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FUNCTIONS OF DEADENYLATION FACTORS IN MRNA DECAY AND MRNA PROCESSING BODY FORMATION

Dinghai Zheng

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**FUNCTIONS OF DEADENYLATION FACTORS IN mRNA DECAY AND
mRNA PROCESSING BODY FORMATION**

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A
DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
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in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2010

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FUNCTIONS OF DEADENYLATION FACTORS IN mRNA DECAY AND mRNA PROCESSING BODY FORMATION

Publication No. _____

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Most newly synthesized messenger RNAs possess a 5' cap and a 3' poly(A) tail. The process of poly(A) tail shortening, also termed deadenylation, is important for post-transcriptional gene regulation, because deadenylation not only leads to mRNA translational inhibition but also is the first step of major mRNA degradation. Translationally inhibited mRNAs can be stored and/or degraded in dynamic cytoplasmic foci termed mRNA processing bodies, or P bodies, which are conserved in eukaryotes.

To shed new light on the mechanisms of P body formation and P body functions, I focused on the link between deadenylation factors and P bodies. I found that the two major deadenylation complexes, Pan3-Pan2 and Ccr4-Caf1, can both be enriched in P bodies. The deadenylase activity of the Ccr4-Caf1 complex is prerequisite for P body formation. Pan3, but not the deadenylase Pan2, is essential for P body formation. While the C-terminal domain of Pan3 is important for interaction with Pan2, Pan3 N-terminal domain is important for Pan3 to form cytoplasmic foci colocalizing with P bodies and to promote mRNA decay. Interestingly, Pan3 N-terminal domain may be phosphorylated to regulate Pan3 localization and functions. Aside from the functions of the two deadenylation complexes in P bodies, I also studied all reported human P body proteins as a whole using bioinformatics. This effort not only has generated a comprehensive picture of the functions of and interactions among human P body proteins, but also has predicted proteins that may regulate P body formation and/or functions.

In summary, my study has established a direct link between mRNA deadenylation and P body formation and has also led to new hypotheses to guide future research on how P body dynamics are controlled.

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Abbreviations

ARE	AU-rich element
BTG	B-cell translocation gene
Caf1	CCR4-associated factor 1
CAT-1	Cationic amino acid transporter 1
Ccr4	Carbon catabolite repressor protein 4
CDK5	Cyclin-dependent kinase 5
CIAP	Calf intestinal alkaline phosphatase
CNOT7	CCR4-NOT transcription complex, subunit 7
CNOT8	CCR4-NOT transcription complex, subunit 8
CPEB1	Cytoplasmic polyadenylation element binding protein 1
DEPP	Disorder Enhanced Phosphorylation Predictor
eIF3	Eukaryotic translation initiation factor 3
eIF4A	Eukaryotic translation initiation factor 4A
eIF4B	Eukaryotic translation initiation factor 4B
eIF4E	Eukaryotic translation initiation factor 4E
eIF4E-T	Eukaryotic translation initiation factor 4E nuclear import factor 1
eIF4G	Eukaryotic translation initiation factor 4G
eRF3	Translation release factor 3
IRES	Internal ribosome entry site
LLR	Leucine-rich repeat
LMB	Leptomycin B
m ⁷ G	7-methylguanosine
mRNP	Messenger ribonucleoprotein
NMD	Nonsense-mediated mRNA decay
NOT	Negative regulator of transcription
NP11	Nonphosphorylatable at eleven sites
P body	mRNA processing body
PABP	Poly(A) binding protein
PAM2	PABP-interacting <i>motif 2</i>
PAN	Poly(A) nuclease
Pan2	Poly(A) nuclease subunit 2
Pan3	Poly(A) nuclease subunit 3
PARN	Poly(A)-specific ribonuclease
PINA	Protein Interaction Network Analysis
PM11	Phosphor-mimetic at eleven sites
PPI	Protein-Protein Interaction
PTC	Premature termination codon
TOB	Transducer of ERBB2
UTR	Untranslated region
Xrn1	5'-3' exoribonuclease 1

Chapter 1: Introduction

1.1 The Importance of Eukaryotic mRNA Poly(A) Tail

In eukaryotic cells, newly synthesized nuclear messenger RNAs generally possess a 5' 7-methylguanosine cap (m⁷G cap) and a 3' polymer of adenosine-5'-monophosphate (poly(A) tail). The poly(A) tail was added to the pre-mRNA by a large cleavage/polyadenylation machinery via a two-step reaction. In metazoan cells, the first step is cleavage of the pre-mRNA between an A(A/U)UAAA hexamer and a U/GU-rich region. After cleavage, the poly(A) polymerase sequentially adds 100-250 adenosine residues to the 3' end of the cleavage product (1). The poly(A) tail is essential for both translation initiation and mRNA stability after the nascent transcripts are exported to the cytoplasm.

1.1.1 Poly(A) tail in mRNA translation initiation

Translation of most eukaryotic mRNAs is initiated in a cap-dependent manner, which requires several eukaryotic initiation factors (eIFs). The cap binding protein eIF4E interacts with eIF4G, which recruits the 43S pre-initiation complex (2), consisting of the ternary complex eIF2-Met-tRNA-GTP, the 40S ribosome subunit, and several initiation factors including eIF3 (3). The recruited 43S pre-initiation complex scans the 5' untranslated region (UTR) of the mRNA in a 5' to 3' direction, until the start codon is recognized by the Met-tRNA, which eventually leads to the joining of the 60S ribosome subunit and formation of the 80S initiation complex ready for translation elongation (4).

By simultaneously interacting with the poly(A) binding protein (PABP) and the cap binding protein eIF4E, the scaffold protein eIF4G physically links the 3' poly(A) tail and the 5' cap (5) (Fig. 1.1). The circularization of mRNA through the cooperative interactions between PABP, eIF4G, and eIF4E enhances the RNA helicase activity of translation initiation factors eIF4A, eIF4B, and eIF-iso4F (6), resulting in increased translation initiation. Moreover, the circularization of mRNA may enhance the recycling of ribosomes from the 3' end to the 5' end of the same mRNA, a notion further supported by the observation of interaction between the eukaryotic release factor 3 (eRF3) and PABP (7). The importance of poly(A) tail in cap-dependent translation initiation is exemplified during oocyte

maturation and early embryogenesis, during which the short poly(A) tails of a set of cytoplasmic mRNAs are elongated by cytoplasmic poly(A) polymerase. The polyadenylation transforms the mRNAs from translationally silent to active (8). A microarray analysis following separation of cellular mRNAs according to their poly(A) tail length indicated that yeast mRNA poly(A) tail length positively correlates with ribosome density (ribosomes per unit length of mRNA), further confirming the importance of poly(A) tail in translation (9).

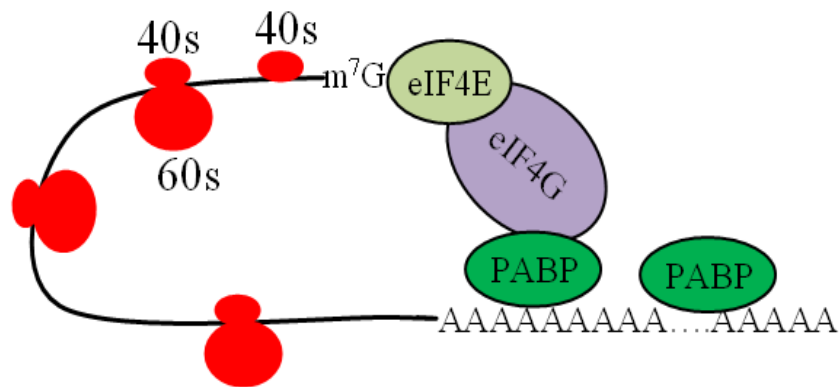


Figure 1.1: The closed-loop model. eIF4G interacts with both the cap binding protein eIF4E and the poly(A) tail binding protein PABP to circularize the mRNA and promote translation initiation.

Translation of some viral and about 10-15% of eukaryotic mRNAs can also be initiated in a cap-independent manner (10, 11) through direct recruitment of the 40S ribosome subunit by internal ribosome entry sites (IRES), which are highly structured regions within the 5' UTR. IRES-mediated translation initiation is generally not affected by conditions that inhibit cap-dependent translation initiation, thus allowing the cells to cope with some stressful situations (3). Surprisingly, IRES-mediated translation initiation of the c-myc and BiP mRNAs is also enhanced by the poly(A) tail, although PABP and eIF4G are not involved (12, 13).

1.1.2 Poly(A) tail in mRNA stability

The poly(A) tail not only enhances translation initiation, but also stabilizes eukaryotic mRNAs in the cytoplasm. The importance of poly(A) tail in stabilizing mRNAs is reflected by the fact that shortening of the poly(A) tail (deadenylation) is a prerequisite for the subsequent degradation of most cytoplasmic mRNAs, regardless of whether they are degraded by the exosome complex from 3' to 5' or by the 5' to 3' exonuclease Xrn1 following decapping (14, 15) (Fig. 1.2). In mammalian cells deadenylation is the first step of the decay of both stable messages, such as beta-globin mRNA (16), and unstable messages, such as mRNAs bearing premature termination codons (PTC) (16), AU-rich elements (AREs) (17, 18), or miRNA targeting sites (19, 20). Consistently, blocking deadenylation by knocking down the enzymes catalyzing the deadenylation process (deadenylases) or by over expression of catalytically inactive deadenylase mutants stabilized mRNAs in mammalian cells (16, 21).

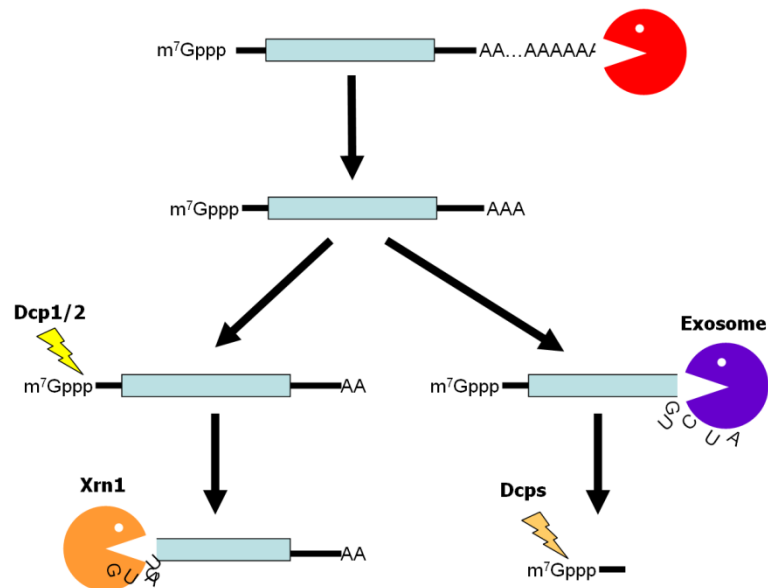


Figure 1.2: Deadenylation is the first step for the degradation of most cytoplasmic mRNAs. After deadenylation, mRNAs can be degraded in two pathways. In one pathway, the mRNAs are decapped and then degraded from 5' to 3' by Xrn1. In the other pathway, the mRNAs are degraded from 3' to 5' by the exosome complex, followed by Dcps-mediated decapping.

Why is the poly(A) tail so important for mRNA stability? The poly(A) tail and the associated cytoplasmic PABP may inhibit the 3' to 5' mRNA decay pathway simply by blocking the access of exosome to the mRNA. On the other hand, the 5' to 3' mRNA decay pathway can be inhibited by the poly(A) tail and associated PABP through several mechanisms. For example, in rabbit reticulocyte lysates, the cooperative interactions between PABPC, eIF4G, and eIF4E significantly strengthened the interaction between the 5' cap and eIF4E (22), which protects the 5' cap from the decapping machinery. PABP can also directly bind to 5' end of capped mRNA (23), which may help to inhibit decapping as well. In addition, the poly(A) tail and PABP can inhibit the recruitment of decapping activators such as the Lsm1-7p complex in yeast (24).

1.2 Major Eukaryotic Deadenylases

The mRNA poly(A) tail can be shortened from 3' to 5' by deadenylases in the cytoplasm. Two deadenylase complexes highly conserved in yeast and human are the PAN and Ccr4-Not complexes. Other deadenylases, such as PARN and Nocturnin, only exist in higher eukaryotes (25). In the following section, these deadenylases will be briefly reviewed.

The PAB-dependent poly(A)-specific ribonuclease (PAN) is a heterodimer composed of the Pan3 and Pan2 subunits. Pan2 is a Mg²⁺-dependent exoribonuclease that releases 5' -AMP from the 3' end of poly(A) tails (26, 27). Sequence analysis using Conserved Domain Database (28) showed that Pan2 contains an exonuclease domain, a peptidase domain, and a WD40 domain. The exonuclease domain is related to the DEDD superfamily and contains four conserved acidic residues that bind the Mg²⁺ ion involved in catalysis (29). Pan3 can interact with both Pan2 and PABP (Pab1p in yeast) to recruit Pan2 to the poly(A) tail. In addition, in vitro experiments further showed that PAN is activated by PABP associated with the RNA substrates (27, 30). Interestingly, the 3' UTR of yeast MFA2 RNA can also stimulate the activity of PAN, suggesting that PAN activity is not solely regulated by PABP (26).

In yeast, Pan3p-Pan2p slowly shortens mRNA poly(A) tails when the major deadenylase Ccr4p-Pop2p (yeast Caf1p) is deleted (31). Yeast strains harboring deficient Pan2p, Pan3p, or Pab1p (yeast homolog of PABP), produce mRNAs with longer poly(A) tails (30, 32, 33). In mammalian cells, the Pan3-Pan2 complex carries out the first phase of deadenylation in the cytoplasm by shortening mRNA tails from over 200 nt to about 110 nt before the Ccr4-Caf1 deadenylase takes over in the second phase of deadenylation (16, 19). Pan3 can be phosphorylated (34), but how Pan3 phosphorylation affects its localization and functions has never been studied.

Yeast Pan3p-Pan2p complex may also play a role in controlling the poly(A) tail length of newly synthesized mRNAs. It has been reported that partially purified RNA 3' -end processing machinery from yeast Pan3 Δ , Pan2 Δ , or Pan3 Δ Pan2 Δ strains produced transcripts with poly(A) tails longer than those from a wild-type yeast (35). Within 10 min after addition of purified Pab1p and Pan3p-Pan2p, the long tails were trimmed to 60-80 residues, a length normally observed in the wild-type extract. The poly(A) tails were then only slowly shortened over the next 20 min in the reaction. Additionally, *in vivo* transcriptional pulse chase experiments showed that the first population of *PGK1* transcripts generated after 4 minutes of galactose-mediated transcription had already undergone Pan3-Pan2 specific processing, indicating that Pan3p-Pan2p mediated rapid trimming of newly synthesized poly(A) tails *in vivo*. Thus, it was proposed that cleaved pre-mRNAs are polyadenylated to a long default length by the poly(A) polymerase machinery and then quickly deadenylated to a message-specific length by Pan3p-Pan2p in a Pab1-dependent manner (35). However, this model was challenged by a recent observation that the poly(A) tail length control of the *CYC1* mRNA in an *in vitro* system was restored by adding the nuclear poly(A)-binding protein Nab2p even in the absence of Pan3p and Pan2p (36).

In eukaryotes, the major deadenylase activity is carried by the CCR4-NOT complex which contains the deadenylases Ccr4 and Caf1 and several different NOT (negative regulator of transcription) proteins (37, 38). Ccr4 contains a leucine-rich repeat (LLR) responsible for interaction with Caf1 (39) and a ribonuclease domain similar to a family of Mg²⁺-dependent nucleases related to *E. coli* exonuclease III. Yeast Ccr4p exhibited poly(A)-specific 3' -exoribonucleases activity *in vitro* even in the absence of the

other subunits of the CCR4-NOT complex (40). Deletion of the CCR4 gene in yeast slowed down the rate of deadenylation and stabilized mRNAs (31). Unlike the Pan3p-Pan2p complex, the in vitro activity of yeast Ccr4p can be inhibited by Pab1p (38).

Although Ccr4p is the major deadenylase in the Ccr4-Not complexes in yeast (38), Caf1p (Pop2p) is also required for efficient mRNA degradation in vivo and has weak deadenylase activity in vitro (41, 42). Like Pan2, Caf1 is a member of the DEDD family of exoribonucleases (43). Human Caf1 has two paralogs, CNOT7 (hCaf1/Caf1a) and CNOT8 (hPop2/Caf1b), both of which are catalytically active and required for efficient proliferation of cultured cells (44).

In addition to Ccr4p and Caf1p, the 1-MDa yeast CCR4-NOT complex contains the five NOT proteins (Not1-5p) as well as Caf40p and Caf130p (45). Deletion of some of the NOT genes in yeast negatively affected reporter mRNA deadenylation (38). A microarray analysis of mRNAs from yeast strains with deletion of individual subunits of the Ccr4-Not complex showed that each of the subunits regulates the expression of a specific group of genes (46). In *Drosophila* Schneider cells, siRNA-mediated silencing of CAF1, NOT1, NOT2, and NOT3, but not CCR4, CAF40, or NOT4, inhibited bulk deadenylation (47). Interestingly, a yeast decapping factor, Dhh1p, co-immunoprecipitates with Not1p, suggesting a physical link between deadenylation and decapping machineries (48). (38)

The Ccr4-Not complex can be recruited to specific mRNAs to promote deadenylation of the mRNAs. TOB, a member of the anti-proliferative BTG (B-cell translocation gene) family, interacts with both PABP and human Ccr4-Not complex to promote mRNA deadenylation (49). TOB ^{-/-} and CNOT7(Caf1) ^{-/-} mice both exhibit increased bone formation (50, 51), suggesting that TOB may recruit the Ccr4-Not complex to promote the decay of transcripts of genes involved in bone formation. Yeast Vts1p, a sequence-specific RNA binding protein, interacts with and recruit the Ccr4-Not complex, resulting in deadenylation of the target mRNAs (52). In mammalian cells, the Ccr4-Not complex also mediates deadenylation of miRNA targets (19) and mRNAs bearing premature termination codon (PTC) or AU-rich elements (AREs) (21).

PARN, another member of the DEDD nuclease family, is a Mg^{2+} -dependent poly(A)-specific 3' exoribonuclease inhibited by PABP (53, 54). PARN contains an RNA-binding domain and can bind both the 5' cap and the poly(A) tail of mRNAs (55, 56). Interestingly, cap binding enhances the activity and processivity of PARN (55, 57). Although not found in yeast, PARN is indispensable for embryogenesis in higher plants and promotes deadenylation of a subset of embryo-specific mRNAs (58). Some studies, mostly using in vitro decay systems, suggested that mammalian PARN was recruited by several ARE binding proteins to promote deadenylation of transcripts containing AREs (59-61). However, direct in vivo evidence showed that unlike Pan2 and Ccr4, PARN did not play a major role in general deadenylation in mouse NIH 3T3 cells (16).

Nocturnin, a deadenylase related to Ccr4, is unique in that it is encoded by a clock-controlled gene whose transcription peaks at night in *Xenopus* retinal phosphoreceptor cells (62) and multiple tissues of mice (63). Interestingly, Nocturnin knockout mice have defects in lipid metabolism and are therefore immune to diet-induced obesity and hepatic steatosis(64).

In summary, different deadenylases may function in unique ways, which may be overlapping sometimes, to shorten mRNA poly(A) tails, leading to translational inhibition and/or degradation of the mRNAs.

1.3 mRNA Processing Bodies

mRNA processing bodies, also named P bodies or GW bodies, are dynamic cytoplasmic aggregates of non-translating mRNAs and factors involved in mRNA translational inhibition and decay. Conserved from yeast to human, P bodies have a diameter of 100-300 nm (65). These membrane-free structures are mainly anchored to microtubules while constantly moving around in the cytoplasm (66).

Under light microscope, P bodies co-localize with translationally inhibited mRNAs and around 30 or 50 different proteins in yeast and human cells, respectively. These P body proteins are either involved in mRNA binding (e.g. eIF4E), translational inhibition (e.g. eIF4ET and CPEB1), mRNA decapping (e.g. Dcp1/2 and Lsm1-7), mRNA 5' to 3' degradation (e.g. Xrn1), miRNA-mediated gene silencing (e.g. Ago1-4 and GW182), or with unknown functions (e.g. ZAR11) (67, 68). Factors involved in nonsense-

mediated decay (NMD) were enriched in P bodies when NMD is interrupted in yeast (69) and in mammalian cells (70). In contrast, most factors involved in mRNA translation, for example PABP, eIF4G, and ribosome subunits, are normally absent from P bodies (71).

P bodies are sites for mRNA translational inhibition, degradation, and storage (Fig. 1.3). Because P bodies are ribosome-free, mRNAs in P bodies cannot be translated. Some mRNAs are likely degraded in P bodies, as inhibition of 5' to 3' decay by knocking down Xrn1 increased P body sizes and mRNA decay intermediates were found in P bodies (72). However, P bodies may also be temporary storage sites for some mRNAs, as suggested by an observation that a P body mRNA encoding cationic amino acid transporter 1 (CAT-1) was released from P bodies and subsequently translated when the cells were subjected to different stresses (73). Therefore, P bodies may function as a “lockup” where the fates of arrested non-translating mRNAs will be determined.

Although P bodies are conserved in eukaryotes, the physiological significance of their formation is unknown. One possibility is that by enriching local concentrations of translational inhibitors and mRNA decay factors, cells can silence unwanted mRNAs more effectively.

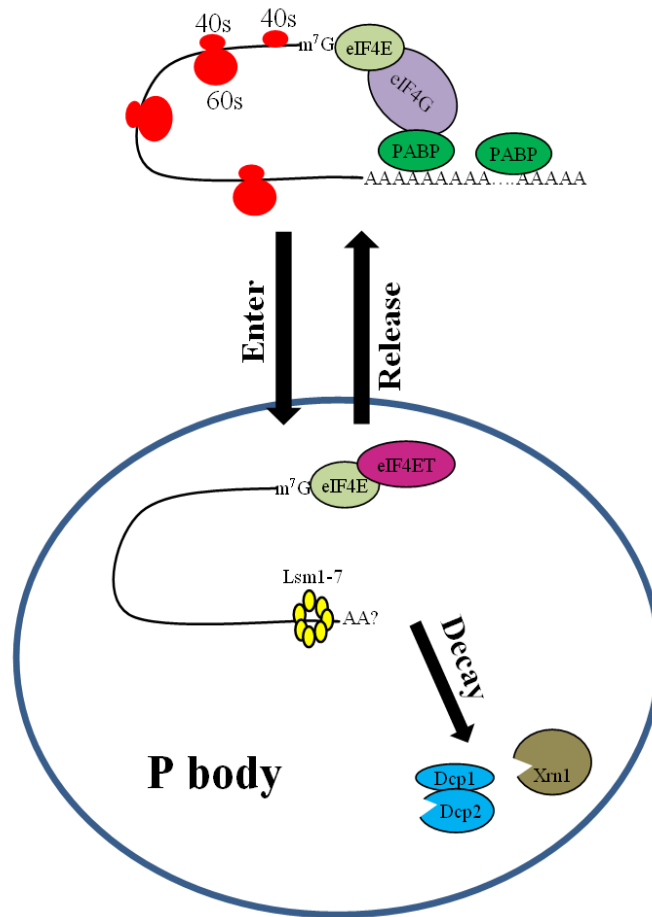


Figure 1.3: Translationally inhibited mRNAs in P bodies can be degraded or released back to the cytosol for translation. Translation factors such as PABP, eIF4G, and ribosome subunits are released from mRNAs targeted to P bodies, where these mRNAs can be decapped by Dcp1/2 and degraded from 5' to 3' by Xrn1. However, mRNAs in P bodies may also be released to the cytosol and translated.

P body formation involves remodeling and subsequent aggregation of mRNAs and their associated proteins, together known as messenger ribonucleoproteins (mRNPs). The mRNP remodeling may include recruitment of translational inhibitors and dissociation of translation activators and translation machinery. siRNA-mediated knockdown of translational inhibitors such as eIF4E-T and RCK/p54 blocked P body formation (74, 75). Cycloheximide treatment, which inhibits the release of ribosomes from mRNAs, also abolished P body formation (72, 76). Since deadenylation often leads to mRNA translational inhibition and mRNA degradation, it is likely that deadenylation is required for P body formation. In HeLa cells, siRNA-mediated knockdown of Ccr4 blocked P body formation (74).

Surprisingly, deletion of Ccr4 or Pop2(Caf1), the major deadenylases in yeast, only slightly reduced P body formation (77). However, it is unclear whether deadenylation was completely inhibited by Ccr4 knockdown. The localization and functions of the Pan3-Pan2 complex and Caf1 with regards to P bodies had not been reported. Therefore the role of deadenylases in P body formation or functions remained elusive.

The aggregation of the translationally inhibited mRNPs into microscopically visible P bodies seems to depend on protein-protein interactions, a notion supported by the discovery that most yeast P body components contain Q/N-rich aggregation-prone regions (78). However, it is not known if the Q/N-rich regions are also enriched in mammalian P body components.

Although hundreds of studies of P body components have been published, a comprehensive list of reported P body proteins is lacking, hampering the studying of P bodies as a whole. A comprehensive list of P body proteins will allow us to predict new P body proteins in mammalian cells, envision how some of the P body components may function together in the same pathways or processes, and analyze how P body proteins interact with each other and with other cellular proteins. Thus, such list will lead to a better understanding of the physiological significance of P-bodies and how P body dynamics may be controlled.

1.4 Significance of my study

The first part of my study, as described in Chapter 2, was focused on the localization of deadenylases with regards to P bodies and their functions in P body formation. This study has shed new light on the mechanisms of P body formation by showing that 1) deadenylase activity of the Ccr4-Caf1 complex is required for mammalian P body formation, and 2) Pan3 is required for P body formation.

The focus of Chapter 3 is functional dissection of Pan3 domains and to test the possible role of Pan3 phosphorylation. This study not only helped us further understand the functions of Pan3 in both deadenylation and P body formation but also suggested that Pan3 localization and functions might be regulated by phosphorylation.

Chapter 4 is a bioinformatics study of all reported P body components, which not only provides a comprehensive picture of P body organizations and potential functions but also predicts new factors important for P body dynamics and functions.

Chapter 2: Deadenylation is Required for mRNA Processing Body Formation

2.1. Introduction

Shortening of mRNA poly(A) tails, a process termed deadenylation, is essential for post-transcriptional inhibition of gene expression in eukaryotes, because the poly(A) tail significantly enhances cytoplasmic mRNA stability and translation initiation. The importance of poly(A) tail in stabilizing mRNAs is reflected by the fact that deadenylation is the first step for the decay of both stable and many unstable messages (16-20). The poly(A) tail also enhances translation through the poly(A) binding protein (PABP) and eIF4G-mediated mRNA circularization, which may facilitate the recycling of ribosomes from the 3' end to the 5' end of the same mRNA (7, 79) and may also enhance the RNA helicase activity of some translation initiation factors (6).

Deadenylation is catalyzed by deadenylases, such as the highly conserved eukaryotic Pan3-Pan2 and Ccr4-Caf1 complexes. In the Pan3-Pan2 complex, Pan2 catalyzes deadenylation while Pan3 can interact with both Pan2 and the PABP to regulate Pan2 activity (27, 30). On the other hand, both Ccr4 and Caf1 have deadenylase activities (37, 38, 42). In yeast, the Ccr4p-Caf1p complex is responsible for major deadenylation in the cytoplasm (31). *In vitro* study showed that the deadenylase activity of Ccr4-Caf1 complex is inhibited by PABP (38). In mammalian cells, deadenylation in the cytoplasm are biphasic. Poly(A) tails are first shortened to about 110 nt by the Pan3-Pan2 complex, followed by a second phase of deadenylation, during which the Ccr4-Caf1 complex catalyzes further shortening of the poly(A) tail to oligo(A) (16).

After deadenylation, the mRNA can either be degraded in a 3' to 5' direction by the exosome complex or be decayed from 5' to 3' by Xrn1 following decapping by Dcp1-Dcp2 decapping complex (80). Translationally inhibited mRNAs, translation inhibitors, decapping factors, and the 5' to 3' ribonuclease Xrn1 are found in cytoplasmic foci named mRNA processing bodies or P bodies, which are membrane-free mRNP aggregates with a diameter of 100-300 nm (65).

P bodies are sites for mRNA translational inhibition, degradation, and storage. Because P bodies are ribosome-free, mRNAs in P bodies cannot be translated (71). P body mRNAs can be degraded, as

inhibition of 5' to 3' decay by knocking down Xrn1 increased P body sizes and mRNA decay intermediates were found in P bodies (72). P bodies may also be temporary storage sites for some mRNAs before they are degraded, as suggested by an observation that an P body mRNA encoding cationic amino acid transporter 1 (CAT-1) was released from P bodies and subsequently translated when the cells were subjected to different stresses (73). Therefore, P bodies provide a location where the fates of non-translating mRNAs (either degradation or release) will be determined pending on the condition.

While decapping and 5' to 3' mRNA decay can impact P body formation, the link between deadenylation and P bodies is unclear. In yeast, both Ccr4p and Pop2p (yeast Caf1) only co-localize with P bodies in strains where decapping or 5' to 3' mRNA decay is inhibited (77). GFP-tagged human Ccr4 can weakly localize to P bodies in HEK293 cells (76). The Pan3-Pan2 complex was reported to localize in the cytoplasm in both yeast and mammalian cells (16, 27); however, it was unknown whether the Pan3-Pan2 deadenylase complex can be found in P bodies.

Deadenylation might be one of the mRNP remodeling steps required for P body formation because it results in mRNA translational inhibition and mRNA decay, both of which can occur in P bodies. The observation that siRNA-mediated knockdown of Ccr4 blocked P body formation in HeLa cells (74) supports this notion. However, it remains to be determined whether the deadenylase activity or the structural entity of Ccr4 is required for P body formation. Besides, deletion of either Ccr4p or Pop2p (yeast Caf1) only had a small effect on P bodies in yeast (77). Therefore, it remains elusive, especially in mammalian cells, whether the deadenylase activities of the Ccr4-Caf1 and Pan3-Pan2 complexes are required for P body formation.

To reveal the link between P body formation and deadenylation and shed new light on the mechanisms of eukaryotic post-transcriptional gene regulation, I examined cellular localizations of the Pan3-Pan2 and Ccr4-Caf1 complexes with regards to P bodies, the importance of deadenylase activity in P body formation, and the underlying mechanisms for the deadenylation-P body formation relationship in mammalian cells. These experiments revealed that the Pan3-Pan2 complex is enriched in mammalian P bodies and that Pan3 helps the Ccr4-Caf1 complex localize to P bodies. In addition,

combining *in vivo* functional analysis performed in our lab, we demonstrated that mammalian P body formation depends on the deadenylase activity of the Ccr4-Caf1 complex, even when mRNAs are rendered ribosome-free by puromycin treatment. Taken together, this study provides new insights into the mechanisms of P body formation and post-transcriptional gene regulation.

2.2. Materials and Methods

2.2.1. Plasmids

To construct a plasmid encoding HA-tagged Pan3 or Caf1, a 2.2-kb Pan3L cDNA amplified by RT-PCR from human testis total RNA (Clontech Laboratories, Inc.) or an 855-bp fragment encoding Caf1 amplified from IMAGE clone 6207987 was inserted between the EcoRV and XhoI sites of pSRHisHA (gift from S. Ohno, Yokohama City University, Yokohama, Japan). The Caf1 cDNA was inserted between the HindIII and XbaI sites of pcDNA6/V5-HisA (Invitrogen) to generate pcDNA6-Caf1-V5. A plasmid encoding catalytic inactive Caf1 mutant (D40A) was created using the QuikChange site-directed mutagenesis kit (Stratagene) with pSR-HA-Caf1 as the template. To construct pcDNA6-Pan2-V5, a 3.6-kb Pan2 cDNA was amplified from IMAGE clone 3357890 and inserted between the AflIII and XbaI sites of pcDNA6/V5-HisA (Invitrogen). GFP-Dcp1a and GFP-Ccr4 (gifts from B. Seraphin, Centre de Génétique Moléculaire, Gif sur Yvette Cedex, France) were described previously (21).

2.2.2. Cell culture and transfection

Either Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche) was used for transient transfections. NIH3T3 B2A2 cells were split to a density of 2.6×10^6 /10-cm dish 24 h before transfection in 100 ng/ml tetracycline. 40 μ l Lipofectamine 2000 and 1.5 ml Opti-MEM were mixed well and incubated for 5 min at RT. DNA (1.3 μ g of reporter plasmid and 1.3 μ g of internal control plasmid, pSV- α -globin-GAPDH) and siRNA (4.86 μ g of nonspecific siRNA or Pan3 siRNA, or 2.43 μ g Caf1 siRNA plus 2.43 μ g Pop2 siRNA [SMARTpool; Thermo Fisher Scientific]) were diluted into 1.5 ml Opti-

MEM, added to the Lipofectamine 2000 mixture, and incubated at RT for 25 min. The final mixture was then added to the cells in a 10-cm dish and incubated at 37°C (5% CO₂). Cells were split 18 h later into 6-cm dishes (1.5 × 10⁶/6 cm dish) and incubated at 37°C (8% CO₂) for 24 h. When using FuGENE 6, NIH3T3 B2A2 cells were split to a density of 0.65 × 10⁶/6-cm dish 24 h before transfection in the presence of 50 ng/ml tetracycline. 6.9 μl FuGENE 6 was diluted in 100 μl DME and mixed with 2.3 μg DNA containing 0.055 μg of reporter plasmid, 0.11 μg of internal control plasmid, and 2.13 μg of DNA encoding HA-tagged proteins. The mixture was incubated at RT for 25 min, added to the culture dish, and incubated at 37°C (8% CO₂) for 42 h. Time-course experiments using the Tet-off system for transcriptional pulsing were performed as described previously (21, 81).

2.2.3. Western blot analysis

Cytoplasmic and nuclear lysates were prepared as described previously (82). Total cell lysates (5–40 μg) were resolved on a 7 or 10% SDS-polyacrylamide gel and analyzed using an ECL Western blotting kit (GE Healthcare). The PVDF blots were probed with specific antibodies as indicated in each figure and detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Membranes were incubated with one of the primary antibodies at the indicated dilution: HRP-conjugated monoclonal Anti-V5 antibody at 1:5,000 (Invitrogen); HRP-conjugated monoclonal anti-HA antibody at 1:1,000 (Roche); rat anti-HA monoclonal antibody at 1:4,000 (Roche); mouse anti-α-tubulin monoclonal antibody at 1:10,000 (Sigma-Aldrich); rabbit anti-Dcp1a serum at 1:4,000 (Bethyl Laboratories, Inc. or gift from S. Ohno); rabbit anti-Rck/p54 serum at 1:1,000 (Bethyl Laboratories, Inc.); mouse monoclonal antibody against GAPDH at 1:10,000 (Research Diagnostics, Inc.); and mouse antibody against lamin A/C at 1:1,000 (Santa Cruz Biotechnology, Inc.). Rabbit anti-PABP antibody (gift from R. Lloyd, Baylor College of Medicine, Houston, TX) was used at a 1:4,000 dilution. To detect endogenous Caf1, Pan2, and Pan3 in mouse NIH3T3 or monkey COS7 cells, the corresponding polyclonal antibodies were generated in rabbits immunized with gene-specific peptides using the custom antibody service from Bethyl Laboratories, Inc. Affinity-purified anti-peptide antibodies were used at

the following dilutions: rabbit anti-Pan3 peptide antibody at 1:2,000; rabbit anti-Caf1 peptide at 1:1,000; or rabbit anti-Pan2 peptide at 1:2,000. HRP-conjugated donkey anti-rabbit IgG antibodies (1:4,000; GE Healthcare) or goat anti-mouse (1:5,000; Bio-Rad Laboratories) were used as secondary antibodies for detection with a chemiluminescence reagent (peroxide/luminol enhancer; Thermo Fisher Scientific) (21).

2.2.4. Immunofluorescence staining and microscopy

NIH3T3 cells were seeded in 6-well plates at a density of 0.4×10^6 cells per well, 24 h before transfection using Lipofectamine 2000 (Invitrogen). At 22–26 h after transfection, cells were reseeded to slide chambers (BD Biosciences) and incubated overnight. For cycloheximide, puromycin, or arsenite treatment, cells were incubated in media containing either 7.5 μ g/ml cycloheximide (Sigma-Aldrich) for 2 h, 100 μ g/ml puromycin (Sigma-Aldrich) for 1 h, or 0.3 mM sodium arsenite (Sigma-Aldrich) for 1 h before fixation. Cells in the slide chambers were fixed for 10 min each with 3.7% (wt/vol) PFA (Sigma-Aldrich) in PBS and then with cold methanol, followed by a 10-min incubation in 0.2% (vol/vol) Triton X-100 in PBS. Each microgram of Zenon-labeled antibodies (see third paragraph in this section) was diluted in 500 μ l of 1% BSA in PBS and incubated with the fixed slides. After incubating for 1 h at RT and washing, the slides were fixed again in 3.7% PFA. For indirect immunofluorescence microscopy, all of the primary and secondary antibodies, except the mouse anti-myc serum, were diluted 1:1,000 with 1% (wt/vol) BSA in PBS. Endogenous Pan2 was detected using rabbit anti-Pan2 labeled with Zenon 555 rabbit IgG labeling reagent. Endogenous Dcp1a, Xrn1, or Pan3 was detected using rabbit anti-Dcp1a, anti-Xrn1, or anti-Pan3 labeled with Zenon 488 rabbit IgG labeling reagent. HA-tagged Pan3, Ccr4, or Caf1 was detected using rat anti-HA monoclonal antibody and Alexa Fluor 350 goat anti-rat IgG. HA-Pan2 or V5-tagged proteins were detected using rat monoclonal anti-HA or mouse monoclonal anti-V5 and Alexa Fluor 555 goat anti-rat IgG. Rabbit anti-Xrn1 antibody was a gift from J. Lykke-Andersen (University of Colorado at Boulder, Boulder, CO), human anti-GW182 antiserum was a gift from M.J. Fritzler (University of Calgary, Calgary, Canada), rabbit anti-G3BP1 was a gift from R. Lloyd (Baylor College of Medicine, Houston, Texas), and rabbit anti-Dcp1a antibody

was a gift from S. Ohno (Yokohama, Japan). The mouse anti-myc monoclonal antibody from culture medium collected from anti-myc monoclonal antibody secreting hybridoma cells (American Type Culture Collection) was used at 1:50 dilution. After incubation with the indicated primary antibodies/sera, cells were washed three times in PBS for 5 min and incubated with fluorescently labeled secondary antibodies (Invitrogen) as indicated in the figures. Fluorescence mounting medium with or without DAPI was added.

Images were obtained at RT by optical z-sectioning (20 sections in total, 0.2- μ m-space between sections) using an objective lens (100 \times /1.35 NA; Olympus) of a deconvolution microscope system (DeltaVision) containing an inverted microscope (IX70; Olympus). Immersion oil ($n = 1.514$) was from Applied Precision, LLC. Images were captured using a digital camera (Coolsnap HQ; Roper Scientific). Stacks of 20 images were projected as a single 2D picture using softWoRx Explorer (version 3.3.6; Applied Precision, LLC). The RGB colors of the resulting pictures were separated using Photoshop CS (Adobe).

When two different primary antibodies raised in rabbits were used together to visualize P-bodies, the following procedure was used. One μ g of rabbit antibody was incubated for 5 min at RT with 5 μ l Zenon rabbit IgG labeling reagent, either Alexa Flour 488- or 555-labeled Fab fragment (Invitrogen). The labeled Fab fragment was bound to the Fc portion of the rabbit IgG, and excess Fab fragment was neutralized by the addition of 5 μ l of nonspecific rabbit IgG, which prevented cross-labeling of the Fab fragment when two rabbit antibodies were used. After a 5-min incubation at RT, the labeled antibodies were used for staining in combination with each other or with labeled secondary antibodies specific for other species. To analyze the changes in P-body number and size after Pan3 knockdown, 87 cells transfected with control nonspecific siRNA and 91 cells transfected with Pan3 siRNA were analyzed using ImageJ software (pixel range for particle brightness between 40 and 215; National Institutes of Health). Dcp1a particles with values >50 square pixels were considered above background staining and were selected for P-body analysis (21).

2.3. Results

2.3.1. Both Pan2 and Pan3 co-localize with P bodies in mammalian cells.

To reveal the link between deadenylation and P bodies, we first determined the subcellular localization of Pan2 and Pan3, the factors involved in initiating mammalian mRNA deadenylation (16), with regards to P-bodies in mammalian cells. The well-characterized P-body components Dcp1a and Xrn1 are used as markers to visualize P bodies by immune-fluorescence microscopy (83). The results show that cytoplasmic foci detected by an anti-Pan2 antibody co-localize with P bodies marked by endogenous Dcp1a (Fig. 2.1 A) in NIH 3T3 cells. Cycloheximide treatment, which blocks P body formation by inhibiting the release of ribosomes from mRNAs (84), also blocked Pan2 foci formation (Fig. 2.1 B). Endogenous Pan2 foci also co-localize with both endogenous Dcp1a foci and endogenous Xrn1 foci in COS7 cells (Fig. 2.1 C). These results indicate that Pan2 is a component of P bodies in mammalian cells.

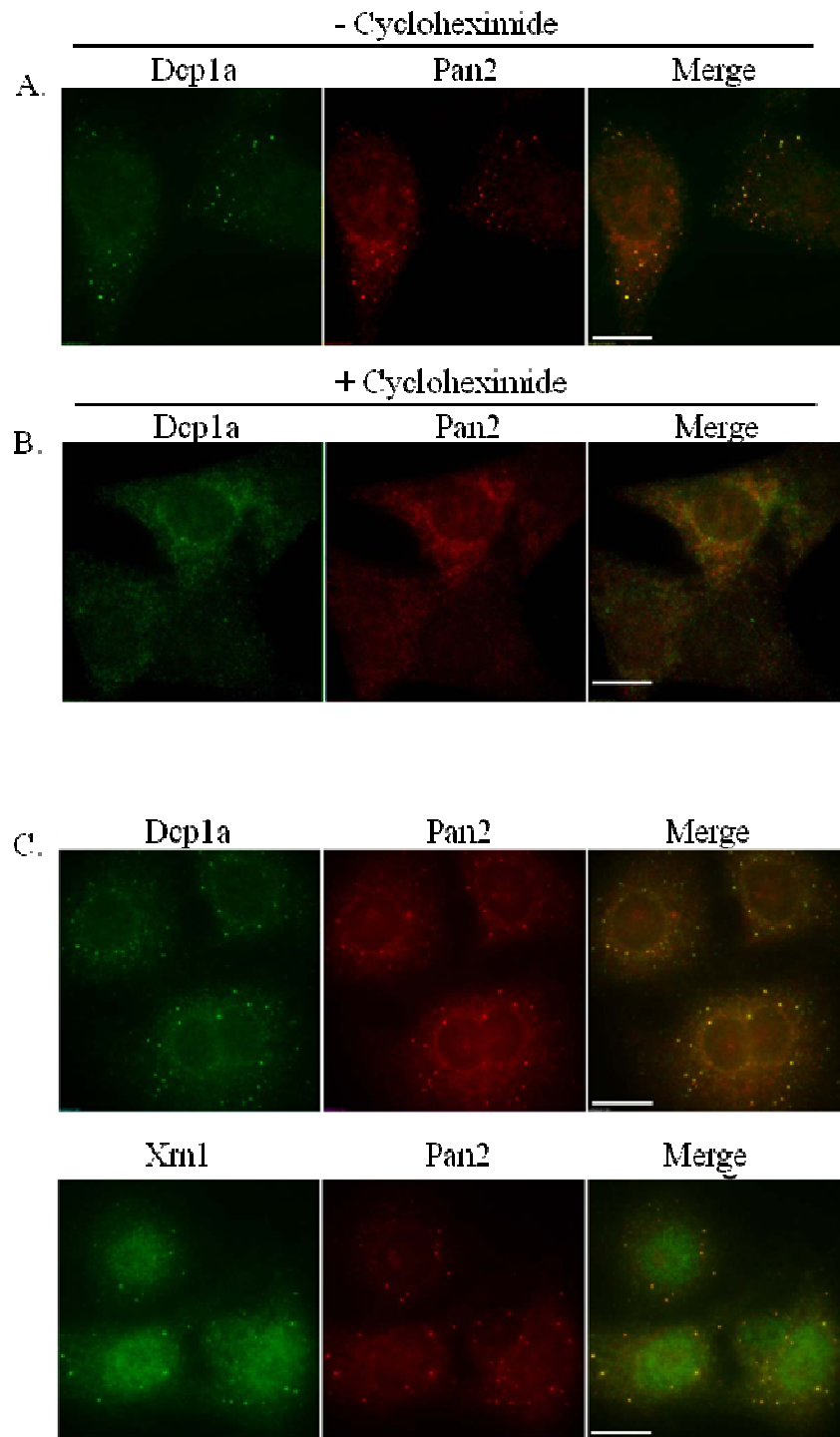


Figure 2.1: Endogenous Pan2 is enriched in P bodies in both NIH 3T3 cells and COS7 cells.

(A) Immuno-fluorescence staining showing endogenous Pan2 colocalizes with Dcp1a in P bodies in NIH 3T3 cells. (B) The Pan2 foci in NIH 3T3 cells are disassembled by cycloheximide treatment, as expected for P bodies. (C) Pan2 foci colocalize with P-bodies detected with anti-Dcp1a (top) or anti-Xrn1 (bottom) in COS7 cells. (The experiments were repeated once.)

Courtesy to The Journal of Cell Biology (21).

Next we checked if Pan3, the other subunit of the Pan3-Pan2 complex, also co-localizes with P bodies. Due to the lack of an anti-Pan3 antibody suitable for immune-fluorescence microscopy, a plasmid encoding HA-tagged Pan3 (HA-Pan3) was delivered into NIH 3T3 cells through transient transfection and an anti-HA antibody was used to study the sub-cellular localization of HA-Pan3. As expected, HA-Pan3 also co-localizes with P bodies labeled with either anti-Dcp1a or GFP-Dcp1a in NIH 3T3 cells (Fig. 2.2 A). Since Pan3 can also interact with PABP (27, 30), which was not found in P bodies previously (71), we checked if PABP can be enriched in HA-Pan3 foci. We found that neither ectopically expressed myc-tagged PABP (Fig. 2.2 B) nor endogenous PABP (data not shown) is concentrated in HA-Pan3 foci. Moreover, the HA-Pan3 foci are not stress granules, as they do not co-localize with the stress granule marker G3BP-1 (71), no matter whether the cells were treated with arsenite to induce stress granule formation (Fig. 2.2 C, top) or not (Fig. 2.2 C, bottom). Collectively, our results identify both Pan2 and Pan3 as two new components of P bodies in mammalian cells.

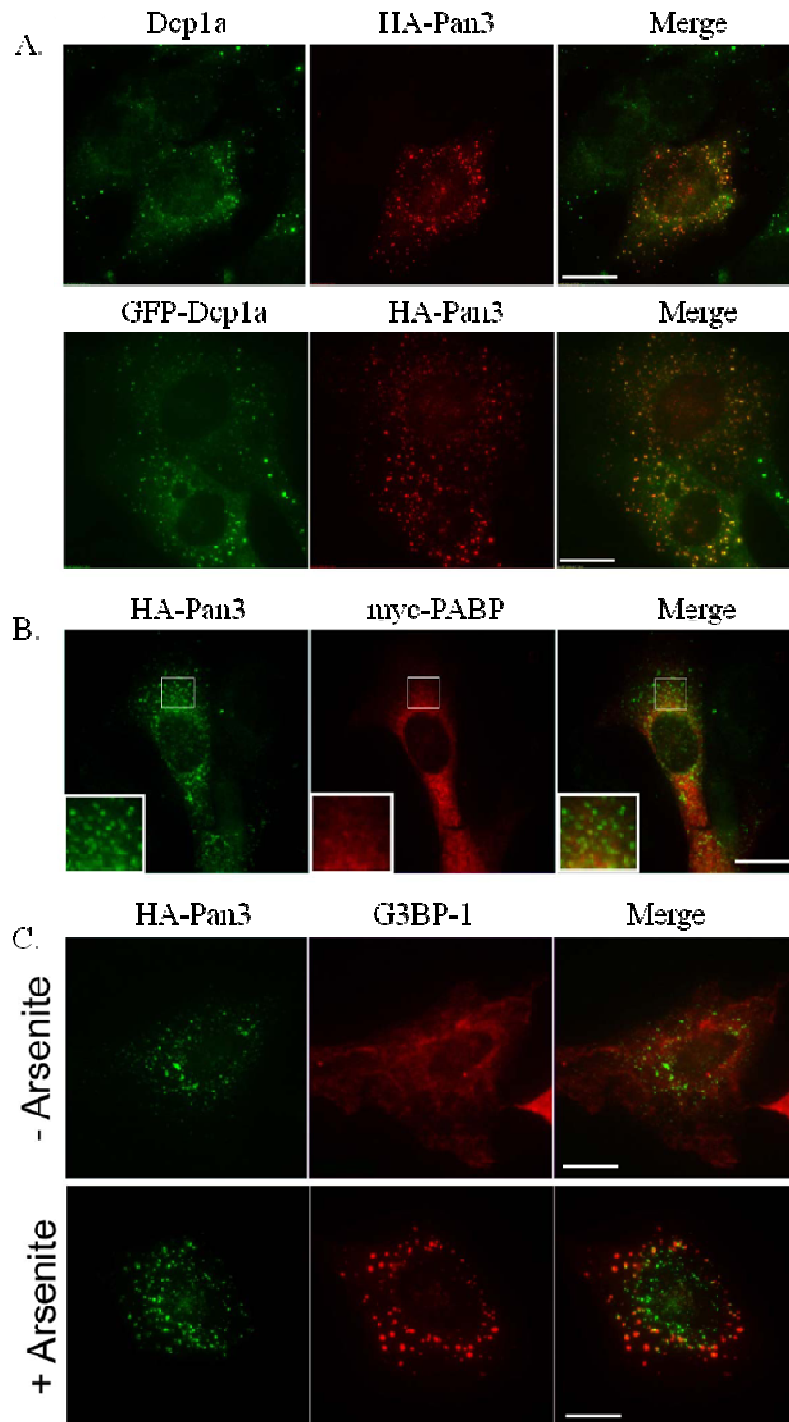


Figure 2.2: PABP is not found in HA-Pan3 foci, which colocalize with P bodies but not stress granules. (A) Immuno-fluorescence staining showing ectopically expressed HA-tagged Pan3 colocalizes with endogenous Dcp1a (top) and EGFP-tagged Dcp1a (bottom) in P bodies of NIH 3T3 cells. (B) HA-Pan3 foci do not colocalize with myc-PABP. (C) HA-Pan3 foci do not colocalize with the stress granule marker G3BP-1 in NIH 3T3 cells with (top) or without (bottom) arsenite treatment. (The experiments were repeated at least once.)

Courtesy to The Journal of Cell Biology (21).

2.3.2. Pan3 is an essential P body component that can recruit the non-essential P body component Pan2 to P bodies

Since both Pan3 and Pan2 are P body components, we next determined whether they are essential for P body formation in NIH 3T3 cells. To this end, NIH 3T3 cells were transfected with either non-specific siRNA (NS siRNA), Pan3 specific siRNAs, or Pan2 specific siRNA, followed by immunofluorescence microscopy and particle analysis using the free NIH software ImageJ (85). siRNA-mediated Pan3 knockdown in NIH 3T3 cells dramatically decreased the number and average size of P bodies in cells labeled with anti-RCK/p54, another popular P body marker (Fig. 2.3). This observation suggests that Pan3 is an essential component of P bodies. On the other hand, siRNA-mediated Pan2 knockdown had little effect on P body formation (Fig. 2.4 A&B). Moreover, overexpression of a catalytic mutant of Pan2 did not block P body formation (Fig. 2.4 C). These observations suggest that Pan2 is not essential for P body formation.

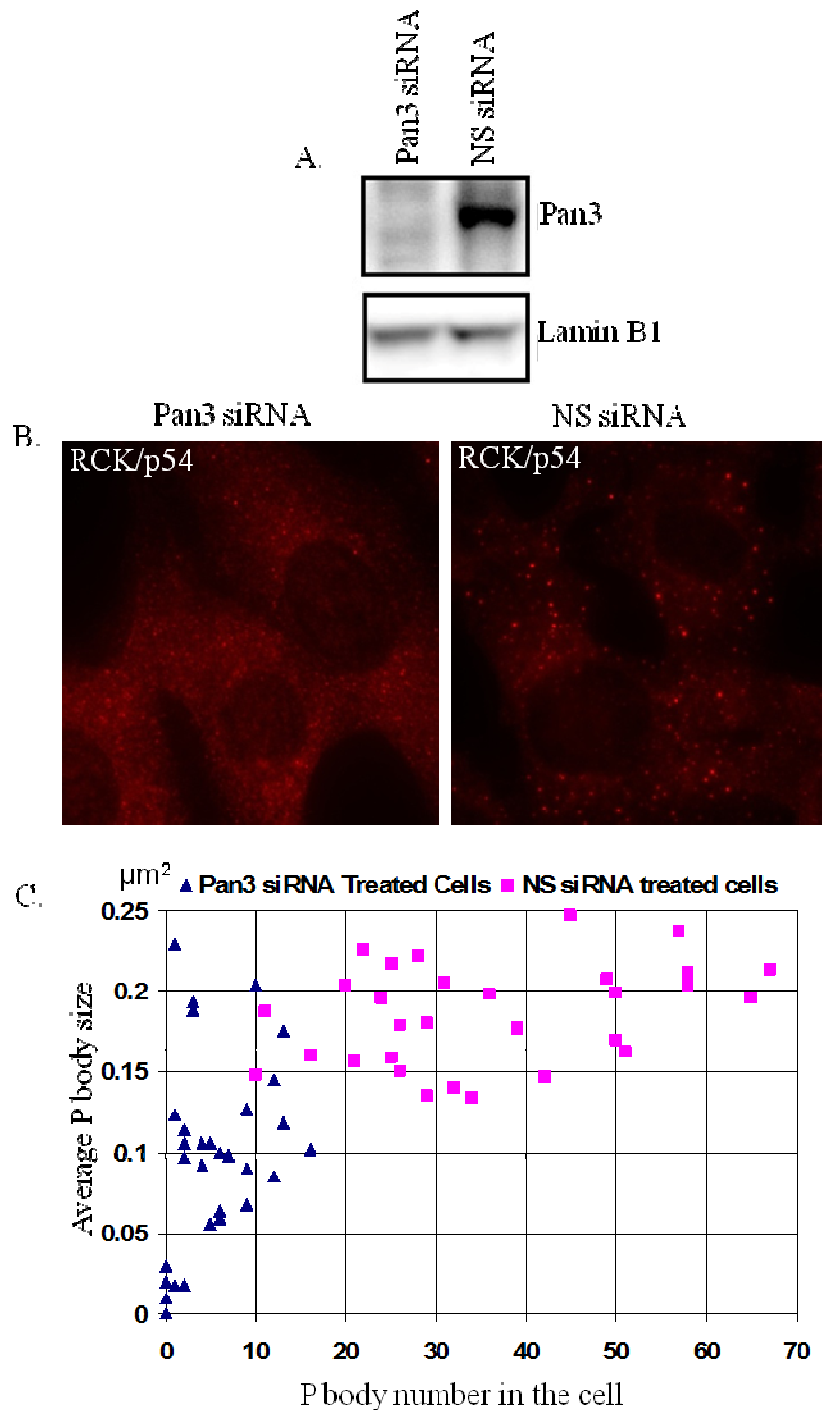


Figure 2.3: siRNA-mediated Pan3 knockdown inhibits P body formation in NIH 3T3 cells.

(A) Western Blotting showing knockdown of Pan3 by siRNA. (B) Immuno-fluorescence staining using anti-RCK/p54 to detect P bodies in NIH 3T3 cells transfected with either Pan3 siRNA or nonspecific siRNA. (C) Analysis of P body numbers and average sizes in NIH 3T3 cells transfected with either Pan3 siRNA or nonspecific siRNA. The experiment was repeated once.

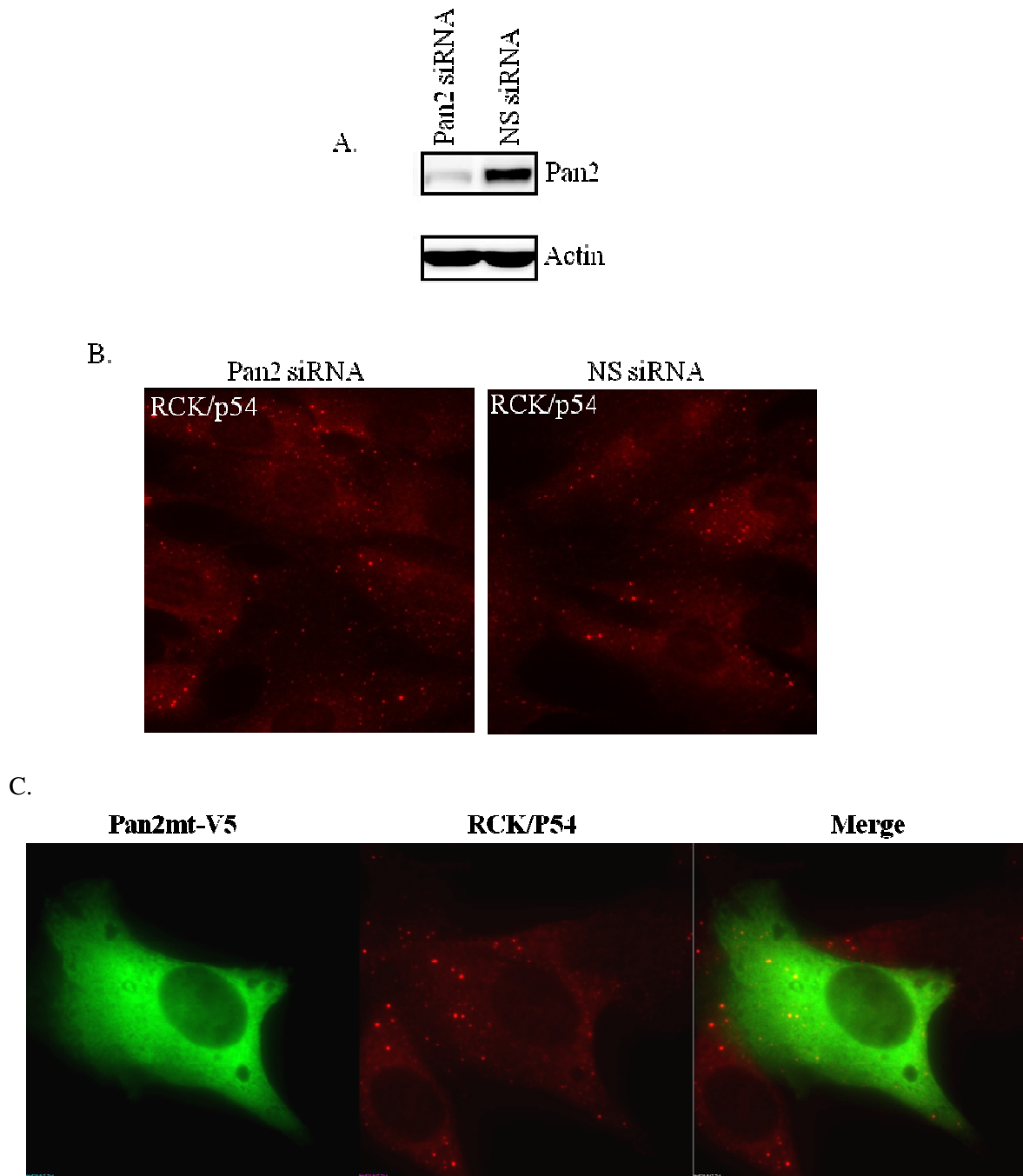


Figure 2.4: siRNA-mediated Pan2 knockdown has little effect on P body formation in NIH 3T3 cells.

(A) Western Blotting showing knockdown of Pan2 by siRNA. (B) Immuno-fluorescence staining using anti-RCK/p54 to detect P bodies in NIH 3T3 cells transfected with either Pan2 siRNA or nonspecific siRNA.(C) Overexpression of a catalytic mutant of Pan2 did not block P body formation. The experiment was repeated once.

The above results are consistent with the following observations. Firstly, HA-Pan3 localizes to P bodies when it is ectopically expressed alone (Fig. 2.2 A), suggesting that Pan3 can associate with P bodies without a coordinated expression of Pan2. Secondly, ectopically expressed Pan2 did not form any cytoplasmic foci when expressed alone (Fig. 2.5 A), but did co-localize with P bodies when co-expressed with HA-Pan3 (Fig. 2.5 B), indicating that Pan2 is recruited to P bodies through its interaction with Pan3. Together, these results indicate that Pan3 is an essential P body component that can recruit the non-essential P body component Pan2 to P bodies.

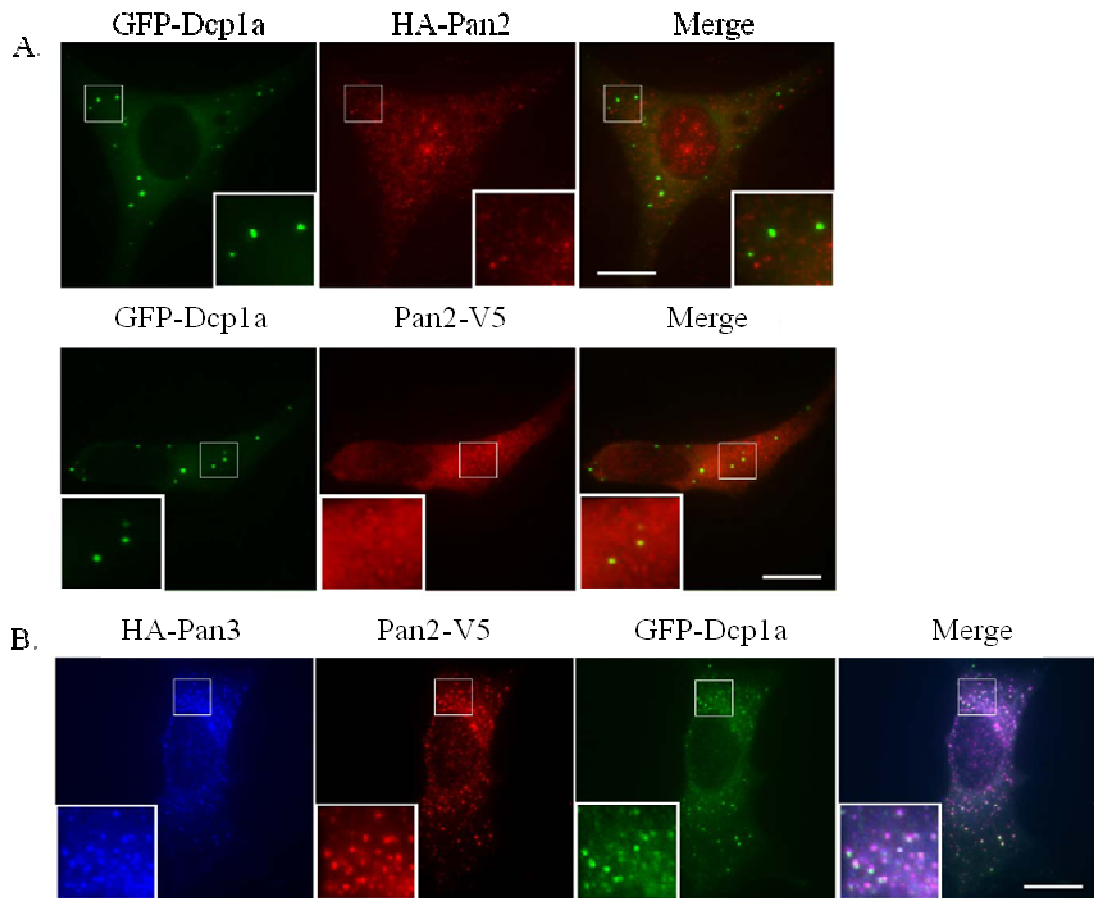


Figure 2.5: HA-Pan3 helps ectopically expressed Pan2 to colocalize with P bodies.

(A) Ectopically expressed HA-tagged Pan2 (top) and V5-tagged Pan2 (bottom) cannot be enriched in P bodies. (B) V5-tagged Pan2 is enriched in P bodies when HA-tagged Pan3 is co-expressed. The experiment was repeated twice.

Courtesy to The Journal of Cell Biology (21).

2.3.3. HA-Pan3 helps co-expressed Caf1 and Ccr4 to be enriched in P bodies.

After the first phase of deadenylation by the Pan3-Pan2 complex, the Ccr4-Caf1 deadenylase complex catalyzes the second phase of deadenylation (16). Since Ccr4 weakly localizes to P bodies in HEK293 cells (76), we asked whether Caf1, which forms a complex with Ccr4, also co-localizes with P bodies. Our results show that ectopically expressed Caf1 was not enriched in P bodies in NIH 3T3 cells, regardless of whether it was expressed alone (Fig. 2.6 A, top) or together with ectopic Ccr4 (Fig. 2.6 A, bottom). GFP-tagged Ccr4 also did not form any foci co-localizing with P bodies in NIH 3T3 cells (Fig. 2.6 C). Our results are consistent with the observations that yeast Ccr4p and Caf1p (Pop2p) normally do not co-localize with P bodies (77). Because the Pan3-Pan2 complex, which catalyzes the first phase of deadenylation, is in P bodies, we suspected that co-expressed HA-Pan3 can help the second phase deadenylases Ccr4 and Caf1 to localize to P bodies. Indeed, both V5-tagged Caf1 and GFP-tagged Ccr4 were enriched in P bodies when HA-Pan3 was co-expressed (Fig. 2.6 B and D). The effect of co-expressed HA-Pan3 on Caf1-V5 and GFP-Ccr4 localizations is not likely due to non-specific recruitment of co-expressed proteins by HA-Pan3 foci, because co-expressed myc-PABP was not enriched in HA-Pan3 foci (Fig. 2.2 B).

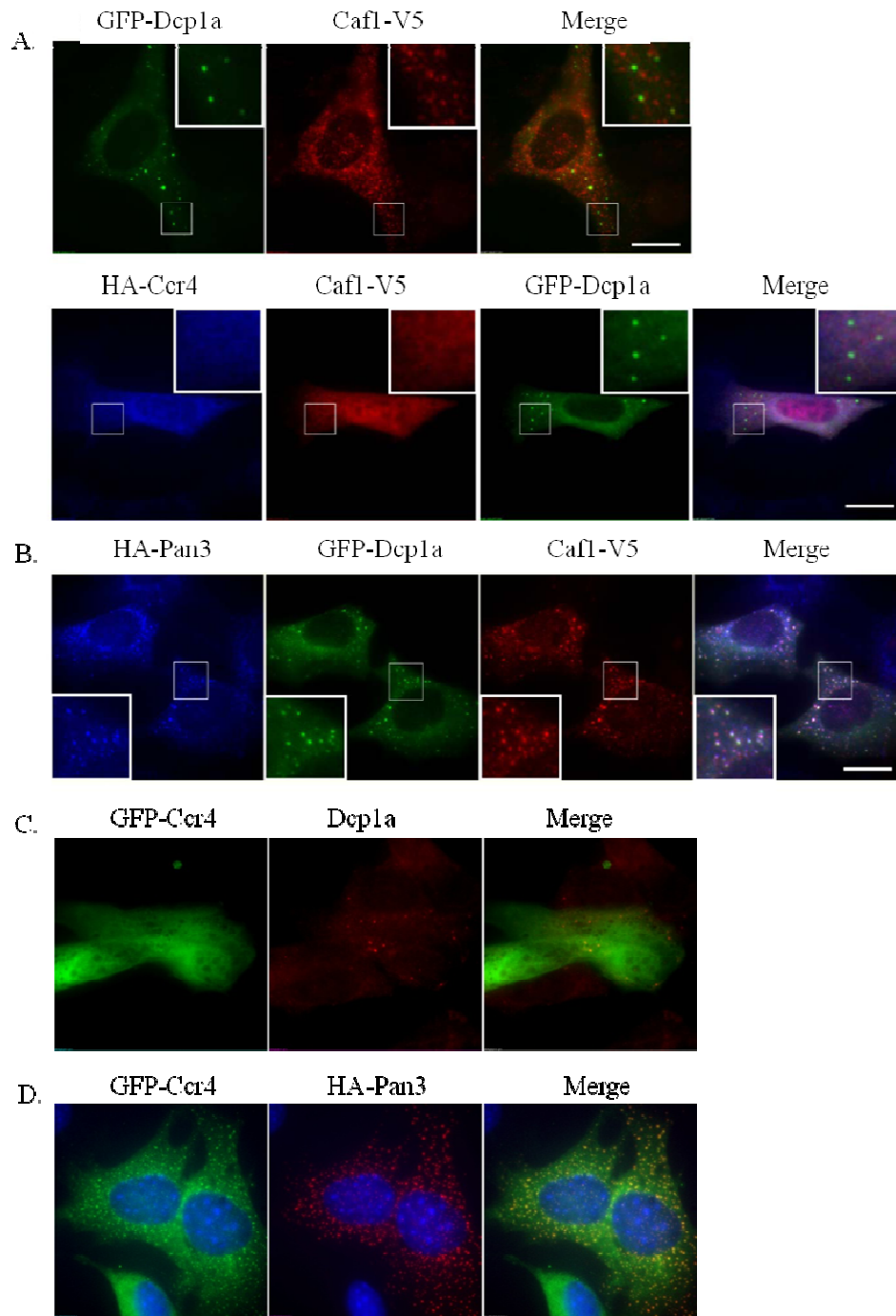


Figure 2.6: HA-Pan3 helps ectopically expressed Caf1 and Ccr4 to colocalize with P bodies.

(A) Ectopically expressed V5-tagged Caf1 (top) cannot be enriched in P bodies, even when HA-tagged Ccr4 is co-expressed (bottom). (B) V5-tagged Caf1 is enriched in P bodies when HA-tagged Pan3 is co-expressed. (C) GFP-tagged Ccr4 can hardly be enriched in P bodies in NIH 3T3 cells. (D) GFP-Ccr4 can be enriched in P bodies when co-expressed with HA-tagged Pan3. The experiment was repeated twice.

Courtesy to The Journal of Cell Biology (21).

2.3.4. Deadenylase activity of the Ccr4-Caf1 complex is required for P body formation

Both Caf1 and Ccr4 can be enriched in P bodies when co-expressed with HA-Pan3. Since siRNA-mediated knockdown of Pan3 inhibited P body formation in NIH3T3 cells (21) and Ccr4 knockdown was reported to block P body formation in HeLa cells (74), we asked if Caf1 is also required for P body formation. siRNA-mediated Caf1 knockdown blocked P body formation (Fig 2.7), indicating that Caf1 is important for the formation of microscopically visible P bodies.

Next, we asked if the deadenylase activity of the Ccr4-Caf1 complex is required for P body formation. To this end, we created a catalytically inactive mutant of Caf1 by changing a critical aspartate residue necessary for metal binding and Caf1 nuclease activity to alanine by site-directed mutagenesis (29). While ectopically expressed wild-type Caf1 promoted deadenylation and subsequent decay of the reporter BBB mRNA (Fig. 2.8 C), mutant Caf1 expression almost completely blocked reporter mRNA deadenylation (Fig. 2.8 D). The reporter mRNA was still degraded, although at a much slower rate, suggesting that mRNA can be degraded in deadenylation-independent manner when deadenylation is blocked. The catalytically mutant, but not the wild-type, Caf1 also blocked P body formation in NIH 3T3 cells (Fig. 2.8 E and F) and COS7 cells (data not shown). Interestingly, co-expression of wild-type Ccr4, but not catalytically mutant Ccr4, with Caf1 mutant restored fast deadenylation of the BBB reporter mRNA (Fig. 2.9 B), suggesting that the deadenylase activity of Ccr4 is complementary to Caf1 in the Ccr4-Caf1 complex. Concomitantly, co-expressed wild-type Ccr4, but not catalytically mutant Ccr4, released the blockage of P body formation by Caf1mt (Fig. 2.9 D and E). Collectively, our observations provided strong evidence that the deadenylase activity of the Ccr4-Caf1 complex is required for P body formation.

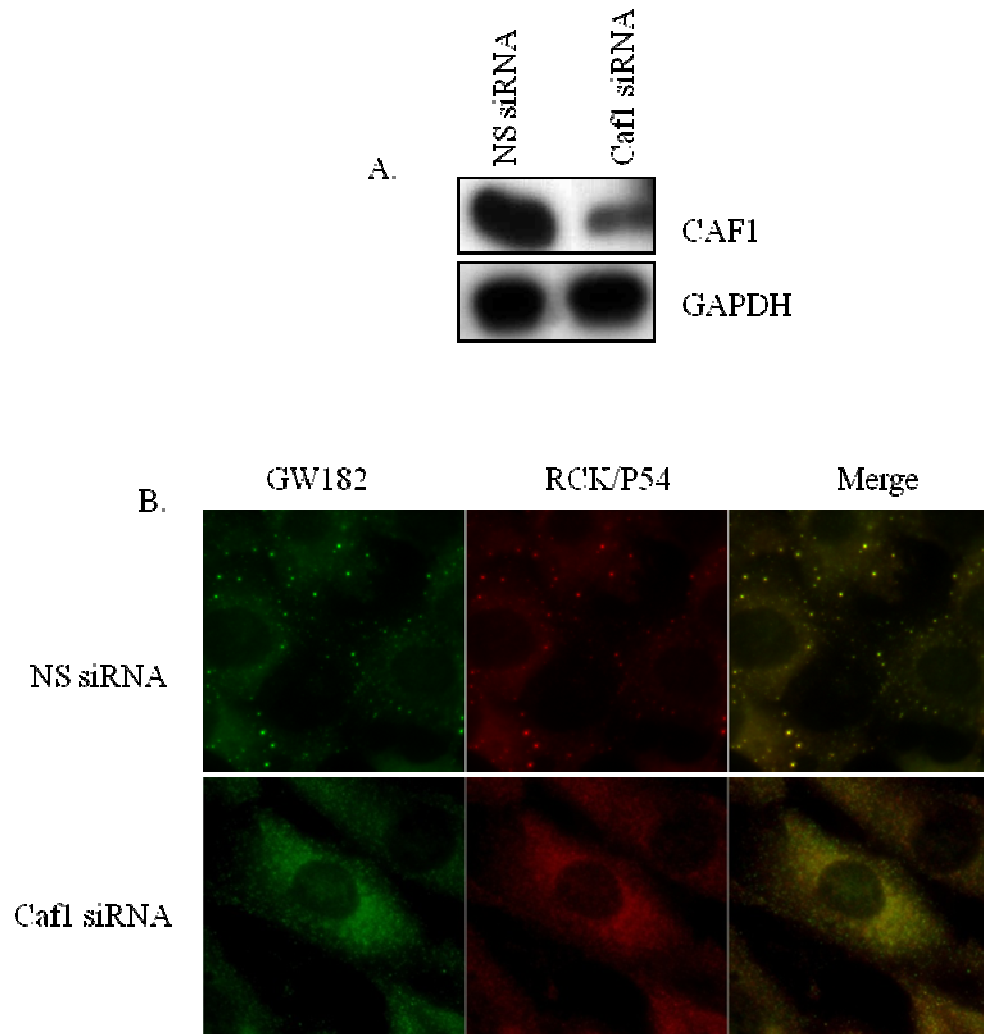


Figure 2.7: siRNA-mediated Caf1 knockdown blocks P body formation in NIH 3T3 cells.

(A) Western showing the knockdown of Caf1 by siRNA. (B) Immuno-fluorescence staining showing the effect of siRNA-mediated Caf1 knockdown on P body formation. The experiments were repeated three times.

Courtesy to The Journal of Cell Biology (21).

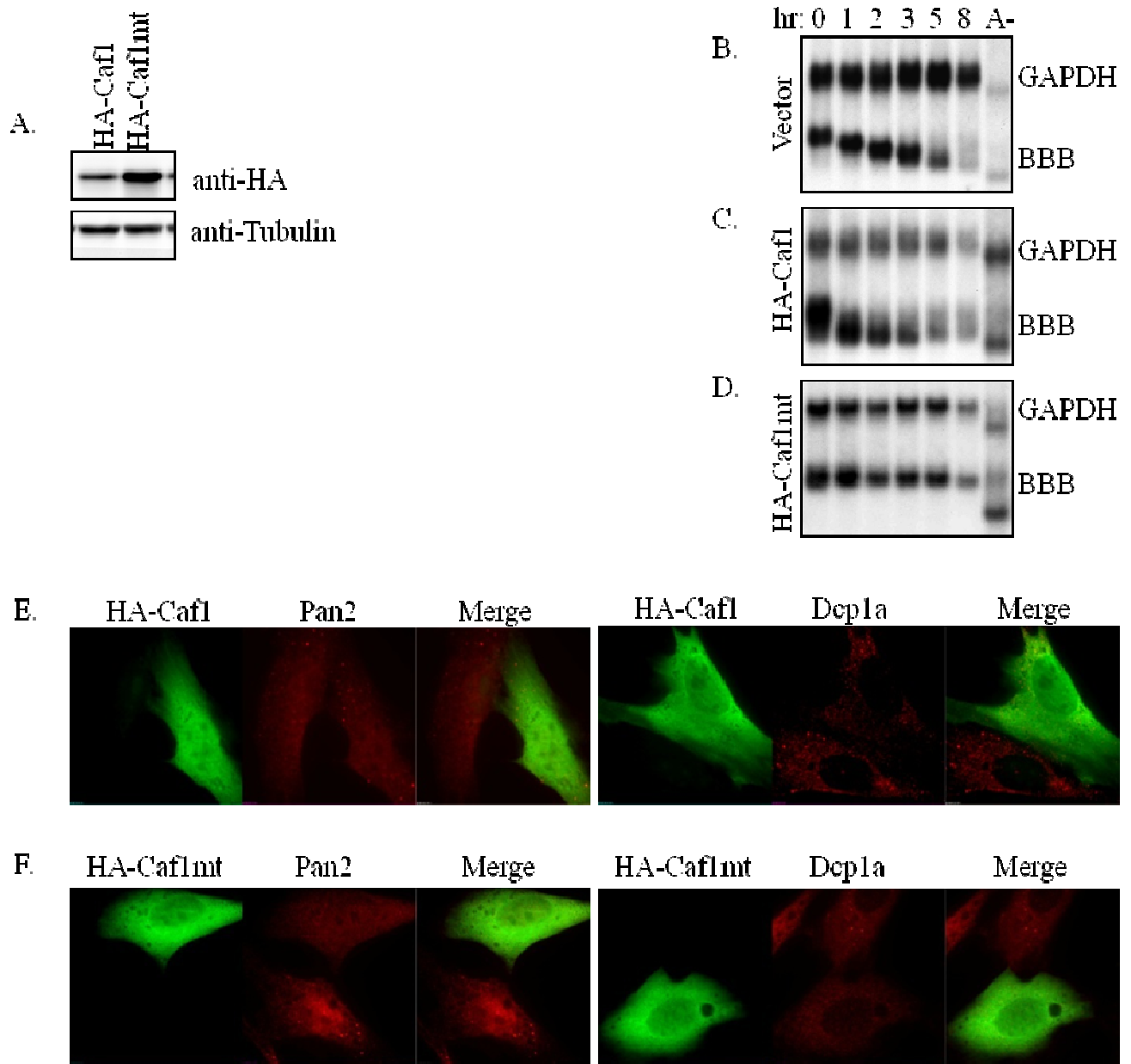


Figure 2.8: Overexpressed Caf1 catalytic mutant, but not wild-type Caf1, inhibits both P body formation and deadenylation.

(A) Western showing the expression of HA-tagged Caf1 and Caf1 mutant. (B-D) Northern blots showing the effects of overexpression of nothing (B), wild-type (C), or mutant (D) Caf1 on the deadenylation and decay of the BBB reporter mRNA. (E-F) Immuno-fluorescence staining showing ectopically expressed HA-tagged Caf1 (E) or Caf1 catalytic mutant (F) on P body formation. The experiment was repeated twice.

Courtesy to The Journal of Cell Biology (21).

Northern analysis (B,C,D) was performed by Wenmiao Zhu.

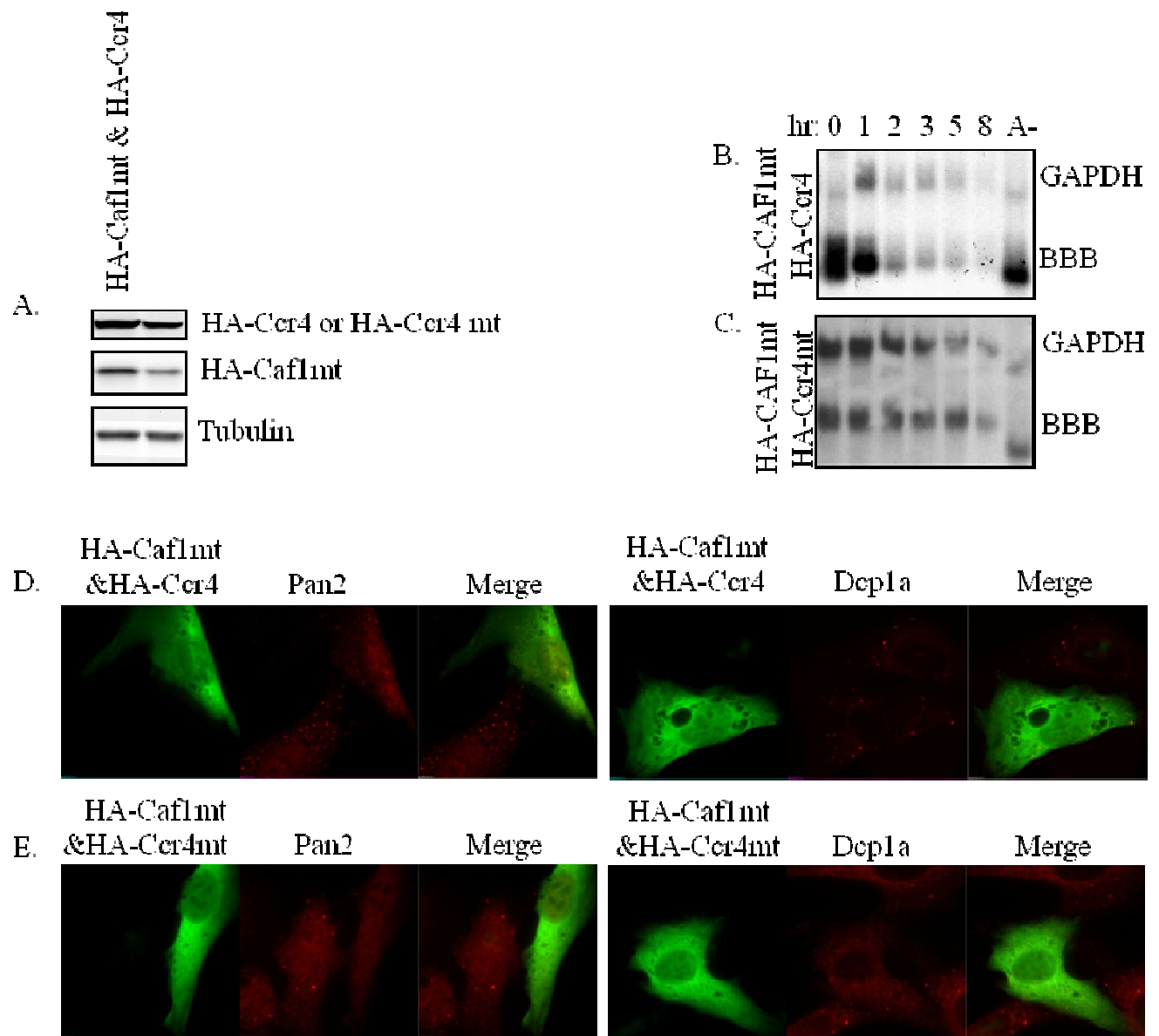


Figure 2.9: P body formation is restored when the blockage of deadenylation is released by co-expressed Ccr4.

(A) Western blotting showing the expression of Caf1 mutant and Ccr4 or Ccr4 mutant. (B-C) Northern blots showing the effects of overexpression of both Caf1 mutant and wild-type Ccr4 (B) or Ccr4 catalytic mutant (C) on the deadenylation and decay of the BBB reporter mRNA. (D-E) Immuno-fluorescence staining showing ectopically expressed Caf1 catalytic mutant and wild-type (D) or catalytically mutant Ccr4 (E) on P body formation. The experiment was repeated twice.

Courtesy to The Journal of Cell Biology (21).

Northern blot analysis (B) and (C) was performed by Wenmiao Zhu and Dr. Nader Ezzeddine, respectively.

2.3.5. Requirement of deadenylation for P body formation cannot be bypassed by puromycin treatment that removes ribosomes from mRNAs

Why is P body formation deadenylation-dependent? Since only ribosome-free mRNAs can enter P bodies and translation of most mRNAs is inhibited by deadenylation (9), it is likely that the requirement of deadenylation for P body formation is simply because deadenylation helps the mRNAs to become ribosome-free. To test this idea, puromycin was used to release ribosomes from cellular mRNAs (86) while deadenylation was inhibited either by overexpression of Caf1 catalytic mutant or siRNA-mediated knockdown of Caf1 in NIH 3T3 cells. Strikingly, puromycin treatment failed to induce P-body formation when deadenylation is inhibited (Fig 2.10), although puromycin indeed promoted P body formation in cells where deadenylation is not impaired. Therefore, deadenylation is required for mRNAs to enter P bodies even if the mRNAs are ribosome-free. In other words, the shortening of poly(A) tails *per se*, not only the release of ribosomes from mRNA, is one of the necessary remodeling steps for mRNPs to form P bodies.

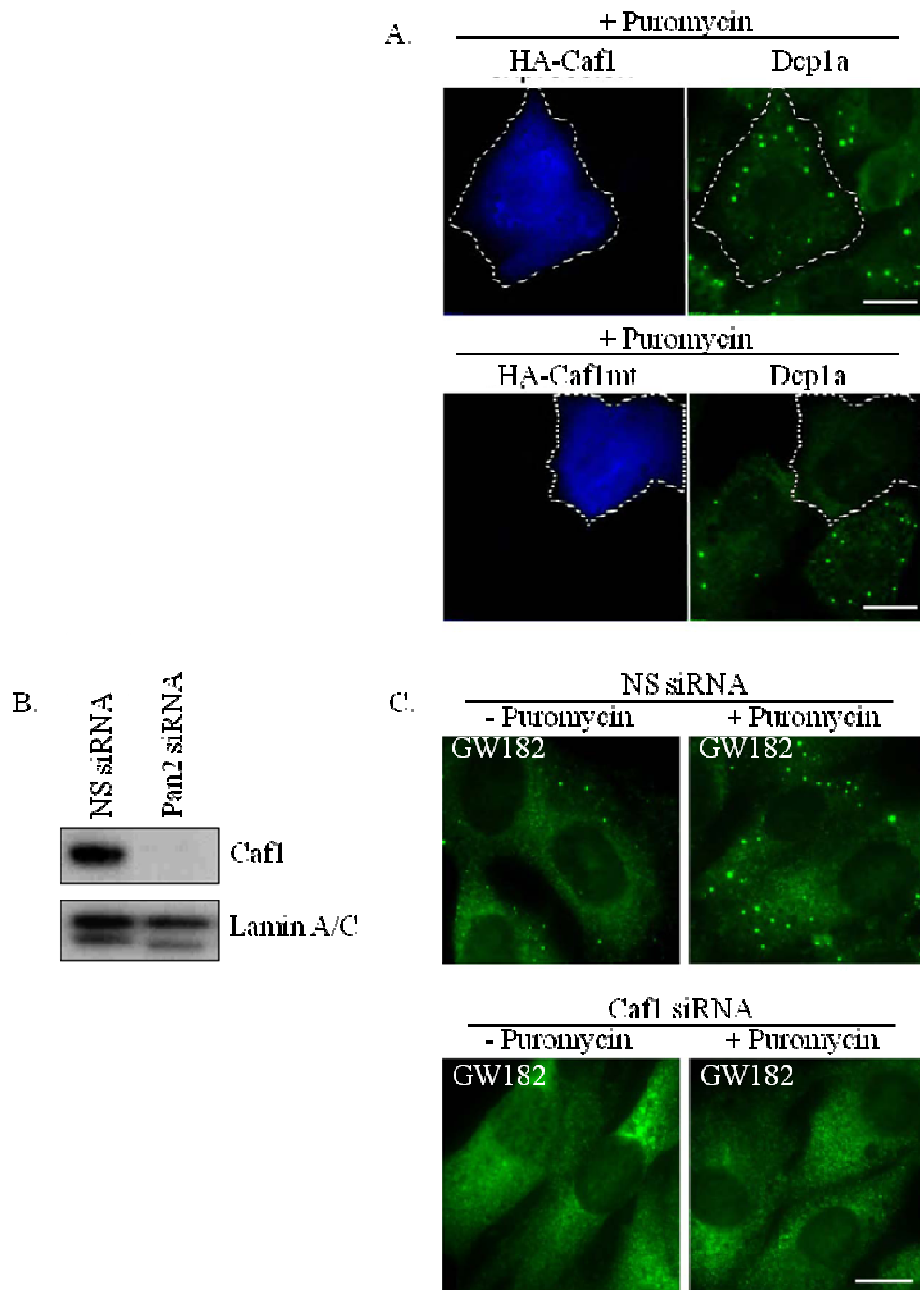


Figure 2.10: Puromycin treatment cannot induce P body formation when HA-Caf1 mt is over expressed or Caf1 is knocked down by siRNAs.

(A) Indirect immuno-fluorescence staining showing the effects of puromycin treatment on untransfected cells and cells expressing either wild-type (upper panel) or catalytically mutant (lower panel) Caf1. (B) Western blotting showing siRNA-mediated Caf1 knockdown. (C) Indirect immuno-fluorescence staining showing the effects of puromycin treatment and/or siRNA-mediated Caf1 knockdown on P body formation detected by GW182 antibody. The experiment was repeated twice.

2.4. Discussion

2.4.1. Deadenylation may also occur in P bodies

Both the Pan3-Pan2 and the Ccr4-Caf1 deadenylase complexes can be enriched in P bodies of mammalian cells (Figs. 2.1, 2.2 & 2.6), suggesting that deadenylation may also occur in P bodies. In yeast, Ccr4p and Pop2p (yeast Caf1) co-localize with P bodies in strains where decapping or 5' to 3' mRNA decay is inhibited (77). It is possible that the Ccr4-Caf1 complex transits through P bodies quickly in yeast and therefore is only enriched in P bodies under special conditions. Moreover, at least some mRNAs in P bodies still have poly(A) or oligo(A) tails. Poly(A)+ transcripts can be detected in yeast P bodies during glucose deprivation and in stationary phase (87). In human cells, the CAT-1 mRNA can be released from P bodies and recruited to polysomes for translation in response to different types of stresses (73). It is possible that some translationally inhibited poly(A)+ RNAs are targeted to P bodies for temporary storage, where they can either be deadenylated, decapped, and subsequently degraded, or released for translation before complete deadenylation.

2.4.2. Deadenylation factors have different roles in P body formation

Our observations also indicate that Pan3, Pan2, and the Ccr4-Caf1 complex play different roles in P body formation. Pan3, which interacts with both PABP and Pan2, is essential for P body formation, as siRNA-mediated Pan3 knockdown greatly decreased P body numbers and sizes in NIH 3T3 cells (Fig. 2.3). Co-expressed HA-Pan3 also helps ectopically expressed Pan2, Ccr4, and Caf1, but not PABP, to be enriched in P bodies (Fig. 2.5 & 2.6). Interestingly, the deadenylase Pan2 seems to be dispensable for P body formation, although it interacts with Pan3 and colocalizes with P bodies. siRNA-mediated knock down of the deadenylase Pan2 had little effect on P body formation (Fig. 2.4 A&B), which is consistent with the observation that over expression of catalytically mutant Pan2 had no effect on P bodies in NIH 3T3 cells (Fig. 2.4 C). Our results suggest that Pan3 has functions independent of Pan2 in P body formation, which will be addressed in Chapter three.

The deadenylase activity of the Ccr4-Caf1 complex is essential for P body formation in mammalian cells. siRNA-mediated knockdown of Caf1 inhibited P body formation in NIH 3T3 cells (21) and Ccr4 knockdown blocked P body formation in Hela cells (74). Over expression of catalytic mutant of either Caf1 or Ccr4 inhibited deadenylation (Fig. 2.8 & (16)) and blocked P body formation (Fig. 2.8 and data not shown). Moreover, restoring deadenylation allowed P body formation (Fig. 2.9). Collectively, these observations indicate that P body formation in mammalian cells requires the deadenylase activity of the Ccr4-Caf1 complex.

It is noteworthy that in yeast deletion of either Ccr4p or Pop2p (yeast Caf1) only had a small effect on P bodies (77), even though the Ccr4p-Pop2p complex is the major deadenylase in yeast. This discrepancy suggests different mechanisms for P body formation in yeast and mammalian cells. Indeed, yeast, but not human, P body components are more likely to contain Q/N-rich aggregation-prone regions (78), which may help the aggregation of associated mRNPs into P bodies.

2.4.3. Deadenylation per se, and not just dissociation of ribosomes from mRNAs, is required for mammalian P body formation

Since mRNAs need to be ribosome-free to form P bodies (72, 76) and the poly(A) promotes translation initiation, it is likely that deadenylation allows P body formation by rendering the mRNAs ribosome-free. To test this possibility, cells were treated with puromycin to release ribosomes from mRNAs while deadenylation was inhibited either by overexpression of Caf1 mutant or siRNA-mediated knockdown of Caf1. To our surprise, P body formation was still blocked in cells treated with puromycin when deadenylation was inhibited. Our observation indicates that the importance of deadenylation in P body formation cannot be simply explained by the inhibitory effect of deadenylation on recruitment of ribosomes to the mRNAs.

Another reason why deadenylation is required for P body formation might be that deadenylation helps removal of the translation activators PABP and eIF4G from the mRNAs. eIF4G can simultaneously interact with both the cap-binding protein eIF4E and the poly(A) tail-binding protein

PABP to circularize poly(A)+ mRNAs, forming a closed-loop conformation that may inhibit P body localization and degradation. Indeed, neither PABP nor eIF4G is enriched in P bodies. In addition, deadenylation may promote the interaction between mRNAs and the Lsm1-7 complex, a decapping activator that prefers to bind deadenylated mRNAs (88). In summary, my experiments demonstrate a pivotal role of deadenylation in P body formation.

2.4.4. A working model linking deadenylation and P body formation

Based on previous and current observations, the following working model is proposed to link deadenylation and P body formation in mammalian cells (Fig. 2.11). The PABPs on mRNA poly(A) tail interact with Pan3 to recruit the deadenylase Pan2, initiating the first phase of deadenylation. After the poly(A) tail is significantly shortened by Pan2, the remaining PABPs associated with the Poly(A) tail is less effective in inhibiting the deadenylase activity of the Ccr4-Caf1 complex, allowing the second phase of deadenylation to occur. During the first phase and/or early second phase of deadenylation, PABP, eIF4G, and ribosomes dissociate from the mRNPs and the mRNPs can loosely attach to P bodies. It may be speculated that these loose mRNPs constitute the outer layer of P bodies. At this stage, the mRNPs can either be released from P bodies for translation or be further deadenylated by the Ccr4-Caf1 complex, which results in recruitment of the Lsm1-7 complex. The resultant mRNPs then form the core of P bodies and its mRNA component can be decapped and degraded within P bodies. One possibility is that when the deadenylase activity of the Ccr4-Caf1 complex is inhibited, the mRNPs cannot form the cores of P bodies, therefore P body formation is inhibited.

The proposed model helps explain why the deadenylation complexes co-localize with P bodies, why deadenylation is required for P body formation, why PABP, eIF4G, and ribosomes are not enriched in P bodies, and why some mRNAs released from P bodies can still be translated.

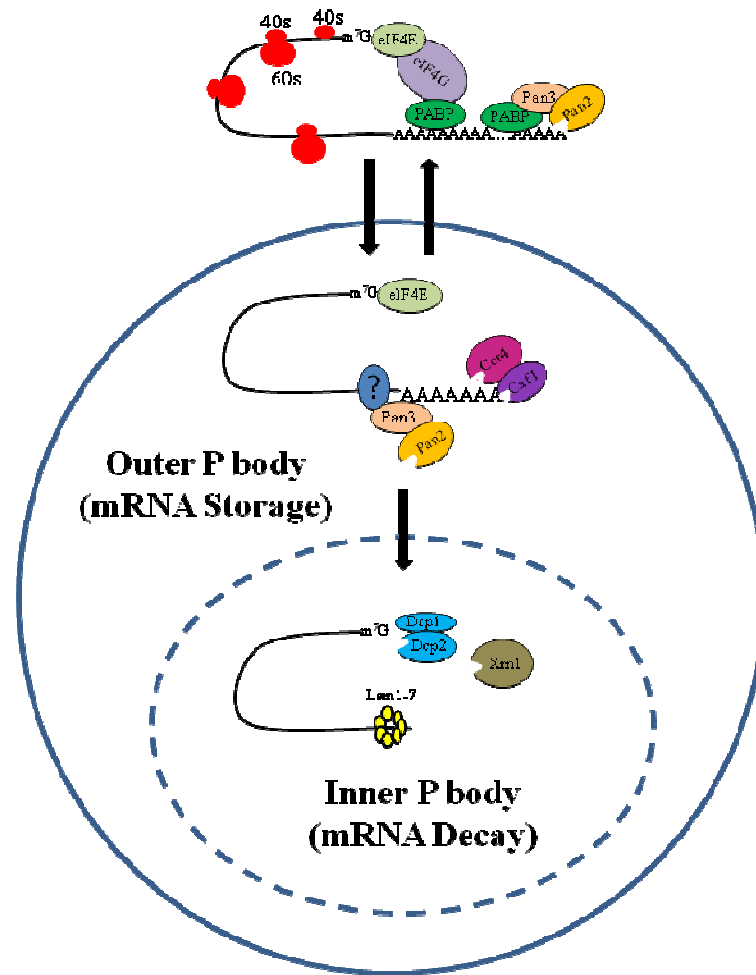


Figure 2.11: A model linking deadenylation and P body formation.

The PABP proteins on mRNA poly(A) tail interact with Pan3 to recruit the deadenylase Pan2, initiating the first phase of deadenylation. After the poly(A) tail is significantly shortened by Pan2, the remaining PABP proteins associated with the Poly(A) tail is less effective in inhibiting the deadenylase activity of the Ccr4-Caf1 complex, allowing the second phase of deadenylation to occur. During the first phase and/or early second phase of deadenylation, PABP, eIF4G, and ribosomes dissociate from the mRNP and the mRNP loosely attach to P bodies, forming the outer layer of P bodies. At this stage, the mRNP can either be released from P bodies for translation or be further deadenylated by the Ccr4-Caf1 complex, which results in recruitment of the Lsm1-7 complex. The resulting mRNP now form the core of P bodies and its mRNA component can be decapped and degraded within P bodies.

Chapter 3: Potential Phosphorylation of Pan3 N-terminal Domain in Regulating Pan3 Localization and Functions

3.1. Introduction

The Pan3-Pan2 deadenylase complex is conserved from yeast to human. In yeast, Pan3p-Pan2p slowly shortens mRNA poly(A) tails when the major deadenylase Ccr4p-Pop2p is deleted (31). In mammalian cells, the Pan3-Pan2 complex carries out the first phase of deadenylation in the cytoplasm by shortening mRNA tails from over 200 nt to about 110 nt before the Ccr4-Caf1 deadenylase takes over in the second phase of deadenylation (16, 19).

The N-terminal domain of Pan3 interacts with the cytoplasmic PABP, while the C-terminal domain of Pan3 binds deadenylase Pan2 (27, 30). By simultaneously interacting with both PABP and Pan2, Pan3 recruits the Pan2 to its substrate, the mRNA poly(A) tail. Interestingly, yeast Pan2p substrate specificity is determined by Pan3p and Pab1p (homolog of PABP), as non-poly(A) sequences bound by Pab1p can also be degraded by purified Pan3p-Pan2p (26). Although it is clear that Pan3 recruits Pan2 to its substrates, Pan3 may also play other roles in regulating Pan2 activity.

Mammalian Pan3 and Pan2 are enriched in cytoplasmic mRNA processing bodies (P bodies) and Pan3 is required for P body formation in NIH 3T3 cells (21). Yeast Pan3p and Pan2p also form cytoplasmic foci (36), although it is unclear whether those foci co-localize with P bodies. The co-localization of Pan3-Pan2 with P bodies is independent of PABP, because PABP is not enriched in mammalian P bodies even when Pan3 is over expressed (21, 71). Ectopically expressed Pan3 helps co-transfected Pan2 to enter P bodies (21), but it's unclear whether Pan3's P body localization is Pan2-dependent.

Interestingly, Pan3 can be phosphorylated at multiple sites. Yeast Pan3p is a substrate of the Pho85-Pcl1 cyclin-dependent kinase (89), a functional homolog of mammalian CDK5. Yeast Pan3p can also be phosphorylated at T57 and S252 upon DNA damage (90). Mouse Pan3 is phosphorylated at T41 (91), while human Pan3 in Hela cells is phosphorylated at S208 during G1 phase of cell cycle (34). It should be noted that all of the reported phosphorylation sites are within the N-terminal domain of Pan3. How phosphorylation affects Pan3's functions and localizations is unknown.

In this study, I generated a series of Pan3 truncation mutants to study the interplay among Pan3-Pan2 interaction, P body localization, and mRNA degradation. Mutations in Pan3 N-terminal domain mimicking hyperphosphorylation or hypophosphorylation were made to study the effects of Pan3 N-terminal domain phosphorylation on Pan3 localization and function.

3.2. Materials and Methods

3.2.1 Plasmids.

Construction of pSR-HA-hPan3 was described in Chapter 2. pSR- λ N-HA-hPan3 was constructed by ligating synthesized λ N DNA flanked by NheI and MluI sticky ends with NheI and MluI-digested PCR product using pSR-HA-hPan3 as template and two primers that amplify HA-Pan3 sequence and the plasmid backbone. The forward and reverse sequences of the NheI- λ N-MluI fragment are CTAGCATGAACGCACGAACACGAC GACGTGAGCGTCGCGCTGAGAAACAAGCTCAAT GGAAAGCTGCAAAC and CGCGTGTTTGCAGCTTTCCATTGAGCTTGTTTCTCAGCGCGACG CTCACGTCGTCGTGTTTCGTGCGTTCAT, respectively. The forward and reverse primers for amplifying HA-Pan3 sequence and the plasmid backbone are actagt(adaptor)ACGCGT(MluI site)-GGTTCATACCCATACGATGTTCCAG and actagt(adaptor)GCTAGC(NheI site)-ATGAGAACCCC GCATGGTG.

pSR-HA-hPan3(166-741), pSR-HA-hPan3(288-741), pSR-HA-hPan3(354-741), pSR-HA-hPan3(1-353), and pSR-HA-hPan3(1-588) were generated by ligating XmaI and XhoI-digested PCR-amplified hPan3 cDNA partial fragments with the larger fragment of XmaI and XhoI-digested pSR-HA-hPan3 plasmid. hPan3(166-741) was PCR amplified from the pSR-HA-hPan3 plasmid template using primers TTCCCGGGTCTGCCTTCTCTCAAGTTTTCTCTCACC and GCTCGAGTCACAACGAAAGCAAACGCAC and GCTCGAGTCACAACGAAAGCAAACGCAC, hPan3(288-741) using primers TTCCCGGGATGCAACGAAAGCAAACGCAC and GCTCGAGTCACAACGAAAGCAAACGCAC, hPan3(354-741) using primers aattatCCCGGG-GGATATATTACATCTTGCTACAAAGCTG and ATCGCTACTTAGCTAGAGCTCG, hPan3(1-353) using primers ATGTTCCAGATTACGCTTTAATTAAGC and

aattatCTCGAGAAAATTACTTGATTTCTGTATCCGGTTG, and hPan3(1-588) using ATGTTCCAGATTACGCTTTAATTAAGC and aataatCTCGAG-AGTCAACAAATACAAAATCAGATTCTTCAG. pSR-HA-hPan3(1-353, 589-741) was created using the Quickchange kit (Stratagene) and primers that allow PCR amplification of the whole pSR-HA-hPan3 plasmid except the cDNA encoding aa 354-588 of hPan3. The sequences of the Quickchange primers are GGATACAGAAATCAAGTAATGACCAAAACAGGATGCGAAGTG and CACTTCGCATCCTGTTTTGGTCATTACTTGATTTCTGTATCC. pSR- λ N-HA-hPan3(166-741), pSR- λ N-HA-hPan3(288-741), pSR- λ N-HA-hPan3(1-353), and pSR- λ N-HA-hPan3(1-588) and pSR- λ N-HA-hPan3(1-353, 589-741) were created similarly using pSR- λ N-HA-hPan3 as template.

pSR- λ N-HA-Pan(PM11) and pSR- λ N-HA-Pan(NP11) were created in three steps. The first step was creation of a λ N-HA-hPan3(PCD) mutant with a truncation in the potentially phosphorylated region which results in a unique recognition site for the blunt end restriction enzyme MscI. This was done using the Quickchange kit (Stratagene) and primers GCCCTGCTACTGCTGGCCAAACTATCATATTTATCCTCC and GTTGGAGGATAAATATGATAGTTTGGCCAGCAGTAGCAGGGC. The second step was PCR amplification of the potentially phosphorylated regions with 11 point mutations either inhibiting or mimicking phosphorylation from minigenes ordered from IDT. The third step was blunt end ligation of the PCR products with the pSR- λ N-HA-hPan3(PCD) linearized with MscI.

3.2.2. Cell culture and siRNA transfection.

Lipofectamine RNAiMAX (Invitrogen) was used for consecutive reverse and forward siRNA transfections of NIH3T3 B2A2 cells. For siRNA reverse transfection, 540 pmol SMARTpool Pan3 siRNA (Thermo Fisher Scientific), nonspecific siRNA (Thermo Fisher Scientific), or Stealth Pan2 siRNA (Duplex #1, Invitrogen) diluted with 3ml Opti-MEM serum-free medium (Invitrogen) was mixed with 36ul of Lipofectamine RNAiMAX (Invitrogen) in 10-cm dishes and incubated at room temperature for 20 minutes. 2.4×10^6 NIH3T3 B2A2 cells re-suspended in 10ml medium containing

130 ng/ml Tetroccline were added to each of the 10-cm dishes containing siRNA-RNAiMAX complexes, mixed well, and incubated at 37°C (5% CO₂) for 24 hrs before changing fresh medium for forward transfection. For the siRNA forward transfection, the siRNA-RNAiMAX complexes were prepared in the same way as reverse transfection, added to the reverse transfected cells in 10-cm dishes, and incubated at 37°C (5% CO₂) for 24 hrs. The cells were then reseeded to new 10-cm dishes, incubated at 37°C (5% CO₂) for 24 hrs, and collected for Western blot analysis and poly(A) tail length distribution profile analysis.

3.2.3. Analysis of poly(A) tail length distribution profiles.

Cytoplasmic RNA was extracted using RNeasy Mini kit (Qiagen) from cytoplasmic lysate of transfected NIH3T3 B2A2 cells, during which the RNA was subject to on-column DNase digestion with the RNase-free DNase set (QIAGEN). One µg of purified cytoplasmic RNA was incubated with 10 µCi (5000 Ci/mmol) Cordycepin-5' Triphosphate (PerkinElmer), 600 U Yeast poly(A) polymerase (Usb) and 20 U Rnasin (Promega) in 1x poly(A) polymerase reaction buffer at 37 °C for 20 minutes. The 3' end labeled RNA was then purified by phenol-chloroform extraction and ethanol precipitation. The radioactivity of the product was assayed by liquid scintillation following TCA precipitation. A constant amount of radioactivity (1000,000 cpm) was mixed and incubated with 20 µg yeast total RNA, 0.02 µg RNase A (Roche), and 87 U RNase T1(Sigma-Aldrich) in a 20 µl reaction at 30 °C for 30 minutes to digest RNA body (RNase A cleaves 3' of U and C residues and Rnase T1 cleaves after G residues). The RNase digestion reaction was stopped by the addition of SDS and Proteinase K and incubation at 37 °C for 30 minutes. The poly(A) RNA was phenol-chloroform extracted and ethanol precipitated. The radioactivity of the product was assayed by TCA precipitation and liquid scintillation again. A constant amount of radioactivity (15,000 cpm) was separated on an 8% denaturing polyacrylamide gel (with 7 M urea). The gel was run at 300 V until the bromophenol blue dye was running out of the gel. Autoradiography of the dried gel was performed at -80°C with an intensifying screen for 5.5 h.

3.2.4. Cell culture and plasmid transfection.

NIH3T3 B2A2 cells were seeded to a density of 2.4×10^6 /10-cm dish in 50 ng/ml tetracycline 24 hrs before transfection. 40 µl Lipofectamine 2000 (Invitrogen) and 1 ml Opti-MEM (Invitrogen) were mixed and incubated at room temperature for 5 minutes. 13 µg total DNA were diluted into 1ml Opti-MEM, mixed with the diluted Lipofectamine 2000, and incubated at room temperature for 25 minutes. The mixture was then added to the cells in a 10-cm dish and incubated at 37°C in 5% CO₂ for 18-20 hours. For co-IP experiments, the transfected cells from each 10-cm dish were split to two 10-cm dishes and incubated at 37°C in 8% CO₂ for 24 hours before the cells were harvest. For immuno-fluorescence staining experiments, the transfect cells were reseeded to glass chamber slides (BD Falcon) and incubated at 37°C in 8% CO₂ for 24 hours before the cells were fixed. For transcriptional pulse-chase and Northern blot analysis, the transfected NIH3T3 B2A2 cells in each 10-cm dish were split to six 6-cm dishes and one 35-mm dish and incubated at 37°C in 8% CO₂ for 20 hours before the cells were grown in tetracycline-free medium for 120 minutes to induce a transcriptional pulse driven by the Tet-off promoter of the reporter plasmid in transfected cells. At various time points after the transcription pulse, cells from the 6-cm dishes were harvested and saved at -80C as pellets for isolating total RNA and subsequent Northern blot analysis. Cells from the 35-mm dish were used for Western blot analysis.

3.2.5. Immunoprecipitation.

Cytoplasmic lysate was prepared by incubating harvested cells for 10 minutes on ice in 600ul of lysis buffer (20 mM Tris-HCl pH 7.4 buffer, 150 mM NaCl, 1% NP-40, 1 mM Na orthovanadate, 1 mM Na pyrophosphate, 1 mM NaF, and 1x protease inhibitor cocktail (Roche)) and spinning down nuclei and cell debris at 400x g for 3 minutes at 4°C. 30 µl of the supernatant was saved as input at -80°C and the rest of the supernatant was incubated with 30 µl of rat monoclonal anti-HA Affinity Matrix (Roche, clone 3F10) in the presence of 90 µg/ml RNase A for 2 hours at 4°C. The beads were washed five times with the lysis buffer and boiled in 30 µl of 2x SDS loading buffer for 5 minutes. The supernatant of the boiled mixture was saved for Western blot analysis.

3.2.6. Western blot analysis.

Either cytoplasmic or total cell lysate was used for Western, as indicated in each figure. Preparation of cytoplasmic lysate was described in “Immunoprecipitation”. Total cell lysate was prepared by lysing the cell pellets in SDS lysis buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 1x protease inhibitor cocktail (Roche), and 1 mM DTT) and filtering the lysate with a QIAshredder column (QIAGEN). Protein lysate concentration was determined by Bio-Rad Dc protein assay (Bio-Rad). Except for the immunoprecipitation experiment, 15 µg proteins were resolved on an 8% SDS-polyacrylamide gel and transferred to a PVDF membrane. The PVDF blots were probed with different antibodies as described in each figure. The following primary antibodies were used at indicated dilution: rabbit anti-Pan2 peptide antibody at 1:2,000 (Bethyl), rabbit anti-Pan3 peptide antibody at 1:1,000 (Bethyl), mouse anti-tubulin monoclonal antibody at 1:50,000 (Sigma-Aldrich), rat anti-HA monoclonal antibody at 1:3,000 (Roche), mouse anti-GAPDH monoclonal antibody at 1:50,000, and goat anti-Lamin B1 polyclonal antibody at 1:200 (Santa Cruz). After washing the membrane in 1xPBST buffer, the membrane was incubated with HRP-conjugated donkey anti-rabbit IgG, goat anti-mouse IgG, or rabbit anti-goat IgG at 1:5,000 for 1 hour at room temperature. After washing in 1xPBST buffer again, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) for 5 minutes at room temperature, and exposed to a SYNGENE Gnome gel documentation system (SYNGENE).

3.2.7. Northern blot analysis.

RNase H treatment of cytoplasmic mRNA after annealing to Oligo dT (Invitrogen) was performed to generate poly(A)⁻ RNA. 10 µg of cytoplasmic RNA with or without RNase H treatment was separated using 1.4% formaldehyde agarose gel electrophoresis, transferred to GeneScreen Hybridization Transfer membrane (PerkinElmer), UV cross-linked to the membrane, and blotted with ³²P-labeled BBB or α GAPDH DNA probe dissolved in ULTRAhyb hybridization buffer (Ambion). The ³²P-labeled probes were prepared by labeling PCR amplified BBB and α GAPDH fragments with α -[³²P]dCTP (>6000 Ci/mmol; PerkinElmer) using the Rediprime II Random Prim Labeling System

(GE Healthcare). The probes were purified using MicroSpin G-25 columns (GE Healthcare) before used for hybridization.

3.2.8. Immunofluorescence microscopy.

This was conducted as described in Chapter 2. Briefly, transfected NIH3T3 cells were seeded to chamber slides (BD Falcon) and cultured at 37°C (8% CO₂) overnight before the cells were fixed. For sodium arsenite (Sigma-Aldrich) or Leptomycin B (Sigma-Aldrich) treatment, cells were cultured in medium containing 0.3mM sodium arsenite for 1 hour or 20 ng/ml Leptomycin B for 4 hours before the cells were fixed. Ectopically expressed HA-tagged proteins were detected using rat anti-HA monoclonal antibody (Roche, clone 3F10) and Alexa Fluor 350 goat anti-rat IgG (Invitrogen). Endogenous P bodies were detected using rabbit anti-RCK/p54 (Bethyl) or human anti-GW182 (a gift from Dr. M.J. Fritzler, University of Calgary, Canada) and Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen) or Alexa Fluor 488 goat anti-human IgG (Invitrogen). Endogenous stress granules were detected using rabbit anti-G3BP1 (a gift from Dr. R.Lloyd, Baylor College of Medicine, USA) and Alexa Fluor 555 goat anti-rabbit IgG. All the primary and secondary antibodies used for immunofluorescence staining were diluted at 1:1,000.

Images were obtained at RT by optical z-sectioning (20 sections in total, 0.2- μ m-space between sections) using an objective lens (100 \times /1.35 NA; Olympus) of a deconvolution microscope system (DeltaVision) containing an inverted microscope (IX70; Olympus). Immersion oil (n = 1.514) was from Applied Precision, LLC. Images were captured using a digital camera (Coolsnap HQ; Roper Scientific). Stacks of 20 images were projected as a single 2D picture using softWoRx Explorer (version 3.3.6; Applied Precision, LLC). The RGB colors of the resulting pictures were separated using Photoshop CS (Adobe) (21).

3.3. Results

3.3.1. siRNA-mediated knockdown of Pan3 or Pan2 in NIH 3T3 cells resulted in cytoplasmic mRNAs with longer poly(A) tails

Although deletion of Pan3 and/or Pan2 in yeast extended poly(A) tail length of bulk mRNAs (30), the effect of Pan3 or Pan2 depletion on the poly(A) tail of bulk mRNA in mammalian cells has never been studied. Cytoplasmic mRNA from NIH 3T3 cells transfected with Pan3 or Pan2-specific or non-specific siRNA was labeled at 3' ends with ³²P-Cordycepin (3' deoxyadenosine) and treated with diluted Rnase T1 and Rnase A to remove the non-poly(A) RNA body. The resultant poly(A) tails were resolved on a denaturing urea-polyacrylamide gel to reveal poly(A) size distribution profile (Fig. 3.1 B). Poly(A) tails of cytoplasmic mRNAs from Pan3 siRNA transfected cells were significantly longer than those from non-specific siRNA transfected cells. Pan2 knockdown only slightly increased bulk mRNA poly(A) tail length, probably because the knockdown of Pan2 was not complete (Fig. 3.1 A). This result confirmed that Pan3 and Pan2 are general deadenylation factors in mammalian cells.

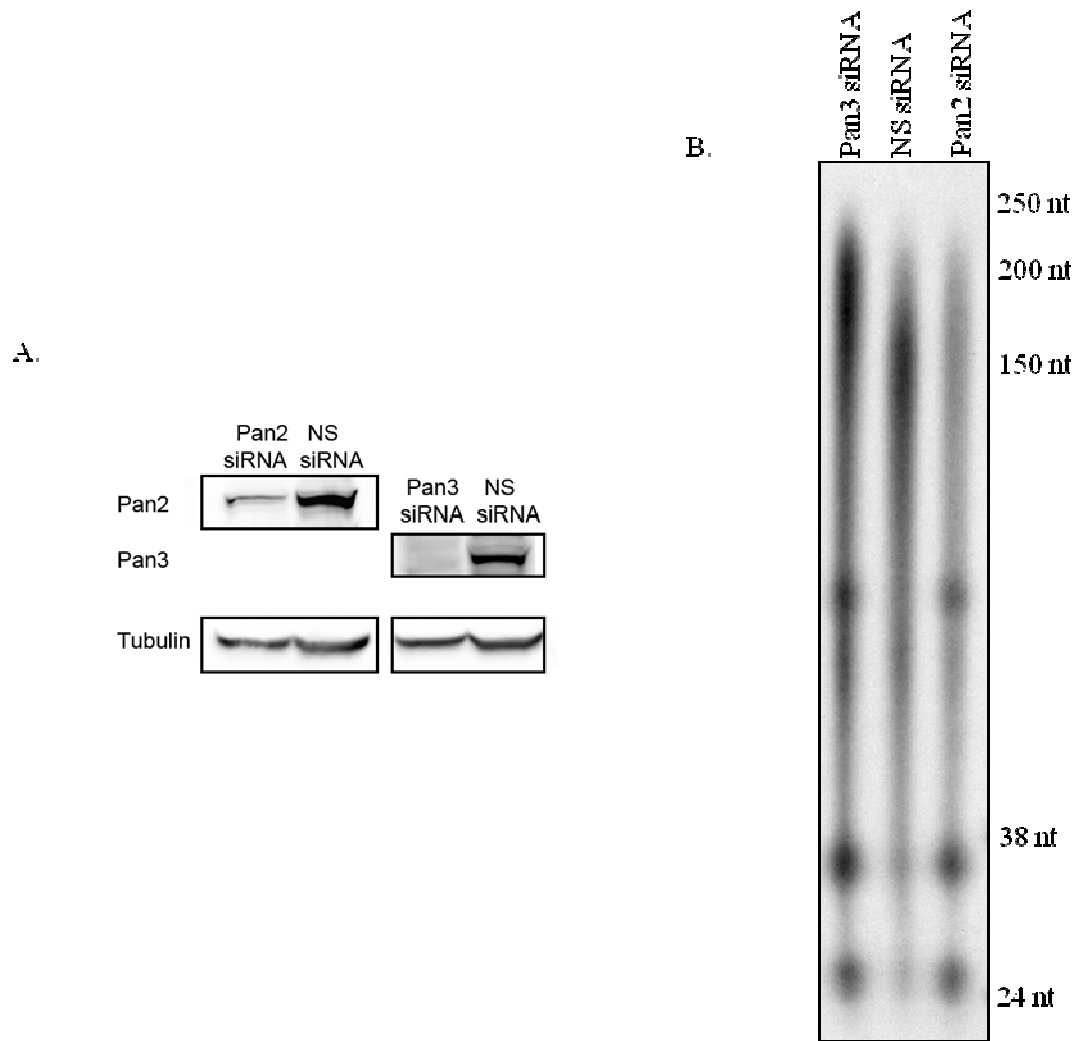


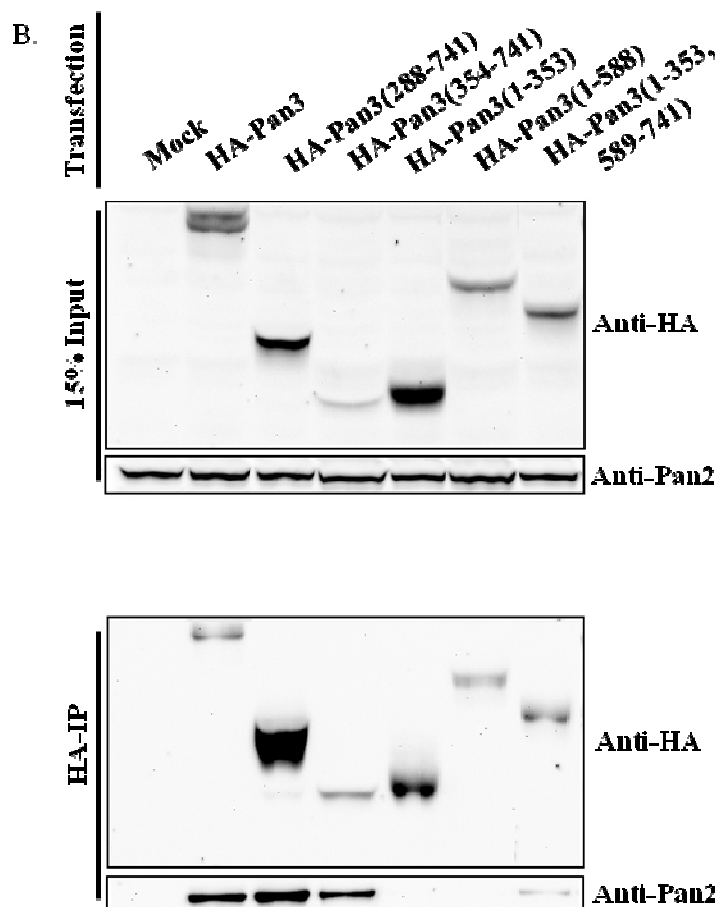
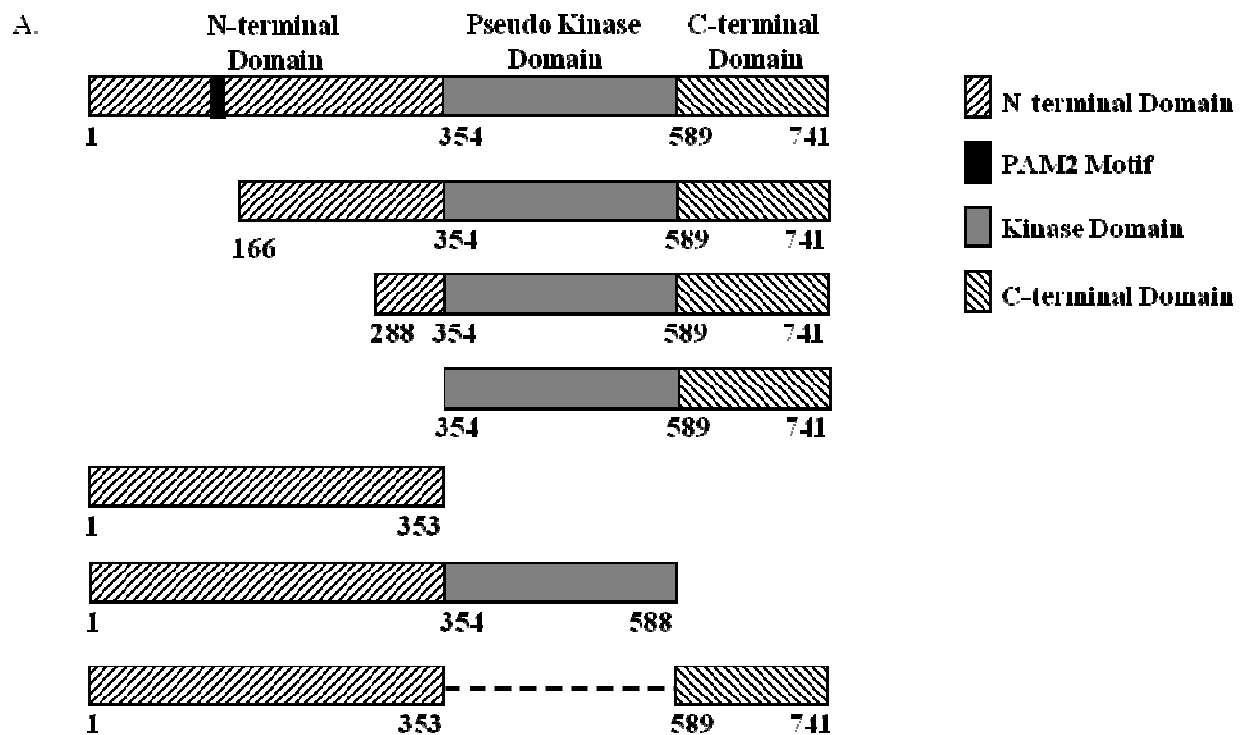
Figure 3.1: Effects of Pan3 knockdown on poly(A) tails of bulk cytoplasmic mRNAs in NIH 3T3 Cells.

(A) Western Blot analysis of total cell lysate showing knockdown of Pan2 or Pan3 by siRNA.

(B) Poly(A) tail length distribution profiles analyzed by denaturing urea-polyacrylamide gel electrophoresis. Following transfection with Pan3 siRNA or nonspecific siRNA or Pan2 siRNA, cytoplasmic RNA was isolated from NIH 3T3 cells. The RNA samples were first labeled with ^{32}P labeled Cordycepin in the presence of yeast PAP, which are then digested with Rnase A and Rnase T1 to remove the RNA body. The resulting poly(A) or oligo(A) tails of RNA were resolved on a denaturing urea-polyacrylamide gel. The bands running at ~70nt, 35nt, and 25nt can sometimes be observed in the sample with NS siRNA treatment. (This siRNA knockdown experiment was repeated twice, while the poly(A) profiling was repeated four times.)

3.3.2. The N-terminal domain of Pan3 is dispensable for interaction with Pan2 in NIH 3T3 cells

Pan3 consists of a less conserved N-terminal domain, a pseudo kinase domain, and a conserved C-terminal domain (Fig. 3.2A). While the PAM2 motif in Pan3 N-terminal domain is responsible for Pan3-PABP interaction (92), in yeast the C-terminal domain of Pan3p is responsible for interaction with Pan2p (33). To map the Pan2-binding domains in human Pan3, I generated a series of HA-tagged Pan3 truncation mutants (Fig. 3.2 A) and tested their ability to pull down endogenous Pan2 in NIH 3T3 cell lysate by co-IP (Fig. 3.2 B). The co-IP experiment showed that deletion of the N-terminal domain (1-353) didn't affect Pan3-Pan2 interaction and that deletion of the pseudo kinase domain (354-588) significantly decreased the Pan3-Pan2 interaction, while deletion of the C-terminal domain (589-741) abolished Pan3-Pan2 interaction. These results indicate that human Pan3 C-terminal domain is required for interaction with Pan2 and that the pseudo-kinase domain enhances Pan2-Pan3 interaction. In contrast, the N-terminal domain is dispensable for Pan3-Pan2 interaction.



: Only the C-terminal Pan3 is required for 1 with Pan2 in NIH 3T3

atic representation of wild and Pan3 truncation mutants. if is responsible for Pan3-raction.

n blot analysis showing HA-teins and endogenous Pan2 in c input (Upper two panels) iplexes precipitated by anti-ly conjugated to agarose ver two panels). Mock cell lysate was used as a ontrol to show the specificity ipitation. (The coIP t was repeated once.)

3.3.3. Domain requirement for tethered Pan3 to promote rapid deadenylation and decay of the reporter mRNA

Pan3 interacts with both PABP and deadenylase Pan2 to recruit Pan2 to the mRNA poly(A) tail associated with PABP (27, 33, 92, 93). To understand whether Pan3 simply recruits Pan2 or also regulates Pan2 nuclease activity, λ N-tagged Pan3 or Pan3 mutants were co-expressed with the reporter mRNA BBB-4boxB and decay kinetics of the reporter mRNA in the cytoplasm of the NIH3T3 B2A2 cells were studied by Tet-off driven transcriptional pulse-chase experiments (Fig. 3.3 A) (81). The λ N tag, which is a 22 amino acid peptide derived from the N-terminus of the lambda bacteriophage N protein, can specifically bind the boxB sequences in the 3' untranslated region (UTR) of the reporter mRNA (94).

As expected, tethering wild type Pan3 but not LacZ promoted deadenylation and decay of the reporter mRNA in the cytoplasm (Fig. 3.3 C, D). In contrast, the stability of the cytoplasmic reporter mRNA was not affected by tethering Pan3 mutants with deletion in N-terminal domain, pseudo kinase domain, or C-terminal domain (Fig. 3.3.E-G). It is not surprising that tethering Pan3(1-588) did not promote rapid deadenylation and decay of the reporter mRNA, since Pan3(1-588) cannot interact with the deadenylase Pan2 (Fig. 3.3 F). However, both Pan3(288-741) and Pan3(1-353, 589-741), which were able to interact with Pan2 (Fig. 3.2 B), also failed to stimulate reporter mRNA deadenylation and decay when tethered (Fig. 3.3 E, G), suggesting that while Pan3 C-terminal domain is responsible for recruiting Pan2, Pan3 N-terminal and pseudo kinase domains are also important for stimulating Pan2 deadenylase activity.

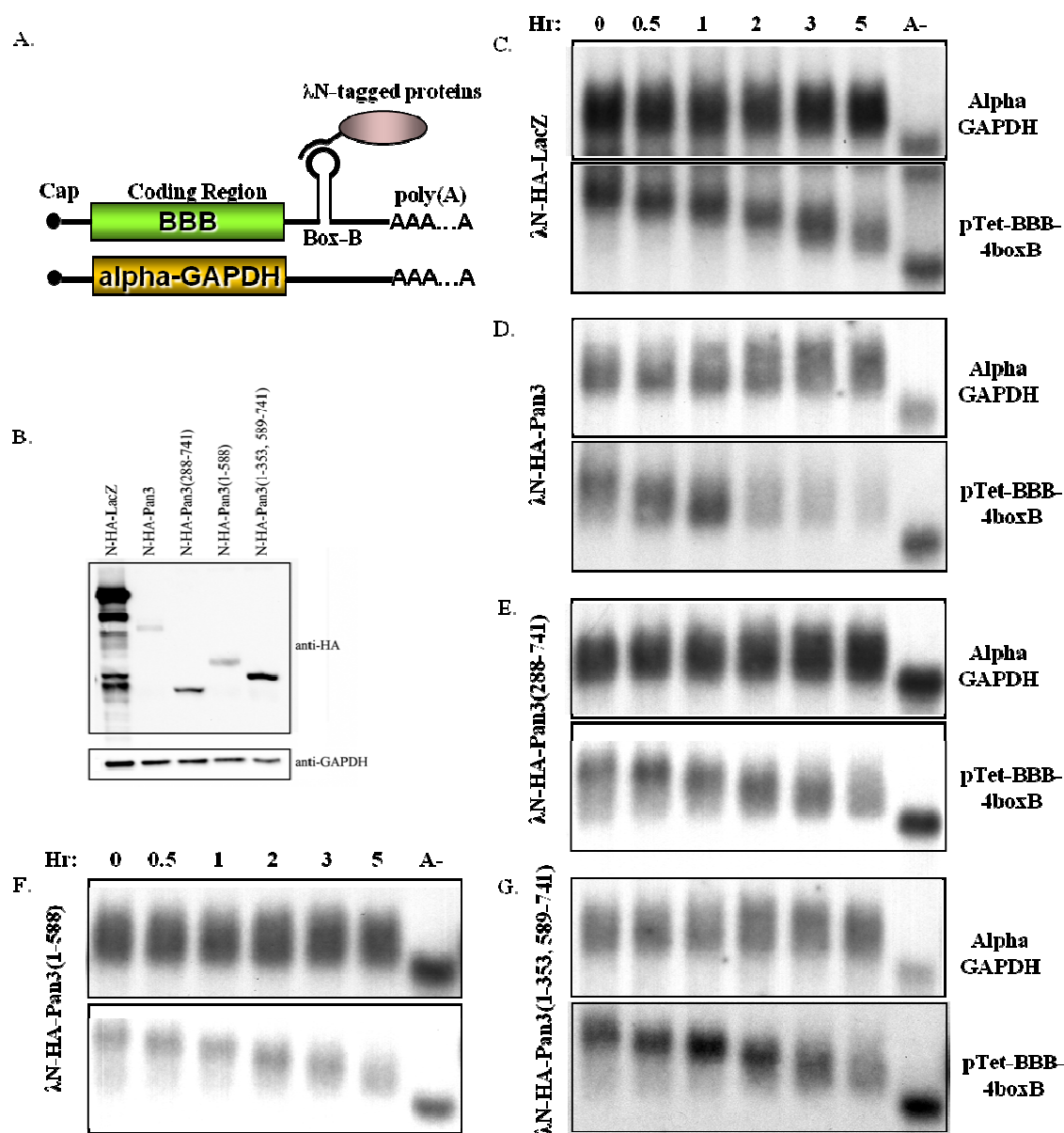


Figure 3.3: Effects of tethered λ N-HA-LacZ, λ N-HA-Pan3, or λ N-HA-Pan3 truncation mutants on the decay kinetics of the reporter mRNA BBB-4boxB.

(A) NIH3T3 B2A2 cells were transiently transfected with a Tet promoter-regulated plasmid encoding the reporter mRNA BBB-4boxB and the plasmids encoding λ N-tagged proteins as indicated. A plasmid encoding constitutively expressed alpha-globin-GAPDH mRNA was also co-transfected to provide an internal standard for transfection efficiency and sample handling. (B) Western blot analysis of total cell lysate showing expression levels of λ N-tagged proteins for the tethering experiment. Endogenous GAPDH was used as a loading control. (C-G) Northern blots showing cytoplasmic decay of the BBB-4boxB mRNA tethered with λ N-HA-LacZ (C), λ N-HA-Pan3(D), λ N-HA-Pan3(288-741) (E), λ N-HA-Pan3(1-588) (F), λ N-HA-Pan3(1-353, 589-741) (G). The times given at the top correspond to hours after tetracycline addition. Poly(A)- RNA (A-) was prepared in vitro by treating an RNA sample from an early time point with oligo(dT) and RNase H. (This experiment was repeated once.)

3.3.4. The N-terminal domain and pseudo kinase domain, but not the C-terminal domain, are important for Pan3 to form many foci in NIH 3T3 cells

Pan3 not only co-localizes with P bodies but also is required for P-body formation in mammalian cells (21). However, it was unclear which domains of Pan3 are important for its P body localization and whether Pan3 is dependent on Pan2 for localizing to P bodies. To address these questions, the aforementioned Pan3 truncation mutants were transiently expressed in NIH 3T3 cells and their sub-cellular localization were examined using indirect immuno-fluorescence microscopy (Fig. 3.4). Anti-HA and anti-RCK/p54 antibodies were used to detect the HA-tagged Pan3 wt or mutants and endogenous P bodies, respectively. Consistent with what we reported before, wild type Pan3 was mainly cytoplasmic and forms many foci co-localizing with endogenous P bodies. Except the kinase domain deletion mutant (Fig. 3.4 F), all other mutants showed increased nuclear distribution and were somewhat able to localize to cytoplasmic P bodies. The expression level of HA-Pan3(354-741) was so low that I did not find cells expressing HA-Pan3(354-741) under microscope. Interestingly, the Pan3 mutant with most of the N-terminal domain deleted only formed a few foci (Fig. 3.4 C), while the Pan3 mutant with C-terminal domain deleted still formed many foci (Fig. 3.4 D), suggesting that the N-terminal domain of Pan3 is responsible for Pan3 colocalization with P-bodies in the cells. This experiment also indicated that interaction with Pan2 is not required for Pan3 to enter P bodies, since the C-terminal domain deletion mutant that no longer interacts with Pan2 could still co-localize with P bodies (Fig. 3.4 D, E). Collectively, these results showed that both N-terminal and pseudo-kinase domains, but not C-terminal domain, are necessary for Pan3 to colocalize with P bodies. Intriguingly, the N-terminal domain by itself was sufficient to localize to P bodies.

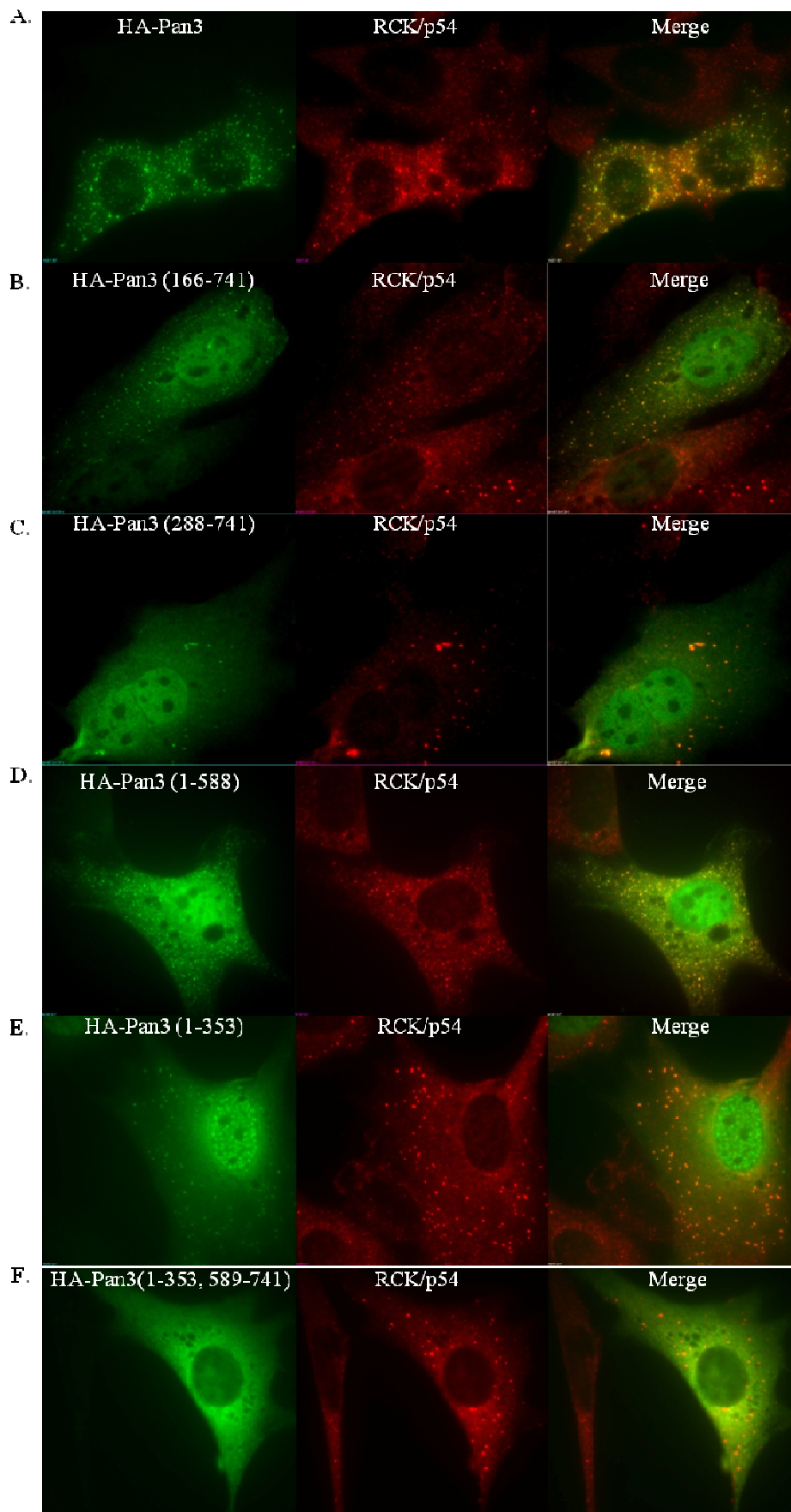


Figure 3.4: Indirect immunofluorescence staining showing sub-cellular localization of HA-tagged Pan3 or Pan3 truncation mutants and the P body marker RCK/p54.

A) Wild-type Pan3 formed many cytoplasmic foci colocalizing with P bodies. **B)** Pan3 could still localize to most P bodies when half of the Pan3 N-terminal domain was deleted. **C)** Deletion of most of the Pan3 N-terminal domain impaired Pan3's ability to form many cytoplasmic foci. **D)** The Pan3 mutant with N-terminal domain deleted was still able to form many cytoplasmic foci. **E)** Pan3 N-terminal domain alone was able to colocalize with P bodies. **F)** Deletion of the pseudo kinase domain completely abolished the ability of Pan3 to colocalize with P bodies. (This experiment was repeated vice.)

3.3.5. Pan3 N-terminal domain may be phosphorylated to regulate Pan3 localization and/or function

Since Pan3 N-terminal domain is important for Pan3 to stimulate Pan2 activity and to localize to P bodies, I decided to take a close look at this domain. An alignment of Pan3 sequences from different species revealed that the N-terminal domain is less conserved than the other domains (Fig. 3.5 A). However, the N-terminal domains of all the analyzed Pan3 sequences are proline-rich. Since the conformation of proline is rigid, proline-rich motifs often tend to form disordered regions that are difficult to characterize by NMR spectroscopy or X-ray diffraction (95). Indeed, the N-terminal domains of both human and yeast Pan3 are predicted to be intrinsically disordered by the VL-XT software(96).

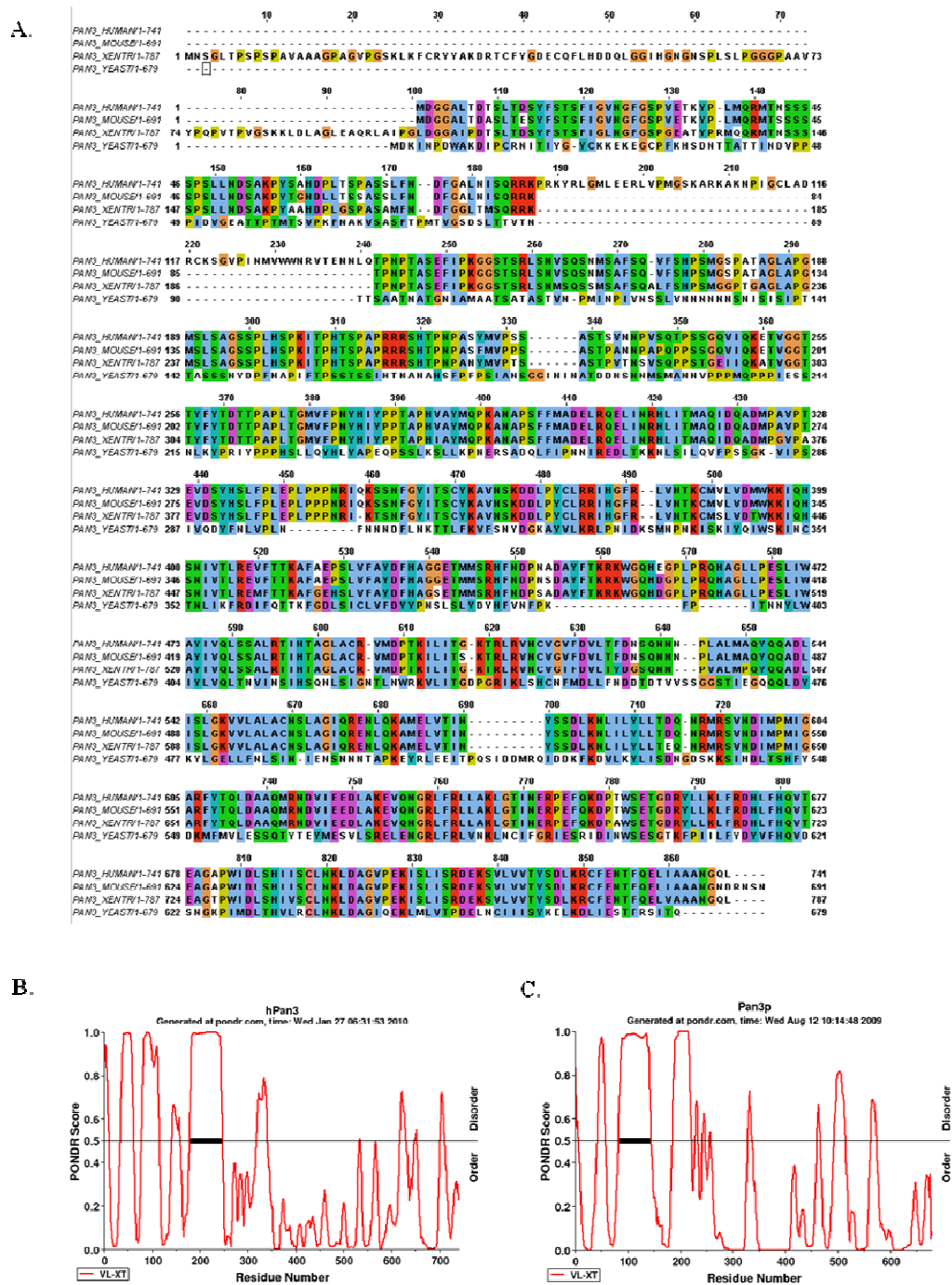


Figure 3.5: Human Pan3 N-terminal domain contains a conserved proline-rich, disordered region.

(A) Alignment of Pan3 protein sequences from yeast, xenopus, mouse and human using ClustalW2. Proline residues are highlighted in yellow. (B-C) Prediction of intrinsically disordered region in human Pan3 (B) and yeast Pan3 (C) using VL-XT from POND3. Regions with a ponder score above 0.5 are considered to be disordered by the VL-XT.

Both proline-rich motifs and intrinsically disordered regions are often target sites for phosphorylation and are involved in protein-protein interaction (95, 97). HA-tagged wild-type Pan3 and Pan3 N-terminal domain appeared as double bands on the Western blot membrane and the upper band was sensitive to calf intestinal alkaline phosphatase (CIAP) treatment (Fig. 3.6 A), suggesting that Pan3 N-terminal domain indeed can be phosphorylated in NIH 3T3 cells.

Two different online tools were used to predict phosphorylated residues in Pan3 N-terminal domain. The first tool is DEEP (Disorder Enhanced Phosphorylation Predictor), which predicts phosphorylation sites based on both disorder information and position-specific amino acid frequencies (98). The second tool, ScanSite 2.0, can identify short protein sequence motifs that are substrates for protein Ser/Thr- or Tyr-kinases (99). Although the two tools use different methods to predict protein phosphorylation sites, they both predicted that a small region around a.a. residue 200 in Pan3 N-terminal domain is likely hyperphosphorylated (Fig. 3.6 B, C).

To study the functional significance of Pan3 N-terminal domain phosphorylation, eleven predicted phosphorylation sites around residue 200 in λ N-HA-tagged Pan3 were mutated to either inhibit or mimic phosphorylation at those sites. The sequences of part of the N-terminal domain of wild type Pan3, Pan3 mutant nonphosphorylatable at the eleven sites (NP11), and phosphor-mimetic Pan3 mutant (PM11) were aligned, with the mutated residues highlighted in white background (Fig. 3.6 D). Western blot analysis showed that both λ N-HA-tagged Pan3(PM11) and λ N-HA-tagged Pan3(NP11) were expressed in NIH 3T3 cells after transient transfection (Fig. 3.6 E).

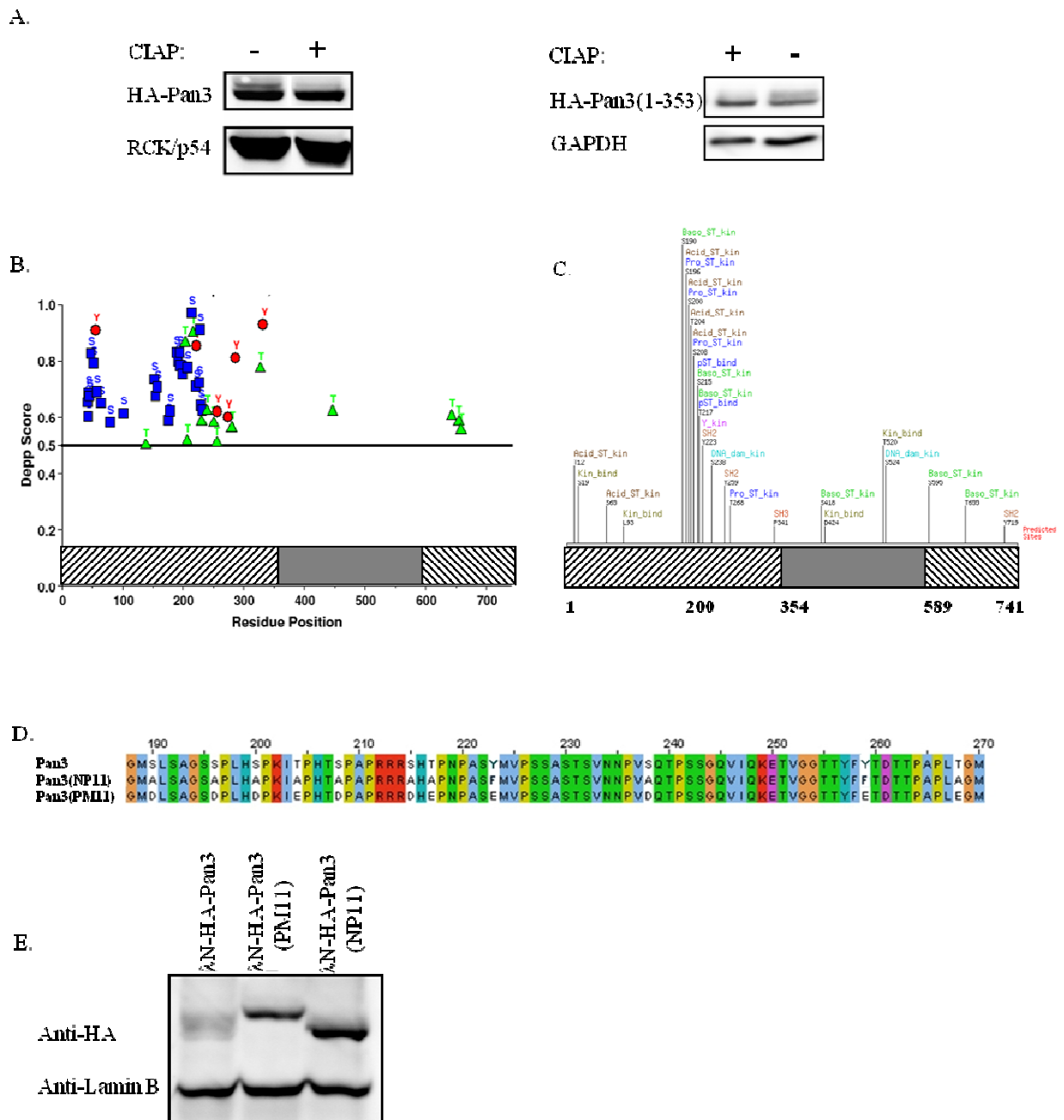


Figure 3.6: Mutagenesis to mimic or inhibit phosphorylation in the disordered region of Pan3.

(A) Western blot analysis of HA-tagged wild-type Pan3 (Left panel) or HA-tagged Pan3 N-terminal domain (Right panel) in CIAP treated or mock treated cell lysate. (CIAP treatment of wild-type Pan3 was repeated once; CIAP treatment of Pan3(1-353) was performed only once.) (B) Prediction of Pan3 phosphorylation sites using PONDR. (C) Prediction of Pan3 phosphorylation sites using ScanSite. (D) Local sequence alignment of wild-type Pan3, Pan3(NP11), and Pan3(PM11) using ClustalW2. The eleven residues with white background are mutated to either mimic phosphorylation (PM11) or inhibit phosphorylation (NP11). (E) Western blot analysis of total cell lysate showing the expression of λ N-HA-tagged wild type Pan3, Pan3(NP11), and Pan3(PM11).

Interestingly, mutations at the eleven potential phosphorylation sites in Pan3 N-terminal domain dramatically changed Pan3 localization. While λ N-HA-tagged Pan3 mostly formed cytoplasmic foci co-localizing with P bodies (Fig. 3.7 A), λ N-HA-tagged Pan3(NP11) mimicking hypo-phosphorylation in Pan3 N-terminal domain induced formation of large, irregularly shaped speckles co-localizing with P bodies (Fig. 3.7 B). On the other hand, λ N-HA-tagged Pan3(PM11) mimicking hyper-phosphorylation in Pan3 N-terminal domain induced formation of nuclear foci (Fig. 3.7 C), similar to the localization of HA-tagged Pan3(1-353) (Fig. 3.4 E). Moreover, λ N-HA-tagged Pan3(PM11) in the cytoplasm no longer enriches in P bodies (Fig. 3.7 C).

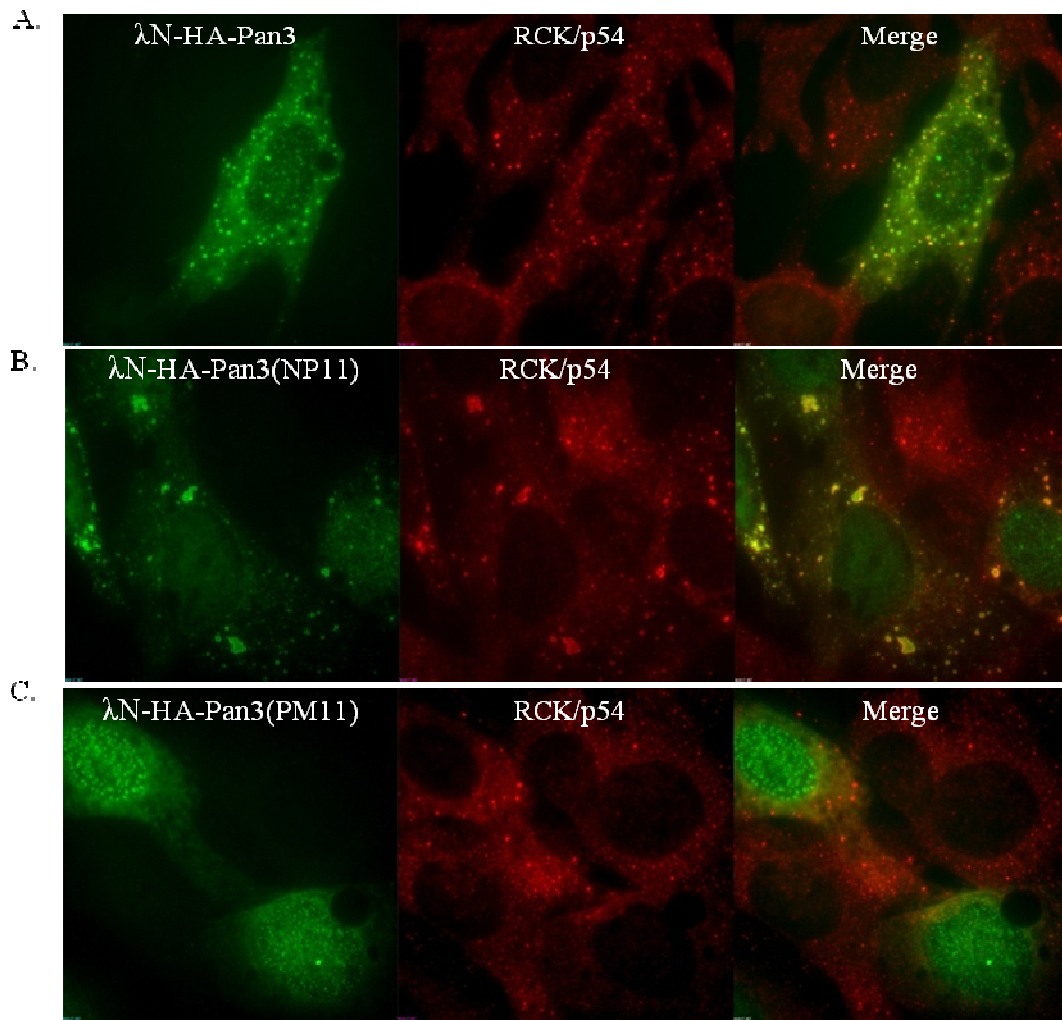


Figure 3.7 Mutations at the potential phosphorylation sites in Pan3 N-terminal domain dramatically changed Pan3 localization.

Immuno-fluorescence staining showing the localization of the P body marker RCK/p54 and λ N-HA-tagged wild type Pan3 (A), Pan3(NP11) (B), or Pan3(PM11) (C). (This experiment was repeated twice.)

The λ N-HA-tagged Pan3(PM11) nuclear foci resemble the nuclear HA-tagged Pan3 foci formed upon leptomycin B (LMB) treatment, which inhibits CRM1-mediated nuclear export (100) (Fig. 3.8 A). Interestingly, LMB treatment also increased the relative intensity of the upper band of HA-Pan3 (Fig. 3.8 B). It is possible that LMB treatment resulted in both Pan3 nuclear localization and hyperphosphorylation.

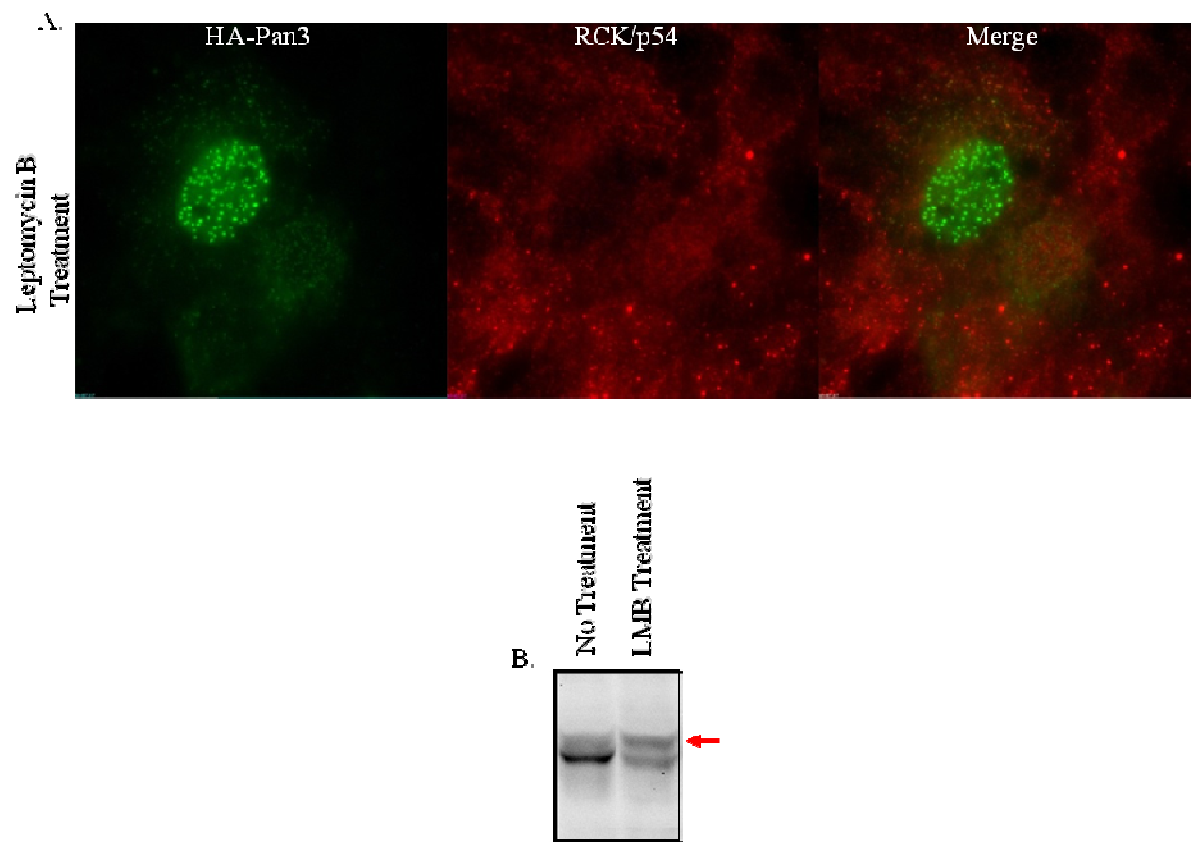


Figure 3.8: Effects of Leptomycin B treatment on HA-tagged Pan3.

(A) Immuno-fluorescence staining showing the localization of HA-tagged Pan3 upon Leptomycin treatment. (B) Western blot analysis of total cell lysate showing that Leptomycin B (LMB) treatment may affect phosphorylation of HA-tagged Pan3. (This experiment was performed once.)

The observation that sub-cellular localizations of λ N-HA-tagged Pan3(PM11) and Pan3(NP11) are distinct from that of λ N-HA-tagged Pan3 suggests that these mutants may have effects different from Pan3 on mRNA decay kinetics. To test this possibility, Pan3(PM11) or Pan3(NP11) was tethered to the 3'UTR of the reporter pTet-BBB-4boxB through specific λ N-boxB interaction, followed by Tet-off driven transcriptional pulse-chase experiments for studying reporter mRNA decay kinetics. The results showed that tethering either one of Pan3, Pan3(PM11), or Pan3(NP11) promoted cytoplasmic deadenylation and decay of the reporter mRNA BBB-4boxB (Fig. 3.9). These results strongly suggest that phosphorylation status of the eleven residues in Pan3 N-terminal domain does not directly affect Pan3's ability to promote rapid deadenylation and decay. Instead, it affects Pan3 subcellular localization.

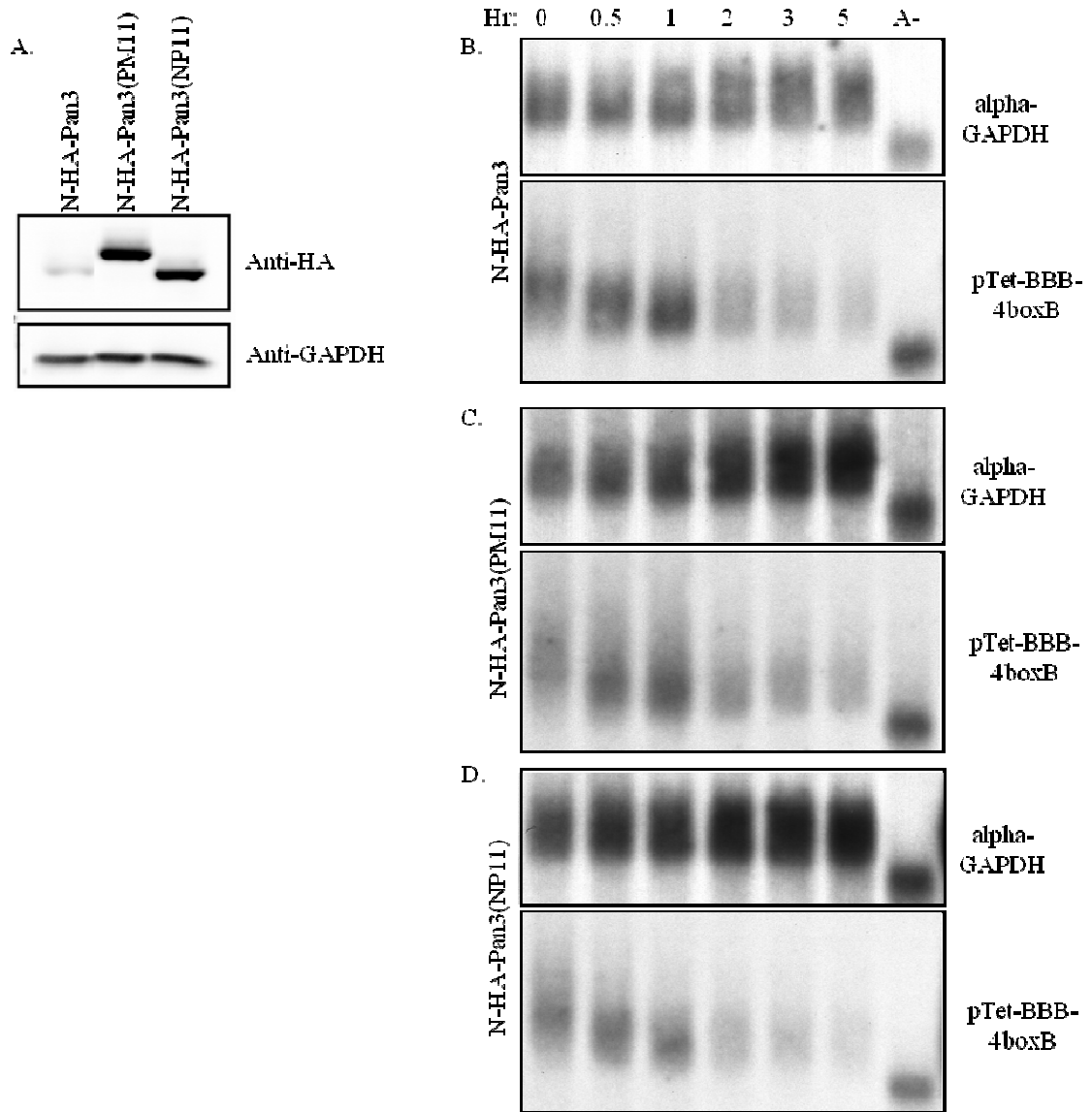


Figure 3.9: Effects of tethered Pan3(PM11) or Pan3(NP11) on reporter mRNA decay.

(A) Western blot analysis of total cell lysate showing expression of λ N-HA-tagged proteins for the tethering experiment. (B-D) Northern blots showing cytoplasmic decay of the BBB-4boxB mRNA tethered with λ N-HA-Pan3 (B), λ N-HA-Pan3(PM11) (C), and λ N-HA-Pan3(NP11) (D). A plasmid encoding constitutively expressed alpha-globin-GAPDH mRNA was also co-transfected to provide an internal standard for transfection efficiency and sample handling. The times given at the top correspond to hours after tetracycline addition. Poly(A)- RNA (A-) was prepared in vitro by treating an RNA sample from an early time point with oligo(dT) and RNase H. (This experiment was performed once.)

3.4. Discussion

In this study, I showed that siRNA-mediated knockdown of Pan3 resulted in bulk cytoplasmic mRNAs with longer poly(A) tails in NIH 3T3 cells (Fig. 3.1). Pan2 knockdown had a similar but weaker effect, probably because the knockdown was not complete (Fig 3.1). This phenotype is reminiscent of that observed in budding yeast, where Pan3p-Pan2p was reported to trim the poly(A) tails of newly synthesized transcripts to message-specific length [35], although it was unclear whether this trimming occurred in the nucleus or in the cytoplasm. Interestingly, my preliminary data showed that HA-tagged wild-type Pan3 formed nuclear foci when the cells were treated with leptomycin B (Fig. 3.8). This observation is consistent with a speculation that the Pan3-Pan2 complex may trim the poly(A) tails of nascent transcripts at the transcription sites.

A novel role for Pan3 in regulating Pan2 poly(A) nuclease activity is also suggested by my domain dissection study on Pan3. Tethering wild-type Pan3, but not the Pan3 mutant deficient in Pan2 interaction, promoted deadenylation and degradation of the reporter mRNAs in NIH 3T3 cells (Fig. 3.2 & Fig. 3.3 F), suggesting that interaction with Pan2 is important for Pan3 to promote deadenylation. Interestingly, tethering Pan3 mutant that lacks N-terminal domain or pseudo-kinase domain but is capable of interacting with Pan2 also did not elicit rapid deadenylation and decay of the reporter mRNA (Fig. 3.2 & Fig. 3.3 E&G). These results suggest that the N-terminal and pseudo-kinase domains are also required for Pan3 to promote the poly(A) nuclease activity of Pan2. Since purified human Pan2 alone can trim oligo(A) in the absence of PABP in an *in vitro* reaction albeit slowly [27], it is possible that the N-terminal domain and the pseudo kinase domain of Pan3 are required to release PABP at the very 3' end of the poly(A) tail, making the poly(A) tail more accessible to Pan2. Consistent with this idea, the Pan3 N-terminal domain indeed contains a PAM2 motif for interaction with PABP [92].

Previously, we have shown that deadenylation is required for mRNAs to enter or form P bodies in mammalian cells and that both Pan3 and Pan2 are enriched in P bodies [21]. Therefore, the Pan3-Pan2 complex may be recruited to P bodies via aggregation of mRNPs that contain mRNAs whose poly(A)

tails just have been shortened by the Pan3-Pan2 complex. However, my experiments showed that localization of Pan3 to P-bodies is independent from its ability to interact with Pan2 or promote deadenylation (Fig 3.4). Moreover, my observation that siRNA-mediated knockdown of Pan3, but not Pan2, inhibited P body formation in NIH 3T3 cells (Fig. 2.3 & 2.4) suggests that Pan3 plays an active role in P body formation. Collectively, my data indicate that in addition to playing a regulatory role in deadenylation, Pan3 also contributes to P body formation by exerting some kind of Pan2-independent function.

My data also suggest that the localization and functions of Pan3 are likely regulated by phosphorylation of a disordered region in Pan3 N-terminal domain. The proline-rich human Pan3 N-terminal domain is predicted to contain an intrinsically disordered region by PONDR. Interestingly, yeast Pan3p also contains an N-terminal disordered region (Fig. 3.5), suggesting that the disordered region may be functionally important. As intrinsically disordered regions are frequently phosphorylated to regulate protein functions, I also searched the kinase target sequences in the intrinsically disordered region of Pan3 N-terminal domain by Scansite (Fig. 3.6). Phosphatase treatment greatly reduced the relative intensity of the upper band of HA-Pan3 and HA-Pan3 N-terminal domain doublet on Western blot membranes (Fig. 3.5 & 3.6), suggesting that the Pan3 N-terminal domain can be phosphorylated. Mutations (NP11) that inhibit phosphorylation at eleven potential phosphorylation sites in Pan3 N-terminal domain induced formation of abnormally large P bodies (Fig. 3.7). The increase of P body size is unlikely caused by inhibition of mRNA decay within the aberrant P bodies, as tethered Pan3(NP11) can still interact with Pan2 and promote decay of the reporter mRNA (Fig. 3.9). Therefore, it is possible that phosphorylation of some of these residues may be required for Pan3 in P bodies to be released back to the cytosol. On the other hand, Pan3(PM11), which contains mutations that mimic phosphorylation at the eleven potential phosphorylation sites, is diffused in the cytoplasm, but highly enriched in nuclear foci (Fig. 3.7). Although the distinct localization phenotypes of Pan3(NP11) and Pan3(PM11) might stem from structural defects caused by the mutations, it is also possible that phosphorylation states of

Pan3 N-terminal domain may determine the localization and function of Pan3. It will be important to confirm that endogenous Pan3 N-terminal domain indeed is hyper-phosphorylated.

Chapter 4: Bioinformatic Analysis of Human mRNA Processing Body Components

4.1. Introduction

mRNA processing bodies (P bodies) are dynamic cytoplasmic foci containing non-translating messenger ribonucleoproteins (mRNPs) as well as proteins for translational inhibition and mRNA degradation. Conserved from yeast to human, P bodies have a diameter of 100-300 nm (65). These membrane-free structures are mainly anchored to microtubules while constantly moving around in the cytoplasm (66). Fluorescence microscopy studies revealed that P bodies contain translationally inhibited mRNAs and many proteins involved in translational inhibition and mRNA degradation (68). In contrast, most factors involved in mRNA translation, for example eIF4G and ribosome subunits, are normally absent from P bodies (71).

P bodies provide another potential layer of post-transcriptional gene regulation because mRNAs in P bodies can be translationally inhibited and either degraded or released for translation. Since P bodies are ribosome-free, mRNAs in P bodies cannot be translated. mRNAs in P bodies can be degraded, as inhibition of 5' to 3' decay by knocking down Xrn1 increased P body sizes and mRNA decay intermediates were found in P bodies (72). However, P bodies may also be temporary storage sites for some mRNAs before they are degraded, as suggested by an observation that a P body mRNA encoding cationic amino acid transporter 1 (CAT-1) was released from P bodies and subsequently translated when the cells were subjected to different stresses (73). Therefore, P bodies may function as a “recycle bin” where the fates of arrested non-translating mRNAs (either degradation or release) will be determined.

Although over 250 papers on P bodies or P body components in different species have been published, the biological role and physiological significance of P-bodies remain unclear. Currently, a comprehensive list of reported P body components is not available, and our ability to study P body components as a whole is limited. For example, how many P body proteins have been found? How can they be classified based on their known functions? How do they interact with each other and other cellular proteins? Answers to these questions are likely to provide key insight into the biological functions of P bodies and thus regulation of P body dynamics.

In this study, a list of P body proteins in different species reported to date is provided. Reported and predicted human P body proteins are then grouped based on their functions. Protein-protein interaction analysis reveals that many P body proteins form complex interaction networks with each other as well as other cellular proteins that have not been reported as P body components yet. Interestingly, some of these cellular proteins can interact with three or even more P body components, suggesting that these proteins may also localize to P bodies or be important for P body dynamics and functions.

4.2. Methods and Results

4.2.1. Currently reported P body protein components in different species

In order to have a comprehensive view of known P body proteins, over 250 Pubmed articles published since 2002 that contain various synonyms of P bodies in their titles or abstracts were identified. After careful examination of immunofluorescence microscopic evidence for P-body localization in each article, a list of currently reported P body protein components in different species was generated (Table. 4.1). Together, 57 human proteins, 10 mouse proteins, 30 yeast proteins, and 17 proteins from plants, worms, or fruit fly were included in the list.

Species	Gene_ID	Uniprot_Ac	Name	Alias	Reference
Arabidopsis	837357	Q9SJF3	DCP1		(101)
Arabidopsis	831201	Q8GW31	DCP2		(101)
Arabidopsis	820530	Q9LTT8	VCS	EDC4, GE-1	(101)
C. elegans	181719	Q17740	AIN-1	GW182	(102)
C. elegans	181504	Q20578	ALG-1		(102)
C. elegans	175680	Q21992	LARP-1		(103)
C. elegans	175654	Q19818	NHL-2		(104)
C. elegans	174808	Q20374	PATR-1	Pat1	(105)
Drosophila	36544	Q32KD4	AGO1		(106)
Drosophila	33934	Q9VMA3	Cup		(107)
Drosophila	39957	Q9VVI2	EDC3		(108)
Drosophila	37528	Q9NFU0	FMRP		(109)
Drosophila	43808	Q8SY33	GW182	Gawky	(106)
				Dhh1,	
Drosophila	34364	P23128	Me31B	RCK/P54	(110)
Drosophila	32926	Q9V3F9	Out		(107)
Drosophila	37065	P25159	Staufen		(109)
				RAP55,	
Drosophila	39430	Q9VTZ0	Tral	LSM14A	(108)
Human	26523	Q9UL18	Ago1	EIF2C1	(111)
Human	27161	Q9UKV8	Ago2	EIF2C2	(111)
Human	192669	Q9H9G7	Ago3	EIF2C3	(112)
Human	192670	Q9HCK5	Ago4	EIF2C4	(113)
Human	200316	Q8IUX4	APOBEC3F		(114)
Human	60489	Q9HC16	APOBEC3G		(114)
Human	29883	Q9UIV1	Caf1	CNOT7	(21)
Human	57472	Q9ULM6	Ccr4	CNOT6	(76)
Human	64506	Q9BZB8	CPEB1		(115)
Human	55802	Q9NPI6	Dcp1a		(71)
Human	196513	Q8IZD4	Dcp1b		(76)
Human	167227	Q8IU60	Dcp2		(116)
Human	80153	Q96F86	Ede3	LSM16	(117)
Human	1977	P06730	eIF4E		(74)
Human	56478	Q9NRA8	eIF4E-T	EIF4ENIF1	(74)
Human	10922	Q14296	FAST	FASTK	(71)
				EDC4,	
Human	23644	Q6P2E9	Ge-1	HEDLS	(118)
Human	25929	Q8TEQ6	Gemin5		(119)
Human	220988	P51991	hnRNP A3		(120)
Human	3064	P42858	Htt		(121)
Human	10526	O15397	Imp8	IPO8	(122)
Human	79727	Q9H9Z2	Lin28	ZCCHC1	(123)
Human	27257	O15116	LSm1		(124)
Human	57819	Q9Y333	LSm2		(124)
Human	27258	P62310	LSm3		(124)
Human	25804	Q9Y4Z0	LSm4		(124)
Human	23658	Q9Y4Y9	LSm5		(124)
Human	11157	P62312	LSm6		(124)
Human	51690	Q9UK45	LSm7		(124)
Human	92312	A1L020	Mex-3A		(125)
Human	84206	Q6ZN04	Mex-3B		(125)
Human	4343	Q9HCE1	MOV10		(126)

Human	340152	A2A288	P58(TFL)		(127)
Human	9924	Q504Q3	Pan2	USP52	(21)
Human	255967	Q58A45	Pan3		(21)
Human	219988	Q86TB9	PatL1		(128)
Human	5093	Q15365	PCBP1	HNRPE1	(129)
Human	5094	Q15366	PCBP2	HNRPE2	(130)
Human	5610	P19525	PKR		(131)
Human	55629	Q9NPJ4	PNRC2		(132)
Human	26065	Q8ND56	RAP55	LSM14A	(133)
Human	1656	P26196	RCK/p54	DDX6	(75)
Human	6737	P19474	Ro52	SSA1	(134)
Human	23381	Q9UPR3	SMG5		(135)
Human	23293	Q86US8	SMG6		(70)
Human	9887	Q92540	SMG7		(135)
Human	27327	Q8NDV7	TNRC6A	GW182	(136)
Human	23112	Q9UPQ9	TNRC6B		(136)
Human	57690	Q9HCJ0	TNRC6C		(137)
Human	10766	Q14106	Tob2		(49)
Human	3842	Q92973	TNPO1	TRN	(138)
Human	7538	P26651	TTP	Tis11, Zfp36	(138)
Human	5976	Q92900	UPF1		(135)
Human	65110	Q9H1J1	UPF3a		(70)
Human	65109	Q9BZI7	UPF3b		(70)
Human	54464	Q8IZH2	Xrn1		(124)
Human	4904	P67809	YB-1	YBX1	(139)
Mouse	15572	Q61701	HuD	Elavl4	(140)
Mouse	232943	Q91W40	KLC3		(141)
Mouse	83557	Q8K3Y3	Lin28		(123)
Mouse	636931	Q1PSW8	mLin41		(142)
Mouse	378430	P60322	Nanos2		(143)
Mouse	244551	P60324	Nanos3		(144)
Mouse	170722	Q80SZ6	NXF7		(120)
Mouse	22695	P22893	TTP	Tis11, Zfp36	(71)
Mouse	317755	Q80SU3	Zar1		(67)
Mouse	545824	C3VD30	Zar1L		(67)
Yeast	854016	Q12517	Dcp1p		(72)
Yeast	855605	P53550	Dcp2p		(72)
Yeast	854344	Q12123	Dcs2		(145)
Yeast	854379	P06634	Ded1p		(146)
Yeast	851394	P39517	Dhh1p		(72)
Yeast	851787	Q03466	Ebs1p		(147)
Yeast	856700	P39998	Edc3p	LSM16	(148)
Yeast	853318	P47017	Lsm1p		(72)
Yeast	854504	P19524	Myo2p		(149)
Yeast	850455	P25655	Not1p		(150)
Yeast	851389	P06100	Not2p		(150)
Yeast	854773	P06102	Not3p		(150)
Yeast	856799	P34909	Not4p		(150)
Yeast	850440	P25644	Pat1p		(72)
Yeast	852391	P38254	Pby1p		(151)
Yeast	855788	P39008	Pop2p		(77)
Yeast	850647	Q07807	Puf3p		(152)
Yeast	852513	P32831	Rbp1p	Ngr1p	(153)

Yeast	853301	P20433	Rpb4p		(154)
Yeast	852013	P34087	Rpb7p		(155)
Yeast	854883	Q02773	Rpm2p		(156)
Yeast	856351	P10080	Sbp1p		(157)
Yeast	855898	P06245	Tpk2		(158)
Yeast	853688	P05986	Tpk3		(158)
Yeast	854889	P09733	Tub1		(151)
Yeast	855104	P30771	Upf1p		(69)
Yeast	856476	P38798	Upf2p		(69)
Yeast	852963	P48412	Upf3p		(69)
Yeast	854541	Q08831	Vts1p		(159)
Yeast	852702	P22147	Xrn1p	Kem1p	(72)

Table 4.1 A comprehensive list of reported P body components in different species. The list was manually generated based on published research articles in Pubmed. NCBI Gene IDs and Uniprot Accession Numbers are provided for the convenience of future bioinformatic analysis.

4.2.2. Functional categorization of reported and predicted human P body components

Most of the P body components are conserved across species, but the localization of some of the P body proteins was only studied in non-human species. Since the functions and localizations of many P body components are conserved across eukaryotes, we assume that human orthologs of P body proteins found in other species will also localize to human P bodies. The NCBI HomoloGene database was searched and 22 potential human P body components whose orthologs have been reported to localize to P bodies in other species were identified (Table 4.2, italic rows). To better understand the functions of human P body components, the reported and predicted human P body proteins were clustered based on their known functions (Table 4.2).

Gene Name	Alias	Homolog in PBs	Function(s)	Reference
ARE-mediated mRNA degradation				
TRN	TNPO1		Nuclear transport receptor; trafficking of TTP between the PBs and SGs and modulates ARE-containing mRNA stability.	(138, 160)
TTP	ZFP36		Binds to and destabilize some mRNAs with AU-rich elements	(161, 162)
Nonsense-mediated decay				
PNRC2			A bridge between the mRNA decapping complex and the NMD machinery	(132)
SMG5	EST1B		Required for the dephosphorylation of UPF1	(135, 163)
SMG6	EST1A		Required for the dephosphorylation of UPF1.	(70, 164, 165)
SMG7	EST1C		Telomere maintenance	
			Binds to phosphorylated UPF1; Triggers mRNA decay	(135, 166)
UPF1			Binds to eRF1 and eRF3; Required for nonsense-mediated mRNA decay	(135, 167)
UPF2		<i>Yeast Upf2p</i>	<i>Bridges Upf1 to the exon junction complex during nonsense-mediated mRNA decay; Stimulate helicase activity of Upf1</i>	(69, 168)
UPF3A			Less effective than Upf3b to induce nonsense-mediated mRNA decay	(70, 169)
UPF3B			Bridges Upf1 to the exon junction complex during nonsense-mediated mRNA decay; Stimulate helicase activity of Upf1; Required for the phosphorylation of UPF1	(70, 167, 168)
Gene silencing by miRNA or siRNA				
EIF2C1	AGO1		mi/siRNA mediated mRNA decay and translational repression	(170)
EIF2C2	AGO2		mi/siRNA mediated mRNA decay and translational repression	(170, 171)
EIF2C3	AGO3		mi/siRNA mediated mRNA decay and translational repression	(112, 170)
EIF2C4	AGO4		mi/siRNA mediated mRNA decay and translational repression	(113, 170)
HTT			Contributes to RNA-mediated gene silencing through association with Argonaute and P bodies	(121)
IPO8	Imp8		Nuclear import; Function in cytoplasmic miRNA-guided gene silencing and affects nuclear localization of Ago proteins	(122, 172)
MOV10			Interacts with Ago1 and Ago2; Required for siRNA-mediated mRNA cleavage	(126)
TNRC6A	GW182		Recruited by Agonaute proteins, important for miRNA-mediated deadenylation and translational repression	(173, 174)
TNRC6B			Recruited by Agonaute proteins, important for miRNA-mediated deadenylation and translational repression	(113, 136)
TNRC6C			Recruited by Agonaute proteins, important for miRNA-mediated deadenylation and translational repression	(19, 113)
UPF1			Interacts with Ago1 and Ago2; Participates in RNA silencing	(175)
Negative regulation of miRNA pathway				
LIN28			Inhibits let-7 miRNA maturation; An RNA binding protein in PBs and SGs.	(123, 176, 177)
LIN41	TRIM71	<i>Mouse Lin41</i>	<i>A stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2.</i>	(142)

Binding to telomere or telomerase			
hnRNPA3			Binding to the telomeric sequence; Cytoplasmic trafficking of RNA. (120, 178, 179)
MOV10			Inhibits production of infectious retroviruses when over expressed; Binds to telomere. (180, 181)
PCBP2	HNRPE2		Interacts with telomeric DNA and telomerase RNA; mRNA stabilization and destabilization (182-184)
SMG6	EST1A		Required for the dephosphorylation of UPF1. Telomere maintenance (70, 164, 165)
Transcription			
<i>CNOT1-4</i>		<i>Yeast Not1-4p</i>	<i>Form complexes with the deadenylases CNOT6 and CNOT7 or CNOT8; Involved in mRNA splicing, transport, and deadenylation.</i> (37, 185)
PCBP1	HNRPE1		Transcription activation; Splicing; mRNA stabilization; cap-dependent mRNA translational inhibition; IRES-driven translation activation (171, 186, 187)
<i>POLR2G</i>	<i>RPB7</i>	<i>Yeast Rpb4p</i>	<i>The seventh largest subunit of RNA polymerase II</i> (154)
YB-1	YBX1		Transcription activation; Splicing enhancement; Translation activation by binding to 5'-UTR (188-190)
Splicing			
PCBP1	HNRPE1		Transcription activation; Splicing; mRNA stabilization; cap-dependent mRNA translational inhibition; IRES-driven translation activation (171, 186, 187)
YB-1	YBX1		Transcription activation; Splicing enhancement; Translation activation by binding to 5'-UTR (188-190)
mRNA trafficking			
<i>FMR1</i>	<i>FMRP</i>	<i>Drosophila FMR1</i>	<i>Involved in translation regulation and trafficking of certain mRNA.</i> (109, 191, 192)
hnRNPA3			Binding to the telomeric sequence; Cytoplasmic trafficking of RNA. (120, 178, 179)
<i>KLC3</i>		<i>Mouse KLC3</i>	<i>May bind cargo and regulate kinesin activity.</i> (141)
<i>MYO5C</i>		<i>Yeast Myo2p</i>	<i>Granule trafficking</i> (149, 193)
<i>NXF2</i>		<i>Mouse NXF7</i>	<i>Nuclear RNA export; Cytoplasmic mRNA localization</i> (120, 194)
<i>STAUI</i>		<i>Drosophila Staufn</i>	<i>mRNA localization and translation regulation; Competes with Upf2 to interact with Upf1 to promote mRNA decay.</i> (109, 195, 196)
<i>TUBA1C</i>		<i>Yeast Tub1p</i>	<i>Tubulin alpha-1C chain</i> (151)
mRNA stabilization			
EIF4E			Binds mRNA 5' cap to stabilize mRNA and promote translation initiation (197)
<i>HUD</i>	<i>ELAVL4</i>	<i>Mouse HuD</i>	<i>Binds to and stabilize some AU-rich element (ARE) containing mRNAs</i> (198-200)
PCBP1	HNRPE1		Transcription activation; Splicing; mRNA stabilization; cap-dependent mRNA translational inhibition; IRES-driven translation activation (171, 186, 187)
PCBP2	HNRPE2		Interacts with telomeric DNA and telomerase RNA; mRNA stabilization and destabilization (182-184)
Translation inhibition or activation			
CPEB1			During the early development, it behaves first as an inhibitor and later as an activator of translation. (115)
DDX6	RCK/p54		Required for microRNA-induced gene silencing (75)
EIF2C2	AGO2		Competes with eIF4E to bind to the 5' cap to inhibit translation (201)
EIF4E			Binds mRNA 5' cap to stabilize mRNA and promote translation initiation (197)
EIF4ENIF1	EIF4T		Mediates the nuclear import of EIF4E. Interacts with the cap binding protein 4E to inhibit translation. (74, 202)
GEMIN5			Inhibits both cap-dependent and IRES-driven translation initiations (203)
PatL1			May be a decapping activator and translation (128)

		repressor	
PCBP1	HNRPE1	Transcription activation; Splicing; mRNA stabilization; cap-dependent mRNA translational inhibition; IRES-driven translation activation	(171, 186, 187)
RAP55	TRAL	Translation inhibition	(204)
YB-1	YBX1	Transcription activation; Splicing enhancement; Translation activation by binding to 5'-UTR	(188)
Deadenylation			
<i>CNOT1-4</i>	<i>Yeast Not1-4p</i>	<i>Form complexes with the deadenylases CNOT6 and CNOT7 or CNOT8; Involved in mRNA splicing, transport, and deadenylation.</i>	(37)
CNOT6	Ccr4	Deadenylase required for second phase of deadenylation	(19, 21, 76)
CNOT7	Caf1	Deadenylase required for second phase of deadenylation	(21)
<i>NANOS2</i>	<i>Mouse Nanos2</i>	<i>Recruits Ccr4-NOT deadenylation complex to mRNAs</i>	(143)
PAN3		Interacts with both PABP and the deadenylase Pan2 to stimulate Pan2 activity	(27, 92)
PAN2		Deadenylase required for first phase of deadenylation	(16, 21)
TOB2		Interacts with PABP and recruit the deadenylase Caf1	(49)
Decapping			
DCP1A		Decapping enzyme subunit	(71)
DCP1B		Decapping enzyme subunit	(76)
DCP2		Catalytic subunit of decapping enzyme	(76, 116)
EDC3		Interact with multiple components of the decapping machinery, including DCP1, DCP2, and DDX6	(205-207)
GE-1	Hedls, EDC4	Promotes complex formation between DCP1A and DCP2. Enhances the catalytic activity of DCP2	(118, 205)
LSM1-7		Sm-like protein complex, decapping activator	(88, 124)
PATL1		May function as a decapping activator and translation repressor	(128)
5' to 3' Exonuclease activity			
XRN1		5' to 3' riboexonuclease	(124, 208)
Helicase activity			
<i>DDX3Y</i>	<i>Yeast Ded1p</i>	<i>Probable ATP-dependent RNA helicase.</i>	(146)
DDX6	RCK/p54	Helicase activity is required for translational inhibition and P body formation.	(209)
MOV10		Probable RNA helicase; interacts with Ago1 and Ago2; Required for siRNA-mediated mRNA cleavage	(126)
UPF1		Helicase activity is required to promote mRNA decay.	(210, 211)
Endonuclease activity			
EIF2C2	Ago2	Cleaves both passenger strand and mRNA targets of siRNAs	(212, 213)
SMG6		Functions in nonsense-mediated decay	(214, 215)
ZC3H12D	P58(TFL)	Probable endonuclease	
Protein kinase activity			
EIF2AK2	PKR	Phosphorylates eIF2alpha to inhibit translation in virus infected cells; Targeted to PBs by the E6 oncoprotein of HPV	(131, 216)
FAST	FASTK	In response to Fas receptor ligation, it phosphorylates TIA1, an apoptosis-promoting nuclear RNA-binding protein.	(217)
<i>PRKACB</i>	<i>Yeast Tpk2p</i>	<i>Mediates cAMP-dependent signaling triggered by receptor binding to GPCRs.</i>	(218)

<i>PRKX</i>		<i>Yeast Tpk3p</i>	<i>A serine threonine protein kinase that has similarity to the catalytic subunit of cyclic AMP dependent protein kinases.</i>	(219)
Ubiquitin ligase activity				
<i>LIN41</i>	<i>TRIM71</i>	<i>Mouse Lin41</i>	<i>A stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2</i>	(142)
TRIM21	Ro52		E3 ubiquitin ligase; interacts with Dcp2 to enhance its decapping activity	(134, 220)
Cap-binding activity				
EIF2C2	AGO2		Compete with eIF4E to bind to the 5' cap to inhibit mRNA translation.	(201)
EIF4E			Binds mRNA 5' cap to stabilize mRNA and promote translation initiation	(197)
GEMIN5			Binds to m ⁷ G cap of mRNAs	(221)
Response to virus				
APOBEC3F			A cytidine deaminase that restricts retroviral replication	(222)
APOBEC3G			A cytidine deaminase that restricts retroviral replication	(222)
EIF2AK2	PKR		Double-stranded RNA protein kinase targeted to PBs by the E6 oncoprotein of HPV	(131, 216)
MOV10			Inhibits production of infectious retroviruses when over expressed; Binds to telomere.	(126, 180, 181)
Miscellaneous				
<i>LARP1</i>		<i>C. elegans LARP-1</i>	<i>RNA binding</i>	(223)
MEX-3A			RNA binding protein; May be involved in post-transcriptional regulatory mechanisms.	(125)
MEX-3B			RNA binding protein; May be involved in post-transcriptional regulatory mechanisms.	(125, 224)
<i>NANOS3</i>		<i>Mouse Nanos3</i>	<i>Germ cell-specific RNA binding protein</i>	(225)
<i>ZAR1</i>		<i>Mouse ZAR1</i>	<i>Zygote arrest protein 1</i>	(67)
<i>ZAR1L</i>		<i>Mouse ZAR1L</i>	<i>ZAR1-like protein</i>	(67)

Table 4.2: Functional categorization of reported and predicted human P body components.

P body proteins predicted based on homology are in italic. Some P body proteins with diverse functions are listed in different groups.

4.2.3. Protein-protein interactions among P body components

Proteins often exert their functions through interactions with other protein molecules. To understand how different P body components aggregate to form microscopically visible foci and how P body components are functionally linked, protein-protein interactions (PPIs) among P body components themselves were analyzed. To this end, 79 gene names of reported and predicted human P body components were submitted to the STRING database version 8.2 to retrieve 90 PPIs among human P body proteins detected by anti-tag coimmunoprecipitation (coIP), yeast two-hybrid, and pulldown experiments. (226). Another 32 PPIs were identified by manually checking recent publications about P body components. The 122 PPIs among 55 of the 79 reported and predicted P body components were then visualized using Cytoscape version 2.6.3 (227). Proteins with similar or related functions are represented with similarly colored nodes, and interactions among the proteins were denoted by lines (Fig. 4.1). It should be noted that the interactions detected by coIP experiments are not necessarily direct.

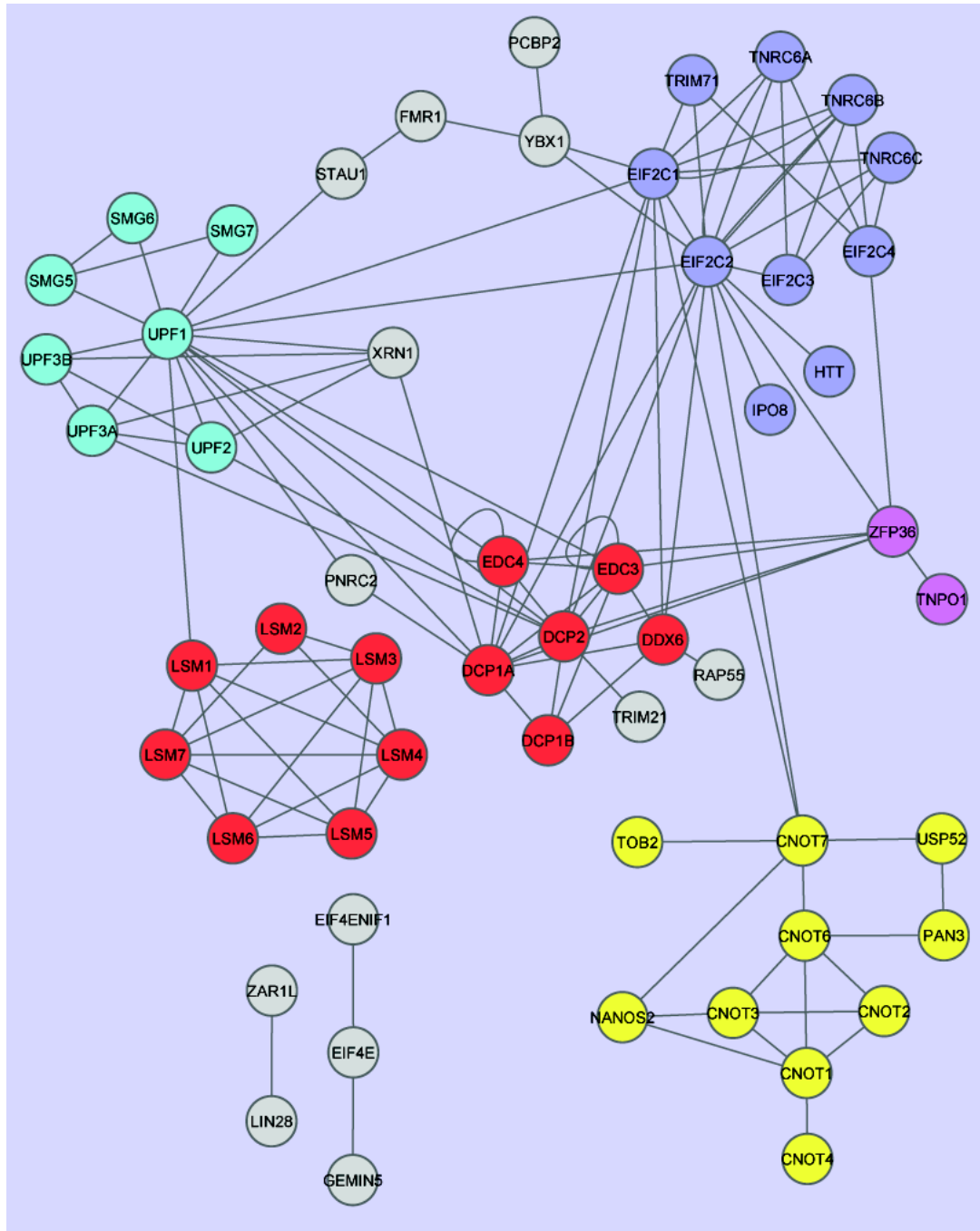


Figure 4.1: Protein-protein interactions among P body components. 120 PPIs among 55 of the 79 reported and predicted P body components were visualized using Cytoscape version 2.6.3. Cyan nodes: nonsense-mediated decay factors; blue nodes: miRNA-mediated gene silencing factors; pink nodes: ARE-mediated decay factors; red nodes: decapping factors; yellow nodes: deadenylation factors. Lines between the nodes represent protein-protein interactions retrieved from STRING or recent publications in Pubmed.

4.2.4. Protein-protein interactions between P body proteins and other cellular proteins

Currently little is known about how P body assembly and disassembly is elicited and regulated and what may be the participating protein factors. Since proteins often alter their functions and subcellular localization through interactions with other proteins, we reasoned that proteins involved in controlling P body dynamics and/or themselves also localizing to P bodies may do so via their interactions with P body component(s). PPIs between human P body proteins and other cellular proteins detected by affinity purification and yeast two hybrid experiments were retrieved from PINA which integrates PPI data from six databases (228). Retrieved PPIs were processed with a PHP script and Excel to remove duplicated records, records with obsolete Uniprot accession numbers, and other unnecessary information. The processed file was imported to Cytoscape version 2.6.3, where interactants of P body proteins were grouped and colored differently based on the numbers of interactions between them and P body factors (Fig. 4.2). P body components were represented with pink nodes in the big circle close to the bottom, while the nodes in the other differently colored circles represent cellular proteins that interact with either one (sky blue ; 410 interactions), two (blue; 85 interactions), three (purple; 32 interactions), four (magenta; 10 interactions), five (red; 5 interactions), six (brown; 2 interactions), seven (yellow; 1 interaction), or eight (green; 2 interactions) P body components (Fig 4.2).

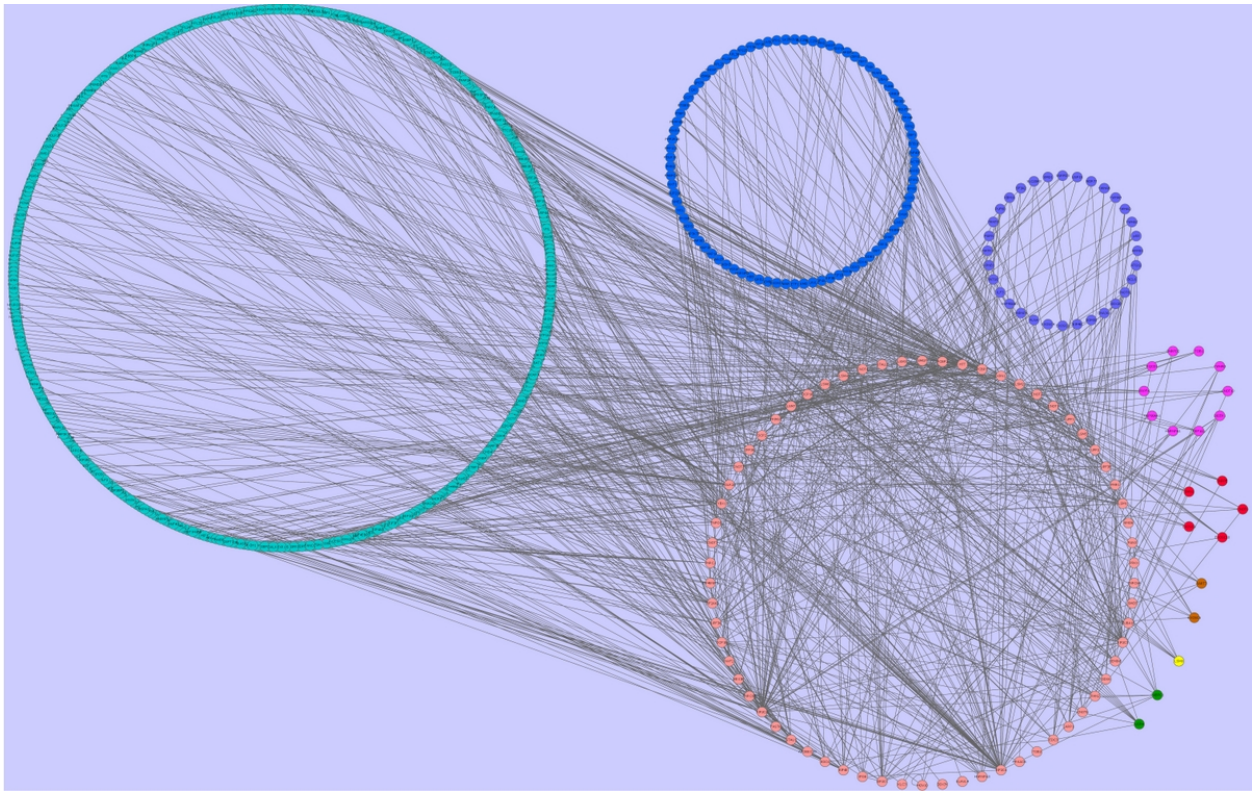


Figure 4.2: Interactions between P body components and other cellular proteins visualized with Cytoscape version 2.6.3. P body components were represented with pink nodes in the big circle close to the bottom, while the nodes in the other differently colored circles represent cellular proteins that interact with either one, two, three, four, five, six, seven, or eight P body components. Lines between the nodes represent protein-protein interactions based on either affinity purification and/or yeast two-hybrid experiments.

I reasoned that proteins that can interact with more P body components are more likely to affect P body dynamics and functions or localize to P bodies. Therefore, I listed the proteins that can interact with three or more P body components in Table 4.3. Their Uniprot Accession number, number of P body interactants, and their corresponding P body interactants were also included.

Protein	UniprotAC	# of Interactants	Interactants
PABPC1	P11940	8	CNOT7,EIF2C1,EIF2C2,EIF2C4,PAN2,PAN3,PCBP1,PCBP2,UPF1
USP4	Q13107	8	DCP1A,DCP1B,EDC3,LSM2,LSM4,LSM6,LSM7,TRIM21
LSM8	O95777	7	DCP1A,LSM2,LSM3,LSM4,LSM6,LSM7,UPF1
EXOSC6	Q5RKV6	6	DCP1B,LSM1,LSM7,UPF2,XRN1,ZFP36
SART3	Q15020	6	EIF2C1,EIF2C2,LSM2,LSM4,LSM6,LSM7
CNOT8	Q9UFF9	5	CNOT1,CNOT2,CNOT3,CNOT6,CNOT7
EXOSC10	Q01780	5	LSM2,UPF1,UPF2,UPF3A,XRN1
EXOSC8	Q96B26	5	LSM1,LSM7,UPF2,XRN1,ZFP36
SMN1	Q16637	5	GEMIN5,LSM2,LSM4,LSM6,LSM7
SNRPE	P62304	5	GEMIN5,LSM2,LSM3,LSM4,LSM5
CNOT6L	Q96LI5	4	CNOT1,CNOT2,CNOT3,NANOS2
CNOT10	Q9H9A5	4	CNOT1,CNOT2,CNOT6,CNOT7
DICER1	A7E2D3	4	EIF2C1,EIF2C2,EIF2C3,EIF2C4
EEF1A1	P68104	4	EIF2C2, EIF2C3, EIF2C4, LSM3
HNRNPA1	P09651	4	EIF2C1, EIF2C2, EIF4E, XRN1
HSP90AA1	P07900	4	EIF2C3, EIF2C4, LSM1, EIF2AK2
PRPF4	O43172	4	LSM2, LSM4, LSM6, LSM7
RQCD1	Q92600	4	CNOT1, CNOT2, CNOT6,CNOT7
SNRPD2	P62316	4	LSM2, LSM6, EIF2C4, GEMIN5
TOB1	P50616	4	CNOT1,CNOT2,CNOT3,CNOT7
YWHAG	P61981	4	LARP1,TRIM21,EDC3,KLC3
ARRB1	P49407	3	HNRNPA3,TUBA1C,YBX1
ATG12	O94817	3	DDX6,HNRNPA3,MOV10
BCL6	P41182	3	DDX6,EIF4ENIF1,LSM1
DDX20	Q9UHI6	3	EIF2C2,GEMIN5,LSM2
DNAJA1	P31689	3	EIF2C3,EIF2C4,LSM2
DOM3Z	O77932	3	DCP2,UPF1,XRN1
EIF4B	P23588	3	EIF2C2,EIF2C3,EIF2C4
EXOSC2	Q13868	3	UPF1,UPF2,UPF3A
EXOSC4	Q9NPD3	3	UPF1,UPF2,UPF3A
FLNA	P21333	3	EIF2C3,PCBP2,PRKACB
HNRNPK	P61978	3	PCBP1,PCBP2,YBX1
HSP90AB1	P08238	3	EIF2C2,EIF2C3,EIF2C4
ILF3	Q12906	3	EIF2C1,EIF2C2,EIF2AK2
INTS6	Q9UL03	3	EIF4E,UPF1,UPF2
KPNB1	Q14974	3	EIF4E,EIF4ENIF1,IPO8
MATR3	P43243	3	EIF2C1,EIF2C2,PCBP1
p53	P04637	3	EIF2AK2,HTT,YBX1
PARN	O95453	3	UPF1,UPF2,UPF3A
PRMT5	O14744	3	EIF2C2,EIF2C3,EIF2C4
PUF60	Q9UHX1	3	HNRNPA3,MOV10,PCBP1
RBM10	P98175	3	EIF2C2,EIF2C3,EIF2C4

SF3A2	Q15428	3	HNRNPA3,PCBP1,YBX1
SLC25A3	Q00325	3	EIF2C2,EIF2C3,EIF2C4
SMG1	Q96Q15	3	UPF1,UPF2,UPF3A
SNRPD3	P62318	3	LSM3,LSM7,GEMIN5
USP15	Q9Y4E8	3	LSM2,LSM4,LSM6
WDR77	Q9BQA1	3	EIF2C2,EIF2C3,EIF2C4
YWHAB	P31946	3	EDC3,LARP1,ZFP36
YWHAH	Q04917	3	KLC3,LARP1,ZFP36
YWHAQ	P27348	3	DDX6,EDC3,LARP1
YWHAZ	P63104	3	EDC3,LARP1,TRIM21

Table 4.3: A list of cellular proteins that are reported to interact with three or more P body components. Proteins in bold have already been reported to be important for P body functions or formation.

Some of the proteins listed in Table 4.3 (e.g. CNOT6L, CNOT8, and TOB1) are paralogs of reported P body proteins; therefore it is not surprising that they are identified. Some are more likely to be involved in functions not directly related to P bodies, such as the 3' to 5' riboexonuclease subunits (EXOSC2, 4, 6, 8, and 10) and the Sm-like protein Lsm8 involved in splicing (124). Interestingly, a few of the proteins in Table 4.3 have already been reported to be important for P body formation or functions (see discussion below), such as Hsp90 and 14-3-3 families of proteins (in bold) (206, 224, 229, 230). Therefore, these reports lend support to the feasibility of using this in silico approach to unraveling potential functions of P bodies and the regulation of P body dynamics. It is very likely that the rest of the proteins in Table 4.3 are functionally related to P bodies.

4.3. Discussion

A comprehensive list of reported P body proteins in different species has been generated during this study (Table 4.1). Together, 114 proteins in different species have been reported to localize to P bodies. Among these P body proteins, 57 are from human, 10 from mouse, 30 from yeast, and 17 from Arabidopsis, C. elegans, or Drosophila. It should be noted that some of these proteins (e.g. C. elegans AIN-1, Drosophila GAWKY, Human TNRC6A, TNRC6B, and TNRC6C) are orthologs or paralogs.

Since orthologs often have similar functions, 22 human proteins whose orthologs have been reported to localize to P bodies in other species are also predicted to be human P body components (Table 4.2). The 79 reported and predicted human P body protein components are clustered based on their functions (Table 4.2). This analysis has yielded some interesting conclusions and/or implications. First, P bodies contain proteins involved in many aspects of mRNA metabolism, including transcription, splicing, mRNA transport, mRNA stabilization, mRNA translational inhibition or activation, ARE or nonsense-mediated mRNA decay, miRNA-mediated gene silencing, mRNA deadenylation, decapping, 5' to 3' decay, and endocleavage. Second, although many P body components are mRNA degradation factors, some P body components are mRNA stabilizers (e.g. eIF4E, HuD, PCBP1, and PCBP2), suggesting that some of the mRNAs in P bodies may be protected from degradation, which is consistent with the notion that P bodies can be temporary mRNA storage sites. Third, besides the 5' to 3' ribonuclease Xrn1, several endonucleases can also be enriched in P bodies; therefore it is possible that decay of some mRNAs in P bodies might involve endonucleolytic cleavage. It will be interesting to see how knocking down these endonucleases or their activities may impact P body dynamics. Finally, colocalization of protein modification factors, including protein kinases and E3 ubiquitin ligases, with P bodies suggests that phosphorylation and ubiquitination may occur in P bodies to directly modulate P body dynamics or functions. Conversely, these post-translational modifications may indirectly lead to changes in the amount or accessibility of P-body proteins for P-body formation.

In this study, I also generate an interactome of P body component. An analysis of protein-protein interactions (PPIs) among human P body protein components shows that at least two thirds of the human P body proteins form dense PPI networks (Fig. 4.1), suggesting that collective PPIs among P body components contribute to the aggregation of mRNPs during P body formation. Moreover, the result indicates that factors involved in the ARE-mediated decay (pink), miRNA-mediated gene silencing (blue), and nonsense-mediated decay (cyan) are physically linked. Indeed, the human nonsense-mediated decay factor Upf1 also participates in small RNA-mediated gene knockdown (175). Similarly, TTP in the ARE-mediated decay pathway binds to Ago2 and Ago4 in the miRNA-mediated

gene silencing pathway and is required for mRNA targeting by miR16 (231). Interestingly, although all of the above three mRNA decay pathways promote deadenylation, only physical interactions between deadenylation factors (yellow) and miRNA-mediated mRNA decay factors (blue) proteins have been reported thus far (174). In light of the demonstration that deadenylation is a pre-requisite for P body formation and that PABP, not found in P body, is involved in the first phase of deadenylation and is removed from mRNA following deadenylation, the PPI analysis provides new insight into the mechanism of P body formation. The following sequential events for P body formation may be envisaged. Following poly(A) shortening via these decay pathways, deadenylases and other deadenylation associated factors remain on the mRNPs. Deadenylation then elicits mRNP remodeling that allows other non-deadenylation factors to join the mRNPs. Remodeled mRNPs aggregate into P bodies via multiple PPIs identified among P body components, including the Pan3 deadenylation factor.

An analysis of protein-protein interactions (PPIs) between human P body protein components and other cellular proteins identified many factors that interact with three or more P body components (Fig 4.2 and Table 4.3). This *in silico* approach apparently represents a valid way of identifying factors potentially involved in regulating P body dynamics. For instance, some proteins belong to non-P body protein families reported to be required for proper P-body formation and/or function (e.g., Hsp90 and 14-3-3 families) were also included in Table 4.3. Our analysis identified two major cytosolic HSP90 proteins, HSP90AA1 and HSP90AB1, and inhibition of Hsp90 chaperon activity was reported to affect miRNA-mediated gene silencing, siRNA-directed cleavage of target mRNA, and P body formation (229, 230). We also identified the 14-3-3 protein family (YWHAG, YWHAB, YWHAH, YWHAQ, and YWHAZ), which bind to phosphoserine-containing proteins. It is reported that binding of 14-3-3 to Edc3 alters P body morphology, inhibits miRNA-mediated gene silencing, and changes Edc3 protein-protein interactions (206). In addition, the sorting of hMex-3B between P bodies and stress granules is regulated by the interaction between hMex-3B and 14-3-3 proteins (224), although this interaction was not retrieved using PINA and therefore not included during the analysis. The interactions between 14-3-3 family proteins and several other reported or predicted P body components (Table 4.3), including

LARP1, KLC3, TRIM21 (Ro52), TTP (ZFP36), RCK/p54 (DDX6), suggest that 14-3-3 family of proteins play more important roles in regulating P body functions than they were previously thought. It is also worth noting that the identification of the interactions between Ago proteins and PABP, which is not enriched in mammalian P bodies (21, 71), is consistent with the recent finding that PABP was required for miRNA-mediated deadenylation (174).

Therefore, PPIs support a hypothesis that the other proteins listed in Table 4.3 may also have P body-related functions or even colocalize with P bodies. It will be interesting to test whether or not knockdown of these proteins will affect P-body dynamics and whether these proteins themselves localize to P bodies as well.

Chapter 5: Perspectives and Prospects

mRNA poly(A) tails, which are essential for both mRNA stability and translation, can be shortened in a process termed deadenylation by deadenylases. In this study, I found that the two major mammalian deadenylase complexes, Pan3-Pan2 and Ccr4-Caf1, can both localize to mammalian mRNA processing bodies (P bodies), which are normally thought to be cytoplasmic sites for mRNA translational inhibition, mRNA decapping, and 5' to 3' mRNA degradation. More importantly, my experiments demonstrated that deadenylation by the Ccr4-Caf1 complex is a prerequisite for P body formation in mammalian cells.

One of the consequences of deadenylation is dissociation of ribosomes from mRNAs. It was known that dissociation of ribosomes from mRNAs to render the mRNAs translationally silent is necessary for the mRNAs to enter or form P bodies. However, it was not clear whether the dissociation step alone is sufficient or whether other steps are also required. My work revealed an intriguing finding that even when ribosomes are forced to be released from cytoplasmic mRNAs by puromycin treatment, deadenylation is still required for P body formation. Given that deadenylation leads to translation repression or formation of non-translatable mRNPs, one possible scenario is as follow: when deadenylation is inhibited, the poly(A) binding proteins (PABPs) associated with the poly(A) tails inhibit mRNAs from forming P bodies by promoting mRNA circularization and preventing the displacement of translation activators by specific translation inhibitors that are also found in P bodies. It will be interesting to test this hypothesis by analyzing protein components of mRNPs from cells whose translation and deadenylation are blocked.

Unlike the Ccr4-Caf1 complex, the roles of the Pan3-Pan2 complex in P body formation appear to be more complicated. Unlike overexpressing the Caf1 catalytically inactive mutant, overexpression of a Pan2 catalytic mutant didn't inhibit P body formation, suggesting that Pan2 deadenylase activity is not essential for P body formation. Consistently, siRNA-mediated knockdown of Pan2 has little effect on P bodies. Interestingly, Pan3 mutants with C-terminal domain truncation, which no longer interact with Pan2, still formed many cytoplasmic foci co-localizing with P bodies. The N-terminal domain and kinase domain of Pan3, although not required for interaction with Pan2, are thus both important for Pan3 to co-localize with P bodies. Moreover, ectopic expression of Pan3 mutants with phosphorylation sites mutated at the N-terminal domain, e.g., NP11, results in P body morphology change. Collectively,

these observations argue that Pan3 possesses Pan2-independent functions that are essential for P body formation.

What could be the Pan2-unrelated functions of Pan3 during P body formation? A clue comes from a discrepancy between a reported observation and my experimental results. *In vitro*, purified Pan2 was reported to shorten oligo(A) in the absence of Pan3 and PABP. However, I observed that tethered Pan3 mutants with deletions in the N-terminal domain or kinase domain failed to induce fast deadenylation of the reporter mRNA, even though these mutants can still interact with Pan2. One possibility is that in my *in vivo* experiment PABPs associated with the poly(A) tail may block the deadenylase activity of Pan2 recruited by Pan3 mutants while in the reported *in vitro* experiment no PABP is present in the reaction. The observation that tethered wild-type Pan3, but not Pan3 truncation mutants, induced fast deadenylation suggests that Pan3 N-terminal and kinase domains may be required to promote dissociation of PABPs from the poly(A) tail. Therefore Pan3 alone might be able to remove PABP from the poly(A) tail and allow these poly(A)+ mRNAs to enter P bodies, which might explain why HA-tagged Pan3 promotes the enrichment of Ccr4-Caf1 and other P body components, but not PABP, in P bodies. This hypothesis can be tested by checking the ability of Pan3 or Pan3 mutants (e.g., deficient in Pan2 binding) to release PABPs from the poly(A) tail of reporter mRNAs using cytoplasmic lysates prepared from cells expressing Pan2 catalytic mutant.

Another possible Pan2-independent function of Pan3 in P body formation may involve the Pan3-Pan3 self-interaction as discovered in yeast (33) and suggested by my preliminary observations in NIH 3T3 cells. The Pan3-Pan3 self-interaction may promote aggregation of mRNPs during P body formation. To further confirm that Pan3 self-interacts within P bodies, the distance between YFP-Pan3 and CFP-Pan3 in P bodies can be measured using fluorescence resonance energy transfer (FRET).

Pan3 N-terminal domain may be phosphorylated to regulate Pan3 localization and functions. Guided by two different methods for phosphorylation site prediction, I focused on eleven potential phosphorylation sites in Pan3 N-terminal domain. NP11, the Pan3 mutant where the eleven potential

phosphorylation sites were replaced by alanine or phenylalanine, induced large and irregularly shaped P body formation. On the other hand, PM11, the Pan3 mutant mimicking phosphorylation at the eleven sites, no longer colocalizes with P bodies. Instead, it forms nuclear foci. Interestingly, HA-tagged wild-type Pan3 can also form nuclear foci and be hyper-phosphorylated upon Leptomycin B treatment. Like the wild-type Pan3, these two mutants are able to promote reporter mRNA deadenylation and decay when tethered to the reporter. It will be interesting to confirm phosphorylation of Pan3 N-terminal domain and identify the phosphorylated residues by mass spectrometry analysis of affinity purified HA-Pan3. Once the phosphorylated sites in Pan3 N-terminal domain are identified, the effects of mutations mimicking or inhibiting phosphorylation at those sites on Pan3 localization and functions can then be determined.

The observation that Pan3(1-353) and Pan3(PM11) formed nuclear foci suggests that Pan3 may play a role in the nucleus. In addition, wild-type Pan3 also formed nuclear foci when the cells were treated with Leptomycin B, an inhibitor of the nuclear export receptor CRM1. In yeast, Pan3p-Pan2p complex trims the poly(A) tails of newly synthesized mRNAs to message-specific length, although it was not clear whether this occurs in the nucleus or in the cytoplasm (35). My observations suggest that Pan3-Pan2 may also trim the poly(A) tails of nascent mRNAs in the nucleus in mammalian cells. To test this possibility, we can check if siRNA-mediated knock down of Pan3 or Pan2 results in formation of longer poly(A) tails of nuclear mRNA. Since Pan3 nuclear localization may correlate with Pan3 hyperphosphorylation, it will also be interesting to determine whether Pan3 phosphorylation affects any potential nuclear function of Pan3.

Aside from focusing on the functions of the two deadenylation complexes in P bodies, I have also compiled a list of reported P body protein components and predicted new human P body proteins based on sequence homology. The reported and predicted human P body components were categorized according to their functions, and their protein-protein interaction networks were visualized and analyzed. This effort not only has helped us to better understand the functions of and interactions among human P body proteins, but also has yielded some interesting predictions. For instance, my study suggests that

cellular proteins able to interact with three or more P body components may also localize to P bodies or play a role in P body functions. In agreement with this prediction, some of the identified proteins have already been reported to be important for P body formation or functions. Future experiments along this line will be to determine P body-related functions of proteins selected in this study. These experiments may reveal novel P body components and post-translational modification pathways that control P body dynamics.

Bibliography

1. Millevoi, S., and S. Vagner. 2009. Molecular mechanisms of eukaryotic pre-mRNA 3' end processing regulation. *Nucleic Acids Research*.
2. Pestova, T. V., V. G. Kolupaeva, I. B. Lomakin, E. V. Pilipenko, I. N. Shatsky, V. I. Agol, and C. U. Hellen. 2001. Molecular mechanisms of translation initiation in eukaryotes. *Proceedings of the National Academy of Sciences of the United States of America* 98:7029.
3. Van Der Kelen, K., R. Beyaert, D. Inzé, and L. De Veylder. 2009. Translational control of eukaryotic gene expression. *Critical Reviews in Biochemistry and Molecular Biology* 44:143.
4. Jackson, R. J., C. U. T. Hellen, and T. V. Pestova. 2010. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nature Reviews. Molecular Cell Biology* 11:113.
5. Mangus, D. A., M. C. Evans, and A. Jacobson. 2003. Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. *Genome Biology* 4:223.
6. Bi, X., and D. J. Goss. 2000. Wheat germ poly(A)-binding protein increases the ATPase and the RNA helicase activity of translation initiation factors eIF4A, eIF4B, and eIF-iso4F. *The Journal of Biological Chemistry* 275:17740.
7. Uchida, N., S.-I. Hoshino, H. Imataka, N. Sonenberg, and T. Katada. 2002. A novel role of the mammalian GSPT/eRF3 associating with poly(A)-binding protein in Cap/Poly(A)-dependent translation. *The Journal of Biological Chemistry* 277:50286.
8. Piccioni, F., V. Zappavigna, and A. C. Verrotti. 2005. Translational regulation during oogenesis and early development: the cap-poly(A) tail relationship. *Comptes Rendus Biologies* 328:863.
9. Beilharz, T. H., and T. Preiss. 2007. Widespread use of poly(A) tail length control to accentuate expression of the yeast transcriptome. *RNA* 13:982-997.

10. López-Lastra, M., A. Rivas, and M. I. Barriá. 2005. Protein synthesis in eukaryotes: the growing biological relevance of cap-independent translation initiation. *Biological Research* 38:121.
11. Spriggs, K. A., M. Stoneley, M. Bushell, and A. E. Willis. 2008. Re-programming of translation following cell stress allows IRES-mediated translation to predominate. *Biology of the Cell / Under the Auspices of the European Cell Biology Organization* 100:27.
12. Thoma, C., G. Bergamini, B. Galy, P. Hundsdoerfer, and M. W. Hentze. 2004. Enhancement of IRES-mediated translation of the c-myc and BiP mRNAs by the poly(A) tail is independent of intact eIF4G and PABP. *Molecular Cell* 15:925.
13. Thoma, C., S. Fraterman, M. Gentzel, M. Wilm, and M. W. Hentze. 2008. Translation initiation by the c-myc mRNA internal ribosome entry sequence and the poly(A) tail. *RNA (New York, N.Y.)* 14:1579.
14. Garneau, N. L., J. Wilusz, and C. J. Wilusz. 2007. The highways and byways of mRNA decay. *Nat Rev Mol Cell Biol* 8:113.
15. Goldstrohm, A. C., and M. Wickens. 2008. Multifunctional deadenylase complexes diversify mRNA control. *Nature Reviews. Molecular Cell Biology* 9:337.
16. Yamashita, A., T.-C. Chang, Y. Yamashita, W. Zhu, Z. Zhong, C.-Y. A. Chen, and A.-B. Shyu. 2005. Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover. *Nature Structural & Molecular Biology* 12:1054.
17. Chen, C. Y., N. Xu, and A. B. Shyu. 1995. mRNA decay mediated by two distinct AU-rich elements from c-fos and granulocyte-macrophage colony-stimulating factor transcripts: different deadenylation kinetics and uncoupling from translation. *Molecular and Cellular Biology* 15:5777.
18. Lai, W. S., E. Carballo, J. R. Strum, E. A. Kennington, R. S. Phillips, and P. J. Blackshear. 1999. Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. *Molecular and Cellular Biology* 19:4311.

19. Chen, C.-Y. A., D. Zheng, Z. Xia, and A.-B. Shyu. 2009. Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. *Nature Structural & Molecular Biology* 16:1160.
20. Piao, X., X. Zhang, L. Wu, and J. G. Belasco. 2010. CCR4-NOT deadenylates mRNA associated with RNA-induced silencing complexes in human cells. *Molecular and Cellular Biology*.
21. Zheng, D., N. Ezzeddine, C.-Y. A. Chen, W. Zhu, X. He, and A.-B. Shyu. 2008. Deadenylation is prerequisite for P-body formation and mRNA decay in mammalian cells. *The Journal of Cell Biology* 182:89.
22. Borman, A. M., Y. M. Michel, and K. M. Kean. 2000. Biochemical characterisation of cap-poly(A) synergy in rabbit reticulocyte lysates: the eIF4G-PABP interaction increases the functional affinity of eIF4E for the capped mRNA 5'-end. *Nucleic Acids Research* 28:4068.
23. Khanna, R., and M. Kiledjian. 2004. Poly(A)-binding-protein-mediated regulation of hDcp2 decapping in vitro. *The EMBO Journal* 23:1968.
24. Tharun, S., and R. Parker. 2001. Targeting an mRNA for decapping: displacement of translation factors and association of the Lsm1p-7p complex on deadenylated yeast mRNAs. *Molecular Cell* 8:1075.
25. Fritz, D. T., N. Bergman, W. J. Kilpatrick, C. J. Wilusz, and J. Wilusz. 2004. Messenger RNA decay in mammalian cells: the exonuclease perspective. *Cell Biochemistry and Biophysics* 41:265.
26. Lowell, J. E., D. Z. Rudner, and A. B. Sachs. 1992. 3'-UTR-dependent deadenylation by the yeast poly(A) nuclease. *Genes & Development* 6:2088.
27. Uchida, N., S.-I. Hoshino, and T. Katada. 2004. Identification of a human cytoplasmic poly(A) nuclease complex stimulated by poly(A)-binding protein. *The Journal of Biological Chemistry* 279:1383.
28. Marchler-Bauer, A., J. B. Anderson, F. Chitsaz, M. K. Derbyshire, C. DeWeese-Scott, J. H. Fong, L. Y. Geer, R. C. Geer, N. R. Gonzales, M. Gwadz, S. He, D. I. Hurwitz, J. D. Jackson, Z.

- Ke, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, S. Lu, G. H. Marchler, M. Mullokandov, J. S. Song, A. Tasneem, N. Thanki, R. A. Yamashita, D. Zhang, N. Zhang, and S. H. Bryant. 2009. CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res* 37:D205-210.
29. Zuo, Y., and M. P. Deutscher. 2001. Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res* 29:1017-1026.
 30. Brown, C. E., S. Z. Tarun, R. Boeck, and A. B. Sachs. 1996. PAN3 encodes a subunit of the Pab1p-dependent poly(A) nuclease in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* 16:5744.
 31. Tucker, M., M. A. Valencia-Sanchez, R. R. Staples, J. Chen, C. L. Denis, and R. Parker. 2001. The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in *Saccharomyces cerevisiae*. *Cell* 104:377.
 32. Chekanova, J. A., R. J. Shaw, and D. A. Belostotsky. 2001. Analysis of an essential requirement for the poly(A) binding protein function using cross-species complementation. *Current Biology: CB* 11:1207.
 33. Mangus, D. A., M. C. Evans, N. S. Agrin, M. Smith, P. Gongidi, and A. Jacobson. 2004. Positive and negative regulation of poly(A) nuclease. *Molecular and Cellular Biology* 24:5521.
 34. Dephoure, N., C. Zhou, J. Villén, S. A. Beausoleil, C. E. Bakalarski, S. J. Elledge, and S. P. Gygi. 2008. A quantitative atlas of mitotic phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America* 105:10762.
 35. Brown, C. E., and A. B. Sachs. 1998. Poly(A) tail length control in *Saccharomyces cerevisiae* occurs by message-specific deadenylation. *Molecular and Cellular Biology* 18:6548.
 36. Dheur, S., K. R. Nykamp, N. Viphakone, M. S. Swanson, and L. Minvielle-Sebastia. 2005. Yeast mRNA Poly(A) tail length control can be reconstituted in vitro in the absence of Pab1p-dependent Poly(A) nuclease activity. *The Journal of Biological Chemistry* 280:24532.

37. Lau, N.-C., A. Kolkman, F. M. A. van Schaik, K. W. Mulder, W. W. M. P. Pijnappel, A. J. R. Heck, and H. T. M. Timmers. 2009. Human Ccr4-Not complexes contain variable deadenylase subunits. *The Biochemical Journal* 422:443.
38. Tucker, M., R. R. Staples, M. A. Valencia-Sanchez, D. Muhlrade, and R. Parker. 2002. Ccr4p is the catalytic subunit of a Ccr4p/Pop2p/Notp mRNA deadenylase complex in *Saccharomyces cerevisiae*. *The EMBO Journal* 21:1427.
39. Clark, L. B., P. Viswanathan, G. Quigley, Y. C. Chiang, J. S. McMahon, G. Yao, J. Chen, A. Nelsbach, and C. L. Denis. 2004. Systematic mutagenesis of the leucine-rich repeat (LRR) domain of CCR4 reveals specific sites for binding to CAF1 and a separate critical role for the LRR in CCR4 deadenylase activity. *J Biol Chem* 279:13616-13623.
40. Chen, J., Y. C. Chiang, and C. L. Denis. 2002. CCR4, a 3'-5' poly(A) RNA and ssDNA exonuclease, is the catalytic component of the cytoplasmic deadenylase. *EMBO J* 21:1414-1426.
41. Andersen, K. R., A. T. Jonstrup, L. B. Van, and D. E. Brodersen. 2009. The activity and selectivity of fission yeast Pop2p are affected by a high affinity for Zn²⁺ and Mn²⁺ in the active site. *RNA (New York, N.Y.)* 15:850.
42. Daugeron, M. C., F. Mauxion, and B. Séraphin. 2001. The yeast POP2 gene encodes a nuclease involved in mRNA deadenylation. *Nucleic Acids Research* 29:2448.
43. Viswanathan, P., T. Ohn, Y.-C. Chiang, J. Chen, and C. L. Denis. 2004. Mouse CAF1 can function as a processive deadenylase/3'-5'-exonuclease in vitro but in yeast the deadenylase function of CAF1 is not required for mRNA poly(A) removal. *The Journal of Biological Chemistry* 279:23988.
44. Aslam, A., S. Mittal, F. Koch, J.-C. Andrau, and G. S. Winkler. 2009. The Ccr4-NOT deadenylase subunits CNOT7 and CNOT8 have overlapping roles and modulate cell proliferation. *Molecular Biology of the Cell* 20:3840.
45. Denis, C. L., and J. Chen. 2003. The CCR4-NOT complex plays diverse roles in mRNA metabolism. *Progress in Nucleic Acid Research and Molecular Biology* 73:221.

46. Azzouz, N., O. O. Panasenko, C. Deluen, J. Hsieh, G. Theiler, and M. A. Collart. 2009. Specific roles for the Ccr4-Not complex subunits in expression of the genome. *RNA* 15:377-383.
47. Temme, C., L. Zhang, E. Kremmer, C. Ihling, A. Chartier, A. Sinz, M. Simonelig, and E. Wahle. 2010. Subunits of the *Drosophila* CCR4-NOT complex and their roles in mRNA deadenylation. *RNA* 16:1356-1370.
48. Maillet, L., and M. A. Collart. 2002. Interaction between Not1p, a component of the Ccr4-not complex, a global regulator of transcription, and Dhh1p, a putative RNA helicase. *J Biol Chem* 277:2835-2842.
49. Ezzeddine, N., T.-C. Chang, W. Zhu, A. Yamashita, C.-Y. A. Chen, Z. Zhong, Y. Yamashita, D. Zheng, and A.-B. Shyu. 2007. Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. *Molecular and Cellular Biology* 27:7791.
50. Chen, D., M. Zhao, and G. R. Mundy. 2004. Bone morphogenetic proteins. *Growth Factors* (Chur, Switzerland) 22:233.
51. Washio-Oikawa, K., T. Nakamura, M. Usui, M. Yoneda, Y. Ezura, I. Ishikawa, K. Nakashima, T. Noda, T. Yamamoto, and M. Noda. 2007. Cnot7-null mice exhibit high bone mass phenotype and modulation of BMP actions. *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research* 22:1217.
52. Rendl, L. M., M. A. Bieman, and C. A. Smibert. 2008. *S. cerevisiae* Vts1p induces deadenylation-dependent transcript degradation and interacts with the Ccr4p-Pop2p-Not deadenylase complex. *RNA* 14:1328-1336.
53. Korner, C. G., and E. Wahle. 1997. Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. *J Biol Chem* 272:10448-10456.
54. Ren, Y. G., J. Martinez, and A. Virtanen. 2002. Identification of the active site of poly(A)-specific ribonuclease by site-directed mutagenesis and Fe(2+)-mediated cleavage. *J Biol Chem* 277:5982-5987.

55. Dehlin, E., M. Wormington, C. G. Körner, and E. Wahle. 2000. Cap-dependent deadenylation of mRNA. *The EMBO Journal* 19:1079.
56. Nilsson, P., N. Henriksson, A. Niedzwiecka, N. A. A. Balatsos, K. Kokkoris, J. Eriksson, and A. Virtanen. 2007. A multifunctional RNA recognition motif in poly(A)-specific ribonuclease with cap and poly(A) binding properties. *The Journal of Biological Chemistry* 282:32902.
57. Martínez, J., Y. G. Ren, P. Nilsson, M. Ehrenberg, and A. Virtanen. 2001. The mRNA cap structure stimulates rate of poly(A) removal and amplifies processivity of degradation. *The Journal of Biological Chemistry* 276:27923.
58. Reverdatto, S. V., J. A. Dutko, J. A. Chekanova, D. A. Hamilton, and D. A. Belostotsky. 2004. mRNA deadenylation by PARN is essential for embryogenesis in higher plants. *RNA (New York, N.Y.)* 10:1200.
59. Gherzi, R., K.-Y. Lee, P. Briata, D. Wegmüller, C. Moroni, M. Karin, and C.-Y. Chen. 2004. A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. *Molecular Cell* 14:571.
60. Lai, W. S., E. A. Kennington, and P. J. Blackshear. 2003. Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease. *Molecular and Cellular Biology* 23:3798.
61. Moraes, K. C. M., C. J. Wilusz, and J. Wilusz. 2006. CUG-BP binds to RNA substrates and recruits PARN deadenylase. *RNA (New York, N.Y.)* 12:1084.
62. Liu, X., and C. B. Green. 2002. Circadian regulation of nocturnin transcription by phosphorylated CREB in *Xenopus* retinal photoreceptor cells. *Molecular and Cellular Biology* 22:7501.
63. Wang, Y., D. L. Osterbur, P. L. Megaw, G. Tosini, C. Fukuhara, C. B. Green, and J. C. Besharse. 2001. Rhythmic expression of Nocturnin mRNA in multiple tissues of the mouse. *BMC Developmental Biology* 1:9.
64. Green, C. B., N. Douris, S. Kojima, C. A. Strayer, J. Fogerty, D. Lourim, S. R. Keller, and J. C. Besharse. 2007. Loss of Nocturnin, a circadian deadenylase, confers resistance to hepatic

- steatosis and diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America* 104:9888.
65. Eystathiou, T., E. K. L. Chan, S. A. Tenenbaum, J. D. Keene, K. Griffith, and M. J. Fritzler. 2002. A phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles. *Molecular Biology of the Cell* 13:1338.
 66. Aizer, A., Y. Brody, L. W. Ler, N. Sonenberg, R. H. Singer, and Y. Shav-Tal. 2008. The dynamics of mammalian P body transport, assembly, and disassembly in vivo. *Molecular Biology of the Cell* 19:4154.
 67. Hu, J., F. Wang, X. Zhu, Y. Yuan, M. Ding, and S. Gao. 2010. Mouse ZAR1-like (XM_359149) colocalizes with mRNA processing components and its dominant-negative mutant caused two-cell-stage embryonic arrest. *Developmental Dynamics: An Official Publication of the American Association of Anatomists* 239:407.
 68. Parker, R., and U. Sheth. 2007. P bodies and the control of mRNA translation and degradation. *Molecular Cell* 25:635.
 69. Sheth, U., and R. Parker. 2006. Targeting of aberrant mRNAs to cytoplasmic processing bodies. *Cell* 125:1095.
 70. Durand, S., N. Cougot, F. Mahuteau-Betzer, C.-H. Nguyen, D. S. Grierson, E. Bertrand, J. Tazi, and F. Lejeune. 2007. Inhibition of nonsense-mediated mRNA decay (NMD) by a new chemical molecule reveals the dynamic of NMD factors in P-bodies. *The Journal of Cell Biology* 178:1145.
 71. Kedersha, N., G. Stoecklin, M. Ayodele, P. Yacono, J. Lykke-Andersen, M. J. Fritzler, D. Scheuner, R. J. Kaufman, D. E. Golan, and P. Anderson. 2005. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *The Journal of Cell Biology* 169:871.
 72. Sheth, U., and R. Parker. 2003. Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science (New York, N.Y.)* 300:805.

73. Bhattacharyya, S. N., R. Habermacher, U. Martine, E. I. Closs, and W. Filipowicz. 2006. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* 125:1111.
74. Andrei, M. A., D. Ingelfinger, R. Heintzmann, T. Achsel, R. Rivera-Pomar, and R. Lührmann. 2005. A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies. *RNA (New York, N.Y.)* 11:717.
75. Chu, C.-y., and T. M. Rana. 2006. Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. *PLoS Biology* 4:e210.
76. Cougot, N., S. Babajko, and B. Séraphin. 2004. Cytoplasmic foci are sites of mRNA decay in human cells. *The Journal of Cell Biology* 165:31.
77. Teixeira, D., and R. Parker. 2007. Analysis of P-body assembly in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* 18:2274.
78. Reijns, M. A. M., R. D. Alexander, M. P. Spiller, and J. D. Beggs. 2008. A role for Q/N-rich aggregation-prone regions in P-body localization. *Journal of Cell Science* 121:2463.
79. Amrani, N., S. Ghosh, D. A. Mangus, and A. Jacobson. 2008. Translation factors promote the formation of two states of the closed-loop mRNP. *Nature* 453:1276-1280.
80. Meyer, S., C. Temme, and E. Wahle. 2004. Messenger RNA turnover in eukaryotes: pathways and enzymes. *Critical Reviews in Biochemistry and Molecular Biology* 39:197.
81. Chen, C.-Y. A., Y. Yamashita, T.-C. Chang, A. Yamashita, W. Zhu, Z. Zhong, and A.-B. Shyu. 2007. Versatile applications of transcriptional pulsing to study mRNA turnover in mammalian cells. *RNA (New York, N.Y.)* 13:1775.
82. Peng, S. S., C. Y. Chen, N. Xu, and A. B. Shyu. 1998. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J* 17:3461-3470.
83. Rehwinkel, J., I. Behm-Ansmant, D. Gatfield, and E. Izaurralde. 2005. A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. *RNA* 11:1640-1647.

84. Ferraiuolo, M. A., S. Basak, J. Dostie, E. L. Murray, D. R. Schoenberg, and N. Sonenberg. 2005. A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay. *The Journal of Cell Biology* 170:913.
85. Collins, T. J. 2007. ImageJ for microscopy. *Biotechniques* 43:25-30.
86. Unsworth, H., S. Raguz, H. J. Edwards, C. F. Higgins, and E. Yague. 2010. mRNA escape from stress granule sequestration is dictated by localization to the endoplasmic reticulum. *FASEB J.*
87. Brengues, M., and R. Parker. 2007. Accumulation of polyadenylated mRNA, Pab1p, eIF4E, and eIF4G with P-bodies in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* 18:2592.
88. Chowdhury, A., J. Mukhopadhyay, and S. Tharun. 2007. The decapping activator Lsm1p-7p-Pat1p complex has the intrinsic ability to distinguish between oligoadenylated and polyadenylated RNAs. *RNA (New York, N.Y.)* 13:998.
89. Dephoure, N., R. W. Howson, J. D. Blethrow, K. M. Shokat, and E. K. O'Shea. 2005. Combining chemical genetics and proteomics to identify protein kinase substrates. *Proceedings of the National Academy of Sciences of the United States of America* 102:17940.
90. Albuquerque, C. P., M. B. Smolka, S. H. Payne, V. Bafna, J. Eng, and H. Zhou. 2008. A multidimensional chromatography technology for in-depth phosphoproteome analysis. *Molecular & Cellular Proteomics: MCP* 7:1389.
91. Pan, C., F. Gnäd, J. V. Olsen, and M. Mann. 2008. Quantitative phosphoproteome analysis of a mouse liver cell line reveals specificity of phosphatase inhibitors. *Proteomics* 8:4534.
92. Siddiqui, N., D. A. Mangus, T.-C. Chang, J.-M. Palermino, A.-B. Shyu, and K. Gehring. 2007. Poly(A) nuclease interacts with the C-terminal domain of polyadenylate-binding protein domain from poly(A)-binding protein. *The Journal of Biological Chemistry* 282:25067.
93. Boeck, R., S. Tarun, M. Rieger, J. A. Deardorff, S. Müller-Auer, and A. B. Sachs. 1996. The yeast Pan2 protein is required for poly(A)-binding protein-stimulated poly(A)-nuclease activity. *The Journal of Biological Chemistry* 271:432.

94. Keryer-Bibens, C., C. Barreau, and H. B. Osborne. 2008. Tethering of proteins to RNAs by bacteriophage proteins. *Biology of the Cell / Under the Auspices of the European Cell Biology Organization* 100:125.
95. Kay, B. K., M. P. Williamson, and M. Sudol. 2000. The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 14:231.
96. Li, Romero, Rani, Dunker, and Obradovic. 1999. Predicting Protein Disorder for N-, C-, and Internal Regions. *Genome Informatics. Workshop on Genome Informatics* 10:30.
97. Romero, P., Z. Obradovic, and A. K. Dunker. 2004. Natively disordered proteins: functions and predictions. *Applied Bioinformatics* 3:105.
98. Iakoucheva, L. M., P. Radivojac, C. J. Brown, T. R. O'Connor, J. G. Sikes, Z. Obradovic, and A. K. Dunker. 2004. The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Research* 32:1037.
99. Obenauer, J. C., L. C. Cantley, and M. B. Yaffe. 2003. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Research* 31:3635.
100. Yashiroda, Y., and M. Yoshida. 2003. Nucleo-cytoplasmic transport of proteins as a target for therapeutic drugs. *Curr Med Chem* 10:741-748.
101. Xu, J., J.-Y. Yang, Q.-W. Niu, and N.-H. Chua. 2006. Arabidopsis DCP2, DCP1, and VARICOSE form a decapping complex required for postembryonic development. *The Plant Cell* 18:3386.
102. Ding, L., A. Spencer, K. Morita, and M. Han. 2005. The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in *C. elegans*. *Molecular Cell* 19:437.
103. Nykamp, K., M.-H. Lee, and J. Kimble. 2008. *C. elegans* La-related protein, LARP-1, localizes to germline P bodies and attenuates Ras-MAPK signaling during oogenesis. *RNA (New York, N.Y.)* 14:1378.

104. Hammell, C. M., I. Lubin, P. R. Boag, T. K. Blackwell, and V. Ambros. 2009. *nhl-2* Modulates microRNA activity in *Caenorhabditis elegans*. *Cell* 136:926.
105. Gallo, C. M., E. Munro, D. Rasoloson, C. Merritt, and G. Seydoux. 2008. Processing bodies and germ granules are distinct RNA granules that interact in *C. elegans* embryos. *Developmental Biology* 323:76.
106. Eulalio, A., S. Helms, C. Fritsch, M. Fauser, and E. Izaurralde. 2009. A C-terminal silencing domain in GW182 is essential for miRNA function. *RNA (New York, N.Y.)* 15:1067.
107. Lee, L., S. E. Davies, and J.-L. Liu. 2009. The spinal muscular atrophy protein SMN affects *Drosophila* germline nuclear organization through the U body-P body pathway. *Developmental Biology* 332:142.
108. Tritschler, F., A. Eulalio, V. Truffault, M. D. Hartmann, S. Helms, S. Schmidt, M. Coles, E. Izaurralde, and O. Weichenrieder. 2007. A divergent Sm fold in EDC3 proteins mediates DCP1 binding and P-body targeting. *Molecular and Cellular Biology* 27:8600.
109. Barbee, S. A., P. S. Estes, A.-M. Cziko, J. Hillebrand, R. A. Luedeman, J. M. Collier, N. Johnson, I. C. Howlett, C. Geng, R. Ueda, A. H. Brand, S. F. Newbury, J. E. Wilhelm, R. B. Levine, A. Nakamura, R. Parker, and M. Ramaswami. 2006. Staufen- and FMRP-containing neuronal RNPs are structurally and functionally related to somatic P bodies. *Neuron* 52:997.
110. Lin, M.-D., S.-J. Fan, W.-S. Hsu, and T.-B. Chou. 2006. *Drosophila* decapping protein 1, dDcp1, is a component of the oskar mRNP complex and directs its posterior localization in the oocyte. *Developmental Cell* 10:601.
111. Sen, G. L., and H. M. Blau. 2005. Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nature Cell Biology* 7:633.
112. Azuma-Mukai, A., H. Oguri, T. Mituyama, Z. R. Qian, K. Asai, H. Siomi, and M. C. Siomi. 2008. Characterization of endogenous human Argonautes and their miRNA partners in RNA silencing. *Proceedings of the National Academy of Sciences of the United States of America* 105:7964.

113. Lazzaretti, D., I. Tournier, and E. Izaurralde. 2009. The C-terminal domains of human TNRC6A, TNRC6B, and TNRC6C silence bound transcripts independently of Argonaute proteins. *RNA (New York, N.Y.)* 15:1059.
114. Wichroski, M. J., G. B. Robb, and T. M. Rana. 2006. Human retroviral host restriction factors APOBEC3G and APOBEC3F localize to mRNA processing bodies. *PLoS Pathogens* 2:e41.
115. Wilczynska, A., C. Aigueperse, M. Kress, F. Dautry, and D. Weil. 2005. The translational regulator CPEB1 provides a link between dcp1 bodies and stress granules. *Journal of Cell Science* 118:981.
116. van Dijk, E., N. Cougot, S. Meyer, S. Babajko, E. Wahle, and B. Séraphin. 2002. Human Dcp2: a catalytically active mRNA decapping enzyme located in specific cytoplasmic structures. *The EMBO Journal* 21:6915.
117. Ling, S. H. M., C. J. Decker, M. A. Walsh, M. She, R. Parker, and H. Song. 2008. Crystal structure of human Edc3 and its functional implications. *Molecular and Cellular Biology* 28:5965.
118. Yu, J. H., W.-H. Yang, T. Gulick, K. D. Bloch, and D. B. Bloch. 2005. Ge-1 is a central component of the mammalian cytoplasmic mRNA processing body. *RNA (New York, N.Y.)* 11:1795.
119. Fierro-Monti, I., S. Mohammed, R. Matthiesen, R. Santoro, J. S. Burns, D. J. Williams, C. G. Proud, M. Kassem, O. N. Jensen, and P. Roepstorff. 2006. Quantitative proteomics identifies Gemin5, a scaffolding protein involved in ribonucleoprotein assembly, as a novel partner for eukaryotic initiation factor 4E. *Journal of Proteome Research* 5:1367.
120. Katahira, J., T. Miki, K. Takano, M. Maruhashi, M. Uchikawa, T. Tachibana, and Y. Yoneda. 2008. Nuclear RNA export factor 7 is localized in processing bodies and neuronal RNA granules through interactions with shuttling hnRNPs. *Nucleic Acids Research* 36:616.
121. Savas, J. N., A. Makusky, S. Ottosen, D. Baillat, F. Then, D. Krainc, R. Shiekhattar, S. P. Markey, and N. Tanese. 2008. Huntington's disease protein contributes to RNA-mediated gene

- silencing through association with Argonaute and P bodies. *Proceedings of the National Academy of Sciences of the United States of America* 105:10820.
122. Weinmann, L., J. Höck, T. Ivacevic, T. Ohrt, J. Mütze, P. Schwill, E. Kremmer, V. Benes, H. Urlaub, and G. Meister. 2009. Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs. *Cell* 136:496.
 123. Balzer, E., and E. G. Moss. 2007. Localization of the developmental timing regulator Lin28 to mRNP complexes, P-bodies and stress granules. *RNA Biology* 4:16.
 124. Ingelfinger, D., D. J. Arndt-Jovin, R. Lührmann, and T. Achsel. 2002. The human LSM1-7 proteins colocalize with the mRNA-degrading enzymes Dcp1/2 and Xrn1 in distinct cytoplasmic foci. *RNA (New York, N.Y.)* 8:1489.
 125. Buchet-Poyau, K., J. Courchet, H. Le Hir, B. Séraphin, J.-Y. Scoazec, L. Duret, C. Domon-Dell, J.-N. Freund, and M. Billaud. 2007. Identification and characterization of human Mex-3 proteins, a novel family of evolutionarily conserved RNA-binding proteins differentially localized to processing bodies. *Nucleic Acids Research* 35:1289.
 126. Meister, G., M. Landthaler, L. Peters, P. Y. Chen, H. Urlaub, R. Lührmann, and T. Tuschl. 2005. Identification of novel argonaute-associated proteins. *Current Biology: CB* 15:2149.
 127. Minagawa, K., Y. Katayama, S. Nishikawa, K. Yamamoto, A. Sada, A. Okamura, M. Shimoyama, and T. Matsui. 2009. Inhibition of G(1) to S phase progression by a novel zinc finger protein P58(TFL) at P-bodies. *Molecular Cancer Research: MCR* 7:880.
 128. Scheller, N., P. Resa-Infante, S. de la Luna, R. P. Galao, M. Albrecht, L. Kaestner, P. Lipp, T. Lengauer, A. Meyerhans, and J. Díez. 2007. Identification of PatL1, a human homolog to yeast P body component Pat1. *Biochimica Et Biophysica Acta* 1773:1786.
 129. Fujimura, K., J. Katahira, F. Kano, Y. Yoneda, and M. Murata. 2009. Selective localization of PCBP2 to cytoplasmic processing bodies. *Biochimica Et Biophysica Acta* 1793:878.
 130. Fujimura, K., F. Kano, and M. Murata. 2008. Identification of PCBP2, a facilitator of IRES-mediated translation, as a novel constituent of stress granules and processing bodies. *RNA (New York, N.Y.)* 14:425.

131. Hebner, C. M., R. Wilson, J. Rader, M. Bidder, and L. A. Laimins. 2006. Human papillomaviruses target the double-stranded RNA protein kinase pathway. *The Journal of General Virology* 87:3183.
132. Cho, H., K. M. Kim, and Y. K. Kim. 2009. Human proline-rich nuclear receptor coregulatory protein 2 mediates an interaction between mRNA surveillance machinery and decapping complex. *Molecular Cell* 33:75.
133. Yang, W.-H., J. H. Yu, T. Gulick, K. D. Bloch, and D. B. Bloch. 2006. RNA-associated protein 55 (RAP55) localizes to mRNA processing bodies and stress granules. *RNA (New York, N.Y.)* 12:547.
134. Yamochi, T., K. Ohnuma, O. Hosono, H. Tanaka, Y. Kanai, and C. Morimoto. 2008. SSA/Ro52 autoantigen interacts with Dcp2 to enhance its decapping activity. *Biochemical and Biophysical Research Communications* 370:195.
135. Unterholzner, L., and E. Izaurralde. 2004. SMG7 acts as a molecular link between mRNA surveillance and mRNA decay. *Molecular Cell* 16:587.
136. Takimoto, K., M. Wakiyama, and S. Yokoyama. 2009. Mammalian GW182 contains multiple Argonaute-binding sites and functions in microRNA-mediated translational repression. *RNA (New York, N.Y.)* 15:1078.
137. Baillat, D., and R. Shiekhattar. 2009. Functional dissection of the human TNRC6 (GW182-related) family of proteins. *Molecular and Cellular Biology* 29:4144.
138. Chang, W.-L., and W.-Y. Tarn. 2009. A role for transportin in deposition of TTP to cytoplasmic RNA granules and mRNA decay. *Nucleic Acids Research* 37:6600.
139. Yang, W.-H., and D. B. Bloch. 2007. Probing the mRNA processing body using protein macroarrays and "autoantigenomics". *RNA (New York, N.Y.)* 13:704.
140. Blumenthal, J., and I. Ginzburg. 2008. Zinc as a translation regulator in neurons: implications for P-body aggregation. *Journal of Cell Science* 121:3253.

141. Chung, S., Y. Zhang, F. Van Der Hoorn, and R. Hawkes. 2007. The anatomy of the cerebellar nuclei in the normal and scrambler mouse as revealed by the expression of the microtubule-associated protein kinesin light chain 3. *Brain Research* 1140:120.
142. Rybak, A., H. Fuchs, K. Hadian, L. Smirnova, E. A. Wulczyn, G. Michel, R. Nitsch, D. Krappmann, and F. G. Wulczyn. 2009. The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. *Nature Cell Biology* 11:1411.
143. Suzuki, A., K. Igarashi, K.-I. Aisaki, J. Kanno, and Y. Saga. 2010. NANOS2 interacts with the CCR4-NOT deadenylation complex and leads to suppression of specific RNAs. *Proceedings of the National Academy of Sciences of the United States of America*.
144. Yamaji, M., T. Tanaka, M. Shigeta, S. Chuma, Y. Saga, and M. Saitou. 2010. Functional reconstruction of NANOS3 expression in the germ cell lineage by a novel transgenic reporter reveals distinct subcellular localizations of NANOS3. *Reproduction (Cambridge, England)* 139:381.
145. Malys, N., and J. E. G. McCarthy. 2006. Dcs2, a novel stress-induced modulator of m7GpppX pyrophosphatase activity that locates to P bodies. *Journal of Molecular Biology* 363:370.
146. Beckham, C., A. Hilliker, A.-M. Cziko, A. Noueiry, M. Ramaswami, and R. Parker. 2008. The DEAD-box RNA helicase Ded1p affects and accumulates in *Saccharomyces cerevisiae* P-bodies. *Molecular Biology of the Cell* 19:984.
147. Luke, B., C. M. Azzalin, N. Hug, A. Deplazes, M. Peter, and J. Lingner. 2007. *Saccharomyces cerevisiae* Ebs1p is a putative ortholog of human Smg7 and promotes nonsense-mediated mRNA decay. *Nucleic Acids Research* 35:7688.
148. Kshirsagar, M., and R. Parker. 2004. Identification of Edc3p as an enhancer of mRNA decapping in *Saccharomyces cerevisiae*. *Genetics* 166:729.
149. Chang, W., R. F. Zaarour, S. Reck-Peterson, J. Rinn, R. H. Singer, M. Snyder, P. Novick, and M. S. Mooseker. 2008. Myo2p, a class V myosin in budding yeast, associates with a large ribonucleic acid-protein complex that contains mRNAs and subunits of the RNA-processing body. *RNA (New York, N.Y.)* 14:491.

150. Muhlrad, D., and R. Parker. 2005. The yeast EDC1 mRNA undergoes deadenylation-independent decapping stimulated by Not2p, Not4p, and Not5p. *The EMBO Journal* 24:1033.
151. Sweet, T. J., B. Boyer, W. Hu, K. E. Baker, and J. Collier. 2007. Microtubule disruption stimulates P-body formation. *RNA (New York, N.Y.)* 13:493.
152. Lee, S.-I., A. M. Dudley, D. Drubin, P. A. Silver, N. J. Krogan, D. Pe'er, and D. Koller. 2009. Learning a prior on regulatory potential from eQTL data. *PLoS Genetics* 5:e1000358.
153. Jang, L.-T., L.-M. Buu, and F.-J. S. Lee. 2006. Determinants of Rbp1p localization in specific cytoplasmic mRNA-processing foci, P-bodies. *The Journal of Biological Chemistry* 281:29379.
154. Lotan, R., V. G. Bar-On, L. Harel-Sharvit, L. Duek, D. Melamed, and M. Choder. 2005. The RNA polymerase II subunit Rpb4p mediates decay of a specific class of mRNAs. *Genes & Development* 19:3004.
155. Lotan, R., V. Goler-Baron, L. Duek, G. Haimovich, and M. Choder. 2007. The Rpb7p subunit of yeast RNA polymerase II plays roles in the two major cytoplasmic mRNA decay mechanisms. *The Journal of Cell Biology* 178:1133.
156. Stribinskis, V., and K. S. Ramos. 2007. Rpm2p, a protein subunit of mitochondrial RNase P, physically and genetically interacts with cytoplasmic processing bodies. *Nucleic Acids Research* 35:1301.
157. Segal, S. P., T. Dunkley, and R. Parker. 2006. Sbp1p affects translational repression and decapping in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* 26:5120.
158. Tudisca, V., V. Recouvreux, S. Moreno, E. Boy-Marcotte, M. Jacquet, and P. Portela. 2009. Differential localization to cytoplasm, nucleus or P-bodies of yeast PKA subunits under different growth conditions. *European Journal of Cell Biology*.
159. Rendl, L. M., M. A. Bieman, and C. A. Smibert. 2008. *S. cerevisiae* Vts1p induces deadenylation-dependent transcript degradation and interacts with the Ccr4p-Pop2p-Not deadenylase complex. *RNA (New York, N.Y.)* 14:1328.
160. Favre, N., M. Camps, C. Arod, C. Chabert, C. Rommel, and C. Pasquali. 2008. Chemokine receptor CCR2 undergoes transportin1-dependent nuclear translocation. *Proteomics* 8:4560.

161. Deleault, K. M., S. J. Skinner, and S. A. Brooks. 2008. Tristetraprolin regulates TNF TNF- α mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways. *Molecular Immunology* 45:13.
162. Franks, T. M., and J. Lykke-Andersen. 2007. TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements. *Genes & Development* 21:719.
163. Ohnishi, T., A. Yamashita, I. Kashima, T. Schell, K. R. Anders, A. Grimson, T. Hachiya, M. W. Hentze, P. Anderson, and S. Ohno. 2003. Phosphorylation of hUPF1 induces formation of mRNA surveillance complexes containing hSMG-5 and hSMG-7. *Molecular Cell* 12:1187.
164. Chiu, S.-Y., G. Serin, O. Ohara, and L. E. Maquat. 2003. Characterization of human Smg5/7a: a protein with similarities to *Caenorhabditis elegans* SMG5 and SMG7 that functions in the dephosphorylation of Upf1. *RNA (New York, N.Y.)* 9:77.
165. Reichenbach, P., M. Höss, C. M. Azzalin, M. Nabholz, P. Bucher, and J. Lingner. 2003. A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. *Current Biology: CB* 13:568.
166. Fukuhara, N., J. Ebert, L. Unterholzner, D. Lindner, E. Izaurralde, and E. Conti. 2005. SMG7 is a 14-3-3-like adaptor in the nonsense-mediated mRNA decay pathway. *Molecular Cell* 17:537.
167. Ivanov, P. V., N. H. Gehring, J. B. Kunz, M. W. Hentze, and A. E. Kulozik. 2008. Interactions between UPF1, eRFs, PABP and the exon junction complex suggest an integrated model for mammalian NMD pathways. *The EMBO Journal* 27:736.
168. Chamieh, H., L. Ballut, F. Bonneau, and H. Le Hir. 2008. NMD factors UPF2 and UPF3 bridge UPF1 to the exon junction complex and stimulate its RNA helicase activity. *Nature Structural & Molecular Biology* 15:85.
169. Kunz, J. B., G. Neu-Yilik, M. W. Hentze, A. E. Kulozik, and N. H. Gehring. 2006. Functions of hUpf3a and hUpf3b in nonsense-mediated mRNA decay and translation. *RNA (New York, N.Y.)* 12:1015.
170. Wu, L., J. Fan, and J. G. Belasco. 2008. Importance of translation and nonnucleolytic ago proteins for on-target RNA interference. *Current Biology: CB* 18:1327.

171. Meng, Q., S. K. Rayala, A. E. Gururaj, A. H. Talukder, B. W. O'Malley, and R. Kumar. 2007. Signaling-dependent and coordinated regulation of transcription, splicing, and translation resides in a single coregulator, PCBP1. *Proceedings of the National Academy of Sciences of the United States of America* 104:5866.
172. Yao, X., X. Chen, C. Cottonham, and L. Xu. 2008. Preferential utilization of Imp7/8 in nuclear import of Smads. *The Journal of Biological Chemistry* 283:22867.
173. Eulalio, A., E. Huntzinger, and E. Izaurralde. 2008. GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat Struct Mol Biol* 15:346.
174. Fabian, M. R., G. Mathonnet, T. Sundermeier, H. Mathys, J. T. Zipprich, Y. V. Svitkin, F. Rivas, M. Jinek, J. Wohlschlegel, J. A. Doudna, C.-Y. A. Chen, A.-B. Shyu, J. R. Yates, G. J. Hannon, W. Filipowicz, T. F. Duchaine, and N. Sonenberg. 2009. Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Molecular Cell* 35:868.
175. Jin, H., M. R. Suh, J. Han, K.-H. Yeom, Y. Lee, I. Heo, M. Ha, S. Hyun, and V. N. Kim. 2009. Human UPF1 Participates in Small RNA-Induced mRNA Downregulation. *Mol. Cell. Biol.* 29:5789.
176. Viswanathan, S. R., G. Q. Daley, and R. I. Gregory. 2008. Selective Blockade of MicroRNA Processing by Lin28. *Science* 320:97.
177. Viswanathan, S. R., J. T. Powers, W. Einhorn, Y. Hoshida, T. Ng, S. Toffanin, M. O'Sullivan, J. Lu, L. A. Philips, V. L. Lockhart, S. P. Shah, P. S. Tanwar, C. H. Mermel, R. Beroukhim, M. Azam, J. Teixeira, M. Meyerson, T. P. Hughes, J. M. Llovet, J. Radich, C. G. Mullighan, T. R. Golub, P. H. Sorensen, and G. Q. Daley. 2009. Lin28 Enhances Tumorigenesis and is Associated With Advanced Human Malignancies. *Nature genetics* 41:843.
178. Huang, P.-R., S.-T. Tsai, K.-H. Hsieh, and T.-C. V. Wang. 2008. Heterogeneous nuclear ribonucleoprotein A3 binds single-stranded telomeric DNA and inhibits telomerase extension in vitro. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1783:193.

179. Ma, A. S. W., K. Moran-Jones, J. Shan, T. P. Munro, M. J. Snee, K. S. Hoek, and R. Smith. 2002. Heterogeneous Nuclear Ribonucleoprotein A3, a Novel RNA Trafficking Response Element-binding Protein. *Journal of Biological Chemistry* 277:18010.
180. Furtak, V., A. Mulky, S. A. Rawlings, L. Kozhaya, K. Lee, V. N. KewalRamani, and D. Unutmaz. 2010. Perturbation of the P-Body Component Mov10 Inhibits HIV-1 Infectivity. *PloS One* 5.
181. Nakano, M., Y. Kakiuchi, Y. Shimada, M. Ohyama, Y. Ogiwara, N. Sasaki-Higashiyama, N. Yano, F. Ikeda, E. Yamada, A. Iwamatsu, K. Kobayashi, K. Nishiyama, S. Ichikawa, K. Kaji, T. Ide, H. Murofushi, and K. Murakami-Murofushi. 2009. MOV10 as a novel telomerase-associated protein. *Biochemical and Biophysical Research Communications* 388:328.
182. Du, Z., J. Yu, Y. Chen, R. Andino, and T. L. James. 2004. Specific Recognition of the C-rich Strand of Human Telomeric DNA and the RNA Template of Human Telomerase by the First KH Domain of Human Poly(C)-binding Protein-2. *Journal of Biological Chemistry* 279:48126.
183. Kiledjian, M., X. Wang, and S. A. Liebhaber. 1995. Identification of two KH domain proteins in the alpha-globin mRNP stability complex. *The EMBO Journal* 14:4357.
184. Waggoner, S. A., G. J. Johannes, and S. A. Liebhaber. 2009. Depletion of the poly(C)-binding proteins alphaCP1 and alphaCP2 from K562 cells leads to p53-independent induction of cyclin-dependent kinase inhibitor (CDKN1A) and G1 arrest. *The Journal of Biological Chemistry* 284:9039.
185. Collart, M. A. 2003. Global control of gene expression in yeast by the Ccr4-Not complex. *Gene* 313:1-16.
186. Adams, D. J., D. J. Beveridge, L. van der Weyden, H. Mangs, P. J. Leedman, and B. J. Morris. 2003. HADHB, HuR, and CP1 Bind to the Distal 3' -Untranslated Region of Human Renin mRNA and Differentially Modulate Renin Expression. *Journal of Biological Chemistry* 278:44894.

187. Pickering, B. M., S. A. Mitchell, K. A. Spriggs, M. Stoneley, and A. E. Willis. 2004. Bag-1 Internal Ribosome Entry Segment Activity Is Promoted by Structural Changes Mediated by Poly(rC) Binding Protein 1 and Recruitment of Polypyrimidine Tract Binding Protein 1. *Molecular and Cellular Biology* 24:5595.
188. Fukuda, T., M. Ashizuka, T. Nakamura, K. Shibahara, K. Maeda, H. Izumi, K. Kohno, M. Kuwano, and T. Uchiumi. 2004. Characterization of the 5'-untranslated region of YB-1 mRNA and autoregulation of translation by YB-1 protein. *Nucleic Acids Research* 32:611.
189. Jurchott, K., S. Bergmann, U. Stein, W. Walther, M. Janz, I. Manni, G. Piaggio, E. Fietze, M. Dietel, and H.-D. Royer. 2003. YB-1 as a cell cycle-regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. *The Journal of Biological Chemistry* 278:27988.
190. Stickeler, E., S. D. Fraser, A. Honig, A. L. Chen, S. M. Berget, and T. A. Cooper. 2001. The RNA binding protein YB-1 binds A/C-rich exon enhancers and stimulates splicing of the CD44 alternative exon v4. *The EMBO Journal* 20:3821.
191. De Diego Otero, Y., L.-A. Severijnen, G. van Cappellen, M. Schrier, B. Oostra, and R. Willemsen. 2002. Transport of Fragile X Mental Retardation Protein via Granules in Neurites of PC12 Cells. *Molecular and Cellular Biology* 22:8332.
192. Wang, H., J. B. Dichtenberg, L. Ku, W. Li, G. J. Bassell, and Y. Feng. 2008. Dynamic Association of the Fragile X Mental Retardation Protein as a Messenger Ribonucleoprotein between Microtubules and Polyribosomes. *Molecular Biology of the Cell* 19:105.
193. Jacobs, D. T., R. Weigert, K. D. Grode, J. G. Donaldson, and R. E. Cheney. 2009. Myosin Vc Is a Molecular Motor That Functions in Secretory Granule Trafficking. *Mol. Biol. Cell* 20:4471.
194. Takano, K., T. Miki, J. Katahira, and Y. Yoneda. 2007. NXF2 is involved in cytoplasmic mRNA dynamics through interactions with motor proteins. *Nucleic Acids Research* 35:2513.
195. Villacé, P., R. M. Marión, and J. Ortín. 2004. The composition of Staufen-containing RNA granules from human cells indicates their role in the regulated transport and translation of messenger RNAs. *Nucleic Acids Research* 32:2411.

196. Gong, C., Y. K. Kim, C. F. Woeller, Y. Tang, and L. E. Maquat. 2009. SMD and NMD are competitive pathways that contribute to myogenesis: effects on PAX3 and myogenin mRNAs. *Genes & Development* 23:54.
197. von der Haar, T., J. D. Gross, G. Wagner, and J. E. G. McCarthy. 2004. The mRNA cap-binding protein eIF4E in post-transcriptional gene expression. *Nat Struct Mol Biol* 11:503.
198. Beckel-Mitchener, A. C., A. Miera, R. Keller, and N. I. Perrone-Bizzozero. 2002. Poly(A) Tail Length-dependent Stabilization of GAP-43 mRNA by the RNA-binding Protein HuD. *Journal of Biological Chemistry* 277:27996.
199. Ratti, A., C. Fallini, L. Cova, R. Fantozzi, C. Calzarossa, E. Zennaro, A. Pascale, A. Quattrone, and V. Silani. 2006. A role for the ELAV RNA-binding proteins in neural stem cells: stabilization of Msi1 mRNA. *J Cell Sci* 119:1442.
200. Wein, G., M. Rössler, R. Klug, and T. Herget. 2003. The 3'-UTR of the mRNA coding for the major protein kinase C substrate MARCKS contains a novel CU-rich element interacting with the mRNA stabilizing factors HuD and HuR. *European Journal of Biochemistry* 270:350.
201. Kiriakidou, M., G. S. Tan, S. Lamprinaki, M. De Planell-Saguer, P. T. Nelson, and Z. Mourelatos. 2007. An mRNA m7G Cap Binding-like Motif within Human Ago2 Represses Translation. *Cell* 129:1141.
202. Dostie, J., M. Ferraiuolo, A. Pause, S. A. Adam, and N. Sonenberg. 2000. A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5' cap-binding protein, eIF4E. *The EMBO Journal* 19:3142.
203. Pacheco, A., S. L. de Quinto, J. Ramajo, N. Fernández, and E. Martínez-Salas. 2009. A novel role for Gemin5 in mRNA translation. *Nucleic Acids Research* 37:582.
204. Tanaka, K. J., K. Ogawa, M. Takagi, N. Imamoto, K. Matsumoto, and M. Tsujimoto. 2006. RAP55, a cytoplasmic mRNP component, represses translation in *Xenopus* oocytes. *The Journal of Biological Chemistry* 281:40096.

205. Fenger-Grøn, M., C. Fillman, B. Norrild, and J. Lykke-Andersen. 2005. Multiple Processing Body Factors and the ARE Binding Protein TTP Activate mRNA Decapping. *Molecular Cell* 20:905.
206. Larance, M., A. F. Rowland, K. Hoehn, D. T. Humphreys, T. Preiss, M. Guilhaus, and D. E. James. 2010. Global phosphoproteomics identifies a major role for akt and 14-3-3 in regulating Edc3. *Molecular & Cellular Proteomics: MCP*.
207. Tritschler, F., J. E. Braun, A. Eulalio, V. Truffault, E. Izaurralde, and O. Weichenrieder. 2009. Structural basis for the mutually exclusive anchoring of P body components EDC3 and Tral to the DEAD box protein DDX6/Me31B. *Molecular Cell* 33:661.
208. Hsu, C. L., and A. Stevens. 1993. Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. *Molecular and Cellular Biology* 13:4826.
209. Minshall, N., M. Kress, D. Weil, and N. Standart. 2009. Role of p54 RNA helicase activity and its C-terminal domain in translational repression, P-body localization and assembly. *Molecular Biology of the Cell* 20:2464.
210. Bhattacharya, A., K. Czaplinski, P. Trifillis, F. He, A. Jacobson, and S. W. Peltz. 2000. Characterization of the biochemical properties of the human Upf1 gene product that is involved in nonsense-mediated mRNA decay. *RNA* 6:1226.
211. Weng, Y., K. Czaplinski, and S. W. Peltz. 1996. Genetic and biochemical characterization of mutations in the ATPase and helicase regions of the Upf1 protein. *Molecular and Cellular Biology* 16:5477.
212. Rand, T. A., S. Petersen, F. Du, and X. Wang. 2005. Argonaute2 Cleaves the Anti-Guide Strand of siRNA during RISC Activation. *Cell* 123:621.
213. Wang, B., S. Li, H. H. Qi, D. Chowdhury, Y. Shi, and C. D. Novina. 2009. Distinct passenger strand and mRNA cleavage activities of human Argonaute proteins. *Nat Struct Mol Biol* 16:1259.

214. Eberle, A. B., S. Lykke-Andersen, O. Muhlemann, and T. H. Jensen. 2009. SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nat Struct Mol Biol* 16:49.
215. Glavan, F., I. Behm-Ansmant, E. Izaurralde, and E. Conti. 2006. Structures of the PIN domains of SMG6 and SMG5 reveal a nuclease within the mRNA surveillance complex. *The EMBO Journal* 25:5117.
216. Garaigorta, U., and F. V. Chisari. 2009. Hepatitis C Virus Blocks Interferon Effector Function by Inducing Protein Kinase R Phosphorylation. *Cell Host & Microbe* 6:513.
217. Anderson P, T. Q. 1995. Fas-activated serine/threonine kinase (FAST) phosphorylates TIA-1 during Fas-mediated apoptosis. *The Journal of Experimental Medicine* 182:865.
218. Hanamoto, T., T. Ozaki, K. Furuya, M. Hosoda, S. Hayashi, M. Nakanishi, H. Yamamoto, H. Kikuchi, S. Todo, and A. Nakagawara. 2005. Identification of Protein Kinase A Catalytic Subunit β as a Novel Binding Partner of p73 and Regulation of p73 Function. *Journal of Biological Chemistry* 280:16665.
219. Klink, A., K. Schiebel, M. Winkelmann, and G. Rappold. 1995. The human protein kinase gene PKX1 on Xp22.3 displays Xp/Yp homology and is a site of chromosomal instability. *Hum Mol Genet.* 4:869.
220. Wada, K., and T. Kamitani. 2006. Autoantigen Ro52 is an E3 ubiquitin ligase. *Biochemical and Biophysical Research Communications* 339:415.
221. Bradrick, S. S., and M. Gromeier. 2009. Identification of Gemin5 as a Novel 7-Methylguanosine Cap-Binding Protein. *PloS One* 4.
222. Holmes, R. K., F. A. Koning, K. N. Bishop, and M. H. Malim. 2007. APOBEC3F Can Inhibit the Accumulation of HIV-1 Reverse Transcription Products in the Absence of Hypermutation. *Journal of Biological Chemistry* 282:2587.
223. Horke, S., K. Reumann, M. Schweizer, H. Will, and T. Heise. 2004. Nuclear Trafficking of La Protein Depends on a Newly Identified Nucleolar Localization Signal and the Ability to Bind RNA. *Journal of Biological Chemistry* 279:26563.

224. Courchet, J., K. Buchet-Poyau, A. Potemski, A. Brès, I. Jariel-Encontre, and M. Billaud. 2008. Interaction with 14-3-3 adaptors regulates the sorting of hMex-3B RNA-binding protein to distinct classes of RNA granules. *The Journal of Biological Chemistry* 283:32131.
225. Qin, Y., H. Zhao, E. Kovanci, J. L. Simpson, Z.-J. Chen, and A. Rajkovic. 2007. Mutation Analysis of NANOS3 in 80 Chinese and 88 Caucasian Women with Premature Ovarian Failure. *Fertility and sterility* 88:1465.
226. Jensen, L. J., M. Kuhn, M. Stark, S. Chaffron, C. Creevey, J. Muller, T. Doerks, P. Julien, A. Roth, M. Simonovic, P. Bork, and C. von Mering. 2009. STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Research* 37:D412-416.
227. Shannon, P., A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research* 13:2498.
228. Wu, J., T. Vallenius, K. Ovaska, J. Westermarck, T. P. Mäkelä, and S. Hautaniemi. 2009. Integrated network analysis platform for protein-protein interactions. *Nature Methods* 6:75.
229. Johnston, M., M.-C. Geoffroy, A. Sobala, R. Hay, and G. Hutvagner. 2010. HSP90 Protein Stabilizes Unloaded Argonaute Complexes and Microscopic P-bodies in Human Cells. *Molecular Biology of the Cell*.
230. Pare, J. M., N. Tahbaz, J. López-Orozco, P. LaPointe, P. Lasko, and T. C. Hobman. 2009. Hsp90 regulates the function of argonaute 2 and its recruitment to stress granules and P-bodies. *Molecular Biology of the Cell* 20:3273.
231. Jing, Q., S. Huang, S. Guth, T. Zarubin, A. Motoyama, J. Chen, F. Di Padova, S.-C. Lin, H. Gram, and J. Han. 2005. Involvement of MicroRNA in AU-Rich Element-Mediated mRNA Instability. *Cell* 120:623.

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Publications:

1. Chen, C-Y A, Zheng D, Xia, Z, Shyu, A-B., 2009. Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. *Nat Struct Mol Biol.* 16(11):1160-6.
2. Zheng D, Ezzeddine N, Chen CY, Zhu W, He X, Shyu AB., 2008. Deadenylation is prerequisite for P-body formation and mRNA decay in mammalian cells. *J Cell Biol.* 182(1):89-101.
3. Ezzeddine N, Chang TC, Zhu W, Yamashita A, Chen CY, Zhong Z, Yamashita Y, Zheng D, Shyu AB., 2007. Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. *Mol Cell Biol.* 27(22):7791-801.
4. Tu X, Wang J, Guo M, Zheng D, Teng M, Niu L, Liu Q, Huang Q, Hao Q., 2004. Purification, partial characterization, crystallization and preliminary X-ray diffraction of two cysteine-rich secretory proteins from *Naja atra* and *Trimeresurus stejnegeri* venoms. *Acta Crystallogr D Biol Crystallogr.* 60 (Pt6):1108-11.