

**Investigating the Components and Assembly of Processing Bodies in Human
Cells**

by

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Abstract

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Investigating the Components and Assembly of Processing Bodies in Human Cells

Thesis directed by Associate Professor Jens Lykke-Andersen

Messenger RNA degradation is important for the control of gene expression. The major mRNA decay pathway requires the coordination of proteins involved in deadenylation, decapping, and exonucleolysis to function properly. Interestingly, many of those proteins, as well as translationally repressed mRNAs, localize to discrete cytoplasmic foci called processing bodies (PBs). It remains unclear how PBs form and their functional significance is, as yet, unknown. To better understand how PB assembly may be regulated, I tested whether the cytoskeleton is required for PB dynamics in human cells. I found that the cytoskeleton is likely not required for overall PB assembly, integrity, or disassociation; moreover, disruption of the cytoskeleton does not inhibit mRNA decay efficiency. However, the localization of AU-rich element (ARE)-containing mRNAs in PBs was inhibited upon cytoskeleton disruption, which suggests a possible role for the cytoskeleton in transcript-specific delivery to PBs.

In an assay designed to identify novel PB factors, I found two proteins (PRMT5 and MEP50) that are known to be involved in splicing as a part of the methylosome complex, to co-purify with PB proteins. PRMT5 is a methyltransferase that has an affinity for methylating arginine residues within GRG-tripeptide repeats. Interestingly, the PB protein Lsm4 contains a large GRG-repeat domain in its C-terminus. I confirmed that the Lsm4 C-terminus is methylated and wished to determine if this methylation was

important for Lsm4 function in PBs and mRNA decay. Unlike yeast Lsm4, I found that the C-terminus of Lsm4 in humans is neither necessary, nor sufficient, to form PBs. Knockdown of Lsm4 revealed it is important for efficient mRNA decay; however, I found that this is not dependent on the GRG-rich c-terminal domain. Taken together, these studies add to our understanding of PBs assembly and mRNA decay in human cells.

Dedication

This thesis is dedicated to my Mom and Dad and to my husband Nathan. I did it!

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Chapter 1
Background and Significance

1.1 Degradation of mRNA and the regulation of gene expression

From DNA to RNA to protein, there are many regulatory steps along the way that are necessary for the control of gene expression. Cells must maintain appropriate protein levels and ensure each is accurately translated at the right time and place in order to function properly. Just as the strict control of DNA replication, transcription, and translation help to ensure this, the regulation of mRNA degradation is equally crucial for proper cell function. The ability to control mRNA stability and degradation allows for a cell to quickly change protein synthesis rates to adapt to its needs.

Depending on the state of the cell and the protein it encodes, mRNA half-lives can vary from transcript to transcript. Proteins that must be constitutively present, such as β -globin in globin-producing cells, have mRNAs that are more stable and have half-lives of greater than 20 hours in human cells. By contrast, proteins that must be tightly regulated and whose concentration changes rapidly, such as cytokines and oncogenes, are encoded by mRNAs that are less stable and have half-lives as short as 10 minutes (Hollams et al., 2002). The importance of mRNA degradation for proper cell function has become more apparent through recent discoveries linking aberrant control of mRNA degradation to several human diseases including cancer, arthritis and Alzheimer's disease (Hollams et al., 2002; Khabar, 2010).

Cis and trans elements control mRNA transcript stability

Many factors can influence the stability of an mRNA transcript. Mature eukaryotic mRNAs contain several intrinsic factors that protect it from degradation. Pre-mRNA processing steps in the nucleus provide a 7-methyl guanosine (m^7G) cap on the 5' end

and a 3' poly-A tail. The 5' cap enhances translation efficiency and is bound by the translation initiation factor eIF4e. The cap effectively blocks 5'-3' exonucleases, which target monophosphate 5' ends (Stevens & Maupin, 1987). The poly-A tail is a string of adenosines, usually 70-250 nt in length in human cells, which is bound by poly-A binding protein (PABP). It prevents 3'-5' exonucleolysis, as well as decapping (Coller & Parker, 2004; Hollams et al., 2002; Stevens & Maupin, 1987).

1.2 Pathways leading to mRNA decay

AU-rich element mediated decay

Certain transcripts also contain internal sequence elements that control stability. The most widely studied sequence element is the AU-rich element (ARE), found in the 3' untranslated region (UTR) of as many as 5-8% of human genes (Bakheet et al., 2003). AREs are commonly seen in mRNAs encoding cytokines, proto-oncogenes, and growth factors, which are usually short-lived transcripts whose translation needs to be tightly regulated. The ARE was first observed as a conserved sequence element found in many cytokine genes (Caput et al., 1986) and was later proven to be responsible for causing mRNA instability after the ARE from the granulocyte macrophage colony stimulating factor (GM-CSF) was inserted into the 3' UTR of β -globin and caused the otherwise stable β -globin mRNA to become unstable (Shaw & Kamen, 1986). AREs are variable in length and sequence, but generally contain numerous repeats of the pentamer AUUUA and can be divided into three classes. Class I AREs contain 1-3 separate copies of the AUUUA pentamer, which are usually in close proximity to a U-rich region. Transcripts containing Class I AREs, such as *c-fos* mRNA, undergo

synchronous, nonprocessive deadenylation. Class II AREs are defined as having at least two overlapping AUUUA pentamers within a U-rich region. The GM-CSF mRNA is an example of a Class II ARE, and they undergoes asynchronous, processive deadenylation. Class III AREs, such as found in *c-jun* mRNA, do not contain the AUUUA pentamer and instead consist only of a U-rich region, and undergo synchronous deadenylation (Chen & Shyu, 1995; Hollams et al., 2002).

Regulation of mRNA decay by AREs occurs through proteins that specifically bind the AU-rich sequence. ARE-binding proteins known to regulate mRNA stability include HuR, AUF1, BRF1, and BRF2. The most well studied ARE-binding protein is Tristetraprolin (TTP) which has been shown to bind the AREs in TNF- α , GM-CSF, IL-2, IL-3, and *c-fos* mRNAs (Carballo et al., 1998; Lai & Blackshear, 2001; Raghavan et al., 2001; Stoecklin et al., 2000) and lead to decay of those transcripts. Recent studies have helped in understanding how TTP causes rapid mRNA decay by finding that TTP interacts with several mRNA decay factors (Chen et al., 1995; Lykke-Andersen & Wagner, 2005) and is responsible for forming an ARE-mRNP that assembles with other repressed mRNPs in cytoplasmic granules (Franks & Lykke-Andersen, 2007).

Nonsense mediated decay

Some mRNA decay pathways serve in quality control by monitoring for mutated or aberrant transcripts, the most well studied of which is nonsense mediated decay (NMD). Transcripts that contain a premature termination codon (PTC) upstream of the normal stop codon can lead to truncated proteins and be potentially harmful to the cell. The NMD pathway recognizes these aberrant transcripts and signals for their rapid decay.

The link between PTCs and mRNA instability was first demonstrated using the *URA 3* gene in *S. cerevisiae* (Losson & Lacroute, 1979). Most PTCs arise from alternative splicing or from transcription errors. Some pathways exploit NMD as a means to eliminate nonsense transcripts created by programmed DNA arrangement, such as T-cell receptors and immunoglobulin genes. They use this to generate a wide variety of antigen receptors and it is estimated that two thirds of the transcripts contain PTCs (Chen et al., 1995).

Many of the proteins involved in triggering NMD are known, including Upf1, the core NMD protein, which binds eukaryotic release factors eRF1 and eRF3 at the PTC. Upf1 also recruits Upf2 and Upf3 and interact with Smg1, Smg5, Smg6, and Smg7 proteins to initiate decay. How PTCs are distinguished from normal termination codons is poorly understood. When ribosomes encounter a PTC, it is thought that the physical distance from the normal 3' UTR triggers NMD through a separation from proteins downstream that normally interact with translation termination machinery, including PABP, which allows the activation of Upf1 and signal for the degradation of the transcript (Chang et al., 2007; Mühlemann et al., 2008; Rebbapragada & Lykke-Andersen, 2009).

mRNA degradation by small RNAs

The miRNA (microRNA) and siRNA (small interfering RNA) pathways use small noncoding RNAs to bind target sequences, usually located in the 3'UTR of an mRNA, and cause silencing or degradation of that transcript. miRNAs originate from endogenously expressed genes with transcription of the pri-mRNA, which forms a stem-loop structure (usually ~75 bp in length) flanked by the rest of the transcript. The stem

loop is excised from the transcript by the enzyme Drosha to form the pre-miRNA. In mammals, this pre-miRNA is exported to the cytoplasm where the loop is excised by the enzyme Dicer. Although first thought to only originate from exogenous sources such as viruses, siRNAs have recently been discovered to occur endogenously as well, and originate from long double stranded RNAs targeted by Dicer. The cleaved miRNA or siRNA duplexes are unwound by Argonaute proteins into single strands and, together with GW182 and other effector proteins, form into the RNA induced silencing complex (RISC). The targets of miRNAs are usually recognized by base-pairing with the 5' end of the miRNA, through the so-called seed sequence, followed by imperfect base-pairing. Depending on the degree of complementarity, transcripts are then either translationally repressed and/or cleaved and degraded. siRNA targets display perfect base pairing, which leads to cleavage of the transcript (Carthew & Sontheimer, 2009; Ghildiyal & Zamore, 2009).

1.3 Mechanisms and machinery of mRNA decay

Deadenylation-dependent decay

Once an mRNA is targeted for decay, there are several mechanisms by which the transcript can be degraded. The major mRNA decay pathway in eukaryotes is deadenylation dependent, and as such begins with removal of the poly-A tail at the 3' end (Figure 1-1). Next, the transcript undergoes decapping to remove m⁷GDP from the 5' end. After the cap is removed, the transcript is then vulnerable to 5'-3' exonucleolytic degradation (Meyer et al., 2004; Parker & Song, 2004; Wilusz & Wilusz, 2004). Pulse-chase experiments in yeast showed that immunoprecipitation of capped mRNAs in

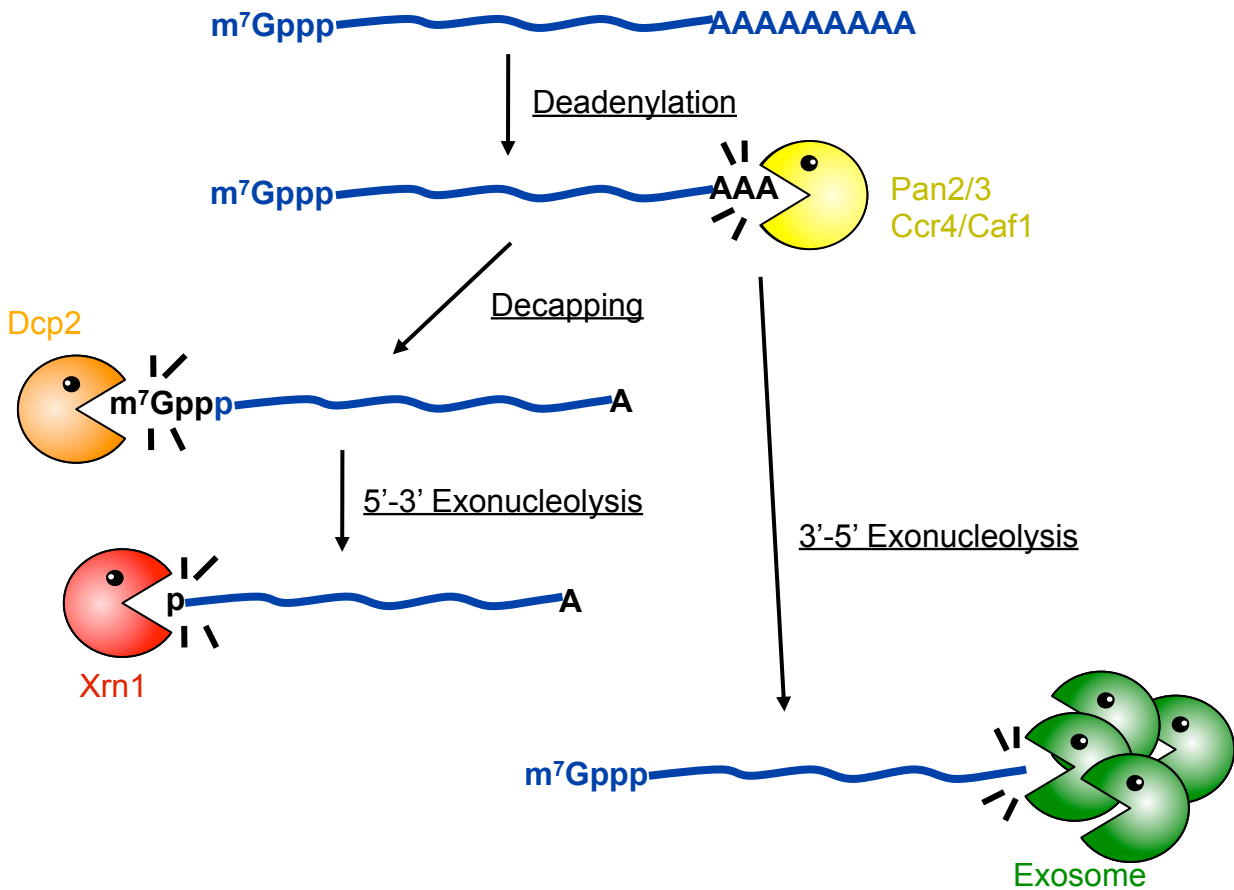


Figure 1-1. The major mRNA degradation pathway

When a transcript is targeted to degrade in the major mRNA degradation pathway, the first step is removal of the poly-A tail by a complex of deadenylases. Next, the transcript can either be degraded in the 3'-5' direction by the exosome, or it is decapped by Dcp2. Following decapping the transcript is vulnerable to 5'-3' exonucleolysis by Xrn1.

strains lacking the 5'-3' exonuclease Xrn1 yielded transcripts with shortened poly-A tails of varying length, and over time results in the stabilization of full length deadenylated and decapped transcripts (Muhlrad et al., 1994) providing evidence that decay proceeds in the 5'-3' direction and requires poly-A tail shortening followed by decapping. A secondary route in this pathway involves degradation in the 3'-5' direction once the poly-A tail is removed. How mRNAs are directed to one pathway or another remains unclear, but they are crucial to cell function as inhibition of both 3'-5' and 5'-3' decay is lethal in yeast (Anderson & Parker, 1998). Interestingly, knocking out either 5'-3' or 3'-5' pathways resulted in no significant change in transcriptome levels, indicating redundancy may exist between the two (He et al., 2003; Houalla et al., 2006).

There are many factors involved in the coordination of each step in mRNA decay, most of which are largely conserved in eukaryotes. Deadenylation, the first and rate-limiting step, is accomplished through the catalytic activity of Ccr4 and Caf1, who along with nine other effector proteins form the Ccr4-Not complex. Other deadenylating enzymes shown to participate in poly-A removal include the PAN2-PAN3 complex. In mammals, PAN2 and PAN3 have been shown to initiate trimming of poly-A tails to ~80 nt, at which point the Ccr4-Not complex takes over to complete deadenylation (Garneau et al., 2007; Yamashita et al., 2005). A third vertebrate-specific major deadenylase is PARN, whose activity is cap-dependent and deadenylates ARE-containing mRNAs through TTP (Lai et al., 2003) and can deadenylate NMD substrates and interact with Upf proteins (Lejeune et al., 2003)

Removal of the 5' cap occurs through the coordination of proteins that catalyze and stimulate decapping. Decapping is catalyzed by the enzyme Dcp2 and, together

with the co-activator Dcp1, forms the major decapping complex in eukaryotes. The decapping reaction irreversibly removes the m⁷GDP from the mRNA and produces a 5' monophosphate which can then be targeted for 5'-3' degradation (Lykke-Andersen, 2002; Parker & Song, 2004; Stevens & Maupin, 1987). DcpS is another decapping enzyme that primarily catalyzes the removal of caps from mRNAs that undergo exosome-mediated 3'-5' exonucleolysis following deadenylation (Wang & Kiledjian, 2001).

Proteins that stimulate decapping include the Lsm1-7 and Pat1 proteins. Lsm1-7 is a 7-member ring that has RNA-binding properties (Khusial et al., 2005). Studies have shown that Lsm1-7 interacts in an RNA-dependent manner with decapping proteins and inhibiting the Lsm1-7 complex results in the accumulation of capped, deadenylated mRNAs, arguing that this complex is important for initiating decapping (Bouveret et al., 2000; Tharun et al., 2000). Pat1 plays a role in translational repression in yeast (Coller & Parker, 2005) and directly interacts with Lsm1-7 to form the Lsm-Pat complex, which preferentially binds to mRNA at the 3' end after deadenylation and protects the 3' end from further 3'-5' exonucleolytic degradation (Bouveret et al., 2000; He & Parker, 2001). Proteins known as enhancers of decapping (Edc) have been found to interact with the decapping machinery and further stimulate decapping. Human enhancers of decapping include Edc3, Hedls (Edc4), and Rck/p54 (homologue of yeast Dhh1) (Fenger-Grøn et al., 2005). Although they are not required for decapping to occur, they interact with the decapping proteins and their presence enhances decapping activity (Coller et al., 2001; Fenger-Grøn et al., 2005; Kshirsagar & Parker, 2004).

The final step in decay is exonucleolysis. The primary cytoplasmic 5'-3'

exonucleolytic enzyme in eukaryotes is Xrn1. Its enzymatic activity is effectively blocked by the cap. Decapping of an mRNA leaves a 5' monophosphate, which is a substrate for Xrn1 (Stevens & Maupin, 1987). In yeast, cells lacking Xrn1 accumulate full-length mRNAs that lack a cap, demonstrating its importance in mRNA degradation (Hsu & Stevens, 1993; Muhlrاد et al., 1994). A homologue of Xrn1, Xrn2, also displays 5'-3' exonucleolytic activity, but is believed to primarily localize to the nucleus where it plays a role in nuclear mRNA processing (Bousquet-Antonelli et al., 2000). The exosome in humans is a 6-subunit ring complex. One subunit, Rrp44, has been shown to display 3'-5' exonuclease activity, as well as endonuclease activity. It degrades substrates in the 3'-5' direction and has been shown to interact with the scavenger decapping enzyme DcpS (Wang & Kiledjian, 2001).

Deadenylation-independent decay and endonucleolytic cleavage

While not a widely observed phenomenon, some mRNAs undergo deadenylation-independent decay. In yeast, the RPS28B and EDC1 mRNAs have both been observed to undergo decapping in the absence of deadenylation. When translation of the deadenylase Ccr4 is repressed, RPS28B mRNA retains its poly-A tail, yet is observed to still undergo efficient degradation and is mediated by Edc3 through an unknown mechanism (Badis et al., 2004). In the case of EDC1 mRNA, when transcripts were blocked from undergoing 5'-3' exonucleolysis, this resulted in decay intermediates that had been decapped, yet retained long poly-A tails (Muhlrاد & Parker, 2005).

Endonucleolytic cleavage is an efficient way to degrade mRNAs as it results in two mRNA fragments, one that can be degraded in the 3'-5' direction, and another that is

degraded from 5'-3'. Transcripts targeted by miRNA or siRNA, when perfectly base-paired, are endonucleolytically cleaved by the Argonaute 2 protein (Liu et al., 2004). Additionally, mRNAs containing a PTC can be cleaved and degraded through the endonucleolytic activity of the NMD factor Smg6 (Eberle et al., 2009).

1.4 Processing bodies and mRNA metabolism

mRNA decay proteins concentrate in cytoplasmic foci

It has been observed that many of the enzymes, effector proteins, and mRNAs involved in mRNA decay localize to discrete cytoplasmic foci called processing bodies (PBs). PBs were first observed over a decade ago when indirect immunofluorescence of the exonuclease Xrn1 revealed it localized to distinct foci in the cytoplasm of mouse fibroblast cells (Bashkirov et al., 1997). Since then, most of the other proteins involved in general mRNA decay have also been found to co-localize to PBs. These factors are summarized in Table 1-1 and include the deadenylases Ccr4, Caf1, PAN2, PAN3, the decapping proteins Dcp1 and Dcp2, and the decapping effector proteins Lsm1-7, Pat1, Edc3, Edc4, and Rck/p54 (for review see Franks & Lykke-Andersen, 2008; Kulkarni et al., 2010; Parker & Sheth, 2007). In addition to general decay machinery factors, proteins involved in specific mRNA decay pathways are also found in PBs. The ARE-binding protein TTP as well as ARE-containing mRNAs (Franks & Lykke-Andersen, 2007; Kedersha et al., 2005), along with NMD factors Upf1, Upf2, Upf3, Smg5, Smg6, and Smg7 and PTC-containing transcripts (Durand et al., 2007; Sheth & Parker, 2006; Unterholzner & Izaurralde, 2004), and the miRNA-associated Argonaute (Ago) and

PB Component	Function
Ago1-4	miRNA associated proteins, subunits of RISC complex, endonucleases
Caf1	deadenylase, 3'-5' exonuclease
Ccr4	deadenylase, 3'-5' exonuclease
Dcp1	enhances decapping
Dcp2	decapping enzyme
Edc3	enhances decapping
Edc4/Hedls/Ge-1	enhances decapping, no known yeast homolog
GW-182	involved in miRNA pathway
Lsm 1-7	enhances decapping, binds RNA
Pan2, Pan3	deadenylases, 3'-5' exonucleases
Pat1	enhances decapping, represses translation in yeast
Rck/p54/DDX6	Dhh1 in yeast, enhances decapping, DEAD-box RNA helicase
Smg5, Smg6, Smg7	activates NMD
TTP	binds AREs and activates ARE-mediated decay
Upf1	core NMD protein, activates NMD, RNA helicase, ATP hydrolysis
Upf2, Upf3	activates NMD
Xrn1	5'-3' exonuclease
mRNA	ARE-containing mRNAs, PTC-containing mRNAs, miRNA target mRNAs

Table 1-1. Summary of processing body components and their function

Human processing body components are listed on the left with their function described on the right. All components have a homolog in yeast unless otherwise noted.

GW182 proteins and miRNA-target mRNAs (Eystathioy et al., 2003; Liu et al., 2005), can all be found concentrated within PBs.

PBs require mRNA to form and consist of translationally repressed mRNPs associated with mRNA decay and translation repression factors. This was demonstrated in studies that found mRNA decay intermediates accumulated in PBs when the decay pathway was inhibited (Cougot et al., 2004; Sheth & Parker, 2003). Additionally, altering the amount of available ribosome-free mRNA causes changes to the appearance of PBs. When the amount of non-translating mRNA is limited by the treatment of cells with cycloheximide, which locks mRNAs into polysomes, PBs disappear. In contrast, PBs grow larger when the amount of ribosome-free mRNA is increased after treating cells with puromycin, which releases mRNAs from polysomes (Cougot et al., 2004; Teixeira et al., 2005; Wilczynska et al., 2005). Finally, inhibiting the decapping enzyme Dcp2 or the exonuclease Xrn1 also results in an increase in PB size (Cougot et al., 2004; Fenger-Grøn et al., 2005; Franks & Lykke-Andersen, 2007; Sheth & Parker, 2003).

PBs are highly dynamic structures, as demonstrated by their increase and decrease in size in response to the amount of available mRNA substrates described above. Further illustration of their dynamic nature is seen in fluorescence recovery after photobleaching (FRAP) experiments showing that many proteins can rapidly cycle in and out of PBs (Andrei et al., 2005; Kedersha et al., 2005). Additionally, mRNAs are seen to exit and then re-enter the pool of translating mRNAs by moving between polysomes and PBs (Bregues et al., 2005).

These observations raise many questions as to what causes the formation and persistence of PBs in cells, and how the ongoing flux of mRNAs and proteins in PBs is

coordinated. Importantly, the functional significance of PBs remains an active area of research and debate. Degradation of mRNA likely occurs in these foci, yet when PBs are eliminated from cells, there is no noticeable effects on the rates of decay (Decker et al., 2007; Eulalio et al., 2007). One current model for the function of PBs suggests that they may simply exist help to enhance kinetics. By concentrating mRNA decay machinery into foci, it encourages more efficient degradation; especially in cases where mRNA substrates are in excess compared to decay enzymes (Franks & Lykke-Andersen, 2008).

Scope of thesis

The work presented here aims to add to our understanding of how PBs function in human cells within the context of mRNA decay. In chapter 2, I explore how the cytoskeleton may be involved in PB assembly, integrity and disassembly and find that PB dynamics occur independently of the cytoskeleton. Next, chapter 3 discusses my search for novel PB proteins and I find that proteins from the methylosome complex interact with PB proteins. Finally, in chapter 4, I present work investigating the c-terminal domain of human Lsm4 and it's rols in mRNA decay and PB formation. Surprisingly, I find that Lsm4 in humans likely plays a different role than that of yeast Lsm4 in that it's C-terminus is neither necessary nor sufficient for PB formation.

Chapter 2

The Cytoskeleton and its Role in Processing Body Dynamics

2.1 Introduction

The cytoskeleton is a network of protein filaments that act as a cellular scaffolding system, enable cell motility, and serve as a means to transport organelles, proteins, and mRNA to specific locations throughout the cell. The two main types of cytoskeletal networks are actin filaments and microtubules; each of which is formed from protein subunits that polymerize into filaments. Actin filaments are formed from actin monomers (G-actin), which polymerize to form filamentous actin (F-actin). F-actin is organized into two-stranded helices, that can then form into a variety of bundled structures. Actin filaments are polarized and contain a rapidly growing “plus” end and a slow growing “minus” end. Microtubules are formed from tubulin, a heterodimer consisting of α -tubulin and β -tubulin, which polymerize to form a protofilament. Like actin filaments, microtubule protofilaments are polarized and have a “plus” and “minus” end. A fully formed microtubule is a rigid, hollow cylinder assembled from 13 protofilaments, and is generally wider, longer, and less flexible than an actin filament (Alberts et al., 2002).

In many organisms, the cytoskeleton has been implicated in the transport and localization of material within the cell. The correct localization of certain mRNAs and protein is often critical for proper cell function. For example, in *S. cerevisiae*, the localization of ASH1 mRNA to the bud tip is dependent on actin and the molecular motor Myo4 (Long et al., 1997). In *Drosophila*, correct formation and patterning of the anterior portion of embryos is determined by the localization of Bicoid mRNAs to the anterior pole by microtubules (Theurkauf & Hazelrigg, 1998). Finally, in mammalian cells, β -actin mRNA has been found to localize to the leading edge of motile fibroblasts and this localization is dependent on actin filaments (Latham et al., 2001).

Processing bodies (PBs) are discrete cytoplasmic foci where many mRNA decay enzymes, effector proteins, and mRNAs targeted for decay co-localize. The proteins and mRNAs within PBs have also been observed to exhibit dynamic properties. The mechanism for how components become localized to PBs remains unclear. Given that the control of mRNA degradation is a highly regulated process, it would make sense for the cell to also regulate the localization of mRNA decay factors. Here, I examine a possible role for actin filaments or microtubules in PB assembly, disassembly, and integrity and also ask whether the rate of mRNA decay depends on an intact cytoskeleton. I found that PB dynamics and efficient mRNA decay is likely controlled independently of the cytoskeleton. However, ARE mRNAs may depend on the cytoskeleton for localization to PBs.

2.2 Results

Processing bodies retain their integrity after cytoskeleton disruption

It has been reported that some PB proteins are dynamic and can rapidly shuttle in and out of PBs (Andrei et al., 2005; Kedersha et al., 2005). Despite this shuttling, PBs retain their shape and remain microscopically visible. One possible role for the cytoskeleton may be to ensure that the proteins and mRNA found in PBs retain their localization within the foci, possibly by the anchoring of non-shuttling proteins to the cytoskeleton. To determine whether PB integrity is dependent on the cytoskeleton, human HeLa cells were treated with cytoskeleton disrupting drugs and then PBs were visualized with anti-hDcp1a and anti-hXrn1 antibodies. In Figure 2-1A, cells were incubated for 1 hour with or without the actin-destabilizing drug cytochalasin D. Cytochalasin D is a fungal toxin

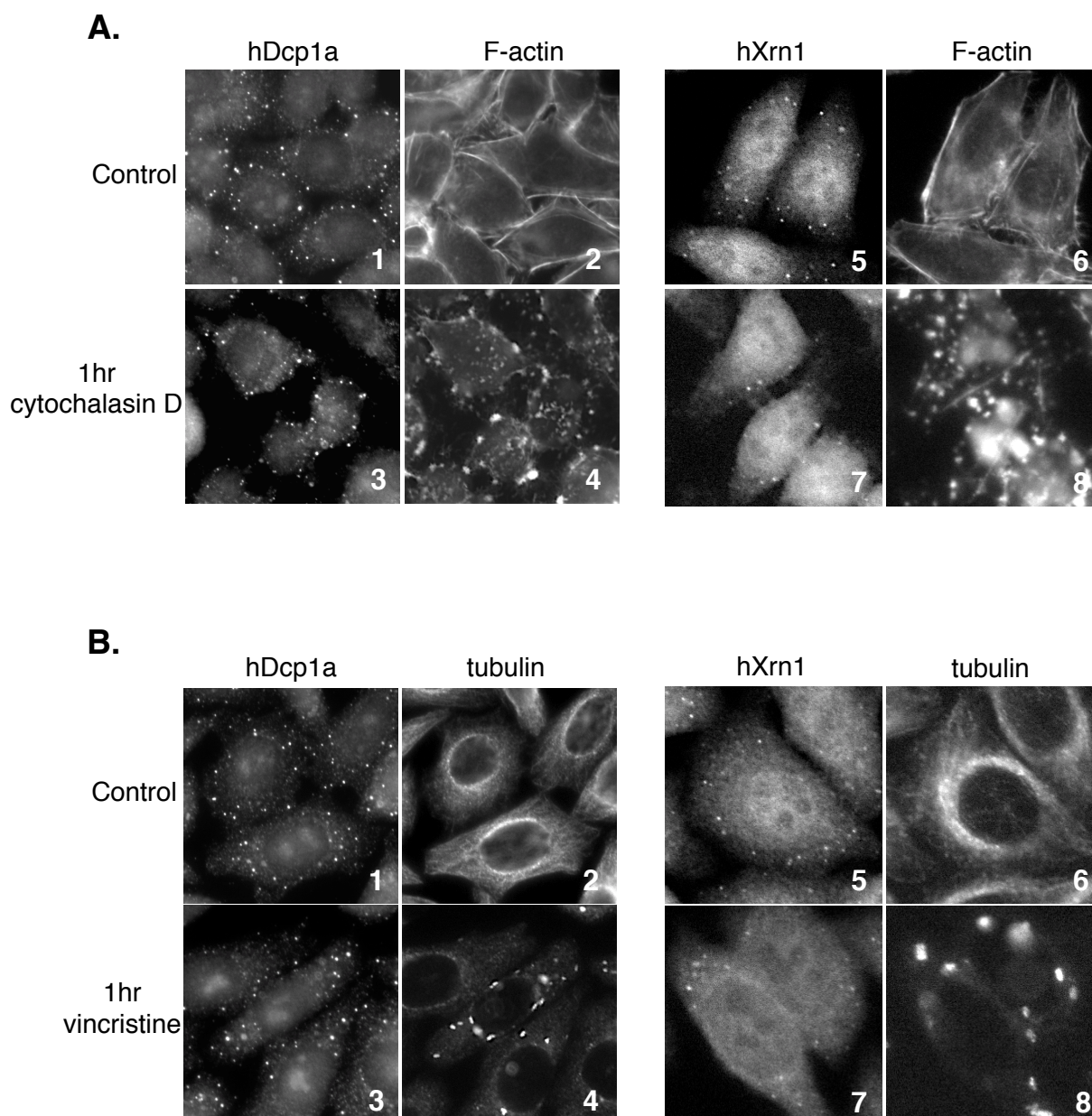


Figure 2-1. Processing bodies retain their integrity after cytoskeleton disruption

An indirect immunofluorescence assay to visualize PBs upon disruption of the cytoskeleton. PBs were visualized with anti-hDcp1a antibodies (panels 1, 3) or anti-hXrn1 antibodies (panels 5, 7). (A) Human HeLa cells were left untreated (panels 1, 2, 5, 7) or treated (panels 3, 4, 7, 8) with cytochalasin D for 1 hour. Actin filaments were visualized with fluorescent phalloidin (panels 2, 4, 6, 8) (B) Cells were left untreated (panels 1, 2, 5, 6) or treated (panels 3, 4, 7, 8) with vincristine for 1 hour. Microtubules were visualized with anti- β -tubulin antibodies (panels 2, 4, 6, 8).

that acts by binding the “plus” end of actin filaments, preventing polymerization and disrupting the actin network organization. In panels 3 and 7, PBs are still clearly visible even after disruption of actin filaments (compare with untreated cells in panels 1 and 5). The disruption of actin filaments by cytochalasin D was confirmed through co-staining with fluorescent phalloidin, a molecule which binds F-actin. Actin filament disruption is visible in panels 4 and 8.

I next tested the effect of depleting microtubules. In Figure 2-1B, cells were incubated for 1 hour with the microtubule disrupting drug vincristine. Vincristine is a plant toxin that binds tubulin monomers and inhibits microtubule polymerization, thereby disrupting the microtubule network. Disruption of microtubules by vincristine was confirmed by staining with anti- β -tubulin antibodies, as seen in panels 4 and 8. After treatment with vincristine, no visible effects on PBs were observed, as can be seen by the presence of PBs in panels 3 and 7. Treatment of cells with either cytochalasin D or vincristine produced no noticeable effect on the number, size or shape of PBs when compared to control conditions (Figure 2-1A/B, panels 1 and 5). Thus, an intact cytoskeleton is not necessary for maintaining PB integrity. However, from these experiments it cannot be ruled out that PB components fail to disassociate from PBs in the absence of the cytoskeleton. The role of the cytoskeleton in PB disassociation is examined later in this section.

Processing body nucleation occurs independent of the cytoskeleton

The next step in determining a possible role for the cytoskeleton in PB dynamics was to test whether nucleation of microscopically visible PBs was dependent on the

cytoskeleton. I tested this by first eliminating PBs from cells so that their initial formation could be observed. To do this, HeLa cells were treated with the translation inhibitor cycloheximide, which acts through blocking the elongation phase of eukaryotic translation by binding the ribosome and inhibiting eEF2-mediated translocation, trapping mRNAs in polysomes (Obrig et al., 1971). Cycloheximide has been previously shown to eliminate PBs (Cougot et al., 2004; Teixeira et al., 2005; Wilczynska et al., 2005). The role of cytoskeleton components in the formation of PBs was subsequently monitored after washing out cycloheximide in cells treated with cytoskeleton inhibitors. First, I tested the role of actin filaments on PB formation and the results are shown in Figure 2-2A. PBs were visualized with hDcp1a antibodies and hXrn1 antibodies (data not shown). Cells were first incubated with cycloheximide for 30 minutes, which resulted in the complete disappearance of PBs from cells (panel 3). Next, cells were briefly washed, and then incubated with cytochalasin D in the absence of cycloheximide for 1 hour to disrupt actin filaments and reactivate translation. In panel 5 it is clearly seen that PBs are present, and were therefore able to re-form in the absence of a functioning actin filament network. Similarly, I tested whether microtubules contributed to PB formation. Figure 2-2B shows that when PBs are eliminated with cycloheximide (panel 3), washed, and then incubated for 1 hour with vincristine in the absence of cycloheximide, PBs are still able to form (Figure 2-2B, panel 5). Interestingly, in this experiment the number of PBs seems to be increased upon cytoskeleton disruption; however, this result was not reproducible. Thus, neither the disruption of actin filaments, nor microtubules, hindered the ability of PBs to form into microscopically visible foci. It is possible however, that after cycloheximide was removed, PBs were able to reform

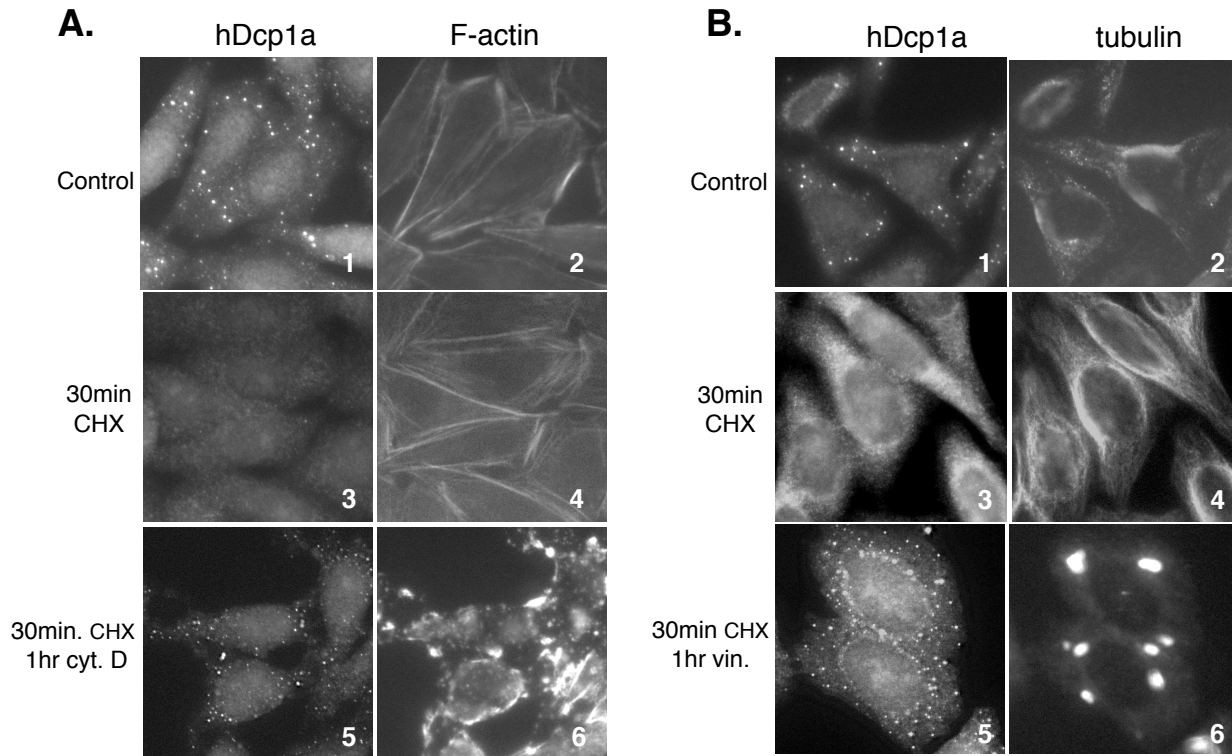


Figure 2-2. Processing body nucleation occurs independent of the cytoskeleton
 Indirect immunofluorescence assays showing that PB formation occurs in the absence of an intact cytoskeleton. PBs were visualized with anti-hDcp1a antibodies. (A) Cells treated with cytochalasin D to disrupt actin are able to recover PBs (panel 5) after cycloheximide-induced PB disassociation (panel 3). Disruption of actin filaments was visualized with fluorescent phalloidin. (B) Cells treated with vincristine to disrupt microtubules are able to recover PBs (panel 5) after disruption with cycloheximide (panel 3). Microtubule disruption was confirmed with anti- β -tubulin antibodies.

during the time before cytochalasin D or vincristine were able to fully disrupt the cytoskeleton. In future experiments, drugs should be added to disrupt the cytoskeleton before cycloheximide is removed to eliminate this possibility.

Disassociation of processing bodies can occur without the cytoskeleton

Processing bodies disappear when translation is inhibited, as seen with cycloheximide, and during mitosis (Cougot et al., 2004). The mechanism for PB disassociation may rely on the cytoskeleton to shuttle proteins and mRNA away from foci. I tested this by asking if PBs in cells with a disrupted cytoskeleton could disassociate normally upon treatment with cycloheximide. Human HeLa cells were incubated with cytochalasin D to impair actin filaments and after 1 hour, cycloheximide was added (Figure 2-3A). If actin filaments are required for the dissolution of PBs, it would be expected to see PBs remain visible after the incubation with cycloheximide. After staining for PBs with anti-hDcp1a antibodies and Xrn1 antibodies (data not shown), I observed that PB were able to dissolve under these conditions (panel 3), despite the loss of the actin cytoskeleton. I was next interested in testing whether PBs were dependent on microtubules for their disassociation. As shown in Figure 2-3B, cells were first incubated with vincristine for 1 hour to cause disruption of microtubules. Then cycloheximide was added for 1 hour in the continued presence of vincristine and cells were monitored for the presence or absence of PBs, as indicated by hDcp1a staining. It was observed that PBs were also able to disassociate without an intact microtubule cytoskeleton (panel 3). As with the integrity and formation of PBs, the data here suggests that cycloheximide-induced dissociation of PBs occurs independently of both

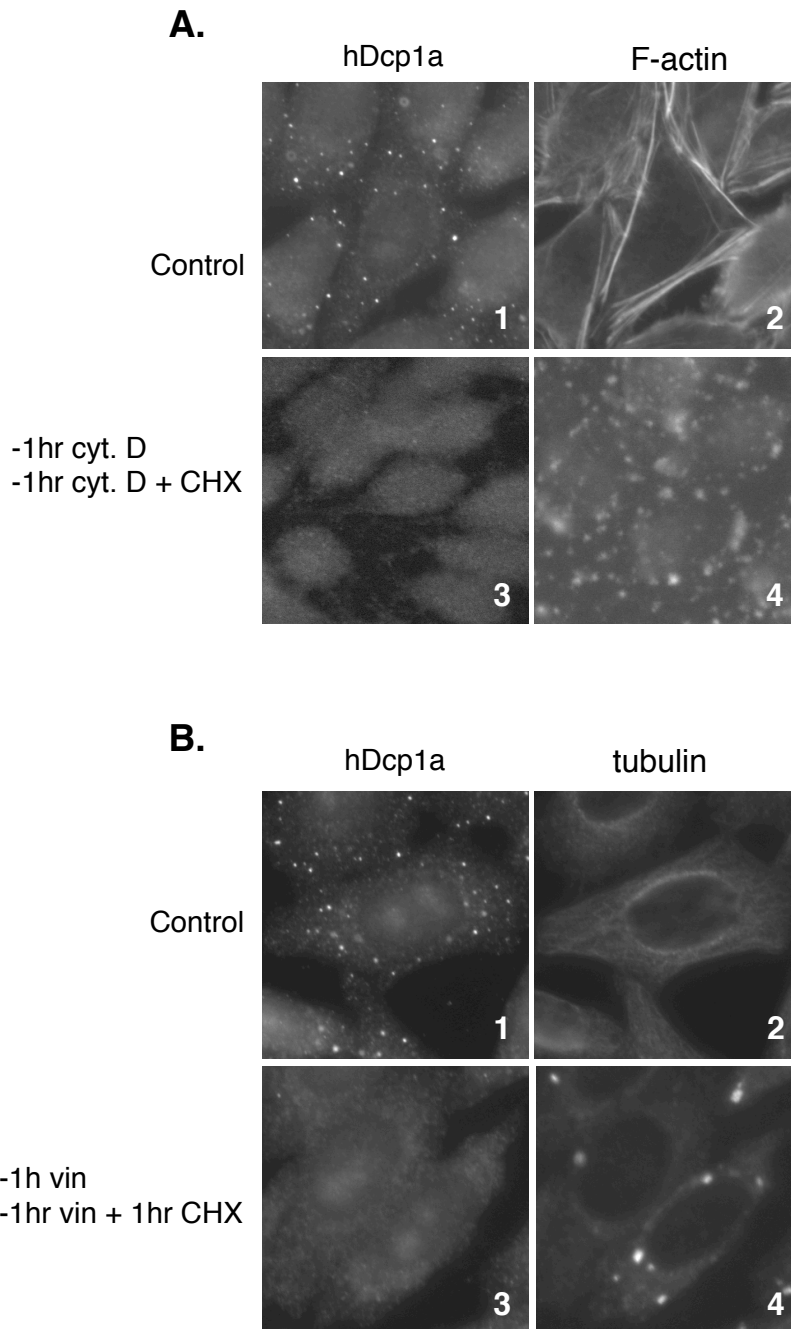


Figure 2-3. Processing bodies can disassociate after cytoskeleton disruption

An immunofluorescence assay visualizing PB disappearance with cycloheximide in the absence of a functioning cytoskeleton. PBs were visualized with anti-hDcp1a antibodies. (A) Actin filaments were disrupted by incubation with cytochalasin D. After 1 hour, cycloheximide was added to induce PB disassociation (panel 3). (B) Microtubules were disrupted by incubation with vincristine. After 1 hour cycloheximide was added to induce PB disassembly (panel 3). Actin filaments were visualized with fluorescence-conjugated phalloidin, and microtubules were visualized with anti- β -tubulin antibodies.

actin filaments and microtubules. Cytoskeleton disruption may cause a delay in PB disassociation that could not be detected when visualizing PBs 1 hour after cycloheximide treatment. It would be necessary in future experiments to observe the appearance of PBs at several time points within that hour after cycloheximide treatment to monitor for this possibility.

Disruption of actin or tubulin causes mislocalization of ARE mRNA

PB formation has been shown to be dependent on RNA, and PBs contain non-translating mRNAs (Teixeira et al., 2005). Our lab has shown that mRNAs containing an ARE in their 3'UTR concentrate in PBs (Franks & Lykke-Andersen, 2007).

As discussed previously, several mRNAs have been shown to require the cytoskeleton for directed localization to specific sites within a cell (Latham et al., 2001; Long et al., 1997; Theurkauf & Hazelrigg, 1998). I assessed whether ARE-containing mRNAs were dependent on the cytoskeleton for their localization to PBs. To test this, a fluorescence in-situ hybridization (FISH) assay was performed in cells transiently transfected with a β -globin reporter mRNA containing the AU-rich element from the 3'-UTR of GMCSF (β -GMCSF). The transcription of this reporter mRNA is controlled by tetracycline.

Transcription is induced by removing tetracycline, which allows for the activation of a tetracycline-repressible activator protein (see Material and Methods). hDcp1a fused to green fluorescent protein (GFP) was co-transfected as a PB marker. Under normal conditions, β -GMCSF is observed to localize to foci (Figure 2-4A, panel 2), which co-localize to PBs in 88% of cells (panel 3). In addition, nuclear ARE-mRNA foci are observed, likely corresponding to sites of transcription (Franks and Lykke-

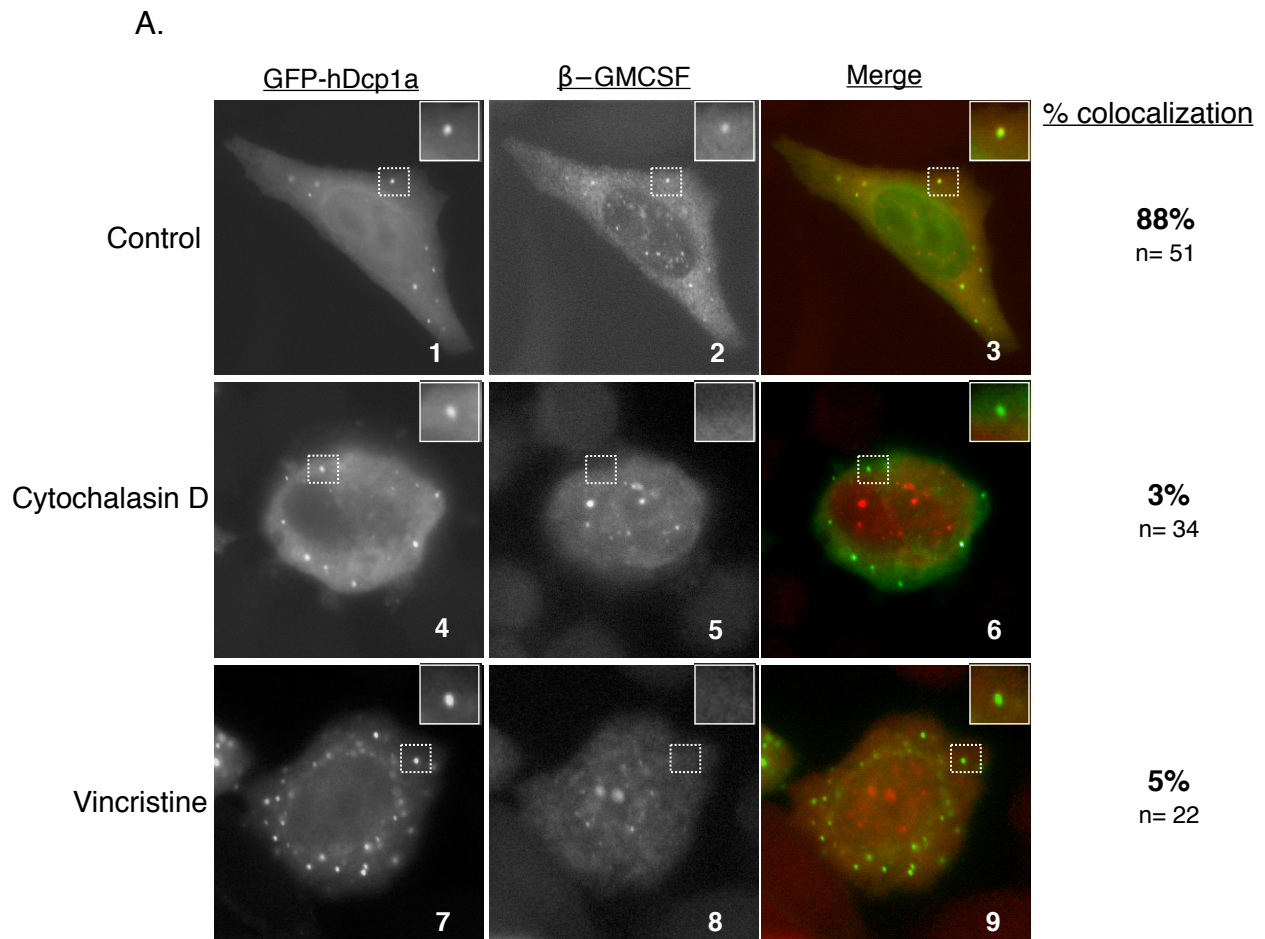


Figure 2-4. Disruption of actin or tubulin causes mislocalization of ARE-containing reporter mRNA

In-situ hybridization assay showing the localization of mRNA in relation to PBs during cytoskeleton disruption. Cells were left untreated (panels 1-3) or treated with cytochalasin D (panels 4-6) or vincristine (panels 7-8). (A) Localization of β -GMCSF mRNA (panels 2, 5, 8) was compared to the localization of GFP-hDcp1a (panels 1,4,7). Images are merged in panels 3, 6, and 9. The percent colocalization of β -GMCSF foci with GFP-hDcp1a foci is shown on the right. An enlarged portion of the cell is shown in the upper right corner of each panel and represents the area in the dotted square.

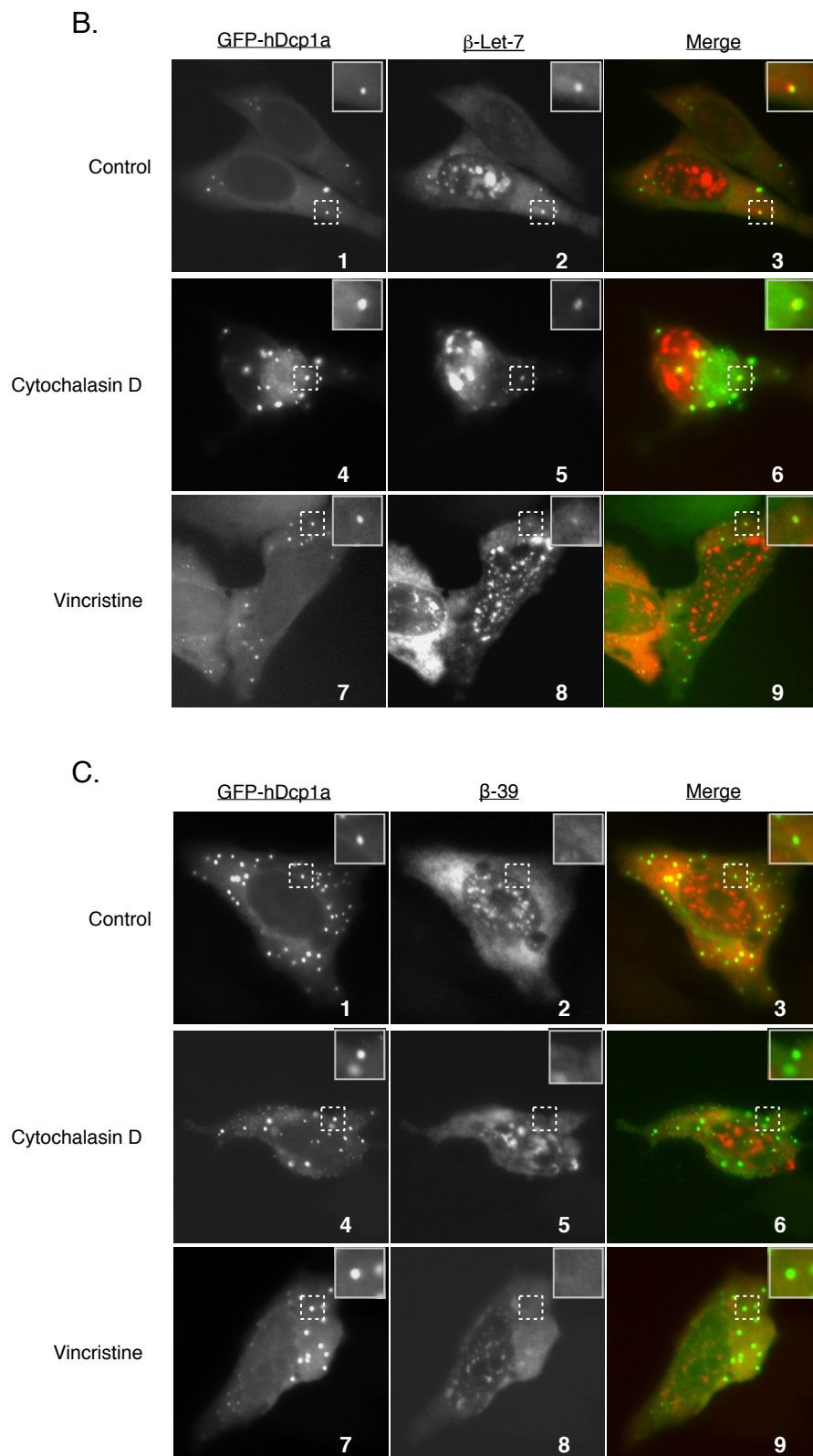


Figure 2-4. Continued

Localization of (B) β -Let-7 and (C) β -39 mRNA (panels 2, 5, 8) was compared to the localization of GFP-hDcp1a (panels 1,4,7). Images are merged in panels 3, 6, and 9. An enlarged portion of the cell is shown in the upper right corner of each panel and represents the area in the dotted square.

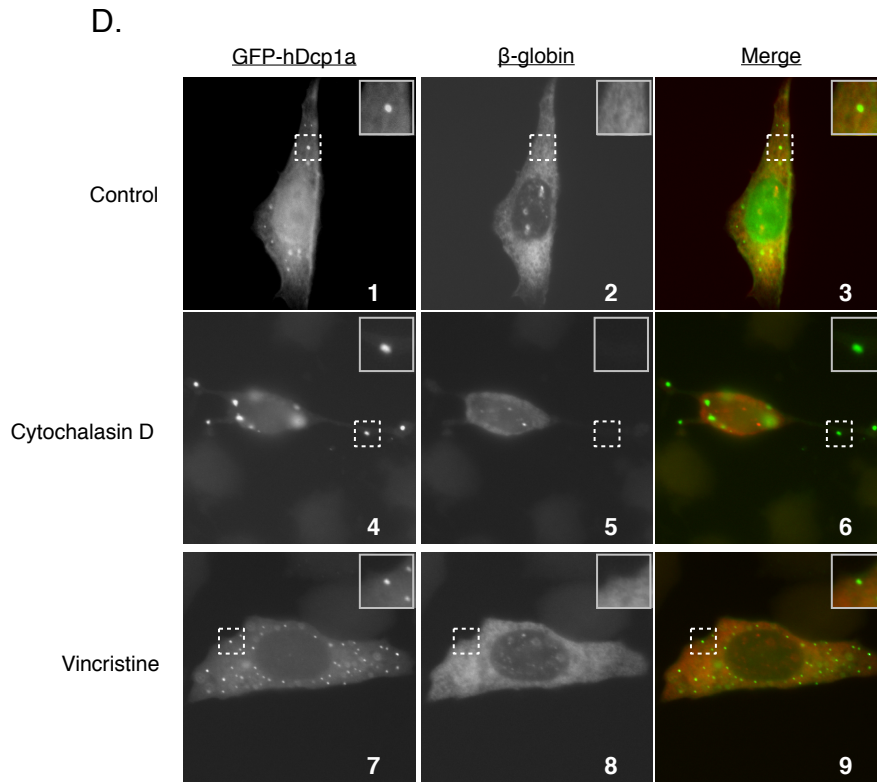


Figure 2-4. Continued

Localization of (D) β -Globin (panels 2, 5, 8) negative control was compared to the localization of GFP-hDcp1a (panels 1,4,7). Images are merged in panels 3, 6, and 9. An enlarged portion of the cell is shown in the upper right corner of each panel and represents the area in the dotted square.

Andersen, 2007). The localization of a reporter expressing only wild-type β -globin mRNA, which does not co-localize to PBs, was used as a negative control for this assay (Figure 2-4D).

If the co-localization of ARE-mRNAs to PBs is dependent on the cytoskeleton, the prediction would be that when the cytoskeleton is disrupted, the ARE-mRNA foci would disappear or become mislocalized away from PBs. In cells that were treated with cytochalasin D, to disrupt actin filaments, β -GMCSF mRNA is observed only in the nuclear foci (panel 5). Foci that colocalize with hDcp1a are observed in only 3% of cells. Similarly, cells incubated with vincristine to disrupt microtubules show β -GMCSF only in the nuclear foci (panel 8); only 5% of cells displayed colocalization with hDcp1a. These results suggest that the cytoskeleton is required for assembling the ARE mRNP with PBs.

The localization of two other mRNA reporters, β -Let-7 and β -39, were also tested in this experiment; however, no effect was seen in their ability to localize to PBs. The β -Let-7 mRNA reporter contains a let-7 micro (mi)RNA binding site in the 3' UTR, which recruits endogenous let-7 miRNA, leading to rapid decay, and localizes to PBs (Liu et al., 2005). As seen in Figure 2-4B, the β -Let-7 reporter is still observed in PBs even after treatment with either cytochalasin D (panel 5) or vincristine (panel 8). The β -39 reporter contains β -globin mRNA that has a premature termination codon at position 39 and is targeted for rapid decay through the NMD pathway (Lykke-Andersen et al., 2000; Franks et al., Cell, 2010). The β -39 reporter only rarely concentrates in PBs under normal conditions and requires expression of mutant Upf1 that is deficient in ATPase

activity, which is critical for efficient NMD, in order to be strongly seen in PBs (Franks et al., Cell, 2010). Figure 2-4C shows diffuse cytoplasmic localization of β -39 mRNA in HeLa cells (panel 2) compared to localization of GFP-hDcp1a in PBs (panel 1), which does not change after treatment with either cytochalasin D (panel 5) or vincristine (panel 8).

Efficient mRNA decay does not require the cytoskeleton

The results described above suggest that the cytoskeleton may be required for assembling ARE-mRNAs into PBs. This raised the question of whether the cytoskeleton is required for the efficient turnover of these mRNAs. To test this, I performed a pulse-chase mRNA decay assay to measure the rates of decay of several β -globin reporter mRNAs in cells incubated with or without cytoskeleton disrupting drugs. The prediction would be that if the cytoskeleton ensures efficient decay of mRNA, then a stabilization and an increase in half-life of the reporter mRNAs when compared to control conditions when the cytoskeleton is disrupted would be observed. The decay of three different reporter mRNAs was measured in this experiment: β -GMCSF, β -Let-7, and β -39 which were described earlier. Half-lives were measured with northern blot analysis by comparing the amount of reporter mRNA to the amount of constitutively expressed control mRNA, β -GAP, for each time point after tetracycline addition. In Figure 2-5A, the half-life of β -GMCSF mRNA in cells incubated with cytochalasin D showed a slight increase in half-life (126 minutes as compared to 82 minutes in untreated cells). However, treatment with vincristine did not show a significant change in half-life in comparison to cells untreated with vincristine. Observed differences in half-lives between untreated samples in individual experiments (top panels), may be due to

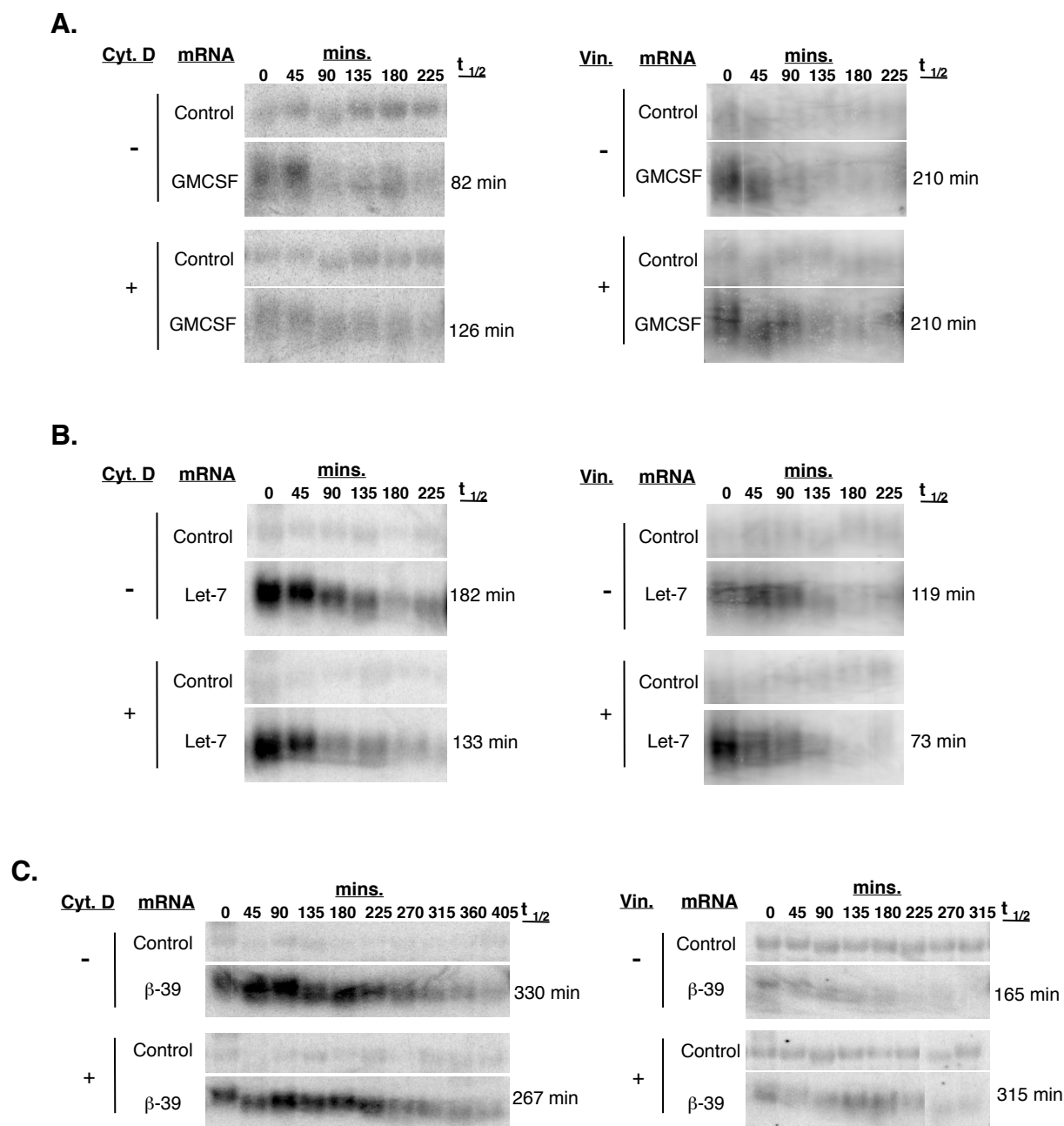


Figure 2-5. Efficient mRNA decay does not require the cytoskeleton

Pulse-chase mRNA decay assays showing the degradation of (A) β -GMCSF, (B) β -Let-7, or (C) β -39 reporter mRNA in the presence (+) or absence (-) of cytochalasin D (left panels) or vincristine (right panels). Expression of each reporter mRNA was pulsed for 6 hours and then stopped, and the decay of mRNA was measured every 45 minutes as indicated. Half-lives ($t_{1/2}$) were measured by comparing the amount of reporter mRNA compared to the amount of the constitutively expressed Control mRNA.

Eystathioy et al., 2003; Sheth & Parker, 2003; Dijk et al., 2002). PBs do not appear to

assays being performed with HeLa Tet-off cells at different confluencies. However, individual experiments should be comparable to their controls as these were always done in parallel and were reproducible. β -Let-7 (Figure 2-5B) and β -39 (Figure 2-5C) mRNAs also showed little effect upon cytoskeleton disruption, although the β -39 reporter mRNA may have been somewhat stabilized in the presence of vincristine.

2.3 Discussion

Many proteins involved in mRNA decay, as well as mRNAs targeted for decay, have been observed to localize in discreet cytoplasmic foci called PBs (Cougot et al., 2004; be static aggregates, as the proteins and mRNA within them display highly dynamic properties (Bregues et al., 2005; Cougot et al., 2004; Teixeira et al., 2005; Wilczynska et al., 2005). The mechanism for how these foci form and what controls the movement of proteins and mRNA to and from PBs is unknown. The cytoskeleton has previously been implicated in mRNP transport (Knowles et al., 1996) and I wished to uncover whether PBs utilized the cytoskeleton for assembly, integrity, disassembly, mRNA localization, and efficient mRNA decay. I demonstrated that when actin filaments or microtubules are disrupted, PBs retained their ability to form de novo, maintained their structure, and can disassociate.

Here, I find that incubating cells with cytochalasin D or vincristine to cause disruption of the cytoskeleton does not have an effect on PB dynamics. The integrity, assembly de novo, and the cycloheximide-induced disassociation of PBs were unaffected when the cytoskeleton was disrupted (Figures 2-1,-2,-3). However, the localization of ARE mRNA to PBs appears to be impaired when actin or microtubules

are disrupted (Figure 2-4). Interestingly, in both the cytochalasin D and vincristine treated cells, there appears to be large nuclear foci containing ARE mRNA. These foci may be sites of transcription of the ARE-mRNA, or they may be the result of the inability of the ARE-mRNA to leave the nucleus when the cytoskeleton is disrupted. Since PBs have been shown to be dependent on mRNA for their formation (Teixeira et al., 2005) and PBs are clearly visible in this experiment as seen with GFP-hDcp1a staining, nuclear export of mRNA is likely not effected by disruption of the cytoskeleton. In addition, diffuse cytoplasmic staining of the ARE-mRNA *in situ* probes is seen in these cells, indicating mRNA is exported to the cytoplasm but is not localized to PBs. When investigating whether the mislocalization of ARE mRNA to PBs results in a decrease in mRNA decay efficiency, I find that mRNA decay is not largely affected in cells where the cytoskeleton is disrupted. It has been reported that the presence of PBs is not required for efficient mRNA decay (Eulalio et al., 2007), therefore, it is not surprising that even though ARE mRNA is mislocalized when the cytoskeleton is disrupted, their decay rate is not greatly affected. This does not eliminate the possibility that this is a transcript-specific phenomenon, and that certain other mRNA transcripts not tested here may indeed rely on the delivery to PBs by the cytoskeleton for their efficient decay.

Similar work has provided varying results in proving a link between PBs and the cytoskeleton. In yeast, microtubule disruption led to an increase in the formation of PBs (Sweet et al., 2007). Sweet *et al.* also observe that microtubule disruption did not interfere with normal mRNA decay rates, similar to what I observed here in human cells. Another study using human cells observed in live cell imaging that PBs exhibit little movement within the cytoplasm, but on occasion display directed movement and

observed PBs in close proximity to actin filaments and microtubules (Aizer et al., 2008). Loschi *et al.* found that knock down of the molecular motors dynein and kinesin effected PB size (Loschi et al., 2009). It is important to note that stress and changes to RNA metabolism have an effect on processing bodies, and that disruption to the cytoskeleton may cause pleiotropic effects. In the future, more direct methods would be useful to draw a definitive conclusion on the role of the cytoskeleton plays in PB dynamics.

The findings here that PB dynamics are not guided by the cytoskeleton network in human cells demonstrates that other mechanisms exist to explain how mRNA decay factors and mRNAs targeted for silencing or decay find themselves reliably within these foci. One possible mechanism is aggregation of PBs from diffusing mRNPs mediated by protein-protein and protein-mRNA interactions. Recent studies have revealed that several known PB factors in yeast contain glutamine/asparagine-rich (Q/N-rich) regions, classified as prion-like domains, and have been shown to be important for PB formation (Decker et al., 2007; Reijns et al., 2008). Analysis reveals that several PB proteins in humans also contain Q/N-rich regions. It may be that these predicted prion-like domains are responsible for controlling PB formation, integrity, and disassociation through aggregation, rather than through the cytoskeleton. Further study into this hypothesis is discussed in Chapter 4 of this thesis.

2.4 Materials and Methods

Plasmids

The plasmid encoding GFP-Dcp1a (pcNEGFP-hDcp1a) used as a processing body marker has been described previously (Franks & Lykke-Andersen, 2007).

Plasmids expressing reporter β -globin mRNAs used in mRNA decay assays, Control (pc β GAP), β -GMCSF (pPC β wtATGMCSF), and β -39 (pPC β 39) have been described previously (Lykke-Andersen & Wagner, 2005). The β -Let-7 reporter was created by cloning the minimal let-7 binding site (Grosshans & Slack, 2002) into the Xba1/Apa1 sites in the pcDNA3 vector (Invitrogen). Then the pcTet2Bwt vector (Singh et al., 2008) was cut with Mlu1 and Xba1 and the resulting fragment was ligated into the vector containing the let-7 binding site. A plasmid encoding a tetracycline-responsive activator protein was used to activate transcription of a reporter mRNA for the in-situ hybridization assay (pTet-TTA; Clontech).

Indirect immunofluorescence and in situ hybridization assays

HeLa cells in DMEM/10% fetal bovine serum (FBS) were split to chamber slides when ~50% confluent. 24 hours later, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) for 15 min, and permeabilized and blocked with PBS/1% goat serum/0.1% Triton X-100 for 20 min. Cells in Fig. 1 were incubated with 2 μ g/mL cytochalasin D (Sigma) or 10 μ g/mL vincristine (Sigma), as indicated, for 1 hour prior to fixation and permeabilization. Cells in Fig. 2 were incubated with 20 μ g/mL cycloheximide (Sigma) for 1 hour, washed twice with PBS, then incubated with either 2 μ g/mL cytochalasin C or 10 μ g/mL vincristine, as indicated, for 1 hour prior to fixation and permeabilization. In Fig. 3, cells were incubated with 2 μ g/mL cytochalasin D or 10 μ g/mL vincristine, as indicated, for 1 hour and then incubated with 20 μ g/mL cycloheximide for 1 hour prior to fixation and permeabilization. After fixation and permeabilization, cells were incubated

for 1 hour with PBS/1% goat serum containing rabbit anti-hDcp1a (1:200) or anti-hXrn1 (1:200) (Lykke-Andersen & Wagner, 2005) and, when staining for microtubules, mouse anti- β -tubulin (Sigma, 1:100) antibodies. Following removal of the primary antibody and three washes with PBS/1% goat serum, cells were incubated for 1 hour with PBS/1% goat serum containing 4 μ g/ml secondary anti-rabbit antibodies or anti-mouse antibodies labeled with either Texas-Red or Alexa 488 fluorophore (Molecular Probes), or Oregon Green 488-conjugated phalloidin (Molecular Probes, 1:100) when co-staining for F-actin. Cells were washed 6 times with PBS/1% goat serum followed by one wash with water and covered with Vectashield (Vector Labs) and a coverslip prior to fluorescence microscopy.

For in-situ hybridization, cells were transfected in the presence of 50 ng/mL tetracycline with 300 ng of reporter mRNA expression plasmid, 300 ng of pTet-tTA, 100 ng of GFP-hDcp1a, and empty pcDNA3-FLAG vector to 1 μ g total DNA. Two days after transfection, transcription of the reporter mRNA was initiated by washing cells in PBS and placing them in DMEM/10% FBS, without tetracycline. After a transcriptional pulse of 0h or 6h cells were fixed in 4% paraformaldehyde for 15 min and permeabilized overnight in 70% ethanol. Cells were then rehydrated for 10 min in 50% formamide and 2 \times SSC (300 mM sodium chloride, 30 mM sodium citrate). Next, cells were incubated overnight at 37°C in a solution containing 50% formamide, 2 \times SSC, 0.02% bovine serum albumin (BSA), 2 mM vanadyl-ribonucleoside complexes, 1 μ g/mL total yeast RNA, and 0.1 mg/mL dextran sulfate. In order to detect the localization of the β -globin mRNA, four Texas-red labeled 50-nucleotide DNA oligo probes (Franks et al., 2010) complementary to sequences in exons 1, 2, and 3 were also added to the mixture at a

concentration of 20 ng/mL each. The sequences of the oligos are as follows:

Exon 1:

5' XTAACGGCAGACTTCTCCTCAGGAGTCAGGTGCACCATGGTGTCTGTTTG 3'

Exon 2-1:

5' XCATAACAGCATCAGGAGTGGACAGATCCCCAAAGGACTCAAAGAACCTCT 3'

Exon 2-2:

5' XGAAGTTCTCAGGATCCACGTGCAGCTTGTTCACAGTGCAGCTCACTCAGTG 3'

Exon 3:

5' XTAGTTGGACTTAGGGAACAAAGGAACCTTTAATAGAAATTGGACAGCAAG 3'

X = Texas Red fluorophore

Cells were washed twice for 30 min at 37°C in 50% formamide and 2× SSC then covered with Vectashield (Vector Labs) and a coverslip prior to fluorescence microscopy.

Pulse-chase mRNA decay assays

Human HeLa Tet-off cells (Clontech) in full media (DMEM, 10% FBS, 1% penicillin/streptomycin solution) at ~30% confluency in 3.5-cm wells were transfected in the presence of 50 ng/ml tetracycline, using TransIT HeLaMonster reagent according to manufacturers protocols (Mirus), with a total of 2 µg of plasmid, including 80 ng internal control expression plasmid, expressing β-GAP-UAC mRNA, and 500 ng reporter plasmid expressing β-GMCSF (Fig. 4A), β-Let-7 (Fig. 4B), or β-39 (Figs. 4C) mRNA. Empty pcDNA3-FLAG plasmid was added to each reaction to 2 µg of total plasmid. 40 hours after transfection, transcription of the reporter mRNAs was initiated by washing

the cells with PBS and adding 2 ml DMEM/10% FBS, containing no tetracycline. Six hours later, tetracycline was added to 1.0 $\mu\text{g/ml}$ to stop transcription and cells were incubated with or without either 2 $\mu\text{g/mL}$ cytochalasin D or 10 $\mu\text{g/mL}$ vincristine as indicated in the figure. Cells were washed with PBS and taken up in 1 ml of Trizol (Invitrogen) starting 30 min after addition of tetracycline (0-min time point), and then every 45 min as indicated. Total RNA was prepared according to manufacturer's protocols and analyzed by Northern blotting, as described previously (Lykke-Andersen et al., 2000).

Chapter 3

Identifying Novel Processing Body Components

3.1 Introduction

Since they were first observed, PBs have been found to contain a wide variety of proteins involved in cytoplasmic mRNA function. Proteins involved in general mRNA degradation were among the first to be recognized as PB factors and include deadenylases, decapping machinery, various effector proteins, and an exonuclease enzyme. In addition, proteins and mRNA involved in specific mRNA decay pathways such as ARE-mediated decay and nonsense-mediated decay have been found in PBs. Other proteins found to localize in PBs include factors involved in miRNA-mediated silencing, translation initiation, and translation repression. Stress granules (SGs), another cytoplasmic mRNP granule observed during cell stress, have also been found to share proteins and be spatially associated with PBs. It has been suggested that PBs and SGs are functionally linked (for review see Franks & Lykke-Andersen, 2008; Kulkarni et al., 2010; Parker & Sheth, 2007).

With all that is known about the many proteins and functions linked to PBs, the functional significance of these cytoplasmic foci remains unclear. PBs have proven to be highly conserved in eukaryotes and have been observed in yeast, mammals, flies, worm, and plants. As such, one would predict that PBs serve a useful and important purpose for the cell. However, no experimental evidence for a function of PBs in mRNA metabolism has been found, as the depletion of PBs does not impair the rate of decay of tested mRNAs in yeast (Decker et al., 2007), or of NMD, or miRNA-mediated silencing in *Drosophila* S2 cells (Eulalio et al., 2007). Current models for the role of PBs suggest they may instead serve as a way to sequester mRNA decay components away from the rest of the cell in order to inhibit promiscuous mRNA degradation. They may

also help to enhance mRNA decay kinetics. When available mRNA decay machinery is limiting compared to the amount of mRNA to be degraded, PBs may serve to focus decay proteins in discreet foci for more efficient processing.

Since their discovery, more than 70 proteins have been identified to localize to PBs and they have been linked to at least 6 different cellular mRNA processes (Kulkarni et al., 2010). By studying the functions and interactions of the many proteins within PBs, much progress has been made to elucidate the functional significance of these foci; however, many questions remain unanswered. Why do they form? How do they form? How do they regulate mRNA turnover? The identification of new PB proteins are continually being reported in the literature; however, the complete inventory of PB proteins remains to be elucidated. Partial PB purification has been achieved in the past (Teixeira et al., 2005) and previous work by our lab (Fenger-Grøn et al., 2005) utilized co-immunoprecipitation and mass spectrometry analysis to identify previously uncharacterized PB proteins Hedls, Rck/p54 (DDX6), and human Edc3. By identifying and characterizing new PB proteins, we may find clues to understanding PBs and the mechanisms that regulate mRNA turnover.

Here, I performed immunoprecipitation assays on HEK 293 cells, which were stably transfected with FLAG-tagged PB factors hDcp1a, Rck/p54, and hXrn1. Mass spectrometry analysis revealed several co-purifying proteins including PABP1, FXMRP-IP, Not3, and Xrn2. Most surprisingly was the discovery that PRMT5 and MEP50, components of the methylosome complex, interact with several of the immunoprecipitated PB factors. This complex methylates Sm proteins and is involved in core snRNP formation in the cytoplasm. The identification of these new PB-interacting

proteins provides a more complete picture of the make-up of PBs and may help to gain insight into to their function.

3.2 Results and Discussion

Purification and isolation of proteins that co-purify with known PB factors

To isolate potential new PB proteins, HEK 293 cells stably transfected with FLAG-tagged hDCP1a, Rck/p54, and hXrn1 were each grown up in 15 cm plates to ~80% confluency (see Materials and Methods). Cells stably transfected with empty FLAG vector were also used as a negative control in three initial trial-run experiments, which were performed to identify consistently co-purifying complexes and to monitor for any non-specific binding to the beads (Figure 3-1A). The day before harvesting, expression at levels equivalent to the corresponding endogenous protein of each FLAG-tagged protein (Fillman, unpublished observations) was induced by the addition of low amounts of tetracycline to the media (see Materials and Methods). The following day, cells were harvested, RNase-treated, and subjected to anti-FLAG immunoprecipitation, elution, and final precipitation steps. Each sample was then run on a 3-layered SDS-PAGE gel (Figure 3-1B) containing a stacking layer, 8%, and 15% polyacrylamide layer to more effectively resolve both large and small proteins that may have been purified. BSA was loaded into the 4th lane to be used as a positive control during mass spectrometry analysis. The resolved gel was stained and protein bands from each sample that had consistently proven specific to individual FLAG-tagged proteins in the trial assays (Figure 3-1A) were excised, extracted from the gel, and analyzed by mass spectrometry.

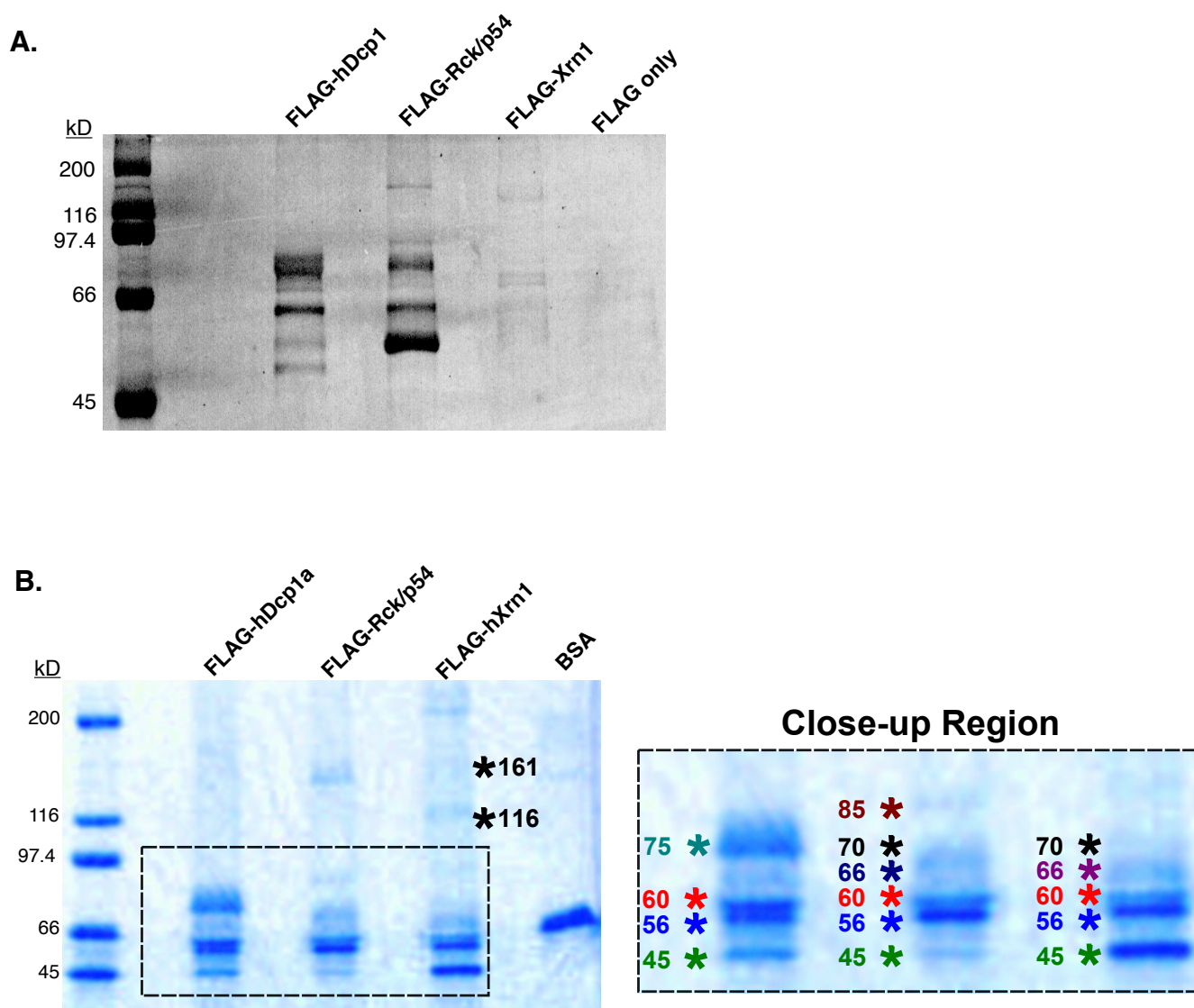


Figure 3-1. Immunoprecipitation of PB proteins yields co-purifying protein complexes that were excised for mass spectrometry analysis

Immunoprecipitations (IPs) of FLAG-hDcp1a, FLAG-Rck/p54, and FLAG-Xrn1 resolved by SDS-PAGE and stained for resulting protein complexes. A) Silver stained gel of one of three trial run IPs performed to identify bands that consistently appeared. IP of a FLAG-only vector in the far right lane shows no protein band staining, indicating that this method of IP for produces no non-specific binding to Flag. B) GelCode Blue stained gel from which the protein bands were excised for subsequent mass spectrometry analysis. The boxed region is enlarged on the right. Each band that produced interpretable mass spectrometry data are labeled with an asterisk with a number corresponding to molecular weight in kD. The proteins identified are listed in Table 3-1. BSA was run in the far right lane and used as a positive control during mass spectrometry analysis.

Identification of potential novel PB factors by mass spectrometry analysis

Each protein sample was analyzed by HPLC MS/MS and were identified using the Mascot MS/MS Ion Search program (Perkins et al., 1999). Table 3-1 lists the proteins that were identified at the corresponding size indicated by asterisks in Figure 3-1B. Several of the proteins have been previously observed to localize in PBs. Rck/p54, identified in all three immunoprecipitations, and Edc3, identified in two immunoprecipitations, were originally identified by our lab to co-purify with hDcp1a and localize to PBs (Fenger-Grøn et al., 2005). Not3, identified here in a pulldown with Xrn1, is part of the Ccr4-Not mRNA deadenylation complex and is known to localize to PBs in yeast (Muhlrad & Parker, 2005). These results confirm that our assay works to pull down PB complexes.

SG, and SG-related proteins, were also identified. PABPC1 co-purifies in a complex with Rck/p54, an RNA helicase involved in mRNA decay. It has been previously reported that PABP-1 is found in SGs but is absent from PBs (Kedersha et al., 2005). Rck/p54, known to primarily localize in PBs, has also been observed to colocalize in SG in immunofluorescence microscopy assays (Wilczynska et al., 2005). Another SG-related protein I identified was Nuclear fragile X mental retardation protein interacting protein (NUFIP2, also called 82-FIP) in a complex with Rck/p54. Fragile X mental retardation protein (FMRP) is a cytoplasmic RNA binding protein, whose absence causes fragile X syndrome, and is also involved in SG formation (Mazroui et al., 2002). NUFIP2 interacts with FMRP and localizes to both the nucleus and cytoplasm (Bardoni et al., 2003). Finally, I identified the Ataxin-2-like protein (ATXN2L) to co-purify in a complex with Xrn1. ATXN2L is a protein of unknown function and is

Protein Identified	Molecular Weight (kD)	FLAG IP	Peptides
MEP50	45	Dcp1a	36-YRSDGALLLGASSLS-52 122-KYEHDDIVSTVSVSSGTQAVSGSK-145
		Rck	4-KETPPPLVPPAARE-15 36-RYRSDGALLLGASSLSGRC-52 122-KYEHDDIVSTVSVLSSGTQAVSGSKD-145
		Xrn1	4-KETPPPLVPPAARE-15 30-RQLEAARY-35 36-RYRSDGALLLGASSLSGRC-52 122-KYEHDDIVSTVSVLSSGTQAVSGSKD-145 165-RAHAAQVTCVAASPHKD-179
Rck/p54	56	Dcp1a	212-LDDTVHVVIATPGR-225 448-SIEEQLGTEIKPIPSNIDK-466 467-SLYVAEYHSEPVEDEKP-483
		Rck/p54	23-RGPVKPTGGPGGGGTQTQQQMNQLKN-46 75-KTLKLPPKD-81 85-RIKTS DVTSTKG-94 95-KGNEFEDYCLKR-104 106-RELLMGIFEMGWEEKPSPIQEEESIPIALSGRD-134 135-RDILARA-139 190-KHMGGAKVMATTGGTNLRD-206 212-RLDDTVHVVIATPGRI-225 226-RILDLIKK-231 347-RVELLAKK-352 448-KSIEEQLGTEIKPIPSNIDKS-466 467-KSLYVAEYHSEPVEDEKP-483
		Xrn1	414-KLAETYLHRI-421 448-KSIEEQLGTEIKPIPSNIDKS-466 467-KSLYVAEYHSEPVEDEKP-483
Edc3	60	Dcp1a	106-KKPASSSSAPQNIPKR-119 244-RYRH DENILESEPIVYRR-259 260-RRIVPHNVSKE-269 270-KEFCTDSGLVVP SISELHKK-288 290-KLLSVAEKH-296 388-KTQQQQVSSLKD-397 398-KDLPTSPVDLVINCLDCPENVFLRD-420 437-RAPVLSIDPPVHEVEQGIDAKW-456 457-KWSLALGLPLPLGEHAGRI-473
		Rck/p54	106-KKPASSSSAPQNIPKR-119 186-KNKDDEC FGDIEEIPD TDFDFEGLNALFDKA-215 244-RYRH DENILESEPIVYRR-259 270-KEFCTDSGLVVP SISELHKK-288 297-KHGLTLERR-303 374-KMLESITNELSLFSKT-387 388-KTQQQQVSSLKD-397 398-KDLPTSPVDLVINCLDCPENVFLRD-420 437-RAPVLSIDPPVHEVEQGIDAKW-456 457-KWSLALGLPLPLGEHAGRI-473

Table 3-1. Proteins identified by mass spectrometry that interact with PB factors

The column on the left lists the proteins identified by mass spectrometry analysis that were found to IP with hDcp1a, Rck/p54, and/or Xrn1, as indicated in the third column. The second column lists the size, in kD, corresponding to the band they were excised and purified from and indicated with asterisks in Figure 3-1B. The column on the right lists the peptides identified by mass spec for each protein.

Protein Identified	Molecular Weight (kD)	FLAG IP	Peptides
PABPC1	66	Rck/p54	51-RSLGYAYVNFQQPADAERA-67 139-KGYGFVHFETQEEAERA-153 214-KFGPALSVKV-221 312-RKEFSPFGTITSAKV-324 357-RIVATKPLYVALAQRK-370 605-RSKVDEAVAVLQAHQAKE-620
Not3	66	Xrn1	536-KAPEPLSSLK-544
PRMT5	70	Rck/p54	69-RSDLLSGRD-76 165-RDDIENAPTTHTTEEYSGEEK-184 349-RVPEEEKDTNVQVLMVLGAGRG-368 459-KDDGVSIPGEYTSFLAPISSSKL-479
		Xrn1	19-RDLNCVPEIADTLGAVAKQ-35 154-RVPLVAPEDLRDDIENAPTTHTTEEYSGEEK-183 202-RIAVALEIGADLPSNHVIDRW-220 348-RVPEEEKDTNVQVLMVLGAGR-367 388-KLYAVEKN-393 459 -KDDGVSIPGEYTSFLAPISSSKL-479
Dcp1a	75	Dcp1a	60-RSASPYHGFTIVNRL-72 400-RLTPQHDQIQTPLGKG-415 520-RKASSPSPLTIGTPESQRK-536
FMRP-IP	85	Rck/p54	123-RVLNGNQVVDTSLKQ-136 582-KSGTTSESGALSLEPSHIGDLQKA-603 618-KDYEIESQNPLASPTNTLLGSAKE-639
Xrn2	116	Xrn1	190-KNLTVILSDASAPGEGEHKI-207 660-RAALEEVYPDLTPEETRR-675 806-RRPVHLDQAAFRT-816
ATXN2L	161	Xrn1	107-KGPPQSPVFEGVYNNSRM-122 499-KISLAPTDVKE-507 554-KLQPSSSPENSLDPFPRI-570

Table 3-1. Continued

The column on the left lists the proteins identified by mass spectrometry analysis that were found to IP with hDcp1a, Rck/p54, and/or Xrn1, as indicated in the third column. The second column lists the size, in kD, corresponding to the band they were excised and purified from and indicated with asterisks in Figure 3-1B. The column on the right lists the peptides identified by mass spec for each protein.

related to the Ataxin-2 protein. Ataxin-2 colocalizes in SGs and has an Lsm/LsmAD domain that mediates an interaction with Rck/p54. Although its exact function is unknown, overexpression of Ataxin-2 leads to a reduction of PBs, while knockdown of Ataxin-2 impairs SG assembly (Nonhoff et al., 2007). These observations point to a possible function in either mRNA decay or translational repression and provide us with clues to a function for ATXN2L, as it also has an Lsm/LsmAD domain and Xrn1 can localize to SGs (Kedersha et al., 2005).

Taken together, some of the proteins identified here likely represent complexes forming on mRNPs that assembly into SGs. These results raise interesting questions as to the connection between mRNPs that localize in SGs and PBs. A functional relationship between mRNPs in SGs and PBs has been proposed in the past as they are known to share a subset of proteins and have been observed in close proximity to each other in the cytoplasm. Both SGs and PBs consist of repressed mRNPs and may work together to coordinate the fate of mRNA, directing them to degrade in PBs or remain translationally repressed in SGs (Buchan et al., 2008). Further study into what role the RNA helicase Rck/p54 and its interactions with PABP-1 and NUFIP-2, as well as the interaction of Xrn1 and ATXN2L, play in mRNP accumulation in SGs may provide insight into the relationship between PBs and SGs and the control of mRNA processing within these granules.

Xrn1 is a cytoplasmic exonuclease that degrades mRNA in the 5'-3' direction and localizes to PBs and SGs. The identification of Xrn2 in the co-immunoprecipitation with Xrn1 is surprising for two reasons. One being that, although related, Xrn1 and Xrn2 have not been shown to interact with each other. Two, Xrn1 is known to localize to the

cytoplasm, while Rat1p, the yeast ortholog of human Xrn2, has been previously shown to primarily localize to the nucleus (Johnson, 1997). These results suggest that Xrn1 and Xrn2 could potentially work together to degrade mRNA.

The methylosome complex interacts with PB proteins

The proteins identified so far have revealed interesting details previously unknown about PB protein interactions. However, considering their known connections to mRNA translational silencing, these findings are not surprising. In contrast, the presence of MEP50 (methylosome protein 50) in all three immunoprecipitations as well as PRMT5 (protein arginine methyltransferase 5) in a complex with Rck/p54 and Xrn1 was unanticipated. MEP50 and PRMT5 are known to interact with each other, and with a third protein pICln, to form the methylosome complex (Friesen et al., 2001). The methylosome is a crucial regulatory component of the assembly of spliceosomal U snRNPs in the cytoplasm. PRMT5 is the catalytically active component and acts by methylating arginine residues of Sm complex proteins. This methylation promotes subsequent binding of the Sm complex to the survival of motor neuron (SMN) protein and is important for efficient assembly of the Sm snRNP core (Meister et al., 2001). The Sm/SMN/snRNP is then transported into the nucleus where spliceosome assembly is completed (Friesen et al., 2001; Friesen et al., 2002; Paushkin et al., 2002).

Intriguingly, there is a link between spliceosome assembly and PBs. Homologs of Sm proteins, called Lsms, localize to PBs and play a crucial role in mRNA degradation (Bouveret et al., 2000; Ingelfinger et al., 2002). Both Sm and Lsm proteins form a heteroheptamer complex that bind RNA. The Lsm complex that localizes in PBs is

composed of the proteins Lsm1 through Lsm7 (Khusial et al., 2005). Studies have shown that when components of Lsm1-7 are knocked down, mRNA degradation is inhibited (Stoecklin et al., 2006). The exact mechanism for how the Lsm1-7 complex regulates mRNA degradation remains unknown; however, the Lsm1-7 complex is thought to bind degrading mRNAs after the poly-A tail is removed and then acts to activate decapping and degradation of the transcript by recruiting the decapping machinery or the 5'-3' exonuclease Xrn1 (He & Parker, 2000).

PRMT5 is known to methylate its protein substrates at arginines of specific GRG peptide repeat residues (Brahms et al., 2001; Liu & Dreyfuss, 1995). Several proteins in the Sm family contain GRG repeats (Brahms et al., 2001; Friesen & Dreyfuss, 2000) and several Sm proteins were found to be methylated by PRMT5 (Friesen et al., 2001). Interestingly, Lsm4, a component of the Lsm1-7 complex, contains a distinct GRG peptide repeat domain in its C-terminus and has been shown, through mass spectrometry analysis, to be methylated (Brahms et al., 2001). It may be that PRMT5 is the methyltransferase responsible for methylating Lsm4, and could explain why methylosome complex proteins co-purify with Dcp1, Rck/p54, and Xrn1. PRMT5 was predicted to be the arginine methyltransferase responsible for dimethylating U6-associated Lsm4 (Brahms et al., 2001), because at the time PRMT5 was the only known methyltransferase to produce symmetric dimethylation, whereas the other 10 known PRMTs produced asymmetric dimethylation (Figure 3-2). Recently, PRMT7 was discovered to also be a symmetric dimethyltransferase (Lee et al., 2005) and it would be interesting to test whether PRMT7 might also interact with Lsm4. In addition, it would be interesting to see if knockdowns of PRMT5, PRMT7, or both, has any effect on PB

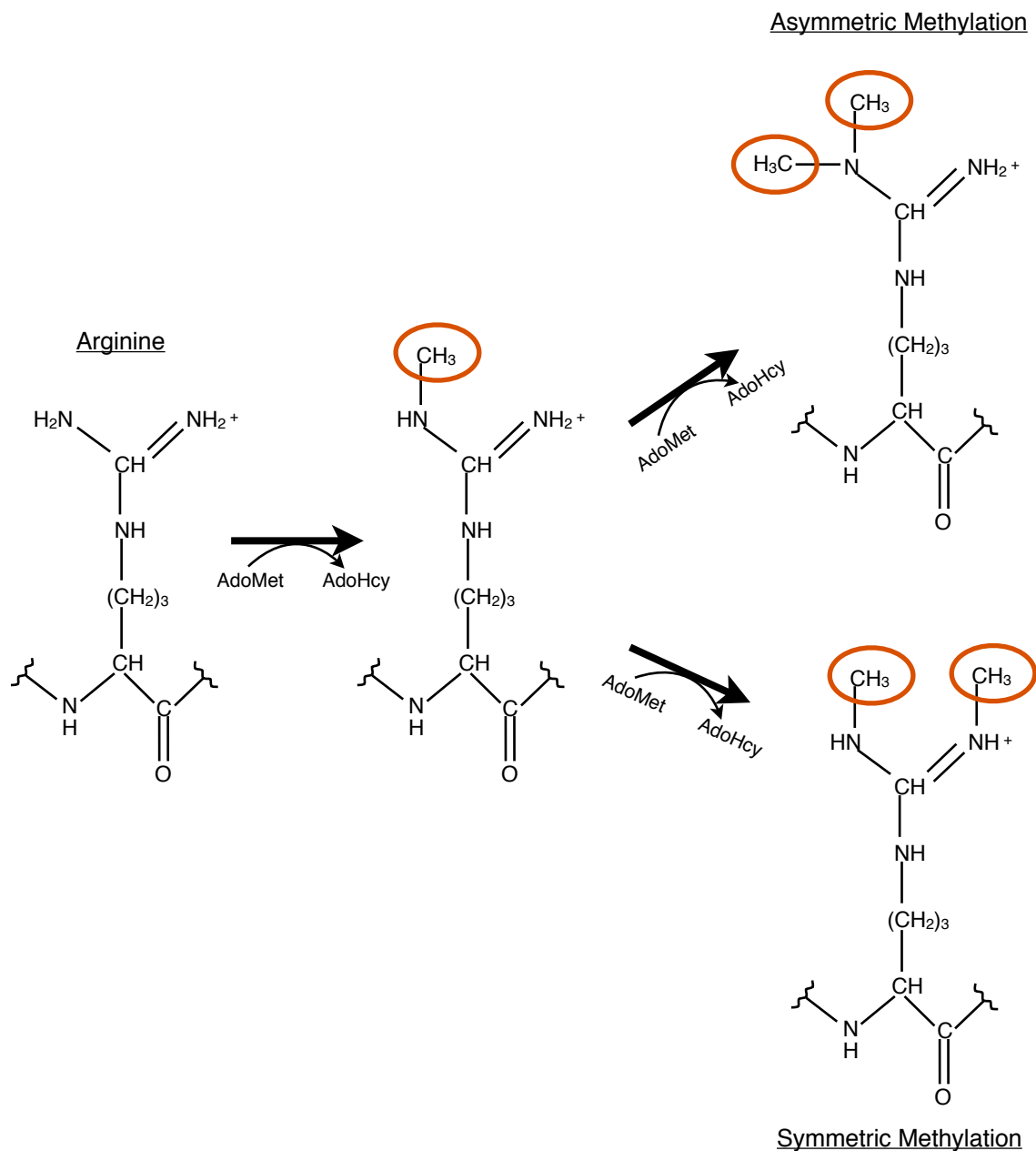


Figure 3-2. Asymmetric versus symmetric arginine methylation

Symmetric or asymmetric di-methylated arginines are produced by protein arginine methyltransferases (PRMTs). PRMT5 produces symmetric dimethylated arginines.

assembly.

The discovery of a component involved in splicing to co-purify with PB proteins links yet another RNA processing event to PBs. The results I presented here demonstrate that the more we examine the proteins that localize to PBs, the more we can learn about the function PBs serve in the cell. Eventually, the entire inventory of PB proteins their interactions may be solved and reveal why the presence of PBs has been preserved among so many species.

3.3 Materials and Methods

Plasmids

Stable HEK 293 cell lines expressing FLAG-tagged hDcp1a were created as described earlier (Fenger-Grøn et al., 2005) using the Flp-In T-Rex system according to the manufacturers' instructions (Invitrogen). Stable cell lines expressing FLAG-Rck/p54 (Fenger-Grøn et al., 2005) and FLAG-Xrn1 (Lykke-Andersen & Wagner, 2005) were created by inserting the open reading frames between the *HindIII* and *NotI* sites (Rck/p54) or *BamHI* and *NotI* sites (Xrn1) of pcDNA5/FRT/TO (Invitrogen).

Immunoprecipitation

To generate samples for mass spectrometry analysis, HEK 293 cells stably transfected with FLAG-tagged hDCP1a, Rck/p54, and hXrn1 were each grown up in five (hDcp1a, Rck/p54) or ten (hXrn1) 15 cm plates to ~80% confluency. Cells stably transfected with empty FLAG vector were also plated in five 15 cm plates and used as a negative control in three initial trial-run experiments, which were performed to ensure

consistency and monitor for any non-specific bands. The day before harvesting, translation of each FLAG-tagged protein was induced by the addition of 30 ng/mL (Dcp1) or 10 ng/mL (Rck/p54, Xrn1) tetracycline to the media, which was optimized to produce near endogenous levels of the exogenous proteins (Fillman, unpublished data). The following day, cells were washed carefully twice with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and the cells were scraped and pelleted. Cells were lysed with 1 mL hypotonic gentle lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml leupeptin, 2 µg/ml of aprotinin) with 1 µg/mL FLAG peptide added. Lysates were incubated on ice for 10 minutes. NaCl was added to 150 mM and RNase A was added to 125 µg/ml, and the extracts were incubated for 5 min on ice. Lysates were cleared by centrifugation at 14,000 rpm at 4°C for 15 minutes, and 800 µl supernatant was loaded onto pre-washed anti-FLAG M2 agarose (Sigma), 20 µl bead volume and nutated at 4°C for 4 hours. Beads were washed eight times with 1 ml of ice-cold NET-2 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Triton X-100). Bound proteins were eluted by gently shaking tubes at 4°C for 1 hour, centrifugation at 10,000 rpm for 10 minutes, and transferring 200 µl supernatant to another tube. 200 µl NET-2 and 200 µg/mL FLAG peptide was added back to beads and elution procedure was repeated 2 times.

Mass Spectrometry

Eluted anti-FLAG M2 agarose immunoprecipitates were subjected to alkylation by heating samples for 5 min with 4 mM DTT and 40 mM Tris-HCl pH 8.0, followed by

cooling to room temperature and incubation with 10 mM iodoacetamide for 30 minutes in the dark. Samples were then precipitated by adding 1/10 volume trichloro-acetic acid (TCA) and incubated on ice for 10 minutes. Samples were pelleted by centrifugation at 14,000 rpm for 10 minutes. The TCA was then removed and the pellets washed in acetone. After washing, the acetone was removed and the pellets were air dried and re-dissolved in 40 μ L SDS sample buffer (100 mM Tris-HCl pH 6.8, 2% Sodium dodecyl sulphate (SDS), 1% mercaptoethanol, 0.02 % bromphenol blue). Samples were run on a 3-layered SDS-polyacrylamide gel containing a stacking layer, an 8%, and a 15% acrylamide layer to more effectively resolve both large and small proteins that may have been purified. 2 μ g of BSA was run in a 4th lane and used for a positive control in mass spectrometry analysis.

The gel was stained with GelCode Blue (Pierce) and specific bands were excised and placed in tubes that were pre-washed with 0.1% trifluoroacetic acid and 50% acetonitrile. Stain was removed from each gel piece by adding 500 μ l HPLC grade water and shaking for 15 minutes at room temperature. The water was then removed and 100 mM sodium thiosulfate and 30 mM potassium ferrocyanide was added and tubes were shaken for 5 minutes at room temperature. This solution was replaced with 1mL HPLC grade water and agitated for 10 minutes for 3 washes. The water was then removed and 1 mL of 1 part 100 mM ammonium bicarbonate to 1 part 100% acetonitrile was added to each tube and agitated for 15 minutes. Gel pieces were then dehydrated in 100% acetonitrile for 15 minutes and dried completely in a sterile hood for 15 minutes. In-gel digestion was carried out by resolubilizing the gel pieces in 40 mM ammonium bicarbonate containing 0.02 μ g/ml of Sequencing Grade Modified Trypsin

(Promega), followed by shaking over night at 37°C. The peptide supernatant was transferred to a new tube and 50 µL of 0.1% TCA was added back to the gel slice and agitated for 30 minutes at 37°C. This second extract was added to the first peptide supernatant and each sample was sent to the University of Colorado, Boulder Department of Chemistry Mass Spectrometry Facility for HPLC MS/MS analysis with a nanospray source and a 3D ion trap mass spectrometer. Peptide sequences were analyzed by the Mascot MS/MS Ion Search program (Perkins et al., 1999).

Chapter 4

Divergent Function of the Lsm4 Carboxy-Terminus in Eukaryotes

4.1 Introduction

Control of mRNA turnover is crucial for the proper regulation of gene expression. To our knowledge, the most common pathway of mRNA decay in eukaryotic cells is deadenylation-dependent decay. This process begins with removal of the poly-A tail from the 3' end of the transcript, followed by decapping by the enzyme Dcp2, along with several decapping effector proteins, and finally the mRNA is degraded in the 5'-3' direction by the exonuclease Xrn1 (for review see (Garneau et al., 2007)). In human cells, mRNAs that contain AU-rich elements (AREs) in the 3' untranslated region (UTR), recruit trans-factors that activate rapid deadenylation-dependent degradation of the mRNA (Barreau et al., 2005).

Many proteins involved in the regulation of mRNA stability have been found to localize to cytoplasmic mRNP granules, known PBs. Such PB factors include proteins involved in general decay such as the decapping enzyme Dcp2, decapping effector proteins Dcp1, Lsm1-7, Hedls/Edc4, Rck/p54, Edc3, and Pat1, and the exonuclease Xrn1 (for review see (Kulkarni et al., 2010; Parker & Sheth, 2007)). Many other proteins involved in cytoplasmic mRNP regulation localize to PBs as well, such as those involved in ARE-mediated decay, miRNA function, translational control, and nonsense-mediated decay (NMD). PBs are highly dynamic structures, as observed through fluorescent recovery after photobleaching (FRAP) analysis (Andrei et al., 2005; Kedersha et al., 2005), and can change in size and number depending on, or caused by manipulation of, rates of translation (Teixeira et al., 2005) or decay enzyme function (Teixeira & Parker, 2007). PBs are also a highly conserved phenomenon and have so far been observed in humans, yeast, *C. elegans*, *Arabidopsis*, *Xenopus*, *Drosophila*, and trypanosomes

(Kulkarni et al., 2010); however, so far the functional significance of P-bodies remains unknown.

The Sm and Sm-Like (Lsm) family of proteins are involved in RNA processing in eukaryotes, bacteria and archaea (Salgado-Garrido et al., 1999). These proteins share a unique Sm domain, containing Sm motifs 1 and 2, which allow for RNA binding and the formation of heptameric rings (Khusial et al., 2005). Lsm proteins are found in two major ring complexes: Lsm1-7 and Lsm2-8, where the presence of Lsm1 or Lsm8 differentiates the two complexes. The Lsm2-8 complex localizes to the nucleus and is involved in splicing (Spiller et al., 2007). The Lsm1-7 complex plays a role in the mRNA decay pathway and in PB formation in yeast (Decker et al., 2007; Ingelfinger et al., 2002, Reijns et al., 2008). Studies have shown that the Lsm1-7 complex localizes to PBs in the cytoplasm and preferentially binds the 3' end of mRNAs with shortened poly-A tails (Tharun & Parker, 2001). Additionally, Lsm1 is known to interact with the mRNA decay proteins Xrn1, Pat1 (Bouveret et al., 2000), and Dcp1/Dcp2 (Tharun et al., 2000; Tharun & Parker, 2001). Knockdown of Lsm1 results in the accumulation of capped mRNAs (Tharun et al., 2000) and inhibits ARE-mediated mRNA decay (Stoecklin et al., 2006).

Recently, the c-terminus of Lsm4 was shown to be important for PB formation in yeast through a Q/N-rich prion-like domain (Decker et al., 2007; Reijns et al., 2008). Interestingly, most organisms do not share this Q/N-rich region of Lsm4, instead they have a GR-rich c-terminus which, in humans, has been shown through mass spectrometry to be a likely site of arginine methylation (Brahms et al., 2001). I investigated the possible role for the human Lsm4 GR-rich c-terminus and how it may

compare or contrast to that of yeast Lsm4. I show that human Lsm4 is symmetrically dimethylated at specific arginine residues within the GR-rich region. After exploring many possible functions for this domain and its arginine methylation, I found that the GR-rich region was not required for Lsm4 to interact with Lsm1, the specific Lsm1-7 factor, and therefore is likely not required for its incorporation into the Lsm1-7 complex. Full-length Lsm4 is important for ARE-mRNA degradation, as knockdown of the Lsm4 protein results in less efficient decay; however, this does not seem to be dependent on the GR-rich region. Importantly, I also find that the human Lsm4 c-terminus does not seem to replicate its function in yeast, as the GR-rich c-terminus of human Lsm4 is neither required nor for the promotion of PB formation. Thus, PBs appear to have evolved to use different protein domains for their formation in different eukaryotes.

4.2 Results

The Lsm4 protein of most metazoans contains GRG-repeats in place of the Q/N-rich prion-like PB localization domain of *S. cerevisiae* Lsm4

Evidence suggests that a C-terminal Q/N-rich prion-like domain of the Lsm4 subunit of the *S. cerevisiae* Lsm1-7-Pat1 complex is important for mRNP assembly into PBs (Decker et al., 2007; Reijns et al., 2008). To begin to understand the mechanism of assembly of mRNPs into PBs in human cells, I compared the sequence of *S. cerevisiae* Lsm4 C-terminus to that of other eukaryotes. Interestingly, as seen in Figure 4-1, the Lsm4 C-terminus of most metazoans lack Q/N-rich regions, but instead contain characteristic GRG-repeats. This was interesting for several reasons. First, G3BP, a protein involved in mRNP assembly into SGs, contains similar C-terminal GRG-repeats,

although the functional significance of this domain is unknown (Tourrière et al., 2003). Second, I had previously observed the presence of protein arginine methyltransferase 5 (PRMT5), which symmetrically dimethylates arginines within GRG repeats (Brahms et al., 2001; Meister et al., 2001), in affinity-purified complexes of various PB components (Chapter 3, Table 3-1). Third, mass spectrometry analyses have previously shown that the C-terminus of human Lsm4 from HeLa nuclear extracts contained dimethylated arginines (Brahms et al., 2001). I therefore wanted to determine whether the C-terminus of human Lsm4 and arginine dimethylation recapitulates the function of the Q/N-rich domain of yeast Lsm4, or if this domain has other functions in the mRNA degradation pathway.

Human Lsm4 is symmetrically dimethylated at arginine residues within the GRG repeats

GR-rich motifs are recognition sites for a subset of PRMTs that, depending on the specific PRMT, symmetrically or asymmetrically dimethylate the arginine residues within or around the GR-rich motifs (Boisvert et al., 2005; Friesen et al., 2001; Pahlich et al., 2006). The Lsm4 c-terminus contains a GR-rich region, specifically several GRG-peptide repeats and evidence from mass spectrometry analyses suggested that nuclear human Lsm4 is dimethylated at arginine residues within its C-terminal GRG-repeats (Brahms et al., 2001). To verify whether human Lsm4 is symmetrically or asymmetrically dimethylated, we asked whether it reacts with antibodies specific for these modifications. Human embryonic kidney (HEK) 293S cell lines that stably express FLAG-tagged human Lsm4, or various mutant versions thereof were created (Figure

4-2A, cell lines created by Christian Damgaard). In these cell lines, expression of the FLAG-tagged Lsm4 proteins is regulated by tetracycline-inducible promoters. FLAG-tagged Lsm4 proteins were immunopurified and subjected to immunoblotting with antibodies specific for dimethylated arginines. As seen in the immunoblots in Figure 4-2B, wild-type human Lsm4 is recognized by the Sym10 antibody, which is specific for symmetrically methylated arginines (lane 1). When all arginine residues within the GRG region are mutated to lysines (GKG), or when the entire C-terminal GRG region is deleted (Δ CT), Lsm4 is no longer recognized by the Sym10 antibody (lanes 2 and 3). No detection was observed with the Asym antibody, which is specific for asymmetrically methylated arginines. These results verify that hLsm4 is symmetrically dimethylated at one or more arginine residues within the C-terminal GRG repeats.

The Lsm4 C-terminus is not required for association with Lsm1

Sm proteins form heptameric complexes via interactions between the Sm domains of individual subunits (Khusial et al., 2005). The C-termini of metazoan Sm proteins SmD1, SmD3, and SmB/B', like human Lsm4, contain GRG-repeats, and in humans and *Drosophila* (SmB) have previously been observed to contain symmetrically dimethylated arginines (Brahms et al., 2001; Brahms et al., 2000; Gonsalvez et al., 2006). Evidence in human cells suggest that Sm protein dimethylation is critical for their association with the survival of motor neurons (SMN) protein and assembly of Sm proteins into snRNP core complexes (Brahms et al., 2001; Friesen & Dreyfuss, 2000), although a similar dependence on methylation of Sm complex assembly was not observed in *Drosophila* (Friesen et al., 2001). Given the evidence that arginine

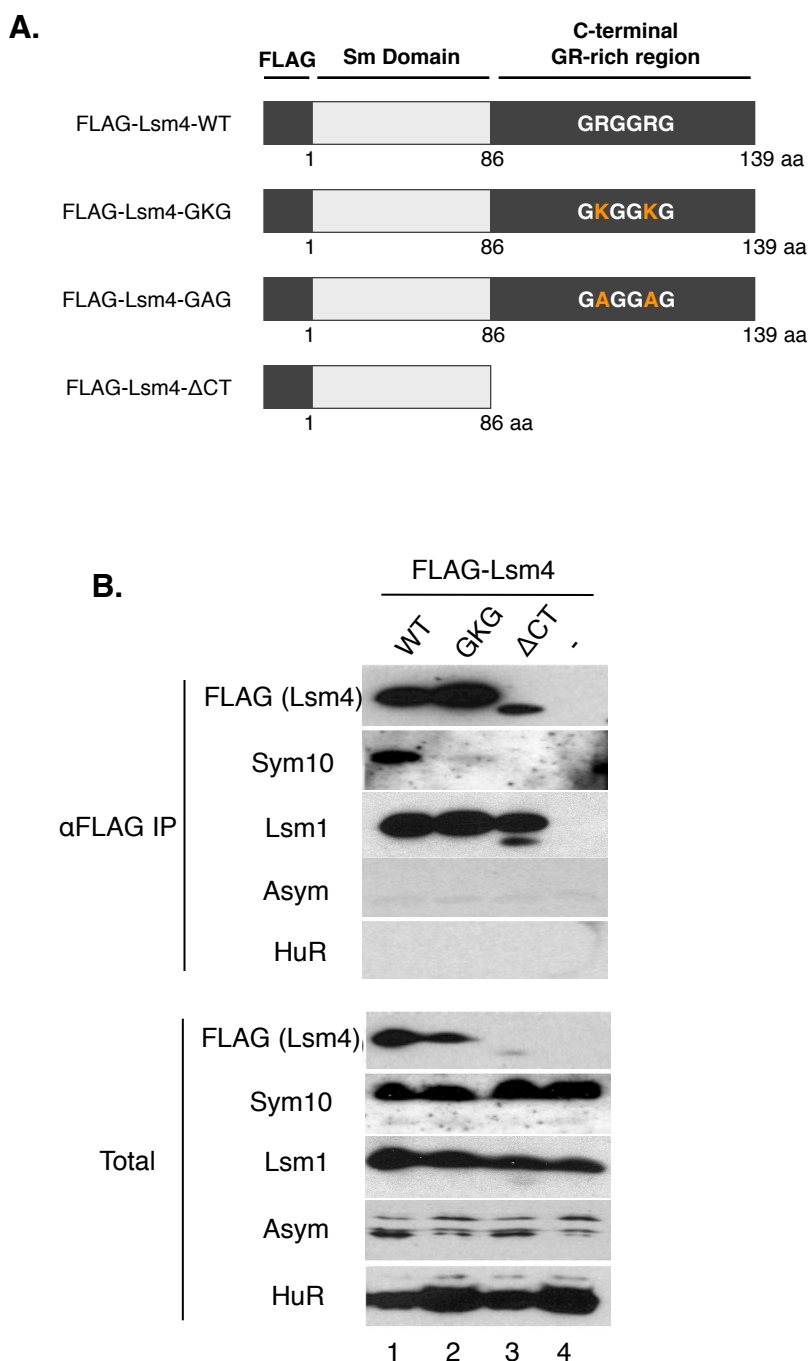


Figure 4-2. Human Lsm4 C-terminus is symmetrically dimethylated and not required for Lsm1 interaction

A) Schematic representations of the Flag-tagged Lsm4 constructs used. B) Western blot showing the anti-Flag immunoprecipitation of stably expressed Flag-Lsm4-WT, -GKG, - Δ CT, and an empty control vector in the top panel, and the total protein samples in the bottom panel. The samples were probed for the expression of the Flag-tagged Lsm4 protein (Flag-Lsm4), the presence of symmetrically dimethylated arginines (Sym10), and asymmetrically dimethylated arginines (Asym). The binding of HuR was probed as a negative control. (Assay done by Christian Damgaard).

methylation may mediate protein-protein interactions, we tested whether the methylation of the Lsm4 c-terminus was required for its incorporation into the Lsm1-7 complex.

We monitored the ability of endogenous Lsm1 to co-immunopurify with wild-type and mutant Lsm4 proteins. We chose to test interaction with Lsm1 because it is the only Lsm protein specific to the Lsm1-7 complex, whereas Lsms 2-7 are also a part of the Lsm2-8 complex. Furthermore, although the precise arrangement of Lsm proteins within the Lsm1-7 complex has not been determined, it has been predicted that Lsm4 and Lsm1 directly interact based on homology with Sm proteins (Beggs, 2005). As seen in the western blot for Lsm1 in Figure 4-2B, neither the Lsm4 C-terminus, nor its dimethylated arginines, is critical for association with Lsm1, as mutants lacking the C-terminus (Δ CT) or lacking the dimethylated arginines (GKG), retain the ability to interact with Lsm1 (compare lanes 2, 3 with lane 1). By contrast, Lsm1 does not co-purify with the beads when no FLAG-tagged Lsm4 is expressed (lane 4). Thus, the human Lsm4 C-terminus and its dimethylated arginines are not required for the association of Lsm4 with Lsm1. Previous work has shown the N-terminal Sm domains of Lsm proteins can mediate protein-protein interaction in core snRNP formation (Cooper et al., 1995; Hermann et al., 1995; Séraphin, 1995). Our results indicate that the same may be true for Lsm1-7 complex protein interactions. This suggests that Lsm4 likely assembles into the Lsm1-7 complex independently of its C-terminus, although we were unable to test other components of the Lsm1-7 complex as antibodies are unavailable.

Lsm4, but not its C-terminus, stimulates ARE-mediated mRNA decay

The Lsm1-7 complex has previously been implicated in human ARE-mediated

mRNA decay, as evidenced by the stabilization of an ARE-containing mRNA reporter upon Lsm1 knock-down (Stoecklin et al., 2006). To test whether Lsm4 and its C-terminus is important in ARE-mediated mRNA decay, three different β -globin ARE-reporter mRNAs were tested in pulse-chase mRNA decay assays. Upon Lsm4 knockdown (knockdown efficiency shown in Figure 4-3E), a moderate level of stabilization is observed for all three reporter mRNAs (Figure 4-3A-C) similar in extent to that observed previously upon Lsm1 knock-down (Stoecklin et al., 2006).

The largest increase in stability upon Lsm4 knock-down was observed for the β -globin reporter mRNA containing the ARE from c-Fos mRNA (reproducibly ≈ 2 -fold; Figure 4-3A). To test whether the C-terminus and/or dimethylated arginines of Lsm4 are required for its ability to stimulate ARE-mRNA decay, the ability of exogenous wild-type and C-terminal mutant Lsm4 proteins to substitute for endogenous Lsm4 in the decay of the β -globin c-fos-ARE mRNA was tested. As seen in Figure 4-3D, both wild-type and C-terminal mutant Lsm4 proteins lacking methylated arginines (GAG) or the entire C-terminus (Δ CT) can fully substitute for endogenous Lsm4 in β -globin c-fos-ARE-mRNA decay. Thus, while Lsm4 stimulates ARE-mRNA decay, the Lsm4 C-terminus and its dimethylated arginines are not required for this activity.

Human Lsm4 does not depend on its C-terminus for processing body association

I next wanted to know whether the dimethylated arginine-containing GRG-rich C-terminus of human Lsm4 plays a similar role in the assembly of mRNPs into PBs as the C-terminal Q/N-rich domain of Lsm4 does in *S. cerevisiae* (Decker et al., 2007; Ingelfinger et al., 2002; Reijns et al., 2008). To this end, I first tested whether global

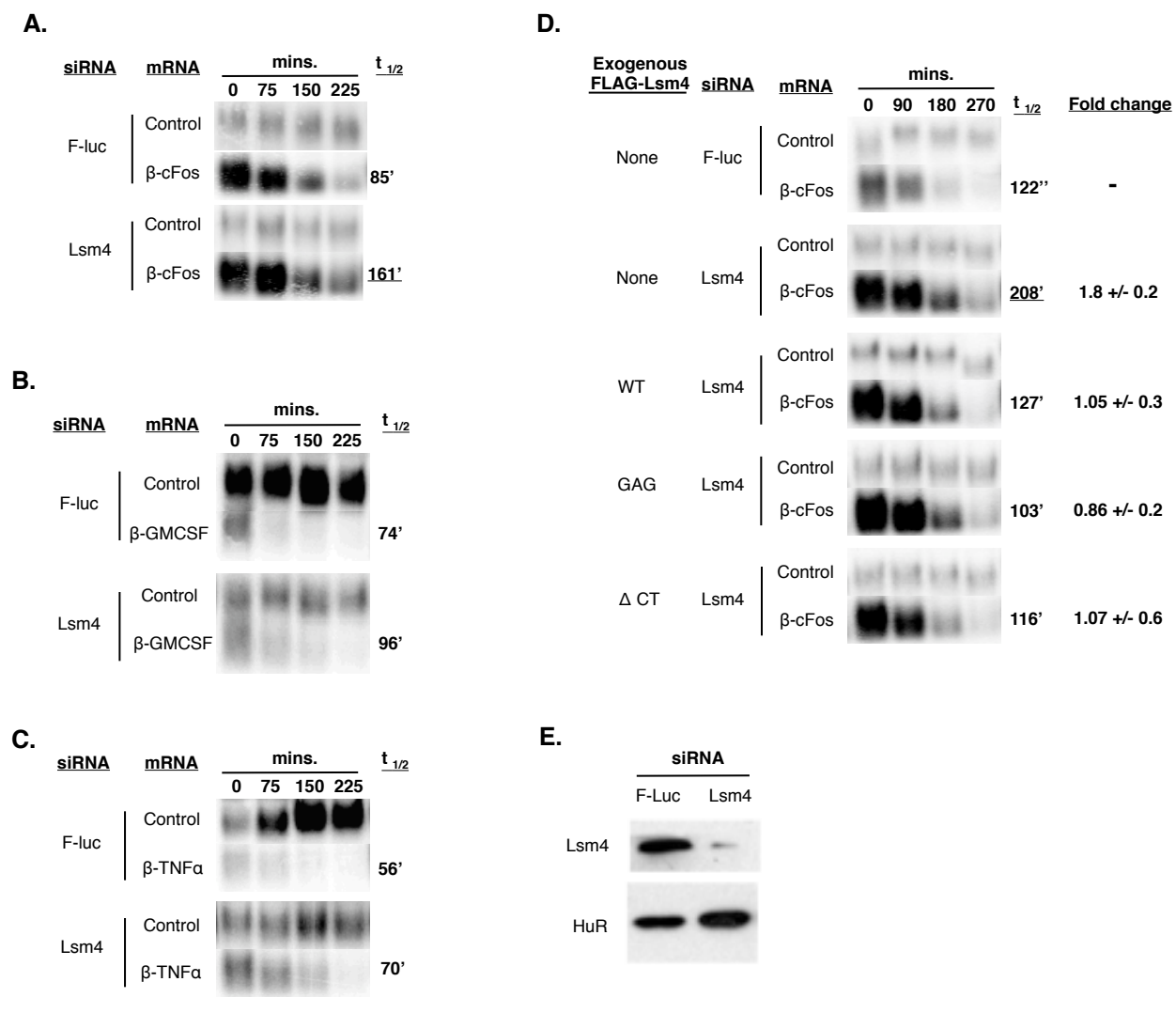


Figure 4-3. Lsm4, but not its C-terminus, is required for efficient ARE-mediated mRNA decay

Northern blot showing the rate of decay of the A) β -cFos, B) β -GMCSF, and C) β -TNF- α reporter mRNA in the presence of control siRNA (F-Luc) or siRNA directed against endogenous Lsm4, as indicated on the left. Reporter mRNA levels were compared and normalized to a stable mRNA reporter encoding full length β -globin (Control) and decay rates were calculated as $t_{1/2}$ as indicated on the right. Timepoints, in minutes, are indicated above the panel and refer to the time after transcription was stopped. D) Northern blot showing the rate of decay of the β -cFos reporter mRNA but with the addition of an “add-back” of transiently expressed siRNA resistant Flag-Lsm4-WT, -GAG, and - Δ CT where indicated, to samples where endogenous Lsm4 is knocked down using siRNA. The fold changes in half-life were calculated over 3 experiments and indicated on the right, +/- the standard deviation. E) Western blot showing siRNA knockdown of endogenous Lsm4 protein.

arginine methylation plays a role in human PB formation, by treating human HeLa cells with the drug AMI-1. AMI-1 is a global arginine methyltransferase inhibitor that binds the arginine-binding pocket of PRMTs (Cheng et al., 2004). As seen in the indirect immunofluorescence assays in Figure 4-4A, treatment for 1 hour with AMI-1 results in a dramatic reduction in the localization in PBs of all tested PB factors, including hDcp1a, Edc4 (as monitored by rabbit anti-Edc4 antibody, as well as human IC-6 serum), Lsm1, Lsm4, and Rck/p54. Thus, AMI-1 impairs PB formation. This could be a direct or an indirect effect of the inhibition of arginine methylation.

I next asked whether the methylated C-terminus of human Lsm4 is important for its ability to accumulate in PBs, as the C-terminal Q/N-rich region of Lsm4 is in yeast. Endogenous Lsm4 was knocked down in the HEK 293S cell lines stably expressing exogenous FLAG-tagged Lsm4 proteins, and exogenous FLAG-tagged wild-type Lsm4, Lsm4-GKG, Lsm4-GAG, or Lsm4- Δ CT (which are all produced from mRNAs resistant to the used siRNA) were expressed by the addition of tetracycline. Figure 4-4B shows that surprisingly, neither the dimethylated arginines nor the C-terminus is important for the localization of Lsm4 in PBs, as mutant Lsm4 proteins lacking the methylated arginines (Lsm4 GKG and GAG, panels 3 and 5, respectively), as well as Lsm4 lacking the entire C-terminus (Lsm4 Δ CT, panel 7) retain their ability to localize in processing bodies (compare with panels 4, 6, and 8). The mutant Lsm4 proteins also support the assembly of the decapping factor Edc4 into PBs as shown by IC-6 staining. These observations suggest that neither the dimethylated arginines, nor any other part of the C-terminus of human Lsm4 is necessary for its localization in PBs. This contrasts the importance of the yeast Lsm4 C-terminal domain in PB formation (Decker et al., 2007; Reijns et al.,

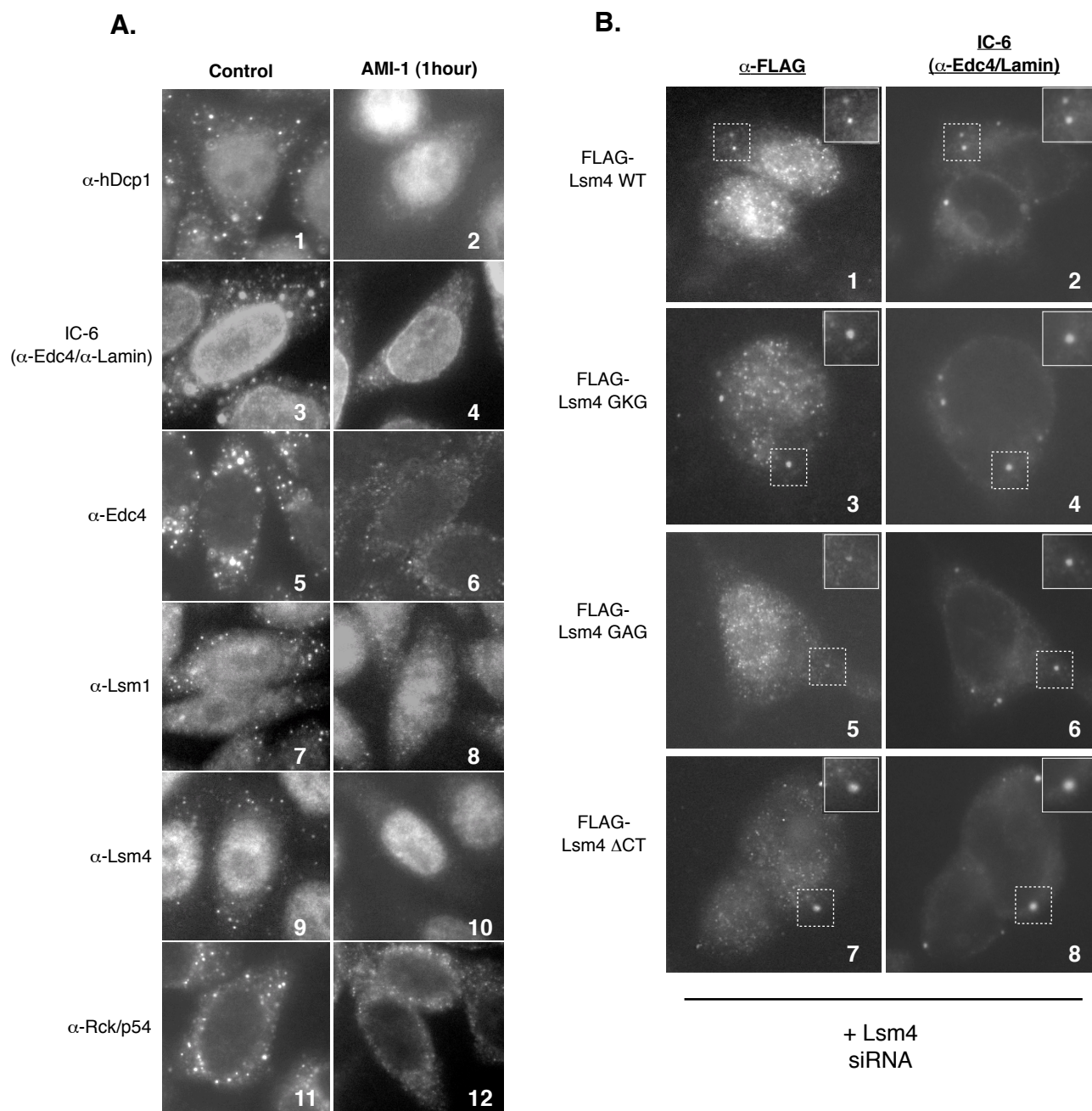


Figure 4-4. Global inhibition of arginine methylation results in PB dissociation and human Lsm4 does not depend on its C-terminus for processing body association

A) Indirect immunofluorescence assay showing HeLa cells that were untreated (panels 1, 3, 5, 7, 9, 11) or treated (panels 2, 4, 6, 8, 10) with 800 mM AMI-1 for 1 hour prior to harvest. The presence of P-bodies was monitored with antibodies directed against P-body components hDcp-1, IC-6, Hedls, Lsm1, Lsm4, and Rck/p54, as indicated to the left. B) Indirect immunofluorescence assay in HEK293S cells stably expressing siRNA resistant Flag-tagged Lsm4-WT, -GKG, -GAK, or -DCT as indicated on the left, in the presence of siRNA directed against endogenous Lsm4. P-bodies in the same cells were visualized using IC-6 serum which recognizes the proteins Hedls (panels 2, 4, 6, 8). Localization of the Flag-tagged Lsm4 constructs was visualized using antibodies against the Flag tag (panels 1, 3, 5, 7).

2008).

The Lsm4 C-terminus is not sufficient for PB localization

To further characterize the human Lsm4 protein and its role in PB formation, it was important to ask whether the C-terminal GR-rich region is sufficient to localize to PBs. To test this, the C-terminus of Lsm4 was fused to green fluorescent protein (GFP) and transfected into HeLa cells for visualization by immunofluorescence microscopy. As a positive control, the prion-related domain (PRD) of TIA-1, a known stress granule (SG) protein, was fused with GFP. The PRD of TIA-1 is a Q/N-rich region that has been shown to be sufficient to form into cytoplasmic foci and is important for SG assembly (Gilks et al., 2004; Kedersha et al., 1999). GFP alone was used as a negative control. As seen in Figure 4-5A, GFP fused to the Lsm4 C-terminal domain exhibits diffuse cytoplasmic staining and fails to localize in foci (panel 1). This should be compared to the ability of TIA-1 PRD to localize to distinct foci (panel 5). I further wanted to examine if arginine methylation of the C-terminus had any effect on its ability to localize to PBs, as arginine methylation of the wild-type Lsm4 c-terminus could possibly exclude it from localizing to foci. The GAG mutant c-terminus was fused to GFP and similarly transfected into HeLa cells for visualization by immunofluorescence microscopy. GFP-Lsm4 GAG-ct was also unable to localize into foci (panel 3). This contrasts with results seen in yeast, where GFP fused to the Q/N-rich Lsm4 c-terminus was sufficient to localize to cytoplasmic foci (Reijns et al., 2008). These data indicate that the c-terminus of human Lsm4 is not sufficient to localize to PBs and this inability is not due to arginine methylation.

Since PBs require mRNA to form, perhaps the inability of the Lsm4 c-terminus to

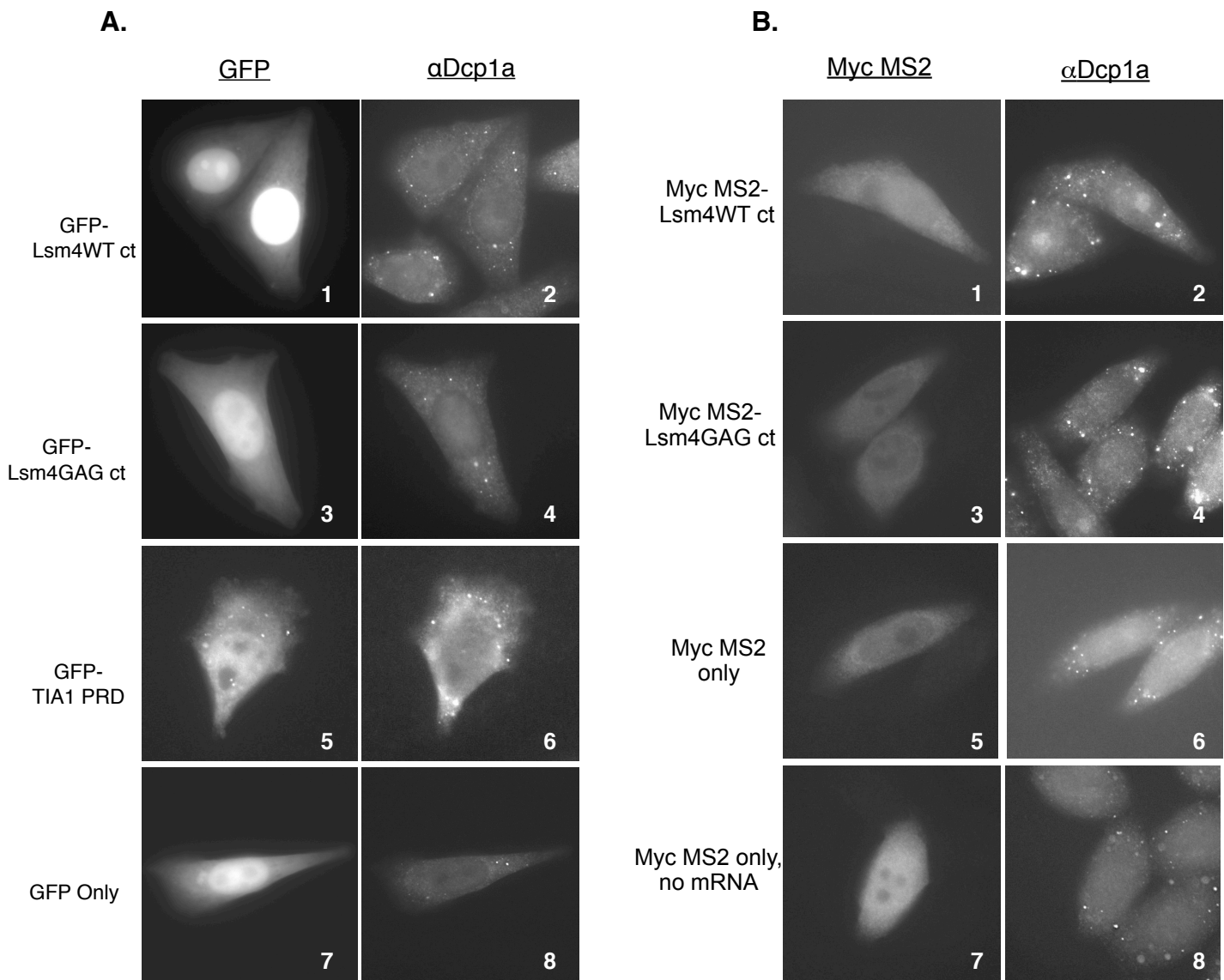


Figure 4-5. The C-terminus of Lsm4 is not sufficient for localization to foci

(A) Indirect immunofluorescence assays performed in HeLa cells to monitor localization of exogenously expressed GFP fused to the wild-type Lsm4 c-terminus (GFP-Lsm4WT ct, panel 1) and mutant Lsm4 c-terminus (GFP-Lsm4GAG ct, panel 3). GFP fused to the prion related domain of TIA-1 was used as a positive control for foci formation (GFP-TIA1 PRD, panel 5). GFP alone was expressed as a negative control (GFP only, panel 7). PBs were visualized with anti-hDcp1a antibodies (panels 2, 4, 6, 8). (B) Indirect immunofluorescence assay in HeLa cells to monitor the localization of exogenously expressed Myc-tagged MS2 coat protein fused to the wild-type Lsm4 c-terminus (GFP-Lsm4WT ct, panel 1) and mutant Lsm4 c-terminus (GFP-Lsm4GAG ct, panel 3) tethered to mRNA containing six MS2 coat binding sites that was co-expressed. Localization of empty Myc-MS2 plasmid was tested as a negative control both with (panel 5) and without (panel 7) co-expression of the MS2 coat binding site mRNA. Myc-MS2 constructs were visualized with anti-myc antibodies (panels 1, 3, 5, 7). PBs were visualized with anti-hDcp1a antibodies (panels 2, 4, 6, 8).

localize to foci is due to its inability to interact with mRNA, and therefore with other potential cofactors that might promote aggregation. To test if the C-terminus requires mRNA to localize to foci, the Lsm4 wild-type c-terminus and GAG mutant fused to Myc-tagged MS2 coat protein were transfected into cells co-expressing mRNA containing six MS2 coat protein binding sites to tether the Lsm4-c-terminus to mRNA. As seen in Figure 4-5B, tethering to mRNA is not sufficient to localize the Lsm4 c-terminus (panel 1) or the GAG-c-terminus mutant (panel 3) to foci. These results confirm that the c-terminus of human Lsm4 is insufficient to form foci, even when tethered to mRNA.

4.3 Discussion

Lsm4 is a component of two 7-member ring complexes: Lsm1-7, which localizes to the cytoplasm and is involved in mRNA decay; and Lsm2-8, which localizes to the nucleus and interacts with the U6 snRNP. It contains numerous GRG tri-peptide repeats in its c-terminus, a motif known to be a target of arginine methyltransferases. A previous study purified Lsm4 from HeLa nuclear extracts by anti trimethyl G-cap immunoaffinity chromatography, and mass spectrometry analysis revealed symmetrical dimethylation at arginine residues within the GR-rich c-terminus (Brahms et al., 2001). Here, through immunoprecipitation and western blotting with the Sym10 antibody, we confirmed that human Lsm4 c-terminus is symmetrically dimethylated.

Proteins involved in mRNA decay localize along with translationally repressed mRNPs in cytoplasmic foci called PBs. Recent work in yeast has shown that the Lsm4 protein contains a Q/N-rich prion-like domain that is important for mediating PB assembly (Decker et al., 2007). Moreover, the yeast Lsm4 c-terminus is sufficient to

aggregate into microscopically visible cytoplasmic foci (Reijns et al., 2008). I wished to assess corresponding functions for the methyl-arginine domain of human Lsm4 in the context of PB assembly and mRNA decay. Here, I show that the c-terminus of human Lsm4 is not necessary for PB localization, as Lsm4 Δ ct in cells depleted of endogenous Lsm4 can localize to PBs. Additionally, I find that the c-terminus of human Lsm4 is not sufficient to localize to foci. These results suggest a divergent role for the Lsm4 protein in human cells. Perhaps, the assembly of PBs has evolved to rely on different protein domains in different eukaryotes.

An interesting observation in Figure 4-4A shows that inhibiting global arginine methylation with AMI-1 resulted in the loss of visible PBs. This may indicate that protein arginine methylation of Lsm4 or other proteins may indeed be important for PB formation. However, since PBs are sensitive to changes in translation, it is therefore possible that inhibiting arginine methylation affects translation rates, thereby affecting PB assembly in a more indirect manner.

There are conflicting reports from yeast as to whether the c-terminus of Lsm4 is important for efficient mRNA decay. Decker et al. found that deletion of the c-terminus resulted in no effect on decay, while a different study by Reijns et al. found decay rates to be inhibited (Decker et al., 2007; Reijns et al., 2008). Here, I find that deletion of the c-terminus of human Lsm4 has no significant effect on the decay of the β -cFos reporter mRNA. I did find, however, that the Lsm4 protein is important for efficient ARE-mediated decay, as knockdown of endogenous Lsm4 resulted in a \approx 2-fold increase in the stability of ARE mRNA reporters. This is comparable with the finding that knockdown of Lsm1 inhibits ARE-mRNA decay (Stoecklin et al., 2006), and adds further evidence that the

Lsm1-7 complex plays an important role in mRNA decay.

Interestingly, the GR-rich domain found in Lsm4 is not present among the other Lsm1-7 and Lsm2-8 complex proteins, yet it is a conserved feature among Lsm4 homologues in most eukaryotes, with the exception of *S. cerevisiae* and *D. melanogaster* (contains only one GRG tripeptide). As such, one would expect this domain to be of functional significance. Indeed, deletion of the Lsm4 c-terminus inhibits its ability to interact with the survival of motor neuron (SMN) protein (Friesen & Dreyfuss, 2000). SMN interacts with Sm proteins to facilitate the formation core U snRNPs and is important for splicing (Brahms et al., 2001; Friesen & Dreyfuss, 2000). In addition, the Lsm4 c-terminus is sufficient to mediate this interaction (Friesen & Dreyfuss, 2000) and in the presence of the methyltransferase inhibitor SAH, interaction of Lsm4 with SMN was inhibited (Brahms et al., 2001). This suggests a role for the Lsm4 c-terminus within the context of splicing and its presence in the nuclear Lsm2-8 complex. Our data shows that the function of cytoplasmic Lsm4, as a part of the Lsm1-7 complex and mRNA decay, relies instead on its N-terminus. Taken together, this implies that the methylation status of the Lsm4 c-terminus may direct its assembly into either the Lsm1-7 or Lsm2-8 complex. It is as yet unclear how Lsm proteins coordinate their assembly into one complex or the other. One could speculate that methylated Lsm4 becomes incorporated into Lsm2-8, and non-methylated Lsm4 is incorporated into Lsm1-7. This would be important to investigate for future studies. Similarly, it would also be important to elucidate when Lsm4 is methylated and what percentage of the protein is methylated at a given time, as this is currently unknown. Here, I was able to test the effects of non-methylatable Lsm4 mutants (Lsm4-GKG and Lsm4-GAG). It has been

previously reported that mutating arginine residues to phenylalanine may mimic constitutive arginine methylation (Mostaqul Huq et al., 2006). It would be worthwhile to test the effects of a constitutively methylated form of Lsm4 in PB assembly and mRNA decay.

4.4 Materials and Methods

Plasmids and stable cell lines

Plasmids expressing FLAG-tagged wild-type Lsm4 protein, FLAG-Lsm4-WT, were created by inserting the open reading frame of human Lsm4 into the vectors pcDNA3-FLAG (Lykke-Andersen, 2002) between *Bam*HI and *Apal* sites. Four point mutations in the Lsm4 coding sequence, which do not alter the protein product (codons 83-86 encoding Val-Val-Ala-Lys were changed from to 5'-GTGGTGGCCAAG-3' to 5'-GTCGTCGCGAAA-3'), were generated using site-directed mutagenesis (Quickchange, Stratagene) to create constructs expressing Lsm4 mRNA resistant to an siRNA targeting endogenous Lsm4 (see below). Plasmids expressing FLAG-tagged Lsm4 containing C-terminal mutations, FLAG-Lsm4-GKG and FLAG-Lsm4-GAG (all GRG motifs changed to GKG or GAG, respectively), were generated by using a 193 nucleotide antisense DNA oligo (IDT) containing the desired mutant Lsm4 C-terminus sequence as a template for PCR. The oligo contains *Hind*III, *Eco*RI and *Not*I sites in the 3' end (corresponding to the 5' end of the coding sequence) and *Sal*I and *Apal* sites in the 5' end (the 3' end of the coding sequence). The 193-mer oligos were amplified by PCR using appropriate 5' and 3' end primers. PCR products were inserted between *Not*I (which occurs immediately upstream of the C-terminal region of human Lsm4 cDNA)

and *Apal* (occurring downstream of the open reading frame) sites of pcDNA3-FLAG-Lsm4-WT. Plasmids expressing FLAG-tagged Lsm4 lacking the entire C-terminus, FLAG-Lsm4- Δ CT, were created by cutting pcDNA3-FLAG-Lsm4-WT with *NotI* followed by religation after gel-purification to remove the C-terminal Lsm4 fragment, which is flanked by *NotI* sites.

Stable HEK293S cell lines were generated using the Flp-In T-Rex system (Invitrogen) by site-specific integration using the pcDNA5-frt-TO expression plasmid. pcDNA3-FLAG-Lsm4-WT, -GKG, -GAG-, or - Δ CT (described above) were cut and inserted into the *HindIII* and *Apal* sites of pcDNA5-frt-TO to create pcDNA5-frt-TO-FLAG-Lsm4-WT, -GKG, -GAG, and - Δ CT plasmids.

Plasmids expressing Green Fluorescent Protein (GFP) N-terminally fused to the Lsm4 wild-type C-terminus, and the Lsm4 GAG mutant C-terminus, were generated by PCR amplification of the Lsm4 C-terminal region (amino acids 86-139) of FLAG-Lsm4-WT and FLAG-Lsm4-GAG constructs described above and insertion into the *EcoRI* and *Apal* sites of the plasmid pcNEGFP (Franks & Lykke-Andersen, 2007). Plasmids expressing Myc-tagged MS2 coat protein fused to the Lsm4 wild-type C-terminus, and the Lsm4 GAG mutant C-terminus, were generated by inserting the same PCR products described above into the plasmid pcNMS2-Myc at the same restriction sites used to generate the GFP fusion proteins. The pcNMS2-Myc plasmid is based on the pcNMS2-Flag plasmid described earlier (Lykke-Andersen & Wagner, 2005), but contains a myc tag in place of a Flag tag.

Plasmids expressing reporter β -globin mRNAs β GAP-UAC (pc- β GAP UAC), β -GMCSF (pPC- β wt-ATGMCSF), and β -TNF- α (pPC- β wt-TNF- α) have been described

previously (Franks & Lykke-Andersen, 2007; Lykke-Andersen & Wagner, 2005). The plasmid expressing reporter mRNA β -cFos (pPC- β wt-cFos) was generated by inserting annealed oligos containing the c-fos ARE (Chen et al., 1995) between *Xba*I and *Apa*I sites of the pPC- β wt plasmid (Fenger-Grøn et al., 2005). The plasmid expressing β globin mRNA containing MS2 coat binding sites (MS2-6bs) has been described previously (Lykke-Andersen et al., 2000).

RNA interference

HEK293S stable cell lines (Figs. 2B and 4B) and HeLa Tet-off cells (Clontech) (Fig. 3) were seeded onto 3.5-cm wells at ~20% confluency in full medium (Dulbeccos modified eagle medium (DMEM), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin solution) for 24 hours prior to the first transfection. The following day, the first transfection was conducted using siLentFect Lipid (Bio-Rad) according to manufacturers protocol and cells were transfected with either control siRNA targeting Luciferase, 5'-CGUACGCGGAAUACUUCGAUU-3' + 5'-AAUCGAAGUAUCCGCGUACG-3', or siRNA targeting Lsm4, 5'-AGGAGGAGGUGGUGGCCAAUU-3' + 5'-AAUUGGCCACCACCUCUCCU-3', at a final concentration of 20 nM. After 24 hours, cells were washed once in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ pH 7.4) and fresh full medium was added to each well. The next day, a second transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturers protocol with siRNA targeting Luciferase or Lsm4 at a final concentration of 20 nM and, for Fig. 3 only, 500 ng ARE-mRNA reporter plasmid, 80 ng control plasmid (pc β GAP),

and 420 ng empty pcDNA3-FLAG plasmid per well. Tetracycline was added to a final concentration of 50 ng/ml for each well to repress ARE-reporter mRNA expression. 24 hours later, cells were washed once in PBS and fresh full medium was added back, with the addition of 50 ng/ml tetracycline per well in the case of the mRNA decay assays. For indirect immunofluorescence assays, cells were split onto 8-well chamber slides. The following day, experiments were carried out as described for indirect immunofluorescence, immunoprecipitation, or pulse-chase mRNA decay assays.

Indirect immunofluorescence

For Figure 5A, HeLa cells were transfected with 1 μ g of pcNEGFP, pcNEGFP-Lsm4 WT CTD, -Lsm4 GAG CTD, or -TIA1 PRD plasmids using TransIT HeLaMonster reagent according to manufacturers protocols (Mirus). In Figure 5B, HeLa cells were transfected with 1 μ g MycMS2, MycMS2-Lsm4 WT CTD, or -Lsm4 GAG CT with (panels 1-6) or without (panels 7,8) co-transfection of 1 μ g of plasmid expressing β -globin mRNA containing MS2 coat binding sites (6bs) also using TransIT HeLaMonster reagent according to manufacturers protocols (Mirus). Empty pcDNA3-FLAG plasmid was added to some reactions to reach 2 μ g of total plasmid. HeLa cells (Fig. 4A and 5) or HEK293S stable cell lines (Fig. 4B) in DMEM/10% FBS were then split to chamber slides when ~50% confluent. For Fig. 4B, tetracycline was added to each well at 50 ng/ml to induce transcription of the stably transfected FLAG-tagged Lsm4 constructs. 24 hours later, cells were fixed with 4% paraformaldehyde/PBS for 15 minutes at room temperature, and permeabilized and blocked with PBS/1% goat serum/0.1% Triton X-100 for 20 minutes. Cells in Figure 4A were incubated with 800 μ M AMI-1

(Calbiochem) for 1 hour prior to fixation and permeabilization. After fixation and permeabilization, cells were incubated for 1 hour with PBS/1% goat serum containing either rabbit anti-hDcp1a (1:200) (Lykke-Andersen & Wagner, 2005), rabbit anti-Hedls (1:8,000) (Fenger-Grøn et al., 2005), human IC-6 serum (1:8,000; a generous gift from Drs. Marv Fritzler and Ed Chan), rabbit anti-Lsm1 (1:200) or -Lsm4 (1:200; generous gifts from Dr. Tilmann Achsel), rabbit anti-Rck/p54 (1:500; DDX6 antibody, Bethyl Laboratories), or mouse anti-Myc (1:2,000; Cell Signaling) antibodies. For Fig. 4B, cells were incubated with rabbit anti-FLAG antibody (Sigma) and human IC-6 serum at 1:500 and 1:8,000 dilutions, respectively. Following removal of the primary antibody and three washes with PBS/1% goat serum, cells were incubated for 1 hour with PBS/1% goat serum containing 4 µg/ml secondary anti-rabbit or anti-human antibodies labeled with either Texas-Red or Alexa 488 fluorophore (Molecular Probes). Cells were washed 6 times with PBS/1% goat serum followed by one wash with water and covered with Vectashield (Vector Labs) and a coverslip prior to fluorescence microscopy.

Immunoprecipitation assays

For the immunoprecipitation of stably expressed FLAG-tagged Lsm4-WT, -GKG, and -ΔCT in Figure 2, HEK293S stable cell lines were grown in 3.5-cm wells to approximately 50% confluency in DMEM/10% FBS and the expression of FLAG-tagged Lsm4 proteins were induced by addition of 50 ng/ml tetracycline 24 hours prior to harvest. Cells were washed and scraped off using a rubber policeman in PBS and, after pelleting, were lysed in 800 µL of hypotonic gentle lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride

(PMSF), 2 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ of aprotinin) for 10 minutes on ice. NaCl was added to 150 mM and RNase A was added to 125 $\mu\text{g/ml}$, and the extracts were incubated for 5 minutes on ice. Lysates were cleared by centrifugation at 14,000 rpm at 4°C for 15 minutes, and 800 μl supernatant was loaded onto pre-washed anti-FLAG M2 agarose (Sigma, 20 μl bead volume) and nutated at 4°C for 4 hours. Beads were washed eight times with 1 ml of ice-cold NET-2 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Triton X-100). Bound protein was eluted by addition of 25 μl of SDS sample buffer (100 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulphate (SDS), 1% mercaptoethanol, 0.02 % bromophenol blue) to the beads. Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using primary antibodies rabbit anti-Lsm1 (1:500), rabbit anti-Lsm4 (1:10,000) (both generous gifts from Dr. Tilmann Achsel), rabbit anti-FLAG (1:1000; Sigma), Sym10 (1:800; Fisher), Asym (1:500; Asym24, Millipore), or mouse anti-HuR (1:20,000; described previously by Gallouzi et al., 2000) in blot buffer (100mM Tris HCL pH 7.5, 150mM NaCl, 0.1% Tween 20), containing 5% milk powder and 0.02% NaAz. Secondary antibodies were HRP-conjugated goat anti-mouse (1:20,000) or donkey anti-rabbit (1:30,000) (Peirce) in blot buffer and 5% milk powder. Blots were then incubated with chemiluminescent substrate (Peirce) according to manufacture's protocol and exposed to film for visualization.

Pulse-chase mRNA decay assay

Human HeLa Tet-off cells (Clontech) in full media at ~30% confluency in 3.5-cm wells were transfected in the presence of 50 ng/ml tetracycline, using TransIT HeLaMonster reagent according to manufacturers protocols (Mirus), with a total of 2 μg

of plasmid, including 80 ng internal control expression plasmid, expressing β -GAP mRNA, and 500 ng reporter plasmid expressing β -cfos (Figs. 3A and D), β -GMCSF (Fig. 3B), or β -TNF- α (Fig. 3C). In addition, either control siRNA targeting Luciferase or siRNA targeting *Lsm4* was transfected as described above (Fig. 3E) and exogenous protein expression plasmids were included at 500 ng for pcDNA3-Flag-*Lsm4*-WT, -GAG, and - Δ CT. Empty pcDNA3-FLAG plasmid was added to each reaction to 2 μ g of total plasmid. 40 hours after transfection, transcription of the reporter mRNAs was initiated by washing the cells with PBS and adding 2 ml DMEM/10% FBS containing no tetracycline. Six hours later, tetracycline was added to 1.0 μ g/ml to stop transcription. Cells were washed with PBS and taken up in 1 ml of Trizol (Invitrogen) starting (0-min time point) 30 min after addition of tetracycline, and then every 75 or 90 minutes as indicated in Figure 3. Total RNA was prepared according to manufacturer's protocols and analyzed by Northern blotting as described previously (Lykke-Andersen et al., 2000).

Chapter 5

Discussion and Future Perspectives

Cells rely upon various levels of regulation to ensure genetic fidelity. The strict control of DNA replication, transcription into mRNA, and its subsequent translation exists to maintain optimal protein levels at a given time. Just as mRNA synthesis is important for the control of gene expression, the degradation of mRNA is similarly crucial to allow the cell to rapidly adapt to its needs. The importance of mRNA decay is highlighted by the various diseases caused by improper control of mRNA decay.

It has been observed that mRNAs interact with various proteins and can assemble into mRNP granules, some of which are microscopically visible. The ability for translationally repressed mRNAs to co-localize with factors involved in mRNA decay in PBs, demonstrates an important role for mRNPs in the control of mRNA fate. Cytoplasmic granules containing other factors involved in the control of post-translational gene expression have been observed in neurons, germ cells, and during cell stress (for review see Anderson & Kedersha, 2009). Given that the ability to form mRNP granules is a conserved phenomenon, it is likely that they serve an important function for the cell; therefore, it is important that we understand how these granules are formed and why.

The mechanism by which PBs assemble is still a point of much research. Given that the control of mRNA degradation is a highly regulated process, it would make sense for the cell to also regulate the localization of mRNA decay factors in PBs. The cytoskeleton is a likely candidate for this role, and when I began this research it was unknown whether or not the cytoskeleton was involved in PB assembly. Recent studies have demonstrated various lines of evidence indicating that the cytoskeleton does play a role in PB dynamics, although it does not seem to be required for overall PB

assembly. Live cell imaging of PBs reveals they are mostly stationary, but on occasion exhibit movements that appear to be cytoskeleton-directed. Microtubule disruption was found to inhibit this movement of PBs in human cells (Aizer et al., 2008), and cause an increase in PB formation in both yeast and human cells (Aizer et al., 2008; Sweet et al., 2007). Investigations into cytoskeletal motor proteins have found that dynein, a minus-end directed motor, and kinesin, a plus end-directed motor, may play opposing roles in enhancing PB assembly (Loschi et al., 2009). Myosin 2p partially colocalizes with PBs and co-purifies with Dhh1 (Rck/p54) and Lsm1 in yeast (Chang et al., 2008).

Here, I have presented further evidence that the cytoskeleton is unlikely to be critical for the regulation of overall PB assembly, but may play other roles. My finding that the localization of GM-CSF ARE mRNA was inhibited upon cytoskeleton disruption argues that localization to PBs by the cytoskeleton may be transcript- or mRNP-specific. This suggests that not all PBs are made equal, and perhaps depending on what mRNPs are localized there, some PBs may be linked to the cytoskeleton. It would be informative for future studies to test a wider range of transcripts for the ability to localize to PBs and then determine whether this is cytoskeleton dependent. If successful, one could compare sequence elements and identify co-purifying proteins in the hopes of establishing domains and/or co-factors responsible for cytoskeleton-dependent PB localization. It is important to note that stress and changes to RNA metabolism have an effect on processing bodies, and that disruption to the cytoskeleton may cause pleiotropic effects. In the future, more direct methods would be useful to draw a definitive conclusion on the role of the cytoskeleton plays in PB dynamics.

Work in yeast provides evidence that protein-protein interactions, specifically domains that promote aggregation or dimerization, may contribute to PB assembly. Several yeast PB factors contain Q/N-rich prion-like domains, such as Lsm4. Additionally, yeast Edc3 contains a Yjef-N dimerization domain in its c-terminus. Both of these domains been found to be required for PB formation (Decker et al., 2007). Interestingly, human Lsm4 does not have the Q/N-domains found in the yeast counterpart. Instead, human Lsm4 has a GR-rich arginine methylation domain in place of the Q/N prion domain. Although protein arginine methylation has been implicated in modulating protein-protein, protein-DNA, and protein-RNA interactions (reviewed in Lee & Stallcup, 2009), I have found that in the case of Lsm4, the arginine methylation domain is not necessary or sufficient for PB assembly. Instead this domain may be an important factor in determining its localization to either the Lsm1-7 or Lsm2-8 complex, although this has to be investigated further.

Taken together, it is likely that human PB assembly is controlled through different protein interactions than in yeast. The function of Lsm4 may have transferred to other proteins in humans, perhaps the proteins Pat1, Edc4, and GW182. These proteins contain Q/N-rich regions, which may play important roles in the assembly of mRNPs into PBs (Behm-Ansmant et al., 2006; Eulalio et al., 2009; Haas et al., 2010; Jinek et al., 2008). Edc4 is especially intriguing given that there is no known yeast homologue. Although the proteins involved may differ, PB assembly in both yeast and humans is likely not controlled by one, or even two, proteins. Rather, it is more likely that PB assembly occurs through the coordination of many proteins. This idea is supported by the fact that translationally repressed mRNAs from several different mRNA decay

pathways are able to localize to PBs. The mRNPs from ARE-, miRNA-, and NMD-mediated decay pathways all can assemble into PBs, and as such might each require unique protein interactions to assemble into a PB.

Another key question that remains to be answered is what overall cellular function do PBs serve. The answer will give us insight into the mRNA decay pathway and may also shed light onto the functional significance of other mRNP granules, such as SGs. PBs require mRNA to form, harbor decay enzymes, and mRNA decay intermediates are found there. This suggests that mRNA decay can actively occur in these granules, however, there is no direct evidence to confirm this. In any case, mRNA decay does not rely on the presence of microscopically visible PBs, as when they are abolished, tested decay rates are unaffected (Decker et al., 2007; Eulalio et al., 2007). This also raises the question as to what defines a functional processing body. Perhaps the reason that efficient mRNA decay can occur without the presence of PBs is that there may actually be smaller sub-microscopic PB assemblies that can still function. If it is true that PBs are not needed for efficient decay, then what other purpose could they serve? Three possible roles have been suggested. PBs may serve to sequester mRNA decay machinery in foci to prevent promiscuous decay. Alternatively, they may be sites of storage for translationally repressed mRNPs when cells lack sufficient decay enzymes to process them. By keeping them to discreet foci, the mRNAs may be effectively inhibited from entering the actively translating pool of mRNA. Finally, they may be a way to ensure more efficient decay by having all of the necessary decay enzymes and enhancer proteins in close proximity to the mRNAs that have been targeted for decay.

As of yet there is no direct evidence that favors either idea. So how does one go

about resolving the issue of PB assembly and function? So far, at least 70 proteins have been identified to localize to PBs (Kulkarni et al., 2010) and the identification of new PB proteins are continually being reported in the literature. By characterizing each new protein, we gain insight into the inner workings of PBs, and this may one day lead to a definitive PB function. To this end, a major goal is to elucidate the complete protein inventory of PBs. Partial PB purification has been achieved in the past (Teixeira et al., 2005), and our lab has been successful in identifying novel PB factors in human cells (Fenger-Grøn et al., 2005). Here, I was able to show that proteins from the methylosome complex interact with known PB proteins as potential new PB factors. This finding could indicate that arginine methylation plays a role in mRNA decay. Although this does not seem to be the case with Lsm4 methylation, it may be that other as yet unidentified PB factors contain similar GR-rich methylation domains that are required for PB assembly or mRNA decay. The recent discovery of Rap55A in PBs supports this idea. Rap55A contains an N-terminal Lsm14 domain and a C-terminal GR-rich domain, and knockdown of Rap55 leads to a loss of PBs (Marnef et al., 2009; Yang et al., 2006). An interesting question for future studies would be to test whether Rap55a is methylated and, if so, ask if it is important for PB assembly. The continued identification and characterization of other such PB components will undoubtedly further our understanding of PBs, mRNA decay, and the overall regulation of gene expression.

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