,232
FBgn0259162	34	7	-2,221
FBgn0004188	321	69	-2,220
FBgn0051143	420	91	-2,213
FBgn0083037	4223	913	-2,210
FBgn0011844	28	6	-2,207
FBgn0038828	20	4	-2,206
FBgn0083015	38098	8318	-2,195
FBgn0263461	20460	4484	-2,190
FBgn0086039	335	74	-2,173
FBgn0053517	30	7	-2,160
FBgn0082929	338	76	-2,155
FBgn0083011	6572	1479	-2,152
FBgn0011935	37	8	-2,149
FBgn0083000	7310	1652	-2,146
FBgn0050254	24	6	-2,144
FBgn0051579	23	5	-2,140
FBgn0264006	291	66	-2,138
FBgn0050407	20	4	-2,134
FBgn0083988	2581	589	-2,131
FBgn0003499	283	65	-2,127
FBgn0263481	10067	2305	-2,127
FBgn0050506	46	11	-2,126
FBgn0011952	31	7	-2,120
FBgn0083033	694	161	-2,109
FBgn0041721	2102	488	-2,107
FBgn0263851	81	19	-2,089
FBgn0015543	475	112	-2,079
FBgn0263467	137	32	-2,075

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FBgn0011866	26	6	-2,071
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FBgn0082950	91	22	-2,062
FBgn0033065	29	7	-2,054
FBgn0082988	8518	2058	-2,049
FBgn0004186	555	136	-2,029
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FBgn0051485	72	18	-2,028
FBgn0086069	731	180	-2,025
FBgn0263476	28369	6998	-2,019
FBgn0262300	65	16	-2,018
FBgn0033867	26	6	-2,018
FBgn0063391	842	213	-1,982
FBgn0083048	1217	309	-1,977
FBgn0002590	381288	98349	-1,955
FBgn0052482	20	5	-1,953
FBgn0011284	41849	10984	-1,930
FBgn0052289	101	27	-1,918
FBgn0035260	237	63	-1,918
FBgn0263471	59449	15771	-1,914
FBgn0083016	24511	6508	-1,913
FBgn0011845	156	42	-1,910
FBgn0051583	53	14	-1,902
FBgn0003651	27	7	-1,900
FBgn0263483	747	201	-1,897
FBgn0086049	2056	553	-1,894
FBgn0063379	315	86	-1,882
FBgn0037616	29	8	-1,872
FBgn0082978	6577	1806	-1,864
FBgn0082998	959	264	-1,863
FBgn0004187	475	132	-1,852
FBgn0083035	60	17	-1,849
FBgn0002733	214	59	-1,848
FBgn0083006	4957	1379	-1,846
FBgn0051568	71	20	-1,841
FBgn0086025	3946	1109	-1,831
FBgn0085384	1056	297	-1,829
FBgn0003938	336	95	-1,828
FBgn0028577	60921	17216	-1,823
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FBgn0030396	28	8	-1,814
FBgn0039911	37	11	-1,794
FBgn0011934	37	11	-1,782
FBgn0011904	26	8	-1,781
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FBgn0063383	696	204	-1,771
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FBgn0082926	495	150	-1,723
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FBgn0030122	1280	398	-1,685
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FBgn0250753	293334	91996	-1,673
FBgn0082969	592	187	-1,660
FBgn0263484	77494	24630	-1,654
FBgn0052479	53097	16882	-1,653
FBgn0083057	8646	2758	-1,649
FBgn0005695	1055	337	-1,648
FBgn0043022	47	15	-1,638
FBgn0037339	78046	25126	-1,635
FBgn0065076	747	241	-1,632
FBgn0029907	35	11	-1,624
FBgn0082937	519	169	-1,620
FBgn0051480	61	20	-1,612
FBgn0011896	560	185	-1,602
FBgn0033927	331	110	-1,597
FBgn0000158	55	18	-1,589
FBgn0011890	520	176	-1,560
FBgn0082979	4882	1660	-1,557
FBgn0003934	293	100	-1,554
FBgn0026533	8132	2773	-1,552
FBgn0083124	451993	155368	-1,541
FBgn0065073	33465	11508	-1,540
FBgn0086663	3073	1060	-1,535
FBgn0051604	158	55	-1,519
FBgn0063449	14021	4896	-1,518
FBgn0020248	33436	11685	-1,517
FBgn0003062	33742	11810	-1,514
FBgn0004192	1313	461	-1,509
FBgn0031628	185	66	-1,491
FBgn0026169	155748	56051	-1,474
FBgn0082989	102	37	-1,473
FBgn0082934+	166378	60452	-1,461
FBgn0086670	31280	11371	-1,460
FBgn0082931	1573	573	-1,457
FBgn0261619	123795	45386	-1,448
FBgn0019936	30765	11317	-1,443
FBgn0001565	2927	1088	-1,428
FBgn0052440	33	12	-1,419
FBgn0036728	307	115	-1,414
FBgn0262993	44	17	-1,401

FBgn0043853	32519	12368	-1,395
FBgn0082942	214	81	-1,391
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FBgn0086710	18485	7081	-1,384
FBgn0037504	32790	12582	-1,382
FBgn0035398	2071	801	-1,371
FBgn0033268	65	25	-1,361
FBgn0086054	42	16	-1,348
FBgn0031384	141	56	-1,340
FBgn0263485	78617	31360	-1,326
FBgn0086603	371654	148895	-1,320
FBgn0033375	372704	149342	-1,319
FBgn0037713	222	89	-1,316
FBgn0038845	99	40	-1,309
FBgn0041579	327	133	-1,302
FBgn0038973	141	57	-1,299
FBgn0262447+	98	40	-1,289
FBgn0086065	391	160	-1,289
FBgn0035777	100	41	-1,285
FBgn0034091	1196	493	-1,279
FBgn0030653	44	18	-1,273
FBgn0051603	77	32	-1,252
FBgn0037110	44	19	-1,234
FBgn0040899	110	47	-1,227
FBgn0263464	166	71	-1,222
FBgn0050339	1236	530	-1,220
FBgn0030794	92	40	-1,218
FBgn0033688	81071	34935	-1,215
FBgn0004842	67	29	-1,214
FBgn0041720	12733	5511	-1,208
FBgn0039713	37842	16440	-1,203
FBgn0083940	365	160	-1,191
FBgn0003930	44237	19413	-1,188
FBgn0025881	3894	1716	-1,183
FBgn0086665+	34903	15408	-1,180
FBgn0032935	44647	19822	-1,171
FBgn0051032	143	64	-1,171
FBgn0037064	99	44	-1,168
FBgn0083047	8604	3837	-1,165
FBgn0036019	402	179	-1,165
FBgn0034012	227	102	-1,160
FBgn0259683	7553	3380	-1,160
FBgn0065055	115080	52068	-1,144
FBgn0050269	52	24	-1,143
FBgn0086042+	260	118	-1,142
FBgn0000242	301	137	-1,142
FBgn0086660	3448	1575	-1,131
FBgn0262881	102	47	-1,126

FBgn0260960	3100	1425	-1,121
FBgn0039359	9839	4544	-1,115
FBgn0082939+	10818	4997	-1,114
FBgn0051126	56	26	-1,112
FBgn0028961	149	69	-1,107
FBgn0086659	726816	337804	-1,105
FBgn0034789	182	85	-1,095
FBgn0040890	119	56	-1,094
FBgn0031980	121471	57124	-1,088
FBgn0066292+	1483	702	-1,080
FBgn0052406	75	36	-1,073
FBgn0034564	95	45	-1,072
FBgn0039481	994	476	-1,064
FBgn0052698	121	58	-1,058
FBgn0004575	662	319	-1,056
FBgn0033813	199	96	-1,046
FBgn0083050	3622	1754	-1,046
FBgn0015298	226	110	-1,045
FBgn0083017	876	426	-1,040
FBgn0085459	201	98	-1,037
FBgn0051051	522	255	-1,034
FBgn0086071+	10145	4953	-1,034
FBgn0003922	3766	1841	-1,032
FBgn0013325	25144	12344	-1,026
FBgn0033782+	221	109	-1,026
FBgn0052499	400	197	-1,024
FBgn0262947	74	37	-1,020
FBgn0261836+	45408	22431	-1,017
FBgn0261792	142	71	-1,013
FBgn0083012	51730	25793	-1,004
FBgn0065048	9921	4959	-1,000
FBgn0260645	90	45	-1,000
FBgn0003923	3470	1738	-0,998
FBgn0025634	274	137	-0,998
FBgn0262593	173	87	-0,990
FBgn0038980	146	74	-0,976
FBgn0000239	2401	1223	-0,973
FBgn0017579	16460	8384	-0,973
FBgn0261599	9668	4929	-0,972
FBgn0035020	239	122	-0,967
FBgn0039184+	3619	1854	-0,965
FBgn0003435	190	97	-0,964
FBgn0035404	318	163	-0,963
FBgn0033134	97	50	-0,962
FBgn0035693	151	78	-0,959
FBgn0038846	148	77	-0,952
FBgn0051388	96	50	-0,951
FBgn0031397	84	43	-0,945

FBgn0065047	17300	8989	-0,945
FBgn0262887	921	479	-0,944
FBgn0036491	119	62	-0,943
FBgn0259705+	925	481	-0,942
FBgn0031529	118	61	-0,941
FBgn0031268	565	294	-0,941
FBgn0030954	123	64	-0,940
FBgn0039500	86	45	-0,927
FBgn0050423	405	214	-0,925
FBgn0028377+	100	53	-0,915
FBgn0034398	6393	3401	-0,911
FBgn0037222+	1190	633	-0,909
FBgn0033128	386	206	-0,907
FBgn0031393	111	59	-0,907
FBgn0000094	99	53	-0,903
FBgn0033104	121	65	-0,892
FBgn0034639	86	46	-0,889
FBgn0003920	3597	1961	-0,875
FBgn0030571	279	152	-0,872
FBgn0038074	86	47	-0,871
FBgn0261454	38980	21340	-0,869
FBgn0041337	482	264	-0,868
FBgn0261244	1166	643	-0,858
FBgn0035035	209	116	-0,850
FBgn0036741	39610	22036	-0,846
FBgn0082938+	1863	1037	-0,845
FBgn0038858	100	56	-0,843
FBgn0028506	358	200	-0,843
FBgn0051777	564	315	-0,842
FBgn0010894	281	157	-0,838
FBgn0033905	217	122	-0,836
FBgn0000546	6135	3441	-0,834
FBgn0005630	23100	12970	-0,833
FBgn0264303+	475	267	-0,832
FBgn0026402	298	167	-0,831
FBgn0034519+	144	81	-0,828
FBgn0032381	269	152	-0,827
FBgn0032002+	319	180	-0,825
FBgn0033240	440	248	-0,823
FBgn0263768	342	194	-0,819
FBgn0029854	356	202	-0,817
FBgn0029526	371	211	-0,815
FBgn0261606	26082	14855	-0,812
FBgn0260451+	224	127	-0,811
FBgn0033032	252	145	-0,800
FBgn0015872	420	242	-0,796
FBgn0035975	1240	717	-0,791
FBgn0053170	612	353	-0,791

FBgn0010409	12224	7067	-0,791
FBgn0030025	150	87	-0,789
FBgn0034800	352	204	-0,789
FBgn0261453	27844	16142	-0,787
FBgn0050108+	638	370	-0,786
FBgn0038425	177	103	-0,782
FBgn0013812	463	269	-0,782
FBgn0019925	1265	736	-0,782
FBgn0030641+	1335	782	-0,772
FBgn0034267+	199	117	-0,770
FBgn0037680	411	241	-0,769
FBgn0032247	157	92	-0,764
FBgn0032408	164	96	-0,763
FBgn0028955	170	100	-0,760
FBgn0034802	3996	2360	-0,760
FBgn0050290+	235	139	-0,759
FBgn0030657	265	157	-0,755
FBgn0031437	206	122	-0,755
FBgn0002542	28417	16844	-0,754
FBgn0016726	10468	6211	-0,753
FBgn0038224	417	248	-0,752
FBgn0036101	604	359	-0,750
FBgn0259728	173	103	-0,746
FBgn0035032	307	184	-0,745
FBgn0030642	459	274	-0,741
FBgn0038421+	460	275	-0,740
FBgn0052600	163	97	-0,740
FBgn0038221	272	163	-0,739
FBgn0040985	676	405	-0,737
FBgn0061515	948	569	-0,735
FBgn0260770+	182	109	-0,735
FBgn0039682	315	190	-0,731
FBgn0036294	156	94	-0,730
FBgn0040696+	153	92	-0,729
FBgn0033570	210	127	-0,727
FBgn0039930	154	93	-0,727
FBgn0016120	653	395	-0,725
FBgn0003941	4547	2765	-0,718
FBgn0028342	215	131	-0,718
FBgn0053002	152	93	-0,716
FBgn0033083	401	245	-0,715
FBgn0053523	1258	768	-0,712
FBgn0035475	212	129	-0,712
FBgn0003138	3008	1837	-0,711
FBgn0262018	388	238	-0,706
FBgn0261373	199	122	-0,702
FBgn0262512	158	98	-0,700
FBgn0261608	7685	4732	-0,700

FBgn0035854	274	169	-0,698
FBgn0027359	143	88	-0,697
FBgn0016119	415	256	-0,696
FBgn0040007	22493	13913	-0,693
FBgn0010265	7123	4411	-0,691
FBgn0004855	155	97	-0,680
FBgn0028663	1073	672	-0,676
FBgn0014342	166	104	-0,675
FBgn0051687	181	114	-0,673
FBgn0053774	329	206	-0,671
FBgn0037747	441	277	-0,671
FBgn0260965	1110	697	-0,671
FBgn0030026	174	109	-0,669
FBgn0015393	352	222	-0,668
FBgn0011640	1099	691	-0,668
FBgn0033261	167	105	-0,667
FBgn0032986	1148	724	-0,664
FBgn0031777	200	126	-0,661
FBgn0037309	372	236	-0,661
FBgn0036848	1335	845	-0,660
FBgn0034921	165	105	-0,658
FBgn0030350	1035	657	-0,656
FBgn0033434+	2444	1553	-0,654
FBgn0035102	345	220	-0,652
FBgn0034763	277	177	-0,651
FBgn0027936	299	191	-0,651
FBgn0002626	7386	4727	-0,644
FBgn0064225	45607	29205	-0,643
FBgn0003741	384	247	-0,635
FBgn0031774	239	154	-0,635
FBgn0032215	459	296	-0,633
FBgn0015229	362	233	-0,631
FBgn0030352	171	111	-0,627
FBgn0031049	806	522	-0,626
FBgn0033716	339	220	-0,626
FBgn0000286	434	281	-0,625
FBgn0039501	150	98	-0,622
FBgn0011272	43045	28080	-0,616
FBgn0001281+	310	203	-0,613
FBgn0031302	204	133	-0,612
FBgn0038149	607	397	-0,611
FBgn0033081	376	247	-0,609
FBgn0038533	486	319	-0,609
FBgn0262362	999	656	-0,608
FBgn0034876	314	207	-0,606
FBgn0030679	565	371	-0,606
FBgn0036096	210	138	-0,605
FBgn0035517	209	137	-0,604

FBgn0037409	5311	3504	-0,600
FBgn0001168	4214	2781	-0,600
FBgn0037723	612	405	-0,597
FBgn0262453+	218	144	-0,595
FBgn0052452	428	284	-0,594
FBgn0036516	257	171	-0,592
FBgn0034253	300775	199918	-0,589
FBgn0082922	300026	199534	-0,588
FBgn0030040	205	136	-0,588
FBgn0039802	520	346	-0,587
FBgn0037822+	495	330	-0,584
FBgn0011945	23	0	-Inf
FBgn0051130	19	0	-Inf
FBgn0011915	16	0	-Inf
FBgn0050298	22	0	-Inf
FBgn0050509	11	0	-Inf
FBgn0052360	18	0	-Inf
FBgn0051885	8	0	-Inf

Table S2: Transcript levels in control cells compare to XRN1 knockdown cells (E4 vs. E6). The table is divided in two parts: Part I lists all 53 transcripts upregulated in XRN1 knockdown cells ($\text{padj} < 0.1$, $\log_2\text{fold change} > 0.58$). Part II lists all 24 transcripts with lower expression in XRN1 knockdown cells ($\text{padj} < 0.1$, $\log_2\text{fold change} < -0.58$)(Table published in Antic et al., 2015).

Gene Name: Flybase ID	Base mean Control cells	Base mean XRN1 KD cells	$\log_2\text{Fold Change}$
Part I. Transcripts upregulated in XRN1 knockdown cells			
FBgn0052702	632	4170	2,723
FBgn0051051	58	358	2,617
FBgn0036181	123	751	2,613
FBgn0052808	34	187	2,440
FBgn0002926	68	348	2,349
FBgn0034182	64	299	2,217
FBgn0029580	35	159	2,185
FBgn0039481	99	407	2,040
FBgn0034012	128	520	2,025
FBgn0050340	68	272	1,998
FBgn0259993	1129	4219	1,901
FBgn0032343	57	207	1,862
FBgn0026403	77	268	1,805
FBgn0036975	221	749	1,762
FBgn0003137	4745	15195	1,679
FBgn0002774	935	2946	1,656
FBgn0030795	326	951	1,543
FBgn0039809	302	879	1,541
FBgn0085774	762	2199	1,529
FBgn0022787	8366	22599	1,434

FBgn0033387	1094	2884	1,399
FBgn0260450	850	2236	1,396
FBgn0016080	170	437	1,362
FBgn0035608	3250	8355	1,362
FBgn0034950	178	455	1,354
FBgn0031077	1267	3210	1,342
FBgn0053196	1345	3367	1,324
FBgn0033313	2269	5629	1,311
FBgn0050339	354	877	1,309
FBgn0004167	4067	9732	1,259
FBgn0014903	224	536	1,256
FBgn0032981	684	1634	1,256
FBgn0034880	562	1334	1,248
FBgn0027499	706	1617	1,195
FBgn0050069	471	1049	1,157
FBgn0262945	2336	5149	1,140
FBgn0036809	261	572	1,134
FBgn0033153	1051	2237	1,089
FBgn0261872	968	2031	1,069
FBgn0038853	1351	2789	1,046
FBgn0039157	732	1489	1,026
FBgn0025808	926	1879	1,021
FBgn0262110	1299	2603	1,003
FBgn0259175	1408	2748	0,965
FBgn0261836	12683	24116	0,927
FBgn0034400	9768	18507	0,922
FBgn0022063	2852	5342	0,906
FBgn0028430	931	1724	0,888
FBgn0035708	1849	3419	0,887
FBgn0035713	2389	4355	0,866
FBgn0036663	5633	10071	0,838
FBgn0003943	13994	24366	0,800
FBgn0035162	11684	19855	0,765
Gene Name: Flybase ID	Base mean Control cells	Base mean XRN1 KD cells	log2FoldChange
Part II. Transcripts downregulated in XRN1 knockdown cells			
FBgn0044811	550	117	-2,237
FBgn0041150	715	163	-2,135
FBgn0052320	575	138	-2,061
FBgn0035343	243	65	-1,907
FBgn0263041	348	101	-1,789
FBgn0037290	438	135	-1,698
FBgn0262508	300	99	-1,594
FBgn0029837	348	117	-1,567
FBgn0036491	271	92	-1,567
FBgn0032364	1780	606	-1,554
FBgn0051690	408	140	-1,540

FBgn0031835	262	91	-1,518
FBgn0032066	473	177	-1,421
FBgn0029639	1118	454	-1,301
FBgn0003091	437	180	-1,281
FBgn0025393	1459	690	-1,081
FBgn0031805	4686	2260	-1,052
FBgn0039682	1213	606	-1,002
FBgn0051092	2584	1319	-0,970
FBgn0035216	1397	718	-0,961
FBgn0028514	2584	1337	-0,950
FBgn0032024	9390	5336	-0,815
FBgn0263865	31570	18750	-0,752

Table S3: Transcript levels in control cells compared to AGO1 knockdown cells (E3 vsE7). The table is divided in two parts: Part I lists all 1115 transcripts upregulated in AGO1 knockdown cells ($\text{padj} < 0.1, \log_2\text{foldchange} > 0.58$). Part II lists all 726 transcripts with lower expression in AGO1 knockdown cells ($\text{padj} < 0.1, \log_2\text{foldchange} < -0.58$). Transcripts highlighted in pink are also regulated by AGO1 in Hong X. et al., PNAS 106, 15085 (Table published in Antic et al., 2015).

Gene Name: Flybase ID	Base mean Control cells	Base mean AGO1 KD cells	log2FoldChange
Part I. Transcripts upregulated in AGO1 knockdown cells			
FBgn0038530	131	5311	5,336
FBgn0041579	70	1669	4,581
FBgn0013279	97	2157	4,472
FBgn0030262	42	912	4,432
FBgn0043578	149	2340	3,975
FBgn0034406	308	4028	3,710
FBgn0264541	12	145	3,637
FBgn0003997	45	522	3,540
FBgn0033495	77	864	3,479
FBgn0030929	36	389	3,451
FBgn0036547	59	584	3,319
FBgn0262631	40	403	3,317
FBgn0037212	64	631	3,296
FBgn0052170	93	894	3,271
FBgn0019643	29	274	3,257
FBgn0036299	745	7008	3,233
FBgn0038595	74	695	3,223
FBgn0051195	791	7349	3,216
FBgn0032420	187	1654	3,148
FBgn0260722	255	2125	3,061
FBgn0037240	1633	13604	3,058
FBgn0030932	71	567	3,005
FBgn0260866	194	1533	2,979
FBgn0035649	41	325	2,971
FBgn0038545	219	1693	2,952
FBgn0038291	181	1376	2,929
FBgn0010894	581	4322	2,894

FBgn0040850	189	1386	2,872
FBgn0035907	48	348	2,871
FBgn0000108	38	275	2,852
FBgn0052311	631	4279	2,762
FBgn0085446	208	1414	2,762
FBgn0052062	449	3028	2,753
FBgn0031268	362	2440	2,753
FBgn0261563	516	3467	2,749
FBgn0035710	1096	7308	2,738
FBgn0036468	26	174	2,726
FBgn0020368	107	707	2,720
FBgn0036198	96	627	2,710
FBgn0013720	421	2743	2,704
FBgn0031461	368	2385	2,697
FBgn0020240	1982	12706	2,681
FBgn0263397	95	608	2,675
FBgn0010388	290	1848	2,671
FBgn0031254	42	262	2,643
FBgn0000535	89	554	2,640
FBgn0011260	723	4504	2,640
FBgn0261560	301	1875	2,640
FBgn0002931	44	272	2,639
FBgn0026592	113	702	2,636
FBgn0038149	538	3320	2,626
FBgn0002968	632	3903	2,626
FBgn0259834	864	5294	2,615
FBgn0030766	175	1047	2,581
FBgn0032810	29	173	2,576
FBgn0037336	1389	8250	2,571
FBgn0037680	581	3436	2,564
FBgn0031760	310	1823	2,558
FBgn0029896	562	3298	2,554
FBgn0259677	677	3977	2,553
FBgn0031086	49	286	2,553
FBgn0005636	349	2050	2,552
FBgn0020493	60	350	2,542
FBgn0262579	9972	57359	2,524
FBgn0044047	932	5298	2,507
FBgn0261451	695	3941	2,504
FBgn0037906	203	1149	2,499
FBgn0021764	552	3076	2,479
FBgn0051431	398	2188	2,460
FBgn0028499	131	709	2,440
FBgn0012042	324	1755	2,438
FBgn0259685	158	848	2,427
FBgn0015568	388	2082	2,425
FBgn0052580	316	1680	2,411
FBgn0028369	102	530	2,373

FBgn0003162	154	797	2,368
FBgn0023531	101	521	2,367
FBgn0000277	294	1506	2,356
FBgn0051361	550	2792	2,343
FBgn0037636	4233	21334	2,333
FBgn0034407	4262	20998	2,301
FBgn0003520	1234	5994	2,281
FBgn0037717	161	780	2,276
FBgn0039916	94	449	2,259
FBgn0083007	1295	6157	2,249
FBgn0259176	8112	38546	2,249
FBgn0039622	46	219	2,239
FBgn0037130	178	840	2,237
FBgn0035140	122	573	2,233
FBgn0083008	1156	5425	2,230
FBgn0261553	1067	4968	2,219
FBgn0039113	905	4203	2,216
FBgn0039356	54	250	2,215
FBgn0052676	640	2955	2,208
FBgn0262730	439	2015	2,197
FBgn0051601	79	362	2,188
FBgn0032421	573	2609	2,187
FBgn0004133	415	1890	2,186
FBgn0013323	89	403	2,186
FBgn0053653	143	645	2,178
FBgn0041581	1044	4711	2,174
FBgn0034225	554	2499	2,174
FBgn0034498	477	2149	2,173
FBgn0038815	102	460	2,173
FBgn0033919	174	777	2,161
FBgn0039914	71	318	2,161
FBgn0031811	177	785	2,152
FBgn0004188	340	1500	2,139
FBgn0082973	242	1067	2,139
FBgn0039900	61	269	2,138
FBgn0000568	834	3638	2,125
FBgn0035142	7593	32739	2,108
FBgn0035959	876	3768	2,104
FBgn0032808	50	214	2,102
FBgn0032156	2822	12053	2,095
FBgn0082954	324	1366	2,076
FBgn0035283	276	1160	2,074
FBgn0037816	54	229	2,072
FBgn0036735	1843	7719	2,067
FBgn0023416	545	2281	2,066
FBgn0051999	61	253	2,061
FBgn0034261	296	1230	2,057
FBgn0036165	487	2010	2,046

FBgn0082962	172	707	2,042
FBgn0030532	287	1171	2,031
FBgn0000337	124	506	2,025
FBgn0035397	1570	6366	2,020
FBgn0262127	569	2304	2,018
FBgn0011674	108	439	2,018
FBgn0013997	2225	9007	2,017
FBgn0033134	183	738	2,016
FBgn0037536	487	1965	2,012
FBgn0015399	703	2835	2,011
FBgn0031738	1815	7309	2,010
FBgn0037614	400	1602	2,001
FBgn0086911	138	551	1,999
FBgn0052185	2149	8577	1,996
FBgn0263993	1446	5762	1,995
FBgn0002973	177	700	1,983
FBgn0034736	567	2210	1,961
FBgn0263352	5729	22301	1,961
FBgn0024732	108	418	1,951
FBgn0037106	142	547	1,950
FBgn0031018	96	368	1,939
FBgn0030510	363	1393	1,939
FBgn0030183	292	1116	1,936
FBgn0082966	712	2709	1,928
FBgn0262881	264	1004	1,924
FBgn0032249	176	668	1,924
FBgn0086686	791	2983	1,914
FBgn0013771	129	483	1,906
FBgn0037092	477	1786	1,904
FBgn0011648	477	1772	1,892
FBgn0038532	1976	7303	1,886
FBgn0033032	520	1920	1,884
FBgn0035151	68	251	1,882
FBgn0030418	1113	4101	1,882
FBgn0036454	173	637	1,878
FBgn0001332	375	1373	1,874
FBgn0031397	190	690	1,862
FBgn0039928	3972	14422	1,860
FBgn0038129	250	904	1,855
FBgn0051641	2410	8713	1,854
FBgn0082967	756	2719	1,846
FBgn0259977	153	547	1,833
FBgn0032817	1015	3611	1,831
FBgn0000276	544	1930	1,827
FBgn0035144	425	1506	1,825
FBgn0003862	1404	4977	1,825
FBgn0040071	213	754	1,825
FBgn0026263	105	373	1,824

FBgn0052544	76	270	1,821
FBgn0004190	231	817	1,820
FBgn0086666	1351	4763	1,818
FBgn0052373	84	296	1,812
FBgn0000464	1053	3695	1,812
FBgn0033033	160	559	1,806
FBgn0028397	870	3038	1,804
FBgn0039863	427	1491	1,802
FBgn0033579	90	312	1,790
FBgn0086698	352	1217	1,790
FBgn0003067	473	1632	1,788
FBgn0038290	2087	7203	1,787
FBgn0027339	1684	5808	1,786
FBgn0262887	304	1047	1,784
FBgn0052767	1432	4924	1,782
FBgn0047205	173	593	1,778
FBgn0011259	140	479	1,775
FBgn0033372	86	295	1,771
FBgn0263490	3476	11822	1,766
FBgn0032666	614	2087	1,764
FBgn0083057	1608	5455	1,762
FBgn0086668	811	2745	1,760
FBgn0031258	678	2290	1,756
FBgn0010825	1159	3912	1,755
FBgn0003900	124	417	1,752
FBgn0004607	9465	31831	1,750
FBgn0038296	160	539	1,749
FBgn0019660	10785	35987	1,738
FBgn0062565	170	566	1,738
FBgn0082976	213	710	1,736
FBgn0025642	138	458	1,730
FBgn0083014	3044	10077	1,727
FBgn0083041	1720	5679	1,723
FBgn0263220	246	809	1,715
FBgn0022355	930	3051	1,715
FBgn0029088	1030	3380	1,714
FBgn0031107	1511	4930	1,706
FBgn0261565	1310	4262	1,702
FBgn0032908	312	1013	1,700
FBgn0051072	509	1652	1,699
FBgn0017581	8303	26862	1,694
FBgn0032218	101	326	1,693
FBgn0004189	224	723	1,692
FBgn0261642	2255	7280	1,691
FBgn0014007	1091	3508	1,685
FBgn0262110	961	3088	1,685
FBgn0086662	648	2079	1,682
FBgn0030710	273	876	1,682

FBgn0005631	1043	3331	1,676
FBgn0033752	217	693	1,672
FBgn0036398	3074	9795	1,672
FBgn0010389	577	1835	1,670
FBgn0035475	276	877	1,670
FBgn0015229	117	373	1,669
FBgn0004395	3307	10510	1,668
FBgn0031319	271	858	1,664
FBgn0012037	698	2196	1,654
FBgn0003423	1335	4195	1,652
FBgn0264495	757	2375	1,650
FBgn0250788	3259	10196	1,646
FBgn0051352	468	1463	1,644
FBgn0033609	314	979	1,639
FBgn0030486	451	1405	1,639
FBgn0036134	743	2311	1,638
FBgn0083050	458	1425	1,637
FBgn0083000	141	438	1,637
FBgn0052512	243	755	1,636
FBgn0261885	4615	14267	1,628
FBgn0034400	7782	23979	1,624
FBgn0030612	220	677	1,623
FBgn0000152	314	965	1,622
FBgn0037856	156	477	1,618
FBgn0085773	617	1892	1,617
FBgn0039738	575	1764	1,616
FBgn0029894	189	580	1,616
FBgn0039560	264	810	1,615
FBgn0035002	288	880	1,612
FBgn0038912	347	1057	1,607
FBgn0010113	1934	5884	1,605
FBgn0082988	1292	3927	1,604
FBgn0039831	1117	3393	1,603
FBgn0030228	288	872	1,599
FBgn0026262	4380	13212	1,593
FBgn0263392	3620	10912	1,592
FBgn0041111	797	2403	1,592
FBgn0261823	1108	3339	1,591
FBgn0031872	218	655	1,590
FBgn0086758	3499	10493	1,584
FBgn0033639	550	1649	1,583
FBgn0016977	5342	16003	1,583
FBgn0000536	1591	4766	1,582
FBgn0263395	733	2195	1,581
FBgn0035986	1129	3377	1,581
FBgn0261259	951	2844	1,580
FBgn0259234	349	1042	1,580
FBgn0039214	1952	5827	1,578

FBgn0261934	875	2609	1,576
FBgn0052756	913	2721	1,576
FBgn0035720	1910	5691	1,575
FBgn0065100	3998	11897	1,573
FBgn0025549	1074	3195	1,573
FBgn0034271	952	2827	1,571
FBgn0030960	517	1536	1,571
FBgn0001301	1511	4488	1,570
FBgn0259745	1659	4903	1,564
FBgn0004049	395	1165	1,562
FBgn0036196	373	1100	1,560
FBgn0083054	208	613	1,557
FBgn0083042	422	1238	1,554
FBgn0033757	152	446	1,551
FBgn0003716	238	697	1,550
FBgn0260748	3412	9986	1,549
FBgn0260744	960	2808	1,549
FBgn0263930	819	2390	1,544
FBgn0037679	180	523	1,543
FBgn0025809	278	810	1,542
FBgn0020762	817	2381	1,542
FBgn0038353	691	2004	1,537
FBgn0015818	128	370	1,535
FBgn0036663	4922	14248	1,534
FBgn0026148	246	713	1,533
FBgn0039696	474	1369	1,531
FBgn0052296	378	1088	1,525
FBgn0034054	173	498	1,524
FBgn0086658	1598	4578	1,519
FBgn0000286	198	567	1,516
FBgn0003353	250	715	1,516
FBgn0260938	758	2168	1,516
FBgn0086671	804	2295	1,514
FBgn0028420	326	929	1,510
FBgn0026318	416	1185	1,509
FBgn0003464	1517	4313	1,507
FBgn0000524	482	1367	1,505
FBgn0082831	808	2287	1,501
FBgn0032400	3569	10091	1,499
FBgn0028487	214	604	1,497
FBgn0016126	2502	7059	1,496
FBgn0250755	227	640	1,496
FBgn0083046	1080	3043	1,495
FBgn0027342	719	2025	1,494
FBgn0032821	1152	3245	1,494
FBgn0039590	491	1381	1,491
FBgn0250789	9916	27876	1,491
FBgn0031127	253	711	1,491

FBgn0032252	212	595	1,488
FBgn0036522	643	1804	1,487
FBgn0033984	1104	3092	1,486
FBgn0259227	349	977	1,484
FBgn0085364	37384	104458	1,482
FBgn0026206	2455	6843	1,479
FBgn0034300	237	660	1,479
FBgn0034501	3052	8476	1,474
FBgn0033177	1449	4016	1,470
FBgn0037917	647	1794	1,470
FBgn0015714	951	2631	1,468
FBgn0030114	1396	3864	1,468
FBgn0035842	688	1899	1,465
FBgn0259240	1708	4705	1,461
FBgn0034389	701	1930	1,461
FBgn0036333	1098	3016	1,458
FBgn0031637	2048	5611	1,454
FBgn0052985	221	604	1,452
FBgn0004583	2382	6512	1,451
FBgn0038220	844	2306	1,451
FBgn0260450	746	2037	1,450
FBgn0036809	281	764	1,444
FBgn0261617	1694	4608	1,444
FBgn0086347	1995	5423	1,442
FBgn0004652	2366	6426	1,441
FBgn0050015	894	2428	1,441
FBgn0033087	709	1926	1,441
FBgn0082971	295	799	1,440
FBgn0034976	297	806	1,438
FBgn0051992	3073	8315	1,436
FBgn0003175	559	1512	1,436
FBgn0264672	211	572	1,436
FBgn0035237	639	1728	1,435
FBgn0039920	1666	4503	1,434
FBgn0262794	437	1179	1,432
FBgn0003396	1264	3410	1,432
FBgn0030137	146	393	1,432
FBgn0083016	192	517	1,429
FBgn0036732	1161	3125	1,428
FBgn0030795	250	672	1,427
FBgn0020416	4510	12106	1,424
FBgn0030237	3809	10208	1,422
FBgn0030680	634	1699	1,422
FBgn0083045	272	729	1,421
FBgn0000547	207	553	1,420
FBgn0263476	3232	8643	1,419
FBgn0085407	642	1715	1,417
FBgn0010238	1854	4945	1,415

FBgn0031285	2924	7787	1,413
FBgn0039908	1956	5208	1,413
FBgn0035688	2444	6501	1,411
FBgn0264305	1896	5035	1,409
FBgn0033392	213	566	1,407
FBgn0032845	161	428	1,405
FBgn0086602	1415	3743	1,403
FBgn0044028	385	1018	1,402
FBgn0261574	6579	17373	1,401
FBgn0243513	155	407	1,397
FBgn0037188	788	2070	1,393
FBgn0083012	169	443	1,390
FBgn0086350	324	850	1,390
FBgn0085436	6429	16819	1,387
FBgn0029504	1072	2803	1,387
FBgn0261836	9551	24943	1,385
FBgn0004957	601	1567	1,384
FBgn0058469	8515	22203	1,383
FBgn0031170	1854	4831	1,382
FBgn0037949	648	1689	1,382
FBgn0039633	792	2057	1,376
FBgn0025740	2194	5687	1,374
FBgn0010414	833	2154	1,370
FBgn0261610	263	679	1,369
FBgn0031885	850	2191	1,365
FBgn0003093	533	1372	1,365
FBgn0000575	624	1607	1,364
FBgn0035953	634	1631	1,363
FBgn0015778	4012	10291	1,359
FBgn0031398	275	706	1,358
FBgn0039538	205	524	1,357
FBgn0005666	149	381	1,357
FBgn0004606	1859	4750	1,353
FBgn0030813	222	568	1,353
FBgn0039994	1443	3685	1,353
FBgn0259212	1182	3017	1,352
FBgn0086448	485	1235	1,348
FBgn0030809	2159	5495	1,348
FBgn0004369	510	1296	1,346
FBgn0032354	326	828	1,345
FBgn0027101	2254	5722	1,344
FBgn0011592	1801	4571	1,344
FBgn0032358	332	842	1,344
FBgn0028509	516	1310	1,343
FBgn0051038	805	2040	1,342
FBgn0033973	238	603	1,341
FBgn0040375	340	862	1,341
FBgn0035148	1699	4301	1,339

FBgn0035689	615	1556	1,339
FBgn0037989	7824	19785	1,338
FBgn0032223	1498	3775	1,334
FBgn0263110	889	2240	1,333
FBgn0037098	1143	2876	1,332
FBgn0065064	19808	49852	1,332
FBgn0033988	894	2249	1,331
FBgn0035542	1345	3375	1,328
FBgn0002121	12616	31660	1,327
FBgn0082972	187	469	1,327
FBgn0029911	923	2311	1,325
FBgn0029825	986	2467	1,323
FBgn0036980	547	1367	1,322
FBgn0035488	931	2327	1,321
FBgn0260400	999	2494	1,319
FBgn0083020	290	724	1,318
FBgn0033233	230	572	1,317
FBgn0051108	871	2164	1,313
FBgn0015838	1699	4213	1,310
FBgn0038564	252	626	1,310
FBgn0082978	329	813	1,307
FBgn0010575	1012	2494	1,301
FBgn0036684	993	2446	1,301
FBgn0022935	436	1072	1,299
FBgn0033718	1405	3453	1,297
FBgn0262735	5169	12698	1,297
FBgn0261793	3951	9701	1,296
FBgn0039026	1571	3856	1,295
FBgn0034542	1216	2984	1,295
FBgn0015520	305	748	1,294
FBgn0017558	267	654	1,290
FBgn0028372	283	692	1,290
FBgn0005632	3707	9045	1,287
FBgn0026313	1932	4712	1,286
FBgn0037120	500	1219	1,285
FBgn0000376	311	756	1,283
FBgn0037814	823	2002	1,283
FBgn0051472	318	772	1,281
FBgn0011300	1518	3687	1,280
FBgn0031817	267	649	1,279
FBgn0262740	1006	2440	1,278
FBgn0034694	840	2037	1,278
FBgn0262684	1915	4642	1,278
FBgn0020309	5178	12542	1,276
FBgn0027498	440	1064	1,274
FBgn0027492	1543	3730	1,273
FBgn0032955	1109	2674	1,270
FBgn0026401	8141	19603	1,268

FBgn0262582	3327	8008	1,267
FBgn0051619	4259	10247	1,267
FBgn0025726	12342	29600	1,262
FBgn0025186	472	1131	1,260
FBgn0083919	1244	2978	1,259
FBgn0264270	1667	3989	1,259
FBgn0038286	1006	2406	1,258
FBgn0036837	578	1382	1,258
FBgn0031011	2103	5031	1,258
FBgn0011224	396	946	1,258
FBgn0029891	1420	3388	1,255
FBgn0030055	571	1362	1,254
FBgn0003891	13346	31830	1,254
FBgn0035411	682	1624	1,252
FBgn0030246	588	1399	1,251
FBgn0030336	1082	2575	1,250
FBgn0026869	5597	13274	1,246
FBgn0000709	279	662	1,245
FBgn0082980	1139	2698	1,244
FBgn0035977	874	2066	1,241
FBgn0086902	9806	23168	1,240
FBgn0013733	3555	8393	1,240
FBgn0033569	430	1015	1,239
FBgn0032974	1967	4638	1,238
FBgn0029688	1381	3256	1,237
FBgn0065055	1633	3844	1,235
FBgn0032515	489	1148	1,230
FBgn0033539	1324	3106	1,230
FBgn0038100	3244	7602	1,229
FBgn0026722	361	847	1,229
FBgn0030892	623	1458	1,227
FBgn0025741	7699	18011	1,226
FBgn0037016	257	601	1,226
FBgn0034009	1002	2344	1,225
FBgn0011232	451	1053	1,224
FBgn0085757	505	1178	1,223
FBgn0086779	3250	7586	1,223
FBgn0037384	248	578	1,222
FBgn0003044	847	1972	1,220
FBgn0262731	884	2059	1,219
FBgn0016641	674	1565	1,216
FBgn0031575	273	634	1,215
FBgn0026597	539	1251	1,214
FBgn0000581	1699	3940	1,214
FBgn0026372	5557	12854	1,210
FBgn0003598	575	1326	1,205
FBgn0013759	949	2188	1,205
FBgn0000541	3441	7922	1,203

FBgn0039632	372	855	1,199
FBgn0000384	1400	3213	1,199
FBgn0014037	1200	2754	1,199
FBgn0011655	505	1157	1,197
FBgn0035523	739	1695	1,197
FBgn0031606	482	1104	1,195
FBgn0026404	3202	7312	1,191
FBgn0029979	2876	6561	1,190
FBgn0004227	401	914	1,190
FBgn0263346	333	759	1,189
FBgn0003345	4668	10640	1,189
FBgn0032876	741	1686	1,186
FBgn0250785	357	811	1,185
FBgn0004875	1209	2749	1,185
FBgn0263093	449	1020	1,184
FBgn0050000	1061	2407	1,182
FBgn0262614	3821	8667	1,182
FBgn0040056	2348	5321	1,180
FBgn0015577	1761	3992	1,180
FBgn0261671	2788	6313	1,179
FBgn0033188	3113	7049	1,179
FBgn0034155	653	1476	1,177
FBgn0085817	241	543	1,174
FBgn0032305	702	1583	1,174
FBgn0003261	7762	17502	1,173
FBgn0030869	466	1049	1,172
FBgn0036988	504	1136	1,171
FBgn0002891	491	1106	1,170
FBgn0036353	481	1082	1,170
FBgn0030456	830	1866	1,169
FBgn0003984	637	1427	1,165
FBgn0032815	922	2065	1,164
FBgn0028476	617	1381	1,162
FBgn0000307	893	1993	1,158
FBgn0038877	1055	2353	1,157
FBgn0036446	1212	2703	1,157
FBgn0263477	5612	12506	1,156
FBgn0032796	591	1316	1,156
FBgn0039977	743	1656	1,156
FBgn0033638	501	1116	1,156
FBgn0031896	680	1515	1,155
FBgn0038593	587	1306	1,155
FBgn0037556	765	1704	1,155
FBgn0035421	671	1493	1,153
FBgn0030266	437	972	1,153
FBgn0262733	1080	2400	1,152
FBgn0030316	398	884	1,151
FBgn0036688	311	691	1,150

FBgn0001965	801	1777	1,149
FBgn0030660	663	1468	1,147
FBgn0036372	1181	2616	1,147
FBgn0030701	1182	2616	1,146
FBgn0020415	5808	12842	1,145
FBgn0031317	1672	3689	1,141
FBgn0035812	583	1284	1,139
FBgn0036824	2230	4898	1,135
FBgn0041094	2371	5205	1,134
FBgn0050007	625	1371	1,134
FBgn0033174	493	1082	1,134
FBgn0035877	701	1536	1,132
FBgn0264491	921	2017	1,132
FBgn0004611	509	1114	1,131
FBgn0259168	1004	2199	1,131
FBgn0263832	648	1418	1,129
FBgn0036032	700	1531	1,129
FBgn0052822	291	635	1,128
FBgn0052579	609	1331	1,128
FBgn0010309	420	918	1,128
FBgn0259824	1462	3193	1,127
FBgn0036059	2144	4677	1,125
FBgn0037074	2986	6509	1,124
FBgn0019968	1755	3826	1,124
FBgn0042083	718	1565	1,124
FBgn0039890	3107	6767	1,123
FBgn0036451	1918	4177	1,123
FBgn0051635	1259	2741	1,123
FBgn0085430	1772	3859	1,123
FBgn0003169	2090	4548	1,122
FBgn0037760	596	1297	1,121
FBgn0001234	14931	32466	1,121
FBgn0037999	1410	3067	1,121
FBgn0032455	782	1700	1,121
FBgn0263115	1071	2327	1,120
FBgn0032295	916	1989	1,118
FBgn0261862	931	2020	1,118
FBgn0004656	3250	7045	1,116
FBgn0005630	7972	17269	1,115
FBgn0011837	3753	8123	1,114
FBgn0014018	4518	9764	1,112
FBgn0031608	288	621	1,109
FBgn0039923	4054	8743	1,109
FBgn0022768	852	1836	1,108
FBgn0052594	2178	4687	1,106
FBgn0004919	2682	5772	1,106
FBgn0030581	685	1473	1,105
FBgn0020304	7289	15666	1,104

FBgn0015575	2853	6132	1,104
FBgn0003969	3566	7659	1,103
FBgn0037347	320	687	1,101
FBgn0037850	452	968	1,099
FBgn0039877	630	1349	1,098
FBgn0030739	406	868	1,097
FBgn0031981	1693	3619	1,096
FBgn0027655	1828	3908	1,096
FBgn0034308	576	1229	1,094
FBgn0030735	1222	2608	1,094
FBgn0027363	1084	2312	1,092
FBgn0030674	5120	10912	1,092
FBgn0031118	1064	2264	1,089
FBgn0051370	520	1104	1,087
FBgn0030603	1166	2478	1,087
FBgn0025676	382	812	1,087
FBgn0028985	2351	4993	1,086
FBgn0030973	643	1365	1,086
FBgn0039189	499	1057	1,084
FBgn0261931	489	1036	1,082
FBgn0053554	7863	16645	1,082
FBgn0034570	1609	3405	1,082
FBgn0001169	775	1640	1,081
FBgn0000247	1820	3848	1,080
FBgn0083962	1948	4118	1,080
FBgn0039532	679	1435	1,080
FBgn0261551	957	2024	1,080
FBgn0015772	717	1515	1,080
FBgn0025455	647	1367	1,080
FBgn0259178	433	915	1,080
FBgn0087039	449	948	1,079
FBgn0024234	3031	6398	1,078
FBgn0015269	950	2004	1,077
FBgn0038381	334	703	1,075
FBgn0033739	302	636	1,075
FBgn0052350	1217	2562	1,074
FBgn0015542	1253	2636	1,073
FBgn0026086	1567	3294	1,072
FBgn0027499	724	1522	1,071
FBgn0004373	2016	4233	1,070
FBgn0041161	1032	2166	1,070
FBgn0027525	2406	5049	1,069
FBgn0041775	1654	3469	1,068
FBgn0264693	2740	5746	1,068
FBgn0001994	1757	3683	1,068
FBgn0027532	1323	2769	1,065
FBgn0030970	616	1288	1,064
FBgn0043884	4093	8550	1,063

FBgn0036546	320	669	1,062
FBgn0005198	634	1322	1,059
FBgn0021873	1685	3510	1,059
FBgn0051755	768	1601	1,059
FBgn0034223	935	1948	1,059
FBgn0082999	379	790	1,059
FBgn0035016	943	1962	1,058
FBgn0025621	865	1800	1,057
FBgn0033010	3573	7432	1,056
FBgn0052485	610	1268	1,056
FBgn0260794	4138	8598	1,055
FBgn0030485	624	1295	1,054
FBgn0002543	909	1887	1,053
FBgn0036374	530	1098	1,052
FBgn0005616	1046	2170	1,052
FBgn0051935	739	1531	1,052
FBgn0028399	1887	3910	1,051
FBgn0035106	875	1811	1,049
FBgn0034046	818	1692	1,049
FBgn0040068	1322	2734	1,048
FBgn0029092	4660	9629	1,047
FBgn0039003	1052	2174	1,047
FBgn0031820	636	1314	1,046
FBgn0033005	3204	6610	1,045
FBgn0005386	568	1171	1,044
FBgn0017414	316	651	1,044
FBgn0034527	596	1229	1,044
FBgn0038108	4206	8664	1,043
FBgn0043001	383	788	1,042
FBgn0004893	2867	5899	1,041
FBgn0041092	5296	10896	1,041
FBgn0082994	1181	2427	1,039
FBgn0034728	1086	2232	1,039
FBgn0031490	1546	3176	1,038
FBgn0037076	1242	2551	1,038
FBgn0014141	7217	14815	1,038
FBgn0051342	2211	4532	1,035
FBgn0033052	1655	3389	1,034
FBgn0033466	763	1562	1,033
FBgn0033313	1751	3582	1,033
FBgn0263470	9200	18813	1,032
FBgn0263112	1523	3114	1,032
FBgn0034987	1546	3160	1,031
FBgn0003557	2777	5670	1,030
FBgn0017549	513	1045	1,027
FBgn0001079	712	1450	1,026
FBgn0085434	562	1144	1,025
FBgn0033038	3537	7196	1,025

FBgn0015278	1413	2865	1,020
FBgn0261444	1324	2684	1,019
FBgn0000317	797	1616	1,019
FBgn0003870	1249	2531	1,019
FBgn0260941	654	1326	1,019
FBgn0085451	860	1742	1,017
FBgn0261854	2072	4190	1,016
FBgn0026192	1946	3936	1,016
FBgn0030087	1679	3396	1,016
FBgn0043900	535	1081	1,015
FBgn0011566	469	948	1,015
FBgn0038651	1411	2849	1,014
FBgn0033224	1486	3001	1,014
FBgn0033453	1639	3307	1,012
FBgn0024806	1237	2494	1,011
FBgn0000382	1670	3364	1,010
FBgn0028473	18912	38058	1,009
FBgn0263461	2088	4191	1,005
FBgn0031882	878	1759	1,002
FBgn0263929	1599	3197	1,000
FBgn0016131	1046	2092	1,000
FBgn0035497	863	1726	0,999
FBgn0017418	1202	2400	0,997
FBgn0261873	1227	2446	0,995
FBgn0036448	3083	6144	0,995
FBgn0003371	2255	4492	0,994
FBgn0031186	2193	4367	0,994
FBgn0039508	1305	2598	0,993
FBgn0052486	771	1534	0,993
FBgn0039907	3624	7213	0,993
FBgn0034113	471	937	0,992
FBgn0033889	5382	10699	0,991
FBgn0032130	622	1234	0,989
FBgn0083005	891	1767	0,988
FBgn0029006	1976	3916	0,987
FBgn0027529	1175	2326	0,985
FBgn0038432	442	875	0,984
FBgn0261802	3570	7062	0,984
FBgn0052016	1706	3370	0,982
FBgn0058439	1403	2762	0,977
FBgn0037007	5165	10161	0,976
FBgn0264707	839	1651	0,976
FBgn0052533	574	1128	0,975
FBgn0019890	2416	4744	0,974
FBgn0003277	2539	4981	0,972
FBgn0262737	1769	3466	0,970
FBgn0034083	2310	4524	0,969
FBgn0031090	1188	2326	0,969

FBgn0033504	977	1912	0,969
FBgn0262117	2526	4940	0,967
FBgn0004406	734	1435	0,967
FBgn0033212	4172	8134	0,963
FBgn0032197	1150	2236	0,960
FBgn0040236	2067	4017	0,959
FBgn0039266	3534	6866	0,958
FBgn0015247	3102	6022	0,957
FBgn0032243	882	1708	0,954
FBgn0030330	643	1243	0,952
FBgn0039157	572	1105	0,951
FBgn0028953	524	1012	0,950
FBgn0020647	2833	5467	0,948
FBgn0046685	1329	2563	0,948
FBgn0011785	1239	2389	0,948
FBgn0037808	6771	13057	0,947
FBgn0264075	629	1213	0,946
FBgn0027539	595	1147	0,946
FBgn0036511	2012	3874	0,945
FBgn0037142	958	1844	0,944
FBgn0038037	1896	3647	0,944
FBgn0033166	546	1049	0,943
FBgn0011766	2122	4079	0,943
FBgn0001565	2798	5377	0,943
FBgn0011747	7815	15018	0,942
FBgn0038735	531	1020	0,942
FBgn0261787	690	1326	0,942
FBgn0025739	625	1200	0,940
FBgn0067779	696	1336	0,939
FBgn0004698	2952	5659	0,939
FBgn0029992	772	1479	0,938
FBgn0013984	2332	4460	0,935
FBgn0039727	1536	2938	0,935
FBgn0004655	649	1240	0,935
FBgn0040324	4129	7887	0,934
FBgn0016081	4563	8706	0,932
FBgn0010300	4689	8937	0,931
FBgn0024921	4434	8440	0,929
FBgn0038870	902	1717	0,928
FBgn0026160	3225	6131	0,927
FBgn0038588	572	1087	0,925
FBgn0026379	1160	2201	0,924
FBgn0034418	1634	3099	0,924
FBgn0022764	5690	10792	0,923
FBgn0083987	28608	54221	0,922
FBgn0031453	4737	8968	0,921
FBgn0086899	2710	5130	0,921
FBgn0010417	692	1309	0,920

FBgn0050497	3458	6535	0,918
FBgn0032363	1848	3489	0,917
FBgn0037235	917	1731	0,917
FBgn0003415	2244	4238	0,917
FBgn0025936	3845	7247	0,914
FBgn0010488	6740	12689	0,913
FBgn0003137	3856	7259	0,913
FBgn0031643	1598	3006	0,912
FBgn0261570	1790	3367	0,912
FBgn0001078	1966	3698	0,911
FBgn0032957	1607	3020	0,910
FBgn0037554	739	1389	0,909
FBgn0261618	5662	10633	0,909
FBgn0262714	1089	2043	0,907
FBgn0036579	567	1062	0,907
FBgn0032341	2154	4036	0,906
FBgn0031897	1931	3618	0,905
FBgn0039055	1683	3149	0,904
FBgn0015562	544	1017	0,903
FBgn0005411	2485	4644	0,902
FBgn0030648	1024	1913	0,902
FBgn0052451	4658	8703	0,902
FBgn0243512	2154	4023	0,901
FBgn0003444	1513	2823	0,900
FBgn0003079	975	1818	0,900
FBgn0261552	3237	6038	0,900
FBgn0003334	1085	2024	0,899
FBgn0002566	2826	5268	0,899
FBgn0030894	780	1452	0,897
FBgn0031216	1514	2819	0,897
FBgn0027611	1335	2485	0,896
FBgn0036897	894	1662	0,895
FBgn0051249	1437	2672	0,895
FBgn0039209	3311	6156	0,895
FBgn0030711	607	1129	0,895
FBgn0023172	2915	5412	0,893
FBgn0085442	2398	4448	0,891
FBgn0034975	656	1217	0,890
FBgn0028406	827	1532	0,890
FBgn0261984	1926	3567	0,889
FBgn0026573	722	1336	0,888
FBgn0040765	1291	2385	0,886
FBgn0036180	1283	2364	0,882
FBgn0026427	1540	2836	0,881
FBgn0028980	517	952	0,881
FBgn0000097	1111	2045	0,880
FBgn0026375	958	1762	0,880
FBgn0004436	2364	4350	0,880

FBgn0004924	2636	4845	0,878
FBgn0052438	1610	2958	0,877
FBgn0033926	117249	215275	0,877
FBgn0039924	2460	4513	0,876
FBgn0031213	2920	5356	0,875
FBgn0261004	1181	2165	0,875
FBgn0040079	1276	2340	0,874
FBgn0035811	1244	2281	0,874
FBgn0034072	3384	6197	0,873
FBgn0003963	1592	2914	0,872
FBgn0082582	4447	8140	0,872
FBgn0004598	2591	4742	0,872
FBgn0052687	1150	2104	0,871
FBgn0036428	659	1206	0,871
FBgn0052113	1201	2192	0,868
FBgn0052521	1720	3138	0,868
FBgn0001123	2713	4947	0,867
FBgn0035150	1366	2490	0,866
FBgn0052627	1307	2382	0,866
FBgn0029944	1285	2341	0,865
FBgn0263998	2174	3959	0,865
FBgn0028703	968	1762	0,864
FBgn0262114	1726	3141	0,864
FBgn0087035	3010	5472	0,863
FBgn0031814	684	1243	0,863
FBgn0043903	965	1752	0,861
FBgn0035540	564	1024	0,861
FBgn0250786	2264	4111	0,861
FBgn0032208	5770	10468	0,859
FBgn0033029	3799	6881	0,857
FBgn0002638	3618	6553	0,857
FBgn0010315	1934	3499	0,856
FBgn0263391	2535	4578	0,853
FBgn0000256	3710	6700	0,853
FBgn0000611	5660	10217	0,852
FBgn0013772	1306	2357	0,851
FBgn0003165	5502	9924	0,851
FBgn0000063	954	1721	0,851
FBgn0037445	1399	2521	0,850
FBgn0037472	1075	1937	0,850
FBgn0085432	3089	5563	0,849
FBgn0038826	2398	4315	0,848
FBgn0010772	710	1277	0,847
FBgn0038809	3284	5906	0,847
FBgn0025683	3305	5943	0,847
FBgn0015402	867	1559	0,846
FBgn0040230	1052	1891	0,846
FBgn0037344	2014	3617	0,845

FBgn0042180	671	1203	0,843
FBgn0030724	765	1373	0,843
FBgn0028539	2200	3946	0,843
FBgn0011764	6590	11811	0,842
FBgn0001624	4739	8485	0,840
FBgn0029893	815	1459	0,840
FBgn0027561	1516	2711	0,839
FBgn0020622	3352	5989	0,837
FBgn0026199	4313	7704	0,837
FBgn0042177	965	1723	0,837
FBgn0003475	3294	5883	0,837
FBgn0026315	2343	4182	0,836
FBgn0261983	1148	2048	0,835
FBgn0024248	1084	1931	0,833
FBgn0037612	1180	2102	0,833
FBgn0039466	1228	2185	0,832
FBgn0042134	2552	4541	0,831
FBgn0052243	3152	5602	0,830
FBgn0085423	1698	3018	0,830
FBgn0036030	3047	5407	0,827
FBgn0261938	1193	2116	0,826
FBgn0005536	2118	3753	0,825
FBgn0025802	1825	3231	0,824
FBgn0260935	778	1378	0,824
FBgn0029824	1854	3283	0,824
FBgn0024807	946	1673	0,823
FBgn0031379	814	1439	0,822
FBgn0051998	4515	7979	0,821
FBgn0052000	9124	16117	0,821
FBgn0040271	2275	4014	0,819
FBgn0000099	25362	44746	0,819
FBgn0003328	756	1334	0,819
FBgn0035806	1585	2792	0,817
FBgn0010303	1282	2259	0,817
FBgn0040813	28630	50411	0,816
FBgn0033260	1078	1897	0,815
FBgn0016797	742	1306	0,815
FBgn0036534	4925	8666	0,815
FBgn0038034	1506	2650	0,815
FBgn0043364	1812	3185	0,814
FBgn0033159	3624	6366	0,813
FBgn0016696	1112	1953	0,813
FBgn0037555	841	1477	0,812
FBgn0032456	3945	6913	0,809
FBgn0262527	1758	3081	0,809
FBgn0039528	1795	3142	0,808
FBgn0004387	946	1656	0,808
FBgn0037252	924	1617	0,807

FBgn0011305	1078	1885	0,806
FBgn0000562	1113	1945	0,805
FBgn0011642	1934	3377	0,804
FBgn0086371	833	1453	0,804
FBgn0262081	2142	3738	0,804
FBgn0040388	2504	4366	0,802
FBgn0040344	1142	1992	0,802
FBgn0001404	3946	6874	0,801
FBgn0004391	2305	4009	0,799
FBgn0005410	1047	1821	0,798
FBgn0032997	1413	2457	0,798
FBgn0037705	926	1608	0,797
FBgn0043070	1882	3267	0,796
FBgn0052115	1120	1944	0,796
FBgn0038244	1448	2512	0,795
FBgn0264307	847	1468	0,794
FBgn0046692	1396	2418	0,793
FBgn0035111	879	1522	0,792
FBgn0030049	827	1429	0,789
FBgn0011236	1043	1800	0,788
FBgn0031190	2491	4295	0,786
FBgn0000163	2485	4284	0,786
FBgn0029819	1373	2366	0,785
FBgn0037109	1183	2038	0,784
FBgn0022699	878	1511	0,784
FBgn0029996	2153	3707	0,784
FBgn0000546	1215	2087	0,780
FBgn0028962	916	1573	0,780
FBgn0042085	1401	2400	0,777
FBgn0014006	1236	2118	0,776
FBgn0039215	1108	1898	0,776
FBgn0051694	4370	7475	0,774
FBgn0052133	958	1638	0,774
FBgn0027505	885	1513	0,773
FBgn0028341	1164	1986	0,770
FBgn0029870	2254	3842	0,769
FBgn0004198	1823	3107	0,769
FBgn0025800	1719	2925	0,767
FBgn0000054	944	1606	0,766
FBgn0031710	987	1678	0,766
FBgn0031450	859	1460	0,766
FBgn0035416	2161	3672	0,765
FBgn0264493	1482	2518	0,765
FBgn0260799	6442	10941	0,764
FBgn0029820	1126	1910	0,763
FBgn0046696	49348	83702	0,762
FBgn0013762	778	1319	0,762
FBgn0031759	2498	4236	0,762

FBgn0031150	1235	2094	0,762
FBgn0001337	1548	2625	0,762
FBgn0051536	2782	4717	0,762
FBgn0035285	1395	2366	0,762
FBgn0261268	2931	4968	0,761
FBgn0030484	2607	4416	0,760
FBgn0031220	19334	32698	0,758
FBgn0032210	930	1572	0,757
FBgn0038889	762	1287	0,756
FBgn0261811	6230	10500	0,753
FBgn0261113	10004	16852	0,752
FBgn0030796	1004	1689	0,751
FBgn0250823	3030	5098	0,751
FBgn0040011	4365	7343	0,750
FBgn0026370	1548	2603	0,750
FBgn0053180	1471	2470	0,748
FBgn0004648	3622	6077	0,746
FBgn0027507	1702	2855	0,746
FBgn0015806	3242	5430	0,744
FBgn0002521	2851	4772	0,743
FBgn0034025	898	1502	0,742
FBgn0039016	1543	2579	0,742
FBgn0039260	2338	3904	0,740
FBgn0260798	1782	2975	0,739
FBgn0038652	836	1394	0,738
FBgn0026076	1297	2163	0,738
FBgn0005672	6625	11041	0,737
FBgn0261387	2459	4095	0,736
FBgn0038533	1989	3311	0,736
FBgn0025743	1304	2169	0,734
FBgn0024811	2528	4204	0,734
FBgn0261530	999	1660	0,733
FBgn0263706	1829	3038	0,732
FBgn0004435	5990	9948	0,732
FBgn0037363	1737	2884	0,732
FBgn0031356	2282	3786	0,730
FBgn0263039	1046	1732	0,728
FBgn0031971	1576	2607	0,726
FBgn0037758	1398	2312	0,726
FBgn0015509	3112	5142	0,724
FBgn0027335	5273	8711	0,724
FBgn0036958	1094	1805	0,722
FBgn0040005	1921	3166	0,721
FBgn0263102	4497	7410	0,721
FBgn0022063	2330	3837	0,720
FBgn0010215	4427	7289	0,719
FBgn0030744	1339	2203	0,718
FBgn0034181	1558	2560	0,716

FBgn0030685	2386	3918	0,715
FBgn0039970	2114	3470	0,715
FBgn0259214	11346	18630	0,715
FBgn0004889	1982	3253	0,715
FBgn0031149	3072	5039	0,714
FBgn0027932	6163	10103	0,713
FBgn0011740	2015	3302	0,712
FBgn0260934	5580	9141	0,712
FBgn0034491	1236	2024	0,712
FBgn0034110	1206	1973	0,711
FBgn0033388	2766	4528	0,711
FBgn0261403	5995	9782	0,706
FBgn0040009	1203	1962	0,705
FBgn0038197	2150	3506	0,705
FBgn0038881	1467	2391	0,704
FBgn0085450	1531	2494	0,704
FBgn0023215	1192	1941	0,703
FBgn0032474	1046	1699	0,699
FBgn0085443	3523	5714	0,698
FBgn0032521	1819	2951	0,698
FBgn0011666	2607	4227	0,697
FBgn0000319	16734	27093	0,695
FBgn0261985	1504	2435	0,695
FBgn0031187	1322	2140	0,694
FBgn0023081	1803	2916	0,693
FBgn0033232	1189	1918	0,690
FBgn0033310	1149	1853	0,689
FBgn0030793	1080	1739	0,687
FBgn0039431	5271	8473	0,685
FBgn0000413	2056	3302	0,684
FBgn0014001	3320	5332	0,684
FBgn0034068	1323	2124	0,683
FBgn0023479	12506	20063	0,682
FBgn0027087	3537	5673	0,682
FBgn0051523	5078	8144	0,682
FBgn0262872	3421	5465	0,676
FBgn0026630	2566	4095	0,674
FBgn0261456	1603	2558	0,674
FBgn0033879	1167	1862	0,674
FBgn0020440	1505	2394	0,670
FBgn0032600	2180	3464	0,668
FBgn0002914	3119	4950	0,666
FBgn0034180	2567	4067	0,664
FBgn0001978	1870	2956	0,660
FBgn0052428	1760	2780	0,660
FBgn0041171	2826	4461	0,658
FBgn0050372	1959	3090	0,658
FBgn0037094	3567	5628	0,658

FBgn0262517	2026	3191	0,655
FBgn0263855	2047	3219	0,653
FBgn0050020	1294	2033	0,652
FBgn0021761	1672	2626	0,651
FBgn0045035	2452	3847	0,650
FBgn0029152	3112	4873	0,647
FBgn0033476	4184	6540	0,645
FBgn0044826	3893	6078	0,643
FBgn0035983	1714	2676	0,643
FBgn0085388	1323	2063	0,641
FBgn0030719	6412	9998	0,641
FBgn0020767	2119	3301	0,639
FBgn0033109	4008	6241	0,639
FBgn0024734	11782	18339	0,638
FBgn0086558	9756	15158	0,636
FBgn0003943	11689	18148	0,635
FBgn0036913	2214	3434	0,634
FBgn0010434	3951	6127	0,633
FBgn0029095	3588	5537	0,626
FBgn0030520	7160	10991	0,618
FBgn0086656	3262	5003	0,617
FBgn0000578	4155	6343	0,610
FBgn0040395	6554	9980	0,607
FBgn0037541	3679	5602	0,607
FBgn0032940	2528	3849	0,607
FBgn0005640	2227	3386	0,605
FBgn0031359	2078	3154	0,602
FBgn0039936	5394	8149	0,595
FBgn0033998	2496	3767	0,594
FBgn0035770	6429	9698	0,593
FBgn0032943	2699	4058	0,589
FBgn0039109	2046	3072	0,587
Gene Name: Flybase ID	Base mean Control cells	Base mean AGO1 KD cells	log2FoldChange
Part II. Transcripts downregulated in AGO1 knockdown cells			
FBgn0038716	5102	25	-7,679
FBgn0031701	36599	212	-7,435
FBgn0264339	348	3	-7,100
FBgn0261362	935	7	-7,018
FBgn0029837	359	4	-6,636
FBgn0032151	218	2	-6,543
FBgn0034885	136	1	-6,543
FBgn0039290	161	2	-6,483
FBgn0039396	259	3	-6,312
FBgn0034943	55	1	-6,238
FBgn0032364	1578	22	-6,134
FBgn0044811	570	9	-5,913

FBgn0032074	69	1	-5,552
FBgn0022800	137	3	-5,478
FBgn0028562	112	3	-5,466
FBgn0032788	64	1	-5,464
FBgn0051092	2239	55	-5,349
FBgn0003285	240	6	-5,290
FBgn0053117	198	5	-5,277
FBgn0035656	136	4	-5,242
FBgn0028396	3301	90	-5,196
FBgn0031563	73	2	-5,055
FBgn0033448	87	3	-5,022
FBgn0041183	999	33	-4,907
FBgn0004910	189	7	-4,856
FBgn0034870	1324	47	-4,821
FBgn0054054	445	16	-4,787
FBgn0052320	428	17	-4,692
FBgn0031835	242	10	-4,650
FBgn0010381	2992	119	-4,649
FBgn0036491	210	8	-4,645
FBgn0030763	118	5	-4,642
FBgn0032066	432	17	-4,634
FBgn0038214	190	8	-4,568
FBgn0038299	461	20	-4,543
FBgn0039321	1742	76	-4,525
FBgn0262588	668	29	-4,514
FBgn0051777	994	44	-4,505
FBgn0038818	106	5	-4,485
FBgn0021767	153	7	-4,472
FBgn0034861	343	17	-4,373
FBgn0262587	1985	98	-4,345
FBgn0262508	273	14	-4,249
FBgn0085465	499	27	-4,218
FBgn0013953	365	20	-4,197
FBgn0042106	1445	79	-4,194
FBgn0031693	786	44	-4,175
FBgn0035623	182	10	-4,149
FBgn0039101	789	45	-4,143
FBgn0014033	532	30	-4,142
FBgn0002962	99	6	-4,136
FBgn0004882	105	6	-4,112
FBgn0031074	915	53	-4,099
FBgn0033635	515	31	-4,051
FBgn0261260	11547	697	-4,051
FBgn0261861	973	59	-4,047
FBgn0002869	7780	482	-4,014
FBgn0260011	280	18	-3,978
FBgn0038213	96	6	-3,959
FBgn0036616	222	15	-3,907

FBgn0035904	2241	156	-3,842
FBgn0032772	160	11	-3,813
FBgn0038114	104	8	-3,759
FBgn0031694	1798	133	-3,755
FBgn0035348	412	31	-3,745
FBgn0034221	1676	126	-3,733
FBgn0030394	93	7	-3,729
FBgn0041087	737	56	-3,723
FBgn0028514	2316	175	-3,723
FBgn0031762	207	16	-3,693
FBgn0051221	423	33	-3,691
FBgn0045852	237	19	-3,654
FBgn0053225	168	14	-3,600
FBgn0039844	1139	94	-3,597
FBgn0038789	384	32	-3,583
FBgn0034408	268	23	-3,549
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FBgn0037153	190	18	-3,433
FBgn0263415	332	31	-3,414
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FBgn0027793	368	36	-3,363
FBgn0010395	1050	103	-3,356
FBgn0053080	147	14	-3,350
FBgn0028863	1271	125	-3,347
FBgn0051769	215	21	-3,332
FBgn0033748	166	17	-3,309
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FBgn0262169	655	67	-3,289
FBgn0262983	210	22	-3,263
FBgn0003890	1377	144	-3,262
FBgn0264343	307	33	-3,236
FBgn0032483	250	27	-3,235
FBgn0026175	1325	141	-3,233
FBgn0040551	716	77	-3,226
FBgn0039114	291	32	-3,179
FBgn0050148	569	63	-3,175
FBgn0051778	399	44	-3,172
FBgn0031613	1249	140	-3,155
FBgn0033875	6350	718	-3,144
FBgn0037934	278	32	-3,131
FBgn0065032	539	62	-3,128
FBgn0038878	455	53	-3,102
FBgn0000116	177	22	-3,004

FBgn0032381	146	19	-2,971
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FBgn0038260	163	22	-2,912
FBgn0036323	240	32	-2,909
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FBgn0024977	366	49	-2,891
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FBgn0037290	499	69	-2,856
FBgn0261989	15130	2093	-2,853
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FBgn0001217	157	24	-2,712
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FBgn0259225	150	24	-2,626
FBgn0000146	796	131	-2,602
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FBgn0034709	41858	6939	-2,593
FBgn0051690	482	80	-2,591
FBgn0003450	179	30	-2,582
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FBgn0035767	1035	173	-2,577
FBgn0034638	1824	306	-2,575
FBgn0262146	3445	581	-2,568
FBgn0053192	3103	538	-2,528
FBgn0033584	438	76	-2,524
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FBgn0028936	330	59	-2,491
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FBgn0032024	10123	1817	-2,478
FBgn0086691	442	80	-2,472
FBgn0036839	263	48	-2,458

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FBgn0039593	1778	326	-2,446
FBgn0016123	227	42	-2,436
FBgn0053461	575	107	-2,421
FBgn0031471	1174	221	-2,408
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FBgn0037205	1283	251	-2,352
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FBgn0024984	431	87	-2,311
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FBgn0086365	4222	860	-2,295
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FBgn0032729	1262	273	-2,210
FBgn0028430	724	159	-2,186
FBgn0038974	782	172	-2,183
FBgn0039809	290	64	-2,183
FBgn0038681	2288	507	-2,175
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FBgn0037727	236	53	-2,144
FBgn0029147	1300	295	-2,140
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FBgn0003091	305	71	-2,110
FBgn0035209	870	202	-2,104
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FBgn0031622	1056	265	-1,996
FBgn0259740	225	57	-1,989
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FBgn0063923	487	125	-1,969
FBgn0034396	776	199	-1,964
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FBgn0026874	379	99	-1,942
FBgn0264475	3388	900	-1,913
FBgn0034614	930	247	-1,909
FBgn0037000	276	74	-1,906
FBgn0038804	467	125	-1,901
FBgn0022774	423	113	-1,901
FBgn0035415	536	144	-1,896
FBgn0262970	622	168	-1,890
FBgn0039094	491	134	-1,873
FBgn0011274	1339	367	-1,866
FBgn0040600	1604	442	-1,859
FBgn0037883	291	80	-1,857
FBgn0039640	2089	582	-1,845
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FBgn0051075	2983	833	-1,839
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FBgn0016122	257	72	-1,827
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FBgn0029639	1064	304	-1,807
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FBgn0011704	4603	1324	-1,798
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FBgn0036145	470	136	-1,791
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FBgn0039464	1939	570	-1,765
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FBgn0010223	383	118	-1,698
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FBgn0039304	795	250	-1,671
FBgn0036765	2354	740	-1,670
FBgn0023214	3787	1195	-1,664
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FBgn0000299	32794	10385	-1,659
FBgn0039494	377	120	-1,654
FBgn0003308	670	214	-1,649
FBgn0015774	648	207	-1,648
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FBgn0053062	397	132	-1,587
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FBgn0034194	2612	874	-1,579
FBgn0035216	1295	434	-1,578
FBgn0037239	777	262	-1,571
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FBgn0011722	40293	13665	-1,560
FBgn0031677	354	121	-1,553
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FBgn0038321	493	168	-1,549
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FBgn0040321	1982	716	-1,469
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FBgn0024994	2750	1206	-1,190
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FBgn0040954	2138	956	-1,161
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FBgn0026089	1465	659	-1,153
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FBgn0034058	1046	472	-1,147
FBgn0001137	932	421	-1,146
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FBgn0039163	1292	588	-1,135
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FBgn0034219	2223	1018	-1,126
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FBgn0039208	4974	2292	-1,118
FBgn0032773	905	417	-1,117
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FBgn0032140	7309	3406	-1,102
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FBgn0029854	2071	1042	-0,991
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FBgn0263510	3125	1600	-0,966

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FBgn0000426	1508	774	-0,963
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FBgn0045842	2788	1486	-0,908
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FBgn0032775	3719	2074	-0,842
FBgn0039868	1031	575	-0,842
FBgn0037537	2421	1353	-0,840
FBgn0046214	8277	4638	-0,836
FBgn0028468	2298	1289	-0,834
FBgn0038224	2452	1375	-0,834
FBgn0263121	4394	2465	-0,834
FBgn0031663	1255	704	-0,834
FBgn0015031	2311	1302	-0,828
FBgn0032925	1164	656	-0,827
FBgn0037955	1256	708	-0,827
FBgn0261975	1637	923	-0,826
FBgn0032725	1974	1115	-0,824
FBgn0037890	1411	797	-0,824
FBgn0011016	6445	3641	-0,824
FBgn0036772	2326	1316	-0,821
FBgn0040340	6338	3588	-0,821
FBgn0031256	6313	3575	-0,821
FBgn0033059	1219	691	-0,819
FBgn0023174	3526	2004	-0,815
FBgn0025678	15528	8833	-0,814
FBgn0031776	2050	1168	-0,812
FBgn0023212	3100	1766	-0,812
FBgn0011509	4378	2497	-0,810
FBgn0028693	3012	1719	-0,809
FBgn0010591	1817	1039	-0,806
FBgn0002780	6518	3731	-0,805
FBgn0025839	1216	696	-0,805
FBgn0039303	6342	3635	-0,803

FBgn0033428	1399	803	-0,802
FBgn0034646	2153	1235	-0,801
FBgn0035771	5878	3373	-0,801
FBgn0023175	3618	2077	-0,801
FBgn0086443	6297	3616	-0,800
FBgn0034050	1321	760	-0,798
FBgn0053303	8670	4988	-0,798
FBgn0261244	1016	585	-0,797
FBgn0086134	4728	2722	-0,796
FBgn0260985	1326	765	-0,792
FBgn0011584	3355	1938	-0,792
FBgn0029134	5161	2982	-0,792
FBgn0015766	13320	7697	-0,791
FBgn0028688	3048	1762	-0,791
FBgn0065046	4413	2555	-0,788
FBgn0020255	13214	7660	-0,787
FBgn0035375	1607	931	-0,787
FBgn0003676	8261	4794	-0,785
FBgn0250848	11793	6859	-0,782
FBgn0034259	2469	1436	-0,782
FBgn0261394	4357	2535	-0,781
FBgn0038738	2563	1494	-0,779
FBgn0015282	3244	1892	-0,778
FBgn0028833	3736	2180	-0,777
FBgn0022160	2737	1602	-0,773
FBgn0037930	4706	2763	-0,768
FBgn0039580	7353	4323	-0,766
FBgn0032480	3415	2012	-0,763
FBgn0040075	1941	1145	-0,761
FBgn0030519	3169	1873	-0,759
FBgn0262515	5633	3328	-0,759
FBgn0051301	1805	1068	-0,757
FBgn0023549	1694	1002	-0,757
FBgn0025457	1652	978	-0,756
FBgn0028684	2247	1339	-0,747
FBgn0014857	4664	2783	-0,745
FBgn0035432	7672	4582	-0,744
FBgn0263852	1713	1023	-0,744
FBgn0032198	4684	2798	-0,743
FBgn0039108	6219	3716	-0,743
FBgn0038196	1356	811	-0,742
FBgn0028425	7687	4597	-0,742
FBgn0030582	2087	1251	-0,739
FBgn0041164	2752	1654	-0,735
FBgn0031389	8440	5094	-0,729
FBgn0038318	5148	3108	-0,728
FBgn0000405	3536	2141	-0,724
FBgn0037354	1538	932	-0,722

FBgn0024196	2771	1682	-0,720
FBgn0016685	5513	3347	-0,720
FBgn0021795	3108	1887	-0,720
FBgn0050344	6846	4160	-0,719
FBgn0003278	1795	1094	-0,715
FBgn0038947	5714	3482	-0,714
FBgn0035400	3954	2412	-0,713
FBgn0003607	4090	2504	-0,708
FBgn0029709	4465	2734	-0,708
FBgn0011638	6637	4067	-0,707
FBgn0033378	1795	1100	-0,706
FBgn0062442	2270	1391	-0,706
FBgn0020369	1457	895	-0,702
FBgn0016691	4693	2885	-0,702
FBgn0010470	3457	2126	-0,701
FBgn0035630	13075	8059	-0,698
FBgn0034628	1711	1055	-0,698
FBgn0028475	2546	1575	-0,693
FBgn0035203	1627	1008	-0,692
FBgn0022343	1876	1164	-0,689
FBgn0029174	4765	2959	-0,687
FBgn0033901	3504	2177	-0,687
FBgn0026380	2948	1834	-0,685
FBgn0010638	3589	2233	-0,684
FBgn0040493	4349	2714	-0,680
FBgn0035772	7142	4458	-0,680
FBgn0012034	4398	2747	-0,679
FBgn0040773	5153	3221	-0,678
FBgn0025724	9895	6189	-0,677
FBgn0263488	3314	2073	-0,676
FBgn0030514	2322	1454	-0,676
FBgn0052626	8789	5507	-0,674
FBgn0003124	5817	3649	-0,673
FBgn0036764	1939	1219	-0,670
FBgn0036623	1745	1097	-0,670
FBgn0010602	5176	3254	-0,670
FBgn0002022	7172	4512	-0,669
FBgn0029849	2036	1281	-0,668
FBgn0039830	11361	7150	-0,668
FBgn0000053	2824	1778	-0,668
FBgn0035321	2055	1294	-0,667
FBgn0014869	1704	1075	-0,665
FBgn0041182	30318	19199	-0,659
FBgn0026170	9980	6327	-0,657
FBgn0000404	6476	4109	-0,656
FBgn0038829	2126	1351	-0,655
FBgn0037834	4946	3143	-0,654
FBgn0037552	3097	1973	-0,651

FBgn0028694	2177	1389	-0,649
FBgn0027496	2794	1783	-0,648
FBgn0039172	3661	2338	-0,647
FBgn0000071	2985	1906	-0,647
FBgn0038065	3674	2347	-0,646
FBgn0262057	2270	1451	-0,646
FBgn0016120	2741	1752	-0,646
FBgn0014879	1610	1030	-0,644
FBgn0015283	2976	1909	-0,640
FBgn0004867	22558	14473	-0,640
FBgn0034345	7990	5133	-0,638
FBgn0034697	2623	1686	-0,638
FBgn0024432	2709	1742	-0,637
FBgn0031260	7720	4967	-0,636
FBgn0004066	5090	3283	-0,633
FBgn0011272	38515	24844	-0,633
FBgn0030658	2682	1730	-0,632
FBgn0032704	9100	5882	-0,630
FBgn0011672	4613	2985	-0,628
FBgn0032216	2071	1341	-0,628
FBgn0028687	5274	3419	-0,625
FBgn0031066	4013	2604	-0,624
FBgn0028491	2242	1456	-0,623
FBgn0025865	5257	3416	-0,622
FBgn0033844	4248	2761	-0,622
FBgn0036213	90022	58768	-0,615
FBgn0028336	2401	1573	-0,610
FBgn0031376	3106	2037	-0,609
FBgn0040529	2709	1778	-0,607
FBgn0013770	24276	15965	-0,605
FBgn0264294	4228	2789	-0,600
FBgn0000084	5016	3313	-0,598
FBgn0029823	6742	4454	-0,598
FBgn0013750	6771	4491	-0,592
FBgn0010348	19778	13174	-0,586
FBgn0263351	2514	1675	-0,586
FBgn0003885	2889	1927	-0,584
FBgn0011361	3610	2410	-0,583
FBgn0024352	7777	5196	-0,582
FBgn0000024	72	0	-Inf
FBgn0264087	61	0	-Inf
FBgn0028573	53	0	-Inf

9. Discussion

9.1 General mRNA degradation on ribosomes

Both translation and decay play a significant role in the regulation of gene expression. For many years it has been postulated that translation and degradation are separate processes. It was believed that degradation requires dissociation of mRNAs from the ribosome followed by their accumulation in P-bodies where most of the degradation factors are stored and where the degradation is taking place (Franks and Lykke-Andersen, 2008; Sheth and Parker, 2003). Recently, we have been noticing the interplay between mRNA translation and mRNA decay suggesting the close temporal and spatial link between these two processes and therefore the possibility of co-translational mRNA degradation on the ribosome.

To test the hypothesis about co-translational mRNA degradation for both general and miRNA-mediated degradation we have tested association of NOT2 with ribosomes, as a component of CCR4-NOT deadenylase complex, HPat, as a decapping activator, and AGO1, as a part of the miRISC complex in *Drosophila* as a higher Eukaryote. First, we have performed the polysome analysis experiments. All those factors have been detected in polysome fractions (Figure 1). These findings are in correlation with results of the other studies found in literature so far. For example, DCP2 decapping enzyme (Wang et al., 2002), Dhh1 decapping activator (Sweet et al., 2012), XRN1 5'-3' exoribonuclease (Mangus and Jacobson, 1999), and even human AGO2 (Landthaler et al., 2008) have been shown to co-sediment with polysomes. Furthermore, Hu et al. demonstrated the decapped nonsense-containing CYH2 mRNA associated with polyribosomes in yeast (Hu et al., 2010). Not only that NMD intermediates were detected, but also intermediates of general mRNA degradation were caught on the polyribosomes. Namely, in yeast, mRNA degradation intermediates of three endogenous genes, CYH2, ADH1, and RPL41a were detected on the polyribosomes (Eystathiou et al., 2003; Hu et al., 2009). Taken these together it is obvious that translation and degradation are tightly coupled on the polyribosomes and polyribosomes could be suggested as a place for degradation of aberrant mRNAs but also of general 5'-to-3' degradation in yeast. Nevertheless, co-translational mRNA degradation could occur not only in yeast but also in *Drosophila* as a representative of higher Eukaryote which unlike yeast contains miRNAs, one of the most crucial regulators of translation and degradation.

Co-sedimentation of degradation factors with polysomes that we have shown by using polysome analysis (Figure 1) was indicating the possibility of co-translational degradation also in *Drosophila*. However, there was a concern that detected factors could co-sedimenting with P-bodies or pseudo-polysomes (Fukaya and Tomari, 2012; Thermann and Hentze, 2007) which are high-molecular-weight

structures that itself could co-sediment with polysomes and give false-positive results. In any case, polysome analysis gave us a good hint to investigate further the possibility of co-translational degradation also in *Drosophila*. Therefore, we have established ribosome affinity purification to test the association of degradation factors with ribosome complexes (Figure 2) depleted from P-bodies and pseudo-polysomes. Using ribosome affinity purification method, we could detect subunits NOT1 and NOT2 of CCR4-NOT deadenylase complex, then several decapping factors as HPat, EDC4 and Me31B, and the exoribonuclease XRN1 (Figure 3). So, all tested deadenylation and decapping factors were found on ribosome complexes. Finding HPat and Me31b on ribosomes was crucial since it was known that they appear to couple translation and decapping. Coupling might be the result of modulation of the mRNP composition on mRNAs. HPat and Me31b create two different classes of mRNAs. One type is associated with translation initiation factors and capable of recruiting ribosomes and initiate translation. The second class is related to translational repressors and the mRNA degradation machinery responsible for translation repression and induction of mRNA degradation (Decker and Parker, 2012; Sharif H et al., 2013). Upon signal for degradation HPat and Me31b are vital proteins responsible for assembling a translation repression complex. This leads to sequestration of the mRNA into a mRNP that is unable to be accessed by translation initiation factors anymore while still associated with ribosome complexes. In that case, mRNPs are remodeled from a translationally active state to allow decapping complexes to form and degrade the mRNA on ribosome complexes. However, a quantitative kinetic analysis of the binding interactions between mRNAs and translation initiation components and decapping factors would shed light on the exact order of events and the apparent competition between translation initiation and decapping on ribosome complexes.

Interestingly, the novel role of Pat1 has been discovered in yeast starting from our observation that HPat is associated with ribosomes. Namely, mutants of Pat1 were not only deficient in decapping, but they were also defective in rRNA processing. (Muppavarrapu et al., 2016). Thus, it was concluded that Pat1 is also required for efficient ribosome biogenesis in yeast. On the other hand, engagement of HPat in ribosome biogenesis confirmed once again the close relationship between Pat1 proteins and ribosomes.

The next experiment we did with limited treatment with micrococcal nuclease and degradation factors did not show co-purification with ribosome complexes (Figure 4). Micrococcal nuclease, a relatively non-specific endo-exonuclease, cleaves unprotected parts of mRNA which may suggest the mRNA-dependent association of factors with ribosome complexes. However, micrococcal nuclease could digest also unprotected regions of rRNA, so the further experiments are needed to test whether factors are associated with mRNA or rRNA of ribosome complexes.

Finding the factors of degradation machinery linked to the ribosomes in mRNA dependent manner is the prerequisite for co-translational mRNA degradation, but for concluding co-translational degradation, it was necessary to detect degradation intermediates co-purifying with the ribosomes complexes. For several reporter constructs and endogenous genes of yeast as CYH2, ADH1, and RPL41a (Hu et al., 2009), mRNA degradation intermediates were found on ribosomes (Hu et al., 2010). We were additionally curious about this phenomenon in *Drosophila* and how general is the process on the transcriptome-wide level. To be able to answer the question whether the degradation is happening on the ribosome only for specific mRNAs or for the bulk mRNAs we have performed high-throughput sequence analysis in *Drosophila* cells. The libraries were made from decapped mRNA intermediates and not from deadenylated since general 5'-to-3' decay begins with loss of PABPC and deadenylation, as the first step (Tharun and Parker, 2001). Thus, decapped mRNA is already deadenylated as well. Secondly, if we have made the deadenylated libraries, it would not have been impossible to detect the degradation intermediates of the mRNAs that contain no poly(A) tails, as in the case of histone mRNAs. 5'-to-3' decay was considered since 3'-to-5' decay deadenylation-dependent pathway is usually minor degradation pathway happening in minor cases. In addition, co-translational 3'-to-5' decay could mainly result in aberrant nascent protein in case the last ribosome did not terminate the translation and the 3'-to-5' decay has already initiated. Thus, decapped libraries, made from decapped intermediates isolated from input lysate and ribosome complexes, have been sequenced. When compared the abundance of decapped intermediates from lysate and from ribosome complexes we could see that 93% of detected transcripts had the same abundance in input and on the ribosome. Only 5% were less abundant on the ribosome, and 2% showed a bit more abundance on the ribosome (Figure 5C). For several transcripts, this was even confirmed by qPCR. Finding the decay intermediates highly abundant on the ribosomes indicated the co-translational mRNA degradation and not only for several mRNAs but it is a general phenomenon for the bulk mRNAs.

Almost at the same time when we have published our observations Pelechano et al. have shown that mRNAs are co-translationally degraded. They have presented a 5PSeq approach which provides a rapid and complementary method to measure ribosome dynamics in vivo, avoiding artifacts caused by translation inhibitors used during ribosome profiling. By applying this method to *Schizosaccharomyces pombe*, they demonstrate not only co-translational decay but also that co-translational decay is a widespread and conserved process affecting most genes. Just highly translated genes are less often co-translationally degraded. Further research is needed to study to what degree this process is conserved in higher eukaryotes (Pelechano et al., 2015).

Bond between translation and decay has been consistently observed for a class of transcripts derived from immediate early genes (IEGs) (Almendral et al., 1988; Yamazaki et al., 2008). IEGs encode

cytokines, transcription factors, or proto-oncogenes that activate downstream signaling and cell growth (Bahrami and Drabløs, 2016). The translation-dependent decay of IEG transcripts ensures transient protein production, which may be essential for preventing uncontrolled cell growth. The mechanism of translation-dependent decay is known only for several transcripts. One of them is a Fos, an IEG, and a critical proto-oncogene. Fos CDS contains a protein-binding sequence called the major coding-region determinant of instability (mCRD) (Schiavi et al., 1994) that stabilizes Fos mRNA before translation (Grosset et al., 2000). The current model is that ribosome transit disrupts or alters a protein complex that bridges the mCRD and poly(A) tail, leading to degradation of the transcript (Bicknell and Ricci, 2017).

Recently, mRNAs with non-optimal codons have emerged as a new class of transcripts manifesting coupling of translation and decay in a translation-dependent decay manner (Presnyak et al., 2015; Radhakrishnan and Green, 2016; Bazzini et al., 2016; Mishima and Tomari, 2016). The term ‘codon optimality’ describes the differential decoding efficiency for the 61 different codons. It has been revealed that codon bias - the uneven use of synonymous codons in the transcriptome - serves as a secondary genetic code: a code that guides the efficiency of protein production, the fidelity of translation and the metabolism of mRNAs (Hanson and Collier, 2018). Codon optimality depends on the abundance of all tRNAs that can recognize a codon, and the nature of the base-pairing interaction between the codon and the tRNA(s) that recognize it (Reis dos et al., 2004). This was first observed for the yeast PGK1 gene, which was expressed from its endogenous locus as wild-type or with non-optimal synonymous codon substitutions (Hoekema et al., 1987). In addition to both slower elongation and reduced protein levels, the non-optimal version exhibited dramatically lower PGK1 mRNA levels, suggesting mRNA destabilization. More recently, Presnyak et al. measured *S. cerevisiae* decay rates transcriptome-wide and found a striking positive correlation between the percentage of optimal codons and mRNA half-life. Interestingly, for Metazoa, the use of non-optimal codons is common for maternal transcripts during embryonic development. During maternal-to-zygotic transition (MZT), maternal mRNAs are degraded when embryonic mRNAs are starting to be synthesized (Tadros and Lipshitz, 2009). The use of non-optimal codons in maternal transcripts may serve to rapidly and tightly link the timing of decay with fertilization via translation-dependent degradation. Beside maternal mRNAs, proteins involved in transient responses to stimuli, such as the pheromone response, are also enriched in non-optimal codons. This may reflect selective pressure to keep the levels of such regulatory gene products low, and the instability conveyed by prominent levels of non-optimal codon content helps to ensure that responses to transient stimuli can be quickly curtailed when the stimulus is withdrawn. One well-established example of this is the clock genes, which control circadian rhythms in various organisms. The mechanism behind linkage of codon optimality and decay is that RNA stability is directly linked to ribosome elongation rates in a Dhh1-dependent manner. Dhh1 senses

elongation rates and triggers decay when slow-moving ribosomes are present (Radhakrishnan et al., 2016).

Beside the co-translational 5'-to-3' deadenylation-dependent decay pathway recently has also been confirmed that 3'-to-5' deadenylation-dependent decay triggered by the exosome is taking place on the ribosome. Schmidt et al. reported that the Ski complex directly associates with 80S ribosomes and guides RNAs to the exosome complex. Therefore, the exosome is in association with a mRNA which is bound to the ribosome (Schmidt et al., 2016). The data reported previously by Bönisch et al. 2007 and by Wahle et al. lead to the following plausible model for the co-translational decay of the Hsp70 mRNA. The Hsp70 mRNA loses its poly(A) tail while it is being translated. The deadenylated decay intermediate accumulates transiently in a capped form (Bönisch et al., 2007) and can be degraded in a 5'-to-3' pathway. Alternatively, while being translated, the deadenylated, capped RNA can be digested from the 3' end. This is a minor pathway (Bönisch et al., 2007) and proceeds through detectable paused intermediates, which are substrates for addition of adenin by Trf4-1 and possibly additional noncanonical poly(A) polymerases. Oligoadenylate facilitates further digestion by the exosome. It is quite similar process as recent analysis of mammalian histone mRNA decay (Slevin et al., 2014) that were found to be degraded by the exosome in 3'-5' direction while being still translated.

9.2 miRNA-mediated mRNA degradation on the ribosomes

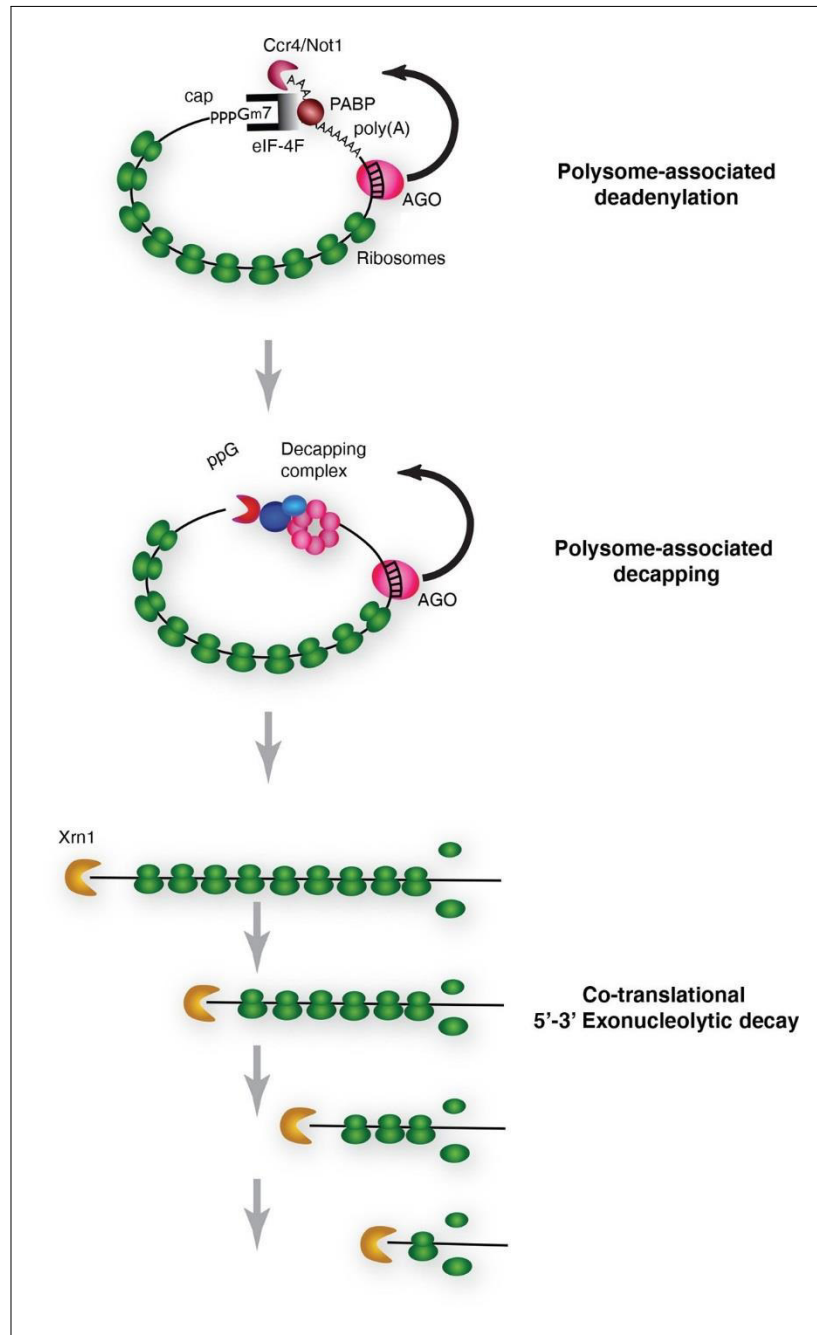
Interestingly, our observation showed that also components of miRISC AGO1 and GW182 are co-purifying with ribosome complexes that are deprived of P-bodies or pseudo-polysomes (Figure 3). Thus, not only general but also miRNA-mediated mRNA degradation is taking place on the ribosome complexes.

Since among 93% of detected transcripts whose degradation intermediates were found on ribosomes were also identified miRNA targets, we could broaden our model to not only general but also miRNA-mediated co-translational degradation. The similar observations were noticed by many other studies. Those studies have been showing that miRNA-targeted mRNAs are entirely associated with polysomes (Olsen and Ambros, 1999; Seggerson et al., 2002). Even several studies indicate that a considerable number of miRNAs themselves associate with actively translating ribosomes (Nelson et al., 2004; Maroney et al., 2008). Tat et al. have analyzed endogenous bantam and a representative member of the miR-2 family (miR-13b) and together with their mRNA targets. They have found the decapped fragments of the reporter mRNA while it was still polysome-bound (Tat et al., 2016). Other observations indirectly showed a need for interaction between miRNA-effector complexes and translating ribosomes to trigger the decay (Bartel, 2009). Namely, they have noticed that MREs (miRNA responsive elements) are less efficient when positioned in open reading frames.

Finally, co-translational mRNA degradation could lead to better understanding of miRNAs working model. It was for a long time believed that general and miRNA-mediated mRNA decay take place when mRNAs are in a ribosome-free state, in subcellular aggregates known as P-bodies which are sites of concentrated RNA degradative activities (reviewed in Valencia-Sanchez et al., 2006). Thus, it was hard to reconcile the large amount of evidence that shows that most miRNA-mediated downregulation occurs making mRNA destabilized and not only translationally repressed. But once it was demonstrated that general mRNA decay could happen co-translationally (Hu and al., 2009) and that miRNAs use the general machinery for degradation (Braun et al., 2011; Fabian et al., 2012; Chekulaeva et al., 2011) we were closer to the solution for this longstanding puzzle. Showing miRNA RISC components, miRNAs and miRNA-targeted mRNAs intermediates on the ribosomes, we could assemble miRNA-mediated mRNA decay model according to which miRNA mediates mRNA decay co-translationally.

A simple model could be made where miRNA complex recruits the CCR4-NOT deadenylase complex through GW182 and sequentially the translational repressor and decapping activator DDX6 which then promotes mRNAs exiting translation leading to either translational repression and mRNA storage or mRNA degradation on the ribosomes which started with deadenylation, followed by decapping and finally degraded by XRN1 (Model 1).

There is an alternative model which suggests that AGOs repress translation by interfering with eIF4A function. This might be happening during 43S ribosomal scanning, and this mechanism could be independent of GW182 and the CCR4-NOT complex in *D. melanogaster* (Meijer et al., 2013).



Model 1. Pathway of miRNA-mediated co-translational mRNA decay. miRNA complex recruits CCR4-NOT deadenylase complex which proceeds with decapping and 5'-3' degradation by XRN1 while still associated with polysomes (Figure derived from Tat et al., Elife, 2016).

Thus, finding components of miRNA complex on the ribosome and components of general mRNA degradation machinery could explain how miRNAs through a combination of translational repression and mRNA destabilization modulate both translation and decay swiftly and efficiently.

9.3 P-bodies

Our findings that decay components and intermediates are associated with ribosomes are consistent with the observations that P-bodies are not the only places for mRNA decay and translational repression (Decker et al., 2007; Sweet et al., 2007). There is currently a lot of evidence that there is no need for assembling of mRNA into P bodies to be silent or degraded. First, decay factors are found in P bodies but less than 10% of their cytoplasmic pool. Most of these factors are diffusely distributed elsewhere in the cytoplasm (Leung et al., 2006; Aizer et al., 2008). Furthermore, P-bodies are not obligatory sites for translational repression and decay in yeast because when the integrity of P-bodies is disrupted through the deletion of EDC3, the translation or degradation of bulk or sequence-specific mRNAs is not interrupted. (Stoecklin et al., 2006; Decker et al., 2007; Eulalio et al., 2007b). The same case was when deadenylation was blocked by knocking down mammalian Caf1 or overexpressing mutant variant that impaired P-body formation, but deadenylation was not reduced (Zheng et al., 2008). miRNA-mediated repression can be carried out without P-bodies in other organisms (Eulalio et al., 2007b). Also, although some studies have shown localization of NMD components in P-bodies (Cho et al., 2006; Durand et al., 2007; Unterholzner and Izaurralde, 2004), depletion of Ge-1, an essential component of P-bodies, does not inhibit NMD (Stalder and Mühlemann, 2009). All together, these observations indicate that P-body formation requires the decay factors and translation repressors, whereas decay and translation repression does not require P-body formation.

Interestingly, novel cell structures were described based on our observation that polysomes are an alternative site for RNA decay (Patel et al., 2016). Namely, P-bodies were also called GW-bodies, since GW182 and AGO1 are often associated with P-bodies and was thought that both general and miRNA-mediated decay takes place there (Eystathiou et al., 2003; Behm-Ansmant et al., 2006). As we have seen above, recently many reports were showing that neither deadenylation regulators nor decapping factors are present in P (GW)-bodies in vivo. That suggested firstly that GW-bodies are perhaps not the same structures as P-bodies and secondly that GW-bodies are not the sites for RNA decay (Patel et al., 2016). For example, use of inhibitor cycloheximide which sequesters RNAs in polysomes does not decrease the GW-bodies (Patel et al., 2016). However, when puromycin was used, and puromycin is known to inhibit protein translation through the premature release of translating RNAs from the ribosome, the pool of non-translating RNAs increased, and it led to an enlargement of GW-bodies. That indicates that GW-bodies are composed of non-translating mRNAs (Patel et al., 2016). Taking all data together was concluded that GW-bodies are not the same as P-bodies plus that effective translational silencing most likely does not take place in GW-bodies. Instead GW-bodies most likely function as a

place for translationally silenced RNAs to store while ribosomes are decay area. Identification of RNAs present in GW-bodies may reveal RNAs important for regulating developmental processes such as cellularization. Furthermore, future characterizations of the RNA contents of GW-bodies will provide valuable insights into the functional role of these structures.

However, P-bodies and stress granules could still have some role in repression and decay. One possibility is that mRNP aggregation is limited to a subset of mRNAs (Decker and Parker, 2012). Another option is that mRNAs aggregate into P-bodies just in specific conditions, for instance in extreme demand of the mRNA degradation machinery and then P-bodies may be a by-product, manifested as visible mRNP aggregates rather than a cause of mRNA degradation (Eulalio et al., 2007b). Many observations have shown enlargement of P-body size and number in the stress environment. For example, in budding yeast, unicellular organism, changes in nutrient availability, osmotic pressure, ultraviolet light exposure, or low pH trigger movement of translating RNAs into P-bodies (Teixeira et al., 2005; Iwaki and Izawa, 2012). Similar roles for P-bodies have also been noticed in multicellular organisms during particular processes. For example, oogenesis in *Drosophila* is regulated by nutrient availability, and starvation of female flies induces a mid-oogenesis checkpoint. It leads to the degradation of late stage egg chambers and an arresting of previtellogenic egg chambers (Drummond-Barbosa and Spradling, 2001; Mazzalupo and Cooley, 2006). This nutrient withdrawal induces P-body formation in arrested nurse cells, which appears to be critical for maintaining oocyte viability (Shimada et al., 2011). Thus in vivo, the P-body formation is also associated with RNA storage during stress-induced cellular quiescence.

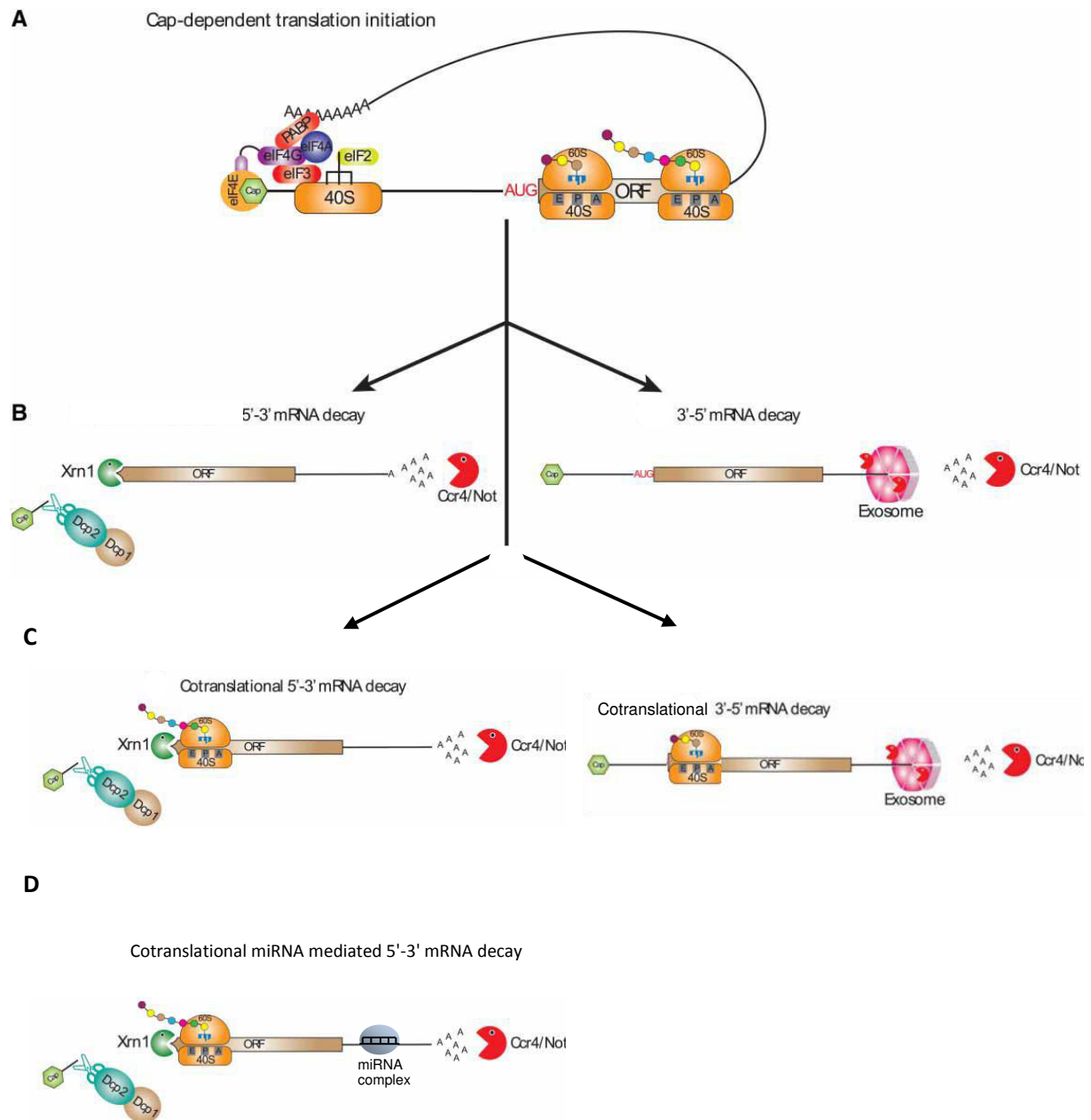
Stress granules could be aggregates of mRNPs stalled in the process of translation initiation caused by stress conditions (Kedersha and Anderson, 2007). Therefore, this message can be stored as stable species so that they can be reutilized once the stress conditions are overcome. Arresting translation and maintaining transcripts in a calm, inert form provides the cell with a complex poised to respond quickly to biological cues.

9.4 Conclusion and perspectives

Translation, initiation of translation, repression, deadenylation, and decapping are in competitive but coordinated relationship to prevent endless translation and to fulfill the needs of the cell. Previously, it was thought that translation of a mRNA prevents its degradation and mRNA surveillance mechanisms, which degrade mRNAs during their translation, were exceptions. Recently, however, it has become clear that many mRNAs, including mi-RNA targets, are degraded co-translationally. Upon signal a conserved set of proteins, Pat1 and Dhh1, function to modulate the transition between mRNAs engaged in translation initiation and a mRNP competent for mRNA decay. They provide at the same

time removal of initiation factors and recruitment of more decapping factors (Decker and Parker, 2012; Sharif et al., 2013) which results in one of two well established decay pathways (Model 2B).

Taking together the studies from the others and our observations we could suggest building up the scheme of mRNA degradation which besides well established 5'-3' and 3'-5' ones can happen co-translationally as well (Model 2C and D).



Model 2. mRNA degradation. **A)** Upon assembling translation initiation complex, mRNA enters translational pool going through a couple of translation cycles until it receives the signal for degradation. **B)** Upon signal for degradation, ribosomes are leaving, and mRNA is degraded in 5'-3' or 3'-5' degradation manner. **C)** Alternatively, mRNA is degraded co-translationally in 5'-3' or 3'-5' degradation manner while mRNA still associated with

ribosomes. **D)** miRNA targets are also degraded co-translationally (The Model adapted from Hanson and Collier, Nat Rev Mol Cell Biol, 2018)

Providing degradation happening on ribosomes it makes a quick response to changes in environmental conditions without losing time and energy for dropping off from the ribosomes and translocation of mRNAs to P-bodies. Only special mRNAs or in different stressful condition ribosomes run off, and mRNAs could accumulate in P-bodies or stress granules waiting for better conditions to re-enter translational pool.

The mode of cross-talk between translation and mRNA decay influences the way protein output from a transcript can be regulated and is likely linked to the function of the encoded protein. We have just begun to scratch the surface in understanding the functional implications of the many ways that the translation and mRNA decay machinery can interact. A better understanding of mechanism and location of both general and miRNA-mediated degradation of mRNAs could improve the understanding not only of the kinetics of mRNA degradation and regulation but also how biological systems with all its complexity respond to various external stimuli to overcome the stressful situation and keep its homeostasis. Application of our approach to other species will help to understand the global principles of mRNA degradation and gene expression regulation in the context of a diverse array of cellular processes, from development and differentiation through proliferation and neoplastic transformation to the response to different stress conditions.

10 References

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