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**MECHANISMS AND CONTROL OF mRNA TURNOVER
IN THE YEAST *SACCHAROMYCES CEREVISIAE***

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To my parents

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Teixeira, D, Sheth, U, Valencia-Sanchez, MA, Brengues, M and Parker, R (2005) Processing bodies require RNA for assembly and contain nontranslating mRNAs. *RNA*. 11(4):371-382.

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No cumprimento do Decreto-Lei mencionado supra, o autor desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação e discussão dos resultados e na sua redacção.

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ABSTRACT

mRNA turnover, is an integral event in gene expression that provides post-transcriptional control of transcript levels and ensures the elimination of faulty mRNAs that could impact on cell function. Over the past decade, examination of mRNA turnover processes in different organisms have succeeded in increasing the appreciation for the large contribution of this process in controlling gene expression. The yeast *Saccharomyces cerevisiae*, owing to the power of genetic and molecular manipulations possible in this organism, has been particularly fruitful in illuminating the mechanisms of mRNA turnover, and for the identification and characterization of the decay enzymes and factors that participate in mRNA degradation. Two major general and functionally redundant pathways of mRNA turnover have been characterized in yeast, and are believed to exist in all eukaryotic cells. The first step for both pathways is the shortening of the mRNA 3' poly(A) tail. Following poly(A) removal, the deadenylated mRNA can be degraded exonucleolytically in a 3' to 5' direction, or more commonly, the mRNA 5' cap structure is removed (decapping), facilitating 5' to 3' exonucleolytic digestion. Recent experiments from our group have defined novel cytoplasmic structures, referred to as processing bodies (P-bodies), wherein mRNA decay factors are concentrated and where mRNA decapping and 5' to 3' decay can occur. The identification of yeast P-bodies represents one of the most important recent discoveries in the field of mRNA turnover. Importantly, P-bodies are conserved in other eukaryotes, they suggest cellular compartmentalization of mRNA turnover, providing a potential point to enforce regulation on mRNA turnover and moreover, they are likely to be sites of additional processes in cytoplasmic mRNA physiology. Notwithstanding these observations, P-body formation, function and biological impact on the cell remain largely unknown.

In this work, the yeast *Saccharomyces cerevisiae* was used to investigate the formation, assembly and function of P-bodies. We examined how P-bodies structures vary under a diversity of cellular conditions. Our results revealed that yeast P-bodies are dynamic structures, which vary in size and abundance between individual cells, and in response to numerous extracellular stresses and growth conditions, suggesting that P-bodies provide an important function during cellular responses to stress in addition to being sites of mRNA degradation. We also investigated the relationship between P-bodies and the translational status of mRNA in the cell. We provide evidence that P-bodies and mRNA translation show a reciprocal relationship; P-body assembly increased with the availability of nontranslating mRNA and decreased when the mRNA is trapped in the polysome pool. These results indicate that P-bodies contain nontranslating mRNA, and is consistent with

the spatial segregation of translating mRNA and P-body structures, providing a possible mechanism for the observed competition between mRNA degradation and translation. We further addressed whether nontranslating mRNAs within P-bodies could exit out of a P-body structure and return to the pool of mRNA that can engage in translation. We provide evidence that mRNAs targeted for P-bodies are not committed for degradation, but can return to translation, thereby suggesting an additional biological role for P-bodies in sequestering translationally repressed mRNAs. Our results have defined an important cycle of mRNA physical movement within the cell wherein mRNAs move from polysomes to P-bodies and then within P-bodies can either be targeted for degradation or returned to active translation from a stored state. This finding has fundamental implications for the control of mRNA translation and degradation, and we propose a specific role for P-bodies in modulating the balance between translating and nontranslating pools of mRNAs. Furthermore, we suggest that mRNA storage within mRNP granules is a widely conserved mechanism for gene regulation.

The mechanism of P-body formation and assembly was investigated, and we provide evidence that P-bodies are dependent on RNA for their formation and integrity. Furthermore, genetic analyses of P-body formation and composition using yeast strains defective in proteins known to accumulate in P-bodies revealed that P-body assembly is redundant and no single known protein component of a P-body is absolutely required for P-body assembly, with partial roles being played by certain P-body components. Moreover, these experiments also revealed specific dependencies for individual proteins association with P-bodies. Our results provide insight both into the mechanism of P-body assembly and the function of individual proteins in mRNA degradation, allowing us to propose a working model for the mechanism by which yeast P-bodies accumulate and function in light of additional aspects of mRNA metabolism.

Taken together, these studies represent a very important contribution to our understanding of the assembly and function of P-bodies in the cell, and ultimately the complex control of mRNA translation and turnover.

SUMÁRIO

A degradação do mRNA, é um processo integrado na expressão genética que permite o controlo pós-transcricional dos níveis dos transcritos celulares e assegura a eliminação de mRNAs defeituosos que podem afectar a função celular. Durante a última década, sucederam-se os estudos sobre os mecanismos de degradação do mRNA em diferentes organismos, juntamente com uma maior apreciação da importância destes processos para o controlo da expressão genética. A levedura *Saccharomyces cerevisiae*, devido às manipulações genéticas e moleculares possíveis neste organismo, tem sido particularmente útil na elucidação dos mecanismos da degradação do mRNA, e na identificação e caracterização das enzimas e dos factores envolvidos na degradação do mRNA. As duas principais, e funcionalmente redundantes, vias de degradação do mRNA foram caracterizadas na levedura, mas também estão presentes em outras células eucarióticas. Em ambas as vias, a degradação do mRNA é iniciada pelo encurtamento da cauda de adeninas na extremidade 3' do mRNA. Depois da remoção da cauda de adeninas, o transcrito pode ser degradado por exonucleases na direcção 3'-5', ou mais comumente, a estrutura cap na extremidade 5' do mRNA é removida permitindo a degradação do transcrito por exonucleases na direcção 5'-3'. Recentemente, experiências realizadas pelo nosso grupo definiram novas estruturas citoplasmáticas, chamadas corpos de processamento (corpos-P), como locais onde os factores que participam na degradação do mRNA estão concentrados, e onde a remoção da 5' cap e a degradação 5'-3' do mRNA pode ocorrer. A identificação dos corpos-P na levedura foi uma das mais importantes descobertas no campo da degradação do mRNA. Notavelmente, os corpos-P são conservados noutros organismos eucariotas, sugerem que o processo de degradação do mRNA ocorre compartimentalizado na célula, adicionando uma potencial etapa para o controlo da degradação do mRNA, e além do mais, são locais potenciais onde outros processos aliados a fisiologia citoplasmática do mRNA podem ocorrer. Não obstante estas afirmações, a formação dos corpos-P, as suas funções e o impacto biológico na célula é largamente desconhecido.

Neste trabalho, usámos a levedura *Saccharomyces cerevisiae* para investigar a formação, montagem e função dos corpos-P. Neste sentido, examinámos como estas estruturas variavam em resposta a diversas condições celulares. Os nossos estudos revelaram que os corpos-P são estruturas dinâmicas, que variam em tamanho e abundância de célula para célula, e em resposta a vários stresses extracelulares e condições de crescimento, sugerindo que os corpos-P desempenham uma importante função durante a resposta celular ao stress, além de funcionarem como locais de degradação do mRNA. Também investigámos a relação entre os corpos-P e o estado de

tradução do mRNA na célula. Os nossos estudos demonstraram que os corpos-P e a tradução do mRNA estão reciprocamente relacionados; a montagem dos corpos-P aumentou com a disponibilidade de mRNA que não está associado a ribossomas, e diminuiu quando o mRNA se encontrava associado a ribossomas. Estes resultados indicam que os corpos-P contém mRNAs que não estão a ser activamente traduzidos, e são consistentes com a segregação celular dos mRNAs entre a tradução associada com polissomas e as estruturas corpos-P, sugerindo um possível mecanismo para a competição existente entre os processos de degradação e tradução do mRNA. Também analisámos se estes mRNAs não traduzidos localizados dentro dos corpos-P, poderiam sair dos corpos-P e retornar para a maquinaria molecular da tradução a fim de serem novamente traduzidos. Os nossos estudos demonstraram que os mRNA acumulados nos corpos-P não estão destinados a ser degradados, podendo também voltar a ser traduzidos, sugerindo assim uma nova função biológica para os corpos-P no armazenamento de mRNAs não traduzidos. Estes resultados permitiram definir um importante ciclo de movimento físico do mRNA dentro da célula, onde os mRNAs se movem dos locais de tradução para os corpos-P e uma vez dentro dos corpos-P podem ser degradados ou sair destas estruturas para voltarem a ser traduzidos. Esta descoberta tem implicações fundamentais para o controlo da tradução e degradação do mRNA, pelo que propomos um papel específico para os corpos-P em modelar o equilíbrio entre mRNAs que são ou não traduzidos. Além do mais, sugerimos que o armazenamento do mRNA em grânulos citoplasmáticos sob a forma de mRNPs é um mecanismo de regulação genética largamente conservado.

Também investigámos o mecanismo de formação e montagem dos corpos-P. Os nossos estudos revelaram que os corpos-P dependem da presença de mRNA para a sua formação e integridade. Além do mais, análises genéticas da formação e composição dos corpos-P, usando estirpes de levedura que não expressam diversos componentes proteicos dos corpos-P, revelaram que a montagem dos corpos-P é redundante, e nenhum componente que se sabe acumular nos corpos-P é absolutamente necessário para a sua formação, apesar de certos componentes desempenharem papéis parciais na montagem dos corpos-P. Estas experiências também identificaram dependências específicas para a associação de proteínas individuais com os corpos-P. Os nossos resultados elucidaram não só o mecanismo de montagem dos corpos-P, mas também a função de proteínas individuais na degradação do mRNA, permitindo-nos propor um possível modelo para o mecanismo de acumulação e função dos corpos-P na levedura à luz de aspectos adicionais do metabolismo do mRNA.

De uma forma global, os nossos estudos representam uma contribuição muito importante para a elucidação da formação e função dos corpos-P na célula, e finalmente para o entendimento do complexo controlo da degradação do mRNA e da sua tradução.

RÉSUMÉ

Le renouvellement des ARNm représente une étape importante dans l'expression des gènes en participant au contrôle post-transcriptionnel du niveau de présence des messagers et en assurant l'élimination des ARNm défectueux qui pourraient avoir un effet sur le fonctionnement cellulaire. Au cours de cette dernière décennie, l'étude du métabolisme des ARNm dans différents organismes a considérablement contribué à l'état des connaissances dans le contrôle de l'expression des gènes. La levure *Saccharomyces cerevisiae*, grâce aux manipulations génétiques et moléculaires possibles dans cet organisme, a participé grandement à la découverte des mécanismes impliqués dans le renouvellement des ARNm, et à l'identification et la caractérisation des enzymes de dégradation et des facteurs participant à la dégradation des ARNm. Deux mécanismes généraux et fonctionnellement redondants de la dégradation des ARNm ont été caractérisés chez la levure, et sont conservés chez les autres eucaryotes. La première étape, commune à ces deux mécanismes est le raccourcissement de la queue poly(A) en 3' du messager. Suite à l'enlèvement de la queue poly(A), l'ARNm peut être dégradé de façon exonucléolytique de 3' vers 5', ou bien de façon plus commune, la coiffe en 5' de l'ARNm est éliminée (décapping), permettant la digestion exonucléolytique dans le sens 5' vers 3'. Des expériences récentes dans notre équipe, ont permis l'identification de nouvelles structures cytoplasmiques, nommés corpuscules-P, à l'intérieur desquels les facteurs de dégradation des ARNm sont concentrés et où le décapping et la dégradation 5' vers 3' ont lieu. L'identification des corpuscules-P chez la levure représente une des plus importantes récentes découvertes dans le domaine du métabolisme des ARNm. Un fait important, ces corpuscules-P sont conservés chez les autres eucaryotes, ce qui suggère une compartimentalisation cellulaire pour le renouvellement des ARNm, ajoutant une étape potentielle à la régulation du renouvellement des ARNm, et de plus, ils représenteraient des sites pour d'autres processus liés à la physiologie cytoplasmique des ARNm. Cependant, la formation des corpuscules-P, leurs fonctions et leurs impacts biologiques sur la cellule restent encore largement inconnus.

Dans ce travail, la levure *Saccharomyces cerevisiae* a été utilisée pour l'étude de la formation, de l'assemblage et de la fonction des corpuscules-P. Nous avons examiné la façon dont la structure des corpuscules-P varie avec les différentes conditions cellulaires testées. Nos résultats ont montré que les corpuscules-P sont des structures dynamiques, dont la taille et l'abondance varient d'une cellule à l'autre chez la levure, ainsi qu'en réponse à de nombreux stress extra-cellulaires et aux conditions de croissance, ce qui suggère que les corpuscules-P accomplissent une fonction importante au cours des

réponses cellulaires à un stress, tout en étant aussi le site de dégradation des ARNm. Nous avons aussi analysé la relation entre les corpuscules-P et l'état de traduction des ARNm dans la cellule. Nous avons démontré que les corpuscules-P et la traduction des ARNm sont étroitement liés; l'assemblage des corpuscules-P augmente avec le taux d'ARNm qui ne sont pas en cours de traduction et diminue quand les ARNm sont engagés sur les polysomes. Ces résultats indiquent que les corpuscules-P contiennent des ARNm qui ne sont pas en cours de traduction, et ceci est consistant avec la ségrégation des ARNm en cours de traduction et avec la structure des corpuscules-P, suggérant un mécanisme possible pour la compétition observée entre la dégradation des ARNm et leur traduction. Nous avons aussi cherché à savoir si ces ARNm présents dans les corpuscules-P et qui ne sont pas en cours de traduction pouvaient exister à l'extérieur de la structure d'un corpuscule-P et retourner ainsi à la machinerie de traduction. Nous avons démontré que les ARNm présents dans les corpuscules-P ne sont pas condamnés à être dégradés, mais qu'ils peuvent être traduits, suggérant une nouvelle fonction biologique pour les corpuscules-P en tant que lieu de stockage des ARNm qui ne sont pas en cours de traduction. Nos résultats ont permis de définir un mouvement cyclique de l'ARNm à l'intérieur de la cellule où les ARNm se déplacent des polysomes vers les corpuscules-P et peuvent ensuite être soit dégradés à l'intérieur des corpuscules-P ou retourner vers la machinerie de traduction à partir de cet état de veille. Cette découverte a des implications fondamentales dans le contrôle de la traduction et de la dégradation des ARNm, et nous proposons un rôle spécifique pour les corpuscules-P dans le maintien de la balance entre les ARNm en cours de traduction et ceux qui ne le sont pas. De plus, nous pensons que le stockage des ARNm dans les granules mRNP est un mécanisme hautement conservé pour la régulation des gènes.

Le mécanisme de formation et d'assemblage des corpuscules-P a été analysé, et nous avons démontré que la formation et l'intégrité des corpuscules-P sont dépendantes de la présence de l'ARN. De plus, les analyses génétiques sur la formation et la composition des corpuscules-P en utilisant des souches de levure dont les gènes codant pour des protéines reconnues pour leur accumulation dans les corpuscules-P ont été mutés, ont révélé que la formation des corpuscules-P est redondante et qu'aucun composant protéique connu n'est absolument nécessaire pour la formation des corpuscules-P, avec des rôles partiels joués par certains composants des corpuscules-P. Enfin, ces expériences ont aussi révélé des dépendances spécifiques pour l'association de protéines individuelles avec les corpuscules-P. Nos résultats apportent des évidences à la fois sur le mécanisme de formation des corpuscules-P et sur la fonction de certaines protéines dans la dégradation des ARNm, nous permettant de proposer un modèle pour le

mécanisme par lequel les corpuscules-P de levure s'accumulent et fonctionnent en parallèle des autres aspects du métabolisme des ARNm.

L'ensemble de ces résultats contribue grandement à notre connaissance de la compréhension de l'assemblage des corpuscules-P et leurs fonctions dans la cellule, ainsi que du contrôle très complexe de la traduction et de la dégradation des ARNm.

ORGANIZATION OF THE THESIS

The present thesis represents part of the work performed at Dr Roy Parker lab at Department of Molecular and Cellular Biology, University of Arizona, Tucson, USA to complete my Ph.D. dissertation. This thesis is organized into five chapters. The first chapter reviews and introduces the main concepts and current understanding of the mechanism of mRNA turnover and its regulation in the yeast *Saccharomyces cerevisiae*. The second chapter addresses the physical nature and properties of yeast cytoplasmic sites of mRNA turnover, P-bodies, with respect to mRNA translation and different cellular growth conditions. In the third chapter, evidence that P-bodies represent sites of additional biological processes, such as storage, is presented. The fourth chapter presents a simplistic model for yeast P-body assembly based on genetic analysis of P-body composition and organization. Finally, general conclusions are presented in chapter five, unifying the published data and suggesting avenues for future research.

ABBREVIATIONS

A	Adenosine
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
AUF1	ARE/poly(U)-binding degradation factor 1
AU-rich element	Adenylate, uridylate-rich element
C	Cytosine
CEN	Centromere
C-terminal	carboxy-terminal
CUP1	Copper-inducible promoter
DEAD	Aspartate-Glutamate-Alanine-Aspartate
DEPC	Diethyl pirocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EF	Elongation factor
eIF	Eukaryotic initiation factor
ELAV	Embryonic lethal abnormal vision
eRF	Eukaryotic release factor
EtOH	Ethanol
G	Guanosine
GAL1	Galactose kinase 1
GPD	Glyceraldehyde-3-phosphate dehydrogenase
GTPase	Guanosine 5'-triphosphatase
hnRNP	Heterogeneous ribonucleoprotein
HRP	Horseradish peroxidase
HuR	Human antigen R
KH	K-Homology domain
LEU	Leucine
Lsm	Like-Sm
m⁷GDP	N7-methylguanosine diphosphate
m⁷GMP	N7-methylguanosine monophosphate
m⁷GTP	N7-methylguanosine triphosphate
N	Nucleotide
N-terminal	Amino-terminal

PARN	Poly(A) ribonuclease
PCR	Polymerase chain reaction
PNPase	Polynucleotide phosphorylase
PUF	Pumilio–Fem
RBP	RNA-binding protein
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNase	Ribonuclease
RRM	RNA-recognition motif
Rrp	Ribosomal RNA-processing
S1	Small 1 domain
Ski	Superkiller
T	Thymidine
TRAMP	Trf4/5p-Air2/1p-Mtr4p polyadenylation
Tris	Tris(hydroxymethyl)aminomethane
TRP	Tryptophan
TTP	Tristetraproline
U	Uracil
Upf	Up-frameshift
VASP	Vasodilator-stimulated phosphoprotein
Δ	Deletion.

CHAPTER I

GENERAL INTRODUCTION

I.1 Transcription to Degradation-The Life Cycle of mRNA

The flow of genetic information from DNA to messenger RNA (mRNA) to protein constitutes the basis for cellular life (Figure I.1). Eukaryotic gene expression is a complex stepwise process that begins in the nucleus with the transcription of protein-encoding genes by RNA polymerase II (RNAP II) and several transcription factors as precursor mRNAs (pre-mRNAs) that need to be processed (Lee and Young 2000; Sims et al. 2004). During and after transcription, nascent transcripts undergo extensive nuclear processing including the addition of a 5' end 7-methylguanosine cap, splicing of introns and, 3' end cleavage and polyadenylate [poly(A)] tail addition (Proudfoot et al. 2002; Kornblihtt et al. 2004; Proudfoot 2004; Bentley 2005). Once processing is complete, mature mRNAs are released from the site of transcription and then exported from the nucleus through the nuclear pore complexes to the cytoplasm (Suntharalingam and Went 2003).

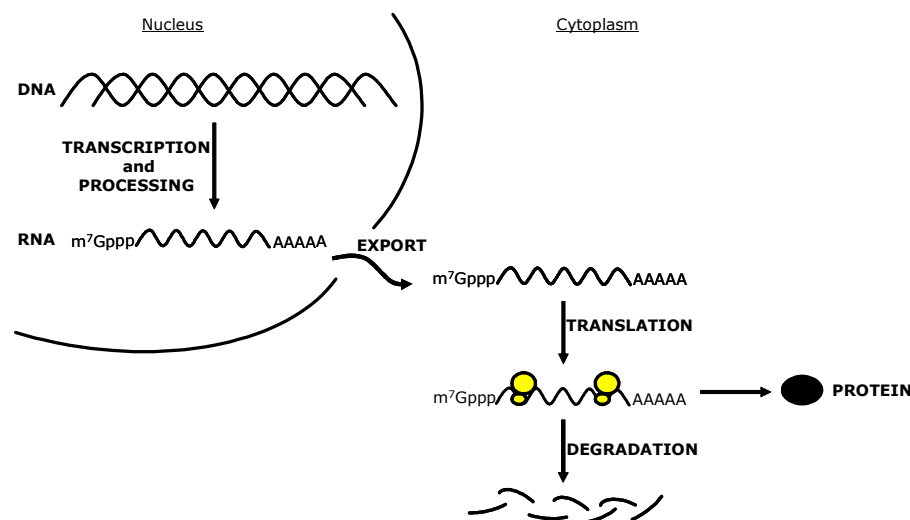


Figure I.1 Simplified view of eukaryotic gene expression.

The pre-mRNA processing steps are interlinked with one another, and with transcription and mRNA nuclear export (Dimaano and Ullman 2004; Vinciguerra and Stutz 2004). This extensive coupling starts as soon as a gene is transcribed, when its mRNA starts a complex and dynamic association with various RNA-binding proteins, including transcription, processing and export factors, forming a large messenger ribonucleoprotein complex (mRNP) (Dreyfuss et al. 2002). Co-transcriptional assembly of mRNPs is also integrated with quality control mechanisms that proofread the transcript at each phase of production, ensuring that only correctly processed mRNAs are released from the nuclear compartment (Saguez et al. 2005; Sommer and Nehrass 2005). Hence, the physical and

functional coupling between all the steps of mRNA biogenesis is important to coordinate the activities of the gene expression machineries and to ensure its efficiency and fidelity (Maniatis and Reed 2002). Once in the cytoplasm, the mature mRNP and the associated mRNA have several potential fates, which can be influenced by the set of proteins associated with the RNA either in the nucleus or in the cytoplasm. They can associate with ribosomes for productive translation of the mRNA into protein, they can be sequestered in specific subcellular locations, these mRNAs are often translationally repressed, or they can be destroyed via cytoplasmic mRNA turnover (Gebauer and Hentze 2004; Huang and Richter 2004; van der Bor and Davis 2004). The majority of mRNAs face the competing activities of translation and degradation. Ultimately, all mRNAs are degraded and the mRNA pool must be replenished by newly processed and exported transcripts.

I.2 Biological Significance of mRNA Turnover

The process of mRNA turnover is important in diverse aspects of eukaryotic mRNA physiology. Although modulation of expression levels can and does occur at all the steps in the mRNA life cycle, specifically, mRNA turnover has been recognized as a major contributor to the regulation of gene expression. The steady-state level of an mRNA in the cell depends on both its rate of synthesis and its rate of decay (the half-life). The rate of mRNA decay not only determines the rate of its disappearance but also its induction. In fact, the steady-state level of an mRNA can vary many-fold following a small change in its rate of decay, without any change in its rate of synthesis. In addition, the rate of mRNA decay also affects the time required for an mRNA to reach a new steady-state level following a change in its rate of synthesis. This means that an unstable mRNA would respond to changes in transcription more rapidly than those that are relatively stable (Hargrove and Schmidt 1989; Ross 1995). Notably, in eukaryotic cells, the rates of decay of individual mRNA species vary by orders of magnitude (Singer and Penman 1973; Spradling et al. 1975; Chia and McLaughlin 1979; Cabrera et al. 1984). For example, the half-lives of individual yeast and mammalian mRNAs vary from as short as 1-5 minutes and 15-30 minutes, respectively, to as long as 30-60 minutes and 10 or more hours, respectively (Volloch and Housman 1981; Shaw and Kamen 1986; Herrick et al. 1990). This diversity of mRNA turnover rates is an important determinant of gene expression contributing significantly to differential levels of expression. Interestingly, global mRNA turnover analysis indicated that mRNAs encoding proteins in the same functional class or complex undergo decay at similar rates, thereby coordinating the expression of genes

involved in specific biological processes (Wang et al. 2002a; Yang et al. 2003). Even though individual mRNAs rates of decay are generally invariant, changes in the rates of decay of a number of mRNAs have been shown to occur in response to a wide variety of stimuli including environmental factors (Mullner and Kuhn 1988; Petersen and Lindquist 1988; Wodnar-Filipowicz and Moroni 1990; Lombardo et al. 1992; Levy et al. 1996), specific hormones (Guyette et al. 1979; Brock and Shapiro 1983; Paek and Axel 1987), stage of cell cycle (DeLisle et al. 1983; Coppock and Pardee 1987; Levy and Hug 1992), or as a consequence of proliferation and differentiation (Mangiarotti et al. 1983, krowczynska et al. 1985; Jack and Wabl 1988) and viral infection (Nishioka and Silverstein 1977; Inglis 1982; Rice and Roberts 1983). These differential rates of decay for the same mRNA under different conditions provide the cell with flexibility in effecting rapid changes in gene expression patterns, which is essential for tight temporal expression. This level of regulation is particularly important for the expression of many clinically relevant genes encoding cytokines, lymphokines, growth factors and proto-oncogenes that are transiently activated by cellular stimuli (Chen and Shyu 1995; Bevilacqua et al. 2003). Consistently, misregulation of mRNA stability has been implicated in disease states, including immune and inflammatory diseases, Alzheimer's disease and cancer (Hollams et al. 2002; Audic and Hartley 2004). Hence, mRNA turnover is a major mechanism controlling gene expression both by determining the level of gene expression and as a site of regulatory responses crucial for cellular homeostasis.

In addition, mRNA turnover plays a critical role in assessing the accuracy of mRNA biogenesis and degradation of aberrant transcripts. These transcripts are recognized and targeted for rapid degradation by mRNA surveillance mechanisms. Such processes block the expression of defective mRNAs, which can have deleterious consequences for the cell (Hilleren and Parker 1999; Maquat and Carmichael 2001; Fasken and Corbett 2005). Consistently, many inherited genetic disorders and cancer can be caused by mutations that generate aberrant mRNAs (Culbertson 1999; Frischmeyer and Dietz 1999; Holbrook et al. 2004).

Finally, mRNA turnover mechanisms play a function in defending the genome against viruses and transposons. This antiviral defense role can include functions of the basic mRNA degradation machinery as well as specialized systems, such as RNA interference (RNAi), a sequence-specific RNA degradation pathway (Waterhouse et al. 2001; Butler 2002; Voinnet 2005). RNAi is also an evolutionarily conserved mechanism of gene silencing, which controls the expression of some endogenous genes that determine fundamental cell fate decisions of differentiation and survival. In addition to its natural

biological functions, RNAi has powerful biotechnological and biomedical applications including its use as a tool for gene-function analysis, and as a new modality for antiviral or other illnesses therapy (Dykxhoorn et al. 2003; Dykxhoorn and Lieberman 2005). Given these functions, it is important to understand the different mechanisms of turnover of mRNAs and how rates of turnover of different mRNAs are controlled.

I.3 Pathways of Eukaryotic mRNA Turnover

Several distinct mechanisms by which eukaryotic mRNAs can be degraded have been defined (Figure I.2). These mechanisms include the deadenylation-dependent decay, endonucleolytic digestion, or specialized quality control pathways. However, the relative contribution of each of the pathways may vary depending on the organism, either in general or for specific transcripts. One important point is that the diversity of decay pathways contributes for a wide spectrum of mRNA rates of decay and for their differential regulation.

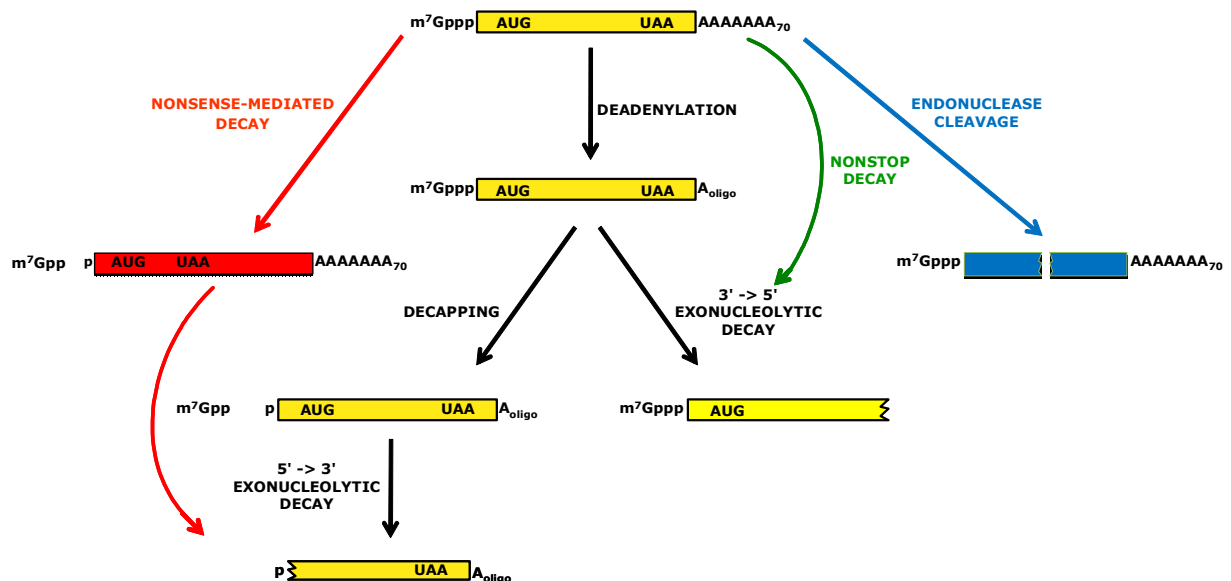


Figure I.2 Pathways of eukaryotic mRNA turnover.

I.3.1 Deadenylation-Dependent mRNA Decay

The deadenylation-dependent decay pathway is a general mechanism of turnover that acts on polyadenylated transcripts (Figure I.2). In yeast, this pathway of mRNA decay is

initiated with the shortening of the poly(A) tail at the 3' end of the mRNA, in a process referred as deadenylation, by a variety of deadenylases (Muhlrad and Parker 1992; Decker and Parker 1993; Anderson and Parker 1998). The predominant deadenylase complex is composed by Ccr4p and Pop2p nucleases and several accessory proteins (Tucker et al. 2001). Deadenylation of the transcript triggers the removal of the 5' cap structure, in a process referred as decapping, by the Dcp1p/Dcp2p decapping complex (Decker and Parker 1993; Muhlrad et al. 1994, 1995; Beelman et al. 1996). The deadenylated, uncapped mRNA is subsequently rapidly degraded by 5' to 3' exonucleolytic degradation performed by the 5' exonuclease Xrn1p (Hsu and Stevens 1993; Muhlrad et al. 1994, 1995). Alternatively, following deadenylation transcripts can be degraded by 3' to 5' exonucleolytic degradation (Muhlrad et al. 1995; Anderson and Parker 1998). This process involves a conserved multiprotein complex, termed the exosome (van Hoof and Parker 1999; Mitchell and Tollervey 2000). In this case, the residual oligonucleotide cap structure is hydrolyzed by the Dcs1p scavenger decapping enzyme (Liu et al. 2002).

In the yeast *Saccharomyces cerevisiae*, at least for mRNAs analyzed so far, decapping and 5' to 3' degradation following deadenylation seem to be the major mechanism of mRNA turnover (Decker and Parker 1993; Hsu and Stevens 1993; Muhlrad et al. 1994, 1995). Accordingly, mutations that affect 3' to 5' decay do not affect mRNAs half-lives when the yeast strain is wild-type (WT) with respect to 5' to 3' decay (Anderson and Parker 1998). In addition, the 5' to 3' pathway degrades mRNAs more rapidly than the 3' to 5' degradation pathway (Muhlrad et al. 1995; Anderson and Parker 1998; Cao and Parker 2001). Interestingly, a genome-wide survey of mRNA abundance indicated that abolishing the 5' to 3' decay by mutating Xrn1p has minimal effect on global mRNA decay rates, suggesting that either the 3' to 5' decay may be more important than previously thought, or it can compensate effectively for 5' to 3' decay when necessary (He et al. 2003). However, the reciprocal experiment of examining mRNA abundance in mutants defective in the 3' to 5' decay showed also minimal effects, which implies redundancy (Houalla et al. 2006). It is possible that the balance between 5' to 3' and 3' to 5' decay differs among mRNAs, different conditions or organisms. For example, in yeast, upon amino acid starvation, the 3' to 5' decay seems to prevail (Benard 2004). In addition, in mammalian cells, the available evidence, based mostly on *in vitro* studies, implies that 3' to 5' decay is more active than 5' to 3' decay (Chen et al. 2001a; Wang and Kiledjian 2001; Mukherjee et al. 2002), although it remains uncertain which pathway predominates in higher eukaryotes. One important point is that, in yeast, simultaneous inactivation of both decay pathways is synthetic lethal and, conditional double mutants leads to almost

complete stability of mRNAs under nonpermissive conditions (Johnson and Kolodner 1995; Anderson and Parker 1998). These observations indicate that these are the two major pathways that degrade most normal mRNAs, and also reinforce the critical importance of the process of mRNA turnover for cell metabolism and division.

I.3.1.1 The Enzymes Involved in the Deadenylation-Dependent Decay

The identification and characterization of the mRNA degradative enzymes was a major contribution toward understanding the mechanisms and control of mRNA turnover.

I.3.1.1.1 The mRNA Deadenylases

Deadenylation occurs via multiple mRNA deadenylases (Figure I.3), although the exact role of each of these enzyme complexes and the relative importance of the individual nucleases for different mRNAs is not fully understood.

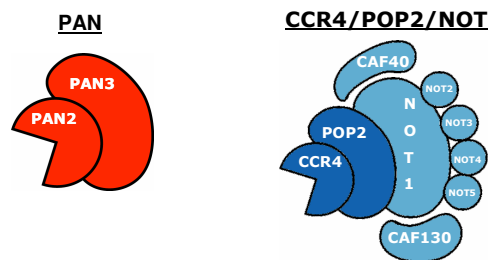


Figure I.3 Multiple mRNA deadenylases.

I.3.1.1.1.1 Ccr4p/Pop2p/Not Complex

In yeast, the major cytoplasmic deadenylase consist of a protein complex composed by two nucleases, Ccr4p and Pop2p/Caf1p, and several associated proteins, Not1p-Not5p, Caf4p, Caf16p, Caf40p and Caf130p (Figure I.3) (Daugeron et al. 2001; Tucker et al. 2001; Chen et al. 2002; Tucker et al. 2002; Denis and Chen 2003). Ccr4p and Pop2p are required for maximal mRNA deadenylation *in vivo* because *ccr4Δ* or *pop2Δ* deletion strains showed defects in both the rate and extent of deadenylation, resulting in mRNA stabilization. In addition, they co-purified with deadenylase activity *in vitro* (Tucker et al. 2001). Both Ccr4p and Pop2p subunits, bear sequence similarity to nucleases, the Ccr4p is a member of the magnesium-dependent exonuclease III (ExoIII)/apurinic (AP) endonuclease family (Dlakic 2000), whereas Pop2p is a member of the RNase D family of 3' exonucleases that uses a two-metal mechanism for catalysis (Moser et al. 1997),

although the predicted catalytic site is not well conserved (Tucker et al. 2001; Thore et al. 2003). This suggested that both Ccr4p and Pop2p could potentially function as the actual catalytic subunit of the yeast deadenylase. However, Ccr4p has been identified as the predominant catalytic activity in this deadenylase complex because mutations in the predicted catalytic residues abolished Ccr4p *in vivo* function and its *in vitro* deadenylase activity under the conditions examined (Chen et al. 2002; Tucker et al. 2002). Moreover, Ccr4p is active as a poly(A)-specific nuclease in the absence of Pop2p (Chen et al. 2002). On the contrary, even though Pop2p can display some deadenylase activity *in vitro* under certain conditions (Daugeron et al. 2001; Thore et al. 2003; Viswanathan et al. 2004), it does not seem to primarily function as an active nuclease *in vivo* within the Ccr4p/Pop2p/Not complex. This is indicated by the observation that although strains completely lacking Pop2p showed a defect in deadenylation, strains containing a full-length but catalytically inactive Pop2p showed no deadenylation phenotype and do not affect the catalytic activity of the Ccr4p/Pop2p complex *in vitro* (Tucker et al. 2002; Viswanathan et al. 2004). This indicates that the nuclease activity of Pop2p is not required for its *in vivo* deadenylation function. A role for Pop2p in deadenylation may be to enhance the function of the Ccr4p deadenylase. Pop2p contacts with Ccr4p C-terminal exonuclease domain and a conserved leucine-rich repeat (LRR) domain, which are both required for its exonuclease activity (Draper et al. 1995; Clark et al. 2004). However, interfering with Pop2p binding to Ccr4p modestly affects Ccr4p deadenylase activity *in vivo* and *in vitro* (Clark et al. 2004; Ohn et al. 2007). This suggests that Pop2p binding to Ccr4p, while contributing to Pop2p function in deadenylation, does not seem to directly regulate Ccr4p activity (Chen et al. 2002; Clark et al. 2004; Ohn et al. 2007). Instead, Pop2p may work either by stabilizing the deadenylase complex or, by perhaps providing additional interactions between the deadenylase complex and the mRNA or, other regulators of deadenylation. Consistent with this, Pop2p seems to be absolutely required for the association of Not1p and the remainder of the Not proteins complex with Ccr4p (Liu et al. 1998; Bai et al. 1999; Chen et al. 2001b). The Ccr4p/Pop2p associated Not1-5p complex are key regulatory proteins for deadenylation that only affect in a minor extent the deadenylation rate of some mRNAs (Tucker et al. 2002). In addition, Pop2p interacts with Puf proteins, and this interaction is required for Puf-induced deadenylation of specific mRNAs by recruiting the Ccr4p enzyme to the mRNA (Goldstrohm et al. 2006, 2007; Hook et al. 2007). Finally, Pop2p seems to play additional functions in deadenylation, possibly through its functional association with translationally important proteins including the poly(A)-binding protein (Pab1p) (Ohn et al. 2007). Additional studies are necessary to clarify how these interactions affect deadenylation. Nevertheless, the nuclease activity of the Pop2p and the conservation of some key residues in the active site do not rule out the

possibility that under specific conditions Pop2p may be a second catalytic subunit of the deadenylase complex (Dangeron et al. 2001; Thore et al. 2003; Jonstrup et al. 2007). Consistent with this, Pop2p has been shown to be phosphorylated in the absence of glucose (Moriya et al. 2001). Thus, the Ccr4p/Pop2p/Not complex would have multiple nucleases, which might be regulated differentially.

Interestingly, Ccr4p and Pop2p are also components of complexes involved in transcription regulation (Denis and Chen 2003). It is not clear whether the connection to transcriptional machinery represents a requirement for an initial interaction of the Ccr4p cytoplasmic deadenylase with the mRNA in a co-transcriptional manner or, whether Ccr4p complexes in the nucleus play an alternative independent role during transcription. Future studies shall determine the nature of the link, if any, between the transcriptional and turnover machinery.

I.3.1.1.1.2 PAN: Pan2p/Pan3p Complex

Another deadenylase complex consists of the poly(A) nuclease, referred to as PAN, shown to be an enzyme complex composed by Pan2p and Pan3p subunits (Figure I.3) (Boeck et al. 1996; Brown et al. 1996). PAN depends on Pab1p for activity (Lowell et al. 1992; Sachs and Deardorff 1992). The Pan2p subunit is likely the catalytic subunit of the complex since it is a member of the RNase D family of 3' exonucleases requiring magnesium for catalysis (Lowell et al. 1992; Boeck et al. 1996; Moser et al. 1997). The role of Pan3p is presumably to enhance or regulate Pan2p activity possibly through its interaction with Pab1p (Brown et al. 1996; Mangus et al. 2004). Deletion of *PAN2* and/or *PAN3* genes results in increases of the average length of mRNA poly(A) tails (Boeck et al. 1996; Brown et al. 1996; Brown and Sachs 1998). However, *pan2Δ* and *pan3Δ* strains showed minimal effects in the rate of mRNA deadenylation (Brown and Sachs 1998). PAN main role seems to be in the early trimming of the poly(A) tail on newly synthesized transcripts from the length of approximately 90-70 to 75-55 A residues. This initial poly(A) shortening phase is necessary for message-specific poly(A) tail length control in *S. cerevisiae* (Brown and Sachs 1998). The role of PAN in cytoplasmic mRNA deadenylation is revealed in *ccr4Δ* strains where the predominant deadenylase is absent. Indeed, the residual deadenylation seen in a *ccr4Δ* strain is ablated in a *ccr4Δ pan2Δ* double mutant strain (Tucker et al. 2001). Notably, both the PAN and Ccr4p/Pop2p/Not complexes must be disrupted to have an appreciable effect on deadenylation rates *in vivo*. This indicates that these are the only significant mRNA deadenylases in yeast. This also suggests that PAN can participate in cytoplasmic deadenylation, although is not the major

activity for cytoplasmic decay. Consistent with this, both Pan2p and Pan3p are located primarily in the cytoplasm (Huh et al. 2003). It is possible that PAN may be the predominant cytoplasmic deadenylase for specific mRNAs or under certain conditions. Indeed, PAN appears to play a role in the specific regulation of the *RAD5* mRNA in response to replicative stress (Hammet et al. 2002).

I.3.1.1.2 The mRNA Decapping Enzymes

One way to provide access for exonucleases is to hydrolyze the 5' cap structure of the mRNA by the decapping enzymes. Two different nucleases with distinct activities have been identified thus far: the Dcp1p/Dcp2p decapping enzyme and the DcpS scavenger decapping enzyme (Figure I.4).

I.3.1.1.2.1 Dcp1p/Dcp2p Decapping Enzyme

A combination of genetic and biochemical analyses indicated that two proteins, Dcp1p and Dcp2p, function in conjunction as a decapping holoenzyme. First, loss of either Dcp1p or Dcp2p subunit leads to an absolute block to decapping *in vivo*, suggesting that no other important decapping enzyme can act on mRNA substrates (Beelman et al. 1996; Dunckley and Parker 1999). The *dcp1Δ* and *dcp2Δ* strains accumulate deadenylated, capped mRNAs. Second, these proteins physically interact with each other (Dunckley and Parker 1999; Dunckley et al. 2001; Steiger et al. 2003). Third, co-expression of recombinant yeast Dcp1p and Dcp2p from *Escherichia coli* yields active decapping enzyme under a variety of conditions (Steiger et al. 2003). Moreover, yeast Dcp2p alone can have decapping activity in the presence of manganese (Steiger et al. 2003) or in high magnesium (van Dijk et al. 2002). This suggested that Dcp2p is the major catalytic subunit in this enzyme complex that is capable of cleaving the cap structure. Consistent with this, Dcp2p contains a nucleotide diphosphate linked to an X moiety (Nudix) motif, which is a catalytic domain found in a particular class of pyrophosphatases designated as the Nudix hydrolases (Koonin 1993; Bessman et al. 1996), central for Dcp2p function in decapping (Dunckley and Parker 1999). Deletion or mutagenesis of the Dcp2p region containing the Nudix motif was sufficient to eliminate the function of Dcp2p in mRNA decapping both *in vivo* and *in vitro* (Dunckley and Parker 1999; Steiger et al. 2003). In addition to the Nudix domain, which appears to be the active site, Dcp2p has additional conserved regions flanking the Nudix domain, termed Box A motif that seems to be required for the efficiency of Dcp2p activity, potentially by facilitating the interaction between Dcp1p and Dcp2p, and Box B motif that seems to be required for both RNA binding and decapping activity (Wang

et al. 2002b; Piccirillo et al. 2003; She et al. 2006). In yeast, Dcp2p has an extensively long C-terminus, which is not seen in other homologs and is not required for decapping (Dunckley and Parker 1999).

Even though Dcp2p is sufficient to catalyze the decapping reaction, Dcp2p activity is highly stimulated in the presence of Dcp1p (Steiger et al. 2003; She et al. 2006). Moreover, Dcp2p cannot function *in vivo* without Dcp1p (Beelman et al. 1996). The simplest interpretation of these observations is that Dcp2p is the catalytic subunit of the decapping complex and cleaves the RNA, and Dcp1p primarily functions to enhance Dcp2p activity by a currently unresolved mechanism. Significantly, Dcp1p belongs to a novel class of functional Ena-VASP homology 1/Wiskott-Aldrich syndrome protein homology 1 (EVH1/WH1) domain proteins (Callebaut 2002; She et al. 2004). The EVH1/WH1 domains are protein-protein interaction modules that mediate interactions with proline-rich containing ligands through a proline-rich sequence (PRS)-binding site, thereby helping in complex assemblies and providing essential links between different proteins (Ball et al. 2002). Mapping the sequence conservation on the molecular surface of Dcp1p revealed two prominent regions. One region corresponds to the (PRS)-binding site and is likely to have a role in contacting decapping regulatory proteins (She et al. 2004). This suggests that Dcp1p may enhance Dcp2p decapping activity by linking the Dcp2p enzyme to proteins that control decapping. In addition, a second region, which is required for the stimulatory role of Dcp1p in Dcp2p activity, without affecting Dcp1p interaction with Dcp2p, suggests a more direct role for Dcp1p (Tharun and Parker 1999; She et al. 2004). Possible mechanisms whereby Dcp1p facilitates Dcp2p activity would include direct interaction with the substrate or assisting metal binding. Moreover, a conserved hydrophobic patch adjacent to these two regions of Dcp1p structure is revealed to be critical for decapping (She et al. 2004). Finally, it is also possible that the interaction between Dcp1p and Dcp2p contributes structurally to form an active decapping enzyme, maybe by Dcp1p inducing a conformational change in Dcp2p, thereby increasing Dcp2p catalytic activity (She et al. 2006). Consistent with this idea, under conditions where expression of either protein alone is insufficient for decapping activity, co-expression of Dcp1p and Dcp2p together allows for robust decapping (Steiger et al. 2003). More insight into the catalytic mechanism of Dcp1p/Dcp2p should arise from further structural studies. There has been uncertainty whether previous observations indicating that Dcp1p purified from yeast could have catalytic activity were somehow misleading or whether Dcp1p can also have decapping activity (LaGrandeur and Parker 1998; Dunckley et al. 2001). The previous results are probably due to undetected Dcp2p present in the yeast extracts and Dcp1p is no longer thought to contain intrinsic decapping activity because the decapping activity of such preparations is absent in strains with mutations in the Dcp2p catalytic site

(Dunckley and Parker 1999; Dunckley et al. 2001). Surprisingly, recombinant Dcp1p has been reported to have decapping activity (Vilela et al. 2000). However, efforts in our and others laboratory to obtain active recombinant Dcp1p have been unsuccessful under a variety of conditions (Steiger et al. 2003). Nevertheless, could be that the Dcp1p/Dcp2p complex contains two different decapping proteins, but there is now no doubt that Dcp2p is the major decapping activity.

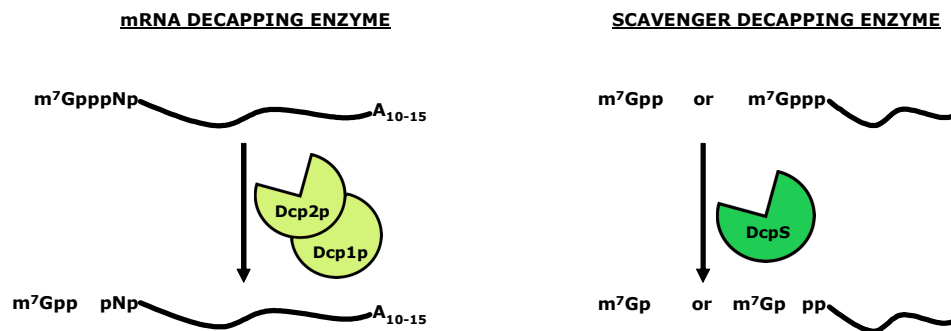


Figure I.4 mRNA decapping enzymes and their products.

The decapping reaction by Dcp1p/Dcp2p, or by Dcp2p alone, cleaves the m^7GpppN (in yeast, N is generally either an A or a G) of mRNAs in a metal-dependent reaction, releasing m^7GDP and a 5'-monophosphate mRNA as products (Figure I.4) (Stevens 1988; Beelman et al. 1996; LaGrandeur and Parker 1998; Dunckley and Parker 1999). The production of a 5'-monophosphate mRNA may be functionally significant for the subsequent rapid digestion by the 5' exonuclease Xrn1p since it preferentially degrades substrates containing a 5'-monophosphate end (Stevens and Maupin 1987).

Several observations suggest that the mRNA substrate specificity of the decapping enzyme is determined both by interactions with the cap 7-methyl group and with the body of the transcript. First, although the 7-methyl group of the cap structure contributes to substrate specificity of the enzyme, the addition of cap analog, m^7GpppG_{OH} , fails to inhibit the decapping reaction to any great extent (LaGrandeur and Parker 1998). Interestingly, Dcp1p/Dcp2p was effectively inhibited *in vitro* by uncapped mRNAs. Indeed, Dcp2p can bind RNA directly (CJ Decker and R Parker, unpublished observation). Moreover, the decapping enzyme prefers substrates longer than 25 nucleotides, with enhanced activity on longer mRNA substrates (Stevens 1988; LaGrandeur and Parker 1998; Steiger et al. 2003). This preference for longer substrates may be biologically relevant because the 5' cap structure is often complexed with a set of proteins involved in translation initiation, referred to as the cap-binding complex or eIF4F. A requirement for a significant length of RNA in addition to the cap structure for mRNA substrate recognition by Dcp1p/Dcp2p may

prevent decapping of mRNAs on which the translation initiation complex has already assembled.

I.3.1.1.2.2 Scavenger Decapping Enzyme

Eukaryotic cells also contain a second type of decapping enzyme referred to as the scavenger decapping enzyme, DcpS, also known as Dcs1p in yeast. The scavenger decapping enzymes were described as enzymes that decap short pieces of RNA (Figure I.4) (Nuss et al. 1975; Nuss and Furuichi 1977; Kumagai et al. 1992). DcpS acts to decap the capped oligonucleotides, m^7GpppX , produced after exosome-mediated 3' to 5' degradation of mRNA, releasing m^7GMP and 5'-diphosphate oligonucleotides (Wang and Kiledjian 2001; Liu et al. 2002). Interestingly, DcpS exists in a complex with the exosome in mammalian cells (Wang and Kiledjian 2001). The formation of a degradation complex consisting of exoribonucleases and a decapping activity may be significant to allow coupled 3' to 5' degradation of the mRNA body and subsequent removal of the cap structure. DcpS is believed to play a role in ensuring that no excess unhydrolyzed cap accumulates, which could sequester the translation initiation factor, eIF4E, away from translated mRNAs (Liu et al. 2004). DcpS can also hydrolyze the m^7GDP produced by mRNA decapping to m^7GMP and phosphate (van Dijk et al. 2003). Interestingly, recent studies suggested that DcpS activity can stimulate the 5' exoribonuclease activity thereby affecting overall mRNA stability (Liu and Kiledjian 2005). In this sense, DcpS influences both the 3' to 5' and 5' to 3' decay probably coordinating the two exonucleolytic mRNA degradation pathways. Thus, although the two decay pathways are generally distinct, it is becoming evident that there is an extensive interplay between them.

Scavenger decapping enzymes are members of the histidine triad (HIT) superfamily of pyrophosphatases that use a histidine triad to perform the metal-independent cleavage of pyrophosphate bonds (Seraphin 1992; Liu et al. 2002). DcpS is a modular protein requiring both the core HIT motif active site at the C-terminal domain and sequences at the N-terminal domain for cap binding and hydrolysis (Liu et al. 2004). Structural analysis revealed that DcpS complexed with a cap moiety is an asymmetric homodimer that simultaneously creates an open non-productive conformation on one side of the protein and a closed productive conformation on the other (Gu et al. 2004; Chen et al. 2005). Interestingly, DcpS is inactive in non-methylated cap analog, and is unable to decap long substrates (Liu et al. 2002, 2004). It has been suggested that long mRNAs, due to greater steric and entropic constraints, might interfere with the formation of a closed productive active site capable of cap hydrolysis (Gu et al. 2004). This may be biologically relevant to prevent DcpS from prematurely decapping mRNAs not targeted for degradation.

I.3.1.1.3 The 5' to 3' Exonuclease: Xrn1p

The *XRN1* gene encodes an abundant cytoplasmic 5' exonuclease, Xrn1p, responsible for the rapid degradation of the mRNA body after decapping (Larimer and Stevens 1990). Xrn1p is not essential for viability, but *xrn1Δ* strains accumulate deadenylated, decapped full length mRNAs (Hsu and Stevens 1993; Muhlrads et al. 1994, 1995). Xrn1p is a member of a large family of conserved exonucleases, although little is known about the catalytic mechanism of its members. Biochemically, the exonuclease processively degrades single-stranded RNA containing a 5'-monophosphate releasing mononucleotides as it proceeds toward the 5' end (Stevens 1980). Xrn1p is inhibited by the cap structure and by secondary structures (Hsu and Stevens 1993; Poole and Stevens 1997).

I.3.1.1.4 The Exosome

The yeast cytoplasmic exosome is involved in the 3' to 5' mRNA degradation pathway following deadenylation (Anderson and Parker 1998). The exosome is a large protein complex with exoribonuclease activity that contains at least ten essential core subunits (Mitchell et al. 1997; Allmang et al. 1999a). These include six subunits with predicted phosphorolytic activity (Rrp41p/Ski6p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p), based on similarity with the 3' exoribonucleases PNPase and RNase PH from *E. coli*, suggesting that they catalyze RNA digestion using phosphate as the attacking group, releasing 5'-diphosphate nucleotides (Mitchell et al. 1997; Moser et al. 1997; Allmang et al. 1999a). Three subunits (Rrp4p, Rrp40p and Csl4p/Ski4p) contain putative S1 and KH RNA-binding motifs, indicating that they can bind RNA (Allmang et al. 1999a; Luz et al. 2007; Oddone et al. 2007). The last subunit (Rrp44p/Dis3p) is predicted to have hydrolytic activity since it is related to the 3' hydrolase RNase II from *E. coli*, suggesting that it uses water as the attacking group during RNA digestion, releasing 5'-monophosphate nucleotides (Mitchell et al. 1997; Moser et al. 1997). Surprisingly, recent studies revealed that the phosphorolytic subunits of the yeast exosome are catalytically inactive, and the hydrolytic subunit Rrp44p is the only detectable active exonuclease in the complex (Liu et al. 2006; Dziembowski et al. 2007). However, defects in any core subunit generally lead to similar phenotypes in exosome-dependent events, indicating that the exosome function as part of a complex (Anderson and Parker 1998; Allmang et al. 1999a, van Hoof et al. 2000a).

A model for yeast exosome structure and organization has been predicted based on similarity to bacterial PNPase and archaeal exosome (Symmons et al. 2000; Aloy et al.

2002; Symmons et al. 2002; Buttner et al. 2005; Lorentzen et al. 2005, 2007), as well as based on structural and biochemical data of yeast and human exosome (Figure I.5) (Ito et al. 2000; Uetz et al. 2000; Ito et al. 2001; Gavin et al. 2002; Ho et al. 2002; Oliveira et al. 2002; Hernández et al. 2006; Liu et al. 2006; Synowsky et al. 2006; Luz et al. 2007). The exosome seems to be a member of an emerging family of nuclease complexes that form ring-like structures, with the six RNase PH-domain proteins (Rrp41p/Rrp45p/Rrp46p/Rrp43p/Mtr3p/ Rrp42p) forming a hexameric PH-ring, which is tightly bound on one side by the three RNA-binding subunits (Csl4p/Rrp4p/Rrp40p), and on the opposite side by the hydrolytic RNase subunit (Rrp44p), which seems to be more loosely attached (Mitchell et al. 2003; Hernandez et al. 2006).

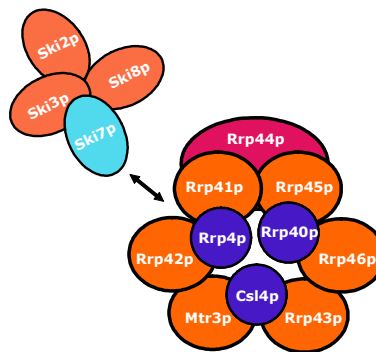


Figure I.5 Schematic putative structure and organization of the exosome.

The current model for the exosome structure suggests that the RNA moves through a constricted central cavity of the ring subunits, from the Csl4p/Rrp4p/Rrp40p face, consistent with the presence of the RNA-binding subunits at the top of the structure, to approach the Rrp44p on the other side of the complex. The formation of an entry pore by this enzyme assembly that is too narrow for structured RNA to enter may be relevant to restrict substrate access and prevent indiscriminate degradation (Buttner et al. 2006). Remarkably, the observation that the exosome core is mostly a hydrolytic enzyme, and its activity is mediated mostly by Rrp44p subunit *in vitro* and *in vivo*, indicates that the central cavity is not used for catalysis (Liu et al. 2006; Dziembowski et al. 2007). Instead, the different subunits that form the core of the exosome may be structurally important to bridge Rrp44p with various exosome-associated proteins and regulators. Consistent with this, Ski7p, which is essential for mRNA degradation by the exosome, has been shown to interact with the PH-ring subunits rather than Rrp44p (Dziembowski et al. 2007). In addition, given the variety of roles of the exosome in RNA processes, they might also play a role in distinguishing between different classes of substrates. Accordingly, Csl4p seems to be involved in RNA substrates recognition and distinction, since mutants in its RNA-

binding domain have been identified that are either defective in mRNA degradation or in ribosomal RNA (rRNA) processing (van Hoof et al. 2000b). Moreover, they might also contribute to cooperative binding of specific RNA substrates by the exosome in comparison with individual subunits, and consequently increase its efficiency of degradation. Nevertheless, it is possible that under specific conditions and/or for specific substrates the yeast central cavity may also be catalytically active, as the human exosome (Liu et al. 2006). Finally, this type of exosome structure also suggests that additional factors might be required to load mRNAs into the central cavity. Interestingly, the exosome interacts with a heterotrimeric complex of Ski2p, Ski3p and Ski8p, which are thought to act as cofactors for the exosome to degrade cytoplasmic mRNA (Anderson and Parker 1998; Brown et al. 2000). Ski2p, a member of the DEAD-box RNA helicase family, may use ATP hydrolysis to remove RNA structures or proteins complexes, and perhaps promote entry of the RNA into the central cavity of the exosome (Tanner and Linder 2001). Ski2p seems to interact with the exosome through Ski3p and Ski8p (Brown et al. 2000). And, the Ski2p/Ski3p/Ski8p complex seems to be recruited to the exosome through interactions with the cytoplasmic specific subunit, Ski7p (Wang et al. 2005). Ski7p is a putative GTPase with homology to translation factors that associates with the exosome and seems to have multiple roles in cytoplasmic mRNA degradation (Bernard et al. 1999; van Hoof et al. 2000b; Araki et al. 2001).

The exosome complex is also present in the nucleus and function in both processing and degradation of various types of nuclear RNA species (Houseley et al. 2006). These include processing and degradation of rRNA, small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA), and degradation of improperly processed stable RNAs and mRNAs (Butler 2002). The exosome function is also required for degradation of normal mRNAs that are retained in the nucleus by a mechanism referred to as degradation of mRNA in the nucleus (DRN) (Das et al. 2003; Kuai et al. 2005), and is involved in the degradation of cryptic RNAP II unstable transcripts (Wyers et al. 2005; Davis and Ares 2006). Although the nuclear and cytoplasmic forms of the exosome share the core components, they are distinguished by the specific cofactors that associate with the core complex. The nuclear exosome is associated with a nucleic acid-binding protein, Rrp47p/Lrp1p (Mitchell et al. 2003; Peng et al. 2003), which cooperates with Rrp6p, another 3' exoribonuclease that is a member of the RNase D family and specifically affects nuclear RNA processing and degradation (Torchet et al. 2002; Das 2003; Milligan et al. 2005; Midtgaard et al. 2006). Similarly to cytoplasmic exosome, the helicase Mtr4p/Dob1p is required for most nuclear activities of the exosome, probably unwinding substrates and/or promoting entrance into the central cavity (de la Cruz et al. 1998;

Allmang et al. 1999b; van Hoof et al. 2000a). It is probable that the association of the exosome with different cofactors may have a key role in directing a specific function on different substrates in both the nucleus and the cytoplasm. One important working area is to determine the nature of interactions between the exosome and its substrate and adaptors.

I.3.1.2 The Deadenylation-Dependent Decay is a Conserved Turnover Mechanism

Studies of the mRNA turnover in other organisms suggested that the deadenylation-dependent mRNA decay mechanisms and the enzymes involved in these pathways are conserved in more complex eukaryotes. Similarly to yeast, deadenylation can be the first step of mRNA degradation in mammals both *in vitro* and *in vivo*, and in *Drosophila melanogaster* (Restifo and Guild 1986; Brewer and Ross 1988; Wilson and Treisman 1988; Shyu et al. 1991; Brewer 1998; Temme et al. 2004; Bönisch et al. 2007). In addition, both the Ccr4p/Pop2p/Not proteins complex and PAN deadenylase are widely conserved (Draper et al. 1995; Albert et al. 2000; Dageron et al. 2001; Dupressoir et al. 2001; Baggs and Green 2003; Temme et al. 2004; Uchida et al. 2004; Bianchin et al. 2005). Moreover, human and *D. melanogaster* Ccr4p/Pop2p, and human Pan2p display deadenylase activity (Chen et al. 2002; Temme et al. 2004; Uchida et al. 2004; Bianchin et al. 2005; Morita et al. 2007). Significantly, like yeast, the CCR4/CAF1 complex represents the major deadenylase in humans and *D. melanogaster*, although PAN also contributes to mRNA deadenylation (Lai et al. 2003; Temme et al. 2004; Yamashita et al. 2005; Bönisch et al. 2007). Furthermore, PAN and CCR4/CAF1 complexes together carry out cytoplasmic deadenylation of mammalian mRNAs in a sequential manner, as in yeast (Yamashita et al. 2005). Interestingly, a third deadenylase, the homodimeric PARN, is conserved in most eukaryotes, but is absent in *S. cerevisiae* and *D. melanogaster*, indicating that this enzyme is not required by all eukaryotes (Korner and Wahle 1997; Korner et al. 1998; Wu et al. 2005). Similar to Pop2p and Pan2p, PARN is also a member of the RNase D family of 3' exonucleases that seems to be required for specialized decay processes (Ren et al. 2002; Lai et al. 2003; Lejeune et al. 2003; Opyrchal et al. 2005).

Subsequent decay of the deadenylated transcript is thought to proceed via mRNA decapping and 5' to 3' exonucleolytic degradation in other eukaryotic cells as well. Indeed, decapping takes place after deadenylation in mammals and *D. melanogaster*, as in yeast (Yamashita et al. 2005; Stoecklin et al. 2006; Bönisch et al. 2007). Moreover, mRNA decay intermediates that have been deadenylated and decapped can be detected in mammalian cells (Couttet et al. 1997). Accordingly, a decapping activity was identified in HeLa cytoplasmic extracts, which is inactive in substrates containing poly(A) tails (Gao et

al. 2001). Significantly, the decapping enzyme proteins, Dcp1p and Dcp2p, are conserved among eukaryotes (Lykke-Andersen 2002; van Dijk et al. 2002; Wang et al. 2002b; Xu et al. 2006). Moreover, human Dcp2p has intrinsic decapping activity *in vitro*, and shares many similarities to yeast decapping activity, including the involvement of the conserved Nudix motif in mRNA decapping (Lykke-Andersen 2002; van Dijk et al. 2002; Wang et al. 2002b; Piccirillo et al. 2003). Two human Dcp1p homologs, hDcp1a and hDcp1b, have been identified, and although possibly redundant, only hDcp1a protein co-purifies with decapping activity and appears to be functionally related to the yeast Dcp1p (Lykke-Andersen 2002). Alternatively, the two versions of Dcp1p might have different functional properties, thereby increasing the diversity of decapping activities in mammals (Lykke-Andersen 2002). Finally, hDcp1 and hDcp2 physically associate (Lykke-Andersen 2002; Wang et al. 2002b), and together with a third component, Hedls/Ge-1, which is a bridging factor required for functional hDcp1-hDcp2 interaction with no homolog in yeast, form the decapping complex in mammals (Fenger-Gron et al. 2005). This suggests that yeast and mammals have diverged to use different mechanisms in decapping. Also, similarly to yeast, in extracts of mammalian cells there are 5' exonuclease activities that act on uncapped RNA substrates (Ross et al. 1987). Moreover, the yeast 5' exoribonuclease Xrn1p is conserved among eukaryotes (Bashkirov et al. 1997; Till et al. 1998; Kastenmayer and Green 2000; Newbury and Woollard 2004; Li et al. 2006). Finally, conservation of the 5' to 3' decay pathway is also supported by the aggregation of factors involved in decapping and 5' to 3' degradation into distinct subcellular structures in different organisms (Eulalio et al. 2007).

Decay of mRNAs in a 3' to 5' direction after deadenylation also appears to be conserved. Moreover, 3' to 5' decay may be the predominant degradation pathway in mammals (Chen et al. 2001a; Wang and Kiledjian 2001; Mukherjee et al. 2002). The individual exosome subunits, and their ability to form a complex, are conserved throughout eukaryotes (Allmang et al. 1999a; Chekanova et al. 2000; Brouwer et al. 2001; Estevez et al. 2001; Andrulis et al. 2002). In addition, in mammals, *in vivo* and in *in vitro* decay systems the exosome degrades RNAs after deadenylation, with a preference for those containing AU-rich elements (AREs) (Brewer 1999; Chen et al. 2001a; Wang and Kiledjian 2001; Mukherjee et al. 2002; Rodgers et al. 2002). Finally, DcpS is present in mammals to hydrolyze the cap structure after exosome degradation (Wang and Kiledjian 2001). However, given the increased need for regulation, it should be expected complex organisms to have additional decay mechanisms that are specific to particular classes of mRNAs or to specific cellular responses.

I.3.2 Endonucleolytic Cleavage as a Mechanism for mRNA Turnover

For some individual mRNAs the process of mRNA turnover can be initiated without prior deadenylation by endonucleolytic cleavage within the transcript body, followed by degradation of the two unstable products (Figure I.2) (Dodson and Shapiro 2002). Presumably, the resulting 5' fragment may be targeted for a 3' to 5' degradation activity, whereas the polyadenylated 3' fragment may be a substrate for a 5' to 3' activity. Example of transcripts that undergo endonuclease-mediated decay include the mammalian 9E3 (Stoeckle and Hanafusa 1989), GRO- α (Stoeckle 1992), insulin-like growth factor II (Meinsma et al. 1991), transferrin receptor (*TfR*) (Binder et al. 1994), *c-myc* (Lee et al. 1998), β -globin (Lim and Maquat 1992) and α -globin (Wang and Kiledjian 2000a), chicken apolipoprotein II (Binder et al. 1989), *Xenopus laevis Xlhbox2B* (Brown and Harland 1990), albumin (Pastory et al. 1991) and vitellogenin mRNAs (Cunningham et al. 2000), and yeast *CLB2* mRNA (Gill et al. 2004). However, it remains to be seen whether endonucleolytic cleavage participates in the degradation of a significant number of yeast mRNAs. Evidence that endonucleolytic cleavage constitutes an early step in the degradation of these transcripts comes from the *in vivo* detection of the 5' and/or 3' cleavage RNA fragments. However, does not appear to be similarities between the cleavage site in these mRNAs and several endonucleases have been identified with different cleavage specificities (Chernokalskaya et al. 1997, 1998; Gallouzi et al. 1998; Cunningham et al. 2001; Hollien and Weissman 2006). For example, the endonuclease suggested to be responsible for β -globin decay appears to preferentially cleave between UG and UC dinucleotides (Bremer et al. 2003), whereas decay of the *c-myc* mRNA appears to occur by cleavage between CA or UA dinucleotides at the coding region determinant (Bergstrom et al. 2006). Endonuclease-mediated decay can be regulated by reversible protection of the endonucleolytic cleavage site by RNA-binding proteins, thereby allowing mRNAs to be transiently destabilized when required (Brown et al. 1993; Doyle et al. 1998; Cunningham et al. 2000; Wang and Kiledjian 2000b; Sparanese and Lee 2007). For example, an iron responsive element (IRE)-binding protein binds to IREs present in the 3' untranslated region (UTR) of the *TfR* mRNA, preventing endonuclease access to the cleavage site in response to cellular iron levels (Klausner et al. 1993).

Endonucleolytic cleavage is also important in RNA-mediated gene silencing processes (Filipowicz et al. 2005; Valencia-Sanchez et al. 2006). The RNA silencing pathway is initiated by the recognition of a double-stranded RNA (dsRNA) sequence by the endonuclease *Dicer*. *Dicer* cleaves the long dsRNA to generate approximately 21-23 nucleotide (nt)-long RNA species, small interfering RNAs (siRNAs) and micro RNAs

(miRNAs), distinguished by their mode of biogenesis. The siRNAs and miRNAs are used to target the multicomponent RNA-induced silencing complex (RISC) to the complementary mRNA, which in some cases facilitates a second endonucleolytic cleavage that triggers the destruction of the mRNA. The 5' fragment appears to be degraded by the exosome, whereas the 3' fragment of the mRNA appears to be degraded by Xrn1p (Souret et al. 2004; Orban and Izaurralde 2005).

I.3.3 mRNA Quality Control Mechanisms

Cells frequently make mistakes. Many types of errors can arise in a transcript as it is expressed; consequently cells have evolved multiple quality control mechanisms to monitor fidelity at each step of gene expression. Remarkably, RNA turnover is a part of specialized surveillance pathways that ensures the recognition and specific elimination of cellular RNAs containing errors, thereby allowing that only high quality RNAs are engaged in protein synthesis and other cellular processes.

As mentioned, quality control systems exist in the nucleus to degrade transcripts in response to defects during mRNA biogenesis (Moore 2002; Vasudevan and Peltz 2003; Saguez et al. 2005). Pre-mRNAs with incomplete transcriptional processes, as well as other abnormal transcripts such as hyperedited dsRNA (Zhang and Carmichael 2001), hypomodified transfer RNA (tRNA) (kadaba et al. 2004) or that possess CUG expansions (Davis et al. 1997) can be retained in the nucleus at or close to the site of transcription and/or ultimately be degraded (Custodio et al. 1999; Hilleren et al. 2001; Jensen et al. 2001; Libri et al. 2002; Thomsen et al. 2003). In yeast, the nuclear degradation pathway appears to primarily involve 3' to 5' degradation by the nuclear exosome, but can also involve 5' to 3' degradation by the nuclear 5' exonuclease Xrn1p homolog, Rat1p (Johnson 1997; Bousquet-Antonelli et al. 2000; Geerlings et al. 2000; Das et al. 2003). The favored pathway appears to be determined by the substrate. Rat1p has been implicated in the maturation of rRNAs and snoRNAs, as well as the degradation of nuclear mRNAs (Petfalski et al. 1998; Bousquet-Antonelli et al. 2000; Fang et al. 2005). Although 5' to 3' degradation presumably requires mRNA deadenylation and decapping, a yeast nuclear deadenylating or decapping activity has not been demonstrated. Interestingly, nuclear 5' decapping seems to be stimulated by the nuclear Lsm2-8p complex (Kufel et al. 2004). Given that the cytoplasmic Lsm1-7p complex stimulates decapping in the cytoplasm (see below), this suggests that decapping also occurs in the nucleus. The nuclear exosome and its nuclear component Rrp6p play a major role in this quality control

step in immediate proximity to transcription foci, both in selecting the aberrant mRNAs for nuclear retention and in its subsequent degradation (Houseley et al. 2006; Vanacova and Stefl 2007). These include unspliced pre-mRNAs, transcripts with abnormal 3' ends, and transcripts that do not form export-competent mRNPs (Bousquet-Antonelli et al. 2000; Burkard and Butler 2000; Hilleren et al. 2001; Libri et al. 2002; Torchet et al. 2002; Zenklusen et al. 2002; Thomsen et al. 2003; Milligan et al. 2005). Interestingly, this nuclear RNA surveillance pathway can be initiated by polyadenylation by the TRAMP complexes, which adds short poly(A) tails to transcripts and then allows rapid exosome-mediated decay (LaCava et al. 2005; Vanacova et al. 2005; Wyers et al. 2005; Egecioglu et al. 2006; Houseley and Tollervey 2006; Kadaba et al. 2006). This contrasts with the stabilizing effect of poly(A) tails in the cytoplasm, and is reminiscent of bacterial mRNA decay.

Interestingly, mRNAs are also monitored when they move from the nucleus to the cytoplasm. A quality control step located at the nuclear pore complex surveys transcripts to assure that only mature, fully processed mRNAs are exported from the nucleus (Galy et al. 2004; Vinciguerra et al. 2005).

Finally, in the cytoplasm, the cell can assess the translatability of the mRNA through specialized quality control mechanisms that identifies and degrades mRNAs that have no translation termination (nonstop) codons (nonstop-mediated mRNA decay, NSD) (Maquat 2002; Vasudevan et al. 2002) or that contain premature translation termination (nonsense) codons (PTCs) (nonsense-mediated mRNA decay, NMD) (Hilleren and Parker 1999; Baker and Parker 2004; Amrani et al. 2006), thereby preventing the accumulation of potentially harmful abnormal protein products (Figure I.2). Recognition of nonsense and nonstop mRNAs as abnormal requires their functional translation by ribosomes, which fail to terminate properly. This improper termination leads to recruitment of the same enzymes responsible for general mRNA decay, but involve specific mechanisms for targeting these enzymes to these mRNAs (see below). Finally, mRNAs impaired in translation elongation are recognized and targeted for degradation by another quality control mechanism, referred to as the no-go mRNA decay (NGD) pathway (Clement and Lykke-Andersen 2006).

I.3.3.1 Nonsense-Mediated mRNA Decay

The NMD pathway degrades not only mRNAs containing PTCs as a result of nonsense or frameshift mutations, but also a number of different mRNA substrates (He et al. 2003).

These include aberrant transcripts containing unspliced introns (He et al. 1993), or abnormal 3' UTRs that escape to the cytoplasm (Muhlrad and Parker 1999a), and wild-type transcripts containing short upstream open reading frames (uORFs) in the 5' UTR (Oliveira and McCarthy 1995; Ruiz-Echevarria and Peltz 2000) or mRNAs in which leaky scanning promotes out-of-frame translation initiation (Welch and Jacobson 1999).

The NMD pathway has been studied extensively in yeast, in which recognition of a nonsense codon targets the aberrant mRNA for rapid degradation primarily by deadenylation-independent decapping mediated by the Dcp1p/Dcp2p complex, wherein the normal prerequisite for poly(A) shortening before decapping is bypassed, followed by 5' to 3' degradation by Xrn1p (Muhlrad and Parker 1994; He and Jacobson 2001). The deadenylation-independent decapping pathway can occur localized in discrete cytoplasmic sites (Sheth and Parker 2006). NMD by accelerated deadenylation, followed by exosome-mediated 3' to 5' exonucleolytic degradation can also occur to less extent (Cao and Parker 2003; Mitchell and Tollervey 2003). NMD substrates are recognized at the end of each round of translation and targeted for degradation as a consequence of premature translation termination (Maderazo et al. 2003; Culbertson and Neeno-Eckwall 2005). Discrimination between normal and premature translation termination seems to involve the cross-talk between the terminating ribosome at the stop codon and other mRNP features, which can be poorly conserved downstream sequence elements (DSEs) located 3' of the stop codon that binds specific marker proteins (Gonzalez et al. 2001; Wang et al. 2006), or an abnormally long 3' UTR that alters the spatial relationship between the termination codon and the poly(A)-bound Pab1p (Amrani et al. 2004, 2006). Aberrant termination at the PTC is thought to allow the assembly of three specific NMD factors, Upf1p, Upf2p/Nmd2p, and Upf3p that comprises the core NMD machinery (Leeds et al. 1991, 1992; Cui et al. 1995; He and Jacobson 1995; Lee and Culbertson 1995). Upf proteins function in promoting NMD by providing a direct link between the premature translation termination event and mRNA degradation. Indeed, the Upf proteins associate with eRF1 and eRF3 translation termination factors, which, in turn, are bound to the terminating ribosome acceptor (A) site, to form the surveillance complex (He et al. 1997; Czaplinski et al. 1998; Wang et al. 2001). In addition, Upf1p interacts with Dcp2p, which seems to recruit the decapping enzyme to the mRNA, thereby promoting decapping and subsequent 5' to 3' decay (He and Jacobson 1995). Moreover, Upf1p interacts with Ski7p to promote exosome-mediated 3' to 5' decay (Takahashi et al. 2003).

NMD is a highly conserved pathway that exists in all eukaryotic cells examined to date, and functional homologues of the yeast Upf NMD factors have been characterized

(Culbertson and Leeds 2003; Maquat 2004a; Conti and Izaurralde 2005). However, key mechanistic details seem to differ between organisms.

In mammalian cells, although controversial, depending on the particular mRNA, NMD has been proposed to occur in association with the nuclei, or in the cytoplasm (Maquat 2004b). Recent evidence suggests however that NMD takes place primarily, if not entirely, in the cytoplasm as in yeast (Singh et al. 2007). Regardless of the cellular place, NMD likely occurs as a result of PTC recognition on newly synthesized mRNAs during a unique pioneer round of translation and requires a splicing event (Ishigaki et al. 2001; Lejeune et al. 2002; Chiu et al. 2004; Maquat 2004b). Generally, during the first round of translation of a mammalian mRNA, recognition a stop codon involves a complex that includes eRF1 and eRF3 termination factors and UPF1 (Kashima et al. 2006). NMD is specifically triggered when UPF1 is allowed to interact with UPF2, which is bound to UPF3 (Hosoda et al. 2005; Kashima et al. 2006). UPF2 and UPF3 are both present in a multiprotein complex, the exon-junction complex (EJC) that is deposited 20-24 nt upstream of each exon-exon junction during pre-mRNA splicing and remains bound to mature mRNAs when they enter the cytoplasm (Le Hir et al. 2000; Lykke-Andersen et al. 2000; Le Hir et al. 2001; Kim et al. 2001; Serin et al. 2001; Gehring et al. 2003; Tange et al. 2004). In a nonsense mRNA, there is typically one EJC deposited at an exon-exon junction located at least 50-55 nt downstream of the nonsense codon, thereby allowing interaction between UPF1 associated with a termination complex at the PTC and UPF2 associated with the downstream EJC (Nagy and Maquat 1998; Maquat 2005; Kashima et al. 2006). Subsequently, a cycle of UPF1 phosphorylation/dephosphorylation appears to be critical to trigger subsequent steps that ultimately degrade the mRNA (Ohnishi et al. 2003), including the recruitment and/or activation of mRNA degradative activities, and movement to cytoplasmic specialized decay bodies (Unterholzner and Izaurralde 2004; Fukuhara et al. 2005). In mammals, similar to yeast, nonsense mRNAs are degraded both by decapping-dependent 5' to 3' decay and exosome-mediated 3' to 5' decay (Chen and Shyu 2003; Lejeune et al. 2003; Couttet and Grange 2004). Interestingly, mammalian NMD can occur by alternative mechanisms that are independent of UPF2 or UPF3 or the EJC, but they all depend on UPF1 to elicit NMD (Gehring et al. 2005; Buhler et al. 2006; Chan et al. 2007).

Finally, in *D. melanogaster* general NMD pathway, nonsense mRNAs are primarily endonucleolytic cleaved in the vicinity of the PTC, presumably by a ribosome-associated endonuclease or by the ribosome itself. The resulting 5' fragment is degraded from the 3' end by the exosome, whereas the 3' fragment is degraded from the 5' end by Xrn1p/Pacman (Gatfield and Izaurralde 2004).

I.3.3.2 Nonstop-Mediated mRNA Decay

Many mRNAs possess consensus internal polyadenylation signals that can be erroneously used to produce an aberrant 3' end upstream of the translation stop codon during RNA processing as a result of premature polyadenylation (Frischmeyer et al. 2002). Interestingly, this seems more probable to occur when transcription aborts (Cui and Dennis 2003). NSD is a specialized decay process that exists to ensure the degradation of these transcripts that lack a stop codon and prevent their expression (Maquat 2002; Vasudevan et al. 2002). In the absence of a stop codon, ribosome transit continues through the 3' UTR and poly(A) tail, displacing Pab1p, and eventually stalls at the 3' end of the mRNA. The NSD mechanism releases ribosomes stalled at the 3' end of the nonstop mRNA and stimulates rapid mRNA degradation (Frischmeyer et al. 2002; van Hoof et al. 2002; Inada and Aiba 2005). In this pathway, the nonstop mRNA can be targeted to 3' to 5' degradation by the cytoplasmic exosome, as well as, 5' to 3' degradation by rapid decapping and exonucleolytic digestion. The exosome seems to be recruited to nonstop mRNAs by an interaction between the ribosome stalled at the 3' end of the transcript and the exosome-associated factor Ski7p (van Hoof et al. 2002). It has been proposed that the C-terminal domain of Ski7p, which closely resembles the GTPase domain of EF1A elongation and eRF3 termination factors, interacts with the empty A site of the stalled ribosome to recruit the exosome as well as the associated Ski2p/Ski3p/Ski8p complex, resulting in degradation of the nonstop mRNA by 3' to 5' decay that begins at the 3' poly(A) tail (Bernard et al. 1999; van Hoof et al. 2002). Remarkably, the form of the exosome that functions in nonstop-triggered decay seems to have the ability to degrade the poly(A) tail and the RNA body. In addition, in the absence of Ski7p, degradation of nonstop mRNAs also occurs by accelerated decapping and 5' to 3' degradation by Xrn1p, probably stimulated by dissociation of Pab1p during translation through the poly(A) tail (Inada and Aiba 2005). It is possible that both pathways function in concert. NSD also occurs in mammals suggesting that it serves an important biological role (Frischmeyer et al. 2002).

I.3.3.3 No-Go mRNA Decay

Another quality control mechanism exists to eliminate mRNAs with stalled translation elongation complexes, the NGD. In this pathway, a ribosome stalled either by an artificial stem-loop structure or other stalls in translation, including those induced by rare codons and pseudoknots, triggers endonucleolytic cleavage of the mRNA near to where the translationally active ribosome stalls, with the resulting 5' fragment being degraded by the

cytoplasmic exosome, whereas the 3' fragment is a substrate for Xrn1p (Doma and Parker 2006). Dom34p together with Hbs1p, which are related to translation factors eRF1 and eRF3, respectively (Carr-Schmid et al. 2002), are probably involved in the recognition of the stalled ribosome on an mRNA, perhaps by interacting with the ribosomal A site, and triggering the initial endonucleolytic cleavage by a currently unknown mechanism. While the endonuclease has not been identified, it is possible that Dom34p is the endonuclease itself because it was shown to have endoribonuclease activity (Lee et al. 2007).

I.4 Mechanisms of Control of mRNA Turnover

Messenger RNA turnover is not a random, indiscriminate nuclease degradation process, but instead a highly regulated process that can be modulated at each step of the pathway either globally or in a sequence-specific manner. Several mechanisms that contribute to the modulation of mRNA turnover will be discussed.

I.4.1 Structural Features that Affect mRNA Turnover

mRNAs contain several different sequences and structures all over the message that can modulate their degradation (Figure I.6). Control of mRNA degradation is achieved in part by orchestrated interactions between these mRNAs elements (*cis*-acting elements), and RNA-binding proteins (*trans*-acting factors) that can be general or mRNA-specific, and which in turn can interact either directly or indirectly with other proteins. An important point is that specific interactions contribute to RNA-specific degradation by nonsequence-specific nucleases activities, determining both the pathway and rate of mRNA turnover. Moreover, these interactions are frequently subjected to regulatory control.

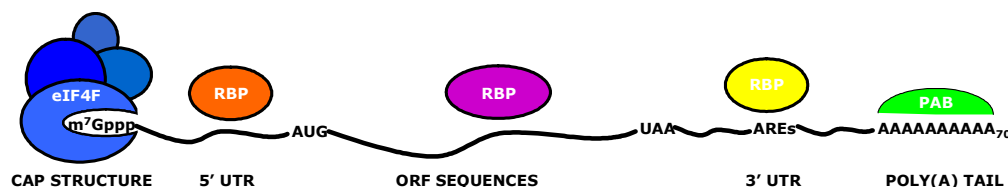


Figure I.6 Location of mRNA sequence elements involved in regulating mRNA turnover.

I.4.1.1 Modulation of mRNA Turnover Rates by Specific *cis*-acting Elements

In yeast, numerous specific *cis*-acting elements, diverse in sequence and structure, have been identified that can modulate the rate of decay of a particular mRNA, either by promoting (destabilizing elements) or by inhibiting (stabilizing elements) degradation, although the mechanism by which these elements modulate mRNA turnover is not fully understood. These elements can be found at different locations in the mRNA, including the 5' UTR, the protein coding region, or the 3' UTR (Figure I.6). Moreover, in some cases, these elements act in concert to regulate mRNA turnover. Specifically the 3' UTRs are well known to contain sequences that control mRNA decay, and serve as binding sites for numerous factors that regulate mRNA stability. For example, sequences present in the 3' UTR of unstable *MFA2* mRNA affects the rate of decay by leading to both faster deadenylation and decapping rates (Muhlrad and Parker 1992; LaGrandeur and Parker 1999). Similarly, the 3' UTR of the unstable *STE3* mRNA contains sequences that specify rapid decay (Heaton et al. 1992). Moreover, *STE3* mRNA also contains a destabilizing element in its coding region (Heaton et al. 1992). Sequence elements have been identified in the coding region of other mRNAs. The best defined of these is a 65 nt sequence that has high content of rare codons in the coding region of *MAT α 1* mRNA that promotes rapid deadenylation and decapping (Parker and Jacobson 1990; Caponigro et al. 1993; Caponigro and Parker 1996). This element stimulates decay in a manner linked to mRNA translation, as destabilization requires translation up to, or through it (Parker and Jacobson 1990; Hennigan and Jacobson 1996). In addition, destabilizing elements present in the coding region of *HIS3* and *SPO13* mRNAs mediate their rapid decay (Herrick and Jacobson 1992; Surosky and Esposito 1992). The destabilizing elements located in the coding region of *SPO13* mRNA seem to be important in keeping meiotic mRNAs to a very low level in growing cells (Surosky and Esposito 1992). In contrast, the constitutively stable *PGK1* mRNA contains sequence information within its coding region that together with the context of the translation start codon (AUG) specifies a slow rate of decapping following deadenylation (Muhlrad et al. 1995; LaGrandeur and Parker 1999). Finally, the 5' UTR of the highly unstable *PPR1* mRNA contains sequences that promote rapid degradation (Pierret et al. 1993). Similarly, the 5' UTR of *Ip* mRNA promotes its degradation in response to different carbon sources (Cereghino et al. 1995).

One important class of sequence elements, AREs, found in the 3' UTRs of most unstable mammalian mRNAs encoding proteins of regulatory significance, can promote rapid mRNA turnover (Chen and Shyu 1995). Although distinct classes of AREs exist, typically, the AU-rich motif contains a variable number of the AUUUA pentanucleotide sequence,

frequently harbored within a U-rich region of the mRNA (Barreau et al. 2006). AREs appear to act as binding sites for diverse ARE-binding proteins (ARE-BPs) that in turn regulate ARE-mediated decay (Bevilacqua et al. 2003). For example, HuR, an ELAV-related RNA-binding protein, mediates stabilization of some ARE-containing mRNAs (Fan and Steitz 1998; Peng et al. 1998; Raineri et al. 2004). In contrast, TTP, a zinc-finger protein, causes destabilization of some ARE-containing mRNAs (Carballo et al. 1998; Lai et al. 1999; Lykke-Andersen and Wagner 2005). While AUF1 (or hnRNP D) can either stabilize or destabilize ARE-containing mRNAs (Xu et al. 2001; Sarkar et al. 2003; Raineri et al. 2004). ARE-BPs seem to modulate the decay of ARE-containing mRNAs either by recruiting and/or stimulating the mRNA degradative activities or, by displacing from the mRNA stabilizing proteins or, by protecting the mRNA from nucleases. *In vitro* RNA decay studies indicated that AREs and/or the ARE-BPs can stimulate mRNA deadenylation (Wilson and Treisman 1988; Shyu et al. 1991; Chen et al. 1994; Lai et al. 2003), followed primarily by exosome-mediated 3' to 5' decay (Chen et al. 2001a; Mukherjee et al. 2002; Gherzi et al. 2004), although decapping has also been described (Gao et al. 2001). However, in mammalian cells, degradation of ARE-containing mRNAs seem to be mediated primarily by decapping and 5' to 3' exonucleolytic decay, associated with discrete cytoplasmic granules (Lykke-Andersen and Wagner 2005; Stoecklin et al. 2006; Lin et al. 2007). Possibly, both 5' to 3' and 3' to 5' pathways are involved in the degradation of ARE-containing mRNAs. Significantly, AREs are also present in yeast mRNAs to regulate decay rates, suggesting conservation of the ARE-mediated decay between yeast and mammals. Indeed, yeast or mammalian AREs can promote degradation of a yeast reporter transcript by inducing rapid deadenylation, followed by decapping and 5' to 3' decay (Vasudevan and Peltz 2001). Moreover, an AU-rich sequence in the 3' UTR of *TIF51A* mRNA determines its differential degradation in response to carbon sources, in a manner dependent on a signal transduction pathway (Vasudevan and Peltz 2001). Furthermore, an AU-rich sequence within the 3' UTR of *MFA2* mRNA regulates its rapid degradation (Muhlrads and Parker 1992; Duttagupta et al. 2003). Finally, the yeast ARE-BP ELAV homologue, Pub1p, as well as the TTP homologue, Cth2p, can regulate the stability of yeast mRNAs containing ARE-like elements (Vasudevan and Peltz 2001; Duttagupta et al. 2005; Puig et al. 2005).

I.4.1.2 Coupling mRNA Turnover and Translation

In addition to these elements that may be diverse in sequence and structure, other more discrete elements also dictate the control of mRNA turnover, including the 7-methylguanosine cap structure present at the 5' end of the mRNA, and the poly(A) tail at

the 3' end of the mRNA (Figure I.6). These are critical protective features common to most mRNAs responsible, at least in part, for their intrinsic stability. Consistent with this, the poly(A) tail is the first target of attack in both general pathways of mRNA turnover, and the cap structure is the second in the major pathway to allow exonucleases access to the body of the mRNA. Significantly, these structures and their associated proteins also have a role in translation (Jacobson and Peltz 1996; Sachs et al. 1997). This dual role suggests that translation of an mRNA may be an important determinant of its stability. In the cytoplasm, the poly(A) tails of mRNAs are bound by Pab1p, a highly conserved protein that stabilizes the poly(A) tail (Mangus et al. 2003). While the cap structure is bound by the cytoplasmic cap binding complex, eIF4F, a complex comprising eIF4E, the cytoplasmic cap-binding protein; eIF4A, DEAD-box RNA helicase; and eIF4G, a modular protein that binds eIF4E and eIF4A, and recruits the small ribosomal subunit by binding eIF3 (Gingras et al. 1999). Moreover, eIF4G binds both the cap-binding protein eIF4E at the 5' end of the mRNA, and Pab1p associated with the poly(A) tail at the 3' end (Tarun and Sachs 1996). This cap/eIF4E-eIF4G-Pab1p/poly(A) tail interaction is thought to result in the functional circularization of the mRNA (Wells et al. 1998), which seems to stabilize the translation initiation complex at the 5' cap and strength the association of Pab1p with the poly(A) tail, consequently stimulating translation and presumably protecting both ends of the transcript from the mRNA degradation machinery (Gallie 1998; Preiss and Hentze 1998; Otero et al. 1999; Gray et al. 2000; Sachs 2000). In fact, there is extensive evidence supporting a close link between the translation status of mRNAs and their rates of degradation. For example, inhibition of translation initiation either in *cis* or in *trans* results in mRNA destabilization. First, mutations in translation initiation factors eIF4E, eIF4G, eIF4A and Prt1p (a component of eIF3 complex), which strongly decrease translation initiation, lead to increased rates of deadenylation and decapping (Schwartz and Parker 1999). Second, decreases in translation initiation of individual mRNAs by the insertion of a stem loop in the 5' UTR, or by a change in the AUG context, also increased the rates of deadenylation and decapping (Muhlrad et al. 1995; LaGrandeur and Parker 1999). Conversely, inhibition of translation elongation results in mRNA stabilization. First, blocking translation elongation with cycloheximide, which traps mRNA in polysomes, greatly stabilized several yeast mRNAs through an inhibition of decapping (Beelman and Parker 1994). Similarly, mutations in factors required for the production of functional tRNAs, which lead to an apparent translation elongation block and an increase in the relative number of ribosomes associated with mRNAs, reduced mRNA decay rates (Peltz et al. 1992). Thus, translation and mRNA degradation appear to be in competition. This suggests that the modulation of the stability of the translation initiation complex on the mRNA may be a critical aspect for specifying mRNA decay rates. Another example of the

link between mRNA translation and degradation is the rapid degradation of aberrant mRNAs that terminate translation prematurely by NMD. In addition, several factors known to promote mRNA decay show links to the translation machinery and also have roles in translation repression (Wyers et al. 2000; Coller et al. 2001; Coller and Parker 2005). Finally, the observation that mRNA turnover is directly influenced by the process of translation predicts that at least some *cis*-acting elements that regulate mRNA decay are likely to modulate translation efficiency, thereby leading to changes in both rates of deadenylation and decapping. Indeed, destabilizing elements can be located in the coding region, and some depend on translation through the element to destabilize the transcript (Parker and Jacobson 1990). In addition, the sequence elements in *PGK1* mRNA that promote its stability, also promote its efficient translation. And, sequence changes that lead to destabilization of the transcript, also reduce its translational efficiency (LaGrandeur and Parker 1999). Moreover, AREs that promote rapid mRNA decay also influence translation (Kruys and Huez 1994; Vasudevan et al. 2005). Therefore, understanding this interrelationship is critical for understanding how mRNA turnover is controlled.

I.4.2 mRNA Deadenylation and Control

There are a number of reasons to believe that deadenylation plays an important role in the modulation of mRNAs function and turnover. For example, shortening of the 3' poly(A) tail is required before the degradation of most yeast mRNAs by either decapping- or exosome-mediated decay (Decker and Parker 1993; Muhlrads et al. 1994,1995; Anderson and Parker 1998). Moreover, deadenylation initiates mRNA degradation in more complex eukaryotes (Wilson and Treisman 1988; Shyu et al. 1991). In addition, different mRNAs can have very different rates of deadenylation, and these variations can lead to overall differences in mRNA decay rates (Decker and Parker 1993). Moreover, computational modeling indicates that changes in deadenylation rates cause the largest alteration in overall mRNA stability (Cao and Parker 2001). Third, many *cis*-acting elements that regulate mRNA decay do so, at least in part, by affecting deadenylation rates (Muhlrads and Parker 1992; Caponigro and Parker 1996; Vasudevan and Peltz 2001). Finally, deadenylation has also a specific role in the maturation of mRNAs when they undergo the initial poly(A) tail trimming to defined lengths (Brown and Sachs 1998). In addition, poly(A) shortening occurs continuous throughout the lifetime of an mRNA. And, cytoplasmic changes in poly(A) tail lengths can alter the translatability of an mRNA (Sachs et al. 1997). The regulation of mRNA translation by both deadenylation and re-adenylation of a transcript is a common strategy in early development in the production of stored maternal

mRNAs (Gray and Wickens 1998). Thus, deadenylation is unique in that it is a reversible step and appears to be a main control point of mRNA turnover and overall gene expression. Given these facts, to determine the mechanisms that control deadenylation is important for understanding mRNA turnover.

I.4.2.1 Regulation of Deadenylation by Poly(A) Shortening Nucleases

Although so far it remains unclear exactly how or when deadenylation is triggered, and little is known about its regulation, the identification of various yeast deadenylases offers the opportunity to understand how specific deadenylation rates are established through the modulation of different 3' poly(A) nucleases. In yeast, polysome-associated shortening of the poly(A) tail seems to occur in at least two phases that are kinetically distinct reactions performed by different deadenylase activities (Figure I.7). First, the poly(A) tail is deadenylated of about 15-20 nt to mRNA-specific lengths in a phase referred to as initial deadenylation by the PAN complex (Decker and Parker 1993; Brown and Sachs 1998). Subsequently, most of the poly(A) tail is shortened at mRNA-specific rates by the Ccr4p/Pop2p/Not complex, which leaves mRNAs with an oligo(A) tail of approximately 10-15 A residues (Muhlrad and Parker 1992; Decker and Parker 1993; Tucker et al. 2001). This oligo(A) tail is then shortened slowly, a process referred to as terminal deadenylation. However, terminal deadenylation does not seem to contribute in a significant way to subsequent events in the decapping-dependent decay. Indeed, 5' to 3' decay products from several mRNAs have oligo(A) tails, indicating that these transcripts are decapped and degraded independently of complete removal of the poly(A) tail (Decker and Parker 1993; Muhlrad et al. 1994, 1995). Nevertheless, this implies that deadenylation of different regions of the poly(A) tail may be mechanistically different.

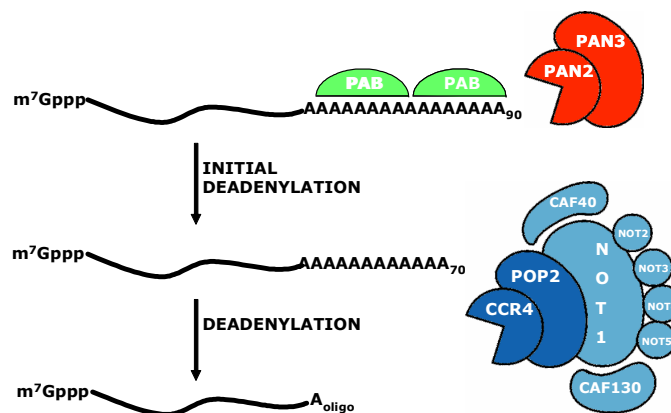


Figure I.7 Model illustrating distinct stages of mRNA deadenylation.

The mechanisms controlling deadenylation by poly(A) nucleases activities are unknown, but it is interesting to note that the different deadenylases have distinct substrate specificities. The Ccr4p complex is inhibited by Pab1p *in vitro* (Tucker et al. 2002), whereas the PAN complex requires Pab1p for activity (Sachs and Deardorff 1992). In addition, Ccr4p complex is not affected by the presence of the cap structure of the mRNA (Tucker et al. 2002; Viswanathan et al. 2003), whereas it is not known whether the cap structure can influence PAN activity. The identification of multiple deadenylase activities with different biochemical properties suggests that each mRNA deadenylase will have a different preferred mRNP substrate. For example, PAN would preferentially deadenylate an mRNA with Pab1p bound to the poly(A) tail. In contrast, Ccr4p complex would preferentially act on an mRNA without Pab1p bound to the poly(A) tail. Thus, mRNAs with different mRNP states may be targeted by different deadenylases. This suggests that one way to control deadenylation can be by changes in mRNP composition and dynamics, which would alter the susceptibility of mRNAs to different deadenylases. Consistent with this view, PAN initial deadenylation may be required to initiate a rearrangement in the translating mRNP into an mRNP state that can then be susceptible to deadenylation by the Ccr4p complex. Finally, this also implies that modulating the activity of a specific deadenylase will allow for differential control of the deadenylation of different pools of mRNAs, perhaps in response to specific regulatory signals.

I.4.2.2 Pab1p and Regulation of Deadenylation

Most mRNAs start their lives with long poly(A) tails associated with multiple Pab1p molecules, which seem to have a role in coupling several processes in the cell, including mRNA translation and stability (Mangus et al. 2003). Pab1p, as a component of a poly(A) mRNP structure that also contains translation factors seems to protect the poly(A) tail against deadenylation. However, *pab1* mutants are defective in deadenylation. For example, deletion of *PAB1* gene results in increased average length of total cellular poly(A) tails and an extended temporal delay before the onset of deadenylation (Caponigro and Parker 1995). This may reflect a defect in the initial deadenylation performed by PAN, which is Pab1p dependent (Sachs and Davis 1989). In fact, the C-terminal domain of Pab1p directly interacts with Pan3p, interaction which has been suggested to be responsible for recruiting the Pan2p/Pan3p complex for the mRNP to initiate limited poly(A) tail shortening (Mangus et al. 2004; Siddiqui et al. 2007). In addition, although the Ccr4p deadenylase complex is inhibited by Pab1p *in vitro* (Tucker et al. 2002), strains lacking Pab1p also show decreased rates of deadenylation (Caponigro and Parker 1995). This suggests that Pab1p can also promote mRNA

deadenylation. Recent studies addressing the role of Pab1p in the deadenylation process revealed that Pab1p seems to promote mRNA deadenylation through its ability to dissociate from the poly(A) tail and self-associate to form Pab1p multimers. Specifically, Pab1p C-terminal regions seem to be directly involved in mRNA deadenylation by altering its dissociation from the poly(A) tail. Deletion of Pab1p C-terminal domains impairs deadenylation *in vivo*, and inhibits Ccr4p deadenylase activity *in vitro* (Simon and Seraphin 2007). In addition, Pab1p ability to self-associate forming circular and multimeric species appear to significantly affect Pab1p binding to the poly(A) tail, and mRNA deadenylation by the Ccr4p deadenylase complex. Pab1p domain mutations that interfere with Pab1p self-association significantly decreased Pab1p removal from the poly(A) tail and deadenylation. Conversely, deletions in Pab1p that promote its self-association increased deadenylation (Yao et al. 2007). This is consistent with a model where Pab1p bound to the poly(A) tail can simultaneously block Ccr4p deadenylase complex function, presumably by impeding Ccr4p progress or association with the mRNA, and stimulate PAN activity. After PAN initial deadenylation, removal of the first Pab1p from the poly(A) tail may cause sequential dissociation of other Pab1p molecules from the poly(A). Pab1p-Pab1p self-association would thus aid in removing Pab1p from the mRNA and allow deadenylation by the Ccr4p deadenylase complex to occur. This suggests that one way to control deadenylation would be by modulating the ability of Pab1p to self-associate. This also implies that specific factors that regulate mRNA deadenylation may function by controlling Pab1p self-association. Moreover, because increases in deadenylation rates are often associated with decreased translation rates this suggests that the interaction between Pab1p and translation initiation factors, which stabilizes Pab1p association with the poly(A) tail, may prevent its self-association and consequently inhibit deadenylation. Notably, the control of mRNA deadenylation and turnover seems to be functionally and physically connected to translation termination. In fact, eRF3 mutations that decrease translation efficiency resulted in mRNA stabilization by altering deadenylation (Kobayashi et al. 2004). Interestingly, eRF3 associates with the C-terminal region of Pab1p (Cosson et al. 2002), and strains in which this interaction is impaired show reduced deadenylation rates (Hosoda et al. 2003). This suggests that eRF3-Pab1p interaction may be involved in initiating deadenylation. Although the precise mechanism is not known, it is possible that eRF3 binding to Pab1p may stimulate PAN deadenylase activity, promoting removal of Pab1p from the poly(A) tail, thereby allowing the Ccr4p complex to subsequently complete the deadenylation process. Alternatively, eRF3 contact to Pab1p may directly modulate the association of Pab1p with the poly(A) tail, perhaps favoring Pab1p self-association, and thereby stimulating deadenylation.

I.4.2.3 Specific Regulators of Deadenylation

Interestingly, recent results demonstrated that deadenylation can be specifically activated by recruitment of Ccr4p/Pop2p deadenylase to a reporter transcript (Finoux and Seraphin 2006). This indicates that deadenylation can be controlled by association of the deadenylase with its substrate. Such modulation could be mediated through interaction with mRNA-specific binding proteins that associate with the mRNA in the mRNP structure. Consistent with this, both yeast Puf4p and Puf5p/Mtr5p, which belong to a family of RNA-binding proteins that bind the 3' UTR of specific mRNAs to control translation and decay (Wickens et al. 2002), were shown to directly bind Pop2p, simultaneously recruiting the Ccr4p/Pop2p deadenylase to *HO* mRNA, thereby stimulating Ccr4p deadenylase activity and promoting *HO* mRNA deadenylation and decay (Goldstrohm et al. 2006, 2007). Similarly, yeast Puf3p binds to the 3' UTR of *COX17* mRNA and stimulates its deadenylation and subsequent decay (Olivas and Parker 2000). In addition, rapid deadenylation of *COX17* mRNA depends on Ccr4p deadenylase complex (Tucker et al. 2002). This suggests that similar to Puf4p and Puf5p, Puf3p may enhance *COX17* mRNA deadenylation by recruiting Ccr4p deadenylase activity through direct interaction with Pop2p. Furthermore, this suggests that Puf proteins are specific regulators of deadenylation that may function by recruiting Ccr4p deadenylase to their target mRNAs, through an interaction with Pop2, thereby stimulating deadenylation. Interestingly, each of the five yeast Puf proteins (Puf1p-Puf5p) appears to be associated with a large distinct set of mRNAs that encode proteins functionally related and very often share a common cellular localization (Gerber et al. 2004). For example, Puf3p preferential interacts with mRNAs that encode mitochondrial proteins, whereas Puf5p preferential associates with mRNAs encoding chromatin modifiers and components of the spindle pole body. This suggests that Puf proteins through their distinct binding specificities are likely to play a role in establishing mRNA-specific deadenylation rates. Moreover, co-regulation of a single mRNA by more than one Puf protein may also occur, since some of these mRNAs can be associated with multiple Puf proteins (Gerber et al. 2004; Hook et al. 2007). Finally, because Puf proteins directly interact with Pop2p homologs in other organisms, recruitment of Ccr4p activity through this interaction may be a conserved mechanism to modulate deadenylation (Goldstrohm et al. 2006). Moreover, it should be expected the identification of other yeast regulatory factors that use a similar strategy to control mRNA deadenylation. For example, Vts1p has been shown to bind the 3' UTR of a specific reporter RNA and induce its destabilization in a Ccr4p-dependent manner (Aviv et al. 2003). Although the mechanism by which recruitment of Ccr4p deadenylase complex to the mRNA enhances deadenylation remains unknown, it is possible that this occurs

through direct stimulation of Ccr4p nuclease activity. In addition, modification of the mRNP structure may allow a more productive access of the deadenylase to the substrate, and thereby enhances its activity. This is consistent with the displacement of Pab1p from the poly(A) tail increasing its susceptibility to Ccr4p deadenylation. And, with the requirement for a certain length of RNA for substrate recognition by Ccr4p deadenylase (Viswanathan et al. 2003). Nevertheless, one important idea is that recruitment of the deadenylase to an mRNA may serve as a mean to activate mRNA turnover in yeast.

I.4.3 mRNA Decapping and Control

Decapping is a central step in the 5' to 3' pathway of mRNA degradation, which is the major mechanism of mRNA turnover in yeast (Decker and Parker 1993; Muhlrads et al. 1994, 1995). The unusual 5'-5' triphosphate linkage that characterizes the 5' cap structure makes the body of the mRNA intrinsically resistant to degradation by 5' exonucleases. Thus, excision of the cap structure (decapping), which is an irreversible step, effectively terminates the cytoplasmic functionality of mRNA because it precedes and permits its extremely rapid degradation by 5' to 3' degradation. Moreover, following deadenylation, mRNAs are also decapped at different rates, contributing for differences in the rates of mRNA decay (Muhlrads et al. 1994, 1995). Another role for decapping is the completion of the 3' to 5' decay pathway by the DcpS activity (Liu et al. 2002). Decapping is also critical in the process of mRNA surveillance that degrades aberrant mRNAs containing nonsense codons (Muhlrads and Parker 1994). For these reasons, decapping is one key event in mRNA turnover that must be carefully controlled by several factors. The decapping process seems to consist of four central and related events (Figure I.8). These include removal, or functional inactivation, of the poly(A) tail as an inhibitor of decapping, exit from active translation, assembly of a decapping complex that assists in the recruitment of the decapping enzyme to the mRNA, and sequestration of the mRNA into discrete cytoplasmic structures. Each of these steps is a potential site for regulation.

I.4.3.1 Poly(A) tails as an Inhibitor of Decapping

A central aspect of mRNA decapping is that poly(A) shortening is required before decapping can occur. Indeed, several observations indicate that the poly(A) tail has the ability to inhibit mRNA decapping. For example, for several yeast mRNAs, including stable and unstable mRNAs, decapping does not occur until the poly(A) tail has been shortened to an oligo(A) length. Moreover, the time required before decapping occurs closely

correlates to the time required for these mRNAs to deadenylate (Decker and Parker 1993; Muhlrads et al. 1994, 1995). Accordingly, decay intermediates produced by the decapping reaction can only be detected after at least some of the population of mRNA has undergone deadenylation. In addition, these decay intermediates possess oligo(A) length tails, indicating that deadenylated mRNAs are the substrate for decapping. Thus, decapping occurs coupled to deadenylation. The observation that mRNAs with poly(A) tails are resistant to decapping in cell-free extracts in a Pab1p-dependent manner (Wilusz et al. 2001), suggested that the poly(A) tail may exert its effect on decapping primarily due to the binding of Pab1p, which then inhibits decapping. In fact, in *pab1Δ* mutants, decapping occurs prematurely before completion of deadenylation (Caponigro and Parker 1995; Morrissey et al. 1999). In this case, mRNA decay intermediates accumulated as decapped, polyadenylated species. This indicates that Pab1p is required for the normal coupling of poly(A) shortening and decapping. Moreover, this indicates that Pab1p in complex with the poly(A) tail normally functions as an inhibitor of mRNA decapping. It is thought that 3' poly(A) tail shortening to an oligo(A) length of approximately 10-15 A residues, may cause the loss of the last Pab1p associated with the transcript, since this is approximately the minimum length necessary for Pab1p binding (Sachs et al. 1987), and thus relieve the inhibition to decapping imposed by the poly(A) tail. However, yeast cell extracts lacking Pab1p still show some poly(A) tail inhibition of decapping, suggesting that there might be unknown factors that bind the poly(A) tail and also inhibits decapping independent of Pab1p (Wilusz et al. 2001). In addition, the block to decapping by Pab1p does not seem to absolutely require the presence of the poly(A) tail. Artificial tethering of Pab1p to mRNAs in a manner independent of the poly(A) tail, causes these mRNAs to accumulate as capped full-length messages but deadenylated (Coller et al. 1998). This indicates that if Pab1p can bind to an mRNA by other interactions, its ability to inhibit decapping becomes independent of its binding to poly(A) tail, and suggests that one role of the poly(A) tail is to recruit Pab1p to the transcript. Hence, deadenylation can be seen as a process that removes the binding sites for Pab1p from the mRNA, relieving Pab1p-dependent inhibition of decapping.

I.4.3.1.1 Pab1p as a Decapping Blocker

Although not fully understood, it appears that no single mechanism is sufficient to explain the effect of Pab1p in inhibiting mRNA decapping, and coupling deadenylation and decapping. Given that Pab1p physically interacts with eIF4G component of the cytoplasmic cap-binding complex (Tarun and Sachs 1996), and thus, indirectly with the cap-binding protein eIF4E, stabilizing their association with the mRNA. One simple

mechanism by which Pab1p interactions with the cap-binding complex might lead to inhibition of decapping is by keeping the cap protected from Dcp1p/Dcp2p, or by enhancing the translation of the mRNA, thereby indirectly inhibiting decapping (Schwartz and Parker 1999, 2000; Wilusz et al. 2001). However, several observations argue that the interaction of Pab1p with eIF4G, though it may contribute, cannot solely explain the Pab1p inhibition of decapping. For example, deletion of the eIF4G interacting domain in Pab1p has no effect on the ability of tethered Pab1p to block decapping (Coller et al. 1998). This indicates that the region within the Pab1p that functions in decapping is distinct from the region that binds eIF4G. In addition, mutations in eIF4E, eIF4G, or other initiation factors do not permit decapping to occur uncoupled from deadenylation, even though translational initiation is severely compromised and decapping rates are increased (Schwartz and Parker 1999). This suggests that inhibition of decapping by Pab1p may also involve interactions with factors other than the translation initiation complex. Finally, it is also possible that Pab1p can inhibit decapping independent of additional factors by direct association with the 5' cap structure, which may inhibit decapping through steric exclusion of the decapping enzyme. *In vitro* studies in mammalian cells demonstrated that Pab1p can bind the 5' cap structure and directly block decapping (Khanna and Kiledjian 2004). However, direct binding to cap was not found for yeast Pab1p.

Another mechanism by which Pab1p could inhibit decapping is by affecting the spatial location of mRNAs within the cell. The observation that decapping can occur in discrete cytoplasmic structures (Sheth and Parker 2003), and that mRNAs trapped in polysomes, by treating cells with cycloheximide, are resistant to decapping (Beelman and Parker 1994), suggests that translating and degrading pools of mRNAs are spatially distinct. Thus, the presence of poly(A)-bound Pab1p might inhibit decapping by preventing mRNAs from entering these sites of decapping, or by promoting rapid exit from these sites. This suggests that Pab1p may work by different, and overlapping, mechanisms possible connecting multiple processes, such as mRNA translation, localization and turnover to modulate decapping.

Interestingly, the observation that nonsense mRNAs, which terminate translation prematurely, can undergo rapid decapping independent of prior deadenylation, suggests that proper translation termination may affect Pab1p function in decapping regulation (Muhlrad and Parker 1994; Cao and Parker 2003). Additional experimental evidence indicated that proper translation termination requires Pab1p close to the ribosome at the stop codon, and this seems to be required for Pab1p role in mediating the dependence of decapping upon prior deadenylation (Coller et al. 1998; Amrani et al. 2004). Thus, one interesting idea is that the ability of Pab1p to block decapping depends on proper translation termination.

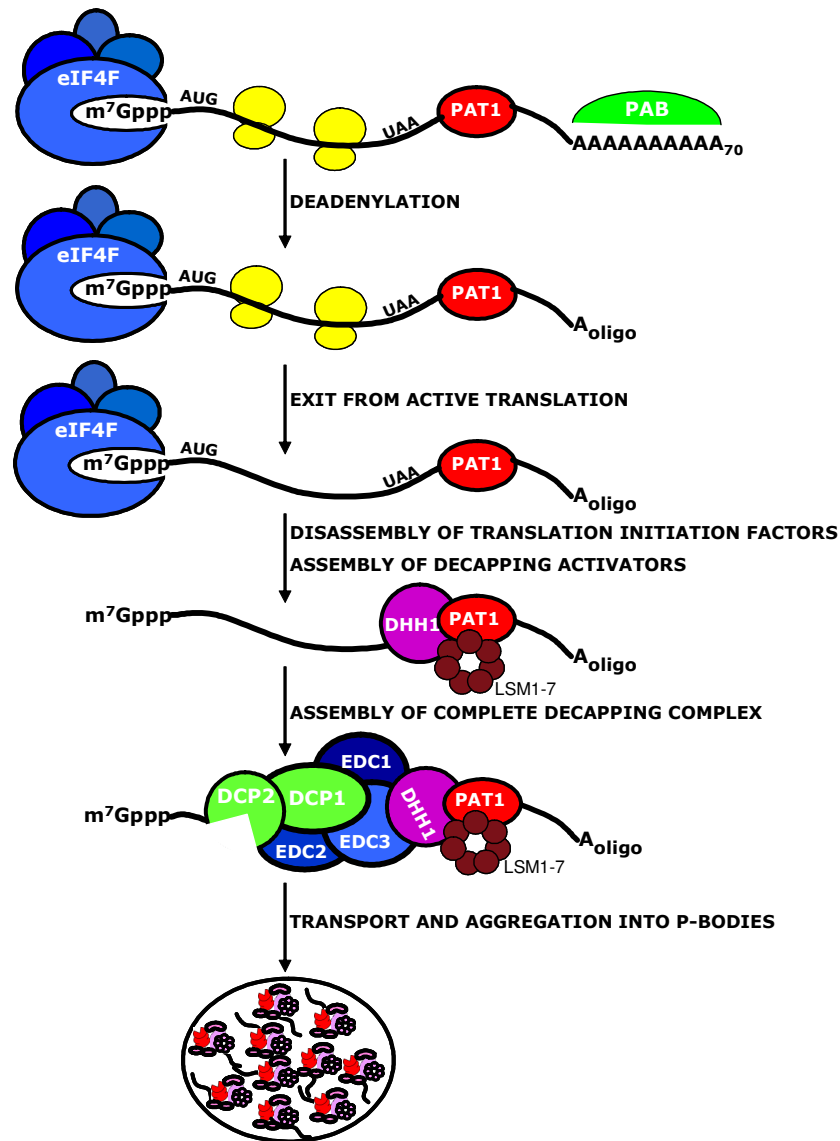


Figure I.8 Simplified model for the process of mRNA decapping.

I.4.3.2 Decapping Requires a Transition to a Non-translation Competent mRNP

The cap structure functions both as the site of assembly for the cytoplasmic cap-binding complex eIF4F and its associated proteins promoting translation initiation, and the site where mRNA decapping occurs. This suggests that decapping may be controlled by access of the Dcp1p/Dcp2p decapping enzyme to the cap structure in competition with the cytoplasmic cap-binding complex. Several observations support the competition between the cytoplasmic cap-binding complex and the decapping enzyme for access to the cap.

For example, any mutation affecting the cap initiation complex known to decrease translation rates also increased the rate of decapping (Schwartz and Parker 1999). In addition, co-expression of the cap binding protein eIF4E and its interacting domain from eIF4G increased mRNA stability, presumably by preventing accessibility of the cap to the decapping enzyme (Vilela et al. 2000). Because eIF4E subunit of eIF4F complex associates specifically with the cap structure, this factor is therefore expected to have a negative effect on the accessibility of the cap to Dcp1p/Dcp2p, and block decapping. Consistent with this, purified eIF4E was shown to inhibit decapping activity *in vitro* (Schwartz and Parker 2000; Vilela et al. 2000). Moreover, mutant forms of eIF4E that fail to bind the cap structure also fail to block decapping with purified decapping enzyme (Schwartz and Parker 2000; Ramirez et al. 2002). Finally, defects in eIF4E restored the decapping defect of a partial loss-of-function *dcp1-1* strain (Schwartz and Parker 2000). Thus, eIF4E is an inhibitor of decapping and dissociation of eIF4E, or eIF4F complex, from the cap structure seems to be a key step before decapping and mRNA turnover can occur. Notably, this provides an explanation for the protection of translating mRNAs from decapping. This also implies that a critical and priori step in mRNA turnover involves a reorganization of the mRNP structure that shifts the mRNA from a translationally active state to being committed for decapping and degradation. This important, but yet poorly understood, transition appears to occur after mRNA deadenylation and include displacement of Pab1p, disassembly of translation initiation factors from the mRNA cap structure, and association of the mRNA with a new set of proteins promoting decapping (Tharun and Parker 2001). Evidence for such mRNP transition has also come from coimmunoprecipitations experiments. Although the decapping activator Lsm1-7p complex coimmunoprecipitated with mRNA molecules and the Dcp1p/Dcp2p decapping enzyme, these mRNPs no longer contained eIF4E, eIF4G and Pab1p, which are generally found on translating mRNAs (Tharun and Parker 2001). Hence, the modulation of the stability of association of the cytoplasmic cap-binding complex with the mRNA cap structure and differences in transitions in mRNP states are likely to contribute to differences in decapping rates.

I.4.3.3 Factors Involved in Decapping

Although Dcp1p and Dcp2p are the only proteins required for the decapping catalytic step itself, decapping is a complex process requiring several protein factors for its proper orchestration and modulation (Table I.1).

Table I.1 Regulators of mRNA decapping.

Protein	Properties	Function	Significant interactions
Pab1p	Contains four N-terminal RRM domains and a proline-rich C-terminus	Major protein associated with poly(A) tail. Blocks mRNA decapping and stimulates translation. Primary coupler of deadenylation and decapping.	eIF4G, eRF3, Pan2/3p
eIF4E	Cap-binding protein	Component of the eukaryotic translational initiation complex, eIF4F. Blocks mRNA decapping by competing with Dcp1p/Dcp2p for access to the cap.	Lsm7p, eIF4G, eIF4A, eIF4B, Pab1p
Lsm1-7p	Sm-like proteins	Required for the efficiency of decapping <i>in vivo</i> . Forms a heteroheptameric ring complex and interacts with the mRNA 3' end after deadenylation. May facilitate the assembly of the decapping complex.	Dcp1p, Dcp2p, Dhh1p, Pat1p, Xrn1p, Upf1p
Pat1p	88 kDa protein with no recognizable sequence motifs	Interacts with both polyadenylated and deadenylated mRNA. Required for efficiency of the decapping and formation of P-bodies <i>in vivo</i> . May "seed" the decapping complex on the mRNA. Involved in translation repression.	Dcp1p, Dcp2p, Lsm1-7p, Dhh1p, Xrn1p, Crm1p
Dhh1p	Member of the ATP-dependent DEAD-box helicase family	Required for the efficiency of decapping <i>in vivo</i> . Involved in translational repression. Homologs across species are required for translational repression during mRNA storage events.	Dcp1p, Dcp2p, Lsm1-7p, Ccr4p, Pop2p, Caf17p, Pbp1p, Edc3p
Edc1p, Edc2p	Small, basic proteins with sequence similarity to each other.	Required for efficient decapping <i>in vitro</i> . Directly binds to the mRNA substrate. Recruits Dcp1p/Dcp2p complex.	Dcp1p, Dcp2p
Edc3p	Contains five conserved domains including a like-Sm, FDF and YjeF-N domains	A general and mRNA-specific regulator of decapping. Regulates the decapping of the <i>RPS28B</i> mRNA and <i>YRA</i> pre-mRNA.	Dcp1p, Dcp2p, Dhh1p, Crm1p, Rps28bp, Nup157p, Lsm8p
Puf proteins	Pumilio-like proteins, contains eight PUF repeats	Messages specific activators of mRNA deadenylation and decapping. Homologs facilitate translational repression.	Pop2p
Upf1p, Upf2p, Upf3p	Upf1p is an ATP-dependent RNA helicase	Required for nonsense-mediated decapping.	eRF1, eRF3, Dcp2p, Upf2p, Upf3, Lsm1p
eIF5A, Vps16p, Mrt4p, Sla2p, Grc5p, Ths1p, Sbp1p		Additional proteins suggested being involved in mRNA turnover but functions, in most cases, remain unclear.	

One class of proteins includes those that inhibit the decapping process. The Pab1p is an important member of this group because it is required to couple decapping to prior deadenylation (Caponigro and Parker 1995). Another example of members that also impede decapping are components of the translation initiation complex. For example, eIF4E is a negative effector of decapping by affecting the accessibility of the cap to the decapping enzyme (Schwartz and Parker 2000).

In addition, several factors required for efficient decapping of most normal mRNAs have been identified, including seven Lsm proteins, Lsm1-7p, which form an RNA-binding complex, Pat1p/Mtr1p, which is of unknown biochemical properties, and Dhh1p, which is a member of the DEAD-box protein family (Hatfield et al. 1996; Boeck et al. 1998; Bonnerot et al. 2000; Bouveret et al. 2000; Tharun et al. 2000; Collier et al. 2001; Fischer and Weis 2002). Indeed, deletion of *LSM1*, *PAT1* or *DHH1* genes affects mRNA degradation primarily by inhibiting decapping of regular mRNAs. However, inactivation of these proteins led to a partial inhibition, not to absolute block of decapping, indicating that they are not a required part of the decapping enzyme, but instead serve as general activators of decapping. Notably, they appear to act at distinct stages of the decapping process. Pat1p and Dhh1p seem to associate with the mRNA early, perhaps while it is still translationally competent to initiate the rearrangements in mRNP organization that leads to decapping and degradation of mRNAs. Consistently, Pat1p can be coimmunoprecipitated with translation factors, eIF4E and eIF4G, and Pab1p in a RNA-dependent manner (Tharun and Parker 2001). In addition, both Pat1p and Dhh1p show links to translation (Wyers et al. 2000; Collier et al. 2001). Moreover, Pat1p and Dhh1p play a role in the translation repression of mRNAs, favoring the exit of an mRNA from a translating to a nontranslating state (Collier and Parker 2005). Significantly, the Dhh1p helicase activity may be important in promoting these conformational changes in the mRNA or mRNP structure. Pat1p, and possibly Dhh1p, seem to play an additional role activating decapping in addition to function as translation repressors, potentially by enhancing the binding of the Dcp1p/Dcp2p enzyme to the mRNA (Collier et al. 2001; Tharun and Parker 2001; Fischer and Weis 2002; Collier and Parker 2005). This indicates that Pat1p and Dhh1p are critical proteins regulating and connecting mRNA translation and degradation. In addition, Dhh1p by interacting with both the decapping enzyme and the deadenylase complex may have a role in coordinating the distinct, but functionally connected deadenylation and decapping steps (Hata et al. 1998; Collier et al. 2001). Although, Pat1p and the Lsm1-7p complex form a complex (Bouveret et al. 2000; Tharun et al. 2000), the Lsm1-7p complex seems to preferentially associate with an mRNP that is targeted for decay but not with mRNPs translationally competent. Consistently, Lsm1-7p can be coimmunoprecipitated with mRNAs that lack translation initiation factors and Pab1p. In addition, the Lsm1-7p complex has been shown to preferentially associate with deadenylated mRNAs (Tharun and Parker 2001). Moreover, Lsm1-7p complex seems to have the intrinsic ability to distinguish between adenylated and nonadenylated mRNAs (Chowdhury et al. 2007). The Lsm1-7p complex function to promote decapping seems to be mediated by its ability to specifically interact with, or near, the 3' end of mRNAs following deadenylation, thereby enhancing the interaction of the decapping enzyme with

the transcript (Tharun and Parker 2001; Tharun et al. 2005; Chowdhury et al. 2007). It is possible that the loss of Pab1p from the mRNA after deadenylation may make the 3' end available for association with the Lsm1-7p complex. In addition to decapping, the direct binding of the Lsm1-7p/Pat1p complex to the 3' end of the oligoadenylated mRNAs seem to have a role in protecting the 3' ends of these mRNAs from a trimming reaction by 10-20 nt (He and Parker 2001; Tharun et al. 2005).

A related class of proteins referred to as enhancer of decapping proteins include the Edc1p, Edc2p, and Edc3p. These proteins are not rate-limiting for mRNA decapping, because *edc1Δ*, *edc2Δ* or *edc3Δ* mutant strains do not affect general decapping rates of mRNAs, unless decapping activity is already compromised (Dunckley et al. 2001; Schwartz et al. 2003; Kshirsagar and Parker 2004). Edc proteins appear to stimulate decapping by binding to both the mRNA substrate and Dcp1p/Dcp2p enzyme, thereby directly enhancing the decapping activity of the enzyme. Consistent with this, Edc proteins associate with the Dcp1p/Dcp2p complex (Dunckley et al. 2001; Gavin et al. 2002). In addition, both Edc1p and Edc2p can bind RNA and are able to stimulate decapping activity *in vitro* either in yeast extracts or with purified enzyme (Schwartz et al. 2003; Steiger et al. 2003). Moreover, overexpression of either Edc1p or Edc2p suppresses loss-of-function mutations in the decapping enzyme (Dunckley et al. 2001). The fact that Edc proteins directly stimulate the decapping enzyme but are not required for decapping suggested that they may function to modulate decapping of specific mRNAs. Indeed, Edc3p specifically affects the decapping rate of the *RPS28B* mRNA and *YRA1* pre-mRNA (Badis et al. 2004; Dong et al. 2007).

Other proteins are specifically required for the decapping of subsets of mRNAs. For example, specific mRNA-binding proteins, such as PUF protein family members, can control the rate of decapping of individual mRNAs (Olivas and Parker 2000). In addition, Upf1p, Upf2p and Upf3p are specifically required for the recognition and rapid decapping of nonsense mRNAs (Maquat 2004b). One important issue for understanding the mechanism of decapping will be to clarify the roles of proteins that activate decapping in specific sub-steps of the process. Notably, this may also provide insight into the mechanism of translation repression.

I.4.3.4 Assembly of Decapping Complexes

Decapping of mRNAs involves the assembly of distinct complexes of proteins on the mRNA that may function, in part, to enhance the interaction of the decapping enzyme with

the transcript, and reflect the variety of turnover pathways or steps along a single pathway. In yeast, the majority of mRNAs seem to assemble a decapping complex consisting of the Pat1p, Dhh1p, Lsm1-7p complex, and Dcp1p/Dcp2p (Bellman et al. 1996; Hatfield et al. 1996; Boeck et al. 1998; Bouveret et al. 2000; Tharun et al. 2000; Coller et al. 2001; Fischer and Weis 2002). In contrast, Upf1p, through its interaction with Dcp2p, is suggested to recruit the decapping enzyme to nonsense mRNAs in the NMD pathway (He and Jacobson 1995). Another specialized decapping complex seems to occur on the *RPS28B* mRNA, where in an autoregulatory process, the Rps28p binds a stem-loop structure in the 3' UTR of its own mRNA and then interacts with Edc3p, which appears to recruit Dcp1p/Dcp2p to the complex for rapid deadenylation-independent decapping of the mRNA (Badis et al. 2004). Similarly, Edc3p also seems to play a role in the autoregulatory degradation of *YRA1* pre-mRNA in the cytoplasm, recruiting the Dcp1p/Dcp2p decapping enzyme, which then leads to 5' to 3' degradation by Xrn1p (Dong et al. 2007). Thus, specific set of physical interactions can recruit the decapping enzyme to the specific mRNAs. One should expect to see more such complexes identified as work in this area progresses, providing the opportunity for differential regulation of decapping on individual mRNAs. For example, the Edc1p and Edc2p may also nucleate mRNA decapping complexes on specific mRNAs yet to be identified. Finally, the *EDC1* mRNA undergoes decapping independent of prior deadenylation although it requires some subunits of the Ccr4p/Pop2/Not complex (Muhlrad and Parker 2005). That *EDC1* mRNA encodes an enhancer of decapping, Edc1p, and its decay is dependent on decapping suggests a possible feedback regulation mechanism.

1.4.3.5 Processing Bodies- Sites of mRNA Decapping and Decay

Strikingly, the proteins involved in mRNA decapping and 5' to 3' exonucleolytic decay are enriched in discrete cytoplasmic foci, termed processing bodies (P-bodies). In yeast, the decapping proteins Dcp1p and Dcp2p, the mRNA degrading factors Lsm proteins, Pat1p, Dhh1p and Edc3p, and the 5' to 3' exonuclease Xrn1p are co-localized in cytoplasmic P-bodies (Sheth and Parker 2003; Kshirsagar and Parker 2004). In contrast, Ccr4p subunit of the yeast deadenylase is more uniformly distributed, and localization within cytoplasmic P-bodies was uncertain (Sheth and Parker 2003). In addition to the concentration of decapping factors and the 5' to 3' exonuclease in P-bodies, several observations suggest that P-bodies are specific sites of mRNA decapping and 5' to 3' degradation. First, the size and the number of P-bodies vary in a manner that correlates with the flux of mRNA molecules through the decapping step. For example, inhibiting mRNA decay at the deadenylation step in a *ccr4Δ* strain leads to a reduction in P-body size and number

(Sheth and Parker 2003). Similarly, inhibiting decapping at an upstream step in a *pat1Δ* strain or by adding cycloheximide leads to a reduction or loss of P-bodies (Beelman and Parker 1994; Sheth and Parker 2003). In contrast, blocking the catalytic steps of decapping in a *dcp1Δ* or 5' to 3' digestion in a *xrn1Δ* strain lead to increased number and size of P-bodies (Sheth and Parker 2003). Moreover, mRNA decay intermediates that accumulate either by the insertion of strong secondary structures that impairs exonucleolytic degradation or by *xrn1Δ*, can be specifically localized to P-bodies (Sheth and Parker 2003). These observations indicate that P-bodies are actual sites where mRNA decapping and 5' to 3' exonucleolytic decay can occur. However, because these mRNA decay factors are also found distributed throughout the cytoplasm, decapping and degradation may also occur outside of P-bodies in submicroscopic complexes. Interestingly, the exosome component, Ski7p does not appear to be localized to P-bodies (Sheth and Parker 2003). This suggests that the 3' to 5' pathway of mRNA degradation may occur at a different subcellular site to the 5' to 3' decay pathway. Hence, these observations suggest that localization of decay factors and mRNAs within P-bodies may have a role in the regulation of decapping and mRNA turnover. Interestingly, the observation that cycloheximide treatment, a translation elongation inhibitor, leads to the dissolution of P-bodies, together with the requirement of a specific mRNP transition where the mRNAs exit translation before decapping, suggests that the pool of mRNAs that have exited translation could be segregated within P-bodies (Tharun and Parker 2001; Sheth and Parker 2003; Kshirsagar and Parker 2004).

Importantly, similar cytoplasmic P-bodies (also referred to as DCP-bodies or GW-bodies), have also been characterized in humans, nematodes, flies and plants, and they also contain enzymes and proteins in the decapping decay pathway (Bashkurov et al. 1997; Ingelfinger et al. 2002; Lykke-Andersen 2002; van Dijk et al. 2002; Eystathioy et al. 2003; Cougot et al. 2004; Temme et al. 2004; Andrei et al. 2005; Fenger-Gron et al. 2005; Ferraiuolo et al. 2005; Yu et al. 2005). It should be noted that the conservation of these structures from yeast to mammals supports their biological significance.

The discovery that mRNA turnover can take place in cytoplasmic P-bodies raises a number of interesting and important questions. For example, what mechanisms are responsible for targeting specific mRNAs and decay factors to P-bodies? What controls their movement to P-bodies? How do P-bodies assemble? What is the composition and organization of P-bodies? What is the relationship between P-bodies and other cellular processes including mRNA biogenesis and translation, or other subcellular RNA granules? Is P-body function limited only to mRNA decapping and exonucleolytic decay or might they have additional functions? Understanding the structure and organization of P-bodies, how they form and their function may prove to be essential for understanding

control of mRNA turnover and lead to significant advances in the field. In addition, given the reciprocal relationship of translation and mRNA degradation, understanding P-bodies is also likely to yield novel insights for understanding mRNA translation and control.

CHAPTER II

P-BODIES REQUIRE RNA FOR ASSEMBLY AND CONTAIN NONTRANSLATING mRNAs

II. P-BODIES REQUIRE RNA FOR ASSEMBLY AND CONTAIN NONTRANSLATING mRNAs

Abstract

Recent experiments have defined cytoplasmic foci, referred to as processing bodies (P-bodies), wherein mRNA decay factors are concentrated and where mRNA decay can occur. However, the physical nature of P-bodies, their relationship to translation, and possible roles of P-bodies in cellular responses remain unclear. We describe four properties of yeast P-bodies that indicate that P-bodies are dynamic structures that contain nontranslating mRNAs and function during cellular responses to stress. First, *in vivo* and *in vitro* analysis indicates that P-bodies are dependent on RNA for their formation. Second, the number and size of P-bodies vary in response to glucose deprivation, osmotic stress, and the stage of cell growth. Third, P-bodies vary with the status of the cellular translation machinery. Inhibition of translation initiation by mutations, or cellular stress, results in increased P-bodies. In contrast, inhibition of translation elongation, thereby trapping the mRNA in polysomes, leads to dissociation of P-bodies. Fourth, multiple translation factors and ribosomal proteins are lacking from P-bodies. These results suggest additional biological roles of P-bodies in addition to being sites of mRNA degradation.

II.1 Introduction

The regulation of mRNA turnover plays a significant role in controlling gene expression. In the last decade, specific pathways of mRNA turnover have been defined that can be controlled to modulate mRNA decay rates. In yeast, the major pathway of mRNA turnover is initiated with the shortening of the 3' poly(A) tail, in a process referred to as deadenylation (Coller and Parker 2004). Shortening of the poly(A) tail leads to a deadenylated transcript that is primarily a substrate for removal of the 5' cap structure by the Dcp1p/Dcp2p decapping complex, thereby exposing the transcript to degradation by the 5' to 3' exonuclease, Xrn1p (Decker and Parker 1993; Hsu and Stevens 1993; Muhlrads et al. 1994). Alternatively, transcripts can be degraded in a 3' to 5' direction following deadenylation (Muhlrads et al. 1995; Mitchell et al. 1997; Anderson and Parker 1998). 3' to 5' degradation is catalyzed by the exosome, a conserved multiprotein complex (van Hoof and Parker 1999). Experiments in mammalian cells, and the conservation of the mRNA decay enzymes and factors, suggest that the major pathways are shared between yeast and mammals (Parker and Song 2004).

Decapping is a critical step in mRNA degradation because it both precedes and permits the degradation of the mRNA body. Several observations support the argument that the processes of mRNA decapping and translation are inversely related. For example, decreasing translation initiation either *in cis* or *in trans* promotes faster decapping. (Muhlrads et al. 1995; LaGrande and Parker 1999; Schwartz and Parker 1999; Ramirez et al. 2002). Moreover, the cap-binding protein, which promotes translation initiation, is an effective inhibitor of decapping *in vitro* (Schwartz and Parker 2000; Ramirez et al. 2002; Khanna and Kiledjian 2004). Conversely, inhibition of translation elongation either by mutations or by treatment with elongation inhibitors (e.g., cycloheximide) leads to a decrease in the rate of decapping (Peltz et al. 1992; Beelman and Parker 1994). Similar experiments suggest translation and mRNA degradation are also related in mammalian cells (Muckenthaler et al. 1997). Together, these observations indicate that mRNA translation and mRNA decapping are in competition.

Insight into the competition between translation and decapping has come from recent experiments that identified discrete cytoplasmic foci in yeast and mammals, referred to as P-bodies. P-bodies include the Dcp1p/Dcp2p decapping enzyme, activators of decapping, Dhh1p (referred to as RCK/p54 in mammals), Pat1p, Lsm1–7p, Edc3p, and the 5' to 3' exonuclease Xrn1p (Bashkurov et al. 1997; Ingelfinger et al. 2002; Lykke-Andersen 2002; van Dijk et al. 2002; Sheth and Parker 2003; Cougot et al. 2004; Kshirsagar and Parker

2004). P-bodies are suggested to be sites where decapping and degradation of mRNAs can occur. P-bodies size is proportional to the flux of mRNAs undergoing the decapping step in turnover, and mRNA decay intermediates trapped in the process of degradation are also localized to these structures in yeast (Sheth and Parker 2003). Similar observations were made in human cells, indicating that these cytoplasmic structures also represent sites of mRNA decay in more complex eukaryotes (Cougot et al. 2004). The conservation of P-bodies from yeast to mammals suggests that they play important roles in the cytoplasmic function of eukaryotic mRNAs including modulating their degradation.

The identification of P-bodies suggested a model wherein these structures represent pools of mRNAs that have exited translation and are destined for degradation (Sheth and Parker 2003). However, it is not known whether P-bodies are dependent on RNA for assembly or can form in an RNA-independent manner. Similarly, the hypothesized reciprocal relationship of P-bodies and a translating pool of mRNA is largely based on the loss of Dhh1p and Edc3p localization to P-bodies upon cycloheximide treatment (Sheth and Parker 2003; Kshirsagar and Parker 2004). In this work we have addressed the role of RNA in P-body formation, the relationship of P-bodies to the translating pool of mRNA, and the variation in P-body size and number under a variety of conditions. The results suggest that P-bodies are dynamic and are affected by a range of cellular perturbations including glucose deprivation, osmotic stress, and the stage of cell growth. Moreover, our observations provide evidence that P-bodies have a reciprocal relationship with translation and represent pools of nontranslating mRNPs. These results suggest a dramatic transition of mRNAs involving significant rearrangement of the mRNP for movement of mRNAs from the translating pool of mRNAs to P-bodies.

II.2 Material and Methods

II.2.1 Yeast Strains and Growth Conditions

The genotypes of all strains used in this study are listed in Table II.1. All strains have *GAL1* upstream activating sequence-regulated *PGK1pG* and *MFA2pG* genes, as well as the *LEU2* gene, collectively termed *LEU2pm*, integrated at the *CUP1* locus unless stated otherwise (Hatfield et al. 1996). Proteins were C-terminal tagged with green fluorescent protein (GFP) following the PCR-based gene modification method described by Longtine et al. (1998). These fusion proteins include the full-length protein and are functional (Sheth and Parker 2003). Yeast crosses were carried out using standard laboratory

procedures. Strains were grown on either standard yeast extract/peptone medium (YP) or synthetic medium (SC) yeast nitrogen base/ammonium sulphate supplemented with appropriate amino acids, and 2% dextrose (Glu) or 2% galactose (Gal) or 3% glycerol (Gly) as carbon source, as indicated. Strains were grown at 30°C except for temperature-sensitive (ts) mutants, which were grown at 23°C.

Table II.1 Yeast strains.

Strain	Genotype	Source
yRP684	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 lys2-201</i>	Hatfield et al. 1996
yRP840	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG</i>	Hatfield et al. 1996
yRP1724	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DHH1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1726	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DCP1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1727	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DCP2-GFP (NEO)</i>	Sheth and Parker 2003
yRP1728	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG PAT1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1729	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG LSM1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1730	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG XRN1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1738	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA DHH1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1824	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 DHH1-GFP (NEO)</i>	This study
yRP1825	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 DCP2-GFP (NEO)</i>	This study
yRP1826	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 prt1-63 cup1::LEU2/PGK1pG/MFA2pG DHH1-GFP (NEO)</i>	This study
yRP1827	<i>MATα leu2-3,112 ura3-52 prt1-63 cup1::LEU2/PGK1pG/MFA2pG DCP2-GFP (NEO)</i>	This study
yRP1828	<i>MATα leu2 ura3 his3 met15 PRT1-GFP (HIS)</i>	Huh et al. 2003
yRP1829	<i>MATα leu2 ura3 his3 met15 TEF4-GFP (HIS)</i>	Huh et al. 2003
yRP1830	<i>MATα leu2 ura3 his3 met15 SUP45-GFP (HIS)</i>	Huh et al. 2003
yRP1911	<i>MATα leu2 ura3 his3 met15 RPL5-GFP (HIS)</i>	Huh et al. 2003
yRP1912	<i>MATα leu2-3,112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG hog1::NEO DHH1-GFP (NEO)</i>	This study
yRP1913	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG hog1::NEO DCP2-GFP (NEO)</i>	This study
yRP1921	<i>MATα leu2-3,112 trp1 his4-539 met1 cyh2 DHH1-GFP (NEO)</i>	This study
yRP1922	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cyh2 DCP2-GFP (NEO)</i>	This study
yRP1923	<i>MATα leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA DCP2-GFP (NEO)</i>	This study
yRP1924	<i>MATα leu2-3,112 trp1 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA LSM1-GFP (NEO)</i>	This study

II.2.2 Plasmids

Plasmid MFA2pG, pRP1081, expressing the *MFA2* mRNA with a poly(G) tract, or MFA2, pRP1083, expressing *MFA2* mRNA with no functional poly(G) tract, were constructed as described in Sheth and Parker (2003). The plasmid DCP2-RFP, pRP1186, was created by PCR-amplification of the *DCP2* gene from yRP840, and the fragment was ligated into the yeast expression vector pRP1084 between the *Sall* and *BamHI* sites (Sheth and Parker 2003) and confirmed by sequencing. For *PGK1* mRNA localization, the plasmid U1A-GFP,

pRP1187, was created by replacement of the *GAL* promoter, in plasmid pPS2042 (Brodsky and Silver 2000), with the *GPD* promoter in between *Sall* sites. The plasmid PGK1-U1A expressing *PGK1* mRNA with U1A-binding sites (pPS2037) was created as described in Brodsky and Silver (2000). The CEN LEU plasmid, pRP1155, expressing DCP2-RFP derives from pRP1186 (Sheth and Parker 2006). Yeast strains were transformed by standard techniques, and plasmids were maintained by growth in selective media.

II.2.3 Preparation of Cells for Confocal Microscopy

The cells were grown to an optical density (OD_{600}) of 0.3–0.35 in the appropriate medium. Cells were washed two times with SC plus amino acids supplemented with the same carbon source used for cell growth. Cells were resuspended in SC containing the appropriate carbon source and amino acids and immediately observed, unless otherwise indicated. Observations were made using a Nikon PCM 2000 Confocal Microscope using a 100x objective with 3x zoom with Compix Software. All images are a Z-series compilation of 6–10 images in a stack, except for colocalization analysis, where images result from a single Z-section.

II.2.4 Preparation of Semipurified P-bodies

For purification of P-bodies, 250 ml of cells was grown to an OD_{600} of 3, harvested by centrifugation, and washed in cold lysis buffer (50 mM Tris at pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 0.1% NP-40, 1 mM β -mercaptoethanol, 1x protease inhibitor complete mini EDTA free [Roche, #1836170], 0.4 U/ μ l RNase inhibitor). All subsequent steps were performed at 4°C. Cells were lysed by vortexing in the presence of cold lysis buffer and an equal volume of glass beads. Lysates were clarified briefly at 2000g for 2 minutes. The supernatant was centrifuged at 10,000g for 10 minutes, and a P-body pellet was formed. Most of the supernatant was removed, and the pellet was resuspended in lysis buffer without RNase inhibitor. The resuspended pellet was then split into two equal portions. One portion was treated with RNase A to a concentration of 1 μ g/ μ l for 30 minutes at room temperature. The other portion was incubated under the same conditions with cold lysis buffer. After incubation, both RNase A treated and untreated fractions were centrifuged at 10,000g for 10 minutes, producing the final supernatant and pellet. For microscopic analysis, 10 μ l of the P-body rich pellet was visualized directly with the confocal microscope. For Western blot analysis, equal volumes of the pellet and supernatant fractions for control and sample were used. Western blot analysis of the GFP-

tagged proteins was carried out following standard protocols using GFP primary antibody (Covance) at a 1:1000 dilution and goat anti-mouse HRP secondary antibody (Sigma-Aldrich) at a 1:5000 dilution.

II.3 Results

II.3.1 P-bodies Vary Under Different Cellular Conditions

Initial studies of P-bodies suggested that there could be variation in the size and number of P-bodies depending on the experimental conditions (Sheth and Parker 2003). Thus, we examined how a variety of different growth and experimental conditions impact the number and size of yeast P-bodies using GFP-tagged fusions of known P-body components. We primarily examined two proteins as P-body markers. First, we examined the distribution of Dcp2p, which is a subunit of the decapping enzyme and is easily observed in P-bodies under all conditions. Second, we also examined the distribution of Dhh1p, which is an activator of the decapping reaction that is weakly observed in P-bodies and generally more diffuse in the cytoplasm than Dcp2p. All pictures presented are collapsed stacks of a Z-series through yeast cells, unless stated otherwise. In addition, all observations described are seen in >80% of the cells examined. The different conditions we examined and the results are discussed below.

II.3.1.1 Stage of Cellular Growth Affects P-body Formation

We examined how P-bodies vary during the stage of growth of a yeast culture, when provided glucose as a carbon source. Yeast cells in culture display a characteristic growth pattern in which a phase of logarithmic (log) growth is followed by a diauxic shift, a slow growth phase, and then by stationary phase. We observed that the size and number of P-bodies varied with the stage of growth. Furthermore, different P-body components have different dynamics in their association with P-bodies. Specifically, in cells in the early-log phase of growth (OD_{600} 0.3), Dcp2p is observed in multiple small P-bodies in the cell (Figure II.1A). In contrast, at this stage of growth, Dhh1p is generally diffuse, although some cytoplasmic foci can be observed (Figure II.1G). Moreover, examination of Dhh1p in a single Z-section from a series of confocal images reveals a clear concentration in P-bodies that is lost when a Z-series is collapsed (data not shown). The P-bodies seen in these experiments for Dcp2p and Dhh1p are smaller than has previously been described for yeast in mid-log growth (Sheth and Parker 2003). This difference is reconciled by the

knowledge that the cells in the earlier publication were undergoing a partial stress response.

As cell density increased and cells underwent diauxic shift, we observed an increase in brightness and number of P-bodies in cells, with clear and dramatic concentrations of Dcp2p and Dhh1p in foci (Figure II.1B–E, H–L). As cells reached even higher densities, P-bodies continued to increase in size and the number per cell decreased (Figure II.1F, M). All known components of P-bodies examined (Pat1p, Lsm1p, Dcp1p, Xrn1p, Edc3p) show these changes in P-bodies with increasing cell density (data not shown). Thus, P-bodies increase in size and decrease in number with increasing cell density.

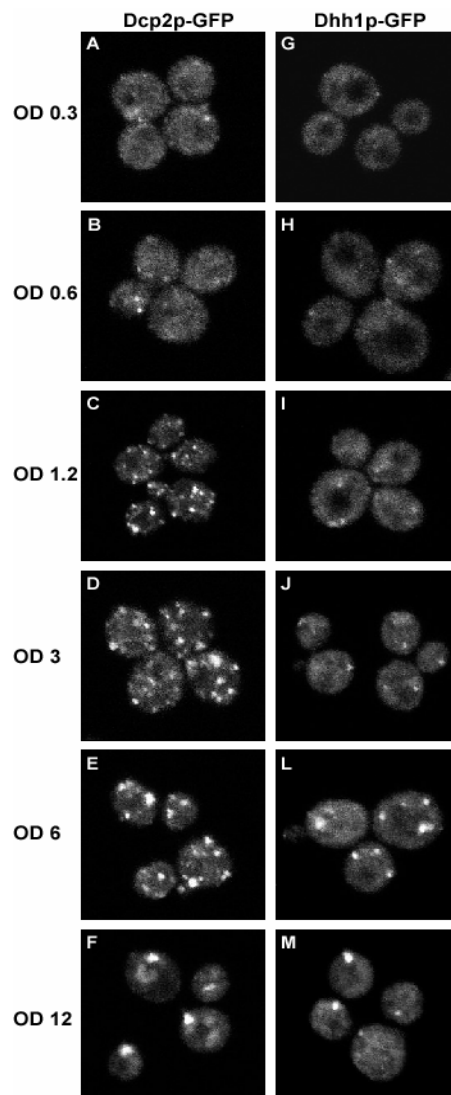


Figure II.1 P-bodies increase in the late stages of cell growth. Cells were grown in YPGlu and observed at different stages of cellular growth, as described. The different OD₆₀₀ units (OD) are indicated at the left side of the figure. Cells expressing a GFP-tagged version of Dcp2p (left panel) and Dhh1p (right panel) are shown.

The changes in P-body size roughly parallel changes in the use of different carbon sources during cell growth. However, P-bodies do not differ significantly when early-log cells are grown in alternative carbon sources. We observed that P-bodies generally appear the same with growth in glucose (Figure II.2A, B), galactose (Figure II.2C, D) and glycerol (Figure II.2E, F). We interpret these results to indicate that changes in cellular metabolism at different cell densities affect the flux of P-body components in and out of P-bodies.

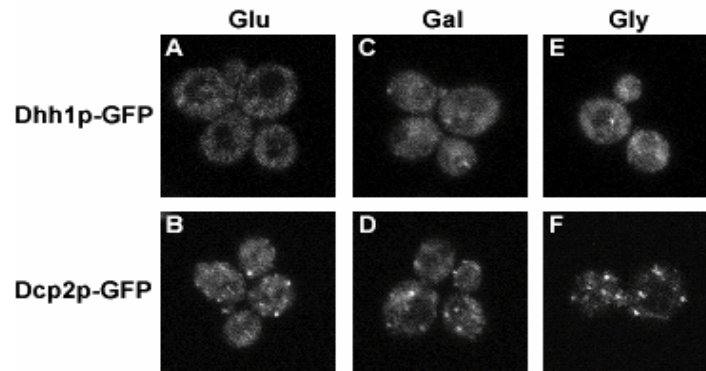


Figure II.2 P-bodies are not affected by carbon source. Cells were grown in YPGlu (A,B), Gal (C,D) or Gly (E,F) and observed as described. Cells expressing GFP-tagged version of Dhh1p (top panel) and Dcp2p (bottom panel) are shown.

II.3.1.2 P-bodies Increase During Glucose Deprivation

In order to examine how P-bodies respond to stress, we first examined how P-bodies changed with glucose depletion. Glucose is the preferred energy source for most eukaryotic cells, and yeast cells respond rapidly to glucose deprivation with changes in a wide variety of cellular processes (Rolland et al. 2002). We observed that when yeast cells were grown in rich medium containing glucose and shifted for 10 minutes to medium lacking glucose, P-bodies increased in brightness and number for both Dcp2p and Dhh1p (Figure II.3A, III, IV). This increase was not seen in cells shifted into fresh media containing glucose (data not shown). Furthermore, we observed that Dhh1p foci colocalize with Dcp2p foci (Figure II.3B, III). Under these conditions, we observed the same increase in the concentration within P-bodies for Lsm1p, Pat1p, Edc3p, Dcp1p, and Xrn1p (data not shown). Interestingly, this increase in P-bodies inversely correlates with the rapid loss of translation induced by glucose deprivation (Ashe et al. 2000; Uesono et al. 2004), and suggests that inhibition of translation initiation leads to an increase in P-body formation.

Consistent with the previous observation, we observe that treatment of cells with 2-Deoxy-D-Glucose, an inhibitor of glycolysis, results in an increase in P-bodies for both Dcp2p and Dhh1p after 30 minutes incubation (Figure II.4C, D).

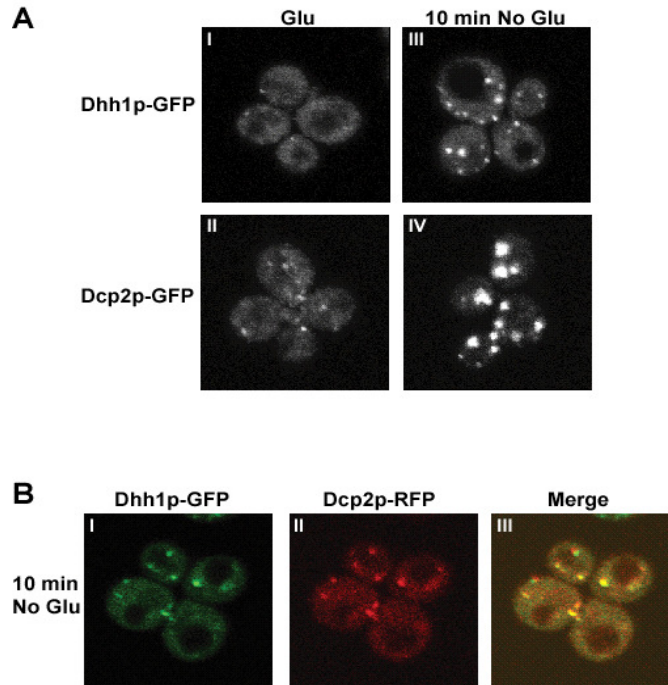


Figure II.3 P-bodies size increases under glucose deprivation. (A) Cells were grown in YPGlu, washed in YP, and resuspended in YP for 10 minutes before being collected. For observation, cells were washed with SC with (I,II) or without (III,IV) Glu, resuspended in the same SC, respectively, and observed. Cells expressing a GFP-tagged version of Dhh1p (top panel) and Dcp2p (bottom panel) are shown. (B) Cells were grown in SC plus Glu, washed, and resuspended in SC for 10 minutes before being collected. For observation, cells were washed and resuspended in SC without Glu. Cells coexpressing a GFP-tagged version of Dhh1p (I) and a RFP-tagged version of Dcp2p (II) are shown. A merged view is shown (III).

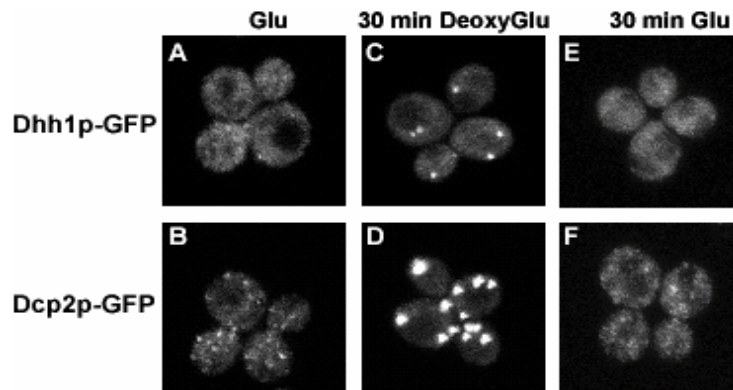
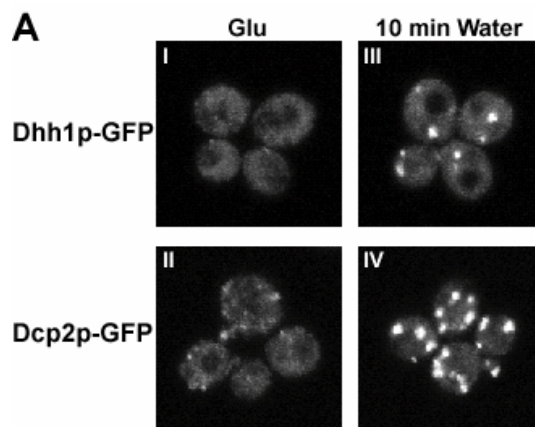


Figure II.4 P-bodies increase under energy depletion conditions. Strains containing GFP-tagged version of Dhh1p (top panel) and Dcp2p (bottom panel) were grown in YPGlu (A,B), washed in YP and divided in two samples. One sample was resuspended in YP plus 2% 2-Deoxyglucose (DeoxyGlu). After 30 minutes treatment with 2-DeoxyGlu cells were washed with SC plus 2-DeoxyGlu, resuspended in the same medium, and observed (C,D). The other sample was resuspended in YPGlu for 30 minutes, cells were then washed and observed as described (E,F).

II.3.1.3 P-bodies Increase Under Osmotic Stress

We next examined the status of P-bodies in cells subject to osmotic stress. In yeast, changes in the extracellular osmolarity affect different signaling pathways (de Nadal et al. 2002), which can lead to a transient inhibition of protein synthesis (Uesono and Toh-e 2002). We first examined how early-log cells grown in glucose respond to hypotonic stress when exposed to water. We observed that induction of hypotonic shock by washing and incubating cells in either water or water supplemented with glucose for 10 minutes led to larger and more numerous P-bodies (Figure II.5A, III, IV; data not shown). In contrast, when cells were incubated in rich media and washed in synthetic media containing glucose, no changes in P-bodies were observed (data not shown). Similarly, P-bodies were examined under hyperosmotic stress conditions. After exposure of cells in the early-log stage of growth to either 1 M KCl (Figure II.5B, V, VII) or 0.8 M NaCl (data not shown) for 15 minutes, we observed a dramatic increase in P-body size and number, whether Dcp2p or Dhh1p was examined. Moreover, the increase in P-bodies in response to 1 M KCl was significantly reduced in a strain lacking Hog1p (Figure II.5B, VI, VIII), which is a critical kinase in the cellular response to osmotic stress (Uesono and Toh-e 2002). Consistent with a relationship between translation and P-body formation (see below), and the substantial but not complete reduction in P-body formation in *hog1Δ* cells, we observed that cells lacking Hog1p also show a partial repression of translation in response to osmotic stress as judged by polysome profiles (data not shown). This suggests that the increase in P-bodies is influenced by the same signal transduction pathway that leads to other changes in cellular metabolism in response to osmotic stress. It should be noted that over time in WT cells, P-bodies returned to the status similar to that observed before the shift in osmolarity (Figure II.5B, IX, X). This transient nature suggests P-bodies may function in an aspect of the stress response.



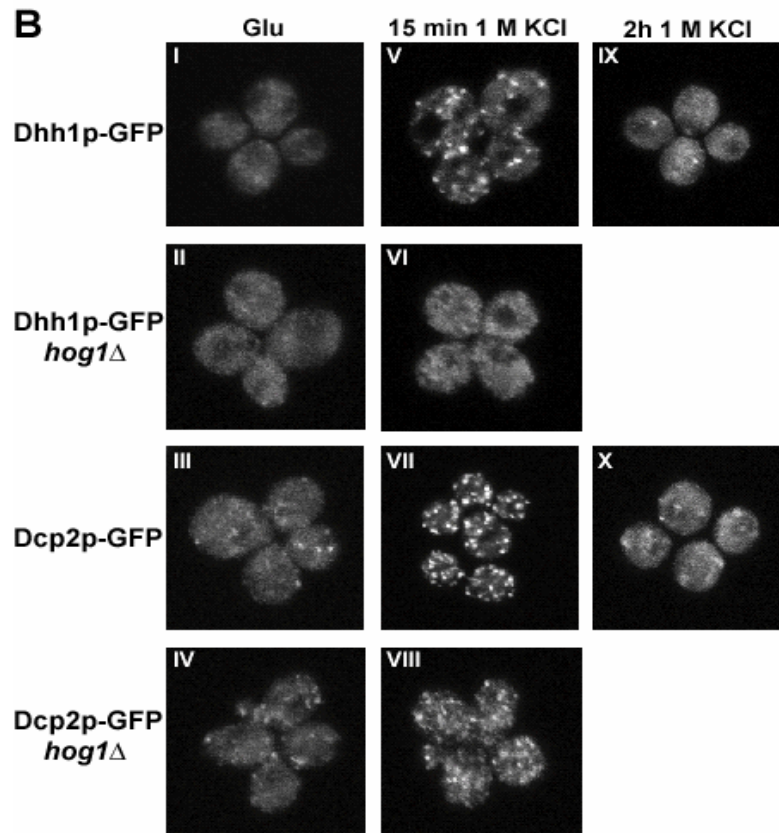


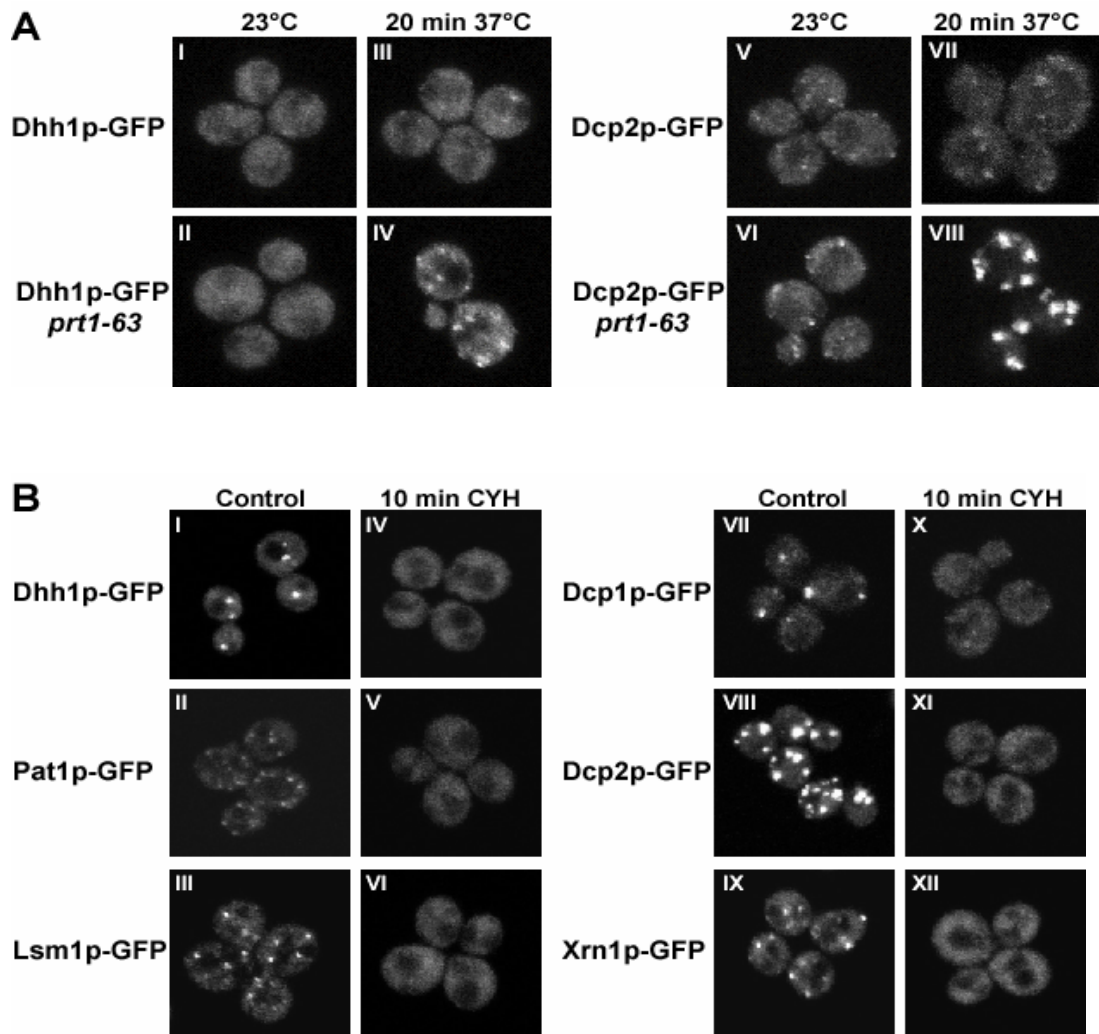
Figure II.5 P-bodies are increased by osmotic stress. (A) Cells were grown in YPGlu, washed, and resuspended in water for 10 minutes before collection. For observation, cells were washed and resuspended in water (III,IV). Cells expressing a GFP-tagged version of Dhh1p (top panel) and Dcp2p (bottom panel) are shown. **(B)** Cells expressing a GFP-tagged version of Dhh1p in a WT (I,V,IX) or *hog1* Δ strain (II,VI), and Dcp2p in a WT (III,VII,X) or *hog1* Δ strain (IV,VIII) after exposure to 1 M KCl for 15 minutes (V–VIII) or 1 M KCl for 2 hours (IX,X) are shown. Cells were washed and resuspended in SC plus Glu supplemented (V–X) or not (I–IV) with 1 M KCl, and observed.

II.3.2 P-bodies and Translation Show a Reciprocal Relationship

The above results indicate that the number and size of P-bodies in the cell increase in response to several cellular stresses and growth limitations. Because many of these conditions affect translation, a likely hypothesis is that mRNAs undergoing translation are excluded from P-bodies, while nontranslating mRNAs can assemble into P-bodies. In order to test this hypothesis, we examined the size and number of P-bodies when translation is directly altered.

II.3.2.1 P-bodies are Affected by Translation Initiation Rates

In order to analyze the effect of defects in translation initiation, we examined P-bodies using a strain carrying a conditional *ts* allele (*prt1-63*) of the translation initiation factor Prt1p, which is a subunit of the eIF3 complex. In the *prt1-63* strain, translation is inhibited at the nonpermissive temperature of 37°C (Evans et al. 1995). We observed that following 20 minutes at 37°C, P-bodies increased in size in the *prt1-63* strain (Figure II.6A, IV, VIII). Similarly, when we shifted for 20 minutes to 37°C a strain carrying a conditional *ts* allele of the translation initiation factor eIF4E, we observed an increase in P-body size (data not shown). No effect was seen when a WT strain was shifted to 37°C for 20 minutes (Figure II.6A, III, VII). These results indicate that P-bodies increase as a consequence of defects in translation initiation.



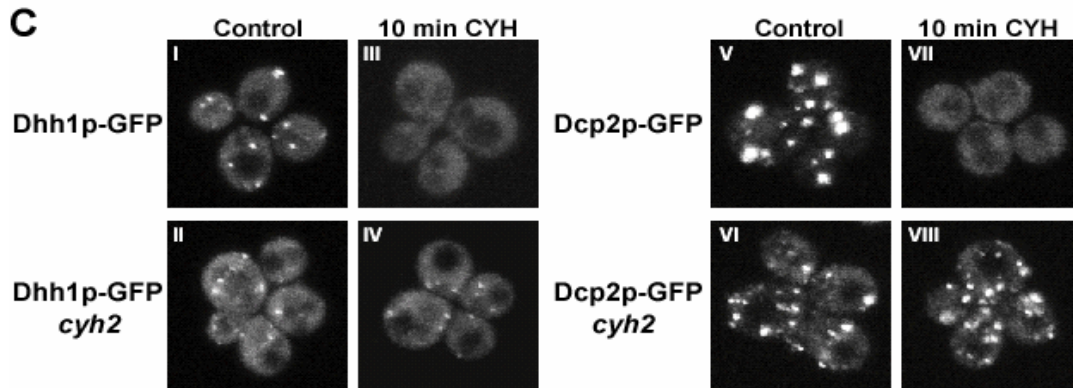


Figure II.6 P-bodies and translation show a reciprocal relationship. (A) P-bodies are affected by translation initiation rates. Cells expressing a GFP-tagged version of Dhh1p carrying the conditional ts allele *prt1-63* or a WT allele (left panel) and cells expressing a GFP-tagged version of Dcp2p carrying the conditional ts allele *prt1-63* or a WT allele (right panel) were grown in YPGlu at 23°C (I,II,V,VI) and then shifted to 37°C for 20 minutes (III,IV,VII,VIII). Cells were collected, washed, and resuspended in SC plus Glu. (B) Inhibition of translation elongation disrupts P-bodies. Cells were grown in YPGlu. The culture was divided into two samples; cycloheximide (CYH) at a concentration of 100 µg/ml was added to one sample. The cells were grown for another 10 minutes before they were collected. The water used for washing and resuspension of the CYH-treated cells contained the same concentration of the drug (IV–VI,X–XII). The other sample was treated similarly but without adding the drug (I–III,VII–IX). Dcp1p, Dcp2p, Dhh1p, Lsm1p, Pat1p, and Xrn1p GFP-tagged version proteins are shown. (C) Inhibition of translation elongation in a strain altered in ribosomal protein L28 does not affect P-bodies. Cells were grown in YPGlu, the culture was divided into two samples; CYH at a concentration of 2 µg/ml was added for 10 minutes to one sample. The cells were washed in water with (III,IV,VII,VIII) or without (I,II,V,VI) CYH before they were collected and observed. Cells expressing a GFP-tagged version of Dhh1p carrying the *cyh2* allele or a WT allele (left panel), and Dcp2p carrying the *cyh2* allele or a WT allele (right panel) are shown.

II.3.2.2 Inhibition of Translation Elongation Leads to Dissociation of all Known Components of P-bodies

The increase in P-bodies when translation initiation is inhibited suggests that P-bodies are forming on a pool of nontranslating mRNPs. Consistent with that result, the presence of Dhh1p and Edc3p in P-bodies is abolished when cells are treated with cycloheximide, which is an inhibitor of translation elongation and leads to mRNA accumulation on polysomes (Sheth and Parker 2003; Kshirsagar and Parker 2004). These findings suggest that either P-bodies themselves dissociate when mRNAs are trapped in polysomes, or that Dhh1p and Edc3p require ongoing translation for their localization in P-bodies. In order to distinguish these two possibilities, we examined the distribution of all known P-body components in response to cycloheximide treatment.

We first examined the effect of cycloheximide on P-bodies under conditions of osmotic stress, where P-bodies are large and numerous. Cells were washed and resuspended in water with, or without, cycloheximide. Every known component of P-bodies was no longer

observed in foci following a 10 minutes treatment with 100 µg/ml of cycloheximide (Figure II.6B, IV–VI, X–XII). Although these results were obtained under osmotic stress conditions, similar results are seen when the cells were washed and resuspended in synthetic medium with glucose with, or without, cycloheximide (data not shown; Figure III.1). Western analysis of the tagged proteins under these conditions indicated that the protein levels in each case remained constant (data not shown). Moreover, in yeast strains resistant to the effect of cycloheximide because of a point mutation in the ribosomal protein L28 (Stocklein and Piepersberg 1980), P-bodies were maintained in the presence of cycloheximide (Figure II.6C, IV, VIII). These observations suggest that cycloheximide is exerting its effect on P-bodies through inhibition of translation elongation. The simplest interpretation of these results is that the assembly of P-bodies requires nontranslating mRNA and when mRNAs are trapped in polysomes there is no significant residual P-body structure that remains. An implication of this model would be that P-bodies require RNA for their assembly. However, we cannot rule out the formal possibility that there is a, yet to be discovered, protein(s) that forms a P-body seed and remains in foci even when mRNAs are no longer available.

II.3.3 P-bodies Require RNA for Assembly

II.3.3.1 P-bodies are Sensitive to RNase A Treatment

The results above raised the possibility that P-bodies require nontranslating mRNA for their assembly. In order to test whether P-bodies are dependent on RNA, we partially purified P-bodies from an *xrn1Δ* strain where they are abundant as compared to WT (Sheth and Parker 2003). In this strain, P-bodies can be highly concentrated and purified by differential centrifugation such that a pellet is obtained that is enriched for P-bodies. Evidence that the pellet fraction contains P-bodies is microscopic identification of fluorescent foci similar to those observed in cells expressing Dcp2p-GFP (Figure II.7A). In addition, these foci increase in *xrn1Δ* as compared to WT cells (Figure II.7A, compare I with II), contain multiple components of P-bodies, and decrease following cycloheximide treatment (MA Valencia-Sanchez and R Parker observations).

To determine if P-bodies require RNA for their integrity, we treated the enriched P-body fraction with RNase A and then repurified the P-bodies by centrifugation. Two assays indicated that RNase A treatment disrupted P-body integrity. First, re-examination of the pellet fraction following RNase A treatment showed a >90% reduction in the number of microscopically identifiable P-bodies as assessed by Dcp2p, Dhh1p, or Lsm1p (Figure

II.7B, IV–VI). Second, analysis of P-body components by Western analysis showed that P-body components that are initially in the pellet are now found in the supernatant following RNase A treatment (Figure II.7C; data not shown). Thus, these partially purified P-bodies are dependent on RNA for their integrity. Although these experiments were done with partially purified P-bodies from an *xrn1Δ* strain, similar results are seen with P-bodies purified from WT strains (data not shown). In addition, previous experiments indicate that the coimmunoprecipitation of different P-body components requires RNA (Tharun and Parker 2001). We interpret these observations to suggest that P-body proteins interact with mRNAs and then the resulting mRNPs aggregate into a larger structure. However, from this experiment we cannot formally distinguish what RNA is required for aggregation of the P-body structure, although the results presented below suggest it is mRNA.

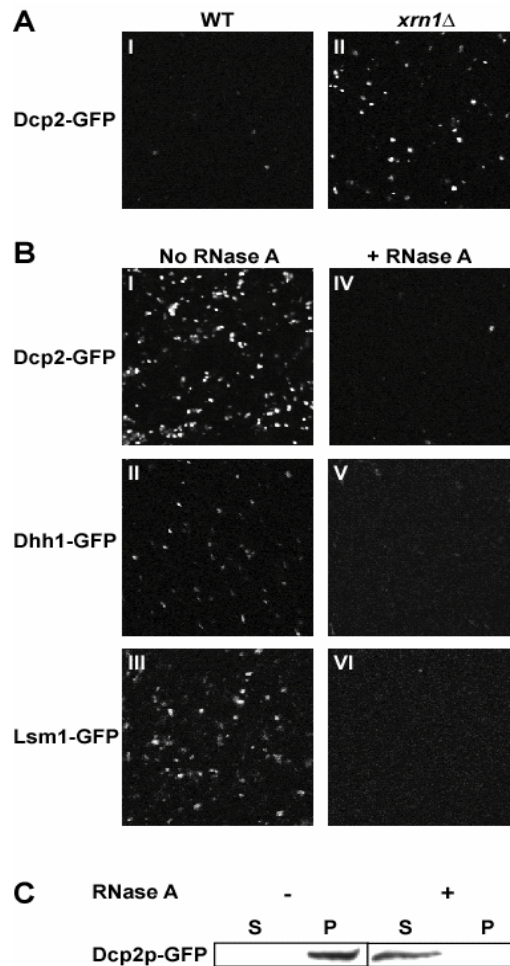


Figure II.7 P-bodies are sensitive to RNase A digestion. (A) Microscopic analysis of P-body concentrated pellet from a WT (I) and an *xrn1Δ* strain (II) expressing a GFP-tagged version of Dcp2p. **(B)** Fluorescence microscopy of the P-body concentrated fractions in the presence (IV–VI) and absence (I–III) of RNase A. Dcp2p (top panel), Dhh1p (middle panel) and Lsm1p (bottom panel) GFP-tagged version proteins in *xrn1Δ* strain are shown. **(C)** Western blot analysis of the supernatant (S) and pellet (P) fractions from a Dcp2p-GFP *xrn1Δ* strain in the presence (+) or absence (–) of RNase A (MA Valencia-Sanchez observations).

II.3.3.2 Increasing the Level of Trapped mRNA Decay Intermediates Increases P-body Size

The work above suggested that the formation and integrity of P-bodies requires RNA. A prediction of this hypothesis is that if we trapped mRNAs in the process of degradation we should observe an increase in P-bodies. To test this prediction, we expressed high levels of an mRNA that produces an RNA fragment trapped in the process of degradation because of a poly(G) track strong secondary structure that blocks the 5' to 3' exonuclease Xrn1p (Decker and Parker 1993). In this experiment, we localized Dcp2p, as well as Dhh1p, in a strain containing a high-copy-number plasmid expressing the *MFA2* mRNA with, or without, a poly(G) tract in its 3' UTR from the very strong *GPD* gene promoter. Cells containing high levels of *MFA2* mRNA decay intermediate accumulated P-bodies (Figure II.8A, V, VI). In contrast, cells void of plasmid (Figure II.8A, I, II), or expressing *MFA2* mRNA without a functional poly(G) tract, did not show an increase in P-body size (Figure II.8A, III, IV). This result indicates that the size and number of P-bodies can be increased by trapping mRNA in decay and is consistent with P-bodies requiring mRNAs for their formation.

We repeated this experiment using a different methodology. In this case, we compared yeast strains that expressed two reporter mRNAs (*PGK1* and *MFA2*) with, or without, poly(G) tracts in their 3' UTRs under the control of a galactose-inducible promoter. Here we examined the appearance of P-bodies after transcription of these genes, and thus the accumulation of the decay intermediate, was induced by the presence of galactose in the growth medium. We observed that in yeast strains expressing mRNAs with the poly(G) in the presence of galactose P-bodies were increased above those seen in glucose (Figure II.8B, compare V, VI to VII, VIII). Moreover, yeast strains expressing mRNAs without the poly(G) did not show a significant increase in P-bodies when grown on galactose media (Figure II.8B, III, IV). This indicates that the effect on P-body size is attributable to the accumulation of the poly(G) mRNAs and not the change in carbon source. We interpret these results to indicate that when mRNAs are trapped in the process of decay, P-bodies accumulate, which provides additional evidence that P-body formation is dependent on mRNA molecules. This result is also consistent with the observations that blocking decapping or 5' to 3' degradation, which traps numerous mRNAs in the process of decay, leads to an increase in P-body size and number (Sheth and Parker 2003).

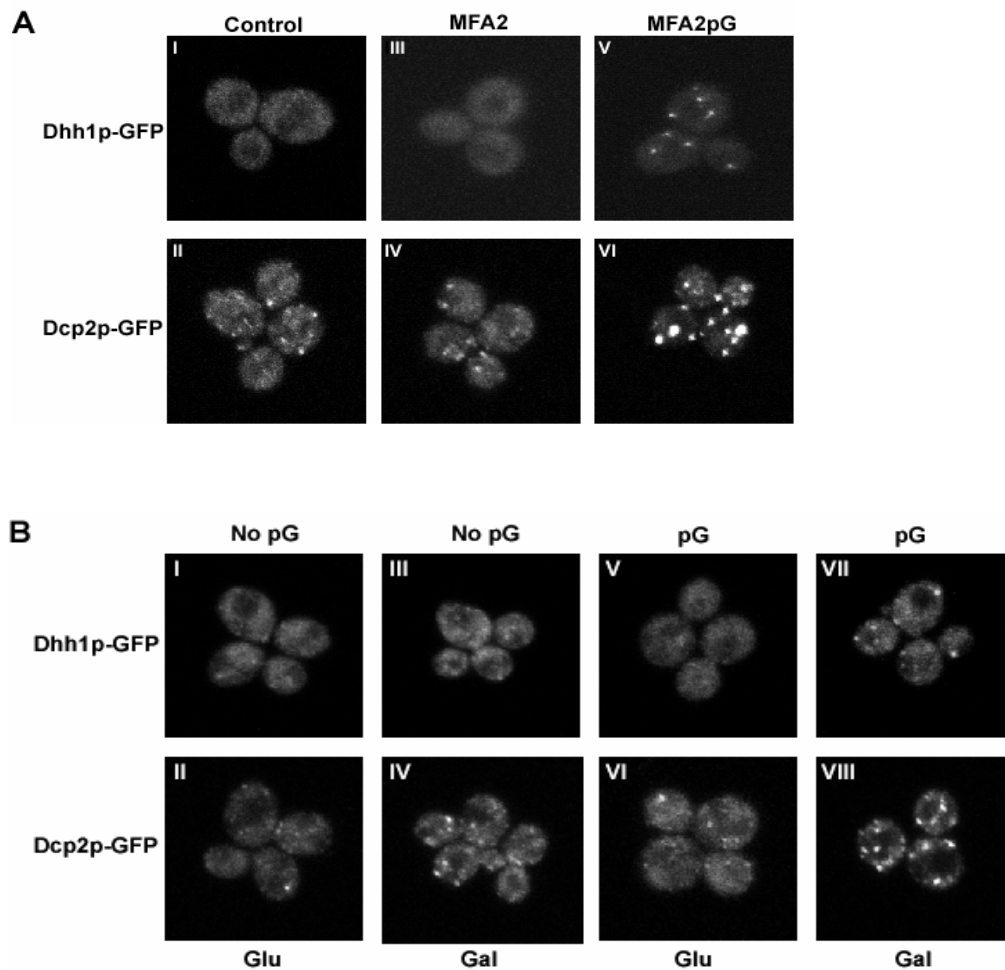


Figure II.8 P-body integrity is dependent on mRNA. (A) Dhh1p (top panel) and Dcp2p (bottom panel) GFP-tagged strain cells transformed with plasmid MFA2 expressing *MFA2* mRNA with no functional poly(G) site (III,IV), or transformed with the plasmid MFA2pG expressing *MFA2* mRNA with poly(G) (V,VI); control cells (I,II) are shown. Cells expressing a GFP-tagged version of Dhh1p (top panel) and Dcp2p (bottom panel) are shown (U Sheth observations). (B) Cells lacking the *GAL-PGK1pG/MFA2pG* constructs (I-IV) and expressing a GFP-tagged version of Dhh1p (top panel) and Dcp2p (bottom panel) grown in Glu (I,II) or Gal (III,IV) are shown. Cells expressing a GFP-tagged version of Dhh1p (top panel) and Dcp2p (bottom panel) containing the *GAL1-PGK1pG/MFA2pG* constructs (V-VIII) grown in Glu (V,VI) or Gal (VII,VIII) are shown.

II.3.4 mRNA Localizes to P-bodies During Translation Repression

The results above suggest that P-bodies form on nontranslating mRNAs. One prediction of this model is that specific mRNAs should be detectable in P-bodies during translation repression. To test this prediction, we localized the *PGK1* mRNA within cells using a transcript containing multiple binding sites in its 3' UTR for a U1A-GFP fusion protein (Brodsky and Silver 2000). This mRNA was distributed throughout the cytoplasm during

cell growth in glucose (Figure II.9A). However, after 10 minutes of glucose deprivation, strains coexpressing the *PGK1* mRNA containing U1A-binding sites and the U1A-GFP fusion protein showed a clear concentration of the GFP signal in cytoplasmic foci, which colocalized with Dcp2p-RFP (Figure II.9B–D). Control strains expressing the U1A-GFP without the target *PGK1* mRNA did not show any discrete concentration of the GFP signal in response to glucose deprivation (Figure II.9F). This provides direct evidence that mRNA molecules can be detected in P-bodies during translation repression.

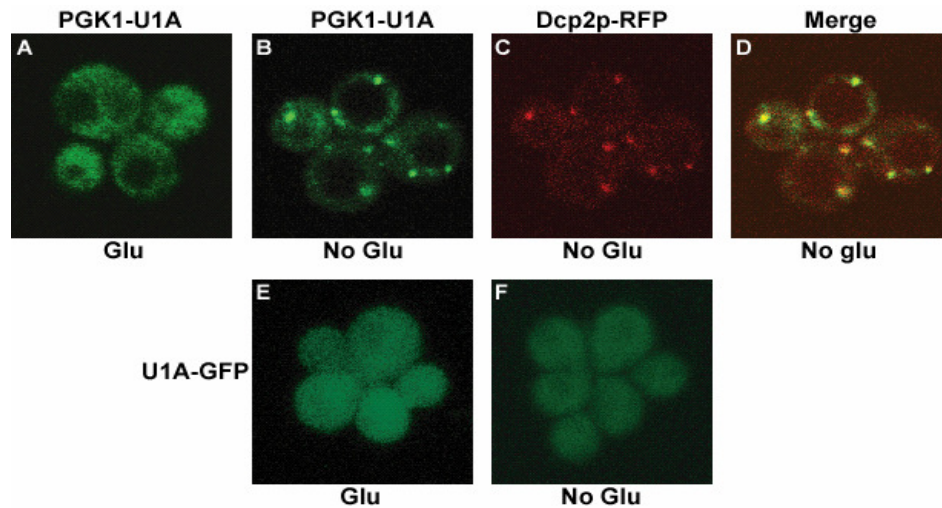


Figure II.9 mRNA localizes to P-bodies during translation repression. WT strain coexpressing plasmids U1A-GFP, PGK1-U1A and DCP2-RFP (A–C), or control plasmid U1A-GFP (E,F) was grown in SC plus Glu. For glucose depletion, cells were washed and resuspended in SC for 10 minutes before collection. For observation, cells were washed and resuspended in SC with (A,E) or without (B,C,F) Glu. Merged view is shown (D).

II.3.5 Translation Factors are not Associated with P-bodies

The results above led to the hypothesis that P-bodies contain pools of nontranslating mRNPs. In order to examine the relationship between translation and P-bodies, we determined the subcellular distribution of translation factors. To this end, we used tagged genes for several translation factors with GFP in their chromosomal location under the control of their own promoter. The proteins examined include the translation initiation factor Prt1p, a subunit of the eIF3 translation initiation complex; TEF4p, a translation elongation factor; Sup45p, a translation termination factor; and Rpl5p, a subunit of the large ribosomal subunit.

We observed that these translation factors were distributed in the cell without any concentration in cytoplasmic foci during log growth (Figure II.10A, II; Figure II.10B, I–III).

Moreover, we did not observe localization of these translation factors to P-bodies under glucose deprivation (Figure II.10A, V; Figure II.10B, IV–VI), conditions where P-bodies are dramatically increased (Figure II.3). Examination of RFP-tagged Dcp2p in the same cells showed that P-bodies were forming efficiently in these experiments (Figure II.10A, IV, VI). We interpret these results to indicate that ribosomes, and multiple translation factors, do not accumulate in P-bodies. This supports the argument that mRNAs within P-bodies are in a nontranslating state.

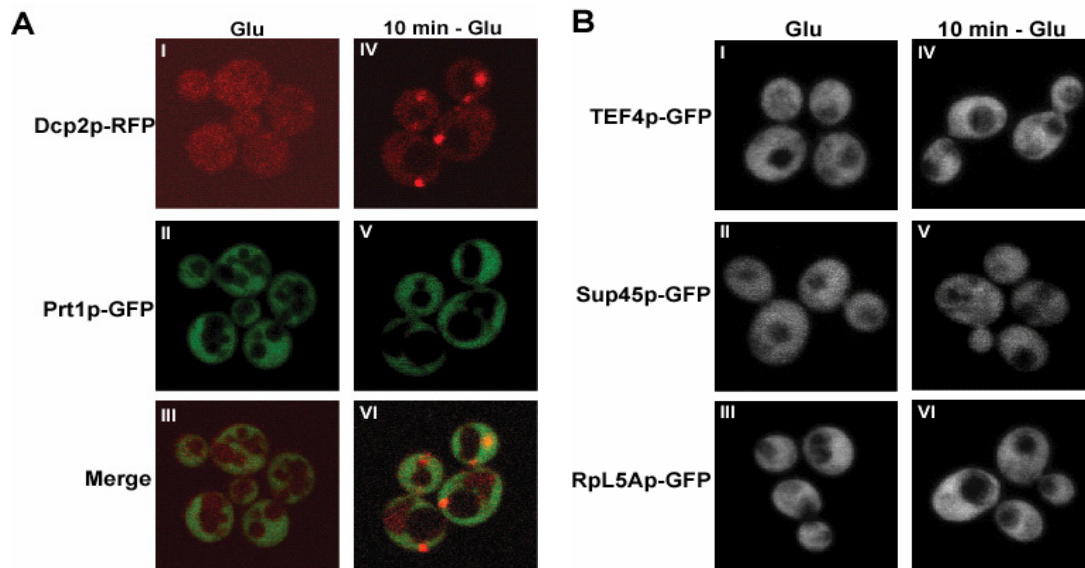


Figure II.10 Translation factors are not associated with P-bodies. (A) Cells were grown in SC plus Glu. For glucose depletion, cells were washed in SC for 10 minutes before collection. For observation, cells were washed and resuspended in SC with (I,II) or without (IV,V) Glu. Cells coexpressing a GFP-tagged version of Prt1p and RFP-tagged version of Dcp2p (I,II,IV,V); and merged views (III,VI) are shown. **(B)** GFP-tagged versions of TEF4 (top panel), Sup45p (middle panel), and Rpl5Ap (bottom panel) were grown in YPGlu and observed as described in Figure II.3. Cells observed in the presence (I–III) or absence (IV–VI) of Glu are shown.

II.4 Discussion

II.4.1 P-bodies Require RNA for Their Assembly

Two types of observations indicate that the formation of P-bodies requires RNA. First, trapping mRNA in the process of decay *in vivo* by *cis*-acting structures leads to the accumulation of P-bodies (Figure II.8). This result is consistent with the observation that inactivation of the decapping enzyme or 5' to 3' exonuclease increases P-bodies (Sheth and Parker 2003). Second, semipurified P-bodies are sensitive to RNase A treatment as assessed both by microscopic and biochemical analysis (Figure II.7). This is also

consistent with previous results showing that coimmunoprecipitation of P-body components is sensitive to RNase A treatment (Tharun and Parker 2001). Based on these observations, we conclude that the formation, or integrity, of P-bodies requires RNA.

Several observations support the argument that nontranslating mRNAs are the RNA underlying P-body formation. First, trapping mRNA molecules in the process of decay by the insertion of a poly(G) tract *in cis* leads to increases in P-body formation (Figure II.8). An important point is that mRNA fragments trapped by the insertion of poly(G) tracts are not translated (Muhlrads et al. 1995), and are not found associated with ribosomes (Hillgren and Parker 2001). Second, driving yeast mRNAs into polysomes by inhibiting translation elongation leads to loss of P-bodies (Figure II.6). Third, inhibition of translation initiation by mutations (Figure II.6) or cellular stresses leads to increases in P-bodies (Figures II.1, II.3-II.5). Fourth, translation factors and ribosome components are not found within P-bodies, even under conditions in which the P-bodies are large (Figure II.10). Finally, *PGK1* mRNA can be detected within P-bodies during glucose deprivation (Figure II.9). While we cannot formally rule out that there is an additional non-mRNA component of P-bodies, the pool of nontranslating mRNA can clearly affect the size and abundance of P-bodies.

The conclusion that P-bodies are dependent on mRNA for their formation implies that mRNAs can be specifically delivered to P-bodies, or that individual mRNPs have the ability to aggregate together. Such aggregation could occur through the interaction of mRNA-binding proteins that have multiple RNA-binding domains, and thereby provide links between individual mRNAs causing aggregation. Alternatively, there may be key RNA-binding proteins that, after binding to the mRNA, have the ability to interact with each other, thereby bringing the mRNPs together. This latter model is particularly appealing since the localization of nontranslating mRNAs within cytoplasmic granules that are formed in response to stress in mammalian cells, stress granules, appears to be dependent on glutamine/asparagine (Q/N)-rich domains that are prion-like aggregation motifs found in the mRNA-binding proteins TIA-1 and TIA-R (Kedersha et al. 2000; Gilks et al. 2004). Interestingly, several P-body components contain Q/N-rich prionlike protein domains, which might have a role in P-body formation. Finally, although the specific mechanism through which RNA drives P-body formation is not clear, one interesting possibility is that the affinity between different P-body proteins may increase when they are bound to RNA, thereby nucleating the formation of P-bodies.

An unresolved issue is whether P-bodies can form *de novo*. For example, there could be a pre-existing structure to which mRNAs and their associated proteins are targeted. Alternatively, mRNAs might form a specific type of mRNP that has the ability to aggregate into higher-order structures, which are then visualized in the light microscope as P-bodies. Our results are most consistent with the a model where P-bodies form by the assembly of a specific mRNP structure on nontranslating mRNAs, which then has the ability to aggregate into larger structures, since all known components of P-bodies disperse when cycloheximide is introduced (Figure II.6). In addition, P-bodies appear to be able to form *de novo* when cells are subject to stresses, and are found throughout the cytoplasm. Experiments addressing P-body formation and organization should provide insight into the specific mechanisms by which P-body assemble.

II.4.2 P-bodies may Function in Stress Responses

Our results suggest the possibility that P-bodies will be important in cellular responses to stress. The critical observation is that the size and number of P-bodies increased in several stress conditions including glucose deprivation, osmotic stress, and late stage of growth (Figures II.1, II.3–II.5). The cellular changes taking place during the stress conditions analyzed suggests a correlation between a decrease in translation rates and an increase in the concentration of P-body components in cytoplasmic foci. For example, glucose deprivation and osmotic stress are known to inhibit translation initiation (Ashe et al. 2000; Uesono and Toh-e 2002; Uesono et al. 2004; data not shown) and both lead to dramatic increases in P-bodies. In contrast, exposure of yeast cells to heat stress or oxidative stress does not seem to have an effect on translation rates (Uesono and Toh-e 2002; data not shown) and does not increase P-bodies (data not shown). We interpret these results to suggest that stresses that lead to decreases in translation initiation lead to an increase in P-bodies due to the movement of mRNAs from the translating, or polysome pool, to P-bodies. An interesting observation is that the mechanism leading to translation initiation inhibition seems to be different between stresses (Ashe et al. 2000; Uesono and Toh-e 2002). Suggesting that although there is variety in the signaling pathways that sense a particular stress condition they all lead to decreases in translation and increases in P-body size. Another cellular stress where P-bodies may have a function is in the response to ultraviolet (UV) light because we observed that P-bodies are also increased by exposure to UV light (Teixeira et al. 2005). Future experiments should address the impact of the signaling pathways under different conditions in P-body assembly, and the significance of those changes.

The ability to sequester mRNAs into a nontranslating pool during stress conditions could be beneficial to the cell. For example, given limiting translation factors during a stress response, by sequestering the pre-stress pool of mRNAs into a nontranslating state, new mRNAs that are required for the cellular stress response might be preferentially translated because of reduced competition from the pre-existing mRNAs. Second, if the mRNAs targeted to P-bodies during stress were also subsequently degraded, their targeting to P-bodies would assist in the reprogramming of the mRNA pool to reflect the new growth conditions. Third, if mRNAs targeted to P-bodies can return to the translating pool, then this system provides a way to transiently arrest translation on a subset of the mRNA population, and then to reuse such mRNAs when the cell has recovered from the stress conditions. This suggests that P-bodies might have another biological role storing mRNAs. The idea that mRNAs can reenter translation following storage in P-bodies during stress response is particularly interesting because of the similarity between P-bodies and the other mRNP granules (see below). In fact, this dynamic would be similar to what has been proposed for stress granules (SGs), which are cytoplasmic particles of nontranslating mRNAs that form in response to various stress, and contain poly(A)⁺ mRNAs, the small ribosomal subunit, some translation initiation factors and RNA-binding proteins including Pab1p, TIA and TIA-R SGs markers (Nover et al. 1983; Anderson and Kedersha 2002; Anderson and Kedersha 2006). SGs have been proposed to store mRNAs during a stress response because SGs decline during stress recovery due to retranslation of the mRNAs in SGs (Wilczynska et al. 2005). Interestingly, in mammalian cells, although P-bodies and SGs appear to be physically distinct and spatially separate cytoplasmic structures (Kedersha et al. 1999; Cougot et al. 2004), there appears to be some functional or temporal relationship between these pools of mRNPs. For example, P-bodies can be physically juxtaposed to SGs, some proteins are found within both SGs and P-bodies, and both have similar dynamics (Kedersha et al. 2005; Wilczynska et al. 2005). Both P-bodies and SGs are increased by decreasing translation initiation and decline when mRNAs are driven into polysomes. However, SGs disperse after stress recovery, while P-bodies are present continually (Cougot et al. 2004; Kedersha et al. 2005; Wilczynska et al. 2005). This suggests that SGs might represent an intermediate state of mRNPs between translating mRNPs and mRNPs within P-bodies and that nontranslating mRNAs may move between these two compartments to regulate the cytoplasmic function of mRNA.

An unresolved issue is the relationship between SGs and P-bodies in yeast. In our studies, we have been unable to visualize structures analogous to SGs in *Saccharomyces cerevisiae* under a variety of conditions (data not shown), although they can be detected in *Schizosaccharomyces pombe* (Dunand-Sauthier et al. 2002). This suggests that this

biochemical compartment is either absent or more transient in yeast than in more complex eukaryotes. Because in yeast mRNAs accumulate in P-bodies when translation initiation is compromised by several stresses (Figure II.1, II.3-II.5), and moreover, yeast P-bodies exhibit many properties of mammalian SGs, this suggests that yeast P-bodies may be related to mammalian SGs. We can speculate that P-bodies may be an ancestral structure that in yeast plays roles both in the presence and absence of stress, while in more complex eukaryotes the function of these aggregates under normal or stress conditions might have segregated into distinct structures, P-bodies and SGs structures. Additional experiments should reveal the manner in which P-body formation contributes to the stress response. Nevertheless, it should be noted that intermediate mRNP states between translation and P-bodies are also likely to exist in yeast. Future studies identifying the different type of mRNPs that may form under different cellular conditions and addressing the composition of these mRNPs should provide insight into this issue.

II.4.3 Movement of mRNA From Translation to P-bodies

Our results indicate that mRNAs exist in two distinct functional pools, one pool associated with polysomes engaged in translation and one sequestered in a nontranslating state, which is concentrated in P-bodies. Moreover, our results argue that movement of mRNAs from the polysome pool to P-bodies is an important and dramatic transition in the state of eukaryotic mRNA. This suggestion is based on P-bodies containing pools of nontranslating mRNAs and the reciprocal nature of the relationship between translation and P-bodies. A critical transition between a translating pool of mRNA and nontranslating mRNAs destined for degradation is also supported by coimmunoprecipitation experiments wherein decapping factors coimmunoprecipitated mRNAs but not translation factors (Tharun and Parker 2001). Thus, a reasonable hypothesis is that mRNAs undergo a transition to a nontranslating competent mRNP state that is both an mRNP precursor to decapping and has the ability to aggregate in subcellular structures, P-bodies. The transition of mRNAs from polysomes to P-bodies is striking in that it involves the loss of ribosomes and translation factors from the mRNA, the addition of the decapping machinery and proteins that promote P-body assembly, as well as physical movement of the mRNA between the polysome and P-body compartments (Figure II.10; Tharun and Parker 2001; Collier and Parker 2005). Thus, we anticipate that several intermediates states between translation, translation repression, and accumulation within P-bodies for mRNA decapping and degradation will exist. The mechanism by which the mRNA makes these transitions requires further study. Given that these transitions are likely to be sites of

biological regulation, an important area of future work will be in determining the signals and mechanisms by which mRNA exit translation and enter the P-body compartment.

Notably, recent results have begun to identify factors that mediate the transition of the mRNA from translation to an mRNP translationally repressed that can undergo decapping and assemble into P-bodies. In yeast, Dhh1p and Pat1p, which activate decapping and are components of P-bodies, have been described to play a role in stimulating general mRNA translation repression, promoting dissociation of the mRNA from polysomes, and thereby facilitating P-body formation (Coller and Parker 2005). This implies that P-bodies may have a role in the translation repression of mRNAs. Moreover, this suggests that other decapping activators may also function to repress translation. An important future goal will be to identify factors involved in the mRNA transition from the translating pool to the nontranslating pool and their function in the process.

CHAPTER III

MOVEMENT OF EUKARYOTIC mRNAs BETWEEN POLYSOMES AND CYTOPLASMIC P-BODIES

III. MOVEMENT OF EUKARYOTIC mRNAs BETWEEN POLYSOMES AND CYTOPLASMIC P-BODIES

Abstract

Eukaryotic cells contain nontranslating mRNA concentrated in P-bodies, which are sites where the mRNA can be decapped and degraded. We present evidence that mRNA molecules within yeast P-bodies can also return to translation. First, inhibiting delivery of new mRNAs to P-bodies leads to their disassembly independent of mRNA decay. Second, P-bodies decline in a translation initiation–dependent manner during stress recovery. Third, reporter mRNAs concentrate in P-bodies when translation initiation is blocked and resume translation and exit P-bodies when translation is restored. The reciprocal movement of mRNAs between polysomes and P-bodies is likely to be important in the control of mRNA translation and degradation. Moreover, the presence of related proteins in P-bodies and maternal mRNA storage granules suggests this mechanism is widely adapted for mRNA storage.

III.1 Introduction

A key aspect of the regulation of eukaryotic gene expression is the control of mRNA translation and degradation, which initiates with deadenylation and often occurs by decapping, followed by 5' to 3' decay (Coller and Parker 2004). Translation and mRNA degradation via decapping are tightly linked due to the mechanisms of mRNA decay. To decap, an mRNA exits translation and assembles into a translationally repressed mRNP lacking translation initiation factors and containing the decapping enzyme and several accessory proteins (Tharun and Parker 2001; Coller and Parker 2005). These translationally repressed mRNPs accumulate within P-bodies (also referred to as GW- or DCP-bodies) (Chapter II; Bashkurov et al. 1997; Ingelfinger et al. 2002; Lykke-Andersen 2002; van Dijk et al. 2002; Eystathiou et al. 2003), where decapping can occur (Sheth and Parker 2003; Cougot et al. 2004). The formation of a P-body mRNP containing nontranslating mRNAs appears to be also important for control of translational repression. For example, yeast cells lacking both Dhh1p and Pat1p, which are components of P-bodies, are defective in P-body formation and are unable to globally repress translation in response to amino acid starvation or glucose deprivation (Coller and Parker 2005). Moreover, it has been suggested that mammalian P-bodies function in miRNA-mediated mRNA translation repression and possibly degradation (Liu et al. 2005a; Sen and Blau 2005). An unresolved issue is whether mRNAs targeted to P-bodies are solely destined for degradation, or whether P-bodies can store mRNAs and later release them to reenter translation. Such a cycle would suggest that P-bodies have additional properties important for translational control.

III.2 Material and Methods

III.2.1 Yeast strains and Growth Conditions

The genotypes of all strains used in this study are listed in Table III.1. Proteins were C-terminal tagged with GFP following the PCR-based gene modification method described previously Longtine et al. (1998). These fusion proteins are full-length and functional (Sheth and Parker 2003). Yeast crosses were carried out using standard laboratory procedures. Strains were grown on either standard YP or SC supplemented with appropriate amino acids and 2% dextrose (Glu) as carbon source. Strains were grown at 30°C except for conditional *ts* mutants, which were grown at 23°C and shifted to 37°C to inactivate the mutant allele. For the strains expressing *MFA2pG* or *MFA2P-U1A* mRNA

reporters under the control of the tetracycline repression promoter (*Tet-Off*) (pRP1192, pRP1291), transcription was blocked by using doxycycline (Sigma) to a final concentration of 2 µg/ml.

Table III.1 Yeast strains.

Strain	Genotype	Source
yRP840	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG</i>	Hatfield et al. 1996
yRP1326	<i>MATa leu2-3,112 trp1 ura3-52 prt1-63 cup1::LEU2/PGK1pG/MFA2pG</i>	Schwartz and Parker 1999
yRP1340	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2 dcp1-2::TRP1</i>	Dunkley and Parker 1999
yRP1724	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DHH1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1727	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DCP2-GFP (NEO)</i>	Sheth and Parker 2003
yRP1736	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 DHH1-GFP(NEO)</i>	Sheth and Parker 2003
yRP1738	<i>MATa leu2-3,112 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrm1::URA3 DHH1-GFP(NEO)</i>	Sheth and Parker 2003
yRP1826	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 prt1-63 cup1::LEU2/PGK1pG/MFA2pG DHH1-GFP(NEO)</i>	Teixeira et al. 2005
yRP1827	<i>MATa leu2-3,112 trp1 ura3-52 prt1-63 cup1::LEU2/PGK1pG/MFA2pG DCP2-GFP(NEO)</i>	Teixeira et al. 2005
yRP1923	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrm1::URA3 DCP2-GFP (NEO)</i>	Teixeira et al. 2005
yRP1932	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2 dcp1-2::TRP1 DCP2-GFP(NEO)</i>	This study
yRP1933	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 lys2 dcp1-2::TRP1 cup1 DHH1-GFP(NEO)</i>	This study
yRP1934	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ski2::LEU DCP2-GFP(NEO)</i>	This study
yRP1935	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 dcp1-2::TRP1 cup1::LEU2/PGK1pG/MFA2pG ski2::LEU DCP2-GFP(NEO)</i>	This study
yRP1936	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 DCP2-GFP (NEO)</i>	This study
yRP1937	<i>MATa leu2 ura3 his3 met15 SKI2-GFP (HIS)</i>	Huh et al. 2003
yRP1938	<i>MATa leu2 ura3 his3 met15 SKI3-GFP (HIS)</i>	Huh et al. 2003
yRP1939	<i>MATa leu2 ura3 his3 met15 SKI4-GFP (HIS)</i>	Huh et al. 2003
yRP1940	<i>MATa leu2 ura3 his3 met15 SKI6-GFP (HIS)</i>	Huh et al. 2003
yRP1941	<i>MATa leu2 ura3 his3 met15 SKI7-GFP (HIS)</i>	Huh et al. 2003
yRP1942	<i>MATa leu2 ura3 his3 met15 SKI8-GFP (HIS)</i>	Huh et al. 2003
yRP1943	<i>MATa leu2 ura3 his3 met15 EIF1A-GFP (HIS)</i>	Huh et al. 2003
yRP1944	<i>MATa leu2 ura3 his3 met15 TIF35-GFP (HIS)</i>	Huh et al. 2003
yRP1945	<i>MATa leu2 ura3 his3 met15 EIF4B-GFP (HIS)</i>	Huh et al. 2003
yRP1946	<i>MATa leu2 ura3 his3 met15 EIF4E-GFP (HIS)</i>	Huh et al. 2003
yRP1947	<i>MATa leu2 ura3 his3 met15 EIF4G1-GFP (HIS)</i>	Huh et al. 2003
yRP1948	<i>MATa leu2 ura3 his3 met15 EIF4G2-GFP (HIS)</i>	Huh et al. 2003
yRP1949	<i>MATa leu2 ura3 his3 met15 EIF5-GFP (HIS)</i>	Huh et al. 2003
yRP1950	<i>MATa leu2 ura3 his3 met15 EIF5B-GFP (HIS)</i>	Huh et al. 2003
yRP1951	<i>MATa leu2 ura3 his3 met15 EIF6-GFP (HIS)</i>	Huh et al. 2003
yRP1952	<i>MATa leu2 ura3 his3 met15 RPG1-GFP (HIS)</i>	Huh et al. 2003
yRP1953	<i>MATa leu2 ura3 his3 met15 NIP-GFP (HIS)</i>	Huh et al. 2003

III.2.2 Plasmids

For *PGK1* and *MFA2* mRNA localization, the 2 μ TRP plasmid U1A-GFP, pRP1187, was created as described previously (Chapter II). The 2 μ LEU U1A-GFP, pRP1194, was created by introducing the U1A-GFP fragment (*SmaI-BglII*) from pRP1187 into yEP351 *SmaI-BamHI* sites. The PGK1-U1A and MFA2P-U1A reporters contain the 5' UTR and coding region of the *PGK1* or *MFA2* mRNAs, respectively, fused to the *PGK1* 3' UTR containing multiple binding sites for a U1A-GFP fusion protein Brodsky and Silver (2000). The plasmid PGK1-U1A expressing *PGK1* mRNA with U1A binding sites (pPS2037) was created as described previously Brodsky and Silver (2000). The plasmid MFA2-U1A expressing *MFA2* mRNA with U1A binding sites (pRP1193) was created by PCR-amplification of the *MFA2* gene from the plasmid pRP1043, and ligation into pPS2037 by replacement of the *PGK1* gene (*XhoI* and *BamHI* sites). The tetracycline repression plasmid Tet-Off MFA2pG (pRP1192) was created by replacing the *BamHI-HindIII* fragment from the Tet-Off PGK1 construct (pRP1103) (Hilleren and Parker 2003) with the *SacI-HindIII* fragment from pRP485, expressing *MFA2*. The tetracycline repression plasmid Tet-Off MFA2P-U1A (pRP1291) was created by replacing the *BamHI-HindIII* fragment from the Tet-Off PGK1 construct (pRP1103) (Hilleren and Parker 2003) with the U1A binding sites fragment (*BamHI-HindIII*) from pPS2037 and with the PCR product (*BglII*) of the *MFA2* gene from the plasmid pRP485 in the *BamHI* site. Yeast strains were transformed by standard techniques and plasmids were maintained by growth in selective media.

III.2.3 Preparation of Cells for Confocal Microscopy

Cells were grown to an OD₆₀₀ of 0.3-0.35 in the appropriate medium. For observation, cells were washed twice and resuspended in SC plus amino acids supplemented with Glu. For glucose depletion, cells were washed in YP or SC supplemented with appropriate amino acids without Glu and resuspended in the same medium for 10 minutes before being collected. Cells were then washed with SC without Glu, resuspended in the same medium, and analyzed. For glucose re-addition experiments, Glu was added to the culture for 5 minutes at 30°C (WT cells) or 10 minutes at 37°C (ts mutant cells). Cells were washed and resuspended with SC plus Glu and analyzed. For experiments involving ts mutant strains, cells were kept at 37°C for observation. Cycloheximide (CYH) was used at a concentration of 100 μ g/ml and cells were grown for another 10 minutes before collection. Medium used for washing and resuspension of CYH-treated cells also contained the same concentration of the drug. Observations were made using a Nikon

PCM 2000 Confocal Microscope using 100X objective with 3X zoom using Compix Software. All images are a Z-series compilation of 6-10 images. All phenotypes are observed in > 80% of the cells examined, unless otherwise stated.

III.2.4 Polysome Analysis

Polysome analysis was performed by a modification of the protocol described previously Kuhn et al (2001). Cell lysates were prepared from 200 ml exponential growth culture (0.3 OD₆₀₀/ml). At the time of harvest, CYH was added to a final concentration of 100 µg/ml in the presence of ice. Cells were pelleted by centrifugation at 4000 rpm for 5 minutes at 4°C and washed in 20 ml of lysis buffer (20 mM Tris-HCl pH8, 140 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 1% Triton X-100, 0.1 mg/ml CYH, 1 mg/ml heparin), followed by freezing in liquid nitrogen. For cell lysis, the pellet was resuspended in 400 µl of lysis buffer. A 400 µl volume of glass beads was added and the reaction mixture was vortexed at full speed for 2 minutes followed by incubation on ice for 2 minutes. This process was repeated 3 times. After cells lysis, excess cells debris and glass beads were removed in a one step clarification performed for 2 minutes at 4000 rpm at 4°C. Approximately 20 A₂₅₄ units were loaded on a 15% to 50% sucrose gradient that contained lysis buffer lacking Triton X-100. The samples were sedimented in a Beckman SW41 rotor at 4°C for 2.5 hours at 39,000 rpm and collected, while the A₂₅₄ value was monitored using a continuous flow cell UV detector.

III.2.5 RNA Analysis

RNA was extracted from polysome fractions (900µl) by adding an equal volume of Phenol/CHCl₃/LET (LET: 100 mM LiCl, 25 mM Tris-HCl [pH8], 20 mM EDTA) and vortexing for 2 minutes at maximum speed. The aqueous phase was re-extracted once with an equal volume of CHCl₃, and RNA was recovered by precipitation with 2 volumes of 100% EtOH and 1/10 volume of 3M NaCH₃CO₂ (pH 5.2). Pellets were washed in 75% EtOH and resuspended in DEPC water. RNA was separated by agarose gel and transferred to nylon membrane (Schleicher & Schuell). Hybridization was achieved using radiolabeled oligonucleotide probes directed against *MFA2P-U1A* mRNA (oRP1250: 5'-TTCGGATAGAAAAGCACTAGTGGATCT AG-3'), *PGK1* mRNA (oRP70: 5'-CGGATAAGAA AGCAACACCTGG-3'), *RPL41A* mRNA (oRP1249: 5'-TTAGAGTTATTTACTCATAATCC GC-3') and the *MFA2pG* mRNA reporter (oRP140: 5'-ATATTGATTAGATCAGGAATTCC-3'). For *CYH2* mRNA, a random-prime radiolabeled DNA probe complementary to *CYH2*

mRNA was used. mRNAs were visualized by Phosphorimager analysis (Typhoon 9410, Amersham Biosciences).

III.3 Results

III.3.1 mRNAs in P-bodies are not Committed to Decapping and Degradation

To determine whether mRNAs in P-bodies were committed to decapping and 5' to 3' degradation, we examined P-bodies in *dcp1Δ* and *xrn1Δ* mutant strains, where decapping and exonucleolytic degradation is blocked, after 10 minutes of cycloheximide treatment, which prevents mRNAs from exiting translation and entering P-bodies (Figure II.6; Sheth and Parker 2003; Cougot et al. 2004; Andrei et al. 2005). If mRNAs in P-bodies are solely committed to decapping and 5' to 3' decay, then P-bodies should persist. Conversely, if mRNAs can exit P-bodies, then P-bodies would decline following cycloheximide addition, despite the block to decapping and 5' to 3' decay. We directly observed P-body proteins by using GFP-tagged versions of the components Dcp2p and Dhh1p, whose presence in P-bodies is dependent on RNA, thus also providing an indirect manner of observing P-body mRNAs (Chapter II). We observed that P-bodies in *dcp1Δ*, *dcp2Δ* and *xrn1Δ* cells declined after 10 minutes of cycloheximide treatment (Figure III.1E, F, L, M; data not shown). These results suggest that mRNPs can exit P-bodies in the absence of decapping and 5' to 3' degradation.

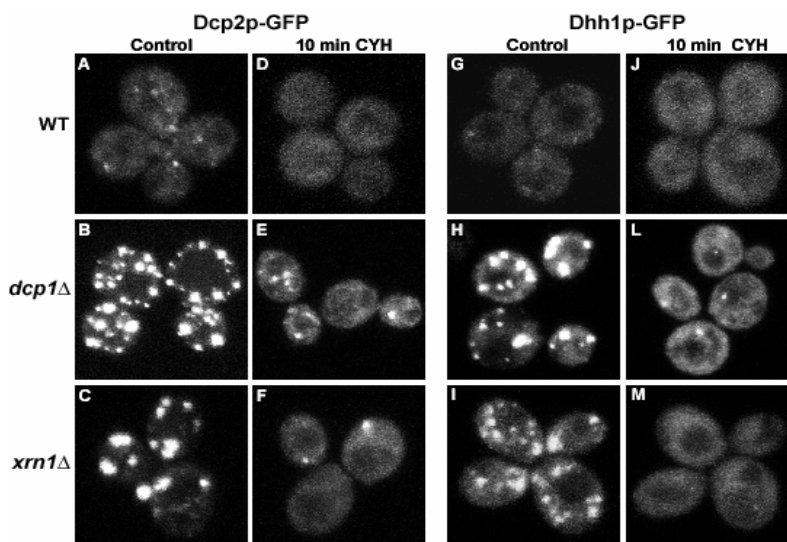


Figure III.1 mRNAs exit P-bodies in the absence of decapping and 5' to 3' degradation. WT, *dcp1Δ* and *xrn1Δ* cells grown in YPGlu at 30°C (A-C,G-I) were treated with cycloheximide (CYH, 100 μg/mL) for 10 minutes (D-F,J-M). Cells expressing a GFP-tagged version of Dcp2p (left panel) or Dhh1p (right panel) are shown.

If mRNAs within P-bodies are committed to degradation, then the reduction in P-bodies seen in the *dcp1Δ* strain could be due to 3' to 5' degradation. Thus, we examined P-bodies in *dcp1* mutant strains lacking Ski2p, which is required for 3' to 5' degradation of mRNAs. Because blocks to decapping and 3' to 5' degradation of mRNA are lethal in combination (Anderson and Parker 1998), we inhibited decapping in the *ski2Δ* strain by using the ts allele *dcp1-2*, which is rapidly inactivated following a shift to the nonpermissive temperature. Results are shown using the P-body component Dcp2p, although similar results were seen with another P-body component, Dhh1p (data not shown). After a shift to 37°C for 30 minutes and because of inhibition of mRNA decapping, P-bodies increased in size and number in the *dcp1-2* and *dcp1-2 ski2Δ* strains (Figure III.2G, H) compared to WT and *ski2Δ* strains (Figure III.2E, F), but still declined after cycloheximide addition (Figure III.2K, L). This indicates that the reduction in P-bodies under these conditions is not due to 3' to 5' degradation. Consistently, proteins required for 3' to 5' degradation of mRNAs are not concentrated in P-bodies (Figure III.3). These results suggest that mRNAs exit P-bodies independent of mRNA degradation, possibly to return to translation.

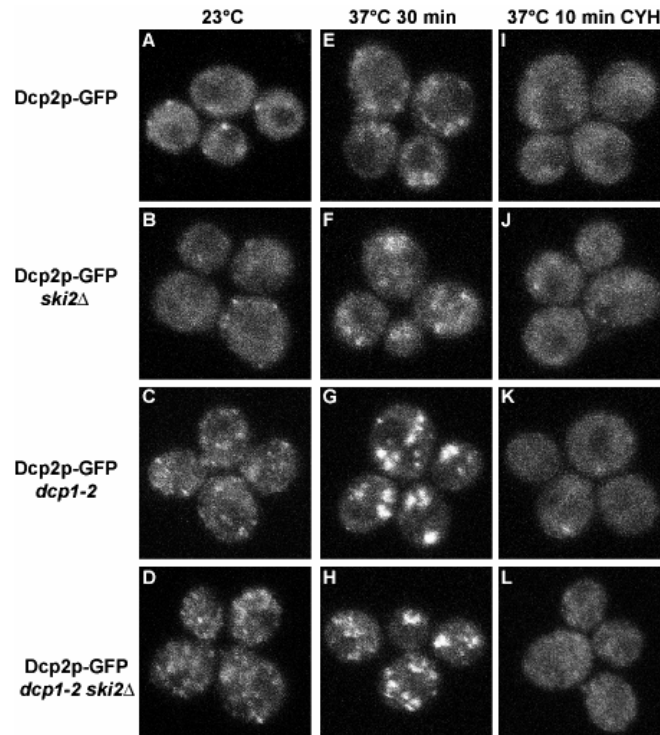


Figure III.2 mRNAs exit P-bodies in the absence of decapping and 3' to 5' degradation. Cells grown in YPGlu at 23°C (A-D) were shifted to 37°C for 30 minutes (E-H) followed by CYH treatment for 10 minutes (I-L). Cells expressing GFP-tagged version of Dcp2p in WT (A,E,I), *ski2Δ* (B,F,J), *dcp1-2* (C,G,K) and *dcp1-2 ski2Δ* (D,H,L) are shown.

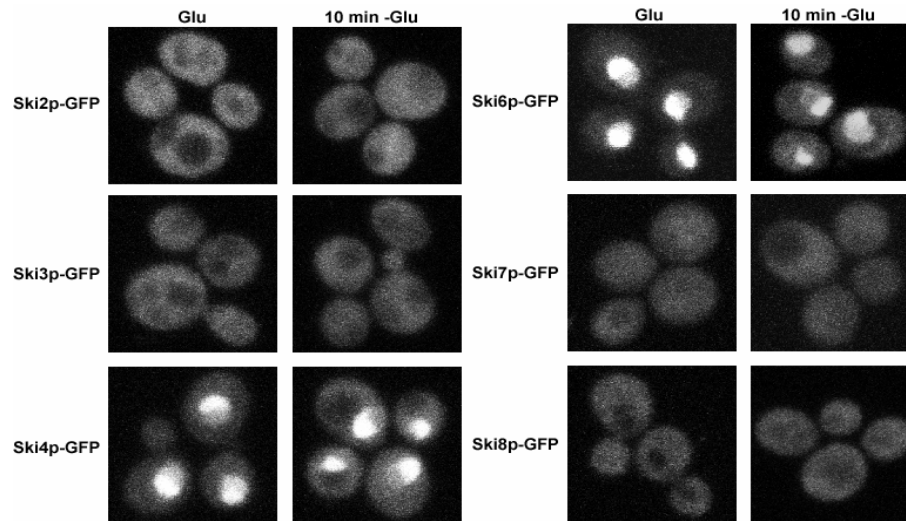


Figure III.3 Exosome components are not present in P-bodies. Cells were grown in YPGlu (Glu), then shifted in YP without Glu for 10 minutes (10 min -Glu). Cells expressing GFP-tagged versions of Ski2p, Ski3p, Ski4p, Ski6p, Ski7p and Ski8p are shown.

III.3.2 P-bodies and Polysomes Show a Reciprocal Relationship During Glucose Deprivation and Restoration

To determine whether P-body mRNAs could enter translation, we examined translation regulation by the presence of glucose (Ashe et al. 2000). In yeast, glucose deprivation rapidly leads to both a decline in polysomes (Ashe et al. 2000) and an increase in P-bodies (Figure II.3), while readdition of glucose rapidly restores translation even in the absence of new transcription (Ashe et al. 2000). Given this, we hypothesized that mRNAs are stored in P-bodies during glucose deprivation and could reenter translation when glucose was restored. To test this, we examined P-bodies and translation during glucose deprivation and restoration. We assessed translation by polysome analysis (D Teixeira). In addition, we assessed P-bodies by following the subcellular distribution of Dhh1p and Dcp2p (D Teixeira) as well as the subcellular distribution of two reporter mRNAs, *PGK1-U1A* and *MFA2P-U1A* (M Brengues). To visualize the localization of these reporter mRNAs, we introduced into their 3' UTR binding sites for the U1A protein, and also expressed a U1A-GFP fusion protein. The binding of the U1A-GFP fusion protein to the RNA U1A sites allow the mRNA to be localized in the cells (Brodsky and Silver 2000). We observed that glucose deprivation leads to a rapid loss of polysomes (Figure III.4F) and an increased accumulation of Dcp2p, Dhh1p, and *PGK1-U1A* and *MFA2P-U1A* mRNAs in P-bodies (Figure III.4G-J). Readdition of glucose leads to rapid restoration of polysomes

(Figure III.4K) and loss of Dcp2p, Dhh1p, and *PGK1-U1A* and *MFA2P-U1A* mRNAs from P-bodies (Figure III.4L-O). Interestingly, the *MFA2P-U1A* mRNA accumulated in P-bodies before glucose deprivation and after glucose restoration (Figure III.4E, O). This is consistent with the poorer translation of the *MFA2* mRNA (~24% untranslated) compared with *PGK1* mRNA (~2% untranslated). These observations demonstrate that restoration of translation after glucose deprivation leads to disassembly of P-bodies, either by decapping and degradation of the mRNA within P-bodies or through return of the mRNAs within P-bodies to polysomes.

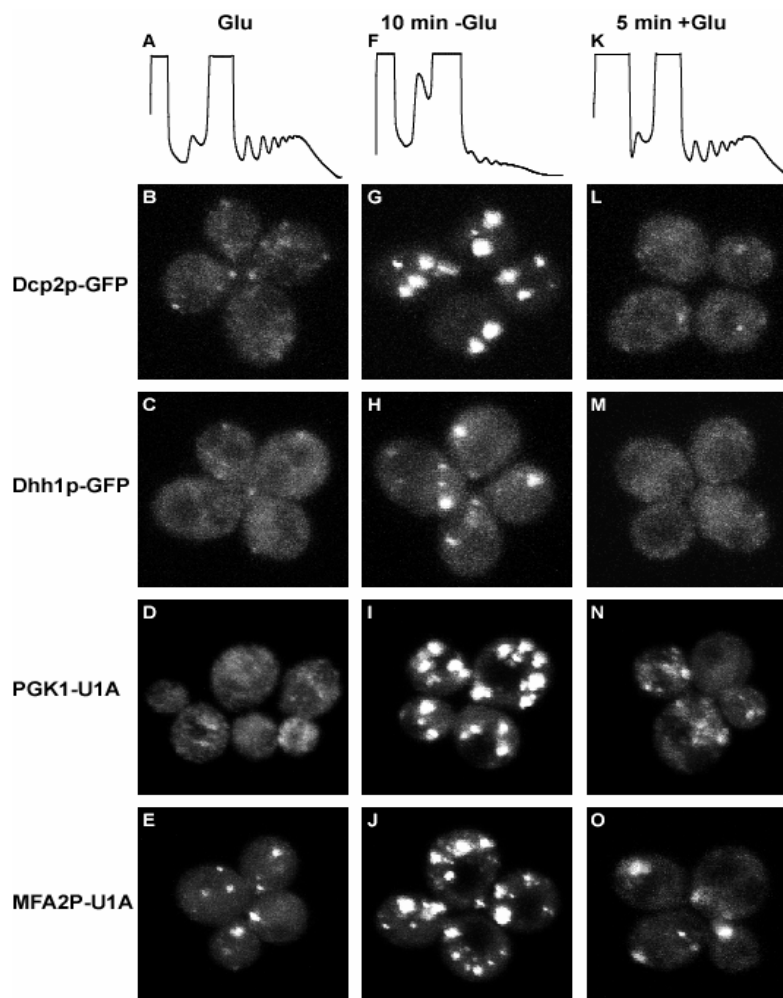


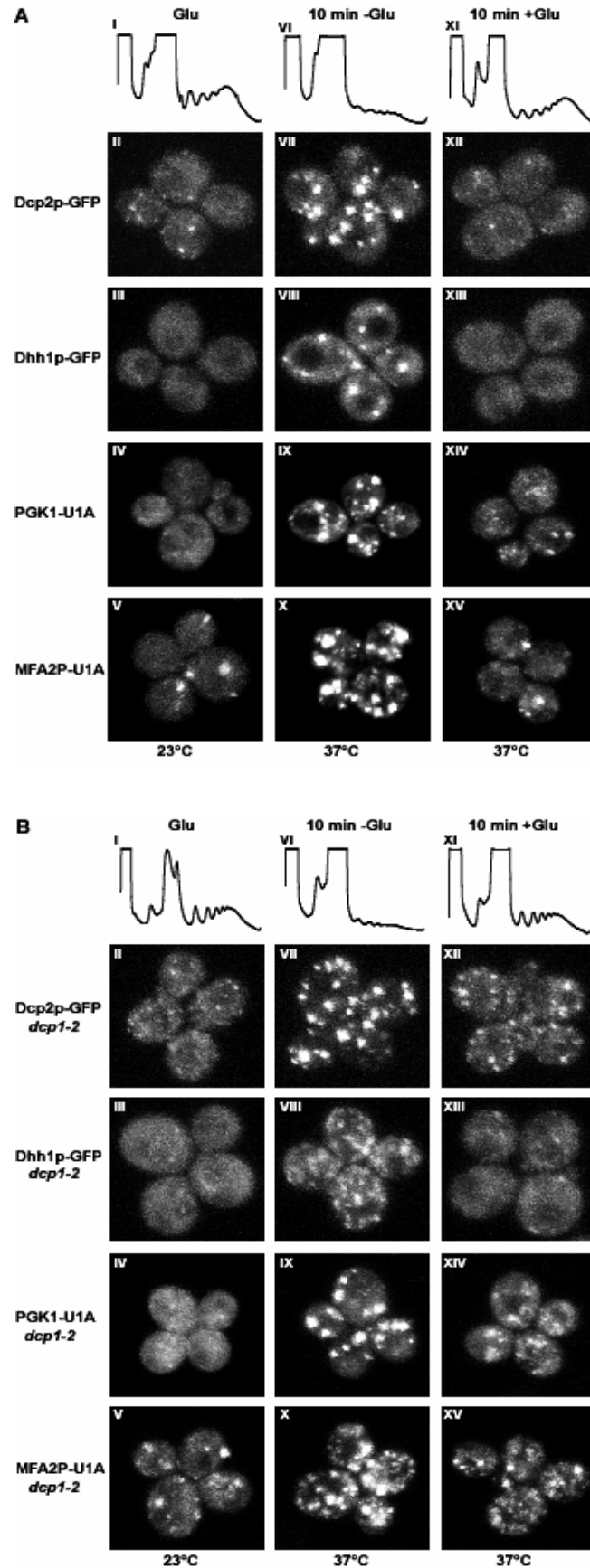
Figure III.4 Disassembly of P-bodies after translation restoration. WT cells expressing a GFP-tagged version of Dcp2p (B,G,L), Dhh1p (C,H,M), or coexpressing plasmids U1A-GFP with *PGK1-U1A* (D,I,N) or *MFA2P-U1A* (E,J,O), were grown in glucose containing medium at 30°C (Glu), shifted for 10 minutes without glucose (10 min -Glu), followed by readdition of glucose for 5 minutes (5 min +Glu). Polysome profiles under each condition are shown (A,F,K).

III.3.2.1 Decrease in P-Bodies Following Glucose Restoration is Largely Independent of Decapping

To evaluate whether decapping was required for the decline of P-bodies after glucose restoration, we examined the response to glucose deprivation and restoration in the *dcp1-2* strain, where decapping is rapidly blocked at the nonpermissive temperature. Similar to WT cells at 37°C (Figure III.5A), glucose deprivation in *dcp1-2* cells led to Dhh1p, Dcp2p, *PGK1-U1A*, and *MFA2P-U1A* mRNAs accumulating in P-bodies (Figure III.5B, VII-X) and a decline in polysomes (Figure III.5B, VI). Readdition of glucose to the *dcp1-2* strain led to reformation of polysomes (Figure III.5B, XI) and a decline in P-bodies (Figure III.5B, XII-XV). This indicates that the disassembly of P-bodies that occurs upon restoration of glucose is therefore not dependent on decapping and degradation of mRNA within P-bodies. However, because P-bodies do not completely return to basal amounts in the *dcp1-2* strain after glucose restoration, some mRNAs may be targeted for decay under these conditions.

III.3.2.2 Decrease in P-Bodies Following Glucose Restoration is Dependent on Translation Initiation

To determine whether translation initiation was required for the disassembly of P-bodies after glucose restoration, we examined the response to glucose deprivation and restoration in a strain carrying a conditional *ts* allele of a subunit of initiation factor eIF3, *prt1-63*. Similar to WT cells (Figure III.5A), glucose deprivation in *prt1-63* cells led to an accumulation of Dcp2p, Dhh1p, and *PGK1-U1A* and *MFA2P-U1A* reporter mRNAs in P-bodies (Figure III.5C, VII-X) and a decline in polysomes (Figure III.5C, VI). Readdition of glucose led to a limited reformation of polysomes (Figure III.5C, XI), and P-bodies largely persisted (Figure III.5C, XII-XV). The small decrease in P-bodies seen under these conditions is likely due to the partial restoration of polysomes in the *prt1-63* strain at 37°C and to some mRNAs being degraded by decapping, consistent with the persistence of small P-bodies in the *dcp1-2* strain after glucose restoration. This indicates that the decline of P-bodies that occurs upon restoration of glucose requires translation initiation, presumably to allow the dynamic movement of mRNAs between polysomes and P-bodies to shift to the translating state. This result argues that mRNAs within P-bodies are reentering translation.



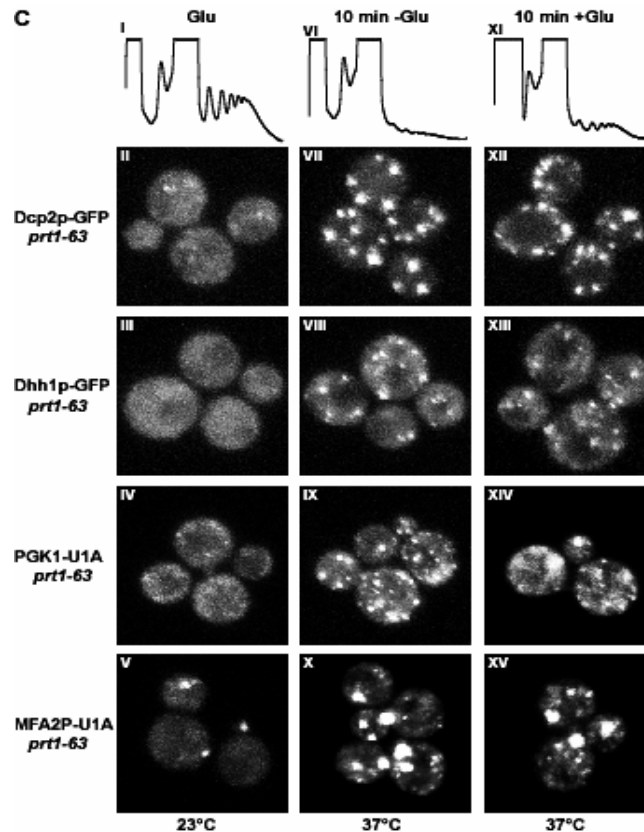


Figure III.5 Translation initiation, but not decapping, is required for mRNAs to exit P-bodies. Cells were grown in glucose-containing medium at 23°C (Glu), shifted to 37°C for 10 minutes without glucose (10 min -Glu), followed by readdition of glucose for 10 minutes at 37°C (10 min +Glu). Polysome profiles under each condition are shown (I,VI,XI). (A) WT, (B) *dcp1-2*, and (C) *prt1-63* cells expressing a GFP-tagged version of Dcp2p (II,VII,XII), Dhh1p (III,VIII,XIII), or co-expressing the plasmids U1A-GFP and PGK1-U1A (IV,IX,XIV), or U1A-GFP and MFA2P-U1A (V,X,XV) are shown.

III.3.3 mRNAs Move Between P-bodies and Polysomes

To demonstrate that P-body mRNAs were reentering translation, we examined the translation status of specific mRNAs on sucrose gradients before and during glucose deprivation, and after glucose restoration. This analysis allows to distinguish mRNAs that are engaged in translation and associated with polyribosomes, from mRNAs that accumulate within P-bodies. This experiment revealed that *MFA2P-U1A* mRNA was primarily associated with polysomes during log growth (~76% polysome-associated) (Figure III.6A, Glu), shifted to nontranslating fractions of the gradient during glucose deprivation (~36% polysome-associated) where P-body components sediment (Figure III.6A, 10 min -Glu) (Hillgren and Parker 2001; Bonnerot et al. 2000; Collier and Parker

2005), and shifted back into the polysome region of the gradient during glucose restoration (~72% polysome-associated) (Figure III.6A, 5 min +Glu). These results are consistent with the subcellular location of the *MFA2P-U1A* mRNA, whose concentration in P-bodies is increased during glucose deprivation and then decreased by glucose restoration (Figure III.4). This result argues that mRNAs can return to translation from a P-body state. Similar shifts were observed with endogenous mRNAs, including *RPL41A*, *PGK1*, and *CYH2* transcripts (Figure III.6B).

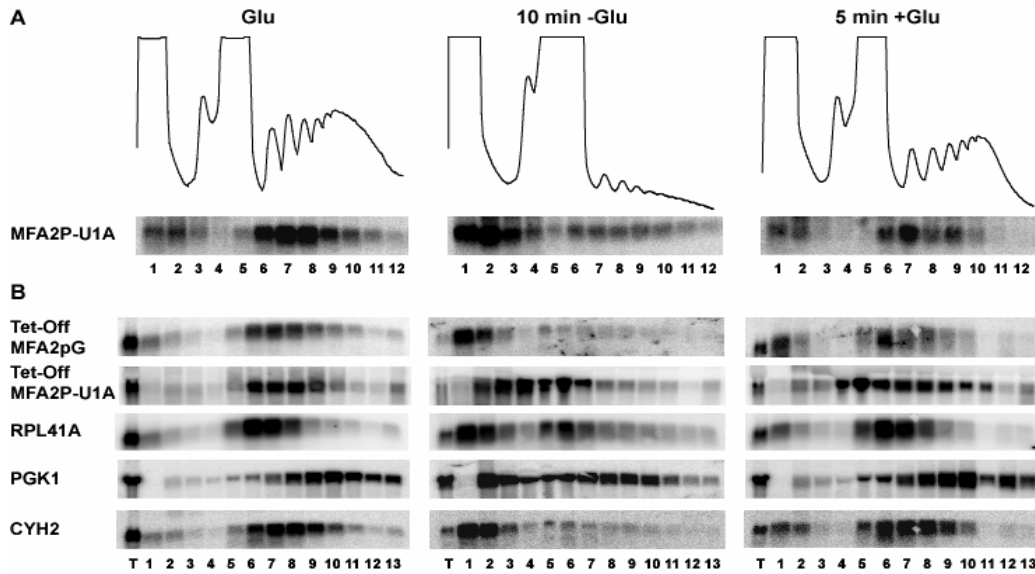


Figure III.6 Movement of mRNAs between a translating and a nontranslating pool. (A) WT cells co-expressing plasmids U1A-GFP with *MFA2P-U1A* or (B) expressing a plasmid containing *MFA2P-U1A* or *MFA2pG* under the control of a tetracycline repression promoter (*Tet-Off MFA2P-U1A* and *Tet-Off MFA2pG*) were grown at 30°C in SC plus glucose (Glu), shifted for 10 minutes to (A) SC with no glucose or (B) SC with no glucose containing doxycycline (10 min -Glu), followed by readdition of glucose for 5 minutes (5 min +Glu). (A) Polysome profiles of the collected sucrose gradients are shown. Fraction 1 corresponds to the top of the gradient. (A and B) Northern blots for the indicated mRNA are shown (M Brengues observations).

In principle, the transcripts associated with polysomes during glucose restoration could be products of new transcription and not those previously localized to P-bodies. To verify that the appearance of mRNAs in the translating pool does not require new transcription, we performed similar experiments by using a tetracycline repression construct (*Tet-Off* promoter) with the *MFA2pG* and the *MFA2P-U1A* reporters, thereby allowing repression of transcription at the time of glucose deprivation. We observed a similar movement of these mRNAs from the polysome pool to the nontranslating pool and back to translation in response to glucose availability (Figure III.6B, *Tet-Off MFA2pG*, *Tet-Off MFA2P-U1A*),

demonstrating that the appearance of *MFA2* transcripts in the translating pool after glucose readdition is caused by the restored translation of preexisting transcripts. We interpret these results to indicate that transcripts can move from polysomes to P-bodies and can then reassociate with polysomes, suggesting that P-bodies can serve as sites of mRNA storage.

III.4 Discussion

III.4.1 Cycling of mRNAs Between Translation and P-bodies

Previous work has demonstrated that mRNAs can move from polysomes to P-bodies when translation is repressed (Chapter II; Collier and Parker 2005). Several observations now indicate that mRNAs can also exit P-bodies to reenter translation. First, P-bodies disperse upon addition of cycloheximide in a manner independent of mRNA degradation (Figure III.1; Figure III.2). Second, when examined either by P-body proteins or reporter mRNAs, polysomes and P-bodies show a reciprocal relationship during glucose deprivation and restoration (Figure III.4), and the disassembly of P-bodies following glucose restoration is independent of decapping and dependent on new translation initiation (Figure III.5). Third, previously transcribed *MFA2* mRNA concentrate in P-bodies and show decreased translation during glucose deprivation, and disperse from P-bodies and show increased translation when glucose is restored (Figure III.6). These results define a bidirectional movement of yeast mRNAs between polysomes and P-bodies. Moreover, these results identify a critical decision point for mRNA fate within P-bodies, where they can either be degraded or return to polysomes.

Because most yeast mRNAs undergo decapping and therefore associate with P-body components to presumably localize to P-bodies (Collier and Parker 2004), and P-body proteins are required for the general repression of translation in response to stress (Collier and Parker 2005), we anticipate that most yeast mRNAs can move between polysomes and P-bodies. Moreover, because P-bodies decline in *dcp1Δ* and *xrn1Δ* cells after cycloheximide addition (Figure III.1), even in cells grown in normal conditions, there is likely to be a continual flux of mRNAs in and out of P-bodies. Finally, mammalian P-bodies are similar to yeast P-bodies (Cougot et al. 2004), suggesting that the movement of mRNAs from P-bodies to polysomes is likely to be a fundamental and conserved property in other eukaryotic cells as well.

The reciprocal movement of mRNAs between polysomes and P-bodies implies that when a nontranslating mRNA enters a P-body, a decision will be made regarding its fate that can lead to either decapping and 5' to 3' degradation, storage, or return to the translating pool. The molecular mechanisms that control this decision within P-bodies remain to be determined. It is possible that specific factors associated with the mRNP might be enough to distinguish between mRNA degradation or retranslation. Future studies addressing the complete biochemical composition of different mRNPs that assemble in P-bodies and the role of individual components that compose these mRNPs should provide insight into this issue.

III.4.2 P-bodies can be Sites of Storage

The ability of mRNAs to return to translation from P-bodies suggests that P-bodies can serve as sites of mRNA storage under certain biological conditions. One example could be yeast in the stationary phase of growth, where cells enter a G0-like state (Gray et al. 2004). Indeed, stationary phase yeast have large P-bodies (Figure II.1), and polysomes are greatly reduced (Brenques et al. 2005). In addition, mRNA accumulated in P-bodies in stationary phase can be stored and return to translation once growth is resumed in the absence of new transcription (Brenques et al. 2005). The storage of yeast mRNAs in P-bodies during stationary phase may provide these cells a mechanism to rapidly resume metabolism in the presence of fresh nutrients. In addition, this suggests that other types of quiescent cells may contain similar pools of stored mRNAs. Thus, mRNA storage in P-bodies is likely to play a significant biological role.

The concept of deadenylated mRNAs being stored in cytoplasmic particles in a translationally repressed state is common in different biological processes including during oogenesis, cell cycle, response to stress, and mRNA transport in neurons. In addition, homologs of the activator of decapping and P-body component Dhh1p, RCK/p54 in mammals, Me31B in *D. melanogaster*, Xp54 in *X. laevis*, and CGH-1 in *C. elegans*, are involved in translation repression and storage of mRNAs in distinct cytoplasmic granules (Weston and Sommerville 2006). This suggests that such mRNP granules could be related to P-bodies, and that these diverse forms of mRNA storage will utilize a conserved mechanism of translation repression and activation in different biological contexts. Moreover, one common property to all these particles is the segregation of mRNAs from the rest of the cytoplasm, suggesting that it may be a mechanism important for translation repression and its regulation.

III.4.2 Why an mRNA Cycle?

This work adds evidence to an emerging theme that translating and nontranslating pools of yeast mRNAs are spatially segregated in the cytoplasm between polysomes and P-bodies (Chapter II). The presence of multiple discrete biochemical and cytological states for cytoplasmic mRNAs strongly implies that mechanisms that control the movement of mRNAs between these states will be important in the regulation of mRNA translation and degradation, and ultimately, cytoplasmic mRNA function.

The cycling of mRNAs between polysomes and P-bodies may confer significant biological advantage to the eukaryotic cell. For example, the ability to sequester mRNAs and then later reuse them allows for a level of rapid and specific translational control of mRNAs. Second, the ability to sequester pools of mRNAs in response to decreased translation initiation rate may maintain a proper balance of translating mRNAs to translation capacity of the cell, thereby providing the cell with a translational capacitor. Finally, because ribosomal components and translation factors are not concentrated in yeast P-bodies, even under stress (Figure II.10; Figure III.7), the movement of mRNAs into P-bodies seems to require the loss of ribosomes and translation initiation factors. Thus, cycling an mRNA through a P-body and back to translation would lead to the reassembly of a new translation initiation complex. Such reassembly may allow for the replacement of any defective translation complexes, thereby providing the cell with an opportunity to restore function to defective translation complexes. Moreover, may allow for specific regulatory mRNA binding proteins present in the mRNP to be exchanged during this process, and thereby update mRNP composition to meet the any new cellular condition. Consistently, it should also be noted that each cycle of mRNA through a P-body may allow for distinction between mRNAs destined for degradation or retranslation. Addressing the biological relevance of mRNA cycling between P-bodies and polysomes will require detailed understanding of the molecular mechanisms by which mRNAs cycle and the properties of such cycle.

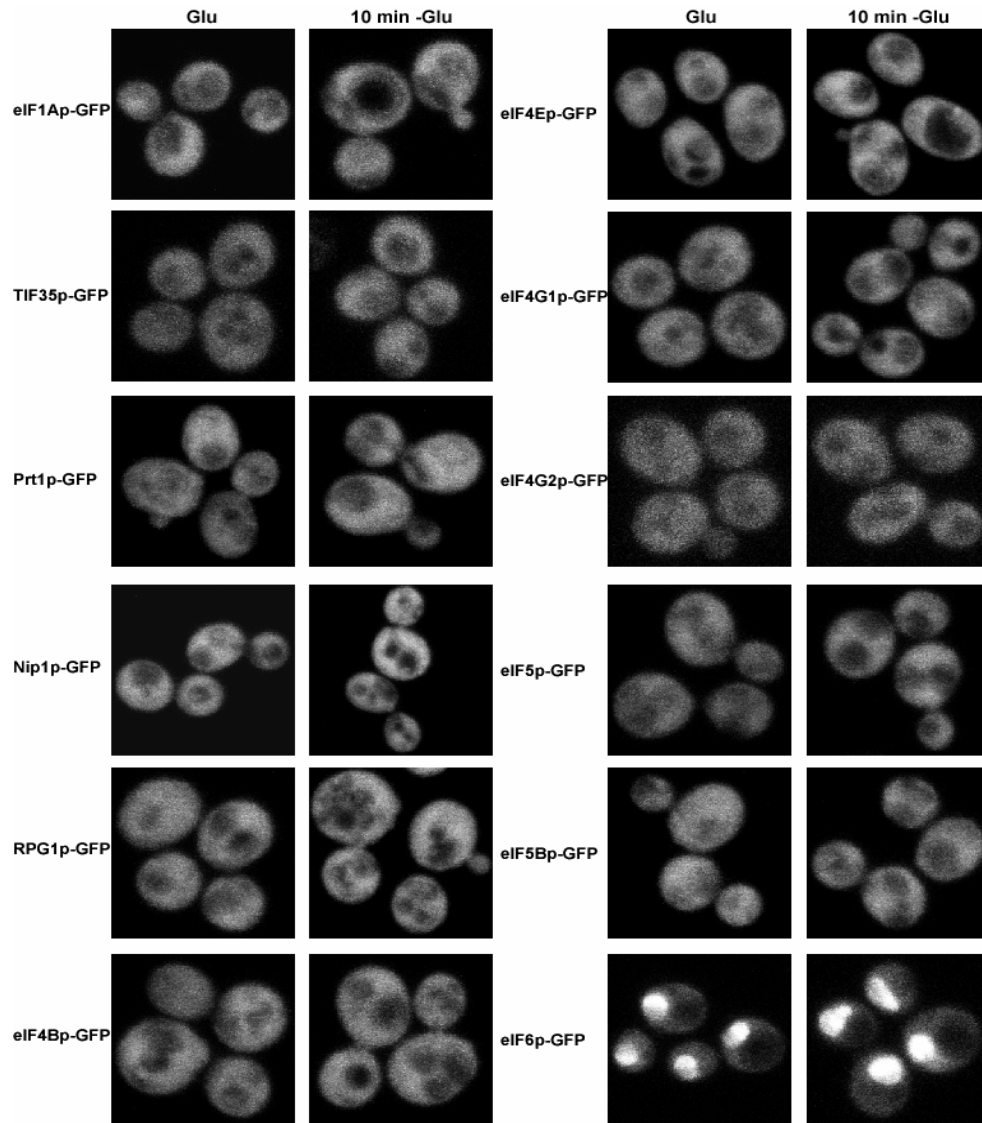


Figure III.7 Translation factors are not associated with P-bodies. Cells were grown in YPGlu (Glu) and then shifted in YP without Glu for 10 minutes (10 min -Glu). Cells expressing GFP-tagged versions of eIF1Ap, TIF35p, Prt1p, Nip1p, RPG1p, eIF4Bp, eIF4Ep, eIF4G1p, eIF4G2p, eIF5p, eIF5Bp and eIF6p are shown.

CHAPTER IV

ANALYSIS OF P-BODY ASSEMBLY IN YEAST

IV. ANALYSIS OF P-BODY ASSEMBLY IN YEAST

Abstract

P-bodies are cytoplasmic foci that contain nontranslating mRNAs in conjunction with proteins involved in translation repression and mRNA decapping and degradation. However, the order of protein assembly into P-bodies and the interactions that promote P-body assembly are largely unknown. To gain insight into how yeast P-bodies assemble, we examined the P-body accumulation of Dcp1p, Dcp2p, Edc3p, Dhh1p, Pat1p, Lsm1p, Xrn1p, Ccr4p, and Pop2p in deletion mutants lacking one or more P-body component. These experiments revealed that Dcp2p and Pat1p are required for recruitment of Dcp1p and of the Lsm1-7p complex to P-bodies, respectively. We also demonstrate that P-body assembly is redundant and no single known component of P-bodies is required for P-body assembly, although both Dcp2p, Pat1p and Edc3p contribute to P-body assembly. In addition, our results indicate that Pat1p can be a nuclear-cytoplasmic shuttling protein and acts early in P-body assembly. In contrast, the Lsm1-7p complex appears to primarily function in a rate limiting step after P-body assembly in triggering decapping. Taken together, these results provide insight both into the function of individual proteins involved in mRNA degradation and the mechanisms by which yeast P-bodies assemble.

IV.1 Introduction

The regulation of mRNA translation and degradation is an important aspect of the control of eukaryotic gene expression. In eukaryotes, two major decay pathways initiate with deadenylation of the 3' poly(A) tail, with the predominant cytoplasmic deadenylase being the Ccr4p/Pop2p/Not1-5p complex (Meyer et al. 2004; Parker and Song 2004). Deadenylation can lead to 3' to 5' degradation, but primarily is followed by removal of the 5' end cap structure by the Dcp1p/Dcp2p decapping enzyme and 5' to 3' degradation of the body of the transcript by the exonuclease Xrn1p. Decapping is a key step of this process, because it precedes and permits the degradation of the body of the mRNA and represents the site of multiple control inputs.

The process of mRNA decapping and translation are mechanistically intertwined and appear to compete with each other, at least in yeast (Beelman and Parker 1994; LaGrandeur and Parker 1999; Muhlrads and Parker 1999b; Schwartz and Parker 1999, 2000). Before decapping, the mRNA must exit translation and assemble into a translationally repressed mRNP complex capable of decapping (Tharun and Parker 2001). These results indicate that before decapping mRNAs cease translation and assemble an mRNP containing the decapping machinery.

Evidence for a discrete population of nontranslating mRNPs has been that nontranslating mRNAs and, the translation repression and decapping machinery accumulate in discrete cytoplasmic foci called P-bodies (also referred as GW- or DCP-bodies) (Bashkirov et al. 1997; Ingelfinger et al. 2002; Lykke-Andersen 2002; van Dijk et al. 2002; Sheth and Parker 2003; Cougot et al. 2004; Collier and Parker 2005; Teixeira et al. 2005). P-bodies have now been observed in yeast, insect cells, nematodes, and mammalian cells and contain various proteins implicated in mRNA degradation, including the decapping enzyme (Dcp1p/Dcp2p), activators of decapping Dhh1p, Pat1p, Lsm1-7p, Edc3p, and the exonuclease Xrn1p (Eulalio et al. 2007). Consistent with the reciprocal relationship between mRNA translation and degradation, the assembly of P-bodies is in a dynamic competition with translation (Chapter II). Moreover, the mRNPs that accumulate in P-bodies have been suggested to be functionally involved in mRNA decapping (Sheth and Parker 2003; Cougot et al. 2004), mRNA storage (Chapter III; Bhattacharyya et al. 2006), general translation repression (Holmes et al. 2004; Collier and Parker 2005), miRNA-mediated repression (Jakymiw et al. 2005; Liu et al. 2005a; Pillai et al. 2005), nonsense-mediated decay (Unterholzner and Izaurralde 2004 ; Sheth and Parker 2006), and possibly viral packaging (Beliakova-Bethell et al. 2006; Beckham et al. 2007). The

existence of P-bodies as a discrete cytoplasmic compartment containing nontranslating mRNAs that can be either degraded or stored and retranslated suggests that understanding the nature of the specific protein–protein and protein–RNA interactions that mediate the assembly of both mRNPs containing the decapping machinery, as well as larger scale P-bodies that are visible microscopically, will be important in understanding the control of mRNA translation and degradation.

Two aspects of the assembly of P-bodies have emerged. First, it has been shown that P-bodies require mRNA for their formation and integrity (Chapter II). Second, coimmunoprecipitation and two-hybrid experiments have revealed a dense network of interactions between the components of the mRNA translation repression, decapping and degradation machinery found in P-bodies (Hata et al. 1998; Collier et al. 2001; Ho et al. 2002; Fenger-Gron et al. 2005; Gavin et al. 2006; Krogan et al. 2006). However, the specific interactions that mediate the assembly of the translation repression and decapping machinery on mRNAs to form an mRNP that is capable of being incorporated into a P-body, as well as the aggregation of individual mRNPs into larger scale structures are largely unknown.

Several proteins have been described as affecting P-body assembly in yeast and mammalian cells. However, the competition between translation and P-body formation suggests that lack of a specific protein can affect P-body formation by either reducing the pool of nontranslating mRNAs, or by decreasing the aggregation of the nontranslating P-body mRNPs. For example, in mammalian cells, P-bodies are greatly reduced by knockdown of GW182, RCK/p54, RAP55, miRNA biogenesis in general, LSM4, Hedls/Ge-1, or 4E-T (Andrei et al. 2005; Ferraiuolo et al. 2005; Jakymiw et al. 2005; Pauley et al. 2006). However, for at least LSM4 knockdowns, P-bodies are restored when translation initiation is inhibited by arsenite (Kedersha et al. 2005). Additionally, depletion of GW182 relieves translational repression by miRNAs (Jakymiw et al. 2005; Liu et al. 2005b; Meister et al. 2005; Rehwinkel et al. 2005; Behm-Ansmant et al. 2006). Moreover, 4-ET and RCK/p54 are known to function in translational repression (Andrei et al. 2005; Ferraiuolo et al. 2005). These observations argues that LSM4p, and possibly these other factors as well, are not required for P-body assembly *per se*, but instead contribute to P-body formation in mammalian cells by increasing the pool of translationally repressed mRNAs. Similarly, in yeast, Dhh1p and Pat1p are required for global translation repression of mRNAs, targeting mRNAs for decapping, and promoting their assembly into P-bodies (Collier and Parker 2005). Strains lacking Dhh1p and Pat1p are defective in translation repression in response to glucose deprivation and amino acid starvation,

mRNA decapping, and consequently in P-body formation (Holmes et al. 2004; Collier and Parker 2005). In contrast, overexpression of Dhh1p or Pat1p results in inhibition of translation and induces P-body formation (Collier and Parker 2005).

Given the limited understanding of how P-bodies assemble our goal in this work was to determine the requirement for numerous yeast proteins in P-body assembly and organization. To this end, we examined P-body formation and composition in strains defective in proteins known to accumulate in P-bodies. These experiments revealed specific dependencies in P-body assembly for individual proteins. In addition, this work argues that P-body assembly is redundant and no single known component of P-bodies is absolutely required for P-body assembly although Dcp2p, Pat1p and Edc3p can affect P-body assembly. Taken together, these results provide insight both into the function of individual proteins involved in mRNA degradation and the mechanisms by which yeast P-bodies assemble.

IV.2 Materials and Methods

IV.2.1 Yeast Strains and Growth Conditions

The genotypes of all strains used in this study are listed in Table IV.1. All strains have *GAL1* upstream activating sequence-regulated *PGK1pG* and *MFA2pG* genes, as well as the *LEU2* gene, collectively termed *LEU2pm*, integrated at the *CUP1* locus (Hatfield et al. 1996). Proteins were C-terminal tagged with GFP following the PCR-based gene modification method described by Longtine et al. (1998). These fusion proteins include the full-length protein and are all at least partially functional (Sheth and Parker 2003). Yeast crosses were carried out using standard laboratory procedures. Strains were grown on YP supplemented with 2% Dextrose (Glu) as carbon source. Strains were grown at 30 °C.

Table IV.1 Yeast strains.

Strain	Genotype	Source
yRP1724	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DHH1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1726	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DCP1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1727	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DCP2-GFP (NEO)</i>	Sheth and Parker 2003
yRP1728	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG PAT1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1729	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG LSM1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1730	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG XRN1-GFP (NEO)</i>	Sheth and Parker 2003

yRP1731	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG CCR4-GFP (NEO)</i>	Sheth and Parker 2003
yRP1736	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 DHH1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1737	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO DHH1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1738	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 DHH1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1739	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 DHH1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1740	MATα <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 DHH1-GFP (NEO)</i>	Sheth and Parker 2003
yRP1758	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG EDC3-GFP (NEO)</i>	Kshirsagar and Parker 2004
yRP1923	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 DCP2-GFP (NEO)</i>	Teixeira et al. 2005
yRP1924	MATa <i>leu2-3,112 trp1 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 LSM1-GFP (NEO)</i>	Teixeira et al. 2005
yRP1936	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 DCP2-GFP (NEO)</i>	Brengues et al. 2005
yRP2213	MATα <i>leu2-3,112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG POP2-GFP (NEO)</i>	This study
yRP2215	MATα <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO DHH1-GFP (NEO)</i>	This study
yRP2216	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 DHH1-GFP (NEO)</i>	This study
yRP2217	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG edc3::NEO DHH1-GFP (NEO)</i>	This study
yRP2187	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO DCP2-GFP (NEO)</i>	This study
yRP2188	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO DCP2-GFP (NEO)</i>	This study
yRP2183	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 DCP2-GFP (NEO)</i>	This study
yRP2181	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 DCP2-GFP (NEO)</i>	This study
yRP2182	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 DCP2-GFP (NEO)</i>	This study
yRP2189	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG edc3::NEO DCP2-GFP (NEO)</i>	This study
yRP2218	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO DCP1-GFP (NEO)</i>	This study
yRP2219	MAT α <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO DCP1-GFP (NEO)</i>	This study
yRP2220	MAT α <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 DCP1-GFP (NEO)</i>	This study
yRP2221	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 DCP1-GFP (NEO)</i>	This study
yRP2222	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 DCP1-GFP (NEO)</i>	This study
yRP2223	MATa <i>leu2-3,112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 DCP1-GFP (NEO)</i>	This study
yRP2224	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 DCP1-GFP (NEO)</i>	This study
yRP2225	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG edc3::NEO DCP1-GFP (NEO)</i>	This study
yRP2226	MATα <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO LSM1-GFP (NEO)</i>	This study
yRP2227	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO LSM1-GFP (NEO)</i>	This study
yRP2228	MATα <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 LSM1-GFP (NEO)</i>	This study
yRP2229	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 LSM1-GFP (NEO)</i>	This study
yRP2230	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 LSM1-GFP (NEO)</i>	This study
yRP2231	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 LSM1-GFP (NEO)</i>	This study
yRP2232	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG edc3::NEO LSM1-GFP (NEO)</i>	This study
yRP2233	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO PAT1-GFP (NEO)</i>	This study
yRP2234	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO PAT1-GFP (NEO)</i>	This study

yRP2235	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 PAT1-GFP (NEO)</i>	This study
yRP2236	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 PAT1-GFP (NEO)</i>	This study
yRP2237	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 PAT1-GFP (NEO)</i>	This study
yRP2238	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 PAT1-GFP (NEO)</i>	This study
yRP2239	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 PAT1-GFP (NEO)</i>	This study
yRP2240	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG edc3::NEO PAT1-GFP (NEO)</i>	This study
yRP2241	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO XRN1-GFP (NEO)</i>	This study
yRP2242	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO XRN1-GFP (NEO)</i>	This study
yRP2243	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 XRN1-GFP (NEO)</i>	This study
yRP2244	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 XRN1-GFP (NEO)</i>	This study
yRP2245	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 XRN1-GFP (NEO)</i>	This study
yRP2246	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 XRN1-GFP (NEO)</i>	This study
yRP2247	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 XRN1-GFP (NEO)</i>	This study
yRP2248	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG edc3::NEO XRN1-GFP (NEO)</i>	This study
yRP2249	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO EDC3-GFP (NEO)</i>	This study
yRP2250	MATa <i>leu2-3,112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO EDC3-GFP (NEO)</i>	This study
yRP2251	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 EDC3-GFP (NEO)</i>	This study
yRP2252	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 EDC3-GFP (NEO)</i>	This study
yRP2253	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 EDC3-GFP (NEO)</i>	This study
yRP2254	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 EDC3-GFP (NEO)</i>	This study
yRP2255	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 EDC3-GFP (NEO)</i>	This study
yRP2256	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 EDC3-GFP (NEO)</i>	This study
yRP2257	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pop2::NEO CCR4-GFP (NEO)</i>	This study
yRP2258	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 CCR4-GFP (NEO)</i>	This study
yRP2259	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 CCR4-GFP (NEO)</i>	This study
yRP2260	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 CCR4-GFP (NEO)</i>	This study
yRP2261	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 CCR4-GFP (NEO)</i>	This study
yRP2262	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 CCR4-GFP (NEO)</i>	This study
yRP2263	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 CCR4-GFP (NEO)</i>	This study
yRP2264	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG edc3::NEO CCR4-GFP (NEO)</i>	This study
yRP2265	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG ccr4::NEO POP2-GFP (NEO)</i>	This study
yRP2266	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 POP2-GFP (NEO)</i>	This study
yRP2267	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 POP2-GFP (NEO)</i>	This study
yRP2268	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 POP2-GFP (NEO)</i>	This study
yRP2269	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 POP2-GFP (NEO)</i>	This study
yRP2270	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 POP2-GFP (NEO)</i>	This study
yRP2271	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG xrn1::URA3 POP2-GFP (NEO)</i>	This study

yRP2272	MATa <i>ade2 leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG edc3::NEO POP2-GFP (NEO)</i>	This study
yRP2273	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp2::TRP1 xrn1::URA3 DCP1-GFP (NEO)</i>	This study
yRP2277	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 dcp2::TRP1 PAT1-GFP (NEO)</i>	This study
yRP2279	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 dcp2::TRP1 DHH1-GFP (NEO)</i>	This study
yRP2280	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3 lsm1::TRP1 PAT1-GFP (NEO)</i>	This study
yRP2283	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 lsm1::TRP1 DCP1-GFP (NEO)</i>	This study
yRP2284	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 lsm1::TRP1 DCP2-GFP (NEO)</i>	This study
yRP2286	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 lsm1::TRP1 XRN1-GFP (NEO)</i>	This study
yRP2289	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 lsm1::TRP1 EDC3-GFP (NEO)</i>	This study
yRP2290	MATa <i>leu2-3,112 trp1 ura3-52 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 lsm1::TRP1 DCP1-GFP (NEO)</i>	This study
yRP2292	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 lsm1::TRP1 DCP2-GFP (NEO)</i>	This study
yRP2294	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 lsm1::TRP1 XRN1-GFP (NEO)</i>	This study
yRP2295	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 lsm1::TRP1 EDC3-GFP (NEO)</i>	This study
yRP2297	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 xrn1::URA3 CCR4-GFP (NEO)</i>	This study
yRP2299	MATa <i>leu2-3,112 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG pat1::LEU2 xrn1::URA3 POP2-GFP (NEO)</i>	This study
yRP2300	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 xrn1::URA3 CCR4-GFP (NEO)</i>	This study
yRP2302	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG dhh1::URA3 xrn1::URA3 POP2-GFP (NEO)</i>	This study
yRP2303	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 xrn1::URA3 CCR4-GFP (NEO)</i>	This study
yRP2304	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 xrn1::URA3 POP2-GFP (NEO)</i>	This study
yRP2382	MATa <i>leu2-3,112 trp1 ura3-52 his4-539 lys2-201 cup1::LEU2/PGK1pG/MFA2pG lsm1::TRP1 xrn1::URA3 PAT1-GFP (NEO)</i>	This study

IV.2.2 Preparation of Cells for Confocal Microscopy

Cells were grown to an OD₆₀₀ of 0.3–0.35 in YPGlu. For observation, cells were washed once and resuspended in SC supplemented with amino acids and Glu and immediately observed. For glucose depletion, cells were washed in YP without Glu and resuspended in the same medium for 10 more minutes before being collected. Cells were then washed with SC plus amino acids without Glu, resuspended in the same medium, and examined. For observation at high cell density, cells were grown in SC to an OD₆₀₀ of 1.0. Cells were then washed and resuspended in SC plus amino acids without Glu before observation. For *in vivo* 4',6-diamidino-2-phenylindole (DAPI, Sigma) staining, 1 ml of cells was harvested by centrifugation and resuspended in 150 µl of medium containing 1.5 µl of DAPI (1 mg/ml). After shaking for 30 minutes at 30°C, cells were again pelleted, resuspended in 150 µl of media alone, and observed. Observations were made using Nikon PCM 2000 confocal microscope (Melville, NY) using a 100x objective with a 3x

zoom using Compix software (Sewickley, PA). All images are a Z-series compilation of 6–10 images in a stack.

IV.2.3 Polysome Analysis

Polysome analysis was performed as described previously in chapter III.

IV.3 Results

IV.3.1 General Strategy

To examine the role of various proteins in P-body assembly, we analyzed the contributions and dependencies to P-body formation of Dcp1p, Dcp2p, Edc3p, Dhh1p, Pat1p, Lsm1p, Xrn1p, Ccr4p, and Pop2p. For each protein a series of strains was constructed lacking one protein and individually containing a GFP fusion version of the other proteins. The GFP fusion proteins are integrated at the relevant genomic locus under the control of the endogenous promoter, include the full length protein C-terminal tagged to GFP, and are all at least partially functional (Sheth and Parker 2003). Each strain was then first examined microscopically for accumulation of each protein in P-bodies in yeast grown in glucose-supplemented rich medium during the mid-log growth phase, wherein P-bodies are small in WT cells (Chapter II). This provides an ideal condition to identify lesions that increase the size of P-bodies. In some cases, strains were also observed under the stress condition of glucose deprivation, which decreases translation initiation and leads to the accumulation of large P-bodies (Chapter II; Chapter III; Ashe et al. 2000). Because P-bodies are large in WT cells undergoing a response to glucose deprivation, this provides an opportunity to identify lesions that reduce or compromise P-body assembly. Finally, where relevant we examined the accumulation of some proteins in strains lacking two proteins. The important results are discussed below.

IV.3.2 Defects in the Catalysis of Decapping or 5' to 3' Exonuclease Digestion Lead to the Accumulation of P-Bodies

Several observations demonstrated that blocking the catalytic events of decapping or 5' to 3' exonuclease digestion led to increased P-bodies. For example, strains lacking the 5' to 3' exonuclease Xrn1p, accumulated Dhh1p, Pat1p, Lsm1p, Dcp1p, Dcp2p, and Edc3p in

P-bodies even in mid-log cultures where yeast P-bodies are generally small (Figure IV.1B, III–VIII). Similarly, during mid-log growth the *dcp1Δ* strain showed high levels of accumulation of Dhh1p, Pat1p, Lsm1p, Dcp2p, Edc3p, and Xrn1p in P-bodies (Figure IV.1C, III–IX). These large P-bodies observed in *xrn1Δ* or *dcp1Δ* strains did not increase significantly when cells were subjected to glucose deprivation, by shifting the cells for 10 minutes to medium lacking glucose (data not shown; Figure IV.2B, IV), which is consistent with the observation that *xrn1Δ* and *dcp1Δ* strains are unable to repress translation during glucose deprivation (Holmes et al. 2004; Collier and Parker 2005).

Because the *xrn1Δ* blocks 5' to 3' degradation and the *dcp1Δ* blocks decapping, these observations indicate that defects in the catalytic steps of decapping or 5' to 3' degradation lead to increased P-bodies. Moreover, because all the proteins examined accumulated in P-bodies under these conditions, it argues that no other known component of P-bodies is strictly dependent on Xrn1p or Dcp1p for accumulation in P-bodies. This is consistent and extends earlier work showing an accumulation of Dhh1p in P-bodies in *xrn1Δ* or *dcp1Δ* strains (Sheth and Parker 2003). Moreover, these observations are consistent with the observation that RNAi-mediated knockdown of the decapping enzyme Dcp2p in mammalian cells leads to an accumulation of P-bodies as assessed by the human ortholog of Dhh1p, RCK/p54, as well as hCcr4p, and hLsm1p (Andrei et al. 2005).

Interestingly, Ccr4p and Pop2p were also clearly observed to accumulate in P-bodies in the *xrn1Δ*, *dcp1Δ*, and *dcp2Δ* mutant strains (Figures IV.1B, I, II; IV.1C, I, II; IV.1D, I, II). Similarly, we observed that Ccr4p and Pop2p accumulated at low levels in P-bodies during glucose deprivation (Figure IV.8A). This indicates that Ccr4p and Pop2p can accumulate in yeast P-bodies. This is consistent with earlier observations that other components of the Ccr4p/Pop2p/Not complex, the Not1-5 proteins, are detected in P-bodies in *dcp1Δ* strains (Muhlrad and Parker 2005) and that yeast Ccr4p can be observed in small foci during a water stress response (Sheth and Parker 2003). In addition, mammalian and *D. melanogaster* Ccr4p also accumulate in P-bodies (Cougot et al. 2004; Temme et al. 2004; Andrei et al. 2005). These results indicate that the Ccr4p/Pop2p/Not1-5p complex accumulates in P-bodies in yeast as well as other eukaryotes. However, the limited amount of Ccr4p-GFP and Pop2p-GFP seen in P-bodies compared with other factors suggests that these proteins are present at reduced stoichiometry.

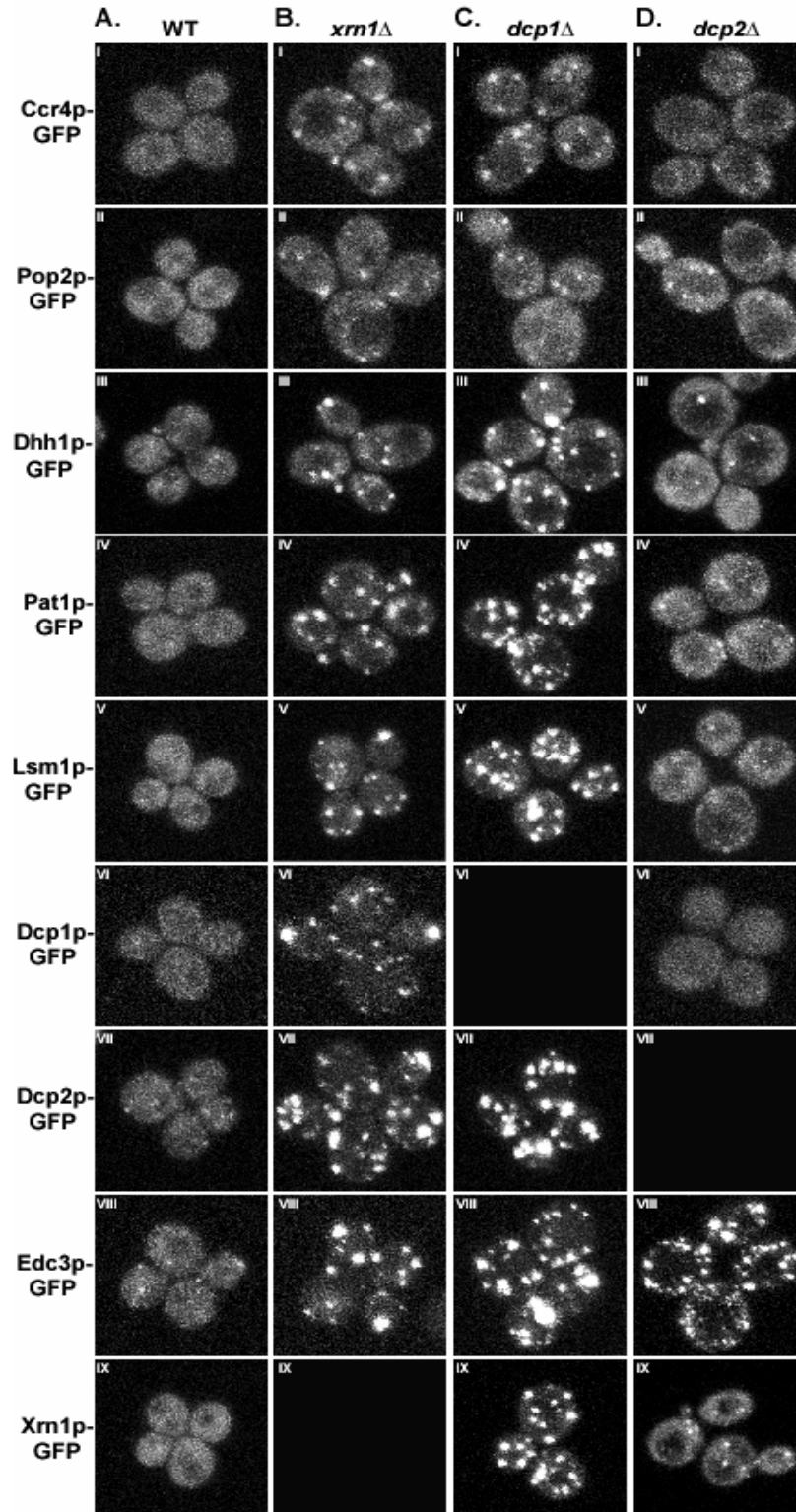


Figure IV.1 Blocks in the catalytic steps of decapping or 5' to 3' degradation lead to increased P-bodies. (A) WT, (B) *xrn1Δ*, (C) *dcp1Δ* and (D) *dcp2Δ* cells expressing a GFP-tagged version of Ccr4p (I), Pop2p (II), Dhh1p (III), Pat1p (IV), Lsm1p (V), Dcp1p (VI), Dcp2p (VII), Edc3p (VIII) and Xrn1p (IX) are shown. All images are shown at the same scale and a 3μm-scale bar is shown in the lower right panel.

IV.3.2.1 Dcp2p Is Required for Dcp1p Accumulation in P-Bodies

Strains lacking Dcp2p also showed increased P-bodies in midlog cultures with accumulations of Ccr4p, Pop2p, Dhh1p, Pat1p, Lsm1p, Edc3p, and Xrn1p in P-bodies (Figure IV.1D, I–V, VIII–IX), but with some differences from *dcp1Δ* or *xrn1Δ* strains. First, despite the increased P-bodies in *dcp2Δ* strains as judged by other protein components, Dcp1p-GFP was distributed throughout the cell and completely absent from P-bodies (Figure IV.1D, VI). Moreover, Western analysis showed that Dcp1p-GFP was still efficiently expressed in the *dcp2Δ* strain (data not shown). Because *dcp1Δ* and *dcp2Δ* strains have identical and complete blocks to decapping (Beelman et al. 1996; Dunckley and Parker 1999) and all other proteins accumulate in P-bodies in the *dcp2Δ* strain, this observation argues that Dcp2p is required to recruit Dcp1p to P-bodies.

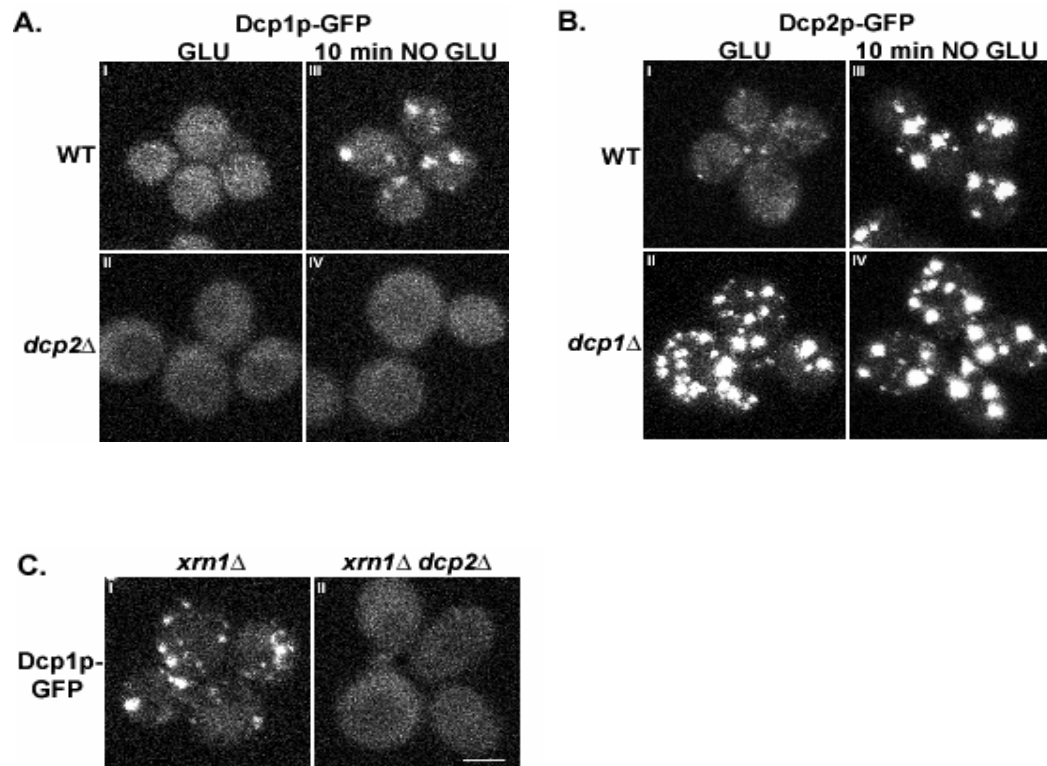


Figure IV.2 Dcp2p is required for Dcp1p recruitment to P-bodies. (A) Distribution of GFP-tagged version of Dcp1p in WT (I,III) and *dcp2Δ* (II,IV) cells is shown. (B) Distribution of GFP-tagged version of Dcp2p in WT (I,III) and *dcp1Δ* (II,IV) cells is shown. Pictures of cells before glucose deprivation (GLU) and after 10 minutes of glucose deprivation (10 min NO GLU) treatment are shown. (C) Distribution of a GFP-tagged version of Dcp1p in *xrn1Δ* (I) and *xrn1Δ dcp2Δ* (II) cells is shown. All images are shown at the same scale and a 3μm-scale bar is shown in the (C) lower right panel.

Two additional observations confirm Dcp2p is required for Dcp1p recruitment to P-bodies. First, even when *dcp2Δ* strains are undergoing a response to glucose deprivation, which increases Dcp1p-GFP accumulation in P-bodies in WT strains, Dcp1p-GFP is completely absent from P-bodies in a *dcp2Δ* strain (Figure IV.2A, IV). In contrast, Dcp2p-GFP accumulates in P-bodies independent of Dcp1p with or without glucose deprivation conditions (Figure IV.2B, II, IV). Moreover, other P-body markers still accumulate in P-bodies in a *dcp2Δ* with or without glucose deprivation conditions (Figure IV.1D; data not shown). Second, although Dcp1p-GFP accumulates in P-bodies to high levels in the *xrn1Δ* strain (Figure IV.2C, I), this accumulation is completely lost in the *xrn1Δ dcp2Δ* double mutant strain (Figure IV.2C, II). The requirement of Dcp2p for Dcp1p recruitment to P-bodies is consistent with the direct physical interaction between Dcp1p and Dcp2p in yeast (Steiger et al. 2003; She et al. 2004, 2006) and with the observation that specific point mutations in residues of Dcp2p that disrupt the physical interaction between Dcp2p and Dcp1p prevent Dcp1p from accumulating in P-bodies (CJ Decker and R Parker, unpublished observation). These results indicate that Dcp1p is recruited to the P-body through interactions with Dcp2p.

IV.3.2.2 Dcp2p has an Additional Role in P-Body Assembly or Persistence

A second interesting result observed with the *dcp2Δ* strain was that P-bodies were reproducibly smaller than in *dcp1Δ* or *xrn1Δ* strains (Figure IV.1B–D). This can be easily seen by comparing the accumulation of Dhh1p, Pat1p, Lsm1p, or Xrn1p in *dcp1Δ* strains compared with *dcp2Δ* strains (compare Figure IV.1C, III–V, IX with IV.1D, III–V, IX). Because both *dcp1Δ* and *dcp2Δ* strains show absolute blocks to decapping (Beelman et al. 1996; Dunckley and Parker 1999), this difference in P-body size must reflect a difference in the function of Dcp1p or Dcp2p in the formation of P-bodies independent of their catalytic role in decapping. Interestingly, the smaller size of P-bodies in the *dcp2Δ* strain relative to the *dcp1Δ* strain was not as pronounced when Edc3p was examined (Figure IV.1D, VIII), suggesting some difference in how Edc3p's accumulation in P-bodies is affected compared with other factors.

Two possible models can explain the difference between P-body size in the *dcp1Δ* and *dcp2Δ* strains, which we distinguished by the analysis of *dcp1Δ dcp2Δ* double mutants. First, it could be that Dcp1p is also an inhibitor of P-body formation and that P-bodies are larger in the *dcp1Δ* strain compared with the *dcp2Δ* strain because of the loss of the Dcp1p inhibitory role on P-body formation. In this model, a *dcp1Δ dcp2Δ* double mutant is predicted to have large P-bodies similar to the *dcp1Δ* strain. Alternatively, Dcp2p might

have an additional role in the assembly or maintenance of P-bodies, possibly through additional protein–protein interactions. In this model, a *dcp1Δ dcp2Δ* strain is predicted to be phenotypically similar to a *dcp2Δ* strain.

To distinguish these models, we examined the accumulation of Dhh1p and Pat1p in *dcp1Δ dcp2Δ* double mutants strains as compared with *dcp1Δ* and *dcp2Δ* single mutant strains. Strikingly, we observed that both Dhh1p-GFP and Pat1p-GFP accumulated in P-bodies in the *dcp1Δ dcp2Δ* double mutant to the same extent seen in the *dcp2Δ* single mutant (Figure IV.3E, F). This provides evidence that Dcp2p has some role in the assembly or maintenance of P-bodies. A likely possibility is that the multiple interactions that Dcp2p has with other components of P-bodies contribute to the assembly of the P-body mRNP or to the interactions that allow P-body aggregation into visible structures in the light microscope.

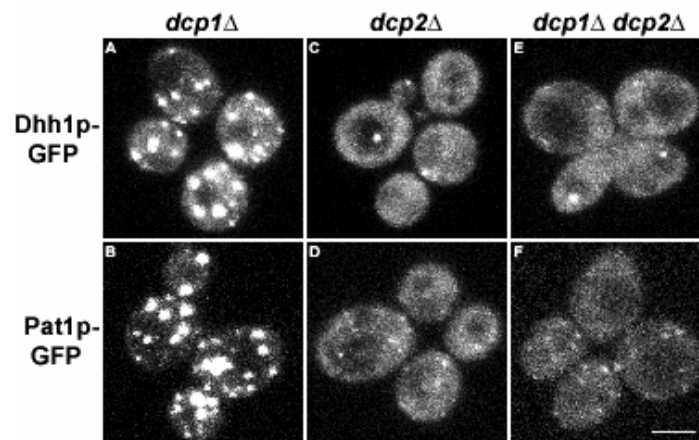


Figure IV.3 Dcp2p plays a role in the formation of P-bodies. Shown are *dcp1Δ* (left panel), *dcp2Δ* (middle panel) and *dcp1Δ dcp2Δ* (right panel) cells expressing a GFP-tagged version of Dhh1p (A,C,E) and Pat1p (B,D,F). All images are shown at the same scale and a 3μm-scale bar is shown in the lower right panel.

IV.3.3 Minor Affects of Ccr4p and Pop2p on P-Body Assembly

We also examined how Ccr4p and Pop2p affect the formation and composition of P-bodies in both mid-log cultures and in response to glucose deprivation. We observed that *ccr4Δ* led to a small, but clear, decrease in the accumulation of Dcp2p-GFP and Edc3p-GFP in P-bodies in mid-log cultures (Figure IV.4B, V, VI). Furthermore, we observed *pop2Δ* led to a small decrease in the accumulation Dcp1p-GFP and Dcp2p-GFP in P-bodies (Figure IV.4C, IV, V). This is consistent with the *ccr4Δ* and *pop2Δ* affecting deadenylation (Tucker et al. 2001) and previous work arguing that in mid-log growth

phase deadenylated mRNAs preferentially associate with P-body components such as Lsm1p (Tharun and Parker 2001). In mid-log cultures, the *ccr4Δ* or *pop2Δ* strains did not show a significant difference in the concentration of Dhh1p-GFP, Pat1p-GFP, Lsm1p-GFP, or Xrn1p-GFP in P-bodies (Figures IV.4B, I–III, VII; IV.4C, I–III, VII). However, because it is difficult to observe these proteins in P-bodies in mid-log growth, we are unable to determine if the *ccr4Δ* or *pop2Δ* affects their accumulation in P-bodies under these conditions.

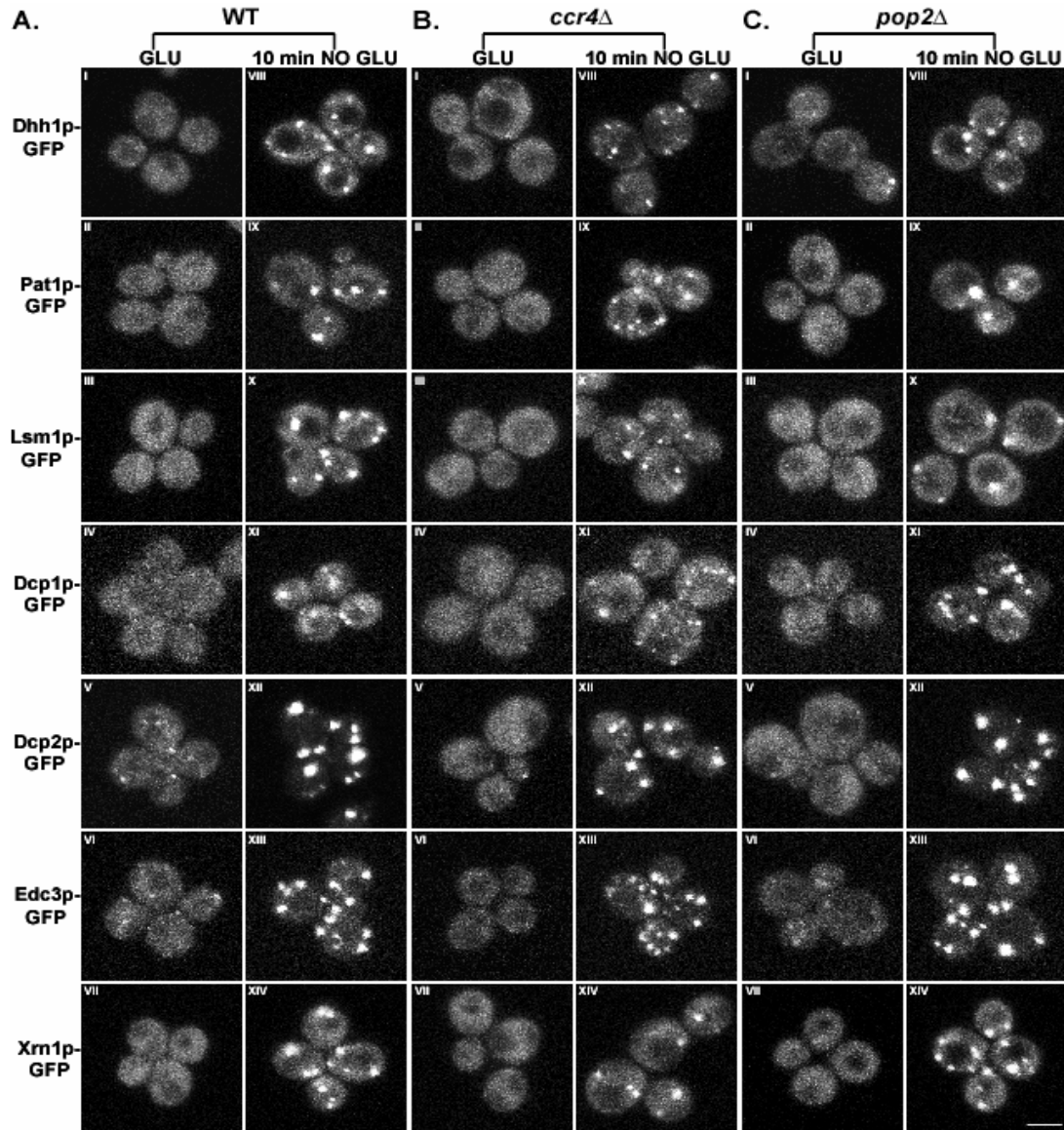


Figure IV.4 Defects in the mRNA deadenylases Ccr4p and Pop2p do not significantly affect P-body formation and composition. (A) WT, (B) *ccr4Δ* and (C) *pop2Δ* cells expressing a GFP-tagged version of Dhh1p (I,VIII), Pat1p (II,IX), Lsm1p (III,X), Dcp1p (IV,XI), Dcp2p (V,XII), Edc3p (VI,XIII) and Xrn1p (VII,XIV). Pictures of cells before glucose deprivation (GLU) and after 10 minutes of glucose deprivation (10 min NO GLU) treatment are shown. All images are shown at the same scale and a 3μm-scale bar is shown in the lower right panel.

In the *ccr4Δ* strains in response to glucose deprivation stress, where decreases in P-bodies are more easily identified, Pat1p-GFP and Edc3p-GFP accumulated in P-bodies in a manner similar to WT cells (Figure IV.4B, IX, XIII), whereas Dhh1p-GFP, Lsm1p-GFP, Dcp1p-GFP, Dcp2p-GFP, and Xrn1p-GFP accumulated but less pronouncedly compared with WT cells (Figure IV.4B, VIII, X–XII, XIV). This slightly reduced accumulation of P-bodies in this mutant may be due to the reduced levels of protein expression in *ccr4Δ* strains, as has been shown to be the case for Dhh1p (Sheth and Parker 2003). In contrast, after glucose deprivation in a *pop2Δ* strain, Dhh1p, Pat1p, Lsm1p, Dcp1p, Dcp2p, Edc3p, and Xrn1p accumulated in P-bodies in a manner similar to WT cells (Figure IV.4C, VIII–XIV). Taken generally, these results indicate that during glucose deprivation, neither Ccr4p nor Pop2p has a large impact on P-body assembly. This is consistent with recent results indicating that poly(A)⁺ mRNAs are translationally repressed during glucose deprivation and accumulate in P-bodies (Brenques and Parker 2007).

IV.3.4 Dhh1p, Pat1p, and Lsm1p Have Different Effects on P-Body Assembly and Composition

The proteins Dhh1p, Pat1p, and Lsm1p are all found in P-bodies and function as general decapping activators (Coller and Parker 2004). To determine the roles of these proteins on P-body assembly we examined the accumulation of GFP-tagged version of Dcp1p, Dcp2p, Edc3p, Xrn1p, Dhh1p, Pat1p, and Lsm1p in *lsm1Δ*, *pat1Δ* or *dhh1Δ* mutant strains. Because results from mammalian cells indicate that the Lsm1-7p complex must be intact to localize to P-bodies (Ingelfinger et al. 2002), and *lsm1Δ* is sufficient to fully inactivate this complex with respect to decapping (Tharun et al. 2000), the *lsm1Δ* should be sufficient to address the contribution of the entire Lsm1p-7p complex to P-body formation. In addition, we examined both the affect of the deletions on P-bodies during mid-log growth, where P-bodies are small and increases are easily seen, and during glucose deprivation, where P-bodies are large, and decreases in P-body assembly are more easily observed. These results are presented in Figure IV.5 and important observations are discussed below.

One important observation was that *lsm1Δ* strains showed increased accumulation of Dcp1p, Dcp2p, Edc3p, Xrn1p, and Dhh1p in P-bodies even in midlog growth cells (Figure IV.5B, I–V). Pat1p did not accumulate in P-bodies in mid-log cultures in the *lsm1Δ* strain, perhaps because of an effect of the *lsm1Δ* on the nuclear-cytoplasmic distribution of Pat1p (Figure IV.5B, VI; see below). The accumulation of all P-body components except

Pat1p in P-bodies in *lsm1Δ* strain is consistent with the decapping defect seen in *lsm1Δ* strains (Boeck et al. 1998; Bouveret et al. 2000; Tharun et al. 2000). Moreover, this observation argues that the rate-limiting step in decapping in *lsm1Δ* strains is after mRNAs assembly into an mRNP that can accumulate in P-bodies. This identifies an important function of the Lsm1-7p complex in triggering decapping after translation repression and accumulation of the mRNA in the P-body mRNP. Consistent with the partial block to mRNA decapping in the *lsm1Δ* strain, the accumulation of P-bodies is not as strong in the *lsm1Δ* strain compared with the *dcp1Δ* strain, where decapping is absolutely blocked (compare Figure IV.1C with IV.5B). Moreover, in *lsm1Δ* strains, P-bodies still increased during glucose deprivation compared with nonstressed *lsm1Δ* cells (Figure IV.5B, VIII–XIII), which suggests that *lsm1Δ* strains do not have a maximal accumulation of P-bodies.

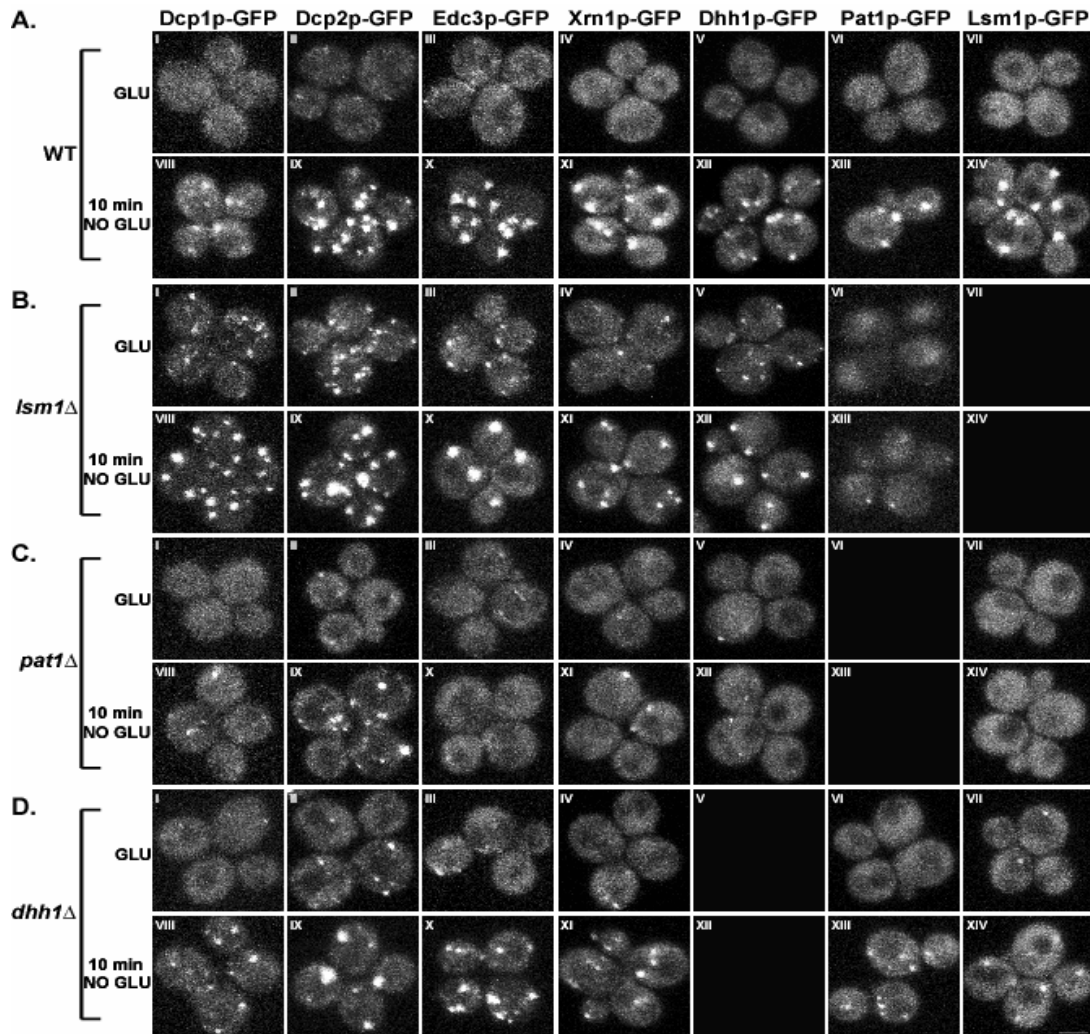


Figure IV.5 Dhh1p, Pat1p, and Lsm1p have different affects in P-body assembly and composition. (A) WT, (B) *lsm1Δ*, (C) *pat1Δ* and (D) *dhh1Δ* cells expressing a GFP-tagged version

of Dcp1p (I,VIII), Dcp2p (II,IX), Edc3p (III,X), Xrn1p (IV,XI), Dhh1p (V,XII), Pat1p (VI,XIII) and Lsm1p (VII,XIV). Pictures of cells before glucose deprivation (GLU) and after 10 minutes of glucose deprivation (10 min NO GLU) treatment are shown. All images are shown at the same scale and a 3µm-scale bar is shown in the lower right panel.

In contrast to the accumulation of P-bodies in the *lsm1Δ* strain, in general the *pat1Δ* and *dhh1Δ* strains showed either minor or no reduction of P-bodies during mid-log growth, respectively, as assessed by Dcp1p, Dcp2p, Edc3p, and Xrn1p (Figure IV.5C, I–IV; IV.5D, I–IV). Interestingly, we did observe a minor increase in P-bodies during mid-log growth for *dhh1Δ* and *pat1Δ* cells in a subpopulation of the cells (Figure IV.5C; IV.5D; data not shown). This suggests that Dhh1p and/or Pat1p can affect the rate of mRNA decay within P-bodies or the rate of mRNA exit from P-bodies in at least some cells. However, during glucose deprivation the *pat1Δ* strain and to a more modest effect the *dhh1Δ* strain showed a reduction in the amount of P-bodies formed (compare Figure IV.5A, VIII–XIV, with IV.5C, VIII–XIV and IV.5D, VIII–XIV). This is consistent with earlier results that *dhh1Δ* and *pat1Δ* strains are partially defective in translation repression during glucose deprivation (Holmes et al. 2004; Collier and Parker 2005). Because both *dhh1Δ* and *pat1Δ* appear to affect translation repression similarly during glucose deprivation (Holmes et al. 2004; Collier and Parker 2005), the stronger reduction of P-bodies observed in *pat1Δ* suggests that Pat1p may have a more significant role in P-body assembly and aggregation than Dhh1p (see below and Discussion).

IV.3.4.1 Pat1p Is Required for Recruitment of Lsm1p to P-Bodies

A second important result from these comparisons was that strains lacking Pat1p failed to accumulate Lsm1p in P-bodies with or without glucose repression (Figure IV.5C, VII, XIV). This argued that Pat1p is required for Lsm1-7p to be recruited to P-bodies. However, because the loss of Pat1p can affect the size of P-bodies during glucose repression, we verified this result under conditions where P-bodies were large even in a *pat1Δ* strain. To do this, we examined whether Lsm1p accumulates in P-bodies in a *pat1Δ* strain at high cell density, where P-bodies are large (Figure II.1). Consistent with Pat1p being required for Lsm1p to enter into P-bodies, we observed that *pat1Δ* cells did not accumulate Lsm1p in P-bodies at high OD₆₀₀ (Figure IV.6A, II). These observations demonstrate that Pat1p is required to recruit the Lsm1-7p complex to P-bodies.

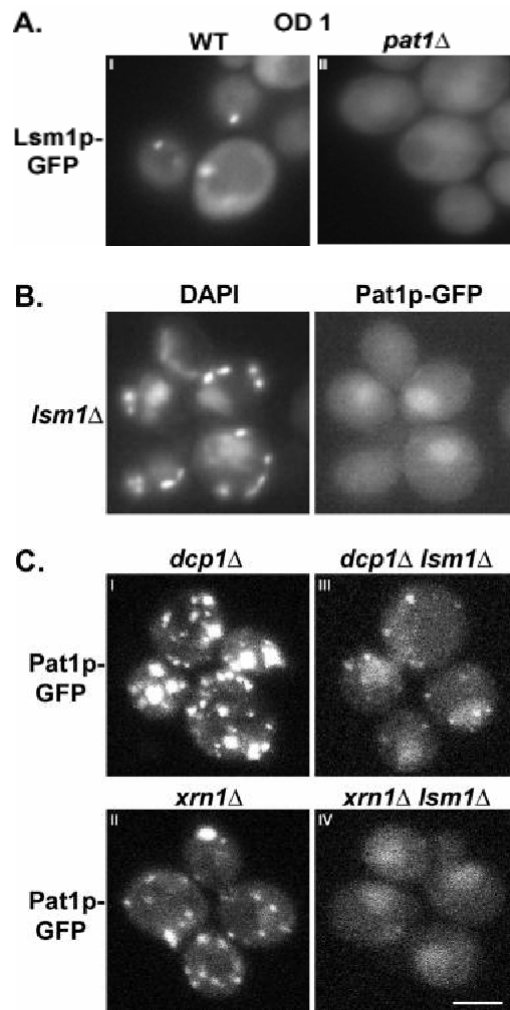


Figure IV.6 Pat1p is required for recruitment of Lsm1p to P-bodies but Lsm1p is not required for Pat1p targeting to P-bodies. (A) Shown are WT (I) and *pat1Δ* (II) cells expressing GFP-tagged version of Lsm1p grown to high cell density of 1 OD₆₀₀ unit (OD 1) as indicated at the top of the figure. (B) Distribution of GFP-tagged version of Pat1p in *lsm1Δ* cells (right panel). The nucleus is visible by DAPI staining of nuclear DNA (left panel). Note that the small foci seen with DAPI staining are due to mitochondrial DNA. (C) Distribution of GFP-tagged version of Pat1p in *dcp1Δ* (I), *dcp1Δ lsm1Δ* (III), *xrn1Δ* (II) and *xrn1Δ lsm1Δ* (IV) cells is shown. All images are shown at the same scale and a 3μm-scale bar is shown in the lower right panel.

IV.3.4.2 Lsm1p Affect the Nuclear-Cytoplasmic Distribution of Pat1p

A third observation from these comparisons was that in the *lsm1Δ* strain Pat1p accumulated in a large circular region of the cell that was similar in size to the nucleus (Figure IV.5B, VI). Subsequent DNA staining with DAPI revealed that this circular region of Pat1p concentration colocalized with the nucleus (Figure IV.6B). This observation indicates that Lsm1p, and presumably the whole Lsm1-7p complex, plays a role in

decreasing the concentration of Pat1p in the nucleus and increasing the cytoplasmic concentration of Pat1p. These results suggest that Pat1p is a likely nuclear-cytoplasmic shuttling protein. This conclusion is consistent with earlier work showing that Pat1p shows physical interactions with the mRNA export factor Crm1p (Jensen et al. 2000).

Despite the nuclear concentration of Pat1p in the *lsm1Δ* mutant, three observations suggest that some Pat1p is still in the cytoplasm and is functioning in decapping. First, even in the *lsm1Δ* strain during mid-log growth, some Pat1p-GFP was observed distributed in the cytoplasm (Figure IV.5B, VI). Second, when *lsm1Δ* cells were glucose deprived some Pat1p was seen to accumulate in P-bodies (Figure IV.5B, XIII). Third, when P-bodies accumulate because of *dcp1Δ*, Pat1p-GFP is still present in P-bodies, even in the absence of Lsm1p, albeit at a reduced level compared with a *dcp1Δ* alone (Figure IV.6C, III). These observations indicate that Lsm1p is not absolutely required for Pat1p to enter P-bodies, although it clearly appears to decrease the amount of Pat1p seen in P-bodies, at least in part by affecting the distribution of Pat1p between the nucleus and the cytoplasm. Interestingly, although P-bodies accumulate due to *xrn1Δ*, Pat1p-GFP was no longer observed in P-bodies in the *xrn1Δ lsm1Δ* double mutant (Figure IV.6C, IV). This further suggests the idea that Pat1p/Lsm1-7p complex/Xrn1p forms a functionally important complex, which is consistent with their previous biochemical copurification (Bouveret et al. 2000).

IV.3.4.3 Double Mutants Indicate Pat1p Functions Before Lsm1p in mRNA Decapping

The above observation suggest a model for P-body assembly wherein Pat1p acts early in the process and is required to recruit the Lsm1-7p complex, which functions later to enhance decapping rate. A prediction of this model is that a *pat1Δ lsm1Δ* double mutant should show the phenotype of the *pat1Δ* single mutant strain and not the *lsm1Δ* strain. To test this prediction, we examined the accumulation of GFP-tagged Dcp1, Dcp2, Edc3p and Xrn1p in *pat1Δ lsm1Δ* strains. For comparison, we also examined the same proteins in a *dhh1Δ lsm1Δ* strain.

A clear and significant observation was that during mid-log growth the *pat1Δ lsm1Δ* strain showed little P-body accumulation of all four proteins similar to a *pat1Δ* strain (Figure IV.7E–H) and unlike a *lsm1Δ* strain where P-bodies are increased (Figure IV.5B). This result provides additional evidence that Pat1p acts upstream of Lsm1p in the process of P-body assembly and mRNA decapping. In contrast, we observed that *dhh1Δ lsm1Δ* leads to increased concentration of Dcp1p, Dcp2p, Edc3p, and Xrn1p in P-bodies (Figure IV.7,

A–D), because P-bodies increased in size and number compared with both WT cells and *dhh1Δ* or *lsm1Δ* single mutant strains (Figure IV.5B; IV.5D). This suggests that Dhh1p, like Lsm1p, can also play a role in the actual rate of decapping after assembly of the mRNP capable of aggregation in P-bodies. Consistent with this, a *dhh1Δ lsm1Δ* double mutant showed an additive effect on mRNA decay compared to *dhh1Δ* or *lsm1Δ* single mutant strains. In contrast, a *pat1Δ lsm1Δ* double mutant affected mRNA decay similar to *pat1Δ* or *lsm1Δ* single mutant (Coller and Parker 2005).

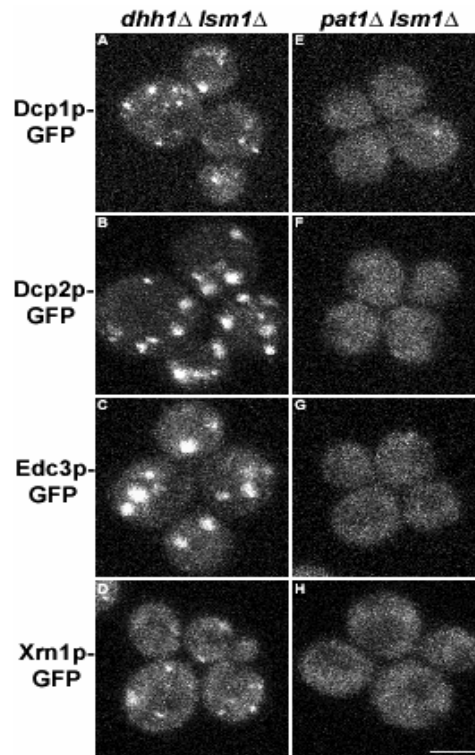


Figure IV.7 Pat1p functions before Lsm1p in P-body assembly. Distribution of GFP-tagged version of Dcp1p (A,E), Dcp2p (B,F), Edc3p (C,G) and Xrn1p (D,H) in *dhh1Δ lsm1Δ* (left panel) and *pat1Δ lsm1Δ* (right panel) cells are shown. All images are shown at the same scale and a 3μm-scale bar is shown in the lower right panel.

IV.3.4.4 Dhh1p, Pat1p, and Lsm1p Affect the Recruitment of Ccr4p and Pop2p to P-Bodies

In examining the function of Dhh1p, Pat1p, and Lsm1p in P-body assembly we also examined how they affected the recruitment of Ccr4p and Pop2p, subunits of the major cytoplasmic deadenylase, to P-bodies during glucose deprivation (Figure IV.8A). We observed that during glucose deprivation the amount of Ccr4p or Pop2p seen in P-bodies

declined in *dhh1Δ*, *pat1Δ* or *lsm1Δ* strains compared with a WT strain, where small but clear accumulation of Ccr4p and Pop2p in P-bodies could be seen during glucose deprivation (Figure IV.8A, III, IV, VII, VIII, XI, XII, XV, XVI). This suggests that the accumulation of Ccr4p and Pop2p in P-bodies requires Dhh1p, Pat1p, and Lsm1p. However, a limitation of this analysis is that the accumulation of Ccr4p and Pop2p in P-bodies in the WT strain is relatively low; thus it is difficult to make a robust conclusion from this experiment alone.

To further support this observation we asked how *dhh1Δ*, *pat1Δ* or *lsm1Δ* affected the accumulation of Ccr4p or Pop2p in P-bodies in combination with *xrn1Δ* where the accumulation of Ccr4p and Pop2p in P-bodies is easily observed (Figure IV.1B). We observed that the *dhh1Δ*, *pat1Δ* or *lsm1Δ* all reduced the accumulation of Ccr4p and Pop2p in P-bodies compared with *xrn1Δ* single mutant (Figure IV.8B, III–VIII). This is consistent with the observations during glucose deprivation and argues that the efficient recruitment of Ccr4p and Pop2p to P-bodies requires Dhh1p, Pat1p, and the Lsm1-7p complex.

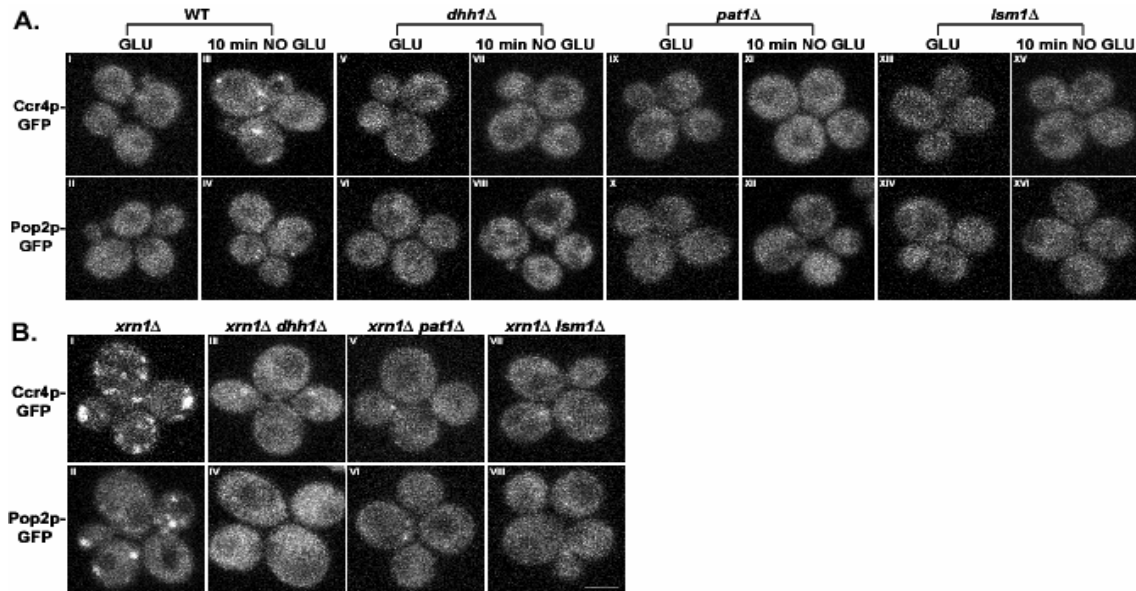
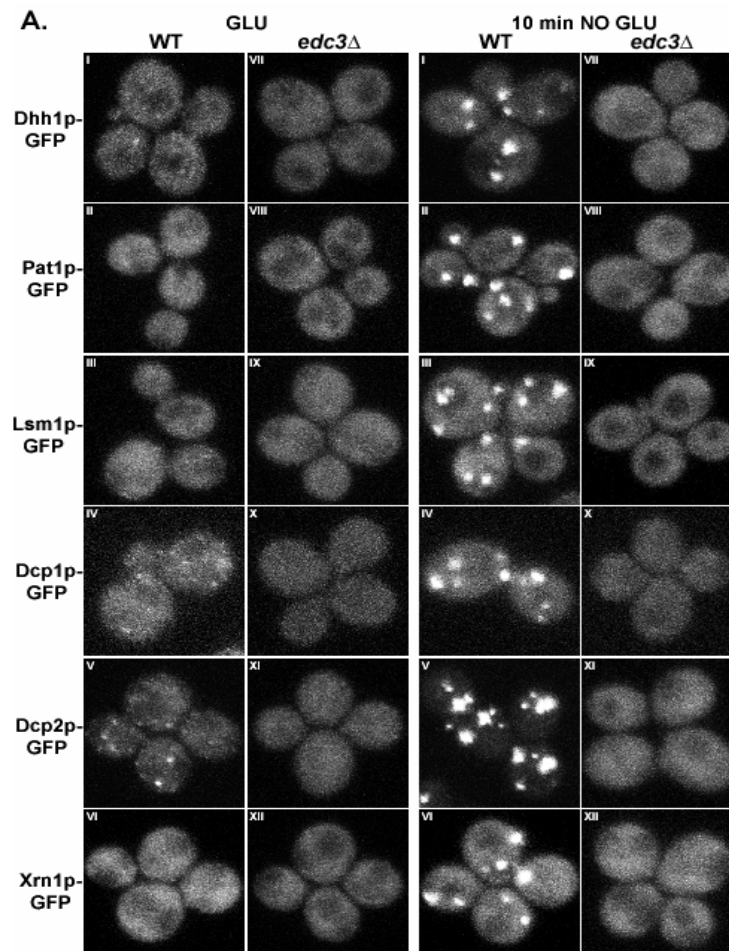


Figure IV.8 Dhh1p, Pat1p and Lsm1p are required to the recruitment of Ccr4p and Pop2p to P-bodies. (A) Distribution of GFP-tagged version of Ccr4p (top panel) and Pop2p (bottom panel) in WT (I-IV), *dhh1Δ* (V-VIII), *pat1Δ* (IX-XII) and *lsm1Δ* (XIII-XVI) cells. Pictures of cells before glucose deprivation (GLU) and after 10 minutes of glucose deprivation (10 min NO GLU) treatment are shown. (B) Shown are *xrn1Δ* (I,II), *xrn1Δ dhh1Δ* (III,IV), *xrn1Δ pat1Δ* (V,VI) and *xrn1Δ lsm1Δ* (VII,VIII) cells expressing a GFP-tagged version of Ccr4p (top panel) and Pop2p (bottom panel). All images are shown at the same scale and a 3μm-scale bar is shown in the lower right panel.

IV.3.5 Edc3p Functions in P-body Formation

Given that Edc3p is found in P-bodies (Kshirsagar and Parker 2004), we examined the ability of *edc3Δ* strains to form P-bodies. In *edc3Δ* strains, Dhh1p-GFP, Dcp1p-GFP and Dcp2p-GFP were no longer seen in the small P-bodies normally observed in mid-log cultures in WT strains (Figure IV.9A, GLU, VII, X, XI). This result suggested that Edc3p might play a role in P-body assembly. However, because of the small size of P-bodies under mid-log growth conditions was difficult to distinguish how significant is the contribution of Edc3p to P-body assembly. Given this, we examined the effect of the *edc3Δ* on P-body formation during glucose deprivation where translation is inhibited and P-bodies are consequently increased (Chapter II). Strikingly, we observed that *edc3Δ* strains showed a strong reduction in the accumulation of microscopically visible P-bodies following glucose deprivation as judged by Dhh1p-GFP, Pat1p-GFP, Lsm1p-GFP, Dcp1p-GFP, Dcp2p-GFP and Xrn1p-GFP (Figure IV.9A, 10 min NO GLU, VII-XII) although some P-bodies could still form (data not shown). The strong reduction of P-bodies indicates that Edc3p plays some important role in the formation of P-bodies.



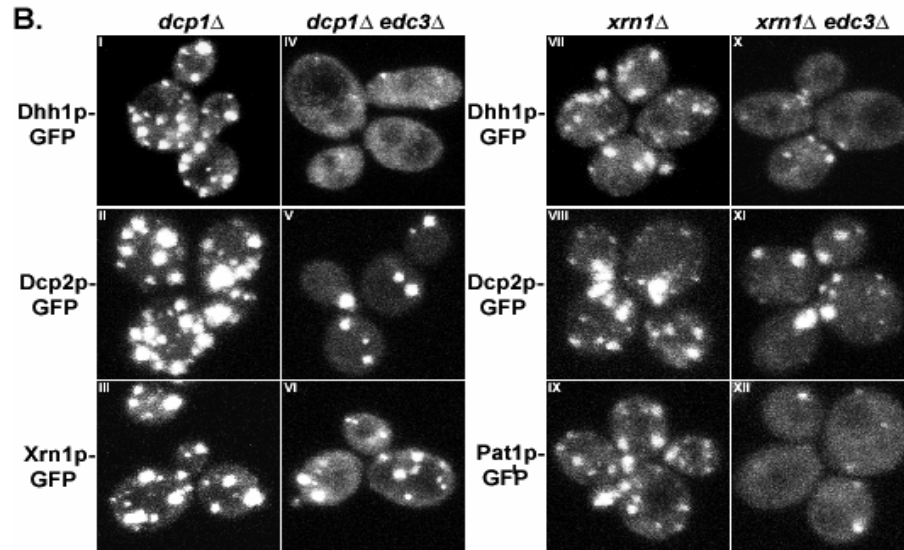


Figure IV.9 Edc3p is important for P-body assembly. (A) Localization of GFP tagged version of Dhh1p, Pat1p, Lsm1p, Dcp1p, Dcp2p, and Xrn1p in WT (I-VI) and *edc3Δ* (VII-XII) strains during exponential growth in YP containing Glu (Glu) or after being deprived of glucose for 10 minutes (10 min NO GLU). (B) Localization of GFP tagged version of Dhh1p, Dcp2p, and Xrn1p in *dcp1Δ* (I-III) or *dcp1Δ edc3Δ* (IV-VI) strains or Dhh1p, Dcp2p and Pat1p in *xrn1Δ* (VII-IX) or *xrn1Δ edc3Δ* (X-XIII) strains during growth in YP containing Glu.

To determine if Edc3p was absolutely required for P-body assembly, we asked how the *edc3Δ* affected the formation of P-bodies in *dcp1Δ* or *xrn1Δ* strains, where very large P-bodies accumulate due to the defects in decapping and 5' to 3' degradation (Figure IV.1). If Edc3p is absolutely required for P-body formation, no P-bodies should be observed in the *dcp1Δ edc3Δ* or *xrn1Δ edc3Δ* double mutants. Conversely, if there are other assembly mechanisms that can contribute to P-body formation, the *dcp1Δ edc3Δ* and *xrn1Δ edc3Δ* double mutants would be expected to contain a reduced level of P-bodies. We observed that the *dcp1Δ edc3Δ* and *xrn1Δ edc3Δ* strains still formed P-bodies as judged by the accumulation of Dcp2p-GFP, Dhh1p-GFP, Pat1p-GFP, or Xrn1p-GFP in P-bodies although P-bodies were dramatically reduced as compared to *dcp1Δ* or *xrn1Δ* strains alone (Figure IV.9B, IV-VI, X-XII). This observation demonstrates that Edc3p functions in the assembly of P-bodies in some manner, but is not absolutely required for P-body formation, suggesting that there is additional mechanism(s) that contribute to P-body assembly.

IV.3.5.1 Edc3p is not Required for Translation Repression

The strong reduction of P-bodies in the *edc3Δ* strain during glucose deprivation suggests that Edc3p could be required for the aggregation of mRNPs into P-bodies. Alternatively, because P-bodies are composed of nontranslating mRNPs that are in balance with the translating pool of mRNA (Chapter II), Edc3p could be necessary for translational repression under these conditions. To distinguish these two possibilities, we examined whether *edc3Δ* strain could repress translation during glucose deprivation as assessed by polysome analysis. We observed that both WT and *edc3Δ* cells showed a similar reduction in polysomes during glucose deprivation (Figure IV.10, III, IV). Since Edc3p strongly affects P-body formation under these conditions, this observation indicates that Edc3p primarily functions in the aggregation of nontranslating mRNPs into P-bodies, without altering translation repression. This result also implies that aggregation of mRNPs into large microscopically visible P-bodies may not be required for translation repression, at least in response to glucose-deprivation.

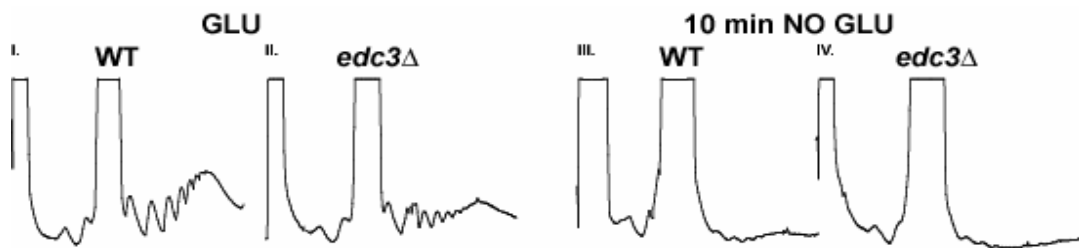


Figure IV.10 Edc3p is not required for translation repression. Polysome profiles obtained from WT (I,III) or *edc3Δ* (II,IV) strains grown in the presence of glucose (GLU) or deprived of glucose for 10 minutes (10 min NO GLU).

IV.4 Discussion

IV.4.1 Multiple Proteins Function in Yeast P-Body Assembly

This work begins to reveal the process by which P-bodies assemble and the possible roles of individual proteins within that assembly. One principle that emerges is that P-body assembly appears to be redundant, with no single protein being absolutely required for formation of P-bodies.

One protein that can affect P-body formation was Dcp2p. This was surprising because strains lacking Dcp2p show a complete block to decapping and therefore, like *dcp1Δ* strains, would be expected to accumulate very large P-bodies. However, *dcp2Δ* strains had reproducibly smaller P-bodies than *dcp1Δ* or *xrn1Δ* strains, although *dcp2Δ* strains still showed enhanced P-bodies compared with WT strains (Figure IV.1). Moreover, because *dcp2Δ* strains have smaller P-bodies than a *dcp1Δ* strain as assessed by seven different proteins, it strongly argues that the difference is in the actual formation of P-bodies themselves and not simply a change in P-body composition. Finally, because *dcp2Δ dcp1Δ* strains are similar to *dcp2Δ* single mutant strains alone when assessed for P-body formation, it argues that Dcp2p is required for optimal P-body accumulation (Figure IV.3). Because Dcp2p shows direct physical interactions with Dcp1p, Dhh1p, Edc3p, and possibly Pat1p (Steiger et al. 2003; She et al. 2004; CJ Decker, G Pilkington and R Parker, unpublished observation), the simplest possibility is that the multiple protein–protein interactions that Dcp2p participates in either stabilizes the P-body monomer mRNP or helps to provide cross-linking interactions between individual mRNPs, thereby contributing to aggregation of multiple mRNPs into a larger P-body. Thus, Dcp2p is not only the catalytic subunit of the decapping enzyme, but also has a second role in promoting P-body assembly or maintenance.

Several observations argue that Pat1p is likely to affect P-body formation in multiple manners. First, because Pat1p overexpression leads to inhibition of translation (Coller and Parker 2005), one possible role of Pat1p is likely to be to inhibit translation initiation in some manner, thereby contributing to P-body formation by affecting the size of the pool of nontranslating mRNA, which can then aggregate into P-bodies. Second, because P-bodies are reduced in *pat1Δ* strains, even during glucose deprivation, where translation is markedly repressed even in a *pat1Δ* strain, it suggests that Pat1p also plays a role in assembly of P-bodies (Figure IV.5). The role of Pat1p in assembly is again likely to be through multiple protein–protein interactions that promote formation of the P-body monomer mRNP, and cross-linking between individual mRNPs. Finally, we suggest that Pat1p has a final role in promoting mRNA decapping after assembly of the P-body mRNP. This is suggested by both the requirement for Lsm1p for efficient decapping after P-body assembly, and the requirement for Pat1p for assembly of Lsm1p into P-bodies.

We also identified Edc3p as a protein that contributes to P-body assembly. The critical observation is that *edc3Δ* show loss of the small P-bodies observed in mid-log growth conditions, as assessed by Dhh1p, Dcp1p and Dcp2p (Figure IV.9A). In addition, *edc3Δ*

strains show essentially no proteins concentrated with P-bodies during glucose deprivation for every component examined, although some small P-bodies could be observed (Figure 9A; data not shown). However, Edc3p is not absolutely required for P-body formation as *edc3Δ dcp1Δ* strains or *edc3Δ xrn1Δ* strains can still form P-bodies, albeit it at reduced levels (Figure IV.9B). Interestingly, *edc3Δ* strains were still able to repress translation during glucose deprivation despite the strong reduction of visible P-bodies. This indicates that Edc3p is not required for translation repression *per se*, and specifically acts after translation repression to promote aggregation of translationally repressed mRNPs into microscopically visible P-bodies. In addition, this result suggests that the formation of large P-body structures visible in the light microscope may not be absolutely required for translation repression, and moreover, because Edc3p is not required for decapping in normal cells (Kshirsagar and Parker 2004), or even under stress conditions (data not shown), it also implies that formation of P-bodies aggregates visible in the light microscope may not be essential for efficient decapping as well. However, it should be noted that because P-bodies can still assemble without Edc3p, it may be that a smaller P-body mRNP is assembling that has an effect on translation repression and decapping efficiency. Because Edc3p show extensive two-hybrid interactions with several decay factors, the contribution of Edc3p to P-body formation is likely to be through protein-protein interactions with other components of the P-body mRNP. Moreover, genome-wide analyses identified the C-terminus of Edc3p as being sufficient to interact with itself by two-hybrid or phage display experiments (Fromont-Racine et al. 2000; Marino-Ramirez and Hu 2002), which suggests that Edc3p may contribute to P-body assembly by aggregating either individual mRNPs or small mRNP complexes into large P-bodies by self-interaction. Finally, these results imply that additional mechanism(s) independent of Edc3p probably contribute to the assembly of P-body.

IV.4.2 Specific Dependencies in P-Body Assembly

Our analysis of yeast P-bodies suggests that there are some clear dependencies in the assembly of specific components. For example, the assembly of Dcp1p in P-bodies is dependent on Dcp2p (Figures IV.1 and IV.2). This is consistent with the direct physical interaction between these components from yeast (Steiger et al. 2003; She et al. 2004, 2006). A second clear dependency is that Pat1p is required for the recruitment of Lsm1p to P-bodies (Figures IV.5 and IV.6). Because Lsm1p is a component of the Lsm1-7p complex and Lsm1-7p complex formation is required for its recruitment to P-bodies and for the Lsm1-7p complex to function in mRNA decay (Bouveret et al. 2000; Tharun et al.

2000, 2005; Ingelfinger et al. 2002), this suggests that Pat1p is required for the recruitment of the entire Lsm1-7p complex into P-bodies. This is consistent with the physical interactions between Pat1p and the Lsm1-7p complex (Bouveret et al. 2000; Tharun et al. 2000).

IV.4.3 Pat1p Acts Early in Translation Repression and mRNA Decapping While Recruiting the Lsm1-7p Complex to Trigger Decapping in a Late Step

Our results also argue that the Lsm1-7p complex has an important role after P-body assembly in the actual triggering of decapping. The key observation is that *lsm1Δ* strains show an accumulation of P-bodies, as judged by the subcellular distribution of Dcp1p, Dcp2p, Edc3p, Xrn1p, and Dhh1p (Figure IV.5). This is consistent with the observed defect in mRNA decapping in the *lsm1Δ* strains (Boeck et al. 1998; Bouveret et al. 2000; Tharun et al. 2000) and indicates that the decapping mRNP can assemble and aggregate in P-bodies in the absence of the Lsm1p, but is then deficient at actual decapping, and hence a pool of mRNAs accumulates in P-bodies. It should be noted that these results do not rule out a potential earlier role of the Lsm1-7p complex in translation repression or assembly of the monomer unit, they just reveal what becomes the rate-limiting step in the absence of Lsm1p.

An important implication of the function of the Lsm1-7p complex in triggering decapping after P-body formation is that this role of the Lsm1-7p complex could be used to alter the fate of mRNAs within P-bodies. Specifically, individual mRNAs that are destined for storage could simply be packaged into an mRNP that is lacking the Lsm1-7p complex and thereby have a reduced rate of mRNA decapping. Similarly, in certain biological contexts where mRNA storage is important, the Lsm1-7p complex may be lacking from P-bodies and thereby converts the fate of mRNAs within P-bodies into storage.

Our data also indicate that Pat1p is required for Lsm1p to assemble into P-bodies. The key observation is that P-bodies formed in *pat1Δ* strains lack Lsm1p, even under glucose deprivation (Figure IV.5) or high OD (Figure IV.6). This is also consistent with earlier work showing that the coimmunoprecipitation of Lsm1p with Dcp2p is greatly reduced in a *pat1Δ* strain (Tharun and Parker 2001). The simplest model is that Pat1p directly interacts with Lsm1-7p complex and recruits it to P-bodies, although one anticipates that Pat1p might also facilitate the interaction of the Lsm1-7p complex with other components of P-bodies.

The phenotypes of *lsm1Δ* and *pat1Δ* strains suggest a model for these proteins function wherein Pat1p participates early in translation repression and the movement of mRNAs into P-bodies and the Lsm1-7p complex has a rate limiting role at a later stage to trigger decapping. The early role of Pat1p is suggested by the observations that overexpression of Pat1p inhibits translation (Coller and Parker 2005) and that *pat1Δ* strains show reduced P-bodies even under glucose deprivation conditions (Figure IV.5). The late role of Lsm1p is indicated by the accumulation of P-bodies in the *lsm1Δ* strain. Moreover, epistasis analysis is consistent with Pat1p acting before Lsm1-7p because *pat1Δ lsm1Δ* double mutant strains show reduced P-bodies similar to *pat1Δ* strains (Figure IV.7).

IV.4.4 Pat1p is a Nuclear-Cytoplasmic Shuttling Protein and its Distribution is Affected by Lsm1p

Our results indicate that Pat1p is likely to be a nuclear-cytoplasmic shuttling protein and its distribution between the nucleus and the cytoplasm is affected by the Lsm1-7p complex. The key observation is that in *lsm1Δ* strains, Pat1p accumulates in the nucleus (Figures IV.5 and IV.6). Consistent with Pat1p being a nuclear-cytoplasmic shuttling protein, previous work has identified a two-hybrid interaction between Pat1p and Crm1p, which is involved in mRNA export (Jensen et al. 2000). In addition, Pat1p was identified in a proteomic analysis of the components of the penta-snRNP, which is a large nuclear complex involved in pre-mRNA splicing (Stevens et al. 2002). It would be interesting to determine if Pat1p indeed shuttles between the nucleus and the cytoplasm. This could be easily addressed by examining Pat1p-GFP localization in nuclear export mutants. Interestingly, this nuclear localization is not unique to Pat1p in *lsm1Δ* strains, because we also detect a clear accumulation of Dhh1p in the nucleus in *lsm1Δ* strains under glucose deprivation conditions (Figure IV.5). In addition, Dhh1p also interacts with components of the mRNA export machinery (Fisher and Weis 2002). This suggests that other components of P-bodies may be nuclear-cytoplasmic shuttling proteins.

In principle, the accumulation of Pat1p in the nucleus in *lsm1Δ* strain could be explained in two manners. First, it could be that interaction of the Lsm1-7p complex with Pat1p in the nucleus is required for efficient export of Pat1p from the nucleus, perhaps in association with mRNAs. This model would imply that an mRNP structure similar to a P-body mRNP may form on some, or all, mRNAs in the nucleus and be transported with them to the

cytoplasm. An alternative, and potentially overlapping, model is that interaction of Pat1p with the Lsm1-7p complex in the cytoplasm limits import of Pat1p into the nucleus. Future experiments should be able to distinguish between these models.

Pat1p might function in the nucleus in one of two manners. First, it could be that Pat1p plays a role in a nuclear process such as splicing or degradation of aberrant mRNAs in the nucleus. Alternatively, Pat1p may enter the nucleus to become assembled into nascent mRNPs before export to the cytoplasm, which might lead to those mRNAs entering the cytoplasm in a translationally repressed mRNP that is directly targeted to P-bodies. Such potential targeting of nascent mRNPs might occur on all mRNAs, or more likely, might be more specific to subclasses of mRNAs, or biological contexts, where translation repression of nascent transcripts is particularly important for translational control. Interestingly, one example of such a context is in the biogenesis of maternal mRNAs, which are exported to the cytoplasm and then stored in maternal germ granules, which are related to P-bodies (Anderson and Kedersha 2006). Here it is worth noting that the *Xenopus* ortholog of Dhh1p is a nuclear-cytoplasmic shuttling protein (Smillie and Sommerville 2002) and may play a role in packaging nascent mRNAs for direct targeting to storage particles. This further suggests that Dhh1p may also shuttle between the nucleus and the cytoplasm. This is also consistent with an emerging theme that many proteins that determine the destinations of mRNAs are recruited on nascent transcripts in the nucleus. An interesting area of future work will be to understand the functional significance of P-body components shuttling between the nucleus and the cytoplasm and the implications to P-body formation. These studies are likely to reveal unanticipated links between mRNA export, localization, translation and decay.

CHAPTER V

CONCLUDING REMARKS

V. CONCLUDING REMARKS

The control of mRNA translation and degradation are intimately linked to regulate gene expression. The relationship between mRNA translation and degradation has been illuminated with the characterization of the pathways of mRNA degradation. In yeast, most mRNAs initiate their degradation with deadenylation of the 3' poly(A) tail, followed by removal of the 5' cap structure (decapping) and 5' to 3' exonucleolytic degradation. Consistent with the mRNA 5' cap being a substrate for both cap-dependent translational initiation and mRNA decapping, the two events of mRNA decapping and translation are inversely regulated. Indeed, efficient decapping requires a key transition to a specific nontranslating mRNP that lacks translation factors and include the decapping machinery (Coller and Parker 2004).

Strikingly, all mRNA decay factors involved in decapping and 5' to 3' degradation of the mRNA, as well as, mRNA decay intermediates were observed to accumulate in cytoplasmic structures called P-bodies in yeast. Functional analyses in yeast described P-bodies as discrete sites of mRNA decapping and 5' to 3' degradation (Sheth and Parker 2003). Notably, P-bodies have been identified in different organisms including yeast, worms, flies, and mammals (Eulalio et al. 2007). P-body components include the mRNA decapping enzyme Dcp1p/Dcp2p, the decapping activators Dhh1p/RCK/p54, Pat1p, Lsm1-7p, Edc3p, and the exonuclease Xrn1p, which seem to compose the core of proteins found in P-bodies (Sheth and Parker 2003; Kshirsagar and Parker 2004; Eulalio et al. 2007). The CCR4/CAF1 deadenylase also accumulates in P-bodies in mammals and *D. melanogaster* (Cougot et al. 2004; Temme et al. 2004; Andrei et al. 2005). In yeast, our studies showed that although Ccr4p and Pop2p subunits of the deadenylase complex are not primarily found in P-bodies, they can accumulate in P-bodies in *dcp1Δ* or *xrn1Δ* cells, where decapping and 5' exonucleolytic degradation is blocked and accumulate large aggregates, suggesting that Ccr4p/Pop2p association with yeast P-bodies may be rapid and transient, but under specific conditions can accumulate in P-bodies. In contrast, our results showed that several components of the cytoplasmic exosome and its associated Ski complex are not concentrated in P-bodies. Moreover, we showed that mRNAs can exit P-bodies independent of 3' to 5' decay. These results suggests the that 3' to 5' mRNA decay pathway probably does not occur localized within P-bodies, and reinforces the compartmentalization of the 5' to 3' mRNA decay pathway in the cytoplasm.

Since the discovery of P-bodies, the number of proteins detected in P-bodies has increased tremendously. In addition to this conserved set of decay proteins, other proteins can be found in P-bodies in an organism-specific manner. For example, mammalian P-bodies contain the RAP55, homolog of yeast Scd6p, which functions in translation repression (Yang et al. 2006), another mammalian P-body component with no ortholog in yeast is Ge-1/Hedls, which functions in decapping (Fenger-Gron et al. 2005; Yu et al. 2005). In addition, mammalian P-bodies (also called GW- or DCP-bodies) can contain factors involved in miRNA/siRNA-mediated mRNA silencing such as Argonaute proteins and GW182, which have not been identified in yeast, indicating a role for P-bodies in RNA silencing (Eystathiou et al. 2003; Jakymiw et al. 2005; Liu et al. 2005a; Meister et al. 2005; Pillai et al. 2005; Sen and Blau 2005). Moreover, proteins that participate in other degradation processes such as NMD can also be localized in human and yeast P-bodies under specific cell conditions or mutants (Unterholzner and Izaurralde 2004; Sheth and Parker 2006). Finally, factors involved in ARE-mediated decay pathway can be localized to P-bodies in mammals (Fenger-Gron et al. 2005; Stoecklin et al. 2006). Although the complete composition of P-bodies remains to be determined, P-body composition seems to be more diverse in higher eukaryotes than in yeast. Additionally, the presence of proteins involved in diverse degradation pathways within P-bodies suggests that P-bodies are sites where mRNAs may be targeted for degradation by a variety of mechanisms. It is important to note that the factors localized in P-bodies are also distributed throughout the cytoplasm, which suggests that mRNA degradation may also occur diffuse in the cytoplasm. One interesting, but difficult question would be the quantification of the fractionation of these proteins between the cytoplasm and P-bodies, and the contribution of P-body components concentrated within aggregates for processes that take place within P-bodies. Nevertheless, P-bodies are anticipated to play a central role in the regulation of gene expression.

Interestingly, our work provided evidence that P-bodies also contain translationally repressed mRNAs, and moreover, P-bodies require mRNA for assembly and integrity. Consistent with this finding, we showed that the pool of nontranslated mRNA is proportional to the size and abundance of P-bodies. For example, we showed that when mRNAs are trapped in polysomes by blocking elongation, P-bodies decline. In contrast, when mRNAs are not associated with polysomes due to inhibition of translation initiation by mutants deficient in translation initiation or cellular stress, P-bodies increased. In addition, we found that several translation and ribosomal factors are excluded from P-bodies. Thus, we conclude that P-bodies accumulate mRNAs that are not engaged in translation. These results identified a dramatic transition of mRNAs involving significant

rearrangement of the mRNPs for movement of mRNAs from the translating pool to P-bodies. Hence, although it has been known for a long time that mRNA translation and degradation processes are functionally linked, these results indicate that they are also physically connected. This suggests that a critical issue understanding mRNA decay and translation will be to determine the nature of transitions between translating and nontranslating states of mRNAs and their accumulation in P-bodies, and moreover, how these transitions are regulated.

Remarkably, several P-body components have been shown to function in translation repression of mRNAs and promote P-body formation. For example, yeast Dhh1p and Pat1p are required for general translation repression in response to certain stresses (Holmes et al. 2004; Collier and Parker 2005). In addition, homologs of Dhh1p function in translation repression of maternal mRNAs in different organisms (Weston and Sommerville 2006). Finally, RAP55/Scd6p has been shown to repress translation of mRNAs (Tanaka et al. 2006). Interestingly, yeast cells overexpressing Dhh1p or Pat1p resulted in inhibition of translation and increased P-bodies. In contrast, yeast cells lacking Dhh1p and Pat1p are blocked in translation repression and lead to P-body loss (Collier and Parker 2005). This further supports our finding that P-bodies are formed by the accumulation of nontranslating mRNPs, and hence, relies on them for their existence. Together this suggests that accumulation of nontranslating mRNAs and the translation repression machinery within P-bodies may be part of the general mechanism of mRNA translation repression required before an mRNA can undergo decapping and degradation. Consistent with P-bodies playing an important role in translation repression, an emerging theme is that mRNAs translationally repressed by different mechanisms, including miRNA-mediated repression or mRNA-specific regulators, can accumulate in P-bodies for repression/degradation (Muhlrad and Parker 1999b; Ding et al. 2005; Fenger-Gron et al. 2005; Jakymiw et al. 2005; Liu et al. 2005a, 2005b; Pillai et al. 2005; Sen and Blau 2005; Goldstrohm et al. 2006; Sheth and Parker 2006). We propose that sequestration of translationally repressed mRNAs within P-bodies may facilitate the removal of the mRNA from the translational pool, as the translation machinery is excluded from P-bodies, and moreover, maintain mRNAs in a repressed state, thereby limiting reentry of the mRNA into the translating pool, and consequently facilitating decapping and degradation. Furthermore, this may also efficiently maintain the degradation machinery away from functional mRNAs, perhaps to prevent premature decapping of mRNAs that are not a substrate for decay. The existence of factors that promote general translation repression implies that the translation repression machinery may be constantly functioning and in competition with the translation machinery for assembly on the mRNA to determine

whether an mRNA is translated or targeted for translation repression or decay. The assembly of the translation repression machinery would thus promote mRNAs exit from translation to a repressed state and aggregation of these nontranslating mRNAs within P-bodies to be subject to decay. Thus, a fundamental aspect of control of mRNA degradation and translation rates would be the interface between mRNA translation in polysomes and mRNA accumulation in P-bodies.

Importantly, in addition to mRNA decapping and degradation, our results showed that translationally repressed mRNAs that accumulate within P-bodies can also exit the P-body structure to return to translation. Indeed, under specific growth conditions we observed that yeast cells have large P-bodies that accumulate nontranslating mRNAs, and these same mRNAs can reenter the polysome-associated pool for active translation following a change in conditions. Hence, P-bodies can function as temporary storage sites for repressed mRNAs, and transcripts associated with P-bodies can either be degraded or return to translation. These results revealed an important cycle of mRNA movement between P-bodies and polysomes anticipated to be important in providing a buffering system to maintain a proper pool of translating mRNAs. Understanding the molecular mechanisms by which mRNAs cycle between polysomes and P-bodies, the dynamics of this cycling, and the function of the factors involved will be important for understanding of mRNA degradation and translation control.

One interesting issue is what determines the fate of an mRNA within a P-body structure. It is possible that the fate of an mRNA within a P-body structure may be determined by the nature of the proteins that compose the mRNPs that assemble in P-bodies, which could be determined either by specific RNA-binding proteins that associate with the mRNA or by the mechanism of translation repression that target a specific mRNA to P-bodies. Interestingly, in mammalian cells, mRNAs can be translationally repressed when the 5' cap structure is complexed with eIF4E bound to eIF4E-transporter (4E-T), which blocks recruitment of eIF4G and consequently inhibits translation initiation. Since eIF4E and 4E-T accumulates in P-bodies, this suggests that translationally repressed mRNAs bound by the eIF4E-4E-T complex may be preferentially stored and not degraded (Andrei et al. 2005; Ferraiuolo et al. 2005). Similarly, in yeast, recent results suggested that an mRNA that is bound by Pab1p, eIF4E and eIF4G2 would be preferentially destined for storage to reenter translation (Brengues and Parker 2007). In contrast, based on our observation that Lsm1-7p complex seems to primarily function to activate decapping after its association with P-bodies, we speculate that an mRNA bound by Lsm1-7p complex would primarily be destined for degradation. This implies that different subpopulations of P-

bodies exist within the cell that may reflect the factors associated with a particular mRNA. An important future goal will be to define these different types of mRNP complexes that may occur during the transition of mRNAs between compartments.

Different cytoplasmic RNA granules found in eukaryotic cells seem to be related to P-bodies because they contain many of the same proteins that are found in P-bodies, and they all accumulate translationally silenced mRNAs stored for later reactivation. These include maternal mRNA granules, neuronal granules and stress granules (Anderson and Kedersha 2006). The similarities between P-bodies and these other RNA granules suggest that the mechanism of mRNA translation repression and accumulation of translationally repressed mRNAs in cytoplasmic structures for later activation has been widely conserved, and most likely of biological relevance. This suggests that although they are clearly distinct structures with distinct composition, formation, and function, a deeper understanding of each single type of RNA granule may be useful to reveal principles that are applicable to the other granules. One important future goal would be to determine the biochemical and functional relationship between these different RNA granules, and to identify the role of mRNA compartmentalization in these diverse RNA granules. Insight into these issues will require biochemical and genetic analysis of their composition, formation, mechanisms of assembly/disassembly, and relationship to other cellular structures and processes.

Although details of P-body formation and assembly are only in its infancy, it is possible to speculate that multiple mechanisms by which P-bodies assemble may exist. Our results are consistent with P-bodies being formed by nontranslating mRNPs, which in fact appear to be the building blocks of P-bodies. This suggests a model for P-body assembly initiated by the association of an mRNA with various proteins (presumably a minimal core of Dcp1p, Dcp2p, Edc3p, Dhh1p, Pat1p, Lsm1p and Xrn1p P-body components) to form the P-body core mRNP that then can aggregate probably through multiple mechanisms into a larger P-body visible by the light microscope. However, it is probable that there are intermediate steps in aggregation of mRNPs that can assemble a number of mRNPs too small to be microscopically visualized. In addition, there might be other RNA-binding proteins bound to the mRNA in this initial complex. Our genetic analysis of the yeast mechanism of P-body formation and aggregation revealed that P-body assembly appear to be redundant with no single known P-body component being absolutely required for their formation, although we identified that Pat1p, Dcp2p and Edc3p all contribute to some degree to the formation and assembly of P-bodies. Two principles have emerged from our studies. First, any given protein may play several roles coupling different processes such

as translation repression, formation of the P-body mRNP, aggregation of individual P-body mRNPs into larger aggregates and mRNA decapping. Second, all decay factors known to accumulate in P-bodies appear to contribute to P-body assembly through multiple interactions either with other P-body components or the RNA, helping to form the P-body mRNP, as well as helping in the subsequent aggregation of these mRNPs into microscopically visible higher order structures. Consistently, coimmunoprecipitation and two-hybrid experiments revealed a dense network of interactions between P-body components (Hata et al. 1998; Coller et al. 2001; Bonnerot et al. 2000; Bouveret et al. 2000; Ho et al. 2002; Steiger et al. 2003; Fenger-Gron et al. 2005; Gavin et al. 2006; Krogan et al. 2006). However, the nature of the protein-protein and protein-RNA interactions that allow the assembly of individual mRNPs containing the P-body components, as well as the aggregation of these mRNPs into larger P-bodies visible by light microscopy are largely unknown. An important goal in the future will be to determine these multiple interactions and how they contribute to P-body assembly. In addition, the actual order of protein assembly into the P-body mRNP is also largely unknown. Our results revealed that Pat1p assembles early in the process of P-body assembly, recruiting the Lsm1-7p complex to P-bodies to function in decapping. Moreover, because the composition of individual P-body mRNPs may be diverse, we should expect specific interactions and mechanisms of aggregation, in addition to general mechanisms of aggregation. A deeper understanding of P-body assembly should provide insight into the mRNA translation repression, storage and decapping processes, and their control.

An unresolved issue is whether the assembly of a microscopically visible higher order P-body structure is essential for the control of mRNA translation and degradation. Our results suggest that aggregation of P-body mRNPs in higher order structures may not be absolutely required for their function in translation repression, storage or mRNA decay. For example, in a yeast strain lacking Edc3p, where P-bodies are dramatically reduced under normal and stress conditions, albeit not completely eliminated, decapping and translation repression remain unaffected. Consistent with this, P-bodies aggregates can be depleted from mammalian cells without affecting miRNA-mediated repression (Chu and Rana 2006), or mRNA decay stimulated by AREs (Stoecklin et al. 2006). This suggests that association of P-body components with the mRNA to form non-detectable complexes (individual mRNA or small aggregates not detected by the light microscope) may be sufficient for basal control of translation repression and/or mRNA degradation, and the presence of microscopically visible P-bodies are visible markers of a pool of nontranslating mRNPs that can aggregate in P-bodies. However, the conservation of aggregation of nontranslating mRNPs into larger structures suggests that it may have

some functional advantage. It is possible that aggregation may increase the efficiency of these processes, perhaps, by helping to reinforce and maintain the translational repressed state by limiting the access of translation machinery to mRNAs. In addition, association of P-body mRNPs may also irreversibly commit an mRNA for degradation. Moreover, aggregation of mRNPs may play a different role in the control of mRNA function, such as aid in subsequent remodeling of mRNP organization, transport of mRNAs to specific subcellular regions. An important goal of future work will be the analysis of strains specifically lacking P-bodies due to defects in mRNP aggregation without altering the pool of nontranslating mRNPs to determine the function of larger P-bodies structures in translation repression, storage and mRNA degradation, or their relevance for cytoplasmic mRNA metabolism.

In summary, the available evidence suggests that P-bodies are sites where proteins involved in mRNA decay, translational repression, mRNA surveillance and RNA-mediated gene silencing, together with their targets accumulate. The present evidence suggests that mRNAs that are not actively being translated and their associated proteins form an mRNP that can aggregate within P-bodies where a decision concerning their fate will be made. They can either be degraded, silenced or retranslated. Despite recent progress, it seems clear that many details remain unknown concerning the structure, composition, formation, dynamics, regulation and function of P-bodies. Moreover, we anticipate that P-bodies may play additional roles in diverse areas of RNA metabolism. For example, our results suggest that Pat1p, and probably Dhh1p, might have a role in mRNA export and directly direct all or a subset of nascent mRNAs to P-bodies for translational control. In addition, the presence of Rbp4p, a subunit of RNAPII, in P-bodies suggests that P-bodies may be linked to transcriptional processes to coordinate mRNA biogenesis and turnover (Lotan et al. 2005). Finally, recent results argue that the Ty3 retrotransposon in yeast may assemble its virus-like particles in association with P-bodies (Beliakova-Bethell et al 2006). In addition, it has been reported that P-bodies may have a role in brome mosaic virus (BMV) replication (Beckham et al. 2007). This suggests that P-bodies may be important sites of specific steps in retrotransposon and viral life cycles. An important area of future research would be to address the role of P-bodies in other aspects of mRNA metabolism. It is likely that yeast will continue to lead the way toward understanding P-bodies, however because many relevant P-bodies components are not found in yeast, such as those involved in RNA-mediated silencing, studies in higher eukaryotes are also essential.

CHAPTER VI

REFERENCES

VI. REFERENCES

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