Title
Investigations of mRNP regulation through post translational modifications to the RNA binding proteins PABP and Lsm4

Permalink
https://escholarship.org/uc/item/33s061qc

Author
Arribas-Layton, Marc

Publication Date
2015

Peer reviewed|Thesis/dissertation
Investigations of mRNP regulation through post translational modifications
to the RNA binding proteins PABP and Lsm4

A dissertation submitted in partial satisfaction of the requirements for
the degree Doctor of Philosophy

in

Biology

by

Marc Arribas-Layton

Committee in Charge:

Professor Jens Lykke-Andersen, Chair
Professor Eric Bennett
Professor Tracy Johnson
Professor James Kadonaga
Professor Miles Wilkenson

2015
The Dissertation of Marc Arribas-Layton is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

______________________________________________________________

______________________________________________________________

______________________________________________________________

______________________________________________________________

______________________________________________________________

Chair
# TABLE OF CONTENTS

SIGNATURE PAGE ......................................................................................................................... iii

TABLE OF CONTENTS ....................................................................................................................... iv

LIST OF FIGURES ............................................................................................................................ vii

LIST OF TABLES ............................................................................................................................... ix

ACKNOWLEDGEMENTS .................................................................................................................... x

VITA ................................................................................................................................................... xi

ABSTRACT OF THE DISSERTATION ................................................................................................. xii

CHAPTER I - The dynamic life of eukaryotic mRNPs ................................................................. 1

CHAPTER II - Structural and functional control of the eukaryotic mRNA decapping machinery .................................................................................................................. 3

Abstract .......................................................................................................................................... 3

Introduction ...................................................................................................................................... 4

The Dcp1-Dcp2 decapping complex ............................................................................................. 4

Catalytic enhancers of decapping ............................................................................................... 5

Cis-elements and trans-factors that promote mRNA-specific decapping by Dcp2 ....................... 9

mRNA Decapping and P Bodies .................................................................................................. 10

Additional decapping enzymes ................................................................................................. 10

Looking forward at decapping .................................................................................................... 11
CHAPTER III - The C-terminal RGG domain of human Lsm4 promotes processing body formation stimulated by arginine dimethylation.................. 15

Abstract .................................................................................................... 15

Introduction .............................................................................................. 16

Results ..................................................................................................... 18

Discussion ............................................................................................... 23

Materials and Methods............................................................................. 27

Acknowledgements.................................................................................. 33

References .............................................................................................. 45

CHAPTER IV - PNRC2 has a decapping activation motif independent of Dcp1 interaction domain ........................................................................................................... 50

Abstract.................................................................................................... 50

Introduction .............................................................................................. 50

Results ..................................................................................................... 52

Discussion ............................................................................................... 54

Materials and Methods............................................................................. 57

Acknowledgements.................................................................................. 62

References .............................................................................................. 63

CHAPTER V - Investigating Post Translational Modifications to PABP .............. 64
LIST OF FIGURES

Figure 2.1. The crystal structures of Dcp1 and Dcp2 proteins......................... 6
Figure 2.2. The structures of the enhancers of decapping Edc3, Dhh1 and Pat1 8
Figure 2.3. General principles of decapping complex recruitment.................. 9
Figure 2.4. P body dynamics ........................................................................ 10
Figure 3.1. The RGG domain of Lsm4 is required for visible PBs .............. 35
Figure 3.2. The RGG domain of Lsm4 is required for interaction with
HAT1/RBBP7 but is not necessary for association with Lsm1-7 components or
decapping factors ................................................................................ 36
Figure 3.3. The RGG domain of Lsm4 is not required for mRNA decay or
translational repression ......................................................................... 37
Figure 3.4. Lsm4 RGG domain arginines are required for interaction with
Hat1/RBBP7 but dispensable for Lsm1-7 and decapping factor association ..... 38
Figure 3.5. Lsm4 RGG domain arginines are required for PB accumulation ..... 39
Figure 3.6. PRMT5 is required for PB formation......................................... 40
Figure 3.7. Hat1 depletion or overexpression does not affect visible PBs ...... 41
Figure 3.8. Lsm4 RGG domain arginines are required for association with
HAT1/RBBP7 but not Lsm1-7 and decapping factor association.................. 42
Figure 3.9. The Lsm4 RGG domain is not required for mRNA decay or
translational repression ......................................................................... 43
Figure 3.10. The Lsm4 RGG domain is not sufficient for granule formation .... 44
Figure 4.1. PNRC2 YAG motif is needed to stimulate decapping .............. 59
Figure 4.2. PNRC2 Y93A co-localizes to PBs ............................................. 60
Figure 4.3. PNRC2 depletion or overexpression does not affect decay of an ARE reporter ............................................................................................................... 61

Figure 5.1. PABP mutants behave as control in sucrose gradients............... 75

Figure 5.2. PABP mutants form SGs in response to heat shock ................. 76

Figure 5.3. PABP R455/R460 methylation under control or stress conditions.... 77

Figure 6.1. PRMT5 depletion increases Hat1 association with Lsm4 WT ....... 91
LIST OF TABLES

Table 5.1. PABP mutants gradients.................................................................... 74
ACKNOWLEDGMENTS

Thanks to my PI, Jens Lykke-Andersen. He is an amazing mentor, and always strives to get the best results out of every situation and person in the lab.

A very heartfelt thanks to every member of the JLA lab during my time there. I would not have succeeded without them, and I will miss seeing them every day.

Thanks to my friends and cohort of students at UCSD. I would not have succeeded without them also.

A special thanks to members of the JEB and JRN groups that I overlapped with.

Thanks to my families, Kaitlyn, Taco & Bandido, and my parents and brothers for always supporting me and encouraging me.

Chapter II, in full, is a reprint of the material as it appears in Arribas-Layton Marc; Wu Donghui, Lykke-Andersen Jens; Song Haiwei. (2013) Structural and functional control of the eukaryotic mRNA decapping machinery, *Biochimica et Biophysica Acta*, 1829(6-7), 580–9. The dissertation author was a primary author of this paper along with Donghui Wu.

Chapter III, in large part, is currently being prepared for publication of the material. Arribas-Layton, Marc; Dennis, Jackie; Bennett, Eric; Damgaard, Christian K; and Lykke-Andersen, Jens. The dissertation author was the primary author of this material.

Chapter IV contains a figure from my collaborators, Jeffery Mugridge and John Gross. The dissertation author was the primary author of this material.
VITA

2002 Bachelor of Science, California Institute of Technology
2004 Master of Science, Columbia University
2015 Doctor of Philosophy, University of California, San Diego

PUBLICATIONS


Chapter I is an introduction to the unifying theme of my thesis, competition between translation initiation and mRNA decay with a focus on my proteins of interest.

Chapter II is a reprint of a review co-authored by myself and Donghui Wu. The review covers structural insights into the mechanisms of decapping, catalytic decapping enhancers, and enhancers that function primarily by repressing translation.

Chapter III focuses on the role of the decay factor Lsm4 in regulating Processing Bodies (PBs). PBs are cytoplasmic foci that contain mRNA decay
factors decay intermediates. PBs are lost when human cells are depleted of Lsm4, which contains an arginine/glycine rich RGG C-terminal domain that has been shown to undergo arginine dimethylation. Using immunofluorescence and Lsm4 mutants, I show that arginine methylation, likely by the methyltransferase PRMT5, in the RGG domain of Lsm4 is critical for PB accumulation. Assays for mRNA decay and translational activity, and mass spectrometry analysis all suggest that the RGG domain is not required for efficient decay, translation inhibition or decay factor recruitment.

Chapter IV shows the results of a collaboration investigating the decapping enhancer PNRC2. PNRC2 can interact with and stimulate the activity of the Dcp1/Dcp2 decapping complex. Disrupting this interaction leads to a loss in decapping stimulation. However, Dr. Jeffery Mugridge (Gross lab, UCSF) discovered that PNRC2 contains a separate YAG motif found in other decapping activators and we wondered if this motif is important for PNRc2 function. Using in vitro decapping assays, Dr. Mugridge shows that mutations in the YAG motif abrogate decapping stimulation, while I used immunofluorescence microscopy to show that a YAG mutant is able to maintain co-localization with Dcp1a in PBs, unlike PNRC2 mutants unable to bind Dcp1a.

Chapter V focuses on PABP, a highly conserved protein that binds to the 3’ poly(A) tail of nearly all mRNA and stimulates translation. We wondered if modifications to PABP could regulate mRNP function similar to how histone modifications regulate transcription. I used PABP mutants and assays designed to identify protein modifications to investigate this question.
Chapter VI is a look at the most pressing questions arising from my research.
CHAPTER I – The dynamic life of eukaryotic mRNPs

Pre-mRNA is transcribed in the nucleus where the majority undergoes addition of a 5’ cap, splicing, and 3’ cleavage and polyadenylation. Following this maturation process, mRNAs are exported to the cytoplasm where they can be actively translated, stored in a translationally repressed state, or targeted for degradation.

Actively translating mRNA is bound on the 5’ cap the eIF4F translation initiation machinery and on the 3’ poly(A) tail by PABP. These proteins act synergistically to stimulate active translation. While there are many quality control pathways that exist to rid cells of aberrant transcripts, the majority of mRNA in eukaryotic cells will undergo turnover through a deadenylation initiated decay process. There is evidence that this process starts with a slow, non-processive shortening of the poly(A) tail that eventually switches to a rapid, processive deadenylation to a short A tract incapable of PABP binding, with a loss of PABP presumably slowing translation initiation. After deadenylation, most transcripts will not be further degraded in the 3’ direction, as the short A tract can be bound and protected by the Lsm1-7 complex, a heptameric ring that can recruit further mRNA decay factors. Decapping enhancers can associate with the deadenylated mRNA decay intermediate and prime it for decapping by the Dcp1/Dcp2 complex. These intermediates can accumulate in concentrated cytoplasmic foci termed processing bodies, and there is evidence that deadenylated transcripts
can somehow reenter active translation. Following decapping, decay can proceed, irreversibly, through 5’ exonucleolytic decay.

While we know about many of the individual mechanisms involved in the deadenylation initiated decay process, there are gaps that need to be addressed. What role does PABP protein play in regulating mRNPs fate, as it can help promote translation, but can also interact with deadenylases? How are PABP and the translation initiation machinery, which can hinder the decapping reaction, removed to allow for decay? What role do processing bodies play in the lifespan of mRNPs, and how are they regulated? My graduate work initially looked at these broad questions. As I began to gather promising data, my project moved from looking at the effects of post translational modifications on PABP regulating its function, to the role of Lsm4, a member of the Lsm1-7 complex, in regulating processing bodies. Though the experience I gained studying the decay pathway I was also in a position to collaborate on a project investigating the properties of a recently characterized decapping enhancer, PNRC2.
CHAPTER II – Structural and functional control of the eukaryotic mRNA
decapping machinery

Abstract

The regulation of mRNA degradation is critical for proper gene expression. Many major pathways for mRNA decay involve the removal of the 5' 7-methylguanosine (m7G) cap in the cytoplasm to allow for 5'-to-3' exonucleolytic decay. The most well studied and conserved eukaryotic decapping enzyme is Dcp2, and its function is aided by co-factors and decapping enhancers. A subset of these factors can act to enhance the catalytic activity of Dcp2, while others might stimulate the remodeling of proteins bound to the mRNA substrate that may otherwise inhibit decapping. Structural studies have provided major insights into the mechanisms by which Dcp2 and decapping co-factors activate decapping. Additional mRNA decay factors can function by recruiting components of the decapping machinery to target mRNAs. mRNA decay factors, decapping factors, and mRNA substrates can be found in cytoplasmic foci named P bodies that are conserved in eukaryotes, though their function remains unknown. In addition to Dcp2, other decapping enzymes have been identified, which may serve to supplement the function of Dcp2 or act in independent decay or quality control pathways. This article is part of a Special Issue entitled: RNA Decay mechanisms.
Review

Structural and functional control of the eukaryotic mRNA decapping machinery

Marcos Arribas-Layton a,1, Donghui Wu b,1, Jens Lykke-Andersen a,*, Haiwei Song b,**

a University of California San Diego, Division of Biological Sciences, 9500 Gilman Drive, La Jolla, CA 92039, USA
b Laboratory of Macromolecular Structure, Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673, Singapore

ARTICLE INFO

Article history:
Received 5 October 2012
Received in revised form 15 December 2012
Accepted 17 December 2012
Available online 31 December 2012

Keywords:
Decapping
Dcp2
Dcp1
Enhancer of decapping
mRNA decay

ABSTRACT

The regulation of mRNA degradation is critical for proper gene expression. Many major pathways for mRNA decay involve the removal of the 5'-7-methylguanosine (m7G) cap in the cytoplasm to allow for 5'-to-3' exonucleolytic decay. The most well studied and conserved eukaryotic decapping enzyme is Dcp2, and its function is aided by co-factors and decapping enhancers. A subset of these factors can act to enhance the catalytic activity of Dcp2, while others might stimulate the remodeling of proteins bound to the mRNA substrate that may otherwise inhibit decapping. Structural studies have provided major insights into the mechanisms by which Dcp2 and decapping co-factors activate decapping. Additional mRNA decay factors can function by recruiting components of the decapping machinery to targeted mRNAs. mRNA decay factors, decapping factors, and mRNA substrates can be found in cytoplasmic foci named P bodies that are conserved in eukaryotes, though their function remains unknown. In addition to Dcp2, other decapping enzymes have been identified, which may serve to supplement the function of Dcx2 or act in independent decay or quality control pathways. This article is part of a Special Issue entitled: RNA Decay mechanisms.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

The removal of the mRNA cap by the process of decapping is a critical step during the degradation of eukaryotic mRNAs. Concurrency with transcription, eukaryotic mRNAs are capped on the 5' end with a 7-methylguanosine (m7G) cap. The m7G cap promotes post-transcriptional gene expression at multiple levels [1]. In the nucleus, the m7G cap associates co-transcriptionally with the nuclear cap-binding complex (CBC), which stimulates pre-mRNA splicing and mRNA export [2]. After nuclear export, the CBC is exchanged for translation initiation factor 4F (eIF4F), which plays a critical role in translation initiation of most mRNAs [3]. In addition to serving as a ligand for the cap-binding proteins, the m7G cap protects mRNAs from nuclear and cytoplasmic 5'-to-3' exonucleases [4]. Thus decapping of mRNA exposes the mRNA to degradation from the 5' end and, in the cytoplasm, simultaneously shuts down translation initiation. Depending on the specific pathway, decapping can be the first, an intermediate, or the last step in mRNA decay [5–7]. Multiple decapping enzymes have been characterized which differ in their cellular localization and substrate specificities. In this review we will discuss current structural and functional insights into the mechanism and control of one of the key complexes in mRNA decapping, the highly conserved Dcp2 decapping machinery.

2. The Dcp1–Dcp2 decapping complex

The most well-characterized and widely conserved eukaryotic decapping enzyme is Dcp2. Dcp2 was originally identified as a decapping cofactor in the budding yeast Saccharomyces cerevisiae [8]. In subsequent biochemical studies, Dcp2 from human, budding yeast, the nematode Caenorhabditis elegans and the plant Arabidopsis thaliana were shown to have intrinsic decapping activity, releasing m7GDP from m7G-capped RNAs [9–12]. Consistent with its role in the 5'-to-3' mRNA decay pathway, purified recombinant Dcp2 is unable to effectively hydrolyze unmethylated cap or free GTP, and shows poor activity on short m7G-capped oligonucleotides [13]. Dcp2 contains a Nudix domain (originally termed MutT) [8,9], which is found in enzymes that hydrolyze nucleoside diphosphates linked to other moieties [14], and is responsible for the catalytic activity of Dcp2 [15,16].

An essential cofactor for Dcp2 in vivo in budding yeast is Dcp1. Dcp1 was originally described as the catalytic decapping protein, and S. cerevisiae strains defective for Dcp1 are highly deficient in decapping [13]. Dcp2 directly interacts with Dcp1 [17,18] and, while recombinant yeast Dcp2 has intrinsic decapping activity in vitro, it is greatly stimulated by Dcp1 [10]. Though the Dcp1–Dcp2 complex is conserved among eukaryotes, metazoans may require additional factors to stimulate Dcp1–Dcp2 interaction [17,18].
2.1. Structural insights into the Dcp1–Dcp2 decapping complex

Dcp2 catalyzes cap hydrolysis in the 5′ to 3′ mRNA decay pathway in a divergent metal ion dependent reaction [9]. Sequence alignment reveals that the N-terminal region of Dcp2 is conserved while its C-terminal region is divergent containing anywhere from ~175 amino acids in human to nearly 700 amino acids in budding yeast [9]. The conserved N-terminal region of Dcp2 is featured by the presence of the Nudix fold which is flanked on both sides by two additional conserved regions known as Box A and Box B [9] (Fig. 1A). Box A has been implicated in facilitating the interaction of Dcp2 with Dcp1 while Box B has been shown to be important in RNA binding [20,21]. This has recently been confirmed by NMR chemical shift perturbation experiments in which residues important for RNA binding have been mapped to Box B in the Nudix domain of S. cerevisiae Dcp2 [15].

Crystallographic analyses revealed that the conserved N-terminal region of Dcp2 from the fission yeast Schizosaccharomyces pombe forms a bilobed architecture with an N-terminal regulatory domain (NTD) preceding a classic Nudix domain [21] (Fig. 1B). Box A lies in the NTD while the Nudix fold together with Box B constitutes the Nudix domain characterized by the canonical α7/α8 sandwich structure [21]. The Nudix motif forms the catalytic core of Dcp2 by acting through conserved glutamate residues within this motif. These glutamate residues are required for coordination of the divalent Mn2+ or Mg2+ ion during the catalytic reaction [10,20]. The NTD alone has no detectable catalytic activity in vitro but can affect the decapping efficiency of Dcp2 and is indispensable for decapping in vivo [21]. Moreover, the NTD mediates the binding of Dcp2 to Dcp1 and is required for Dcp1 to stimulate Dcp2 activity.

Dcp2 is an RNA binding protein and prefers longer RNA substrates for efficient decapping [10,16,20]. Structural analysis of the Dcp1–Dcp2 complex reveals that the RNA substrate is bound in a channel on the surface of Dcp2 with the cap structure in the active site and the body of the RNA wrapping across the Nudix domain and a channel along the Box B. The minimum length of the RNA substrate was predicted to be 12 nucleotides for efficient binding to Dcp2 [17]. The requirement for a longer RNA in addition to the cap structure for substrate recognition by Dcp1–Dcp2 has been suggested to prevent accidental decapping of translating mRNAs on which translation initiation complexes are assembled [19].

Dcp1 is a small protein containing an EHV1 domain [22], which generally serves as a protein–protein interaction module [23]. Three conserved patches have been identified on the crystal structure of yeast Dcp1 with patch 1 structurally corresponding to the proline-rich sequence (PRS) recognition site of EHV1 domains in other proteins [24–27] (Fig. 1C). Mutations of the conserved residues in patch 1 do not affect either the physical interaction of Dcp1 with Dcp2 or the decapping activity of the Dcp1–Dcp2 complex in vitro [22]. Instead, patch 1 serves as a binding site for a subset of decappers of decapping (see below). In addition to the well-conserved EHV1 domain, Dcp1 contains a C-terminal trimerization domain which is conserved in metazoans but absent in fungi (Fig. 1A). Crystal structures of the Dcp1–trimerization domain from human and Drosophila melanogaster reveal an antiparallel assembly comprised of three kinked alpha-helices [28]. Mutations that disrupt trimerization prevent Dcp1 from being incorporated into active decapping complexes and impairs mRNA decapping in vivo [28]. This finding reveals an unexpected connection and complexity of the mRNA decay network in metazoans.

The cocystal structure of the S. pombe Dcp1–Dcp2 complex (Fig. 1E and F) shows that Dcp2 binds to Dcp1 through the Dcp2 NTD and that the three conserved patches in Dcp1 are not involved in Dcp2 binding [17]. Instead, Dcp1 uses the N-terminal helix α1 and the loop between β5 and β6 that are only conserved in yeast species to recognize Dcp2. Consistent with this observation, mutations in S. cerevisiae Dcp1 that affect decapping in vivo are mapped to the N-terminal helix of Dcp1 [29] and yeast two-hybrid assays revealed that mutations in the N-terminus of the yeast Dcp1 disrupt its interaction with Dcp2 and hence decapping [17]. These results explain why stable, direct interactions between Dcp1 and Dcp2 in human and nematode are not observed [12,18] and why additional factors such as Edc4 (also called Hedls or Ge-1, or VARICOS in Arabidopsis) stimulate the interaction between Dcp1 and Dcp2 in higher eukaryotes [30,31].

The S. pombe Dcp1–Dcp2 complex was shown to exist in open and closed conformations (Fig. 1E and F) within the crystal, both of which were confirmed to be functionally relevant states as their existence in solution was verified by small angle X-ray scattering (SAXS) [17]. In the open or extended conformation, Dcp2 in the complex resembles the structure of the apo-Dcp2 [21] and adopts a dumbbell conformation. In contrast, in the closed complex Dcp1 and the Dcp2 NTD were shown to be in close proximity to the Nudix domain to form a compact structure. Structural and biochemical data indicated that the closed complex is, or closely resembles, the catalytically more active form of the enzyme [17]. Most recently, NMR spectroscopy [32] showed that Dcp2 exists in a conformational equilibrium in solution between the open and closed states in the absence of ligand, and the open-to-closed conformational switch is mediated by a gatekeeper tryptophan (Trp43). Further enzyme kinetics and NMR experiments [33] revealed that Trp43 promotes formation of the composite active site (see below) and allows Dcp1 and bound coactivators to enhance the catalytic step.

Several lines of evidence suggest that the conformational switch between open and closed states is required for efficient decapping. First, Dcp1 and the NTD of Dcp2 are essential for decapping in budding yeast [21,34]. Second, mutations in prolines of the interdomain linker block closure, hinder decapping in vitro, and cause an accumulation of Dcp2 in P bodies (see Section 5) [21]. Third, the NTD of Dcp2 and Dcp1 together contribute a factor of 1,000 to the catalytic step [15,20] but are distinctly located from the active site in the open state of the Dcp1–Dcp2 complex [17]. Kinetic analyses indicate that Dcp1 affects the chemistry of the decapping reaction but not substrate binding [15]. Structural analysis suggests that Dcp1 activates Dcp2 by promoting and/or stabilizing the formation of the closed complex. Mutation of residues that block the formation of the closed complex result in the loss of decapping activity and the inability of Dcp1 to stimulate Dcp2 [17], thus confirming that efficient activation of Dcp2 by Dcp1 requires formation of the closed complex. However, how closure of the Dcp1–Dcp2 complex is linked to efficient cap hydrolysis remains elusive. Recently, Gross and colleagues [33] showed that cap recognition involves both the NTD and Nudix domains of Dcp2. Mutations in the cap-binding site on the NTD block closure and retard the catalytic step by two orders of magnitude whereas Dcp1 enhances the catalytic step by a factor of 10 and promotes closure, likely by stabilizing the closed conformation. These findings suggest that the conversion to the active form of Dcp2 is driven through specific cap recognition by the NTD. The resultant active form of Dcp2 harbors a composite active site with both domains sandwiching the cap.

3. Catalytic enhancers of decapping

The Dcp1–Dcp2 decapping complex is stimulated by multiple enhancers of decapping. The first enhancers of decapping to be described were the S. cerevisiae proteins, enhancer of decapping (Edc) 1 and Edc2, which were identified as high copy suppressors of Dcp1 and Dcp2 mutants, though deletion of Edc1 or Edc2 did not impair in vivo mRNA decay rates of tested reporter mRNAs [35]. Subsequent studies showed that Edc1 and Edc2 bind directly to RNA and can stimulate decapping in vitro [36].

No homologs of Edc1 and Edc2 have been identified in eukaryotes outside of budding yeast. By contrast, another enhancer of decapping, Edc3, is highly conserved among eukaryotes. Edc3 directly interacts with the Dcp1–Dcp2 complex [37]. Yeast Edc3 stimulates the decapping
Fig. 1. The crystal structures of Dcp1 and Dcp2 proteins. (A) Schematic diagrams of the domain organization of Dcp1 and Dcp2 proteins. (B) The crystal structure of the S. pombe Dcp2 (residues 1–266) in the apo form. (C) Crystal structure of S. cerevisiae Dcp1 (green) with the patch 1 region marked. (D) Crystal structure of the EVH1 domain of human Dcp1a (green) in complex with the PNRC2 peptide (pink). (E) and (F) The open and closed conformations of the Dcp1–Dcp2 complex with Dcp1 in green, Dcp2 NTD in orange, Nudix domain in cyan, and Nudix motif in red.

reaction in vitro [38,39] and yeast strains deleted for the Edc3 gene have decreased decapping rates [40]. Another enhancer of decapping, Edc4, appears to be restricted to metazoans [30–41]. The specific mechanism by which Edc4 stimulates decapping is unknown, but human and Arabidopsis Edc4 promotes the association between Dcp1 and Dcp2, and stimulates decapping by Dcp2 in vitro [30,31].

Another enhancer of decapping, Pat1, was initially identified in a yeast mutational screen as a protein that stimulates mRNA decapping [42], and later found to interact directly with Dcp1 and stimulate decapping by recombinant Dcp1–Dcp2 in vitro [39,43]. Pat1, and its orthologs in human and Drosophila, has been found in association with multiple decapping factors [43–45]. One of these is another
enhancer of decapping, the Lsm-1-7 complex [39]. In S. cerevisiae, Lsm1-7 associates with mRNAs that have undergone deadenylation [46], which is generally associated with activation of decapping [47]. Consistent with a role in deadenylation-mediated decapping, lsm1Δ and pat1Δ S. cerevisiae strains show an accumulation of deadenylated mRNA [43]. The purified Lsm1-7-Pat1 complex has intrinsic affinity for the 3' end of oligoadenylated mRNAs over polyadenylated mRNAs and binds preferentially to deadenylated mRNAs carrying a U-tract at their 3’ terminus over those that do not [48]. Another enhancer of decapping, the DEAD Box superfamily 2 helicase Dbh1 (called DXD6 or Rck/p54 in human and Me31B in Drosophila) was first identified as a decapping activator in S. cerevisiae as its depletion leads to the accumulation of capped reporter mRNA decay intermediates [40-51]. Dbh1, and its orthologs in other eukaryotes, have been identified in complex with multiple decapping factors, including Dcp2, Edc3 and Pat1 [39,44,45].

An important unresolved question is whether enhancers of decapping are globally involved in cellular decapping, or whether they act in mRNA-specific manners. Microarray studies analyzing mRNA expression in Drosophila S2 cells depleted for Dcp1, Edc3, or Edc4 indicated that Edc4 and Dcp1 generally regulate the same mRNAs, whereas only a subset of these mRNAs were regulated by Edc3 [52]. Thus, some enhancers of decapping might be specific for a subset of decapping substrates, whereas others may act more generally.

3.1. Structural insights into enhancers of decapping

Multiple structural studies have provided insights into the mechanisms by which enhancers of decapping stimulate the Dcp1-Dcp2 complex. The budding yeast decapping coactivators Edc1 and Edc2 were shown to enhance decapping activity of the Dcp1-Dcp2 complex by a 1000-fold through interaction with the EVH1 domain of Dcp1 [53]. These findings suggest that a crucial function of Dcp1 is to couple the binding of coactivators to substrate recognition and activation of Dcp2. Consistent with this notion, crystal structure of human Dcp1a in complex with proline-rich nuclear receptor coactivator 2 (PNRC2), a protein that stimulates decapping in the nonsense-mediated decay (NMD) pathway, shows that the proline-rich region of PNRC2 is bound to the EVH1 domain of Dcp1a with patch 1 mediating their interaction [54] (Fig. 1D). Structural and biochemical analyses of the human Dcp1a-PNRC2 complex [54] showed that both PNRC2 and Dcp1a in isolation stimulate decapping, and that PNRC2 works in synergy with Dcp1a to promote the decapping activity of Dcp2. In contrast to the mode of action of Edc1 and Edc2 [53], which stimulate decapping through Dcp1-mediated recruitment to Dcp2, PNRC2 stimulates decapping through direct binding to Dcp2. These results indicate that PNRC2 is a bona fide decapping coactivator in addition to its adaptor role in NMD.

The conserved enhancer of decapping Edc3 consists of an N-terminal divergent Lsm domain, a central FDP domain and a C-terminal Yef-N domain [55,56] (Fig. 2A). The N-terminal Lsm domain of Edc3 adopts a noncanonical Sm fold, which lacks the characteristic N-terminal α helix and remains monomeric in solution [57]. The crystal structure of human Edc3 revealed that the FDP domain is unstructured and the Yef-N domain adopts a divergent Rossmann fold that forms a dimer [58] (Fig. 2B). Residues involved in Edc3 dimerization are highly conserved in eukaryotes. Structure-based mutagenesis demonstrates that Edc3 dimerization is of functional importance for RNA binding. P-body formation and possible degradation of Rps28 mRNA, a specific substrate of Edc3 [59]. S. cerevisiae Sc65 (Lsm13), vertebrate RAP55 (Lsm14), D. melanogaster Trailer Hitch (Lsm15 or Tral), and C. elegans CAR-1 belong to the Scd6 family of proteins and share a number of sequence and functional features with Edc3 [55-57,59,60]. However, in contrast to Edc3, Scd6 shows only mild stimulation of the Dcp1-Dcp2 complex in vitro [37,61].

Moreover, the recent structure of yeast Edc3 Lsm domain in complex with a short helical leucine-rich motif (HLM) from Dcp2 was determined [37] (Fig. 2C). The HLM motif is located close to the C-terminal end of the Nudix domain and identification of its interaction with Edc3 highlights the regulatory role of Edc3 in decapping. Structure-based sequence analysis of the C-terminal unstructured extension of S. cerevisiae Dcp2 reveals additional HLMs, which can also interact with Edc3 and are essential for the localization of the Dcp1-Dcp2 complex to P bodies [37]. Not surprisingly, the Scd6 Lsm domain competes with Edc3 for the interaction with these HLMs [37]. Unexpectedly, in contrast to yeast, the HLM that mediates Edc3 binding is present in metazona Dcp1 but absent from Dcp2 [37].

The crystal structure of S. cerevisiae Dhh1 (Fig. 2D) shows that it consists of two RecA-like α/β domains with a unique arrangement in such a way that the two domains are linked through the interactions of the canonical helicase motifs [62]. Electrostatic potential mapping combined with mutagenesis revealed that motifs I, V, and VI are involved in RNA binding. Limited proteolysis of Dhh1 suggested that ATP binding enhances an RNA-induced conformational change. However, how the conformational change of Dhh1 upon ATP and RNA binding affects the decapping activity of the Dcp1-Dcp2 complex remains elusive. One possibility is that Dhh1 utilizes the energy of ATP hydrolysis to unwind RNA with secondary structures and/or to displace bound translation factors, thereby facilitating access of the Dcp1-Dcp2 complex to the cap structure of the mRNA substrate.

A previous study showed that fragments of Edc3 and Tra1 proteins covering the FDP motifs can coimmunoprecipitate with the C-terminal RecA-like domain of Me318, the Drosophila ortholog of S. cerevisiae Dhh1 [60]. The crystal structure of the C-terminal RecA-like domain of DDX5 (Fig. 2E), the human ortholog of Dhh1, in complex with the FDP motif of Edc3 [59] shows that the FDP motif adopts an α-helical conformation upon binding to DDX5, occupying a shallow groove opposite to the DDX6 surface involved in RNA binding and ATP hydrolysis. The interaction of DDX6 with the Edc3 FDP motif is required for its P body localization and its role in translation repression [59] (see below). Similarly, Tra1 also employs its FDP motif to interact with Me318, thus competing the interaction of Edc3 with Me318 in a mutually exclusive manner [59]. The biological significance of this competitive interaction remains to be characterized. Conceivably, the competition between Tra1 and Edc3 to associate with DDX6/Me318 may facilitate the formation of distinct decapping complexes that have different functions and/or target distinct sets of mRNAs.

The Lsm1-7-Pat1 complex plays important roles in mRNA decapping. Recently, a crystal structure of the C-terminal domain of human Pat1 (Pat1-C), which is the only structured domain of Pat1, was determined. Pat1-C folds into an α-α superhelix [63] (Fig. 2F). Structure-based mutagenesis shows that a conserved and basic surface patch of Pat1-C is required for its binding to RNA, Dcp2, Edc4 and Lsm1-7 [63]. While the Lsm1-7 complex is predicted to form a heteroheptameric ring [43,64-67], its subunits may form various subcomplexes. A crystal structure of Lsm3 from S. cerevisiae shows that it forms an octameric ring structure [68]. Recent crystallographic analysis combined with analytical ultracentrifugation of Lsm3, Lsm4 and a Lsm5/6/7 subcomplex from S. pombe indicates that these exist in solution as a heptamer, a monomer and a hexamer, respectively [69,70]. Moreover, like Lsm3 alone, the S. pombe Lsm2/3 subcomplex is also in a heptameric state in solution. Given that substructures or individual subunits of Lsm1-7 proteins have a propensity to form oligomers [68-70], it is unclear how the seven Lsm proteins assemble into the ring structure and whether subcomplexes exist in vivo as functional units. In addition, how Pat1 interacts with the Lsm1-7 complex and how this hetero-octameric complex modulates decapping remains to be understood.

3.2. Stimulation of mRNA decapping through mRNP remodeling leading to repression of translation initiation

The mRNA m1G cap is generally not free, but bound in the nucleus by the CBC and in the cytoplasm by the elf4E complex. The elf4E
cap-binding subunit of eIF4F can inhibit decapping by yeast Dcp2 in vitro [71,72], and eIF4G, which stimulates eIF4E binding to the cap, can further decrease the in vitro decapping rate [73]. This suggests that the translation initiation machinery can protect mRNA from decapping and that a competition between the translation initiation complex and decapping exists. Evidence supporting this can be seen in vivo with yeast strains harboring defective translation initiation factors. Expression of mutant translation initiation factors, including eIF4E and eIF4G, which slow the rate of translation, generally increases the rate of mRNA decay [74]. Thus, an important mechanism for enhancing decapping likely involves remodeling of the translation initiation complex associated with the mRNA 5' end to allow access for Dcp2 to the m^G cap. Indeed, a subset of enhancers of decapping also acts as repressors of translation.

One such decapping factor that functions as a translational repressor is Pat1. Budding yeast strains lacking Pat1 are unable to effectively inhibit translation in response to glucose deprivation, and overexpression of Pat1 leads to general translation repression [75]. These observations suggest that in addition to playing a possible role in directly recruiting or activating the decapping complex, Pat1 may promote decapping by stimulating remodeling or release of translation initiation components. Interestingly, in budding yeast Pat1 shows RNA-dependent association with the translation initiation factors eIF4E and eIF4G, as well as with the poly(A)-binding protein Pat1 [46]. By contrast, the Lsm1-7 complex fails to associate with eIF4E and eIF4G, even in strains lacking Dcp1, Dcp2, or Pat1 [46]. This is consistent with the idea that the association of the Lsm1-7 complex with mRNPs is accompanied by an eIF4F release step distinct from the catalytic activity of Dcp2. Recent observations that the human [45,76] and Drosophila [44] homologs of Pat1 also exists in complex with decapping factors, including Dcp2 and the Lsm1-7 complex, and stimulate decay of a tethered reporter mRNA shows that some of these functions have been conserved throughout evolution.
Human and Drosophila Pat1, unlike the budding yeast homolog, also interact with deadenylation factors in an RNA-independent manner, suggesting a function in both deadenylation and decapping [44,45]. Collectively, these observations suggest a possible role of the Pat1-5sml-1 complex in the release of translation initiation factors in preparation for decapping. However, given their link to deadenylation, an alternative possibility is that the release of eIF4G occurs as a secondary consequence of removal of the poly(A)-binding protein, which is known to stimulate cap-binding by the eIF4F complex [77].

Another enhancer of decapping, the DEAD box helicase Dhh1, may play an important role in mRNP remodeling. Dhh1 deletion and overexpression in budding yeast has similar effects as those observed for Pat1, namely decreased and increased translational repression, respectively [75], and Dhh1 inhibits translation in vitro [75]. The exact mechanism by which Dhh1 represses translation and how this relates to enhancement of decapping is not fully understood. In yeast, the effect of Dhh1 on translation may be to inhibit formation of a stable 48S translation pre-initiation complex [75], or it may function to slow the movement of ribosomes along the mRNA [78]. Surprisingly, tethered Dhh1 is able to repress translation and stimulate decay in yeast even when its ATPase activity is abolished, suggesting a conformational change may not be necessary for these activities [79].

In vitro studies have shown that mutant DDXX, the human ortholog of Dhh1, deficient in ATP hydrolysis can relax structured RNA in the presence of ATP but not ADP, suggesting that ATP hydrolysis may trigger a switch from an active to an inactive state [80]. DDXX function may also depend on its ability to oligomerize on mRNA, which has been observed in cells from both human and the frog Xenopus laevis [80,81]. In yeast, decapping of long non-coding (Inc) RNAs, which are capped and polyadenylated, is mediated by Dcp2 but unaffected by deletion of Dhh1 or Lsm1 suggesting these cofactors are not needed for decapping of these non-translated RNA species [82].

A number of decapping enhancers can also be found in complexes that appear to promote translation repression in the absence of decapping. For example, in D. melanogaster, C. elegans, and X. laevis, the Dhh1 orthologs Me31B, CGH-1, and Xp54, respectively, play important roles in translational repression and storage of mRNAs during development of the oocyte [81,83,84]. In D. melanogaster this involves a complex that also contains the eIF4E-binding protein CUP [83], and competitive binding of Me31B by either Trailer Hitch (Tral) or Edc3 may determine if an mRNA is translationally repressed or decapped [60]. Contrasting the activity of Dhh1 in yeast, Xp54 ATPase mutants stimulate the translation of a tethered reporter in X. laevis oocytes [85]. Homologs of these proteins have also been characterized in complex with decapping factors in other eukaryotes (see Section 3.1), but what dictates which complexes form on mRNAs under different conditions remains unclear.

Thus, multiple lines of evidence support that decapping complexes interfere with translation initiation. However, while decapping is generally in competition with translation initiation, evidence suggests that decapping can occur while mRNAs are still engaged with elongating ribosomes [86,87]. The specific mechanism by which decapping factors promote remodeling of translation initiation complexes, and what dictates whether complexes containing decapping factors repress translation only, or also activate decapping, remains to be answered.

4. Cis-elements and trans-factors that promote mRNA-specific decapping by Dcp2

Though decapping factors can stimulate the function of Dcp2 to initiate decapping and eventual 5′-to-3′ decay of mRNA substrates, regulation of Dcp2 is important to control which transcripts are decapped and degraded. One mechanism by which decapping by Dcp2 is thought to be regulated is through substrate-dependent recruitment. Several ways by which Dcp2 can be enlisted to decap specific substrates have been uncovered, including direct recruitment of Dcp2 to mRNA cis-elements, and recruitment via RNA binding proteins that interact with Dcp2 or other members of the decapping complex (Fig. 3).

The observation that Dcp2 has a preference for capped RNA substrates over free cap raised the possibility that Dcp2 has a preference for specific RNA sequences [20]. Consistent with this, the mRNA encoding the human exosome component Rrp41 was found to contain an element in the 5′ UTR which stimulates Dcp2 binding and rapid decapping when in close proximity to the mRNA cap [5]. This region was predicted to have a stem-loop structure and compensatory mutational analysis suggested that the secondary structure is important for Dcp2 recruitment and activation [68]. An interesting question is if this direct recruitment of Dcp2 to an mRNA is a widespread mechanism of mRNA decay regulation. A genome-wide analysis predicted hundreds of human mRNAs that could harbor similar stem loop structure in their 5′UTRs, and several of these showed enhanced decapping in vitro by Dcp2 [88]. Contrasting this, in C. elegans 70% of mRNA transcripts contain a 22-nucleotide splice leader sequence, which reduces their in vitro decapping rate compared to transcripts without the splice leader [12]. The sequence is not predicted to have a strong secondary structure and the mechanism by which decapping is inhibited is unknown. This is independent of the non-canonical trimethylated m7G cap found on trans-spliced C. elegans mRNAs, which is effectively hydrolyzed by budding yeast, human, and nematode Dcp2 [12].

In addition to direct RNA recruitment, the decapping machinery can be recruited to target mRNAs through RNA binding proteins. For example, as discussed in Section 3.1, evidence suggests that the Lsm1-7-Pat1 complex serves a general role in recruiting the decapping complex to deadenylated mRNAs, in what is thought to be a major pathway of mRNA decay in eukaryotes [47]. More recent studies have also presented evidence that the Lsm1-7 complex serves to recruit decapping factors to RNAs, including histone mRNAs, that have undergone tailing by unconventional terminal ribonucleotidytransferases [80,90].

Additionally, multiple examples exist for mRNA-specific RNA binding proteins that activate the decapping complex. For example, in S. cerevisiae, ribosomal protein (rp) S28b autoregulates its own mRNA by binding to the 3′UTR and activating decapping independently of deadenylation, through interaction of rpS28b with Edc3.

---

**Fig. 3.** General principles of decapping complex recruitment. mRNA cis-elements, such as the hairpin structure in Rrp41 mRNA, can recruit Dcp2 directly. RNA binding proteins (RNA-BPs) can stimulate decapping by recruiting the Dcp1-Dcp2 complex directly or through decapping enhancers.
and Dcp1 [91]. Another example in budding yeast is the Puf5 protein, a member of the PUF family of proteins that generally target substrate mRNAs for translational repression and/or mRNA decay [92,93]. Puf5 associates with the deadenylase Caf1/Pop2 and the decapping factor Dcp1 [94], and stimulates the deadenylation and decapping of target mRNAs [95].

In mammalian cells, multiple trans-factors are involved in degrading mRNAs containing destabilizing 3'UTR AU-rich elements (AREs) [96]. One such protein is tristetraprolin (TTP), which interacts with multiple mRNA decay factors [97-99], including Edc3 and Dcp2 components of the decapping complex, and stimulates the decapping activity of Dcp2 in vitro on ARE-containing RNA [30]. There is also evidence to suggest that decapping plays a role in mRNA silencing by micro (mi)RNAs. Experiments in Drosophila S2 cells indicated that depletion of Dcp2 resulted in an increase in both steady state miRNA-target reporter mRNA levels and translation [100,101]. In murine embryonic fibroblast cells hypomorphic for Dcp2, no distinguishable defect in silencing of a miRNA reporter mRNA was observed, unless the cells were also depleted for another cytoplasmic decapping enzyme, Nudt16 [102]. In human cells, DDX6 interacts with the mRNA effectors Ago1 and Ago2 in an RNA independent manner, and depletion of DDX6 diminishes repression of a miRNA target reporter [103]. Decapping is also activated in the nonsense mediated decay (NMD) pathway. The central factor in NMD, Upf1, interacts with Dcp2 and activates decapping [18,104]. In yeast, depletion of the decapping factor Dcp1 leads to a major defect in NMD [105,106]. In human cells, the interaction of Upf1 with Dcp2 is bridged by the protein PARN [107], and appears to serve as one of many mechanisms by which Upf1 can initiate mRNA degradation, which also includes endonucleolytic cleavage and deadenylation [108,109].

While a number of examples of recruitment of the decapping machinery by trans-factors have been documented, in most cases it remains to be established how the trans-factors communicate with decapping complexes (Fig. 3). Do they all communicate with a common decapping holoenzyme complex, or are there multiple inroads into the decapping machinery, for example through alternative decapping sub-complexes all leading to decapping by Dcp2? Moreover, in most cases, how critical decapping is as compared to the two other mechanisms of initiation of mRNA decay, deadenylation and endonucleolytic cleavage, remains poorly understood. Finally, could mechanisms other than mRNA-specific recruitment contribute to substrate specificity by the decapping complex, such as mRNA remodeling events that expose the mRNA cap?

5. mRNA decapping and P bodies

Dcp2 and many of its enhancers and cofactors can be seen to accumulate in discrete foci in the cytoplasm termed P bodies [110], which are conserved across eukaryotes [111,112]. Accumulating evidence suggests that P bodies are self-assembly RNA granules that primarily assemble from RNA decay intermediates that accumulate with decapping factors [111] (Fig. 4). Consistent with this, the depletion of factors that activate mRNA decay prior to the decapping step, such as deadenylasomes, reduces the number of visible P bodies in cells, while depleting cells of factors, such as Dcp2 or Xrn1, that enhance decapping or the subsequent 5'-to-3' decay step generally increases the size or number of detectable P bodies [75,110,113]. Moreover, mRNA decay intermediates have been localized to P bodies, and RNase treatment disrupts P bodies, suggesting that RNA is a necessary component for P body integrity [113-116]. In budding yeast, the decapping stimulator Edc3 and the Lsm4 subunit of the Lsm1-7 complex play critical roles in assembling mRNPs into P bodies, likely by promoting intermolecular contacts between mRNPs assembles with decapping complexes [49,117]. Ribosomal proteins, poly(A)-binding protein, and translation initiation factors, with the exception of eIF4E, are absent from P bodies [118,119], and translation elongation inhibitors generally rapidly disassemble P bodies [120], suggesting that mRNPs that assemble into P bodies need to be free of ribosomes. While P bodies may primarily consist of intermediates of mRNA decay, pulse transcribed reporter mRNAs that accumulate in P bodies during glucose starvation in budding yeast have been observed to reenter translation after glucose restoration, suggesting that decay is not the only fate for mRNAs that accumulate in P bodies [118]. Despite ongoing interest and research on P bodies, and despite their conservation in all tested eukaryotes, their function remains unclear, and there is currently little evidence that the assembly of RNA decay intermediates into P bodies plays a rate-limiting role in mRNA degradation.

6. Additional decapping enzymes

In addition to Dcp2, there are other eukaryotic proteins capable of hydrolyzing the mRNA cap. While Dcp2 was believed to be the only factor that catalyzed decapping in the 5'-to-3' mRNA decay pathway, a hypomorphic Dcp2 mouse showed no reduction in mRNA decay rates in cells where Dcp2 was no longer detectable [121]. By testing the ability of other mammalian Nudix domain-containing proteins to hydrolyze capped RNA, Nudt16 was discovered to have decapping activity similar to Dcp2 [121]. Nudt16 is the mammalian ortholog of the X. laevis protein X29, which was initially described to hydrolyze the m⁷G cap of the U8 snRNA and may function in the turnover of other nuclear capped RNAs [122]. Interestingly, unlike its Xenopus homolog, mammalian Nudt16 appears to be primarily cytoplasmic [121]. Further studies in mammalian cells suggest that Nudt16 and Dcp2 may be responsible for regulating distinct subsets of mRNAs with some shared targets [102].
In human cell lysates, a decapping activity was found to cosegregate with the 3′-to-5′ degradation machinery, the exosome. This activity, termed DcpS (Dcp Scavenger), is able to hydrolyze free mG-cap, which is the product of a complete mRNA degradation reaction by the exosome [123]. By contrast, DcpS shows no activity towards unmethylated GpppG. Contrasting with the Dcp2 reaction, which has a preference for long capped substrates and releases m7GDP, the product of the scavenger decapping reaction is m7GMP and DcpS is most effective in hydrolyzing free cap, though it shows some decapping activity on substrates of up to ten nucleotides [7].

Further decapping enzymes exist that may perform specialized functions rather than act as part of general mRNA decay pathways. The yeast factor Ral1, which exists in complex with the nuclear 5′-to-3′ exonuclease Ratt1 [124], is able to remove unmethylated GpppG from RNA substrates in vitro, and Ratt15 strains accumulate aberrantly capped mRNA substrates under certain stress conditions [125], suggesting that Ral1 has a role in a nuclear capping quality control mechanism. Recently, a yeast homolog of Ral1 has been identified that appears to have both decapping and exonucleolytic activity and was named Dso1 [126]. Ral1Δ,Dso1Δ double deletion mutants show an increase in endogenously aberrantly capped mRNA, but normal levels of properly capped mRNA, suggesting that like Ral1, Dso1 might function in capping quality control [126]. Recent work in humans has provided evidence that Dcp2 also has a role in nuclear decapping, in which it is in complex with Xrn2, the human ortholog of Ratt1, promotes premature transcription termination by activating 5′-to-3′ degradation of nascent transcripts [127].

7. Looking forward at decapping

Biochemical, cellular, and structural analyses have led to the identification and characterization of many of the central components involved in mRNA decapping allowing for the understanding of the basic principles of cytoplasmic decapping. While a lot has been learned in recent years about the mechanisms and regulation of these factors, there are many interesting open questions that need to be addressed. For example, although the structures of S. pombe Dcp2 in the apo form and in complex with Dcp1 have been determined, how the 5′ cap of the mRNA substrate is recognized and cleaved by Dcp2 still remains unclear. Because cap recognition occurs during the catalytic step and requires the presence of the RNA body, a high resolution crystal structure of Dcp2, or the Dcp1–Dcp2 complex, with a bound capped RNA would be required to elucidate the mechanism of cap recognition. In addition, what are the mechanisms by which the enhancers of decapping stimulate the decapping activity of Dcp2? Since the assembly of the decapping complex is a dynamic and complex process and decapping enhancers may use different mechanisms to bind Dcp2 and activate decapping, an important area of future study will be directed towards understanding the interactions of various decapping enhancers with Dcp2 and their functional significance for regulating mRNA decapping. In addition to these structural questions, there are a number of important functional questions for future study. For example, studies in D. melanogaster, C. elegans, and X. laevis have identified mRNPs containing decapping factors that appear to repress translation and store mRNA, rather than targeting them for decay. What dictates whether an mRNA is stored or degraded and how are decapping factors involved in both of these seemingly contrasting functions? There is substantial evidence that suggests translation initiation and decapping are in competition. How is the mRNP remodeled to allow for translational repression and access to the 5′ cap by Dcp2? While decapping was long thought to be an irreversible process that triggered the rapid decay of mRNA, the recent identification of a cytoplasmic capping activity has challenged this view [128,129]. How widespread is cytoplasmic capping and is cap homoeostasis an important mechanism for translation regulation? P bodies are conserved cytoplasmic foci containing many components of the decapping machinery as well as mRNA in various states of decay. What role do P bodies play in translational repression, mRNA decapping and decay, and what determines their composition and formation? Nudt16 has been identified as a nuclear and cytoplasmic factor capable of decapping and its function appears to at least partially overlap with Dcp2. What role does Nudt16 play in mRNA decapping and what factors regulate its function? Insights into these and other questions regarding decapping will allow us to better understand this important mechanism of eukaryotic post-transcriptional gene regulation.

References


[89] Y. Mullen, W.J. Marzluff, Degradation of histone mRNA requires oligouridylation followed by decapping and simultaneous degradation of the mRNA both 5′ to 3′ and 3′ to 5′, Genes Dev. 22 (2008) 56–68.


Chapter II, in full, is a reprint of the material as it appears in Arribas-Layton Marc; Wu Donghui, Lykke-Andersen Jens; Song Haiwei. (2013) Structural and functional control of the eukaryotic mRNA decapping machinery, *Biochimica et Biophysica Acta*, 1829(6-7), 580–9. The dissertation author was a primary author of this paper along with Donghui Wu.
CHAPTER III - The C-terminal RGG domain of human Lsm4 promotes processing body formation stimulated by arginine dimethylation

Abstract

Processing Bodies (PBs) are conserved cytoplasmic aggregations of translationally repressed mRNAs assembled with mRNA decay factors. The aggregation of mRNA-protein (mRNP) complexes into PBs involves interactions between low complexity regions of protein components of the mRNPs. In *Saccharomyces cerevisiae*, the carboxy- (C-)terminal Q/N-rich domain of the Lsm4 subunit of Lsm1-7 plays an important role in PB formation, but the C-terminal domain of Lsm4 in most eukaryotes are RGG domains rather than Q/N-rich. Here we show that the Lsm4 RGG domain promotes PB accumulation in human cells, and that symmetric dimethylation of arginines within the RGG domain stimulates this process. An Lsm4 mutant lacking the RGG domain fails to rescue PB formation in cells depleted of endogenous Lsm4, despite this mutant retaining the ability to assemble with Lsm1-7, associate with decapping factors, and promote mRNA decay and translational repression. Mutation of the symmetrically dimethylated arginines within the RGG domain to alanines or lysines, respectively fully, or partially abrogated the ability of Lsm4 to promote PB accumulation. Depletion of PRMT5, the primary factor responsible for symmetric arginine dimethylation, resulted in loss of PBs. We also uncovered the HAT1-RBBP7 lysine acetylase complex as an interaction partner of the Lsm4 RGG domain, but found no evidence for a role of this complex in PB metabolism.
Together our findings suggest a stimulatory role for post-translational modifications in PB accumulation and raise the possibility that mRNP dynamics could be post-translationally regulated.

Introduction

Post-transcriptional gene regulation is critical for maintaining proper cellular function. The combination of proteins interacting with mRNAs making up the messenger ribonucleoproteins (mRNPs) determines the state of the mRNP, whether it is actively translated, targeted for mRNA decay, or stored in a translationally repressed state. These alternative fates for mRNPs can be regulated globally or mRNP specifically in response to intracellular or extracellular cues\textsuperscript{1,2}.

Eukaryotic cytoplasmic mRNAs are bound on the 5’ cap by the translation initiation factor eIF4F and on the 3’ poly(A)-tail by poly(A)-binding protein (PABP), which act synergistically to stimulate translation\textsuperscript{3}. A major pathway of mRNA turnover in eukaryotes initiates by mRNA deadenylation\textsuperscript{4,5}. This is followed by decapping by the Dcp2 decapping complex and ultimately 5’ to 3’ exonucleolytic decay by Xrn1\textsuperscript{6}. The Lsm1-7 complex together with its co-factor Pat1 (known as PatL1 in human) binds to 3’ ends of deadenylated mRNAs and promotes decapping by a mechanism that is not fully understood but involves association of Lsm1-7-Pat1 with decapping enhancers\textsuperscript{7,8,9,10,11}.

mRNAs that are targeted for deadenylation-initiated mRNA decay can accumulate in the cytoplasm in RNP granules known as Processing Bodies.
These granules are highly dynamic and sensitive to the level of intermediates of mRNPs undergoing deadenylation-initiated mRNA decay in the cell. For example, manipulations that inhibit accumulation of mRNPs targeted for deadenylation-initiated decay, such as trapping mRNAs with ribosomes using translation elongation inhibitors\textsuperscript{13,14}, or depleting factors acting early in the pathway results in loss of visible PBs\textsuperscript{15}. Contrasting this, depletion of factors acting late in the pathway leads to accumulation of decay intermediate mRNPs, which in general results in increased size and/or number of PBs in cells\textsuperscript{16}.

In \textit{S. cerevisiae}, mRNP aggregation into PBs is known to involve the glutamine-asparagine (Q/N)-rich C-terminal domain of the Lsm4 subunit of the Lsm1-7 complex\textsuperscript{17,18}. In human cells, depletion of Lsm4 also leads to a loss of PBs\textsuperscript{19}, but the C-terminus of human Lsm4, as in most metazoans, is divergent from that of \textit{S. cerevisiae} Lsm4, and consists of an arginine-glycine (R/G)-rich RGG domain rather than a Q/N-rich region. Recent studies have implicated low complexity polypeptide regions of proteins, including R/G-rich regions, in protein polymerization and aggregation\textsuperscript{20}. The arginines of the human Lsm4 RGG domain have been shown to be symmetrically dimethylated\textsuperscript{21}, raising the intriguing question of whether post-translational modifications (PTMs) play a role in PB formation. Here we show that the RGG domain of human Lsm4 stimulates PB formation. While the RGG domain does not appear to play a role in formation of the Lsm1-7 complex, association with mRNA decapping factors, or in translational repression or mRNA decay, our findings identify the arginines of the RGG domain and their dimethylation to promote PB formation. This is evidenced...
by the inhibition of PB accumulation as a result of mutating the dimethylated arginines of Lsm4, as well as of inhibiting symmetric arginine dimethylation by depletion of the arginine methyl transferase PRMT5. Interestingly, we also discover a novel interaction of the Lsm4 RGG domain with HAT1 and RBBP7, components of a cytoplasmic lysine acetyl transferase complex, leading to the possibility of a PTM network involved in mRNP regulation.

Results

The RGG domain of human Lsm4 is required for PB formation

To determine if the C-terminal RGG domain of human Lsm4 plays a role in mRNA regulation, we created stable human embryonic kidney (HEK) 293 T-REx cell lines expressing FLAG-tagged wild-type (WT) Lsm4 or Lsm4 deleted for its RGG domain (ΔRGG) (Figure 1A) under control of a tetracycline-regulated promoter. Silent mutations in the exogenous Lsm4 coding region allowed for complementation assays in which endogenous Lsm4 could be specifically depleted using an siRNA and exogenous Lsm4 expressed at near endogenous levels using low concentrations of tetracycline (Figure 1B). To determine if the Lsm4 C-terminus is important for PB formation, we depleted cells of endogenous Lsm4 and expressed Lsm4 WT or ΔRGG at near endogenous levels and visualized PBs by indirect immunofluorescence for the endogenous PB component Dcp1a (Figures 1C, D). As expected, Lsm4 depletion led to loss of Dcp1a localization in PBs, which could be rescued by expression of exogenous
Lsm4 WT. By contrast, the Lsm4 ΔRGG mutant failed to rescue Dcp1a localization. This observation, which is further supported using additional Lsm4 mutants and monitoring for additional PB factors shown below, suggests that the C-terminal RGG domain of human Lsm4 plays an important role in PB accumulation.

**The RGG domain of Lsm4 is not required for Lsm4 assembly with Lsm1-7 and decapping factors**

A possible explanation for the failure of Lsm4 ΔRGG to support PB accumulation is that it fails to form Lsm1-7 complexes. To determine if this was the case, we performed immunoprecipitation (IP) for Lsm4 and Lsm4 ΔRGG followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). IPs were performed in the presence of RNase A to specifically monitor for interactions that are independent of RNA. These assays showed similar levels of association of Lsm4 WT and ΔRGG with Lsm1-7 subunits (Figure 2A and Supplemental Table S1; Lsm5 was below detection in either sample). Similarly, the IP-LC-MS/MS assays revealed that the C-terminal RGG domain is not required for association of Lsm4 with decapping enhancers PatL1, DDX6, or Edc3 (Figure 2B; other decapping components were not observed over background). These observations suggest that the loss of PB accumulation associated with Lsm4 ΔRGG is not a result of defects in Lsm1-7 complex or decapping factor association, a conclusion that was further supported by IP-Western assays shown below.
While the RGG domain of Lsm4 was not important for Lsm4 association with Lsm1-7 or decapping factors, our LC-MS/MS assays did reveal two proteins, HAT1 and RBBP7, which showed an RGG-dependent association with Lsm4 (Figure 2C). This was intriguing given that HAT1 and RBBP7 are known to form a lysine acetylase complex that functions in both the cytoplasm and nucleus\(^\text{22}\) and the mouse orthologs were previously reported to inhibit formation of PB-like mRNP granules known as Chromatoid Bodies in mouse germ cells\(^\text{23}\). However, we found no defect in PB accumulation upon co-depletion of HAT1 and RBBP7 or overexpression of WT or catalytic mutants E187Q and W119A of HAT1\(^\text{24}\), suggesting that HAT1 does not play a limiting role in the accumulation of PBs in HeLa cells (Figure 7A, B).

**Lsm4 represses translation and activates mRNA decay independently of the RGG domain**

The inability of Lsm4 \(^\Delta\)RGG to support accumulation of visible PBs could reflect a role for the RGG domain in translation repression or mRNA decay. We therefore tested the ability of Lsm4 \(^\Delta\)RGG to complement Lsm4 depletion in these processes. The Lsm1-7 complex is limiting for the degradation of mRNAs containing AU-rich elements (AREs)\(^\text{26}\) and of replication-dependent histone mRNAs\(^\text{26}\). As expected, depletion of Lsm4 caused an increase in the half-life of a \(\beta\)-globin reporter mRNA containing the ARE from c-fos mRNA in the 3’ UTR, which was rescued by exogenous expression of Lsm4 WT (Figure 3A). Lsm4 \(^\Delta\)RGG also fully rescued degradation of the reporter mRNA suggesting that the
Lsm4 RGG domain is not limiting for its degradation. Lsm4 deleted of its C-terminal 5 or 10 amino acids was previously observed to be impaired in histone mRNA degradation\textsuperscript{26}. However, Lsm4 WT and ΔRGG were both fully able to complement the defect of depleting endogenous Lsm4 in degradation of histone H2A mRNA induced by hydroxyurea treatment (Figure 3B). Thus, the Lsm4 RGG domain is not limiting for degradation of histone H2A mRNA; deletion of the entire RGG domain appears to rescue the defect associated with deleting the C-terminal 5-10 amino acids only.

In yeast, several proteins containing RGG domains were shown to interact with eIF4G and repress translation initiation\textsuperscript{27}. To determine if the Lsm4 RGG domain could be acting in a similar manner, we conducted a tethering assay using MS2 coat protein-Lsm4 fusion proteins and a firefly luciferase reporter mRNA containing six MS2 coat protein binding sites. When the MS2-Lsm4 fusion proteins were co-expressed with the luciferase reporter, Lsm4 WT and ΔRGG were able to repress the luciferase reporter to a similar extent as compared to the negative control of expressing MS2 coat protein alone (Figure 3C). Expression of an MS2 coat protein fused to the Lsm4 RGG domain alone had no effect on luciferase expression. Collectively, these observations suggest that the RGG domain is neither necessary nor sufficient for mRNA repression by Lsm4.

**Arginines in the Lsm4 RGG domain promote PB accumulation**

R/G-containing domains have been identified as targets for arginine methylation, and the RGG domain of Lsm4 specifically has been identified as a
target for symmetric arginine dimethylation\textsuperscript{26}. To test the importance in PB accumulation of the arginines of Lsm4 undergoing symmetric dimethylation, we created mutants of Lsm4 where all arginines that undergo methylation were mutated to either alanines (Lsm4 AGG) or lysines (Lsm4 KGG). As expected, these mutations prevented detection of Lsm4 by an antibody specific for symmetric dimethylated arginines (Sym10; Figure 4A). Similar to Lsm4 \( \Delta \)RGG, Lsm4 AGG and KGG expressed at close to endogenous Lsm4 levels supported interaction with Lsm1-7 components and decapping factors as monitored by co-IP (Figures 4A and 4B) and IP-LC-MS/MS (Figure 8A, B) and both showed impaired association with HAT1 and RBBP7 (Figure 5C and Figure 8), with Lsm4 KGG being only partially impaired for HAT1-RBBP7 association. Lsm4 AGG and KGG could also fully complement the depletion of endogenous Lsm4 in degradation of the tested ARE-reporter and Histone H2A mRNAs and in repression of tethered luciferase mRNA (Figure 10). When monitoring for effects on PBs of complementing endogenous Lsm4 with the mutant Lsm4 proteins expressed at near endogenous levels, Lsm4 AGG was unable to support PB accumulation as monitored by three different PB markers (Figure 5A-C). Lsm4 KGG also showed impaired ability to support PB accumulation, but in contrast to Lsm4 AGG, could partially rescue PBs (Figures 6A, B). Thus, the arginines of the Lsm4 RGG domain that undergo symmetric dimethylation play an important role in PB accumulation.
**PRMT5 depletion disrupts PB accumulation**

To test whether symmetric arginine dimethylation plays a role in PB formation, we tested the effect of inhibiting the enzyme responsible for this activity. Indeed, treatment of HeLa cells with the global methylation inhibitor AMI-1 for 1 hour resulted in reduced PB accumulation compared to mock treated cells (J.D., unpublished observations). Symmetric arginine dimethylation as found in Lsm4 is carried out by the type II PRMTs, PRMT5 and PRMT7. A study quantifying the HeLa cell proteome showed PRMT5 to be expressed 20-fold over PRMT7; we therefore focused on PRMT5. When HeLa cells were depleted of PRMT5 using two different siRNAs, PBs were strongly reduced, as monitored by two different PB markers, compared to cells treated with a control siRNA (Figure 6). This demonstrates that PRMT5 is important for PB accumulation, and together with the Lsm4 mutational studies, suggests that symmetric arginine dimethylation of the Lsm4 C-terminal RGG domain stimulates PB accumulation.

**Discussion**

In this study we demonstrate that the C-terminal RGG domain of Lsm4 promotes accumulation of PBs in human cells. This is evidenced by the impaired ability of Lsm4 deleted of or mutated in the RGG domain to support PB accumulation (Figures 1 and 5), despite retaining the ability to associate with Lsm1-7 and decapping factors (Figures 2 and 4). Two lines of evidence suggest that symmetric arginine dimethylation of the Lsm4 RGG domain stimulates PB accumulation. First, depletion of PRMT5, the primary arginine methyl transferase
producing symmetrically dimethylated arginines, results in loss of PBs (Figure 6).
Second, a mutant version of Lsm4 in which all arginines in the RGG domain that undergo symmetric dimethylation are mutated to alanines is unable to form visible PBs (Figure 5). The observation that a mutant version of Lsm4 with the RGG arginines mutated to lysines modestly rescues PB formation, suggests a contribution also from the positive charge of the arginines of the RGG domain to PB accumulation and that methylation of the Lsm4 arginines stimulates PB accumulation but is not essential. PB components other than Lsm4 might also undergo methylation to stimulate PB accumulation, which could explain the dramatic effect on PB accumulation of PRMT5 depletion (Figure 4); for example the PB core component Lsm14A (also known as RAP55) also contains a C-terminal RGG domain.

What is the molecular mechanism by which the RGG domain of Lsm4 stimulates PB accumulation? PB formation requires the accumulation of translationally repressed mRNAs associated with mRNA decay machinery. Several yeast RGG-domain proteins were shown to inhibit translation in a manner involving interaction between the RGG domain and eIF4G. However, we found no evidence for the human Lsm4 RGG domain playing a role in translation repression as monitored by tethered luciferase assays (Figures 3C and Figure 9), though we cannot rule out an effect on specific endogenous targets. Additionally, the RGG domain is not limiting for the decay of an ARE-reporter mRNA or of histone H2A mRNA (Figures 3A, 3B and Figure 9). This is congruent with the observation that the RGG domain does not affect the
interaction of Lsm4 with known decay factors as evidenced by co-IP and IP-LC-MS/MS experiments (Figures 2, 4). Thus, the most likely role of the Lsm4 RGG domain in PB accumulation is that it promotes the aggregation of repressed mRNPs. Consistent with this, RGG domains of human RNA-binding proteins and of trypanosome Scd6 (a homolog of human LSM14A) have previously been implicated in granule formation, but it is unknown whether arginine methylation plays a role in those cases. Interestingly, previous studies have presented evidence that repeats of (G/S)Y(G/S) can promote the formation of hydrogels that resemble dynamic RNA granules. An intriguing possibility is that GRG repeats have the same property by a process that is stimulated by arginine dimethylation. A similar role in PB aggregation was previously identified for the C-terminal domain of budding yeast Lsm4, which contains a Q/N-rich prion-like primary sequence, different from the human Lsm4 RGG domain C-terminus. Thus, the functionality of the Lsm4 C-terminal domain in PB accumulation may be evolutionarily conserved despite a great degree of variation in the primary sequence. However, in contrast to observations of the budding yeast Lsm4 C-terminal Q/N-domain forming foci when fused to GFP, we observed no granule formation for a GFP-human Lsm4 RGG fusion protein, suggesting that in the case of human Lsm4, the RGG domain is not sufficient for protein aggregation (Figure 10).

There is increasing evidence that mRNP modification plays an important role in mRNA regulation. Post-transcriptional modifications of mRNAs, including nucleoside modification and mRNA tailing, have been found to impact mRNA
translation and decay\textsuperscript{34,35}. In addition, PTMs are abundant in protein components of mRNPs, but to date only a few have been identified to play regulatory roles\textsuperscript{36}. Interestingly, our co-IP and IP-LC-MS/MS experiments revealed an RGG-dependent association of Lsm4 with the HAT1-RBBP7 lysine acetylase complex (Figures 2 and 4), which has previously been implicated in chromatoid body formation in murine germ line cells\textsuperscript{23}. The observation of a correlation between Lsm4 association with the HAT1-RBBP7 complex and ability to promote PB accumulation suggests a link between these events, although HAT1 does not appear to be limiting for PB accumulation under conditions tested here (Figure 7). The identification of an interaction of Lsm4 with PTM machinery via post-translationally modified residues raises the exciting possibility of mRNP dynamics being regulated by a network of PTMs, similar to what we now know about the regulation of chromatin dynamics through histone PTMs.
Materials and Methods

Plasmid Constructs

The full coding sequence (CSD) of human Lsm4 was inserted into pcDNA5/FRT/TO (Invitrogen) encoding an N-terminal FLAG epitope and mutated at nucleotide 248 to CGTCGCAGAAA (mutations in bold) using site directed mutagenesis (SDM) to confer siRNA resistance. The ∆RGG construct was created by cleaving the pcDNA5/FRT/TO-Lsm4 plasmid with NotI followed by religation; NotI cleaves at the end of the Lsm domain of the Lsm4 CDS and in the plasmid polylinker downstream of the Lsm4 CDS, leaving the first 270 nucleotides of the Lsm4 coding sequence. AGG and KGG mutants were generated by inserting DNA oligonucleotides between the two NotI sites in the pcDNA5/FRT/TO-Lsm4 plasmid, with the DNA oligonucleotides encoding the final 49 amino acids of Lsm4 with all arginines mutated to alanines (AGG) or lysines (KGG). The CDS of human Hat1 was amplified from cDNA reverse-transcribed using Superscript II (Invitrogen) from total RNA of HeLa cells isolated using Trizol (Thermo Fisher) and inserted into pcDNA5/FRT/TO-FLAG and pcDNA3-FLAG and mutated using SDM to create E187Q and W119A mutants. Lsm4 and mutants as well as the NMS2 sequence from pcFLAG-NMS2 were cloned into pcDNA5/FRT/TO using a Gibson Assembly Kit (New England Biolabs). Firefly and renilla luciferase were subcloned from pGL2 and pRL (Promega) into pcDNA3 and pcDNA3-3xMS2, a plasmid containing 6 MS2 RNA stem loops prior to the BGH poly(A) site. The pcTET2 β-GAP plasmid has been
previously described\textsuperscript{39}. pcTET2 β-c-fos was created by inserting the c-fos AU-rich element following the β-globing CDS of pcTET2-β\textit{wt}β plasmid described previously\textsuperscript{40}. Sequences are available upon request.

**Cell Culture**

HeLa or HEK 293 Flp-In T-REx (Thermo Fisher) cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco) with 10% heat inactivated fetal bovine serum (FBS). Stable cell lines were generated from Flp-In T-REx 293 cells according to the manufacturer’s recommendations (Thermo Fisher). Briefly, cells were transfected with 1 ng pcDNA5/FRT/TO containing wild-type or mutant Lsm4, Lsm4-NMS2, or Hat1 CDSs and 9 ng pOG44 (Thermo Fisher) using Transit 293 transfection reagent (Mirus). 48 hours after transfection, cells were split to ≈10% confluency and grown in DMEM/10% FBS with 100 μg/ml hygromycin and 15 μg/ml blasticidin until visible colonies formed. Colonies were selected and tested by Western blotting (WB) for protein expression by titration with tetracycline to achieve expression at near endogenous level.

**Antibodies**

Antibodies used for WB and immunofluorescence (IF) were obtained from the following sources and used at the indicated concentrations. Rabbit anti-Dcp1a\textsuperscript{41} (IF, 1:100), anti-Edc3\textsuperscript{42} (WB, 1:1,000), and anti-Hedls42 (WB, 1:1,000) as previously described. Human IC-6 (IF, 1:9,000), which recognizes Edc4 and Lamin proteins, graciously provided by Ed Chan and Donald Bloch\textsuperscript{43}
Hat1 (sc-376268; IF, 1:100), mouse anti-HuR (sc-5261; WB, 1:1000), and goat anti-Hat1 (sc-8752; WB, 1:1,000) were obtained from Santa Cruz Biotechnology. Rabbit anti-Rbbp7 (ab109285; WB, 1:1,000) and rabbit anti-PABP (ab21060; WB, 1:1,000) were obtained from Abcam. Rabbit anti-DDX6 (A300-461A; WB, 1:1,000) from Bethyl Laboratories. Rabbit anti-Lsm4 (PA5-25731; WB, 1:500) from Thermo Fisher. Rabbit polyclonal antisera were raised (Cocalico Biologicals Inc.) against PatL1 (amino acids 1-240), fused to an N-terminal glutathione S-transferase tag (WB 1:1000).

siRNAs and depletions

For all depletions, cells were treated with siRNA using siLentFect (Bio-Rad) according to manufacturer’s protocol. For Lsm4 and PRMT5 depletions, cells were treated 96 and 48 hrs prior to harvest at 20 or 30 nM final siRNA concentration, respectively. Hat1 depleted cells were transfected once at 20 nM final siRNA concentration 72 hrs prior to harvest. All siRNA are from GE Dharmacon with UU overhangs with the following sequences: siLsm4 – AGGAGGAGGUGGUGGCCAA, siPRMT5-1 – GGCCAUCUAUAAAUGUCU44, siPRMT5-2 – ACCGCUAUUGCACCU45, HAT1 – GUUUAGAGUUUAUGAGCAU, RBBP7 – AGAGAAGAAGUUGCUUAA.
Co-immunoprecipitation (co-IP) and IP followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) assays

10-cm plates seeded with cells stably expressing Lsm4 were treated with tetracycline (WT 7.5 ng/ml, ΔRGG 100 ng/ml, AGG 7 ng/ml, KGG 7 ng/ml) to express exogenous Lsm4 and treated with siRNA to deplete endogenous Lsm4 (see above). Cells were washed with phosphate-buffered saline (PBS; 136 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH7.4), and lysed on the plate with 1 ml isotonic lysis buffer (ILB; 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 2 μM aprotinin, 2 μM leupeptin, 1 ng/mL FLAG peptide (Sigma)) and incubated 10 minutes on ice with 50 μg/ml RNase A. Cell debris was pelleted by spinning at 21,000 g for 10 min at 4ºC and the supernatant was nutated with 50 μl anti-FLAG M2 agarose beads (Sigma) for 4 hours to overnight depending on the experiment. Beads were washed 8 times with NET-2 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Triton X-100) and resuspended in SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue, and 200 mM DTT). For IP-LC-MS/MS assays, cells were treated as above with the following modifications: two 15-cm plates of cells were used and lysed with 2.5 ml per plate of ILB containing 2.5 ng/ml FLAG peptide. Clarified lysates were incubated with 125 μl anti-FLAG M2 agarose per plate and washed 6 times with NET-2. Beads were eluted 3 times with 125 μl of NET-2 containing 300 ng/ml FLAG peptide. Eluents were combined and precipitated by adding trichloroacetic acid (TCA) to 20% and incubating on ice for 1 hour or overnight at -20º C. Protein
was collected by spinning at 21,000 g for 30 min at 4°C and washed with ice cold 10% TCA followed by 3 washes with ice cold acetone, spinning at 21,000 g for 10 min at 4°C between each wash. LC-MS/MS was carried out as previously described.

**Luciferase assays**

Cells stably expressing Lsm4-NMS2 were seeded in 3.5-cm dishes with 2 ml DMEM/10%FBS and treated with tetracycline to induce near endogenous levels of Lsm4 (WT 8 ng/ml, ΔRGG 11 ng/ml, AGG 10 ng/ml, KGG 17 ng/ml). HEK 293 Flp-In T-Rex parent line cells were also seeded in 3.5-cm dishes for transient transfection with pcDNA3-NMS2 and pcDNA3-RGG-NMS2 (RGG only). 24 hours later, cells were transfected with 0.25 µg pc-FLuc-3xMS2, 0.05 µg pc-RLuc, 0.04 µg pcDNA3-RGG-NMS2 (RGG only), 0.04 µg pcDNA3-NMS2 (None) and pcDNA3 to a total of 2.0 µg using TransIT HeLa (Mirus) according to manufacturer’s recommendations. Cells were lysed with 0.5 ml Passive Lysis Buffer (Promega) at room temperature with gentle rocking for 20 minutes. 10 µl of lysate was analyzed for luciferase activity using Dual-Luciferase Reporter Assay (Promega) reagents and a NOVOstar plate reader.

**Indirect immunofluorescence assays**

HEK293 T-REx cells stably expressing Lsm4 or Hat1 were seeded in 3.5-cm wells with 2 ml DMEM/10%FBS containing a 12 mm coverslip pre-treated with poly-D lysine (Corning BioCoat) and treated with siRNA and tetracycline to
deplete endogenous Lsm4 and rescue with exogenous Lsm4 as described above (for Hat1 overexpression there was no siRNA treatment). Treated cells were fixed in 3 to 4% formaldehyde in PBS for 15 minutes at room temperature, permeabilized in 0.5% Triton-X100 in PBS containing 1% goat serum (GS, Life Technologies) for 15 minutes, and incubated with primary antibodies at concentrations listed above in PBS/1% GS for 1 hour. Cells were then washed 2 times for 5 minutes with PBS/1% GS and treated with secondary antibodies in PBS/1% GS for 1 hour (anti-rabbit Texas Red (Invitrogen, 1:1,000), anti-mouse Alexa Fluor 488 (Thermo Fisher, 1:1,000), and anti-human FITC (Jackson ImmunoResearch, 1:500) followed by treatment with 4′,6-diamidino-2-phenylindole (5 μg/ml in PBS/1% GS, Sigma) for 2 minutes and three washes with PBS/1% GS and one wash with distilled water. Coverslips were briefly air dried and mounted on slides. Images are from a Zeiss AX10 microscope using a 60x objective. Hat1 mutant overexpression are from HeLa cells grown in 3.5-cm wells transfected with 2 μg of pcDNA3-Hat1 E187Q or W199A vector using TransIT HeLa according to the manufacturer’s recommendation (Mirus). 24 hours after transfection cells were trypsinized and transferred to 8-well chamber slides (Thermo Scientific) for 24 hours and treated as above. PBs were counted using CellProfiler47, project file available upon request.

mRNA decay assays

For ARE-mRNA decay assays, HeLa Tet-Off cells (Clontech) were grown in 3.5-cm wells with DMEM/10%FBS containing 50 ng/ml tetracycline. 48 hours
prior to time point 0, cells were transfected with 0.6 µg pcTET2-β-c-fos, 0.1 µg pcDNA3-β-GAP and 1.3 µg pcDNA3 using TransIT HeLa (Mirus). 6.5 hours prior to time point 0, reporter mRNA expression was induced by changing media to fresh medium lacking tetracycline. 30 minutes prior to time point 0, reporter expression was halted by changing to fresh medium containing 1 µg/ml tetracycline. Cells were then harvested in 1 ml Trizol (Thermo Fisher) at indicated time points. For Histone mRNA decay assays, HEK293 T-REx cells stably expressing exogenous Lsm4 were grown in 3.5-cm wells. 30 minutes prior to time point 0, cells were changed into fresh medium containing 5 mM hydroxyurea. Cells were then harvested in 1 ml Trizol at indicated time points. Total RNA was prepared according to manufacturer’s recommendations and resolved in 1.1% agarose-formaldehyde gels followed by Northern Blotting, visualization on a Typhoon Trio (Amersham Biosciences) and quantification using ImageJ.

Acknowledgements

We thank Drs. Marv Fritzler and Ed Chan for human IC-6 serum. Dr. Tilmann Achsel is thanked for anti-Lsm1 and anti-Lsm4 antibodies. This work was supported by the National Institutes of Health R01 GM077243 to J. L-A, F31 GM106655 to M.A-L. and F31 GM083624 to J. D., and by the Alfred Benzon Foundation, Copenhagen, Denmark to C.K.D.

Chapter III, in large part, is currently being prepared for publication of the material. Arribas-Layton, Marc; Dennis, Jackie; Bennett, Eric; Damgaard,
Christian K; and Lykke-Andersen, Jens. The dissertation author was the primary author of this material.
Figure 3.1. The RGG domain of Lsm4 is required for visible PBs

(A) Schematic showing Lsm4 WT and Lsm4 ΔRGG proteins. (B) Western blot showing depletion of endogenous Lsm4 and expression of exogenous Lsm4 WT at near-endogenous levels in stable HEK 293 T-REx cells. (C) HEK 293 T-REx cells were treated with siRNA against GFP (control) or Lsm4 and induced to express FLAG-tagged Lsm4 WT or ΔRGG and stained for Dcp1a (red). DAPI staining is blue. (D) Quantification of PB numbers from three independent experiments. Error bars represent standard deviation (S.D.). **: P<0.01 (student’s paired two-tailed t-test).
Figure 3.2. The RGG domain of Lsm4 is required for interaction with HAT1/RBBP7 but is not necessary for association with Lsm1-7 components or decapping factors. Graphs showing number of detected peptides per 1000 total co-precipitating with FLAG-tagged Lsm4 WT or ΔRGG for (A) Lsm1-7 complex members, (B) decapping factors, or (C) HAT1 complex members. None is a negative control IP performed from parental cells containing no FLAG-Lsm4 protein.
Figure 3.3. The RGG domain of Lsm4 is not required for mRNA decay or translational repression

(A) Northern blots showing the decay of an ARE containing reporter mRNA (β-c-fos) in HeLa Tet-off cells treated with indicated siRNAs and transiently expressing indicated Lsm4 proteins. β-c-fos half-lives ($t_{1/2}$) were calculated using the constitutively transcribed β-GAP internal control mRNA for normalization. Fold stabilization was calculated relative to the siLuc condition from 3 experiments, with Standard Error of the Means (S.E.M) indicated. (B) Northern blots showing decay of endogenous H2A mRNA induced by treatment with 5 mM hydroxyurea in HEK 293 T-REx cells treated with indicated siRNAs and stably expressing indicated Lsm4 proteins. GAPDH mRNA served as normalization control. (C) Luciferase luminescence assays from cells transiently transfected with plasmids encoding indicated MS2 fusion proteins as well as with a Firefly luciferase reporter with 6 MS2 coat protein binding sites in its 3` UTR (F-Luc-6xMS2) and a Renilla luciferase reporter (R-Luc) as an internal control. F-Luc-6xMS2 was normalized to R-Luc and all samples were normalized to cells transfected with MS2 and the reporters alone.
Figure 3.4. Lsm4 RGG domain arginines are required for interaction with Hat1/RBBP7 but dispensable for Lsm1-7 and decapping factor association

Western blots showing proteins co-immunoprecipitating with Lsm4 WT and mutants. Input samples are shown on the left. (A) Lsm proteins. Sym10 is specific for symmetrically dimethylated arginines, Asym is specific for asymmetrically dimethylated arginines. (B) Decapping factors. (C) HAT1 complex members. * indicates cross reaction with IgG.
Figure 3.5. Lsm4 RGG domain arginines are required for PB accumulation

(A) Indirect immunofluorescence assays for Dcp1a (red) in HEK 293 T-Rex cells treated with siRNA against Lsm4 and induced to express Lsm4 AGG and KGG. DAPI staining is shown in blue to mark nuclei. (B) and (C) Quantification of PBs for indicated PB markers from three independent experiments. Error bars represent standard deviation (S.D.). **: P<0.01, *: P<0.05 (student’s paired two-tailed t-test).
Figure 3.6. PRMT5 is required for PB formation

(A) Indirect immunofluorescence assays for Dcp1a (red) and Edc4 (IC-6, green) in HeLa cells treated with siRNA against luciferase (control) or PRMT5 (2 different siRNAs). DAPI staining is shown in blue to mark nuclei. Merged images are on the right. (B) Quantification of PBs for Dcp1a and Edc4. Error bars represent standard deviation (S.D.). (C) Western blot showing PRMT5 depletion.
Figure 3.7. Hat1 depletion or overexpression does not affect visible PBs

(A) Indirect immunofluorescence assays for endogenous PB markers Dcp1a (red) and Edc4 (IC-6, green) in HeLa cells treated with siRNA against HAT1. DAPI staining is shown in blue to mark nuclei. Merged images are on the right. (B) Indirect immunofluorescence assays for Dcp1a (red) and HAT1 (green) in HEK 293 T-REx cells stably expressing tetracycline inducible HAT1 WT, either uninduced or induced to overexpress HAT1. DAPI staining is shown in blue, and a merged image is on the right. (C) Indirect immunofluorescence assays for Dcp1a (red) and HAT1 (green in) HeLa cells transiently expressing either HAT1 E187Q or W119A. DAPI staining is shown in blue, and a merged image is on the right.
Figure 3.8. Lsm4 RGG domain arginines are required for association with HAT1/RBBP7 but not Lsm1-7 and decapping factor association
Graphs showing the number of detected peptides per 1000 total co-precipitating with Lsm4 WT or mutants as indicated.
Figure 3.9. The Lsm4 RGG domain is not required for mRNA decay or translational repression

(A) Graphs showing histone H2A and β-c-fos mRNA half-lives in the presence of Lsm4 WT and mutants monitored as in Figures 3A, B. Error bars represent S.E.M. (B) Graphs showing luciferase activity from tethering of MS2-Lsm4 WT and mutant proteins monitored as in Figure 3C. Error bars represent S.E.M. (C) Western blot indicating even expression of MS2-Lsm4 proteins mutants as detected by the FLAG antibody. * indicates a cross-reacting band.
Figure 3.10. The Lsm4 RGG domain is not sufficient for granule formation
GFP fusion proteins with the RGG domain of Lsm4 (GFP-RGG), the prion-like PRD domain of the stress granule marker TIA-1 (GFP-TIA1-PRD) or GFP alone transiently expressed in cells that were then stained for Dcp1a. Quantification is % GFP signal that overlaps with Dcp1a signal.
References


suggests plays an important role in co-ordinating its activities. The Biochemical Journal, 441(3), 803–12.


CHAPTER IV - PNRC2 has a decapping activation motif independent of Dcp1 interaction domain

Abstract

PNRC2 has been identified as a decapping activator that can interact with Dcp1/Dcp2 and stimulate decapping as part of the NMD pathway. PNRC2 co-localizes in PBs with Dcp1, but not if the Dcp1-interacting domain is mutated. Interestingly PNRC2 shares a YAG motif with the yeast enhancer of decapping EDC1 independent of the Dcp1-interaction domain. Here we show that this YAG motif is required for stimulation of the catalytic decapping activity of Dcp1/Dcp2. In vitro decapping reactions with PNRC2 Y93A show a loss of decapping stimulation, though the mutant maintains co-localization with Dcp1 in PBs.

Introduction

Human proline-rich nuclear receptor coregulatory protein 2 (PNRC2) was first implicated as an mRNA decay factor by studies suggesting it is involved in nonsense mediated decay (NMD), a surveillance pathway that promotes the decay of mRNA containing premature termination codons (PTCs) by activating decapping and 5’ to 3’ degradation as well as endonucleolytic decay. PNRC2 co-precipitates with both Upf1, the primary effector of NMD, and Dcp1a, a major component of the Dcp2 decapping complex, independent of RNA. PNRC2 also colocalizes to cytoplasmic foci known as Processing Bodies (PBs) along with other mRNA decay factors. Depletion of PNRC2 has been shown to stabilize
mRNA reporters containing a PTC and endogenous NMD targets. Furthermore, PNRC2 interacts more strongly with a hyperphosphorylated form of Upf1, which may be a more potent stimulator of NMD.¹

PNRC2 has also been implicated in Staufen mediated decay (SMD), a decay process where Staufen1 binds to the 3’-UTR of target mRNA to promote rapid degradation. In SMD, there is evidence that PNRC2 is recruited to Staufen in an Upf1 dependent manner.²

Structural studies have shown that PNRC2 interacts with the EVH1 domain of Dcp1a via a proline rich region³. Mutations to the proline rich region of PNRC2 disrupt the interaction with Dcp1a and result in a loss of localization of PNRC2 to PBs and loss of the ability to stimulate degradation of a tethered reporter. It is possible that PNRC2 acts to stimulate the interaction of Dcp1a with Dcp2, the catalytic component of the decapping complex.³

The interaction of Dcp1a with PNRC2 is similar to the interaction of the yeast protein Edc1 with Dcp1a.⁴ Edc1 can stimulate the catalytic activity of Dcp1/Dcp2 in a manner dependent on its C-terminus. Interestingly, PNRC2 shares a YAG motif found within the C-terminus of Edc1. We asked whether the PNRC2 YAG motif was involved in decapping, and if this motif influenced Dcp1 binding and PNRC2 localization. We also sought to identify if PNRC2 is required for efficient NMD in human cells, where decapping is not thought to be limiting, and if it can act as a general decapping activator in other decapping dependent mRNA decay pathways.
Results

The YAG motif of PNRC2 stimulates in vitro decapping by Dcp1/Dcp2

In vitro, WT PNRC2 is able to stimulate the catalytic activity of Dcp1/Dcp2. However, in experiments performed by our collaborators Drs. Jeffrey Mugridge and John Gross, a mutant form of PNRC2 where the tyrosine of the YAG motif is mutated to an alanine (PNRC2 Y93A) is completely unable to stimulate decapping. Other mutations to the YAG motif also abrogate in vitro decapping, though to a lesser extent (Fig. 1, Mugridge). In vitro, these mutant forms of PNRC2 are still able to interact with Dcp1a (Mugridge and Gross, unpublished observations). These results suggest that PNRC2 contains a motif that is able to stimulate the decapping reaction independent of the proline rich region known to be responsible for the PNRC2-Dcp1a interaction.

Mutation of the YAG motif does not disrupt PNRC2 localization

Mutations in the region of PNRC2 shown to interact with Dcp1a, such as the W114A mutant, have been shown to lose their localization to PBs\(^3\). We were interested in investigating the localization of a PNRC2 mutant that was unable to stimulate decapping activity because of mutations to the YAG motif. By creating 5xMyc tagged versions of PNRC2 WT, PNRC2 Y93A, and PNRC2 W114A, I was able to monitor the localization of transiently expressed PNRC2 using immunofluorescence in human HEK293T cells (Fig. 2). As a positive control, PNRC2 WT shows strong co-localization with the PB marker Dcp1a. As
previously reported, the PNRC2 W114A mutant fails to co-localize with Dcp1a foci. It has a predominantly nuclear localization, though even in cells highly expressing the mutant construct, it shows general cytoplasmic localization with no enrichment in Dcp1a foci. Interestingly, the PNRC2 Y93A mutant, despite being completely inactive in decapping stimulation, does show co-localization with Dcp1a in PBs. This result is perhaps not unexpected, as mutations in the YAG motif did not appear to affect in vitro association of PNRC2 and Dcp1a. Because we lack an antibody against endogenous PNRC2, the amount of transfected plasmid was titrated and one of the lowest visible amounts of PNRC2 was used to avoid possible effects of overexpression. Results were comparable with or without treatment with siRNA against PNRC2, and with three times the amount of reported plasmid transfected.

**Overexpression of PNRC2 WT or mutants does not affect AMD reporter decay**

As PNRC2 interacts with the Dcp1/Dcp2 holoenzyme and catalytically stimulates the decapping reaction, we also wondered if disruption of PNRC2 in cells could disrupt deadenylation initiated decay. ARE mediated decay (AMD) is a deadenylation initiated decay pathway, and AMD reporters can be stabilized in a deadenylated state by disruption of decapping through overexpression of the decapping factor Edc4\(^5\). To determine if PRNC2 might act in a similar manner, we observed the decay of an ARE reporter mRNA in cells depleted of PNRC2 or overexpressing PNRC2 WT or mutants (Fig. 3C). As a positive control, we
depleted or overexpressed Edc4. While cells overexpressing Edc4 did indeed show accumulation of a deadenylated decay intermediate, no other conditions, including depletion of Edc4, showed accumulation of a deadenylated band. This may be because of ineffective siRNA against PNRC2 or, despite depletion, the small portion of remaining PNRC2 is sufficient for efficient decapping. This is the observation for Edc4 where a typically robust depletion still fails to show a defect in the ARE reporter decay. For overexpression of PNRC2 WT or mutants a deadenylated band may not be observed because overexpression is not dominantly negative, as is with the case of overexpression of the decapping factor Edc4\(^5\), or the proteins may not be expressing at sufficiently high levels.

**Discussion**

It has previously been reported that PNRC2 can act as a decapping activator. Structural studies have shown that PNRC2 can directly interact with the catalytic subunit of the Dcp2 decapping complex, Dcp2, as well as Dcp1. Current studies have focused on the interaction of PNRC2 with Dcp1, showing that a proline-rich region of PNRC2 interacts with the EVH1 domain of Dcp1, and that mutations in these regions can abolish the ability of PNRC2 to stimulate decapping\(^3\). Here by mutating a conserved decapping activation motif, the YAG sequence, we present evidence for an additional interaction important in decapping activation. In vitro studies performed by our collaborators in the Gross lab show that mutations in the YAG domain abrogate the ability of PNRC2 to stimulate decapping, with a mutation of the tyrosine acting as a full null mutant
(Fig. 1, Mugridge). However, in contrast to previously reported mutants of PNRC2 in the proline rich region, such as the W114A mutation, mutations to the YAG domain are still able to interact with Dcp1a, as in vitro pulldowns of PNRC2 Y93A coprecipitate Dcp1 (Mugridge and Gross, unpublished observations). The ability of PNRC2 YAG mutants to interact with decapping cofactors is also suggested by its localization. PNRC2 WT co-localizes with Dcp1a and other decapping activators to PBs. Disruption of the Dcp1a interaction motif, such as in the W114A mutant, cause PNRC2 to lose PB co-localization. The W114A mutant shows a predominantly nuclear localization, though even cytoplasmic localization does not appear to be enriched in PBs (Fig. 2). The nuclear localization of PNRC2 is not unusual, as PNRC2 was originally identified as a nuclear receptor co-activator. Contrasting the localization of the W114A mutant, the PNRC2 Y93A mutant strongly co-localizes with Dcp1a, again suggesting its ability to interact with Dcp1a is not disrupted. While these results suggest the YAG motif of PNRC2 is critical for stimulating decapping, the mechanism by which this occurs is unknown. As PNRC2 Y93A can still interact with Dcp1 and localizes to PBs that appear normal in size and quantity, it is likely that this mutation does not disrupt the formation of a larger decapping complex. In the absence of other stresses to the mRNA decay pathway, this larger complex may allow for decapping efficient enough to maintain a wildtype PB phenotype. Another possibility is that endogenous PNRC2 is still able to function in these conditions despite the presence of exogenous PNRC2 Y93A.
While we have shown some evidence that PNRC2 has an ability to stimulate decapping independent of its Dcp1 interacting domain, we were interested if PNRC2 and its YAG motif played a role in NMD or general mRNA turnover. There have been varying reports as to the role of PNRC2 in NMD\textsuperscript{6,7}. Unfortunately, my experiments investigating PNRC2 in these roles remain inconclusive. We sought to determine if PNRC2 acts as a general activator of decapping. My attempts to detect defaults in the decapping process by looking for the accumulation of a deadenylated intermediate of ARE stimulated decay only showed negative results. However, this assay is sensitive only to a strong block of decapping, and only conditions where Edc4 is overexpressed show an accumulation of the deadenylated intermediate (Fig. 3C). Furthermore, overexpression of exogenous PNRC2 in HeLa derivative cells (such as the HeLa Tet-Off used for these decay experiments) is difficult, as observed by difficulty in detecting Myc-tagged proteins via Western blotting or immunofluorescence, meaning that even if PNRC2 mutants are able to act as dominant negatives, they may not be expressed at sufficient levels in these experiments. Decay assays investigating if PRNC2 plays a role in NMD decay were uninterpretable. Treatment of cells with siRNA against Upf1 resulted in an increase in detectable reporter and half-lives similar to previous results from our lab, but cells treated with siRNA against PNRC2 or a control showed little reporter mRNA, indicating experimental failure rather than a failure to disrupt NMD.
Materials and Methods

Plasmids

pc-Myc-PNRC2 WT and mutants were received from our collaborators Jeffrey Mugridge and John Gross. Pc-5xMyc-PNRC2 WT, Y93A, and W114A were created by subcloning using BamHI and NotI restriction sites. µg pcTET2-β-ARE (pcTET2-β-GMCSF-ARE in text), pcDNA3-β-GAP, and pcDNA3-Myc-Edc4 (pcDNA3-Myc-Hedls in reference) have been previously described.

Indirect immunofluorescence assays

HEK293T cells were seeded in 3.5-cm wells with 2 ml DMEM/10%FBS containing a 12 mm coverslip pre-treated with poly-D lysine (Corning BioCoat). 24 hours after seeding, cells were transfected with 1.9 µg of pcDNA3 and 0.1 µg of pcDNA3-5xMyc-PNRC2 WT, Y93A, or W114A using TransIT 293 (Mirus) according to manufacturer’s protocol. 48 hours after transfection cells were fixed in 3 to 4% formaldehyde in PBS for 15 minutes at room temperature, permeabilized in 0.5% Triton-X100 in PBS containing 1% goat serum (PBS; 136 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH7.4, GS, Life Technologies) for 15 minutes, and incubated with primary antibodies in PBS/1% GS for 1 hour (Ms anti-Myc 1:100, Sigma M4439, Rb anti-Dcp1a 1:100 Cocalico Labs). Cells were then washed 2 times for 5 minutes with PBS/1% GS and treated with secondary antibodies in PBS/1% GS for 1 hour (anti-rabbit Texas Red (Invitrogen, 1:1,000), anti-mouse Alexa Fluor 488 (Thermo Fisher, 1:1,000))
followed by treatment with 4′,6-diamidino-2-phenylindole (DAPI, 5 μg/ml in PBS/1% GS, Sigma) for 2 minutes and three washes with PBS/1% GS and one wash with distilled water. Coverslips were briefly air dried and mounted on slides. Images are from a Zeiss AX10 microscope using a 60x objective.

**mRNA decay assays**

For ARE-mRNA decay assays, HeLa Tet-Off cells (Clontech) were grown in 3.5-cm wells with DMEM/10%FBS containing 50 ng/ml tetracycline. 48 hours prior to time point 0, cells were transfected with 0.6 μg pcTET2-β-ARE, 0.1 μg pcDNA3-β-GAP, 1 μg pcDNA3-Myc-Edc4 or pcDNA3-5xMyc-PNRC2 mutants, and pcDNA3 up to 2 μg total DNA using TransIT HeLa (Mirus) according to manufacturer's protocol. 6.5 hours prior to time point 0, reporter mRNA expression was induced by changing media to fresh medium lacking tetracycline. 30 minutes prior to time point 0, reporter expression was halted by changing to fresh medium containing 1 μg/ml tetracycline. Cells were then harvested in 1 ml Trizol (Thermo Fisher) at indicated time points. Total RNA was prepared according to manufacturer's recommendations and resolved in 1.1% agarose-formaldehyde gels followed by Northern Blotting, visualization on a Typhoon Trio (Amersham Biosciences) and quantification using ImageJ.
Figure 4.1 – PNRC2 YAG motif is needed to stimulate decapping (Jeffrey Mugridge, UCSF)
In vitro decapping reactions using recombinant protein purified from bacteria. All experiments contain Dcp1/Dcp2 in addition to the labeled PNRC2 mutants.
Figure 4.2 – PNRC2 Y93A co-localizes to PBs
Indirect immunofluorescence assays for Dcp1a (red) and Myc (green) in HEK 293 T-REx cells transfected with the labeled pcDNA3-5xMyc-PNRC2 constructs. DAPI staining is shown in blue to mark nuclei.
Figure 4.3 – PNRC2 depletion or overexpression does not affect decay of an ARE reporter
Northern blots showing the decay of a transiently expressed ARE containing reporter mRNA (β-ARE) in HeLa Tet-off cells treated with indicated siRNAs. β-GAP was co-transfected as a control.
Acknowledgements

Chapter IV contains a figure from my collaborators, Jeffery Mugridge and John Gross. The dissertation author was the primary author of this material.
References


CHAPTER V - Investigating Post Translational Modifications to PABP

Abstract

PABP is bound to the majority of eukaryotic mRNA and helps stimulate translation through interactions with the eIF4F translation initiation complex. It can also interact with proteins that inhibit its ability to stimulate translation or that may promote mRNA decay. Recent high throughput studies identifying post translational modifications across the proteome have identified numerous possible sites of modifications to PABP. We investigated if post translational modifications to PABP could regulate its function. We found no effect of single point mutations to PABP on its ability to associate with actively translating mRNA, nor did we detect a difference in post translational modifications to PABP from actively translating or translationally repressed populations.

Introduction

A defining characteristic of most eukaryotic mRNAs is a 3' poly(A) tail which is bound by poly(A) binding proteins for the majority of the mRNA's lifespan. mRNA export from the nucleus may be aided by the cytoplasmic PABPC1 (PAB1 in yeast, subsequently referred to as PABP), which is a shuttling protein\(^1\) and has been shown to interact with members of the nuclear pore\(^2\). In the cytoplasm, PABP can interact with many different proteins to produce differing effects.
PABP promotes translation by several mechanisms. It interacts with eIF4G to help stimulate translation initiation in various ways, including increasing the affinity of the eIF4F complex for the 5’ cap and increased 80S formation efficiency\(^3,4\). Some models hypothesize that a PABP-eIF4G interaction can circularize mRNA and allow for ribosomal recycling, though PABP has been shown to also stimulate translation via a *trans*-poly(A) tail in vitro\(^5\). In addition to stimulating the rate-limiting step of translation initiation, PABP is also able to stimulate translation by promoting efficient translation termination through an interaction with the termination factor eRF3\(^6\). Conversely, interactions with the PABP interacting protein 2 (PAIP2) can inhibit the stimulation of translation by PABP by blocking the ability of PABP to bind to mRNA and translation factors\(^7\).

In addition to its role in promoting translation, PABP can act to protect mRNA from degradation, much of which is thought to occur via a deadenylation initiated pathway\(^8\). PABP can inhibit in vitro 3’ exonucleolytic decay\(^9\), and tethered PABP can inhibit mRNA degradation independent of a poly(A) tail\(^10\). Despite serving to protect the 3’ end of mRNA from degradation, PABP can actually act to stimulate mRNA degradation in certain cases. Studies have shown that GW182, a component of the RISC complex responsible for miRNA mediated degradation, requires PABP to efficiently initiate miRNA mediated deadenylation\(^11\). PABP can also directly interact with PAN3, a member of the PAN2/PAN3 deadenylase complex which may promote initial, non-processive deadenylolation of transcripts\(^12\).
Because of the large number of interacting proteins as well as the diverse outcomes PABP can promote, PABP is a strong candidate focal point for mRNA regulation. Interestingly, high throughput proteomic studies have identified many potential sites of post translational modification (PTM) on PABP that could serve to regulate the function of PABP, much as modifications to histones regulate chromatin structure and transcription.

My research on how PTMs might regulate PABP function used 2 approaches: (1) creating PABP mutants inhibiting or mimicking PTMs and assaying their effects, and (2) isolating PABP in different states, such as PABP bound to actively translating mRNAs compared to free PABP, or PABP bound to mRNA not associated with ribosomes, and attempting to identify PTMs associated with different pools of PABP. My attempts to pursue these approaches failed to identify regulatory PTMs in PABP; more effort is needed to test whether mRNA activity is regulated by PTMs in PABP or other mRNP components.

Results

Use of mutants to investigate possible PTM-modulated PABP regulation

As a tool to investigate the effects of PTMs on PABP and its effect on mRNA translation, I created a series of FLAG tagged mutants that were unable to be modified as well as mutants that may mimic a PTM. For lysines, mutation to an arginine would maintain a positive charge and would resist acetylation by
lysine specific acetyl transferases. Conversely, mutation to a glutamine could possibly mimic a constitutively acetylated lysine. For tyrosine, mutation to an alanine precludes the possibility of being phosphorylated albeit without mimicking the chemical properties of a tyrosine. Mutation of a tyrosine to glutamic acid can mimic the negative charge of a phosphorylation event. Single mutants were created for 5 lysine and 5 tyrosine residues identified as possible sites of PTM identified through global mass spectrometry experiments \textsuperscript{13,14,15,16}, as well as a double mutant for lysines 104 and 108 (Table 1). I then used the mutants in a series of experiments to determine if they behaved differently than WT PABP.

To determine if the mutants had a similar capacity to stimulate translation as wildtype PABP, I exogenously expressed the FLAG-PABP constructs in HeLa cells by transient transfection followed by polysome fractionation over a sucrose gradient and Western blotting for the tagged PABP. If a particular PTM was important for stimulating translation, mutation of that amino acid could potentially cause mRNA bound by the mutant PABP to remain in lighter sedimentation fractions rather than being associated with the heavier, actively translating fractions (Fig. 1A), and hence the Western blot would show a signal enriched in the lighter fractions. By making mutants that are unable to be modified as well as mutants that potentially mimic a PTM, the constitutively modified mutant could potentially verify results by having an opposing effect. For the mutants created, there did not seem to be any difference in PABP sedimentation for WT or mutant proteins and both wildtype and mutant PABP showed a similar distribution after fractionation (Fig. 1B, shown for 104/108 K→R and K→Q, other data not shown).
During stress, translationally repressed mRNAs accumulate with PABP in cytoplasmic foci known as stress granules (SGs)\textsuperscript{17}. We considered whether PTMs to PABP could influence whether a bound mRNA entered a SG by affecting its translational state, or through another mechanism. To test this hypothesis, I transiently expressed PABP WT and mutants in HeLa cells and monitored their localization by indirect immunofluorescence under normal conditions, or after subjecting the cells to heat shock. If a specific PTM regulated PABP and its ability to enter into or promote SG formation, the corresponding mutant could promote SGs under normal conditions, or evade SG in stress conditions. Additionally, as PABP is a shuttling protein, it was possible that PTMs to PABP could act to regulate its nuclear/cytoplasmic localization, which would be observable in this experiment. Overall, all tested mutants behaved as WT with general cytoplasmic localization during normal conditions and cytoplasmic granule formation under heat shock conditions (Fig. 2). Occasionally, rare cases of cells with PABP foci under normal conditions were observed, but this could also occasionally be seen for cells transfected with a WT PABP construct, indicating this may occur due to overexpression of PABP, a result of transfection reagents, or as a rarely occurring natural phenomenon. Taken together, these results suggest that the tested sites for PTMs to PABP do not regulate the ability of PABP to affect translation or to assemble into SGs.
Identifying differences in PTMs on distinct pools of PABP

As a second approach to examine if PTMs to PABP regulate its function, I designed experiments that would allow me to probe the PTMs of endogenous or exogenous WT PABP in different translational states. If I could detect distinct PTMs to PABP in these different states, it would suggest they may play a role in regulating mRNA translation. For these experiments I took advantage of a commercially available antibody specific to PABP containing methylated arginines at positions 455 and 460 (Cell Signaling), which had been identified as targets of methylation by CARM118.

To test whether PABP bound to actively translating mRNA showed different modification status at R455/R460 than PABP not associated with polysomes, I sedimented cell lysate over sucrose polysome gradients. I then performed Western blotting for fractions of the gradient using the methylation-specific PABP antibody as compared to a general PABP antibody. Cells subjected to global translation repression by heat shock (42°C, 2 hours), serum starvation (72 hours), or treatment with puromycin (500 ng/ml, 20 minutes) were compared to control cells in normal growth conditions. These assays revealed the expected shift of PABP from heavy to light polysomal fractions (Fig. 3, left panels) but showed the same pattern for the R455/R460-methylation specific antibody as for the general antibody against PABP (Fig. 3, compare left to right panels), indicating that either the monitored methylation events did not regulate PABP association with polysomes or that the methylation specific antibody lacked specificity. Treatment of cells with the methyltransferase inhibitor AMI-1
showed no change for the methylation specific antibody (Fig. 3), but I have no positive control to determine if AMI-1 treatment was successful in inhibiting methylation.

Using antibodies against global acetylated lysines to monitor for generally acetylated PABP was attempted, but turned out not feasible in these experiments. Probing entire fractions from the sedimentation yielded too much background from other proteins to identify bands corresponding to PABP. I attempted to immunoprecipitate PABP from the fractions, but IPs were not possible as sepharose beads did not mix or sediment adequately in the high concentration of sucrose, leading to poor depletion of PABP from the samples. Moreover, testing these general antibodies against immunoprecipitated PABP from cells grown under normal conditions showed no signal, indicating that the antibodies did not react with PABP at detectable levels.

Discussion

Because of the central role of PABP in mRNA regulation, its potential for PTMs, and the fact that PABP is bound to mRNAs in the nucleus to near the beginning of decay, it seems likely that mRNA activity can be regulated by PABP PTMs. However, it is difficult to draw many conclusions from the experiments I performed investigating the role of PTMs in PABP function. Experiments involving mutant versions of PABP failed to show any change on PABP association with polysomal fractions of a sucrose gradient or subcellular location from endogenous PABP. However, there are pitfalls to consider in these
experiments. One important factor is that all the tested mutants, except for the 104/108 double mutant, are single point mutants. It is possible that any PTMs regulating PABP act in a combinatorial fashion, where more than one PTM is needed in order to have a quantifiable effect. Another consideration is that all of the mutant experiments were conducted in the presence of endogenous PABP. PABP has a minimal binding site of around 12 amino acids\textsuperscript{19}, and a footprint of around 25 amino acids\textsuperscript{20}. mRNA in humans are initially adenylated to tails of around 250 nucleotides in length, but in the cytoplasm tails may be shorter. A recent high-throughput sequencing study suggested that the tail length for many mRNA in humans may be between 50-100 nucleotides in length\textsuperscript{21}. This would allow for 2-4 molecules of PABP per mRNA. Because of this, using a mutant PABP may not have a detectable effect if an endogenous PABP on the same transcript is able to rescue complete function. As for the experiments conducted using modification specific antibodies, the assay is only as reliable as the antibodies. Several of the antibodies I used were created specifically to observe histone modifications, and were made using short, modified peptide sequences from histone tails. They may have limited cross-reactivity with PABP, as noted by my inability to see a signal even on immunoprecipitated PABP. Similarly, though the 455/460 arginine methylation antibody was created with the exact PABP sequence, I lack a negative control to show that it does not cross react with unmethylated PABP. Despite these pitfalls, the role of PTMs to PABP and how PABP might control the fate of mRNAs remains an interesting question for future research.
Materials and Methods

Plasmids

pcDNA3-FLAG-PABP has been previously described (PABPC1 in text)\textsuperscript{22}. Mutants were created using site directed mutagenesis.

Western blotting and antibodies

Proteins were separated on 10\% SDS-PAGE gels and transferred to nitrocellulose. Primary antibodies were rabbit anti-FLAG (Sigma F7425, 1:1000), rabbit anti-PABP (Abcam ab21060, 1:1000), rabbit anti-PABP 455/460 (Cell Signaling c60a10, 1:1000).

Polysome Profiling

10 cm plates were seeded with HeLa cells at 10\% confluency. 24 hours after plating, cells were transfected with 3 \( \mu \)g pcDNA3-FLAG-PABP WT or mutants and 12 \( \mu \)g pcDNA3 using TansIT HeLaMONSTER (Mirus) according to manufacturer’s protocol. 48 hours after transfection, polysome profiles were performed as previously described\textsuperscript{23}, with the following modifications. Cells were lysed in 1 ml, and 800 \( \mu \)l of lysate were sedimented. After fractionation, protein was precipitated from samples by addition of 1:10 1\% Sodium Deoxycholate (SIGMA) as a carrier and 100\% cold trichloroacetic acid (w/v in water, TCA) was added to 20\% final concentration. Samples were kept on ice for 20 minutes, then spun at 13,000 g for 30 minutes at 4\(^\circ\)C. Pellets were washed once with ice cold
20% TCA followed by 2 washes with acetone (-20°C) with 5 minute spins at 4°C in between washes. Pellets were resuspended in 100 µl 2x SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (Sigma), 0.2% bromophenol blue (Sigma), 20% glycerol (Sigma), 200 mM dithiothreitol (Sigma)) and 100 µl 1M Tris-HCl pH 8.8 for Western blotting.

**Indirect immunofluorescence assays**

HeLa cells were seeded at 10% confluency in 3.5-cm wells with 2 ml DMEM/10%FBS containing. 24 hours after seeding, cells were transfected with 1 µg of pcDNA3 and 1 µg of pcDNA3-FLAG-PABP WT or mutants using TransIT HeLaMONSTER (Mirus) according to manufacturer’s protocol. 24 hours after transfection cells were transferred to 8-well chamber slides (Thermo Scientific). 24 hours later cells were fixed in 3 to 4% formaldehyde in PBS for 15 minutes at room temperature, permeabilized in 0.5% Triton-X100 in PBS containing 1% goat serum (PBS; 136 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH7.4, GS, Life Technologies) for 15 minutes, and incubated with anti-FLAG antibodies (1:100) in PBS/1% GS for 1. Cells were then washed 2 times for 5 minutes with PBS/1% GS and treated with secondary antibodies in PBS/1% GS for 1 hour (anti-rabbit Texas Red, Invitrogen, 1:1,000), followed by treatment with 4’,6-diamidino-2-phenylindole(DAPI, 5 µg/ml in PBS/1% GS, Sigma) for 2 minutes and three washes with PBS/1% GS and one wash with distilled water. Images are from a Zeiss AX10 microscope using a 60x objective.
Table 5.1. PABP mutants
Table indicating single mutants made for this study. * indicates amino acids mutated for a double mutant.

<table>
<thead>
<tr>
<th>Sequence Number</th>
<th>Amino Acid</th>
<th>Non-PTM mimic</th>
<th>PTM mimic</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>Y</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>54</td>
<td>Y</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>104*</td>
<td>K</td>
<td>R</td>
<td>Q</td>
</tr>
<tr>
<td>108*</td>
<td>K</td>
<td>R</td>
<td>Q</td>
</tr>
<tr>
<td>116</td>
<td>Y</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>213</td>
<td>K</td>
<td>R</td>
<td>Q</td>
</tr>
<tr>
<td>259</td>
<td>K</td>
<td>R</td>
<td>Q</td>
</tr>
<tr>
<td>364</td>
<td>Y</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>382</td>
<td>Y</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>512</td>
<td>K</td>
<td>R</td>
<td>Q</td>
</tr>
</tbody>
</table>
Figure 5.1 – PABP mutants behave as control in sucrose gradients

(A) Typical trace for a sedimentation experiment showing relative unbound, monosomal, and polysomeal fractions

(B) Western blot of indicated FLAG-PABP WT or mutants. Indicative of all generated mutants.
Figure 5.2 – PABP mutants form SGs in response to heat shock
Indirect immunofluorescence assay for FLAG-PABP mutants (red) in transfected cells grown in control or heat shock (42º C, 30 min). DAPI staining of nuclei in blue.
Figure 5.3 – PABP R455/R460 methylation under control or stress conditions
Western blot for protein isolated from cells grown under control, heat shock (42º C, 2 hours), serum starvation (72 hours), puromycin treatment (500 ng/ml 20 min), or AMI-1 treatment (300 μM, 24 hours) and subjected to sedimentation on sucrose gradients.
References


CHAPTER VI – Discussion  

Future Directions – Lsm4 

While my work on Lsm4 showed evidence that arginine methylation of the Lsm4 C-terminal RGG domain is important for PB formation, there are still many interesting questions that can be investigated. For me, the most interesting outstanding question is what is the role of the association of Hat1 with Lsm4? The initial novel finding of Hat1 as an interacting partner of Lsm4 dependent on the RGG domain, along with published literature showing a role of the mouse homolog of Hat1 regulating chromatoid bodies in the murine germ line, makes Hat1 seem like an ideal candidate for further PB regulation by PTMs in mammalian cells. If Hat1 were to act in a similar manner in human cells, an increase in the association of Hat1 could lead to a loss of PBs. Initial experiments suggest that the Lsm4 association with Hat1 does change with RGG methylation. Hat1 association with Lsm4 appears to increase with PRMT5 depletion (Fig. 1). As a control, Hat1 association with Lsm4 KGG does not appear to change in PRMT5 knockdown conditions, suggesting that the change is based on arginine methylation and not pleiotropic effects of PRMT5 depletion. In the mouse germ line, Hat1 targets DDX4, an RNA helicase, and acetylation of DDX4 lysines renders it unable to bind to mRNA and results in a disruption of chromatoid bodies. Given that it like DDX4 is an RNA helicase, another possible candidate for regulation by Hat1 could be eIF4A, a member of the eIF4F complex that allows for efficient ribosomal scanning. Lsm4 could serve to inhibit translation.
initiation by acetylating eIF4A and disrupting its function. However, depletion or overexpression of Hat1 does not seem to affect PBs as measured by several assays (see Chapter 3). Furthermore preliminary experiments IPing DDX6, as well as other candidate decay factors, from cells +/- Hat1 depletion followed by western blotting using acetylated lysine specific antibodies showed no obvious changes. It is possible that deacetylation occurs rapidly in cell lysates, and repeating the experiments in the presence of deacetylase inhibitors may identify Hat1 targets. These results may also indicate that there is redundancy in the system. Yeast HAT1 null strains show no phenotype, and there are other cytoplasmic acetyltransferases, such as P300, ATF2, and Elp3, in yeast and humans. Finding the target or targets of Hat1 will be critical for understanding its possible role in mRNA regulation.

Hat1 could also associate with Lsm4 for reasons other than PB regulation or mRNA decay. Lsm4 is known to associate with histone mRNA. It is possible that Lsm4 association with histone mRNA recruits the Hat1 complex to allow for efficient histone modification as soon as histone proteins are translated. Lsm4 and Hat1 also have roles in the nucleus, Lsm4 as a splicing factor and Hat1 can be associated with nuclear H3/H4. It is possible that the Lsm4-Hat1 interaction has a role in some nuclear function.

Another question that my research has been unable to resolve is the mechanism for the RGG domain functioning in PB regulation. We ruled out general roles of the RGG domain in mRNA decay, decapping factor recruitment, and translation inhibition, leaving us to believe that the RGG domain of Lsm4
acts as the yeast Lsm4 C-terminal Q/N-rich domain and plays a role in protein-protein aggregation. Initial experiments with GFP-RGG domain fusion proteins did not show foci, something that can be seen with GFP fused to the C-terminal domain of yeast Lsm4\(^8\), suggesting the RGG domain is necessary but not sufficient for PB formation (Jackie Dennis, Figure 3.10). It is possible that Lsm4 may have to be bound to mRNA to form PBs. This could be tested by expressing the MS2-RGG construct with an mRNA containing MS2 binding sites and observing cells for MS2 foci. It is also possible that the RGG domain plays a role in the nucleus that may be important for PB formation. There is some evidence that RGG domains and methylation may play a role in the nuclear import of proteins; methylation of the RGG domain of Npl3 influences its association with Mtr10 which is required for nuclear import\(^9\). However, I have not observed an obvious change in Lsm4 localization between mutants in my experiments. Other experiments also suggest that the RGG domain is not necessary for Lsm4 to associate with the U6 snRNA (Jackie Dennis, unpublished observations).

However, further experiments could be conducted to look for general splicing defects in cells that only express Lsm4 \(\Delta\)RGG. It is also possible that the RGG domain of Lsm4 shows no role in mRNA decay or translational repression because these systems are very robust in human cells. It is possible that testing decay or translational repression under conditions that already stress the decay pathway could reveal a role for the RGG domain in these activities in certain conditions.
Future Directions – PNRC2

PNRC2 has been shown to act as a decapping activator that can stimulate the catalytic activity of Dcp1/Dcp2 in vitro\textsuperscript{10}. Some studies have also implicated PNRC2 stimulating decapping in vivo as part of the NMD pathway\textsuperscript{10,11}. While these studies suggest that PNRC2 is able to stimulate decapping by interacting with both Dcp1 and Dcp2 to stabilize certain conformations, our collaborators, the Gross lab at UCSF, have shown that PNRC2 has a YAG motif similar to Edc1, separate from the Dcp1-interaction motif, that is required for PNRC2 to activate the decapping reaction (Figure 4.1). Disruption of this YAG motif does not affect the localization of PNRC2 to PBs, unlike disruption of the Dcp1 interaction region, which causes a loss of PB localization (Figure 4.2). While cells expressing a PNRC2 YAG mutant were able to form PBs, there could still be some defects to PB function. It’s possible that the YAG mutant causes a defect in decapping that may result in an increase in time it takes Dcp1 to cycle in and out of PBs, which can be measured by FRAP experiments using a fluorescently tagged Dcp1.

As previous reports implicating PNRC2 in NMD in human cells have relied mainly on tethering assays or monitoring the steady state levels of target mRNA\textsuperscript{10,11}, it could be interesting to see if PNRC2 depletion can affect the decay rate of NMD targets. Because decapping is not always rate limiting in NMD in human cells\textsuperscript{12}, it is possible that to see an effect of PNRC2 disruption of the endonucleolytic decay may be necessary. This could be done by depleting cells of SMG6\textsuperscript{13}. It is also possible that PNRC2 acts outside of NMD as a general...
decapping factor. Our initial experiments to see if PNRC2 disruption blocked decapping of an ARE reporter failed to give positive results. However, decapping in human cells is a robust process, and it is difficult to trap decapping intermediates. These experiments could be repeated in a system where decapping is already impaired, such as with a knockdown of the major 5’ to 3’ exonuclease Xrn1 which leads to an increased mRNA half-life\textsuperscript{14}, to see if disruption of PNRC2 can amplify any defects. Some decapping factors, such as Edc3, are thought to affect only a subset of mRNAs, and this could be true for PNRC2. A more global approach, such as RIP-seq, could identify specific targets of PNRC2 if a candidate approach fails.

The identification of PNRC2 as a decapping activator is also interesting because while PNRC2 shares a YAG motif capable of stimulating decapping, they are not homologs, as there are no known homologs of yeast decapping activators Edc1 and Edc2 in humans. It raises the question of if there are homologs of Edc1 and Edc2 in metazoans and just how many decapping factors there are. PNRC2 is a small (139 aa), unstructured protein and it is possible that there are similar proteins that have yet to be identified, or proteins that are too small to have been properly catalogued.

**Future Directions – PABP**

PABP, the protein bound to the 3’ poly(A) tails of the vast majority of eukaryotic mRNA, interacts with a wide range of partners. Many of these, such as eIF4G, work synergistically with PABP to enhance binding to mRNA and
stimulate translation. Conversely, PABP is known to interact with a smaller subset of proteins that can stimulate mRNA decay, such as the deadenylase PAN3. As many sites of PTMs have been identified on PABP, it is possible that PABP modification regulates the state of the mRNP by dictating what partners it interacts with. Initial experiments using point mutants designed to block or mimic single PTMs on PABP failed to show activity different from WT PABP (Figure 5.1). One of the reasons for this may be that they were tested in vivo in the presence of endogenous PABP. As multiple copies of PABP bind each mRNA, it is possible any effects of the mutants were counteracted by the endogenous PABP. To get around this, the ability of PABP mutants to stimulate translation in vitro in the absence of PABP could be conducted using a dual reporter luciferase assay with recombinant proteins. As PTMs to PABP could influence its ability to interact with eIF4F complex members, these other factors would have to be included in the in vitro reactions. It could also be possible to test the ability of PABP mutants to stimulate translation of a tethered reporter in cells. To avoid endogenous PABP from interacting with the reporter, a reporter lacking a poly(A) tail could be used. There are several 3’ ends from viral systems that could be added to the reporter to allow for stability in the absence of a poly(A) tail. In a system such as this, it may be necessary to mutate or delete the RNA binding domains of PABP to preclude mutants from binding control reporter mRNA. Another problem with initial studies was that almost all mutants tested were single mutants. It could help to make combinations of the mutants, perhaps mutating all possible PTMs in a single domain at a time.
While mutational analysis may give some insights into how PABP is regulated through PTMs, the best approach to the question may be through proteomics. It could be possible to isolate large populations of PABP, and mRNPs, which are in different states of translational competency either through sucrose sedimentation or by globally affecting translation in cells. Once isolated, PABP and its interacting partners could be sequenced. This would not only identify PTMs to PABP, but could potentially reveal new interacting partners that are important for PABP function. The results from this approach could then be validated through mutational studies that could elucidate the mechanisms of mRNP regulation.

The role of Processing Bodies

Since PBs were discovered nearly 20 years ago as mouse Xrn1 foci\textsuperscript{18}, there has been much speculation as to their function\textsuperscript{19,20,21}. As decay factors and mRNA decay intermediates were discovered to co-localize to PBs, a straightforward hypothesis would be that they are sites of active mRNA decay\textsuperscript{19}. This could be advantageous as mRNA decay could be segregated, preventing off target degradation, and it could be efficient as there would be a very high local concentration of decay factors. However, further investigation into mRNA dynamics and PBs have shown evidence that PBs are dispensable for many decay pathways\textsuperscript{20}. Depletion of mRNA decay factors that reduce visible PBs in cells can often have no effect on mRNA decay rates or steady state levels\textsuperscript{20}. 
Also, there is evidence that mRNA from PBs can re-enter the actively translating pool\textsuperscript{22}.

An important consideration may be that Dcp2, the catalytic enzyme for decapping, is thought to be one of the most regulated members of the mRNA decay pathway\textsuperscript{23, 24, 25}. Global proteomic studies suggest that Dcp2 is present in cells at a lower copy number (10 fold less than Dcp1, as much as 100 fold less than DDX6 and Lsm1) than most other decay factors\textsuperscript{26}. Studies from our lab also show that Dcp2 has a short half-life compared to other decay factors, and that uncomplexed Dcp2 is targeted for rapid proteosomal degradation\textsuperscript{24}. Because of this, it may be that Dcp2 is normally the limiting component of mRNA decay. In this case, depletion of other decay factors, even by 90-95\%, could still leave stoichiometric amounts for Dcp2 to function normally and have no effect on decay rates or steady state levels of mRNA. However, even though there is a sufficient amount of these factors to promote decay, there may not be enough to either aggregate and form PBs, or maintain mRNA targeted for decay in a translationally repressed state. Both of these would result in a loss of PBs, but maintain proper decay. This leaves the question as to what PBs are for.

I think PBs arise as a consequence of normal cellular function, and act as a repository of mRNA that can be used if needed. There is evidence that because of the interaction of PABP with translation termination factors, mRNA may lose PABP proteins gradually as translation occurs, leading to poly(A) tail trimming and eventually loss of translation initiation\textsuperscript{27}. These mRNAs could then enter the mRNA decay pathway by association of the Lsm1-7-PatL1 complex to
the deadenylated 3’ end. As Dcp2 is limiting, these mRNAs can recruit further decay factors that repress translation and promote aggregation into PBs without yet committing decapping and final 5’ to 3’ degradation. Also, as they are bound on the 3’ end and protected from 3’ exonucleolytic degradation, they maintain an entire ORF capable of making a functional protein. Even though these mRNAs bound by decapping enhancers are primed for degradation, work from our lab suggests that mRNA quality control pathways, such as NMD, targeting nascent transcripts can still maintain priority for decay factors or utilize non decapping dependent decay, such as endonucleolytic cleavage, to prevent aberrant protein production (Sebastien Durand et al, in submission). Depending on the availability of free Dcp2 not needed for quality control, PB localized mRNA undergo continual degradation. If transcription rates drop, PBs may be triggered to disassemble, and the increased free eIF4F can outcompete decay factors to promote the return of mRNA to a translating pool.

Aggregation of these decay intermediates into PBs may be a consequence of conserved protein-protein interaction domains on translational repressors that serve multiple purposes. For example, in germline cells, early development, or senescent neuronal cells, protein expression is often highly regulated post-transcriptionally\(^ {28, 29, 30} \). In these cases mRNA localization is often important for proper gene expression. Translational repressors, such as DDX6, may aggregate primarily for these reasons, and as a consequence aggregate in PBs to no adverse effect. There is also some evidence that PBs and stress
granules may be able to undergo vacuole or autophagosome degradation, which could be a reason why the aggregation is still favored in differentiated cells.
Figure 6.1 – PRMT5 depletion increases Hat1 association with Lsm4 WT
Western blot showing proteins isolated from FLAG immunoprecipitation from cells expressing FLAG-Lsm4 WT or KGG treated with siRNA against Lsm4 (all cells) and PRMT5 (indicated cells). Depletion of PRMT5 shown below IP with Hat1 as loading control. All images are from different exposures of the same Western blot.
References


